HHS Public Access

Author manuscript

Curr Biol. Author manuscript; available in PMC 2024 January 23.

Published in final edited form as:

Curr Biol. 2023 January 23; 33(2): 351–363.e3. doi:10.1016/j.cub.2022.12.025.

Polyphasic circadian neural circuits drive differential activities in multiple downstream rhythmic centers

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Summary

Circadian clocks align various behaviors such as locomotor activity, sleep/wake, feeding, and mating to times of day that are most adaptive. How rhythmic information in pacemaker circuits is translated to neuronal outputs is not well understood. Here we used brain-wide, 24-hr *in vivo* calcium imaging in the *Drosophila* brain and searched for circadian rhythmic activity among identified clusters of dopaminergic (DA) and peptidergic neurosecretory (NS) neurons. Such rhythms were widespread and imposed by the PERIOD-dependent clock activity within the ~150 cell circadian pacemaker network. The rhythms displayed either a morning, an evening, or mid-day phase. Different sub-groups of circadian pacemakers imposed neural activity rhythms onto different downstream non-clock neurons. Outputs from the canonical M and E pacemakers converged to regulate DA-PPM3 and DA-PAL neurons. E pacemakers regulate the evening-active DA-PPL1 neurons. In addition to these canonical M and E oscillators, we present evidence for a third dedicated phase occurring at mid-day (MD): the l-LNv pacemakers present the MD activity peak and they regulate the MD-active DA-PPM1/2 neurons and three distinct NS cell types. Thus, the *Drosophila* circadian pacemaker network is a polyphasic rhythm generator. It presents dedicated M, E, and MD phases that are functionally transduced as neuronal outputs to organize diverse daily activity patterns in downstream circuits.

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Liang *et al*. study circadian neurophysiology in the *Drosophila* brain *in vivo*. Here they extend focus to dopaminergic and peptidergic neurons, many of which exhibit spontaneous daily rhythms of activity with diverse phases. These patterns are imposed by specific circadian oscillators including the Morning, Evening and Mid-Day pacemakers.

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Author Contributions

X.L., T.H.E., and P.H.T. conceived the experiments; X.L. performed and analyzed all experiments; X.L. and P.H.T. wrote the manuscript.

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Declaration of Interests

The authors have no financial interests or positions to declare. T.E.H. has a patent on OCPI microscopy.

Introduction

Animals display daily rhythms in a variety of physiological processes and behaviors, such as locomotor activity, sleep/wake, feeding, and mating behaviors[1, 2]. Many such rhythms are controlled by circadian timing mechanisms and they exhibit a variety of phases throughout the solar day. Furthermore, the daily spectrum of circadian phases is itself regulated by daily changes in the presentation of environmental zeitgebers, especially light and temperature. Under laboratory conditions, the locomotor activity of the fruit fly *Drosophila* peaks twice a day, in the morning and in the evening. During the morning peak, the fly shows a daily peak of feeding behavior [3] and mating behavior [4, 5]. Following each of these two activity peaks, the fly exhibits two separate sleep bouts: one around mid-day and the other generally throughout the night. These behavioral rhythms are driven by synchronous clock gene oscillations (molecular clocks) in ~150 circadian pacemaker neurons [6]. How a small population of circadian neurons, sharing a mono-phase molecular clock, regulates all the different phases of behavioral rhythms required for fitness of a species remains poorly understood.

Previously we reported that molecular clocks generate circadian neural activity rhythms with diverse phases among five major circadian neuron groups [7]. Each group peaks at a specific time of day. Three laterally-localized circadian neuron groups: s-LNv, l-LNv, and LNd display spontaneous activity peaks in the morning (M), at mid-day (MD), and in the evening (E), respectively. Two dorsally-localized circadian neuron groups the DN3 and DN1 cells are sequentially active during the nighttime (N1, CT18) and (N2, CT20). Genetic analyses revealed that the molecular clock dictates a default morning phase onto the pacemakers, but the polyphasic activity pattern ensues due to the delaying activities of light and neuropeptide modulation within the circadian neuron circuit [8]. This reproducible series of phasic activity periods displayed across the circadian pacemaker network may therefore represent dedicated phasic timepoints by which different downstream output circuits could achieve temporal order. This general problem - how output circuits are regulated by circadian pacemaker circuits - is a fundamental problem in the field of biological rhythms.

We previously showed that the morning and evening phases (defined by activity peaks in the s-LNv and LNd/5th s-LNv neurons respectively), drive in biphasic activity patterns in the Ring Neurons of the Ellipsoid Body (RN-EB) and in a subset of the PPM3 dopaminergic neurons[9]. Both the RN-EB and PPM3 neurons are downstream neural circuits responding to clock signals to promote locomotor activity. Thus, the M and E phases of activity in the pacemaker circuit underlie authentic circadian phasic information that shapes premotor output to drive daily rhythmic locomotion.

Here we ask whether other phasic activity periods presented by the pacemaker circuit – those of the mid-day (MD - l-LNv group), the early night (N1 - DN3 group) and the late night (N2 - DN1 group) likewise direct daily rhythmic activity in downstream responsive non-pacemaker circuits. The problem could be approached by testing specific populations known to regulate different physiological and behavioral daily rhythms, as candidate responders of MD, N1 or N2 phasic information. For instance, a recent study [10] revealed a candidate neural output circuit that mediates the N1 and N2 phasic information

to promote nighttime sleep. However, more such candidate neural output circuits remain to be characterized [11, 12]. As an alternative approach, we measured spontaneous daily activity patterns *in vivo* across two populations of chemically-defined neurons that are known to influence physiology and behavior – dopaminergic (DA) neurons and peptidergic neurosecretory (NS) neurons. We report that both populations exhibit daily periods of spontaneous activity whose phases align with either the M, the E or MD phases of circadian pacemaker neurons. We focus on MD phase regulation and present evidence from both experimental manipulations and from normal developmental progression to support the hypothesis that the MD activity phase is dictated by l-LNv pacemaker group activity. Together these findings extend the hypothesis that polyphasic timing information from the *Drosophila* pacemaker circuit has broad functional significance. It spreads widely and independently through parallel downstream pathways to generate phase-diverse patterns of physiological and behavioral rhythms.

Results

Daily neural activity rhythms of dopaminergic neurons

Several aspects of fly physiology and behavior, such as locomotor activity, sleep/wake, feeding, and mating behaviors are regulated by neuromodulatory systems in the fly's brain. Prominent among these systems is the diverse collection of dopaminergic (DA) neurons [13, 14]. Therefore, we extended our previous measurements of spontaneous activity in the DA-PPM3 neurons [9] and asked whether the neural activity of other DA neurons might also display temporal bias in the first day of constant darkness, following 12:12 LD entrainment. Using *TH (tyrosine hydroxylase)-GAL4*, we imaged five spatially-distinct clusters of DA neurons in the fly's dorsal protocerebrum: PAM, PAL, PPL1, PPM1/2, and PPM3 [15, 16]. With the exception of the PAM, each cluster displayed a unique circadian-rhythmic Ca^{2+} activity pattern (Figure 1AB). Three clusters showed prominent single Ca^{2+} activity peaks, but at different times of day: PAL neurons peaked around dawn, followed by the PPM1/2 cluster, which peaked around mid-day, and later, the PPL1 peaked around dusk. As we previously reported [9], PPM3 exhibits a bimodal activity pattern, with a peak at dawn and a second at dusk. These distinct and stereotyped daily activity patterns in DA neuron clusters were confirmed by using two independent genetic drivers: *TH-C-GAL4* and *TH-D-GAL4* (Figure S1AB), which separately label largely non-overlapping DA neuron clusters. Together they recapitulate most of the *TH-GAL4* expression pattern [17]. Using highly specific, split-GAL4 drivers [14], we found that a specific subset of DA-PPL1 neurons, which project to the dorsal fan-shape body (dFSB) and suppress dFSB sleep-promoting neurons [17–23], showed a prominent evening Ca^{2+} activity peak (Figure S2AC). The Ca^{2+} activity of PPL1-dFSB subset was narrower than averaged Ca^{2+} peak from entire PPL1 cluster (Figure S3A-C) and dropped immediately at the beginning of subjective night, consistent with the onset of nighttime sleep. Another specific design targeted a pair of *fruitless*-positive DA neurons from the PAL cluster (Fru^{+} PAL, Figure S2B), which has been suggested to encode the internal drive of male mating behavior [24]. We found that these two identified *Fru+* PAL neurons revealed a spontaneous morning Ca^{2+} peak (Figure S2D and Figure S3DE), suggesting alignment with the daily behavioral mating activity pattern [4]. Together, these

observations suggest the distinct daily activity patterns in DA neuron clusters may contribute to the mechanistic basis that differentially times modulation of diverse behavioral rhythms.

We also measured DA Ca^{2+} activities at high frequency (1 Hz) for short periods *in vivo* in brains exposed acutely at different times of day. We found that DA neurons, while at the peak of their daily slow Ca^{2+} fluctuations, also displayed a more dynamic fast Ca^{2+} activity than those at the trough time (Figure S1D-K). These observations were motived by high-frequency sampling of calcium fluctuations in pacemaker neurons [25]. That study revealed that each of the five pacemaker groups exhibits co-phasic peaks of slow and fast Ca^{2+} oscillations that are mechanistically-distinct. Importantly, the spontaneous daily neural activity patterns of DA neurons were completely arrhythmic in circadian-defective per^0 mutant flies (Figure 1C and Figure S1C). Lastly, we noted clear alterations in spontaneous DA activity patterns in the absence of PDF signaling, which also speaks to the importance of the circadian pacemaker network (Figure 1D). In a severe *pdfr* mutant background, the PPM1/2 and PPL1 activities were undisturbed. However, the morning peak of the PPM3 cluster was lost and that of the PAL cluster was displaced to the evening phase. In summary, many neurons of the *Drosophila* dopaminergic system exhibit clock-dependent and cluster-specific spontaneous circadian neural activity patterns. Notably, the *TH*-gal4+ DA neurons do not express oscillatory clock proteins [26]: together the findings suggest that diverse daily rhythms of DA neuronal activity are imposed by neuronal activity from the circadian pacemaker network.

Daily neural activity rhythms of peptidergic neurosecretory neurons

Neurosecretory (NS) cells release diverse neuropeptides and peptide hormones and represent a second major neuromodulatory system [27]. Many neurosecretory cell bodies in insects localize in the *Pars Intercerebralis* (PI) (Figure 2AB), a homolog of the mammalian hypothalamus [28]. They have been implicated in regulating sleep [29, 30], locomotion [31], and metabolism [32, 33]. We identified these neurosecretory cells by a peptidergic neuron marker DIMMED, which is a bHLH transcription factor associated with neurosecretory or neuroendocrine cell differentiation [34]. Using the *c929-GAL4 driver* that reports on the *dimm* promotor [34], we simultaneously imaged from all PI cells, from the pair of large laterally leukokinin neurons [35], and also from the DIMM-positive circadian neuron group, l-LNv [36]. We found that on average, PI neurons displayed a daily Ca^{2+} activity peak around mid-day, with a phase very similar to that of the l-LNv (Figure 2C). NS cells in the PI are heterogeneous: different neurons release different peptide hormones [34] which regulate different physiological and behavioral functions. We genetically dissected PI neurons for imaging based on the types of neuropeptides they released, including DH44, dromyosuppressin (DMS), SIFamide (SIFa [32]), and insulin-like peptides (dILPs). Most PI groups, when analyzed with these more specific Gal4 drivers, had activity peaks prominently at mid-day (Figure 2B, E-G), consistent with the averaged signal from the entire PI group (Figure 2C). We noted one exception: the insulin-producing cells (IPCs, labeled by *dILP2-GAL4*), which peaked in the morning (Figure 2D). This pattern is consistent with previous observations that IPC display a higher electrophysiological firing rate when recorded *in vitro* in the morning, than at other times of day [32]. Other studies suggest they might be regulated by the outputs of M cells [37]. IPC activity peaks in the morning, suggesting that the release

of insulin-like peptides peaks in the morning, and could coincide with the peak phase of the fly's daily feeding rhythm [3]. Outside of the PI, a prominent pair of neurosecretory neurons releasing leucokinin (LK) regulate locomotor activity rhythms [38] and feeding behavior [35, 39]. We found the LK neurons showed a daily activity peak in the evening, different from the other NS cells we studied, but consistent with the prediction that LK neurons are suppressed by the morning-active PDF neurons [38]. Together, our results show that many NS cells produce spontaneous circadian rhythms of neural activity and, like the DA neurons group described above, the rhythms exhibit diverse phases of peak activity (i.e., M, E, and MD). This is consistent with the hypothesis that the release of diverse peptidergic modulators and hormones are under complex, polyphasic circadian regulation.

Circadian neurons dictate phases of output circuits

The different output circuits we surveyed produced distinct phases of daily neural activity patterns. We developed several lines of evidence to test whether and how these patterns are temporally-organized by molecular clocks through the polyphasic outputs of the pacemaker network [7]. We first asked whether different groups of circadian neurons regulate different downstream output circuits. For instance, do circadian neurons that peak in the morning generate corresponding morning output peaks? Likewise, do circadian neurons that peak at other phases of the 24-hr day produce corresponding co-phasic outputs? To do so, we selectively shift the activity phase of a subset, or even of single circadian neuron groups, by applying selective over-expression of Shaggy (SGG, *Drosophila* GSK3), a kinase for the clock protein TIM, that accelerates the molecular clocks when over-expressed [40]. We measured the consequences by comparing the daily activity patterns of multiple output circuits. Driving SGG expression with *dvpdf-GAL4* [41] accelerated molecular clocks in M-pacemakers (s-LNv), MD-pacemakers (l-LNv), and E pacemakers (5^{th} s-LNv and LNds) (Figure 3A). In these flies, the Ca^{2+} peaks of M cells and E cells, as well as the morning and evening peaks of locomotor activity were advanced (Figure S4A and S4D), while the MD-cell (l-LNv) Ca^{2+} peak was not affected. Corresponding to the behavioral phenotype, we found that the morning and evening peaks of PPM3 were significantly advanced ($p = 0.029, 0.014$; Watson-Williams test). Likewise, the daily activity peaks of PAL neurons in the morning and PPL1 neurons in the evening were also advanced (Figure 3A; PAL, $p = 0.0097$; PPL1, $p = 0.028$, Watson-Williams test). The phase of the Mid-Day PPM1/2 neurons was not affected.

We extended the analysis of changing clock phase in pacemaker subsets by using either of two more restrictive Gal4 lines. *pdf*-Gal4 restricted SGG over-expression to just the M and MD pacemakers: this caused a selectively advance of the M-cell (s-LNv) Ca^{2+} peak and the morning peak of locomotor activity (Figure 3B and Figure S4BE; [42]). However, as found above with *dvpdf-GAL4,* the MD-cell (l-LNv) Ca^{2+} peak was not affected by over-expressing SGG. Among downstream circuits, we found that the morning phase of PPM3 neurons were selectively advanced ($p = 0.00056$; Watson-Williams test), while the PAL neuron morning phase was not. The second Gal4 line (the split-GAL4 driver *MB122B*, cf. [83]) restricted SGG over-expression to the LNd and 5^{th} s-LNv E pacemakers. We observed a selectively advance in the E-cell (LNd) Ca^{2+} peak and in the evening peak of locomotor activity (Figure 3C and Figure S4CF; cf. [9]). Likewise, the evening peaks of PPM3 neurons

and the evening peaks of PPL1 neurons were both selectively advanced (PPM3, p = 0.027; PPL1, p = 0.01; Watson-Williams test). Unexpectedly, the morning peaks of PAL neurons were also advanced (p = 0.05; Watson-Williams test). Together, these results suggested that E cells may help signal proper activity phases for multiple downstream neuron groups, namely the PAL, PPL1, and the evening phase of the PPM3.

We were struck by the alignment of the l-LNv and PPM1/2 clusters, yet no SGG manipulation that we tested could alter the Ca^{2+} activity phase of either of these MD-active cell groups. Therefore, to ask if the phase of l-LNv neuron activity can influence daily activity patterns of any downstream output neurons, we turned to l-LNv mis-expression of PDFR which delays its Ca^{2+} peak by as much as 6 hr [8]. We used *pdf*-Gal4 to drive *pdfr* in the l- and s-LNv. This manipulation also slightly advanced the Ca^{2+} phase in s-LNv (Figure 4D), likely as a result of earlier termination of Ca^{2+} activation by autonomous PDFR [8]. In downstream circuits, we found that the morning peak of PAL neurons was advanced (Figure 4E), while the morning peak of PPM3 became smaller. PDFR over-expression also substantially delayed the Ca^{2+} peak in the l-LNv away from its MD phase by 5.7 hr (Figure 4D). Notably, the MD phase of the DA-PPM1/2 neurons displayed a comparable multi-hr delay, such that they remained in synchrony with the l-LNv (Figure 4E). Thus PPM1/2 neurons are normally synchronous with the l-LNv at MD, and they remain aligned when the phase of the l-LNv is delayed as much as ~ 6 hr by experimental manipulation.

The l-LNv normally express little if any PDFR [26, 43], nor do they respond pharmacologically to PDF *in vivo* [11, 44]. Likewise, loss of *pdfr* (*han* mutant flies) does not affect the normal l-LNv activity phase, whereas activity phases of the LNd and DN3 pacemakers are broadly phase advanced in the *han* background [7]. Recently, Klose and Shaw [45] reported that on the first day of adult stage (the day of eclosion, termed Day 0), l-LNv express PDFR and respond pharmacologically to PDF. This transient period of PDFR expression declines by the third day to the low levels normally associated with the adult. We therefore measured the phases of Ca^{2+} activity of the entire pacemaker system and of selected downstream neurons on Day 0 to ask if the transient expression of PDFR (now due to a normal developmental progression, not an experimental consequence) had effects. We found that on Day 0, the s-LNv, LNd, DN1 and DN3 groups all displayed the same phases as found in mature adults, but that the phase of the l-LNv was delayed by 6.7 hr (Figure 5AB). Among the downstream DA and NS neurons that we had measured in later adult stages, all maintained their normal phases, with the exceptions of those that normally peak at the MD phase. Ca^{2+} peak phases of DA-PPM1/2 neurons (Figure 5CD) and the *Dimm+* PI NS neurons (Figure 5EF) also displayed a substantial delay and so remained aligned with the l-LNv during this specific developmental stage. Thus, during normal developmental progression at the earliest times in the adult stage, l-LNv transiently delay their activity peak as they transiently express PDFR. In conjunction, the activity peaks of downstream neurons that are normally synchronized with l-LNv at MD are also transiently delayed to the late day/early evening.

Finally, we asked how pacemaker neurons communicate with non-clock downstream followers to shape their specific activity periods. We had already seen complex changes in spontaneous activity by the diverse DA neuronal groups lacking PDF signaling (*han* mutants, Figure 1D): the MD-phased (PPM1/2) and E-phased (PPL1) DA neurons sustained

their normal times of activity. However, the PPM3 which are biphasic, sustained their E peak but lost their distinct M peak, and the M-phased PAL group was now active in the evening. We next turned to imaging from DA neurons as we selectively activated different pacemaker groups that expressed ATP-gated P2X2 receptors with ATP application [46]. Selective PDF neuron activation using *pdf-GAL4* excited all four DA neuron clusters that had circadian Ca^{2+} activity rhythms (PAL, PPL1, PPM1/2, and PPM3; Figure 6A-C). In contrast, the PAM cluster, which did not produce circadian Ca^{2+} activity rhythms, was reproducibly inhibited by PDF neuron activation. When we selectively activated E cells using the same method, only the DA neuron clusters that showed evening activity peaks, PPL1 and PPM3, were excited by E-cell activation; PAL and PAM were inhibited by E-cell activation (Figure 6D-F). The effect of E-cell activation on PAL was modest and with a multi-minute latency, possibly via more complex neuropeptidergic signaling (cf. [8]). Together these results indicated broad sensitivity of DA clusters to M cell activation and somewhat less to E cell activation. In addition, it suggested that the daily activity pattern of PAL neurons might be shaped by a combination of excitatory inputs from M cells in the morning and modulatory/inhibitory inputs from E cells in the evening. Thus, many of the circadian pacemaker groups have neuronal connectivity patterns that could support them regulating the phases of activity in downstream non-pacemaker DA neurons.

Discussion

We performed a directed search among dopaminergic (DA) and neurosecretory (NS) neurons across the whole fly brain for cells that display circadian neural activity rhythms. We reasoned that subsets of these groups may exhibit circadian timing patterns, as some DA neurons relate to sleep-wake regulation [20], and because in mammals the neuroendocrine system is heavily reliant on circadian regulation [47, 48, 49]. In *Drosophila*, these two different neuronal complements show diverse daily activity patterns, with different NS and DA neural centers exhibiting activity peaks at different times of day. DA-PPM3 neurons display daily bimodal rhythms and they contribute to normal locomotor activity rhythms [9]. *Fru+* PAL DA neurons display a morning activity peak, which is consistent with their driving a morning-biased mating rhythm [4, 5]. PPL1-dFSB DA neurons displayed an evening activity peak, which is consistent with their promotion of arousal around dusk. In the *Pars Intercerebralis* (PI), insulin-producing cells (IPCs) had activity peaks in the morning, consistent with their involvement in feeding rhythms [28, 31]. Other PI NS cells displayed daily activity rhythms that peaked around mid-day, and which likely underlie rhythms of hormone secretion for multiple peptidergic neurosecretory/neuroendocrine systems. Daily neural activity rhythms of these output circuits were dependent on the molecular clock and driven by activity derived in the circadian pacemaker circuit. Based on these findings, we hypothesize that multiple, sequential neuronal outputs from the polyphasic circadian pacemaker circuit are used to assign diverse phases to different physiological processes and behaviors as illustrated in Figure 7.

We found that the spontaneous activity patterns of three distinct groups of DA neurons (PAL, PPL1, and PPM1/2) are all under circadian control, similar to that displayed by the DA-PPM3 group [9]. Previous studies have described synaptic connections between DA neurons and circadian neurons [50], and suggested that DA regulates circadian neuron activity.

Our findings, as well as other recent studies [51, 52], argue that circadian pacemakers also regulate DA neuron activity. DA neurons responded to circadian neuron activation (Figure 6) and showed circadian neural activity rhythms (Figure 1B). DA neural activity rhythms required functional clock gene oscillations (Figure 1C) and normal circadian pacemaker neurotransmission (Figure 1D). Lastly, different phases of DA neural activity rhythms were dictated by phases of different circadian neuron groups (Figure 3-5).

Neuropeptides released by NS cells regulate multiple aspects of *Drosophila* physiological states and behaviors [27]. We found several groups of NS cells that exhibit circadian neural activity rhythms, including those expressing dILP2, SIFa, DMS, and DH44 in the PI, and LK neurons in lateral horns (Figure 2). dILP2 neurons (a.k.a. insulin-producing cells, IPC), which promote feeding and suppress sleep [29, 32], peaked in the morning and may be controlled by M cells (Figure 2D) [53]. The other PI neurons peaked around mid-day, including the SIFa, DMS, and DH44 neurons. SIFa neurons can promote sleep [30] and mating [54], and also suppress feeding [32]. DH44 neurons together with a pair of LK neurons regulate locomotor activity rhythms [31, 38]. LK neurons are also involved in metabolism and regulate behavior associated with daily feeding rhythms [35, 39]. We found that DH44 neuron activity peaked around mid-day, whereas that of LK neurons peaked in early evening (Figure 2): these data are consistent with the activity patterns of these two groups of peptidergic neurons when measured previously in acutely dissected brains [38]. The evening activation of LK neurons might be associated with the second feeding peak occurring around the evening, which might be suppressed by light under LD [55]. Together, dILP2, SIFa, and LK neurons, with different activation phases and effects [3, 55], help shape the daily feeding pattern (Figure 7). However, the activity patterns of DH44 and LK neurons were different from the profile of locomotor activity. Further studies are required to determine how the DH44 and LK neuronal activity patterns specifically contribute to the daily bimodal pattern of locomotor rhythms.

More generally, these studies prompt consideration of how polyphasic circadian timing information is normally transmitted from clock-expressing pacemakers to non-clock-expressing “downstream neurons”. In mammals, numerous hormones are released in circadian patterns and at different times of day. For example, melatonin is uniformly released in the night, while glucocorticoids are normally released in anticipation of waking, a phase-point that varies widely among different species. Moreover, circadian regulation over daily hormone release depends on direct connectivity with the neurons of the suprachiasmatic nucleus (SCN) [56, 57]. Jones *et al.* [58] recently studied circadian corticosterone production and showed that VIP-secreting neurons of the SCN delay corticotropin-releasing hormone (CRH) release by inhibiting CRH neurons of the paraventricular nucleus. The inhibition is two-fold: VIP neuron activation entrains the Period-based molecular clock intrinsic to the CRH neurons. In addition, VIP neurons acutely suppress CRH neuron activity by regulating basal Ca^{2+} levels. The latter is a phenomenon very similar to the effect of neuropeptide PDF in *Drosophila*: PDF suppresses neuronal activity in the LNd Evening pacemakers by regulating basal Ca^{2+} levels for many hours [8].

We term the *Drosophila* pacemaker system “polyphasic” because its constituent neural groups produce at least five distinct and stereotyped phases of neuronal activity across

the solar day: the M, MD, E, N-1 (Night-1) and N-2 phases [7]. Different subsets of DA and NS neurons exhibit similar polyphasic activity patterns with different subsets aligning unambiguously with the different phases of the pacemaker network. For example (i) M phase activity is displayed by DA-PAL and NS-dILP2, (ii) E phase activity is displayed by the DA-PPL1 and NS-LK, (iii) both M and the E phase activity is displayed by the DA-PPM3, or (iv) the MD phase is displayed by the DA-PPM1/2, NS-SIFa, NS-DMS, and NS-DH44. The simplest hypothesis would suggest a one-to-one relationship between the driver for a particular circadian phase and the followers for that phase point. To some extent, there is support for that possibility: the M and E pacemakers independently regulate the morning and evening phases of activity in the biphasic DA-PPM3 and EB-RNs [9]. However, in other cases, phasic control may be more complex: Here we found that the DA-PAL is normally active in the morning and aligned with M (s-LNv) pacemakers. However, advancing the phase of either the M or the E pacemakers advanced the PAL phase (Figure 3), suggesting the PAL morning phase is normally the product of at least two different sources of pacemaker input. We found complexity also in the regulation of MD-active downstream neurons. The MD phase point is represented by the activity of the l-LNv and its ability to control the phase of neurons normally active at MD was shown by experimental manipulation (Figure 4D) and importantly also by tracking normal developmental progression (Figure 5). The l-LNv are themselves NS neurons that secrete the neuropeptide PDF. There is at present no strong evidence to support the possibility of additional l-LNv transmitters, suggesting PDF is the basis by which the MD phase is relayed from the pacemaker system to downstream centers in the instances we documented MD phase alterations. However, with loss of PDF signaling (as measured in a *pdfr* gene mutation in mature *Drosophila*), the MD phase remains intact (Figure 1D). Hence the cellular-molecular basis that defines the MD phase in the mature adult remains enigmatic, both within the pacemaker circuit [7] and outside it. Both are insensitive to loss of function for PDF signaling, yet both respond with multi-hr phase delays to greater PDFR expression by l-LNv.

Irrespective of its basis, our results clearly show that the MD (mid-day) timepoint is a third *bona fide* phase marker produced by the circadian pacemaker circuit. This finding extends the definition of functional neuronal oscillators in *Drosophila* beyond the two canonical Morning and Evening ones (e.g., [7, 9, 42, 59–62]). In summary, we found multiple neural pathways relating the circadian pacemaker system with daily rhythms of behaviors. Different groups of circadian neurons, acting alone and/or in concert, impose diverse neural activity rhythms onto different groups of downstream DA and NS neurons. These downstream neurons then separately or synergistically regulate the daily rhythms in locomotor activity, sleep/wake, feeding, and mating behaviors. Notably, several groups of downstream neurons have been suggested to be involved in the interaction between different rhythmic behaviors - sleep and mating [63–64], and sleep and feeding [35, 65]. Our findings suggest parallel and over-lapping control from circadian neurons to downstream functional circuits which may be a substrate to regulate such interactions. Future studies will help to define the precise nature of the cellular and molecular signals by which the polyphasic circadian timing system is translated across a wide array of physiological outputs.

STAR Methods

RESOURCE AVAILABILITY

Lead contact—Further information and requests for reagents may be directed to and will be fulfilled by the Lead Contact, Paul H. Taghert (taghertp@wustl.edu).

Materials availability—This study did not generate new unique reagents.

Data and code availability

- Microscopy data reported in this paper will be shared by the lead contact upon request.
- All original code is be publicly available; the main code has been deposited at Github.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Fly stocks.—Flies were reared on standard cornmeal/agar food at room temperature. Before imaging experiments, flies were entrained under 12 h light: 12 h dark (LD) cycles at 25°C for at least 3 days. All experiments used male flies older than three days after eclosion except for Figure 5, in which flies within one day after eclosion were used.

The following fly lines have been described previously: *tim(UAS)-GAL4* [66], *TH-GAL4* [15], *TH-C-GAL4* and *TH-D-GAL4* [17], *dILP2-GAL4* [69], *DH44-GAL4* [31], *DMS-GAL4* [4], *SIFa-GAL4* [4], *Lk-GAL4* [70], *c929-GAL4* [67], *dvpdf-GAL4* [41]; split-GAL4 lines: *R76F01-DBD* and *R76F02-AD* [14]; *MB122B*: *R12G04-AD and R18D09-DBD* [83] *;TH-LexA* [75], *dimm-LexA* [76], *cry-LexA* [8], *pdf-LexA* [20]; *TH-Flp* [14]; *Fru-Flp* [81]; *UAS-SGG* [73], *UAS-pdfr* [73], *UAS-P2X2* and *LexAop-P2X2* [74], *UAS-GCaMP6s* and *LexAop-GCaMP6s* [71]; per^{01} [77] and $pdfr^{han5403}$ [78].

***In vivo* fly preparations.—**The surgical procedures were as previously described [1, 3]. The flies were mounted by inserting the neck into a narrow cut in a piece of aluminum foil. Thus, the foil separated the head from the body and permitted the immersion of exposed brain by saline, while leaving the body in an oxygen-normal environment. During brain-exposing surgery and in vivo imaging, the head was immersed in saline, while the body remained in an air-filled enclosure. To access circadian neurons, one antenna, a portion of the dorso-anterior head capsule, and a small part of one compound eye were removed. To access dopaminergic (DA) neurons and Pars Intercerebralis (PI), a portion of the dorsal head capsule and the ocelli were removed, while the compound eyes and antennae remained intact. The orientation of fly was then tilted for a more optimal view of DA neurons in the posterior brain.

***In vivo* calcium imaging.—**For long-term (24-hr) *in vivo* imaging, a custom horizontal-scanning Objective Coupled Planar Illumination (OCPI) microscope [81] was used, as

described in Liang *et al.* [7, 8]. Briefly, the ~5 μm thick light sheet was scanned across the fly brain through a small cranial window every 10 min with a step size of 10 microns to acquire 20 to 40 separate images. Exposure time of each image was less than 0.1 s. For short-term high-frequency imaging, a custom high-speed OCPI-2 microscope [82] was used, acquiring volumetric images with 0.1–1Hz, as described in Liang *et al.* [9, 25]. All imaging was performed under constant darkness and fresh HL3 saline (5 mM KCl, 1.5 mM CaCl2, 70 mM NaCl, 20 mM MgCl2, 10 mM NaHCO3, 5 mM trehalose, 115 mM sucrose, and 5 mM HEPES; pH 7.1) was perfused continuously (0.1–0.2 mL/min). For pharmacological tests, after 5-min baseline recordings, 10 mM ATP solution (pH was adjusted to 7) was manually added into a 9 ml static HL3 bath over a ~2 s period.

Data reporting.—No statistical methods were used to predetermine sample sizes. The selection of flies from vials for imaging and behavioral tests were randomized. The investigators were not blinded to fly genotypes.

QUANTIFICATION AND STATISTICAL ANALYSIS

Imaging data analysis.—Calcium imaging data was analyzed as described previously [7, 9, 25]. Images were acquired by custom software (Imagine) [81] and processed in Julia 0.6, including non-rigid registration, alignment and maximal projection along z-axis. Then ImageJ-based Fiji [80] was used for rigid registration (plugin: Template Matching) and to manually select regions of interest (ROIs) over individual cells or groups of cells. Average intensities of ROIs were measured through the time course and divided by the average of the whole image to subtract background noise.

For spontaneous calcium transients, each time trace was calculated as $dF/F=(F-F_{min})/F_{mean}$. For 24-hr time traces, traces of certain cell type ROIs were firstly aligned based on Zeitgeber Time and then averaged across different flies. The averaged traced was plotted as mean ± SEM. n value (number of animals or cells) can be found in the corresponding figure legend. The phase relationship between traces was estimated by cross-correlation analysis. The 24-hr-clock circular plot of phases reflected both mean peak time and phase relationship of the same cell-group traces from different flies. For neurons with daily bimodal patterns (PPM3 DA neurons), each trace was split into two parts: ZT18-ZT6 (morning) and ZT6-ZT18 (evening) to estimate the morning and evening peak phases respectively. Watson-Williams test was used to compare phase difference of the same type of cells among different genotypes or developmental stages.

For pharmacological calcium responses, each time trace was normalized by the initial intensity (F/F_0). The maximum change was calculated by the maximum difference of normalized intensities between baseline and following drug application. Trace analysis and statistics were performed using R 3.3.3 and Prism 7 (GraphPad, San Diego CA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Holy and Taghert laboratories and the Washington University Center for Cellular Imaging (WUCCI) for advice and technical support; Mark Wu, Margaret Ho, and Tingting Xie for guidance in selecting driver lines for dopaminergic neurons; Orie Shafer, Francois Rouyer, Larry Zipursky, Michael Rosbash, and Bloomington Stock Center provided fly stocks and reagents. The work was supported by the Washington University McDonnell Center for Cellular and Molecular Neurobiology and by NIH grants R01 NS068409 and R01 DP1 DA035081 (T.E.H.), R01 NS099332 and R01 GM127508 (P.H.T.), and R24 NS086741 (T.E.H. and P.H.T.).

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HIGHLIGHTS

- Non-pacemaker neurons exhibit circadian rhythmic activity *in vivo*
- Their phases are diverse and match ones of the circadian pacemaker circuit
- Morning and Evening pacemakers drive co-phasic follower groups
- Mid-Day pacemakers define a novel functional circadian timeline

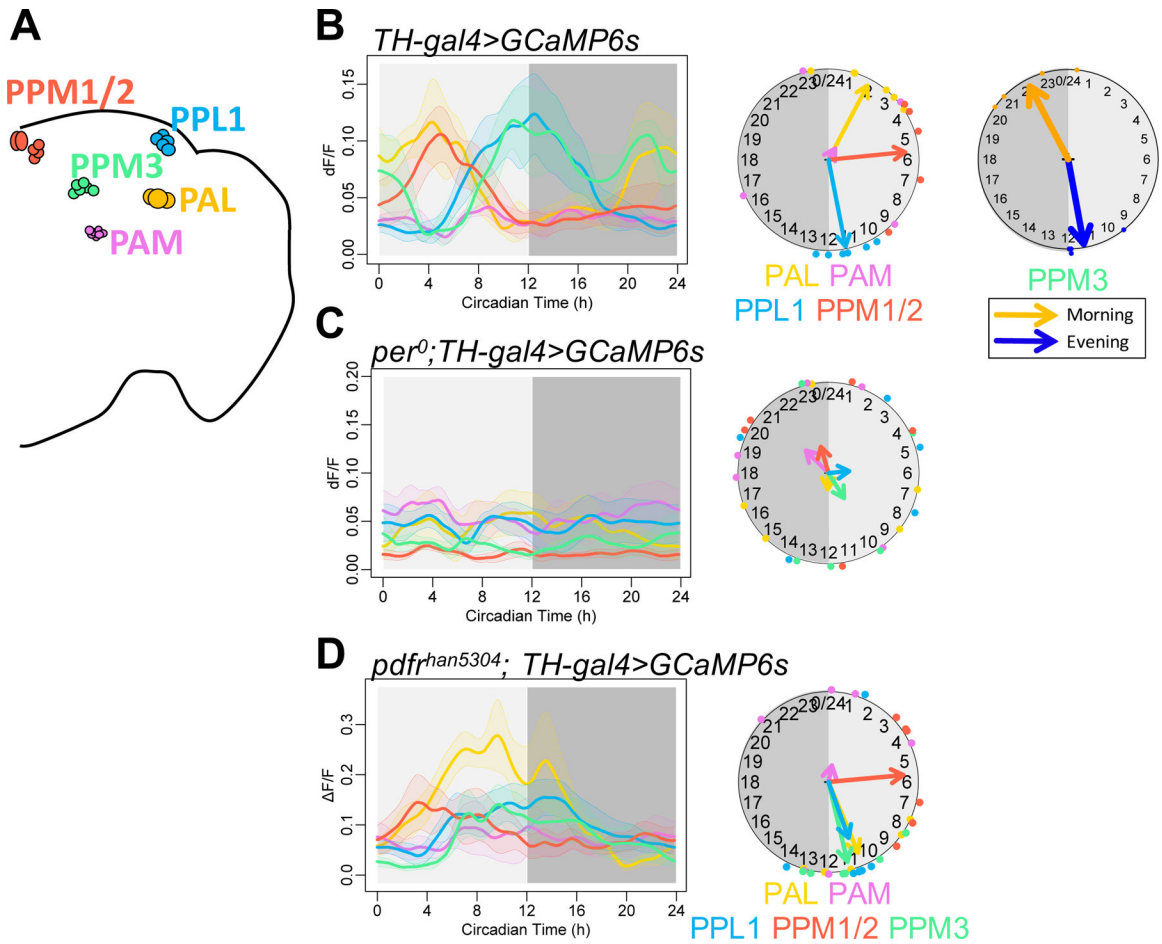

Figure 1. Diverse daily Ca^{2+} activity patterns of DA neuron clusters.

(A) Map of the five DA neuron clusters accessible via *in vivo* imaging. PAL, protocerebral anterior lateral cluster; PAM, protocerebral anterior medial cluster; PPL, protocerebral posterior lateral clusters; PPM, protocerebral posterior medial clusters.

(B) Left, daily Ca^{2+} activity patterns of DA neuron clusters under DD (n = 6 flies). Middle, Ca^{2+} phase distribution of DA neuron clusters showing single peaks (PAL, PPL1, and PPM1) or arrhythmic activity (PAM). Right, Ca^{2+} phase distribution of PPM3 for both morning peaks (orange) and evening peaks (blue): this data was collected from four flies; the appearance of only two data points (two blue dots) reflects the highly overlapped nature of three of the evening peaks (around CT12).

(C) Arrhythmic Ca^{2+} activity patterns of DA neuron clusters under DD in *per*^{01} mutants (n = 5 flies).

(D) Altered patterns of Ca^{2+} activity patterns of DA neuron clusters under DD in *pdf* ^{han5304} mutants (n = 5 flies).

See also Figures S1, S2, and S3.

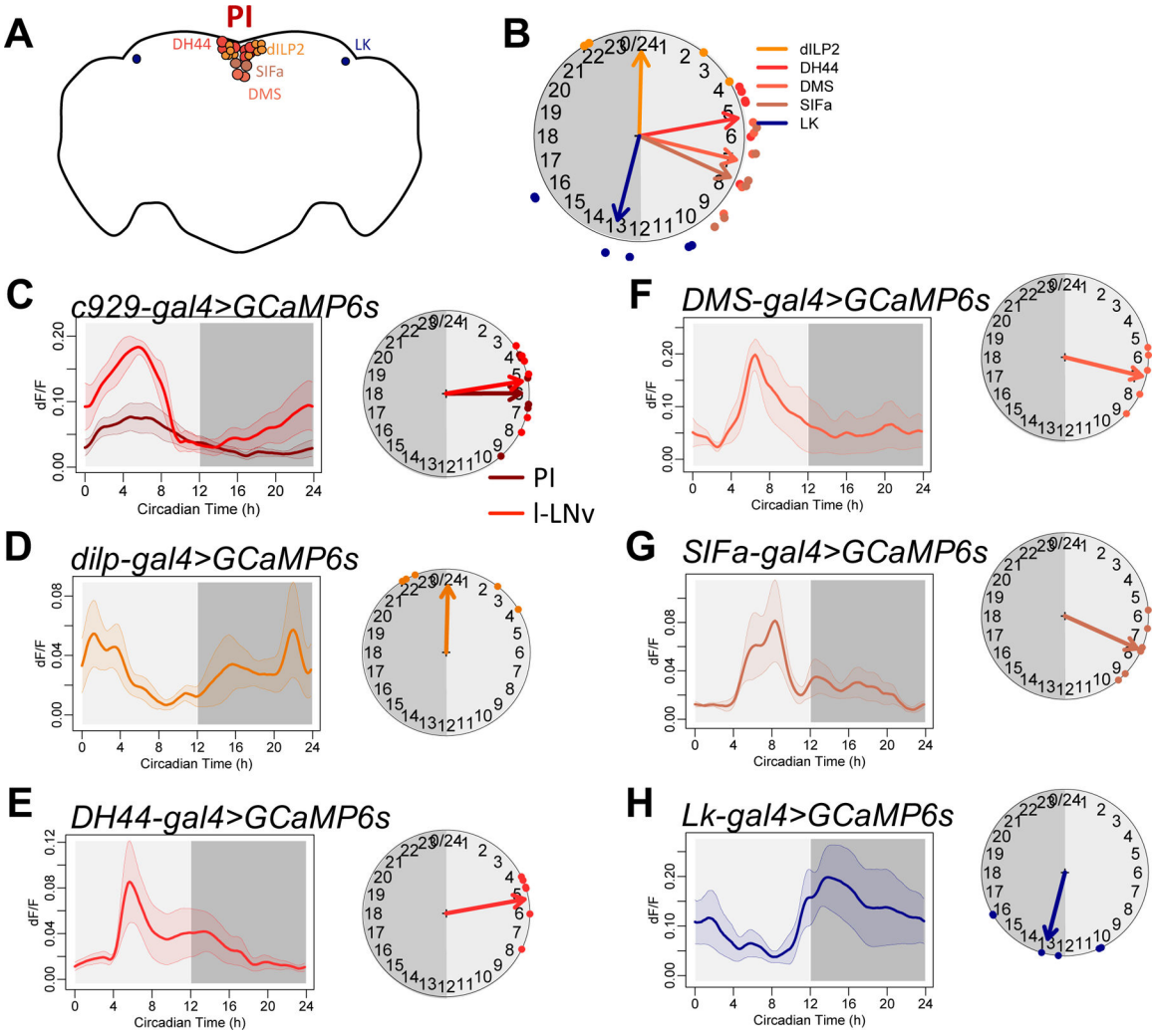

Figure 2. Daily neural activity patterns of peptidergic NS neurons.

(**A**) Diagram of some of the major peptidergic NS neurons in the brain.

(**B**) Summary of phase distributions of different NS neurons in (D-H).

(**C**) Averaged Ca^{2+} activity rhythms of Pars Intercerebralis (PI) NS neurons and circadian neurons l-LNv, all labelled by *dimm(c929)-gal4* (n = 6 flies).

(**D-G**) Daily neural activity patterns of four different of PI subgroups: insulin producing cells (labelled by *dilp2-gal4*), diuretic hormone 44 (DH44) neurons, dromyosuppressin (DMS) neurons, and SIFamide (SIFa) neurons (n = 5, 6, 5, and 6 flies).

(**H**) Daily neural activity patterns of leucokinin (LK) neurons (n = 6 flies).

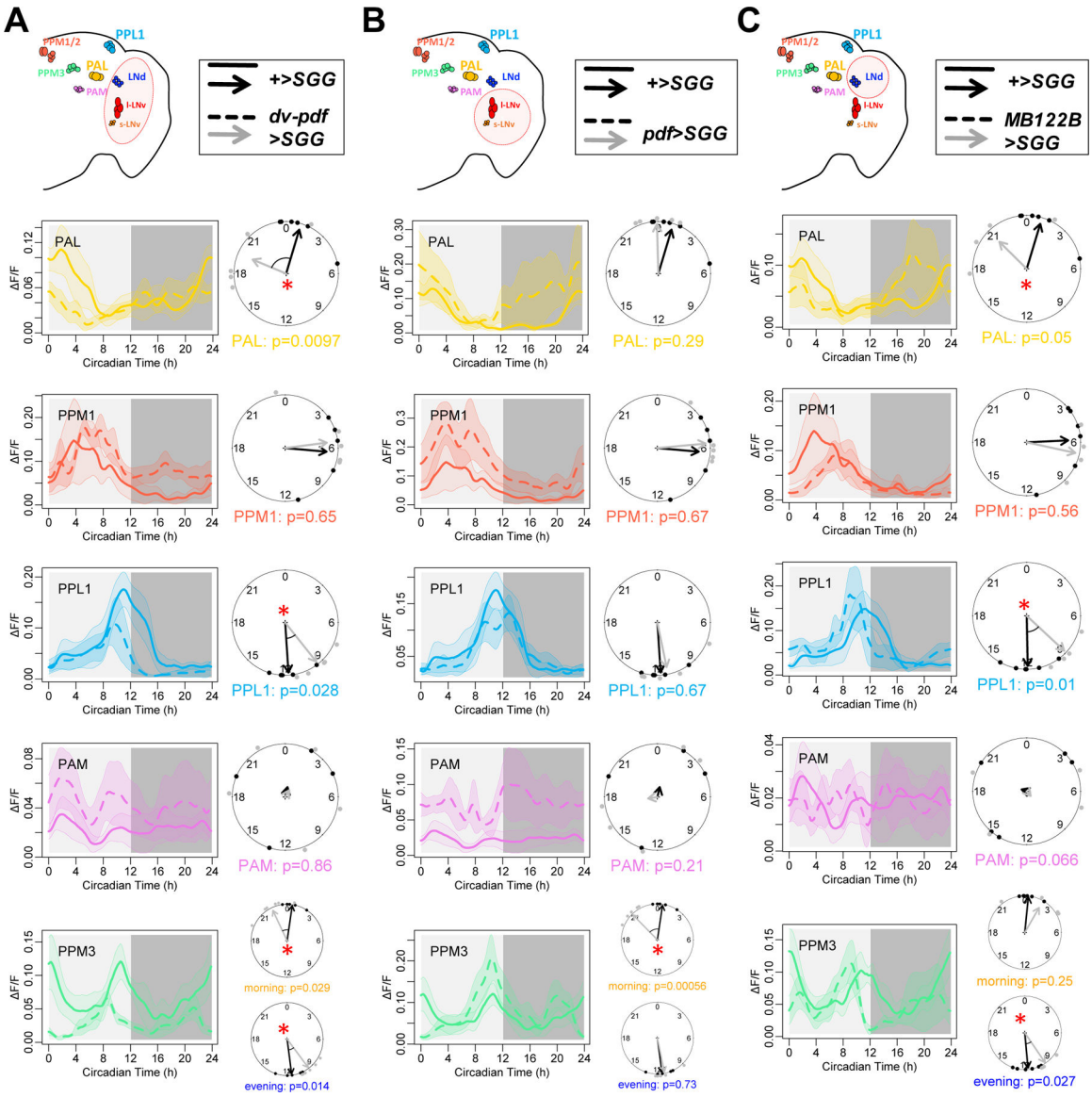

Figure 3. Daily activity phases of the PAL, PPL1, and PPM3 DA neuron groups are dictated by M and E cells.

(A) Daily Ca^{2+} activity patterns and phase comparison of DA neurons between WT flies under DD (solid lines, *+>SGG;TH-lexA>GCaMP6s,* n = 4 flies) and *dvpdf>SGG* flies under DD (dash lines, *dvpdf-GAL4>SGG;TH-lexA>GCaMP6s,* n = 6 flies). The daily peak phases of PAL and PPL1 and both morning and evening phases of PPM3 were advanced in *dvpdf>SGG* flies (* P < 0.05, Watson-Williams test).

(**B**) Daily Ca^{2+} activity patterns and phase comparison of DA neurons between WT flies under DD (solid lines, n = 4 flies) and *pdf>SGG* flies under DD (dashed lines, *pdf-GAL4>SGG;TH-lexA>GCaMP6s,* n = 5 flies). Morning peak of PPM3 were significantly advanced in *pdf>SGG*. (bottom panel replotted from [9], Liang *et al.,* 2019)

(**C**) Daily Ca^{2+} activity patterns and phase comparison of DA neurons between WT flies under DD (solid lines, n = 4 flies) and *MB122B>SGG* flies under DD (dashed lines,

MB122B-splitGAL4s>SGG;TH-lexA>GCaMP6s, n = 5 flies). The daily peak phases of PPL1, PAL, and the evening peak of PPM3 were advanced in *MB122B>SGG*.
See also Figure S4.

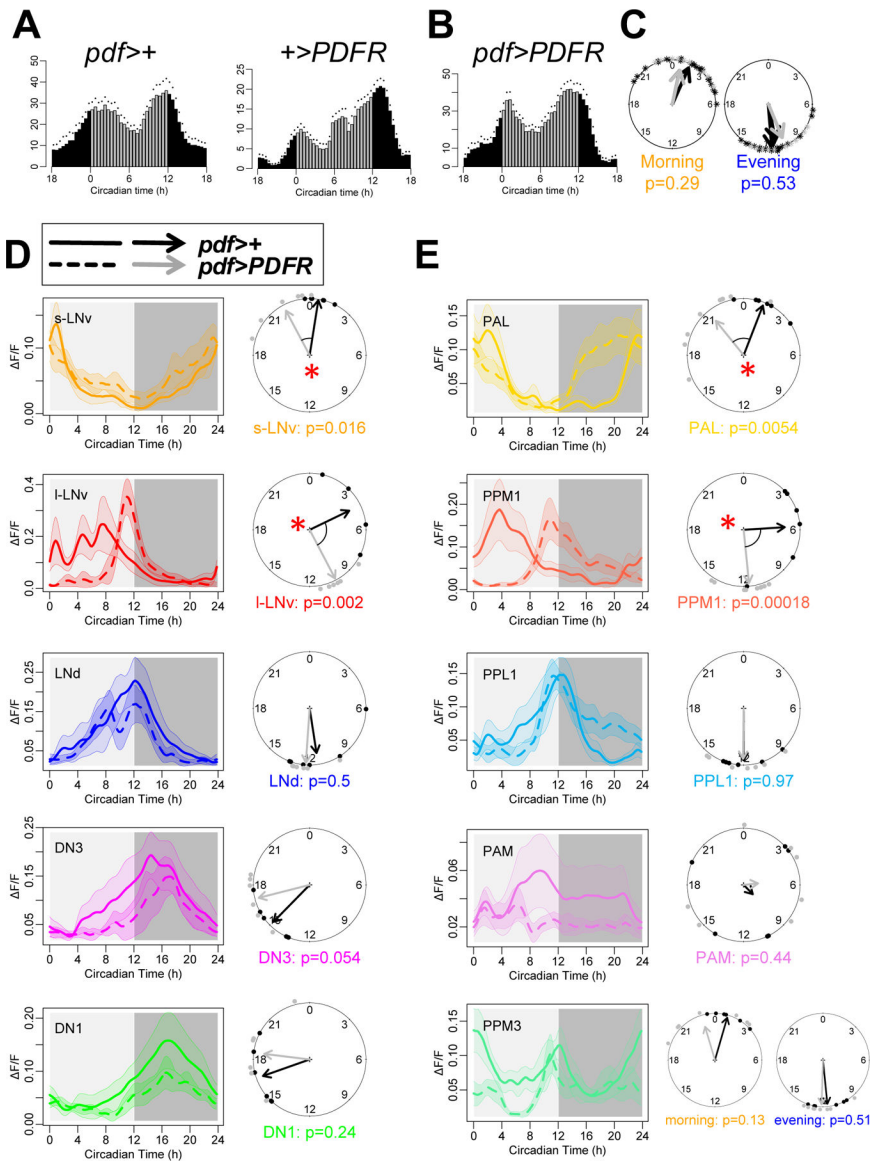

Figure 4. Daily activity phase of the PPM1 DA neuron group is dictated by circadian neurons l-LNv.

(**A**) Average locomotor activity in DD1 of wild type flies (WT, n = 16).

(**B**) Average locomotor activity in DD1 of flies expressing *PDFR* in PDF neurons using *pdf-GAL4* (n = 16 flies).

(**C**) Phase comparisons of morning and evening activity between WT and *pdf>PDFR*.

(**D**) Daily Ca^{2+} activity patterns and phase comparison of circadian neurons between WT flies (solid lines, *pdf-GAL4;cry-LexA>GCaMP6s*, n = 4 flies) and *pdf>PDFR* flies under DD (dashed lines, *pdf-GAL4>PDF;cry-LexA>GCaMP6s*, n = 5 flies). The daily peak phases of s-LNv were advanced while the daily peak phases of l-LNv were delayed in *pdf>PDFR* flies (* $P < 0.05$, Watson-Williams test).

(**E**) Daily Ca^{2+} activity patterns and phase comparison of DA neurons between WT flies (solid lines, *pdf-GAL4;TH-LexA>GCaMP6s*,n = 4 flies) and *pdf>PDFR* flies under DD (dashed lines, *pdf-GAL4>PDF;TH-LexA>GCaMP6s*, n = 5 flies). The daily peak phases of

PAL were advanced while the daily peak phases of PPM1 were delayed in *pdf>PDFR* flies (* $P < 0.05$, Watson-Williams test).

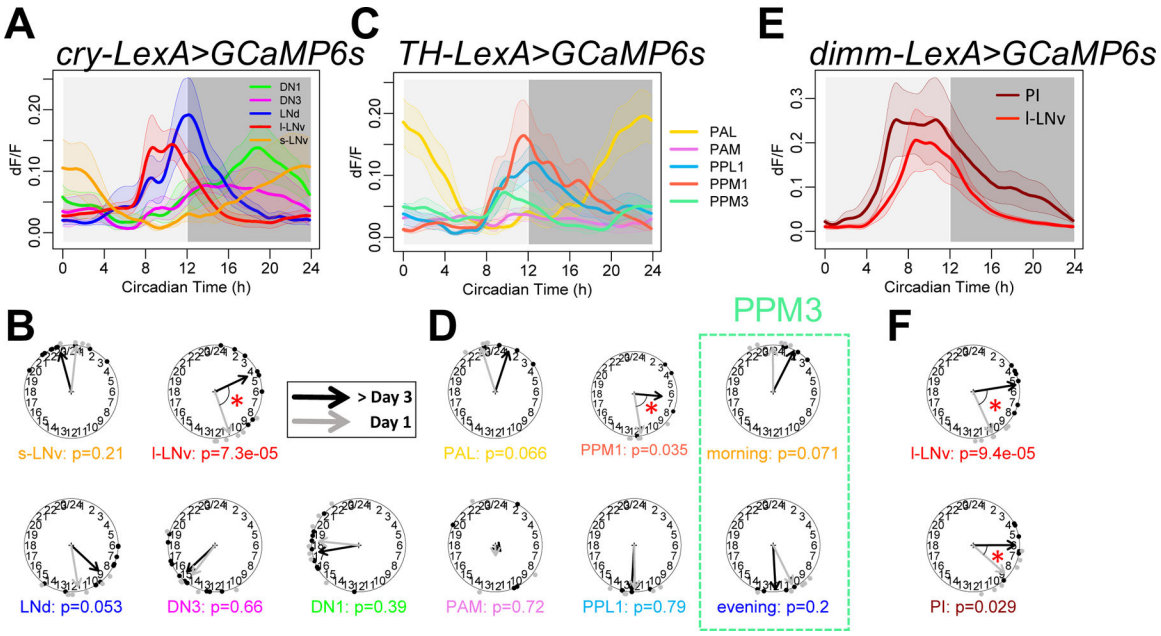

Figure 5. Daily activity patterns of circadian neurons and output circuits in the first day after adult eclosion.

(**A**) Daily Ca^{2+} activity patterns of circadian neurons in flies within one day after eclosion (n = 7 flies).

(**B**) Phase comparison between aged and newly eclosed flies. Note that l-LNv phases in newly eclosed flies were significantly latter than that in aged flies. (*p < 0.05, Watson-Williams test).

(**C**) Daily Ca^{2+} activity patterns of DA neurons in newly eclosed flies (n = 5 flies).

(**D**) PPM1 phases in newly eclosed flies were significantly latter than that in aged flies.

(**E**) Daily Ca^{2+} activity patterns of l-LNv and PI cells in newly eclosed flies (n = 6 flies).

(**F**) l-LNv and PI cell phases in newly eclosed flies were significantly latter than that in aged flies.

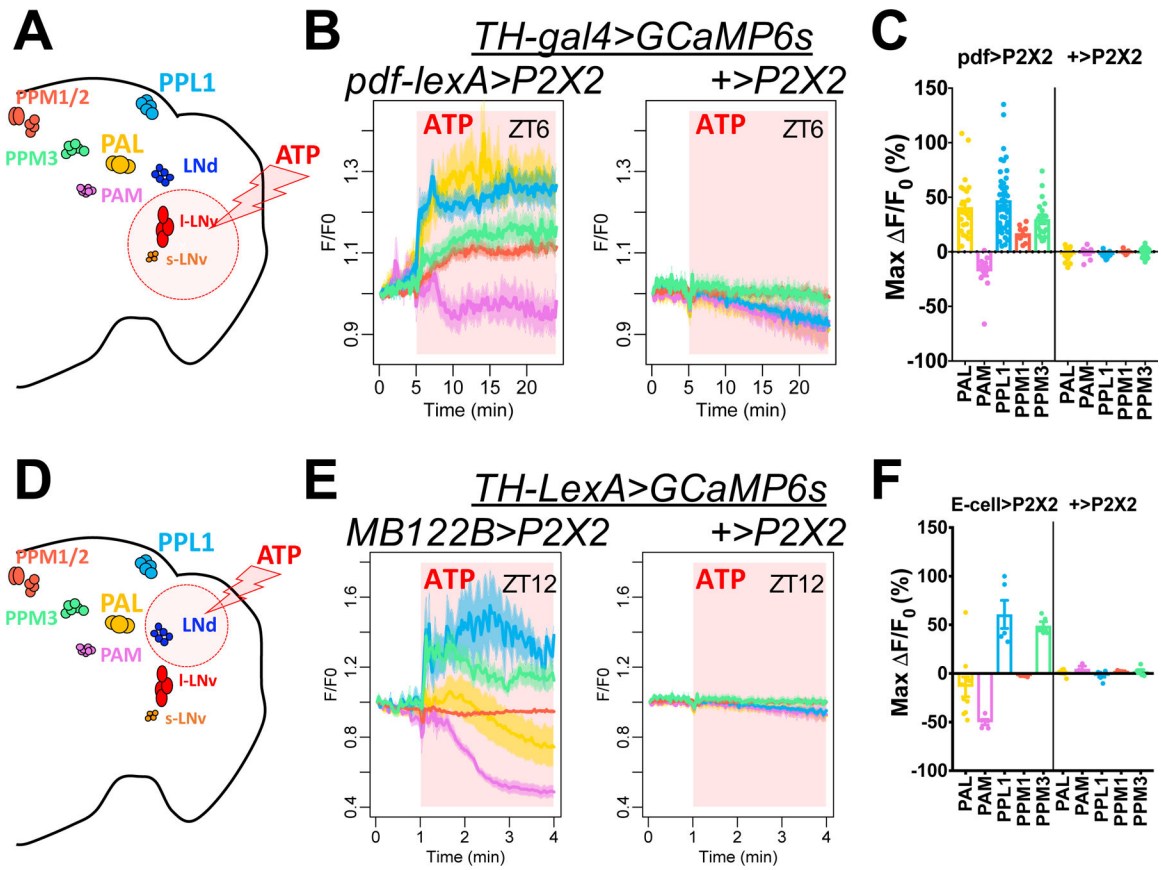

Figure 6. Functional connections from M and E cells to DA neuron clusters.

(**A**) Illustration of pharmacological activation of PDF-positive neurons (sLNv and lLNv).

(**B**) Average traces of DA neuron clusters responding to ATP application in flies with P2X2 expressing in PDF neurons (left, n = 7 flies) and in control flies without P2X2 expression (right, n = 4 flies). Red area indicates duration of ATP application.

(**C**) Maximum Ca^{2+} signal changes after ATP application in individual cells in (B).

(**D**) Illustration of pharmacological activation of E cells (the 5^{th} sLNv and 3 LNd).

(**E**) Average traces of DA neuron clusters responding to ATP application in flies with P2X2 expressing in E cells (left, n = 3 flies) and in control flies without P2X2 expression (right, n = 3 flies).

(**F**) Maximum Ca^{2+} signal changes after ATP application in individual cells in (E).

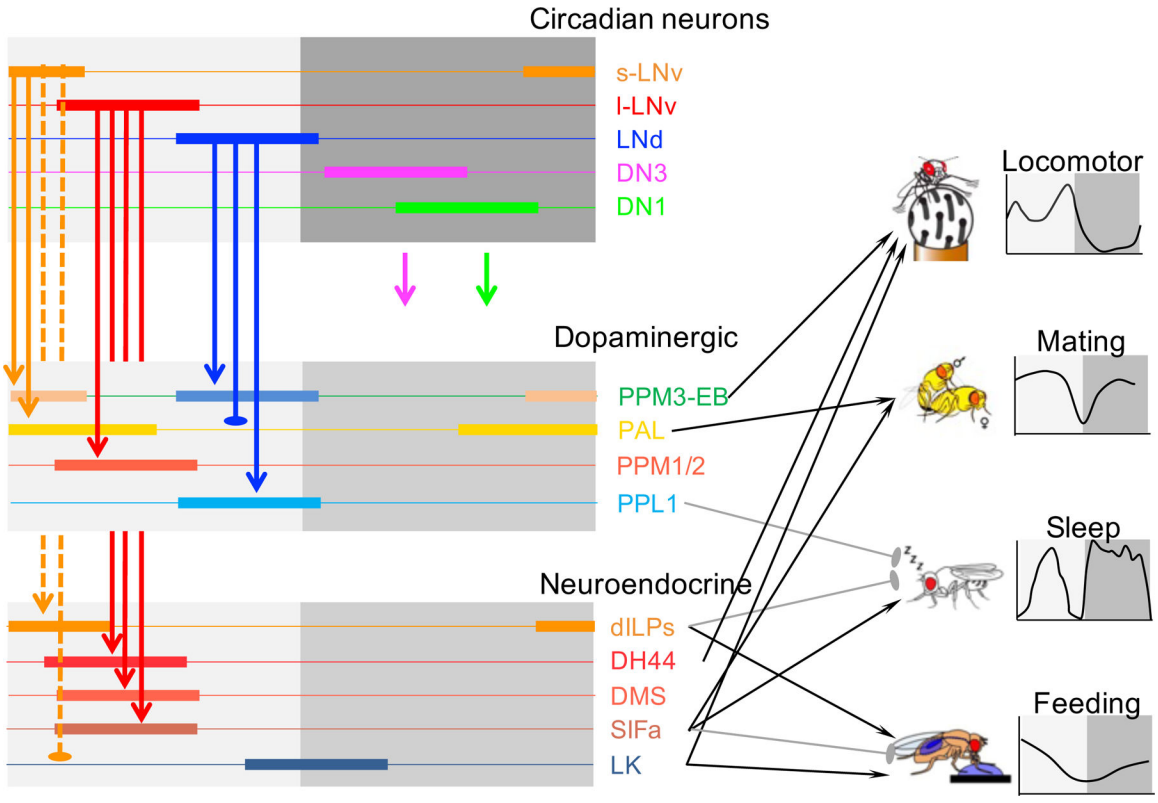

Figure 7. A model of the polyphasic circadian output pathways in *Drosophila*.

Groups of circadian neurons peaking at different times of day sent outputs to different downstream circuits to generate diverse phases. Circadian pacemaker M cells (orange arrows) and E cells (blue arrows) independently activate PPM3 neurons around dawn and dusk, which drive the locomotor activity rhythms [9]. Fru+ PAL neurons might be activated by M cells and inhibited by E cells and drive mating behavior (the daily mating pattern was redrawn from [4]. E cells activate PPL1-dFSB neurons to suppress sleep around dusk (the daily sleep pattern was redrawn from Liu *et al.,* [17]. M cells activate PI neurons that produce insulin-like peptides (dILPs), which then promote (black arrow) feeding and suppress (gray arrow) sleep around dawn; the daily feeding pattern was redrawn from Xu *et al.* [3]. M cells might inhibit Leucokinin (LK) neurons [38], which regulate locomotor rhythms and associated with the evening feeding peak[55]. Lastly, l-LNv (red arrows) controls the mid-day phase of dILP-negative PI neurons and PPM1 DA neurons.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
Adenosine 5′-triphosphate	Sigma	A5394
Experimental Models: Organisms/Strains		
Drosophila: tim(UAS)-GAL4	(Blau and Young, 1999 [66])	NA
Drosophila: c929-GAL4	Taghert Lab (Hewes *et al.,* 2003[67])	RRID: BDSC_25373
Drosophila: pdf-GAL4	Taghert Lab (Renn *et al.,* 1999[69])	NA
Drosophila: TH-GAL4	Jay Hirsh (Friggi-Grelin *et al.,* 2003[15])	NA
Drosophila: TH-C-GAL4	Mark Wu (Liu *et al.,* 2012[17])	NA
Drosophila: TH-D-GAL4	Mark Wu (Liu *et al.,* 2012[17])	NA
Drosophila: dILP2-GAL4	Bloomington (Rulifson *et al.,* 2002[69])	RRID: BDSC_37516
Drosophila: DH44-GAL4	Bloomington (Cavanaugh *et al.,* 2014[31])	RRID: BDSC_51987
Drosophila: DMS-GAL4	Taghert lab (Park *et al.,* 2008[34])	NA
Drosophila: SIFa-GAL4	Jan Veenstra (Terhzaz *et al.,* 2007[54])	NA
Drosophila: Lk-GAL4	Bloomington (de Haro *et al.,* 2010[70])	RRID: BDSC_51993
Drosophila: dvpdf-GAL4	Bloomington (Bahn *et al.,* 2009[41])	NA
Drosophila:20XUAS-IVS-GCaMP6s(attP40)	Bloomington (Chen *et al.,* 2013[71])	RRID: BDSC_42746
Drosophila:13XLexAop2-IVS-GCaMP6s-p10(su(Hw)attP1)	Bloomington (Chen *et al.,* 2013[71])	RRID: BDSC_44274
Drosophila: UAS-SGG	Bloomington (Martinek *et al.,* 2001[72])	RRID: BDSC_5435
Drosophila: UAS-pdfr-16	Taghert Lab (Mertens *et al.,* 2005[73])	NA
Drosophila: UAS-P2X2	Orie Shafer (Yao *et al.,* 2014[74])	NA
Drosophila: LexAop-P2X2	Orie Shafer (Yao *et al.,* 2014[74])	NA
Drosophila: cry-LexA::GAD	F. Rouyer (CNRS Gyf, Paris) Liang *et al.,* 2017[8])	NA
Drosophila: TH-LexA	(Berry *et al.,* 2015[75])	NA
Drosophila: dimm-LexA	Orie Shafer (Shao *et al.,* 2019[76])	NA
Drosophila: R76F01-DBD	Mark Wu (Xie *et al.,* 2018[14])	NA
Drosophila: R76F02-AD	Mark Wu (Xie *et al.,* 2018[14])	NA
Drosophila: GMR_MB122B-GAL4: R12G04-AD and R18D09-DBD	Gifts from Drs. Dionne, Nern and Rubin (Janelia Research Center, VA) (Liang *et al.,* 2017[8])	NA
Drosophila: per^{01}	(Konopka and Benzer, 1971[77])	NA
Drosophila: pdfr^{han5403}	(Hyun *et al.,* 2005[78])	NA
Drosophila: TH-Flp	Mark Wu (Xie *et al.,* 2018[14])	NA
Drosophila: Fru-Flp	Bloomington (Yu *et al.,* 2010[81])	RRID: BDSC_66870
Software and Algorithms		
R	http://www.R-project.org/	Version: 3.3.3
Julia	https://julialang.org/	Version: 0.6
Prism 9	GraphPad	https://www.graphpad.com/

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Fiji	(Schindelin *et al.,* 2012[80])	https://fiji.sc/
Imagine	(Holekamp *et al.,* 2008[81])	http://holylab.wustl.edu/