Dominant-Negative CK2α Induces Potent Effects on Circadian Rhythmicity

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Circadian clocks organize the precise timing of cellular and behavioral events. In Drosophila, circadian clocks consist of negative feedback loops in which the clock component PERIOD (PER) represses its own transcription. PER phosphorylation is a critical step in timing the onset and termination of this feedback. The protein kinase CK2 has been linked to circadian timing, but the importance of this contribution is unclear; it is not certain where and when CK2 acts to regulate circadian rhythms. To determine its temporal and spatial functions, a dominant negative mutant of the catalytic alpha subunit, CK2αTik, was targeted to circadian neurons. Behaviorally, CK2αTik induces severe period lengthening (~33 h), greater than nearly all known circadian mutant alleles, and abolishes detectable free-running behavioral rhythmicity at high levels of expression. CK2αTik, when targeted to a subset of pacemaker neurons, generates period splitting, resulting in flies exhibiting both long and near 24-h periods. These behavioral effects are evident even when CK2αTik expression is induced only during adulthood, implicating an acute role for CK2α function in circadian rhythms. CK2αTik expression results in reduced PER phosphorylation, delayed nuclear entry, and dampened cycling with elevated trough levels of PER. Heightened trough levels of per transcript accompany increased protein levels, suggesting that CK2αTik disturbs negative feedback of PER on its own transcription. Taken together, these in vivo data implicate a central role of CK2α function in timing PER negative feedback in adult circadian neurons.

Introduction

Circadian rhythms that orchestrate daily fluctuations in biochemistry, physiology and behavior are observed across distinct phylogenetic kingdoms. Underlying the evolutionary importance of these clocks, the molecular processes that drive circadian rhythms are also highly conserved. At the core of the circadian pathway is a transcriptional feedback loop. In Drosophila melanogaster, CLOCK (CLK) and CYCLE (CYC) activate expression of target genes such as period (per) and timeless (tim) [1–3]. PER and TIM ultimately translocate to the nucleus and inhibit CLK/CYC transcription [3–6]. Notably, the overall architecture of this feedback loop, as well as some of the molecular players, are observed in organisms as diverse as fungi, plants, and mammals [3].

In addition to transcriptional influence in circadian rhythms, posttranslational modification, particularly for PER, has been shown to play a critical role in normal and disordered circadian timing [7–10]. The most well studied kinase CK1/Dblonduenselme (DBT) is hypothesized to regulate PER nuclear entry, repression, and degradation [7,11,12]. A second enzyme, glycogen synthase kinase (GSKβ)/SHAGGY (SGG), regulates phosphorylation of TIM protein, levels, and nuclear entry [13]. These rhythmic phosphorylation cycles also necessarily include the activity of a phosphatase, and protein phosphatase 2A (PP2A) has been implicated in the rhythmic dephosphorylation of PER [14], while protein phosphatase 1 (PP1) has been implicated in the dephosphorylation of both TIM and PER [15].

Our laboratory has been investigating the function of the protein kinase CK2 in circadian clock function [16,17]. The CK2 holoenzyme is a heterotetramer consisting of two alpha catalytic and two beta regulatory subunits [18,19]. Mutant CK2α and CK2β alleles result in period lengthening phenotypes (~3 h long), consistent with their proposed clock role [16,20]. The manner in which CK2 is important for setting circadian period remains unclear. CK2 also functions in various developmental processes [18], consistent with the pre-adult lethality observed in CK2α and CK2β mutants [16,21]. This developmental function raises the question that CK2 phenotypes may derive from its activity during maturation rather than in adults. While both CK2 subunits are expressed in pacemaker neurons [16,20], it is uncertain if CK2 functions in these neurons to regulate circadian rhythms. RNAi studies in S2 cells suggest that the role of CK2 phosphorylation is to promote transcriptional repression by PER [22]; however, it is not clear if this is true in vivo.

To better address these questions, we expressed a dominant negative CK2α Timekeeper (Tik) mutant [16] in a spatially and temporally controlled manner and queried the effects on behavior, PER protein levels, phosphorylation, repression, and nuclear entry in core pacemaker neurons of adult D. melanogaster. Taken together, these findings reveal remarkably potent effects of manipulating CK2 activity in adult circadian rhythms.
neurons and uncover a role consistent with the regulation of PER nuclear localization and feedback repression.

**Results**

**Induction of the Dominant CK2\(x^{Tik}\) Mutation in Circadian Neurons Dramatically Lengthens Circadian Period**

Prior studies implicate CK2 in the control of circadian function in *Drosophila, Arabidopsis*, and *Neurospora* [16,20,23,24]. Testing of the strongest homozygous mutants alleles is limited by developmental lethality [16,21]. More modest period phenotypes raised questions as to the functional significance of CK2 action in the circadian clocks. To determine the consequences of suppressing CK2 activity, we used the GAL4/UAS system to drive expression of CK2\(a\) bearing the dominant *Tik* mutation (CK2\(a^{Tik}\)) [25]. The CK2\(a^{Tik}\) allele contains two missense mutations, one of which introduces a charged residue into the putative hydrophobic binding pocket for the phosphodonor nucleotide [16,19]. In vitro analysis indicates that these mutations eliminate most catalytic activity [26]. The molecular lesion, the loss of biochemical activity and the dominant behavioral phenotype suggest that *Tik* encodes a dominant negative form of CK2."}

"To examine the behavioral consequences of CK2\(a^{Tik}\) expression, we crossed flies bearing UAS-driven CK2\(a^{Tik}\) (UASTik) with *timGal4*-62 driver flies [27] and assayed circadian behavior in the progeny (*timGal4/+; UASTik\(^{T/T}\), "timTik"). The *Drosophila* circadian network consists of six bilateral groups of cells: large and small ventral lateral neurons (lg- and sm-LNv), dorsal lateral neurons (LNd), and three clusters of dorsal neurons (DN1–3) [28]. The *tim* promoter induces GAL4 expression in all of these key neuronal clusters that coordinate circadian behavior [29]. To our surprise, these timTik flies display extraordinarily long periods averaging ~33 h relative to control periods of ~24 h (Figure 1, compare Figure 1A and Figure 1B; Table 1). Moreover, the influence on period is dose-dependent; by increasing Gal4 dosage in timTik flies with a second ciricadian driver, *cry16Gal4* [30], the period is further lengthened to ~37 h (Table 1). Confirming the circadian specificity of this result, expression of UASTik only in photoreceptor neurons with the GMRGal4 driver [31] does not result in period lengthening (data not shown). Heterozygous *Tik/+* mutant flies display period ~2–3 h longer than wild-type controls with a reduction of ~50% in CK2 activity [16]. The magnitude of the period effects strongly argues that CK2 activity is more gravely inhibited in timTik flies. The fact that the magnitude of period effects exceeds that of nearly all circadian mutant alleles suggests that CK2 activity is critically important for setting circadian period.

By increasing dosage of the dominant allele with double copies of both the broad circadian *timGal4* driver and the UASTik transgene (*timGal4; UASTik\(^{T/T}\), "timTik2x"), rhythmicity is undetectable in constant darkness (Figure 1C; Table 1). The above results suggest that CK2\(x\) function in central pacemaker neurons is essential for wild type behavioral rhythms. Thus, CK2\(x\) and DBT appear to be the only core circadian kinases demonstrated to be obligatory for free-running behavioral rhythms [11,32]. Mutations in a catalytic subunit of the cAMP-dependent protein kinase (PKA) also result in behavioral arrhythmicity [33]; however, as this lesion leaves core molecular cycling of the clock intact, it is likely to function in an output capacity."

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**Figure 1. Circadian CK2\(x\) Loss of Function Alters Period and Rhythmicity**

(A–E) Representative double-plotted actograms of indicated genotypes. The y-axis symbolizes two consecutive 24-h time frames; gray bars, subjective day; black bars, subjective night. The y-axis represents consecutive days and vertical black bars represent activity of a single fly. (A) The UASTik alone control shows a normal 24-h period. (B) Expression of a single copy of UASTik with *timGal4*, a broad circadian driver, lengthens period while (C) increased transgene dosage induces behavioral arrhythmity. (D) Overexpression of dominant-negative CK2\(x\) in PDF-positive clock neurons causes long periods or (E) period splitting.

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Expression of CK2α^Tik in PDF+ Pacemaker LNv Leads to Robust Period Lengthening and Complex Rhythms

The neuropeptide Pigment-Dispersing Factor (PDF) mediates transmission of timing information from core LNv pacemaker neurons to downstream neural circuits [34]. The CK2α and β subunits are strongly expressed in the pacemaker LNv [16,20]. To test the hypothesis that CK2α functions in pacemaker neurons, CK2α^Tik was induced in the LNv using a pdfGal4 driver [34]. Similar to timTik flies, CK2α expression in PDF+ neurons (pdfGal4/+; UASTik^T1/+; “pdfTik”) also results in dramatically long periods (~32 h; Figure 1D; Table 1). Again, these effects are dose-dependent, as adding an additional Gal4 driver, cry16Gal4, increased the period length to ~37 h (Table 1). We previously identified a spontaneous revertant allele, TikR, which deletes a portion of the Tik coding region, largely reverts the dominant circadian phenotype but still lacks catalytic activity, consistent with its characterization as a recessive loss-of-function allele [16]. Supporting this hypothesis, pdfGal4 expression of independent UASTikR lines had no significant effect on circadian rhythms (Table 1). These results confirm the hypothesis that the Tik mutation acts as a dominant-negative to inhibit function of endogenous wild type CK2α.

PDF neurons communicate with and reset the clocks in non-PDF pacemaker neurons to synchronize different clusters in the network [35]. The CK2α^Tik period effects were blocked in a pdf null [34] background or by coexpressing an inwardly rectifying potassium channel that hyperpolarizes the LNv (UASKIR.1; [36], Table 1), indicating that CK2α^Tik period effects are transmitted by LNv activity and PDF output. These manipulations alone (pdf^null mutant expression of UASKIR with pdfGal4) result in short, weak periods (Figure S1; Table 1) ([34,36]). These data provide functional evidence that CK2α operates in pacemaker LNv to regulate circadian period, consistent with published expression data.

While pdfTik flies show a long period phenotype, we also noted variability in the period measurement and reduction of the strength of the rhythm in these flies (Table 1). It was hypothesized that “wild-type” non-PDF clock neurons were unable to entrain to the long period program in PDF+ cells, and were expressing a secondary rhythm. To see if flies were exhibiting more than one period, we performed periodogram analysis using the Lomb-Scargle method [37,38]. This approach eliminates misidentification of periods that are simply multiples of a true period. This analysis reveals that approximately 45% of pdfTik animals display two significant periods (Figures 1E and 2A). The dominant period is 35.3 (+/− 0.3) h while a secondary peak indicates an average period of 23.2 (+/− 0.1) h (Figures 2B and S2). When UASTik is expressed as a heterozygote with the broader expressing timGal4 driver, reduced rhythm strength and splitting is not detectable (Figure 2A; Table 1), suggesting that hyper-elongating period only in PDF-positive LNv causes uncoupling of clock cell groups. We propose that non-PDF neurons are unable to maintain synchrony with PDF clocks with extreme periods. To our knowledge, this is the first example of complex rhythmicity due to altering period length in a subset of pacemaker neurons.

| Genotype          | Perioda | Strengthb | %Rc  | nd  |
|-------------------|---------|-----------|------|-----|
| UASTik^T1/+       | 23.9    | 150.4     | 97   | 38  |
| timGal4/+         | 24.3    | 118.7     | 100  | 42  |
| timGal4/+; UASTik^T1/+ | 33.0 | 93.8      | 92   | 53  |
| timGal4/+; cry16Gal4/UASTik^T1 | 36.8 | 35.7      | 50   | 16  |
| timGal4; UASTik^T1 | n/a     | 2.0       | 0    | 24  |
| pdfGal4/+         | 24.3    | 119.4     | 100  | 25  |
| pdfGal4/+; UASTik^T1/+ | 32.2 | 21.1      | 48   | 105 |
| pdfGal4/+; cry16Gal4/UASTik^T1 | 36.7 | 63.6      | 85   | 33  |
| pdfGal4/+; UASTik^RT2/+ | 24.0 | 95.9      | 100  | 8   |
| pdfGal4/+; UASTik^RT2+/+ | 23.9 | 112.5     | 100  | 8   |
| UASKIR/+          | 23.5    | 121.1     | 100  | 10  |
| pdfGal4/+; UASKIR/+ | 22.0 | 10.7      | 32   | 22  |
| pdfGal4/+; UASTik^RT3/+; UASKIR | 22.6 | 15.8      | 42   | 31  |
| pdfGal4/UASTik^T1; pdf^Tik  | 24.1    | 29.9      | 71   | 56  |

aMean period (+/− standard error of the mean).
bMean rhythm strength (“p<” value, see Methods, +/- standard error of the mean).
cPercent rhythmic.
dSample size for the indicated genotypes.

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during development. Flies were then tested at either permissive (18 °C) or restrictive (29 °C) temperatures and period was calculated during constant conditions. A cardinal feature of circadian clocks is their temperature compensation, i.e., period is roughly invariant over a broad temperature range [40,41]. Consistent with this idea, the control strain here (tubGal80ts/+, UASTik T1+/+) shows little period change between 18 °C and 29 °C (Figure 3A and 3B, top panels). Constitutively inhibiting CK2α in pdfTik flies again demonstrates the severe period lengthening effect at both temperatures (Figure 3A and 3B, middle panels); splitting of rhythms in these flies is observed at levels similar to those described above, but only at 29 °C (unpublished data).

Interestingly, when dominant-negative UASTik is selectively activated at 29 °C during testing of adult flies, the extreme long period phenotype (>30 h) is still manifested (Figure 3A and 3B, bottom panels); splitting of rhythms in these flies is observed at levels similar to those described above, but only at 29 °C (unpublished data). Interestingly, when dominant-negative UASTik is selectively activated at 29 °C during testing of adult flies, the extreme long period phenotype (>30 h) is still manifested (Figure 3A and 3B, bottom panels); splitting of rhythms in these flies is observed at levels similar to those described above, but only at 29 °C (unpublished data). These results indicate that CK2α plays a direct role in adult circadian rhythms, and its loss of function in Tik and UASTik animals is not likely due to some developmental artifact. Consistent with this idea, inspection of LNv structure and PDF labeling in UASTik-expressing brains reveals no gross abnormalities of circadian pacemaker anatomy (unpublished data). To our knowledge, this is one of the few temporal investigations of clock gene function demonstrating an acute role of a circadian gene during adulthood [42,43].

Inhibition of CK2α Activity in Pacemaker Neurons Delays PER Rhythms

To determine the effects of CK2α loss of function on core molecular clock rhythms, we tested whether expression of UASTik in PDF-positive LNv altered cycling of the core clock protein PER. Levels and cellular distribution of PER protein in smlLNv were examined quantitatively on the first day of DD in pdfTik or control Ga4 flies. Although we do observe splitting in these flies, behavior remains largely synchronous on the first day of DD (Figure 4A). Control flies show the
typical evening peak of activity at ~CT12 while the long-period pdfTik flies have a delayed evening activity peak, regardless of whether they exhibit split periods or not (Figure 4A, pdfTikL v. pdfTikS). Measurements of pixel intensity indirectly report the amount of PER protein in smLNv [44]; as seen in Figure 4B and 4C, PER levels are elevated in smLNv of pdfTik during the subjective day relative to controls. Wild type PER levels wane from CT4–8 and begin accumulating again in the subjective evening (CT16–20); in contrast, a prolonged decline in PER throughout the day (CT4–12) is evident in pdfTik flies, and levels only disappear during subjective evening (CT12–20), consistent with a long period phenotype. Peak and trough PER levels are also elevated in pdfTik flies relative to controls ($p < 0.001$ comparing pdfGal4/+ CT0 to pdfTik CT4 for peak and pdfGal4/+ CT12 to pdfTik CT16 for trough, Figure 4C). PER typically transitions from the cytoplasm to a predominantly nuclear distribution during the middle of the night, and such a pattern is observed in pdfGal4/+ control flies (Figure 4B and 4D). However, the amplitude of the localization rhythm (as quantified by the nuclear:cytoplasmic ratio) is seriously reduced in pdfTik flies (Figure 4D, $p < 0.001$ at CT0, CT4, CT8, and CT20 pdfGal4/+ v. pdfTik). The timing of nuclear localization is also delayed in pdfTik flies; while PER never becomes predominantly nuclear, the time at which the most PER is localized to the nucleus occurs later from CT4–12 in pdfTik smLNv, rather than CT0–4 for the GAL4 control (Figure 4D and 4E). This finding is supported by analysis of nuclear PER levels in pdfGal4/+ and pdfTik smLNv. Nuclear PER levels accumulate to a similar degree in pdfTik as in the GAL4 control; however, nuclear levels do not rise until later in the subjective day relative to control (Figure 4E). The overall fraction of nuclear PER is lower (Figure 4D), as more of the PER protein in pdfTik neurons remains sequestered in the cytoplasm (Figure 4F). Indeed, the reduced nuclear PER levels in the face of elevated cytoplasmic PER levels at CT0 provide the most compelling evidence that CK2α is important for nuclear PER localization independent of regulating its cytoplasmic abundance. These results are consistent with prior reports that reduction of CK2 activity inhibits nuclear entry [16].

**CK2αTik Expression Reduces PER Phosphorylation and Increases PER Trough Levels**

To quantitatively examine the effect of CK2αTik on PER cycling and phosphorylation, we used western blots of whole head extracts on the first day of DD. The far majority of PER

![Figure 4](image_url)
Negative Feedback Repression Is Altered by CK2α^{Tik} Expression

Previous studies have implicated CK2 in promoting PER repression of CLK activation [22]. However, these studies were performed in cultured Drosophila S2 cells which do not harbor functioning circadian clocks. To test the hypothesis that CK2 promotes PER repression in vivo, we examined circadian transcription in UASTik expressing flies. Levels of two CLK-activated transcripts, per and vri (vri) [46] were analyzed using quantitative real-time reverse-transcriptase polymerase chain reaction (qRT-PCR). We hypothesized that if negative feedback is unaffected in UASTik expressing flies, then elevated PER levels would strongly repress CLK, reducing per and vri transcript levels. If negative feedback is disrupted, then elevated PER levels would fail to appropriately repress per and vri transcription. Expression of dominant-negative UASTik in circadian neurons in timTik flies postpones the decline in per transcript until early subjective night (Figure 5A), consistent with the effect of CK2 loss of function in the heterozygous Tik/+ mutant. Whereas wild type per transcript peaks around CT9–13, per levels do not achieve maximum until CT13–17 in timTik flies, and a similar pattern emerges from analysis of the vri transcript (Figure 5B).

The most informative result becomes apparent in timTik2x flies. Further reductions in CK2α activity in timTik2x result in per and vri transcript levels with a severely reduced amplitude rhythm (Figure 5A and 5B). Importantly, per and vri never reach wild-type trough levels (per: p < 0.01 for y w at CT1 relative to timTik and timTik2x at CT5 and p < 0.01 for vri at the same time points), consistent with the hypothesis that elevated PER protein levels are unable to fully repress CLK target genes in UASTik-expressing flies. Taken together, the magnitude of the observed effects suggests that CK2 not only promotes PER repression activity in vivo, but that it has a sizable impact on transcriptional repression.

Discussion

The role of posttranslational modification in regulating precise circadian timing is well established [9], and indeed may be principally responsible for molecular cycling [8]. CK2 has been implicated in regulating circadian rhythms, PER modification and metabolism [16,17]. The present study sought to determine if CK2α activity is required in adult core pacemaker neurons for molecular and behavioral rhythmicity. Broad spatial expression of UASTik in pacemaker neurons with the timGal4 driver causes severe length-
that CK2 activity may also regulate morning behavior, as functional molecular clock. As CK2 functions to dictate would be expected to regulate feedback in all cells that have a true component of the core transcriptional pacemaker, it could favor a strong morning or evening activity phase. The contribution of CK2 activity to morning and evening behavior is currently under investigation.

Further evidence that CK2 activity is important in LNv derives from the finding that inhibition by CK2αTak causes delayed nuclear entry of PER in these core pacemaker cells. An unanticipated consequence of pdfTik expression is splitting of the behavioral rhythm into long (~35 h) and short (~23 h) components. All of the above behavioral effects are due to acute CK2α activity as adult-specific inhibition is able to induce the rhythm phenotypes. At the molecular level, elevated levels and diminished phosphorylation of PER protein is associated with reduced CK2 function; this effect on PER protein is further correlated with elevated and delayed transcription of per and vri clock genes.

The severity of the observed behavioral phenotype places CK2 as a critical regulator of circadian rhythms. Of known circadian kinases, only mutants of doubletime (dbt) and PKA are also capable of completely eliminating rhythmicity as is observed in timTik2x flies [32,33]. As the core molecular clock is unperturbed by PKA mutations, this kinase is proposed to function in circadian locomotor output [32,33], leaving DBT and CK2 as the only critical core circadian kinases. Originally, DBT was found to regulate PER stability and electrophoretic mobility; this initial study concluded that DBT-mediated phosphorylation led to PER degradation [11,49]. Subsequent studies suggested that DBT may retard the ability of PER to enter the nucleus and repress transcription [12,22,50]. Many other gene mutations that result in arrhythmicity affect either input or output of the circadian system. As the core molecular feedback loop is disrupted in UASTikh-expressing flies, CK2 appears to regulate timing of the core clock and shows phenotypes similar to mutants of other core circadian genes such as per, tim, and Clk. However, the magnitude of the period phenotype in both pdfTik and timTik flies is greater than nearly all circadian mutants. The only other alleles which produce a similar degree of period lengthening include the tim17 mutation [51] and a novel dominant-negative kinase dead dbt allele whose expression also results in period lengthening or arrhythmicity [52].

We present numerous pieces of evidence to support the hypothesis that CK2α acutely functions in the PDF+ LNv neurons. The long period phenotype observed when CK2αTak is expressed in PDF-positive LNv is associated with splitting of the behavioral rhythm into two components: a predominant, long, ~35 h period and a weak shorter period of approximately 23 h. The splitting is reflected in the low strength of behavioral rhythms observed in pdfTik flies. Splitting was originally observed in Syrian hamsters maintained under constant light; this finding was the foundation for a two-oscillator model whose coordinated output is manifested as an overt circadian rhythm [33]. It has similarly been shown that non-conventional entrainment conditions can induce multi-period splitting and desynchronization of circadian neurons in mammals [54,55]. Early reports indicated splitting of the Drosophila circadian period in sine oculis mutants that have disrupted optic development, suggesting that dual periods may arise from entrainment

ekening of circadian period to ~33 h, a degree even greater than that of the heterozygous Tik mutant. Radical reductions in CK2α activity by increasing copy number of the transgenes in timTik2x flies ultimately result in behavioral arrhythmicity, demonstrating that CK2α is an obligatory component of circadian rhythms. Previous work demonstrated that overexpression of wild type CK2 mildly lengthens period [17]; taken together, these data indicate that period is highly sensitive to CK2 activity. Expression of UASTik in PDF+ LNv is also sufficient to lengthen period; indeed, the effect requires LNv activity and output of the PDF neuropeptide. That the period length is not exacerbated by additional clock neuron expression in timTik versus pdfTik flies implies that the phenotype originates largely from the LNv; however, the possibility that CK2α additionally functions in other circadian cells cannot be excluded. Given that splitting is eliminated when the genetic programs of both LNv and downstream circadian neurons are identical with respect to UASTik expression, the data imply that this manipulation affects CK2 function in other clock cells. Indeed, if CK2 is a true component of the core transcriptional pacemaker, it would be expected to regulate feedback in all cells that have a functional molecular clock. As CK2 functions to dictate period in LNv cells during constant conditions, it is possible that CK2 activity may also regulate morning behavior, as these cells drive morning activity, while downstream neurons dictate evening activity [47,48]. However, as CK2 may also function in cells responsible for evening behavior, some balance of CK2 between LNv and non-LNv neurons could favor a strong morning or evening activity phase. The contribution of CK2 activity to morning and evening behavior is currently under investigation.

Further evidence that CK2α activity is important in LNv derives from the finding that inhibition by CK2αTak causes delayed nuclear entry of PER in these core pacemaker cells. An unanticipated consequence of pdfTik expression is splitting of the behavioral rhythm into long (~35 h) and short (~23 h) components. All of the above behavioral effects are due to acute CK2α activity as adult-specific inhibition is able to induce the rhythm phenotypes. At the molecular level, elevated levels and diminished phosphorylation of PER protein is associated with reduced CK2 function; this effect on PER protein is further correlated with elevated and delayed transcription of per and vri clock genes.

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![Figure 6. Circadian CK2a Loss of Function Impacts Clock Gene RNA Levels](image.png)

(A) Overexpression of one copy of UASTik leads to elevated levels of per transcript during early subjective night, while further diminishing CK2a function with two copies each of timGal4 and UASTik greatly diminishes the amplitude of per RNA cycling. (B) Analysis of vri transcript reveals a similar pattern of delayed and dampened transcript persistence when CK2a activity is inhibited in circadian cells.+/y w, timTik: timGal4/+; UASTik1/+; timTik2x: timGal4; UASTik1. CT: circadian time. Values obtained from two to three independent experiments. doi:10.1371/journal.pgen.0040012.g006
through different input pathways [56]. Similarly, both wild type flies under low light and mutants of cryptochrome, the major circadian photoreceptor, exhibit split rhythms under constant light [57,58]. These periods include short (~22 h) and long (~25 h) components that alternatively decrease or increase with light intensity, respectively, again implicating variation of the oscillator system input pathway. Ectopic misexpression of the PDF output neuropeptide induced multiple periods during DD (of ~22 h and ~25 h) [59]. Nitabach et al. [38] further identified complex rhythmicity by activating LNv neurons; at least two periods of ~22 or ~25–26 h lengths are observed (with an occasional 3rd, shorter ~20–21 h peak). Elevated PDF levels and desynchronization of circadian neurons are detected in these flies [38]. Both of the above cases suggest that split periods arise from misregulation of neuronal output from the core pacemaker neurons. The result presented here is the first demonstration of splitting as a consequence of altering a core clock component.

It is hypothesized that driving the oscillator period to such an extreme only in the LNv uncouples them from non-UASTik expressing, PDF-negative “wild type” circadian neurons (i.e., LN/dDNs) that then contribute the shorter, weaker behavioral rhythm (~23 h), such as that seen in pdf^0^ mutants [34]. This notion is further supported by the behavior of timTik flies that express UASTik in all circadian neurons; in this case, when the genetic programs of all clock cells are identical, no such splitting is observed and rhythm strength returns to normal levels. These results begin to examine the limits of entrainment of one oscillator by a coupled oscillator in a circadian pacemaker network.

CK2 has a number of roles in cellular biology [18]. It is required at multiple transitions during the cell cycle including mitosis and functions to regulate caspase-mediated apoptosis and cell survival [18]. Developmentally, CK2 regulates proliferation and cell fate decisions [25,60]. Not surprisingly, it is an essential gene, as homozygous Tik mutants are not viable as adults [16]. We were able to utilize the TARGET system [39] to conditionally induce dominant-negative CK2α in adult flies. Interestingly, when CK2α activity was inhibited in LNv solely during adulthood, the behavioral phenotypes are still manifested. Thus, an acute CK2α loss of function impacts rhythmicity in the adult circadian system, presenting it as a critical and direct regulator of the circadian clock. While it has been shown that such adult-specific rescue of per is able to restore rhythmicity in Drosophila [43], it will be important to investigate the life-stage properties of other circadian genes; for example, Clk is also known to have developmental roles [61].

Thus, CK2α is an acute, direct, and essential component of circadian rhythms; we propose that CK2α regulates the core oscillator by phosphorylating PER to promote nuclear entry and repression. There is abundant evidence for PER as a bona fide CK2α substrate. CK2α can phosphorylate PER in vitro at specific predicted CK2α sites [16,17]; moreover, mutation of these CK2α target residues causes period phenotypes similar to the Tik mutation when expressed in vivo, demonstrating the functional relevance of this modification on PER activity [17]. Finally, we present here the clear defects in PER mobility observed with a deficiency in CK2α activity. Expressing UASTik singly or in double dosage with the timGal4 driver results in increased, hypophosphorylated PER. Indeed, the amplitude of PER cycling appears completely diminished when CK2α is severely inhibited in timTik2x flies. Again, the molecular PER phenotype mirrors the behavioral effect of these manipulations; timTik2x flies are arrhythmic under constant conditions, supporting the idea that CK2α activity is critical for the maintenance of a molecular and behavioral clock.

A further consequence of CK2α loss of function in core pacemaker neurons is a pattern of delayed PER decline, consistent with the long period phenotype observed in these flies. Despite increases in overall and cytoplasmic PER levels, nuclear PER levels are lower relative to wild type during the early subjective day, providing further evidence that nuclear translocation is not strictly driven by protein accumulation [50,62]. The dampened and delayed nuclear entry of PER protein of CK2α^Tik^ expressing smLNv provides support that CK2α normally functions to promote nuclear translocation. A second possibility is that the high levels of PER protein saturate the nuclear entry pathway, preventing the majority of PER from localizing to the nucleus in pdfTik flies. Yet, the delay in nuclear accumulation is consistent with the hypothesis that CK2α activity typically functions to permit timely PER nuclear entry.

Previous evidence indicated that knock-down of CK2 levels in cultured Drosophila S2 cells limits the ability of PER to repress a Clk-driven luciferase reporter [22]. It is critical to validate such studies in vivo to determine the true function of the kinase in the circadian system. While one may expect that the increased levels of PER associated with CK2α^Tik^ expression (particularly at trough time points) would lead to enhanced clock gene repression, we do not see such an effect. Conversely, CK2α inhibition results in delayed per and vri transcription, and elevated trough transcript levels, confirming that CK2α normally operates to promote repression of clock gene transcription. The features of CK2α function are both in opposition with and complementary to those put forth for the DBT kinase. DBT is thought to retard PER nuclear entry [12] and signal its degradation [11]; in contrast, CK2α appears to promote nuclear entry of PER (and hence repression), but may also influence its turnover.

We have outlined a model of the way in which CK2 promotes repression of circadian transcription developed from existing and currently presented data. We speculate that effects of CK2 on PER nuclear localization may operate through the proposed interval timer described in S2 cells. Based on the interval timer model, PER and TIM heterodimerize in the cytoplasm in a time-insensitive manner [63]; after some lag or upon some signal, they dissociate and enter the nucleus independently [44,63] where PER mediates transcriptional repression. The role of nuclear TIM is yet unclear. Recent work indicates that repression is not achieved merely by physical association of PER with CLK, but perhaps by PER acting as a scaffold to bridge CLK and DBT [64]. It is hypothesized that phosphorylation of CLK by DBT diminishes its transactivating capabilities [64], similar to the model proposed in Neurospora [23]. Nawathean et al. conclude that the ability of PER to repress transcription is primarily a function of its nuclear localization, which is, in turn, dependent on phosphorylation [65]; however, they also acknowledge that phosphorylation may secondarily modulate the intrinsic ability of PER to enact repression. While PER is predominantly cytoplasmic in S2 cells (e.g., [65]), altering its
subcellular localization by adding a nuclear localization signal or blocking nuclear export increases transcriptional repression; however, this activity is reduced in mutants that lack a critical DBT binding site shown to be important for PER phosphorylation [64, 65]. Thus, the data from S2 cells does not resolve whether activity primarily regulates PER nuclear entry or repression, but provides evidence for both functions. The conflicting S2 cell results, particularly for DBT analysis, support the use of our in vivo approach to determine the role of kinase modification on molecular cycling.

We propose that the effects of CK2 loss of function on feedback repression are due, in part, to the inability of PER to properly translocate to the nucleus. Phosphorylation of the PER-TIM heterodimer in the cytoplasm by CK2 may act as the interval-time signal [63] to dissociate this complex and/or facilitate nuclear entry. Indeed, with reduced CK2α function, PER nuclear accumulation is delayed, and correlates with delayed repression of circadian transcription. DBT functions to induce degradation of free cytoplasmic PER, as well as hyperphosphorylated nuclear PER. We propose that increased and lingering levels of PER in UASTik-expressing brains is due to an inability of CK2 to signal dissociation of the PER-TIM heterodimer. Persistence of PER in this complex and its failure to independently enter the nucleus protects it from DBT-mediated phosphorylation. The lack of DBT phosphorylation would reduce PER degradation and repress or activity, leading to increased cytoplasmic PER and altered feedback repression. Intrinsic PER repressor function does not appear to be greatly compromised by CK2 loss of function, as liberation of PER after light-induced TIM degradation in timTik2x flies results in robust suppression of per RNA (R. Meissner, J. Lin, unpublished observations). As nuclear entry is a critical step in feedback repression, CK2 function is important for the maintenance of a functional molecular and behavioral clock. Alternatively, CK2 may promote PER-TIM dimerization and subsequent nuclear entry. CK2 may facilitate PER’s interaction with CLK and thereby enhance repression. Lastly, CK2 could function to modulate DBT phosphorylation of PER either by modulating DBT activity or by providing a phosphorylated substrate for recognition by DBT, thus controlling PER abundance.

Conditional spatio-temporal expression of this dominant-negative CK2α mutation is a useful tool for in vivo exploration of the myriad roles of CK2 in all aspects of biology. The UASTik transgene has already been used to dissect features of CK2 function during Drosophila eye development [25]. Here, the use of such a strong CK2α allele permits dissection of the molecular, genetic, and neuro-anatomical clock. Ultimately, these studies provide a model in which CK2α activity in the core adult pacemaker is critical for the proper functioning of the circadian feedback loop. The critical role of CK2α in pacemaker function highlights the importance of a concerted post-translational modification scheme to regulate cycling of core clock components in order to manifest precise circadian rhythms at the molecular and behavioral level. Indeed, mutation of a phosphorylation site in the human per2 gene is responsible for Familial Advanced Sleep Phase Syndrome [10]. As CK2 is highly conserved across kingdoms and has been shown to function in the circadian pathway of other species [23, 24], it is a priority to investigate its role in mammalian circadian rhythms.

Materials and Methods

Fly stocks. For generation of UASTik lines, we cut the BglII and XhoI fragment containing Tik from pET-Tik [16], and cloned it into pUAST to create transgenic flies. All transgenic DNA constructs were done by Applied Biologicals (Pond, BC). Fly stocks were maintained at 25°C. UAS-Tik flies [39] were ordered from Bloomington Stock Center, Indiana University. Flies were maintained in standard cornmeal-molasses-agar food at 25°C unless otherwise noted.

Behavior. As described [66], male flies aged 2–7 days old were entrained to 2–5 days of 12 h light:12 h dark (LD) and exposed to 7–10 days of constant darkness (DD). Activity patterns were monitored by the Drosophila Activity Monitoring system (TriKinetics) in 30 min intervals. As a result, activity patterns were evaluated using a chi-square periodogram (x = 0.01). Rhythm strength was measured as the power of each record minus the significance (p<0.01). Flies were considered rhythmic if they exhibited a p<0.01 value of >10. Visual inspection of actograms was performed to confirm rhythmicity (or lack thereof). Rhythms that were weakly rhythmic or variable periodograms were constructed using ClockLab to score period splitting as previously described [38] except significance was set at x = 0.01. Period peaks were considered if they crossed the significance line; the percent of flies exhibiting a single peak of ~24 h versus a single long period or two peaks was calculated.

For developmental analysis (tubGal80ts crosses), flies were mat and progeny raised at 18°C. Flies were then placed into the behavior apparatus and exposed to 5 days of LD and 7–10 days of DD at either 18°C or 29°C such that the LD phase allowed Gal4 induction (at 29°C). Circadian behavior was calculated during the DD phase.

Immunostaining. Adult male flies of the indicated genotypes aged 2–10 days were entrained to at least 3 days of LD and shifted to DD. Brains were dissected on the first day of DD as follows (modified from [44]): following brief anaesthetization, flies were pinned to a Sylgard dish and the proboscis was removed. Cold PBS was placed around the wound until all flies for that time point were processed, PBS was replaced with 4% formaldehyde/PBS to fix for 15 min. The brain was then dissected away from the head capsule under cold PBS, fixed again for 20 min, and stored in 4°C overnight. Brains were washed 5×3 min in PBSTx (PBS+0.1% Triton-X100), blocked for 10 min in PBSTG (PBSTx+10% normal goat serum), and incubated in the following primary antibodies overnight: rabbit anti-PER 1:4,000 and rat anti-PDF 1:1,000 [61, 67], gifts from M. Rosbash, Brandeis University. Antibody was removed and brains were washed 4 × 10 min in PBSTx, then incubated with secondary goat anti-rabbit-Alexa488 (Molecular Probes, Invitrogen) and donkey anti-rat-Cy3 (Jackson ImmunoResearch) at 1:500 dilutions for at least 2 h at room temperature. Final washes of 4 × 10 min in PBSTx were performed, brains were rinsed in PBS, and placed in 80% glycerol overnight at 4°C before mounting.

Immunofluorescence was imaged with a Nikon C1 confocal microscope and analyzed as reported [44]. Briefly, PDF staining was used to determine cytoplasmic/nuclear compartments, and the average pixel intensity for PER staining was measured with Image J (National Institutes of Health). Background levels in nearby pixels were 1:10,000 and 1:2,000 in TBST buffer (ECL protocol; Amersham Biosciences). For generation of UASTik lines, we cut the BglII and XhoI fragment containing Tik from pET-Tik [16], and cloned it into pUAST to create transgenic flies. All transgenic DNA constructs were done by Applied Biologicals (Pond, BC). Female flies were crossed with tubGal80ts flies [39] obtained from Grae Davis, UCSF. TubGal80ts flies [39] were ordered from the Bloomington Stock Center, Indiana University. Flies were maintained in standard cornmeal-molasses-agar food at 25°C unless otherwise noted.

Western blotting. Quantification of PER western blotting was performed as described previously with NIH Image J [16]. Equal loading and transfer were confirmed with Ponceau S staining of membranes. The dilutions for the primary and secondary antibodies were 1:10,000 and 1:2,000 in TBS1 buffer (BCA protocol; Amersham Biosciences). Five TBST buffer washes lasting 5 min each were performed following the primary and the secondary antibody incubations. Blocking was achieved with TBST+5% milk (Bio-Rad).
The incubation times for blocking, primary, and the secondary antibody incubations were as follows: 1 h at room temperature, overnight at 4 °C, and 1 h at room temperature, respectively. ECL reagents were used for immunoassay signals. A single-factor ANOVA compared the effect of genotype at trough time points.

**Quantitative real-time PCR.** Total RNA was isolated from frozen whole heads using TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. DNA was removed from RNA extracts using RQ1 DNase from Promega. Real-time PCR reactions were run using the Applied Biosystems 7900HT fast real-time PCR instrument. Data were collected using SDS software version 2.2.1. Data were analyzed using the 2^-ΔΔCt method [68] using RP49 expression values to normalize for differences in RNA amount among samples. Statistical significance was evaluated for trough transcript levels by comparing the effect of genotype at the indicated CT using a single-factor ANOVA.

For PCR reactions, ~100 ng RNA were used per reaction. Reactions were prepared using the reagents from the Qiagen QuantiTect SYBR Green RT-PCR kit. Total reaction volume was 25 μl and reactions were run in 96-well plates. Primer sets used were ordered from Integrated DNA Technologies. Primer sequences are as follows: per forward primer is 5'-CACAGAGGGTAATG-3', and the reverse primer is 5'-GAGTCCGACACTTGG-3'. vri forward primer is 5'-TTTTTTTGGCGTGTTTGCA-3', and the reverse primer is 5'-TTAGCACAACACGGATAGA-3'. RP49 forward primer is 5'-GAGGCTTTGAAGGACGAC-3', and the reverse primer is 5'-TCCAGAAGTTGAAAGACTGCT-3'. RT-PCR cycling parameters were as follows: 50 min at 50 °C, 15 min at 95 °C, and 30 cycles of 15 sec at 94 °C, 30 sec at 55 °C, and 30 sec at 72 °C.

**Supporting Information**

**Figure S1.** Short, Weak Rhythms Persist in LNv Silenced and pdf Null Flies Regardless of UAS/T Expression of pef3/CA.

Sample actograms and P-S rhythmicity values are presented for flies of the following genotypes: pefGAL4/+; UASKr64A (pdfKIR), pdfGal4/+; UASTik71/+; UASKr64A (pdfTKIKIR), and pdfGal4/+UASTik81; pdfIf4 (pdfTkIf4P).

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