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A recessive mutant of *Drosophila Clock* reveals a role in circadian rhythm amplitude

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The transcription factor *Clock* (*Clk*) plays a critical role in animal circadian rhythms. Genetic studies defining its function have relied on two dominant negative alleles, one in *Drosophila* and one in mice. Here we describe a novel recessive allele of *Drosophila Clock*, Clk^{ar}. Homozygous Clk^{ar} flies are viable and behaviorally arrhythmic. The Clk^{ar} phenotype is caused by a splice site mutation that severely disrupts splicing and reduces *Clk* activity. Despite the behavioral arrhythmicity, molecular oscillations are still detectable in Clk^{ar} flies. Transcription analysis indicates potent effects of Clk^{ar} on levels and amplitude of transcriptional oscillations. Taken together with other data, we propose that *Clk* makes a major contribution to the strength and amplitude of circadian rhythms.

Keywords: amplitude/circadian rhythms/*Clock*/mutant allele/transcription

Introduction

The persistence of daily rhythmic behaviors without external temporal cues is a manifestation of circadian clocks. Although only approximating 24 h periodicity under constant conditions, these clocks achieve precise 24 h cycles by synchronizing to solar light cycles. Circadian pacemakers facilitate anticipation of these and other environmental events, and rhythmicity present throughout the animal and plant kingdoms punctuates its value to species fitness.

The investigation of fruit fly (*Drosophila melanogaster*) clocks has revealed many evolutionarily conserved pacemaker components (Allada *et al*., 2001). Studies on the biochemistry of the timekeeping mechanism have focused on transcriptional regulation. It is believed that the basic helix–loop–helix (bHLH) transcription factors CLOCK (CLK) and CYCLE (CYC) directly bind to upstream E boxes (CACGTG) and activate transcription of the *period* (*per*) and *timeless* (*tim*) genes (Hao *et al*., 1997; Allada *et al*., 1998; Darlington *et al*., 1998; Rutila *et al*., 1998; Wang *et al*., 2001). This view is based on strong biochemical evidence and on the phenotype of one semi-dominant allele of *Clk*. PER and TIM proteins subsequently feed back and inhibit transcriptional activation by CLOCK and CYCLE (Darlington *et al*., 1998; Lee *et al*., 1998, 1999). A similar feedback loop exists in mammals, including humans. Of note, circadian transcription studies *in vivo* have relied heavily on two dominant negative (antimorphic) alleles of *Clock*, one in *Drosophila* (Clk^{Jrk}) and one in mouse (King *et al*., 1997a,b; Allada *et al*., 1998).

Further studies have implicated a second feedback loop in circadian timing. Like *per* and *tim*, *Clk* and *cry* RNA levels also oscillate with respect to time of day (Bae *et al*., 1998; Darlington *et al*., 1998; Emery *et al*., 1998). However, these oscillations are antiphase to those of *per* and *tim*, suggesting that they are indirect targets of the *Clk–cyc* system. This is consistent with the levels of the *Clk* and *cry* RNAs in Clk^{Jrk} and cyc^{0} mutants; they are high, whereas the levels of *per* and *tim* RNAs are low (Emery *et al*., 1998; Glossop *et al*., 1999). It has been proposed that these genes, *per* and *tim* on the one hand and *Clk* on the other, define two interdependent transcriptional feedback loops. Two *Clk*-dependent transcription factors VRILLE and PDP1 are thought to mediate rhythmic *Clk* transcription (Cyran *et al*., 2003; Glossop *et al*., 2003). Transcriptional oscillations are thought to emerge from the dynamic interplay of these feedback loops, leading to behavioral and physiological rhythms.

Several aspects of circadian gene expression are also subject to post-transcriptional control, including RNA and protein stability, as well as protein phosphorylation (Dembinska *et al*., 1997; So and Rosbash, 1997; Kim *et al*., 2002). Protein levels and phosphorylation states of PER and TIM oscillate with time of day (reviewed in Allada *et al*., 2001). *Doubletime* (*Dbt*), a casein kinase I epsilon homolog; *shaggy*, a glycogen synthase kinase-3 homolog; and *casein kinase 2*(*CK2*), appear to phosphorylate PER and TIM (Kloss *et al*., 1998, 2001; Price *et al*., 1998; Martinek *et al*., 2001; Lin *et al*., 2002). These additional layers of feedback make it difficult to untangle the roles of different mechanisms in determining rhythm period, phase and amplitude.

Here we describe a second mutant allele of *Drosophila Clk*, Clk^{ar}, that is fully viable and recessive. Although behaviorally arrhythmic, molecular oscillations persist with reduced levels and amplitude, consistent with the reported role of *Clk* in transcriptional regulation. However, the phase of these oscillations is not substantially altered. These data are consistent with previous reports of Clk^{Jrk} heterozygotes, which exhibit modest changes in period in combination with reductions in rhythmicity as well as a reduced amplitude of gene expression (Allada *et al*., 1998). We propose that PER/TIM and its associated kinases primarily regulate period

Table I. Circadian locomotor rhythms in *Clk* mutants

Genotype	%R[a]	Period[b]	*n*[c]
+/+	97	23.98 ± 0.05	34
Clk^{ar}/+	94	24.19 ± 0.06	51
Clk^{ar}/Clk^{ar}	1[d]	AR[e]	161
D1/+	65	25.62 ± 0.11	23
Clk^{ar}/*D1*	0	AR	26
Clk^{Jrk}/+	75	24.67 ± 0.13	16
Clk^{ar}/Clk^{Jrk}	0	AR	80
crygal4-Clk^{ar}/*UASClock-*Clk^{ar}	60	22.58 ± 0.07	25
Clk^{Jrk}/Clk^{Jrk}	0	AR	23
crygal4-Clk^{Jrk}*UASClock-*Clk^{Jrk}	18	22.33 ± 0.13	37

[a]% rhythmic.
[b]± indicates SEM.
[c]Number of flies analyzed.
[d]Single weakly rhythmic fly.
[e]AR = arrhythmic.

and phase, whereas CLK/CYC primarily regulates rhythmic amplitude.

Results

Identification of a novel recessive Clock allele

As part of a search for novel genes involved in circadian rhythmicity, we screened ethyl methane sulfonate (EMS) mutagenized per^L flies for alterations in circadian locomotor activity (Rutila *et al*., 1996). One line homozygous for a mutagenized third chromosome, first called *1(7)*, was arrhythmic. Although initially described in a per^L background, *1(7)* is similarly arrhythmic in a wild-type background (Table I; see Clk^{ar}). This phenotype maps to the third chromosome and is recessive, i.e., homozygotes do not exhibit robust rhythms, whereas heterozygotes are virtually indistinguishable from wild type (Table I; see Clk^{ar}).

Meiotic recombination mapping placed the *1(7)* phenotype near the circadian rhythm gene *Clock* (*Clk*; data not shown). We were able to separate a female recessive sterile found in the original *1(7)* stock from the circadian rhythm phenotype by meiotic recombination. However, we were unable to identify wild-type recombinants between *1(7)* and Clk^{Jrk} (0/112 recombinants). To determine if *1(7)* is an allele of *Clk*, we performed complementation analysis with a deletion (*D1*), that removes the *Clk* locus and Clk^{Jrk}. Neither *D1* nor Clk^{Jrk} complemented the arrhythmicity of *1(7)*, consistent with the notion that *1(7)* is an allele of *Clk*. As a result, we renamed *1(7)* Clk^{ar} (Table I). The observation that the heterozygous *D1* period phenotype (~25.5 h) is more severe than that of heterozygous Clk^{ar} further suggests that Clk^{ar} is a hypomorphic allele, although alternative interpretations must also be considered (see Discussion).

Rescue of Clk^{ar} using GAL4/UAS

To confirm this assignment we rescued the arrhythmic phenotype of Clk^{ar} using the GAL4/UAS system. We generated a single transgenic *Clk* line driven by the upstream activating sequence (*UAS*) for GAL4 (*UASClk*) and combined it with various rhythm-relevant *gal4* drivers. Overexpression of *Clk* driven by *period promoter*-GAL4 (*pergal4*) or *timeless promoter*-GAL4 (*timgal4*) resulted in early larval lethality (Emery *et al*., 1998; Kaneko and Hall, 2000). Heterozygous lines expressing *Clk* driven by *pigment dispersing factor promoter*-GAL4 (*pdfgal4*) or *cryptochrome* promoter-GAL4 (*crygal4*) were adult viable and therefore were assessed for behavioral rescue of Clk^{ar} (Emery *et al*., 2000; Park *et al*., 2000). *pdfgal4* is expressed in the central brain in pacemaker neurons (small and large ventral lateral neurons; *sLNv*, *lLNv*), whereas *crygal4* is expressed in a slightly broader distribution, extending to a dorsal group of lateral neurons (*LNd*; Emery *et al*., 2000; Park *et al*., 2000).

Expression of *Clk* by *pdfgal4* in a Clk^{ar} background did not result in significant rescue of rhythmicity (data not shown). On the other hand, *crygal4*-driven expression of *Clk* resulted in rescue in the rhythmicity of a majority of these flies (Table I). The rescued flies exhibited a slightly short period, similar to periods in flies with *crygal4*-driven expression of *Clk* in a wild-type background (data not shown). The period shortening with increased *Clk* expression is consistent with the long periods of flies with only a single dose of *Clk* (Table I; see *D1*/+). BAC transgenic mice containing extra copies of *Clock* also exhibit short periods (Antoch *et al*., 1997). We obtained similar results in a Clk^{Jrk} background: *crygal4*-driven *Clk* expression was able to rescue the rhythmicity of Clk^{Jrk} (18% rhythmic), although more weakly than Clk^{ar} (60% rhythmic), consistent with the antimorphic effects of Clk^{Jrk}.

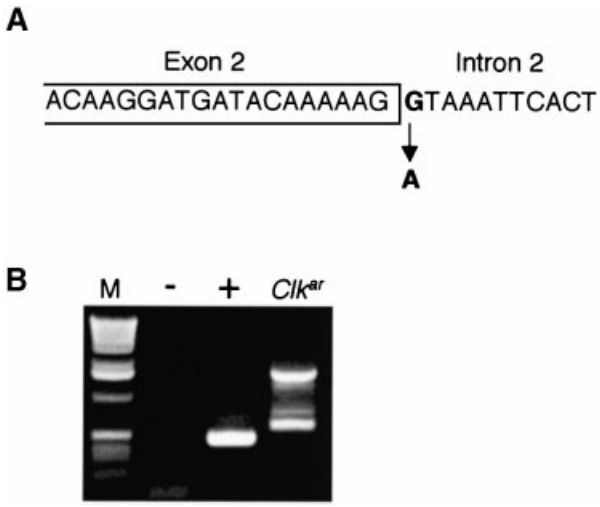

Fig. 1. Alterations in *Clk* DNA and RNA in Clk^{ar} flies. (**A**) Sequence change present at the 5′ splice site of intron 2. (**B**) Altered splicing of intron 2 in Clk^{ar} as shown by RT–PCR across intron 2. – indicates negative control (no RNA). + indicates single band in wild type corresponding to RNA without intron 2 (spliced).

Altered Clock sequence, transcripts and function in Clk^{ar}

Given the strong evidence that Clk^{ar} is an allele of *Clk*, we searched coding exons and exon–intron boundaries for EMS-induced base changes, comparing Clk^{ar} with sibs. We identified a single mutation at the 5′ splice site of the second intron, destroying the GT dinucleotide required for efficient splicing (Figure 1A). The mutation is a G to A transition classically found in EMS-induced alleles (Bentley *et al*., 2000). We examined *Clk* spliceforms across the second intron in the Clk^{ar} mutant using reverse

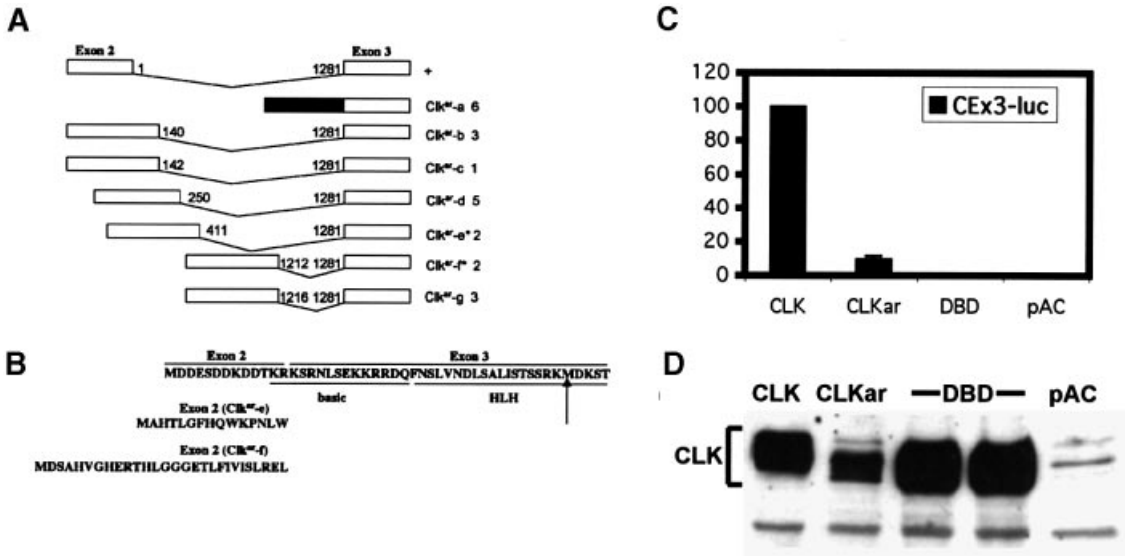

Fig. 2. Analysis of splice junctions in *Clk*^{ar} transcripts. (**A**) Schematic of splicing of exons 2 and 3 flanking intron 2. Black box indicates the presence of intron and absence of any detectable splicing of intron 2. Altered transcripts present in *Clk*^{ar} alphabetically are indicated as well as the number of clones of that type identified out of 22 clones analyzed. Nucleotide numbers of intron (1–1281 in wild type) indicate sites of splice junctions. Asterisks indicate clones with potential in-frame methionines from the altered upstream exon 2. (**B**) Conceptual translation of altered transcripts. Top line indicates translation of wild-type transcript from first methionine located in exon 2. Basic DBD (basic) and helix–loop–helix DNA binding and dimerization motif is indicated (HLH) in the translated product. Potential new N-termini from altered exon 2 in Clk^{ar}-e and -f are indicated from *Clk*^{ar} transcripts. (**C**) Activation of a triplicated *per* circadian enhancer by CLK^{ar} in S2 cells. CLK, CLOCK; DBD, CLOCK without a DBD; pAC, empty pActin vector; CEx3-luc is a triplicated circadian enhancer derived from the *per* promoter; *y*-axis indicates luminescence relative to CLK where CLK = 100%. Measurements displayed are averages of two independent experiments. Error bars indicate SEM. Some error bars were not visible due to low variability. (**D**) Expression of CLOCK^{ar} in transfected cells.

transcriptase–polymerase chain reaction (RT–PCR). RT–PCR across this intron identified a single band of the appropriate size in wild-type flies (Figure 1B). In *Clk*^{ar}, multiple bands are observed, none of which correspond by electrophoretic migration to that seen in wild type, consistent with the observed splice site mutation. Splice junctions between other coding exons were not grossly perturbed as assayed by RT–PCR (data not shown). Exon 2 encodes for the N-terminal 13 amino acids, including the first two amino acids of the basic region (Figure 2B; KR). The exons beyond exon 2 encode the remainder of the CLK protein, including most of the basic region, the PAS dimerization motif and the glutamine-rich activation domain (Allada *et al*., 1998). To determine if these altered *Clk*^{ar} transcripts can produce functional CLK protein, we sequenced *Clk*^{ar} cDNAs. To produce essentially full-length CLK, altered transcripts must splice properly into exon 3. We therefore sequenced *Clk*^{ar} cDNAs to determine if there were any intact open reading frames upstream of exon 3. In 18/22 cases, there is a stop codon in-frame and upstream of the remaining *Clk* gene in the *Clk*^{ar} cDNA (Figure 2A). Assuming initiation from the first downstream methionine within exon 3, translation would result in a CLK protein lacking the basic DNA binding domain (DBD) and a portion of the helix–loop–helix dimerization motif. In 4/22 clones, an upstream methionine codon is in-frame with the remainder of the *Clk* gene (Figure 2A and B; see Clk^{ar}-e and f). Assuming initiation from this methionine, translation of these transcripts would result in a CLK protein with novel N-termini: two of 15 amino acids and two of 28 amino acids (Figure 2B). In all four cases, only the first two amino acids of the basic DBD are altered.

To determine the functional consequence of the splice site mutation, the *Clk*^{ar} gene, including the mutant second intron, was subcloned into an S2 tissue culture expression vector under the control of the *Actin* promoter (pAc). Consistent with this mutation, transfection of the *Clk*^{ar} clone resulted in a substantial reduction in transcriptional activation relative to wild-type *Clk*. Nonetheless, this *Clk*^{ar} clone did activate transcription well above that achieved with pAc alone (Figure 2C). We observed comparable levels of activation by *Clk*^{ar} with a full-length *per* promoter construct (*BG-luc*) and an enhancer derived from the *tim* promoter (*timenh-LUC*; see below; data not shown). Given that a large fraction of *Clk*^{ar} transcripts should produce *Clk* without its DBD, we sought to determine if this form can activate transcription. However, cloning of this particular isoform into the pAc vector gave rise to little activation relative to *Clk*^{ar} (Figure 2C). Because differences in activation may derive from differences in protein levels, we compared expression of each of these forms by western blot analysis (Figure 2D). The wild-type and DBD forms of CLK are comparably expressed, whereas the CLK^{ar} clone has reduced protein levels. Inefficient splicing to the AG of exon 3 might hinder expression of CLK^{ar}, which probably contributes to the reduced activity of CLK^{ar} relative to wild-type CLK (~10%; Figure 2C). Nonetheless, CLK^{ar} has low but significant activity, and the data suggest that this activity derives from a minor, essentially full-length form of the protein, such as those from *Clk*^{ar}-*e* and *f* or perhaps other unidentified forms (Figure 2B). We conclude that *Clk*^{ar} is a hypomorphic allele with residual activity.

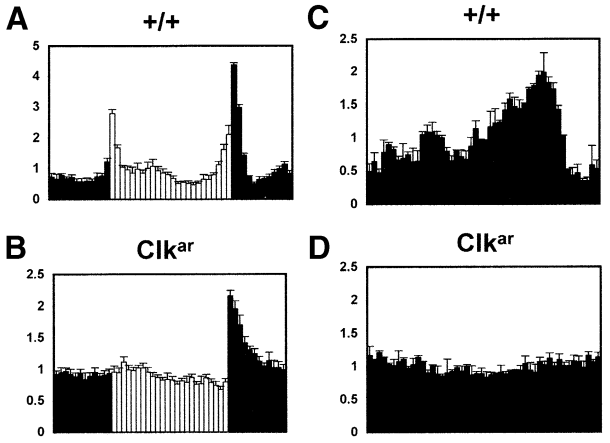

Fig. 3. Abnormal diurnal and circadian locomotor behavior in *Clk*^{ar} flies. Normalized plots of locomotor activity in LD (**A** and **B**) and first two days of constant darkness (**C** and **D**; see Materials and methods) for wild type (+; A and C; $n = 16$) and *Clk*^{ar} flies (B and D; $n = 16$). Light and dark bars correspond to times of lights-on and -off. Error bars indicate SEM.

No evident behavioral rhythmicity in Clk^{ar}

Consistent with the hypomorph interpretation was a more detailed analysis of the homozygous *Clk*^{ar} behavioral rhythms (Figure 3). Under light: dark conditions (Figure 3A and B), these mutant flies manifested a highly abnormal activity pattern: no lights-on startle response but a robust lights-off startle response. This feature was indistinguishable from that of *Clk*^{Jrk} flies and substantiates the connection between *Clk* and responses to light–dark (LD) transitions (Allada *et al*., 1998). In contrast, wild-type flies demonstrate anticipation of lights-on and lights-off peaks, evidence of an underlying endogenous clock, as well as 'startle' responses to both lights-on and lights-off events. There was also no measurable rhythmic activity of *Clk*^{ar} in constant darkness (DD), even during the first two days of DD (Figure 3C and D). This contrasts markedly with the rhythmic activity of *pdf*^{01} flies during the first two days of DD (Renn *et al*., 1999). In sum, the homozygous *Clk*^{ar} behavioral data showed no evidence of rhythmicity and were essentially identical to those of homozygous *Clk*^{Jrk} flies, consistent with a strong hypomorphic etiology.

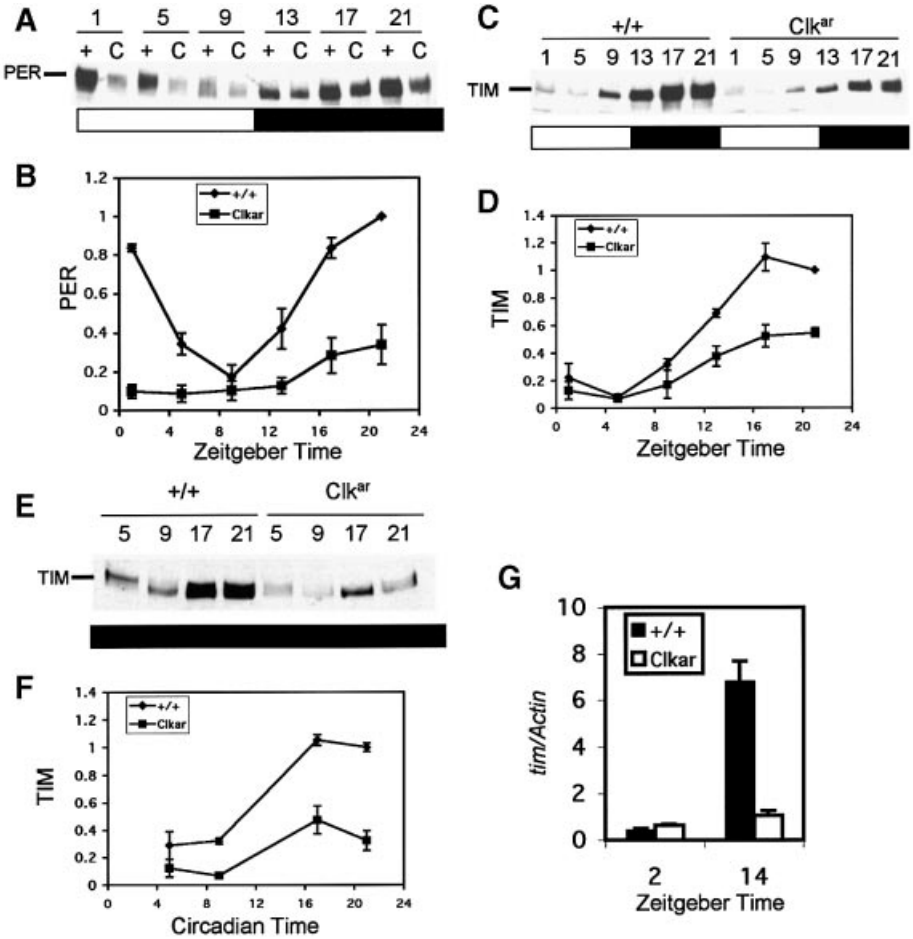

Fig. 4. Molecular oscillations in *Clk*^{ar}. Western blots of PERIOD (PER; **A** and **B**) and TIM (**C**–**F**) under 12 h light:12 h dark (A–D) and constant darkness (E and F) conditions. Zeitgeber Time (ZT; B and D) 0 is lights-on and 12 is lights-off. Circadian time (F) indicates time in constant darkness where Circadian Time 0 is 12 h after lights-off. PER quantification (B) is from four independent experiments (three for ZT 1 and 13). TIM quantification in LD (D) is from three independent experiments (two for ZT 1 and 13) and in DD (F) from two independent experiments. Error bars indicate SEM tim transcript oscillations as measured by real-time quantitative PCR (see Materials and methods). (**G**) Levels of *tim* transcript measured with respect to *Actin* control (*tim/Actin*).

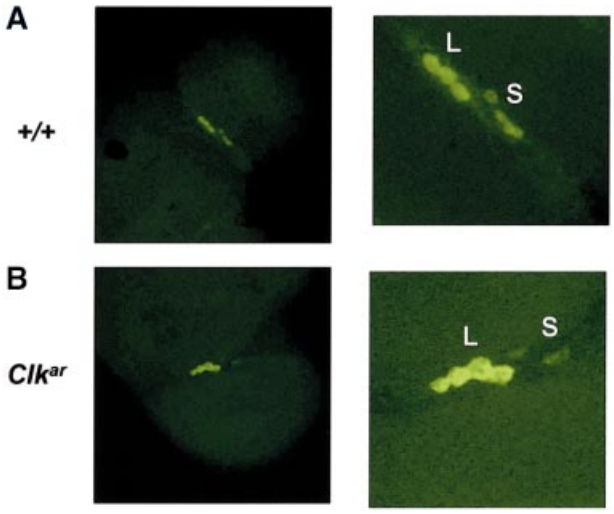

Fig. 5. *pdf* expression in Clk^{ar}. Fluorescent *in situ* hybridization for *pdf* transcript in wild type (**A**) and Clk^{ar} (**B**) reveals labeling of large (L) and small (S) ventral lateral neurons (LNs) under low (left panel) and high (right panel) magnifications. These two groups of neurons can be detected in both wild type and Clk^{ar}, although levels are reduced in the small neurons in Clk^{ar}. High magnification image of Clk^{ar} is enhanced to ease visualization of the small LNs.

Persistent molecular oscillations in Clk^{ar}

To determine the effect of Clk^{ar} on molecular oscillations, we examined *timeless* and *period* protein by western blotting under LD conditions (Figure 4). In Clk^{ar}, PER and TIM cycle robustly under LD conditions, despite a decrease in protein levels relative to wild-type flies (Figure 4A–D). As light has previously been shown to suppress TIM levels and contribute to protein oscillations, we also assayed TIM under DD conditions (first full day of DD). To our surprise, TIM cycling persisted (Figure 4E and F), indicating the presence of a circadian clock not evident at the behavioral level (Figure 3D). We made similar observations of reduced but oscillating PER levels in DD in the Clk^{ar} mutant (data not shown). The reduction in peak levels of PER and TIM was consistent with a reduction in *Clk* transcriptional activation. As confirmation, we measured *tim* transcript levels using real-time quantitative PCR. Consistent with our western results, we found a strong reduction in peak levels of *tim* (Figure 4G; Zeitgeber time 14).

The absence of behavioral rhythms in the context of intact molecular rhythms has been previously observed in mutants that lack PDF-expressing lateral neurons (Hardin *et al*., 1992). We therefore performed fluorescent *in situ* hybridization for *pdf* transcripts to assess the presence of large and small pacemaker lateral neurons. Consistent with previous reports in Clk^{Jrk} mutants, we found reductions in *pdf* expression specifically in the small ventral lateral neurons (Figure 5). Nonetheless, we were able to visualize both large and small groups of *pdf* neurons. Thus, the absence of behavioral rhythms is not due to a complete failure of pacemaker lateral neurons to develop.

Reduction in cycling amplitude and levels of per and tim enhancer activity

To more directly address CLK activity, we examined the cycling of *tim* and *per* enhancers in Clk^{ar} mutants, using luciferase-mediated bioluminescence as a reporter (Brandes *et al*., 1996). The *tim* promoter contains an E-box target site for CLK/CYC that is required for full rescue and high level activity (McDonald *et al*., 2001; Wang *et al*., 2001). We cloned a small fragment (–756 to –604) encompassing this E-box upstream of DNA encoding the firefly luciferase gene (*timenh-LUC*). We observed robust cycling, demonstrating the presence of a cycling enhancer (Figure 6A). Consistent with the notion that the E-box is a functional target of CLK, we found that the bioluminescence levels are dramatically reduced in Clk^{ar} (Figure 6B). However, they are not as low as a version of this enhancer in which the E-box has been mutated (CACGTG to CTCGAG; *timenhmut-LUC*) consistent with the notion that Clk^{ar} is not a null allele (Figure 6C).

Despite the large reduction in expression levels, cycling bioluminescence was still apparent in *timenh-LUC*, Clk^{ar} flies. There was a large reduction in cycling amplitude relative to wild-type flies as measured by peak-to-trough ratios (Figure 6F), consistent with the large effect on cycling amplitude (Figure 5G). However, the phase was not dramatically affected as measured by times of peak and trough luminescence each day (Figure 6G). We also examined the activity of a triplicated circadian enhancer from the *per* promoter (*CEx3-LUC*; Hao *et al*., 1997; So *et al*., 2000). This enhancer contains a functional E-box and confers robust cycling on the luciferase reporter (Figure 6D). When assayed in a Clk^{ar} background, we observed luciferase cycling, also with reduced levels and amplitude (Figure 6E and F). Again, phase is not strongly affected (Figure 6G). These data are similar to those shown above for PER and TIM levels, suggesting that Clk^{ar} rhythms have reduced amplitudes and expression levels but only modest effects on phase.

Discussion

Here we describe the first recessive allele of *Clock*. The only previously reported allele, Clk^{Jrk}, is semi-dominant and behaves as a dominant negative (Allada *et al*., 1998). As a result, it is possible that Clk^{Jrk} may exert its effects through a gain-of-function effect. The strongest genetic evidence for a *Clk* requirement for normal rhythms is the long period of flies heterozygous for a deletion of the *Clk* locus (Allada *et al*., 1998). However, homozygous *D1* flies do not live to adulthood, and behavioral rhythms therefore cannot be assessed in *Clk*-null animals. Even if they were arrhythmic, an essential contribution of *Clk* to rhythms would remain uncertain because the *D1* deletion removes several genes. These most likely include *Pdp1* and *Hn*, which are candidate circadian rhythm genes and are located within 40 kb (right) and 10 kb (left) of *Clk*, respectively (Neckameyer and White, 1992; Florez *et al*., 1996; McDonald and Rosbash, 2001). The *D1* deletion was initially identified by its failure to complement the eye pigment phenotype of *Hn*, which lies just to the left of *Clk* (Grasso, 1996). Furthermore, *D1* fails to complement mutant loci to the right of *Pdp1* and is therefore likely to remove this gene as well (Allada *et al*., 1998). The heterozygous phenotype of the *D1* deletion may therefore be due to the absence of one or more of these genes. A similar ambiguity exists in mammals, as there is only a single semi-dominant allele and no hypomorphic or null

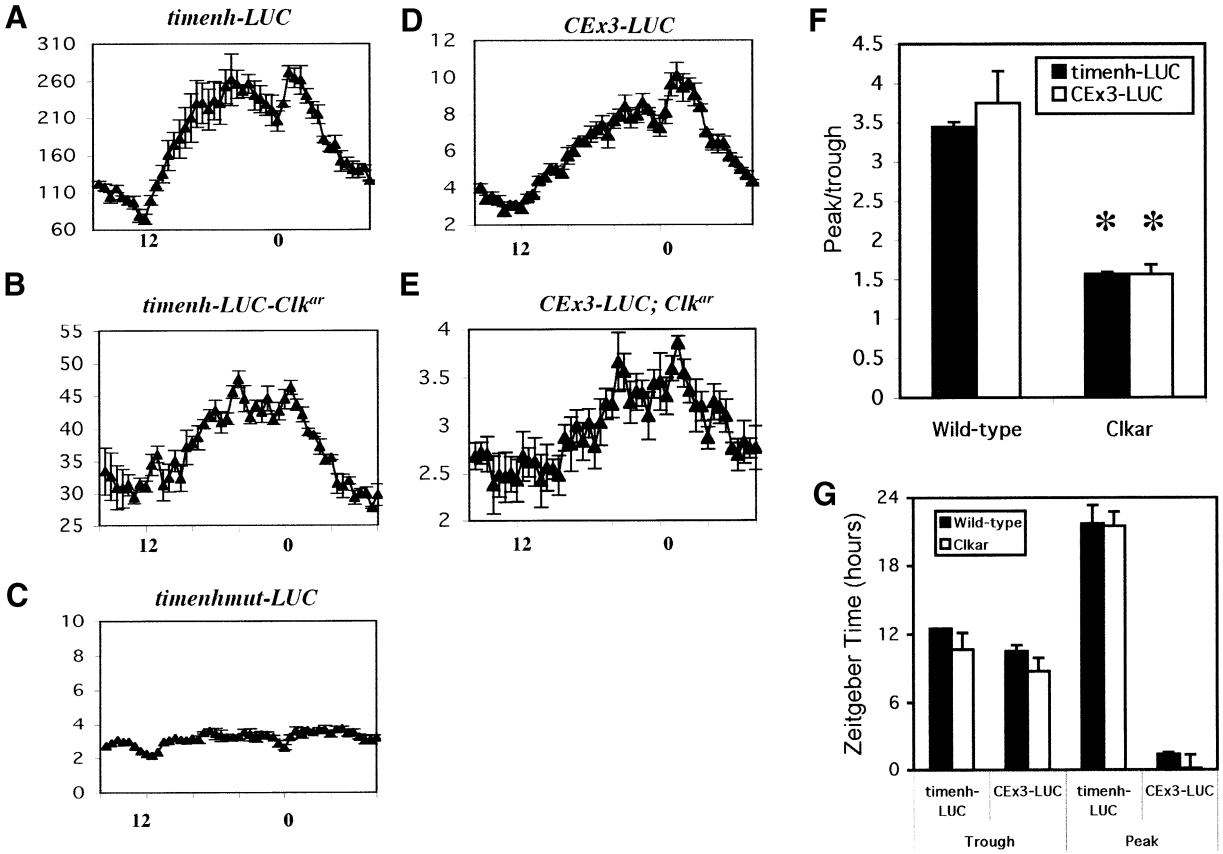

Fig. 6. Reduction in cycling levels and amplitude of *per* and *tim* enhancers in Clk^{ar}. *timenh-LUC* indicates transgenic flies containing a *tim* enhancer fused to luciferase. *timenhmut-LUC* is the same *tim* enhancer with the E-box site (CACGTG) mutated. *CEx3-LUC* indicates a 69 bp circadian enhancer from the *per* promoter triplicated and fused to luciferase. Luciferase activity is expressed in thousands of counts per minute for the *tim* enhancers (*timenh-LUC*, *timenhmut-LUC*) and millions of counts per minute for the *per* enhancer (*CEx3-LUC*). Error bars indicate SEM. Zeitgeber time is indicated on the *x*-axis with ZT0 as time of lights-on and ZT12 as time of lights-off. For *timenh-LUC* (**A**), $n = 13$, for *timenh-LUC-*Clk^{ar} (**B**), $n = 16$, for *timenhmut-LUC* (**C**), $n = 17$, for *CEx3-LUC* (**D**), $n = 7$ and for *CEx3-LUC*; Clk^{ar} (**E**), $n = 5$. (**F**) Amplitude assessment in wild type and Clk^{ar}. Error bars indicate the SEM across days. An asterisk indicates statistically significant result ($P = 0.0001$ for timenh; $P = 0.002$ for CEx3). (**G**) Phase assessment in wild type and Clk^{ar}. Times of average peak and trough values are indicated. SEM over several days indicated. No statistically significant differences detected between wild type and Clk^{ar}.

alleles of the mouse *Clock* locus. All of these considerations make this new recessive arrhythmic *Clk* allele important and indicate that *Clk* is indeed required for behavioral rhythmicity.

Part of the proof that Clk^{ar} is truly a *Clk* mutant comes from the rescue with heterozygous combinations of *crygal4* and *UASClk*. We were, however, unable to effect successful rescue with other rhythm-relevant gal4 drivers: *timgal4* and *pergal4* were lethal in combination with *UASClk*, and *pdfgal4* had no effect on the arrhythmic Clk^{ar} phenotype. The *pdfgal4* driver was also unable to rescue the arrhythmic Clk^{Jrk} background. We also tested the rescuing ability of *pdfgal4* in flies trans-heterozygous for the *Clk* mutants and the *Clk* deletion, *D1*: in these genetic backgrounds, *pdfgal4* also failed to rescue either Clk^{Jrk} or Clk^{ar} (data not shown). The most obvious difference between *crygal4* and *pdfgal4* is a more widespread expression pattern in the case of *crygal4*, which extends to another more dorsal group of neurons, the LNds (Renn *et al*., 1999; Emery *et al*., 2000). Locomotor activity rhythms may therefore require wild-type *Clk* expression in more than just the restricted *pdf* locations.

Another possible difference between the *cry* and *pdf* drivers is RNA cycling: *cry* mRNA cycles with a roughly similar amplitude and phase to *Clk*, whereas the *pdf* gene undergoes no substantial transcriptional fluctuations (Park and Hall, 1998). Although *Clk* and *cry* mRNA cycling may contribute to circadian function, we do not favor this explanation. The stability of GAL4 should prevent significant oscillations in protein levels, even if mRNA levels undergo robust cycling. This suggests that rhythmic transcription of *Clk* is unnecessary for behavioral rhythmicity and is consistent with other experiments indicating that the timing and levels of *Clk* oscillation can be substantially altered without strong effects on circadian behavior (Kim *et al*., 2002). This conclusion is also consistent with the notion that the *Clk* transcriptional loop is less important than the original *per–tim* loop for behavioral rhythms.

We note, however, that GAL4-mediated rescue of *Clk* is only partial, with 60% rhythmicity and shortened periods. This is comparable to the GAL4-mediated rescue of per^0; tim^0 double mutants in which rhythmicity is 40–50% and periods range from 24–27 h (Yang and Sehgal, 2001). The

similarly poor % rhythmicity values may reflect comparable contributions of mRNA cycling, *per* and *tim* in one case and *Clk* in the other. Alternatively, mRNA cycling may be largely irrelevant to penetrance; the poor rescue may result from inappropriate GAL4 levels (too high or too low) or from some other inadequate feature of GAL4-mediated expression.

The molecular assays in Clk^{ar} indicate bona fide rhythms with a predominant effect on circadian rhythm amplitude and no more than a modest effect on phase or period. With circadian *per* and *tim* enhancers, we observed reduced enhancer activity and a reduced cycling amplitude in a Clk^{ar} background, consistent with the role of *Clk* in regulating these enhancers (Figure 6). Nonetheless, the phase of oscillating bioluminescence is similar to that of wild-type flies. The presence of molecular rhythms contrasts with the absence of detectable behavioral rhythms. We favor the notion that this reflects a level or amplitude reduction below a critical threshold for behavioral rhythmicity. The absence of anticipation of LD transitions makes it very unlikely that an effect restricted to the lateral neurons, the absence of the neuropeptide PDF for example, is primarily responsible for the phenotype. This is also because LD behavioral rhythms are largely normal in flies devoid of PDF or the pacemaker lateral neurons (Renn *et al*., 1999). Moreover, we demonstrate both large and small PDF-expressing lateral pacemaker neurons are present in Clk^{ar} (Figure 5). However, we did observe a reduction in *pdf* expression in the small lateral neurons that may contribute to Clk^{ar} arrhythmicity in constant darkness.

Previous results with Clk^{Jrk} also support a role for *Clk* in defining rhythmic amplitude. Clk^{Jrk} heterozygotes reveal a dominant reduction in the amplitude of molecular rhythms with little apparent change in phase (Allada *et al*., 1998). These heterozygotes also exhibit reductions in rhythmic behavior with only slightly long periods. Indeed, *Clk* overexpression results in a selective increase in the amplitude of *per* RNA oscillations (Kim *et al*., 2002). This modest effect on period or phase of varying *Clk* activity is similar to the phenotype of transgenic strains missing the *per* promoter or expressing *per* and *tim* from constitutive promoters (Frisch *et al*., 1994; Yang and Sehgal, 2001). These strains also have reasonable periods (22–26 h) with poor rhythm amplitudes, as evidenced by the poor penetrance of rhythmicity. One argument for a role for *Clk* in period control is the phenotype of the D1 heterozygote (~25.5 h period). However, even this altered period is within the limited range of altering *per* or *tim* transcription. Taken together, these data suggest that substantial changes in *Clock* gene transcription have limited effects on circadian period. Separate control of circadian rhythm amplitude on the one hand and period (or phase) on the other is also consistent with anatomical experiments in both the fly and mammalian system (Liu *et al*., 1991; Low-Zeddies and Takahashi, 2001).

We propose that the post-transcriptional phosphorylation turnover feedback loop involving several Clock components (e.g. *per*, *tim*, the protein kinase *Dbt*) is predominantly responsible for period determination. Excluding null alleles that are either arrhythmic or lethal, Flybase lists mutant alleles of *per*, *tim* and *Dbt* which exhibit period alterations ranging from 16–30 h for *per* (8 mutant alleles), 21–33 h for *tim* (8 mutant alleles) and 18–29 h for *Dbt* (5 mutant alleles; Flybase Consortium, 2002). Indeed, the only *Dbt* allele that fails to exhibit rhythmicity as a homozygote, displays a potent period-altering phenotype as a heterozygote (Rothenfluh *et al*., 2000). More recent additions to this list are the protein kinases *shaggy* (Martinek *et al*., 2001) and *CK2*. Indeed, one mutant allele of *CK2*α, $CK2\alpha^{Tik}$, exhibits one of the strongest dominant period effects of any rhythm mutant (Lin *et al*., 2002). These large period effects contrast with the transcription factor mutants (*Clk* and *cyc*). Their phenotypes indicate that near-normal periods are maintained despite large protein level changes.

Materials and methods

Fly stocks, mutagenesis, mapping and behavioral analysis

per^{L};ry^{506} flies were used for mutagenesis (Rutila *et al*., 1996). *pdfGAL4*, *crypiGAL4*, *timGAL4* have been previously described (Emery *et al*., 2000; Kaneko and Hall, 2000; Park *et al*., 2000). Flies were entrained for 2–5 days of 12 h light: 12 h dark before monitoring of activity in constant darkness for at least 5 days (Hamblen *et al*., 1986). Circadian periods were determined by χ-square periodograms analysis. For high-resolution analysis of diurnal and circadian behavior, data for individual flies is normalized to total activity and then the normalized activity of several flies and days is averaged together to produce single-day activity plots.

Construction of transgenic flies

Clock was first tagged with hemagglutinin (HA) epitope by PCR cloning. Briefly, a C-terminal fragment of *Clock* was PCR-amplifed using pSK(–) *Clock* cDNA and an oligonucleotide with HA epitope and *Xho*I site and an internal oligonucleotide. The amplified fragment was digested with *Cla*I/*Xho*I and ligated to *Cla*I/*Xho*I-digested pSKClock vector to generated pSK(–) ClockHA. ClockHA was subsequently ligated into pUAST (*Eag*I/*Xho*I) to generate pUAS-ClockHA.

tim enhancer luciferase flies were constructed by PCR and subcloning into luciferase vector. A *Not*I/*Eco*RI fragment containing the E-box region of the timeless promoter (position –756 to –604; McDonald *et al*., 2001) and a *Eco*RI/*Sal*I fragment containing the heat-shock minimal promoter (hs43) were obtained by PCR and enzymatic digestion, and cloned in front of the luciferase cDNA in pBluescript (Stanewsky *et al*., 1997). From this intermediate construct, a *Not*I/*Kpn*I fragment was excised and cloned in pCasperR4. The resulting construct is called pCasp-timenh-luc. The pCasp-timenhmut-luc construct is identical to pCasp-timenh-luc, expect that the E-box (CACGTG) is mutated to an *Xho*I site (CTCGAG). These two luciferase constructs were sequenced prior to injection to ensure their integrity. *y w*; *Ki* p^{p} (ry^{+} *delta 2–3)*/+ embryos were injected with DNAs. A single line (*UASClk*) was obtained as a third chromosome insert. HA epitope is not immunologically detectable using western blotting or immunoprecipitation with anti-HA antibodies.

Genomic DNA sequencing and transcript analysis

Genomic DNA was prepared from Clk^{ar} flies and wild-type sibs (Allada *et al*., 1998). *Clk* coding region exons and intron–exon boundaries were amplified by PCR and subjected to DNA sequencing. Total RNA was prepared from adult wild-type and Clk^{ar} heads (Rutila *et al*., 1996). Superscript II RNase H^{-} and primers flanking intron 2 of *Clk* were used to generate cDNA by RT–PCR. PCR fragments were subcloned by TA cloning into pCR (Invitrogen). Individual clones were prepared and subjected to DNA sequencing to analyze intron–exon structure.

Transfection assays

Transfection assays performed as in McDonald *et al*. (2001). PAcClock (250 ng), pAc-Bgal (10–100 ng) and CEx3-LUC (100–500 ng) were used for transfections.

Western analysis

Western analysis was performed using rabbit anti-PER (1:10 000) and rat anti-TIM (1:5–10 000) antibodies (Allada *et al*., 1998). Rabbit anti-CLK antibody was obtained commercially (Alpha Diagnostic, San Antonio, TX). Cells transfected with CLK were pelleted and boiled in sample buffer. Samples were run on SDS–PAGE transferred and membranes were blocked in 5% milk/TBST. Blots were probed at 1 μg/1 ml in 1%

milk/TBST overnight at 4°C and subsequently probed with 1:500 anti-rabbit secondary antibodies for 2 h at room temperature.

Real-time quantitative RT–PCR for tim transcript levels

Total RNA was prepared from adult wild-type and Clk^{ar} heads (Rutila *et al*., 1996). cDNA derived from this RNA was used as template for a quantitative real-time PCR assay performed on the Corbett Research Rotor-Gene 2000 real-time cycler. The PCR mixture contained Platinum Taq polymerase (Life Technologies), optimized concentrations of Sybrgreen and the following primers: *tim* primers, 5′-CCTTTTCGTA-CACAGATGCC-3′ and 5′-GGTCCGTCTGGTGATCCCAG-3′; *actin* primers, 5′-TGCAGCGGATAACTAGAAACTACTC-3′ and 5′-CAA-AGGAGCCTCAGTAAGCAAG-3′. Cycling parameters were 95°C for 3 min, followed by 40 cycles of 95°C for 30 s, 55°C for 45 s and 72°C for 45 s. Fluorescence intensities were plotted against the number of cycles by using an algorithm provided by the manufacturer. *tim* and *actin* mRNA levels were quantitated using a calibration curve based upon dilution of concentrated cDNA from wild-type flies at ZT14. *tim* values/levels were normalized with the *actin* signal. For each condition three (in the case of wild-type ZT14, Clk^{ar} ZT2 and ZT14) or two (for wild-type ZT2) independent RNA samples were analyzed. For each RNA sample, four independent RT–PCR experiments were performed, in each real-time PCR experiment the samples were evaluated in triplicate. The mean of the four measurements for each RNA sample was determined and the *tim/actin* ratio was calculated. The *tim/actin* ratios for each of the three independent experiments (or two in the case of wild type at ZT2) were also averaged to give the data shown in Figure 4G.

pdf in situ mRNA hybridization on adult brain whole mounts

Adult fly brains were dissected in phosphate-buffered saline and fixed in 4% paraformaldehyde for 30 min at room temperature. After pre-hybridization for minimum of 2 h in Hybrix (50% formamide, 5× SSD, 100 µg/ml tRNA, 100 µg/ml ssDNA, 0.1% Tween-20) at 55°C, the brains were incubated with probes overnight at 55°C. The *pdf* probe used corresponds to nucleotides 282–570. Antisense RNA probes were synthesized and labeled using digoxigenin (*pdf*) RNA labeling kit from Boehringer Mannheim. The probes were hydrolyzed in sodium bicarbonate buffer and stored in Hybrix at –20°C until use. The hybridized RNA signals were detected using fluorescent tyramides (NEN LifeScience). Brains were mounted in glycerol with 4% *n*-propyl gallate and examined by confocal microscopy.

Luciferase reporting and analysis

Single fly luciferase activity reporting was performed under 12 h light: 12 h dark conditions (Brandes *et al*., 1996). Data from several flies were averaged together. Due to the decrement in bioluminescence activity observed over several days, we then performed detrending analysis as follows. We fit the data to a linear curve and obtained a slope and intercept. We then calculate the location of the fitted curve at the end of the experiment [y_{end} = slope*number of measurements (n) + y-intercept (y_{int}; luminescence)]. Where the fitted line starts (the y_{int}) and where the line ends (y_{end}) are used to calculate the % increase over the course of the experiment required to correct for the decrement [(y_{int} /y_{end} –1)*100%]. The % correction is then divided by the total number of measurements in the experiment (n). Each value is then corrected by a percentage, depending on the time from the beginning of the experiment. Thus based on a linear change in the % correction, early time points are increased by a small percentage and measurements late in the experiment are increased by a relatively large percentage. As a result, the detrended data would have a slope of approximately zero, i.e., no decrement. The averaged and detrended data from three to four days were then averaged together to produce a single day of data to facilitate phase comparisons. Standard error of the mean (SEM) is that of these 3–4 days averaged.

Assessments of amplitude and amplitude are performed on the data averaged from several flies without detrending. For each day, the peak and trough values are noted and peak/trough ratios are calculated for each day and averaged over 3–4 days of the experiment. This ratio reflects the amplitude of the oscillation. The times at which these peak and trough values are reached are also averaged over 3–4 days of the experiment to get an assessment of the phase of the oscillation. The SEM is that of these days averaged. Statistical comparisons were performed using Student's *t*-test.

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