Transcriptional and Translational Mechanisms Controlling Circadian Rhythms in Drosophila: A Dissertation

Jinli Ling
University of Massachusetts Medical School

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Transcriptional and translational mechanisms controlling circadian rhythms in *Drosophila*

A Dissertation Presented

By

Jinli Ling

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

DOCTOR OF PHILOSOPHY

June 14th, 2013

Program in Neuroscience
Transcriptional and translational mechanisms controlling circadian rhythms in *Drosophila*

A Dissertation Presented

By

Jinli Ling

The signatures of the Dissertation Defense Committee signify completion and approval as to style and content of the Dissertation

__________________________

Patrick Emery, Ph.D., Thesis Advisor

__________________________

William Schwartz, M.D., Member of Committee

__________________________

Hong-Sheng Li, Ph.D., Member of Committee

__________________________

Neal Silverman, Ph.D., Member of Committee

__________________________

F. Rob Jackson, Ph.D., Member of Committee

The signature of the Chair of the Committee signifies that the written dissertation meets the requirements of the Dissertation Committee

__________________________

David Weaver, Chair of Committee

The signature of the Dean of the Graduate School of Biomedical Sciences signifies that the student has met all graduation requirements of the school

__________________________

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

Program in Neuroscience
June 14th, 2013
Dedicated to my husband Zhifeng,
and my beloved family in China.
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ABSTRACT

Circadian rhythms are self-sustained 24-hour period oscillations present in most organisms, from bacteria to human. They can be synchronized to external cues, thus allowing organisms to anticipate environmental variations and optimize their performance in nature.

In *Drosophila*, the molecular pacemaker consists of two interlocked transcriptional feedback loops. CLOCK/CYCLE (CLK/CYC) sits in the center and drives rhythmic transcription of *period (per)*, *timeless (tim)*, *vrille (vri)* and *PAR domain protein 1 (Pdp1)*. PER and TIM negatively feedback on CLK/CYC transcriptional activity, forming one loop, while VRI and PDP1 form the other by regulating Clk transcription negatively and positively, respectively. Posttranscriptional and posttranslational regulations also contribute to circadian rhythms. Although much has been learned about these feedback loops, we are still far from understanding how stable 24-hour period rhythms are generated.

My thesis work was to determine by which molecular mechanisms *kayak-α (kay-α)* and *Ataxin-2 (Atx2)* regulate *Drosophila* circadian behavior. Both genes are required for the precision of circadian rhythms since knocking down either gene in circadian pacemaker neurons results in long period phenotype.

The work on *kay-α* constitutes the first half of my thesis. We found that the transcription factor KAY-α can bind to VRI and inhibit VRI’s repression on the Clk promoter. Interestingly, KAY-α can also repress CLK’s transcriptional activity on
its target genes (e.g., *per* and *tim*). Therefore, KAY-α is proposed to bring precision and stability to the molecular pacemaker by regulating both transcriptional loops.

The second half of my thesis focuses on ATX2, an RNA binding protein whose mammalian homolog has been implicated in neurodegenerative diseases. We found that ATX2 is required for PER accumulation in circadian pacemaker neurons. It forms a complex with TWENTY-FOUR (TYF) - a crucial activator of PER translation - and promotes TYF’s interaction with Poly(A)-binding protein. This work reveals the role of ATX2 in the control of circadian rhythms as an activator of PER translation, in contrast to its well-established role as a repressor of translation. It also further demonstrates the importance of translational regulation on circadian rhythms. Finally, it may help understanding how ATX2 causes neuronal degeneration in human diseases.
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LIST OF ABBREVIATIONS

3' UTR: 3' untranslated region
ALS: Amyotrophic Lateral Sclerosis
aMe: accessory medulla
ATX2: Ataxin-2 (*Drosophila*)
ATXN2: ataxin-2 (*human*)
bHLH: basic-helix-loop-helix
bZip: basic leucine zipper
ch: chordotonal
CKII: casein kinase II
CLK: CLOCK
CRY: CRYPTOCHROME
CT: circadian time
CWO: CLOCKWORK ORANGE
CYC: CYCLE
DBT: DOUBLE-TIME
DD: constant darkness
DN1s: Dorsal Neurons 1
DN2s: Dorsal Neurons 2
DN3s: Dorsal Neurons 3
E-cells: evening cells
eIF4F: eukaryotic translation initiation factor F
E-peak: evening peak
es: external sensory
FRP: free running period
H-B eyelet: Hofbauer-Buchner eyelet
hid: head involution defective
KAY: KAYAK
LD: 12hr light: 12hr dark cycle
LL: constant light
ILNvs: large ventral Lateral Neurons
LNds: dorsal Lateral Neurons
LPNs: Lateral Posterior Neurons
Lsm domain: Sm like domain
LsmAD: Lsm-associated domain
M-cells: morning cells
miRNA: microRNA
M-peak: morning peak
nocte: no circadian temperature entrainment
norpA: no receptor potential A
PABP: Poly(A)-binding protein
PAS: PER-ARNT-SIM
PDF: pigment dispersing factor
Pdp1 ε: PAR-domain protein 1ε
PER: PERIOD
polyQ: polyglutamine
PP1: protein phosphatase 1
PP2A: protein phosphatase 2A
RNAi: RNA interference
SAD: seasonal affective disorder
SCA2: Spinocerebellar Ataxia type 2
SCN: Suprachiasmatic Nucleus
SG: stress granules
SGG: SHAGGY
sLNvs: small ventral Lateral Neurons
TC: temperature cycles
TPR: temperature preference rhythms
UAS: upstream activator sequence
PREFACE

Parts of the dissertation have been published.

CHAPTER II has been published as:

CHAPTER III has been published as:
* These authors contributed equally to this work.

APPENDIX has been published as:

Contributions of authors are addressed at the beginning of each chapter.
CHAPTER I

Introduction

In this chapter, I will first introduce the basic concepts and features of circadian rhythms and the advantages of using *Drosophila* as a model organism. Then I will review the current knowledge about the molecular mechanisms underlying circadian rhythms, upon which Chapter II and III are based and contribute to. At last, the clock neurons and the synchronization of circadian rhythms by light and temperature will be introduced to give a comprehensive view of the circadian system and provide additional background for Chapter II, III and appendix.

A. Biological clocks and circadian rhythms

As the Earth rotates about its axis, light and temperature fluctuate in a rhythmic fashion every 24 hours. Organisms ranging from cyanobacteria to human (Ouyang et al., 1998; Czeisler et al., 1999), have evolved biological clocks to accommodate their physiology and behavior to these daily environmental changes. The earliest reports on an endogenous clock can be traced back to 1729, when Jean-Jacques d’Ortous de Mairan observed that the daily leaf movements of heliotrope plants continue in constant darkness. Then in 1832, Augustin Pyramus de Candolle found that the rhythm actually run with an
approximately 22-23 hour period in constant light which is significantly shorter than the Earth’s day/night cycle, strongly indicating the existence of endogenous clocks. In 1959, Franz Halberg termed these daily rhythms as circadian (Latin: about a day) to emphasize their endogenous nature. By 1960s, circadian rhythms had been found in various species, including insects, birds, rodents, primates and human. It was a descriptive era of circadian rhythms, yet little was known about its nature further than Erwin Bünning’s finding in 1935 that the period length is inheritable in bean plants and thus circadian rhythms have a genetic basis.

The understanding of circadian rhythms was revolutionized by Ronald Konopka and Seymour Benzer, who identified three clock mutants in fruit fly with long, short period or arrhythmia in 1971 and mapped the mutants to the same gene, period (per) (Konopka and Benzer, 1971). This marks the beginning of the molecular era of circadian rhythms. Shortly after that, the gene frequency was identified as essential for circadian rhythms in Neurospora crassa (Feldman and Hoyle, 1973). Subsequently, mutants affecting circadian rhythms in hamsters and mice were found (Ralph and Menaker, 1988; Vitaterna et al., 1994) and the genes were identified (Antoch et al., 1997; Lowrey et al., 2000), and the list went on to include both prokaryotic and eukaryotic systems, e.g., cyanobacteria, fungi, plants, insects and mammals.
A functional biological clock consists of three components: input pathways, pacemaker and output pathways. Pacemaker is an oscillator or a network of oscillators. It generates ~24-hour circadian rhythms and translates this temporal information into downstream behavioral and physiological rhythms through output pathways. For the pacemaker to be synchronized with local environment, information such as daily variation of light and temperature (Zeitgeber, German for “time giver”) is incorporated into the pacemaker via input pathways.

There are three fundamental features of the biological clock: 1) it is self-sustained or endogenous- the rhythm persists even without environmental inputs; 2) it is entrainable- the oscillator can be reset or phase shifted by transient exposure to time cues such as light and temperature, and be synchronized to an external cycle; 3) it is temperature compensated- the period of circadian rhythms changes very little during different temperatures within the organism's physiological range.

The biological clocks impact cellular functions, physiological processes and behaviors. For animals, clocks allow them to anticipate environmental variations and thus optimize their performance in nature. For instance, cyanobacteria strains with functional clocks have reproductive advantages over stains that have defective clocks in rhythmic environment (Woelfle et al., 2004). As for humans, though survival in the wild is no longer critical, circadian rhythms still affect our lives and have immense medical implications (Edery, 2000). Firstly,
acute or chronic changes of time cues that disturb the circadian timing system could have adverse effects on otherwise healthy individuals (e.g. nurses on shift work schedule are more likely to misdiagnose and have more severe depressive symptoms) (Gold et al., 1992; Asaoka et al., 2013). Secondly, circadian rhythms could impact the efficacy of certain drugs since various physiological and behavioral variables manifest daily rhythms (Klevecz et al., 1987; Levi, 1999). By optimizing the timing of drug delivery, it is possible to maximize the therapeutic potential of drugs and minimize their side effects. For example, drugs used in chemotherapy for cancer treatment affects the function and replication of both normal and malignant cells. If drugs are administrated at times when normal cells are less likely to undergo DNA synthesis, higher levels of drugs can be tolerated and the effectiveness of the therapy can be increased (Klevecz et al., 1987; Smaaland, 1996). Last but not the least, malfunctions in the circadian timing system are associated with several disorders, including chronic sleep disturbance, manic-depression and seasonal affective disorders (SAD or winter depression) (Copinschi et al., 2000), the symptoms of which can be alleviated by light treatment (Terman et al., 1995). Therefore, it is of great importance to fully understand the mechanisms of circadian rhythms.

B. Using *Drosophila* as a model organism to study circadian rhythms
Ever since the discovery of the *period* mutant flies, *Drosophila melanogaster* has been one of the most popular and best studied organisms for circadian rhythms. It manifests circadian rhythms in eclosion (adult emergence from pupal case), locomotor behavior, as well as courtship behavior (Fujii et al., 2007). Molecular oscillations have been found in fly heads as well as in periphery body parts such as wings, legs, and Malpighian tubules (Emery et al., 1997; Giebultowicz and Hege, 1997; Plautz et al., 1997).

The advantage of using *Drosophila* includes but is not limited to the following aspects. First, compared to mammals, it has a compact genome (1.8X10^8 v.s. 2.9X10^9 base pairs) with much less gene redundancy.

Second, its robust circadian locomotor behavior and short generation time (10-14 days at 25°C) facilitate genetic screens aimed at understanding various aspects of circadian rhythms. Figure 1.1 shows the locomotor behavioral assay commonly used in laboratories. Flies manifest bimodal locomotor behavior in a standard 12hr light: 12hr dark cycle (LD), with a morning anticipation or M-peak before the light on and an evening anticipation or E-peak before light off. This behavioral rhythm persists in constant darkness (DD) indicating its endogenous nature. The free running period (FRP) or simply period, is defined as the time to complete one cycle in constant conditions. It is usually measured from peak to peak or trough to trough. This behavior assay enables screens for long, short
period or arrhythmia, defects in morning or evening anticipation, and abnormal phasing of the peaks.

Third, powerful genetic approaches allow the manipulation of gene expression and neuronal properties with great spatial and temporal resolution. One of the widely used techniques is the GAL4/upstream activator sequence (UAS) binary system (Brand et al., 1994). Transgenic flies expressing GAL4- the yeast transcription factor- under the control of a promoter are crossed to transgenic lines carrying a gene of interest controlled by UAS- the GAL4 binding site- to generate progeny carrying both transgenes. These progeny will express the gene of interest in a spatial pattern determined by the promoter controlling GAL4. The expression pattern can be further refined by co-expressing GAL80, a repressor of GAL4, under a different promoter (Lee and Luo, 1999). The temporal control of gene expression can be achieved using GAL80ts, a temperature sensitive form of GAL80, which is inactive above 29ºC and active below 18ºC (McGuire et al., 2004). An alternative to the GAL4/UAS system is the Q-system which consists of the QF transcription factor, the QS suppressor and the QUAS that contains binding sites for QF. QF binds to the QUAS and induces downstream gene expression, while QS inhibits QF (Potter et al., 2010). The temporal control of the Q-system can be achieved by feeding flies a diet containing quinic acid which inhibits the QS repressor (Potter et al., 2010). The GAL4/UAS system and/or the Q-system combined with the RNA interference
(RNAi) enables tissue-specific knockdown of gene expression, which is widely used in genetic screens in *Drosophila*.

**C. The molecular pacemaker of *Drosophila***

1. **The two interlocked transcriptional feedback loops.**

During the past 40 years, dozens of genes have been found regulating circadian rhythms in *Drosophila melanogaster*. Interplay of these genes constitutes an elaborated map of the molecular pacemaker. In the center of the map are two interlocked transcriptional feedback loops (Figure 1.2).

In one loop, CLOCK (CLK) (Allada et al., 1998) and CYCLE (CYC) (Rutila et al., 1998), containing basic-helix-loop-helix (bHLH) DNA-binding domains and PER-ARNT-SIM (PAS) dimerization domains, form a heterodimer, bind to the E-boxes in the promoter regions of *period (per)* and *timeless (tim)*, and promote their transcription (Darlington et al., 1998). PER and TIM form a complex in the cytoplasm, with TIM stabilizing PER (Vosshall et al., 1994; Gekakis et al., 1995). After translocation to the nucleus, the PER/TIM complex inhibits its own gene transcription by repressing the activity of CLK/CYC, probably by reducing CLK/CYC’s affinity to the E-boxes (Darlington et al., 1998; Lee et al., 1999; Yu et al., 2006; Menet et al., 2010). This loop generates rhythmic expression of *per* and *tim* and is particularly important for the rhythm generation.
A modulator of this loop is CLOCKWORK ORANGE (CWO), a transcription factor with an Orange domain commonly found in bHLH repressors. CWO is rhythmically expressed under the transcriptional control of CLK/CYC. It recognizes the same E-boxes as CLK/CYC and thus represses CLK/CYC target genes by competing for E-box binding (Kadener et al., 2007; Lim et al., 2007; Matsumoto et al., 2007). cwo mutants have long periods, and mRNA profiles of CLK/CYC targets (e.g., *per*, *tim*, *vrille*) manifest reduced amplitude with higher trough values. It was hypothesized that CWO functions preferentially in the late night and in synergy with PER to terminate CLK/CYC mediated activation, and is required for generating high amplitude transcriptional oscillations (Kadener et al., 2007; Lim et al., 2007; Matsumoto et al., 2007; Richier et al., 2008).

In the second loop, CLK/CYC activates transcription of *vrille* (*vri*) and *PAR-domain protein 1ε* (*Pdp1ε*), both encoding basic leucine zipper (bZip) transcription factors (Blau and Young, 1999; McDonald and Rosbash, 2001; Cyran et al., 2003). mRNA expressions of *vri* and *Pdp1ε* reach peaks in the early night and late night respectively, ~ 3 hours apart. In the cytoplasm, VRI accumulates first and enters the nucleus to repress *Clk* transcription by directly binding to its promoter region (named V/P box); PDP1ε accumulates later when VRI dissipates, and promotes *Clk* transcription by competing with VRI for the access to the V/P box (Cyran et al., 2003; Glossop et al., 2003). This loop controls rhythmic transcription of *Clk*. Although the function of *Clk* mRNA oscillation does not seem to be critical for 24-hour rhythms and remains
uncertain (Kim et al., 2002), flies do require VRI and PDP1ε for proper circadian behaviors: VRI overexpression and PDP1ε mutant flies manifest long periods or arrhythmicity, with disrupted PER and TIM cycling (Blau and Young, 1999; Cyran et al., 2003; Zheng et al., 2009).

2. Posttranslational regulation of the molecular pacemaker.

The transcriptional feedback loops described above are commonly thought to be the major forces in generating rhythms. However, posttranscriptional and posttranslational regulations (e.g., mRNA or protein stability, protein activity, and timing of protein translocation) also contribute to circadian rhythms.

A set of kinases and phosphatases have been found to regulate the exact pace of the feedback loops posttranslationally. For example, PER protein stability is regulated by the kinase DOUBLE-TIME (DBT) - a homolog of human casein kinase 1ε/δ, casein kinase II (CKII), proline-directed kinase NEMO and protein phosphatase 2A (PP2A). DBT and CKII phosphorylate PER and promote its phosphorylation-induced degradation (Kloss et al., 1998; Price et al., 1998; Lin et al., 2002; Akten et al., 2003). In contrast, phosphorylation by NEMO delays the DBT-dependent progressive phosphorylation events at sites required for degradation recognition thus may stabilize PER (Chiu et al., 2011), and PP2A stabilizes PER by dephosphorylation (Sathyarayanan et al., 2004). In the nucleus, DBT and PP2A also regulate CLK phosphorylation and stability (Kim
and Edery, 2006; Yu et al., 2006). TIM phosphorylation is regulated by SHAGGY (SGG) and protein phosphatase 1 (PP1) (Martinek et al., 2001; Fang et al., 2007). Translocation of PER/TIM is promoted by SGG and CKII (Martinek et al., 2001; Lin et al., 2002).

3. Posttranscriptional regulation of per mRNA stability and splicing.

Regulation of mRNA stability also exists in pacemaker machinery. When per mRNA profile was compared with its transcriptional rate curve, it seems that the transcription alone was not sufficient to explain the mRNA cycling pattern (So and Rosbash, 1997). Indeed, per mRNA profile showed a significant delay during the rising phase (ZT9 to ZT15, where ZT0-Zeitgeber Time 0- refers to the light on transition in LD) but coincide with the transcriptional curve during the declining phase (after ZT15), suggesting a temporal regulation of per mRNA half-life: per mRNA is more stable in the rising phase than in the declining phase (So and Rosbash, 1997). Posttranscriptional regulation was also observed with a promotorless per transgene (7.2) which shows no circadian transcription, but has a 2-3 fold cycling of mRNA (Frisch et al., 1994; So and Rosbash, 1997). Moreover, Suri et al. found the heat-shock induced TIM could increase per mRNA levels. This increase cannot be fully explained by the transcriptional mechanism nor does it depend on the transcription factor CYC, and it may thus be achieved through a posttranscriptional mechanism such as mRNA stabilization (Suri et al., 1999).
Regulation of *per* mRNA splicing was observed by Majercak *et al.* (1999). They found a thermosensitive splicing of an intron in the 3’ untranslated region (3’UTR) of *per* mRNA: low temperature enhances the splicing of this intron and thus advances *per* mRNA accumulation, which contributes to the accelerated PER protein accumulation and probably leads to the advanced phase of the evening peak activity under cold temperature (Majercak *et al.*, 1999).

4. Translational regulation of the molecular pacemaker.

In addition to the posttranslational and posttranscriptional regulation described above, accumulating evidence suggest that translational control also plays a role in circadian oscillations, and may be especially important in circadian pacemaker neurons, the small ventral Lateral Neurons (Lim *et al.*, 2011; Bradley *et al.*, 2012). The gene *twenty four* (*tyf*) was recently identified as a regulator of *per* translation (Lim *et al.*, 2011). TYF expression was primarily found in pacemaker neurons, and *tyf* mutant has weak rhythms but long periods with significantly reduced PER level. TYF is associated with the 5’-cap-binding complex, poly(A)-binding protein (PABP) and *per* transcripts. Moreover, TYF activates reporter luciferase expression when tethered to a reporter RNA, and the activation is significantly more efficient when *per* 3'UTR is present. Thus, it was proposed that TYF regulates circadian period by promoting *per* mRNA’s cap-dependent translation, presumably through facilitating the circularization of *per* mRNA (Lim *et al.*, 2011). Shortly after this study, an RNAi screen targeting
translation and RNA factors was conducted to further investigate the role of translation in the *Drosophila* circadian system, and the noncanonical translation initiation factor NAT1 (an eIF4G paralog) was found to be important in pacemaker neurons for *per* translation, probably through a cap-independent mechanism (Bradley et al., 2012) which usually take place under conditions of cellular stress (Marr et al., 2007). Thus, both cap-dependent and cap-independent translational regulations contribute to the precision of the *Drosophila* pacemaker. Besides *Drosophila*, translational regulations were also suggested in mammals: mouse LARK was found to bind the 3’UTR of *Per1* and was indicated to activate *Per1* translation in a cap-dependent manner (Kojima et al., 2007).

Taken together, a functional and precise pacemaker requires transcriptional feedback loops as well as posttranscriptional and posttranslational regulations. Despite the elaborated “clock map” we have achieved now, our understanding of the molecular pacemaker is far from complete.

5. Ataxin-2, a regulator of mRNA metabolism and translation, and its link to circadian rhythms.

Expansion of a polyglutamine (PolyQ) repeat in human ataxin-2 (ATXN2) protein is responsible for Spinocerebellar Ataxia type 2 (SCA2) and is associated with increased risk of Amyotrophic Lateral Sclerosis (ALS) (Lastres-Becker et al., 2008; Elden et al., 2010). ATXN2 contains a Sm like domain (Lsm domain) and a Lsm-associated domain (LsmAD). The Lsm domain is capable of RNA binding
and Lsm proteins have been shown to regulate pre-mRNA splicing and mRNA degradation (He and Parker, 2000). ATXN2 also has a PAM2 motif, capable of binding Poly(A)-binding protein (PABP), a regulator of translation initiation and mRNA decay (Kahvejian et al., 2005). Although how pathogenic expansions in ATXN2 causes SCA2 is far from being well understood, accumulating evidence from analysis of ATXN2 and several closely related homologs suggest a role of this gene family in regulating mRNA metabolism and translation.

For example, ATX-2, the *C. elegans* ortholog of ATXN2, forms a complex with poly(A)-binding protein and plays a role in GLD-1- and MEX-3-mediated translational repression in the germline (Ciosk et al., 2004). The yeast ATXN2 homolog Pbp1 was also found in association with yeast poly(A)-binding protein - Pab1, and disruption of Pbp1 suppresses the lethality caused by Pab1 deletion. Since Pab1 appears to promote translation, Pbp1 was proposed to negatively regulate translation by repressing Pab1 (Mangus et al., 1998). Moreover, Ralser *et al.* (2005) generated a comprehensive protein interaction map for Pbp1 based on publicly available database, unveiling the potential functions of Pbp1 in diverse RNA-processing pathways, e.g. mRNA export and degradation (Ralser et al., 2005). This map provided insightful information on human ATXN2 function and some interactions were indeed recapitulated using ATXN2 in mammalian cells. For instance, ATXN2 was found associated with DEAD/H-box RNA helicase DDX6, a component of P-bodies and stress granules (SG) - structures regulating mRNA turnover and translation (Nonhoff et al., 2007). Altered level of
ATXN2 interferes with P-body and SG assembly. Notably, both P-bodies and SG have been linked to translational repression via microRNAs (miRNAs), a class of small non-coding RNAs that base pair with complementary sequences usually in the 3'UTR of mRNA and induce gene silencing (Leung et al., 2006; Eulalio et al., 2007). Indeed, genetic analysis suggests that Ataxin-2 (ATX2), the *Drosophila* homolog of ATXN2, functions with miRNA-pathway components—Argonaute1 and Me31B—for long-term olfactory habituation in *Drosophila*. Moreover, ATX2 is required for optimal repression of several miRNA target mRNAs *in vivo* (McCann et al., 2011). Several models have been proposed for ATX2’s function in translational repression (e.g. ATX2 disrupts PABP and eIF4G binding and thus inhibits translation initiation, or ATX2 via Me31B recruits the deadenylase complex which may enhance mRNA recruitment to P-bodies for storage or degradation) (Satterfield and Pallanck, 2006; McCann et al., 2011). Taken together, studies on ATXN2 and its homologs converged to suggest its role in RNA metabolism and translational repression.

A potential link between ATXN2 and circadian rhythms comes from the fact that patients affected with SCA2 suffer from sleep disruptions, particularly disruption of rapid eye movement sleep (REM sleep) which is under circadian regulation (Rodriguez-Labrada et al., 2011; Velazquez-Perez et al., 2011). Specifically, patients with SCA2 have reduced REM sleep percentage and REM density, both of which are closely related to the increase in ataxia scores (Velazquez-Perez et al., 2011). Moreover, significant disruption of REM sleep
was also found in presymptomatic gene carriers, making REM sleep pathology a prominent herald sign of SCA2 as well as a potentially important link to understand disease pathophysiology (Rodriguez-Labrada et al., 2011). Interestingly, REM sleep is known to be regulated by circadian rhythms: the time course of REM sleep is strongly dependent on circadian phase with the maximum REM sleep located shortly after the minimum of the endogenous core body rhythms (Dijk and Czeisler, 1995). In addition, circadian timing of REM sleep was shown to be coupled with oscillators in the dorsomedial SCN of rats (Lee et al., 2009). Thus, SCA2 patients with disrupted REM sleep reveal a potential link between ATXN2 and circadian rhythms.

With this potential link, the function of ATXN2 in circadian rhythms becomes very intriguing and could be helpful to understand the sleep symptoms as well as the pathogenesis of SCA2. Therefore, I started to investigate whether and how ATX2 regulates circadian rhythms in *Drosophila* (See chapter III).

**D. The clock neurons in *Drosophila***

**1. Identification of clock neurons.**

In *Drosophila melanogaster*, rhythmic expression of *per* and *tim* are found in approximately 150 neurons in the adult central brain (Kaneko et al., 1997; Kaneko and Hall, 2000; Shafer et al., 2006). They are important for the rhythmic
locomotor activity, and are referred to as clock neurons (Taghert and Shafer, 2006). Based on their anatomical location and size, these neurons are grouped into large and small ventral Lateral Neurons (ILNvs and sLNvs), dorsal Lateral Neurons (LNds), 3 groups of Dorsal Neurons (DN1s, DN2s and DN3s) and Lateral Posterior Neurons (LPNs) (Figure 1.3).

This anatomical grouping is only very superficial, as many of these groups are heterogeneous, as revealed by neurohistochemical and genetic studies. Examples of heterogeneity within anatomical groups is revealed by the expression patterns of the neuropeptide pigment dispersing factor (PDF) and the blue light photoreceptor CRYPTOCHROME (CRY). PDF is required for free-running behavior (Renn et al., 1999) and CRY mediates circadian light response (Emery et al., 1998; Stanewsky et al., 1998). Among the 5 sLNvs of each brain hemisphere, 4 neurons express PDF but one is PDF negative (the so called 5th sLNvs). PDF expression is also found in ILNvs, but not in LNds or any of the dorsal neurons (Helfrich-Forster, 1995). CRY is expressed in almost all clock neuron groups, except the 2 DN2s (no or very weak expression) (Emery et al., 2000; Klarsfeld et al., 2004; Benito et al., 2008; Yoshii et al., 2008). While all LNvs express CRY, only half of the LNds are strongly CRY positive, with the rest expressing little or no CRY. The DN1s consists of approximately 17 neurons in each hemisphere. The DN1s- two neurons located in an anterior position and thus separated from the others - show CRY immunoreactivity, while the rest of the DN1s are heterogeneous in CRY expression. The DN3 cluster, about 40
neurons in each hemisphere, has only two neurons labeled by cry-GAL4 line #13 (Helfrich-Forster et al., 2007a).

The arborizations of the clock neurons form a complex network (Figure 1.3) (Helfrich-Forster et al., 2007b). Interconnection of clock neurons allows crosstalk between them. For example, the sLNvs send PDF positive fibers to the dorsal protocerebrum and terminate close to the DN1s and DN2s. Most DNs indeed respond acutely to PDF (Shafer et al., 2008). In addition to interconnections within the circadian neuronal network, clock neurons also project to other brain regions. The two main targets are the accessory medulla (aMe) and the dorsal brain (Helfrich-Forster et al., 2007b). The aMe appears to be the principal pacemaker center of insects and the dorsal brain houses the hormonal center (pars intercerebralis and pars lateralis) and also has connections to most brain regions (Homberg et al., 2003; Siga, 2003). Therefore, these connections could provide information flow from upstream input to clock neurons and to downstream output.

2. Functions of clock neurons.

Among the ~150 clock neurons, the LNvs are the most important pacemaker neurons. They were also the first groups of clock neurons being studied, largely due to the availability of GAL4 drivers that enable manipulation of these neurons as well as the PDF staining that nicely traces these neurons. Pdf-GAL4 combined with UAS-head involution defective (hid) ablates PDF
expressing LNvs including 4 sLNvs and the ILNvs. As a result, flies lose the M-peak in LD and become arrhythmic in DD, demonstrating the necessity of the LNvs in generating circadian behavior (Renn et al., 1999; Stoleru et al., 2004). The sufficiency of LNvs was demonstrated by Grima et al. when they restored per expression only in the PDF expressing LNvs in per\(^0\) flies, and found it was sufficient to generate the M-peak and 24-hour rhythmic locomotor behavior in DD (Grima et al., 2004). The responsible LNvs were even narrowed down to the PDF expressing sLNvs: PER restoration in only the sLNvs (R6-GAL4), but not the ILNvs (C929-GAL4) is sufficient to restore M-peak and rhythmicity in DD (Cusumano et al., 2009). Therefore, the PDF expressing sLNvs are referred as M-cells for their responsibility of generating M-peak, and as pacemaker neurons for they are necessary and sufficient to generate rhythms in constant conditions.

The M-cells require PDF for their circadian function. Pdf\(^0\) flies, like M-cell ablated flies, have no M-peak, and are predominantly arrhythmic in DD with rare escapers having short periods (Renn et al., 1999). Levels of PDF can be reduced posttranslationally by VRI or promoted posttranscriptionally by CLK/CYC and PDP1 by an unknown mechanism (Blau and Young, 1999; Park et al., 2000; Cyran et al., 2003). PDF staining exhibits daily cycling within the terminals of projections from sLNvs to the vicinity of DNs (Park et al., 2000), which is thought to reflect rhythmic secretion. Actually, most DNs respond acutely to PDF peptide (Shafer et al., 2008). It is thought that PDF is required to coordinate the phase
and amplitude of circadian rhythms among diverse circadian neurons (Peng et al., 2003; Lin et al., 2004).

Similar to the M-cells, the E-cells were also sorted out based on ablation and PER rescue experiments: ablation of LNds, 5th-sLNv and 2DN1s using cry-GAL4; Pdf-GAL80 totally abolished E peaks (Stoleru et al., 2004); limiting PER expression in LNds and 5th-sLNv is sufficient to restore the E-peaks (Helfrich-Forster et al., 2007b). In addition, the phase of PER and TIM oscillations in the larger LNds and the 5th s-LNv has been associated with the phase of the evening peak (Rieger et al., 2006; Bachleitner et al., 2007). Therefore, the E-cells consist of LNds, the 5th sLNv, and possibly a subset of DN1s. Interestingly, in constant light conditions (LL), the E-cells and a subset of DN1s are sufficient to generate robust rhythmicity (Murad et al., 2007; Stoleru et al., 2007).

The functions of DNs and LPNs are less well defined, but at least a subset of them seem to be important for temperature entrainment. Miyasako et al. found that when subjected to light dark cycle (LD) combined with a 6-hour advanced temperature cycle (TC), the DNs and LPNs shifted their phase of TIMELESS expression toward the TC, while the LNvs basically maintained their original phase with the LD (Miyasako et al., 2007). Thus, the LNs seem to be preferentially light-entrainable while the DNs and LPNs seem to be preferentially temperature-entrainable or temperature sensitive. In an independent study, these temperature sensitive circadian neurons were shown to contribute to late evening activity when TC is present (Busza et al., 2007). Specifically, when LNs are
ablated using *cry-GAL4*, *UAS-hid*, the locomotor behavior exhibits a weak evening peak of circadian nature under a long thermophase TC. This peak exist under TC in either DD or constant light (LL) conditions but is absent in LD cycle with constant temperature, excluding a possible negative masking effect by light in LD and further emphasizing the temperature specific function of DNs and LPNs in the control of circadian behavior (Busza et al., 2007). Moreover, the temperature sensitive neurons are also necessary for properly timing the activity of the E cells: they repress the mid-day activity generated by E cells under TC (Busza et al., 2007).

The function of the DN1s in LL was discovered by Murad et al., (2007). LL usually makes fly arrhythmic after a day or two (Konopka et al., 1989). Murad et al., found that overexpressing *per* or *morgue* in all clock neurons using *tim-GAL4* makes flies rhythmic in LL, and surprisingly, the rhythms seemed to be driven by a subset of DN1s rather than the LNvs, because molecular rhythms were only observed in a subset of DN1s. This finding suggests flies could rely on either the LNvs or the DN1s to control circadian behavior, depending on the presence or absence of light (Murad et al., 2007). Similar observations were made with SGG overexpression (Stoleru et al., 2007). It was proposed that the DN1s would play a more prominent role during long days of summer, while the LNvs would be more important when photoperiods are shorter (Murad et al., 2007; Stoleru et al., 2007).
The function of the DN1s was further investigated by Zhang et al. (2010) using $Clk4.1M$-GAL4, which specifically drives expression in a subset of DN1 neurons. Misexpression of PER with $Clk4.1M$-GAL4 in per$^0$ flies is able to restore PER rhythms in those DN1s, and these rescued flies have the potential to exhibit both morning and evening activities. However, this DN1-driven morning and evening activity is under tight environmental control, with high light intensity and high temperature inhibiting evening activity and low temperature inhibiting morning activity (Zhang et al., 2010). This thermal and photic modulation of DN1 output provides a simple mechanism for daily adaptations to the changes of temperature and light intensity due to daily or seasonal weather patterns.

DN2-specific functions were until recently studied mostly in the larval brain, which has a much simpler clock network than the adult brain: four PDF positive LNvs, one PDF negative LNv, two DN1s and two DN2s (Kaneko et al., 1997). Only the DN1s and PDF positive LNvs express CRY - the blue light photoreceptor mediating light entrainment in Drosophila (Klarsfeld et al., 2004). Thus, the DN2s are “blind” and requires PDF signaling from the LNvs to be entrained to LD cycles, but with an almost antiphase relationship with the LNvs (Picot et al., 2009). However, TC appears to act directly on the DN2s but not on the PDF positive LNvs. Indeed, LNvs require signals from DN2s to be entrained to TC and possess a phase corresponding to that of the DN2s but different from all the other clock neurons (Picot et al., 2009). Therefore, the hierarchy of clock
neurons thus appears very different during entrainment of the clock network by light or temperature.

A novel function of the DN2s was very recently identified in adult flies (Kaneko et al., 2012) (I contributed to this work, see Appendix). When allowed to distribute along a thermal gradient (e.g. 18° C to 32° C), flies prefer ~25° C (Sayeed and Benzer, 1996). Kaneko et al. found that the temperature preference exhibits daily rhythms: the preferred temperature starts to increase in the morning (ZT1-3), reaches its peak in the evening (ZT10-12), gets to trough at ZT13-15 and then reaches a second smaller peak at ZT19-21. This temperature preference rhythms (TPR) is controlled by an endogenous clock and requires the clock genes per and tim. Interestingly, the M-cells and E-cells controlling locomotor behavior are neither necessary nor sufficient for TPR, while the DN2s are sufficient to drive TPR. Importantly, DN2s alone are not sufficient to drive locomotor rhythms in either LD or TC, suggesting the DN2s regulate TPR independently from circadian locomotor behavior, and that locomotor rhythms and TPR are controlled by different sets of clock neurons (Kaneko et al., 2012).

E. Light and temperature can reset the pacemaker

The organisms’ ability to be synchronized to external environment is both necessary and beneficial. First of all, since the free running periods are close to, but not exactly, 24 hours, circadian rhythms must be reset each day in order to
avoid falling out of phase with the external environmental cycle. Secondly, circadian rhythms need to be adjustable to daily weather pattern and seasonal changes in day length for adaptation. In nature, light and temperature variations are the two most common Zeitgebers for synchronization.

1. *Drosophila* circadian rhythms can be entrained by light.

Light is one of the most powerful Zeitgebers for synchronization. For *Drosophila melanogaster*, light intensity as low as 0.03 lux (equivalent to light from a quarter moon) during the light phase of LD is able to synchronize circadian behavior (Bachleitner et al., 2007). And when using blue light, intensity that is 3–4 orders of magnitude lower is sufficient (Hirsh et al., 2010). However, the biological clock is not equally sensitive to light at all time of the day. In general, a brief light pulse in the early night or “subjective” night (if flies are in DD prior to light pulse) produces a phase delay, whereas light pulse in the late night or “subjective” night produces a phase advance shift. Light pulses during the day or “subjective” day produce very small or no phase shifts. A phase response curve can be generated by plotting the magnitude and direction of these responses with respect to the time at which the light pulse is administered (Suri et al., 1998).

Light can be perceived by *Drosophila* in at least three ways (Helfrich-Forster et al., 2001). The most intensively studied is the CRY-dependent pathway. CRY is a blue light photoreceptor, member of the Cryptochrome/6-4
Photolyase Protein Family (Emery et al., 1998). It has a photolyase homology domain sufficient for light transduction, and a C-terminal domain regulating CRY’s proteasome-dependent degradation, thus determining the photosensitivity of the circadian clock (Busza et al., 2004). Upon light stimuli, CRY binds directly to TIM and triggers proteasomal degradation of TIM (Ceriani et al., 1999; Naidoo et al., 1999; Busza et al., 2004). CRY itself undergoes degradation when exposed to light (Emery et al., 1998). TIM degradation resets the phase of the molecular clock, resulting in phase shifts of locomotor behavior (Hunter-Ensor et al., 1996; Lee et al., 1996; Myers et al., 1996; Zeng et al., 1996; Suri et al., 1998). In LL, flies immediately become arrhythmic, presumably because CRY is constantly activated in light and TIM is continuously degraded. In the severely hypomorphic cryb mutants, where a missense mutation affects the highly conserved Flavin Adenine Dinucleotide binding region in the photolyase homology domain, CRY is not functional with extremely low protein level and cannot trigger TIM degradation in response to light. As a result, cryb flies are rhythmic in LL with a ~24-hour period (Emery et al., 2000). In addition, cryb flies are unable to phase shift their internal clock after short light pulse, nor do they have PER and TIM cycling in peripheral tissues (e.g., eyes) under LD cycles (Stanewsky et al., 1998). LNv-restricted CRY expression partially rescues all circadian behavior defects of cryb flies, and CRY overexpression in clock neurons increases photosensitivity (Emery et al., 1998; Emery et al., 2000).
Nevertheless, CRY is not the only photosensor for synchronizing locomotor behavior to LD. The fact that $cry^b$ flies can still be entrained to LD with synchronized molecular oscillations in the LNvs (Stanewsky et al., 1998) suggests there are CRY-independent photoreceptors. The possible candidates are structures expressing opsin-based photopigments: the compound eyes, the ocelli, and the Hofbauer-Buchner eyelet (H-B eyelet) (Figure 1.3). Among the three, the compound eyes and the ocelli utilize no receptor potential A ($norpA$)-dependent phototransduction cascade, constituting the second photoreception pathway for circadian rhythms. $norpA$ mutants that affect phopholipase C in the phototransduction cascade and thus impair compound eye and ocelli function are unentrainable at very low light intensities (Pearn et al., 1996). However, $norpA^{P41}; cry^b$ double mutants can still be entrained to LD cycles although they need many more days than single mutants (Stanewsky et al., 1998; Emery et al., 2000). This indicates a third light input pathway that is independent of $norpA$ or $cry$. Then, Helfrich-Forster et al. (2001) found that $glass^{60j} cry^b$ double mutants - which lack all known retinal eye structures, H-B eyelets and have no functional CRY - are completely blind to light. They do not entrain to LD cycles and exhibit free-running behavior. Even molecular oscillations in LNvs seem to free-run (Helfrich-Forster et al., 2001). Therefore, the third light input pathway probably involves the H-B eyelets and utilizes a $norpA$-independent phototransduction cascade. H-B eyelets are a pair of putative extraretinal photosensory structures underneath the retina and project directly to the aMe where the LNvs reside (Yasuyama and
Meinertzhagen, 1999), making them anatomically suited to transmit light to the LNvs. They express the photopigment Rhodopsin 6 and thus seem to have photoreceptive properties (Yasuyama and Meinertzhagen, 1999). Indeed, the function of H-B eyelets as a circadian photoreceptor was later confirmed by Veleri et al.. They show that synaptic communication between the H-B eyelet and clock neurons contributes to synchronization of molecular and behavioral rhythms (Veleri et al., 2007).

Taken together, the three light transmitting pathways - CRY, compound eyes and ocelli, and H-B eyelets - seem capable of entraining locomotor activity to LD cycles on its own. Nevertheless, the CRY-mediated pathway is essential for the phase shift response to short light pulse, and the compound eyes are especially important for entrainment at extreme photoperiods (Stanewsky et al., 1998; Rieger et al., 2003).

In addition to the three pathways described above, a putative 4th pathway was suggested by Veleri et al. (2003). They found that even in glass60j cryb double mutants, bioluminescence from a promoterless per-luciferase reporter can still be synchronized to the light dark cycle in a subset of dorsal neurons (mostly the DN3s), suggesting a light input route that is independent of CRY and the visual photoreceptors (the H-B eyelets, compound eyes and ocelli). Although circadian behavioral rhythms of the double mutants cannot be synchronized to LD, they do exhibit immediate locomotor activity changes at light /dark or dark/light transition, suggesting some light sensing ability of these flies.
Nevertheless, the photopigments mediating this 4\textsuperscript{th} pathway are currently unknown (Veleri et al., 2003).

2. \textit{Drosophila} circadian rhythms can be entrained by temperature.

Temperature is another important Zeitgeber for synchronizing \textit{Drosophila} locomotor behavior. Temperature cycles with amplitude as little as 2°C are able to induce rhythmicity in LL which otherwise causes both behavioral and molecular arrhythmicity (Yoshii et al., 2005). It takes flies 6-10 cycles to reach a steady-state behavioral synchronization to TC, with an anticipatory peak of circadian nature during the late thermal phase (Yoshii et al., 2005). PER and TIM cycling in fly heads is also restored under TC in LL (Yoshii et al., 2005). Similar temperature entrainment was observed by Busza et al. (2007) in the absence of light. They found that TC is able to synchronize locomotor behavior in DD, and flies stay rhythmic after being released in constant conditions (DD and constant temperature) with a phase determined by TC (Busza et al., 2007). Taken together, temperature is unambiguously a circadian Zeitgeber for locomotor behavior.

Moreover, similar to the phase shift response to brief light pulse, flies can phase shift their circadian clock in response to a 30 min 37°C heat pulse during early evening (Edery et al., 1994). Interestingly, this response is also CRY-dependent, like the response induced by short light pulses. Heat pulse promotes CRY binding to the PER/TIM dimer, triggers their proteasome-dependent
degradation, and cry\textsuperscript{b} mutants have severely impaired heat-induced phase shifts (Kaushik et al., 2007). In addition, low temperature (15°C) keeps flies rhythmic in LL while warm temperature (25°C) makes them arrhythmic, suggesting temperature can fine-tune the light-dependent interaction between CRY and PER/TIM (Kaushik et al, 2007). However, CRY is dispensable for long term circadian entrainment in more physiologically relevant temperatures (e.g. 12 hr 29°C:12hr 20°C cycles) (Glaser and Stanewsky, 2005; Busza et al., 2007). Hence, there must be other mechanisms underlying temperature entrainment.

In contrast to light entrainment, the molecular mechanisms underlying temperature entrainment are largely unknown. The conventional thermal sensor in \textit{Drosophila melanogaster} is the outer segment of antenna, without which flies fail to orient properly in a temperature gradient (18°C to 31°C) (Sayeed and Benzer, 1996; Zars, 2001). However, the antenna is dispensable in circadian temperature entrainment and thus is not the dedicated structure for circadian thermal sensing (Glaser and Stanewsky, 2005). In fact, fly body parts can be synchronized to TC in an autonomous fashion: isolated body parts show robust circadian rhythms of \textit{per} luciferase in LL under TC (Glaser and Stanewsky, 2005). The exception is the isolated brain. It oscillates with a phase different from other body parts and the whole fly, and is not properly entrained by TC in LL (Sehadova et al., 2009). Regardless of the brain, the autonomous fashion suggests the temperature entrainment may rely on molecules or pathways common in those body parts.
To identify the molecules required for circadian thermal sensing, a mutagenesis screening was done and _nocte_ (*no circadian temperature entrainment*) was isolated as a novel gene involved in temperature entrainment of *Drosophila* (Glaser and Stanewsky, 2005; Sehadova et al., 2009). _nocte_, encoding a Glutamine-rich protein, is expressed in chordotonal (ch) organs known as stretch receptors, as well as in external sensory (es) organs with putative chemoreceptive and mechanoreceptive neurons. Behavioral entrainment to TC in LL is disrupted in _nocte^1_ mutants and in flies with impaired ch or es organs. Since isolated brains seem unable to be entrained to TC in LL, it is proposed that the circadian thermal sensing in *Drosophila* depends on _nocte_ and ch/es organs, and clock neurons in the brain rely on those peripheral organs to be properly entrained, probably through neuronal communication (Sehadova et al., 2009). This idea is in part parallel with the findings in adult mice: Suprachiasmatic Nucleus (SCN)- the pacemaker center in mouse brain- is resistant to temperature fluctuations while the peripheral tissues are cell-autonomously temperature sensitive (Buhr et al., 2010), although SCN’s temperature insensitivity is under debate (Ruby, 2011). Notably, individual neurons in the SCN are nevertheless entrainable to temperature. It is the neuronal cross talk that protects SCN as a whole from responding to temperature fluctuations (Buhr et al., 2010). Moreover, in contrast to what is proposed in flies, mice use SCN to drives circadian rhythms in body temperature which then act as a universal cue for the entrainment of cell autonomous oscillators throughout the
body (Buhr et al., 2010). Taken together, the studies in *Drosophila* so far emphasize the importance of chill organs and nocte in temperature entrainment, which shares similarity as well as poses distinction from mammals.
Figure 1.1 Rhythmic locomotor behavior of *Drosophila melanogaster*. 

**A,** To study the locomotor behavior of adult flies in the lab, single male flies are placed in glass tubes with one end covered with cotton and one end filled with food. Tubes are then placed into monitors where infrared beams cross the tubes. 

**B,** Each time a fly interrupts the beam, this will be recorded by the computer and the number of interruptions during a 30min interval will be used as the magnitude of locomotor activity for that time. An actogram can be generated by plotting the magnitude of locomotor activity with respect to time. 

**C,** Double-plotted actograms showing the average locomotor activity of a group of wild-type flies entrained to a 12 hour light : 12 hour dark cycle (LD), and then released in constant darkness (DD). Days 2-4 are plotted twice: first on the right half of the actogram and then on the left half of the next line. Gray shading indicates darkness. Zeitgeber Time (ZT) 0 equals the time of lights on. The arrowheads show the increase in activity that anticipates the light-on and light-off transitions. The approximately 24-hour rhythms persist in DD. This figure is modified from Dubruille and Emery, 2008 with permission.
Figure 1.2 Simplified model of the molecular pacemaker of *Drosophila melanogaster*. The two interlocked transcriptional feedback loops are shown here. CLOCK/CYCLE (CLK/CYC) sits in the center and drives rhythmic transcription of *period* (*per*), *timeless* (*tim*), *vrille* (*vri*) and *PAR domain protein 1* (*Pdp1*). PER and TIM negatively feed back on CLK/CYC transcriptional activity, forming one loop, while VRI and PDP1 form the other by regulating *Clk* transcription negatively and positively, respectively.
Figure 1.3 Location, arborization of clock neurons, and light sensing organs in adult *Drosophila* brain. Based on anatomical location and size, clock neurons are grouped into large and small ventral Lateral Neurons (ILNvs and sLNvs), dorsal Lateral Neurons (LNds), 3 groups of Dorsal Neurons (DN1s, DN2s and DN3s) and Lateral Posterior Neurons (LPNs). Each group of neurons and their arborizations are depicted in different colors. The accessory medulla (aMe) and the dorsal brain are the two main projection targets. Compound eyes, ocelli and H-B eyelets are CRYPTOCHROME-independent light sensing organs for circadian light entrainment. This figure is adopted from Helfrich-Forster *et al.*, 2007 with permission.
CHAPTER II

KAYAK-α modulates circadian transcriptional feedback loops in *Drosophila* pacemaker neurons

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R. Dubruille first observed that knockdown of *kayak*-α in pacemaker neurons generates long period phenotype. P. Emery found that *kayak*-α represses CLK’s activation of *per* and *tim* promoters in a cell culture assay. All other research was done by me. P. Emery wrote the body of the text, while I made the Figures, Materials and Methods and provided feedback on the other sections.
A. Abstract

Circadian rhythms are generated by well-conserved interlocked transcriptional feedback loops in animals. In *Drosophila*, the dimeric transcription factor CLOCK/CYCLE (CLK/CYC) promotes period (*per*), timeless (*tim*), vriile (*vri*), and PAR-domain protein 1 (*Pdp1*) transcription. PER and TIM negatively feed back on CLK/CYC transcriptional activity, whereas VRI and PDP1 negatively and positively regulate Clk transcription, respectively. Here, we show that the α isoform of the *Drosophila* FOS homolog KAYAK (KAY) is required for normal circadian behavior. KAY-α downregulation in circadian pacemaker neurons increases period length by 1.5 h. This behavioral phenotype is correlated with decreased expression of several circadian proteins. The strongest effects are on CLK and the neuropeptide PIGMENT DISPERSING FACTOR, which are both under VRI and PDP1 control. Consistently, KAY-α can bind to VRI and inhibit its interaction with the Clk promoter. Interestingly, KAY-α can also repress CLK activity. Hence, in flies with low KAY-α levels, CLK derepression would partially compensate for increased VRI repression, thus attenuating the consequences of KAY-α downregulation on CLK targets. We propose that the double role of KAY-α in the two transcriptional loops controlling *Drosophila* circadian behavior brings precision and stability to their oscillations.
B. Introduction

Circadian rhythms synchronize animal physiology and behavior with the day/night cycle. They are generated by a complex transcriptional network of interlocked feedback loops. The architecture of this network and many of its components are conserved between insects and mammals (Emery and Reppert, 2004).

In *Drosophila*, the dimeric transcription factor CLOCK/CYCLE (CLK/CYC) is at the center of this network (Hardin, 2005; Zhang and Emery, 2012). It promotes *period (per)* and *timeless (tim)* transcription. PER and TIM form dimers that are phosphorylated by several kinases: DOUBLETIME (DBT), CASEIN KINASE II, NEMO, and SHAGGY (Kloss et al., 1998; Price et al., 1998; Martinek et al., 2001; Lin et al., 2002; Akten et al., 2003; Ko et al., 2010). Once properly phosphorylated, PER and TIM move into the nucleus in which they interact with CLK/CYC. They initiate repression first on the chromatin and then displace CLK/CYC from their binding sites (E-boxes) (Menet et al., 2010). PER/TIM repression requires DBT, which stably binds PER (Kloss et al., 2001; Yu et al., 2009). This first transcriptional loop plays a particularly important role in the generation of 24 h period rhythms. Modulating this loop is the transcriptional repressor CLOCKWORK ORANGE (CWO), which recognizes the same E-boxes as the CLK/CYC dimer (Kadener et al., 2007; Lim et al., 2007; Matsumoto et al., 2007; Richier et al., 2008). *cwo* transcription is itself regulated by CLK/CYC.
The second feedback loop is somewhat simpler. CLK/CYC transactivate the *vrille* (*vri*) and *PAR-domain protein 1* (*pdp1*) genes (Cyran et al., 2003; Glossop et al., 2003). PDP1 feeds back positively on the *Clk* promoter, whereas VRI antagonizes the activity of PDP1 by competing for the same binding sites. The phase of PDP1 and VRI protein rhythms differ by a few hours, hence permitting *Clk* transcription to oscillate. The importance of *Clk* transcription rhythms remains uncertain, because they are not necessary for 24 h period behavioral rhythms (Kim et al., 2002). However, PDP1 and VRI levels are crucial for proper CLK expression (Blau and Young, 1999; Zheng et al., 2009). For example, a mutation that specifically abolishes the PDP1ε isoform results in low CLK levels, loss of PER and TIM cycling, and arrhythmic behavior (Zheng et al., 2009). Interestingly, forced expression of CLK in *pdp1ε* mutants restores PER and TIM rhythms, without restoring behavioral rhythms (Zheng et al., 2009). Thus, the CLK/VRI/PDP1 loop is particularly important for circadian output pathways.

This transcriptional network is present in all tissues with circadian rhythms, including the neurons driving circadian behavior. There are ~150 circadian neurons in the fly brain (Nitabach and Taghert, 2008). For the control of rhythmic locomotor activity, the most important are the PIGMENT DISPERSING FACTOR (PDF)-positive small ventral lateral neurons (sLNvs). Indeed, these cells drive circadian locomotor behavior in constant darkness (DD) (Renn et al., 1999; Blanchardon et al., 2001; Stoleru et al., 2005). In their absence, or in the
absence of the neuropeptide PDF, flies are usually arrhythmic or show weak short period rhythms. Here we show that KAYAK (KAY)—the *Drosophila* homolog of the transcription factor c-FOS—modulates the circadian transcriptional network in sLNvs.

C. Results

1. *kay-α* downregulation lengthens circadian behavioral rhythms

In a previous misexpression screen, we looked for flies that remained rhythmic under constant light (LL) with the idea of isolating novel genes that regulate the CRYPTOCHROME (CRY) input pathway (Dubruille et al., 2009). CRY is a key circadian photoreceptor that binds to TIM and triggers its proteasomal degradation after blue-light photon absorption, hence resetting the circadian molecular pace (Emery et al., 1998; Stanewsky et al., 1998; Lin et al., 2001; Busza et al., 2004). Wild-type flies are arrhythmic in LL because CRY is constantly activated, whereas *cry* mutant flies remain rhythmic, as if they were in DD (Emery et al., 2000). However, it is known that overexpressing pacemaker genes can also make flies rhythmic in LL (Murad et al., 2007; Stoleru et al., 2007; Dubruille et al., 2009). Thus, our screen had the potential of identifying both pacemaker and light input genes. We previously identified the chromatin remodeling protein KISMET as a regulator of CRY photoresponses (Dubruille et
The present study focuses on the role of *kay*—another candidate gene isolated with our LL screen—in the circadian pacemaker.

*kay*, the *Drosophila* homolog of *c-fos*, encodes a bZip transcription factor (Zeitlinger et al., 1997). To understand the role *kay* might play in the regulation of *Drosophila* circadian rhythms, we decided to test *kay* loss-of-function flies. Because severe *kay* mutants are embryonic lethal (Zeitlinger et al., 1997), we used an RNAi approach to knock down *kay*. There are five KAY isoforms in *D. melanogaster* (Figure 2.1A) (Hudson and Goldstein, 2008). Full-length isoforms α, β, γ, and δ are generated through alternative promoters. The four alternative first exons encode specific N-terminal domains. The other exons are common to all four full-length isoforms, which thus share the same DNA binding domain (bZIP domain). The fifth isoform is encoded only by the exon specific to the α isoform and thus does not contain the bZIP domain. This truncated isoform was previously called KAY–SRO, because the *shroud* (*sro*) mutation was initially mapped to its encoding exon (Hudson and Goldstein, 2008). Because it is now known that the *sro* mutation affects another gene (Niwa et al., 2010), we renamed this isoform KAY–TRUNC. The P-element [EP(3)3084] we isolated in our LL screen is inserted about 1 kb upstream of the exon specific to the KAY-α/TRUNC isoforms. We therefore expressed a dsRNA targeting specifically these isoforms, encoded by the NIG15507 transgene. Expression of this dsRNA was lethal with widely expressed drivers, such as *tim–GAL4* (Kaneko et al., 2000). We therefore used the *Pdf–GAL4* driver, which is expressed only in the circadian
neurons that drive circadian behavior in DD, the sLNvs (Renn et al., 1999). We observed a \(\sim 1\)-h-long period phenotype in DD with two independent NIG15507 insertions (kayR2 and kayR4) (Table 2.1). Period was slightly longer with the kayR2 insertion. For the rest of this study, we used this insertion. Coexpressing DICER2 (DCR2) with dsRNAs is known to enhance RNAi effects (Dietzl et al., 2007). Consistently, the period phenotype was slightly enhanced when DCR2 was coexpressed with the dsRNAs. These RNAi flies showed a period lengthening of \(\sim 1.5\) h (Figure 2.1B, bars 1 and 2, C). The proportion of arrhythmic flies was also increased in the presence of DCR2.

We generated an antibody directed against the KAY-\(\alpha\) N-terminal region. Unfortunately, this antibody was not sensitive enough to detect endogenous KAY-\(\alpha\) expression by Western blotting or immunohistochemistry. Thus, to verify that the NIG15507 dsRNAs are indeed able to downregulate KAY-\(\alpha\) expression, we overexpressed KAY-\(\alpha\) specifically in the eyes with the \textit{Rhodopsin1–GAL4} (\textit{Rh1-GAL4}) driver (Mollereau et al., 2000) in the presence or absence of the kay-\(\alpha\) dsRNAs. KAY-\(\alpha\) overexpression was dramatically reduced in the presence of the dsRNAs (Figure 2.1D). Thus, the NIG15507 dsRNAs efficiently inhibit KAY-\(\alpha\) expression.

Although the NIG15507 dsRNAs lengthened circadian period by \(\sim 1.5\) h, dsRNAs targeting all full-length KAY isoforms resulted only in modest period lengthening (up to 0.5 h; Table 2.2). Thus, either these dsRNAs are inefficient at
repressing KAY expression or repression of the truncated KAY isoform plays a major role in the long period phenotype. To determine whether the long period phenotype is attributable to KAY-α or to KAY–TRUNC knockdown, we performed a cross-species rescue experiment (Figure 2.1A). We generated UAS–kay-α and UAS–kay–trunc transgenes expressing kay mRNAs resistant to the NIG15507 dsRNAs by replacing the region targeted by these dsRNAs with homologous but sufficiently divergent D. pseudoobscura sequences (Langer et al., 2010). We expressed these constructs in flies expressing NIG15507 dsRNAs. We found that the chimeric pseudoobscura/melanogaster KAY-α rescued the long period phenotype (6 of 10 lines tested fully rescued, one rescued partially), although the pseudoobscura KAY–TRUNC did not (none of the nine lines tested could rescue) (Figure 2.1B,C; Table 2.3). Thus, we conclude that the long period phenotype observed in flies expressing the NIG15507 dsRNAs in PDF-positive LNvs is caused by KAY-α downregulation. Therefore, this KAY isoform regulates circadian behavior. The increase in arrhythmicity was not rescued, however, and might thus be attributable to off-target effects. For the rest of the manuscript, flies expressing NIG15507 dsRNAs (and DCR2) in PDF neurons will be simply referred to as kay-αRNAi flies.

2. Reduced PER and PDF levels in the sLNvs of kay-αRNAi flies

To begin to understand how KAY-α might control circadian behavior, we measured PER levels in PDF-positive sLNvs of kay-αRNAi flies. These flies and
control flies were first entrained to a standard LD cycle for 3 d and then released in DD. Fly brains were dissected at different circadian times (CT) during the fourth subjective night and fifth subjective day and immunostained with PER and PDF antibodies. In control flies, PER peaked near CT24 as expected (Figure 2.2A,B). However, in kay-αRNAi flies, the peak was between CT6 and CT10. This delay on day 4–5 of DD fits well with the ∼1.5 h period lengthening observed behaviorally with these flies. We therefore conclude that the long period phenotype of kay-αRNAi flies is attributable to a slow-running pacemaker in the sLNvs. We also noted that overall PER levels were reduced by approximately half at peak level in kay-αRNAi flies. KAY-α thus positively affects PER levels.

Interestingly, we also observed that PDF levels were severely reduced at all time points in the sLNvs of kay-αRNAi flies (Figure 2.2A). Nevertheless, PDF was detectable in the dorsal projections of sLNvs, and these projections appeared anatomically normal, indicating that the sLNvs develop normally when KAY-α is downregulated (Figure 2.2C). As a circadian neuropeptide, PDF is required for normal rhythm amplitude and circadian period length in DD (Renn et al., 1999). Absence of PDF leads to arrhythmicity or short behavioral period. It thus seemed unlikely that the long period phenotype we observed would be caused by low PDF levels. To confirm this, we restored PDF levels in the sLNvs of kay-αRNAi flies with a UAS–Pdf transgene (Renn et al., 1999). Circadian
behavior kept its long period (Figure 2.2D). This demonstrates that low PDF levels are not the cause of the long period phenotype seen in kay-αRNAi flies.

3. KAY-α represses CLK transactivation of the tim promoter and the per E-box

KAY is a bZip transcription factor. This class of transcription factors uses their leucine Zipper domains to form homodimers or heterodimers. The best-known partner of KAY is JRA (Bohmann et al., 1994), with which it forms the AP-1 complex that recognizes the consensus sequence TGAGTCA. In most cases, AP-1 functions as a transcriptional activator. Intriguingly, we found a perfect AP-1 binding site in the tim promoter. If KAY-α were to activate transcription through this site, it could explain the phenotypes seen in kay-αRNAi flies. Indeed, low TIM levels result in long period phenotypes and low PER levels (McDonald et al., 2001).

We tested this idea using a luciferase reporter assay. The proximal tim promoter was fused to a luciferase reporter gene and transfected into Drosophila S2R+ cells. Unexpectedly, coexpression of KAY-α and JRA did not activate the tim promoter at all (Figure 2.3A). We reasoned that CLK might be needed as well, because it forms a crucial tim transactivator with CYC, which is endogenously expressed in S2R+ cells (Darlington et al., 1998). As expected, we saw a robust transactivation of the tim promoter when CLK was expressed. However, we did not see additional activation when coexpressing KAY-α and
JRA with CLK. On the contrary, we actually observed a decrease in the activation of the *tim* promoter with CLK (Figure 2.3A). Also unexpectedly, the AP-1 binding site in the *tim* promoter was dispensable for KAY-α repression of *tim* (Figure 2.3A). Because *per* is another target gene of CLK/CYC, we tested a construct that only contains multimerized *per* E-boxes in front of the luciferase reporter gene. We found that KAY-α could also repress the activation of these E-boxes by CLK (Figure 2.3A). This clearly shows that KAY-α represses CLK transactivation independently of AP-1 binding sites. Importantly, KAY-α-mediated repression was not the result of reduced CLK levels, nor was it attributable to retention of CLK in the cytoplasm (Figure 2.3B,C). In conclusion, KAY-α represses the transactivation potential of CLK and might thus regulate a large subset of CLK target genes.

Given that AP-1 sites are not required for KAY-α repression, we wondered whether JRA is required. We observed that it made no difference to cotransfect or not a plasmid encoding JRA (Figure 2.3D, bars 3 and 5). This suggests that JRA is not required for repression. To exclude the possibility that KAY-α uses endogenously expressed JRA to form the AP-1 complex, we treated the S$_2$R$^+$ cells with dsRNAs targeting *Jra*. This led to increased CLK transactivation, a phenomenon that we did not study further. Importantly, however, KAY-α could still repress efficiently the activation of the *tim* promoter by CLK (Figure 2.3D, bars 6 and 8). Consistently, *Jra* downregulation by RNAi in flies did not lengthen
circadian period (Table 2.4). Together, our data show that KAY-α represses CLK activation independently of JRA and AP-1 binding sites.

To determine whether KAY-α might directly bind to the E-box and compete for CLK binding, as does CWO (Kadener et al., 2007; Lim et al., 2007; Matsumoto et al., 2007; Richier et al., 2008), we added a VP16 activation domain to KAY-α. In the case of CWO, this repressor protein is transformed into an activator by the addition of VP16 (Kadener et al., 2007; Lim et al., 2007). This was not the case with KAY-α. The VP16–KAY-α protein was unable to transactivate *tim–luc* and still functioned as a repressor in the presence of CLK (Figure 2.3E). Thus, KAY-α probably does not bind directly the *tim* or *per* E-boxes. Nevertheless, its DNA binding domain is required for repression (Figure 2.3F,G), because deletion of the basic region of the bZIP domain—responsible for interaction with DNA—completely eliminated repression (see Discussion).

We then asked whether the repression was specific to the KAY-α isoform. We therefore tested whether KAY-β, KAY-γ, and KAY–TRUNC could also repress CLK. None of these isoforms were able to repress CLK, although they were expressed at similar or higher levels than KAY-α (Figure 2.4A,B). Thus, CLK repression is specific to the α isoform. This specificity strongly strengthens the notion that the repression of CLK by KAY-α is important to determine circadian period length, because specifically downregulating this KAY isoform is sufficient *in vivo* to lengthen circadian behavioral rhythms.
We next determined whether KAY-α repression was specific to CLK or could happen with any activator recognizing E-boxes. As mentioned previously, CWO is a repressive helix–loop–helix protein that competes with CLK for binding to E-boxes (Kadener et al., 2007; Lim et al., 2007; Matsumoto et al., 2007; Richier et al., 2008). When CWO is fused to the strong VP16 transactivation domain, CWO becomes an activator. KAY-α could not repress the activation of the tim promoter by VP16–CWO (Figure 2.4C). This indicates that KAY-α repression is specific to CLK. In summary, our transcription assays show that KAY-α specifically represses CLK-mediated transcription.

4. Both circadian transcriptional feedback loops are altered in kay-αRNAi flies

Our results in S2R+ cells appear to be paradoxical. If KAY-α is a CLK repressor, then its downregulation should increase PER levels rather than decrease them as observed in sLNvs (Figure 2.2A,B). Thus, the role played by KAY-α might not be limited to repressing CLK activity. To understand better the impact of KAY-α on the circadian molecular pacemaker, we measured PER, TIM, PDP1, and VRI levels at expected peak times in kay-αRNAi flies during the first day of DD. We also measured CLK levels at two time points: CT12 and CT24. Overall CLK levels do not oscillate, but its phosphorylation does. CT12 and CT24 correspond to the peak for the hypo-phosphorylated and hyper-phosphorylated CLK isoforms, respectively (Yu et al., 2006). As expected, PER levels were
reduced by \( \sim 50\% \) in \( kay\)-\alphaRNAi flies, as observed on days 4–5 of DD (Figure 2.2). When PER was expressed independently of its own promoter with \( Pdf\)-GAL4 in a \( per^0 \) background, KAY-\alpha downregulation had only weak effects on PER levels, further supporting the notion that KAY-\alpha regulates PER transcriptionally (Figure 2.5B). The small reduction in PER levels, significant only in one experiment, could indicate that KAY-\alpha also weakly affects PER posttranscriptional regulation. This could be because two subunits of protein phosphatase 2A, which regulates PER phosphorylation and thus its stability, are under circadian control (Sathyanarayanan et al., 2004) and might therefore be misregulated in \( kay\)-\alphaRNAi flies. PDP1 levels were also clearly reduced (\( \sim 40\% \) on average), but VRI was not affected (Figure 2.5A). TIM levels were lower in all three experiments that we performed (Figure 2.5A), but this was statistically significant in only one of them. Overall, the strongest effects of KAY-\alpha downregulation were on CLK levels, which were reduced by \( \sim 60\% \) in \( kay\)-\alphaRNAi flies (Figure 2.5A). The \( Clk \) promoter might thus be an important target of KAY-\alpha.

5. KAY-\alpha modulates VRI activity on the \( Clk \) promoter

PDP1 and VRI are critical activator and repressor of the \( Clk \) promoter, respectively. Interestingly, they have similar positive and negative effects on PDF expression, through as yet unknown mechanisms. In \( Pdp1\epsilon \) mutants, both CLK and PDF levels are severely reduced in sLNs (Zheng et al., 2009). PDF expression is also decreased when VRI is overexpressed, although \( Pdf \) mRNA
appears unaffected. This indicates that VRI regulates the expression of a gene controlling PDF posttranscriptionally (Blau and Young, 1999). Because the strongest effects of \textit{kay-}\textalpha{} downregulation are on CLK and PDF, we wondered whether KAY-\textalpha{} might interact with VRI and/or PDP1 and modulate their activity.

We turned to a well-established luciferase reporter assay in HEK293 cells, in which PDP1 and a VRI–VP16 fusion protein can both activate the \textit{Clk} promoter. We cotransfected KAY-\textalpha{} with either PDP1 or VRI–VP16 (Cyran et al., 2003). Strikingly, we found that KAY-\textalpha{} could repress the activation of the \textit{Clk} promoter by VRI–VP16 in a dose-dependent manner. Conversely, it was unable to block PDP1 activity (Figure 2.6A). These results fit remarkably well with those of the dimerization prediction study of Fassler et al. (Fassler et al., 2002), in which they anticipated that KAY-\textalpha{} would interact with VRI, but not with PDP1, based on the amino acid content of their leucine Zipper.

To confirm that KAY-\textalpha{} indeed interacts with VRI, we performed a coimmunoprecipitation experiment. MYC–KAY\textalpha{} and VRI–VP16 were coexpressed in HEK293 cells. KAY-\textalpha{} was immunoprecipitated with an anti-MYC antibody. As expected, we found VRI–VP16 to coimmunoprecipitate with KAY-\textalpha{} (Figure 2.6B). These results lead us to propose that KAY-\textalpha{} negatively regulates the repressive activity of VRI by forming a complex that is unable to recognize its targets, such as the \textit{Clk} promoter.
D. Discussion

Transcriptional feedback loops play a critical role in the generation of circadian rhythms in most organisms. In *Drosophila*, as in mammals, two interlocked feedback loops produce antiphase circadian transcriptional oscillations (Zhang and Emery, 2012). In this study, we have identified a novel transcriptional regulator of circadian rhythms in *Drosophila*: KAY-α. Our results indicate that it modulates both circadian feedback loops: it is both a repressor of the CLK/CYC transactivation complex and an inhibitor of VRI-mediated repression (Figure 2.7).

Interestingly, our results indicate that KAY-α directly binds to VRI. KAY and VRI are two of the 27 bZip proteins encoded by the *D. melanogaster* genome (Fassler et al., 2002). The leucine zipper domain is typically composed of four to five heptad repeats of amino acids, with the seven unique positions in the heptad labeled “a,” “b,” “c,” “d,” “e,” “f,” and “g.” These positions are critical for dimerization stability and specificity. By evaluating (1) the presence of attractive or repulsive interhelical “g–e” electrostatic interface and (2) the presence of polar or charged amino acid in the “a” and “d” positions of the hydrophobic interface, Fassler et al. (2002) predicted that KAY can dimerize with VRI but not PDP1. Our results confirm functionally this prediction. KAY-α blocks the ability of VRI to transactivate the Clk promoter when VRI is made an activator by the addition of a VP16 domain, but it does not interfere with PDP1 transactivation. Moreover, it
forms a complex with VRI. By taking together our data and Fassler's prediction, we propose that KAY and VRI form a dimer through leucine Zipper interactions and that this dimer is not able to bind to the *Clk* promoter and other VRI/PDP1 target sequences. This model would explain why CLK and PDF levels are low in the sLNvs of *kay*-αRNAi flies. There would be an increase in VRI repressive activity in these flies (Figure 2.7). It is interesting to note that PDF levels seem to be particularly low in the small LNvs but much less dramatically affected in the large LNvs (Figure 2.2C). Thus, it appears that PDF levels are regulated through different mechanisms in small and large LNvs. This could be a reflection of the different role played by these cells in the control of circadian behavior and arousal (Shang et al., 2008; Sheeba et al., 2008).

The mechanism by which KAY-α regulates CLK/CYC transactivation is not yet clear. We could not detect a direct interaction between KAY-α and CLK/CYC by coimmunoprecipitation when these proteins were expressed in cell culture. Of course, this does not exclude the possibility that they interact *in vivo* or that they do so transiently. However, because these proteins belong to two different families of transcription factors (bZip and bHLH–PAS), direct interactions would not be predicted. It also seems unlikely that KAY-α competes with CLK/CYC for binding to the E-box. Indeed, a VP16–KAY-α fusion protein was not able to transactivate E-box containing promoters, whereas a CWO–VP16 fusion protein could (CWO does compete with CLK/CYC for E-box binding) (Kadener et al., 2007; Lim et al., 2007). In fact, KAY-α–VP16 still functioned as a CLK/CYC
repressor. Nevertheless, the basic region necessary for DNA binding is needed for KAY-α repression. This suggests that KAY-α controls the expression of a gene important for CLK/CYC activity. Most likely, it promotes the expression of a CLK/CYC repressor. In any case, this repressive role for KAY-α probably explains why the expression of CLK/CYC targets is mildly affected by KAY-α downregulation and the resulting increased VRI repression. The decrease in repressive activity on the CLK/CYC transcription factor would compensate for the stronger VRI repression of the Clk promoter (Figure 2.7). CLK/CYC targets include PDP1, which positively activates Clk. Particularly high affinity of PDP1 for the Clk promoter could explain why CLK levels are less affected than PDF by KAY-α downregulation. Also supporting an important role of CLK/CYC repression by KAY-α is the fact that this repression is specific to the α isoform. This result implies that the N-terminal domain of KAY proteins plays an important role in defining their respective function and the genes and proteins they are regulating. However, we note that repression of VRI–VP16 transactivation occurred with all full-length KAY isoforms tested (Figure 2.6C). This is not entirely surprising, because this repression is most likely mediated through the leucine Zipper domain common to all isoforms, as discussed above.

A question that remains to be answered is whether KAY-α levels are regulated by the circadian clock. Our antibodies were not sensitive enough to detect endogenous KAY-α protein levels, and, because of low mRNA levels in head extracts, we could not reliably measure kay-α mRNA levels by quantitative
real-time PCR. Interestingly, the \textit{kay-\alpha} promoter actually contains a perfect E-box, and this promoter can be transactivated by CLK/CYC in cell culture (Figure 2.6D). However, Abruzzi et al. (Abruzzi et al., 2011) did not detect CLK/CYC binding to the \textit{kay-\alpha} promoter in whole-head chromatin immunoprecipitation experiments, although CLK/CYC binds rhythmically to the \textit{kay-\beta} promoter. CLK/CYC could thus influence at distance the \textit{kay-\alpha} promoter \textit{in vivo}. It is also possible that CLK/CYC only bind to the \textit{kay-\alpha} promoter in specific tissues, such as the PDF-positive sLNvs.

This brings us to the question of the site of action of KAY-\alpha. Our study focused on the sLNvs, because we could not broadly drive dsRNAs against KAY-\alpha. KAY plays an important role during development and in many signal transduction cascades. Not surprisingly, lethality was observed with \textit{tim–GAL4}, which is expressed in all peripheral circadian tissues. It is thus possible that KAY-\alpha plays a role not just in pacemaker neurons but also in peripheral tissues. However, recent whole-genome expression studies have revealed a striking enrichment of transcription factors in PDF-positive circadian neurons (Nagoshi et al., 2010). This suggests that transcriptional regulation is particularly important for the function of these neurons. KAY-\alpha might thus be specifically recruited in this complex transcriptional network to provide the sLNvs with their striking characteristic of being self-sustained pacemaker neurons that drive rhythms of other clock neurons in DD (Stoleru et al., 2005). By affecting both transcriptional
loops, KAY-α might bring stability to the circadian pacemaker, particularly if it proved to be itself clock-controlled.

The transcriptional network controlling circadian rhythms is well conserved between mammals and Drosophila. Could FOS family members also control circadian period in mammals? There are four mammalian members of the FOS family: c-FOS, FOSB, FRA-1, and FRA-2. Light exposure during the subjective night strongly increases expression of c-FOS, FOSB, and FRA-2 in the suprachiasmatic nucleus (SCN), the brain structure that controls circadian rhythms in mammals (Aronin et al., 1990; Kornhauser et al., 1990; Schwartz et al., 2000). This induction might be important to phase shift circadian rhythms. Indeed, c-fos knock-out mice reset their circadian behavioral rhythms to a phase-shifted LD cycle more slowly (Honrado et al., 1996), and antisense oligonucleotides targeting both jun-b and c-fos block circadian responses to short light pulses in rats (Wollnik et al., 1995). Interestingly also, c-FOS and FRA-2 are rhythmically expressed in DD in the dorsomedial region of the SCN, which is particularly important for circadian rhythm generation (Schwartz et al., 2000). To our knowledge, only c-fos knock-out mice have been tested in DD, and the period length of their circadian behavior is normal (Honrado et al., 1996). This could be explained by redundancy with FRA-2, which can compete with c-FOS for the same DNA binding sites (Takeuchi et al., 1993). If FOS family members were important for generating circadian rhythms, it would be unlikely that they function identically to KAY-α in Drosophila. Indeed, although constructed with the
same logic in flies and mammals, the feedback loop interlocked with the PER feedback loop is based on different transcription factors: bZIPs (VRI and PDP1) in flies (Blau and Young, 1999; Cyran et al., 2003) and nuclear receptors (RORα and REVERBα) in mammals (Preitner et al., 2002; Sato et al., 2004). It is thus unlikely that FOS family members would be able to directly bind to REVERBα as they do with VRI in Drosophila. However, it would be interesting to determine whether mammalian FOS proteins can modulate CLK/BMAL1 transactivation.

In summary, we have identified KAY-α as a novel transcriptional regulator of the circadian pacemaker that modulates both circadian transcriptional feedback loops. KAY-α contributes to the precision of the circadian timekeeping mechanisms and possibly also to its stability.

E. Materials and Methods

Plasmid constructs

The DNA sequence of the exon encoding the N-terminal region of KAY-α was amplified by PCR from Drosophila melanogaster genomic DNA with the following primers: kay-α forward, 5′-CGTAGCGAATTCTATGATTGCACTAAAGGCCACC-3′; and kay-α–BstBI reverse, 5′-TCGAACCTTCGAGTTGCCGAGTTGTCTGCCCATTGAAGGG-3′. The rest of the KAY-α coding sequence was amplified from BDGP cDNA clone SD04477
with the following primers: **kay-α**–BstB1 forward, 5′-GATCTGTTGAAACCAGGCCAGGTTCTC-3′; and **kay-α** reverse, 5′-CATCTCCTCGAGTTATAAGCTGACCAGCTTGGA-3′. The two BstB1 primers introduce silent mutations that create a BstB1 restriction site that was used to clone in a three-fragment ligation the whole **kay-α** cDNA in the EcoRI and XhoI restriction sites of **pAc5.1/V5–HisB** (**pAc** for short). This construct (and all the other constructs generated by PCR) was confirmed by sequencing. We noticed two coding differences between our sequence and that of Flybase: six additional nucleotides are found in a stretch of glutamine-encoding codons in our sequence. This adds two glutamines to that glutamine repeat (Glu124–129 becomes Glu124–131). In addition, the Ala15 codon is changed to Pro15. These coding changes were found in independent clones and must thus represent polymorphisms. The **kay-α** cDNA was subsequently transferred to **pUAST** to make a **pUAST–kay-α** construct.

Genomic *Drosophila pseudoobscura* DNA was used to amplify the exon coding for KAY–trunc and KAY-α. The following primers were used: Psesro-5.1, 5′-GTCGAATTCATGATTCCATC-3′; Psesro-3.1, 5′-GAGTTACTCGAGCTAGGGCATACTTACATGTCT-3′; and Psesro-3.2, TCGAACTTCGAGGTGGCCAGATGCTTTGTATCAGCTTGCCG. Note that we amplified an extra 24 nucleotides compared with the predicted *Drosophila pseudoobscura* KAY-α coding region, because those eight N-terminal codons are obviously conserved between *Drosophila melanogaster* and *pseudoobscura*. To
generate the chimeric kay-α construct in pUAST, a three-fragment ligation was made with the PCR product obtained with Pseso-5.1 and Pseso-3.2. The pUAST–kay–trunc construct was generated by cloning the PCR product obtained with Pseso-5.1 and Pseso-3.1. These two constructs were injected in embryos to generate transgenic flies.

per–Ebox–luc, pAc–Clk, pAc–β-galactosidase, pAc–VP16–CWO, Clk–luc, CMV–vri–VP16, CMV–Pdp1ε, and CMV–Renilla were described previously (Darlington et al., 1998; Cyran et al., 2003; Lim et al., 2007). tim–luc and tim–mut–luc were generated by amplifying the proximal promoter region of the tim promoter with the following primers: 5′tim, 5′-CTAGCTGGTACCGAGTGCACAGAAACGTTCTG-3′; 3′tim, 5′-GCTACGACGCGTCTGAAAGTAGTTTTAAGAATATTG-3′; ap1mut1, 5′-CTGCGACTCGAGGTGTAAGCACTCTTTAAGAT-3′; and ap1mut2, 5′-CGTCGACTCGAGCTGGTCTTTCTCTCAGT-3′. The amplification products were cloned in the KpnI and MluI sites of pGL3. The kay–α–promoter–luc construct was made as follows. A 323 bp fragment ~300–600 bp upstream of the transcription start site of kay-α was amplified from genomic DNA and ligated into pGL3 vector through KpnI and MluI restriction sites: primer forward, 5′-CGGGTACCATGTCTGGCTAGCGAAAAGC-3′; primer reverse, 5′-CCGACGCATTTCACGCTGATAATGAGTCAACC-3′.
pAc–Jra was made by ligating the Jun-related antigen (Jra) cDNA (from BDGP clone LD25202) into pAc. The kay-γ cDNA was obtained from BDGP clone SD04477. pAc–kay-γ was made by ligating this cDNA into the EcoR1 and Xhol sites of pAc. pAc–kay-α was already described above. pAc–myc–kay-α was made by ligating a myc–kay-α PCR fragment into pAc through EcoRI and Xhol. Primers used for PCR were 5’-GACGAATTTCATGGAGGAGCAGAAGCTGATCTCAGAGGAGGACCTGGCGGCGCAATTGCACTAAAGGCCACCCGA-3’ and kay-α reverse. A NotI site was introduced between the myc tag and kay-α. pAc–myc–kay-β was made by ligating a kay-β fragment to the NotI and BstB1 site of pAc–myc–kay-α. Primers to amplify the kay-β fragment were as follows: NotI–kay-β forward, 5’-CAACGCGGCCGCGAAAGTCAAAGTGGAGCGC-3’; and kay-β–BstBI reverse, 5’-TCGAACCTTCCAAGTTGCGAGGATAAGATTGCAGCGTCGGTG-3’. To make pAc–kay–trunc–V5, kay–trunc with mutated stop codon was amplified from genomic DNA using the following primers: kay-α forward primer; and kay–trunc–V5 reverse, 5’-CGGCCTCGAGTATCGTACGCACTTAACTA-3’. This fragment was ligated into the EcoRI and Xhol restriction sites of pAc. The stop codon was mutated in a way such that the V5 in the pAc vector is in-phase with kay–trunc. In a similar way, pAc–kay-α–V5 was made using reverse primer 5’-CTAGACTCGAGTATAAGCTGACCAGCTTG-3’. CMV–myc–kay-α, CMV–myc–kay-β were made by ligating the corresponding kay sequence in pAc–myc–kay-α and pAc–myc–kay-β into pcDNA3.1 vector through EcoRI and Xhol restriction.
sites. *kay*-γ sequence from *pAc–kay*-γ was ligated into *pcDNA3.1* through EcoRI and XhoI to make *CMV–kay*-γ.

*pAc–VP16–kay*-α was made by ligating *kay*-α sequence into the *pAc–VP16* vector (generous gift from Dr. R. Allada, Northwestern University, Evanston, IL) using EcoRI and XbaI.

*pAc–kay*-α–basicΔ was made by ligating two PCR fragments amplified from *pAc–kay*-α into *pAc* vector through EcoRI, Acc65I, and XhoI restriction sites. This generates an ~60 bp deletion of the basic region. Primers used were as follows: *kay*-α forward, 5′-TAGACTGGTACCCTGCTCCTCCTCCGGGGTCA-3′; and *kay*-α reverse, 5′-ATCGATGGTACCGTGGACCAGACCAACGAGCT-3′.

*Drosophila* stocks and transgenic flies

Flies were reared on a standard cornmeal/agar medium at 25°C under a light/dark (LD) cycle. *Pdf–GAL4* and *Rh1–GAL4* flies were described previously (Renn et al., 1999; Mollereau et al., 2000). *Pdf–GAL4* was meiotically recombined with *UAS–dicer2* (Dietzl et al., 2007) to generate a *Pdf–GAL4, UAS–dicer2* stock (PGD). *Pdf–GAL4* or *PGD* was then crossed with NIG15507–R2 (*kayR2*) or NIG15507–R4 (*kayR4*) from the National Institute of Genetics (NIG) Stock Center (Mishima, Japan) to get *Pdf–GAL4 /+; kayR2/+* or *Pdf–GAL4 /+; kayR4/+* and *PGD /+; kayR2/+* flies. A stable *PGD/CyO; kayR2/TM6B* line was made and crossed to different *kay-α/trunc* rescue lines for the cross-species
rescue experiments. We also tested RNAi transgenes directed against all full-length KAY isoforms: NIG15509–R1 and R2 (NIG stock center) and VDRC6212 (Vienna Drosophila RNAi Center, Vienna, Austria). Jra RNAi and luciferase RNAi (luc RNAi) lines are from the Transgenic RNAi Project at Harvard Medical School (Boston, MA). A stable PGD/CyO; lucRNAi/TM6B line was generated and crossed to per^{Δ};;UAS–per (Grima et al., 2004) to generate per^{Δ}; PGD/+; UAS–per/lucRNAi flies. per^{Δ}; PGD/+; UAS–per/kayR2 flies were generated similarly.


**Behavioral assays**

Locomotor activities of adult males were monitored individually in Drosophila activity monitors (Trikinetics) in Percival I-36LL incubators (Percival Scientific). For LD cycle, light intensity was ~500 lux in the light phase. Behavior data were collected using Drosophila Activity Monitor program (Trikinetics). Behavioral period was determined for each fly by analyzing the data from the first day to the fifth day in DD using FaasX (courtesy of F. Rouyer, Centre National de la Recherche Scientifique, Gif-sur-Yvette, France). Average actograms were generated by a signal processing toolbox for MATLAB using function
“dam_panels” (MathWorks) (courtesy of J. Levine, University of Toronto, Mississauga, Ontario, Canada) (Levine et al., 2002). Only flies that survived the whole run were analyzed.

**Adult brain immunocytochemistry and quantification of fluorescent signal**

Fly brains were immunostained and imaged essentially as described previously (Zhang et al., 2010). Primary antibodies included the following: mouse anti-PDF (1:400), rabbit anti-PER (1:1500; generous gift from Dr. M. Rosbash, Brandeis University, Waltham, MA), an affinity-purified guinea pig anti-TIM (1:100) (Rakshit et al., 2012), anti-rabbit PDP1 (1:400; generous gift from Dr. J. Blau, New York University, New York, NY), guinea pig anti-VRI (1:10,000), and guinea pig anti-CLK (1:2500) (generous gifts from Dr. P. Hardin, Texas A&M University, College Station, TX). Secondary antibodies included the following: anti-mouse Cy5, anti-rat Cy3, anti-rabbit FITC, and anti-guinea pig Cy3 (1:200 dilution; Jackson ImmunoResearch). Mounted brains were scanned using a Carl Zeiss LSM5 Pascal confocal microscope using 40× water lens. Images are single Z sections in Figures 2A,D and 5 and are digitally projected Z stacks in Figure 2.2C.

Fluorescent signals for circadian proteins were quantified using NIH ImageJ v1.42q (http://rsb.info.nih.gov/ij). We subtracted background signal from the signal intensity of each neuron. Background signal was determined by taking the mean signal of three surrounding fields of each neuron. Brains with similar
background intensities were quantified. For each time point, at least eight neurons from three independent brain hemispheres were quantified.

**Cell culture, cell transfection, and luciferase assay**

*Drosophila* S$_2$R$^+$ cells were maintained in SFX growth medium (HyClone) supplemented with 9% fetal bovine serum and 1% penicillin–streptomycin (Invitrogen). Cells were seeded in 12-well plates. When reaching 70–90% confluence, they were transiently transfected using Cellfectin II (Invitrogen) as detailed in the instructions of the manufacturer. Luciferase reporter constructs were transfected at 50 ng/well. β-Galactosidase (100 ng) was transfected in each well to normalize transfection efficiency. Empty pAc vector was added so that each well was transfected with same amount of total DNA. Two days after transfection, cells were washed and lysed. Luciferase activity and β-galactosidase activity were measured separately in a 96-well plate using a Microtiter Plate Luminometer. β-Galactosidase activity was measured with the Galacto-light Plus kit (Invitrogen). When involving RNAi in S$_2$R$^+$ cells, 7.5 μg/well double-stranded RNAs (dsRNAs) were added to cell culture medium 1 d before transfection. For each data point, at least three independent experiments were performed.

HEK293 cells were maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum. Cells were seeded in 24-well plates. When reaching 70–90% confluence, they were transiently transfected using Lipofectamine 2000
reagent (Invitrogen) according to the instructions of the manufacturer. \textit{Clk–luc} was transfected at 20 ng/well. \textit{CMV–Renilla} (3 ng) was transfected in each well to normalize transfection efficiency. Empty \textit{pcDNA3.1} DNA was added so that each well was transfected with the same amount of total DNA. One day after transfection, dual luciferase assay were performed according to the instructions of the manufacturer (dual luciferase reporter assay system catalog \#32788; Promega) in a 96-well plate using Veritas Microplate Luminometer. For each experiment, at least three independent experiments were performed.

\textit{S}_2 \textit{R}^+ \textit{cell immunocytochemistry}

\textit{S}_2 \textit{R}^+ \textit{cells} were seeded on coverslips in six-well plates and transfected with \textit{pAc–Clk} and/or \textit{pAc–kay-α}. Two days after transfection, cells were washed twice in PBS and then fixed in 4\% paraformaldehyde in PBS for 20 min. Cells were then washed in PBT (PBS plus 0.1\% Triton X-100) and blocked in 10\% normal donkey serum for 30 min at room temperature. After that, each coverslip was incubated with 150 μl of primary antibodies [guinea pig anti-CLK at 1:3000, rabbit anti-KAY-α/TRUNC at 1:50, and mouse anti-lamin at 1:50 (generous gift from V. Budnik, University of Massachusetts Medical School, Worcester, MA)] at 4°C overnight. Coverslips were then washed and incubated with secondary antibodies (anti-guinea pig Cy3, anti-rabbit FITC, and anti-mouse Cy5 at 1:200 dilution) for 4 h at room temperature. After washing in PBT, coverslips were mounted onto slides in Vectashield Mounting Media and visualized under
confocal microscope using a 25× oil lens. Figure 2.3C shows digitally projected Z stacks.

*dsRNA synthesis*

We produced dsRNAs by *in vitro* transcription of a PCR-generated DNA template containing the T7 promoter sequence on both ends. A ∼500 bp *Jra* fragment was amplified from genomic DNA with the following primers: forward, 5′-TTAATACGACTCTATAGGGAGAGGCTCGCTGGATCTGAAC-3′; and reverse, 5′-TTAATACGACTCTATAGGGAGAGATGCAGCCACACCGTAA-3′.

We performed *in vitro* RNA transcription with Ambion MEGAscript T7 kit (catalog #AM1334) following the instructions of the manufacturer. After 2 h of 37°C incubation of the reaction, 1 μl of DNase was added to 20 μl of reaction and incubated for 15 min to degrade DNA template. Then, the reaction was stopped and single-stranded RNAs were precipitated, washed, and resuspended in water. Annealings of single-stranded RNAs were done by performing the following program: (1) 65°C, 30 min; (2) 65°C, 1 min, 1°C/cycle for 50 cycles. We confirmed the quality and size of dsRNAs on agarose gel.

*Antibody production, coimmunoprecipitation, and Western blot*

A synthesized peptide corresponding to amino acids 186–200 of KAY-α was used for the immunization of rabbits at Cocalico. Affinity-purified antisera were used for KAY-α detection.
For coimmunoprecipitation experiments, HEK293 cells were seeded in 100 mm Petri dishes. When reaching 70–90% confluence, cells were transfected with 5 μg of CMV–vri–VP16, 6 μg of CMV–myc–kay-α, or both. Empty pcDNA3.1 DNA was added to equalize the total amount of DNA transfected. One day after transfection, cells were lysed in Passive Lysis Buffer (1 ml of buffer per Petri dish; Promega) for 20 min, and supernatant was collected after centrifugation. Protein G Sepharose beads (catalog #17-0618-01; GE Healthcare) were incubated with anti-MYC antibody (catalog #11667149001; Roche) for 1 h at room temperature (2.5 μl of antibody per 15 μl of beads). Then 15 μl of these beads were added to 1 ml of supernatant and incubated overnight at 4°C with gentle agitation. Beads were then washed and resuspended in 1× SDS-PAGE loading buffer and boiled, ready for Western blotting.

Western blot were performed essentially as described previously (Emery et al., 1998). Equal loading and quality of protein transfer were first verified by Ponceau Red staining and then by the intensity of cross-reacting bands. Primary antibodies included the following: mouse anti-MYC at 1:1000, rabbit anti-KAY-α/TRUNC at 1:1000, rabbit anti-KAY main body (Dfos-112AP; FabGennix) at 1:500, mouse anti-V5 (Invitrogen) at 1:5000, and guinea pig anti-VRI at 1:5000. Secondary antibodies conjugated with HRP from Jackson ImmunoResearch were used at 1:10,000 dilution, except for goat anti-guinea pig IgG–HRP (1:5000; Abcam).
In Figure 2.3B, signals on the film were digitalized using IR-LAS-1000 Lite V2.12 (Fujifilm) and quantified with Image Gauge V4.22. CLK–V5 signals were normalized to the cross-reacting signals, and the ratio without *kay-α*–V5 was set to 100.
Table 2.1 KAY-α downregulation.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>% of rhythmic flies</th>
<th>Period average ± SEM</th>
<th>Power average ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>y w</td>
<td>122</td>
<td>74.59</td>
<td>23.81 ±0.03</td>
<td>76.08 ±3.76</td>
</tr>
<tr>
<td>w^{1118}</td>
<td>23</td>
<td>91.30</td>
<td>23.96 ±0.05</td>
<td>99.51 ±6.78</td>
</tr>
<tr>
<td>Pdf-GAL4/+</td>
<td>103</td>
<td>89.32</td>
<td>24.45 ±0.03</td>
<td>70.59 ±2.86</td>
</tr>
<tr>
<td>kayR2/+</td>
<td>103</td>
<td>72.82</td>
<td>24.29 ±0.04</td>
<td>69.62 ±4.60</td>
</tr>
<tr>
<td>Pdf-GAL4/+; kayR2/+</td>
<td>73</td>
<td>91.78</td>
<td>25.62 ±0.06***</td>
<td>76.78 ±3.91</td>
</tr>
<tr>
<td>kayR4/Y</td>
<td>63</td>
<td>85.71</td>
<td>24.01 ±0.05</td>
<td>80.48 ±3.91</td>
</tr>
<tr>
<td>kayR4/Y; Pdf-GAL4/+</td>
<td>32</td>
<td>71.88</td>
<td>25.20 ±0.08***</td>
<td>37.70 ±2.92</td>
</tr>
</tbody>
</table>

Behavior of control flies and flies expressing dsRNAs targeting kay-α/trunc in DD at 25°C. One-way ANOVA, $p < 0.0001$. Tukey's multiple comparison test, ***$p < 0.001$ when compared with Pdf–GAL4/+.
Table 2.2  Downregulation of all full-length KAY isoform.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>% of rhythmic flies</th>
<th>Period Average ± SEM</th>
<th>Power Average ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGD/+</td>
<td>31</td>
<td>83.87</td>
<td>24.95 ±0.07</td>
<td>67.66 ±4.32</td>
</tr>
<tr>
<td>PGD/+; NIG15509R-1/+</td>
<td>46</td>
<td>97.83</td>
<td>25.46 ±0.06***</td>
<td>34.20 ±5.10</td>
</tr>
<tr>
<td>NIG15509R-1/+</td>
<td>30</td>
<td>96.67</td>
<td>24.23 ±0.10</td>
<td>77.57 ±4.49</td>
</tr>
<tr>
<td>PGD/+; NIG15509R-2/+</td>
<td>31</td>
<td>83.87</td>
<td>25.32 ±0.08*</td>
<td>77.75 ±5.27</td>
</tr>
<tr>
<td>NIG15509R-2/+</td>
<td>15</td>
<td>60.00</td>
<td>24.23 ±0.18</td>
<td>69.88 ±8.53</td>
</tr>
<tr>
<td>PGD/+; VDRC6212/+</td>
<td>8</td>
<td>62.50</td>
<td>25.34 ±0.06</td>
<td>38.62 ±7.28</td>
</tr>
<tr>
<td>VDRC6212/+</td>
<td>19</td>
<td>78.95</td>
<td>24.27 ±0.09</td>
<td>81.63 ±7.36</td>
</tr>
</tbody>
</table>

Behavior of control flies and flies expressing dsRNAs targeting all full-length kay isoforms in DD at 25°C. One-way ANOVA, \( p<0.0001 \). Tukey’s multiple comparison test, ***\( p<0.001 \), *\( p<0.05 \) when compared with PGD/+ (Pdf-GAL4, UAS-dcr2)
Table 2.3 Cross-species rescue of KAY-α downregulation.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>% of rhythmic flies</th>
<th>Period average ± SEM</th>
<th>Power average ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>y w</strong></td>
<td>76</td>
<td>84.21</td>
<td>23.74 ±0.05</td>
<td>69.47 ±3.01</td>
</tr>
<tr>
<td>PGD/+</td>
<td>80</td>
<td>87.50</td>
<td>24.39 ±0.04</td>
<td>55.07 ±2.69</td>
</tr>
<tr>
<td>PGD/+; kayR2/+</td>
<td>86</td>
<td>51.16</td>
<td>25.75 ±0.10</td>
<td>50.96 ±3.98</td>
</tr>
<tr>
<td>PGD/α1; kayR2/+</td>
<td>22</td>
<td>59.09</td>
<td>25.56 ±0.21</td>
<td>45.49 ±5.33</td>
</tr>
<tr>
<td>PGD/+; kayR2/α2</td>
<td>20</td>
<td>60.00</td>
<td>25.42 ±0.14</td>
<td>64.13 ±8.43</td>
</tr>
<tr>
<td>PGD/α3; kayR2/+</td>
<td>31</td>
<td>51.61</td>
<td>24.72 ±0.10*</td>
<td>53.29 ±4.35</td>
</tr>
<tr>
<td>PGD/+; kayR2/α4</td>
<td>22</td>
<td>77.27</td>
<td>24.68 ±0.06*</td>
<td>70.38 ±6.05</td>
</tr>
<tr>
<td>PGD/+; kayR2/α5</td>
<td>34</td>
<td>85.29</td>
<td>25.17 ±0.07**</td>
<td>55.41 ±3.51</td>
</tr>
<tr>
<td>PGD/α6; kayR2/+</td>
<td>40</td>
<td>52.50</td>
<td>24.22 ±0.08*</td>
<td>44.76 ±4.75</td>
</tr>
<tr>
<td>PGD/+; kayR2/α7</td>
<td>16</td>
<td>62.50</td>
<td>25.18 ±0.10</td>
<td>55.12 ±5.95</td>
</tr>
<tr>
<td>PGD/α9; kayR2/+</td>
<td>23</td>
<td>56.52</td>
<td>24.56 ±0.10*</td>
<td>49.52 ±5.60</td>
</tr>
<tr>
<td>PGD/+; kayR2/α10</td>
<td>53</td>
<td>75.47</td>
<td>24.59 ±0.07*</td>
<td>47.06 ±2.14</td>
</tr>
<tr>
<td>PGD/α11; kayR2/+</td>
<td>18</td>
<td>33.33</td>
<td>24.62 ±0.23*</td>
<td>48.68 ±5.95</td>
</tr>
<tr>
<td>PGD/trunc1; kayR2/+</td>
<td>23</td>
<td>52.17</td>
<td>25.75 ±0.18</td>
<td>52.35 ±6.53</td>
</tr>
<tr>
<td>PGD/+; kayR2/trunc2</td>
<td>35</td>
<td>37.14</td>
<td>25.40 ±0.21</td>
<td>46.11 ±2.98</td>
</tr>
<tr>
<td>PGD/+; kayR2/trunc3</td>
<td>31</td>
<td>74.19</td>
<td>26.11 ±0.17</td>
<td>39.49 ±3.11</td>
</tr>
<tr>
<td>PGD/+; kayR2/trunc4</td>
<td>18</td>
<td>44.44</td>
<td>26.04 ±0.46</td>
<td>54.59 ±7.03</td>
</tr>
<tr>
<td>PGD/trunc5; kayR2/+</td>
<td>14</td>
<td>50.00</td>
<td>26.03 ±0.24</td>
<td>52.19 ±10.52</td>
</tr>
<tr>
<td>PGD/trunc6; kayR2/+</td>
<td>13</td>
<td>23.08</td>
<td>25.57 ±0.17</td>
<td>37.00 ±3.38</td>
</tr>
<tr>
<td>PGD/+; kayR2/trunc7</td>
<td>22</td>
<td>18.18</td>
<td>26.53 ±0.29</td>
<td>34.18 ±5.76</td>
</tr>
<tr>
<td>PGD/trunc8; kayR2/+</td>
<td>35</td>
<td>37.14</td>
<td>26.77 ±0.19</td>
<td>39.82 ±4.80</td>
</tr>
<tr>
<td>PGD/+; kayR2/trunc9</td>
<td>36</td>
<td>63.89</td>
<td>25.73 ±0.11</td>
<td>45.67 ±3.31</td>
</tr>
</tbody>
</table>
Behavior of *kay*-αRNAi flies expressing chimeric *pseudoobscura/melanogaster kay*-α or *kay–trunc* in DD at 25°C. In bold are the lines that show statistically significant rescue of the period lengthening caused by *kay*-α/trunc downregulation (7 of 10 α lines, 0 of 9 trunc lines). One-way ANOVA, *p*<0.0001. Bonferroni’s multiple comparison test was done on the following pairs: all rescue lines versus *PGD/+* and all rescue lines versus *PGD/+; kayR2/+*. *α*=0.05. Lines with mean periods that are not significantly different from that of *PGD/+* are considered as fully rescued (†). One line with mean period that is significantly different from both *PGD/+* and *PGD/+; kayR2/+* and is less than that of *PGD/+; kayR2/+* is considered as partially rescued (‡).
Table 2.4 Knockdown of \textit{Jra} in pacemaker neurons did not result in long period.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>% of rhythmic flies</th>
<th>Period average ± SEM</th>
<th>Power average ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{y w}</td>
<td>16</td>
<td>62.50</td>
<td>23.84 ± 0.16</td>
<td>46.93 ± 6.53</td>
</tr>
<tr>
<td>\textit{PGD/+}</td>
<td>30</td>
<td>96.67</td>
<td>24.92 ± 0.04</td>
<td>56.96 ± 4.90</td>
</tr>
<tr>
<td>\textit{PGD/+; JraRNAi\textsuperscript{JF01184/+}}</td>
<td>32</td>
<td>93.75</td>
<td>24.47 ± 0.05</td>
<td>57.71 ± 2.98</td>
</tr>
<tr>
<td>\textit{PGD/+; JraRNAi\textsuperscript{JF01379/+}}</td>
<td>16</td>
<td>93.75</td>
<td>24.73 ± 0.11</td>
<td>65.37 ± 7.27</td>
</tr>
</tbody>
</table>

Behavior of control flies and flies expressing dsRNAs targeting \textit{Jra} in DD at 25°C.
Figure 2.1

A

B

From D. pseudoobscura

From D. melanogaster
Figure 2.1 Downregulating kay-α lengthens the period of free-running circadian behavior in DD. A, Organization of the kay locus in *D. melanogaster*. kay is predicted to produce five isoforms. The dark boxes indicate coding sequences, and open boxes indicate noncoding sequences. Transgene NIG15507 generates dsRNAs targeting both the α and trunc isoforms. The red line indicates the region targeted by dsRNA. Constructs for cross-species rescue experiments are shown on the bottom of the panel. α9, α10, trunc3, and trunc9 are insertions of UAS-controlled transgenes that generate kay mRNAs resistant to the NIG15507 dsRNAs. The region targeted by NIG15507 dsRNAs was replaced with homologous *D. pseudoobscura* sequences. B, Downregulation of kay-α lengthens circadian behavior period. Bars 1 and 2, Flies expressing dsRNAs targeting kay-α and kay–trunc under the Pdf–GAL4 driver have ~26-h-long period rhythms (control, 24.4 h). PGD: Pdf-GAL4, UAS-dcr2. Bars 3–6, The kay RNAi phenotype can be rescued with the kay-α construct resistant to the dsRNAs but not with the kay–trunc construct. Bars 7 and 8, The rescue is not explained by a period shortening caused by expression of the chimeric kay-α, because its expression in wild-type flies does not shorten circadian behavioral rhythms (it actually slightly lengthens them). Error bars correspond to SEM. Digits in the bar are the numbers of tested flies. Percentage of rhythmicity is indicated above the bars. One-way ANOVA, $p < 0.0001$. Tukey’s multiple comparison test. ***$p < 0.001$; n.s., not significant at level of 0.05. C, Double-plotted actograms showing the average activity for each genotype. Flies were entrained in standard LD cycle for 3 d and then released in DD. D, The NIG15507-R2 transgene can inhibit KAY-α expression *in vivo*. Fly head extracts were immunoblotted with an anti-KAY-α/TRUNC antibody. Strong immunoreactivity was observed when KAY-α was misexpressed in the eyes with the Rh1–GAL4 driver (lane 1), whereas endogenous KAY-α was undetectable (lane 2). dsRNAs generated by NIG15507-R2 transgene were able to dramatically knock down the overexpression of KAY-α (lane 3).
Figure 2.2

A

<table>
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<tr>
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<th>Day 4 in DD</th>
<th>Day 5 in DD</th>
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B

PER Signal (arbitrary unit) vs Circadian Time (hr)

C

PGD/+  
PGD/+; kayR2/+  
PMDL/LNv  s-LNv  s-LNv  s-LNv

D

Period (hr)

- PGD/+  
- PGD/+; kayR2/+  
- PGD/UAS-PDF; kayR2/+  
- PGD/UAS-PDF
Figure 2.2 Altered PER rhythms and reduced PDF levels in pacemaker neurons of kay-αRNAi flies. A, Confocal images of brains from control and kay-αRNAi (PGD/+; kayR2+) flies immunostained with PER and PDF antibodies. Flies were entrained to a LD cycle for 3 d and then released in DD. Fly brains were dissected at indicated CTs during the fourth subjective night and fifth subjective day. Representative sLNvs are shown. B, Quantification of PER signals after subtraction of background signal. At CT14 for control flies and CT17 for kay-αRNAi flies, PER signals are indistinguishable from background; thus, they are set to “0” on the plot. Error bars correspond to SEM. C, The sLNvs develop normally in kay-αRNAi flies. Fly brains were dissected at CT24 on the first day in DD after 3 d of standard LD cycle and were immunostained for PDF and PER. Images are Z-stack projections of confocal images. Neuronal processes from the sLNvs to the dorsal brain (arrows) appear indistinguishable between PGD/+ and PGD/+; kayR2/+ flies in morphology. Circled are the regions containing cell bodies of large or small LNvs. PGD/+; kayR2/+ flies have only very weak PDF staining in the cell bodies of sLNvs. D, Restoring PDF levels does not rescue the long period phenotype. Overexpressing Pdf in kay-αRNAi flies restored PDF levels in sLNvs (the third panel, white arrow) but did not rescue the long period phenotype (bar 3), which is thus not attributable to low PDF levels but to a defective pacemaker. One-way ANOVA, p < 0.0001. Tukey's multiple comparison test. ***p < 0.001.
Figure 2.3
**Figure 2.3 KAY-α represses CLK/CYC transactivation.** *Drosophila* S$_2$R$^+$ cells were transfected as indicated. Luciferase activity was measured 2 d after transfection. A β-galactosidase-expressing plasmid was cotransfected to normalize transfection efficiency. The relative luciferase activity with *Clk* was set to 100 on each graph. **A**, KAY-α represses CLK activation of the *tim* promoter and the *per* E-box. The proximal *tim* promoter with a wild-type or mutagenized AP-1 binding site and multimerized *per* E-boxes were cloned in the pGL3 luciferase reporter vector to make *tim*–luc, *tim*–mut–luc, and *per*–Ebox–luc. Jra was cotransfected with *kay*-α. **B**, KAY-α does not alter CLK expression level in S$_2$R$^+$ cells. Cell lysates were immunoblotted with anti-V5 antibody. CLK–V5 protein level was quantified. CLK–V5 levels did not change whether or not *kay*-α was transfected in three independent experiments. CLK–V5 levels in the absence of KAY-α were set at 100. In the presence of KAY-α, relative CLK amount was 108 ± 20, *n* = 3. **C**, KAY-α does not alter CLK subcellular localization in S$_2$R$^+$ cells. Representative immunostaining showing CLK localization in the nucleus in the presence or absence of KAY-α. More than 95% of cells expressing or not KAY-α show this primarily nuclear CLK localization. Very rarely, and in both cells with or without KAY-α, CLK showed both nuclear and cytoplasmic localization. Lamin stains inner nuclear membrane. Circles outline the cell bodies. **D**, The repression of CLK activation by KAY-α does not require JRA. S$_2$R$^+$ cells were treated with dsRNAs targeting *Jra* 1 d before transfection to knock down endogenous JRA. Even in the presence of these *Jra* dsRNAs, KAY can still repress CLK/CYC activation. **E**, VP16–KAY-α cannot activate the proximal *tim* promoter. The activation domain of VP16 was fused to the N terminal of KAY-α to generate VP16–KAY-α. The fusion protein could not activate *tim*–luc but still repressed the activity of CLK. **F**, KAY-α DNA binding domain is required for CLK repression. The basic region of KAY-α was deleted to generate a *kay*-α–basicΔ construct, which was not able to repress CLK activity. **G**, The removal of the DNA binding domain of KAY-α does not affect its stability. Expression of wild-type and *kay*-α–basicΔ was comparable on Western blots.
**Figure 2.4**

CLK repression is specific to KAY-α. *Drosophila* S₂R⁺ cells were transfected as indicated. A β-galactosidase-expressing plasmid was cotransfected to normalize transfection efficiency. The relative luciferase activity with Clk or VP16–cwo was set to 100 on the graph. **A**, CLK activation in the presence of different KAY isoforms. Neither KAY-β, KAY-γ, nor KAY–TRUNC can repress CLK activation of the *tim* promoter or the *per* E-box. **B**, KAY-β, KAY-γ and KAY–TRUNC are well expressed in S₂R⁺ cells. Cell lysates were immunoblotted with anti-KAY-main body antibody (left) or anti-KAY-α/TRUNC antibody (right). **C**, KAY-α does not repress the activation of the *tim* promoter by VP16–CWO.
Figure 2.5

A

- CLK-CT24
- CLK-CT12
- PER-CT24

Fluorescence signal (arbitrary unit)

- PGD/+ vs. PGD/+; karyR2/+ (****)

TIM-CT24

PDP1-CT18

VRI-CT15

Fluorescence signal (arbitrary unit)

- PGD/+ vs. PGD/+; karyR2/+ (n.s.)

B

- PER-CT24

- perP; PGD/+; UAS-per/lucRNAi
- perP; PGD/+; UAS-per/karyR2

Fluorescence signal (arbitrary unit)

- Exp.1 vs. Exp.2 (n.s.)
**Figure 2.5 Altered circadian protein expression in kay-αRNAi flies.** A, KAY-α downregulation alters expression of several circadian proteins. Flies were entrained to a standard LD cycle for 3 d and then released in DD. Fly brains were dissected on the first day of DD at the expected peak time point of the protein measured, followed by immunocytochemistry (kay-αRNAi flies were dissected ~1–1.5 h after control flies to correct for differences in period length). Representative staining images and quantifications are shown. Arrows point to sLNvs in the focal plane. Quantification of protein levels are represented by boxes and whiskers in which whiskers show the minimum and maximum values, boxes show the middle 50% of the values, and horizontal lines in the boxes show the median. Two to four independent experiments were performed. CLK, PER, and PDP1 levels were markedly reduced in kay-αRNAi in all experiments. VRI levels were unaffected. TIM levels were reduced in kay-αRNAi flies in all three experiments, but only in one experiment was that decrease statistically significant. Student's t test. ***p < 0.001; n.s., not significant. B, KAY-α regulation of PER levels is strongly dependent on the per promoter. When PER was expressed independently of its promoter with Pdf–GAL4 in a per^0 background, KAY-α downregulation had weak effects on PER levels. Quantifications of two independent experiments are shown. In the first one, the average 13% decrease in PER level in kay-αRNAi flies was not statistically significant, but the average 22% decrease in the second experiment was marginally significant (Student's t test, p = 0.04). Thus, posttranscriptional regulation of PER might have a weak contribution to its protein decrease in kay-αRNAi flies.
Figure 2.6 KAY-α interacts with and inhibits VRI. A, KAY-α blocks specifically VRI–VP16 activation of Clk promoter. HEK293 cells were transfected as indicated. Renilla luciferase was transfected to normalize transfection efficiency. Luciferase activity was measured 1 d after transfection. Relative luciferase activity with vri–VP16 was set to 100. VRI–VP16 activates the Clk promoter, as described previously (Cyran et al., 2003). The activation of the Clk promoter by VRI–VP16 was inhibited in a dose-dependent manner by KAY-α, but the activation of the Clk promoter by PDP1ε was unaffected. Error bars are SEM. B, KAY-α interacts with VRI–VP16 in HEK293 cells. HEK293 cells were transfected as indicated. Cell lysates were immunoprecipitated with anti-MYC antibody. Bound proteins were probed with anti-MYC and anti-VRI antibodies. VRI–VP16 was coimmunoprecipitated with MYC–KAY-α. C, KAY-β and KAY-γ can also repress the activation of the Clk promoter by VRI–VP16. HEK293 cells were transfected as indicated. A Renilla-expressing vector was cotransfected to normalize transfection efficiency. The normalized luciferase activity with vri–VP16 was set to 100 on the graph. Error bars are SEM. D, CLK can activate the kay–α promoter. A ~300 bp kay–α promoter fragment containing an E-box was cloned in the pGL3 vector to generate kay–α-Ebox–luc. It can be activated by CLK. The normalized luciferase activity without Clk was set to 1 on the graph. Error bars are SEM.
Figure 2.7

\[\text{kay-}\alpha\text{ knockdown}\]
Figure 2.7 A model for the role of KAY-α in the control of circadian behavior. Our results indicate that KAY-α affects both circadian transcriptional feedback loops. It inhibits VRI through direct physical contact and also represses CLK transactivation (top). When KAY-α is absent (bottom), VRI repression is enhanced (thicker red lines), which results in decreased CLK and PDF levels (lighter filling color). However, CLK activity also increases (zigzags). This mitigates the effects of increased VRI repression, and CLK/CYC targets are either weakly (TIM) or moderately (PER, PDP1) affected or not at all (VRI).
Chapter III

A role for *Drosophila* ATX2 in activation of PER translation and circadian behavior

This chapter has been published as


R. Dubruille first observed the long period phenotype in ATX2 knockdown flies. Y. Zhang further characterized this phenotype by knocking down ATX2 in different groups of neurons using different RNAi lines. He did ATX2 conditional knockdown experiment and PER misexpression experiment. He also found genetic interactions between ATX2 and TYF. Yong and I together characterized the changes of circadian proteins in central circadian neurons in ATX2 knockdown flies as well as the RNA binding property of ATX2. I performed the cell culture assays indicating ATX2's role in the activation of PER translation. I also did the co-immunoprecipitations showing the physical interaction between ATX2 and TYF and the requirement of ATX2 for the stability of TYF/PABP complex. In addition, I made the chimeric construct for the behavioral rescue experiment. C. Yuan generated the antigen for ATX2 antibody and provided technical assistance for immunoprecipitation. P. Emery wrote the body of the text, while Y. Zhang and I contributed to the Figures, Tables, Materials and Methods and provided feedback on the other sections.
A. Abstract

A negative transcriptional feedback loop generates circadian rhythms in Drosophila. PERIOD (PER) is a critical state-variable in this mechanism, and its abundance is tightly regulated. We found that the Drosophila homolog of ATAXIN-2 (ATX2)—an RNA-binding protein implicated in human neurodegenerative diseases—was required for circadian locomotor behavior. ATX2 was necessary for PER accumulation in circadian pacemaker neurons and thus determined period length of circadian behavior. ATX2 was required for the function of TWENTY-FOUR (TYF), a crucial activator of PER translation. ATX2 formed a complex with TYF and promoted its interaction with polyadenylate-binding protein (PABP). Our work uncovers a role for ATX2 in circadian timing and reveals that this protein functions as an activator of PER translation in circadian neurons.

B. Results and Discussion

In animals, circadian pacemakers synchronize a wide range of processes with the day-night cycle, from basic cellular metabolism to complex behaviors. These molecular clocks are composed of highly conserved transcriptional feedback loops (Yu and Hardin, 2006). In Drosophila, PERIOD (PER) is a critical component of the circadian pacemaker and is under tight transcriptional and posttranslational control (Yu and Hardin, 2006). PER, with the help of TIMELESS (TIM), negatively regulates its own gene expression by displacing the dimeric
transactivator CLOCK-CYCLE from its promoter (Menet et al., 2010). PER phosphorylation—regulated by several kinases (NEMO, DOUBLETIME, and Casein Kinase II) and by the phosphatase PP2A (Sathyanarayanan et al., 2004; Ko et al., 2010)—controls PER stability, activity, and nuclear entry. per mRNA stability and translation are also regulated (So and Rosbash, 1997; Suri et al., 1999; Stanewsky et al., 2002; Lim et al., 2011; Bradley et al., 2012). The protein TWENTY-FOUR (TYF) (Lim et al., 2011) promotes PER translation in the Pigment-Dispersing Factor (PDF)—containing small ventral lateral neurons (sLNvs), which play a particularly important role in the control of circadian behavior (Renn et al., 1999; Stoleru et al., 2005). TYF binds both polyadenylate-binding protein (PABP) and eukaryotic translation initiation factor 4F (eIF4F), thus presumably promoting per mRNA circularization and translation. However, TYF does not appear to bind per mRNA directly (Lim et al., 2011).

ATAXIN-2 (ATX2) is an RNA-binding protein that is proposed to regulate translation. It interacts with PABP, is found in stress granules, and in Drosophila has an important role in miRNA silencing (Satterfield and Pallanck, 2006; Nonhoff et al., 2007; McCann et al., 2011). In an RNA interference (RNAi) screen aimed at identifying previously unknown regulators of circadian behavior, Atx2 was among the genes linked to miRNA silencing that were down-regulated with long or short double-stranded RNAs (dsRNAs). The expression of these dsRNAs is controlled by Upstream Activating Sequence (UAS)—binding sites (Dietzl et al., 2007; Ni et al., 2011) and can thus be activated with tissue-specific GAL4
transgenes. When Atx2 dsRNAs (Atx2\textsuperscript{RNAi-1}) (Figure 3.S1A) were expressed in all circadian neurons with tim-GAL4 (Kaneko et al., 2000), the period of circadian locomotor behavior under constant darkness (DD) was lengthened to ~26.7 hours (Figure 3.1A and Table 3.S1). We also noticed increased arrhythmicity and lower amplitude of rhythms (Table 3.S1, "power"). These data indicate a crucial role for ATX2 in the circadian molecular pacemaker. Depletion of GW182, a protein essential for miRNA silencing (Eulalio et al., 2009), resulted in a distinct circadian phenotype (Zhang and Emery, 2013), indicating that ATX2 may regulate circadian behavior independently of miRNA silencing.

Because the PDF-containing sLNvs control circadian behavior in DD (Renn et al., 1999; Stoleru et al., 2005), we restricted expression of Atx2 dsRNAs with the Pdf-GAL4 driver. The phenotype in DD was as severe as that observed with tim-GAL4 (Figure 3.1A and Table 3.S1). Furthermore, no phenotype was observed when we restricted expression of Atx2 dsRNAs by combining tim-GAL4 with Pdf-GAL80, which blocks GAL4-mediated transcription in PDF cells (Figure 3.1A and Table 3.S1) (Stoleru et al., 2005). To exclude off-target effects, we expressed a second dsRNA in PDF-containing circadian neurons, which targets a different region of the Atx2 mRNA (Atx2\textsuperscript{RNAi-2}) (Figure 3.S1A). A similar long-period phenotype was observed (Figure 3.1A and Table 3.S1). Moreover, when dsRNAs were expressed with tim-GAL4, ATX2 was depleted in all circadian neurons examined [sLNvs, large LNvs (ILNvs), dorsal Lateral Neurons (LNds) and Dorsal Neurons 1 (DN1s)] (Figure 3.1B). The long-period phenotype was
prevented by expression of a chimeric UAS-Atx2 (UAS-Dm/pAtx2), in which the region targeted by one of the dsRNAs was from D. pseudoobscura and thus sufficiently divergent to be resistant to RNAi (Figure 3.1A, Figure 3.S1A, and Table 3.S1) (Langer et al., 2010). We therefore conclude that ATX2 is required in PDF-containing sLNvs for normal circadian behavior in DD.

No obvious anatomical defects were observed in sLNvs when ATX2 was depleted (Figure 3.S2A), but subtle developmental defects cannot be excluded. We therefore restricted expression of the dsRNAs either to development or to adulthood with GAL80\textsuperscript{ts}, a temperature-sensitive repressor of GAL4 (McGuire et al., 2004). Flies that developed at 29°C and were transferred after eclosion to 18°C to prevent further production of Atx2 dsRNAs behaved like control flies in DD. (Figure 3.S2B and Table 3.S1). Thus, developmental expression of Atx2 dsRNAs does not appear to affect circadian behavior in adult flies. On the contrary, flies that developed at 18°C and were transferred to 29°C after eclosion showed a ~1-hour increase in period (Figure 3.S2B and Table 3.S1). The depletion of ATX2 in adults thus appears to be sufficient to lengthen circadian behavioral rhythms. The weaker effect on period as compared with that of life-long ATX2 down-regulation (Figure 3.1A and Table 3.S1) may reflect less effective depletion of ATX2 in the presence of GAL80\textsuperscript{ts}, either because this protein still weakly represses GAL4 even at high temperature or because less time has elapsed for the sLNvs to accumulate dsRNAs. We conclude that ATX2 is required acutely in adult sLNvs.
To understand the consequences of ATX2 depletion on the circadian pacemaker, we measured abundance of PER in circadian neurons on the 4th day of DD. PER rhythms were delayed by ~8 hours in the sLNvs (Figure 3.2) and in the LNds (Figure 3.S3A) of flies with low ATX2 amounts, which is consistent with the long-period behavioral phenotype. Unexpectedly, in the DN1s peak PER concentrations were not delayed, although its minimum concentrations were (Figure 3.S3A). The reason for this partial delay in DN1s is not clear. Amounts of PER in the sLNvs were low (Figure 3.2) but close to normal in the DN1s and the LNds (Figure 3.S3A). Amounts of TIM were not affected in any of these neurons (Figure 3.S3B). Low abundance of PER appeared to be sufficient to explain the long-period phenotype because overexpression of PER restored normal period length in flies with depleted ATX2 (Figure 3.2C).

The long-period phenotype, increased behavioral arrhythmicity, and low abundance of PER in the sLNvs are reminiscent of the phenotypes observed in tyf mutant flies (Lim et al., 2011). To determine whether ATX2 and TYF might work together in the circadian cycle, we tested double loss-of-function mutants. The double-mutants showed no additive effects compared with the single mutants (Figure 3.3A), which indicates that ATX2 and TYF regulate the same step of the circadian cycle: PER translation.

TYF does not bind RNA on its own. Thus, to assay TYF’s translational activity in cell culture, TYF was fused to the MS2 RNA-binding protein, and MS2-
binding sites were placed in the 3′ untranslated region (3′UTR) of a luciferase reporter gene (Lim et al., 2011). MS2-TYF promoted luciferase expression, and this transactivation was increased in the presence of per 3′UTR sequences (Figure 3.3B), as previously reported (Lim et al., 2011). If ATX2 was depleted with dsRNAs, TYF transactivation was decreased (Figure 3.3B). However, basal luciferase expression was unaffected. We therefore conclude that TYF requires ATX2 to promote PER translation.

To further test cooperative action of ATX2 and TYF to regulate PER translation, we determined whether they form a complex in vivo. We overexpressed a V5-tagged TYF and either isolated TYF complexes with an antibody to V5 or immunoprecipitated endogenous ATX2 with a polyclonal antibody to this protein. In both cases, ATX2 and TYF coimmunoprecipitated (Figure 3.3C). Moreover, the ATX2-TYF complexes were resistant to RNase A treatment (Figure 3.3D). Because TYF is associated with per mRNA, we tested whether ATX2 is also associated with per mRNA. We immunoprecipitated ATX2 and found per mRNA to be enriched in ATX2 immunoprecipitates compared with that of the control (Figure 3.3E and Figure 3.S4A). Although TYF appears to bind per mRNAs preferentially (Lim et al., 2011), ATX2 also associated with other mRNAs. Thus, how TYF is specifically recruited to per mRNAs remains unclear. A rhythm in ATX2-per mRNA complex formation was observed. It appears to be largely driven by mRNA abundance rhythms (Figure 3.S4, B and C).
TYF interacts with PABP and eIF4F (Lim et al., 2011). Because ATX2 also
binds to PABP (Satterfield and Pallanck, 2006), we tested whether ATX2
contributes to the formation of a stable TYF complex that bridges eIF4F and
PABP and thus stimulates PER translation through mRNA circularization
(Sonenberg and Hinnebusch, 2009). We immunoprecipitated TYF-V5 with an
antibody to V5 from whole-head extracts of control flies, or from heads of flies
expressing Atx2 dsRNAs (Figure 3.3F). Depletion of ATX2 reduced the amount
of both ATX2 and PABP in the V5-TYF immunoprecipitate. Thus, ATX2 is
necessary for the formation of a stable complex between TYF and PABP (Figure
3.S5).

Our results implicate ATX2 in the control of circadian rhythms as a critical
activator of PER translation. They support the emerging notion that translational
control is critical for the function of circadian pacemaker neurons (Lim et al., 2011;
Bradley et al., 2012). Our results also emphasize that ATX2 can function as a
translational activator as well as a translational repressor (Satterfield and
Pallanck, 2006; Nonhoff et al., 2007; McCann et al., 2011). Whether ATX2
functions as an activator or repressor may depend on the combination of proteins
associated with a specific mRNA. In the case of per, ATX2’s association with
TYF might explain its positive effect on PER translation.

In humans, extension of ATAXIN-2’s Poly-Q stretch to pathological lengths
is responsible for spinocerebellar ataxia and is associated with increased risk of
amyotrophic lateral sclerosis (Lastres-Becker et al., 2008; Elden et al., 2010). Patients affected with spinocerebellar ataxia, and even asymptomatic carriers of a pathological Atx2 allele, suffer from sleep disruption, particularly disruption of rapid eye movement (REM) sleep (Boesch et al., 2006; Tuin et al., 2006; Rodriguez-Labrada et al., 2011; Velazquez-Perez et al., 2011), which is under circadian regulation (Dijk and Czeisler, 1995; Lee et al., 2009). Decreased ATX2 activity in the circadian clock perhaps contributes to these sleep disorders.

C. Materials and Methods

_Drosophila stocks_

The following strains were used: _w_^{1118}, _y_ _w_; _tim-GAL4/CyO_ (Kaneko et al., 2000), _y_ _w_; _Pdf-GAL4/CyO_ (Renn et al., 1999); _y_ _w_; _tim-GAL4/CyO_; _Pdf-GAL80/TM6B_ (Murad et al., 2007), _y_ _w_; _tim-GAL4 UAS-dcr2/CyO_ (Dubruille et al., 2009), _y_ _w_; _Pdf-GAL4 UAS-dcr2/CyO_ (Dubruille et al., 2009), _y_ _w_; _tim-GAL4-16_ (Kaneko et al., 2000) was obtained from R. Stanewsky, _UAS-per24_ (Kaneko, 1998) and _tubP-GAL80^{ts}_ (McGuire et al., 2004) were combined with _tim-GAL4_ in the lab. For Atx2 RNAi, the KK100423 (Atx2^{RNAi-1}) and GD11562 (Atx2^{RNAi-2}) lines were obtained from VDRC. GD11562 was meiotically recombined with PD2 on the 2nd chromosome to perform rescue experiments. Recombinants were selected based on the long period phenotype, and confirmed by PCR. _tyf_ mutants and _UAS-tyf-V5_ flies (Lim et al., 2011) were obtained from R. Allada. All
the flies were raised on cornmeal/agar medium at 25 °C under LD conditions, except when testing for potential developmental effects of ATX2 downregulation.

**DNA constructs and transgenic flies**

*pAc-Atx2* was generated by ligating a PCR product containing the Atx2 isoform B coding sequence (*Drosophila* Genomics Research Center) into the NotI/XbaI site of a *pAc-MYC* vector so that a MYC tag is added to the N-terminus of ATX2. Primers used were NotI-Atx2-for: 5’-AACCAGCGGCGCAACAATAGCAAGCGGAA-3’. Atx2-XbaI-rev-new: 5’-TGGCTCTGAGCTACTGTGGCTGATGCTGCTGATC-3’.

*pAc-Dm/pAtx2* was generated by ligating two PCR fragments using In-Fusion HD Cloning System (Clontech). One fragment includes all the sequence of *pAc-Atx2* except the region targeted by *Atx2RNAi-2*. It was generated by PCR using *pAc-Atx2* as a template and the following primers: vector-for: 5’-CGGGCCCAGCACCCTC-3’, and vector-rev2: 5’-TGTATGGTAAATTGGCAACGGCTG-3’. The other fragment includes sequences from *Drosophila pseudoobscura* that are homologous to the *Atx2RNAi-2* targeted region. It was generated by PCR using *Drosophila pseudoobscura* genomic DNA as a template and the following primers: Dp-for-3: 5’-GCCAATTTACCATAATGCCACCCACCTACCAGAC-3’, and Dp-rev-2: 5’-CGGGGTGCTGGGCCCGCCGCGGCTAAAAGGCTTGGC-3’.
pAc-Dm/pAtx2 was then digested by NotI and XbaI and the Dm/pAtx2 fragments was ligated into a pUAST-MYC vector (which adds a MYC tag to the N terminal of Dm/pATX2) to generate pUAST- Dm/pAtx2. The construct was verified by sequencing and then introduced in flies by Genetic Services, Inc (Cambridge, MA) using standard P element-mediated germ-line transformation. Three transformants were obtained and labeled as #3, #18 and #53.

**Behavioral analysis**

2-5 days old single male flies were entrained under 12hr: 12hr light-dark cycle (LD) for 3 days and then released to constant darkness (DD) at 25°C for at least 5 days. The locomotor activity of single males was monitored in Trikinetics Activity Monitors (Waltham, MA) using I-36LL Percival incubators (Percival Scientific, Perry IA). Each behavioral experiment was repeated at least three times. Data analysis was done with FaasX software (Picot et al., 2007). Actograms were generated with a toolbox for Matlab (Math Works, Natick, MA) (Levine et al., 2002).

**Whole-mount brain immunostaining and quantification**

Flies were entrained under standard light-dark cycles for 3 days at 25°C and then released in constant darkness. Brains were dissected at the indicated Circadian Times (CT) during the 4th subjective day and night. Fly brains were immunostained and imaged essentially as previously described (Zhang et al., 2010). Primary antibodies used were: mouse anti-PDF (1:400), rabbit anti-PER
(1:1500, generous gift from Dr. M. Rosbash), an affinity purified guinea pig anti-TIM (1:100) (Rakshit et al., 2012) and a guinea pig anti-Atx2 (1:400). This antibody was raised at Cocalico Biologicals Inc. (Reamstown, PA) against the 150 N-terminal end of the ATX2-PC isoform (Flybase). This region is common to all three predicted ATX2 isoforms. Secondary antibodies were: anti-mouse-Cy5, anti-rabbit-FITC, and anti-guinea pig-Cy3 (Jackson ImmunoResearch Inc. 1:200 dilution). Mounted brains were scanned using a Zeiss LSM5 Pascal confocal microscope. Images are single Z sections in Figure 3.1 and 3.2, and are digitally projected Z-stacks in Figure 3.S2.

Fluorescent signals for circadian proteins were quantified using ImageJ v1.42q (http://rsb.info.nih.gov/ij). We subtracted background signal from the signal intensity of each neuron. Background signal was determined by taking the mean signal of three surrounding fields of each neuron. Brains with similar background intensities were quantified. Neurons from at least 4 independent brain hemispheres were quantified for each data point. TIM levels were quantified at CT0 and CT8. For ATX2 quantifications, since ATX2 is mainly expressed in the cytoplasm of cell bodies, we subtracted background signals in brain region without cell bodies from the signal measured either in circadian neurons or in neighboring non-circadian neurons. We then normalized the subtracted signal from clock neurons with the average from three neighboring noncircadian neurons. Each experiment was done at least twice, showing reproducible results.
**Immunoprecipitations and Western blot**

The aforementioned anti-ATX2 antibody was used to immunoprecipitate ATX2 from head extracts. A pre-immunization bleed from the same guinea pig was used as negative control. Coimmunoprecipitation was essentially done as described in Lim et al. (Lim et al., 2011). For each genotype, 200 fly heads were homogenized in 200μl Lysis buffer (25mM Tris-HCl, pH 7.5, 300mM NaCl, 10% glycerol, 1mM EDTA, 1mM DTT, 0.5% NP-40, 1X protease inhibitor) and incubated at 4°C for 20 min with gentle agitation. After two clarifications by centrifugation, soluble extracts were diluted to adjust the salt concentration to 100mM. Sepharose 4 fast flow beads with protein A or protein G were incubated with anti-ATX2 or anti-V5 (Invitrogen) antibodies respectively for 1.5hr. After a brief wash, these beads were added to the extract and incubated overnight at 4°C. Beads were then washed and bound proteins were subjected to Western blot. Where indicated, RNase A was added to the soluble extract at a concentration of 100μg/ml and extracts were incubated at room temperature for 15 min before adding the beads. In Figure 3.4B, fly heads were lysed similarly and V5 conjugated agarose beads were added to the soluble extracts and incubated for 2hr at 4°C before washing. Each experiment was repeated at least twice, except the experiment with RNase A treatment.

Western blots were performed essentially as described previously (Busza et al., 2004). Primary antibodies used were: rabbit anti-PABP 1:12000 (Roy et al.,
2004), mouse anti-V5 1:5000, guinea pig anti-ATX2 1:4000. Secondary antibodies conjugated with HRP from Jackson ImmunoResearch were used at 1:10,000 dilution, except for goat anti-guinea pig IgGHRP (Abcam, 1:5000).

For RNA-immunoprecipitations, 200 fly heads were similarly lysed in 400ul Lysis Buffer (300mM NaCl) without dilution, and RNAs were coimmunoprecipitated with either ATX2 antibody or pre-immune sera. The results were obtained from four independent experiments.

*Real-time quantitative PCR*

RNAs from ATX2 immunoprecipitation or input (from head collected at ZT3 and ZT15) were extracted in three steps first with pure Phenol pH4.5, then a 1:1 Phenol: Chloroform mix and finally pure Chloroform. Total RNAs were then precipitated with 100% ethanol and Glycoblu (Ambion). Reverse transcriptions were done with random hexamers (Promega) using Superscript III (Invitrogen). SYBR Green dye (Biorad) was used to perform Real-time PCR analysis with an ABI SDS 7000 apparatus (Applied Biosystems). The sequences of primer sets were described previously (Dubruille et al., 2009). In Figure 3.3E, the mRNA enrichment index was calculated by subtracting the relative amount measured by RT-qPCR in the control immunoprecipitation from the relative amount found in the immunoprecipitation performed with the antibody to ATX2. In Figure 3.S4A, enrichment was normalized to input, as described in (Lim et al., 2011).

*dsRNA synthesis*
We produced dsRNAs by in vitro transcription of a PCR generated DNA template containing the T7 promoter sequence on both ends. A ~500 bp Atx2 fragment was amplified from genomic DNA with the following primers: For: 5’-TTAATACGACTCACTATAGGG GGTGACTTCCAGTTGAGGGA-3’ and Rev: 5’-TTAATACGACTCACTATAGGGTTGCGAGTTACCCTGGTAGG-3’.

We performed in vitro RNA transcription with Ambion MEGAscript T7 kit (Cat# AM1334) as per manufacturer’s instructions. After 2hr of 37ºC incubation of the reaction, 1μl DNase was added to 20μl reaction and incubated for 15min to degrade DNA template. Then, the reaction was stopped and ssRNAs were precipitated, washed and resuspended in water. Annealing of ssRNAs were done by performing the following program: 1) 65ºC 30min; 2) 65ºC 1min -1ºC /cycle for 50 cycles. We confirmed the quality and size of dsRNAs on agarose gel.

Cell culture, cell transfection and luciferase assay

Drosophila S₂R⁺ cells were maintained in SFX growth medium (HyClone) supplemented with 9% fetal bovine serum and 1% penicillin-streptomycin (Invitrogen). Cells were seeded in 12-well plates. When reaching 70-90% confluence, they were transiently transfected using Cellfectin II (Invitrogen) as per manufacturer’s instructions. Luciferase reporter constructs FLUC and FLUC-3’UTR (Lim et al., 2011) were transfected at 20ng/well. Expression constructs pAc-MS2 and pAc-TYF-MS2 (Lim et al., 2011) were transfected at 400ng/well. 50ng β-galactosidase was transfected in each well to normalize transfection
efficiency. Two days after transfection, cells were washed and lysed. Luciferase activity and β-galactosidase activity were measured separately in a 96-well plate using a Microtiter Plate Luminometer. β-galactosidase activity was measured with the Galactolight Plus kit (Life Technology). When involving RNAi in S_{2}R^{+} cells, 7.5μg/well dsRNAs were added to cell culture medium two days before transfection. For each data point, at least three independent experiments were performed.
Table 3.S1 Locomotor behavior of control flies, and flies expressing dsRNAs targeting ATX2 in constant darkness.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>N</th>
<th>% Rhythmicity</th>
<th>Period (hr)±S.E.M</th>
<th>Power* ±S.E.M</th>
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<tr>
<td>25°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tim-GAL4,UAS-dicer2(=TD2)/+</td>
<td>31</td>
<td>90</td>
<td>24.6±0.06</td>
<td>69.3±6.0</td>
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<td>79</td>
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<td>63.2±3.5</td>
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<tr>
<td>tim-GAL4/++;Pdf-GAL80/+</td>
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<td>94</td>
<td>24.4±0.05</td>
<td>50.1±6.1</td>
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<td>26.7±0.10</td>
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<tr>
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<td>26.6±0.10</td>
<td>39.4±3.1</td>
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<tr>
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<td>28.8±2.9</td>
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<td>43.9±3.8</td>
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<td>tim-GAL4/ Atx2 RNAi-;Tub-GAL80ts/+</td>
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<tr>
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<td>18</td>
<td>24.4</td>
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<tr>
<td>tim-GAL4/ Atx2 RNAi-;Tub-GAL80ts/+</td>
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<td>25.2</td>
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<td>tim-GAL4/+;Tub-GAL80ts/+</td>
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<td>24.2</td>
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<td>23.8</td>
<td>0.08</td>
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* Power is a measure of rhythm amplitude and corresponds to the height of the periodogram peak above the significance line (Liu et al., 1991).
Figure 3.1

(A) Graph showing the period (hr) and percentage values for different experimental conditions. The graph includes bars indicating statistical significance (***, n.s.) for each condition.

(B) Images of tissue sections stained with markers for ATX2, PDF, and PER, showing expression patterns in various structures like sLNv, ILNv, LNd, and DN1. The right side of the figure includes a graph showing relative PER signal levels for different conditions with statistical significance (***).
Figure 3.1 Requirement of ATX2 in LNvs for normal circadian behavior. (A) Effects of depleting ATX2 in pacemaker neurons on period length of circadian behavior. (Top) Bar graph showing period length (y axis), percentage of rhythmic flies, and number of flies tested (in the bars). Error bars indicate SEM. TG4, *tim-GAL4*; TD2, *tim-GAL4,UAS-dicer2*; PD2, *Pdf-GAL4,UAS-dicer2*; and PG80, *Pdf-GAL80*. Dicer2 increases RNAi effects (15). (Bottom) Double-plotted actograms of control and *TG4/Atx2^{RNAi-1}* flies showing the last day of the light-dark (LD) cycle and 5 days of DD. (B) (Top) ATX2 is severely down-regulated in circadian neurons expressing *Atx2* dsRNA. Fly brains were dissected at Zeitgeber Time (ZT) 0 (ZT0 corresponds to the beginning of the light phase of the LD cycle) and immunostained with antibodies to ATX2, PER, and PDF. (Bottom) Quantification of ATX2 levels in sLNvs, iLNvs, LNds, and DN1s. Between 11 to 20 neurons were quantified per data point. ***P < 0.001 as determined with Tukey’s multiple comparison test after one-way analysis of variance; n.s., not significant at the 0.05 level, in both (A) and (B).
Figure 3.2 Regulation of PER accumulation in sLNvs by ATX2. (A) sLNvs of brains from wild-type and Atx2 RNAi flies, dissected at different time points (Circadian Time, CT) during the 4th day of DD and stained with antibodies to PDF and PER. (B) Quantification of PER staining in 12 to 16 sLNvs per data point. (C) PER overexpression corrects the period-lengthening induced by means of Atx2 knockdown. Statistics and abbreviations as in Figure 3.1.
Figure 3.3
Figure 3.3 Functional interaction between Atx2 and tyf. (A) Effects of Atx2 RNAi and tyf mutations on circadian period. Statistics and abbreviations are as in Figure 3.1. (B) Requirement of ATX2 for TYF to promote per translation. MS2- and TYF-MS2–expression plasmids were cotransfected in Drosophila S2R+ cells with firefly luciferase reporter plasmids (FLUC) controlled by MS2-binding sites, in the presence or absence of the per 3’UTR. Luciferase activity with MS2 was set to 1. dsRNAs targeting Atx2 were added 2 days before transfection and strongly reduced ATX2 levels (see insertion). n = 3 biological repeats. Error bars indicate SEM. *P < 0.05, ***P < 0.001; n.s., not significant (P > 0.05) as determined with Student’s t test with Bonferroni correction. (C) Physical association of ATX2 with TYF. Protein from head extracts of flies expressing (+) or not (–) TYF-V5 were immunoprecipitated (IP) with an antibody to ATX2 (left) or V5 (right). A serum obtained before immunization with ATX2 antigens was used as the negative control (pre-imm). Asterisk indicates cross-reacting band. (D) The association between ATX2 and TYF is resistant to ribonuclease (RNase) A treatment and thus independent of RNA. (E) Association of ATX2 with per and other transcripts. The RNA enrichment index (y axis) was calculated by subtracting the relative amount of RNA found “immunoprecipitated” with the preimmunization control from the relative amount found in the immunoprecipitation with the antibody to ATX2; n = 4 biological repeats. (F) ATX2 promotes TYF-PABP interactions. Much less PABP is coimmunoprecipitated with TYF-V5 when ATX2 is down-regulated by means of RNAi.
Figure 3.S1 Dm/pAtx2 restores ATX2 expression in sLNvs. (A) (Upper) Two independent Atx2 dsRNAs were used in this study; targeting non-overlapping sequences common to all three Atx2 isoforms (the exon structure of the longest one is shown). (Lower) A chimeric Atx2 transgene resistant to the second dsRNA was generated by replacing the targeted region with the homologous region from *D. pseudoobscura* (orange box). This transgene contains an N-terminal MYC tag (red circle). (B) Representative confocal images showing the level of ATX2 in sLNvs in Atx2 RNAi flies rescued or not by coexpression of Dm/pAtx2. Flies were entrained in LD cycles and dissected at ZT0, stained with anti-ATX2 and anti-PDF antibodies. Images are from a single focal plane. (C) Quantification of ATX2 signals in sLNvs. ATX2 levels are knocked down to about 36% by RNAi and rescued to about 4.3 folds by co-expression of Dm/pAtx2, compared to neighboring PDF negative neurons. 17-19 sLNvs from 6 different brains were quantified. Error bars = SEM. **** = P<0.0001 as determined by Student’s t test.
Figure 3.S2 ATX2 is required in adult flies for normal circadian behavior. 
(A) The sLNvs (and ILNvs) are anatomically normal when ATX2 is downregulated. TD2/Atx2RNAi-1 and Atx2RNAi-1/+ fly brains were stained with anti-PDF. PDF signals in 8-9 sLNvs from 4 brains were quantified. No significant difference was observed (p>0.05, Student’s t test). (B) ATX2 is required during adulthood, but not during development. Left, flies were grown at 29°C to inactivate GAL80ts and thus allow expression of Atx2 dsRNAs during development, and then transferred to 18°C to block ATX2 expression after eclosion. Right, flies were grown at 18°C to inhibit dsRNA expression during development and transferred at 29°C after eclosion to allow ATX2 downregulation in adult flies. Flies were entrained for 3 days of LD cycles and released into DD for at least 5 days. Error bars indicate SEM. ***= P< 0.001 as determined by One-way ANOVA, Tukey’s multiple comparison test. Percentage of rhythmicity is indicated above the bars, number of flies tested in the bars.
Figure 3.S3 PER and TIM oscillations in wild-type flies and flies with low ATX2 levels. (A) (Left) Quantification of PER levels in DN1s (Right) Quantification of PER levels in LNds. (B) Quantifications of TIM levels in DN1s, sLNvs and LNds. For DN1s, TIM levels at CT0 were compared since in both genotypes TIM peaked at this time point, like PER. For sLNvs and LNds, TIM peak levels were CT0 in control flies and CT8 in \( TD2^{+/}\)Atx2\( ^{RNAi-1} \). There were no significant differences between control and \( TD2^{+/}\)Atx2\( ^{RNAi-1} \) flies. Error bars indicate SEM. *** = \( P < 0.001 \) as determined by One-way ANOVA, Tukey’s multiple comparison test. n.s. not statistically significant (p>0.05). In both A and B, between 15-20 neurons from at least 4 brains were quantified for each data point.
Figure 3.S4 ATX2 binds to per transcripts as well as other transcripts. (A) y axis showing the fold enrichment of mRNA binding to ATX2, calculated as the difference between the amount of mRNA immunoprecipitated with the anti-ATX2 antibody and the amount found “immunoprecipitated” in the pre-immunization control normalized by the input level of each mRNA (9). (B) The amount of RNAs associated with ATX2 oscillates between ZT3 and ZT15 for per, tim and cry. y axis shows the ratio of bound mRNAs between ZT15 and ZT3. (C) The amount of per, tim and cry mRNAs in the input oscillates between ZT3 and ZT15 and are thus largely responsible for the oscillations in ATX2-mRNA complex formation. The y axis shows the ratio of mRNA in the input between ZT15 and ZT3. Error bars are SEM and N=4 in all panels.
**Figure 3.S5 ATX2 stabilizes the TYF complex.** ATX2 promotes the interaction between PABP and TYF, and thus circularization and translation of *per* mRNA since TYF can also interact with the cap-binding complex eIF4F. For simplicity, only two subunits of this complex are shown: eIF4E, which directly binds the 5’cap, and eIF4G, which interacts with PABP.
CHAPTER IV

Conclusions and Discussion

In this thesis, I identified two genes, kay-α and Atx2, controlling the period of Drosophila circadian behavior. I also investigated the transcriptional and translational mechanisms by which these two genes regulate the circadian pacemaker.

Chapter II focused on the role of KAY-α in the control of circadian behavior. The Drosophila molecular pacemaker consists of two interlocked feedback loops with CLK sitting in the center and connecting these two loops. We found that KAY-α regulates the transcriptional activity of CLK as well as the transcription of Clk, thus regulating both feedback loops.

Specifically, KAY-α represses CLK’s transcriptional activation of its target genes (e.g., per and tim). In addition, KAY-α inhibits VRI’s repression on Clk transcription, thus promoting the transcription of Clk. The two seemly conflicting events result in more CLK protein with less transcriptional activity. Nevertheless, this may constitute a nice buffering system for the pacemaker. For example, in a situation where CLK is made hyperactive, KAY-α may shift its balancing function towards repression of CLK’s activity, leading to less KAY-α available for promoting Clk transcription. As a result, CLK activity is more intensely inhibited
and less CLK is produced, both of which counteract the hyperactive CLK, thus bringing stability to the pacemaker. Moreover, assigning multiple functions to one protein is economic to a system. Indeed, dual functions are not rare in circadian time keeping system. In a very recent study, dual roles of FBXL3 have been found in the mammalian circadian feedback loops: FBXL3 inhibits the repressor activity of Rev-Erbα:HDAC3 complex on $Bmal$ transcription, as well as regulates the amplitudes of E-box-driven gene expression under BMAL/CLOCK. (Shi et al., 2013).

The detailed mechanism of how KAY-α represses CLK activity remains unknown. Our studies suggest that the repression is indirect: KAY-α does not seem to compete with CLK/CYC for binding to the E-box (Figure 2.3E), and physical association between KAY-α and CLK was neither predicted nor detected. Since the DNA-binding region of KAY-α is required for repression (Figure 2.3F), it is more likely that KAY-α promotes the expression of an unknown CLK/CYC repressor.

Then the question becomes what could be that repressor. To address this question, an RNA-sequencing of $S_2R^+$ cells that overexpressing KAY-α or KAY-β could be done. Since only the α isoform of KAY represses CLK activity (Figure 2.4A), the candidate repressor could be among the genes that are differentially regulated in the two conditions. However, the candidate repressors isolated by $S_2R^+$ cell screen may not be expressed in vivo in pacemaker neurons, or may not
function in the same way \textit{in vivo}. Therefore, they must be tested further \textit{in vivo}, e.g., by overexpressing or knocking down the candidate repressor in pacemaker neurons to see whether CLK activity is changed. An alternative to the S$_2$R$^+$ cell screen is an \textit{in vivo} RNA-sequencing screen. Because knockdown of \textit{kay-}$\alpha$ in all clock neurons results in lethality, \textit{kay-}$\alpha$’s function was only studied in pacemaker neurons, i.e., the sLNvs. Therefore, the RNA-sequencing screen has to be done specifically in those neurons. The technique of isolating pacemaker neurons from the brain is tricky but doable (Kula-Eversole et al., 2010; Nagoshi et al., 2010). Because KAY-\alpha and KAY-\beta can both regulate \textit{Clk} transcription but only KAY-\alpha represses CLK activity (Figure 2.6C, 2.4A), the candidate repressor would probably only be regulated by KAY-\alpha and thus could be among the genes that are differentially regulated by knocking down either \textit{kay-}$\alpha$ or \textit{kay-}$\beta$ in pacemaker neurons.

Chapter III focused on the role of ATX2 in regulating circadian behavior. We found that knockdown of ATX2 in pacemaker neurons leads to ~60% reduction of PER oscillation and flies manifest a ~2 hour longer period. Firstly, we believe that the long period is due to reduced PER levels since overexpressing PER in pacemaker neurons is able to rescue the long period of \textit{Atx2 RNAi} flies (Figure 3.2C). Indeed, PER levels was associated with period length in several studies. In general, decreased PER leads to a long period and increased PER shortens circadian period (Cote and Brody, 1986; Baylies et al., 1987). Secondly, that knockdown of ATX2 or TYF (Lim et al., 2011) in pacemaker neurons is
sufficient to generate behavioral long period reemphasizes the notion that manipulating the pacemaker neurons alone is sufficient to affect circadian behavior in DD.

Moreover, we found genetic interactions and physical associations between ATX2 and TYF. Results from our luciferase reporter and biochemistry assays suggest that ATX2 stabilizes the TYF-PABP-eIF4F complex that presumably promotes *per* mRNA circularization, thus enhancing *per* translation (see also Lim et al., 2013). With TYF’s association with the 5’ cap complex eIF4F, this mechanism more likely promotes the cap-dependent translation of *per* instead of the cap-independent or the internal ribosome entry site-mediated translation (Lopez-Lastra et al., 2005) regulated by atypical translation factor NAT1 (Bradley et al., 2012). In mice, the cap-dependent translation of *Per1* was indicated to be activated by mouse LARK which binds to the 3’UTR of *Per1* (Kojima et al., 2007). Interestingly, mouse LARK can also activate cap-independent translation under cell stress (Lin et al., 2007). Therefore, it is not unlikely that ATX2 and TYF could also regulate *per*’s cap-independent translation under certain circumstances.

In all, our work on ATX2’s function in *Drosophila* circadian system has made significant contributions in the following aspects. First, we identified a novel biochemical function of ATX2 as a translational activator, in contrast to its previously characterized role as a translational repressor (See Chapter I, C.5).
The switch between the two roles may depend on the proteins and mRNAs with which it associates. In the case of per, the association with TYF might explain ATX2’s positive effects on PER translation. Provided the two distinct functions of ATX2, it will be very interesting to examine whether these functions are mediated by different domains or not. Our cross-species rescue experiment has provided a good platform to answer this question. Since we successfully rescued the Atx2 RNAi phenotype by expressing a chimeric Dm/pATX2 (Figure 3.1A), we can express different chimeric Dm/pATX2 with deletions to see whether the rescue remains. Any failure to rescue the phenotype would suggest the requirement of the deleted region for ATX2’s function in circadian rhythms. The conserved Sm domain, LsmAD, PolyQ region and PAM2 motif are good candidate regions to start with. Using a similar approach, we can map the region (s) required for ATX2’s function in miRNA-mediated repression (McCann et al., 2011), and thus determine whether the two distinct functions depend on the same domain or not.

Second, our study supports the emerging notion that translational control is critical for the function of Drosophila circadian pacemaker neurons (Lim et al., 2011; Bradley et al., 2012; Lim and Allada, 2013). Translation is one of the posttranscriptional events besides RNA splicing, polyadenylation, deadenylation etc. that have been shown controlled by the circadian clock (Kojima et al., 2011). In Drosophila, three proteins besides ATX2 have been indicated in the translation regulation of the clock. One is the RNA binding protein LARK which affects circadian locomotor behavior and eclosion rhythms, probably through affecting
the output pathways, e.g., altering neuronal excitability and PDF release from sLNvs’ dorsal terminals (Newby and Jackson, 1993; Huang et al., 2009; Sundram et al., 2012). Another is TYF which was found to promote per translation presumably by facilitating per mRNA circularization (Lim et al., 2011). The last one is NAT1 which was identified to regulate the cap-independent translation of per (Bradley et al., 2012). Among them, TYF and NAT1 were shown to be particularly important in PDF positive neurons: knocking down either TYF or NAT1 in those neurons was sufficient to generate the same arrhythmic or long period phenotype as tyf hypomorphic mutants or knocking down NAT1 in all clock neurons (Lim et al., 2011; Bradley et al., 2012). Although LARK’s function in regulating circadian locomotor behavior may not be limited in PDF positive neurons, knockdown of LARK in these neurons was able to generate arrhythmic phenotype. Moreover, altered PDF signals in the dorsal terminals of sLNvs and altered PER levels in ILNvs were observed in those flies (Sundram et al., 2012). Therefore, translational control, whether it regulates the pacemaker or the output, may be particularly important for the function of PDF positive neurons or more precisely the sLNVs since those are the pacemaker neurons responsible for rhythmic locomotor behavior in DD. Our finding that ATX2 regulates per translation specifically in pacemaker neurons provides additional support to this notion.

Moreover, translational mechanism may allow integration of nutrient signals into the pacemaker. In eukaryotes, the cap-dependent translational
initiation can be stimulated by TOR (target of rapamycin) pathway, a well-known nutrient sensing pathway (Sonenberg and Hinnebusch, 2009). The TOR pathway has been shown to impact *Drosophila* brain pacemakers: increased TOR signaling in pacemaker neurons results in a longer locomotor period (Zheng and Sehgal, 2010). Although this effect appears to be mediated by regulating SHAGGY activity and thus regulating TIM/PER nuclear accumulation (Zheng and Sehgal, 2010), translational control may provide an alternative mechanism by which nutrient signals are integrated into the pacemakers. Indeed, blocking TOR kinase activation of cap-dependent translation which potentially promotes cap-independent translation has been shown to slightly increase cycling amplitude in cultured wings (Bradley et al., 2012). In the future, it would be interesting to know whether and how the TOR pathway regulates ATX2.

Finally, since ATXN2, the mammalian homolog of ATX2, has been implicated in neurodegenerative diseases (e.g., SCA2, ALS) (Lastres-Becker et al., 2008; Elden et al., 2010), and patients with SCA2 suffer from disruption of REM sleep which is under circadian control (Dijk and Czeisler, 1995; Lee et al., 2009; Rodriguez-Labrada et al., 2011; Velazquez-Perez et al., 2011) (also see Chapter I, C.5), our study on ATX2's role in *Drosophila* circadian system may help to explain and even in the future to alleviate the sleep symptoms of SCA2 patients. Moreover, although the pathogenesis of SCA2 is largely unknown, studies based on diseases also triggered by unusual polyQ stretch (spinal and bulbar muscular atrophy, SCA1 and Huntington disease) suggest that the polyQ
expansion may exaggerate a native function of the wild type protein, contributing to the pathology (Orr, 2012). This mechanism might also account for the pathogenesis of SCA2, emphasizing the importance of studying the native physiological function of wild type ATXN2. The novel biochemical function of ATX2 in promoting translation we found in *Drosophila* may thus provide a new perspective to understand the pathogenesis of SCA2. In the future, it will be worthwhile to go one step further to investigate whether *ataxin 2* knockout mice exhibit any circadian rhythms defects and what is the role of ATXN2 in the mammalian circadian system. Based on the general conservation of the molecular pacemaker, the translation machinery and the *ataxin 2* gene, it is possible that the mammalian ATXN2 would also regulate *Per1* and/or *Per2* translation. However, since no *tyf* ortholog has yet been found in mammals, ATXN2 may have a different partner, or ATXN2 may function via a totally different mechanism to control circadian gene expression. In addition, it is also worthwhile to examine mice that carry ATXN2 with pathogenic stretch of polyQ. If these mice have different circadian phenotype as the ATXN2 knockout mice, the pathogenic ATXN2 may function as a gain of function in the clock network. If similar circadian phenotypes are observed, then it may function as dominant negative.

What remains unaddressed in Chapter III is how ATX2’s function is limited to the pacemaker neurons and to the *per* transcripts, given that ATX2 is expressed in all circadian neurons and no significant preference for *per* mRNA
binding was found (Figure 3.1 and 3.3). The first half of the question could be answered by TYF’s limited expression to the LNvs (and more weakly in LNds) and the second half could be explained by TYF’s preference for \textit{per} mRNA binding (Lim et al., 2011). However, TYF probably does not bind RNA itself, and would thus need to be recruited to the mRNA by a specific RNA binding protein (Lim et al., 2011). Therefore, TYF’s specificity for \textit{per} mRNA should come from its unknown recruiter, which is the big missing element in our model. An RNA-binding protein screen could be the first step to identify the recruiter. There are about \~200 genes predicted or proved to encode mRNA binding proteins in \textit{Drosophila} genome. Each of the genes will be knocked down by RNAi either in all clock neurons using \textit{tim-GAL4} or in pacemaker neurons using \textit{Pdf-GAL4}, and flies will be screened for behavioral period phenotype. Among the \~200 candidates, TYF and/or ATX2 associated proteins (based on coimmunoprecipitation in \textit{S}_2\textit{R}^+ cells, \textit{Drosophila} Protein interaction Mapping project) will be given priority.

Taken together, two genes were identified as novel transcriptional and translational regulators of the \textit{Drosophila} circadian behavior. Notably, both genes were initially identified from RNAi screens (Dubruille et al., 2009; Zhang et al., 2013). Despite the limitations of possible off-target effects or inefficient knockdown, the RNAi screen has become a powerful and widely used genetic tool. When combined with the GAL4/UAS system or the Q-system, it allows for tissue-specific knockdown which not only may bypass the lethality encountered
by knocking out essential genes ubiquitously, but also enables study of gene function in specific tissues. Moreover, even the strength of the knockdown can be manipulated by introducing \textit{dicer2}. An alternative to the RNAi screen is the misexpression screen that manipulates gene expression in the other direction. However, the interpretation of results from misexpression screen can be limited by gene’s possible gain of novel function. In summary, I am confident that the RNAi approach will unveil the mysteries of more genes regulating the \textit{Drosophila} circadian rhythms as well as other biological systems.
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APPENDIX

Circadian rhythms of temperature preference and its neural control in

*Drosophila*

Haruna Kaneko, Lauren M. Head, Jinli Ling, Xin Tang, Yilin Liu, Paul E. Hardin,

Patrick Emery and Fumika N. Hamada

This appendix is reprinted from an article published in Current Biology on October 9, 2012 (Kaneko et al., 2012). This work is the result of collaboration between our Laboratory, the Hamada Laboratory and the Hardin Laboratory. For this study, I characterized the expression pattern of *Clk9M-GAL4* in adult brain and performed behavior experiments suggesting that the DN2s are not sufficient to generate either morning or evening peaks under light-dark or temperature cycles.

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Circadian Rhythm of Temperature Preference and Its Neural Control in *Drosophila*

Haruna Kaneko,1 Lauren M. Head,1 Jinli Ling,2 Xin Tang,1 Yilin Liu,2,5 Paul E. Hardin,1 Patrick Emery,2 and Fumika N. Hamada1,4,6

1The Visual Systems Group, Division of Pediatric Ophthalmology, Cincinnati Children’s Hospital Medical Center, 3333 Burnet Avenue, Cincinnati, OH 45229, USA
2Department of Neurobiology and Program in Neuroscience, University of Massachusetts Medical School, Worcester, MA 01605, USA
3Department of Biology and The Center for Biological Clocks Research, Texas A&M University, College Station, TX 77845-3259, USA
4Japan Science and Technology Agency, PRESTO, 4-1-8 Honcho kawaguchi, Saitama 332-0012, Japan

Summary

A daily body temperature rhythm (BTR) is critical for the maintenance of homeostasis in mammals. Whereas mammals use internal energy to regulate body temperature, ectotherms typically regulate body temperature behaviorally [1]. Some ectotherms maintain homeostasis via a daily temperature preference rhythm (TPR) [2], but the underlying mechanisms are largely unknown. Here, we show that *Drosophila* exhibit a daily circadian clock-dependent TPR that resembles mammalian BTR. Pacemaker neurons critical for locomotor activity are not necessary for TPR; instead, the dorsal neurons 2 (DN2s), whose function was previously unknown, is sufficient. This indicates that TPR, like BTR, is controlled independently from locomotor activity. Therefore, the mechanisms controlling temperature fluctuations in fly TPR and mammalian BTR may share parallel features. Taken together, the results indicate the existence of a novel DN2-based circadian neural circuit that specifically regulates TPR, thus understanding the mechanisms of TPR will shed new light on the function and neural control of circadian rhythms.

Results

Mammalian body temperature varies rhythmically throughout the day. This body temperature rhythm (BTR) contributes to their homeostasis by regulating sleep and metabolic energy usage [2–4]. Whereas mammals control body temperature by generating heat, ectotherms use behavioral strategies to regulate body temperature [1]. Because ectotherms adapt their body temperature to their surrounding temperature, temperature preference rhythm (TPR) is believed to be a strategy for ectotherms to achieve homeostasis [2]. For example, slugs, pill bugs, crayfish, and goldfish have been shown to exhibit TPR. Some lizards also exhibit TPR, which causes the temperature of their skin to exhibit a daily rhythm [5]. Although BTR and TPR are well-known phenomena in animal physiology, the mechanisms by which the circadian clock controls rhythms in body temperature are largely unknown in both mammals and ectotherms. Fly-based research has contributed to the discovery of many genes crucial for circadian rhythms, which has subsequently resulted in fundamental discoveries relevant to the workings of molecular clocks in several animals including mammals [6, 7]. Therefore, we sought to understand the molecular and cellular mechanisms that control TPR in *Drosophila*. As a first step, we determined whether flies exhibit TPR.

*Drosophila* exhibit a Circadian Clock-Dependent Temperature Preference Rhythm

We previously showed that *Drosophila* exhibit a robust temperature preference behavior [9]. To determine whether this preference is rhythmic, we performed behavioral assays at different times of day. Control fly strains w1118, Canton-S (cs) and yw were raised under 12 hr light/12 hr dark cycles (LD) at 25°C, mimicking natural day and night cycles. During each ZT (zeitgeber time) zone (ZT1–3, ZT4–6, ZT7–9, ZT10–12, ZT13–15, ZT16–18, ZT18–21, and ZT22–24), temperature preference behavioral assays were performed for 30 min each using a temperature gradient ranging from −17–33°C (Figure 1A). We found that the distribution of preferred temperature shifted from colder to warmer temperatures and vice versa, depending on the time of day (see Figures S1A–S1D available online). By plotting their average preferred temperature, we found that their preferred temperature remained constant over the course of 24 hr (ANOVA, p < 0.0001) (Figure 1B). The preferred temperature gradually increased from morning (ZT1–3) to evening (ZT10–12) and reached its peak in the evening at ZT10–12. The preferred temperature was lowest at ZT13–15 and had a second small peak at ZT19–21 (Figure 1B). Thus, we conclude that the fly displays TPR.

TPR Is under Clock Control

To assess whether TPR is clock-regulated or driven by light-dark cycles, we tested flies in “free-running” conditions in DD (constant dark) and LL (constant light) (Figure 2). We found that w1118 control flies still exhibited TPR during DD day 2 (ANOVA, p = 0.0004) (Figure 2A; Table S1) and DD day 4 (ANOVA, p < 0.0001) (Figure 2B; Table S1). The phase of these TPR oscillations in DD was the same as under LD condition (Figures 2A and 2B). Thus, TPR is controlled by an endogenous clock. Previous studies using locomotor activity have shown that oscillator functions are abolished by day 4 in LL conditions [9, 10]. Nonetheless, we found that flies kept in LL for 4 days and 8 days still exhibited TPR (Figures 2C and 2D; Table S1), although the oscillations amplitude was lower on day 8 (Figure 2D; Table S1). Next, we investigated whether the essential circadian clock genes *per* and *timeless* (tim) are required for TPR. We found that null mutant, *tim*074, showed arrhythmic TPR in DD day 2 and in LL day 4 (Figure 2G; Table S1), whereas the null mutant *per*074, TPR was also profoundly disrupted under DD and LL. A weak rhythm appeared to be present in LL day 4, but the preferred temperatures in each time zone were not statistically different from ZT1–3 (Figure 2E; Table S1).
The Morning and Evening Oscillators Are Neither Necessary Nor Sufficient for Daytime TPR

There are ~150 central pacemaker cells in the fly brain, which are functional homologs of mammalian SCN neurons [12]. The pacemaker cells are divided into groups of lateral neurons (s-LNv, l-LNv, 5th-6th-LNv, LNup, and LPN) and dorsal neurons (DN1, DN2, and DN3) based on their brain location (Figure S2B) [13]. *Drosophila*’s locomotor activity has two peaks in the morning and evening. Whereas the morning activity is controlled by morning (M) oscillators (s-LNv), the evening activity is controlled by evening (E) oscillators (5th-6th-LNv, LNup). The function of the DN and LPNs is not yet well understood, but they appear to play a particularly important role in the detection of environmental inputs such as temperature and light [13-18].

In TPR, we found that the preferred temperature started to rise in the morning (dawn) and reached its peak in the evening (dusk) when locomotor activity is high (Figure 1), indicating that TPR is synchronized with locomotor activity and might therefore be controlled by the same pacemaker neurons. Therefore, we first determined whether the morning and evening oscillators are necessary and/or sufficient for daytime TPR by targeting subsets of pacemaker cells with well-characterized Gal4 lines with specific spatial expressions (Figure S2A).

During the daytime, per005 and tim005 flies constantly preferred ~28 °C and 26.5 °C respectively and did not exhibit daytime TPR in LD (Figures 3A and 3B). For locomotor activity, tissue-specific expression of PER by either elav-Gal4 (a pan-neuronal driver) or tim-Gal4 (a driver for all circadian cells) is known to rescue the per005 arrhythmic phenotype in LD and DD [19, 20]. For TPR, we found that both the elav- and tim-Gal4 drivers were able to rescue daytime TPR in per005 under LD conditions (Figures 3C, 3D, and 3G). This result indicates that PER expression in circadian neurons is sufficient for daytime TPR.

We then tried to rescue per005 flies only in the M cells with pdf-Gal4 and then in both the M and E cells with cry13-Gal4 [19, 21-23]. pdf-Gal4 is well known to rescue morning activity, whereas cry13-Gal4 restores both morning and evening activities [21]. We reexamined the locomotor activity in LD and reached the same conclusion (Figures S3A, S3B, S3E, and S3F). Surprisingly, however, neither driver was able to rescue daytime TPR under LD conditions, although pdf-Gal4 was able to drive a weaker-amplitude rhythm with an abnormal phase (Figures 3E-3G). Thus, the M and E cells are not sufficient for daytime TPR. Furthermore, to determine whether morning and evening oscillators are necessary, we abladed them by expressing the proapoptotic gene hid with pdf-Gal4 and cry13-Gal4. We confirmed that pdf-neuron ablated flies exhibited loss of the morning peak of locomotor activity and advanced evening activity, whereas cry-neuron ablated flies exhibited loss of both the morning and evening peak of locomotor activity (Figures S3G-S3K), as previously reported [19, 22]. However, interestingly, both morning oscillators ablated (pdf-Gal4/UAS-hid) and morning/evening oscillators ablated (cry13-Gal4/UAS-hid) flies were still able to increase their preferred temperature to similar levels as in the control flies during LD (Figure 3H). This data indicates that morning and evening oscillators are neither necessary nor sufficient for daytime TPR in LD.
Figure 2. The Fly’s TPR is Circadian Clock-Dependent

(A–H) Comparison of TPR over the course of 24 hr in w1118, perp31, and timp31 flies kept in LD (red lines), DD2 (blue lines), DD4 (light blue lines), LL4 (green lines), and LL8 (black lines). w1118 flies kept in LD and DD2 (A), LD and DD4 (B), LD and LL4 (C), and LD and LL8 (D). The same LD data from Figure 1B were used in (A–D). perp31 flies kept in LL4 and DD2 (E), and LD and DD2 (F). The same DD2 data for perp31 were used for the comparison in (E) and (F). timp31 flies kept in LL4 and DD2 (G), and LD and DD2 (H). The same DD2 data for timp31 were used for the comparison in (G) and (H). Numbers represent the number of assays. CT is circadian time in DD or LL. Tukey-Kramer test comparison to ZT1–3. **p < 0.001, *p < 0.01, or *p < 0.05 is shown. More detailed statistics are shown in Table S1.

(i) Schematic of experimental conditions. w1118 flies were raised under LD conditions and were transferred to DD or LL conditions. Then, TPR assays were performed over the course of 24 hr in LD, DD2, DD4, LL4, or LL8.

(ii) The bar graph shows daytime temperature increases (ZT or CT1–12) for w1118, perp31, and timp31 in the daytime TPR in LD (red), DD2 (blue), DD4 (light blue), and LL4 (green) conditions. The value of temperature increases during the daytime was calculated by subtracting the preferred temperature at ZT or CT1–3 from the preferred temperature at ZT or CT10–12. ANOVA was performed on the following groups: w1118 (LD, DD2, DD4, and LL4); perp31 (LD, DD2, and LL4) and w1118 (LD); and timp31 (LD, DD2, and LL4) and w1118 (LD). Tukey-Kramer test comparison to w1118 in LD.

Data are presented as mean ± SEM.

The neuropeptide PDF is a key molecule expressed in s-LNv and I-LNv cells. Both PDF and its receptor PDF-Receptor (PDFR) are required for normal circadian locomotor behavior in LD and DD [22, 24, 25]. However, the null mutants of these genes, pdfp31 and pdfp31null, exhibited daytime TPR similar to those of wild-type (WT) flies during LD (Figure 3B; Figures
S2C and S2D), which suggests that PDF and PDF-R are also dispensable for daytime TPR in LD. This result is consistent with the data in Figures 3E-3H that suggested s-LN_s and l-LN_s cells are not required for daytime TPR.

**DN2 Neurons Are Sufficient for Daytime TPR**

We then determined whether a subset of dorsal pacemaker neurons might control daytime TPR. DN2 neurons are suspected to be important for temperature entrainment of
circadian neurons in larvae [15]. However, the functions of the DN2s in the adult are largely unknown, although they still seem particularly sensitive to temperature cycles [16]. To analyze the functions of DN2 cells in daytime TPR, we created new Ga4 lines by using a DNA fragment containing the first three Cik introns and exons 2 and 3. We found that Cik9M-Ga4H is selectively expressed in both s-LNv and DN2s (Figure 4A; Figure S2A). Because pdf-Gal80 can eliminate Gal4-dependent expression in the s-LNv, expression indicates that PER expression in DN2 neurons is sufficient for daytime TPR.

DN2s Regulate TPR Independently from Circadian Locomotor Behavior

To determine whether the DN2s also influence circadian locomotor behavior, we tested locomotor activity using perRDl; Cik9M-Gal4UAS-per flies. We found that these flies are not able to rescue the perRDl locomotor activity in circadian neurons in larvae [15]. However, the functions of the DN2s in the adult are largely unknown, although they still seem particularly sensitive to temperature cycles [16]. To analyze the functions of DN2 cells in daytime TPR, we created new Ga4 lines by using a DNA fragment containing the first three Cik introns and exons 2 and 3. We found that Cik9M-Ga4H is selectively expressed in both s-LNv and DN2s (Figure 4A; Figure S2A). Because pdf-Gal80 can eliminate Gal4-dependent expression in the s-LNv, expression indicates that PER expression in DN2 neurons is sufficient for daytime TPR.

DN2s Regulate TPR Independently from Circadian Locomotor Behavior

To determine whether the DN2s also influence circadian locomotor behavior, we tested locomotor activity using perRDl; Cik9M-Gal4UAS-per flies. We found that these flies are not able to rescue the perRDl locomotor activity.
phenotypes under either light-dark (LD) or temperature (TC) cycles (Figures S3A–S3D and S2L–S2O). These flies were also arrhythmic in DD (data not shown). Therefore, our data indicates that adult DN2 only regulates TPR, not locomotor activity. Taken together, our data indicate that locomotor activity and TPR are controlled by distinct pacemaker cells in adult flies.

Discussion

Here, we show that Drosophila exhibit a daily TPR—low in the morning, high in the evening—that follows a similar pattern as in humans [26]. This study is not only the first demonstration of fly TPR, but also the first systematic analysis of the molecular and neural mechanisms underlying TPR. We found that TPR is controlled by the DN2s, which might explain why temperature preference remains rhythmic in LL in Figures 2C and 2D. The DN2s do not express CRYPTOCHROME (CRY), a blue-light sensor crucial for circadian photoreception [27–29]. Arrhythmicity in LL is caused by constant activation of CRY and thus constant degradation of TIM [23, 30]. Therefore, CRY-negative DN2 neurons may maintain residual rhythms in LL for a longer period of time than CRY-positive circadian neurons. To explore this possibility, we performed immunostaining of brains with TIM-antibody and analyzed the staining levels of DN2 cells in LL 4 days. Although we found TIM signal to be weakly rhythmic in DN2 neurons, these oscillations were not statistically significant. Further studies will thus be needed to verify that DN2 neurons maintain residual rhythm in LL. Because locomotor activity is controlled by CRY-positive circadian neurons and rapidly becomes arrhythmic in LL, the maintenance of TPR rhythms in LL also supports the conclusion that locomotor activity and TPR are controlled by independent circadian neural pathways.

Our data reveals striking parallel features between fly TPR and mammalian BTR, although the modes of heat production are not the same. We demonstrate here that flies exhibit robust temperature increases during the daytime, which is the same phenomenon as diurnal mammalian BTR. Furthermore, ablation studies in rats show that BTR is controlled by SCN neurons that target a different subset of subparaventricular zone neurons than those that control locomotor activity [31]. Thus, both fly TPR and mammalian BTR exhibit circadian clock-dependent temperature fluctuations, independently regulated from locomotor activity. Taken together, our data raises the possibility that mammalian BTR and fly TPR are evolutionarily conserved, which may be because temperature fluctuation in an animal's body is fundamental for maintenance of its homeostasis.

Why do flies exhibit TPR? Flies probably exhibit TPR primarily to maintain homeostasis, like mammals [2]. Mammalian BTR has been shown to have a clear interaction with sleep [4], and it has been reported that mechanisms controlling fly sleep are analogous to those controlling mammalian sleep [6, 32–34]. Therefore, fly TPR may also have a relationship with sleep. Intriguingly, we did observe that PER expression limited to pdf neurons can generate a weak TPR with an abnormal phase in per(1) mutants (Figure 3F), suggesting that pdf neurons may have a role in the TPR circuits. pdf-positive (sLNV and ILNV) neurons regulate sleep [34] and sLNVs are known to project near the DN2s [35]. Therefore, pdf neurons may be able to modulate DN2 activity even when these neurons are arrhythmic and may represent the neural basis for an interaction between TPR and sleep.

Additionally, TPR may provide feedback to circadian pacemakers. Ambient temperature fluctuations can entrain not only peripheral clocks in mammals [36, 37] and flies [38] but also circadian pacemaker neurons in the fly brain, which contribute to morning and evening locomotor activity [14, 16, 39, 40]. Because TPR can generate temperature fluctuations in the fly body, the output of TPR may thus reinforce or refine circadian rhythm entrainment. For circadian locomotor behavior, the DN2s could actually participate in the reinforcement, because in the larval brain the DN2s help the sLNVs entraining to temperature cycles [15]. Therefore, by further exploring this newly discovered circadian rhythm, Drosophila TPR might not only help understanding the mechanisms underlying body temperature control in animals but also contribute to a greater understanding of circadian rhythm's plasticity.

Supplemental information

Supplemental Information includes three figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2012.08.006.

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Supplemental Information

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Haruna Kaneko, Lauren M. Head, Jinli Ling, Xin Tang, Yilin Liu, Paul E. Hardin,
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Supplemental Inventory

1. Supplemental Figures and Tables
   - Figure S1, related to Figure 1
   - Figure S2, related to Figures 3 and 4
   - Figure S3, related to Figures 3 and 4
   - Table S1, related to Figures 1 and 2

2. Supplemental Experimental Procedures

3. Supplemental References
Figure S1. Distribution of Temperature Preference Behavior in w^{1118}, Canton-S (cs) and yw Flies, Related to Figure 1

(A and B) Comparison of the distribution of w^{1118} in temperature preference behavior between ZT1-3 and ZT10-12 (A) and between ZT10-12 and ZT13-15 (B). ZT is Zeitgeber Time (12h light/dark cycle; ZT0 is lights-on, ZT12 is lights-off). The w^{1118} flies preferred a warmer temperature at ZT10-12 than at ZT1-3 and a colder temperature at ZT13-15 than at ZT10-12.

(C and D) Distribution of cs and yw in temperature preference behavior at ZT1-3 and ZT10-12. The cs and yw flies preferred a warmer temperature at ZT10-12 than at ZT1-3. Data are shown as mean, the error bars indicate ± s.e.m, and numbers represent number of assays. Unpaired t-test, are compared to flies at 26-28°C, ***P<0.0001 and *P<0.05. Data are shown as mean, error bars indicate s.e.m and n represents number of assays.

(E and F) Comparison of preferred temperatures of w^{1118}, Canton-S (cs) and yw flies during the day. Numbers in the figures represent number of assays. Although the preferred temperatures of each fly line were different, the daytime temperature increases were ~1-1.5°C for all three lines.
Figure S2. Gal4/Gal80 Lines which Label Pacemaker Cells (A), Schematic of Pacemaker Cells in the Brain (B), and TPR of pdf^01 and pdfR^pan(53.94) Flies during the Day (C and D), Related to Figures 3 and 4.

(C and D) The line graph shows preferred temperatures of wild type and mutants pdf^01 and pdfR^pan(53.94) (D). The bar graph shows daytime TPR (ZT1-12) in LD condition for each genotype.
Figure S3. The DN2s Are Not Sufficient to Generate Either Morning and Evening Anticipation under LD (A-K) and Temperature (L-O) Cycles, Related to Figures 3 and 4

(A-K) Average activity of flies entrained to a standard LD cycle. Day activity is shown with white bars and night activities with grey bars. per^{lt}; Clk9M-Gal4/+; UAS-per/+ show morning anticipation since they have clocks in sLNvs.

(L-O) Flies were first entrained to a light-dark cycles for 3 days and then transferred to temperature cycles (29°C/20°C) in constant darkness for 5 days. Activities were averaged over the last three days of temperature cycles for each genotype. White bars represented the thermophase, grey bars the cryophase. per^{lt}; Clk9M-Gal4/+; UAS-per/+ show morning anticipation since they have clocks in sLNvs.
Table S1. Statistics of Figures 1 and 2

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AVOVA, P (tim^II DD2) = 0.3939, P (tim^II LL4) = 0.7271.

ANOVA and Tukey-Kramer test compared to ZT1-3 and ZT 13-15, ***P<0.001, **P<0.01 or *P<0.05.
Supplemental Experimental Procedures

Fly Lines

Pdf-Gal4 and tim-Gal4 were obtained from Bloomington Drosophila stock center. cry13-Gal4, per01 and tim01 were obtained from Dr. Paul Taghert. Clk9M-Gal4 were created by the first three Clk introns, a ~3kb fragment was amplified via PCR from genomic DNA using a forward primer that spanned the exon 1/intron 1 border (5’ TATAACCGCGGACCCGAAATAGACGAC 3’) and a reverse primer that spanned the intron 3/exon 4 border (5’ CCCTGGATCTCTAACATCTTTGGGAACG 3’) [1], and inserted into the Sac II and Bam HI sites of the pChs-Gal4 vector [2]. The resulting plasmid (Clkint1-3-Gal4) was injected into w118 embryos (Duke University Model System Genomics Facility) to generate multiple transformant lines. The M9 Clkint1-3-Gal4 line (abbreviated Clk9M-Gal4) was used for further analysis.

Temperature Preference Behavioral Assay

Temperature preference assays were performed as described previously [3] but with some modifications. Each behavioral assay was performed for 30 min during different time zones (ZT or CT 1-3, 4-6, 7-9, 10-12, 13-15, 16-18, 19-21 and 22-24) in an environmental room maintained at 25°C/65-70%RH. Since phenotype variations were expected right before and after light was turned ON (ZT0) or OFF (ZT12), we did not examine the temperature preference behavior during these times (ZT or CT 0-1, 11.5-13 and 23.5-24). For each trial, 20-25 adult flies were used and were not reused in subsequent trials. The flies were raised under LD conditions and were transferred to DD or LL conditions for 2-4 days. The light intensity is 500-1000 lux. When we used these flies for the behavioral assay, the flies we used in LD conditions were 1-4 days old, 2 days old for DD2, 4 days old for LL4 and DD4, and 8 days old for LL8. In the LD conditions, temperature preference behavior was performed in the lightness during the daytime (ZT 1-3, 4-6, 7-9 and 10-12) and the darkness during the nighttime (ZT13-15, 16-18, 19-21 and 22-24). In the DD conditions, the temperature preference behavior was performed in the darkness and in the LL conditions, the temperature preference behavior was performed in the lightness. Pictures were taken with a flash after 30 min. After each experiment, the flies were discarded.

Whole-Mount Immunohistochemistry

Adult flies were entrained to a 12hr light- 12hr dark cycles for 3 days. At ZT21-22 on the third day, fly brains were dissected, immunostained and imaged essentially as described in Zhang et al., 2010. Primary antibodies used were: mouse anti-GFP (from Invitrogen 3E6, 1:200), rat anti-PDF (1:400) and rabbit anti-PER (1:1500). Both anti-PDF and anti-PER antibodies were generous gifts from Dr. M. Rosbash. Secondary antibodies were: anti-mouse-FITC (1:200), anti-rat-Cy5 (1:200) and anti-rabbit-Cy3 (1:200) (all from Jackson ImmunoResearch Inc.). Mounted brains were scanned using a Zeiss LSM5 Pascal confocal microscope. Images are digitally projected Z stacks.
**Data Analysis**

*Measuring temperature along the behavioral apparatus:* Temperature was measured at different locations along the gradient using six temperature probes connected to a Fluke 52 II thermometer. The temperature probes were positioned in the air space between the cover and aluminum plate. Using the locations of the probes as well as their corresponding recorded temperatures, the locations of temperatures to the whole number between ~17-33°C were calculated. The distribution of temperature between the temperature probes was estimated to be linear.

*Calculating a mean preferred temperature for each behavioral trial:* In each behavioral trial, 20-25 flies were used. After the 30 min temperature preference behavioral assay, the numbers of flies located between each one degree temperature interval were counted. Flies that were partially or completely on the walls of the apparatus cover were not counted. Data points were plotted as a percentage of flies within one degree temperature. A mean preferred temperature was calculated by summing the products of each interval’s percentage of flies and temperature, as shown below:

\[ \% \text{ of flies} \times 18.5^\circ C + \% \text{ of flies} \times 19.5^\circ C + \% \text{ of flies} \times 20.5^\circ C \ldots + \% \text{ of flies} \times 31.5^\circ C + \% \text{ of flies} \times 32.5^\circ C. \]

*Calculating averaged preferred temperature in each time zone:* Temperature preference behavior was performed >5 times during each time zone (ZT 1-3, 4-6, 7-9, 10-12, 13-15, 16-18, 19-21 and 22-24). To calculate averaged preferred temperature in each time zone, the mean preferred temperature of each trial were then all averaged together. The s.e.m error bars are equal to the error between the trials.

*Calculating preferred TPR during daytime:* After calculating the preferred temperature in each time zone, we subtracted preferred temperature in one time zone from preferred temperature in another time zone in order to obtain temperature changes between time zones. In order to calculate daytime TPR the preferred temperature at ZT10-12 was subtracted from the preferred temperature at ZT1-3.

*Statistical analysis:* Statistical significance was determined by a one-way ANOVA using GraphPad InStat followed by the Tukey-Kramer test to compare with the controls.

**Author Contributions**

F.N.H and H.K designed research. F.N.H, H.K, L.M.H and X.T performed TPR behavioral experiments. J.L and P.E performed locomotor activity behavioral experiments and *Ck9M-Gal4* staining. Y.L and P.E.H made *Ck9M-Gal4* fly lines. F.N.H, H.K and L.M.H wrote the manuscript.
Supplemental References

