REVIEW ARTICLE

The *Drosophila melanogaster* circadian pacemaker circuit

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Abstract

As an experimental model system, the fruit fly *Drosophila melanogaster* has been seminal in shaping our understanding of the circadian clockwork. The wealth of genetic tools at our disposal over the past four decades has enabled discovery of the genetic and molecular bases of circadian rhythmicity. More recently, detailed investigation leading to the anatomical, neurochemical and electrophysiological characterization of the various neuronal subgroups that comprise the circadian machinery has revealed pathways through which these neurons come together to act as a neuronal circuit. Thus the *D. melanogaster* circadian pacemaker circuit presents a relatively simple and attractive model for the study of neuronal circuits and their functions.

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Introduction

The fruit fly *Drosophila melanogaster* has served as a model system for the study of circadian rhythms primarily due to the availability of molecular genetic tools that enabled identification of genes, proteins and neuronal groups that are essential components of the circadian machinery. Further, *D. melanogaster* exhibits robust and relatively easily measurable rhythms in behaviours such as activity/rest, adult emergence (eclosion), mating and egg-laying (reviewed in Sakai and Ishida 2001; Myers *et al*. 2003; Howlader and Sharma 2006; Rosato and Kyriacou 2006). The underlying molecular basis for some of these rhythms has been extensively studied over the past four decades and is known to be composed of several interlocked transcriptional–translational feedback loops (TTL) along with post-translational modifications of proteins which enable the persistence of near-24-h rhythms even in the absence of environmental cycles that can serve as *Zeitgebers* (time cues) (reviewed by Hardin 2005; Sheeba *et al*. 2008a; Zheng and Sehgal 2008). The circadian oscillation in levels of mRNA and protein of some of the core components of the TTL such as *period (per), timeless (tim)* and *par-domain-protein-1 (pdp1)* are now considered molecular markers of the selfsustained circadian oscillator. Oscillation in *per* transcription has been shown to occur in a large variety of tissues in the fly such as the eye, brain, proboscis, antennae, wings, abdomen, Malpighian tubules and testes (Plautz *et al*. 1997; Giebultowicz 2001). Although cell-autonomous circadian function is attributed to several tissues in *Drosophila*, circadian pacemaker neurons located in the brain have been shown to be essential for the regulation of behavioral rhythms (Helfrich-Förster 1998). In this review, I will discuss the results of studies that have led to the consensus that circadian pacemaker neurons are functionally interconnected and influence each other so as to function as a neuronal circuit that ultimately governs behavioral circadian rhythmicity.

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Circadian pacemaker neuronal subgroups: the nodes of the circuit

A number of neuronal subgroups in the fly brain have been identified as circadian pacemaker neurons (figure 1,A) based on their expression of proteins PERIOD (PER), TIMELESS (TIM) and the neurotransmitter Pigment Dispersing Factor (PDF). The first appearance of neurons with possible circadian function occurs in the early first instar larval. Ventrally located bilateral clusters each consisting of four neurons called small ventral lateral neurons (s-LN_v) express both PER and PDF (Helfrich-Förster 1997; Kaneko *et al*. 1997). Towards the late first larval instar, two more pairs of neurons,

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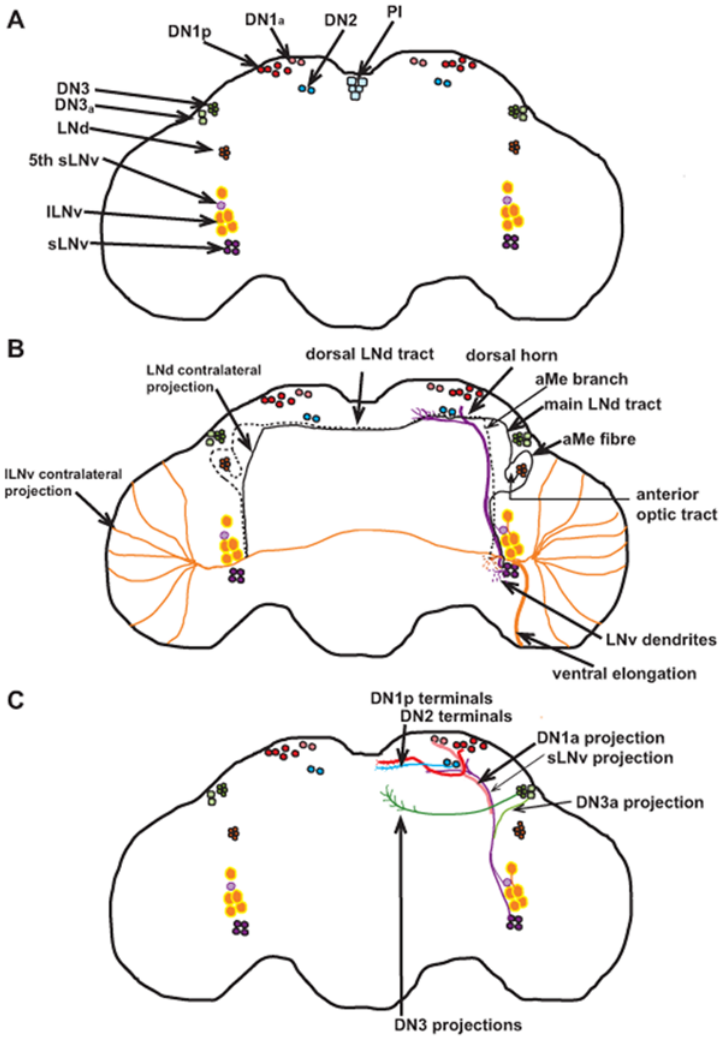

Figure 1. Schematic representation of the circadian pacemaker circuit in adult *Drosophila melanogaster*. (A) The known members of the various neuronal subgroups are represented as bilaterally placed coloured dots. (B) The projections from the LN_v and LN_d of the right hemisphere are denoted. All four PDF positive and the 5th PDF negative s-LN_v show similar projection pattern and arborize in the dorsal protocerebrum (purple trace). l-LN_v send out projections that innervate the optic medullary surface of both the ipsilateral and contralateral hemispheres (orange traces). In the ipsilateral lobe the l-LN_v also arborize ventrally to form the ventral elongation. Dendritic fibres from both LN_v in the accessory medulla (aMe) are shown as thin dashed lines (purple and orange). Projections from the LN_d (black solid line) first form around the anterior optic tract and a thin bundle of fibres splits from the main tract and moves ventrally in the ipsilateral hemisphere (aMe fibres). Main LN_d moves towards the $DN3_a$ and travels close to the terminals of s-LN_v. Another bundle of fibres (aMe branch) is found to break off acutely from the main LN_d tract (black dotted line) and joins the aMe fibres. The aMe branch is likely to be the axonal projections from the LN_d of the contralateral brain hemisphere and is thought to terminate in the region of the aMe where the LN_v dendritic arbors are also seen. (C) $DN1_a$ project ventrally (pink) in parallel to the s-LN_v tract (purple) while the majority of the DN1 project towards the dorsal fusion commissure (red) and do not cross the midline similar to the DN2 (blue). $DN3_a$ project ventrally (pale green) and parallel to the s-LN_v tract while the other DN3 project towards the dorsal commissure in the dorsal protocerebrum more ventrally than the other DNs (dark green). s-LN_v, small ventral lateral neurons; l-LN_v, large ventral lateral neurons; LN_d, dorsal lateral neurons; DN, dorsal neurons; LPN, lateral posterior neurons; PI, pars intercerebralis.

DN1 and DN2 that express PER are observed in the dorsal brain region (Kaneko *et al*. 1997; Kaneko and Hall 2000; Klarsfeld *et al*. 2004). During the third larval instar and early pupa, another cluster of about six cells appears bilaterally in the dorso–lateral position and these neurons are denoted LN_d (Kaneko and Hall 2000). During the same time in the preadult developmental stages, a set of four ventral lateral neurons that are distinctly larger than s-LN_v also differentiate bilaterally (l-LN_v; Kaneko *et al*. 1997; Helfrich-Förster *et al*. 2007). A fifth pair of s-LN_v that does not express PDF also differentiates at the late third larval instar (Kaneko *et al*. 1997; Helfrich-Förster *et al*. 2007). The adult fly brain has many more dorsal neurons including a bilaterally located cluster of ~40 DN3 neurons. A subset of the DN3 group are designated $DN3_a$ due to their anterior location in comparison to the other members of this group (Shafer *et al*. 2006). Further, in adults, the DN1 cluster differentiates into an anterior $DN1_a$ subset consisting of two neurons that originated in the larval stage and a posterior subset consisting of 12–15 neurons ($DN1_p$) located about 15–30 microns away from the $DN1_a$ cluster (Shafer *et al*. 2006). Unlike the circadian neurons described above, which express both PER and TIM, a bilateral cluster of 3–4 neurons located in the posterior lateral location expresses TIM but not PER (denoted as LPN, Kaneko and Hall 2000; Shafer *et al*. 2006). While the LN_v can be easily classified into small and large subgroups based on size, other circadian pacemaker neurons show less obvious but nevertheless distinct diversity in size. The six LN_d and ~16 $DN1_p$ show a smooth gradation in cell body size (Shafer *et al*. 2006). Among the 40 DN3 neurons, three to five are clearly larger than the rest (Shafer *et al*. 2006). It is highly likely that future research will reveal even greater functional diversity among members of the currently known circadian pacemaker neuronal subgroups.

Projection patterns of circadian pacemaker neurons: the anatomical connections of the circuit

The projection patterns of the neuronal subgroups mentioned above have been extensively studied at various developmental stages using many different approaches, such as antibody staining against circadian neuropeptides (Helfrich-Förster and Homberg 1993; Helfrich-Förster 1995, 1997), expression of markers such as *lacZ* or green fluorescent protein (GFP) using promoters of circadian genes (Kaneko and Hall 2000; Shafer *et al*. 2006), and by cell filling with fluorescent dyes (Park and Griffith 2006). Based on the findings of these studies, I summarize in this section our current understanding of the neuro-anatomical circuitry of circadian pacemakers in the brain of adult *D. melanogaster* (figure 1,B&C).

The majority of the projections from the known circadian pacemaker neurons terminate in the dorsal protocerebral region, which is the site for neurosecretory cells in dipterans (Shiga 2003; reviewed in Helfrich-Förster 2005). In addition, the large and small LN_v and LN_d densely innervate the accessory medulla region (aMe; Helfrich-Förster *et al*. 2007) that has been shown to be a circadian pacemaking centre in other insects (reviewed in Helfrich-Förster *et al*. 1998). The four pairs of s-LN_v send out projections (that are thought to function as axons) ipsilaterally toward the dorsal protocerebrum and terminate near the mushroom body calyces (figure 1,B). In some cases, a small branch extends dorsally to form the dorsal horn (Helfrich-Förster and Homberg 1993; Helfrich-Förster *et al*. 2007). The axonal projections from the PDF negative fifth s-LN_v are indistinguishable from those of the other four PDF positive s-LN_v (Helfrich-Förster *et al*. 2007). All l-LN_v send out axonal projections in opposite directions and arborize both on the ipsilateral and contralateral medullary surface (figure 1,B). In the ipsilateral aMe, the l-LN_v projections extend ventrally to form the ventral elongation (Helfrich-Förster *et al*. 2007). Both l-LN_v and s-LN_v densely arborize in the aMe region forming what appear to be dendritic arbors (Helfrich-Förster *et al*. 2007).

The projections from $DN1_a$ (the subset of DN1 already present in larvae) project towards aMe (figure 1,C). The $DN1_p$ send projections to the dorsal fusion commissure where they show dense arborization and cross the midline but do not extend to aMe (Helfrich-Förster *et al*. 2007). The DN2 project to the dorsal commissure and terminate in the ipsilateral dorsal brain hemisphere close to the pars intercerebralis (PI, Helfrich-Förster *et al*. 2007). The DN3, with the exception of two neurons ($DN3_a$), send out projections to the dorsal commissure slightly more ventral than the DN1 and DN2 projections, and remain ipsilateral but do not cross the midline (Helfrich-Förster 2003; Helfrich-Förster *et al*. 2007). The $DN3_a$ pair sends projections ipsilaterally to the aMe (Helfrich-Förster *et al*. 2007). The LN_d neurons send out a bundle of projections that form a loop around the anterior optic tract as they first emerge from the cell bodies and run along the outer surface of the lateral horn neuropil and then a thin bundle of fibres splits off from the main LN_d tract and moves latero-posteriorly and ventrally towards the ipsilateral aMe (aMe fibres). The main LN_d tract moves close to the region of the $DN3_a$ and loops again towards the median posterior part of the brain close to the terminals of the s-LN_v. Another branch of fibres designated as the aMe-branch breaks off from the main LN_d tract at this point at an acute angle and moves ventrally to join the aMe fibres, which had split from the main LN_d tract earlier (black dotted line, figure 1,c). Together, they move ventrally in parallel to the s-LN_v tract. The main LN_d tract moves dorsally and in parallel to the DN3 projections; extensively arborizes in the dorsal protocerebrum and crosses the midline in the dorsal fusion commissure (Helfrich-Förster *et al*. 2007). Based on the comparison of thickness of neuronal fibres in mutant flies that lack subsets of DNs (using fluorescent markers of neuronal projections), it has been suggested that the LN_d neurons send projections both ipsilaterally and contralaterally. Therefore, it is likely that LN_d projections are another means of

communication between the two brain hemispheres (Kaneko and Hall 2000; Helfrich-Förster *et al*. 2007). The projection patterns of LPN are not yet known.

A recent study has shown that an allele of the *roundabout* gene which is involved in axon guidance (*robo*^{hy}) leads to shortening of free-running period accompanied by early nuclear translocation of PER protein (Berni *et al*. 2008). This study suggests that circuit assembly and maintenance is controlled by *robo*. Further, this research group also detected a circadian oscillation in terminal axonal arborization of the s-LN$_v$ in the dorsal protocerebrum of wild-type flies (Fernández *et al*. 2008). They propose that this phenomenon may be responsible for efficient coupling among the circadian pacemaker neurons. Glial cells expressing ebony have also been shown to contribute to the production of rhythmic activity/rest behaviour (Suh and Jackson 2007). Although this study found that ebony function is downstream of and independent of known circadian pacemakers, their study also revealed that communication between glia and neurons is necessary for normal circadian rhythms. Further, this study showed that *ebony* expressing glia lie in close proximity to dopaminergic cells and circadian neurons s-LN$_v$, LN$_d$, DN1 and DN3 leading to the possibility that glia take up dopamine and conjugate it to β-alanine to produce N-β-alanine dopamine whose release may in turn control neuronal activity of circuits that regulate rhythmic locomotor activity (Suh and Jackson 2007).

Neurotransmitters: the chemical signals of the circuit

In *D. melanogaster* a few neurotransmitters have been identified to play a role in circadian pacemaker function, among which, the neuropeptide PDF is the best characterized. PDF, a homologue of crustacean Pigment Dispersing Hormone (PDH; Rao *et al*. 1985), was the first circadian neurotransmitter discovered in *Drosophila* (Helfrich-Förster and Homberg 1993). In other insects, such as cockroaches, PDH expression is found in regions of the brain where circadian clocks are believed to be present (Homberg *et al*. 2003). PDF is produced by four out of the five s-LN$_v$ and all l-LN$_v$ (Helfrich-Förster 1995) and its absence causes the disruption of free-running rhythms as well as alteration in the ability to entrain to environmental cycles of light–dark and temperature changes (Renn *et al*. 1999; Busza *et al*. 2007; Miyasako *et al*. 2007; Tomioka *et al*. 2008), confirming its importance in the regulation of circadian rhythms. PDF secreted by the s-LN$_v$ is released in the dorsal protocerebral region in a circadian manner (Park *et al*. 2000). When PDF is ectopically expressed in this region of the brain it results in complex rhythmicity or arrhythmicity in locomotor activity and disruption of adult emergence rhythms under DD (Helfrich-Förster *et al*. 2000). These studies suggested that since the dorsal protocerebral region contains cell bodies and terminals of many of the other members of the circadian pacemaker circuit, it is likely that PDF is the molecule through which sLN$_v$ modulates circadian molecular oscillation in other pacemaker neurons.

Very recently, a study using a tethered-toxin technique showed that the phase of PDF release rhythm from the LN$_v$ determines the phase of the molecular oscillation of circadian proteins in other circadian pacemaker neurons (Wu *et al*. 2008a). Another recent study of mutants that show abnormal arborization of PDF expressing l-LN$_v$ has demonstrated that PDF can cause differential effects on target neurons depending on their location in the brain (Wulbeck *et al*. 2008). The *sine oculis* mutant (*so*^{1}), which shows abnormally high number of PDF expressing fibres in the aMe also exhibits significantly longer free running period (24.6 h) compared to controls. On the other hand the mutant *so*^{mda} and double mutant *small optic lobes sol*;*so*^{1} which show abnormally high number of arbors in the dorsal protocerebrum exhibit both a long period behavioral rhythm and a short period rhythm that appears to emerge out of the long period rhythm. The behavioral rhythm abnormalities in both cases disappeared in *pdf*^{01} background suggesting that both the shortening and lengthening of free-running period occurs via PDF. Future studies are expected to reveal which neurons undergo period shortening and which undergo lengthening in response to PDF.

Oscillations of *per* and *tim* mRNA in the circadian pacemaker neurons of *pdf*^{01} flies are found to rapidly dampen under constant darkness (DD), whereas they persisted for at least eight days in wild-type flies, leading to the conclusion that PDF is essential for communication among and between the LN$_v$ and other circadian neurons located in the dorsal protocerebrum (Peng *et al*. 2003). This idea was further strengthened by the findings of a study showing that cellular localization of PER protein in the s-LN$_v$ and LN$_d$ are kept in tight synchrony among and within the members of these two neuronal groups by the PDF neuropeptide (Lin *et al*. 2004).

Almost a decade after the discovery of PDF as an essential circadian neuropeptide, studies by three independent research groups identified a class II peptide G-protein coupled receptor variously denoted as Han, groom-of-pdf, and PDFR (Hyun *et al*. 2005; Lear *et al*. 2005a; Mertens *et al*. 2005) as the receptor of PDF. Yet the *in vivo* site of the receptor expression (henceforth referred to as PDFR) is not yet fully resolved since the above three studies came to slightly different conclusions. While the results of *in situ* hybridisation suggested that PDFR is expressed in LN$_d$ and some dorsal neurons (Lear *et al*. 2005a), antibodies raised against the N and C terminal amino acids of PDFR suggest different expression patterns. The N-terminus-directed antibody suggests that PDFR is expressed in all l-LN$_v$, one LN$_d$, seven DN1 and one DN3 (Hyun *et al*. 2005) whereas the C-terminus-directed antibody shows the presence of immunoreactive puncta around all three lateral neuron groups: s-LN$_v$, l-LN$_v$ and LN$_d$, although only two DN1 and three DN3 cell bodies are labelled. More recently, another study used a novel method of genet-

ically encoded sensors of cAMP levels to localize the cells on which PDF may act (Shafer *et al*. 2008). This study detected PDF neuropeptide-induced increase in cAMP levels in all non-PDF expressing groups (LN_d, DN1, DN2 and $DN3_a$) as well as s-LN_v (but not l-LN_v) and showed that this effect is mediated via PDFR. Taken together these studies suggest that the neuropeptide PDF may feedback on to the neurons that produce it in addition to acting on other members of the circadian pacemaker circuit. Such feedback mechanism has been shown to exist in the mammalian circadian pacemaker the suprachiasmatic nucleus (SCN), where more than half of the neurons which produce the output molecule vasoactive intestinal polypeptide (VIP) also express its receptor $VPAC_2$ (Kalamatianos *et al*. 2004).

Recent studies reveal that the $DN1_a$ subgroup expresses the peptide IPNamide that is a product of the gene *neuropeptide-like-precursor1* (Shafer *et al*. 2006), and that in male flies a subset of the LN_d neurons express neuropeptide F (Lee *et al*. 2006), thus giving rise to the possibility of two more candidate circadian neurotransmitters. Another study showed that subsets of DN1 and DN3 neurons in the adult brain express vesicular glutamate transporters and that both types of LN_v express the metabotropic glutamate receptor type A (Hamasaka *et al*. 2007). Using calcium imaging of dissociated s-LN_v in culture, Hamasaka and colleagues showed that glutamate provides inhibitory signals to the s-LN_v making glutamate a likely candidate for communication between a subset of the dorsal neuronal groups and the s-LN_v.

A combination of site directed expression of GFP along with antibodies against biogenic amines serotonin, histamine and dopamine (tyrosine hydroxylase was targeted) showed that these three monoamines are likely to play a role in the circadian circuit of adult flies (Hamasaka and Nassel 2006). Serotonin immunoreactivity was seen in neurites that are adjacent to dendrites and axonal terminals of s-LN_v. The l-LN_v terminals in the medulla of adults also overlap with serotonin inmmunoreactive neurites. The dendrites of the Hofbauer–Buchner eyelets (larval photoreceptors that persist in the adult) are histaminergic and terminate in the aMe and overlap with dendritic arbors of both LN_v. Tyrosine hydroxylase immunoreactivity is seen around the dorsal terminals of the s-LN_v and the medullary terminals of the l-LN_v. Thus all three monoamines studied are found in the vicinity of neurites of circadian pacemaker cells and not in the cell bodies. Another study suggests that GABA (γ amino butyric acid) is an input molecule through which s-LN_v perceive slow inhibitory stimuli (Hamasaka *et al*. 2005). This study used calcium imaging of dissociated s-LN_v from larval brain and immunohistochemical methods to detect GABA and one of its receptors ($GABA_B$-R2) on the s-LN_v. Thus researchers have begun to unravel the underlying neurochemical bases of communication among the components of the circadian neuronal circuit.

Electrophysiological properties of pacemaker neurons

We can now distinguish at least eight different neuronal subgroups and the anatomical connections between them in the brain of the adult fly. Further, at least one neurotransmitter and several putative neurotransmitters for both inputs and outputs to and from these neuronal groups are known. Nevertheless, electrophysiological characterization of the different neuronal subgroups and the physiological nature of communication between the various neuronal subgroups resulting in a neuronal circuit that drives behavioral rhythms is still in its infancy. In this section, I summarize our current understanding of this issue.

Among the earliest studies of electrical activity of circadian pacemaker cells, recordings made on basal retinal neurons of the marine molluscs *Aplysia californica* and *Bulla gouldiana* showed that action potential firing frequency oscillates with high firing rate during the day and low frequency during the night (Block 1981; Block and McMahon 1983). Prior to these studies, extracellular recordings in the mammalian hypothalamus had also revealed a day–night oscillation in electrical activity both under light:dark (LD) cycles and in DD (Inouye and Kawamura 1979). Thus, it is generally believed that membrane electrical properties are integral to the functioning of circadian pacemaker cells. Conclusive evidence for this was provided by studies in *D. melanogaster* where the targeted expression of voltage-gated potassium channels in a small subset of pacemaker neurons, the LN_v resulted in hyperpolarisation and consequent electrical silencing of those neurons and a loss of both behavioral and molecular oscillations (Nitabach *et al*. 2002, 2005; figure 2 in Holmes *et al*. 2007). Interestingly, when synaptic transmission is blocked in the circadian pacemaker neurons by targeted expression of tetanus toxin light chain there is no effect on the molecular oscillations of circadian proteins while behavioral rhythms under DD are severely affected (Kaneko *et al*. 2000; Nitabach *et al*. 2005). This suggests that the persistence of circadian molecular oscillation is a cell-autonomous property of LN_v.

The electrophysiological characterization of *Drosophila* circadian pacemaker neurons *in vivo* has thus far been mainly limited to the l-LN_v and to a smaller extent to the s-LN_v (Park and Griffith 2006; Holmes *et al*. 2007; Sheeba *et al*. 2008a–d; Cao and Nitabach 2008). These studies showed that like mammalian and molluscan pacemaker neurons, the LN_v also show a diurnal variation in the ability to fire action potentials, firing frequency and resting membrane potential (RMP, Park and Griffith 2006; Cao and Nitabach 2008; Sheeba *et al*. 2008b&d). The pattern of l-LN_v spontaneous action potential firing shows some distinctive features based on which they are classified into two categories: (i) tonic firing pattern, with a stable RMP and steady action potential firing frequency, and (ii) burst firing pattern, where

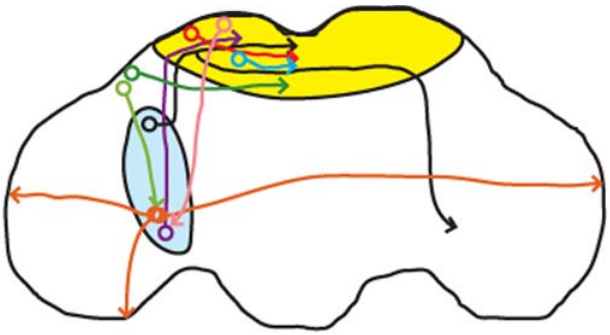

Figure 2. Schematic representation of the putative neuronal circuit in the brain of adult *D. melanogaster*. The two brain areas which appear to be the centres of communication between members of the neuronal circuit the dorsal protocerebrum (shaded yellow) and the accessory medulla (aMe, shaded blue) are the regions where maximum overlap between axonal projections and/or dendritic projections from the different members of the pacemaker circuit occur. The dorsal protocerebrum is the site of several hormonal release centres such as the pars intercerebralis (PI) while the aMe has been shown in other insects to be the region for communication between the two brain hemispheres suggesting similar function in *D. melanogaster*. Putative axonal termini are shown as arrows and putative dendritic regions are shown by circles.

membrane potential shows a low amplitude, low frequency oscillation, with bursts of action potentials riding the crest of the oscillation. The spontaneous firing of action potentials and slow wave oscillations in RMP are found to be dependent on voltage gated sodium channels (Sheeba *et al*. 2008b). Two sizes of action potentials one with large amplitude originating ipsilaterally and another with smaller amplitude originating from the contralateral lobe have been reported from patch clamp recordings of the l-LN_v (Cao and Nitabach 2008). As with the cockroach where circadian clocks in aMe of the bilateral brain hemispheres were found to be synchronized via gap junctions (Schneider and Stengl 2006), so also in adult *D. melanogaster*, gap junctions are likely to modulate membrane excitability since application of gap junction blockers reduces firing frequency of both large and small size action potentials (Cao and Nitabach 2008). Under LD, the l-LN_v show maximum membrane depolarization and highest rate of firing immediately after lights-on. This is followed by a decrease in RMP and firing frequency as the day progresses which remains low during the early part of night followed by a gradual increase as dawn approaches. This diurnal oscillation in membrane properties was lost in per^{01} mutant l-LN_v, suggesting the involvement of circadian proteins in the regulation of membrane electrophysiological properties. The s-LN_v show similarity with l-LN_v in that they have highest RMP around lights on (Cao and Nitabach 2008). Cao and Nitabach (2008) did not detect a circadian oscillation in membrane electrical properties on the first day in DD. Recordings of l-LN_v after long term exposure to DD (day 14) at four circadian phases based on the locomotor activity of individual flies showed oscillation in both RMP and firing frequency (Sheeba *et al*. 2008b). This may reflect the dependence of circadian oscillation in membrane electrical activity on the molecular oscillator encoded by the TTLs as *tim* mRNA oscillations are known to re-emerge in the l-LN_v upon prolonged exposure to DD (Peng *et al*. 2003).

Both RMP and firing frequency of the l-LN_v neurons respond to light. When light intensity is increased from 0 to 5-7 klux the RMP was depolarized and firing frequency increased (Sheeba *et al*. 2008b). This sensitivity to light occurs via the circadian photoreceptor CRYPTOCHROME (CRY) as evidenced by a partial loss of light sensitivity of l-LN_v in the hypomorphic mutant cry^b. Significantly lower change in RMP and firing frequency is seen in cry^b l-LN_v compared to controls in response to a 5-7-klux increase in light intensity.

Very little is known about the ion channels that are endogenous to the circadian pacemaker neurons in *Drosophila*. A putative ion channel gene *narrow abdomen (na)* has been shown to play a role in regulating membrane activity of circadian pacemaker neurons (Lear *et al*. 2005b). *na* mutants show weak rhythms in DD and elevated daytime activity under LD cycles. However, oscillation of circadian proteins in pacemaker neurons is intact suggesting a role for this putative ion channel in output pathways from the central clock machinery (Lear *et al*. 2005b). A recent study suggests a role for a potassium channel *Shaw*, mostly in the dorsal subsets of the circuit (Hodge and Stanewsky 2008). This study finds that increase in *Shaw* expression in dorsal neurons including the LN_d causes increased nocturnal activity and arrhythmia in DD, but there is no effect on the molecular oscillations of clock proteins. On the other hand, when *Shaw* expression is repressed in DN groups alone, then activity/rest rhythm free runs in DD with a long period. Importantly *Shaw* expression is crucial for the rhythmic accumulation of PDF in the dorsal terminals of s-LN_v. The sodium channel *para* is also suggested to be involved in the phasing between the circadian molecular clock and PDF release by the LN_v based on a study that used a tethered toxin to inactivate the *para* channel (Wu *et al*. 2008a). *para* inactivation caused phase advance in behavioral rhythms in LD, and arrhythmicity in DD. Further, the circadian molecular oscillator in various nontargeted pacemaker neurons were found to be phase advanced, and this was attributed to the advance in release of PDF from terminals of the LN_v. Further studies that unravel the other key endogenous channels that regulate the resting membrane potential, firing frequency and pattern of firing in the various neurons of the pacemaker circuit will enable a better understanding of how the circuit functions.

Circadian pacemaker neurons form a neuronal circuit

In the recent past, several studies using different genetic tools have shown that manipulation of circadian oscillation in a subset of the pacemaker neurons affects circadian oscillations even in nontarget pacemaker neurons (Peng *et al*. 2003;

Grima *et al*. 2004; Stoleru *et al*. 2004; Murad *et al*. 2007) suggesting that circadian pacemaker neurons form an integrated circuit. Evidence for the influence of membrane electrical activity of a small subset of the circadian pacemaker circuit on the other pacemaker neurons was obtained by ectopically expressing a voltage-gated sodium channel (NaChBac) in the PDF-expressing LN_v subgroups. This causes profound changes in the electrical activity of the membrane as revealed by whole-cell patch clamp recordings of the l-LN_v (Sheeba *et al*. 2008c). The locomotor activity/rest behaviour of these transgenic flies showed very distinct patterns such as the emergence of multiple bouts of activity free running under DD with distinct free running periodicities (Nitabach *et al*. 2006; Sheeba *et al*. 2008c), as well as increased nocturnal activity and consequent loss of sleep at night under LD cycles (Sheeba *et al*. 2008d). In the presence of NaChBac the cell membrane of l-LN_v exhibit high amplitude action potentials resulting in greater depolarization of the membrane (Sheeba *et al*. 2008c&d) and constitutive expression of the neuropeptide PDF in the terminals of s-LN_v under DD (Nitabach *et al*. 2006). Further, the behavioral abnormalities were accompanied by corresponding changes in molecular oscillations both in the target neurons and in those pacemaker neurons that were themselves not expressing the NaChBac channel. When introduced into DD, initially (for up to five days), these flies are arrhythmic. This is also reflected in the lack of synchrony in oscillation of PER and PDP1 both of which are considered as molecular markers of circadian oscillation (Nitabach *et al*. 2006). After five days, the locomotor activity starts to get consolidated such that one bout of activity free-runs with a period shorter than 24 h, while a second bout free-runs with a period longer than 24 h. When these two bouts of activity were maximally apart, the state of the molecular oscillation in the different neuronal groups are found to have re-grouped such that s-LN_v peak coincides with the faster running activity bout while the LN_d and 5th s-LN_v peaks are in synchrony with each other and with the slower running activity bout. The DN1 and DN2 show two peaks per cycle coinciding with the two activity bouts. Whole-cell current clamp recordings made on the l-LN_v after prolonged exposure to DD and appearance of the novel behavioral rhythms revealed no change in terms of the RMP or firing frequency or the pattern of firing compared to recordings made at earlier stages. Thus there is no evidence for cell autonomous homeostatic compensation for the changes induced by NaChBac in the LN_v as the firing properties of the l-LN_v are not altered after long-term exposure to DD. Instead, the study concluded that the circadian pacemaker circuit is capable of circuit wide homeostatic compensation in response to perturbations in a small part of the circuit as evidenced by the emergence of a novel pattern of synchrony in molecular oscillations among the members of the circuit and a corresponding emergence of novel pattern of behavioral rhythmicity.

Electrical silencing of the LN_v by targeted expression of Kir2.1 a voltage gated potassium channel also resulted in disruption of circadian molecular oscillation in other neurons of the circuit (Wu *et al*. 2008b). While the s-LN_v are themselves hyperpolarized and show loss of PDP1 oscillation, other pacemaker neurons that are likely to receive inputs from the s-LN_v (either directly through synapses or through neurotransmitters) such as the LN_d, DN1 and DN2 show significantly phase advanced oscillation in PDP1 expression. Thus we now have multiple lines of evidence for interdependence among various neuronal subgroups of circadian pacemaker circuit for normal expression of circadian rhythms.

Another approach to understand the effect of pacemaker neuron membrane electrical activity on circadian function has been via the study of mutants of ion channel genes. One such study using mutants of the ion channel gene *slowpoke (slo)*, which encodes a calcium activated potassium channel reveals that multiple aspects of the circadian pacemaker circuit function are affected (Fernández *et al*. 2007). Fernández and colleagues report that although SLO is not expressed within the neurons of any known circadian pacemaker neurons the SLO positive neurites lie in close proximity to DN1 and s-LN_v axonal terminals in the dorsal protocerebrum as well as the l-LN_v axonal terminals in the optic lobe. The mutant slo^4 is arrhythmic in DD but PER and TIM oscillations persist in s-LN_v. On the other hand axonal projections of s-LN_v show abnormal terminal arborization and a loss of circadian oscillation in PDF levels. Further, subcellular localization of TIM in the DN1 and LN_d subsets is also disrupted in slo^4 mutants thus providing one more piece of evidence for a neuronal circuit operating between s-LN_v and the dorsal neurons DN1 and LN_d via PDF as was previously suggested (Peng *et al*. 2003; Lin *et al*. 2004).

The circadian pacemaker circuit of the adult fly exhibits membrane electrical activity dependent plasticity in function based on behaviour, underlying molecular oscillators and neuroanatomical markers. Thus the *Drosophila* circadian pacemaker circuit with its relatively small number of neurons, availability of molecular markers of clock function and a robust behavioral read-out offers the opportunity to better understand the mechanisms through which neuronal circuits function. Future studies aimed at characterization of electrophysiological properties of all the known neuronal groups should reveal how different pacemaker neurons interact with each other to function as a circuit. Investigations of the neurochemical basis of inhibitory and excitatory signals between the pacemaker neurons will enable us to derive more accurate models of the underlying neuronal circuit. Identification of the ion channels involved in regulating the electrical activity of these pacemaker neurons may help in devising genetic means to override rhythm defects and thus provide clues to treat circadian rhythm disorders in more complex circuits.

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References

Berni J., Beckwith E. J., Fernández M. P. and Ceriani M. F. 2008 The axon-guidance *roundabout* gene alters the pace of the *Drosophila* circadian clock. *Eur. J. Neurosci*. **27**, 396–407.

Block G. D. 1981 *In vivo* recording of the ocular circadian rhythm in *Aplysia. Brain. Res*. **222**, 138–143.

Block G. D. and McMahon D. G. 1983 Localized illumination of the *Aplysia* and *Bulla* eye reveals new relationships between retinal layers. *Brain Res*. **265**, 134–137.

Busza A., Murad A. and Emery P. 2007 Interactions between circadian neurons control temperature synchronization of *Drosophila* behaviour. *J. Neurosci*. **27**, 10722–10733.

Cao G. and Nitabach M. N. 2008 Circadian control of membrane excitability in *Drosophila melanogaster* lateral ventral clock neurons. *J. Neurosci*. **28**, 6493–6501.

Fernández M. P., Chu J., Villella A., Atkinson N., Kay S. A. and Ceriani M. F. 2007 Impaired clock output by altered connectivity in the circadian network. *Proc. Natl. Acad. Sci. USA* **104**, 5650–5655.

Fernández M. P., Berni J. and Ceriani M. F. 2008 Circadian remodelling of neuronal circuits involved in rhythmic behavior. *PLoS Biol*. **6**, e69.

Giebultowicz J. M. 2001 Peripheral clocks and their role in circadian timing: insights from insects. *Philos. Trans. R. Soc. Lond. Ser. B* **356**, 1791–1799.

Grima B., Chelot E., Xia R. and Rouyer F. 2004 Morning and evening peaks of activity rely on different clock neurons of the *Drosophila* brain. *Nature* **431**, 869–873.

Hamasaka Y. and Nassel D. R. 2006 Mapping of serotonin, dopamine, and histamine in relation to different clock neurons in the brain of *Drosophila. J. Comp. Neurol*. **10**, 314–330.

Hamasaka Y., Wegener C. and Nassel D. R. 2005 GABA modulates *Drosophila* circadian clock neurons via GABA-B receptors and decreases in calcium. *J. Neurobiol*. **65**, 225–240.

Hamasaka Y., Rieger D., Parmentier M. L., Grau Y., Helfrich-Forster C. and Nassel D. R. 2007 Glutamate and its metabotropic receptor in *Drosophila* clock neuron circuits. *J. Comp. Neurol*. **505**, 32–45.

Hardin P. E. 2005 The circadian timekeeping system of *Drosophila. Curr. Biol*. **15**, R714–R722.

Helfrich-Förster C. 1995 The period clock gene is expressed in central nervous system neurons which also produce a neuropeptide that reveals the projections of circadian pacemaker cells within the brain of *Drosophila melanogaster. Proc. Natl. Acad. Sci. USA* **92**, 612–616.

Helfrich-Förster C. 1997 Development of pigment-dispersing hormone-immunoreactive neurons in the nervous system of *Drosophila melanogaster. J. Comp. Neurol*. **380**, 335–354.

Helfrich-Förster C. 1998 Robust circadian rhythmicity of *Drosophila melanogaster* requires the presence of lateral neurons: a brain-behavioral study of disconnected mutants. *J. Comp. Physiol. A* **182**, 435–453.

Helfrich-Förster C. 2003 The neuroarchitecture of the circadian clock in the brain of *Drosophila melanogaster. Microsc. Res. Tech*. **62**, 94–102.

Helfrich-Förster C. 2005 Organization of endogenous clocks in insects. *Biochem. Soc. Trans*. **33**, 957–961.

Helfrich-Förster C. and Homberg U. 1993 Pigment-dispersing hormone-immunoreactive neurons in the nervous system of wild-type *Drosophila melanogaster* and of several mutants with altered circadian rhythmicity. *J. Comp. Neurol*. **337**, 177–190.

Helfrich-Förster C., Stengl M. and Homberg U. 1998 Organization of the circadian system in insects. *Chronobiol. Intl*. **15**, 567–594.

Helfrich-Förster C., Tauber M., Park J. H., Muhlig-Versen M., Schneuwly S. and Hofbauer A. 2000 Ectopic expression of the neuropeptide pigment-dispersing factor alters behavioral rhythms in *Drosophila melanogaster. J. Neurosci*. **20**, 3339–3353.

Helfrich-Förster C., Shafer O. T., Wulbeck C., Grieshaber E., Rieger D. and Taghert P. 2007 Development and morphology of the clock-gene-expressing lateral neurons of *Drosophila melanogaster. J. Comp. Neurol*. **500**, 47–70.

Hodge J. J. and Stanewsky R. 2008 Function of the Shaw potassium channel within the *Drosophila* circadian clock. *PLoS ONE* **3**, e2274.

Holmes T. C., Sheeba V., Mizrak D., Rubovszky B. and Dahdal D. 2007 Circuit breaking and behavioral analysis by molecular genetic manipulation of neural activity in *Drosophila*. In *Invertebrate neurobiology* (ed. G. North and R. Greenspan), pp. 19–52. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Homberg U., Reischig T. and Stengl M. 2003 Neural organization of the circadian system of the cockroach *Leucophaea maderae. Chronobiol. Intl*. **20**, 577–591.

Howlader G. and Sharma V. K. 2006 Circadian regulation of egg-laying behavior in fruit flies *Drosophila melanogaster. J. Insect Physiol*. **52**, 779–785.

Hyun S., Lee Y., Hong S. T., Bang S., Paik D., Kang J. *et al*. 2005 *Drosophila* GPCR Han is a receptor for the circadian clock neuropeptide PDF. *Neuron* **48**, 267–278.

Inouye S. T. and Kawamura H. 1979 Persistence of circadian rhythmicity in a mammalian hypothalamic "island" containing the suprachiasmatic nucleus. *Proc. Natl. Acad. Sci. USA* **76**, 5962–5966.

Kalamatianos T., Kallo I., Piggins H. D. and Coen C. W. 2004 Expression of VIP and/or PACAP receptor mRNA in peptide synthesizing cells within the suprachiasmatic nucleus of the rat and in its efferent target sites. *J. Comp. Neurol*. **475**, 19–35.

Kaneko M. and Hall J. C. 2000 Neuroanatomy of cells expressing clock genes in *Drosophila*: transgenic manipulation of the *period* and *timeless* genes to mark the perikarya of circadian pacemaker neurons and their projections. *J. Comp. Neurol*. **422**, 66–94.

Kaneko M., Helfrich-Forster C. and Hall J. C. 1997 Spatial and temporal expression of the *period* and *timeless* genes in the developing nervous system of *Drosophila*: newly identified pacemaker candidates and novel features of clock gene product cycling. *J. Neurosci*. **17**, 6745–6760.

Kaneko M., Park J. H., Cheng Y., Hardin P. E. and Hall J. C. 2000 Disruption of synaptic transmission or clock-gene-product oscillations in circadian pacemaker cells of *Drosophila* cause abnormal behavioral rhythms. *J. Neurobiol*. **43**, 207–233.

Klarsfeld A., Malpel S., Michard-Vanhee C., Picot M., Chelot E. and Rouyer F. 2004 Novel features of *cryptochrome*-mediated photoreception in the brain circadian clock of *Drosophila. J. Neurosci*. **24**, 1468–1477.

Lear B. C., Merrill C. E., Lin J. M., Schroeder A., Zhang L. and Allada R. 2005a A G protein-coupled receptor, groom-of-PDF, is required for PDF neuron action in circadian behavior. *Neuron* **48**, 221–227.

Lear B. C., Lin J. M., Keath J. R., McGill J. J., Raman I. M. and Allada R. 2005b The ion channel narrow abdomen is critical for neural output of the *Drosophila* circadian pacemaker. *Neuron* **48**, 965–976.

Lee G., Bahn J. H. and Park J. H. 2006 Sex- and clock-controlled expression of the neuropeptide F gene in *Drosophila. Proc. Natl. Acad. Sci. USA* **103**, 12580–12585.

Lin Y., Stormo G. D. and Taghert P. H. 2004 The neuropeptide pigment-dispersing factor coordinates pacemaker interactions in the *Drosophila* circadian system. *J. Neurosci*. **24**, 7951–7957.

Mertens I., Vandingenen A., Johnson E. C., Shafer O. T., Li W., Trigg J. S. *et al*. 2005 PDF receptor signalling in *Drosophila* contributes to both circadian and geotactic behaviours. *Neuron* **48**, 213–219.

Miyasako Y., Umezaki Y. and Tomioka K. 2007 Separate sets of cerebral clock neurons are responsible for light and temperature entrainment of *Drosophila* circadian locomotor rhythms. *J. Biol. Rhythms* **22**, 115–126.

Murad A., Emery-Le M. and Emery P. 2007 A subset of dorsal neurons modulates circadian behavior and light responses in *Drosophila. Neuron* **53**, 689–701.

Myers E. M., Yu J. and Sehgal A. 2003 Circadian control of eclosion: interaction between a central and peripheral clock in *Drosophila melanogaster. Curr. Biol*. **13**, 526–533.

Nitabach M. N., Blau J. and Holmes T. C. 2002 Electrical silencing of *Drosophila* pacemaker neurons stops the free-running circadian clock. *Cell* **109**, 485–495.

Nitabach M. N., Sheeba V., Vera D. A., Blau J. and Holmes T. C. 2005 Membrane electrical excitability is necessary for the free-running larval *Drosophila* circadian clock. *J. Neurobiol*. **62**, 1–13.

Nitabach M. N., Wu Y., Sheeba V., Lemon W. C., Strumbos J., Zelensky P. K. *et al*. 2006 Electrical hyperexcitation of lateral ventral pacemaker neurons desynchronizes downstream circadian oscillators in the fly circadian circuit and induces multiple behavioral periods. *J. Neurosci*. **26**, 479–489.

Park D. and Griffith L. C. 2006 Electrophysiological and anatomical characterization of PDF-positive clock neurons in the intact adult *Drosophila* brain. *J. Neurophysiol*. **95**, 3955–3960.

Park J. H., Helfrich-Förster C., Lee G., Liu L., Rosbash M. and Hall J. C. 2000 Differential regulation of circadian pacemaker output by separate clock genes in *Drosophila. Proc. Natl. Acad. Sci. USA* **97**, 3608–3613.

Peng Y., Stoleru D., Levine J. D., Hall J. C. and Rosbash M. 2003 *Drosophila* free-running rhythms require intercellular communication. *PLoS Biol*. **1**, e13.

Plautz J. D., Kaneko M., Hall J. C. and Kay S. A. 1997 Independent photoreceptive circadian clocks throughout *Drosophila. Science* **278**, 1632–1635.

Rao K. R., Riehm J. P., Zahnow C. A., Kleinholz L. H., Tarr G. E., Johnson L. *et al*. 1985 Characterization of a pigment-dispersing hormone in eyestalks of the fiddler crab *Uca pugilator. Proc. Natl. Acad. Sci. USA* **82**, 5319–5322.

Renn S. C., Park J. H., Rosbash M., Hall J. C. and Taghert P. H. 1999 A *pdf* neuropeptide gene mutation and ablation of PDF neurons each cause severe abnormalities of behavioral circadian rhythms in *Drosophila. Cell* **99**, 791–802.

Rosato E. and Kyriacou C. P. 2006 Analysis of locomotor activity rhythms in *Drosophila. Nat. Protoc*. **1**, 559–568.

Sakai T. and Ishida N. 2001 Time, love and species. *Neuro. Endocrinol. Lett*. **22**, 222–228.

Schneider N. L. and Stengl M. 2006 Gap junctions between accessory medulla neurons appear to synchronize circadian clock cells of the cockroach *Leucophaea maderae. J. Neurophysiol*. **95**, 1996–2002.

Shafer O. T., Helfrich-Förster C., Renn S. C. and Taghert P. H. 2006 Re-evaluation of *Drosophila melanogaster's* neuronal circadian pacemakers reveals new neuronal classes. *J. Comp. Neurol*. **498**, 180–193.

Shafer O. T., Kim D. J., Dunbar-Yaffe R., Nikolaev V. O., Lohse M. J. and Taghert P. H. 2008 Widespread receptivity to neuropeptide PDF throughout the neuronal circadian clock network of *Drosophila* revealed by real-time cyclic AMP imaging. *Neuron* **58**, 223–237.

Sheeba V., Kaneko M., Sharma V. K. and Holmes T. C. 2008a The *Drosophila* circadian pacemaker circuit: Pas de Deux or Tarantella? *Crit. Rev. Biochem. Mol. Biol*. **43**, 37–61.

Sheeba V., Gu H., Sharma V. K., O'Dowd D. K. and Holmes T. C. 2008b Circadian- and light-dependent regulation of resting membrane potential and spontaneous action potential firing of *Drosophila* circadian pacemaker neurons. *J. Neurophysiol*. **99**, 976–988.

Sheeba V., Sharma V. K., Gu H., Chou Y. T., O'Dowd D. K. and Holmes T. C. 2008c Pigment dispersing factor-dependent and -independent circadian locomotor behavioral rhythms. *J. Neurosci*. **28**, 217–227.

Sheeba V., Fogle K.J., Kaneko M., Rashid S., Chou Y. T., Sharma V. K. and Holmes T. C. 2008d Large ventral lateral neurons modulate arousal and sleep in *Drosophila. Curr. Biol*. **18**, 1537–1545.

Shiga S. 2003 Anatomy and functions of brain neurosecretory cells in Diptera. *Microsc. Res. Tech*. **62**, 114–131.

Stoleru D., Peng Y., Agosto J. and Rosbash M. 2004 Coupled oscillators control morning and evening locomotor behaviour of *Drosophila. Nature* **431**, 862–868.

Suh J. and Jackson F. R. 2007 *Drosophila* ebony activity is required in glia for the circadian regulation of locomotor activity. *Neuron* **55**, 435–447.

Tomioka K., Miyasako Y. and Umezaki Y. 2008 PDF as a coupling mediator between the light-entrainable and temperature-entrainable clocks in *Drosophila melanogaster. Acta. Biol. Hung*. **59**, suppl. 149–155.

Wu Y., Cao G., Pavlicek B., Luo X. and Nitabach M. N. 2008a Phase coupling of a circadian neuropeptide with rest/activity rhythms detected using a membrane-tethered spider toxin. *PLoS Biol*. **6**, e273.

Wu Y., Cao G. and Nitabach M. N. 2008b Electrical silencing of PDF neurons advances the phase of non-PDF clock neurons in *Drosophila. J. Biol. Rhythms* **23**, 117–128.

Wulbeck C., Grieshaber E. and Helfrich-Forster C. 2008 Pigment-dispersing factor (PDF) has different effects on *Drosophila's* circadian clocks in the accessory medulla and in the dorsal brain. *J. Biol. Rhythms* **23**, 409–424.

Zheng X. and Sehgal A. 2008 Probing the relative importance of molecular oscillations in the circadian clock. *Genetics* **178**, 1147–1155.

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