10-25-2007

Molecular and Neuronal Analysis of Circadian Photoresponses in *Drosophila*: A Dissertation

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A Dissertation Presented

By

Alejandro D. Murad

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences,
Worcester, MA 01605

In partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

October 25th, 2007

Biomedical Sciences
Program in Neuroscience
Molecular and Neuronal Analysis of Circadian Photoresponses in

*Drosophila*

A Dissertation Presented
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ACKNOWLEDGMENTS

I would like to thank the UMass Neuroscience community, for creating an amazing environment to learn science. In particular, I want to thank Dr. Patrick Emery, my mentor, for giving me the opportunity to learn about flies and clocks. I would also like to thank the members of my committee for taking time between grants, classes and Canadian Thanksgivings to read about flies and clocks. I also want to thank my present and past lab-mates: Ania, Raph, Dianne, Diana, Myai, Sheeba, Betty and Neethi for making the Emery Lab such a great learning/fun space.

The members of the Freeman, Waddell and Budnik labs taught me all I know about confocal microscopy; and the members of the Reppert, Weaver and Alkema labs allowed me to “borrow” many useful reagents. Thank you!

Finally, I would like to thank those that helped me survive the grad school years in Worcester: Ania, Raph, Ruth, Alex, Dennis, Rami, Brett, Martin, Natalia, Paola, Mariela, Carla, Manolis, Daniel, Al, Catalina, the City of Boston, the City of Cambridge, the City of Somerville, Sol, Mariana, New York City, The Chinatown Bus, Trader Joe’s, Cardullo’s, Rami’s roommates, the Massachusetts Bay Transportation Authority, Santiago’s Plaza, the Town of Provincetown, Boston Harbor Cruises (P-Town Ferry), Greg, Wagner, Fabiola, Vanuda, Erich, The GSBS office, YMCA of Greater Worcester, the Massachusetts Institute of Technology (MIT), The Lebanese Club at MIT (LCM), Au Bon Pain, Miranda
Café, Starbucks Coffee, the Provincetown Art Association and Museum (PAAM) and Diesel Café.
ABSTRACT

Most organisms, from cyanobacteria to humans are equipped with circadian clocks. These endogenous and self-sustained pacemakers allow organisms to adapt their physiology and behavior to daily environmental variations, and to anticipate them. The circadian clock is synchronized by environmental cues (i.e. light and temperature fluctuations).

The fruit fly, Drosophila melanogaster, is well established as a model for the study of circadian rhythms. Molecular mechanisms of the Drosophila circadian clock are conserved in mammals. Using genetic screens, several essential clock proteins (PER, TIM, CLK, CYC, DBT, SGG and CK-II) were identified in flies. Homologs of most of these proteins are also involved in generating mammalian circadian rhythms. In addition, there are only six neuronal groups in the adult fly brain (comprising about 75 pairs of cells) that express high levels of clock genes. The simplicity of this system is ideal for the study of the neural circuitry underlying behavior.

The first half of this dissertation focuses on a genetic screen designed to identify novel genes involved in the circadian light input pathway. The screen was based on previous observations that a mutation in the circadian photoreceptor CRYPTOCHROME (CRY) allows flies to remain rhythmic in constant light (LL), while wild type flies are usually arrhythmic under this condition. 2000 genes were overexpressed and those that showed a rhythmic
behavior in LL (like cry mutants) were isolated. The candidate genes isolated in the screen present a wide variety of biological functions. These include genes involved in protein degradation, signaling pathways, regulation of transcription, and even a pacemaker gene. In this dissertation, I describe work done in order to validate and characterize such candidates.

The second part of this dissertation focuses on identifying the pacemaker neurons that drive circadian rhythms in constant light (LL) when the pacemaker gene period is overexpressed. We found that a subset of pacemaker neurons, the DN1s, is responsible for driving rhythms in constant light. This attractive finding reveals a novel role for the DN1s in driving behavioral rhythms under constant conditions and suggests a mechanism for seasonal adaptation in Drosophila.
## TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>COVER PAGE</td>
<td>i</td>
</tr>
<tr>
<td>SIGNATURE PAGE</td>
<td>ii</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>iv</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>v</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xi</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xii</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>xiv</td>
</tr>
</tbody>
</table>

### CHAPTER I: Introduction 1

#### A. Circadian Clocks 1

1. Definition and fundamental properties 1
2. Circadian parameters 4

#### B. Circadian rhythms in the fruit fly *Drosophila melanogaster* 5

1. The fruit fly as a model for the study of circadian rhythms 5
2. The *Drosophila* circadian clock is a transcriptional feedback loop 8
3. How does the *Drosophila* clock sense light? 10
4. Circadian organization of the *Drosophila* brain 13
5. Manipulation of gene expression in different neuronal clusters

CHAPTER II: A circadian gain-of-function screen under constant light

Abstract

A. Introduction

B. Results

1. 30 lines show circadian rhythmicity in constant light

2. Is morgue part of the light input pathway?

3. A secondary PRC screen for genes involved in the light input pathway

4. A secondary RNAi-based loss-of-function screen

C. Discussion

D. Materials and Methods

1. Drosophila stocks and transgenics

2. Behavioral analysis

3. Real-Time PCR

4. Protein extracts and Western blots

E. Acknowledged contributions

CHAPTER III: A subset of dorsal neurons modulates circadian behavior and light responses in Drosophila.
Abstract

A. Introduction

B. Results

1. Flies overexpressing *per* are rhythmic under constant light

2. Non-PDF circadian neurons maintain circadian behavioral rhythms under constant light

3. Circadian oscillations persist in DN1 neurons in LL when *per* is overexpressed

4. The DN1s also drive circadian rhythms in flies overexpressing *morgue* under constant light

5. PDF is not required for circadian behavioral rhythms under constant light

6. Inhibition of the CRY input pathway allows the DN1s to remain rhythmic in LL

C. Discussion

D. Materials and Methods

1. *Drosophila* stocks and transgenics

2. Behavioral analysis

3. Whole-mount immunohistochemistry and quantitation

E. Acknowledged contributions

Addendum to Chapter III
CHAPTER IV: Final thoughts. 120
  A. General discussion 120
  B. Future directions 125

Bibliography 127

APPENDIX I: Interactions between circadian neurons control temperature
  Synchronization of Drosophila behavior 142

APPENDIX II: PER-TIM interactions with the photoreceptor
  CRYPTOCHROME mediate circadian temperature responses in
  Drosophila 155

APPENDIX III: Ectopic CRYPTOCHROME renders TIM light sensitive
  In the Drosophila ovary 166
LIST OF TABLES

Table 2-I  Behavior of the selected EP-lines crossed to *timGAL4* under constant light (1000 lux).

Table 2-II  Candidate genes sorted by biological function.

Table 2-III  Behavior of the selected EP lines crossed to *timGAL4* under constant darkness.

Table 2-IV  Behavior of RNAi lines for *kismet, CG30152, CG10459, slimb* and *morgue* crossed to *timGAL4* in LL and DD.

Table 2-V  Behavior of *morgue* deletion mutants in LL of different decreasing light intensities.

Table 3-I  Behavior of flies overexpressing *per* and *morgue* under LL conditions.

Table 3-II  Behavior of *pdf*<sup>01</sup> flies overexpressing (or not) *morgue* under LL.

Table 3-III  Behavior of flies overexpressing *per* and *morgue* under DD.

Table 3-IV  Behavior of *pdf*<sup>01</sup> flies overexpressing (or not) *morgue* under DD.

Table 3-V  Quantification of PDP-1 staining in LNv-rescued *cry*<sup>b</sup> flies.
LIST OF FIGURES

1-1. Circadian parameters.
1-3. The circadian negative feedback loop of *Drosophila*.
1-4. The *Drosophila* brain: clock cells and light sensing organs.
1-5. The GAL4/GAL80 system.

2-1. A misexpression screen in constant light.
2-4. The effect of overexpressing *morgue* is specific.
2-5. CRY and TIM levels in heads of flies that overexpress *morgue*.
2-6. Phase response at decreasing light intensities for the deletion mutant *morgue126*.
2-7. *miR-282* phase response curve (PRC).

3-1. Flies overexpressing *per* are rhythmic in LL.
3-2. The LNv and LNd neurons do not show molecular circadian oscillations in LL when *per* is overexpressed.
3-3. A subset of DN1 neurons show molecular circadian oscillations in LL when *per* is overexpressed.
3-4. LL rhythmicity is dependent on the DN1s in flies overexpressing *morgue*, but does not require PDF.

3-5. Inhibition of the CRY input pathway allows the DN1s to remain rhythmic in LL.

3-6. Genetic manipulations of PER expression in the LNvs.

3-7. Effects of *morgue* tissue-specific overexpression in PRC.
LIST OF ABBREVIATIONS

CK2: casein kinase 2
CLK: clock
CRY: cryptochrome
CYC: cycle
CT: circadian time
DBT: doubletime
DIAP1: *Drosophila* Inhibitor of Apoptosis Protein 1
DNs: dorsal neurons
DN1s: dorsal neurons 1
DN2s: dorsal neurons 2
DN3s: dorsal neurons 3
DD: constant darkness
E-cells: evening cells
E-peak: evening peak
LD: light/dark cycles
LL: constant light
LNvs: ventral lateral neurons
LNds: dorsal lateral neurons
LPN: lateral posterior neurons
M-cells: morning cells
M-peak: morning peak

NIG: National Institute of Genetics (Japan)

PCR: polymerase chain reaction

PDF: pigment dispersing factor

PDF (+): PDF positive

PDF (-): PDF negative

PDP1: PAR domain protein 1 epsilon

PER: period

PI: Pars Intercerebralis

PP1: protein phosphatase 1

PP2a: protein phosphatase 2a

RNAi: RNA interference

SD: standard deviation

SGG: shaggy

TIM: timeless

UAS: Upstream Activating Sequence

VDRC: Vienna Drosophila RNAi Center

VRI: vrille

ZT: Zeitgeber time
CHAPTER I

INTRODUCTION

A. Circadian clocks

1. Definition and fundamental properties of a circadian clock.

From the daily rising and setting of the sun to the waxing and waning of the moon, periodical changes in the geophysical world have been observed and recorded since earliest human times. Physiological and behavioral cycles closely related with changes in the environment were also long observed. These cycles include wake/sleep patterns, menstrual cycles, the seasonal appearance and disappearance of certain plants, and the migration of birds.

In order to adapt their physiology and behavior to changes in the environment, virtually all organisms are equipped with circadian clocks (from the Latin *circa dies*, which means: about a day). These clocks exist within the organism and reveal an evolutionary adaptation to life on a rotating planet.

Circadian clocks run with a periodicity of about 24 hours, sometimes
shorter, sometimes longer. These almost perfect timekeepers would be useless without their ability to be entrained by the environment: a properly entrained circadian clock will be more effective at anticipating daily variations. Various synchronizing cues from the surrounding environment, known as *Zeitgebers* (German for time-givers), are able to effectively entrain the circadian clock. The daily light/dark cycle is considered to be the primary *Zeitgeber*. Other cues include temperature fluctuations (Pittendrigh, 1954) (Liu et al., 1998) (Glaser and Stanewsky, 2005) (Yoshii et al., 2005), food availability (Schibler et al., 2003) (Castillo et al., 2004) (Davidson et al., 2005) and social interactions (Levine et al., 2002) (Fujii et al., 2007)

Since circadian rhythms are generated endogenously, they persist in the absence of any environmental cue. The ability to produce behavioral and physiological rhythms in constant conditions, or in other words, the ability to "free-run", is the most important property of a circadian clock. This was first reported in 1729 by the French astronomer Jean Jacques d’Ortous de Mairan, when he devised a now-classic circadian experiment. Intrigued by the daily opening and closing of the leaves of the heliotrope plant *mimosa pudica*, de Mairan decided to test whether this biological "behavior" was simply a response to the sun. To do so, he confined the plant to the darkness of a closet to observe that the daily rhythmic motions of the heliotrope’s leaves persisted even in the absence of sunlight. The astronomer concluded that heliotropes have internal clocks. His findings might have gone unnoticed, had a colleague not published
the results for him (de Mairan, 1729).

Unlike other metabolic processes, which are affected by changes in temperature, circadian rhythms are temperature compensated. The period of the circadian clock stays constant (~24 hr) through a wide range of physiologically relevant temperatures (Pittendrigh, 1954). Although the circadian clock is temperature compensated, temperature can act as a potent Zeitgeber entraining the clock to daily temperature fluctuations.

In sum, circadian rhythms have three fundamental properties: 1) they are generated endogenously and persist in constant conditions, with a periodicity of ~24 hr; 2) they are entrainable by environmental stimuli, such as light and temperature; and 3) circadian clocks are temperature compensated.

Circadian rhythms have two additional important properties: 1) their ubiquitous nature: circadian rhythms are present in most organisms and control a broad variety of biological processes; and 2) circadian rhythms are generated at a cellular level: unicellular organisms display circadian rhythms (i.e. algae or the dinoflagellate Gonyaulax) and these rhythms share mechanistic similarity to those of highly complex mammals (reviewed in (Lloyd, 1998)). Moreover, mouse fibroblasts in cell culture contain cell-autonomous circadian clocks with properties similar to those present in adult SCNs (Nagoshi et al., 2004).
2. Circadian parameters.

Amplitude, phase and period.

Figure 1-1 illustrates a circadian oscillation under a daily light:dark cycle. The Y axis represents the measurement of a particular circadian variability (i.e. levels of a given hormone, locomotor activity, body temperature). The difference in levels between the peak and trough values is called the amplitude of the rhythm (A). The phase (Φ) represents a time location during the cycle (i.e. the peak, or the beginning of the night). The period (τ) of a rhythm is defined as the time interval between two recurrences (i.e. two peaks). Circadian rhythms persist in constant conditions (constant darkness, Figure 1-1A), and this is referred to as free-running.

Resetting of the circadian clock.

Certain signals, such as a light pulse during the night, can reset the circadian clock, either by advancing the phase or delaying it (Figure 1-1B). In the case of light pulses, whether the phase is advanced or delayed will depend on the time at which the light pulse is administered. This response to light pulses can be expressed as a Phase Response Curve (PRC) (Figure 1-1C).

Exposure to a light pulse during the early part of the night causes a phase delay, while exposure to light in the later part of the animal’s night causes an advance. Figure 1-1C illustrates a typical Drosophila type 1 PRC. In a type 1
PRC, the response to the stimulus is in the form of “slow resetting”, with phase shifts that are smaller than 6 hr, and the transition from the delay zone to the advance zone is continuous. A type 0 PRC is characterized by “fast resetting”. Whether Type 1 or Type 0 resetting is exhibited often depends on the strength of the stimulus. In flies and mosquitoes, increasing the light dose of the stimulus converts Type 1 into Type 0 resetting (Peterson, 1980) (Pittendrigh, 1960) (Saunders, 1978)

B. Circadian rhythms in the fruit fly *Drosophila melanogaster*

1. The fruit fly as a model for the study of circadian rhythms.

*Drosophila* offers many advantages as a model system for the study of genetic basis of behavior. These include: a short generation time, a completely sequenced genome, the availability of a wide variety of tools that allow the manipulation of gene expression and rapid mutant analysis. Also, the *Drosophila* genome shares great homology to that of humans in a fraction of the size, and many molecular pathways are highly conserved between these two (reviewed in (Sokolowski, 2001)).

To date, we understand more about the biology of the fruit fly than any other multicellular organism. The use of *Drosophila* as a model to study genetics dates back to the early 1900s, when Thomas Hunt Morgan and his collaborators
uncovered some of the basic principles of eukaryotic genetics, including the demonstration that genes are physically located on chromosomes and are distributed along these as a linear array.

Decades later, in 1968, Ron Konopka carried out the first studies in clock mechanisms in *Drosophila*. Before Konopka’s studies, circadian rhythms have been described in almost every organism, in the shape of locomotor activity rhythms, hormonal and body temperature fluctuations and, in the case of *Drosophila*, pupal eclosion rhythms, but nothing was known about the molecular mechanisms that generated such oscillations. In 1954, Colin Pittendrigh described the rhythmic pattern of *Drosophila* pupal eclosion (Pittendrigh, 1954). Every day, there is a peak of pupal eclosion at dawn, and this rhythm persists in constant darkness. Based on this behavior, Konopka mutagenized flies and systematically screened for mutants that showed an alteration in their temporal pupal eclosion patterns in light:dark (LD) cycles. He found three mutant strains that were arrhythmic, or showed either advanced or delayed eclosion peaks (Konopka and Benzer, 1971). He then tested the mutants in constant darkness (DD) and observed that the mutants had short (19 h) and long (29 h) free-running *periods*. All three mutations were mapped in the same locus, which was then called *period* (*per*); the mutant alleles were called *per*\(^{01}\) (aperiodic), *per*\(^{S}\) (Short) and *per*\(^{L}\) (Long) (Konopka and Benzer, 1971). Konopka’s work prompted *per* cloning and molecular analysis, and similar approaches have identified several new clock genes.
1.1 Locomotor activity rhythms.

The measurement of activity/rest cycles (locomotor activity) has become the method of choice of fly chronobiologists when it comes to evaluating circadian rhythms. This is due, in part, to the efforts in the field that have lead to the development of automated systems for monitoring locomotor activity in individual flies over many days (Figure 1-2A).

In standard experimental conditions of 12h light: 12h dark (or 12:12 L:D), at 25C, fruit flies display a bimodal pattern of locomotor activity. This "crepuscular" behavior is characterized by a peak of activity in the morning, right after the lights-on transition (morning peak / M-peak), followed by a period of low activity or "siesta". The second peak of activity occurs around the lights-off transition, thus called evening peak (E-peak). During the dark phase, flies rest, which is the reason why Drosophila is considered a diurnal animal (Figure 1-2B, (Rosato E, 2006)).

Although these artificially generated light-dark transitions are different from the gradual variations in light intensity that characterize dawn and dusk, they provide a simple, robust and reproducible behavioral assay.
2. The *Drosophila* circadian clock is a transcriptional feedback loop.

All circadian clocks that have been studied to date at the molecular level run on negative feedback loops.

In *Drosophila*, the negative feedback loop centers in the activity of the products of the genes *period* (PER) and *timeless* (TIM) and the inhibition of their own transcription (extensively reviewed in (Collins and Blau, 2007) and (Hardin, 2004)). The basic helix-loop-helix (bHLH) transcription factors CLOCK (CLK) and CYCLE (CYC) activate the transcription of *per* and *tim* by binding to E-box regulatory sequences in their promoters (Fig. 3A) (Darlington et al., 1998) (Allada et al., 1998) (Rutila et al., 1998) (Hao et al., 1997). PER and TIM do not reach peak levels until the mid-late night (Zeng et al., 1996) (Zerr et al., 1990) (Hunter-Ensor et al., 1996), since TIM is light sensitive and required for PER stabilization (Price et al., 1995) (Gekakis et al., 1995) (Zeng et al., 1996). PER and TIM form heterodimers which accumulate in the cytoplasm and enter the nucleus <6 h after lights off. Once in the nucleus, PER interacts with the CLK/CYC heterodimer and prevents it from binding to the *per* and *tim* promoters, inhibiting their transcription (Lee et al., 1998). A recent study implies a role for DOUBLETIME (DBT, an ortholog of the mammalian Casein Kinase I) in regulating CLK, suggesting that PER alone is not enough to inhibit transcription, but acts as a bridge to deliver DBT and possibly other factors that regulate CLK transcriptional activity (Kim et al., 2007).
A series of post-translational events regulate PER/TIM stability, and their timely entry into the nucleus. DBT destabilizes monomeric PER and delays the accumulation of the PER/TIM heterodimer until enough TIM is accumulated (Price et al., 1998) (Kim et al., 2007). Casein Kinase 2 (CK2) also regulates PER phosphorylation (Lin et al., 2002). Shaggy (SGG), an ortholog of the mammalian Glycogen Synthase Kinase-3 (GSK-3), regulates TIM phosphorylation (Martinek et al., 2001).

Two phosphatases, PP2A and PP1, are also important in regulating PER and TIM stability and nuclear accumulation. De-phosphorylation of TIM by PP1 promotes TIM accumulation by preventing its degradation in the 26S proteasome, resulting in further stabilization of PER. TIM/PER is then subjected to phosphorylation/de-phosphorylation events by SGG and PP2A respectively, regulating the nuclear accumulation of the heterodimer (Sathyanarayanan et al., 2004) (Fang Y, 2007).

Finally, the F-box protein SLIMB preferentially interacts with DBT-hyper-phosphorylated PER and stimulates its degradation by the 26S proteasome (Ko H, 2002) (Grima B, 2002).

Although much less understood, there is evidence for the existence of a second interlocked positive feedback loop. In this positive feedback loop, CLK/CYC activates the transcription of the vri lle (vri) and pdp1 genes. VRI and PDP-1 are two transcription factors that repress and activate clk transcription, respectively (Cyran et al., 2003). Recent data question the role of PDP-1 in
regulating clk mRNA levels, and suggests a role for PDP-1 in output pathways of the circadian clock instead (Benito et al., 2007).

3. **How does the *Drosophila* clock sense light?**

Many environmental cues (*Zeitgebers*) can efficiently synchronize the circadian clock. These include: light, social cues, temperature fluctuations and food availability. The daily light/dark cycle is the strongest of the environmental cues and the best characterized *Zeitgeber*.

In *Drosophila*, light can reach the circadian neurons deep in the brain through two different photoreception pathways: 1) Opsin-based photoreception and 2) the cell-autonomous circadian photoreceptor CRYPTOCHROME (CRY) (Helfrich-Forster et al., 2001).

3.1 **Opsin-based photoreception.**

Opsin-based photoreception occurs through three peripheral light-sensing organs: 1) compound eyes, 2) ocelli and 3) Hofbauer-Buchner eyelets (H-B eyelets) (Figure 1-4, extensively reviewed in (Rieger et al., 2003)).

All three photoreceptive tissues contribute significantly to entrainment of the circadian clock, but the compound eyes are especially important for
entrainment to extreme photoperiods (i.e. extremely long days) (Rieger et al., 2003).

The H-B eyelet lays between the retina and the medulla in both fly optic lobes and recent data show that synaptic communication between the H-B eyelet and clock neurons contributes to the synchronization of molecular and behavioral rhythms (Veleri et al., 2007).

Although these tissues appear to be important in circadian photoreception, CRY is considered to be the primary circadian photoreceptor.

3.2 CRY-based photoreception.

The circadian photoreceptor CRYPTOCHROME (CRY) was identified in a mutagenesis screen looking for mutations that altered per expression (Stanewsky et al., 1998) as well as in an overexpression approach (Emery et al., 1998). The isolated mutant, called cry\textsuperscript{b}, showed absence of PER and TIM cycling in light:dark conditions (LD), although PER and TIM oscillations could persist in constant darkness after the mutants were entrained to temperature cycles (Stanewsky et al., 1998). In addition, cry\textsuperscript{b} mutants are insensitive to the phase shifting effects of a brief light pulse administered during the night. Also, these mutants are behaviorally rhythmic under constant light with a period of \textasciitilde24h, a condition that renders wild type flies arrhythmic (Stanewsky et al., 1998) (Emery et al., 2000b). These phenotypes can be rescued by expressing the wild
the type form of cry in clock neurons alone, suggesting that CRY is a cell-
onautonomous circadian photoreceptor (Emery et al., 2000b). cry mRNA levels are
under circadian control and, once translated, CRY accumulates in the dark and
degrades upon exposure to light in a proteasome-dependent manner (Emery et

CRYs are found in both plants and animals, and belong to a family of
flavin-containing blue-light photoreceptors related to photolyases (Cashmore,
2003). Photolyases have a flavin and a pterin cofactor, the latter acts as a
chromophore that absorbs photons and transfers energy to the flavin cofactor
through a chain of redox reactions. The energy is then used to repair UV-
damaged DNA. Unlike photolyases that use the absorbed energy to repair UV-
damaged DNA, it is believed that CRYs use the energy to undergo a
conformational change (Cashmore, 2003) (Green, 2004).

How does CRY transfer the light information into the clock? The steps that
connect the conformational change in Drosophila CRY with the resetting of the
circadian clock remain a major question in the field. Evidence points at TIM, a
key pacemaker component that degrades upon exposure to light in a CRY-
dependent manner, as the connection between CRY and the resetting of the
circadian clock (Myers et al., 1996) (Lee et al., 1996) (Yang et al., 1998) (Suri et
al., 1998). In cryb mutants, TIM is not degraded by light (Lin et al., 2001).
Moreover, ectopic expression of cry in the ovary, which does not normally
express cry and where TIM is not degraded by light, promotes TIM light
degradation (Rush et al., 2006).

It is believed that upon exposure to light, CRY transiently binds to TIM and irreversibly commits TIM to degradation in the 26S proteasome (Naidoo et al., 1999) (Ceriani et al., 1999) (Busza et al., 2004). This event is thought to be crucial in the resetting of the clock by light.


The *Drosophila* brain contains ~150 neurons that express clock genes (Figure 1-4). These neurons are divided in two major groups that have been named according to their location in the brain: 1) dorsal neurons (DNs), 2) lateral neurons (LNs) (Ewer et al., 1992) (Frisch et al., 1994) (Kaneko and Hall, 2000). The DNs are divided in three groups: DN1 (~16 neurons), DN2 (2 neurons) and DN3 (~40 neurons). The LNs include the dorsal-lateral neurons (dLNs) (~6 neurons), the small ventral-lateral neurons (sLNvs) (5 neurons) and the large ventral-lateral neurons (lLNvs) (4 neurons). Except one, all LNvs express the neuropeptide Pigment Dispersing Factor (PDF), which is essential for circadian behavior (Renn et al., 1999) (Helfrich-Forster, 1995).

Recent studies have identified a new cluster of pacemaker cells where PER and TIM oscillations are also observed, the lateral posterior neurons (LPNs) (Shafer et al., 2006).
4.1 The LNvs drive rhythms in constant conditions.

In mammals, the suprachiasmatic nucleus (SCN) is considered to be the "master clock", controlling the pace of all peripheral oscillators (or "slave clocks") (Klein et al.). Without the SCN, these "slave clocks" can only sustain rhythms for a few days (reviewed in (Reppert and Weaver, 2002)).

In Drosophila, the LNvs are considered to be the “main oscillator”, although unlike mammalian peripheral clocks, the fly’s peripheral tissues can still entrain to light:dark cycles because they express CRY.

Numerous studies support a role for the LNvs as “master clocks”. Disconnected (disco) mutants that lack the LNvs, as well as flies where the LNvs have been ablated by expressing apoptotic genes in a tissue-specific manner, are both arrhythmic in DD (Helfrich-Forster, 1998) (Blanchardon et al., 2001) (Renn et al., 1999). The LNvs maintain circadian behavioral rhythms in constant conditions through the circadian expression of the neuropeptide PDF. Flies that have intact LNvs but lack PDF (pdf01: pdf null mutants) are also arrhythmic in DD (Renn et al., 1999). Also, rescuing per expression in the LNvs of per0 mutants alone is enough to rescue rhythmicity. Moreover, the period length in DD is related to the period length of the molecular oscillations in the small LNvs, further suggesting that the LNvs, and in particular the small LNvs, are the cells responsible for maintaining behavioral rhythms in constant darkness (Stoleru et al., 2005).
Altogether, this collection of data strongly suggests a role for the LNvs as the main oscillator that controls DD rhythmicity.

4.2 One clock is not enough: the M-cells and the E-cells.

Animals, ranging from insects to mammals, display differential morning and evening peaks of activity (M-peak and E-peak, respectively) (Dunlap et al., 2003) (Aschoff, 1966) (Helfrich-Forster, 2001). In 1976, Pittendrigh and Daan proposed a dual-oscillator model which explains this dual-activity trend (Pittendrigh and Daan, 1976). In the Pittendrigh-Daan model, the circadian clock consists of two groups of oscillators with different responsiveness to light, one governing the morning (M) and the other the evening (E) activity of the animal. The power of this model is that it explains observed adaptations to seasonal changes in day length. The model predicts that the M-peak will occur earlier and the E-peak will occur later in long summer days, helping diurnal animals avoid the high temperatures around midday.

As mentioned above, under standard experimental conditions of 12h light: 12h dark (or 12:12 L:D), at 25C, fruit flies display a surge of activity around the lights-on transition (morning peak / M-peak), followed by a period of low activity or “siesta”. The second surge of activity occurs around the lights-off transition, and it is called evening peak (E-peak) (Rosato E, 2006). More importantly, the morning and evening activity begin before the lights-on and lights-off transition.
This phenomenon is called “anticipation” and reveals the importance of an internal timekeeping mechanism in the adaptation to environmental changes. 

$pdf^{01}$ null mutants are arrhythmic in DD, and display an abnormal pattern of entrainment to light:dark cycles. In LD, these mutants lack anticipation to the M-peak of activity, while they normally anticipate the E-peak. This suggests a specific role for the PDF (+) LNvs in controlling the M-peak, in particular the small LNvs. Therefore, these cells are referred to as the M-cells (Grima et al., 2004) (Stoleru et al., 2004).

Tissue-specific ablation and rescue experiments have revealed a role for the dLNs and possibly a subset of DNs and the PDF (-) small LNv in driving the E-peak of activity. These cells are referred to as the E-cells (Grima et al., 2004) (Stoleru et al., 2004) (Rieger et al., 2006).

In addition, the M-cells not only drive rhythms in constant darkness, but also determine the period of both, the M- and E- peaks of activity. Also, they are able to determine the period of the molecular oscillations in the E-cells (Stoleru et al., 2005), supporting a role as the “master clock”.

Studies of locomotor activity under constant light and long photoperiods in flies overexpressing $per$ and $sgg$ revealed a role for the E-cells, more specifically a subset of DN1s, in controlling behavior (Chapter II) (Murad et al., 2007) (Stoleru et al., 2007). These recent findings support the existence of a dual-oscillator system, where the small PDF (+) LNvs (M-cells) are the main pacemaker in constant darkness, while a group of E-cells are able to dominate
the system during extended periods of light. The role of the DN1s in driving rhythms in constant light conditions is discussed in more detail in Chapter II.

5. Manipulation of gene expression in different neuronal clusters.

Powerful tools are available for *Drosophila* geneticists in order to manipulate gene expression. At the top of the list is the yeast-based GAL4/UAS system, which allows the expression of a gene of interest in a tissue-specific manner (Brand and Perrimon, 1993). This binary expression system utilizes a yeast transcription factor, GAL4, and its target sequence, UAS (Upstream Activating Sequence), to which GAL4 binds in order to activate gene transcription. GAL4 can be expressed in a tissue-specifically by placing it under the control of a promoter sequence of a gene that is only expressed in the tissue of interest. When flies that carry this GAL4 construct are crossed to flies that carry UAS sites upstream of a given gene, the latter will be expressed only in the targeted tissue (Figure 1-5). For example, the *timGAL4* driver will direct expression of a given gene in all clock cells, which express *tim*. Alternatively, when the *pdfGAL4* driver is used, expression occurs only in the PDF (+) LNvs.

The introduction of an additional tool made possible the functional dissection of the circadian network: the GAL80 repressor (Lee, T. and Luo, L., 2001). GAL80 inhibits the activity of the transcription factor GAL4, refining expression of transgenes driven by the binary GAL4/UAS system (Figure 1-5).
For example: as mentioned above, the \textit{timGAL4} driver will direct expression of a given gene in all clock cells, which express \textit{tim} (including the PDF (+) LNvs). If now we add \textit{pdfGAL80} into the system (\textit{timGAL4/pdfGAL80}), expression will be prevented in the PDF (+) LNvs. Therefore, all clock cells, except the LNvs, will be expressing the gene of interest. Different activator/repressor (GAL4/GAL80) combinations allow expression in different clusters of neurons. There is a caveat with this system, which is that one must know the identity of a gene that is expressed in the tissue of interest.
Figure 1-1: Circadian parameters.

A, B. The plots represent a given circadian oscillation (i.e. daily changes in hormonal levels, body temperature fluctuations, locomotor activity). The parameters amplitude ($A$), phase ($\phi$), period ($\tau$) are represented in the plots. Also a phase change is illustrated. Note that the rhythms persist in constant darkness. 

C. A representation of a *Drosophila* phase response curve (PRC). Light pulses administered at the beginning of the night cause a phase delay, while pulses administered at the end of the night cause an advance.
Figure 1-2: Measuring locomotor activity rhythms in *Drosophila*. 

A. Individual flies are loaded into small glass tubes that contain food in one end and enough space for the fly to walk back and forth. The tubes are placed in boards that are equipped with infrared beams that detect movement. The boards are stored in incubators where the temperature and light conditions are controlled. Data collected over many days is analyzed and represented as “actograms”.

B. A representative actogram. Each day is plotted twice: once on the right and once on the left on the next line. Flies are entrained to light:dark (LD) cycles (white/grey boxes), where they display the characteristic bimodal behavior (M and E). Once flies are released into constant conditions (DD, grey box), the rhythms persist for many days.
Figure 1-3: The circadian negative feedback loop of *Drosophila*.
The cartoon illustrates the current model for the *Drosophila* molecular clock, a negative feedback loop. CLOCK (CLK) and CYCLE (CYC) activate *period* and *timeless* transcription through binding to E-box sequences in their promoter regions. PER and TIM accumulate in the cytoplasm and form heterodimers. A set of kinases (SGG, DBT, CKII) and two phosphatases (PP2A, PP1) control the phosphorylation levels of PER and TIM, regulating their stability and timely nuclear entry. Once in the nucleus, they inhibit their own transcription. CRY, the circadian photoreceptor, is sensitive to light and interacts with TIM in a light-dependent manner. Upon exposure to light, both CRY and TIM undergo degradation in the 26S proteasome. TIM degradation occurs via the activity of the F-box protein JET (JETLAG). This event is critical in resetting the phase of the clock.
Figure 1-4: The *Drosophila* brain: clock cells and light sensing organs.
The cartoon illustrates the position of the circadian neurons and their projections relative to the brain: Dorsal neurons (DN1, DN2, DN3), ventral-lateral neurons (vLNs, small and large), dorsal-lateral neurons (LNd) and lateral-posterior neurons (LPN). Light sensing organs are also illustrated: compound eye, ocelli and the Hofbauer-Buchner eyelet (HB-eyelet). Figure adapted from Helfrich-Forster, 2003.
**Figure 1-5: The GAL4/GAL80 system.**

A. The cartoon illustrates the GAL4/GAL80 system. The yeast transcriptional activator GAL4 is expressed in a tissue-specific manner. When combined with a UAS line, GAL4 will activate expression of the gene positioned downstream of the UAS binding sites. GAL80 is a GAL4 repressor that is used to refine expression.

B. An example of the use of these tools in driving gene expression in different circadian neuronal clusters. *timGAL4* will drive expression in all clock cells, while *pdfGAL4* will only drive expression in the PDF (+) LNvs. A combination of *timGAL4* and *pdfGAL80*, can achieve expression only in PDF (-) clock neurons.
CHAPTER II:

A CIRCADIAN GAIN-OF-FUNCTION SCREEN UNDER CONSTANT LIGHT

Abstract:

The photoreceptor CRYPTOCHROME is the primary circadian photoreceptor in *Drosophila*. In its absence, flies remain behaviorally rhythmic instead of becoming arrhythmic under constant illumination. Here I describe a gain-of-function screen under constant light aimed at isolating components of the CRY light input pathway into the circadian pacemaker and follow-up experiments aimed at validating the isolated candidate genes. This screen has identified specific genes that can modulate circadian light responses when overexpressed. Among the genes isolated is a pacemaker gene (*slimb*), and genes involved in diverse biological processes, including at least five genes involved in signal transduction pathways. Although the screen was designed to isolate components of the circadian light input pathway, it can also isolate pacemaker genes.
A. Introduction

Circadian rhythms allow most organisms to adapt their physiology and behavior to periodic changes in the environment. These rhythms are generated by an endogenous, self-sustained molecular pacemaker (Dunlap, 1999). The *Drosophila* molecular pacemaker is composed of two feedback loops: a negative one and a positive one. In the negative feedback loop, the products of the *period* and *timeless* genes (PER and TIM) repress their own gene transcription by directly interacting and blocking the activity of two transcriptional activators: CLK and CYC. This transcriptional cycle is built to last 24 hours. In order to do so, PER and TIM activity and stability are finely regulated by a group of kinases (DBT, CKII, SGG) and two phosphatases (PP1, PP2A) (Chapter 1, Figure 1-3).

The second feedback loop regulates CLK expression. PDP1 is a positive regulator of *clk* while VRI is a negative transcriptional regulator. CLK/CYC dimers positively regulate their circadian expression (Hardin, 2005). The specific function of the positive feedback loop remains to be understood. The mammalian molecular circadian clock shares high similarity to that of *Drosophila* (Shearman, 2000).

*Drosophila* locomotor activity rhythms display two peaks of activity: around dawn and before dusk. Two separate groups of cells control these two peaks of activity: the ventral Lateral Neurons (LNvs) - that express the neuropeptide PDF - control the morning peak of activity, while the dorsal Lateral Neurons (dLNs) and
possibly a subgroup of Dorsal Neurons (DNs) and a 5th LNv that does not express PDF, control the evening peak (Grima et al., 2004) (Stoleru et al., 2004) (Rieger et al., 2006). The PDF (+) LNvs are also responsible for maintaining circadian rhythms in the absence of environmental cues (constant darkness and constant temperature) (Renn et al., 1999). These PDF (+) LNvs are also believed to orchestrate the other groups of circadian neurons (Renn et al., 1999) (Grima et al., 2004) (Stoleru et al., 2004) (Stoleru et al., 2005) (Peng et al., 2003) (Lin et al., 2004).

Unlike constant darkness, constant light disrupts circadian rhythms in most species. In the presence of constant light, wild-type flies become totally arrhythmic, while under constant darkness they would remain rhythmic for weeks. This circadian response to constant light is dependent on the cell-autonomous circadian photoreceptor CRYPTOCHROME (CRY). A severely hypomorphic cry mutant (cry<sup>b</sup>) remains rhythmic under constant light, with a periodicity of about 24 hours, as if it was in constant darkness (Emery et al., 2000b).

Before I joined the Ph.D. program at UMass Medical School, Dr. Patrick Emery screened a collection of mutant flies that contain randomly inserted P-elements in their genome (Rørth, 1996) (Rørth et al., 1998). These P-elements, called EP, contain UAS binding sites that are recognized by the yeast transcription factor GAL4. By crossing these EP fly lines to flies expressing
GAL4 under the control of the *timeless* promoter (*tim-GAL4* flies), one can overexpress, or in rare case downregulate, the genes that have been targeted by the EP element specifically in tissues with circadian rhythms (Figure 2-1). Whether the targeted gene is up- or down-regulated depends on the orientation of the EP element insertion. A sense RNA is usually produced, which results in overexpression of the targeted gene. Sometimes, however, the EP element generates an antisense RNA.

The original purpose of this screen was to isolate genes involved in the CRY light input pathway by looking for genes that, when overexpressed, produced a *cry*\(^{b}\)-like phenotype. 30 EP-lines were isolated in this screen. These candidates represent genes that have a wide variety of biological functions, as discussed below.

**B. Results**

1. **30 lines show circadian rhythmicity in constant light.**

2300 EP lines located on the 2\(^{rd}\) and 3\(^{rd}\) chromosome have been screened. The EP lines were part of the Pernille Rørth collection (Rørth, 1996). 4 flies were screened for each line initially. The light intensity was set at around 1000 lux. Since even control flies sometimes show weak residual rhythmicity under constant light, only those lines for which at least two flies were rhythmic
were selected. Each selected line was then retested under constant light (LL) at least twice, usually three times, with a minimum of 8 flies per experiment. 30 lines were consistently rhythmic in LL (Table 2-I). In constant darkness (DD) however, none of these lines showed any obvious defect. They were all robustly rhythmic, with a period length close to that of control flies (~24 hours) (Table 2-III). In LL however, the period length was usually not that of normal flies in DD, or of cry$^b$ flies in LL. Periods were in most cases long, with most lines (20/30) showing a period range that was centered around 26.5-27 hours (Figure 2-2 and Table 2-I). In LL, power was weaker than under DD, and the variability of period within lines was higher (see (Ewer et al., 1992) for power definition). This shows that the amplitude of circadian rhythms is reduced, which is not surprising since LL is disruptive to circadian rhythms. Therefore, most lines appear not to be completely insensitive to LL, unlike cry$^b$.

A small number of flies in several lines showed a complex behavior. Most of them displayed two components in their circadian behavior, one with a periodicity of 24 hours, and the other one 26.5 hours. This complex behavior was very rare, and probably occurred randomly. However, one line (EP(2)2356) was strikingly different: about one quarter of rhythmic flies showed a complex behavior. The short component had a periodicity that varied between 18 and 21 hours, while the long component was either in the 26.5 hr range, or in the 24 hr range. The rest of the flies showed only one component (approximately half of them had a $\tau<22$hr, the rest had a $\tau\sim24$ hr or $\sim26$ hr; data not shown). Complex
behavior has been observed before in cry\textsuperscript{b} flies under specific light conditions, where the short period component had a period of approximately 22 hr and a long component of \( \sim \) 25 hr (Rieger et al., 2003). In a recent study, M. Nitabach and colleagues expressed a bacterial depolarization-activated sodium channel (NaChBac) in the LNvs. This channel increases membrane excitability, and abolishes PDF cycling in LNv nerve terminals, which results in free-running complex behavior (Nitabach et al., 2006).

Virtually all EP insertions of the Rørth collection have been mapped on the genome (see Flybase). The location of the insertions has been verified for 8 lines, all 8 locations were identical to the insertion sites given in Flybase. For two genes (\textit{lk6} and \textit{morgue}), we also verified that they were indeed overexpressed as predicted by real-time PCR. We can therefore predict which genes should be misexpressed in the selected lines (Table 2-II).

1.1 Function of the isolated genes.

\textit{Regulators of gene expression.}

The screen identified 6 genes that regulate gene expression (Table 2-II). Two transcription factors were isolated: \textit{elB} which is known for its role in tracheal and wing development, and might regulate opsin expression (Dorfman et al., 2002); and \textit{kay} which is the \textit{Drosophila} homologue of \textit{fos} and is implicated in many developmental processes, including the development of the central
nervous system (Souid and Yanicostas, 2003). couch potato (cpo) also turned out in our screen (EP(3)661). CPO is an RNA binding protein that regulates different aspects of adult Drosophila behavior. cpo mutants show an aberrant flight behavior, recover slowly from anesthesia and are overall hypoactive (Bellen et al., 1992) (Hall, 1994). We also found cpo mutant flies (cpo\textsuperscript{3} allele) to be arrhythmic in constant darkness (data not shown). In our hands, these mutants rarely survive to adulthood, and those that survive frequently die during behavioral monitoring. The very small number of viable flies we could obtain precluded further investigation on a potential role of cpo in regulating circadian rhythms. It is thus unclear whether the absence of circadian rhythms is due to a developmental defect, their poor health, or to an actual specific defect in their circadian system.

An interesting set of lines that resulted in LL rhythmicity affected microRNAs. Strikingly, among the 6 lines with the strongest phenotype were three lines that are predicted to overexpress the microRNA miR-282. A fourth line affected the same gene, and also showed a robust phenotype.

Micro-RNAs (miRNAs) were first found in the worm C. elegans, associated with cell-fate specification (Lee et al., 1993). Since then, they have been found in most species. miRNAs are small RNA molecules that are complementary to a part of one or more mRNAs. The annealing of the miRNA to the mRNA then inhibits protein translation, but sometimes facilitates cleavage of the mRNA (like RNAi) (Meister, 2007). miRNAs have been implicated in pathways controlling
development and disease. Interestingly, recent work supports the role of two miRNAs (miR-219 and miR-132) as key regulators of the mammalian circadian clock (Cheng et al., 2007). miR-219 appears to be a target of the CLOCK-BMAL1 heterodimer and regulates the circadian period length: injection of an inhibitor that decreases the levels of miR-219 resulted in significantly longer periods (Cheng et al., 2007). CLOCK and BMAL1 are transcription activators that heterodimerize to induce the expression of clock-controlled genes in mammals, such as per (per1, per2, per3) and cry (cry1, cry2) (Gekakis et al., 1998) (Bunger et al., 2000) (Travnickova-Bendova et al., 2002). On the other hand, miR-132 appears to be induced by photic entrainment cues and its reduction in levels significantly enhances light-induced behavioral phase shifts (PRC) (Cheng et al., 2007). The specific targets of miR-219 and miR-132 remain unknown: Cheng et al. report a list of more than 30 predicted targets for each of the two miRNAs.

Further studies with Drosophila miR-282 may reveal a role for this miRNA in regulating circadian rhythms in flies.

**Genes under circadian control.**

Among the genes isolated, we found several genes that are circadianly regulated: lk6, akap200, calpB and morgue. The last three were shown to cycle in fly heads by DNA microarray studies (Claridge-Chang et al., 2001), while lk6 was shown to oscillate in fly bodies (Ceriani et al., 2002). RNase protection and
Northern Blot revealed that \textit{lk6} expression is also under circadian oscillations in heads, with, interestingly, a phase identical to \textit{cry} (PE unpublished data). Thus \textit{lk6} and \textit{cry} appear to be circadianly co-regulated.

Using Real-Time PCR I was unable to reproduce the Claridge-Chang et al. findings that suggest that \textit{morgue} mRNA is under circadian regulation in fly heads (data not shown). This finding would make \textit{morgue} land within the small percentage of false positives in the Claridge-Chang published screen. I did not find this as discouraging, since \textit{morgue} does not necessarily need to be under circadian regulation in order to play a specific role in CRY degradation. Also, the Real-Time PCR experiments were performed in whole-head extracts, thus it is still possible that \textit{morgue} mRNA or protein abundance are under circadian regulation in pacemaker cells specifically, and these changes could not be detected in our experiments.

\textit{Regulators of protein stability.}

Two genes that are implicated in proteosomal degradation (\textit{slimb} and \textit{morgue}) were identified in the LL screen. This is an attractive finding, since the degradation of both TIM and CRY occurs via the proteosome (Naidoo et al., 1999) (Lin et al., 2001) (Busza et al., 2004). Both SLIMB and MORGUE are F-box proteins. The F-box is a 50 amino acid motif present in adaptor proteins that provide substrate targeting functions within the \textit{Drosophila} SCF (Skp-Cullin-F-box) E3 Ligase complex (Jin and Harper, 2002) (Kipreos and Pagano, 2000)
(Patton et al., 1998). The SCF complex contains a scaffold Cullin protein that binds to both a RING domain protein and a Skp protein (Zheng et al., 2002). The RING domain binds an E2 ubiquitin conjugase allowing the E2 to donate ubiquitin to the substrate. Skp binds to an F-box protein, which in turn binds to the substrate. Thus, the F-box protein provides substrate discrimination. Consistent with this role, there is a large number of F-box proteins that contain diverse protein interaction domains.

*slimb* was previously identified as a component of the circadian molecular pacemaker, controlling the levels of both PER and TIM. SLIMB preferentially interacts with hyperphosphorylated PER and stimulates its degradation by the 26S proteasome (Grima B, 2002) (Ko HW, 2002).

MORGUE (*modifier of reaper and grim, ubiquitously expressed*) contains an E2 ubiquitin conjugase domain, in addition to its F-box domain. Originally, *morgue* was isolated in parallel from two different genetic modifier screens, aimed at searching for genes that regulate cell death (Hays et al., 2002) (Wing et al., 2002). MORGUE binds DIAP1 (*Drosophila Inhibitor of Apoptosis Protein 1*) and targets it to degradation in the 26S proteasome, thus promoting cell death. The F-box in MORGUE binds to *Drosophila* SkpA, which also enhances GRIM-REAPER-mediated apoptosis (Wang et al., 2000) (Schreader et al., 2003). This finding supports a role for MORGUE in a specific SCF E3 ligase complex. Since MORGUE also contains a ubiquitin conjugase domain, it may also possess E2 enzymatic activity. The conjugase domain present in E2 enzymes is composed
of 140 highly conserved amino acids. Conjugase domains present an active-site cysteine that binds to ubiquitin monomers via a thioester linkage. MORGUE lacks the active-site cystein, bearing a glycine residue instead (reviewed in Schreader et al., 2003). This substitution makes MORGUE unable to bind to Ubiquitin. Although unable to bind to ubiquitin, MORGUE could still offer E2 enzymatic activity by acting as a ubiquitin enzyme E2 Conjugase Variant or “UEV”. UEVs belong to a conserved family of E2-like proteins that, as MORGUE, lack the active-site cysteine that is necessary to bind to ubiquitin. Instead of binding directly to ubiquitin, these proteins recruit a “true” E2 conjugase into the complex, providing “indirect” E2 activity (Bailly et al., 1994) (Sung et al., 1991).

In addition to morgue and slimb, we isolated other genes involved in protein turnover and phosphorylation. Two of theses are CALPB, a calcium activated protease of the calpain family, and SDA, a protease known to be important for normal nervous system function, since sda mutants are prone to seizures (Zhang et al., 2002). A kinase (lk6) and a phosphatase (cg9801) were also isolated.
2. Is *morgue* part of the light input pathway?

Altogether, (i) the involvement of MORGUE in targeting proteins to proteasomal degradation, (ii) the fact that *morgue* displays the most robust of the phenotypes of the candidates that were isolated in the LL screen, and (iii) the microarray data suggesting that *morgue* mRNA is under circadian regulation, made *morgue* a good candidate for being involved in the light input pathway, possibly targeting CRY to degradation in the 26S proteasome. Here I describe a series of experiments aimed at analyzing the potential role of *morgue* in the light input pathway to the circadian clock.

2.1 The phenotype observed is truly due to *morgue* misexpression.

We performed a series of experiments aimed at determining whether the phenotype observed in LL was truly due to *morgue* overexpression and whether the effect was specific to *morgue*. Flies that overexpress *morgue* (*tim-GAL4/UAS-morgue*) show an increase in *morgue* mRNA levels of about 10-fold as compared to controls, measured by Real-Time PCR (Figure 2-3). We also generated an independent strain of transgenic flies that bear a *UAS-morgue* insertion. When tested in LL, the independent insertion displayed a rhythmic phenotype that was almost indistinguishable from that of EP2367/*timGAL4* (data not shown).
2.2 The phenotype observed is specific to *morgue*.

Since both *morgue* and *slimb* contain an F-box, we wondered whether any protein with an F-box or involved in proteasomal degradation could, when overexpressed, render flies resistant to constant light. This was not the case: overexpressing the ubiquitin conjuguase (UBCD1) or the F-box containing E3 ligase AGO did not result in rhythmicity under constant light (Figure 2-4). This demonstrates the specificity of the constant light phenotype to MORGUE and SLIMB. These results plus the data obtained with the independent *UAS-morgue* insertion suggest that the phenotype observed when overexpressing *morgue* is truly due to up-regulation of *morgue*, and that this effect is specific.

2.3 Does *morgue* promote CRY or TIM degradation?

We performed a series of experiments aimed at determining whether MORGUE acts in the light input pathway, and in particular, whether MORGUE promotes CRY or TIM proteasomal degradation. We measured CRY and TIM levels in head extracts from flies that overexpress *morgue* with the *tim-GAL4* driver. If the role of MORGUE is to promote TIM and/or CRY proteasomal degradation, we expect to observe a dramatic decrease of TIM and/or CRY protein levels when *morgue* is overexpressed. TIM levels in flies that
overexpress *morgue* did not show any difference in abundance or circadian oscillation when compared to control flies (Figure 2-5B), suggesting that MORGUE does not promote TIM degradation in the 26S proteasome. During the early phase of this project we were lacking a key reagent: a CRY antibody. Our laboratory, as well as other groups in the field, had difficulties at generating such reagent. Therefore, we used transgenic flies that overexpress a tagged version of CRY (Myc-CRY) under the the control of the *tim-GAL4* driver: *tim-GAL4-UAS-Myc-cry* (TMC). These flies constitutively express high levels of Myc-CRY in all *tim*(+) cells. Myc-CRY is a functional protein that is able to rescue the severely hypomorphic mutant *cry*<sup>h</sup> (Busza et al., 2004). Levels of Myc-CRY were measured by western blot at 6 time-points spanning along the entire day in TMC-UAS-*morgue* flies (which overexpress both: *morgue* and *myc-cry*) vs. TMC flies (which only overexpress Myc-CRY). Myc-CRY levels were consistently decreased at all time-points assayed (Figure 2-5A). We also measured *myc-cry* mRNA levels by Real-Time PCR in order to determine whether the reduction in Myc-CRY observed when combined with morgue overexpression was truly due to degradation of the mature protein or to a decrease in transcript levels due to a saturation of the GAL4 system, which was not the case (data not shown). This result was extremely encouraging and suggested that MORGUE targeted CRY to proteasomal degradation. Although this was an interesting observation, I was unable to confirm this results once we successfully generated a CRY antibody: when *morgue* was overexpressed in a wild type background (without
overexpression of Myc-cry), endogenous CRY protein levels were indistinguishable from the controls (data not shown).

This result can be attributed to the fact that MORGUE may be part of a non-CRY-specific degradation complex that the cell recruits in order to deal with excessive amounts of Myc-CRY, after the specific complex reaches saturation. Alternatively, since these experiments were performed using whole head extracts, the possibility of a reduction in CRY levels in clock cells alone cannot be excluded. Many other tissues, such as the compound eye—which express cry, are present in the samples. These tissues could be obscuring a clock-cell-specific effect of MORGUE overexpression on CRY abundance.

2.4 Does overexpression of morgue affect a different CRY-mediated response?

We measured a CRY-dependent response to light other than rhythmicity in LL, the phase response curve (PRC) to brief light pulses. In a PRC, individuals are entrained to a light:dark cycle (LD) and then released in constant dark. During the last dark phase of the LD cycle, flies are exposed to a 5 minutes light pulse of about 1,000 lux. Flies that have been pulsed will experience a delay or advance of the phase of the circadian rhythm, measured over several days in DD. Whether the flies experience a phase delay or advance of their rhythms will depend on whether they receive the light pulse early or late in the night. Light pulses at the beginning of the night will generate phase
delays, while light pulses perceived at the end of the night will generate phase advances. Pulses administered in the middle of the night will cause no effect (inflection point) (Chapter I, Figure 1-1C). Wild-type flies experience phase delays and advances of several hours, while $cry^b$ flies do not respond. This is due to the fact that this phase response is mediated by the photoreceptor CRY, and $cry^b$ flies are blind to the stimulus.

When flies that overexpress $morgue$ with the $timGAL4$ driver were tested in a PRC paradigm, they performed poorly, almost like $cry^b$ flies. This suggests that overexpression of $morgue$ is by some means affecting the CRY light input pathway. Flies that overexpress $morgue$ behaved very similarly to $cry^m$ mutant flies (Busza et al., 2004) (Chapter III, (Murad et al., 2007)). $cry^m$ flies contain a premature stop codon that truncates CRY’s last 19 amino acids, leaving the photolyase domain intact. When tested in a PRC paradigm, $cry^m$ flies show a minor response to light pulses. This observation indicates that these mutants, as well as flies overexpressing $morgue$, are not completely blind to light pulses, unlike $cry^b$.

2.5 $morgue$ loss-of-function.

John Nambu and Barbara Schr"{e}ader (UMASS Amherst) generated $morgue$ deletion mutants by imprecise excision of the EP2367 insertion (Schreader B and Nambu J, unpublished), and kindly provided these reagents to
We tested two deletions: morgue126 and morgue457. Both deletions remove morgue’s promoter region. In morgue126 one third of the morgue genomic region is deleted, leaving the F-box domain and the conjugase domain. morgue457 carries a larger deletion that expands to the F-box domain (Figure 2-6A). We used as a control morgue100, a clean P-element excision where only the P-element is deleted, leaving morgue sequence intact (Figure 2-6A). We also received a complete deletion (morgue19), which was not viable. The latter deletion also affects a gene of unknown function that is adjacent to morgue, the predicted gene CG15432 (Figure 2-6A).

We wondered whether these morgue loss-of-function mutants would provide information that would further suggest the involvement of morgue in the CRY input pathway. If MORGUE acts in the CRY input pathway by promoting CRY proteasomal degradation, the hypothesis was that in a morgue loss-of-function background, CRY protein levels would be increased. An increase in CRY levels would make flies more sensitive to light pulses in a PRC, reaching maximum response even at lower light intensities, similar to flies that overexpress cry (Emery et al., 1998) (Emery et al., 2000b). Also, these mutants were expected to be arrhythmic in LL, even at extremely low light intensities (below 10 lux) at which wild type flies start showing a rhythmic behavior (Konopka et al., 1989). In other words, I expected morgue a loss-of function mutants to be hypersensitive to light.
We tested the response of $morgue_{126}$ to light pulses of different light intensities (PRC). The hypothesis was that if $morgue$ acts in the light input pathway by affecting CRY-dependent photoresponses (i.e. promoting CRY degradation), deletion mutants would be hypersensitive to light. In other words, a brief light pulse would cause a greater effect in the mutants than in control flies, even at very low light intensities. We tested the response of the mutant $morgue_{126}$ to 5 min. light pulses of 100, 20, 10, 5 and 2 lux administered at ZT15 (delay zone). No obvious enhancement of the response was observed compared to control flies (Figure 2-6B). $morgue_{126}$ mutants showed a subtle decrease of the response at extremely low light intensity (5 lux) when compared to the $yw$ controls, suggesting a diminished sensitivity. We also used as a control flies that overexpress $cry$, which showed an enhancement of the response, as expected (data not shown).

We then tested $morgue_{126}$ and $morgue_{457}$ mutants in LL of decreasing light intensities. Flies were entrained to 12:12 h LD cycles and then released in constant light. Monitors were covered with filters that allow to decrease the light intensity to 10, 3, 1 and 0.3 lux. As expected, a higher proportion of control flies ($morgue_{100}$) become rhythmic as the light intensity decreases (Table 2-V). A similar effect is observed for $morgue_{126}$ mutants: about half of the flies are rhythmic at 0.3 lux, with a period of 27.78 h, characteristic of wild type flies under these conditions. On the other hand, the majority of $morgue_{457}$ mutants remained arrhythmic at 0.3 lux, suggesting an increased sensitivity to light.
Although interesting, this effect is subtle and mostly observed at extremely low light intensities. Also, the effect was opposite to that observed in the *morgue*126 phase response (Figure 2-6A).

Since the previous two experiments showed subtle opposite effects and were difficult to interpret, we decided to test the effect of *morgue* loss-of-function in a sensitized background: *morgue*126/cry"m double mutants. Unlike cry"b mutants, that are circadianly blind to light, cry"m mutants are partially blind to the effects of light in the circadian clock (Busza et al., 2004). cry"m mutants not only have a partially depleted PRC (like *morgue* overexpressing flies) but they also lengthen their period in LL as the light intensity decreases: at 200 lux LL, their period is 25.1 h, while at 25 lux LL their period is 26.6 h (Busza et al., 2004), further demonstrating their circadian “partial blindness”. We sought to use this low light intensity LL paradigm to test *morgue/cry"m* double mutants, and see whether introducing a *morgue* mutation in this already sensitized background would repress or enhance the cry"m phenotype. The *morgue*126/cry"m double mutants were rhythmic in 40 lux LL (46.15% R, \(\tau=27.6\) h), similar to the cry"m control flies (31.2% R, \(\tau=27.1\) h). Thus, introducing a *morgue* mutation did not rescue the rhythmic phenotype nor did it rescue the long period displayed by cry"m in 40 lux LL. Also, *morgue*126 single mutants were arrhythmic in LL, suggesting that loss-of-function of *morgue* does not disrupt the light input pathway (data not shown).
Finally, since slimb is a pacemaker gene that encodes an F-box protein, we wondered whether morgue could also affect the circadian clock, instead of the light input pathway. morgue126 mutants were tested for locomotor activity in constant darkness where they displayed normal locomotor activity rhythms: the majority of the flies were rhythmic in DD and the period was ~24h, just like control flies (data not shown). Additionally, we generated morgue126/perL double mutants. perL mutants carry a valine to aspartic acid missense mutation (Baylies et al., 1987) in the PAS dimerization domain that causes period length and temperature compensation defects (Konopka et al., 1989) (Ewer et al., 1990). As a result, perL mutants have a “broken clock” that runs slower than usual, with a periodicity of ~29 h in DD. Our hypothesis was that introducing the morgue deletion in a perL sensitized background could unveil an effect of morgue in the clock by making the flies arrhythmic or by affecting their period. morgue126/perL double mutants were rhythmic in DD (62.5% R, τ=29.2 h), similar to perL single mutants (81.25% R, τ=29.8 h). Therefore, the pacemaker appears not to be affected by these mutations, suggesting that morgue does not play a crucial role in regulating the function of the clock.

The lack of an unambiguous phenotype of morgue deletion mutants could be attributed to the fact that morgue is not an essential component of the CRY input pathway or the pacemaker, or that there are other proteins that have a redundant function and can compensate for the loss of morgue. The absence of
clear evidence for a role of morgue in the light input pathway or in the pacemaker discouraged us from perusing any further analysis of this candidate gene.

Although the phenotype observed when morgue is misexpressed is robust and specific, whether morgue plays a role in the light input pathway or not remains unclear.

3. A Secondary PRC screen for genes involved in the light input pathway

Encouraged by the PRC results presented in the previous section and in Chapter III, showing a robust effect of morgue overexpression in decreasing phase shifting after brief light pulses, we decided to perform a PRC for each of the candidates that were isolated in the original LL screen. As mentioned before, even though the original intention of the LL screen was to identify new candidate genes involved in the light input pathway, a known pacemaker gene, slimb, was isolated. Also overexpression of per resulted in rhythmicity in LL (Murad et al., 2007) (Chapter II). Interestingly, when tested in a PRC, overexpression of slimb did not affect the ability of flies to phase shift in response to a brief light pulse, unlike the effects observed when overexpressing morgue (data not shown). We therefore hypothesized that a PRC sub-screen could give us an idea of which of the isolated candidates are truly involved in the CRY-light input pathway, since phase change in response to brief light pulses is another CRY-mediated circadian response. Those genes that affected the PRC when overexpressed
would more likely be involved in the light input pathway, while the rest of the candidates could be affecting output pathways or the pacemaker itself, or be just false positives.

24 EP-lines that displayed a rhythmic behavior in LL in the original screen were crossed to *timGAL4* and tested in a PRC paradigm: flies were entrained to 12:12h LD cycles and light-pulsed during the last night (ZT15 and ZT21, 5 min light pulse of 1,000 lux) before release into constant darkness (DD). Although most of the EP-lines (19 lines) did not showed altered phase responses, the micro-RNA *miR-282* (EP3041) showed a robustly diminished phase delay (ZT15) and advance (ZT21) (Figure 2-7). The effect of the other 3 EP-lines in the PRC is unclear, since there were variations in the response between independent experiments.

The fact that in addition to LL rhythmicity, overexpression *miR-282* led to a robust decrease in the phase response to light pulses (comparable to *cry* mutants) suggests a role for this miRNA in regulating photoresponses in *Drosophila*. Interestingly, it has been shown in mammals that a decrease in levels by competitive inhibition of the light-induced *miR-132* resulted in a robust increase in phase delays after a short light pulse (Cheng et al., 2007). Additional experiments will be needed in order to determine the specific role of *miR-282* in the circadian light-input pathway, such as the effects of decreasing *miR-282* levels by competitive inhibition and the identification of potential targets.
It remains uncertain whether the high light intensity pulses used in these experiments obscured a PRC effect of some of these candidates due to saturation in the system. Previous studies showed that overexpressing CRY in pacemaker neurons makes flies hypersensitive to light pulses of very low light intensities, suggesting that at lower light intensities CRY levels are normally limiting (Emery et al., 1998) (Emery et al., 2000b). Therefore, a PRC with light pulses of lower intensities may offer a more sensitive system to test the EP-lines that did not show an effect, and those that showed ambiguous results, in order to unveil a potential PRC effect that could have been masked by the saturating pulses used. These experiments will be followed up by the Emery Lab.

4. A secondary RNAi-based loss-of-function screen

RNA-interference (RNAi) uses double stranded RNA molecules (dsRNA) to potently knock-down the translation of a particular gene by the specific degradation of its mRNA (Mello and Conte, 2004). The power of RNAi knock-down combined with the availability of the entire genome sequence of *Drosophila* offers an enormous advantage in functional genomic approaches, allowing the identification of components of diverse molecular pathways and understanding of their biological function.
The Japanese National Institute of Genetics (NIG) and the Vienna Drosophila RNAi Center (VDRC) have independently generated collections of fly stocks that carry UAS-RNAi insertions targeting every single fly gene (Dietzl et al., 2007). A partial NIG collection became publicly available in early 2007, while the complete VDRC collection became available in May, 2007.

The available NIG RNAi lines that target a subset of the genes that were isolated in our LL screen were tested for behavior in constant light (LL) and in constant darkness (DD). These RNAi constructs targeted kismet, slimb, morgue kayak and the predicted genes CG30152 and CG10459. The UAS-RNAi constructs were combined with the timGAL4 driver for tissue-specific knock-down in clock cells (Table 2-IV).

One of the RNAi mutants showed a robust phenotype in LL (UAS-kis(RNAi), 62.5 % of the flies remained rhythmic, Table 2-IV), while two others showed a weaker rhythmic phenotype in LL (CG30152 and CG10459, Table 2-IV). The rest of the lines were arrhythmic in LL and all of the lines tested displayed a normal behavior in DD (Table 2-IV and data not shown). UAS-kay(RNAi) flies failed to survive when crossed to timGAL4, possibly due to kayak’s role during embryonic development (Giesen et al., 2003). Since UAS-kis(RNAi)/timGAL4 flies displayed a rhythmic behavior in LL, while the behavior in constant darkness was normal (Table 2-IV), down-regulation of kis appears to have an effect in the light input pathway but not in the pacemaker itself.

Interestingly, the P-element insertion used in the original screen that targeted kis
*kismet* is an antisense insertion. An antisense P-element insertion is predicted to generate an antisense RNA transcript. Instead of a *kis* gain-of-function, this antisense RNA could be mimicking the down-regulating effects of an RNAi molecule, leading to a *kis* down-regulation. This is likely the reason why the *kis* RNAi mutant phenocopies the results observed with the P-element insertion.

*kismet* is a member of the Trithorax (TrxG) gene family and was identified in a screen for extragenic suppressors of *polycomb* (*pc*) (Kennison and Tamkun, 1988). The trxG group of genes encodes activators that regulate segmentation during *Drosophila* embryogenesis (Simon and Tamkun, 2002), while the Polycomb (PcG) family encodes repressors. *kis* encodes two major nuclear proteins: Kis-L (long) and Kis-S (short). Kis-L contains an ATPase domain that shares high homology to those present in chromatin remodeling proteins, suggesting a role for Kis-L in ATP-dependent chromatin alterations. Mutations in *kis* have also been isolated in screens for genes involved in the Notch and Ras signaling pathways, suggesting a broader function for *kis* that is beyond the control of embryonic segmentation (Go and Artavanis-Tsakonas, 1998) (Verheyen et al., 1996) (Therrien et al., 2000). Recent studies suggest that Kis-L might play a global role in transcription by Polymerase II (Srinivasan et al., 2005). Kis-S lacks the ATPase domain and might act as a naturally occurring dominant-negative (Srinivasan et al., 2005).

Studies in mammals revealed a direct link between chromatin remodeling events and circadian control of transcription. In mammals, the activation of
clock-controlled genes occurs via CLOCK and BMAL1. CLOCK and BMAL1 are basic-helix-loop-helix (bHLH)-PAS transcription activators that heterodimerize to induce the expression of clock-controlled genes, such as \textit{per} and \textit{cry}. The activation of clock-controlled genes by CLOCK-BMAL1 is coupled to changes in histone acetylation at the promoter sites (Curtis et al., 2004) (Etchegaray et al., 2003) (Naruse et al., 2004) (Ripperger and Schibler, 2006). Moreover, CLOCK itself has histone acetyltransferase activity (HAT) (Doi et al., 2006). Different histone modifications, such as phosphorylation and acetylation appears to interplay, suggesting a dynamic interaction between these events (reviewed in (Nakahata et al., 2007)). Also, histone methylation appears to be important for circadian transcriptional regulation, since mammalian cell-culture studies suggest that EZH2, a member of the polycomb group of proteins, is required for circadian clock functions (Etchegaray et al., 2003).

Even though it is suggested that \textit{kismet} may play a general role in the regulation of transcription in \textit{Drosophila}, its down-regulation in clock cells specifically resulted in rhythmic behavior in constant light. This effect appears to be specific for the light-input pathway, since locomotor activity rhythms in constant darkness are normal, suggesting that the pacemaker is unaffected. Moreover, the period of \textit{kis} RNAi flies in LL is 24.5 h, just like \textit{cry}^b, further suggesting a role for \textit{kis} in mediating light responses specifically. Therefore, KIS-mediated chromatin modifications could be affecting \textit{cry} or other components of the light input pathway.
The complete VDRC RNAi collection is now publicly available. These valuable reagents offer the possibility of performing an RNAi subscreen of all the candidate genes isolated in the original LL screen. These experiments will be followed up by the Emery Lab.

C. Discussion

Although the constant light screen presented in this chapter was originally designed to isolate components of the CRY-input pathway, the isolated genes represent a wide variety of biological functions, including the pacemaker gene slimb. Further validation and analysis of these candidate genes might reveal not only components of the light input pathway, but also possibly core clock genes.

Some of the isolated candidates are part of signal transduction pathways (i.e. MAPK and PKA pathways). Previous work suggests a role for MAPK and PKA in regulating clock outputs. Williams et al. found that a null mutation of the neurofibromatosis-1 (Nf1) gene, which leads to increased MAPK activity, resulted in abnormal circadian behavior, although molecular oscillations in the brain were unaffected. This circadian phenotype was rescued by loss-of-function mutations in the Ras/MAPK pathway, linking MAPK with circadian outputs (Williams et al., 2001). Majercak et al. found that mutations in a gene that encodes the major catalytic subunit of PKA renders flies arrhythmic, although the molecular clock still oscillates, suggesting that PKA is also involved in pathways downstream of
the circadian clock (Majercak et al., 1997). These signaling pathways are also linked to circadian inputs and transcription mechanisms that govern the circadian clock. *Drosophila dunce* mutants (*dnc*), in which a cAMP specific phosphodiesterase is affected, show an increase in light pulse-induced phase delays and have short circadian periods in DD, suggesting a role for cAMP in the light input pathway, as well as the clock (Levine et al., 1994). Also, Weber et al. 2006 observed that PKA and the Ras/MAPK signaling pathways regulate CLK/CYC mediated transcription, through phosphorylation of CLK (Weber et al., 2006). Thus, the candidates isolated in the LL screen could reveal components of the circadian output pathways, in addition to input and clock genes.

We originally focused on *morgue*, since both CRY and TIM undergo proteasomal-dependent degradation upon exposure to light (Naidoo et al., 1999) (Busza et al., 2004). Overexpression of *morgue* using both the original EP line belonging to the Rørth collection, as well as an independent UAS-*morgue* line that I have generated, produced the most robust rhythmic phenotype in LL. Also, overexpression of *morgue* made flies almost insensitive to light pulses, mimicking the response of *cry* loss-of-function mutants, and suggesting a role for *morgue* in the light input pathway. Whether this role is specific to CRY or to components of the light input pathway, remains unclear. Studies looking at specific molecular interactions between MORGUE, CRY and other clock components will be needed in order to address this point. Also, expression studies could reveal the sites of MORGUE action. Regarding this last point, *morgue* mRNA is broadly
expressed in almost all cells during embryogenesis, and it is transcribed during all stages of the *Drosophila* life cycle, revealed by *in situ* hybridization and RT/PCR assays (Hays et al., 2002) (Wing et al., 2002), suggesting that its action expands beyond the boundaries of the circadian pacemaker.

As with overexpression of *morgue*, overexpression of the micro-RNA *miR-282* also resulted in a poor response to light pulses, measured by PRC. The PRC of these animals is similar to that of *cry*<sup>b</sup> mutants, suggesting a role for *miR-282* in circadian photoresponses. Recently, a technique based on competitive inhibition was successfully used to “trap” small RNAs in mammalian cells, providing a method to study loss-of-function of miRNAs (Ebert et al., 2007). Using similar techniques, loss-of-function of *Drosophila* miR-282 phenotypes could be studied, which will help elucidate whether or not *miR-282* is an essential component of the *Drosophila* circadian system. Also, localization studies will reveal the pattern of expression of this miRNA and whether it is restricted to circadian tissues or not. Even if *miR-282*, and the other miRNAs isolated in the screen, turn out not to be required for circadian rhythms, the study of their potential targets could help identifying new circadian genes. The large number of predicted targets for a given miRNA makes identifying specific targets a difficult enterprise. Improved target prediction software versions are now becoming available, this new tools will be of great value at narrowing down the large number of potential targets, allowing their verification *in vivo* or in cell culture.
Besides *slimb*, it remains to be determined whether the genes we isolated do play a role in generating or controlling circadian rhythms. We tested classic loss-of-function mutants for two genes (*lk6* and *morgue*, unpublished results), but did not find any circadian defects in terms of light sensitivity and circadian period length. This does not necessarily mean that these two genes are not involved in the control of circadian rhythms. First, there might be redundant pathways or molecules that can substitute for the products of these genes. Second, these molecules might regulate circadian rhythms only in specific tissues, which may not necessarily be involved in regulating circadian behavior. The isolation of *slimb* strongly suggests that other genes found in our screen will turn out to be relevant to circadian rhythms when loss-of-function mutations are analyzed.

New tools are now available for the study of loss-of-function mutations in *Drosophila*: a genome-wide collection of RNAi fly lines available through the VDRC (Austria). These reagents will be of great value in the validation of the candidate genes isolated in our LL screen. Studies done with a small set of RNAi lines available through a partial collection (NIG, Japan) allowed us to reveal a potential role for *kismet* in regulating circadian photoresponses. Since rhythms in constant dark were normal in these mutants, the effect of *kismet* down-regulation appears to be specific to circadian photoresponses.

In summary, the work presented in this Chapter has identified new potential genes that might regulate several aspects of circadian rhythms. These
genes might be involved in mediating circadian light responses, in regulating pacemaker function or even regulating circadian outputs.

D. Materials and methods

1. Drosophila Stocks and Transgenics.

The following Drosophila strains were used in this study: y w; tim-GAL4 (Emery et al., 1998). The P-element insertions belong to the Rorth collection (Rørth et al., 1998; Wing et al., 2002). cry<sup>m</sup> mutant flies were previously described (Busza et al., 2004). TMC (timGAL4-myc-cry) flies were previously described. morgue deletion mutants 100, 126 and 457 were generously provided by John Nambu and Barbara Schreader, UMASS Amherst (unpublished). RNAi lines were purchased from the Japanese National Institute of Genetics (http://www.shigen.nig.ac.jp/fly/nigfly/).

2. Behavioral Analysis.

Locomotor activity of male flies (1-5 days old) were measured with Trikinetics Activity Monitors (Waltham, MA, USA) for 3 full days under 12 hr light: 12 dark conditions (LD) followed by 6 full days of either constant light or constant darkness at 25°C. For almost all experiments, a light intensity of ca. 200 lux was used, unless specifically described. Data analysis was performed with the FAAS
software (Grima et al., 2002). Rhythmic flies were defined by $\chi^2$ periodogram analysis with the following criteria: power $\geq 10$, width $\geq 2$ (Ewer et al., 1992). The group activity actograms were generated using a signal-processing toolbox (Levine et al., 2002) implemented in MATLAB (MathWorks). Phase Responses Curves were generated essentially as described in Busza et al. (2004).

3. **Real-Time PCR.**

Real-Time PCR was performed essentially as described in Busza et al. (2004). Primers and probes: *cry* forward primer: 5’-

AGTACGTCCCGGAGTTGATGA-3’, *cry* reverse primer: 5’-

TGCTGCTCGGCAGACATTC-3’, *cry* probe: 5’-6 –FAM-

CAGGGCTCGTAACAAATTCTT-TAMRA-3’, *morgue* forward primer: 5’-

CTACGAAGGCGGCAAGTTCT-3’, *morgue* reverse primer: 5’-

CTGTGGGGCGGCCTCAT-3’, *morgue* probe: 5’-6 –FAM-

CTGTTCATATACTTCCCGGAGCGATATCC-TAMRA-3’.

4. **Protein extracts and Western blots.**

Protein extracts and western blots were performed as described in Busza et al. 2004. Anti-MYC monoclonal antibody was purchased from Roche.


E. Acknowledged contributions

The initial phase of the screen was performed by Patrick Emery and Myai Emery-Le at Michael Rosbash’s Lab, Brandeis University. Professor John Nambu and Barbara Schreader (UMass Amherst) provided the *morgue* deletion mutants. The PRC secondary screen was performed in collaboration with Dr. Raphaëlle Dubruille.
Table 2-I: Behavior of the selected EP-lines crossed to *tim-GAL4* under constant light (1000 lux).

<table>
<thead>
<tr>
<th>Genotype (lines crossed to timgal4)</th>
<th>Gene predicted to be affected</th>
<th># of flies</th>
<th>% rhythmic flies</th>
<th>Period average (± sd)</th>
<th>Power average (± sd)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EP(2)2367</td>
<td>morgue</td>
<td>30</td>
<td>87</td>
<td>26.4±0.6</td>
<td>37.5±22.8</td>
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<tr>
<td>EP(3)714</td>
<td>mir-282</td>
<td>43</td>
<td>83</td>
<td>27.5±0.7</td>
<td>54.8±26.5</td>
</tr>
<tr>
<td>EP(3)3718</td>
<td><em>mir-282</em></td>
<td>31</td>
<td>78</td>
<td>27.5±1.0</td>
<td>37.8±23.0</td>
</tr>
<tr>
<td>EP(2)670</td>
<td>GstS1</td>
<td>33</td>
<td>73</td>
<td>26.5±1.0</td>
<td>49.1±23.2</td>
</tr>
<tr>
<td>EP(3)703</td>
<td>cg8165/8176</td>
<td>74</td>
<td>71</td>
<td>26.6±1.8</td>
<td>32.5±17.9</td>
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<tr>
<td>EP(3)3041</td>
<td><em>mir-282</em></td>
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<td>70</td>
<td>28.5±1.1</td>
<td>37.3±21.3</td>
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<td>EP(2)965</td>
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<td>31.3±20.5</td>
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<td>EP(3)972</td>
<td>calpB</td>
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<td>65</td>
<td>26.5±1.2</td>
<td>33.3±15.6</td>
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<tr>
<td>EP(3)902</td>
<td>kay</td>
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<td>64</td>
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<td>63</td>
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<td>sip1</td>
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<td>61</td>
<td>26.6±1.2</td>
<td>27.4±14.8</td>
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<tr>
<td>EP(3)3617</td>
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<td>61</td>
<td>27.0±1.4</td>
<td>32.7±17.0</td>
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<td>EP(2)323</td>
<td>cg8735</td>
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<td>24.7±11.3</td>
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<td>EP(3)662</td>
<td>slmb</td>
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<td>31.8±13.2</td>
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<td>EP(2)2345</td>
<td>dap (as) or CG10459 (as)</td>
<td>44</td>
<td>59</td>
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<td>26.0±17.0</td>
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<tr>
<td>EP(3)3084</td>
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<td>29</td>
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<td>44.0±28.3</td>
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<td>EP(2)575</td>
<td>cg13791 (as)</td>
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<td>27.0±14.7</td>
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<td>EP(2)813</td>
<td>dpld (as) or cg1621 (as)</td>
<td>24</td>
<td>58</td>
<td>27.2±1.0</td>
<td>31.2±13.2</td>
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<tr>
<td>EP(3)1141</td>
<td>sda</td>
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<td>EP(2)2241</td>
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<td>26.4±0.7</td>
<td>30.3±10.8</td>
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<td>56</td>
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<td>34.9±16.4</td>
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<td>akap200</td>
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<td>55</td>
<td>26.4±1.1</td>
<td>27.1±14.2</td>
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</table>
|                | Gene     | Count | Controls | Tm | Genotype
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<td>cg31184/cg33108</td>
<td>36</td>
<td>53</td>
<td>26.5±1.5</td>
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<td>EP(2)2356</td>
<td>mir-310/311/312//313</td>
<td>34</td>
<td>53</td>
<td>complex</td>
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<td>EP(2)2098</td>
<td>cg30152</td>
<td>35</td>
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<td>29.5±16.0</td>
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<tr>
<td>EP(2)2402</td>
<td>mir-8</td>
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<td>23.5±0.7</td>
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<td>EP(3)3094</td>
<td>lk6</td>
<td>20</td>
<td>50</td>
<td>28.5±2.2</td>
<td>22.9±8.9</td>
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<td><strong>Controls</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>y w; timgal4/+</td>
<td></td>
<td>59</td>
<td>1.7</td>
<td>25.1</td>
<td>17.0</td>
</tr>
<tr>
<td>cry°</td>
<td></td>
<td>76</td>
<td>83</td>
<td>24.1±0.7</td>
<td>40.2±21.0</td>
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</tbody>
</table>
Table 2-II: Candidate genes sorted by biological function. Only the genes that are unambiguously identified are mentioned in this table.

<table>
<thead>
<tr>
<th>Biological function</th>
<th>Genes</th>
<th>Molecular activity</th>
<th>Connection to circadian rhythms</th>
<th>Signal transduction pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regulation of gene expression</td>
<td><em>elB</em></td>
<td>Transcription factor</td>
<td></td>
<td>JNK cascade</td>
</tr>
<tr>
<td></td>
<td><em>kay</em></td>
<td>Transcription factor</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>cpo</em></td>
<td>RNA binding protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>mir-282</em></td>
<td>MicroRNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>mir-310-313</em></td>
<td>MicroRNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>mir-8</em></td>
<td>MicroRNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein degradation</td>
<td><em>morgue</em></td>
<td>E2 ubiquitin conjuguase</td>
<td>Under circadian regulation</td>
<td>HH and WG pathway</td>
</tr>
<tr>
<td></td>
<td><em>slimb</em></td>
<td>E3 ubiquitin ligase</td>
<td>Regulates PER and TIM levels</td>
<td>Calcium signalling</td>
</tr>
<tr>
<td></td>
<td><em>calpB</em></td>
<td>calcium activated protease</td>
<td>Under circadian regulation</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>sda</em></td>
<td>protease</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein modification</td>
<td><em>lk6</em></td>
<td>Serine/threonine kinase</td>
<td>Under circadian regulation</td>
<td>MAPK signaling cascade</td>
</tr>
<tr>
<td></td>
<td><em>cg9801</em></td>
<td>Serine/threonine phosphatase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytoskeleton regulation</td>
<td><em>sip1</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein localization</td>
<td>akap200</td>
<td>PKA interacting protein, actin binding</td>
<td>Under circadian regulation</td>
<td>cAMP signaling cascade</td>
</tr>
<tr>
<td>----------------------</td>
<td>---------</td>
<td>----------------------------------------</td>
<td>---------------------------</td>
<td>-----------------------</td>
</tr>
<tr>
<td>Metabolism</td>
<td>Gst-S1</td>
<td>Glutathione-S-transferase</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>cg12173</td>
<td>Amino acid metabolism</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>cg10082</td>
<td>Inositol metabolism</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No putative function</td>
<td>cg30152</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>cg8735</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2-III: Behavior of the selected EP lines crossed to *tim-GAL4* under constant darkness.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Gene predicted to be affected</th>
<th>Number of flies</th>
<th>% rhythmic flies</th>
<th>Period average (± sd)</th>
<th>Power average (± sd)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EP(2)2367</td>
<td><em>morgue</em></td>
<td>12</td>
<td>83</td>
<td>24.5±0.3</td>
<td>72.9±27.2</td>
</tr>
<tr>
<td>EP(3)714</td>
<td><em>mir-282</em></td>
<td>11</td>
<td>55</td>
<td>24.9±0.4</td>
<td>59.6±35.6</td>
</tr>
<tr>
<td>EP(3)3718</td>
<td><em>mir-282</em></td>
<td>10</td>
<td>80</td>
<td>24.2±0.5</td>
<td>47.1±23.6</td>
</tr>
<tr>
<td>EP(2)670</td>
<td><em>GstS1</em></td>
<td>10</td>
<td>100</td>
<td>24.9±0.2</td>
<td>56.7±11.3</td>
</tr>
<tr>
<td>EP(3)703</td>
<td><em>cg8165/8176</em></td>
<td>22</td>
<td>100</td>
<td>24.8±0.4</td>
<td>54.2±21.7</td>
</tr>
<tr>
<td>EP(3)3041</td>
<td><em>mir-282</em></td>
<td>10</td>
<td>100</td>
<td>24.3±0.4</td>
<td>46.7±21.1</td>
</tr>
<tr>
<td>EP(2)965</td>
<td><em>elB</em></td>
<td>22</td>
<td>100</td>
<td>24.9±0.3</td>
<td>68.2±24.5</td>
</tr>
<tr>
<td>EP(3)972</td>
<td><em>calpB</em></td>
<td>11</td>
<td>55</td>
<td>25±0.3</td>
<td>33.3±10</td>
</tr>
<tr>
<td>EP(3)902</td>
<td><em>kay</em></td>
<td>9</td>
<td>100</td>
<td>24.5±0.4</td>
<td>38.8±16</td>
</tr>
<tr>
<td>EP(3)614</td>
<td><em>cg12173</em></td>
<td>17</td>
<td>88</td>
<td>24.8±0.6</td>
<td>56.3±29</td>
</tr>
<tr>
<td>EP(2)506</td>
<td><em>sip1</em></td>
<td>19</td>
<td>95</td>
<td>24.8±0.4</td>
<td>62.8±26</td>
</tr>
<tr>
<td>EP(3)3617</td>
<td><em>mir-282</em></td>
<td>nd</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EP(2)323</td>
<td><em>cg8735</em></td>
<td>19</td>
<td>95</td>
<td>24.6±0.2</td>
<td>60.3±26.6</td>
</tr>
<tr>
<td>EP(3)662</td>
<td><em>slmb</em></td>
<td>7</td>
<td>85</td>
<td>25.1±0.3</td>
<td>42.3±9.4</td>
</tr>
<tr>
<td>EP(2)2345</td>
<td><em>dap (as) or CG10459 (as)</em></td>
<td>11</td>
<td>100</td>
<td>24.6±0.4</td>
<td>70.5±30.6</td>
</tr>
<tr>
<td>EP(3)3084</td>
<td><em>kay</em></td>
<td>nd</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EP(2)2319</td>
<td><em>cg10082</em></td>
<td>10</td>
<td>100</td>
<td>25.1±0.4</td>
<td>67.1±26.4</td>
</tr>
<tr>
<td>EP(2)575</td>
<td><em>cg13791 (as)</em></td>
<td>11</td>
<td>91</td>
<td>24.6±0.3</td>
<td>59.4±20.7</td>
</tr>
<tr>
<td>EP(2)813</td>
<td><em>dpld (as) or cg1621 (as)</em></td>
<td>10</td>
<td>80</td>
<td>24.5±0.3</td>
<td>59.3±26.9</td>
</tr>
<tr>
<td>EP(3)1141</td>
<td>sda</td>
<td>10</td>
<td>70</td>
<td>25±0.5</td>
<td>47.9±26.8</td>
</tr>
<tr>
<td>-----------</td>
<td>--------</td>
<td>----</td>
<td>----</td>
<td>--------</td>
<td>-----------</td>
</tr>
<tr>
<td>EP(3)1110</td>
<td>cg9801</td>
<td>12</td>
<td>100</td>
<td>24.7±0.4</td>
<td>44.8±20.7</td>
</tr>
<tr>
<td>EP(2)2241</td>
<td>Dg</td>
<td>19</td>
<td>100</td>
<td>24.7±0.3</td>
<td>71.8±24.3</td>
</tr>
<tr>
<td>EP(3)661</td>
<td>cpo</td>
<td>10</td>
<td>90</td>
<td>25±0.3</td>
<td>57.2±28.5</td>
</tr>
<tr>
<td>EP(2)2469</td>
<td>kis (as)</td>
<td>10</td>
<td>80</td>
<td>25±0.3</td>
<td>57.2±28.5</td>
</tr>
<tr>
<td>EP(2)2254</td>
<td>akap200</td>
<td>11</td>
<td>100</td>
<td>24.8±0.2</td>
<td>68.3±25.2</td>
</tr>
<tr>
<td>EP(3)996</td>
<td>cg31184/cg33108</td>
<td>11</td>
<td>64</td>
<td>25.1±1.4</td>
<td>33.6±12.6</td>
</tr>
<tr>
<td>EP(2)2356</td>
<td>mir-310/311/312//313</td>
<td>11</td>
<td>100</td>
<td>24.1±0.3</td>
<td>54.5±17.7</td>
</tr>
<tr>
<td>EP(2)2098</td>
<td>cg30152</td>
<td>11</td>
<td>100</td>
<td>24.6±0.3</td>
<td>81.8±35.9</td>
</tr>
<tr>
<td>EP(2)2402</td>
<td>mir-8</td>
<td>11</td>
<td>100</td>
<td>25.3±0.4</td>
<td>60±29</td>
</tr>
<tr>
<td>EP(3)3094</td>
<td>lk6</td>
<td>15</td>
<td>93</td>
<td>25.3±0.4</td>
<td>73.5±25.1</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>y w;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>timgal4/+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cry</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>100</td>
<td>24.8±0.4</td>
<td>52.9±18.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>75</td>
<td>23.9±0.4</td>
<td>84.7±40.4</td>
<td></td>
</tr>
</tbody>
</table>
Table 2-IV: Behavior of RNAi lines for *kismet*, CG30152, CG10459, *slimb* and *morgue* crossed to *timGAL4* in LL and DD.

<table>
<thead>
<tr>
<th>Gene</th>
<th>RNAi line</th>
<th>% Rhythmicity in LL (period ±SD)</th>
<th>% Rhythmicity in DD (period ±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>kismet</td>
<td>3696-R1</td>
<td>62.5% $\tau=24.5 \pm 1.4$</td>
<td>92.9% $\tau=25.1 \pm 0.2$</td>
</tr>
<tr>
<td></td>
<td>30152-R1</td>
<td>22.9% $\tau=25.2 \pm 2.7$</td>
<td>81.3% $\tau=25.1 \pm 0.3$</td>
</tr>
<tr>
<td></td>
<td>30152-R2</td>
<td>29.8% $\tau=25.7 \pm 5.5$</td>
<td>100% $\tau=25.2\pm0.2$</td>
</tr>
<tr>
<td>CG10459</td>
<td>10459-R1</td>
<td>20.8% $\tau=28.4 \pm 3.4$</td>
<td>100% $\tau=25.2 \pm 0.2$</td>
</tr>
<tr>
<td>slimb</td>
<td>3412-R1</td>
<td>0%</td>
<td>66.7% $\tau=24.9 \pm 0.5$</td>
</tr>
<tr>
<td></td>
<td>3412-R3</td>
<td>0%</td>
<td>72.7% $\tau=25.2 \pm 0.4$</td>
</tr>
<tr>
<td></td>
<td>3412-R1</td>
<td>12.5%</td>
<td>93.8% $\tau=25.0 \pm 0.29$</td>
</tr>
<tr>
<td></td>
<td>10459-R1</td>
<td>0%</td>
<td>100% $\tau=25.3 \pm 0.4$</td>
</tr>
<tr>
<td>morgue</td>
<td>15437-R1</td>
<td>0%</td>
<td>69% $\tau=23.6 \pm 0.2$</td>
</tr>
<tr>
<td></td>
<td>15437-R2</td>
<td>12.5%</td>
<td>93.8% $\tau=25.0 \pm 0.29$</td>
</tr>
<tr>
<td>yw</td>
<td>control</td>
<td>6.2%</td>
<td>96.8% $\tau=24.6 \pm 0.3$</td>
</tr>
<tr>
<td>yw;;cry</td>
<td>control</td>
<td>61.3% $\tau=24.4\pm0.5$</td>
<td>ND</td>
</tr>
<tr>
<td>tim-Gal4/+</td>
<td>control</td>
<td>6.2% $\tau=25.0\pm0.5$</td>
<td>ND</td>
</tr>
</tbody>
</table>
Table 2-V: Behavior of *morgue* deletion mutants in LL of different decreasing light intensities (10, 3, 1 and 0.3 lux).

<table>
<thead>
<tr>
<th>deletion</th>
<th>10 lux</th>
<th>3 lux</th>
<th>1 lux</th>
<th>0.3 lux</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>#Rhyth. flies</td>
<td>Period ± SD</td>
<td>#Rhyth. flies</td>
<td>Period ± SD</td>
</tr>
<tr>
<td>m126</td>
<td>2/31</td>
<td>25.00 ±0.28</td>
<td>2/32</td>
<td>24.95 ±0.78</td>
</tr>
<tr>
<td>m457</td>
<td>0</td>
<td>ND</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>m100</td>
<td>1/32</td>
<td>25.5</td>
<td>5/32</td>
<td>28.58 ±2.37</td>
</tr>
</tbody>
</table>
Figure 2-1: A misexpression screen in constant light.
The yeast-based GAL4 system is a fine tool that allow tissue-directed expression in *Drosophila*. GAL4 is expressed in a tissue-specific manner and when it binds to UAS (GAL4-binding sites) activates the transcription of a targeted gene.

In the screen, flies that carry a random P-element insertion, that contains UAS sites in its promoter (P-element), were crossed to flies carrying a tissue-specific *gal4* driver (*tim-GAL4*), in order to drive expression of the targeted genes in clock neurons.
Figure 2-2: Period length distribution of EP lines crossed to *tim-GAL4* in constant light (LL).
The plot represents the period distribution (in period ranges of 0.5 h) of all the EP lines crossed to *tim-GAL4* in constant light (LL). The majority of the lines land in the 26.25-27.25 zone. Only few lines showed very short or very long periods, while two lines showed a period that is closer to that characteristic of DD behavior (24.75-25.25)
Figure 2-3: *morgue* mRNA levels at ZT 9 and ZT 21 in LD in *timGAL4/UAS-morgue* flies.

Real-Time PCR was performed using heads of *timGAL4/UAS-morgue* (*morgue OE*), *UAS-morgue/+* as a control (*morgue +*) and *Canton-S* flies. > 50 Flies were entrained to 12:12 h light:dark (LD) and equal amounts of heads were collected at ZT 9 and ZT 21 (ZT 0 = lights on). In *morgue OE* fly heads, *morgue* mRNA levels are increased > 10 fold. See materials and methods.
Figure 2-4: The effect of overexpressing MORGUE is specific.
Flies that overexpress UBCD-1 (SCF-ubiquitin conjugase) and AGO (SCF-ubiquitin ligase) were compared in LL to those that overexpress MORGUE. While flies overexpressing MORGUE remain strongly rhythmic in LL, those overexpressing either UBCD-1 or AGO showed strong arrhythmicity, just like wild-type flies normally behave. This suggests that the effect observed for MORGUE is specific.
Figure 2-5: CRY and TIM levels in heads of flies that overexpress *morgue*.
Flies that overexpress *morgue* and a tagged version of *cry* (M = *timGAL4-UAS-MYCCry / UAS-morgue*) were entrained to 12:12 h light:dark at 25°C (LD). Heads were collected every 4 hours (ZT0 = lights on) and were analyzed by Western-Blot. A. anti-MYC monoclonal antibody was used to detect MYC-CRY. Tubulin was used as a loading control. MYC-CRY levels were decreased at all time-points, as compared to the control flies (+ = *timGAL4-UAS-MYCCry / +*). B. Unlike MYC-CRY levels, TIM levels were unaffected.
**Figure 2-6: Phase response at decreasing light intensities for the deletion mutant morgue126.**

A. Schematic representation of the morgue genetic region and extension of the deletions generated at the Nambu Lab. Morgue P element mutant alleles: morgue100 is a precise excision allele; morgue126 and morgue457 delete portions of morgue; while morgue19 deletes all of morgue and a portion of CG15432. Morgue126 and morgue457 deletions start before the FB domain and between the FB and CD domains respectively. FB: F-box. CD: conjugase domain.

B. Mutant flies (morgue126) and control flies (yw) were entrained to 12:12 h LD cycles and then released in DD. At the beginning of the last night in LD (ZT15) different groups of flies were light pulsed using different light intensities (100, 20, 10, 5 and 2 lux). Morgue126 mutants did not show an increase of the response compared to control flies, suggesting that the mutants are not hypersensitive to light. This experiment was done once.
**Figure 2-7: EP3041 (miR-282) phase response curve (PRC).**
Phase response curve at ZT15 and ZT21 for EP3041 (miR-282) crossed to *timGAL4*. Flies were entrained to 12:12 h LD cycles, and then released into DD. During the last night of LD, flies were light pulsed at ZT15 or ZT21, and the phase of the behavior was analyzed in DD. A significant decrease in phase response was observed for *EP3041/timGAL4* at ZT15, comparable to that of *cry* mutants. *p=0.007, using Student’s test.*
CHAPTER III:

A SUBSET OF DORSAL NEURONS MODULATES CIRCADIAN BEHAVIOR AND LIGHT RESPONSES IN Drosophila

Alejandro Murad, Myai Emery-Le, Patrick Emery

Note: This chapter has been published in Neuron in 2007 (Murad et al., 2007) and it is the result of a collaboration between M.E., P.E, and myself. The studies that revealed a rhythmic behavior for flies that overexpress PER were initiated by M.E., and the EP line used to overexpress MORGUE was isolated in a screen carried by P.E. All other research done by A.M. P.E. wrote the body of the text, while I contributed to the Materials and Methods and provided feedback on the other sections.

Abstract:

A fundamental property of circadian rhythms is their ability to persist under constant conditions. In Drosophila, the ventral Lateral Neurons (LNvs) are the pacemaker neurons driving circadian behavior under constant darkness. Wild type flies are arrhythmic under constant illumination, but flies defective for the circadian photoreceptor CRY remain rhythmic. We found that flies overexpressing the pacemaker gene per or the morgue gene are also behaviorally rhythmic under constant light. Unexpectedly, the LNvs do not drive these rhythms: they are molecularly arrhythmic, and PDF—the neuropeptide
they secrete to synchronize behavioral rhythms under constant darkness—is dispensable for rhythmicity in constant light. Molecular circadian rhythms are only found in a group of Dorsal Neurons: the DN1s. Thus, a subset of Dorsal Neurons shares with the LNvs the ability to function as pacemakers for circadian behavior, and its importance is promoted by light.

A. Introduction

Circadian rhythms give the sense of time to cyanobacteria and most eukaryotes, so that these organisms can adapt their physiology and behavior to daily environmental variations. These rhythms are generated by an endogenous, self-sustained molecular pacemaker (Dunlap, 1999). In Drosophila, this pacemaker is a transcriptional feedback loop (Hardin, 2005). Two proteins, PER and TIM, repress their own gene transcription by blocking the activity of two transcription factors: CLK and CYC. A set of kinases (DBT, CKII, SGG) and a phosphatase (PP2A) regulate PER and TIM phosphorylation, and therefore their stability and activity, so that the cycle lasts 24 hours. A second feedback loop regulates CLK expression. PDP1 and VRI are positive and negative transcriptional regulators of the clk gene, respectively, while CLK regulates positively their circadian expression (Hardin, 2005). The first loop is absolutely essential for circadian rhythms, but the function of the second loop still needs to be established. It might be important for the robustness of circadian rhythms, or
their stability (Emery and Reppert, 2004). A strikingly similar molecular architecture that involves two interlocked feedback loops is also found in mammals (Shearman, 2000).

Recent studies have begun to elucidate the neural circuitry underlying circadian rhythms in *Drosophila*. This crepuscular animal shows two peaks of activity: around dawn and before dusk. Two separate groups of cells control these two peaks of activity: the ventral Lateral Neurons (LNvs) - that express the neuropeptide PDF - control the morning peak of activity, while the dorsal Lateral Neurons (LNds) and possibly two Dorsal Neurons (DNs) control the evening peak (Grima et al., 2004) (Stoleru et al., 2004). A recent study suggests that a specific LNv that does not express PDF might also contribute to the evening peak (Rieger et al., 2006). The PDF positive LNvs have another crucial function: they maintain circadian rhythms in constant environmental conditions (constant darkness and constant temperature to be precise; Renn et al., 1999). These cells are believed to synchronize the other groups of circadian neurons through the rhythmic secretion of PDF (Park et al., 2000) (Stoleru et al., 2005). The absence of the LNvs or of PDF results in rapid loss of behavioral rhythmicity under constant darkness, severely reduced amplitude of *tim* mRNA oscillations, and desynchronization of PER cycling within different groups of circadian neurons (Lin et al., 2004) (Peng et al., 2003) (Renn et al., 1999).

Since the LNvs control the oscillations of other circadian cells, they must be properly synchronized with the environment. The light:dark (LD) cycle is a
crucial environmental cue. The LNvs receive two kinds of photic input. First, these cells are directly blue-light sensitive because they express the photoreceptor CRY (Emery et al., 2000b). Second, photoreceptive organs that express rhodopsins (eyes, ocelli, Hofbauer-Buchner eyelets) all contribute to a certain degree to the synchronization of the LNvs (Helfrich-Forster et al., 2001) (Rieger et al., 2003).

CRY is thought to be the primary circadian photoreceptor, because it functions within circadian neurons (Emery et al., 2000b). Flies defective for CRY show very severe circadian photoreceptive defects. They cannot respond to short light pulses, while pulses as short as 1 minute can change the phase of circadian rhythms by several hours in wild-type flies (Stanewsky et al., 1998) (Egan et al., 1999). They also react abnormally to constant light. Under these conditions, wild-type flies are arrhythmic, but flies without a functional CRY input pathway have a 24-hr period rhythm, as if they were in constant darkness (Emery et al., 2000b). Rescuing CRY function only in the LNvs is sufficient to significantly restore circadian behavioral light responses (Emery et al., 2000a). This indicates an important autonomous role of the LNvs in CRY dependent light responses. However, since these responses are not completely restored to normal, there might be other cells that contribute to CRY photoreception.

Here, we show that a gain-of-function mutation in the circadian pacemaker can also protect flies from the disruptive effects of constant light. Indeed, flies overexpressing the key pacemaker gene per are robustly rhythmic under
constant illumination. Interestingly, our results demonstrate that the cells maintaining these behavioral rhythms are not the LNvs, but a subset of Dorsal Neurons of the DN1 group. Thus, these poorly characterized neurons play a central role in the control of circadian rhythms and the modulation of circadian responses to constant light.

B. Results

1. Flies overexpressing per are rhythmic under constant light.

The circadian behavior of wild-type flies is dramatically affected by the presence of constant light. The flies become arrhythmic after a day or two, while under constant darkness they would remain rhythmic for weeks (Konopka et al., 1989). This circadian response to constant light is dependent on the circadian photoreceptor CRY. \textit{cry}^{b} flies, that carry a severely hypomorphic \textit{cry} mutation (most likely a null mutation), remain rhythmic under constant light, with a periodicity of 24 hours, as if they were under constant darkness (Figure 3-1A; (Emery et al., 2000b)).

Interestingly, we found that when we overexpressed \textit{per} with the \textit{tim-GAL4} driver (Emery et al., 1998) (Kaneko and Hall, 2000) (Kaneko et al., 2000), which is active in every cell with circadian rhythms (genotype: \textit{y w;tim-GAL4/+; uas-PER/+}), almost all flies showed a robust ca. 26.8-hr period phenotype under 200
lux constant light (LL; Figure 3-1B and C, and Table 3-I). The vast majority of control flies were arrhythmic (Table 3-I). Only a few flies showed residual rhythmicity of weak amplitude; their period was similar to that observed in constant darkness (DD). Under DD, *per* overexpressing flies had a longer period than their control (25.7 hr vs 24.8 hr; Table 3-III), but that period length was shorter than under LL (25.7 hr vs 26.8 hr). Thus, manipulating the level of PER expression, a central element of the molecular circadian pacemaker, protects flies from the disruptive effects of constant light. However, while severe mutations in the CRY input pathway result in flies that are blind to constant light (Emery et al., 2000a) (Koh et al., 2006), flies overexpressing *per* are still partially responsive to LL.

2. Non-PDF circadian neurons maintain circadian behavioral rhythms under constant light.

The LNvS are the cells maintaining circadian rhythms under constant darkness (Lin et al., 2004) (Peng et al., 2003) (Renn et al., 1999). In their absence, flies become rapidly arrhythmic, within 2-3 days. Moreover, CRY expression in the LNvS has been reported to significantly restore responses to constant light in *cry*^b^ flies (Emery et al., 2000b). Thus, the simplest explanation for why flies overexpressing *per* remain rhythmic in LL is that somehow the LNvS have lost most of their light sensitivity. Therefore, we tested whether restricting
per overexpression to these cells would result in LL rhythmicity. We drove per overexpression with pdf-GAL4, a driver that is specifically expressed in the LNvs in the adult fly brain (Renn et al., 1999). Unexpectedly, this restricted per expression did not result in LL rhythmicity (Figure 3-1B and C, and Table 3-I). This suggests that the LNvs are not the critical cells for circadian rhythms in LL. To verify that this result was not due to a lower level of per expression in flies with the pdf-GAL4 driver compared to those with the tim-GAL4 driver, we drove per overexpression in flies with the tim-GAL4 driver, but excluded this overexpression from the LNvs with the pdf-GAL80 repressive transgene (Stoleru et al., 2004). These flies no longer overexpress per in the LNvs (Figure 3-6), but still do so in most (if not all) other clock neurons (data not shown). They also have a normal period length in DD, which indicates that the period lengthening was due to overexpression of per in the LNvs (Table 3-III). Nevertheless, the tim-GAL4 / pdf-GAL80/ UAS-per flies were as rhythmic as the tim-GAL4 / UAS-per flies in LL, and the period length of their behavior was identical (Figure 3-1B and C, Table 3-I).

Moreover, when tim-GAL4 was used in combination with cry-GAL80, rhythmicity was greatly reduced, and the period of the few remaining rhythmic flies was shortened to 25.3 hours (Figure 3-1B and C, and Table 3-I). cry-GAL80 blocks tim-GAL4 expression in the LNds and the PDF negative LNv, in addition to the PDF positive LNvs (Stoleru et al., 2004). Most likely, it also represses tim-GAL4 driven expression in all the other DNs since cry is expressed in these cells,
but this repression is not as complete. *cry* is also expressed in the eyes, and *cry-GAL80* could thus potentially block *tim-GAL4* in this tissue as well. However flies with overexpression of *per* driven by the eye-specific *gmr-GAL4* driver remained completely arrhythmic in constant light (Table 3-I).

Taken together, these results indicate that dorsally located circadian neurons (or possibly the unique PDF-negative LNv) modulate the responses to constant light and share with the PDF-positive LNvs the ability to maintain circadian rhythms over a long period of time under constant conditions. To confirm that the LNvs were not rhythmic in flies overexpressing *per* with *tim-GAL4* in LL, we measured PDP1 levels in these cells by immuno-histochemistry. PDP1 shows robust circadian oscillations with a very narrow concentration peak between ZT18 and ZT21 under LD conditions (Cyran et al., 2003). Thus, PDP1 is an excellent phase marker. We monitored *tim-GAL4/UAS-per* flies behaviorally in LL and dissected the brains of the flies that were rhythmic to determine whether PDP1 oscillate in their LNvs. As shown on Figure 3-2, PDP1 did not oscillate in the LNvs of flies collected at CT2 and 17, which are the predicted peak and trough time points for PDP1 staining in flies with 26.8-hour period rhythms on the 3rd day of LL. As expected, we did not observe oscillations at CT10 and 21 either, which would have occurred had the LNvs continued to oscillate with a period close to that of wild-type flies (data not shown). This proves that the LNvs are not circadianly functional under LL, even when *per* is overexpressed. This strengthens the notion that circadian neurons
other than the PDF-positive LNvs can maintain circadian rhythms in LL on their own.

3. Circadian oscillations persist in DN1 neurons in LL when *per* is overexpressed.

To determine which cells might generate behavioral rhythms under constant light, we studied PDP1 staining in non-PDF circadian neurons of *tim*-GAL4/UAS-*per* flies. We first focused on the LNds, since these cells are believed to be the E-cells critical for the control of the evening activity (Grima et al., 2004) (Stoleru et al., 2004). No oscillations of PDP1 staining could be detected in this group of neurons in *per* overexpressing flies under LL. PDP1 was constantly high, as 5-6 cells with PDP1 nuclear staining were detected at all 4 time points tested (Figure 3-2 and data not shown). A PDF-negative LNv has recently been implicated in the control of the evening peak of activity as well, and could underlie the long period behavioral rhythms observed in *cry*^b^ flies under LL when these flies split their behavior into a short and long period component (Rieger et al., 2006). In several brains dissected either at CT2 and CT17, we observed high PDP1 levels in a cell closely associated with the LNvs that was PDF negative, but we cannot be certain that this cell was the PDF-negative LNv. Indeed, additional PDP1 positive, PDF negative cells were seen in the vicinity of
the LNvs. These cells did not appear to show circadian oscillations of PDP1 either.

However, when we looked at the DN1 group, we clearly saw a much larger number of positive cells for PDP1 staining at CT2 compared to CT17, which are the predicted PDP1 peak and trough for per overexpressing flies, taking into account their long period phenotype in LL (Figure 3-3). On average, we saw ca. 8 positive neurons at the predicted trough for PDP1, while there were ca. 13 positive neurons at the predicted peak. Thus, a subset of DN1s oscillates in LL when per is overexpressed. Importantly, the number of PDP1 positive cells was low at both CT10 and 21, even though during the LD cycle there were low at ZT10 and high at ZT21 (Figure 3-3). Therefore, the period of the molecular oscillations in the oscillating subset of DN1s is not 24 hours, but is longer by several hours. This fits well with the period of the circadian behavior of per overexpressing flies in LL. This result strongly supports the idea that it is a subset of dorsal neurons that controls circadian behavior under constant light.

We also examined the DN2 and DN3 groups in LL with per overexpression (Figure 3-3). The DN2s did not oscillate; both DN2 neurons were PDP1 positive in most brains at all 4 time points. In the DN3 group, PDP1 did not appear to oscillate either, even though we cannot exclude that a small subset of these ca. 40 neurons were rhythmic. In conclusion, robust molecular oscillations are restricted to a subset of DN1s under constant illumination in per overexpressing flies. This result, combined with our genetic data, indicates that
these are the neurons maintaining circadian behavior in LL. Therefore, they play an important role in the neural circuits regulating circadian rhythms.

4. The DN1s also drive circadian rhythms in flies overexpressing morgue under constant light.

To obtain an independent confirmation of the important role of the DN1s in the circadian neural circuits, we turned to flies overexpressing morgue (Wing et al., 2002). In the genetic screen is described in details in Chapter III, we isolated several genes that can protect flies from the disruptive effects of constant light when overexpressed with the tim-GAL4 driver. The strongest phenotype was observed with morgue, and was very similar to that observed with per overexpression: a long period phenotype of 26.2 hr (Figure 3-4A and C, and Table 3-I). In DD, circadian behavior was normal (Table 3-III).

As shown on Figure 3-4 and Table 3-I, flies overexpressing morgue were very robustly rhythmic in LL conditions when tim-GAL4 was used, but not when pdf-GAL4 was used. The addition of pdf-GAL80 to tim-GAL4 flies had no effect, further demonstrating that non-PDF cells are important for constant light rhythmicity. Finally, as with per overexpression, blocking morgue overexpression with cry-GAL80 led to arrhythmicity in constant light.

We then determined which circadian cells are oscillating at a molecular level in the brains of morgue overexpressing flies. We used a PER antibody for
these experiments. The staining was done on the 3rd day of LL at CT6 and CT17, which are the predicted peak of nuclear PER accumulation and its concentration trough, respectively, based on the period length of the behavior (Shafer et al., 2002). The results were strikingly similar to those observed with PDP1 staining in per overexpressing flies (Figure 3-4B). The LNvs did not oscillate, including the PDF-negative LNv that was this time unambiguously identified. The LNds did not cycle either. However, there were very clear molecular oscillations in a subset of DN1s. Both DN2s were strongly stained at CT6. Staining was more variable at CT17, but some brains still had both DN2s that were PER positive. The DN3s did not appear to cycle. Thus, as observed with per overexpression, robust molecular oscillations are limited to a subset of DN1s when morgue is overexpressed. These results indicate that the DN1s are maintaining circadian behavioral rhythms in LL when morgue is overexpressed, and strongly support the notion that these cells play a central role in the control of circadian rhythms.

5. PDF is not required for circadian behavioral rhythms under constant light.

In DD, the LNvs synchronize behavior and the other brain circadian neurons such as the DN1s through the rhythmic secretion of PDF from their dorsal projection (Park et al., 2000) (Peng et al., 2003) (Lin et al., 2004) (Stoleru
et al., 2005). Since the LNvs are molecularly arrhythmic in *morgue* or *per* overexpressing flies, PDF secretion should be arrhythmic too. However, the DN1s might be able to induce rhythmic PDF secretion even when there is no functional circadian clock in the LNvs. Indeed, the DN1s send projection toward the LNvs (Kaneko and Hall, 2000), and *per*\(^0\) flies in which PER expression (and thus circadian rhythms) has been rescued in every neuron except the LNvs show morning anticipation, even though this anticipatory behavior is normally controlled by the LNvs (Stoleru et al., 2004). Rhythmic PDF secretion driven by the DN1s could even feedback and help the DN1s to remain rhythmic in LL. To determine whether PDF secretion is required for rhythmic behavior in LL, we overexpressed *morgue* in *pdf*\(^0 \gamma\) mutant flies (Renn et al., 1999). Under a light:dark cycle, these flies showed the typical advance in the phase of the evening activity found in *pdf*\(^0 \gamma\) flies. As expected, most of them became arrhythmic in DD (Table 3-IV), although we observed more rhythmicity than in *pdf*\(^0 \gamma\) control flies (the degree of residual rhythmicity varies in PDF deficient flies of different genetic background; see Renn et al., 1999). The period of the rhythmic *morgue* overexpressing *pdf*\(^0 \gamma\) flies was short, as previously observed with the rhythmic *pdf*\(^0 \gamma\) flies (Renn et al., 1999). In LL however, ca. 60\% of *morgue* overexpressing *pdf*\(^0 \gamma\) flies remained rhythmic (Table 3-II and Figure 3-4C). This demonstrates that output from the LNvs is dispensable for rhythmicity in constant light, and reinforces the notion that the DN1s can function independently of the LNvs when light is present. However, it should be noted
that the behavioral rhythms observed in LL without PDF are not as robust as those observed in the presence of PDF (higher degree of arrhythmicity, lower amplitude) and their period is about one hour shorter than control, as observed in DD. Thus, although PDF is not needed for LL rhythms, it influences their property.

6. Inhibition of the CRY input pathway allows the DN1s to remain rhythmic in LL.

CRY is responsible for the arrhythmic behavior observed under constant light, presumably because under these conditions it constantly degrades the pacemaker molecule TIM (Emery et al., 2000b) (Stanewsky et al., 1998). Thus, the mechanism that allows the DN1s of flies overexpressing per or morgue to escape the disruptive effects of constant light might be a repression of the CRY input pathway. If this hypothesis were correct, we would expect that other behavioral circadian responses to light would be affected in these flies. Wild-type flies delay their clock after a short early night light pulse, while they delay their behavior with a late night light pulse. In flies with cry mutations, these responses are severely reduced or absent (Busza et al., 2004) (Stanewsky et al., 1998). We therefore tested the ability of flies overexpressing morgue to respond to short light pulses. These flies responded to short light pulses like flies with a hypomorphic mutation in cry (cry\textsuperscript{m}; Busza et al., 2004): phase shifts could be
detected, but they were very severely reduced compared to control (Figure 3-5A). This result strongly suggests that the CRY input pathway is inhibited in morgue overexpressing flies.

In an earlier study, it was shown that cry\textsuperscript{b} mutant flies expressing wild-type CRY in the LNvs only (genotype: y w; pdf-GAL4/UAS-cry; cry\textsuperscript{b}) are partially rhythmic under constant light (Emery et al., 2000b). About half of the LNv-rescued cry\textsuperscript{b} flies were rhythmic. We wondered whether the DN1s might be the pacemaker neurons in these flies. We first monitored LNv-rescued cry\textsuperscript{b} under our current experimental conditions and found that about 50% of them were rhythmic in LL for at least 6 days (Figure 3-5B and data not shown). As expected, CRY expression with tim-GAL4 fully rescued the cry\textsuperscript{b} phenotype under LL (i.e. all the flies were arrhythmic). To determine whether the DN1s are the cells generating LL rhythms, we measured PDP1 levels in the brains of LNv-rescued cry\textsuperscript{b} flies. Since these flies exhibit ca. 24-hr period rhythms, we dissected the brains at CT21 (predicted peak) and CT10 (predicted trough). As expected, no oscillations could be detected in the LNvs of LNv-rescued cry\textsuperscript{b} flies since they express CRY (Table 3-V). PDP1 levels were lower than those observed in flies overexpressing per or in wild-type flies, suggesting that the clock in the LNvs is frozen at a different time point in LNv-rescued cry\textsuperscript{b} flies. This could be due to a more extensive degradation of TIM, since CRY should be overexpressed in these cells. We could not identify the LNds in these brains, presumably because PDP1 levels were very low. PDP1 levels were also
constantly low in a subset of DN1s (Figure 3-5C and Table 3-V). The number of DN2 positive cells was higher at CT21 than at CT10 (Table 3-V), but this oscillation was not statistically significant. Finally, staining in the DN3 was low in all brains at CT10, but the number of PDP1 positive cells varied considerably at CT21 (Table 3-V). This suggests that the DN3 might be oscillating, but that after three days in LL their oscillations are not synchronized properly any more, probably because they do not get synchronization signals from the LNvs. However, we observed robust, coherent PDP1 oscillations in ca. 6-7 DN1s (Figure 3-5C).

These results are very important. First, they confirm that a subset of DN1s play the role of pacemaker cells for circadian behavior in LL. Second, since this last set of results is obtained in flies with a cry loss-of-function mutation, rather than flies overexpressing a specific gene, the conclusion is that the DN1s are intrinsically able to control and generate self-sustained circadian behavioral rhythms when light is present. Their ability to do so when overexpressing morgue or per is thus not due to a gain-of-function that would have given them a property that they do not usually have. The DN1s thus play an important role in the control of circadian behavior and its responses to light.
C. Discussion

Recent studies have shown that two groups of cells control circadian behavior. The PDF positive LNvs are called morning cells (M-cells), and the LNds evening cells (E-cells), because they control the anticipatory behavior observed before dawn and dusk respectively (Grima et al., 2004) (Stoleru et al., 2004). In addition, the LNvs are the cells maintaining circadian behavior in constant darkness and controlling the phase of most circadian neurons of the brain (Lin et al., 2004) (Peng et al., 2003) (Renn et al., 1999) (Stoleru et al., 2005). In their absence, circadian behavior rhythms are lost after a few days in DD. Surprisingly, our results show that a functional circadian clock in the LNvs is actually not necessary for long-term behavioral rhythms. In flies overexpressing PER, the LNvs are no longer circadianly functional under constant illumination. No oscillation of the circadian protein PDP1 can be detected and yet these flies remain rhythmic for at least 7 days. Moreover, limiting per overexpression to circadian neurons that do not express PDF is sufficient to obtain circadian behavioral rhythms under constant environmental conditions.

We believe that the neurons maintaining circadian behavior independently of the LNvs are not the E-cells. Indeed, when per is overexpressed, we did not see any sign of circadian oscillation in the neurons that are thought to control the evening activity: the LNds (Grima et al., 2004) (Stoleru et al., 2004). In addition, the PDF negative LNv that might also contribute to the evening activity (Rieger et
al., 2006) did not cycle in LL when morgue was overexpressed. Moreover, flies with per overexpression driven by cry-GAL4 were completely arrhythmic under constant light (Table 3-I). cry-GAL4 is one of the critical GAL4 driver used to define the E-cells (Stoleru et al., 2004). Importantly, we actually detected molecular circadian oscillations in only one group of cells when per was overexpressed: the DN1s. Due to the high number of DN3s, we cannot rule out that a few cells in the DN3 groups also oscillate. Interestingly, Veleri et al. (2003) have previously shown that a subset of DN3 neurons can maintain their own circadian oscillations in DD, in the absence of circadianly functional LNvs. However, these DN3 cells were not able to generate rhythmic behavior in DD. While it is possible that light is a necessary co-factor for these self-sustained DN3s to participate in the control circadian behavior, we favor the hypothesis that it is the DN1s that maintain circadian rhythmicity in LL. This idea is strongly supported by several additional findings. First, the phase of PDP1 molecular oscillations in the DN1s on the 3\textsuperscript{rd} day of LL fits well with the long period of the circadian behavior observed under these conditions in per overexpressing flies. Second, the behavioral observations made with morgue overexpression also suggest that the critical cells for rhythmicity are not the LNvs, and PER staining in morgue overexpressing flies gave us an independent confirmation that robust circadian molecular oscillations are restricted to the DN1s in LL. Finally, in LNv-rescued cry\textsuperscript{b} flies, only the DN1s show robust, coherent circadian rhythms in phase with the behavioral rhythms. Remarkably, the DN1s can maintain
circadian behavior in LL even when PDF is absent. This indicates that they can work autonomously of LNv output. Interestingly, not all DN1s do oscillate in LL, only about 6-7 cells most likely. This shows that the DN1 group is heterogeneous. This is not surprising, since the different groups of circadian neurons were named based on their location in the brain, not on their function or developmental lineage. There is ample evidence for heterogeneity of morphology, gene expression and behavior within these different groups of cells, including the DN1s (see for example Rieger et al., 2006; Shafer et al., 2006).

Thus, a subset of DN1s can control and generate circadian behavioral rhythms. They must therefore play an important role in the circadian neuronal circuits. Since ablation of the M cells and E cells results in flies with no morning and evening activity, and no self-sustained rhythms in DD (Stoleru et al., 2004), this could mean that the DN1s are usually functioning downstream of the M and E cells. This is further supported by the fact that in the absence of the neuropeptide PDF - believed to be the critical synchronizing signal secreted by the M cells – the DN1s cannot maintain their circadian rhythms in the long run in DD (Lin et al., 2004) (Peng et al., 2003). The DN1s can thus probably function as a relay connecting the LNvs with the neurosecretory cells of the pars intercerebralis (PI), believed to play an important role in the control of locomotor behavior (Helfrich-Forster et al., 1998) (Kaneko and Hall, 2000). A LNvs-DN1-PI pathway has also been suggested based on the anatomical studies of the projections of the small LNvs and the DN1s (Kaneko and Hall, 2000). The
expression of the receptor for PDF in at least a subset of DN1s also supports the existence of a functional connection between them and the LNvs (Mertens et al., 2005) (Hyun et al., 2005) (Lear et al., 2005). The implication of this connection is that in wild-type flies under LL, the LNvs should constantly send a disruptive signal to the DN1s, presumably the non-oscillating secretion of PDF.

This leaves us with the following question: if the LNvs and rhythmic PDF secretion are normally required for the DN1s to be rhythmic, why are the DN1s able to free themselves from the disruptive effects of constant light, while at the same time becoming independent of the LNvs? Our results show that an important mechanism is the inhibition of the CRY-dependent light input pathway. Indeed, morgue overexpressing flies are defective in the CRY-dependent behavioral responses to short light pulses, and cry loss-of-function mutations also result in rhythms driven by the DN1s. In the case of per overexpression, we presume that TIM role is reduced, since one of its major functions is to protect PER from proteasomal degradation (Grima B, 2002) (Ko HW, 2002) (Price et al., 1995). TIM is the target of CRY, thus its reduced importance would result in DN1s that are less sensitive to the CRY input pathway. In addition, overexpression of SHAGGY, which inhibits CRY signaling, also results in LL rhythms driven by dorsal neurons (Stoleru et al., 2007, #56292). However, under natural environmental conditions, inhibition of the CRY input pathway is probably not required for the DN1s to participate in the control circadian rhythms. Indeed, even in the polar regions of the globe that experience constant light conditions
during the summer, the elevation of the sun varies during the day, and this 
should result in variations of temperature sufficient to synchronize the DN1 
circadian clock (Yoshii et al., 2005).

The mechanism by which the DN1s avoid to become arrhythmic in LL as a 
result of the molecular arrhythmicity of the LNvs, which should result in constant 
PDF secretion, is not clear yet. It is possible that the presence of light inhibits 
PDF signaling and thus promotes the role of the DN1s. Light input could come 
from the eyes, ocelli, or from the DN1s themselves (Rieger et al., 2003). 
Alternatively, as mentioned in the result section, the DN1s could induce rhythmic 
PDF secretion. The fact that PDF is not required for LL behavioral rhythms does 
not exclude this possibility, particularly since the robustness of the rhythms is 
improved by the presence of PDF.

Interestingly, per and morgue overexpression results in a very similar long 
period phenotype under LL, which could suggest that these two molecules 
coincidentally affect the period length of the circadian molecular pacemaker in 
the same way. In DD however, per overexpression does affect behavioral period 
length, while morgue does not. The long period phenotype observed in LL 
actually probably reflects the fact that the CRY input pathway is not completely 
blocked in the DN1s of per or morgue overexpressing flies. Indeed, under very 
low light intensity, wild-type flies exhibit a long period phenotype as well 
(Konopka et al., 1989). In addition, morgue overexpression does not completely 
block the CRY-dependent responses to short light pulses (Figure 3-5A). Finally
and most importantly, LNv-rescued cry<sup>b</sup> flies - in which the CRY input pathway is completely non-functional in the DN1s – have 24-hr period rhythms. The LNv-rescued cry<sup>b</sup> flies show nevertheless a higher degree of arrhythmicity than normal cry<sup>b</sup> flies, or than flies overexpressing morgue or per. This might be due to the desynchronization observed within the DN3 group of circadian neurons. Indeed, the DN3s do not appear to be desynchronized in per or morgue overexpressing flies.

A previous report had already shown that LNv-rescued cry<sup>b</sup> flies are partially rhythmic (Emery et al., 2000b), and this was interpreted as evidence for a functional role of CRY directly in the LNvs. Our new results show that expression of CRY in the LNvs is probably not very important for the response to constant light. The DN1s are the important cells for this response. Does this mean that CRY is not a photoreceptor in the LNvs? We believe it actually does function as a photoreceptor in the LNvs as well. CRY is expressed in these cells (Emery et al., 2000b) (Klarsfeld et al., 2004), and LNv-rescued cry<sup>b</sup> flies show very significantly rescued responses to short light pulses. Preliminary experiments with morgue overexpression limited to the LNvs confirm a predominant role of these cells for light pulse responses (see Addendum to chapter III). Thus, the CRY input pathway might mediate response to short light pulses by its action in the LNvs, and constant light responses by its action in the DN1s.
In summary, our work underscores the importance of the DN1s in the control of circadian behavior and responses to light. Earlier genetic studies have indicated that the DN1s modulate the sensitivity of the circadian network to light:dark cycles of very low light intensity (Klarsfeld et al., 2004). Our results significantly extend this observation by showing the profound impact the DN1s have on the response to constant light and by demonstrating that these cells not only modulate circadian light responses, but can also become the driving force controlling circadian locomotor behavior, and this in the absence of environmental cues and functional LNvs. This confers upon them a unique status among non-PDF circadian neurons. One of our striking results is that genetically identical flies rely either on the LNvs or the DN1s for the control of their circadian rhythms, depending on the presence or absence of light. Indeed, the LNvs determine period length in our experiments with per overexpression in DD, but in LL the DN1s set the pace. That the presence or the absence of light can so remarkably shift the dominance from one cell group to the other strongly suggests that the relative contribution of the LNvs and DN1s to the control of circadian rhythms change during the course of the year, particularly at high latitude. The DN1s, which interestingly generate evening activity (Figure 3-1,3-4 and 3-5), would play a more prominent role in the control of circadian behavior during the long days of the summer, while the LNvs would be more important when photoperiods are shorter.
D. Materials and Methods

1. Drosophila stocks and transgenics.

The following Drosophila strains were used in this study: y w; UAS-per 24 (Kaneko et al., 2000), cry^b ss (Stanewsky et al., 1998). y w; tim-GAL4 (Emery et al., 1998), y w; pdf-GAL4 (Renn et al., 1999). cry^b flies with rescued wild-type CRY expression have been described previously (Emery et al., 2000b). To overexpress morgue, we used a P-element containing UAS binding sites inserted in front of the morgue gene: EP(2)2367 (Rorth et al., 1998; Wing et al., 2002). EP(2)2367 and tim-GAL4 were separately combined with the pdf^01 mutation (Renn et al., 1999) in y w background, so that pdf^01 mutant flies overexpressing morgue could be generated. A y w; tim-GAL4, pdf-GAL80/Cyo; pdf-GAL80/TM6B strain was generated in two steps to be able to drive per overexpression in every clock cells except the LNvs. First the 2^nd chromosome tim-GAL4 insertion was meiotically recombined with a chromosome containing two 2^nd chromosome insertions of the pdf-GAL80 transgene (Stoleru et al., 2004). Recombinants with darker eye colors than the parental strains were selected, which should contain both tim-GAL4 and at least one copy of pdf-GAL80. The presence of both pdf-GAL80 and tim-GAL4 in the recombinants was confirmed by PCR. Then, we added two copies of the pdf-GAL80 transgene carried on the 3^rd chromosome. The final stock thus contains at least three copies of pdf-GAL80, which ensure a
very strong repression of GAL4 in the LNvs (see Figure3-6). To further restrict per overexpression, a y w; tim-GAL4/CyO; cry-GAL80/TM6B strain was generated by combining a 2nd chromosome insertion of tim-GAL4 with two cry-GAL80 insertions on the 3rd chromosome (Stoleru et al., 2004).

2. Behavioral analysis.

Locomotor activity of male flies (1-5 days old) were measured with Trikinetics Activity Monitors (Waltham, MA, USA) for 3 full days under 12 hr light: 12 dark conditions (LD) followed by 6 full days of either constant light or constant darkness at 25°C. For almost all experiments, a light intensity of ca. 200 lux was used. Data analysis was performed with the FAAS software (Grima et al., 2002). Rhythmic flies were defined by $\chi^2$ periodogram analysis with the following criteria: power $\geq 10$, width $\geq 2$ (Ewer et al., 1992). The group activity actograms were generated using a signal-processing toolbox (Levine et al., 2002) implemented in MATLAB (MathWorks). Phase Responses Curves were generated essentially as described in Busza et al. (2004).

3. Whole-mount immunohistochemistry and quantitation.

Adult Drosophila (1-5 days old) were entrained to 3 days of LD and then released in LL. Adult brains were dissected during the second and third day of LL. Immunotainings for PER were performed as previously described (Lear et al., 2005). The same protocol was used for PDP-1 staining. The anti-PDP1
antibody was a generous gift from Justin Blau and was used at a concentration of 1:5,000. For the PDF staining, anti-PDF (generous gift from Michael Rosbash) was used at a concentration of 1:400 and then visualized using a FITC-anti-rat secondary antibody (Jackson Immunoresearch, PA, USA). All samples were mounted in BioRad antifade reagent, and viewed on a Zeiss LSM5 Pascal confocal microscope. At least two experiments for each time points were performed and produced comparable results. The images presented in the figures are overlays of several confocal stacks (1μm) obtained using ImageJ software (freely available at http://rsb.info.nih.gov/ij/). After discriminating between the different neurons on the overlays, the brains were scored for the number of PDP1 or PER positive cells and staining intensity by an observer who was blind for genotypes and time points. Staining intensity was subjectively scored from 0 (no staining) to 5 (high staining) for all PDP1 positive cells of two to three representative brain hemispheres, except for the DN3s. For this group, ca. 10 representative neurons were scored.

E. Acknowledged Contributions

The morgue gene was isolated in a screen initiated by Patrick Emery in M. Rosbash’s lab. I would like to thank A. Busza for help with behavioral experiments and analysis, D. Stoleru and M. Rosbash for the pdf-GAL80 and cry-GAL80 flies, M. Boudinot and F. Rouyer for the FAAS behavior analysis
software. J. Blau generously provided the anti-PDP1 antibody and M. Rosbash kindly provided the anti-PDF and anti-PER antibodies. I would like to thank Diane Szydlik for technical help. This work was supported by an R01 NIH grant to P.E. (# 5R01GM066777).

This chapter has been published:

Addendum to Chapter III

NOTE: The data presented in this addendum is the result of research done after Chapter III was already published.

In Chapter III we report results suggesting that the CRY input pathway is inhibited in morgue overexpressing flies. We tested the ability of flies overexpressing morgue to respond to short light pulses (phase response curve, PRC). These flies responded similarly to like cry/m mutants (Busza et al., 2004): phase shifts could be detected, but they were severely attenuated compared to controls (Figure 3-5A). In these experiments, overexpression of morgue was driven using the timGAL4 driver, achieving expression in all clock neurons. We therefore wondered: Is this response also mediated by the DN1s, like rhythmicity in LL? Or is it mediated by the PDF (+) LNvs?

It has been shown before that rescuing cry expression in cry/b mutants in all clock cells (with the timGAL4 driver) completely rescues phase responses to wild type levels (Emery et al., 2000b). When rescuing cry expression only in the PDF (+) LNvs (with the pdfGAL4 driver), the authors observe a complete rescue of the phase advance (ZT21), but only a partial rescue of the phase delay (ZT15)
cry expression in the PDF (+) LNvs alone also partially rescues arrhythmicity in LL to about 50% of wild type levels, while rescuing cry expression with the timGAL4 driver brings LL arrhythmicity back to wild type levels (Emery et al., 2000b). The authors concluded that the PDF (+) LNvs are important for circadian photoreception.

In a more recent study by Stoleru et al., the authors use the GAL4/GAL80 system to rescue the expression of cry in cry\textsuperscript{b} mutants in all clock cells except the PDF (+) LNvs (timGAL4-UAS-myc-cry/pdfGAL80, cry\textsuperscript{b}). These E-cell-rescued cry\textsuperscript{b} mutants show normal phase shift responses (delay and advance) comparable to the control (timGAL4-UAS-myc-cry, cry\textsuperscript{b}) (Stoleru et al., 2007).

Moreover, the PRC is affected in flies that overexpress the kinase sgg in an E-cell-specific manner (Stoleru et al., 2007): these flies show a severely attenuated phase delay and an early breakpoint between the delay and advance zones. The authors conclude that the PDF (-) E-cells are important for this circadian photoresponse (PRC), in contrast with Emery et al. 2000b conclusions that the PDF (+) LNvs are important in controlling the response to light brief light pulses.

In order to test whether the severe attenuation in phase response observed in flies that overexpress morgue was mediated by the PDF (+) LNvs or by PDF (-) neurons (including the E-cells), we used a combination of GAL4/GAL80 drivers to restrict morgue expression to these two neuronal groups. Figure 3-7 compares the phase response after a short light pulse (5 min, 1,000 lux) at ZT15 and ZT 21 of timGAL4/UAS-morgue flies to: 1) flies that overexpress
morgue in PDF (+) neurons alone (pdfGAL4/UAS-morgue) and 2) flies where morgue expression is restricted to PDF (-) clock neurons, including the E-cells (timGAL4-pdfGAL80/UAS-morgue). When morgue is only overexpressed in the PDF (+) neurons, flies experience a severely attenuated phase delay (ZT15), comparable to that of timGAL-4/UAS-morgue (Figure 3-5A and 3-7), but show almost normal phase advance (ZT21). Interestingly, when we overexpress morgue in all clock neurons, except the LNvs (timGAL4-pdfGAL80/UAS-morgue), phase delay and advance are both normal. These results suggest a role for the PDF (+) LNvs, but not for other groups of dorsal neurons including the E-cells, in controlling this particular photoresponse, at least during the delay zone and when morgue is overexpressed. These results differ from those presented in Stoleru et. al. 2007, where they suggest that both photoresponses, arrythmicity in LL and PRC, are under the exclusive control of PDF (-) cells (including a subgroup of DN1s) (Stoleru et al., 2007). The results presented here also differ from those presented by Emery et al. 2000b, where the authors attribute the control of both, delay and advances to the PDF (+) LNvs (Emery et al., 2000b).

Since rescuing cry expression in LNvs alone rescues phase responses (Emery et al., 2000b) and since in our hands, overexpressing morgue in the LNvs alone is enough to trigger a decrease in the PRC delay zone (comparable to that of cry$^{77}$), we conclude that the PDF (+) LNvs are important pacemaker cells that regulate this response to light. Taken together, both groups of cells appear to be capable of controlling phase photoresponses. The different results that are
observed in different studies can be attributed to the fact that both groups of neurons are differentially sensitive to a particular genetic manipulation. In addition, the differences in responses between advance and delay zones of the PRC could be explained by the possibility that these two mechanisms use different signal transduction pathways.
Table 3-I: Behavior of flies overexpressing *per* and *morgue* under LL conditions (200 lux). Genotypes with robust rhythms are highlighted in bold.

Rhythmic flies have a power greater than 10 and a width greater than 2. See Ewer et al. (Ewer et al., 1992) for power and width definition.

<table>
<thead>
<tr>
<th>gene</th>
<th>GAL4 driver</th>
<th>GAL80 driver</th>
<th># flies</th>
<th># rhythmic flies</th>
<th>Period average (±st.dev.)</th>
<th>Power average (±st.dev.)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>per</em></td>
<td><em>tim</em>-GAL4</td>
<td>-</td>
<td>31</td>
<td>28</td>
<td>26.8±1.5</td>
<td>51.1±31.1</td>
</tr>
<tr>
<td><em>per</em></td>
<td><em>pdf</em>-GAL4</td>
<td>-</td>
<td>32</td>
<td>1</td>
<td>25.5</td>
<td>41.9</td>
</tr>
<tr>
<td><em>per</em></td>
<td><em>tim</em>-GAL4</td>
<td><em>pdf</em>-GAL80</td>
<td>26</td>
<td>25</td>
<td>26.7±1.7</td>
<td>56.1±32.2</td>
</tr>
<tr>
<td><em>per</em></td>
<td><em>cry</em>-GAL80</td>
<td>-</td>
<td>30</td>
<td>7</td>
<td>25.2±0.9</td>
<td>33.9±12.0</td>
</tr>
<tr>
<td><em>per</em></td>
<td><em>gmr</em>-GAL4</td>
<td>-</td>
<td>15</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>morgue</em></td>
<td><em>tim</em>-GAL4</td>
<td>-</td>
<td>30</td>
<td>29</td>
<td>26.2±0.6</td>
<td>52.7±16.6</td>
</tr>
<tr>
<td><em>morgue</em></td>
<td><em>pdf</em>-GAL4</td>
<td>-</td>
<td>30</td>
<td>1</td>
<td>16.4</td>
<td>17.0</td>
</tr>
<tr>
<td><em>morgue</em></td>
<td><em>tim</em>-GAL4</td>
<td><em>pdf</em>-GAL80</td>
<td>32</td>
<td>32</td>
<td>26.4±0.3</td>
<td>65.8±18.3</td>
</tr>
<tr>
<td><em>morgue</em></td>
<td><em>cry</em>-GAL80</td>
<td>-</td>
<td>32</td>
<td>5</td>
<td>24.7±1.7</td>
<td>25.4±12.7</td>
</tr>
<tr>
<td>-</td>
<td><em>tim</em>-GAL4</td>
<td>-</td>
<td>28</td>
<td>6</td>
<td>24.6±2.6</td>
<td>29.1±16.3</td>
</tr>
<tr>
<td>-</td>
<td><em>pdf</em>-GAL4</td>
<td>-</td>
<td>28</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td><em>tim</em>-GAL4</td>
<td><em>pdf</em>-GAL80</td>
<td>28</td>
<td>7</td>
<td>25.1±1.8</td>
<td>28.3±13.0</td>
</tr>
<tr>
<td>-</td>
<td><em>tim</em>-GAL4</td>
<td><em>cry</em>-GAL80</td>
<td>27</td>
<td>4</td>
<td>23.2±1.7</td>
<td>13.8±2.2</td>
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<tr>
<td>-</td>
<td><em>cry</em>-GAL4</td>
<td>-</td>
<td>16</td>
<td>2</td>
<td>21.3±0.2</td>
<td>13.8±0.4</td>
</tr>
<tr>
<td>-</td>
<td><em>gmr</em>-GAL4</td>
<td>-</td>
<td>14</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 3-II: Behavior of pdf\(^{01}\) flies overexpressing (or not) morgue under LL conditions (200 lux). Genotypes with robust rhythms are highlighted in bold.

<table>
<thead>
<tr>
<th>Genotype</th>
<th># flies</th>
<th># of rhythmic flies</th>
<th>Period average (±st.dev.)</th>
<th>Power average (±st.dev)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pdf(^{01})</td>
<td>20</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>y w; tim-GAL4/+; pdf(^{01})</td>
<td>25</td>
<td>5</td>
<td>25.2±1.7</td>
<td>27.0±21.6</td>
</tr>
<tr>
<td>y w; +/UAS-morgue; pdf(^{01})</td>
<td>25</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>y w; tim-GAL4/UAS-morgue; pdf(^{01})</td>
<td>65</td>
<td>37</td>
<td>25.0±1.0</td>
<td>29.2±12.3</td>
</tr>
<tr>
<td>y w; tim-GAL4/ UAS-morgue; +</td>
<td>16</td>
<td>15</td>
<td>26.2±0.7</td>
<td>40.5±14.2</td>
</tr>
</tbody>
</table>
Table 3-III: Behavior of flies overexpressing *per* and *morgue* under DD conditions. Rhythmic flies have a power greater than 10 and a width greater than 2. See Ewer et al (1992) for power and width definition.

<table>
<thead>
<tr>
<th>gene</th>
<th>GAL4 driver</th>
<th>GAL80 driver</th>
<th>Number of flies</th>
<th>Number of rhythmic flies</th>
<th>Period average (±st.dev.)</th>
<th>Power average (±st.dev)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>per</em></td>
<td>tim-GAL4</td>
<td>-</td>
<td>32</td>
<td>25</td>
<td>25.7±0.7</td>
<td>40.7±23.1</td>
</tr>
<tr>
<td><em>per</em></td>
<td>pdf-GAL4</td>
<td>-</td>
<td>32</td>
<td>27</td>
<td>24.6±0.6</td>
<td>54.1±32.1</td>
</tr>
<tr>
<td><em>per</em></td>
<td>tim-GAL4</td>
<td>pdf-GAL80</td>
<td>30</td>
<td>21</td>
<td>24.5±0.7</td>
<td>42.2±22.5</td>
</tr>
<tr>
<td><em>per</em></td>
<td>tim-GAL4</td>
<td>cry-GAL80</td>
<td>31</td>
<td>23</td>
<td>23.9±0.6</td>
<td>44.7±20.5</td>
</tr>
<tr>
<td><em>morgue</em></td>
<td>tim-GAL4</td>
<td>-</td>
<td>16</td>
<td>16</td>
<td>24.8±0.2</td>
<td>99.1±37.3</td>
</tr>
<tr>
<td><em>morgue</em></td>
<td>pdf-GAL4</td>
<td>-</td>
<td>14</td>
<td>14</td>
<td>25.2±0.4</td>
<td>110.5±56.3</td>
</tr>
<tr>
<td><em>morgue</em></td>
<td>tim-GAL4</td>
<td>pdf-GAL80</td>
<td>14</td>
<td>9</td>
<td>24.1±0.4</td>
<td>88.3±54.3</td>
</tr>
<tr>
<td><em>morgue</em></td>
<td>tim-GAL4</td>
<td>cry-GAL80</td>
<td>12</td>
<td>11</td>
<td>24.2±0.2</td>
<td>63.9±21.0</td>
</tr>
<tr>
<td>-</td>
<td>tim-GAL4</td>
<td>-</td>
<td>15</td>
<td>14</td>
<td>24.6±0.3</td>
<td>113.6±36.7</td>
</tr>
<tr>
<td>-</td>
<td>pdf-GAL4</td>
<td>-</td>
<td>16</td>
<td>16</td>
<td>24.2±0.3</td>
<td>111.6±42.2</td>
</tr>
<tr>
<td>-</td>
<td>tim-GAL4</td>
<td>pdf-GAL80</td>
<td>15</td>
<td>15</td>
<td>23.9±0.2</td>
<td>132.1±45.4</td>
</tr>
<tr>
<td>-</td>
<td>tim-GAL4</td>
<td>cry-GAL80</td>
<td>16</td>
<td>15</td>
<td>23.8±0.1</td>
<td>135.4±41.4</td>
</tr>
</tbody>
</table>
Table 3-IV: Behavior of *pdf*\(^{01}\) flies overexpressing (or not) *morgue* under DD conditions.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of flies</th>
<th>Number of rhythmic flies</th>
<th>Period average (±st.dev.)</th>
<th>Power average (±st.dev)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>pdf</em>(^{01})</td>
<td>18</td>
<td>2</td>
<td>22.1±1.2</td>
<td>12.0±0.7</td>
</tr>
<tr>
<td><em>y w; tim-GAL4/UAS-morgue; pdf</em>(^{01})</td>
<td>29</td>
<td>8</td>
<td>22.7±0.7</td>
<td>39.7±22.9</td>
</tr>
<tr>
<td><em>y w; tim-GAL4/UAS-morgue; +</em></td>
<td>19</td>
<td>18</td>
<td>24.1±0.5</td>
<td>51.7±14.0</td>
</tr>
</tbody>
</table>
Table 3-V: Quantification of PDP-1 staining in LNv-rescued cry^{b} flies. The number of PDP-1 positive neurons and their staining intensity were blind-scored for each group of circadian neurons in y w; pdf-GAL4/UAS-cry; cryb flies in LL, at circadian time (CT) 10 and 21. The LNds could not be identified, presumably because of constant low PDP1 expression. Statistically significant differences are indicated in bold.

<table>
<thead>
<tr>
<th></th>
<th>Number of PDP1 positive cells</th>
<th>Staining intensity in PDP1 positive cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CT10</td>
<td>CT21</td>
</tr>
<tr>
<td>small LNvs</td>
<td>0.3±0.4</td>
<td>0.6±0.5</td>
</tr>
<tr>
<td>large LNvs</td>
<td>0.3±0.5</td>
<td>0.4±0.5</td>
</tr>
<tr>
<td>LNds</td>
<td>Not identified</td>
<td>Not identified</td>
</tr>
<tr>
<td>DN1s</td>
<td>1.7±1.5</td>
<td>8.3±2.0</td>
</tr>
<tr>
<td>DN2s</td>
<td>0.6±0.8</td>
<td>1.1±0.4</td>
</tr>
<tr>
<td>DN3s</td>
<td>1.1±2.2</td>
<td>9.7±9.6</td>
</tr>
</tbody>
</table>
A

Control

$cry^b$

B

tg4-per
ga4-per
tg4-pg80-per
tg4-cg80-per

C

% of Rhythmic Flies

26.8 ± 1.5

26.7 ± 1.7

per

Control

tg4

pg4
tg4-pg80
tg4-cg80
**Figure 3-1:** Flies overexpressing *per* are rhythmic in LL.  

**A.** Average double-plotted actograms of 16 wild-type (control) and 16 *cry* flies under constant light. Both groups of flies were first synchronized to a light dark (LD) cycle (grey shadings indicate the dark phase), and then released under constant light (LL, indicated with an arrow head). Wild-type flies become rapidly arrhythmic under constant light, while *cry* flies are robustly rhythmic, with a 24-hr period. Each day (except the first) is plotted twice: on the right half of the actogram and then on the left half, on the next line. The first day is plotted only once on the left half of the first line. Arrhythmic flies are included in all average actograms, including those of figure 4 and 5.  

**B.** Average double-plotted actograms of flies overexpressing *per* in different groups of circadian cells under LL conditions (16 flies per genotype). *per* was overexpressed in different groups of circadian neurons using a combination of tissue-specific GAL4 and GAL80 drivers to drive UAS-*per*. *per* overexpression was driven by either the *tim*-GAL4 driver (*tg4-per*) or the *pdf*-GAL4 driver (*pg4-per*). *tim*-GAL4 was also combined with the *pdf*-GAL80 (*tg4-pg80-per*) and *cry*-GAL80 (*tg4-cg80-per*) repressive transgenes (see the Material and Methods section for details). Note that overexpression of *per* in the PDF positive LNvs is neither sufficient, nor required for rhythmicity under constant light.  

**C.** Percentage of rhythmic flies for each genotype shown in (B), and for their controls (the driver and repressor transgenes without uas-*per*). The average period is indicated above the bars for the robustly rhythmic genotypes. See also Table I for more details.
The LNv and LNd neurons do not show molecular circadian oscillations in LL when per is overexpressed. The LNv and LNd neurons were immunostained with anti-PDP1 (red) in the adult brain of flies overexpressing per (per O/E, genotype: yw;tim-GAL4/++; UAS-per/+), and control (yw) flies. Anti-PDF (green) was used to localize the PDF (+) small and large LNvs.

A. Expression of PDP1 under a Light:Dark cycle (LD). PDP1 protein is highly concentrated in the nuclei of the small LNvs (s), the large LNvs (L) and the LNds at ZT21 in adult brains of control flies. As expected, PDP1 (+) cells are not detected at ZT10. A similar pattern of staining was observed in adult brains of flies that overexpress per (per O/E), suggesting normal circadian oscillations under this light regime. There was a notable exception (indicated with asterisks) in the large LNvs (L), which did not express detectable PDP1 levels at ZT21. The LNvs might be particularly sensitive to PER dosage, which is a repressor of pdp1 transcription.

B. Expression of PDP1 in constant light (LL). Adult brains were dissected during the third day in LL at the predicted peak (CT2) and trough (CT17) of PDP1 abundance, taking into consideration the long circadian period of flies overexpressing PER. PDP1 protein is highly concentrated in the nuclei of the small LNvs (s), the large LNvs (L) and the LNds at both CTs in adult brains of the control as well as the per O/E flies, even though these flies are behaviorally rhythmic. Subjective scoring did not reveal any significant variations of staining intensity in any of the LN subtypes (data not shown).
Figure 3-3: A subset of DN1 neurons show molecular circadian oscillations in LL when per is overexpressed. DN neurons were identified by anti-PDP1 (red) immunostaining in the adult brain of per overexpressing (per O/E, genotype yw; tim-GAL4/+; UAS-per/) and control (yw) flies. Projections coming from the LNvs are stained with anti-PDF (green).

A. Expression of PDP1 in the DNs under a Light:Dark cycle (LD). PDP1 protein is highly concentrated in the nuclei of the DN1, the 2 DN2 and the DN3 neurons in both control and per O/E brains at ZT21. As expected, PDP1 (+) cells are not detected at ZT10.

B. Expression of PDP1 in the DNs in constant light (LL). PDP1 protein is highly concentrated in the nuclei of the 2 DN2 and the DN3 neurons at all CTs tested for both the control and the per O/E brains. As shown in the quantification to the right of the confocal sections, about 15 DN1s are PDP1 (+) at the two time points tested in the control brains (Number of brain hemispheres quantified: 4 for CT2 and 4 for CT17). On the other hand, per O/E brains show robust PDP1 oscillation in a subgroup of DN1 neurons with a peak at CT2, which is the predicted peak of PDP1 expression for per overexpressing flies (Number of brain hemispheres quantified: 8 for CT21, 11 for CT2, 8 for CT10 and 14 for CT17). Student’s t tests show that the differences between the number of PDP1 positive cells at CT2 and the other time points is statistically significant (P < 10^{-4}, indicated with an asterisk), while there are no statistically significant differences between the other time points. This strongly suggests that this group of oscillating DN1s is responsible for the rhythmic behavior observed in LL. Subjective scoring did not reveal any significant variations of staining intensity in PDP1 positive DNs (data not shown).
Figure 3-4: LL rhythmicity is dependent on the DN1s in flies overexpressing morgue, but does not require PDF.

A. morgue was overexpressed with the same combinations of transgenes described in figure 1. As with per overexpression, the PDF cells are not the cells critical for LL rhythmicity. The average period is given for the genotypes that were robustly rhythmic. See also Table I for more details.

B. The LNv, LNd and DN neurons were immunostained with anti-PER (red) in the adult brain of flies overexpressing morgue (genotype: yw;tim-GAL4/UAS-morgue) in constant light (LL). Anti-PDF (green) was used to localize the PDF (+) small and large LNvs. Adult brains were dissected during the third day in LL at the predicted peak (CT6) and trough (CT17) of PER nuclear abundance, taking into consideration the long circadian period of these flies. PER protein is highly concentrated in the nuclei of the small LNvs (sLNvs), the large LNvs (LLNvs), the 5th PDF(-)LNv (*), the LNds, the 2 DN2s and the DN3s at both CTs. Staining intensity does not show significant oscillations in these cell groups (data not shown). However, as shown in the quantification to the right of the confocal sections, morgue overexpressing brains show robust PER oscillation in a subgroup of DN1 neurons with a peak at CT6 (Number of brain hemispheres quantified: 8 at CT6, 5 at CT17). The difference in the number of PER positive DN1s between the two time points is statistically significant (asterisk, P < 0.005).

C. Average double-plotted actograms of flies overexpressing morgue with the tim-gal4 driver (genotype yw; tim-GAL4/UAS-morgue; +, n=16), flies overexpressing morgue with the tim-gal4 driver in a pdf01 background (genotype yw; tim-GAL4/UAS-morgue; pdf01, n=28) and control flies (genotype yw; UAS-morgue/+; pdf01, n=8) under LL conditions. The arrowhead indicates when the flies were released in LL.
Figure 3-5: Inhibition of the CRY input pathway allows the DN1s to remain rhythmic in LL.

A. Phase response curve for wild-type flies (Canton-S strain, cs, solid black line) and flies overexpressing morgue with the tim-gal4 driver (genotype yw; tim-GAL4/UAS-morgue; +, solid red line). For comparative purposes, the response of cry<sup>m</sup> flies (adapted from Busza et al. 2004, dashed line) has been included. 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 Zeitgeber Time (ZT) of the light pulse. Data are averages of three independent experiments; Standard Deviations between experiments are shown.

B. Average double-plotted actograms of cry<sup>b</sup> flies in which cry expression was rescued either with the pdf-gal4 driver (genotype yw; pdf-GAL4/UAS-cry; cry<sup>b</sup>, n=13) or the tim-gal4 driver (genotype yw; tim-GAL4/UAS-cry; cry<sup>b</sup>, n=15) under LL conditions. The arrowhead indicates when the flies were released in LL.

C. Expression of PDP1 in the DNs of LNv-rescued cry<sup>b</sup> in constant light (LL) at CT10 and CT21. DN neurons were identified by anti-PDP1 (red) immunostaining in the adult brain of cry<sup>b</sup> flies in which cry expression was rescued with the pdf-gal4 driver (genotype yw; pdf-GAL4/UAS-cry; cry<sup>b</sup>). Projections coming from the LNvs are stained with anti-PDF (green). As shown in the quantification on the right of the confocal sections we observed robust coherent PDP1 oscillations in ca. 6-7 DN1s (Number of brain hemispheres quantified: 7 for both time points). Student’s t tests show that the difference between the number of PDP1 positive cells between the two time points is statistically highly significant (P<10<sup>-4</sup>, indicated with an asterisk). Only the DN1s showed coherent circadian oscillations (see Table S-III). This indicates that this group of circadian neuron is responsible for the rhythmic behavior observed in LL.
Figure 3-6: Genetic manipulations of PER expression in the LNvs.
Adult fly brains were immunostained using anti-PER (red) and anti-PDF (green). Control flies show normal PER oscillations in LD, with a peak of nuclear localization at ZT1 and a trough at ZT13 in both small (s) and large (L) LNvs. When PER is overexpressed using tim-GAL4 (tg4-per), PER is highly concentrated in both the nucleus and cytoplasm of both s- and l-LNvs at both time points. When PER overexpression is repressed in the PDF (+) cells with pdf-GAL80 (tg4-pg80-per), these neurons show normal PER oscillations as in control flies.
Figure 3-7: Effects of *morgue* tissue-specific overexpression in PRC.

Phase response curve at ZT15 and ZT21 for flies overexpressing *morgue* in all clock cells (*timGAL4*), in PDF (+) cells alone (*pdfGAL4*) and in PDF (-) clock neurons (*timGAL4/pdfGAL80*). Flies were entrained to 12:12 h LD cycles, and then released into DD. During the last night of LD, flies were light pulsed at ZT15 or ZT21, and the phase of the behavior was analyzed in DD. A robust decrease in phase delay (ZT15) was observed when *morgue* was overexpressed in PDF (+) cells alone, comparable to that of *cryb* mutants. P values were calculated using Student’s test.
CHAPTER IV

FINAL THOUGHTS

A. General discussion

Understanding the genetic and neuronal basis of complex behaviors is one of the utmost challenges in neurobiology. Circadian rhythms offer an ideal model for the study of these aspects of behavior. The main circadian oscillator in mammals, the suprachiasmatic nucleus (SCN), is composed of about 20,000 neurons. Drosophila can achieve comparable levels of fine circadian regulation with a little over 100 neurons that express clock genes. The simplicity and efficiency of the fly brain combined with a tremendous arsenal of genetic tools, makes Drosophila an invaluable model system for the study of the molecular and cellular aspects of circadian rhythms. In the past few years, research in Drosophila has revealed important aspects of the neuronal circuits behind the hands of the circadian clock. We know the location of clock neurons in the brain, their neuronal projections, and the pattern of expression of clock genes. Drosophila is a crepuscular animal, showing a surge of activity at dawn (M-peak)
followed by a surge of activity at dusk (E-peak). Different clusters of circadian neurons drive these two peaks of locomotor activity. The PDF (+) vLNs (most likely the small) drive the M-peak (consequently called M-cells), while the E-cells (LNds, and possibly a subset of DNs) drive the E-peak (Grima et al., 2004) (Stoleru et al., 2004). The M-cells are also important in driving rhythmic behavior under constant darkness. Moreover, the M-cells appear to be the main oscillator in the fly brain, since manipulations of the circadian period in this cluster alone can alter the period of other clock cells (Stoleru et al., 2004).

How does light entrain the circadian clock? What are the components of the light input pathway into the clock besides CRY and TIM? How do these components interact to transfer the resetting information? Do different groups of circadian neurons react to light differentially? How do these different neurons functionally interact with each other in order to generate a rhythmic behavior? This dissertation investigates some of these questions and the work presented here adds to a growing body of research aimed at elucidating these aspects of circadian photoreception and functional circadian neuronal circuitry.

Chapter II describes a screen aimed at isolating new components of the CRY light input pathway, by looking for genes that, when overexpressed, result in a rhythmic behavior in constant light conditions. The logic of the screen was based on previous observations that a severely hypomorphic mutation in cry
(which encodes the main circadian photoreceptor) renders flies rhythmic in
constant light (Emery et al., 2000b). A number of candidate genes have been
isolated in the screen, which display a variety of biological functions. These
include genes involved in protein turnover, transcriptional regulation, chromatin
modification and signal transduction. Surprisingly, the screen also isolated a
pacemaker gene: slimb. Further analysis of these candidate genes might reveal
new components of the light input pathway, as well as new core clock genes and
possibly give insight into clock output pathways.

The observation that overexpression of a pacemaker gene \( (slimb) \)
produced a rhythmic behavior in constant light raised a question: will
overexpression of other pacemaker genes do the same? To address this
question, we decided to overexpress \( per \). When \( per \) was overexpressed we
observed a strikingly similar phenotype to that observed with the candidate
genes isolated in the LL screen: flies were rhythmic in constant light, with a
similar periodicity. This observation raised many questions. Why does
overexpression of a pacemaker gene make flies almost blind to the disruptive
effects of constant light? What is the molecular mechanism behind this
response? Which pacemaker cells are responsible for driving these rhythms? Is
this response mediated by the M-cells, which are responsible for driving
rhythmicity in constant dark? Is this response PDF-mediated?

Chapter III describes our efforts at trying to answer these questions. We
focused on understanding which subset of circadian neurons drive these
rhythms, by manipulating *per* expression in the different known groups of circadian cells and looking at molecular oscillations in each group.

The work described in Chapter III underscores the role of a subgroup of dorsal neurons (DN1s) in driving circadian behavior under specific conditions (constant light, overexpression of PER and MORGUE). These DN1s possibly overlap with those that control the evening peak of activity (E-cells). The ability of these DN1s to drive rhythms in LL is independent of the M-cells and of their neuropeptide PDF. This important finding adds to a growing body of work in the field that focuses on understanding how the M-oscillator and the E-oscillator interact with each other to produce daily rhythms. Furthermore, how these two oscillators cross-talk in order to adapt to seasonal changes in day length is also a current question of interest.

Shortly after the work presented in Chapter III was published, colleagues in the field found strikingly similar results when *sgg* was overexpressed (Stoleru et al., 2007). Stoleru and colleagues started their studies based on previous observations that overexpression of kinase-encoding gene *sgg* in clock cells speeds up the pace of the intracellular oscillations (Martinek et al., 2001). Speeding-up the M-clock with SGG resulted in faster E-clocks and shorter periodicity, while overexpression of SGG in the E-cells alone did not change the intrinsic pace of the M-clock. This indicates that the M-clock controls the network, at least under constant darkness conditions (Stoleru et al., 2007).
When tested in constant light, a subgroup of DN1s (E-cells) took over the system and was responsible of driving rhythmicity, replicating our observations with per and morgue overexpression (Stoleru et al., 2007) (Murad et al., 2007).

Both sets of data suggest that the two oscillators that control overall rhythms in Drosophila (M- and E-clock) are able to take turns at dominating the system depending on the length of the light phase, suggesting a mechanism for seasonal adaptation. Stoleru et al. further demonstrates this by analyzing the alternating dominance of each clock in artificial long and short photoperiods, designed to mimic the changes of day length in the summer and winter.

The specific molecular mechanisms by which overexpression of PER and MORGUE make the clock partially blind to constant light and allow the E-cells to freely drive rhythms in constant light remain to be understood.

In sum, the work presented in this dissertation has the potential to identify novel components of the light-input pathway, as well as clock components. The identification of these components is important for understanding the steps that follow light-induced CRY and TIM degradation, which functions to synchronize the circadian clock to the surrounding environment. This work also highlights the function of a subgroup of DN1s in controlling circadian behavior in constant light, and suggests a role for these pacemaker cells in adaptation to seasonal changes. These DN1s might overlap with those controlling the E-peak of activity, although that is a question that remains to be answered.
B. Future directions

As for the LL screen, further validation and characterization of the candidates that have been isolated is needed in order to unveil a role for them in the light input pathway to the circadian clock, or in the clock itself. Two comprehensive RNAi collections are available now through the Japanese NIG and the Austrian VDRC. This will allow the study of loss-of-function mutations in the candidate genes and their effect in rhythmic behavior. Also, expression studies (such as in situ hybridization) will reveal if these genes generate transcripts in clock cells. Dr. Raphaelle Dubruille is performing these studies in the Laboratory of Patrick Emery.

Although we speculate that the DN1s that are able to drive rhythms in LL overlap with the E-cells, this subject remains unclear. This is due in part to the fact that we do not have a specific GAL4 driver for DN1s, or for a subgroup of them. DNs appear to be a heterogeneous group of cells. For example, only a subset of them appears to express glass (Klarsfeld et al., 2004). In addition, it remains unclear exactly which cells form the E-oscillator. It is suggested that the LNds, together with possibly some DN1s and a small vLN that does not express PDF form the E-oscillator, but there are some discrepancies regarding this issue. It will be necessary to first unambiguously identify the E-cells and then see
whether or not the DN1s that oscillate in LL overlap. This is a difficult task that will require the development of new tools to be able to manipulate gene expression in DN1s.

Expanding our knowledge on how the Drosophila circadian clock entrains to light and how the circadian neuronal network wires in order to control this complex behavior will add to our understanding on how complex organisms adapt to changes in the environment. Drosophila has been extensively used as a model system to study genetic basis of behavior, from circadian rhythms to learning and memory, and more recently sleep. The interactions and contributions of genes to the behavior of Drosophila can provide important insights in the role of genes in human behavior.
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APPENDIX I

Interactions between circadian neurons control temperature synchronization of *Drosophila* behavior

Ania Busza, Alejandro Murad and Patrick Emery

This appendix is reprinted from an article published in The Journal of Neuroscience in October, 2007. This work is the result of a collaboration between Ania Busza, Patrick Emery and myself. For this study, I performed the immunohistochemistry experiments for the staining of clock neurons in temperature cycles.

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Interactions between Circadian Neurons Control Temperature Synchronization of Drosophila Behavior

Ania Busza, Alejandro Murad, and Patrick Emery

Introduction

Like most organisms, 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 are identified. The products of the period (per), timeless (tim), clock (clk), and cycle genes form a transcriptional feedback loop; a set of kinases and phosphatases adjusts the period of this oscillator to 24 h (Hardin, 2005). Intracellular signaling through the photoreceptor CRYPTOCHROME (CRY) and synaptic input from visual organs synchronize the circadian clock to the light cycle (Helfrich-Forster et al., 2001). Drosophila exhibit a bimodal locomotor activity pattern under a light/dark (LD) cycle, with morning (M) and evening (E) surges of activity. Specific circadian neurons contribute to these peaks of activity (Grima et al., 2004; Stoleru et al., 2007). Under constant darkness (DD), the LNvs are necessary and sufficient for behavioral rhythms (Renn et al., 1999; Grima et al., 2004) and dictate their period (Stoleru et al., 2005). However, genetic studies revealed that, under constant light (LL), the E cells and a subset of DN1s can also 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 cycles when these cues are simultaneously present (Miyasako et al., 2007).

Most of the work on how Drosophila synchronizes its clock to environmental cycles has centered on light input pathways. How-
ever, temperature fluctuations can also reset circadian clocks. Temperature can dominate light input in *Neurospora* (Liu et al., 1998). In mammals, temperature cycles mimicking body temperature fluctuations help keep peripheral clocks synchronized (Brown et al., 2002). In *Drosophila*, temperature cycles synchronize eclosion rhythms (Pittendrigh, 1954), locomotor activity rhythms (Wheeler et al., 1993; Yoshii et al., 2002, 2005; Glaser and Stanewsky, 2005), and molecular oscillations in peripheral tissues (Glaser and Stanewsky, 2005) and brain neurons (Yoshii et al., 2005). To understand further how temperature synchronizes *Drosophila* behavior, we studied the contribution of different circadian neurons and the intercellular network connecting them to thermal entrainment.

**Materials and Methods**

*Drosophila strains and transgenics.* Flies with targeted neuronal ablation of the M cells or both the M and E cells were obtained as described previously (Renn et al., 1999; Stoleru et al., 2004) from the following stable stocks: y w; UAS-hid/CyO;_b_ plus flies, y w; pdf-GAL4;_b_; and y w;_b_; cry-GALA–13/TM6b. The pdfo_0_ mutant flies were described previously (Renn et al., 1999). For neuronal ablation in per mutants, UAS-hid was introduced into perE_0_; perE_0_; and perE_0_; backgrounds, and these lines were then crossed to y w; pdf-GAL4;_b_; or y w;_b_; cry-GALA–13/TM6b flies. perE_0_ flies were rescued by expressing per with the pdf-GAL4 driver as described previously (Grima et al., 2004), as well as with cry-GALA–13 and a cry-GALA–13/pdf-GAL80 combination (Stoleru et al., 2004). To make y w; pdf-GAL4;_b_; UAS-per/; flies, we crossed y w; pdf-GAL4;_b_; UAS-per; males with perO_0_ w;_b_; UAS-per/; females (Grima et al., 2004). CB104 heterozygote flies were made by crossing y w females with ;_b_;; TM2 w; male flies (Allada et al., 1998).

**Behavioral assays and analysis.** To record daily locomotor activity, individual male flies (1–6 d old) were placed into *Drosophila* activity monitors (Trikinetics, Waltham MA). All experiments were done in Percival I-36L incubators (Percival Scientific, Perry IA). The thermophase/ cryophase (TC) cycles were performed at a temperature of 29°C for the thermophase and 20°C for the cryophase. Temperature during runs was monitored with a Fluke SII 53 digital thermometer. Shifting temperature from 20°C to 29°C took _~_30 min in our incubators. Once the system had reached the correct temperature, it remained stable within ±0.4°C. A light intensity of _~_150–200 lux was used for LD cycles and for one of the LL experiments. For the other (LL) experiment, some light bulbs were turned off in the incubator to obtain a light intensity of _~_75 lux. For each experiment, details on the number of days in LD, TC, and constant conditions (CC) (dark, 20°C) are explained in the text or in the figure legends.

Behavioral data collected with the *Drosophila* Activity Monitoring program (Trikinetics) was analyzed with FaasX (courtesy of F. Rouyer, Centre National de la Recherche Scientifique, Gif-sur-Yvette, France) or a signal processing toolbox for Matlab (MathWorks, Natick, MA) (courtesy of J. Levine, University of Toronto, Mississauga, Ontario, Canada). All actograms are group averages plotted using Matlab "dam_panels" function (Levine et al., 2002). Histograms are group averages plotted using Matlab "dam_panels" function (see Fig. 7) (supplemental Fig. S5, available at www.jneurosci.org as supplemental material) or are group average eductions using FaasX (see Figs. 4, 6).

To determine the phase of the daily evening peak before, after, or during temperature cycles (see Figs. 1B, 8A, B) (supplemental Fig. S1, available at www.jneurosci.org as supplemental material), we analyzed all flies that had survived the entire run. Phase was calculated for each individual fly for each day in Matlab with the "peakphaseplot" function (using an 8 h Butterworth filter and manual removal of non-evening peaks), and the group mean and SE was calculated and plotted in Excel (Microsoft, Seattle, WA).

For experiments measuring the phase shift of TC-entrained circadian behavior after release into CC (Figs. 1C, 2, 8C) (supplemental Fig. S3, available at www.jneurosci.org as supplemental material), we calculated the phase of the evening peak on the second day after release in CC for each experimental group. To calculate relative phase shift of the TC-exposed group of flies, mean phase of the no-TC control group was subtracted from mean phase of the TC-exposed group, and the combined SE of the two groups of flies was calculated. Arrhythmic flies were excluded from all phase-shift analysis except in Figure 8C, for which all flies with a clear evening peak on the second day were used. *Immunohistochemistry.* Brain immunostainings for PER and PDF were performed as described by Murad et al. (2007).

**Results**

**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 DD. These conditions allow us to study specifically temperature synchronization without the confounding effects of light input and also to monitor the impact of temperature cycles on the circadian pacemaker underlying rhythmic behavior. This cannot be done under 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 attributable 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 persist after temperature entrainment. Using 12 h/12 h 29°C/20°C thermophase/cryophase temperature cycles in DD, we phase-advanced or phase-delayed wild-type flies (Fig. 1A) that had been previously synchronized to an 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 (for example, Fig. 1A, right) but was usually of much smaller amplitude than that observed in an LD cycle and sometimes not detectable. This might be because morning activity is suppressed at lower temperatures, as shown previously in LD studies at different constant temperatures (Miyasako et al., 2007). Therefore, to begin our analysis of the phase-shifting effects of TC cycles on circadian behavior, we measured each fly’s daily evening peak and compared it with the evening peaks of control flies left in constant conditions (20°C DD) (Fig. 1B). On the last day of LD, the fly’s evening activity peaked at approximately Zeitgeber time 12 (ZT12) (in which ZT0 refers to the lights-on time during LD). The evening activity peak then drifted to approximately ZT11 by the fourth day of DD (day 6). After 5 d of TC, flies exposed to a 9 h advanced TC cycle had evening peaks 8 h earlier than the control flies, showing that their behavior had been re-synchronized. Conversely, flies exposed to a 6 h delay TC cycle had evening peaks 6 h later than controls. All fly groups showed a daily advance when released into constant 20°C DD because our y w wild-type flies have a period slightly shorter than 24 h 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 cycle is much slower than under an LD cycle.
When it experiences the warm temperature, wild-type flies exhibit a different response to a temperature cycle depending on the current temperature, the peak of activity in period rhythms. This probably explains why, on release to constant temperature, flies that have been exposed to 12 h/12 h 29°C/20°C TC cycles that were either advanced by 9 h (left; n = 16) or delayed by 6 h (right; n = 12) compared with the LD cycle. After 5 d in TC, the flies were released into CC. The light phase of the LD cycle is represented in white, and the dark phase is in gray. The warm phase of the TC cycle is shaded in orange, and the cold phase is in gray. The orange shading indicates the days during which the flies were exposed to the TC. When the flies were returned to constant conditions, the orange shading indicates the days during which the flies were exposed to the TC.

Figure 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 2 d of 12 h/12 h LD cycles at 20°C and then released into CC (darkness at 20°C) for 4 d. The flies were then exposed to 12 h/12 h 29°C/20°C TC cycles that were either advanced by 9 h (left; n = 16) or delayed by 6 h (right; n = 12) compared with the LD cycle. After 5 d in TC, the flies were released into CC. The light phase of the LD cycle is represented in white, and the dark phase is in gray. The warm phase of the TC cycle is shaded in orange, and the cold phase is in gray. The orange shading indicates the days during which the flies were exposed to the TC. 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 lights-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 (20°C 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 the TC. C, To determine the effect of TC cycles on the phase of the circadian oscillator underlying circadian behavior, the phase of the free-running behavior was determined for wild-type flies (y w) after 1, 2, 3, or 4 d of exposure to an 8 h advanced TC (x-axis) and compared with the phase of flies left under constant conditions (for details, see Materials and Methods). The phase difference (y-axis) represents the magnitude of the phase shift induced by the TC on the endogenous circadian oscillator (number of rhythmic flies ranged from 10 to 13 per fly group; rhythmicity ranged from 73 to 93%).

We observed slow displacement of the evening peak between day 1 and day 6 (transients) (supplemental Fig. S1 E, available at www.jneurosci.org as supplemental material). Wondering whether the phase of this peak reflects the state of synchronization of the underlying circadian pacemaker, we entrained flies for 1, 2, 3, or 4 d to an 8 h advanced TC cycle and then released them in constant temperature to determine the phase of their free-running behavior (Fig. 1C). We also observed a progressive phase shift with increasing numbers of temperature cycles, similar to that observed with the evening peak under TC cycles (Fig. 1 B) (supplemental Fig. S1 E, available at www.jneurosci.org as supplemental material). Thus, the evening peak is a close reflection of the state of the underlying oscillator under TC cycles.

One more feature of the results shown in Figure 1 should be noted. On return to constant temperature, flies that have been advanced or delayed by temperature cycles shift their behavioral phase by 2–3 h toward the subjective morning. We presume that this phase change is attributable to the temperature-dependent per splicing that advances behavioral phase at 20°C and delays it at 29°C (it would thus do so during temperature entrainment) (Majercak et al., 1999). In addition, our y w flies show short period rhythms. This probably explains why, on release to constant temperature, the peak of activity in y w flies is shifted toward the early part of the day (see Figs. 1, 3, 6).

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, 1960). For example, flies respond to short light pulses by delaying their clock after 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 whether 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 h 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) (Fig. 2 A). Maximum phase shifts were elicited when the 29°C exposure began in late subjective day (delay of 3 h at ZT9 and ZT11) and in the mid/late subjective night (advance of 2–2.5 h at ZT17 and ZT19). Strikingly, 12 h, 29°C exposure beginning at ZT13 versus ZT15 elicits very different phase shifts (2 h delay and 1 h advance, respectively), despite 10 h of thermophase overlap. The 29°C exposure starting early in the subjective day (e.g., ZT1) elicited almost no phase shift, presumably because it coincided with the time of day when the animal expects its environment to become warmer. A 6 h pulse PRC was also generated and showed a similar shape with greater responses from pulses initiated at ZT11 and ZT19 (Fig. 2 B), although the amplitude of the delay responses was smaller than with a 12 h 29°C pulse. Previous studies on Drosophila pseudoobscura eclosion rhythms show a similar phase-shifting curve in response to 12 h at 28°C, 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
them to only a single 6-h-long 29°C pulse. Both advance and response at ZT19, but, at ZT23, mutant flies (Fig. 2).

Figure 2. Phase response curve to 12 and 6 h 29°C warm pulses.

We therefore decided to further challenge (Stanewsky et al., 1998; Kaushik et al., 2007) (data not shown).

It has been shown recently that CRY binds to the PER/TIM dimer to mediate specific behavioral responses to temperature, such as the phase-shifting effects of a 1 h high-temperature heat pulse (37°C) (Edery et al., 1994; Kaushik et al., 2007). Under 29°C/20°C TC cycles, we did not observe any obvious entrainment defects in cryb mutant flies, although these flies have no functional CRY and do not respond to 37°C heat pulses (Stanewsky et al., 1998; Kaushik et al., 2007) (data not shown). We therefore decided to further challenge cryb flies by exposing them to only a single 6-h-long 29°C pulse. Both advance and delay responses to these single pulses were preserved in cryb mutant flies (Fig. 2B). We noticed a reduction in the advance response at ZT19, but, at ZT23, cryb flies strongly phase advanced their clock. This result and previous molecular studies (Stanewsky et al., 1998; Glaser and Stanewsky, 2005) show that CRY is not essential for temperature entrainment of circadian rhythms at moderate temperature (18–29°C) range. This suggests that, besides CRY, the PER/TIM dimer, proposed to be thermosensitive (Kaushik et al., 2007), can bind other molecules in a temperature-dependent manner to synchronize circadian rhythms with moderate temperature cycles. Higher temperature might either promote these putative interactions (as shown for CRY) or, on the contrary, destabilize complexes formed at low temperature.

The PDF-positive M cells are necessary for persistence of temperature-synchronized circadian behavior

Under an LD cycle, PDF-positive (PDF+) LNvs are primarily responsible for the anticipatory behavior of flies at dawn and are thus called M cells, whereas a set of more dorsally located E cells are primarily responsible for the evening activity (Grima et al., 2004; Stoleru et al., 2004). In addition, the M cells maintain circadian rhythms in constant darkness (Renn et al., 1999; Grima et al., 2004). We used genetic techniques to ablate specific subsets of circadian neurons or rescue their clock function in an arhythmic genetic background to determine the respective function of these groups of cells under dark TC cycles. To study the role of the PDF+ cells, or M cells, we first ablated the LNvs by driving the proapoptotic gene hid (head involution defective) in these neurons with the pdf-GAL4 driver (Fig. 3B) (Renn et al., 1999). A brief surge of activity at the beginning of the thermophase was observed. Because it was present in all genotypes, even those without a functional clock, it is a noncircadian response to the temperature transition. 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 under TC conditions because, 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 on return to constant temperature (Fig. 3B). Thus, the M cells are critical for long-term behavioral rhythmicity after temperature synchronization. Because pdf+ flies have the same phenotype as flies without M cells (Fig. 3C), the PDF neuropeptide is required for the function of the M cells under TC cycles.

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-driven circadian behavioral rhythms, we rescued PER expression only in these cells in per0 flies (Grima et al., 2004) (Fig. 3E). The midday peak seen in these mosaic flies is also seen in per0 flies (Yoshii et al., 2002) (Fig. 3D). It is thus not generated by the rescued M cells but is actually caused by the improper activity of the E cells in the middle of the day because this peak is absent in flies without E cells (see below and Fig. 7). M-cell-rescued per0 flies were able to remain rhythmic under constant dark after TC (Fig. 3E), whereas the per0 control flies could not (Fig. 3D). Notably, the activity was clearly resynchronized 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, re-
mains to be determined. We noted that the persistent activity after synchronization to a TC cycle was concentrated in the subjective morning, as observed after an LD cycle (Grima et al., 2004). This suggests that, similarly to what has been observed during or after an LD cycle, the M cells are mostly generating morning activity after exposure to TC cycles. Thus, the difficulty of detecting the anticipatory morning behavior under TC may be attributable to an inhibitory effect of the colder temperature (negative masking) (Miyasako et al., 2007) or possibly to the onset of this peak being slightly later in TC than in LD and thus positively masked by the surge of activity at the beginning of the thermophase.

The evening activity 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, in DD, this peak persists for ~2 d (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 (supplemental Fig. S3, available at www.jneurosci.org as supplemental material), 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: per0, per5, and perL (Konopka and Benzer, 1971). 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 h 29°C, 6 h 20°C). The longer thermophase prevents the peak of activity of perL flies from occurring in the cryophase and being suppressed by cold phase negative masking, as it would in a standard 12 h/12 h TC. Indeed, a peak is present in all three genotypes during the 18-h-long thermophase (Fig. 4). Its location relative to the temperature cycle is earlier in per5 than in wild-type flies but later in perL, demonstrating that the evening peak is gated by a per-dependent time-keeping mechanism. A peak of activity is also observed in per0 under this particular TC cycle, as well as under 12 h/12 h TC (Fig. 3D) as described previously (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 perL. It is eliminated when the E cells are ablated (see Fig. 7). 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 after TC entrainment to determine which circadian neurons were ablated (Fig. 5A). As described previously (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 likely that at least two DN1s are ablated. The LPNs were not ablated. As expected, the LNvs were missing.
Most LNds were missing, although we cannot entirely exclude that a subset of LNds escape ablation (the LNds are close to the DN3 groups, and one or two residual LNds could thus be mistaken for DN3 cells). Importantly, behavioral data verify that the evening peak is already completely missing during LD (Fig. 6B), which means that all E cells have been eliminated. The cry-GALA4/UAS-hid flies showed no morning or evening peak of activity in TC, other than the brief startle response after the temperature increase (Fig. 6B). 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 flies in the M and E cells with the cry-GALA4 and UAS-per transgenes. We determined in which cells PER was expressed and found, as expected from Stoleru et al. (2004), that the LNvs showed robust rescued PER oscillations under TC (Fig. 5B). We also found that PER expression was rescued in three to four LNds and two DN1s and that it cycled robustly. This pattern of PER expression was sufficient to fully rescue LD behavior: both the M and the E peak looked similar to wild type (Fig. 6A, D). In addition, like PER rescue in only the M cells, cry-GALA driven PER rescue restored rhythmicity in constant conditions after both LD and TC cycle. The circadian pacemaker thus functions normally in the M and E cells of these rescued flies, as suggested by the strong molecular oscillations observed by immunohistochemistry. However, during TC, per flies with rescued M and E cells 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 (Fig. 6, compare A, C, D). Thus, in TC as in LD, the E cells play an important role in controlling the evening activity peak. As expected, rescuing per expression only in the E cells restored a similar evening peak (Fig. 6E), although for unclear reasons it appeared sharper than in flies with both the M and E cells rescued. As expected from the M cell ablation experiment, this peak did not persist after release in 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.

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; cry-GALA4/UAS-per flies), circadian behavior is not normal in phase and duration under TC (Fig. 6D). More specifically, under TC, the evening peak begins earlier and lasts longer, as if the network regulating activity is not well tuned. This abnormal behavior is not attributable to abnormal pacemaker function, because robust PER oscillations and normal LD and DD behaviors are observed in M- and E-cell-rescued per flies (Figs. 5B, 6D). This suggests the intriguing possibility that, in wild-type flies, additional circadian neurons may contribute to regulating locomotor behavior specifically under TC.

We therefore reexamined our cry-GALA4/UAS-hid fly data and found that a small increase of activity could be detected in some of our experiments at the very end of the 12 h thermoperiod. However, it was difficult to distinguish this slight increase in activity from the relatively high masking activity seen when both the E and M cells are ablated (data not shown). To better visualize any residual evening activity in M and E cell ablated flies, we used the long thermoperiod/short cryoperiod assay to avoid the masking effects of cold temperature after ZT12. Interestingly, we con-
sistently observed an evening peak of small amplitude in flies without M and E cells under 18 h/6 h TC, but this peak was not present in LD with an 18-h-long photoperiod (Fig. 7D,E). Importantly, a similar peak was seen in 18 h/6 h TC conditions under constant light (Fig. 7F), which demonstrates that the absence of evening activity in LD is not attributable to negative light masking. To determine whether the evening peak is of circadian nature, we monitored per mutant flies without M and E cells under 18 h/6 h TC conditions (Fig. 7) (supplemental Fig. S5, available at www.jneurosci.org as supplemental material). The evening peak is more apparent in the per^S and per^E backgrounds than in wild type. Its timing is earlier in the per^S background and later in per^E, whereas it is absent in per^0 flies. There is again no sign of evening activity in LD in any of these genotypes. Together, these data provide strong evidence that circadian neurons specifically sensitive to temperature contribute to late evening activity.

The M cells modulate the response of the E cells to temperature cycles
Having demonstrated that, at physiological temperature ranges, TC cycles phase shift circadian rhythmicity much more slowly than LD cycles (supplemental Fig. S2, available at www.jneurosci.org as supplemental material), 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, because we have shown that this peak is controlled by the circadian clock and is not attributable to a masking phenomenon. Unexpectedly, this activity peak resynchronized very rapidly to TC cycles when the M cells were absent (Fig. 8A). After one day, the phase of the evening peak was already strongly shifted, although it varied significantly from individual to individual (Fig. 8A, error bars), and its amplitude was smaller than that observed after 2 or more days (data not shown). After 2 d, virtually all flies were perfectly and fully synchronized. 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, because flies without this neuropeptide also rapidly synchronize to temperature cycles (Fig. 8A) (supplemental Fig. S3, available at www.jneurosci.org as supplemental material).

Altering the oscillator in the M cells leads to abnormally fast entrainment to temperature cycles
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. To test this hypothesis further, we exposed y w; pdf-GAL4/UAS-per flies to temperature cycles that were 8 h advanced relative to the LD entrainment and measured their evening peak during TC. Strikingly, synchronization to temperature cycle was considerably accelerated in these flies, which overexpress PER only in the LNvs and are otherwise wild type (Fig. 8B). This confirms that the LNvs are the cells determining the rate of synchronization to temperature, slowing 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. Excessive PER levels are known to be disruptive to circadian rhythms (Zeng et al., 1994; Kaneko et al., 2000), and a weaker oscillator is predicted to be more responsive to environmental perturbation (Pittendrigh et al., 1991). We observed that y w; pdf-GAL4/UAS-per flies have a 1 h longer period phenotype (24.8 ± 0.1) compared with control flies (y w, 23.8 ± 0.2), suggesting that the M cell oscillator is indeed altered. To investigate further whether oscillators with attenuated molecular oscillations are more vulnerable to temperature entrainment, we measured the rate of entrainment of Clk^Kr^+ heterozygotes, because these mutants have decreased amplitude of oscillations in per and tim transcription and PER
and TIM protein cycling (Allada et al., 1998). We noticed that ClkJrk/H11001 heterozygote flies have higher activity during the cryophase than control flies (data not shown). This increase in activity partially masked the evening peak during TC entrainment. Therefore, instead of determining the phase of the evening peak during TC, we measured the phase of the free-running rhythms after 1, 2, 3, or 4 d of temperature synchronization. We found that, similarly to flies overexpressing PER in the LNvs, the ClkJrk/H11001 heterozygotes had completely entrained to the TC cycle after 2 d (Fig. 8C). They even overreacted to the TC cycle, particularly on the second and third days of entrainment. Combined, our results indicate that robust molecular oscillations in the M cells protect Drosophila from reacting excessively to temperature cycles.

**Discussion**

Circadian rhythms are generated by cell-autonomous molecular pacemakers (Dunlap, 1999). In Drosophila, even circadian environmental inputs can be detected cell autonomously. Light is detected by the intracellular photoreceptor CRY in brain neurons and peripheral tissues (Emery et al., 2000). Moreover, dissected peripheral tissues can also detect temperature cycles (Glaser and Stanewsky, 2005), suggesting the existence of a cell-autonomous circadian thermosensor. It was proposed recently that the PER/TIM dimer itself is thermosensitive (Kaushik et al., 2007).

If circadian rhythms can be synchronized and function cell autonomously, why are circadian neurons organized in a network? In Drosophila, the circadian neuronal network appears nec-
necessary to maintain stable circadian rhythms in constant conditions. Indeed, without a proper neural circuitry, individual neurons cannot keep a proper amplitude and phase for their circadian oscillations in the absence of external inputs (Peng et al., 2003; Lin et al., 2004). However, because only a minority of organisms actually experience constant conditions during their lifetime, the importance of this function under natural conditions is unclear. 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 (Inagaki et al., 2007; VanderLeest et al., 2007). In *Drosophila*, this adaptation results from 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 (Fig. 9).

The first interaction involves the aforementioned M and E cells and determines the pace at which circadian behavior is synchronized by temperature input. *Drosophila* behavior responds slowly to temperature cycles. Nevertheless, specific neurons can respond rapidly. The E cells are very rapidly synchronized to TC cycles if they are disconnected from the M cells. It is actually the M cells that (predominantly) set the pace of behavioral synchronization to TC, at least in DD conditions. Indeed, increasing PER levels only in the M cells results in a considerably accelerated synchronization. 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 changes. This is probably important in a natural environment in which *Drosophila* can experience erratic variations in temperature attributable to weather changes. Because the E cells can also influence the M cells (Stoleru et al., 2007), it is likely that their sensitivity to temperature cycles can be used to fine-tune the synchronization of the M cells to the environment, particularly under long photoperiod.

As mentioned above, we found that specifically manipulating the circadian pacemaker of the M cells accelerates synchronization to TC. We increased 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 M cell pacemaker with this manipulation, and the 1 h period lengthening observed is consistent with this notion. Indeed, high PER levels increase transcriptional repression in the circadian molecular feedback loop and can in some cases completely eliminate molecular and behavioral rhythms (Zeng et al., 1994; Kaneko et al., 2000). Moreover, a weakened oscillator is predicted to respond more strongly to environmental input (Pittendrigh et al., 1991). In mammals, mutants with attenuated oscillators have stronger circadian responses to light pulses (Vitaterna et al., 2006). It should be noted that we cannot exclude that acceleration of TC synchronization is attributable at least in part to the temperature sensitivity of the GAL4/UAS system. This sensitivity could create a temperature-induced per mRNA cycling that could contribute to accelerate synchronization. However, this possibility seems unlikely. First, the kinetic of synchronization is accelerated with both advanced and delayed TC cycles (data not shown). It is unlikely that a GAL4/UAS-mediated temperature effect would affect both directions of resynchronization similarly. Second, there is little phase difference in behavior between wild-type and heterozygotes under TC. Because CK(6) 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 (20°C DD) in flies exposed to 1, 2, 3, or 4 d TC. Error bars indicate ± SEM.

Figure 9. Model for the control of behavioral responses to temperature cycles by the circadian neuronal network. We have identified three groups of cells that contribute to behavioral responses to temperature entrainment: the M, E, and temperature-sensitive (TS) cells. Each group is represented by one oscillator-containing cell for simplicity. The three groups are sensitive to temperature, and they interact with each other to properly time circadian behavior in response to temperature cycles. The M cells have a robust pacemaker (shown in bold) that is relatively slow at responding to temperature cycles. Through rhythmic PDF secretion, the M cells slow down the response of the highly sensitive E cells. It is however likely that the E cells can also influence the M cells (dashed arrow), particularly in the presence of light (Stoleru et al., 2007). The combination of highly sensitive E cells and relatively resistant M cells is probably important for the balance between behavioral adaptability to temperature changes and resistance to random variations of temperature. In addition to the M–E cell interactions, the temperature-sensitive cells also interact with the E cells, inhibiting their behavioral output in the middle of the day.

Figure 8. 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 ( yw; dashed line), pdf mutants ( pdf01; solid line with open circles), and M cell ablated flies ( yw; pdf-GAL4/UAS-hid; solid line with filled triangles). Flies were synchronized to 2 d LD and 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 indicate ± SEM. B, Kinetics of TC synchronization in wild-type flies ( yw; dashed line) and flies with PER overexpression only in the M cells ( yw; pdf-GAL4/ UAS-per; solid line) in 4 d of TC (experiment and analysis same as in A). C, Kinetics of TC entrainment in wild-type flies ( yw; black bars) and CK(6) heterozygotes ( yw; +; CK(6)+/+; gray bars). Because CK(6) 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 (20°C DD) in flies exposed to 1, 2, 3, or 4 d TC. x-Axis, Total number of days in TC before release in constant conditions. Error bars indicate ± SEM.
and PER-overexpressing flies once stable entrainment is reached. Flies overexpressing PER only have a slightly delayed phase, which is expected because they have a 1 h longer period, and this would not help advancing the circadian clock under TC. Thus, any effects that the temperature sensitivity of the GAL4/UAS system could have on the phase of PER cycling is most likely superceded by the circadian regulation of PER levels and the circadian synchronization resulting from temperature cycling. In addition, we obtained independent confirmation that flies with an attenuated pacemaker are much more sensitive to temperature. Indeed, flies heterozygous for the ClkJrk mutation also phase shift their clock very rapidly when exposed to a temperature cycle.

Thus, the picture emerges 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 h advanced relative to the light cycle (Miyasako et al., 2007). The PDF-positive LNvs (M cells), and the LNds and the PDF-negative LNv (E cells) followed the light cycle. Our interpretation is however different from that of Miyasako et al. (2007). 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 by Stoleru et al., 2004) are actually highly sensitive to temperature cycles. Second, the M cells also clearly detect and respond to temperature cycles, because they are sufficient for persistent temperature synchronization. Moreover, they determine the response of circadian behavior to temperature cycles.

We also present behavioral evidence that circadian neurons distinct from the M and E cells are involved in the control of circadian behavior specifically when temperature cycles are present. An E cell- and M cell-independent peak was observed under a long thermophase TC cycle. The circadian clock controls this TC-specific peak of activity, because 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 attributable to a negative masking effect of light. Because the LPNs and the DN2a are not ablated in our experiments and are particularly sensitive to TC (Yoshi et al., 2005; Miyasako et al., 2007), they are strong candidates for playing a temperature-specific function in the control of circadian behavior. Although it was initially proposed that the LPNs oscillate only under TC (in LL conditions) (Yoshii et al., 2005), it was recently reported that they also show molecular oscillations in LD (Shafer et al., 2006). Under our conditions, we failed to detect PER cycling in the LPNs in LD (data not shown), which might thus be of low amplitude. However, we detected robust PER cycling under TC (Fig. 5A). The LPNs therefore probably require temperature cycles to exhibit robust molecular oscillations and participate in the control of circadian rhythms.

Interestingly, non-M, non-E temperature-sensitive neurons are also necessary for properly timing the activity of the E cells, and this brings us to the second important circadian neuronal interaction that our study uncovered. In per^w flies, a peak of activity is present in the middle of the day. This peak is caused by improper activity of the E cells, because it is present in per^w 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^w flies, circadian activity is not normal under TC, despite being perfectly rescued in LD. There are 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 TC. 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 TC might be responsible for properly phasing E cell output. The LPNs are strong candidates for playing this dual role. However, it is also possible that two distinct groups of cells are responsible for activation of locomotor behavior under TC cycles.

In conclusion, our results demonstrate that the functional coupling of different groups of circadian 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. 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 (Inagaki et al., 2007; Stoleru et al., 2007; VanderLeest et al., 2007).

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APPENDIX II

PER-TIM interactions with the photoreceptor CRYPTOCHROME mediate circadian temperature responses in Drosophila

Rachna Kaushik, Pipat Nawathean, Ania Busza, Alejandro Murad, Patrick Emery and Michael Rosbash

This appendix is reprinted from an article published in PLoS in June, 2007. This work is the result of a collaboration between our Laboratory and the Rosbash Laboratory. For this study, I worked in collaboration with Myai-Emery-Le in the generation of anti-CRY antibodies and then tested the different antisera and optimized the western blot conditions for its usage.
PER-TIM Interactions with the Photoreceptor Cryptochrome Mediate Circadian Temperature Responses in Drosophila

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Drosophila cryptochrome (CRY) is a key circadian photoreceptor that interacts with the period and timeless proteins (PER and TIM) in a light-dependent manner. We show here that a heat pulse also mediates this interaction, and heat-induced phase shifts are severely reduced in the cryptochrome loss-of-function mutant cry\(\text{\textsuperscript{\textcircled{\textsuperscript{\circ}}}}\). The period mutant per\(\text{\textsuperscript{\textcircled{\textsuperscript{\circ}}}}\) manifests a comparable CRY dependence and dramatically enhanced temperature sensitivity of biochemical interactions and behavioral phase shifting. Remarkably, CRY is also critical for most of the abnormal temperature compensation of per\(\text{\textsuperscript{\textcircled{\textsuperscript{\circ}}}}\) flies, because a per\(\text{\textsuperscript{\textcircled{\textsuperscript{\circ}}}}\); cry\(\text{\textsuperscript{\textcircled{\textsuperscript{\circ}}}}\) strain manifests nearly normal temperature compensation. Finally, light and temperature act together to affect rhythms in wild-type flies. The results indicate a role for CRY in circadian temperature as well as light regulation and suggest that these two features of the external 24-h cycle normally act together to dictate circadian phase.

Introduction

Most organisms have circadian rhythms of gene expression and behavior that are controlled by endogenous clocks. A few studies have verified that these systems increase fitness and help organisms adapt to the physical and ecological environment in which they live [1]. At the molecular level, the central pacemaker of animals is proposed to consist of auto-regulatory feedback loops that regulate the expression of key clock genes [2]. An admittedly simplified view of the Drosophila central clock posits a core system of four interacting regulatory proteins. A circadian cycle begins when a CLOCK (CLK) and CYCLE (CYC) heterodimer activates the expression of two other proteins, PERIOD (PER) and TIMELESS (TIM). PER and TIM levels slowly accumulate over time, and these two proteins also heterodimerize. At some point, PER-TIM complexes enter the nucleus and inactivate CLOCK-CYCLE activity, slowing their own production and signaling the end of a cycle. Importantly, kinases and phosphatases modify PER, TIM, and CLK and play critical roles in circadian rhythms [3–7].

Endogenous periods are usually different from the precise 24-h rotation of Earth. Nonetheless, circadian clocks keep precise 24-h time under normal conditions and are reset every day by environmental signals like light and temperature, which are the dominant entraining cues in nature. In Drosophila, circadian light perception is well-understood, and a major fraction of it is mediated by the circadian photoreceptor molecule cryptochrome (CRY) [2,8]. Cryptochromes are related to photolyases, a family of blue-light-sensitive DNA repair enzymes, and also play important roles in photoreception and circadian rhythms of other animals as well as plants [9,10].

Drosophila CRY is prominently expressed in pacemaker neurons [11–13]. Moreover, a mutant cry strain (cry\(\text{\textsuperscript{\textcircled{\textsuperscript{\circ}}}}\)) manifests severe molecular and behavioral problems. These include a lack of PER and TIM molecular cycling in peripheral tissues under light-dark cycles and an inability to undergo phase resetting in response to short light pulses [14]. cry\(\text{\textsuperscript{\textcircled{\textsuperscript{\circ}}}}\) flies are also rhythmic in constant light, i.e., the characteristic arrhythmicity of Drosophila and many other animals in constant light is absent [15]. Finally, there is strong evidence that CRY contributes to standard entrainment by light-dark cycles [16].

At the biochemical level, photon capture by CRY leads to an interaction with TIM or with the PER-TIM complex [17–20]. CRY also interacts with and blocks the function of the PER-TIM complex in a light-dependent manner in an S2 cell-based assay [17]. The current view is that the CRY:TIM interaction leads to TIM degradation, which results in phase-resetting in response to a light pulse [21–25].

In addition to light, other factors such as social interactions, activity, and especially temperature can modulate free-running rhythms. Indeed, temperature is generally regarded as secondary only to light as an entrainment cue.
Circadian rhythms profoundly affect the physiology and behavior of most organisms. These rhythms are generated by a self-sustained molecular clock, which is largely conserved between fruit flies and mammals and synchronizes to the day/night cycle. This synchronization is achieved in most organisms by a daily resetting caused by light and/or temperature fluctuations. The molecular mechanisms underlying light synchronization are reasonably well understood, but an understanding of how temperature affects the circadian clock is lacking. This study demonstrates a striking and unanticipated relationship between light and temperature resetting mechanisms in Drosophila. An interaction between the circadian photoreceptor CRYPTOCHROME (CRY) and a complex composed of the key circadian regulators PERIOD (PER) and TIMELESS (TIM) are critical for circadian temperature responses as well as a circadian light responses. Moreover, the data not only indicate that light and temperature reset the clock through similar mechanisms but also that these two inputs can act synergistically. An interaction between light and temperature may fine-tune the dawn and dusk response of the clock and even contribute to seasonal adaptation of clock function, an emerging area of research in circadian biology.

Results

Heat Pulse–Mediated Phase Delays of Wild-Type Flies Require 37 °C

To investigate the effect of heat on Drosophila locomotor activity rhythms, we first compared a heat phase response curve (PRC) to a standard light PRC. In both cases, the pulses lasted for 30 min, either with saturating light or with a shift from 25 °C to 37 °C. We used a modified PRC protocol, called the anchored PRC (APRC; [32–34]). The pulses are applied to wild-type flies during the night half of a light-dark cycle (zeitgeber time [ZT]12–24) and then during the first 12 h of the subsequent “day” in constant darkness (circadian time [CT]0–12). Locomotor activity phases were then measured after several subsequent days in constant darkness.

A typical PRC was obtained for light, with maximum phase advances about 2.5 h in the early morning.

Figure 1. Heat Pulse–Mediated Phase Delays of Wild-Type Flies Require 37 °C

Phase response curves for CS flies after heat (circle) versus light pulse (square). Flies were entrained for 3 d in 12 h:12 h LD cycles and pulsed for 30 min of light and 30 °C, 34 °C, and 37 °C heat pulse (HP) during the last night of the LD entrainment cycle, after which the flies were released in constant darkness for 5 d. Phase changes were calculated by comparing behavioral offsets of light or HP treated flies 3 d after the pulse to the behavior of the control group of the same genotype that did not receive a pulse. The calculations were made by MATLAB software using previously described methods [46]. Phase delays and phase advances are plotted (± SEM) as negative and positive values respectively. In all cases, the experiments were repeated at least twice with similar results. Data were pooled from the following number of flies (each pair of values referring to wild-type light-pulsed and wild-type 37 °C heat-pulsed): control: 32, 32; pulse at ZT12: 32, 22; pulse at ZT15: 32, 26; pulse at ZT18: 23, 26; pulse at ZT21: 24, 19; and pulse at ZT24: 29, 27. doi:10.1371/journal.pbio.0050146.g001

The weak heat-mediated delay and absence of a substantial advance makes it uncertain whether there is a relationship between the heat and light PRCs. We therefore assayed the biochemical effects of a heat pulse and compared them to those of a light pulse. The strategy was based on the interaction of CRY with TIM and/or PER, which is a light-dependent event (e.g., [20]). There is also substantial evidence...
that these events are crucial to clock resetting after short light pulses [19,37,38]. To assay CRY interactions in flies, we used a previously described strain that expresses N-terminal MYC-tagged CRY [20]. We subjected flies to either light or heat pulses and then assayed CRY complexes via immunoprecipitation with anti-MYC anti-sera. Remarkably, an interaction between CRY and PER-TIM was observed at ZT15 after a 37 °C heat pulse as well as after a light pulse. There was no detectable interaction if the ZT15 heat pulse was at 30 °C (Figure 2A), nor was there a robust 37 °C heat-mediated interaction at ZT21, despite a canonical light-mediated interaction at this time (Figure 2B). These results mirror the behavioral observations, namely, a 37 °C phase shift and no 30 °C phase shift at ZT15, with no 37 °C phase shift at ZT21 (Figure 1) [35]. The data indicate that a CRY:PER-TIM interaction correlates with heat-mediated phase shifts and suggest that it might underlie the behavioral phase shifts.

CRY Is Required for Heat-Mediated Phase Shifts

These results predict that heat PRCs should be affected in the severe loss-of-function mutant cryb. Indeed, these flies show little to no response to a heat pulse, i.e., an almost flat PRC (Figure 3A). The results are very similar to those observed for a light PRC in cryb [14]. To verify that this result is not due to a strain differences unrelated to the cry locus, we rescued the cryb mutation by expressing CRY in clock-pacemaker cells using pdf-GAL4 [13,39,40]. pdf-GAL4-mediated CRY expression partially rescued the cryb heat delay at ZT15 (Figure 3B) as well as the

Figure 2. CRY interacts with PER/TIM in a 37 °C Heat Pulse-Dependent Manner at ZT15 but Not at ZT21

Heat- and light-dependent interactions among CRY, TIM, and PER were measured three times with similar results. (A) TMC flies (Myc-CRY) were subjected to standard 12:12 LD conditions referred as control, pulsed with 37 °C (2), pulsed with 30 °C (3), light-pulsed (4), or not (1) for 30 min at ZT15, collected, and frozen. Head extracts (HE) were immunoprecipitated with antibody to MYC (IP), all as previously described [20]. CRY, PER, and TIM levels were measured by Western blotting. (B) Exactly as above but pulses were at ZT21.

doi:10.1371/journal.pbio.0050146.g002

Figure 3. 37 °C Heat Pulse-Mediated Phase Responses of cryb and Rescued Strains

(A) Phase response curves for wild-type (circle) flies and cryb (square) mutant flies. The experiment was performed as described in Figure 1. Phase delays and phase advances are plotted (± SEM) as negative and positive values, respectively. Data were pooled from the following number of flies (each pair of values referring to wild-type heat pulsed and cryb heat pulsed): control: 32, 32; pulse at ZT12: 22, 39; pulse at ZT15: 26, 40; pulse at ZT18: 26, 39; pulse at ZT21: 19, 36; and pulse at ZT24: 27, 15. (B and C). Bottom left and right panels show the phase changes observed at ZT15 (B) and ZT21(C), respectively. On the x-axis, the Zeitgeber 37 °C heat (HP) or light pulse (LP) is indicated. Phase delays and advances are described in Figure 1 and plotted on the y-axis (± SEM) as negative and positive values, respectively. The genotype of the flies is indicated on the x-axes: the first row shows the transgenes present (plus sign corresponds to a chromosome without a transgene), whereas the second row indicates the genetic background (wild-type [WT] or cryb).

doi:10.1371/journal.pbio.0050146.g003
cryb light delay as previously described [13]. We also compared the response of these strains to heat and light pulses at ZT21 (Figure 3C). As predicted from the wild-type heat PRC pattern (Figure 3A), the addition of pdf-GAL4-mediated CRY expression to the cryb background had no effect on the essentially nonexistent heat-phase shift at ZT21, whereas it rescued the cryb ZT21 light-phase shift (Figure 3C) [13]. In contrast, tim-GAL4-mediated CRY-B expression was unable to rescue either light- or heat-mediated cryb phase shifts (Figure S2), consistent with the strong hypomorphic cryb mutation. Taken together with the heat-mediated physical interaction between CRY and PER-TIM (Figure 2), the results indicate that CRY is important for circadian clock heat responses as well as light responses.

per4 Flies Are Hypersensitive to Heat

The perA genotype shows aberrant temperature compensation, with dramatically increased periods at elevated constant temperatures [41,42]. We speculated that this phenomenon might be related to heat-pulse responses and even light pulse-mediated phase shifts. To examine this possibility, we first assayed a standard light PRC of perA flies. It is very similar to that for wild-type flies, except that the perA curve is delayed by several hours (Figure 4A) [34]. There are essentially indistinguishable phase delays 18 h after the last DL (dark-light) transition for perA flies and 15 h after the last DL transition for wild-type flies. Moreover, there are similar phase advances, about 26 h after the last DL transition in perA and 21 h after the last DL transition in wild-type (compare Figure 4A with Figure 1).

Consistent with the notion that perA flies are more heat sensitive than wild-type flies, there is essentially no difference between the perA heat and light PRCs in the delay zone (Figure 4A), in contrast to the magnitude of the wild-type heat-mediated delay, which is clearly less than that of the wild-type light-mediated delay (Figure 1) [35]. Even more impressive is the heat-mediated advance for perA flies, which is indistinguishable from the light-mediated maximal advance (Figure 4A); there is little or no heat-mediated advance in wild-type flies (Figure 1). Finally, perA flies are sensitive to a 30 °C heat pulse, whereas wild-type flies are insensitive even to a 34 °C pulse (Figures 1 and 4B) [36].

The heat-mediated phase advance of perA flies suggested that there might be an interaction between CRY and PERL-TIM at these times, e.g., at CT2 (ZT26 = CT2). Indeed, we confirmed such an interaction after a 30 °C as well as a 37 °C heat pulse (Figure 4B). With minor differences, the interaction was similar to that elicited by a light pulse at this same time, and no interaction was observed without a heat or a light pulse (Figure 4B). There is no detectable heat-mediated interaction between CRY and wild-type PER-TIM in the advance zone or at 30 °C (Figures 1 and 2), i.e., the interactions between CRY and PERL-TIM correlate well with the behavioral observations (Figure 4A) and further indicate that they are important for the observed heat-mediated phase shifts.

per4 Heat-Mediated Phase Shifts, CRY, and Temperature Compensation

To verify that the CRY:PERL-TIM interaction is functionally relevant, we generated perA; cryb double mutant flies. They have a long free running period of ~28 h, characteristic of perA, and are rhythmic in light-dark (LD), characteristic of cryb (Figure 5A). These flies also show much smaller phase shifts in response to 37 °C heat pulses in the delay zone at ZT18 as well as in the advance zone at ZT26 (ZT26 = CT2; Figure 5B). The exaggerated perA heat-mediated phase shifts are therefore CRY dependent.

Finally, to establish a link between the exaggerated heat-mediated phase shifts and the temperature compensation defect of perA flies, we assayed the free-running period of perA; cryb double mutant flies at constant temperatures (Figure 5C). The results indicate that this genotype shows much less period change with temperature, in striking contrast to perA.
flies. This indicates that a temperature-sensitive CRY:PERL-TIM interaction underlies most of the perL− loss of temperature compensation. It also connects the free-running period phenotype assayed at constant temperatures with the response to a heat pulse. Indeed, there is also a CRY:PERL-TIM interaction after incubation of perL− flies at a constant temperature of 29°C (Figure S3). Moreover, the fact that the perL− strain has an altered period compared to the perL−; cryb double mutant strain at 15°C (Figure 5C) suggests that even at low temperatures, the PERL-TIM complex interacts with
CRY. We suggest that advances predominate (an aggregate shorter period) at 15 °C, whereas delays predominate (an aggregate longer period) at temperatures ≥ 25 °C.

A Model

These data suggest that the perL misense mutation facilitates a PER-TIM conformational change (Figure 6A; 1 → 2). Heat facilitates the same change in wild-type PER-TIM, although higher temperatures are required and a smaller fraction of PER-TIM is affected. If CRY interacts predominantly with TIM, then the per mutation and heat must also help promote a TIM conformational change (Figure 6A; 3). We imagine that this altered PER-TIM conformation could also facilitate an interaction with active CRY, which is a conformational state similar to that promoted by illumination, i.e. by CRY photon capture (Figure 6A; CRY*).]

Light and Temperature Can Act Together on Wild-Type Fly Rhythms

These observations suggest that even in wild-type flies, temperature and light can synergize to affect CRY:PER-TIM complex formation at physiologically normal temperatures. To test this hypothesis, we subjected Canton-S (CS) flies to constant illumination at 10 and 100 lux. Constant light even at low intensities render most flies arrhythmic at a standard incubation temperature of 25 °C (Figure 6B) [41], and constant light arrhythmicity requires CRY [15]. The results and model (Figure 6A) suggest that low temperatures might reduce complex formation and arrhythmicity, and constant light arrhythmicity has not been assayed at 15 °C. Indeed, we observed substantially larger numbers of arrhythmic flies at 25 °C than at 15 °C, at 100 as well as at 10 lux of constant light (Figure 6B). We interpret the result to indicate more CRY:PER-TIM complex formation at 25 °C than at 15 °C, indicating that light and temperature can act together in wild-type flies at physiologically relevant temperatures. The convergence induces phase shifts as well as causes arrhythmicity in constant light. We speculate that it also serves to fine tune the dawn and dusk response of the clock when light and temperature increase and decrease together.

Discussion

We show here that the photoreceptor CRY and its interaction with the PER-TIM complex is critical for heat shock-mediated phase shifts as well as for the loss of temperature compensation in the perL mutant strain. Heat-induced phase delays take place in a wild-type strain, and they are severely reduced in the cry loss-of-function mutant cryL. Moreover, there is a physical interaction between CRY and PER-TIM at circadian times that correspond to phase delays. More robust heat-mediated phase delays and even phase advances occur in perL mutant flies. The perL behavioral results are mirrored by CRY:PERL-TIM interactions, which occur in the advance zone and also in response to 30 °C temperature pulses. perL phase shifts like wild-type phase shifts are severely reduced by the addition of cryL to the perL background. These perL; cryL double mutant flies also have largely restored temperature compensation, indicating that an interaction between CRY and PER-TIM is responsible for the loss of temperature compensation in the perL strain as well as for heat-mediated phase shifts of wild-type as well as perL flies. The similarity between heat-mediated and light-mediated phase shifts suggests that light and temperature can synergize to cause phase shifts, and an experiment in wild-type flies supports this notion.

The temperature-induced complex formation between CRY and PER-TIM parallels the substantial evidence that a similar interaction is critical for light-mediated phase shifts. Biochemical as well as genetic data indicate that complex formation between light-activated CRY and TIM, or between light-activated CRY and PER-TIM, leads to TIM degradation, which is believed to advance or delay the clock (e.g., [25]). Although some data indicate a physical interaction between CRY and PER, most observations indicate that physical contact is predominantly between CRY and TIM; for example, PER usually requires the presence of TIM to interact with CRY, but a TIM:CRY interaction can take place without PER (e.g., [20]). Because much of TIM is in complex with PER, especially in the early night [22], a CRY-TIM interaction is effectively a CRY:PER-TIM interaction. All of this begins with CRY photon capture, which activates CRY by causing a conformational change and a subsequent interaction with PER-TIM. Indeed, experimental studies on Drosophila CRY as well on other related proteins provide a coherent view of a CRY-centric light-initiation event [10].

Although a connection between light pulse- and heat pulse-initiated interactions appeared enigmatic, previous studies in wild-type flies suggested that heat phase shifts are like light pulses and are due to posttranscriptional events that influence PER and/or TIM [36]. The failure to elicit a phase shift with a 34 °C pulse (Figure 1) indicates that a heat shock may be required [43]. This is accompanied by numerous changes in cell physiology and gene expression, which could perturb the dynamics of an oscillatory system [44]. However, perL flies show robust phase shifts and CRY:PER-TIM complex formation after a 30 °C heat pulse, making it unlikely that a heat-shock response is generally required for heat pulse-mediated phase shifts in Drosophila. Extrapolation to wild-type flies makes two assumptions: (i) perL flies do not have an unprecedented heat-shock response triggered at much lower temperatures and (ii) the failure to observe 30 °C behavioral phase shifts and biochemical interactions in wild-type reflects quantitative rather than a qualitative differences between 30 °C and 37 °C and between wild-type and perL genotypes. Indeed, the convergence of light and temperature on wild-type fly behavior at physiological temperatures (Figure 6B) suggests that these CRY:PER-TIM interactions are normally difficult to detect at lower temperatures, because they are quantitatively minor.

The perL behavioral and biochemical results indicate that the missense mutation causes a large increase in the fraction of PERL-TIM interacting with CRY at normal temperatures (Figure 4B). This suggests that the PERL-TIM structure is temperature sensitive (Figure 6A), an interpretation consistent with the period of the perL; cryL double mutant strain being somewhat temperature sensitive (Figure 5C; see below).
CRY Promotes Circadian Temperature Responses

A 1

PER$^L$ TIM (temp; mutation)

which then promotes these steps

B

10 Lux

100 Lux

Actogram Autocorrelation Actogram Autocorrelation

15C (LL)

25C (LL)

time (h) Lag (h) time (h) Lag (h)
Moreover, this strain has a substantially longer period than the perL single mutant strain at 15 °C (Figure 5C), suggesting that PERL-TIM manifests an enhanced interaction with CRY at all physiologically relevant temperatures. These experiments cannot definitively rule out CRY as the temperature-sensitive component; in this case, the perL mutation would only cause an increased interaction between PER-TIM and CRY. In either case, the close correspondence between the 37 °C heat and light PRCs (Figure 4A) indicates that the CRY photocycle is insensate for CRY:PER-TIM interactions and behavioral phase shifts in Drosophila. We speculate that heat activation of PER-TIM causes the same CRY conformational change as does light—albeit indirectly (Figure 6A).

The heat-induced interactions between PERL-TIM and CRY as well as the perL; cryb genotype make a strong link between the circadian response to temperature pulses and incubations at constant temperatures, analogous to non-parametric and parametric light entrainment, respectively. This is because a persistent CRY:PERL-TIM interaction affects the perL period like the enhanced phase-shift response of perL to a heat pulse. This recalls the hypersensitivity of perL to incubation at constant low light intensities, which lengthens the perL period more severely and at lower intensities than is required to lengthen wild-type periods [41]. Our results explain this observation and suggest that the more CRY-interactive PERL-TIM requires less CRY light activation than does wild-type PER-TIM. Moreover, the similarities between light and heat inspired the experiment suggesting that light and temperature function together, even on wild-type flies (Figure 6B). This synergy might fine-tune the dawn and dusk response of the clock and even contribute to seasonal adaptation of clock function [45].

The circadian problem of temperature compensation has gained little traction since the discovery more than 15 y ago that the per missense mutants manifest aberrant temperature compensation [41]. Our results here suggest that the timSL allele suppresses the temperature compensation defect of perL by failing to interact with CRY [42]. The observations suggest that the same PERL-TIM structure that facilitates a CRY interaction in response to a phase-shifting perturbation (heat- or light-mediated CRY activation) keeps time in a temperature-sensitive manner under constant conditions. Characterization of this altered PERL-TIM structure is an important goal for the near future.

Materials and Methods

Drosophila genetics. Wild-type CS, perL; and cryb flies were used for average activity and phase response analyses (see below) and as controls for the locomotor activity analyses. The perL mutation was combined with cryb to generate perL; cryb flies. The pdf-GAL4 and UAS-cry transgenic flies have been described previously [15]. The y; tim-GAL4 UAS-mycRycyO line (TMC) was previously described [20]. The TMC transgenes were introduced into perL to obtain perL; tim-GAL4 UAS-mycRycyO (abbreviated as perL; TMC). The UAS-cry and pdf-GAL4 transgenes were introduced in cryb backgrounds to produce y w; pdf-GAL4/UAS-cry; cryb flies.

Phase shift protocol and behavioral analysis. In all experiments unless stated otherwise, CS males were collected at 1–3 d old and reared in LD 12:12 at 25 °C for 3 d. In the APRC protocol, flies were given a 10-min saturating white light pulse (2000 lux) during the third dark phase of the cycle, at the indicated times during the night and the following subjective day. A separate control group of flies was not given a pulse. Flies were then put into constant darkness for another 3 d. For the heat pulse PRGs, flies were placed in activity monitors in LD 12:12 at 25 °C for 3 d. During the third dark phase of the cycle, one monitor of untreated flies was retained as a control. For the heat treatment, behavior tubes containing flies were removed from the monitors, held upright, and an elastic band placed around them to hold them tightly together. The entire package was then placed in a 50-ml conical tube, so that the flies would stay upright. All flies were in a water-tight environment but small enough for efficient heat transfer from the water bath to the tubes. The top of the activity tubes were always an inch below the top of the 50-ml conical tube, so the water level would be above the tubes. Incubation was in the water bath for 30 min at 37 °C. The 50-ml tube was then removed, and the behavior tubes placed back in the monitors. Each tube had been marked on the top with a number and then placed back in the same monitor channel. A second control set of flies was handled identically except that they were just kept upright (with the elastic band) in 50-ml tubes in the incubator but not placed in a water bath. In all cases, the experiments were repeated at least twice with essentially identical results. For each genotype an average phase shift from 15–32 flies is shown. Locomotor activities of individual flies were monitored using Trikinetics Drosophila activity monitors (TriKinetics Inc, Waltham, Massachusetts, United States). The analysis was done with a signal processing toolbox implemented in MATLAB (Mathworks; http://www.mathworks.com) as described [46].

Autocorrelation it is a measure of how well a signal matches a time-shifted version of itself as a function of the amount of time shift. In our analysis, autocorrelation and spectral analysis were used to assess rhythmicity and to estimate period. The phase information was obtained with circular statistics [46]. The column in Figure 5A labeled autocorrelation shows correlograms for the data. Correlation coefficients are plotted on the ordinate with a range of values from −1 to 1. The gray region centered around 0 defines a 95% confidence interval. The lag of the autocorrelation function is plotted on the abscissa. An asterisk is placed above the third peak of the autocorrelation function. The value at that point defines the rhythmicity index (RI), an estimate of the strength of rhythmicity. Weak asterisks are not significant; the absence of a correlation function indicates a lack of rhythmicity. Values for the RI appear in the lower left corner of these plots along with a related number called the absolute value of the confidence line. This metric indicates that the rhythmicity described by the correlogram is statistically significant when the value is greater than 1 [46].

The MESA analysis is a spectral analysis of the data that provides an estimate of period. Spectral density is given in arbitrary units on the ordinate, and the range of assessed periods is shown on the abscissa. Asterisks are placed over the highest peak shown in a range between 18–30 h. Although this value is generally taken as the estimate of circadian period, there may be other periodicities present within the horizontal range (the width) of the peak or elsewhere on the plot, and these additional rhythmic components are also present in the data. Absence of an asterisk indicates either the absence of a peak or that a peak within the cited plot occurs outside the circadian range. Note that the autocorrelation plot is used to determine rhythmicity, and meso is used to provide an estimate of the period only when warranted by correlogram [46].

Immunoprecipitation. About 250 adult flies were entrained to a 12-h light: 12-h dark cycle for 3 d. At ZT15, ZT21, or CT02, they were pulsed with bright white light for 15 min and 30 °C or 37 °C for 30 min before being collected and frozen. Head extracts were prepared and homogenized in Extraction Buffer (20 mM Hepes, pH 7.3, 100 mM NaCl, 10% glycerol, 1% Triton X-100, 0.5% deoxycholic acid).
significant differences were found for CS flies at ZT12 and ZT15 and group of flies are significantly different, \( p < 0.01 \) Statistically actions among CRY, TIM, and PER were measured twice with similar results.

Protein extracts and Western blots. Fly heads extracts were prepared and Western blots were performed as described [22]. 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 \( \alpha \)-tubulin antibody (clone DM1A, Sigma, 1:1000 dilution; http://www.sigmaldiirch.com). The anti-CRY rabbit antibody was used at 1:500 dilution [47]. The anti-PER antibody is previously described and used at 1:1500 dilution, whereas the anti-TIM antibody was made in rat and used at 1:3000 dilution [22].

Supporting Information

Figure S1. 37 °C Heat Pulses Result in Robust Phase Shifts of Wild-Type and per \(^{-}\) Flies but Not of cry \(^{-}\} or per \(^{-}\} \times \) cry \(^{-}\) Flies

(A) Circular analysis figures of locomotor behavior in wild-type CS and cry \(^{-}\} \times \) per \(^{-}\} \times \) flies after a 37 °C heat pulse (HP). (B) The same circular analyses for per \(^{-}\} \times \) per \(^{-}\} \times \) cry \(^{-}\} \times \) after a 37 °C HP. On these plots, time moves forward in a clock-counter-clockwise direction. The behavioral phase estimates for each rhythmic specimen are plotted just outside the unit circle and a mean vector summarizes the phase of the group. The direction of the vector indicates the behavioral phase, whereas its length reflects the dispersion (variability) of the individual estimates (see [46] for more details). The Rayleigh's test was used to determine whether each vector is significantly different (\( p < 0.05 \)) from the null vector (random distribution). Then, the Watson-Williams-Stevens test was used to obtain an \( F \)-statistic that determined whether the two vectors obtained from the nonpulsed control and the experimental group of flies are significantly different, \( p < 0.01 \). Statistically significant differences were found for CS flies at ZT12 and ZT15 and for per \(^{-}\} \times \) at ZT18 and CT2. cry \(^{-}\} \times \) and per \(^{-}\} \times \) cry \(^{-}\} \times \) flies did not significantly shift their phase at any time points. For the estimates of the phase differences see Figure 1.

References


APPENDIX III

Ectopic CRYPTOCHROME renders TIM light sensitive in the *Drosophila* ovary.

Brandy Rush, Alejandro Murad, Patrick Emery and Jadwiga Giebultowicz

This appendix is reprinted from an article published in The Journal of Biological Rhythms in August, 2006. This work is the result of a collaboration between our Laboratory and the Giebultowicz Laboratory. For this study, I worked on the generation of anti-CRY antibodies, tested the different antisera and optimized the western blot conditions for its usage.

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Ectopic CRYPTOCHROME Renders TIM Light Sensitive in the Drosophila Ovary

Brandy L. Rush, Alejandro Murad, Patrick Emery, and Jadwiga M. Giebultowicz

Abstract The period (per) and timeless (tim) genes play a central role in the Drosophila circadian clock mechanism. PERIOD (PER) and TIMELESS (TIM) proteins periodically accumulate in the nuclei of pace-making cells in the fly brain and many cells in peripheral organs. In contrast, TIM and PER in the ovarian follicle cells remain cytoplasmic and do not show daily oscillations in their levels. Moreover, TIM is not light sensitive in the ovary, while it is highly sensitive to this input in circadian tissues. The mechanism underlying this intriguing difference is addressed here. It is demonstrated that the circadian photoreceptor CRYPTOCHROME (CRY) is not expressed in ovarian tissues. Remarkably, ectopic cry expression in the ovary is sufficient to cause degradation of TIM after exposure to light. In addition, PER levels are reduced in response to light when CRY is present, as observed in circadian cells. Hence, CRY is the key component of the light input pathway missing in the ovary. However, the factors regulating PER and TIM levels downstream of light/cry action appear to be present in this non-circadian organ.

Key words circadian clock, cryptochrome, ovary, period, timeless

Organisms are equipped with circadian clocks, which generate molecular, physiological, and behavioral rhythms and synchronize them with the solar cycle. Circadian clocks operate via interacting molecular feedback loops that show substantial homology between Drosophila and mammals (Stanewsky, 2003). The period (per) and timeless (tim) genes play a central role in the Drosophila clock mechanism. They are positively regulated by the transcription factors CLOCK (CLK) and CYCLE (CYC), which bind to and activate the per and tim promoters. The PERIOD (PER) and TIMELESS (TIM) proteins accumulate in the cytoplasm and then translocate to the nucleus, where PER disrupts the positive activity of CLK and CYC. This results in the repression of per and tim transcription. For continual oscillations to occur, TIM and PER are degraded, allowing CLK and CYC to reactivate the tim and per promoters.

Circadian rhythms persist under constant external conditions; however, in nature, they are entrained by environmental cues. LD cycles are particularly important for the synchronization of circadian rhythms. When Drosophila are exposed to light, the levels of TIM decline rapidly via proteasomal degradation (Hunter-Ensor et al., 1996; Lee et al., 1996; Myers et al., 1996; Zeng et al., 1996; Naidoo et al., 1999). This response is mediated by a light-activated protein encoded by the cryptochrome (cry) gene. Cryptochromes are flavin-containing photoreceptors with a high degree of similarity to DNA photolyase.
A missense mutation within the flavin-binding region of *Drosophila* cry (cry’), interferes with light entrainment of locomotor activity (Stanewsky et al., 1998). cry’ mutants are unable to reset their clocks in response to short light pulses and have disrupted PER and TIM oscillations in most tissues, with the exception of the pacemaker neurons that control circadian behavior; these cells receive additional light inputs from visual photoreceptors (Stanewsky et al., 1998). Flies overexpressing the cry gene are behaviorally hypersensitive to light (Emery et al., 1998; Emery et al., 2000b). Interestingly, cry’ flies remain behaviorally rhythmic under intense constant light (LL) (Emery et al., 2000a), a condition that renders wild-type flies arrhythmic (Konopka et al., 1989). Previous reports demonstrated interactions between CRY, TIM, and PER (Ceriani et al., 1999; Lin et al., 2001; Rosato et al., 2001). More recently, light-activated CRY was shown to bind to TIM, leading to its rapid degradation; however, no interaction was detected between CRY and PER without TIM (Busza et al., 2004).

The circadian mechanism operates in both the *Drosophila* brain and various peripheral organs (Giebultowicz, 2000; Glossop and Hardin, 2002). PER and TIM show daily brain-independent oscillations in renal Malpighian tubules, seminal vesicles, and other organs (Giebultowicz and Hege, 1997; Hege et al., 1997; Beaver et al., 2002). Furthermore, TIM is light sensitive in the Malpighian tubules, and CRY is required for TIM degradation in response to short light pulses (Giebultowicz et al., 2000; Ivanchenko et al., 2001). In addition to photoreceptor function, CRY appears to be an essential component of peripheral clocks (Ivanchenko et al., 2001; Krishnan et al., 2001); this role was confirmed by a recent study showing that CRY functions as a corepressor protein in photoreceptor cells (Collins et al., 2006). In mammals, cryptochromes are negative elements of circadian clocks and may also be involved in circadian photoreception (Partch and Sancar, 2005a), suggesting that the dual role of these proteins may be common for insects and mammals.

Among fly organs, a prominent exception to the rhythmic expression of per exists in the ovary. Levels of per mRNA do not cycle in the ovary, and PER protein appears cytoplasmic at all times of the day in ovarian follicle cells (Liu et al., 1988; Saez and Young, 1988; Liu et al., 1992; Hardin, 1994). These data suggest that per plays non-clock roles and is regulated differently in the ovary than in other body cells. To understand why PER behaves in a dramatically different way in the ovary than in clock cells, we began asking whether other clock-associated molecules are expressed in the ovary. We recently reported that TIM is colocalized with PER in the follicle cells of previtellogenic egg chambers; however, both proteins were cytoplasmic at all times, and their levels did not show daily fluctuations (Beaver et al., 2003). We also made the intriguing observation that, in contrast to circadian oscillators, TIM (and PER) in follicle cells was not degraded by light, even after 3 days in constant light (Beaver et al., 2003). In this article, we sought to understand why ovarian TIM does not respond to light. We report that endogenous CRY is absent in the ovary. We then demonstrate that forcing cry expression in the ovary is sufficient to render TIM light sensitive in this noncircadian tissue.

**MATERIALS AND METHODS**

**Rearing Conditions and Fly Lines**

*Drosophila melanogaster* were reared on cornmeal-molasses-yeast diet. Flies were maintained in 12-h LD cycles at 25 °C and then kept in LD or transferred to LL. By convention, the time of lights-off is denoted as ZT12; in LD 12:12, the time of lights-on is at ZT0. Wild-type flies used were Canton-S. The following transgenic lines were used: *wttim* (UAS)Gal4; (Blau and Young, 1999), *y w; cry*’; *y w*; UAS-*cry*:24, and *y w; UAS-cry*:31 (Emery et al., 1998; Emery et al., 2000b). In addition, independently generated *y w crypGal4-24*; and *y w; crypGal4-16* were used; they contain 5 kb of promoter region and the fraction of the first exon of the cry gene in front of a Gal4 coding sequence (Zhao et al., 2003).

**Western Blotting**

For ovary collections, flies were placed in a dish with cold phosphate-buffered saline (PBS). Ovaries were dissected and quickly frozen in 1.5-mL tubes on dry ice. For ovary collections during the dark phase, dissections were conducted using dim red light. For head collections, whole flies were frozen and heads collected with a sieve on dry ice. Whole-head and ovarian extracts were obtained and Western blotting performed as described in Edery et al. (1994), with some modifications listed below. Protein concentrations were determined using BCA Protein Assay (Pierce, Rockford, IL) to ensure equal loading, and MemCode Reversible Protein Stain Kit (Pierce) was...
used to ensure equal transfer. For analysis of CRY protein, membranes were blocked for 1 h with TBST/5% milk (Bio-Rad, Hercules, CA). Two CRY antisera were used both at 1:1000 dilution in TBST/5% milk (Bio-Rad). The anti-CRY antibody used in Figure 1 was generated by injecting rabbits with CRY’s N-terminal region (amino acid 1-183) as an antigen. The antigen was produced in BL21 bacteria with an N-terminal 6-HIS tag and purified on Nickel beads. After imidazole elution, the antigen solution was dialyzed against decreasing concentration of Urea in PBS. Animal immunization was carried out at Cocalico Biologicals (Reamstown, PA). Animals were tested for immunoreactivity to the antigen by Western blots after the third antigen injection, and animals reacting to the antigen were exsanguinated after the fourth antigen injection. The rabbit anti-CRY antibody #14 gave the strongest signal on Western blots and can detect endogenous CRY levels; this antibody was used to generate data shown in Figure 1. The anti-CRY antibody used in Figure 2 was generated in guinea pig and described in Busza et al. (2004). It can only detect overexpressed levels of CRY. Blots were incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated antirabbit (Molecular Probes, Eugene, OR) and anti–guinea pig (Southern Biotech, Birmingham, AL) secondary antibodies, which were diluted in TBST/1% milk at 1:1000 and 1:8000, respectively. Blots were then incubated in Immune-Star HRP Substrate Kit (Bio-Rad) and exposed to film.

For analysis of TIM protein, we used anti-TIM UPR #41 at 1:1000 from A. Sehgal on the blots shown in Figures 2B and C. Following transfer, the membranes were blocked for 1 h in TBS/5% milk (Bio-Rad). TIM antisera were diluted in TBST/5% milk and incubated overnight at 4 °C. Secondary antibody was antirat IRDye 800 (Rockland, Gilbertsville, PA) diluted 1:10,000 in TBST/2% milk; blots were incubated for 1 h at room temperature. Membranes were scanned and proteins quantified using LI-COR Odyssey Infrared Imaging System. Immunocytochemistry for TIM and PER was performed as described in Beaver et al. (2003).

RESULTS

Cryptochrome Is Not Expressed in Ovarian Cells Positive for TIM and PER

We have shown previously that high levels of TIM (and PER) are present in Drosophila ovarian follicle cells throughout the light phase of the LD cycle and remain high even after 3 days in LL (Beaver et al., 2003). The lack of TIM’s degradation in response to light could be caused by one or many deviations from the clock mechanism. For example, the photoreceptor CRY could be either not expressed or not able to interact with TIM. Alternatively, components targeting TIM to the proteasome could be missing in the ovary. To address these questions, we first tested whether the photoreceptor protein CRY is present in the ovary. CRY levels were examined in the ovaries and heads by Western blotting. In agreement with previous reports (Emery et al., 1998; Emery et al., 2000b), CRY was present in heads during the dark phase at ZT20 and light phase (ZT8) in high and low levels, respectively. In contrast, we did not detect CRY in the ovary at ZT20 or ZT8 (Fig. 1A).

To verify that the cry gene is not transcriptionally active in the ovary, we used a UAS-gfp reporter driven by the cry promoter region fused to Gal4 (Brand and Perrimon, 1993). The Gal4 lines crypGal4-16 and crypGal4-24 drive expression in all brain clock cells and some ectopic locations in the fly brain (Zhao

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Figure 1. CRY is not expressed in the Drosophila ovary. (A) Western blot analysis detected CRY at high levels at night (ZT20) and low levels during the day (ZT8) in fly heads, but CRY was absent in the ovaries at both time points. Heads of the cry

mutant were used as negative control. An equal amount (85 µg) of total protein was loaded in each lane. Western blots were repeated 3 times with similar results. (B) Green fluorescent protein (GFP) expression in Malpighian tubules (M. tubule) and ovarioles driven by either timGal4 or cryGal4. Arrows indicate ovarian somatic follicle cells. timGal4 drove expression in both M. tubules (a known circadian peripheral oscillator) and in ovarian follicle cells, while cryGal4 showed strong signal in M. tubules but not in the ovary.
in ovarian follicle cells, the cry gene is not active in these cells.

Ectopic Expression of cry Renders Ovarian TIM Light Sensitive

To determine whether the persistent presence of TIM in follicle cells of light-exposed females is related to the absence of CRY, we used the Gal4/UAS system to express cry in the ovary. Ectopic expression of cry or cry\textsuperscript{b} was achieved using the tim(UAS)Gal4 driver (Blau and Young, 1999), which drives expression of target genes into all TIM-positive cells, including the follicle cells of previtellogenic egg chambers (Fig. 1B). We used UAS-cry or UAS-cry\textsuperscript{b} (Emery et al., 1998; Emery et al., 2000b) transgenic lines to drive functional or defective CRY into these cells. Western blot confirmed high ectopic CRY levels in the ovaries of flies expressing the wild-type cry transgene (Fig. 2A), CRY was not detected in control flies expressing the cry\textsuperscript{b} transgene because the mutant protein is unstable (Fig. 1A) (Stanewsky et al., 1998).

We next tested whether ectopic CRY could function as a photoreceptor in the ovary and trigger TIM degradation in response to light. TIM levels were monitored in the ovaries of tim(UAS)Gal4/UAS-cry (cry-ec) and control tim(UAS)Gal4/UAS-cry\textsuperscript{b} (cry\textsuperscript{b}-ec) flies after 3 days in LL; at this time, TIM is still present in the ovary of wild-type flies (Beaver et al., 2003). Analysis of TIM by Western blot confirmed high levels of this protein in the ovaries expressing defective photoreceptor protein from the cry\textsuperscript{b} transgene, both during the dark phase of LD (ZT20) and after 3 days in LL (Fig. 2B). TIM was also detected during the dark phase of LD (ZT20) in ovaries expressing the wild-type version of the CRY protein (cry-ec). Importantly, TIM was no longer detected in cry-ec ovaries held in LL for 3 days. Thus, ectopic ovarian cry expression is sufficient to render TIM sensitive to ambient light. To determine whether ectopic CRY requires long light exposure to degrade TIM or whether TIM could be degraded during the light phase of an LD cycle, we tested levels of TIM in cry-ec females 4 h after lights-on (ZT4). TIM levels were already dramatically reduced 4 h into the light phase (Fig. 2C). Thus, prolonged light exposure is not necessary to commit TIM to degradation in the ovary of cry-ec females. Rather, ectopic ovarian CRY seems to act within a physiological time frame similar to peripheral tissues with circadian oscillations (Giebultowicz et al., 2000).
Degradation of TIM via Ectopic CRY Destabilizes PER in the Ovary

In our final experiment, we asked whether light-induced degradation of TIM leads to PER instability, as was previously observed in bona fide clock cells (Price et al., 1995). Immunocytochemistry using anti-TIM and anti-PER antibodies was performed on ovaries from cry-ec and wild-type females during the dark phase of LD (ZT20) and at the equivalent time point in LL. TIM and PER were detected in ovarian follicle cells from wild-type control females during the dark phase as well as in LL (Fig. 3). High levels of TIM and PER were also present in cry-ec ovaries fixed during the dark phase. In contrast, both proteins were absent in the follicle cells of cry-ec egg chambers taken from females held in LL. These data are consistent with our Western results (Fig. 2). They are also consistent with our previous demonstration that PER is absent in the ovary of tim null mutants (Beaver et al., 2003). It appears that TIM provides stability to the PER protein in the ovary, as in clock cells. In support of this notion, we recently determined by coimmunoprecipitation that PER and TIM interact in the ovary (Rush and Giebultowicz, unpublished data), similar as in clock cells.

DISCUSSION

Our data demonstrate that CRYPTOCHROME is a pivotal clock-associated element missing in the ovary. Remarkably, ectopic expression of cry in the ovary is sufficient to cause rapid light-dependent TIM degradation in this non-circadian tissue. Thus, except for CRY, all the elements necessary for TIM degradation appear to be present in the ovary. This shows that the mechanisms regulating TIM and, indirectly, PER levels may be common in all tissues, even those in which PER and TIM have a non-circadian role. What differ from tissue to tissue are the molecules that connect TIM to the environment. For tissues expressing circadian rhythms, CRY is a mediator of light input. In the ovary, the environmental cues regulating TIM levels and their mediators remain to be identified. This is an important question because ovarian PER and TIM appear to regulate fecundity (Beaver et al., 2003). In addition to ovaries, CRY is also absent in a group of larval circadian neurons, the DN2s. In these cells, PER cycles antiphase to the other circadian neuronal groups (Kaneko et al., 1997). When CRY is ectopically expressed in the DN2s, PER cycles in phase with the other neuronal groups (Klarstfeld et al., 2004), suggesting that these cells need to be blind to light to show antiphase oscillations.

Our results pose an interesting evolutionary question regarding the origin of PER and TIM expression in the ovary. On one hand, the presence of PER and TIM in the ovary could represent a vestigial clock mechanism from which CRY was lost. On the other hand, the ovary may represent a preclock situation requiring the continuous presence of PER and TIM, perhaps in conjunction with some as yet unknown signaling function. One piece of evidence in support of the vestigial clock mechanism is that ovarian TIM has clock-like stabilizing effects on PER (Fig. 3). If CRY was the only missing element, it should be possible to “jumpstart” the clock function in the ovaries by CRY. Perhaps, once ovarian TIM and PER are degraded in response to light, the rhythmicity in the ovaries could continue even in constant darkness. However, there is evidence that the ovarian PER and TIM show other deviations from the clock mechanism: they remain cytoplasmic at all times in previtellogenic follicles, and their accumulation does not depend on positive clock elements encoded by Clk and cyc (Beaver et al., 2003). Presumably, transcription of per and tim in the ovary does not require Clk and cyc; therefore, it is unlikely that ectopic cry could elicit clocklike oscillations in the ovary.

Ovarian expression of PER and TIM resembles expression of clock genes in developing murine sperm cells, in which the levels of mPER1 are nonrhythmic
and not dependent on the positive circadian element mClock (Alvarez et al., 2003; Morse et al., 2003). Thus, it appears that a non-circadian function for circadian genes in the developing gonads of the animal might be an ancient feature. Further comparative studies on molecular behavior of clock proteins in their circadian and non-circadian roles may help to resolve the enigma of the evolutionary origins of circadian clocks. In addition, our work offers a novel simple model system to elucidate the mode of CRY action. Despite the important role of this molecule as a photoreceptor in Arabidopsis, Drosophila, and possibly mammals, the primary photochemical reaction carried out by CRY is not yet understood (Partch and Sancar, 2005b).

ACKNOWLEDGMENTS

We thank B. Gvakharia for helpful discussions; M. Emery-Le for preparing the CRY antigen for rabbit immunization; R. Stanewsky and J. Hall for anti-PER; A. Sehgal, L. Saez, and M. Young for anti-TIM; and R. Allada for cry-Gal4 lines. Data are based on work supported by the National Science Foundation grant no. 0446339 and the National Institutes of Health (NIH) grant no. R01GM073792 to J.M.G.; P.E. is supported by the NIH (grant no. R01 GM66777).

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