Circadian rhythms in neuronal activity propagate through output circuits

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Twenty-four hour rhythms in behavior are organized by a network of circadian pacemaker neurons. Rhythmic activity in this network is generated by intrinsic rhythms in clock neuron physiology and communication between clock neurons. However, it is poorly understood how the activity of a small number of pacemaker neurons is translated into rhythmic behavior of the whole animal. To understand this, we screened for signals that could identify circadian output circuits in Drosophila melanogaster. We found that leucokinin neuropeptide (LK) and its receptor (LK-R) were required for normal behavioral rhythms. This LK/LK-R circuit connects pacemaker neurons to brain areas that regulate locomotor activity and sleep. Our experiments revealed that pacemaker neurons impose rhythmic activity and excitability on LK- and LK-R-expressing neurons. We also found pacemaker neuron-dependent activity rhythms in a second circadian output pathway controlled by DH44 neuropeptide–expressing neurons. We conclude that rhythmic clock neuron activity propagates to multiple downstream circuits to orchestrate behavioral rhythms. Innate behaviors such as circadian rhythms are hardwired into the nervous system, making them particularly useful for studying how information flows through neuronal circuits to generate behavior. Circadian rhythms in behavior help animals anticipate predictable daily changes in the environment1,2 and are controlled by circadian pacemaker neurons. These neurons contain molecular clocks that drive rhythmic gene expression and set up 24-h rhythms in pacemaker neuron resting membrane potential, spontaneous firing rate and overall excitability3. Communication between clock neurons synchronizes their molecular clocks and adds robustness to the system2. Specific subgroups of clock neurons have peak neuronal activity at different times of day from each other and presumably regulate distinct output circuits to drive numerous rhythmic behaviors, including locomotor activity, sleep and feeding4. However, how the clock neuronal network controls different output circuits remains poorly understood.

Rhythms in pacemaker neurons can propagate to downstream cells via two mechanisms. Clock neurons can act on distant cells via rhythmic hormonal signals which entrain and synchronize molecular clocks in peripheral tissues. For example, clock neurons control rhythmic glucocorticoid release from the adrenal gland into the bloodstream, which then helps reset the molecular clocks in peripheral organs such as the liver4. Clock neurons could also impose rhythmic activity on downstream neurons via direct neuronal communication. Although many neurons fire rhythmically in the mammalian brain, widespread clock gene expression in mammals makes it difficult to exclude a role for local clocks in these rhythms5.

Studies of Drosophila have been instrumental in dissecting the molecular and neuronal bases of circadian rhythms2. However, how clock outputs are mediated in Drosophila remains poorly understood. Although peripheral clocks control rhythms in eclosion6 and feeding7, the clock output circuits controlling locomotor activity rhythms and sleep remain elusive. These outputs probably converge on the central complex8, pars intercerebralis (PI)9,10 and mushroom bodies11–13. One output pathway links the small LNv principal pacemaker neurons (s-LNv) to DN1p clock neurons, which then innervate a subset of pars intercerebralis neurons that express the DH44 neuropeptide. These DH44-expressing neurons are required for circadian rhythms10, but how their activity is regulated by the clock network has not been addressed. A second likely clock output pathway involves central complex neurons that respond to pigment-dispersing factor (PDF) released from LNv-s, although the function of these central complex neurons in circadian behavior has not yet been determined14. A third output pathway involves DH31 release from DN1 clock neurons to regulate sleep, but the relevant targets remain to be characterized15.

Here we identify an additional circadian output circuit connecting clock neurons to locomotor and sleep centers in the brain. This circuit comprises a pair of non-clock neurons expressing the neuropeptide leucokinin (LK) and a set of downstream neurons expressing the leucokinin receptor (LK-R) that project to the central complex. Using calcium imaging, we demonstrate that clock neurons impose 24-h rhythms on the excitability and activity of LK and LK-R neurons by neuronal communication. We also show that LK and LK-R neurons control the rhythmicity and levels of locomotor activity and sleep. In addition, we found that clock neurons also impose activity rhythms on the previously characterized DH44 circadian output neurons. Thus propagation of clock neuron electrical rhythms is a general mechanism for organizing circadian rhythms of behavior via multiple circuits.

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RESULTS
Leucokinin signaling is required for circadian rhythms
We hypothesized that we could identify a novel circadian output circuit by screening for circadian behavioral defects in flies mutant for a signaling molecule and/or its relevant receptor. We chose neuropeptides because they usually have more restricted distributions than neurotransmitters and because many neuropeptides modulate neuronal activity to regulate specific behaviors, as PDF does in circadian rhythms\(^{16,17}\).

We used transgenic RNA interference lines expressed via the pan-neuronal driver elav-Gal4 to knock down Drosophila neuropeptides in the whole brain and then assayed adult locomotor rhythms in constant darkness (DD; Fig. 1a and Supplementary Table 1). We found four RNAi transgenes that weakened behavioral rhythms: Bursicon (Burs), SIHamide (SIHa), Leucokinin (Lk) and Neuropeptide-like precursor 3 (Nlp3) (Fig. 1a,b). We focused on Lk because it does not seem to be involved in development and has an intriguing expression pattern in the brain (see below).

To further test whether Lk signaling is important in circadian behavior, we used RNAi to knock down expression of Leucokinin receptor (Lkr). This also weakened behavioral rhythms (Fig. 1b and Supplementary Table 2). To complement these RNAi experiments, we assayed the behavior of Lk\(^{275}\) and Lkr\(^{003}\) hypomorphic flies, which have reduced Lk peptide and Lk-R protein levels\(^{18}\). These mutants had weaker rhythms than heterozygous controls. Lkr\(^{003}\) and additional Lkr alleles gave similar behavioral phenotypes as hemizygotes (Fig. 1c,d, Supplementary Fig. 1a and Supplementary Table 2).

Quantifying Lk peptide levels and Lkr RNA in the different mutants revealed that the strength of behavioral phenotypes correlates with the extent of knockdown: Lk levels were reduced much more strongly by LkRNAi than by Lkr\(^{275}\), with only 3% of wild-type Lk levels in LkRNAi flies and stronger effects on behavior (Supplementary Fig. 1b,c and Supplementary Table 2). Lkr RNA levels were reduced to similar levels in Lkr\(^{003}\) hypomorphs and LkrRNAi knockdowns (Supplementary Fig. 1d) and these genotypes gave behavioral phenotypes similar in strength (Supplementary Table 2).

Together, these RNAi and mutant analyses indicate that Lk signaling is important in adult circadian rhythms. We were not able to test for a specific function of Lk in adult neurons, as restricting LkRNAi expression to adulthood did not reduce Lk peptide levels (data not shown). Below we describe how manipulating the activity of Lk- and Lk-R-expressing neurons in adults altered rhythmic locomotor activity.

Lk and Lk-R neurons are not clock neurons
Next we tested whether Lk and Lk-R neurons are clock neurons themselves. Lk is expressed in only four neurons in the adult brain\(^{19}\): one pair of neurons (SELKs) in the subesophageal ganglion and another...
Figure 2 The anatomy of LHLK LK-R neurons suggests they lie downstream of clock neurons. (a–e) White arrowheads indicate LHLK cell bodies. (a–c) Upper panels, z projections; lower panels, single confocal sections of the regions indicated by white dashed rectangles in upper panels. LHLKs project close to s-LNv (PDF staining, a), DN1p (Clk4.1-Gal4;CD8::GFP, b) and LNv (Mai179-Gal4;Pdf-Gal80>CD8::GFP, c). (d) LHLK dendrites (Lkr>DenMark) and s-LNv projections (PDF) are found in similar planes (anterior brain sections, left), whereas LHLK axon terminals (Lkr>Syt::GFP) are enriched in more posterior sections (right). (e) Single confocal section from dashed rectangle in d shows that LHLK dendrites intermingle with s-LNv dorsal projections. (f) Lkr-Gal4>CD8::GFP labels neurons in the lateral horn (LH), ellipsoid body (EB) and pars intercerebralis (PI). Inset: Lkr-Gal4 is not expressed in LHLK neurons (single confocal section). (g) Lkr>FLEXAMP clone labels LK-R neurons in the LH projecting to the FSB. (h) Single confocal section from the dashed square in g showing overlap of LK-R projections and LHLK arborizations, and several potential contacts. (i) LHLK processes are close to LK-R dendrites (Lkr>DenMark, single confocal section). (j,k) LK-R outputs (Syt::GFP) are found predominantly in the EB (j) and FSB (k). Scale bars: 20 µm, except 100 µm in f,g. More than 8 brains examined for each anatomical observation. See also Supplementary Figure 3.

pair in the lateral horn, the lateral horn LK neurons (LHLKs; Fig. 1e). Since the DN1 clock neurons are close to LHLK cell bodies, we examined LK staining with clock neuron markers. LHLK neurons did not produce the essential clock protein Timeless (TIM, Fig. 1f) nor did they express tim- or per-Gal4 (Fig. 1g and Supplementary Fig. 2a). We also expressed a dominant negative cycle transgene (UAS-cycΔ) to block the molecular clock. UAS-cycΔ completely abolished behavioral rhythms when expressed in clock neurons via tim-Gal4, but had no effect when expressed in LK neurons (Supplementary Fig. 2b and Supplementary Table 2).

To examine Lkr expression, we used an Lkr-Gal4 line that recapitulates endogenous LK-R expression18. Lkr is more widely expressed in the brain than LK and is also present in regions where clock neurons are located (Fig. 1h). However, we did not detect TIM expression in Lkr-Gal4-expressing neurons (Fig. 1i) and locomotor rhythms were unaffected by expressing UAS-cycΔ in LK-R neurons (Supplementary Fig. 2b and Supplementary Table 2). Thus we conclude that LK and LK-R neurons are not clock neurons.

Finally, we tested whether LK signaling affects the molecular clock in pacemaker neurons. We measured levels of the TIM and Vrille (VRI) core clock proteins in strong LkRNAi knockdown flies on the second and third days in DD and found that their rhythms were very similar to the rhythms in control flies (Supplementary Fig. 2c). Thus LK signaling is likely downstream of the clock since LkRNAi disrupts behavior without affecting molecular clock rhythms.

LHLK neurons project near clock neurons

To test whether LK and LK-R neurons are outputs of the clock, we wanted to determine whether they communicate with clock neurons. We first analyzed their anatomy and found that LHLK projections (marked by LK staining) are very close to the dorsal projections of the s-LNv, clock neurons (Fig. 2a). To more clearly visualize LHLK projections, we used an Lk-Gal4 line that recapitulates endogenous LK expression19 and the GFP amplification cassette FLEXAMP21. This revealed many sites of potential contact between s-LNv and LHLK projections (Fig. 2a). Several clock neuron classes converge at the s-LNv, dorsal projections, and we also found LK staining very close to projections of DN1p and LNv clock neurons (Fig. 2b,c). These data are consistent with LHLKs communicating with one or more classes of clock neurons.

Next we examined the location of synaptic and dendritic markers in LK neurons. We expressed DenMark23 and Syt::GFP24 in LHLK neurons to simultaneously label LHLK input and output areas, respectively, and used immunohistochemistry for PDF to label the clock network output region. We found that DenMark accumulated in LHLK neurons close to s-LNv projections, with LHLK dendrites often intermingling with s-LNv projections in single confocal sections (Fig. 2b,c). In contrast, mainly we found Syt::GFP in more posterior sections of LHLK neurons, which do not contain s-LNv projections (Fig. 2d). Since s-LNv has presynaptic markers all along their dorsal projections25, our observations are consistent with the idea that LHLK neurons lie downstream of clock neurons.

Given the proximity of clock neurons and LHLKs, we wanted to test whether LK regulates circadian rhythms via LHLKs rather than the other LK-expressing neurons19,26. We used apterous-Gal4 to express LkRNAi in LHLKs but not in SELKs or abdominal LK neurons (ABLks)26. Since apterous-Gal4>LkRNAi flies had weaker rhythms than control flies (Supplementary Table 2), we propose that LK functions as a clock output specifically in LHLK neurons. This function appears
distinct from those of LK signaling in feeding and diuresis, which are likely mediated by the SELKs and ABLKs.18,27

The LK/LKR circuit connects to locomotor and sleep areas
Since LHLK neurons contact LK-R neurons,18,27 we examined the projections of LK-R-expressing neurons to determine which brain regions are the likely target of the LK/LKR circuit. LK-R neurons form a dense and complex meshwork. However, a subset of LK-R neurons either project to or have cell bodies in brain regions implicated in controlling locomotion and/or sleep—specifically, the pars intercerebralis and two regions of the central complex: the ellipsoid body and fan-shaped body (FSB)8,9 (Fig. 2f, g). To visualize subsets of LK-R neurons, we generated flip-out FLEXAMP clones with labeled LK-R cell bodies in the lateral horn (Fig. 2g). These lateral horn LK-R neurons arborized in the posterior part of the brain and overlapped extensively with LHLK projections (Fig. 2h). Moreover, these LK-R arborizations likely represent inputs because they were enriched for DenMark staining (Fig. 2i) and because the presynaptic marker Syt::GFP localized in LHLKs in these posterior regions of the brain (Fig. 2d). These data suggest that most LK-R neurons projecting to the FSB receive inputs from LHLKs. In contrast, LK-R outputs marked by Syt::GFP were located primarily in the ellipsoid body and FSB (Fig. 2j, k). LK-R outputs were also present in the subesophageal ganglion, where SELKs are found (data not shown).

We found a second lkr;Gal4 line (lkr^{R65C07};Gal4) that also labeled neurons in the lateral horn with presynaptic termini in the FSB (Supplementary Fig. 3). Using a Lkr^{R65C07};LexA driver, we found that LK-R^{R65C07} projections in the FSB intermingled with projections from neurons labeled by three other FSB-neuron Gal4 lines that affect locomotor activity and sleep.28 (Supplementary Fig. 3f). Thus LHLKs and these lateral horn LK-R neurons have the appropriate anatomy to connect clock neurons with locomotor activity and sleep control centers.

LHLK neuron excitability is regulated by clock neurons
Next we used a functional approach to directly test connectivity and identify the direction of information transfer. We first manipulated s-LN_s activity by expressing the mammalian ATP-gated cation channel P2X_2. Since Drosophila neurons do not express endogenous ATP-gated channels, ATP activates only neurons expressing the P2X_2 transgene.29 We determined how this affects LHLK neuronal responses, using the genetically encoded calcium indicator GCaMP6s30 as a proxy for neuronal activity.

As a control, we first expressed P2X_2 and GCaMP6s in LN_s and detected robust calcium transients in s-LN_s after perfusing ATP onto explanted brains (Supplementary Fig. 4a). We saw similar responses in the large LN_s (l-LN_s) that regulate arousal.31,32 To measure responses in LHLKs, we used Lk::Gal4 to express GCaMP6s and Pdf::LexA (ref. 32) to express P2X_2 in LN_s. However, LN_s...
activation did not detectably change GCaMP6S fluorescence in LHKL neurons (Supplementary Fig. 4a).

To test whether LN₃s inhibit LHKL neurons, we first needed to identify a way to activate LHKLs. We found that the acetylcholine agonist carbacol (CCh) induced calcium transients in LHKLs in a dose-dependent manner (Supplementary Fig. 4b). We preincubated brains with tetrodotoxin (TTX) to determine whether this response was direct. TTX blocks most communication via neural circuits by preventing action potentials, although graded potentials are probably unaffected. The LHKL response to CCh persisted in the presence of TTX, suggesting that CCh directly activates LHKLs (Supplementary Fig. 4b).

We then tested whether the response of LHKLs to CCh is inhibited by LN₃ activation, using a lower CCh concentration to be able to detect inhibition. We found that inducing LN₃ firing almost completely abolished the LHKL response to CCh (Fig. 3a). This inhibition was specific, as it was not observed in s-LN₃s (PDF-Dti; Lk>GCaMP6S) (Supplementary Fig. 4c) or in LN vs (CT9–12 versus CT16–19; P = 0.383, D = 0.219). (g) Baseline GCaMP6S intensity per LHKL cell body in brains from 1-h time windows on day 1 in DD (n = 32; for each data point). Baseline GCaMP6S levels are rhythmic in wild-type brains (P < 0.0001, H = 28.69, 4 d.f.), but not in per⁰ mutants (P = 0.464, H = 2.585, 2 d.f.). (h) No rhythms are observed with Lk-Gal4 expressing destabilized GFP (PDF-Dti; Lk>GCaMP6S) (P = 0.2778, D = 0.219). (i) The LHKL GCaMP6S rhythm (P = 0.0005, H = 15.41, 2 d.f.), Kruskal-Wallis ANOVA, is lost in brains lacking LN₃s (PDF-Dti; Lk>GCaMP6S) (P = 0.6386, H = 0.897, 2 d.f., n = 32; for each data point). (j) PDF treatment (100 µM 30 min incubation before imaging) reduces baseline LHKL GCaMP6S during their peak phase (CT11–14; P = 0.0343, D = 0.344 by KS test, n = 32; for each data point).

Next, we asked whether PDF neuropetide, the main LN₃ output, is responsible for inhibiting LHKLs. PDF increases cyclic AMP by activating the PDF receptor (PDFR) in several classes of clock neurons, including s-LN₃s and DN₁₅,16.17 We found that a 30-s PDF perfusion gradually increased intracellular calcium levels in s-LN₃s, consistent with PDF depolarizing s-LN₃s,17. This response was specific, as it was not observed in i-LN₃s (Supplementary Fig. 4c), which do not express PDFR.34

Although LHKL neurons were not detectably activated by PDF perfusion (Supplementary Fig. 4c), preincubating brains with PDF markedly inhibited their CCh response (Fig. 3c). PDF inhibition was transient and disappeared after 15 min washout (Supplementary Fig. 4d). Thus PDF signaling can inhibit LHKLs, further evidence that LHKLs are downstream of the clock network.

However, since PDF can activate s-LN₃s and other clock neurons16,17,34 (Supplementary Fig. 4c), these data do not determine whether PDF directly controls LHKL excitability. To test this, we used two approaches. First, we used TTX to block action potentials while applying PDF. TTX was added for 20 min before PDF and also through-out the experiment. We found that TTX treatment largely eliminated LHKL inhibition by PDF (Fig. 3c), indicating that PDF acts indirectly on LHKLs. Second, we preincubated brains in PDF but this time with LN₃s ablated via a PDF–diphtheria toxin (PDF-Dti) transgene.35 This also prevented PDF from inhibiting LHKLs (Fig. 3d). Since PDF requires LN₃s to inhibit LHKLs, we interpret this to mean that PDF activates s-LN₃s (Supplementary Fig. 4c) which then signal to LHKLs either via an additional s-LN₃ neurotransmitter or indirectly via the clock network. Identifying the neurons that directly regulate LHKLs will require finding the signal that modulates LHKL excitability.

LK peptide does not modulate LN₃ excitability

We then determined how LK affects its target neurons. First we tested whether LK-R neurons respond to LK peptide, focusing on the LK-R neurons with cell bodies in the lateral horn that project to the FSB. Adding LK to Lkr>GCaMP6S brains did not activate these LK-R neurons (Fig. 4a–f). LHKL responses to 10 µM CCh on day 1 in DD during 3-h time windows indicated above the line graphs by colored bars; dark gray and black bars show subjective day and night, respectively. n = 28; for each sample in a–d, n = 32; for e,f, taken from ≥2 independent experiments. (a) LHKL excitability is rhythmic in wild-type brains (P = 0.0029, D = 0.464, KS test). (b) Quantification of a: CT0–3 versus CT4–7 P = 0.917, D = 0.143; CT9–12 versus CT16–19 P = 0.917, D = 0.143; CT4–7 versus CT9–12 P = 0.0029, D = 0.464. LHKL excitability is not rhythmic in per⁰ mutants (c,d, P = 0.987, D = 0.125 or PDF-Dti brains (e,f, P = 0.383, D = 0.219). (g) Baseline GCaMP6S intensity per LHKL cell body in brains from 1-h time windows on day 1 in DD (n = 32; for each data point). Baseline GCaMP6S levels are rhythmic in wild-type brains (P < 0.0001, H = 28.69, 4 d.f.), but not in per⁰ mutants (P = 0.2746, H = 2.585, 2 d.f.). (h) No rhythms are observed with Lk-Gal4 expressing destabilized GFP (PDF-Dti; Lk>GCaMP6S) (P = 0.2778, D = 0.219). (i) The LHKL GCaMP6S rhythm (P = 0.0005, H = 15.41, 2 d.f.), Kruskal-Wallis ANOVA, is lost in brains lacking LN₃s (PDF-Dti; Lk>GCaMP6S) (P = 0.6386, H = 0.897, 2 d.f., n = 32; for each data point). (j) PDF treatment (100 µM 30 min incubation before imaging) reduces baseline LHKL GCaMP6S during their peak phase (CT11–14; P = 0.0343, D = 0.344 by KS test, n = 32; for each data point).
neurons (Supplementary Fig. 4e). However, they were activated by CCh, and this response was strongly reduced by preincubation in LK peptide (Fig. 3e). This contrasts with non-neuronal stellate cells, where LK increases intracellular calcium, and could be explained by differential G protein coupling in distinct cell types. We conclude that the LK-R neurons in the lateral horn are bona fide LK-responsible neurons. In addition, their projection patterns strongly suggest they are downstream of LHLLs but not SELKs. Therefore we propose that the LK/LK-R network connects clock neurons to the FSB and possibly also to the ellipsoid body and pars intercerebralis.

We also tested whether LK feeds back on clock neurons. Since we found no evidence for LK activating LNₖₛ in Pdf-GCaMP6S brains (data not shown), we tested whether LNₖₛ can be inhibited by LK. LNₖₛ respond to CCh, but this response was unaffected by preincubating brains with LK (Fig. 3f and Supplementary Fig. 4f). Thus LNₖₛ does not affect s-LNₖ activity and LHLLK neurons seem to act as outputs of the clock network.

Clock neurons impose rhythms on LHLLK activity

LNₖₛ and DN₁ₖₛ are most depolarized and have highest spontaneous firing rates around dawn, but LHLLK neuronal excitability is controlled by LNₖ, firing, we speculated that LHLLK neuron activity is also rhythmic (Fig. 3). However, the timing of peak LNₖ, and LHLLK activity should differ since LNₖ inhibits LHLLKs.

To test these ideas, we first measured LHLLK responses to CCh at two different times in DD. We measured LHLLK responses when LNₖ activity is high during the subjective morning (CT0–3, where CT reflects circadian time in DD after entrainment to a 12:12 light:dark (LD) cycle), and in the subjective evening (CT9–12), when LNₖ activity is low. We maintained individual flies in the dark until dissection to minimize exposure to light. We found that the LHLLK response to CCh was two-fold lower in the subjective morning than subjective evening, and additional time points revealed a 24-h rhythm (Fig. 4a,b). Low LHLLK excitability when LNₖ, activity is high is consistent with LNₖ, inhibiting LHLLK neurons. Oscillations in explanted brains indicate that these rhythms are not driven by locomotor activity.

LHLLK excitability rhythms are likely to be clock controlled because they persist in DD. To test this, we measured CCh responses in period null mutant (per⁰), flies in which the molecular clock has stopped. We found that changes in LHLLK excitability were lost in per⁰ mutants, showing that these rhythms require intact molecular clocks (Fig. 4c,d). This suggests that LHLLK rhythms are imposed by circadian pacemaker neuron, as LHLLK neurons do not contain molecular clocks. We thus measured LHLLK excitability rhythms in brains with LNₖ, ablated and found this also eliminated LHLLK rhythms (Fig. 4e,f).

To determine whether these LHLLK rhythms reflect endogenous neuronal activity, we quantified baseline GCaMP6S fluorescence in living explanted brains as a measure of spontaneous activity. We observed a robust oscillation of GCaMP6S intensity with a peak around subjective dusk (CT11) and a trough at subjective dawn (CT0 and CT23; Fig. 4g). We did not see any changes in GFP intensity between CT0 and CT11 using a destabilized GFP transgene expressed with Lk-Gal4 (Fig. 4h, as in ref. 43). Thus the GCaMP6S oscillation is not due to rhythmic Lk-Gal4 expression and presumably reflects changes in spontaneous LHLLK activity over 24 h.

We also measured baseline GCaMP6S in LHLLK neurons in per⁰ mutants (Fig. 4g) and when LNₖ, were ablated (Fig. 4i). Rhythms were lost in both situations, confirming that LHLLK excitability is clock-controlled and driven by pacemaker neurons. We also found that artificialy activating s-LNₖ activity in the evening by applying PDF peptide decreased baseline GCaMP6S in LHLLKs (Fig. 4j). Thus we conclude that s-LNₖ firing reduces LHLLK neuronal activity. PDF did not reduce LHLLK GCaMP6S to the trough levels observed at dawn. This could mean that either s-LNₖ firing is required for >30 min to fully inhibit LHLLKs or that weaker s-LNₖ synaptic outputs at dusk prevent complete LHLLK inhibition. In conclusion, these results demonstrate that LHLLK excitability rhythms are generated non-cell-autonomously by rhythmic signaling of the clock network.

LHLLK activity rhythms propagate to LK-R neurons

Next we tested whether LHLLK activity rhythms are transmitted to LK-R neurons. We measured LK-R responses to CCh in DD and found that LK-R neurons were more excitable at dawn than dusk (Fig. 5a,b). Furthermore, LK-R excitability rhythms were abolished in per⁰ mutants (Fig. 5c,d). We also measured LK-R excitability in Lkr⁰ hypomorphs to test whether LK peptide itself transmits LHLLK activity

Data are from ≥ 2 independent experiments. (a) Lateral horn LHLLK neuron excitability (response to 100 μM CCh) is rhythmic (n = 80;10 for each sample, P < 0.0001, D = 0.375). (b) Quantification of a,d,e. The rhythm is lost in per⁰ mutants (n = 80;10 each, P = 0.798, D = 0.1). (c) The rhythm is dampened in Lkr⁰ hypomorphs, although still significant (n = 80;10 each, P = 0.0036, D = 0.275). LHLLK excitability is significantly higher in Lkr⁰ mutants compared to wild type in both time windows (CT0–3 P = 0.0001, D = 0.337, CT9–12 P < 0.0001, D = 0.512). (e) Lateral horn LK-LK-R-axononal stellate cells, where LK increases intracellular calcium, and could be explained by differential G protein coupling in distinct cell types. We conclude that the LK-R neurons in the lateral horn are bona fide LK-responsible neurons. In addition, their projection patterns strongly suggest they are downstream of LHLLKs but not SELKs. Therefore we propose that the LK/LK-R network connects clock neurons to the FSB and possibly also to the ellipsoid body and pars intercerebralis.

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Next we tested whether LHLLK activity rhythms are transmitted to LK-R neurons. We measured LK-R responses to CCh in DD and found that LK-R neurons were more excitable at dawn than dusk (Fig. 5a,b). Furthermore, LK-R excitability rhythms were abolished in per⁰ mutants (Fig. 5c,d). We also measured LK-R excitability in Lkr⁰ hypomorphs to test whether LK peptide itself transmits LHLLK activity...
Figure 6  

**LK and LK-R neuron signaling controls locomotor activity levels.** (a) Representative actograms of flies maintained 6 d in DD at 19 °C and then 5 d at 28 °C (red shaded area). Gray and black bars: subjective day and night, respectively. Rhythms of control flies (LK-Gal4/+ and UAS-TrpA1+) become stronger at 28 °C than at 19 °C but become weaker when activating LK neurons with TrpA1 at 28 °C (LK>TrpA1). See Supplementary Table 2 for details and for LK-R and DH44 neuron activation data. (b,c) Acute (24 h, red shaded area) activation (b) and inhibition (c) of LK and LK-R neurons. Population average locomotor activity over 3 d in DD, with the first 12 h of activation magnified below (error bars, s.e.m.). *P < 0.05 for differences between experimental flies and both parental controls (KS test). LK neuron activation decreased locomotor activity (b, left), while LK-R (Lk>TrpA1) neuron activation increased locomotor activity during the first 6 h (b, right). Locomotor activity recovered to normal levels on day 3 in both experiments. Lk-Gal4/+ n = 62; TrpA1/+ n = 62; Lk>TrpA1 n = 63; LkR65C07, Gal4/+ n = 94; TrpA1/+ n = 94; LkR65C07>TrpA1 n = 93. Data are from 2 (left) or 3 (right) independent experiments. Inhibiting synaptic transmission from LK neurons had minimal effects on locomotor activity (c, left), while inhibiting LK-R R65C07 neurons reduced locomotor activity through most of the subjective day (c, right). Lk-Gal4/+ n = 63; shiP2 n = 62; Lk>shiP2 n = 63; LkR65C07, Gal4/+ n = 62; LkR65C07>shiP2 n = 62. Data are from 2 independent experiments. See also Supplementary Figures 5 and 6.

Rhythms to LK-R neurons. We found that LK-R excitability oscillations were dampened in LkrR65C07 mutant flies (Fig. 5b,d), consistent with the hypomorphic nature of this allele and with LK modulating LK-R neurons. Thus LK-R neuron excitability is rhythmic, clock-controlled and in antiphase to LHLK, consistent with LK peptide inhibiting LK-R neuronal activity.

We also tested whether these LK-R excitability rhythms reflect endogenous rhythms in neuronal activity by measuring baseline GCaMP6S levels. We observed a robust 24-h oscillation (Fig. 5e) in antiphase to LHLKs (compare Fig. 4g), demonstrating that LHLK rhythms originate from pacemaker neurons.

We also used LkrR65C07, Gal4 line to drive GCaMP6S in the LK-R neuronal subset that projects to the FSB (Supplementary Fig. 3). These neurons also responded to LK peptide (Supplementary Fig. 4g), and their baseline GCaMP6S levels oscillated in phase with Lkr-Gal4 (Supplementary Fig. 4h), confirming that both Lkr-Gal4 lines label the same neurons. Thus rhythmic pacemaker neuron activity is propagated at least two layers deeper into the brain to generate non-cell-autonomous rhythms in LK-R neurons via LK signaling.

**LK and LK-R neurons control locomotor activity and sleep**

Rhythms in LHLK and LK-R activity suggest that the clock network imposes rhythmic neuronal activity on locomotor and sleep control centers. To test whether these neuronal rhythms are important for behavioral rhythms, we manipulated LK and LK-R neuronal activity. To manipulate the subset of LK-R neurons most likely to receive LHLK inputs, we used the more restricted LkrR65C07, Gal4. We activated LK and LK-R R65C07 neurons for 4 d using a UAS transgene expressing the heat-activated cation channel TrpA1, which is inactive below 25 °C (ref. 44). After entraining to LD cycles at 19 °C, flies were assayed in DD for 4 d at 19 °C and then for 4 d at 28 °C. Control flies had stronger rhythms at 28 °C than at 19 °C, as seen previously (ref. 44). After entraining to LD cycles at 19 °C, flies were assayed in DD for 4 d at 19 °C and then for 4 d at 28 °C. Control flies had stronger rhythms at 28 °C than at 19 °C, as seen previously (ref. 44). In contrast, activating LK-R R65C07 neurons blocked the increase in rhythm strength at 28 °C (Fig. 6a and Supplementary Table 2). These data suggest that LK and LK-R neuronal activity rhythms are required for normal behavioral rhythms.

To explore the effect of LK and LK-R neurons in more detail, we performed 1-d activation experiments and also used the temperature-sensitive dominant negative Dynamin (UAS-shiP2) to block synaptic outputs (ref. 45). Control flies increased their activity levels in response to heat (Fig. 6b). This was due to increased locomotor activity while awake and decreased sleep (Supplementary Fig. 5a-d). In contrast, activating LK neurons reduced locomotor activity levels (Fig. 6b), by increasing the amount of sleep and reducing activity levels while awake (Supplementary Fig. 5a,c). Since Lk>TrpA1 flies recovered similar activity and sleep levels to those of control flies on returning to 19 °C, activating LK neurons does not permanently alter locomotor and sleep circuit function or render flies unhealthy (Fig. 6b and Supplementary Fig. 5a,c).

Activating LK-RR65C07 neurons had the opposite effect to activating LK neurons, increasing locomotor activity and decreasing sleep compared to controls (Fig. 6b and Supplementary Fig. 5b). This effect was shorter lived than for LK neurons and was most apparent during the first 6 h of the temperature shift (Fig. 6b and Supplementary Fig. 5d). The opposite effects of activating LK and LK-R neurons are consistent with LK inhibiting LK-R excitability (Fig. 3) and indicate that LK neurons control locomotor activity and sleep levels by inhibiting LK-R neurons.

We then inhibited synaptic transmission from LK and LK-R R65C07 neurons with shiP2. Surprisingly, inhibiting LK neuron synaptic transmission had almost no effect on locomotor activity or sleep (Fig. 6c and Supplementary Fig. 5e). One possible explanation is that LK neurons control these behaviors via neuromodulator signaling, which may be independent of Dynamin (ref. 46). Indeed, constitutively hyperpolarizing LK neurons with the inward rectifier potassium channel Kir2.1 (ref. 47) reduced locomotor rhythm strength (Supplementary Table 2).
In conclusion, our experiments reveal a mechanism to temporally gate LK-R neuron activity is also imposed by pacemaker neurons. We propose that nonautonomous propagation of neuronal rhythms is a general mechanism for transmitting pacemaker neuron information.

Figure 7 Clock electrical rhythms propagate through multiple output circuits. Baseline GCaMP6S levels oscillate in DH44 neurons (P = 0.0033, H = 11.45, 2 d.f.; CT0 n = 115;23, CT11 n = 125;23, CT23 n = 124;23). This rhythm was lost in brains lacking LN₅s (Pdf-Δti, P = 0.2585, H = 2.705, 2 d.f.; CT0 n = 103;19, CT11 n = 96;19, CT23 n = 100;19). a.u., arbitrary units. Error bars show s.e.m. Statistics as in Figures 4 and 5. Data are from 3 independent experiments.

In contrast, inhibiting synaptic transmission from LK-R₉⁶₅₇ neurons reduced locomotor activity and increased sleep (Fig. 6c and Supplementary Fig. 5f). This effect was the opposite of the effect of activating LK-R₉⁶₅₇ neurons and similar to the effect of activating LN₅s (Pdf-Δti) (Fig. 6b). These data further support the model that LN₅s inhibit LK-R neurons, which normally promote locomotor activity and inhibit sleep. These results also show that LK-R neuron signaling is required by day for normal levels of locomotor activity and sleep.

We repeated the experiments with LK-R₉⁶₅₇ neurons with a heat pulse starting at CT12 and obtained very similar results to those of CT0–24 heat pulses: LK-R₉⁶₅₇ neuron activation and inhibition mainly affected behavior during subjective day (data not shown). Thus LK-R neurons seem competent to control locomotor and sleep only at times when they are most excitable (see Fig. 5). The absence of phenotypes at night could be due to masking effects by heat and/or interactions with other neural pathways that override the effects of LK-R signaling during subjective night. Indeed, light, slightly delayed the effects of LK-R neuron activation and inhibition on locomotor activity during the day (Supplementary Fig. 6a). This suggests that one or more pathways downstream of light at least partially suppress the effects of interfering with LK-R neuron signaling. Thus LK-R neuron outputs are likely integrated with other pathways to shape behavioral rhythms.

LK-R expressed in Malphigian tubule stellate cells responds to circulating LK peptide released from ABLKs to regulate diuresis. To test whether the TrpA1 locomotor activity phenotypes require neuronal expression, we added elav–Gal80 to eliminate TrpA1 expression from LK>TrpA1 and LK₉⁶₅₇>TrpA1 flies. Restricting TrpA1 expression to non-neuronal tissues abolished the effect on locomotor activity and sleep (Supplementary Fig. 6b). Thus the LK and LK-R cells controlling locomotion and sleep are neurons. Together with LK/LK-R anatomy and our functional imaging experiments, these behavioral data implicate the brain LK/LK-R circuit as a critical circadian output that regulates rhythmic locomotor activity and sleep.

Rhythms propagate in a second clock output circuit

Finally we examined a second group of clock output neurons: the DH44-expressing neurons in the pars intercerebralis that receive inputs from DN₁₅ clock neurons but do not express molecular clock components. We found that baseline GCaMP6S levels oscillated in DH44 neurons and this required LN₅s (Fig. 7). Thus rhythmic DH44 neuron activity is also imposed by pacemaker neurons. We propose that nonautonomous propagation of neuronal rhythms is a general mechanism for transmitting pacemaker neuron information.

DISCUSSION

How does the clock network regulate downstream circuits? We show that the LHLK, LK-R and DH44 neurons downstream of the *Drosophila* clock network display clock-dependent activity rhythms in explanted brains, although these neurons have no molecular clocks themselves. The loss of LHLK, LK-R and DH44 neuronal activity rhythms after LN₅ ablation demonstrates that these rhythms originate from pacemaker neurons (Supplementary Fig. 7). In addition, PDER-expressing neurons in the ellipsoid body display a circadian rhythm in their cAMP response to acetylcholine that partly depends on PDE. Thus clock output pathways relay rhythmic information to several different brain regions using diverse signals.

Function of LK/LK-R signaling

Behavioral analyses of *Lk* and *Lkr* mutants and neuronal manipulations implicate the LK/LK-R circuit in organizing locomotor activity and sleep over time. Specifically, we found that LK-R neurons promote locomotor activity and reduce sleep (Fig. 6). This function seems distinct from the diuretic function of LK/LK-R signaling, which is likely controlled by LK release from ABLKs. Indeed, we found that locomotor behavior was disrupted with RNAi targeting LK only in LHLKs but not in SELKs and ABLKs, and also when manipulating LK-R specifically in neurons. Other functions of LK/LK-R signaling such as regulating feeding are unlikely to affect locomotor rhythms, since *Lk* and *Lkr* mutants ingest normal amounts of food and since blocking feeding rhythms does not alter locomotor activity rhythms. However, we cannot completely rule out the possibility that LK-LK-R regulation of feeding affects locomotor and sleep behaviors given the precedent of orexins (hypocretins) regulating both energy intake and arousal in vertebrates.

LK-R neurons intermingle with neurons that promote locomotor activity in the FSB. However, LK-R neurons projecting to other locomotor centers such as the pars intercerebralis and ellipsoid body might also contribute to circadian behavior. The locomotor activity–promoting role of LK-R neurons is consistent with their neuronal activity profile determined by GCaMP: they are more excitable and active around dawn, when flies have high locomotor activity. Together with our analysis of LHLK and pacemaker neuron connections, these observations suggest a model in which signaling from the clock network inhibits LHLK neurons at dawn to allow LK-R neurons to signal and promote locomotor activity (Supplementary Fig. 7). Supporting this model, downregulating *Lk* and *Lkr* by RNAi interfered with morning anticipatory behavior but had no effect in the evening (data not shown).

In addition to being inhibited by LHLKs at dusk and night, we found evidence suggesting that LK-R neuron outputs are blocked by additional unidentified signaling pathways, as activating them at night did not affect locomotor behavior. Some of these pathways may be downstream of light, which partially suppresses LK-R-driven locomotor activity during the day. The exact timing of LK and LK-R firing is also likely to depend on additional non-circadian inputs and probably differs from the windows of excitability imposed by clock neurons. More work will be required to determine how the different circuits downstream of the clock interact to organize circadian behaviors.

In conclusion, our experiments reveal a mechanism to temporally control behavior: Pacemaker neuron electrical rhythms are propagated through downstream neuronal circuits that control specific components of circadian behavior.
METHODS
Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
M.C. and B.C. performed the RNAi screen. M.C. performed all other experiments and analyses except the immunostaining in Supplementary Figure 3, which was done by C.B. M.C. and J.B. wrote the manuscript, with comments from B.C. and C.B.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Fly strains. The following Drosophila melanogaster fly strains have been described previously: y++; Lk(27s) (ref. 18), y++; Lk(11s) (ref. 18), y++; Lkmet1 (M60336), y++; Lkmet2 (M80660), y++; Df(3L)BSC371 (ref. 49), y++; Df(3L)BSC372 (ref. 49), y++; Df(3L)BSC557 (ref. 49), y+w; Lk-Gal4 (ref. 19), y+w; Lk-Gal4 (ref. 18), Pdfl-Gal4 (ref. 50), tim>UAS-Gal4 (referred to here as tim-Gal4, ref. 51), per>Gal4 (ref. 52), Ap-Gal4M244 (ref. 53), UAS-nlsGFP (from C. Desplan), elav-Gal4, UAS-Dcr-2 (refs. 54, 55), the UAS-Dcr-2 transgene was included to enhance RNAi effectiveness, y++; UAS-LkRNAi (TRIP [JF01956]), y++; UAS-LkRNAi (TRIP [JF01956]), y++; UAS-mcd38:GFP (ref. 56), FLEX CASSette: y++; UAS-Flyt; tub-Gal80P/CyO; act-FRT-stop; y++; FRT-LexA, 13lexaap-myr:GFP (ref. 21), Ct2a1-Gal4 (ref. 57), Mai179-Gal4; PdfGal80 (refs. 58, 59), y++; L1cyO; UAS-Syt: GFP, UAS-DenMark (ref. 23), w++; UAS-cycD (ref. 20), w++; UAS-GCaMP6S (ref. 30), w++; Pf6-Lexa, parP2X2–FL (ref. 60), y++; perw; (ref. 61), Pf6-Dti (ref. 35); we verified that Pf6-Dti ablates all adult LNvs by immunostaining, n = 8 brains, data not shown), UAS-dsGFP (ref. 62), DH44–VT-Gal4 VDCR (ref. 10), w1118; UAS-Kir2.1 (ref. 47), w++; UAS-TrpA1 (ref. 44), u++; UAS-shits; UAS-shits (ref. 45), w1118; LkrR8307; Gal4 and u1218; LkrR8307; LexA (ref. 63, Janelia R6307), 121Y-Gal4, CS–Gal4, c584-Gal4 (refs. 8, 28) and elav-Gal4 (from S. Sweeney, ref. 64).

Behavioral analyses. Flies were raised on standard cornmeal medium and entrained to 12:12 h LD cycles for at least 3 d before transfer to DD. Flies were raised and assayed at 25 °C except for experiments involving UAS-TrpA1 or UAS-shits, in which flies were raised at 19 °C and assayed at 19 °C and 28 °C. Male flies assayed for behavior were ~5–10 d old. No randomization or blinding was used when preparing and analyzing behavioral experiments, but controls were performed in parallel. Locomotor activity was recorded using the DAM system (TriKinetics, Waltham, MA). Manual inspection of actograms was performed for each fly to exclude flies that died during an experiment. Rhythm strength (power) and period were analyzed by ClockLab in Matlab using chi-squared analysis. All other analyses (actograms, activity and sleep profiles, total activity and waking activity) and statistical tests were performed using custom-written scripts in IgorPro (WaveMetrics) as in ref. 65. Sleep was defined as at least 5 min of inactivity: 0 beam crossings in a 5-min data window.

Minimum required sample sizes were determined empirically; no statistical methods were used. Behavioral experiments were repeated at least twice. The RNAi screen was initially performed with 8 flies, but genotypes with potentially interesting phenotypes were repeated to obtain >16 flies. For TrpA1 and shits experiments, larger sample sizes were required because most experimental treatments lasted only 1 d. Thus these experiments were performed with 32 flies at least twice. To determine which statistical test with which to compare experimental flies to parental controls, we first ran Levene’s test to determine whether variance was equal and found that they were often unequal. Thus we used the non-parametric Kolmogorov-Smirnov test (KS test) to determine whether experimental flies differed from controls. TrpA1 or shits manipulations were considered to have a significant effect when experimental flies were statistically different from both controls (P < 0.05).

Immunocytochemistry. Adult brains were dissected in PBS, fixed for 45 min in 4% formaldehyde in PBS, rinsed three times in PBS + 1% Triton and washed for ~2 h in PBS + 1% Triton. Primary antibodies were incubated in PBS + 0.5% Triton + 4% horse serum overnight at 4 °C. Secondary antibodies were incubated for 2 h at room temperature and rinsed overnight at 4 °C. Brains were mounted in SlowFade (Invitrogen). Primary antibodies used were as follows: rabbit anti-LK at either 1:1,000 or 1:10,000 for quantification24, mouse anti-PDPh1 1:50, rat anti-TIM 1:250 (from A. Sehgal)26, guinea pig anti-VRI 1:1,500 (from P. Hardin)28, sheep anti-GFP 1:500 (Novus Biologicals NB100-62262), rabbit anti-RFP 1:500 (Invitrogen R10367) and mouse anti-RFP 1:100 (MBL 8D6). Alexa Fluor secondary antibodies (Invitrogen A-21206, A-10042, A-31573, A-21434, A-11015, A-31571, A-11073, A-10036) were all used at 1:200. Confocal stacks were acquired with a Leica SP5 confocal microscope with a 20× water immersion objective and processed in ImageJ. Anatomical analyses were performed on male and female flies ~2–10 d old, with a minimum of 8 brains imaged on both left and right sides for each experiment. Anatomical observations were highly reproducible from brain to brain.

TIM staining in Figure 1 was performed when TIM levels are high in clock neurons. Brains were dissected at ZT22 (Zeitgeber time, time in a 12:12 LD cycle)

to examine all neurons except in the case of DN5s, where brains were dissected at ZT10 (Fig. 11). Flip-out FLEXAMP clones (Fig. 2g) were generated with tub-Gal80B; Lkr-Gal4 by transferring developing larvae to 29 °C for ~3 h to allow transient UAS-Flip expression from Lkr-Gal4.

Protein quantification was performed on male flies ~3–5 d old entrained to LD cycles. Quantification of LK, VRI and TIM in wild-type and Lk mutants and of dsGFP in wild-type flies was performed using IgorPro (WaveMetrics) on eight-bit images (that is, pixel intensities ranging from 0 to 255). Average pixel intensity (integrated intensity/area) was measured for individual cell bodies using manually defined ROIs on x projections. Background intensity was measured for each image and subtracted from the corresponding cell bodies. Sample sizes were determined empirically and were based on the relatively low variability observed between brains, as noted previously65. We imaged 8 brains (16 LHKL cell bodies) to quantify LK in Supplementary Figure 1bc and 9 or 10 brains (>60 cell bodies) for TIM and VRI in Supplementary Figure 2c–e for each data point. To quantify LK in LNvRNAi brains, LHKL cell bodies were first identified using higher laser power, as they were almost undetectable, and then imaged using standard acquisition settings. s-LNvs were identified using PDF staining and distinguished from 1-LNvs by size. No randomization or blinding was used when preparing and analyzing immunostaining.

Quantitative real-time PCR (qPCR). Lkr mRNA was measured by qPCR using a standard curve constructed as in ref. 69. RNA was extracted from male whole heads (~40 heads per extraction, flies ~3–5 d old) using PureLink RNA Mini Kit (Ambion). Sample sizes were based on ref. 69. Each data point consisted of 2 biological replicates with 3 technical replicates (6 samples total). No randomization or blinding was used when preparing and analyzing qPCR. Reactions were performed using the LightCycler RNA Master Hybrid Probe kit (Roche) with Calmodulin as a loading control43. Primers and probes were synthesized by TIB Molbiol (Adelphi, NJ).

Calcium imaging. We chose the GCaMP6S variant for two reasons. First, GCaMP6S has slower kinetics than GCaMP6F and GCaMP6M20, which makes it easier to detect calcium transients in many neurons in a large field of view at slow scanning speed, even though the slow time course of GCaMP6S responses (~5 min) is not physiological. Second, GCaMP6S is more sensitive than GCaMP6F and GCaMP6M20, making it easier to detect subtle changes in neuronal activity, especially for baseline GCaMP6S fluorescence.

Adult male flies ~3–5 d old entrained to LD cycles were anesthetized on ice and dissected in hemolymph-like saline (HL3)34. For measuring GCaMP6S responses to drugs, brains were gently pressed against a glass slide coated with poly-1-lysinse (Sigma) and mounted in a Bioteps FCS3 perfusion chamber. HL3 flow across the brain was established and maintained at ~1 ml/min by gravity. Brains were allowed to recover for ~5 min in the chamber before an experiment. Test compounds (0.5 ml) were injected into the tubing system using a syringe and three-way stopcock. Compounds were perfused for ~30 s and started at slightly different times depending on experiments because of small changes in flow rate and tubing length. The timing of drug perfusion is indicated by the position of the gray bar on GCaMP6S line graphs. To measure baseline GCaMP6S intensity, live brains were mounted in HL3 medium on glass slides coated with poly-1-lysinse and imaged immediately in one z-stack. No randomization or blinding was used when preparing and analyzing calcium imaging experiments.

ATP (Sigma) and carbachol (Sigma) were dissolved directly in HL3. KCl was used at 35 mM, after ref. 70. PDF and LK peptides were synthesized by PolyPeptide Group (San Diego, CA), dissolved in DMSO, diluted to final concentration in HL3 and used within 1 d. Three different batches of PDF were used during this study and had different efficacies, as previously noted44. The batch used in Supplementary Figure 4d was more potent and thus used at lower concentration (20 μM) than the batch used in Figure 5c.d (100 μM).

TTX (tetrodotoxin citrate, Abcam) was dissolved in HL3 and included in the main HL3 flow throughout the relevant experiments and while test compounds...
were injected. Electrophysiological recordings of l-LNv neurons showed that TTX completely eliminates action potentials within 1 min of application on brain explants46. We used a high TTX concentration to completely inhibit action potentials, as in refs. 38, 71. TTX works in our preparation because it eliminates the inhibitory effect of PDF on LHLKs (see Fig. 3c).

Preincubation with PDF, LK or TTX was in a 1-μl drop per well of HL3 before mounting in the chamber. For the PDF + TTX experiment (Fig. 3c), brains were first incubated for 20 min in TTX and then for another 20 min in PDF + TTX (or vehicle + TTX). PDF washout (Supplementary Fig. 4d) was performed in the perfusion chamber after the first CCh stimulation.

GCaMP6S imaging was performed with an Olympus two-photon system with a Mai-Tai laser (Spectra Physics) at 920 nm and a 10× water immersion objective. z stacks (~20 slices at 5-μm intervals) were acquired every 30 s for 10 min. Maximal z projections were used to quantify fluorescence in individual neuronal cell bodies over time. Twelve-bit images were used (that is, pixel intensity ranging from 0 to 4,095). Subsequent data processing was performed using custom-written scripts in IgorPro (Wavemetrics). Individual traces were normalized to initial fluorescence (F0) and averaged across samples. The line graphs show the average GCaMP6S fluorescence (thick line) ± s.e.m. (thin vertical lines) plotted versus time. The mean maximum change in GCaMP6S fluorescence (∆F/∆F0) was calculated by averaging the peak F/F0 determined for each trace of a given sample. For each experiment, the sample sizes are indicated as n = n1/n2, where n1 and n2 are the total number of cell bodies and brains quantified per sample, respectively. No statistical method was used to determine minimum required sample sizes; they were based on ref. 38 and also determined empirically. Generally, 8 brains were imaged for each data point over at least 2 experiments performed on different days. More brains were imaged when the results were variable from one day to another and all results pooled for analysis. We verified that the difference in maximum ∆F/∆F0 between samples was due to differences in absolute peak GCaMP6S intensity, not to differences in initial GCaMP6S intensity for each experiment.

Statistics were analyzed in IgorPro (WaveMetrics). Since samples often had unequal variances, we used the KS test to compare responses to drugs to avoid the problem of requiring normal distributions and equal variances. For baseline GCaMP6S experiments, we used Kruskal–Wallis ANOVA to determine whether there was a significant rhythm in the data.

Low expression levels from Lk-Gal4 and UAS-GCaMP6S in LHLKs, except in Pdf-Dti experiments, were used Kruskal–Wallis ANOVA to determine whether there was a significant rhythm in the data.

Code availability. All IgorPro scripts are available upon request.

A Supplementary Methods Checklist is available.