AN ABSTRACT OF THE DISSERTATION OF

Kuntol Rakshit for the degree of Doctor of Philosophy in Molecular and Cellular Biology presented on April 17, 2013.
Title: Exploring Functional Links Between Circadian Clocks, Neurodegeneration, and Aging in *Drosophila melanogaster*.

Abstract approved: _____
Jadwiga M. Giebultowicz

Circadian clocks are endogenous molecular mechanisms that coordinate daily rhythms in gene expression, cellular activities, and physiological functions with external day/night cycles. Breakdown of circadian rhythms such as sleep/wake cycles is associated with the onset of several neurological diseases; however, it is not clear whether disruption of rhythms is a symptom or cause of neurodegeneration, or both. To address this important question, circadian rhythms were disrupted by both genetic and environmental manipulations in *Drosophila* mutants prone to neurodegeneration. This led to shortening of lifespan, premature accumulation of oxidative and nervous damage during aging, and overall decline in healthspan, suggesting that circadian clocks may be causally involved in neuroprotective pathways in aging *Drosophila*.

Recent evidence suggests bidirectional relationships between circadian rhythms and aging. While disruption of the clock mechanism accelerates aging and age-related pathologies in mammals, output rhythms of sleep and hormonal fluctuations tend to deteriorate during aging in humans, rodents, and fruit flies. To understand whether this decay is caused by defects in the core transcriptional clock, or weakening of the clock output pathways, a comprehensive study on age-related changes in the behavioral and molecular circadian rhythms was conducted using the fruit fly as a model organism. Aging caused disruption of rest/activity patterns and lengthening of the free-running period of the circadian locomotor activity rhythm. Transcriptional oscillations of four genes involved in the clock mechanism, *period*, *timeless*, *Par domain protein 1ε,* and *vrille,* were significantly reduced in heads, but not in bodies of aging flies. It was further

determined that reduced transcription of these genes is not caused by the deficient expression of their activators, encoded by *Clock* and *cycle* genes. Moreover, transcriptional activation by CLOCK-CYCLE complexes is impaired despite reduced levels of the PERIOD repressor protein in old flies. These data suggest that aging alters the properties of the core transcriptional clock in flies such that both the positive and the negative limbs of the clock are attenuated.

In fruit flies, the protein CRYPTOCHROME (CRY) acts in a cell-autonomous manner to synchronize circadian oscillations with light-dark cycles. The oscillatory amplitude of CRY is significantly dampened in heads of old flies at both mRNA and protein levels. Rescue of CRY using the binary GAL4/UAS system in old flies significantly enhanced the dampened molecular oscillations of several clock genes, and also strengthened the locomotor activity rhythms. There was a remarkable extension of healthspan in flies with elevated CRY. Conversely, CRY deficient mutants accumulated greater oxidative damage and showed accelerated functional decline. Interestingly, rescue of CRY in central clock neurons alone was not sufficient to restore rest/activity rhythms or extend healthspan. These data suggest novel anti-aging functions of CRY and indicate that peripheral clocks play an active role in delaying behavioral and physiological aging.

Taken together, research conducted for this dissertation is a first attempt to elucidate functional links between circadian clocks, neurodegeneration, and aging. While previous evidence linking these processes was of correlative nature, functional studies conducted in this dissertation demonstrate that disruption of circadian clocks causes neurodegeneration and aging. While aging disrupts circadian rhythms at the molecular and behavioral levels, restoration of these rhythms can delay aging and improve healthspan in *Drosophila*. Owing to the conserved nature of clocks, novel insights obtained from this research can illuminate future translational research aimed to extend human healthspan.

# Exploring Functional Links Between Circadian Clocks, Neurodegeneration, and Aging in *Drosophila melanogaster*

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Kuntol Rakshit

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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# CONTRIBUTION OF AUTHORS

In chapter 2, Dr. Natraj Krishnan was involved in the experimental design, protein carbonyl assay, and lifespan analysis. Dr. Doris Kretzschmar and Jill S. Wentzell conducted brain sectioning studies, and Eileen S. Chow conducted the RING assay.

In chapter 3, Dr. Elżbieta Pyza and Elżbieta M. Guzik performed immunofluorescence studies on brain sections, and Dr. Natraj Krishnan helped in troubleshooting the Western Blotting protocol.

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# Exploring functional links between circadian clocks, neurodegeneration, and aging in *Drosophila melanogaster*

## CHAPTER 1

## Introduction and Background

### 1.1 General overview of circadian clocks

Organisms ranging from bacteria to humans synchronize their functions with the earth's 24 h periodicity in day/night cycles. This synchrony is maintained by internal time-keeping mechanisms known as circadian clocks (Latin *circa*, meaning "around"; *diem*, meaning "day"). These clocks generate daily rhythms in behavior, physiology, metabolism, and gene expression, which help organisms anticipate time of the day. Clocks can be set to local time or entrained by external stimuli called Zeitgebers (German "time giver" or "synchronizer") most commonly light, temperature, food, etc. Circadian rhythms persist in wide range of temperatures and even in the absence of external cues, implying a temperature compensated and endogenous timing mechanism. We experience jetlag while traveling across time zones when phases of endogenous rhythms set to local time at the place of departure, are in process of adjusting to the shifted light dark cycles at the destination.

There exists a hierarchy in the basic organization of clocks in which the "central" or "master" clock, entrained by inputs obtained from Zeitgebers, regulates downstream oscillators in other cells and tissues, comprising the "peripheral" clocks. In mammals including humans, the master circadian clock is located in the suprachiasmatic nucleus (SCN), which is a collection of ~20,000-100,000 neurons in the anterior hypothalamus of the brain (Hofman & Swaab 2006). In the fruit fly *Drosophila melanogaster*, a network of ~150 pacemaker neurons in the brain regulates rest/activity rhythms, comprising the central clock (Nitabach & Taghert 2008). Most mammalian organs and tissues like liver, lungs, heart, etc. have their own peripheral clocks, which can be synchronized by humoral and electrical signals from the SCN, but also show autonomous oscillations in the absence of SCN (Schibler 2007). Fruit flies also have peripheral clocks in many cells of the nervous system, including retinal photoreceptors, glia, sensory neurons, and other non-neural tissues in the head, in addition to many body tissues such as Malpighian tubules, gut, fat body, etc. (Giebultowicz 2001; Glossop & Hardin 2002; Giebultowicz 2004).

**1.2 Molecular basis of circadian clocks**

Genetic experiments in fruit flies helped to uncover the molecular circuitry of circadian clocks. The first clock gene *period* (*per*) was discovered when mutations at this locus altered the rhythms of locomotor activity and eclosion (Konopka & Benzer 1971). Thereafter, several studies investigating molecular mechanisms that control circadian rhythms in different model organisms, led to the discoveries of additional clock genes. According to the current model, the core clock mechanism consists of clock genes and proteins organized into 24 h negative feedback cycles. There is substantial conservation of the circadian system between different organisms with respect to the network properties of clock genes. In particular, the identity of clock genes and multi-oscillatory organization of the central and peripheral clocks are remarkably conserved between insects and mammals (Reppert & Weaver 2000; Stanewsky 2003; Yu & Hardin 2006).

In *D. melanogaster*, *Clock* (*Clk*) and *cycle* (*cyc*) genes encode transcription factors, which form CLK-CYC activator complexes and stimulate the expression of *per* and *timeless* (*tim*) genes at early night (Figure 1.1). PER and TIM proteins form heterodimers that subsequently accumulate in the cell nuclei and repress CLK-CYC transcriptional activity, thus suppressing their own transcription (Hardin 2011). In another negative feedback loop, CLK-CYC complexes also induce the expression of transcription factors *Par domain protein 1ε* (*Pdp1ε*) and *vrille* (*vri*) that act as an activator and repressor of *Clk* transcription respectively (Cyran *et al.* 2003; Glossop *et al.* 2003; Zheng *et al.* 2009).

The sub-cellular localization and activities of clock proteins are controlled by post-translational modifications, such as phosphorylation status, mediated by specific kinases and phosphatases (Bae & Edery 2006). Degradation of clock proteins is necessary for progression of the circadian cycle, and is mediated by the ubiquitin-proteasome complex (Naidoo *et al.* 1999; Grima *et al.* 2002; Ko *et al.* 2002). Synchronization of the clock phase to external light/dark (LD) cycles is accomplished by a blue light photoreceptive flavin-binding protein CRYPTOCHROME (CRY) (Emery *et al.* 1998; Stanewsky *et al.* 1998). CRY targets TIM for proteasome-mediated degradation

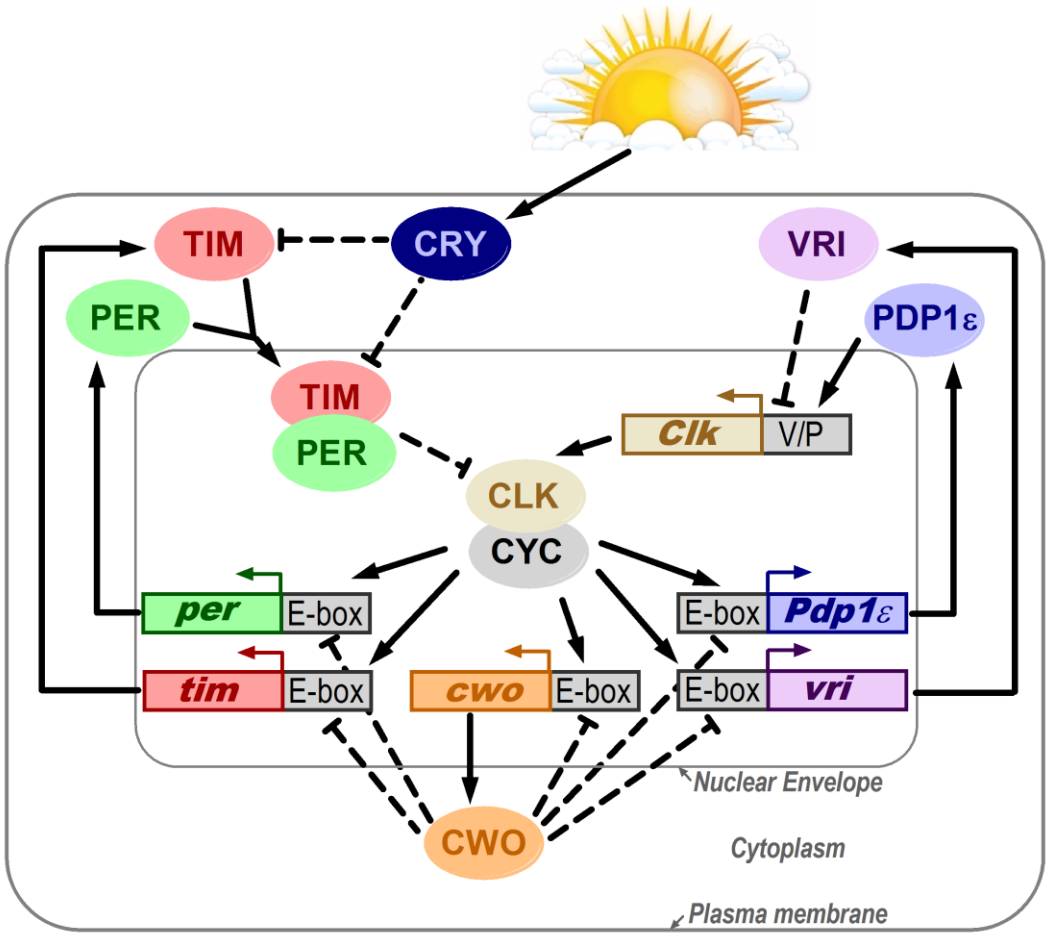


**Figure 1.1 Molecular mechanism of the *Drosophila* circadian clock.** CLOCK (CLK) and CYCLE (CYC) heterodimers bind to E-boxes in the upstream promoter regions of genes *period* (*per*) and *timeless* (*tim*) to activate their transcription during the day and early evening. PER and TIM proteins accumulate at night, and translocate into the nucleus to repress their own activators CLK/CYC. Next morning, CRYPTOCHROME (CRY) targets TIM for degradation, allowing a new transcription cycle to start. CLK/CYC also activate the transcription of *Par Domain Protein 1ε* (*Pdp1ε*), *vrille* (*vri*), and *clockwork orange* (*cwo*). As VRI and PDP1ε proteins accumulate, they translocate into the nucleus and bind to VRI/PDP1-binding sites (V/P) to inhibit and activate *Clk* transcription respectively. CWO feeds back to repress CLK/CYC activation by binding to E-box elements.

after lights on, rendering PER unstable and incapable of suppressing CLK-CYC mediated transcription (Busza *et al.* 2004).

### 1.3 Circadian system defects and neurodegenerative diseases

Neurodegeneration is the progressive loss of structure or function of neurons including their death. This can occur at different levels of the neuronal circuitry ranging from molecular to systemic, and contributes to the onset of several neurodegenerative diseases such as Alzheimer's (AD), Parkinson's (PD), and Huntington's diseases (HD). While every neurodegenerative disease has its unique neuro-pathological signature, sleep-disruption is one of the earliest signs of their onset (Barnard & Nolan 2008; Kondratova & Kondratov 2012). Deteriorations in other measurable circadian output parameters such as circulating hormone levels are also among common symptoms, and those worsen with age and disease progression. The negative effect of neurodegeneration on the circadian clock has been confirmed in mouse models of AD (Sterniczuk *et al.* 2010; Kudo 2011), and HD (Oakeshott *et al.* 2011). It is hypothesized that disruption in the pattern of neurotransmitter release as a result of age or neurodegeneration may contribute to defects in sleep and circadian clock activity (Wulff *et al.* 2011).

Neurodegeneration is often associated with the accumulation of age-related oxidative damage in the nervous system (Sayre *et al.* 2001). Major neuro-pathological diseases are characterized by some form of oxidative stress-induced damage to molecules tend to accumulate as plaques and neurofibrillary tangles in AD, and Lewy bodies in PD. Potential mechanistic links between the circadian clocks and oxidative stress have been studied in model organisms. On one hand, the circadian clock plays a role in antioxidant defense to remove the reactive oxygen species (ROS) (Hardeland *et al.* 2003; Beaver *et al.* 2012); on the other hand, disruption of the circadian clock decreases the organism's ability to withstand oxidative stress (Krishnan *et al.* 2008; Krishnan *et al.* 2009). The correlation between neurodegeneration, oxidative stress, and the circadian clock raises an important question - Is the circadian system involved in the development of

neurodegenerative diseases, and if so, how? This question has been addressed in chapter 2 of this dissertation.

### 1.4 Defects in the circadian system and early aging

Several recent studies in different animals reported that the disruption of circadian rhythms has negative effects on health, longevity, and aging. Chronic jetlag increased the risk of cancer and mortality in mice (Filipski *et al.* 2004; Davidson *et al.* 2006), while other means of non-invasive disruption of rhythmicity significantly reduced the longevity in normal golden hamsters and Syrian hamsters suffering from cardiomyopathy (Hurd & Ralph 1998; Penev *et al.* 1998). Mice deficient in clock proteins PER1 and PER2 are arrhythmic in behavior and gene expression, and display a faster age-related decline in fertility and loss of soft tissues (Lee *et al.* 2005). Exposure of *Per2* knockout ($Per2^{-/-}$) mice to non-lethal radiation further provoked the development of early aging phenotypes and lymphoma (Fu *et al.* 2002). Homozygous *Clock* mutant mice are similarly predisposed to tumor formation and reduced lifespan on exposure to radiation (Antoch *et al.* 2008). Adding further to the list, $Bmal1^{-/-}$ mice show strikingly premature aging phenotype that includes reduction in muscle mass and adipose tissues, osteoporosis, shrinkage of kidney and spleen, and other age-related pathologies (Kondratov *et al.* 2006). Early onset of age-related pathologies in $Bmal1^{-/-}$ mice is attributed to increased ROS levels in various tissues, given that BMAL1 directly regulates ROS homeostasis and protects tissues from oxidative damage (Kondratov *et al.* 2006). Similar observations were reported in *per*-null ($per^{01}$) *Drosophila* mutants that show increased accumulation of oxidatively damaged proteins and lipids compared to the wild-type controls (Krishnan *et al.* 2008). Although lifespan of $per^{01}$ males is very similar to the wild-type under normal conditions, they are less resilient when exposed to mild non-lethal oxidative stress at middle-age (Krishnan *et al.* 2009). This suggests that the absence of circadian clocks may affect or even reduce healthspan, which is the period of an individual's life when one is generally healthy and free from serious or chronic illness.

### 1.5 Effects of aging on circadian rhythms

Aging is the progressive accumulation of deleterious changes that may reduce an organism's ability to survive. It is a complex process that commonly involves gradual breakdown of physiological, biochemical, and cellular homeostasis over the life of an individual. While arrhythmic animals exhibit accelerated aging, it is also established that organisms with an otherwise functional clock behave less rhythmically with age. One of the hallmarks of aging is the disruption of sleep/activity patterns and dampened melatonin oscillations in aged rodents and humans (Turek *et al.* 1995; Huang *et al.* 2002; Oster *et al.* 2003; Hofman & Swaab 2006). Fragmentation of sleep/activity was also reported in aged flies (Koh *et al.* 2006), suggesting that the effects of aging on the circadian system are evolutionarily conserved.

The mechanisms underlying age-related dampening of circadian rhythms are not fully understood. Several studies were performed in aging mammals to test the expression of selected clock genes in different tissues. However, it was difficult to obtain a clear picture because of reports of either reduced or normal gene expression of different clock genes depending on the organs and species examined. While *rCry1* was reduced with age in the SCN, there was no change in *rPer1* and *rPer2* in rats (Asai *et al.* 2001). In golden hamsters, *Per1* and *Per2* were not affected, but *Clock* and *Bmal1* were severely reduced in the SCN (Kolker *et al.* 2003). In contrast, a reduction in *Per2* but unchanged *Bmal1* expression was reported in the pituitary gland of old monkeys (Sitzmann *et al.* 2010). There was a tissue-specific phase advance of *Per1*-luciferase activity in cell cultures of old mouse (Yamazaki *et al.* 2002). The effect of aging on clock gene expression was also studied in the zebra fish brain, and it was reported that *zBmal1* and *zPer1* were dampened but *zClock* was unaffected (Zhdanova *et al.* 2008). Taken together, it appears that aging affects the molecular circadian oscillations in a tissue- and species-specific manner. Since clock genes and proteins affect each other's expression via interconnected feedback loops, it is important to study age-related effects on all clock genes in a single species to obtain further insights into the mechanisms involved. Chapter 3 of this dissertation encompasses a comprehensive study on how aging affects the

oscillations of several clock genes and proteins in *D. melanogaster*, and further generates hypotheses into the mechanisms involved.

### 1.6 Strong circadian clocks and healthy aging

Bidirectional relationships between circadian clocks and aging have been suggested for quite some time; however, there is little molecular data available to substantiate them. Also, the correlative evidence available at present generates the classical "cause or effect" paradox. Is the aging process contributing to the dampening of rhythms or does breakdown of the circadian clock lead to aging? While one can argue that aging often results in the progressive weakening of several biological systems including the circadian system, an important question of interest to circadian biologists and gerontologists is whether a weakened clock is detrimental for longevity and healthspan? If strong clocks are important for healthspan, then restoration of the circadian system in old age should improve the health and longevity of the individual. However, this hypothesis has not been rigorously tested in any organism. Transplantation of fetal SCN into the brains of young and old Syrian hamsters with ablated SCN helped to restore circadian rhythmicity (Viswanathan & Davis 1995) and also extended the lifespan of aged hamsters (Hurd & Ralph 1998). In chapter 4 of this dissertation, circadian oscillations are restored in old fruit flies at both molecular and behavioral levels using genetic tools, and the effects of these manipulations are examined on fly healthspan.

### 1.7 *Drosophila* as a model to study circadian clocks, neurodegeneration, and aging

Studies of the circadian clock mechanism are challenging in mammals because most clock genes have multiple paralogs with partially overlapping functions such that one can compensate for the absence of the other (Ko & Takahashi 2006; Dibner *et al.* 2010). In contrast, there is a single ortholog for every clock gene in fruit flies and each one cycles with its characteristic phase in all the cells due to direct light sensitivity (Giebultowicz 2000). The ubiquitous nature and high conservation of circadian clocks, including their basic multi-oscillatory organization, make flies a useful model to study links between

circadian system, neurodegeneration, and aging. Besides the ease of maintenance, flies live for ~60-70 days compared to mammalian models that have a mean lifespan of several years. Thus, several sets of experiments on aging can be performed in *Drosophila* over a short period, giving high statistical confidence in the results. Additionally, several symptoms of aging including the fragmentation of sleep/activity cycles are very similar in flies and humans. Not only is *Drosophila* genetics very well understood, it is easy to generate mutants, and availability of tools such as the binary GAL4/UAS system and RNAi make it possible to design and conduct functional studies in flies.

## 1.8 Hypotheses, aims, and objectives of the dissertation

### *1.8.1 Determine whether absence of the circadian clock accelerates aging and neurodegeneration*

Recent evidence suggests that animals without a functional circadian system show greater oxidative damage with age. Accumulation of oxidatively damaged proteins and lipids in the nervous system result in several neuro-pathological conditions. Also, most neurological diseases have circadian rhythm disruption as a common early symptom; however, it is not clear whether loss of rhythms is the cause or result of neurodegeneration, or both. To address this important question, double mutant *Drosophila* lines were generated, which were both arrhythmic as well as prone to neurodegeneration. In addition to the genetic disruption, circadian rhythms were also impaired by environmental means to study the effects on aging, longevity, and healthspan in neurodegeneration-prone mutants. Results of this study have been described in chapter 2.

### *1.8.2 Determine the effects of aging on the circadian clock at the molecular level*

While absence of the circadian clock accelerates the aging process, output rhythms of sleep/wake and hormone cycling are also dampened during normal aging. However, it is not clear whether this decay is caused by defects in the core transcriptional clock, or weakening of the clock-output pathways, or both. To determine whether expression of

clock components is altered during aging, amplitude and levels of several clock genes were monitored in young, middle-aged, and old *Drosophila* in a tissue-specific manner. Results of this study have been described in chapter 3 and allow several hypotheses to be generated regarding the mechanisms involved during aging.

*1.8.3 Determine whether restoration of the circadian system at the molecular and behavioral levels delays aging and improves healthspan*

While aim 1.8.1 investigates how clock deficiency accelerates aging and neurodegeneration, aim 1.8.2 provides several novel insights into the molecular defects that may cause dampening of the circadian oscillatory network with age. It becomes important to test whether this dampening is detrimental for longevity and healthspan. If true, then restoration of circadian rhythms would be predicted to improve the health of an organism. There is ample evidence that an impaired circadian system reduces life expectancy in mammals by increasing incidences of cancer and other diseases. Longevity in mammals is shortened with a noninvasive disruption of rhythmicity, suggesting pro-aging effects of disrupted clocks. By extension, this suggests that strong circadian clocks are important for longevity and health, but this hypothesis has not been tested in any organism. In a first attempt, levels and amplitude of the clock gene *cry* were restored in old *Drosophila* using the binary GAL4/UAS system, and effects on circadian rhythms were tested at both behavioral and molecular levels, in addition to healthspan and longevity. Results of this study have been described in chapter 4.

# Exploring functional links between circadian clocks, neurodegeneration, and aging in *Drosophila melanogaster*

## CHAPTER 2

## Loss of circadian clock accelerates aging in neurodegeneration-prone mutants

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## 2.1 Abstract

Circadian clocks generate rhythms in molecular, cellular, physiological, and behavioral processes. Recent studies suggest that disruption of the clock mechanism accelerates organismal senescence and age-related pathologies in mammals. Impaired circadian rhythms are observed in many neurological diseases; however, it is not clear whether loss of rhythms is the cause or result of neurodegeneration, or both. To address this important question, we examined the effects of circadian disruption in *Drosophila melanogaster* mutants that display clock-unrelated neurodegenerative phenotypes. We combined a null mutation in the clock gene *period* (*per*$^{01}$) that abolishes circadian rhythms, with a hypomorphic mutation in the carbonyl reductase gene *sniffer* (*sni*$^{1}$), which displays oxidative stress induced neurodegeneration. We report that disruption of circadian rhythms in *sni*$^{1}$ mutants significantly reduces their lifespan compared to single mutants. Shortened lifespan in double mutants was coupled with accelerated neuronal degeneration evidenced by vacuolization in the adult brain. In addition, *per*$^{01}$ *sni*$^{1}$ flies showed drastically impaired vertical mobility and increased accumulation of carbonylated proteins compared to age-matched single mutant flies. Loss of *per* function does not affect *sni* mRNA expression, suggesting that these genes act via independent pathways producing additive effects. Finally, we show that *per*$^{01}$ mutation accelerates the onset of brain pathologies when combined with neurodegeneration-prone mutation in another gene, *swiss cheese* (*sws*$^{1}$), which does not operate through the oxidative stress pathway. Taken together, our data suggest that the *period* gene may be causally involved in neuroprotective pathways in aging *Drosophila*.

## 2.2 Introduction

Circadian clocks are endogenous timekeeping mechanisms that generate rhythms with circa-24 h periodicity. At the molecular level, circadian clocks consist of cell autonomous networks of core clock genes and proteins engaged in transcriptional-translational feedback loops, which are largely conserved between *Drosophila* and mammals (Yu & Hardin 2006). Rhythmic activities of clock genes generate daily fluctuations in the

expression level of many target genes that underlie cellular, physiological and behavioral rhythms (Schibler 2007; Allada & Chung 2010). Disruption of circadian rhythms by environmental manipulations or mutations in specific clock genes lead to various age-related pathologies and may reduce lifespan in mice (Davidson *et al.* 2006; Kondratov *et al.* 2006; Lee 2006; Antoch *et al.* 2008).

Functional links between circadian rhythms and aging are supported by observations that an impaired circadian system may predispose organisms to neurodegenerative diseases (Gibson *et al.* 2009). However, the evidence linking disruption of circadian rhythms to premature neurodegeneration is of correlative nature and the mechanisms involved are not yet understood. Studies in the model organism, *Drosophila melanogaster,* showed that a null mutation in the clock gene *period* (*per*$^{01}$) is associated with increased susceptibility to oxidative challenge (Krishnan *et al.* 2008; Beaver *et al.* 2010). Furthermore, exposure of aging *per*$^{01}$ flies to mild oxidative stress increased their mortality risk, accelerated functional senescence, and increased signs of neurodegeneration compared to the age-matched controls (Krishnan *et al.* 2009). Together, these data suggest that the clock gene *period* may protect the health of the nervous system in aging animals.

Neurodegeneration is a detrimental aging phenotype affecting homeostasis, motor performance, and cognitive functions. Several mutants uncovered in *Drosophila* show these phenotypes (Kretzschmar 2005); one of them affects the gene *sniffer (sni)* that encodes for a carbonyl reductase in fruit flies. Carbonyl reductases catalyze the detoxification of lipid peroxides generated by reactive oxygen species (ROS) and help to prevent protein carbonylation (Maser 2006). Loss of *sni* function leads to a progressive neurodegenerative phenotype with the formation of spongiform lesions in the brain neuropil, and apoptotic cell death of glia and neurons (Botella *et al.* 2004). Similar to *sni,* mutation in the *swiss cheese (sws)* gene produces age-dependent lesions in the neuropil that are accompanied by apoptotic neuronal death (Kretzschmar *et al.* 1997). However, the *sws* gene encodes a phospholipase that interacts with Protein Kinase A (PKA) and it has not been connected with oxidative stress (Muhlig-Versen *et al.* 2005).

Neurodegeneration is often associated with accumulated oxidative damage in the nervous system (Sayre *et al.* 2001). We previously reported that arrhythmic $per^{01}$ flies show significantly increased levels of lipid peroxidation and protein carbonylation during aging (Krishnan *et al.* 2009). We therefore hypothesized that the circadian system may contribute to cellular homeostasis by curtailing oxidative damage in the nervous system. To test this hypothesis, we examined aging phenotypes in flies carrying mutations in the clock gene *per* and carbonyl reductase encoded by *sni*. We report that such double mutants show significantly shortened lifespan, accelerated neurodegeneration, and a decline in climbing ability. Interestingly, these effects were not restricted to the *sni* gene alone, because arrhythmia due to loss of *per* function also accelerated neurodegeneration in the *sws* mutant. Together, our data suggest that the core clock gene *period*, functions in neuroprotective pathways that may delay the progression of brain pathologies during aging.

## 2.3 Materials and Methods

### *2.3.1 Fly rearing and creation of double mutants*

*D. melanogaster* were reared on 1% agar, 6.25% cornmeal, 6.25% molasses, and 3.5% Red Star yeast at 25°C in 12 h light: dark (LD 12:12) cycles (with an average light intensity of ~2000 lx). All experiments were performed between 4 and 8 h after lights-on (or equivalent time in constant light (LL)) in male flies of different ages, as specified in results. To determine lifespan, 3-4 cohorts of 100 mated males of a given genotype were housed in 8 oz round bottom polypropylene bottles (Genesee Scientific) inverted over 60 mm Falcon Primaria Tissue culture dishes (Becton Dickinson Labware) containing 15 ml of diet. Diet was replaced on alternate days without anesthesia after tapping flies to the bottom of the bottle, and mortality was recorded at this time. The $per^{01}$ mutants were previously backcrossed to the Canton S (CS) for 8 generations and $sni^{1}$ mutants were backcrossed to *yellow white* (*y w*). The $per^{01}$ $sni^{1}$ double mutants were created by recombination using $per^{01}$ *w* crossed to *y w* $sni^{1}$ and selecting flies that were $per^{01}$ *w* $sni^{1}$ ($sni^{1}$ was detected by the orange eye color). *y* is localized at 1A5, *per* at 3B1, *w* at 3B6

and *sni* at 7D22. Similarly, the *per*$^{01}$ *sws*$^{1}$ double mutants were created by recombination with *per*$^{01}$ *w* and *y w sws*$^{1}$ *Appl*-GAL4 (as a visible marker proximal of *sws*, which is localized at 7D1, detectable by the orange eye color) and selecting flies that were *per*$^{01}$ *w sws*$^{1}$ *Appl*-GAL4. The correct genotype was confirmed by external markers, mutant phenotype, and PCR. To determine circadian rhythmicity for each genotype, locomotor activity patterns were monitored in 2-3 independent experiments using the Trikinetics monitor (Waltham, MA). Flies were entrained to LD for 3 d and then recorded for 7 d in constant darkness. Fast Fourier Transform (FFT) analysis was conducted using the ClockLab software (Actimetrics, Coulbourn Instruments). Flies with FFT values >0.04, which showed a single well-defined peak in the periodogram, were classified as rhythmic and included in the calculation of free-running period using the ClockLab software. The *y w* flies served as control for *sni*$^{1}$ and *sws*$^{1}$ single mutants and double mutants carrying *per*$^{01}$allele.

*2.3.2 Neuronal degeneration*

Paraffin-embedded sections of heads were processed as previously described (Tschape *et al.* 2002; Bettencourt da Cruz *et al.* 2005). Briefly, heads were cut in 7 µm serial sections, the paraffin was removed in SafeClear (Fisher Scientific), sections were embedded in Permount, and analyzed with a Zeiss Axioscope 2 microscope using the auto-fluorescence caused by the eye pigment (no staining was used). Experimental and control flies were put next to each other in the same paraffin block, cut, and processed together. Microscopic pictures were taken at the same level of the brain, the vacuoles (identified by being unstained and exceeding 50 pixels in size) were counted and vacuolized area was calculated using our established methods (Tschape *et al.* 2002; Bettencourt da Cruz *et al.* 2005). For *sws*, the pictures were taken at the level of the great commissure (z=-1; http://web.neurobio.arizona.edu/Flybrain/html/atlas/silver/horiz/index.html) and the holes in the deutocerebral neuropil were measured as described (Bettencourt Da Cruz *et al.* 2008). For *sni*, the pictures were taken from sections that contained the ventral deutocerebral neuropil (z=-6;

http://web.neurobio.arizona.edu/Flybrain/html/atlas/silver/horiz/index.html), and the vacuoles in all four optic neuropils (lamina, medulla, lobula, and lobula plate) were counted and measured. In both cases, each side of the brain was scored independently (the number of brain hemispheres analyzed for each genotype is indicated in the figures). For a double blind analyses, pictures were taken and numbered, vacuoles were counted, and the area of vacuoles was measured in pixels in Photoshop and subsequently converted into $\mu m^2$ (Bettencourt da Cruz *et al.* 2005). Statistical analysis was done using one-way ANOVA.

*2.3.3 Rapid iterative negative geotaxis (RING) and oxidative damage assays*

Vertical mobility was tested using the RING assay as described (Gargano *et al.* 2005). Briefly, 2 groups of 25 flies of each genotype were transferred into empty vials without anesthesia, and the vials were loaded into the RING apparatus. The apparatus was rapped three times in rapid succession to initiate a negative geotaxis response. The flies' movements in tubes were videotaped and digital images captured 4 s after initiating the behavior. The climbed distance was calculated for each fly and expressed as average height climbed in the 4 s interval. The performance of flies in a single vial was calculated as the average of 5 consecutive trials (interspersed with a 30 s rest). To assess oxidative damage, protein carbonyls were measured in male head homogenates of the various genotypes at 370 nm after reaction with 2,4-Dinitrophenylhydrazine (DNPH) using a BioTek Synergy 2 plate reader, as described previously (Krishnan *et al.* 2008). Results were expressed as $nmol.mg^{-1}$ protein using an extinction coefficient of 22,000 $M^{-1}cm^{-1}$.

*2.3.4 Gene expression by qRT-PCR*

The expression of *sni* gene was measured in $per^{01}$ mutants and CS control flies collected at 4 h intervals around the clock in LD. Total RNA was extracted from fly heads using TriReagent (Sigma). The samples were purified using the RNeasy mini kit (Qiagen) with on-column DNAse digestion (Qiagen), and cDNA was synthesized with iScript (Bio-Rad). Real-time PCR (qRT-PCR) was performed on the StepOnePlus (Applied

Biosystems) under default thermal cycling conditions with a dissociation curve step. Every reaction contained iTaq SYBR Green Supermix with ROX (Bio-Rad), 0.6 ng cDNA, 80 nM primers. Primer sequences are available upon request. Data were analyzed using the $2^{-\Delta\Delta CT}$ method with mRNA levels normalized to the gene *rp49*. Relative mRNA levels were calculated with respect to the trough levels set as 1 for control flies.

*2.3.5 Statistical analyses*

Lifespan and survival curves were plotted using Kaplan Meier survival curves and statistical significance of curves assessed using the Log-Rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon tests (GraphPad Prism v5.0; GraphPad Software Inc., San Diego, CA). For statistical analysis of biochemical and gene expression results, one-way ANOVA with post-hoc tests were conducted (GraphPad Instat v3.0).

**2.4 Results**

*2.4.1 Loss of circadian rhythms shortens the lifespan of flies mutant for carbonyl reductase*

To determine the effects of *per* on longevity of *sni*-deficient flies, we created $per^{01}$ $sni^{1}$ double mutants. Since both genes are localized on the X chromosome (*per* at 3B and *sni* at 7D), we achieved this by recombination using $per^{01}$ *w* and *y w* $sni^{1}$and selecting flies that lost the *y* marker but were orange due to the P-element in $sni^{1}$. The double mutants were confirmed by phenotype and by PCR using primers within the P-element and *sni*. Several double mutant lines were generated and two lines (referred to as $per^{01}$ $sni^{1}$ line 1 and 2) were selected for further analysis. As expected, both double mutant lines exhibited loss of circadian rhythms due to $per^{01}$ mutation, while single mutants were mostly rhythmic in DD indicating that they had a functional circadian clock (Table 2.1).

The lifespan of $per^{01}$ $sni^{1}$ flies was compared to the $sni^{1}$ and $per^{01}$ single mutants, as well as *y w* controls. There was no difference in mean lifespan between both the single mutants ($per^{01}$ and $sni^{1}$) and the *y w* control (Figure 2.1A, Table 2.2). In contrast, $per^{01}$ $sni^{1}$ double mutants showed very significant ($p<0.001$) reduction of their mean lifespan

**Table 2.1** Table showing the percentage of rhythmic flies and mean period length in flies of indicated genotypes. Locomotor activity was monitored in 2-3 independent experiments and the total number of flies (n) analyzed for each genotype is indicated.

| Genotype | n | % Rhythmic | Period |
|---|---|---|---|
| *y w* | 30 | 93 | 23.87 |
| $sni^{1}$ | 30 | 70 | 23.77 |
| $per^{01}$ | 30 | 0 | - |
| $sws^{1}$ | 31 | 97 | 23.70 |
| $per^{01}$ $sni^{1}$ (1) | 14 | 0 | - |
| $per^{01}$ $sni^{1}$ (2) | 21 | 0 | - |
| $per^{01}$ $sws^{1}$ | 23 | 13 | 24.11 |

**Table 2.2** Disruption of circadian rhythms shortens lifespan in $sni^{1}$ mutants. Median and mean ± SEM lifespan (days) is shown for indicated genotypes with n=sample size. Statistical comparison was conducted using one-way ANOVA with Tukey-Kramer multiple comparison's test. Values with different superscripts are significantly different at $p<0.001$.

| Genotype | Regime | n | Median | Mean ± SEM |
|---|---|---|---|---|
| *y w* | LD | 300 | 55 | $55.2 \pm 0.7^{a}$ |
| *y w* | LL | 300 | 52 | $53.8 \pm 0.3^{a}$ |
| $per^{01}$ | LD | 295 | 51.5 | $55.7 \pm 0.4^{a}$ |
| $sni^{1}$ | LD | 197 | 49 | $55.1 \pm 0.9^{a}$ |
| $sni^{1}$ | LL | 303 | 37 | $37.1 \pm 0.4^{b}$ |
| $per^{01}$ $sni^{1}$ (1) | LD | 194 | 37 | $35.7 \pm 1.0^{b}$ |
| $per^{01}$ $sni^{1}$ (2) | LD | 268 | 25 | $27.1 \pm 0.5^{c}$ |

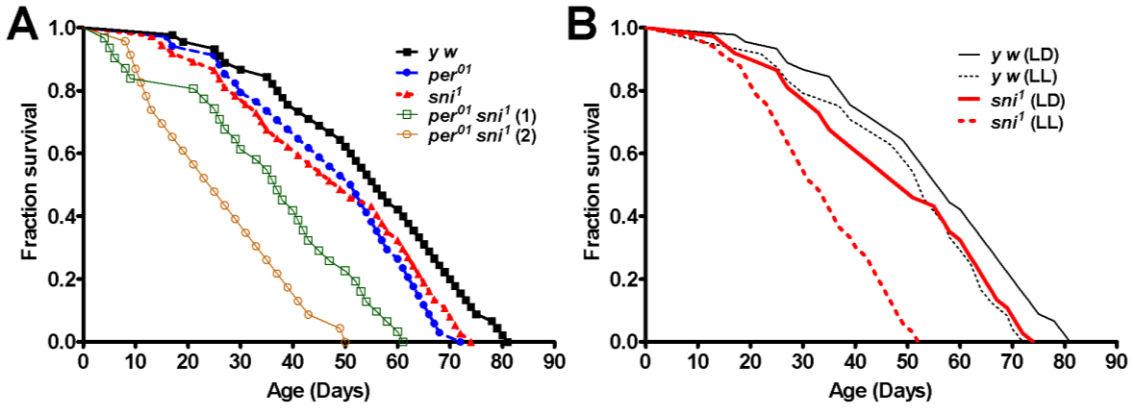


**Figure 2.1 Loss of circadian rhythms dramatically shortens the lifespan of *sni*$^{1}$ mutants. A)** Survival curves for *y w*, *per*$^{01}$, *sni*$^{1}$, and *per*$^{01}$ *sni*$^{1}$ double mutant lines **B**) Lifespan of *sni*$^{1}$ and *y w* in 12 h light: dark (LD) cycles and constant light (LL), which disrupts the circadian clock function.

compared to single mutants. While both double mutant lines were short-lived, we observed significant difference in their lifespan with 32% reduction in recombinant line 1, and 50 % reduction in line 2 (Figure 2.1A, Table 2.2).

We next tested whether disruption of circadian rhythms by non-genetic interventions affect longevity in *sni*$^{1}$ single mutants. Adult *sni*$^{1}$ flies were reared in constant light (LL) which interferes with the circadian clock mechanism and causes behavioral arrhythmia (Price *et al.* 1995). The lifespan of *sni*$^{1}$ flies maintained in LL was significantly shortened ($p<0.0001$) compared to *sni*$^{1}$ flies reared in LD 12:12, while *y w* flies showed similar lifespan in both LD and LL (Figure 2.1B, Table 2.2).

### *2.4.2 Double per$^{01}$ sni$^{1}$ mutants show increased neurodegeneration and reduced climbing ability*

Due to its neuroprotective role, loss of carbonyl reductase in *sni*$^{1}$ mutants results in progressive degeneration with small vacuoles appearing in the first 7-9 days of adult life, and becoming larger and more numerous with progressing age (Botella *et al.* 2004). We demonstrate that neurodegeneration is dramatically increased in *per*$^{01}$ *sni*$^{1}$ double mutants (Figure 2.2). Vacuoles were rarely detected in 9 day-old *per*$^{01}$ fly brains (Figure 2.2A), while we consistently observed a few small vacuoles in *sni*$^{1}$ mutants (Figure 2.2B). The vacuolization of the brain was markedly exacerbated in the age-matched *per*$^{01}$ *sni*$^{1}$ double mutant flies (Figure 2.2C). Both, the area taken up by vacuoles and average vacuole number increased very significantly in each of the two *per*$^{01}$ *sni*$^{1}$ lines examined at 9 days of age (Figures 2.2D-E). More pronounced vacuolization was also maintained in 19 day-old *per*$^{01}$ *sni*$^{1}$ compared to *sni*$^{1}$ alone (Figure 2.2F). In the next experiment, we asked whether disruption of circadian rhythms by LL, which shortens lifespan of *sni*$^{1}$ mutants, affects the levels of neurodegeneration. Brains of 10 day-old *sni*$^{1}$ males maintained in LD or LL were sectioned and examined for vacuole formation. Disrupting the circadian clock by constant light significantly increased brain vacuolization in *sni*$^{1}$ (Figure 2.3), although the effects were less severe than in *per*$^{01}$ *sni*$^{1}$ double mutants.

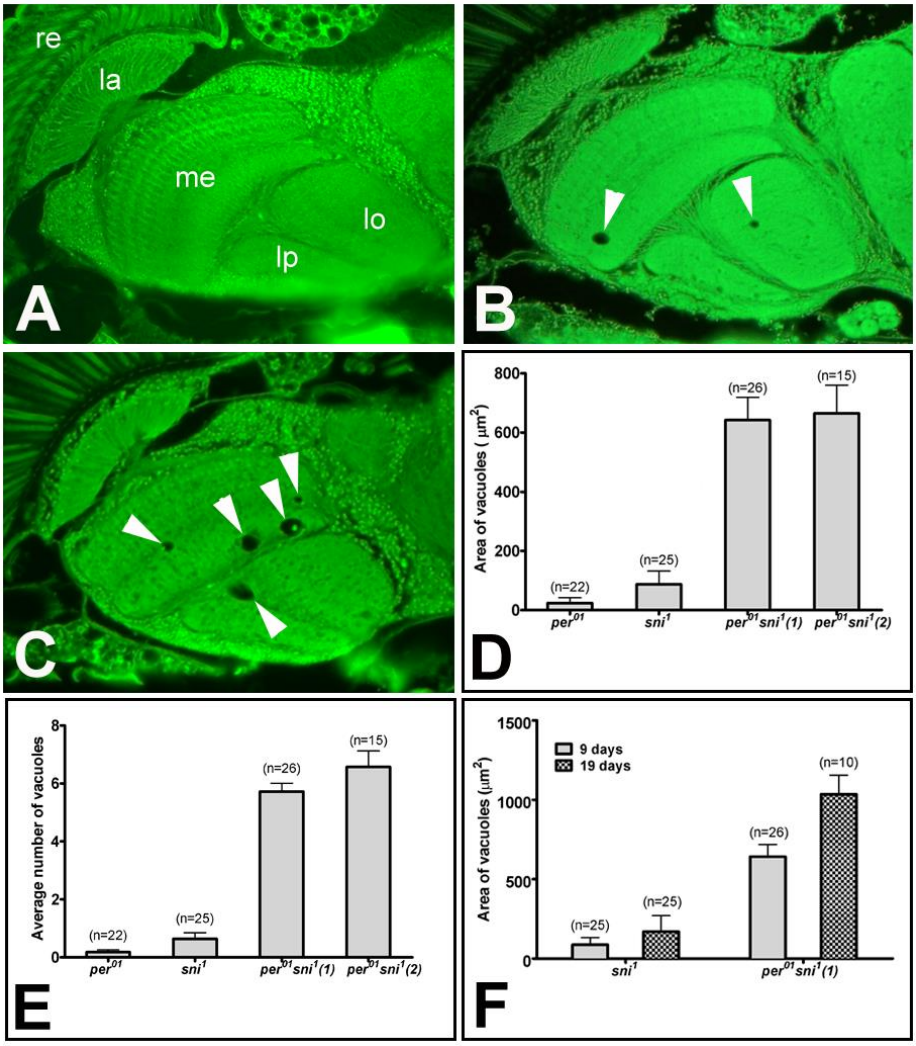


**Figure 2.2 Interfering with the clock increases neurodegeneration in *sni*$^{1}$ mutants. A-C)** Paraffin head sections from 9 day-old males (scale bar=25µm, re=retina, la=lamina, me=medulla, lo=lobula, lp=lobula plate). **A)** No vacuoles are detectable in the brain of a *per*$^{01}$ fly. **B)** A *sni*$^{1}$ fly brain shows a few vacuoles (arrows). **C)** Brains of *per*$^{01}$ *sni*$^{1}$ double mutant show increase in the size and number of vacuoles. **D)** Bar graph showing the mean ± SEM area of all vacuoles /brain hemisphere. There is a significant difference between the *sni*$^{1}$ and *per*$^{01}$ *sni*$^{1}$ line 1 ($p$=2.9x10$^{-6}$), and *sni*$^{1}$ and *per*$^{01}$ *sni*$^{1}$ line 2 ($p$=1.6x10$^{-5}$). **E)** The mean number of vacuoles/brain hemisphere is increased in *per*$^{01}$ *sni*$^{1}$ compared to *sni*$^{1}$ alone [*sni*$^{1}$ to *per*$^{01}$ *sni*$^{1}$ (1): $p$=8.42x10$^{-12}$; *sni*$^{1}$ to *per*$^{01}$ *sni*$^{1}$ (2): $p$=6.5x10$^{-8}$]. **F)** Comparison of the vacuolization between 9 and 19 day-old flies shows that the phenotype is progressive with age for both *sni*$^{1}$ ($p$=0.036) and *per*$^{01}$ *sni*$^{1}$ line 1 ($p$=0.03). **D-F)** The number of brain hemispheres (n) examined to calculate the average values are indicated on the top of each bar.

To test whether increased neurodegeneration is associated with altered motor abilities, we conducted the RING assay on 10 day-old single and double mutants along with their controls. The climbing ability of $per^{01}$ flies did not differ significantly from their CS control, while $sni^{1}$ mutants showed modest but significant ($p<0.05$) impairment of climbing ability compared to their *y w* control. Importantly, the average climbing distance was dramatically reduced ($p<0.001$) in both $per^{01}$ $sni^{1}$ double mutant lines compared to the single $sni^{1}$ mutants (Figure 2.4).

*2.4.3 Protein carbonyl levels are elevated in $per^{01}$ $sni^{1}$ double mutants*

Since mutation in the *sni* gene severely attenuates carbonyl reductase expression (Botella *et al.* 2004), we tested the levels of oxidatively damaged proteins in heads of $per^{01}$ and $sni^{1}$ single mutants as well as $per^{01}$ $sni^{1}$ double mutants by measuring protein carbonyls. Levels of protein carbonyls were significantly increased ($p<0.01$) in heads of both $per^{01}$ and $sni^{1}$ single mutants compared to CS and *y w* controls. Importantly, a significant increase in the protein carbonyl accumulation ($p<0.01$) was detected in both recombinant lines of the $per^{01}$ $sni^{1}$ double mutants compared to the controls and single mutants (Figure 2.5).

*2.4.4 Expression of sni is not affected in $per^{01}$ mutants*

The gene *per* encodes transcriptional co-regulators that may affect the expression of downstream target genes (Claridge-Chang *et al.* 2001). Because $per^{01}$ alone increases protein carbonylation (Krishnan *et al.* 2008); Figure 2.5), we tested whether *sni* expression might be clock-controlled and therefore altered by the loss of *per* function. We measured the levels of *sni* mRNA around the clock in CS and $per^{01}$ flies by qRT-PCR. The expression of *sni* did not show a daily rhythm in CS flies, and was not significantly reduced in $per^{01}$ mutants compared to controls (Figure 2.6). These data suggest that *sni* is not a downstream target of *per*, rather both mutants appear to act through independent pathways causing additive effects.

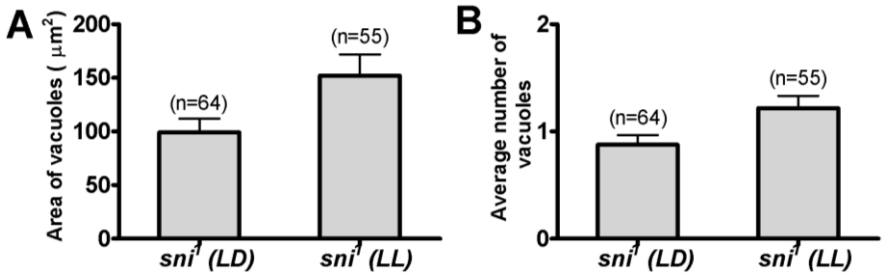


**Figure 2.3 Disrupting the circadian clock by constant light increases vacuolization in *sni*$^{1}$ mutant. A)** 9 day-old *sni*$^{1}$ flies maintained in constant light (LL) show significant increase in the mean area of vacuoles /brain hemisphere compared to 9 day-old *sni*$^{1}$ flies in 12h light: dark (LD 12:12) cycles ($p$=0.024). **B)** The mean number of vacuoles is also significantly higher in *sni*$^{1}$ mutant kept in LL ($p$=0.018). **A-B)** The number of brain hemispheres (n) examined to calculate the average values are indicated on the top of each bar.

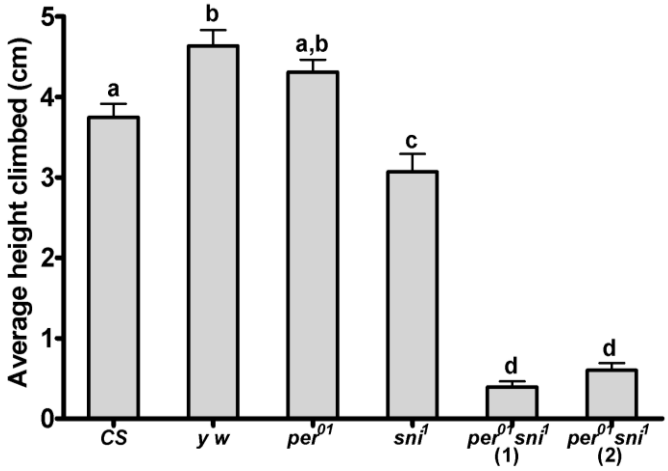


**Figure 2.4 *per*$^{01}$ *sni*$^{1}$ double mutants show accelerated mobility impairment.** Vertical mobility was measured by the RING assay in 10 day-old males of the indicated genotypes. Bars represent mean height climbed (± SEM), based on testing 2 vials per genotype, each containing 25 flies. Bars with different superscripts are significantly different at $p$<0.01.

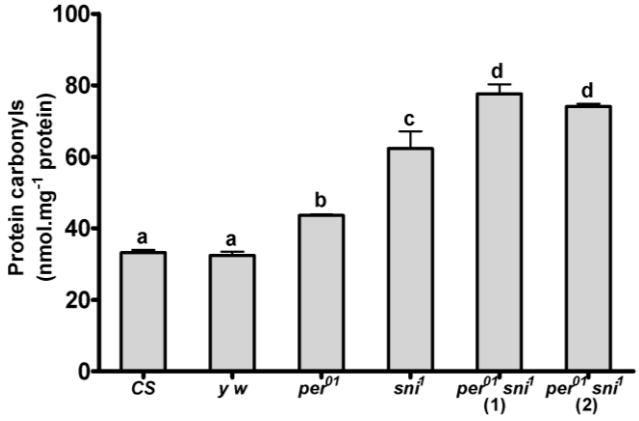


**Figure 2.5 Oxidative damage in the form of protein carbonyls accumulates to higher levels in *per*$^{01}$ *sni*$^{1}$ flies.** Protein carbonyl levels were measured in heads of 10 day-old males of the indicated genotypes. Both *per*$^{01}$ and *sni*$^{1}$ single mutants had higher protein carbonyls than their respective CS and *y w* controls. Protein carbonyls were further elevated in *per*$^{01}$ *sni*$^{1}$ double mutants, compared to *per*$^{01}$ or *sni*$^{1}$ single mutants. Bars represent mean carbonyl levels (± SEM), based on testing 3 independent sets of flies each containing 75 flies in 3 technical repeats of 25 flies each. Bars with different superscripts are significantly different at $p<0.01$.

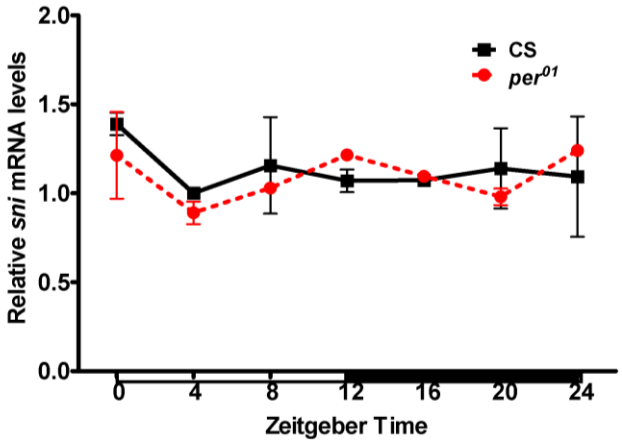


**Figure 2.6 Relative *sni* mRNA levels are not significantly different between CS and *per*$^{01}$ mutants.** Expression profile of *sni* was analyzed by qRT-PCR in heads of flies collected at 4 h intervals in LD 12:12 cycles. White and black horizontal bars indicate periods of light and darkness, respectively.

*2.4.5 Neurodegeneration in sws mutant is substantially increased in a $per^{01}$ background*

To investigate whether arrhythmicity may affect another neurodegeneration-prone mutant, we compared the neurodegenerative changes caused by a mutation in the *swiss cheese* (*sws*) gene in the wild-type and $per^{01}$ background. The $per^{01}$ $sws^{1}$ double mutants were created by recombination between $per^{01}$ *w* and *y w* $sws^{1}$ *Appl*-GAL4 (see Methods). Locomotor activity assays indicated that 97% of $sws^{1}$ single mutants showed rhythmicity with a free-running period similar as in control *y w* flies, but rhythmicity was mostly lost in $per^{01}$ $sws^{1}$ (Table 2.1). As reported previously (Kretzschmar *et al.* 1997), 14 day-old $sws^{1}$ mutant displayed characteristic symptoms of neurodegeneration evidenced by vacuoles in the dorsal neuropil (Figures 2.7A, B), which does not occur in *y w* control flies at this age (Figure 2.7D). Importantly, the age-matched $per^{01}$ $sws^{1}$ double mutants showed marked increase in the size and number of vacuoles (Figure 2.7C arrows) compared to the $sws^{1}$ single mutants. The three-fold increase in vacuolization was highly significant (Figures 2.7E, F).

## 2.5 Discussion

Our study demonstrates that disruption of circadian rhythms accelerates aging in two independent mutants that display neurodegenerative phenotypes. We show that lifespan of $sni^{1}$ flies is reduced by 32-50% in a $per^{01}$ background, which abolishes molecular and behavioral rhythms. Significant lifespan shortening was also observed in $sni^{1}$ flies reared in LL, which is known to disrupt circadian systems (Price *et al.* 1995). Lifespan reduction resulting from the disruption of the clock by either genetic or environmental manipulations strongly suggests that this phenotype is caused by the loss of rhythmicity. However, we cannot exclude that clock-unrelated pleiotropic effects of *per* may be involved in accelerated aging, since PER protein is unstable in LL (Price *et al.* 1995). Interestingly, studies in mammals have also shown that interfering with the circadian clock mechanism by the knock-out of specific clock genes may lead to shortened lifespan (Davidson *et al.* 2006; Yu & Weaver 2011). Premature aging was observed in mice with mutant core clock genes *Bmal1* or *Clock*, which together form the positive feedback loop

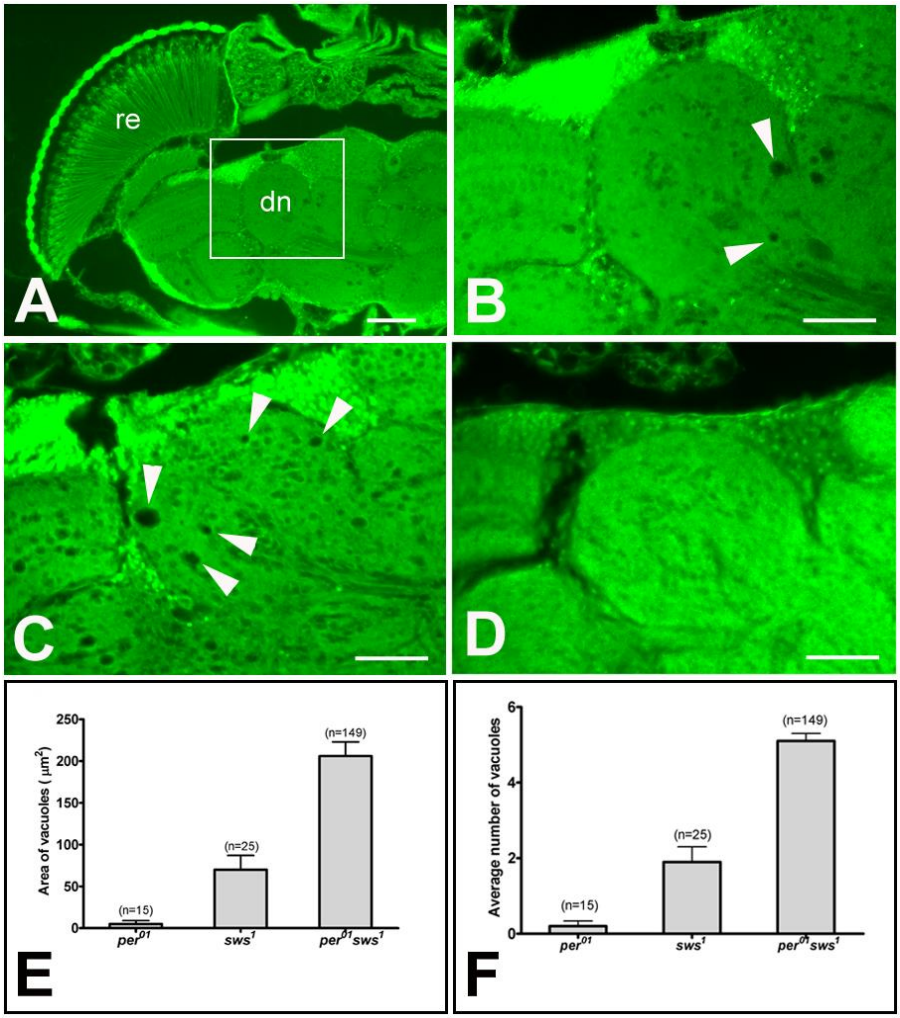


**Figure 2.7 Loss of *per* function increases neurodegeneration in *sws* mutants. A)** Paraffin head sections from a 14 day-old old $sws^1$ fly show widespread degeneration (arrows) characteristic for this mutant (scale bar=50µm, re=retina, ol=optic lobes, dn=deutocerebral neuropil). **B)** Magnification from A (box), showing the deutocerebral neuropil that was used for measurements. **C)** Age-matched $per^{01}$ $sws^1$ double mutants show increase in the size and number of vacuoles compared to $sws^1$ single mutants. **D)** Age-matched *y w* control does not show vacuoles in this area. **B-D)** Scale bar=25µm**. E)** Bar graph showing significant difference in the mean area of all vacuoles in the deutocerebral neuropil between $sws^1$ and $per^{01}$ $sws^1$ ($p$=1.9x10$^{-8}$). **F)** The mean number of vacuoles/brain hemisphere is also significantly higher in the double mutant compared to $sws^1$ alone ($p$=0.0033). **E-F)** The number of brain hemispheres (n) examined to calculate average values are indicated on the top of each bar.

of the circadian clock (Kondratov *et al.* 2006; Antoch *et al.* 2008). Genetic ablation of *per* gene homologs in mice resulted in some aging phenotypes and a significant increase in cancer incidence after gamma-radiation challenge (Lee 2006). Our previous study showed that exposure of $per^{01}$ flies to external oxidative challenge, significantly increased their mortality risk (Krishnan *et al.* 2009). Here we show that lifespan is compromised even further when loss of *per* function is combined with an internal oxidative stress caused by carbonyl reductase deficiency. Taken together, these data suggest that intact circadian clocks promote longevity under various homeostatic challenges.

We show that *per*-null related arrhythmia is associated with premature loss of neuronal integrity. A significant increase in the number and size of vacuoles was observed in the brains of $per^{01}$ $sni^{1}$ double mutants in LD, or the $sni^{1}$ mutant in LL compared to $sni^{1}$ males kept in LD. The increased deterioration of the nervous system might be the cause of shortened lifespan in these flies because it has been shown that several neurodegenerative mutants are short-lived (Kretzschmar *et al.* 1997; Tschape *et al.* 2002). Consistent with the neuronal damage, $per^{01}$ $sni^{1}$ flies showed precipitous loss of climbing ability at the age of 10 days, whereas $sni^{1}$ flies with a functional clock showed only modest (albeit significant) climbing impairment at this age. These data provide experimental evidence suggesting that the disruption of circadian rhythms, which is also observed in human neurodegenerative diseases, may be a causative factor contributing to these pathologies. Indeed, we showed previously that the loss of the clock by itself can lead to neurodegenerative symptoms in $per^{01}$ mutants later in life (Krishnan *et al.* 2009). Interestingly, there is also evidence for a reverse relationship such that progressive neurodegeneration may contribute to the loss of clock function in both flies and mice (Morton *et al.* 2005; Rezaval *et al.* 2008).

Carbonyl reductase encoded by *sni,* acts as a neuroprotective enzyme against oxidative stress (Botella *et al.* 2004); therefore, we tested the levels of oxidatively damaged proteins in heads of single and $per^{01}$ $sni^{1}$ double mutants. Consistent with our previous report (Krishnan *et al.* 2009), there was a significant increase in the protein

carbonyl levels in *per*$^{01}$ mutants, and these levels were even higher in *sni*$^{1}$ mutants (Figure 2.4). While protein carbonyl levels were further elevated in *per*$^{01}$ *sni*$^{1}$ flies, this was not as dramatic as the increase in brain damage observed in these double mutants at the same age (Figure 2.2). It is possible that other oxidatively damaged species might accumulate to higher levels in *per*$^{01}$ *sni*$^{1}$ flies since deficiency in carbonyl reductase activity may also contribute to increased lipid peroxidation (Sgraja *et al.* 2004; Martin *et al.* 2011).

To address the nature of *per*$^{01}$ and *sni*$^{1}$ interactions, we tested daily profiles of *sni* expression in heads of wild-type CS flies and clock-deficient *per*$^{01}$ mutants. The levels of *sni* mRNA did not change significantly across circadian time points in control flies and neither were they different in *per*$^{01}$ mutants. These data suggest that *per* does not regulate *sni* expression, consistent with the exacerbated neurodegeneration observed in double mutants. The effects of *per* mutation appear indirect and additive suggesting more general protective functions of this clock gene. This is further supported by the fact that loss of *per* function resulted in accelerated neuronal damage in *sws* mutant, which increases neurodegeneration via different mechanisms. This gene encodes a phospholipase, thereby interfering with the phospholipid homeostasis (Muhlig-Versen *et al.* 2005). These data show that detrimental effects of *per*-null allele are not specific to the *sni*$^{1}$ mutant with increased oxidative stress, but also extend to the *sws*$^{1}$ mutant, which does not appear to act via the oxidative stress pathway. However, the increase in vacuolization was greater in *sni*$^{1}$ than in *sws*$^{1}$ mutant (Figures 2.2, 2.7), suggesting that *per* mutation may affect *sni* via multiple pathways that may or may not be related to oxidative damage.

While our results suggest that a functional circadian system improves the performance of neurodegeneration-prone mutants, the mechanisms involved remain to be investigated. We hypothesize that the circadian clocks slow down the accumulation of neuronal damage in aging organisms by synchronizing the activities of enzymes involved in cellular homeostasis. Indeed, microarray studies of daily gene expression profiles suggested synchronous circadian fluctuations in the expression of some protective enzymes such as glutathione-S-transferases, in fly heads (Wijnen & Young 2006). Our

previous data demonstrated daily fluctuations in the levels of mitochondrial ROS and carbonylated proteins in flies with a functional clock, indicative of a daily rhythm in the removal of oxidative damage (Krishnan *et al.* 2008). In the absence of the circadian clock, enzymes working in a specific pathway may become dysregulated, leading to the impaired removal of oxidative damage. Consistent with this idea, we reported increased levels of oxidatively damaged lipids and proteins in $per^{01}$ mutants during aging (Krishnan *et al.* 2009). It was also shown that oxidative stress may impair the clock function in *Drosophila* (Zheng *et al.* 2007).

Circadian clocks are involved in the regulation of response to genotoxic stress and xenobiotics in both mice and fly (Antoch *et al.* 2008; Beaver *et al.* 2010; Gachon & Firsov 2011). Therefore, increased neurodegeneration in arrhythmic $sni^{1}$ and $sws^{1}$ mutants may involve additional pathways beyond ROS homeostasis. In mice, the loss of an essential clock component encoded by *Bmal1* causes a variety of premature aging phenotypes (Kondratov 2007). Interestingly, treatment with antioxidants reversed some aging symptoms, but had no effect on other age-related pathologies such as sarcopenia (Kondratov *et al.* 2009). This suggests that both ROS-dependent and independent mechanisms may contribute to neuroprotection in organisms with functional clocks.

In summary, we show that disrupting clocks in flies has a profound impact on neurodegeneration-prone mutants. Circadian rhythm disturbances such as sleep disorders, which are commonly observed during aging, are exacerbated in neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington disease (Wu & Swaab 2007; Sterniczuk *et al.* 2010). Our functional study, which involved the manipulation of a clock gene to assess its neuroprotective role, substantiates the possibility that arrhythmia is not a mere correlation, but may actually contribute to the onset of neurodegenerative disorders. Conversely, intact circadian clocks appear to promote the health of the nervous system during aging.

## 2.6 Acknowledgements

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# Exploring functional links between circadian clocks, neurodegeneration, and aging in *Drosophila melanogaster*

## CHAPTER 3

## Effects of aging on the molecular circadian oscillations in *Drosophila*

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## 3.1 Abstract

Circadian clocks maintain temporal homeostasis by generating daily output rhythms in molecular, cellular, and physiological functions. Output rhythms such as sleep/wake cycles and hormonal fluctuations tend to deteriorate during aging in humans, rodents, and fruit flies. However, it is not clear whether this decay is caused by defects in the core transcriptional clock, or weakening of the clock output pathways, or both. We monitored age-related changes in behavioral and molecular rhythms in *Drosophila melanogaster*. Aging was associated with disrupted rest/activity patterns and lengthening of the free-running period of the circadian locomotor activity rhythm. The expression of core clock genes was measured in heads and bodies of young, middle-aged, and old flies. Transcriptional oscillations of four clock genes, *period*, *timeless*, *Par domain protein 1ε,* and *vrille,* were significantly reduced in heads but not in bodies of aging flies. It was determined that reduced transcription of these genes was not caused by the deficient expression of their activators, encoded by *Clock* and *cycle* genes. Interestingly, transcriptional activation by CLOCK-CYCLE complexes was impaired despite reduced levels of the PERIOD repressor protein in old flies. Our data suggest that aging alters the properties of the core transcriptional clock in flies such that both the positive and the negative limbs of the clock are attenuated.

## 3.2 Background

Circadian rhythms at the molecular, behavioral, and physiological levels are important for maintaining temporal homeostasis (Reddy & O'Neill 2010). While robust high-amplitude circadian rhythms are observed in young individuals, these often lose their strength with age. Daily rhythms in hormone levels, body temperature, sleep/wake cycles, and other physiological and behavioral variables are diminished during aging (Touitou & Haus 2000; Weinert & Waterhouse 2007; Zhdanova *et al.* 2011). Loss of temporal coordination in humans is correlated with a variety of diseases, including Alzheimer's disease and cancer (Van Someren & Riemersma-Van Der Lek 2007; Wu & Swaab 2007). Functional studies in mice demonstrated that disruption of circadian

rhythms by the knockout of specific clock genes or chronic jet-lag, accelerates the onset of age-related pathologies and may reduce lifespan (Davidson *et al.* 2006; Kondratov *et al.* 2006; Lee 2006; Antoch *et al.* 2008). Similar to vertebrates, the strength of sleep/wake rhythms is also reduced in aged *Drosophila*, indicated by fragmentation of sleep and decreased length of activity bouts in light/dark cycles (Koh *et al.* 2006). These data suggest that links between the circadian system and aging are evolutionarily conserved. Given that circadian coordination has a pronounced impact on physiological functions, overall health, and disease susceptibility, it is important to determine why circadian rhythms diminish during aging, and whether this process could be reversed. Consequently, there is a need to understand how the core clock mechanism is altered during aging.

At the molecular level, the circadian clock is based on transcription-translation feedback loops that are largely conserved from *Drosophila* to mammals (Stanewsky 2003; Yu & Hardin 2006). In fruit flies, the key activator complex is composed of two transcription factors encoded by genes *Clock* (*Clk*) and *cycle* (*cyc*), the latter known as *Bmal1* in mammals. The CLK-CYC complex stimulates the expression of genes *period* (*per*) and *timeless* (*tim*) in the early night. PER and TIM proteins accumulate in cell nuclei late at night, and PER represses CLK-CYC transcriptional activity, resulting in the suppression of *per* and *tim* transcription (Hardin 2004). CLK-CYC complexes also induce the expression of transcription factors *Par domain protein 1ε* (*Pdp1ε*) and *vrille* (*vri*), which contribute to the rhythmic expression of *Clk* (Cyran *et al.* 2003; Glossop *et al.* 2003; Zheng & Sehgal 2008). Another gene *clockwork orange* (*cwo*) was recently shown to regulate core clock gene expression (Kadener *et al.* 2007; Lim *et al.* 2007; Matsumoto *et al.* 2007; Richier *et al.* 2008). Post-translational modifications that affect the phosphorylation status and degradation of clock proteins are also important modulators of period and amplitude of circadian oscillations (Bae & Edery 2006; Allada & Chung 2010).

While the core transcriptional clock is well understood in young animals, much less is known about changes in the oscillations of clock genes during aging. Studies

performed in aging mammals and zebra fish reported either reduced or normal expression of different clock genes, depending on the organs and species examined (Asai *et al.* 2001; Yamazaki *et al.* 2002; Kolker *et al.* 2003; Zhdanova *et al.* 2008). Studies of age-related changes in the vertebrate clock mechanism are challenging, because most clock genes have paralogs with partially overlapping functions (Ko & Takahashi 2006; Dibner *et al.* 2010). In flies, there is a single ortholog for every clock gene, and each one cycles with its characteristic phase in all the cells due to direct light sensitivity (Giebultowicz 2000). Here, we investigated the effects of age on the molecular clock mechanism in heads and bodies of *Drosophila melanogaster.*

We have previously shown that *per* mRNA oscillations become attenuated in old flies (Krishnan *et al.* 2009). To obtain further insights into age-related changes in the clock mechanism in *Drosophila,* we investigated the expression profiles of seven clock-related genes acting in circadian feedback loops. We report that the expression levels of four genes regulated by the CLK-CYC activator complex are reduced in fly heads but not in bodies. We then investigated whether the expression of *Clk* or *cyc* is affected by age. Finally, we measured profiles of PER and TIM proteins in aging flies to elucidate changes in the repressive phase of the transcriptional feedback loop. Our study suggests that aging weakens the positive limb of the circadian feedback loop in a manner that may also affect the negative limb of the clock.

## 3.3 Methods

All the experimental protocols conform to international ethical standards (Portaluppi *et al.* 2010).

### *3.3.1 Fly stocks and rearing*

*D. melanogaster* were reared on 1% agar, 6.25% cornmeal, 6.25% molasses, and 3.5% Red Star yeast at 25°C in 12 h light: dark (LD 12:12) cycles (with an average light intensity of ~2000 lux). By convention, lights-off is denoted as Zeitgeber time (ZT) 12. For experiments on aging flies, cohorts of 100 Canton S (CS) mated males were housed

in 8 oz round bottom polypropylene bottles (Genesee Scientific, San Diego, CA) inverted over 60 mm Falcon Primaria Tissue culture dishes (Becton Dickinson Labware, Franklin Lakes, NJ) containing 15 ml of diet. Diet dishes were replaced every other day without $CO_2$, after tapping flies to the bottom of the bottle.

*3.3.2 Locomotor Activity Analysis*

Flies were entrained in LD 12:12 at 25°C. Locomotor activity of 5, 35, and 50 day-old males was recorded for 3 d in LD 12:12, followed by 7 d in constant darkness (DD) using the Trikinetics locomotor activity monitor (Waltham, MA). For a quantitative measure of circadian rhythmicity, Fast Fourier Transform (FFT) analysis was conducted using the ClockLab software (Actimetrics Coulbourn Instruments, Whitehall, PA). Flies with FFT values <0.04 were classified as arrhythmic, 0.04-0.08 were classified as weakly rhythmic, while flies with FFT values >0.08 were considered strongly rhythmic. Flies with both weak and strong rhythms, which showed a single peak in the periodogram, were included in the calculation of the free-running period using the ClockLab software (Actimetrics, Wilmette, IL).

*3.3.3 Quantitative Real-Time PCR*

Three independent bio-replicates of flies were collected at 4 h intervals around the clock on days 5, 35 and 50. Total RNA was extracted from fly heads and bodies separately, using TriReagent (Sigma, St. Louis, MO). The samples were purified using the RNeasy mini kit (Qiagen) with on-column DNAse digestion (Qiagen, Valencia, CA). Synthesis of cDNA was achieved with iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Quantitative Real-time PCR (qRT-PCR) was performed on the StepOnePlus Real-Time machine (Applied Biosystems, Carlsbad, CA) under default thermal cycling conditions with a dissociation curve step. Every reaction contained iTaq SYBR Green Supermix with ROX (Bio-Rad), 0.6-1ng cDNA, 80nM primers. Primer sequences are available upon request. Data were analyzed using the $2^{-\Delta\Delta CT}$ method with mRNA levels normalized

to the gene *rp49*. Relative mRNA amplitude was calculated with respect to the trough levels set as 1 for each age.

*3.3.4 Western Blotting*

Three independent bio-replicates of 5 and 50 day-old males were collected at ZT 16, 20, 0, and 4. About 5-10 fly heads/time point were homogenized on ice in Laemmli Buffer, sonicated, boiled at 100˚C for 5 min, and centrifuged at 12,000g at 4˚C. A constant ratio of the buffer (7µl/head) was used to ensure equal protein loading and separation on 5.7% acrylamide gel. Proteins were transferred to the 0.45 µm PVDF Immobilon-FL membrane (Millipore, Billerica, MA), and incubated in TBST (10mM Tris, 0.15M NaCl, 0.1% Tween-20, pH 7.5) + 5% milk (for PER) or Odyssey Blocking buffer (LI-COR Biosciences, Lincoln, NE) (for TIM) for 2 h, then overnight at 4˚C with 1:15,000 anti-PER (Muskus *et al.* 2007) or 1:2,500 anti-TIM (Giebultowicz & Emery, unpublished) in their respective buffers. Membranes were treated for 2 h with 1:20,000 goat anti-rabbit IRDye680 (LI-COR Biosciences) and 1:5,000 goat anti-guinea pig IRDye700 (LI-COR Biosciences), respectively. Proteins were quantified using the LI-COR Odyssey Infrared Imaging System software (v. 3.0).

*3.3.5 Immunofluorescence*

PER immunofluorescence (IF) in the retinal photoreceptors was examined in 5 and 50 day-old males collected at ZT 8, 20, and 24. Heads were fixed in 4% paraformaldehyde in phosphate buffer saline (PBS; 0.1 M, pH 7) for 2 h, cryoprotected in 12.5% sucrose for 10 min followed by 25% sucrose overnight at 4˚C, embedded in Tissue Tek, frozen in liquid nitrogen, and cut into 20 µm cryo-sections. Sections were washed in PBS, then PBST (PBS with 0.2% of TritonX 100), and incubated in 5% Normal Goat Serum in PBST and 0.5% Bovine Serum Albumin (BSA) for 1 h at room temperature. Sections were incubated with 1:1000 polyclonal rabbit anti-PER (Muskus *et al.* 2007) for 48 h at 4˚C, followed by overnight incubation at 4˚C with the 1:800 secondary goat anti-rabbit conjugated with Cy3 (Jackson ImmunoResearch Laboratories, Westgrove, PA). For

negative control, *per*$^{01}$ mutants were used. Sections were mounted in Vectashield with 4'6'-Diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA) and examined on the Zeiss Meta 510 LSM confocal microscope. PER levels were evaluated by measuring the fluorescence intensity in photoreceptor cell nuclei after converting the mean level of fluorescence to the mean gray value that was quantified using ImageJ (v.1.4, NIH, http://rsb.info.nih.gov/ij/) software. The same brightness parameters and other image settings were used for comparison of young and old tissues. For whole mounts, dissected abdominal organs were fixed and processed as described (Kotwica *et al.* 2009), and stained with anti-PER followed by Alexa 488 (Invitrogen, Carlsbad, CA) secondary antibodies.

### *3.3.6 Statistical Analysis*

Data were statistically analyzed with GraphPad Prism (v.5.0) and GraphPad Instat (v.3.0, San Diego, CA). For analysis of locomotor activity data in 5, 35, and 50 day-old flies, average FFT values and period were subjected to one-way analysis of variance (ANOVA) with Tukey's post-hoc test. For qRT-PCR data, statistical significance was evaluated by two-way ANOVA with Bonferroni's post-hoc test. For Western data, the relative strength of the signals was quantified using LI-COR Image analysis software (v.3.0) and subjected to two-way ANOVA with Bonferroni's post-hoc test. For immunofluorescence (IF) data, mean fluorescence levels were converted to the mean gray value, which was quantified using the ImageJ software (v.1.4). The numerical values obtained were subjected to Wilcoxon matched pairs test.

## 3.4 Results

### *3.4.1 Aging lengthens the free-running period of locomotor activity rhythms*

The free-running period of locomotor activity provides a sensitive measure of the circadian clock mechanism. The median lifespan of CS males is approximately 62 d (Krishnan *et al.* 2009), and we recorded locomotor activity rhythms in 5-15 day-old (young), 35-45 day-old (middle-aged), and 50-60 day-old (old) flies to assess age-related

changes in period and other circadian parameters. Males were monitored for 3 d in LD 12:12 followed by 7 d in DD. We observed that the activity of aging flies was fragmented and extended into the night in LD (Figure 3.1A), similar to previous reports (Koh *et al.* 2006; Rezaval *et al.* 2008). Analysis of fly activity in DD revealed decreased proportion of rhythmic flies in 35 and 50 day-old flies (Figure 3.1B). We observed a significant decrease in rhythm strength [$F_{2,22}$=6.35, $p$<0.01] in middle-aged and old flies (Figure 3.1C). Flies that remained rhythmic on days 35 and 50, showed significant age-dependent lengthening of the free-running period [$F_{2,22}$=20.13, $p$<0.001], compared to day 5 (Figure 3.1D).

*3.4.2 Expression of CLK-CYC transcriptional targets dampen with age in fly heads*

Decrease in the strength of behavioral rhythms prompted us to examine whether the molecular cycling of clock genes might be altered during aging. Hence, we obtained daily expression profiles of two core clock genes, *per* and *tim*, in the heads and bodies of 5, 35, and 50 day-old CS males. In heads of young flies, *per* mRNA showed expected daily oscillations, with trough at ZT 4 and peak at ZT 16. The trough to peak amplitude of *per* mRNA oscillations was significantly dampened in 35 day-old flies and further reduced on day 50 (Figure 3.2A, Table 3.1). Similar changes were detected in the expression profile of *tim*, which encodes TIM protein that forms heterodimers with PER. High amplitude oscillations of *tim* mRNA observed in young flies were significantly dampened on day 35, and further significant reduction was observed on day 50 (Figure 3.2A, Table 3.1). Reduced oscillations of both *per* and *tim* suggest that CLK-CYC driven transcription may be weakened during aging. To test this, we examined the expression of *Pdp1ε* and *vri*, which are also activated by the CLK-CYC complex and oscillate with a phase similar to *per* and *tim*. We observed that the amplitude of mRNA oscillations for both genes was considerably dampened at the peak (ZT 12-16) in heads of middle aged and old flies (Figure 3.2A, Table 3.1). Interestingly, the mRNA for *per*, *tim,* and *Pdp1ε,* initially increased with a similar slope in heads of both young and old flies until ZT 12 (or until ZT 8 in case of *vri*), suggesting that the initiation of cyclic transcription is not

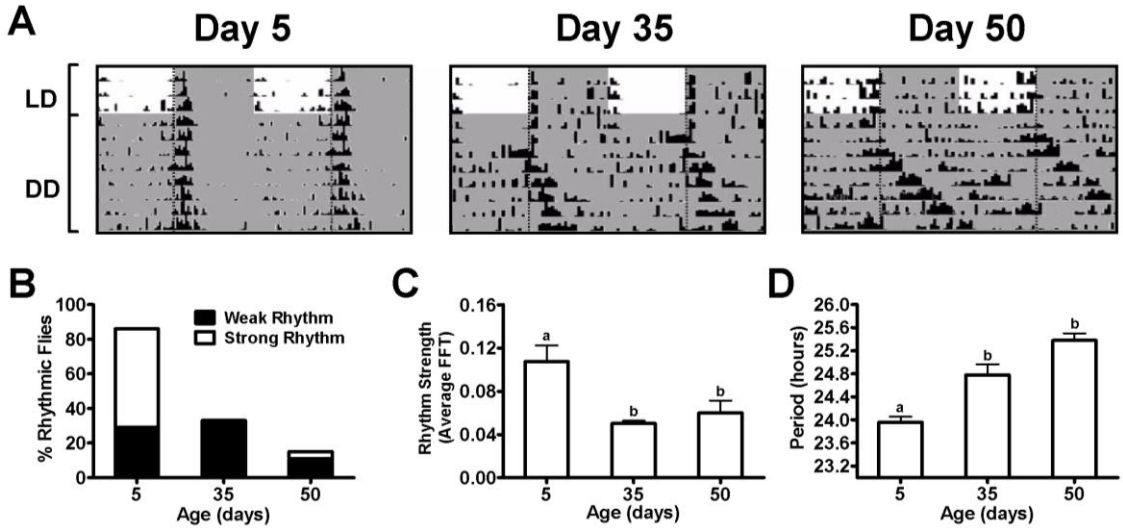


**Figure 3.1 A)** Locomotor activity profiles of representative 5, 35, and 50 day-old CS males. Flies of each age were monitored in LD 12:12 for 3 d, followed by 7 d in DD at 25°C. Shaded areas represent periods of darkness. Vertical dotted lines indicate time of lights off (ZT/CT 12). **B)** Percentage of rhythmic flies on days 5, 35, and 50. Flies with FFT values >0.08 were considered strongly rhythmic while flies with FFT values 0.04-0.08 were classified as weakly rhythmic. **C)** Average rhythm strength on days 5, 35, and 50. Values are mean ± SEM (n=14 for day 5, n=27 for day 35, and n=28 for day 50). Statistical significance was determined using one-way ANOVA with Tukey's post-hoc test, and bars with different letters are significantly different (5 vs. 35 $p<0.01$; 5 vs. 50 $p<0.01$; 35 vs. 50 $p>0.05$). **D)** Average free-running period of locomotor activity on days 5, 35, and 50. Values are mean ± SEM (n=14 for day 5, n=27 for day 35, and n=28 for day 50). Statistical significance was determined using one-way ANOVA with Tukey's post-hoc test, and bars with different letters are significantly different (day 5 vs. 35 $p<0.001$; 5 vs. 50 $p<0.001$; 35 vs. 50 $p>0.05$).

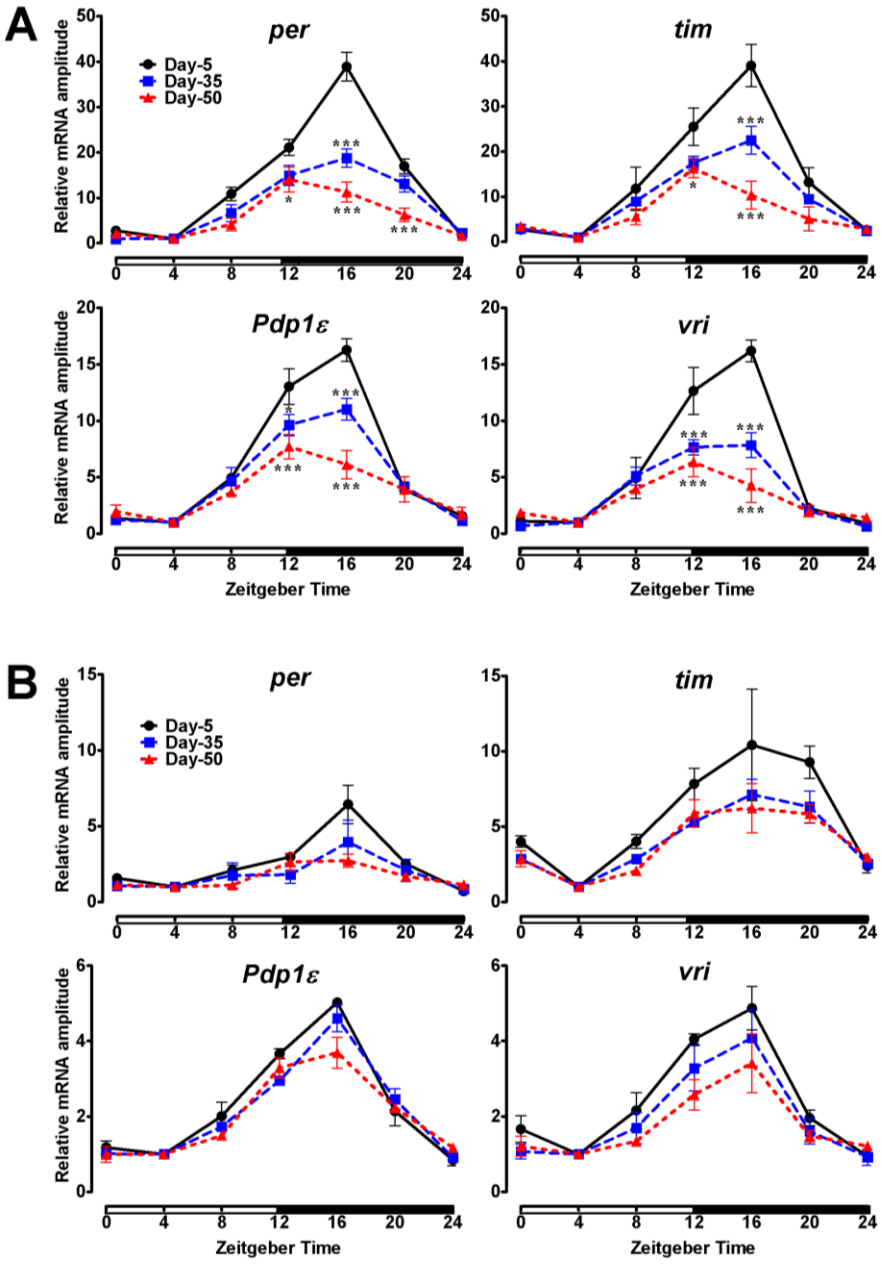


**Figure 3.2** Daily mRNA profiles of four CLK-CYC controlled transcripts on days 5, 35, and 50 in **A)** heads, and **B)** bodies of CS males, normalized to the trough (ZT 4) values set at 1 for each age. White and black horizontal bars mark periods of light and dark, respectively. Each data point represents mean ± SEM for three independent RNA samples. Statistical significance between day 5 vs. 35, and day 5 vs. 50 values was determined using two-way ANOVA with Bonferonni's post-hoc test, and is denoted by ***$p$<0.001 and *$p$<0.05.

**Table 3.1** Statistical analysis of gene expression (qRT-PCR) data by two-way ANOVA with Bonferroni's post-hoc test. Subscripted values indicate the degrees of freedom in numerator ($DF_n$) and denominator ($DF_d$) respectively.

| Gene | Effects of ZT | | Effects of age | |
|---|---|---|---|---|
| **Heads** | | | | |
| *per* | $F_{6,42} = 253.29$ | $p < 0.0001$ | $F_{2,42} = 134.60$ | $p < 0.0001$ |
| *tim* | $F_{6,42} = 45.56$ | $p < 0.0001$ | $F_{2,42} = 18.63$ | $p < 0.0001$ |
| *Pdp1ε* | $F_{6,42} = 93.04$ | $p < 0.0001$ | $F_{2,42} = 15.97$ | $p < 0.0001$ |
| *vri* | $F_{6,42} = 58.06$ | $p < 0.0001$ | $F_{2,42} = 17.62$ | $p < 0.0001$ |
| *cwo* | $F_{6,42} = 21.81$ | $p < 0.0001$ | $F_{2,42} = 1.71$ | $p = 0.1926$ |
| *Clk* | $F_{6,42} = 20.27$ | $p < 0.0001$ | $F_{2,42} = 0.41$ | $p = 0.6685$ |
| *cyc* | $F_{6,42} = 0.17$ | $p = 0.9843$ | $F_{2,42} = 1.32$ | $p = 0.1970$ |
| **Bodies** | | | | |
| *per* | $F_{6,42} = 14.76$ | $p < 0.0001$ | $F_{2,42} = 1.74$ | $p = 0.0926$ |
| *tim* | $F_{6,42} = 19.04$ | $p < 0.0001$ | $F_{2,42} = 0.81$ | $p = 0.6495$ |
| *Pdp1ε* | $F_{6,42} = 122.83$ | $p < 0.0001$ | $F_{2,42} = 2.25$ | $p = 0.0663$ |
| *vri* | $F_{6,42} = 31.43$ | $p < 0.0001$ | $F_{2,42} = 0.85$ | $p = 0.6022$ |
| *Clk* | $F_{6,42} = 23.26$ | $p < 0.0001$ | $F_{2,42} = 0.55$ | $p = 0.5817$ |
| *cyc* | $F_{6,42} = 1.68$ | $p = 0.1486$ | $F_{2,42} = 0.61$ | $p = 0.8176$ |

affected with age. However, the peak expression of all four genes was prematurely truncated in old flies (Figure 3.2A).

In parallel with heads, we measured the expression profiles of the same clock genes in male bodies. As previously reported, the amplitude of *per* oscillations was lower in bodies compared to the heads of young flies (Hardin 1994). We determined that mRNA for other clock genes *tim*, *vri*, and *Pdp1ε* also cycled with a lower amplitude in bodies than in heads of young males (Figure 3.2B). Importantly, in contrast to heads, there was no statistically significant effect of age on the oscillatory amplitude of these genes ($p>0.05$), although there was a declining trend in the peak levels of all four genes (Figure 3.2B, Table 3.1).

Reduced expression of the CLK-CYC target genes was reported in young flies with disrupted *clockwork orange* (*cwo*) (Kadener *et al.* 2007; Lim *et al.* 2007; Matsumoto *et al.* 2007; Richier *et al.* 2008). It is not known whether the expression of this gene changes during aging; therefore, we examined *cwo* mRNA profile as a function of age. As reported previously, *cwo* mRNA cycled in the heads of young flies with a trough at ZT 4 and a peak at ZT 12 (Figure 3.3) (Kadener *et al.* 2007; Lim *et al.* 2007; Matsumoto *et al.* 2007; Richier *et al.* 2008). There was no significant effect of age on *cwo* cycling, albeit individual comparison of 5 vs. 50 day-old samples revealed that levels of *cwo* were significantly lower ($p<0.05$) at ZT 16 (Figure 3.3, Table 3.1).

### *3.4.3 Aging does not alter the expression of Clk and cyc mRNA*

Since our data suggest that aging is associated with reduced activity of the CLK-CYC complexes, we next measured the expression levels of *Clk* and *cyc* mRNA in young, middle-aged, and old flies. Young flies showed expected *Clk* mRNA oscillations, with peak levels at ZT 4 and trough at ZT 16. Similar oscillations were detected in the heads of middle aged and old flies, and there was no significant reduction in *Clk* mRNA amplitude with age (Figure 3.4A, Table 3.1).

The expression of *cyc* is non-rhythmic in young flies (Bae *et al.* 2000), and we determined that the same was true in heads of old flies (Figure 3.4A, Table 3.1). There

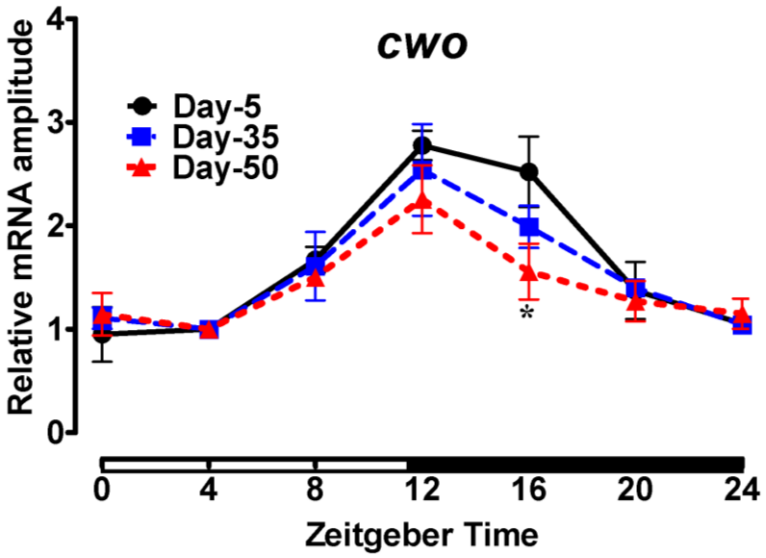


**Figure 3.3** Daily mRNA profiles of *cwo* in the heads of 5, 35, and 50 day-old CS males, normalized to the trough (ZT 4) values set at 1 for each age. Each data point represents mean ± SEM for three independent RNA samples. Statistical significance between day 5 vs. 35, and day 5 vs. 50 values was determined using two-way ANOVA with Bonferonni's post-hoc test, and is denoted by *$p$<0.05.

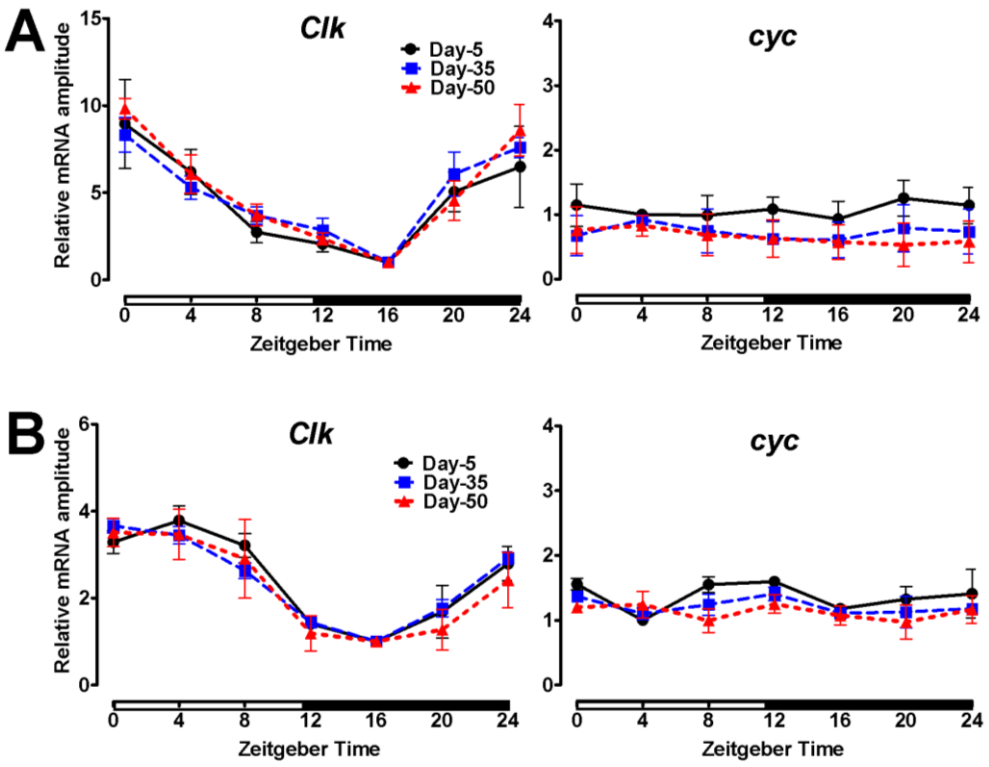


**Figure 3.4** Daily mRNA profiles of *Clk* and *cyc* on days 5, 35, and 50 in **A)** heads, and **B)** bodies of CS males, normalized to ZT 16 (*Clk*) and ZT 4 (*cyc*) values set at 1 for each age. Each data point represents mean ± SEM for three independent RNA samples. Statistical significance between day 5 vs. 35, and day 5 vs. 50 values was determined using two-way ANOVA with Bonferonni's post-hoc test ($p$>0.05).

was a declining trend in *cyc* mRNA in heads of old flies; however, the reduction was not statistically significant. Similar as in heads, *Clk* and *cyc* expression did not change significantly ($p>0.05$) in bodies of old flies (Figure 3.4B, Table 3.1).

*3.4.4 PER and TIM protein levels are substantially reduced in old flies*

Given the age-related decline in *per* and *tim* transcription (Figure 3.2), we compared PER and TIM protein profiles in head extracts of 5 and 50 day-old flies. In young flies, PER showed expected cycling, with peak at ZT 20 and decline at ZT 0-4, with retarded band migration indicative of progressive phosphorylation (Figure 3.5). By comparison, PER levels were lower in old flies across all time points, with an especially significant reduction observed at ZT 20 [Effect of age: $F_{1,16}=59.77$, $p<0.0001$; effect of ZT: $F_{3,16}=26.02$, $p<0.0001$]. Interestingly, the retarded migration of residual PER was observed at ZT 0-4 in old flies, suggesting that PER phosphorylation was similar as in young flies. In addition to PER, we analyzed TIM by Western blotting and found that levels of this protein also declined in old flies, with the most significant difference observed at ZT 20 [Effect of age: $F_{1,16}=59.96$, $p<0.0001$; effect of ZT: $F_{3,16}=35.01$, $p<0.0001$] (Figure 3.5). Consequently, the peak of both PER and TIM was prematurely truncated in old flies, consistent with age-related changes in the mRNA profiles for both genes (Figure 3.2A).

PER and TIM proteins detected by Western blotting in whole-head homogenates are derived from both central and peripheral oscillators, but the bulk of both proteins are from retinal photoreceptors (Cheng & Hardin 1998). Therefore, we examined the pattern of PER in photoreceptor cells of the retina by immunofluorescence on fly-head sections. In both young and old flies, PER was localized in cell nuclei; however, PER levels were significantly reduced in 50 day-old flies at ZT 20 and 24 (Figure 3.6A). The highest signal from individual photoreceptor nuclei in old flies did not reach the levels observed in the young flies, suggesting loss of PER amplitude in individual cells. In addition to photoreceptors, we also compared PER in several abdominal organs of young and old flies and determined that the levels of PER protein were not altered significantly with

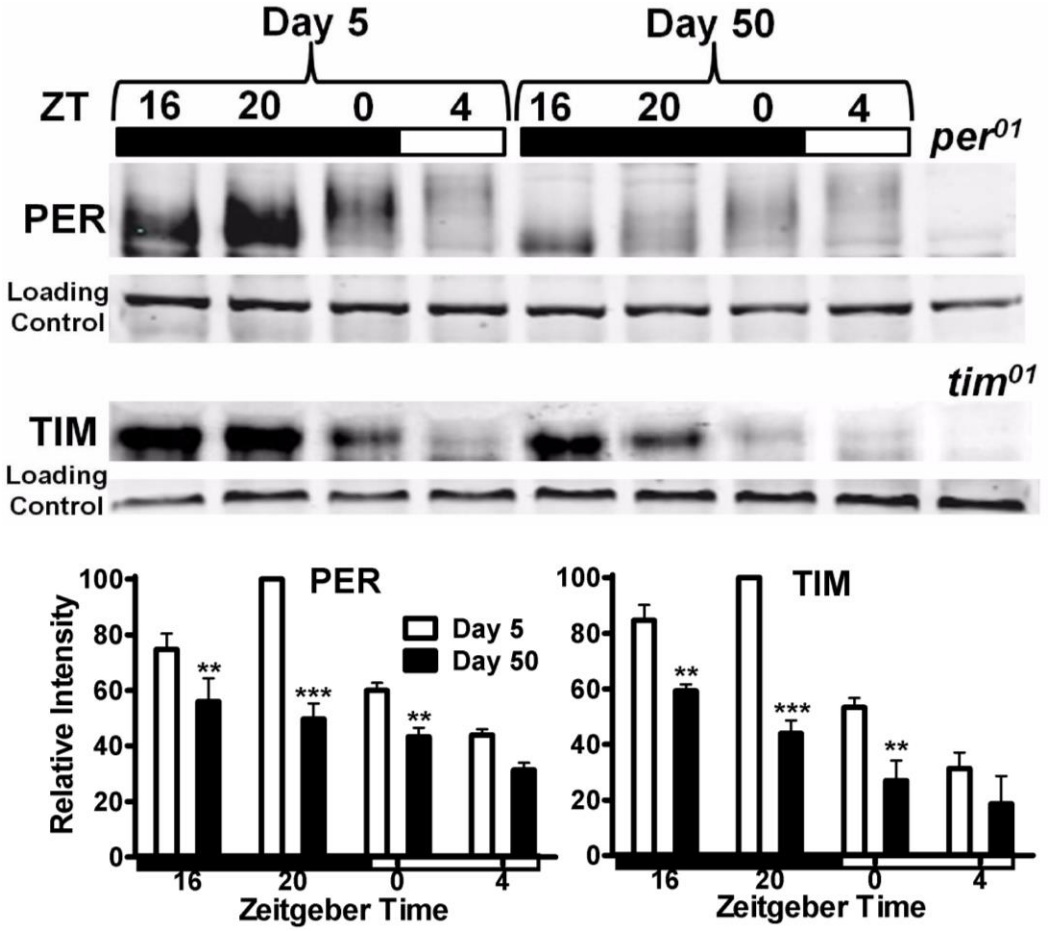


**Figure 3.5** Western blots showing PER and TIM protein profiles and phosphorylation status on days 5 and 50. Bar graphs (below) indicate the relative band intensity for different ages and time points, with signal intensity at peak (ZT 20) in 5 day-old flies set as 100. Values are mean ± SEM of three independent bio-replicates. Statistical significance between day 5 vs. 50 values was determined using two-way ANOVA with Bonferonni's post-hoc test, and is denoted by ***$p$<0.001 and **$p$<0.01.

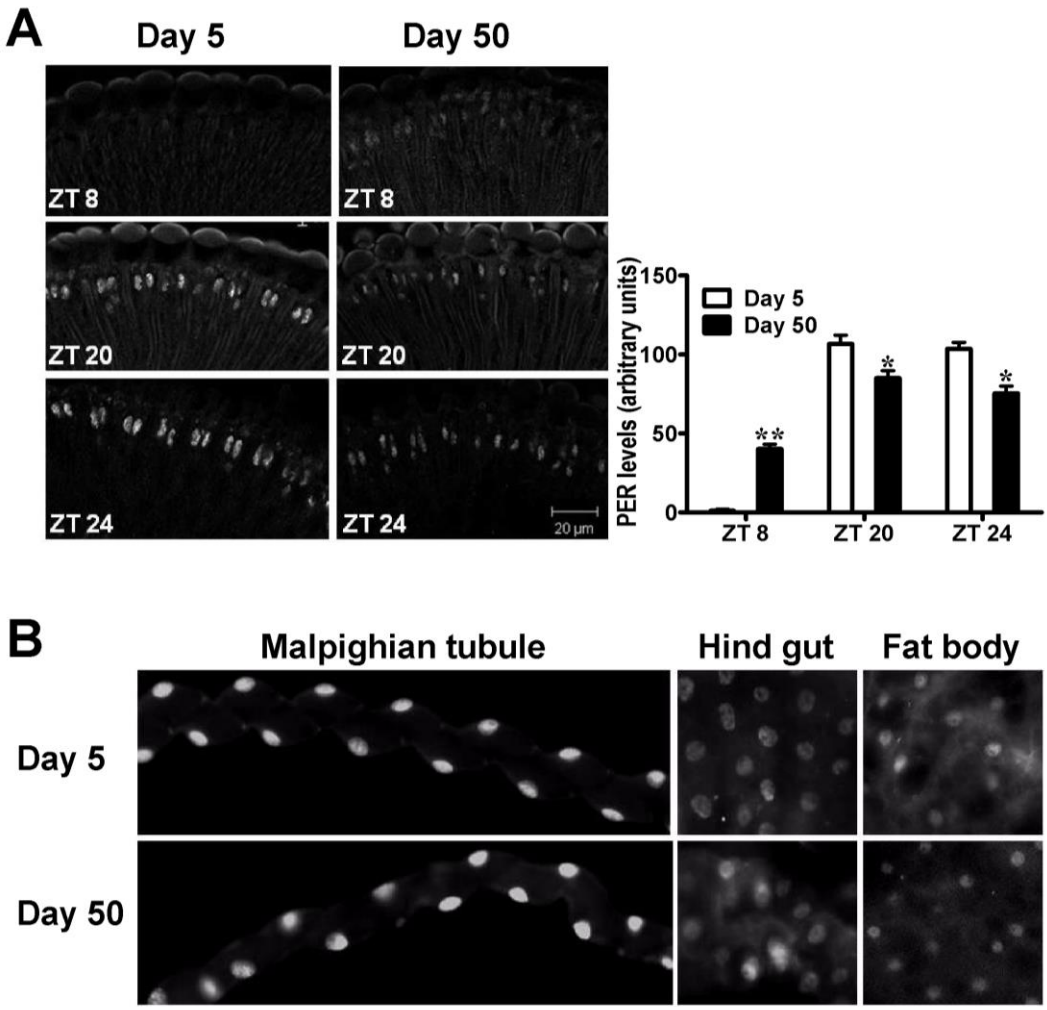


**Figure 3.6 A)** PER protein levels in the nuclei of retinal photoreceptors of 5 and 50 day-old males at ZT 8, 20, and 24. Bar graph (right) shows quantification of the signal averaged from >20 flies for each time point and age. Statistical significance was determined using Wilcoxon matched pairs test, and is denoted by $^{**}p<0.01$ and $^{*}p<0.05$. **B)** PER protein levels in the nuclei of Malpighian tubule, hind gut, and abdominal fat body cells of 5 and 50 day-old males at ZT 24. Images represent typical staining based on 6-8 flies analyzed for each age.

age. Similar levels of nuclear PER were detected in Malpighian tubules, gut, and abdominal fat of young and old flies at ZT 24 (Figure 3.6B), while PER staining was absent at ZT 8 (not shown). These data agree with *per* mRNA profiles, which did not show significant decline with age in fly bodies (Figure 3.2B).

### 3.5 Discussion

It has been widely observed that circadian output rhythms decay during aging in different animals, including humans. This decay has been suggested to have detrimental effects on health, and promote aging; therefore, it is important to determine how aging alters the clock mechanism at the molecular level. We addressed this question using *Drosophila*, which are short-lived and have a well understood circadian system. First, we confirmed previous reports of deterioration of the circadian locomotor activity rhythm in aging flies (Koh *et al.* 2006; Rezaval *et al.* 2008). In our study, flies that remained rhythmic in DD showed significant lengthening of the free-running period of locomotor activity. While the previous study on aging flies reported a similar tendency, period length did not show a statistically significant difference (Rezaval *et al.* 2008). Interestingly, one of the studies examining the period of wheel-running activity as a function of age in mammals reported significant lengthening of the free-running period in 2 year-old mice (Valentinuzzi *et al.* 1997).

In addition to the behavioral changes in old flies, we observed dampening of the molecular clock oscillations, manifested as significantly reduced peak levels of *per*, *tim*, *Pdp1ε*, and *vri* mRNA in heads of aging flies. Given that we analyzed gene expression in whole-head extracts (or retinal photoreceptors), while the circadian locomotor activity rhythms are regulated by ~150 central brain pacemaker cells, a direct link between the observed behavioral and molecular changes cannot be made. Nevertheless, previous studies have shown that flies with lower amplitude of *per* and *tim* oscillations in head extracts, show a tendency to lengthen the period of behavioral rhythms (Allada *et al.* 1998; Kadener *et al.* 2007; Lim *et al.* 2007; Matsumoto *et al.* 2007; Richier *et al.* 2008). On the other hand, flies expressing CYC-VP16 have strongly enhanced transcriptional

activity relative to that of wild type CLK–CYC, and display shortened period, implicating the strength of circadian transcription in period determination (Kadener *et al.* 2008).

In young flies, the transcription of *per*, *tim*, *Pdp1ε,* and *vri* is activated by the CLK-CYC complex that forms the positive limb of the clock. Synchronous dampening of CLK-CYC target genes in aging flies suggests weakened transcriptional activity of the CLK-CYC complexes. We determined that this is not caused by *Clk* and *cyc* deficiency, as the expression of *Clk* mRNA did not differ between young and old flies. While we show that the expression of both the activator (*Pdp1ε*) and the repressor (*vri*) of *Clk* transcription is dampened during aging, the net effect may be negligible due to their opposing action. Expression of *cyc* showed a declining trend in old flies, but these changes were not statistically significant. In addition to *Clk* and *cyc*, we analyzed the expression levels of *cwo* during aging, because the transcription of CLK-CYC target genes is diminished in *cwo* mutants, while the *cwo* gene is itself a target of CLK-CYC complexes (Kadener *et al.* 2007; Lim *et al.* 2007; Matsumoto *et al.* 2007; Richier *et al.* 2008). We detected subtle, albeit, statistically significant reduction of *cwo* levels in 50 day-old flies at ZT 16.

Our data do not reveal strong age-related changes in *Clk, cyc*, and *cwo* expression that could account for >50% reduction of CLK-CYC transcriptional targets observed in old flies. Further studies would be needed to identify factors contributing to the dampening of the positive limb of the clock with age. These could include changes in CLK or CYC protein levels, post-translational modifications (particularly the phosphorylation status of CLK), binding of CLK-CYC complexes to DNA, as well as age-related alterations in chromatin modifications that result from CLK-CYC binding to DNA (Bae *et al.* 2000; Yu & Chung 2001; Yu *et al.* 2006; Taylor & Hardin 2008; Menet *et al.* 2010). Interestingly, dampened amplitude of *per*, *tim*, *Pdp1ε,* and *vri* was consistently observed in heads of aging flies but not in bodies, despite similar profiles of *Clk* and *cyc* mRNA in both tissues. This could be connected to our previous findings that levels of oxidative damage are substantially higher in the heads than in bodies of aging flies (Krishnan *et al.* 2009). Consistent with this idea, a strong oxidative stressor reduced

the amplitude of *per*-reporter oscillations in young flies and prevented CLK from activating transcription from *per* E-box in transfected cells (Zheng *et al.* 2007). The relative insensitivity of clocks to aging in abdominal tissues could be related to feeding rhythms identified in *Drosophila* (Xu *et al.* 2008). It has been shown recently that feeding affects the phase of rhythmic gene expression in the fat body, without affecting clocks in the brain (Xu *et al.* 2011). If the feeding rhythm remains strong in aging flies, it could support clock gene oscillations in abdominal tissues.

Robust molecular circadian oscillations in the heads of young flies are generated by transcription-translation negative feedback loops (Zeng *et al.* 1994). PER/TIM proteins accumulate in cell nuclei late at night and PER represses the CLK-CYC activating complexes, resulting in the inhibition of *per* and *tim* transcription until PER is removed (Hardin 2005; Zheng & Sehgal 2008). Based on this model, a plausible cause of weakened CLK-CYC activity in old flies could have been the persistence of PER, perhaps due to age-related defects in phosphorylation, or degradation of this protein (Grima *et al.* 2002; Ko *et al.* 2002; Bae & Edery 2006). However, a quantitative measure of protein levels by Western blotting showed significant reduction of PER as well as TIM in the heads of old flies, in agreement with their mRNA profiles. Additionally PER, albeit significantly reduced, appeared to have normal phosphorylation profile in heads and displayed normal nuclear localization in the retinal photoreceptor cells of old flies.

Taken together, the above data demonstrate that aging weakens both the positive and negative limbs of the clock feedback loop, such that reduced *per* mRNA expression results in reduced PER protein levels. It remains to be determined whether inadequate repression due to PER deficiency may contribute to the dampening of CLK-CYC activity during aging. This line of reasoning is supported by studies highlighting the complex involvement of PER in sequestering DNA-bound CLK, which leads to the nighttime decrease of CLK-CYC activity. Later, the complex is released from DNA, leading to the re-activation of CLK-CYC activity in the next circadian cycle (Yu *et al.* 2009; Menet *et al.* 2010; Sun *et al.* 2010).

Our analysis of the expression of clock genes and proteins across the lifespan determined that aging affects the molecular oscillations in heads more strongly than in the body tissues of *Drosophila*. Thus, the extent of age-related changes in clock gene expression in flies depends on the specific clock component and tissue examined, similar to what was previously reported in vertebrates (Asai *et al.* 2001; Yamazaki *et al.* 2002; Kolker *et al.* 2003; Zhdanova *et al.* 2008; Jud *et al.* 2009). Importantly, weakened transcriptional activity of the CLK-CYC complexes during aging in *Drosophila* provides a foundation to investigate how this dampening affects clock-controlled pathways. Previous microarray studies identified several oscillating genes in fly heads, whose expression became arrhythmic in $Clk^{Jrk}$ mutants (McDonald & Rosbash 2001). Further, several studies have shown widespread gene activation by CLOCK-BMAL1 complexes in mammals (Panda *et al.* 2002; Rey *et al.* 2011). Dampening of the positive limb of the clock during aging is likely to impair the rhythmic expression of clock-controlled effector genes that are involved in many pathways maintaining temporal homeostasis.

### 3.6 Acknowledgements

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# Exploring functional links between circadian clocks, neurodegeneration, and aging in *Drosophila melanogaster*

## CHAPTER 4

## *Cryptochrome* restores dampened circadian rhythms and promotes healthspan in aging *Drosophila*

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## 4.1 Summary

Circadian clocks generate daily rhythms in molecular, cellular, and physiological functions providing temporal dimension to organismal homeostasis. Recent evidence suggests two-way relationship between circadian clocks and aging. While disruption of the circadian clock leads to premature aging in animals, there is also age-related dampening of output rhythms such as sleep/wake cycles and hormonal fluctuations. Decay in the oscillations of several clock genes was recently reported in aged fruit flies, but mechanisms underlying these age-related changes are not understood. We report that the circadian light-sensitive protein CRYPTOCHROME (CRY), is significantly reduced at both mRNA and protein levels in heads of old *Drosophila melanogaster*. Restoration of CRY using the binary GAL4/UAS system in old flies significantly enhanced the mRNA oscillatory amplitude of several genes involved in the clock mechanism. Flies with replenished CRY in all clock cells maintained strong rest/activity rhythms in constant darkness late in life when rhythms were disrupted in most control flies. There was a remarkable extension of healthspan in flies with elevated CRY. Conversely, CRY deficient mutants showed accelerated functional decline and accumulated greater oxidative damage. Interestingly, rescue of CRY in central clock neurons alone was not sufficient to restore rest/activity rhythms or extend healthspan. Together, these data suggest novel anti-aging functions of CRY and indicate that peripheral clocks play an active role in delaying behavioral and physiological aging.

## 4.2 Introduction

Circadian clocks are intrinsic mechanisms that generate daily rhythms in behavior, physiology, and cellular processes, ensuring temporal homeostasis coordinated with day/night cycles (Reddy & O'Neill 2010). Increasing evidence suggests bidirectional relationships between circadian clocks and aging. On one hand, genetic or environmental disruptions of the clock function accelerate physiological aging, onset of late life diseases, and mortality risk in mammals (Davidson *et al.* 2006; Kondratov *et al.* 2006; Lee 2006; Antoch *et al.* 2008; Yu & Weaver 2011). On the other hand, aging is also

known to impair circadian rhythms as evidenced by disruption of sleep/wake cycles, dampening of hormonal rhythms, and weakening of clock gene oscillations (Valentinuzzi *et al.* 1997; Huang *et al.* 2002; Hofman & Swaab 2006; Kondratova & Kondratov 2012). Age-related decline in temporal coordination of metabolic, physiological and neurological functions have profound effects on health and disease susceptibility, yet the mechanisms underlying the decay of the circadian system are not understood.

Bidirectional relationships between circadian clocks and aging have also been established in *Drosophila melanogaster*. Disruption of the circadian clock increases the susceptibility of aging fruit flies to oxidative stress and neurodegeneration (Krishnan *et al.* 2009; Krishnan *et al.* 2012). Conversely, aging flies show fragmented sleep/activity patterns and dampened clock gene oscillations (Koh *et al.* 2006; Luo *et al.* 2012a; Rakshit *et al.* 2012). In the current study, we used *Drosophila* to address mechanisms underlying the decay of the circadian system, and investigate whether this decay could be reversed in aging flies.

The molecular mechanism of the circadian clock is based transcription-translation feedback loops that are evolutionarily conserved from flies to mammals (Stanewsky 2003; Yu & Hardin 2006). In fruit flies, *Clock* (*Clk*) and *cycle* (*cyc*) genes encode transcription factors that form CLK-CYC activator complexes and stimulate the expression of genes *period* (*per*) and *timeless* (*tim*) early at night. Subsequently, PER and TIM proteins form heterodimers and accumulate in the cell nuclei repressing CLK-CYC transcriptional activity, and thus suppressing their own transcription (Hardin 2011). In another negative feedback loop, CLK-CYC complexes induce the expression of transcription factors *Par domain protein 1ε* (*Pdp1ε*) and *vrille* (*vri*) that act as an activator and repressor of *Clk* (Cyran *et al.* 2003). This basic clock mechanism is cell-autonomous and operates in the central and peripheral clock cells. The central clock is formed by a network of ~150 pacemaker neurons in the fly brain, which regulate rest/activity rhythms (Nitabach & Taghert 2008). Peripheral clocks are found in many cells of the nervous system, such as retinal photoreceptors, glia, sensory neurons, and in non-neural tissues in the head and body (Hardin 2011; Xu *et al.* 2011).

High-amplitude oscillations of *per*, *tim*, *Pdp1ε*, and *vri* mRNA as well as PER and TIM proteins observed in young flies, are significantly reduced in heads of aging flies (Luo *et al.* 2012a; Rakshit *et al.* 2012). Interestingly, there were strong oscillations of PER in central clock neurons (Luo *et al.* 2012a), suggesting that peripheral oscillators in fly heads are affected by aging more than the central clock, and are therefore responsible for reduced clock gene oscillations observed in the heads. The causes of clock gene oscillation dampening in peripheral clocks are not clear. In young flies, the blue light photoreceptive flavin-binding protein CRYPTOCHROME (CRY), is critical for the synchronization of individual oscillator cells (Emery *et al.* 1998; Stanewsky *et al.* 1998). CRY targets TIM for degradation after lights on and synchronizes the circadian oscillations with external day/night cycles (Busza *et al.* 2004). In addition to acting as circadian photoreceptor that mediates light input into the clock, CRY appears to function as the central clockwork component in peripheral clocks. Indeed, in hypomorphic $cry^b$ mutants, rhythmic expression of *per* and *tim* at mRNA and protein levels is abolished in the peripheral clock cells but not in the central clock neurons (Stanewsky *et al.* 1998). Furthermore, CRY is necessary for circadian clock functions in other peripheral oscillators under constant darkness (Ivanchenko *et al.* 2001; Krishnan *et al.* 2001), but its role is not yet understood. Mammalian mCry1 and mCry2 are not photoreceptive and act as circadian transcriptional regulators necessary for clock function (Kume *et al.* 1999). Interestingly, a recent report shows that human HsCRY-1 protein confers light-independent biological activity in transgenic *Drosophila* (Vieira *et al.* 2012), suggesting functional similarities between fly and human CRY. More unexpectedly, it was shown that endogenous fly CRY also regulates considerable light-independent transcriptional activity in *Drosophila* (Vieira *et al.* 2012). An increased repertoire of CRY functions in flies including effects on metabolism (Fogle *et al.* 2011; Seay & Thummel 2011; Kumar *et al.* 2012), suggest that CRY may act via multiple mechanisms that remain to be understood.

As mentioned above, rhythmic expression of *per* and *tim* is abolished in peripheral clock cells such as photoreceptors and glia of hypomorphic $cry^b$ mutants

(Stanewsky *et al.* 1998). Age-related dampening of *cry* mRNA oscillations was recently reported in heads of flies, along with significantly reduced mRNA oscillations of *per*, *tim*, *Pdp1ε*, and *vri* (Luo *et al.* 2012a; Rakshit *et al.* 2012). We reasoned that reduced levels of *cry* in heads of old flies may be responsible for diminished cycling of *per* and *tim* mRNA, similar as in young $cry^b$ mutants (Stanewsky *et al.* 1998).

In this study, we observed that CRY is reduced with age at both mRNA and protein levels, and attempted to replenish CRY in old flies using the binary GAL4/UAS system. We report that overexpression of CRY in all clock expressing cells improves the mRNA oscillatory amplitude of several genes involved in the clock mechanism, and restores strong circadian rest/activity rhythms in old flies. We further show that flies with elevated CRY levels have significantly improved healthspan during aging. Conversely, flies deficient in CRY show accelerated functional decline and accumulate greater oxidative damage, together suggesting novel anti-aging role of CRY. Interestingly, restored rest/activity rhythms and healthspan benefiting effects are only observed when CRY is constitutively restored in all clock cells, but not in the central clock neurons alone, suggesting that peripheral clocks play an active role in maintaining organismal health during aging.

## 4.3 Results

### *4.3.1 CRY is reduced at both mRNA and protein levels in heads of old flies*

We obtained daily mRNA expression profiles of *cry* in the heads and bodies of adult Canton S (CS) males on day 5 (young), 35 (middle aged), and 50 (old). In heads of young flies, *cry* mRNA showed expected daily oscillations, with peak at Zeitgeber time (ZT) 4-8 and trough at ZT 16 (Figure 1A). The levels and amplitude of *cry* mRNA oscillations were significantly dampened in 35 day-old flies and further reduced on day 50 (Figure 4.1A, Table S4.1), similar to a recent report (Luo *et al.* 2012a). In contrast to heads, there was no statistically significant effect of age on *cry* oscillations in bodies (Table S4.1).

Given the age-related decline in *cry* mRNA levels, we compared CRY protein profiles in head extracts of 5 and 50 day-old CS flies. In young flies, the highest levels of

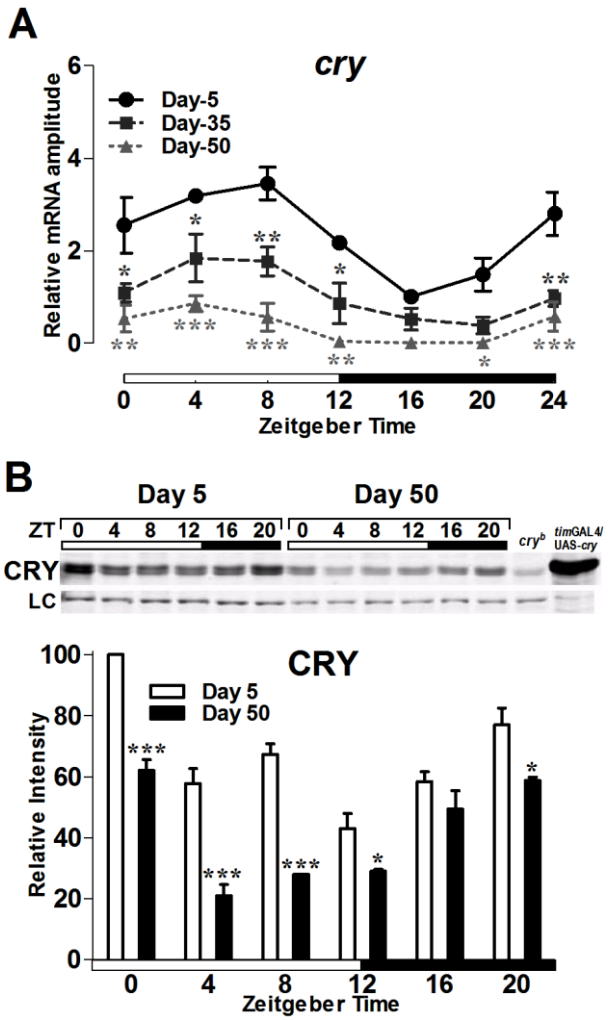


**Figure 4.1 CRY is reduced with age at both mRNA and protein levels. A)** Daily mRNA profiles of *cry* on days 5, 35, and 50 in heads of CS males, normalized to the trough (ZT 16) values set at 1 for day 5. White and black horizontal bars mark periods of light and dark respectively. Each data point represents mean ± SEM of three independent RNA samples. Statistical significance between day 5 vs. 35 and day 5 vs. 50 values was determined by two-way ANOVA with Bonferonni's post hoc test (***$p$<0.001, **$p$<0.01, and *$p$<0.05). **B)** Western blot showing CRY protein profile in head extracts of 5 and 50 day-old CS males with *cry*$^b$ and *tim*>CRY serving as negative and positive controls. Bar graph (below) indicates the relative band intensity for different ages and time points, with signal intensity at peak (ZT 0) in 5 day-old flies set as 100. Values are mean ± SEM of three independent bioreplicates. Statistical significance between day 5 vs. 50 values was determined using two-way ANOVA with Bonferonni's post hoc test, and is denoted by ***$p$<0.001 and *$p$<0.05 (LC-Loading control).

CRY protein were detected near the end of the dark phase at ZT 0/24. CRY declined during the day to a trough at ZT 12. By comparison, CRY levels were significantly lower in old flies across all time points except ZT 16 (Figure 4.1B, Table S4.1).

*4.3.2 Old flies with elevated CRY have increased oscillatory amplitude of several genes involved in the clock mechanism*

We and others recently reported that the oscillatory amplitude of *per*, *tim*, *Pdp1ε*, and *vri* is dampened during aging (Luo *et al.* 2012a; Rakshit *et al.* 2012); however, the underlying mechanisms are not fully understood. Since *per* and *tim* transcription is reduced and non-rhythmic in young $cry^b$ mutants (Stanewsky *et al.* 1998), we investigated whether CRY deficiency may contribute to the dampened oscillations of clock genes in old flies. We increased wild type CRY levels in all central and peripheral clock cells by combining the *tim*GAL4 driver with the UAS-*cry* responder (*tim*>CRY). As a control, *tim*GAL4 flies were crossed to UAS-$cry^b$ (*tim*>$CRY^B$), which carries a missense mutation at the putative flavin-binding residue, rendering $CRY^B$ protein light-insensitive and unstable (Stanewsky *et al.* 1998; Emery *et al.* 2000; Busza *et al.* 2004). As a second control, *tim*GAL4 was crossed to $w^{1118}$ (*tim*GAL4/+).

We first determined that CRY protein was significantly elevated in heads of both young and old *tim*>CRY flies, compared to unstable mutant protein in *tim*>$CRY^B$ (Figure S4.1). At both ages, CRY levels in heads of *tim*>CRY were lower during the day (ZT 4) than at night (ZT 16) suggesting that the ectopic CRY undergoes light-dependent degradation similar as the endogenous CRY. To determine whether higher CRY levels affected the expression of clock genes, we obtained daily expression profiles of *per* and *tim* in heads of old flies. The control *tim*GAL4/+ flies showed considerably dampened daily oscillations of *per* mRNA with trough at ZT 4 and a shallow peak between ZT 12-16 on day 50, consistent with recent reports (Luo *et al.* 2012a; Rakshit *et al.* 2012). In contrast, age-matched *tim*>CRY flies had significantly higher amplitude of *per* mRNA expression with a well-defined peak at ZT 16 (Figure 4.2, Table S4.2), when a peak is usually observed in young flies (Rakshit *et al.* 2012). Expression of mutant $CRY^B$ protein

did not increase *per* levels in old *tim*>CRY$^{B}$ flies, and they had a very similar mRNA profile as the *tim*GAL4/+ control. We next measured the expression profile of *tim*, which encodes TIM protein that forms heterodimers with PER. Flies overexpressing CRY had higher amplitude of *tim* cycling due to significantly high levels at the peak time point (ZT 16), compared to the age-matched controls (Figure 4.2, Table S4.2).

To determine whether other components of the clock mechanism rather than *per* and *tim* alone are enhanced by the replenishment of CRY, we examined the mRNA expression profiles of *Pdp1ε* and *vri* that are also activated by CLK/CYC complexes and oscillate in phase with *per* and *tim*. We observed that mRNA oscillations for the two genes were significantly higher in heads of 50 day-old *tim*>CRY flies than in both controls (Figure 4.2, Table S4.2). Together, these data suggested that flies overexpressing CRY could have increased levels of *Clk* and *cyc* genes leading to increased CLK/CYC mediated activation of *per*, *tim, Pdp1ε* and *vri*. To begin addressing this question, we examined the expression levels of *Clk* and *cyc* mRNA in heads of old males. Control flies showed expected *Clk* mRNA oscillations, with peak levels at ZT 4 and trough at ZT 16. There was no significant difference in *Clk* mRNA profiles in heads of *tim*>CRY flies compared to both controls (Figure 4.2, Table S4.2). The expression of *cyc* is non-rhythmic (Bae *et al.* 2000), and does not significantly change with age in flies (Rakshit *et al.* 2012). Similar as *Clk*, there was no difference in *cyc* mRNA levels in heads of *cry* overexpressing flies and controls (Figure 4.2, Table S4.2).

*4.3.3 CRY overexpression prevents age-related weakening of rest/activity rhythms*

Higher oscillations of several clock genes in heads of old CRY overexpressing flies prompted us to examine the strength of behavioral rhythms in these flies. We recorded locomotor activity rhythms in *tim*>CRY and control flies on days 5-15, 35-45, and 50-60, to assess age-related changes in rhythm strength and other circadian parameters. In addition, we also monitored activity in two lines of *cry*-null flies ($cry^{01}$ and $cry^{02}$) (Dolezelova *et al.* 2007). Males were monitored for 3 d in LD 12:12 followed by 7 d in DD. Young control flies showed bimodal activity distribution in LD with morning and

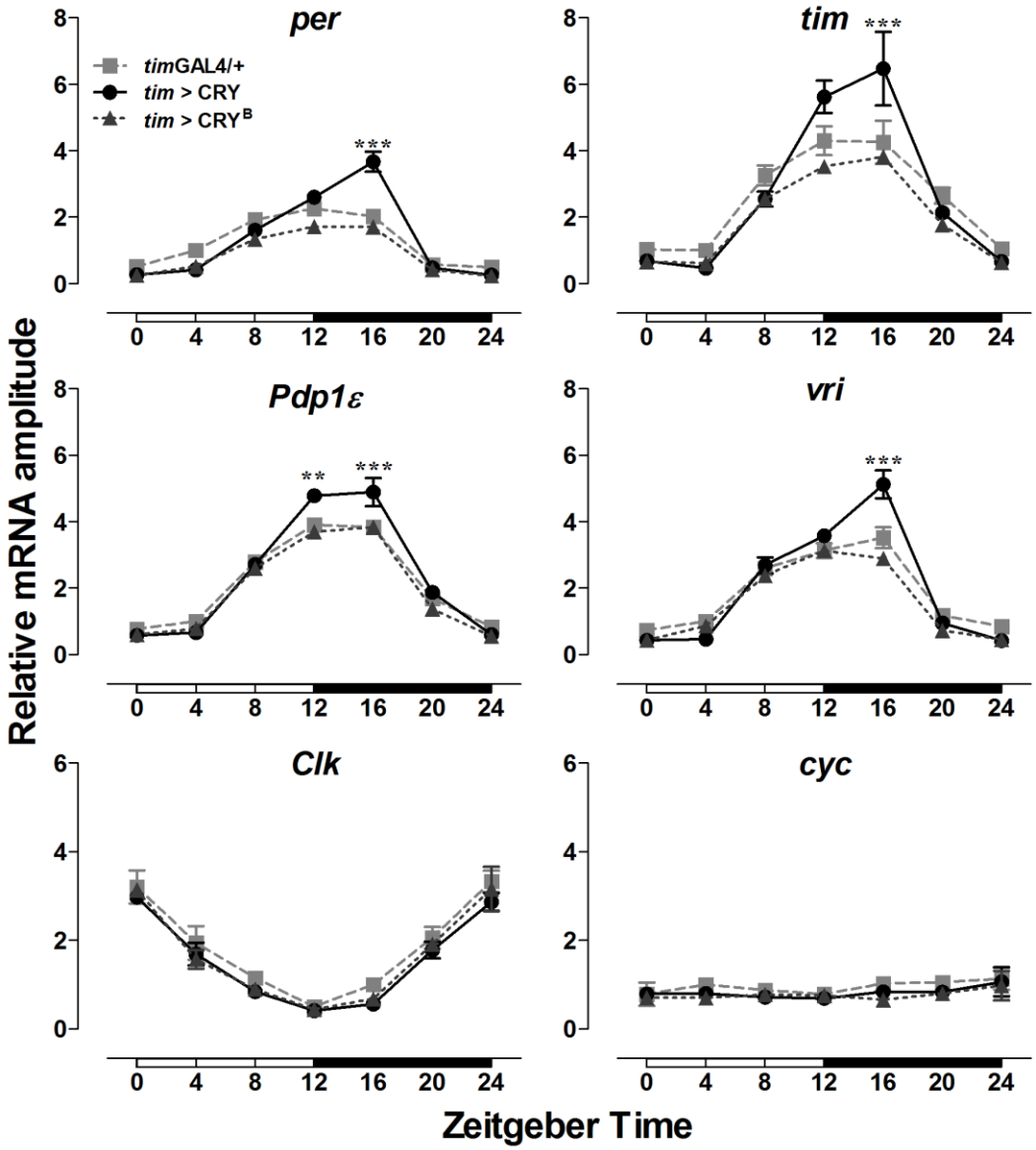


**Figure 4.2 Old flies with elevated CRY have increased clock gene oscillations.** Daily mRNA profiles of clock genes in heads of *tim*GAL4/+, *tim*>CRY, and *tim*>CRY$^{B}$ males on day 50, normalized to the trough (ZT 4/ZT 16 for *Clk*) values set at 1 for *tim*GAL4/+ control. White and black horizontal bars mark periods of light and dark respectively. Each data point represents mean ± SEM of three independent RNA samples. Statistical significance between the genotypes was determined by two-way ANOVA with Bonferonni's post hoc test, and is denoted by ***$p$<0.001 and **$p$<0.01.

evening peaks and anticipation of lights on and off (Figure S4.2, Table S4.3). There was a general age-related reduction in average LD activity counts accompanied by the loss of anticipation. However, 50 day-old *tim*>CRY flies showed pronounced morning and evening peaks while one or both of these peaks were attenuated in control or *cry*-null flies at this age (Figure S4.2).

Our analysis of free-running rhythms in DD revealed that 50-60 day-old *tim*>CRY flies maintained remarkably strong rest/activity rhythms while those rhythms decayed in most control flies (Figure 4.3). Young $cry^{01}$ flies had strong activity rhythms as expected (Dolezelova *et al.* 2007), but the rhythms weakened at middle age similar to controls (Figure 4.3A and 4.3B). The behavior of $cry^{02}$ flies changed across lifespan in a similar manner as $cry^{01}$ (not shown). Overall calculation of rhythmic flies pooled from two independent experiments demonstrated that *tim*>CRY flies were still 82% rhythmic on days 50-60 when the proportion of rhythmic flies decreased below 40% in control and *cry*-null lines (Figure 4.3C, Table S4.3). Persistence of behavioral rhythms in old *tim*>CRY in DD suggests that a durable intrinsic circadian system is maintained in these flies.

### *4.3.4 Replenishment of CRY in all clock-expressing cells improves the healthspan of aging flies*

Correlational data suggest links between degradation of circadian rhythms and accelerated aging; however, it is not known whether rejuvenation of the circadian system is associated with extended healthspan. Our finding that replenishment of CRY restores molecular and behavioral rhythms in aging flies provided a unique opportunity to examine the relationship between strength of circadian rhythms and the rate of aging. One of the important biomarkers of aging in *Drosophila* is the decline of climbing ability, which can be measured via the RING assay that utilizes negative geotaxis to assess vertical mobility (Rhodenizer *et al.* 2008). We used the RING assay to compare climbing ability in flies with altered CRY levels across lifespan. On day 5, the climbing ability of *tim*>CRY flies was not different from the *tim*GAL4/+ *or tim*>$CRY^{B}$ controls; however,

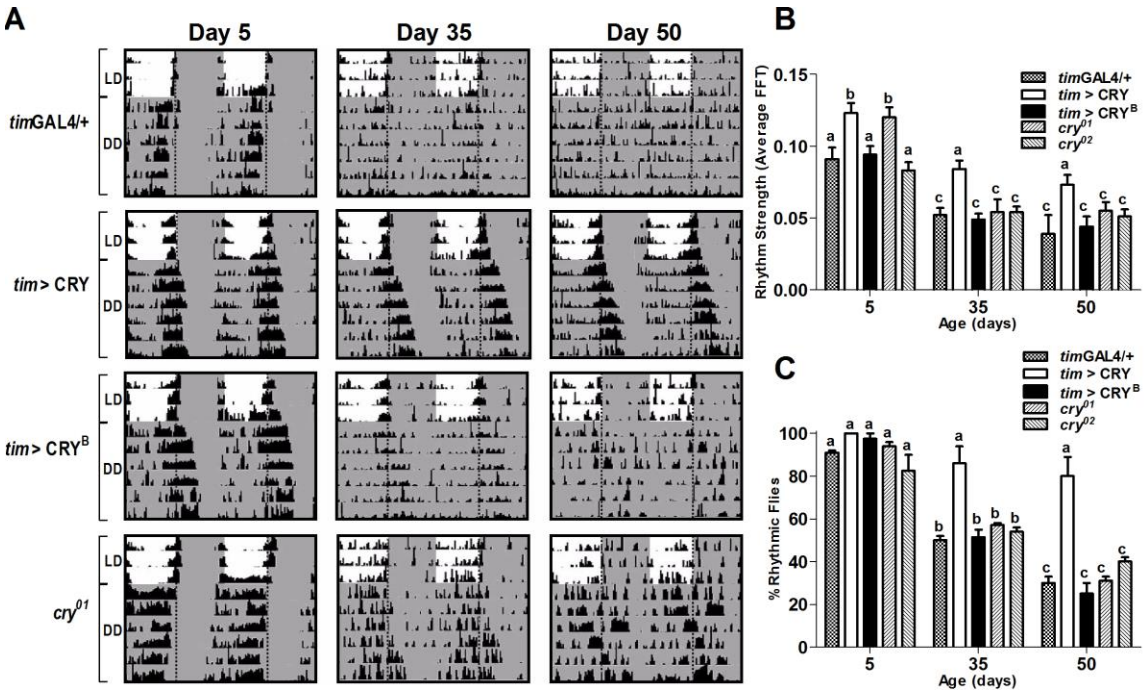


**Figure 4.3 CRY overexpression prevents disruption of locomotor activity rhythms. A)** Locomotor activity profiles of representative 5, 35, and 50 day-old males of the indicated genotypes. Flies of each age were monitored in LD 12:12 for 3 d, followed by 7 d in DD at 25°C. Shaded areas represent periods of darkness. Vertical dotted lines indicate time of lights-off (ZT/CT 12). **B**) Average rhythm strength, and C**)** Percentage of rhythmic flies on days 5, 35, and 50. Flies with FFT values >0.04 were considered rhythmic. Values are mean ± SEM of two independent activity analyses. Total number of flies analyzed for each genotype is indicated in Table S4.3. Statistical significance was determined using two-way ANOVA with Bonferroni's post hoc test, and bars with different letters are significantly different at $p<0.05$.

both $cry^{01}$ and $cry^{02}$ mutants had significantly lower climbing ability even at this young age (Figure 4.4A, Table S4). The climbing ability declined significantly in both controls and *cry*-null flies on day 35. In contrast, *tim*>CRY flies maintained significantly higher vertical mobility that was not different from day 5 ($p>0.05$). Flies of all genotypes further lost their vertical mobility with progressing age; however, *tim*>CRY flies retained significantly higher climbing ability than controls even on day 50 (Figure 4.4A, Table S4.4).

Another biomarker of aging is the accumulation of oxidatively damaged proteins, which can be biochemically measured as levels of protein carbonyls (PC) in the total protein extract (Krishnan *et al.* 2009). Since alterations in CRY levels affected climbing ability, we tested whether the accumulation of oxidative damage in old age was also affected. We quantified PC in heads of 50 day-old flies at ZT 8, which is the peak of PC accumulation (K. Rakshit, unpublished). Indeed, *tim*>CRY flies had significantly lower PC accumulation in heads, compared to the controls (Figure 4.4B). On the other hand, both $cry^{01}$ and $cry^{02}$ flies had higher PC than the controls and this difference reached statistical significance in $cry^{02}$ flies. Together, these data suggest that CRY may have novel anti-aging functions.

An important aspect of healthy aging is the ability to withstand homeostatic insults, such as oxidative stress. We reported that flies with disrupted clocks showed significantly increased mortality risk after short-term oxidative challenge (24 h of 100% hyperoxia) in the middle or old age (Krishnan *et al.* 2009). We used this assay here to test whether flies with elevated CRY levels are capable of buffering short-term oxidative challenge later in life, by exposing 50 day-old flies to 24 h hyperoxia. This treatment significantly shortened the lifespan of *tim*GAL4/+ and *tim*>$CRY^{B}$ flies. In contrast, *tim*>CRY flies were resilient to this stress and had very similar survival curves as their normoxia controls, despite a few initial deaths (Figure 4.4C, Table S4.5). The lifespan of *tim*>CRY flies under normoxia was ~7% longer than control flies, albeit this difference was not statistically significant.

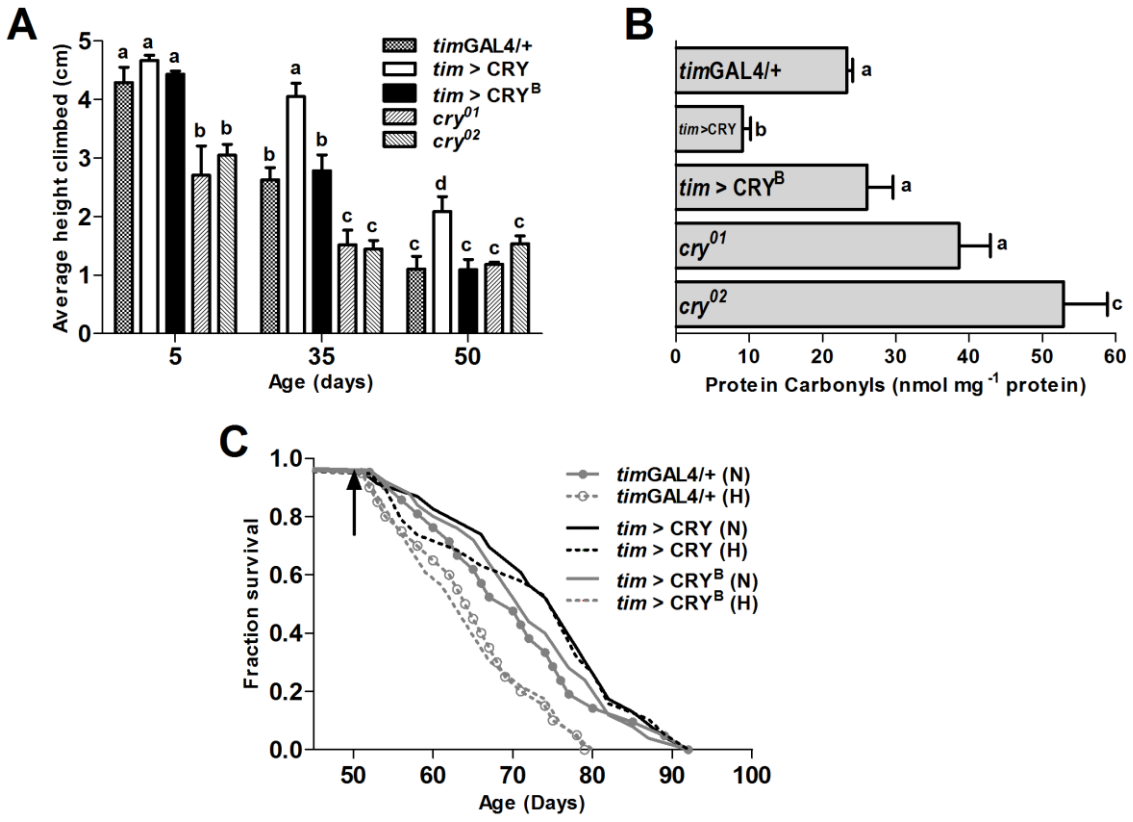


**Figure 4.4 Flies overexpressing CRY have improved healthspan. A)** Vertical mobility was measured by the RING assay in 5, 35, and 50 day-old males of the indicated genotypes. Bars represent mean height climbed (± SEM), based on testing 4 vials per genotype, each containing 25 flies. Statistical significance was determined using two-way ANOVA with Bonferroni's post hoc test, and bars with different letters are significantly different at $p<0.05$. **B**) Oxidative damage in the form of protein carbonyls (PC) were measured in heads of 50 day-old males of the indicated genotypes at ZT 8. *tim*>CRY flies had significantly lower PC compared to the *tim*GAL4/+ and *tim*>CRY$^B$ controls, and *cry*$^{01}$. There was significantly higher PC accumulation in heads of *cry*$^{02}$ flies compared to *tim*GAL4/+ control. Bars represent mean carbonyl levels (± SEM), based on testing 3 independent sets of flies, each containing 75 flies in 3 technical repeats of 25 flies each. Statistical significance was determined using one-way ANOVA with Tukey's post hoc test, and bars with different letters are significantly different at $p<0.05$. **C)** Lifespan of *tim*GAL4/+, *tim*>CRY, and *tim*>CRY$^B$ flies in normoxia (solid lines) and following 24 h hyperoxia (dotted lines) on day 50 (marked by arrow). In normoxia, there was no significant difference in mean survival curves among the genotypes. Hyperoxia on day 50 significantly shortened the lifespan of *tim*GAL4/+, and *tim*>CRY$^B$ controls ($p<0.05$) but not *tim*>CRY flies.

*4.3.5 Overexpression of CRY in central pacemaker neurons does not improve rest/activity rhythms during aging*

In young flies, locomotor activity rhythms are controlled by ~150 central pacemaker neurons in the brain (Nitabach & Taghert 2008). The *tim*GAL4 driver increases CRY in both central and peripheral clock cells. Therefore, we next examined whether overexpression of CRY in central pacemaker neurons alone is sufficient to restore locomotor activity rhythms during aging. We initially used the *Pdf*GAL4 driver to overexpress CRY or $CRY^B$ in PDF-positive lateral ventral neurons ($LN_vs$), which are crucial for DD activity rhythms in young flies (Nitabach & Taghert 2008). Also, *Pdf*GAL4 driven UAS-*cry* can rescue behavioral photoresponses in $cry^b$ mutants (Emery *et al.* 2000). Surprisingly, we found that *Pdf* driven expression of CRY in $LN_vs$ is not sufficient to prevent age-related decay of behavioral rhythms. *Pdf*>CRY flies showed weakening and age-related fragmentation of rest/activity rhythms similar as the *Pdf*>$CRY^B$ controls (Figure 4.5A). There was no significant difference in the average daily activity in LD between *Pdf*>CRY and *Pdf*>$CRY^B$ flies (Figure S4.3, Table S4.3). Analysis of fly activity in DD revealed very similar age-related decline in the rhythm strength (Figure 4.5B) and the percentage of rhythmic flies (Figure 4.5C) in both *Pdf*>CRY and *Pdf*>$CRY^B$ flies, with no statistical difference at any tested age (Table S4.3).

We then overexpressed CRY or $CRY^B$ using another driver line *cry*GAL4-39, which is active in some dorsal (DNs) and lateral dorsal neurons ($LN_ds$) in addition to $LN_vs$ (Klarsfeld *et al.* 2004). Despite that a larger subset of central pacemaker neurons was targeted, rhythms of locomotor activity decayed at a similar rate with in *cry*39>CRY as in *cry*39>$CRY^B$ controls (Figures 4.5D-F and S4.3, Table S4.3).

*4.3.6 Rescue of CRY in central pacemaker neurons alone does not improve healthspan*

We tested whether rescue of CRY in the central pacemaker neurons affects biomarkers of aging that were improved by CRY overexpression in both central and peripheral clocks.

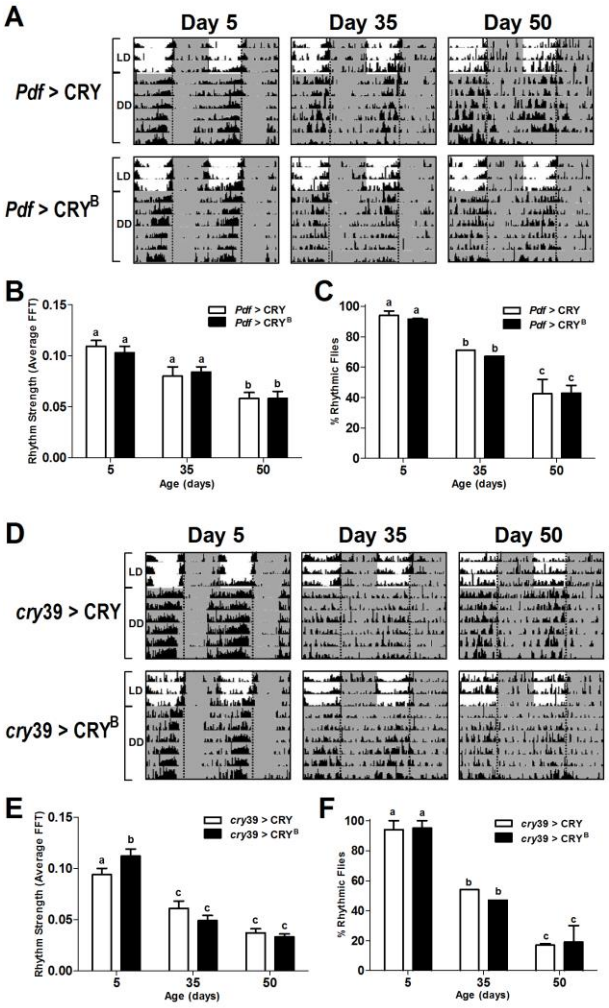


**Figure 4.5 Rescue of CRY in central pacemaker neurons alone does not improve locomotor activity rhythms. A)** Locomotor activity profiles of representative *Pdf*>CRY and *Pdf*>CRY$^{B}$ males on days 5, 35, and 50. Flies of each age were monitored in LD 12:12 for 3 d, followed by 7 d in DD at 25°C. Shaded areas represent periods of darkness. Vertical dotted lines indicate time of lights-off (ZT/CT 12). **B**) Average rhythm strength, and C**)** Percentage of rhythmic *Pdf*>CRY and *Pdf*>CRY$^{B}$ flies on days 5, 35, and 50. **D)** Representative locomotor activity profiles, **E**) Average rhythm strength, and **F)** Percentage of rhythmic *cry*39>CRY and *cry*39>CRY$^{B}$ flies on days 5, 35, and 50. (**B,C,D,F** - Flies with FFT values >0.04 were considered rhythmic. Values are mean ± SEM of two independent activity analyses. Total number of flies analyzed for each genotype is indicated in Table S3. Statistical significance was determined using two-way ANOVA with Bonferroni's post hoc test, and bars with different letters are significantly different at $p<0.05$.)

There was no statistically significant difference in climbing ability of *Pdf*>CRY or *cry*39>CRY flies compared to their respective controls at any physiological age (Figure 4.6A). Neither was there any statistically significant difference in PC accumulation in heads of old *Pdf*>CRY/*cry*39>CRY flies compared to their *Pdf*>$CRY^{B}$/*cry*39>$CRY^{B}$ controls (Figure 4.6B).

**4.4 Discussion**

The decline of circadian output rhythms is a common signature of aging in animals including humans. It was recently reported in *Drosophila* that aging is associated with dampened molecular oscillations of core clock genes in peripheral but not central oscillators (Luo *et al.* 2012a; Rakshit *et al.* 2012). Given the persistence of molecular oscillations in the central clock, there is currently no explanation as to why rest/activity rhythms lose their robustness during aging. The results of our study suggest that enhancement of peripheral clocks may prevent degradation of the circadian output. Furthermore, our data suggest a cause and effect relationship between the molecular and behavioral decay of circadian rhythms and fly healthspan.

We observed reduction in *cry* mRNA levels in heads of old flies, consistent with a previous study (Luo *et al.* 2012a). Interestingly, *cry* decline was observed in the heads but not in bodies similar to our results on other clock genes (Rakshit *et al.* 2012), suggesting that the peripheral clocks in fly head are more susceptible to aging than clocks in the body tissues. We also report that CRY protein is significantly reduced in heads of 50 day-old CS flies at most time points, although it still oscillates in a phase similar to young flies. This suggests that the circadian system is severely attenuated yet functional in old flies.

Our data shows that age-related CRY decline is functionally linked to decay of the circadian system, as restoration of wild type CRY significantly improved the amplitude of *per*, *tim*, *Pdp1ε*, and *vri*, genes in heads of old flies. These results may seem surprising as it was reported that overexpression of CRY together with PER using the

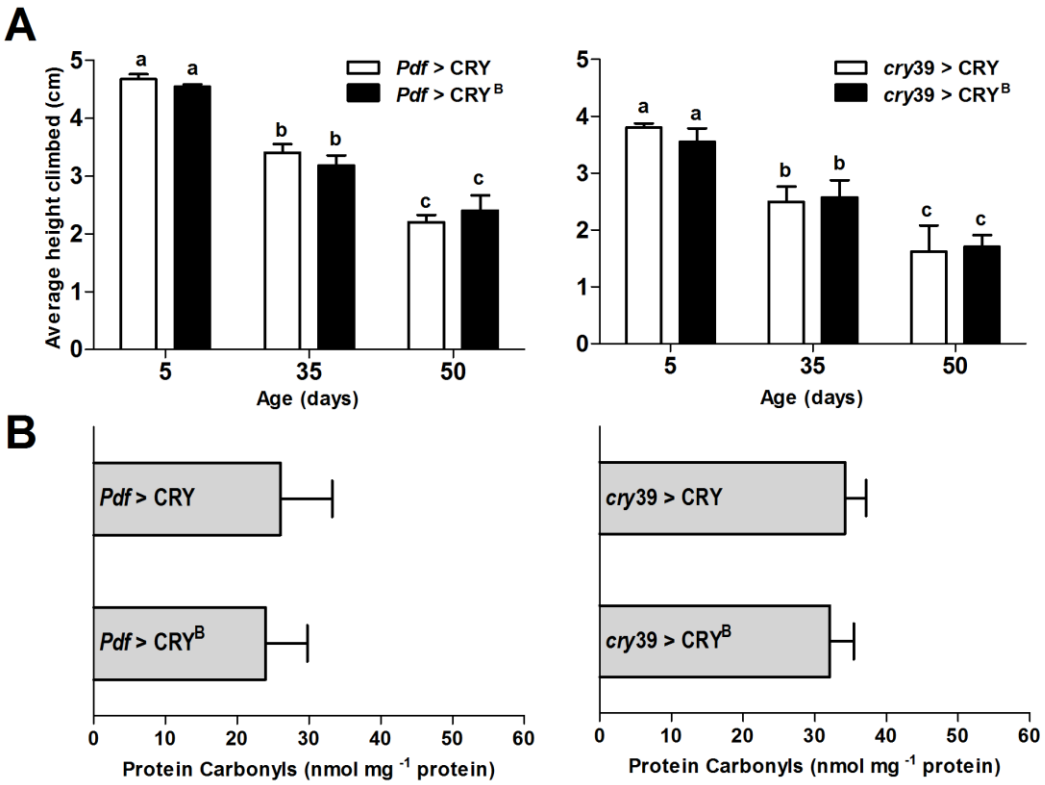


**Figure 4.6 CRY-restoration in central pacemaker neurons alone does not improve healthspan. A)** Vertical mobility was measured by the RING assay in 5, 35, and 50 day-old males of the indicated genotypes. Bars represent mean height climbed (± SEM), based on testing 4 vials per genotype, each containing 25 flies. Statistical significance was determined using two-way ANOVA with Bonferroni's post hoc test, and bars with different letters are significantly different at $p<0.05$. **B**) Oxidative damage in the form of protein carbonyls (PC) were measured in heads of 50 day-old males of the indicated genotypes at ZT 8. Bars represent mean carbonyl levels (± SEM), based on testing 3 independent sets of flies, each containing 75 flies in 3 technical repeats of 25 flies each. Statistical significance was determined using one-way ANOVA with Tukey's post hoc test.

eye-specific *gmr*GAL4 driver suppressed *tim* mRNA expression (Collins *et al.* 2006). However, this study also reported that overexpression of CRY alone significantly increased the amplitude of *tim* and *vri* in young flies (Collins *et al.* 2006), consistent with our current data in old flies. Further studies are required to determine whether replenishment of CRY in peripheral clock cells may enhance the transcriptional activity of CLK-CYC complexes in the heads. Although we show that neither aging nor CRY overexpression altered *Clk* and *cyc* mRNA levels, downstream post-translational mechanisms or even epigenetic modifications could be conceivably affected in old flies, and restored by CRY overexpression. In support of this notion, it was reported that CLK protein levels are constitutively low in heads of $cry^b$ flies (Collins *et al.* 2006). Further experiments would be required to obtain insights into the mode of action of CRY in maintaining molecular circadian rhythms. Interestingly, age-related decay of the circadian clock at molecular and behavioral levels was reported in mPer1 and mCry2 knockout mice (Oster *et al.* 2003).

Our study reveals that restoration of CRY in all clock expressing cells prevented the decay of locomotor activity rhythms with age. While rhythms in LD were slightly improved in *tim*>CRY flies, remarkable differences were observed in DD where 35 and 50 day-old flies with replenished CRY showed almost young-like rest/activity rhythms suggesting that the free-running circadian system was intact in those flies. We also found that augmentation of CRY in central clock neurons alone was not sufficient to restore rest/activity rhythms. While only central clocks are required for behavioral rhythms in young flies (Hardin 2011), our data suggest that peripheral clocks may be necessary for maintaining neuronal homeostasis and preventing the degradation of circadian output pathways in aging individuals. Interestingly, decay of clock output pathways despite strong mPer2 oscillations in the central clock (SCN) has been recently reported in mouse (Nakamura *et al.* 2012).

Our data show that CRY-mediated strengthening of circadian rhythms is associated with deceleration of functional decline normally observed in aging flies. Namely, CRY overexpressing flies maintained significantly higher climbing ability than

their controls at every age tested, suggesting that these flies had a slower rate of aging. Furthermore, these flies had significantly lower oxidative damage in old age. Consistent with these findings, they were also able to withstand and recover from homeostatic insults such as exposure to mild non-lethal hyperoxia. In contrast, *cry*-null flies showed accelerated functional decline and higher accumulation of oxidatively damaged proteins. Together, these results suggest that strong circadian rhythms are important for maintaining the health of an organism during aging, and also point toward novel anti-aging functions of CRY. While several studies demonstrated that genetic or environmental disruptions of the clock function accelerate physiological aging and age-related diseases, it is not clear whether rejuvenation of the circadian system can delay aging. Here, we achieved circadian rejuvenation by reversing the age-related CRY decline, and demonstrate that this treatment extends healthspan in flies. CRY supplementation and conversely, CRY deficiency at the same chronological age, shifted the readouts of aging in opposite directions. Namely, *tim*>CRY flies showed better climbing vigor and reduced oxidative damage than age-matched controls, while climbing was impaired even in the young *cry*-null flies and they accumulated greater oxidative damage during aging. Taken together, our data suggest that CRY-mediated improvement of circadian rhythms may delay functional aging. This could be linked to the enhanced expression of clock-controlled effector genes involved in several pathways maintaining temporal homeostasis (Wijnen & Young 2006; Krishnan *et al.* 2008). On the other hand, we cannot exclude that healthspan benefiting effects of CRY overexpression may be uncoupled from circadian rhythms and instead linked to its pleiotropic effects. Several novel non-circadian functions of CRY have been recently suggested in *Drosophila* (Fogle *et al.* 2011; Seay & Thummel 2011; Kumar *et al.* 2012). Intriguingly, recent *in vivo* analysis comparing the transcriptional activity of human and fly cryptochromes in transgenic *Drosophila* suggests that these proteins may share common signaling pathways in DD, regulating genes implicated in stress response (Vieira *et al.* 2012).

In summary, we demonstrate that age-related dampening of clock gene oscillations and daily activity rhythms can be significantly improved by the genetic

manipulation of a protein that is best known for its role in circadian photo-transduction. We provide evidence that overexpression of CRY helps slow down the aging process and reverse age-associated phenotypes. Our study suggests novel anti-aging role for the gene *cryptochrome* in *Drosophila,* as it has profound effects on the health and fitness most likely by improving clock-controlled effector genes. However, the mode of CRY action as an anti-aging factor will require future studies.

## 4.5 Experimental Procedures

### *4.5.1 Fly rearing*

*D. melanogaster* were reared on 1% agar, 6.25% cornmeal, 6.25% molasses, and 3.5% Red Star yeast at 25°C in 12 h light:12 h dark (LD) cycles (with an average light intensity of ~2000 lux). By convention, lights-off is denoted as Zeitgeber time (ZT) 12. For experiments on aging flies, cohorts of 50-75 mated males, were housed in 8 oz round bottom polypropylene bottles (Genesee Scientific, San Diego, CA) inverted over 60 mm Falcon Primaria Tissue culture dishes (Becton Dickinson Labware, Franklin Lakes, NJ) containing 15 mL of diet. Diet dishes were replaced daily without $CO_2$, after tapping flies to the bottom of the bottle. For other assays, cohorts of 25 mated males were reared in narrow vials (Genesee Scientific).

### *4.5.2 Fly Stocks*

Canton S mated males were used for RNA and protein measurements of *cry* during aging. For the *cry* overexpression studies, $w^{1118}$ served as the wild type control to which driver and responder lines were backcrossed for 8 generations. Driver lines $w^{1118}$;+;BsR-B (*Pdf*GAL4) (Park *et al.* 2000), *w*;*cry*Gal4$^{\#39}$;+ (*cry39*GAL4) (Klarsfeld *et al.* 2004), *w*;*tim*Gal4(62);+ (*tim*GAL4) (Kaneko & Hall 2000) were crossed to either responder line $w^{1118}$;UAS-*cry*24;+ (UAS-*cry*) (Emery *et al.* 1998), or $w^{1118}$;$w^{+}$ UAS$cry^{b}$31;+ (UAS-$cry^{b}$) (Emery *et al.* 2000) as a control. Additionally, $cry^{01}$ and $cry^{02}$ mutants (Dolezelova *et al.* 2007) were backcrossed to $w^{1118}$ and used in some experiments.

*4.5.3 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)*

Three independent bio-replicates of flies were collected at 4 h intervals around the clock on days 5, 35, and 50. Total RNA was extracted from fly heads and bodies separately using TriReagent (Sigma, St. Louis, MO). The samples were purified and treated with Takara Recombinant DNase I (Clontech Laboratories Inc., Mountain View, CA). Synthesis of cDNA was achieved with the iScript cDNA synthesis kit (BioRad, Hercules, CA). qRT-PCR was performed on the StepOnePlus Real-Time machine (Applied Biosystems, Carlsbad, CA) under default thermal cycling conditions, with a dissociation curve step. Every reaction contained Power SYBR Green (Applied Biosystems), 10 ng cDNA, and 400 nM primers. Primer sequences are available upon request. Data were analyzed using the $2^{-\Delta\Delta CT}$ method with mRNA levels normalized to the gene *rp49*. Relative mRNA amplitude was calculated with respect to the trough levels on day 5 set as 1 (for aging study) or trough levels of *tim*GAL4/+ (control) set as 1 on day 50 (for *cry* overexpression studies).

*4.5.4 Western Blotting*

Three independent bioreplicates of 5 and 50 day-old males of different genotypes were collected at specific ZT points. About 5-10 fly heads/time point were homogenized on ice in Laemmli buffer, sonicated, boiled at 100°C for 5 min, and centrifuged at 12000 g at 4°C. A constant ratio of the buffer (7 μL/head) was used to ensure equal protein loading and separation on 10% acrylamide gel. Proteins were transferred to the 0.45 μm polyvinylidene fluoride (PVDF) Immobilon-FL membrane (Millipore Billerica, MA) and incubated in 1X TBST (10mM Tris, 0.15M NaCL, 0.1% Tween-20, pH 7.5) + 5% milk for 2 h, then overnight at 4°C with 1:2,000 anti-CRY (Rush *et al.* 2006) in blocking buffer. Membranes were treated for 2 h with 1:20,000 goat anti-rabbit IRDye680 (LI-COR Biosciences, Lincoln, NE). Proteins were quantified using the LI-COR Odyssey Infrared Imaging System software (v. 3.0).

*4.5.5 Locomotor Activity Analysis*

Flies were entrained in LD 12:12 at 25°C. Locomotor activity of 5, 35, and 50 day-old males was recorded or 3 d in LD 12:12, followed by 7 d in constant darkness (DD) using the Trikinetics locomotor activity monitor (Waltham, MA). For a quantitative measure of circadian rhythmicity in DD, Fast Fourier Transform (FFT) analysis was conducted using ClockLab software (Actimetrics; Coulbourn Instruments, Whitehall, PA). Flies with FFT values <0.04 were classified as arrhythmic, ones with values of 0.04-0.08 were classified as weakly rhythmic, whereas flies with FFT values >0.08 were considered strongly rhythmic. Flies with both weak and strong rhythms that showed a single peak in the periodogram were included in the calculation of the free running period using the ClockLab software (Actimetrics, Wilmette, IL).

*4.5.6 Rapid iterative negative geotaxis (RING) assay*

Vertical mobility was tested using the RING assay as described (Rhodenizer *et al.* 2008; Krishnan *et al.* 2012). Briefly, 4 groups of 25 mated males of each age and genotype were transferred into empty vials without anesthesia, and the vials were loaded into the RING apparatus. The apparatus was rapped three times in rapid succession to initiate a negative geotaxis response. The flies' movements in tubes were videotaped and digital images captured 4 s after initiating the behavior. The climbed distance was calculated for each fly and expressed as average height climbed in the 4 s interval. The performance of flies in a single vial was calculated as the average of 5 consecutive trials (interspersed with a 30 s rest).

*4.5.7 Protein Carbonyl (PC) Assay*

To assess oxidative damage, protein carbonyls were measured in head homogenates of 50 day-old males of various genotypes at 370 nm after reaction with 2,4-Dinitrophenylhydrazine (DNPH), using a Synergy 2 plate reader (BioTek, Winooski, VT), as described previously (Krishnan *et al.* 2008). Results were expressed as nmol $mg^{-1}$ protein using an extinction coefficient of 22,000 $M^{-1}$ $cm^{-1}$.

*4.5.8 Hyperoxia treatment and lifespan analysis*

For hyperoxia exposure, 4 cohorts of 25 males in narrow vials with diet were placed in a Plexiglass chamber filled with oxygen (100% medical grade) flowing at a constant rate (300ml/min) for 24 h, as described (Krishnan *et al.* 2009). Control flies were transferred to narrow vials with diet and kept under normoxia. After the treatment, flies were transferred from narrow vials to bottles as described in the fly rearing section. Diet was replaced on alternate days without anesthesia, and mortality was recorded daily.

*4.5.9 Statistical Analysis*

Data were statistically analyzed with GraphPad Prism (v.5.0) and GraphPad Instat (v.3.0; San Diego, CA). The qRT-PCR, locomotor activity analysis and RING data were evaluated by two-way analysis of variance (ANOVA) with Bonferroni's post hoc test. PC data were evaluated by one-way ANOVA with Tukey's post hoc test. For Western data, the relative strength of the signals was quantified using LI-COR Image analysis software (v.3.0) and subjected to two-way ANOVA with Bonferroni's post hoc test. Life span and survival curves were plotted following Kaplan Meier survival analysis and statistical significance of curves assessed using the Log-Rank (Mantel-Cox) test.

## 4.6 Acknowledgements

We are grateful to Eileen Chow for help with fly rearing. We thank Patrick Emery for anti-CRY antibody and fly stocks. Other fly lines were kindly shared by Paul Hardin, Paul Taghert, and Jeff Hall. This research was supported by NIH R21 AG038989 and R21 NS075500 grants to JMG. KR is supported by NSF IGERT in Aging Sciences Fellowship at Oregon State University (DGE 0965820).

## 4.7 Author Contributions

KR and JMG designed the study. KR conducted all the experiments and wrote the manuscript. JMG provided critical feedback and revised the first draft. The authors declare no conflict of interest.

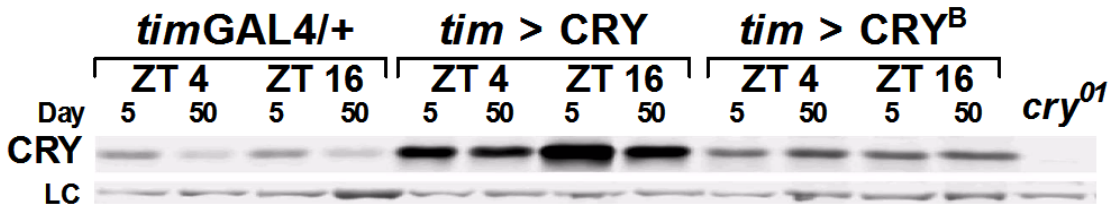


**Figure S4.1** Western blot showing CRY protein levels in head extracts of 5 and 50 day-old *tim*>CRY flies and controls at ZT 4 and 16. LC represents the loading control. CRY levels were lower in the morning (ZT4) than at night (ZT16) in both young and old *tim*>CRY flies, suggesting light sensitivity of ectopic CRY.

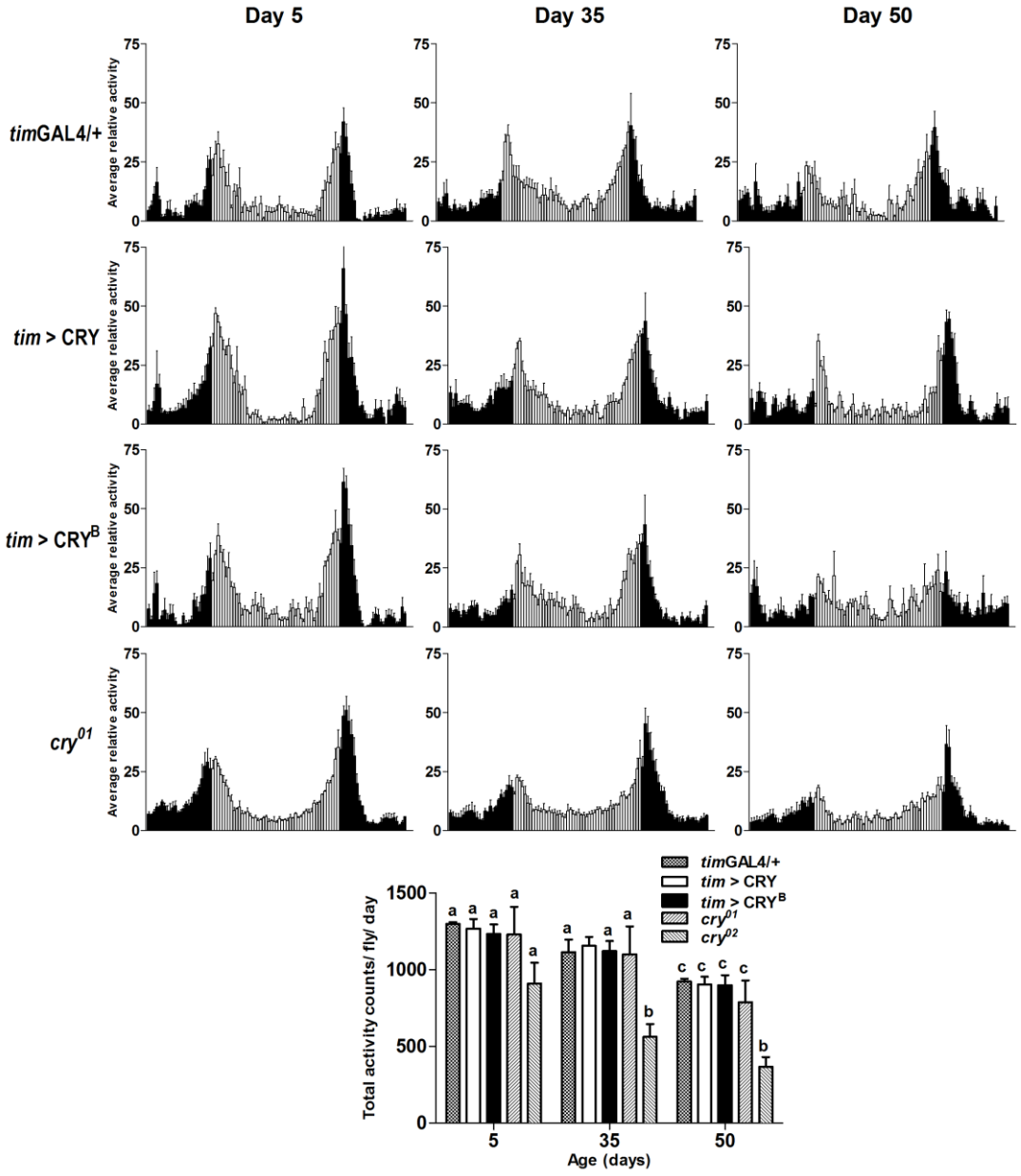


**Figure S4.2** Average daily activity profiles of 5, 35, and 50 day-old males of the indicated genotypes in LD 12:12. Each bar represents daily activity counts in a 15 min bin averaged for 3 days. White and black bars represent period of light and dark respectively. Bar graph (below) indicates total activity counts per fly per day. Statistical significance was determined using two-way ANOVA with Bonferroni's post hoc test, and bars with different letters are significantly different at $p<0.05$.

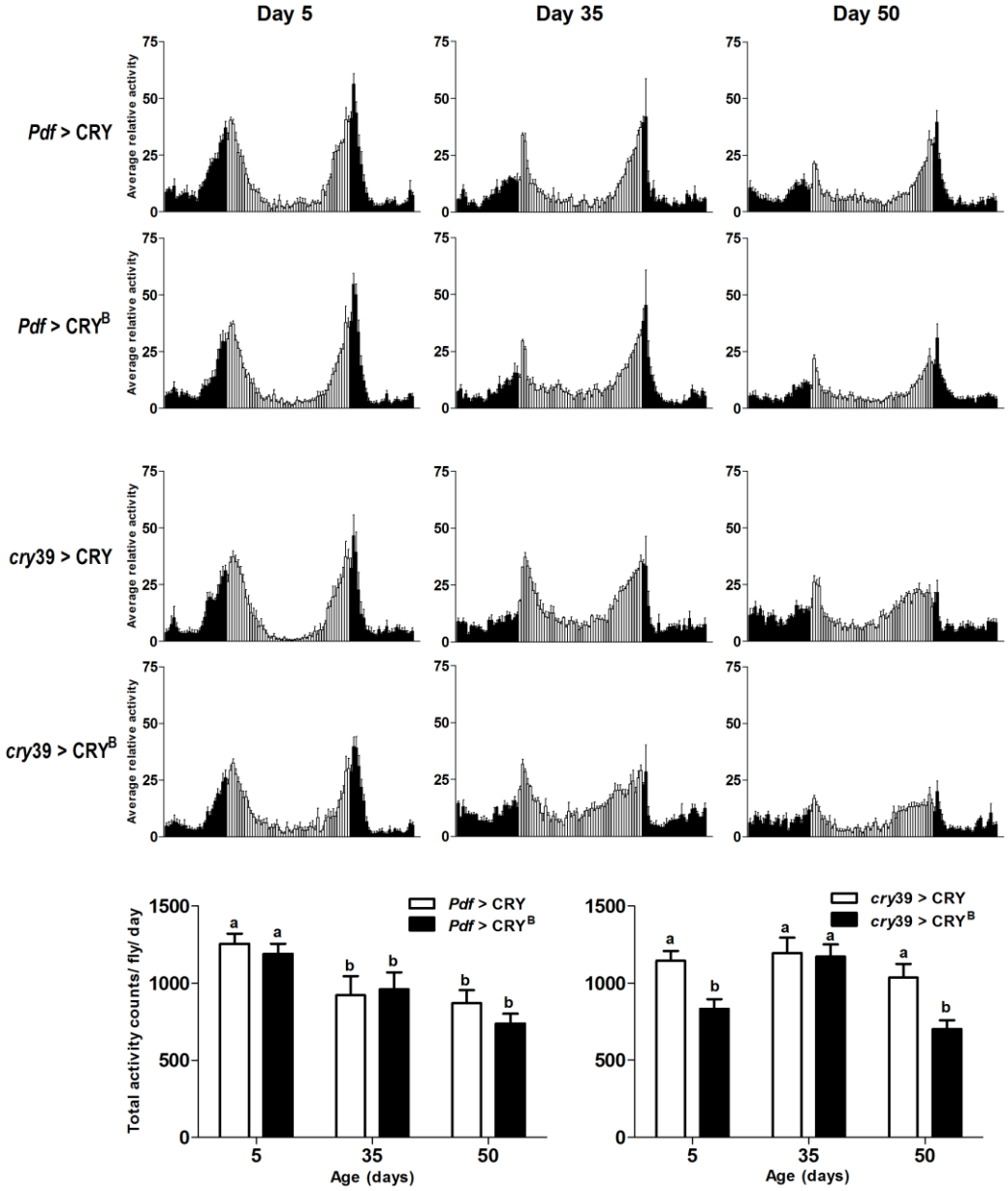


**Figure S4.3** Average daily activity profiles of 5, 35, and 50 day-old males of the indicated genotypes in LD 12:12. Each bar represents daily activity counts in a 15 min bin averaged for 3 days. White and black bars represent period of light and dark respectively. Bar graphs (below) indicate total activity counts per fly per day. Statistical significance was determined using two-way ANOVA with Bonferroni's post hoc test, and bars with different letters are significantly different at $p<0.05$.

**Table S4.1** Statistical analysis of gene expression (qRT-PCR) and protein (Western Blotting) data by two-way ANOVA with Bonferroni's post-hoc test

| | **Effect of ZT** | | **Effect of age** | |
|---|---|---|---|---|
| *cry* (Heads) | $F_{6,42} = 11.26$ | $p < 0.0001$ | $F_{2,42} = 81.64$ | $p < 0.0001$ |
| *cry* (Bodies) | $F_{6,42} = 54.15$ | $p < 0.0001$ | $F_{2,42} = 0.40$ | $p = 0.6729$ |
| CRY (Heads) | $F_{5,24} = 43.29$ | $p < 0.0001$ | $F_{1,24} = 145.16$ | $p < 0.0001$ |

Subscripted values indicate the degrees of freedom in numerator ($DF_n$) and denominator ($DF_d$), respectively.

**Table S4.2** Statistical analysis of gene expression (qRT-PCR) data in heads of 50 day-old flies by two-way ANOVA with Bonferroni's post-hoc test

| **Gene** | **Effect of ZT** | | **Effect of genotype** | |
|---|---|---|---|---|
| *per* | $F_{6,42} = 246.40$ | $p < 0.0001$ | $F_{2,42} = 39.16$ | $p < 0.0001$ |
| *tim* | $F_{6,42} = 83.13$ | $p < 0.0001$ | $F_{2,42} = 9.00$ | $p < 0.001$ |
| *Pdp1ε* | $F_{6,42} = 481.95$ | $p < 0.0001$ | $F_{2,42} = 16.68$ | $p < 0.0001$ |
| *vri* | $F_{6,42} = 234.22$ | $p < 0.0001$ | $F_{2,42} = 11.95$ | $p < 0.0001$ |
| *Clk* | $F_{6,42} = 82.11$ | $p < 0.0001$ | $F_{2,42} = 3.65$ | $p = 0.0544$ |
| *cyc* | $F_{6,42} = 1.52$ | $p = 0.1953$ | $F_{2,42} = 2.73$ | $p = 0.0768$ |

Subscripted values indicate the degrees of freedom in numerator ($DF_n$) and denominator ($DF_d$), respectively.

**Table S4.3** Locomotor activity analysis of flies overexpressing CRY

| Genotype | Age (Days) | n | % Rhythmic (Strong + Weak)* | Rhythm Strength (Avg. FFT) | Period (DD) | Avg. Daily Activity (LD) |
|---|---|---|---|---|---|---|
| *tim*GAL4/+ | 5 | 13 | 92% (69% + 23%) | 0.09 ± 0.01 | 23.5 ± 0.1 | 1300 ± 12 |
| | 35 | 21 | 52% (10% + 42%) | 0.05 ± 0.01 | 23.6 ± 0.1 | 1116 ± 82 |
| | 50 | 9 | 33% (11% + 22%) | 0.04 ± 0.01 | 24.0 ± 0.4 | 924 ± 18 |
| *tim* > CRY | 5 | 36 | 100% (92% + 8%) | 0.12 ± 0.01 | 23.9 ± 0.1 | 1269 ± 63 |
| | 35 | 41 | 85% (51% + 34%) | 0.08 ± 0.01 | 24.2 ± 0.1 | 1057 ± 59 |
| | 50 | 33 | 82% (36% + 45%) | 0.07 ± 0.01 | 24.6 ± 0.1 | 950 ± 50 |
| *tim* > CRY$^{B}$ | 5 | 37 | 97% (62% + 35%) | 0.09 ± 0.01 | 24.1 ± 0.1 | 1233 ± 62 |
| | 35 | 43 | 51% (15% + 40%) | 0.05 ± 0.00 | 24.1 ± 0.1 | 1023 ± 65 |
| | 50 | 27 | 26% (11% + 15%) | 0.04 ± 0.01 | 24.9 ± 0.3 | 899 ± 67 |
| *cry*$^{01}$ | 5 | 48 | 94% (77% + 17%) | 0.12 ± 0.01 | 23.5 ± 0.0 | 1231 ± 180 |
| | 35 | 38 | 58% (29% + 29%) | 0.05 ± 0.01 | 23.9 ± 0.1 | 1102 ± 181 |
| | 50 | 32 | 32% (13% + 19%) | 0.05 ± 0.01 | 24.3 ± 0.1 | 789 ± 142 |
| *cry*$^{02}$ | 5 | 47 | 83% (57% + 26%) | 0.08 ± 0.01 | 23.5 ± 0.0 | 911 ± 134 |
| | 35 | 46 | 54% (15% + 39%) | 0.05 ± 0.00 | 23.7 ± 0.0 | 563 ± 84 |
| | 50 | 35 | 40% (17% + 23%) | 0.05 ± 0.01 | 23.9 ± 0.1 | 368 ± 63 |
| *Pdf* > CRY | 5 | 45 | 96% (80% + 16%) | 0.11 ± 0.01 | 23.7 ± 0.0 | 1256 ± 66 |
| | 35 | 21 | 71% (48% + 24%) | 0.08 ± 0.01 | 23.7 ± 0.1 | 922 ± 123 |
| | 50 | 35 | 47% (25% + 22%) | 0.06 ± 0.01 | 24.0 ± 0.1 | 871 ± 84 |
| *Pdf* > CRY$^{B}$ | 5 | 47 | 91% (85% + 6%) | 0.10 ± 0.01 | 23.6 ± 0.0 | 1190 ± 66 |
| | 35 | 21 | 67% (29% + 38%) | 0.08 ± 0.00 | 23.4 ± 0.0 | 961 ± 109 |
| | 50 | 33 | 45% (24% + 21%) | 0.06 ± 0.01 | 24.1 ± 0.1 | 738 ± 64 |
| *cry*39 > CRY | 5 | 44 | 91% (64% + 27%) | 0.09 ± 0.01 | 23.6 ± 0.0 | 1146 ± 63 |
| | 35 | 22 | 54% (23% + 31%) | 0.06 ± 0.01 | 23.5 ± 0.1 | 1195 ± 99 |
| | 50 | 36 | 17% (11% + 6%) | 0.04 ± 0.00 | 24.4 ± 0.2 | 1037 ± 87 |
| *cry*39 > CRY$^{B}$ | 5 | 40 | 93% (73% + 20%) | 0.11 ± 0.01 | 24.0 ± 0.1 | 833 ± 62 |
| | 35 | 18 | 49% (11% + 38%) | 0.05 ± 0.01 | 23.5 ± 0.1 | 1173 ± 79 |
| | 50 | 36 | 14% (0% + 14%) | 0.03 ± 0.00 | 23.9 ± 0.2 | 703 ± 57 |

*Flies with FFT values >0.08 were considered strongly rhythmic, 0.04-0.08 as weakly rhythmic, while <0.04 were classified as arrhythmic.

**Table S4.4** Statistical analysis of Rapid Iterative Negative Geotaxis (RING) data by two-way ANOVA with Bonferroni's post-hoc test

| Genotype | Effect of age | | Effect of genotype | |
|---|---|---|---|---|
| *tim*GAL4/+<br>*tim* > CRY<br>*tim* > CRY$^{B}$<br>*cry*$^{01}$<br>*cry*$^{02}$ | $F_{2,45} = 142.86$ | $p < 0.0001$ | $F_{4,45} = 29.17$ | $p < 0.0001$ |
| *Pdf* > CRY<br>*Pdf* > CRY$^{B}$ | $F_{2,18} = 110.84$ | $p < 0.0001$ | $F_{2,18} = 0.16$ | $p = 0.6974$ |
| *cry*39 > CRY<br>*cry*39 > CRY$^{B}$ | $F_{2,18} = 25.50$ | $p < 0.0001$ | $F_{2,18} = 0.01$ | $p = 0.9051$ |

Subscripted values indicate the degrees of freedom in numerator ($DF_n$) and denominator ($DF_d$), respectively.

**Table S4.5** Median lifespan (days) is shown for indicated genotypes with n=sample size. Statistical analysis was conducted using Log Rank (Mantel-Cox) test. Values with different superscripts are significantly different at $p<0.05$.

| Genotype | Treatment | n | Median lifespan (days) |
|---|---|---|---|
| *tim*GAL4/+ | Normoxia | 77 | 70.0$^{a}$ |
| *tim*GAL4/+ | D50 Hyperoxia | 77 | 64.5$^{b}$ |
| *tim* > CRY | Normoxia | 84 | 75.0$^{a}$ |
| *tim* > CRY | D50 Hyperoxia | 72 | 75.0$^{a}$ |
| *tim* > CRY$^{B}$ | Normoxia | 82 | 71.0$^{a}$ |
| *tim* > CRY$^{B}$ | D50 Hyperoxia | 86 | 63.0$^{b}$ |

**Exploring functional links between circadian clocks, neurodegeneration, and aging in *Drosophila melanogaster***

# CHAPTER 5

## General Discussion and Conclusions

## 5.1 General conclusions

An intrinsic circadian system helps organisms adapt to the earth's day/night and temperature cycles by providing temporal dimension to their behavioral, physiological, and molecular processes. It is believed that this temporal homeostasis of life functions contributes to the organism's health and fitness; however, the prevalent evidence is of correlative nature. Multiple studies in humans and animal models suggest that aging alters the circadian system resulting in the decay of clock controlled processes, and eventually contributing to age-related pathologies including cognitive decline and neurodegenerative disorders (Hofman & Swaab 2006; Kondratov & Antoch 2007; Kondratova & Kondratov 2012). While several phenotypes of aging do not directly affect lifespan, they negatively affect healthspan, the duration of an organism's lifespan during which it is healthy and free from any disease. Aging is a complex process involving detrimental cellular changes and decay in the several physiological systems. While aging cannot be avoided, the rate of aging can be manipulated in model organisms, showing promise that biological factors promoting healthy aging would be better understood.

The overall goal of this research was to investigate functional links between the circadian system, neurodegeneration, and aging using *Drosophila* as a model system. To that end, it was demonstrated that both genetic and environmental disruption of circadian rhythms accelerated aging and shortened lifespan in two fly mutants that displayed neurodegenerative phenotypes independently (Chapter 2). This study suggests that circadian clocks are involved in neuro-protective pathways which may curb damage to the nervous system during aging. Previous research indicates that aging negatively affects the nervous system, and disruption of circadian rhythms during aging could be one of the potential mechanisms for neurodegeneration. Therefore, the effects of aging on the circadian system were studied at both behavioral and molecular levels, and it was demonstrated that aging affects both the negative and positive limbs of the circadian clock (Chapter 3). Aging not only weakened the rest/activity rhythms, daily oscillations of several core clock genes and proteins were also dampened tissue-specifically. Weakened behavioral and molecular circadian rhythms could be restored in old fruit flies

by using genetic tools to overexpress the clock protein CRY, better known for its role as a photoreceptor (Chapter 4). While it is still not clear whether restoration of circadian rhythms is due to supra-physiological elevation of CRY protein, or its pleiotropic clock-independent effects, old flies with improved circadian rhythms had better healthspan, fitness and resilience to short-term stress (Chapter 4). These data suggest that a strong circadian system at both behavioral and molecular levels supports the health of an organism during aging.

Taken together, novel findings of this dissertation research are a first attempt at answering the cause or effect paradox. While aging affects different physiological systems including the circadian system, clocks are equally important in delaying the process of aging in an organism, particularly that restoring dampened clocks improved the health and longevity of aged flies. The circadian system possibly delays the aging process by eliminating cellular ROS, lowering oxidative stress-induced damage to biomolecules, and preventing neurodegeneration. This may be achieved via clock controlled output pathways, or even clock-unrelated pleiotropic effects of certain clock proteins that are as yet unknown. It is likely that a combination of several processes interacting at the environmental, physiological, genetic, and even epigenetic levels together support healthspan and healthy aging. Further studies are required to explore these hypotheses.

### 5.2 Future directions

This dissertation provides several mechanistic insights into the relationships between circadian clocks, neurodegeneration, and aging, yet there are many unanswered questions that require further investigation. It was demonstrated that disruption of circadian rhythms by addition of *per* mutation led to functional decline, lifespan shortening, and increased brain damage in $sni^{1}$ mutants (Chapter 2). Some of these phenotypes were also observed when $per^{01}$ was combined with another neurodegeneration-prone $sws^{1}$ mutant, suggesting that effects of *per* mutation are indirect and additive. As a next step, it would be interesting to test if rescuing *per* in the $per^{01}$ $sni^{1}$ or $per^{01}$ $sws^{1}$ double mutant

backgrounds can partially restore brain damage and the functional decline. To obtain further insights into the mechanisms of *per*-mediated neuroprotective effects, PER can be overexpressed by the binary GAL4/UAS system using driver lines specific to all clock expressing cells, eyes, nervous system, glia, or specific clock neurons to test if age-related neurodegeneration can be delayed. PER can also be tissue-specifically overexpressed in a $sni^{1}$ or $sws^{1}$ background to reduce neurodegenerative phenotypes of these mutant flies that otherwise show pronounced brain damage even at younger ages. To further investigate if the neuroprotective effects of *per* are pleiotropic rather than its role in the circadian clock, it would be important to tests age-related neurodegeneration in transgenic fly lines that are defective in forming the PER-PER complexes (Landskron *et al.* 2009). It was recently reported that PER can exist as homodimers in addition to the known heterodimer PER-TIM complexes (Landskron *et al.* 2009), suggesting pleiotropic roles of PER that aim to explain why certain phenotypes are stronger in $per^{01}$ flies compared to other clock gene mutants.

If prevention of brain damage with age is mediated by strong circadian rhythms, then flies having stronger behavioral and molecular circadian rhythms would be predicted to have significantly lower neurodegeneration compared to age-matched controls. One approach to restoring the circadian system in middle-aged and old flies is the overexpression of CRY by the constitutive *tim*GAL4 driver (Chapter 4). It would be interesting to test if those flies have significantly lower brain damage than controls at similar age. If true, then it would suggest that supporting the health of the nervous system is one of the roles of circadian clocks in extending healthspan. These results can be translated by restoring circadian rhythms in mammalian models of neurodegenerative diseases using therapeutic treatments such as melatonin, lipoic acid, or light therapy, to limit disease progression. It would be equally fascinating to investigate if drugs such as minocycline used for the treatment of oxidative stress-induced neurodegeneration (Bonilla *et al.* 2006), function by improving the rhythms of sleep/wake or other physiological processes.

Aging alters the properties of the core transcriptional clock in flies such that both the positive and the negative limbs of the clock are attenuated. The mRNA oscillatory amplitude of clock genes *per*, *tim*, *Pdp1ε*, and *vri*, are severely dampened in heads of old flies, while *Clk* and *cyc* remain unchanged (Chapter 3). PER and TIM proteins also decline significantly, eliminating the constant repression of CLK-CYC transcriptional activation as a potential cause of low mRNA levels. There are several other hypotheses for low levels of these clock gene transcripts in heads of old flies.

One of the prime candidates for further mechanistic investigation is the status of CLK protein. While *Clk* mRNA levels were unchanged, CLK protein levels could still be reduced owing to defects in the translational machinery, thereby making it rate-limiting. Further, post-translational modifications such as the phosphorylation and ubiquitylation status of CLK greatly determines its stability and the ability to activate transcription of downstream genes (Yu *et al.* 2006; Luo *et al.* 2012b). While hypo-phosphorylated CLK is stable, hyper-phosphorylated CLK is unstable and unable to bind to DNA (Yu *et al.* 2006). It was also reported recently that deubiquitylation of CLK by a ubiquitin-specific protease 8 (USP8) greatly inhibits CLK-CYC transcription in *Drosophila* (Luo *et al.* 2012b). Technical limitations prevented the investigation of CLK protein levels and its phosphorylation status in the heads of old flies.

Future investigation can also be directed toward binding of CLK-CYC complexes to upstream promoter regions of target genes at different ages using chromatin immunoprecipitation (Ch-IP) studies. This binding in young flies is followed by several events including epigenetic chromatin modifications like $H_3K_4$ tri-methylation and $H_3K_9$ acetylation (Taylor & Hardin 2008). While RNA Polymerase II (Pol II) is constitutively bound to DNA, rhythmic phosphorylation of Ser-5 residue at the C-terminal domain results in transcriptional activation (Taylor & Hardin 2008). Any of these processes may be affected with age leading to the parallel dampening of mRNA oscillations of CLK-CYC controlled genes.

Post-transcriptional mRNA processing in eukaryotes includes three events namely 5’ capping, removal of introns by splicing, and 3’ cleavage of RNA followed by

polyadenylation (the addition of a poly(A) tail). Upon translocation into the cytoplasm, some mRNAs are immediately translated into proteins, some undergo deadenylation and degradation, while others are stored in cytoplasmic processing bodies or stress granules until they are degraded or re-polyadenylated (Kojima *et al.* 2011). Temporal coordination of mRNA stability is mediated by *cis*-elements commonly found in 5' or 3' untranslated regions (UTRs) that serve as specific binding sites for *trans*-acting factors such as RNA-binding proteins (RBPs) or microRNAs (miRNAs) (Kojima *et al.* 2011). The composition and timing of such *trans*-acting factors greatly determine the fate of the mRNA. Rhythmic mRNA expression of *per* has been attributed to post-transcriptional regulation in heads of young *Drosophila*, where *per* mRNA was more stable during the rising phase of the mRNA curve (ZT 9 - 15), and less stable during the declining phase (after ZT 15) (So & Rosbash 1997). While the initiation of cyclic transcription during the day appears to be unaffected in heads of middle-aged and old flies, there is significant reduction in peak mRNA levels (Chapter 3). An age-related change in mRNA stability of clock genes caused by the deadenylation and early degradation (during ZT 8-12) of the nascent mRNA may explain the significantly low mRNA accumulation at peak (ZT 16).

It has been recently reported that most introns from nascent mRNA are efficiently spliced co-transcriptionally in heads of young flies (Khodor *et al.* 2011). Another mechanistic hypothesis behind the reduction of clock gene mRNA amplitude could be a change in the efficiency of co-transcriptional splicing with age, resulting in degradation of nascent mRNA. The rate of elongation by RNA Polymerase II also plays an important role in determining the efficiency of co-transcriptional splicing (Khodor *et al.* 2011). Dissociation of pol II from DNA during the middle of the transcriptional cycle in old flies could result in premature termination of transcription as well as early dissociation of the spliceosome complex leading to the degradation of newly formed mRNA.

A recent parallel comparison of Nascent-Seq and RNA-Seq datasets of circadian clock genes in heads of young wild type *Drosophila* indicates substantial transcriptional regulation as well as a notable post-transcriptional contribution to the amplitude of rhythmic mRNA cycling (Rodriguez *et al.* 2013). The daily amplitude of mRNA cycling

of *per*, *tim*, *vri*, and *cry* genes were significantly higher than the oscillatory amplitude of the respective nascent mRNAs, indicating significant post-transcriptional contribution to mRNA cycling (Rodriguez *et al.* 2013). Therefore, any defects in the processing of nascent mRNA may explain their lower oscillatory amplitude with age.

Age-related changes in the electrophysiological properties of central clock neurons could lead to potential desynchrony of different oscillator cells. While PER was reported to be unchanged (Luo *et al.* 2012a), levels of TIM and the principal fly neurotransmitter Pigment-dispersing factor (PDF) were severely attenuated with age in the central clock neurons (Umezaki *et al.* 2012). Despite strong cycling of clock proteins in the fruit fly pacemaker neurons, there can be age-related defects in the neuronal firing rates or synaptic transmission, desynchronizing the central clock with peripheral clocks in different tissues.

A novel discovery in Chapter 4 is that overexpression of CRY by the ubiquitous *tim*GAL4 driver, but not pacemaker neuron-specific *Pdf*GAL4 and *cry39*GAL4 drivers, improved circadian rhythms and healthspan of middle-aged and old flies. An extension of this study could involve restoration of CRY using tissue-specific drivers (eyes, glia, etc.) to determine which tissues are involved in aging and healthspan. Also, CRY can be conditionally overexpressed in middle-aged and old flies using the RU gene switch to determine the specific physiological age when CRY demonstrates its anti-aging function. To determine whether anti-aging effects of CRY are mediated via the circadian clock, CRY can be overexpressed in arrhythmic flies (with a $per^{01}$ background) to test if they still show increased vertical mobility and resilience to oxidative stress. It may also be interesting to test the effects of CRY overexpression on healthspan in constant light (LL) and constant darkness (DD), and also whether old flies with elevated CRY have greater ability for entrainment to shifted LD cycles. This is particularly important because CRY is known to function as a photoreceptor and yet, CRY restoration improves behavioral activity rhythms in DD. CRY may also mediate increased activity of CLK-CYC complexes by enhancing protein-protein interactions or recruiting novel factors to

enhance CLK-CYC stability. Although highly speculative, this hypothesis may be tested using the co-IP technique.

Several other alternative approaches can be used to further determine relationships between the strength of clock gene oscillations, longevity, and healthspan. These may include testing circadian rhythms in long-lived fly mutants, and flies whose lifespan in increased by dietary restriction.

### 5.3 Significance

Human lifespan has dramatically increased over the past several years; however, increasing evidence suggests that lifespan may be uncoupled from healthspan, the fit and healthy period of one's lifespan. Age-related health issues deeply impact both individuals and society in the USA and increased lifespan has created a new level of challenges for biomedical sciences. There is an urgent need to better understand factors supporting healthy aging so as to limit the progression of age-related diseases and improve healthspan.

The incidences of neurodegenerative diseases and pathologies of the aging brain are on the rise and attract significant attention. Identification of the molecular pathways associated with these pathologies is essential for their prevention and treatment. With increasing evidence, the circadian system is being strongly linked to the control of aging, and age-related pathologies including neurodegenerative diseases. On one hand, aging severely affects several biological systems including the circadian and nervous systems at behavioral, physiological, and cellular levels. On the other hand, a weak circadian system also makes an organism prone to premature aging and neurodegeneration.

There is emerging evidence that aging-related pathologies could be mitigated in humans by therapies directed toward enhancement of the circadian clock (Bhattacharjee 2007). A fundamental understanding of the reciprocal links between the circadian system and aging must be achieved in order to design effective therapies. Research accomplished for this dissertation provides several lines of novel results toward achieving this goal. This dissertation not only supports neuroprotective effects of circadian clocks, it also

provides a comprehensive picture of aging of the behavioral and molecular circadian system in a single model organism. It additionally highlights the importance of strong peripheral clocks in promoting healthy aging, as opposed to the central pacemaker clock that has been traditionally known to orchestrate daily rhythms. Some clock genes and proteins may also have pleiotropic effects that are independent of the circadian clock function and as yet unknown.

Basic research conducted in a short-lived model, as reported in this dissertation, provides significant insights into the molecular mechanisms by which circadian clocks retard aging and neurodegeneration in fruit flies, and are likely to illuminate respective mechanisms in humans. A novel direction for the diagnosis and treatment of neurodegenerative and other age-related disorders could be targeting the circadian system especially the peripheral clocks.

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