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Heating and cooling the *Drosophila melanogaster* clock

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Abstract

Most biological phenomena are under control of a circuit known as the ‘molecular circadian clock.’ Over the past forty years of research in *Drosophila melanogaster*, studies have made significant advances in our understanding of the molecular timing mechanism of this circuit, which is determined by a core inhibitory feedback loop. While the timing mechanism of the molecular circadian clock is endogenous, it is well established that exogenous cues such as light and temperature modulate its timing. In the following article, we summarize our current understanding of how temperature interacts with the molecular circadian clock in adult *Drosophila*.

Keywords

circadian rhythm; *Drosophila melanogaster*; limit of rhythmicity; temperature entrainment; temperature compensation

1. Introduction

Most biochemical, physiological, and behavioral phenomena exhibit daily oscillations that are under control of a circadian timing system which functions to synchronize these processes to peak at critical moments of the 24 hr day. The ability to coordinate biological rhythms according to the light:dark transitions of the environment confers a fitness advantage in several species [1–3], which is presumably why the circadian timing system is conserved from bacteria to mammals. In the adult fruit fly (*Drosophila melanogaster*), the circadian network consists of ~150 neurons in the brain that exhibit molecular oscillations of a specialized genetic circuit known as the ‘molecular circadian clock,’ the core mechanism

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of which consists of a negative feedback loop in which ‘clock’ proteins inhibit the transcription of their own mRNAs (Figure 1). In the principal feedback loop, two transcription factors (CLK and CYC) bind to E-boxes upstream of the *period* (*per*) and *timeless* (*tim*) genes and transcribe *per* and *tim* mRNAs. These mRNAs are then translated into proteins, which accumulate in the cytoplasm, form heterodimer complexes, and translocate into the nucleus. Phosphorylation plays a central role in the accumulation of these proteins as well as their nuclear transfer. Once PER-TIM complexes translocate into the nucleus, they bind to CLK-CYC, inhibiting their binding affinity for the *per* and *tim* E-boxes, thereby negatively regulating the transcription of their own mRNAs. The negative inhibition is removed once other proteins degrade the PER-TIM complexes, allowing the cycle to begin again. For a detailed review of the molecular circadian clock in *Drosophila*, refer to Zheng and Sehgal [4].

While the molecular circadian cycle is an endogenous oscillator, meaning that the internal timing mechanism persists without input from the environment, it can be modulated by external cues such as light and temperature. Over the recent years, a good understanding of the molecular circadian clock’s response to light has emerged: in adult *Drosophila*, multiple photosensitive pathways converge onto clock neurons in which the photoreceptor CRYPTOCHROME (CRY) mediates the interaction between light and the molecular clock [5–7]. However, in contrast to what is known about light, little is understood about how temperature modulates the clock. This is interesting considering that temperature is also important for how the molecular clock adapts to seasonal changes in the environment [8–10]. In the following article, we review the three ways in which the molecular clock in adult *Drosophila* responds to temperature when light conditions are held constant: 1) the molecular clock entrains to heat pulses or temperature cycles, 2) the molecular clock compensates for changes in temperature to maintain circadian periodicity, 3) the molecular clock dampens under low temperature stress. In discussing how the molecular clock responds to these temperature cues, we will also review what is known about the input pathways that sense and relay temperature information to clock neurons. To date, we do not fully understand how the clock neurons transmit signals to circadian-controlled processes, so we do not cover this information here.

2. The molecular clock entrains to heat pulses or temperature cycles

In nature, the molecular circadian clock synchronizes to natural cycles of daily light versus darkness and associated temperature fluctuations. When holding light conditions constant, the phase of the endogenous molecular clock and thus its output rhythms entrains to heat pulses [11,12] or temperature cycles [13–19] through independent mechanisms.

2.1.1. The molecular mechanism of clock phase-resetting by heat pulses—

Sidote et al. [11**] were the first to observe that *Drosophila* subjected to 37°C pulses of heat show rapid decreases in PER and TIM protein levels, an effect that resets the phase of the molecular clock. Because this condition activates the heat-shock response pathway [20], it was an intriguing possibility that this pathway contributes to the enhanced degradation of PER and TIM. To test this, Sidote and Edery [21] conditionally suppressed the heat-shock response pathway during 37°C pulses but interestingly found that PER and TIM are still

degraded. As it turns out, there is a striking connection in the circadian clock's response to both heat and light pulses: both rely on the actions of CRY – the circadian photoreceptor molecule that is related to photolyases, a family of DNA repair enzymes. The severe loss-of-function *cry^b* mutant shows no response to heat pulses, a phenotype that is partially rescued when driving *cry⁺* expression in circadian pacemaker cells [12**]. Biochemically, heat pulses may mediate PER and TIM degradation via CRY if they structurally modify and thus enhance the binding efficacy of the PER-TIM complexes to active CRY proteins.

2.1.2. The molecular mechanism of clock phase-resetting by temperature cycles

—While it is molecularly unknown how the clock synchronizes to temperature cycles, Glaser and Stanewsky [15**] found that the gene *no receptor potential A* (*norpA*) – which encodes phospholipase C (PLC) – is necessary for the response. Previous work has shown that *norpA* mediates the thermosensitive splicing of *per*, which is functionally important for determining the phase of behavioral activity under warm versus cold days: under cold conditions, *norpA* mediated splicing of *per* advances *per* mRNA accumulation, protein, and thus behavioral activity [8*,22*]. This finding motivated Glaser and Stanewsky [15**] to test whether *norpA*-mediated *per* splicing was also important for the clock's synchronization to temperature cycles. Interestingly, the authors found that *norpA* mediated splicing of *per* is not relevant under these conditions because wild-type animals contain equal amount of spliced and unspliced forms of the *per* transcript regardless of temperature cycling conditions. This suggests that PLC mediates molecular synchronization of the clock through a novel mechanism.

2.2. Heat pulses and temperature cycling input pathways to the clock

How do clock neurons receive information about heat pulses and temperature cycles? At present, it is unknown whether clock neurons themselves are capable of sensing heat pulses or temperature cycling cues ¹. However, data from Sehadova et al. [24**] show that the brain cannot synchronize to temperature cycles when separated from peripheral tissues. Instead, they rely on the action of external sense organs as well as chordotonal organs, the latter of which are mechanosensory structures (Figure 2). Thus, knocking-down structural proteins [such as *nocte* (*no circadian temperature entrainment*)] in these organs prevents behavioral entrainment to temperature cycles [15**]. Work by Wolfgang et al. [25] suggests that chordotonal organs contain different types of thermoreceptors specialized to sense different ranges of temperature cycling, such as the TRP channel encoded by the *pyrexia* (*pyr*) gene, which senses and transmits temperature signals within a low (16–20°C) but not high (21–29°C) range.

Clock neurons in the adult brain are divided into 8 groups based on their location, size, projection pattern, and neuropeptide content [26] (Figure 1). Some of these groups, such as the lateral posterior neurons (LPNs) and some dorsal neurons (DN) groups, are highly sensitive to temperature cycles because the molecular clocks in these neurons preferentially entrain to temperature cycles over light cycles when the two Zeitgebers are out-of-phase

¹Lee and Montell [23] state that pacemaker neurons entrain to temperature cycles via TRPA1 channels. However, it is unclear whether their behavioral assays show proper entrainment.

[27]. However, studies have shown that clocks in these cells are not necessary to entrain *Drosophila* behavior to temperature cycles because when Busza et al. [14] restored *per* to CRY-positive or PDF-positive cells (which do not include the DN_s and dorsal lateral neurons (LND_s)) in a *per*⁰¹ background, these flies could be entrained to temperature cycles.

At present, it is unknown whether peripheral organs transmit heat pulse cues to clock neurons nor which clock neurons are responsible for controlling heat pulse resetting behavior.

3. The molecular clock compensates for changes in temperature to maintain circadian periodicity

One generic property of the molecular clock is its ability to maintain a ~24 hr periodicity over a permissive temperature range, a property known as ‘temperature compensation.’ This was first demonstrated by Colin Pittendrigh [28*], who observed that the eclosion rhythm (which is controlled by the molecular clock) has a constant period when assayed over a wide range of temperatures. Temperature compensation is an interesting kinetic property because biochemical reactions are predicted to speed up or slow down according to warm or cool temperatures respectively, and so the constancy of circadian period implies a unique underlying biochemistry. To date, theoretical models have been most successful in explaining the mechanism of temperature compensation: thus far, studies have found the most support for the ‘antagonistic principle’ [29,30], which states that circadian period will remain stable over a range of temperatures if the reaction rates that decrease period length are balanced by those that increase it. Later, Bodenstein et al. [31] showed through theoretical modeling that this balance occurs by choosing appropriate activation energies for each reaction. From a molecular genetic perspective, it is unlikely that there is a *bona fide* temperature compensation gene, although some circadian molecules are clearly more important than others² because several circadian mutants compensate temperature, while some are completely defective. For a complete list of these mutants, see Hong et al. [33].

4. The molecular clock dampens under low temperature stress

One property of circadian rhythms is that they compensate for changes in temperature. However, temperature compensation only occurs under a physiologically-relevant range of temperature. When bringing circadian organisms such as *Drosophila* under extreme high or low temperature conditions, rhythmic cycles are abolished. Until recently, the cellular and molecular mechanism underlying the loss of circadian behavior under cold temperature stress was unknown. The challenge inherent in studying this circadian property is the difficulty in identifying genetic lines capable of maintaining rhythm under low temperature stress. In our most recent article, we overcame this challenge by sampling from natural populations of *Drosophila* that are evolving under divergent temperature selection pressures of the environment [34**]. When we compared molecular clock cycling in circadian cells we found that the maintenance of rhythm is associated with robust clock function (Figure 3).

²Sawyer et al. [32] found that the length of threonine-glycine (Thr-Gly) encoding repeats of *per* is functionally important for temperature compensation, but to date it is unknown how this occurs molecularly.

Consistent with previous studies [35,36], our data suggest that a threshold level of PER protein is required to drive rhythmic behavior and it is likely that some lines lose their ability to maintain rhythm under cold temperature stress because circadian cells do not reach sufficient PER expression. Overall, our work highlights the importance of using lines derived from natural populations to identify clock mechanisms, especially those that influenced by temperature-based selection pressure from the environment.

4. Discussion

The information reviewed here suggests that the molecular circadian clock in adult *Drosophila* displays an impressive diversity in response to temperature. For example, while heat pulses and temperature cycles modulate the phase of the clock, they do so through distinct mechanisms. In addition, we have observed variability in how the clock responds to a temperature ranges: within a permissive temperature range, the clock resists the effects of temperature to maintain a consistent periodicity, whereas the clock dampens under conditions of low temperature stress. In addition, while it is unclear how temperature signals are transmitted to the circadian clock, there are likely several types of pathways specialized to distinct temperature cues as well. For instance, the circuits underlying temperature entrainment and temperature preference in *Drosophila* are distinct: antennal thermosensors are important for thermal preference [37] whereas external sense and chordotonal organs are necessary for behavioral entrainment to temperature cycles [24**]. It is therefore likely that *Drosophila* rely on distinct receptors, signaling pathways, and circuits to modulate behavior. The extreme sensitivity that *Drosophila melanogaster* exhibit to temperature might be one reason why they have been so successful in colonizing temperately diverse environmental regimes as opposed to other *Drosophila* species [38*].

Acknowledgments

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*of special interest

**of outstanding interest

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Research highlights

- The circadian clock shows impressive diversity in how it responds to temperature.
- The clock entrains to temperatures pulses and cycles via independent mechanisms.
- The clock displays variability in response to temperature ranges.
- The clock's temperature sensitivity could underlie a species' colonization pattern.

Figure 1. The *Drosophila* circadian network

~150 neurons in the *Drosophila* brain constitute a circadian network that controls the 24 hr timing of rhythms. These cells are identified by their expression of PERIOD (PER) protein, which is shown in green. Clock neurons can be further subdivided into 8 groups based on their location, size, and neuropeptide content: dorsal lateral neurons (LNDs), large ventral lateral neurons (I-LNVs), small ventral lateral neurons (s-LNVs), the 5th small ventral lateral neurons (5th s-LNVs), lateral posterior neurons (LPNs), and three dorsal neuron groups (DN1s, DN2s, DN3s). Only the s-LNVs and the I-LNVs express the neuropeptide pigment-dispersing factor (PDF), which is outlined in magenta.

Figure 2. Temperature cycling input pathways

a) Circadian rhythms entrain to temperature cycles under conditions where light is held constant: either constant darkness (DD) or light (LL). Entrainment is typically established after three days (72 hrs) **b)** Peripheral tissues in *Drosophila* sense temperature cycles. These sensory inputs primarily consist of chordotonal (ch) and external sense organs (both shown in green) and include the labial, maxillary palps, antennae, antennal ch organs, wing base, ventral wing margin, wing veins, wing ch organs, haltere, and leg ch organs. Temperature cycling signals from the periphery are transmitted to the brain, which does not entrain in isolation of these inputs. At present, it is unclear which clock neurons underlie circadian entrainment to temperature cycles in adult *Drosophila*.

Figure 3. The maintenance of rhythm under low temperature stress is clock-based

In the *Drosophila melanogaster* brain, PER expression (green) in the small ventral-lateral neurons (s-LNVs) is essential for the maintenance of rhythmic behavior in DD. The s-LNVs can be identified by their expression of pigment-dispersing factor (PDF), shown in magenta.

a) Rhythmic, cold-adapted circadian strains have stronger PER oscillations than **b)** generic, wild-type strains at 15°C. As shown previously, a threshold amount of PER expression is required for the expression of rhythmic behavior [35,36] and so it is likely that generic, wild-type strains are arrhythmic because their s-LNVs do not reach sufficient PER expression to achieve rhythmicity.