CIRCADIAN CLOCK DEVELOPMENT AND INITIATION IN *DROSOPHILA MELANOGASTER*

A Dissertation

by

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ABSTRACT

In *Drosophila*, time-keeping is based on a ~24h transcription feedback loop, in which CLOCK-CYCLE (CLK-CYC) heterodimers activate transcription of genes encoding the feedback repressors PERIOD (PER) and TIMELESS (TIM). Despite the progress that has been made to define the molecular mechanisms that govern feedback loop function in animals, we do not understand how/when clock cells develop, how the clock network is organized, and how the feedback loop is initiated.

To determine when pacemaker neurons arise in *Drosophila*, I used a *Clk*-GFP transgene because *Clk* is the first clock gene to be expressed. I found that CLK is expressed in the late pacemaker neuron clusters (neurons with active rhythms in *per* expression during metamorphosis) by L3 larvae. This delay in *per* expression is not due to the lack of *cyc* expression as a GFP-*cyc* transgene is expressed exclusively in *Clk*-expressing pacemaker neurons, indicating novel factors are needed to activate circadian oscillator function during metamorphosis.

In addressing how *Clk* promotes CYC expression to initiate clock function, I found that *Clk* controls CYC accumulation by stabilizing CYC protein in cultured *Drosophila* Schneider 2 (S2) cells. Likewise, CYC accumulates specifically in ectopic cells expressing *Clk*, indicating that CLK also stabilizes CYC *in vivo*. CLK and CYC are, however, not sufficient for clock function in ectopic cells; *cryptochrome* (*cry*) is also required to entrain and/or maintain these clocks.

To determine how *Clk* affects the formation of clock network, I generated a *Clk21*-Gal4 transgene to mark pacemaker neurons in Clk^{out} flies. I found that *Clk* is essential for the maintenance of small ventral lateral neurons (sLN_vs) in adults without affecting the development of other groups of pacemaker neurons, which provides a new role of *Clk* in the sLN_v development and/or maintenance besides its role in initiating circadian clock function.

Taken together, my work has demonstrated clock cell development, network formation, as well as the mechanisms by which the functions of clock are initiated in *Drosophila*. Since these features appear to be conserved in eukaryotic clocks, my research will hopefully provide new insights into the development and function initiation of the clock in other organisms.

DEDICATION

This work is dedicated to my parents for making who I am, and my husband, Shaoyong, who accompanied me through the whole process, and gave me constant support, strength and love, that have motivated me to achieve my dream.

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CONTRIBUTORS AND FUNDING SOURCES

Contributors

Part 1, faculty committee recognition

This work was supervised by a dissertation committee consisting of Dr. Paul Hardin (advisor), Dr. David Earnest, Dr. Christine Merlin of the Department of Biology and Dr. Hubert Amrein of the Department of Molecular and Cellular Medicine.

Part 2, student/collaborator contributions

The *attB*-P[acman]-*Clk*V5-EGFP (*Clk*-GFP) transgene (Chapter II) and the pMK33-*cyc*-Flag plasmid (Chapter III) were generated by a post-doctoral fellow in the Hardin lab, Dr. Guruswamy Mahesh (Chapter II)(Liu et al., 2015). The N-terminal eGFP tagged *cycle* (*cyc*) transgene (GFP-*cyc*) was generated by a post-doctoral fellow in the Hardin lab , Dr. Jerry Houl (Chapter II)(Liu et al., 2015). Gal4-kanamycin plasmid was constructed by a research scientist, Dr. Wangjie Yu in Hardin Lab (Chapter IV).

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CHAPTER I

INTRODUCTION[1]

BACKGROUND

Circadian clocks are essential for wellbeing

One of the most fascinating aspects of life on earth is that many organisms have a circadian clock (~24h) that enables them to synchronize physiological, metabolic, and behavioral rhythms with daily environmental cycles. This synchrony is essential to wellbeing since desynchronization causes metabolic diseases (e.g. obesity and metabolic syndrome), sleep disorders and even cancer (Laposky et al., 2008; Turek et al., 2005). Thus it is of clinical importance to understand how/when clock cells develop and how the clock network is organized. These studies will provide a foundation that will enable us to determine which cells will contain a clock and what requirements must be satisfied for cells to contain a functional clock. Since the core clock components are well conserved from insects to mammals, understanding these requirements may uncover novel clock activators in model systems that may function in other animals, which may become novel targets for treatment for clock disorders (e.g. Familial Advanced Sleep Phase Syndrome (Laposky et al., 2008)).

[1] Several paragraphs of this chapter are reprinted from Liu, T., Mahesh, G., Houl, J. H., and Hardin, P. E. (2015). Circadian activators are expressed days before they initiate clock function in late pacemaker neurons from *Drosophila*. Journal of Neuroscience, 35(22), 8662-8671, and the paper that has been submitted to PNAS and under review as Liu, T., Mahesh, G., Yu, W., and Hardin, P. E. (2017). CLOCK stabilizes CYCLE to initiate clock function in *Drosophila*

Timekeeping is based on a transcription feedback loop

In animals, circadian clocks keep circadian time via transcriptional feedback loops, which use conserved transcriptional activators and repressors to drive rhythms in transcription. The components are well conserved from insects to mammals (Bell-Pedersen et al., 2005; Yu and Hardin, 2006). Therefore, what we learn from *Drosophila* could provide insights into the mechanism underlying circadian clock function in mammals.

The identification of clock genes in *Drosophila* revealed that time keeping is based on a transcriptional feedback loop. In the core feedback loop (Figure 1), the transcriptional activators CLOCK (CLK) and CYCLE (CYC) form a heterodimer that binds to the E-boxes of the *period* (*per*) and *timeless* (*tim*), and activates *per* and *tim* transcription. *per* and *tim* mRNAs accumulate during the day, and PER and TIM proteins begin to accumulate in the cytoplasm at ~ZT12 (Zeitgeber Time, or ZT, refers to time in hours during a light–dark cycle, where ZT0 is lights on and ZT12 is lights off), and enter into the nucleus in the middle of the night at ~ZT18. When PER protein is produced, it is bond and phosphorylated by DBT, which is a homolog of mammalian casein kinase 1. At the same time the PER-DBT protein complex is stabilized by TIM protein, which forms a PER-TIM-DBT complex in the cytoplasm. The phosphorylation of PER by casein kinase 2 (CK2) and the phosphorylation of TIM by SHAGGY (SGG) promotes PER-TIM-DBT nucleus localization. Meanwhile, PER-TIM-DBT complex is dephosphorylated by protein phosphatase 1 and protein phosphatase 2. When PER-TIM-DBT enters the nucleus, the complex (PER-DBT and/or PER-TIM-DBT) binds to CLK

and promotes CLK phosphorylation. The CLK-CYC heterodimer is released from binding to the E-boxes in the *per* and *tim* promoter regions, therefore inhibiting *per* and *tim* transcription. When lights are coming on at ZT0, TIM protein is rapidly degraded. Without protection by TIM, PER is phosphorylated by DBT at PER serine 47 (S47) and bound by E3 ubiquitin ligase SLIMB, which leads to PER ubiquitination and rapid degradation (Chiu et al., 2008). When PER is degraded, CLK is dephosphorylated by an unknown mechanism before it binds to E-boxes, therefore activating the transcription feedback loop again. In addition, in the feedback loops, multiple levels of post-translation controls (e.g. phosphorylation and dephosphorylation) (Chiu et al., 2011; Yu et al., 2011), which affect a variety of clock components on their synthesis, nuclear localizations, and degradations, are thought to delay the cycles so that the cycles will take ~24h.

Besides the core feedback loop, an interlocked transcriptional feedback loop that is mediated by VRILLE (VRI) and PAR Domain Protein 1 (PDP1) also contributes to the regulation of rhythmic transcription in *Drosophila* (Cyran et al., 2003; Glossop et al., 2003). In the interlocked loop, the CLK-CYC complex binds to the E-boxes of *vri* and *Pdp1* separately to activate the transcription of *vri* and *Pdp1* (Blau and Young, 1999; Cyran et al., 2003). VRI protein accumulates in phase with its mRNA, and binds to the D-box found in the *Clk* promoter and represses *Clk* transcription. On the other hand, PDP1 protein accumulates and peaks several hours later than VRI protein, and binds to D-box found in the *Clk* promoter to activate *Clk* transcription. PDP1 protein also directly

regulates the output pathway in a circadian rhythm (Benito et al., 2008; Benito et al., 2007a; Zheng et al., 2009).

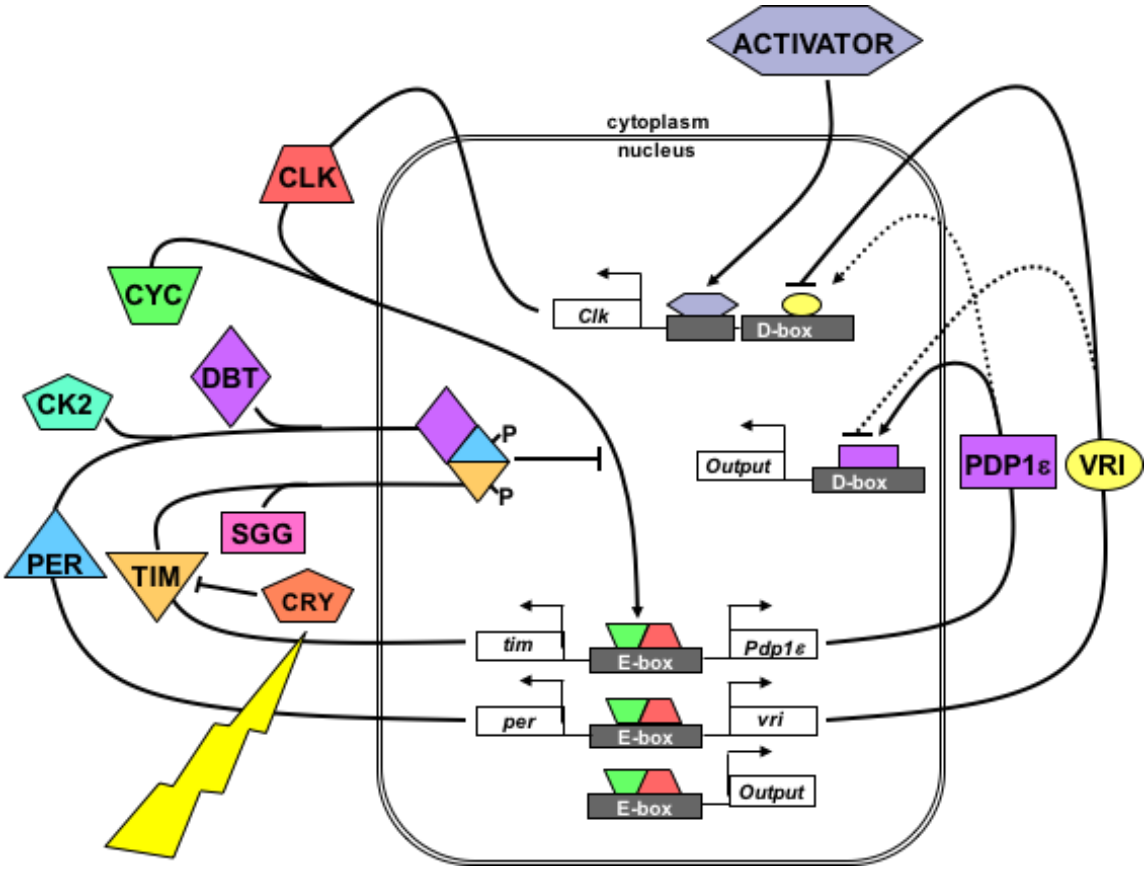

Figure 1. Model of the transcription feedback loops that keep circadian time in *Drosophila*. All gene, regulatory element, and protein names are as defined in the text. Double line, nuclear envelope; solid arrows, synthesis, assembly and/or localization steps; blocked line, repression; dash line, possible activation or repression; P, phosphorylation site(s). (Adapted from Benito et al. 2007)

The transcriptional feedback loop works in many tissues in both the head and body of *Drosophila*

Oscillators distributed in different tissues are roughly divided into two groups: the central oscillator which is composed of several groups of neurons in the brain, and peripheral oscillators, which exist in other parts of the body (Bell-Pedersen et al., 2005; Yu and Hardin, 2006). In contrast to the hierarchical model in mammals, where only the central oscillators are capable of being entrained by light and then mediate entrainment of peripheral oscillators, both central and peripheral oscillators in *Drosophila* can be directly entrained by light and function as pacemakers. In this case, the feedback loops in different tissues will drive rhythmic transcription of output genes that regulate physiology, metabolism and behavior (Bell-Pedersen et al., 2005; Yu and Hardin, 2006).

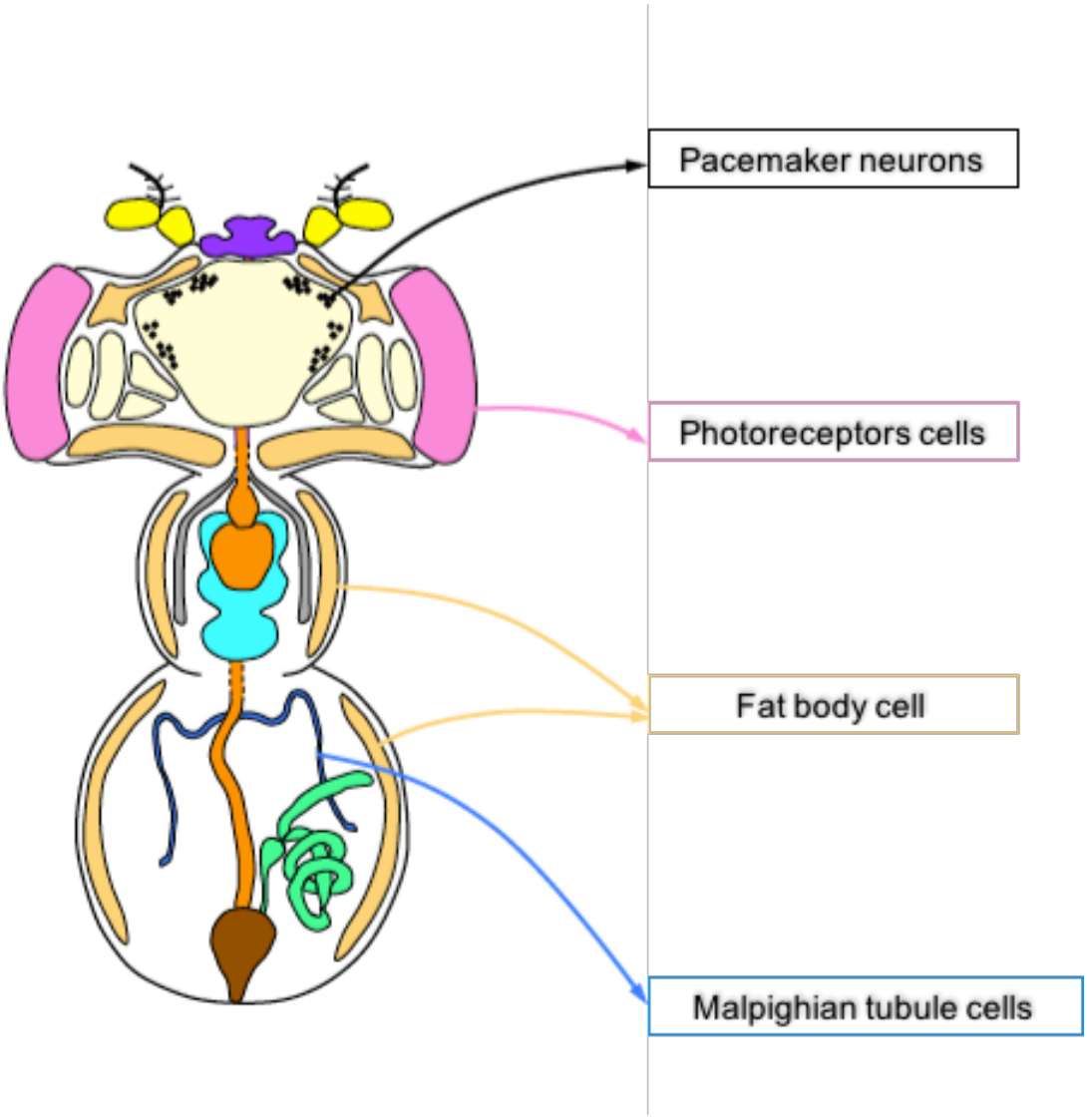

Figure 2. Clock gene-expressing tissues in an adult *Drosophila*. Yellow, antennae; cream, brain; tan, fat body; purple, proboscis; black, pacemaker neurons; pink, photoreceptors; orange, digestive tract; gray, salivary glands; aqua, ventral nerve chord; blue, Malpighian tubules; green, male reproductive tract; brown, rectum. Arrows denote regulation of clock gene and output gene expression in brain pacemaker neurons, photoreceptor cells, fat body cells and Malpighian tubules (right). Pacemaker neuron-specific factor binding site, PN-FBS; photoreceptor-specific factor binding site, PR-FBS; fat body-specific factor binding site, FB-FBS; Malpighian tubule-specific factor binding site, MT-FBS. (Adapted from Menet and Hardin, 2014)

CRY has different functions in brain pacemaker neurons and in peripheral tissues

Cryptochrome (CRY), which is an ortholog of cryptochrome in plants, acts as a cell-autonomous photoreceptor in *Drosophila* (Egan et al., 1999; Emery et al., 2000)(Figure 3). During entrainment to light-dark cycles, although TIM is degraded fast once lights are turned on, TIM does not sense the light itself. In response to short light-pulses, CRY is activated and becomes a substrate for JETLAG (JET) protein. Activated CRY binds to TIM, and JET binds to TIM protein to promote TIM degradation in the proteasome (Emery et al., 1998; Emery et al., 2000; Stanewsky et al., 1998). In the cry^{b} mutant, which is a point mutation that disrupts light sensing, the flies show no response to short light pulses, but the pacemaker can still entrain to light through other photoreceptors e.g. Hofbauer–Buchner (H–B) eyelet, and external photoreceptors in the compound eye and ocelli in the head (Stanewsky et al., 1998). However, circadian oscillators in peripheral tissues cannot entrain to light, which suggests that CRY is required for the light entrainment of peripheral tissues (Stanewsky et al., 1998). In addition, CRY works as a transcription repressor that is required for peripheral circadian clocks, while it is not a repressor in pacemaker neurons (Krishnan et al., 2001) (Ivanchenko et al., 2001) (Collins et al., 2006).

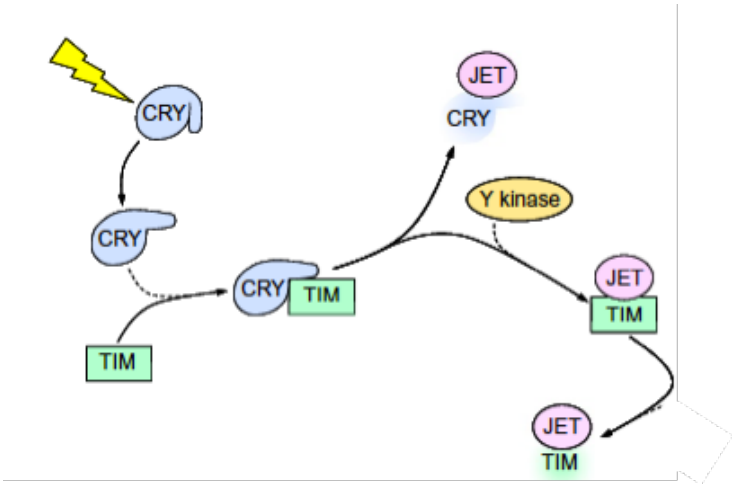

Figure 3. Light-induced phase resetting mechanism. All gene, regulatory element, and protein names are as defined in the text. Y kinase, tyrosine kinase (Adapted from Hardin 2011)

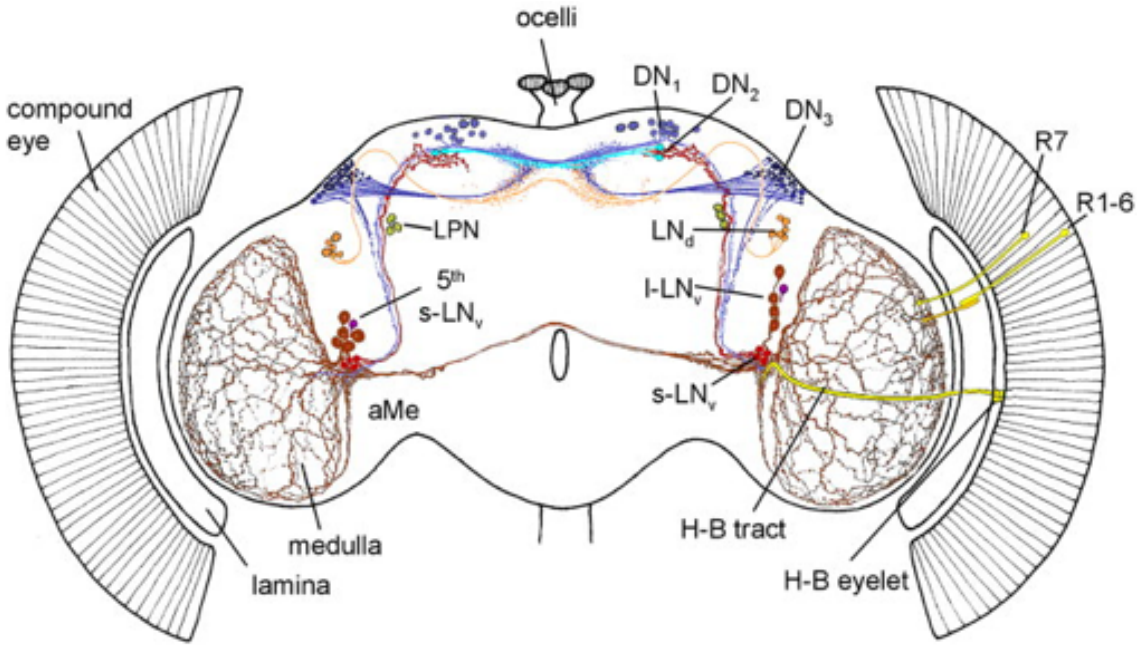

Figure 4. Pacemaker neurons communicate with neuropeptide to form a network in *Drosophila*. (Reprinted from Helfrich-Förster et al., 2007)

Pacemaker neurons in the brain control different aspects of daily behavior

In the brain, the feedback loop operates in ~150 pacemaker neurons that function to drive locomotor activity rhythms in adults. According to their location, size, and neuropeptide expression, they are divided into seven groups. The pacemaker neurons include 4 anterior dorsal neurons 1s (DN_{1a}s) and ~30 posterior dorsal neuron 1s (DN_{1p}s), 4 dorsal neuron 2s (DN_2s), and around 80 dorsal neuron 3s (DN_3s), eight pigment dispersing factor (PDF)-expressing small ventral lateral neurons (sLN_vs), two PDF-negative sLN_vs, eight large ventral lateral neurons (lLN_vs), 12 dorsal lateral neurons (LN_ds), and six lateral posterior neurons (LPNs) (Helfrich-Förster, 2005; Nitabach and Taghert, 2008) (Figure 4). The different groups of pacemaker neurons communicate using neuropeptides and form a network that determines the pattern of locomotor activity based on environmental inputs (Helfrich-Förster, 2003; Peschel and Helfrich-Förster, 2011; Taghert and Nitabach, 2012) (Kalsbeek et al., 2012). Among the pacemaker neurons, a subset of DN_1s have been shown to regulate the circadian rhythm of locomotor activity in LL conditions (Picot et al., 2007); DN_2s and LPNs are important for temperature entrainment (Kaneko et al., 2012); LN_ds and 5th LN_vs, that are categorized as evening cells, drive the evening peak in the activity rhythm (Grima et al., 2004; Stoleru et al., 2004; Yao and Shafer, 2014); PDF-positive small sLN_vs drive the morning peak in the activity rhythm and are essential for activity rhythm in constant darkness (DD) (Grima et al., 2004; Renn et al., 1999; Stoleru et al., 2004; Yao and Shafer, 2014); lLNvs play significant roles in the arousal rhythm regulation (Shang et al., 2008; Sheeba et al., 2008). The key roles in regulating different aspects of behavior

emphasize the importance of understanding the organization of the clock network in *Drosophila*. Several important questions arise: when do different clusters of pacemaker neurons develop? What are the factors that determine their fates? When does the network form? How is the function of clock initiated?

Development of pacemaker neurons in the brain

During the life cycle of *Drosophila*, embryos hatch into 1st instar larvae 24h after fertilization at 25 ºC. After 24h as 1st instar larvae (L1) and 24h as 2nd instar larvae (L2), they will spend 48 hours as 3rd instar larvae (L3) before metamorphosis. During the four days of the pupal stage, they will go through metamorphosis and emerge as adult flies (Ashburner)(Figure 5).

During development, CLK is detected in 8–10 sLN_vs, a subset of four $DN_{1a}s$, and four DN_2s several hours before PER is detected in late embryos and early first instar (L1) larvae (Helfrich-Förster et al., 2007; Houl et al., 2008). Rhythmic PER expression persists throughout larval and pupal development and in adults (Kaneko et al., 1997) (Kaneko and Hall, 2000), demonstrating that circadian clocks operate continuously in "early" pacemaker neurons after embryogenesis (Figure 5). The remaining pacemaker neurons (e.g., LN_ds, $DN_{1p}s$, LPNs, DN_3s, lLN_vs), which account for 85% of all brain pacemaker neurons, are not detected until 50–90% of pupal development (>48h after pupal formation), when they initiate rhythmic PER expression (Kaneko and Hall, 2000; Kaneko et al., 1997) (Figure 5). This result suggests that "late" pacemaker neurons do not develop until the last half of metamorphosis.

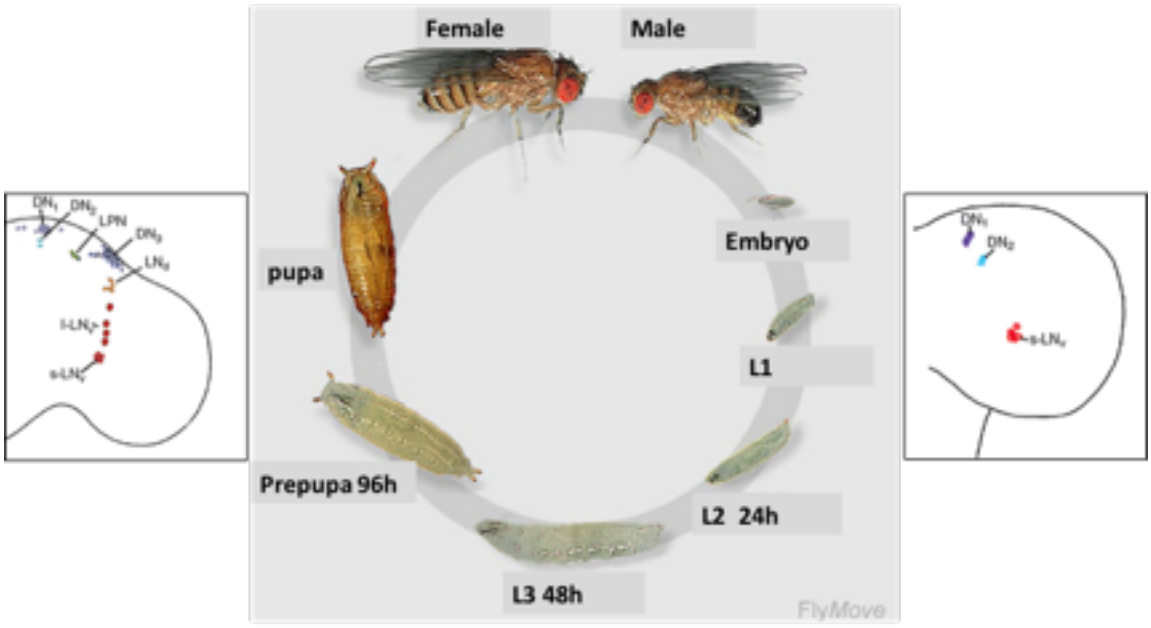

Figure 5. *per mRNA* and protein expression marks cells with functional circadian clocks. Center figure, a life cycle of *Drosophila* at 25 ºC. (Adapted from FlyMove (Weigmann et al., 2003)). Side figures, brain pacemaker neurons in each hemisphere marked by PER staining in L3 larvae and adult. (Adapted from Helfrich-Förster, 2005)

I hypothesized that late pacemaker neurons including DN_{1p}, DN_3, LN_d, lLN_vs appear at different pupal stages during development. To determine when late pacemaker neurons arise during metamorphosis, a *Clk*-GFP transgene was used because *Clk* is the first clock gene to be expressed and CLK-GFP can be detected with high sensitivity. If CLK-GFP expression faithfully marks all the groups of pacemaker neurons that express PER in the adult brain as well as rescue locomotor activity in a *Clk* null mutant, this will indicate that the CLK-GFP protein is functional and can be used for further analysis. If the transgene tool works, I would like to address the following questions: (1) Are there ~8 CLK-GFP expressing neurons by L3 larval stage that are consistent with the PER

expressing pacemaker neurons? (2) When do CLK-GFP pacemaker neurons develop during pupal development? (4) Do CLK-expressing neurons always express PER during development? (5) Does PER protein oscillate in all the CLK-expressing neurons? By tracing CLK-GFP expressing neurons during development, I hypothesized that DN_{1p}, DN_3, LPN, LN_d, lLN_vs begin to appear during the late-pupal stage. I hypothesized that CLK-GFP expression is co-localized with PER-expressing neurons. If CLK-GFP expresses earlier than PER expression during development, it will indicate that CLK-expressing neurons develop earlier than they are expected to have functional clock, and then further studies will be conducted to determine what factors are lacking in those pacemaker neurons during development, and how is circadian clock function initiated in clock neurons. I will address these important questions in Chapter II, and hope my research will help provide novel insights into the development of pacemaker neurons in *Drosophila*, as well as the clock cell developments in other organisms.

The initiation of clock function in *Drosophila*

Since CLK-CYC initiates clock function as a differentiated feature of brain pacemaker neurons that control activity rhythms (Liu et al., 2015), activation of *Clock* (*Clk)* and *cycle* (*cyc)* is thought to determine which cells and tissues will contain circadian clocks. Although a lot of studies have shown the molecular mechanism of the feedback loop, it is not known how CLK and CYC initiate the feedback loop. Circadian clocks normally operates in ~150 brain pacemaker neurons and many peripheral tissues in the head and body, but can also be induced by expressing CLK in cells that normally lack clock

function (Zhao et al., 2003). In previous studies, *Clk* is overexpressed by *cry*-Gal4 (*cry*-Gal4/ UAS-*Clk*), which is expressed in both canonical clock cells as well as non-clock cells (Zhao et al., 2003). Ectopic clocks in the brain show robust rhythms in *per* and *tim* mRNA and protein cycling in LD that dampen in DD (Kilman and Allada, 2009; Nagoshi et al., 2010). Like canonical clock cells, these ectopic clocks require *cyc* expression(Kilman and Allada, 2009; Nagoshi et al., 2010), which suggests that *cyc* is expressed in both clock cells and those ectopic locations.

The activation of *Clk* has been well documented in brain pacemaker neurons (Houl et al., 2008; Houl et al., 2006; Liu et al., 2015), but comparatively little is known about *cyc* expression. The Hardin lab recently found that a fully functional GFP-*cyc* transgene expresses GFP-CYC protein exclusively in circadian pacemaker neurons in brains of larvae and adults (Liu et al., 2015), suggesting that CYC expression is limited to clock cells. However, mRNA enrichment analysis in the *Drosophila* brain indicates that *cyc* mRNA is not enriched in the clock cells (<2-fold), while mRNA of all the known clock genes were within the top 36 clock cell enriched mRNAs (for instance, *Clk*, *per*, *tim*, *cry*) (Nagoshi et al., 2010). This result suggests that *cyc* mRNA is broadly expressed. Since *cyc* mRNA is broadly expressed, yet CYC protein is only detected in canonical clock cells (Liu et al., 2015), these observations suggest that *Clk* is required for CYC expression to initiate clock function, but how *Clk* promotes CYC accumulation and whether these clock components are sufficient to initiate clock function are not known. In Chapter III, I will focus on the mechanism underlying how CLK promotes

CYC protein expression, which will provide insights into the mechanisms of the initiation of the feedback loop.

The role of *Clk* in the development of pacemaker neurons

Since *Clk* is only expressed in post-mitotic cells, pacemaker neurons presumably exist before *Clk* is expressed (Liu et al., 2015). Important questions arise from this evidence. How early do the pacemaker neurons appear during development? What are the factors involved in clock cell determination? What is the role of the *Clk* gene in pacemaker neuron development and network formation?

Among the ~150 pacemaker neurons in a fly brain, a group of only 4-5 small ventral lateral neurons in each hemisphere are necessary and sufficient to drive locomotor activity (Renn et al., 1999). Thus it is essential to understand the development of the sLN_vs. In the *Drosophila* brain, lateral neurons are usually identified by their location as well as the neurotransmitter they produced. PDF is the main neurotransmitter in 4 sLN_vs and lLN_vs in the adult brain (Renn et al., 1999). PDF is expressed in the dorsal projection from sLN_vs and in posterior optic tract (POT) projections and medulla arborizations from lLN_vs in adults (Helfrich-Förster, 2003). Larval lateral neurons also express PDF and have a similar projection in adults, and will become adult sLN_vs during development (Helfrich-Förster, 2003) (Figure 4). Previous studies showed that PDF expression in sLN_vs was not detectable in the larval LN_v and adult sLN_v of a *Clk* mutant, while the PDF expression in lLN_vs was not affected (Park et al., 2000). An important question that arises from these results is how does the *Clk* gene regulate the development

of sLN_vs? There are two possibilities: one possibility is that *Clk* affects PDF expression or processing specifically in the sLN_v of adults and the precursors in larval brains (Park et al., 2000). The other possibility is that *Clk* is required for the development and/or maintenance of the sLN_v cells. Are the sLN_vs cells still present in the brains of *Clk* mutant flies? Is this phenomenon specific to sLN_vs? If so, in which stage does *Clk* affect the development and/or maintenance of sLN_vs? Does *Clk* effect the development of other groups of pacemaker neurons in the brain? I will address these questions in Chapter IV, and hope this research will provide new insights into the role of *Clk* in the pacemaker neuron network formation.

CHAPTER II

DEVELOPMENT OF CIRCDIAN PACEMAKER NEURONS IN THE *DROSOPHILA* BRAIN[2]

BACKGROUND

Circadian pacemaker neurons in the *Drosophila* brain control daily rhythms in locomotor activity. These pacemaker neurons can be classified as early or late depending on whether they activate rhythms in *period* (*per*) and *timeless* (*tim*) expression at the L1 larval stage or during metamorphosis, respectively (Houl et al., 2008; Kaneko et al., 1997). Previous studies on pacemaker neurons based on PER immunostaining suggests that late pacemaker neurons do not develop until the last half of metamorphosis (Kaneko et al., 1997).

Since *Clk* is the first clock gene to be expressed, a *Clk*-GFP transgene was generated to detect when late pacemaker neurons arise during metamorphosis. Since CLOCK-CYCLE (CLK-CYC) heterodimers activate *per* and *tim* transcription, I was surprised to see CLK-GFP expressed in the late pacemaker neurons including DN_{1p}, DN_3, LPN, LN_d at the L3 larval stage. CLK-GFP is only detected in post-mitotic neurons from L3 larvae, suggesting that these late pacemaker neurons are formed prior to the L3

[2] This chapter is reprinted from Liu, T., Mahesh, G., Houl, J. H., and Hardin, P. E. (2015). Circadian activators are expressed days before they initiate clock function in late pacemaker neurons from *Drosophila*. Journal of Neuroscience, 35(22), 8662-8671

larval stage. However, PER protein only accumulates in the ~8 late pacemaker neurons (DN_{1a}, DN_2, LN_v), indicating that late pacemaker neurons don't have a functional clock. Since CLK and CYC formed a heterodimer, I hypothesized that the lack of PER is due to the lack of CLK partner CYC. To test this hypothesis, a GFP-CYC transgene was generated and I found that CYC is expressed exclusively in pacemaker neurons, demonstrating that CLK-CYC is not sufficient to activate *per* and *tim* in late pacemaker neurons from L3 larvae. These results suggest that late pacemaker neurons develop days before they have functional clock, and novel factors are needed to activate circadian oscillator function during metamorphosis.

RESULTS

Essentially all brain pacemaker neurons from adults are present in L3 larvae

Given that *Clk* and *cyc* initiate circadian oscillator function (Hardin, 2011), I used CLK protein expression to mark developing brain pacemaker neurons. To detect CLK expression with high sensitivity, a ~15kb genomic DNA fragment was used to generate a transgene that expresses CLK fused to a C-terminal GFP tag (*Clk*-GFP) (see Materials and Methods). The *Clk*-GFP transgene effectively rescues locomotor activity rhythms of Clk^{out} null mutant flies with a period of ~24h in constant darkness (DD) (Figure 6), demonstrating that the CLK-GFP fusion protein supports circadian oscillator function. To determine whether CLK-GFP is expressed specifically in clock cells, brains from adults collected at ZT21 were dissected and immunostained with antibodies against GFP and the clock cell marker PER. These experiments show that CLK-GFP is expressed

exclusively in brain pacemaker neurons (Figure 7), demonstrating that CLK-GFP marks brain pacemaker neurons in adults and thus accurately reflects CLK protein expression (Houl et al., 2008).

A

Genotype	N	% Rhythmic	Period +/-SEM	Power +/-SEM
Clk^{out}	16	0	N/A	N/A
Clk-GFP; *Clk*^{out}	16	93.75 (15)	24.5 +/- 0.0	77.1 +/- 9.1
VK18	16	100	23.9 +/- 0.1	89.4 +/- 12.6

B

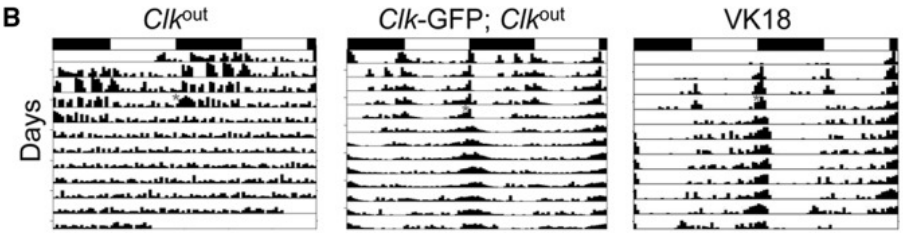

Figure 6. The *Clk*-GFP transgene rescues activity rhythms and expression in brain pacemaker neurons. A. *Clk*^{out} and *Clk*-GFP; *Clk*^{out} flies were entrained in LD cycles for three days and transferred to constant darkness. Activity in constant darkness was analyzed as described in Materials and Methods. N, number of animals tested; % Rhythmic, percentage of flies that showed significant rhythmicity; Period +/- SEM, rhythm period +/- standard error of the mean; Power +/- SEM, rhythm power +/- standard error of the mean. B. Representative actograms of flies tested in A. Flies of the indicated genotypes were entrained in LD cycles, then placed in DD for at least 7 days. The actograms show two LD cycles per line, where the second cycle on a line is the same as the first cycle on the following line. White boxes, lights-on period; black boxes, lights-off period; asterisk, time when flies were placed in DD; vertical bars, fly activity. The height of vertical bars indicates relative level of activity.

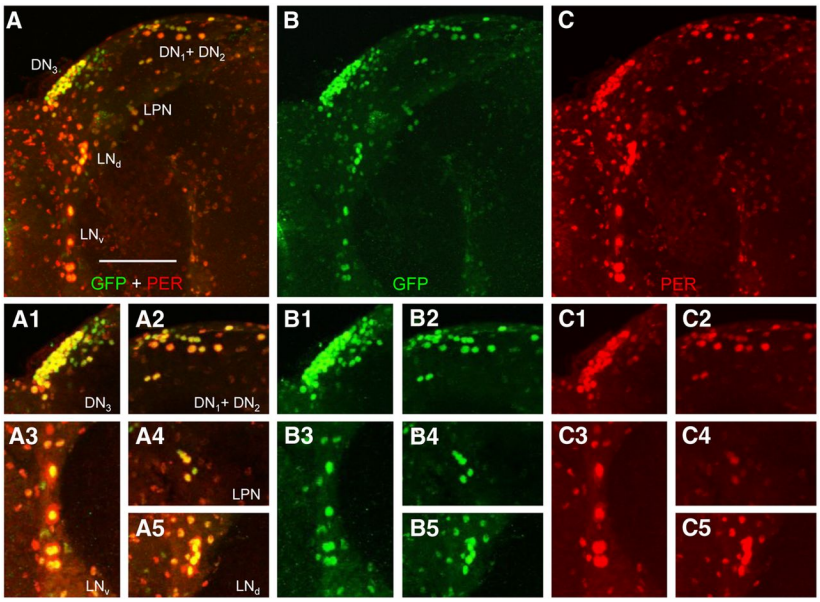

Figure 7. Spatial expression of CLK-GFP in brains from adults. Brains were dissected from *Clk*-GFP; *Clk*^{out} adults collected at ZT21, immunostained with GFP and PER antisera, and imaged by confocal microscopy. (A-C) A 94 µm projected Z-series image of the right brain hemisphere from an adult fly, where lateral is right and dorsal is top. GFP + PER (A), GFP (B) and PER (C) immunostaining is detected in DN_1s, DN_2s, DN_3s, LN_ds, LPNs, and LN_vs. Co-localization of GFP (green) and PER (red) is shown as yellow. (A1-C5) Magnified view of pacemaker neuron subgroups from panel A (A1-A5), panel B (B1-B5) and panel C (C1-C5) imaged at a higher laser intensity. (A1, B1, C1) Images of a 48 µm projected Z-series of DN_1s + DN_2s. (A2, B2, C2) Images of a 36 µm projected Z-series of DN_3s. (A3, B3, C3) Images of a 10 µm projected Z-series of LN_ds. (A4, B4, C4) Images of a 10 µm projected Z-series of LPNs. (A5, B5, C5) Images of an 18 µm projected Z-series of LN_vs. All images are representative of 12 or more brain hemispheres.

Since *Clk* is required for *per* expression (Allada et al., 1998; Darlington et al., 1998), I immunostained brains at Zeitgeber Time 21 (ZT21, where ZT0 is lights on and ZT12 is lights off in an LD cycle) from L3 larvae to confirm that CLK-GFP was expressed in ~8 PER positive pacemaker neurons per hemisphere. To my surprise, CLK-GFP was detected in ~60 brain neurons per hemisphere (Figure 8A-C). These brain pacemaker neurons encompassed almost all pacemaker neuron clusters that had previously been detected in late pupae and adults using PER as a marker (Kaneko and

Hall, 2000; Kaneko et al., 1997; Shafer et al., 2006). To confirm that the expanded pattern of CLK-GFP expression reflects that of endogenous CLK, wild-type L3 larvae were immunostained with CLK antiserum. CLK is detected in ~60 brain neurons per hemisphere that correspond spatially to the same clusters of pacemaker neurons seen with GFP-CLK (Figure 8D-F). In contrast, PER is present in only a small subset of CLK expressing pacemaker neurons in each hemisphere at ZT21 corresponding to approximately 4-5 LN_vs that show strong PER immunostaining and 2 anterior DN_{1a}s that show weak PER immunostaining (Figure 8C, F). PER is also expressed in 2 DN_2s in each hemisphere of L3 larval brains, but PER levels in DN_2s cycle in antiphase to the LN_vs and DN_{1a}s and are thus not detectable at ZT21 (Kaneko et al., 1997). These results demonstrate that L3 larvae express CLK in nearly all brain pacemaker neurons seen in adults, but that PER expression is limited to ~8 pacemaker neurons/hemisphere.

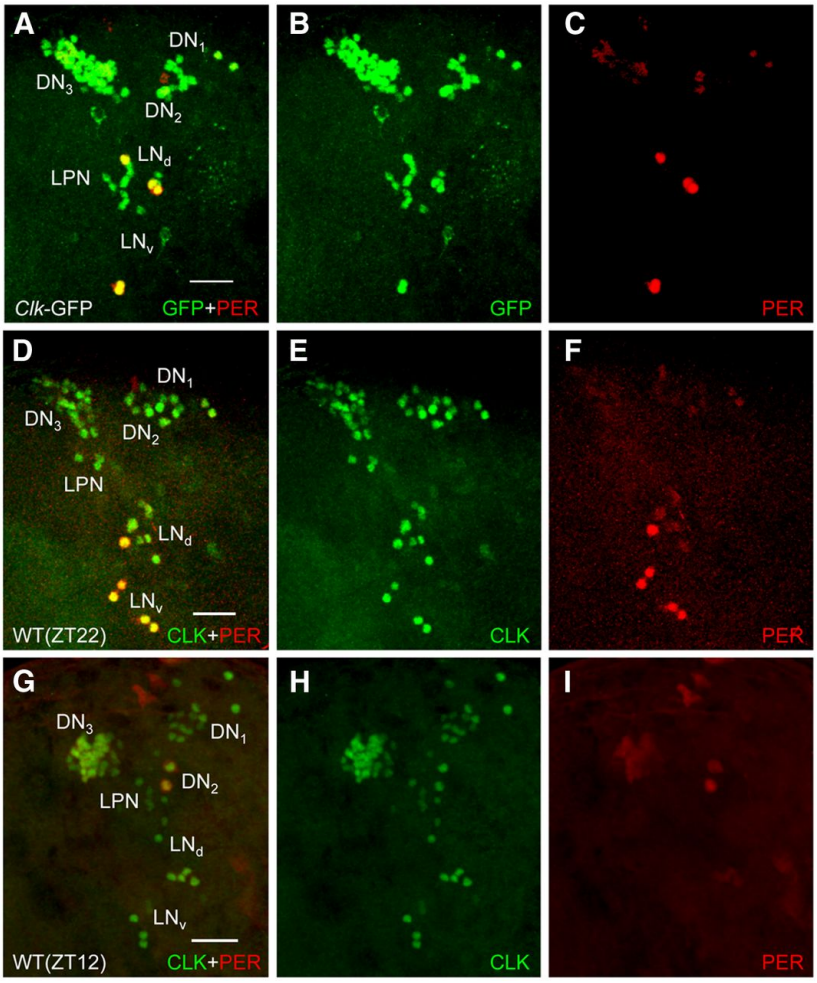

Figure 8. Spatial expression of CLK-GFP in brains from L3 larvae. CNSs were dissected from *Clk*-GFP; Clk^{out} and WT L3 larvae collected at ZT21, immunostained with GFP and PER antisera (for *Clk*-GFP; Clk^{out} larvae) or CLK (dC-17) and PER antisera (for WT larvae), and imaged by confocal microscopy. (A-C) A 52 μm projected Z-series image of the left hemisphere of a *Clk*-GFP adult fly brain, where lateral is left and dorsal is top. GFP + PER (A), GFP (B) and PER (C) immunostaining is detected in DN_1s, DN_2s, DN_3s, LPNs, LN_ds and LN_vs. Co-localization of GFP (green) and PER (red) is shown as yellow. (D-F) A 50 μm projected Z-series image of the left hemisphere of a WT adult fly brain, where lateral is left and dorsal is top. CLK + PER (A), CLK (B) and PER (C) immunostaining is detected in DN_1s, DN_2s, DN_3s, LPNs, LN_ds and LN_vs. Co-localization of CLK (green) and PER (red) is shown as yellow. All images are representative of 12 or more brain hemispheres.

GFP-CLK expression in pacemaker neurons expands in L3 larvae

Since CLK is expressed in ~60 neurons/hemisphere in L3 larvae, I wondered whether this expanded pattern of CLK expression was present at earlier larval stages. To test this possibility, dissected CNSs from L1 and L2 *Clk*-GFP larvae were immunostained with GFP antibody. In the CNS of both L1 and L2 larvae, CLK-GFP is expressed in ~8 brain neurons per hemisphere. The location of these CLK-GFP expressing neurons corresponds to sLN_vs, $DN_{1a}s$ and DN_2s (Figure 9A, B; Table 1), consistent with previous reports of PER localization in L1 and L2 larvae (Kaneko and Hall, 2000; Kaneko et al., 1997).

When *Clk*-GFP larvae are immunostained near the L2-L3 transition (48h post-hatching), additional CLK-GFP positive neurons are detected in brain regions populated by LN_ds and LPNs (Figure 9C; Table 1). Later on during the L3 stage (60h after hatching), additional CLK-GFP neurons are detected in the LN_d and DN_3 brain regions (Figure 9D; Table 1). By late L3 stage (72h post-hatching), CLK-GFP is detected in locations corresponding to all clusters of brain pacemaker neurons seen in adults except the lLN_vs (Figure 9E, F; Table 1). The absence of lLN_vs was confirmed by immunostaining with an antibody to the neuropeptide PDF, which is expressed in dorsal projections from sLN_vs and in posterior optic tract (POT) projections and medulla arborizations from lLN_vs in adults (Figure 10A-C) (Helfrich-Förster, 2003). In late L3 larvae, PDF is only detected in dorsal projections (Figure 10D-F), which argues that lLN_vs are not yet present at this time during development. Indeed, lLN_vs are not yet present at 20% pupal development (Figure 11A-C), but by 40% pupal stage CLK-GFP is

detected in four additional PDF positive neurons (Figure 11D-F), which likely correspond to lLN_vs even though they lack POT projections and medulla arborizations. By 50% pupal stage, these CLK-GFP and PDF positive lLN_vs show the characteristic POT projections and medulla arborizations (Figure 11G-I), consistent with previous results (Kaneko et al., 1997), and the intensity of PDF immunostaining increases in POT projections and medulla arborizations by the 60% pupal stage (Figure 11J-L). Although CLK-GFP is detected in all pacemaker neurons except lLN_vs in late L3 larvae, there are fewer $DN_{1p}s$ and DN_3s at this stage than in adults (Figure 9D-F; Table 1), suggesting that all the neurons in these groups are either not yet present or that CLK is expressed in a subset of these neurons at this developmental stage. Analysis of CLK-GFP expression during larval development revealed that CLK expression expands beyond PER expressing sLN_v, DN_{1a} and DN_2 neurons in L3 larvae to encompass all the clusters of brain pacemaker neurons except lLN_vs.

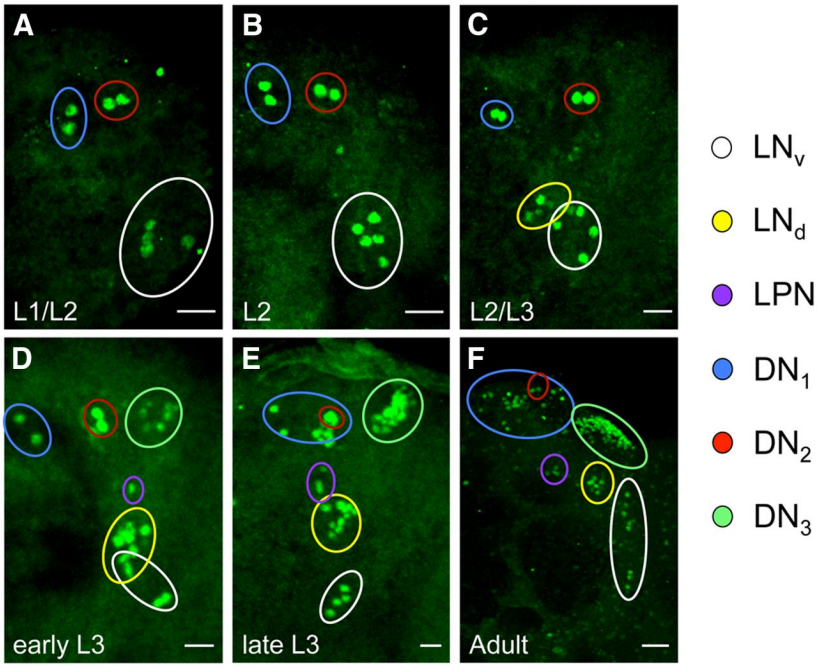

Figure 9. Expression of CLK-GFP in the brain during larval development. *Clk*-GFP; *Clk*^{out} flies were grown to the indicated stage (see Materials and Methods) and collected at ZT21. CNSs from these larvae were dissected, immunostained with GFP antibody, and imaged by confocal microscopy. (A-F) Images of 24 h L1-L2 (A), 36 h L2 (B), 48 h L2-L3 (C), 60 h early L3 (D), >72 h late L3 (E), and adult (F) brains. For each developmental stage, a 42 µm (L1-L2), 46 µm (L2), 46 µm (L2-L3), 44 µm (early L3), 50 µm (late L3) or 78 µm (adult) projected Z-series image is shown. The colored circles denote pacemaker neuron subgroups according to the key on the right. All images are representative of 12 or more brain hemispheres.

Table1. Quantifying CLK-GFP expression in brains during development

	No. stained cells						
Stage (h post-hatching)	DN1a	DN1p	DN2	DN3	LNv	LNd	LPN
L1-L2 (24h)	2.0 ± 0	0	2.0 ± 0	0	4.0 ± 0	0	0
L2 (36h)	2.0 ± 0	0	2.0 ± 0	0	4.0 ± 0	0	0
L2-L3 (48h)	2.0 ± 0	0	2.0 ± 0	0	4.3 ± 1.3	2.0 ± 1.2	1.4 ± 0.8
L3 (60h)	2.0 ± 0	0	2.0 ± 0	8.9 ± 3.8	6.1 ± 1.2	5.3 ± 2.0	1.6 ± 0.5
L3 (72h)	2.0 ± 0	6.6 ± 0.9	2.0 ± 0	23.8 ± 5.2	4.9 ± 0.8	6.6 ± 1.2	2.3 ± 1.2
Adult	2.0 ± 0	15.4 ± 1.1	1.9 ± 0.4	32.4 ± 5.9	8.5 ± 1.9	5.0 ± 0.5	3.0± 1.0

Table 1. Quantifying CLK-GFP expression in brains during development. Developing or adult flies carrying the CLK-GFP transgene were used, as procedure described in Materials and Methods. The "No. stained cells" in each entry are quoted ± SEM. The different life cycle stages are indicated by L1-L2 (24 hrs after embryonic hatching); mid-L2 (36 hrs after embryonic hatching); L2-L3 (48 hrs after embryonic hatching); mid-L3 (60 hrs after embryonic hatching); Late L3 (>72 hrs after embryonic hatching). Data are based on 7-8 hemispheres from 5 brains at each stage.

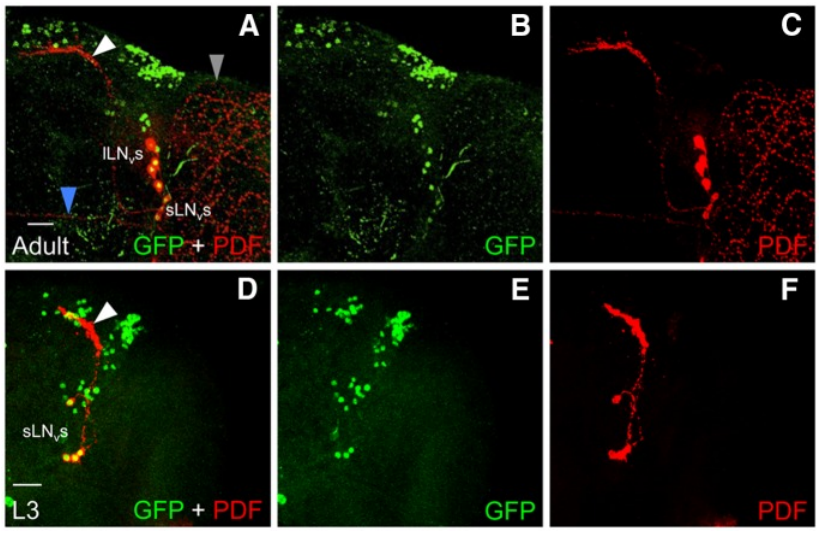

Figure 10. PDF expression in LN_vs from L3 larvae and adults. *Clk*-GFP; *Clk*^{out} L3 larvae and adults were collected at ZT2. CNSs from larvae and brains from adults were dissected, immunostained with GFP and PDF antibodies, and imaged by confocal microscopy. Co-localization of GFP (green) and PDF (red) is shown as yellow. A-C, an 88 µm projected Z-series image of the right hemisphere of a *Clk*-GFP adult fly brain, where lateral is right and dorsal is top. D-F, a 60 µm projected Z-series image of the right hemisphere of a *Clk*-GFP L3 larval fly brain, where lateral is right and dorsal is top. sLN_v and lLN_v denote the position of LNv cell bodies. White arrowhead, sLNv dorsal projection; blue arrowhead, lLNv posterior optic tract projection; gray arrowhead, lLNv medulla arborizations. All images are representative of 12 or more brain hemispheres.

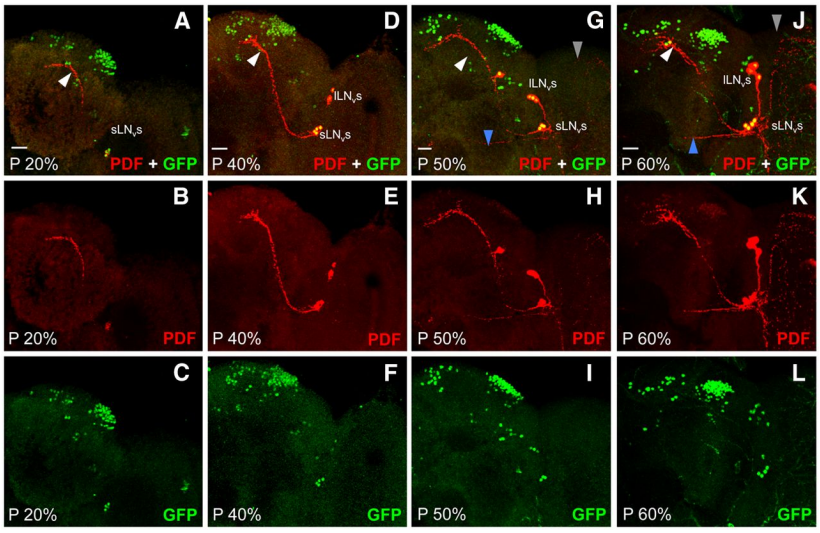

Figure 11. PDF expression in LN_vs from pupae. *Clk*-GFP; *Clk*^{out} pupae at the indicated developmental stages were collected at ZT2. Brains from pupae were dissected, immunostained with GFP and PDF antibodies, and imaged by confocal microscopy. (A-C) A 66 µm projected Z-series image of GFP + PDF (A), PDF (B) and GFP (C) immunostaining. (D-F) A 76 µm projected Z-series image of GFP + PDF (D), PDF (E) and GFP (F) immunostaining. (G-I) A 56 µm projected Z-series image of GFP + PDF (G), PDF (H) and GFP (I) immunostaining. (J-L) A 76 µm projected Z-series image of GFP + PDF (J), PDF (K) and GFP (L) immunostaining. A right hemisphere is shown in each image, where lateral is right and dorsal is top. sLN_v and lLN_v denote the position of LNv cell bodies. Co-localization of GFP (green) and PDF (red) is shown as yellow. White arrowhead, sLNv dorsal projection; blue arrowhead, lLNv posterior optic tract projection; gray arrowhead, lLNv medulla arborizations. All images are representative of 12 or more brain hemispheres.

CLK-GFP is expressed exclusively in post-mitotic neurons of L3 larvae

By the L3 larval stage early pacemaker neurons already extend projections and express the post-mitotic neuronal marker Embryonic Lethal Abnormal Visual system (ELAV)(Kaneko and Hall, 2000; Kaneko et al., 1997; Robinow and White, 1991), thus I reasoned that *Clk*-GFP is activated in preexisting groups of cells in L3 larvae that are fated to become late pacemaker neurons. To determine if this is the case, early and late L3 larvae were immunostained with GFP and ELAV antibodies. In each case, I find that CLK-GFP is only detected in cells that express ELAV, including early brain pacemaker

neurons (Figure 12) and newly emerging DN_{1p}, LN_d, DN_3 and LPN brain pacemaker neurons in L3 larval brains (Figure 12). From these results, I conclude that CLK-GFP is expressed in post-mitotic neurons, which suggests that DN_{1p}s, LN_ds, DN_3s and LPNs derive from presumptive pacemaker neurons already present in L3 larvae.

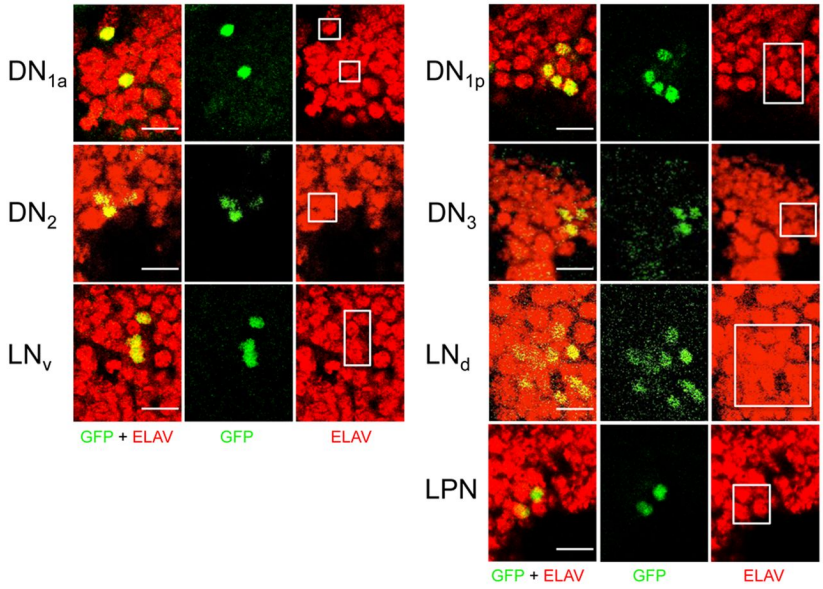

Figure 12. CLK-GFP is expressed in post-mitotic neurons from L3 larvae. *Clk*-GFP; *Clk*^{out} L3 larvae were collected at ZT21. CNSs from larvae were dissected, immunostained with GFP and ELAV antibodies, and imaged by confocal microscopy. GFP + ELAV, GFP, and ELAV immunostaining is shown for the indicated groups of pacemaker neurons. For each group of pacemaker neurons, a 2 µm (DN_2, LN_v, DN_{1p}, DN3), 4 µm (DN_{1a}), or 6 µm (LN_d, LPN) projected Z-series image is shown. Co-localization of GFP (green) and ELAV (red) is shown as yellow. The white boxes indicate the region containing ELAV positive cells that co-immunostained with GFP. All images are representative of 12 or more brain hemispheres.

CYC-GFP is expressed in all CLK-expressing pacemaker neurons in L3 larvae

The lack of PER expression in late brain pacemaker neurons from L3 larvae was unexpected because CLK initiates circadian oscillator function by activating the

feedback loop repressors *per* and *tim* even when expressed ectopically (Kilman and Allada, 2009; Zhao et al., 2003). Since CLK requires CYC to initiate oscillator function (Allada et al., 1998; Darlington et al., 1998; Rutila et al., 1998), the lack of PER expression in late brain pacemaker neurons from L3 larvae could be due to the absence of CYC expression. Unfortunately, antibodies capable of detecting CYC in tissue are not available, so a transgene (GFP-*cyc*) that expresses CYC fused to a N-terminal GFP tag was constructed to determine CYC spatial expression (see Materials and Methods). The GFP-*cyc* transgene effectively rescues locomotor activity rhythms in cyc^{01} null mutants with a long (~26h) period in DD (Figure 13). These results show that the GFP-CYC fusion protein is functional in the context of the circadian oscillator.

Since *cyc* is required for *Clk* to generate ectopic clocks in a wide array of brain neurons (Kilman and Allada, 2009), I expected GFP-CYC to be widely expressed in the adult brain. However, my results show that GFP-CYC is expressed exclusively in brain pacemaker neurons that co-express CLK (Figure 14A-C). This result demonstrates that like the other core clock components (e.g. CLK, PER and TIM), CYC is expressed exclusively in circadian oscillator cells, at least in brains from adults. In L3 larval brains, CYC is also expressed specifically in CLK-expressing neurons (Figure 14D-F), indicating that the lack of oscillator function in all but the early pacemaker neuron subset of the ~130 pacemaker neurons in L3 larval brains is not due to the absence of CYC expression. The lack of circadian oscillator function in late pacemaker neurons from L3 larvae may result from the inability of CLK-CYC to activate *per* and *tim* transcription. A previous study tested whether *per* is transcribed in L3 larvae using a *per* circadian

regulatory sequence (CRS) enhancer driven *lacZ* reporter gene, which is activated by CLK-CYC and faithfully reports *per* spatial expression in adult brains (Darlington et al., 1998; Hao et al., 1999). This work found that ß-galactosidase is only detected in early pacemaker neurons from L3 larvae (Hao et al., 1999), indicating that *per* is not transcribed in late pacemaker neurons at the L3 larval stage. The CYC-GFP and *per* CRS-*lacZ* reporter results indicate that CLK and CYC are not sufficient to initiate oscillator function in late pacemaker neurons, which implies that other factors are required to activate *per* and *tim* transcription, and thus oscillator function, in these neurons during metamorphosis.

A

Genotype	N	% Rhythmic	Period +/-SEM	Power +/-SEM
cyc^{01}	16	0	N/A	N/A
GFP-*cyc*; cyc^{01}	32	84.3 (27)	25.9 +/- 0.09	71.7 +/- 6.8
attP40	15	73.3 (11)	23.6 +/- 0.1	35.9 +/- 4.6

B

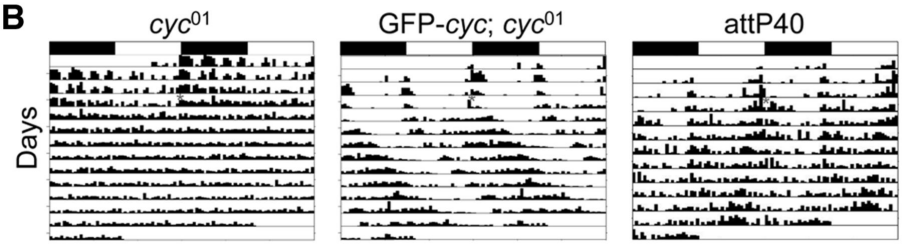

Figure 13. The GFP-*cyc* transgene rescues activity rhythms and expression in brain pacemaker neurons. A. cyc^{01} and GFP-*cyc*; cyc^{01} flies were entrained in LD cycles for three days and transferred to constant darkness. Activity in constant darkness was analyzed as described in Materials and Methods. N, number of animals tested; % Rhythmic, percentage of flies that showed significant rhythmicity; Period +/- SEM, rhythm period +/- standard error of the mean; Power +/- SEM, rhythm power +/- standard error of the mean. B. Representative actograms of flies tested in A. Flies of the indicated genotypes were entrained in LD cycles, then placed in DD for at least 7 days. The actograms show two LD cycles per line, where the second cycle on a line is the same as the first cycle on the following line. White boxes, lights-on period; black boxes, lights-off period; asterisk, time when flies were placed in DD; vertical bars, fly activity. The height of vertical bars indicates relative level of activity.

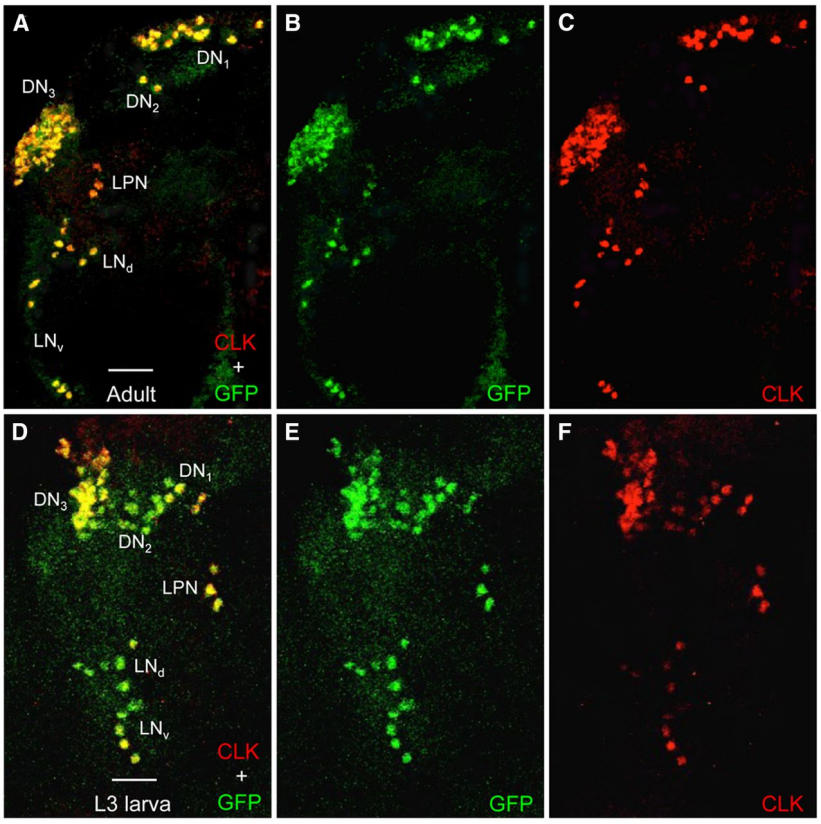

Figure 14. GFP-CYC expression is restricted to CLK expressing neurons in adult and L3 larval brains. Brains were dissected from GFP-*cyc*; cyc^{01} L3 larvae and adults collected at ZT21, immunostained with GFP and CLK antisera, and imaged by confocal microscopy. Co-localization of GFP (green) and CLK (red) is shown as yellow. (A-C) A 75 µm projected Z-series image of the left hemisphere of a GFP-*cyc*; cyc^{01} adult fly brain, where lateral is left and dorsal is top. GFP + CLK (A), GFP (B) and CLK (C) immunostaining is detected in the indicated groups of pacemaker neurons. (D-F) A 60 µm projected Z-series image of the left hemisphere of a GFP-*cyc*; cyc^{01} L3 larval brain, where lateral is left and dorsal is top. GFP + CLK (D), GFP (E) and CLK (F) immunostaining is detected in the indicated groups of pacemaker neurons. All images are representative of 12 or more brain hemispheres.

CONCLUSIONS

In this study I show that the late developing DN_{1p}, LN_d, DN_3 and LPN pacemaker neurons, which were previously detected only during the second half of metamorphosis and in adults, are present >2 days earlier at the L3 larval stage (Figure 8). The presence

of late pacemaker neurons in L3 larvae was first detected in transgenic flies that express CLK-GFP, and confirmed in wild-type L3 larval brains immunostained with CLK antibody (Figure 8). One notable absence among the late pacemaker neurons in L3 larvae were the lLN_vs, which do not appear until the last half of metamorphosis regardless of the clock neuron marker used (Figure 10, 11). Unlike the early sLN_v, DN_{1a} and DN_2 pacemaker neurons, the late pacemaker neurons do not express PER at the L3 larval stage, indicating that the circadian oscillator in these pacemaker neurons is not functional (Figure 8). The lack of PER expression in late pacemaker neurons from L3 larvae is not due to the absence of CYC, which is expressed in all CLK positive neurons in L3 larvae (Figure 14). These results suggest that CLK and CYC positive late pacemaker neurons in L3 larvae remain in a "primed" state until the last half of metamorphosis, when other factors apparently activate oscillator function.

I was surprised to see that CLK-CYC expressing neurons lacked circadian oscillator function given that ectopic expression of CLK is capable of initiating oscillator function in many adult tissues (Figure 14) (Kilman and Allada, 2009; Zhao et al., 2003). The ability of *Clk* to produce ectopic oscillators is dependent on *cyc* (Kilman and Allada, 2009), thus I predicted that *cyc* is broadly expressed. However, I found that CYC-GFP is expressed exclusively in CLK positive brain neurons at the L3 larval and adult stages (Figure 14), which suggests that CLK somehow promotes CYC expression.

Brain pacemaker neurons are first detected at three different stages of *Drosophila* development. The sLN_v, DN_{1a} and DN_2 early pacemaker neurons are detected in late embryos (Houl et al., 2008), and are present in all subsequent developmental stages and

in adults (Helfrich-Förster, 1997; Kaneko et al., 1997). The sLN_v cluster of pacemaker neurons extend projections and express PDF as early as the L1 larval stage (Helfrich-Förster, 1997; Kaneko and Hall, 2000; Kaneko et al., 1997), indicating that these cells are fully differentiated. The DN_{1a}s and DN_2s extend projections by the L3 larval stage (Kaneko and Hall, 2000), but when these projections are first formed is not known. I show that CLK is co-expressed with the neuronal differentiation marker ELAV in early pacemaker neurons from L3 larvae (Figure 12), strongly arguing that these pacemaker neurons do not give rise to late pacemaker neurons. CLK is also detected in ELAV positive late pacemaker neurons from L3 larvae (Figure 12), which shows that these neurons are already differentiated by the time CLK is expressed. This result implies that the circadian pacemaker neuron network is established well before CLK is expressed in late pacemaker neurons.

METHODS

Fly strains

The following *Drosophila* strains were used in this study: w^{1118}*, w; Cyo/Sco;* TM2/TM6B, cyc^{01} (Rutila et al., 1998), and Clk^{out} (Mahesh et al., 2014).

Generating the *Clk*-GFP and GFP-*cyc* transgenes

To detect CLK with high sensitivity, an *attB*-P[acman]-*Clk*V5-EGFP (*Clk*-GFP) transgene that expresses a CLK-GFP fusion protein was generated by a post-doctoral fellow in the Hardin lab, Dr. Guruswamy Mahesh. To accomplish this, GFP was fused in

frame to the C- terminal V5 epitope tag of CLK-V5 within the *attB*-P[acman]-*Clk*V5 plasmid (Mahesh et al., 2014). To introduce GFP, a 3' genomic fragment of *Clk*V5 (from 351bp upstream to 1580bp downstream of the translation stop) was first cloned into the TA vector (Invitrogen) using SphI and NotI to form *Clk*V5-TA. An FseI site was introduced before the *Clk*V5 stop codon to form *Clk*V5Fse-TA using the Quickchange site directed mutagenesis kit (Stratagene). GFP coding sequence was amplified from PL-452-C-EGFP vector (Addgene) using primers containing FseI sites and inserted into the TA vector (Invitrogen). The GFP fragment was excised using FseI and inserted into *Clk*V5Fse-TA at the FseI site to form *Clk*V5GFP-TA. The 3' *Clk* SphI-NotI genomic fragment in *attB*-P[acman]-*Clk-V5* was swapped with the *Clk* SphI-NotI genomic fragment from *Clk*V5GFP-TA to form *attB*-P[acman]-*Clk*V5-GFP. This transgene, which I refer to as *Clk*-GFP*,* was inserted into the VK00018 attP site on chromosome 2 via PhiC31-mediated transgenesis (Groth et al., 2004; Venken et al., 2006). The *Clk*-GFP transgene was moved into Clk^{out} for behavioral analysis and immunostaining.

An N-terminal eGFP tagged *cycle* (*cyc*) transgene (GFP-*cyc*) was generated by a post-doctoral fellow in the Hardin lab, Dr. Jerry Houl. The transgene was constructed via recombineering (Venken et al., 2006). Phusion DNA polymerase (New England BioLabs, Inc.) was used to amplify the eGFP-LoxP-kanamycin cassette from plasmid PL-452 N-eGFP (Addgene) using primer *cyc*-L (5'-gtcggaaagggcttaatttttcataagcaaacgtcaccgattggcgATGGTGAGCAAGGGCGAGGAGCTG-3'), which contains 45 nucleotides of *cyc* sequence upstream of the translation start (lower case) and the first 24 nucleotides of the GFP translated sequence (upper case),

and *cyc*-R (5'-gtaatttgcaatgcacttttccagtgaaactcaccagaaactcctgaacttcACTAGTGGATCCCCTCGAGGGAC-3'), which contains 52 nucleotides from *cyc* exon 1 (lower case) and 23 nucleotides from the 3' end of the eGFP cassette (upper case). This fragment was used to transform SW102 cells harboring the BAC clone C322-08N23 (BAC-PAC Resources Center), which contains the *cyc* genomic region, and recombinants containing the eGFP-LoxP-kanamycin cassette inserted into *cyc* were selected on plates containing kanamycin. The kanamycin gene was removed by inducing recombination at the LoxP sites (Venken et al., 2009; Venken et al., 2008), resulting in the chloramphenicol-resistant eGFP-*cyc* p(ACMAN) clone. eGFP-*cyc* was amplified in EPI 300 cells (Epicentre), and sequenced to confirm the N-terminal GFP-CYC fusion. The eGFP-*cyc* transgene was inserted into attP40 on chromosome 2 via PhiC31-mediated transgenesis (Groth et al., 2004). The eGFP-*cyc* transgene was moved into cyc^{01} for behavioral analysis and immunostaining.

Staging of larvae and pupae

Clk-GFP; Clk^{out} and eGFP-*cyc*; cyc^{01} flies were maintained in light 12h: dark 12h (LD) for three cycles at 25°C. Larvae and pupae were staged as previously described(Helfrich‐Förster, 1997; Kaneko et al., 1997). Larvae were collected at the following times: L1-L2, 24 h after hatching; L2, 36h after hatching; L2-L3, 48h after hatching; early L3, 60h after hatching; late L3, 72h after hatching. P20%, P40%, 50%, 60% of pupal (P) development: P20%, 24h after pupa formation; P40%, 48h after pupa formation; P50%, 60h after pupa formation; P60%, 72h after pupa formation.

Immunostaining larval CNSs and adult brains

Antibody staining of adult brain and larval CNSs was carried out as previously described (Houl et al., 2008). Larvae were staged and collected as above. Larval CNSs and adult brains were dissected and fixed with 3.7% formaldehyde. Samples were washed and incubated with primary and secondary antibodies in a solution containing 1X PBS, 5% BSA, 3% Goat Serum (Sigma) (3% Donkey serum for primary antibodies raised in goat), 0.03% sodium deoxycholate, 0.03% TritonX100. The following primary antibodies were used: Guinea pig anti-CLK GP50 1:3,000, Goat anti-CLK dC-17 (Santa Cruz Biotechnology, Inc) 1:100, rabbit anti-GFP ab6556 (Abcam) 1:500, rabbit anti-GFP ab290 (Abcam) 1:2000, mouse anti-ELAV (DSHB) 1:100, pre-absorbed rabbit anti-PER (gift from Michael Rosbash, Brandeis University) 1: 15,000, rat anti-PER (gift from Orie Shafer, University of Michigan) 1:500, and mouse anti-PDF (DSHB) 1:500. The following secondary antibodies were used: goat anti-rabbit Alexa 647 (Molecular

Probes), donkey anti-rabbit Alexa 647 (Molecular Probes), goat anti-guinea pig Cy-3 (Jackson ImmunoResearch Laboratories, Inc.), goat anti-mouse Cy-3 (Jackson ImmunoResearch Laboratories, Inc.), goat anti-rat Cy-5 (Jackson ImmunoResearch Laboratories, Inc.), donkey anti-rat Cy-5 (Jackson ImmunoResearch Laboratories, Inc.), goat anti-mouse Alex488 (Molecular Probes), and donkey anti-goat Alex488 (Molecular Probes).

Confocal microscopy

Confocal stacks were imaged using an Olympus FV1000 confocal microscope equipped with 20 X /0.85 NA and 100 X 1.40 NA oil-immersion objectives. For double-labeling experiments, sequential scans of the argon ion 488 nm and HeNe (543 nm for Cy3, 633 nm for Alexa Fluor 647 and Cy5) lasers were used to avoid bleed-through between channels. For imaging Alexa Fluor 488 and Cy3, 488 and 543 nm lasers were used, with the 405/488/543nmdichroic mirror for excitation. Fluorescence signals were separated by a beam splitter (560 nm long pass) and recorded on spectral detectors set to 500–530 and 555–655 nm for Alexa Fluor 488 and Cy3, respectively. For imaging Alexa Fluor 488 and either Alexa Fluor 647 or Cy5, Argon 488 and HeNe 633 nm lasers were used, with the 488/543/633 nm dichroic mirror for excitation. Fluorescence signals were separated by a dichroic beam splitter (560 nm long-pass). A spectral detector set to 500–555 nm was used for Alexa Fluor 488 and a detector with 650 nm long-pass filter was used for Alexa Fluor 647 or Cy5 signals. The Fluoview “Hi-Lo” look-up table was used to set the maximal signal below saturation and set the background to near zero using the

high voltage and offset controls. Z-series were obtained at 2 μm step size, and Kalman-averaging was not used. Original Olympus images were saved as 12-bit oib format and processed using FV1000 confocal software to generate maximum intensity projections (Z-projections). Images were adjusted for brightness and contrast using Adobe Photoshop. For each genotype and developmental stage, brain images were acquired using the same settings (power, gain, offset) at the
same time.

Assignment of pacemaker neuron subgroups

Projected Z-series of CLK-GFP, CLK, PER, and GFP-CYC immunostaining were generated along the anterior–posterior (A–P) axis for dissected larval and adult brains. In larvae, DN_3s and DN_1 as could be readily distinguished from other pace pacemaker neurons by their locations along the dorsal–ventral (D–V) and lateral– contralateral (L–C) axis, whereas scans through the A–P axis were necessary to distinguish the more posterior DN_2s from DN1ps, the more posterior LPNs from LN_ds, and the more posterior LN_ds from LN_vs. Brains were only used for
pacemaker neuron quantification if all subgroups could unambiguously be assigned. In adults, pacemaker neuron subgroups were assigned as described for larvae, except that there was no overlap between the more ventral sLN_vs and LN_ds. The lLN_vs often intermingled with LN_ds, but could typically be distinguished from LN_ds because they have larger cell bodies. When lLN_vs and sLN_vs were not marked with PDF, there was some ambiguity in discriminating between lLN_vs and LN_ds.

Circadian locomotor activity monitoring

Two to three day old male flies were entrained for three days in LD and transferred to constant darkness (DD) for 7 days at 25 °C. Locomotor activity was monitored using the *Drosophila* Activity Monitor (DAM) system (Trikinetics). Locomotor activity during DD was analyzed using ClockLab (Actimetrics) software as described (Pfeiffenberger et al., 2010).

CHAPTER III

DETERMINING HOW CLOCK PROMOTES CYCLE EXPRESSION TO INTIATE CIRCADIAN CLOCK FUNCTION[3]

BACKGROUND

The *Drosophila* circadian clock keeps time via transcriptional feedback loops. These feedback loops are initiated by CLOCK-CYCLE (CLK-CYC) heterodimers, which activate transcription of genes encoding the feedback repressors PERIOD (PER) and TIMELESS (TIM). Circadian clocks normally operate in ~150 brain pacemaker neurons and many peripheral tissues in the head and body, but can also be induced by expressing CLK in non-clock cells (Kilman and Allada, 2009; Zhao et al., 2003). These ectopic clocks also require *cyc* (Kilman and Allada, 2009), yet CYC expression is restricted to canonical clock cells despite evidence that *cyc* mRNA is widely expressed(Liu et al., 2015; Nagoshi et al., 2010).

I hypothesized that CLK promotes CYC protein expression to initiate circadian clock function. Since CLK and CYC are heterodimers, I hypothesized that CLK binding to CYC stabilizes CYC protein, which helps CYC protein accumulation. Here I found that CLK stabilizes CYC protein in cultured *Drosophila* Schneider 2 (S2) cells. Likewise, CYC accumulates specifically in ectopic cells expressing *Clk*, indicating that CLK also stabilizes CYC *in vivo*. CLK and CYC are, however, not sufficient for clock

[3] This chapter has been submitted to PNAS and under review as Liu, T., Mahesh, G., Yu, W., and Hardin, P. E. (2017). CLOCK stabilizes CYCLE to initiate clock function in *Drosophila*

function in ectopic cells; *cry* is also required to entrain and/or maintain these clocks. This work reveals genes that are required to initiate circadian clock function, defines mechanisms underlying the accumulation of activator complexes that appear to be a conserved feature of eukaryotic clocks, and suggests that *Clk*, *cyc* and *cry* expression are sufficient to program clock function in naïve *Drosophila* cells.

RESULTS

CYC protein is stabilized by CLK

Previous work showing that *cyc* mRNA is not enriched in pacemaker neurons suggests that *cyc* is also expressed in non-clock cells (Nagoshi et al., 2010). Broad *cyc* expression is consistent with the ability of *Clk* to generate clocks in non-clock brain neurons (Kilman and Allada, 2009; Zhao et al., 2003), but contrasts with the pacemaker neuron-specific accumulation of GFP-CYC in the brain (Liu et al., 2015). To reconcile these data, I propose that *cyc* mRNA is broadly expressed, but that CYC only accumulates in cells that also express *Clk*. If CYC accumulation is dependent on *Clk*, then loss of *Clk* in clock neurons should also eliminate CYC. Indeed, GFP-CYC was not detectable in pacemaker neurons from Clk^{out} flies (Figure 15A-B), which lack CLK (Mahesh et al., 2014). To determine if *Clk* is required for CYC accumulation in whole heads, where most clock gene expression emanates from retinal photoreceptors (Glossop and Hardin, 2002), I employed a *cyc*-FLAG transgene that fully rescues clock function (Abruzzi et al., 2011). The levels of CYC-FLAG in Clk^{out} fly heads was reduced >10-fold compared

to controls bearing intact clocks (Figure 15C-D). These results show that *Clk* controls CYC protein accumulation.

The reduced levels of CYC in Clk^{out} flies could result from decreased synthesis or decreased stability. Although there is evidence that transcription factors such as BMAL1 and HIF2α act in the cytoplasm to generally enhance translation (Lipton et al., 2015; Uniacke et al., 2012), I favor the possibility that CYC is stabilized as a product of CLK-CYC heterodimer formation, which is known to stabilize other heterodimeric transcription factors (Cheng et al., 2002; Hattori et al., 2003). To test whether CLK stabilizes CYC, I first determined the half-life of FLAG-tagged CYC protein in *Drosophila* Schneider 2 (S2) cells. S2 cells were transfected with pMK33-*cyc*-FLAG plasmid, CYC-FLAG expression was induced, translation was inhibited using cycloheximide (CHX), and samples were collected as described (see Materials and Methods). The levels of CYC-FLAG declined rapidly after CHX addition, with a half-life of ~1h (Figure 16A, D). To identify the pathway that mediates CYC degradation, I measured CYC-FLAG half-life after treatment with the 26S proteasome inhibitor MG132. CYC-FLAG levels were not detectably reduced in the presence of MG132, indicating that CYC is degraded by proteasome (Figure 16B, D). To determine the impact of CLK on CYC protein stability, I measured CYC levels in presence of V5-tagged CLK. CYC-FLAG was stabilized in presence of CLK-V5 with a half-life ~9h, demonstrating that CLK stabilizes CYC (Figure 16C, D). When CYC-FLAG and CLK-V5 were co-expressed in S2 cells, CYC-FLAG was immunoprecipitated by CLK-V5, demonstrating that CLK and CYC are in the same complex (Figure 16E). These results

show that CLK stabilizes CYC by forming a complex that protects CYC from proteasomal degradation.

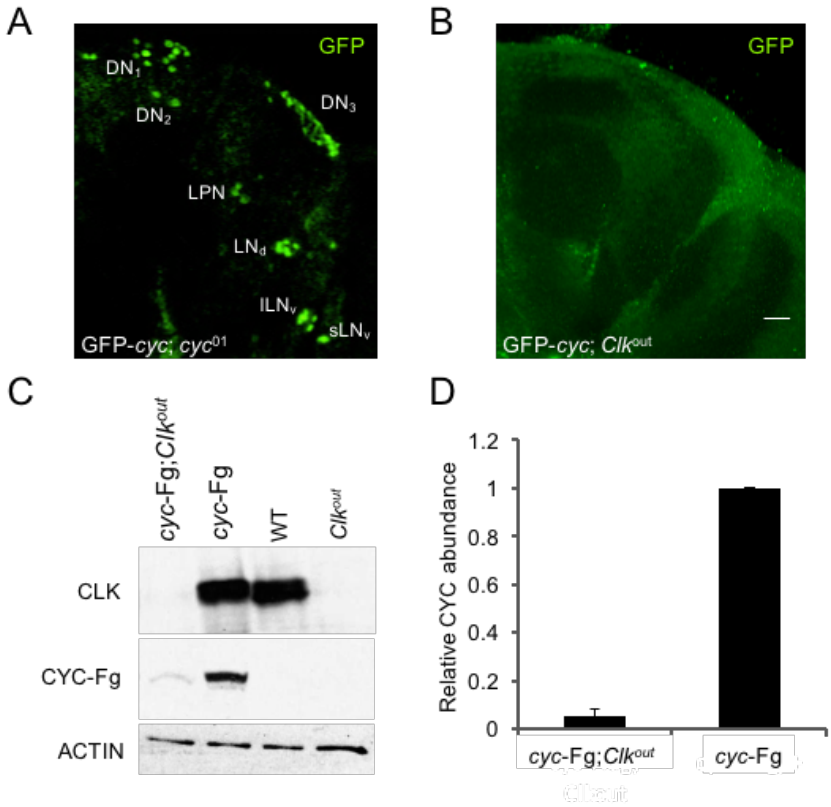

Figure 15. CYC protein is expressed at low-levels in *Clk*^{out} flies. A, B. GFP-CYC expression in brain pacemaker neurons was assessed in GFP-*cyc*; *cyc*^{01} and GFP-*cyc*; *Clk*^{out} flies that were entrained in LD for at least three days and collected at ZT2. Immunostaining was performed on dissected brains using anti-GFP antibody and imaged by confocal microscopy as described (see Materials and Methods). A. 84µm projected Z-series image of a right brain hemisphere from a GFP-*cyc*; *cyc*^{01} fly. B. 104µm projected Z-series image of a right brain hemisphere from a GFP-*cyc*; *Clk*^{out} fly. Brains are oriented where lateral is to the right and dorsal is at the top. DN_1, DN_2, DN_3, LPN, LN_d, lLN_v and sLN_v refer to pacemaker neuron groups as defined in the text. Scale bar, 10µm. All images are representative of 6 or more brains. C, D. CYC-FLAG expression in heads from wild-type (WT), *cyc*-FLAG (*cyc*-Fg), *cyc*-FLAG; *Clk*^{out} (*cyc*-Fg; *Clk*^{out}) and *Clk*^{out} flies collected at ZT14 were assessed on western blots. C. Western blot probed with CLK, FLAG and β−ACTIN antibodies to measure the levels of CLK, CYC-FLAG (CYC-Fg) and β−ACTIN (ACTIN). β−ACTIN was used as a loading control. D. Relative levels of CYC-FLAG were determined by measuring band intensities using Image J software (see Materials and Methods). Values represent mean ± SEM from three independent experiments.

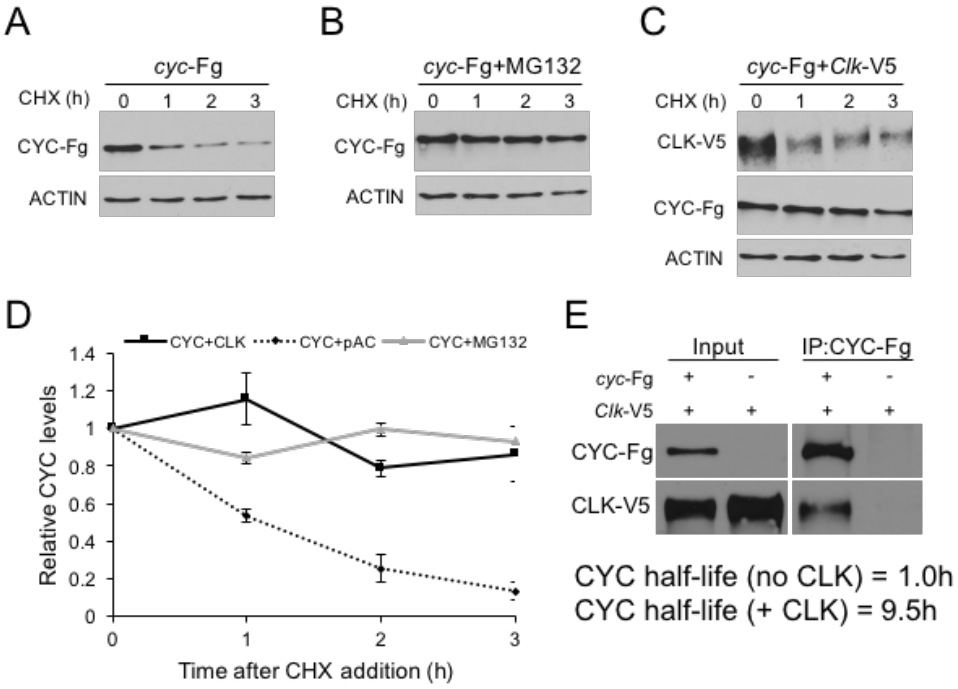

Figure 16. CYC protein is stabilized when co-expressed with CLK. S2 cells transfected with pMK-*cyc*-FLAG (*cyc*-Fg) plasmid alone or in combination with pAct-*Clk*-V5 (*Clk*-V5) plasmid were incubated with $CuSO_4$ for 1h to induce *cyc*-Fg expression, then treated with cycloheximide (CHX) to inhibit translation. (A) S2 cells co-transfected with *cyc*-Fg. (B) S2 cells transfected with *cyc*-Fg and treated with MG132 at 0h. (C) S2 cells co-transfected with *cyc*-Fg and *Clk*-V5. Proteins were extracted from cells harvested at the indicated times after CHX addition and used to prepare western blots that were probed with anti-FLAG, anti-CLK, and anti-β–ACTIN antibodies. (D) Relative CYC-Fg levels were quantified using Image J software as described (see Materials and Methods) and plotted as the mean value ± SEM from four independent experiments. (E) Protein extracts from cells transfected with *Clk*-V5 alone or *Clk*-V5 and *cyc*-Fg were subjected to immunoprecipitation (IP) using anti-FLAG antibody. Western blots containing cell extracts (Input) or immune complexes (IP) were probed with anti-FLAG and anti-CLK antibodies.

Clk promotes CYC accumulation in ectopic cells, but is not sufficient for clock function in all ectopic cells

If CLK stabilizes CYC in vivo as it does in S2 cells, I predict that CYC will accumulate in cells that ectopically express CLK. To test this prediction, *Clk* was driven in cry-

expressing clock and non-clock neurons using the 3.0*cry*-Gal4 driver (Zheng et al., 2008) and in non-clock-expressing Mushroom Body neurons using the hormone activated MB-GeneSwitch (MB-GS) driver (Mao et al., 2004). I first confirmed the spatial expression pattern of these drivers by using them to activate UAS-*lacZ*, which expresses nuclear localized ß-galactosidase (BDSC). As expected, the 3.0*cry*-Gal4 driver is expressed in a subset of pacemaker neurons including ~8 DN_1s, ~2 DN_3s, s-LN_vs, l-LN_vs, and ~6 LN_ds and several groups of non-clock cells including the new 1, new 2 and dorsal optic lope (DOL) neurons (Figure 17A-C). Likewise, the MB-GS driver was strongly expressed in Mushroom Body neurons in the presence, but not the absence, of the RU486 inducer (Figure 17D-I). The 3.0*cry*-Gal4 and MB-GS drivers were then used to determine whether CLK stabilizes CYC in non-clock cells. For this, flies were generated that contain either the 3.0*cry*-Gal4 or MB-GS driver, a UAS-*Clk* responder and the GFP-*cyc* transgene, collected at ZT2, and immunostained with GFP to detect CYC and PER to mark CLK-CYC dependent gene expression.

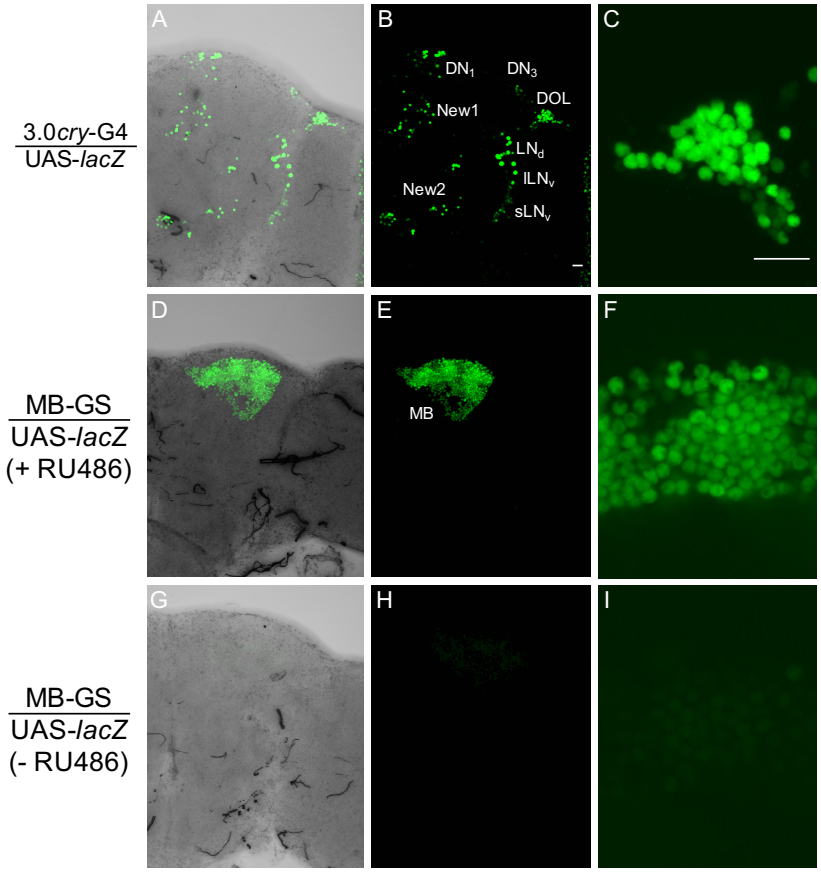

Figure 17. Localization of GAL4-dependent expression in brain. A UAS responder expressing nuclear targeted *lacZ* (UAS-*lacZ*) was expressed under the control of the 3.0*cry*-Gal4 (3.0*cry*-G4) and mushroom body-GeneSwitch (MB-GS) drivers. Immunostaining with LacZ antibody was performed on dissected adult fly brains and imaged by confocal microscopy. A projected Z-series image of the right brain hemisphere, where lateral is right and dorsal is top. Pacemaker neuron groups are as defined in Figure 1. 3.0*cry*-G4 drives expression in some pacemaker neurons plus New 1, New 2 and dorsal optic lobe (DOL) cells, and MB-G4 drives expression in mushroom body neurons (MB). (A-C) 136 µm projected Z-series image of the right brain hemisphere from a 3.0*cry*-G4/UAS-*lacZ* fly. (A) Image shown in B that includes the transmitted light view to show the outline of the brain hemisphere. (C) Magnified 58 µm projected Z-series image of the DOL region from B. (D-F) 120µm projected Z-series image of the right brain hemisphere from an MB-GS/UAS-*lacZ* induced with RU486. (D) Image shown in E that includes the transmitted light view to show the outline of the brain hemisphere. (F) Magnified 2µm image of the MB region from E. (G-I) 128µm projected Z-series image of the right brain hemisphere from MB-GS/UAS-*lacZ* without RU486 induction. (G) Image shown in H that includes the transmitted light view to show the outline of the brain hemisphere. (I) Magnified 2µm image of the MB region from H. Scale bar, 10µm. All images are representative of 6 or more brains.

When the 3.0*cry*-Gal4 driver was used to express *Clk*, GFP-CYC expression was not only detected in pacemaker neurons, but also in non-clock cells (Figure 18D-F). Among the different non-clock cell groups, I focused on DOL cells since they comprise ~20 cells that are spatially segregated from pacemaker neurons and other 3.0*cry*-Gal4 expressing cells. GFP-CYC was detected in DOL cells in the presence, but not the absence, of *Clk* expression (Figure 18A-C), demonstrating that CLK promotes CYC accumulation in vivo. Moreover, PER also accumulates in pacemaker neurons and DOL cells (Figure 18E), indicating that CLK-CYC activates downstream target genes. Consistent with previous results (Kilman and Allada, 2009; Zhao et al., 2003), PER levels cycle in DOL cells during 12h light: 12h dark (LD) cycles (Figure 19B, E, G, H), though PER cycling amplitude in DOL cells is less robust than in sLN_v pacemaker neurons (Figure 19B, E, G, H). These results demonstrate that *Clk* expression promotes CYC accumulation and PER cycling in DOL cells.

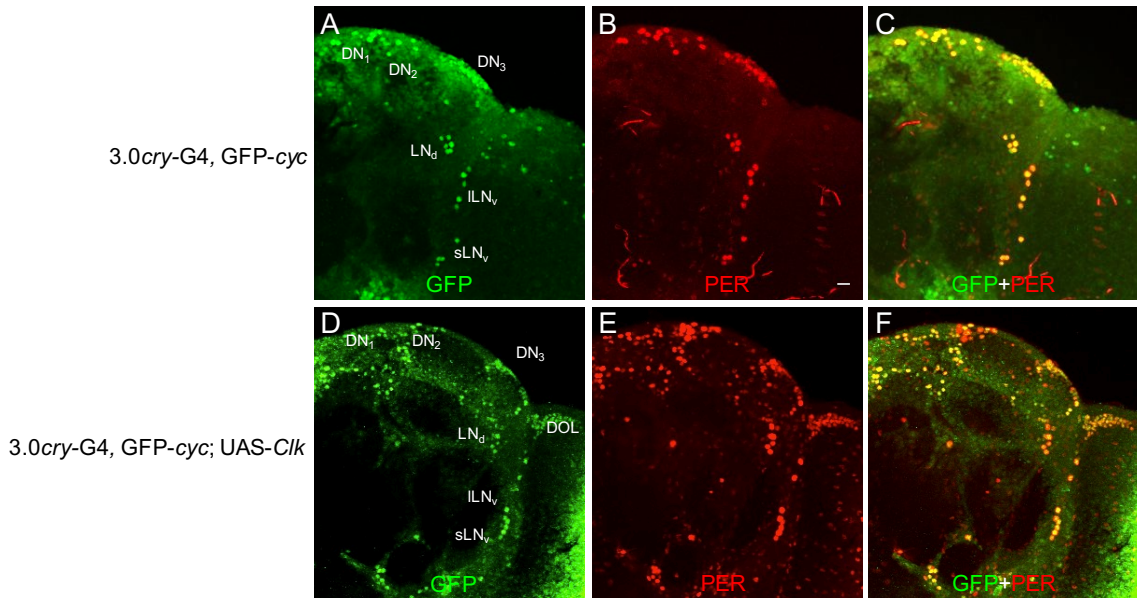

Figure 18. *Clk* expression in DOL cells promotes GFP-CYC accumulation. 3.0*cry*-Gal4, GFP-*cyc* (3.0*cry*-G4, GFP-*cyc*) and 3.0*cry*-Gal4, GFP-*cyc*; UAS-*Clk*/+ (3.0*cry*-G4, GFP-*cyc*; UAS-*Clk*) flies were entrained in LD for at least three days and collected at ZT2. Immunostaining with GFP and PER antibodies was performed on dissected adult brains and imaged by confocal microscopy. Projected Z-series images of right brain hemispheres are shown, where lateral is right and dorsal is top. Pacemaker neuron groups are as defined in Figure 1, and DOL cells are as defined in Figure S1. Co-localization of GFP (green) and PER (red) is shown as yellow. (A-C) 76µm projected Z-series image of a 3.0*cry*-G4, GFP-*cyc* fly brain immunostained with GFP (A), PER (B), or GFP and PER (C). (D-F) 86µm projected Z-series image of a 3.0*cry*-G4, GFP-*cyc*; UAS-*Clk* fly brain immunostained with GFP (D), PER (E), or GFP and PER (F). GFP and PER immunostaining is detected in the indicated groups of pacemaker neurons and DOL cells, as well as additional ectopic locations. Scale bar, 10µm. All images are representative of 6 or more brains.

To determine if PER cycling in DOL cells is driven by LD cycles, I monitored PER rhythms in DOL cells and sLN_v pacemaker neurons during constant darkness (DD). Flies containing 3.0*cry*-Gal4 and UAS-*Clk* were entrained to LD cycles for 3 days, transferred to DD, and collected every 12 hours for two days starting at Circadian Time 0 (CT0), which corresponds to subjective lights on. In sLN_v neurons, PER abundance showed significant ($P<0.05$) circadian cycling with high levels at CT0, CT24 and CT48

and low levels at CT12 and CT36 (Figure 20A, B). In DOL cells, PER abundance was not significantly rhythmic, though PER levels at CT0 and CT24 were significantly ($P<0.01$) higher than at CT12 (Figure 20C, D), indicative of a rapidly dampened rhythm.

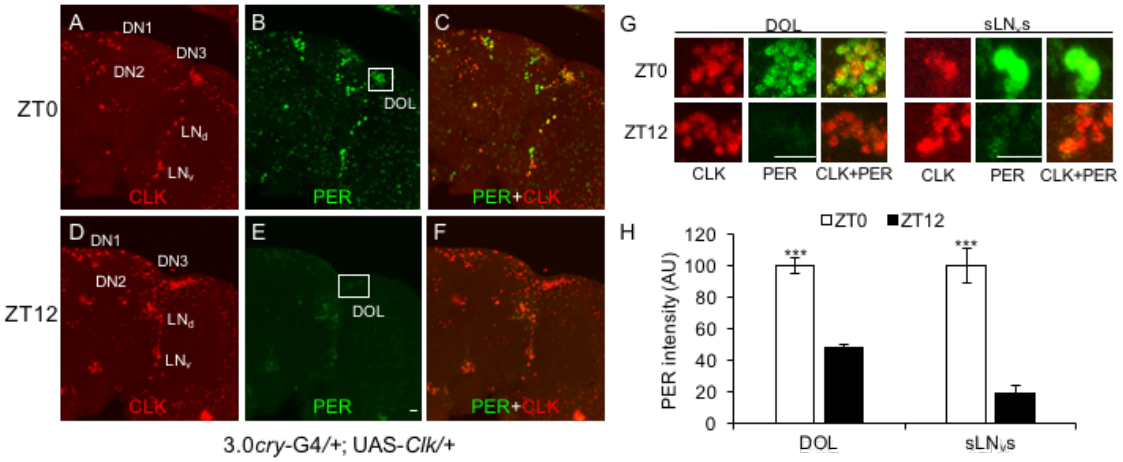

Figure 19. *Clk* expression in DOL cells is sufficient for PER cycling in LD. 3.0*cry-Gal4/+;* UAS-*Clk*/+ flies were entrained in LD for at three days and collected at ZT0 and ZT12. Immunostaining with CLK and PER antibodies was performed on dissected adult brains and imaged by confocal microscopy. Projected Z-series images of right brain hemispheres are shown, where lateral is right and dorsal is top. Pacemaker neuron groups are as defined in Figure 1, and DOL cells are as defined in Figure S1. Co-localization of CLK (red) and PER (green) is shown as yellow. (A-C) 88 µm projected Z-series image of a brain from flies collected at ZT0 and immunostained with CLK (A), PER (B), or CLK and PER (C). (D-F) 76 µm projected Z-series image from flies collected at ZT12 and immunostained with CLK (D), PER (E), or CLK and PER (F). (G) Magnified 24 µm projected Z-series images of DOL cells (left) or magnified 12 µm projected Z-series images of sLN_vs (right) from flies collected at ZT0 in panels A-C or ZT12 in panels D-F. Scale bar, 10 µm. All images are representative of six or more brains. (H) PER immunostaining intensity was quantified in DOL cells and sLN_vs from flies collected at ZT0 and ZT12. AU; arbitrary units. Error Bars indicate +/- SEM. PER intensity was significantly (***$P<0.001$) higher both DOL cells and sLN_vs at ZT0 than at ZT12 by two tailed student t test.

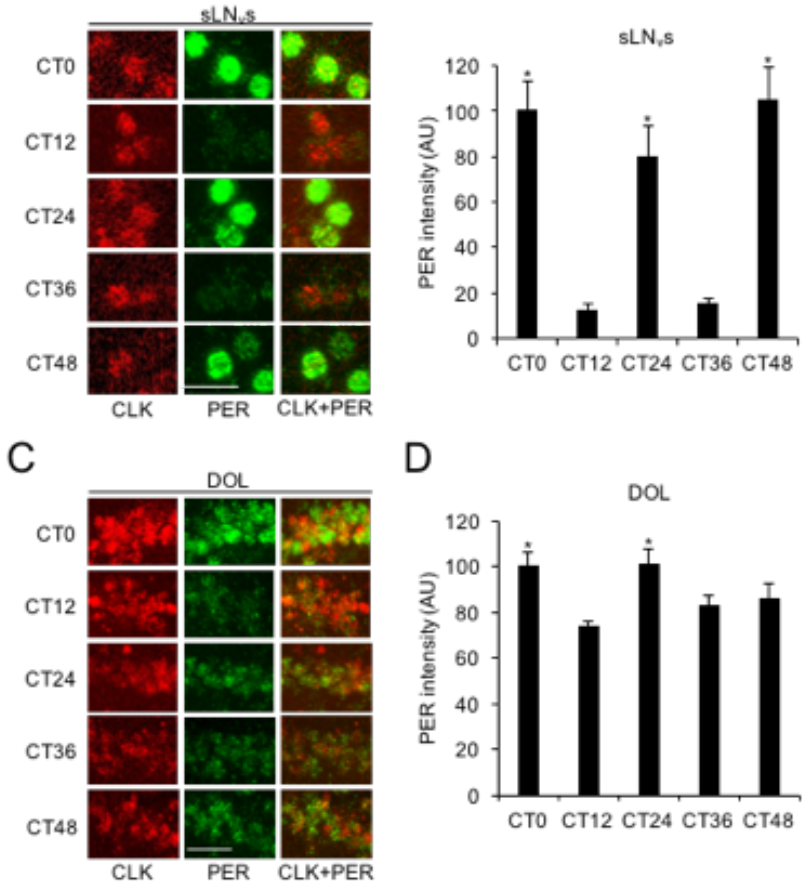

Figure 20. *Clk* expression in DOL cells supports PER cycling that rapidly dampens in DD. 3.0*cry-Gal4/+;* UAS-*Clk*/+ flies were entrained in LD cycles for three days, transferred to constant darkness, and collected at CT0, CT12, CT24, CT36 and CT48. Immunostaining with anti-PER and anti-CLK antibodies was performed on adult brains and imaged by confocal microscopy. Co-localization of CLK (red) and PER (green) is shown as yellow. (A) 22µm projected Z-series images of sLN_vs from flies collected at the indicated times and immunostained with CLK (left column), PER (middle column) or CLK and PER (right column). (B) PER immunostaining intensity from sLN_vs was quantified in arbitrary units (AU) as described in Materials and Methods. Error Bars indicate +/- SEM. The overall effects of time of day were significant ($P<0.0001$) by one-way ANOVA. Time-dependent cycling was significant ($P<0.05$) by Tukey post hoc analysis. Asterisks denote significant ($P< 0.05$) increase in PER in sLN_vs at CT0, CT24, CT48 compared to CT12 and CT36. (C) 26µm projected Z-series images of DOL cells from flies collected at the indicated times and immunostained with CLK (left column), PER (middle column) or CLK and PER (right column). (D) PER immunostaining intensity from DOL cells was quantified in arbitrary units (AU) as described above. Error Bars indicate +/- SEM. The overall effects of time of day were significant ($P<0.01$) by one-way ANOVA. Time-dependent cycling was not significant by Tukey post hoc analysis. Asterisks denote significant ($P< 0.05$) increase in PER in DOL cells at CT0 and CT48 compared to CT12. Scale bar, 10 µm. All images are representative of 6 or more brains.

When *Clk* was driven by the MB-GS driver, GFP-CYC was detected in both pacemaker neurons and MB neurons (Figure 21D, F, D1, F1), but only in pacemaker neurons in controls lacking MB-GS driven *Clk* (Figure 21A, C). As in DOL cells, *Clk* expression supports PER accumulation in MB neurons (Figure 21E, E1), indicating that CLK engages CYC to drive target gene expression. However, PER levels remained constant in MB neurons at ZT0 and ZT12 (Figure 22B, E, G, H), in contrast to the robust rhythms PER staining intensity seen in pacemaker neurons (Figure 22B, E, G, H), or in DOL cells during LD (Figure 19B, E, G, H). From these results, I conclude that even though *Clk* expression in MB neurons promotes CYC accumulation, it is not sufficient to support clock function.

I then determined whether PER protein oscillations in MB neurons co-expressing *Clk* and *cry* persisted in constant darkness (DD). Although PER levels in sLN_v pacemaker neurons from these flies showed significant ($P<0.05$) circadian cycling with peaks at CT0, CT24 and CT48 and troughs at CT12 and CT36 (Figure 24A, B), the levels of PER in MB neurons did not show significant ($P<0.05$) cycling (Figure 24C, D). However, PER levels in MB neurons at CT0 and CT24 were significantly ($P<0.01$) higher than at CT12 (Figure 24D), indicating that PER oscillations rapidly dampen in DD. Thus, ectopic clocks in MB neurons, like those in DOL cells, show a robust rhythm in PER cycling that quickly dampens in the absence of LD cycling.

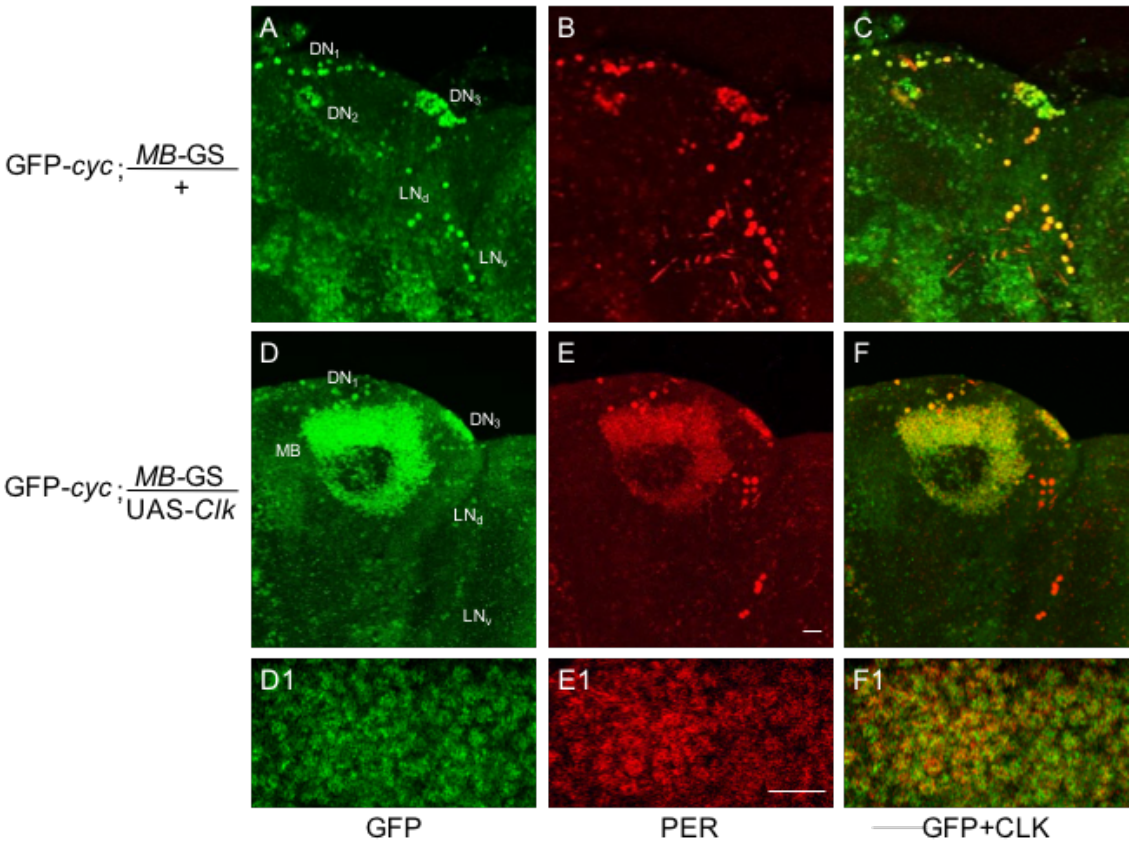

Figure 21. *Clk* expression in MB neurons promotes GFP-CYC accumulation. GFP-*cyc;* MB-GS and GFP-*cyc; MB-GS/* UAS-*Clk* flies were entrained and collected as described in Figure 18. Immunostaining with GFP and PER antibodies was performed on dissected brains and imaged by confocal microscopy. Projected Z-series images of right brain hemispheres are shown, where lateral is right and dorsal is top. Pacemaker neuron groups are as defined in Figure 15, and MB neurons are as defined in Figure S1. Co-localization of GFP (green) and PER (red) is shown as yellow. (A-C) 106µm projected Z-series image of a GFP-*cyc;* MB-GS fly brain immunostained with GFP (A), PER (B), or GFP and PER (C). (D-F) 118µm projected Z-series image of a GFP-*cyc; MB-GS/* UAS-*Clk* fly brain immunostained with GFP (D), PER (E), GFP and PER (F). GFP and PER immunostaining are detected in the indicated groups of pacemaker neurons and in Mushroom Body neurons. (D1-F1) Magnified 2µm image of MB neurons shown in panels D-F. Scale bar, 10µm. All images are representative of 6 or more brains.

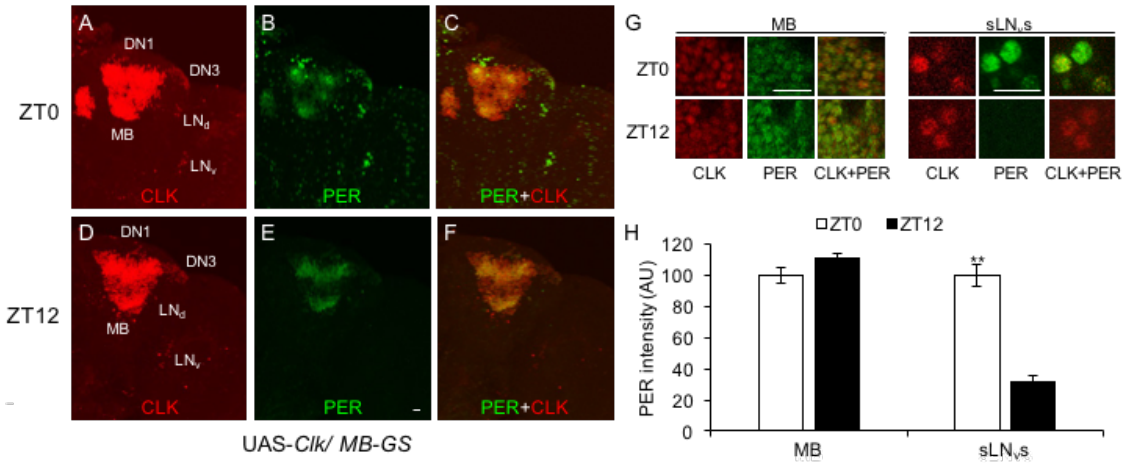

Figure 22. *Clk* expression in MB neurons does not support PER cycling in LD. MB-GS/+; UAS-*Clk*/+ flies were entrained and collected as described in Figure 19. Immunostaining with CLK and PER antibodies was performed on dissected adult brains and imaged by confocal microscopy. Projected Z-series images of right brain hemispheres are shown, where lateral is right and dorsal is top. Pacemaker neuron groups are as defined in Figure 15, and MB neurons are as defined in Figure 17. Co-localization of CLK (red) and PER (green) is shown as yellow. (A-C) 136µm projected Z-series image of a brain from flies collected at ZT0 and immunostained with CLK (A), PER (B), or CLK and PER (C). (D-F) 136µm projected Z-series image of a brain from flies collected at ZT12 and immunostained with CLK (D), PER (E), or CLK and PER (F). (G) Magnified 2µm images of MB neurons (left) and magnified 18µm projected Z-series images of sLN_vs (right) from flies collected at ZT0 in panels A-C or ZT12 in panels D-F. Scale bar, 10 µm. All images are representative of 6 or more brains. (H) PER immunostaining intensity was quantified in MB neurons and sLN_vs from flies collected at ZT0 and ZT12. AU; arbitrary units. Error Bars indicate +/- SEM. PER intensity was significantly (**$P<0.01$) higher in sLN_vs at ZT0 than at ZT12 by two tailed student t test.

CRY is required for ectopic clock function

The ability of 3.0*cry*-Gal4 driven *Clk* expression, but not MB-GS driven *Clk* expression, to generate ectopic clocks likely results from differences in gene expression in these target cell populations. The most obvious difference is that the 3.0*cry*-Gal4 driver is only expressed in CRY positive cells, whereas no CRY is detected in MB neurons targeted by the MB-GS driver (Benito et al., 2008; Yoshii et al., 2008). Importantly, *cry* expression

is required for the light entrainment and/or operation of the circadian clock in multiple peripheral tissues (Ivanchenko et al., 2001; Krishnan et al., 2001; Levine et al., 2002), and may also be required for ectopic clock function. To test this possibility, I used the MB-GS driver to express both *Clk* and *cry* in the presence of RU486, and assessed PER levels in MB neurons at ZT0 and ZT12. Co-expression of *Clk* and *cry* in MB neurons supported PER cycling, with high levels at ZT0 and low levels at ZT12 that paralleled PER rhythms in pacemaker neurons (Figure 23). These experiments demonstrate that cry is necessary for oscillator function in ectopic MB clocks.

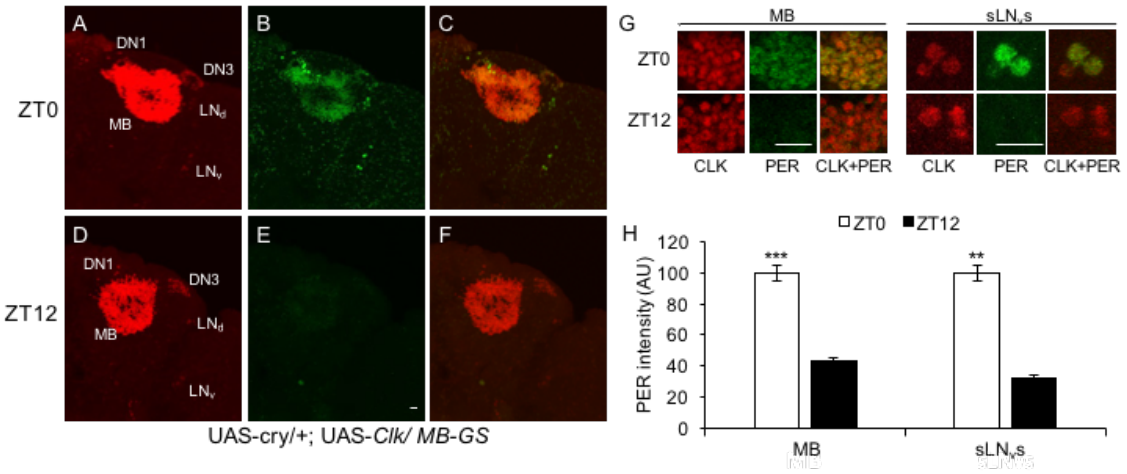

Figure 23. *Clk* and *cry* expression are required to support PER cycling in MB neurons during LD. MB-GS/UAS-*cry*; UAS-*Clk*/+ flies were entrained and collected as described in Figure 19. Immunostaining with CLK and PER antibodies was performed on dissected adult brains and imaged by confocal microscopy. Projected Z-series images of the right brain hemispheres are shown, where lateral is right and dorsal is top. Pacemaker neuron groups are as defined in Figure 1, and MB neurons are as defined in Figure S1. Co-localization of CLK (red) and PER (green) is shown as yellow. (A-C) 122µm projected Z-series image of a brain from flies collected at ZT0 and immunostained with CLK (A), PER (B), or CLK and PER (C). (D-F) 122µm projected Z-series image of a brain from flies collected at ZT12 and immunostained with CLK (D), PER (E), or CLK and PER (F). (G) Magnified 2µm images of MB neurons (left) and magnified 16µm projected Z-series images of sLN_vs from flies collected at ZT0 in panels A-C or ZT12 in panels D-F. Scale bar, 10 µm. All images are representative of 6 or more brains. (H) PER immunostaining intensity was quantified in MB neurons and sLN_vs from flies collected at ZT0 and ZT12. AU; arbitrary units. Error Bars indicate +/- SEM. PER intensity was significantly (***P<0.001) higher in MB neurons and significantly higher in sLN_vs (**P<0.01) at ZT0 than at ZT12 by two tailed student t test.

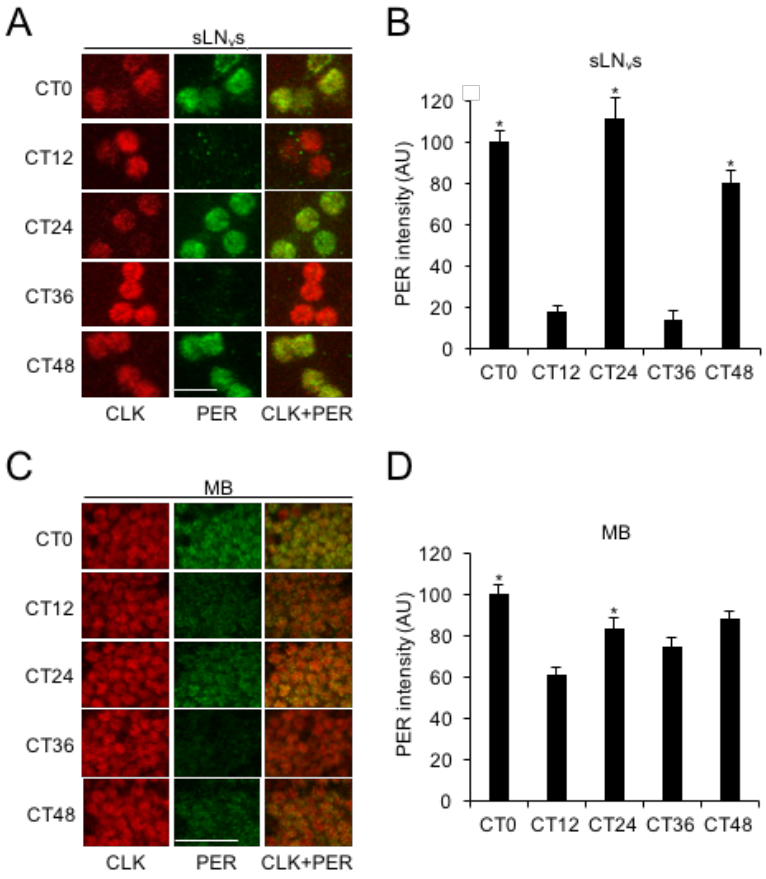

Figure 24. *Clk* and *cry* expression in MB neurons supports PER cycling that rapidly dampens in DD. MB-GS/UAS-*cry*; UAS-*Clk*/+ flies were entrained in LD cycles for three days, transferred to constant darkness, and collected at CT0, CT12, CT24, CT36 and CT48. Immunostaining with PER and CLK antibodies was performed on adult brains and imaged by confocal microscopy. Co-localization of CLK (red) and PER (green) is shown as yellow. (A) 22µm projected Z-series images of sLNvs from flies collected at indicated times and immunostained with CLK (left column), PER (middle column) or CLK and PER (right column). (B) PER immunostaining intensity from sLN_vs was quantified in arbitrary units (AU) as described in Materials and Methods. Error Bars indicate +/- SEM. The overall effects of time of day were statistically significant ($P<0.0001$) by one-way ANOVA. Time-dependent cycling was significant ($P<0.05$) by Tukey post hoc analysis. Asterisks denote significant ($P< 0.05$) increase in PER in sLN_vs at CT0, CT24, CT48 compared to CT12 and CT36. (C) 2µm images of MB neurons from flies collected at indicated times and immunostained with CLK (left column), PER (middle column) or CLK and PER (right column). (D) PER immunostaining intensity from MB neurons was quantified in arbitrary units (AU) as described above. Error Bars indicate +/- SEM. The overall effects of time of day were significant ($P<0.01$) by one-way ANOVA. Time-dependent cycling was not significant by Tukey post hoc analysis. Asterisks denote significant ($P< 0.05$) increase in PER in DOL cells at CT0 and CT48 compared to CT12. All images are representative of 6 or more brains. Scale bar, 10 µm. All images are representative of 6 or more brains.

CONCLUSIONS

CLK and CYC form heterodimers to initiate transcriptional feedback loops that keep circadian time in *Drosophila* (Hardin, 2011). During development, *Clk* is expressed as a differentiated feature of brain neurons destined to contain circadian clocks (Liu et al., 2015). Regulatory sequences that direct *Clk* expression in DN_1 or LN_v plus DN_2 pacemaker neurons have been identified (Kaneko et al., 2012; Zhang et al., 2010a; Zhang et al., 2010b), which suggests that additional regulatory elements are present at the *Clk* locus that direct expression in other subsets of pacemaker neurons and in peripheral clock tissues. Since *cyc* is also required for molecular clock function and behavioral rhythms (Rutila et al., 1998), a fully functional GFP-*cyc* transgene was used to show that GFP-CYC fusion protein in fly brains was present exclusively in pacemaker neurons (Liu et al., 2015). This result was somewhat surprising since *cyc* is not enriched in clock neurons (Nagoshi et al., 2010), and *cyc*-dependent ectopic clocks could be generated by *Clk* expression elsewhere in the brain (Kilman and Allada, 2009; Zhao et al., 2003).

Here I showed that clock neuron specificity of CYC protein expression in wild-type fly brains and the generation of *Clk*-dependent ectopic clocks in brain neurons is due to the stabilization of CYC by CLK. In pacemaker neurons and whole fly heads, CLK is required for the accumulation of CYC protein (Figure 15), demonstrating that *Clk* is required for CYC accumulation in clock cells. Experiments in S2 cells showed that CYC has an extremely short (~1h) half-life due to proteasomal degradation that is lengthened >9-fold by when *Clk* is co-expressed (Figure 16). Since CLK and CYC-

FLAG form complexes in S2 cells, the most parsimonious conclusion is that CLK-CYC heterodimerization stabilizes CYC via protection from proteasomal degradation.

Since *cyc* mRNA expression is not restricted to pacemaker neurons in the fly brain (Nagoshi et al., 2010), and ectopic clock generation by *Clk* is *cyc*-dependent (Kilman and Allada, 2009), CLK is predicted to stabilize CYC in non-clock cells. Indeed, *Clk* expression promotes CYC accumulation in *cry*-expressing brain neurons that lack circadian clocks and in MB neurons in the fly brain (Figures 19, 21).

Once CYC is stabilized by CLK in non-clock cells, CLK-CYC complexes can activate target gene transcription. In *cry*-expressing DOL cells, *Clk* expression induces ectopic clock function as measured by rhythms in PER accumulation that parallel those in pacemaker neurons during LD (Figure 19). Rhythms in PER accumulation persist during the first day of DD, but at a greatly reduced amplitude, and cycling is lost by day two of DD (Figure 20). This inability to maintain a robust rhythm is reminiscent of clocks in peripheral fly tissues that also display lower amplitude rhythms than those seen in brain pacemaker neurons (Mezan et al., 2016; Weiss et al., 2014), which maintain high amplitude molecular rhythms via reinforcing neuronal signaling (Mezan et al., 2016; Weiss et al., 2014). Nevertheless, *Clk*-induced ectopic clocks maintain high amplitude PER oscillations under natural conditions of environmental cycling (Figure 19), indicative of a functional molecular clock.

Although *Clk* is sufficient to generate ectopic clock function in DOL cells, this was not the case in MB neurons, where *Clk* expression led to constant levels of PER expression during LD cycles (Figure 22). Thus, although CLK-CYC is capable of

activating target genes in MB neurons, including the key feedback regulator *per*, the ability of these genes to support feedback loop function was compromised. One difference in the drivers used to express *Clk* in non-clock cells is that *cry*-Gal4 is by definition expressed in cells that express CRY, whereas MB neurons lack CRY expression (Benito et al., 2008; Yoshii et al., 2008). Since CRY mediates light entrainment in many pacemaker neurons and is necessary for both light entrainment and clock function in all peripheral tissues that have been examined (Egan et al., 1999; Emery et al., 1998; Emery et al., 2000; Ivanchenko et al., 2001; Krishnan et al., 2001; Stanewsky et al., 1998), my inability to generate an ectopic clock in MB neurons may be due to the lack of CRY. Indeed, expressing both *Clk* and *cry* in MB neurons resulted in robust cycling in PER levels during LD (Figure 23), indicative of ectopic clock function. These *Clk* and *cry* induced PER rhythms in MB neurons mirror those in pacemaker neurons during LD, but rapidly dampen during DD (Figure 24). The rapid dampening of PER rhythms in MB neurons is similar to that seen in DOL cells during DD (Figure 20), and is much faster than the rhythm dampening seen in peripheral clocks using *per*-luciferase or *tim*-luciferase reporter assays (Ivanchenko et al., 2001; Krishnan et al., 2001).

My experiments demonstrate that *cry*, like *Clk*, is required for ectopic clock function. Since *cyc* is also necessary for ectopic clock function, it is possible that naïve *Drosophila* cells can be programmed to express molecular clocks that function in LD by expressing *Clk*, *cyc* and *cry*. If such clock programming is possible, this work could lead to the development of *Drosophila* cell lines that possess clocks that operate in LD. The

resulting cell lines would be analogous to monarch DpN1 cells, which possess a robust molecular clock that only operates in LD (Zhu et al., 2008), yet represent a valuable tool for understanding the molecular machinery required for feedback loop function.

METHODS

Fly strains

The following *Drosophila* strains were used in this study: w1118, w; Cyo/Sco; TM2/TM6B, cyc^{01} (Rutila et al., 1998), and Clk^{out} (Mahesh et al., 2014), GFP-*cyc*; cyc^{01} (Liu et al., 2015), w; *cyc*-Flag (Abruzzi et al., 2011), 3.0*cry*-Gal4 (Zheng et al., 2008), MB-*GeneSwitch* (Roman et al., 2001), UAS-*Clk* (Zhao et al., 2003), UAS-*lacZ* (BDSC #3955) and UAS-*cry* (Emery et al., 1998).

Plasmid construction

The pMK33-*cyc*-Flag plasmid was generated by a post-doctoral fellow in the Hardin lab, Dr. Guruswamy Mahesh. pMK33-*cyc*-Flag was constructed by inserting the *cyc* ORF into the pMK33-TAP-3XFLAG-6XHis expression vector (Mahesh et al., 2014). The *cyc* ORF was amplified from genomic DNA using XhoI *cyc* forward (5'- ctattc CTC GAG ATG GAA GTT CAG GAG TTC TGC G -3') and SpeI *cyc* reverse (5'- aattcc ACT AGT TTA TAA GAA CAC GGA ATT CTT GGC GA -3') primers, and subcloned into TA to form TA-*cyc* ORF (Invitrogen). The *cyc* ORF was removed from TA-*cyc* ORF with XhoI and SpeI and inserted into pMK33-TAP-3XFLAG-6XHis to form pMK33-*cyc*-Flag.

S2 cell experiments

S2 cells were maintained in Schneider's *Drosophila* medium (Invitrogen) containing 10% fetal bovine serum with (100 units/ml) penicillin and streptomycin (100 g/ml) (Invitrogen). Cells were transiently transfected at 40–80% confluence according to company recommendations using Effectene Transfection Reagent (QIAGEN) (6.4ul of Enhancer, 10ul of Transfection Reagent, 0.8ug of total DNA). S2 cells were transfected with pMK33-*cyc*-Flag and pAc-*Clk*-V5 or pAc-V5, and 36h after transfection cells were induced with copper sulfate (500 μM) as described (Mahesh et al., 2014). After a 1h induction, cells were treated with cycloheximide (10ug/ml, C7698, Sigma) or cycloheximide plus MG132 (50 μM, Sigma) and harvested 0h, 1h, 2h, 3h later to measure proteasomal degradation and protein half-life (Liu et al., 2000; Mahesh et al., 2014).

Western blot analysis

S2 cells and fly head extracts were prepared using radioimmunoprecipitation assay (RIPA) buffer and used to prepare western blots as described (Mahesh et al., 2014). 10μg (for probing with CLK antibody) or 1μg (for probing with FLAG and ACTIN antibodies) of S2 cell extract and 10μg of fly head extract was loaded on gels for western blotting. Blots were probed with guinea pig anti-CLK (gp50,1:3000), mouse anti-FLAG (Sigma, 1: 30,000), and mouse anti-actin (Abcam, 1: 50,000) antibodies. Horseradish peroxidase conjugated secondary antibodies (Sigma) were diluted 1:1,000. Immunoblots

were visualized using ECL plus reagent (GE Life Sciences). The ImageJ program was used to quantify protein abundance as described (Mahesh et al., 2014).

Immunoprecipitation

Cell extracts were prepared using lysis buffer (50mM Tris HCl, PH7.5, with 150mM NaCl, 1mM EDTA, and 1% TRITON X100), containing 0.5 mM PMSF (phenylmethylsulfonyl fluoride), 1 mM Na_3VO_4, and 1 mM NaF) and complete EDTA-free protease inhibitor mixture (Roche Applied Science). Proteins were immunoprecipitated using Anti-FLAG M2 Magnetic Beads according to manufacturer's instructions (Sigma-Aldrich). 1mg protein was added to TBS-washed beads and incubated with agitation at 4ºC overnight. Beads were collected using appropriate magnetic separator and the supernatant was kept on the side. Beads were then washed three times with lysis buffer, transferred to a new tube, mixed with 20ul of 2 x Sample buffer (125mM Tris HCl, PH 6.8, 4% SDS, 20% (v/v) glycerol, and 0.004% bromophenol blue, 10% 2-mercaptoethanol), and boiled for 5 min. The supernatant was used for Western blotting as described above.

Immunostaining adult brains

Antibody staining of adult brain was carried out as previously described (Liu et al., 2015). Adult brains were dissected and fixed with 3.7% formaldehyde. Samples were washed and incubated with primary and secondary antibodies in a solution containing 1X PBS, 5% BSA, 5% Goat Serum (Sigma) (5% Donkey serum for primary antibodies

raised in goat), 0.03% sodium deoxycholate, 0.3% TritonX100. The following primary antibodies were used: goat anti-CLK dC-17 (Santa Cruz Biotechnology, Inc) 1:100, goat anti-PER (Santa Cruz Biotechnology, Inc) 1:500, mouse anti-Beta-Galactosidase (Promega Corporation) 1:200, rabbit anti-GFP ab6556 (Abcam) 1:500, pre-absorbed rabbit anti-PER (gift from Michael Rosbash, Brandeis University) 1:15000. The following secondary antibodies were used: goat anti-rabbit Alexa 647 (Molecular Probes), goat anti-rabbit Alexa 488(Molecular Probes), donkey anti-rabbit Alexa 488 (Jackson ImmunoResearch Laboratories), donkey anti-mouse Alexa 488 (Jackson ImmunoResearch Laboratories), and donkey anti-goat Cy5 (ImmunoResearch Laboratories).

Imaging and quantification

Adult fly brains were imaged using an Olympus FV1000 confocal microscope as described previously (Liu et al., 2015). Confocal stacks were imaged using an Olympus FV1000 confocal microscope equipped with 20 X/0.85 NA and 100 X1.40 NA oil-immersion objectives. For double-labeling experiments, sequential scans of the argon ion 488 nm and HeNe (633 nm for Alexa Fluor 647 and Cy5) lasers were used to avoid bleed-through between channels. For imaging Alexa Fluor 488 and either Alexa Fluor 647 or Cy5, Argon 488 and HeNe 633 nm lasers were used, with the 488/543/633 nm dichroic mirror for excitation. Fluorescence signals were separated by a dichroic beam splitter (560 nm long-pass). A spectral detector set to 500–555 nm was used for Alexa Fluor 488 and a detector with 650 nm long-pass filter was used for Alexa Fluor 647 or

Cy5 signals. The Fluoview "Hi-Lo" look-up table was used to set the maximal signal below saturation and set the background to near zero using the high voltage and offset controls. Z-series were obtained at 2 um step size, and Kalman-averaging was not used. Original images were saved as 12-bit oib format and processed using FV1000 confocal software to generate maximum intensity projections (Z-projections). Images were adjusted for brightness and contrast using Adobe Photoshop. For each genotype and time points, brain images were acquired using the same settings (power, gain, offset) at the same time.

Data of sLN_vs and ectopic clocks were quantified from digital images using the Fiji version of ImageJ (Schindelin et al., 2012). CLK staining was used to mark the ectopic clock cell and sLN_v nuclei, and PDF staining was used to identify sLN_vs (data not shown). For ectopic clocks cells, the Fiji plugin 3D counter was used to measure the total number of cells (Bolte and Cordelieres, 2006). The sum of the fluorescence intensity for all cells was measured, and the average intensity for each cell was calculated. An average was then calculated for each time point and scaled so that the typical peak, usually ZT0/CT0 for ectopic clocks, was 100 AU (arbitrary units) as described (Kilman and Allada, 2009). AU = arbitrary units, Error Bar +/- SEM. One-way ANOVA with post hoc Tukey test was used to identify time-dependent cycling ($p < 0.05$). A Student T-test was performed to compare data between two time points.

CHAPTER IV

DETERMINING THE ROLE OF CLOCK GENE IN CIRCADIAN CLOCK NETWORK FORMATION

BACKGROUND

Different groups of pacemaker neurons in the *Drosophila* brain communicate using neuropeptides to form a network, which controls the locomotor activity of adults. Since *Clk* is only expressed in post-mitotic cells, pacemaker neurons presumably exist before *Clk* is expressed (Liu et al., 2015). Thus I seek to determine how *Clk* affects the fates of clock cells as well as what role *Clk* plays in pacemaker neuron development and network formation. To address these questions, I designed experiments to determine whether pacemaker neurons are present in Clk^{out} flies. Previous evidence showed that PDF expression in sLN_vs was not detectable in both the adult sLN_v of the *Clk* mutant and the larval LN_vs which become adult sLN_vs during development (Helfrich-Förster, 1997; Park et al., 2000). It is possible that *Clk* affects the specific PDF expression or processing specifically in sLN_v of adults and the precursors in the larval brain (Park et al., 2000). It is also possible that *Clk* is required for the development and/or maintenance of sLN_v cells. Up to now, it is not clear how *Clk* affects sLN_v development and/or function.

I hypothesize that *Clk* is required for sLN_v development therefore sLN_v cells are missing in the absence of *Clk.* To test the hypothesis, two Gal4 promoters were employed: *Clk21*-Gal4, a transgene (generated by Tianxin Liu) that is expressed in all

groups of pacemaker neurons, and MZ520-Gal4, a published transgene that is expressed in sLNvs and lLN_vs (Grima et al., 2004; Head et al., 2015; Ito et al., 1995). These two lines were crossed with UAS-lacZ.NZ, a reporter gene in the nucleus and 10XUAS-IVS-mCD8::GFP (UAS-CD8-GFP), a reporter gene in the cell membrane, and the adult progeny were assayed for brain lacZ staining and GFP staining in wild-type or in Clk^{out} mutants.

In this chapter, I found that in adult brains, almost all the groups of pacemaker neurons (DN_1s, DN_2s, DN_3s, LN_d, lLN_vs) are present in Clk^{out} null mutants, while sLN_vs cells are missing, indicating that *Clk* is essential for sLN_v development and/ maintenance. However, larval LN_vs are still present in the Clk^{out} null mutant, suggesting that *Clk* plays an important role in the maintenance of sLN_vs. These results reveal that *Clk* is not required for the development of pacemaker neurons in the adult brain except sLN_vs, which distinguishes the sLN_vs from all the other groups of pacemaker neurons. Furthermore, my findings reveal a new role of *Clk* in sLN_vs development, and will hopefully encourage more studies on sLN_v development and maintenance, as well as investigations on other functions of *Clk* that are independent of circadian oscillation.

RESULTS

Generating a *Clk*-Gal4 transgene that expresses in all pacemaker neurons

To determine whether pacemaker neurons are present in Clk^{out} flies, a marker which is still expressed for these cells in the absence of *Clk* is needed. Since this requirement eliminates all *Clk*-dependent genes and *Clk* is the first clock gene expressed in clock

cells, *Clk*-Gal4 will be used to mark all the pacemaker neurons in Clk^{out} flies. Since multiple enhancers are required to drive *Clk* expression (Gummadova et al., 2009), a *Clk*-driven Gal4 was generated using a 21kb Bac containing *Clk* as well as all the *Clk* enhancers and parts of surrounding genes (Figure 25).

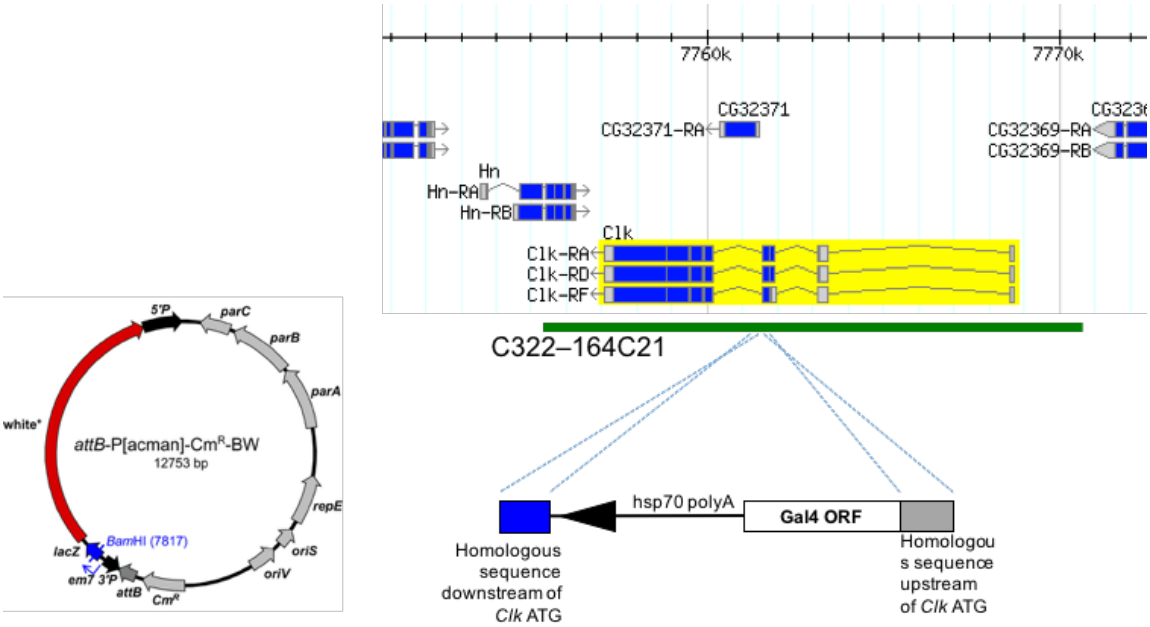

Figure 25. Generation of *Clk21*-Gal4 in a P[acman] clone via homologous recombineering. Gal4 was amplified using primer *Clk*-L which contains 70 nt of *Clk* sequence upstream of the translation start and the first 28 nt of the Gal4 translated sequence, and *Clk*-R primer which contains 70nt from *Clk* intron1, and 21nt from the 3' end of the Gal4-Kan cassette. This fragment was used to transform EL350 cells harboring the BAC clone C322–164C21, which contains the *Clk* genomic region (See in Method section)

Clock-Gal4 (*Clk*21-Gal4) was generated as described in this chapter's Method section. To test the spatial expression of the *Clk21*-Gal4, *Clk21*-Gal4 flies were crossed with UAS-lacZ.NZ flies. Adult brains that carry *Clk21*-Gal4/UAS-lacZ were collected at ZT21 and immunostained with β-gal to mark *Clk*21-Gal4 expression and anti-VRI to

mark all the pacemaker neurons in the adult brain. As shown in the Figure 26, *Clk*21-Gal4 drives UAS-lacZ expression only in DN_1, DN_2, DN_3, LN_d, LN_v, and LPN pacemaker neurons marked by VRI staining, indicating that the *Clk21*-Gal4 faithfully marked all groups of pacemaker neurons in the adult brain. Compared with previous *Clk*-Gal4 drivers which were generated with different regions of *Clk* promoter and expressed only in subgroups of pacemaker neurons in fly brains (Glossop et al., 2003; Gummadova et al., 2009), *Clk*21-Gal4 that I generated will be a better tool to mark all the groups of pacemaker neurons.

Clk is required for sLN_v development in adults

To define the role of *Clk* in pacemaker neuron development, I used the *Clk21*-Gal4 transgene to mark pacemaker neurons in Clk^{out} flies which lack *Clk* expression (Mahesh et al., 2014). I found that almost all the groups of pacemaker neurons including DN_{1+2}, DN_3, LPN, LN_d, LN_v are still present in *Clk21*-Gal4/UAS-lacZ; Clk^{out} flies (Figure 27A). Although most groups of pacemaker neurons are present in Clk^{out} flies, I found that the sLN_v subgroup of LN_vs were absent compared to wild-type controls, which is confirmed by the PDF staining (Figure 27). To insure that the lack of sLN_vs was not simply due to the loss of *Clk*-Gal4 driven expression, I used another Gal4 line that drives expression specifically in both adult LN_v subsets, called MZ520-Gal4, that is regulated by enhancers independent of *Clk* (Grima et al., 2004; Head et al., 2015; Ito et al., 1995). MZ520-Gal4 driven lacZ is only detected in lLN_vs in Clk^{out} flies (Figure 28), but in both sLN_v and lLN_vs subgroups in wild-type controls (Figure 28), which suggests that sLN_v

development is compromised. To confirm the absence of sLN_vs in *Clk*^{out} flies bearing *Clk21*-Gal4 or MZ520-Gal4 driven lacZ, immunostaining with PDF antibody revealed only lLN_v projections (Figure 27D, 28F), and using MZ520-Gal4 to drive membrane bound CD8-GFP showed that only lLN_v projections are present (Figure 28E). These results are consistent with previous results showing that PDF expression in the sLN_v dorsal projection is absent in *Clk*^{Jrk} flies (Park et al., 2000), and suggest that sLN_v development is compromised in the absence of *Clk*.

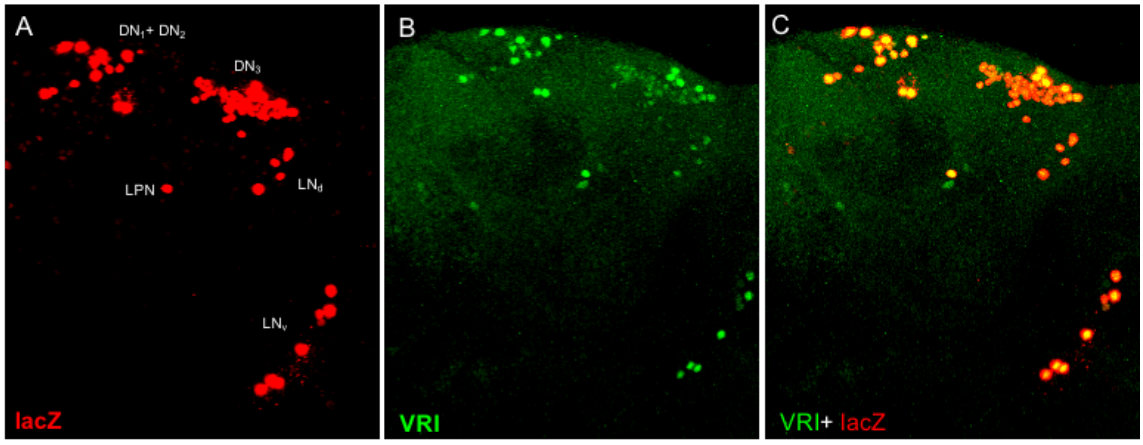

Figure 26. *Clk21*-Gal4 drives expression exclusively in clock neurons in adult brains. Brains from *Clk21*-Gal4/UAS-lacZnls adults were collected at ZT21, immunostained with β-gal and VRI antisera, and imaged by confocal microscopy. (A-C) projected Z-series of the right hemisphere from an adult fly, where lateral is right and dorsal is top. Right hemisphere stained with the lacZ (A), VRI (B), lacZ + VRI (C) immunostaining is detected in DN_1s, DN_2s, DN_3s, LN_ds, LPNs, and LN_vs. Co-localization of lacZ (red) and VRI (green) is shown as yellow. Scale bar, 10μm. All images are representative of 6 or more brains.

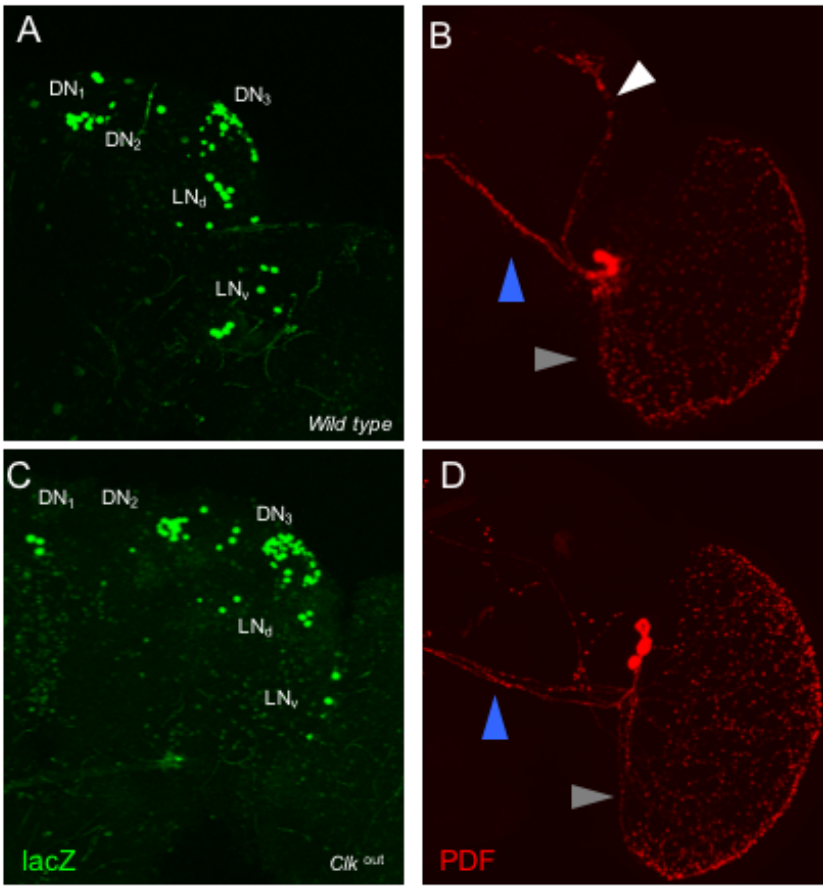

Figure 27. Expression of *Clk21*-Gal4 in brains from *Clk*^{out} flies. Brains from *Clk21*-Gal4/UAS-lacZnls and *Clk21*-Gal4/UAS-lacZ; Clkout flies were collected at ZT21, immunostained with β-gal (A, C) (green) and anti-PDF antisera (B, D) (red), and imaged by confocal microscopy. A. projected Z-series of the right hemisphere from *Clk21*-Gal4/UAS-lacZnls flies stained with β-gal. B. projected Z-series of the right hemisphere from *Clk21*-Gal4/UAS-lacZnls flies stained with PDF. C. projected Z-series of the right hemisphere from right hemisphere from *Clk21*-Gal4/UAS-lacZ; *Clk*^{out} stained with β-gal. D. projected Z-series of the right hemisphere from right hemisphere from *Clk21*-Gal4/UAS-lacZ; Clkout stained with PDF. Brains are oriented where lateral is to the right and dorsal is at the top. DN_1, DN_2, DN_3, LPN, LN_d, lLN_v and sLN_v refer to pacemaker neuron groups as defined in the text. White arrowhead, sLN_v dorsal projection; blue arrowhead, lLN_v posterior optic tract projection; gray arrowhead, lLN_v medulla arborizations. All images are representative of 6 or more brains.

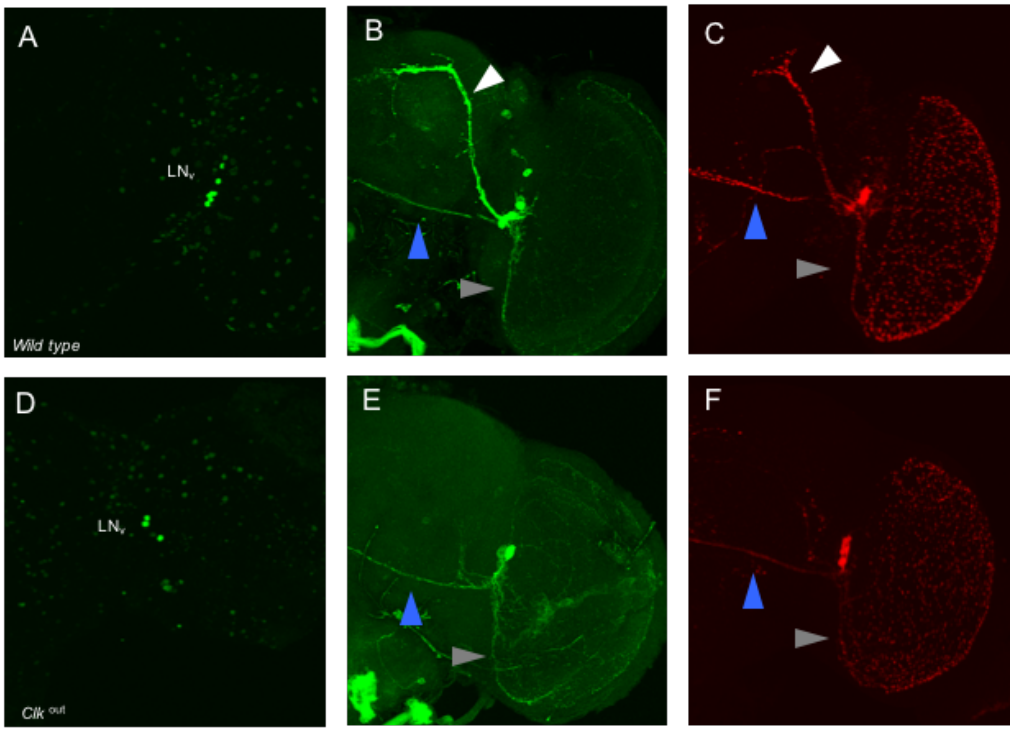

Figure 28. *Clk* is required for the development of sLN_v. Adult brains were collected at ZT21, immunostained with β-gal (A, D) (green), GFP (B, E) (green) and anti-PDF antisera (C, F) (red), and imaged by confocal microscopy. A. projected Z-series of the right hemisphere from MZ520-Gal4/UAS-lacZnls flies stained with β-gal. B. projected Z-series of the right hemisphere from MZ520-Gal4/UAS-CD8-GFP flies stained with GFP. C. projected Z-series of the right hemisphere from right hemisphere from MZ520-Gal4/UAS-lacZnls stained with PDF. D. projected Z-series of the right hemisphere from right hemisphere from MZ520-Gal4/UAS-lacZ; Clk^{out} stained with β-gal. E. projected Z-series of the right hemisphere from MZ520-Gal4/UAS-CD8-GFP; Clk^{out} flies stained with GFP. F. projected Z-series of the right hemisphere from right hemisphere from MZ520-Gal4/UAS-lacZnls; Clk^{out} stained with PDF. Brains are oriented where lateral is to the right and dorsal is at the top. White arrowhead, sLN_v dorsal projection; blue arrowhead, lLN_v posterior optic tract projection; gray arrowhead, lLN_v medulla arborizations. All images are representative of 6 or more brains.

The sLN_v dorsal projection is present in larvae, but absent in adults

Early pacemaker neurons, encompassing 4-5 larval LN_vs, 2 DN_2s and 2 DN_{1a}s, begin to express CLK in stage 16 and 17 embryos (Houl et al., 2008). In L1 larvae, PDF begins to be expressed in the larval LN_vs, which send a projection into the dorsal brain in the vicinity of the DN_2s and DN_{1a}s (Helfrich-Förster, 1997). During metamorphosis,

circadian clock function is initiated in lLN_vs, and larval LN_vs continue on as sLN_vs in adults (Helfrich-Förster, 1997; Kaneko et al., 1997; Liu et al., 2015). Since I found that *Clk*21-Gal4 and MZ520-Gal4-driven expression in sLN_vs is absent in Clk^{out} adults, I expected that there would be no *Clk*-driven expression in their larval LN_vs. Surprisingly, there are ~4 neurons in both *Clk*-Gal4/UAS-lacZ; Clk^{out} and MZ520/UAS-lacZ; Clk^{out} that correspond to larval LN_vs (Figure 29 A, C). Given that sLN_vs are derived from larval LN_vs, and they are absent in brains from Clk^{out} adults, these results indicate that *Clk* is not required for larval LN_v development, but is required for the maintenance of these cells during later developmental stages.

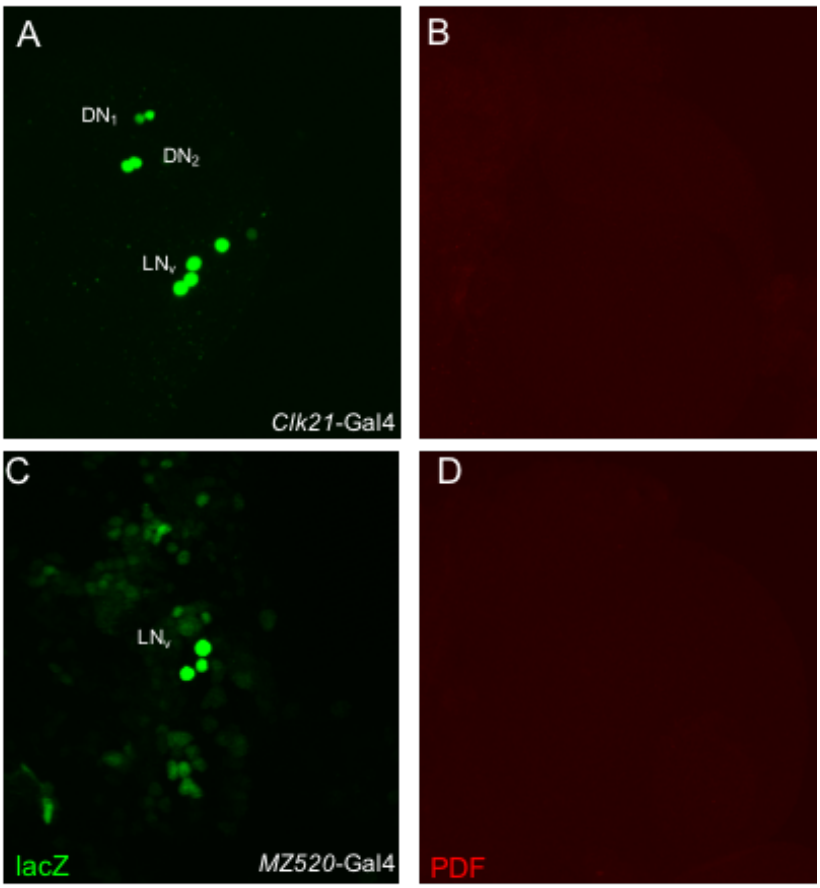

Figure 29. *Clk* is not required for the existence of sLN_v during early development. Laval brains from *Clk21*-Gal4/UAS-*lac*Znls; Clk^{out} and MZ520-Gal4/UAS-lacZ; Clk^{out} were collected at ZT21, immunostained with β-gal (A, C) (green) and anti-PDF antisera (B, D) (red), and imaged by confocal microscopy. A. projected Z-series of the right hemisphere from *Clk21*-Gal4/UAS-lacZnls flies stained with β-gal. B. projected Z-series of the right hemisphere from *Clk21*-Gal4/UAS-lacZnls flies stained with PDF. C. projected Z-series of the right hemisphere from right hemisphere from MZ520-Gal4/UAS-lacZ; Clk^{out} stained with β-gal. D. projected Z-series of the right hemisphere from right hemisphere from MZ520-Gal4/UAS-lacZ; Clk^{out} stained with PDF. Brains are oriented where lateral is to the right and dorsal is at the top. DN_1, DN_2, LN_vs refer to larval pacemaker neuron groups as defined in the text. All images are representative of 6 or more brains.

CONCLUSIONS

Since sLN_vs are essential for locomotor activity in *Drosophila*, it is important to know the factors that affect LN_vs development. Previous evidence showed that PDF is

undetectable in the sLN_v of a *Clk* mutant (Park et al., 2000), without providing clear explanations on whether it is caused by the effect of PDF expression or sLN_v development. In addition, there is no clear evidence about the role of *Clk* in all the groups of pacemaker neurons during development. In this chapter, I developed a tool, *Clk*21-Gal4 that accurately reports *Clk* expression and is active in *Clk* mutant flies. I showed that *Clk* is not required for the development of the most groups of pacemaker neurons in *Drosophila* (e.g. DNs, LN_ds, LPNs) (Figure 27). However, the absence of *Clk* leads to reduced numbers of lateral neurons (Figure 27, 28). Immunostaining of PDF that mark sLN_vs and lLN_vs (Figure 27, 28) indicates that PDF in sLN_vs is missing, which is consistent with previous PDF staining results in Clk^{jrk} mutants. In addition, the data from MZ520-Gal4, which is a promoter independent of *Clk*, showed that sLN_vs are absent, which argues that the sLN_vs are missing rather than the *Clk* reporter expression is disrupted in Clk^{out} flies. These results indicate that *Clk* is required for sLN_v presence in the brain and *Clk* has a special role in sLN_v development. The presence of sLN_vs in early development (Figure 29) suggests that *Clk* plays a key role in maintenance of the sLN_v, which provides a new role for *Clk* in sLN_v development and/or maintenance in addition to its role in initiating circadian clock function.

METHODS

Fly strains

UAS-lacZ.NZ, 10XUAS-IVS-mCD8::GFP, Clk^{out}, MZ520-Gal4

Generation of *Clk21*-Gal4

A C-terminal Gal4 tagged *Clock* (*Clk*) transgene (*Clk*21-Gal4) was constructed via recombineering (Venken et al., 2006). Q5 DNA polymerase (New England BioLabs) was used to amplify the Gal4-kanamycin from Gal4-Kan plasmid (Gal4-Kan was constructed by Dr. Wangjie Yu in Hardin Lab) using primer *Clk*-L (5_ TTCCCATTTTTTTTTCGGCATTTGTTTAAAAATTGTGCGCCTTGTAGTGGACT ATCCTACAGACCCGAAA*ATGAAGCTACTGTCTTCTATCGAACAAG*-3), which contains 70 nt of *Clk* sequence upstream of the translation start (italized) and the first 28 nt of the Gal4 translated sequence (underline), and *Clk*-R (5-ATGGCCAACGGATTTGTGCTATCATCTAACGAATGATTGAGTGTGGGTACTA AATGTCTAGTGAATTTACCGTCGTCATCCTTGTAATCCA), which contains 70nt from *Clk* intron1, and 21nt from the 3' end of the Gal4-Kan cassette (underline).This fragment was used to transform EL350 cells harboring the BAC clone C322–164C21(BAC-PAC Resources Center), which contains the *Clk* genomic region, and recombinants containing the Gal4-LoxP-kanamycin cassette inserted into *Clk* were selected on plates containing kanamycin. The kanamycin gene was removed by inducing recombination at the LoxP sites (Venken et al., 2009; Venken et al., 2008), resulting in the chloramphenicol-resistant *Clk*-Gal4 PACMAN clone. *Clk*-Gal4 was amplified in EPI 300 cells (Epicenter), and sequenced to confirm the C-terminal and N-terminal fusion. The *Clk21*-Gal4 transgene was inserted into attP40 on chromosome 2 via PhiC31-mediated transgenesis (Groth et al., 2004). The *Clk*-Gal4 transgene was used for immunostaining.

Immunohistochemistry

Antibody staining of adult brain was carried out as previously described (Liu et al., 2015). Adult brains were dissected and fixed with 3.7% formaldehyde. Samples were washed and incubated with primary and secondary antibodies in a solution containing 1X PBS, 5% BSA, 5% Goat Serum (Sigma), 0.03% sodium deoxycholate, 0.3% TritonX100. The following primary antibodies were used: mouse anti-Beta-Galactosidase (Promega Corporation) 1:200, Guinea pig anti-VRI 1:25000, rabbit anti-GFP ab290 (Abcam) 1:2000, mouse anti-PDF (DSHB) 1:500. The following secondary antibodies were used: goat anti-rabbit Alexa 647 (Molecular Probes), goat anti-rabbit Alexa 488(Molecular Probes), goat anti-mouse Alexa 488 (Jackson ImmunoResearch Laboratories), goat anti-guinea pig Cy-5 (Jackson ImmunoResearch Laboratories, Inc.).

Imaging

Adult fly brains and larval brain were imaged using an Olympus FV1000 confocal microscope as described previously (3). Confocal stacks were imaged using an Olympus FV1000 confocal microscope equipped with 20 X/0.85 NA oil-immersion objectives. For double-labeling experiments, sequential scans of the argon ion 488 nm and HeNe (633 nm for Alexa Fluor 647 and Cy5) lasers were used to avoid bleed-through between channels. For imaging Alexa Fluor 488 and either Alexa Fluor 647 or Cy5, Argon 488 and HeNe 633 nm lasers were used, with the 488/543/633 nm dichroic mirror for excitation. Fluorescence signals were separated by a dichroic beam splitter (560 nm

long-pass). A spectral detector set to 500–555 nm was used for Alexa Fluor 488 and a detector with 650 nm long-pass filter was used for Alexa Fluor 647 or Cy5 signals. The Fluoview “Hi-Lo” look-up table was used to set the maximal signal below saturation and set the background to near zero using the high voltage and offset controls. Z-series were obtained at 2 um step size, and Kalman-averaging was not used. Original images were saved as 12-bit oib format and processed using FV1000 confocal software to generate maximum intensity projections (Z-projections). Images were adjusted for brightness and contrast using Adobe Photoshop. For each genotype and time points, brain images were acquired using the same settings (power, gain, offset) at the same time.

CHAPTER V

SUMMARY AND DISCUSSION[4]

SUMMARY

A major objective of my research was to study clock cell development and function initiation in *Drosophila.* Since the mechanisms underlying the development and function initiation of circadian clocks that are likely conserved in other eukaryotes, my work will hopefully uncover novel clock activators, and unravel the mechanistic links between clocks and development in other animals. In my studies, I have employed various tools including transgenetic manipulations, immunochemistry analysis, behavioral and biochemical analysis to address the following questions: When do clock cells develop? How do clock cells develop? How is the clock network organized? What are the requirements for cells to contain a functional clock?

In tracing the development of pacemaker neurons in the *Drosophila* brain, I demonstrated that late pacemaker neurons expand dramatically during L3 larval development but they lack circadian clock function until metamorphosis which implies that a novel activation event is required to initiate circadian clock function. In addition, I found that CLK is expressed exclusively in post-mitotic cells in *Drosophila*, suggesting that early pacemaker neurons do not give rise to late pacemaker neurons (Chapter II).

[4] Several paragraphs of this chapter are reprinted from Liu, T., Mahesh, G., Houl, J. H., and Hardin, P. E. (2015). Circadian activators are expressed days before they initiate clock function in late pacemaker neurons from *Drosophila*. Journal of Neuroscience, 35(22), 8662-8671, and the paper that has been submitted to PNAS and under review as Liu, T., Mahesh, G., Yu, W., and Hardin, P. E. (2017). CLOCK stabilizes CYCLE to initiate clock function in *Drosophila*

These results bring up important questions: When do late pacemaker neurons arise? What determine their fate? How and when do the late pacemaker neurons integrate with early pacemaker neurons?

Also, I found that CYC protein is expressed in all CLK-expressing brain pacemaker neurons in larvae, providing an evidence that CLK promotes CYC expression (Chapter II). Following these findings, I demonstrated that CLK stabilization of CYC protein to promote CYC protein expression (Chapter III). My data account for the accumulation of CYC only in *Clk*-expressing neurons, and further define the first molecular events required to initiate clock function in *Drosophila*. In addition, my results, together with previous evidence that *cyc* mRNA is widely expressed, should stimulate more studies on several important questions: Why is *cyc* mRNA widely expressed? What is the function of CYC protein besides the circadian clock function? Does CYC protein partner with other proteins than CLK?

In addition, I have demonstrated that *Clk*, *cyc*, *cry* could activate circadian clock function in naïve cells (Chapter III). My work defines the genetic architecture and molecular mechanisms required for clock initiation that are likely conserved in other eukaryotes. In addition, my studies suggest that naïve cells might be reprogrammed to clock cells by *Clock*, *cycle* and *cryptochrome* expression. My research will encourage studies to determine if non-clock cells retain a program for activating tissue-specific CLK-CYC output genes, and if naïve cells can be reprogrammed to support oscillator function. Finally, my research will enable the development of clock-containing cell lines

in *Drosophila*, which will be a valuable tool for understanding the molecular machinery required for feedback loop function.

Lastly, I found that *Clk* is specifically required for the maintenance of sLN_vs, which elucidates the role of *Clk* in clock cell development that is independent of its circadian clock function (Chapter IV). In addition, together with my data on the development of pacemaker neurons (Chapter II), my work will provide insights into how the neural network is designed to ultimately control locomotor activity rhythms in adult flies.

DISCUSSION

Late pacemaker neurons expand dramatically during L3 larval development, but lack circadian clock function until metamorphosis

Previous research on the development of pacemaker neurons showed that the majority of the pacemaker neurons (DN_{1p}, DN_3, LN_d, LPN) developed during the second half of metamorphosis (Kaneko et al., 1997). Surprisingly, I found that almost all the groups of pacemaker neurons in the adult brain (DN_{1p}, DN_3, LN_d, LPN) already exist in late L3 larval brains (Figure 8). For the first time, I showed that late pacemaker neurons expand dramatically during L3 larval development (Figure 9), but they don't have a functional clock based on PER immunostaining (Figure 8). The lack of PER expression in late pacemaker neurons from L3 larvae is not due to the absence of CYC, which is expressed in all CLK-positive neurons in L3 larvae (Figure 14). Circadian oscillator function is initiated in these late pacemaker neurons during the second half of metamorphosis

(Kaneko et al., 1997), which suggests that additional factors act during metamorphosis to promote robust CLK-CYC-dependent transcription of *per*, *tim*, and possibly other target genes. The ultimate triggers of transcriptional activity during metamorphosis are likely to be either the steroid hormone ecdysone, which binds to nuclear receptors to regulate target gene transcription, or the sesquiterpenoid Juvenile Hormone (JH), which binds to the bHLH-PAS transcription factors Methoprene-tolerant (Met) or germ-cell expressed (gce) to activate transcription of target genes (Di Cara and King-Jones, 2013). Ecdysone and JH titers rise and fall multiple times during larval and pupal development (Di Cara and King-Jones, 2013), thereby altering the transcriptional activity of their receptors. Because CLK-CYC is present well before metamorphosis in most late pacemaker neurons, the proximate triggering factor may activate CLK-CYC-dependent transcription via post-translational (i.e., adding or removing protein modifications) or transcriptional (i.e., chromatin remodeling or cooperative activator DNA binding) mechanisms.

The only cluster of late pacemaker neurons that do not express CLK and CYC in L3 larvae are the lLN_vs (Figure 10). Because these neurons begin to express CLK and PER around the same time PER starts to be expressed in other late pacemaker neurons (Kaneko et al., 1997) (Figure 11) , the triggering mechanism may be the same. The significance of the difference in the timing of CLK expression in the lLN_vs and other late brain pacemaker neurons is not clear, and should be studied in future research.

In my studies, I found that both the early pacemaker neurons (DN_{1a}, DN_2, LN_v) and newly emerged late pacemaker neurons (DN_{1p}, DN_3, LN_d, LPN), marked by CLK-GFP, express the neuronal differentiation marker ELAV, indicating that CLK-GFP is

only expressed in post-mitotic neurons (Figure 12). These results suggest that early pacemaker neurons do not give rise to late pacemaker neurons, and these four late pacemaker neuron clusters are formed and the network is established before *Clk* is expressed. Future work is needed to detect the development of late pacemaker neurons and identify genes that determine the fate of late pacemaker neurons. For this purpose, a marker for the pacemaker neuron precursor, which is expressed before *Clk*, needs to be identified. Genes that are highly expressed in larval clock neurons might be good candidates (e.g. *Fer2*) (Nagoshi et al., 2010). A potential problem is that since *Clk* is the first gene that is expressed in clock cells, these late pacemaker neuron clusters could only be identified by their location while the common pacemaker neuron markers (e.g. CLK, VRI) could not be used. To confirm that these cells are actually missing in the Clk^{out} adult brain, the Gal4 technique for real time and clonal expression (G-TRACE) system cell tracing method can be employed (Evans et al., 2009). This technique has been employed to trace Gal4 expression during development. As shown in the Figure 30, Gal4 will activate the expression of FLP recombinase, which will remove an FRT-flanked transcriptional termination cassette inserted between an Ubiquitin-p63E (Ubi-p63E) promoter fragment and EGFP open reading frame. After initiating the cell tracing tool by Gal4, GFP expression will be driven by the Ubi-p63E promoter, and is not dependent on the continuous expression of the Gal4 during development. Therefore, GFP expression will be detected in all the subsequent daughter cells. Using the G-trace tool and the Gal4 driven by potential clock precursor genes, spatial expression of the precursor genes during development will be detected. If GFP is expressed earlier than

CLK in late pacemaker neurons, this gene is a clock precursor gene and may determine the fate of late pacemaker neurons the fate of clock cells. Further test using RNAi or mutant versions of this gene will be needed to determine the effect of this gene on clock cell determination. The loss of specific groups of clock neurons in the RNAi or mutant of the gene will indicate that this gene affects the fate of those clock neurons.

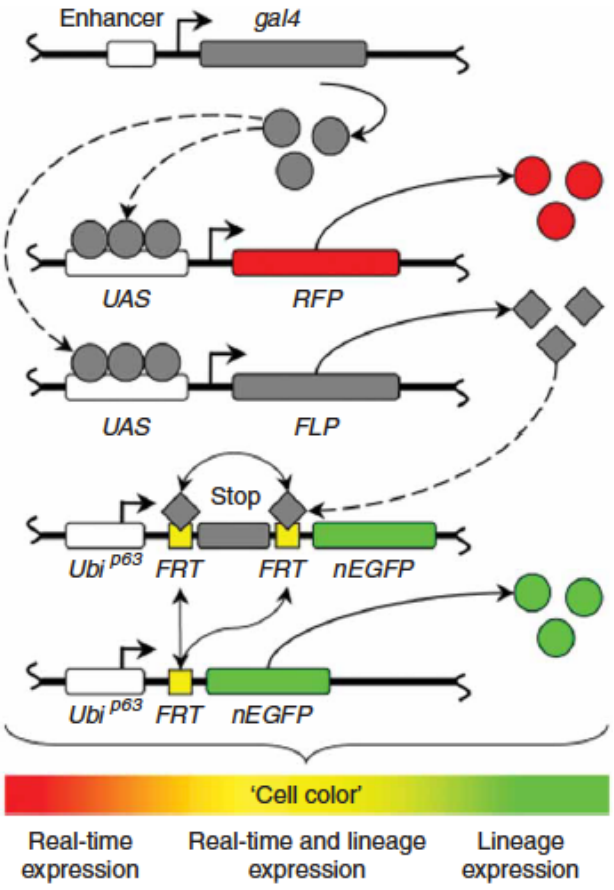

Figure 30. Schematic of the molecular mechanisms of the G-TRACE system. Gal4 activates the expression of RFP and FLP recombinase. Cells expressing FLP recombinase then excise the FRT-flanked stop cassette separating the Ubi-p63E promoter and nuclear EGFP (nEGFP) open reading frame. This initiates expression of EGFP, which is heritably maintained in all daughter cells. (Reprinted from Evans et al., 2009)

CLK protein is expressed exclusively in post-mitotic cells in *Drosophila*, which explains the absence of *Drosophila* cell lines that contain a functional circadian clock

None of the *Drosophila* cell lines currently available are known to possess a functional clock. My initial proposal was to generate a clock cell line by using the UAS-ras^{v12} to immortalize clock cells and UAS-GFP to mark the expression of Gal4 (Mittnacht et al., 1997; Simcox et al., 2008). Various Gal4 lines have been tested (e.g. *Amos*-Gal4, a promoter which is expressed in olfactory sensory neurons (Goulding et al., 2000), was used to generate clock cell lines from clock cell precursors. However, I found that the cells from primary culture either stopped dividing once they began to express GFP or could not attach to the flask to reach the correct confluency. Therefore, no clock cell lines have been generated so far. In tracing the development of pacemaker neurons in the *Drosophila* brain, I found that all the groups of late pacemaker neurons that express *Clk* have already differentiated, as revealed by expression of the differentiated neuron marker ELAV (Figure 12). Consistent with this result, I also found that the all the clock neurons in the brain lack expression of the cell division marker PH3(Singh et al., 2012)(data not shown), suggesting that circadian clock function is not compatible with the dividing cells in the *Drosophila* brain.

Another approach that I used to generate clock cell lines was by using *Drosophila* intestine stem cells (ISCs). The intestinal epithelium is a peripheral clock tissue in *Drosophila*. This tissue is of particular interest with potential for generating clock cell lines because recent work has demonstrated that ISCs, which are important for

regeneration after intestinal damage (Amcheslavsky et al., 2009), contain a circadian clock based on rhythmic PER expression (Karpowicz et al., 2013). However, similar to the situation in the brain, I found that intestinal stem cells that do have the potential to divide (PH3 positive) lack circadian clocks, suggesting that only differentiated cells in *Drosophila* are compatible with circadian clock function. In addition, my work with *Drosophila* cell lines is consistent with work done with mammals where most of their stem cells lack a robust clock until they differentiate (Paulose et al., 2012; Seron-Ferre et al., 2007). Taken together, my work indicates that dividing cells in *Drosophila* don't have functional clocks, and for the first time, explains the absence of *Drosophila* cell lines that contain a functional circadian clock.

However, a number of cell lines in mammals do have circadian clocks, including central clock neurons from the suprachiasmatic nucleus (SCN) and peripheral clock cells from fibroblasts, kidney and smooth muscle (Chalmers et al., 2008; Earnest et al., 1999; Yagita et al., 2001), thereby demonstrating that immortalized clock cells are still capable of clock function in mammals. These made me wonder why mammalian cell lines have circadian clock but available *Drosophila* cell lines lack functional clocks. SCN lines, for instance SCN2.2 line, is immortalized with E1A oncogene, and all cells in this line proliferate. In addition, the cells show varying degrees of differentiation, so circadian rhythm could be measured from a mixed population of cells with different degrees of differentiation (Earnest et al., 1999). It is possible that the rhythm came from only highly differentiated cells, but it is not clear what is the optimal degree or the transition point of differentiation for initiation of functional circadian clocks. On the other hand, a number

of studies on the circadian clock in stem cells has been conducted in mammals. The gene expression studies in embryonic stem cells (ESC) have shown that undifferentiated stem cells do not have a robust circadian clock based on expression of clock genes (Paulose et al., 2012; Seron-Ferre et al., 2007). Although they have cyclic metabolic activity in glucose up-taking, the robust rhythms in mRNA and protein could only be detected after the cells are directed towards neuronal fate (Paulose et al., 2012; Seron-Ferre et al., 2007). However, previous paper on dormant hair-follicle stem cells showed that both clock stem cells and epidermis basal cells showed circadian rhythmicity (Janich et al., 2011). However, the term "functional clocks" is not simply equal to the rhythms in molecular circadian clocks, but may also refer to the rhythms in metabolic or other output pathways. In this case, it is possible that *Drosophila* cell lines are also compatible with circadian clocks that could activate rhythms in specific output pathways. Further studies that address the relationship between cell cycles and circadian clocks, and the generation of new tools to observe rhythms other than molecular rhythms, may provide new insights into this issue.

CLK stabilization of CYC protein initiates circadian clock function

CLOCK requires its heterodimeric partner CYCLE to initiate clock function in canonical groups of brain neurons and peripheral tissues (Rutila et al., 1998), but clock expression can also induce clocks in ectopic locations. It is known that ectopic clocks also need *cyc*, but the mechanism of how *Clk* promotes *cyc* expression in ectopic locations is not known. Previous research showed that *cyc* mRNA is expressed both in clock and non-

clock neurons in the *Drosophila* brain (Nagoshi et al., 2010), however, I observed that CYCLE (CYC) protein is expressed in all CLK-expressing brain pacemaker neurons in *Drosophila* larvae and adults (Figure 14 in Chapter II), indicating that CLK promotes CYC expression in ectopic location. Using vitro experiment, I have demonstrated that CLK-CYC heterodimerization stabilizes CYC via protection from proteasomal degradation (Figure 16). In addition, I found that CYC accumulates specifically in ectopic cells expressing *Clk*, where CYCLE is normally rapidly degraded, indicating that CLK also stabilizes CYC in vivo (Figures 15, 18, 21 in Chapter III). Co-stabilization of heterodimeric transcription factors is not common, but two C/EBP family members, Ig/EBP and CHOP, are stabilized upon heterodimer formation (Hattori et al., 2003), and the *Neurospora* zinc-finger-PAS circadian activator White Collar 1 (WC1) is stabilized by its transcriptional partner White Collar 2 (WC2) upon WC1-WC2 heterodimer formation (Cheng et al., 2002). My data account for the accumulation of CYC only in *Clk*-expressing neurons, and further define the first molecular events required to initiate clock function in *Drosophila*.

In mammals, *Bmal1* mRNA is expressed at high levels, but BMAL1 protein levels are low in the *Clock*-/- mutant (DeBruyne et al., 2006). Given that *Clock* and *Bmal1* are orthologs of *Drosophila Clk* and *cyc*, respectively (reviewed in (Bell-Pedersen et al., 2005; Dunlap, 1999; Young and Kay, 2001), the stabilization of BMAL1 by CLOCK binding may be a conserved property of these proteins. Given that WC2 stabilizes WC1 in *Neurospora*, stabilization of one circadian transcriptional activator by its partner may be a conserved feature of eukaryotic clocks.

Also, CYC protein only accumulates in CLK expressed cells while *cyc* mRNA is widely expressed (Figure 15). One possibility is that CYC is generated and rapidly degraded in many non-clock cell types, but CYC is protected from degradation by other binding partners that are only present, for instance, in response to environmental stress, where CYC complexes mount a transcriptional response to the stressor. Alternatively, *cyc* mRNA may function on its own, independent of producing CYC protein, in non-clock cells. Further studies will be necessary to define the function of *cyc* mRNA in non-clock cells. First, the special expression pattern of *cyc* mRNA should be determined. A *cyc* transcriptional reporter was generated to test *cyc* mRNA spatial expression (data now shown). The 21Kb genomic pacman clone that used to generate GFP-*cyc* transgene was modified by fusing Gal4 coding sequence in frame after the *cyc* translation initiation codon via recombineering and inserting the resulting *cyc*(Gal4) transgene into the attp40 sites. I have used this strategy previously to generate *Clk* (Gal4), which faithfully reports *Clk* gene expression (Figure 26 in Chapter IV). However, none of the *cyc*-Gal4 transgenes (sequencing results of the constructed plasmids are correct and two different genomic pacman clones have been tested) generated was successful to show the spatial pattern of *cyc* mRNA. It is possible that some regions that are essential for *cyc* mRNA transcription exists after the start codon, which is different from other genes transcription (e.g. *Clk, dbt*). Also, it is probable that *cyc* mRNA endogenous level is very low, and cannot be detected by immunostaining even with the reporters. Further experiments will be conducted to generate a *cyc*-Gal4 that can faithfully mark all the *cyc*-expression cells. Once this tool works, it will be used to determine the spatial pattern of *cyc* mRNA.

According to the distribution of *cyc* mRNA, the function of *cyc* in non-clock cells will be analyzed through knockout *cyc* in specific tissues (e.g. RNAi, CRISPR-Cas9(Gratz et al., 2013; Yu and Hardin, 2006) in both normal condition and environment-stress condition.

Defining the genetic architecture and molecular mechanisms required for circadian clock function

Previous studies on ectopic clocks indicated that CLK expression in ectopic location is sufficient to initiate the circadian clocks in a wide range of locations (Zhao et al., 2003). However, later evidence showed that not all ectopic locations could generate clocks, while in some of the ectopic locations PER protein only accumulates, but does not oscillate (Kilman and Allada, 2009). I met the similar situation when I tried to generate ectopic clocks. When *Clk* is overexpressed in ectopic locations, in the DOL region driven by *cry*-Gal4, PER protein oscillates in same phase with sLN_vs (Figure 19) in the LD cycle, however, PER protein does not oscillate in mushroom body neurons (Figure 22). Thus, although CLK-CYC is capable of activating target genes in MB neurons, including the key feedback regulator *per*, the ability of these genes to support feedback loop function was compromised. In my studies, one difference in the drivers used to express *Clk* in non-clock cells is that *cry*-Gal4 is by definition expressed in cells that express CRY, whereas MB neurons lack CRY expression (Benito et al., 2008; Yoshii et al., 2008). Since CRY mediates light entrainment in many pacemaker neurons and is necessary for both light entrainment and clock function in all peripheral tissues that have

been examined (Egan et al., 1999; Emery et al., 1998; Emery et al., 2000; Ivanchenko et al., 2001; Krishnan et al., 2001; Stanewsky et al., 1998), my inability to generate an ectopic clock in MB neurons may be due to the lack of CRY. Indeed, I am excited to find that expressing both *Clk* and *cry* in MB neurons resulted in robust cycling in PER levels during LD, indicative of ectopic clock function (Figure 23), which is in phase with sLN_vs. My results indicate that CLK-CYC activates key feedback repressors to drive ectopic clock function in the presence of CRY. In addition, since ectopic clocks are functional, other clock components including post-translational regulators (e.g. kinases, phosphatases, glygosyltransferases, glycosylases) must be expressed in these ectopic cells (reviewed in (Tataroglu and Emery, 2015)). These post-translational clock regulators are likely to be widely expressed since they are involved in many regulatory pathways, though some of these components could be activated via ectopic *Clk* expression since they contain E-box regulatory elements that are bound by CLK-CYC (Abruzzi et al., 2011).

Although ectopic clocks have robust PER protein rhythms during LD (Figure 19, Figure 23), these rhythms rapidly dampen during DD (Figure 20, Figure 24), while PER rhythms in sLN_vs persist several days in DD (Figure 20, Figure 24). The rapid dampening of PER rhythms in ectopic location is much faster than the rhythm dampening seen in peripheral clocks using *per*-luciferase or *tim*-luciferase reporter assays (Ivanchenko et al., 2001; Krishnan et al., 2001). The inability of DOL cells and MB neurons to sustain clock function in DD likely stems from multiple factors including the suboptimal or non-rhythmic expression of genes that contribute to timekeeping (e.g.

Clk, *cry*, post-transcriptional regulators) and a lack of intercellular coupling that sustains high amplitude rhythms in pacemaker neurons (Mezan et al., 2016; Weiss et al., 2014). These results suggest that the properties of molecular clocks in different cell types, regardless of whether the cells naturally express clock genes or are induced to generate an ectopic clock, will differ depending on their function and gene expression characteristics.

However, there is an apparent contradiction between my results showing that *Clk*, *cyc,* and *cry* are sufficient to initiate circadian clocks and the results of my chapter II showing that CLK and CYC are expressed in late pacemaker neurons for a few days before a clock starts operating. Why aren't CLK and CYC not sufficient to drive clock function in late pacemaker neurons? Is it possible that CRY is the key factor to initiate clock function in these neurons and the absence of functional clock in late pacemaker neurons is due to lacking *cry* expression? Even if that is the case, no PER expression is detected in these late pacemaker neurons before mid-pupal stage (Kaneko et al., 1997). In addition, both at ZT0 and ZT12, PER is lacking in these cells in L3 larval brains, indicating PER is never expressed in these cells. Therefore, the absence of circadian clocks in late pacemaker neurons in L3 larval brains is simply caused by lacking CRY expression. I think the contradiction could be explained from the following aspects. On one hand, the mechanisms underlying the initiation of circadian clock function during development might be different from those in adults. During development, other factors (e.g. JH, *Met*, *gce*) (Di Cara and King-Jones, 2013) that mediate transcriptional or post-translational activities might be needed to initiate the feedback loop during

metamorphosis, while these factors are not needed for the initiation of circadian clocks in adults. On the other hand, the organization of clocks in pacemaker neurons and ectopic clocks are different. *cry* is required for the circadian clock function and light entrainment in peripheral tissues in adult stage, while *cry* in not necessary for the entrainment and clock function in pacemaker neurons (Collins et al., 2006; Emery et al., 1998; Emery et al., 2000; Ivanchenko et al., 2001; Krishnan et al., 2001). The mechanisms underlying circadian clocks in ectopic clocks may be similar to those in peripheral tissues, thus *Clk*, *cyc*, *cry* are sufficient to initiate circadian clock function in ectopic clocks, which is not the case in pacemaker neurons. Therefore, my results from the initiation of clock function in late pacemaker neurons during development and non-clock cells in adults could be compatible.

Taken together, my work defines the genetic architecture and molecular mechanisms required for clock initiation that are likely conserved in other eukaryotes. In addition, my studies suggest that naïve cells might be reprogrammed to clock cells by *Clock*, *cycle* and *cryptochrome* expression. My research will encourage studies to determine if non-clock cells retain a program for activating tissue-specific CLK-CYC output genes, and if naïve cells can be reprogrammed to support oscillator function. Finally, my research will enable the development of clock-containing cell lines in *Drosophila*, which will be a valuable tool for understanding the molecular machinery required for feedback loop function.

A potential method for programming clock function and output in naïve cells

Drosophila skeletal muscle cells do not have a functional clock, while mammalian skeletal muscle cells have a circadian clock (Lefta et al., 2011; McCarthy et al., 2007). If *Clk*, *cyc*, and *cry* expression are induced in *Drosophila* skeletal muscle, can the muscle cells reprogram to become clock cells? Do they still have the conserved CLK-CYC output genes? One future direction is to test whether *Drosophila* skeletal muscles retain the program of clock cells by the induction of *Clk*, *cyc*, *cry*, and then analyze the potential CLK-CYC output genes that might be conserved from mammals. To test the first question, a specific skeletal muscle driver will be used to drive the expression of the *Clk*, *cyc*, *cry*, and then PER protein accumulation and oscillation will be detected. From my work on the genetic architecture of ectopic clock cells (Chapter III), I expect that PER will accumulate upon the expression of *Clk* and *cyc*, and PER protein levels will oscillate after the induction of the 3C genes (*Clk*, *cyc, cry*). If ectopic clocks can be generated by the induction of the 3C genes, *Drosophila* skeletal muscle genes that are orthologs of *Bmal1* specific targets in mammalian skeletal muscles will be identified and analyzed. Analysis will focus on testing whether key CLK-CYC targets have retained E-box sequences and the muscle cell output program is conserved in *Drosophila*. However, given that *Drosophila* have diverged from vertebrates around 900 millions of years ago (Peterson et al., 2004), it is unlikely that key CLK-CYC targets retain E-box sequence to support the muscle cell output program. Although RNA-seq data in mammalian skeletal muscles shows that skeletal muscle tissue specific genes have circadian rhythms in mRNA expression (Lefta et al., 2011; McCarthy et al., 2007), ChIP-seq by *Bmal1* data in

mammalian skeletal muscle is still unavailable. Future ChIP-seq experiments will determine whether E-boxes upstream of rhythmically expressed genes in muscle cells are bound by CLOCK-BMAL1.

In addition, my research on programming naïve cells to have a clock will enable generation of *Drosophila* cell lines that have a functional circadian clock. My work outlines two critical requirements that may be useful for creating clock-containing cell lines. First, *Clk*, *cyc*, *cry* should be expressed, and second, cells need to differentiate once the 3C genes are expressed. For future directions, *Drosophila* cells lines (e.g. SCN neuron cell line) will be transfected with 3C genes (*Clk*, *cyc*, *cry*) that are driven by inducible promoters (e. g. pMT). 3C gene expression will be induced once the cells differentiate. In addition, *per*-luciferase will be transfected and used as marker to detect the oscillator function in the cells. If the cell line is capable of a functional clock, I expect *per* mRNA to cycle.

Characterize the role of the *Clk* gene in the development of brain pacemaker neurons in *Drosophila*

Previous research showed that PDF in sLN_vs is absent in Clk^{Jrk} mutants (Park et al., 2000). However, it is not known whether *Clk* promotes PDF expression in the cells or *Clk* regulates the development of the sLN_vs. I found that both sLN_v cell bodies and the dorsal projection are absent in the Clk^{out} mutant, suggesting that *Clk* is required for the sLN_vs development (Figure 27, 28 in Chapter IV). It was also possible that the *Clk* promoters were turned off in Clk^{out} adults. If the *Clk* promoters were turned off, this

should not impact the MZ520 result. Since the cells are not detectable using either driver in adults, it argues that it is not a promoter downregulation issue, but more likely a cell viability issue.

To confirm that these cells are actually missing in the Clk^{out} adult brain, the Gal4 technique for real time and clonal expression (G-TRACE) system cell tracing method will be employed (Evans et al., 2009). After initiating the cell tracing tool by Gal4, GFP expression will be detected in all the subsequent daughter cells (Figure 30). In future studies, the G-trace tool will be used to detect the presence of the sLN_vs in the Clk^{out} null mutant. *Clk21*-Gal4 and MZ520-Gal4 will initiate the G-trace tool in early development, and GFP expression in sLN_vs will be assayed in adults. The absence of GFP staining in the sLN_vs in the Clk^{out} adult brains will indicate that the sLN_v cells are missing in adults, which confirms that *Clk* is responsible for the maintenance of the sLN_v. These results also make sLN_vs a unique group of pacemaker neurons.

The comparison of the sLN_v in larvae and adults indicates a special role of *Clk* in sLN_v development. For the first time, my evidence showed that *Clk* is not required for the original production of sLN_v, but is necessary for the maintenance of sLN_vs. sLN_v may be eliminated by apoptosis without *Clk* during late larval development. Recent data indicates that *Clk* has a special role in neuron protection, which helps maintain a subset of dopaminergic neurons by protecting them from oxidative stress and cell death (Vaccaro et al., 2017). In addition, studies showed that *Clk* post-transcription control plays important roles in the proper development of pacemaker neurons, especially the PDF-expressing cells including sLN_vs (Lerner et al., 2015). The deletion of the bantam

binding site in the *Clk* 3'UTR contributes to the variable and increased number of PDF-expressing cells, and the number of PDF-expressing cells does not increase until the pupal stage. The time window for increasing the number of PDF-expressing cells coincides with the missing of sLN_vs in the Clk^{out} mutant of my study. Whether there is a relationship between these two events needs to be tested further.

Taken together, my work on the development and function initiation of clock cells, and the formation of clock network will help solve important questions in the development of circadian clock, and will hopefully stimulate more exciting research on the development of circadian clock in other organism, and the evolution of circadian clock in the future!

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APPENDIX A

NOMENCLATURE

CLK	CLOCK
CYC	CYCLE
PER	PERIOD
TIM	TIMELESS
CRY	CRYPTOCHROME
DBT	DOUBLE-TIME
SGG	SHAGGY
VRI	VRILLE
PDP1	PAR Domain Protein 1
JET	JETLAG
DNs	dorsal neurons
sLN_vs	small ventral lateral neurons
lLN_vs	large ventral lateral neurons
LN_ds	dorsal lateral neurons
LPNs	lateral posterior neurons
ZT	zeitgeber time
CT	circadian time
LD	light: dark
DD	constant darkness