Phase Coupling of a Circadian Neuropeptide With Rest/Activity Rhythms Detected Using a Membrane-Tethered Spider Toxin

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Drosophila clock neurons are self-sustaining cellular oscillators that rely on negative transcriptional feedback to keep circadian time. Proper regulation of organismal rhythms of physiology and behavior requires coordination of the oscillations of individual clock neurons within the circadian control network. Over the last decade, it has become clear that a key mechanism for intercellular communication in the circadian network is signaling between a subset of clock neurons that secrete the neuropeptide pigment dispersing factor (PDF) and clock neurons that possess its G protein-coupled receptor (PDFR). Furthermore, the specific hypothesis has been proposed that PDF-secreting clock neurons entrain the phase of organismal rhythms, and the cellular oscillations of other clock neurons, via the temporal patterning of secreted PDF signals. In order to test this hypothesis, we have devised a novel technique for altering the phase relationship between circadian transcriptional feedback oscillation and PDF secretion by using an ion channel-directed spider toxin to modify voltage-gated Na\(^+\) channel inactivation in vivo. This technique relies on the previously reported “tethered-toxin” technology for cell-autonomous modulation of ionic conductances via heterologous expression of subtype-specific peptide ion channel toxins as chimeric fusion proteins tethered to the plasma membrane with a glycosylphosphatidylinositol (GPI) anchor. We demonstrate for the first time, to our knowledge, the utility of the tethered-toxin technology in a transgenic animal, validating four different tethered spider toxins in Drosophila. Focusing on one of these toxins, we show that GPI tethered to the plasma membrane of spider toxin α-ACTX-Hv1a inhibits Drosophila para voltage-gated Na\(^+\) channel inactivation when coexpressed in Xenopus oocytes. Transgenic expression of membrane tethered α-ACTX-Hv1a in vivo in the PDF secreting subset of clock neurons induces rhythmic action potential bursts and depolarized plateau potentials. These in vitro and in vivo electrophysiological effects of membrane tethered α-ACTX-Hv1a are consistent with the effects of soluble α-ACTX-Hv1a purified from venom on Na\(^+\) channel physiological and biophysical properties in cockroach neurons. Membrane-tethered α-ACTX-Hv1a expression in the PDF secreting subset of clock neurons induces an approximately 4-h advance of the rhythm of PDF accumulation in their terminals relative to both the phase of the day:night cycle and the phase of the circadian transcriptional feedback loops. As a consequence, the morning anticipatory peak of locomotor activity preceding dawn, which has been shown to be driven by the clocks of the PDF secreting subset of clock neurons, phase advances coordinately with the phase of the PDF rhythm of the PDF-secreting clock neurons, rather than maintaining its phase relationship with the day:night cycle and circadian transcriptional feedback loops. These results (1) validate the tethered-toxin technology for cell-autonomous modulation of ion channel biophysical properties in vivo in transgenic Drosophila, (2) demonstrate that the kinetics of para Na\(^+\) channel inactivation is a key parameter for determining the phase relationship between circadian transcriptional feedback oscillation and PDF secretion, and (3) provide experimental support for the hypothesis that PDF-secreting clock neurons entrain the phase of organismal rhythms via the temporal patterning of secreted PDF signals.

Introduction

Drosophila clock neurons are self-sustaining cellular oscillators that rely on negative transcriptional feedback and cellular signaling pathways to keep circadian time. PERIOD (PER) and TIMELESS (TIM) clock proteins constitute the core of a negative transcriptional feedback loop, in which these clock proteins inhibit transcription of the genes that encode them (for review, see [1,2]). The transcription factors Vrille (VRI), par domain protein 1 (PDP1), along with PER and TIM, form two transcriptional feedback loops interconnected through CLOCK/CYCLE-mediated transcriptional activation [3–8]. These feedback loops interact to produce daily rhythms of clock gene mRNA and clock protein accumulation, which

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Abbreviations: AP, action potential; cRNA, complementary RNA; CT, circadian time; DD, constant darkness; DN, dorsal neuron; E, evening cell; GPI, glycosylphosphatidylinositol; LD, light:dark; LNV, large ventral lateral neuron; LND, dorsal lateral neuron; mRMP, resting membrane potential; PDF, pigment dispersing factor; PDFR, par domain protein 1; SEM, standard error of the mean; sLNV, small ventral lateral neuron; WT, wild-type; ZT, zeitgeber time

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Author Summary

The regulation of the daily fluctuations that characterize an organism’s physiology and behavior requires coordination of the cellular oscillations of individual “clock” neurons within the circadian control network. Clock neurons that secrete a neuropeptide called pigment dispersing factor (PDF) calibrate, or entrain, both the phase of organismal rhythms and the cellular oscillations of other clock neurons. In this study, we tested the hypothesis that phase of PDF secretion rhythms entrains phase of non-PDF neurons and locomotor rhythms using the tethered-toxin technique (which affixes toxins to the cell membrane) to express ion channel–specific peptide toxins in PDF neurons. A particular toxin inhibits inactivation of the Drosophila para sodium (Na\(^{+}\)) channel. Inhibition of Na\(^{+}\) channel inactivation in PDF neurons of transgenic flies induces phase advance of PDF rhythm, and correlated phase advance of lights-on anticipatory locomotor activity, suggesting that phase of morning activity is determined by phase of PDF oscillation. Therefore, voltage-gated Na\(^{+}\) channels of Drosophila clock neurons play a key role in determining the phase relationship between circadian transcriptional feedback oscillation and PDF secretion, and PDF-secreting clock neurons entrain the phase of organismal rhythms via the temporal patterning of secreted PDF signals.

are required for rhythmic physiology and behavior (for review, see [2,9–11]). Recent studies have also implicated depolarization-dependent ionic conductances and intracellular Ca\(^{2+}\) signals in cellular oscillation in the fly [12,15].

Proper regulation of organismal rhythms of physiology and behavior requires coordination of the cellular oscillations of the individual clock neurons within the circadian control network [14–21]. A key role has been suggested for pigment dispersing factor (PDF) secretion by the lateral ventral subset of clock neurons in this coordination, as pdf\(^{01}\)-null mutant flies, ventral lateral neuron (LN\(_{V}\))-ablated flies, or LN\(_{V}\)-electrically silenced flies all exhibit disrupted free-running locomotor rhythms [12,16,17]. It has been proposed that cyclic release of PDF from the dorso medial terminals of small PDF neurons (sLN\(_{V}\)) provides an important circadian signal to PDF-sensitive downstream targets [18,20–22]. In the absence of PDF, pdf\(^{01}\)-null flies exhibit dispersed and desynchronized phase relationships of independent cellular oscillators [21]. Hyperexcitability of LN\(_{V}\)s via expression of a slowly inactivating bacterial Na\(^{+}\) channel disrupts cyclic release of PDF, and results in desynchrony among subsets of clock neurons and complex behavioral rhythms [18]. These findings suggest that the PDF-secreting LN\(_{V}\)s synchronize independent clock oscillators via clock-regulated PDF secretion. The PDF receptor (PDFR) G protein-coupled receptor appears to be functionally active in many clock neurons, and PDFR mutant flies exhibit disrupted free-running locomotor rhythms similar to pdf\(^{01}\)-null mutant flies [23–26]. These studies further confirm the key role of PDF neuropeptide in circadian control of locomotor activity, indicating the importance of intercellular communication in the circadian network between PDF-secreting LN\(_{V}\)s and clock neurons that express PDFR (for review, see [11]).

Furthermore, the specific hypothesis has been proposed that PDF-secreting clock neurons entrain both the phase of organismal rhythms and cellular oscillations of other clock neurons [17,27–29]. PDF injected into the cockroach brain resets the phase rather than the period of free-running behavioral rhythms [29], suggesting that PDF mediates a nonphotic entraining signal to the circadian clock. In the absence of PDF in pdf\(^{01}\)-null mutant flies, phase of LN\(_{V}\) and non-LN\(_{V}\) clock neurons disperses, and these flies exhibit no morning anticipatory peak of locomotor activity and a phase-advanced evening anticipatory peak [17,21]. This suggests that non-LN\(_{V}\) clock neurons free-run when not receiving entraining PDF signal from LN\(_{V}\)s and that PDF-dependent intercellular communication in the circadian control network is required to maintain the proper phase relationship between morning and evening activity peaks. Morning and evening anticipatory peaks are thought to be controlled by “morning” cells (M cells), the PDF-secreting LN\(_{V}\)s, and “evening” cells (E cells), the dorsal LN\(_{D}\)s/DN1s, respectively [30,31]. In constant darkness (DD), M cells dominate the clock network and entrain the phase of E cells and organismal rhythms by sending the E cells a daily resetting signal [27]. In constant light, genetically modified E cells can drive organismal locomotor rhythms [28,32,33], in some situations independently of PDF signals [32,33]. The daily resetting signal that M cells send to E cells has been hypothesized to be PDF itself [27].

In order to test the hypothesis that phase of PDF secretion rhythms entrains phase of E cells and locomotor rhythms, we have devised a novel technique for altering the phase relationship between circadian transcriptional feedback oscillation and rhythmic PDF secretion by LN\(_{V}\)s using an ion channel–specific spider toxin to modify voltage-gated para Na\(^{+}\) channel inactivation in vivo. This technique relies on the previously reported “tethered-toxin” technology for cell-autonomous modulation of ionic conductances via heterologous expression of subtype-specific peptide ion channel toxins as chimeric fusion proteins tethered to the plasma membrane with a glycosylphosphatidylinositol (GPI) anchor [34]. Membrane-tethered toxins act only on ion channels present in the membrane of the cell that is expressing the toxin, and not on identical ion channels present on neighboring cells that do not express the toxin. In this study, we demonstrate for the first time, to our knowledge, the utility of the tethered-toxin technology in a transgenic animal, validating four different tethered spider toxin ion channel modifiers for use in Drosophila. Focusing on one of these toxins, GPI-tethered Australian funnel-web spider toxin δ-ACX-T-Hv1a, which inhibits Drosophila para voltage-gated Na\(^{+}\) channel inactivation when coexpressed in Xenopus oocytes, we (1) validate the tethered-toxin technology for cell-autonomous modulation of ion channel biophysical properties in vivo in transgenic Drosophila, (2) demonstrate that the kinetics of para Na\(^{+}\) channel inactivation is a key parameter for determining the phase relationship between circadian transcriptional feedback oscillation and PDF secretion, and (3) provide experimental support for the hypothesis that PDF-secreting clock neurons entrain organismal and cellular rhythms via the temporal patterning of secreted PDF signals.

Results

Screen for Membrane-Tethered Ion Channel Toxins Active in Drosophila Circadian Clock Neurons

It has recently been demonstrated that peptide ion channel toxins from venomous predators can be heterologously
expressed as fusion proteins covalently tethered to GPI anchors inserted in the extracellular leaflet of the plasma membrane [34]. Membrane-tethered toxins are expressed as chimeric fusion protein comprising (from N to C termini) a secretory signal sequence, peptide toxin sequence, glycine-asparagine repeat hydrophilic linker, and GPI targeting sequence. Membrane-tethered toxins are targeted to the secretory pathway, where the signal sequence is cleaved, and the GPI targeting sequence is substituted by covalent bond to GPI, thus retaining the toxin on the surface of the cell in which it is expressed. The modulatory effects of these tethered toxins are limited to ion channels on the surface of the cells in which the toxin is expressed, and do not influence channels on neighboring cells not expressing membrane-tethered toxin [34].

We selected 19 different peptide ion channel toxins from the venoms of cone snails, scorpions, bees, and spiders (Table 1) for testing in vivo in membrane-tethered form in the nervous system of transgenic Drosophila melanogaster. Of the 19 membrane-tethered toxins tested, four of them cause complete embryonic/larval lethality when expressed pan-neuronally using an elav-GAL4 driver (Figure 1), indicating activity against functionally essential fly ion channel subtypes. Three of these four membrane-tethered toxins are derived from the venom of a fish-eating cone snail [42], has no effect when expressed pan-neuronally (Figure 1). Lack of effect of µO-MrVIA when expressed pan-neuronally indicates absence of activity against fly Na\(^+\) channels, at least in its tethered form. We thus utilize tethered µO-MrVIA as a negative control for all experiments. The fact that the only four toxins showing signs of bioactivity when expressed in membrane-tethered form in the Drosophila nervous system are derived from spiders, and not from ocean-dwelling cone snails, makes sense in light of the natural prey species of spiders and cone snails.

To determine whether these four tethered spider toxins are active against ion channels important for circadian pacemaker neuron function, we expressed membrane-tethered toxins solely in the LN\(_V\) subset of approximately 20 clock neurons that secrete PDF and are considered key pacemakers of the clock circuit [12,16,17]. Three of the four potent membrane-tethered toxins substantially disrupt free-running circadian rhythms of locomotor activity when expressed in PDF neurons using a pdf-GAL4 driver (Figure 2, Table S1). µ- ACTX-Hv1a or k-ACTX-Hv1c expression of any of these four toxins induces embryonic/early-larval lethality when expressed from any one of multiple independent chromosomal insertions. In contrast, µO-MrVIA, a blocker of vertebrate voltage-gated Na\(^+\) channels from the venom of a fish-eating cone snail [42], has no effect when expressed pan-neuronally (Figure 1).

| Toxin               | Origin                              | Ion Channel Specificity | Lethality* |
|---------------------|-------------------------------------|-------------------------|------------|
| µO-MrVIA            | Mollusc-hunting cone snail          | Na\(^+\)                 | –          |
| HnTXI               | Tarantula spider                    | Na\(^+\)                 | –          |
| HnTXII              | Tarantula spider                    | Na\(^+\)                 | –          |
| δ-ACTX-Hv1a         | Australian funnel-web spider        | Na\(^+\)                 | +          |
| Apamin              | Honey bee                           | SK-type Ca\(^{2+}\)-activated K\(^+\) | –          |
| Tertiapin           | Honey bee                           | Inward rectifier K\(^+\) | –          |
| BmBKTxI             | Asian scorpion                      | BK-type Ca\(^{2+}\)-activated K\(^+\) | –          |
| BmSkTxI             | Asian scorpion                      | SK-type Ca\(^{2+}\)-activated K\(^+\) | –          |
| Charybdotoxin       | North African scorpion              | Voltage-gated K\(^+\)    | +          |
| κ-ACTX-Hv1c         | Australian funnel-web spider        | T-type Ca\(^{2+}\)       | –          |
| Kurtxin             | South African scorpion              | L-type Ca\(^{2+}\)       | –          |
| o-TxVII             | Mollusc-hunting cone snail          | N-type Ca\(^{2+}\)       | –          |
| o-MVIIA             | Fish-hunting cone snail             | P/Q-type Ca\(^{2+}\)     | –          |
| o-MVIIc             | Fish-hunting cone snail             | Slow-inactivating Ca\(^{2+}\) | –          |
| o-ACTX-Hv2a         | Australian funnel-web spider        | Ca\(^{2+}\)              | +          |
| o-ACTX-Hv1c         | Australian funnel-web spider        | Ca\(^{2+}\)              | +          |
| o-AgalIII           | American funnel-web spider          | P-type Ca\(^{2+}\)       | –          |
| o-AgalIVA           | American funnel-web spider          | Insect presynaptic Ca\(^{2+}\) | +          |
| PLTXII              | Plectreurys tristis spider          | Insect presynaptic Ca\(^{2+}\) | +          |

*Minus signs (−) indicate the toxin is not lethal; plus signs (+) indicate the toxin is lethal.

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The table includes the Peptide Ion Channel Toxins Expressed Pan-Neuronally in Membrane-Tethered Form. The toxins are classified by their origin, ion channel specificity, and lethality. The lethality is indicated by minus signs (−) for non-lethal and plus signs (+) for lethal effects. The table also highlights the circadian phase couples peptide and behavior in Drosophila.
**Membrane-Tethered \( \delta \)-ACTX-Hv1a Dose-Dependently Inhibits Inactivation of *Drosophila* para \( Na^+ \) Channel When Coexpressed in *Xenopus* Oocytes**

\( \delta \)-ACTX-Hv1a has been shown to inhibit inactivation of insect and mammalian voltage-gated \( Na^+ \) currents [35]. The predominant *Drosophila* voltage-gated \( Na^+ \) channel is encoded by the *para* gene [45,46]. Functional expression of *para* in *Xenopus* oocytes is enhanced by coexpression of the tipE accessory subunit [47,48]. We coexpressed membrane-tethered \( \delta \)-ACTX-Hv1a with para/tipE to examine the effect of the tethered toxin on *para* voltage-gated \( Na^+ \) currents. Uninjected oocytes showed no voltage-gated inward currents (unpublished data). The inward \( Na^+ \) current recorded from oocytes injected with para/tipE complementary RNA (cRNA) exhibits voltage-dependent activation, with rapid and complete inactivation occurring within several milliseconds (Figure 3A). Coexpression of tethered \( \delta \)-ACTX-Hv1a dose-dependently inhibits the inactivation of *para* \( Na^+ \) current. We used the ratio of steady-state current to peak current during 40 ms of depolarization to \(-30 \) mV to compare the extent of inhibition by membrane-tethered \( \delta \)-ACTX-Hv1a of *para* \( Na^+ \) current inactivation. Coinjection of para/tipE and either \( H_2O \) or tethered PLTXII \( Ca^{2+} \) channel toxin cRNAs (1:1) induces inward \( Na^+ \) current with the current ratio of 0.023 ± 0.015 (\( n = 4 \)) or 0.029 ± 0.004 (\( n = 16 \)), respectively (mean ± standard error of the mean [SEM]). A low concentration of 1:1,000 tethered \( \delta \)-ACTX-Hv1a cRNA significantly decreased the inactivation of \( Na^+ \) current to a current ratio of 0.169 ± 0.018 (\( n = 21, p < 0.001 \)). A high concentration of toxin (1:10) completely abolishes inactivation of \( Na^+ \) current resulting in a current ratio of 1.005 ± 0.003 (\( n = 8, p < 0.001 \); Figure 3). In contrast, tethered \( \delta \)-ACTX-Hv1a has no effect on kinetics and amplitude of *Drosophila Shaker K\(^+\) current, which also exhibits fast activation and inactivation (Figure 3C), indicating retained specificity of membrane-tethered \( \delta \)-ACTX-Hv1a for inhibiting inactivation of voltage-gated \( Na^+ \) channels.

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**Figure 1. Pan-Neuronal Expression of Membrane-Tethered Spider Toxins Induces Lethality in Transgenic *Drosophila***

Flies homozygous for an *elav-GAL4* transgene were mated to flies heterozygous for the indicated UAS-toxin transgenes and a phenotypically marked balancer chromosome, such that one half of the progeny receive both *elav-GAL4* and UAS-toxin transgenes, while the other half of the progeny receive *elav-GAL4* and the balancer chromosome. Lethality was assessed as percent relative mortality, which is 100% if there are no viable adult flies with both transgenes, and 0% if there are the same number of viable adult flies with both transgenes as with *elav-GAL4* and the balancer. 

2). Membrane-tethered \( \omega \)-ACTX-Hv1c expression in PDF neurons using the *pdf-GAL4* driver, which is active in both PDF-expressing clock neurons as well as in neurosecretory cells in the thoracic ganglion starting in early larval development [8,17], causes death by approximately 1 wk posteclosion, suggesting the possibility of toxin-induced developmental defects. To eliminate such possible developmental effects of toxin expression, we exploited temperature-dependent temporal control of GAL4-driven transgene expression using a temperature-sensitive mutant form of GAL80 transcriptional repressor (GAL80<sup>ts</sup>), which binds to GAL4 and inhibits downstream transcription that would otherwise be activated by GAL4 [43,44]. At 18 °C, GAL80<sup>ts</sup> dominates GAL4’s activator function and is active at repressing transcription, but at 30 °C, it is inactive. Thus, GAL4-mediated transgene expression can be temporally activated by shifting flies that also express GAL80<sup>ts</sup> from 18 °C to 30 °C. Flies expressing membrane-tethered \( \omega \)-ACTX-Hv1c with both *pdf-GAL4* and *tub-GAL80<sup>ts</sup>* were allowed to develop to adulthood at 18 °C, and then were shifted to 30 °C to induce membrane-tethered \( \omega \)-ACTX-Hv1c expression. These flies have a normal lifespan, but exhibit circadian rhythm defects of arrhythmicity or complex rhythmicity, as shown in Figure 2 and Table S1. These results indicate the presence of functionally important ion channels sensitive to \( \kappa \)-ACTX-Hv1c, \( \omega \)-ACTX-Hv1c, and \( \delta \)-ACTX-Hv1a in PDF-secreting LN<sub>V</sub> clock neurons.

**Functional Expression of Membrane-Tethered \( \delta \)-ACTX-Hv1a in PDF-Secreting LN<sub>V</sub> Pacemaker Neurons Dramatically Alters Spontaneous Electrical Activity**

To confirm membrane targeting of membrane-tethered \( \delta \)-ACTX-Hv1a in vivo, we examined the expression of Myc-tagged \( \delta \)-ACTX-Hv1a in PDF-secreting clock neurons. The ten-amino acid Myc epitope tag is located in the middle of the glycine-asparagine repeat hydrophilic linker domain. Brains of flies with two, four, or six copies of UAS-\( \delta \)-ACTX-Hv1a and *pdf-GAL4* were double immunostained with anti-Myc and anti-PDF fluorescence, with parental 4\( \times \)UAS-\( \delta \)-ACTX-Hv1a flies as negative control for anti-Myc staining. As shown in Figure 4, control UAS-\( \delta \)-ACTX-Hv1a flies show no anti-Myc immunofluorescence in the LN<sub>V</sub> cell bodies or terminals. Anti-PDF labels the cell bodies of PDF neurons (LN<sub>V</sub> neurons) and their processes, including dorsalmedial terminals of sLN<sub>V</sub> (Figure 4A) and projection of large LN<sub>V</sub> s (ILN<sub>V</sub>s) to the contralateral optic lobe through posterior optic tract.
toxin from different insertions are statistically significant by χ² test; for each of the χ² values, p < 0.0001. pdf > PLTXII flies were not statistically distinguishable from control, n, number of flies tested.

(C) A summary of the percentages of pdf,tub > δ-ACTX-Hv1c flies exhibiting behavioral phenotypes at 30°C. The difference in proportion of behavioral phenotypes between control pdf,tub > μ-MtVIA flies and experimental pdf,tub > δ-ACTX-Hv1c from different insertions are statistically significant by χ² test; for each of the χ² values, p < 0.0001. n, number of flies tested.

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Figure 2. Behavioral Arrhythmicity or Complex Rhythmicity Induced by Membrane-Tethered Toxin Expression

(A) Double-plotted locomotor actograms of representative individual male progeny of the indicated genotypes are shown, spanning 14 d in DD after release from diurnal 12-h:12-h LD entraining conditions. The bar above each actogram indicates subjective day (gray) and subjective night (black). pdf,δ-ACTX-Hv1c or pdf,tub > μ-MtVIA control flies expressing μ-MtVIA in PDF neurons (LN₃s) exhibit a single statistically significant free-running rhythm of locomotor activity. tub indicates the presence of a tub-GAL80ts transgene. tub-GAL80ts is a temperature-sensitive mutant form of the GAL80 transcriptional repressor. At the permissive temperature of 18°C, GAL80ts is active at repressing GAL4-mediated transcription, but at the restrictive temperature of 30°C, it is inactive. Therefore, GAL4-mediated toxin expression can be temporally activated by shifting adult pdf,tub > μ-MtVIA or pdf,tub > δ-ACTX-Hv1c flies from 18°C to 30°C. In contrast, pdf > δ-ACTX-Hv1a flies or pdf > δ-ACTX-Hv1c flies expressing toxin in the LN₃s frequently exhibit behavioral arrhythmicity or complex rhythmicity of locomotor activity. PLTXII expression in the LN₃s has no behavioral effect on locomotor activity. pdf,tub > δ-ACTX-Hv1c flies expressing δ-ACTX-Hv1c driven by pdf > GAL4 and tub-GAL80ts were allowed to develop to adult stage at 18°C and tested for behavior at 30°C, and exhibit behavioral arrhythmicity or complex rhythmicity in comparison to control pdf,tub > μ-MtVIA flies. The asterisk (*) in the bottom row of actograms indicates 2 d during which the locomotor monitoring system was inoperative.

(B) A summary of the percentages of flies exhibiting arrhythmic (red), complex rhythmic (yellow), or single rhythmic (blue) locomotor activity asayed over the first 14 d in DD. The difference in proportion of behavioral phenotypes between control pdf > μ-MtVIA flies and experimental pdf > δ-ACTX-Hv1a or pdf > δ-ACTX-Hv1a flies expressing (unpublished data). Flies expressing δ-ACTX-Hv1a in LN₃s exhibit red anti-Myc immunofluorescence colocalized with anti-PDF green fluorescence in the cell bodies of LN₃s. δ-ACTX-Hv1a expression dose-dependently increases anti-Myc immunofluorescence detected in the cell bodies of both LN₃s and sLN₂s, as well as sLN₅ dorso-medial terminals (Figure 4). Anti-Myc immunofluorescence in sLN₃ dorso-medial terminals is not seen in brain hemispheres of pdf > δ-ACTX-Hv1a or pdf > δ-ACTX-Hv1a flies. In pdf > δ-ACTX-Hv1a flies, 11 out of 16 hemispheres (68.8%) exhibit detectable anti-Myc immunofluorescence in the sLN₅ dorso-medial terminals (Figure 4B). Projection of sLN₃ to the opposite optic lobes through the posterior optic tract is also visualized with anti-Myc immunofluorescence in these brains (unpublished data). These results establish that membrane-tethered δ-ACTX-Hv1a is expressed in LN₅ clock neurons and is transported throughout their neuronal arbors. Membrane-tethered ω-ACTX-Hv1c, a Ca²⁺ channel blocker, expression in LN₅s is shown in Figure S1. High-resolution confocal images of the dorso-medial terminals of sLN₅s expressing membrane-tethered ω-ACTX-Hv1c demonstrate green anti-Myc fluorescence signal surrounding “puncta” of red anti-PDF signal. This is consistent with targeting of ω-ACTX-Hv1c to the plasma membrane of the terminals that encloses regions of concentration of PDF-containing dense-core vesicles. These results indicate that membrane-tethered spider toxins are expressed by LN₅ clock neurons and targeted to the plasma membrane where they can interact with their target ion channels.

In order to confirm functional expression of membrane-tethered δ-ACTX-Hv1a, we performed whole-cell electrophysiological recordings on LN₅s clock neurons expressing δ-ACTX-Hv1a. Previous studies indicate that LN₅ RMP and AP firing rate is regulated by the circadian clock to encode time of day [49,50]. In 12-h:12-h light:dark (LD) conditions, LN₅ RMP varies within the range of -40 mV to -70 mV. For any given cell steady state, LN₅ RMP remains relatively stable, albeit frequently with approximately 5-10-mV membrane potential oscillation. In the representative example shown in Figure 5A, this WT LN₅ RMP is relatively stable at -40 mV. Membrane-tethered δ-ACTX-Hv1a-expressing LN₅ RMP exhibits dramatically different membrane-activity from WT LN₅s. A representative example is shown in Figure 5B. Membrane-tethered δ-ACTX-Hv1a-expressing LN₅ membrane potential oscillates in a very wide range from approximately -140 to approximately 0 mV. Membrane-tethered δ-ACTX-Hv1a-
expressing ILNv RMP becomes gradually depolarized, and spontaneous AP firing rate increases as the membrane potential level increases. When the RMP reaches approximately -40 mV, a burst of 5–30 APs lasting approximately 0.2–0.5 s occurs. After the burst, the membrane potential remains at a depolarized plateau (-15–0 mV) for 2–7 s. Following the plateau, membrane potential repolarizes rapidly down to approximately -140 mV within 2 s. Following this huge hyperpolarization, membrane potential gradually depolarizes to eventually initiate another cycle of this unique behavior. We have recorded from 53 membrane-tethered δ-ACTX-Hv1a expressing ILNv-s, and 36 of them exhibit this basic cyclic temporal pattern of membrane potential. However, the exact frequency of these cycles, the duration of AP bursts and depolarized plateau, and the number of APs during the AP burst, exhibit substantial variation from cell to cell, even when expressing the same number of copies of δ-ACTX-Hv1a transgene. ILNv-s expressing different numbers of δ-ACTX-Hv1a transgene do not show consistent differences in their membrane potential dynamics. Since membrane-tethered δ-ACTX-Hv1a inhibits inactivation of para voltage-gated Na⁺ channels (Figure 3), the AP burst and prolonged depolarized plateau in membrane-tethered δ-ACTX-Hv1a-expressing ILNv-s most likely involve sustained opening of para channels, substantial Na⁺ influx, and increased intracellular [Na⁺]. We thus tested the hypothesis that the massive postplateau hyperpolarization well below the reversal potential for K⁺ is induced by electrogenic Na⁺/K⁺-ATPase pump.
currents expelling all that intracellular Na\(^+\), by blocking those currents with ouabain [51]. In the representative example shown in Figure 5C, the application of 0.1 mM ouabain for 1 min substantially reduces the massive postplateau hyperpolarizations in membrane-tethered \(\delta\)-ACTX-Hv1a–expressing LN\(_V\)s, and this effect is at least partially reversible. These results suggest that Na\(^+\)/K\(^+\)-ATPase underlies the postplateau hyperpolarization.

**LN\(_V\) Expression of Membrane-Tethered \(\delta\)-ACTX-Hv1a Disrupts Free-Running Rhythms of Locomotor Activity**

To determine whether these physiological modifications of LN\(_V\)s induced by expression of membrane-tethered \(\delta\)-ACTX-Hv1a affect the function of the circadian control network, we examined free-running locomotor activity patterns of flies expressing either membrane-tethered \(\delta\)-ACTX-Hv1a or \(\mu\)-MrVIA specifically in the PDF-expressing LN\(_V\)s using pdf-GAL4 driver. Control pdf\(\rightarrow\mu\)-O-MrVIA flies expressing membrane-tethered \(\mu\)-O-MrVIA exhibit rhythmic free-running circadian behavior of locomotor activity with a single statistically significant period of approximately 24.4 h (Figure 6A), of 298 \(\mu\)-O-MrVIA–expressing flies tested, 93% of them exhibited a single statistically significant periodogram peak (Figure 6, Table S1). In contrast, substantial numbers of experimental flies expressing membrane-tethered \(\delta\)-ACTX-Hv1a in the LN\(_V\)s exhibit arrhythmicity or complex rhythmicity (i.e., multiple superimposed free-running circadian rhythms of locomotor activity with different periods: one of approximately 25 h and one of approximately 22 h). Free-running behavioral effects induced by membrane-tethered \(\delta\)-ACTX-Hv1a expression in the LN\(_V\)s, either with single independent chromosomal insertion, or with multiple chromosomal insertions are relatively similar. The remaining rhythmic \(\delta\)-ACTX-Hv1a–expressing flies exhibit single-period free-running circadian rhythms with period corresponding to either the long or short period of complex rhythmicity, suggesting a similar effect on the circadian control network in these flies, albeit with a different behavioral manifestation (unpublished data). Arrhythmicity or complex rhythmicity is only rarely observed in control pdf\(\rightarrow\mu\)-O-MrVIA flies, which almost always exhibit single free-running rhythms (Figure 6, Table S1). The differences in proportion of behavioral phenotypes between membrane-tethered \(\delta\)-ACTX-Hv1a–expressing flies and control (either membrane-tethered \(\mu\)-O-MrVIA–expressing flies or parental UAS flies) were all statistically significant (\(\chi^2; p < 0.001\)).

To exclude a role for developmental effects of membrane-tethered \(\delta\)-ACTX-Hv1a expression in inducing these behavioral phenotypes, we exploited the GAL80\(^{ts}\) system for temporal control of GAL4-driven transgene expression. Flies possessing UAS-\(\delta\)-ACTX-Hv1a, pdf-GAL4, and tub-GAL80\(^{ts}\) transgenes were allowed to develop at 18 °C from egg to adult in the absence of \(\delta\)-ACTX-Hv1a expression, and then shifted to 30 °C to induce membrane-tethered \(\delta\)-ACTX-Hv1a expression. Experimental pdf\(\rightarrow\delta\)-ACTX-Hv1a flies exhibit arrhythmicity or complex rhythmicity at 30 °C similar to pdf\(\rightarrow\delta\)-ACTX-Hv1a flies (Figure S2), indicating that the behavioral phenotype caused by membrane-tethered \(\delta\)-ACTX-Hv1a expression in the LN\(_V\)s is not due to developmental effects. The percentage of arrhythmic or complex rhythmic flies expressing \(\delta\)-ACTX-Hv1a from two or four copies of UAS-\(\delta\)-ACTX-Hv1a is significantly greater
Figure 6. PDF-Secreting LNv Clock Neuron Expression of Membrane-Tethered δ-ACTX-Hv1a Disrupts Free-RunningBehavioral Rhythms

Flies expressing membrane-tethered μO-MvIA or δ-ACTX-Hv1a from indicated numbers of UAS transgenes using pdf-GAL4 driver, as well as indicated nonexpressing parental UAS and pdf-GAL4 flies, were entrained in 12-h:12-h LD conditions before release into DD. Double-plotted normalized actograms depict averaged locomotor activity for indicated genotypes of flies from a representative experiment. Bar graph shows proportions of flies with the indicated behavioral phenotypes as assessed by Lomb-Scargle periodogram analysis of all pooled experiments. Control flies expressing membrane-tethered μO-MvIA or parental transgenic lines not expressing any membrane-tethered peptide exhibit rhythmic locomotor activity with a single free-running rhythm. In contrast, most flies expressing membrane-tethered δ-ACTX-Hv1a exhibit arrhythmicity or complex rhythmicity. The averaged actograms reveal this complex rhythmicity as multiple superimposed free-running rhythms of different periods.

(A) The averaged actograms of all flies (including rhythmic and arrhythmic flies) of the indicated genotypes from a representative experiment are shown. pdf>μO-MvIA flies exhibit locomotor rhythm with a single period of approximately 24.4 h. In contrast, pdf>δ-ACTX-Hv1a flies exhibit complex rhythmicity with simultaneously superimposed rhythms with periods of approximately 25 h and approximately 22 h.

(B) A summary of the percentages of flies exhibiting arrhythmic (red), complex rhythmic (yellow) and single rhythmic (blue) locomotor activity assayed over the first 14 d in DD. The difference in proportion of behavioral phenotypes between each of the control flies, either pdf>μO-MvIA or UAS-toxin strain or pdf-GAL4 driver, and experimental pdf>δ-ACTX-Hv1a flies are each statistically significant by χ² test; for each of the χ² values, p < 0.0001. n, number of flies tested.

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than control pdf>μO-MrVIA or UAS-toxin flies (χ²; p < 0.0001). The exact manifestation of complex rhythmicity in pdf,tub>δ-ACTX-Hv1a flies is somewhat different than in pdf>δ-ACTX-Hv1a flies. The latter tend to simultaneously manifest strong short- and long-period rhythms immediately after transfer from 12-h:12-h LD into DD, while the former tend to initially manifest mainly a long-period rhythm and then, after about a week in DD, transition to predominately just a short-period rhythm, as seen in published studies of complex rhythmic flies [20]. The most likely interpretation of this particular form of complex rhythmicity at the cellular level is—as in the case in which strong short- and long-period rhythms manifest themselves simultaneously—that there are independently free-running subsets of clock neurons running with short and long periods, with the difference that their respective ability to gate locomotor activity waxes and wanes reciprocally over time in DD. These results indicate that the physiological changes induced by LNv expression of membrane-tethered δ-ACTX-Hv1a interfere with the ability of the circadian control network to drive coherent free-running behavioral rhythms in constant darkness.

Membrane-Tethered δ-ACTX-Hv1a Expression in LNv Clock Neurons Phase Advances Both PDF Cycling in Dorsomedial sLNv Nerve Terminals and Morning Anticipatory Locomotor Activity

PDF-secreting LNv are important not only for driving free-running locomotor rhythms in DD, but also for generating the “morning” peak of locomotor activity that anticipates lights-on in 12-h:12-h LD conditions [12,16,17,30,31]. To determine whether LNv expression of membrane-tethered δ-ACTX-Hv1a interferes with the LNv-dependent morning activity peak, we performed a refined analysis of the average relative activity in LD of flies expressing membrane-tethered μO-MrVIA or δ-ACTX-Hv1a using pdf-GAL4 driver. As shown in Figure 7A, control μO-MrVIA–expressing flies exhibit robust morning and evening circadian anticipatory peaks of locomotor activity. Membrane-tethered δ-ACTX-Hv1a–expressing pdf>δ-ACTX-Hv1a flies also exhibit robust morning and evening circadian anticipatory peaks of locomotor activity, albeit with phase-advanced morning activity (Figure 7A) compared to control pdf>μO-MrVIA flies. We quantified phase advance of morning activity peaks by computing an anticipation phase score for each fly, defined as the ratio of total relative activity in the 3-h period before lights-on to that after. Increase in anticipation phase scores reflects greater anticipatory activity before lights-on compared to after, thus quantifying phase advances in anticipation. As seen in Figure 7B, membrane-tethered δ-ACTX-Hv1a expression induces dose-dependent increase in morning anticipation phase score (p < 0.001), indicating dose-dependent phase advance of morning anticipation caused by inhibition of para Na⁺ channel inactivation in LNv morning cells. The dose-dependent phase advance is also apparent in the shape of envelope of relative activity occurring before lights-on, being concave for pdf>μO-MrVIA flies and then becoming progressively more convex with increasing dose of membrane-tethered δ-ACTX-Hv1a. These effects are observed in multiple independent replicate experiments (unpublished data).

PDF has been hypothesized to be a daily phase signal transmitted from LNvS to other clock neurons (and potentially non-clock targets) that entrains both clock neuron cellular oscillation and locomotor rhythms [27]. To determine whether advanced phase of morning activity in flies expressing membrane-tethered δ-ACTX-Hv1a is due to altered phase of PDF secretion rhythms, we compared the daily rhythm of accumulation of PDF in the sLNv dorsomedial terminals (thought to reflect daily rhythm of secretion; [18,29]) of flies expressing either δ-ACTX-Hv1a or μO-MrVIA in the LNvS in LD. There is a recent study demonstrating absence of detectable PDF cycling in the sLNv terminals of a particular transgenic strain of flies that nonetheless still exhibit normal circadian locomotor activity [52]. However, this result is still consistent with a key role for cyclic secretion in circadian rhythms, and with cyclic PDF accumulation as a readout of cyclic secretion, as steady-state accumulation will only reflect secretion rhythms when synthesis rate and secretion rate are
similar in magnitude; under conditions in which synthesis rate outpaces secretion rate, a rhythm of secretion may not result in a detectable rhythm of steady-state accumulation. Furthermore, there is functional, albeit indirect, evidence in the literature that rhythmic-activity–dependent PDF secretion is important for circadian rhythmicity [18,20,49,50]. Therefore, it is reasonable to conclude that rhythmic steady-state accumulation of PDF reflects rhythmic PDF secretion.

In LD conditions, control flies expressing membrane-tethered μO-MrVIA in the LNVs exhibit significantly greater anti-PDF immunofluorescence in the LNv dorso medial terminals at zeitgeber time (ZT)2 and ZT6, in comparison to the night at ZT14 and ZT18 (p < 0.001; Figure 7C). In contrast, PDF accumulation in the LNv dorso medial terminals of experimental flies expressing membrane-tethered δ-ACTX-Hv1a in the LNv cycles with advanced phase, peaking late night, rather than early in the morning, with peak anti-PDF immunofluorescence at around ZT22 (p < 0.001). This approximately 4-h phase advance of PDF cycling induced by LNv expression of membrane-tethered δ-ACTX-Hv1a, in combination with the induced phase advance of morning anticipation, provides experimental support for the hypothesis that the phase of rhythmic PDF secretion determines the phase of morning anticipation. Figure S4 shows an independent replicate of this experiment, confirming δ-ACTX-Hv1a–induced phase advance of PDF accumulation.

We also examined the effect of membrane-tethered δ-ACTX-Hv1a on accumulation of PDF in the sLNv dorso medial terminals in constant darkness. Anti-PDF immunoreactivity in the dorso medial terminals was assayed on the 2nd, 4th, or 6th d after release from LD entraining conditions into DD. As shown in Figure 8, LNv expression of membrane-tethered δ-ACTX-Hv1a induces phase advance of PDF accumulation in DD consistent with the effect in LD. On the 2nd d in DD (DD-D2), anti-PDF immunofluorescence in the sLNv dorso medial terminals of control membrane-tethered μO-MrVIA–expressing flies peaks at CT2 to CT6 (p < 0.001). In contrast, experimental flies expressing membrane-tethered δ-ACTX-Hv1a exhibit phase-advanced peak PDF accumulation at circadian time (CT)22 to CT2 (p < 0.001). On DD-D4, control pdf>μO-MrVIA flies exhibit peak PDF accumulation at CT6–CT10 (p < 0.001). Experimental
pdf-Δ-ACTX-Hv1a flies exhibit phase advance of PDF cycling with peak at CT22 (p < 0.001). On DD-D6, control pdf-μO-MrVIA flies exhibit peak PDF accumulation at CT10 (p < 0.001), while experimental pdf-Δ-ACTX-Hv1a flies exhibit peak PDF accumulation around CT22 (p < 0.001). The gradual accumulation of a phase delay relative to the 24-h d of PDF oscillation in control pdf-μO-MrVIA flies over 6 d in DD is consistent with their free-running behavioral period of greater than 24 h (Figures 2 and 6). The phase advance of PDF accumulation in sLNv terminals of membrane-tethered Δ-ACTX-Hv1a-expressing flies in LD and DD relative to μO-MrVIA-expressing control and the correlated phase advance of morning anticipatory locomotor activity support the hypothesis that phase of LNv PDF secretion rhythms determines phase of morning anticipation.

Membrane-Tethered Δ-ACTX-Hv1a Expression in LNv Neurons Induces a Phase Shift between Par Domain Protein 1 (PDP1) and PDF Oscillations in sLNv's

The results described above show that membrane-tethered Δ-ACTX-Hv1a expression in PDF-secreting LNv's induces a phase-advance of both sLNv PDF secretion rhythms and morning anticipatory locomotor behavior in LD, and disrupts free-running locomotor rhythms in DD. In order to determine the relationship between these phenotypes and cellular transcriptional feedback oscillation, par domain protein 1 (PDP1) clock protein levels were assayed in the sLNv, dorsal lateral neuron (LNvD), dorsal neuron (DN1), and DN2 neurons of flies expressing membrane-tethered Δ-ACTX-Hv1a in the LNv's. PDP1 is an oscillating transcription factor that has been implicated in a second interlocking circadian transcriptional feedback loop [8]. Regardless of the validity of the assertion that PDP1 does not participate in the cellular time-keeping mechanism and is solely a clock output factor [53], PDP1 is a high-fidelity phase marker for circadian transcriptional feedback oscillation and has been used as such by multiple laboratories [18,32,54,55]. Flies expressing membrane-tethered μO-MrVIA or Δ-ACTX-Hv1a are entrained in LD conditions and then released into DD. Brains are fixed at different circadian times, followed by anti-PDP1 immunofluorescence. Anti-PDP1 immunofluorescence is detected in nuclei of clock neurons, and fluorescence levels are quantified by counting the number of stained nuclei in the various anatomical cell layers. To confirm the reliability of this counting method for assessing cellular transcriptional oscillation, we compared it to the established background-subtracted pixel intensity method [13,18,54]. As shown in Figure S3, these two methods reveal the identical temporal patterns of PDP1 oscillation on DD-D4 in DN1 neurons of flies expressing either membrane-tethered μO-MrVIA or Δ-ACTX-Hv1a in LNv's. This establishes the reliability of counting PDP1-stained nuclei as a much less labor-intensive method for assessing transcriptional feedback oscillation at the cellular level.

Consistent with previous studies [8,13,18,54], control pdf-μO-MrVIA flies expressing membrane-tethered μO-MrVIA in the LNv's exhibit PDP1 oscillation in sLNv's with peak levels late at night or subjective night and trough levels...
during day or subjective day (Figure 9). In LD or on DD-D2, sLNv exhibit peak of PDP1 accumulation at CT18–CT22 with approximately four stained sLNVs detected ($p < 0.001$). PDP1 accumulation in sLNv of control pdf-/O-MrVIA flies peaks at CT18–CT22 and at CT2 by DD-D4 and CT22 to CT2 by DD-D6 ($p < 0.001$). PDP1 accumulation in the sLNv of pdf-/d-ACTX-Hv1a flies exhibits temporal pattern similar to that of control pdf-/O-MrVIA flies in LD and on DD-D2 and DD-D4, with peak at CT18–CT22 and trough at CT6–CT10 ($p < 0.001$). On DD-D6, peak PDP1 accumulation in the sLNv of pdf-/d-ACTX-Hv1a flies is slightly advanced to CT18 compared with CT22–CT2 of control pdf-/O-MrVIA flies ($p < 0.001$). Differences among different genotypes at different circadian times were compared using ANOVA with Tukey-Kramer multiple comparisons. *n* = 22 brain hemispheres for each experimental group.

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Figure 9. Membrane-Tethered d-ACTX-Hv1a Expression in LNv Neurons Induces No Phase Advance of PDP1 Oscillation in the sLNv in LD

Red anti-PDP1 immunofluorescence (A) reveals PDP1 accumulation in sLNv nuclei. Bar graphs (B) show the number of neurons labeled with anti-PDP1 immunofluorescence (mean ± SEM). Representative images of sLNv from flies of the indicated genotypes at the indicated time points are shown. N.S. indicates complete absence of sLNv anti-PDP1 staining above background for that time point and genotype. In LD or on DD-D2, sLNv of control pdf-/O-MrVIA flies exhibit peak PDP1 staining at ZT/CT18–ZT/CT22 ($p < 0.001$). Peak of PDP1 accumulation in sLNv expressing O-MrVIA is slightly delayed to CT18–CT22 and CT2 by DD-D4 and CT22 to CT2 by DD-D6 ($p < 0.001$). PDP1 accumulation in the sLNv of pdf-/d-ACTX-Hv1a flies exhibits temporal pattern similar to that of control pdf-/O-MrVIA flies in LD and on DD-D2 and DD-D4, with peak at ZT/CT18–ZT/CT22 and trough at ZT/CT6–ZT/CT10 ($p < 0.001$). On DD-D6, peak PDP1 accumulation in the sLNv of pdf-/d-ACTX-Hv1a flies is slightly advanced to CT18 compared with CT22–CT2 of control pdf-/O-MrVIA flies ($p < 0.001$). Differences among different genotypes at different circadian times were compared using ANOVA with Tukey-Kramer multiple comparisons. *n* = 22 brain hemispheres for each experimental group.

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Membrane-Tethered d-ACTX-Hv1a Expression in LNv Neurons Induces Short-Period PDP1 Oscillation in Non-LNv Neurons

We used anti-PDP1 immunofluorescence to assess the effects of phase-advanced PDF cycling in d-ACTX-Hv1a-expressing PDF-secreting LNv on cellular oscillation of non-LNv clock neurons that express functional PDF receptor [26].
Control pdf\textgreater\muO-MrVIA flies exhibit a similar temporal pattern of PDP1 accumulation in the LN\textsubscript{D} cell group in both LD and DD conditions, with peak levels late at night or subjective night, and trough levels during day or subjective day (Figure 10; \textit{p}, 0.001). Experimental pdf\textgreater\delta-ACTX-Hv1a flies exhibit similar temporal pattern of PDP1 oscillation to control pdf\textgreater\muO-MrVIA flies on DD-D2 with peak at CT22 and trough at CT10. On DD-D4, LN\textsubscript{S}s of pdf\textgreater\delta-ACTX-Hv1a flies exhibit damped peak of PDP1 oscillation at CT22 and trough at CT22CT6 (\textit{p} < 0.001). However, by DD-D6, a phase advance of PDP1 accumulation in the nuclei of LN\textsubscript{S}s induced by \delta-ACTX-Hv1a expression manifests, with a peak at CT10 and trough at CT22-CT2 (\textit{p} < 0.001). \delta-ACTX-Hv1a expression in LN\textsubscript{S}s does not affect PDP1 oscillation in LN\textsubscript{D} neurons in LD. Differences among different genotypes at different circadian times were compared using ANOVA with Tukey-Kramer multiple comparisons. \textit{n} = 22 brain hemispheres for each experimental group, and error bars indicate SEM.

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Control pdf\textgreater\muO-MrVIA flies exhibit a similar temporal pattern of PDP1 accumulation in the LN\textsubscript{D} cell group in both LD and DD conditions, with peak levels late at night or subjective night, and trough levels during day or subjective day (Figure 10; \textit{p} < 0.001). This constant phase of PDP1 accumulation over 6 d in DD indicates that LN\textsubscript{D} cellular oscillation free-runs with approximately 24-h period in control flies. Experimental pdf\textgreater\delta-ACTX-Hv1a flies exhibit a similar temporal pattern of LN\textsubscript{D} PDP1 oscillation to control pdf\textgreater\muO-MrVIA flies in LD and on DD-D2. However, by DD-D4, PDP1 oscillation in LN\textsubscript{S}s of experimental pdf\textgreater\delta-ACTX-Hv1a flies has begun to phase shift relative to control, with blunting of peak accumulation late in subjective night (\textit{p} < 0.001). By DD-D6, the peak of LN\textsubscript{D} PDP1 accumulation in flies expressing membrane-tethered \delta-ACTX-Hv1a in LN\textsubscript{S}s has phase advanced to CT10. This phase advance indicates that membrane-tethered \delta-ACTX-Hv1a expression in the LN\textsubscript{S}s induces an increase in the pace of LN\textsubscript{D} cellular oscillation. The approximately 12-h phase advance that accumulates after 6 d in DD is consistent with the approximately 22-h free-running period of the short-period component of the complex locomotor rhythms exhibited by many pdf\textgreater\delta-ACTX-Hv1a flies.

In control pdf\textgreater\muO-MrVIA flies, DN1s exhibit a peak of PDP1 accumulation in LD around ZT18 (Figure 11). In DD conditions, peak of PDP1 accumulation in DN1s of control flies broadens, but does not exhibit any phase shift. Experimental pdf\textgreater\delta-ACTX-Hv1a flies expressing membrane-tethered \delta-ACTX-Hv1a in LN\textsubscript{S}s exhibit a similar pattern of PDP1 oscillation as control pdf\textgreater\muO-MrVIA flies in LD, with peak around ZT18 (\textit{p} < 0.001). In DD, however, PDP1 oscillation of DN1s in pdf\textgreater\delta-ACTX-Hv1a flies gradually phase advances with accumulation of an approximately 12-h delay after 6 d in DD, consistent with the approximately 22-h free-running locomotor rhythm component of complex rhythmic pdf\textgreater\delta-ACTX-Hv1a flies.

The DN2s of control pdf\textgreater\muO-MrVIA flies exhibit peak PDP1 accumulation at ZT18–ZT22 in LD (Figure 12). The DN2s of control flies exhibit a peak of PDP1 accumulation at CT14 on DD-D2, CT10 on DD-D4, and CT6 on DD-D6. This gradual phase advance of DN2 PDP1 oscillation out of synchrony with the other cell groups after release into DD (Figures 9–12) is consistent with published observations of
DN2 cellular oscillation assayed with anti-PER, anti-TIM, or anti-PDP1 immunostaining [18,54,56] and reflects inherent accelerated cellular oscillation in DN2s in DD. Experimental pdf\textsuperscript{-}\textit{d-ACTX-Hv1a} flies also exhibit gradual phase advance of PDP1 oscillation after release into DD, peaking around CT0 on DD-D6, but this advance is greater than in control flies, which peak around CT6. Acceleration of non-LNV clock neurons in free-running DD conditions has also been observed in flies in which LNV membrane excitability is altered by ectopic ion channel subunit expression [18,54]. Taken together, all these results indicate that altered membrane activity of LN\textsubscript{V}s induced by expression of membrane-tethered \textit{d-ACTX-Hv1a} (1) phase advances sLN\textsubscript{V} PDF secretion rhythms and morning anticipatory locomotor activity in LD without phase advance of sLN\textsubscript{V} PDP1 oscillation and (2) accelerates free-running PDP1 oscillation of LN\textsubscript{V}, DN1, and DN2 PDF receptor-expressing clock neurons in DD.

Discussion

In order to begin to dissect the contribution of particular ionic conductances to the cellular physiological function of the circadian control network, we have employed an adaptation of the tethered-toxin methodology in which peptide ion channel toxins are expressed as chimeric fusion proteins with an N-terminal secretory signal sequence and a C-terminal GPI anchor targeting signal [34]. Membrane-tethered toxins derived from blockers of vertebrate ion channels have been demonstrated to cell-autonomously block their target ion channels with expected pharmacological specificity, when transiently expressed in \textit{Xenopus} oocytes or zebrafish muscle fibers [34]. Here, we extend this system to the use of spider toxins active against insect ion channels in transgenic \textit{Drosophila melanogaster}, developing and validating four membrane-tethered spider toxins with activity against particular voltage-gated Na\textsuperscript{+}, K\textsuperscript{+}, and Ca\textsuperscript{2+} channels. Each of these four toxins causes embryonic/larval lethality when expressed pan-neuronally (Figure 1), and three of them induce behavioral phenotypes when expressed in PDF-secreting LN\textsubscript{V} pacemaker neurons (Figure 2, Table S1). One of these four toxins, \textit{d-ACTX-Hv1a}, is a known inhibitor of inactivation of voltage-gated Na\textsuperscript{+} channels [35].

In order to elucidate mechanisms of intercellular communication in the circadian control network, we employ this...
A novel membrane-tethered spider toxin δ-ACTX-Hv1a as a tool for cell-autonomously inhibiting para Na\(^+\) channel inactivation in vivo in PDF-secreting LN\(_V\) pacemaker neurons of transgenic Drosophila melanogaster. When coexpressed in Xenopus oocytes with para voltage-gated Na\(^+\) channel, membrane-tethered δ-ACTX-Hv1a dose-dependently inhibits rapid inactivation, with complete abolition at the highest dose (Figure 3). When expressed in PDF-secreting LN\(_V\) pacemaker neurons, membrane-tethered δ-ACTX-Hv1a induces a dramatic change in LN\(_V\) membrane excitability (Figure 5). Wild-type (WT) LN\(_V\) neurons generally exhibit tonic or brief burst action potential firing patterns on a relatively stable baseline resting membrane potential (Figure 5; [49,50]). In contrast, LN\(_V\) clock neurons expressing membrane-tethered δ-ACTX-Hv1a exhibit a repetitive pattern of prolonged burst firing followed by a sustained plateau potential, which is in turn followed by a massive hyperpolarization that is slowly recovered from (Figure 5). The massive hyperpolarization is likely mediated by ouabain-sensitive Na\(^+\)/K\(^+\)-ATPase pump current (Figure 5). The physiological effect of membrane-tethered δ-ACTX-Hv1a is explicable as the consequence of induction of a noninactivating toxin-bound subpopulation of para Na\(^+\) channels.

We have performed these electrophysiological experiments on ILN\(_V\)s, despite the fact that it is the sLN\(_V\)s that are thought to underlie the morning anticipatory peak of locomotor activity and dominate free-running rhythmicity in DD (for review, see [11]). This is for technical reasons relating to the very small size of the sLN\(_V\)s and the difficulty of obtaining whole-cell electrophysiological recordings from them. It is, nonetheless, reasonable to consider the effects of membrane-tethered δ-ACTX-Hv1a on sLN\(_V\)s as a proxy for their effects on sLN\(_V\)s, given that the sLN\(_V\)s are certain to possess para Na\(^+\) channels (the only known Na\(^+\) channel encoded in the Drosophila genome[57,58]) and the Na\(^+\)/K\(^+\)-ATPase.

The alteration of LN\(_V\) membrane activity induced by expression of membrane-tethered δ-ACTX-Hv1a has profound effects on circadian rhythms at the cellular and organismal level. In LD conditions, LN\(_V\) expression of membrane-tethered δ-ACTX-Hv1a induces phase advance of both sLN\(_V\) terminal PDF cycling and the morning anticipatory peak of locomotor activity (Figure 7, Figure S4), but does not alter phase of sLN\(_V\) transcriptional feedback oscillation (Figure 9, Figure S4). In DD, LN\(_V\) expression of membrane-tethered δ-ACTX-Hv1a induces arrhythmic and complex behavioral rhythms (Figures 2 and 6), phase advance
of sLNv PDF rhythms (Figures 7 and 8), and acceleration of cellular oscillation in non-sLNv clock cells (Figures 10–12). It is interesting that the same manipulation induces a phase advance of morning activity in LD, while inducing complex and arrhythmic phenotypes in DD. The likely reason for this is that in LD, phase of transcriptional oscillation is dominated by cell-autonomous CRYPTOCHROME-dependent entrainment to the LD cycle [59–61]—thus synchronizing transcriptional rhythms in all clock neurons—whereas in DD, this strong synchronous entraining cue is absent.

These results lead to two important novel conclusions about the relationship between transcriptional feedback oscillation, membrane activity, rhythmic PDF secretion, and behavioral rhythms. First, our results support the conclusion that transcriptional feedback oscillation sets phase of rhythmic PDF secretion via modulation of membrane activity, as manipulation of sLNv membrane activity by inhibition of Na+ channel inactivation alters the phase relationship between sLNv transcriptional oscillation and sLNv PDF rhythms. The specific mechanism by which inhibition of para inactivation alters the phase relationship between transcriptional and peptide output rhythms is an important issue for future studies, as it is likely to shed light on the mechanisms by which transcriptional oscillation influences membrane activity [49]. Interestingly, sLNv expression of membrane-tethered δ-ACTX-Hv1a also induces an increase in the amplitude of sLNv PDF rhythms (Figures 7 and 8); the reason for an increase in amplitude likely relates to this mechanism of transcription-activity coupling.

Second, our results provide experimental support for the hypothesis that sLNv M cells communicate phase information to non-sLNv clock cells, and set the phase of behavioral rhythms, using rhythmic PDF secretion as the phase signal. Previous studies have shown that injected PDF can alter phase of cockroach locomotor rhythms [29] and that Drosophila PDF-secreting LNvs reset the phase daily both of some non-sLNv clock neurons and of locomotor rhythms [27]. Here, we show that phase of sLNv PDF cycling determines phase of morning anticipation and non-sLNv clock neuron cellular oscillation, thus establishing rhythmic PDF secretion by sLNvs as an entraining phase signal in the circadian control network.

The behavioral and cellular effects of δ-ACTX-Hv1a in DD raise the interesting question as to the precise cellular basis for the complex behavioral rhythms also induced by other manipulations of PDF signaling in the circadian control circuit [18,20,62]. Although it does not appear that any of the cell groups we have assayed via anti-PDP1 immunostaining exhibit detectable long-period cellular rhythms that would be consistent with the approximately 25-h long-period component of the complex behavioral rhythms (Table S1), it is possible that some subtypes of LNvs and/or DN1s are differentially affected by PDF signals, so as to induce short-period or long-period oscillations in few, or even single, neurons that are undetectable in the context of the anatomical group as a whole. As a corollary, it is possible that PDF has period-lengthening effects on some clock neurons and period shortening effects on others. Differences in the cellular consequences of manipulations that result in similar behavioral complex rhythms may thus be explained by the possibility that there are multiple free-running configurations of the circadian control circuit that result in similar behavioral complex rhythms—such that in different configurations, it is different particular subgroups of clock neurons that control long or short period locomotor rhythms.

These studies have a number of implications for further inquiry. The specific mechanism by which inhibition of para inactivation alters the phase relationship between transcriptional and peptide output rhythms is an important issue for future studies. In relation to the role of PDF as a phase signal from sLNv M cells to non-sLNv clock neurons and, possibly, other downstream targets, future studies are required to determine the identity of the particular PDF receptor-expressing cells that transduce the phase of sLNv PDF secretion into phase of morning anticipation. In relation to the tethered-toxin technology, our studies now establish the feasibility of this approach to cellular dissection of ion channel function in genetically targeted cellular subsets in the nervous system of a transgenic animal. The four membrane-tethered spider toxins we have developed and validated can now be applied to analysis of ion channel function in other circuits in the Drosophila nervous system. Future studies are also required to determine the cellular and biophysical basis of interference with circadian function by sLNv expression of membrane-tethered δ-ACTX-Hv1c and κ-ACTX-Hv1c.

Materials and Methods

Fly strains. pdf-GAL4 and pdf-gal4;UAS-DsRedII fly lines are as described previously [17,54,63]. pdf-GAL4 was recombined with tub-GAL80ts (from Bloomington Drosophila Stock Center) on the second chromosome. UAS-tox-in fly strains are generated via standard P element transformation techniques [64]. Membrane-tethered toxin transgenes were chemically synthesized with optimal Drosophila codon usage and an optimal Drosophila Kozak translation initiation sequence [AAA]. Encoded chimeric membrane-tethered toxin sequence is based on the long-linker version of Iban˜ ez-Tallon et al. [34], except Myc epitope tag is substituted for FLAG. pdf-GAL4 or pdf-GAL4;tub-GAL80ts virgin flies were crossed to UAS males to generate progeny for behavioral analysis or immunostaining. pdf-GAL4;UAS-DsRedII virgins were crossed with UAS males to generate progeny for electrophysiological recordings. Multiple independent chromosome insertions of the UAS-toxin transgene were recombined using classical genetic methods to generate second and third chromosomes bearing two independent insertions each for dose-dependent experiments.

Circadian behavioral analysis. Locomotor activity of individual adult male flies (1–4 d posteclosion) was measured at 25 °C using the TriKinetix infrared beam-crossing system. The flies were first monitored for 5–6 d in 12-h:12-h LD conditions, and free-running locomotor activity was then monitored in constant darkness (DD) for two further weeks. Total infrared beam crossings in 10-min bins were recorded and circadian rhythms were analyzed in 20-min blocks by Lomb-Scargle periodogram using Actinmetrics Clocklab software [65]. Significant circadian rhythmicity was defined as presence of a peak in periodogram power that exceeds the p = 0.05 significance line, and the percentages of arrhythmic flies were compared using chi-square analyses. Rhythm strength is defined by the height of the relevant periodogram peak, expressed in arbitrary units. Average numbers of activity events per half-hour bin per fly were also calculated, and histograms for relative activities were generated for LD behavior of the flies among 5 d. In addition, the morning anticipation phase score in LD was computed as relative activity in 3-h period before versus after lights-on transition for individual flies, and these peak phases among different genotypes were statistically analyzed by the ANOVA with Tukey-Kramer multiple comparisons.

Two-electrode voltage clamp recording of Xenopus oocytes. δ-ACTX-Hv1a and PLTXII were subcloned into pCS2þ plasmid for Xenopus oocyte expression. The gene constructs for cloning Drosophila voltage-gated Na+ channel τ subunit (para) and its auxiliary βII subunit (tipE) were from M. Wijffels [47,48] and Wijffels and Tipper (Rothamsted Research, Harpenden, United Kingdom). pBSC-ShH37 for Drosophila voltage-gated K+ channel, Shaker, is from L. Salkoff (Washington University, St. Louis, Missouri). Xenopus laevis oocytes

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were prepared, injected, and recorded using standard methods in ND96 solution [60]. Oocytes were injected with less than 50 nl in vitro transcribed cRNAs encoding para/tipE, and either β-ACTX-Hv1a or PLTXII. The full-strength (1:1) of para, tipE, β-ACTX-Hv1a, and PLTXII cRNAs were mixed at 1:1 ratio of volume, and injected into the oocytes. Two days after para/tipE cRNA injection, inward Na+
expression was determined using ANOVA with Tukey-Kramer multiple comparisons.

Clock neuron electrophysiology. Whole-cell recordings on ILNv of fly brain explants are performed as described [49,54]. Briefly, the brains of flies 3–7 post-dissection are dissected in isometric recording solution, which consists of (in mM): 101 NaCl, 3 KCl, 1 CaCl2, 4 MgCl2, 1.25 NaH2PO4, 5 glucose, 20.7 NaHCO3 (pH 7.2) with osmolarity of 290 mmol/kg. The brain is placed ventral side up and secured in a recording chamber with a mammalian brain-slice “hapt” holder and is continuously perfused with external solution bubbled with 95% O2/5% CO2 at room temperature. LNv are visualized by DsRed fluorescence, and the immediate area surrounding the ILNvs is enzymatically digested with floc placement of primate II (2 mg/ml, Sigma). Whole-cell recordings are performed using borosilicate standard-wall capillary glass pipettes (Sutter Instrument Company), and data are acquired with Axopatch 200B amplifier, Digidata 1200 A/D hardware, and pClamp 8.0 software (Axon Instruments). Recording pipettes are filled with internal solution consisting of (in millimolar): 102 potassium gluconate, 17 NaCl, 0.85 CaCl2, 4 Mg-ATP, 0.5 Na-GTP, 0.94 EGTA, and 8.5 HEPES (pH 7.2) and osmolarity of 235 mmol/kg. Gigaohm seals are achieved before recording in cell-attached configuration in voltage-clamp mode, followed by break-in to whole-cell configuration. Then current-clamp is employed to monitor RMP. All cell analysis was conducted in CellMaster.

Brain immunocytochemistry. Adult fly brains were dissected and processed for anti-PDP1 (1:2,000, rabbit) and anti-PDF (1:50, mouse) immunocytochemistry as described previously [18,54]. PDPI and PDF were visualized using a Cy3-conjugated anti-rabbit secondary antibody (1:200) and Cy2 (1:200) and Texas-red (1:300)-conjugated antibodies, respectively.

Supporting Information

Figure S1. Membrane-Tethered β-ACTX-Hv1c Localization in sLNv Dorosomedial Terminals

df -GALA driver flies were crossed to flies with UAS encoding Myc-tagged membrane-tethered-β-ACTX-Hv1c transgene. Adult brains of df -GALA flies or UAS-β-ACTX-Hv1c flies were processed for immunofluorescence with anti-Myc and anti-PDF antibodies. Green anti-Myc staining in the brains of β-ACTX-Hv1c-expressing flies colocalized with red anti-PDF neuropetide in the LNv cell bodies and throughout their terminals. Control UAS-β-ACTX-Hv1c fly brains exhibited no LNv anti-Myc staining. High resolution confocal images of the dorosomedial terminals of LNvs for the inset area show that green anti-Myc fluorescence strongly "puncta" of red anti-PDF signal. This is consistent with targeting of β-ACTX-Hv1c to the plasma membrane that encloses regions of concentration of PDF-containing dense-core vesicles.

Figure S2. Disrupted Circadian Behavior Caused by Membrane-Tethered β-ACTX-Hv1c Expression Is Not Due to Developmental Effects

df-GALA:tf-GAL80 driver flies were crossed to flies-UAS-toxin transgenic flies to generate progeny. Membrane-tethered β-ACTX-Hv1a or µ-MyVIA expression was induced by transferring progeny from 18°C to 30°C after eclosion, the temperature that inactivates tf-GAL80 and allows for GAL4-induced UAS-toxin expression. (A) The average actograms of all flies of the indicated genotypes are shown. Flies expressing µ-MyVIA exhibit a single rhythm of free-running locomotor activity. In contrast, flies expressing β-ACTX-Hv1c display free-running rhythms. (B) Percentages of flies exhibiting arrhythmic (red), complex rhythmic (yellow), and single rhythmic (blue) locomotor activity assayed over the first 14 d in DD at 30°C. n, number of flies tested. The difference in proportion of behavioral phenotypes between each of control flies, either df -tfub > µ-MyVIA or parental UAS-toxin strain df -tfub driver flies, and experimental df -tfub > β-ACTX-Hv1c flies are all statistically significant; for each of the x2 values, p < 0.001.

Figure S3. Cell-Counting Method Reveals Identical Time Course of Rhythmic PDPI Accumulation as Background-Subtracted Pixel Intensity Method

Bar graphs show normalized integrated anti-PDP1-staining intensities (mean ± SEM) in DN1s (A) and anti-PDF-stained DN1 nuclei counts (B) of the indicated genotypes at the indicated time points on DD-D4. Control df -tfub > µ-MyVIA flies exhibit PDPI oscillation in DN1s with peak at CT18 and trough at CT2 (p < 0.001). PDPI accumulation in DN1s of experimental df -tfub > β-ACTX-Hv1c flies exhibit peak at CT10–CT14 and trough at CT2 (p < 0.001).

Figure S4. Repeat Experiment Confirms Phase Shift between PDF and PDPI Rhythms Induced by Membrane-Tethered β-ACTX-Hv1a Expression in PDF-Secreting LNv Neurons in LD Conditions

Bar graphs show anti-PDP1-staining intensities in LNv nuclei counts and normalized integrated anti-PDF-staining intensities in sLNv terminals (mean ± SEM) of the indicated genotypes at the indicated time points in DD. Control df -tfub > µ-MyVIA flies exhibit PDPI oscillation in LNv nuclei with peak at CT18 and trough at CT2 (p < 0.001). PDPI accumulation in experimental df -tfub > β-ACTX-Hv1a flies exhibit peak at CT10–CT14 and trough at CT2 (p < 0.001).

Table S1. Period and Power of Rhythmicity in Different Genotypes of Flies

Table S2. Period and Power of Rhythmicity in Different Genotypes of Flies

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References

1. Sehgal A, Price JL, Man B, Young MW (1994) Positional cloning and sequence analysis of the Drosophila clock gene, timeless. Science 265: 1693–1696.
2. Myers MP, Wager-Smith K, Wesley CS, Young MW, Sehgal A et al. (1995) Isolation of timeless by PER protein interaction: defective interaction between timeless protein and long-period mutant PERL. Science 270: 811–813.
3. Myers MP, Wager-Smith K, Wesely CS, Young MW, Sehgal A (1995) Positional cloning and sequence analysis of the Drosophila clock gene, timeless. Science 270: 805–808.
4. Blanchard E, Grima B, Klarsfeld A, Chelot E, Hardin PE, et al. (2001) Electrical hyperexcitation of lateral ventral pacemaker neurons desynchronizes downstream circadian oscillators in the fly circadian circuit. J Neurosci 21: 12489–12499.
5. Helfrich-Forster C (1998) Robust circadian rhythmicity of Drosophila melanogaster requires the presence of lateral neurons: a brain-behavioral study of disconnected mutants. J Comp Physiol [A] 182: 435–453.
6. Blanchard E, Grima B, Klarsfeld A, Chelot E, Hardin PE, et al. (2001) Electrical hyperexcitation of lateral ventral pacemaker neurons desynchronizes downstream circadian oscillators in the fly circadian circuit and induces multiple behavioral periods. J Neurosci 21: 479–489.
7. Sehgal A, Price JL, Man B, Young MW, Sehgal A (1995) Drosophila free-running rhythms require intercellular communication. PLoS Biol 1: e13. doi:10.1371/journal.pbio.0000013
8. Helfrich-Forster C, Tauber M, Park JH, Muhlig-Versen M, Schneuwly S, et al. (1999) Voltage-gated calcium channels. Eur J Biochem 264: 488–494.
9. Maggio F, King GF (2002) Scanning mutagenesis of a Janus-faced spider toxin, delta-atractotoxin-HV1a, on insect voltage-gated Na+ channels. J Exp Biol 205: 721–721.
10. Wang X, Hayes JL, Sollod B, Wu J, He Y, et al. (2007) The Drosophila circadian network is a seasonal timer. Cell 130: 869–873.
11. Rose EL, Standen J, Lees M, Saul DJ, Murphy-Furber A, et al. (2000) Isolation of timeless by PER protein interaction: defective interaction between timeless protein and long-period mutant PERL. Science 270: 811–813.
12. Taghert PH, Helfrich-Forster C, Gendre F, Migeon J, Chardon P, et al. (2000) Electrical hyperexcitation of lateral ventral pacemaker neurons desynchronizes downstream circadian oscillators in the fly circadian circuit and induces multiple behavioral periods. J Neurosci 20: 4317–4324.
13. Taghert PH, Helfrich-Forster C, Gendre F, Migeon J, Chardon P, et al. (2000) Electrical hyperexcitation of lateral ventral pacemaker neurons desynchronizes downstream circadian oscillators in the fly circadian circuit and induces multiple behavioral periods. J Neurosci 20: 4317–4324.
14. Taghert PH, Helfrich-Forster C, Gendre F, Migeon J, Chardon P, et al. (2000) Electrical hyperexcitation of lateral ventral pacemaker neurons desynchronizes downstream circadian oscillators in the fly circadian circuit and induces multiple behavioral periods. J Neurosci 20: 4317–4324.
15. Taghert PH, Helfrich-Forster C, Gendre F, Migeon J, Chardon P, et al. (2000) Electrical hyperexcitation of lateral ventral pacemaker neurons desynchronizes downstream circadian oscillators in the fly circadian circuit and induces multiple behavioral periods. J Neurosci 20: 4317–4324.
16. Taghert PH, Helfrich-Forster C, Gendre F, Migeon J, Chardon P, et al. (2000) Electrical hyperexcitation of lateral ventral pacemaker neurons desynchronizes downstream circadian oscillators in the fly circadian circuit and induces multiple behavioral periods. J Neurosci 20: 4317–4324.
17. Taghert PH, Helfrich-Forster C, Gendre F, Migeon J, Chardon P, et al. (2000) Electrical hyperexcitation of lateral ventral pacemaker neurons desynchronizes downstream circadian oscillators in the fly circadian circuit and induces multiple behavioral periods. J Neurosci 20: 4317–4324.
18. Taghert PH, Helfrich-Forster C, Gendre F, Migeon J, Chardon P, et al. (2000) Electrical hyperexcitation of lateral ventral pacemaker neurons desynchronizes downstream circadian oscillators in the fly circadian circuit and induces multiple behavioral periods. J Neurosci 20: 4317–4324.
19. Taghert PH, Helfrich-Forster C, Gendre F, Migeon J, Chardon P, et al. (2000) Electrical hyperexcitation of lateral ventral pacemaker neurons desynchronizes downstream circadian oscillators in the fly circadian circuit and induces multiple behavioral periods. J Neurosci 20: 4317–4324.
20. Taghert PH, Helfrich-Forster C, Gendre F, Migeon J, Chardon P, et al. (2000) Electrical hyperexcitation of lateral ventral pacemaker neurons desynchronizes downstream circadian oscillators in the fly circadian circuit and induces multiple behavioral periods. J Neurosci 20: 4317–4324.
21. Taghert PH, Helfrich-Forster C, Gendre F, Migeon J, Chardon P, et al. (2000) Electrical hyperexcitation of lateral ventral pacemaker neurons desynchronizes downstream circadian oscillators in the fly circadian circuit and induces multiple behavioral periods. J Neurosci 20: 4317–4324.
22. Taghert PH, Helfrich-Forster C, Gendre F, Migeon J, Chardon P, et al. (2000) Electrical hyperexcitation of lateral ventral pacemaker neurons desynchronizes downstream circadian oscillators in the fly circadian circuit and induces multiple behavioral periods. J Neurosci 20: 4317–4324.
23. Taghert PH, Helfrich-Forster C, Gendre F, Migeon J, Chardon P, et al. (2000) Electrical hyperexcitation of lateral ventral pacemaker neurons desynchronizes downstream circadian oscillators in the fly circadian circuit and induces multiple behavioral periods. J Neurosci 20: 4317–4324.
24. Taghert PH, Helfrich-Forster C, Gendre F, Migeon J, Chardon P, et al. (2000) Electrical hyperexcitation of lateral ventral pacemaker neurons desynchronizes downstream circadian oscillators in the fly circadian circuit and induces multiple behavioral periods. J Neurosci 20: 4317–4324.
25. Taghert PH, Helfrich-Forster C, Gendre F, Migeon J, Chardon P, et al. (2000) Electrical hyperexcitation of lateral ventral pacemaker neurons desynchronizes downstream circadian oscillators in the fly circadian circuit and induces multiple behavioral periods. J Neurosci 20: 4317–4324.
Drosophila melanogaster lateral ventral clock neurons. J Neurosci 28: 6493–6501.

50. Sheeba V, Gu H, Sharma VK, O’Dowd DK, Holmes TC (2008) Circadian- and light-dependent regulation of resting membrane potential and spontaneous action potential firing of Drosophila circadian pacemaker neurons. J Neurophysiol 99: 976–988.

51. Lebovitz RM, Takeyasu K, Fambrough DM (1989) Molecular characterization and expression of the (Na+ + K+)2-ATPase alpha-subunit in Drosophila melanogaster. EMBO J 8: 195–202.

52. Kula E, Levitan ES, Pyza E, Roshbash M (2006) PDF cycling in the dorsal protocerebrum of the Drosophila brain is not necessary for circadian clock function. J Biol Rhythms 21: 104–117.

53. Benito J, Zheng H, Hardin PE (2007) PDP epsilon functions downstream of the circadian oscillator to mediate behavioral rhythms. J Neurosci 27: 2539–2547.

54. Wu Y, Cao G, Nitabach MN (2008) Electrical silencing of PDF neurons advances the phase of non-PDF clock neurons in Drosophila. J Biol Rhythms 23: 117–128.

55. Lim C, Chung BY, Pitman JL, McGill JJ, Pradhan S, et al. (2007) Clockwork orange encodes a transcriptional repressor important for circadian-clock amplitude in Drosophila. Curr Biol 17: 1082–1089.

56. Veleri S, Brandes C, Helfrich-Forster C, Hall JC, Stanewsky R (2003) A self-sustaining, light-entrainable circadian oscillator in the Drosophila brain. Curr Biol 13: 1758–1767.

57. Littleton JT, Ganetzky B (2009) Ion channels and synaptic organization: analysis of the Drosophila genome. Neuron 29: 35–43.

58. Zhou W, Chung I, Liu Z, Goldin AL, Dong K (2004) A voltage-gated calcium-selective channel encoded by a sodium channel-like gene. Neuron 42: 101–112.

59. Stanewsky R, Kaneko M, Emery P, Beretta B, Wager-Smith K, et al. (1998) The cryb mutation identifies cryptochrome as a circadian photoreceptor in Drosophila. Cell 95: 681–692.

60. Emery P, Stanewsky R, Helfrich-Forster C, Emery-Le M, Hall JC, et al. (2000) Drosophila CRY is a deep brain circadian photoreceptor. Neuron 26: 493–504.

61. Emery P, So WV, Kaneko M, Hall JC, Roshbash M (1998) CRY, a Drosophila clock and light-regulated cryptochrome, is a major contributor to circadian rhythm resetting and photosensitivity. Cell 95: 669–679.

62. Sheeba V, Sharma VK, Gu H, Chou YT, O’Dowd DK, et al. (2008) Pigment dispersing factor-dependent and -independent circadian locomotor behavioral rhythms. J Neurosci 28: 217–227.

63. Brand AH, Perrimon N (1995) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118: 401–415.

64. Rubin GM, Spradling AC (1982) Genetic transformation of Drosophila with transposable element vectors. Science 218: 548–553.

65. Van Dongen HP, Olafsen E, van Hartevelt JH, Kruijt EW (1999) A procedure of multiple period searching in unequally spaced time-series with the Lomb-Scargle method. Biol Rhythm Res 30: 149–177.

66. Goldin AL (1992) Maintenance of Xenopus laevis and oocyte injection. Methods Enzymol 207: 266–279.