Perturbing Dynamin Reveals Potent Effects on the 
Drosophila Circadian Clock

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Abstract

Background: Transcriptional feedback loops are central to circadian clock function. However, the role of neural activity and membrane events in molecular rhythms in the fruit fly Drosophila is unclear. To address this question, we expressed a temperature-sensitive, dominant negative allele of the fly homolog of dynamin called shibire²⁵¹ (sh²⁵¹), an active component in membrane vesicle scission.

Principal Findings: Broad expression in clock cells resulted in unexpectedly long, robust periods (>28 hours) comparable to perturbation of core clock components, suggesting an unappreciated role of membrane dynamics in setting period. Expression in the pacemaker lateral ventral neurons (LNv) was necessary and sufficient for this effect. Manipulation of other endocytic components exacerbated sh²⁵¹’s behavioral effects, suggesting its mechanism is specific to endocytic regulation. PKA overexpression rescued period effects suggesting sh²⁵¹ may downregulate PKA pathways. Levels of the clock component PERIOD were reduced in the sh²⁵¹-expressing pacemaker small LNv of flies held at a fully restrictive temperature (29°C). Less restrictive conditions (25°C) delayed cycling proportional to observed behavioral changes. Levels of the neuropeptide PIGMENT-DISPERSING FACTOR (PDF), the only known LNv neurotransmitter, were also reduced, but PERIOD cycling was still delayed in flies lacking PDF, implicating a PDF-independent process. Further, sh²⁵¹ expression in the eye also results in reduced PER protein and per and vri transcript levels, suggesting that shibire-dependent signaling extends to peripheral clocks. The level of nuclear CLK, transcriptional activator of many core clock genes, is also reduced in sh²⁵¹ flies, and CLK overexpression suppresses the period-altering effects of sh²⁵¹.

Conclusions: We propose that membrane protein turnover through endocytic regulation of PKA pathways modulates the core clock by altering CLK levels and/or activity. These results suggest an important role for membrane scission in setting circadian period.

Introduction

Daily rhythms of sleep and wake are driven by transcriptional feedback loops in pacemaker neurons. In Drosophila, the transcription factor Clock (Clk) heterodimerizes with cycle (cyc) to directly activate key components of a principal feedback loop, period (per) and timeless (tim) [1], and of a secondary feedback loop, par domain protein 1 (pdp-1) and vrille (vri) [2,3]. PER and perhaps TIM feed back and repress CLK/CYC DNA binding leading to molecular oscillations in clock components. VRI feeds back to repress transcription of Clk [3], while PDP may regulate clock output [4]. CLK also activates clockwork orange (cwo), which represses CLK-activated transcription of its targets [5–7]. These molecular feedback loops are thought to operate in a cell-autonomous manner [8]. Several components of these feedback loops are conserved with mammals [1].

Molecular clocks are evident in many peripheral tissues, such as the eye, as well as the central brain [9]. Brain clocks are divided into 7 anatomical clusters: small and large ventral lateral neurons (sLNv, ILNv), dorsal lateral neurons (LNd), three groups of dorsal neurons (DN1, DN2, DN3), and the lateral posterior neurons (LPN) [10–14]. The neuropeptide Pigment Dispersing Factor (PDF) [15] is expressed uniquely by and is the only known transmitter of the LNv. Mutants of PDF or its receptor display short period damping rhythms [16–19]. pdfr− pacemaker molecular oscillations are eventually low amplitude or phase-dispersed, indicating PDF feeds back to maintain synchrony [17,20,21]. Mammalian rhythms are also lost in mutants of the Vasoactive Intestinal Peptide (VIP) system [22,23], indicating a conserved role for neuropeptidergic signaling in clocks. Under light-dark conditions (LD), the PDF+ sLNv mediate behavioral anticipation of the transition from dark to light (“morning”) while “evening” anticipation is mediated by PDF- clocks: the DN1, LNd, and one sLNv [24,25]. Under constant darkness (DD), the LNv dominate behavioral period determination and reset non-PDF clocks [24,26,27]. PDF neurons may also receive a number of other

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Results

**shibire** Potently and Selectively Affects Circadian Period

To test the role of intercellular communication in the *Drosophila* circadian pacemaker, we expressed *shibire* throughout the pacemaker neuron system. Initial experiments utilized GALA lines, crypGAL4-16 (cry16) and crypGAL4-24 (cry24), that drive expression throughout the key circadian neuronal groups (LN and DN, [14,47]) and assayed behavior near the restrictive temperature at 29°C [45]. Under these conditions, most flies died or were arrhythmic consistent with the behavior near the restrictive temperature at 29°C. In flies expressing either the LN or in peripheral eye clock cells also drastically reduces Ckr target gene levels. Ckr itself is reduced in the sLNv and the long period is suppressed by Ckr overexpression. These results suggest that modulation of cell membrane processes such as receptor signaling pathways may powerfully affect the molecular clock.

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To map the neurons involved in this regulation, we expressed *shibire* only in the PDF+ pacemaker LNv. With expression driven by pdfGAL4, the period was nearly 2 hours longer than control, again with equivalent rhythmicity, at 25°C (Fig. 1B, 1C, Table 1), indicating that *shibire* expression in PDF neurons is sufficient for robust period effects. Nonetheless, some of the *shibire* period effects may be derived from its expression in non-PDF neurons, since the period effects of pdfGAL4-driven *shibire* were less than those driven by cry24 and cry16. We then expressed *shibire* broadly in all pacemaker neurons but blocked expression in the LNv using the GALA repressor GAL80 (Table 1, cry24; pdfGAL4/+/UASshibire+/+, [24]). Period was not different from the GALA control, indicating that expression in PDF neurons is necessary and sufficient for period lengthening effects.

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**shibire** Expression Delays PERIOD Rhythm Phase and Suppresses Rhythmic Amplitude

Because LNv expression of *shibire* is necessary and sufficient for behavioral period changes, we next focused on the cellular effects of pdfGAL4-driven *shibire* in the sLNv, crucial for setting period in
DD conditions [25,56]. We set out to examine sLNv PER cycling on the third day of constant dark conditions (DD3) following two days of LD entrainment at 25°C. We reasoned that this would allow us to more easily detect changes in PER cycling resulting from a 2-hour daily period change. To verify the magnitude of changes on DD3 we plotted the behavior of shib1 expressing flies and their GAL4 controls on this day (Fig. 2A). Interestingly, both sets of flies show similar slow increases in activity but control activity then drops relatively abruptly. In the pdfGAL4/+ controls, activity level peaked at about CT13 followed by a rapid decline to a trough around CT17. The activity peak is broader in shib1 expressing flies and the activity reduction is delayed several hours consistent with the observed behavioral period change. These results indicate that the PDF neuron output is intact as PDF

Figure 1. shib1 expression in pacemaker neurons lengthens period. A) Broad expression of shib1 including most circadian neurons induces long behavioral periods. Activity is plotted over two days on each horizontal line, with the second day repeated on the next line. The first four lines show behavior in LD. DD begins at the arrow in each graph. Relative to cryGAL4-16/+ controls, cryGAL4-16/+; Ushib1/+ period is robust and up to 4 hours long (see table 1, table S1). B) Behavioral period is temperature sensitive in shib1 expressing flies. Driven by cryGAL4-16, period increases with temperature until flies become arrhythmic or die at 29°C. pdfGAL4/+; Ushib1/+ behavioral period is about 1.8 hours longer than control at 25°C (see text). C) Activity plot of more restricted expression in PDF+ LNV at 25°C, (as in A). D) shib1 effects on circadian period are modified by other endocytic components. Broad circadian expression (cryGAL-24; Ushib1/+4) of clathrin light chain (Uclc) or a dominant negative version of a Rab5GTPase (URab5S43N), both involved in vesicle trafficking, have no effect on period themselves. However both exacerbate period-lengthening effects when expressed in combination with shib1. (**p<0.01). doi:10.1371/journal.pone.0005235.g001
neurons can still influence evening activity. Moreover, they suggest that shi\textsuperscript{shits1} expressed in PDF neurons may specifically block termination rather than onset of evening activity.

Under the same conditions, (DD3, 25°C) PER staining in the sLNv cycles robustly in control flies (Figure 2B). In the shi\textsuperscript{shits1}-expressing flies PER rhythms are phase-shifted relative to the control (both peak and trough occur later) and are apparently undamped, consistent with the behavior and with published reports of the critical role of the sLNv in setting DD period [25,56]. Thus, not only does shi\textsuperscript{shits1} alter behavioral period but also alters in a parallel manner the rhythms of the core molecular oscillator in the master pacemaker sLNv.

USHITS1 is temperature-sensitive and has been used to disrupt synaptic transmission in specific neurons at 29°C [45]. Though the average behavioral period of pdfGAL4/+; Ushits1/+ flies at 29°C was not longer than at 25°C (Fig. 1B, Table 1, Table S1), the standard error was high (Table S1); some shi\textsuperscript{shits1}-driven periods were so short as to be outside the range of controls. When we examined PER cycling at 29°C, the sLNv on DD3 were delayed relative to controls, similar to the case at 25°C (Fig. 2C). However in addition peak PER levels were substantially reduced. Increases in PER levels measured at timepoints throughout DD3. C) sLNv PER levels throughout DD3 in flies held at 29°C. Peak PER levels were reduced by shi\textsuperscript{shits1} dose-dependently relative to the control. At both 29°C and 25°C, pdfGAL4/4; Ushits1/+ PER cycling also appears delayed (**p<0.01, ***p<0.005). doi:10.1371/journal.pone.0005235.g002

*Figure 2. shi\textsuperscript{shits1} expression in pacemaker neurons perturbs the molecular clock. A) Averaged activity of pdfGAL4/+ and pdfGAL4/+; Ushits1/+ flies on the third day of constant darkness (DD3). B) sLNv PER levels measured at timepoints throughout DD3. C) sLNv PER levels throughout DD3 in flies held at 29°C. Peak PER levels were reduced by shi\textsuperscript{shits1} dose-dependently relative to the control. At both 29°C and 25°C, pdfGAL4/4; Ushits1/+ PER cycling also appears delayed (**p<0.01, ***p<0.005). doi:10.1371/journal.pone.0005235.g002

**Table 1. Circadian Behavior Table.**

| Genotype                        | Period/SEM | Power/SEM | %R | n  |
|---------------------------------|------------|-----------|----|----|
| cyaGAL4-16/+                    | 25.1+/-0.1 | 57.1+/-3.4 | 92 | 106|
| cyaGAL4-16/pUAsiGAL41            | 28.4+/-0.1 | 103.9+/-5.9 | 93 | 91 |
| cyaGAL4-24                       | 24.8+/-0.0 | 83.9+/-4.7 | 92 | 106|
| cyaGAL4-24/pUAsiGAL41           | 27.1+/-0.1 | 105.0+/-5.2 | 98 | 100|
| pdfGAL4/+                       | 24.0+/-0.0 | 73.2+/-5.5 | 90 | 72 |
| pdfGAL4/+; Ushits1/+            | 25.8+/-0.0 | 74.8+/-5.2 | 92 | 93 |
| UAsiGAL41/+                     | 23.9+/-0.0 | 82.7+/-5.3 | 88 | 93 |
| pdfGAL4UShi1/; UAsiGAL41        | 24.0+/-0.1 | 51.1+/-6.6 | 74 | 43 |
| cyaGAL4-24/pdfGAL40/+; UAsiGAL41 | 24.6+/-0.1 | 110.0+/-13.1 | 94 | 17 |
| pdfGAL4/+; pUAsiGAL42/+         | 24.0+/-0.0 | 79.9+/-7.7 | 100 | 24 |
| pdfGAL4/+; UAsiGAL41/pUAsiGAL42 | 23.0+/-0.1 | 73.0+/-9.0 | 88 | 25 |
| pdfGAL4/+; UAsiGAL41/pUAsiGAL42 | 24.3+/-0.1 | 41.0+/-5.1 | 68 | 60 |
| pdfGAL4/+; UAsiGAL41/pUAsiGAL42 | 24.8+/-0.1 | 60.2+/-10.4 | 78 | 27 |
| y w; pdfGAL4/+                  | 23.3+/-0.7 | 4.2+/-0.9 | 23 | 52 |
| pdfGAL4/+; UAsiGAL41/+          | 23.7+/-0.1 | 93.4+/-8.1 | 100 | 22 |
| cyaGAL4-24; pdfGAL4/+           | 24.1+/-0.1 | 82.2+/-9.9 | 100 | 15 |
| cyaGAL4-24; pdfGAL4/+; UAsiGAL41 | 23.0+/-0.2 | 19.3+/-4.2 | 52 | 44 |
| pdfGAL4/+; pdfGAL4/+            | 23.6+/-0.1 | 63.9+/-12.1 | 88 | 8 |
| pdfGAL4/+; pdfGAL4/+; UAsiGAL41 | 22.8+/-0.1 | 6.8+/-1.8 | 25 | 32 |
| pdfGAL4/+; UAsiGAL41/+          | 23.1+/-0.1 | 72.7+/-8.5 | 96 | 24 |
| pdfGAL4/+; UAsiGAL41/+          | 23.3+/-0.1 | 90.4+/-7.4 | 95 | 43 |
| pdfGAL4/+; UAsiGAL41/+          | 24.0+/-0.1 | 23.2+/-6.6 | 36 | 38 |
| pdfGAL4/+; UAsiGAL41/+; UAsiGAL41 | 24.3+/-0.2 | 48.0+/-8.1 | 85 | 26 |
| pdfGAL4/+; UAsiGAL41/+; UAsiGAL41 | 24.0+/-0.1 | 42.4+/-5.5 | 76 | 37 |
| pdfGAL4/+; UAsiGAL41/+; UAsiGAL41 | 24.2+/-0.1 | 81.5+/-8.8 | 87 | 38 |

Flies listed here were tested at 25°C. The average period+/−standard error of the mean (SEM) is listed, followed by the power of the rhythm (calculated as % of flies rhythmic (%R) n = total number of this genotype tested.

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**PDF is Required for Behavioral Output, not for Delay of the sLNv Molecular Clock.**

PDF is the only known neurotransmitter output of the core LNv pacemaker cells and is thought to mediate synchrony among the sLNv and between pacemaker groups [16,21]. We hypothesized...
that shb" may modulate PDF signaling and in turn, affect PDF feedback, thus affecting circadian period. To test this model, we assayed the effects of expressing shb in a pdf" mutant background. Both cry2; UASshb"-pdf"/+pdf" and pdfGAL4/+; UASshb"-pdf"/+pdf" flies exhibited weak short period (~23 hour) rhythms, comparable to y; pdf" mutants and completely distinct from the longer periods observed in shb" expressing PDF-positive flies (Table 1). These results indicate that the behavioral effects of shb" require PDF.

Some studies show that PDF levels in the dorsal brain terminals of the sLNv cycle in a circadian fashion [37] (but see also [38]) and are altered by chronic manipulations of electrical activity in the circuit [17,35,59–61]. These findings suggest changes in neuronal activity may affect PDF levels in the dorsal terminals, perhaps by regulating PDF release. We measured PDF levels in the sLNvs as a crude measure of potential changes in PDF signaling and release. PDF levels in pdfGAL4/+; Ushb"/+ flies were consistently lower than in pdfGAL4/+ (Fig. 3A, see methods). The combined data show a significant reduction in the level of PDF in the sLNvs.

We then asked if PDF is required for shb" effects on the molecular clock in the sLNv. To assay the state of the molecular clock, flies lacking PDF were collected at six timepoints on DD3 as before. To our surprise, sLNv PER cycling in the absence of PDF (~23 hour) was delayed relative to y; pdf" mutants and completely distinct from the longer periods observed in shb" expressing PDF-positive flies (Table 1). These results indicate that this leads to reductions in PER levels. Fly heads expressing shb" in the eye were collected as before on DD1 and analyzed for per mRNA levels. per in controls cycled robustly, peaking at CT9 with roughly 2.5-fold amplitude. By contrast per in pdfGAL4/+; UASshb"/+ heads was lower at the beginning of DD and quickly fell to low baseline levels, remaining relatively stable throughout DD1. This dramatic effect on PER levels was similar to that seen in the sLNv of pdfGAL4/+; Ushb"/+ flies at 29°C (Fig. 2C), indicating that shb"s effects are not specific to networked pacemaker clocks.

Reduced PER could be due to increased turnover or reduced expression that this leads to reductions in PER levels.

CLK is Reduced in shb"-expressing Pacemaker sLNv

It is notable that per and vri are both direct targets of the transcriptional activator CLK [26,63,64]. One mechanism by which both mRNAs could be reduced coordinately is through reductions of CLK-mediated transcription. To assess whether shb" might affect CLK, we quantified CLK levels in the sLNvs of flies expressing high doses of shb" [pdfGAL4/+; Ushb"/+ Ushb"] at 29°C, conditions under which the sLNv was stably reduced 5-fold from the control peak (Fig. 2C). Under the same conditions, we found nuclear CLK in the sLNv was also reduced to approximately 60% of the control level (Fig. 5). Thus it is plausible that CLK expression is reduced by shb" leading to reductions in per and other clock transcripts. If so, we might expect that artificially increasing CLK levels could mitigate period lengthening in shb"-expressing flies. We coexpressed CLK with shb" and assayed behavioral rhythms. Flies expressing CLK in the PDF+ sLNv show behavioral rhythms approximately one hour shorter than the GAL4 control but of equivalent rhythmicity (Table 1). Coexpression of CLK and shb" resulted in nearly identical behavior to expression of CLK alone; i.e. about one hour short. Thus CLK expression in the sLNv either compensated for or completely blocked the effect of shb" on behavioral rhythms. Interestingly, coexpression of the CLK targets per or tim with shb" was also able to return period to control levels (Table 1), supporting the interpretation that shb" modulates period through CLK’s transcriptional activation of per and tim.
Taken together these data suggest that shits1 may affect clocks by reducing CLK levels and/or transcriptional activity and slowing feedback in the molecular clock. As our work indicates that shits1’s effects operate by compromising endocytic pathways, we suggest that membrane events leading to PKA signaling may modulate the clock by regulating expression, turnover, and/or activity of CLK.

Discussion

Our data suggest an important function for membrane events, specifically endocytosis, in circadian timing. While previous studies have demonstrated roles for neural activity in circadian output [59,60], in sustaining molecular rhythms [34], and in synchrony [21,35,36,61], our work strongly suggests a substantial role in circadian timing. Expression of shits1 in pacemaker neurons results in strikingly long periods, suggesting potent effects on circadian timing through perturbing vesicle scission. These effects are enhanced by co-expression of other components of endocytic pathways leading to early endosomes, consistent with shi function in traffic, recycling, and turnover of cell membrane components. PKA expression rescues period defects induced by shits1, suggesting a functional link between the membrane, PKA, and behavioral period. The LNv-expressed shits1 results in delays in the phase of PER molecular rhythms in the sLNv sufficient to account for the delay in behavior. While shits1 effects on behavior require PDF, those on the molecular clock of the sLNv are PDF-independent, implicating a novel pathway. In fact these perturbations of the molecular clock are not specific to locomotor pacemakers, but appear in peripheral clocks as well, suggesting membrane-clock interactions are a general property of clock cells. Reductions in the levels of CLK and CLK-activated transcripts are consistent with the hypothesis that membrane events regulate the molecular clock through CLK.

The importance of a clock component can be inferred by the magnitude of its phenotype when perturbed. By this criterion, the magnitude of the shits1 period effects argues for a critical role for endocytosis in setting circadian period. shits1 overexpression can lead to period changes over three hours longer than controls at 25°C. Indeed, the observed period effects are comparable to or even greater than those when overexpressing per, tim or Clk, suggesting that perturbation of cell membrane recycling may be as important as these canonical core clock elements to period determination [47,65].

shits1 effects are modulated by components of endocytic pathways. Coexpression of a clathrin light chain-GFP (ccl-GFP) or a dominant negative form of Rab5 results in ~1 hour lengthening of
Shits1 period effects and overexpression of wild-type arr2 fully rescues shits1 period lengthening. Given that we predict that shits1 blocks endocytosis, the enhancement of effects by CLC seems paradoxical. One possibility is that overexpression of these components titrates away key endocytic components and thus further enhances the shits1 effects. Alternatively, as we co-expressed a CLC-GFP fusion, this chimeric protein may not be fully functional and thus may impair vesicle scission further. The period enhancing effects of the dominant negative form of Rab5 suggest an important role for endosomal intermediates, while rescue by arr2 or PKA suggests involvement of intracellular signaling pathways. At least two genes involved in endocytosis, syndapin and beta-adaptin (Bap), have been shown to be rhythmic by microarray studies [66]. Of note, syndapin is thought to directly interact with dynamin [67]. Rhythms of endocytosis could reflect or even amplify rhythms of intercellular communication. In addition, intracellular signaling pathways induced by these inputs could alter the intracellular oscillator. Taken together, rhythmic regulation of endocytic pathways may define a novel feedback loop in the circadian clockwork.

Potential effects of shits1 are evident by reductions in CLK expression and/or function. Expression of shits1 in the eye results in reductions in CLK-activated transcripts, per and vri. Similarly, reduced PER levels are also evident in shits1 expressing sLNv. These reductions in CLK-activated genes are accompanied by reductions in CLK levels in the sLNv. Prior studies have shown that CLK expression and activity is primarily regulated posttranscriptionally [68,69]. PER is thought to deliver DBT as well as reductions in CLK levels in the sLNv. Prior studies have shown that under conditions of overexpression, the temperature threshold for various phenotypes may differ from paralysis. Our finding of slight period lengthening relative to controls even at 18°C is consistent with a modest defect, with core clock effects getting stronger gradually as the temperature increases. Our evidence that shits1 is not perturbing outputs (at least at 25°C) raises that possibility that other membrane scission-sensitive processes, such as receptor endocytosis, may have a lower threshold for disruption than synaptic transmission.

What might be the nature of the membrane perturbation evoked by shits1? More broadly, endocytosis regulates membrane protein turnover, and a variety of targets could influence neuronal activity, including ion channels, pumps, and transporters, which in turn could feedback to regulate the core clock. Endocytosis has a well-established role in down-regulation of metabotropic or ionotropic receptors. In the sLNv, potential receptors include (but are not limited to) acetylcholine, GABA, serotonin, dopamine, histamine ([28–30]), and neuropeptides such as IPNamide [14]. Ion channel density may also be modulated by endocytosis [75–77] and could influence core clock rhythms [34,59,60]. On the other hand, our finding that PKA overexpression can suppress shits1 effects on period provide evidence that down regulation of G-protein coupled receptors that stimulate cAMP and PKA may be a mechanism for shi action. The identification and functional analysis of the relevant membrane targets of shits1 will be critical to understanding the role of the membrane in circadian function.

Materials and Methods

Behavior

Crosses were maintained at 21°C until behavior. Male flies were then entrained at 25°C (unless otherwise indicated) for 2 or 5 days in 12-hour light:12 hour dark conditions before release into constant darkness. Activity was monitored with the Trikinetics DAM system. LD activity averages were compiled with in-house DCM software. DD behavior was analyzed using Clocklab software (Actimetrics). Period differences were evaluated with t-test.

Immunostaining

Flies were entrained for 2–3 days in LD before release into DD. On the third day of DD flies were collected and 6–8 were dissected at the indicated timepoints. Brain tissue was fixed 40 minutes in 4% formalin in phosphate buffer (PB). Primary rabbit anti-PER (sometimes embryo pre-adsorbed) at 1:4000 diluted in PBT (PB with 0.3% Triton) with 10% normal serum was applied overnight. A secondary goat anti-rabbit Alexa 594 antibody (Molecular Probes) was applied at 1:600 overnight. Final washes in PBT and PB were followed by mounting in 80% glycerol/PB. For experiments with PDF-positive genotypes, tissue was double-labeled for PER and PDF to aid cell identification. Mouse anti-PDF (1:800, DSHB #C7) and 1:500 goat anti-mouse Alexa 488 (Molecular Probes) were applied with the PER primary and secondary, respectively. For CLK staining, guinea pig anti-CLK

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Quantitation

Slides were coded to allow blind imaging, data collection, and analysis. Experiments were run at least twice and in each experiment staining from 4-6 hemispheres/condition was imaged on a Nikon C1 confocal (PER and PDF, 60X oil objective, PDF terminals 20×, 1 μm steps). PER, CLK quantitation: Cells were identified by position, size, and PDF double-labeling when applicable. The entire soma (nucleus only for CLK) was manually circled in the single confocal section of maximum diameter and digitally quantified using ImageJ (NIH). A nearby non-stained region was quantified for background subtraction. Average intensity for each cell was used to compute a mean for the group. Experiments were repeated at least twice and results were consistent. Subsequently data from individual cells was scaled to the typical peak control average for each experiment (CT1 of pdfG4/+ in figure 2; in figure 3 CT9 of pdfGAL4/+; Ushipdf-/pdf+). PDF quantitation: The terminal segment of sLNv neurites in the dorsal brain was quantified as a single number representing the mean intensity of pixels on a hand-drawn line in ImageJ. Measurements were averaged across genotype within four timepoints for each replication. PDF levels in were lower in

Flies

Fly stocks were obtained from the following sources: M. Rosbash (cry16, cry24, pdfGAL4, pdfGAL20, pdfP1); T. Kitamoto (UASAb1); I. Mellman (UASl); M. Gonzalez-Gaitan (UASAb8); and L. Zweibel (p[art2]); M. Ramaswami (UASTB2-I; UAST27-25 and UAST114-5 and UAST114-2); D. Kalderon (UAS-PKACI). All other fly stocks were obtained from Bloomington Stock Center.

Westerns

Crosses were raised at 18°C. Western blotting was performed as reported elsewhere [79]. After two days of entrainment at 29°C, heads from entrained flies were collected on dry ice at four-hour interval timepoints throughout DD1. Protein extracts were made from 20–30 heads (same within experiment) and run on a 6% SDS-PAGE gel. After Ponceau staining confirmed equal loading, nitrocellulose blots were probed with 1:10000 rabbit anti-PER followed by 1:2000 HRP anti-rabbit. Signal was detected with ECL following kit directions (Amersham). Autoradiographs were scanned and quantified using NIH ImageJ.

Quantitative real-time PCR. Flies were collected as for Westerns. Total RNA was isolated from frozen whole heads using InVitrogen’s TRIzol reagent and manufacturer’s protocol. DNA was removed from RNA extracts using RQ1 DNase from Promega (5 μl DNase in 120 μl total reaction volume). DNase was removed from RNA extracts using the following method: 120 μl of phenol:chloroform:isoamyl alcohol (25:24:1 from Amresco) was added to each sample. Samples were shaken by hand for 1 minute and centrifuged at top speed at room temperature for 5 minutes. 100 μl of the aqueous top phase was transferred to a new tube. 10 μl of 3M sodium acetate and 300 μl of ethanol were added to each sample and incubated at −70°C for 1 hour. Samples were spun down at top speed at 4°C for 15 minutes. Supernatants were removed and the pellet washed with 1 ml 75% ethanol. Samples were spun down at top speed at 4°C for 15 minutes. Supernatants were removed, and the pellet was dried upside-down for 5–10 minutes. RNA pellets were dissolved in 30–50 μl water. Real-time PCR reactions were run using the Applied Biosystems 7900HT fast real-time PCR instrument. Data were collected using SDS software v2.2.1. Data were analyzed using the 2−ΔΔCt method [80] using RPlp expression values to normalize for differences in RNA amount among samples. For PCR reactions, ~100 ng RNA were used per reaction. Total reaction volume was 10 μl and reactions were run in 384-well plates. Primer sets (forward, reverse respectively) are as follows: per (5′-CAGGCGGGCGCTTCAATTG-3′, 5′-GAGTCCAGGACCTTGG-3′), tri (5′-TTGGTTTTTGGCCCGTTCGGTTCA-3′, 5′-TTACGACACACACACGACGTA-3′, 5′-TGCAGACAGGGACAGTATC-3′, 5′-TGGACAGACAGTTACAGAGCTC-3′). PCR cycling parameters were as follows: 30 minutes at 50°C, 15 minutes at 95°C, and 30 cycles of 15 seconds at 94°C, 30 seconds at 55°C, and 30 seconds at 72°C.

Supporting Information

Table S1 Temperature-dependent behavior effects. Table arranged as Table 1. Found at: doi:10.1371/journal.pone.0005235.s001 (0.03 MB DOC)

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Author Contributions

Conceived and designed the experiments: VLK LZ RA. Performed the experiments: VLK LZ RAM EB. Analyzed the data: VLK LZ RAM EB RA. Contributed reagents/materials/analysis tools: VLK LZ EB RA. Wrote the paper: VLK RAM RA.

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