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Drosophila CRY Is a Deep Brain Circadian Photoreceptor

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Summary

***cry* (*cryptochrome*) is an important clock gene, and recent data indicate that it encodes a critical circadian photoreceptor in *Drosophila*. A mutant allele, *cry*^{b}, inhibits circadian photoresponses. Restricting CRY expression to specific fly tissues shows that CRY expression is needed in a cell-autonomous fashion for oscillators present in different locations. CRY overexpression in brain pacemaker cells increases behavioral photosensitivity, and this restricted CRY expression also rescues all circadian defects of *cry*^{b} behavior. As wild-type pacemaker neurons express CRY, the results indicate that they make a striking contribution to all aspects of behavioral circadian rhythms and are directly light responsive. These brain neurons therefore contain an identified deep brain photoreceptor, as well as the other circadian elements: a central pacemaker and a behavioral output system.**

Introduction

The properties of our planet's motion around the sun impose several adaptive challenges to living organisms. They not only need to anticipate and adapt their physiology and behavior to the 24 hr changes in the environment but also to make daily adjustments to the changes in day length that occur at most latitudes. Circadian clocks, which are present in cyanobacteria as well as most eukaryotes, help deal with these persistent environmental oscillations. Some clock features have been strongly conserved during evolution. First, circadian rhythms are largely cell autonomous, even in higher eukaryotes, like *Drosophila* or mammals (Tosini and Menaker, 1996; Emery et al., 1997; Giebultowicz and Hege, 1997; Plautz et al., 1997; Balsalobre et al., 1998; Earnest et al., 1999). In flies, the ventral lateral brain neurons (LNvs) are likely pacemakers for behavioral circadian rhythms (Helfrich-Förster, 1998; Kaneko, 1998). But autonomous clocks are also present in a wide variety of peripheral tissues, and circadian oscillations can be observed in isolated organs (Emery et al., 1997; Giebultowicz and Hege, 1997; Plautz et al., 1997). Second, all studied circadian clock models are based on an intracellular molecular pacemaker consisting of a transcriptional negative feedback loop (Dunlap, 1999). In *Drosophila*, at least five genes are necessary to generate the oscillations of the molecular pacemaker: *period* (*per*), *timeless* (*tim*), *Clock* (*Clk*), *cycle* (*cyc*), and *double-time* (*dbt*) (Young, 1998; Edery, 1999). PER and TIM, whose concentrations and phosphorylation states oscillate during the day, negatively regulate their own transcription, probably by interacting with CLK and CYC and downregulating their positive transcriptional activity (Allada et al., 1998; Darlington et al., 1998; Lee et al., 1998, 1999; Rutila et al., 1998a). There is important post-transcriptional regulation, and the DBT kinase phosphorylates PER and influences its lifetime (Kloss et al., 1998; Price et al., 1998). It is likely that similar mechanisms, involving homologous proteins, generate circadian rhythms in mammals (Dunlap, 1999).

Although circadian rhythms can persist in constant conditions, they are of course under the control of environmental cues, principally light and temperature. Light, in particular, plays a prominent role, and the molecular basis of the *Drosophila* light input pathway is beginning to clarify. Several studies have shown that TIM levels are light sensitive (Hunter-Ensor et al., 1996; Lee et al., 1996; Myers et al., 1996; Zeng et al., 1996), and genetic evidence indicates that this light effect is likely to be relevant to clock resetting (Suri et al., 1998; Yang et al., 1998). TIM is therefore probably the pacemaker element targeted by the light input pathway.

Cryptochrome (CRY) was identified as a second contributor to circadian photoreception (Emery et al., 1998; Stanewsky et al., 1998). CRY belongs to a family of blue light–sensitive proteins, which includes photolyases and plant blue light photoreceptors (Kanai et al., 1997; Cashmore et al., 1999). Flies overexpressing CRY are behaviorally hypersensitive to light pulses (Emery et al., 1998). In addition, a mutant allele of the *cry* gene (*cry*^{b}) causes profound molecular and behavioral photoresponse problems: the disruption of PER and TIM molecular cycling in peripheral tissues under light/dark (LD) conditions and an inability of the mutant flies to reset their clock after a short light pulse (Stanewsky et al., 1998). The *cry*^{b} mutation affects a highly conserved amino acid probably involved in binding flavine adenine dinucleotide (FAD), one of the two cofactors necessary for cryptochrome and photolyase functions. As a result, the mutant protein is probably unstable, as well as inactive (Stanewsky et al., 1998). Consistent with the notion that CRY transmits light information to TIM, these two proteins interact in a light-dependent manner in a heterologous system (Ceriani et al., 1999). Very recent data show that *cry*^{b} flies are rhythmic in intense constant light, indicating that CRY is probably the only dedicated circadian photoreceptor (Emery et al., 2000).

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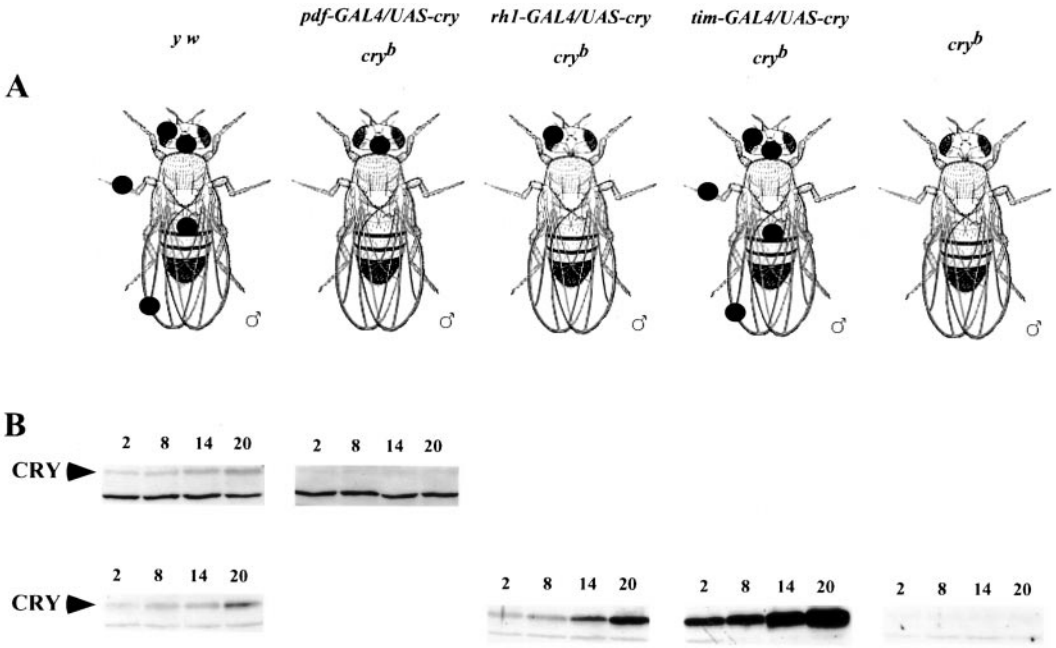

Figure 1. CRY Tissue-Specific Overexpression

(A) The *timeless* (*tim*), *rhodopsin 1* (*rh1*), and *pdf* promoters fused to the GAL4 coding sequence were used to drive CRY expression in all TIM-expressing cells, R1–R6 photoreceptor cells, and LNvs, respectively. The closed circles show schematically the localization of CRY expression in the different indicated genotypes.

(B) Western blots showing the level of CRY expression in wild-type (*y w*) and cry^b fly heads, as well as those obtained with the different combinations of transgenes in a cry^b background. The two rows of Western blots represent separate experiments. The genotypes are indicated in (A). Time of sample collection ([ZT], light is on between ZT 0 and ZT 12, off between ZT 12 and ZT 24) is also indicated. These experiments were performed three times with similar results.

Despite all of these alterations in circadian photoresponses, cry^b flies can still entrain behaviorally to different LD cycles, and the PER and TIM molecular oscillations entrain to light cycles in a subset of the aforementioned LNvs (Stanewsky et al., 1998). This may reflect some residual activity of the CRY^B protein, i.e., cry^b may not be a complete loss-of-function mutant. Alternatively, there may be another CRY-independent pathway, specifically for behavioral photoentrainment. This may involve the eyes, which have been shown to contribute to circadian photosensitivity. Vitamin A–depleted flies have reduced circadian light sensitivity, suggesting a role of opsin-based photopigments (Ohata et al., 1998). In addition, the *norpA; cry*^{b} double mutant shows reduced entrainment (Stanewsky et al., 1998; *norpA* mutations affect a phospholipase-C involved in the visual transduction pathway). The precise nature of this second, putative opsin-based pathway is unclear. It is possible that the eyes send signals to the LNvs or that the eyes directly influence fly behavior; in the latter case, locomotor activity could feed back on the circadian pacemaker cells (Emery et al., 2000). In any case, the eyes are unnecessary for circadian photoentrainment and photoresponses (Wheeler et al., 1993; Yang et al., 1998), raising the question of which other clock-relevant tissues are light sensitive and whether they are CRY dependent.

Interestingly, the LNvs are candidate light-sensitive cells. As mentioned above, these neurons are probably crucial for circadian locomotor behavior (Ewer et al., 1992; Frisch et al., 1994; Helfrich-Förster, 1998), and they were first identified by their strong PER expression (Zerr et al., 1990). They were subsequently shown to express pigment-dispersing factor (PDF; Helfrich-Förster, 1995), a neuropeptide that plays an important role as an output molecule in modulating circadian locomotor activity (Renn et al., 1999). When the LNvs are genetically eliminated, most flies are arrhythmic in constant darkness (DD) (Dushay et al., 1989; Hardin et al., 1992; Helfrich-Förster, 1998; Renn et al., 1999). These neurons can be divided into two groups, according to the size of their somata (Helfrich-Förster, 1996): the large cells send projections mainly into the optic lobe and contralaterally, whereas the small cells send projections to the dorsal part of the brain. Previous studies indicate that the small cells are probably most important for circadian behavior (Helfrich-Förster, 1998). Strikingly, only the small LNvs show robust PER and TIM cycling in cry^b flies (Stanewsky et al., 1998).

In this study, we assigned CRY function to clock-relevant tissues, i.e., the peripheral oscillators and the LNvs, and used genetic approaches to determine the contribution of different tissues to circadian rhythm photoresponses. The results reinforce the remarkable role of the LNvs in all aspects of circadian function, from input to output.

Results

The *Drosophila* GAL4/UAS system is frequently used to overexpress a protein of interest in a specific tissue (Brand et al., 1994). Two transgenic lines are required: the first contains the yeast transcription factor GAL4

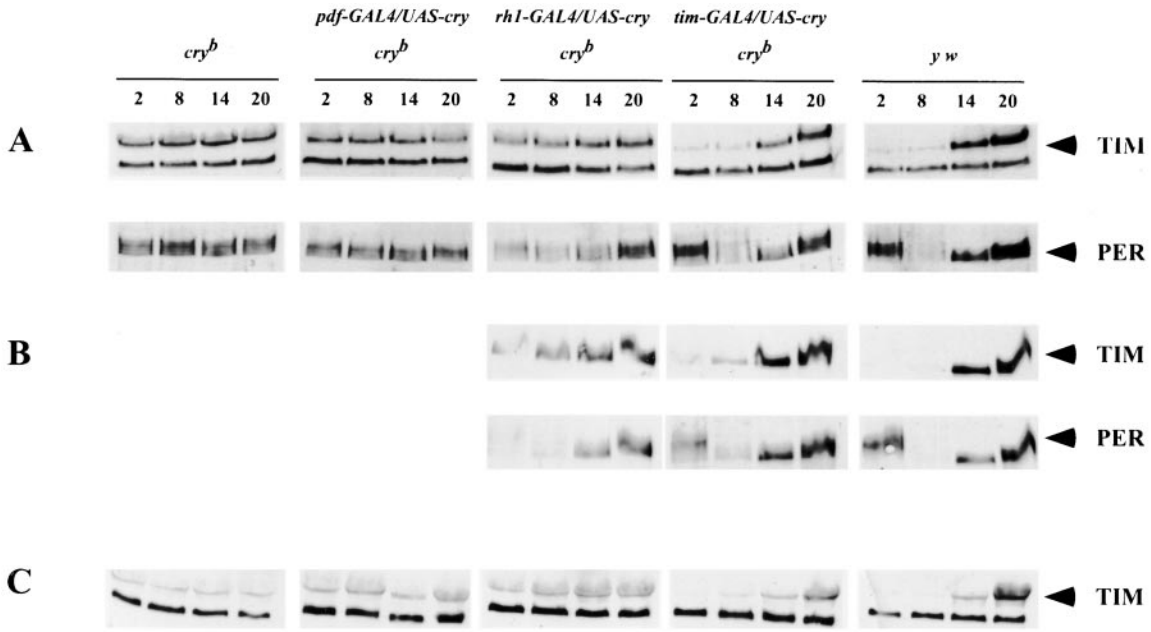

Figure 2. Rescue of PER and TIM Cycling in *cry*^{b} Flies

PER or TIM levels in whole heads (A) eyes (B), and bodies (C) of the indicated genotypes assayed by Western blots. Zeitgeber time of sample collection is also indicated. These experiments were performed two (B and C) or three (A) times with similar results. PER and TIM levels were not cycling in *cry*^{b} and in *pdf-GAL4/UAS-cry; cry*^{b} eyes (data not shown).

coding region under tissue-specific control, and the second contains the coding region of interest under the control of GAL4 binding sites (UAS). A cross between these two lines will overexpress the protein in the desired tissues.

We used three different GAL4 transgenes to overexpress CRY in clock-relevant tissues: in all clock-relevant cells with *tim-GAL4* (Emery et al., 1998; Kaneko and Hall, 2000), in the rhodopsin 1–expressing cells of the eyes with *rh1-GAL4* (Mollereau et al., 2000), and in the LNvs with *pdf-GAL4* (Park et al., 2000; Figure 1A). These transgenes were introduced into a normal *y w* background and into *y w; cry*^{b} flies. Although we cannot exclude some weak CRY expression without a GAL4 driver, CRY cannot be detected when only the *UAS-cry* transgene is present in the *cry*^{b} background. Any potential basal expression level has no detectable effect on *cry*^{b} phenotypes and is therefore negligible (Figure 4; data not shown). With *rh1-GAL4* and *tim-GAL4*, we observed ~3 and ~30-fold overexpression of CRY compared with wild-type head extracts, respectively (Figure 1B). With both drivers, CRY levels cycle due to CRY light sensitivity, with a higher level late at night (Emery et al., 1998). In contrast, we were unable to detect any increase in CRY levels with *pdf-GAL4* (Figure 1B). This is probably because this driver restricts expression to a small subset of brain cells (Park et al., 2000), although it is also possible that it is a weaker driver.

We also overexpressed the mutant CRY^B protein with the *tim-GAL4* driver. CRY^B was detectable and was close to trough levels of wild-type CRY when overexpressed with the same driver. There was also no cycling (data not shown), presumably because the *cry*^{b} mutation affects a crucial amino acid involved in FAD binding (Stanewsky et al., 1998); as a result, the protein is probably insensitive to light and unstable.

Widespread CRY Expression Is Necessary for Rescuing the Peripheral Oscillators

Cell-autonomous oscillators have been described in several *Drosophila* organs, such as the prothoracic glands, malpighian tubules, wings, limbs, and antennae (Emery et al., 1997; Giebultowicz and Hege, 1997; Plautz et al., 1997). By manipulating tissue-specific expression in *cry*^{b} flies, we determined the importance of CRY to these peripheral rhythms. A salient *cry*^{b} phenotype is the absence of the PER and TIM abundance and phosphorylation cycles in these peripheral oscillators, even under LD conditions (Edery et al., 1994; Zeng et al., 1996; Stanewsky et al., 1998). Expression of functional CRY in TIM-expressing cells rescued both biochemical cycling features (Figure 2A). At Zeitgeber time 23 (ZT 23), the TIM phosphorylation rescue was more obvious (data not shown). Neither abundance nor phosphorylation cycling was rescued by overexpressing the CRY^B protein (data not shown). We were also unable to rescue either facet of the biochemical cycling by expressing CRY only in the LNvs (Figure 2A), despite the rescue of behavioral outputs (see below). Therefore, the LNvs appear unable to rescue the peripheral oscillators, suggesting that CRY expression is required in a cell-autonomous fashion.

To test this hypothesis, CRY expression was restricted to a single peripheral tissue. We chose the eyes, because they show molecular cycling in the absence of LNvs (Hardin et al., 1992) and because CRY can be detected by Western blotting in eyes (data not shown). CRY expression with the *rh1-GAL4* driver restored partial TIM protein cycling, as assayed in whole-head extracts (Figure 2A). We observed a reproducible 2- to 2.5-fold cycling of TIM abundance, with a higher concentration late at night, as predicted. We interpret the incomplete rescue in part to the absence of TIM cycling

in brain tissues that do not express CRY. Indeed, a more robust 3- to 4-fold TIM cycling was obtained from eye extracts (Figure 2B).

Autonomous clocks are also present in body tissues (Giebultowicz and Hege, 1997; Plautz et al., 1997). In extracts from bodies, *tim-GAL4*-driven CRY expression rescued TIM cycling; the other two drivers were without effect (Figure 2C). The rescued cycling amplitude was lower than that commonly observed in wild-type flies (Figure 2C). A lower TIM amplitude was also observed in rescued head extracts (Figure 2A), but the body rescue was less complete than the head rescue. PER oscillations were also rescued, but a high background in the Western blots precluded a precise measure of PER abundance changes (data not shown). Taken together, the results suggest that CRY is needed in a cell-autonomous fashion for most if not all of these cellular clocks.

Flies Overexpressing CRY in Lateral Neurons Are Circadianly Hypersensitive to Light

CRY plays a prominent role in behavioral photoresponses and photosensitivity (Emery et al., 1998, 2000; Stanewsky et al., 1998). In response to brief light pulses, wild-type flies manifest phase delays during the early night (ZT 15), and phase advances late at night (ZT 21) (Levine et al., 1994; Saunders et al., 1994; Rutila et al., 1998b; Suri et al., 1998). At both times, *tim-GAL4*-driven CRY overexpression renders flies hypersensitive to low-intensity light pulses (Emery et al., 1998). We extended this approach by restricting CRY overexpression to specific tissues.

With CRY overexpression in the LNvs, flies were hypersensitive to low-intensity illumination, particularly at ZT 15 (Figure 3). At ZT 21, there was also a reproducible but more modest increase in light sensitivity. These results are similar to those previously reported for widespread overexpression (Emery et al., 1998) and suggest that the LNvs play a particularly important role for behavioral photosensitivity. In contrast, there was no significant change in low-light sensitivity by overexpressing CRY only in the eyes (data not shown). This suggests that the eyes do not contribute to light pulse circadian photosensitivity, that they use a CRY-independent pathway, or that CRY concentration in this tissue is not limiting in wild-type flies. To help distinguish between these possibilities, we used cry^b flies to reduce background CRY activity.

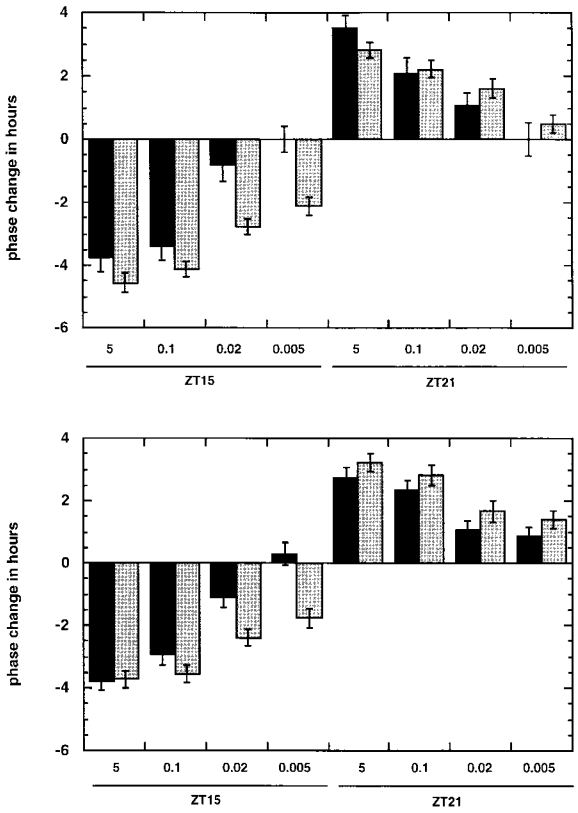

Figure 3. Effects of CRY Overexpression in the LNvs on Circadian Light Sensitivity

Circadian behavior phase change of control flies (*y w; UAS-cry/+*, closed bars) and flies overexpressing CRY in LNvs (*y w; pdf-GAL4/UAS-cry*, shaded bars) in response to light pulses of different intensities at ZT 15 and ZT 21. Two independent experiments are shown. Data were pooled from an average of 13 flies per point. On the x axis, the Zeitgeber time (ZT) and the intensity (in mW/cm^2) of the light pulse are indicated. Phase delays and advances are plotted on the y axis (±SEM) as negative and positive values, respectively. All low light (0.02 and 0.005 mW/cm^2) sensitivity differences between flies with or without LNv-specific overexpression were statistically significant (ZT 15, p is always much smaller than 10^{-3}; ZT 21, $0.02 < p < 0.05$), except for 0.005 mW/cm^2 at ZT 21, in the top graph.

LNv-Restricted CRY Expression Rescues All cry^b Behavioral Photoresponse Defects

Most cry^b behavioral photoresponses are weak or absent; the flies cannot phase shift in response to short light pulses, and they are rhythmic in constant light instead of the arrhythmicity normally observed in wild-type flies (Stanewsky et al., 1998; Emery et al., 2000). Neither mutant phenotype was rescued with *rh1-GAL4*-mediated expression. Taken together with the absence of light hypersensitivity in wild-type flies overexpressing CRY in R1–R6 (data not shown), the results indicate that CRY does not contribute significantly to the behavioral function of the eye photoreceptor cells. In contrast, both defects were fully rescued by *tim-GAL4*-mediated CRY expression and partially rescued with *pdf-GAL4*-mediated CRY expression (Figure 4). In the latter case, there was a robust rescue of the short light pulse ZT 21 phase advance but only a partial rescue of the ZT 15 phase delay. In response to constant light, 50% of the flies showed wild-type-like arrhythmic behavior. The rescue indicates that the LNvs are important cells not only for central pacemaker function but also for circadian photoreception.

To extend this conclusion, we examined other cry^b phenotypes, specifically entrainment. cry^b flies entrain well to regular 24 hr LD cycles, despite the poor response to short light pulses (Stanewsky et al., 1998). per^S flies also entrain well, with little or no manifestation of their endogenous, 19 hr periodicity under imposed 24 hr LD cycles. But the per^S; cry^b double mutant flies manifest pseudo free-running behavior under LD conditions, indicative of an entrainment deficit (Stanewsky et al., 1998: spectral and periodogram analyses detect

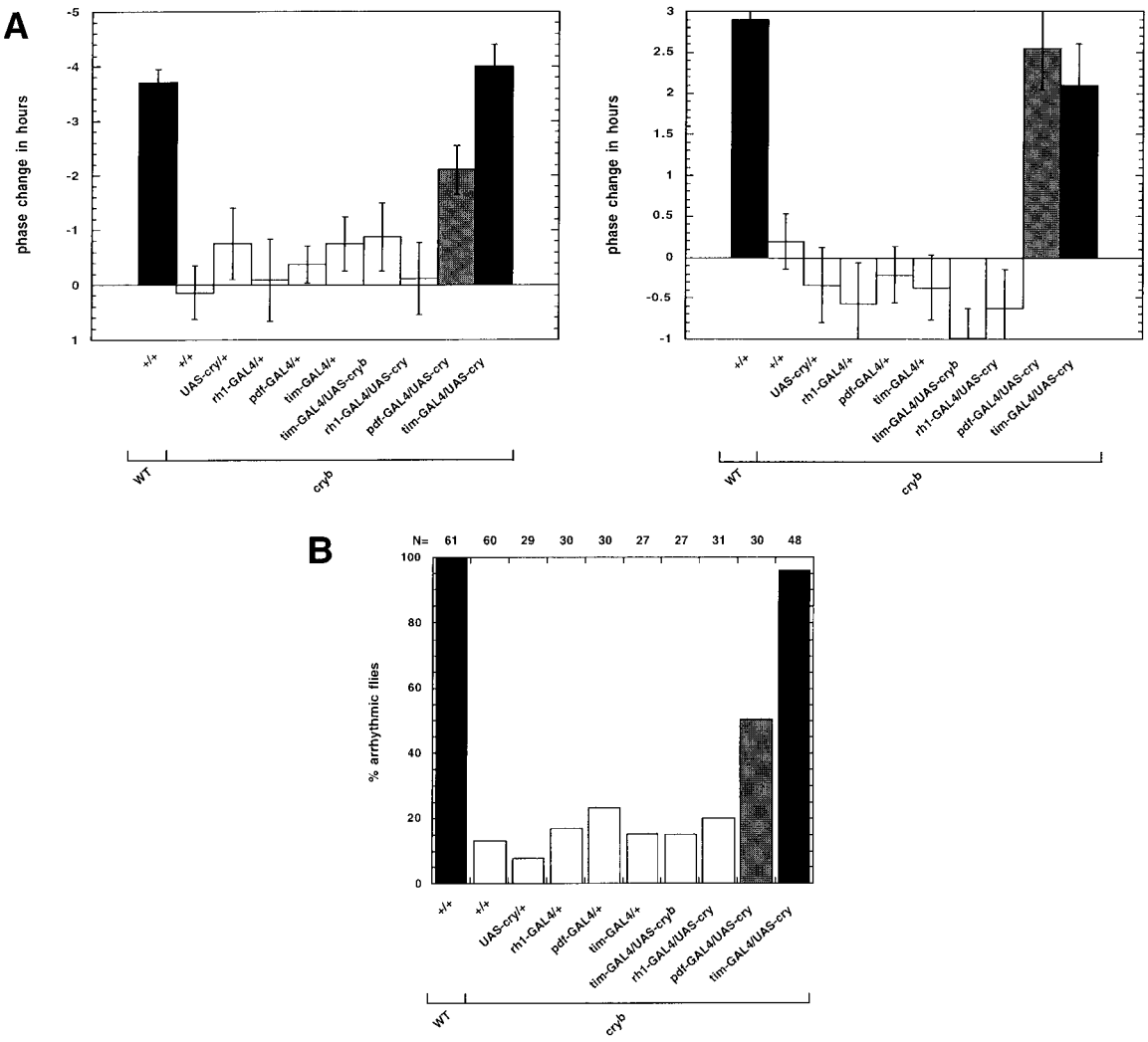

Figure 4. Rescue of Behavioral Photoresponse Defects of *cry*^{b} Flies

(A) Rescue of the circadian phase-resetting defect after short light pulses. Left and right panels show the phase changes observed at ZT 15 and 21, respectively; y axes are as in Figure 3. The genotype of the flies is indicated on the x axes: the first row shows the transgenes present (plus sign, corresponds to a chromosome without transgene), while the second row indicates the genetic background (wild-type [WT] or *cry*^{b}). (B) Rescue of wild-type arrhythmic behavior under constant light. The number of flies analyzed is indicated on the top x axis, the genotype of the flies on the bottom x axis, as in (A). The y axis represents the percentage of arrhythmic flies (cut off, power $<$10 or width $<$2, see Ewer et al. [1992] for power and width definitions).

In both (A) and (B), closed bars represent wild-type or fully rescued photoresponses, open bars, mutant or nonrescued photoresponses, and shaded bars, partially rescued photoresponses.

potent 19 as well as 24 hr components (Figure 5). With the addition of CRY to the LNvs, the *per*^{S}*; cry*^{b} flies showed an activity pattern that resembles very closely that of *per*^{S} flies: 66% of the transgenic flies only had a 24 hr period component, and the 19 hr component of the others was of very low amplitude (Figure 5). This indicates robust rescue of the *cry*^{b} LD entrainment defect by expression of CRY in the LNvs.

norpA^{P41}*; cry*^{b} double mutant flies also entrain poorly. This was interpreted to indicate that two photic pathways contribute to entrainment, one emanating from CRY, and the other from the visual phototransduction cascade (Stanewsky et al., 1998). The single *cry*^{b} mutant flies need on average 3 more days than wild-type flies do to adjust their evening peak to a new LD regime, an entrainment phenotype not noticed in our previous study (Stanewsky et al., 1998; Figure 6). We also noticed an entrainment defect in the single mutant *norpA*^{P41} flies: they need, on average, 1–1.5 days longer to adjust their morning and evening peaks compared with wild-type flies (for a similar observation, see Wheeler et al., 1993). This effect is more pronounced on the morning peak (Figure 6), whereas CRY appears to synchronize predominantly evening activity. (Note that the morning peak is not affected at all by the *cry*^{b} mutant; Figure 6.) In the *norpA*^{P41}*; cry*^{b} double mutant, both activity peaks took very long to reentrain, and some flies did not even reach the new phase by the eleventh day after the shift. This suggests that the mutants have synergistic effects and that both inputs are not completely distinct (Figure 6;

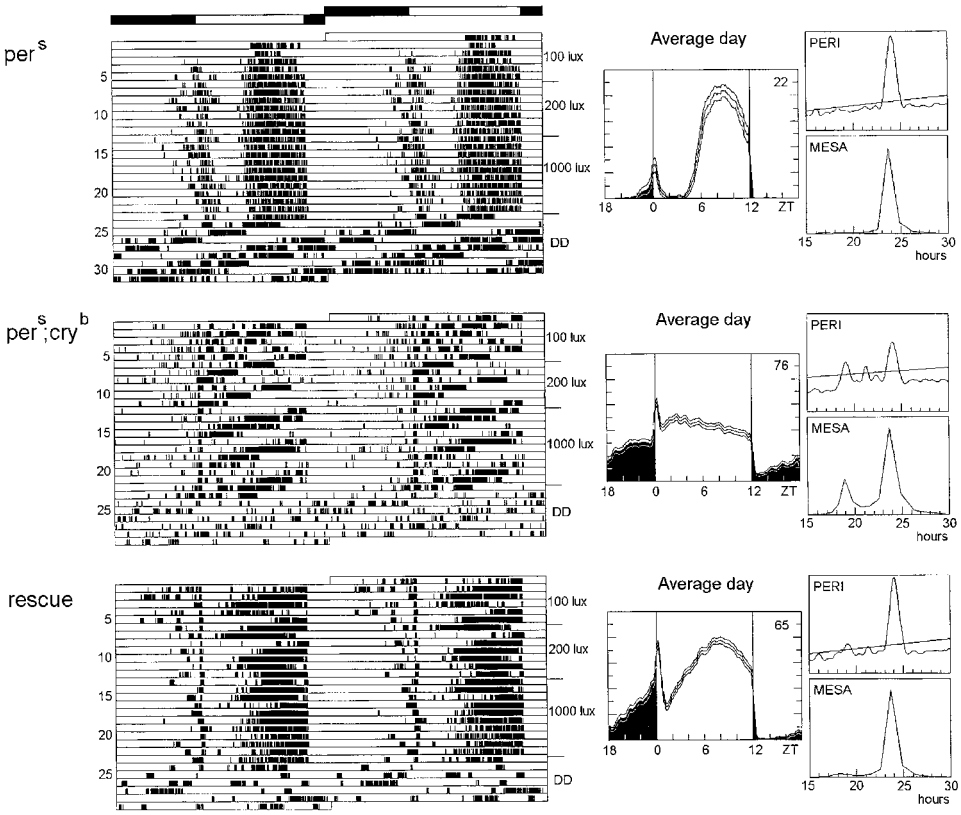

Figure 5. CRY Expression in the LNvs Rescues Entrainment Defects of *per^S; cry^b* Double Mutants

Mutant *per^S*, double mutant *per^S; cry^b*, and double mutants expressing CRY in the LNvs only (*per^S; pdf-GAL4/UAS-cry; cry^b* = rescue) were subjected to a 12 hr:12 hr LD cycle with increasing light intensities (7 days, 100 lux; 7 days, 200 lux; 10 days, 1000 lux), after which they were released into DD. Representative actograms of individual flies for the LD and DD parts of the experiment are shown. The open and closed bars above the actograms indicate when the lights were "on" or "off" during the LD portion of the experiment, respectively. Average activity plots (for the 100 lux LD part of the experiment) were generated for all flies tested of a given genotype (n is indicated in the upper right corner of each average activity plot). The upper and lower lines in each plot indicate the standard error (SE) of the average activity (center line), closed areas represent "night," open areas, "day" (Zeitgeber time [ZT] [hr] is indicated on the x axes, the y axes indicate the relative amount of activity). Average activity plots for the other light intensities looked very similar to the ones shown here for 100 lux (data not shown); t values were calculated for the combined LD portion of the run using periodogram (PERI) and MESA analyses (Stanewsky et al., 1998). The PERI and MESA plots shown here correspond to those individuals whose actograms are plotted. Peaks determined by both methods show the most probable t value(s) of each individual; the height of each peak is proportional to the amplitude of the observed rhythms. In addition, periodogram analysis indicates the significance of an obtained t value by the difference of the peak from the significance line ($p < 0.05$) termed "power" (see Experimental Procedures). In this experiment, all of the 22 *per^S* flies showed high-amplitude rhythms with t = 24 hr. In contrast, 71 of 77 *per^S; cry^b* double mutants showed both 19 hr (*per^S* free-running period) and 24 hr period values. Only 27 of 65 double mutant flies, in which CRY was expressed in the LNvs, showed both 19 and 24 hr periods; and the 19 hr components were of very low power (see example in this Figure). The remaining 43 flies of the rescue cross showed only the 24 hr component, indicating a robust rescue of the double mutant phenotype.

see Discussion). When CRY was expressed in the LNvs, the double mutant phenotype was very similar to that of the single *norpA^{P41}* mutant; only the evening peak took about 1 day longer in the double mutant to adapt to the new phase (Figure 6).

CRY and PDF Expression Colocalize to the LNvs

To show that CRY is expressed in the LNvs, we generated flies containing both *cry-GAL4* and *UAS-EGFP* transgenes (see Experimental Procedures). In the adult brain, two groups of cells strongly expressed the enhanced green fluorescent protein (EGFP) marker (Figure 7C), one with small and the other with larger somata. These are in the correct location to be the large and small LNvs (Helfrich-Förster, 1996; Kaneko, 1998; see also Egan et al., 1999, for a similar observation by in situ mRNA hybridization). To prove that this is the case, we double stained adult brains with an anti-PDF antibody. This reagent specifically recognizes both the large and small LNvs (Renn et al., 1999; Park et al., 2000), and there was perfect colocalization of the intense EGFP and PDF signals (Figure 7D). A weaker EFGP signal was also detected in more dorsal neurons. These may correspond to the dorsal lateral neurons, which also express PER and TIM but not PDF (Kaneko, 1998).

In addition, we detected by in situ mRNA hybridization several *cry*-positive cells in each hemisphere of third instar larval brains (Figure 7A). At least four cells were located in the middle of the brain that almost certainly correspond to the precursors of the small LNvs (Kaneko

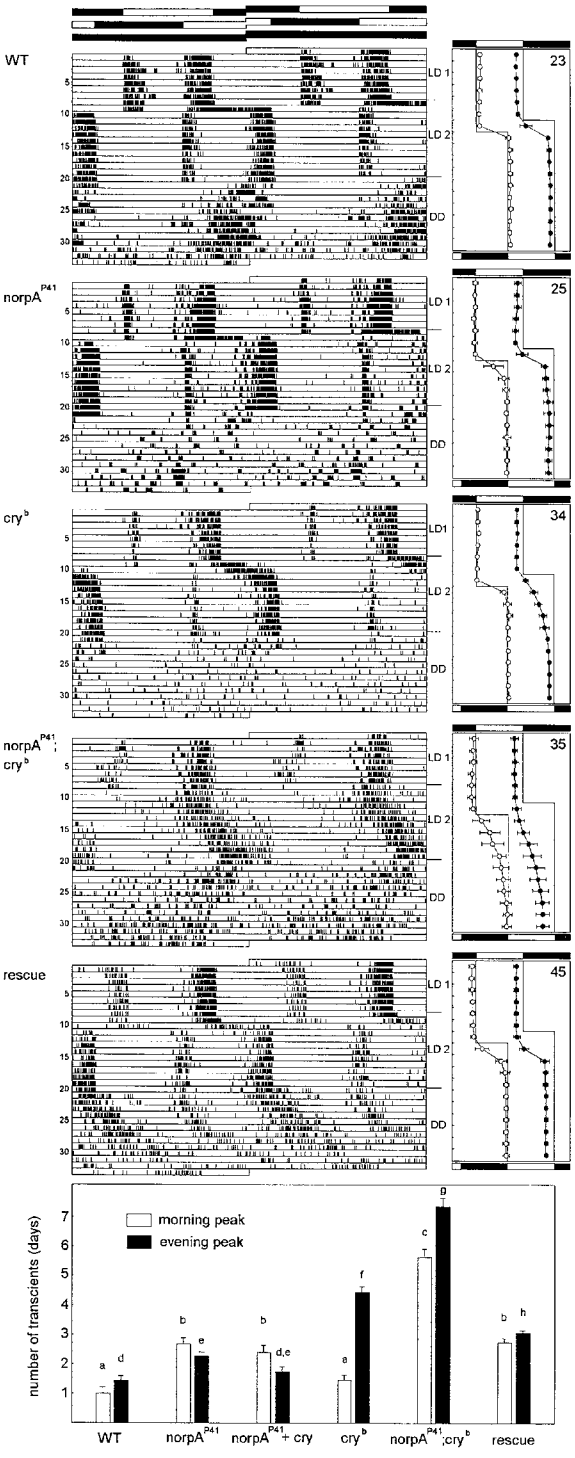

Figure 6. CRY Expression in the LNvs Rescues Reentrainment Defects of *norpA; cry*^{b} Double Mutants

Typical actograms, average phase plots, and a stack plot. These illustrate the number of days wild-type (Canton S) and visually impaired mutants need to reentrain to a phase delay of the 12 hr:12 hr LD cycle by 8 hr. The mutants were *norpA*^{P41}*; cry*^{b}, a double mutant homozygous for *norpA*^{P41} and *cry*^{b} (*norpA*^{P41}*; cry*^{b}), and such a double mutant with normal CRY expressed in the LNvs (*norpA*^{P41}*; pdf-GAL4/UAS-cry; cry*^{b} = rescue). The initial LD cycle (LD 1) was delayed on day 9 of recording by extending the light period for 8 hr (see faint lines in the phase plot). The flies were then recorded for 11 days under the new LD cycle (LD 2) and subsequently released into DD (on day 22). Light intensity for both LD cycles was 1000 lux. The different light regimes during the course of the experiment are also indicated by the closed (lights off) and open (lights on) bars above the actograms: upper row, LD 1; middle row, LD 2; and bottom row, DD. Under LD conditions, all flies showed a bimodal activity with maximal activity around lights on (morning peak) and lights off (evening peak). The maxima of daily morning and evening peaks were determined in individual flies for 6 days prior to and 10 days after the phase shift, as described previously (Helfrich-Förster, 2000). Mean phases (±SD) of morning (open circles) and evening (closed circles) peaks of all flies in a given strain were plotted in the average phase plots. The number in the upper right corner of the phase plots indicates the number of individuals averaged in the plot. The number of transient cycles needed to achieve the original phase relationship to LD was determined for morning and evening peaks separately, and mean values (±SE) for each strain were plotted in the histogram plot. Data were tested for significant differences by the nonparametric Kolgorov-Smirnov two sample test. Values not significantly different from each other ($p > 0.05$) are marked by the same letters. In the double mutant *norpA*^{P41}*; cry*^{b}, both the morning and evening peak required several days to reentrain, and some flies did not even reach their original phase at the eleventh day after the phase shift. For such flies, the number of transient cycles was determined as 11. One fly did not reentrain at all and was excluded from the present analysis. The histogram plot shows data of an additional genotype in which CRY was expressed in the LNvs of *norpA*^{P41} flies, as in the rescue flies (*norpA*^{P41} + *cry*). This overexpression of CRY did not result in a significant change of *norpA*^{P41} behavior (n = 23).

et al., 1997). *cry*-positive cells were also visible in the antero–dorsal part of the brain, where additional PER- and TIM-positive cells were previously described (Kaneko et al., 1997). We observed a similar in situ mRNA hybridization pattern on whole mounts of adult brains, which also resembled closely the adult EGFP pattern (data not shown).

Discussion

The mutant *cry*^{b} gene causes profound circadian light–response problems. Only entrainment to 24 hr LD cycles still takes place in the mutant background (Stanewsky et al., 1998). We show here, however, that even this aspect of *cry*^{b} circadian light perception is aberrant, as the mutant flies need much longer to entrain to a new cycle (Figure 6). A detailed examination of these LD entrainment data suggests that CRY contributes principally to adjusting the evening activity peak. The other major source of entrainment light information, the eyes, contributes principally to adjusting the morning peak. This fits with previous LD activity profile observations indicating that the phase of the evening activity peak is under clock control, whereas the phase of the morning peak is less sensitive to central clock mutations and is probably timed relative to some fixed environmental signal, e.g., the lights on or lights off transition (Hamblen-Coyle et al., 1992; Figure 5). There is, however, a caveat, as the *norpA; cry*^{b} double mutant phenotype suggests some interplay between the two light input pathways (Figure 6). The dual light input pathway for LD entrainment contrasts with the apparently unitary CRY input pathway for all other circadian photoresponses. This underscores the different nature of parametric (LD cycle)

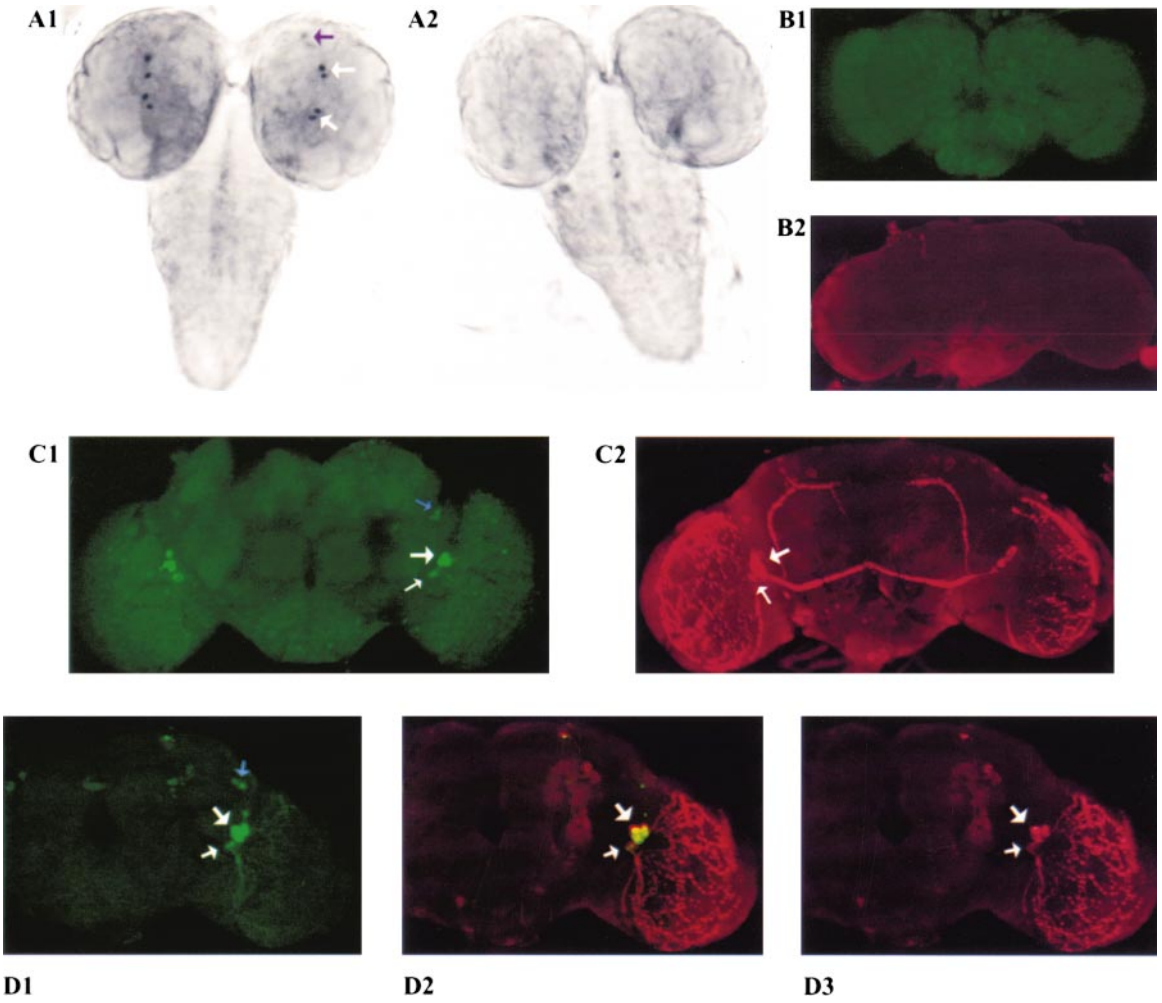

Figure 7. CRY and PDF Expression Colocalize in LNvs

(A) *cry* mRNA was detected by in situ antisense mRNA hybridization in whole third instar larval brains (A1). The white arrows indicate four *cry*-positive cells, located in the middle of the brain, that are likely to be precursors of the small LNvs. The purple arrow indicates antero–dorsal cells that are out of focus in this picture. No signal was detected with the *cry* sense probe control (A2).

(B) Negative controls for EGFP staining (+/*UAS-EGFP* adult brain, [B1]) and anti-PDF staining (adult brain stained without primary antibody, [B2]).

(C) EGFP signal from a whole *cry-GAL4/UAS-EGFP* adult brain (C1). Similar results were obtained with both *cry-GAL4* lines. The large open arrow indicates the large LNvs, the small open arrow, the small LNvs. The blue arrow indicates a group of EGFP-positive cells that could correspond to the dorsal lateral neurons. A whole adult brain was stained with an anti-PDF antibody and a Texas Red–labeled secondary antibody under optimal conditions for PDF staining (C2) (see Experimental Procedures). Large and small arrows are as above. Note the optic lobe and contralateral projections coming from the large LNvs and the dorsal projections from the small LNvs. Part of the dorsal projections is also coming from the large LNvs.

(D) EGFP (D1) and PDF (D3) signals from the same *cry-GAL4/UAS-EGFP* brain. (D2) shows the superimposition of the two signals, which demonstrates the colocalization of CRY and PDF expression in the small and large LNvs. The projections from the small LNvs are not visible in this brain, because we could not use optimal conditions for detecting PDF in this experiment (see Experimental Procedures). Arrows are as in (C).

and nonparametric (short light pulse) entrainment (Stanewsky et al., 1998). Taken together with the absence of any biological effect of CRY^B overexpression and the absence of CRY^B abundance cycling under LD conditions, cry^b is probably a strong hypomorphic allele that encodes a protein without photoreceptor activity. This conclusion is consistent with the results of CRY^B expression studies in heterologous systems (Ceriani et al., 1999; P. E. et al., unpublished data).

The behavioral rescue experiments show that all known photoresponse defects of cry^b are substantially rescued by expressing CRY only in the LNvs. The CRY–LNvs rescue is partial for the light pulse and constant light phenotypes, whereas it is almost complete for the LD entrainment defects of per^S; cry^b and $norpA^{P41}$; cry^b double mutant genotypes. Our in situ mRNA hybridization results suggest further that larvae also rely on CRY expression in the LNv precursor cells for circadian photoreception. There is no behavioral effect of CRY overexpression in the eyes, suggesting that the visual entrainment pathway is CRY independent.

There are several possible explanations for the incomplete rescue of phase resetting and constant light arrhythmicity. *pdf-GAL4* may be a relatively weak driver,

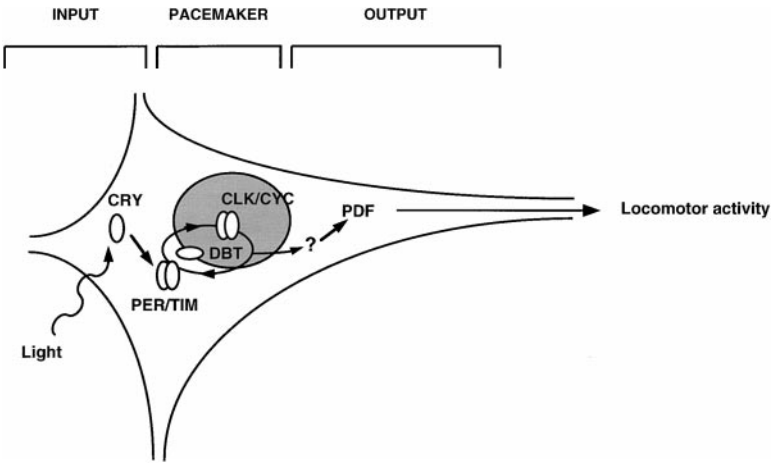

Figure 8. All Three Clock Components Are Present in the *Drosophila* Circadian Pacemaker Cells

To circadianly control behavioral locomotor activity, three elements are required: an input pathway, a pacemaker, and an output pathway. All three are present within the same cells in *Drosophila*: the LNvs. By expressing CRY, the LNvs are directly sensitive to light. CRY controls the phase of the pacemaker, which is composed of a transcriptional feedback loop involving PER/TIM and CLOCK/CYC dimers. The former regulates the transactivation potential of the latter in the nucleus (in gray). DBT, a kinase, is also necessary for central pacemaker function. This transcriptional loop regulates, through poorly understood mechanisms that may involve VRILLE (Blau and Young, 1999), the expression and the release of the neuropeptide PDF, which is an important output element of this circadian system.

such that CRY expression does not reach a required threshold level for full rescue. CRY may play a developmental role that is not fulfilled with *pdf-GAL4*-driven expression. There may also be other CRY-relevant cells, in addition to the LNvs, that contribute to circadian behavior. Consistent with this view, *disco* mutant flies lack LNvs but stay rhythmic for 1–3 days in DD (Wheeler et al., 1993; Helfrich-Förster, 1998). CRY expression studies should identify these accessory pacemaker cells. Good candidates are the dorsal lateral neurons and the dorsal neurons, both of which express PER and TIM and send projections to the same region of the brain as the LNvs (Helfrich-Förster, 1996; Kaneko, 1998; Kaneko and Hall, 2000). Consistent with this notion, cells that could correspond to the dorsal lateral neurons express the EGFP marker driven by *cry-GAL4* (Figures 7C and 7D).

Why does CRY expression seem to be so high in LNvs, compared with other tissues, like the eyes (Egan et al., 1999)? One possibility is that these neurons, located deep inside the brain, need to express high CRY levels to detect low light intensities. For example, this would allow the clock to respond at dawn and adjust its phase every day. Another explanation, more provocative perhaps, is that a high CRY concentration contributes to special pacemaker cell properties of the LNvs. In cry^b, only the LNvs manifest TIM and PER cycling (Stanewsky et al., 1998). This might reflect the high CRY levels in these cells, as well as a second, nonphotoreceptor contribution of CRY to pacemaker function. This CRY dark function could be to maintain the circadian oscillations of the molecular pacemaker, e.g., by contributing directly to the negative feedback loop, as shown in mammals (Griffin et al., 1999; Kume et al., 1999; van der Horst et al., 1999; Vitaterna et al., 1999). A true *cry* null mutation might therefore result in arrhythmicity, as observed in mammals (van der Horst et al., 1999; Vitaterna et al., 1999).

Our evidence suggests that CRY contributes in a cell-autonomous manner to the PER/TIM molecular cycles. This presumably reflects independent photoreception of cells and tissues (Emery et al., 1997; Giebultowicz and Hege, 1997; Plautz et al., 1997; Stanewsky et al., 1998). In the periphery, CRY is absolutely required for light-dependent TIM degradation (Stanewsky et al., 1998), and recent data suggest a direct CRY–TIM interaction (Ceriani et al., 1999). PER, TIM, and CRY colocalization is not yet documented, but several studies have shown that *cry* is expressed in the body, as well as in different fly organs that contain autonomous clocks (Emery et al., 1998; Egan et al., 1999; Ishikawa et al., 1999; Okano et al., 1999). We have not been able to use our anti-CRY antibodies for immunohistochemical studies but are now in the process of a detailed analysis of CRY expression in the new *cry-GAL4* lines.

The rescued cycling in eyes is clearly less strong than that in wild-type eyes or in *tim-GAL4*-driven CRY-expressing flies. Surprisingly, the PER rescue is more robust than that of TIM. The PER:TIM ratio might be higher in *rh1*-expressing cells than in the other PER/TIM-expressing cells of the head. It is unlikely, however, that the relatively poor TIM rescue with *rh1-GAL4* is due to excessive CRY expression in eyes, because *tim-GAL4* drives much higher CRY expression in this tissue (data not shown). The failure to drive expression in R7 and R8 photoreceptor cells is almost certainly relevant to the inefficient rescue (e.g., Zeng et al., 1994). There is a similar inefficient rescue of PER/TIM cycling in bodies. This may be due to excessive CRY levels, which may adversely affect TIM stability. Alternatively, the *tim-GAL4* driver may give rise to some developmental defect, i.e., proper CRY expression during development may be necessary for a full rescue of PER and TIM cycling. This might even be relevant to the relatively poor TIM cycling rescue when CRY is expressed with the *rh1-GAL4* driver.

Evidence is accumulating that the cell-autonomous property of circadian rhythms is universal, but with interesting differences between systems (Tosini and Menaker, 1996; Emery et al., 1997; Giebultowicz and Hege, 1997; Plautz et al., 1997; Balsalobre et al., 1998; Earnest et al., 1999). The neuro-hormonal regulation of physiology may limit the autonomy of peripheral oscillators in mammals, where the suprachiasmatic nucleus (SCN) appears to be the principal central mammalian pacemaker organ (Ralph et al., 1990; Klein et al., 1991). But the SCN, as well as most internal clocks and tissues, is

probably not directly light sensitive. It receives photic cues from the eyes, where the circadian photoreceptor molecule has not been identified. Moreover, it is unclear whether mammalian CRYs ever function as cell-autonomous photoreceptors, e.g., in cultured retina cells that exhibit circadian oscillations (Tosini and Menaker, 1996).

Mammalian peripheral oscillators are probably under SCN control, largely through humoral connectors. This scheme accounts for the 4 hr phase difference between peripheral and SCN molecular cycles in vivo (Lopez-Molina et al., 1997; Balsalobre et al., 1998). The lack of any reported phase difference in *Drosophila*, i.e., between the molecular cycles in the periphery and LNvs, is consistent with our rescue experiments and presumably reflects independent cell-autonomous connections by CRY to environmental light cues. Remarkably, this includes even the LNvs, as CRY expression within these brain pacemaker cells controls every known aspect of circadian behavioral photosensitivity. Although there is evidence in other systems for deep brain photoreceptors (see, for example, Menaker et al., 1970; Okano et al., 1994; Blackshaw, 1999), CRY is the only functionally identified light sensor of this kind. Taken together with the recent identification of a behavioral output factor within the LNvs (Renn et al., 1999; Park et al., 2000), they are now known to contain all three components of a functional, cell-autonomous circadian clock: photoreception, a central pacemaker, and well-defined output (Figure 8).

Experimental Procedures

Fly Strains and Transgenes

pdf-GAL4, *tim-GAL4*, *rh1-GAL4*, and *UAS-cry* transgenes were introduced into both *y w* and *y w; cry^b* backgrounds. *tim-GAL4, pdf-GAL4* and *UAS-cry* flies were as described (Emery et al., 1998; Kaneko and Hall, 2000; Park et al., 2000). *rh1-GAL4* flies (Mollereau et al., 2000) were kindly provided by C. Desplans. The *UAS-cry^b* transgene is identical to *UAS-cry*, except for the *cry^b* D410–N410 missense mutation (Stanewsky et al., 1998). GAL4 transgenic strains were then crossed with *UAS-cry* and *UAS-cry^b* or control *y w* and *y w; cry^b* flies. *UAS-cry* flies were also crossed to the control backgrounds. The progeny were used for behavioral or Western Blot analysis. *norpA; cry^b* and *per^S; cry^b* flies were previously described (Stanewsky et al., 1998).

The *cry-GAL4* transgene was obtained as follows. A cosmid library (kindly provided by J. Tamkun) was screened with a probe corresponding to the 5′ end of the *cry* cDNA. One genomic clone was isolated that contains part of the *cry* gene. The 3′ end is missing. From this cosmid, an ~5.5–6.0 kb BamHI fragment was subcloned in pBluescript KSM13(+) (Stratagene). Partial sequence confirmed that the whole first exon and intron, as well as part of the second exon, are present; ~4.0–4.5 kb upstream of the putative initation region is also present in this fragment. The transcriptional initiation site(s) was not mapped precisely, but the 5′ ends of 7 of 9 cDNAs and expressed sequence tags (ESTs)—present in sequence databases or sequenced by us—are located between 115–125 base pairs upstream of the initial ATG. Thus, we assume that this region contains the main transcriptional initiation site(s). One EST sequence is shorter, and one cDNA longer, with a 5′ end 200 bp upstream of the initial ATG. There is therefore probably a second minor transcriptional initiation region.

A NotI-BstXI fragment, containing the whole upstream sequence with the first intron and part of the second exon, was then excised and fused in-frame to the GAL4 coding region. This fusion was introduced into the pPT-GAL4 plasmid, kindly provided by D. Eberl. *y w; Ki p^p [ry$^+$ Δ2–3]/+* flies were transformed with this construct using P element–mediated transformation. Two lines were obtained.

Protein Extracts and Western Blots

Whole-head protein extracts were obtained as described (Edery et al., 1994). For eye extracts, heads were collected on dry ice and incubated in acetone for 2 hr on dry ice and then overnight at −20°C. Heads were then dried for 30 min at room temperature. Eyes were removed from the heads by dissection and placed in Eppendorf tubes. Proteins were then extracted as described (Edery et al., 1994).

PER, TIM, and CRY Western blots were performed as described, except that a nonpurified anti-CRY antiserum was used at a 1:1000 dilution (Zeng et al., 1996; Emery et al., 1998).

Behavioral Analysis

Circadian phase resetting and photosensitivity were determined by submitting flies to light pulses of different intensities at ZT 15 or ZT 21 as described (Emery et al., 1998). Constant light behavior was studied as in Emery et al. (2000). Recordings for circadian photoentrainment were performed in specially prepared photometer cuvettes as described in Helfrich-Förster (1998). Halogen photooptic lamps (Osram, XENOPHOT^R) served as the light source, and light intensity was adjusted to 100, 200, or 1000 lux with neutral density filters. The phases of morning and evening peaks were determined exactly as described previously (Helfrich-Förster, 2000).

EGFP Histological Detection and Anti-PDF Immunostaining

y w; cry-GAL4 females were crossed with *w; UAS-EGFP* males kindly provided by C. Desplans (EGFP refers to the F64L and S65T GFP double mutant). Whole mounts of adult brains from the progeny were obtained by dissection in phosphate-buffered saline (PBS) (8 g/l NaCl, 0.2 g/l KCl, 1.15 g/l Na_2HPO_4, and 0.2 g/l KH2PO4). EGFP signal could be detected by confocal microscopy without any further treatment.

PDF protein was detected as follows. After dissection, brains were fixed in paraformaldehyde fixative (4% paraformaldehyde and 7% saturated picric acid solution in 0.1 M phosphate buffer [39% 0.1 M NaH_2PO_4 and 61% 0.1 M Na_2HPO_4]) for 3 hr at room temperature. After having been washed five times in PBT (PBS with 0.1% BSA, 0.1% Triton X-100, and 0.02% sodium azide) for 5 min, brains were permeabilized with PBT + 1% Triton X-100. They were then blocked with PBT + 3% normal goat serum for 30 min and incubated overnight with a rat antiserum directed against the PDF peptide (dilution of 1:200 in PBT + 3% normal goat serum). After being washed ten times for 5 min in PBT, brains were incubated with a Texas red–coupled anti-rat secondary antibody (Jackson Immunoresearch, Pennsylvania; dilution of 1:100 in PBT + 3% normal goat serum) for 1 hr. Brains were then washed ten times for 5 min in PBT. The results of applying anti-PDH (Park et al., 2000) were visualized by confocal microscopy.

To detect in the same brain both the anti-PDF and EGFP signals, the anti-PDF staining procedure had to be modified. Picric acid was removed from the fixative to improve the EGFP signal. As a result, the anti-PDF signal was weaker and was lost in the dorsal and contralateral projection.

In Situ mRNA Hybridization on Whole Mounts of Adult and Third Instar Larval Brains

In situ mRNA hybridization on dissected adult and third instar larval brains was preformed essentially as described in Tautz and Pfeifle (1989), except that the temperature of hybridization was 55°C. The sense and anti-sense DIG-labeled *cry* mRNA probes were generated from full-length cDNA.

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