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The *cry*^{b} Mutation Identifies Cryptochrome as a Circadian Photoreceptor in *Drosophila*

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Summary

A new rhythm mutation was isolated based on its elimination of *per*-controlled luciferase cycling. Levels of *period* or *timeless* clock gene products in the mutant are flat in daily light–dark cycles or constant darkness (although PER and TIM oscillate normally in temperature cycles). Consistent with the fact that light normally suppresses TIM, *cry*^{b} is an apparent null mutation in a gene encoding *Drosophila*'s version of the blue light receptor cryptochrome. Behaviorally, *cry*^{b} exhibits poor synchronization to light–dark cycles in genetic backgrounds that cause external blindness or demand several hours of daily rhythm resets, and it shows no response to brief light pulses. *cry*^{b} flies are rhythmic in constant darkness, correlating with robust PER and TIM cycling in certain pacemaker neurons.

Introduction

Circadian photoreception has special physiological properties (reviewed by Roenneberg and Foster, 1997; Foster, 1998). Moreover, distinct tissue structures can subserve circadian photoreception, compared to the standard ones that negotiate light inputs involved in image-forming vision. In circadian systems, extraocular photoreceptors are commonly sufficient to get light to the clock, notably in nonmammalian vertebrates (reviewed by Yoshikawa and Oishi, 1998). Even in mammals, for which the eyes are usually necessary for light-to-clock transmission (Foster, 1998; but see Campbell and Murphy, 1998), orthodox photoreceptor cells in the retina may be unnecessary for light inputs relevant to rhythms (Foster, 1998; Miyamoto and Sancar, 1998; but see Yoshimura and Ebihara, 1998).

Insects can use extraocular photoreception for their circadian system (Page, 1982). In *Drosophila*, the presence or function of external eyes is not necessary to synchronize the fly's rhythms by light input (e.g., Wheeler et al., 1993; Yang et al., 1998). However, the compound eyes are involved, because the sensitivities of genetically eyeless, opsin-depleted, or blind *Drosophila* to light-mediated synchronization of behavioral rhythmicity are much lower than normal (Helfrich-Förster, 1997, and this work). The photosensitive molecules involved in circadian photoreception include one or more of the rhodopsins functioning in *Drosophila*'s external eyes; a mutant (*ninaE*) devoid of the fly's major opsin, as well as *norpA* mutants, are among those whose light sensitivity to entraining light–dark (LD) cycles is poor. The phospholipase C (PLC) encoded by *norpA* functions downstream of light absorption in the phototransduction cascade (reviewed by Zuker, 1996). In another behavioral entrainment study involving an eyeless mutant, dietary depletion of carotenoids did not cause a further decrement in sensitivity of the flies' synchronization to LD cycles; moreover, no retinal was detectable in these doubly defective flies (Ohata et al., 1998). This implicates another kind of circadian photoreceptive substance functioning along with rhodopsin. Periodic emergence of *Drosophila* into adulthood (eclosion) has been suggested to be subserved entirely by nonopsin photoreception; vitamin A–deprived pupae of *D. pseudoobscura* had their peak times of eclosion phase shifted by pulses of light, with no reduction in sensitivity (Zimmerman and Goldsmith, 1971). The maximal sensitivities for such clock resets occurred in the blue range (Frank and Zimmerman, 1969; Klemm and Ninneman, 1976). Light entrainment of pupae with subnormal eye function has not been studied, but behavioral entrainment of adults to LD cycles has, using externally blind mutants (Helfrich-Förster, 1997; Ohata et al., 1998); the nominal peaks for such spectral sensitivities were again at shorter wavelengths than that at which *Drosophila*'s major opsin maximally absorbs (Zuker, 1996) and which most effectively entrains the behavior of adults with eyes (Ohata et al., 1998).

We report here the isolation of a *Drosophila* mutant involving the blue light receptor cryptochrome (see Cashmore, 1998 for a review of those molecules' functions and of *cry* mutants in other organisms); it was found in a novel screen for rhythm variants that involved real-time monitoring of clock gene expression in peripheral tissues. The mutation causes behavioral synchronization defects in addition to molecular cycling ones and affects molecules functioning within the circadian clock. This includes the *timeless* gene product, whose levels are regulated by light, a phenomenon almost certainly linked to entrainment of the fly's rhythms to environmental cycles (reviewed by Young, 1998).

Results

Isolation of a Mutant that Abolishes Cycling of *per* and *tim* Products in LD Cycles

To identify genes involved in the light input pathway entraining the circadian pacemaker, or in the clock itself, we screened for mutants that alter or abolish rhythmic gene expression of *per*. This was made possible by establishing the *luciferase* gene as a reporter for automated

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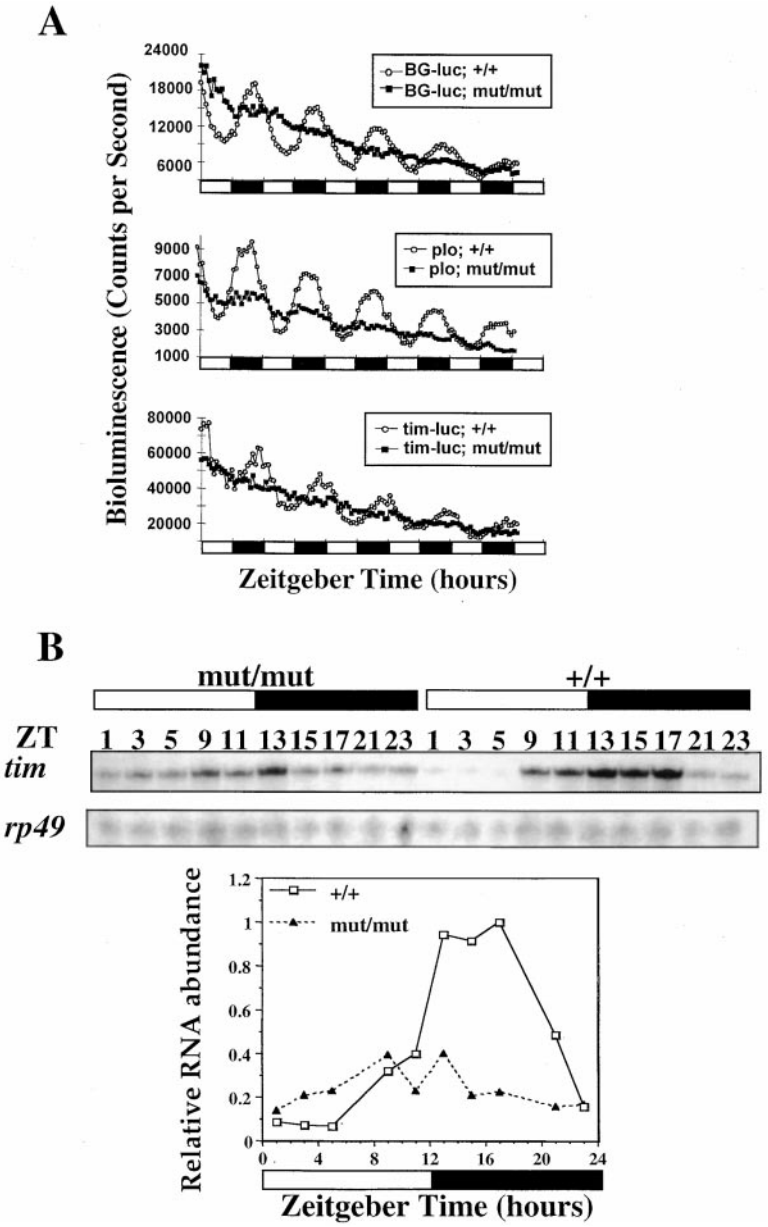

Figure 1. *per* and *tim* Are Expressed Arrhythmically in Flies Homozygous for a Mutation Isolated Using a *per*-Luciferase Fusion Gene

(A) Bioluminescence recordings of individually monitored flies carrying *per*- and *tim*-luciferase fusion genes. Upper panel: average of 21 control flies and 16 mutant ones carrying the X-chromosomally located BG-*luc*60 transgene (*per* 5′-flanking region and PER-encoding sequences fused to *luc*). Middle panel: average of 15 control and 12 mutant flies carrying *per*-luciferase only, in which *per*'s 5′-flanking region is fused directly to *luc*. Bottom panel: average of 16 control and 18 mutant flies carrying a *tim-luc* reporter gene (*tim*'s 5′-flanking region fused directly to *luc*). For flies homozygous for the new mutation, the "flat" averaged time courses (albeit with monotonically decreasing signals, owing to substrate depletion; Brandes et al., 1996) reflects the fact that nearly all individual animals also led to essentially arrhythmic *luc* expression. During meiotic mapping of this mutation, lines were selected that had the BG-*luc* transgene recombined away; those lines were used to cross in the different *luc*-containing transgenes indicated above. Horizontal white and black bars indicate when the lights were on (ZT0 to 12) and off (ZT12 to 24), respectively.

(B) Temporal RNase protection assays of *tim* RNA. RNA of both mutants and controls were separated on the same gel to allow comparison of transcript levels. Signals were standardized against the constitutively transcribed RNA of the ribosomal protein gene *rp49* (e.g., Stanewsky et al., 1997b) and normalized to the peak expression value of the control, set = 1.0.

real-time measurement of rhythmic gene expression in *Drosophila* (Brandes et al., 1996). We chemically mutagenized transgenic flies expressing a PER-LUC fusion protein and monitored descendants of these flies for bioluminescence rhythms. Such rhythms closely reflect native *per* expression in flies carrying a transgene (BG-*luc*) that encodes the N-terminal two-thirds of PER fused to LUC (Stanewsky et al., 1997b). To find recessive or dominant autosomal mutants in a BG-*luc* genetic background, 5137 strains were monitored automatically in 12 hr:12 hr LD and examined for PER-LUC anomalies (see web site version of Experimental Procedures [http://www.cell.com/cgi/content/full/95/5/681/DC1]).

A recessive third chromosomal mutation that abolishes bioluminescence rhythms was identified (Figure 1A). To determine the mutation's effects on *per* and *tim* transcription (cf. Stanewsky et al., 1997b; So and Rosbash, 1997), we introduced a *per-luc* or a newly generated *tim-luc* fusion gene (each encoding luciferase sequences only) into homozygous mutant genetic backgrounds. *luc*-reported expression in both cases was arrhythmic (Figure 1A). In contrast to other recently identified mutations affecting *per* and *tim* expression (Allada et al., 1998; Rutila et al., 1998b), the new mutant does not exhibit profound subnormalities in its overall levels of *per* and *tim* expression (Figures 1–3).

Western blot analyses using head extracts of mutant flies maintained in LD showed that the levels of TIM and PER remained at high levels throughout the day and night, relative to the very low troughs observed during the daytime in wild type (Figures 2A and 2B). In addition, TIM and PER proteins were anomalously present in both

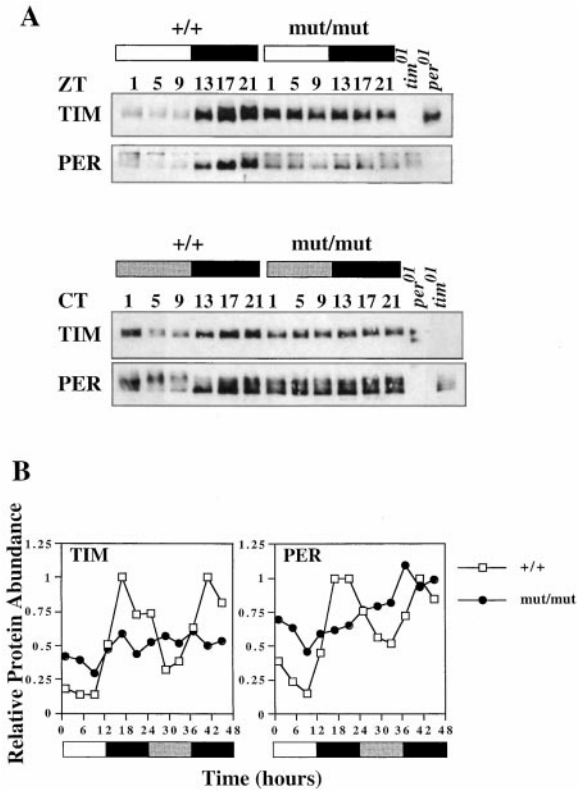

Figure 2. PER and TIM Protein Expression Is Arrhythmic in Homozygous Mutant Flies during and after Light Entrainment

(A) Flies were collected at 4 hr intervals during the third day of a 12 hr:12 hr LD cycle and during the first subsequent day in constant darkness (DD). Equal amounts of head protein extracts were electrophoresed, and the same blot was probed separately with anti-TIM and anti-PER antibodies. The specificity of the antibodies is revealed in extracts of *per*^{01} and *tim*^{01} mutants. White bars, lights were on; black, off; gray, subjective day in constant darkness (lower panel).
(B) Quantification of blots shown in (A). Peak levels of expression in (+/+) were set equal to 1.0 for normalization of data from LD and DD experiments ([+/+] and mutant extracts separated on the same gel). The protein level in the mutant varied between 30% and 60% of the (+/+) maximum. Similar results were obtained in two additional LD experiments and one additional DD time course.

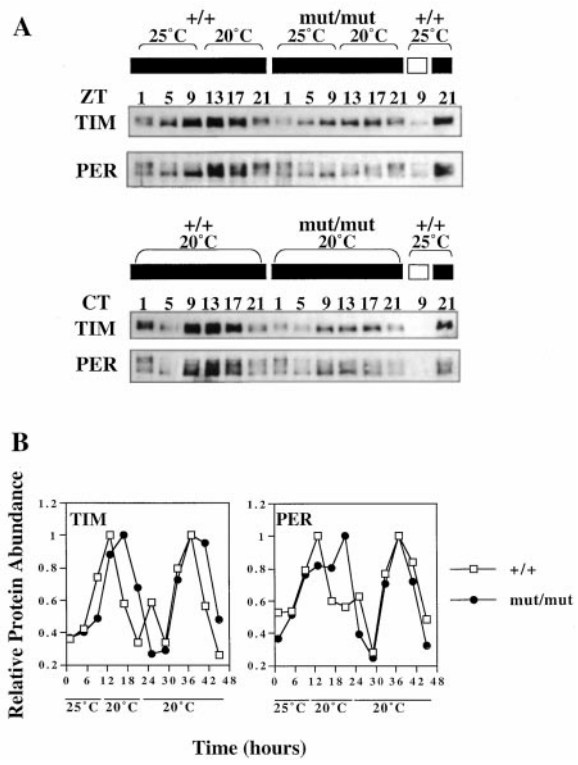

Figure 3. PER and TIM Protein Expression Oscillates during and after Temperature Entrainment

(A) Flies were raised in constant darkness at 25°C. Adults were transferred to new food bottles in the dark and then entrained to a 12 hr:12 hr 25°C:20°C cycle in constant darkness. Flies were collected at 4 hr intervals during the fourth day of entrainment and the first day in constant temperature (20°C). Equal amounts of head protein extracts were electrophoresed and incubated with anti-TIM and anti-PER. To compare the effects of temperature and LD cycling on PER and TIM expression, extracts of flies collected at ZT 9 and ZT 21 in an LD cycle (cf. Figure 2) were loaded on the same gel. White bars, the lights were on; black, off.
(B) Quantification of the blots shown in (A). Peak expression levels for the (+/+) and the mutant were each set equal to 1.0. The overall expression levels of PER and TIM are reduced at any time point by ca. 50% compared to (+/+), but the cycling amplitudes are essentially identical. The same results as those shown were obtained in an independent experiment, in which the temperature cycled between 18°C and 25°C.

hypo- and hyperphosphorylated forms in a temporally unchanging manner (cf. Zeng et al., 1996; Price et al., 1998). That TIM stays at the same levels during the day and night in the mutant is especially interesting, because the rapid disappearance of this protein in response to light is the earliest response to this stimulus of a known component of *Drosophila*'s rhythm system. Yet the absence of rhythmic clock gene transcription (Figure 1B) indicates that the mutant is doubly defective. This is because either of two regulatory phenomena is sufficient to drive TIM cycling (reviewed by Young, 1998): oscillating *tim* expression (which occurs in the absence of environmental fluctuations) or light suppression of TIM (in the absence of *tim* mRNA cycling). Against this background, the absence of effects of some (but not all) orthodox visual mutations on light-induced TIM degradation is notable (Yang et al., 1998), as is the fact that peak sensitivity for this light effect is in the blue range (Suri et al., 1998). Thus, the new mutation might uniquely affect elements of the light entrainment pathway, which would include extraocular reception and processing of blue light inputs. Alternatively, the mutation could affect a protease whose targets include TIM and PER.

If that is not the case, and the new mutation causes a specific defect in the light entrainment pathway, protein oscillations in temperature cycles should not be affected (cf. Crosthwaite et al., 1997; also note that *Drosophila* entrain to temperature cycles [Wheeler et al., 1993]). Western blots of extracts from mutant and normal heads showed that PER and TIM fluctuated robustly in 12 hr:12 hr, 25°C:20°C cycles (Figure 3); such cyclings continued in constant conditions. The daily mobility shifts of PER and TIM are apparent in both genetic backgrounds (Figure 3), indicating that the phosphorylation program can function in the mutant.

Table 1. A Third Chromosomal Deletion Uncovers a Mutation that Affects Clock Gene Cycling

Genotype	# Rhy/Tested (% rhythmic)	Period (h)	Phase ZT (h)	Rel. Amp. Error
+/+ (m)	26/26 (100)	24.4 ± 0.1	19.4 ± 0.2	0.21 ± 0.01
mut/mut (m)[a]	2/16 (13)	23.7 ± 0.2	2.5 ± 0.6	0.55 ± 0.03
mut/*Df(3R)* (m)	24/31 (77)	23.3 ± 0.2	4.3 ± 0.7	0.52 ± 0.02
mut/+ (m)	19/19 (100)	24.3 ± 0.1	19.6 ± 0.2	0.20 ± 0.01
Df(3R)/+(m)	19/19 (100)	24.3 ± 0.1	20 ± 0.2	0.22 ± 0.01
mut/*Df(3R)* (f)	0/11 (0)	—	—	—
Df(3R)/+(f)	9/10 (90)	24.8 ± 0.3	18.3 ± 1.0	0.48 ± 0.05

Bioluminescence rhythms of individual flies were recorded as in Stanewsky et al. (1997b). Flies carried either one copy of BG-*luc* on the third chromosome (used in screening for EMS-induced mutants) or on the X chromosome (BG-*luc*60). Data values are means ± SEM. Phase indicates the peak time of expression (computed as in Plautz et al., 1997b). Significantly rhythmic (Rhy) flies had relative amplitude (rel. amp.) values <0.7 (see web site version of Experimental Procedures).

[a]The averaged bioluminescence expression of these 16 flies is plotted in Figure 1A. mut, mutant; *Df(3R)*, *Df(3R)DI-BX12*; (m), male flies; (f), females (which in general exhibit relatively weak *per-luc* rhythms). The mediocre cyclings in homozygous mutant or *Df*/mut males (high rel. amp. errors) had phases 6 to 8 hr discrepant from those of heterozygous mutants or wild types (cf. Stanewsky et al., 1997b).

The Mutation Maps within the ORF of a Cryptochrome-Encoding Gene

The mutation was meiotically mapped to a position approximately in the middle of the right arm of chromosome *3*, concomitant with separating it from the BG-*luc* transgene (see web site version of Experimental Procedures). A cryptochrome gene (*cry*) was found to be at a nearby locus that is removed by a *3R* deletion (Emery et al., 1998 [this issue of *Cell*]). Flies heterozygous for the mutation and this deletion exhibited poor bioluminescence rhythms (in a BG-*luc* background, that transgene having been brought in via the X chromosome; Table 1). Although a higher proportion of mutation-over-deletion males was rhythmic compared to homozygous mutant flies, formal analysis of these rhythms indicated that they were very weak and exhibited anomalous phases (Table 1; cf. Stanewsky et al., 1997b); mutation-over-deletion females were thoroughly arrhythmic (Table 1).

We sequenced the *cry* in the mutant strain and the starting one used for mutagenesis. A missense mutation was detected (Figure 4A) in a relatively C-terminal position within the cryptochrome polypeptide (Figure 4B). We therefore refer to this mutant as cry^{baby} (cf. Ragovoy and Berns, 1971). The Asp residue at the mutated position is highly conserved among Class I photolyases as well as blue light cryptochrome photoreceptors (Figure 4C) and has been shown to be involved in flavin binding in *E. coli* photolyase (Park et al., 1995; Kanai et al., 1997). Western blotting data (cf. Emery et al., 1998) indicated that cry^{baby} (cry^{b}) is a null mutant or a barely leaky one (Figure 4D). With regard to the mRNA that encodes this protein, the mutation causes this transcript not to cycle, as it does normally (Emery et al., 1998). *cry* RNA was constitutively at 65%–70% of the wild-type peak level (n = 3 RNase protection time courses; data not shown); this is not nearly enough of a decrement to explain the lack of Western blot signal (Figure 4D). That cry^{b} leads to no detectable protein is not a result of the Asp→Asn substitution eliminating a relevant antigenic determinant, because the immunogen used to produce anti-CRY was a polypeptide fragment upstream of the mutated site (Emery et al., 1998). Therefore, the lack of signal in the mutant extract (Figure 4D) seems not to be the result of a change that would leave CRY^{b} at normal levels but not antigenic. It seems instead that alteration of a flavin-binding site in cry^{b} (Figure 4C) deranges the integrity of the protein, causing it to be degraded as it tries to accumulate.

Behavioral Entrainment of cry^{b} Flies to Light–Dark Cycles

Given the drastic effects of this mutation on PER and TIM expression (Figure 2), we expected the locomotor behavior of cry^{b} also to be abnormal. Surprisingly, cry^{b} flies were rhythmic in both LD and DD conditions with ca. 24 hr periods (Table 2). In further LD experiments, flies were first exposed to 5 days of 12 hr:12 hr LD (white light, intensity 640 lux), followed by a second 5 day LD regime in which the lights came on 4 hr later and were changed to dim blue (see web site version of Experimental Procedures). cry^{b} flies entrained to the initial LD cycles, since all the mutant individuals exhibited characteristic bimodal activity peaks (exemplified in Figure 5A, upper left; cf. Hamblen-Coyle et al., 1992). This mutant individual also had no trouble reentraining to the second light regimes, even though a much lower light intensity was used. The same behavior was observed for cry^{+} and cry^{b} flies at all light intensities (Table 3, and data not shown).

Externally blind flies behaviorally entrain to LD cycles but are less sensitive to the synchronizing effects of light in terms of their behavioral rhythmicity (see Introduction and the web site version of Experimental Procedures). Thus, we tested the light responsiveness of $norpA^{P41}$;cry^{b} double mutants; this *norpA* loss-of-function mutation causes the compound eyes and ocelli to be completely unresponsive to light (Pearn et al., 1996). The doubly mutant flies already exhibited entrainment problems in the initial LD regime (Table 3). Subsequently, only about 50% of the flies that entrained to the initial LD cycle were able to synchronize to a new light regime that applied 16-lux blue light; and at still lower light intensities, nearly all of the double mutants failed to entrain to the new LD cycles (Table 3). Figure 5A (upper right) shows an individual actogram of a $norpA^{P41}$;cry^{b} fly, which entrained to the initial LD regime but failed to do so in the new condition (delayed onset of 16-lux blue

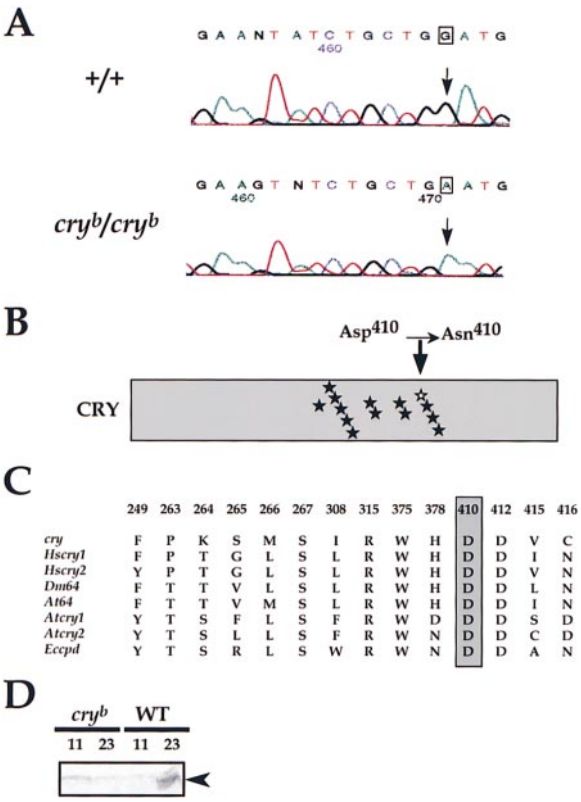

Figure 4. *cry*^{b} Is a Missense Mutation at a Highly Conserved Flavin-Binding Residue within *Drosophila*'s CRY Protein

(A) Results of sequencing the *cry* ORF in the mutant. Electropherogram of the starting strain used in mutagenesis (+/+) compared to that of *cry*^{b}. Arrows indicate the G→A transition.

(B) Schematic representation of the 542 aa CRY protein (cf. Emery et al., 1998). The positions of all 14 residues potentially involved in flavin binding (cf. Park et al., 1995) are indicated by stars (some of which are stacked semivertically because such sites are so near each other in the linear sequence); the white star designates the Asp→Asn substitution in *cry*^{b}.

(C) Conservation of flavin-binding residues among different Class I photolyases and cryptochromes (cf. Park et al., 1995; Kanai et al., 1997). Numbers above the single-letter aa abbreviations refer to positions of potential flavin-binding residues in CRY. Compared are (top to bottom) *D. melanogaster* cryptochrome (*cry*), human cryptochromes (2 genes' worth), *D. melanogaster* and *Arabidopsis thaliana* (6-4) photolyases, *A. thaliana* cryptochromes (2 genes' worth), and *E. coli* (CPD Class I) photolyase.

(D) CRY protein is absent or at very low levels in the *cry*^{b} mutant. Arrow, Western blot signal detected in extracts from *cry*$^+$ adult heads (cf. Emery et al., 1998). Times (ZTs) during an LD cycle when the flies were taken for extractions were near the end of the day (ZT11) and of the night (ZT23). This mutant versus normal comparison was repeated and gave the same result as depicted in the blot shown.

light). This effect can also be seen in average activity plots for a group of doubly mutant flies (Figure 5A, middle and lower right). The activity offsets remained in synchrony with the lights off time of the initial entrainment regime, indicating that the externally blind and *cry*-mutated flies did not resynchronize to the new LD cycles. Note that the light intensity in this example is 100-fold higher compared to that which readily phase shifted the *cry*^{b} single mutant (Figure 5A, left). Since *norpA* and *cry*^{b} mutations by themselves cause minimal or no entrainment problems at these low light intensities, the synergistic effect observed in the double mutant suggests that the two light entrainment pathways operating in *Drosophila*'s rhythm system are now genetically defined.

A Fast Clock *period* Mutation also Uncovers Entrainment Defects in *cry*^{b}

per^S flies have a free-running period of 19 hr (Konopka and Benzer, 1971) but entrain to 12 hr:12 hr LD (bright light) cycles and are simply "driven into" 24 hr behavioral periodicities (Hamblen-Coyle et al., 1992). However, double mutant *per*^{S};*cry*^{b} flies exhibited complex behavior under LD conditions. Figure 5B (upper right) shows a typical actogram of a double mutant, which is different from that of the *per*^{S} control (Figure 5B, upper left); the latter's behavior was synchronized by the LD cycles and thus had 24 hr periodicity. In contrast, the double mutant showed both entrained and free-running behavioral components: late-in-the-day activity onsets that were terminated by lights off, and active phases whose offsets ended several hours earlier during successive 3 day periods (Figure 5B, upper right), similar to the ca. 19 hr periodicity observed after the lights went off for good (below the arrows in Figure 5B, upper). We applied spectral and autocorrelation programs to the LD portions of the mutant records (cf. Dowse and Ringo, 1993): 16/39 of the *per*^{S};*cry*^{b} records were found to contain ca. 19 hr periodic components, whereas none of the 19 *per*^{S} flies did (cf. Hamblen-Coyle et al., 1992). Since the double mutants were active at different times essentially every day (Figure 5B, upper right), their average activity plot shows a monotonic decrease in behavior throughout the daytime (Figure 5B, lower right), whereas singly mutant *per*^{S} flies exhibited a pronounced midday peak of activity (lower left).

The *cry*^{b} Mutant Does Not Exhibit Phase Shifts in Response to Light Pulses

To assess clock resetting by brief pulses of light in (otherwise) constant darkness, phase response curves (PRC) were generated. Wild-type flies (and organisms in general) show phase delays after light pulses are given in the early subjective night, advances in late subjective night, and little or no phase shifting following pulses during the subjective day (*Drosophila* example in Saunders et al., 1994). When *cry*^{b} flies were subjected to light pulses, no clear phase shifts resulted (Figure 5C). This seems to contradict the fact that *cry*^{b} flies tested for entrainment to different (phase shifted) LD cycles were able to "shift over" even at much lower light intensities (Figure 5A). The apparent discrepancy could be explained by differences between the two experimental designs: in one case, flies are exposed to 12 hr of light, and in the PRC case, only to 10 min worth. In a very different kind of behavioral test involving responses to visual stimuli—using short exposures of *cry*^{b} flies to a relatively high light level, as in the PRC experiment—the mutant exhibited normal optomotor behavior (see Experimental Procedures).

Table 2. Diel and Free-Running Locomotor Rhythmicities are Normal in *cry*^{b}-Variant Flies Carrying the Mutation, a Deletion Encompassing the Locus, or both

Genotype	Period[a] in LD (n)	% Rhythmic	Period[a] in DD (n)	% Rhythmic
cry$^+$/*cry*$^+$	24.0 ± 0.0 (68)	93	24.0 ± 0.1 (63)	81
cry^b/*cry*^{b}	24.1 ± 0.0 (124)	84	24.1 ± 0.1 (116)	66
Df(3R)/+	24.1 ± 0.0 (31)	97	23.6 ± 0.1 (30)	93
Df(3R)/*cry*^{b}	24.0 ± 0.0 (38)	68	23.8 ± 0.1 (35)	83

Period values (or the lack thereof) were determined separately for the LD and DD portions of a given fly's record by χ^2 periodogram; only flies showing periods in combination with a "power" value ≥20 and a time bin "width" of ≥2 were considered rhythmic (cf. Ewer et al., 1992). The lower-than-wild-type percentages of rhythmicity for flies expressing certain of the *cry*^{b}-containing genotypes are probably due to genetic background effects and are not a consequence of the *cry*^{b} mutatution (see the web site version of Experimental Procedures).

[a] Period values are h ± SEM. *Df(3R)*: *Df(3R)DI-BX12*. Flies were tested under 12 hr:12 hr light–dark cycling conditions for 7 days (leading to "diel" behavior), with monitoring of locomotor activity continuing during 7 subsequent days in constant darkness (DD).

The *cry* Mutation Does Not Eliminate TIM and PER Cycling within Certain Clock Gene–Expressing Neurons

The *cry*^{b} mutant exhibits rhythmic behavior in constant darkness (Table 2; Figure 5B) in spite of the fact that no rhythmic protein expression during and after light entrainment was detectable (Figure 2). In Western blots involving head extracts, PER and TIM are measured mainly in photoreceptor cells (ca. 90% of the anterior PNS and CNS cells expressing these genes; Kaneko, 1998). We thought that rhythmic clock gene expression in the central pacemaker cells—the lateral neurons (LNs) that subserve behavioral rhythmicity (Kaneko, 1998)—could be masked by constitutive PER and TIM levels (Figure 2) in the *cry*^{b} mutant's eyes. The LNs consist of two groups of cells in each side of the brain, ca. 6 neurons in a relatively dorsal cluster (LN_ds) and ca. 10 such cells in a more ventrally located one (LN_vs). Clock functions in the LN_vs (along with the relevant molecular, physiological, and anatomical outputs) are probably sufficient to generate rhythmic behavior (Kaneko, 1998).

Table 3. Behavior of *cry*^{b} and/or *norpA* mutants in LD Phase Shift Experiments

	No. Synchronized to New LD Regime/No. Tested (Arrhythmic)		
Genotype	16 lux	1.6 lux	0.16 lux
norpA$^+$;*cry*$^+$	31/34 (2)	23/25 (1)	26/27 (1)
cry^b/*cry*^{b}	27/28 (1)	27/27 (0)	29/31 (2)
norpA^{P24}	10/10 (0)	4/6 (1)	ND
norpA^{P41}	17/18 (1)	6/7 (1)	22/31 (3)
norpA^{P41}, *cry*^{b}/*cry*^{b}	7/26[a] (3)	3/25[b] (5)	0/27[a] (4)

After being entrained in a 12 hr:12 hr LD cycle using bright white light (640 lux), males (hence, only one dose of a given X-chromosomal *norpA* allele) were exposed to a 4 hr delayed 12:12 LD cycle, using blue light (385–488 nm) of the indicated intensity.

The ability of a fly to synchronize to the new blue LD regime was judged by inspecting the individual's actogram; only flies that displayed an obvious activity rhythm throughout the experiment (cf. Figure 5) were included in the numerators. Arrhythmic individuals are included in total flies tested, their numbers indicated in parentheses. ND, not done.

[a] In each of the two experiments represented by the data in the first and third data columns, only 16 of the 23 rhythmic flies entrained to the initial LD cycle.

[b] Only 18 out of the 20 rhythmic flies (second data column) entrained to the initial LD cycle; non-entrained (but rhythmic) flies therefore free-ran in LD; for flies expressing the other genotypes, all rhythmic individuals entrained to the first LD cycle.

We examined TIM and PER expression in the CNS (and in other cells of fly heads) by performing antibody stainings on sections of wild-type and *cry*^{b} tissues. These were stained at two time points at which PER and TIM are at trough and peak levels (Figure 6A; cf. Zerr et al., 1990; Hunter-Ensor et al., 1996). The staining intensities for different PER- and TIM-expressing cell types (compound-eye photoreceptors, glia, LN_ds, and LN_vs) were scored blindly (see web site version of Experimental Procedures). Both proteins were observed to cycle in the LNs of *cry*^{b} mutant flies, although with reduced amplitude compared to wild type (Figure 6B). Temporally constitutive, intermediate-level signals were observed in the eyes and glial cells, which explains the Western blot results obtained from extracts of *cry*^{b} heads.

In scoring these stained sections, we did not distinguish between the two different types of LN_vs, which consist of four to five large and four to five smaller neurons; the latter tend to be located in a cluster just ventral to the larger cell bodies (Kaneko, 1998) and are especially important for the control of locomotor rhythmicity (Helfrich-Förster, 1998). To better visualize cycling in the different types of LNs, we performed anti-TIM antibody stainings on whole-mounted preparations of adult brains (Figure 6C). At ZT21, all the LN groups were stained, both in wild type and *cry*^{b}; however, the number of cells per cluster was reduced in mutant preparations except in the small ventrally located LNs; here, ca. 4 cells were strongly stained in each of the 20 brain hemispheres observed. The smaller LN_vs of the mutant were not labeled at ZT9 (Figure 6C), indicating robust cycling of TIM and PER in this subgroup of pacemaker neurons (note, however, that staining for these proteins in *cry*^{b} has not been performed in DD). The large LN_vs and neurons in other clock gene–expressing clusters were anomalously stained at both ZT9 and ZT21. *cry*^{b} also abolishes rhythmic PER and TIM expression in the retina as well as in glia (Figures 6A and 6B). Moreover, in the external photoreceptors the signals were not nuclear, as they normally are at ZT21 (reviewed by Kaneko, 1998).

Discussion

The behavioral effects of *cry*^{b} (Figure 5; Table 3) indicate that the normal gene's function is involved in light-mediated entrainment and clock resettability of the pacemaker that underlies *Drosophila*'s daily rhythms of locomotion. Yet the protein encoded by *cry* is not the only

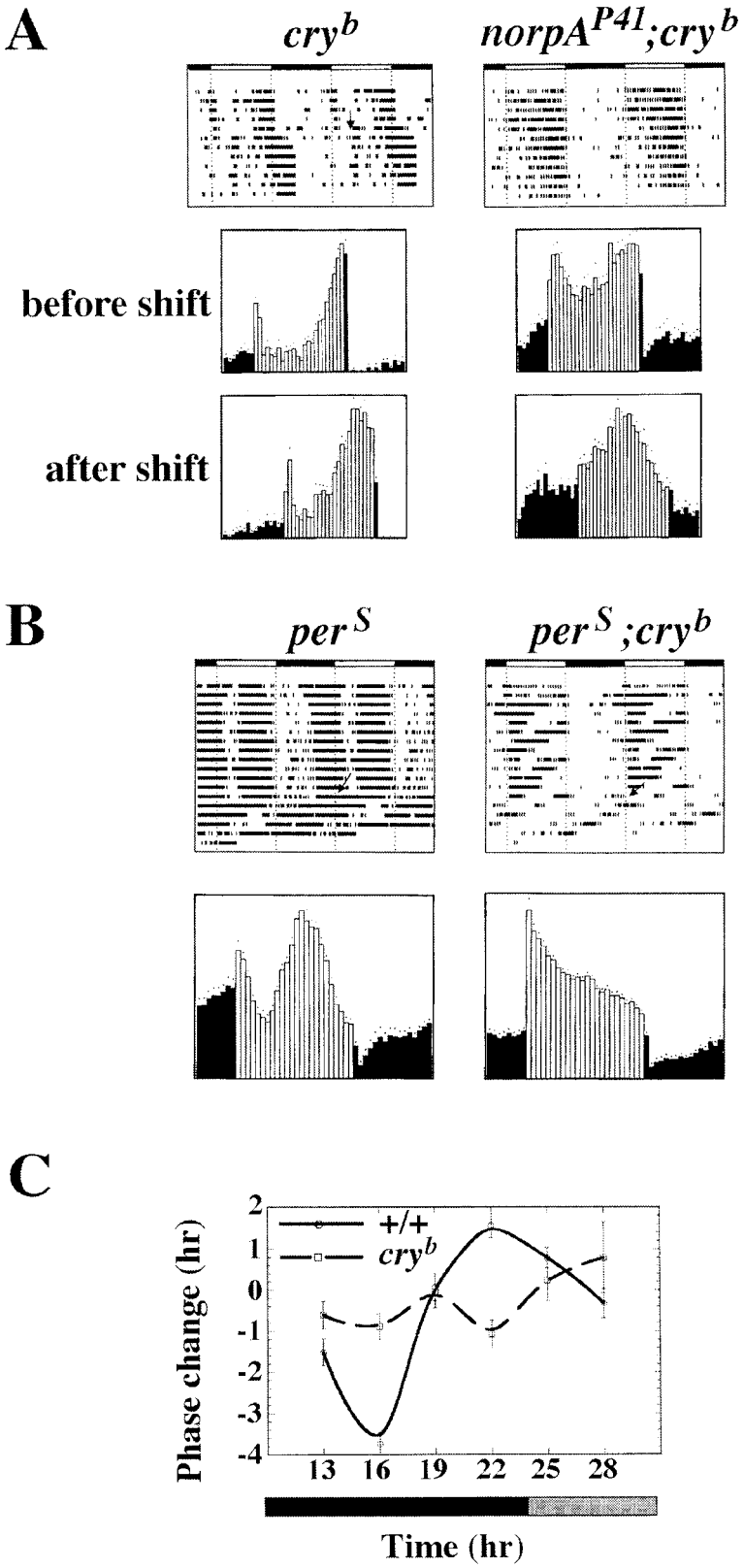

Figure 5. Rhythm Resetting Defects Caused by *cry*^{b}

(A) Behavior in LD cycles was monitored for *cry*^{b} and *norpA*^{P41};*cry*^{b} flies. Adults were entrained for 5 days (12 hr:12 hr LD) using 640-lux white light, followed by a phase shift. On day 6, the lights came on 4 hr later (arrow), and L in the new regime was changed blue light at 0.16-lux (*cry*^{b}) or 16-lux (*norpA*^{P41}; *cry*^{b}). Flies were kept in the second regime for 5 days, and then all the activity data for each individual were inspected by scrutiny of actograms (upper row); average activity plots depicting the overall behavior of flies of a given genotype were also generated (bottom two rows). In the actograms, each hash mark represents 20 activity events/30 min. The data are double plotted (activity from day 1 and 2 on the first line, day 2 and 3 on the second, and so on). These actograms are representative of how flies expressing these genotypes behaved, except that some of the doubly mutant flies did not entrain to the initial LD regime, and some entrained to both regimes (Table 3). In the average activity plots, the ordinates (which are dimensionless, owing to the interfly normalizations; cf. Hamblen-Coyle et al., 1989) reflect locomotor event averages for a given group of flies (n = 13 for *cry*^{b}; n = 15 for *norpA*^{P41};*cry*^{b}). On the abscissas (of actograms as well as average activity plots), the black and white bars indicate when the lights were on (ZT0 to 12) and off (ZT12 to 24), respectively. The generally higher activity level of *norpA*^{P41};*cry*^{b} flies is most likely a genetic background effect and not caused by either one of the mutations (see web site version of Experimental Procedures). The flies whose behavior is summarized via the average activity plots all entrained to the new light regime in the case of *cry*^{b}, whereas the plot for *norpA*^{P41};*cry*^{b} includes six flies that thoroughly entrained and six flies that did not, two that did not entrain to the initial LD cycle (i.e., they free-ran in LD), and one arrhythmic individual.

(B) Behavior in 12 hr:12 hr LD cycles of *per*^{S} and *per*^{S};*cry*^{b} mutants. Flies were monitored for 13 days in LD (using bright white light), followed by 4 days in constant darkness (arrow, time of transition to DD). The two actograms (generated as in [A]) are representative for the 20 *per*^{S} and the 40 *per*^{S};*cry*^{b} flies tested. This *per*^{S} fly had a 24 hr period in LD and a 20.5 hr one in DD; the *per*^{S};*cry*^{b} individual, 24 hr (principal component in LD) and 19 hr (DD), determined by periodogram. This double mutant's behavior yielded an additional 19 hr period during the LD portion of its record (determined by MESA and autocorrelation). Average activity plots were generated for the LD portion of the run (n = 20 for behavioral records for both *per*^{S} and *per*^{S};*cry*^{b}).

(C) Phase response curves for wild-type and the *cry*^{b} mutant. Flies were pulsed with 10 min of bright white light (1400 lux) during the last dark phase of the LD entrainment cycles (black bar) and during the first subjective day of DD (gray bar) at the times indicated on the abscissa. Phase changes were calculated by comparing behavioral offsets of light-pulsed flies, 2 days after the pulse, to the behavior of the control group that did not receive a pulse. Phase delays and advances are plotted (± SEM) as negative and positive values, respectively. Data were pooled from the following number of flies (each pair of values referring to wild-type and homozygous *cry*^{b}): control: 13, 5; pulse at ZT13: 13, 10; ZT16: 14, 9; ZT19: 15, 11; ZT22: 15, 11; CT1: 14, 10; CT4: 9, 8. An additional mutant versus normal comparison gave very similar results: *cry*^{b} flies showed minor phase advances (1 hr) at ZT16, when substantial phase delays are observed in wild type; at all other time points, no significant phase advances or delays were observed for *cry*^{b}. The PRC for wild type in this separate experiment looked identical to the one shown. Number of flies in the second experiment (as above): for the nonpulsed control: 14, 10; for pulses at ZT13: 16, 13; ZT16: 13, 10; ZT 19: 14, 15; ZT22: 11, 14; CT1: 13, 11; CT4: 11, 10.

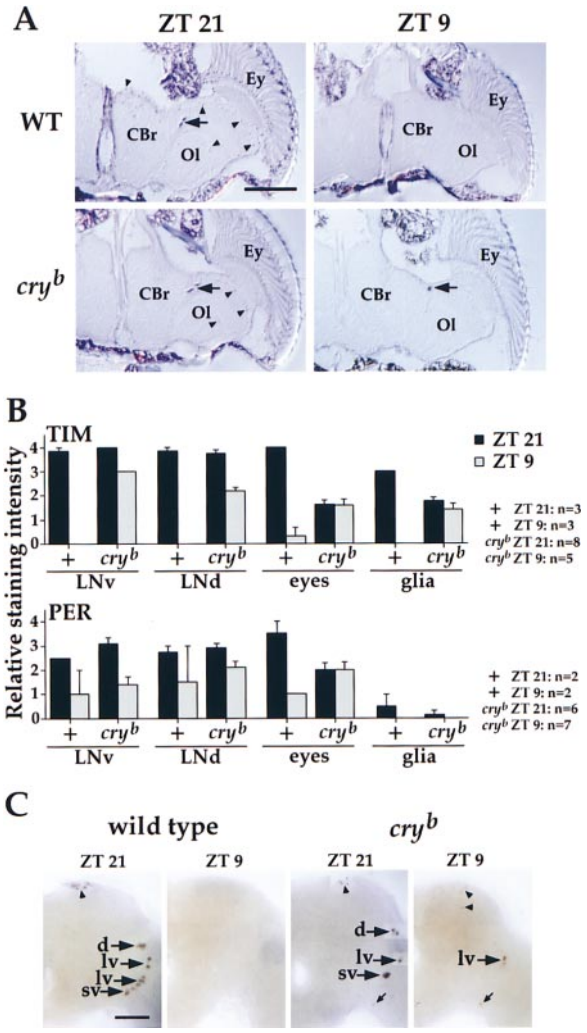

Figure 6. *cry*^{b} Abolishes Rhythmic TIM and PER Expression in Photoreceptor and Glial Cells, But Not within Certain Pacemaker Neurons of Adults Brains

(A) Anti-TIM antibody stainings on head sections of *cry*$^+$ [here called wild-type (WT) but actually *y Df(1)w*] and *cry*^{b} mutant flies at peak (ZT21) and trough (ZT9) levels of TIM expression (cf. Hunter-Ensor et al., 1996). Arrows point to the ventral group of the lateral pacemaker neurons (LN_vs), and arrowheads to glia. At ZT 21, LN_vs with relatively small somata are shown here, whereas in *cry*^{b} at ZT 9, an LN_v with a larger neuronal cell body is shown. Note that almost no nuclear signals are seen in photoreceptors of *cry*^{b} at both time points. CBr, central brain; OL, optic lobe; Ey, eye. Scale bar: 100 μm.

(B) Histograms showing quantitation of staining intensities for anti-TIM immunohistochemistry (cf. [A]) and for a similar set of data stemming from anti-PER staining (images not shown). The numbers of flies scored for each genotype, time points, and proteins detected (TIM or PER) are shown on the right side of each graph. Average staining intensities (plotted ± SEM) were determined subjectively but by blind scoring. For certain cell types and genotypes no histogram bars appear, which does not mean "not done," but that staining scores of 0.0 were assigned to all preparations examined. Glial signals were almost undetectable by anti-PER in both *cry*$^+$ (WT) and mutant. The lower amplitude of TIM and PER cyclings in the LN_vs of *cry*^{b} are most likely due to the noncyclical expression of the proteins within a subgroup of these neurons (the large LN_vs; see Results and [C]). The distinctly noncycling results in *cry*^{b} eyes were inferred from more than the equivalent averaged values for the two time points; thus, photoreceptor staining scores did not range down to zero among specimens but were decoded as intermediate values for essentially all individuals; no appreciable intercellular heterogeneities were observed in the retina within a given sectioned head.

(C) Anti-TIM-mediated stainings on whole-mounted brains of *cry*$^+$ (WT) and *cry*^{b} mutant flies. Ten brains were stained for each time point and genotype. Images from several focal planes were pasted together to give an overview of different neuronal cell types that express this protein. Arrows marked with "d" point to relatively dorsal cluster of lateral neurons (LN_ds). Arrows marked with "lv" and "sv" point to clusters of LN_v cells possessing relatively large and small somata, respectively. Arrowheads point to PER- or TIM-expressing neuronal clusters located in the dorsal protocerebrum (DNs; cf. Ewer et al., 1992; Kaneko, 1998). The small arrows point to a faintly stained cell cluster that was detected only in *cry*^{b}. In *cry*^{b} at ZT21, TIM immunoreactivity was detected in all the classes of *tim*-expressing neurons. However, the numbers of cells stained were reduced compared to *cry*$^+$ (e.g., 5.2 ± 0.2 LN_ds in WT and 2.9 ± 0.2 in *cry*^{b}), except the small LNs, for which ca. 4 cells were consistently stained in both genetic types (3.8 ± 0.1 in WT and 3.9 ± 0.1 in *cry*^{b}). At ZT 9, almost no staining was observed in *cry*$^+$, whereas all classes of neurons but small LN_vs were labeled in more than nine *cry*^{b} brains. In this particular brain hemisphere, LN_ds do not appear because the overall staining was weak, and the failure to detect glial expression of TIM was due to the generally nonintense staining that occurred in this experiment. Scale bar: 50 μm.

photoreceptor involved in this process, as rhodopsin molecules functioning upstream of *norpA*'s product in the canonical phototransduction cascade are a part of the input pathway to the fly's clock (Figure 7A). *norpA*'s PLC could be functioning within the anatomical elements of such pathways located in the CNS as well as within the external eyes, because *norpA*'s product is found within the brain as well as in the eyes (Zhu et al., 1993). This PLC could even act downstream of CRY in extraocular locations, but then a *norpA* mutation would be sufficient to block entrainment in medium-dim light, which is not the case (Figure 5A; Table 3). Therefore, the combined effects of *norpA* and *cry*^{b} mutations on entrainment are likely to be the result of these two functions acting separately in the eye and in the CNS, respectively (Figure 7). This would mean that there are anatomically independent light input pathways to the pacemaker that controls behavior. One of these might involve brain neurons that feed light into the clock. Or, the extraocular pathway could function intracellularly within clock neurons themselves, by analogy to pinealocytes in lower vertebrates being both circadian pacemaker cells and photoreceptors (Yoshikawa and Oishi, 1998). But the fact that anatomical eyelessness causes the same decrement in circadian light sensitivity as do *norpA* mutations (Helfrich-Förster, 1997, and this work) suggests that the latter's effects are acting only through external photoreceptors. *norpA* would participate merely as part of an eye-to-brain throughput pathway, via the compound eyes and optic ganglia, eventually reaching the CNS pacemaker (Figure 7A).

That CRY is not the only important input factor in the fly's circadian system (Figure 7) fits with results from other systems, ranging from microbes to mammals. Multiple input pathways operate with regard to both anatomical structures and physiological processes (e.g., Millar et al., 1995; Roenneberg and Foster, 1997; Yoshikawa and Oishi, 1998). The adaptive value of this complexity is that it permits entraining organisms to sample light of several different wavelengths. Indeed, the quality of light

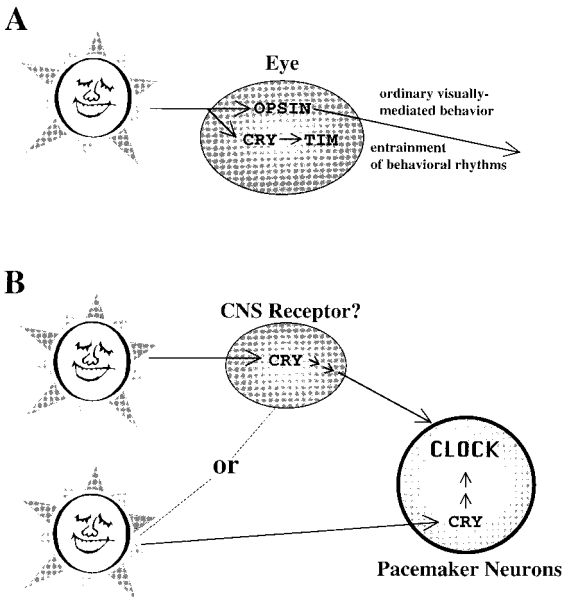

Figure 7. The *cryptochrome* Mutation Affects Peripheral and Central Clock Function in Different Ways: Two Inputs Differentially Regulating Biochemical and Behavioral Rhythms

(A) Visual mutations affecting photoreceptor responses in the eye (*ninaE, norpA*), or vitamin A–deprivation, diminish the fly's ability to synchronize its behavior to light–dark (LD) cycles, thus the arrow proceeding out of the eye. Neither *ninaE* nor *norpA* affects light effects on TIM in the eye, whereas the *cry*^{b} mutation dramatically does (Figure 2), hence the lower branch of the arrow entering the Eye. Visual throughput initiated by OPSIN light absorption—sent from the eye, eventually reaching the clock (see [B]) in order to help entrain it—is hypothesized to be unaffected by *cry*^{b}. Thus, the mutation only blocks TIM responsiveness within the retina, such that light-induced degradation of this clock protein is not involved in the eye-to-brain, pacemaker-entraining throughput. That that pathway has chronobiological significance is revealed by the synergistic effect of combining *cry*^{b} with *norpA* null mutations in LD cycle experiments; thus, when both the eyes (in terms of opsin-mediated reception) and extraocular photoreception (see [B]) are simultaneously defective, the flies do not entrain to (dim blue) light (Figure 5A; Table 3).

(B) *cry*^{b} by itself essentially eliminates the effects of acute light stimuli (brief pulses) on resetting the clock underlying rhythmic behavior (Figure 5C), thus the arrow entering a hypothetical CNS receptor, which would communicate with pacemaker neurons (lower righthand arrow); such CNS receptors could be comprised solely of brain cells expressing the *timeless* gene, owing to the light-induced behavioral phase shifts caused by engineering overexpression of *cry* in these cells (Emery et al., 1998). In this regard, an alternative possibility for inputting the light pulses is that they directly enter the pacemaker neurons underlying behavioral rhythmicity. Such neurons, which may normally express *cry*, consist (or may be entirely comprised) of the small, ventrally located LNs (Figure 6); these are a small fraction of the many *tim*-expressing neurons. Light can stimulate the LNs in the absence of external eyes (Yang et al., 1998) and, hypothetically, autonomous reception by the small LN_v cells is (under this alternative) necessary to cause light-induced phase shifts of the pacemaker: functional eyes in the *cry*^{b} flies that were given brief pulses did not help (Figure 5C).

reaching the earth's surface changes dramatically during dawn and dusk, in particular being enriched in the blue during the dimmest portions of these daily transition times (e.g., Smith, 1982; Roenneberg and Foster, 1997).

cry^{b} has dramatically different effects on clock protein cyclings in gross head extracts (Figure 2) compared to histochemically detected signals in certain brain neurons (Figure 6). Yet it appears to be a null mutant (Figure 4D), so why are TIM and PER oscillations not disturbed in these small, ventral LN cells? That such brain neurons can be entrained by LD cycles (Figure 6), and in a behaviorally meaningful manner (Figure 5; Table 3), is probably because light stimuli reach the small LN_vs via anatomical input pathways that communicate with them (Figure 7). The pathway could start at the external eyes (possessed by all the flies whose brains were examined for Figure 6), or possibly within extraocular photoreceptive structures. The clock gene–expressing LNs can respond to light (by exhibiting a decrease in TIM immunoreactivity) in the absence of external eyes (Yang et al., 1998). CRY may be the only light-absorbing molecule that functions in the extraocular structures so implied. Such structures could include the large, *cry*^{b}-affected LN_v cells (Figure 6), which may possess behaviorally relevant clock functions (Helfrich-Förster, 1998). However, putative deep-brain photoreceptors in other organisms are not necessarily located within pacemaker structures (Yoshikawa and Oishi, 1998; cf. Figure 7), and in *Drosophila* there is a hypothetical light sensor underneath the retina (Hofbauer and Buchner, 1989). At all events, any photoreceptor outside the eyes is likely to possess no cryptochrome function in the *cry*^{b} mutant (Figure 4D). If there is another rhythm-relevant photoreceptive substance in the fly brain, then it would seem not to be a hypothetical "*cry2*" (given the genomic blotting results described in the web site version of Experimental Procedures); nor is it likely to be a brain opsin (given the effects of eyelessness combined with carotenoid depletion; Ohata et al., 1998). This reinforces the notion that light received by the eyes sends signals into the clock, particularly with regard to the small LN_v cycling shown for *cry*^{b} in Figure 6.

Biochemical temperature entrainment time courses and behavioral tests (Figure 3; Table 2) showed that the clockworks are at least partly functional in *cry*^{b}. Therefore, it may be only a synchronization mutant and not defective in pacemaker functions. The flatness of *per* and *tim* product levels—in whole-fly luciferase monitorings or whole-head extracts of *cry*^{b}—could be caused exclusively by inter- or intraanimal asynchronies. But this was not the case. First, all individual *cry*^{b}-expressing flies were very weakly rhythmic or arrhythmic in their *luc*-reported *per* and *tim* fluctuations; the time courses in Figure 1 did not come out flat because of averaging (see figure legend). Second, in the sections and anti-PER or -TIM mediated stainings of individual animals, the asynchrony hypothesis predicts that we should have seen immunohistochemical signals at trough levels in, for example, the eyes of certain specimens and at peak levels in others, or even observed phase differences among separate photoreceptor cells. But the temporal invariances plotted (Figure 6B) reflected similar staining intensities among all flies and the cells therein (see figure legend). These results suggest that the mutant is subnormal in actual pacemaker functioning under LD cycling conditions. This is reinforced by *cry*^{b}'s severe abnormalities of rhythmic clock gene expression at the transcriptional level (Figure 1).

Findings from other systems indicate that light reception and processing are tied to pacemaker functioning (e.g., Millar et al., 1995; Crosthwaite et al., 1997). And

in *Drosophila*, certain clock mutants—isolated as "DD arrhythmic" behavioral variants—turned out to affect the light responsiveness of adult *Drosophila* whose locomotor activity was monitored in LD cycles (Allada et al., 1998; Rutila et al., 1998b). Also consider the molecular effects of these *Clock* and *cycle* mutations, as well as *period* and *timeless* ones, on *cry* expression (Emery et al., 1998). Taken together with cry^b's effects on *per* and *tim* cycles (in LD)—and the fact that the mutation eliminates *cry*'s own mRNA cycling—such interactions suggest connections between CRY function and the central pacemaking mechanism. How intimate these interactions are should be revealed by elucidating the manner in which blue light reception is transduced along a pathway (cf. Zhao and Sancar, 1997; Ahmad et al., 1998) that functions to reset the circadian clock.

Experimental Procedures

A more detailed version of this section can be found on the *Cell* web site and is also available from the authors upon request.

Drosophila Strains and Genetic Methods

The transgenic strains are described in Stanewsky et al. (1997b), except for the new *tim-luc* transcription fusion (methods of construction essentially as in Stanewsky et al. [1997b], using a 6 kb 5′-flanking *tim* fragment described by [Rutila et al., 1998a]). *Drosophila* mutants are described in Lindsley and Zimm (1992), EMS mutagenesis methods (and the basic manner in which subsequent crosses were performed) in Allada et al. (1998).

Luciferase Monitoring

These assays were performed essentially as in Stanewsky et al. (1997b), with the automatically collected data analyzed as in Plautz et al. (1997b).

Bio- and Histochemistry

Western blotting experiments to detect TIM and PER (Figures 2 and 3) or CRY (Figure 4D) were performed essentially as in Zeng et al. (1996), Stanewsky et al. (1997b), and Emery et al. (1998). RNase protection assays were performed and quantified as in Stanewsky et al. (1997b) and Emery et al. (1998).

To detect TIM and PER in situ (Figure 6), 3-day-old adults were processed and scored essentially as in Stanewsky et al. (1997a) and Kaneko et al. (1997).

Chromosomal Mapping and Molecular Genetics

Meiotic mapping of cry^b applied the third chromosomal markers *spineless* and *red* and w^+ (contained in BG-*luc*, inserted in cytogenetic region 80A). cry^b was found to be 6/31 (×100) map units to the right of *ss* and 5/35 (×100) to the right of *red*. The cry^- deletion subsequently applied (Tables 1 and 2) is missing a nearby cytogenetic region (91F1-2 to 92D3-6). The intragenic site of cry^b was mapped using cDNAs (via RT-PCR) and PCRed genomic DNA from mutant flies and cry^+ ones (the mutagenized starting strain). The genome of *D. melanogaster* was examined for a putative *cry2* gene by use of high-density P1 blot (Genome Systems, Inc.) probed at low stringency; only one *cry*-like gene was detectable.

Behavioral Analyses

Locomotor activity of adult males was monitored automatically and analyzed as in Hamblen et al. (1986), Hamblen-Coyle et al. (1989, 1992), Ewer et al. (1992), and Dowse and Ringo (1993). Performance of LD to shifted LD experiments, and/or application of blue light, were essentially as in Wheeler et al. (1993) and Suri et al. (1998) (also see Figure 5A and Table 3). Additional LD to shifted LD experiments were performed to determine how well visual mutants could reentrain to very dim white light: initial LD regime, 5 days, 0.11 lux white light. Each genetic type was split into two groups for the next 5 day cycle, with a 4 hr advanced light onset; L in the second regime was white light of 0.011 lux for one group and 0.0011 lux for the other. Thirty-eight of 64 blind $norpA^{P24}$ or *norpAP41* flies entrained to the first regime, then 1/32 to 0.011 lux and 0/32 to 0.0011. For externally eyeless double mutant (*oc*;*eya*), the corresponding values were 11/31, 0/15, and 0/16; $ninaE^{OI17}$ (rhodopsin-less in R1-6 photoreceptors, among the 8 "R" cells in each compound-eye facet): 21/33, 10/17, and 4/16; wild-type control: 66/68, 37/37, and 31/31.

Phase response curves (Figure 5C) were generated essentially as described in Suri et al. (1998) and Rutila et al. (1998), using brief pulses of relatively bright white light (Figure 5C). Optomotor tests of individual adults, involving freely walking movements in response to rotating vertical stripes, were performed as in Stanewsky et al. (1996); the optomotor score for cry^b was 70 ± 3. Controls: wild-type, 76 ± 2; $optomotor\text{-}blind^{H31}$, 47 ± 3 (n = 10 for each genotype).

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References

Ahmad, M., Jarillo, J.A., Smirnova, O., and Cashmore, A.R. (1998). The CRY1 blue light photorecetor of *Arabidopsis* interacts with phytochrome A in vitro. Mol. Cell *1*, 939–948.

Allada, R., White, N.E., So, V.W., Hall, J.C., and Rosbash, M. (1998). A mutant *Drosophila* homolog of mammalian *Clock* disrupts circadian rhythms and transcription of *period* and *timeless*. Cell *93*, 791–804.

Brandes, C., Plautz, J.D., Stanewsky, R., Jamison, C.F., Straume, M., Wood, K.V., Kay, S.A., and Hall, J.C. (1996). Novel features of Drosophila *period* transcription revealed by real-time luciferase reporting. Neuron *16*, 687–692.

Campbell, S.S., and Murphy, P.J. (1998). Extraocular photoreception in humans. Science *279*, 396–399.

Cashmore, A.R. (1998). The cryptochrome family of blue/UV-A photoreceptors. J. Plant Res. *111*, 267–270.

Crosthwaite, S.K., Dunlap, J.C., and Loros, J.J. (1997). *Neurospora wc-1* and *wc-2*: transcription, photoresponses, and the origins of circadian rhythmicity. Science *276*, 763–769.

Darlington, T.K., Wager-Smith, K., Ceriani, M.F., Staknis, D., Gekakis, N., Steeves, T.D.L., Weitz, C.J., Takahashi, J.S., and Kay, S.A. (1998). Closing the circadian loop: CLOCK-induced transcription of its own inhibitors *per* and *tim*. Science *280*, 1599–1603.

Dowse, H.B., and Ringo, J.M. (1993). Is the circadian clock a "meta-oscillator"? Evidence from studies of ultradian rhythms in *Drosophila.* In Molecular Genetics of Biological Rhythms, M.W. Young, ed. (New York: Marcel Dekker), pp. 195–220.

Emery, P., So, W.V., Kaneko, M., Hall, J.C., and Rosbash, M. (1998). CRY, a *Drosophila* clock and light-regulated cryptochrome, is a major contributor to circadian rhythm resetting and photosensitivity. Cell *95*, this issue, 669–679.

Ewer, J., Frisch, B., Hamblen-Coyle, M.J., Rosbash, M. and Hall, J.C. (1992). Expression of the *period* clock gene within different cell types in the brain of *Drosophila* adults and mosaic analysis of these cells' influence on circadian behavioral rhythms. J. Neurosci. *12*, 3321–3349.

Foster, R.G. (1998). Shedding light on the biological clock. Neuron *20*, 829–832.

Frank, K.D., and Zimmerman, W.F. (1969). Action spectra for phase shifts of a circadian rhythm in Drosophila. Science *163*, 688–689.

Hamblen, M., Zehring, W.A., Kyriacou, C.P., Reddy, P., Yu, Q.,

Wheeler, D.A., Zwiebel, L.J., Konopka, R.J., Rosbash, M., and Hall, J.C. (1986). Germ-line transformation involving DNA from the *period* locus in *Drosophila melanogaster*: overlapping genomic fragments that restore circadian and ultradian rhythmicity to per^0 and per^- mutants. J. Neurogenet. *3*, 249–291.

Hamblen-Coyle, M., Konopka, R.J., Zwiebel, L.J., Colot, H.V., Dowse, H.B., Rosbash, M., and Hall, J.C. (1989). A new mutation at the *period* locus of *Drosophila melanogaster* with some novel effects on circadian rhythms. J. Neurogenet. *5*, 229–256.

Hamblen-Coyle, M.J., Wheeler, D.A., Rutila, J.E., Rosbash, M., and Hall, J.C. (1992). Behavior of period-altered circadian rhythm mutants of *Drosophila* in light:dark cycles (Diptera: Drosophilidae). J. Insect Behav. *5*, 417–446.

Helfrich-Förster, C. (1997). Photic entrainment in *Drosophila*'s activity rhythm occurs by retinal and extraretinal pathways. Biol. Rhythm Res. *28* (Suppl.), 119.

Helfrich-Förster, C. (1998). Robust circadian rhythmicity of *Drosophila melanogaster* requires the presence of lateral neurons: a brain-behavioral study of *disconnected* mutants. J. Comp. Physiol. A *182*, 435–453.

Hofbauer, A., and Buchner, E. (1989). Does *Drosophila* have seven eyes? Naturwissenschaften *76*, 335–336.

Hunter-Ensor, M., Ousley, A., and Sehgal, A. (1996). Regulation of the Drosophila timeless protein suggets a mechanism for resetting the circadian clock by light. Cell *84*, 677–685.

Kanai, S., Kikuno, R., Toh, H., Ryo, H., and Todo, T. (1997). Molecular evolution of the photolyase-blue-light photoreceptor family. Mol. Evol. *45*, 535–548.

Kaneko, M. (1998). Neural substrates of *Drosophila* rhythms revealed by mutants and molecular manipulations. Curr. Opin. Neurobiol. *8*, 652–658.

Kaneko, M., Helfrich-Förster, C., and Hall, J.C. (1997). Spatial and temporal expression of the *period* and *timeless* genes in the developing nervous system of *Drosophila*: newly identified pacemaker candidates and novel features of clock gene product cycling. J. Neurosci. *17*, 6745–6750.

Klemm, E., and Ninnemann, H. (1976). Detailed action spectrum for the delay shift in pupae emergence of *Drosophila pseusoobscura*. Photochem. Photobiol. *24*, 369–371.

Konopka, R.J., and Benzer, S. (1971). Clock mutants of *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA *68*, 2112–2116.

Lindsley, D.L., and Zimm, G.G. (1992). Genetic Variations of *Drosophila melanogaster* (San Diego, CA: Academic Press).

Millar, A.J., Straume, M., Chory, J., Chua, N.-H., and Kay, S.A. (1995). The regulation of circadian period by phototransduction pathways in *Arabidopsis*. Science *267*, 1163–1166.

Miyamoto, Y., and Sancar, A. (1998). Vitamin B_2-based blue-light photoreceptors in the retinohypothalamic tract as the photoactive pigments for setting the circadian clock in mammals. Proc. Natl. Acad. Sci. USA *95*, 6097–6102.

Myers, M.P., Wager-Smith, K., Wesley, C.S., Young, M.W., and Sehgal, A. (1995). Positional cloning and sequence analysis of the *Drosophila* clock gene, *timeless*. Science *270*, 805–808.

Ohata, K., Nishiyama, H., and Tsukahara, Y. (1998). Action spectrum of the circadian clock photoreceptor in *Drosophila melanogaster*. In Biological Clocks: Mechanisms and Applications, Y. Touitou, ed. (Amsterdam: Elsevier), pp. 167–170.

O'Tousa, J.E., Baehr, W., Martin, R.L., Hirsh, J., Pak, W.L., and Applebury, M.L. (1985). The Drosophila *ninaE* gene encodes an opsin. Cell *40*, 839–850.

Page, T.L. (1982). Extraretinal photoreception in entrainment and photoperiodism in invertebrates. Experientia *38*, 1007–1013.

Park, H.-W., Kim, S.-T., Sancar, A., and Deisenhofer, J. (1995). Crystal structure of DNA photolyase from *Escherichia coli*. Science *268*, 1866–1872.

Pearn, M.T., Randall, L.L., Shortridge, R.D., Burg, M.G., and Pak, W.L. (1996). Molecular, biochemical, and electrophysiological characterization of *Drosophila norpA* mutants. J. Biol. Chem. *271*, 4937–4945.

Plautz, J.D., Kaneko, M., Hall, J.C., and Kay, S.A. (1997a). Independent photoreceptive clocks throughout *Drosophila*. Science *278*, 1632–1635.

Plautz, J.D., Straume, M., Stanewsky, R., Jamison, C.F., Brandes, C., Dowse, H.B., Hall, J.C., and Kay, S.A. (1997b). Quantitative analysis of *Drosophila period* gene transcription in living animals. J. Biol. Rhythms *12*, 204–217.

Price, J.L., Blau, J., Rothenfluh, A., Abodeely, M., Kloss, B., and Young, M.W. (1998). *double-time* is a new *Drosophila* clock gene that regulates PERIOD protein accumulation. Cell *94*, 83–95.

Ragovoy, J., and Berns, B. (1971). Cry baby. On The Pearl Album, Joplin, J., voc. (New York: Columbia Records).

Robertson, H.M., Preston, C.R., Phillis, R.W., Johnson-Schlitz, D.M., Benz, W.K., and Engels, W.R. (1988). A stable genomic source of *P*-element transposase in *Drosophila melanogaster*. Genetics *118*, 461–470.

Roenneberg, T., and Foster, R.G. (1997). Twilight times: light and the circadian system. Photochem. Photobiol. *66*, 549–561.

Rosato, E., Trevisan, A., Sandrelli, F., Zordan, M., Kyriacou, C.P., and Costa, R. (1997). Conceptual translation of *timeless* reveals alternative initiating methionines in *Drosophila*. Nucleic Acids Res. *25*, 455–457.

Rutila, J.E., Maltseva, O., and Rosbash, M. (1998a). The tim^{SL} mutant affects a restricted portion of the *Drosophila melanogaster* circadian cycle. J. Biol. Rhythms, in press.

Rutila, J.E., Suri, V., Le, M., So, W.V., Rosbash, M., and Hall, J.C. (1998b). CYCLE is a second bHLH-PAS clock protein essential for circadian rhythmicity and transcription of *period* and *timeless*. Cell *93*, 805–814.

Saunders, D.S., Gillanders, S.W., and Lewis, R.D. (1994). Light-pulse phase response curves for the locomotor activity rhythm in *period* mutants of *Drosophila melanogaster*. J. Insect Physiol. *40*, 957–968.

Sehgal, A., Rothenfluh-Hilfiker, A., Hunter-Ensor, M., Chen, Y., Myers, M., and Young, M.W. (1995). Circadian oscillations and autoregulation of *timeless* RNA. Science *270*, 801–810.

Smith, H. (1982). Light quality, photoperception and plant strategy. Annu. Rev. Plant Physiol. *33,* 481–518.

So, W.V., and Rosbash, M. (1997). Post-transcriptional regulation contributes to *Drosophila* clock gene mRNA cycling. EMBO J. *16*, 7146–7155.

Stanewsky, R., Fry, T.A., Reim, I., Saumweber, H., and Hall, J.C. (1996). Bioassaying putative RNA-binding motifs in a protein encoded by a gene that influences courtship and visually mediated behavior in Drosophila: in vitro mutagenesis of *nonA*. Genetics *143*, 259–275.

Stanewsky, R., Frisch, B., Brandes, C., Hamblen-Coyle, M.J., Rosbash, M., and Hall, J.C. (1997a). Temporal and spatial expression patterns of transgenes containing increasing amounts of the *Drosophila* clock gene *period* and *lacZ* reporter: mapping elements of the PER protein involved in circadian cycling. J. Neurosci. *17*, 676–696.

Stanewsky, R., Jamison, C.F., Plautz, J.D., Kay, S.A., and Hall, J.C. (1997b). Multiple circadian-regulated elements contribute to cycling *period* gene expression in *Drosophila*. EMBO J. *16*, 5006–5018.

Suri, V., Qian, Z., Hall, J.C., and Rosbash, M. (1998). Evidence that the TIM light response is relevant to light-induced phase shifts in *Drosophila melanogaster*. Neuron *21*, 225–234.

Wheeler, D.A., Hamblen-Coyle, M.J., Dushay, M.S., and Hall, J.C. (1993). Behavior in light-dark cycles of *Drosophila* mutants that are blind, arrhythmic, or both. J. Biol. Rhythms *8*, 67–94.

Yang, Z., Emerson, M., Su, H.S., and Sehgal, A. (1998). Reponse of the Timeless protein to light correlates with behavioral entrainment and suggests a nonvisual pathway for circadian photoreception. Neuron *21*, 215–223.

Yoshikawa, T., and Oishi, T. (1998). Extraretinal photoreception and circadian systems in nonmammalian vertebrates. Comp. Biochem. Physiol. B Biochem. Mol. Biol. *119*, 65–72.

Yoshimura, T., and Ebihara, S. (1998). Decline of circadian photosensitivity associated with retinal degeneration in CBA/J-*rd*/*rd* mice. Brain Res. *779*, 188–193.

Young, M.W. (1998). The molecular control of circadian behavioral rhythms and their entrainment in *Drosophila*. Annu. Rev. Biochem. *67*, 135–152.

Zeng, H., Qian, Z., Myers, M.P., and Rosbash, M. (1996). A light entrainment mechanism for the *Drosophila* circadian clock. Nature *380*, 129–135.

Zerr, D.M., Hall, J.C., Rosbash, M., and Siwicki, K.K. (1990). Circadian fluctuation of *period* protein immunoreactivity in the CNS and the visual system of *Drosophila*. J. Neurosci. *10*, 2749–2762.

Zhao, S., and Sancar, A. (1997). Human blue-light photoreceptor hCRY2 specifically interacts with protein serine/threonine phosphatase 5 and mediates its activity. Photochem. Photobiol. *66*, 727–731.

Zhu, L., McKay, R.H., and Shortridge, R.D. (1993). Tissue-specific expression of phospholipase C encoded by the *norpA* gene of *Drosophila melanogaster*. J. Biol. Chem. *268*, 15994–16001.

Zimmerman, W.F., and Goldsmith, T.H. (1971). Photosensitivity of the circadian rhythm and of visual receptors in carotenoid-depleted Drosophila. Science *171*, 1167–1169.

Zuker, C.S. (1996). The biology of vision in *Drosophila*. Proc. Natl. Acad. Sci. USA *93*, 571–576.