



Timed receptor tyrosine kinase signaling couples the central and a peripheral circadian clock in *Drosophila*

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Edited by Ori T. Shafer, Advanced Science Research Center, City University of New York, NY; received May 16, 2023; accepted February 1, 2024
by Editorial Board Member Michael Rosbash

Circadian clocks impose daily periodicities to behavior, physiology, and metabolism. This control is mediated by a central clock and by peripheral clocks, which are synchronized to provide the organism with a unified time through mechanisms that are not fully understood. Here, we characterized in *Drosophila* the cellular and molecular mechanisms involved in coupling the central clock and the peripheral clock located in the prothoracic gland (PG), which together control the circadian rhythm of emergence of adult flies. The time signal from central clock neurons is transmitted via small neuropeptide F (sNPF) to neurons that produce the neuropeptide Prothoracicotropic Hormone (PTTH), which is then translated into daily oscillations of Ca^{2+} concentration and PTTH levels. PTTH signaling is required at the end of metamorphosis and transmits time information to the PG through changes in the expression of the PTTH receptor tyrosine kinase (RTK), TORSO, and of ERK phosphorylation, a key component of PTTH transduction. In addition to PTTH, we demonstrate that signaling mediated by other RTKs contributes to the rhythmicity of emergence. Interestingly, the ligand to one of these receptors (Pvf2) plays an autocrine role in the PG, which may explain why both central brain and PG clocks are required for the circadian gating of emergence. Our findings show that the coupling between the central and the PG clock is unexpectedly complex and involves several RTKs that act in concert and could serve as a paradigm to understand how circadian clocks are coordinated.

prothoracic gland | eclosion | PTTH | neuropeptide | circadian rhythms

Circadian rhythms allow multicellular organisms to anticipate daily changes in the environment such as the arrival of dawn or dusk. In animals, these behavioral and physiological rhythms are generated by multi-oscillator systems composed of a central pacemaker housed in the brain as well as of peripheral pacemakers located in a wide variety of tissues. The coordination of these clocks is critical for the organism to express a unified circadian time (1). In mammals, the so-called “master pacemaker” located in the suprachiasmatic nucleus (SCN) synchronizes to the environmental day–night cycles and coordinates peripheral oscillators through neural, endocrine, behavioral, and thermal signals (2). In turn, a variety of peripheral signals feedback to adjust and stabilize SCN rhythmicity (3, 4). However, our understanding of the cellular and molecular mechanisms and rules that mediate this coupling is still fragmentary (5).

In the fly, *Drosophila melanogaster*, the central clock is comprised of about 150 neurons that are critical for imposing a daily periodicity to behaviors such as locomotor activity and sleep (6). Key components of the central clock are the small and large ventral lateral neurons (s- and lLN_vs, respectively), which produce the neuropeptide pigment-dispersing factor (PDF) and are critical for circadian timekeeping (7). In addition, peripheral clocks reside in a variety of tissues and are mostly autonomous and entrained directly by external inputs (8–10). However, in other cases, and similarly to mammals, the central clock can coordinate its activity with peripheral oscillators. In particular, the rhythm of emergence of adult flies (used here interchangeably with the term eclosion), is mediated by the coupling of the brain clock and a peripheral oscillator that resides in the prothoracic gland (PG), the endocrine gland that produces the steroid molting hormone, ecdysone (11–13). We previously reported that this coupling is mediated by a peptidergic signaling pathway in which sNPF from the sLN_v clock neurons inhibits the neurons that express PTTH (13). In turn, the PTTH neurons (PTTH_n) secrete PTTH, which binds to the receptor tyrosine kinase (RTK) encoded by *torso* in cells of the PG, to control the biosynthesis of the molting hormone, ecdysone (13, 14). Although the titers of this steroid must fall below a threshold level to trigger emergence (15, 16), we recently showed that the clock does not exert its action by regulating the levels of ecdysone but by controlling its actions, which are mediated by the ecdysone receptor in the PG (17). Indeed, although injections

Significance

Circadian clocks impose daily periodicities to behavior, physiology, and metabolism, and are synchronized to provide the organism with a unified time through mechanisms that are poorly understood. In holometabolous insects, the circadian control of adult emergence depends on the coupling between the central clock and a peripheral clock located in the prothoracic gland (PG). Here, we identify the cellular and molecular mechanism that transmits time information from the central clock to the PG clock. This process is unexpectedly complex and involves a number of receptor tyrosine kinases (RTKs). Such a mechanism may add robustness to the coupling between the two clocks and serve as a paradigm for understanding how circadian clocks are coordinated.

Author contributions: J.C.-L., E.A., R.S., C.W., and J.E. designed research; J.C.-L., E.A., M.Z., and J.E. performed research; D.R.N. contributed new reagents/analytic tools; J.C.-L., E.A., R.S., C.W., and J.E. analyzed data; and J.C.-L., E.A., D.R.N., R.S., C.W., and J.E. wrote the paper.

The authors declare no competing interest.

This article is a PNAS Direct Submission. O.T.S. is a guest editor invited by the Editorial Board.

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This article contains supporting information online at <https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2308067121/-/DCSupplemental>.

Published March 5, 2024.

of 20E delay the time of eclosion, they do not affect its circadian gating. By contrast, disabling 20E actions in the PG renders arrhythmic the pattern of adult emergence.

Although the neuropeptide circuit that connects the brain clock to the PG clock has been identified, how and when during development the time signal is transmitted from the central circadian pacemaker to this peripheral clock remains unclear. In order to understand how and when the time signal is transmitted from the central clock to the PG clock, we examined the anatomical, cellular, and molecular basis of the central clock–PTTHn–PG axis. We first determined the connectivity between sLNv clock neurons and the PTTHn by pre- and postsynaptic tracing and found that this connection is unidirectional and likely to be exclusively peptidergic. We also show that PTTHn exhibit daily changes in intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) and of PTTH immunoreactivity at the terminals of PTTHn in the PG. Unexpectedly, we found that PTTH is required for time of day-dependent changes in *torso* expression and PTTH transduction in the PG. Direct imaging of the activity of PTTHn and the PG revealed that the final peak of activity in PTTHn occurred around 16 h before eclosion and was followed 6 h later by a peak of activity in the PG. This timing is consistent with our recent findings on the events that control the timing of eclosion (17), and we further show here that PTTHn signaling is required at the end of metamorphosis for the expression of a circadian rhythm of adult emergence. In addition, the presence of activity peaks in the PG that occurred prior to the activation of PTTHn suggests that the PG responds to additional factors, and we show here that PDGF- and VEGF-receptor related (*Pvr*) and Anaplastic lymphoma kinase (*Alk*), two RTKs that are also expressed in the PG (18), contribute to the circadian control of emergence. Thus, our detailed characterization of the transduction pathway from the sLNv clock neurons to the PG clock reveals that the transmission of time information between clocks is surprisingly complex and involves multiple actors. It also defines a role for RTK signaling in the coordination of circadian clocks. Our work may provide a general mechanism for understanding how circadian clocks are coordinated.

Results

All PDF-Positive sLNv Signal Non-Synaptically to the PTTHn. The dorsal projections of PDF-expressing LNv clock neurons terminate in close proximity to PTTHn arborizations (Fig. 1 *A–A'*). We previously showed that sLNvs transmit time information to PTTHn via peptidergic sNPF signaling, but not PDF (13). To determine which LNv neurons signal to PTTHn and whether this contact includes synaptic transmission, we performed a connectomic analysis by GFP reconstitution across synapses (*syb-GRASP*) (19), BAcTrace (20), and *trans-Tango MkII* (21), in adult pharate animals. This work was necessary because the PTTHn can no longer be detected starting shortly after eclosion (22) and are therefore not included in the available EM-based connectomic datasets that are based on older adult flies (23). Despite the close proximity of the sLNv terminals to the dendritic arborizations of PTTHn, the reconstituted GFP signal between these neurons using *syb-GRASP* was weak and restricted to the primary process (Fig. 1*B*) suggesting that sLNv transmit time information to the PTTHn primarily via non-synaptic connections. In contrast, the reconstituted *syb-GRASP* signal between PTTHn and other subsets of clock neurons (e.g., DN1a, DN2, LNd, and LPN neurons) was much more branched and of varicose nature (Fig. 1 *B'* and *B''*), suggesting that other clock neurons provide synaptic input to the dendritic arborizations of the PTTHn. This suggests that sLNv transmit time information to the PTTHn primarily via non-synaptic connections. We then used BAcTrace and *trans-Tango MkII*, which are retrograde and anterograde neuronal

tracers, respectively, to determine the directionality of the connection and to identify the LNvs that provide input to the PTTHn. As shown in Fig. 1 *C* and *D*, BAcTrace labeled all sLNv and none of the ILNv, whereas *trans-Tango MkII* labeled both pairs of PTTHn. Finally, we used receptor-specific intragenic driver lines to show that PTTHn express the receptor for sNPF (sNPF_R) but not that for PDF (PDF_R) (Fig. 1 *E* and *F*), consistent with our previous finding that transmission is mediated via sNPF and not PDF (13).

Ca^{2+} Signaling in PTTHn Is Relevant to the Circadian Control of Adult Emergence. Since sNPF released from sLNv reduces Ca^{2+} levels $[Ca^{2+}]_i$ in PTTHn (13), we explored whether $[Ca^{2+}]_i$ in PTTHn changed in a time-dependent manner. For this, we used the genetically encoded Ca^{2+} sensor, GCaMP6M (25), to measure changes in $[Ca^{2+}]_i$ in PTTHn during the course of the day (and subjective day) at the beginning of metamorphosis [white pre-pupal stage (WPP)]. (WPP animals were used because the circadian clock is intact and fully functional at this time (11). By contrast, the PG of animals close to emergence is undergoing apoptosis (26), making them difficult to image.) As shown in Fig. 2 *A–C*, the cell bodies of PTTHn expressed a circadian rhythm in $[Ca^{2+}]_i$ under a 12-h light:12-h dark regime (LD) (see [Dataset S1](#) for all statistical analyses of expression data), with maxima at the beginning of the night [Zeitgeber time (ZT) 12] and minima 4 h after lights-on (ZT4). Differences in the amplitude of the GCaMP signal were maintained under constant darkness (DD), but the peak was delayed by up to 6 h [circadian time (CT) 18, where CT12 is the start of the subjective night] (Fig. 2*E*); this delay may occur because photic inputs can set the Ca^{2+} phase in a group of clock neurons (27). Importantly, differences in the amplitude of the GCaMP signal measured at different times were not observed in animals bearing null alleles for the *period* gene (*per⁰*), a core component of the clock, under both LD (Fig. 2*D*) and DD conditions (Fig. 2*F*). Expression of GCaMP in the PTTHn did not affect the rhythm or period of emergence (see [SI Appendix, Table S1](#) for all statistical analyses of emergence rhythmicity).

In order to determine whether there is a temporal relationship between the rhythm of sNPF release from sLNv and the Ca^{2+} oscillations in PTTHn, we measured the temporal pattern of neuropeptide abundance at the terminals of sLNv neurons. Available antibodies did not allow us to reliably quantify sNPF immunoreactivity; thus, we used instead the transgenic neuropeptide reporter, ANF-GFP (28), which we drove in PDF-expressing LNvs. As shown in [SI Appendix, Fig. S1 A and B](#), and similar to previous findings for PDF and *Drosophila* insulin-like peptide 2-GFP in adult flies (29, 30), the ANF-GFP signal in the terminals of sLNv at the WPP stage was higher at the beginning of the day than at the end of the day, which is coincident with the time when $[Ca^{2+}]_i$ levels in PTTHn were minimal (Fig. 2*C*). Thus, our results suggest that the phase of the Ca^{2+} rhythm in PTTH neurons is set by the inhibitory sNPF input from sLNv (although *pdf*> ANF-GFP reports the abundance of both sNPF and PDF, PTTHn do not express PDF_R).

In order to evaluate the relevance of Ca^{2+} in the PTTHn to the circadian control of adult emergence, we knocked down elements involved in Ca^{2+} signaling in PTTHn and determined the consequences on the rhythm of adult emergence (Fig. 2 *G–J*). We found that the knockdown of voltage-gated Ca^{2+} channels (Ca^{2+} - α 1D, Ca^{2+} - α 1 T, and *cacophony*) and of Ca^{2+} -binding proteins (CasK and PKC) did not affect the rhythm or the periodicity of eclosion (Fig. 2*J*). By contrast, knocking down the expression of RyR (ryanodine receptor), an endoplasmic reticulum Ca^{2+} channel, caused a significant weakening of the circadian pattern of emergence (Fig. 2 *G* and *J*). (In these and all RNAi-mediated knockdown experiments, at least two different RNAi transgenes were tested; see [Materials and Methods](#).)

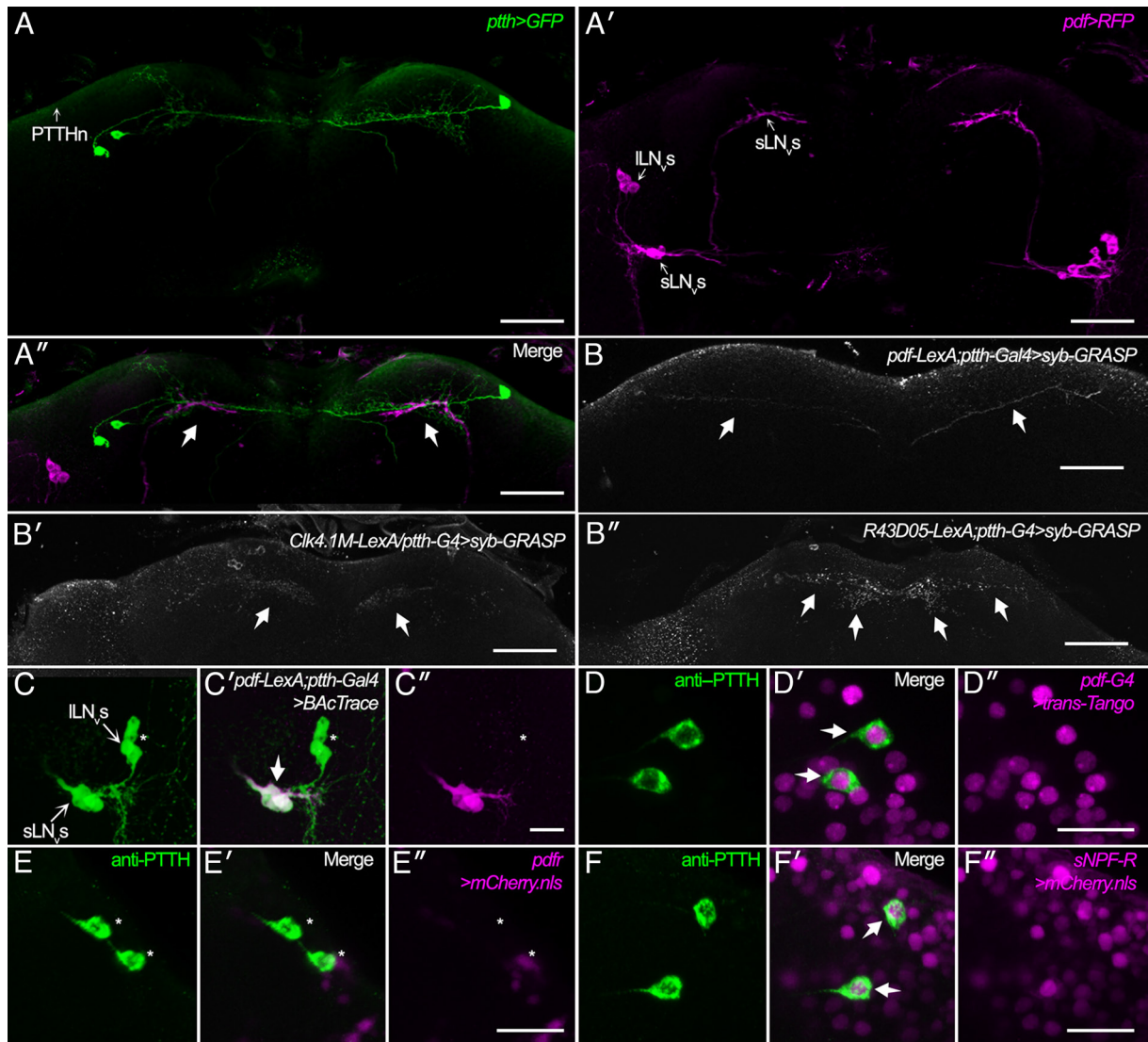


Fig. 1. Connectivity between PDF-expressing sLNv clock neurons and PTTHn. (A–A'') Cell bodies and projections from PTTHn in the superior protocerebrum (A). PDF-expressing sLNv clock neurons project to the superior protocerebrum (A') in close proximity to the projections of PTTHn (arrows in merged image, A''). (B) syb-GRASP reconstruction between PTTHn and DN1p clock neurons labeled by *Clk4.1 M-LexA* produced a faint and sparse reconstruction of GFP along the primary neurite (arrows), which did not include small branches in the contact area between PTTHn and sLNv located in the superior protocerebral region. (B') syb-GRASP reconstruction between PTTHn and DN1a, DN2, LNd, and LPN clock neurons labeled by *R43D05-LexA* (24) produced a varicose branched signal. In comparison (B–B'), the sparse vs. varicose syb-GRASP signals suggest that PTTHn receive non-synaptic input from the sLNvs, and synaptic input from other clock neurons. (C–C'') BACTrace-labeling showing that all PDF-expressing sLNv but not the large ILNv synapse onto the PTTHn. Whereas *pdf-LexA* drives GFP expression in the sLNv and ILNv (C), the BACTrace signal (C') was only detected in the sLNv (C''). (D–D'') *trans-Tango* MkII labeling showed that all PTTHn are downstream to the sLNv since the positive *trans-Tango* MkII signal was visible in PTTHn present in each hemisphere (D'). (E–E'') A PDF receptor-specific intragenic driver line (E') did not label the PTTHn (E) in pharate adults, indicating that the PDF receptor is not expressed (E'). Asterisks mark the position of the PTTHn. (F–F''). By contrast, an sNPF receptor-specific intragenic driver line (F''), labeled the PTTHn (F) in pharate adults (F), indicating sNPF receptor expression. (Scale bars: A and B: 50 μ m; C–F: 20 μ m.)

A similar result was obtained following the knockdown of SERCA (sarco/endoplasmic reticulum calcium ATPase) (Fig. 2 H and J). Importantly, knockdown of RyR or SERCA did not affect the gross morphology of PTTHn (SI Appendix, Fig. S2 A–C) suggesting that the weakening of the rhythm of the emergence may be caused by alterations in Ca^{+2} homeostasis in PTTHn. Together, these results reveal that Ca^{+2} signaling in PTTHn through the RyR and SERCA transporters is part of the cellular mechanism that imposes a daily rhythm to the pattern of adult emergence.

The PTTH/*torso* Axis Is under Clock Control. Although PTTH transmits time information from the sLNv to the PG clock, this timing signal does not involve the circadian regulation of *ptth* transcript levels (13). However, whether PTTH abundance

cycles in the PTTHn axon terminations in the PG is unknown. To explore this possibility, brain-PG complexes from WPP were dissected at different times of day and immunostained for PTTH. As shown in SI Appendix, Fig. S3A, PTTH immunosignal in the cell bodies showed significant changes in amplitude during the course of the day. However, these changes persisted in *per⁰* animals, indicating that they are not dependent on the clock; they may instead be dependent on photic inputs, similar to the role of PTTH in light avoidance during larval stages (21, 31). By contrast, PTTH immunoreactivity of the axonal terminals onto the PG showed clock-dependent changes in intensity during the course of the day. Indeed, PTTH immunoreactivity was maximal during the night and early morning and minimal during the day (Fig. 3 A–C), persisted under DD conditions, and was abolished in *per⁰* null

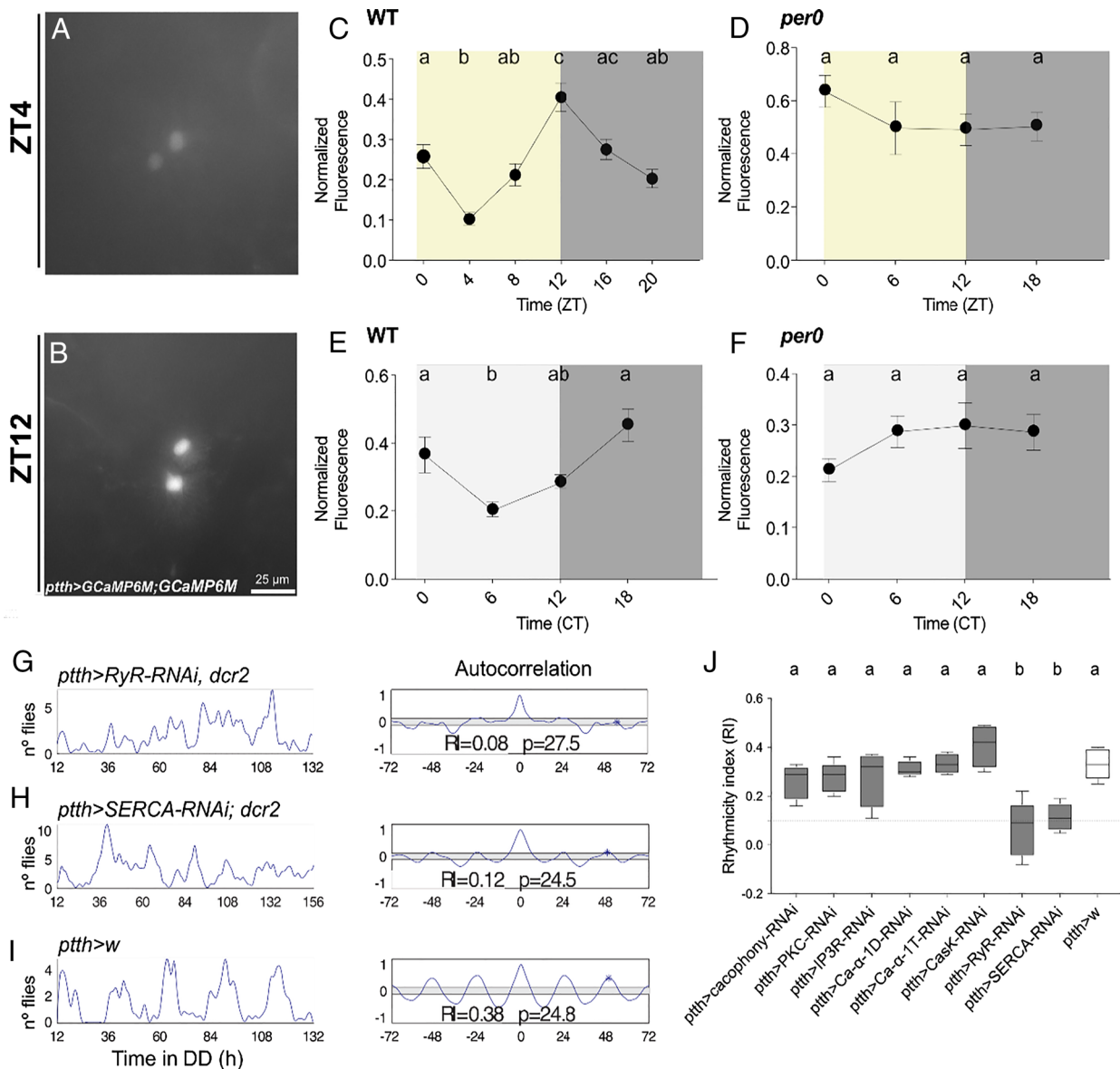


Fig. 2. Ca^{2+} signaling in PTTHn is relevant to the circadian control of adult emergence. (A and B) Representative images of GCaMP fluorescence in PTTHn in the brains of WPP examined at ZT4 (A) and ZT12 (B). (C–F) Levels of GCaMP fluorescence in PTTHn of wild-type (WT) animals at different times of day under LD (C) and DD conditions (E), and in the soma of PTTHn of *per0* mutants under LD (D) and DD (F) conditions. Different letters indicate statistically different groups (ANOVA with Tukey's post hoc test). Between 8 and 10 animals were analyzed for each timepoint. ZT: zeitgeber time. CT: Circadian time. (G–I) Records showing the time course of emergence of a single population of flies under DD conditions (Left) and corresponding autocorrelation analysis (Right) of populations bearing a knockdown in PTTHn of Ryanodine Receptor (RyR) (G), SERCA (H), and in corresponding controls. (I) Periodicity (p, in hours) and associated rhythmicity index (RI) are indicated. (J) Average RI values from knockdown in PTTHn of mediators of Ca^{2+} signaling. The dashed line at RI = 0.1 indicates cutoff below which records are considered arrhythmic. Different letters indicate statistically different groups ($P < 0.05$; one-way ANOVA, Tukey's post hoc multiple comparison analyses; see *SI Appendix, Table S1* and *Dataset S1* for all statistical analyses of emergence rhythmicity and of expression data, respectively).

mutant animals under both LD and DD conditions (Fig. 3 D–F). Together, these results indicate that the circadian clock acts at the post-translational level to impose time of day-dependent changes in PTTH accumulation at the site of innervation of the PG and suggests that PTTH release onto the PG may be modulated by the circadian clock.

Another critical step in the transmission of time information from the brain clock to the PG clock is the PTTH receptor, TORSO (13). To test whether *torso* expression is regulated by the clock, we first examined *torso* mRNA abundance in the PG at different times of day, in WT and in *per⁰* null mutant animals. As shown in Fig. 3 G and H and *SI Appendix, Fig. S3B*, *torso* transcript levels differed between day/subjective day and night/subjective night in WT but not in *per⁰* null animals. Intriguingly, transcript levels were highest during the day/subjective day and were minimal at the beginning

of the night/subjective night, which is in antiphase relative to the oscillations observed for the PTTH-immunoreactivity of the terminals of PTTHn onto the PG. Interestingly, *torso* mRNA levels did not vary over time in the PG of *ptth* null mutant animals under DD condition (Fig. 3I). Taken together, our findings suggest that the changes in the levels of *torso* transcript abundance are dependent on the central clock input and depend on a signal that is transmitted by its ligand, PTTH. As expected, given that knockdown of *torso* in the PG causes the expression of an arrhythmic eclosion pattern (13), *ptth* null mutant animals showed an arrhythmic pattern of emergence (*SI Appendix, Fig. S3C*).

Circadian Regulation of PTTH Transduction in the PG. Our findings reveal that PTTH immunoreactivity of PTTHn terminals and *torso* transcript levels change in antiphase. In order to obtain

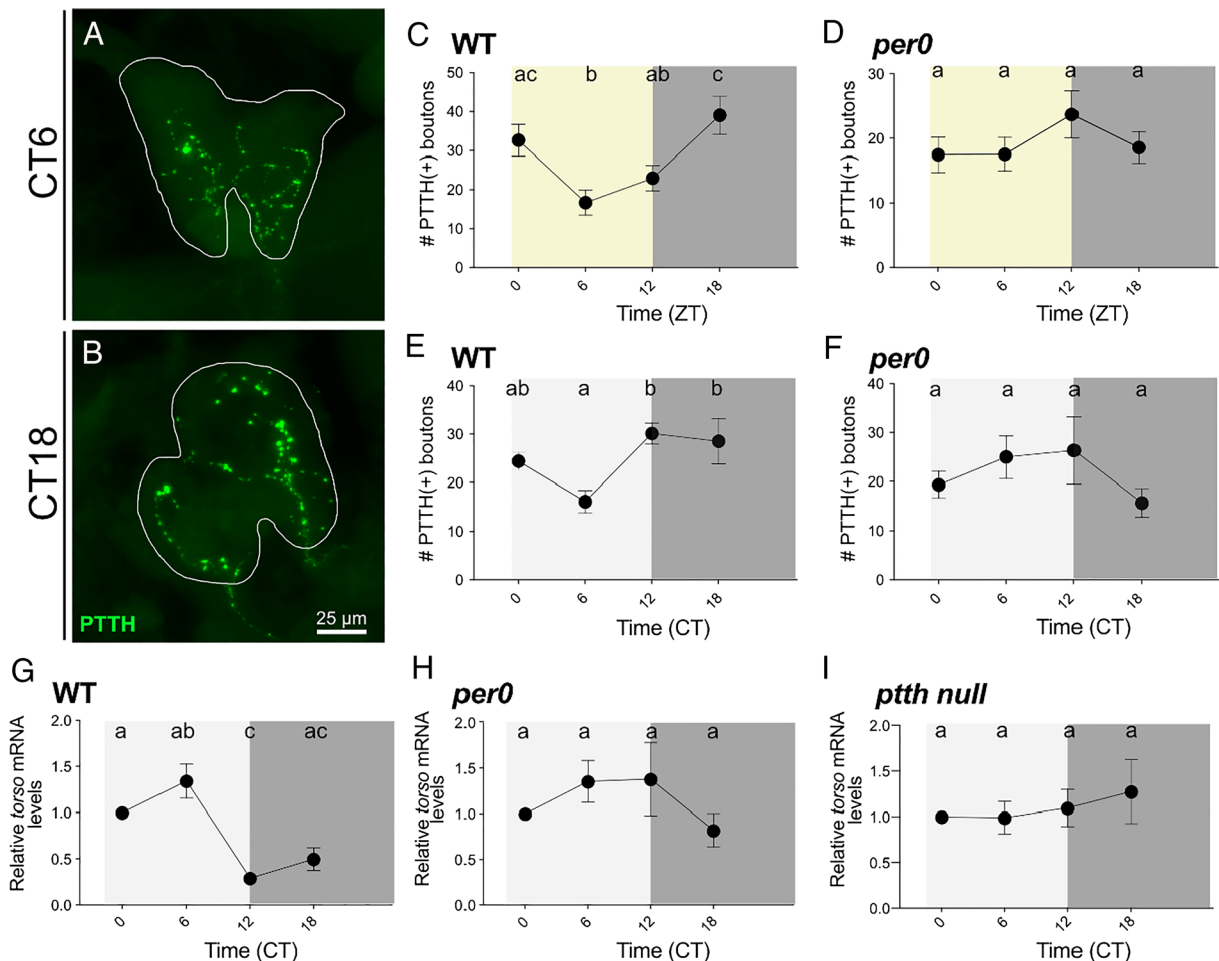


Fig. 3. Clock control of PTTH/TORSO axis. (A and B) Pattern of PTTH-immunoreactivity of terminals of PTTHn on the PG of WPP animals at CT6 (A) and CT18 (B). The white line delimits the PG. (C–F) Average number of PTTH-immunoreactive boutons showing above-threshold intensity on the PG at different times of day in WT (C and E) and in *per0* mutants (D and F) under LD (C and D) and DD (E and F) conditions. Eight to ten brains were analyzed per time-point. (G–I) Relative expression of mRNA levels of *torso* in PGs isolated from WPP stage WT (G), *per0* (H), and *ptth* null mutant (I) animals under DD conditions. Different letters indicate statistically different groups ($P < 0.05$; one-way ANOVA, Tukey's post hoc multiple comparison analyses; see Dataset S1 for all statistical analyses of expression data).

a readout of the temporal pattern of the resulting activation of the TORSO pathway, we asked whether the levels of phosphorylated ERK (phosphoERK), a key component of PTTH transduction (32), change during the course of the day. For this, we expressed in the PG a genetically encoded ERK reporter (ERK-SPARK), which is based on phase separation–based kinase activity (33) (SI Appendix, Fig. S4A). As shown in Fig. 4, total ERK activity was highest during the day and subjective day under LD conditions (Fig. 4G) and exhibits a circadian rhythm under DD conditions (Fig. 4A, B and H, and Dataset S1 for all statistical analyses of expression data). Importantly, these differences in phosphoERK levels were lost in *per0* null mutant animals (Fig. 4I) indicating that total ERK activity in the PG cells is dependent on the circadian clock. They were also lost when *torso* was knocked down in the PG (Fig. 4C, D, and J) (A SPARK signal was still detected under these conditions, as would be expected because this pathway can be activated by other ligands, e.g., insulins). Our results show that PTTH immunoreactivity in the PTTHn was lowest during the day whereas *torso* transcript levels and total phosphoERK levels in the PG were highest at this time of day; this suggest that PTTH release and TORSO activation occur at this time of day. Expression of the ERK reporter in the PG did not affect the circadian rhythmicity of emergence (SI Appendix, Fig. S4 B–D).

ERK shuttling between cytoplasm and nucleus is relevant for ecdysone biosynthesis and for the control of developmental timing during the larval stages (34). Despite the fact that the majority of droplets in the PG were cytoplasmic, the nuclear SPARK signal was also higher during the day than during the night (SI Appendix, Fig. S5C). These daily changes persisted under DD conditions, although the maximum levels were phase-advanced (SI Appendix, Fig. S5 A, B, and E), which is consistent with the time of day-dependent changes in total SPARK signal in the PG cells. In addition, *per0* and PG>*torso*-RNAi animals showed an arrhythmic pattern of nuclear SPARK signal (SI Appendix, Fig. S5 D and F) suggesting that the daily change in phosphoERK shuttling between the cytoplasm and nucleus in the PG is dependent on a functional circadian clock and of daily changes in PTTH input.

Neuronal Activity of PTTHn Is Required at the End of Pupal Development for the Circadian Gating of Eclosion. To determine when during pupal development the rhythmic activity of PTTH is required for a circadian gating of emergence, we investigated the consequences on the timing of emergence of conditionally silencing the PTTHn. To do so, we expressed the inward rectifying K⁺ channel, Kir2.1, at different times of development using the temperature-dependent TARGET system (35). As shown in Fig. 5 A and B, silencing the PTTHn during the embryonic and

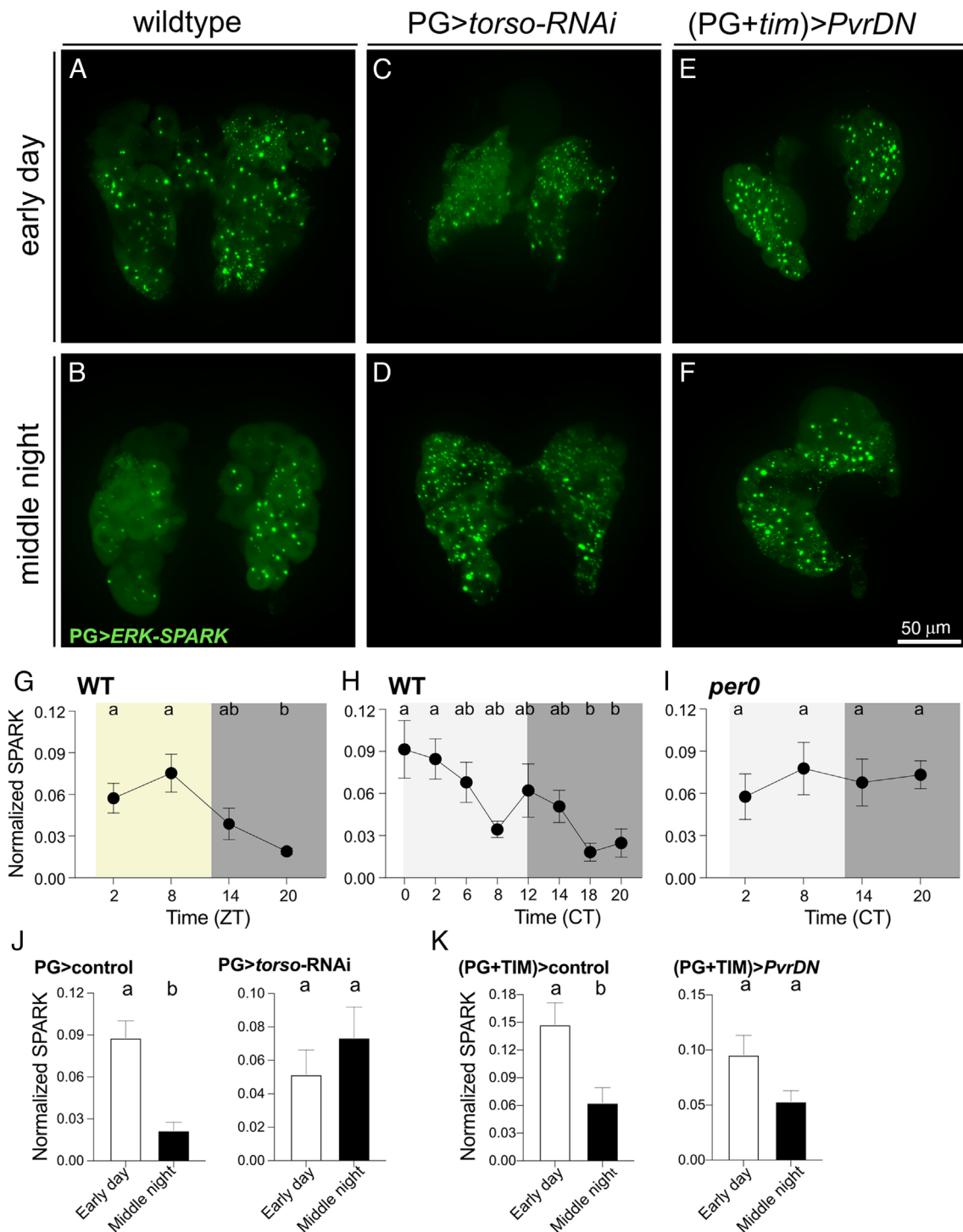


Fig. 4. ERK phosphorylation in the PG shows daily variations that are dependent on *torso* and *Pvr*. (A–F) ERK-SPARK signal in whole mount PGs (*phm>ERK-SPARK*) of WT, *PG>torso-RNAi*, and *(PG+tim)>PvrDN* WPP animals at CT0-2 (early subjective day) and CT18-20 (middle of subjective night). (G–I) Average of normalized ERK-SPARK signal in the PG at different times of day in WT PG under LD (G), DD (H) conditions, and in *per0* mutants (under DD condition; I). (J and K) Average normalized ERK-SPARK signal in (J) *PG>w* and *PG>torso-RNAi* and (K) *(PG+tim)>w* and *(PG+tim)>PvrDN* animals, at CT0-2 (early subjective day) and CT18-20 (middle of subjective night). Eight to ten brains were analyzed per time-point. Different letters indicate statistically different groups ($P < 0.05$; one-way ANOVA, Tukey's post hoc multiple comparison analyses; see Dataset S1 for all statistical analyses of expression data).

larval stages or during early pupal development did not affect the circadian rhythmicity of emergence. Determining the role of PTTH at the end of metamorphosis requires raising temperature at this time, which accelerates metamorphosis and causes animals to emerge over a much shorter period of time. This makes it difficult to evaluate the rhythmicity of the resulting records. Nevertheless, both by visual inspection, and by Lomb–Scargle (LS) (36) and eJTK_Cycle (37) analyses, silencing these neurons

during the entire or only the second half of metamorphosis caused an arrhythmic pattern of emergence. These results suggest that the activity of PTTHn is required during the final stages of metamorphosis for the clock to impose circadian rhythmicity to the temporal pattern of adult emergence.

In order to directly assess the activity of PTTHn and of the PG during the second half of metamorphosis, and considering that we are not able to perform *in vivo* Ca^{2+} imaging of the PG at this time,

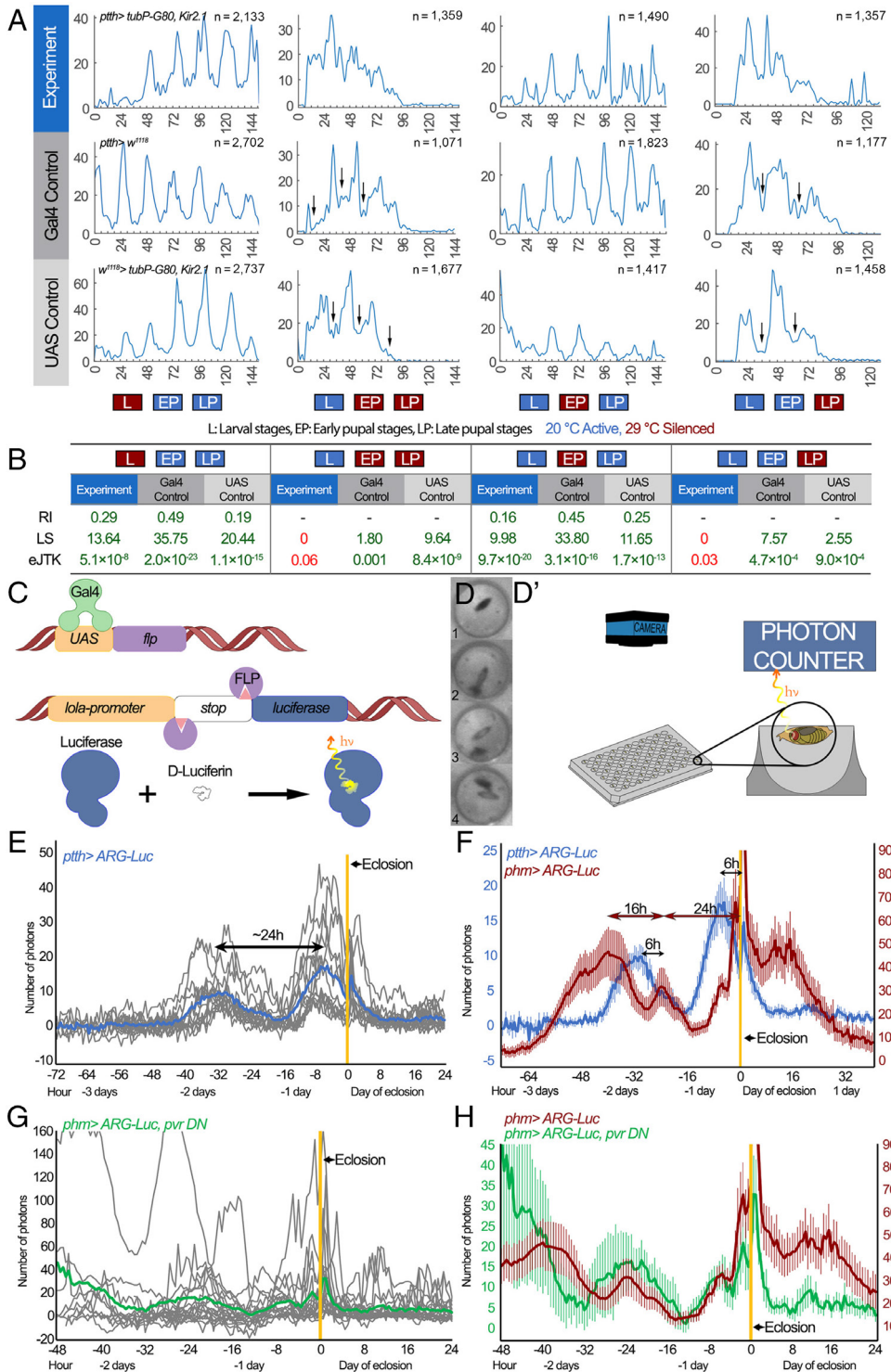


Fig. 5. PTTHn activity is required during the end of pupal development. (A) Record of the time course of emergence in *pttH>tubP-G80^{Δ5}, Kir2.1* flies with PTTHn conditionally silenced at 29 °C during larval (L), entire pupal (EP+LP), early pupal (EP), and late pupal stages (LP), and in corresponding controls. (B) RI, Lomb-Scargle (LS), and eJTK_Cycle values of experiments shown in (A). Rhythmic records are coded in green, arrhythmic ones in red. Raising the temperature to 29 °C during metamorphosis accelerates development, and when this period included the late pupal stage (LP), it causes emergence to occur over a short period of time. This renders autocorrelation inappropriate for rhythmicity analysis. Hence, values are given for LS and eJTK_Cycle analysis only. In addition, rhythmic records show peaks followed by valleys that are separated by ~24 h (arrows); see *Materials and Methods* for further details. (C–F) In vivo imaging of ARG-Luc signal in PTTHn and PG prior to emergence. (C–D') Schematic representation of the ARG-Luc system and in vivo imaging setup. (D) Images of intact animals captured at different stages: pharate [1]; eclosed [2]; prior to [3], and after [4], wing expansion. (E) ARG-Luc activity of PTTHn and PG plotted relative to the time of emergence (yellow line); the blue line corresponds to the average trace. (F) Average ARG-Luc activity for the PTTHn (blue) and for the PG (red, $n = 22$). (G) ARG-Luc activity of PGs expressing the dominant negative form of *Pvr* (*PvrDN*) plotted relative to eclosion (yellow line) ($n = 19$). The mean activity is plotted in green. (H) Mean activity of the PG expressing *PvrDN* [green, same curve as in (G)] is plotted against the mean of normal PG activity [same curve as in (F)]. Error bars indicate SEM. Expression of *PvrDN* in the PG strongly reduced the amplitude of the ARG-Luc signal (note different scales for Y-axes) without significantly affecting its timecourse. Error bars indicate SEM. For PTTHn, 21 animals were recorded, but the records of only 14 were included in E and F; the remaining 7 animals were excluded because their signal did not change during the recording period, most likely because they were either located too far from the sensor or were not correctly oriented. The peaks immediately following eclosion in (E–H) are likely artifacts due to higher photon yields once flies have left their absorbing puparium.

we turned to an Activity-Regulated Gene-Luciferase reporter (ARG-Luc) (38) for long-term monitoring of neuronal activity in intact developing pupae (Fig. 5 C and D). Strikingly, we found that PTTHn under DD conditions express a monophasic circadian rhythm of activity that started 2 d prior to adult emergence, with a second peak of activity preceding eclosion by 6 h (Fig. 5 E and F). This timing is consistent with the peak in intracellular Ca^{2+} signaling detected in the middle of the subjective night in DD at the WPP stage (Fig. 2E), as well as with the increased signal detected just before emergence using the calcium-dependent sensor, CaLexA (39) (SI Appendix, Fig. S6). In parallel experiments, we also used ARG-Luc to monitor the activity of the PG during pupal development. As shown in Fig. 5 F and G, the PG expressed a peak of activity at around -25 h and a large peak at around the time of eclosion, each lagging PTTHn activity by around 6 h. In addition, our records showed an earlier peak of activity in the PG at around -40 h that was not preceded by a peak in PTTHn activity, suggesting that activity in the PG depends on other factors in addition to PTTH but that its phase close to the time of emergence may be set by PTTHn activity. In the PG, increases in ARG-Luc signal may be due to changes in Ca^{2+} levels (11), but we cannot rule out that they may be caused by a different mechanism (38).

PTTH-Independent RTK Signaling Pathways Contribute to the Rhythm of the Adult Emergence. Although PTTH is the best-known ecdysteroidogenic factor, the peak of activity detected in the PG 40 h before eclosion preceded the first peak of activity in PTTHn, which may occur in response to other signals that act on the PG (18, 40, 41). In particular, a recent study reported that anaplastic lymphoma kinase (*Alk*) and PDGF and VEGF receptor-related (*Pvr*), two RTK that are expressed in the PG, act in an additive manner to regulate the timing of metamorphosis (18). To determine whether these RTKs contribute to the rhythm of adult emergence, we determined the consequences on the timing of eclosion of knocking down or of expressing dominant-negative versions of these receptors, in the PG. As shown in Fig. 6 A, B, and I, overexpression of a dominant-negative version of *Pvr* in the PG weakened the rhythm of the emergence relative to its control. A similar phenotype was produced by expressing an *Alk* RNAi construct (Fig. 6C), as well as when both receptors were knocked down together (Fig. 6 D and I). (Reducing the expression of *Alk* and *Pvr* in all clock tissues except for the PG led to a rhythmic pattern of emergence; SI Appendix, Fig. S7 A and B.) Flies bearing only UAS-RNAi transgenes for *Alk* and *Pvr* expressed normal circadian rhythmicity of emergence (Fig. 6I). A role for *Pvr* in the regulation of the timing of emergence is also consistent with the impact on SPARK-ERK signal of disabling PVR signaling in the PG. Indeed, as shown in Fig. 4 E, F, and K, expressing the dominant-negative version of *Pvr* in the PG eliminated the difference between the signal measured during the day vs. the night. Similarly, expressing a dominant version of *Pvr* severely affected the levels of ARG-luc signal in the PG, while the rhythmicity and phase seem unaffected (Fig. 5H). Thus, PVR signaling may act to amplify the endogenous PG signal rather than to alter the phase or rhythmicity of the PG clock.

It has been shown that ALK acts via both PI3K and Ras/Erk signaling pathways (18). Although we previously showed that Ras/Erk is critical for the rhythm of emergence (13), we found that knockdown of *pi3k* in the PG did not affect the rhythm or period of emergence (SI Appendix, Table S1). These results suggest that *Alk* contributes to the circadian control of eclosion exclusively via the Ras/Erk signaling pathway.

Recent work has revealed that PTTHn express the ALK ligand, JEB (Jelly belly), and one of three known ligands for PVR, Pvf3

(18). Consistent with the consequences of knocking down the ALK receptor in the PG, knockdown of its ligand, JEB, in PTTHn using the strong *NP423-GAL4* driver (31) weakened the rhythmicity of emergence (Fig. 6 E and J). By contrast, the corresponding knockdown of *Pvf3* was without effect (Fig. 6 F and J). Finally, given that Pvf2 (another ligand for PVR) and Pvf3 are expressed in the PG (18), we explored the possibility that these ligands might regulate the circadian rhythmicity of emergence in an autocrine manner. Interestingly, knockdown of *Pvf2* (but not of *Pvf3*) in the PG weakened the rhythmicity of adult eclosion (Fig. 6 G, H, and K) suggesting that *Pvf2* contributes to the circadian rhythm of emergence via an autocrine pathway. Together, these results reveal that the PTTH/TORSO axis is a major, but not exclusive, regulator of the rhythmicity of emergence. Indeed, our results indicate that JEB/ALK and autocrine signaling involving Pvf2/PVR also contribute to the rhythmicity of eclosion.

We also explored whether other ecdysteroidogenic signals including Allatostatin A (42), Corazonin (43), insulin signaling (40), as well as Gq proteins encoded by CG30054 and CG17760 (44), might contribute to the circadian control of adult emergence. As shown in SI Appendix, Table S1, RNAi knockdown or null mutations of components of these signaling pathways in the PG did not affect the rhythmicity or the periodicity of adult eclosion.

Since peptidergic neurons often co-express small molecule neurotransmitters (45), we also investigated whether any of such signaling molecules are involved in the circadian control of emergence. As shown in SI Appendix, Fig. S8, we found that PTTHn do not express the signature markers of signaling by glutamate (VGlu), GABA (VGAT), or acetylcholine (ChAT), suggesting that PTTHn may be exclusively peptidergic. Finally, we found that stopping the clock located in the fat body (46) by overexpressing a dominant negative form of the *cycle* gene (*cyc*[Δ 901]) (47) did not affect the timing of adult fly emergence (SI Appendix, Table S1), indicating that the fat body, which also houses a circadian clock and could influence the PG via endocrine signaling, does not contribute to the circadian regulation of adult emergence.

Discussion

Circadian clocks impose daily periodicities to behavior, physiology, and metabolism (5, 48, 49). This control is mediated by the activity of a central clock and of peripheral clocks, which are housed in a variety of relevant organs. Although these various clocks are known to be synchronized to provide the organism with a unified time, how this coordination occurs is not fully understood. Here, we characterized the cellular and molecular mechanisms involved in coupling two circadian clocks in *Drosophila*, the central clock of the brain and the peripheral clock located in the PG, which together restrict the time of emergence of the adult fly to the early part of the day. We showed that time information is propagated from the central sLN_v pacemaker neurons to PTTHn through a peptidergic, non-synaptic, connection mediated by sNPF, and is then translated into a circadian regulation of Ca^{+2} levels and PTTH accumulation in PTTHn. Although these features were measured in animals initiating metamorphosis (white puparium stage, WPP), we found that Ca^{+2} levels in PTTHn also varied in the pharate adult and peaked just before adult emergence. (The fact that PTTHn showed a similar timecourse in Ca^{+2} levels at both developmental stages also suggests that the WPP is a relevant stage for investigating the clock control of behaviors that occur many days later, at adult emergence.) Importantly, we found that neuronal activity of PTTHn at the end of pupal development

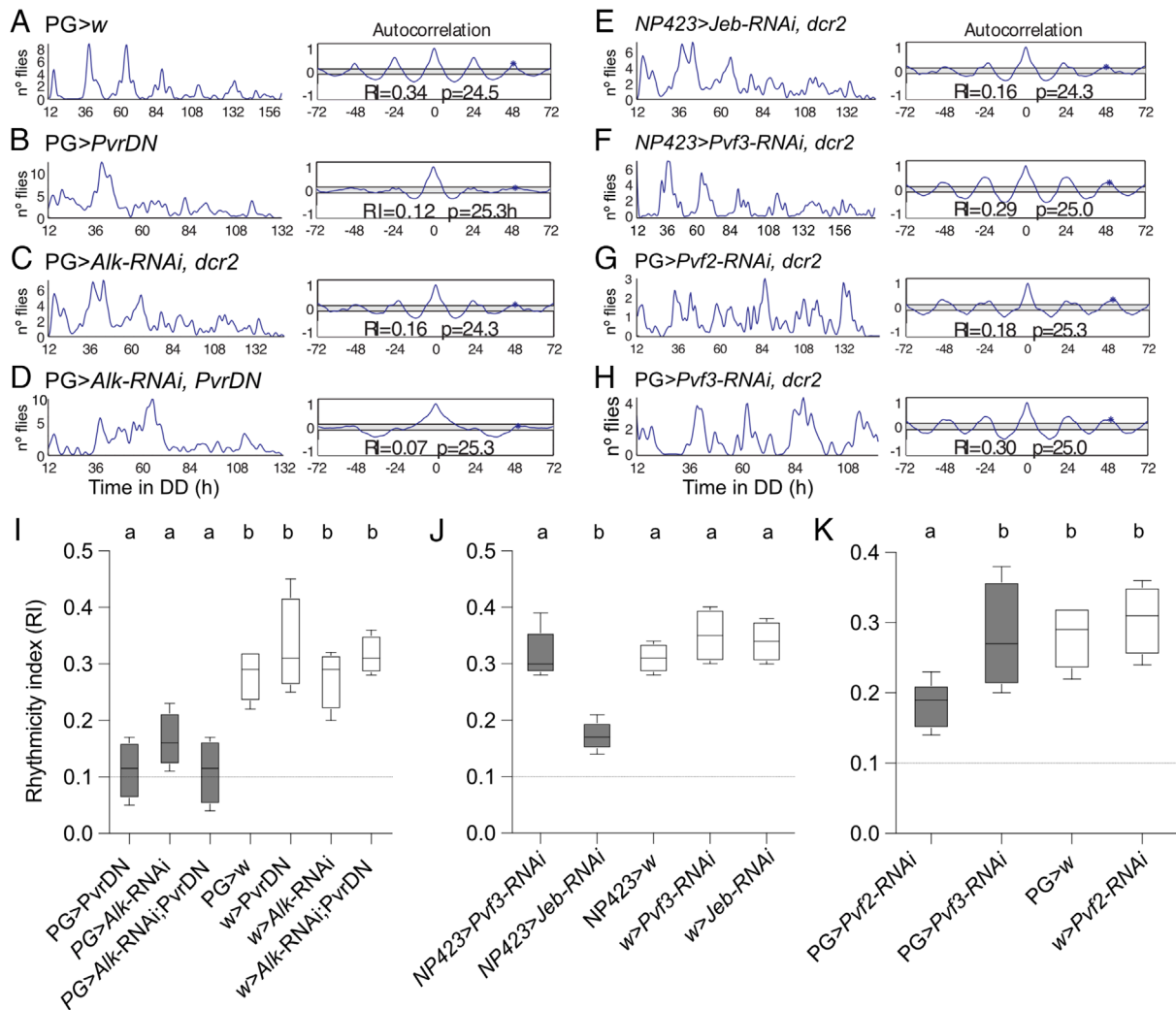


Fig. 6. *Alk* and *Pvr* contribute to the circadian rhythmicity of adult emergence. (A–H) Records showing the time course of emergence under DD (Left) and corresponding autocorrelation analysis (Right) of a single population of flies expressing: in the PG, a dominant negative form of *Pvr* (B), *Alk* RNAi (C), simultaneous knockdown of *Pvr* and *Alk* receptors (D), *Pvf2* RNAi (G) and *Pvf3* RNAi (H); and expressing in PTTHn: *Jeb* RNAi (E) and *Pvf3* RNAi (F). Periodicity (p, in hours) and associated RI are indicated. (I–K) Average RI values for results shown in (A–H) and their respective controls; dashed line indicates cutoff below which records are considered arrhythmic. Different letters indicate statistically significant differences. NP423 is a strong driver that includes PTTHn. * $P < 0.05$; one-way ANOVA, Tukey post hoc analysis; *SI Appendix, Table S1* for all statistical analyses of emergence rhythmicity.

is necessary for the circadian gating of eclosion. The PTTHn then transmit time information to the PG, where we show that PTTH imposes time of day-dependent changes to the transcript levels of *torso* and ERK phosphorylation, a key component of PTTH transduction. Although PTTH plays a critical role in the coupling between the central and the PG clock, we demonstrate here that JEB/ALK and Pvf2/PVR signaling also contribute to regulate the rhythm of emergence, revealing that this circadian behavior is the result of timed signals from several RTKs that converge onto the PG.

The Transduction of the Time Signal in the PG Is Unexpectedly Complex. PTTH accumulation in terminals and somatic Ca^{2+} rhythms have similar phases, suggesting that the increases in Ca^{2+} may cause PTTH release onto the PG. (However, the timing of PTTH release remains hypothetical and will need to be directly demonstrated.) Beyond that point, our findings reveal a complex relationship between the timing of PTTH abundance in the terminals of PTTHn, and the transduction of PTTH action in the PG. Indeed, PTTH immunoreactivity in PTTHn terminals and somatic Ca^{2+} levels were lowest during the subjective day,

coincident with the highest *torso* mRNA expression and a transient maximum of phosphoERK in the PG. In turn, PTTH levels were highest in the terminals of PTTHn during the middle of the night, a time when *torso* mRNA expression and phosphoERK levels in the PG reached their lowest levels. Assuming that TORSO receptor expression follows *torso* mRNA levels (there are currently no reagents to reliably measure TORSO protein levels in situ), this temporal relationship is consistent with an anti-cooperative model proposed for the PTTH/TORSO interaction in the silkworm (50), where the actions of the ligand are dampened by low receptors levels, leading to a low sustained output, whereas high receptor levels induce a transient output burst. A similar regulation has also been described in *Drosophila* and in mammalian cells (51) for the insulin receptor, InR, another RTK family member. Indeed, under high nutrient conditions, a known inductor of insulin secretion, cells down-regulate *InR* expression via the transcription factor FOXO. Although we do not provide a mechanistic explanation for *torso* cycling and its potential implications as a feedback mechanism to PTTH input to the PG, various signals including TGF β /activin signaling (52), Kdm5 (53) and endocytosis mediated by Hrs (54) have shown a regulation of *torso* expression. Further studies will be

required to determine whether the PG undergoes daily change in the responsiveness to PTTH. Importantly, a similar mechanism could also apply to the clock control of glucocorticoid (GC) production by the mammalian adrenal gland, where the clock of the adrenal gland regulates the sensitivity to adrenocorticotropic hormone (ACTH) (55).

Convergence of RTK Signaling onto the PG. Across insects including *Drosophila* (56), RTK signaling plays a major role in the control of growth and the timing of the molts. In the PG, PTTH/TORSO, JEB/ALK, and Pvf/PVR signaling, act to regulate the timing of ecdysteroid production (18), which causes the animal's developmental transitions. Here, we showed that all three RTK signaling pathways also contribute to the circadian control of emergence. Eclosion requires ecdysteroid titers to fall below a certain threshold level (57–59). However, the timing of emergence is not determined by the time when ecdysteroid titers drop below this threshold (60, 61). Instead, it is PTTH (this work) and ecdysteroid signaling (17) that control the circadian gating of eclosion in *Drosophila* by committing the insect to complete metamorphosis around 14 h before emergence (17). Since we observed peaks of activity in PTTHn around 31 h and 6 h before emergence, it may be the timing of the first rather than the second peak of activity in PTTHn that is critical for determining the timing of emergence. In this scenario, PTTH/JEB signaling may not only promote ecdysteroid production, but also regulate the phase of the PG clock. In this regard, it has been shown that RTK/MAPK signaling serves as both an output and an input pathway to the mammalian circadian clock (62). Furthermore, a mutation in *Alk* was found to significantly alter the circadian period of locomotor activity in *Drosophila* (63). In addition, it is known that *Pvr* and *Alk* expression are under circadian control in ventral and dorsal lateral clock neurons in the brain of the adult fly (64), suggesting that these RTKs are downstream of the clock. It is therefore possible that the central-clock driven PTTHn-derived RTK signaling ensures proper eclosion timing by aligning the phases of the central and PG clocks. With respect to the role of *Pvr* in the PG, we found that expression of *PvrDN* in the PG did not alter significantly the timecourse of ARG-luc activity; by contrast, the amplitude of this signal was drastically reduced (by a factor of around 20), and eclosion was only weakly rhythmic. These results suggest that autocrine PVF/PVR signaling (and perhaps also paracrine JEB/ALK signaling) amplifies PTTH/TORSO-dependent ERK signaling in the PG, thereby stabilizing and strengthening rhythmic PG activity and of eclosion but in itself not driving rhythmicity. A similar interpretation has been put forward for PVF/PVR signaling in larval/pupal developmental timing (18).

Insulins are another class of ligands that act on the PG via an RTK (40). An RNAseq study reported that insulin signaling modulates the expression of clock genes in the PG, which in turn regulates *torso* expression (65). Nevertheless, we found that insulin signaling in the PG does not affect the rhythm of emergence. Thus, not all signaling pathways mediated by RTKs are involved in the circadian control of adult emergence. Similarly, even though ALK acts through Ras/ERK and PI3K signaling pathways in the PG (18), we showed that PI3K signaling is not involved in the circadian gating of emergence, suggesting that, similar to TORSO, ALK and PVR contribute to the rhythm of emergence via the Ras/ERK pathway. Interestingly, the involvement of multiple signals in the transmission of the time information is consistent with the general principle of coherent coupling between multiple oscillators, which helps to stabilize and strengthen period, phase distribution, and amplitude in the SCN, and between central and peripheral body clocks (5, 66).

Autonomous Role of the PG Clock in the Timing of Eclosion. We found that knockdown of the ligand, Pvf2, in the PG itself reduced the rhythmicity of emergence. In addition, expression of a dominant negative version of *Pvr* in the PG, caused a loss in the daily variation of SPARK and greatly reduced the levels of the ARG-luc signal. Together, these findings identify elements of the circadian control of emergence that are autonomous to the PG, and may be responsible for the peak of ARG-LUC activity detected in the PG two days prior to eclosion, which occurred before the activation of PTTHn. These results may explain why a functional clock is required in both the central brain and the PG for the daily gating of eclosion (12). Indeed, since *torso* levels do not cycle in the absence of PTTH, it appears that the PG clock does not control the sensitivity to PTTH. In addition, the period and/or phase of eclosion is set by the brain clock (13). Taken together, these findings suggest a plausible yet testable model for the neuroendocrine and molecular coupling between the central and the PG clock. In this model, the central clock controls the period and phase of the PG clock via the PTTHn; the PG clock in turn regulates Pvf/PVR signaling, which would be rhythmically driven by the PG clock itself. When both clocks are synchronized, the joint action of the central and the PG clock would lead to a stable relationship between rhythmic PTTH/TORSO and Pvf/PVR signaling. And since both pathways converge to act intracellularly via ERK, their combined signal would cause a strong increase in the level of PG activity.

Much is currently known about how the circadian clock functions in a variety of multicellular organisms (67). By contrast, much less is known about how the clocks housed in different organs are coordinated. Interestingly, although animal clocks are universally controlled by intracellular transcriptional/translational feedback loops (68, 69), the mechanisms that mediate the coupling of clocks appear to be diverse and fine grained, such that in some cases some genes within a peripheral clock cycle autonomously whereas the cycling of others depends on a central input (70–72). In addition, the relationship between central and peripheral clocks is even more complex if the integration of external stimuli such as light, temperature, or feeding is considered (1, 8–10). In the case of the circadian gating of adult emergence, we provide here a detailed analysis of the mechanism that couples a central and a peripheral clock, which may provide principles that apply to other circadian clocks. Knowing how clocks are coordinated will be critical to understand how circadian rhythmicity is generated at the cell, systems, and organism levels.

Materials and Methods

Fly Stocks and Husbandry. Flies were raised on standard cornmeal/yeast media and maintained at room temperature (20 to 22 °C) under a 12 h:12 h LD schedule. All UAS and GAL4 stocks have previously been described and are listed under *SI Appendix*.

Emergence Monitoring. The timecourse of adult emergence was recorded using Trikinetics eclosion monitors (Trikinetics) and analyzed using the tools described in ref. 73. See *SI Appendix* for further details.

Ex Vivo Measurements of Ca²⁺, Phosphorylated ERK Signals, and CaLexA Ca²⁺ Imaging. In order to measure Ca²⁺ levels in PTTHn, we measured the GFP fluorescence of these neurons expressing the Ca²⁺ sensor, GCaMP6m. Levels of phosphorylated ERK in the PG were determined by driving expression of the SPARK (33) sensor in the PG and measuring the GFP fluorescence emitted by individual PG cells. See *SI Appendix* for further details.

ARG-Luc Imaging. ARG-Luc imaging was performed expressing 20XUAS-*lfp; lola-frt-stop-frt-luc* under control of the appropriate GAL4 driver. Bioluminescence was recorded using a TopCount Multiplate Reader (Perkin

Elmer) and imaged using a sCMOS camera (DMK33UX178, ImagingSource). Rhythmicity of the ARG-Luc signals was analyzed using MESA and JTK_Cycle implemented in BioDare2 (74). See *SI Appendix* for further details.

PTTH Antiserum Production. Rabbit polyclonal PTTH antiserum to PTTH was produced against the synthetic peptide CQSDHPYSWMNKDQPWamide (residues 207 to 221, with an N-terminal Cys added), not amidated which was coupled to thyroglobulin via the N-terminal Cys residue using maleimide. The immunization was carried out by Pineda-Antikörper Service and lasted over 3 mo, with a booster before the final bleed. The specificity of the PTTH antiserum was demonstrated by the lack of PTTH-immunoreactivity in the brains of PTTH null mutants (*SI Appendix, Fig. S9*).

Other Methods. Please see *SI Appendix* for descriptions of methods used for Immunocytochemistry (including *trans*-Tango, BaCTrace, syb-GRASP, and CaLexA Ca²⁺), ARG-Luc imaging, qRT-PCR, and Statistical analyses.

Data, Materials, and Software Availability. This study generated a new polyclonal rabbit antiserum against PTTH. Requests should be directed to D.R.N. All other data are included in the manuscript and/or [supporting information](#).

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