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Neuronal circuitry controlling circadian photoreception in *Drosophila*

A Dissertation Presented

By

Pallavi Lamba

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 29th, 2017

Program in Neuroscience

Neuronal circuitry controlling circadian photoreception in *Drosophila*

A Dissertation Presented
By
Pallavi Lamba

This work was undertaken in the Graduate School of Biomedical Sciences
Program in Neuroscience

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validation of the Dissertation content

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that the student has met all graduation requirements of the School.

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Anthony Carruthers, Ph.D.,
Dean of the Graduate School of Biomedical Sciences

August 29th, 2017

Dedicated to my pillars of strength,
My mother and father

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Abstract

Circadian clocks are endogenous timekeeping mechanisms, which give the sense of time-of-day to most organisms. To help the organisms to adapt to daily fluctuations in the environment, circadian clocks are reset by various environmental cues. Light is one of the cardinal environmental cues that synchronize circadian clocks.

In a standard 12:12 light-dark condition, *Drosophila* exhibits bimodal activity pattern in the anticipation of lights-on and -off. The morning peak of activity is generated by Pigment Dispersing Factor (PDF) positive small ventro-lateral neurons (sLNvs) called the M-oscillators, while the evening peak of activity is generated by the dorsolateral neurons (LNds) and the 5th sLNv together referred to as the E-oscillators. Since the *Drosophila* circadian clock is extremely sensitive to light, a brief light exposure can robustly shift the phase of circadian behavior. The model for this resetting posits that circadian photoreception is cell-autonomous: the photoreceptor CRYPTOCHROME (CRY) senses light, binds to TIMELESS (TIM) and promotes its degradation via JETLAG (JET). However, it was more recently proposed that interactions between circadian neurons are also required for phase resetting.

The goal of my thesis was to map the neuronal circuitry controlling circadian photoreception in *Drosophila.* In the first half of my dissertation (Chapter II), using a novel severe *jet*^{set} mutant and JET RNAi, we identified M- and E-oscillators as critical light sensing neurons. We also found that JET functions cell-autonomously to promote TIM degradation in M- and E-oscillators, and non-autonomously in E-oscillators when expressed in M-oscillators. However, JET expression was required in both groups of

neurons to phase-shift locomotor rhythms in response to light input. Thus M- and E-oscillators cooperate to shift circadian behavior in response to photic cues.

In chapter III, unexpectedly, we found that light can delay or advance circadian behavior even when the M- or E-oscillators are genetically ablated or incapacitated suggesting that behavioral phase shifts in response to light are largely a consequence of cell autonomous light detection by CRY and governed by the molecular properties of the pacemaker. Nevertheless, neural interactions are integral in modulating light responses. The M-oscillator neurotransmitter, PDF was important in coordinating M- and E-oscillators for circadian behavioral response to light input. Moreover, we uncover a potential role for a subset of Dorsal neurons in control of phase advances specifically. Hence, neural modulation of cell autonomous light detection contributes to plasticity of circadian behavior and facilitates its adaptation to environmental inputs.

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List of symbols, abbreviations, or nomenclature

aMe:	Accessory medulla
bHLH:	Basic-helix-loop-helix
BO:	Bolwig organs
cAMP:	Cyclic AMP
CK:	Casein kinase
CLK:	Clock
CRY:	Cryptochrome
CYC:	Cycle
DBT:	Doubletime
DD:	Constant darkness
disco:	Disconnected
DNs:	Dorsal neurons
dsRNA:	Double-stranded RNA
E:	Evening
EMS:	Ethylmethanesulfonate
GRASP:	GFP Reconstitution Across Synaptic Partners
HB eyelet:	Hofbauer-Buchner eyelet
HID:	Head involution defective
ITP:	Ion Transport Peptide
JET:	Jetlag
LD:	Light-dark

LL:	Constant light
LNds:	Dorso-lateral neurons
LNvs:	Ventro-lateral neurons
LPNs:	Lateral posterior neurons
LRR:	Leucine Rich Repeats
ls-*tim*:	Long-short *tim*
M:	Morning
NPF:	Neuropeptide F
PAS:	Per-Arnt-Sim
PDF:	Pigment dispersing factor
PDP1:	PAR domain protein 1
PER:	PERIOD
PI:	Pars intercerebralis
PKA:	Protein kinase A
PP2A:	Protein phosphatase 2A
PRC:	Phase response curve
PTC:	Phase transition curve
QSM:	Quasimodo
Rh:	Rhodopsin
RNAi:	RNA interference
s-tim:	Short *tim*
SCF:	Skp1/Cullin/F-box

SGG:	SHAGGY
sNPF:	Small Neuropeptide F
TIM-LUC:	TIM-LUCIFERASE
TIM:	TIMELESS
UAS:	Upstream Activation Sequence
VRI:	VRILLE
ZT:	Zeitgeber time

Preface

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Contributions of the authors are addressed at the beginning of each chapter

Chapter I

Introduction

1. Circadian rhythms and their fundamental properties

Daily rotation of the Earth around its axis results in cyclical variations in the physical properties of the environment such as light intensity and temperature. Similarly, most organisms also display daily changes in their physiological and behavioral processes such as sleep-wake cycles, oscillation in hormone levels and reproductive cycles. These every day changes in the physiology and behavior are not a mere reaction to daily fluctuations in the environment, but arise from internal time keeping mechanisms referred to as circadian clocks or rhythms (from the Latin term *circa dies*, meaning – about a day) which run with a periodicity of about 24 hours. The earliest published study on circadian rhythms dates back to 1729, when a French astronomer Jean-Jacques d'Ortous de Mairan observed a 24 hour pattern in opening and closing of leaves of a heliotrope plant in continuous darkness, suggesting that leaf movements were controlled by an internal clock.

Circadian rhythms are characterized by three important features, which are as follows:

- Endogenous: Circadian rhythms are self sustained and persist with a period of approximately 24 hours even in the absence of any environmental inputs.
- Temperature compensated: These rhythms maintain their 24-hour periodicity over a broad range of physiologically relevant temperatures unlike other biochemical processes, which increase their rate with rise in temperature. This feature allows circadian clocks to precisely measure time despite seasonal changes in temperature.
- Entrainable: Despite being inherent, these rhythms are synchronized and reset by

various environmental time cues such as light (Pittendrigh 1967), temperature (Glaser and Stanewsky 2005; Liu 1998; Pittendrigh 1954; Yoshii et al. 2005), social (Fujii et al. 2007; Levine et al. 2002a) and olfactory cues (Castillo et al. 2004; Schibler et al. 2003) This ability of circadian clocks to entrain to the environment allows appropriate timing of behavior and physiology and confers maximum survival advantage to the organisms.

1.1 Circadian parameters

Figure 1.1 represents a physiological or behavioral rhythm (e.g., body temperature or locomotor activity respectively) generated by a circadian clock and the various parameters of a rhythm, which can be studied:

Period length (represented by τ) – refers to the time taken to complete one oscillation i.e., the time interval between two peaks or two troughs. Period length is measured under constant conditions in the absence of any environmental inputs, also referred to as free-running period.

Amplitude (A) – refers to the difference in the level between the peak and trough values of the oscillation. Amplitude usually gives a measure of the strength of the rhythm.

Phase (Φ) – represents a stage or time location in an oscillation. Phase can be measured by timing of the peak, trough, onset or offset of the peak.

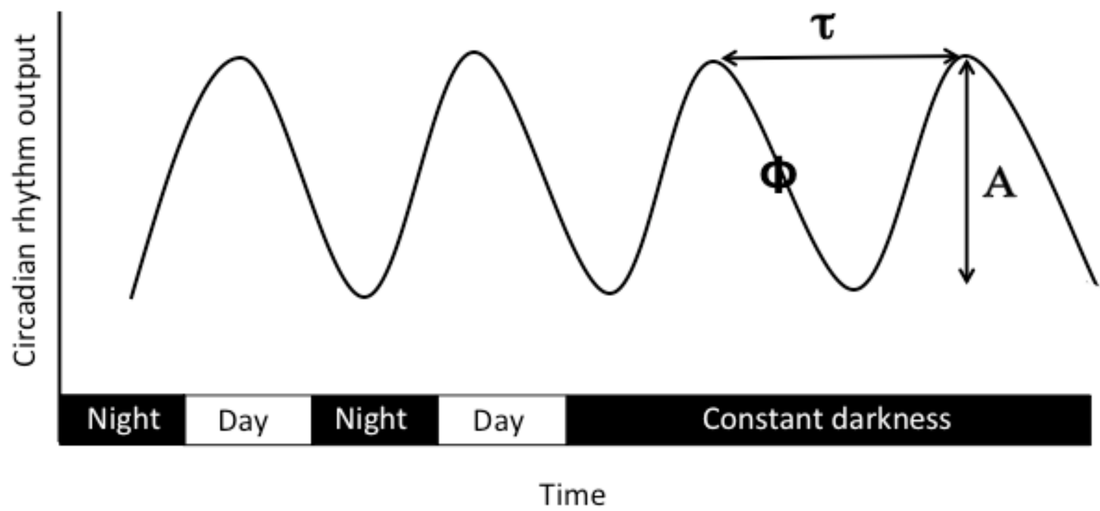

Figure 1.1 Parameters of circadian oscillation

The plot represents a circadian rhythm in which the level of a particular output (e.g., body temperature or locomotor activity) is plotted on y-axis as a function of time. Different circadian parameters are indicated on the plot. A - amplitude, Φ - phase, τ - period length. Note that the rhythm persists in constant darkness, a condition under which period length is generally measured.

1.2 Significance of circadian rhythms

Circadian clocks are nearly ubiquitously present in organisms ranging from unicellular cyanobacteria to highly complex mammals such as human beings (Czeisler et al. 1999; Ouyang et al. 1998). These clocks allow the organisms to anticipate and prepare for changes in the environment associated with the day-night cycle. For example, in cyanobacteria, the rhythmic strains have enhanced reproductive fitness over arrhythmic strains when placed in a day-night cycle (Woelfe et al. 2004, Ouyang et al. 1998). Timing behavior according to the external environment is also crucial for other organisms such as predators that hunt prey when they are most active and organisms feeding on similar diet avoid competition by foraging at different times (Rusak and Zucker 1975). Ostriches provide an example for advantage of circadian rhythms in the context of camouflage, where the black colored male sits on the nest at night while the pale brown colored female sits during the day to incubate the eggs (Rusak and Zucker 1975). Hence, circadian rhythms help organisms to cope up with the daily challenges in the environment and maximize their chances of survival.

Circadian rhythms have immense implications on human health as well. First, misalignment of the internal circadian clock to the external time results in negative health outcomes such as increased risk for cancer (Baldwin and Barrett 1998; Blask et al. 2005; Levi et al. 2000; Erren and Reiter 2008), obesity and type - 2 diabetes (Scheer et al. 2009; Spiegel et al. 2008) observed in night shift workers. Another unfavorable outcome of desynchronized circadian clocks is observed while travelling across different time zones commonly known as jetlag. It is characterized by feelings of fatigue, sleep difficulties,

digestive troubles and reduced efficiency and it takes a few days to recover while the circadian clocks get adjusted to the new time zone (Barion et al., 2009). Second, alteration of circadian rhythms due to seasonal fluctuations in the environment leads to mood disorders such as seasonal affective disorders or winter depression (Lewy et al., 20060. Third, circadian rhythms also determine susceptibility to various pathologies. For instance, its been shown that myocardial infarction has a three fold higher chance of occurrence during morning compared to late evening (Braunwald 2012). Fourth, disruption of circadian rhythms has been linked to increased vulnerability to and progression of neurodegeneration (Hood and Amir 2017). Hence, understanding the mechanisms that generate circadian rhythms and synchronize them to the environment could facilitate in developing new therapeutic treatments for diseases associated with dysfunctional circadian clocks and designing new strategies to alleviate health condition of shift workers.

2. *Drosophila* as a model for studying circadian rhythms

The insect, *Drosophila melanogaster* commonly known as fruit fly has been a seminal model organism in dissecting the genetic, molecular and neural mechanisms that generate circadian rhythms and synchronize them to the environment. In fact a variety of behaviors in *Drosophila* are regulated by circadian clock such as - eclosion (emergence of adult fly from pupa) (Konopka and Benzer 1971), period of rest and activity, olfactory sensitivity (Krishnan et al. 1999), egg laying (Manjunatha et al. 2008), courtship (Fujii et al. 2007; Fujii et al. 2010), gustatory sensitivity (Chatterjee et al. 2010) and learning and memory (Lyons et al. 2009).

The use of *Drosophila* as a model organism for circadian rhythm research dates back to work of Colin Pittendrigh who used various species of *Drosophila* to demonstrate that eclosion (which peaks around dawn) is controlled by a true biological clock and satisfy the criteria of being endogenous, entrainable and temperature compensated (Pittendrigh 1954, 1993).

In fact the first circadian gene – *period* was identified by Seymour Benzer and his student Ron Konopka in fruit flies (Konopka and Benzer 1971). After chemical mutagenesis, they screened flies for abnormalities in eclosion rhythms. They isolated three mutant strains - one that had a longer period (29 hours), one with a shorter period (19 hours) and one, which did not show any rhythm in eclosion (Konopka and Benzer 1971). All three mutations mapped to the same genetic locus located on the X-chromosome. This genetic locus was called *period* (*per*) and the mutant fly strains were termed per^{Long}, per^{Short} and per^{0}. Later, *per* gene was also found to be conserved in higher organisms such as mice and humans (Sun et al. 1997; Tei et al. 1997; Zylka et al. 1998).

2.1 Advantages of using *Drosophila* as a model organism

Drosophila has a short generation time of 10-14 days at 25°C and is easy to maintain in lab. Despite a smaller genome of 175Mbp, about 60% of the genes are conserved between *Drosophila* and humans. Additionally, it is relatively simple to create transgenic fly lines and achieve efficient gene silencing by RNA interference (RNAi). Another powerful tool that has accelerated the use of *Drosophila* as a model organism is the development of GAL4/UAS system, which allows spatio-temporal regulation of gene expression.

2.2 The GAL4/UAS system

The GAL4/UAS system enables expression of genes of interest in a cell/tissue specific manner (Duffy 2002; Brand et al. 1994). It consists of two components:

1. The driver transgenic line expressing the yeast transcriptional factor, GAL4, under the control of a *Drosophila* cell/tissue specific promoter.

2. The responder transgenic line carrying the gene of interest controlled by the GAL4 binding sites - Upstream Activator Sequences (UAS). Target gene expression is achieved by crossing the driver GAL4 and the responder UAS lines together. In the progeny, GAL4 binds to the UAS sites and promotes expression of gene of interest in a spatial pattern determined by the promoter of GAL4 (Fig. 1.2 A).

This bi-partite system where the parental driver and responder lines can be maintained separately allows the analysis of single UAS-transgene in multiple tissues or cells at the same time using different GAL4 drivers. Also, since the UAS responder line is transcriptionally silent without the GAL4, transgenics expressing toxic proteins such as the proapoptotic gene – "head involution defective" (*hid*) can be generated. Rescue experiments by restoring endogenous gene expression in a mutant background or gene silencing by expression of dominant negative or RNAi constructs can be efficiently achieved.

To further refine the spatial expression pattern of GAL4 driven transgene, sometimes GAL80, which binds to the transactivation domain of GAL4 and prevents GAL4 from activating transcription, is also included under a different promoter (Lee and Luo 1999) (Fig 1.2 B). Further, temporal control of GAL4 driven expression of the gene

of interest is possible by expressing a temperature sensitive version of GAL80 ($GAL80^{ts}$) under a ubiquitous promoter such as *tubulin*. $GAL80^{ts}$ is active at 18°C but not at 29°C or beyond, hence, $GAL80^{ts}$ represses GAL4 driven transgene expression at lower temperature and allows expression only at higher temperature (McGuire et al. 2004). For instance, allowing $GAL80^{ts}$ activity at lower temperatures during development and inactivating it at higher temperature only after eclosion, provides a way to avoid developmental defects and conditionally express a toxic protein such as HID specifically during adulthood. Various modifications of GAL4/UAS system are now available to precisely control transgene expression. Another dual binary system, which is commonly used in combination with the GAL4/UAS, is LexA/LexAop system for improved spatial restriction (Lai and Lee 2006). Lex A is a bacterial transcriptional factor, which binds to and activates the LexA operator (LexAop) and functions in a manner analogous to the GAL4/UAS system.

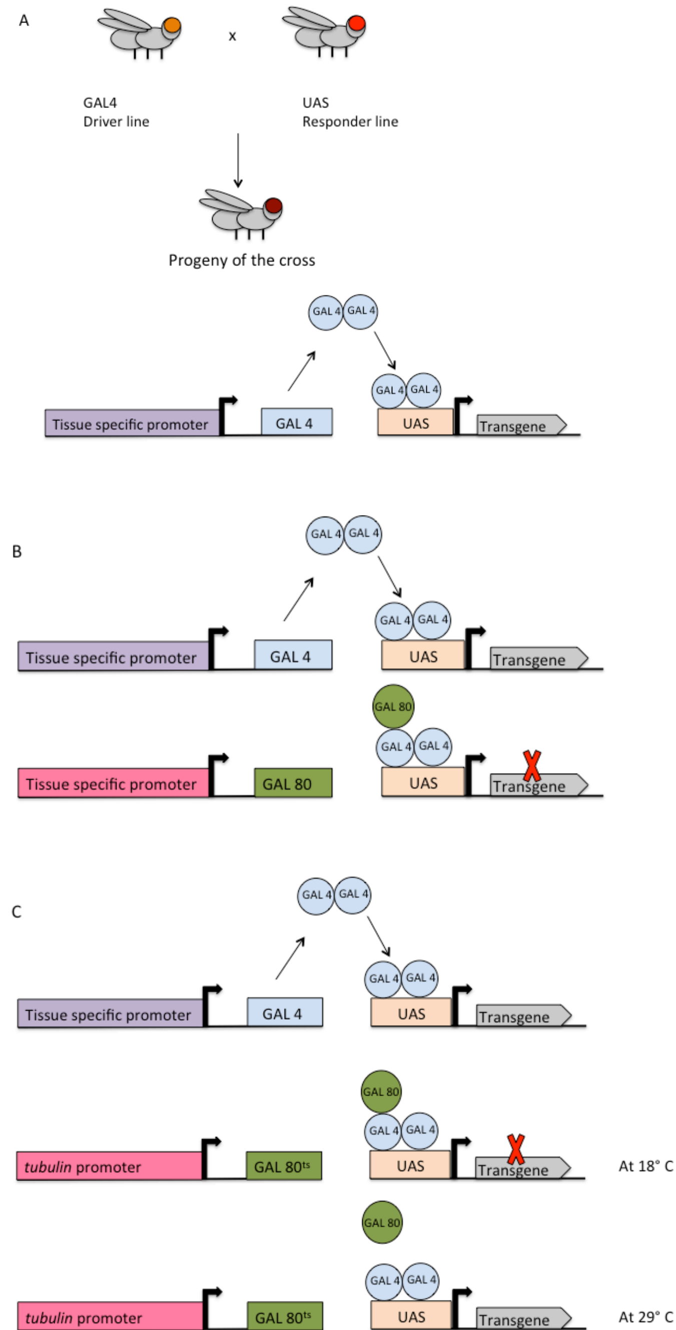

Figure 1.2 The GAL4/UAS system

A. The driver line expressing GAL4 under a tissue specific promoter is crossed to the responder line carrying the transgene under the control of UAS. In the progeny of this cross, GAL4 binds to UAS sites and promotes transcription of the transgene in a spatial manner determined by the GAL4 promoter (purple).

B. GAL80 is a repressor of GAL4. It is can be combined with GAL4 and UAS lines to refine the spatial expression pattern of the transgene. GAL80 is expressed under a promoter (in pink) different from GAL4 promoter (purple). Transgene expression is prevented in tissues where GAL80 is expressed.
C. For temporal control of transgene expression, a temperature sensitive version of GAL80 is often expressed under a ubiquitous promoter. At 18°C, GAL80 is active, represses GAL4 and inhibits transgene expression. At 29°C, GAL80 is inactive and allows transgene expression.

2.3 Assaying locomotor rhythms in *Drosophila*

Even though eclosion rhythm has a historical significance in establishing the field of chronobiology, it is a population rhythm and occurs only once in a lifetime of an individual fly. Hence, a more robust behavioral read out commonly used for study of circadian rhythms is locomotor activity that also occurs in a rhythmic fashion.

Circadian locomotor behavior in *Drosophila* is monitored by placing individual flies in a small glass tube with food at one end and a stopper at the other end (Fig 1.3). These tubes are loaded into activity monitors produced by Trikinetics and housed in incubators with controlled light, temperature and humidity conditions, which are then connected to a computer. The activity monitors are equipped with infrared emitters and receivers. An infrared beam, which is aimed at the center of the glass tube, is broken every time the fly moves across the tube. Locomotor activity is measured by number of infrared beam breaks per unit time (we usually measure locomotor activity in our laboratory in a half an hour window) (Rosato and Kyriacou 2006; Chiu et al. 2010).

Locomotor activity rhythm in laboratory conditions is assessed by subjecting flies to 12 hours of light and 12 hours of darkness (12:12 LD) to mimic a natural day-night cycle. Under 12:12 LD conditions, flies display two peaks of locomotor activity, one in the anticipation of lights-on which is referred to as the Morning (M) peak and one 3-4 hours before the lights are turned off which is referred to as the Evening (E) peak. The M and the E peaks of activity are not a response to lights-on and lights-off respectively because flies start increasing their activity before the lights are turned on or off suggesting that the bi-modal pattern of activity in a LD cycle is under the circadian clock

control. This locomotor activity pattern under LD can be plotted as a function of time and is best visualized by a histogram known as “Eduction”.

However, to gain insight into the functioning of the endogenous clock, which persists in the absence of any external input, flies after being synchronized to 3-4 days of 12:12 LD cycle are released into constant darkness (DD) for 5-6 days. In DD conditions, wild type flies generally exhibit a unimodal activity peak, which occurs approximately at the same time every day reflecting the 24-hour period length of locomotor activity rhythm. This activity pattern can be represented via a double plotted “Actogram”. Actogram is a plot of daily activity with the time of the day on x-axis and number of days in an experiment on the y-axis. In a double plotted actogram, each day is plotted twice, first on the right half and then on successive line on the left half of the plot. Actograms can sometimes be plotted as single or as triple plots. Circadian parameters such as period length, amplitude and phase can be observed using an actogram. Activity pattern of flies with a period length shorter than 24 hours seems to drift towards the left whereas that of the long period flies drifts towards the right on successive days of the record.

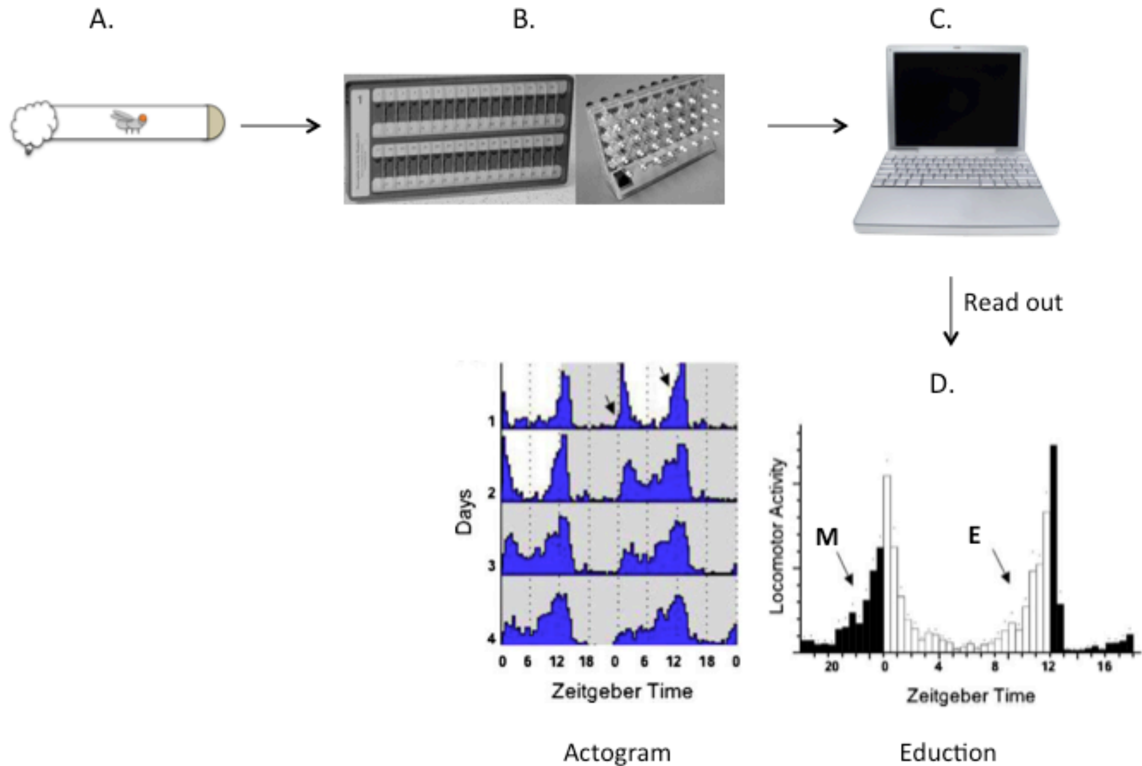

Figure 1.3 Assay for monitoring locomotor activity rhythms in *Drosophila*

(A) Individual flies are loaded into glass tubes with food at one end and a cotton plug at the other end. (B) These glass tubes are then placed into *Drosophila* activity monitors The monitors are placed into an incubator (with controlled light, temperature and humidity conditions) which is connected to a computer. (C) Each time a fly moves in the tube, there is a break in the infrared beam, which is recorded by the computer. (D) Locomotor activity can be represented as a double-plotted Actogram (left) or Eduction (right). In the actogram, the white portion indicates light and grey portion indicates darkness, after entrainment to LD cycles, flies are released into constant darkness (grey portion). In a double-plotted Actogram, the locomotor activity records of 2 days are plotted next to one another on each horizontal line. The activity on the "second day" is first plotted on the far right of each line and then at the start of the subsequent horizontal line resulting in the duplication of displayed data. This double plotting helps to visualize deviations from the 24-hour period length (e.g. short and long period). The activity in the LD cycle is best visualized by Eduction. The black bars show activity in night and white bars indicate activity during the day. Increase in the activity in anticipation of lights-on is indicated by morning (M) peak and anticipation of lights-off is indicated by evening (E) peak.

3. Molecular basis for circadian rhythms

In *Drosophila*, circadian rhythms are generated by an endogenous molecular clock, which is based on a negative feedback transcription-translation loop that drives the coordinated oscillation of two clock proteins – PERIOD (PER) (Konopka and Benzer 1971) and TIMELESS (TIM) (Myers et al. 1995; Sehgal et al. 1994). Two basic-helix-loop-helix (bHLH)/Per-Arnt-Sim (PAS) domain containing transcription factors - CLOCK (CLK) (Allada et al. 1998) and CYCLE (CYC) (Rutila et al. 1998) heterodimerize and bind to E-box sequences in the promoter of *per* and *tim* to initiate their transcription (Darlington et al. 1998) (Figure 1.4). The transcript levels of *per* and *tim* peak during early night but the protein levels do not peak until late in the night. This delay in the peaking of protein levels is generated by phosphorylation of PER by a kinase, DOUBLETIME (DBT) (Price et al. 1998) which is a mammalian ortholog of CASEIN KINASE 1ε/δ (CK 1ε/δ) (Kloss et al. 1998) and makes PER prone to degradation by SLIMB an E3 ubiquitin ligase (Grima et al. 2002; Ko et al. 2002).

Hence, PER is unstable on its own until TIM accumulates in the cytoplasm, binds and stabilizes phosphorylated PER which is still bound to DBT (Gekakis et al. 1995; Vosshall et al. 1994). PER is also stabilized by protein phosphatase 2A (PP2A) (Sathyanarayanan et al. 2004). TIM-PER-DBT complex then enters the nucleus upon SHAGGY (SGG) dependent phosphorylation of TIM and CKII mediated phosphorylation of PER (Martinek et al. 2001; Lin et al. 2002).

Once inside the nucleus, the PER/TIM complex prevents the CLK-CYC heterodimer from binding to the *per* and *tim* promoter, by reducing CLK-CYC

heterodimer's affinity for E-boxes in the promoter and thus inhibit their own gene transcription (Darlington et al. 1998; Lee et al. 1999; Menet et al. 2010; Yu et al. 2006). There is a sharp decrease in TIM levels early in the morning, due to which PER is no longer stable, becomes prone to phosphorylation by DBT and ultimately gets degraded. This lifts the repression caused by TIM-PER-DBT COMPLEX on CLK-CYC binding to the E-boxes and begins a new round of *per* and *tim* transcription. This entire cycle takes about 24 hours to complete and hence generates circadian rhythms.

It is clearly evident that there are several lags in this molecular cycling of PER and TIM. First, PER is unstable on its own and only accumulates after binding to TIM and hence protein levels peak several hours after the mRNA levels. Another lag in this loop is the delay in the nuclear entry of the TIM-PER-DBT complex, which occurs after phosphorylation by SGG and CK2. These delays ensure that mRNA synthesis and repression are never in equilibrium and thus generate molecular oscillation of PER and TIM. These lags are mainly due to phosphorylation events, which affects protein stability as mentioned above, although recently, O-GlcNAcylation of serine/threonine residues on PER and CLK has been shown to provide another level of post-translational control. This modification can compete with phosphorylation of PER and affect both its stability and nuclear entry (Kaasik et al. 2013; Kim et al. 2012).

The molecular oscillation of PER and TIM (with low levels during the day and high levels during the night) has been detected by assays such as immunohistochemistry in photoreceptors, subsets of neurons and groups of glia in the central nervous system and in several peripheral tissues such as alimentary tract, Malpighian tubules and parts of the

reproductive system (Giebultowicz 2000). Assays based on reporter gene expression driven by clock gene promoters have revealed that the peripheral tissues possess cell autonomous oscillators, which can generate endogenous rhythms even when kept in culture conditions (Plautz et al. 1997; Hege et al. 1997; Emery et al. 1997).

A second loop, which is interlocked with the major feedback loop controls *clk* transcription levels. CLK-CYC heterodimers bind to E-box in the promoter of two additional genes – *vrille (vri)* and PAR *domain protein 1(pdp1)* during late day to early night (Blau and Young 1999; Cyran et al. 2003; McDonald et al. 2001). VRI is a repressor and PDP1 is an activator of *clk* transcription (Cyran et al. 2003; Glossop et al. 2003). VRI levels accumulate in phase with its mRNA levels and binds to VRI/PDP1ε (V/P) regulatory element in the promoter region of *clk* to inhibit its transcription. PDP1 accumulates in a delayed manner and high PDP1 levels displace VRI from V/P region and promote *clk* transcription. This second *clk* feedback loop is believed to bring greater stability to the major feedback loop. Other proteins such as CLOCKWORK ORANGE (Kadener et al. 2007; Lim et al. 2007; Matsumoto et al. 2007) and KAYAK α (Ling et al. 2012) have been shown to regulate transcription in both the feedback loops and hence provide greater precision to the molecular pacemaker.

The negative transcription feedback loop lies at the heart of the circadian molecular pacemaker. This core mechanism of clock proteins inhibiting transcription of their own genes is conserved across all the organisms ranging from cyanobacteria to humans (Dunlap, 1999). In fact, not only the basic principle but also some of the clock genes and their functions are conserved. For example, *Drosophila* homologs of CLK,

CYC (BMAL1 in mammals), PER and DBT (CK 1ε/δ in mammals) serve similar function in mammals (Helfrich-Förster 2004). However, there are important distinctions between the two systems. In mammals, CRYPTOCHROME 1 and 2 (CRY1 and CRY2), which belong to a class of flavoproteins interact with mammalian PERs (Griffin et al. 1999; Kume et al. 1999) and repress CLK/BMAL1 mediated transcription (Kume et al. 1999). This is in contrast to *Drosophila*, where CRY (Type I) mainly acts as an intracellular photoreceptor (Stanewsky et al. 1998; Emery et al. 1998) (See section 5.1.2.2). Interestingly, in non-drosophilid insects such as Monarch butterflies (*Danaus plexippus*), their circadian pacemaker is like a hybrid between the "mammalian" and *Drosophila* type pacemaker. Light is perceived by CRY1 (similar to *Drosophila*) and CRY2 is a part of PER/TIM complex and represses the CLK/CYC mediated transcription (like mammals) (Zhu et al. 2005, 2008).

Even though the molecular mechanism controlling circadian rhythms in different organisms can vary, the basic principle of negative transcription-translation feedback loop is conserved, thus suggesting the importance of circadian rhythms in controlling physiology and behavior across different life forms.

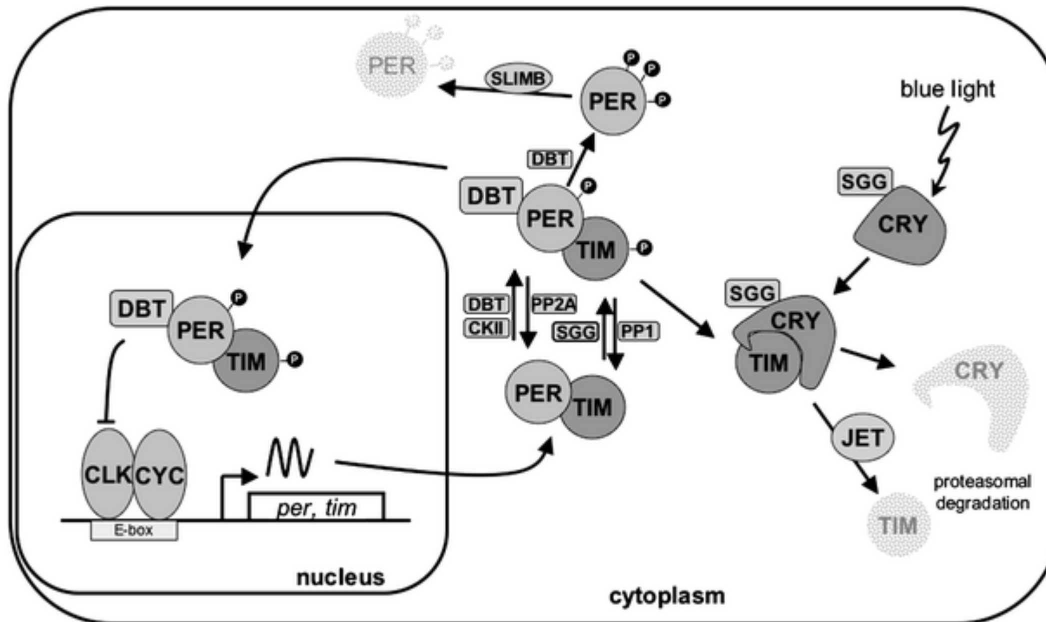

Figure 1.4 The *Drosophila* circadian molecular pacemaker and the CRY light input pathway

CLOCK (CLK) and CYCLE (CYC) heterodimerize and bind to E-boxes to promote transcription of *period (per)* and *timeless (tim)*. P, represents phosphate groups. Different kinases and phosphatases regulate stability and timely nuclear entry of PER and TIM which then inhibit their own transcription. The photoreceptor CRYPTOCHROME (CRY) changes conformation upon exposure to light and promotes degradation of TIM via JETLAG (JET). The dotted shapes indicate proteins undergoing proteasomal degradation. This figure is adapted from (Dubruille and Emery 2008) with permission.

4. Neuronal circuitry controlling circadian rhythms in *Drosophila.*

The above-mentioned molecular clocks are located in a set of 150 neurons (out of 250,000) in *Drosophila* brain, which drive circadian behavioral rhythms including locomotor activity (Kaneko and Hall 2000; Kaneko et al. 1997; Shafer et al. 2006). These circadian neurons were identified based on the rhythmic expression of clock proteins or reporters driven by promoters of clock genes such as *per* and *tim*. These clock neurons are classified into six major groups based on their anatomical location. – the ventro-lateral neurons (LNvs), the dorso-lateral neurons (LNds), three groups of dorsal neurons (DNs) and lateral posterior neurons (LPNs). The LNvs are further subdivided into large LNvs (l-LNvs) and small LNvs (s-LNvs) based on the their soma size. All the LNvs except the fifth s-LNv, express a neuropeptide – Pigment dispersing factor (PDF) (Helfrich-Förster 1995; Renn et al. 1999). Figure 1.5 shows the location of the circadian neurons in *Drosophila* brain and Table 1.1 shows the expression pattern of GAL4 drivers used in this dissertation to manipulate gene expression in different groups of clock neurons.

4.1 Arborization pattern of circadian neurons

Neuroanatomical studies suggest that almost all the circadian neurons except l-LNvs project towards the dorsal protocerebrum (Helfrich-Förster 2005), which houses the pars intercerebralis (PI) neurons - the neurosecretory center of the adult fly that are functionally and developmentally analogous to mammalian hypothalamus (Helfrich-Förster 2005; Helfrich- Förster et al. 1998). Cyclical release of hormones from PI neurons contribute to locomotor behavioral rhythms. The dendritic fibers of s-LNvs and

l-LNvs arborize in the accessory medulla (aMe) region and l-LNvs connect both the aMe by sending projections in the vicinity of contralateral l-LNvs (Helfrich-Förster 2005; Kaneko and Hall 2000). Some of the DN1s and LNds project ventrally towards the dendrites of s-LNvs as well (Johard et al. 2009; Shafer et al. 2006; Zhang et al. 2010). Neurites of clock neurons extensively overlap with each other suggesting cross communication amongst these neurons. In fact, s-LNvs have been shown to form synaptic connections with the LNds and DN1s based on GFP Reconstitution Across Synaptic Partners (GRASP) studies (Gorostiza et al. 2014; Guo et al. 2014).

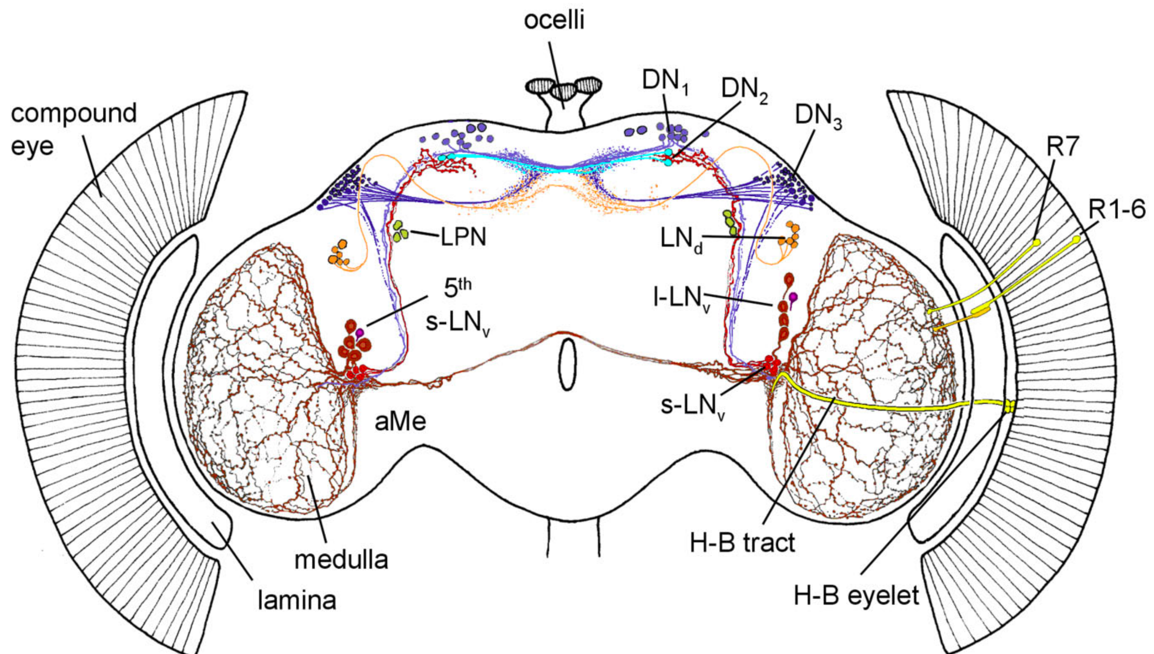

Figure 1.5 Neuronal circuitry controlling circadian rhythms in *Drosophila*

The figure depicts *Drosophila* brain with a set of 150 circadian neurons and their arborization pattern. The lateral neurons are depicted in red/orange, dorsal neurons in blue and LPNs in green. aMe - accessory medulla, is the region where the PDF positive LNvs are present. The CRY independent, opsin based visual system organs are also indicated. The photoreceptors R1-6 and R7 appear to contact the PDF positive dendritic arbor in the optic lobe and HB eyelet seems to contact the s-LNvs. This figure is adapted from (Helfrich-Förster et al. 2007) with permission.

Table 1.1 GAL4 and GAL4/GAL80 combination driven gene expression pattern in different groups of clock neurons

Driver *GAL 4* lines/repressor transgenes	s-LNvs (4)	5th s-LNv (1)	l-LNvs (5)	LNds (6)	DN1s (~17)	DN2s (2)	DN3s (~40)
tim-GAL4	+	+	+	+	+	+	+
Pdf-GAL4	+	-	+	-	-	-	-
Mai179-GAL4	+	+	-	+ (3)	-	-	-
Mai179-GAL4; Pdf-GAL80	-	+	-	+ (3)	-	-	-
DvPdf-GAL4	+	+	-	+ (4)	-	-	-
DvPdf-GAL4; Pdf-GAL80	-	+	-	+ (4)	-	-	-
cry-GAL4 (13)	+	+	+	+	+ (4)	-	+ (2)
Pdf-GAL80; cry-GAL4 (13)	-	+	-	+	+ (4)	-	+ (2)
c929-GAL4	-	-	+	-	-	-	-
Clk4.1M-GAL4	-	-	-	-	+ (6-7)	-	-

The GAL4 drivers used in this dissertation to target gene expression in different groups of circadian neurons are indicated. The total number of neurons in each group per brain hemisphere is indicated below the neuron type. "+" means that the GAL4 driver is expressed in the neuronal group , number in the bracket indicates number of neurons amongst the group with positive expression the "-" means no expression. *Mai179-GAL4* is very weakly expressed in four DN1s (Picot et al. 2007). The expression of *cry-GAL4(13*) is as reported by Shafer et al. 2006. Amongst the DNs, *cry-GAL4(13*) is expressed in 2 DN1as and 2 DN1ps.

4.2 Role of circadian neurons in controlling behavior

4.2.1 The small ventro-lateral neurons (s-LNvs) or the Morning oscillators

Amongst all the clock neurons, the PDF positive s-LNvs are the most important group of neurons for maintaining rhythms under constant conditions (Renn et al. 1999). These neurons are also responsible for driving the morning peak of activity in a LD cycle and hence are also referred to as the Morning (M) cells or oscillators (Grima et al. 2004; Stoleru et al. 2004). The intracellular Ca^{2+} levels (reflecting neuronal activity) peak in the s-LNvs about 2 to 4 hours before the morning peak of activity further confirming their role as M-oscillators (Liang et al. 2016). The evidence for the importance of PDF positive s-LNvs as M-oscillators in LD and pacemaker neurons in constant conditions comes from various studies. First, rescue of PER expression specifically using *Pdf-GAL4* (which is expressed in the 4 s-LNvs and all the l-LNvs) in an arrhythmic per^0 mutant was sufficient to restore rhythmicity in DD and the M-peak in LD cycle (Grima et al. 2004). The large PDF neurons do not contribute towards M-peak or 24 hour rhythms in DD because *per* expression restricted to s-LNvs (using *Mz520-GAL4* and *R6-GAL4*) and not l-LNVs (using *c929-GAL4*) was sufficient to rescue both LD and DD behavioral defects (Cusumano et al. 2009; Grima et al. 2004). Second, genetic ablation of s-LNvs by expressing the proapoptotic genes (such as *hid* and *bax*) (Blanchardon et al. 2001; Renn et al. 1999) or electrically silencing them by expressing a constitutively open K^+ channel (Nitabach et al. 2002) rendered flies arrhythmic in DD after several days and flies could no longer anticipate the lights-on transition in LD. Third, the *disconnected (disco)* mutants which retain the dorsal neurons but lack the lateral neurons due to a

developmental defect are behaviorally arrhythmic (Dushay et al. 1989; Helfrich-Förster 1998). Fourth, s-LNvs maintain molecular rhythms in their core clock machinery, which persists for several days in DD, whereas the l-LNvs, which are also PDF positive do not seem to contribute to rhythmic activity in LD or DD and their molecular oscillations dampen rapidly in DD (Shafer et al. 2002; Veleri et al. 2003; Yang and Sehgal 2001).

4.2.1.1 Pigment dispersing factor (PDF)

The s-LNvs require a neurotransmitter, PDF, to maintain rhythmicity under constant conditions. *Pdf*^{0} null mutant flies exhibit a phenotype identical to flies with genetically ablated s-LNvs i.e., no M-peak in LD and arrhythmicity after 1-3 days in DD (Renn et al. 1999). s-LNvs rhythmically release PDF from their axonal terminals to propagate the time of the day information to their downstream target neurons in the dorsal brain (Park et al. 2000). In fact overexpression of PDF in certain brain regions results in arrhythmicity or complex locomotor behavior (Helfrich-Förster et al. 2000). Also the axonal morphology of the PDF circuit is under circadian control with extensive arborization in early morning and a closed conformation during night. Hence, s-LNvs form synaptic contacts with the downstream neurons in a time-dependent manner to regulate their rhythmic activity (Ceriani et al. 2008).

PDF is a highly conserved 18 amino acid α-amidated neuropeptide present in various insects' nervous system to control behavior and physiology, but its circadian timekeeping function is best understood in *Drosophila.* PDF is crucial for synchronizing the molecular oscillation of clock proteins in other circadian neurons including s-LNvs and thus generating coherent rhythmic output under constant conditions (Lin et al. 2004;

Renn et al. 1999; Yoshii et al. 2009). Loss of PDF causes the molecular clock to run faster in some clock neurons and dampens in others resulting in desynchronization between individual cells as they fall out of phase with each other (Stoleru et al. 2005; Yao and Shafer 2014) resulting in arrhythmicity in behavior.

The receptor for PDF (*PdfR*) is a G-protein coupled receptor of the secretin receptor subfamily. PDF binding to the PDFR activates Gαs (Choi et al. 2012; Zhang and Emery 2013) which in turn activates adenylyl cyclases and increases the levels of cyclic AMP (cAMP) (Mertens et al. 2005; Shafer et al. 2008). Although the exact mechanism by which PDF signaling sets the pace of molecular clock in downstream neurons is still not very clear, biochemical and genetic evidence suggests that increases in cAMP may act through Protein kinase A (PKA) to enhance the stability of PER and TIM, thus changing the phase and period of molecular clocks in the target neurons (Li et al. 2014; Seluzicki et al. 2014). Real-time live imaging using a cAMP sensor shows that most neuronal clusters in the circadian network including s-LNvs respond acutely to bath applied PDF (Shafer et al. 2008). Flies with mutation in *PdfR* behave similarly to Pdf^{0} flies with compromised endogenous circadian rhythms and rescue of *PdfR* expression in circadian neurons was sufficient to restore rhythmicity in DD (Hyun et al. 2005; Lear et al. 2005). This evidence further suggested that PDF is important for coordinating the neurons in the circadian network to generate robust endogenous rhythms. However, PDFR expression is not homogenous amongst the circadian circuit. It is expressed in only half of the LNds, 5th s-LNv, less than half of the DN1ps and DN3s, both DN1as and DN2s and in PDF positive LNvs (all s-LNvs and 2 l-LNvs) themselves (Im and Taghert

2010; Yao and Shafer 2014).

In addition to PDF, s-LNvs also express another neuropeptide - small Neuropeptide F (sNPF) (Johard et al. 2009) and have been shown recently to be glycinergic (Frenkel et al. 2017) as well. sNPF is known to have a sleep promoting role and can function as an inhibitory signal to the motor centers, possibly working as an output of the circadian system (Beckwith and Ceriani 2015). Frenkel et al. (2017) showed that, glycine contributes to synchronization of the circadian network and maintaining rhythmicity in DD. However, the effect of both sNPF and glycine on locomotor activity rhythms is not as prominent as PDF.

4.2.2 The dorsolateral neurons (LNds) and the 5th s-LNv or the Evening oscillators

The LNds and the PDF negative 5th sLNv are together referred to as the evening (E) cells because they drive the evening peak of activity in the anticipation of lights-off in an LD cycle (Grima et al. 2004; Stoleru et al. 2004). The LNds are a group of six neurons per hemisphere that are heterogeneous in terms of their neuropeptide and PDFR expression pattern (Yao and Shafer 2014). There is one PDF negative 5th s-LNv in each brain hemisphere. Rescue of PER expression using E cells specific drivers *Mai179-GAL4* or *DvPdf-GAL4* (Table 1.1) in *per^0* flies restored the evening anticipation in a LD cycle (Grima et al. 2004; Guo et al. 2014). Ablation of the E cells results in loss of evening anticipation with no effect on the M peak (Stoleru et al. 2004). However, recently it has been shown that electrically silencing the E cells disrupts the evening as well as the morning anticipation in LD and reduces overall rhythmicity in DD indicating that in addition to pacemaker M cells, E cells also play an important role in maintaining

endogenous rhythms (Guo et al. 2014). In fact, these E cells also display sustained molecular oscillations in DD for several days (Veleri et al. 2004). E cells have also been shown to control both the morning and evening peaks of activity in long photoperiod/summer like LD conditions and light/moonlight cycles instead of LD. Such ability of E cells might be important for adaptation to seasonal fluctuations in day length (e.g., summer vs. winter) in the environment (Stoleru et al. 2007).

The identity of the signals released from the E cells to drive the evening peak or to maintain the rhythmicity in DD is not very well established. However, Neuropeptide-F (NPF) has been implicated in regulating the timing as well as the amplitude of evening anticipation (Hermann et al. 2012). Another neuropeptide - Ion Transport Peptide (ITP) has been shown to modulate the evening peak of activity in LD and activity rhythms in DD. Knocking down ITP reduces the evening peak of activity and over expression results in rhythmicity defects in DD (Hermann-Luibl et al. 2014).

4.2.3 Dorsal neurons (DNs)

There are three groups of dorsal neurons - DN1s, DN2s and DN3s. The DN1s can be further divided into two groups based on their location - DN1as (anterior) and DN1ps (posterior). There are two DN1as and are the only known circadian neurons that express the neuropeptide IPNamide (Shafer et al. 2006). On the other hand, there are approximately 15 DN1ps which express a transcription factor GLASS that is necessary for visual photoreceptor differentiation (Klarsfeld et al. 2004; Moses et al. 1989; Veleri et al. 2003). *glass*^{60j} mutants which lack all the DN1ps have lower amplitude rhythms suggesting that DN1ps might be required for robustness of the rhythms in DD (Helfrich-

Förster et al. 2001) . However, the molecular rhythms in the DN1s dampen very quickly in DD and they cannot generate behavioral rhythms independently in DD (Veleri et al. 2003). Nevertheless, they are still considered an important class of neurons in circadian control of locomotor activity. The pacemaker s-LNvs make synaptic contacts with the DN1s (Cavanaugh et al. 2014; Gorostiza et al. 2014), which then form synapses with the PI neurons that ultimately control the locomotor activity (Beckwith and Ceriani 2015; Cavanaugh et al. 2014; Charlotte Helfrich-Förster 2005). Hence DN1s might relay the signal from the s-LNvs to the PI neurons.

Also, under different environmental conditions such as constant light, DN1s function as pacemaker neurons to drive rhythmicity. Constant light (LL) exposure makes wild type flies arrhythmic but certain genetic manipulation such as over-expression of PER or mutation of genes involved in the photoreceptive pathway can make flies rhythmic in LL (Murad et al. 2007). DN1s have been shown to display molecular rhythms and generate behavioral rhythmicity under LL conditions (Murad et al. 2007; Stoleru et al. 2007). DN1s can also modulate the output of M and E cells in LD conditions. A functional molecular clock in DN1ps in an otherwise arrhythmic *per^{0}* fly is sufficient for morning anticipation and can also drive evening anticipation under certain temperature conditions (Zhang et al. 2010). Also, recently DN1s have been shown to feedback on M and E cells via inhibitory action of glutamate to promote mid-day siesta and night time sleep (Guo et al. 2016). Similar inhibitory glutamatergic feed back from DN1s to the M cells was also observed in the larval circadian network (Collins et al. 2012). Thus, even though DN1s are not sufficient to generate endogenous rhythms in

DD, they help flies to synchronize their activity to different environmental conditions and hence contribute to the plasticity of the circadian network.

DN2s - The molecular clocks in DN2s run in antiphase to the other circadian neurons (Kaneko et al. 1997). The exact function of the DN2s is not very well known but these neurons are also present in larval stages and persist through fly development. Along with Lateral posterior neurons (LPNs), DN2s have been implicated in temperature entrainment (Busza et al. 2007; Yoshii et al. 2005).

DN3s - There are approximately 40 DN3s per brain hemisphere. Veleri et al. (2003) have shown that DN3s can generate molecular rhythms in DD in the absence of pacemaker sLNvs, but are not sufficient to generate sustained behavioral rhythmicity in DD. The precise function of DN3s in behavioral rhythms is not known yet.

5. Input pathways

Even though an endogenous clock can persist in constant conditions, such conditions almost never exist in the environment. Hence despite being self-sustained, a clock that is unable to adjust to variations in the environment will eventually fall out of phase and will be maladaptive to organisms. Synchronization of the circadian clock to environmental cycles is referred to as entrainment and an environmental stimulus that can entrain circadian clocks is called as “Zeitgeber” (ZT) (meaning “time giver” in German). Thus, every circadian system consists of the following three components:

Various environmental cues can entrain the endogenous clock, which then generate output rhythms in behavior and physiology.

5.1 The light input pathway

Light is the most potent synchronizer of circadian clock. In lab conditions, flies can synchronize their locomotor activity to 12:12 LD cycles even with light intensities as low as 0.03 lux (Bachleitner et al. 2007) reflecting the strength of light as a cue to the circadian clock. Not only behavior, but also most *Drosophila* tissues with their cell autonomous oscillator are light sensitive. Cultured dissociated organs such as wings and legs show *per* promoter driven bioluminescence rhythms that are entrainable by LD cycles (Plautz et al. 1997).

5.1.1 Effect of light on molecular and behavioral rhythms

Light mediates its effect on the molecular pacemaker by promoting degradation of TIM (Hunter-Ensor et al. 1996; Lee et al. 1996; Myers et al. 1996; Zeng et al. 1996). The mechanism of TIM degradation is discussed together with the light sensing pathways in the next section. In the absence of TIM, PER is no longer stable, is subsequently phosphorylated and undergoes proteasomal degradation (Grima et al. 2002; Ko et al. 2002; Lee et al. 1996; Zeng et al. 1996). Hence, the molecular pacemaker is reset. This effect on the molecular clock underlies the behavioral response to light (Suri et al. 1998; Yang et al. 1998).

5.1.1.1 Phase Response Curve (PRC)

The *Drosophila* clock is extremely sensitive to light. Exposure to brief light

pulses even for a minute in the night, shifts the phase of circadian behavior by several hours (Egan et al. 1999; Levine et al. 1994; Pittendrigh 1967; Suri et al. 1998). In nature, the transition from dark to light during dawn and light to dark during dusk are key entrainment stimuli to the clock and in laboratory conditions such dawn and dusk transitions are mimicked by light pulses. For entrainment to occur, light resets the endogenous clock until its internal phase is in equilibrium with its external LD cycle. As a result of which, a circadian clock responds differently to light applied at different phase of its cycle.

The directionality of the phase shift depends on the time at which the light pulse is given (Edery et al. 1994; Levine et al. 1994; Pittendrigh 1967; Suri et al. 1998). A light pulse administered early in the night mimics a delayed dusk and hence generates a phase delay whereas a light exposure late in the night resembles an advanced dawn and results in a phase advance. At the molecular level, this light generated delay or advance depends on the *tim* mRNA levels. Early in the night when *tim* mRNA levels are still high, TIM levels can recover after light mediated degradation thus shifting the circadian cycle back by a few hours and generating a delay. Late in the night, *tim* mRNA levels are low and TIM can not be replenished after photic degradation thus advancing the clock to the next cycle.

Such phase shifting effects of light can be represented by a phase response curve (PRC) (Figure 1.6). A PRC is generated by plotting the magnitude and the direction of the phase shift in the endogenous clock caused by a light pulse as a function of time at which the light is applied in the circadian cycle. As a convention, phase delays are plotted as

negative values and advances as positive values. In a wild type fly, a maximum phase delay response is obtained when a light pulse is given at three to four hours after the lights off at ZT15 (where ZT 0 refers to lights-on in a 12:12 LD cycle and thus ZT15 corresponds to a time 3 hours after lights off) (Suri et al. 1998). Similarly the maximum phase advance response is observed at three to four hours before the lights come on in a subjective day at ZT21. Light pulses administered during the day do not result in any phase shifts because of low TIM levels during the day and hence is referred to as the dead zone. A light PRC is valuable in understanding how circadian pacemakers are entrained to daily environmental LD cycles. However, a PRC is not restricted to light stimulus and can be generated for any environmental stimulus such as temperature, pulses of drugs or chemicals and food availability. The magnitude of the phase shift depends on the strength of the stimulus which in the case of light PRC is determined by the intensity and duration of light pulse.

A PRC can also be plotted as a Phase Transition Curve (PTC), in which the new phase to which the circadian clock is shifted, is plotted on the y-axis and the initial phase at which the light pulse was given is plotted on the x-axis (Figure 1.7). In this type of plot if a light stimulus causes no shift (for e.g., during the day) the original and the new phase will be the same and hence results in a diagonal line with a slope of 1. A very strong light stimulus will always drive the clock to the same phase regardless of the time of delivery resulting in a horizontal line with a slope of 0. A weak phase response curve with relatively smaller phase shifts (usually less than 6 hours) yields a Type 1 PTC with an average slope of 1. A strong phase response curve with greater magnitude phase shifts

yields a Type 0 PTC with an average slope of 0. In Type 1 resetting, there is a continous transition between delay and advance zone when plotted as a PRC whereas in Type 0 resetting which shows larger phase shifts, there is a discontinuity between the delay and advance zone when plotted as a PRC. The occurrence of Type 1 or 0 resetting depends on the strength of the stimulus. For example, in flies and mosquitoes increasing the light dose of the stimulus converts Type 1 to Type 0 phase resetting (Peterson 1980; Pittendrigh 1960; Saunders 1978).

Another commonly studied behavior under laboratory conditons is the response to LL conditions. Wild type flies become arrhythmic on exposure to 24 hours of light (Konopka et al.1989) presumably due to continous degradation of TIM (Emery et al. 2000). Even though LL conditions are artificial, mutations that abolish this effect of LL can reveal components of light sensing pathways that synchronize the endogenous clock to the environmental LD cycles.

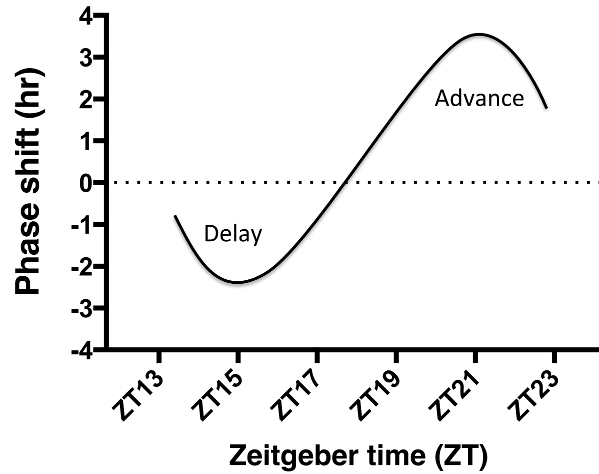

Figure 1.6 A representative Phase Response Curve (PRC) of wild type *Drosophila*

The magnitude and direction of the phase shift (in hours) is plotted as a function of time (ZT) at which the light pulse is administered. Phase delays are represented as negative values and advances are represented as positive values.

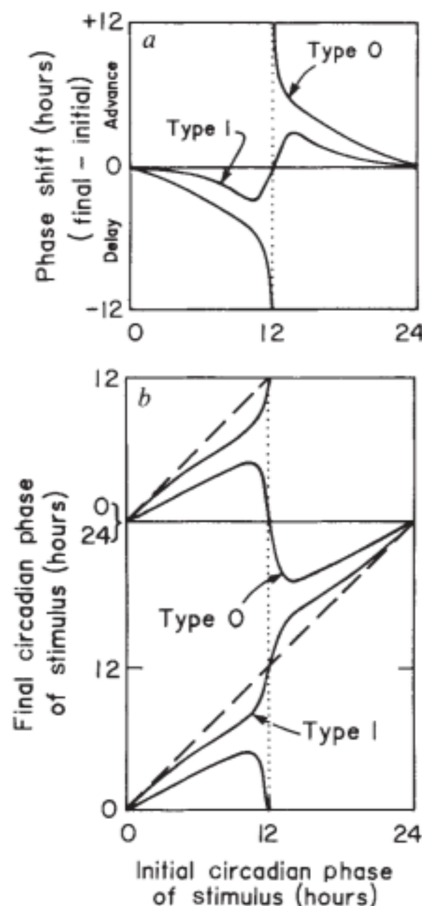

Figure 1.7 A Schematic representation of Type 1 and Type 0 phase resetting.

Top: A phase response curve (PRC). The phase shift is plotted on the y-axis as a function of time at which the stimulus (light pulse) is given.
Bottom: A phase transition curve (PTC). The new (final) phase to which the circadian clock is shifted is plotted on the y-axis and the old (initial) phase at which the stimulus was administered is plotted on the x-axis. If the stimulus (light pulse) causes no phase shift, the new and the old phase will be the same resulting in a diagonal line with a slope of 1 as represented by the dashed line. In, Type 1 resetting, due to weak response to the stimulus, the curve deviates only slightly from the diagonal line resulting in an average slope of 1. Whereas in Type 0 resetting, the response to the stimulus is so strong that the light pulse resets the circadian clock to the same new phase when administered at different old phases resulting in an average slope of 0. (Figure adapted from Jewett et al. 1991 with permission)

5.1.2 *Drosophila* light sensing pathways

Adult *Drosophila* has three different pathways for light perception - compound eyes and ocelli (Rieger et al. 2003); the extra-ocular photoreceptor known as Hofbauer-Buchner (H-B) eyelet (Helfrich-Förster et al. 2002) and an intracellular blue light photoreceptor – CRYPTOCHROME (CRY) (Figure 1.5). Additionally, there also exists a yet unidentified photoreceptor which synchronizes a subset of dorsal neurons to LD cycles even in the absence of visual and CRY photoreceptors (Veleri et al. 2003). More recently, Ni et al. (2017) found that the circadian M-oscillators, in addition to CRY, express another photoreceptor – Rhodopsin 7 (Rh-7). All these photoreceptors contribute in synchronizing the clock to LD cycles. Mutation in one or two of the photoreceptors makes the fly's circadian clock less light sensitive, but a fly is completely blind to light only when all the photoreceptors are eliminated (Helfrich-Förster et al. 2001).

Furthermore, *Drosophila* larva prefer darkness and exhibit photoavoidance behavior to reduce body exposure. The larvae use primitive eye structures called Bolwig organs (BO) and class IV dendritic arborization neurons tiling their body wall to sense light for photoavoidance behavior (Xiang et al. 2010)

5.1.2.1 Light inputs to the circadian clock via visual system

Amongst the visual photoreceptors, compound eyes are the most important for light entrainment. Each compound eye consists of 800 ommatidia, each of which consists of 8 photoreceptor cells – R1 to R8 expressing the different photoreceptor rhodopsins (Rh1-6) (Yoshii et al. 2016). Eyes have been demonstrated to play a major role in entrainment during long day conditions in a LD cycle (Rieger et al. 2003) or in conditions

where there is moonlight instead of complete darkness during the night (Bachleitner et al. 2007, Schlichting et al. 2014). Simulating twilight (i.e., change in light intensity around dawn or dusk) conditions in lab can also affect behavioral rhythms. This effect of twilight on actvity rhythms is mediated by eyes especially the inner R7 and R8 photoreceptor cells (Schlichting et al. 2015). The contribution of ocelli in light entrainment is not very well studied.

The HB eyelets are remnants of a larval photoreceptor, the bolwig organs (BO). HB-eyelet express Rh 5 and 6 (Yasuyama and Meinertzhagen 1999). Flies with triple mutation in *rh 5*, *rh 6* and *cry* take much longer to re-synchronize to a 6 hour shifted LD cycle compared to single *cry* mutant flies suggesting that HB eyelets play a role in entrainment (Szular et al. 2012). Projections of both BO and HB eyelets contact the dendrites of PDF positive LNvs (Helfrich-Förster et al. 2002; Malpel et al. 2002; Yuan et al. 2011). In fact in larvae, both BO and CRY are essential for light entrainment (Klarsfeld et al. 2011). But, the role of HB eyelet in light input to the adult circadian clock is not very clear.

Thus, the role of opsin-based photoreception is evident in entraining the circadian clocks, but the neural and molecular mechanism by which light information through visual system resets the circadian clock in the neurons is not known.

5.1.2.2 CRY mediated photoreception

Genetic ablation of eyes or mutation in the visual phototransduction pathways such as *ninaE* (*rh1* mutation) or *norpA1* (*phospholipase C* mutation) does not block circadian entrainment to LD cycles (Helfrich-Förster et al. 2001). Early studies on

eclosion rhythms showed that blue light (400-500nm) preferentially can shift the phase of peak timing of eclosion (Frank and Zimmerman 1969) which was later confirmed in locomotor activity rhythms as well (Suri et al. 1998). The action spectra of both eclosion and locomotor activity rhythms was significantly different from that of *Drosophila* vision (Stark et al. 1976; Zuker et al. 1996) .All these studies suggested the presence of an additional non-opsin photoreceptor which maximally absorbs light in the blue region of the visible spectrum.

In *Drosophila*, CRY is an intracellular blue light circadian photoreceptor. CRY belongs to a family of blue light sensitive flavoproteins which includes a class of DNA repair enzymes – photolyases (Cashmore 2003). CRY does not have DNA repair activity and its role as blue light photoreceptor was first identified in *Arabidopsis thaliana. Drosophila* CRY is a type I cryptochrome. The type II CRYs are an integral component of the circadian molecular pacemaker and function as transcriptional repressors in mammals as well as in some invertebrates (Kume et al. 1999; Van der Horst et al. 1999). Since, the focus of my thesis is on the light input pathway in *Drosophila*, hereafter, I will only discuss about *Drosophila* CRY and its role as a photoreceptor. CRY has a core domain that shows high homology to photolyases. Photolyases have conserved binding sites for two chromophores, flavin and pterin. Pterin acts as the main light harvesting chromophore which upon absorbing photons transfers the activation energy to flavin via redox reactions. Flavin then acts as a catalytic chromophore to transfer the electron to the thymidine dimer (formed in DNA by ultra-violet light exposure) to repair it (Cashmore 2003). In case of *Drosophila* CRY, light is absorbed in a similar manner but instead of

DNA repair, the energy is used for a conformational change, thus activating CRY (Green 2004).

5.1.2.2.1 How does activated CRY reset the molecular pacemaker and behavioral rhythms?

The conformational change in CRY upon light exposure increases its affinity for TIM. Activated CRY binds to TIM and triggers its proteasomal degradation (Busza et al. 2004; Ceriani et al. 1999; Naidoo et al. 1999) via an Skp1/Cullin/F-box (SCF) E3 ubiquitin ligase which contains a F-box protein called JETLAG (JET) (Koh et al. 2006) (Figure 1.4).

CRY is the primary sensor for circadian photoreception. As mentioned above, the visual photoreceptors are dispensable for synchronization to LD cycles. On the other hand, cry^{baby} (cry^{b}) which is a mutation in a conserved amino acid residue involved in flavin binding, prevents degradation of TIM in response to short light pulses (Stanewsky et al. 1998). Light does not shift the phase of behavior in cry^{b} flies. The oscillation in TIM levels in a 12:12 LD cycle is disrupted in the peripheral tissues (such as eyes) of cry^{b} flies (Stanewsky et al. 1998). Also on exposure to LL conditions, cry^{b} flies stayed rhythmic (as if they can not perceive light) whereas the wild type flies become arrhythmic presumably because CRY is constantly activated resulting in continous degradation of TIM (Emery et al. 2000). Furthermore, over-expression of wild type CRY in clock neurons makes the flies hypersensitive to light pulses of low light intensity (Emery et al. 1998; Emery et al. 2000). Therefore, CRY plays a crucial role in molecular and behavioral responses of circadian clock to light.

5.1.2.2.2 Role of JETLAG in CRY dependent photoresponses

JETLAG is a member of the family of SCF E3 ubiquitin ligases. JETLAG consists of a N-terminal F-box domain that binds to Skp1 of the SCF complex and Leucine Rich Repeats (LRR) domain which is involved in substrate recognition. JETLAG's role in CRY dependent photoresponses was first identified when two mutants – jet^c (common) and jet^r (rare) were found to be behaviorally rhythmic in LL as opposed to wild type flies which are arrhythmic (Koh et al. 2006). TIM degradation and phase shifts in reponse to brief light pulses was also reduced in these *jet* mutants (Koh et al. 2006). In S2 cells, JET interacts with CRY in a light dependent manner and co-expression of CRY, JET and TIM is sufficient to promote ubiquitination and proteasomal degradation of TIM upon exposure to light (Peschel et al. 2009).

Interestingly, in addition to TIM, JET also promotes photic degradation of CRY (Peschel et al. 2009). The sequential order in which TIM and CRY are degraded by JET depends on the kind of *tim* allele present in the fly. Naturally occuring *tim* polymorphism results in two types of *tim* variants with different photosensitivity (Rosato et al. 1997; Sandrelli et al. 2007). Due to an insertion of a nucleotide, the *long-short (ls) tim* variant, has two start codons in frame and produces a long and short isoform of *tim* through the use of alternative start codons. The *s-tim* variant produces only the short isoform. The long isoform (L-TIM) has 23 extra N-terminal amino acids, binds poorly to CRY and is less light sensitive. The short isoform (S-TIM) binds strongly to CRY and is much more photosensitive (Sandrelli et al. 2007). In the presence of S-TIM, which is strongly bound to CRY, S-TIM is preferentially degraded by JET. Whereas, in the presence of L-TIM,

which is weakly bound to CRY, L-TIM is less efficiently degraded by JET, as a result of which CRY is readily available JET mediated degradation. (Peschel et al. 2006).

Even though JET participates in the CRY-TIM dependent light entrainment, the absolute role of JET was not very clear in CRY mediated photoresponses. First, *jet*^{c} mutants were rhythmic in LL conditions only when associated with *ls-tim* allele. *jet*^{c} mutation with the *s-tim* allele behaved like wild type flies in LL (were arrhythmic) (Peschel et al. 2006). Second, TIM degradation and phase shifting response was only partially disrupted in *jet* mutants. Hence the effect of *jet*^{c} and *jet*^{r} were only observed in a less light sensitive *ls-tim* background. This suggested that either other ubiquitin ligases can mediate light responses in the absence of JET or stronger loss of function *jet* mutations were required to ascertain if JET was essential for photoresponses.

In chapter II of this dissertation, I present data for a severe loss of function *jet* mutation in a highly sensitive tim (*s-tim*) background and show that JET is critical for all CRY dependent circadian light responses.

5.1.2.3 Additional CRY independent components of light input pathway

There is evidence for an additional membrane anchored Zona Pellucida domain protein – QUASIMODO (QSM), which is activated after illumination and promotes degradation of TIM in a CRY independent manner (Chen et al. 2011). However, the photoreceptor and the mechanism for TIM degradation is not known.

Another opsin based photoreceptor, Rh 7 was recently identified in the brain pacemaker neurons (Ni et al. 2017). Rh7 was not expressed in the compound eyes but was found to be expressed in the PDF positive LNvs. Hence, in addition to CRY, Rh7

also plays a role in circadian photoentrainment in the LNvs.

5.2 Additional Zeitgebers for the circadian clock

Light is not the only modality that can synchronize the circadian clock. For example, temperature is the best-studied non-photic circadian zeitgeber to entrain the clock. Similar to LD cycles, flies can also entrain to warm and cold temperature cycles during the day. Temperature difference of as low as 2°C can synchronize molecular as well behavioral rhythms (Wheeler et al. 1993). The peripheral thermosensors responsible for moderate temperature sensing in the antennae seem dispensable for thermoreception (Glaser and Stanewsky 2005). Recently, ionotropic receptors on the chordotonal organs (peripheral sensory structure) have been found to mediate behavioral as well as molecular entrainment in some clock neurons to temperature cycles (Chen et al. 2015).

Additionally, social and olfactory cues can also contribute to entrainment. Flies when monitored individually in DD show slight individual differences in period and phase of entrainment and hence become more desynchronized eventually. It has been shown that flies housed in groups rather than in isolation prior to monitoring locomotor activity are more synchronized. Furthermore, if wild type flies are housed with per^0 flies, they show greater desynchronization of locomotor activity in DD suggesting the cues from per^0 flies confuse the clocks of wild type flies (Levine et al. 2002a). Such social cues were shown to be olfactory in nature.

Fuji et al. (2007) have demonstrated that mating between the male-female fly couples can also influence their circadian locomotor behavior. Socially interacting male-female fly couples exhibit a brief rest phase around dusk and high activity throughout the

night and early morning as opposed to socially isolated flies which show a peak of activity around dusk and rest during the night. This distinct locomotor activity rhythm was linked with the courtship rhythms (assayed by visualizing the approaches made by male to female fly). The authors further showed that exposure to stimuli derived from the female flies reset the central and antennal circadian oscillators in the males and thus result in a shift in the locomotor activity of socially interacting flies.

Since, the focus of my thesis is on the light input pathway, other entraining cues will not be discussed further.

6. Cell autonomous *vs.* non autonomous photoreception

CRY binds directly to TIM in a light dependent manner in head protein extracts, in cell culture and even in yeast two-hybrid assay (Zhang and Emery 2012). The model for CRY and JET dependent photic degradation of TIM posits that CRY mediated photoreception functions in a cell autonomous manner. There is ample evidence in support of this model. First, CRY serves as an independent photoreceptor in several peripheral tissues. Body segments such as wings, legs, antennae etc. when separated from the brain, in culture, can still sense light and even re-entrain to a new LD cycle shifted by 6 hours (Plautz et al. 1997). Second, in Malphigian tubules (kidney in flies), a light pulse in the night induces TIM degradation which was disrupted in *cry*^{b} mutants (Ivanchenko et al. 2001; Stanewsky et al. 1998). Third, the cuticle deposition rhythm which is controlled by a peripheral oscillator in the epidermis is entrainable by LD cycles in culture without any cross talk with brain or eyes. Also, this rhythm was not entrained by LD cycles in *cry*^{b} mutants and was rescued by local over expression of CRY (Ito et al. 2008). Fourth,

tissue specific expression of CRY in the eyes of *cry*^{b} mutant flies rescues local TIM cycling but not the behavioral phase shifting response to light. Whereas CRY expression in the PDF positive LNvs was sufficient to rescue behavioral response to light but not TIM cycling in the eyes. Hence, it was believed that CRY functions as a cell autonomous photoreceptor (Emery et al. 2000).

However, CRY is not uniformly expressed in all the circadian neurons that ultimately drive behavioral rhythms. CRY is expressed in all LNvs, 3 (out of 6) LNds , 6 (out of 15) DN1ps and the 2 DN1as. It is not expressed in DN2s, DN3s and LPNs (Yoshii et al. 2008). Moreover, levels of CRY expression also vary amongst different clock neurons (Yoshii et al. 2008). This raises the question that whether light perception occurs cell autonomously or relies on neural interactions between the CRY positive and CRY negative neurons to synchronize behavior to LD cycles. There are several studies which suggest that circadian photoreception also depends on non cell autonomous mechanisms. First, CRY expression restricted to the PDF positive LNvs only partially rescues the behavioral phase response defects of *cry*^{b} flies, suggesting that additional CRY positive circadian neurons contribute to behavioral photoresponses (Emery et al. 2000). I obtained similar results which are shown in chapter II of this dissertation. Second, Yoshii et al. (2008) observed that TIM levels were strongly reduced on exposure to light even in the CRY negative neurons, thus implying that light information is transmitted by intercellular communication between CRY positive and negative neurons. Third, specific groups amongst the 150 clock neurons were implicated to be involved in light response. Shang et al. (2008) found that l-LNvs are necessary for light dependent resetting late in the night

(dawn) and ablation of l-LNvs abolished the phase advance response to a late night light pulse. Tang et al. (2010) showed that TIM degradation in s-LNvs (which are the pacemaker neurons) is neither necessary nor sufficient for behavioral light responses in the early night, and instead the DN1s were proposed to be important for these phase delay responses.

Hence, even though the molecular model very strongly suggests that circadian photoreception via CRY is cell autonomous, the studies cited above imply that neuronal interactions impinges on photoresponses as well. However, if circadian light responses rely on a neuronal network, it was not known which clock neurons were critical in sensing light and relaying the light information to the circadian network. To map the neuronal circuitry controlling circadian photoresponses, we have focussed on the phase resetting response mediated by CRY photoreceptive pathway upon exposure to brief light pulses. In chapter II of my dissertation, we first verified the essential role of JET in circadian photoresponses and used the newly identified *jet* mutant in our lab as a tool to map the neuronal circuitry controlling circadian photoresponses. Then, we also address the question of cell- autonomous vs. non cell-autonomous photoreception by looking at TIM degradation in relevant neurons. In chapter III, we reconcile the two different views of cell-autonomous and non-autonomous photoreception and show that neural interactions modulate cell autonomous light detection by CRY.

Chapter II

Morning and Evening oscillators cooperate to reset circadian behavior in response to light input

This chapter has been published as

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Bilodeau-Wentworth, D. first identified the *jet^{set}* mutant and found it to be rhythmic in constant light. I performed the western blots and all the other behavioral experiments. Zhang Y. and I together conducted the immunocytochemistry experiments. Emery P., Zhang Y. and I wrote the manuscript.

1. Abstract

Light is a crucial input for circadian clocks. In *Drosophila*, short light exposure can robustly shift the phase of circadian behavior. The model for this resetting posits that circadian photoreception is cell-autonomous: CRYPTOCHROME senses light, binds to TIMELESS (TIM) and promotes its degradation, mediated by JETLAG (JET). However, it was recently proposed that interactions between circadian neurons are also required for phase resetting. We identify two groups of neurons critical for circadian photoreception: the Morning (M)- and the Evening (E)-oscillators. These neurons work synergistically to reset rhythmic behavior. JET promotes acute TIM degradation cell-autonomously in M- and E-oscillators, but also non-autonomously in E-oscillators when expressed in M-oscillators. Thus, upon light exposure, the M-oscillators communicate with the E-oscillators. Since the M-oscillators drive circadian behavior, they must also receive inputs from the E-oscillators. Hence, although photic TIM degradation is largely cell-autonomous, neural cooperation between M- and E-oscillators is critical for circadian behavioral photoresponses.

2. Introduction

In *Drosophila*, the self-sustained pacemaker that generates molecular and behavioral circadian rhythms is a negative transcriptional feedback loop: PERIOD (PER) and TIMELESS (TIM) repress CLOCK (CLK) and CYCLE (CYC), which are activators of *per* and *tim* transcription (Zhang and Emery, 2012). This mechanism is present in ca. 150 brain neurons (Nitabach and Taghert 2008). In a standard 12hr light: 12hr dark (LD) cycle, *Drosophila* exhibits two peaks of activity. The morning (M) peak is driven by the

Pigment Dispersing Factor (PDF) positive small ventrolateral neurons (s-LNvs), also referred to as the M-oscillators (Grima et al. 2004; Stoleru et al. 2004). The evening (E) peak is driven by six dorsolateral neurons (LNds), two PDF negative s-LNvs called “5th s-LNvs”, and perhaps a few Dorsal Neurons (DN1s) (Cusumano et al. 2009; Grima et al. 2004; Picot et al. 2007; Stoleru et al. 2004). These cells are known as the E-oscillators. The M-oscillators also function as pacemaker neurons: they maintain behavioral rhythms under constant darkness (DD) and control their pace and phase (Renn et al. 1999; Stoleru et al. 2005).

Circadian rhythms are only beneficial if they are synchronized with the day/night cycle. Light is a crucial cue to entrain the circadian clock. In *Drosophila*, a brief light pulse in the early night, mimicking a delayed dusk - leads to a phase delay, whereas a late night light pulse resembling an early dawn causes a phase advance (Levine et al. 1994). Light promotes rapid TIM degradation, which is critical to reset the circadian pacemaker and behavioral rhythms (Suri et al. 1998; Yang et al. 1998). Upon light exposure, the intracellular blue-light photoreceptor CRYPTOCHROME (CRY) changes its conformation, binds to TIM and triggers its proteasomal degradation by recruiting a JETLAG (JET)-containing E3 ubiquitin ligase (Busza et al. 2004; Ozturk et al. 2011; Peschel et al. 2009; Koh et al. 2006).

Loss of CRY results in severe photoreception defects: light-induced TIM degradation and behavioral phase shifts are abolished (Dolezelova, Dolezel, and Hall 2007; Lin et al. 2001; Stanewsky et al. 1998). *cry* mutant flies also remain rhythmic in constant light (LL), while wild-type flies are arrhythmic under these conditions (Emery,

Stanewsky, Hall, et al. 2000). Two *jet* mutants (*jet*^{c} and *jet*^{r}) are also rhythmic in LL (Peschel et al. 2006; Koh et al. 2006) However, this and other circadian photoresponse phenotypes are only observed in flies carrying the long-short *tim* variant (*ls-tim*) (Rosato et al. 1997). The long TIM isoform encoded by this variant has reduced affinity for CRY, making flies much less sensitive to light compared to flies carrying the short *tim* allele (*s-tim*) (Sandrelli et al. 2007). Thus, although JET promotes TIM degradation, whether it is actually required for TIM degradation and circadian photoresponses remains to be determined.

Although strong evidence supports a cell-autonomous model for circadian photoreception, recent studies indicate that such a mechanism is not sufficient to explain photic resetting of circadian behavior. Indeed, TIM degradation in M-oscillators appears to be neither necessary nor sufficient for phase delays (Tang et al. 2010). Based on the pattern of TIM degradation at Zeitgeber Time (ZT) 15, it was proposed that the DN1s would be important for phase delays (Tang et al. 2010). Moreover, the large (l)-LNvs have been implicated in phase advances (Shang et al. 2008). Ultimately, the DN1s and the l-LNvs would have to communicate with the M-oscillators, since these cells drive circadian behavior in DD, the condition in which phase is measured after exposing flies to a light pulse. Neuronal circuits would thus be important for circadian behavioral photoresponses. Acute TIM degradation in CRY-negative LNds also indicates the existence of non-autonomous photoreceptive mechanisms in the brain (Yoshii et al. 2008).

We used a novel, severe *jet* mutant and *jet* RNA interference (RNAi) to map the

neuronal circuits controlling circadian photoreception. Our results indicate that both cell-autonomous and non-autonomous photoreception take place within the circadian neural network, and that the M- and E-oscillators are crucial for sensing light and resetting circadian locomotor behavior.

3. Results

The jet^{set} mutation profoundly disrupts circadian photoresponses

In a screen for mutants affecting *Drosophila* circadian behavior, we identified a strain that remains robustly rhythmic in LL (Figure 2.1A, Table 2.1). This mutant did not complement jet^{c} and jet^{r} (Table 2.1), and a point mutation causing a Threonine to Isoleucine substitution in JET's Leucine-Rich Repeats (LRR) was identified (Figure 2.1B). However, while jet^{c} and jet^{r} show circadian light response defects only with *ls-tim* (Peschel et al. 2006; Koh et al. 2006) our mutant carries the highly light-sensitive *s-tim* allele (Sandrelli et al. 2007). It is thus a much more severe loss-of-function mutant, which was named jet^{set}. Furthermore, jet^{set} flies showed almost no behavioral phase shifts when challenged with 5-min light pulses applied early (ZT15) or late (ZT21) at night. Phase shift defects were fully rescued by expression of wild-type JET driven by *tim-GAL4,* a pan-circadian driver (Figure 2.1C) (Kaneko et al. 2000). The mutation in the *jet* gene is thus responsible for jet^{set}'s defective photoresponses. TIM undergoes acute light-dependent degradation after short light pulses at night, and oscillates robustly under LD cycles (reviewed in Zhang and Emery, 2012). TIM did not degrade after a light pulse (10 mins at 1500 lux) at ZT21 in jet^{set} mutants (Figure 2.1D). However, TIM cycling under LD was not abolished, although its amplitude was reduced (Figure 2.1E). This is

probably because JET^{SET} retains residual activity detectable with long exposure to light. Thus, we conclude that both molecular and behavioral circadian photoresponses are affected by *jet*^{set}. JET is therefore critical for CRY-dependent circadian behavioral photoresponses and for acute TIM degradation.

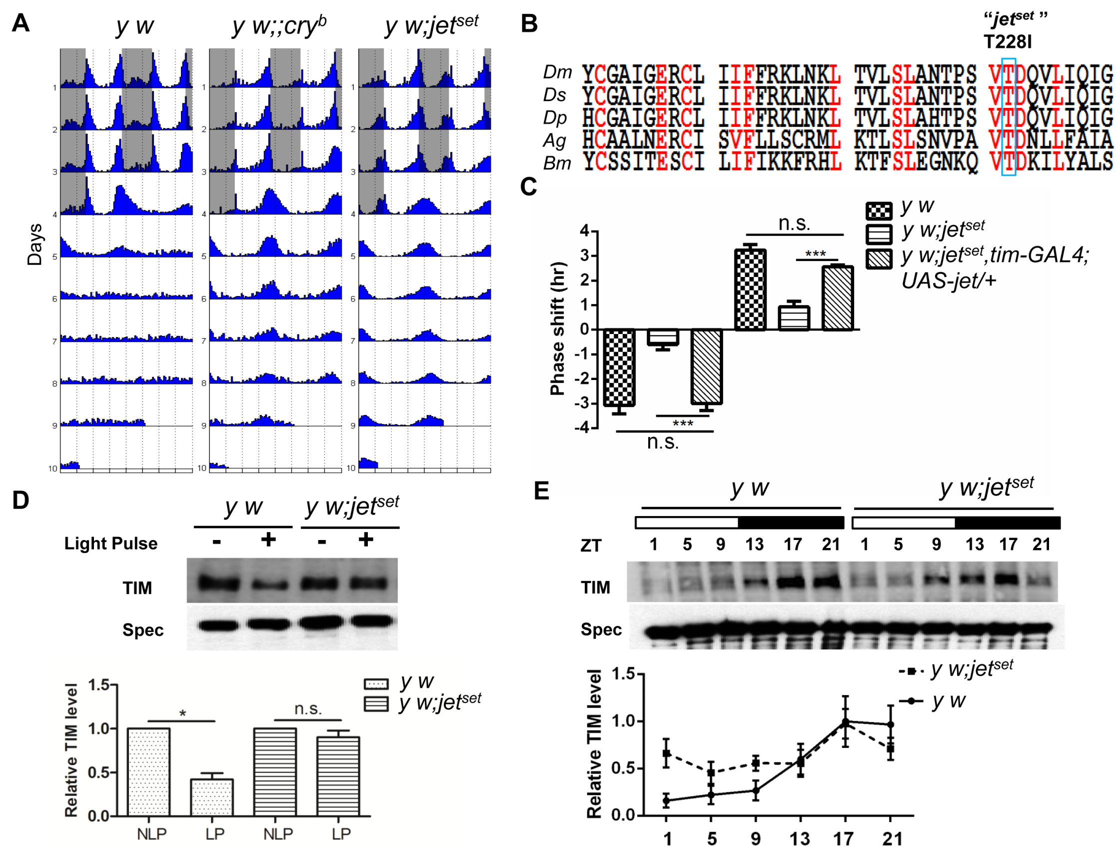

Figure 2.1 Identification and characterization of *jet*^{set}

(A) *y w; jet*^{set} flies are rhythmic under LL. Representative double-plotted actograms of *y w*, *cry*^{b} and *y w; jet*^{set} flies. (white indicates the light phase and gray indicates the dark phase). (B) Sequence alignment of the LRR region of insect JET proteins. The blue box indicates the *jet*^{set} mutation. Dm: *Drosophila melanogaster*; Ds: *Drosophila simulans*; Dp: *Drosophila pseudoobscura*; Ag: *Anopheles gambiae*; Bm: *Bombyx mori* (C) Behavioral phase shifts after short light pulses are profoundly disrupted in *jet*^{set} mutants. Phase delays and advances are plotted as negative and positive values respectively. Phase shifts were almost completely abolished compared to control (*y w*) flies. Phase shifting defects were fully rescued by expression of *UAS-jet* with *tim-GAL4*. 16 flies were used per genotype for analysis, N=3. Error bars correspond to S.E.M. ***, $p < 0.001$, n.s., not significant at the 0.05 level as determined by one-way analysis of variance (ANOVA) coupled to post hoc Tukey's test for multiple comparisons, $F (5, 12) = 121.9$ with p value < 0.0001. (D) *jet*^{set} is defective for acute TIM degradation in response to short light

pulses. Upper panel: representative Western blot showing TIM degradation after light pulse in *y w* and *y w; jet^{set}*. A light pulse (LP) was given at ZT21 and non-light pulsed (NLP) flies were used as controls. Lower panel: quantification of TIM levels. Upon light pulse, *y w* flies showed about 50% TIM degradation while *jet^{set}* did not show any obvious TIM degradation. N=3. For each genotype the LP values are normalized to their NLP control values. Data are plotted as mean ± S.E.M, *, $p < 0.05$; n.s. – not significant as determined by comparing the LP and NLP groups for each genotype by student's t test. (E) TIM oscillations in *jet^{set}* are dampened under LD conditions. Upper panel: representative Western blots showing TIM oscillation in whole heads at indicated ZT times under a LD cycle. The white bars represent the day and the black bars represent the night. TIM levels were normalized to the SPECTRIN levels. N=5. Lower panel: quantification of TIM levels. TIM expression levels for *y w* at ZT17 were set to 1 and other values were normalized to it. Data represents mean ± S.E.M.

Table 2.1 Circadian locomotor behavior under constant light and constant darkness

Genotype	Number of flies (n)	Percent rhythmic	Period average (±SEM)	Power average* (±SEM)
Constant light				
w^{1118}	40	0	NA	NA
y w	32	3	20.5	11.5
y w;jet^{set}	30	100	24.36 ± 0.11	73.15 ± 4.23
y w;cry^{b}	32	91	23.9 ± 0.11	63.2 ± 4.49
y w;jet^{set}*/jet*^{c}	16	100	24.3 ± 0.09	101.8 ± 4.56
y w;jet^{set}*/jet*^{r}	14	88	24.9 ± 0.12	104.4 ± 6.78
Constant darkness				
y w	58	76	23.7 ± 0.06	56.8 ± 3.59
y w;jet^{set}	51	84	24.1 ± 0.06	64.3 ± 3.36

*Power is a measure of rhythm amplitude and corresponds to the height of the periodogram peak above the significance line (Ewer et al. 1992).

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JET expression in M- and E-oscillators controls light-dependent phase resetting

Given its severe phase response defects, we used *jet^{set}* to map the neural circuit controlling circadian entrainment. *GAL4* drivers active in potentially relevant circadian neurons were used to express wild-type JET in *jet^{set}* flies. When we expressed JET with *Clk4.1M-GAL4* (Zhang et al. 2010) only in posterior DN1s – proposed to play a role in phase delays (Tang et al. 2010) - or with *c929-GAL4* (Grima et al. 2004) specifically in the l-LNvs – which are important for phase advances (Shang et al. 2008) - phase responses were not rescued, suggesting that these neurons are not sufficient to reset locomotor behavior (Figure 2.2A). However, JET expression in both M- and E-oscillators with *Mai179-GAL4* (Grima et al. 2004) completely restored phase shifts in *jet^{set}* flies. This indicates that JET expression in these two groups of neurons is critical to phase resetting. To determine the individual contribution of the M- and E-oscillators, we expressed JET only in PDF-positive LNvs (M-oscillators and l-LNvs) using *Pdf-GAL4* (Renn et al. 1999). We could only slightly improve the phase delays. Phase advances were not rescued at all. We then combined *Mai179-GAL4* with *Pdf-GAL80* (Stoleru et al. 2004) to express JET only in the E-oscillators. Unexpectedly, this also could not rescue phase shifts (Figure 2.2A). Hence, JET must be rescued in both M- and E-oscillators for circadian behavior to be responsive to light pulses.

Mai179-GAL4 is weakly expressed in four DN1s (Picot et al. 2007) (Figure 2.3A). To determine if these neurons are required for phase shifts, we used *DvPdf-GAL4,* which is expressed in the M-oscillators, l-LNvs, and a subset of *Mai179-GAL4* positive E-oscillators, but not in the DN1s (Bahn et al. 2009) (Figure 2.3B). This

driver rescues the E-peak of activity in *per*^{0} flies (F. Guo and M. Rosbash, personal communication). We could rescue the phase shifting defects of *jet*^{set} with this driver (Figure 2.3C). Thus JET expression in the DN1s is not required for JET-dependent phase shifts.

To ensure that our identification of the M- and E-oscillators as key neurons for circadian light responses was not the result of a gain-of-function from JET overexpression, we downregulated JET with RNAi (Figure 2.2B). Consistent with our rescue data, JET knockdown in both M- and E-oscillators severely reduced the amplitude of phase delays and advances. This was observed with *Mai179-GAL4* and *DvPdf-GAL4* (Figure 2.2B, 2.3C). The effects of JET downregulation were more evident at ZT15, probably because CRY levels are lower at this time point (Emery et al. 1998; Yoshii et al. 2008) and flies are thus more sensitive to JET downregulation. Since both *Mai179-GAL4* and *DvPdf-GAL4* are expressed in l-LNvs (Bahn et al. 2009; Grima et al. 2004) (Figure 2.3A–B), we also knocked down JET specifically in the l-LNvs with *c929-GAL4* (Figure 2.3C). No effects on phase delays and advances were observed. Thus, JET expression in the l-LNvs is neither necessary nor sufficient for phase shifts. The M- and E-oscillators are therefore essential for behavioral phase shifts.

Also in agreement with our rescue experiments, knocking down JET only in PDF-positive neurons reduced the amplitude of phase shifts, although not to the same degree as knocking down JET in both groups, probably because RNAi does not reduce JET activity as efficiently as the *jet*^{set} mutation. Surprisingly, when we knocked down JET only in the E-oscillators, no effect on phase responses was observed (see explanation

below). Importantly however, the impact of downregulating JET in both M- and E-oscillators on phase shifts is greater than the sum of the effects of knocking down JET in the M- and E-oscillators separately. Thus, both our rescue and RNAi approaches reveal that the M- and E-oscillators collaborate to reset circadian locomotor behavior.

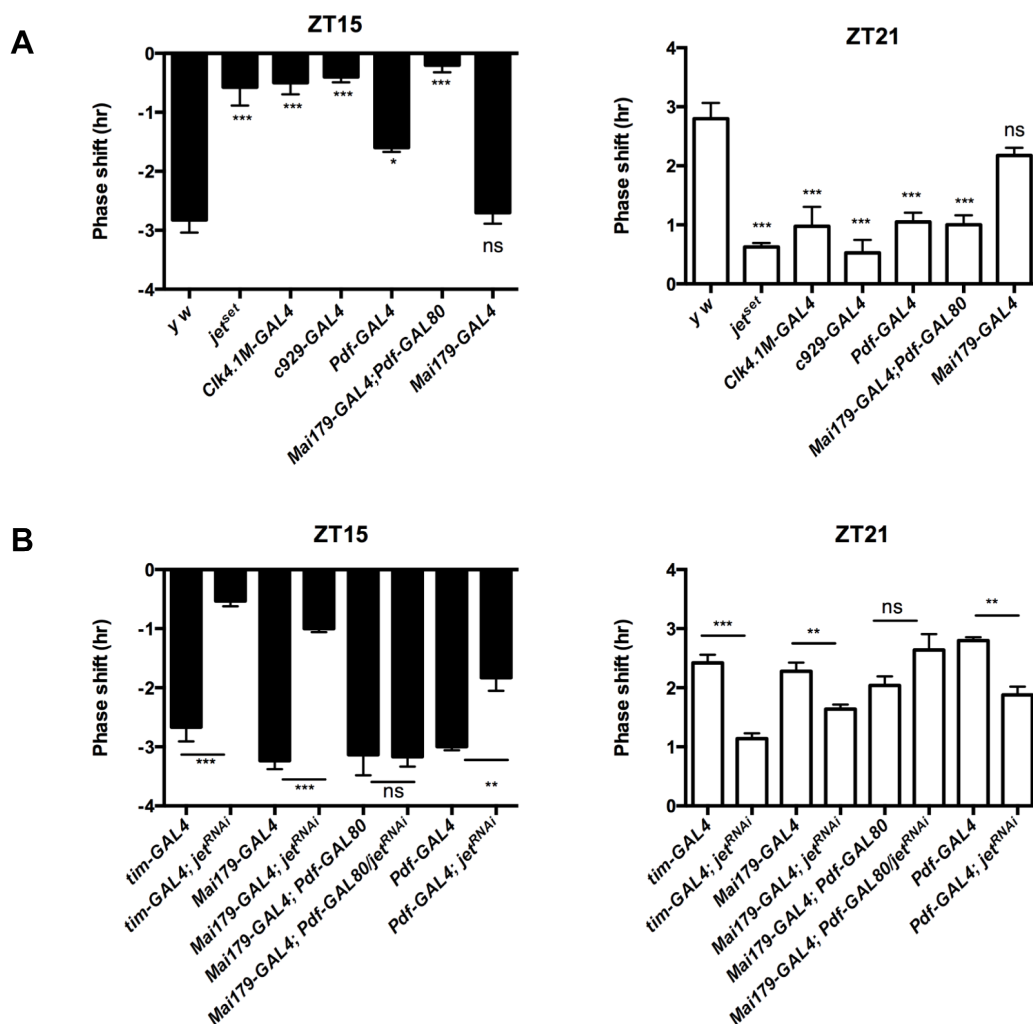


Figure 2.2 JET expression in the M- and E-oscillators is critical for circadian photoresponses

(A) JET expression in the M- and E-oscillators is sufficient to rescue both phase delay and advance defects in *jet*^{set}. Phase shift in response to light pulse at ZT15 is shown on the left and the phase shift at ZT21 is shown on the right. All genotypes were compared to *y w* control. Note that both phase delay (ZT15) and advance (ZT21) were completely rescued only when wild-type JET is expressed in both the M- and E- oscillators using the *Mai179-GAL4* driver. With *Pdf-GAL4*, partial rescue was observed at ZT15. 16 flies per genotype were used and each experiment was repeated at least four times. Error bars represent S.E.M. ***, $p < 0.001$; * $p < 0.05$; n.s., not significant at the 0.05 level as determined by ANOVA coupled to post hoc Tukey's test, $F(6, 33) = 24.77$ for phase delay and $F(6, 33) = 21.54$ for phase advance with p value < 0.0001. (B) Knocking down JET expression in the M- and E-oscillators disrupts phase shifts. Phase delays are plotted

on the left and advances on the right. The controls are the different *GAL4* driver lines crossed to *y w*. All the *GAL4* drivers were combined with *UAS-Dcr2* to enhance RNAi (Dietzl et al. 2007). Each genotype is compared to its *GAL4* driver control. ***, $p < 0.001$; **, $p < 0.01$; n.s., not significant at the 0.05 level, tested using Student's t-test. See Figure 2.4 for additional experiments.

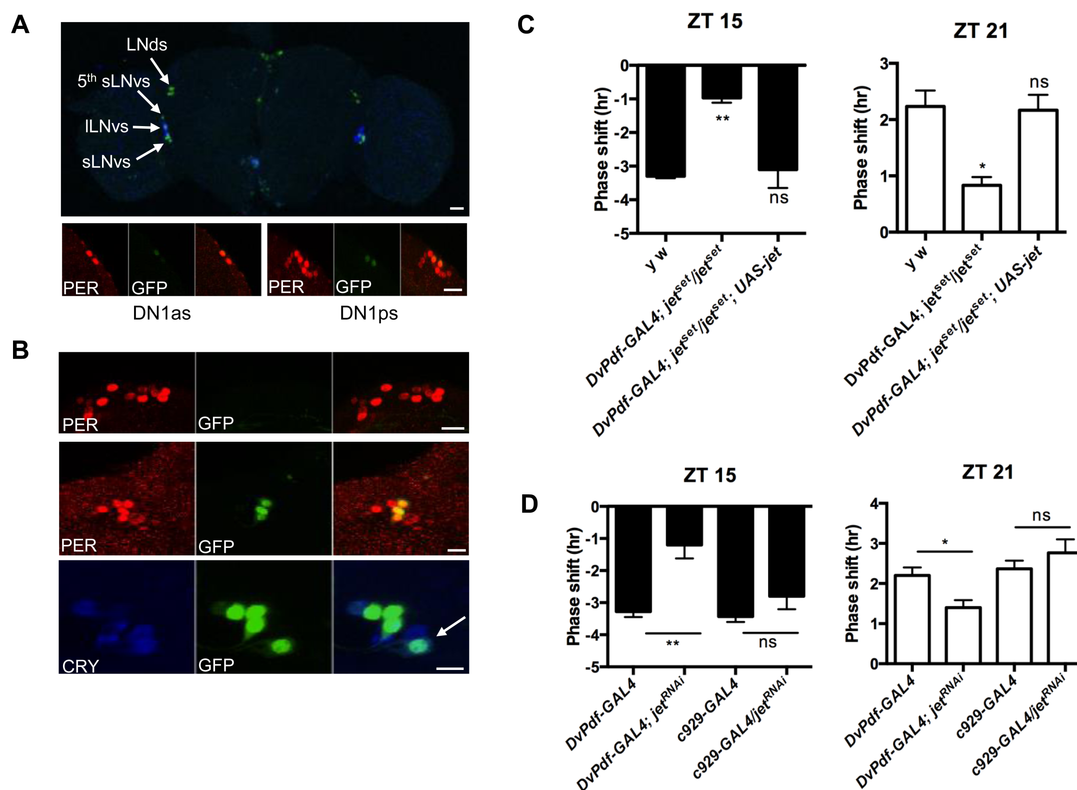


Figure 2.3 DN1s and l-LNvs are not required for phase shifts.

(A) Expression pattern of the *Mai179-GAL4* enhancer trap line. The brains of flies expressing GFP under the control of *Mai179-GAL4* were dissected and stained for anti-GFP (green), anti-PDF (blue) and anti-PER (red). Upper panel shows the whole brain, and bottom panel shows a very weak expression of GFP when driven by *Mai179-GAL4* in DN1as (left) and two DN1ps (right). This pattern of expression is very similar to that described previously (Cusumano et al. 2009). (B) Expression pattern of the *DvPdf-GAL4* enhancer trap line. Upper panel shows the dorsal region, where there is no GFP expression in the DN1s. Middle panel shows the expression in the LNds. *DvPdf-GAL4* is expressed in four LNds. Bottom panel shows that one of these *DvPdf-GAL4* positive LNds (green, pointed by an arrow) expresses CRY (blue). The CRY-positive and *Mai179-GAL4* positive LNds are the same neurons (Yoshii et al. 2008). Thus, *Mai179-GAL4* and *DvPdf-GAL4* expression overlap in one LNd in addition to the 5th sLNv (Bahn et al. 2009). All images are Z-stacks. Scale bars indicate 10 µm. (C and D) The DN1s and l-LNvs are not required for behavioral phase shifts. Upper panel shows that rescue of JET expression using *DvPdf-GAL4* restores the phase shifting defects of *jet^{set}* mutants at both ZT15 and 21, indicating that JET expression is not required in the DN1s for circadian behavioral photoresponses. 16 flies per genotype were used for all the

behavioral analyses and each experiment was repeated three times. Error bars represent S.E.M. **, $P < 0.01$, n.s., not significant at the 0.05 level as determined by one-way analysis of variance (ANOVA) coupled to post hoc Tukey's test for multiple comparisons, $F(2, 6) = 15.31$ and $P = 0.0044$ for phase delay, and $F(2, 6) = 10.59$ and $P = 0.0108$ for phase advance. Lower panel shows *jet* downregulation using *DvPdf-GAL4* and *c929-GAL4*. The *jet*^{RNAi} flies were compared to their *GAL4* control. Downregulating JET expression in the l-LNvs using *c929-GAL4* has no effect on phase shifts, indicating that these cells are not required for JET dependent photoresponses. Error bars represent S.E.M. **, $P < 0.01$; *, $P < 0.05$ tested using student's t-test. n.s., not significant at the 0.05 level tested using student's t-test.

JET controls photic TIM degradation cell-autonomously in M- and E-oscillators, but also non-autonomously in E-oscillators

To understand our rescue and RNAi results, we measured TIM degradation after light pulses at ZT15 and 21 in the M- and E-oscillators. In *jet*^{set} mutants, TIM degradation was abolished in the M-oscillators (Figure 2.4A–B, 2.6A). JET rescue in the M-oscillators with both *Mai179-GAL4* and *Pdf-GAL4* restored photic TIM degradation in these cells. However, expressing JET only in the E-oscillators did not. JET downregulation restricted to the M-oscillators inhibited TIM degradation in M-cells, but E-oscillator downregulation had no effect (Figure 2.4C–D, 2.6B). Knocking down JET using *Mai179-GAL4* also blocked TIM degradation in the M-oscillators, but less severely than with *Pdf-GAL4,* probably because *Mai179-GAL4* - a weaker driver than *Pdf-GAL4* (data not shown) - is less effective in reducing JET activity. Taken together, these results show that JET acts cell-autonomously to trigger TIM degradation in M-oscillators.

In the E-oscillators of *jet*^{set} flies, TIM degradation was also eliminated, and rescued by JET expression in these cells, further supporting the cell-autonomous role of JET in TIM degradation (Figure 2.5A–B, 2.6A). Unexpectedly however, JET expression restricted to the M-oscillators rescued partially, but significantly TIM degradation in the E-oscillators. These results indicate that JET can function non-autonomously when expressed in the M-oscillators. Moreover, TIM degradation appears to be rescued in most LNds when using *Mai179-GAL4*, even though this driver is expressed in only three of the six LNds (Grima et al. 2004; Picot et al. 2007) (Figure 2.5A, 2.7). Indeed, the intensity of TIM signal in individual light-pulsed LNds overlapped only with that observed in 12% of

LNds in non-pulsed control (Figure 2.7). Similar results were obtained even when *Mai179-GAL4* was combined with *Pdf-GAL80*. This suggests that JET in the E-oscillators can non-autonomously trigger TIM degradation in the three *Mai179-GAL4*-negative LNds. Downregulating JET in the M- and E-oscillators with *Mai179-GAL4* attenuated TIM degradation in the E oscillators (Figure 2.5C–D, 2.6B). Interestingly, TIM degradation appeared to be compromised in most LNds (Figure 2.5C, 2.7). This suggests again that the *Mai179-GAL4*-negative LNds, which express low or no CRY (Yoshii et al. 2008), rely predominantly on a JET-dependent non-autonomous mechanism to degrade TIM

Importantly, downregulating JET with *Mai179-GAL4* did not completely block TIM degradation in the E-oscillators (Figure 2.5C–D, 2.6B), while the jet^{set} mutation did. Thus, the E-oscillators retained residual JET activity in *jet* RNAi flies. This explains an apparent paradox in our behavioral results. On one hand, rescuing JET expression in M-oscillators only weakly rescues phase shifts in jet^{set} flies. On the other hand, downregulating JET specifically in E-oscillators has no effect on phase shifts. In the latter case, residual JET activity in E-oscillators and non-autonomous JET activity from M-oscillators result in full TIM degradation in E-oscillators. Hence normal phase shifts are observed. In the former situation, non-autonomous JET activity from the M-oscillators is not sufficient to trigger full TIM degradation, because there is not enough autonomous JET activity in E-oscillators. Thus, phase shifts are poorly rescued. This illustrates the importance of both autonomous and non-autonomous JET activity, and the role played by interactions between M- and E-oscillators in circadian photoreception.

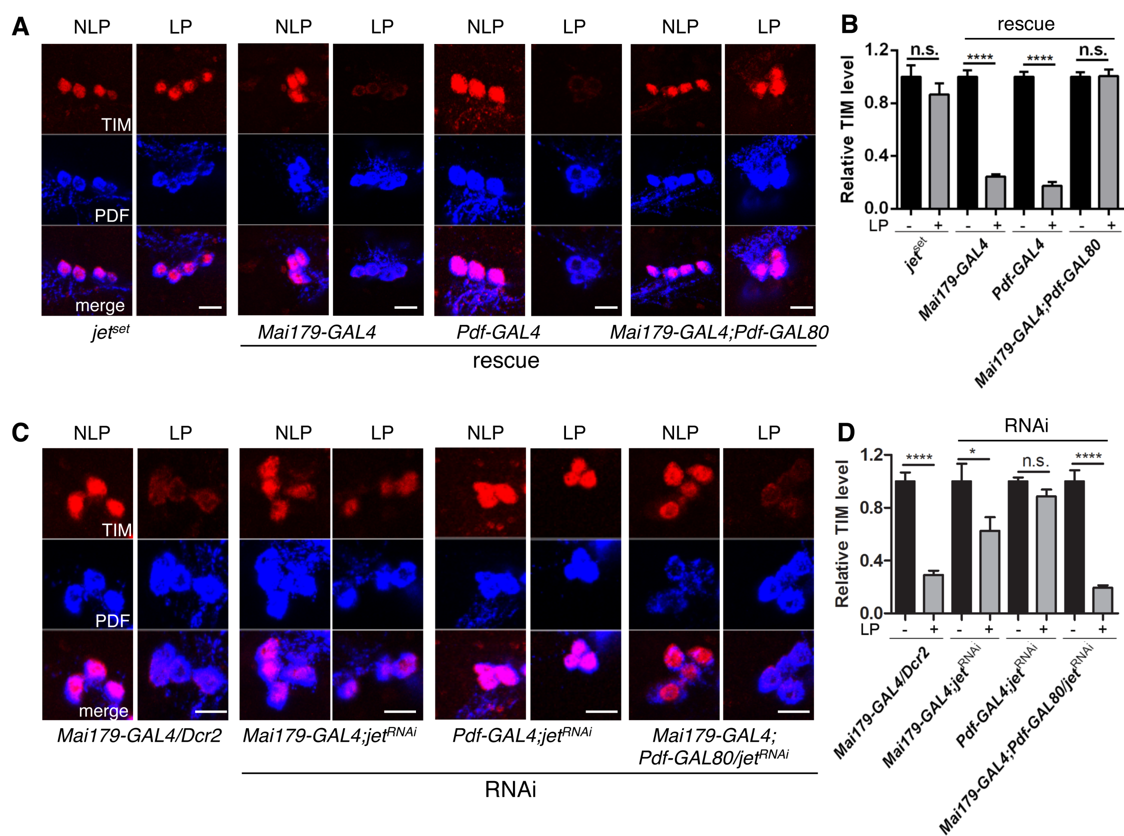


Figure 2.4 Cell-autonomous role of JET in M-oscillators

(A) Representative confocal images showing TIM degradation in M-oscillators of *jet^{set}* flies rescued in M- and/or E-oscillators after a light pulse at ZT21. The brains were stained with anti-TIM antibody (red) and anti-PDF antibody (blue). LP represents light pulse, while NLP means no light pulse. From left to right, fly genotypes are 1) *jet^{set}*, 2) *Mai179-Gal4*, *jet^{set}/jet^{set}*; *UAS-jet*/+, 3) *Pdf-Gal4*, *jet^{set}/jet^{set}*; *UAS-jet*/+, 4) *Mai179-Gal4*, *jet^{set}/jet^{set}*; *UAS-jet*/*Pdf-GAL80*. Scale bars indicate 10 µm. (B) Quantifications of TIM level y-axis shows the relative TIM level in M-oscillators, normalized to NLP controls for each genotype. Error bars correspond to S.E.M. n.s. - no significance, ****, p < 0.0001 was determined by t-test. (C) Representative confocal images showing TIM degradation in M-oscillators when JET dsRNAs are expressed in M and/or E-oscillators. From left to right, fly genotypes are 1) *Mai179-Gal4/UAS-Dcr2*, 2) *Mai179-Gal4/UAS-Dcr2; jet^{RNAi}*/+, 3) *Pdf-Gal4/UAS-Dcr2; jet^{RNAi}*/+, 4) *Mai179-Gal4/UAS-Dcr2; jet^{RNAi}/Pdf-GAL80*. (D) Quantifications of TIM level. y-axis shows the relative TIM level in M-oscillators, normalized to NLP controls. Error bars correspond to S.E.M. n.s. - no significance, *, p < 0.05, ****, p < 0.0001 was determined by t-test. See also Figure 2.6 for the similar results obtained at ZT15.

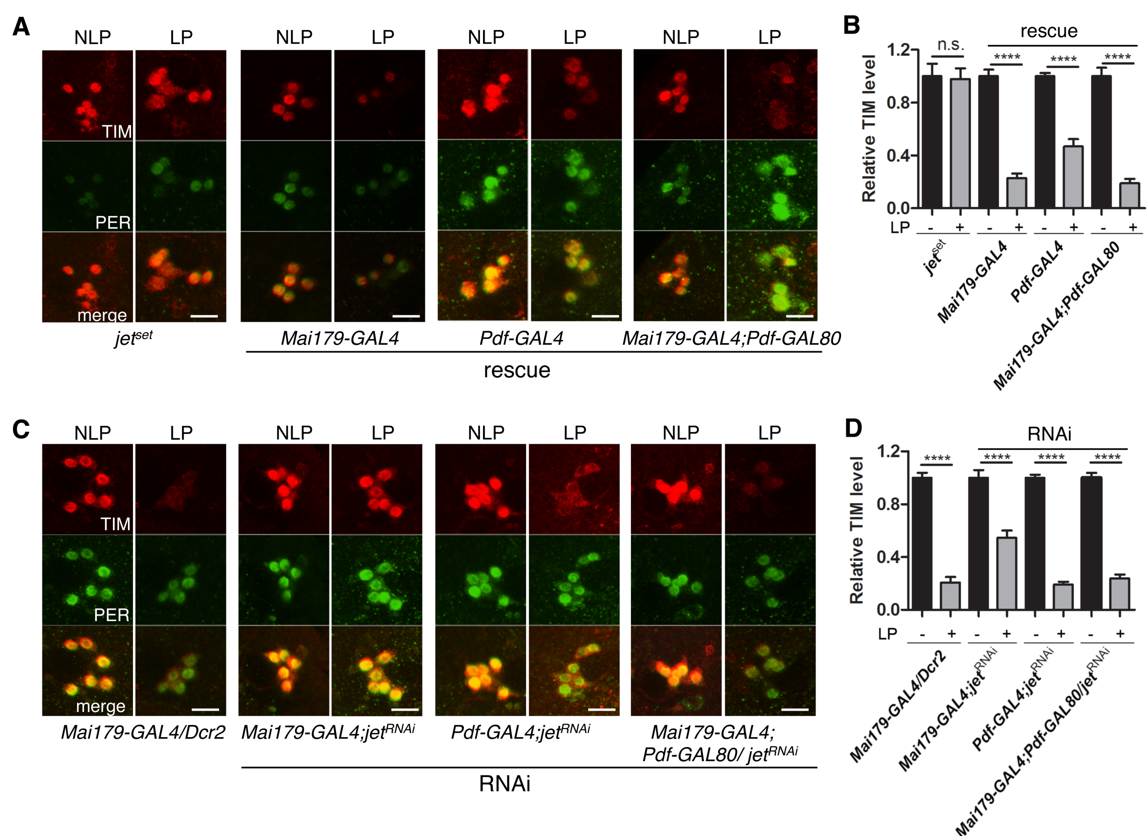


Figure 2.5 Cell-autonomous and non-autonomous role of JET in E-oscillators

(A) Representative confocal images showing TIM degradation in LNds of *jet^{set}* flies rescued in M- and/or E-oscillators, after a light pulse at ZT21. The brains were stained with anti-TIM antibody (red) and anti-PER antibody (green). From left to right, fly genotypes are 1) *jet^{set}*, 2) *Mai179-Gal4, jet^{set}/jet^{set}; UAS-jet/+*, 3) *Pdf-Gal4, jet^{set}/jet^{set}; UAS-jet/+*, 4) *Mai179-Gal4, jet^{set}/jet^{set}; UAS-jet/Pdf-GAL80*. Scale bars indicate 10 µm. (B) Quantifications of TIM level. y-axis shows the relative TIM level in LNds, normalized to the NLP controls. Error bars correspond to S.E.M. ****, $p < 0.0001$ was determined by t-test. Note that TIM is degraded in the LNds of *Pdf-Gal4, jet^{set}/jet^{set}; UAS-jet/+* flies, even though JET is only expressed in M-oscillators (see also Figure 2.6C for additional controls). (C) Representative confocal images showing TIM degradation in LNds when JET dsRNAs are expressed in M and/or E-oscillators, after a light pulse at ZT21. From left to right, fly genotypes are 1) *Mai179-Gal4/UAS-Dcr2*, 2) *Mai179-Gal4/UAS-Dcr2; jet^{RNAi}/+*, 3) *Pdf-Gal4/UAS-Dcr2; jet^{RNAi}/+*, 4) *Mai179-Gal4/UAS-Dcr2; jet^{RNAi}/Pdf-GAL80*. (D) Quantifications of TIM level. y-axis shows the relative TIM level in LNds compared with the average level in three neighboring non-circadian neurons. TIM levels are normalized to NLP controls. Error bars correspond to S.E.M. ****, $p < 0.0001$ was determined by t test. Note that down-regulating JET only in E-oscillators does not affect TIM degradation, but blocking JET expression in both M and E-oscillators does.

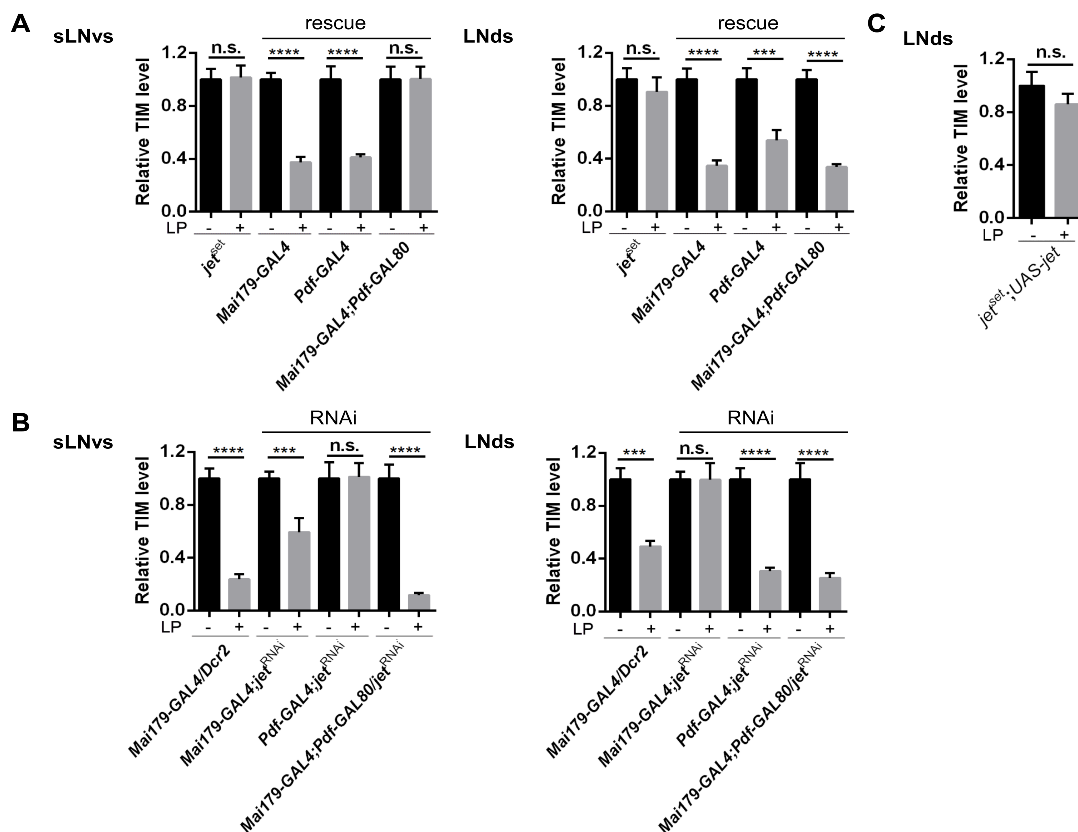
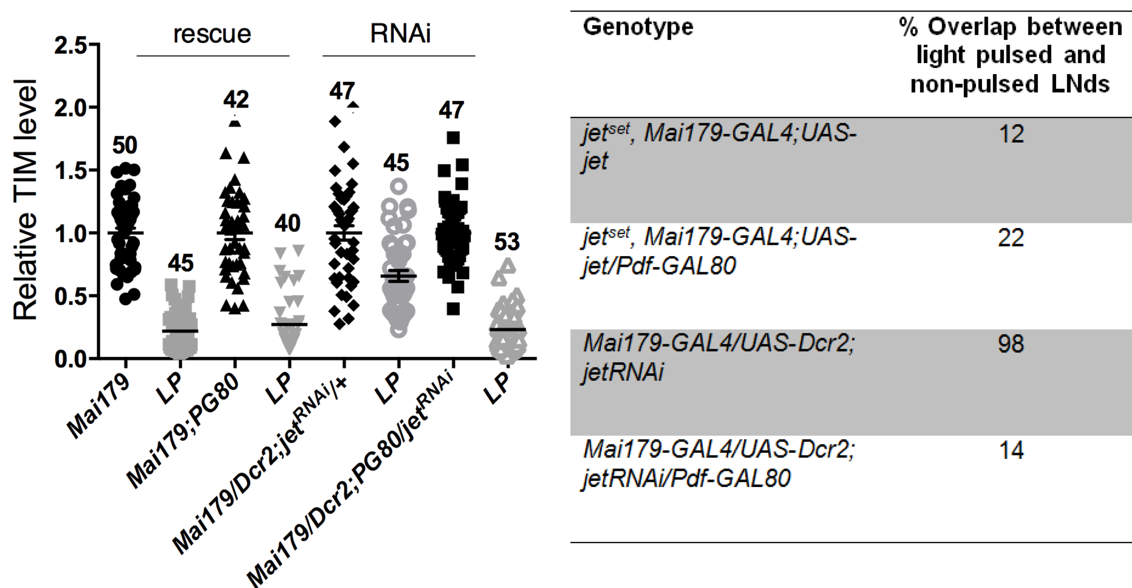


Figure 2.6 TIM degradation in the M- and E- oscillators after a ZT15 light pulse

(A) Quantification of TIM levels in the M-oscillators (left) and E-oscillators (right) in neuron-specific rescued *jet*^{set}. y-axis shows relative TIM levels normalized to no light pulse controls for each genotype. Error bars correspond to S.E.M. n.s. - no significance, ****, P < 0.0001; ***, P < 0.001 as determined by t-test. (B) TIM levels in M-oscillators (left) and E-oscillators (on right) when *jet* is knocked down using RNAi. Relative TIM levels normalized to no light pulsed control are plotted on the y-axis. Statistics are the same as in Fig 2.6A. Abbreviations of the genotypes are the same as in Fig 2.4C. (C) TIM levels in the LNds of *jet*^{set} flies carrying *UAS-jet* but no *GAL4 driver. UAS-jet* alone does not rescue the TIM degradation *jet*^{set} phenotype. Thus there is no leaky expression of JET in LNds. LP was given at ZT21



Genotype	% Overlap between light pulsed and non-pulsed LNds
jet^{set}, Mai179-GAL4;UAS-jet	12
jet^{set}, Mai179-GAL4;UAS-jet/Pdf-GAL80	22
Mai179-GAL4/UAS-Dcr2; jetRNAi	98
Mai179-GAL4/UAS-Dcr2; jetRNAi/Pdf-GAL80	14

Figure 2.7 Distribution of TIM signals in individual LNds with or without light pulses at ZT21.

Left: Each spot represents the relative TIM signal in an individual LNd. Note that most LNds appear to behave similarly within a genotype (and within a brain), which shows that TIM degradation in *Mai179-GAL4* negative LNds is triggered by non-autonomous signals. Error bars correspond to S.E.M. The fly genotypes are 1) *Mai179-Gal4*, *jet^{set}/jet^{set}*; *UAS-jet*/+, 2) *Mai179-Gal4*, *jet^{set}/jet^{set}*; *UAS-jet/Pdf-GAL80,* 3) *Mai179-Gal4/UAS-Dcr2; jet^{RNAi}*/+, 4) *Mai179-Gal4/UAS-Dcr2; jet^{RNAi}/Pdf-GAL80*. LP is abbreviated for light pulse. Number of neurons quantified are indicated.

Right: Percentage overlap of TIM staining intensity between light-pulsed and non-pulsed LNds. If *Mai179*-positive and –negative LNds behaved as separate groups, overlap should be 50%, since 3 of the 6 LNds are *Mai179*-positive. Indeed, only the rescued cells should show TIM degradation, or only the LNds that do not express *jet* dsRNAs. This is clearly not the case. In each case the percentage clearly differed from 50% suggesting that most LNds behaved as a single population. Chi-square test with Yate's correction confirms our interpretation that the LNds do not behave as two equally divided populations: $p < 0.0001$ for all four genotypes.

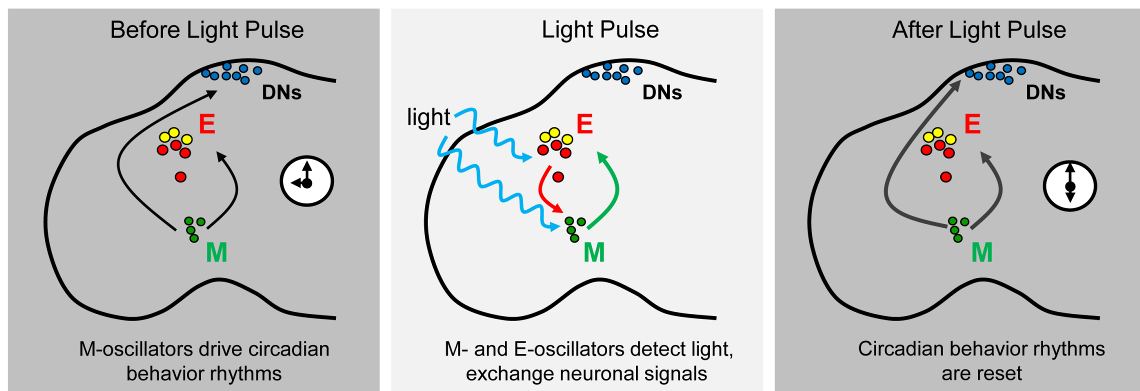


Figure 2.8 Model depicting that M- and E- oscillators cooperate to reset the circadian behavior in response to light input.

Before the light pulse, M-oscillators, which are the pacemaker neurons, maintain rhythmicity under constant conditions. During a light pulse, M- and E- oscillators detect light and interact with each other. Once the M- and E-oscillators are reset, M-oscillators synchronize the oscillations in other clock neurons to generate coherent behavioral rhythms. In this case, a light pulse given in the early night (time depicted by the clock before light pulse) delays the phase of circadian rhythms by three hours (represented by the clock time after the light pulse).

Table 2.2 TIM degradation in M- and E- oscillators and behavioral phase shifts after light pulses

Genotype	JET expression	TIM degradation after light pulse ZT15/ZT21		Phase shift
		s-LNvs (M-oscillator)	LNds (E-oscillator)	ZT15/ZT21
jet^{set}	No JET expression	-/-	-/-	-/-
jet^{set}*, Mai179-GAL4;UAS-jet*	JET expression in both M and E oscillators	++/++	++/++	++/++
jet^{set}*, Pdf-GAL4;UAS-jet*	JET expression only in M oscillator	++/++	+/+	+/-
jet^{set}*, Mai179-GAL4;UAS-jet/Pdf-GAL80*	JET expression only in E oscillator	-/-	++/++	-/-
Mai179-GAL4/UAS-Dcr2	JET expression in both M and E oscillators	++/++	++/++	++/++
Mai179-GAL4/UAS-Dcr2; jetRNAi	JET expression knocked down in M and E oscillators	+/+	+/+	-/+
Pdf-GAL4/UAS-Dcr2; jetRNAi	JET expression knocked down in M oscillator	-/-	++/++	+/+
Mai179-GAL4/UAS-2; jetRNAi/Pdf-GAL80	JET expression knocked down in E oscillator	++/++	++/++	++/++

"++" represents full TIM degradation or phase shift, "+": partial, "-": none

4. Discussion

Circadian photoreception is based on a cell-autonomous mechanism. However, recent studies indicate that resetting circadian behavior in response to light input requires neural interactions (Shang et al. 2008; Tang et al. 2010). Our results show that the M- and E-oscillators are critical for circadian photoresponses and act synergistically to shift the timing of the locomotor rhythms in response to light. Indeed JET is required in both the M- and E-oscillators, whereas individually, these neuronal groups cannot, or only weakly, phase-shift locomotor rhythms. Moreover, JET promotes both cell-autonomous and non-autonomous acute TIM degradation in circadian neurons. Thus, circadian behavior relies heavily on network interactions during its photic resetting (Figure 2.8).

The identification of the E-oscillators as critical cells for both phase delays and advances was unexpected. Indeed, Shang et al. (2008) found that phase advance response was abolished when the l-LNvs were ablated and Tang et al. (2010) based on TIM degradation pattern after low light intensity pulse, proposed that the DN1s were important for phase delays. However, our experiments indicate that JET is neither required, nor sufficient in DN1s and l-LNvs for phase shifts. The l-LNvs might thus secrete a neurotransmitter in a JET-independent manner, and this only happens when the light pulse is administered late at night.

Our finding that JET in the M-oscillators can non-autonomously trigger TIM degradation in the E-oscillators was also unanticipated. How JET does so is unclear, but it must involve rapid communication between the M- and E-oscillators, because we measured TIM degradation only one hour after the light pulse. JET might regulate acutely

neuronal activity, possibly with CRY's help. Indeed, this photoreceptor influences neuronal activity in a light-dependent manner, and is required for phase-shifts in M-oscillators (Fogle et al. 2011; Tang et al. 2010). Interestingly, the reverse is not true: JET in the E-oscillators has no effect on TIM degradation in the M-oscillators. Since the E-oscillators are essential for phase shifts and the M-oscillators drive circadian behavior (Stoleru et al. 2005), the formers have to communicate with the latter through a JET-independent mechanism. Although JET in the E-oscillators cannot promote TIM degradation in M-oscillators, our rescue experiments suggest that it can do so in the *Mai179-GAL4*-negative LNds. Indeed, JET expression restricted to the E-oscillators restored TIM degradation in most LNds (Figure 2.7). In addition, JET expression in M-oscillators promoted TIM degradation in most LNds as well. The non-E-oscillator LNds are CRY negative, which suggests that they rely on a non-autonomous mechanism for TIM degradation (Yoshii et al. 2008). Our results indicate that JET's non-autonomous function in TIM degradation might be critical to spread light information broadly in the circadian neural network.

Strong evidence supports the idea that acute TIM degradation is required for circadian behavioral photoresponses (Suri et al. 1998; Yang et al. 1998). However, a recent study has challenged the notion that TIM degradation in M-oscillators is critical for phase shifts, or at least for phase delays (Tang et al. 2010). Our results suggest that TIM degradation is critical in E-oscillators, whether it is achieved cell-autonomously or not, since partial block of TIM degradation in E-oscillators is associated with compromised phase advances and delays (Figure 2.2, 2.5, Table 2.2). In the M-

oscillators, the requirement for TIM degradation remains uncertain. On one hand, JET is required in these neurons and promotes TIM degradation cell-autonomously. On the other hand, this JET-dependent TIM degradation could be unnecessary for behavioral phase-shifts: JET in M-oscillators could contribute to phase shifts entirely non-autonomously. We note that TIM degradation is severely blocked in M-oscillators when JET is downregulated, but phase delays are only partially disrupted (Table 2.2). This would fit with the idea that TIM degradation in M-oscillators is not required for phase shifts, although we cannot rule out that TIM degradation occurred with slower kinetics. In any case, we propose that after light pulses, TIM degradation in E-oscillators resets their molecular pacemaker, which allows them to help the M-oscillators to resynchronize their own circadian pacemaker. The M-oscillators then readjust the whole circadian neural network. This bears similarities with light synchronization in mammals. The Suprachiasmatic Nucleus (SCN) - the mammalian neural circadian pacemaker - receives light input through dedicated retinal ganglion cells in the retina (Hattar et al. 2006). Cells in the core of the SCN appear to be particularly sensitive to this light input. They communicate with robust pacemaker neurons of the shell, which then reset the whole circadian neural network (Yan et al. 2007)

5. Material and Methods

Fly stocks

All the flies were raised on cornmeal/agar medium at 25ºC under a light: dark (LD) cycle. The following *Drosophila* strains were used in this study: *y w; jet^{set}*

(identified in an ethyl methanesulfonate (EMS) screen), *jet*^{c}*, jet*^{r} and *UAS-jet* (Koh et al. 2006), *y w, cry*^{b} (Stanewsky et al. 1998), *tim-GAL4* (Kaneko et al. 2000), *Pdf-GAL4* (Renn et al. 1999), *c929-GAL4* (Hewes et al. 2003), *Clk4.1M-GAL4* (Zhang et al. 2010), *Mai179-GAL4* (Grima et al. 2004), *DvPdf-GAL4* (Bahn et al. 2009), *Pdf-GAL80* (Stoleru et al. 2004), *jet RNAi* (TRiP.JF01506, Bloomington Drosophila Stock Center), *UAS-myc-cry; cry*^{b} (Busza et al. 2004), *Pdf-GAL4; cry*^{b} (Emery et al. 2000). *UAS-jet* and the *GAL4* lines were genetically recombined with *y w; jet*^{set}. *UAS-Dcr2* was combined with the *jet* RNAi lines to increase the efficiency of knockdown. The presence of both *GAL4* and *jet*^{set} in the recombinants was confirmed by PCR and behavior analysis. The presence of the *s-tim* allele in *jet*^{set} mutants and recombinants was determined by PCR and sequencing. All the flies used for *jet*^{set} rescues are homozygotes for the *s-tim* allele. In the RNAi experiments, most flies are *s-tim/ls-tim* heterozygotes, with the exception of the flies expressing *jet* RNAi with *tim-GAL4*, and their *tim-GAL4/UAS-Dcr2* control, which are homozygous for the *ls-tim* allele.

Protein extracts and Western blotting

The *y w; jet*^{set} and *y w* flies were entrained in a 12:12 LD cycle for three days and fly heads were collected on the fourth day at six Zeitgeber times – 1, 5, 9, 13, 17 and 21. For the acute response of light pulse on TIM levels, one group of flies was exposed to a 10 minutes light pulse (1500 lux) at ZT21 and then kept in dark for 1 hour. Protein extraction from the heads of the pulsed and the no light pulsed flies was performed as described previously (Emery et al. 1998). The samples were then run on a 6% SDS

polyacrylamide gels and transferred to nitrocellulose membrane using a semi-dry electroblotting apparatus. Quality of protein transfer was verified by Ponceau red staining. The membranes were probed with 1:1000 dilution of guinea pig anti-TIM. The signal on the film was digitalized using IR-LAS-1000 Lit V2.12 (Fujifilm) and quantified using Image J software. TIM levels were normalized to α-Spectrin.

Behavioral monitoring and analysis

For constant light behavior (LL), locomotor activity of single adult male fly (2-5 days old) was measured with Trikinetics Activity Monitors (Waltham, MA) for 3 days in a 12 hour light: 12 hour dark (LD) cycle at 25°C followed by six days in constant light at an intensity of ca. 200 lux. Data was analyzed using the FaasX software (courtesy of F. Rouyer, Centre National de la Recherche Scientifique, Gif-sur-Yvette, France). Rhythmicity was defined by the criteria – power >10, width >2 using the $\chi 2$ periodogram analysis. Group activity actograms were generated by signal-processing toolbox (Levine et al. 2002b) for MATLAB (MathWorks). For phase shift experiments, flies were entrained to a 12:12 LD cycle for 5 days and were exposed to a 5-minute pulse of a white fluorescent light (1500 lux) at ZT15 and ZT21 on the 5th day. A separate control group of flies was not light-pulsed. Following the light pulse, flies were released in DD and their locomotor activity was monitored for six days. We found the mid-point of the off-set of subjective evening activity to be the most reliable phase marker across genotypes. It is defined as the time at which the activity of each group of flies (averaged between day 2-6 after the light pulse) reaches 50% of peak value. For the statistical analysis of the

phase shifting behavioral experiments, in rescue situation, we used one way analysis of variance (ANOVA) coupled to Tukey's post hoc test for comparison amongst the genotypes. For the jet RNAi experiments; we compared each genotype and its GAL4 driver control using student's t test.

Whole Mount Immunocytochemistry

For TIM staining, adult flies (2-5 day old) were entrained for 3 days in a 12:12 LD cycle and were subjected to a 5 minutes light pulse of 1500 lux at ZT15 and ZT21 and returned to darkness for an hour before dissection. Flies were then anesthetized and fixed in 4% paraformaldehyde for 45 minutes in darkness. The fixed fly heads were then removed and dissected in PBS. The whole brains were then rinsed and washed with PBT (PBS + 0.1% Triton) three times for 10 mins. For CRY staining, flies were entrained for three days and kept in constant darkness for three days and dissected on the third day at CT23 (peak time for CRY levels). Whole-mount immunohistochemistry for fly brains were then done as previously described (Zhang et al. 2010). The brains were incubated with 10% normal goat serum diluted in PBT for 40 mins at room temperature and then incubated with primary antibodies at 4 ºC overnight. Primary antibodies used were: mouse anti-PDF (1:400, from the Developmental Studies Hybridoma Bank), rabbit anti-PER (1:1500, generous gift from Dr. R. Stanewsky), an affinity purified guinea pig anti-TIM (1:100) (Rakshit et al. 2012), rabbit anti-CRY (1:200, generous gift from Dr. C. Helfrich-Forster) and mouse anti-GFP (1:200). Brains were incubated with the relevant secondary antibody at 4ºC

overnight followed by another round of six washes with PBT. All samples were viewed on a Zeiss LSM5 Pascal confocal microscope. Up to eight fly brains for each genotype were dissected for imaging. Representative images are shown. ImageJ software (NIH) was used for TIM quantification in 18-24 LNds, 12-18 sLNvs and 10-12 DN1as from at least five brains. For quantification, signal intensity in each neuron was measured and then the average signals in three neighboring non-circadian neurons were subtracted. For each genotype, the light pulsed group was compared to its no light pulsed group using a student's t-test. TIM degradation was measured in two independent experiments for both ZT15 and 21 and for all genotypes. Very similar results were obtained. Figures show one of these two independent experiments.

Chapter III

Neural network interactions modulate CRY-dependent photoresponses in *Drosophila*

This chapter is currently under review at *The Journal of Neuroscience* as:

Lamba, P. and Emery, P.

I performed all the experiments. Emery P. and I wrote the manuscript

1. **Abstract**

Light is one of the chief environmental cues that reset circadian clocks. In *Drosophila*, CRYPTOCHROME (CRY) mediates acute photic resetting of circadian clocks by promoting the degradation of TIMELESS (TIM) in a cell-autonomous manner. Thus, even circadian oscillators in peripheral organs can independently perceive light in *Drosophila*. However, there is substantial evidence for non-autonomous mechanisms of circadian photoreception in the brain. We have previously shown that the Morning (M) and Evening (E) oscillators are critical light sensing neurons that cooperate to shift the phase of circadian behavior in response to light input. However, we show here that light can efficiently phase-delay or phase-advance circadian behavior when either the M- or the E-oscillators are ablated, suggesting that behavioral phase shifts and their directionality are largely a consequence of the cell-autonomous nature of CRY photoreception. Our observations that the Phase Response Curve (PRC) of brain and peripheral oscillators are remarkably similar further support this notion. Nevertheless, the neural network modulates circadian photoresponses. We show that the M-oscillator neurotransmitter Pigment Dispersing Factor plays a critical role in the coordination between M- and E-oscillators after light exposure, and we uncover a potential role for a subset of dorsal neurons in the control of phase advances. Thus, neural modulation of autonomous light detection might play an important role in the plasticity of circadian behavior.

Significance statement

Input pathways provide circadian rhythms with the flexibility needed to harmonize their phase with environmental cycles. Light is the chief environmental cue that synchronizes circadian clocks. In *Drosophila*, the photoreceptor CRYPTOCHROME (CRY) resets circadian clocks cell-autonomously. However, recent studies indicate that in the brain, interactions between clock neurons are critical to reset circadian locomotor behavior. We present evidence supporting the notion that the ability of flies to advance or delay their rhythmic behavior in response to light input essentially results from cell-autonomous photoreception. However, because of their networked organization, we find that circadian neurons have to cooperate to reset the phase of circadian behavior in response to photic cues. Our work thus helps reconciling cell-autonomous and non-cell-autonomous models of circadian entrainment.

2. Introduction

Circadian rhythms are endogenous time-keeping mechanisms that drive rhythms in behavior, physiological functions and gene expression. The ability of circadian clocks to be reset by various environmental cues allows organisms to anticipate changes in the environment and capitalize on available resources. Light is the cardinal synchronizer of circadian clocks, which entrains them to 24-hour solar cycles. In a 12:12 light dark (LD) cycle, *Drosophila* exhibits a bimodal activity pattern. A morning peak of activity in anticipation of lights-on is generated by small ventrolateral neurons (sLNvs), also referred to as the Morning (M) oscillators (Grima et al. 2004; Stoleru et al. 2004). The sLNvs are also the pacemaker neurons that maintain free-running behavioral rhythms in constant conditions by rhythmically secreting Pigment Dispersing Factor (PDF) to synchronize the other circadian neurons (Renn et al. 1999; Stoleru et al. 2005). The evening peak of activity in anticipation of lights-off is governed by the dorso-lateral neurons (LNds) and the PDF negative 5th sLNvs, together referred to as the Evening (E) oscillators (Grima et al. 2004; Stoleru et al. 2004).

Drosophila can perceive light via three input pathways - the compound eyes and the ocelli, the extra-ocular photoreceptor known as the Hofbauer-Buchner (H-B) eyelet and the intracellular blue-light photoreceptor CRYPTOCHROME (CRY) (Emery et al. 1998; Helfrich-Förster et al. 2001; Stanewsky et al. 1998; Rieger et al. 2003). Mechanistically, the CRY-dependent input pathway is best understood. CRY resets the pacemaker by promoting the rapid proteasomal degradation of the key pacemaker protein TIMELESS (TIM) (Lin et al. 2001). Upon exposure to light, CRY undergoes a

conformational change that enables its binding to TIM and promotes degradation of TIM via the F-box protein JETLAG (JET), which is a component of an E3 ubiqitin ligase (Busza et al. 2004; Koh et al. 2006). The *Drosophila* circadian clock is actually very sensitive to light. A brief light pulse in the early night - mimicking a belated dusk - delays the phase of circadian behavior, while a late night pulse is perceived as an early dawn and thus advances the phase of locomotor activity (Levine et al. 1994). Severe *cry* or *jet* mutants are unable to respond to short pulses of light, and acute TIM degradation is impaired in these mutants (Lamba et al. 2014; Lin et al. 2001; Stanewsky et al. 1998).

The molecular mechanism underlying CRY-dependent photoresponses is as described above cell-autonomous. In fact, it has been shown that circadian oscillators in isolated body segments such as wings, legs, antennae etc. can sense light independently and even re-entrain to a new LD cycle shifted by 6 hours (Plautz et al. 1997). However, in the brain, there is strong evidence for additional non-autonomous mechanisms. First, CRY expression limited to the M-oscillators is not sufficient to fully rescue circadian phase shifts in *cry* mutant flies (Emery et al. 2000; Lamba et al. 2014). Second, not all circadian neurons express CRY, yet even CRY-negative circadian neurons can acutely degrade TIM in a CRY-dependent manner (Yoshii et al. 2008). Third, specific groups of circadian neurons might promote phase delays or phase advances (the DN1s and l-LNvs, respectively) (Shang et al. 2008; Tang et al. 2010). Fourth, we have recently shown that the M- and E-oscillators are the critical light sensing neurons, working together to reset the phase of circadian behavior upon light exposure (Lamba et al. 2014). Moreover, we found that JET in M-oscillators could promote non cell-autonomously acute TIM

degradation in E-oscillators. Since neural cooperation between M- and E-oscillators is required for photic resetting, we surmised that these circadian neurons are exchanging signals during or after light exposure to phase shift circadian behavior.

Unexpectedly, we show here that neither the M-oscillators, nor the E-oscillators are required for behavioral phase delays or phase advances in responses to light pulses. However, when both cell groups are present, the M-oscillator neurotransmitter PDF functions as a modulator of CRY-dependent light detection. Our results also suggest that additional circadian neurons – a subset of Dorsal Neurons (DNs) are able to modulate phase advances specifically.

3. Results

Light can reset the phase of circadian behavior in the absence of M-oscillators

We wanted to determine how the M-oscillators communicate with the E-oscillators during circadian photoresponses. PDF is the principal circadian neurotransmitter responsible for synchronizing different groups of neurons and for persistence of rhythmicity under free-running conditions (Renn et al. 1999; Lin et al. 2004). Moreover, Guo *et al* have shown that induction of firing in PDF-positive neurons generates behavioral phase shifts reminiscent of those induced by light pulses. and triggers TIM degradation in E-oscillators in a PDF-receptor dependent manner (Guo, Cerullo, et al. 2014). Hence, PDF would be an ideal candidate for the signal transmitted from M- to E-oscillators during light-dependent phase resetting. *Pdf*^{0} flies show an advanced evening peak of activity in LD conditions and gradually become arrhythmic in constant darkness (DD) (Renn et al. 1999). However, a small fraction of flies (16 % in

our hands) remain rhythmic in DD with a short period of approximately 22 hours (Table 3.1). Hence, it was possible to test the ability of these flies to shift their behavior in response to delaying and advancing light pulses (Fig 3.1 A). We analyze phase shifts only during the days when the flies still clearly showed rhythmic activity as a population. We were sometimes able to measure phase shifts over five days, but more frequently 1-3 days were measured. Phase shifts showed no obvious transients, even during the first day and were stable when measured over several days. (Fig 3.2 A) This was actually the case for all the genotypes used in this study. This was the case for almost all genotypes used in this study, except for two genotypes discussed below. We therefore feel confident that although for some genotypes we frequently had shorter measurements, we can compare these genotypes to those with robust rhythms measured systematically over 5 days.

The photic Phase Response Curve (PRC) of wild-type flies (entrained in a 12:12 LD cycle) show phase delays that are maximal around ZT15-17, and phase advances that are maximal around ZT21 (Levine et al. 1994) (Suri et al. 1998). Surprisingly, *Pdf*^{0} flies could shift the phase of their behavior very efficiently: both robust phase advances and phase delays could be observed (Fig 3.1A). This result fits with recent observations made with calcium imaging in living flies (Liang et al. 2017) where light exposure both in the delay as well as advance zone induced shifts in calcium rhythms in *Pdf*^{0} null mutant flies. Since M-oscillators express an additional neuropeptide - short Neuropeptide-F (sNPF) and are also glycinergic (Frenkel et al. 2017; Helfrich-Förster et al. 2007; Liang et al. 2017), we decided to simply ablate the PDF positive cells by expressing the proapoptotic gene *hid*. As expected, the morning peak of activity was lost in *Pdf-GAL4/UAS-hid* flies

(Fig 3.1B). These flies showed greater arrhythmicity than *Pdf*^{0} (Table 3.1). As a result, phase shifts could only be reliably quantified over 1-3 days. LNv-ablated flies shifted the phase of locomotor behavior by approximately 3.5 hours in response to both delaying and advancing light pulses, demonstrating that light-mediated behavioral phase resetting can occur in the absence of M-oscillators (Fig 3.1C and 3.2B). This result, combined with our previous work (Lamba et al. 2014), also implies that the E-oscillators can shift the phase of circadian behavior without the M-oscillators.

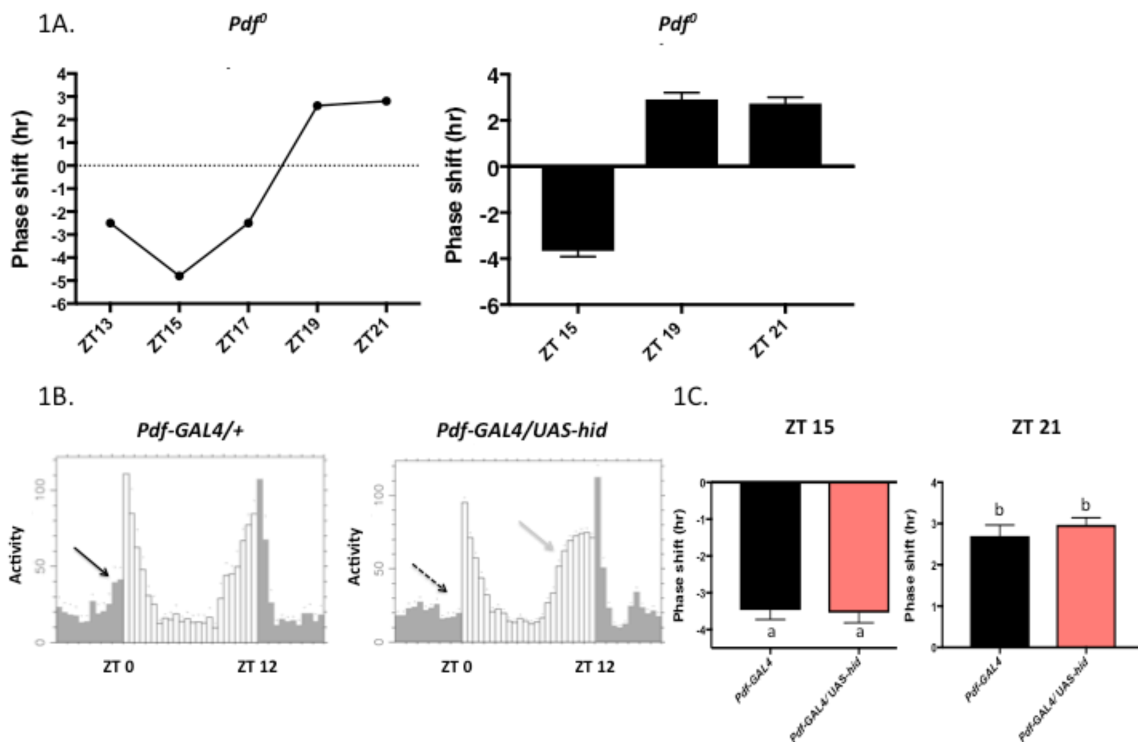


Figure 3.1 Light can reset the phase of locomotor activity in the absence of PDF positive M-oscillators or their neuropeptide PDF.

(A) *Pdf⁰* flies can undergo phase delay as well as phase advance in response to 5 minutes light pulse (1500 lux). The graph on the left shows a single experiment generating a phase response curve for *Pdf⁰* flies. Phase change is plotted on the y-axis; phase delays and advances are represented as negative and positive values respectively. The x-axis represents the zeitgeber (ZT) time of the light pulse. The graph on the right shows the average phase shifting response of *Pdf⁰* flies when exposed to light in the delay zone at ZT15 or in the advance zone at ZT19 and ZT21; $N \geq 4$. Subsequent studies focused on responses at ZT15 and ZT21 because maximal responses occurred at these times.

(B) Ablation of M-oscillators abrogates the morning anticipation of lights-on and advances the evening peak of activity under a 12:12 LD cycle. Activity is plotted as a function of time. Grey bars in the histogram represent activity levels in the night and the white bars represent activity during the day. For *Pdf-GAL4* control flies, the solid arrow shows the morning anticipatory behavior. For *Pdf-GAL4/UAS-hid flies*, the dashed arrow indicates the lack of morning anticipation and the grey arrow indicates the advanced evening peak of activity.

(C) Flies with ablated M-oscillators can respond to brief light pulses. Phase delay in response to light pulse at ZT15 is shown on the left and phase advance in response to light pulse at ZT21 is on the right. Both the phase delay and advance responses in *Pdf-GAL4/UAS-hid* flies (Pink bars) were similar to the *Pdf-GAL4*

control flies (Black bars). Data is plotted as mean $\pm$ S.E.M. Bars with same letters do not differ significantly at $p \leq 0.05$ level as determined by student's t-test.

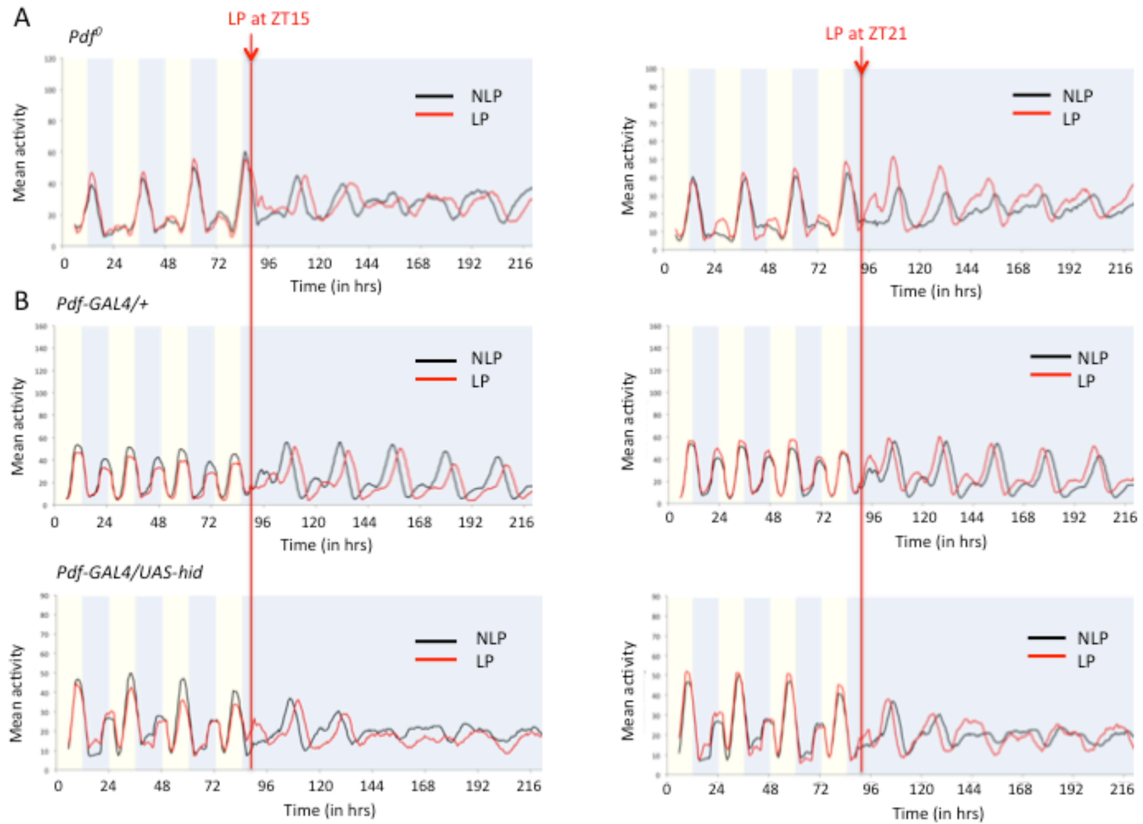


Figure 3.2 Ablation of PDF positive M cells or loss of PDF does not compromise phase shifting response

(A) Representative activity profiles of *Pdf⁰* flies that were subjected to light pulses. Black line: non pulsed control flies; red line: light pulsed flies. Light region represents day and dark region represents night. Light pulse is indicated by red arrow.

(B) Representative activity traces of M cells' ablated flies and their genotypic control. Black line: non pulsed control flies; red line: light pulsed flies.

Table 3.1 Circadian locomotor behavior under constant darkness

Genotype	Number of flies (n)	Percent rhythmic flies	Period average (±SEM)	Power average (±SEM)
pdf^{0}	448	16	22.6 ± 0.15	36.52 ± 2.33
Pdf-GAL4/UAS-hid	61	5	23 ±0.05	36.1 ± 10.95
Pdf-GAL4/+	33	93	24.6 ± 0.06	57.03 ± 2.54
PD2/+	89	87	24.4 ± 0.07	50.06 ± 3.43
UAS-jet RNAi	91	85	24 ± 0.09	62.2 ± 5.21
PD2/UAS-jet RNAi	120	88	24.2 ± 0.08	49.9 ± 2.34
PD2, pdf^{0}; UAS-jet RNAi, pdf^{0}	240	7	22.9 ± 0.15	28.05 ± 1.26
UAS-hid; Pdf-GAL80/+	54	92	23.9 ± 0.05	83.8 ± 14.6
cry-GAL4(13)/+	40	77	23.8 ± 0.03	58.6 ± 14.9
UAS-hid; Pdf-GAL80/cry-GAL4(13)	108	38	23.9 ± 0.12	48.05 ± 7.95
tub-GAL80^{ts}; UAS-kir/+	123	77	26.1 ± 0.05	49.9 ± 3.95
DvPdf-GAL4; Pdf-GAL80	121	94	24.9 ± 0.09	76.85 ± 5.96
tub-GAL80^{ts}; UAS-kir/DvPdf-GAL4; Pdf-GAL80	220	32	25.8 ± 0.13	36.27 ± 3.96
Pdf-GAL80; cry-GAL4(13)	38	100	23.5 ± 0.05	95.45 ± 6.93
tub-GAL80^{ts}; UAS-kir/Pdf-GAL80; cry-GAL4(13)	73	16	26.7 ± 0.11	29.3 ± 6.1

M-oscillators utilize PDF as a signal to modulate photic behavioral phase shifts

The observation that the M-oscillators are dispensable for photic phase shifts seems at odd with our previous work, which had shown that CRY photoreception in the M-oscillators is required for photic behavioral phase shifts (Lamba et al. 2014). In this study, we had found that downregulation of *jet* only in the PDF positive cells compromised the ability of M-oscillators to respond to light and to trigger TIM degradation, but TIM degradation in the E-oscillators was unaffected. As a result of this manipulation, photic behavioral phase shifts were significantly weakened. To explain both these previous and our current observations, we hypothesized that M-oscillators lacking CRY photoreception, by not degrading TIM and thus not shifting the phase of their local oscillators, do not reset the rhythmic secretion of PDF, and thus act as a brake on the resynchronization of the E-oscillators and the rest of the circadian neural network. If this were correct, flies with disrupted *jet* expression in the M-cells would phase-shift normally if they lack PDF.

As previously reported (Lamba et al. 2014), downregulation of *jet* with *Pdf*-GAL4 compromised both phase delays and phase advances (Fig 3.3A, 3.3B and 3.4). Importantly, the absence of PDF in flies with *jet* being knocked-down in M-oscillators completely restored normal shifts, suggesting that the circadianly blind M-oscillators indeed put the brakes on photic behavioral resetting via untimely secretion of PDF (Fig 3.3A, 3.3B and 3.4).

We noticed that at ZT19 and 21, *Pdf*-GAL4/UAS-*jet* RNAi flies usually showed a transient phase shift during the first day or two after the light pulse, which then tended to

disappear (Fig. 3.4). This does not affect our conclusions. Indeed, phase shifts are stable in the experimental flies lacking PDF (as assayed by quantification when possible, or visual inspection of the traces). If anything, differences are stronger than shown on Fig. 3.3B once a stable phase is reached.

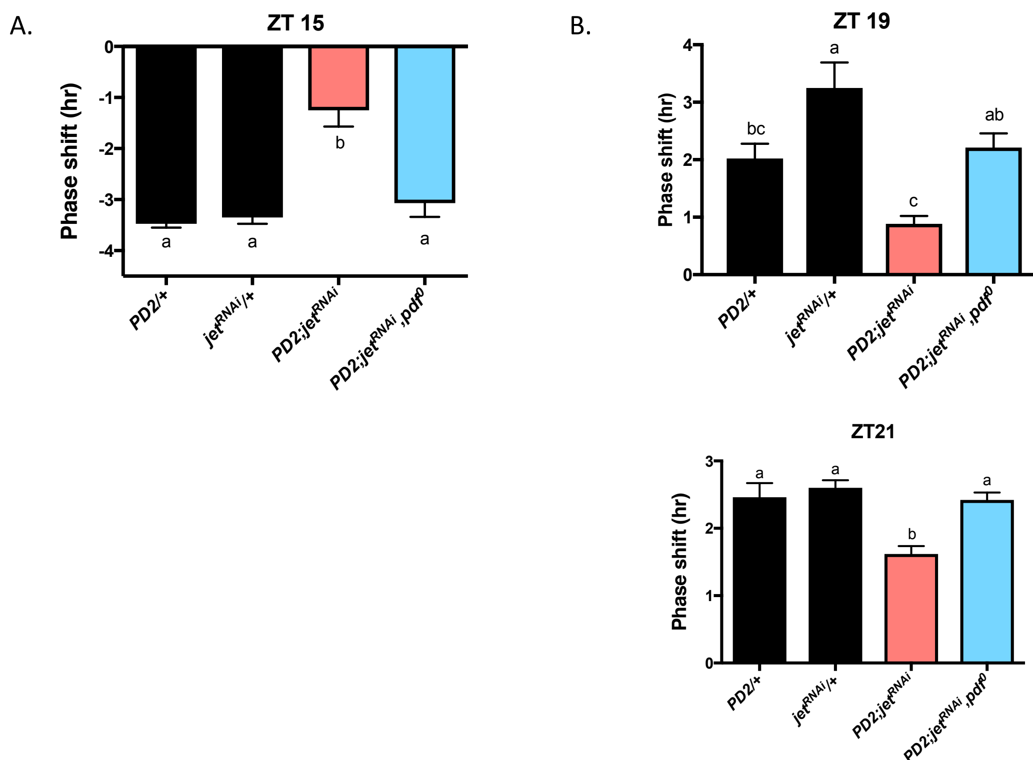


Figure 3.3 M-oscillators utilize PDF to modulate light-mediated phase responses.

(A) Knocking down *jet* in the M-oscillators (pink bar) reduces the phase delay responses compared to the *Pdf-GAL4* and *jet* RNAi control lines (black bars) Loss of PDF restores normal phase shifts in flies with *jet* downregulation in M-oscillators (blue bar). N = 4. Data represents mean $\pm$ S.E.M. Different letters above the bars indicate significant differences revealed by one-way analysis of variance (ANOVA) coupled to post hoc Tukey's test for multiple comparisons. (p <0.05)

(B) Downregulating *jet* in the M-oscillators in Pdf^0 flies (blue bar) restores the phase advance responses of *PD2; jet* RNAi flies (pink bar) when light pulse was given both at ZT19 and ZT21. The controls are in black bars. N = 6. The control *PD2* was not statistically different from *PD2; jet* RNAi at ZT19 because phase shift response of *PD2* flies was dramatically reduced in one of the experiments due to unknown reasons, but the difference in the phase response is still 1 hour between the two genotypes. Statistical significance was tested as above.

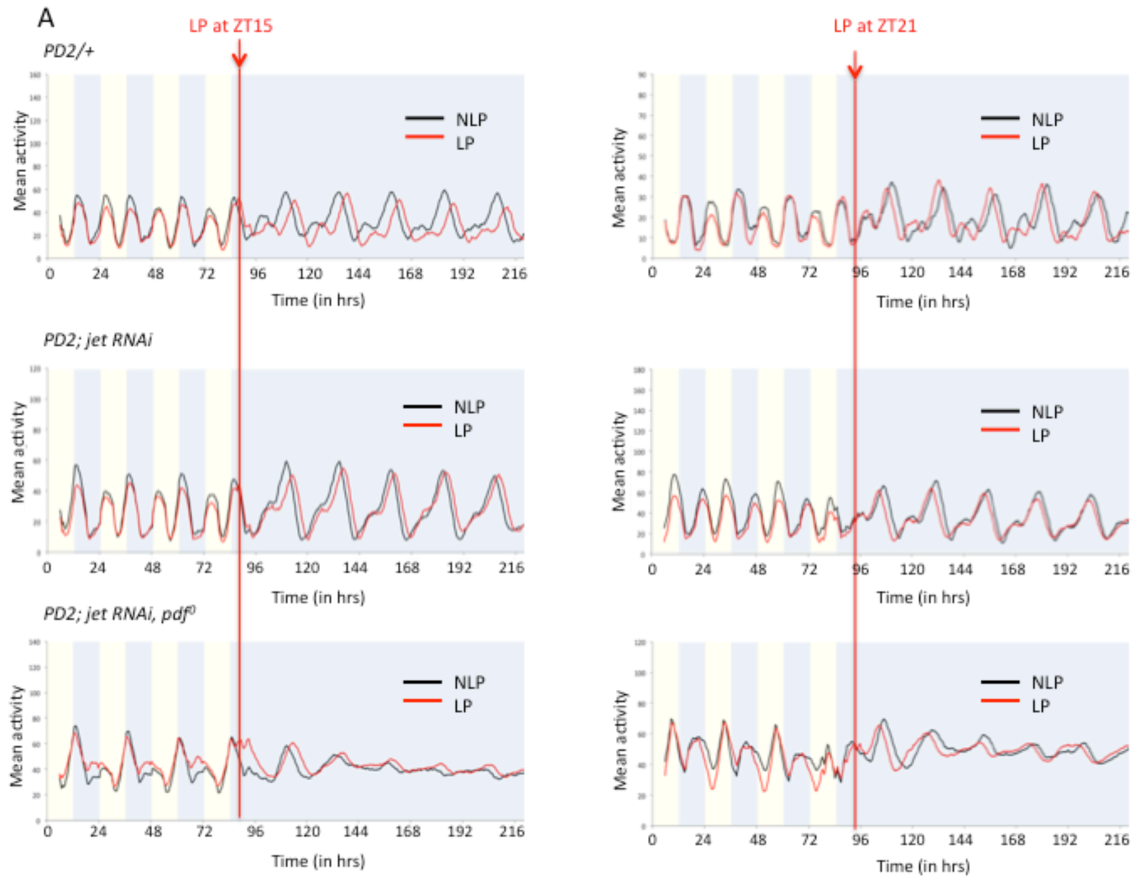


Figure 3.4 PDF modulates the light-mediated phase shifting response.

(A) Upper panel: *PD2/+* control flies; middle panel: *PD2; jet*^{RNAi} and bottom panel: *PD2; jet*^{RNAi}*,Pdf*^{0}. Black line: non pulsed control flies; red line: light pulsed flies. See figure 3.3 for additional details.

Light can reset circadian behavior without the E-oscillators

Recent studies on behavioral entrainment to light/dark cycles and response of the circadian neural network in a dish to light exposure suggest a hierarchy amongst the circadian neurons, with the E-oscillators leading the entrainment of the network (Roberts et al. 2015; Yoshii et al. 2015). To test the significance of the E-oscillators during photoresponses, we ablated the E-oscillators by expressing *UAS-hid* using the *cry-GAL4(13)* driver combined with the *pdf-GAL80* repressor (Stoleru et al. 2004), and administered light pulses both at ZT15 and ZT21. The efficiency of E-oscillators' ablation was verified by the suppression of the Evening peak of activity in a 12:12 LD cycle (Fig 3.5A). We found that flies with ablated E-oscillators showed high arrhythmicity (Table 3.1) consistent with the important role played by these cells in the control of circadian behavior (Guo et al. 2014). Surprisingly, ablation of the E-oscillators did not compromise the ability of flies to sense light circadianly as they underwent a phase shift of approximately 3 hours both in the delay as well as in the advance zone (Fig 3.5B and 3.6). We noticed however a transient during the first day after a pulse at ZT21 in the E-oscillator ablated flies: the phase shift was much less pronounced on day 1 compared to the other days (Fig 3.6, bottom). This is probably because activity is concentrated in the morning in these flies, and the circadian network has not yet fully resynchronized. This does not impact our conclusion that the E-oscillators are not required for phase shift, since this transient slightly decrease the average phase shifts shown in Fig 3.5B.

In summary, neither the M-oscillators nor the E-oscillators are required for photic behavioral phase shifts, but if present both neuronal groups need to detect light for phase shifts to occur properly. This reinforces the notion that the circadian neural network modulates cell-autonomous circadian photoresponses.

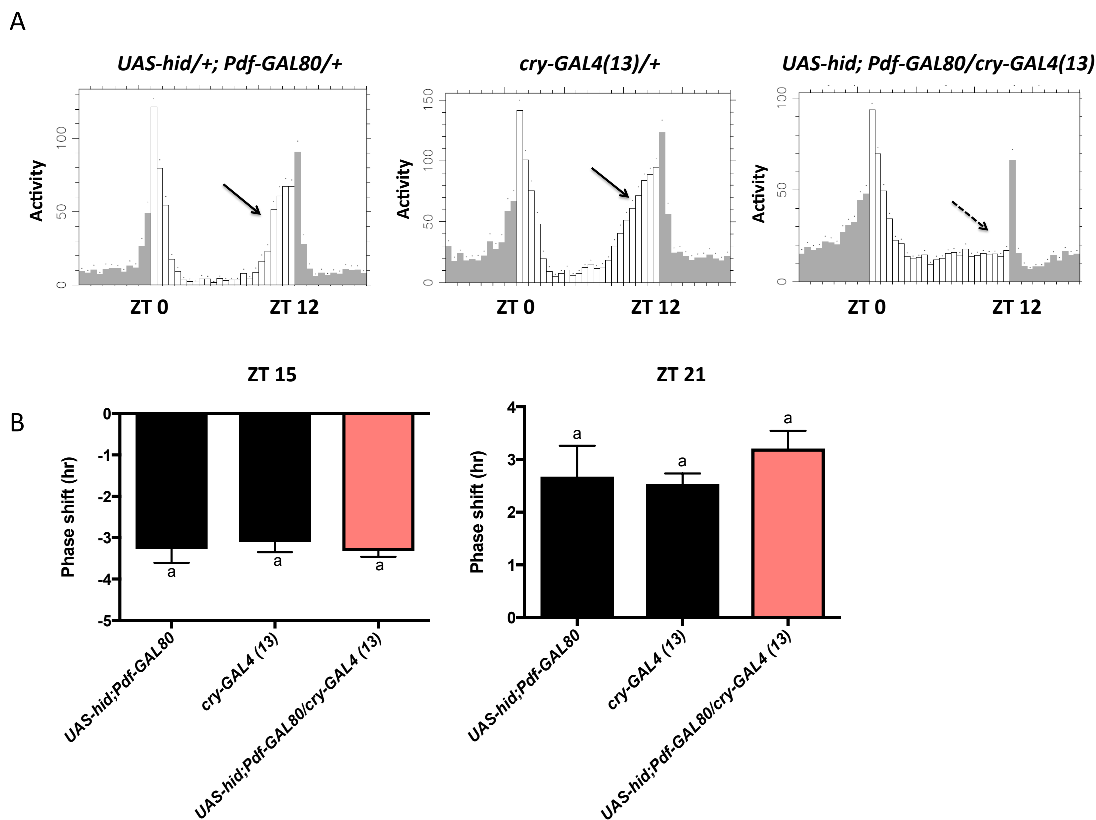


Figure 3.5 Light can reset the phase of circadian behavior in the absence of E-oscillators.

(A) Ablation of E-oscillators abrogates the evening peak of activity in a 12:12 LD cycle. The first two graphs on the right show the LD activity profiles of the control flies. Solid arrows indicate the evening anticipatory behavior. Dashed arrow in the last graph on the right shows the disruption of evening activity peak upon ablation of E-cells.

(B) Ablation of E-oscillators (pink bar) had no effect on phase delay or advance responses. Black bars are the UAS and GAL4 control flies. N=4. Error bars represent S.E.M. Same letters indicate not significant as determined by one way ANOVA and post hoc Tukey's test.

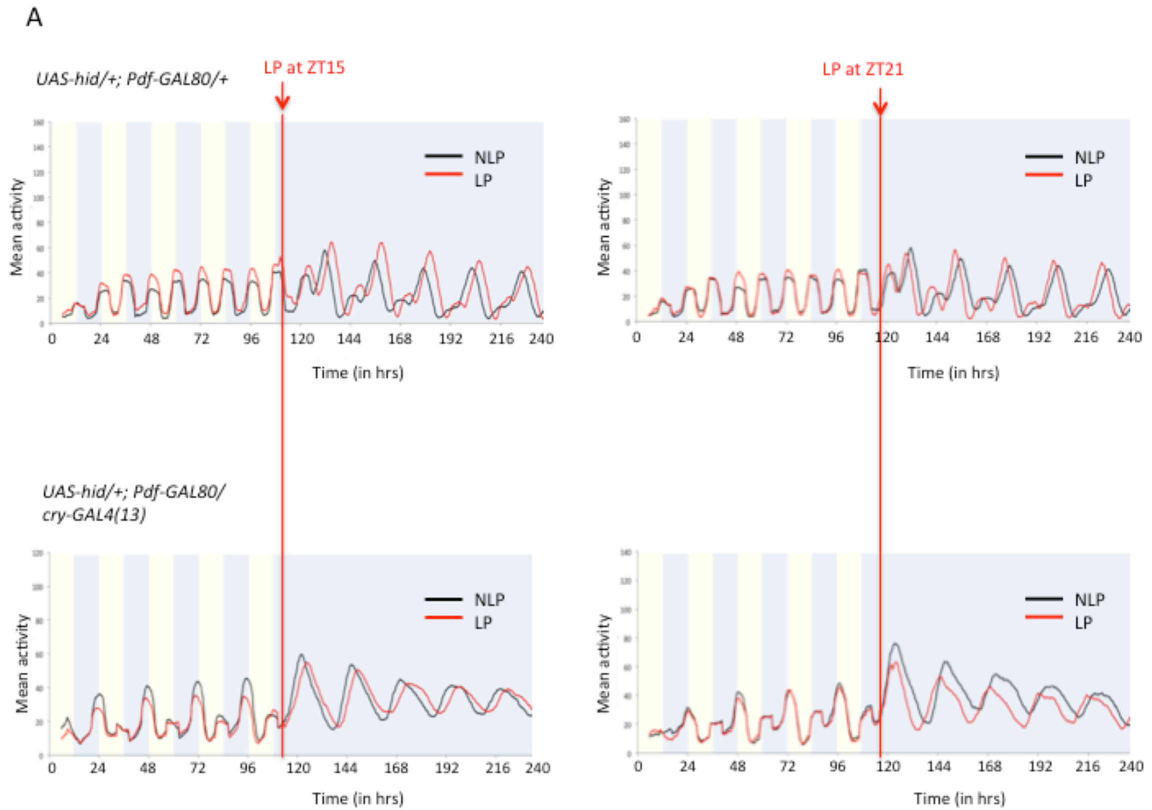


Figure 3.6 Light can reset the phase of behavior in the absence of E-oscillators.

(A) Upper panel: control flies. Bottom panel: E oscillator ablated flies. Black line: non pulsed control flies; red line: light pulsed flies.

Dorsal Neurons can impact photic behavioral phase advances

Since the *cry-GAL4(13)* driver is also expressed in some of the dorsal neurons, including the DN1as, a subset of DN1ps and a few DN3s (Shafer et al. 2006), we decided to use a more specific *GAL4* driver, *DvPdf-GAL4 (*combined with *Pdf-GAL80*), to manipulate the E-oscillators. *DvPdf-GAL4; Pdf-GAL80* is specifically expressed in 3-4 LNds and the single 5th sLNv per brain hemisphere (Guo et al. 2014; Bahn et al. 2009; Lamba et al. 2014). Unfortunately, we were not able to monitor flies with E-oscillators ablated with *DvPdf-GAL4; Pdf-GAL80* because these flies died 2-3 days after eclosion. Hence, we electrically silenced E-oscillators during adulthood through conditional expression of the open rectifying potassium channel *kir* using *tubulin (tub)-GAL80*^{ts} to prevent the neurons from firing action potential at high temperature of 29^{0}C.As previously noted (Guo et al. 2014), *tub-Gal80*^{ts}*; DvPdf-GAL4/UAS-Kir; Pdf-GAL80* flies as well as *tub-Gal80*^{ts} *; UAS-Kir* control flies exhibited a long period of approximately 26 hours in DD (Table 3.1). Thus, we confirmed the expected absence of the E-peak of activity by exposing flies to a long photoperiod (16:8) LD cycle rather than a regular 12:12 LD cycle. The evening peak was observed in the control flies but not in the E-oscillator silenced flies (Fig 3.7A). To determine the effect on phase shifts, we administered light pulses not just at ZT15 and 21, but also at ZT17 and 23 to ascertain that the maximum phase delay and advance are not missed due to the slow pace of the circadian pacemaker.

Flies with electrically silenced E-oscillators phase-shifted their circadian behavioral rhythms in response to delaying (ZT15, ZT17) light pulses with only a slight,

though statistically significant, 0.5 hour reduction in phase shift at ZT17, a time when phase shift was maximal in the experimental flies and the control flies with the same period length (Fig 3.7B and 3.8A). Unexpectedly, flies with electrically silenced E-oscillators showed little or no phase shifts in response to advancing light pulses at ZT21 and ZT23 (Fig 3.7C and 3.8A).

The difference between the results obtained with HID and KIR expression in E-oscillators could be caused by the different methods employed to manipulate the E-oscillators, or by the use of two drivers with slightly different expression patterns. Thus, we also electrically silenced the E-oscillators using *Pdf-GAL80; cry-GAL4(13)* combination (Fig 3.7D). However, these flies still underwent normal phase resetting in response to both phase delaying (ZT17) as well as phase advancing (ZT21) light pulses (Fig 3.7E and 3.8B). Light pulse was administered at ZT17 instead of ZT15 for phase delay responses because of the long period length for *tub-Gal80^{ts}; UAS-Kir/Pdf-GAL80; cry-GAL4(13)* and the *tub-Gal80^{ts}; UAS-Kir* control flies. For phase advance responses light pulse was still administered at ZT21, since even the experimental flies showed robust phase shifts. Thus, silencing the E-oscillators as well as a subset of dorsal neurons does not impact circadian phase shifts, while sparing the dorsal neurons from silencing reduces photic behavioral phase shifts, particularly phase advances. Thus, under certain circumstances, a subset of dorsal neurons can inhibit behavioral phase shifts.

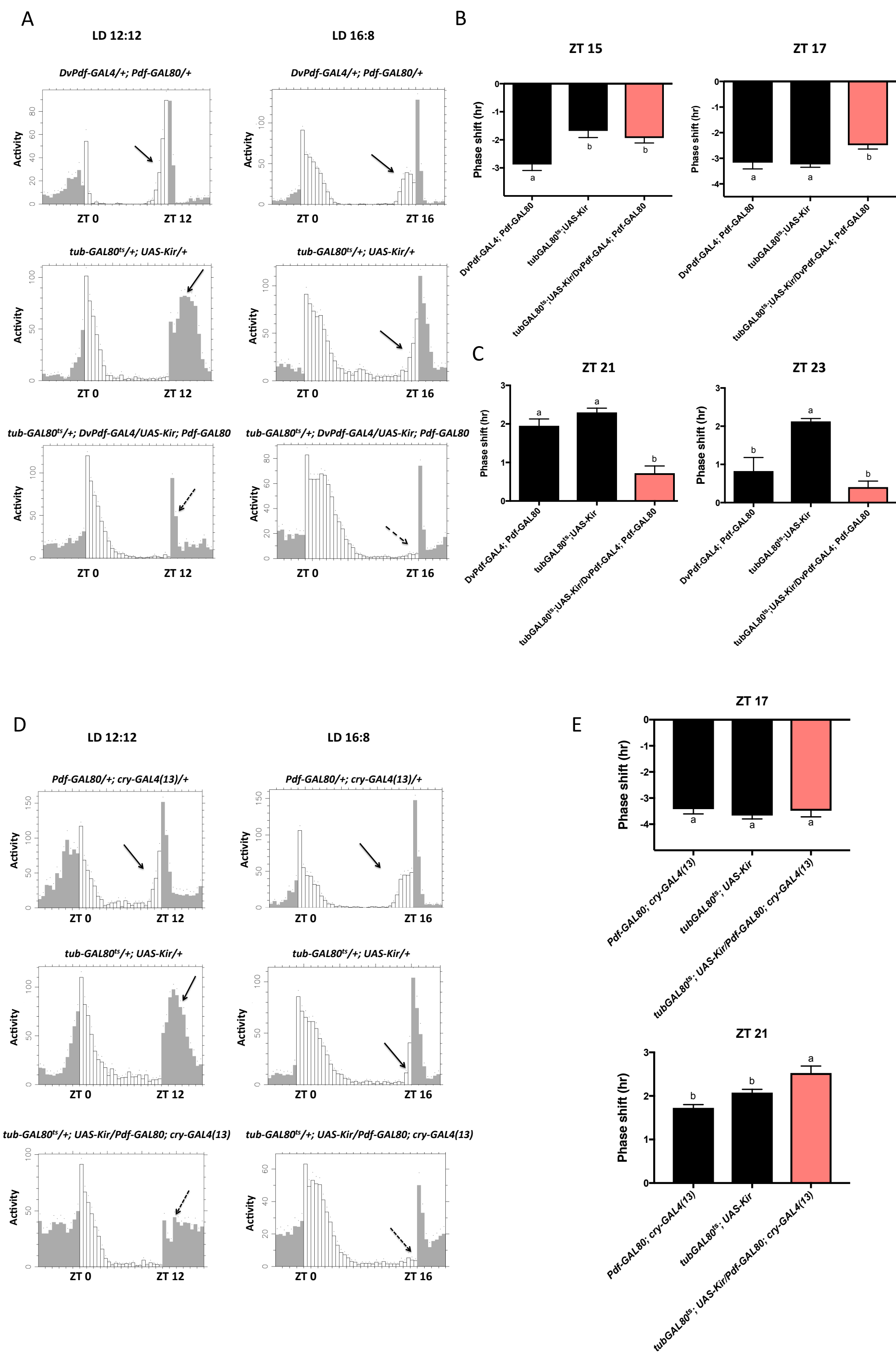

A
LD 12:12
LD 16:8
DvPdf-GAL4/+; Pdf-GAL80/+
tub-GAL80ts/+; UAS-Kir/+
tub-GAL80ts/+; DvPdf-GAL4/UAS-Kir; Pdf-GAL80
Activity
ZT 0
ZT 12
ZT 16
B
ZT 15
ZT 17
Phase shift (hr)
DvPdf-GAL4; Pdf-GAL80
tubGAL80ts;UAS-Kir
tubGAL80ts;UAS-Kir/DvPdf-GAL4; Pdf-GAL80
C
ZT 21
ZT 23
D
Pdf-GAL80/+; cry-GAL4(13)/+
tub-GAL80ts/+; UAS-Kir/+
tub-GAL80ts/+; UAS-Kir/Pdf-GAL80; cry-GAL4(13)
E
ZT 17
ZT 21
Pdf-GAL80; cry-GAL4(13)
tubGAL80ts; UAS-Kir
tubGAL80ts; UAS-Kir/Pdf-GAL80; cry-GAL4(13)

Figure 3.7 Dorsal neurons can modulate phase advance responses when E-oscillators are electrically silenced.

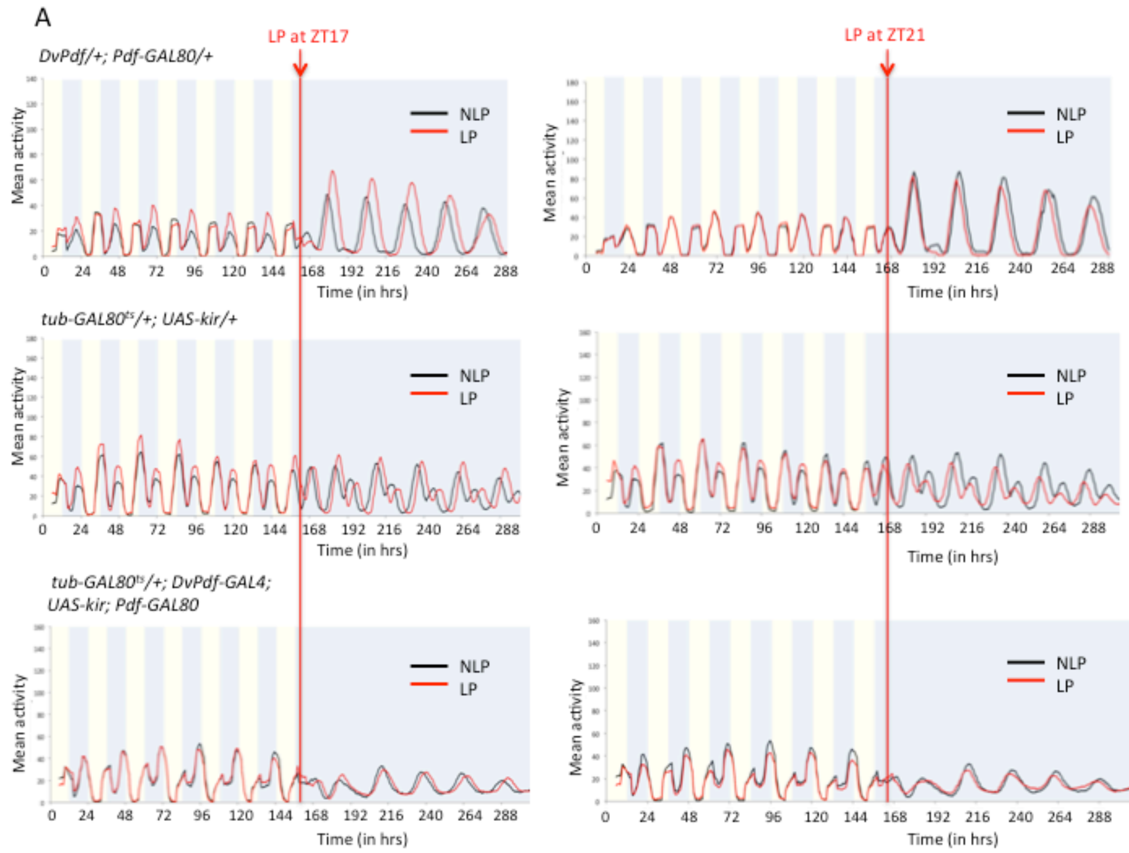
(A) Electrical silencing of E-oscillators disrupts the evening peak of activity. The first column on the left shows the locomotor activity in a 12:12 LD cycle. Solid arrow indicates the evening anticipation, which is disrupted in the electrically silenced flies (dashed arrow). The evening peak of the UAS control and the experimental flies is shifted in the dark phase of LD cycle because of the long period of 26 hours. Second column on the right: locomotor activity rhythms in a 16:08 LD cycle.

(B) Abrogation of firing from the E-oscillators does not affect the response to early night light pulses. Black bars: control flies; pink bars: E-oscillators' electrically silenced flies. Phase shift in response to light pulse at ZT15 is shown on the left and ZT17 on the right. Note that at ZT15 both the UAS control and the experimental flies have reduced phase shift due to their long period phenotype. At ZT17, both the GAL4 and UAS controls shift normally despite the difference in period length. The E-oscillator silenced flies still phase shifted at ZT17 light pulse with a 0.5 hour reduction compared to the controls which was statistically significant as determined by one way ANOVA followed by Tukey's test. N = 5. Different letters above the bars represent significant difference between genotypes. ($p<0.05$).

(C) Dorsal neurons can affect phase response to late night light pulses. Phase response to ZT21 light pulse is on the left and ZT23 on the right. Silencing the E-oscillators with *DvPdf-GAL4; Pdf-GAL80* driver (pink bar) disrupts the phase advance response. Note that the firing from dorsal neurons is still intact. ZT23 is not the time point for maximum advances; therefore, GAL4 control shows reduced response. However, electrically silenced E-oscillators and the UAS control flies with a similar endogenous long period were statistically different. N = 5. Statistical analysis was performed same as above.

(D) Inhibition of firing from E-oscillators using broader *Pdf-GAL80; cry-GAL4(13)* driver also prevented the evening anticipation peak. The first column on the left shows the locomotor activity in 12:12 LD cycle and the second column on the right shows activity in 16:08 LD cycle. Solid arrows indicate evening anticipation and dashed arrows indicate loss of evening peak.

(E) Silencing the E-oscillators by *Pdf-GAL80; cry-GAL4(13)* does not reduce phase delay response to ZT17 light pulse (above) or phase advance response to ZT21 light pulse (below). Black bars: control flies; pink bar: electrically silenced flies. N = 4. Error bars represent S.E.M.


A
LP at ZT17
LP at ZT21
DvPdf/+; Pdf-GAL80/+
tub-GAL80ts/+; UAS-kir/+
tub-GAL80ts/+; DvPdf-GAL4; UAS-kir; Pdf-GAL80
Mean activity
NLP
LP
Time (in hrs)
0 24 48 72 96 120 144 168 192 216 240 264 288

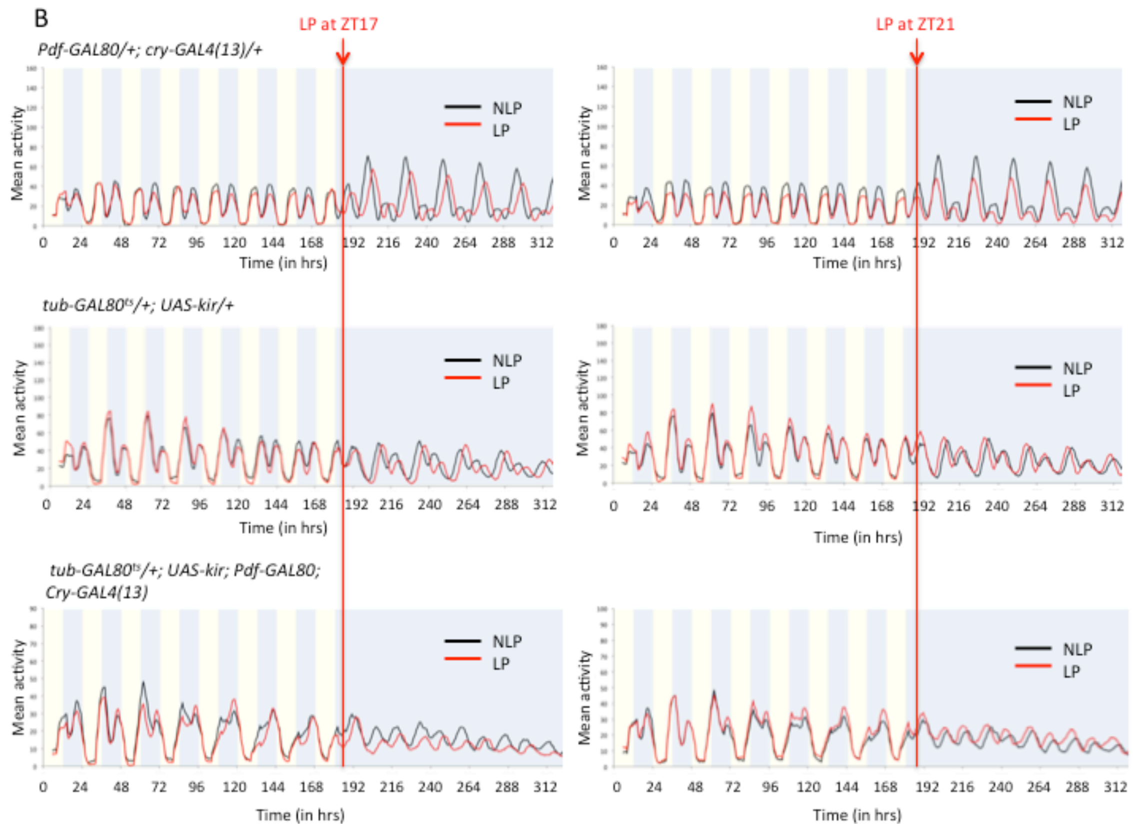


Figure 3.8 Dorsal neurons impact phase advance responses in flies with electrically silenced E-oscillators

(A) Upper and middle panels: GAL4 and UAS control flies respectively. Bottom panel: E-cells' silenced flies.

(B) Upper and the middle panels: genotypic control flies. Bottom panel: Electrically silenced E-cells with broader *Pdf-GAL80; cry-GAL4* driver. Black line: non pulsed control flies; red line: light pulsed flies.

The photic phase response curve (PRC) of peripheral oscillators is similar to the behavioral PRC

The behavioral PRC of *Drosophila* has been well characterized (Levine et al. 1994; Suri et al. 1998). It is a type I PRC with a cross-over point in the middle of the night (ZT18). The PRC is certainly shaped by cell-autonomous TIM degradation (Suri et al. 1998; Yang et al. 1998), but could also be influenced by neural interaction. The results presented above show that neither the M-oscillators nor the E-oscillators are required for phase delays or advances, which suggest that the PRC's properties are essentially driven by cell-autonomous mechanisms, and neural network interaction has little impact on the shape of the PRC. Furthermore, the PRC of *Pdf* null mutant flies is essentially the same as that of wild-type. To test the notion that the shape of the PRC is essentially a cell-autonomous property, we turned to peripheral oscillators, known to respond to light in a tissue-autonomous, brain-independent manner (Emery et al. 2000; Plautz et al. 1997). Flies expressing a TIM-LUCIFERASE (TIM-LUC) fusion gene under the control of the *tim* promoter were subjected to light pulses at different times of the night and luciferase rhythm phase shifts were measured for whole flies. LUC signal primarily comes from peripheral tissues in whole flies (Fig 3.9A) and those peripheral oscillators are entrained in CRY-dependent manner independently from the circadian brain neurons (Plautz et al. 1997; Emery et al. 2000 and Levine et al. 2002). However, since, we used intact flies to measure the TIM-LUC signal, we cannot exclude the possibility that central oscillators contribute to TIM-LUC-rhythms as well. LUC rhythm amplitude rapidly decreases in constant conditions, so we used only the first peak of

luciferase activity to measure phase shifts. However, in most traces, the subsequent trough and peak also showed a similar phase shift. The photic PRC for peripheral TIM-LUC rhythms was strikingly similar to the behavioral PRC, showing clear Type I PRC properties (Fig 3.9B and 3.10). We measured light responses at ZT18, and observed severely attenuated TIM-LUC rhythms after the light pulses, with no clear phase shift (Fig 3.10B). Interestingly, rhythm amplitude was also reduced at ZT19, but they were clearly phase advanced. A small decrease in amplitude was also noted with pulses at ZT17 and ZT21 (Fig 3.9C). The similarity between behavioral and peripheral PRCs strengthen the notion that properties of the *Drosophila* photic PRC rely largely on cell-autonomous resetting mechanisms.

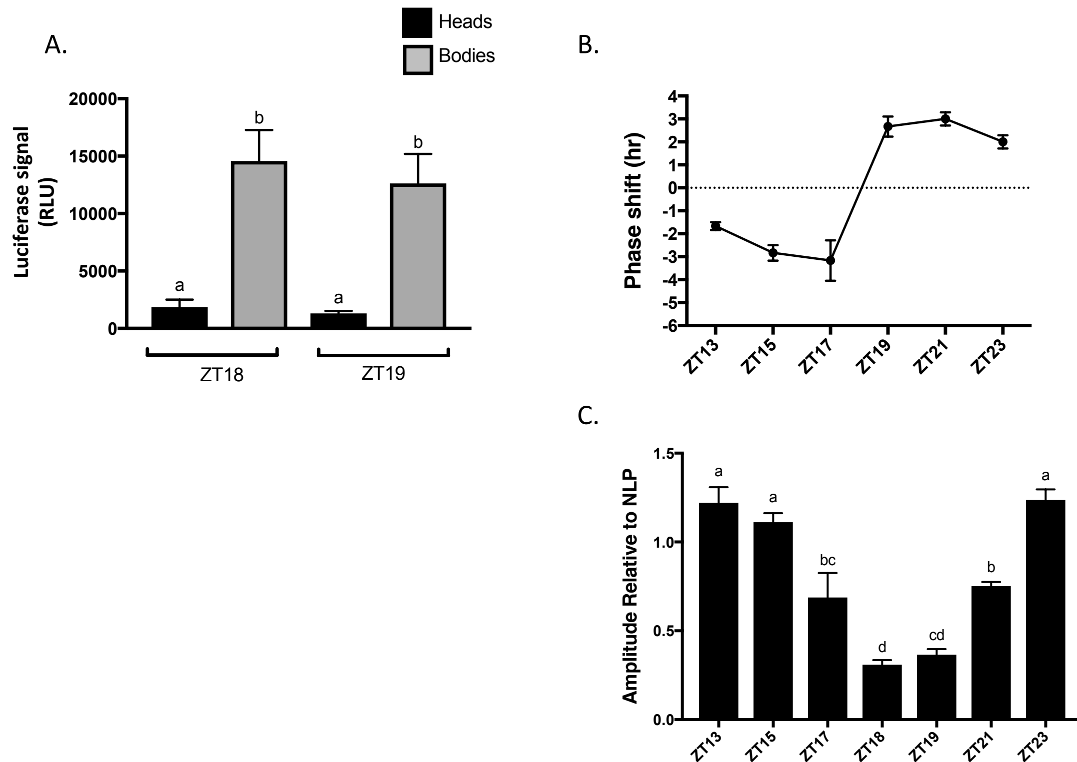


Figure 3.9 Photic phase responses in peripheral oscillators resembles those of circadian behavior

(A) Whole fly LUC signal primarily comes from peripheral tissues. Both at ZT 18 and ZT 19 (time point for the peak TIM-LUC levels), majority of the TIM-LUC signal is emitted from the bodies and there is very little contribution from the heads. The difference between the TIM-LUC signal from heads and bodies is statistically significant as determined by student's t test for each time point separately.

(B) Phase response curve of *ptim*-TIM-LUC flies. 16 flies were tested for each time point. Phase shifts in the TIM-LUC levels are plotted on the y-axis and the time at which light pulse was administered is on the x-axis. N = 3

(C) Amplitude of TIM-LUC rhythms. The amplitude of the light pulsed flies is plotted relative to non light pulsed (NLP) flies on the y-axis. x-axis – different ZTs for light pulse. Note: amplitude is significantly reduced when light pulse is administered at time points closer to the middle of the night. Different letters indicate significant difference as determined by ANOVA followed by post hoc Tukey's test, $p < 0.05$.

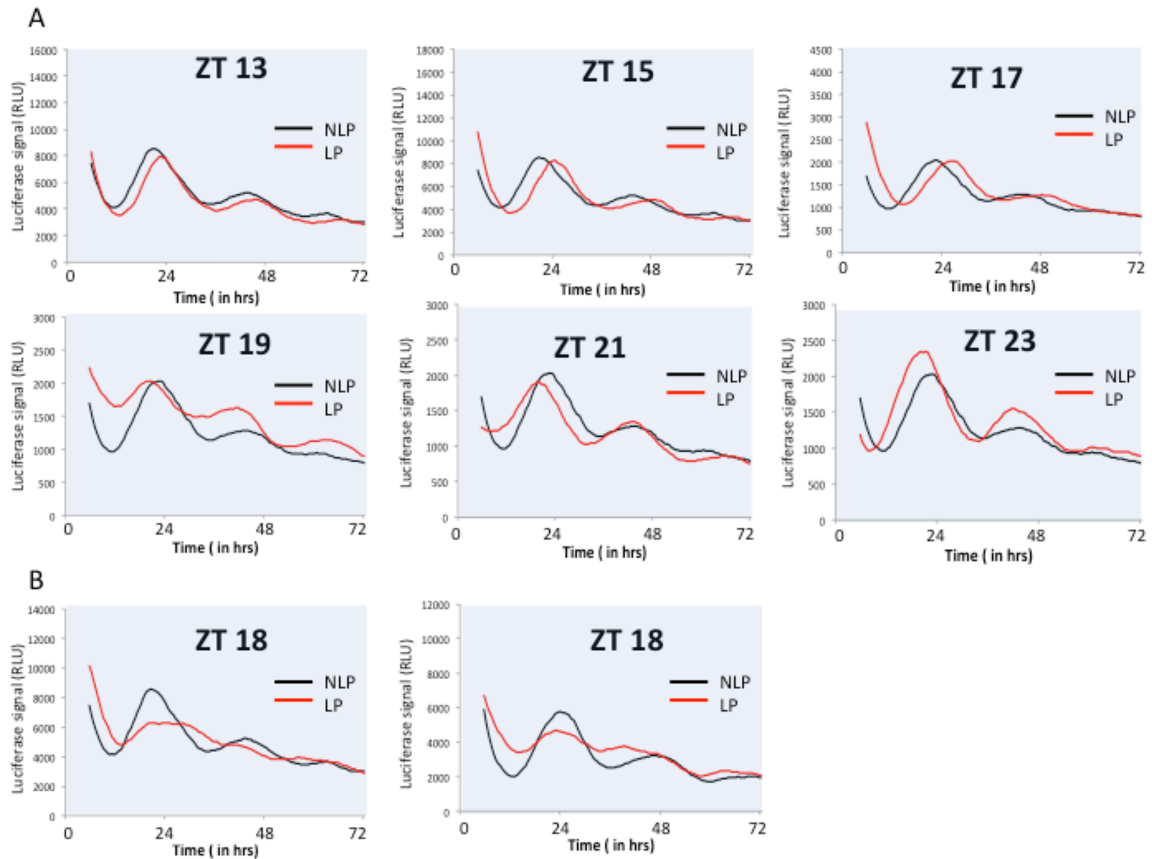


Figure 3.10 Photic phase responses of TIM-LUC rhythms at different times of the day

(A) Representative luciferase recordings of whole TIM-LUC flies showing that a light pulse during the early night generates phase delay, while a phase advance is observed with a late-night light pulse.

(B) Two independent traces as observed with mid-night (ZT18) light pulse. Note the strong loss of amplitude in TIM-LUC rhythm, with no evident phase shift.

4. Discussion

The model for light-mediated resetting of the *Drosophila* molecular circadian pacemaker posits that circadian photoreception is cell-autonomous. Indeed, *tim* mRNA and protein cycles combined with CRY-dependent TIM degradation provides a simple and reasonably satisfactory explanation for how circadian phase delays and advances can be achieved cell-autonomously (Ashmore and Sehgal, 2003). In fact, ectopic expression of CRY in the ovaries can cause TIM degradation upon exposure to light, which is otherwise light-insensitive in the ovaries (Rush et al. 2006), and adding CRY to blind larval Dorsal Neurons 2 (DN2s) anchor the phase of their molecular oscillator to the LD cycle (Klarsfeld et al. 2004). However, others and we have recently shown that neural interactions are required for proper circadian resetting of brain clocks and circadian behavior (Lamba et al. 2014; Shang et al. 2008; Tang et al. 2010; Yoshii et al. 2015; Roberts et al. 2015). It was actually proposed that defined groups of circadian neurons might promote specifically phase advances or delays (Shang et al. 2008; Tang et al. 2010). Our present results support however the notion that photic phase advances and delays are derived from the molecular properties of the circadian clock and CRY photoreception. We had previously shown that CRY photoreception is required in both the M- and E-oscillators, and that these neurons cooperate to reset circadian behavior (Lamba et al. 2014). However, we find here that neither ablation of the M-oscillators, nor elimination of the E-oscillators (with a few Dorsal Neurons, see below), impacts the ability of the flies to advance or delay their circadian behavior. This appears to imply that the M-oscillators or the E-oscillators, on their own, can generate both phase advances or

phase delays, although we cannot exclude the possibility that Dorsal Neurons can substitute for the loss of either group of cells. However, our finding that the PRC of peripheral oscillators - which are not part of a neuronal network and are able to entrain to light autonomously through CRY (Plautz et al. 1997; Emery et al. 2000) – is so similar to that of the behavioral PRC strongly supports the notion that the properties of CRY entrainment are essentially derived from its cell-autonomous nature, including in the brain. Even though we show that the LUC signal is primarily derived from the bodies in whole flies, we cannot exclude the possibility that light acts through the central oscillators to shift the peripheral rhythms because we measured the TIM-LUC signal from intact flies. We are currently undertaking experiments to isolate peripheral tissues from flies and measure phase shifting response to brief light pulses. Given the fact the peripheral oscillators are known to autonomously maintain endogenous rhythms and can synchronize to light inputs (Plautz et al. 1997; Hege et al. 1997; Ivanchenko et al. 2001; Ito et al. 2008), we would expect the shape of the PRC to be similar to the one derived from phase response of intact flies. Of particular interest is the response of peripheral oscillators to light pulses administered at ZT18. There is no clear phase shift, which supports the notion that the peripheral PRC, similar to behavior (brain) PRC, is a type 1 PRC (a type 0 PRC would have a strong phase response in the middle of the night). There is however another striking feature to the ZT18 response: an important loss in oscillation amplitude. This can be explained in two ways. First, a pulse at ZT18 dampens circadian oscillations in peripheral oscillators. Indeed, limit cycle theory predicts that a pulse of the appropriate magnitude at the correct time can stop circadian oscillations, and the

existence of such singularities have been observed in multiple organisms, including *Drosophila pseudoobscura, Neurospora* and humans (Winfree, 1970; Huang et al. 2006; Jewett et al. 1991). Alternatively, the loss of amplitude could be caused by phase dispersion, with some oscillators delaying, while some advance their phase.

Although our results support the notion that the properties of the behavioral PRC emanate from the cell-autonomous nature of circadian photoreception, it is clear that in the brain neuronal interactions play an important role.

First, non-autonomous acute TIM degradation is observed in the brain upon light exposure (Yoshii et al. 2008; Lamba et al. 2014).

Second, proper interaction in the M- and E-oscillator network is clearly critical for light-mediated phase resetting. Indeed, combined with our previous work (Lamba et al. 2014), our present results indicate that both M- and E-oscillators need to be properly reset, and this is very likely explained by conflicting signals being sent if either the M- or the E-oscillator does not reset their circadian clocks. Indeed, removing PDF in flies in which JET expression is compromised in M-oscillators restores normal phase shift. In these flies, PDF thus acts as a brake on circadian behavioral photoresponses. The nature of the neurotransmitters from the E-oscillators that would similarly act as a brake on behavioral phase shifts when those cells are defective for JET remains to be determined. Additionally, it is also possible that the M- and E-oscillators communicate directly via gap junction. However, an electrical synapse between these two groups of neurons is yet to be demonstrated. Thus, we propose that in a normal circadian circuit, proper synchronization of local phase resetting in M and E-oscillators is critical to coordinate

circadian behavioral photoresponses. The latter proposal seems to be at odds with observations made by Guo et al (2014). Indeed, these authors found that thermogenetic activation of the M-oscillators using the temperature sensor TRPA1 is sufficient to trigger phase advances and delays similar to those triggered by light pulses. These phase shifts were PDF-dependent. It is however entirely possible that thermogenetic activation of M-oscillators triggers a much more intense firing activity than light pulses and thus greater PDF release, which could override the absence of direct activation of the E-oscillators.

Third, although circadian photoreception is neither necessary nor sufficient in the lLNvs (Lamba et al. 2014), these cells appear to be required for phase advances (Shang et al. 2008). Moreover, we uncover evidence for an implication of dorsal neurons in phase advances as well. Indeed, while silencing the E-oscillators and a few dorsal neurons with the *cry-GAL4(13)* driver had no impact on phase advances or delays, silencing just the E-oscillators unexpectedly strongly reduced phase advances (a very weak effect on phase delays was also noted). Thus, it appears that a few dorsal neurons - in the absence of active E-oscillators - can inhibit phase shifts. Anatomically, some of the DN1s' fibers project ventrally and are in close association with the dorsal projections of the M-oscillators (Zhang et al. 2010). Moreover, it has been shown that the larval DN1s, which form the 2 DN1as in adult flies, inhibit sLNv neuronal activity via glutamate to modulate light-avoidance behavior (Collins et al. 2012). Also, a functional clock only in the DN1ps can rescue the morning anticipation peak in the *per*^{0} flies and suppress the morning peak of activity during low temperature suggesting that the DN1ps can feed back on the M-oscillators to regulate their output (Zhang et al. 2010). Thus, there is ample evidence that

the DN1s can influence activity of the M-oscillators. Under which circumstances dorsal neurons intervene in the regulation of circadian light responses in a wild-type fly is not clear at this point, but such function might contribute to the plasticity of circadian behavior to environmental inputs.

In summary, properties of photic (CRY-dependent) circadian entrainment in *Drosophila* emerge from the cell-autonomous nature of light perception, but circadian behavior resetting requires coordination between M- and E-oscillators. Interestingly, in the case of temperature also, the M-oscillators on their own are poorly sensitive to temperature inputs and rely on dedicated circadian neurons, which are in this case CRY negative (Busza et al. 2007; Chen et al. 2015; Yoshii et al. 2010). Such reliance on non-autonomous mechanisms and separate population of circadian neurons might allow the pacemaker M-oscillators to integrate multiple modalities, and thus generate circadian behavioral outputs that are as properly synchronized as possible with environmental cycles.

5. Materials and Methods

Fly stocks

Flies were raised on cornmeal/agar medium at 25ºC under a light: dark (LD) cycle. For the experiments in which E-oscillators are conditionally silenced only during adulthood using *tubGAL80^{ts}*, the GAL4 and UAS controls as well as the experimental flies were raised at 18ºC (permissive temperature for *GAL80^{ts}*) until eclosion, after which the flies were transferred to 29ºC. The following *Drosophila* strains were used in this study: *y w* --

w^{1118} -- *y w*; *Pdf-GAL4* -- *y w*; *Pdf-GAL4 UAS-dicer2* -- *y w*; *Pdf-GAL80; cry-GAL4(13)* -- *y w; UAS-hid^{14}/Cyo* -- *y w; UAS-hid; Pdf-GAL80* -- *y w; cry-GAL4-13* -- *jet* RNAi (TRiP.JF01506, Bloomington Drosophila Stock Center). *Pdf^{0}* flies were a generous gift from Dr. Paul Taghert (Washington University, St-Louis, MO). *DvPdf-GAL4; Pdf-GAL80* and *$tubGAL80^{ts}$/FM7a; UAS-kir/Cyo* lines were a generous gift from Dr. Michael Rosbash (Brandeis University, Waltham, MA). *jet* RNAi line was genetically recombined with *Pdf^{0}*. Transgenic flies expressing a TIM-LUCIFERASE fusion gene under the *tim* promoter (ca. 5kb) and 1st intron (*ptim*-TIM-LUC) were used for luciferase experiments.

Behavioral monitoring and analysis

Single adult male (2-5 days old) flies were used to test locomotor activity. Groups of 16 flies per genotypes or 32 flies for genotypes with high arrhythmicity were entrained to a 12:12 LD cycle for 4-5 days at 25°C, except for the conditional silencing experiments using *UAS-kir* and *$tubGAL80^{ts}$*. For the latter experiments, flies were entrained to a LD cycle for 6-7 days at 29°C to ensure that KIR was sufficiently expressed. After entrainment, flies were exposed to a 5-minute pulse of white fluorescent light (1500 lux) at different time points on the last night of the LD cycle. A separate control group of flies was not light-pulsed. Following the light pulse, flies were released in DD and their locomotor activity was monitored using the Trikinetics Activity Monitors (Waltham, MA) for six days. Rhythmicity and period length were analyzed using the FaasX software (courtesy of F. Rouyer, Centre National de la Recherche Scientifique, Gif-sur-Yvette, France). Rhythmicity was defined by the criteria – power $\geq$10, width $\geq$2 using the $\chi 2$

periodogram analysis. To determine the amplitude of photic phase shifts, data analysis was done in Excel (Microsoft) using activity data from all flies, including those arrhythmic according to periodogram analysis. Activity was averaged within each groups of 16 or 32 flies, plotted in Excel, and then fitted with an 8-hour moving average. A genotype-blind observer identified the peaks of activity, which were found to be the most reliable phase marker. Phase shifts were calculated by subtracting the average peak phase of the light-pulsed group from the average peak phase of non-light pulsed group of flies. For genotypes with high arrhythmicity in DD, phase was measured only from the days during which the flies showed clear rhythmic activity as a population.

Luciferase experiments

The luciferase activity of *ptim*-TIM-LUC flies on luciferin (Gold-Biotech) containing agar/sucrose medium (170µl volume, 1% agar, 2% sucrose, 25mM luciferin), was monitored in Berthold LB960 plate reader (Berthold Technologies, USA) in I-36LL Percival incubators with 90% humidity (Percival Scientific, Perry IA). Flies in 96-well white plates were covered with needle-poked Pattern Adhesive PTFE Sealing Film (Analytical Sales & Services, catalog no. 961801). The distance between the agar and film was such that the flies were not able to move vertically. Light pulse protocol and phase analysis was the same as for locomotor activity. Raw bioluminescence data were treated as described for behavioral data, and phase shifts determined by comparing the phase of the 1st peak of bioluminescence in DD in pulsed and non-pulsed flies. Amplitude in pulsed (Ap) and non-pulsed flies (Anp) was calculated by dividing the

bioluminescence peak value with the average of the value of the first two troughs in DD. The relative amplitude (RA) shown on the y-axis of Figure 3.9C was calculated as follow: RA = (Ap-1)/(Anp-1). The subtraction from Ap and Anp accounts for the fact that an arrhythmic trace would have an amplitude equal to 1.

Statistical analysis

Statistical analysis of the phase shifting behavioral experiments was done using the GraphPad Prism 7.0 software. Student's t-test was used to compare means between two groups, and one way analysis of variance (ANOVA) coupled to Tukey's post hoc test was used for multiple comparisons. Same letters indicate no significant difference between genotypes and different letters above the bars represent significant difference between genotypes ($p<0.05$).

Chapter IV

Discussion

Circadian clocks are equipped with a remarkable flexibility to harmonize their internal phase according to the environmental cycle. This ability of circadian clocks not only helps organisms to tell the time of the day but also assists them in adapting to the seasonal fluctuations in the environment. The physical factors of the environment are almost never static. The 24-hour rotation of the earth generates daily variations in the environment and the light-dark cycle is the most evident cyclical change observed on a daily basis. In addition to the daily light-dark cycles, the circadian clock also needs to adjust to the seasonal changes in the photoperiod, such as the short days in winter and long days in summer. Light is the most potent signal to the circadian clocks. Subsequently, circadian clocks are reset and ensure survival of organisms.

In mammals, the master circadian pacemaker, which generates overt rhythms, is the hypothalamic Superchiasmatic Nucleus (SCN) that consists of approximately 20,000 neurons whereas the *Drosophila* brain clock consists of 150 clock neurons (Jackson 2011). In spite of the difference in the number of neurons, the molecular mechanism that generates circadian rhythms is evolutionarily conserved between mammals and *Drosophila* (Dunlap 1999; Weaver & Emery 2013). Hence, *Drosophila* with its relatively small number of neurons and powerful genetic tools, is a good model organism to tease apart the light input pathway to the circadian clocks.

Entrainment is the process of synchronization to time cues such as LD cycles. In the entrained state, the period length of the internal biological rhythm is equal to the environmental LD cycles and therefore, there is a stable phase relationship between the internal rhythm and the external LD cycle. Two classes of models have been proposed to

explain the entrainment of circadian clocks to the environmental LD cycles: The continuous (or parametric) model focuses on the gradual changes in the environment and anticipates that the free running period is continuously modulated throughout the day.In contrast, the discrete (or non-parametric) model focuses on the environmental transitions such as at dawn and dusk. In the discrete model, the endogenous free-running period is taken to be constant and the abrupt light transition during dawn and dusk cause a shift in the phase of the internal clock until the internal free running period is equal to the external light cycle period. In the laboratory, these light transitions at dawn and dusk are mimicked by administering square wave light pulses which can then shift the phase of the internal clock (either delay or advance depending on the time of the day). The discrete phase shift resulting from a single light-pulse allows quantitative prediction and can explain entrainment for a large number of species. Analogous quantitative metrics do not exist for continuous entrainment.

Drosophila can perceive light information via external photoreceptors such as the compound eyes, ocelli, extra-retinal - HB eyelet and also through an intracellular blue light photoreceptor, CRY (Yoshii et al. 2016). These multiple photoreceptors are advantageous for entrainment to different wavelengths of light because there are daily changes in the spectral composition and light intensity during dawn and dusk and seasonal variations in day length (Helfrich-Förster et al. 2005). Each light input pathway is alone capable of entraining circadian rhythms, both at the molecular as well as behavioral level, and only when all three input pathways are completely abolished (such

as with a *glass*^{60J}*; cry*^{b} mutation; the *glass*^{60J} mutation results in lack of both external and internal eye structures), flies are unable to respond to light (Helfrich-Förster et al. 2001).

At the molecular level, how light information is transduced to the circadian clocks is well studied for CRY. CRY and JET (F-box protein of ubiquitin ligase) mediate TIM degradation in a light dependent manner and thus reset the clock (Koh et al. 2006; Peschel et al. 2009). But, it is not very well understood how a heterogeneous set of 150 clock neurons, both CRY positive and CRY negative, senses light to generate coherent behavior that is synchronized to the environment. Does each CRY positive neuron sense light independently? If yes, then how do the CRY negative clock neurons reset their clock? Is there intercellular communication among clock neurons for sensing light? Which are the critical neurons for photoreception? The goal of this dissertation was to investigate these questions and to elucidate the neural mechanisms of CRY-dependent circadian photoresponses. We particularly focused on the phase resetting of circadian clocks by short pulses of light to address the above questions. In *Drosophila*, this phase shifting effect due to acute exposure to light is mediated by the CRY-dependent photoreceptive pathway and not by the visual system. The *cry*^{b} mutant flies fail to respond to acute light pulses, suggesting that photic phase shifting in *Drosophila* predominantly relies on the CRY-mediated light input pathway (Stanewsky et al. 1998). However, these mutant flies are not completely blind to the environmental light input and can still entrain to 12:12 LD cycles (long-term light exposure) through an intact visual system (Stanewsky et al. 1998; Helfrich-Förster et al. 2001). The cellular and molecular

mechanisms of how light perceived by the visual system is transduced to the circadian clock are not known yet.

In chapter II, we used a novel, severe loss-of-function *jet* mutant, *jet*^{set} to map the neuronal circuitry critical for circadian photoreception. We preferred using a *jet* mutant over a *cry* mutant to delineate the circadian network involved in photoreception because CRY over-expression has been shown to render flies behaviorally hypersensitive to light pulses (Emery et al. 1998, 2000) and ectopic expression of CRY in the ovaries can induce light-mediated TIM degradation in the ovaries which are otherwise not sensitive to light (Rush et al. 2006). Additionally, CRY has been proposed to play a role other than TIM degradation in the pacemaker neurons (Tang et al. 2010) and in regulation of molecular clock oscillation in peripheral oscillators (Collins et al. 2006). Hence manipulating CRY levels to delineate the neuronal circuitry could yield ambiguous results.

The *jet*^{set} mutant, which was in a highly light sensitive *tim* (*s-tim*) genetic background, was defective in TIM degradation as well as behavioral phase shifts in response to brief light pulses (Note: the *jet*^{set} mutant flies can still entrain to 12:12 LD cycles through an intact visual system). The amplitude of cycling of TIM levels in a LD cycle was also reduced in the *jet*^{set} flies (Fig 2.1 E). The residual cycling of TIM levels in LD in the *jet*^{set} mutant could be explained by several possibilities: 1. *jet*^{set} is not a null mutant. 2. There are additional ubiquitin ligases, which can serve redundant function. CULLIN-3 is a good candidate, which has been shown to participate in light dependent TIM degradation while TIM is cytoplasmic in the early night (Grima et al. 2012). 3. QSM, another protein that has been shown to be involved in TIM degradation in a light

dependent but CRY independent manner may play a role (Chen et al. 2011). However, knocking down QSM in all circadian neurons in a *jet*^{set} mutant background also did not abolish LD cycling (my unpublished data). Nevertheless, in *jet*^{set} flies both behavioral and molecular CRY dependent light responses were severely disrupted and hence it was a useful tool to dissect the neuronal circuitry controlling light responses.

Using *jet*^{set} and *jet* RNAi, we identified that two groups of circadian neurons - Morning (M) (PDF positive s-LNvs, the pacemaker neurons) and Evening (E) (LNds and the 5th s-LNv) oscillators are critical in sensing light. JET expression was sufficient and required in these two groups to generate both phase delay as well as advance in response to light pulses. The M-oscillators had been previously implicated in photoresponses as well (Emery et al. 2000). More recently, Ni et al. (2017) found that M-oscillators in addition to CRY express another photoreceptor, Rhodopsin-7 (Rh-7). They found that loss of Rh-7 resulted in impaired circadian photoresponse especially under dim light and double mutant *cry*^{b} and *rh7*^{1} flies exhibited severe photoentrainment deficits. Hence, the presence of both CRY and Rh-7 in the M-oscillators might provide mechanisms for sensing both bright and dim light, respectively. Additionally, Rh-7 could play a role in signal amplification similar to the canonical rhodopsins functioning in the phototransduction pathway. Phospholipase C β has been implicated to play a role in the Rh-7 dependent signaling cascade but the mechanism is not entirely understood (Ni et al. 2017). Nevertheless, the presence of two light sensors highlights the importance of M-oscillators in light entrainment.

Our finding that E-oscillators are critical in light sensing was quite unexpected because the l-LNvs were found to be necessary for phase advances (Shang et al. 2008) and DN1s were proposed to be important for phase delays. However, we found that JET expression in the l-LNvs and DN1s was neither required nor sufficient for behavioral phase shifting responses. Our conclusion of M- and E-oscillators being important in light perception has recently been corroborated by another group as well by using a different paradigm for studying light entrainment. Yoshii et al. (2015) rescued wild type CRY expression in different groups of neurons in a *cry*^{01} mutant genetic background (in a manner similar to our *jet* rescue experiments), entrained the flies to an LD cycle for a few days followed by a shifted LD cycle in which the lights came on 8 hours later. While, wild type flies re-entrained (shifted their activity phase) to the new LD cycle in one day, the *cry*^{01} mutant flies took several days to shift their activity to the new LD cycle. *cry*^{01} mutant flies were still able to adjust to the new LD cycle because of the intact visual system which also participates in light entrainment under long term light exposure. Strikingly, CRY expression in the M- and E-oscillators enabled *cry*^{01} mutant flies to re-entrain to an 8-hour shifted LD cycle like the wild type flies, again suggesting that M- and E-oscillators are critical for light entrainment regardless of the regime or duration of the light exposure. Hence, we are confident that M- and E-oscillators play a key role in photoreception.

We also found that JET plays a cell autonomous as well as non-cell autonomous role in TIM degradation. JET expression in the M- or E-oscillators can cell autonomously degrade TIM after a light pulse. But JET expression only in the M-oscillators can also

trigger TIM degradation in the E-oscillators. Hence, JET functions non-autonomously to transmit light information from M- to E-oscillators. The mechanistic details for JET's non-autonomous function are not known yet. However, JET expression in the E-oscillators did not have any effect on TIM degradation in M-oscillators, but was still required in E-cells for complete phase shifts suggesting that E-cells communicate with the M-cells in a JET independent manner.

Within the M-cells, it is possible that upon exposure to light, CRY and JET bind to each other and together regulate the neuronal activity of M-cells. In fact, it has been shown that CRY mediates action potential firing upon exposure to light in the l-LNvs (Fogle et al. 2011). Therefore, a similar mechanism might be functioning in M-cells. One way to test this would be to perform electrophysiological recordings from M-cells upon exposure to a brief light pulse and compare response of M-cells between wild type and jet^{set} flies. But, given the small size and the relatively deep anatomical location of the s-LNvs in the brain, electrophysiological studies are technically challenging. As an alternative, genetically encoded fluorescent voltage indicators such as "Arclight" (Cao et al. 2013) can be used to measure electrical activity in the M-cells. Membrane depolarization causes a decrease and hyperpolarization causes an increase in the fluorescence intensity (Cao et al. 2013) which can be quantified to measure the response of M-cells to light exposure.

In conclusion, in Chapter II we demonstrate that M- and E-oscillators cooperate to reset circadian behavior in response to light input. This study suggested that M- and E-oscillators exchange signals during or after light exposure. What remains unaddressed in

this work is the demonstration of a physical or functional connection between the M- and E-oscillators. The projections of M- and E- oscillators are in close apposition with each other (Helfrich-Förster et al. 2007, Stoleru et al. 2004, Johard et al. 2009, Guo et al. 2014) and recently Gorostiza et al. (2014) using a technique called GRASP have described putative synaptic contacts between the PDF positive projections of M-oscillators (as presynaptic neurons) and the CRY positive E-oscillators (LNds) (as postsynaptic neurons). Functionally the connectivity between M-oscillators and E-oscillators as target neurons has been predicted several times (Im and Taghert 2010; Shafer et al. 2008). In fact Yao et al. (2012) have conducted functional imaging studies and demonstrated a physiological connection by acute excitation of M-oscillators and measuring increase in cAMP levels in E-oscillators expressing a genetically encoded cAMP sensor – Epac1. However, the evidence for reverse connectivity from E- to M-oscillators is only indirect. The E-oscillators have been shown to project ventrally towards the accessory medulla region where the M-oscillators are located, but no pre-synaptic terminals of E-oscillators in this region were identified (Helfrich-Förster et al. 2007). Functional imaging studies by exciting E-oscillators and following Ca^{2+} (using GCaMP) or cAMP (using Epac1) dynamics in the M-oscillators can be done to demonstrate a physiological connection where M-oscillators are the targets of E-oscillators. To determine direct connectivity from E- to M-oscillators would require GRASP or electron microscopy studies. Indirect connectivity between M- and E-oscillators is also possible and could contribute to functional responses while not being detected by GRASP and other anatomical methods.

Nevertheless, these anatomical and functional lines of evidence support the idea that M- and E-oscillators communicate with each other. The question then becomes, what is the basis for M- and E- oscillators' communication and what is the nature of such signals during photoreception?

In chapter III, we show that M-oscillators utilize the neuropeptide PDF to coordinate with the E-oscillators during light responses (Fig 3.3). Interestingly even in mammals, a light pulse in the night causes release of Vasoactive intestinal peptide (VIP) (Francl et al. 2010), a homolog of PDF, suggesting some degree of conservation between *Drosophila* and mammalian light responses.

Unexpectedly we also found that light can reset circadian behavior in response to both early and late night light pulses in the absence of either M- or E-oscillators (Fig 3.1 and 3.5). This result suggested that the individual cellular oscillators are capable of generating both phase delay as well as phase advance thus reinforcing the notion that CRY dependent light detection is cell autonomous and the phase shift in behavior is generated from the molecular property of the pacemaker rather than neural interactions.

One way to assess the ability of M- and E-oscillators to drive the behavioral phase shifts in isolation would be to analyze phase shifting response of flies with a functional clock only in M- or E-oscillators (For instance, reconstituting PER expression either in M- or E-oscillators in a clock mutant per^0 fly). Unfortunately, due to complete arrhythmicity of these flies in our hands, we could not perform phase shifting analysis. Hence to support the idea that individual oscillators can undergo light generated phase delay and advance autonomously, we assayed light-mediated phase resetting in peripheral

tissues which are known to maintain endogenous rhythms and entrain to LD cycles independently of the brain. We used TIM-LUC (a TIM-LUCIFERASE fusion expressed under *tim* promoter) bioluminescence levels as peripheral oscillator phase reporter because the luciferase signal in whole flies primarily is derived from the peripheral tissues. The light mediated phase response curve (PRC) of whole-body TIM-LUC rhythms was strikingly similar to the PRC of behavioral rhythms (Fig 3.9 B) further strengthening the notion that the properties of light dependent phase resetting are derived from the molecular pacemaker rather than neuronal network interactions. Both peripheral and behavioral PRCs were Type 1 PRC with smaller magnitude of phase shifts and a continuous transition between phase delay and advance as opposed to Type 0 PRC which is characterized by large phase shifts and a discontinuity between delay and advance zones. However, increasing the strength of the light stimulus can convert Type 1 to Type 0 PRC (Peterson 1980; Pittendrigh 1960; Saunders 1978). Therefore, whether a light pulse of higher intensity and a longer duration generates Type 0 resetting similarly in both behavioral and peripheral PRCs remains to be determined.

Type 0 PRC is commonly found in lower organisms such as bacteria and fungi (Johnson 1999). Similar to *Drosophila*, photic stimuli in mammals as well generally result in Type 1 PRC with smaller magnitude phase shifts (Takahashi et al. 1984; Comas et al. 2006). Interestingly, Pulivarthy et al. (2007) showed that individual fibroblasts (stably expressing the photopigment melanopsin) from PER2-LUC mice, when exposed to light at a critical phase in the night resulted in reduced amplitude of PER2-LUC rhythm and a Type 0 phase resetting characterized by large magnitude phase shifts.

Vitaterna et al. (2006) have also demonstrated that mice heterozygous for the *Clock-delta-19* mutation, which reduces the oscillator amplitude, enhances the sensitivity to light and thus results in Type 0 phase resetting. Our observation of a Type 1 PRC in the peripheral oscillators (Chapter III) suggests the presence of robust circadian oscillators in *Drosophila* peripheral tissues, which are relatively resistant to phase perturbations and thus result in a weak Type 1 resetting. Like *Drosophila* and mice, weak type 1 phase resetting by a single light pulse has been reported in humans as well (Jewett et al. 1994). Similar to our observations that light pulses at ZT18 and 19 can reduce the amplitude of circadian oscillation (Figure 3.9 C), Jewett et al. (1991) had also observed that light exposure at a critical time point could repress circadian amplitude in human subjects.

In figure 3.9, we show that the LUC signal comes primarily from peripheral tissues. Nevertheless, there could still be communication between the central and the peripheral oscillators because we measure LUC-rhythm phase shifts from intact flies. To overcome, this potential caveat we are currently conducting experiments to isolate peripheral tissues (such as wings, legs and antennae) *in vitro* from TIM-LUC flies and measuring phase shifts in response to 5-minutes light pulses. Since, the peripheral oscillators are known to respond to light in a cell-autonomous and brain independent manner (Plautz et al. 1997; Hege et al. 1997; Ivanchenko et al. 2001; Ito et al. 2008), we expect the PRC to be similar to the one observed with intact TIM-LUC flies. Another way to assess the autonomy of peripheral oscillators' photic resetting would be to measure TIM-LUC rhythm phase shifts in flies which are behaviorally arrhythmic i.e. by knocking down one of the core clock components in the PDF positive M-oscillators or by

expressing a pro-apoptotic gene such as *hid* to ablate the M-oscillators. In these flies, the defective central circadian clock would presumably be unable to communicate a phase-dependent signal to the periphery. If the shape of the TIM-LUC PRC is similar to the one observed with whole, clock-competent TIM-LUC flies, it would confirm our findings that peripheral oscillators are capable of autonomously resetting their clock in response to light.

The similarity between the behavioral and peripheral PRC results presented in Chapter III demonstrate that cell-autonomous light detection by CRY and subsequent degradation of TIM forms the basis of circadian behavioral response to light input. It is therefore somewhat surprising that our results also indicate that both M- and E-oscillators need to be properly reset to shape the behavioral responses to light. For instance, in both Chapters II and III, disrupting CRY dependent photoreception only in M-oscillators (by down regulating *jet*) results in severely attenuated behavioral phase shifts (Fig 2.2B and 3.3). Knocking down *jet* only in M-oscillators compromises their TIM degradation and prevents their resetting, but E-oscillators with intact JET expression undergo TIM degradation and are reset. If the oscillatory groups are not in unison, conflicting signals being sent from the M- and E-oscillators may weaken the behavioral phase shifting response. In fact removing the M-oscillator's key neurotransmitter, PDF, from flies with compromised JET expression in M-oscillators (these flies detect light through CRY only in the E-cells) restored normal phase shifts (Fig 3.3). This finding suggests that untimely secretion of PDF puts a brake on resynchronization of the E-cells and behavioral resetting.

Hence, in an intact neural circuit, M- and E-oscillators need to stay in harmony so that the timing of the release of their critical neurotransmitters is coordinated and thus ensures proper behavioral resetting. This result also suggested that PDF plays an important role in coordination of M- and E-oscillators during photoresponses.

To strengthen the conclusion in Fig. 3.3, that eliminating PDF signaling when *jet* is knocked down in M-oscillators results in normal phase shifts, we could also analyze the phase shifting response in a PDF-receptor mutant background while JET expression is compromised in the M-oscillators. PDF is the principal circadian neurotransmitter and elimination of PDF signaling causes desynchronization among circadian neurons, resulting in a high frequency of arrhythmicity One important aspect to consider in Figures 3.1 to 3.4 is that our phase shifting analysis used Pdf^0 flies that displayed low levels of rhythmicity (about 16%) in DD. From the data presented in Chapter III, we cannot distinguish whether the small subset of flies that scored as rhythmic was due to compensatory mechanisms in Pdf^0 flies or due to the criteria (power > 10, width > 2) used by the $\chi 2$ periodogram analysis to define rhythmicity. Studies from several groups have suggested that this residual rhythmicity in the mutant flies emanates from the PDF negative circadian neurons such as the DNs (Klarsfeld et al. 2004; Blanchardon et al. 2001; Dushay et al. 1989) and perhaps the LNds as well (reviewed in Helfrich-Förster 2005). However, we cannot exclude the possibility that another neurotransmitter or neuropeptide secreted from the PDF positive M-oscillators might be compensating for the loss of PDF and thus contributing to rhythmicity. But ablation of PDF positive M-oscillators (Chapter III) results in a similar behavioral phenotype as Pdf^0 mutation i.e.

low rhythmicity with a short period length which makes it unlikely that another neurotransmitter or neuropeptide from the M-oscillators contributes to the residual rhythmicity of *Pdf*^{0} flies. Additionally, it is also possible that due to developmental and chronic absence of the PDF positive M-oscillators or the neuropeptide PDF, these flies possess a different circadian network and thus retain rhythmicity in constant conditions. But, acute adult-specific electrical silencing of the PDF positive M-oscillators by expressing *kir2.1* using an inducible version of GAL4, GeneSwitch (transcriptionally active in the presence of a progesterone analog) also resulted in high levels of arrhythmicity, with a residual rhythmicity of about 20% and a short period phenotype similar to the *Pdf*^{0} flies and flies with ablated PDF neurons (Depetris-Chauvin et al. 2011). Importantly, this reduced rhythmicity was reversible once flies were transferred to normal food with no progesterone analog (Depetris-Chauvin et al. 2011). Therefore, it seems unlikely that the residual rhythmicity in the *Pdf*^{0} and the PDF-positive neurons ablated flies is due to developmental compensation, but we cannot completely exclude this possibility.

How PDF functions to modulate the response of E-oscillators to acute light exposure is not very clear. E-oscillators express the receptor for PDF and there is evidence for functional as well as physical connections between the PDF positive M-oscillators and E-oscillators, as mentioned above. Guo et al. (2014) showed that thermogenetic activation of M-oscillator firing can induce TIM degradation in E-oscillators in a PDF dependent manner. This degradation of TIM was found to be mediated by CULLIN-3. Hence, it is possible that exposure to light also induces firing of

PDF positive M-oscillators, resulting in subsequent release of PDF which then could target TIM for degradation in the E-oscillators. Recently, Liang et al. (2017) have shown that brief light pulses can induce phase shifts in Ca^{2+} activity pattern which was proposed to be partially modulated by PDF signaling. Hence, PDF could act in concert with light to regulate Ca^{2+} activity in E-oscillators. Alternatively, binding of PDF to its receptor leads to an increase in cAMP levels, which could subsequently activate a cyclic nucleotide gated channel to acutely depolarize and activate E-cells (Seluzicki et al. 2014).

The signals released from the E-oscillators to modulate the light responses still remain to be determined. E-oscillators are known to express neuropeptides such as NPF, sNPF, and ITP. Further, some of them are cholinergic as well. Hence, the E-oscillators can transmit either peptidergic or cholinergic signals during light responses. Yoshii et al. (2015) found that rescue of CRY in NPF positive neurons of *cry^{01}* flies could partially restore the light entrainment. Also, E-oscillators might employ more that one neuropeptide to drive the phase shifting responses. ITP (Hermann-Luibl et al. 2014) and acetylcholine (Johard et al. 2009) have been proposed to regulate the evening activity during light to dark transition in a LD cycle. These neurotransmitters could be acting in conjunction with NPF in the E-oscillators to modulate the phase shifting response. Alternatively, E-cells could utilize s-NPF as their output signal. Recently, Liang et al. (2017) have suggested that sNPF from E-cells can modulate Ca^{2+} rhythms in the dorsal neurons.

A strategy similar to what we used for M-oscillators to identify PDF as the neuromodulator for photoresponses (in Chapter III, Fig 3.3) can be applied to E-

oscillators as well. A situation in which E-oscillators are circadianly blind while functional JET remains only in M-oscillators also weakens the behavioral phase shift response (Chapter II, Fig 2.2). Analyzing phase shifting response with JET expression only in M- but not in E-oscillators to assess the contribution of an E-cell candidate neurotransmitter or its receptor could unveil the identity of the E-oscillator signal. However, due to E-oscillators' heterogeneous neurotransmitter expression pattern, it could be more challenging compared to M-oscillators. For instance, we tried this strategy with an NPF receptor mutant but did not observe improved phase shifts suggesting that either NPF is not the signal or another neuropeptide serves a redundant function.

Another future experiment to identify the signal from E-oscillators could be combining their candidate neurotransmitter or their receptor mutants with *Pdf*^{0} null mutants and analyze the photic behavioral resetting. *Pdf*^{0} mutants alone do not compromise phase shifts presumably due to cell autonomous light detection in E-oscillators, but disrupting the signals from both M- and E-oscillators together should abrogate phase shifts. Double mutant flies are likely to be completely arrhythmic, however, in which case phase shifting analysis would not be possible.

Another line of evidence that implies modulation of cell autonomous light detection by neural interactions is the potential role of a subset of Dorsal Neurons in inhibition of phase advance specifically when the E-oscillators were electrically silenced (Chapter III, Fig 3.7). This kind of feedback from DN1s onto the M-oscillators is not without precedent. DN1s inhibit M-oscillators via glutamatergic modulation in larvae to modulate photo-avoidance behavior (Collins et al. 2012). Recently, DN1s have been

shown to feedback on M and E cells even in adults via inhibitory action of glutamate to promote mid-day siesta and night time sleep (Guo et al. 2016). Moreover, DN1s can also integrate light and temperature inputs to regulate outputs from both M- and E-oscillators (Zhang et al. 2010). In addition to glutamate, DN1s also express IPNamide and DH-31. The signals secreted from DN1s to modulate phase advances response remains to be determined. However, we cannot exclude the role of DN3s as well in control of phase advance response.

Another aspect of the light input pathway that is not well understood is how the light from the visual system reaches the clock neurons. It is well known that, in addition to the CRY mediated photoreception; the opsin-based visual system also feeds light information to the circadian network. But it is not known how the CRY mediated light inputs interact with the input from external photoreceptors. Also, the neural mechanisms of visual system mediated circadian photoreception are not very well understood. It seems likely that PDF positive LNvs relay the light information from the visual system to the circadian network. The extra-retinal HB eyelets' projections directly contact the dendritic fibers of the PDF positive LNvs. In fact, in the larvae, Bolwig organs (precursor of HB eyelet) and CRY together contribute to light entrainment of clock neurons (Helfrich-Förster et al. 2002). It has also been proposed that the excitatory effect of light can be modulated by the HB eyelet via cholinergic excitation of s-LNvs and histaminergic inhibition of l-LNvs (Schlichting et al. 2016). Therefore, it is possible that light information from the visual organs converges onto the LNvs, but the nodes of the neural circuit remain to be determined.

In conclusion, the data presented in this dissertation demonstrates that CRY mediated light resetting of the molecular pacemaker is cell autonomous and that the phase shift in circadian behavior is determined by the molecular properties of the pacemaker rather than neural interactions or specific neurons controlling the directionality of phase shift (Chapter III). Despite this ability to perceive light autonomously, neurons are designed to function as "team players" in an intact circadian circuit (Fig. 4.1). Hence, neural interactions are integral in modulating circadian photoresponses. We have shown that after light pulses, TIM degradation in E-oscillators resets their molecular pacemaker, which allows them to help the M-oscillators to resynchronize their own circadian pacemaker (Chapter II). The M-oscillators being the pacemaker neurons then readjust the whole circadian neural network. This bears similarities with light synchronization in mammals. The Suprachiasmatic Nucleus (SCN) - the mammalian neural circadian pacemaker - receives light input through dedicated retinal ganglion cells in the retina (Hattar et al. 2006). Cells in the core of the SCN appear to be particularly sensitive to this light input. They communicate with robust pacemaker neurons of the shell, which then reset the whole circadian neural network (Yan et al. 2007). The pacemaker neurons in the shell region also control the downstream oscillators in the peripheral tissues. Evans et al. (2015) by using a long photoperiod regime to dissociate the rhythms of the SCN core and shell have demonstrated that the peripheral clocks in the mammals receive the time of the day cues from the pacemaker neurons of the shell region specifically. This is in contrast to the *Drosophila* peripheral tissues, which can maintain and synchronize their rhythms independent of the brain (Plautz et al.

1997; Ivanchenko et al. 2001; Ito et al. 2008). Even though, so far it has not been shown that the peripheral tissues in mammals can detect light autonomously, there is some evidence that the mammalian peripheral clocks can also sustain rhythmicity in isolation (Yoo et al. 2004), indirectly perceive photic signals (Kiessling et al. 2014) and synchronize to the LD cycles (Husse et al. 2014) independent of the SCN neural network.

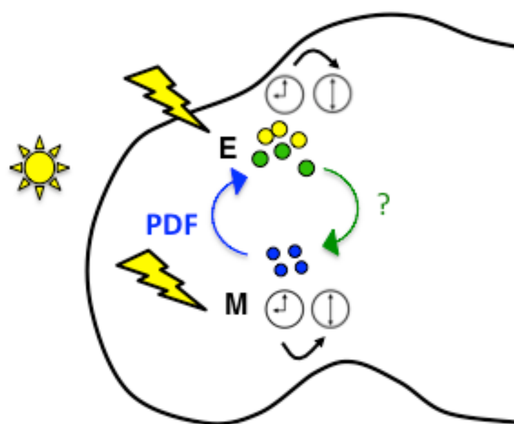


Figure 4.1 M- and E-oscillator network interactions influence CRY dependent cell autonomous light detection.

This model depicts that M- and E- oscillators perceive light independently and reset their individual molecular pacemaker that results in release of PDF from the M-oscillator and an unknown neurotransmitter from the E-oscillator to modulate CRY mediated cell autonomous phase shifting response.

We show that the two groups of oscillators controlling the M- and E-peak of activity in *Drosophila* are critical for light perception and coordination between these neurons is required for synchronizing the circadian behavior to the environmental light input. In fact this M- and E- dual oscillator model was first proposed by Pittendrigh and Daan (1976) to explain the effect of different photoperiods on nocturnal rodent circadian behavior. The E-oscillator, which drives the activity onset in nocturnal rodents, entrains

to dusk and the M-oscillators, which drives activity end, entrains to dawn. The E-oscillator has a shorter intrinsic period length than the M-oscillator and therefore, the two oscillators respond differently to light. The M-oscillator is accelerated by light and decelerated by darkness and the E-oscillator is decelerated by light and accelerated by darkness and thus entrains to dusk. Therefore, under long summer days the M-peak will occur earlier and E-peak later and under short winter days the M-peak will occur later and E-peak will occur earlier. This dual-oscillator model helps in explaining how the organisms track the day length and adapt to different seasons.

By measuring PER1-LUC rhythms from individual cells and tissue explants of SCN, Honma et al. (2008) have proposed the existence of two oscillatory cell groups that control activity onset and end separately under different photoperiods and thus correspond to the E- and M-oscillators respectively in the SCN. Moreover, it has also been suggested that the E-oscillators reside in the anterior region of the SCN and the M-oscillators in the posterior region (Inagaki et al. 2007; Yoshikawa et al. 2017). Evans et al. (2013) have also demonstrated that the SCN network can be reorganized into two subpopulations (the shell and core compartments) that cycle out of phase upon exposure to long day length. Further, they also showed that these SCN core and shell neurons re-synchronize to establish a steady-state network organization through phase-dependent resetting responses. Even, though there is evidence that M- and E-oscillators exist in the SCN in mammals, it was proposed that the M-oscillators control the phase advances and the E-oscillators control phase delays in response to brief light pulses (Daan et al. 2001), which is in contrast to our findings in *Drosophila*, where we propose that the phase

resetting in response to short light pulses is cell-autonomous and is determined by the molecular properties of the pacemaker. Nevertheless, in our studies as well, neural coordination between the M- and E-oscillators was critical for resetting the fly circadian behavior to light input. Therefore, both in flies as well as in mammals, M- and E-oscillators might be contributing to plasticity of circadian behavior and in facilitating its adaptation to environmental inputs. Even in human sleep-wake cycles, under different photoperiods the sleep onset is locked onto dusk and end is entrained to dawn along with melatonin profiles suggesting that the M- and E-oscillator model could explain seasonal changes in sleep time and melatonin rhythms in humans as well (Daan et al. 2001; Wehr et al. 1995).

Light has a profound impact on human health. The human circadian pacemaker is also extremely sensitive to light; in fact a short bright light exposure of 15 seconds can induce significant phase shifts (Rahman et al. 2017). Humans have been shown to share the same characteristic response to timing of light exposure as *Drosophila*. Retinal light exposure in the early subjective night delays the timing of the clock whereas late night exposure fast-forwards the phase of the internal clock (Czeisler et al. 1989; Johnson 1990). Such bright light exposure in the night could have real world implications in circadian rhythms disruption. Photic phase resetting may be an unrecognized risk factor for insomnia and circadian rhythm sleep disorders. Alternatively, effectiveness of short light pulses in resetting the circadian clock may have potential therapeutic value in treatment of disorders associated with circadian misalignment such as jetlag, shift-work dyssomina and delayed sleep phase syndrome. It has been shown that it takes more than 9

days for the human body temperature rhythm to realign to the new sleep-wake cycle following a 6-hour phase advance shift (such phase shifts can be induced due to trans meridian travel or rotating shift-work) (Monk et al. 1988). It has been proposed that with a properly timed light exposure, physiological adaptation to such phase shifts can be complete within 2-3 days (Czeisler et al. 1989) Therefore, transmeridian travelers who spend more time outdoors show faster adaptation of their behavioral rhythms to the new time zone compared to the travelers who stay indoors, suggesting an important role of light (outdoors) in human circadian phase resetting (Klein et al. 1974). Lingjaerde et al. (1985) have used morning bright light to treat patients with delayed sleep phase syndrome. Light-therapy has also been found to be effective during seasonal (fall-winter) depression (Czeisler et al. 1988; Lewy et al.1988) Hence, studying the neural and molecular mechanisms controlling phase resetting by light may advance our understanding and allow for effectively treating disorders arising from desynchronization of the clocks with the environment.

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Appendix

miR-124 Regulates the Phase of *Drosophila* Circadian Locomotor Behavior

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This work is a result of collaboration between our laboratory and Zhang laboratory. I performed the PDF staining of wild type and *miR-124* knock out flies and observed mild anatomical defects in the *miR-124* knock out flies.

Abstract

Animals use circadian rhythms to anticipate daily environmental changes. Circadian clocks have a profound effect on behavior. In *Drosophila*, for example, brain pacemaker neurons dictate that flies are mostly active at dawn and dusk. miRNAs are small, regulatory RNAs (≈22 nt) that play important roles in posttranscriptional regulation. Here, we identify *miR-124* as an important regulator of *Drosophila* circadian locomotor rhythms. Under constant darkness, flies lacking *miR-124* (*miR-124*^{KO}) have a dramatically advanced circadian behavior phase. However, whereas a phase defect is usually caused by a change in the period of the circadian pacemaker, this is not the case in *miR-124*^{KO} flies. Moreover, the phase of the circadian pacemaker in the clock neurons that control rhythmic locomotion is not altered either. Therefore, *miR-124* modulates the output of circadian clock neurons rather than controlling their molecular pacemaker. Circadian phase is also advanced under temperature cycles, but a light/dark cycle partially corrects the defects in *miR-124*^{KO} flie*s.*Indeed, *miR-124*^{KO} shows a normal evening phase under the latter conditions, but morning behavioral activity is suppressed. In summary, *miR-124* controls diurnal activity and determines the phase of circadian locomotor behavior without affecting circadian pacemaker function. It thus provides a potent entry point to elucidate the mechanisms by which the phase of circadian behavior is determined.

SIGNIFICANCE STATEMENT In animals, molecular circadian clocks control the timing of behavioral activities to optimize them with the day/night cycle. This is critical

for their fitness and survival. The mechanisms by which the phase of circadian behaviors is determined downstream of the molecular pacemakers are not yet well understood. Recent studies indicate that miRNAs are important regulators of circadian outputs. We found that *miR-124* shapes diurnal behavioral activity and has a striking impact on the phase of circadian locomotor behavior. Surprisingly, the period and phase of the neural circadian pacemakers driving locomotor rhythms are unaffected. Therefore, *miR-124* is a critical modulator of the circadian output pathways that control circadian behavioral rhythms.

Introduction

Most organisms use circadian clocks to anticipate daily environmental changes and thus maximize their chances of survival. Circadian clocks govern most basic bodily functions, including sleep/wake cycles, hormone secretion, and metabolic rates. In animals, the molecular mechanism underlying circadian rhythms is a highly conserved transcriptional–translational feedback loop (Weaver and Emery, 2013). In flies, the heterodimeric transcription factor CLOCK/CYCLE (CLK/CYC) binds to the promoter region of many genes through E-box sequences. Among these genes, *period* (*per*) and *timeless* (*tim*) encode for CLK/CYC repressors. PER and TIM form a heterodimer, accumulate in the cytoplasm, and enter into the nucleus to suppress their own gene transcription by inhibiting CLK/CYC. A series of kinases and phosphatases regulate the phosphorylation state, stability, and nuclear entry of PER/TIM. The oscillations of this molecular clock are maintained in constant conditions and synchronized by

environmental inputs such as light and temperature. In flies, light changes the conformation of the blue-light photoreceptor CRYPTOCHROME (CRY). CRY binds to TIM and an E3-ubiquitin ligase containing JETLAG (JET) to trigger proteasomal TIM degradation (Zhang and Emery, 2012). TIM degradation exposes PER to phosphorylation and proteasomal degradation and thus resets the circadian pacemaker. Temperature increases also trigger TIM degradation to reset circadian clocks in flies, but this molecular response is mediated by calcium and the atypical protease SOL (Tataroglu et al., 2015). Circadian clock neurons can also receive thermal and photic inputs nonautonomously (Helfrich-Förster et al., 2001; Chen et al., 2015).

miRNAs are small, noncoding RNAs that regulate many important biological processes through posttranscriptional repression of specific target genes (Bartel, 2004). Recent studies have shown that miRNAs play an important role in the control of circadian rhythms in both flies and mammals. Several miRNAs are reported to be under circadian control (Cheng et al., 2007; Yang et al., 2008; Vodala et al., 2012). In mice, two rhythmically expressed miRNAs are important for circadian clocks: *miR-132* is induced by light and regulates circadian photoresponses and *miR-219* modulates the circadian pacemaker (Cheng et al., 2007). Dicer proteins are critical for miRNA biogenesis (Bartel, 2004). In Dicer-deficient mice, shortened circadian rhythms have been observed mainly because of faster translation of PERIOD1 and PERIOD2, two critical pacemaker proteins (Chen et al., 2013). In flies, knocking down DICER-1 decreases the amplitude of locomotor rhythms (Kadener et al., 2009). We have also found that GW182, a protein crucial for miRNA function, controls locomotor rhythms through modulation of the

pigment-dispersing factor (PDF) signaling pathway (Zhang and Emery, 2013). Recently, a cluster of miRNAs (*miR959–miR964*) was found to be under circadian regulation and to affect the timing of feeding, immune response, and circadian period (Vodala et al., 2012). In addition, *miR-279* affects the amplitude of circadian behavioral rhythms (Luo and Sehgal, 2012), whereas *bantam* and let-7 control the expression of pacemaker genes (Kadener et al., 2009; Chen et al., 2014). Here, we reveal that *miR-124* specifically modulates the phase of circadian locomotor behavior without affecting the circadian pacemaker of the brain neurons that control this complex behavior. In addition, we show that *miR-124* affects morning activity and acute behavioral response to light.

Materials and methods

Fly stocks

Flies were raised on standard cornmeal/agar medium at 25°C under light/dark (LD) cycles. The following strains were used: w^{1118}, *miR-124*^{KO}*/CyO* (Sun et al., 2012), *miR-124*^{KO}, and genomic rescue *39N16/CyO* (Sun et al., 2012). The deficiency stocks BL7836 and BL7837 were obtained from the Bloomington *Drosophila* stock center.

Behavior experiments and analysis

Adult male flies (2–5 d old) were used to test locomotor activity rhythms. Flies were entrained under LD for 3 d and released into constant darkness (DD) for at least 5 d at 25°C. For temperature cycles (TCs), flies were entrained to 12 h: 12 h 29°C: 20°C thermocycle for 3 d in the dark and released at constant 25°C for at least 5 d. Locomotor

activity was recorded with *Drosophila* activity monitors (Trikinetics) in Percival I36-LL incubators. FaasX software was used to analyze behavioral data (Grima et al., 2002). For actograms, a signal-processing toolbox implemented in MATLAB was used (Levine et al., 2002). Three days of activity in LD were used to generate average activity bar graphs; 5 d of activity in DD were used to analyze phase.

Electroretinogram recordings

Electroretinograms (ERGs) were performed as described previously (Ni et al., 2008). Flies were immobilized with tapes. Glass recording and reference microelectrodes filled with Ringer's solution containing the following (in mM): 130 NaCl, 5 KCl, 2 $CaCl_2$, 2 MgCl2, 36 sucrose, and 5 HEPES, pH 7.3, were inserted into small drops of electrode cream applied on the compound eye surface and the thorax, respectively. Light was provided by a 100 W halogen lamp and was delivered to the compound eye by fiber optics. Signals were amplified by a Warner IE210 intracellular electrometer, sampled, and analyzed using pCLAMP software. Five-second orange light pulses (1800 lux) were used to stimulate the eye after adapting the fly in the dark for 1 min.

Whole-mount immunohistochemistry and quantification

Whole-mount immunohistochemistry was done as described previously (Lamba et al., 2014). For PDF stainings, flies were entrained to LD for 3 d and dissected at Zeitgeber time (ZT) 1 or 13. For PER stainings, flies were entrained to LD for 3 d and then released in DD. Brains were dissected on the second day of DD at six time points. Rabbit anti-

PER (1:1500) and mouse anti-PDF (1:400) antibodies were used. All samples were imaged with a Zeiss LSM5 Pascal confocal microscope with a constant laser setting for each time point. ImageJ software was used for PER quantification. The average signal of three background areas were subtracted from signal intensity in each circadian neurons. At least five brains for each time point were used for quantification.

Results

Loss of *miR-124* disrupts acute response to light in the morning

Under an LD cycle, wild-type flies increase their activity before the lights-on (morning anticipation or morning peak) and before the lights-off transition (evening anticipation or evening peak) (Fig. 1*A*). Wild-type flies also show a sharp increase in activity at the lights-on and lights-off transition, which is called a startle response. These startle responses are direct reactions to environmental changes because they are independent of the circadian clock and happen even in otherwise arrhythmic flies (*per*^{0} for example). However, the timing of morning and evening anticipation is under circadian clock control. The morning peak is driven by the PDF-positive small ventral lateral neurons (sLNvs), whereas the evening peak is driven by a subset of dorsal lateral neurons (LNds) and a PDF-negative sLNv (Grima et al., 2004; Stoleru et al., 2004). Dorsal neurons (DN1s) appear to work downstream of the sLNvs (Zhang et al., 2010a; Cavanaugh et al., 2014). Because *miR-124* is a highly conserved miRNA with specific expression in the CNS (Sun et al., 2012; Weng and Cohen, 2012), we decided to test whether it could influence circadian or diurnal behavior. *miR-124*^{KO} flies showed a

striking behavioral defect under an LD cycle: morning anticipation and the startle response to lights-on were severely blunted (Fig. 1*A*,*B*). However, an evening peak was clearly present under LD, albeit with reduced amplitude, suggesting that the molecular pacemaker is intact at least in the evening oscillators (Fig. 1*B*). To verify that this evening anticipatory behavior was indeed controlled by the circadian clock and was not merely a direct response to environmental input, we combined *miR-124*^{KO} with the short period *per*^{S} mutation (Konopka and Benzer, 1971). The phase of the evening peak was advanced in *per*^{S};*miR-124*^{KO}double-mutant flies, as in *per*^{S} flies. This clearly demonstrates that the evening peak observed in *miR-124*^{KO} flies is under circadian control (Fig. 1*D*).

To determine whether the phenotypes observed in LD are caused by loss of *miR-124*, we first crossed *miR-124*^{KO} flies to a deficiency line carrying a chromosome lacking the *miR-124* genomic region and tested heterozygous *miR-124*^{KO}*/Df* flies (Fig. 1*A*,*B*). These flies showed similar behavioral profiles as *miR-124*^{KO} homozygous flies. The acute response to lights-on was absent. The amplitude of the morning peak of activity was reduced. We noted that the phase of the residual morning peak seemed delayed compared with wild-type. We then tested *miR-124*^{KO} homozygous flies rescued with a genomic construct containing *miR-124* (Sun et al., 2012; Fig. 1*A*,*B*). The morning startle response was restored, but the amplitude of morning anticipation showed little improvement. This is probably because of a general lower level of activity in *miR-124*^{KO}homozygous flies carrying or not carrying the rescue transgene. *miR-124*^{KO}*/Df* flies did not show this lower activity, which thus does not appear to be caused by loss of *miR-124*. Moreover, the

morning peak of activity was restored in *miR-124*^{KO}*/Df* flies rescued with the genomic construct (Fig. 1*E*). In summary, *miR-124* is required for acute response to light in the morning and contributes to the timing and amplitude of morning anticipatory behavior.

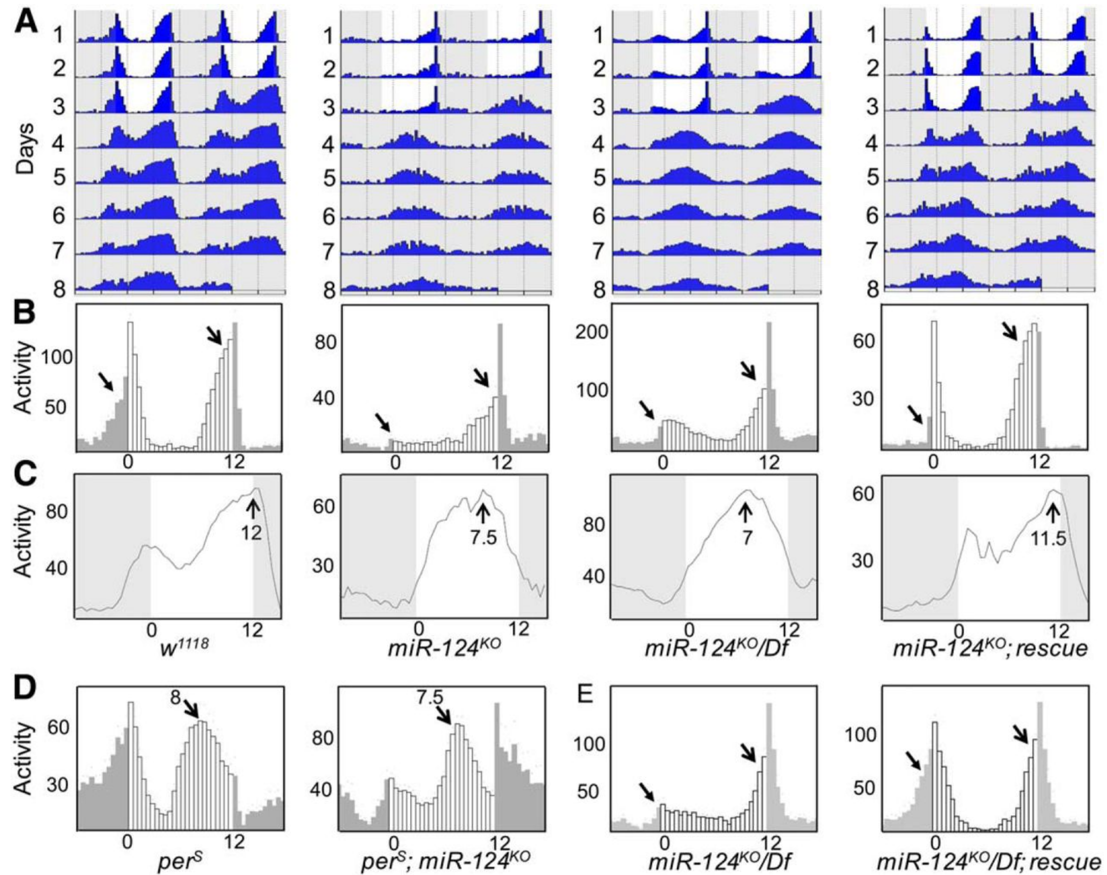

Figure 1. Loss of *miR-124* advances circadian phase under constant darkness.
A, Locomotor behavior under LD cycle and constant darkness. Representative double plotted actograms of w^{1118}, *miR-124*^{KO}, *miR-124*^{KO}*/Df*, and *miR-124*^{KO} rescue flies. White indicates the light phase, gray indicates the dark phase. *B*, Morning anticipation (small arrows) and lights-on startle response are eliminated in *miR-124*^{KO} flies under the LD cycle. Evening anticipation is indicated with large arrows. White bars represent activity during the day, gray bars at night. (*C*) Circadian behavior profile in DD. Circadian phase is dramatically advanced in *miR-124*^{KO} flies in constant darkness. Circadian time of peak activity is indicated on the graph. Gray shades indicate the subjective night. *D*, Phase of the evening peak observed in *miR-124*^{KO} flies is advanced by the *per*^{S} mutation and is thus under circadian control. *E*, Morning anticipation and lights-on startle response are restored in *miR-124*^{KO}*/Df* flies rescued with a genomic *miR-124* construct.

Loss of *miR-124* advances circadian phase in constant darkness

Under DD conditions, the morning and evening peaks of activity persist in wild-type flies with a phase determined by the previous LD (or temperature) cycle, but the amplitude of the morning peak tends to decrease over time. *miR-124*^{KO} flies were rhythmic in DD with a completely normal period of 24.1 h. To our surprise, however, the phase of circadian behavior was dramatically different in *miR-124*^{KO} flies compared with wild-type flies. A single bout of activity was observed with a peak near subjective midday (Fig. 1*C*). This peak likely corresponds to a 4.5 h advanced evening peak of activity based on its sustained large amplitude and the fact that very little morning activity was detected under LD conditions in *miR-124*^{KO}. However, we cannot exclude a small contribution from morning neurons. A similar phenotype was observed in *miR-124*^{KO}*/Df* flies. Both (subjective) morning and evening activity were restored with the genomic construct, with phases close to those observed in wild-type flies. Therefore, *miR-124* plays an important role in the control of morning activity and determines the phase of evening activity under constant conditions.

Loss of *miR-124* advances circadian phase under and after temperature cycles

Although the phase of evening activity was advanced in constant darkness, it was normal under the LD cycle (Fig. 1). Therefore, light input can correct this phase defect of *miR-124*^{KO} flies. We therefore wondered whether temperature, another critical input to the clock, could do the same. We recorded the circadian behavior of *miR-124*^{KO} flies under and after exposure to a 29°C/20°C TC. To our surprise, unlike LD, TC could not correct circadian phase. *miR-124*^{KO} flies showed advanced evening phase under TC and

after release to constant temperature (Fig. 2). Therefore, the phase defect is corrected specifically by light.

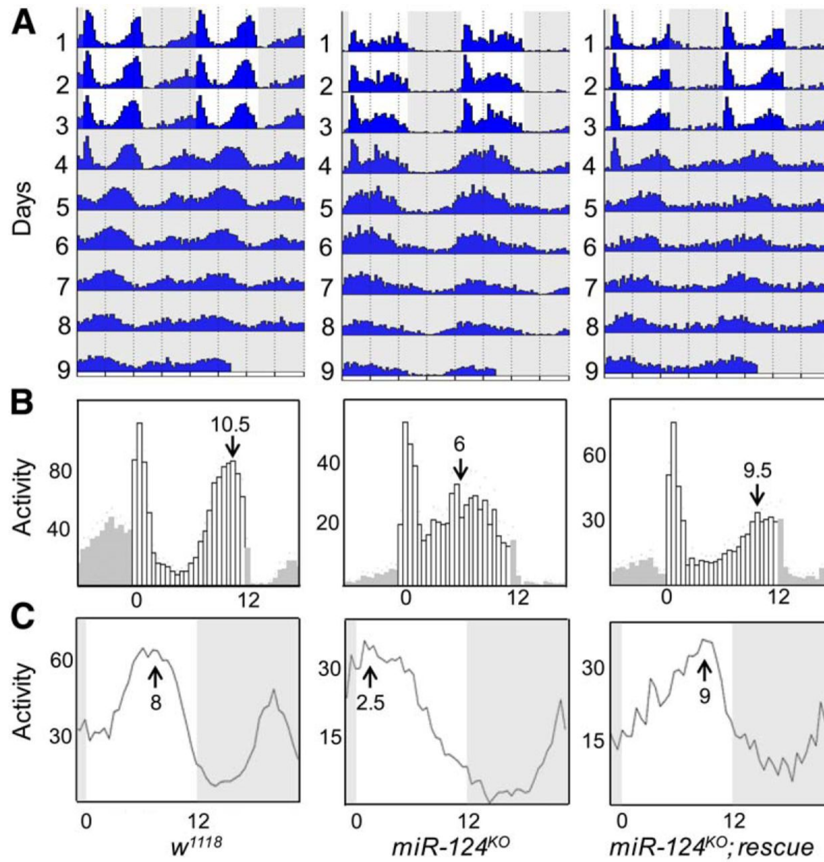

Figure 2. Loss of *miR-124* advances circadian phase under and after temperature cycles. *A*, Locomotor behavior under TCs and constant darkness. White indicates the warm phase (29°C), gray the cold phase (20°C) or the release in constant conditions (25°C). *B*, Evening peak is advanced in *miR-124*^{KO} flies under the TC cycle. *C*, Phase is dramatically advanced in *miR-124*^{KO} flies after release in constant temperature. Gray shades indicate the subjective night (subjective cold phase).

Visual photoreception and the molecular pacemaker of circadian neurons are functional in miR-124^{KO} flies

Because the startle response to the lights-on transition is blunted, we wondered whether visual photoreception is affected in miR-124^{KO} flies. We therefore recorded the light response of visual photoreceptors to light with an ERG. The ERG of wild-type flies shows quick transients representing hyperpolarization and repolarization of laminar neurons postsynaptic to photoreceptors, as well as a sustained depolarization corresponding to the activation of the photoreceptor cells. No ERG defect was found in miR-124^{KO} flies, indicating that the phototransduction cascade and synaptic transmission between photoreceptors and postsynaptic neurons in the eyes are normal (Fig. 3A). Therefore, the defect in light response in the morning is downstream of or unrelated to vision. Next, we tested whether the advance in circadian behavioral phase could be caused by an abnormal phasing of the molecular pacemaker in the sLNvs, LNds, or DN1s, the three groups of circadian neurons known to control locomotor behavior. However, the amplitude and phase of PER oscillations were essentially normal (Fig. 3B,C). Therefore, miR-124 regulates circadian behavior phase by modulating circadian output mechanisms.

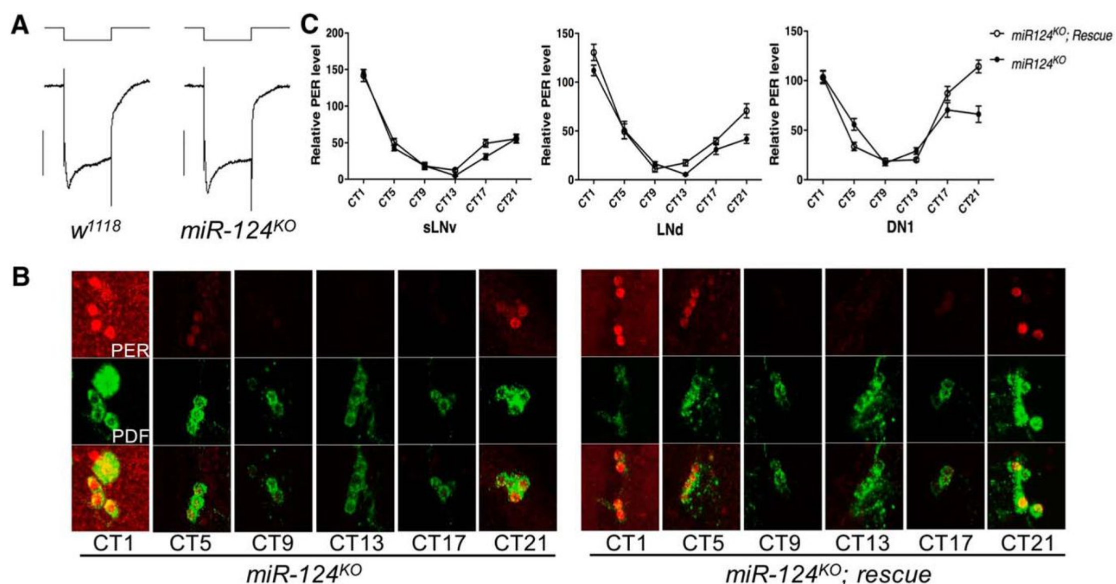

Figure 3. The molecular pacemaker is not affected in *miR-124*^{KO} flies. *A*, ERG recordings do not show any obvious light response defect in the visual photoreception cascade of *miR-124*^{KO} flies. Scale bar, 5 mV. *B*, sLNvs of brains from *miR-124*^{KO} and genomic rescue flies dissected at different time points (circadian time, CT) during the second day of DD and stained with anti-PDF (green) and anti-PER (red) antibodies. *C*, Quantification of PER staining in sLNvs, LNds, and DN1s at different circadian time points. Error bars indicate SEM.

Mild developmental abnormalities in the PDF neural network of *miR-124*^{KO} flies

We also wondered whether the development of pacemaker neurons controlling circadian behavior might be defective in *miR-124*^{KO} flies. *miR-124* has been reported to have a mild effect on neural development, especially bouton numbers at neuromuscular junctions (Sun et al., 2012; Wang et al., 2014). Therefore, we looked at the anatomy of PDF-positive neurons (Fig. 4). Indeed, PDF-positive sLNvs use PDF as a crucial neuropeptide for synchronizing other circadian neurons and to control morning activity (Renn et al., 1999). In all *miR-124*^{KO} brains, the expected set of LNvs with their projections was present: dorsal sLNv projection terminating in the dorsal protocerebrum and contralateral and optic lobe projections from the large LNvs (lLNvs) were observed (cf. Fig. 4*A*–*C*, *D*–*F*). However, in a small fraction (22%) of *miR-124*^{KO} brains, we observed one to two additional LNv projections. These either branched out of the sLNv dorsal projection bundle to terminate more ventrally than normal sLNv projections (Fig. 4*G*) or appeared to be lLNv projections dorsally branching out of the contralateral bundle toward the midline. *miR-124* might thus have a minor impact on sLNv and lLNv projection pathfinding. We also noticed that the lLNv cell bodies tended to be positioned more dorsally than in wild-type flies (Fig. 4*A*,*D*). Finally, we observed in a majority of *miR-124*^{KO} brains (68%) the persistence of projections from the tritocerebral PDF (PDF-Tri)-positive neurons (Fig. 4*D*,*G*), a noncircadian group of neurons that in wild-type flies is eliminated after eclosion through programmed apoptosis (Renn et al., 1999; Gatto and Broadie, 2011). Therefore, elimination of these cells is either delayed or

defective in *miR-124*^{KO} flies. As discussed below, the *miR-124*^{KO} anatomical defects observed in the PDF network are unlikely to account for the behavioral phenotypes.

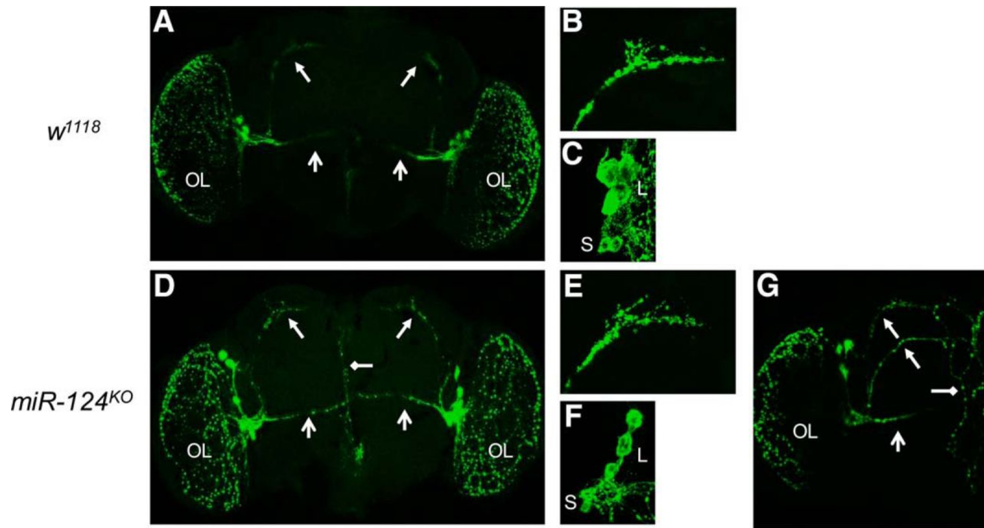

Figure 4. PDF neural network in wild-type and *miR-124*^{KO} flies. PDF staining (green) in w^{1118} (*A*–*C*) and *miR-124*^{KO} (*D*–*G*) brains. *A*, *D*, *G*, Open arrows indicate the sLNv dorsal projections, OL the lLNv optic lobes projections, and closed arrows the lLNv contralateral projections. These projections were all present and normal in *miR-124*^{KO} brains (*D*), but a small fraction of mutant brains showed additional LNv projections, such as more ventral sLNv projections (*G*). Persistence of PDF-Tri projections (diamond arrows) was observed in most mutant brains (*D*, *G*). *B*, *E*, Terminal ends of PDF-positive sLNv projections in the dorsal protocerebrum at ZT1. *C*, *F*, Cell bodies of PDF-positive sLNvs (S) and lLNvs (L).

Discussion

We demonstrate here that *miR-124* is crucial for regulating the phase of circadian behavior, as well as acute behavioral response to light. Unexpectedly, although circadian phase is advanced by up to 5 h, the circadian pacemaker is unaffected. Its period is still ~24 h. Most mutants with a phase defect have altered circadian period. For example, the *per*^{S} phase is considerably advanced under LD conditions, but this reflects the fast pace of the circadian pacemaker. Notable exceptions are mutants that affect *per* thermosensitive splicing, which helps flies adapt the phase of their evening peak to different ambient temperatures (Majercak et al., 1999). However, because PER oscillations are not affected at all in pacemaker neurons, it is highly unlikely that *miR-124* regulates PER splicing. *miR-124* thus works downstream of the circadian pacemaker to determine the circadian phase of locomotor behavior. It is therefore becoming increasingly clear that miRNAs play critical functions in the control of various circadian outputs. Indeed, miRNAs have also been implicated in the modulation of behavioral output amplitude (Luo and Sehgal, 2012), PDF signaling (Zhang and Emery, 2013), timing of feeding, and the immune response (Vodala et al., 2012). Interestingly, the latter rhythmic function was phase shifted in *miR-959–miR964* cluster mutants, but whether this shift was caused by alterations of circadian pacemaker function or output pathways in relevant tissues is not yet known.

The task now is to define in which tissues *miR-124* functions and which mRNAs it regulates. For the latter question, one can turn to target prediction. There are over 100

genes predicted by targetscan. In this list, a few targets are known to regulate circadian behavioral rhythms, including Mef2 and matrix metalloproteases (MMP1). Mef2 overexpression has been shown to lengthen circadian period, cause complex rhythms, and affect PER oscillations (Blanchard et al., 2010). The fact that we did not see such phenotypes makes it unlikely that *miR-124* affected circadian behavior through Mef2. MMP1 has been shown to regulate circadian rhythms by affecting PDF levels at the dorsal terminals of sLNv neurons (Depetris-Chauvin et al., 2014). Acute overexpression of MMP1 during adulthood decreased PDF levels and the rhythmicity of flies in constant darkness. Again, these phenotypes do not fit those observed with *miR-124*^{KO}. Several positive components of BMP signaling pathway are targeted by *miR-124* (Sun et al., 2012). However, constitutive activation of BMP pathway in circadian neurons caused a significant long period (Beckwith et al., 2013), whereas loss of *miR-124* had no effect on period. Identifying relevant *miR-124* targets is thus a priority and will require either genetic screening or gene expression profiling in relevant neurons.

This brings us to the important question of the site of *miR-124* action. First, could the mild anatomical defects that we observed in PDF neurons account for the behavioral phenotypes? We cannot exclude this possibility, but believe it to be unlikely. The lLNv defects were very subtle, with mostly a tendency to have more dorsally located cell bodies. In addition, the lLNvs do not control circadian behavior in DD nor are they required for morning activity and lights-on startle responses (Renn et al., 1999; Grima et al., 2004; Stoleru et al., 2004; Stoleru et al., 2005; Cusumano et al., 2009). Abnormalities in sLNv or lLNv projections were too rare to account for the behavioral phenotypes that

we observed. Finally, PDF-Tri cells, when preserved in adults through inhibition of apoptosis, have no impact on circadian behavior in LD or DD (Renn et al., 1999). Therefore, the persistence of these cells in *miR-124*^{KO} flies again cannot explain the behavioral phenotypes. Finally, because light can correct the phase of *miR-124*^{KO} flies in DD, a developmental defect appears unlikely to cause this phenotype. Interestingly, both the additional sLNv projections and the persistence of PDF-Tri cells are phenotypes observed in *fmr1* mutant flies (Gatto and Broadie, 2011). Moreover, FMR1 regulates *miR-124* levels (Xu et al., 2008). Combined with these previous studies, our current results thus suggest that the anatomical phenotypes observed in *fmr1* mutants are at least in part caused by reduction in *miR-124* expression.

To map the anatomical requirement of *miR-124* for circadian behavior, we attempted to use a rescue approach with the GAL4/UAS system, but our efforts were unsuccessful, in part because overexpression caused unwanted phenotypes (including lethality). However, output from DN1s or neurons downstream of these circadian neurons are potential candidate sites for *miR-124* action. Indeed, the DN1s function downstream of the PDF-positive sLNvs and are critical for circadian rhythms in DD, as well as for morning activity and the lights-on startle response under LD (Zhang et al., 2010a; Cavanaugh et al., 2014). Interestingly, their output is modulated by light (Zhang et al., 2010b) and light was able to correct the phase defect of *miR-124*^{KO} flies. TCs, however were, not able to do so, showing that a specific light input pathway reaches the neural circuit affected by *miR-124*. Future work using more refined approaches to disrupt *miR-124* function or to restore it in a mutant background should identify this

circuit and thus ultimately help to resolve the critical question of how circadian pacemaker and neural output are coupled.

Footnotes

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