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Molecular and Behavioral Analysis of *Drosophila* Circadian Photoreception and Circadian Thermoreception: A Dissertation

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A Dissertation Presented

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Dedicated to my mother and father.

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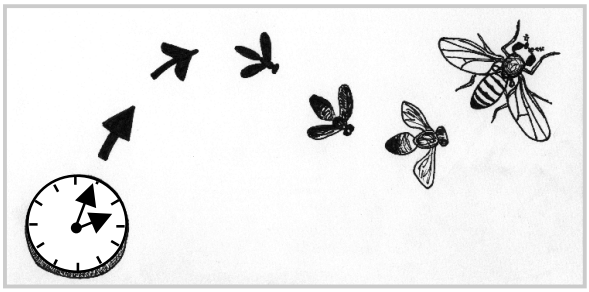

ABSTRACT

Circadian clocks are biological timekeepers that help maintain an organism's behavior and physiological state optimally timed to the Earth's day/night cycle. To do this, these internal pacemakers must accurately keep track of time. Equally importantly, they must be able to adjust their oscillations in response to external time cues to remain properly synchronized with the environment, and correctly anticipate environmental changes. When the internal clock is offset from its surrounding day/night cycle, clinically relevant disruptions develop, ranging from inconveniences such as jet-lag to more severe problems such as sleep disorders or mood disorders. In this work, I have used the fruit fly, *Drosophila melanogaster*, as a model organism to investigate how light and temperature can synchronize circadian systems.

My initial studies centered on an intracellular photoreceptor, CRYPTOCHROME (CRY). CRY is a blue light photoreceptor previously identified as a major component of the primary light-input pathway into the *Drosophila* circadian clock. We used molecular techniques to show that after light-activation, CRY binds to the key circadian molecule TIMELESS (TIM). This interaction irreversibly targets TIM, but not CRY, for degradation. Further studies characterizing a newly isolated *cry* mutant, cry^{m}, showed that the carboxyl-terminus of CRY is not necessary for CRY's ability to impart photic information to the molecular clock. Instead, the C-terminus appears to be necessary for normal CRY stability and protein-protein interactions. Thus, we conclude that in contrast to previous reports on CRYs of other species, where the C-terminal domain was required

for transduction of photic information, the C-terminus of *Drosophila* CRY has a purely modulatory function.

During the second part of my dissertation work, I focused my studies on circadian thermoreception. While the effects of light in synchronization of the *Drosophila* clock to environmental cycles have been extensively characterized, significantly less is known about temperature input pathways into the circadian pacemaker. I have used two approaches to look at how temperature affects the circadian system. First, I conducted a series of behavioral analyses looking at how locomotor rhythms can be phase-shifted in response to temperature cycles. By examining the behavior of genetically ablated flies, we determined that the well-characterized neurons controlling morning and evening surges of activity during light/dark cycles are also implicated in morning and evening behaviors under temperature cycles. However, we also find evidence of cells that contribute to modulating afternoon and evening behavior specifically under temperature cycles. These data contribute to a growing number of studies in the field suggesting that pacemaker cells may play different roles under various environmental conditions. Additionally, we provide data showing that intercellular communication plays an important role in regulating circadian response to temperature cycles. When the morning oscillator is absent or attenuated, the evening cells respond abnormally quickly to temperature cycles. My work thus provides information on the roles of different cell groups during temperature cycles, and suggests that beyond simply synchronizing individual oscillating cells, intercellular network activity may also have a role in modulating proper response to environmental time cues.

Finally, I present some preliminary work looking at effects of temperature on known circadian molecules. Using a combination of *in vivo* and cell culture techniques, I have found that TIM protein levels decrease at higher temperatures. My cell culture data suggest that this is a proteasome-independent degradation event. As TIM is also a key molecule in the light-input pathway, the stability of TIM proteins may be a key point of integration for light and temperature input pathways. While additional research needs to be conducted to confirm these effects *in vivo* in wild-type flies, these preliminary results identify a possible avenue for further study.

Taken together, my work has contributed new data on both molecular and neuronal substrates involved in processing light and temperature inputs into the *Drosophila* circadian clock.

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LIST OF ABBREVIATIONS

AMe: accessory medulla
CE: cell extract
CHX: cycloheximide
CK2: casein kinase 2
CLK: clock
CRY: cryptochrome
CYC: cycle
CT: circadian time
DBT: double-time
dLNs: dorsal lateral neurons
DNs: dorsal neurons
DN1s: dorsal neurons group 1
DN2s: dorsal neurons group 2
DN3s: dorsal neurons group 3
DD: constant dark conditions
E-peak: evening peak
HE: head extract
LD: light/dark cycles
LL: constant light conditions
LPN: lateral posterior neurons
M-peak: morning peak
PDF: pigment-dispersing factor
PDP1: PAR domain protein 1epsilon
PER: period
PI: Pars intercerebralis
PP2a: protein phosphatase 2a
SD: standard deviation
SEM: standard error of the mean
SGG: shaggy
S2: Schneider 2 cells
TC: thermophase/cryophase (temperature cycles)
TIM: timeless
vLNs: ventral lateral neurons
VRI: vrille
ZT: Zeitgeber time

CHAPTER I

INTRODUCTION

At its most basic level, this dissertation is about studying the biology underlying behavior. Understanding the relationship between activity in the brain and outwardly manifested behaviors has been called "one of the holy grails of modern neuroscience research" (Byrne and Suzuki, 2006). Although we are still a long way off from understanding the biological basis of human behaviors, the past 40 years have seen significant progress made into dissecting the anatomical and molecular basis of simple behavioral responses in model organisms. In my work, I have attempted to improve our understanding of how behaviors are generated by using a simple model organism (the fruitfly, *Drosophila melanogaster*) to study a very basic mechanism of regulating behavior (circadian rhythms).

Behaviors, or actions displayed by an organism in response to its environment, can range from very simple to extraordinarily complex. The simplest of behaviors are merely direct responses to events occurring in the environment, such as when a unicellular organism swims away from a noxious stimulant. In these cases, the interaction with the environment is purely reactive. Such responses help organisms survive, but those that develop proactive ways of engaging with the environment are likely to be better off. Successful engagement in proactive behaviors requires some

ability to predict environmental conditions. The most basic and dependable change in environmental conditions occurs daily: the 24-hour day/night cycle on our spinning Earth. Given that the planet rotates once per every day, causing drastic changes in external conditions, it is not surprising that almost all organisms have found a way to keep track of this cycle and use it to make predictive and proactive changes in their behavior and physiology (Dunlap, 1999).

In this introduction, I will explain several key concepts about the biological clocks that help time daily behavior, and then review what is currently known about the molecular clock and neuronal networks underlying the *Drosophila* circadian behavior. Finally, as my dissertation work has focused on environmental inputs into the circadian system, I will summarize current knowledge of input pathways into the clock.

A. Circadian Rhythms

Circadian clocks are the biological time-keeping mechanisms that allow organisms to keep track of the Earth's day/night cycle and optimally adapt their behaviors to this changing environment. The circadian system can be conceptualized as a simplified three component linear system (Eskin, 1979):

Environmental inputs can be as diverse as daily changes in temperature, food availability, or the periodic day/night cycle. These Zeitgebers (German for "time-givers") feed into the self-sustaining circadian clock and synchronize it with the environment. This

synchronization, or circadian entrainment, is one of several remarkable features about the circadian system. Other features include: (1) A stable periodicity: even in the absence of external time cues, the circadian clock of an organism will oscillate with a period length of approximately 24 hours; (2) Compensation: Most biological rates are affected by the organism's current condition. For example, higher temperatures usually increase chemical reaction rates. However, circadian rhythms maintain a remarkably constant period length in a variety of conditions. Therefore it appears that there are compensatory mechanisms built into the system to minimize variations due to changes in metabolic or environmental state. Having a timekeeping mechanism that is both inherently stable and yet exquisitely sensitive to environmental time cues is essential for proper orchestration of physiological states and behavioral activities.

B. The *Drosophila* circadian system

1. Studying *Drosophila* circadian rhythms.

By the late 1960s, circadian rhythms already had been extensively studied. Behavioral and physiological rhythms had been reported in a myriad of organisms ranging from bread molds to humans, and most biologists agreed that there was an internal pacemaker capable of independently tracking time. Despite numerous behavioral studies and a growing understanding of the anatomical centers responsible for regulating rhythms, however nothing was known about the molecular substrates that comprised the clockwork under the rhythms. It was the work done by Seymour Benzer and his graduate

student Ron Konopka on the fruitfly, *Drosophila melanogaster*, that identified the first circadian gene and launched the field of molecular circadian biology.

Previous research showed that *Drosophila* eclosion, or emergence from the pupal case, peaks at dawn (Pittendrigh, 1954). When entrained to a 12 hour light: 12 hour dark cycle and then placed in constant darkness, a fly population will continue to have a sharp eclosion peak at "subjective" dawn (corresponding to when dawn would be during the light:dark cycle). Konopka screened the progeny of mutagen-exposed flies for genotypes with altered eclosion rhythmicity and found the first clock mutants: three alleles of the period (*per*) gene (Konopka and Benzer, 1971). These mutants were also found to have disruptions of their locomotor activity rhythms, suggesting that one central clock mechanism could be responsible for many different types of circadian behaviors. Further research has identified many other genes central to the circadian clock.

Drosophila melanogaster continues to be a popular model organism because it is highly amenable to genetic manipulation and because it has well characterized circadianly-regulated behaviors. Furthermore, there are well-established techniques for reading these clock outputs and over thirty years of research on *Drosophila* circadian rhythms has given us substantial knowledge of the core clock mechanisms. There are several ways to observe the circadian clock in a fruit fly. At the molecular level, protein cycling in individual tissues can be observed through immunocytochemistry, through luciferase reporter genes using circadian promoters, or measuring proteins or mRNA levels in tissue extracts over several time points. At the behavioral level, circadian rhythms have been observed in several ways. Originally, rhythmicity was observed by

measuring when the majority of a population of pupae eclosed, or hatched, from their pupal cases. However, this approach has limitations in that whole populations of flies have to be used, and each fly only exhibits its behavior once. Therefore, most studies now measure circadian rhythms in locomotor activity of adult *Drosophila.* Normally, wild-type flies have a "crepuscular", or bimodal activity pattern: they become restless in the hour before dawn, have a burst of activity after daylight has begun, and then become less active during the afternoon. Then, in the late afternoon they become more active again with an even larger evening peak of activity. At night, they have limited activity and are presumed to be in a "sleep-like" state. In the laboratory, we measure locomotor activity rhythms by using a commercial device described in Figure 1-1. Over 30 years of studying the molecular and behavioral rhythms of genetically manipulated flies has lead to a significant amount of knowledge of the substrates underlying *Drosophila* circadian behavior.

2. The *Drosophila* circadian clock is made of a molecular feedback loop.

The fundamental time-keeping mechanism at the crux of all circadian systems is a molecular clock. In most organisms, from cyanobacteria to humans, interlocking transcriptional feedback loops form the core oscillator (Hardin, 2004; Dunlap, 1999). However there is increasing evidence of an equally important role for post-translational modifications, including phosphorylation and ubiquitination, which regulate timing through effects on protein stability, localization, and complex formation (Gallego and Virshup, 2007; Nakajima et al., 2005).

In *Drosophila,* the molecular clock is made up of two feedback loops in which transcriptional repressors accumulate and negatively regulate transcription of their own transcripts [Figure 1-2]. At the center of the system are two transcription factors: CLOCK (CLK) and CYCLE (CYC). CLK and CYC both contain a PAS domain (a protein-dimerization domain first found in PER, ARNT and SIM) with which they interact to form heterodimers. They also both have a basic-helix-loop-helix domain with which they bind to E-box regulatory elements (CACGTG) (Allada et al., 1998; Hao et al., 1997; Rutila et al., 1998b). During the day, CLK/CYC heterodimers bind to E-boxes and activate transcription of circadianly-regulated genes, including *period* (*per*), *timeless* (*tim*), *vrille* (*vri*), and *PAR domain protein 1ε* (*Pdp1*) (Blau and Young, 1999; Cyran et al., 2003; Darlington et al., 1998). *per* and *tim* mRNA levels accumulate and peak in the early evening (Hardin et al., 1990; Sehgal et al., 1995). However, PER and TIM protein accumulation lags behind mRNA and only peaks in the mid/late night (Zerr et al., 1990; Hunter-Ensor et al., 1996). This is probably due to the protein products being initially vulnerable to degradation. During the daytime, TIM is continuously targeted for degradation through the activity of an intracellular photoreceptor CRYPTOCHROME (CRY), which will be discussed in further detail later in this chapter (Emery et al., 1998a; Lin et al., 2001; Stanewsky et al., 1998). PER is phosphorylated and destabilized by a homolog of mammalian casein kinase 1ε, DOUBLE-TIME (DBT) (Price et al., 1998). After sun-down, TIM accumulates and binds to the PER-DBT complex and stabilizes PER (Gekakis et al., 1995; Zeng et al., 1996). Two other kinases, CASEIN KINASE 2 (CK2) and SHAGGY (SGG), a homolog of mammalian glycogen synthase kinase 3,

phosphorylate PER and TIM, respectively, and promote nuclear localization (Lin et al., 2002; Martinek et al., 2001). Immunocytochemical staining both *in vivo* and in cell culture suggests paradoxically that although PER requires the presence of TIM for nuclear localization to occur (Vosshall et al., 1994), the two proteins can enter the nucleus separately (Meyer et al., 2006; Shafer et al., 2002). Once in the nucleus, PER (either bound or unbound to TIM) physically interacts with CLK and inhibits CLK/CYC mediated transcription, thereby suppressing its own transcription (Ceriani et al., 1998; Lee et al., 1998; Nawathean and Rosbash, 2004). DBT also enters the nucleus, probably in association with PER (Kloss et al., 2001), and further regulates the *per*/*tim* feedback loop by phosphorylating and destabilizing CLK (Kim and Edery, 2006; Yu et al., 2006). DBT activity is counter-balanced by PROTEIN PHOSPHATASE 2A (PP2A) dephosphorylation of CLK and PER (Kim and Edery, 2006; Sathyanarayanan et al., 2004). This may help ensure that transcriptional inhibition by nuclear PER is restricted to late evening/ early morning. (Kim and Edery, 2006; Hardin, 2006). In the morning, CRY-mediated TIM instability and DBT phosphorylation of PER promotes their degradation, CLK/CYC can activate transcription again, and the cycle repeats.

This *per*/*tim* feedback loop might be sufficient for maintaining rhythms in *Drosophila*. However, there is a secondary feedback loop that might contribute to ensuring precise timing and robust oscillations via the regulation of *Clk* mRNA levels. The circadianly regulated gene *vrille (vri)* encodes a basic leucine zipper protein that binds to the *Clk* promoter and represses *Clk* transcription (Blau and Young, 1999; Cyran et al., 2003). *In vitro* assays found that another circadianly-regulated protein, *PAR*

DOMAIN 1ε (PDP1), can compete with VRI for binding to the same site in the *Clk* locus. This suggested that *Clk* transcription is regulated by the VRI:PDP1 ratio, with higher PDP1 levels dislodging VRI from the *Clk* promoter in the late evening to permit *Clk* transcription (Cyran et al., 2003). However, a recent report found that drastically increasing or decreasing PDP1 levels had no effect on *Clk* mRNA cycling *in vitro* even though it did lead to disrupted behavioral rhythmicity (Benito et al., 2007). The authors proposed that PDP1 does not function as a regulator of *Clk* transcription but instead has a role in mediating oscillator output. Further research will be required to determine the role of PDP1 in generation of circadian rhythms, and how *Clk* and *per*/*tim* feedback loops work together to generate stable molecular oscillations.

3. What are the minimal requirements for a 24-hour timekeeper in *Drosophila*?

Every day, in hundreds of clock-gene expressing cells of the fly, the molecular cycle repeats itself. Transcription factors activate transcription, proteins are produced, and some inhibit their own transcription to form a central oscillator. Other proteins determine the timing of the cycle by regulating stability and location of the circadian molecules. Together, a 24-hour molecular oscillator is created. But how do we know that this observed cycle is really a self-sustaining oscillator that underlies the circadian behavior that can persist for months without external cues? Could the molecular "clock" itself be merely another oscillation driven by some other pacemaker, such as one encoded in a neural electrical circuit or a daily hormonal signaling loop between two glands? The currently available answer is only partially satisfactory. Studies of flies with mutations in

core clock genes demonstrate that the period length of behavioral rhythms is altered when clock protein stability, and thus period of the molecular cycle, is altered (Zerr et al., 1990). Rescuing production of wild-type protein in certain brain regions of circadian mutants can revert the activity rhythms to normal (Ewer et al., 1992). Finally, a clock in the form of a full-body oscillation is unlikely, as isolated tissues can show independent molecular oscillations (Emery et al., 1997; Giebultowicz and Hege, 1997; Plautz et al., 1997). Indeed, circadian rhythms have been found in single-cellular organisms, indicating that a molecular feedback loop can be sufficient for a stable pacemaker (Williams, 2007). In *Drosophila,* however, there is some evidence suggesting that there are other as-yet undiscovered clock components necessary for a persistent oscillator, at least in behavioral rhythms. For example, evidence from transgenic flies with electrically silenced clock cells indicate that membrane excitability is necessary for sustained molecular cycles (Nitabach et al., 2002; Nitabach et al., 2005). Additional contributions into the functioning of the molecular clock, or at least modification of other inputs, may occur via neurotransmitter receptor cascades (Yuan et al., 2005). Larger intracellular molecular cascades or intercellular communication, therefore, may be required for persistent circadian oscillations in *Drosophila.* That a roughly 24-hour molecular cycle is present in many *Drosophila* tissues is definite; what components are essential for self-sustaining oscillations, and how these minimal requirements vary between different tissue types, remains to be determined.

4. The molecular clock is expressed in many tissues, including ~150 brain neurons.

The *per*/*tim* molecular oscillator is present in many but not all tissues of the *Drosophila*. Cyclic co-expression of PER and TIM proteins is seen in the visual photoreceptors, alimentary tract, rectum, fat body, renal (Malpighian) tubules and parts of the reproductive system, as well as in a set of neurons and glia in the central nervous system (Siwicki et al., 1988; Zerr et al., 1990; Kaneko and Hall, 2000; Hunter-Ensor et al., 1996; Giebultowicz and Hege, 1997; Giebultowicz et al., 2001). Not much is known about the role of the molecular clock in peripheral tissues, but presumably the pacemaker helps coordinate optimally timed physiological processes. One example of clock-dependent effects on physiology is in the antennal lobe, where the circadian clock modulates olfactory responses in olfactory neurons (Tanoue et al., 2004). Unlike the hierarchical organization of the mammalian circadian system, where a circadian pacemaker in the central nervous system synchronizes molecular cycling in peripheral tissues (Reppert and Weaver, 2002), *Drosophila* peripheral clocks receive synchronizing input directly from the environment. These photo- and thermo-responsive oscillations persist in the absence of input from the central nervous system, as demonstrated in *in vitro* studies measuring cyclic *per*-driven or *tim*-driven luciferase activity in isolated tissues (Plautz et al., 1997; Giebultowicz et al., 2000; Glaser and Stanewsky, 2005). Eventually, however, the molecular cycles dampen in peripheral tissues without environmental inputs. It is unclear if the eventual dampening of peripheral rhythms is due to individual cells becoming out-of-phase with each other, like in mammalian tissues, or if it is because their molecular oscillator lacks some components necessary for robust

rhythmicity in constant conditions. In contrast, a small group of neurons in the brain appear to be able to sustain cyclic clock gene expression for many days without external time-cues (Peng et al., 2003). Many of these ~150 neurons appear to play a role in regulating circadian rhythms in locomotor activity, and thus are considered the main pacemaker cells of the *Drosophila* (Chang, 2006) .

The circadian gene expressing neurons were originally identified by immunohistochemical stainings for *per* expression. Six clusters of neurons in each hemisphere, as well as hundreds of glial cells, were found to express cycling *per* (Ewer et al., 1992; Frisch et al., 1994; Kaneko and Hall, 2000)[Figure 1-3]. The cell groups are named according to anatomical location. The lateral neurons (LNs) are found at the lateral edges of the protocerebrum in the accessory medullae (aMe), at the base of the optic lobe. The Dorsal Neurons (DNs) are located in clusters along the dorsal edge of the brain. Both the LNs and DNs were originally divided into three subclasses. The LNs consist of 5-8 dorsolateral neurons (dLNs) and 5 small and 4-6 large ventrolateral neurons (vLNs). These vLNs, except for one small vLN on each side, are the only circadian neurons that express Pigment Dispersing Factor (PDF), a neuropeptide known to be important for circadian behavior (Helfrich-Förster, 1995; Renn et al., 1999). The DNs are composed of ~15 DN1s and 2 DN2s along the dorsal superior brain, and ~40 very small DN3s in the lateral dorsal brain, closest to the dLNs (Helfrich-Förster, 2003). A recent study using an enhancer trap marker further expanded the classes of circadian pacemakers (Shafer et al., 2006). Shafer and colleagues demonstrated PER/TIM oscillations in the lateral posterior neurons (LPNs), a group of 3-4 cells located in the

posterior lateral central brain, which had been previously thought to be TIM-expressing but non-pacemaker cells (Kaneko and Hall, 2000). Additionally, more detailed immunocytochemical visualization indicated that two of the DN1s are consistently located anterior to the remaining DN1s. Colocalization data showing that these anterior DN1s are the only DNs that express the neuropeptide IPNamide (IPNa) further support a functional distinction between anterior and posterior DN1s (Shafer et al., 2006).

5. The pacemaker neurons are inter-connected via a complex network of neural projections.

As clock gene products are located mainly in the cell bodies of neurons (Saez and Young, 1988; Siwicki et al., 1988), original stains for clock proteins such as PER or TIM did not provide information on cellular projections. However, neuropeptide-specific stains as well as transgenic flies using *perGAL4* or *timGAL4* have been used to drive marker genes such as green fluorescent protein (GFP) labeled the neurites in detail (Helfrich-Förster, 1995; Helfrich-Förster, 1997; Kaneko and Hall, 2000; Helfrich-Förster, 2003). The main projections of many clock-gene expressing neurons terminate in the dorsal protocerebrum, near the Pars Intercerebalis (PI)(the neurosecretory center of the insect) (Helfrich-Förster, 2004). Many pacemaker neurons also project towards each other. The small vLNs send dense PDF+ arborizations to a small neuropil at the base of the optic lobe, called the accessory medulla (aMe) (Helfrich-Förster, 2003). Their other PDF+ projections curve upwards to the dorsal central brain and terminate in close proximity to the DN1s and DN2s. The large vLNs also send PDF+ neurites towards the

aMe, which then branch out into widespread arborizations throughout the medulla of the optic lobe. The vLNs from the two hemispheres of the brain are connected via the posterior optic tract that contains fibers from the large vLNs to the contralateral aMe. This tract has been shown to be crucial for locomotor rhythmicity in cockroaches (Page et al., 1977). The dLNs, along with all three DN groups (and small vLNs), project to the dorsal central brain. The projections from the dLNs run dorsally and then split into two branches. The dorsal branch joins fibers from the DN3s and follows a route anterior and lateral to the projections of the small vLNs, with terminations just ventral to the PI. The ventral dLN branch crosses the midline to the contralateral dorsal protocerebrum. Fibers from the DN1s and DN2s also cross the midline in the dorso-anterior commissure. Projections from the DN1s terminate in the dorsal brain or continue ventrally, retracing the path of projections from the small vLNs to terminate in the contralateral aMe. The two anteriorly-located DN1s may project to the ipsilateral aMe. A few DN1s seem to project directly ventrally, towards the esophagus. The DN2 fibers, after crossing over the midline in the dorso-anterior commissure, terminate near the PI in the contralateral dorsal brain (Helfrich-Förster, 1995; Helfrich-Förster, 1997; Helfrich-Förster, 2003; Kaneko and Hall, 2000; Shafer et al., 2006). In summary, most projections from circadian neurons either project to the neurosecretory center of the insect, where they presumably provide output to circadianly regulated systems, or they send neurites toward other circadian cells. The inter-clock cell communication probably acts to synchronize and reinforce rhythmicity within the cells. In the cockroach, for example, the posterior optic commisure (which connects the contralateral aMe regions) needs to be intact for

locomotor rhythmicity (Page, 1982). In *Drosophila*, the PDF+ projections are necessary for synchronized pacemaker neurons. In the absence of PDF and external time cues, individual neurons become out-of-phase with each other and the animal loses rhythmicity in constant conditions (Lin et al., 2004; Murad et al., 2007; Renn et al., 1999).

How exactly cell-to-cell communication occurs and what type of information is being processed remains to be determined. To understand how these pacemaker neurons function together to coordinate circadian behavior, we need to map the cellular connections and determine how information is transmitted between cells. Electrophysiological recordings aimed at tackling the latter task have only just begun (Park and Griffith, 2006). Although we know that the vLNs express PDF and a subset of the DN1s produce IPNamide, little is known about what other neurotransmitters and neuropeptides are used in the system.

6. The clock-gene expressing neurons are a heterologous group of cells.

The observation that certain groups of clock cells have distinct neuropeptides highlights the fact that despite all exhibiting oscillations in clock molecules, the pacemaker neurons are a heterogeneous group of cells that have differences in soma size, peptide expression, as well as phase and robustness of circadian molecular rhythms. As we further identify these differences, we may find that they correlate with differences in function or response to environmental inputs. Under light/dark cycles (LD) the previously described clock neurons show strong oscillations in PER and TIM staining. Late in the light-phase, PER/TIM proteins are barely visible. During early evening, a

faint but growing cytoplasmic staining is visible, and intense nuclear staining peaks in the late night/early morning (Shafer et al., 2002). This pattern was carefully studied in the LNs (Shafer et al., 2002) but is presumed to occur in all clock cells. However, the phase and robustness of PER/TIM molecular rhythms show differences between cell groups. The large vLNs, for example, have peak PER/TIM nuclear accumulation up to 4 hours earlier than the small vLNs (Shafer et al., 2002). Ironically, although the small vLNs appear to lag behind the large vLNs in LD, large vLNs do not maintain oscillations well under constant conditions. In just over one day of constant dark the large vLNs appear to "stop" at a state that corresponds to ZT 8-10 in LD (Yang and Sehgal, 2001; Shafer et al., 2002). Several days later oscillations are again seen in the large vLNs, presumably from synchronizing input from other cells such as the DN2s (Peng et al., 2003; Stoleru et al., 2005). On the other hand, the small vLNs and at least some of the cells in the dLNs and DNs consistently show persistent rhythmicity even after 5 days in constant conditions (Veleri et al., 2003). The oscillations in the DN1s may have an intermediate resilience to constant conditions: molecular rhythmicity persists for up to 3 days of constant darkness (DD) (Klarsfeld et al., 2004), but there are conflicting reports as to whether oscillations persist in later days of DD (Veleri et al., 2003; Peng et al., 2003; Stoleru et al., 2005). Even more startling are the data suggesting that DN2s may have a completely different inherent phase. In DD, and in larval *Drosophila* (when the DN2s do not yet express the circadian photoreceptor CRY) under LD, nuclear PER oscillations cycle in anti-phase to the small vLNs and DN3s (Klarsfeld et al., 2004; Veleri et al., 2003). This inherent phase appears to be over-ridden in adults under LD, where the light-dark cycle (presumably

through CRY) forces the DN2s to be synchronized with the other clock cells (Klarsfeld et al., 2004).

7. Early studies emphasized a principal pacemaker role for the lateral neurons under constant dark conditions.

Early anatomical studies in the cockroach indicated an important circadian role in the cockroach aMe; destruction of this region leads to arrythmicity, and transplant experiments demonstrate that the period length of circadian behavior depends on the periodicity of the aMe donor (Page, 1982; Sokolove, 1975). In *Drosophila*, the aMe is less anatomically defined than in the cockroach, but appears to play an equally important role in regulating locomotor rhythmicity. Several studies strongly suggest that the vLNs, located in the vicinity of the *Drosophila* aMe, can act as "master clocks." *Disconnected* (*disco*) mutants lack the large and small vLNs due to a developmental defect and are behaviorally arrhythmic (Helfrich-Förster, 1997). Flies that have had their vLNs genetically ablated by expressing pro-apoptotic genes [*rpr*, *hid*, *bax*] become arrhythmic after several days of DD (Blanchardon et al., 2001; Renn et al., 1999). The vLN-specific neuropeptide, PDF, is necessary for vLN-maintained rhythmicity in DD: pdf^{01} null mutants are also incapable of sustaining robust locomotor rhythmicity in DD (Renn et al., 1999). Indeed, in the absence of both PDF signaling and external cues (such as a LD cycle) the phase and amplitude of clock neurons becomes de-synchronized (Lin et al., 2004). However, expression of the *per* gene in the LNs rescues rhythmicity in per^{0} mutants (Frisch et al., 1994). As *per* rescue in only the small vLNs is sufficient to rescue

rhythmicity in per^0 flies (Grima et al., 2004), and as the period length in DD depends on the period length of the molecular cycle in the small vLNs (Stoleru et al., 2005), it is now believed that the small vLNs are the main cells for DD rhythmicity. This view is supported by the fact that the small vLNs are the only cells that express both PER and TIM strongly throughout the flies life (Kaneko et al., 1997) and that they maintain robust clock protein oscillations for more than 5 days in DD (Lin et al., 2004; Veleri et al., 2003). Other clock cells, however, also play an important role in maintenance of rhythmicity under constant conditions. In pdf^{01} mutants, residual behavioral rhythmicity persists for the first 2-3 days of DD (Renn et al., 1999) and in a small subset of flies weak rhythmicity is discernable for 9 days (Lin et al., 2004), suggesting that other cells can temporarily maintain locomotor behavior. Additionally, *cyc* expression in the vLNs of a *cyc* mutant (cyc^{01}) background is not sufficient to rescue rhythmicity (Peng et al., 2003). This apparent contradiction with Grima and colleagues' work (Grima et al., 2004) may be because *CLK* and *CYC* proteins are necessary for proper development of neural circuitry. Alternatively, it could mean that CLK/CYC mediated transcription is required in other cells for vLN-mediated rhythmicity, even though a fully functional clock is not necessary.

8. Evidence for a duo-oscillator system.

As previously mentioned, *Drosophila* circadian behavior is crepuscular – flies have two peaks of locomotor activity: one at dawn (referred to as the Morning-peak or "M-peak") and one at dusk (the Evening-peak or "E-peak"). Both of these surges of

activity begin before the environmental lights-on or lights-off transition has occurred, providing evidence that the underlying time-keeper anticipates the impending transition. More impressively, the M-peak and E-peak are differentially affected by environmental inputs; both high temperature and longer daytimes have stronger effects on the E-peak phase than on the M-peak (Helfrich-Förster, 2001; Majercak et al., 1999). How does a single intracellular molecular clock create this divisible bimodal activity? An explanation comes from the Dual Oscillator Model, first proposed by Pittendrigh and Daan to explain the effects of different light regimes on rodent circadian behavior (Pittendrigh and Daan, 1976). The model suggests the existence of two connected oscillators, differentially sensitive to light, that control the M- and E-peaks respectively. Long exposure to light would cause the M-oscillator to accelerate and the E-oscillator to decelerate, helping the animal adjust its activity to longer/shorter days in different seasons (Pittendrigh and Daan, 1976). In the past 5 years careful tissue-specific manipulations using the UAS-GAL4 system has strongly suggested separate M- and E-oscillator roles for some of the circadian neurons in the fly (Grima et al., 2004; Stoleru et al., 2004; Stoleru et al., 2005). The PDF+ vLNs, the same cells necessary for rhythmicity to persist under constant DD conditions, are responsible for the M-peak. Flies with vLNs ablated and pdf^{01} mutants have E-peak anticipation before dusk, but only a startle response to the dawn with no anticipatory increase in activity (Grima et al., 2004; Stoleru et al., 2004). In contrast, when *per* cyclic expression is rescued in the small vLNs of per^{0} mutants, the flies have M-peak anticipatory increases in locomotor activity before dawn during LD (Grima et al., 2004). Similar experiments using genetic techniques to ablate

or specifically rescue function in different subsets of cells suggest that the dLNs and possibly a subset of DNs drive the E-peak under LD (Grima et al., 2004; Stoleru et al., 2004). Under constant dark conditions, the M-cells dominate: the molecular period of the M-cells is what determines the period of both the M- and E-peaks, and can even enforce the same period onto the molecular oscillations of the dLNs, DN1s, and DN3s. In these conditions, the E-cell period only determines the length of the day, i.e., timing of the E-peak relative to the M-peak (Stoleru et al., 2005). However, two recent papers have found that under certain conditions, the E-cells, or at least a subset of them, can dominate over M-cell activity. While wild-type flies are arrhythmic in constant light conditions, genetic manipulations leading to light-resistant molecular rhythms only in the DN1s rescue behavioral rhythmicity (Murad et al., 2007). Additionally, under conditions of long days and short nights the dLNs, DN1s, and DN3s set the phase of both the evening and the morning activity (Stoleru et al., 2007). Finally, evidence of several behavioral components in wild-type flies exposed to very low-light constant conditions (or photoreceptor mutants in constant light) suggest that sometimes the M-cells can contribute to both Morning and Evening activity (Rieger et al., 2006; Yoshii et al., 2004). It thus appears that there are two main pacemakers in the *Drosophila* circadian cell network. The small PDF+ vLNs are the primary pacemakers in darkness, and possibly in very low light conditions, and a group of dorsal cells including the dLNs and some of the DN1s and DN3s dominate in extended periods of bright light. Together, these cells help to properly time morning and evening bursts of activity in the seasonal changes of longer and shorter day lengths.

How outputs from these pacemaker cells eventually lead to modulations in activity levels is still not well understood. Electrophysical recordings from circadian neurons has only just begun (Park and Griffith, 2006), but hopefully will eventually provide information about how information is integrated within the circadian cell network. Additionally, further characterization is needed about which classical neurotransmitters and/or neuropeptides are secreted by these cells and where their receptors are expressed (Shafer et al., 2006). Presumably, output from the circadian neurons eventually leads information being sent to centers regulating locomotor behavior, possibly in the PI. As my dissertation work focused on inputs, and not outputs, in the *Drosophila* circadian system, this will not be further discussed in my introduction.

C. Inputs into the *Drosophila* Clock

The *Drosophila* circadian clock, like that of other animals, is exquisitely sensitive to changes in the environment. These Zeitgebers, or external time-givers, keep the clock synchronized to the day/night cycle. The environmental light/dark cycle provides the strongest input into the circadian system (Pittendrigh, 1960; Oishi et al., 2004), but temperature cycles and social cues have also been shown to synchronize circadian behavior (Levine et al., 2002a; Wheeler et al., 1993). I will focus on photoreceptive and thermoreceptive circadian input pathways, as little is known about how social cues affect the circadian clock, and my work has focused on molecular and behavioral analysis of light and temperature inputs.

1. Light input pathways into the circadian clock.

Daily light/dark cycles are the strongest environmental inputs, and also the best characterized of all the Zeitgebers in *Drosophila.* The effect of light on the circadian system is manifest in several ways. Most obviously, alternating light/dark cycles [LD] can synchronize and entrain (see glossary) locomotor behavior and eclosion rhythms (Pittendrigh, 1954; Wheeler et al., 1993). Light/dark cycles also synchronize the oscillations of the molecular clock, both in peripheral clocks and in the pacemaker cells (Myers et al., 1996; Plautz et al., 1997; Yang et al., 1998). Brief light pulses given during the subjective night-time can phase-advance or phase-delay fly behavioral rhythms depending on the time they are administered (Saunders et al, 1994). Finally, exposure to continuous light (LL) leads to altered period lengths and arrythmicity (Konopka et al., 1989). Studies have revealed that there are four photoreceptors/photopigments that contribute to circadian photoreception in the fly (reviewed in Rieger et al., 2003). Three of these are peripheral light-sensing organs (the compound eyes, the ocelli, and the Hofbauer-Buchner eyelets) [Figure 1-3]. There is also some evidence suggesting an as yet un-identified photopigment expressed in the Dorsal Neurons (Rieger et al., 2006; Rieger et al., 2003; Veleri et al., 2003). However the main circadian photoreceptor (and the focus of one third of this dissertation) is the intracellular photoreceptor CRYPTOCHROME.

Early studies of *eyeless* mutants lacking compound eyes found that, surprisingly, eclosion rhythm of these flies remained synchronized to the LD cycle (Engelmann and Honegger, 1966). Indeed, although input from the compound eyes triggers the lights-on

LD startle response in *Drosophila* locomotor behavior (Rieger et al., 2003), flies lacking functional compound eyes and ocelli still entrain their locomotor rhythms to LD cycles (Yang et al., 1998). As studies in isolated tissues show that the molecular pacemaker can be re-set by light/dark cycles, there must be a light-sensing mechanism which synchronizes circadian tissues all over the body, independent of input from the eyes (Plautz et al., 1997).

In 1998, the intracellular circadian photoreceptor CRYPTOCHROME (CRY) was identified as the primary light-sensing input for the *Drosophila* circadian clock. A CRY mutant, cry^{baby} (cry^{b}) was isolated in a chemical mutagenesis screen looking for flies with abnormal *per* expression in LD cycles (Stanewsky et al., 1998). Flies carrying the cry^{b} mutation lack PER and TIM cycling in peripheral clocks under LD. However, molecular rhythms can be seen in constant conditions after being initiated by temperature cycles, indicating that the underlying clock is capable of maintaining rhythms (Stanewsky et al., 1998). Behaviorally, cry^{b} mutants are partially circadianly "blind": they are unresponsive to the phase-shifting effects of bright light pulses and they retain rhythmic behavior under LL conditions (which disrupts rhythmicity in wild-type flies) (Emery et al., 2000b; Stanewsky et al., 1998). When combined with a mutation in the *norpA* gene (encoding phospholipase C necessary for functional external photoreceptors) they show severe deficits in entrainment to LD cycles (Emery et al., 2000a; Stanewsky et al., 1998). In contrast, over-expression of the wild-type *cry* gene leads to an increase in circadian sensitivity to light pulses of low light intensity (Emery et al., 1998a). As *cry* is expressed in pacemaking neurons, and the phenotypic deficits of flies can be rescued by

expressing wild-type *cry* exclusively in these neurons, it appears that CRY functions as a cell-autonomous circadian photoreceptor (Emery et al., 2000a).

Structurally, *Drosophila* CRY is part of a larger family of blue-light sensitive flavoproteins (Cashmore et al., 1999). Cryptochromes are found in many species, and play a role in plant and animal circadian clocks (reviewed in Green, 2004). The *Drosophila* CRYPTOCHROME molecule shares with these other CRYs a core domain that shows high homology to photolyases (DNA repair enzymes). This domain has two conserved binding sites for a flavin cofactor (FADH) and a pterin cofactor (5,10-methenyl tetrahydrofolate, MTHF). The pterin acts as a "light-harvesting" chromophore. It absorbs photons and transfers excitation to the flavin through a series of redox reactions. In photolyases, the excited flavin then acts as a "catalytic" chromophore and uses an electron to repair pyrimidine dimers in UV-damaged DNA (Cashmore, 2003). *Drosophila* and plant CRYs are thought to absorb photons similarly, but instead of facilitating DNA repair, the energy is used for a change of conformation (Green, 2004).

How then, does this predicted light-induced conformation change in *Drosophila* CRY lead to re-setting the molecular clock? The answer is not yet known, but an overwhelming amount of evidence suggests that the re-setting mechanism works via the key circadian protein TIMELESS (TIM). Molecular analysis of head extracts collected during LD showed that TIM levels plummet at dawn, preceding the decline in PER (Zeng et al., 1996). A similar decrease in TIM is seen in the pacemaker neurons after a light pulse (Yang et al., 1998). This light-activated TIM degradation is correlated to the behavioral effects of light-pulses: both molecular and behavioral effects have similar

light spectral sensitivity and *tim*^{SL} mutants that have increased TIM light-induced degradation are correspondingly more behaviorally sensitive to light pulses (Suri et al., 1998). Finally, a light-pulse given at a time that results in phase-advances in the TIM molecular cycle lead to behavioral phase-advances, and vice versa (Myers et al., 1996).

In *cry*^{b} mutants, TIM is not degraded by light (Lin et al., 2001). Sequence analysis of *cry*^{b} uncovered a missense mutation in the flavin cofactor binding domain (Stanewsky et al., 1998). Thus, having a functional CRY protein is necessary for light-induced TIM degradation. Intriguingly, CRY itself also undergoes light-dependent degradation. Although *cry* mRNA expression is regulated by the circadian clock, the protein accumulates in constant darkness and degrades upon exposure to light (Emery et al., 1998a). Both TIM and CRY are degraded via the proteasome and TIM degradation is probably preceded by tyrosine phosphorylation and ubiquitination (Lin et al., 2001; Naidoo et al., 1999). How CRY initiates self-degradation and TIM degradation is unknown, but it appears to require electron transport and flavin co-factor binding (Lin et al., 2001; Froy et al., 2002). Perplexingly, *in vitro* studies have provided conflicting evidence over whether or not CRY and TIM interact. A yeast two-hybrid assay found CRY/TIM AND CRY/TIM/PER interactions under light (but not dark) conditions. CRY-PER interactions were not seen under light or dark conditions, and CRY^{B} did not have the light-dependent interaction with TIM or the PER/TIM complex (Ceriani et al., 1999). However, another yeast two-hybrid study found a CRY/PER interaction that was independent of light, and this was supported by co-immunoprecipitation of CRY and PER in *Drosophila* S2 cell culture (in constant darkness). In Chapter II of this

dissertation, I present data from transgenic flies expressing a tagged version of CRY to clarify these proposed interactions. I also report molecular and behavioral studies of a novel *cry* mutant that provide insights into the role of the C-terminal domain in *Drosophila* CRY.

2. Evidence that temperature fluctuations affect the *Drosophila* circadian clock.

While relatively little is known about circadian thermoreception relative to circadian photoreception, a substantial number of studies show that temperature affects the *Drosophila* circadian clock. Both at the behavioral and at the molecular level, temperature fluctuations have an effect on circadian oscillations.

Early during his influential career characterizing *Drosophila* circadian behavior, C.S. Pittendrigh found that a 4-hour 26ºC heat pulse could synchronize eclosion rhythms in normally un-synchronized populations of *Drosophila pseudoobscura* maintained at 16ºC DD (Pittendrigh, 1954). In cultures previously synchronized to a LD cycle, a single temperature shift could alter the circadian phase of the population eclosion rhythm by several hours: temperature increases result in phase-advances and temperature drops lead to phase-delays (Pittendrigh, 1954; Zimmerman et al., 1968). These preliminary observations are complicated by temperature's effect on *Drosophila* pupal development independent of the circadian clock (Pittendrigh, 1954). Further experiments suggested that these temperature responses are more than just physiological effects on rate of development, however, as 12-hour long warm pulses could generate both phase advances and delays depending on the timing of the warm pulse during the circadian cycle

(Zimmerman et al., 1968). The phase-shifting properties of a single heat-pulse have also been observed in adult locomotor activity. A 1-hour heat-pulse of 37ºC given 9 to 17 hours after subjective dawn causes a phase delay of 1-2 hours (Edery et al., 1994). This behavioral effect coincides with dramatic changes at the molecular level in key components of the circadian clock: a 30 minute 37ºC heat-pulse given 9 to 17 hours after subjective dawn results in a rapid decrease in whole head extract PER and TIM levels (Sidote et al., 1998). Intriguingly, these high-temperature effects on the clock appear to be CRY-dependent (Kaushik et al., 2007). This is also seen in per^{L} mutant flies at 30ºC. However, since these effects are not seen in wild-type flies at more physiologically relevant temperatures (under 34ºC), it is unclear what role, if any, this mechanism has in wild-type flies at cooler temperatures (Sidote and Edery, 1999; Sidote et al., 1998). It may be that CRY-dependent temperature effects on the clock involves heat-shock or stress-response pathways, or it may be that the same mechanism occurs at lower temperatures, but because the effects are more subtle, rapid changes in protein levels were not seen.

Indeed, both behavior and molecular rhythms can respond to temperature cycles at cooler temperatures. Temperature cycles of 12hr:12hr 25ºC/30ºC can synchronize and re-synchronize behavioral rhythms of wild-type flies in both LL and DD (Matsumoto et al., 1998). In another study, behavioral rhythms have even been shown to synchronize to recurrent temperature changes of only 2 or 3ºC (Wheeler et al., 1993)! However, while these locomotor activity entrainment experiments show a correlation between the temperature cycle and activity patterns, they do not demonstrate true entrainment of the

circadian pacemaker as the synchronizing effects could be due to a non-circadian effect of warmer temperatures on fly activity level. The fact that molecular oscillations can persist in peripheral clocks of cry^b flies after initiation by 25ºC/30ºC 12hr:12hr temperature cycles (Stanewsky et al., 1998) strongly suggests that these behavioral effects are due to an effect of temperature cycles on the circadian clock itself. Further research demonstrating temperature's lasting effects on locomotor rhythms is needed, however, to definitively state that temperature cycles entrain, as opposed to merely synchronize, *Drosophila* circadian behavior. This is addressed, at least for under DD conditions, in Chapter III of this dissertation.

Two other temperature-related phenomena deserve to be mentioned when discussing the effect of temperature on the circadian clock. The first is temperature-sensitive *per* mRNA splicing. Even when held constant, the temperature level affects circadian behavior. In warm temperatures (25ºC and 29ºC) the activity curve is bimodal as previously described. However, wild-type flies maintained at cooler temperatures (18ºC) have locomotor activity that peaks at mid-day; the evening peak is much earlier and has merged with the morning peak. This altered locomotor pattern is due to enhanced splicing of an intron in the 3'Un-Translated Region of *per* RNAs at lower temperatures (Majercak et al., 1999). The low-temperature splicing of *per* mRNA is theorized to increase efficiency in 3' end cleavage and polyadenylation which could result in an early upswing in *per* mRNA abundance and thus an earlier evening peak. How temperature controls *per* RNA splicing is still not understood, however recent papers suggest that the phospholipase C *norpA* plays an inhibitory role in regulation of this splicing (Collins et

al., 2004; Majercak et al., 2004). As *norpA* is part of the classic visual signal transduction cascade, and activated by light, this *per* splicing/ evening peak effect may be a mechanism by which long daylight and warmer temperatures combine inputs to appropriately delay the evening peak in long summer days.

Finally, the most puzzling temperature-related phenomenon in circadian rhythms is probably best described as a "non-effect" of temperature: the tantalizing observation of "temperature compensation". According to Transition State theory, chemical reactions occur more rapidly at higher temperatures. As circadian clocks are composed of molecular feedback loops, one would expect that a temperature increase in non-homeothermic organisms such as insects would accelerate the circadian reactions and result in a decrease in period length to less than 24 hours. However, Drosophila eclosion patterns and locomotor activity rhythms have a surprisingly consistent period at temperatures ranging from 10ºC to 29ºC (24 hours +/- 0.7 hours) (Pittendrigh, 1954; Zimmerman et al., 1968). It is possible that the circadian system is resistant to temperature because it is comprised of antagonistic feedback loops. If higher temperature increases both positive and negative regulation then the net effect might be neutral. Alternatively, it has been suggested that a reduction in PER accumulation because of temperature-dependent splicing or protein instability could compensate for generally faster reactions at higher temperatures (Kurosawa and Iwasa, 2005).

Whether all of the above temperature-related phenomena are due to separate mechanisms or are simply many facets of one temperature-dependent process remains to

be determined. What seems clear, however, is that the *Drosophila* circadian system is affected by the organism's environmental temperature, and that these effects can be seen at both the behavioral and molecular level. During the second half of my dissertation research, I aimed to gain new insights on the role of temperature in entraining the *Drosophila* circadian clock to environmental day/night cycles. As described in Chapter III, we verified that temperature cycles can act as a true Zeitgeber for circadian behavior. Then, we examined the role of known pacemaker neurons in circadian behavioral responses to temperature cycles. In Chapter IV, we further investigated possible temperature inputs into the clock by looking at the effect of removing peripheral thermoreceptive organs. We also performed molecular studies looking for effects of physiologically relevant temperature fluctuations on circadian protein levels.

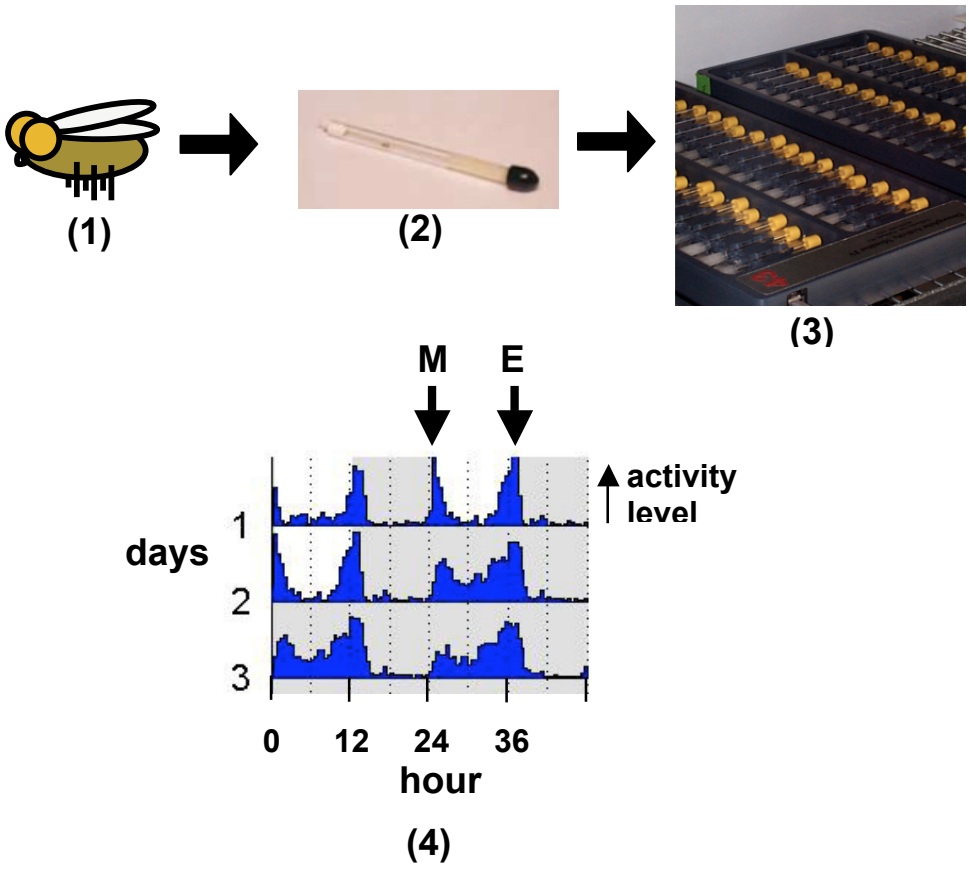


Figure 1-1. Studying locomotor activity of adult *Drosophila.*
We use an automated system to monitor circadian locomotor activity. Adult male flies (1) are placed into glass tubes with food (2) and then into monitors (3) that use infrared sensors to measure activity over several days. We then plot the average activity per 30 minutes in actograms (4). To better view the entire 24-hour cycle, each day of data is plotted twice on the actogram: once on the right half and once on the left half on the next line. In this actogram (4), the average activity of 16 flies is shown during 2 days of LD (12 hours in the light, as indicated by the white background, then 12 hours in the dark, as indicated by the grey background). After the 2nd day, the flies were released into constant dark conditions and their activity patterns persist with roughly the same timing. The morning (M) and evening (E) peaks of activity are indicated above the second day of LD.

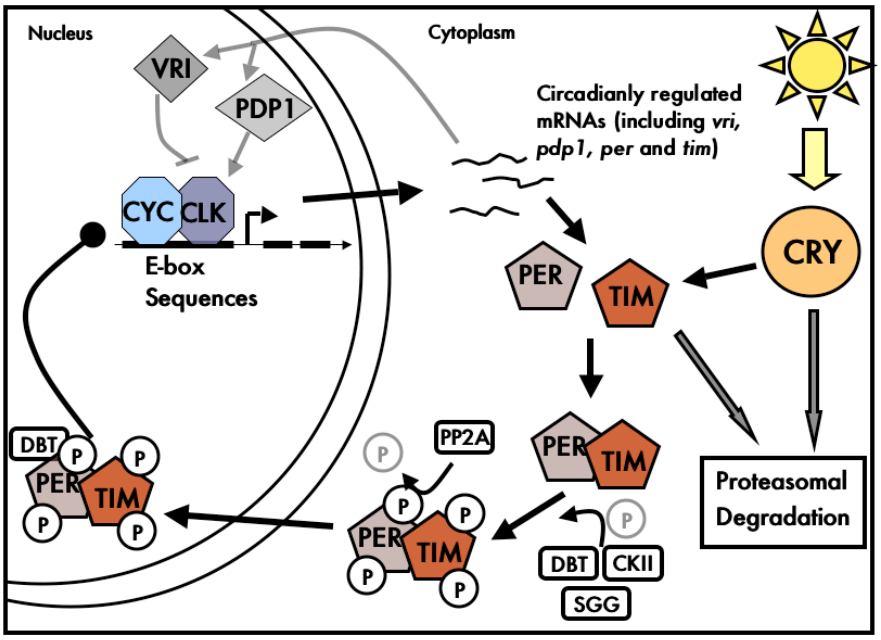


Figure 1-2. The Molecular Clock of *Drosophila melanogaster*. This cartoon model illustrates the main transcriptional feedback loops of the molecular pacemaker: CLOCK (CLK) and CYCLE (CYC) initiate transcription of *period* (*per*) and *timeless* (*tim*) genes. PER and TIM proteins are phosphorylated by a set of kinases (DOUBLETIME [DBT], SHAGGY [SGG], and CASEIN KINASE II [CKII]) and dephosphorylated by PROTEIN PHOSPHATASE 2A (PP2A) in the cytoplasm. TIM, PER, and DBT then enter the nucleus to inhibit the activity of CLK/CYC. A secondary feedback loop, in which VRILLE (VRI) and PAR DOMAIN PROTEIN 1ε (PDP1) regulate *Clk* transcription, is shown in grey. The intracellular light-input pathway is shown on the right: after being activated by light, CRYPTOCHROME (CRY) acts on TIM to target it for ubiquitination and degradation, via the activity of an F-Box protein (JETLAG [JET]) activity. Ultimately, both CRY and TIM are degraded via the proteasome.

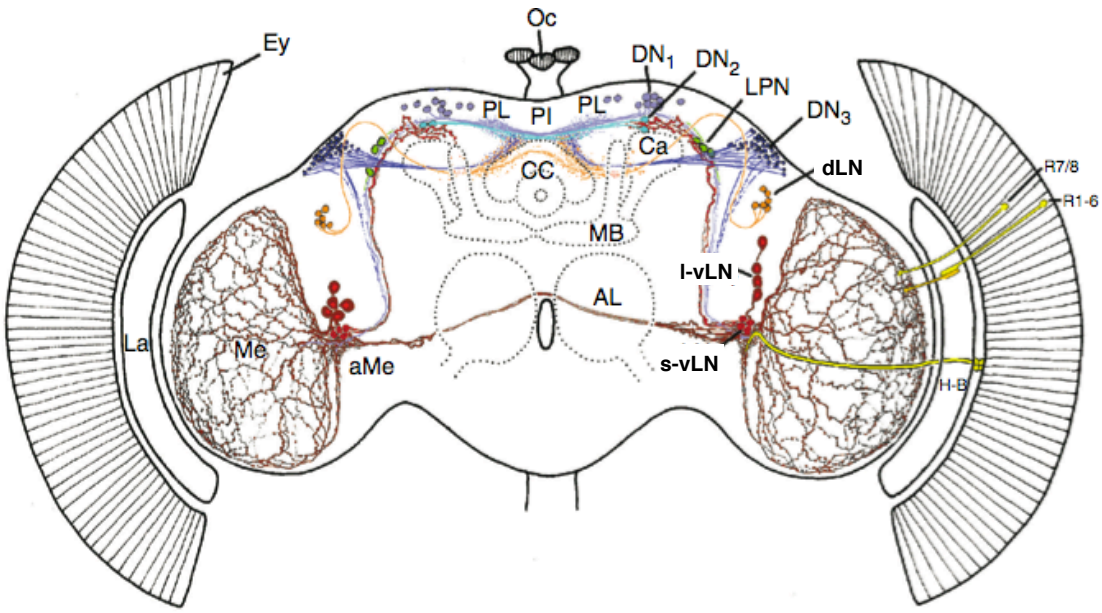


Figure 1-3. Pacemaker cells in the brain of *Drosophila melanogaster*. This section through the *Drosophila* brain shows the clock-gene expressing neurons and their projections. The Lateral Neurons (dLNs, and small (s) and large (l) vLNs) are shown in orange and red, the Dorsal Neurons (DN1, DN2, DN3) are shown in blue, and the lateral posterior neurons (LPNs) in green. For details on the neuronal projections, see the text. On the right side of this figure, the light input pathways from photoreceptor cells in the compound eyes and from the Haufbauer-Buchner eyelet (H-B) are shown. Another peripheral light-sensing organ, the ocelli (Oc) are seen above the Pars Intercerebalis / Lateralis (PI and PL). The Central Complex (CC), Mushroom Bodies (MB) and their calyces (Ca), Antennal Lobes (AL) and Medulla (Me) and accessory Medulla (aMe) are also shown for orientation. Figure from Helfrich-Förster, 2005.

CHAPTER II

ROLES OF THE TWO *DROSOPHILA* CRYPTOCHROME STRUCTURAL DOMAINS IN CIRCADIAN PHOTORECEPTION

This chapter is reprinted from an article published in Science on June 4, 2004 (Busza et al., 2004). This study provides behavioral and molecular evidence that CRY's photolyase domain is sufficient for light-mediated resetting of the circadian clock, while its C-terminus regulates CRY stability and CRY-TIM interactions. The work reported here represents a collaborative effort between the authors: Myai Emery-Le, Michael Rosbash, Patrick Emery and me. Myai Emery-Le isolated the cry^m mutant and did subsequent behavioral analyses. Patrick Emery measured CRY, TIM, and PER levels in cry^m head extracts. While working in Michael Rosbash's lab at Brandeis University, he also did the *in vivo* and *in vitro* studies looking at the kinetics of CRY and TIM degradation during exposure to light. My contribution to this work consisted of *in vivo* and *in vitro* immunoprecipitations demonstrating a light-dependent CRY-TIM interaction. I also used cell culture techniques to show that the CRY^M protein is constitutively degraded by the proteasome. Finally, I performed immunoprecipitations indicating that CRY^M binds to TIM in both light and dark conditions, which demonstrated that the C-terminal domain is necessary for the light-specificity of the CRY-TIM interaction. Patrick Emery wrote the body of the text, while I contributed to the Materials and Methods and provided feedback on the other sections.

A. Abstract

CRYPTOCHROME (CRY) is the primary circadian photoreceptor in *Drosophila*. We show that CRY binding to TIMELESS (TIM) is light-dependent in flies and irreversibly commits TIM to proteasomal degradation. In contrast, CRY degradation is dependent on continuous light exposure, indicating that the CRY-TIM interaction is transient. A novel *cry* mutation (cry^{m}) reveals that CRY's photolyase homology domain is sufficient for light detection and phototransduction, whereas the carboxyl-terminal domain regulates CRY stability, CRY-TIM interaction, and circadian photosensitivity. This contrasts with the function of *Arabidopsis* CRY domains and demonstrates that insect and plant cryptochromes use different mechanisms.

B. Results and Discussion

Cyanobacteria and eukaryotes adapt to the daily physical and ecological changes in their environment with the help of circadian pacemakers. In most organisms, these pacemakers are based on a 24-hour period transcriptional negative feedback loop (Dunlap, 1999). In *Drosophila melanogaster*, PERIOD (PER) and TIMELESS (TIM) dimerize and function as negative transcription factors by interfering with the positive activity of CLOCK (CLK) and CYCLE (CYC), which together bind to and activate the *per* and *tim* promoters (Stanewsky, 2002). A set of kinases (SHAGGY, DOUBLETIME, and CASEIN KINASE-II) regulates PER and TIM stability and activity to ensure that the

cycle lasts 24 hours (Akten et al., 2003; Stanewsky, 2002).

Circadian pacemakers require input pathways to synchronize with the environment. Cryptochromes are blue light–sensitive proteins related to photolyases, a family of DNA repair enzymes. They play important roles in plants and animal circadian photoreception (Cashmore, 2003). There is good evidence that *Drosophila* CRYPTOCHROME (CRY) is the primary circadian photoreceptor, although opsin photoreception also helps to synchronize circadian behavior (Helfrich-Förster, 2001). CRY overexpression increases the sensitivity of the circadian clock, and all circadian photoresponses are affected in the severely hypomorphic cry^b mutant (Emery et al., 2000a; Emery et al., 1998b). However, the mechanisms by which CRY synchronizes the circadian pacemaker are still unclear. The primary target of the CRY input pathway appears to be TIM. TIM light-dependent degradation requires CRY and is crucial to reset the circadian pacemaker after short light pulses (Hunter-Ensor et al., 1996; Lee et al., 1996; Myers et al., 1996; Stanewsky et al., 1998; Suri et al., 1998; Yang et al., 1998; Zeng et al., 1996). Both CRY and TIM are degraded by the proteasome after illumination (Lin et al., 2001; Naidoo et al., 1999), and they interact in a light-dependent manner in yeast (Ceriani et al., 1999). In *Drosophila* S2 cells, however, the CRY-TIM interaction is apparently light-independent (Ceriani et al., 1999). Moreover, CRY interacts with PER, which suggests that PER might also be a pacemaker target of CRY. Like TIM, PER undergoes a light-dependent interaction with CRY in yeast but interacts with CRY in the dark in S2 cells (Rosato et al., 2001).

To study CRY and its interactions directly in flies, we generated flies expressing an N-terminal MYC-tagged CRY (*y w; tim-GAL4 UAS-myccry/CyO*) (*tmc* flies). MYC-CRY is fully functional, as it rescues PER and TIM cycling and arrhythmic constant-light behavior in cry^b flies. *tmc* and *y w* control flies were light-pulsed for 15 min in the late night when PER and TIM levels are high [zeitgeber time (ZT) 21], and CRY binding to PER and TIM was assayed by immunoprecipitation with antibody to MYC. A strong light-dependent interaction was evident among CRY, TIM, and PER [Figure 2-1A, and comparable results were observed at earlier times, at ZT 15 and ZT 17. A weak TIM and PER signal was visible in the dark, but a comparable background signal was visible in *y w* control flies, indicating that there is no detectable binding of CRY to PER and TIM in the dark. Thus, if CRY interacts with PER and TIM in the dark as previously suggested (Ceriani et al., 1999; Rosato et al., 2001), it might be limited to the specific tissues in which CRY contributes to circadian oscillations in constant conditions (Ivanchenko et al., 2001; Krishnan et al., 2001).

Because TIM and PER interact strongly, we examined whether CRY binds to TIM or PER individually. We clearly detected a light-dependent CRY-TIM interaction in per^0 flies [Figure 2-1B]. Similar results were obtained in S2 cells [Figure 2-1C], in contrast to a previous report (Ceriani et al., 1999). These findings, together with the original yeast data (Ceriani et al., 1999), imply that the CRY-TIM light-sensitive interaction occurs in all expression systems. We failed to detect any interaction between CRY and PER without TIM, either in tim^0 flies or in S2 cells (Ceriani et al., 1999)[Figure 2-S3]; thus, TIM appears to be CRY's primary target after light activation. The PER

immunoprecipitation from wild-type flies reflects the strong PER-TIM interaction.

To understand the consequences of the TIM-CRY interaction, we assayed TIM and CRY degradation kinetics after light exposure [Figure 2-2A]. We first expressed CRY alone in S2 cells and monitored its degradation. CRY was very stable in the dark but rapidly degraded in the light, with a half-life of about 25 min. However, after 10 min of light exposure and then 50 min of darkness, CRY levels were only slightly lower than after the 10-min light pulse and much higher than after the 60-min light pulse [Figure 2-2A, left; the difference in the number of photons delivered with the different light protocols does not account for these results (supporting material)]. The same phenomenon was observed in vivo [Figure 2-2A, center] in the presence of PER and TIM. CRY degradation therefore requires continuous light exposure, and CRY can rapidly revert to a stable conformation when returned to darkness. We then assayed TIM after light exposure in wild-type flies. The TIM degradation time course was virtually indistinguishable from that of CRY, with a half-life of 20 min. However, the 10-min light pulse plus 50-min dark protocol produced TIM levels close to those seen after a 60-min light pulse [Figure 2-2A, right]. A 10-min light pulse therefore commits TIM, but not CRY, to degradation. This finding indicates that different mechanisms govern TIM and CRY degradation and suggests that the two proteins interact transiently.

To strengthen the notion that TIM and CRY interact after CRY absorbs light, we measured the wavelength sensitivity of CRY in S2 cells, using its own light-dependent degradation as a readout. Previous experiments in flies have shown that the spectral

sensitivity of TIM's light-dependent degradation closely matches the spectral sensitivity for behavioral phase resetting. Maximal effects are observed between 400 and 500 nm, and virtually no response is detected above 550 nm (Fig. 2-2B) (Suri et al., 1998). The spectral sensitivity of CRY degradation in S2 cells (Fig. 2-2B) is remarkably consistent with the two previous spectral sensitivities in flies. This result strongly reinforces the notion that CRY is the primary photoreceptor for TIM degradation and phase resetting.

CRY contains a photolyase homology domain and a C-terminal domain, like most cryptochrome family members [Figure 2-S2] (Cashmore, 2003). To determine the contribution of these two domains, we studied a *cry* variant isolated in a novel genetic screen for mutants remaining rhythmic in constant 3000-lux light. Wild-type *Drosophila* circadian period lengthens under constant low light intensity (<10 lux), but flies are arrhythmic at higher intensities (Konopka et al., 1989). In contrast, cry^b flies maintain robust 24-hour rhythms even in intense constant light (Emery et al., 2000a). We isolated a rhythmic mutant line with a period slightly longer than 24 hours under constant light. In constant darkness, both the mutant and the parental strain also showed a slightly long circadian period [Figure 2-3A, table 2-S1]. The new mutant failed to complement the cry^b constant-light defect, which indicated that the new mutation was in *cry* [Figure 2-3A]. This new *cry* allele, which we named cry^m, contains a single change from the wild-type line used for the mutagenesis: a premature stop codon that truncates CRY's last 19 amino acids, leaving the photolyase domain intact [Figure 2-S2].

To understand how the loss of CRY's C-terminal domain so profoundly affects

constant-light behavior, we measured CRY^M levels in fly heads [Figure 2-3B, top]. Weak bands were visible, at the position of CRY and just below, which might correspond to the truncated CRY^M protein. Mutant protein levels appeared lower than those of cry^b flies. When expressed in S2 cells, CRY^M was clearly visible, but at levels lower than those of wild-type CRY by a factor of at least 20 [Figure 2-3B, bottom]. A 2-hour light pulse did not noticeably change CRY^M levels. However, addition of the proteasome inhibitor MG-132 strongly increased CRY^M and CRY^B stability [Figure 2-3C]. Because MG-132 also inhibits CRY light-dependent degradation (Lin et al., 2001), the data show that both mutant proteins are constitutively degraded by the proteasome, rather than being degraded in a light-dependent manner.

These results suggest that cry^m flies, like cry^b flies, are blind to constant light because the CRY^M protein is unstable and nonfunctional. However, exposure of cry^m flies to lower constant-light intensities lengthened the circadian period: At 200 lux, the cry^m period lengthened to 25.1 hours. It lengthened even further to 26.6 hours at 25 lux [Table S1]. cry^b flies were unaffected under identical conditions. This behavior of cry^m flies indicates that their circadian clock is not blind and that the CRY^M protein provides light signal to the pacemaker. This is possibly because CRY^M can accumulate to slightly higher levels under low light intensity in the neurons controlling circadian behavior (supporting material).

We then measured other CRY-dependent circadian photoresponses in cry^m flies. In whole head extracts, most PER and TIM signals come from the eyes, a tissue with no

detectable circadian oscillations in cry^b flies under light:dark conditions. In contrast, PER and TIM oscillations were clearly present in cry^m flies, although the amplitude was reduced relative to wild-type flies [Figure 2-4A]. We then tested the effect of the cry^m mutation on the ability of the circadian pacemaker to respond to short light pulses. We briefly illuminated wild-type, cry^b, and cry^m flies at different times of the night and determined the effect on circadian behavioral phase [Figure 2-4B]. For wild-type flies, as expected, there were phase delays in the early night and phase advances in the late night. cry^b flies showed little or no responses, whereas cry^m flies were still able to advance or delay their clock but with a reduced magnitude. Because we had observed that cry^m flies detect constant light better at low intensity, we reduced the intensity of the light:dark regime to 25 lux instead of 1000 lux. The intensity of the light pulses was unchanged (3000 lux). The results were striking, as the magnitude of the cry^m phase changes was almost as robust as that of wild-type flies [Figure 2-4C]. In contrast, the low-intensity regime had no effect on the cry^b responses, confirming previous studies showing that cry^b is a null allele, or nearly so (Ceriani et al., 1999; Emery et al., 2000b; Emery et al., 2000a; Stanewsky et al., 1998). These results indicate that the CRY^M protein is fully functional for circadian phototransduction, despite the absence of the C-terminal domain.

In support of our conclusions, we found that CRY^M can bind TIM as strongly as wild-type CRY can [Figure 2-S3]. This is also true for CRY^B. However, CRY^M and CRY^B bind TIM equally well in the dark or after a light pulse, whereas wild-type CRY binds TIM only when light is present. The mutant proteins cannot bind PER, which

indicates that they retain specificity for TIM (Rosato et al., 2001). These results suggest that there are two separable consequences of CRY photon capture that induce circadian photoresponses. The first is CRY binding to TIM, which is presumably necessary but not sufficient for signal transduction. The second is the irreversible modification of TIM for its targeting to proteasomal degradation, probably with the help of a tyrosine kinase (Naidoo et al., 1999). CRY^{M} can achieve the second step because its photolyase domain is unaltered and sensitive to light. CRY^{B} fails to trigger light-dependent TIM degradation because its mutation probably results in an inability to bind flavin adenine dinucleotide properly [Figure 2-S2] (Stanewsky et al., 1998). We interpret the failure of CRY^{B} to act as a dominant negative mutant as a consequence of its low expression level.

In sum, we propose that CRY's photolyase domain is fully responsible for phototransduction and that the C-terminal domain is not required for this activity. In contrast, the C-terminal domain inhibits the CRY-TIM interaction in the dark and determines the photosensitivity of the circadian clock by regulating CRY proteasomal degradation. This view of CRY photoreception in *Drosophila* contrasts sharply with what we know about *Arabidopsis thaliana* CRYs: In these molecules, the C terminus is the active phototransduction domain, and the photolyase region modulates its signaling function (Yang et al., 2000). Strikingly different mechanistic strategies have therefore emerged during evolution to transmit light information and regulate CRY activity. Interestingly, *Drosophila* TIM is related to molecules involved in various aspects of DNA metabolism—including chromosome segregation and DNA repair—in various organisms. The CRY-TIM interaction, mediated by the DNA-repair photolyase homology domain,

may thus be evolutionarily ancient and may have been central to the origin of circadian rhythms.

C. Materials and Methods

1. Plasmid constructs and transgenic flies.

The *hs-cry* plasmid was described previously (Emery et al., 1998a). A *myc* tag sequence was added to the 5' end of the *cry* cDNA. This *myc-cry* cDNA was introduced into the EcoRI and StuI sites of pCasper-hs and in the EcoRI and XhoI sites of pUAST and pAcV5/HisB (Invitrogen). The resulting constructs are called *hs-myccry*, *UAS-myccry* and pAc*myccry* respectively. *myc* tagged cry^b and cry^m cDNAs were also introduced in the EcoRI and XhoI sites of pAcV5/HisB (pA*c-myccry*^{b} and pAc-*myccry*^{m}) and the Eco RI and XbaI sites of the pCasper-hs vector (*hs-myccry*^{b} and *hs-myccry*^{m}). The pAc-*timha* and pAc-*per* vectors containing the *tim* cDNA fused to an HA tag (NotI/XhoI fragment)(Rutila et al., 1998a) and the *per* cDNA (EcoRI/XbaI fragment) respectively were kindly provided by Ravi Allada and Vipin Suri. The *tim-GAL4* and *UAS-cry* transgenic flies have been described previously (Emery et al., 1998a). *yw*; *Ki pp*[*ry+ 2-3*]/+ embryos were injected with the *UAS-myccry* construct to obtain germline transformants. A 2nd chromosome *y w; UAS-myccry* transgenic line was recombined genetically with *y w; tim-GAL4/CyO*. The resulting *y w*; *tim-GAL4 UASmyccry/CyO* line is abbreviated *tmc*. The *UAS-myccry* and *tim-GAL4* transgenes were also introduced in tim^0 and per^0 backgrounds, to produce per^0*w sn; tim-GAL4 UASmyccry/CyO* (abbreviated

per^0 tmc) and *y w; tim-GAL4 tim^0/ UAS-myccry tim^0* (abbreviated *tim^0 tmc*) flies. *y w per^0 ; ry506* and *yw; tim^0* fly lines were used as controls.

2. S2 cell transfection and drug treatment.

S2 cells were grown in SFX (HyClone) medium supplemented with 10% fetal bovine serum, penicillin (100U/µl) and streptomycin (100 µg/µl). For transfection with HS-*cry*, 3 million cells were transfected with 2 µg of plasmid. Transfections were performed with 10 µl Lipofectin (GibcoBRL) according to manufacturer's instructions (non-adherent cell protocol, 40 minutes of Lipofectin and SFX medium preincubation, 6 hours of transfection). The cells were left at 25ºC to recover for 48 hours. They were then heat-shocked for 1 hour at 37ºC, and left in the dark to recover for another 24 hours. With the other constructs used in this study, S2 cells were transfected using Cellfectin (Invitrogen). For immunoprecipitations (see below), 3x2ml-wells/sample of 60-80% confluent cells were transfected with 1.5 µg total DNA (15 minutes of DNA, Cellfectin, and SFM medium [Gibco/Invitrogen] supplemented with 2mM L-Glutamine preincubation, 4-6 hours of transfection). For the light pulse experiment, each well was transfected with 1.0 µg of pAc constructs using Cellfectin. Thirty-six hours later, the plates were light pulsed or not for 2 hours and then harvested for Western blots. For the proteasome inhibitor experiments, cells were transfected with 1.0 µg of pAc or pHS constructs using Cellfectin. With the pHS constructs, cells were heatshocked for 1 hour at 37ºC, then returned to 25ºC for 2 hours before adding to the cell culture medium MG132 (50µM) and cycloheximide (0.5mg/ml), or DMSO and cycloheximide (0.5mg/ml) as

control. The plates were kept in the dark at all times, and samples were harvested 0, 2, and 4 hours after addition of the drugs.

3. Immunoprecipitations.

Adult flies were entrained to a 12 hour-light: 12 hour-dark cycle for three days. At ZT21, they were pulsed with bright white light for 15 minutes before being collected and frozen. Head extracts were prepared and homogenized in Extraction Buffer (20 mM Hepes pH 7.5, 100mM KCl, 1mM Dithiothreitol, 5% glycerol, 0.05% Nonidet P40, 1x Complete Protease Inhibitor [Roche]). Protein G Sepharose fast flow beads (Amersham) were coated with anti-MYC antibody (2µl anti-MYC antibody (Covance Inc.) + 10 µl beads/sample) and incubated with the head extracts for 4 hours at 4ºC. Pulled-down beads were washed 4 times with 750 µl Extraction Buffer before being resuspended in 40µl 1x SDS loading buffer for Western blot. Head homogenization, incubation, and immunoprecipitation for the light-pulsed samples were done under normal laboratory lighting, while the non-pulsed samples were processed under red light (700nm) and incubated in the dark. For immunoprecipitations with S2 cell samples, cells were light pulsed for 15 minutes 36 hours after transfection, harvested and resuspended in 300 µl Extraction Buffer. They were then homogenized and immunoprecipitated as described for head extracts.

4. CRY and TIM degradation kinetics and CRY degradation action spectrum.

To study the kinetics of CRY and TIM degradation in S2 cells and flies, bright white light (5 mW/cm^2, equivalent to approximately 1000 lux) was used. *hs-cry* transfected S2 cells (about 500,000 per sample) were light pulsed in microcentrifuge tubes. *y w* and *y w; tim-GAL4/UAS-cry* flies were light pulsed in glass vials. To determine CRY's light-dependent degradation action spectrum, *hs-cry* transfected S2 cells (about 300,000 per sample) were placed in small open cups, without any obstacle for the light, and pulsed for one hour. Monochromatic light was generated at an irradiance of 2.5x1017 photons/cm^2 s^2 with 50 nm bandwidth filters as described previously (Suri et al., 1998).

5. Protein extracts and Western blots.

Fly heads extracts were prepared as described previously (Zeng et al., 1996). S2 cell protein extracts were prepared as follows: Cells were harvested with a 1-minute centrifugation, cell culture medium was discarded, cells were resuspended in 1x SDS-PAGE loading buffer and boiled. Western blots were performed as described previously (Emery et al., 1998a; Zeng et al., 1996). With S2 cells, the equivalent of about 150,000 cells/lane was loaded. A sample of S2 cells prior to addition of SDS-PAGE buffer was used for Bradford analysis to normalize the amount of protein loaded. Equal loading and quality of protein transfer were first verified by Ponceau Red staining, and then by the intensity of cross-reacting bands on the Western blots or by reprobing the membrane with a monoclonal α-tubulin antibody (clone DM1A, Sigma, 1:1000 dilution). The anti-CRY antibody is either a previously described rat antibody (Zeng et al., 1996), or a new anti-

serum produced in Guinea Pig. This new anti serum can detect CRY only when overexpressed (data not shown). Both antibodies are directed against the N-terminal region of the protein and their immunoreactivity is therefore not affected by the *cry*^{m} mutation.

6. EMS mutagenesis and constant light screen.

Male Canton-S flies were fed overnight with a 1% sucrose solution containing 10mM Ethyl-Methane-Sulfonate (EMS). These males were crossed to 3rd chromosome TM2/MKRS balancer females. Individual males in the resulting F1 progeny were crossed to TM2/MKRS females. At the next generation, F2 males and females carrying the same mutagenized chromosome were crossed together. F3 males homozygous for mutant 3rd chromosomes were screened for rhythmicity in constant light. They were first subjected to three days of light:dark cycle, and then left under constant light for 6 days. The light intensity was approximately 3000 lux.

7. Behavior phase response assays.

Flies were subjected to a 12-hour light: 12-hour dark cycle for four days and pulsed during the last night with bright white light (3000 lux) for 5 minutes. The entrainment light intensity was either 1000 lux or 25 lux. The pulsed flies were then left in constant darkness for five days. For each of the 6 time points of the phase response curve, done with a 1000 lux light:dark cycle, 16 flies per genotype were used. 32 flies per genotype and per time point were used for the experiments in which different light

intensities were used during entrainment. The average phase of these groups of flies were determined using Levine et al.'s analysis software (Levine et al., 2002b). Arrhythmic flies were excluded from the analysis. For each data point, three or four independent experiments were averaged.

8. Real-Time PCR.

Wild-type Canton-S and cry^m flies were entrained to a 12-hour light: 12-hour dark cycle for three days and then collected and frozen at ZT5 and ZT17. Total RNA was extracted from 20 heads using TRIZOL (Invitrogen), following manufacturer's protocol. Contaminating DNA was removed by incubating the samples with DNase I at 37ºC for 2 hours. cDNA was synthesized from total RNA, using random hexamers. A specific set of primers (*cry* forward primer: 5'-AGTACGTCCCGGAGTTGATGA-3', *cry* reverse primer: 5'-TGCTGCTCGGCAGACATTC-3') and a probe (5'-6 –FAM-CAGGGCTCGTGAACAAATTCCTT-TAMRA-3') located in *cry*'s 2nd exon were designed to determine *cry* levels. Real-time PCR and result analyses were performed following manufacturer's instructions (Applied Biosystems). 6-FAM (6-carboxyfluorescein) was used as a reporter dye, TAMRA (Tetramethylrhodamine) as a quencher, and ROX (Carboxy-X-rhodamine) as the reference dye.

D. Supporting Text

We found the CRY-TIM interaction to be light-dependent *in vivo*, as well as in S2 cells. A previous report failed to detect this light-dependent interaction in S2 cells (Ceriani et al., 1999). The authors of this earlier study pulsed the cells for an hour, but we pulsed the cells for only 15 minutes. A one-hour light pulse may have degraded CRY too extensively to detect its interaction with TIM. CRY was shown to bind PER in a light-dependent manner in yeast (Rosato et al., 2001). We failed to detect such a light-dependent interaction in S2 cells or *in vivo*. This interaction might be too weak to be detected by our approach. However, in yeast, the ÇRY-PER interaction is dependent on light only in the presence of an as yet unidentified yeast factor. Thus, CRY may actually interact with PER in the dark, and this only in specific tissues as discussed in the main text. While studying CRY degradation kinetics, we observed that a 10-minute light pulse followed by 50 minutes of darkness results in higher CRY levels than a 60-minute light pulse [Figures 2-2A and 2-S1]. In order to confirm that CRY degradation requires continuous light exposure, we also pulsed S2 cells and flies overexpressing CRY for 60 minutes at a light intensity 6 times lower than that used for the 10 minutes light – 50 minutes dark protocol. Even though the number of photons delivered was now equal in the two protocols, CRY levels were still substantially lower with the 60-minute low light intensity pulse than with the 10-minute high light intensity plus 50-minute darkness protocol (data not shown). This confirms that CRY degradation requires constant illumination.

To identify the *cry*^{m} mutation, we sequenced CRY's whole coding region and introns, with the exception of a portion of the large first intron. The *cry*^{m} mutation leaves the photolyase domain intact (amino acids 1-513), as well as 10 additional amino acids. The first five of them are not conserved at all in insects and are therefore unlikely to have any important function. The next three are prolines, two of them being highly conserved. These prolines are probably a structural element separating the C-terminal domain from the photolyase domain. The *cry*^{m} mutation thus almost completely deletes CRY's C-terminal domain (only 2 of its 21 amino acid are left). We therefore assume that the only potentially functional domain in the CRY^{M} protein is its photolyase domain. We observed very low CRY protein signal in *cry*^{m} head extracts [Figure 2-3B]. As our antibodies are directed against the N-terminal region of CRY (see material and methods), the low signal does not result from the loss of a critical antigen but reflects low protein concentration. We determined *cry* mRNA levels in *cry*^{m} flies at ZT 5 and 17 by Real-Time PCR and found them to be between wild-type peak and trough mRNA concentration (around 60% of peak value, data not shown). Hence, overall mRNA levels are not significantly affected by the *cry*^{m} mutation. The low CRY protein levels are due to protein instability and not to low mRNA concentration. Our results have shown that the behavioral circadian photoresponses of *cry*^{m} flies improve under low light intensity conditions [see Table S-I and Figure 2-4C]. We presume that this reflects a higher accumulation of CRY^{M} proteins in the cells controlling circadian behavior - the ventral lateral neurons (vLNs) – under low light intensities. The weakness of our CRY antibodies makes this hypothesis difficult to test. This hypothesis seems to contradict our

observations in S2 cells, where CRY^M appears to be constantly degraded by the proteasome [Figure 2-3C]. However, we may not have been able to detect a weak residual dependence of CRY^M degradation on light. The cry^m phenotype might also be more severe in S2 cells. Interestingly, residual light-dependent changes in CRYΔ mutant protein levels have been observed *in vivo* (E. Rosato, personal communication). Since this mutant protein is almost exactly identical to CRY^M (Rosato et al., 2001), it is likely that CRY^M levels also respond weakly to light *in vivo*. J. Aschoff studied in detail the effect of constant light on animal circadian rhythms. One of the rules he derived is that the higher the light intensity is, the stronger is its effect on circadian period length. Arrhythmicity ultimately occurs at high light intensity. However, in cry^m flies, the effect on the period is stronger at low light intensity, at least within the range of light intensities we tested. Thus, the behavior of cry^m flies in constant light breaks one of Aschoff's canonical circadian rules. No obvious light:dark synchronization defect was observed in cry^m flies. Even when the cry^m mutation was combined with the per^s mutation, which strongly affects the ability of cry^b flies to remain synchronized with the light:dark cycle (Stanewsky et al., 1998), no defect was observed (data not shown). This further demonstrates that CRY^M is functional in terms of phototransduction, unlike CRY^B.

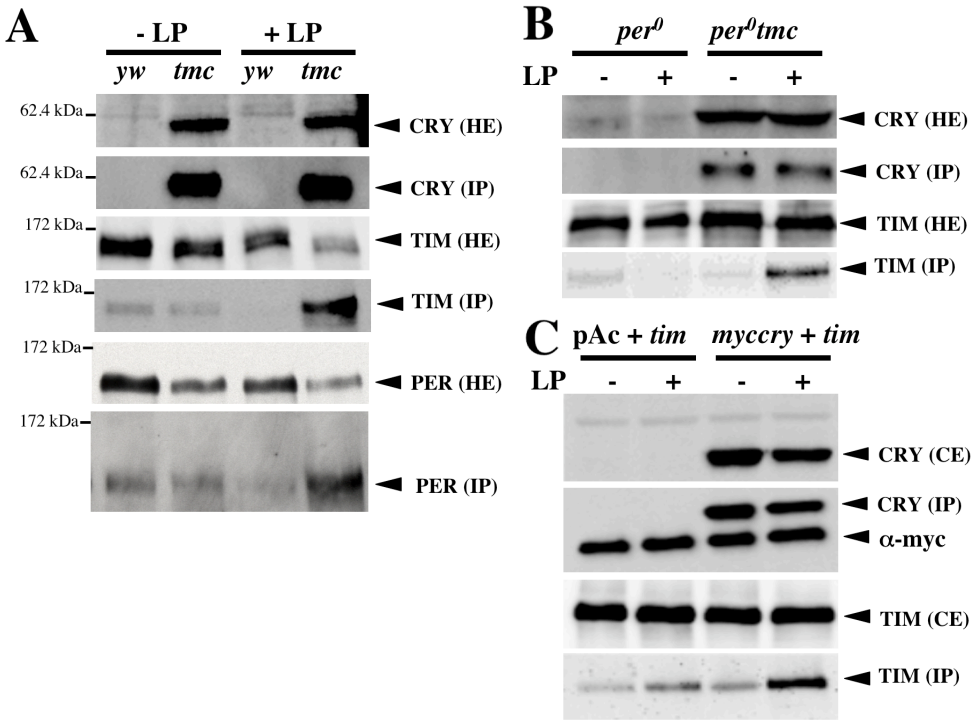
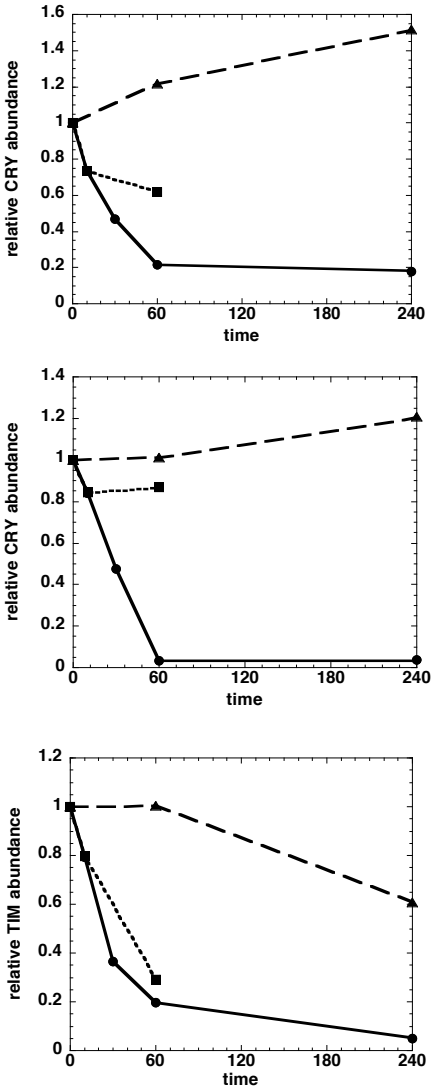


Figure 2-1. Light-dependent interactions among CRY, TIM, and PER *in vivo* and in S2 cells. (**A**) *tmc* flies (see text) and *y w* control flies were subjected to light:dark conditions, light-pulsed (LP) or not for 15 min at ZT 21 (lights are turned on at ZT 0 and turned off at ZT 12), collected, and frozen. Head extracts (HE) were immunoprecipitated with antibody to MYC (IP), and CRY, TIM, and PER levels were measured by Western blot. (**B**) CRY and TIM levels after anti-MYC immunoprecipitation (IP) with per^0 *tmc* and per^0 fly head extracts (HE) light-pulsed or not at ZT 21. (**C**) S2 cells transiently transfected with pAc, pAc-*myc-cry* (*myccry*), and pAc-*tim-HA* (*tim*) were light-pulsed or not, harvested, and their cell extracts (CE) immunoprecipitated with antibody to MYC (IP). CRY and TIM levels were measured by Western blot.

A.


relative CRY abundance
time
relative CRY abundance
time
relative TIM abundance
time

B.

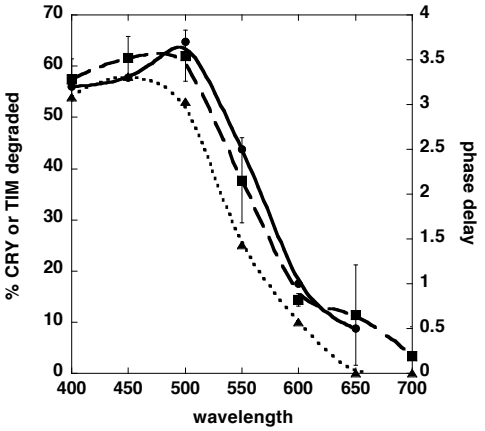
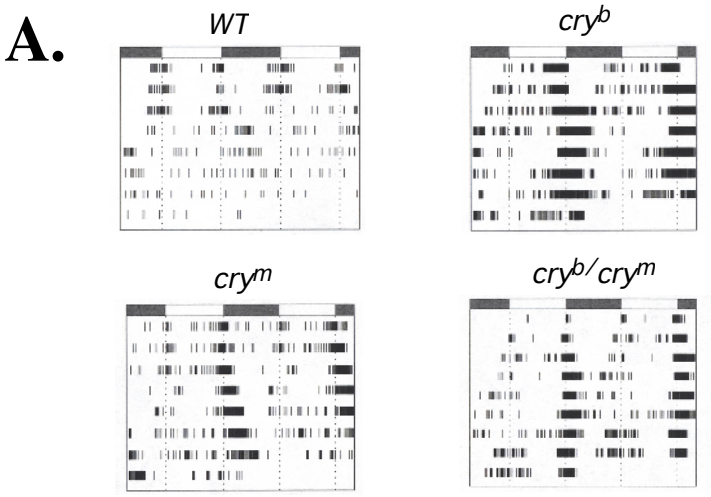
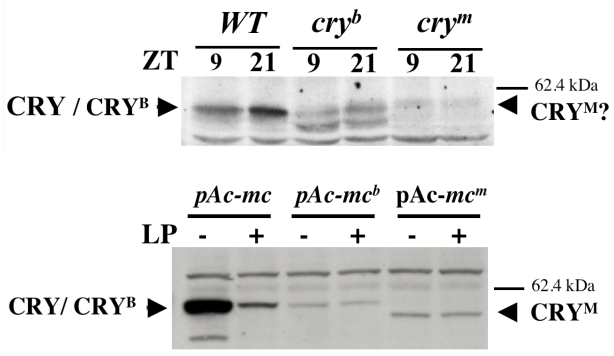

% CRY or TIM degraded
phase delay
wavelength

Figure 2-2. CRY and TIM light-dependent degradation kinetics and spectral sensitivities. (**A**) Flies or CRY-expressing S2 cells were subjected to light pulses of different length (solid lines), subjected to a 10-min light pulse and then placed back in the dark for 50 min (dotted lines), or left in the dark (dashed lines). In flies, the experiments were started at ZT 21. Time after the beginning of the light pulses is indicated. CRY and TIM protein levels were measured by Western blot quantification (fig. S1). Top Panel: CRY degradation in heat-shocked *hs-cry* transfected S2 cells. Center Panel: CRY degradation in flies overexpressing CRY (*y w; tim-GAL4/UAS-cry* flies). CRY overexpressing flies were used here because our CRY antibody has become too weak to accurately quantify CRY levels in wild-type flies. Nonetheless, the qualitative results observed in wild-type flies were similar. Bottom Panel: TIM degradation in wild-type (*y w*) flies. These experiments were reproduced with similar results. (**B**) Spectral sensitivity of CRY light-dependent degradation in S2 cells at an irradiance of 2.5×10^{17} photons cm^{-2} s^{-1} (dashed line). Heat-shocked *hs-cry* transfected cells were pulsed for 1 hour with monochromatic lights of different colors. CRY levels after the pulse were determined by Western blot quantification and compared with the levels obtained in cells that were not pulsed. Data are averages of three independent experiments; SDs are shown. The left *y* axis indicates the percentage of CRY degraded after the pulse; the *x* axis indicates the wavelength of the monochromatic light. The spectral sensitivities of TIM degradation (dotted line) and of behavioral phase response to short light pulse at ZT 15 (solid line) are also shown. The left *y* axis also indicates the percentage of TIM degraded after a light pulse, and the right *y* axis the phase change in hours after a short light pulse at ZT 15.


A.
WT
cry^b
cry^m
cry^b/cry^m


B.
WT
cry^b
cry^m
ZT
9
21
9
21
9
21
CRY / CRY^B
62.4 kDa
CRY^M?
pAc-mc
pAc-mc^b
pAc-mc^m
LP
-
+
-
+
-
+
CRY/ CRY^B
62.4 kDa
CRY^M

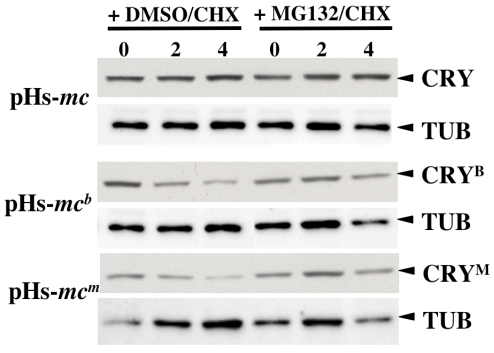
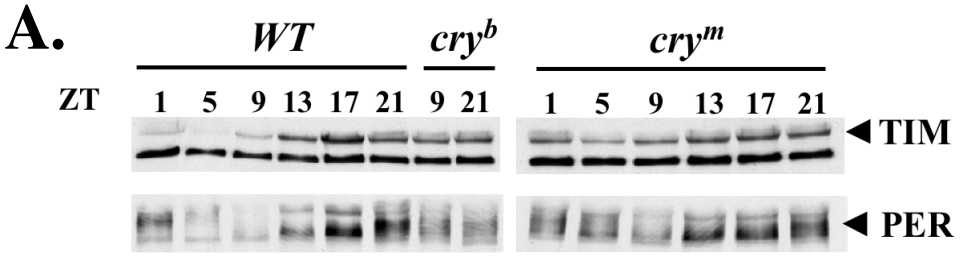
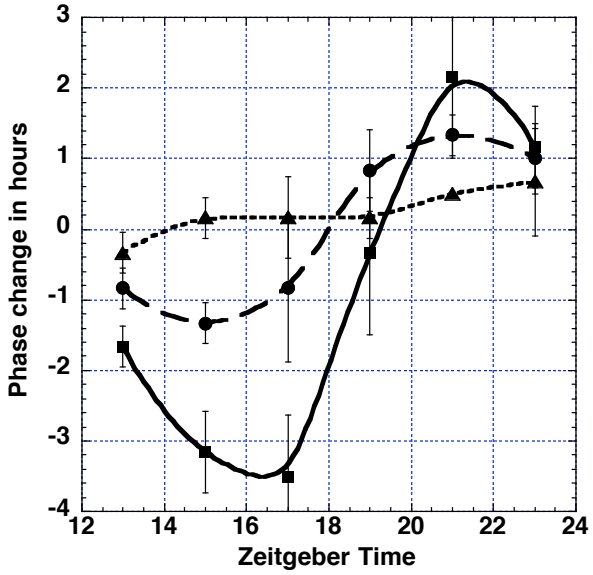

C.
+ DMSO/CHX
+ MG132/CHX
0
2
4
0
2
4
pHs-mc
CRY
TUB
pHs-mc^b
CRY^B
TUB
pHs-mc^m
CRY^M
TUB

Figure 2-3. Isolation of a new *cry* variant: *cry^m*. (**A**) Representative actograms for wild-type (Canton-S strain, *WT*), *cry^b*, *cry^m*, and heterozygous *cry^m/cry^b* flies. Flies were subjected to a light:dark cycle for 3 days and then left under constant light. The arrows indicate when the lights were left on instead of being turned off. (**B**) Top: CRY protein levels in wild-type (Canton-S, *WT*), *cry^b*, and *cry^m* flies measured by Western blot. Flies were kept under light:dark conditions. Flies were collected at the indicated ZTs. CRY^M levels are very low, and the band corresponding to CRY^M could not be identified with certainty. Bottom: CRY, CRY^B, and CRY^M protein levels in S2 cells transfected with pAc-*myccry* (*pAc-mc*), pAc-*myccry^b* (*pAc-mc^b*), and pAc-*myccry^m* (*pAc-mc^m*) vectors, light-pulsed (+) or not (-) for 2 hours. (**C**) CRY, CRY^B, and CRY^M protein levels in heat-shocked S2 cells transfected with pHS-*myccry*, pHS-*myccry^b*, and pHS-*myccry^m* vectors and treated with 50 μM MG132 diluted in dimethyl sulfoxide (DMSO), or with DMSO only. All transfected cells were also treated with cycloheximide (0.5 mg/ml) to block protein synthesis. The drugs were added 2 hours after the heat shock. Time after addition of the drug is indicated in hours. α-Tubulin (TUB) levels were used as loading control. Similar results were obtained when CRY, CRY^B, and CRY^M were expressed with the constitutive actin promoter (pAc vector) (data not shown). Antibodies to CRY [(B), top] and to MYC [(B), bottom, and (C)] were used for the Western blots.


A.
WT
cry^b
cry^m
ZT
1 5 9 13 17 21 9 21
1 5 9 13 17 21
TIM
PER


B.
Phase change in hours
Zeitgeber Time
3
2
1
0
-1
-2
-3
-4
12
14
16
18
20
22
24

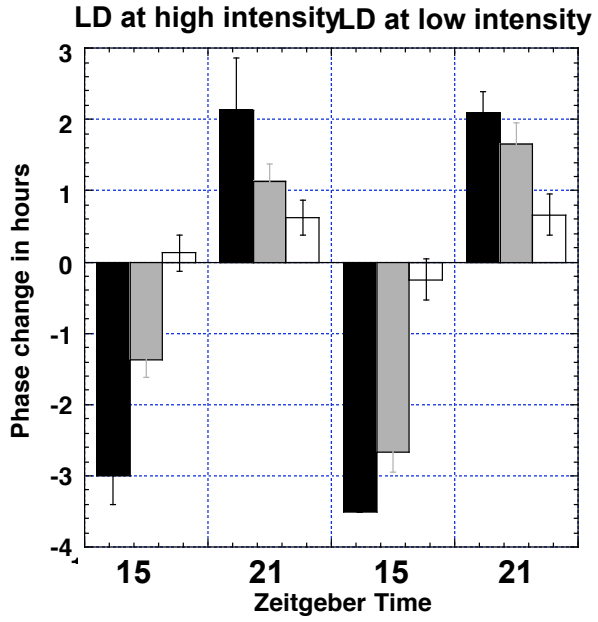

C.
LD at high intensity
LD at low intensity
Phase change in hours
Zeitgeber Time
3
2
1
0
-1
-2
-3
-4
15
21
15
21

Figure 2-4. Circadian photoresponses in *cry^m* flies. (A) TIM and PER levels in wild-type (Canton-S strain, *WT*), *cry^b*, and *cry^m* head extracts. Flies were subjected to three light:dark cycles and collected at the indicated ZTs. These results were reproduced four times. TIM levels were very similar in all experiments, but we noticed a higher degree of variability in PER levels in *cry^m* flies. (**B**) Phase response curve for wild-type (Canton-S strain, *WT*, solid line), *cry^b* (dotted line), and *cry^m* flies (dashed line). Flies were entrained under a 12-hour light:12-hour dark regime. The light intensity during the day was 1000 lux. The flies were then pulsed during the last night of the light:dark regime at 3000 lux for 5 min, and then left in constant darkness. Their phase was compared to those of flies that had not been pulsed. Phase change is plotted on the *y* axis; phase delays and advances are shown as negative and positive values, respectively. The *x* axis represents the ZT of the light pulse. Data are averages of four independent experiments; SDs are shown. (**C**) The responses to short 3000-lux light pulses were measured at ZTs 15 and 21 in wild-type (Canton-S strain, *WT*, black bars), *cry^m* (gray bars), and *cry^b* flies (white bars) exposed to a light:dark regime (LD) with high (1000 lux, left) or low (25 lux, right) light intensities. The *x* and *y* axes are as in (B). Data are averages of three independent experiments; SDs are shown.

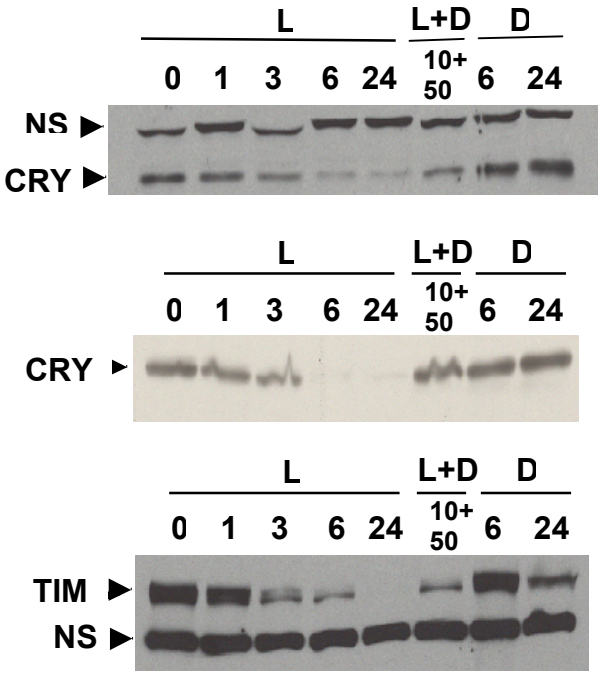


Figure 2-S1. CRY and TIM degradation kinetics. Western blots used for the quantifications shown in Figure 2A. Top: CRY degradation kinetics in S2 cells; Middle: CRY degradation kinetics in flies overexpressing CRY; Bottom: TIM degradation kinetics in wild-type flies. L: protein levels after light pulses of different lengths (in minutes). L+D: protein levels after 10 minutes of light and 50 minutes of darkness. D: protein levels in constant darkness. A non-specific band (NS) is visible in both the upper CRY blot and the TIM blot.

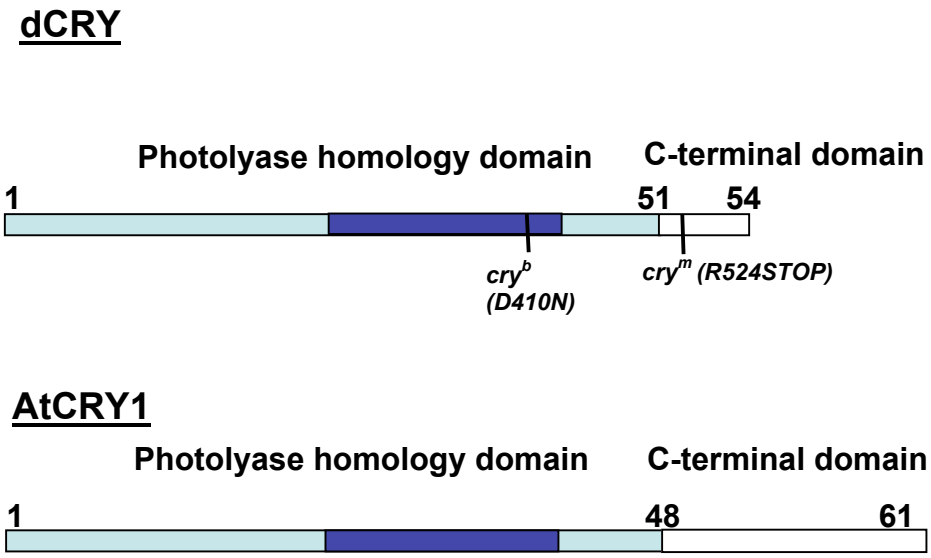


Figure 2-S2. CRY structural domains and mutations. Like most cryptochromes, *Drosophila* CRY (dCRY) and *Arabidopsis thaliana* CRY1 (AtCRY1) contain a photolyase domain (in blue) that includes the Flavin Adenine Dinucleotide (FAD) binding region (dark blue), and a C-terminal domain. The cry^b missense mutation affects a highly conserved FAD binding residue: an ASP residue is mutated to an ASN (D410N). The cry^m mutation changes the ARG524 codon into a STOP codon (R524STOP) that truncates the C-terminal domain.

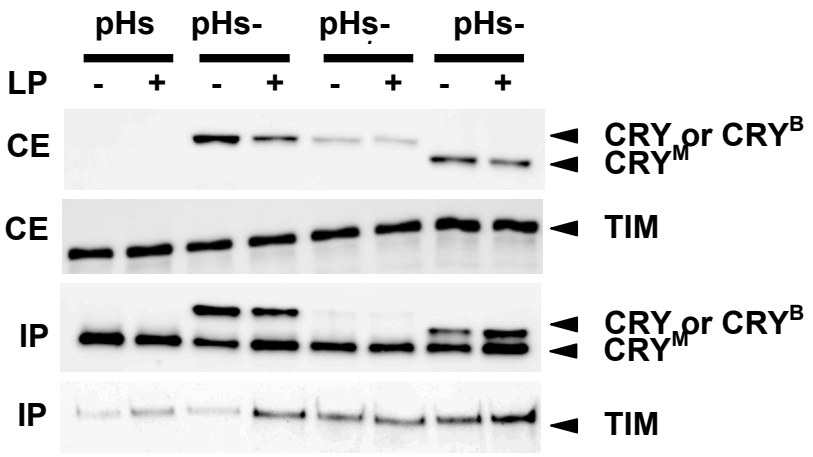


Figure 2-S3. Light-independent interactions between CRY^M or CRY^B and TIM. Heat-shocked S2 cells transiently transfected with pAc-*tim-HA* and either pHs, pHs-*myccry (mc),* pHs-*myccry^m (mc^m)* or pHs-*myccry^b (mc^b)* were light-pulsed or not (LP), harvested, and their cell extracts (CE) immunoprecipitated with anti-MYC antibody (IP). CRY and TIM levels were measured by Western blots. Only a weak CRY^B signal could be detected after immunoprecipitation. Overexposure of the membrane shows that the amount of immunoprecipitated CRY^B is similar in the dark and light-pulsed samples (data not shown). The amount of immunoprecipitated TIM was quantified from four independent experiments, and normalized with the amount of TIM present in the cell extract, and with the amount of immunoprecipitated CRY (if CRY was present). The ratio between the normalized immunoprecipitated TIM amounts under light and dark condition was determined. In the absence of CRY, the ration was 1.2. In the presence of wild-type CRY, CRY^B and CRY^M, the ratio was 4.9, 1.2 and 1.1, respectively. Thus, only with wild-type CRY is the binding to TIM regulated by light.

Genotype	DD			LL-3000 lux			LL-200 lux			LL-25 lux		
	N	% AR	Tau (hours)	N	% AR	Tau (hours)	N	% AR	Tau (hours)	N	% AR	Tau (hours)
WT	32	16	24.6 +/- 0.4	18	100	-	29	100	-	11	100	-
cryb	33	30	23.7 +/- 0.3	25	36	24.2 +/- 0.6	39	18	24.1 +/- 0.6	12	25	23.9 +/- 0.3
crym	24	8	24.5 +/- 0.4	24	25	24.5 +/- 0.4	46	11	25.1 +/- 0.5	12	8	26.6 +/- 0.6

Table 2-1. Circadian behavior of wild-type, cry^b and cry^m flies. The circadian behavior period lengths (Tau) of wild-type (WT, Canton-S strain), cry^b and cry^m flies were measured under constant darkness (DD) and under constant light (LL) at different intensities. N is the number of flies analyzed and %AR is the percentage of arrhythmic flies (power<10, width <2).

CHAPTER III

INTERACTIONS BETWEEN FUNCTIONALLY COUPLED CIRCADIAN NEURONS CONTROL TEMPERATURE SYNCHRONIZATION OF *DROSOPHILA* BEHAVIOR

Ania Busza, Alejandro Murad, and Patrick Emery

This chapter represents a collaborative effort between Alejandro Murad, Patrick Emery, and me. It is an adapted version of a manuscript that was submitted to the Journal of Neuroscience on May 31st, 2007, and is currently under review. The manuscript provides evidence that *Drosophila* can entrain to temperature cycles in constant dark conditions, and that specific circadian neuronal groups function together to regulate different aspects of circadian temperature entrainment. I designed, carried out and analyzed the experiments under the supervision of Patrick Emery. Alejandro Murad provided the immunohistochemical data verifying our neuron-specific genetic rescues and ablations. Patrick Emery and I wrote the text together.

A. Abstract

Most animals rely on circadian clocks to synchronize their physiology and behavior with the day/night cycle. Light and temperature are the major physical variables that can synchronize circadian rhythms. While the effects of light on circadian behavior have been studied in detail in *Drosophila*, the neuronal mechanisms underlying temperature synchronization of circadian behavior have received less attention. Here, we show that temperature cycles synchronize and durably affect circadian behavior in *Drosophila* in the absence of light input. This synchronization depends on the well-characterized and functionally coupled circadian neurons controlling the morning and evening activity under light/dark cycles - the M-cells and E-cells. However, circadian neurons distinct from the M and E-cells are implicated in the control of rhythmic behavior specifically under temperature cycles. These additional neurons play a dual role: they promote evening activity and negatively regulate E-cell function in the middle of the day. We also demonstrate that although temperature synchronizes circadian behavior more slowly than light, this synchronization is considerably accelerated if the M-cell oscillator is absent or attenuated. Thus, while the E-cells show great sensitivity to temperature input, the M-cells and their robust self-sustained pacemaker act as a resistance to behavior synchronization by temperature cycles. In conclusion, the specific cellular and molecular properties of individual neurons and their organization in a network determine the responses of circadian behavior to temperature cycles, and the precise timing of locomotor activity.

B. Introduction

Like most organisms, the fruit fly *Drosophila melanogaster* uses a circadian clock to synchronize its physiology and behavior with the day/night cycle. Many of the molecular and cellular components of this internal pacemaker have been identified. The products of the clock genes *period*, *timeless*, *clock*, and *cycle* form a transcriptional feedback loop; a set of kinases and phosphatases adjusts the period of this oscillator to approximately 24 hours (Hardin, 2005). Intracellular signaling through the photoreceptor CRYPTOCHROME and synaptic input from visual organs synchronize the circadian clock to the light cycle (Helfrich-Förster et al., 2001). At a cellular level, over 150 neurons in the *Drosophila* brain have been shown to express oscillating circadian gene products (Kaneko and Hall, 2000; Shafer et al., 2006).

Under a light:dark (LD) cycle, *Drosophila* exhibit a bimodal locomotor activity pattern with Morning (M) and Evening (E) surges of activity. Specific circadian neurons contribute to these peaks of activity (Grima et al., 2004; Rieger et al., 2006; Stoleru et al., 2004; Stoleru et al., 2005; Yoshii et al., 2004). The ventral lateral neurons (vLNs) control the morning activity peak and are consequently also referred to as "M-cells". The dorsal lateral neurons (dLNs), the PDF negative vLN and possibly some Dorsal Neurons 1 (DN1s) are responsible for the evening peak and are thus called "E-cells" (Grima et al., 2004; Rieger et al., 2006; Stoleru et al., 2004). Other circadian cell groups include the

lateral posterior neurons (LPNs) and other dorsal neurons (DN2s and DN3s), about which much less is known (Kaneko and Hall, 2000; Shafer et al., 2006).

Intriguingly, environmental inputs affect the relative contribution of different brain neurons to specific attributes of circadian activity, and this regulation may aid *Drosophila* to adapt to seasonal changes in day length (Miyasako et al., 2007; Murad et al., 2007; Stoleru et al., 2007). Under constant dark (DD) conditions, the vLNs are necessary and sufficient for behavioral rhythmicity (Grima et al., 2004; Renn et al., 1999) and dictate the period of circadian behavior (Stoleru et al., 2005). Under constant light, however, the E-cells and a subset of DN1s can function as pacemaker neurons (Murad et al., 2007; Stoleru et al., 2007). Moreover, the respective contribution of the M and E-cells to the control of circadian behavior depends on the length of the photoperiod (Stoleru et al., 2007). In addition, separate cell groups may be differentially sensitive to temperature or light/dark inputs when these cues are simultaneously present (Miyasako et al., 2007).

Most of the work on how *Drosophila* synchronizes their clocks to environmental cycles has centered on light input pathways. However, temperature fluctuations can also reset circadian clocks. For example, temperature can dominate light input in the model organism *Neurospora* (Liu et al., 1998). In mammals, temperature cycles that mimic body temperature oscillations help keeping peripheral clocks synchronized (Brown et al., 2002). In *Drosophila*, temperature cycles synchronize eclosion rhythms (Pittendrigh, 1954) and locomotor activity rhythms in constant darkness (Wheeler et al., 1993; Yoshii et al., 2002) and constant light (Stanewsky et al., 1998; Glaser and Stanewsky, 2005;

Yoshii et al., 2005). Additionally, temperature cycles synchronize molecular oscillations in peripheral clock tissues (Glaser and Stanewsky, 2005) and in brain circadian neurons (Yoshii et al., 2005). To understand further how temperature synchronizes *Drosophila* behavior, we studied how circadian neural groups and the intercellular network that connects them contribute to thermal entrainment.

C. Results

1. Temperature is a Zeitgeber for circadian locomotor rhythms.

To study the neuronal mechanisms underlying synchronization of *Drosophila* behavior by temperature cycles, we decided to perform the majority of our experiments in constant darkness (DD). These conditions allow us to study specifically temperature synchronization in the absence of light input, and also to monitor the effects of temperature cycles on the circadian pacemaker underlying rhythmic behavior (see below). This cannot be done under constant light (LL), which was used in past studies (Glaser and Stanewsky, 2005; Yoshii et al., 2005), because circadian rhythms immediately degenerate under LL conditions after return to constant temperature.

Temperature cycles of as little as 3°C have been shown to synchronize locomotor activity in DD (Wheeler et al., 1993). To verify that this is due to a genuine effect on the circadian clock as opposed to a temporary "masking" effect of temperature variations on behavior, we looked for changes in circadian phase that persisted after temperature entrainment. Using 12hr/12hr 29ºC/20ºC thermophase/cryophase (TC) temperature

cycles in DD, we phase-advanced or phase-delayed wild-type flies [Figure 3-1A] that had been previously synchronized to a light:dark (LD) cycle. After several days of TC, we observed a robust evening peak of activity that anticipated the temperature transition, suggesting that the circadian clock underlying the evening activity had been re-entrained by the temperature cycle. Anticipation of the morning temperature transition was visible in some of our experiments (see for example the right panel of Figure 3-1A), but was usually of much smaller amplitude than that observed in an LD cycle, and sometimes not detectable. This is probably because morning activity is dampened at lower temperatures, as previously shown in LD studies at different constant temperatures (Miyasako et al., 2007). Therefore, to begin our analysis of phase-shifting effects of TC on circadian behavior, we measured each fly's daily evening peak and compared it to the evening peaks of control flies left in constant conditions (20ºC DD) [Figure 3-1B]. On the last day of LD, the fly's evening activity peaked at approximately ZT12 (or "Zeitgeber Time" 12, where ZT0 refers to the Lights-On time during LD). The evening activity peak then drifted to approximately ZT 11 by the 4th day of DD (day 6). After 5 days of TC, flies exposed to a 9-hr advance TC had evening peaks 8 hours earlier than the control flies, showing that their behavior had been re-synchronized. Conversely, flies exposed to a 6-hr delay TC had evening peaks 6 hours later than controls. All fly groups showed a daily advance when released into constant 20ºC DD because our *y w* wild-type flies have a period slightly shorter than 24 hours in constant conditions. Importantly, however, the phase advance and delay of the TC-exposed groups is maintained after releasing the flies into constant conditions. This demonstrates that the circadian clock,

and not just behavior, has been synchronized by the temperature cycles. Temperature is thus a Zeitgeber (time-giver) for adult circadian behavior, i.e. it is an input that can durably affect the phase of circadian behavior, even after return to constant conditions. It also provides evidence that temperature fluctuations can affect the clock in the absence of any light input. Interestingly, synchronization under a TC is much slower than under an LD cycle. With an 8-hr advanced TC, it takes over 6 days to reach a completely stable behavior phase, while it takes 5 days with an 8-hr delay ([Figure 3-S1]. Light synchronization is significantly faster and takes at most two days [Figure 3-S2], at least in the conditions used here (200 lux during the day, total darkness during the night). An additional difference between light and temperature entrainment is the final phase of the evening peak of activity if the lights-on and the temperature-up transitions are used as time references. We found that during a TC cycle in constant darkness, the evening peak is a few hours earlier than in an LD cycle. This is consistent with the fact that in nature, temperature variations usually lag behind the light cycle (Boothroyd et al., 2007).

We observed slow displacement of the evening peak between day 1 and day 4 of TC (transients). Wondering if the phase of this peak reflects the state of synchronization of the underlying circadian pacemaker, we released flies entrained to 1-4 days of TC in constant temperature and determined the phase of their free-running behavior [Figure 3-1C]. The phase of the free running behavior after 1-4 days of TC matches the phase of transients of the evening peak observed during TC synchronization [Figure 3-1B]. Thus, we conclude that the phase of the evening peak of activity accurately reflects the state of synchronization of the underlying circadian oscillator.

Depending on the relative time an animal is exposed to an environmental input, its circadian clock may advance, delay, or not respond to the stimulus (Bruce et al., 1960). For example, flies respond to short light pulses by delaying their clock following an early night pulse, and by advancing it in response to a late night pulse. A light pulse during the subjective day does not phase shift the clock (Pittendrigh, 1967). To determine if the *Drosophila* clock has a different response to a temperature cycle depending on when it experiences the warm temperature, wild-type flies entrained at constant 20°C were exposed to 29°C for 12 hours at different times of subjective night and day during constant darkness. The resulting phase-shifts (relative to control flies continuously maintained at 20°C) were graphed as a Phase Response Curve (PRC)[Figure 3-2A]. Maximum phase shifts were elicited when the 29°C exposure began in late subjective day (delay of 3 hours at ZT 11) and in the mid/late subjective night (advance of 2.5 hours at ZTs 17, 19, 21). Strikingly, 12-hour 29ºC exposure beginning at ZT13 versus ZT15 elicits very different phase shifts (2 hours delay and 1 hour advance, respectively), despite 10 hours of thermophase overlap. 29ºC exposure starting early in the subjective day (eg, ZT 1) elicited almost no phase shift, presumably because it coincided with the time of day when the animal expects its environment to become warmer. Warm temperature pulses of as little as 3 hours 29°C at the maximally sensitive times (ZTs 11 and 19) were sufficient for eliciting phase-shifts, however not as effectively as 9 or 12 hour warm pulses (data not shown). A 6 hour pulse PRC was also generated, and showed a similar shape: greater responses occurred with pulses initiated at ZT11 and ZT19 [Figure 3-2B]. Previous studies on *Drosophila pseudoobscura* eclosion rhythms show a

similar phase-shifting curve in response to 12 hours at 28ºC, although with a reduced phase-delay during the subjective day (Zimmerman et al., 1968). That exposure to warm temperature can elicit responses with directionality and amplitude dependent on the state of the circadian clock reinforces the notion that temperature is a proper Zeitgeber for circadian behavior in *Drosophila*.

Since it has recently been shown that CRY mediates specific responses to temperature, such as high temperature heat pulse (37ºC; Kaushik et al., 2007), we tested whether CRY is necessary for responses to 29ºC pulses or for temperature entrainment. These circadian thermal responses were normal in cry^b mutant flies (Stanewsky et al., 1998), which have no functional CRY [Figure 3-2B]. Thus, CRY is not required for temperature entrainment at physiological range.

2. The PDF+ M-cells are necessary for persistence of temperature-synchronized circadian behavior.

Under an LD cycle, the PDF positive vLNs are primarily responsible for the anticipatory behavior of flies at dawn and are thus called M-cells, while a set of more dorsally located E-cells are responsible for the evening activity (Grima et al., 2004; Stoleru et al., 2004). In addition, the M-cells maintain circadian rhythms in constant darkness (Grima et al., 2004; Renn et al., 1999). We used genetic techniques to ablate specific subsets of circadian neurons or rescue their clock function in an arrhythmic genetic background to determine the respective function of these groups of cells under TCs in constant darkness. To study the role of the PDF+ cells, or M-cells, we first

ablated the vLNs by driving the pro-apoptotic gene *hid* in these neurons with the *pdf-GAL4* driver [Figure 3-3B](Renn et al., 1999). A brief surge of activity at the beginning of the thermophase was observed. Since it was present in all genotypes, even those without a functional clock, it is a non-circadian response to the temperature transition (masking). No anticipation of the morning temperature change could be detected, but this absence of anticipation is not definitive proof that the M-cells play a role in morning anticipatory activity, since even in wild-type flies this activity cannot be reliably detected. In contrast, the surge of activity in late thermophase in the flies lacking M-cells strongly anticipates the temperature transition and is thus probably a circadian evening peak. This was also observed in a previous study using temperature cycles in constant light conditions (Yoshii et al, 2005). We will discuss the nature and the control of the evening peak in detail below. Importantly, there was no persistence of circadian behavioral rhythms upon return to constant temperature [Figure 3-3B]. Thus, the M-cells are critical for long-term behavioral rhythmicity after temperature synchronization. As pdf^{01} flies have the same phenotype as flies without M-cells [Figure 3-3C], the PDF neuropeptide is required for the function of the M-cells under TC.

3. The PDF+ M-cells are sufficient for long-term synchronization of circadian behavior after exposure to temperature cycles.

To determine whether the M-cells can independently maintain TC-entrained circadian behavioral rhythms, we rescued PER expression only in these cells in per^{0} flies (Grima et al., 2004). These mosaic flies were able to remain rhythmic under constant

dark after a TC [Figure 3-3E], while the *per*^{0} control flies could not [Figure 3-3D] because of a single point mutation leading to a premature stop codon in *per* (a null allele). Notably, the activity was clearly re-synchronized by the temperature entrainment. Therefore, a functional clock in the M-cells is sufficient to maintain circadian rhythms after exposure to TC and for the phase-shifting effects of temperature cycles. These cells can thus receive information about temperature, but whether this information is obtained through a cell-autonomous thermoreceptor or synaptic input from peripheral sensors, or both, remains to be determined. We noted that the persistent rhythmic activity after synchronization to a TC was concentrated in the subjective morning, as observed after an LD cycle (Grima et al., 2004). This suggests that the M-cells are mostly generating morning activity after exposure to TC cycles, as during or after exposure to an LD cycle. Thus, although the difficulty in detecting the anticipatory morning behavior under TC may be due to an inhibitory effect of the colder temperature (negative masking), it could also indicate that the onset of this peak is slightly later in TC than in LD, and is positively masked by the surge of activity at the beginning of the thermophase.

4. The evening activity peak is controlled by the circadian clock and the E-cells under temperature cycles.

As mentioned above, an evening peak of activity is present in flies without M-cells and in flies missing PDF under TC. A similar evening peak is seen in flies of the same genotypes exposed to an LD cycle, and it has been shown that after exposure to LD cycles this peak persists for about two days (Renn et al., 1999). For unknown reasons,

we had difficulties detecting this short-term persistence of the evening peak after both LD and TC cycles when PDF or the M-cells were missing. In some experiments, however, evening peak persistence after TC was clearly visible, which suggests that the circadian clock controls this peak. To confirm that this peak is indeed regulated by components of the circadian system, we ablated the M-cells in flies carrying arrhythmic or period-altering mutations in the circadian gene *period*: per^0, per^S and per^L. In order to easily visualize the evening peak in the long period mutant, we used a temperature cycle with a longer day and a shorter night (18 hours 29°C, 6 hours 20°C). The longer thermophase prevents the peak of activity of per^L flies from occurring in the cryophase and being suppressed by cold phase negative masking, as it would in a standard 12-hr:12-hr TC. Indeed, a peak is present in all three genotypes during the 18-hr long thermophase [Figure 3-4]. Its location relative to the temperature cycle is earlier in per^S than in wild-type flies, but later in per^L, demonstrating that the evening peak is timed by a *per*-dependent mechanism, presumably some component of the molecular clock. A peak of activity is also observed in per^0 under this particular TC, as well as under 12 hr/12hr TC [Figure 3-3D and Figure 3-4D] as previously described (Yoshii et al., 2002). The phase of this peak is abnormal; it is much earlier than the peak observed in any other *per* allele, even per^S. Thus, the *per*-dependent circadian molecular machinery plays an important role in properly gating the activity of neurons controlling evening locomotor activity under TC cycles.

We then used a combination of *cry-GAL4* and *UAS-hid* transgenes to create flies with both the "M" and "E" cells ablated (Stoleru et al., 2004). Immunocytochemical

staining for PER was performed to determine which circadian neurons were ablated (data not shown). As previously described (Stoleru et al., 2004), the DN1s, DN2s and DN3s are still present in these flies. However, the large number of DN1 and DN3 cells do not allow us to rule out that a subset of Dorsal Neurons is ablated. Based on Stoleru et al. (2004), it is actually likely that at least 2 DN1s are ablated. The LPNs were not ablated. As expected, the vLNs were missing. Most dLNs were missing, although we cannot entirely exclude that a subset of dLNs escape ablation (the dLNs are close to the DN3 groups, one or two residual dLNs could thus be mistaken for DN3 cells). Importantly, behavioral data verify that the evening peak is completely missing in LD [Figure 3-5B], which means that all E-cells have been eliminated. The *cry-GAL4*/*UAS-hid* flies showed no morning or evening peak of activity in TC, other than the brief startle response after the temperature increase [Figure 3-5B]. They had however a relatively high level of activity throughout the day.

To further confirm the role of the E-cells in TC, we rescued per^0 flies in the M and E-cells with the *cry-GAL4* and *UAS-per* transgenes. We determined in which cells PER was expressed and found as expected from Stoleru et al. (2004) that the vLNs had rescued PER oscillations [data not shown]. We found that PER expression was rescued in 3-4 dLNs, and 2 DN1s. This pattern of PER expression was sufficient to fully rescue LD behavior: both the M and the E peak look similar to wild-type. In addition, like PER rescue in only the M-cells, *cry-GAL4* driven PER rescue restored rhythmicity in constant conditions after both LD and TC cycle. However, during TC, these flies had a later evening peak of activity than that observed in M-cell rescued flies, with a phase closer to

that of the wild-type evening peak [compare panels A, C and D of Figure 3-5]. Thus, in TC as in LD, the E-cells play an important role in controlling the evening activity peak. Rescuing *per* expression only in the E-cells restored the evening peak [Figure 3-5], which for unknown reason was sharper than in flies with both the M and E-cells rescued. As expected, this peak does not persist after release to constant temperature. Therefore, the cells controlling evening activity under LD cycle play an important role in controlling evening activity under TC, but cannot independently maintain rhythmicity in constant conditions.

5. Circadian neurons that are neither the M-cells nor the E-cells contribute to the control of circadian behavior.

The previous sections demonstrate that the cells controlling circadian behavior during and after LD also play an important role during and after TC. However, we noted that when PER is expressed in the M and E-cells only (in per^{0}*; cry-GAL4/UAS-per* flies), circadian behavior is not normal in phase and duration under TC, despite appearing fully rescued under LD cycles [Figure 3-5D]. More specifically, under TC the evening peak begins earlier and lasts longer, as if the network regulating activity is not well tuned. This suggests the intriguing possibility that in wild-type flies other circadian neurons may contribute to regulating locomotor behavior specifically under TC.

We re-examined our *cry-GAL4/UAS-hid* fly data and found that individual flies sometimes exhibited a small surge of activity in late TC. To better investigate the evening activity in these flies and avoid the inhibitory masking effects of the cold phase, we

repeated the experiment using the long thermoperiod/short cryoperiod assay. Again, we saw a subtle but consistent minor peak in the evening of individual flies in three independent experiments. Could such a subtle yet consistently appearing peak be evidence of activity of non-M, non-E pacemaker cells during TC? To investigate the nature of this peak, we examined per^S and per^L mutant flies with both M and E-cells ablated (with *cry-GAL4* and *UAS-hid*) under these 18hr/6hr TC conditions. The minor peak is even more apparent in the per^S and per^L backgrounds, and its timing is earlier in the per^S background and later in per^L. Most strikingly, it is absent in per^0, which strongly suggests that it is controlled by circadian cells [Figure 3-6A]. Additionally, a similar peak was seen in 18hr/6hr TC conditions under constant light [Figure 3-6E]. Thus, we believe that by removing the well-characterized M and E-cells, we have uncovered indications of circadian neurons that use a *per*-dependent timing mechanism to contribute to the evening activity specifically under temperature cycles.

6. The M-cells modulate the E-cells' response to temperature cycles.

Having demonstrated that at physiological temperature ranges, TCs phase-shift circadian rhythmicity much more slowly than LD cycles [Figure 3-S2], we decided to use the relative rate of entrainment to study how responsive the M and E oscillators are to temperature. We examined how flies without M-cells synchronize to a temperature cycle. Determining the phase of behavior after TC was not possible, because these flies very rapidly become arrhythmic. We therefore measured the evening peak phase during TC in M-cell ablated flies, since we have shown that this peak is controlled by the

circadian clock and is not due to a masking phenomenon. Unexpectedly, this activity peak re-synchronized very rapidly to TCs when the M-cells were absent [Figure 3-7A]. Therefore, intact intercellular communication from the M-cells is necessary to modulate the response of evening oscillator clocks to temperature cycles, and thus prevents circadian behavior from excessively rapidly responding to temperature inputs. This modulation requires PDF, since flies without this neuropeptide also rapidly synchronize to temperature cycles [Figure 3-7A].

7. Attenuating the oscillator in the M-cells leads to abnormally fast entrainment to TCs.

The results described above strongly suggest that the M-cells play an important role in determining the pace at which circadian behavior is synchronized by temperature. Strikingly, we observed that when we overexpress PER with *pdf-GAL4* in the vLNs of otherwise wild-type flies, synchronization to temperature cycle was considerably accelerated [Figure 3-7B]. We exposed *y w; pdf-GAL4/UAS-per flies* to 2 days of temperature cycles that were 8 hours advanced relative to the LD entrainment. After they were released into constant conditions we measured their behavioral phase and compared it to flies that had not been exposed to temperature cycles or that had been exposed to 6 days of temperature cycles. We found that unlike wild-type flies that had only phase-advanced 3 hours after two days of TC, flies overexpressing PER only in the vLNs had almost completely entrained to the TC cycle and had the same phase as flies that had been exposed to 6 days of TC [Figure 3-7B]. This confirms that the vLNs are the cells

determining the rate of synchronization to temperature, slowing it down in wild-type flies.

A likely explanation for the effect of PER overexpression on the kinetics of synchronization is a reduction in the robustness of the molecular circadian pacemaker. We actually observed that *y w*; *pdf-GAL4/UAS-per* flies have a 1hr longer period phenotype (24.8±0.1) compared to control flies (*y w*: 23.8±0.2). Excessive PER levels are known to be disruptive to circadian rhythms (Kaneko and Hall, 2000; Zeng et al., 1994), and a weaker oscillator is predicted to be more responsive to environmental perturbation (Pittendrigh et al., 1991). To investigate further whether oscillators with attenuated molecular oscillations are more vulnerable to temperature entrainment, we measured the rate of entrainment of Clk^{Jrk}/+ heterozygotes, since these mutants have decreased oscillations in *per* and *tim* transcription and PER and TIM protein cycling (Allada et al., 1998). We exposed Clk^{Jrk}/+ heterozygotes to the same protocol as that used for *pdfGAL4/UAS-per* flies. We found that similarly to flies overexpressing PER in the vLNs, the Clk^{Jrk}/+ heterozygotes were much more responsive to temperature and had almost completely entrained to the TC cycle after 2 days of TC [Figure 3-7C]. Combined, our results indicate that robust molecular oscillations in the M-cells protect *Drosophila* from reacting excessively to temperature cycles.

D. Discussion

Over the past ten years, studies have demonstrated that the cell-autonomous nature of circadian rhythms is not restricted to unicellular organisms, but is also a fundamental characteristic in multicellular organisms (Dunlap, 1999). In the case of *Drosophila*, evidence strongly suggests that even circadian environmental inputs can be detected in a cell-autonomous manner. Light, for example, is detected by the intracellular photoreceptor CRY in brain neurons and peripheral tissues (Emery et al., 2000a). Even tissues that are "circadianly blind" can become light sensitive if they are forced to express CRY (Rush et al., 2006). Moreover, dissected body parts such as wings or legs are not only sensitive to light, but can also detect temperature cycles (Glaser and Stanewsky, 2005; Plautz et al., 1997). It is therefore likely that a cell-autonomous thermosensor provides the circadian clock with temperature information. A candidate for such function is CRY, since it is needed for specific behavioral thermal responses (Kaushik et al., 2007). However, CRY is not required for temperature entrainment: cry^b flies can molecularly entrain to temperature cycles (Glaser and Stanewsky, 2005; Stanewsky et al., 1998), and we did not find any behavioral entrainment defects when using physiological temperature. CRY's function in temperature responses might thus be more relevant to integration between light and temperature inputs and the phenomenon of temperature compensation at physiological temperature in wild-type flies (Kaushik et al., 2007).

If *Drosophila* circadian neurons can be synchronized and function cell-autonomously, then why are they organized in a network with cells influencing each

other? A previous proposal is that the circadian neuronal network is necessary to maintain stable circadian rhythms in constant conditions. This is supported in *Drosophila* by data showing that without proper neural circuitry, individual neurons cannot keep a proper amplitude and phase for their circadian oscillations in the absence of external inputs (Lin et al., 2004; Peng et al., 2003). However, as only a minority of organisms actually experience constant conditions during their lifetime, it is unclear what importance this function has in natural conditions. Recent results demonstrate the importance of the circadian neuronal network for adaptation to changes in photoperiod lengths in *Drosophila* (Stoleru et al., 2007) and mammals (VanderLeest et al., 2007). In *Drosophila*, this adaptation is the result of the interactions between two groups of functionally coupled circadian neurons: the M-cells and E-cells. Our results suggest that a robust self-sustained pacemaker is important for mitigating the resetting effects of inputs such as temperature, and further demonstrate the importance of the circadian network in the response to environmental cues. Indeed, we have identified two neuronal interactions between groups of circadian cells that are essential for proper responses to temperature cycles [Figure 3-8].

The first interaction involves the aforementioned M and E-cells and determines the pace at which circadian behavior is synchronized to temperature. *Drosophila* behavior responds slowly to temperature cycles (TC). Nevertheless, specific neurons can respond much more rapidly. The E-cells are very rapidly synchronized to TC if they are disconnected from the M-cells. It is actually the M-cells alone that set the pace of behavioral synchronization to temperature cycles, at least in DD conditions. Indeed,

increasing PER levels only in the M-cells results in a considerably accelerated synchronization of circadian rhythms. Thus, circadian clocks can be highly sensitive to temperature input, but the pacemakers of some specific cells are more resilient. They prevent flies from overreacting to temperature change. This is probably important in a natural environment in which *Drosophila* can experience erratic variations in temperature due to weather changes. Since the E-cells can also influence the M-cells (Stoleru et al., 2007), it is likely that their sensitivity to temperature cycle can be used to fine-tune the synchronization of the M-cells to the environment under specific conditions, particularly under long photoperiod.

As mentioned above, we found that manipulating the circadian pacemaker of the M-cells accelerates synchronization. We did this by increasing PER levels with the *pdf-GAL4* driver, which should at least double PER levels in the M-cells (Grima et al., 2004). Our interpretation is that we have weakened the pacemaker with this manipulation, and 1hr period lengthening observed is consistent with this notion. Indeed, high PER levels result in increased transcriptional repression in the molecular circadian feedback loop that can in some case even completely eliminate molecular and behavioral rhythms (Kaneko and Hall, 2000; Zeng et al., 1994). Moreover, a weakened oscillator is predicted to respond more strongly to environmental input (Pittendrigh et al., 1991). In mammals, mutants with attenuated oscillators have been found to have stronger circadian responses to light pulses (Vitaterna et al., 2006).

There is at least one alternative interpretation. Since the GAL4/UAS system is more active at higher temperature, we could have artificially created a temperature

induced *per* mRNA cycling that could have contributed to accelerate the rate of synchronization. However this possibility is highly unlikely. First, the 8 hour advanced temperature cycle we used in Figure 3-7 would be predicted to delay the phase of *per* mRNA oscillation, not advance it. Thus, synchronization should have been slower had temperature significantly affected PER expression. Second, we also observed that the kinetics of synchronization is accelerated with a delayed TC (data not shown). That the two opposite directions of resynchronization are affected similarly by PER overexpression virtually excludes the possibility that the effect of temperature on the GAL4 system could have significantly affected the kinetics of synchronization. Moreover, we do not see any obvious phase change in behavior when comparing wild-type and flies overexpressing PER once stable entrainment is reached, again strongly arguing against a significant effect of temperature on the phase of PER cycling. Thus, any effects that the temperature sensitivity of the GAL4/UAS system could have on the phase of PER cycling is most likely superseded by the circadian regulation of PER level and the circadian synchronization of temperature cycling. In addition, we obtained confirmation that flies with attenuated pacemaker are much more sensitive to temperature. Indeed, flies heterozygous for the Clk^{Jrk} mutation also phase shift their clock very rapidly when exposed to a temperature cycle.

Thus, the picture emerge that a strong, self-sustained pacemaker in the M-cells is required for proper response to temperature cycle. It keeps other oscillators controlling circadian behavior from responding excessively to temperature changes. These results fit well with those of a recent study in which flies were exposed simultaneously to

temperature and light cycles, with the temperature cycle 6 hours advanced relative to the light cycle (Miyasako et al., 2007). The PDF positive vLNs (M-cells) and dLNs and the PDF negative vLN (E-cells) followed the light cycle. Our interpretation is however different from that of Miyasako and colleagues. These authors concluded that the M- and E-cells are light sensitive, but not, or only weakly, temperature sensitive. First, our results show that the E-cells (as defined in Stoleru et al., 2004) are actually highly sensitive to temperature cycles. Second, the M-cells also clearly detect and respond to temperature cycles, since they are sufficient for persistent temperature synchronization. Moreover, they determine the response of circadian behavior to temperature cycles.

We also present behavioral evidence suggesting that some non-M, non-E-cells are specifically involved in the control of circadian behavior when temperature cycles are present. An E-cell independent, M-cell independent peak was clearly detected under a temperature cycle, although only with a long thermophase. This could suggest that these cells are active only during long warm days. However, close examination of some of our experiments under regular 12hr:12hr TC indicates that this peak is actually present, but difficult to distinguish from the relatively high masking activity seen when both the E and M-cells are ablated (data not shown). The circadian clock controls this TC specific peak of activity, since PER mutations displace its phase. The peak was observed under both constant darkness and constant light, indicating that its absence in an LD cycle is not due to a negative masking effect of light. There are therefore circadian neurons that contribute to the control of the evening activity, but specifically under temperature cycle. Previous ICC data have suggested that some circadian neurons may be specifically

temperature specific: Miyasako and colleagues also showed that there are cells that do respond strongly to TC when exposed simultaneously to LD and TC, and that a subset of them might influence circadian behavior. Since the LPNs and the DN2s are not ablated in our experiments and are particularly sensitive to temperature cycles (Yoshii et al., 2005; Miyasako et al., 2007), they are strong candidate for playing a temperature specific function in the control of circadian behavior.

Interestingly, non-M, non-E temperature sensitive cells are also necessary for properly timing the activity of the E-cells. In per^0 flies, a peak of activity is present in the middle of the day (see also Yoshii et al., 2002). This peak is caused by improper activity of the E-cells, since it is present in per^0 flies without M-cells, but entirely disappears when both the M and E-cells are ablated. When PER expression is rescued in the M and E-cells of per^0 flies, circadian activity is not normal under a temperature cycle, despite being perfectly rescued in LD. There is still abnormally high levels of activity in the middle of the thermoperiod. These results indicate that a group of circadian neurons are necessary for properly timing the activity of the E-cells under temperature cycle. They probably inhibit the E-cell output pathway, although we cannot exclude that they adjust the phase of the molecular E pacemaker. The same cells that positively participate in the evening peak under temperature cycles might be responsible for properly phasing the output of the E-cells, or two distinct groups of cells might be responsible for activation and inhibition of locomotor behavior under TC.

In conclusion, our results demonstrate that the functional coupling of different circadian groups of neurons is essential to the proper timing of behavioral activity under

temperature cycles, as well as modulating the pace of synchronization so that *Drosophila* do not overreact to temperature changes. It will be interesting to determine whether light synchronization is similarly modulated by such neural interactions in *Drosophila*. Our data add evidence to the emerging notion that the neural circuitry connecting circadian neurons is essential to the adaptation of behavior to the environment (Stoleru et al., 2007; VanderLeest et al., 2007).

E. Materials and Methods

1. *Drosophila* strains and transgenics.

Flies with targeted neuronal ablation of the M-cells or both the M and E-cells were obtained as previously described (Renn et al., 1999; Stoleru et al., 2004) from the following stable stocks: *y w; UAS-hid/cyo; +* flies, *y w; pdf-GAL4; +*, and *y w; +; cry-GAL4-13/TM6b*. The pdf^{01} mutant flies were previously described (Renn et al., 1999). For neuronal ablation in *per* mutants, *UAS-hid* was introduced into per^{0}, per^{S}, and per^{L} backgrounds and these lines were then crossed to *y w; pdf-GAL4; +* or *y w; +; cry-GAL4-13/TM6b* flies. per^{0} flies were rescued by expressing *per* as previously described (Grima et al., 2004). To make *y w; pdf-GAL4/+; UAS-per/+* flies, we crossed *y w* virgin females with per^{0}*; +; UAS-per* males (Grima et al., 2004). Similarly, Clk^{Jrk} heterozygote flies were made by crossing *y w* females with *+; +;* Clk^{Jrk}*/TM2* males (generously given by the Rosbash lab).

2. Behavioral assays and analysis.

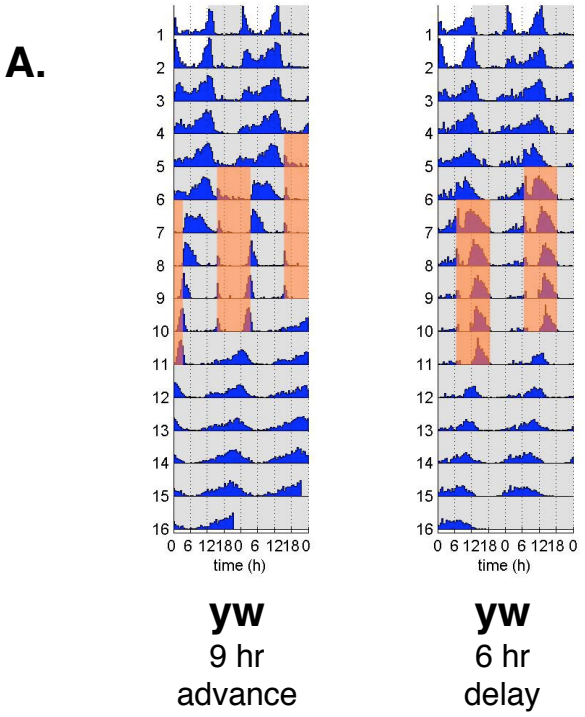
To record daily locomotor activity, individual male flies (1-6 days old) were placed into *Drosophila* activity monitors (Trikinetics, Waltham MA). All experiments were done in Percival I-36LL incubators (Percival Scientific, Perry IA). The Thermophase/Cryophase (TC) cycles were performed at a temperature of 29ºC for the thermophase and 20ºC for the cryophase. Temperature during runs was monitored with a Fluke SII 53 digital thermometer. Shifting temperature from 20ºC to 29ºC took approximately 30 minutes in our incubators. Once the system had reached the correct temperature, it remained stable within ± 0.4ºC. A light intensity of ca. 150-200 lux was used for light:dark (LD) cycles and for one of the constant light (LL) experiments. For the other (LL) experiment, some light bulbs were turned off in the incubator to obtain a light intensity of ca. 75 lux. For each experiment, details on the number of days in LD, TC, and CC (Constant Conditions: dark, 20ºC) are explained in the text or in the figure legends.

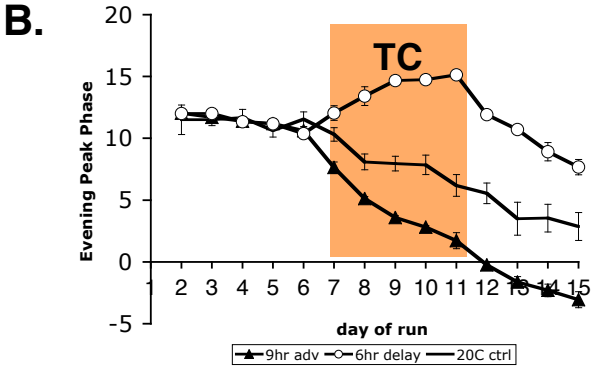
Behavior data collected with the Drosophila Activity Monitoring program (Trikinetics, Waltham MA) was analyzed with FaasX (courtesy of F. Rouyer) or a signal processing toolbox for MATLAB (Mathworks; courtesy of J. Levine). All actograms and histograms are group averages plotted using MATLAB "*dam_panels"* function (Levine et al., 2002).

To determine the phase response of the daily evening peak before, after, or during temperature cycles (Figures 3-1B, 3-7A, 3-7B and 3-S1) we analyzed all flies that had

survived the entire run. Phase was calculated for each individual fly for each day in MATLAB (using an 8-hour Butterworth filter and manual removal of non-evening peaks), and the group mean and standard error was calculated and plotted in Excel (Microsoft).

For experiments measuring the phase of TC-entrained circadian behavior after release into constant conditions (CC; Figures 3-1C, 3-2, and 3-7C), we calculated the difference in average phase between TC exposed flies relative to the no-TC control group on the 2nd day after release in CC. The combined standard error of the two groups of flies was calculated. Arrhythmic flies were excluded from analysis for all figures except 3-7C, for which all flies with a clear peak on the 2nd day were used.


A.
time (h)
time (h)
yw
9 hr
advance
yw
6 hr
delay


B.
TC
Evening Peak Phase
day of run
9hr adv
6hr delay
20C ctrl

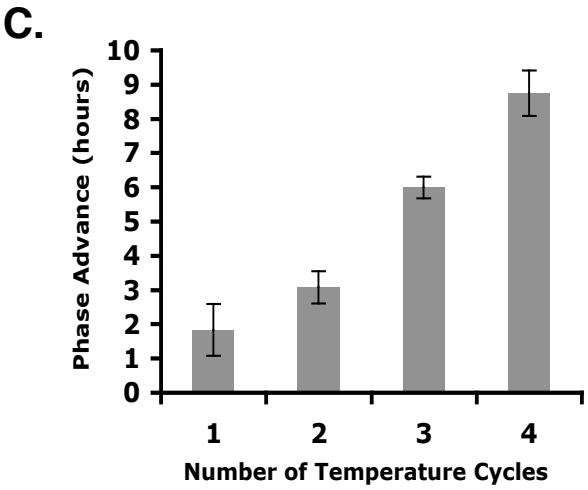

C.
Phase Advance (hours)
Number of Temperature Cycles

Figure 3-1. Temperature is a Zeitgeber for *Drosophila* circadian behavior. **(A)** Actograms showing the average locomotor behavior of groups of *y w* flies. Adult flies were exposed to two days 12hr/12hr light/dark cycles (LD) at 20ºC and then released into constant conditions (CC: darkness at 20ºC) for four days. The flies were then exposed to 12hr/12hr 29ºC/20ºC thermophase/cryophase cycles (TC) that were either advanced by 9 hours (left panel, n=16) or delayed by 6 hours (right panel, n=12) compared to the LD cycle. After five days in TC, the flies were released into CC. The light phase of the LD cycle is represented in white, the dark phase in grey. The warm phase of the TC cycle is shaded in orange, the cold phase in grey **(B)** Phase of the evening peak of locomotor activity during the temperature entrainment assay shown in (A). The phase of the evening peak is plotted on the y-axis (0 corresponds to the light-on transition of the LD cycle) for each day (x-axis). Flies not exposed to a TC (maintained in CC after day 2) were used as controls ("20ºC ctrl", n=11). The difference in post-TC phase was maintained after release into constant conditions. The orange shading indicates the days during which the flies were exposed to TC **(C)** The kinetics of synchronization of M cells to TC. Wild-type flies (*y w*) were exposed to 1, 2, 3, or 4 days of TC (x-axis) to assess the state of the underlying oscillator by measuring phase of behavior after release into constant conditions (y-axis). To determine the amplitude of the phase shift, the average activity post-TC in constant conditions (20ºC DD) was compared between each TC fly group and the no TC control flies. Error bars refer to the average phase variation between days (see materials and methods for details). Each bar refers to a group of at least 10 flies.

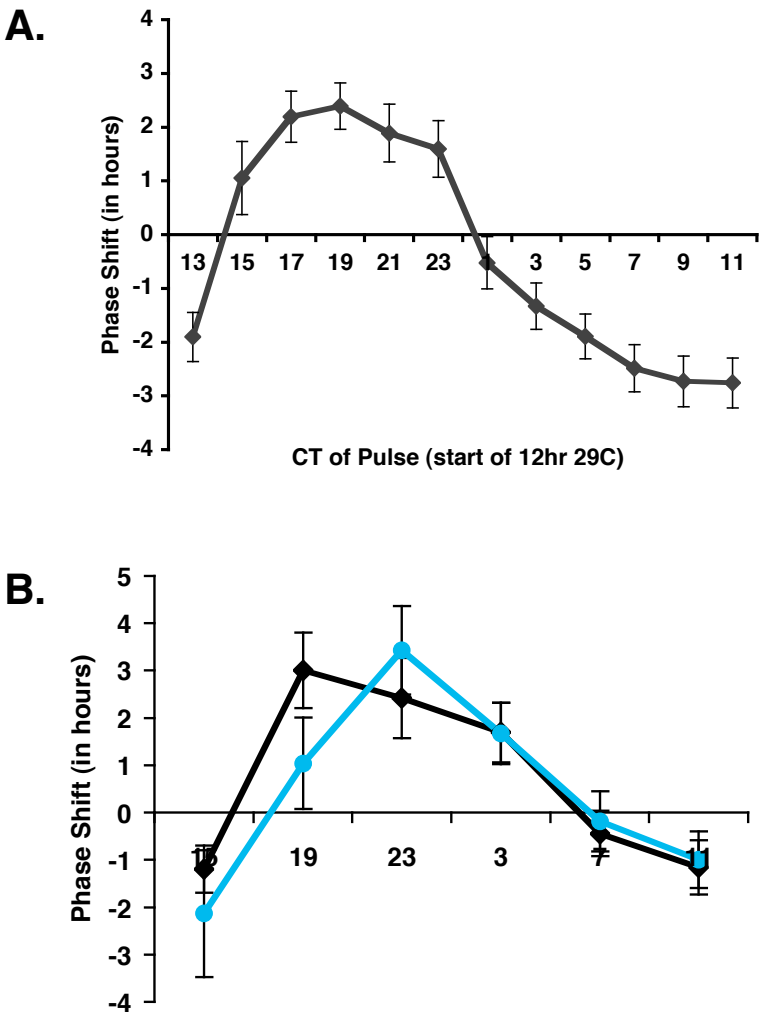


Figure 3-2. Exposure to 29ºC "warm pulses" phase-shifts wild-type and *cry*^{b} flies. Phase Response Curve to temperature pulses. *y w* flies and *cry*^{b} flies were synchronized to a LD cycle at 20ºC and then exposed to 29ºC for 6 or 12 hours at different times of the night and the first subjective day. They were then kept in DD at 20ºC to determine the phase of their locomotor behavior. **(A)** *y w* flies (16-24 flies per time point) exposed to 12-hour warm pulses. **(B)** *y w* flies (9-15 flies per time point) and *cry*^{b} flies (12-16 flies per time point) exposed to 6-hour warm pulses. Both wild-type flies and flies without functional CRY show phase-shifted locomotor activity in response to 6-hours in 29ºC. x-axis: start time of the exposure to 29ºC, in circadian time CT. y-axis: phase shift (in hours) of the evening peak relative to control flies not exposed to 29ºC. Error bars: +/- standard error of the mean.

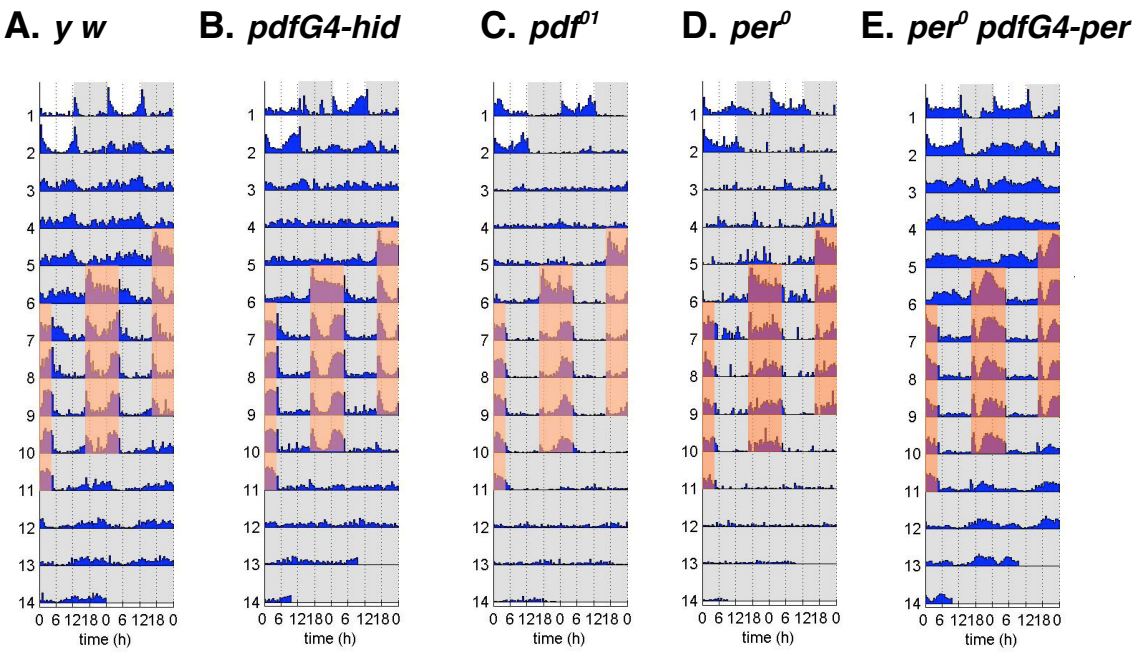


Figure 3-3. The PDF+ M-cells are necessary and sufficient for long-term synchronization of circadian behavior after exposure to temperature cycles. Flies with or without functional PDF+ cells were exposed to 2 days of 20ºC LD, four days of CC, five days of 29ºC/20ºC TC (8 hour advance) and then three days of CC. **(A)** Wild type controls *(y w; +; +,* n=12). **(B)** *pdfG4-hid*: flies without M-cells (*y w; pdf-GAL4/UAS-hid; +,* n=15). **(C)** Flies missing the neuropeptide PDF (*pdf*^{01}, n=30). **(D)** *per*^{0} flies: flies with a null mutation in the *per* gene (*per*^{0} , n=6) **(E)** *per*^{0} *pdfG4-hid*: *per*^{0} flies with PER expression rescued only in the M cells (*per*^{0} *w; pdf-GAL4/+; UAS-per/+,* n=16). Note the persistence of circadian rhythms after TC in flies with the M cells being the only functional circadian neurons (E). Circadian rhythms are not maintained when these cells are either absent (B) or do not produce PDF (C).

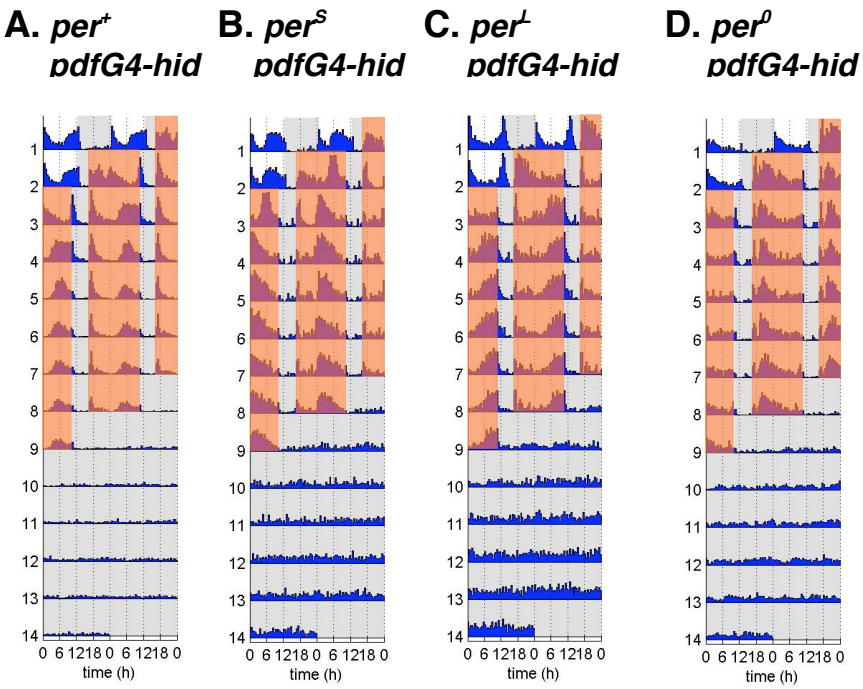


Figure 3-4. The evening peak is regulated by the circadian clock under TC. The M-cells were genetically ablated in flies with short (per^S), long (per^L), or null (per^0) *per* alleles. After two days of 20ºC LD, the flies were exposed to a long thermophase/short cryophase TC (18 hours at 29ºC, 6 hours at 20ºC, with the start of the thermophase occurring 8 hours earlier than the lights-on transition had been during LD). The phase of the M-cell independent evening peak is earlier in the per^S background, later in the per^L background, and very abnormal in the per^0 background, demonstrating that it is under the control of the circadian clock. **(A)** *y w*; *pdf-GAL4*/*UAS-hid*; +, n=14, mean phase=ZT12.8±0.4; **(B)** per^S; *pdf-GAL4*/*UAS-hid*; +, n=7, mean phase=ZT10.0±0.4; **(C)** per^L; *pdf-GAL4*/*UAS-hid*; +, n=12, mean phase=ZT15.5±0.2; **(D)** per^0; *pdf-GAL4*/*UAS-hid*; +, n=10, mean phase=ZT4.2±0.4. ZT=0 is at onset of thermophase, and mean phase refers to mean ZT of evening/afternoon activity peak on the last day of TC±SEM.

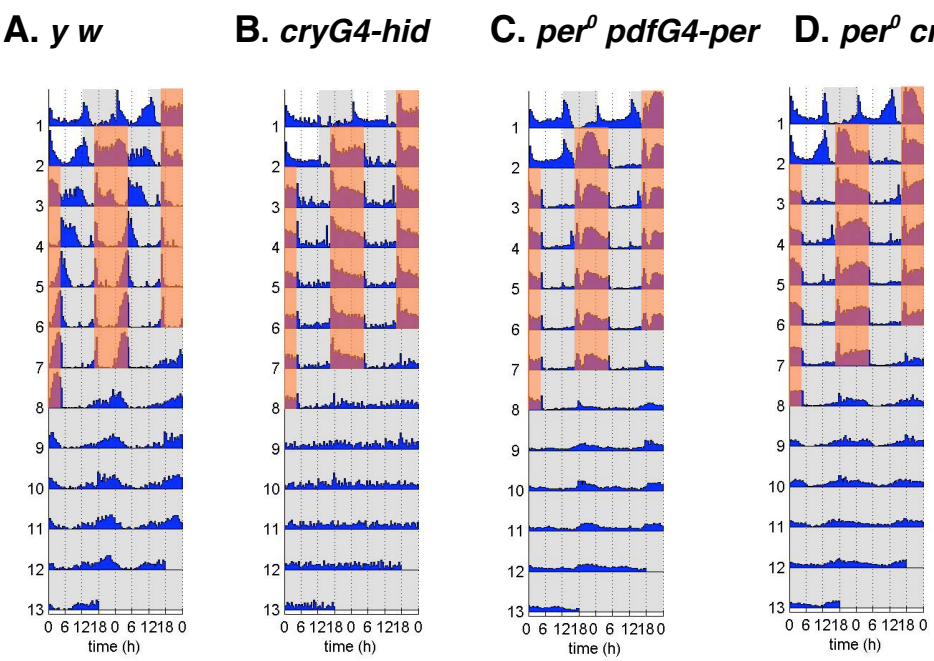


Figure 3-5. The PDF-negative E-cells control the evening peak during temperature cycles. Flies with or without functioning M- and E-cells were exposed to 2 days of 20ºC LD, 6 days of 29ºC/20ºC TC (8-hour advance) and then 6 days of CC. **(A)** Wild type flies (*y w*, n=12). **(B)** Flies in which both the M- and E-cells were ablated (*y w; cry-GAL4/UAS-hid; +,* n=15). **(C)** Flies in which PER expression is limited to the M cells (per^0 *w; pdf-GAL4/+; UAS-per*/+, n=15). **(D)** Flies in which PER is only expressed in the M- and E-cells (per^0 *w; +; cry-GAL4/UAS-per,* n=15). The evening peak of activity cannot be detected when both the M- and E-cells are ablated (B), and is abnormally early when only the M-cells have a functional clock (C). When PER expression is rescued in both the M- and E-cells (D) a later evening peak is present in TC and there is more activity during the subjective afternoon under constant conditions.

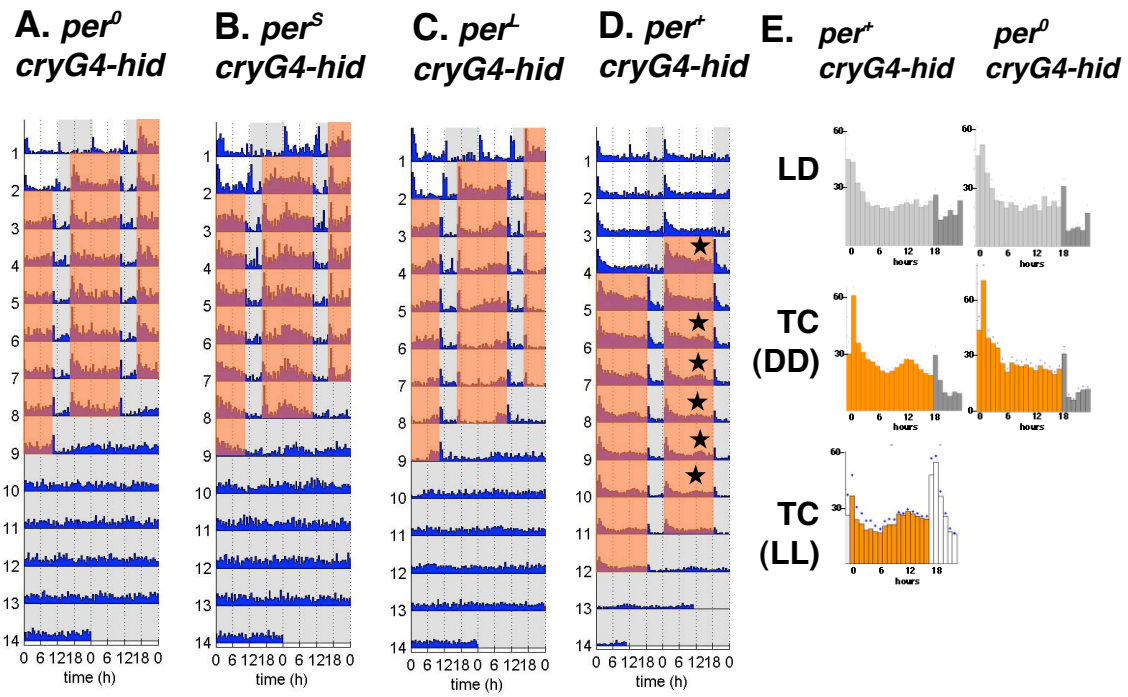


Figure 3-6. Neurons other than the M- and E-cells contribute to the evening peak of activity under TC. (A-D): The M- and E-cells were ablated using *cry-GAL4* and *UAS-hid* in flies with different *per* alleles. per^0, per^S, per^L flies were first exposed to 12-hr:12-hr LD cycles and then to TC cycles with a 16-hr thermophase and an 8-hr cryophase. In the case of per^+, the LD and TC cycles had a 16-hr light phase and an 8-hr dark phase **(D)**. On the per^+ actogram, stars indicate the evening peak when it is clearly visible. Number of flies were 8, 6, 10 and 23 for per^0, per^S, per^L and per^+, respectively. **(E)** Average activity of per^+ and per^0 flies without M- and E-cells over 3 days of 16:8 LD cycles (upper graphs; grey bars: light phase, black bars, dark phase) and 6 days of 16:8 TC cycles in DD or LL (lower graphs; orange bars: thermophase; black/white bars: cryophase).

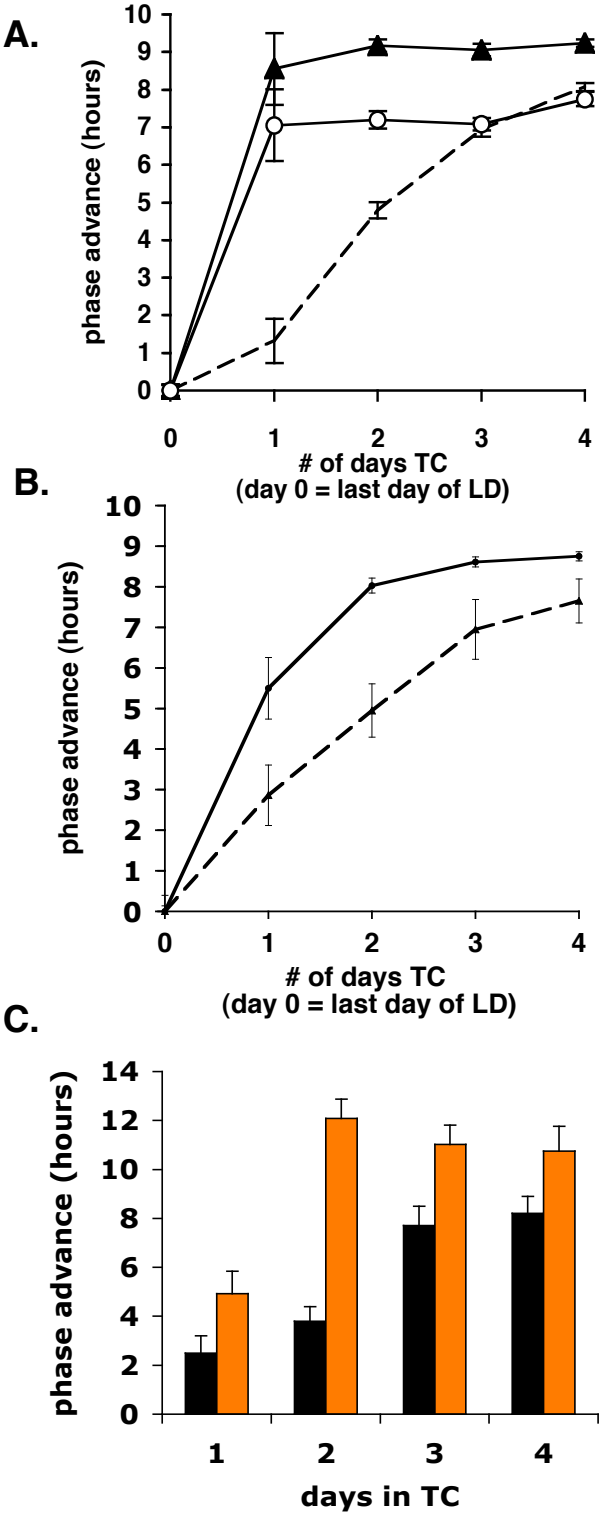

A.
phase advance (hours)
# of days TC
(day 0 = last day of LD)
B.
phase advance (hours)
# of days TC
(day 0 = last day of LD)
C.
phase advance (hours)
days in TC

Figure 3-7. The E-peak shows rapid synchronization in response to temperature cycles when the M-cell oscillator is disrupted or genetically altered. (**A)** Kinetics of synchronization of the cells that regulate the evening peak to TC in wild-type flies (*y w*, dashed line), *pdf* mutants (pdf^{01}, solid line with circles) and M cell ablated flies (*y w; pdf-GAL4/UAS-hid*, solid line with filled triangles). Flies were synchronized to 2 days LD then exposed to 4 consecutive days of TC. The phase-advance of the evening peak was calculated for each day in TC (in hours, relative to the phase in the last day of LD) and is plotted on the y-axis. x-axis: number of days under TC (day 0 corresponds to the last day of LD). Error bars: ± SEM. **(B)** Kinetics of TC synchronization in wild-type flies (*y w*, dashed line) and flies with PER over-expression only in the M cells (*y w; pdf-GAL4; UAS-per,* solid line) in 4 days of TC (experiment and analysis same as in (A)). **(C)** Kinetics of TC entrainment in wild-type flies (*y* w, black bars) and Clk^{Jrk} heterozygotes (*y w; +;* Clk^{Jrk}/+, orange bars). Since Clk^{Jrk} heterozygotes are highly active during the cryophase under TC, phase advances were measured by comparing the phase of the evening peak after release into constant conditions (20ºC DD) in flies exposed to 1, 2, 3, or 4 days TC. y-axis: phase-advance (in hours) relative to no TC control flies. x-axis: total number of days in TC prior to release in constant conditions. Error bars: ± SEM.

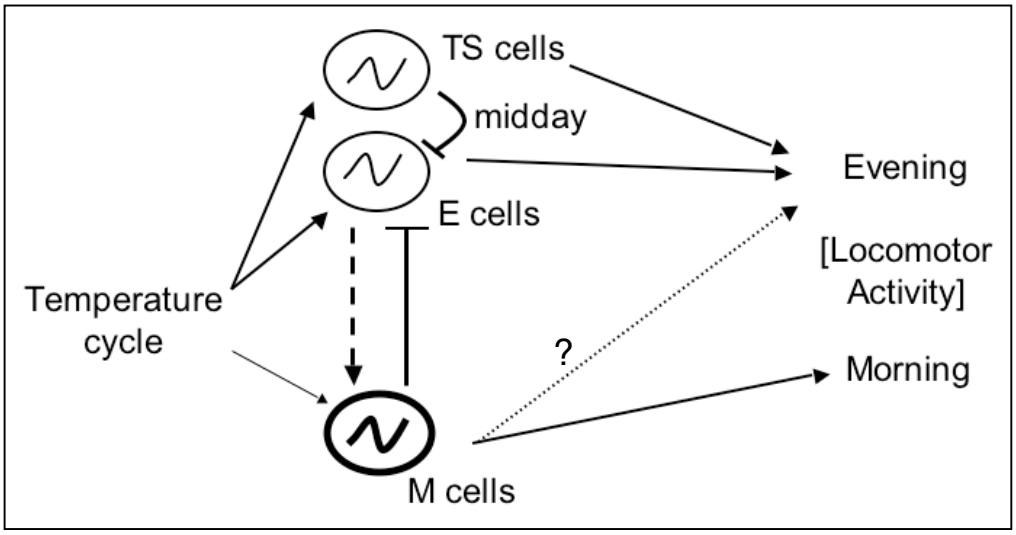


Figure 3-8. Model for the control of behavioral responses to temperature cycles by the circadian cell neuronal network. For simplicity, each group of cells is represented by one cell. Intercellular connections between the M- and E-cells are indicated with a neuronal projection in the shape of an arrow, and additional arrows indicating the cell group's role in modulating circadian locomotor behavior. Other cells also contribute to the proper timing of behavior under temperature cycles, especially during the evening, and possibly during the midday (as an inhibitory signal).

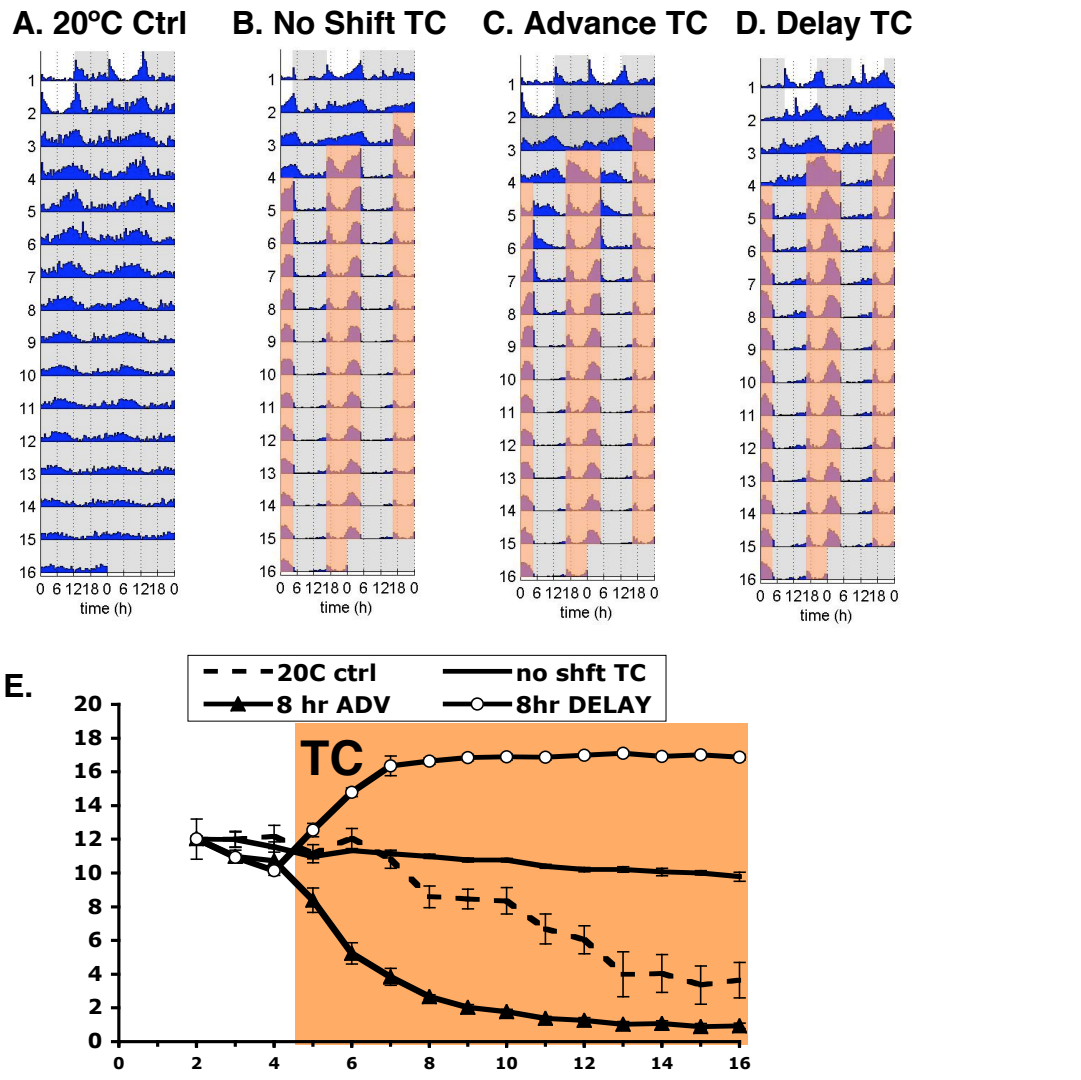


Figure 3-S1. Temperature cycles slowly resynchronize circadian behavior of wild-type flies. The phase of the evening peak of activity was measured to determine precisely how many days were required for *y w* flies to reach a stable phase relationship with the TC. **(A)** Control flies not exposed to a TC (*y w*, n=12). **(B)** Flies exposed to a TC in phase with the LD cycle (*y w*, n=29). **(C)** Flies exposed to an 8-hour advanced TC (*y w*, n=27). **(D)** Flies exposed to an 8-hour delayed TC (*y w*, n=28). **(E)** The evening peak phase was measured daily for each fly in the four experimental groups (A-D). The average phase of each group of flies was plotted (y-axis, +/- standard error, 12hr corresponds to a peak of activity in phase with the light-off transition of the LD cycle) for each day of the experiment (x-axis).

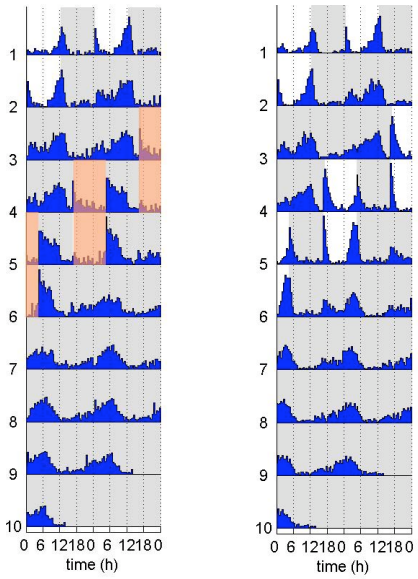


Figure 3-S2. An LD cycle resynchronizes circadian behavior faster than a TC cycle. *y w* flies were first synchronized to an LD cycle, two days in CC and then exposed either to two days of TC (left panel, n=10) or two days of LD cycle (right panel, n=10) advanced by 8 hours. Note that after two days the flies have fully synchronized to the LD cycle, but are far from being fully synchronized to the shifted TC cycle.

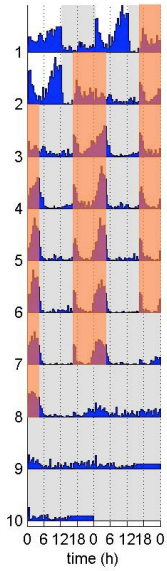


Figure 3-S3. Persistence of the evening peak for a day or two in *pdf*^{01} flies after TC. For unknown reasons, we had some difficulties detecting persistent rhythms after both LD or TC cycles with flies missing the M cells or not expressing PDF, even though it has been reported by several groups that these flies are rhythmic for about two days after an LD cycle. However, in some experiments we could see persistent rhythms for a day or two, as shown on this *pdf*^{01} actogram (n=20).

CHAPTER IV

TIMELESS MAY ACT AS A KEY MOLECULE IN INTEGRATING LIGHT AND TEMPERATURE INPUTS INTO THE *DROSOPHILA* CIRCADIAN CLOCK

The data presented in this chapter are preliminary investigations into possible mechanisms of circadian thermoreception. This data has not yet been published. I designed, carried out and analyzed the experiments under the supervision and guidance of Patrick Emery. I wrote the text by myself, with critical feedback from Patrick Emery, Raphaelle Dubruille, and Alejandro Murad.

A. ABSTRACT

Most organisms use circadian clocks to adapt their behavior and physiology to the changes occurring every day in their environment. The basic mechanism underlying these biological clocks is a network of transcriptional feedback loops that generates ca. 24-hour period rhythms. Even though these rhythms persist under constant conditions, they need to be synchronized every day by external cues such as light and temperature to remain properly in phase with the environment. Disrupted or abnormally synchronized circadian rhythms result in significant adaptive disadvantages in the wild and clinical pathologies in humans.

In *Drosophila melanogaster,* the effect of light on the circadian pacemaker is well described. However, even though temperature fluctuations have been reported to affect circadian behavior in *Drosophila* (Edery et al., 1994; Pittendrigh, 1954; Wheeler et al., 1993; Miyasako et al., 2007; Yoshii et al., 2005), little is known about the mechanism by which they affect the *Drosophila* circadian clock. In this chapter, we investigate possible thermosensation input-pathways into the circadian system. We demonstrate that thermoreception via peripheral thermosensors does not appear to be necessary for behavioral synchronization to temperature cycles. We also provide evidence suggesting that TIMELESS (TIM) protein accumulation is temperature-dependent both *in vivo* and in cell culture. Indeed, TIM appears to undergo proteasome-independent degradation at higher temperatures. We propose that TIM, a key molecule in circadian photoreception, may also play an important role in circadian thermosensation.

B. Introduction

In a world with limited resources and the continual threat of physical dangers, organisms that can successfully predict availability of food and safety are more likely to survive and reproduce. To take full advantage of favorable conditions that reliably repeat, most organisms have evolved internal mechanisms to track time and optimally cue behaviors and physiological states. Termed "circadian" clocks (from the Latin *circa dies*, meaning about a day), these internal timekeepers exhibit a periodicity of approximately 24 hours that persists under constant external conditions. The daily oscillations of these pacemakers must match the phase of the daily solar cycle and have a mechanism to "entrain", or synchronize, to the proper environmental schedule. However the synchronizing mechanism must not be over-sensitive; it must differentiate between important cues, such as the glow and warmth of dawn, and anomalous stimuli, such as lightning or a dark rainstorm. Circadian clocks have evolved to use multiple stimuli in the natural environment as temporal cues, including light-dark cycles, temperature changes and food availability (Panda et al., 2002). Light-dependent synchronization of the circadian clock has been extensively studied. Relatively little, however, is known about the role of temperature in clock entrainment. Here we use *Drosophila* as a model organism to examine possible temperature-input pathways into the circadian system.

The *Drosophila* molecular clock is comprised of interlocking transcriptional feedback loops. At the center of the molecular pacemaker are two transcription factors,

CLOCK (CLK) and CYCLE (CYC). CLK and CYC heterodimerize and bind to E boxes of circadianly regulated genes, activating transcription. In the first feedback loop, the CLK/CYC dimer regulates transcription of the *period* (*per*) and *timeless* (*tim*) genes (reviewed in Stanewsky, 2002). PER and TIM proteins accumulate in the cytoplasm and heterodimerize. They then enter the nucleus and inhibit CLK/CYC mediated transcription. VRILLE (VRI) and PAR DOMAIN PROTEIN 1 (PDP1) are also transcriptionally regulated by the CLK/CYC dimer and form another regulatory loop that feeds back on *Clk* transcription (Blau and Young, 1999; Cyran et al., 2003). The VRI/PDP1 and PER/TIM feedback loops coordinate a continuous oscillation of transcription activation and repression. Other proteins modulate the period length of this molecular clock: the kinases CASEIN KINASE II (CKII), SHAGGY (SGG), and DOUBLETIME (DBT) and a protein phosphatase (PP2A) regulate PER and TIM phosphorylation, affecting stability and nuclear entry (reviewed in Hardin, 2006). Together, these mechanisms maintain the molecular clock's oscillation on a 24-hour cycle.

A rhythmic molecular clock is apparent in many cells of the adult *Drosophila*, including in peripheral tissues such as the Malpighian tubules and the compound eyes (Giebultowicz, 2001). Only specific groups of neurons in the brain, however, express oscillating clock genes. These approximately 150 neurons regulate circadian rhythms in the fly's locomotor behavior. Recent evidence suggests that these cells are a heterogeneous group of neurons, with varying sensitivity to light and temperature cycles, and differing contributions to behavioral activity depending on the environmental

conditions (Collins and Blau, 2007; Miyasako et al., 2007; Murad et al., 2007; Stoleru et al., 2007). How environmental inputs feed into this cellular network, and how the neurons communicate with each other to create a synchronized output has been a recent focus in the *Drosophila* circadian field.

Light and temperature cycles are the major synchronizing cues for circadian rhythms in most species. In mammals, central pacemaker entrainment occurs primarily through photic information from specialized receptors in the eyes (Berson, 2003). Natural body temperature rhythms can also affect circadian gene expression in mammalian cells (Brown et al., 2002). In *Drosophila melanogaster*, light can entrain the molecular clock from both intracellular pathways and synaptic transmission. CRYPTOCHROME (CRY), a blue light photoreceptor, plays a central role in circadian photoreception. This intracellular photoreceptor activates TIM degradation upon exposure to light, and in doing so presumably re-sets the molecular oscillator (Emery et al., 1998b; Lin et al., 2001; Stanewsky et al., 1998). Light input also feeds into the pacemaker neurons controlling circadian behavior via synaptic transmission from opsin-based photoreceptors (such as the compound eyes, the Hofbauer-Buchner eyelet and the ocelli) (Helfrich-Förster et al., 2001). While these *Drosophila* light input pathways are relatively well characterized, the mechanisms by which temperature cycles synchronize the clock remain unknown.

The environmental temperature affects circadian behavior in several ways. When the temperature is held constant, it determines how early flies begin their evening surge of activity. At lower temperatures (18ºC) there is enhanced splicing of a temperature-

sensitive intron in the 3'UTR of *per* RNA which increases the transcript's stability (Majercak et al., 1999). The earlier accumulation of *per* mRNA and then PER protein leads to an earlier evening activity peak. At higher temperatures (25ºC and 29ºC) the evening peak is delayed, presumably reflecting an evolutionary advantage of avoiding activity and dessication during the hottest times of the day (Collins et al., 2004; Majercak et al., 2004).

Under constant light (LL) or constant dark (DD) conditions, temperature cycles can synchronize *Drosophila* locomotor behavior (Wheeler et al., 1993; Yoshii et al., 2005; Yoshii et al., 2002). The fact that this synchronization is affected by circadian gene mutations (Yoshii et al., 2002), and that synchronized behavior persists upon release in constant conditions [Chapter III of this dissertation], strongly suggests that temperature cycles can truly entrain the underlying circadian oscillator. Indeed, several recent studies indicate that temperature fluctuations act on the molecular clock. Immunohistochemical studies examining molecular oscillations of PER and TIM in the pacemaker neurons of the brain show that temperature cycles during constant light, light/dark cycles, or constant darkness can affect the phase of molecular oscillations (Miyasako et al., 2007; Yoshii et al., 2005). Western blot analysis of PER and TIM proteins in peripheral tissues and *per-luciferase* studies in isolated tissues confirms that temperature cycles can cause lasting shifts in molecular oscillations (Glaser and Stanewsky, 2005; Stanewsky et al., 1998). The fact that oscillations in isolated body parts can be entrained by temperature cycles in these studies suggests that circadian thermosensation occurs at least in part by a cell-autonomous thermoreceptive pathway. This is supported by identification of a circadian

thermosensation mutant, *nocte*, in a constant light temperature cycle screen (Glaser and Stanewsky, 2005). *nocte* mutants show both abnormal behavioral synchronization to temperature cycles and attenuated PER/TIM oscillations in peripheral circadian tissues during 12 hr: 12hr temperature cycles. It is currently unclear how the *nocte* pathway feeds into the circadian clock, however the activity of a phospholipase C *norpA* may also be involved in this temperature-input pathway (Glaser and Stanewsky, 2005).

Heat-pulse experiments implicate TIM as a target for thermoreceptive pathways, in addition to its known role as target for the circadian photoreceptor CRY. A 30 minute heat-pulse of 37ºC given in late day/early evening causes a a rapid decrease in whole head extract TIM levels; a one hour 37ºC heat-pulse given at the same time causes behavioral phase delays of 1-2 hours (Edery et al., 1994; Sidote et al., 1998). This effect appears to be dependent upon the activity of CRY (Kaushik et al., in press) and is not seen with brief heat-pulses of less than 34ºC in wild-type flies. Whether the more physiologically relevant effects of temperatures under 30ºC on behavior use similar or currently unknown mechanisms, remains undetermined.

In this chapter we use behavioral and molecular techniques to further study the circadian temperature input pathway. First, we investigate what role, if any, peripheral thermosensation has on circadian entrainment to temperature cycles. Then we look at the effect of physiologically relevant temperatures on the accumulation of PER and TIM proteins in peripheral clock tissues and in cell culture. We find evidence supporting the idea that the TIM protein functions as a major environmental input molecule for temperature fluctuations, in addition to its role in intracellular photoreception.

C. Results

1. Peripheral thermosensation is not necessary for behavioral synchronization to temperature cycles.

Light input into the *Drosophila* circadian system occurs both via peripheral light-sensing organs and via direct effects on the intracellular photoreceptor CRYPTOCHROME (Helfrich-Förster et al., 2001). To investigate the circadian role of peripheral thermosensation, we studied temperature entrainment in flies with temperature-sensing tissues surgically removed. Thermosensitivity assays in *Drosophila* using temperature step gradients indicate the presence of two thermosensors (Sayeed and Benzer, 1996; Zars, 2001). High-temperature thermosensing receptors have not been located to a specific tissue in adult flies. However, low-temperature thermosensors have been found to be located in the third antennal segments (Sayeed and Benzer, 1996). Wild-type flies with bilateral removal of the third antennal segments and aristae lose the wild-type behavioral preference for 24°C on a temperature gradient of 18°C to 31.5°C. However, these flies still manifest high-temperature thermosensation, avoiding surfaces heated above 33°C. Having demonstrated that temperature cycles within these lower ranges are sufficient for entraining the *Drosophila* circadian clock (Chapter III), we tested locomotor activity entrainment of wild-type flies with surgically removed third antennal segments [Figure 4-1]. Flies with only one third antennal segment and arista removed were used as controls, as one residual third antennal segment is enough to

maintain normal temperature preference response (Sayeed and Benzer, 1996). First, a 20ºC/29ºC 12hr: 12hr temperature cycle was used. Flies lacking the third antennal segments appeared to entrain normally to temperature [Figure 4-2, panel A]. As we have previously shown that the phase of the evening peak of locomotor activity can be used to quantify entrainment to temperature cycles, we measured the mean evening peak phase for each day of the behavioral run for all three fly groups [Figure 4-2, panel B]. We found no difference between intact wild-type flies and flies lacking one or both 3rd antennal segments. This could mean that peripheral thermosensation through the third antennal segment does not contribute to circadian thermosensation. Indeed, a previous study reported that full-body circadian protein rhythms were still entrained in intact flies that lack the antennal lobe (Glaser and Stanewsky, 2005). The possibility remained, however, that the pacemaker neurons controlling circadian behavior use multiple temperature input pathways. If so, it is possible that a deficit in one pathway would not be apparent unless the fly was exposed to more "subtle" temperature cycles of only 3ºC difference between cryo- and thermophases. To investigate this possibility, the experiment was repeated with temperature cycles of 18ºC/21ºC or 26ºC/29ºC [Figures 4-3 and 4-4]. During these experiments, we found that flies after surgical removal of antennal segments (both, or just unilaterally) appeared more erratically active. We also observed that the active period for flies of all three groups was more spread out during the experiment with lower temperatures (18ºC/21ºC) leading to less precise quantification of the evening peak. Indeed, the quantification obtained in this experiment is misleading as the flies had abnormally high amounts of activity during the warm phase. It is difficult

to distinguish between the slowly transitioning evening peak and this "masking" behavior. However, when comparing flies lacking both antennal segments and control flies with unilateral ablation, no marked difference was visible in rate of entrainment or phase of evening peak after release into constant conditions on the actogram. We are thus not able to observe evidence of peripheral thermosensors being necessary for thermic input into the *Drosophila* circadian system.

2. TIM and PER protein cycling in *cry*^{b} head extracts is initiated during one temperature cycle.

Previous studies have shown that *cry*^{b} mutants, lacking a functional intracellular circadian photoreceptor, do not show molecular oscillations in their peripheral clocks during LD or DD (Stanewsky et al., 1998). It is unclear if this is due to individual cell oscillators becoming desynchronized without environmental inputs or if it is because the oscillations cannot be sustained without CRY's activity as a transcriptional repressor (Collins et al., 2006). However, temperature cycles can initiate protein rhythms in *cry*^{b} peripheral clocks: after three 12hr: 12hr 25ºC/ 20ºC temperature cycles, PER and TIM protein oscillations are visible in head extracts and persist for at least one day after return to constant conditions (Stanewsky et al., 1998). We began our investigation into the molecular targets of circadian thermoreception by examining how the PER/TIM protein oscillations are initiated. *cry*^{b} flies were behaviorally synchronized to 25ºC LD, and then exposed to a 12hr:12hr 29ºC/20ºC temperature cycle. Every four hours after the initiation of the temperature cycle flies were collected and frozen, and PER and TIM protein levels

in the head extracts were measured by Western Blotting. Already during the first temperature cycle, fluctuations could be seen in PER and TIM protein levels [Figure 4-5]. During the first cool phase (20ºC, ZTs 13-21) PER and TIM protein levels were greater than the 25ºC controls, and peaked at ZT17 before decreasing again. By the second day of the temperature cycle, a clear rhythm can be seen in the head extracts, with both proteins having maximal levels at ZT 17, and PER protein showing rhythms in phosphorylation. Stanewsky and colleagues observed similar oscillations in both cry^b and wild-type head extracts after three full days of 25ºC/20ºC temperature cycles (Stanewsky et al., 1998). We repeated the experiment three times and quantified the PER and TIM proteins over days 1-3 with results consistently showing a clear oscillation by the end of the first day [Figure 4-5, bottom panel]. In some Western blots [Figure 4-5, top panel], there also seemed to be a decrease in PER and TIM levels during the first warm phase, but this was too subtle to be seen in the average quantification. Preliminary studies on *per* and *tim* mRNA levels (using RealTime-PCR) did not find a clear difference between the timing of changes in mRNA levels or protein levels [data not shown]. We thus concluded that the mechanism by which temperature fluctuations are sensed by the molecular clock must be fairly rapid as changes in protein levels can already be seen during the first 12 hours after an increase in temperature.

3. TIM protein levels are affected by temperature cycles in head extracts of circadian mutants.

Our results using cry^b head extracts show a response in PER and TIM protein levels during the first 24 hours of a temperature cycle. As PER protein is unstable when not associated with TIM (Price et al., 1995), however, it is unclear if the PER and TIM protein oscillations seen during the first day of a temperature cycle are due to an effect on both protein levels or primarily on TIM, with PER levels rising following increases in TIM production or decreases in TIM degradation. Therefore, to look separately at the effects of temperature fluctuations on TIM and PER protein levels *in vivo*, we looked at the effects of temperature cycles in per^0 and tim^0 mutants [Figure 4-6]. per^0 mutants lack PER protein and tim^0 mutants lack TIM protein, therefore any effect on the other protein in these flies would not be confounded by an indirect effect via the stability conferred by PER/TIM heterodimers. We found that in tim^0 mutant flies, PER protein levels did not depend on temperature [Figure 4-6]. However, TIM protein levels were clearly affected by temperature in per^0 flies, with TIM levels being approximately 30% lower during the warm phase (ZT 1-9) than during the cold phase (ZT 13-21) of a 29ºC/20ºC temperature cycle. In flies without a functional molecular clock, therefore, TIM levels are dependent upon temperature and show a clear response to temperature changes of 9ºC.

4. *In vitro* proteasome-independent TIM degradation at 29ºC.

Our results suggest that *in vivo*, TIM levels respond to temperature fluctuations, and PER levels are only affected indirectly via TIM/PER heterodimers stabilization of

PER. To further investigate the temperature-dependent nature of TIM levels, we looked at TIM and PER protein expression in a *Drosophila* embryonic cell line: Schneider2 (S2) cells. In these cells, the circadian transcription factor *Clock* is not expressed (or is expressed at extremely low levels). Therefore, to study TIM protein in a cell culture system, we constitutively expressed *Clk* in S2 cells under the regulation of the *Drosophila* actin promotor (pAc-*Clk*). First, we looked for any temperature effect on TIM in S2 cells. As S2 cells survive best at 25ºC, we began our experiments with a 20ºC/25ºC 12hr: 12hr cycle [Figure 4-7]. Our preliminary data showed a slight oscillation in TIM levels (normalized to TIM levels in transfected control cells maintained in constant 20ºC). We repeated the experiment using the same temperature cycle as in our *in vivo* studies (29ºC/20ºC) and included several controls. In pAc-*Clk* transfected cells exposed to three days of 29ºC/20ºC temperature cycle and in cells maintained at constant 29ºC or 20ºC, TIM levels were higher at 20ºC. During the temperature cycle, TIM levels increased during the cold phase and decreased during the warm phase. At constant temperatures, the difference between TIM levels at 29ºC or 20ºC was even more extreme, presumably because the levels had stabilized. Similar effects were seen when the *tim* gene was directly expressed using pAc-*tim*, suggesting that this temperature-dependent difference in TIM levels is not due to an effect of temperature on the ability of CLK to initiate *tim* transcription [Figure 4-8]. It is also unlikely that the differences in TIM protein accumulation are due to a temperature effect on the efficiency of the pAc plasmid, as pAc-ßgal was transfected in other cells as a control and ßgal-V5 protein levels were similar during a temperature cycle. It is possible

that ßgal-V5 is more stable and that therefore an effect of temperature on pAc plasmid expression would not be seen during a temperature cycle. If that were the case, however, one would still expect to see a difference between ßgal-V5 accumulation after several days of continuous 29ºC or 20ºC, which was not seen. Therefore, in S2 cells, as well as *in vivo*, it appears that there is an effect of temperature on TIM protein levels.

Is the change in TIM protein levels in cool and warm temperatures due to a change in TIM production or stability? To study this, we transfected S2 cells with *pAc-tim* and maintained them for several days at 20ºC. On the third day, we added a translation inhibitor (cycloheximide) or a proteasome inhibitor (MG-132) to block protein synthesis or protein degradation and then exposed the cells to 12 hours at 29ºC to observe the resulting effect on TIM levels [Figure 4-9]. Control cells were left at 20ºC. DMSO-treated cells were used as controls at both 20ºC and 29ºC. In DMSO-treated cells, we saw some decrease in TIM levels at the 12-hour time point, possibly indicating that the cells were starting to die. However, in the first three time points, the levels at 20ºC were constant. By contrast, TIM levels rapidly decreased in the control cells placed at 29ºC, again demonstrating that higher temperatures lead to decreases in TIM protein levels. Adding a proteasome inhibitor, MG-132, did not block TIM protein levels from falling. This suggested that either the effect on TIM is at the protein production level, or that TIM is degrading via a temperature-independent manner. If the temperature effect was on translation, then blocking protein production with cycloheximide should eliminate the difference in protein levels between cells exposed to 20ºC and 29ºC. We found that this was not the case; in cycloheximide-treated cells, TIM levels fell in both 29ºC and 20ºC

control conditions. However, they were lower at each time point at the warmer temperature. Therefore, there is increased protein degradation at 29ºC. Finally, we confirmed that this protein degradation is proteasomally independent by concurrently blocking both protein synthesis and proteasomal degradation in the same cells. Again, we see an decrease in TIM at 29ºC that cannot be explained by proteasomal degradation or effects on translation. Cycloheximide and MG-132 were tested for efficacy, by blocking heat-pulse initiation of protein synthesis of CRY in pHS-*cry* transfected S2 cells with cycloheximide, and blocking light-pulse induced proteasomal degradation of CRY in pAc-*cry* transfected S2 cells with MG-132. This *in vitro* data suggest that there is proteasome-independent TIM degradation at higher temperatures.

D. Discussion

In this chapter we have provided evidence suggesting that unlike light, environmental temperatures are only sensed by the circadian molecular clock via intracellular pathways. Additionally, we show a temperature-dependent proteasome-independent degradation of the key circadian molecule TIMELESS, and suggest that TIM may function as a common target of both light and temperature input pathways.

First, we asked if the circadian system receives input from peripheral thermosensors to synchronize locomotor behavior with environmental temperature cycles. Flies without 3rd antennal segments have been previously shown to lack normal behavioral preference to temperature between 18ºC and 31.5ºC (Sayeed and Benzer,

1996). However, we find that they have no defect in circadian thermoreception. They entrain as rapidly to 20ºC/29ºC, 18ºC/21ºC, or 26ºC/29ºC temperature cycles as intact flies. We were therefore not able to demonstrate that peripheral thermoreception is necessary for circadian thermosensation. It is possible that there are several redundant temperature input pathways. If, like light, temperature affects the clock via both intracellular and synaptic input pathways, then it is possible that we would not see the effect of ablating peripheral thermosensors until it is combined with an intracellular thermosensitive mutation. It is also possible that peripheral thermosensation is necessary for circadian thermoreception but that the peripheral input comes from tissues other than those which we ablated.

Our assay is limited by the following: it observes the synchronization of the underlying circadian oscillator by measuring the phase-shifting of the evening peak. In our conditions of temperature cycles under constant darkness, this activity peak is affected by a fairly large group of circadian cells (Grima et al., 2004; Miyasako et al., 2007; Rieger et al., 2006; Stoleru et al., 2004; Stoleru et al., 2005). It is possible that input from peripheral thermosensors does reach circadian neurons, but not the neurons which dominate and control the evening peak under these conditions. In fact, it has been reported that other cells (that do not provide significant input to the evening activity under these conditions) are particularly sensitive to temperature cycles (Miyasako et al., 2007). To fully exclude the possibility of peripheral thermosensory input, we would need to examine the molecular oscillations of all of pacemaker neurons after removal of the 3rd antennal segments and verify that there are no changes in individual cells' response to

temperature cycles. We could also develop other ways of measuring temperature entrainment. Perhaps peripheral thermosensation affects other aspects of circadian behavior under temperature cycles, such as rate of entrainment of the morning peak, which we did not measure.

It is also possible, however, that temperature entrainment occurs solely at the cell autonomous level. Indeed, cell autonomous thermosensation is suggested by previous studies demonstrating that the peripheral clocks, which presumably do not all have access to information from thermosensitive tissues, synchronize their molecular rhythms in response to temperature cycles (Boothroyd et al., 2007; Glaser and Stanewsky, 2005; Stanewsky et al., 1998; Yoshii et al., 2007). Thus, temperature input via intracellular pathways is sufficient for circadian temperature entrainment.

How do temperature fluctuations affect the molecular clock in peripheral tissues? Recent studies indicate that temperature shifts can increase or decrease rates of transcription of circadian genes both in DD and in LL (Boothroyd et al., 2007; Yoshii et al., 2007). Our results point to another possible effect of temperature on clock components: we see a temperature-dependent effect on TIM protein degradation. Having concluded that the primary effect of temperature fluctuations on circadian behavior likely occurs via a cell-autonomous mechanism on the pacemaker neurons, we attempted to identify circadian molecules that show rapid changes in levels after a temperature shift. Our initial studies looked at protein cycling in peripheral clocks in response to temperature fluctuations by measuring circadian proteins in processed head extracts. We saw perturbations in both PER and TIM levels within the first day of the

temperature cycle, showing that already within the first cool phase the molecular clock is being affected by the temperature cycle. Molecular changes during this time-scale presumably must be what underlie our previously reported behavioral phase-shifts in response to one 12-hr 29ºC heat pulse (CHAPTER III). Unfortunately, the resolution of our Western Blot PER and TIM protein level changes is not enough to identify if TIM or PER protein levels change first. To determine if both protein levels are affected by temperature, we looked at TIM and PER levels in circadian mutants where we could see direct effects on protein levels unmasked by a constantly changing molecular oscillation. We found that PER levels remained constant in *tim* null mutants exposed to a 20ºC/29ºC temperature cycle. In contrast, TIM levels were clearly higher at 20ºC and lower in 29ºC phases in *per* null mutants. The TIM levels in per^0 flies showed a step-like high/low shape during a temperature cycle. Thus, without an underlying functional clock TIM levels rapidly respond to changes in temperature. The observation in this mutant that TIM levels are capable of being so quickly influenced by environmental temperatures suggests that there may be a similarly rapid effect on TIM in wild-type flies, which is buffered by the ongoing activity of the rest of the molecular oscillator. Small effects, however, could still change the state of the clock. Indeed, it is likely that the PER oscillation we see in cry^b head extracts is due to PER stability being tightly connected to TIM levels, and that temperature's effect on TIM levels leads to a corresponding effect on PER levels.

How then, are TIM protein levels being altered by temperature during the temperature cycle? We believe that our cell culture data provides a possible mechanism.

In S2 cells, TIM protein degradation at higher temperatures does not depend on the proteasome. Several future studies must be conducted to confirm and further expand upon this finding. First, similar experiments should be done with another TIM antibody against another segment of TIM, to evaluate the possibility of TIM protein being cleaved, and hence invisible to our TIM antibody, but not fully degraded. Then, *in vivo* studies should be performed to confirm that this finding is not an artifact of cell culture. We plan to expand upon this observation by using inhibitors on Malpighian tubules or larval brains in culture. If confirmed *in vivo*, subsequent analysis will be focused on identifying possible proteases and other regulatory components responsible for warm-temperature TIM degradation.

Several other studies have also suggested temperature-dependent TIM degradation that depends on CRY (Kaushik et al., 2007; Sidote and Edery, 1999). However, we believe that our observed TIM degradation is via a separate mechanism for several reasons: (1) Our S2 cells have no observable endogenous *cry* expression; (2) we see similar kinetics in temperature-specific effects on TIM levels in cry^b mutant flies, which only express a severely hypomorphic CRY protein; (3) our experiments were done at much lower temperatures. Neither previous study found behavioral effects at temperatures lower than 34ºC in wild-type flies. While per^L mutant flies appear to have CRY-dependent phase-shifting effects at lower temperatures (Kaushik et al., in press), this may be a misleading gain-of-function due to an abnormally temperature sensitive mutant protein. We feel that it is important to consider a physiologically relevant temperature range: flies typically avoid high temperatures (Sayeed and Benzer, 1996)

and thus temperatures within the heat-shock range may be eliciting non physiologically-relevant reactions. We use 20ºC/29ºC temperature cycles because these temperature ranges are reported as average minimum and maximum temperatures in summer days in Canton, Ohio. However, as fruit flies in the wild presumably are able to regulate their temperature by selecting their location, this temperature range may not be appropriate either. Additionally, we used two alternating temperature steps, as opposed to a gradually increasing and decreasing temperature cycle that is probably more similar to natural circadian inputs in the environment (Boothroyd et al., 2007). Thus, while we believe our data are relevant to exposure to natural environments, they clearly do not accurately mimic what a fly would be exposed to in the wild.

In conclusion, our data suggest that TIM plays a double role in environmental synchronization of the *Drosophila* clock, and provide the first evidence to our knowledge of an effect on protein stabilization at lower and more physiologically relevant temperatures. Thus, in addition to its well-studied role as the primary target of the intracellular light-input pathway, TIM may be an initial clock target for a temperature input pathway. This would place TIM into the central role as an integrator of both light and temperature input pathways, and would provide an explanation for why light inputs usually, but not always, dominate over temperature inputs: in alternating cycles, LD cycles dominate over temperature cycles via rapid TIM degradation. In constant light conditions, CRY and TIM are continuously degraded. Here, however, the effects of temperature on transcription can gently override the constant light input and generate temperature cycle behaviors.

E. Materials and Methods

1. *Drosophila* strains.

The *y w* wild-type flies as well as cry^b, per^0, and tim^0 mutant flies were all generously given by Michael Rosbash. Fly stocks were maintained at 20ºC-25ºC in bottles with media made of corn meal, molasses, yeast, and agar.

2. Behavioral assays and analysis.

For locomotor behavior assays, adult male flies (1-5 days old) were placed into *Drosophila* Activity Monitoring boards [Trikinetics, Waltham MA]. To generate flies lacking the known peripheral thermosensing tissues, wild-type flies (*y w*) were anesthetized with CO_2 and surgical tweezers were used to pull off the 3rd antennal segment, as previously described (Sayeed and Benzer, 1996). Flies with damaged antennae or more than the 3rd antennal segment removed were not used for the experiment. Behavioral monitoring was done in Percival I-36LL incubators (Percival Scientific, Perry IA) and incubator temperature was calibrated with a Fluke SII 53 digital thermometer. Temperature shifts between 20ºC and 29ºC took approximately 30 minutes in our incubators. Once the system had reached the correct temperature, it remained stable to within +/- 0.4ºC. Light during the initial light/dark synchronizing cycle was approximately 200 lux of fluorescent lights (GE "cool white" 20W fluorescent lamps). For each experiment, details on the number of days in LD, TC, and constant conditions are explained in the figure legends. Behavioral data were collected with the Drosophila

Activity Monitoring program (Trikinetics, Waltham MA). Fly activity data were analyzed with a signal processing toolbox (courtesy J. Levine, modified with help from R. Haefner) on MATLAB 6.0 (Mathworks, Framingham MA). All actograms are group averages plotted using MATLAB "*dam_panels*" function (Levine et al., 2002b). Phase-graphs were created by measuring the phase of the evening peak for individual flies on each day of the behavior run using the "*peakphaseplot*" function (using an 8-hour Butterworth filter and manual removal of non-evening peaks). Group mean and Standard Errors of the Mean were calculated and graphed in Excel (Microsoft) (Levine et al., 2002b).

3. Fly entrainment for molecular assays.

For analysis of molecular oscillations in head extracts, 20-40 flies per time point were placed into plastic vials with 2ml of fly food. Exposure to temperature cycles was done in the same Percival incubators as for behavioral analysis (see above for information about incubator settings). Flies were previously exposed to 2-3 days of 20ºC light/dark cycles and then placed into a 20ºC/29ºC temperature cycle. See figure legend for exact details of number of days of temperature entrainment. Fly samples were collected into vials on dry ice in the dark (under red light). Head extracts were prepared and homogenized as previously described (Busza et al., 2004).

4. Plasmid constructs.

pAc-*Clk* and pAc-*tim* were kindly given by Steven Reppert and Michael Rosbash, respectively. pAc-*cry* and pHS-*cry* plasmids are as previously reported (Busza et al., 2004).

5. S2 cell transfection and drug treatment

S2 cells were grown in SFX (HyClone) medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 mg/ml). S2 cells were transfected using Cellfectin (Invitrogen). For transfections, each well of 60-80% confluent cells were transfected with 1 µg total DNA (15 minutes of DNA, Cellfectin, and SFX preincubation, 4-6 hours of transfection). Cells were then maintained at 25ºC in constant darkness for 12 hours and a 750 µl/ sample was placed into small tubes and wrapped in foil to prevent light-exposure during temperature cycles. See figure legends for more details on temperature cycle exposure. For the inhibitor experiments, cells were transferred to centrifuge tubes and then MG132 (50µM), cycloheximide (0.5µg/ml), or DMSO were added to the medium.

6. Protein extracts of S2 cells.

S2 cell protein extracts were prepared as follows: cells were harvested with a 1-minute centrifugation at 800g, then cell culture medium was discarded, and cells were resuspended in 30 µl of Extraction Buffer as previously described (Busza et al., 2004). 5µl was diluted in 4ml of water and then used for protein quantification (Bradford Assay,

using commercial kit from Biorad). The remaining cell extract was resuspended with 1x SDS-PAGE loading buffer and boiled. For Western Blots the equivalent of about 25µg of protein/lane was loaded. Equal loading and quality of protein transfer were verified by Ponceau Red staining, and by the intensity of cross-reacting bands on the Western Blots or by reprobing the membrane with a monoclonal anti-spectrin antibody (obtained from the Developmental Studies Hybridoma Bank maintained by The University of Iowa, Department of Biological Sciences, Iowa City). The anti-TIM and anti-PER antibodies were generously provided by the Rosbash lab.

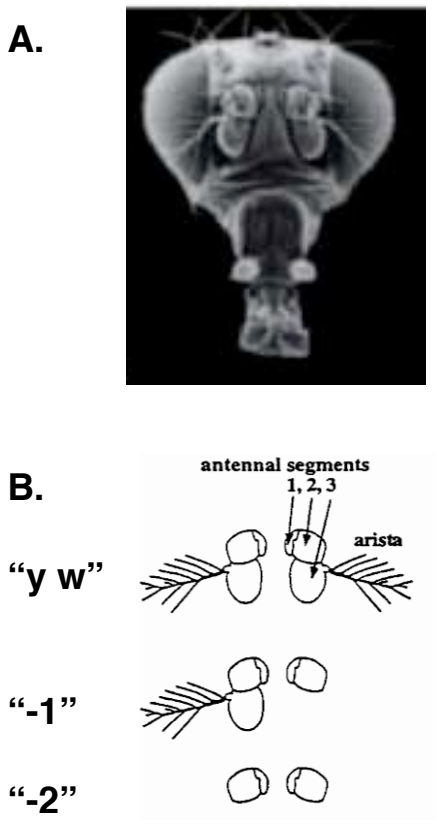


Figure 4-1. Removal of the known thermosensory organs. A. Image of a fly head with white arrow pointing to antennae. **B.** To remove the known thermosensory organs, adult flies were anesthetized with CO_2 and one antennal segment (“-1”), both antennal segments (“-2”) or no antennal segments (as control, “y w”) were removed. (Images from *http://scienceblogs.com/pharyngula/upload/2007/03/stalk_disc.jpg* and Sayeed and Benzer 1996).

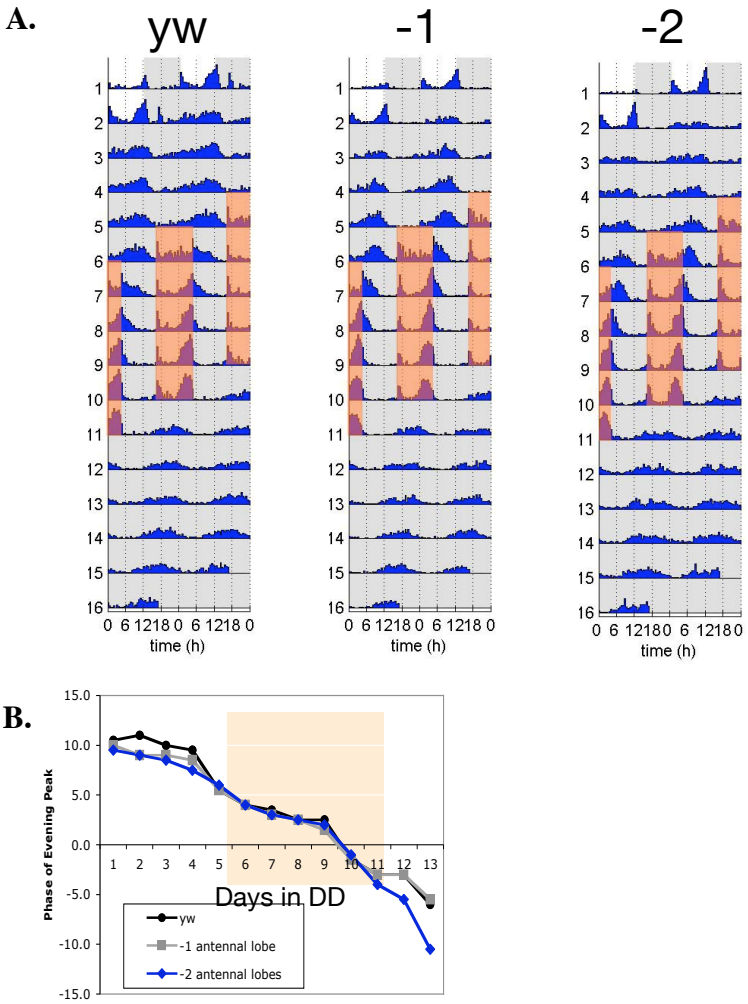


Figure 4-2. Temperature entrainment in flies lacking peripheral temperature sensors. **A**. Average behavioral actograms of *y w* control flies (left panel), *y w* flies after removal of one 3rd antennal lobe (middle panel), or both 3rd antennal lobes (right panel). Flies were entrained to a 12AM Lights On/ 12PM Lights Off at 20ºC for 2 days and then monitored in constant conditions (20ºC DD). On day 6 at 4PM, they were exposed to temperature cycles of 20ºC/29ºC (orange rectangles refer to warm periods) that were 8 hours earlier than the previous LD cycle. **B.** Quantification of the evening peaks for the experiment shown in A. **A,B**: Sample size 16/group (yw & -1) and 32/group (-2) respectively.

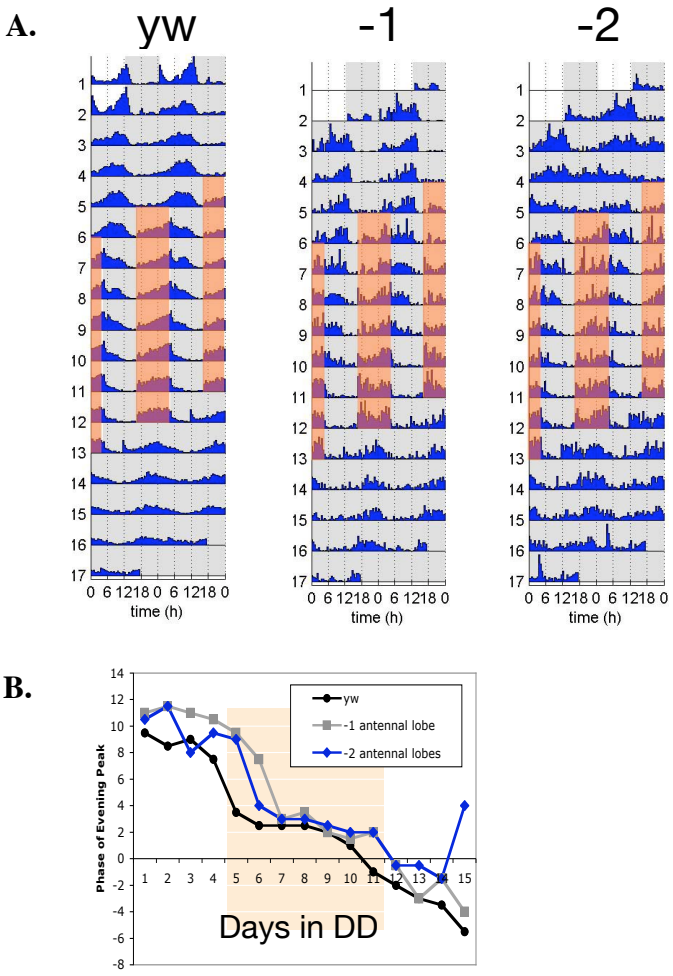


Figure 4-3. Flies lacking peripheral temperature sensors entrain to 18ºC/21ºC temperature cycles. **A**. Average behavioral actograms of *y w* control flies (left panel), *y w* flies after removal of one 3rd antennal lobe (middle panel), or both 3rd antennal lobes (right panel). Flies were entrained to a 12AM Lights On/ 12PM Lights Off at 18ºC for 2 days and then monitored in constant conditions (18ºC DD). On day 6 at 4PM, they were exposed to temperature cycles of 18ºC/20ºC (orange rectangles refer to warm periods) that were 8 hours earlier than the previous LD cycle. **B.** Quantification of the evening peaks for the experiment shown in A. As behavior showed particularly strong masking effects during the warm phase the quantification was difficult to accurately assess during the first few days of TC . **A,B**: Sample size 16/group (yw), 8 (-1) and 24 (-2).

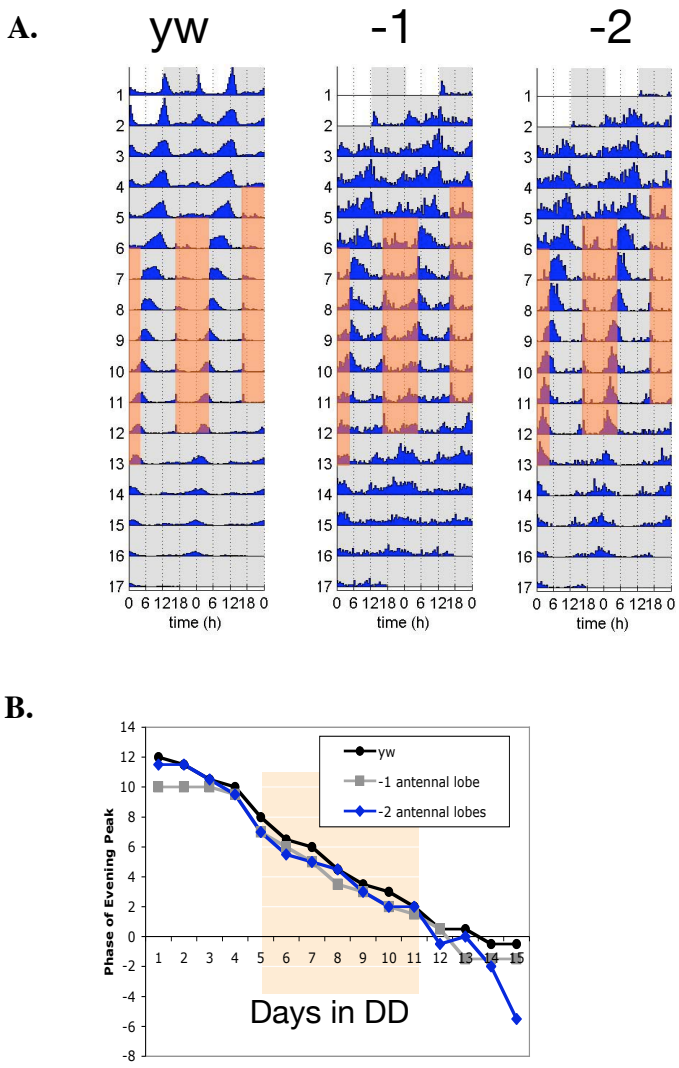


Figure 4-4. Flies lacking peripheral temperature sensors entrain to 26ºC/29ºC temperature cycles. **A**. Average behavioral actograms of *y w* control flies (left panel), *y w* flies after removal of one 3rd antennal lobe (middle panel), or both 3rd antennal lobes (right panel). Flies were entrained to a 12AM Lights On/ 12PM Lights Off at 26ºC for 2 days and then monitored in constant conditions (26ºC DD). On day 6 at 4PM, they were exposed to temperature cycles of 26ºC/29ºC (orange rectangles refer to warm periods) that were 8 hours earlier than the previous LD cycle. **B.** Quantification of the evening peaks for the experiment shown in A. **A,B**: Sample size 16/group (yw), 16 (-1) and 8 (-2).

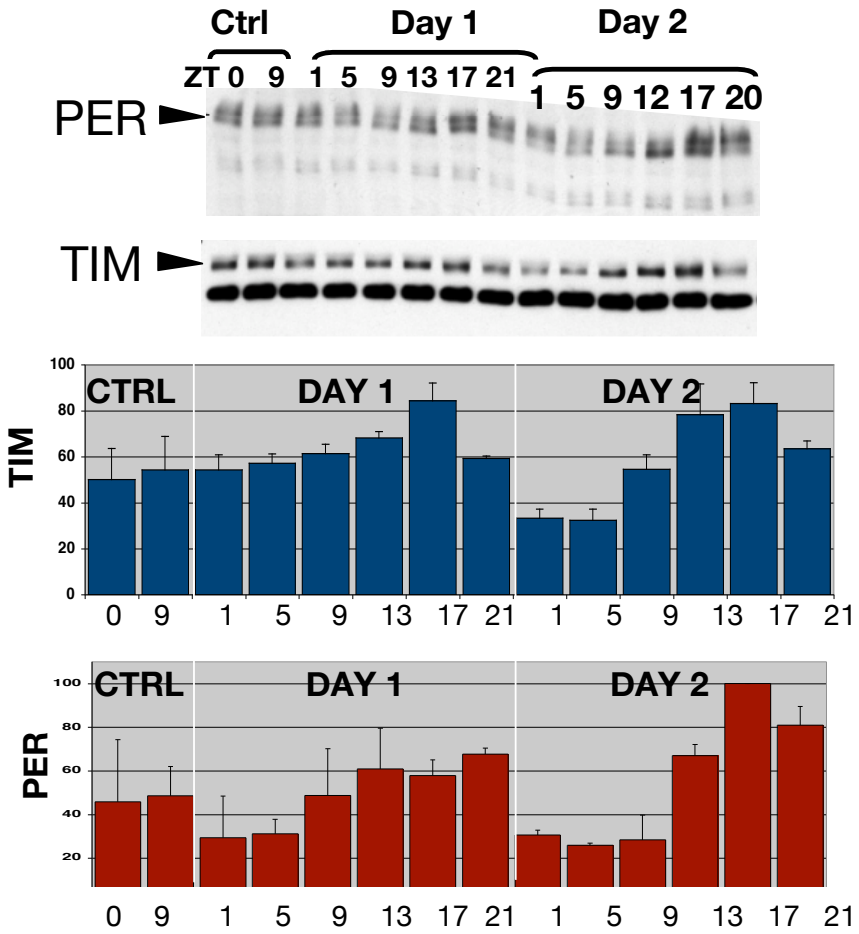


Figure 4-5. Temperature entrainment of PER and TIM cycling in *cry^b* head extracts. *Cry^b* flies were placed into a 12hr:12hr 29ºC/20ºC temperature cycle, with "ZT 0" corresponding to onset of warm period. Controls (flies maintained at constant 25ºC) were collected at ZTs 0 and 9 on the first day. During the first day, there may already be a decrease in PER and TIM in the warm period (ZT 1, 5, 9), and then increase during the first cold period (ZT 13, 17, 21). By the second day of temperature entrainment, robust molecular oscillations are clearly present. Top 2 panels: PER and TIM Western Blots of fly head extracts. Bottom 2 panels: Quantification of 3 similar Western Blots. Note that ZTs 12, 17 and 20 in day 2 of the Western Blot were averaged with ZTs 13, 17 and 21 in the other experiments in the lower 2 panels.

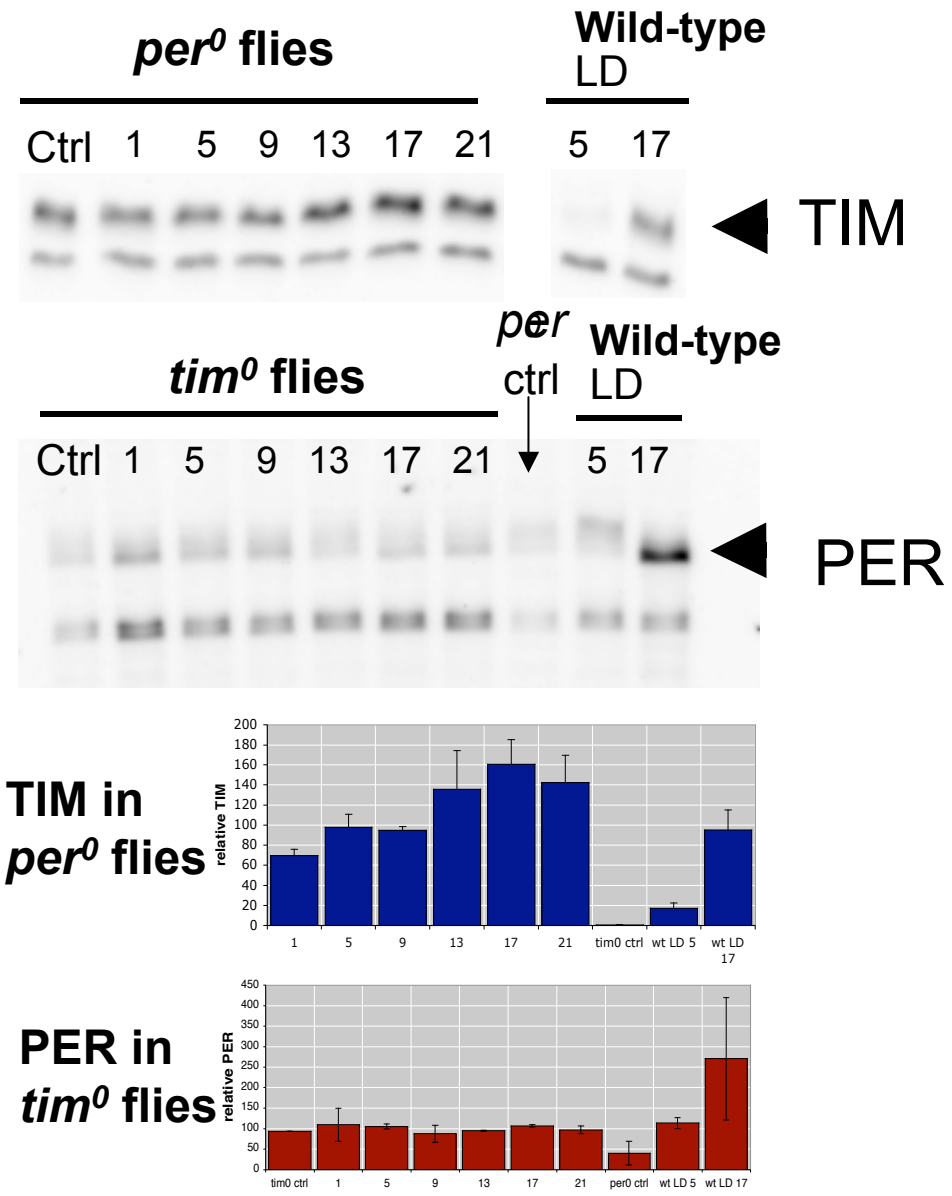


Figure 4-6. Temperature may target TIM *in vivo*: TIM levels are higher at lower temperatures in *per*^{0} head extracts, but PER levels are not affected by temperature in *tim*^{0} head extracts. *per*^{0} and *tim*^{0} flies were entrained to LD for 2 days at 20ºC, and then placed into a 20ºC/29ºC TC in DD (warm phase: ZTs 0-12). After 3-5 days, they were collected and TIM and PER levels assessed with Western Blotting. Control flies include mutant flies maintained at constant 20ºC DD, or wild-type flies in LD. Top panels: anti-TIM and anti-PER Western Blots, bottom panels: Quantification of 2 experiments.

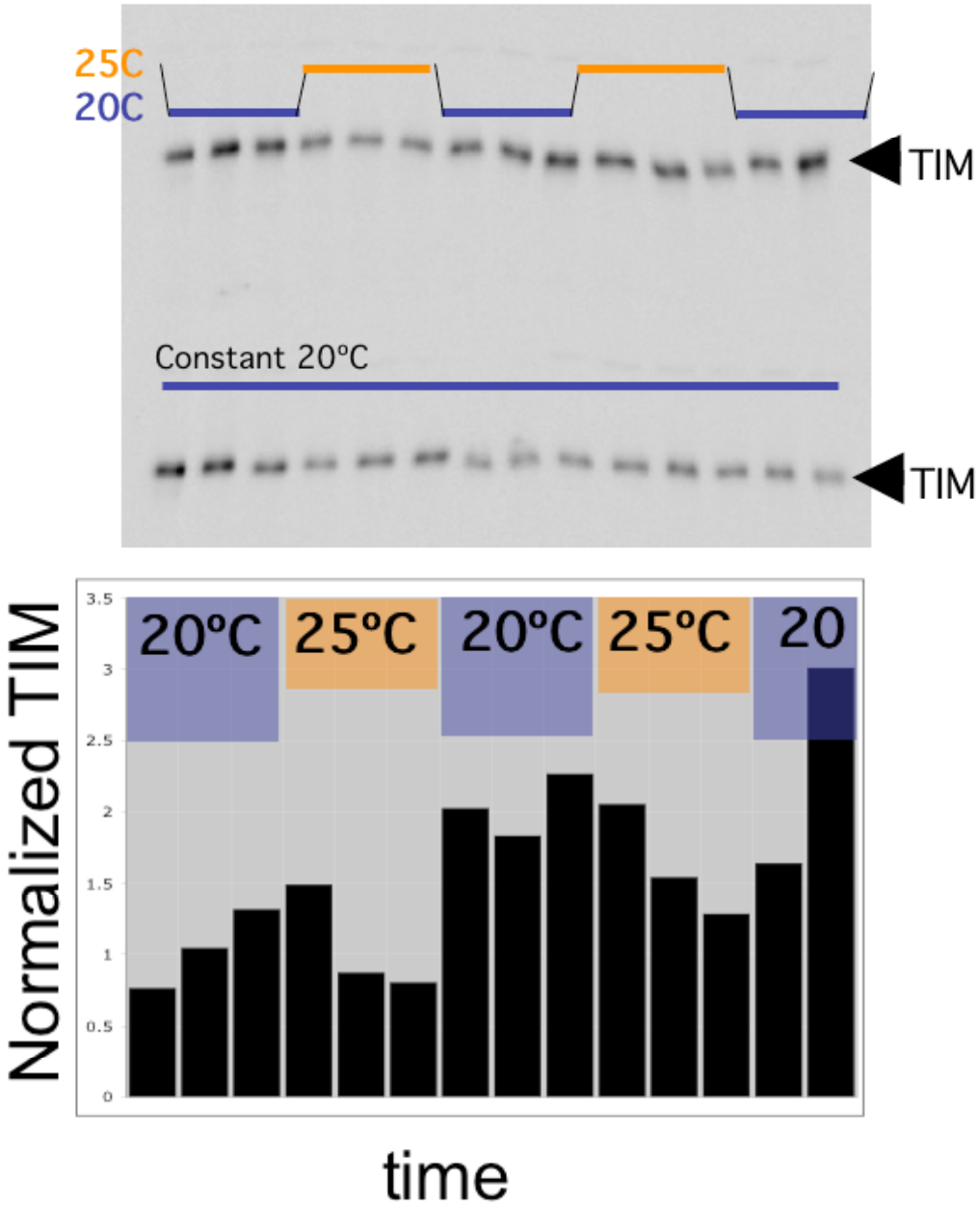


Figure 4-7. Preliminary data suggesting temperature cycles affect TIM levels in cell culture. Drosophila S2 cells were transfected with pAc-*clk,* kept at 25ºC DD for several days, and then divided into two flasks. Half of the cells were exposed to several days of 12hr: 12hr 20ºC/25ºC TC in DD , the other was maintained at 20ºC. Immediately after the temperature cycle began, cells were collected and processed every 4 hours. TIM levels were assessed with Western Blotting, and quantified. Top panel: TIM western blot over 2.5 days TC, bottom panel: quantification of TIM levels during TC (normalized by TIM levels in constant conditions, i.e. each TC level divided by corresponding constant conditions level, to adjust for decreasing transfection efficiency over time).

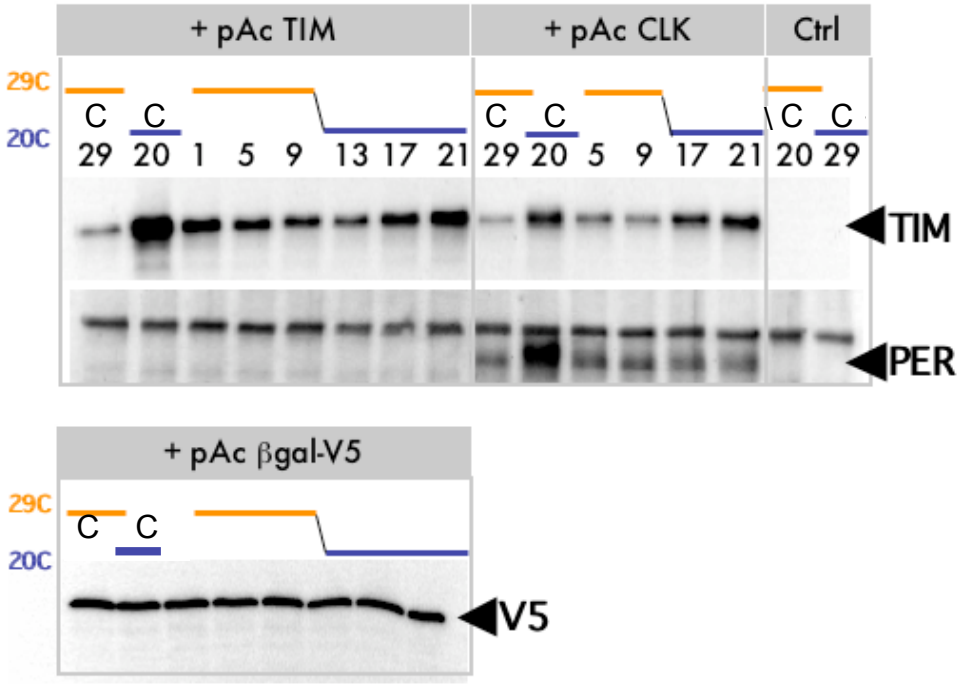


Figure 4-8. Temperature-dependent effects on TIM levels in S2 cells is not an artifact of the pAc-*clk* plasmid. *Drosophila* S2 cells were transfected with pAc-*clk,* pAc-*tim, or* pAc-*βgal-V5* and exposed to several days of 12hr: 12hr 20ºC/29ºC TC in DD (warm phase: ZTs 0-12). After 3 days of TC, cells were collected and TIM or *β*gal-V5 levels measured with Western Blotting (pAc-*clk* ZT 1 and ZT 13 time points were lost during sample processing). Ctrl: non-transfected S2 cells (exposed to 3 days constant 20ºC or constant 29ºC). C: control samples of transfected S2 cells (exposed to 3 days constant 20ºC or constant 29ºC. All control samples were collected during the first collection point of TC-exposed cells. TIM levels appear to be temperature dependent regardless if expressed directly via pAc-*tim* or indirectly via CLK expression. In contrast, *β*gal-V5 appears constant in both warm and cold temperatures.

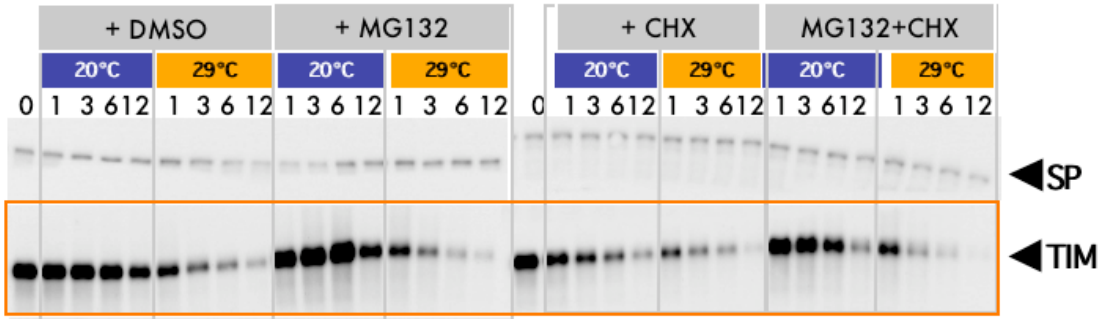


Figure 4-9. Proteasome-independent TIM degradation at 29ºC in *Drosophila* S2 cells. The cells were transfected with pAc-*tim* and then maintained at 20ºC in the dark. At time 0, they were treated with proteasome inhibitor (MG-132), protein synthesis inhibitor (CHX), or both (MG-132 + CHX) and then placed in 20ºC or 29ºC, and harvested after 1, 3, 6, or 12 hours. Western blots were stained with an anti-TIM antibody (TIM) or anti-SPECTRIN (SP) as a loading control. MG-132 did not prevent the decrease in TIM levels at 29ºC. In addition, CHX treated cells still show relatively higher levels of TIM at 20ºC. Taken together, this suggests that there is a proteasome-independent mechanism of TIM degradation at 29ºC that contributes to the difference in TIM levels at warm and cold temperatures.

CHAPTER V

FINAL CONCLUSIONS

A. General Discussion

In this dissertation I have investigated the neuronal and molecular substrates of light and temperature inputs into the *Drosophila* circadian clock.

In Chapter II, I provide data that demonstrates that CRY binds to TIM in a light-dependent manner, and that this irreversibly commits TIM to proteasomal degradation. This study provides new information about how CRY acts as an intracellular circadian photoreceptor. After being activated by light, CRY interacts specifically with TIM, or with the TIM/PER dimer via TIM. Somehow, this interaction results in TIM, but not CRY, being irreversibly tagged for degradation. This could be through phosphorylation of TIM by CRY; there is some evidence that cryptochromes in other species have kinase activity (Ozgur and Sancar, 2006). However, it is likely that CRY's effect on TIM is due to other molecules that CRY recruits when interacting with TIM. Recent studies show that JETLAG, an F-Box protein and putative component of a Skp1/Cullin/F-box (SCF) E3 ubiquitin ligase complex, is necessary for CRY to transmit light-information to TIM

(Koh et al., 2006; Peschel et al., 2006). Thus, a possible picture emerges in which CRY, after a light-induced conformational change, binds to TIM and recruits a kinase that phosphorylates TIM (Naidoo et al., 1999). Phosphorylated TIM would then be targeted by the SCF Complex and ubiquitinated. It is unknown if CRY itself is ubiquitinated. It could possibly be this difference in regulation of ubiquitination that allows CRY, but not TIM, to avoid degradation if returned to dark conditions after a light-pulse. But what are the other proteins involved in these degradation pathways, and what regulates CRY degradation?

Additional studies will be needed to identify other molecules that interact with CRY and TIM and to determine their function in the light-input pathway. However, our analysis of the cry^{m} mutant in Chapter II provides a partial answer to what regulates CRY degradation - the Carboxy-terminal domain (C-terminus) of the protein. Our studies on these mutant flies that express a truncated version of CRY indicate that the C-terminal domain of CRY is not necessary for circadian light detection but instead regulates CRY stability. Putting these data together, we propose a new model of CRY function involving separate roles of its two main structural domains in which the photolyase domain is sufficient for circadian function and the C-terminus plays a regulatory role. Another research group found that over-expressing a truncated CRY with wild-type photolyase domain and no C-terminus resulted in constant-light-like phenotype and therefore reached a similar conclusion about the functional importance of the photolyase domain (Dissel et al., 2004). Intriguingly, this model is very different from the proposed role of these domains in CRYs of other species. In *Arabidopsis*, the CRY1 C-terminal domain is

what transduces the light signal to the circadian clock, as over-expression of the C-terminus alone leads to a constitutively active constant-light-like phenotype (Yang et al., 2000). In contrast with the *Drosophila* CRY, the CRY1 core photolyase domain acts as a regulator and not as a signal-provider. In the dark, the photolyase domain recruits an inhibitory protein, COP1. The CRY1 C-terminus interacts with COP1 and is prevented from binding and signaling to other circadian molecules (Yang et al., 2001). In vertebrates, it is again the photolyase domain that interacts with downstream circadian components, however the modulatory effect of the C-terminus is via regulation of CRY protein localization as opposed to protein stability (Zhu et al., 2003). In these animals, however, the role of the photolyase domain is very different: vertebrate CRYs function as transcriptional repressors, and form part of the core feedback loop of the clock (Stanewsky, 2003). Indeed, CRY has recently been shown to have transcriptional repression activity in many other insect species (Yuan et al., 2007). In the monarch butterfly there are two *cry* genes, one of which has photosensitive properties and the other that acts as a potent transcriptional repressor (Zhu et al., 2005). There is even a growing body of evidence suggesting that *Drosophila* CRY can also act as a transcriptional repressor, at least in peripheral clocks (Collins et al., 2006; Krishnan et al., 2001; Ivanchenko et al., 2001). One can speculate that the original CRY proteins were modified versions of the ancient photolyases around which were formed the basis for anticipatory clock gene networks (Collins et al., 2006). These proteins could have had both light-sensing and repressive functions. Then in different species, these CRYs evolved in separate ways. For many organisms, as direct intracellular photoreception was

no longer useful, circadian photoreception was forced to rely on peripheral photosensors, and CRY's role became strictly repressive. During the course of this evolution from the photolyase, the C-terminal domain has acquired differing functions in various organisms to aid with regulating and specifying the diverse roles it plays in different species. *Drosophila* CRY may be a rare example of a cryptochrome that still retains both functions of the ancestral protein.

While studies have accumulated a substantial amount of knowledge regarding the mechanism of the intracellular circadian light-input pathway, understanding of temperature-inputs into the circadian clock is still extremely limited. The molecular data in Chapter 4 demonstrate that TIM protein levels can be temperature-dependent, at least in per^0 mutants and *in vivo* in cell culture. Whether the TIM degradation I see at 29ºC is via the same mechanism as previous studies reporting acute TIM degradation at higher temperatures is unclear (Sidote et al., 1998). High-temperature behavioral effects appear to be CRY-dependent (Kaushik et al., 2007). In contrast we see TC-induced TIM cycling in *cry*-deficient situations: in cry^b fly head extracts (where the CRY is severely hypomorphic and expressed at very low levels) and in our S2 cells (which do not express detectable levels of CRY protein) (unpublished results). Future experiments using *cry* RNAi to further knockdown *cry* expression in S2 cells could be used to investigate the role of CRY in TIM instability at higher temperatures.

Recently, two articles were published showing an effect of temperature on transcription of circadian genes (Boothroyd et al., 2007; Yoshii et al., 2007). Boothroyd and colleagues used microarray analysis to show that temperature cycles in constant

darkness can entrain circadian transcripts. They found that the set of thermocycle-entrained transcripts overlaps significantly with previously reported photocycle-entrained transcripts. Their analysis used flies never exposed to light/dark cycles, and was measuring transcription rhythms that persisted after the temperature cycles had stopped; the data suggest that temperature cycles can entrain a circadian oscillator that has never been synchronized by light dark cycles. Additionally, they report a temperature-sensitive TIM mRNA splice form: tim^{cold}. Predominantly seen during the cold phase (18ºC), tim^{cold} is a longer *tim* transcript with the last *tim* intron retained. As this last intron contains a premature stop codon, the predicted protein is truncated and missing a fragment of the cytoplasmic domain. It is unclear how this affects the function of the protein, however it may contribute to the observation that overall *tim* transcript levels appear to be increased at 18ºC relative to 25ºC. This temperature-sensitive splicing could regulate temperature entrainment, or could help coordinate timing of the evening peak similarly to the previously reported temperature-sensitive *per* splicing (Majercak et al., 1999). In the other recent article, Yoshii and colleagues show effects of temperature-shifts on transcription of several circadian genes (Yoshii et al., 2007). In this study, the authors found that after a single temperature change between 20ºC and 30ºC, transcript levels of *per*, *tim*, *Clk*, *vri* and *Pdp1* mRNA rose or fell depending on the direction of the temperature shift. The authors suggest that alternating 12-hour temperature steps-up and steps-down would repeatedly produce opposing effects on clock gene transcription rates that could eventually result in stable molecular oscillations in synchrony with the temperature cycle. Together with my results suggesting post-translational temperature

effects, these studies on temperature and transcription indicate that temperature entrainment of the circadian clock may be much more complicated than photic entrainment. As opposed to having several dedicated light-input pathways, temperature may affect many or all reactions in the molecular pacemaker. This will make it very difficult to tease out the relative role of each temperature effect and to determine which are the most important. Careful work using inhibitors to block transcriptional effects or post-translational effects, however, may help us determine which molecular pathways show the most temperature sensitivity. Indeed, there is some evidence that suggests there could be a dedicated temperature-input pathway (Glaser and Stanewsky, 2005). Using a chemical mutagenesis screen, Glaser and colleagues isolated *nocte*, a mutant that entrains normally to light/dark cycles but is deficient in behavioral and molecular synchronizations to temperature cycles in constant light conditions. It is thus possible that future screens could identify a set of molecules that participate in input pathways for temperature entrainment of the molecular clock.

At the cellular level, several advances have been made in our understanding of how the known pacemaker neurons function to regulate circadian responses to temperature.In Chapter III, behavioral analysis of locomotor activity to compare temperature entrainment between wild-type flies and flies lacking subsets of circadian neurons. We found that the cells previously characterized as M (Morning) cells and E (Evening) cells under light/dark cycles play similar roles under temperature cycles in the absence of light. Recent data suggest that environmental conditions can dictate which cell groups dominate the circadian network (Murad et al., 2007; Stoleru et al., 2007). In

our experimental paradigm, the M-cells appear to be necessary for rhythmicity to persist in constant darkness after temperature cycles. This suggests that even after temperature cycles, the M-cells continue to be the primary dark-dominant pacemaker cells. It is unknown if temperature conditions, like light, can modulate the relative importance of different cell groups. However, it appears that under certain conditions there may be cells that have a temperature cycle specific activity. We present evidence that other, non-M and non-E-cells may contribute to evening activity during temperature cycles, both in constant darkness and constant light. These may be the LPNs, a neuronal group reported to show molecular oscillations specifically under temperature cycles but not light/dark cycles (Yoshii et al., 2005). To further verify this, immunocytochemical analysis of flies lacking M and E-cells will have to be done to demonstrate that molecular oscillations persist specifically in temperature cycles in the LPNs (or other cells implicated in temperature-specific behavior).

As we collect a more detailed picture of the roles of individual cell groups under different conditions, the next task will be to better understand the role of communication between these cells in the production of properly timed circadian behavior. Our work in Chapter IV suggests that one possible function of the intercellular network is to modulate sensitivity to external inputs: when M-cell output is disrupted or the M-cell molecular oscillator is altered, E-cells become overly reactive to temperature fluctuations. There is some evidence suggesting that attenuated oscillators exhibit stronger phase-shifting in response to environmental fluctuations (Pittendrigh et al., 1991; Vitaterna et al., 2006). Our data may support this, as over-expressing *per* in the M-cells alone led to faster re-

setting of the entire system. We believe that the resetting is due to an attenuated oscillator in the M-cells generated by higher overall levels of PER, and support this theory by showing that we see similar temperature sensitivity in Clk^{Jrk} heterozyotes, which have decreased amplitude in molecular oscillations (Allada et al., 1998). Further work, possibly using immunocytochemical analysis, is needed to verify that *per* overexpression does indeed lead to a dampened molecular oscillator. However, it is tempting to wonder if intercellular communication in general increases the robustness of the entire circadian system. A recent publication looking at mammalian clock cells expressing mutant proteins suggested that intercellular coupling could compensate for genetic perturbations and increase robustness of the oscillations of the system (Liu et al., 2007). Much remains to be determined as to what effects network communication has, and what type of information is being communicated. Our work supports a growing view that the network may have more functions than just synchronizing individual cell oscillators.

B. Future Directions

Our data add to an understanding of the circadian cell network where individual cell groups contribute to different aspects of circadian behavior, and the cell-to-cell communication is necessary for proper maintenance and response of rhythms. However, our assays are still fairly crude: we used tissue-specific drivers that affect several groups of neurons at the same time, and our behavioral assay is not able to measure subtle

differences in behavior because of the noisiness of behavior that occurs during individual flies' reactions to the temperature cycles. Hopefully, the development of new drivers to target smaller subsets of neurons, as well as further characterization of cell phase under different environmental regimes using immunocytochemistry, can help us further elucidate the details of this network.

Eventually, such studies may lead to a better understanding of how environmental inputs act together to synchronize circadian systems. This will not only deepen our understanding of how circadian behaviors respond to environmental conditions; it may also provide information to help us better manage clinical disorders created when human circadian systems are de-synchronized from the external day/night cycle.

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APPENDIX I:

Glossary of Circadian Terms

Actogram: A graph of daily activity. In this thesis, the actograms show fly locomotor activity levels on the y-axis and the time-of-day on the x-axis. Subsequent days are stacked vertically, and the data is double-plotted such that each line shows the activity over 2 consecutive days.

Circadian Clock: an internal biological time-keeper which maintains a roughly 24 hours, is entrained by external day/night signals, and regulates daily changes in physiology and behavior.

Circadian Time (CT): The time-of-day when an animal is free-running in constant conditions, relative to the beginning of the animal's active phase. In *Drosophila,* this is usually expressed in hours passed since the time that would correspond to dawn if there was an external day/night cycle (as *Drosophila* are diurnally active and maintain a period length of approximately 24 hours).

Clock: a time-keeper that provides output indicating what time it is.

to Entrain: to synchronize the circadian system to an external environmental day/night cycle, or other regularly occurring environmental stimulus, by causing lasting effects on the phase and/or period of the underlying circadian oscillator (see "to synchronize" for comparison).

Free-running period: The innate period length of an organism's circadian cycle. This is assessed by observing the period length of a circadian cycle (i.e., circadian behavior or circadian molecular oscillations) in conditions with no external time-cues. In this dissertation, "Tau" is used as an abbreviation for the free-running period.

Masking: a non-circadian effect that an external stimulus has on a circadianly-regulated output. This can be due to the stimulus acting downstream of the circadian clock, or could be due to the stimulus having an effect on a completely parallel process.

Oscillator: a self-contained system whose state varies in a recurring fashion.

Pacemaker: a device that establishes and maintains the rate of a rhythmic activity.

Period: the length of time it takes for a defined state in an oscillation to re-occur.

Phase: the state an oscillatory system is in relative to the rest of its cycle. In this dissertation, the phase is described by measuring the time that has passed from an external reference point to a specified point of a circadian rhythm.

to Synchronize: to cause to occur at the same frequency and phase. In this thesis, "synchronization" of behavior refers to situations where an external cycle in the environment (such as a temperature cycle) has caused the outward manifestation of a circadian rhythm (such as circadianly regulated locomotor activity) to have a similar phase and period as the external cycle. The term "synchronized" refers to the final effect of the activity pattern being similarly timed as the external cycle; it does not necessarily imply that the phase or period of the underlying circadian oscillator has also been adjusted (see "to entrain" for comparison).

Time-keeper: a system whose state contains information about the time duration since some reference point (e.g. since it was started).

Zeitgeber: (from the German, time-giver) an environmental input that can entrain or re-set a biological clock.

Zeitgeber Time (ZT): The time-of-day, expressed in hours passed since dawn, during a day/night cycle. "ZT 0" is the onset of the day, usually meaning when the lights were turned on. In reference to thermocycles, "ZT 0" refers to the onset of the warm phase.

APPENDIX II:

Development of a Quantitative Temperature Entrainment Assay

1. The importance of quantification in behavioral genetics.

When studying the mechanisms regulating behavior, it is important to develop objective ways of defining and measuring manifestations of the behavior. Just like careful molecular biology requires quantification of protein levels, fluorescent staining, etc., to draw meaningful conclusions from experiments, the study of behavioral analysis also greatly benefits from quantification. Ideally, the behavior should be broken down into several components, and each component should have a way to be objectively measured. Then, "normal" behavior can be defined in wild-type animals under varying stimuli and environmental conditions. Once the normal wild-type behavior has been characterized, the behavior of mutant animals can be objectively compared to that of wild-type animals and conclusions can be made about the effect of genetic manipulations on this particular behavior. Without defined ways of describing and measuring the behavior it is difficult to assess and report how a mutant is responding abnormally. In this appendix, I will discuss how we evaluated temperature cycle entrainment in *Drosophila*. I will then briefly discuss some of the problems of our behavioral quantification techniques and consider which changes, with hindsight, I would recommend for future experiments.

2. The Temperature Entrainment Assay.

In many of the experiments in Chapters III and IV of this dissertation, my goal was to investigate if a particular group of flies exhibited normal entrainment to temperature cycles. To do this, we developed an assay where flies were first observed free-running after entrainment to LD, were then exposed to a phase-shifting TC, and finally released again to free-running conditions [Figure AII-1]. This experimental protocol was chosen for the following reasons: (1) We used an experimental design where the free-running circadian behavior was observed both prior and post exposure to TC so that we could evaluate the effect of TC exposure on the phase of the underlying circadian oscillator. In this way, we could distinguish between genuine circadian entrainment and the non-circadian ("masking") effects of TCs on activity levels. (2) We chose to use a box-step temperature cycle, as opposed to gradually changing temperature fluctuations because it has the benefits of being a simple stimulus with only two conditions (thermophase and cryophase). However, the caveat should be noted that it is less similar to the gradually fluctuating temperature cycle occurring in natural environments. (3) We used 29ºC for the thermophase and 20ºC for the cryophase because according to online weather records, the temperature on an average August day in Canton, OH (where the wild-type *c s* strain was originally collected) fluctuates between 20ºC and 29ºC.

3. Analysis of Behavior.

Our initial method of analyzing *Drosophila* circadian response to temperature was a rough visual analysis of actograms. We have used two different programs to create actograms: the Fly Activity Analysis Suite, or "Faas" (Brandeis Rhythm Package), and the *dam_plot* function in the Levine circadian MATLAB toolbox (Levine et al., 2002b). In the Faas actogram, the time of every 20th crossing of a light-beam by a fly is marked by a vertical line, or "hash", on a one-dimensional graph. As a result the density of the hash-marks is a measure of the fly activity during that time of the day [Figure AII-2]. The individual fly actograms we plotted provided reassurance that the fly behavior was indeed responding to temperature cycles [Figure AII-2]. By using the informal technique humorously referred to by circadian biologists as the "hold the paper up and squint at it till it becomes blurry" strategy, one can see that the darker parts of the actogram (corresponding to increased behavioral levels) phase-shift in response to temperature and then persist in a similar phase post-TC. However, this method is far from ideal: first of all, it relies on subjective decisions of what qualifies as a true behavioral shift, secondly, it has limited usefulness for identifying different components of behavior during TC (such as the morning or evening peaks), and finally, it plots individual flies and not averages. To solve some of these problems, we began using a MATLAB toolbox to plot actograms (Levine et al., 2002b). These actograms [Figure AII-2] provide several benefits: the relative amount of activity per 30-minutes is graphed on the Y-axis for each day, and this activity-curve can more easily be analyzed than hash-marks. Additionally, the average activity of a group of flies can be plotted to look for more subtle trends and

decrease individual noisiness. Furthermore, errorbars can be derived from the fly-to-fly variation.

The MATLAB toolbox we used contains several programs for quantifying phase and phase-shifts. The one we used, "peakphaseplot", first smooths the data using an 8-hour filter, and then identifies all peaks in activity in each actogram. We manually select which of these identified peaks are the evening peaks. The time of each evening peak relative to midnight (or some other external time reference point) is calculated and plotted for each experimental day. From the amount by which the peak shifted from day to day we can determine if the circadian system entrained to the external stimulus, and at what rate [see for example Figure 3-1 in Chapter III].

4. Discussion of our Methods.

Our method proved sufficient in quantifying the response of wild-type flies to temperature cycles. However, experiments with genotypes with more complex/abnormal behaviors demonstrated certain limitations to this method. Indeed, when the assay was used on flies with shorter or longer period lengths or with variable activity peaks, it became a lot more difficult to assess temperature entrainment. Several improvements to our techniques, both at the experimental and analytical levels, would greatly improve our ability to assess temperature entrainment, especially in non-wild-type flies.

(a) Experimental design:

Since our goal of the experiment is to quantify the phase shift in circadian behavior attributable to the stimulus, it is crucial to establish what the phase would be if the flies had not been exposed to the stimulus. In most of our experiments, we did this by comparing the phase after TC with the phase prior to TC. However it is possible that in some flies (especially in some short or long period mutants) the free-running activity of flies not exposed to TC would change during the course of the experiment. Therefore comparing a post-TC phase with a prior-TC phase would not accurately take into account some non-TC related phase changes. Theoretically, we could improve our phase assessment by an extrapolation from the pre-stimulus regime: the free-running period prior to TC exposure presumably predicts where activity should occur if the animals had not been exposed to TC. However, since the circadian rhythm is not necessarily constant over the duration of the experiment (even in the absence of the stimulus), I would strongly advice the use of a non-stimulus control. This non-stimulus control group of flies, left in constant conditions for the duration of the experiment, would help differentiate between phase-changes due to the TC and phase-changes due to natural differences in different genotypes due to genotype-specific or age-specific variations in period length. Furthermore, since the before-after within-group comparison is replaced by a concurrent between-groups comparison, entrainment can be recorded throughout the experiment. In addition to a more robust analysis of the total entrainment effect, it would also provide more accurate information on its day-by-day time course.

(b) Choice of phase marker

The phase of the circadian rhythm is a measurement of the location of the behavioral pattern relative to absolute time. Out of convenience, it is typically measured by the timing of a single feature alone. We chose the evening peak as our phase marker as it is a clear component of the *Drosophila* circadian behavior and there are several programs available for identifying it. However, we found that masking effects sometimes confounded the evening peak. For example, higher overall activity during the warm phase, or startle responses after a temperature change, can shift or obscure the location of the activity peak. Furthermore, genotypes with complex behavior might have less pronounced or multiple evening peaks. An improved way to quantify the phase could involve verifying the measured phase-shifts by re-measuring the phase using other markers. Using additional markers, such as the trough of activity or the offset of activity (such as the midpoint of the rapid decrease of activity at the beginning of subjective night), might help in situations where the evening peak is unclear or affected by masking. Eventually, development of a program that searches each day for patterns that fit the genotype-specific pattern of behavior and uses the entire pattern to measure phase would probably avoid many of these problems.

(c) Marker identification

As mentioned above, we manually selected the behavioral component we were using as the phase-marker (the evening peaks). While I tried to select the evening peaks without looking at the time of the peak, it is possible that when two peaks were

ambiguous, I subconsciously chose the peak that seemed to have the "right" time given the other flies I had looked at. Ideally, an automated system should be developed to avoid creating biased data due to subjectively choosing evening peaks. Currently, our system is only partly automatic; the Matlab *peakphaseplot* program selects all peaks automatically. Evening peak selection could be automated by deciding on a specific definition for "evening peak" in this assay. For example, the system could identify peaks only within a specific time frame. Alternatively, it could identify the last peak before the lowest trough. Possibly, the best would be to include all peaks that fit the criteria, as opposed to selecting only one, and to include both in the data set. Further investigations into determining an objective way to identify phase-markers in the activity record would hopefully improve data consistency.

5. Final thoughts.

Our current strategy provides a method of objectively comparing TC entrainment between different fly groups. However, I suggest additional modifications of both the experimental design and evening peak selection, as well as using an additional phase-marker. Together, these improvements should result in a more robust assay and would improve our ability to screen for abnormal temperature entrainment.

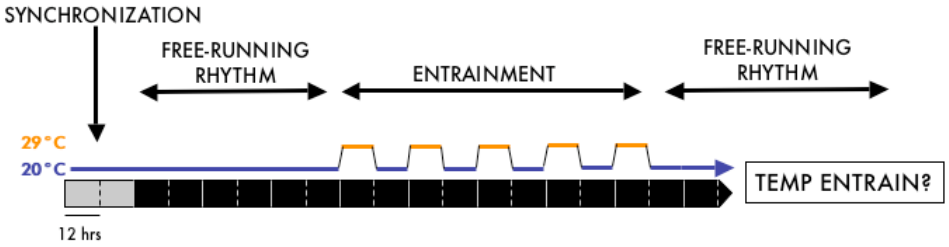

Figure AII-1. Experimental protocol for assessing temperature entrainment. Flies are synchronized to several days light/dark cycles prior to experiment (SYNCHRONIZATION) and then allowed to free-run in the dark in the absence of all time cues (FREE-RUNNING RHYTHM, left side). Then, they are exposed to 12hr:12hr 29ºC/20ºC temperature cycles for 5 days (ENTRAINMENT) and returned to constant conditions again (FREE-RUNNING RHYTHM, right side). In this way, the change in phase of their free-running rhythms prior and post temperature cycle can be assessed.

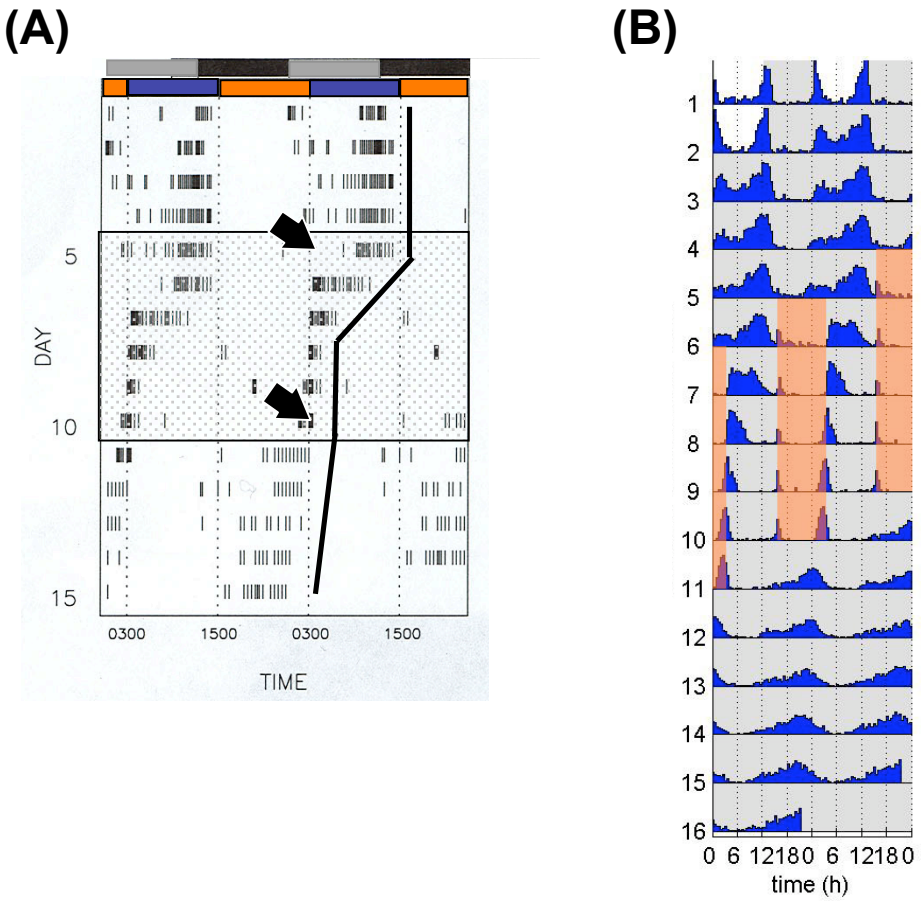

Figure AII-2. Two different styles of actograms showing fly locomotor activity entrainment to temperature cycles. (A) Actogram produced using the Fly Activity Analysis Suite (Brandeis Rhythm Package). Black arrows indicate beginning and end of temperature cycle exposure. In this actogram, relative activity levels are indicated by hash-mark density. Black line shows rough assessment of phase by "eye-balling" the changes in activity. (B) Actogram produced using *dam_plot* MATLAB function (Levine et al., 2002b). Orange boxes indicate exposure to thermophase. In this actogram, activity levels are plotted on the y-axis of the plot. (see text and Figure 1-1 for more details).