Disruption of Cryptochrome partially restores circadian rhythmicity to the arrhythmic *period* mutant of *Drosophila*

Ben H. Collins*†, Stephane Dissel*, Edward Gaten‡, Ezio Rosato*, and Charalambos P. Kyriacou*§

Departments of *Genetics and ‡Biology, University of Leicester, Leicester LE1 7RH, United Kingdom

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The *Drosophila melanogaster* circadian clock is generated by interlocked feedback loops, and null mutations in core genes such as *period* and *timeless* generate behavioral arrhythmicity in constant darkness. In light–dark cycles, the elevation in locomotor activity that usually anticipates the light on or off signals is severely compromised in these mutants. Light transduction pathways mediated by the rhodopsins and the dedicated circadian blue light photoreceptor cryptochrome are also critical in providing the circadian clock with entraining light signals from the environment. The *cry^b^* mutation reduces the light sensitivity of the fly's clock, yet locomotor activity rhythms in constant darkness or light–dark cycles are relatively normal, because the rhodopsins compensate for the lack of cryptochrome function. Remarkably, when we combined a *period*-null mutation with *cry^b^*, circadian rhythmicity in locomotor behavior in light–dark cycles, as measured by a number of different criteria, was restored. This effect was significantly reduced in *timeless*-null mutant backgrounds. Circadian rhythmicity in constant darkness was not restored, and TIM protein did not exhibit oscillations in level or localize to the nuclei of brain neurons known to be essential for circadian locomotor activity. Therefore, we have uncovered residual rhythmicity in the absence of *period* gene function that may be mediated by a previously undescribed *period*-independent role for *timeless* in the *Drosophila* circadian pacemaker. Although we do not yet have a molecular correlate for these apparently iconoclastic observations, we provide a systems explanation for these results based on differential sensitivities of subsets of circadian pacemaker neurons to light.

anticipation | phase shift | oscillator | hourglass | *timeless*

The PERIOD (PER) and TIMELESS (TIM) proteins are core components of the negative feedback loop that drives circadian oscillations in *Drosophila*. TIM stabilizes PER, and the PER and TIM proteins cooperate in nuclear entry and retention in key pacemaker cells at night (1). TIM degrades rapidly in response to light, releasing PER to repress *per* and *tim* transcription (1). *D. melanogaster* individuals carrying the null mutation per^{01} display arrhythmic locomotor activity under constant darkness (DD), whereas in light–dark (LD) cycles, their locomotor activity is elevated under illumination and reduced in darkness, reflecting the "masking" effect of light (2, 3). Another mutation in the gene encoding the blue-light photoreceptor *cryptochrome*, cry^b, gives attenuated light responses in a circadian context (4–7), but normal circadian locomotor behavior in 24-h LD cycles. As part of another study of seasonal behavioral responses to temperature and light effected via changes in *per* 3′ splicing, we generated a per^{01}; cry^b double mutant (8). We noted that a modest cycle in wild-type *per* 3′ splicing in LD cycles at 29°C was exaggerated in per^{01} and cry^b mutants, yet in the double mutant, the amplitude of the splicing reverted back to approximately wild-type levels (8). A previous observation in mammalian clocks found that disruption of *mCry2* restored circadian rhythmicity to *mPer2* mutant mice (9). This remarkable result was explained in terms of the other *mPer* and *mCry* paralogues compensating for the defective molecules. Therefore, we were interested in examining whether other aspects of circadian behavior of the per^{01}; cry^b double mutant display a similar reversion to a wild-type phenotype. Here we report that the *Drosophila* per^{01}; cry^b double mutant does show some very surprising "wild-type" circadian phenotypes that, superficially, are similar to those found in the double mutant mouse. However, because *Drosophila* has only a single copy of *per* and *cry*, the explanation to which we are driven for explaining these effects is quite different.

Materials and Methods

Strains. Flies were raised on standard media at 25°C in LD 12:12 cycles. per^{01}; cry^b, per^{01}; tim^{01}; cry^b, per^{01}; tim^{UL}; cry^b, and tim^{01}; cry^b flies were generated by crossing preexisting laboratory stocks, and all were marked with *white*. Flies expressing the β-galactosidase gene under control of *Rhodopsin3* (*Rh3*), *Rhodopsin5* (*Rh5*), and *Rhodopsin6* (*Rh6*) promoters were a gift of C. Desplan.

Locomotor Activity and T Cycle Experiments. Locomotor activity in LD 12:12 was recorded as described with data transformed to reduce contributions from unusually active/inactive flies (8, 10). For light pulse and T cycle experiments, 1-day-old flies were placed into the locomotor activity apparatus. For light pulses, activity was recorded as flies entrained to the subsequent LD regime for 5 days, then a 1-h light pulse was administered at either Zeitgeber time (ZT) 15 or 21 and locomotor activity recorded for the following 5 days. For T cycle experiments, flies were monitored as they entrained to their new LD regime. Day 5 was chosen for comparisons as on inspection the evening locomotor peaks of all genotypes had entrained to the new LD regime by this point. In this case, the time bin with the highest mean activity value for each fly was used as the metric for comparing evening peak position between genotypes, with data collected during the first hour after "light on" discarded to avoid any startle effect. Between 12 and 32 individual males were analyzed for each genotype in each experiment. Regression analysis was carried out by using EXCEL (Microsoft).

Immunocytochemistry. For each time point, control flies were processed in parallel in a 30-min window centered on each reported ZT. At least 10 brain hemispheres were studied for each genotype. The procedure was carried out as described (11). Primary antibodies were as follows: 1:1,000 rat anti-TIM (UPR41) (12), 1:2,500 rabbit anti-cPDH (13), 1:1,000 mouse anti-β-galactosidase (Pro-

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Abbreviations: DD, constant darkness; LD, light–dark; ZT, Zeitgeber time; LN, lateral neuron; LN_v, ventral LN; LN_d, dorsal LN; l-LN_v, large LN_v.

†Present address: Department of Biology, New York University, 100 Washington Square East, New York, NY 10003.

§To whom correspondence should be addressed. E-mail: cpk@le.ac.uk.

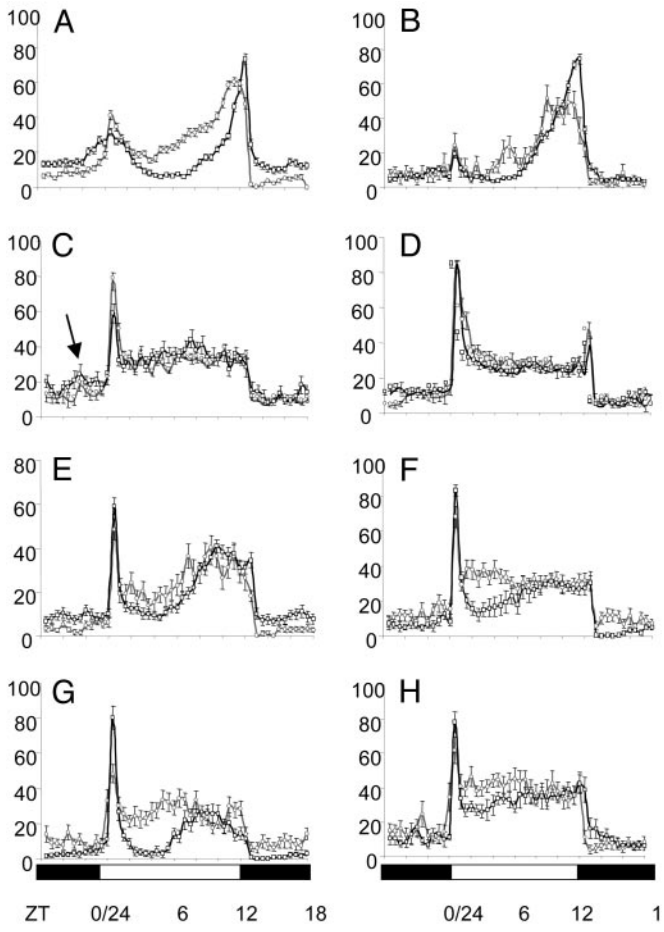

Fig. 1. Locomotor activity in LD 12:12 cycles. (*A*) Canton-S. (*B*) cry^b. (*C*) per^{01}. (*D*) tim^{01}. (*E*) per^{01}; cry^b. (*F*) tim^{01}; cry^b. (*G*) per^{01}; tim^{UL}; cry^b. (*H*) per^{01}; tim^{01}; cry^b. Strains are shown at 18°C (gray) and 29°C (black). The *y* axis shows activity counts ± SEM. Black and white bars represent the LD regime, with lights on at ZT0 and off at ZT12. *A* and *B* are reproduced from ref. 8. Arrow in the per^{01} panel shows the ''morning peak'' described by Helfrich-Förster (16).

mega). Fluorescent secondary antibodies were as follows: 1:400 goat anti-rat IgG-Cy3 (Jackson ImmunoResearch), 1:200 goat anti-rabbit IgG-Cy3 (Jackson ImmunoResearch), 1:400 goat anti-rabbit IgG-Cy2 (Jackson ImmunoResearch), and 1:200 goat anti-mouse IgG-Cy2 (Jackson ImmunoResearch). Optical sections were imaged on a Zeiss LMS510 confocal microscope.

Western Blotting. Heads were collected every 4 h, and protein was extracted from per^{01}, Canton-S, and per^{01}; cry^b flies. Blots were carried out as in ref. 14 with antibodies raised to TIM (1:1:000) (15) and to HSP70 (Sigma, 1:50,000) as a loading control. Secondary horseradish peroxidase (HRP)-conjugated antibodies were goat anti-rat (Sigma, 1:8,000) and goat anti-mouse (Sigma, 1:6,000). Signals were obtained by chemiluminescence and quantified with SCION image analysis software (Scion, Frederick, MD).

Results

We monitored the locomotor activity of genotypes carrying mutations in *per*, *tim*, and *cry*, singly and in combination in LD 12:12 cycles at 18 and 29°C (Fig. 1). Wild-type and cry^b mutants show characteristic clock-controlled evening locomotor activity, peaking between ZT8 and ZT11 (ZT0 = lights on), and subsiding by lights off (ZT12) (Fig. 1 *A* and *B*). In contrast, per^{01} and tim^{01} show no circadian regulation of locomotor behavior under LD conditions beyond a preference for being active during the light phase (Fig. 1 *C* and *D*) (3). Interestingly, we also observe a subtle small peak in activity before ''lights on'' in all *per–null* and *tim–null* genotypes at at least at one temperature, described by Helfrich-Förster for per^{01} as the ''morning peak'' (16). However, for our purposes, a functional clock generates a well defined evening activity peak anticipating lights off that is absent from per^{01} (17, 18).

Using this as an operational definition for the circadian regulation of LD behavior, per^{01}; cry^b has clearly regained clock function as it shows a dramatic anticipation of lights off in LD (Fig. 1*E*). The evening peak in activity also arrives earlier during the light phase than the wild-type, possibly suggesting an underlying short-period oscillation, because a similar pattern has been reported in LD conditions for the per^s 19-h mutant (19). This anticipatory locomotor activity is at least partially TIM-dependent, because tim^{01}; cry^b double mutants and per^{01}; tim^{01}; cry^b triple mutants do not show any locomotor anticipation of lights off at 18°C, although some residual behavior is recovered at 29°C, particularly in the former genotype. In addition, when we crossed in the long-period tim^{UL} allele (20) into the per^{01}; cry^b background, at 18°C, per^{01}; tim^{UL}; cry^b locomotor activity showed some anticipation beyond that seen in tim^{01} or per^{01} (Fig. 1*G*). Again, as in the tim^{01}; cry^b genotypes outlined above, at 29°C, locomotor activity showed a much clearer peak before lights off.

Thus, it appears that simultaneously damaging CRY and PER function allows flies to regain clock function in LD, the strength of which at least partly depends on the response of the different *tim* alleles with temperature. Furthermore, it is also clear that the temperature difference in locomotor profiles that is evident in wild-type, less marked in cry^b, and absent in per^{01}, is also partly restored in the double mutant.

Under DD and constant light, the rhythmicity of per^{01}; cry^b breaks down during the first day (Fig. 6, which is published as supporting information on the PNAS web site), presenting no evidence for a self-sustaining clock in the absence of the light Zeitgeber. This makes it impossible to study the ''clock'' of per^{01}; cry^b by probing the oscillator with short pulses of light and then examining whether behavior has phase shifted under constant conditions (e.g., see ref. 7). Therefore, we investigated whether per^{01}; cry^b responded to brief light pulses at 18°C administered during the night phase at ZT15 and ZT21 in LD 12:12 cycles (Fig. 2). In wild type, a light pulse at ZT15 significantly delays the phase of locomotor activity, whereas a pulse at ZT21 gives a modest but nonsignificant advance (Fig. 2*A*); this is analogous to the phase-shifting effect of light pulses delivered at ZT15 and ZT21 followed by DD (7). Light pulses have no significant effect on the timing of the locomotor activity peak in cry^b, either at ZT15 or ZT21, reflecting the defect in cry^b photoreception (Fig. 2*B*) (7). In per^{01}; cry^b, light pulses give advances at both ZT15 and ZT21, significantly so in the former (Fig. 2*C*). Thus the ''clock'' of these flies ''remembers'' a previously administered light pulse, even in LD cycles. All other *per*- or *tim*-null genotypes failed to show significant phase shifts in this paradigm (Fig. 7, which is published as supporting information on the PNAS web site). Thus, per^{01} provides a partial restoration of the light phase shifting ability that is lost in cry^b, and vice versa.

To further probe the clock of per^{01}; cry^b, we carried out a series of experiments at 18°C where we systematically varied the length of the LD cycle (T cycle) from LD 6:6 to LD 20:20. This variation allows us to distinguish whether the locomotor anticipation of lights-off is (*i*) caused by circadian entrainment via an oscillator, (*ii*) generated by an ''hourglass'' mechanism where peak activity always occurs after a set number of hours, or (*iii*) whether locomotor behavior simply responds to light (21, 22). In wild-type flies, as T increases from LD 6:6 to LD 12:12 the locomotor activity peak moves progressively later with small interfly variability, particularly at LD 12:12. However, from LD 12:12 to

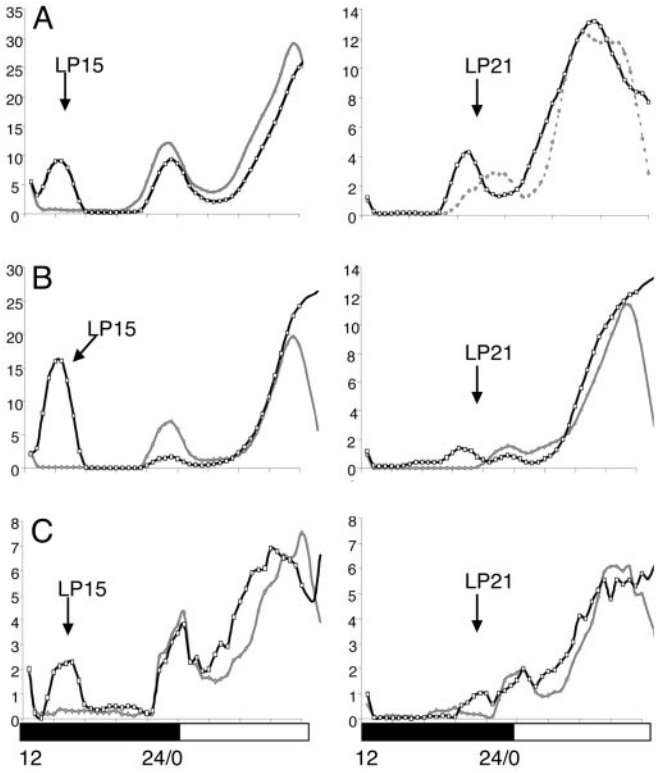

Fig. 2. Locomotor response to light pulses in LD cycles. A 1-h light pulse (LP, black arrows) was administered on the 5th day of entrainment in wild-type (*A*), cry^b (*B*), and per^{01}; cry^b (*C*). Black/white bars represent the LD regime. The average locomotor activity for each group of flies from each genotype is shown with a smoothing five-point moving average on the day before (gray) and after (black) the light pulse. Error bars are omitted for clarity. ANOVA on the raw data revealed significant delays for wild-type at ZT15 ($F_{1,540} = 22.56$, $P \ll 0.001$) and a nonsignificant advance at ZT21 ($F_{1,612} = 2.63$, $P = 0.1$). There were no significant phase shifts for cry^b. per^{01}; cry^b gave significant advances at ZT15 ($F_{1,540} = 3.91$, $P = 0.048$) and nonsignificant advances at ZT21 ($P = 0.45$) (see also Fig. 7).

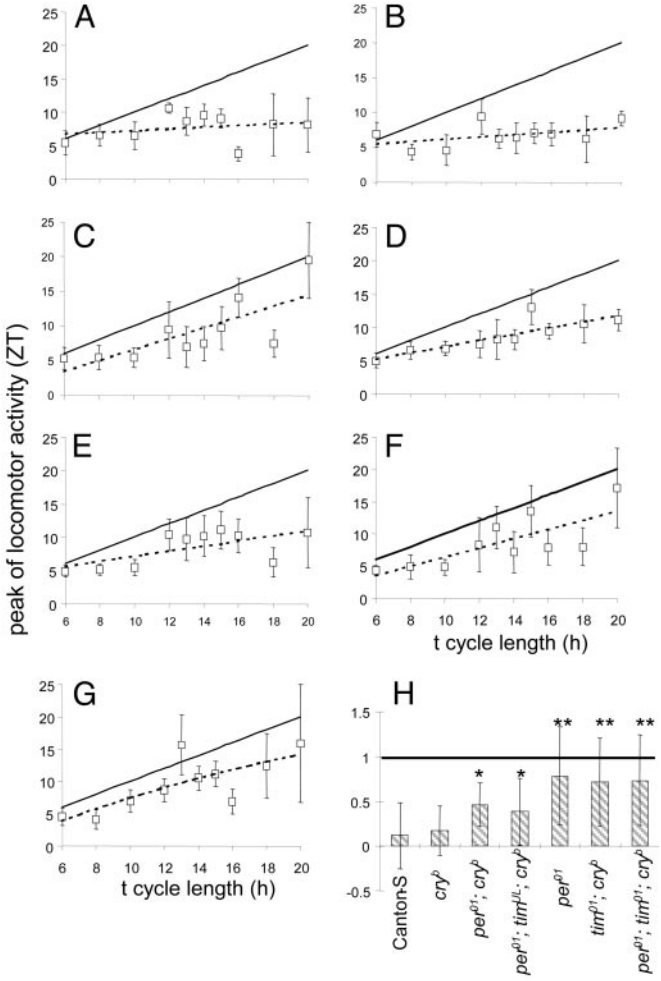

Fig. 3. Locomotor activity peaks in different T cycles. Average peak time for groups of flies on the 5th day (LD16:16 in *B* and *C* from 4th day) is shown for Canton-S (*A*), cry^b (*B*), per^{01} (*C*), per^{01}; cry^b (*D*), per^{01}; tim^{UL}; cry^b (*E*), tim^{01}; cry^b (*F*), and per^{01}; tim^{01}; cry^b (*G*), ±95% confidence limits. ''Lights on'' occurs at 0 on *y* axis, and ''lights off'' is represented by a diagonal black line. The dotted line represents a linear best-fit to the locomotor peaks at each light-dark regime. (*H*) Slope ± 95% confidence limit of regression lines in *A*–*G*. Regression significance: *, $P < 0.05$; **, $P < 0.01$.

LD 20:20, the average peak of activity moves progressively earlier and variation is amplified as the 24-h clock struggles to cope with long T cycles (Fig. 3*A*).

In per^{01} flies, there is no change in the phase relationship of the locomotor peak to the lights off signal (Fig. 3*C*), with peak activity moving progressively later as it tracks increasing T. The same phenomenon, tracking lights off and high variation, is observed in tim^{01}; cry^b and per^{01}; tim^{01}; cry^b (Fig. 3 *F* and *G*). The response of cry^b flies is clearly different from these arrhythmic genotypes and, as in wild type, timing of evening activity falls away from the lights off signal with increasing T, reflecting the robust circadian oscillations in both cry^b and wild type (Fig. 3*B*). Thus, cry^b not only suppresses the effect of per^{01}, but also generates a profile that is not inconsistent with residual circadian activity in the double mutant. This finding is confirmed by the similar behavior of per^{01}; tim^{UL}; cry^b to per^{01}; cry^b (Fig. 3 *D* and *E*).

The response to altered T cycles of per^{01}; cry^b appears intermediate between that of cry^b or wild type and the other *per–null* or *tim–null* genotypes. Because of their robust circadian oscillators, a simple linear regression line through all of the locomotor peaks in the T cycle experiment is not significant for wild-type ($P = 0.48$) or cry^b ($P = 0.18$), whereas all other genotypes show significant regressions (Fig. 3). Therefore, we compared the regression slopes and their associated confidence limits for each of the significant genotypes, aware that these statistics are not informative for wild type and cry^b (Fig. 3*H*). For the other genotypes, if the 95% confidence limits for the gradient overlaps a value of 1, this would suggest that the genotype is simply tracking the lights off slope, which also has a value of unity. Alternatively, if the confidence limits overlap zero, this would mean that irrespective of the T cycle, the locomotor peak falls at the same time after the lights on signal, suggesting an hourglass mechanism. Fig. 3*H* reveals that, except for wild-type and cry^b, the per^{01}; cry^b and per^{01}; tim^{UL}; cry^b genotypes have the smallest gradients (indicative of residual circadian rhythmicity), and confidence limits that neither overlap the lights off signal (the value of unity, suggesting tracking the light), nor the zero value (hourglass). The other *per*- and *tim*-null genotypes all have large slopes overlapping unity. Thus, in a number of metrics, including locomotor peak time, slope and variation, per^{01}; cry^b and per^{01}; tim^{UL}; cry^b show a profile consistent with residual circadian rhythmicity in varying T cycles.

If TIM is partly responsible for providing anticipation of the lights off signal in per^{01}; cry^b and mediating phase advances (Fig. 2), we might expect that it enters the nucleus of clock neurons in the absence of PER and CRY. Therefore, we examined and quantified the cellular localization of TIM in wild-type, cry^b, per^{01}, and per^{01};

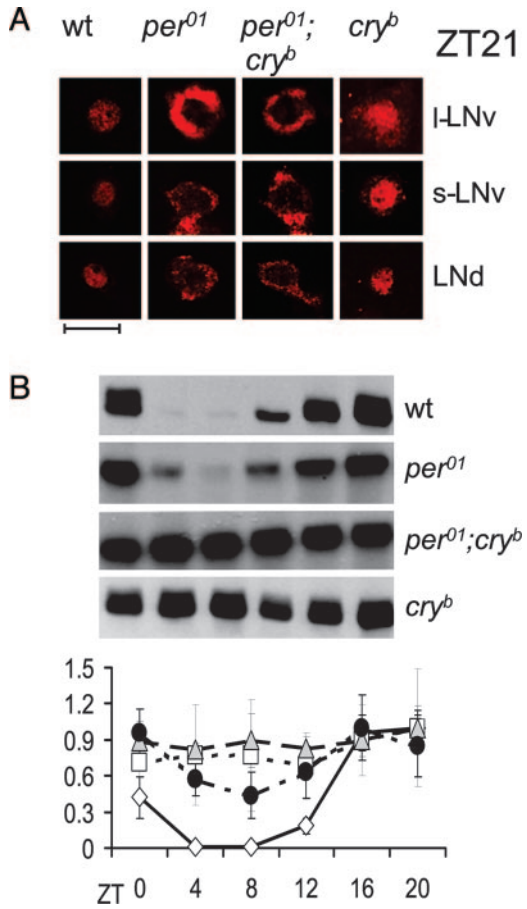

Fig. 4. Nuclear localization and TIM levels in wild-type, per^{01}, per^{01}; cry^b and cry^b. (*A*) The localization is shown of TIM (in red) in the l-LN_vs, s-LN_vs, and LN_ds at ZT21, whereas the other clock neurons showed nuclear staining. These results are representative of 10 brain hemispheres. (Scale bar, 10 μm.) (*B*) TIM cycles in wild-type and per^{01}, but does not cycle in cry^b or per^{01}; cry^b heads in LD cycles. The high molecular weight TIM bands seen at all time points in per^{01}; cry^b but only present at ZT0 in per^{01} heads, are indicative of hyperphosphorylated forms of TIM. The graph shows the normalized mean ± SEM for three independent replicate blots using HSP70 as the loading control (*y* axis) against ZT. Wild-type (white diamonds, continuous line) per^{01} (black dots, hatched and dotted line), per^{01}; cry^b (gray triangles, hatched lines), and cry^b (white squares, dotted line) blots are shown. Wild-type and cry^b data are from ref. 11.

cry^b flies at ZT21, but observed no significant difference in the subcellular distribution of TIM between per^{01} and per^{01}; cry^b, in either the large or small ventral lateral neurons (LN_v), nor LN_d or dorsal neuron pacemaker cells, where TIM was predominantly cytoplasmic at all time points (Fig. 4*A*). We also monitored the levels of the different isoforms of TIM present in fly heads through Western blot analysis in these genotypes (Fig. 4*B*). In per^{01}, although TIM levels cycle in response to the underlying LD cycle, the hyperphosphorylated form of TIM is only present at the end of the night (ZT24/0), as reported (23, 24). In wild type, TIM levels again cycle, and the hyperphosphorylated form of TIM is found only during the night (ZT20–24) (23). In contrast, there is a suggestion that this isoform of TIM may be present at all times in per^{01}; cry^b (Fig. 4) as also reported for cry^b (7). Because CRY is believed to interact with hyperphosphorylated TIM at dawn and mediate its degradation, we can understand why this isoform is stabilized in the absence of CRY. That this "late-night" isoform of TIM may be constitutively present in the double mutant could explain why per^{01}; cry^b only shows phase advances (the late-night response) to light pulses.

Discussion

We have revealed a surprising and intriguing restoration of circadian rhythmicity in LD cycles in per^{01}; cry^b flies. This partial rescue can even be extended to the adaptive thermal change in locomotor behavior mediated by 3′ UTR splicing of the *per* transcript (8, 25, 26). We have subsequently used a number of criteria to dissect rhythmic behavior, including phase shifting in response to light pulses in LD and the use of T cycles to suggest that a residual oscillation, rather than an hourglass, underlies the behavior of the double mutant. The phase shifting of the per^{01}; cry^b oscillator is particularly informative because per^{01} is effectively rescuing this phenotype in cry^b. This can be understood in terms of the robust, high-amplitude oscillator in cry^b, being less "perturbable" by light as CRY photoreception is lost, whereas the damped oscillator in per^{01}; cry^b is more sensitive to the environmental stimulus, precisely because of its low amplitude (27). The damped oscillation in the per^{01}; cry^b double mutant can be eliminated by removing *tim* function, but this is temperature dependent, so *tim* cannot supply the full explanation for these residual cycles. Although our experiments have focused on the "evening" oscillator, of related interest is that we also observe the residual "morning" oscillator that anticipates the lights-on signal in per^{01} revealed by Helfrich-Förster (16). It is clear that both of our studies raise again the possibility of an underlying rhythmicity in per^{01} flies that was initially suggested from statistical analyses of mutant locomotor records (28).

The entrainment of a *frequency*-less oscillator in *Neurospora crassa* has been the subject of some recent debate (21, 22, 29), and the parallels with a residual rhythmicity in *per*-null *Drosophila* are striking. Furthermore, the rescue of per^{01} behavior by cry^b would appear, at least superficially, to be similar to the situation in mammals in which a *Cry* mutation restores free-running rhythms to the arrhythmic *mPer2* mutant mouse (9); this has been explained in terms of the freeing up in the double mutant of other *mPer* and *Cry* paralogues to interact and restore the original behavior. The fly does not have paralogues of *per* and *cry*, so we must seek an explanation elsewhere. The only other genotypes identified so far with an anticipatory locomotor activity peak in LD and loss of rhythmicity in DD are *disconnected* (*disco*) and Pdf^0 (30–33). Neither mutation affects the molecular core of the circadian clock, rather the network of pacemaker neurons is disrupted. PDF is required for the functional integration of several clock neuronal groups within the brain (30, 31), suggesting that disruption of interneuronal signaling causes arrhythmic behavioral output in the absence of synchronizing cues. In arrhythmic *disco* mutants, the clock gene expressing lateral neurons (LN_vs and LN_ds) are usually absent, whereas the dorsal neurons are still present (34), thus indicating that the former are necessary for self-sustained rhythmicity, whereas the latter can only mediate rhythmic behavior under LD conditions (35).

This networking of clock neurons provides a basis for possible models to explain LD behavioral anticipation in the absence of PER, based on functional differences between the three groups of clock genes expressing LNs. Of these, only the small ventral LNs (sLN_vs) and dorsal LNs (LN_ds) have a self-sustaining molecular clock when initially released into DD (12, 30, 35, 36), although the latter depends on the former for synchronization (30). The third group, the large ventral LNs (l-LN_vs) do not have a self-sustaining clock, although after a few days, *tim* mRNA again begins to accumulate rhythmically in these cells (31). Furthermore, rhythmic TIM expression is more sensitive to disruption by *cry* mutations in the l-LN_vs, than in the s-LN_vs or the LN_ds under LD conditions (5, 7, 11, 30), suggesting that rhythmic output from the l-LN_vs are compromised in a cry^b

background. In turn, this may contribute to the peculiar defects of cry^b that includes robust entrainment to LD cycles, but significantly reduced behavioral phase shifts to brief light pulses, and, unlike wild-type, the maintenance of rhythmic behavior in constant light (7, 37).

In the model we favor, the robust s-LN_v and LN_d oscillators in cry^b "resist" the effects of brief light pulses (ref. 7 and Fig. 2*B*), because of the impaired light input that is relayed to the s-LN_vs, and from the s-LN_vs to the LN_ds, by the more light-relevant l-LN_vs. In per^{01}, the molecular clock is severely dampened in all clock neurons, more so in the s-LN_vs and LN_ds that have an endogenous cycle than the l-LN_vs that do not. Thus, the light-mediated input from the l-LN_v neurons into the s-LN_vs, and indirectly to the LN_ds, is no longer resisted, and now overwhelms the residual damped per^{01} oscillator in these neurons, stimulating light-induced non-rhythmic locomotor behavioral output. However, when cry^b and per^{01} are combined, the weak oscillator of per^{01} is no longer overcome by the light input because it is attenuated by cry^b and mediated via the l-LN_vs. Thus, rhythmic behavior is observed in LD cycles, providing a glimpse of the residual PER-independent, partly TIM-regulated clock. We favor this model over a simpler one in which only the s-LN_vs are involved, because previous studies have shown that the only direct photoreceptive input into these neurons is from the Hofbauer–Buchner eyelet, which is a very weak photoreceptor at best and cannot, in the absence of other photoreceptors, entrain the fly's behavior (38). Thus, it is difficult to see how light information would be received by the s-LN_vs to entrain the per^{01}; cry^b double mutant so effectively, unless it is transmitted from another neuronal source: the l-LN_vs.

In support of the model, there appears to be both direct and indirect neural connections between the compound eyes and the l-LN_vs, suggesting that the l-LN_vs may act as the light "amplifier" (16). We have now extended these earlier observations by showing that photoreceptor cells expressing the rhodopsin genes, *Rh3* and *Rh5* (39), send their axons through the medulla terminating in close proximity to the general region where the l-LN_vs likely extend their dendritic arborizations (Fig. 5). Although not definitive, these results support earlier claims that the photoreceptors may directly (or indirectly) synapse with the l-LN_vs (40). As stated above, these molecular and proposed functional differences between s- and l-LN_vs may also contribute to explaining the loss of light responsiveness in cry^b mutant flies, which are blind to constant light and brief light pulses, despite retaining light input from the canonical visual transduction pathway (7, 11, 41, 42). Thus CRYPTOCHROME, aside from being a photoreceptor in its own right, also appears to control a gateway for rhodopsin-mediated light input into the clock.

Although the disruption of neural networks in this way probably explains the light responses of the clock in per^{01}; cry^b, it offers no molecular basis for the observed behavior. The loss of anticipation in *tim*-null-bearing genotypes suggests that TIM may play a key role. Although we did not detect significant nuclear TIM in the LN_vs or LN_ds of per^{01}; cry^b, the latter neurons being particularly relevant for providing the evening peak of locomotor activity present in the double mutants (17, 18), we suspect that TIM is shuttling continually in and out of the nucleus because TIM can enter the nucleus alone, but requires PER for nuclear retention, at least in larval clock neurons (43). Once in the nucleus, TIM is presumably interacting with as yet unidentified protein(s) in a light-dependent manner, generating behavioral rhythms in the double mutants. A microarray study found that 18 of the 72 genes that cycled in LD in wild-type also cycle in per^{01} (44). Any one or more of these light-controlled proteins could therefore interact with TIM, contributing to the light-dependent oscillator of per^{01}; cry^b. In fact, it has been noted by others that a glutamine-rich transcriptional activator domain found within TIM may allow it to regulate other genes in a PER-independent manner (43).

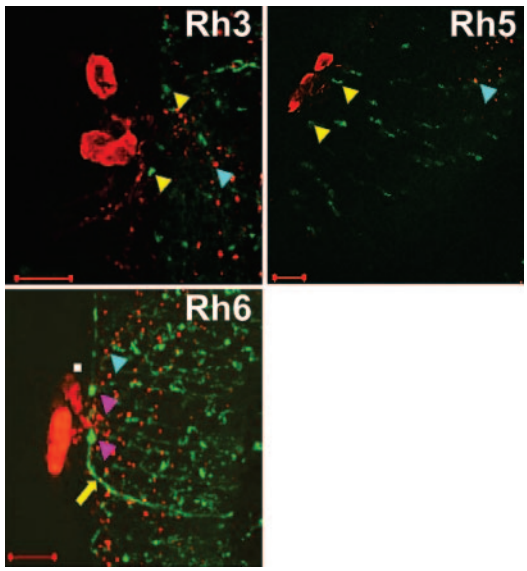

Fig. 5. Photoreceptor connections to l-LN_vs. Projections of confocal *z* stacks. Axons of a subset of visual photoreceptors end in close proximity to the l-LN_vs. Photoreceptor cells expressing β-galactosidase (green) under control of *Rhodopsin3* (*Rh3*), *Rhodopsin5* (*Rh5*), and *Rhodopsin6* (*Rh6*) promoters extend their axons through the medulla in close proximity of l-LN_vs varicosities (blue arrowhead) labeled with α-PDH antibody (red). The *Rh6*-driven expression of *lacZ* also shows projections (arrow) from the Hofbauer–Buchner (HB) eyelets (38). Some neurites from *Rh3*- and *Rh5*-expressing cells end in close proximity (yellow arrow heads) to the l-LN_vs. HB projections engage in synapses (magenta arrow heads) with fibers connecting l-LN_vs and s-LN_vs (white square) as described (38). (Scale bar, 20 μm.)

In conclusion, our results provide evidence for a skeletal *per* independent clock in LD cycles that involves TIM, although not exclusively, and which may represent a PER-independent role for TIM (43, 45). In addition, our "systems" explanation for the residual rhythmicity in per^{01}; cry^b places the l-LN_vs in a central role for transducing CRY-mediated light input into the clock.

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