PER-TIM Interactions with the Photoreceptor Cryptochrome Mediate Circadian Temperature Responses in Drosophila

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Drosophila cryptochrome (CRY) is a key circadian photoreceptor that interacts with the period and timeless proteins (PER and TIM) in a light-dependent manner. We show here that a heat pulse also mediates this interaction, and heat-induced phase shifts are severely reduced in the cryptochrome loss-of-function mutant cry0. The period mutant per4 manifests a comparable CRY dependence and dramatically enhanced temperature sensitivity of biochemical interactions and behavioral phase shifting. Remarkably, CRY is also critical for most of the abnormal temperature compensation of per4 flies, because a per3; cryb strain manifests nearly normal temperature compensation. Finally, light and temperature act together to affect rhythms in wild-type flies. The results indicate a role for CRY in circadian temperature as well as light regulation and suggest that these two features of the external 24-h cycle normally act together to dictate circadian phase.

Introduction

Most organisms have circadian rhythms of gene expression and behavior that are controlled by endogenous clocks. A few studies have verified that these systems increase fitness and help organisms adapt to the physical and ecological environment in which they live [1]. At the molecular level, the central pacemaker of animals is proposed to consist of auto-regulatory feedback loops that regulate the expression of key clock genes [2]. An admittedly simplified view of the Drosophila central clock posits a core system of four interacting regulatory proteins. A circadian cycle begins when a CLOCK (CLK) and CYCLE (CYC) heterodimer activates the expression of two other proteins, PERIOD (PER) and TIMELESS (TIM). PER and TIM levels slowly accumulate over time, and these two proteins also heterodimerize. At some point, PER-TIM complexes enter the nucleus and inactivate CLOCK-CYCLE activity, slowing their own production and signaling the end of a cycle. Importantly, kinases and phosphatases modify PER, TIM, and CLK and play critical roles in circadian rhythms [3–7].

Endogenous periods are usually different from the precise 24-h rotation of Earth. Nonetheless, circadian clocks keep precise 24-h time under normal conditions and are reset every day by environmental signals like light and temperature, which are the dominant entraining cues in nature. In Drosophila, circadian light perception is well-understood, and a major fraction of it is mediated by the circadian photoreceptor molecule cryptochrome (CRY) [2,8]. Cryptochromes are related to photolases, a family of blue-light–sensitive DNA repair enzymes, and also play important roles in photoreception and circadian rhythms of other animals as well as plants [9,10].

Drosophila CRY is prominently expressed in pacemaker neurons [11–13]. Moreover, a mutant cry strain (cry0) manifests severe molecular and behavioral problems. These include a lack of PER and TIM molecular cycling in peripheral tissues under light-dark cycles and an inability to undergo phase resetting in response to short light pulses [14]. cry0 flies are also rhythmic in constant light, i.e., the characteristic arrhythmicity of Drosophila and many other animals in constant light is absent [15]. Finally, there is strong evidence that CRY contributes to standard entrainment by light-dark cycles [16].

At the biochemical level, photon capture by CRY leads to an interaction with TIM or with the PER-TIM complex [17–20]. CRY also interacts with and blocks the function of the PER-TIM complex in a light-dependent manner in an S2 cell-based assay [17]. The current view is that the CRY:TIM interaction leads to TIM degradation, which results in phase-resetting in response to a light pulse [21–25].

In addition to light, other factors such as social interactions, activity, and especially temperature can modulate free-running rhythms. Indeed, temperature is generally regarded as secondary only to light as an entrainment cue.

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Abbreviations: CRY, cryptochrome; CS, Canton-S; CT, circadian time; DL, dark-light; LD, light-dark; LL, light-light; PRC, phase response curve; ZT, zeitgeber time

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Remarkably, these PERL function, an emerging area of research in circadian biology. The clock and even contribute to seasonal adaptation of clock function and critical to maintaining dependable time keeping despite fluctuations in ambient temperature. C. We used a modified PRC protocol, called the anchored PRC (APRC; [32–34]: the pulses are applied to wild-type flies during the night half of a light-dark cycle (zeitgeber time [ZT]12–24) and then during the first 12 h of the subsequent “day” in constant darkness (circadian time [CT]0–12). Locomotor activity phases were then measured after several subsequent days in constant darkness.

A typical PRC was obtained for light, with maximum phase advances of about 2.5 h in the late night. For heat, early night delays were more modest, about 2.5 h, whereas late night advances were very small or absent (Figures 1A and S1). The data are essentially indistinguishable from the only published examples of 37 °C heat pulse-mediated phase shifts in Drosophila [35,36]. Moreover, there was little or no behavioral phase shift after 30 °C or 34 °C heat pulses (Figure 1A) [36].

Cry Promotes Circadian Temperature Responses

Cry promotes circadian temperature responses, which are generally insensitive to alterations in (constant) incubation temperature, i.e., Q10 (the relative rate enhancement corresponding to a 10 °C rise in temperature) ≈ 1.0 [31]. It is believed that temperature compensation is integral to circadian clock function and critical to maintaining dependable time keeping despite fluctuations in ambient temperature.

We found a surprising relationship between the response of the Drosophila clock to heat pulses and light pulses as well as between heat pulses and temperature compensation; the connector is the photoreceptor Cry. The heat-induced phase delays that take place in a wild-type strain are paralleled by a physical interaction between Cry and Period and timeless. In perΔ mutant flies, heat-phase shifts are more robust and occur at lower temperatures, which are mirrored by parallel Cry-Perl-Tim interactions. perΔ Δ phase shifts are also severely reduced by the addition of cryΔ to the genetic background. Remarkably, these perΔ Δ; cryΔ Δ double-mutant flies have largely restored temperature compensation. The results indicate that a more potent interaction between Cry and Perl-Tim causes most of the temperature compensation defects of perΔ Δ as well as the more robust heat-mediated phase shifts of these mutant flies.

Results

Heat Pulse–Mediated Phase Delays of Wild-Type Flies Require 37 °C

To investigate the effect of heat on Drosophila locomotor activity rhythms, we first compared a heat phase response curve (PRC) to a standard light PRC. In both cases, the pulses lasted for 30 min, either with saturating light or with a shift from 25 °C to 37 °C. We used a modified PRC protocol, called the anchored PRC (APRC; [32–34]: the pulses are applied to wild-type flies during the night half of a light-dark cycle (zeitgeber time [ZT]12–24) and then during the first 12 h of the subsequent “day” in constant darkness (circadian time [CT]0–12). Locomotor activity phases were then measured after several subsequent days in constant darkness.

A typical PRC was obtained for light, with maximum phase advances of about 2.5 h in the early night and maximum phase advances of about 2.5 h in the late night. For heat, early night delays were more modest, about 2.5 h, whereas late night advances were very small or absent (Figures 1A and S1). The data are essentially indistinguishable from the only published examples of 37 °C heat pulse-mediated phase shifts in Drosophila [35,36]. Moreover, there was little or no behavioral phase shift after 30 °C or 34 °C heat pulses (Figure 1A) [36].

Cry-Perl-Tim Heat-Dependent Interactions Parallel the Behavioral Responses

The weak heat-mediated delay and absence of a substantial advance makes it uncertain whether there is a relationship between the heat and light PRCs. We therefore assayed the biochemical effects of a heat pulse and compared them to those of a light pulse. The strategy was based on the interaction of Cry with Tim and/or Per, which is a light-dependent event (e.g., [20]). There is also substantial evidence

Figure 1. Heat Pulse–Mediated Phase Delays of Wild-Type Flies Require 37 °C

Phase response curves for CS flies after heat (circle) versus light pulse (square). Flies were entrained for 3 d in 12 h:12 h LD cycles and pulsed for 30 min of light and 30 °C, 34 °C, and 37 °C heat pulse (HP) during the last night of the LD entrainment cycle, after which the flies were released in constant darkness for 5 d. Phase changes were calculated by comparing behavioral offsets of light or HP treated flies 3 d after the pulse to the behavior of the control group of the same genotype that did not receive a pulse. The calculations were made by MATLAB software using previously described methods [46]. Phase delays and phase advances are plotted (± SEM) as negative and positive values respectively. In all cases, the experiments were repeated at least twice with similar results. Data were pooled from the following number of flies (each pair of values referring to wild-type light-pulsed and wild-type 37 °C heat-pulsed); control: 32, 32; pulse at ZT12: 32, 22; pulse at ZT15: 32, 26; pulse at ZT18: 23, 26; pulse at ZT21: 24, 19; and pulse at ZT24: 29, 27.

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that these events are crucial to clock resetting after short light pulses [19,37,38]. To assay CRY interactions in flies, we used a previously described strain that expresses N-terminal MYC-tagged CRY [20]. We subjected flies to either light or heat pulses and then assayed CRY complexes via immunoprecipitation with anti-MYC antisera.

Remarkably, an interaction between CRY and PER-TIM was observed at ZT15 after a 37 °C heat pulse as well as after a light pulse. There was no detectable interaction if the ZT15 heat pulse was at 30 °C (Figure 2A), nor was there a robust 37 °C heat-mediated interaction at ZT21, despite a canonical light-mediated interaction at this time (Figure 2B). These results mirror the behavioral observations, namely, a 37 °C phase shift and no 30 °C phase shift at ZT15, with no 37 °C phase shift at ZT21 (Figure 1) [35]. The data indicate that a CRY:PER-TIM interaction correlates with heat-mediated phase shifts and suggest that it might underlie the behavioral phase shifts.

**CRY Is Required for Heat-Mediated Phase Shifts**

These results predict that heat PRCs should be affected in the severe loss-of-function mutant cryb. Indeed, these flies show little to no response to a heat pulse, i.e., an almost flat PRC (Figure 3A). The results are very similar to those observed for a light PRC in cryb [14].

To verify that this result is not due to a strain differences unrelated to the cry locus, we rescued the cryb mutation by expressing CRY in clock-pacemaker cells using pdf-GAL4 [13,39,40]. pdf-GAL4-mediated CRY expression partially rescued the cryb heat delay at ZT15 (Figure 3B) as well as the
cry<sup>b</sup> light delay as previously described [13]. We also compared the response of these strains to heat and light pulses at ZT21 (Figure 3C). As predicted from the wild-type heat PRC pattern (Figure 3A), the addition of pdf-GAL4-mediated CRY expression to the cry<sup>b</sup> background had no effect on the essentially nonexistent heat-phase shift at ZT21, whereas it rescued the cry<sup>b</sup> ZT21 light-phase shift (Figure 3C) [13]. In contrast, tim-GAL4-mediated CRY-B expression was unable to rescue either light- or heat-mediated cry<sup>b</sup> phase shifts (Figure S2), consistent with the strong hypomorphic cry<sup>b</sup> mutation. Taken together with the heat-mediated physical interaction between CRY and PER-TIM (Figure 2), the results indicate that CRY is important for circadian clock heat responses as well as light responses.

**per<sup>f</sup> Flies Are Hypersensitive to Heat**

The *per<sup>f</sup>* genotype shows aberrant temperature compensation, with dramatically increased periods at elevated constant temperatures [41,42]. We speculated that this phenomenon might be related to heat-pulse responses and even light pulse-mediated phase shifts. To examine this possibility, we first assayed a standard light PRC of *per<sup>f</sup>* flies. It is very similar to that for wild-type flies, except that the *per<sup>f</sup>* curve is delayed by several hours (Figure 4A) [34]. There are essentially indistinguishable phase delays 18 h after the last DL (dark-light) transition for *per<sup>f</sup>* flies and 15 h after the last DL transition for wild-type flies. Moreover, there are similar phase advances, about 26 h after the last DL transition in *per<sup>f</sup>* and 21 h after the last DL transition in wild-type (compare Figure 4A with Figure 1).

Consistent with the notion that *per<sup>f</sup>* flies are more heat sensitive than wild-type flies, there is essentially no difference between the *per<sup>f</sup>* heat and light PRCs in the delay zone (Figure 4A), in contrast to the magnitude of the wild-type heat-mediated delay, which is clearly less than that of the wild-type light-mediated delay (Figure 1) [35]. Even more impressive is the heat-mediated advance for *per<sup>f</sup>* flies, which is indistinguishable from the light-mediated maximal advance (Figure 4A); there is little or no heat-mediated advance in wild-type flies (Figure 1). Finally, *per<sup>f</sup>* flies are sensitive to a 30 °C heat pulse, whereas wild-type flies are insensitive even to a 34 °C pulse (Figures 1 and 4B) [36].

The heat-mediated phase advance of *per<sup>f</sup>* flies suggested that there might be an interaction between CRY and PERL-TIM at these times, e.g., CT2 (ZT26 = CT2). Indeed, we confirmed such an interaction after a 30 °C as well as a 37 °C heat pulse (Figure 4B). With minor differences, the interaction was similar to that elicited by a light pulse at this same time, and no interaction was observed without a heat or a light pulse (Figure 4B). There is no detectable heat-mediated interaction between CRY and wild-type PERL-TIM in the advance zone or at 30 °C (Figures 1 and 2), i.e., the interactions between CRY and PERL-TIM correlate well with the behavioral observations (Figure 4A) and further indicate that they are important for the observed heat-mediated phase shifts.

**per<sup>f</sup>** Heat-Mediated Phase Shifts, CRY, and Temperature Compensation

To verify that the CRY:PERL-TIM interaction is functionally relevant, we generated *per<sup>f</sup>*; cry<sup>b</sup> double mutant flies. They have a long free running period of ~28 h, characteristic of *per<sup>f</sup>*; and are rhythmic in light-light (LL), characteristic of cry<sup>b</sup> (Figure 5A). These flies also show much smaller phase shifts in response to 37 °C heat pulses in the delay zone at ZT18 as well as in the advance zone at ZT26 (ZT26 = CT2); Figure 5B). The exaggerated *per<sup>f</sup>* heat-mediated phase shifts are therefore CRY dependent.

Finally, to establish a link between the exaggerated heat-mediated phase shifts and the temperature compensation defect of *per<sup>f</sup>* flies, we assayed the free-running period of *per<sup>f</sup>*; cry<sup>b</sup> double mutant flies at constant temperatures (Figure 5C). The results indicate that this genotype shows much less period change with temperature, in striking contrast to *per<sup>f</sup>*; cry<sup>b</sup>
flies. This indicates that a temperature-sensitive CRY:PERL-TIM interaction underlies most of the perLT loss of temperature compensation. It also connects the free-running period phenotype assayed at constant temperatures with the response to a heat pulse. Indeed, there is also a CRY:PERL-TIM interaction after incubation of perLT flies at a constant temperature of 29 °C (Figure S3). Moreover, the fact that the perLT strain has an altered period compared to the perLT; cryb double mutant strain at 15 °C (Figure 5C) suggests that even at low temperatures, the PERL-TIM complex interacts with...
CRY. We suggest that advances predominate (an aggregate shorter period) at 15 °C, whereas delays predominate (an aggregate longer period) at temperatures ≥ 25 °C.

A Model
These data suggest that the \( \text{per}^l \) missense mutation facilitates a PER-TIM conformational change (Figure 6A; 1 → 2). Heat facilitates the same change in wild-type PER-TIM, although higher temperatures are required and a smaller fraction of PER-TIM is affected. If CRY interacts predominately with TIM, then the \( \text{per} \) mutation and heat must also help promote a TIM conformational change (Figure 6A; 3). We imagine that this altered PER-TIM conformation could also facilitate an interaction with active CRY, which is a conformational state similar to that promoted by illumination, i.e. by CRY photon capture (Figure 6A; CRY*). The key phase-shifting complex can then be promoted by increasing the concentration of either component, activated PER-TIM by temperature/mutation or activated CRY by light (Figure 6A; 2–3 or CRY*, respectively). Importantly, a temperature-sensitive PERL-TIM complex is consistent with a slightly longer average period (~1 h) of the \( \text{per}^l \); \( \text{cry}^b \) strain at 29 °C relative to 15 °C (Figure 5C).

Light and Temperature Can Act Together on Wild-Type Fly Rhythms
These observations suggest that even in wild-type flies, temperature and light can synergize to affect CRY:PER-TIM complex formation at physiologically normal temperatures. To test this hypothesis, we subjected Canton-S (CS) flies to constant illumination at 10 and 100 lux. Constant light even at low intensities render most flies arrhythmic at a standard incubation temperature of 25 °C (Figure 6B) [41], and constant light arrhythmicity requires CRY [15]. The results and model (Figure 6A) suggest that low temperatures might reduce complex formation and arrhythmicity, and constant light arrhythmicity has not been assayed at 15 °C. Indeed, we observed substantially larger numbers of arrhythmic flies at 25 °C than at 15 °C, at 100 as well as at 10 lux of constant light (Figure 6B). We interpret the result to indicate more CRY:PER-TIM complex formation at 25 °C than at 15 °C, indicating that light and temperature can act together in wild-type flies at physiologically relevant temperatures. The convergence induces phase shifts as well as causes arrhythmicity in constant light. We speculate that it also serves to fine tune the dawn and dusk response of the clock when light and temperature increase and decrease together.

Discussion
We show here that the photoreceptor CRY and its interaction with the PER-TIM complex is critical for heat shock–mediated phase shifts as well as for the loss of temperature compensation in the \( \text{per}^l \) mutant strain. Heat-induced phase delays take place in a wild-type strain, and they are severely reduced in the \( \text{cry} \) loss-of-function mutant \( \text{cry}^b \). Moreover, there is a physical interaction between CRY and PER-TIM at circadian times that correspond to phase delays. More robust heat-mediated phase delays and even phase advances occur in \( \text{per}^l \) mutant flies. The \( \text{per}^l \) behavioral results are mirrored by CRY:PERL-TIM interactions, which occur in the advance zone and also in response to 30 °C temperature pulses. \( \text{per}^l \) phase shifts like wild-type phase shifts are severely reduced by the addition of \( \text{cry}^b \) to the \( \text{per}^l \) background. These \( \text{per}^l \); \( \text{cry}^b \) double mutant flies also have largely restored temperature compensation, indicating that an interaction between CRY and PER-TIM is responsible for the loss of temperature compensation in the \( \text{per}^l \) strain as well as for heat-mediated phase shifts of wild-type as well as \( \text{per}^l \) flies. The similarity between heat-mediated and light-mediated phase shifts suggests that light and temperature can synergize to cause phase shifts, and an experiment in wild-type flies supports this notion.

The temperature-induced complex formation between CRY and PER-TIM parallels the substantial evidence that a similar interaction is critical for light-mediated phase shifts. Biochemical as well as genetic data indicate that complex formation between light-activated CRY and TIM, or between light-activated CRY and PER-TIM, leads to TIM degradation, which is believed to advance or delay the clock (e.g., [25]). Although some data indicate a physical interaction between CRY and PER, most observations indicate that physical contact is predominantly between CRY and TIM; for example, PER usually requires the presence of TIM to interact with CRY, but a TIM:CRY interaction can take place without PER (e.g., [20]). Because much of TIM is in complex with PER, especially in the early night [22], a CRY-TIM interaction is effectively a CRY:PER-TIM interaction. All of this begins with CRY photon capture, which activates CRY by causing a conformational change and a subsequent interaction with PER-TIM. Indeed, experimental studies on *Drosophila* CRY as well on other related proteins provide a coherent view of a CRY-centric light-initiation event [10].

Although a connection between light pulse– and heat pulse–initiated interactions appeared enigmatic, previous studies in wild-type flies suggested that heat phase shifts are like light pulses and are due to posttranscriptional events that influence TIM and/or PER [36]. The failure to elicit a phase shift with a 34 °C pulse (Figure 1) indicates that a heat shock may be required [43]. This is accompanied by numerous changes in cell physiology and gene expression, which could perturb the dynamics of an oscillatory system [44]. However, \( \text{per}^l \) flies show robust phase shifts and CRY:PER-TIM complex formation after a 30 °C heat pulse, making it unlikely that a heat-shock response is generally required for heat pulse–mediated phase shifts in *Drosophila*. Extrapolation to wild-type flies makes two assumptions: (i) \( \text{per}^l \) flies do not have an unprecedented heat-shock response triggered at much lower temperatures and (ii) the failure to observe 30 °C behavioral phase shifts and biochemical interactions in wild-type reflects quantitative rather than a qualitative differences between 30 °C and 37 °C and between wild-type and \( \text{per}^l \) genotypes. Indeed, the convergence of light and temperature on wild-type fly behavior at physiological temperatures (Figure 6B) suggests that these CRY:PER-TIM interactions are normally difficult to detect at lower temperatures, because they are quantitatively minor.

The \( \text{per}^l \) behavioral and biochemical results indicate that the missense mutation causes a large increase in the fraction of PERL-TIM interacting with CRY at normal temperatures (Figure 4B). This suggests that the PERL-TIM structure is temperature sensitive (Figure 6A), an interpretation consistent with the period of the \( \text{per}^l \); \( \text{cry}^b \) double mutant strain being somewhat temperature sensitive (Figure 5C; see below).
A 1

PER^L TIM

(temp; mutation)

which then promotes these steps

2

PER^L TIM

3

PER^L TIM

CRY^+ CRY

B

10 Lux

100 Lux

Actogram

Autocorrelation

15C (LL)

15C (LL)

25C (LL)

25C (LL)

time (h)

Lag (h)

time (h)

Lag (h)
Moreover, this strain has a substantially longer period than the per<sup>−/−</sup> single mutant strain at 15 °C (Figure 5C), suggesting that PERL-TIM manifests an enhanced interaction with CRY at all physiologically relevant temperatures. These experiments cannot definitively rule out CRY as the temperature-sensitive component; in this case, the per<sup>−/−</sup> mutation would only cause an increased interaction between PER-TIM and CRY. In either case, the close correspondence between the 37 °C per<sup>−/−</sup> heat and light PRGs (Figure 4A) indicates that the CRY photocycle is inessential for CRY-PER-TIM interactions and behavioral phase shifts in Drosophila. We speculate that heat activation of PER-TIM causes the same CRY conformational change as does light—albeit indirectly (Figure 6A).

The heat-induced interactions between PERL-TIM and CRY as well as the per<sup>−/−</sup>; cry<sup>−/−</sup> phenotype make a strong link between the circadian response to temperature pulses and incubations at constant temperatures, analogous to non-parametric and parametric light entrainment, respectively. This is because a persistent CRY:PERL-TIM interaction affects the per<sup>−/−</sup> period like the enhanced phase-shift response of per<sup>−/−</sup> to a heat pulse. This recalls the hypersensitivity of per<sup>−/−</sup> to incubation at constant low light intensities, which lengthen the per<sup>−/−</sup> period more severely and at lower intensities than is required to lengthen wild-type periods [41]. Our results explain this observation and suggest that the more CRY-interactive PERL-TIM requires less CRY light activation than does wild-type PER-TIM. Moreover, the similarities between light and heat inspired the experiment suggesting that light and temperature function together, even on wild-type flies (Figure 6B). This synergy might fine-tune the dawn and dusk response of the clock and even contribute to seasonal adaptation of clock function [45].

The circadian problem of temperature compensation has gained little traction since the discovery more than 15 y ago that the per<sup>−/−</sup> allele suppresses the temperature compensation defect of per<sup>−/−</sup> by failing to interact with CRY [42]. The observations suggest that the same PERL-TIM structure that facilitates a CRY interaction in response to a phase-shifting perturbation (heat- or light-mediated CRY activation) keeps time in a temperature-sensitive manner under constant conditions. Characterization of this altered PERL-TIM structure is an important goal for the near future.

Materials and Methods

Drosophila genetics. Wild-type CS, per<sup>−/−</sup>; and cry<sup>−/−</sup> flies were used for average activity and phase response analyses (see below) and as controls for the locomotor activity analyses. The per<sup>−/−</sup> mutation was combined with cry<sup>−/−</sup> to generate per<sup>−/−</sup>; cry<sup>−/−</sup> flies. The pdf-GAL4 and UAS-cry transgenic flies have been described previously [15]. The y w; tim-GAL4 UAS-myc/cryCyO line (TMC) was previously described [20]. The TMC transgenes were introduced into per<sup>−/−</sup> to obtain per<sup>−/−</sup>; tim-GAL4 UAS-myc/cry (abbreviated as per<sup>−/−</sup>; TMC). The UAS-cry and pdf-GAL4 transgenes were introduced in cry<sup>−/−</sup> backgrounds to produce y w; pdf-GAL4/UAS-cry; cry<sup>−/−</sup> flies.

Phase shift protocol and behavioral analysis. In all experiments unless stated otherwise, CS males were collected at 1–3 d old and reared in LD 12:12 at 25 °C for 3 d. In the APRC protocol, flies were given a 10-min saturating white light pulse (2000 lux) during the third dark phase of the cycle, at the indicated times during the night and the following subjective day. A separate control group of flies was not given a pulse. Flies were then put into constant darkness for another 3 d. For the heat pulse PRGs, flies were placed in activity monitors in LD 12:12 at 25 °C for 3 d. During the third dark phase of the cycle, one monitor of untreated flies was retained as a control. For the light treatment, behavior tubes containing flies were removed from the monitors, held upright, and an elastic band placed around them to hold them tightly together. The entire package was then placed in a 50-ml conical tube, so that the tubes would stay upright in a water-tight environment but small enough for efficient heat transfer from the water bath to the tubes. The top of the activity tubes were always an inch below the top of the 50-ml conical tube, so the water level would be above the tubes. Incubation was in the water bath for 30 min at 37 °C. The 50-ml tube was then removed, and the behavior tubes placed back in the monitors. Each tube had been marked on the top with a number and then placed back in the same monitor channel. A second control set of flies was handled identically except that they were just kept upright (with the elastic band) in 50-ml tubes in the incubator but not placed in a water bath. In all cases, the experiments were repeated at least twice with essentially identical results. For each genotype an average phase shift from 15–32 flies is shown. Locomotor activities of individual flies were monitored using Trikinetics Drosophila activity monitors (TriKinetics Inc, Waltham, Massachusetts, United States). The analysis was done with a signal processing toolbox implemented in MATLAB (Mathworks; http://www.mathworks.com) as described [46].

Autocorrelation is a measure of how well a signal matches a time-shifted version of itself as a function of the amount of time shift. In our analysis, autocorrelation and spectral analysis were used to assess rhythmicity and to estimate period. The phase information was obtained with circular statistics [46]. The column in Figure 5A labeled autocorrelation shows correlograms for the data. Correlation coefficients are plotted on the ordinate with a range of values from 0–1. The gray region centered around 0 describes a 95% confidence interval. The lag of the autocorrelation function is plotted on the abscissa. An asterisk is placed above the third peak of the autocorrelation function. The value at that point defines the rhythmicity index (RI), an estimate of the strength of rhythmicity. When the asterisk is not present, the autocorrelation function indicates a lack of rhythmicity. Values for the RI appear in the lower left corner of these plots along with a related number called the rhythmicity statistic (RS). The RS value is the ratio of the RI to the absolute value of the confidence line. This metric indicates that the rhythmicity described by the correlogram is statistically significant when the value is ≥ 1 [46].

The MESA analysis is a spectral analysis of the data that provides an estimate of period. Spectral density is given in arbitrary units on the ordinate, and mesa is used to provide an estimate of the period. The phase information was obtained with circular statistics [46]. The column in Figure 5A labeled autocorrelation shows correlograms for the data. Correlation coefficients are plotted on the ordinate with a range of values from 0–1. The gray region centered around 0 describes a 95% confidence interval. The lag of the autocorrelation function is plotted on the abscissa. An asterisk is placed above the third peak of the autocorrelation function. The value at that point defines the rhythmicity index (RI), an estimate of the strength of rhythmicity. When the asterisk is not present, the autocorrelation function indicates a lack of rhythmicity. Values for the RI appear in the lower left corner of these plots along with a related number called the rhythmicity statistic (RS). The RS value is the ratio of the RI to the absolute value of the confidence line. This metric indicates that the rhythmicity described by the correlogram is statistically significant when the value is ≥ 1 [46].

The MESA analysis is a spectral analysis of the data that provides an estimate of period. Spectral density is given in arbitrary units on the ordinate, and mesa is used to provide an estimate of the period. The phase information was obtained with circular statistics [46]. The column in Figure 5A labeled autocorrelation shows correlograms for the data. Correlation coefficients are plotted on the ordinate with a range of values from 0–1. The gray region centered around 0 describes a 95% confidence interval. The lag of the autocorrelation function is plotted on the abscissa. An asterisk is placed above the third peak of the autocorrelation function. The value at that point defines the rhythmicity index (RI), an estimate of the strength of rhythmicity. When the asterisk is not present, the autocorrelation function indicates a lack of rhythmicity. Values for the RI appear in the lower left corner of these plots along with a related number called the rhythmicity statistic (RS). The RS value is the ratio of the RI to the absolute value of the confidence line. This metric indicates that the rhythmicity described by the correlogram is statistically significant when the value is ≥ 1 [46].
mM KCl, 1mM Dithiothreitol, 5% glycerol, 0.05% Nonidet P40, 1X Complete Protease Inhibitor [Roche; http://www.roche.com]. Protein G sepharose fast flow beads (Amersham; http://www.amersham.com) were coated with anti-MYC antibody (2 μl; Santa Cruz Biotechnologies; clone 9E10; http://www.scbt.com) plus 20 μl beads/sample for 1 h. The beads were then washed twice and incubated with the head extracts at 4 °C. Pulled-down samples were washed four times with 750 μl extraction buffer before being resuspended in 40 μl SDS loading buffer for Western blot analysis. Head homogenization, incubation, and immunoprecipitation for the light-pulsed samples were done under normal laboratory lighting, whereas the nonpulsed and heat-pulsed samples were processed under red light (700 nm) and incubated in the dark.

**Protein extracts and Western blots.** Fly heads extracts were prepared and Western blots were performed as described [22]. Equal loading for equal quality of protein transfer were first verified by Poncet Red staining and then by the intensity of cross-reacting bands on the Western blots, or by reprobing the membrane with a monoclonal α-tubulin antibody (clone DM1A, Sigma, 1:1000 dilution; http://www.sigmaaldrich.com). The anti-CRY rabbit antibody was used at 1:500 dilution [47]. The anti-PER antibody is previously described and used at 1:1000 dilution whereas the anti-TIM antibody was made in rat and used at 1:3000 dilution [22].

**Supporting Information**

Figure S1. 37 °C Heat Pulses Result in Robust Phase Shifts of Wild-Type and per<sup>l</sup> Flies but Not of cry<sup>l</sup> or per<sup>l</sup>; cry<sup>l</sup> Flies

(A) Circular analysis figures of locomotor behavior in wild-type CS and cry<sup>l</sup> flies after a 37 °C heat pulse (HP). (B) The same circular analyses for per<sup>l</sup> and per<sup>l</sup>; cry<sup>l</sup> after a 37 °C HP. On these plots, time moves forward in a counter-clockwise direction. The behavioral phase estimates for each rhythmic specimen are plotted just outside the unit circle and a mean vector summarizes the phase of the group. The direction of the vector indicates the behavioral phase, whereas its length reflects the dispersion (variability) of the individual estimates (see [46] for more details). The Rayleigh’s test was used to determine whether each vector is significantly different (p < 0.05) from the null vector (random distribution). Then, the Watson-Williams-Stevens test was used to obtain an F-statistic that determined whether the two vectors obtained from the nonpulsed control and the experimental group of flies are significantly different, (p < 0.01) Statistically significant differences were found for CS flies at ZT12 and ZT15 and for per<sup>l</sup> at ZT18 and CT2. cry<sup>l</sup> and per<sup>l</sup>; cry<sup>l</sup> flies did not significantly shift their phase at any time points. For the estimates of the phase differences see Figure 1.

**References**