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Title: Decoding *Drosophila* circadian pacemaker circuit

Anatoly Kozlov[1] and Emi Nagoshi[1]*

[1]Department of Genetics and Evolution, University of Geneva, Sciences III, 30 Quai Ernest-Ansermet, CH-1211, Geneva-4, Switzerland

*Corresponding author (e-mail: Emi.Nagoshi@unige.ch)

Abstract

Drosophila circadian circuit is one of the best described neural circuits but is complex enough to obscure our understanding of how it actually works. Animals' rhythmic behavior, the seemingly simple outcome of their internal clocks, relies on the interaction of heterogeneous clock neurons that are spread across the brain. Direct observations of their coordinated network interactions can bring us forward in understanding the circuit. The current challenge is to observe activity of each of these neurons over a long span of time –hours to days– in live animals. Here we review the progress in circadian circuit interrogation powered by *in vivo* calcium imaging.

Circadian pacemaker circuit

Circadian rhythms drive organism's intrinsic capacity to anticipate daily and seasonal changes of the environment and to accordingly adapt their behavior and physiology. Circadian clocks, present in most species from protozoa to mammals, are built using a conserved principle of transcriptional-translational feedback loops (TTFL) [1, 2]. Molecular studies of circadian clocks were pioneered using *Drosophila melanogaster* [3–5] and continue to bring new discoveries to this day. Major players of *Drosophila* TTFL are transcriptional activators CLOCK

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(CLK) and CYCLE (CYC) and two genes encoding repressors, *period* (*per*) and *timeless* (*tim*). CLK/CYC heterodimer activates the expression of *per* and *tim*. Following transcription, they undergo a series of post-transcriptional regulations and phosphorylation, which allow PER and TIM to accumulate in the cytoplasm with a delay of approximately 6-8 h after the transcription, then dimerize and enter the nucleus and suppress the transcriptional activity of CLK/CYC. CLK and CYC also activate transcription of genes encoding the basic-zipper regulators PAR DOMAIN PROTEIN 1 $\mathcal{E}$ (PDP-1$\mathcal{E}$) and VRILLE (VRI), which activates and inhibits *Clk* gene expression, respectively. These interlocked positive and negative feedback loops stabilize the main loop and ensure the generation of 24-h rhythms [2]. The molecular clock rhythmically controls the expression of a large number of genes and ultimately produces rhythms in diverse cellular functions, including neuronal activity [6].

Drosophila also offers a proven, powerful tool to study neuronal mechanism of circadian behavior. *Drosophila melanogaster* belongs to the class of crepuscular animals, which are physically active at dawn and dusk. In the laboratory conditions, this feature is observed using automatic recording of infrared beam crossing of single flies. In 12h:12h light-dark cycles (LD), flies show a stereotypic pattern of activity, in which the peaks of activity are aligned with the moment of light/dark transitions in the morning and the evening. Gradual increments of activities precede the light transition, which display the endogenous origin of their behavior controlled by their internal clocks. When flies are released into constant darkness (DD), they free-run with similar bimodal pattern of activity that repeats roughly every 24 hr, showing another evidence of endogenous clocks [7]. Approximately 75 pairs of neurons in the brain containing a full set of clock genes are the pacemaker neurons that coordinate this circadian behavior. Circadian pacemaker neurons are classified into several groups according to their location in the brain: the large- ventral lateral neurons (l-LNvs, four per hemisphere), small-ventral lateral neurons (four s-LNvs that express neuropeptide pigment dispersing factor (PDF) and one PDF-negative 5th s-LNv), dorsal-lateral neurons

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(LNds, 6 per hemisphere) and three groups of dorsal neurons (DN1s (anterior and posterior subgroups, 16 in total), DN2s (two) and DN3 (circa 30)) [8, 9].

Back in the 70s it was proposed that morning and evening activity peaks of crepuscular animals are produced by two coupled but independent morning (M) and evening (E) circadian oscillators, which track dawn and dusk, respectively, throughout the season[10]. It took about 30 years until the physical existence of such oscillators was first demonstrated in flies. The pioneering studies by the groups of M. Rosbash [11] and F. Rouyer [12] independently showed that s-LNvs represent the M-oscillator that drives the morning anticipation and rhythmic locomotor activity in DD. They also identified E-oscillator that drives the evening anticipation activity, which consists of the pacemaker neurons expressing circadian photoreceptor Cryptochrome (CRY) but not PDF. Subsequent studies by many groups have verified the basic principle of this dual-oscillator model and later defined LNds and 5th s-LNv as the main constituents of the E-oscillator [13, 14].

Decoding the pacemaker circuit

Whereas the essence of the dual-oscillator model stands the test of time, comprehension of the circuit operation requires identifying individual components, drawing wiring diagram, describing neurochemistry and the nature of communication between the components. Pursuing these questions, a number of studies have added evidence to the complexity of the pacemaker circuit organization. Several lines of evidence indicate that DN1ps, the posterior subgroup of DN1, contribute to both morning and evening activity peaks [15–18]. DN1ps control morning activity peak downstream of the s-LNvs [15, 18], whereas their role in controlling evening peak runs parallel to the E-oscillator and is regulated by light, which inhibits DN1p output [16]. DN1s make physical contact with the neurosecretory cells in the pars intercerebralis (PI). Signaling from the s-LNvs via DN1s to the PI appears to be one of the multiple routes to circadian locomotor output [17, 19, 20].

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Neurochemistry of the pacemaker circuit adds another dimension in the circuit organizational logic. At least several neuropeptides, including PDF, neuropeptide F (NPF), short neuropeptide F (sNPF) and, ion transport peptide (ITP), and several neurotransmitters, such as glutamate, acetylcholine, glycine [21] and GABA, are expressed within or act on pacemaker neurons (reviewed in [22, 23]). Findings from several studies highlight the importance of neuropeptidergic signaling in the fly circadian pacemaker circuit and its evolutionary significance, as evidenced by the parallel discoveries on the critical roles of neuropeptides in mammalian pacemaker circuit [24–26]. Works with flies in this domain have consistently indicated that all its main functions of the M-oscillator, s-LNvs, are mediated by PDF, as any manipulation altering its production or expression of its cognate receptor leads to the same set of phenotypes: the advance of the evening peak in LD, absence of the morning anticipation and very low rhythmicity in DD [27–32]. Manipulation of the s-LNvs neuronal excitability, which presumably controls the rate of PDF secretion, leads to the phenotypes similar to those induced by up or down regulation of PDF signaling [33–37]. sNPF is another neuropeptide expressed in the s-LNvs, as well as in the LNds and l-LNvs [38, 39]. LNds additionally produce NPF and ITP [9, 35]. The expanding panel of neuropeptides found within the pacemaker circuit and the diversity of neuropeptide function in general pose further challenges.

A breakthrough came in 2016 when the group of P. Taghert recorded for the first time the Ca^{2+} dynamics of individual groups of pacemaker neurons over 24 h in head-fixed live flies [40, 41]. Intracellular Ca^{2+} levels, a convenient surrogate of neural activity, provides the critical information to decipher the functional interaction among pacemaker neuron subgroups. This was made possible by the use of genetically encoded Ca^{2+} sensor GCaMP and the Objective-Coupled Planar Illumination microscopy (OCPI), a type of light sheet microscopy, which overcomes the limitations of the standard confocal or two-photon microscopes in temporal resolution, sectioning, size of detection field and photobleaching [42, 43].

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How does the information flow through the pacemaker circuit, whose intrinsic rhythmicity is genetically underpinned? How do the environmental (light) stimuli transform the information to shape the behavioral output? The group addressed these questions in three consecutive studies [20, 40, 41]. The first, perhaps unexpected, observation was that each pacemaker subgroup produces circadian Ca^{2+} wave that peaks at a distinct temporal window. This is strikingly different from the ticking of their molecular clocks, which are roughly in-phase in normal conditions [31, 44, 45]. The timing of the Ca^{2+} peak of the s-and l-LNvs was in a rough accordance with electrophysiological measurements shown in prior works [46–48], validating their experimental setup. Ca^{2+} waves of the s-LNvs (M-oscillator) and LNds (E-oscillator) keep track of the morning and evening light transitions whether it is in the standard 12h: 12h LD cycles or in the photoperiod mimicking the short-day, winter condition (8h: 16h LD) or the long-day, summer condition (16h: 8h LD). This property of Ca^{2+} activity patterns of the M- and E- oscillators is concordant with the morning and evening behavioral peaks. Ca^{2+} rhythms are abolished in the "clockless" per^0 mutants and phase-advanced in the short-period per^s mutants (Fig. 1). Therefore, Ca^{2+} rhythms are generated by the molecular clocks and connect the clocks of the M- and E-oscillators to the behavioral output.

How do the molecular clocks ticking in phase produce Ca^{2+} rhythms that are up to 12 h out of phase? The answer turned out to be the action of two neuropeptides, PDF and sNPF. In the null mutant for Pdf (Pdf^{01}) and severe hypomorphic mutant of PdfR (han^{5304}), Ca^{+2} peaks of the M- and E- oscillators and DN3s coincided around dawn. Bath application of synthetic PDF to Pdf^{01} mutants restored the sequential order of the Ca^{+2} rhythms in the 24-h recordings. The immediate response of PDF bath application at ZT7 (zeitgeber time 7, 7h after lights-on) was a decrease of Ca^2 levels at least in the s-LNVs and LNds. Therefore, PDF/PDFR signaling plays a major role in delaying the Ca^{2+} peaks in the LNds and DN3s by suppressing their Ca^{2+} levels.

Strikingly, neuropeptide signaling not only adjusts the phase but can non-cell-autonomously drive Ca^{2+} rhythms. This is the case in the DN1s, which also display Ca^{2+}

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oscillations in a rough accordance with their membrane excitability [49]. However, their Ca^{2+} rhythms are abolished when sNPF secretion from the s-LNvs and LNds is blocked. As shown in [41], sNPF bath application decreases Ca^{2+} levels in the DN1s, consistent with the notion that sNPF is generally inhibitory [50]. Therefore, analogous to the role of PDF in coordinating Ca^{2+} waves by inhibiting the onset of Ca^{2+} rise, sNPF imposes Ca^{2+} rhythms in the DN1s via an inhibitory mechanism (Fig. 2).

The phase of free-running locomotor activity can be delayed or advanced by a light pulse delivered at early or late night, respectively. *In vivo* Ca^{2+} imaging also helped advance the study of neuronal mechanisms underlying this well-established but poorly understood phenomenon. In the head-fixed flies, phase-delaying light pulses given at ZT17 delayed Ca^{2+} rhythms of all pacemaker neurons within the first day, although it took two more days to re-establish the normal Ca^{2+} phase pattern of the pacemaker network. In contrast, ZT21 phase-advancing light pulses initially phase-advanced only the s-LNvs, and the rest of the pacemaker neurons shifted their Ca^{2+} phase on the second day. The gradual phase advance of non-s-LNv pacemakers may be mediated by PDF released from the s-LNvs, as all pacemaker neurons shifted their Ca^{2+} phases at once in response to phase-advancing pulses in Pdf^{01} flies [41]. These results suggest an intrinsic capacity of all pacemaker neurons to rapidly respond to light stimuli, which is modulated by PDF signaling.

Activities of pacemaker neurons should be relayed to the area controlling locomotion. Extending *in vivo* Ca^{2+} imaging beyond circadian pacemakers, Taghert's group recently identified an output circuit of the M- and E-oscillators [20]. They found that Ring Neurons of the Ellipsoid Body (EB-RNs) and the PPM3-EB subclass of DA neurons presynaptic to the EB-RNs exhibit Ca^2 rhythms with two peaks, coinciding with those of the M - and E- oscillators. The experimental set up in this study included one interesting addition, an infrared detector that monitors leg movements during *in vivo* Ca^{2+}imaging. Leg movements of the head-fixed flies displayed bimodal patterns, which were similar to the locomotor activity of freely moving

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flies and matched the Ca^{2+} activities of the identified output circuit. Together, these results demonstrate that activities of the M- and E-oscillators are relayed by dopamine signaling to the EB-RNs, a known pre-motor center, thereby generating morning and evening peaks of locomotor behavior.

The series of discoveries from *in vivo* Ca^{2+} imaging brought us forward in mechanistic understanding of pacemaker circuit, but not without conundrums. Whereas different experiments in Taghert group's papers have consistently shown that PDF/PDFR signaling reduces Ca^{2} levels in pacemaker neurons, several previous studies demonstrated the elevation in Ca^{2} and/or cAMP levels upon PDF signaling activation [32, 50–54]. PdfR is a G-protein coupled receptor, which generally increases cAMP levels upon activation, leading to depolarization, increased firing rates and elevated Ca^{2} [53]. Nonetheless, two opposing findings are not necessarily a contradiction, as the timescale of these phenomena are quite different, from seconds (in acute pharmacological experiments) to hours (in 24-h recordings). It should be noted that Ca^{2+} levels were recorded from the cell bodies of pacemaker neurons. Ca^{2+} concentration in the cell bodies represents more than just the neuronal activity; it results from the combination of the processes involving membrane calcium channels and intracellular calcium sources. Another point that should not be ignored is the heterogeneity within the neuronal classes. Because of the technical limitation, *in vivo* Ca^{2+} imaging lacked cellular resolution. The DN1s, LNds and DN3s contain subclasses of neurons with different receptivity towards neuronal signals and environment (light and temperature) and produce different pools of neuropeptides/transmitters [9, 31, 55]. This means that some important features of Ca^{2+} activity may not have been detected. Therefore, studies using complementary approaches, such as short-term *in vivo* Ca^{2+} imaging [56], recording of bioluminescence Ca^{2+}-reporter in freely moving flies [57], live-imaging of molecular clockwork in cultured brains using bioluminescent [45] or fluorescent clock gene reporters [58–60], are warranted to fill the knowledge gaps.

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PDF/PDFR signaling triggers multiple downstream processes, which are cell-type-dependent, temporally gated and occur at different time scales. It affects membrane electrical status, calcium levels and molecular clocks. One pathway is through the increase in cAMP levels and protein kinase A (PKA) activity, leading to the increase in the stability of PER and TIM [53, 54, 58]. It was also shown that PDF signaling upregulates *per* transcription in *ex vivo* brain culture [58]. The mechanism of the third downstream effect, inhibition of Ca^{2+} levels, is currently unknown and should entail more complex processes than hyperpolarization. Together with the new finding that sNPF can impose Ca^{2+} rhythms that are independent of the molecular clocks, a large body of work centered on the role of PDF casts further challenges in decoding the pacemaker circuit. Network communication and the molecular clockwork intertwine at different time scales and modulate membrane electrical status and cellular processes; only when we understand each of these components and mechanisms, can we get how flies display seemingly simple two peaks of activity.

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Figure legends

Figure 1. Phases of the Ca^{2+} peaks in different pacemaker groups.

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Peak times of pacemaker neuron subgroups in different experimental conditions, summarized from the results in [40]. LD12:12, LD16:8 and LD8:16 are the profiles of wild-type flies in different photoperiods. PerS, PDFR mut and Rescue indicate the Ca^{2+} peaks in per^{S}, *PdfR* mutants (han^{5304}) and *PdfR* mutants with genetic rescue in 12h:12h LD photoperiod, respectively. Each neuronal group has its distinct peak phase. Ca^{2+} peaks of the M-oscillator (s-LNvs) and E-oscillator (LNds) track morning and evening light-dark transitions, respectively. Long-and short-day photoperiod experiments suggest that circadian pacemaker circuit indeed can work as a seasonal adaptation system. Ca^{2+} rhythms in per^{S} are phase-advanced and have a shorter period. In *pdfR* mutants, LNd and DN3 Ca^{2+} peaks crowd around dawn, in phase with the s-LNvs. Restoration of PDFR re-establishes the proper order of the peaks.

Figure 2. Synchronous circadian pacemakers produce asynchronous Ca^{2+} waves. Molecular clocks in all subgroups tick roughly in sync (arrows in the colored circles indicate PER phase). The s-LNvs enforce delays in Ca^{2+} waves of the LNds and DN3s through PDF-mediated inhibition (orange lines). Similar mechanism shortens the time of their own active phase. The s-LNvs and LNds sequentially secrete sNPF and induce circadian variation of the DN1 Ca^{2+} levels, which are otherwise constantly upregulated. In coordination with PDF, light delays Ca^{2+} rhythms in the LNds (yellow dotted line). Blue dotted line indicates sNPF released from the LNds, orange dotted line indicates sNPF from the s-LNvs. As a consequence, the s-LNvs drive morning anticipation behavior and the LNds induce evening anticipation.

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Figure 1

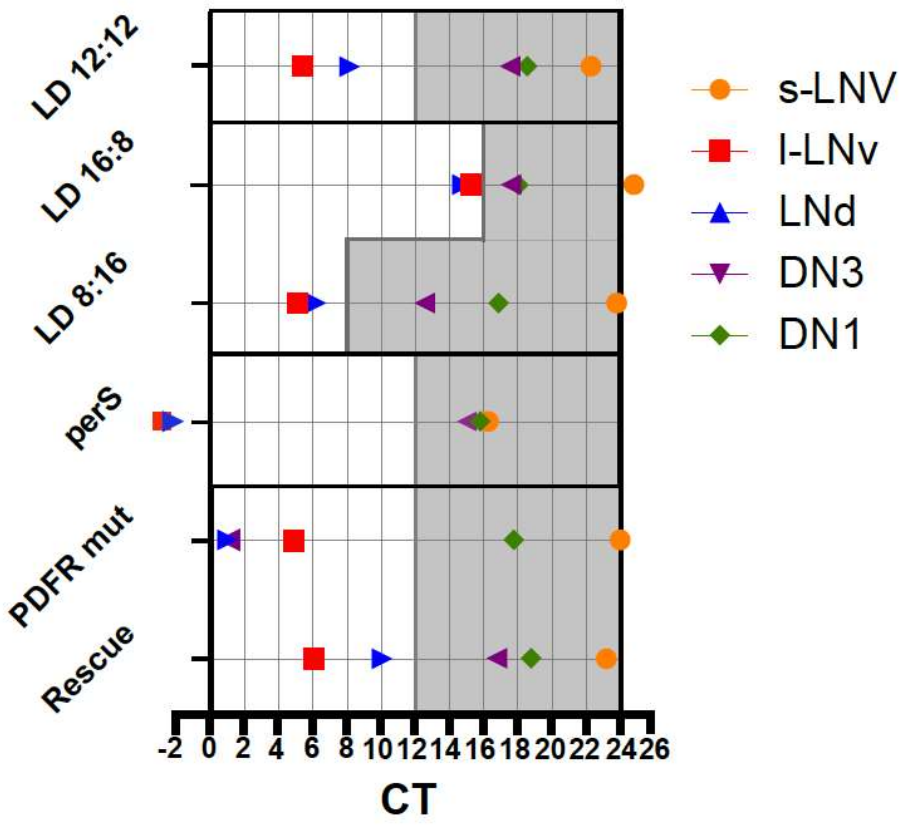

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Figure 2