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Molecular and circuit mechanisms mediating circadian clock output in the *Drosophila* brain

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Abstract

A central question in the circadian biology field concerns the mechanisms that translate ~24-hour oscillations of the molecular clock into overt rhythms. *Drosophila melanogaster* is a powerful system that provided the first understanding of how molecular clocks are generated and is now illuminating the neural basis of circadian behavior. The identity of ~150 clock neurons in the *Drosophila* brain and their roles in shaping circadian rhythms of locomotor activity have been described before. This review summarizes mechanisms that transmit time-of-day signals from the clock, within the clock network as well as downstream of it. We also discuss the identification of functional multisynaptic circuits between clock neurons and output neurons that regulate locomotor activity.

Abstract

Circadian pacemaking is a result of individually rhythmic clock neurons synchronized across a circuit. In the circadian system of *Drosophila melanogaster*, rhythmic neuronal activity also propagates downstream of the central clock neurons, s-LNvs, to output circuits that regulate behavioral rhythms.

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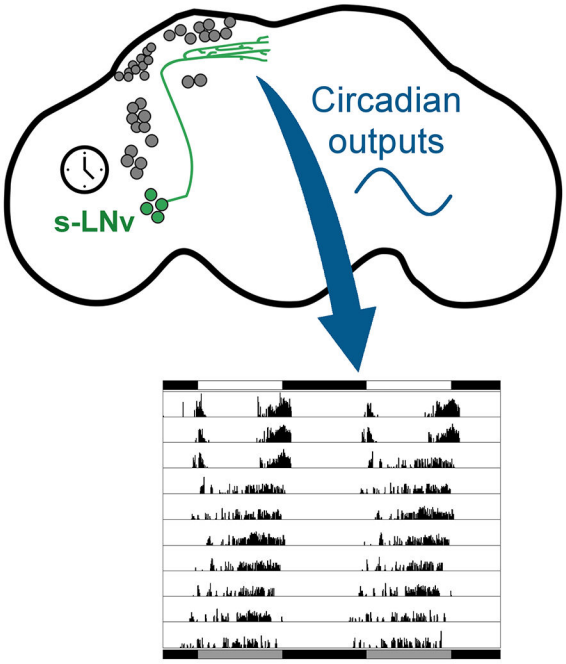

Keywords

Circadian rhythm; Rest:activity rhythms; Neuropeptide; Neuronal activity

Introduction

Circadian (~24 hour) rhythms allow animals to anticipate daily changes in their environment and coordinate their behavior and physiology with time of day. These rhythms are generated by an internal timing mechanism, which is synchronized to environmental cycles of light and temperature imposed by the rotation of earth. In its simplest form, a circadian system is modeled with three basic components: the clock, input pathways, and output pathways. The clock maintains ~24-hour rhythms even in constant darkness. Input pathways synchronize the clock to external signals such as light. Output pathways receive and translate circadian signals from the clock to produce biological rhythms.

Much of our molecular knowledge of circadian clocks came from genetic studies in the fruit fly, *Drosophila melanogaster*. In flies, circadian rhythms are typically studied using locomotor activity as the output. Under a 12-hour light:12-hour dark cycle, the fly exhibits a bimodal pattern in locomotor activity, with activity peaks anticipating the light-to-dark (evening) and dark-to-light (morning) transitions. Locomotor activity rhythms are dependent on **endogenous clocks** and persist in constant darkness (DD), albeit with a different pattern. In DD, the fly's locomotor activity free-runs with the periodicity of the endogenous clock, which is about (but usually not exactly) 24-hours, such that activity each day generally occurs during the subjective day and rest occurs during the subjective night. Besides rest:activity rhythms, flies also exhibit rhythms in eclosion (emergence of adult flies from pupae), feeding, temperature preference, and sleep. Besides behavior, there are circadian

rhythms at the cellular level, such as electrical activity of neurons, gene expression, and metabolic processes. A basic molecular clock mechanism regulates all output rhythms. **Yet compared to our relatively advanced understanding of the molecular clock mechanism, the output mechanisms that turn molecular clock oscillations into diverse behavioral and physiological rhythms are not well understood.**

Here, we review output mechanisms of the circadian clock in the *Drosophila* brain. We will start by describing the circadian clock network and the output mechanisms that occur within the network. Then, we will move beyond the circadian clock network and review recent work that identified output circuits regulating circadian rhythms of behavior and physiology.

The circadian clock network in *Drosophila* brain

The basic molecular oscillator in eukaryotes consists of transcriptional activators and repressors in a feedback loop. In *Drosophila*, the co-activator complex, CLOCK-CYCLE, drives transcription of the co-repressors, *period* (*per*) and *timeless* (*tim*). Accumulated PER and TIM proteins feed back to inhibit CLOCK-CYCLE activity. Delays are built into the basic molecular oscillator at multiple steps, and include post-transcriptional and post-translational mechanisms, which ensure 24-hr rhythms in PER and TIM expression [reviewed in (Zheng & Sehgal, 2012)].

Oscillations in the circadian clock are self-sustained. However, the clock is usually synchronized to external cues through a process called entrainment, which is crucial for adaption to the environment [reviewed in (Yoshii *et al.*, 2016)]. Light is the primary entrainment cue, and in flies it involves a dedicated circadian photoreceptor, Cryptochrome (CRY). Upon light exposure, CRY binds TIM and targets TIM for ubiquitination by the E3 ligase, JETLAG, and then degradation. In addition to the CRY mechanism, light-input circuits from the visual system to the central clock neurons are also important for light entrainment.

In the *Drosophila* brain, there are ~150 clock neurons subdivided into six groups based on neuroanatomy. **The six groups of PER-TIM-expressing neurons include the large and small ventral lateral neurons (l-LNvs and s-LNvs), the dorsal lateral neurons (LNds), the lateral posterior neurons (LPNs), and three groups of dorsal neurons (DN1s, DN2s and DN3s) (Figure 1)** (Kaneko & Hall, 2000; Helfrich-Förster *et al.*, 2007). Clock neurons between and within groups use a heterogenous set of neuropeptides and neurotransmitters for signaling [reviewed in (Beckwith & Ceriani, 2015)].

The LNvs are comprised of two groups of neurons, s-LNvs and l-LNvs, and are genetically identified by expression of neuropeptide Pigment-Dispersing Factor (PDF) (Helfrich-Förster, 1995). There are four s-LNvs and four l-LNvs in each hemisphere of the fly brain. **An additional pair of cells called the "5th s-LNvs" also has molecular clocks but is PDF-negative.** The *Pdf*$^{+}$ LNvs hold an important role in regulating rest:activity rhythms. Flies with ablated or electrically silenced LNvs have arrhythmic rest:activity behavior in DD (Renn *et al.*, 1999; Nitabach *et al.*, 2002; Depetris-Chauvin *et al.*, 2011), and *period* null

mutant flies with *period* restored in LNvs display normal rest:activity rhythms in DD (Grima *et al.*, 2004).

Although the *Pdf*$^{+}$ LNvs appear to have the primary role, robust rest:activity rhythms are a result of clock network coordination. When molecular clocks in the network are mismatched with one another, arrhythmicity, complex rhythms (comprised of multiple rhythmic components of different period lengths), or weak rest:activity rhythms emerge in the fly behavior (Yao and Shafer 2014). The network is often simply modeled as a system of dual oscillators, where oscillators in *Pdf*$^{+}$ LNvs control the morning peak of locomotor activity, and oscillators in LNds and the 5th s-LNv control the evening peak **(Grima *et al.*, 2004; Stoleru *et al.*, 2004; Rieger *et al.*, 2006; Guo *et al.*, 2014).** The LNd group is comprised of six neurons per hemisphere. Blocking neurotransmission from a LNd subset results in a large proportion of arrhythmic flies in DD (Guo *et al.*, 2014). In addition, molecular clocks in a subset of LNds drive transcriptional rhythms of a set of metabolic genes in the fat body, a peripheral tissue analogous to adipose/liver tissue, through Neuropeptide F signaling (Erion *et al.*, 2016).

The DN1 group is comprised of 2 anterior (DN1a) and 15 posterior (DN1p) neurons. DN1ps serve diverse functions as integrators of light, temperature, and circadian cues as well as effectors of locomotor activity, sleep, and mating. DN1s have molecular clocks that can be entrained to temperature (Yoshii *et al.*, 2010), and calcium (Ca^{2+}) activity in DN1ps is regulated by temperature (Guo *et al.*, 2016; Yadlapalli *et al.*, 2018). DN1ps integrate temperature and light information to promote robust rest:activity rhythms (Zhang, Chung, *et al.*, 2010; Zhang, Liu, *et al.*, 2010) and also regulate sleep at specific times of day, through different circuits using either DH31 neuropeptide or glutamate signaling (Kunst *et al.*, 2014; Guo *et al.*, 2016). In addition, DN1ps mediate rhythms in male sex drive (Fujii *et al.*, 2017).

The DN2s also have temperature-entrainable molecular clocks and regulate rhythms of temperature preference, namely the tendency of flies to seek different temperatures at different times of day (Yoshii *et al.*, 2010; Kaneko *et al.*, 2012). A circuit for temperature preference at dawn has been mapped from the thermosensory anterior cells to s-LNvs to DN2s (Tang *et al.*, 2017). The molecular clocks in LPNs are also strongly synchronized to temperature cycles (Miyasako *et al.*, 2007).

Finally, glial cells in the brain also express PER and TIM (Zerr *et al.*, 1990). Astrocytes are important for rest:activity rhythms, although the molecular clock in these cells is dispensable (Ng *et al.*, 2011). Glial cells are proposed to regulate outputs of clock neurons, but the signaling mechanisms remain to be uncovered (Ng & Jackson, 2015; Herrero *et al.*, 2017). In the *Drosophila* blood-brain barrier (BBB), molecular clocks in glial cells drive circadian rhythms in BBB permeability (Zhang *et al.*, 2018).

PDF is an important clock output factor in the clock network

In the clock network, pigment-dispensing factor (PDF) is an important clock output factor [reviewed in (Shafer & Yao, 2014)]. Loss or overexpression of *Pdf* causes arrhythmic rest:activity behavior (Renn *et al.*, 1999; Helfrich-Förster *et al.*, 2000), and mutations in the

PDF receptor (PDFR) phenocopy *Pdf* mutants (Hyun *et al.*, 2005; Lear, Merrill, *et al.*, 2005; Mertens *et al.*, 2005). PDFR is a G-protein coupled receptor that activates cAMP production upon binding of PDF peptide. An important function of PDF/PDFR signaling is to maintain coherent and synchronized molecular oscillations in the clock network (Lin *et al.*, 2004; Yoshii *et al.*, 2009). All the groups of clock neurons, except the l-LNvs, express PDFR and respond to PDF application (Shafer *et al.*, 2008; Im & Taghert, 2010). Since PDFR in also expressed in Pdf^{+} s-LNv, PDF may feed back to cell-autonomously regulate the clock itself or output from the clock (Choi *et al.*, 2012). **Outside the clock network, PDFR expression is sparse in the brain (Im & Taghert, 2010), and while PDF does signal to non-clock neurons implicated in behavior (Pírez *et al.*, 2013; Chen *et al.*, 2016), it is unclear whether PDF signaling in these circuits confers circadian timing to behavior.**

PDF levels cycle across the day at s-LNv terminals in the dorsal protocerebrum, indicating that PDF may be secreted in a circadian manner (Park *et al.*, 2000). **In addition, CLOCK regulates PDF expression at the post-transcriptional level through VRILLE, a repressor that functions in a second feedback loop interlocked with the core clock (Blau & Young, 1999; Gunawardhana & Hardin, 2017). CLOCK also regulates *Pdf* at the transcriptional level through a nuclear receptor called Hormone receptor-like 38 (Mezan *et al.*, 2016).** However, it is unclear whether rhythmic PDF levels or secretion are important for rest:activity rhythms (Kula *et al.*, 2006). Instead, rhythmic PDF levels in the s-LNv terminals may be a secondary consequence of rhythmic neuronal firing or remodeling of the projections (discussed below). Furthermore, rhythmic PDF signaling may also occur through circadian-gated sensitivity to PDF in target neurons, mediated by PDFR and a small GTPase, Ral A (Klose *et al.*, 2016). In summary, PDF is important for circadian rhythms, and its effect on circadian behavior is largely localized within the clock network.

Glycine and glutamate mediate reciprocal inhibition between the s-LNvs and DN1ps

Compared to neuropeptides, less is known about fast neurotransmitters in the clock network. However, within the s-LNv-DN1p circuit, the inhibitory neurotransmitter, glycine, is used in addition to PDF (Frenkel *et al.*, 2017). Knockdown of the glycine transporter or disrupting glycine synthesis in the Pdf^{+} LNvs lengthens the period of rest:activity rhythms, suggesting LNvs are glycinergic. In addition, glycine application on DN1ps reduces their firing frequency, and knockdown of glycine receptors subunits in the DN1ps reduces the power of rest:activity rhythms in flies, confirming functional glycine signaling in the s-LNv to DN1 circuit.

In the reciprocal direction, DN1ps signal to the s-LNvs through glutamate, which appears to be an inhibitory neurotransmitter in this circuit (Hamasaka *et al.*, 2007; Guo *et al.*, 2016). A subset of the DN1ps expresses vesicular glutamate transporter (VGlut), and s-LNvs and LNds express the metabotropic glutamate receptor, mGluRA. Consistent with an inhibitory effect, glutamate application decreases Ca^{2+} levels in s-LNvs and LNds. Glutamate signaling is also relevant for behavioral rhythms—glutamate from non-LNv clock neurons is required for robust rest:activity rhythms and knockdown of *mGluRA* in Pdf^{+}

LNvs lengthens the period of rest:activity rhythms (Hamasaka *et al.*, 2007; Collins *et al.*, 2012)

Neuropeptides sNPF and PDF set different phases of Ca^{2+} rhythms in clock network

Intercellular signaling is not only essential for synchronizing molecular clock rhythms but also coordinating neuronal activity rhythms in the clock network. It is thought that the molecular clock regulates the excitability of clock neurons, such that the neurons are more active at certain times of day than other times. Electrophysiological recordings from s-LNv, l-LNv, and DN1 have shown that the molecular clock drives these cells to be more active at dawn than at dusk (Table 1) (Cao & Nitabach, 2008; Sheeba, Gu, *et al.*, 2008; Flourakis *et al.*, 2015). Recent studies use genetically encoded Ca^{2+} sensors to perform longitudinal imaging of neuronal activity in the entire clock network over 24 hours, with the added advantages of obtaining more temporal information and precise determination of when clock neurons are most active (Liang *et al.*, 2016, 2017). We review this work reported in a pair of papers by Liang, Holy, and Taghert.

Intracellular calcium (Ca^{2+}) ions are important secondary messengers for many signaling pathways, and Ca^{2+} levels rise during electrical activity in neurons. In circadian regulation, Ca^{2+} signaling is both an input and output of the molecular clock (Ikeda, 2004; Harrisingh *et al.*, 2007), with all groups of clock neurons displaying 24-hr Ca^{2+} rhythms. Despite synchrony of the molecular oscillator across the clock network, Ca^{2+} rhythms are asynchronous among the different groups of clock neurons (Table 1) (Liang *et al.*, 2016). Ca^{2+} peaks in clock neurons occur at times that match with their roles in behavior. For example, s-LNvs control morning locomotor activity and have peak Ca^{2+} levels at dawn, and LNds control evening locomotor activity and have peak Ca^{2+} levels preceding the evening.

How does the clock network coordinate different phases of Ca^{2+} rhythms? To discover the mechanisms, Liang *et al.* (2017) focused on neuropeptides, such as PDF. In the absence of PDF, the Ca^{2+} peaks in LNds and DN3s are shifted from ~CT 8 and ~CT 16, respectively, to dawn (~CT 0). (CT is the circadian time is defined by an organism's endogenous circadian clock in constant conditions; CT 0 corresponds to the start of subjective day and CT 12 to the start of subjective night). To determine if the shift in Ca^{2+} rhythms is a phase advance or delay, they applied synthetic PDF and found that Ca^{2+} levels decreased in LNds and DN3s, and importantly, the Ca^{2+} levels remained depressed for several hours. Therefore, PDF delays the Ca^{2+} peaks in LNds and DN3s and does so by staggering their Ca^{2+} peaks to two different times of the day. How one neuropeptide produces two different effects on phase is not known. The authors also determined that sNPF (short Neuropeptide F) inhibits Ca^{2+} and delays the Ca^{2+} peak in DN1s. sNPF in the clock network is required for rhythmic Ca^{2+} rhythms but not molecular clock oscillations in DN1s. Therefore, for certain clock neurons, circuit mechanisms may dominate over the cell-autonomous molecular clock in shaping Ca^{2+} rhythms. **This study reported an inhibitory effect for PDF, while others have noted acute depolarization (Seluzicki *et al.*, 2014; Vecsey *et al.*, 2014) or increased Ca^{2+} levels in target cells (Seluzicki *et al.*, 2014). In fact, PDF can apparently increase or decrease**

Ca^{2+} levels in different neurons within the DN1p cluster (Chatterjee *et al.*, 2018). Opposing effects of PDF are also seen in the cockroach *Rhyparobia maderae*, such that responses to it vary across circadian clock neurons (Wei *et al.*, 2014; Gestrich *et al.*, 2018). Neuropeptides have complex roles in the clock network, as they synchronize the phases of molecular clocks and Ca^{2+} rhythms; in addition, acute and long-term effects of neuropeptides on target neurons may be different. How neuropeptides serve diverse functions is the clock network is still not well understood but likely involves divergent downstream signaling mechanisms (Duvall & Taghert, 2013; Seluzicki *et al.*, 2014).

Circadian regulation of structural plasticity in s-LNvs

Circadian structural plasticity in the fly brain was first reported in the lamina, the first optic neuropil of the visual system [reviewed in (Górska-Andrzejak *et al.*, 2015)]. In the lamina, many structures undergo circadian rhythms in morphological plasticity, including the retinal photoreceptor terminals, monopolar cells, and synapses **(Pyza & Meinertzhagen, 1995; Weber *et al.*, 2009; Górska-Andrzejak *et al.*, 2013).** Circadian plasticity of these structures is complex and involves multiple inputs from phototransduction pathways, clock neurons, and peripheral clocks in glia and photoreceptor cells.

Circadian structural plasticity has also been extensively studied in the terminal projections of s-LNvs in the dorsal protocerebrum. The s-LNv projections include both presynaptic and postsynaptic sites and are near most other clock neurons, implicating s-LNv projections as major sites for communication in the clock network (Helfrich-Förster *et al.*, 2007; Yasuyama & Meinertzhagen, 2010). In the morning, the s-LNv terminals display greater complexity, with more arbors, branching, and volume, than at night (Fernández *et al.*, 2008; Petsakou *et al.*, 2015). Presumably, the increased terminal complexity indicates more synaptic connections. Indeed, using the GRASP (GFP reconstitution across synaptic partners) assay, which labels synaptic contacts between two populations of neurons, the number of contacts between s-LNv and their partners were found to be higher during the day than in the evening (Gorostiza *et al.*, 2014; Tang *et al.*, 2017). As such, the structural plasticity of s-LNv projections is a circadian output rhythm, regulated by the molecular clock and maintained in constant darkness (Fernández *et al.*, 2008).

Circadian remodeling of s-LNv projections appears to be important for behavior, as disrupted remodeling of these projections is associated with compromised overt rest:activity rhythms. When the *Pdf^{+}* LNvs are acutely silenced, the s-LNv projections have reduced axonal complexity throughout the day (Depetris-Chauvin *et al.*, 2011) and the flies display arrhythmic rest:activity behavior, although the s-LNv molecular clock still runs with a normal 24-hr period, suggesting that electrical activity is an output of the molecular clock and regulates remodeling of s-LNv projections. The circadian remodeling of s-LNv projections is also regulated by cell-autonomous expression of PDF and Mmp1, a matrix metalloproteinase that processes PDF (Depetris-Chauvin *et al.*, 2014).

Other genes that affect rest:activity rhythms by dysregulating circadian remodeling of s-LNv projections, include *Mef2* (Myocyte enhancer factor 2), a transcription factor

expressed in all groups of clock neurons (Blanchard *et al.*, 2010). *Mef2* transcription is directly regulated by the CLOCK-CYCLE transcription factor complex, and Mef2, in turn, regulates transcription of many genes, including Fasciclin 2 (Fas2), the *Drosophila* ortholog of neural cell adhesion molecule, NCAM (Sivachenko *et al.*, 2013). **In s-LNvs, Mef2 promotes branching of the dorsal projections, while Fas2 represses branching through fasciculation, a phenomenon where axons stick to each other as they grow. From this study, a clock output mechanism emerges for circadian remodeling of s-LNv projections: CLOCK-CYCLE → Mef2 → Fas2 → s-LNv remodeling (Sivachenko *et al.*, 2013).** Dysregulation of *Mef2* in *Pdf*$^{+}$ LNvs leads to decreased power of rest:activity rhythms or complex rhythms (Blanchard *et al.*, 2010; Sivachenko *et al.*, 2013), but these behavioral changes are also correlated with altered molecular clocks in s-LNvs, suggesting Mef2 may also **feedback** onto the molecular clock (Blanchard *et al.*, 2010).

Another pathway that regulates s-LNv projections involves Rho1, a member of the Rho family of GTPase signaling proteins. Rho1 activity cycles in the s-LNv projections and is highest in the evening (ZT12), when the projections are most condensed, which is consistent with the role of Rho1 in promoting retraction of projections (Petsakou *et al.*, 2015). A Rho Guanine Nucleotide Exchange Factor (GEF), Puratrophin-1-like (Pura), activates Rho1 by promoting its association with GTP rather than GDP. *Pura* transcription cycles in s-LNvs and may be a direct target of CLOCK. Petsakou *et al.* (2015) proposed that clock-regulated *Pura* imposes rhythms in Rho1 activity, and the *Rho-ROCK*-myosin light chain pathway regulates actomyosin retraction of s-LNv projections in a circadian manner. When *Rho1* is overexpressed in the *Pdf*$^{+}$ LNv, the s-LNv projections do not branch in the morning, and flies display arrhythmic rest:activity behavior. At the molecular level, the s-LNv molecular clocks are normal, but in downstream DN1s, the molecular clocks are phase-shifted by up to 12 hours. Thus, remodeling of the s-LNvs has effects on other clock neurons.

The findings described above implicate two different pathways in circadian s-LNv remodeling, both of which are linked to the circadian clock. Both *Mef2* and *Pura* are targets of CLOCK, but they are maximally expressed at different times of day, suggesting they regulate s-LNv projections in different ways (Blanchard *et al.*, 2010; Sivachenko *et al.*, 2013; Petsakou *et al.*, 2015). Mechanisms of Mef2-Fas2 and Pura-Rho1 interaction are not known, and it is possible they act in parallel pathways, where Mef2-Fas2 regulate axon fasciculation cycles and Pura-Rho1 regulate axon growth- contraction cycles to together drive the circadian structural remodeling of s-LNv projections.

Clock output genes

Less is known about the circadian output pathways that transmit timekeeping signals from central clock cells to other parts of the brain to produce rest:activity rhythms. An output component is defined as a molecule or cell population that is regulated by the circadian clock but is not an intrinsic part of the clock mechanism. Several output genes have been implicated in behavioral rhythms, including *na*, *slo, miR-279, Nf1, wake,* and *ebony*. Dysregulation of these genes disrupts behavioral rhythms in animals but does so without affecting oscillations of the molecular clock. Many of these clock output genes exhibit clock-dependent diurnal variation in expression or function.

***Na* (*narrow abdomen*) encodes an ion channel with homology to the mammalian sodium leak channel, nonselective (NALCN),** and is required broadly in the clock network for normal rest:activity rhythms (Lear, Lin, *et al.*, 2005). In the posterior DN1 (DN1p) and l-LNv clock neurons, *na* is required for cycling of a sodium leak current, which contributes to oscillations in firing frequency and resting membrane potential (Flourakis *et al.*, 2015). *Nlf-1* (also known as *Mid1*) is a NA localization factor that is rhythmically expressed and clock-controlled. *Nlf-1* is also required for robust rest:activity rhythms (Ghezzi *et al.*, 2014; Flourakis *et al.*, 2015). Together, NLF-1 and NA are part of a cell-autonomous clock output mechanism to ensure robust rhythms of neuronal activity.

The SLO (slowpoke) potassium channel was identified as an output factor, because its binding partner, SLOB (slowpoke binding protein) is coded by a clock-controlled gene with robust transcriptional rhythms (Claridge-Chang *et al.*, 2001; McDonald & Rosbash, 2001; Ceriani *et al.*, 2002). *slo* mutants are arrhythmic in constant darkness but have intact s-LNv molecular clocks. Instead, *slo* mutants have altered levels of PDF in s-LNv projections and desynchronized clocks in the DN1s (Fernández *et al.*, 2007). *slo* may also have an important role outside the clock network, since clock neuron-specific rescue of *slo* only partially rescues rest:activity rhythms. Furthermore, *dyschronic,* a factor that regulates http://flybase.org/search/sloSLO expression, is required in non-clock neurons for rest:activity rhythms (Jepson *et al.*, 2012).

miR-279 is a microRNA that regulates rest:activity rhythms by targeting and downregulating expression of *Unpaired 1* (*Upd1*), a ligand of the JAK/STAT pathway (Luo & Sehgal, 2012). JAK/STAT signaling constitutes a critical pathway for development and immunity, but disrupting this pathway only in adulthood impairs rest:activity rhythms. *miR-279* and *Upd1* were found to be required in clock neurons for rest:activity rhythms, although their cellular requirements were not precisely mapped. Given findings that UPD1 is a fly analog of leptin and expressed in the Pdf^{+} LNvs, UPD1 could be an output of the s-LNvs (Beshel *et al.*, 2017).

Wake (*wide awake*) is a clock output molecule that regulates the timing of sleep onset. *wake* mutants have a delayed sleep onset at night but normal rest:activity rhythms (Liu *et al.*, 2014). WAKE levels cycle in the l-LNvs, peaking near dusk, when they are required to promote sleep. Previously, the l-LNvs were shown to promote arousal and respond to inhibition by GABA (Parisky *et al.*, 2008; Shang *et al.*, 2008; Sheeba, Fogle, *et al.*, 2008). In l-LNvs, WAKE upregulates membrane localization of RDL, a GABA(A) receptor, which would inhibit the excitability of arousal-promoting l-LNvs. Indeed, in *wake* mutants, the l-LNvs show decreased GABA sensitivity and increased excitability (Liu *et al.*, 2014). RDL also cycles in l-LNvs and is regulated by rhythmic degradation though the E3 ligase Fbxl4, whose transcription is clock-controlled. As expected, *Fbxl4* mutants have the opposite phenotype of *wake* mutants, with a shorter latency to sleep onset at dusk (Li *et al.*, 2017).

Nf1 (*neurofibromatosis-1*) encodes a Ras-specific GTPase activating protein required for rest:activity rhythms (Williams *et al.*, 2001). *Nf1* mutants have increased Ras/mitogen-activated protein kinase (MAPK) signaling, and loss-of-function mutations in the MAPK pathway can rescue rest:activity rhythms in *Nf1* mutants. Restoring *Nf1* in clock cells does

not rescue the behavioral deficits. Instead, *Nf1* is required broadly in the brain, presumably in multiple circadian neurons that regulate rest:activity rhythms (Bai *et al.*, 2018). Not only does *Nf1* regulate PDF levels in the s-LNv projections, it regulates Ca^{2+} and neuropeptide levels in circadian output neurons that are downstream of clock neurons (discussed below).

Ebony encodes a β-alanyl-biogenic amine synthase that controls the levels of free biogenic amines. EBONY is expressed exclusively in glial cells, where it functions to regulate rest:activity rhythms (Suh & Jackson, 2007). At least some of the glial expression of EBONY co-localizes with PER and TIM clock proteins, suggesting that *ebony* is an output molecule of glial clock cells.

All the clock output genes identified so far, except for WAKE, appear to broadly function in multiple groups of circadian clock neurons or glial cells. Expression of many of these genes, including *nlf-1*, *slob*, *wake*, and *ebony*, is controlled by the clock and so they directly link the circadian clock to neuronal activity or cell signaling. Despite arrhythmic behavior in the clock output mutants, molecular oscillations in the s-LNv central neurons remain unaffected. However, there may be examples where mechanisms that affect the electrical activity of clock neurons also feedback onto the molecular clock (Nitabach *et al.*, 2002; Ruben *et al.*, 2012), thus assigning output and input functions to some genes is not so clear-cut. Finally, since the output genes described above have only been studied in the context of the known circadian network, their additional action in as-yet unidentified circadian neurons cannot be excluded.

Circadian output circuits that regulate rhythms of behavior/physiology

In the last 5 years, with advances in circuit mapping tools, we have identified multisynaptic output circuits that regulate circadian rhythms (Figure 1). These circuits consist of non-clock neurons that convey circadian timing information from clock neurons to sites that control behavior or physiology. Output neurons receive inputs from clock neurons, either directly or indirectly through another group of output neurons. To date, assays of output neurons have revealed cycling of neural/cellular activity in a clock-dependent fashion (Table 1). Disruption of this neuronal activity disrupts the output rhythm without affecting the molecular clock. Therefore, most phenotypes from manipulating circadian output neurons are effects on rhythmicity of rest:activity rather than changes in circadian period, which is an intrinsic property of the clock. However, output neurons could feedback onto the clock to affect periodicity. As output circuits identified thus far are peptidergic and neuromodulatory in nature, and possibly also redundant, their disruption tends to weaken the amplitude of the rest:activity rhythm and not eliminate it altogether as would loss of molecular clock oscillations.

The pars intercerebralis (PI) has been proposed as a clock output region for many years. Ablation studies in cockroaches showed that the PI is required for locomotor activity rhythms (Nishiitsutsuji-Uwo *et al.*, 1967; Matsui *et al.*, 2009). Furthermore, in *Drosophila,* nearly all the circadian clock neurons, except for the l-LNvs, project to the PI (Helfrich-Förster, 1995; Kaneko & Hall, 2000; Helfrich-Förster *et al.*, 2007). The PI is a major neurosecretory center with a high degree of neurochemical heterogeneity, as such

functionally analogous to the mammalian hypothalamus (de Velasco *et al.*, 2007). PI neurons regulate various behaviors in flies including sleep (Foltenyi *et al.*, 2007; Crocker *et al.*, 2010), feeding (Zhan *et al.*, 2016), nutrient sensing (Dus *et al.*, 2015), courtship (Terhzaz *et al.*, 2007), and aggression (Davis *et al.*, 2014). Thus, the PI may be a major output center for regulating circadian timing of behaviors.

Our group identified populations of PI neurons relevant for circadian rhythms. Three different PI groups, those that express DH44 (Diuretic hormone 44), SIFa (SIFamide), or DILP2 (*Drosophila* insulin-like peptide 2), synapse with DN1p clock neurons (Cavanaugh *et al.*, 2014; Barber *et al.*, 2016). The three PI groups are largely distinct from one another, with the exception that a pair of the *Dh44*$^+$ neurons expresses low levels of DILP2 (Ohhara *et al.*, 2018). Currently, it is not known whether s-LNvs or other clock neurons directly signal to the PI. Furthermore, we do not know the identity of the signaling molecules that mediate the DN1p to PI communication.

DH44→Hugin: A neuropeptidergic output circuit regulates rest:activity rhythms

The six *Dh44*$^+$ neurons of the PI receive clock input through a multisynaptic circuit comprised of s-LNv → DN1 → *Dh44*$^+$ PI (Cavanaugh *et al.*, 2014). Activation or ablation of *Dh44*$^+$ PI neurons reduces the power (or amplitude) of rest:activity rhythms without affecting the molecular oscillation of clock proteins in s-LNvs, demonstrating that *Dh44*$^+$ PI neurons are output neurons downstream of the clock. In *Dh44*$^+$ PI neurons, Ca^{2+} levels cycle across the 24-hr day, with peak activity occurring around evening and trough activity in the morning. Ca^{2+} cycling in *Dh44*$^+$ neurons requires the *Pdf*$^+$ LNvs, suggesting that rhythmic Ca^{2+} levels propagate from the s-LNvs to *Dh44*$^+$ neurons (Cavey *et al.*, 2016). In addition, the *Nf1* circadian output gene cell-autonomously regulates Ca^{2+} cycling in *Dh44*$^+$ neurons (Bai *et al.*, 2018).

What about the role of the DH44 neuropeptide in rest:activity rhythms? DH44 and one of its receptors, DH44-R1, are required for strong rest:activity rhythms (Cavanaugh *et al.*, 2014; King *et al.*, 2017). Our group also mapped the circuit downstream of *Dh44*$^+$ PI neurons to another set of neuropeptidergic neurons in the subesophageal zone. Knockdown of *Dh44-R1* in *hugin*$^+$ neurons reduces the power of rest:activity rhythms. In addition, *hugin* and its encoded neuropeptides, Hugin-γ and/or Prokynin-2, are required for robust rest:activity rhythms. *hugin*$^+$ neurons themselves display clock-dependent cycling of neuropeptide vesicle release from their axon termini. A subset of *hugin*$^+$ neurons projects back to the PI, potentially providing feedback regulation, while another subset of *hugin*$^+$ neurons projects to the ventral nerve cord (VNC), where the circuit potentially modulates motor circuits driving locomotor activity (King *et al.*, 2017). For the first time, we have a minimal, linear circuit between clock neurons and output neurons regulating locomotor activity.

SIFa$^+$ PI neurons regulate rest:activity rhythms

In the same screen that identified *Dh44*$^+$ PI neurons, the *SIFa*$^+$ PI neurons were also found to regulate rest:activity rhythms (Cavanaugh *et al.*, 2014). Ablation of all four *SIFa*$^+$ neurons

in the brain disrupts rest:activity rhythms but spares the s-LNv molecular clock. Loss of SIFa peptide itself produces a weaker effect on rest:activity rhythms than neuronal ablation, suggesting that other or co-neurotransmitters from *SIFa*$^{+}$ neurons regulate rest:activity rhythms (Bai *et al.*, 2018). Finally, circadian phenotypes in *Nf1* mutants may be due to dysregulation of *SIFa*$^{+}$ neurons. In *Nf1* mutants with arrhythmic rest:activity behavior, both Ca^{2+} levels in *SIFa*$^{+}$ neurons and mRNA levels of *SIFa* are elevated (Bai *et al.*, 2018).

Dilp2^{+} PI neurons integrate circadian timing and metabolic signals

The fourteen *Dilp2*$^{+}$ PI neurons and the insulin-like peptides have well-described roles in feeding and metabolism (Nässel *et al.*, 2013). Similar to the *Dh44*$^{+}$ and *SIFa*$^{+}$ neurons, *Dilp2*$^{+}$ neurons receive inputs from DN1p clock neurons (Barber *et al.*, 2016). However, unlike their PI counterparts, *Dilp2*$^{+}$ neurons do not appear to control rest:activity rhythms. Activation of *Dilp2*$^{+}$ neurons in the adult fly is not sufficient to impair rest:activity rhythms (Cavanaugh *et al.*, 2014). However, *Dilp2*$^{+}$ neurons and insulin signaling may be important for development of circadian output circuits (Monyak *et al.*, 2017). A set of *Dilp2*$^{+}$ neurons project out of the brain and into the aorta, where circulating insulin-like peptides may be released to affect peripheral tissues, like the fat body. *Dilp2*$^{+}$ neurons and insulin signaling regulate transcriptional rhythms of *sxe2*, a lipase in the fat body (Barber *et al.*, 2016). As circadian output neurons, *Dilp2*$^{+}$ neurons show cycling in electrical activity (Barber *et al.*, 2016). *Dilp2*$^{+}$ neurons exhibit higher electrical activity in the morning compared to the night, specifically increased firing frequency and burst firing events. These differences in electrical activity are lost in a *period* null mutant, demonstrating that cycling of *Dilp2*$^{+}$ neuronal activity is clock-dependent. Cycling of electrical activity in *Dilp2*$^{+}$ neurons is in phase with cycling in upstream clock neurons, DN1s and LNvs (Cao & Nitabach, 2008; Sheeba, Gu, *et al.*, 2008; Flourakis *et al.*, 2015). In addition to clock-regulation, firing in *Dilp2*$^{+}$ neuron is regulated by feeding, since restricted feeding can shift the nighttime firing pattern of *Dilp2*$^{+}$ neurons to the daytime firing pattern (Barber *et al.*, 2016). Thus, *Dilp2*$^{+}$ PI neurons integrate both circadian timing and metabolic signals.

Leucokinin regulates rest:activity rhythms

Leucokinin (*Lk*)-expressing neurons in the lateral horn are circadian output neurons that regulate sleep and rest:activity rhythms (Cavey *et al.*, 2016). Both *Lk* and *Lk receptor* (*Lk-R*) mutants have reduced power of rest:activity rhythms. s-LNv clock neurons project to *Lk*$^{+}$ lateral horn neurons, and firing of *Pdf*$^{+}$ LNv neurons indirectly inhibits Ca^{2+} in *Lk*$^{+}$ lateral horn neurons. LK-R is expressed broadly in the brain, including the lateral horn, ellipsoid body, and fan-shaped body, which are all areas implicated in locomotor control. The cellular requirement of LK-R for rest:activity rhythms has not been mapped. However, both *Lk*$^{+}$ and *Lk-R*$^{+}$ neurons in the lateral horn display cycling of Ca^{2+} levels that is dependent on the molecular clock and *Pdf*$^{+}$ LNvs. Cycling of Ca^{2+} levels occurs with opposite phases in *Lk*$^{+}$ and *Lk-R*$^{+}$ neurons, since LK peptide decreases Ca^{2+} levels in *Lk-R*$^{+}$ neurons. *Lk*$^{+}$ and *Lk-R*$^{+}$ lateral horn neurons also exhibit rhythms in excitability to carbachol, a cholinergic receptor agonist, that tracks with baseline Ca^{2+} rhythms. In summary, rhythmic neuronal activity can propagate to output neurons that are at least two synapses removed from clock neurons.

PTTH+ neurons regulate eclosion rhythms

Eclosion (adult emergence from pupae) occurs only once in the life of a fly, but rhythms of eclosion can be monitored in a population, with peaks of emerging flies typically observed around dawn. The prothoracic gland (PG) is an endocrine gland that produces ecdysone, the steroid hormone that controls molting. Eclosion rhythms are controlled by central brain clocks and peripheral clocks in the PG (Myers *et al.*, 2003), but the brain clock has a dominant role over the PG clock (Selcho *et al.,* 2017). The central clock transmits timing information to the PG clock through a s-LNv → *PTTH*+ neurons → PG circuit (Selcho *et al.*, 2017). PTTH (prothoracicotropic hormone) is expressed in two pairs of brain neurons that receive input from s-LNvs via short Neuropeptide F. In turn, PTTH from the brain signals onto the PG through the PTTH receptor, *torso*. Knockdown of *torso* in the PG disrupts eclosion rhythms but has no effect on adult rest:activity rhythms (Selcho *et al.*, 2017). These works highlight that s-LNvs control rest:activity rhythms and eclosion rhythms through different output circuits.

Conclusion

The molecular mechanism of the circadian oscillator has been worked out in detail. For many years, we knew much less about how oscillations of the molecular clock are translated into overt rhythms in behavior and physiology. Only recently has the field begun to identify functional connections within and downstream of the clock network, thus providing a neural basis for circadian rhythms. The primary focus of the field has been dissecting functional circuits that control rest:activity rhythms, and so the circuits that control other rhythmic behaviors in adult flies are underexplored. For all circadian circuits, clock-regulated cycling of neuronal activity appears to be the output mechanism for timekeeping and can propagate from clock neurons to output neurons along multisynaptic circuits. Longitudinal recording of neuronal activity over 24 hours remains a challenge in flies but will be informative for precisely studying how cycling of activity in circadian circuits is shaped by the molecular clock and neurotransmission.

The knowledge provided by dissection of circadian circuits will likely establish principles applicable to the function of circuits in general. The circadian model allows study of the neuromodulatory role of neuropeptides, the mechanisms coordinating release of a peptide and a fast neurotransmitter at the same synapse, and the use of different signaling mechanisms in response to the same neuromodulator, all in the context of robust behavioral outputs. Once the basic mechanisms governing these processes have been determined, their adaptation to more complex or nuanced behaviors can be ascertained. Moreover, while the emphasis in the circadian field is on 24-hour rhythms, the principles driving transmission of temporal signals may also be relevant for understanding those that occur on a shorter timescale.

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Abbreviations

BBB	blood-brain barrier
Ca^{2+}	intracellular calcium ion
CRY	Cryptochrome
CT	circadian time
DD	constant darkness
DH31	Diuretic Hormone 31
DH44	Diuretic Hormone 44
DH44-R1	diuretic hormone 44 receptor 1
DILP2	Drosophila insulin-like peptide 2
DN1	dorsal neuron (group 1)
DN1p	posterior dorsal neuron (group 1)
DN2	dorsal neuron (group 2)
DN3	dorsal neuron (group 3)
Fas2	Fasciclin 2
GRASP	GFP reconstitution across synaptic partners
LHLK	Leucokinin-expressing lateral horn neurons
LK	Leucokinin
LK-R	Leucokinin receptor
l-LNv	large ventral lateral neuron
LNd	dorsal lateral neuron
LNv	ventral lateral neuron
LPN	lateral posterior neuron
Mef2	Myocyte enhancer factor 2
mGluRA	metabotropic Glutamate Receptor
na	narrow abdomen
Nf1	Neurofibromatosis-1
PDF	Pigment-Dispersing Factor
PDFR	Pigment-Dispersing Factor receptor

PER	PERIOD
PG	prothoracic gland
PI	pars intercerebralis
PTTH	prothoracicotropic hormone
Pura	Puratrophin-1-like
SEZ	subesophageal zone
SIFa	SIFamide
s-LNv	small ventral lateral neuron
slo	slowpoke
sNPF	short Neuropeptide F
TIM	TIMELESS
VGlut	Vesicular glutamate transporter
VNC	ventral nerve cord
wake	wide awake
ZT	Zeitgeber Time

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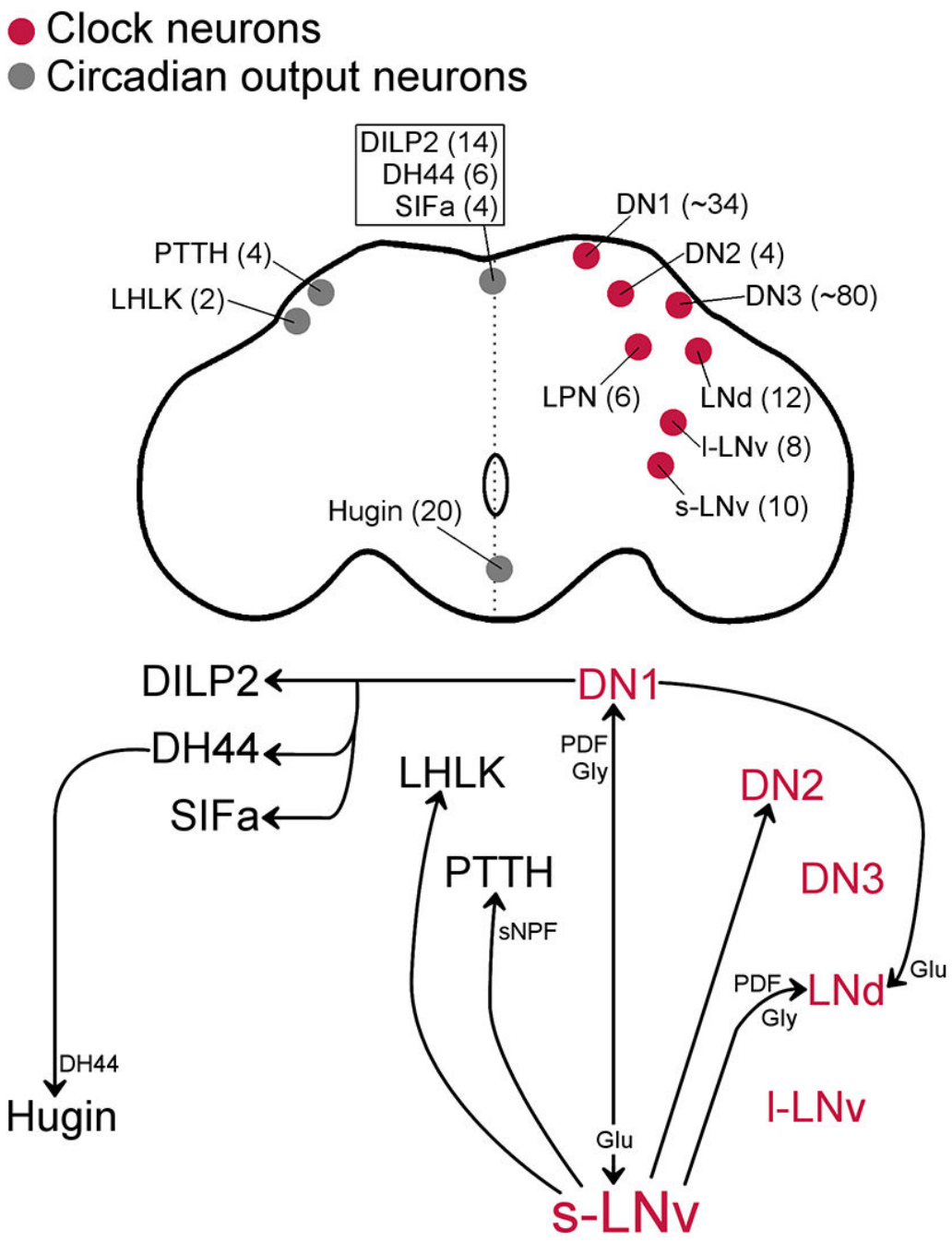

Figure 1: Circadian circuits in the fly brain.

Top. Schematic representation of a fly brain with neuroanatomical locations of clock neurons (red, right hemisphere) and circadian output neurons (gray, left hemisphere or midline). Bilaterally represented neurons are labeled in only one of the hemispheres. Approximate total number of cells in the brain is indicated in parentheses. **Bottom.** Arrows represent the paths of communication between groups of circadian neurons. Circuits were mapped using neuronal activation and functional imaging and/or GRASP (GFP reconstitution across synaptic partners) methods. The neuropeptide/neurotransmitters that

signal in the circuits were genetically identified by removing the peptide or neurotransmitter transporter in the presynaptic neuron and removing the receptor in the postsynaptic neuron. PDF mediates s-LNv communication to LNd, DN1, and LHLK (indirectly) (Leucokinin^{+} lateral horn). Glycine (Gly) also signals from s-LNv to DN1 and LNd. Short neuropeptide F (sNPF) signals in the s-LNv to PTTH circuit. Glutamate (Glu) signals from the DN1 to s-LNv and LNd. The molecules that signal between DN1 and PI neurons (DH44/SIFa/Dilp2) are unknown. DH44 neuropeptide signal from *Dh44*$^{+}$ to *hugin*$^{+}$ neurons.

Table 1:

Cycling in Circadian Circuits

Neuronal group:	Cycling:	Highest at: (hours since lights-on)	Cycles in constant darkness?	Cycle lost in a clock mutant?:	Reference
Clock neurons					
s-LNv	Electrical activity	~0	N.d.	N.d.	(Cao & Nitabach, 2008)
	Intracellular calcium (Ca^{2+}) levels	23-24	Yes	Yes	(Liang *et al.*, 2016)
	Complexity of projections	~0	Yes	Yes	(Fernández *et al.*, 2008)
	Synapse contacts	~2	Yes	N.d.	(Gorostiza *et al.*, 2014)
	Rho1 activity	~12	Yes	Yes	(Petsakou *et al.*, 2015)
	PDF levels in projections	0-6	Yes	Yes	(Park *et al.*, 2000)
	PDF and dopamine sensitivity	~0	Yes	N.d.	(Klose *et al.*, 2016)
l-LNv	Electrical activity	1-6	No (DD day 1); Yes (DD day 14)	Yes	(Cao & Nitabach, 2008; Sheeba, Gu, *et al.*, 2008)
	Ca^{2+} levels	5-6	Yes	Yes	(Liang *et al.*, 2016)
	GABA sensitivity	Evening	N.d.	N.d.	(Li *et al.*, 2017)
LNd	Ca^{2+} levels	~12	Yes (highest at CT 8-9)	Yes	(Liang *et al.*, 2016)
DN1	Electrical activity	0-4 or 20-24	N.d.	Yes	(Flourakis *et al.*, 2015)
	Ca^{2+} levels	18-20	Yes	Yes	(Liang *et al.*, 2016)
DN2	Synaptic contacts with s-LNvs	22-24	N.d.	N.d.	(Tang *et al.*, 2017)
DN3	Ca^{2+} levels	17-18	Yes	Yes	(Liang *et al.*, 2016)
Circadian output neurons					
$DILP2^{+}$ PI	Electrical activity	0-4	No	Yes	(Barber *et al.*, 2016)
$DH44^{+}$ PI	Ca^{2+} levels	7-12	Yes	Yes	(Cavey *et al.*, 2016; Bai *et al.*, 2018)
$Hugin^{+}$ SEZ	Neuropeptide vesicle release	Night	N.d.	Yes	(King *et al.*, 2017)
LK^{+} LH	Ca^{2+} levels	Night	Yes	Yes	(Cavey *et al.*, 2016)
	Carbachol sensitivity	Night	Yes	Yes	(Cavey *et al.*, 2016)
LK $Receptor^{+}$ LH	Ca^{2+} levels	Day	Yes	Yes	(Cavey *et al.*, 2016)
	Carbachol sensitivity	Day	Yes	Yes	(Cavey *et al.*, 2016)

N.d. = not determined

CT = circadian time

DD = constant darkness

LH = lateral horn

PI = pars intercerebralis

SEZ = subesophageal zone