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

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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 cryb 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 cryb, circadian rhythmicity in locomotor behavior in light–dark cycles, as measured by a different number of 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.

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-c-PDH (13), 1:1,000 mouse anti-β-galactosidase (Pro-
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 one temperature, described by Helfrich-Förster for per01 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 per01 (17, 18).

Using this as an operational definition for the circadian regulation of LD behavior, per01; cryb has clearly regained clock function as it shows a dramatic anticipation of lights off in LD (Fig. 1E). 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 per01 19-h mutant (19). This anticipatory locomotor activity is at least partially TIM-dependent, because tim01; cryb double mutants and per01; tim01; cryb; cryy 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 tim01 allele (20) into the per01; cryb background, at 18°C, per01; tim01; cryb locomotor activity showed some anticipation beyond that seen in tim01 or per01 (Fig. 1G). Again, as in the tim01; cryb 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 cryb, and absent in per01, is also partly restored in the double mutant.

Under DD and constant light, the rhythmicity of per01; cryb 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 per01; cryb 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 per01; cryb 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 and B). Arrow in the per01 panel shows the “morning peak” described by Helfrich-Förster (16).

Western Blotting. Heads were collected every 4 h, and protein was extracted from per01, Canton-S, and perb 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-IgG (Jackson ImmunoResearch). Optical sections were imaged on a Zeiss LMS510 confocal microscope.

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 cryb 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, per01 and tim01 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 one temperature, described by Helfrich-Förster for per01 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 per01 (17, 18).

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To further probe the clock of per01; cryb, 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...
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. 3A).

In per01 flies, there is no change in the phase relationship of the locomotor peak to the lights off signal (Fig. 3C), with peak activity moving progressively later as it tracks increasing T. The same phenomenon, tracking lights off and high variation, is observed in tim01; cryb and per01; tim01; cryb (Fig. 3F and G). The response of cryb 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 cryb and wild type (Fig. 3B). Thus, cryb not only suppresses the effect of per01, 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 per01; timUL; cryb to per01; cryb (Fig. 3D and E).

The response to altered T cycles of per01; cryb appears intermediate between that of cryb 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 cryb (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 cryb (Fig. 3H). 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. 3H reveals that, except for wild-type and cryb, the per01; cryb and per01; timUL; cryb 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, a number of metrics, including locomotor peak time, slope and variation, per01; cryb and per01; timUL; cryb, 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 per01; cryb 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, cryb, per01; and per01;
cry\textsuperscript{b} flies at ZT21, but observed no significant difference in the subcellular distribution of TIM between \textit{per}\textsuperscript{01} and \textit{per}\textsuperscript{01}; \textit{cry}\textsuperscript{b} in either the large or small ventral lateral neurons (LN\textsubscript{S}) nor LN\textsubscript{D} or dorsal neuron pacemaker cells, where TIM was predominantly cytoplasmic at all time points (Fig. 4). We also monitored the levels of the different isoforms of TIM present in fly heads through Western blot analysis in these genotypes (Fig. 4B). In \textit{per}\textsuperscript{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 delay in the l-LN\textsubscript{S} but not in other neurons. In an independent experiment using a different \textit{cry}\textsuperscript{b} strain, TIM was predominantly cytoplasmic in the l-LN\textsubscript{S} 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 \textit{per}\textsuperscript{01}, but does not cycle in \textit{cry}\textsuperscript{b} or \textit{per}\textsuperscript{01}; \textit{cry}\textsuperscript{b} heads in LD cycles. The high molecular weight TIM bands seen at all time points in \textit{per}\textsuperscript{01}; \textit{cry}\textsuperscript{b} but only present at ZT0 in \textit{per}\textsuperscript{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) \textit{per}\textsuperscript{01} (black dots, hatched and dotted line), \textit{per}\textsuperscript{01}; \textit{cry}\textsuperscript{b} (gray triangles, hatched lines), and \textit{cry}\textsuperscript{b} (white squares, dotted line) blots are shown. Wild-type and \textit{cry}\textsuperscript{b} data are from ref. 11.

Discussion

We have revealed a surprising and intriguing restoration of circadian rhythmicity in LD cycles in \textit{per}\textsuperscript{01}; \textit{cry}\textsuperscript{b} flies. This partial rescue can even be extended to the adaptive thermal change in locomotor behavior mediated by 3’ UTR splicing of the \textit{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 \textit{per}\textsuperscript{01}; \textit{cry}\textsuperscript{b} oscillator is particularly informative because \textit{per}\textsuperscript{01} is effectively rescuing this phenotype in \textit{cry}\textsuperscript{b}. This can be understood in terms of the robust, high-amplitude oscillator in \textit{cry}\textsuperscript{b}, being less “perturbable” by light as CRY photoreception is lost, whereas the damped oscillator in \textit{per}\textsuperscript{01}; \textit{cry}\textsuperscript{b} is more sensitive to the environmental stimulus, precisely because of its low amplitude (27). The damped oscillation in the \textit{per}\textsuperscript{01}; \textit{cry}\textsuperscript{b} double mutant can be eliminated by removing \textit{tim} function, but this is temperature dependent, so \textit{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 \textit{per}\textsuperscript{01} revealed by Helfrich-Förster (16). It is clear that both of our studies raise again the possibility of an underlying rhythmicity in \textit{per}\textsuperscript{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 \textit{per}-null Drosophila are striking. Furthermore, the rescue of \textit{per}\textsuperscript{01} behavior by \textit{cry}\textsuperscript{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 \textit{mPer2} mutant mouse (9); this has been explained in terms of the freeing up in the double mutant of other \textit{mPer} and CRY paralogues to interact and restore the original behavior. The fly does not have paralogues of \textit{per} and \textit{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 (\textit{disco}) and \textit{Pdf}\textsuperscript{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 \textit{disco} mutants, the clock gene expressing lateral neurons (LN\textsubscript{S} and LN\textsubscript{D}) 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 \textit{PER}, based on functional differences between the three groups of clock genes expressing LNs. Of these, only the large ventral LNs (sLN\textsubscript{S}) and dorsal LNs (LN\textsubscript{S}) 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\textsubscript{S}) do not have a self-sustaining clock, although after a few days, \textit{tim} mRNA again begins to accumulate rhythmically in these cells (31). Furthermore, rhythmic TIM expression is more sensitive to disruption by \textit{cry} mutations in the l-LN\textsubscript{S}, than in the s-LN\textsubscript{S} or the LN\textsubscript{D} under LD conditions (5, 7, 11, 30), suggesting that rhythmic output from the l-LN\textsubscript{S} are compromised in a \textit{cry}\textsuperscript{b}}
background. In turn, this may contribute to the peculiar defects of cryb 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-LNv and LNds oscillators in cryb 'resist' the effects of brief light pulses (ref. 7 and Fig. 2B), because of the impaired light input that is relayed to the s-LNvs, and from the s-LNvs to the LNds, by the more light-relevant l-LNvs. In per01, the molecular clock is severely dampened in all clock neurons, more so in the s-LNvs and LNds that have an endogenous cycle than the l-LNvs that do not. Thus, the light-mediated input from the l-LNvs, the s-LNvs, and indirectly to the LNds, is no longer resisted, and now overwhelms the residual damped per01 oscillator in these neurons, stimulating light-induced non-rhythmic locomotor behavioral output. However, when cryb and per01 are combined, the weak oscillator of per01 is no longer overcome by the light input because it is attenuated by cryb and mediated via the l-LNvs. Thus, rhythmic behavior is observed in LD cycles, providing a glimpse of the residual PER-independent, partly TIM-regulated clock. We favor this model overall a simpler one in which only the s-LNvs 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-LNvs to entrain the per01 oscillator; cryb double mutant so effectively, unless it is transmitted from another neuronal source: the l-LNvs.

In support of the model, there appears to be both direct and indirect neural connections between the compound eyes and the l-LNvs, suggesting that the l-LNvs 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-LNvs 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-LNvs (40). As stated above, these molecular and proposed functional differences between s- and l-LNvs may also contribute to explaining the loss of light responsiveness in cryb 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 per01; cryb, 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 LNvs or LNds of per01; cryb, 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 per01 (44). Any one or more of these light-controlled proteins could therefore interact with TIM, contributing to the light-dependent oscillator of per01; cryb. 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).

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 per01; cryb places the l-LNvs in a central role for transducing CRY-mediated light input into the clock.

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