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Aging and the Clock: Perspective from Flies to Humans

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Abstract

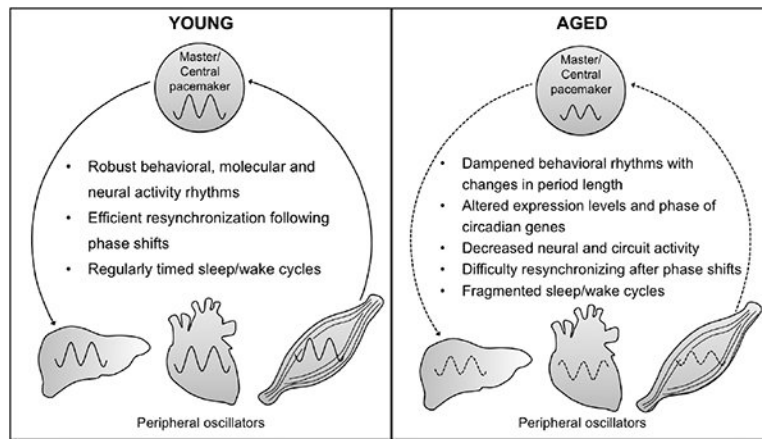
Endogenous circadian oscillators regulate molecular, cellular and physiological rhythms, synchronizing tissues and organ function to coordinate activity and metabolism with environmental cycles. The technological nature of modern society with round-the-clock work schedules and heavy reliance on personal electronics has precipitated a striking increase in the incidence of circadian and sleep disorders. Circadian dysfunction contributes to an increased risk for many diseases and appears to have adverse effects on aging and longevity in animal models. From invertebrate organisms to humans, the function and synchronization of the circadian system weakens with age aggravating age-related disorders and pathologies. In this review, we highlight the impacts of circadian dysfunction on aging and longevity and the reciprocal effects of aging on circadian function with examples from *Drosophila* to humans underscoring the highly conserved nature of these interactions. Additionally, we review the potential for using reinforcement of the circadian system to promote healthy aging and mitigate age-related pathologies. Advancements in medicine and public health have significantly increased human lifespan in the past century. With the demographics of countries worldwide shifting to an older population, there is a critical need to understand the factors that shape healthy aging. *Drosophila melanogaster*, as a model for aging and circadian interactions, has the capacity to facilitate the rapid advancement of research in this area and provide mechanistic insights for targeted investigations in mammals.

Graphical abstract

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Increasing longevity necessitates understanding the factors that negatively impact healthy aging. The circadian clock weakens with age and circadian dysfunction exacerbates age-related pathologies. In this review, we highlight *Drosophila* as a valuable model for studying circadian-aging interactions, examine the interplay between the circadian system and aging with examples from *Drosophila* to humans, and review studies in which reinforcement of the circadian system promotes healthier aging.

Keywords

Drosophila; circadian; rhythms; biological clock

Scope of Problem

The Aging Population

Over the past century, improvements in public health and scientific advances have significantly increased human longevity in countries around the world. In the United States, the average life expectancy for a baby born at the start of the 20th century was 47.3 years compared to recent projections of life expectancy for a child born in 2014 at 78.9 years (Crimmins, 2015; Arias *et al.*, 2017). Globally, increases in lifespan are predicted to continue rising with life expectancy anticipated to be greater than 85 years for individuals in many countries by 2030 (Kontis *et al.*, 2017). Consequently, the proportion of the adult population comprised of older individuals (greater than 65) has significantly increased with approximately 46 million people age 65 and older in the United States constituting 15% of the total population. By 2050, the number of older individuals is expected to almost double and will represent more than 22% of the population (Vincent & Velkoff, 2010; Ortman *et al.*, 2014). With the increase in life expectancy and the changing demographics, the importance of age-related diseases for public health has escalated rapidly.

Broadly speaking, age-related diseases refer to those diseases that increase in prevalence with age. Extending lifespan significantly increases the prevalence of cancer, neurodegenerative and cognitive disorders including age-related dementia, Alzheimer's and Parkinson's Disease (White *et al.*, 2014; Brown, 2015). In addition to the impacts on

individual health, age-related diseases intensify societal burdens with increased demands on family members and caregivers as well as escalating the costs of health care services. Alzheimer's disease alone affects more than 5.5 million Americans and represents the most economically costly disease in the United States with annual costs estimated at \$277 billion (Alzheimer's Association, 2018). By 2050, between 13.8 – 16 million individuals in the United States are predicted to develop Alzheimer's disease (Alzheimer's Association, 2018). With an increase in lifespan, individuals also must manage chronic conditions such as chronic inflammatory diseases, osteoarthritis and diabetes for longer periods potentially affecting the quality of life (Frasca *et al.*, 2017; Franceschi *et al.*, 2018). A recent study found that more older Americans suffer from three or more chronic conditions (36%) than older individuals in other developed countries including New Zealand, Australia, Norway, Switzerland and the Netherlands (rates range from 13% - 17%) with the differences attributed to lower affordability and access to medical care (Osborn *et al.*, 2017). With lifespans increasing, the ability to manage chronic conditions poses a serious issue. Thus, there is a critical need for systems level studies to identify factors that exacerbate age-related diseases and identify therapeutic options to manage healthy aging.

The endogenous circadian clock regulates metabolic, physiological and behavioral rhythms with a 24 hour cycle providing the ability to coordinate physiological functions and anticipate regularly occurring environmental events. Evolutionarily, the light-dark cycle provides the strongest entrainment signal for the circadian clock (Roenneberg & Merrow, 2016), although feeding, activity, and social interactions can also provide input to the circadian system as well as being rhythmically regulated outputs (Webb *et al.*, 2014). In the past two decades, the role of the circadian clock in health and disease has become increasingly clear. Disruptions or desynchronization of the circadian system increases the risk and incidence of many diseases including cancers, metabolic disorders, cardiovascular diseases, neurodegenerative and cognitive disorders, and neuropsychiatric disorders (Scheer *et al.*, 2009; Schlosser Covell *et al.*, 2012; Evans & Davidson, 2013; Golombek *et al.*, 2013; James *et al.*, 2017). In this review, we highlight the use of *Drosophila* as a model system for studying the interactions of the circadian clock with aging and underscore the conservation of these interactions between *Drosophila* and mammalian systems.

Versatility of the *Drosophila* model system

Drosophila is as an excellent genetic model to study the complexity of circadian and aging interactions, offering the ability to combine the characterization of single-gene mutations with circuit and system level analysis. The fruit fly has persisted for over a century as a model organism due to its conserved signaling pathways that make it applicable for the study of animal development and behavior, neurobiology, and human diseases. Logistically, the short lifespan (60–80 days post-eclosion) of *Drosophila* coupled with its capability of generating large populations within 12–14 days make fruit flies a practical model for studies of aging. Genetic tools available in flies permit the analysis of systems level tissue interactions, using spatial and temporal regulation of gene expression, with the effects of environmental interventions such as oxidative stress, diet, and inflammation that influence longevity. The fly genome is approximately 5% of the size of human genome (132 billion base pairs for the fly compared to 3.2 billion base pairs in human) with nearly 14,000 genes

on its four chromosomes (Adams *et al.*, 2000) compared to the estimated 22,000 genes on the 23 chromosome pairs of humans. Importantly, both flies and humans have retained homologous genes for over 60% of their genome, including genes related to disease traits (Reiter *et al.*, 2001; Harrison *et al.*, 2002; Lloyd & Taylor, 2010; Pandey & Nichols, 2011). Approximately 75% of genes associated with human cancers and genetic diseases have homologs in the fly genome (Chien *et al.*, 2002; Lessing & Bonini, 2009). These factors render *Drosophila* an ideal “simple model system” for the analysis of aging-related diseases and pathologies.

The cost-effectiveness, genetic tractability, high throughput assays and availability of tools to manipulate neuronal properties with spatiotemporal accuracy of *Drosophila* have propelled it to the forefront as a competitive model for rapid identification of the cellular and molecular mechanisms underlying aging-related diseases including neurodegenerative diseases (Krishnan *et al.*, 2009; Blake *et al.*, 2015; Gerstner *et al.*, 2016); cardiovascular diseases (Piazza & Wessells, 2011; Yu *et al.*, 2013; Zarndt *et al.*, 2015), cancer (Rudrapatna *et al.*, 2012; Christofi & Apidianakis, 2013); diabetes and obesity (Trinh & Boulianne, 2013; Park *et al.*, 2014; Alfa & Kim, 2016). As a model, *Drosophila* has also been used for evaluating the efficacy of potential therapeutic drugs to treat aging-related diseases and disorders (Das & Cagan, 2013; Palandri *et al.*, 2018). Despite the obvious differences in anatomical structure and levels of behavioral complexity, the physiological mechanisms underlying most biological processes between *Drosophila* and mammals are remarkably well conserved (Pandey *et al.*, 1998; Pandey, 1998; Pandey & Nichols, 2011). Flies have analogous organs or structures that perform the functional equivalent of mammalian organs including the heart (Seyres *et al.*, 2012; Brandt & Paululat, 2013; Ma, 2016), lung (Roeder *et al.*, 2012; Zuo *et al.*, 2013), kidney (Dow & Romero, 2010; Miller *et al.*, 2013), gut (Erkosar & Leulier, 2014; Katzenberger *et al.*, 2015a; Katzenberger *et al.*, 2015b; Pasco *et al.*, 2015) and liver (Buchon *et al.*, 2011; Maruyama & Andrew, 2012; Ugur *et al.*, 2016).

The molecular, cellular, and electrophysiological properties underlying neuronal behavior and synaptic plasticity are remarkably well conserved between flies and higher organisms even with the considerable neuroanatomical differences (Kazama, 2015; Maximino *et al.*, 2015). The *Drosophila* brain is on the order of 100,000 neurons compared to the 100 million neurons of the mouse brain. The *Drosophila* connectome has revealed 43 discreet neural subregions within the fly brain organized by modality into olfaction, auditory/mechanosensation, right vision, left vision and pre-motor modules (Chiang *et al.*, 2011; Kaiser, 2015; Shih *et al.*, 2015). Distinct neural circuits have been identified for the regulation of complex behaviors including sleep (Potdar & Sheeba, 2013; Parisky *et al.*, 2016; Artiushin & Sehgal, 2017), learning and memory (Busto *et al.*, 2016; Kaun & Rothenfluh, 2017), grooming and feeding (Joseph & Carlson, 2015; Landayan & Wolf, 2015), circadian rhythms (Helfrich-Förster, 2014), aggression and courtship (Yamamoto & Sato, 2013; Kravitz & Fernandez, 2015). Recently, whole brain imaging combined with targeted neurogenetics has generated neural maps of locomotor and social behaviors in *Drosophila* (Robie *et al.*, 2017) facilitating systems level research.

***Drosophila* as a Model for the Circadian Clock**

Colin Pittendrigh's study of eclosion rates of fruit flies in the 1950s established *Drosophila* as a prominent model for circadian research allowing scientists to define the theoretical, molecular and genetic framework for the circadian clock (Pittendrigh *et al.*, 1973; Konopka *et al.*, 1989; Pittendrigh & Takamura, 1989; Pittendrigh *et al.*, 1991). Understanding the molecular mechanisms of the circadian oscillator began with the seminal discovery by Konopka and Benzer in 1971 of the first genetic mutants (*period* gene) that changed the length of the circadian period. Genetic transformation of *per⁰* arrhythmic flies using DNA encoding the wild-type *period* gene restored rhythms in eclosion and locomotor behavior in these flies confirming the role of *period* in the circadian oscillator (Bargiello *et al.*, 1984; Zehring *et al.*, 1984). Subsequent studies using genetic screens and analysis of locomotor activity rhythms identified numerous genes that form the core clock mechanism in flies including *timeless*, *clock*, *cycle*, *doubletime*, *shaggy*, *casein kinase 2* subunits and *cryptochrome* (Sehgal *et al.*, 1994; Reppert & Sauman, 1995; Allada *et al.*, 1998; Kloss *et al.*, 1998; Price *et al.*, 1998; Rutila *et al.*, 1998; Martinek *et al.*, 2001; Lin *et al.*, 2002; Akten *et al.*, 2003; Fan *et al.*, 2013). In the late 1990s, the *per* and *clock* genes were the first mammalian circadian genes to be identified and sequenced (King *et al.*, 1997; Tei *et al.*, 1997; Kloss *et al.*, 1998).

The central pacemaker of *Drosophila* spans approximately 150 neurons, anatomically organized into seven neuronal groups comprising an interactive network of oscillators. The small and large ventral lateral neurons control the morning activity peak and express pigment dispersing factor (PDF; fly orthologue of mammalian neuropeptide VIP), a neuromodulator important for the internal synchronization and output pathways of the fly's circadian clock. The lateral dorsal and dorsal neurons control the evening activity peak (Kaneko & Hall, 2000; Helfrich-Förster, 2003; Yao & Shafer, 2014; Yoshii *et al.*, 2016).

A set of transcription-translation feedback loops drive the circadian rhythms in physiology and behavior for both mammals and flies (Ko & Takahashi, 2006; Dibner *et al.*, 2010; Ozkaya & Rosato, 2012; Hardin & Panda, 2013). Although differences in individual genes exist between the core oscillators of *Drosophila* and mammals, the underlying mechanisms of clock function are similar. Transcription factors in the positive limb activate transcription of genes in the negative limb that then repress the transcriptional activators (Glossop *et al.*, 1999; Morse & Sassone-Corsi, 2002; Reppert & Weaver, 2002). In *Drosophila*, *clock* (*dClk*) and *cycle* (*dCyc*) act as transcriptional activators which heterodimerize and bind to the E-box-regulated promoters of *period* (*per*) and *timeless* (*tim*) to activate transcription of the core circadian genes, *per* and *tim* (Sehgal *et al.*, 1994; Reppert & Sauman, 1995; Rutila *et al.*, 1998) and other clock controlled genes (Menet *et al.*, 2010; Abruzzi *et al.*, 2011). In the cytoplasm, PER monomers are phosphorylated by *doubletime* (DBT) rendering them unstable. When the levels of dPER and dTIM increase, these two proteins form a dPER/dTIM/DBT complex and translocate back to the nucleus, interrupting further transcription by *dClk* and *dCyc* (Kloss *et al.*, 1998; Price *et al.*, 1998; He *et al.*, 2017). In mammals, the proteins CLOCK (CLK) and brain and muscle ARNT-like protein1 (BMAL1) are the members of the family of basic helix loop helix – PAS transcription factors that form the positive limb of the feedback loop (Vitaterna *et al.*, 1994; Gekakis *et al.*, 1998). CLK and

BMAL1 heterodimerize and bind to the promoters of the *per* (*mPer1*, *mPer2* and *mPer3*) and *cryptochrome* (*mCry1* and *mCry2*) genes, activating their transcription along with many other clock genes (Huang *et al.*, 2012; Partch *et al.*, 2014). Following translation of mPER and mCRY proteins, mPER and mCRY heterodimerize and form a repressor complex, translocating to the nucleus to inhibit CLK-BMAL1-mediated activation of *mPer* and *mCry* genes (Partch *et al.*, 2014). Unlike mammals, in the central brain pacemakers of *Drosophila* CRY has an important role in circadian photoreception and triggers light-induced TIM degradation (Stanewsky *et al.*, 1998; Emery *et al.*, 2000). In both mammals and flies, posttranscriptional and translational elements regulate the core clock proteins to influence their cellular localization and nuclear stability fine-tuning period length (Martinek *et al.*, 2001; Ko & Takahashi, 2006; He *et al.*, 2017). These include kinases such as *doubletime* (*casein kinase 1 epsilon*, CK1E in mammals) that regulate phosphorylation of PER that targets the protein for ubiquitination and proteasome degradation. More detailed information on the circadian clock can be found in the following recent review articles (Tataroglu & Emery, 2015; Yoshii *et al.*, 2016; He *et al.*, 2017; Mendoza-Viveros *et al.*, 2017).

Aging-related behaviors and physiological changes are similar between *Drosophila* and mammals

Given the widespread changes that occur with aging across tissues and systems, it can be difficult to distinguish the primary impacts of aging on cellular function and tissue damage from secondary consequences and subsequent disease states. Recently, there has been growing emphasis on the use of ‘simple’ model systems for the study of systems level interactions. The fruit fly *Drosophila* has long been appreciated as a genetic model for the identification of highly conserved molecular and cellular signaling processes.

Although *Drosophila* have a relatively short life cycle with a maximum lifespan of 60 – 80 days post-eclosion depending upon the strain and development conditions, many of the physiological and molecular changes associated with aging parallel those processes in mammals. In flies as in humans, aging differentially affects behavioral responses, neural circuits and organs distinguishing age-related changes from general physiological decline. The adult *Drosophila* is almost entirely post-mitotic from the time of eclosion until death (Bozcuk, 1972; Ito & Hotta, 1992) making the fruit fly suitable for studies of aging and cellular senescence (Grotewiel *et al.*, 2005). Similar to humans, fruit flies exhibit age-related declines in behavioral phenotypes from motor skills to learning and memory. In the following sections, we highlight a few age-related phenotypic changes to demonstrate equivalencies in aging from *Drosophila* to mammals and humans.

Senescence of muscles and motor activity

A marked feature of aging in humans and other mammals is the progressive loss of skeletal muscle mass known as sarcopenia (Fried *et al.*, 2004). Muscle dysfunction as a result of aging has been extensively studied in *Drosophila* (Fisher, 2004; Augustin & Partridge, 2009; Demontis *et al.*, 2013; Rai *et al.*, 2014). *Drosophila* display muscle deterioration with increasing age (Taylor, 2006; Piccirillo *et al.*, 2014). Older flies exhibit sarcomere-related defects including reduced length and increased disorganization of sarcomeres as well as

changes in myofibrillar protein composition and function (D'Antona *et al.*, 2007; Miller *et al.*, 2008).

Commonly used assays to assess motor function with aging in *Drosophila* include negative geotaxis, exploratory walking and locomotor activity (Gargano *et al.*, 2005; Grotewiel *et al.*, 2005; Liao *et al.*, 2017). Individual and longitudinal studies in *Drosophila* have shown that negative geotaxis declines as flies reach middle and old age reflecting age-related changes in motor function. (Miquel *et al.*, 1976; Leffelaar & Grigliatti, 1984; Cook-Wiens & Grotewiel, 2002; Goddeeris *et al.*, 2003; Rhodenizer *et al.*, 2008; Ratliff *et al.*, 2015). Walking velocity, exploratory activity, and the duration of individual motor activity bouts also decrease with age (Grotewiel *et al.*, 2005; Liao *et al.*, 2017). Phototactic behavior and flight ability have also been used to measure age related declines in motor activity (Leffelaar & Grigliatti, 1984; Palladino *et al.*, 2002).

Age-related declines in learning and memory

Age-associated cognitive decline encompasses numerous memory impairments with increased forgetfulness, the inability to maintain focus and a lowered problem solving capability starting as early as age 50 in humans (Albert & Heaton, 1988; Aartsen *et al.*, 2002; Salthouse, 2009). Studies of hippocampal function in rodent models suggest that aging induces changes in synaptic plasticity rather than neuronal loss (Burke & Barnes, 2006; Shetty *et al.*, 2017). In *Drosophila*, aging has been found to impact the acquisition of learning with significant differences in the rate of habituation of the proboscis extension reflex observed between young flies (3 days old) and middle-aged flies (35 days old; Fois *et al.*, 1991). Age-related impairments in the acquisition of learning also become apparent during middle age for associative learning paradigms including conditioned suppression of the proboscis extension reflex and classical olfactory conditioning (Brigui *et al.*, 1990; Fois *et al.*, 1991; Tamura *et al.*, 2003; Grotewiel *et al.*, 2005). Similarly, deficits in acquisition are seen when visual cues are paired with quinine during inhibitory conditioning of the proboscis extension reflex in middle aged (30 day) and older flies (50 days old) with these flies requiring extended training to learn the association (Fresquet & Médioni, 1993).

In addition to age-related deficits in learning acquisition, olfactory conditioning has been used to define age-related changes in multiple forms of memory. Middle-aged flies (20 or 30 days old) exhibit significant decrements in intermediate or middle-term memory measured one hour after training (Tamura *et al.*, 2003; Yamazaki *et al.*, 2007; Tonoki & Davis, 2012; Yamazaki *et al.*, 2014). Functional imaging of neural circuit activity found that the memory trace for intermediate-term memory was impaired in 30 day old flies suggesting deficits in synaptic transmission (Tonoki & Davis, 2012). Long-term memory, dependent upon transcription and protein synthesis, is also subject to age-related impairments as observed for olfactory conditioning (Mery *et al.*, 2007) with age-related defects apparent in synaptic connectivity (Tonoki & Davis, 2015). Aged flies demonstrate an accelerated rate of memory extinction, an active inhibition of memory processes, and impaired memory restoration (Chen *et al.*, 2015). As in humans, the appearance and degree of age related memory impairments in *Drosophila* may be dependent upon the type of learning and the neural circuits involved. For example, learning and memory of conditioned courtship suppression

remains robust in middle-aged flies (Savvateeva *et al.*, 1999; Savvateeva *et al.*, 2000). Aging also appears to have a minimal effect on a protein synthesis independent form of memory, anesthesia resistant memory, in *Drosophila* (Tamura *et al.*, 2003; Mery, 2007; Tonoki & Davis, 2012). With the ability to distinguish multiple facets of learning and memory at the behavioral level, *Drosophila* presents an excellent model for studying age-related memory impairments.

Age-related changes in sensory systems

Olfactory sensitivity noticeably decreases with age in humans (Doty *et al.*, 1984; Zhang & Wang, 2017). Deterioration of olfactory sensitivity has been identified as an early predictor of cognitive impairment in later years (Royall *et al.*, 2002; Swan & Carmelli, 2002; Wilson *et al.*, 2007; Schubert *et al.*, 2008). Similarly, studies in *Drosophila* have shown that older flies exhibit a characteristic decline in olfactory avoidance behavior to aversive odors with olfactory deficits first appearing in middle age (25 days; Cook-Wiens & Grotewiel, 2002; Grotewiel *et al.*, 2005). The decline in olfactory abilities can also be observed in middle aged males affecting nocturnal courtship activities (Ratliff *et al.*, 2015). Age-dependent changes in the *Drosophila* olfactory system occur both at the molecular level with significantly decreased mRNA expression for many olfactory pathway components (Ratliff *et al.*, 2015) and at the structural level with aging-induced structural changes within neurons of the olfactory circuit (Hussain *et al.*, 2018). Age-related declines in sensory perception appear to be system specific as there are no observable effects of age on behavioral responses to electric shocks or the slow phototactic attraction response to light (Cook-Wiens & Grotewiel, 2002; Tamura *et al.*, 2003; Grotewiel *et al.*, 2005; Simon *et al.*, 2006; Hussain *et al.*, 2018).

In humans, photoreception is particularly susceptible to age-related changes with changes in light sensitivity for circadian entrainment occurring with age (Zhang *et al.*, 1996; Benloucif *et al.*, 1997; Duffy *et al.*, 2007; Zuurbier *et al.*, 2015) and the development of age-related diseases such as macular degeneration (Ardeljan & Chan, 2013; DeAngelis *et al.*, 2017). More than 20% of elderly Americans suffer from visual impairments (Klein & Klein, 2013). While several research studies concluded that aging did not affect *Drosophila* photoreception, more detailed analysis has found decreased fast phototactic responses in older flies (Simon *et al.*, 2006; Hall *et al.*, 2017). Although no significant retinal degeneration has been observed in flies up to 40 days of age (Kurada & O'Tousa, 1995; Hall *et al.*, 2017), studies in *Drosophila* have identified changes in photoreceptor gene expression associated with visual senescence at the cellular level using transcriptome profiling (Hall *et al.*, 2017).

Age-dependent changes in cardiac function

Cardiac performance declines progressively with increased age across many organisms (Ocorr *et al.*, 2007a; Nishimura *et al.*, 2011). In mammals, cardiac aging includes the decline in cardiomyocyte number, decreased diastolic function, fibrosis and accumulation of collagen as well as an increase in prevalence of atrial fibrillation and left ventricular hypertrophy (Olivetti *et al.*, 1991a; Olivetti *et al.*, 1991b; Khan *et al.*, 2002; Chiao & Rabinovitch, 2015; Steenman & Lande, 2017). Unlike mammals, *Drosophila* has an open

sleep consolidation compared to younger 10 day old flies (Vienne *et al.*, 2016). Middle-aged male flies (25–27 days old) also have significantly more sleep fragmentation during the night (increased number of sleep bouts with shorter duration per bout) compared to young males (Williams *et al.*, 2016). Older flies exhibit decreased arousal thresholds and increased sensitivity to neuronal stimuli compared to younger flies (Vienne *et al.*, 2016). Age-dependent changes in sleep homeostasis also occur in *Drosophila* with reduced recovery sleep following sleep deprivation in older flies (Vienne *et al.*, 2016). Similarly, older people (60 years and older) exhibit decreased total sleep duration, poorer sleep quality and increased sleep latency (Pandi-Perumal *et al.*, 2005; Ohayon, 2010). Older adults also are more easily aroused from sleep than young adults (Zepelin *et al.*, 1984). Reduced sleep rebound with aging occurs in humans and in mammalian models (Carrier *et al.*, 2001; Landolt & Borbély, 2001; Dijk *et al.*, 2010; Zimmermann, 2011).

Sleep fragmentation and mis-timed sleep induces inflammatory responses and activates pathways reflecting oxidative stress in both young (5–7 days old) and middle-aged flies (25–27 days old); however, the continued elevation of reactive oxidative species occurs only in middle-aged flies and not in young flies following recovery sleep (Williams *et al.*, 2016). ER-chaperone genes are expressed at low levels in both normal sleeping young and middle-aged flies but are significantly increased in middle-aged flies following recurrent sleep fragmentation, implying that older flies are more susceptible to neuronal stress and that aging affects the molecular consequences of sleep disturbances (Williams *et al.*, 2016).

Understanding Circadian and Aging Interactions

From *Drosophila* to humans, the weakening of the circadian system with age results in lower amplitude circadian rhythms as well as fragmentation of these rhythms (Weinert, 2000; Gibson *et al.*, 2009; Zuurbier *et al.*, 2015; Mattis & Sehgal, 2016). Furthermore, circadian perturbations and the weakening of the circadian system can increase sleep fragmentation with aging. Circadian dysfunction and sleep disorders aggravate many age-related cognitive and neurodegenerative disorders (Musiek & Holtzman, 2016; Liu & Chang, 2017) contributing to declining quality of life in old age. Recent research suggests that circadian rhythm fragmentation is associated with pre-clinical Alzheimer's disease and may exacerbate disease development (Musiek *et al.*, 2018). Disrupted circadian rhythms and sleep patterns have also been identified as a predictive factor for the development of cognitive impairment and dementia (Schlosser Covell *et al.*, 2012; Shi *et al.*, 2017). Below, we highlight research from *Drosophila* and mammalian models that has facilitated our understanding of the reciprocal interactions between the circadian system starting with examples of how aging weakens the circadian system and then examining ways through which circadian dysfunction contributes to aging pathologies. Finally, we review studies in which reinforcement or strengthening of the circadian system mitigates age-related pathologies.

Impact of Aging on Circadian Oscillators

In humans and other mammals, the suprachiasmatic nucleus (SCN) in the hypothalamus houses the 'master' circadian clock responsible for synchronizing many tissue specific

circadian oscillators in a hierarchical manner (Albrecht, 2012). At the molecular level, aging results in changes in the phase and amplitude of rhythms in core clock genes in the SCN (Weinert *et al.*, 2001; Kolker *et al.*, 2003; Bonaconsa *et al.*, 2014; Nakamura *et al.*, 2015) and in other brain regions (Chen *et al.*, 2016). Neural activity rhythms of SCN cells are also drastically altered by aging with significantly decreased amplitudes of the rhythms observed (Nakamura *et al.*, 2011). In hamsters, the transplant of fetal SCN tissue to aged animals significantly increased longevity suggesting that a robust functioning circadian clock contributes to healthy aging (Hurd & Ralph, 1998).

However, aging does not uniformly affect molecular rhythms in gene expression as age-related impacts appear to differ between core circadian genes. When SCN function was analyzed in rats using a *Per1*-luciferase reporter, the free running period was significantly shortened in older animals although robust cycling was observed (Yamazaki *et al.*, 2002). In Syrian hamsters, aging decreases *Bmal1* expression in the SCN (Kolker *et al.*, 2003; Duncan *et al.*, 2013) and core clock gene expression of *Bmal1*, *Clk* and *per2* are significantly attenuated in multiple brain regions of older mice and hamsters including the hippocampus and cingulate cortex (Duncan *et al.*, 2001; Wyse & Coogan, 2010; Duncan *et al.*, 2013). Similarly, age-related discrepancies in *per2* mRNA rhythms in the SCN have been reported in mice (Weinert *et al.*, 2001). However, other studies have failed to find any age-dependent weakening of the SCN molecular oscillator. In a recent gene expression profiling study of aging using a non-human primate model, no differences were found in the cycling of core clock genes or genes involved in SCN signaling between young and old animals (Eghlidi *et al.*, 2018). When extra-SCN brain regions are examined, significant effects of age on molecular circadian rhythms can be observed similar to the impacts seen in peripheral brain oscillators in *Drosophila*. In a pioneering study of the effect of aging on circadian gene expression in human brain tissue, circadian rhythmicity was found to be altered in an age-dependent manner for approximately 1200 genes with changes in rhythmicity found for genes involved in cognition, sleep and mood regulation (Chen *et al.*, 2016). More detailed reviews of changes in circadian function and their implications for health and disease in mammalian systems are available (Kondratov, 2007; Tevy *et al.*, 2013; Arellanes-Licea *et al.*, 2014; Banks *et al.*, 2016; Hood & Amir, 2017).

At the behavioral level, locomotor activity rhythms are frequently used as a proxy to assess the state and function of the central brain circadian clock in *Drosophila* or the SCN in mammals. Aging lengthens the free-running period of locomotor activity rhythms in *Drosophila* and weakens rhythmicity. In addition to increasing the percentage of arrhythmic flies, more phase instability and rhythm fragmentation is observed in older flies (Koh *et al.*, 2006; Luo *et al.*, 2012; Rakshit *et al.*, 2012). These changes can be observed starting in middle-age with continuous declines as flies get older (Koh *et al.*, 2006; Luo *et al.*, 2012; Rakshit *et al.*, 2012; Umezaki *et al.*, 2012). It can be difficult to distinguish the effects of aging from the adverse physiological impacts of cumulative environmental exposures to toxins. In developmental studies in which extended multi-generational selection was done to establish a population of faster developing flies, it was found that faster developing flies exhibited age-related phenotypes in circadian rhythms significantly earlier than wild-type flies including a lengthening of the circadian period and increased arrhythmicity (Yadav & Sharma, 2014). Thus, aging of the circadian system appears due to physiological and

developmental mechanisms rather than chronological age or cumulative exposure to environmental factors. Deterioration of circadian rhythms with age are phylogenetically conserved with rodent models also displaying age-dependent changes in free-running period length, increased fragmentation and decreased rhythm strength (Weinert, 2000; Weinert *et al.*, 2001; Yamazaki *et al.*, 2002; Froy, 2011). Age-dependent changes in the free-running period of activity rhythms also occur in non-human primate models (Aujard *et al.*, 2006).

Early molecular studies in *Drosophila* investigating the principles through which the core circadian oscillator functions relied upon analysis of RNA and protein levels in whole fly heads (Hardin *et al.*, 1990; 1992), and molecular analysis of whole fly heads remains a frequent starting point for *Drosophila* circadian studies. With aging, a decline in the amplitude of the RNA rhythm for the *per* gene in whole fly heads becomes apparent starting in middle age (35 day flies) with greater suppression of the RNA rhythms seen in older flies (50 days; Krishnan *et al.*, 2009). More widespread analysis of core circadian clock genes in aging *Drosophila* found that *tim*, *cry*, *Pdp1* and *vri* mRNA rhythms also dampened with significant reductions in the amplitude of the molecular rhythm in the heads of middle-aged and older flies (Rakshit *et al.*, 2012; Rakshit & Giebultowicz, 2013). In contrast, aging does not alter the expression levels of *Clk* and *cyc* mRNAs with *Clk* mRNA remaining robustly rhythmic in older flies (Rakshit *et al.*, 2012). In conjunction with the declining amplitude in *per* and *tim* mRNA oscillations, PER and TIM protein levels are markedly reduced in the heads of old flies compared to young flies (Rakshit *et al.*, 2012).

Given the complexity of the circadian system even in a relatively simple model such as *Drosophila*, one cannot directly ascribe changes in behavioral rhythms to alterations in core oscillator function of pacemaker neurons when molecular rhythms are measured in whole fly heads. The circadian pacemaker neurons only make up a small fraction of the neurons in the brain with the central circadian clock consisting of 150 neurons grouped into seven neuronal groups (Helfrich-Förster *et al.*, 2011; Helfrich-Förster, 2014; Yoshii *et al.*, 2016). As peripheral oscillators in the head, *Drosophila* photoreceptors comprise the largest population of rhythmic neurons when whole heads are examined. When *Drosophila* photoreceptors alone are examined, significant age-related declines in core circadian genes are observed (Luo *et al.*, 2012; Rakshit *et al.*, 2012) raising the possibility that age-related changes in photoreceptor circadian gene expression may obscure the true state of pacemaker neurons. More specific analysis of the circadian pacemaker neurons found circadian PER protein expression in key groups of clock neurons, the small ventral lateral neurons, the dorsal lateral neurons and the group 1 dorsal neurons in late middle-aged flies (40 days; Luo *et al.*, 2012). The robust cycling of PER continued in older flies (60 days and 80 days old), although the phase was shifted compared to younger flies providing a possible mechanism for the longer free-running periods of older flies (Luo *et al.*, 2012). It should be noted that not all studies have found similar results potentially due to genetic background differences in the fly strains examined. In a contrasting study, when the peak protein levels for PER and TIM were examined in all seven groups of pacemaker neurons in the central brain, the levels were significantly reduced in late middle age (40 days old) and older flies (50 days old; Umezaki *et al.*, 2012). At the cellular level, aging results in the loss of structural plasticity in the pacemaker cells of the ventral lateral neurons with no increase of synaptic terminals in response to social enrichment observed in middle-aged flies (Donlea *et al.*, 2014b). Thus,

while the effects of aging on pacemaker neurons are not fully understood at the molecular level in either flies or mice, the evidence is clear that age-related changes occur in neural activity and structural plasticity in the central clock affecting circadian outputs.

Aging, Circuit Connectivity and Synchronization

Individually the neurons within the mammalian SCN maintain autonomous rhythms; however, the period and phase of the rhythms of individual cells varies significantly (Welsh *et al.*, 1995). Synchronization and intercellular coupling between SCN neurons is necessary for robustness of the circadian clock (Aton & Herzog, 2005). During phase shifts (changes in time zones) of the circadian system, resetting to the new phase requires synchronization between central circadian pacemaker neurons and peripheral oscillators. As one ages it becomes more difficult to reset the circadian system resulting in more severe jetlag (Monk *et al.*, 1993; Monk *et al.*, 2000). Aging appears to impact multiple mechanisms affecting the ability of the SCN neurons to respond to phase shifts. Advancing age decreases the sensitivity of the circadian system to light resetting in humans and other animals making it more difficult for the circadian system to respond to phase shifts (Zhang *et al.*, 1996; Benloucif *et al.*, 1997; Duffy *et al.*, 2007; Zuurbier *et al.*, 2015). Furthermore, aging decreases synchronization between SCN neurons as well as affecting the neural activity of individual cells resulting in a weakening of the master pacemaker (Farajnia *et al.*, 2012; Duffy *et al.*, 2015; Hood & Amir, 2017).

At the molecular level, aging weakens SCN output activity with decreased rhythms in the circadian neuropeptide vasopressin (VIP) and age-dependent reductions in its receptor observed in rodent models (Kawakami *et al.*, 1997; Krajnak *et al.*, 1998; Duncan *et al.*, 2001; Kalló *et al.*, 2004; Nakamura *et al.*, 2016; Allen *et al.*, 2017). In non-human primates, significant age-dependent phase-shifts are also seen in VIP expression as well as in arginine-vasopressin, another neuropeptide with circadian functions, suggesting age-related deficits in transmission of circadian information from the SCN to downstream neuronal targets (Cayetanot *et al.*, 2005; Aujard *et al.*, 2006). In addition to synchronization within the SCN neurons, there must be a resynchronization throughout the hierarchical circadian system with peripheral oscillators becoming realigned to the new phase. Aging also impairs the resynchronization between central and peripheral oscillators following phase-shifts (Davidson *et al.*, 2008; Sellix *et al.*, 2012). Thus, it appears that the aging induces impairments in circadian neural output activity and circuit connectivity resulting in decreased circuit synchronization. Desynchronization between the central oscillator and peripheral oscillators can exacerbate disease pathology and accelerate mortality (Zuurbier *et al.*, 2015; Chauhan *et al.*, 2017).

In *Drosophila*, pigment dispersing factor (PDF) is the primary circadian neurotransmitter connecting the evening and morning oscillators as well as providing a mechanism for circadian output to many neurons throughout the brain (Renn *et al.*, 1999; Taghert & Shafer, 2006; Yasuyama & Meinertzhagen, 2010) similar to the role of the VIP in the mammalian brain (Jin *et al.*, 1999). Age-dependent changes in circadian activity rhythms in *Drosophila* have been correlated with reduced PDF signaling in older flies suggesting a weakening of the circadian neural circuit or weakened communication to downstream target neurons

(Umezaki *et al.*, 2012). Overexpression of PDF in older flies strengthens circadian activity rhythms and buffers the age-induced change in period length (Umezaki *et al.*, 2012). Moreover, PDF overexpression increases TIM cycling in pacemaker neurons removing the age-related suppression of peak protein abundance in the older flies (Umezaki *et al.*, 2012). Thus, the effect of aging on circadian output and connectivity appear conserved across species.

In addition to neuronal pacemakers, glial cells have circadian oscillators and function in the regulation of circadian rhythms in *Drosophila* (Ng *et al.*, 2011; Jackson *et al.*, 2015; You *et al.*, 2018) and mammals (Prolo *et al.*, 2005; Marpegan *et al.*, 2009; Barca-Mayo *et al.*, 2017; Brancaccio *et al.*, 2017; Tso *et al.*, 2017). One mechanism through which aging may modulate the circadian system is through alterations in the rhythms of the expression of clock genes in glial cells or subsets of glial cells. The GAL4/UAS system, a bipartite transgene expression system commonly used in *Drosophila*, permits spatial manipulation of gene expression in specific cell types (Brand & Perrimon, 1993; Osterwalder *et al.*, 2001; Roman *et al.*, 2001; McGuire *et al.*, 2004) and has been used to determine the impact of aging on molecular circadian rhythms in glial cells (Long & Giebultowicz, 2017). While PER protein abundance is rhythmic in multiple glial subtypes including perineural glia, subperineural glia, cortex glia, central brain ensheathing glia and medulla giant glia in young flies (Suh & Jackson, 2007; Long & Giebultowicz, 2017), age does not affect PER protein rhythms equally in all cell types. Older flies show significantly dampened PER protein levels in all rhythmic glial populations examined with the exception of the central brain ensheathing glia and no rhythmic expression was found in either young or aged flies in central brain astrocytes (Long & Giebultowicz, 2017). Aging also affects circadian modulation of glial function in mammals. In rats, hippocampal microglia exhibit circadian mRNA rhythms in the core clock genes as well as downstream targets of the microglia oscillator TNF α and IL-1 β (Fonken *et al.*, 2016). Aged rats (25 months) display nearly flat *per1* mRNA expression and low amplitude cycling of *per2* in hippocampal microglia with non-rhythmic high expression levels of downstream targets (Fonken *et al.*, 2016). Aging related changes in the circadian regulation of glial function may alter mechanisms involved in neuronal homeostasis both synaptically and non-synaptically.

Impact of Aging on Peripheral Oscillators and Tissue Function

Similar to the differential physiological impacts of aging on organ and tissue function, aging also appears to affect peripheral oscillators to varying degrees. In *Drosophila*, peripheral oscillators throughout the body can function independently from the central brain oscillator (Plautz *et al.*, 1997; Giebultowicz, 2001). When an *in vivo per* promoter luciferase reporter system was used to assess peripheral circadian rhythms, robust circadian rhythms were present in young flies (11 days) but significantly lower expression levels of the reporter with weak cycling was observed in older flies (51 days; Luo *et al.*, 2012). Analysis of circadian expression for *per*, *tim*, *vri* and *Pdp1e* mRNA levels in older male bodies (50 day) found that rhythmic gene expression was apparent for all four genes, although there were lower expression levels and a seemingly reduced amplitude in the rhythms particularly for *per* and *tim* (Rakshit *et al.*, 2012). However, PER protein levels remain rhythmic in the peripheral oscillators in the Malpighian tubules, gut and abdominal fat bodies in aged flies (50 day old)

(Rakshit *et al.*, 2012). Similarly, no differences were observed between young and aged mice in *mPer1/mPer2* mRNA expression in either LD or constant darkness, or when a PER2-luciferase reporter was used to measure circadian rhythms in the kidneys, liver and submandibular gland (Tahara *et al.*, 2017).

Age dependent changes in circadian regulation also disturb the oscillations of clock-controlled genes in peripheral tissues and target organs affecting system function. For example, the circadian clock regulates many genes involved in the responses to oxidative stress and cellular redox (Krishnan *et al.*, 2008; Giebultowicz, 2018). Aging significantly alters the rhythms of clock-controlled genes regulating the cellular redox cycle (Klichko *et al.*, 2015). Young flies (5 days old) display strong diurnal patterns glutathione biosynthesis with rhythms apparent in both *Gclc* and *Gclm* mRNA and protein levels, the genes encoding the catalytic and modulatory subunits of glutamate cysteine ligase (GCL; (Beaver *et al.*, 2012). Older flies (50 days old) display an absence of diurnal glutathione rhythms and have significantly higher levels of *Gclc* mRNA and protein throughout the 24-hour cycle with irregular peaks and troughs as well as alterations in the expression and activity of other redox involved molecules reflecting impaired temporal redox homeostasis (Klichko *et al.*, 2015). In mammals, oxidative stress responses are regulated by the circadian clock and deletions of *Bmal1* accelerates neuronal damage and impairs the expression of genes involved in oxidative stress responses in aging mice (Musiek *et al.*, 2013).

The circadian clock regulates drug and alcohol-induced toxicity from flies to mammals. Studies from the 1950s and 1960s demonstrated that the toxicity of alcohol in mice was dependent upon the time of delivery (Haus & Halberg, 1959) as was the toxicity of amphetamines (Scheving *et al.*, 1968) and other drugs (Carlsson & Serin, 1950b; a). The consequences of drug and alcohol abuse appear higher in aging populations (Kendler *et al.*, 2016) in which circadian and sleep disruption is common. Studies from our lab have shown increased behavioral sensitivity to alcohol and increased alcohol-induced toxicity correlated with decreased circadian function in *Drosophila* (Van der Linde & Lyons, 2011; De Nobrega & Lyons, 2016; De Nobrega *et al.*, 2017). Aging increases the behavioral sensitivity to binge-like alcohol exposures as well as slowing recovery following alcohol exposure and increasing toxicity (De Nobrega *et al.*, 2017). Intriguingly, increased alcohol sensitivity is phase-specific suggesting that circadian regulation provides a protective buffer to alcohol-induced toxicity that declines with aging and decreased circadian function (De Nobrega *et al.*, 2017). Aging, in animal models and humans, has also been shown to significantly increase the sensitivity to other drugs including nicotine and benzodiazepines (Cherry & Morton, 1989; Okamoto *et al.*, 1994; Dowling *et al.*, 2008). Additional research is needed to identify the mechanisms through which the circadian clock mediates drug and alcohol-induced toxicity and determine the impact of these interactions on age-related increased drug susceptibility.

The core circadian clock as a mediator of lifespan

The links between the circadian clock and the processes of aging are bidirectional as circadian dysfunction appears to hasten cellular aging and age-related disease processes (Grosbellet *et al.*, 2015). Given the high degree of circadian regulation of metabolic and

physiological rhythms, one can hypothesize that alterations in the functioning of the circadian system could adversely affect an organism's longevity. Studies in *Drosophila* have investigated the physiological relevance of core clock genes to lifespan and healthy aging finding that mutations in *per* altered lifespan with *per⁰¹* flies having shorter lifespans (Ewer *et al.*, 1990; Krishnan *et al.*, 2012). Likewise, flies with mutations that significantly altered period length also showed reduced longevity (Klarsfeld & Rouyer, 1998). Similar observations have been made in mammals with circadian mutants exhibiting decreased longevity. For example, BMA11 knockout mutant mice have shorter lifespans and exhibit premature aging in many systems (Kondratov *et al.*, 2006). Mice with free-running circadian periods close to 24 hours exhibit 20% greater longevity compared to mice that had circadian periods that were longer or shorter (Libert *et al.*, 2012). When the light-dark cycle becomes dissociated from a 24 hour period, alterations in longevity are also observed in *Drosophila* (Pittendrigh & Minis, 1972) and wild-type mice housed in extremely short 4 h: 4 h LD cycles exhibit increased mortality (Park *et al.*, 2012).

The link between rhythmicity in gene function and lifespan also extends to clock controlled genes. Although the specific genes and proteins regulated by the circadian clock differ between organs and tissues, the circadian clock appears to regulate between 5 – 15% of genes at the transcriptional level or with post-transcriptional circadian regulation to regulate rhythmic translation or protein activity (Duffield *et al.*, 2002; Panda *et al.*, 2002). Clock-controlled genes also appear to play an important role in determining lifespan. In *Drosophila*, the recently characterized clock controlled gene *Achilles* regulates the immune system and provides a signaling link between neurons and immunological tissues (Li *et al.*, 2017). *Achilles* is highly rhythmic in the brain with peak mRNA expression during the late dark period and trough expression during the late phase of the light cycle (Li *et al.*, 2017). Even in the absence of an immune challenge, reduction of *Achilles* expression shortens lifespan (Li *et al.*, 2017). In mammalian models, numerous studies have demonstrated the interrelationship between circadian function and immune system responses (Scheiermann *et al.*, 2013; Curtis *et al.*, 2014; Geiger *et al.*, 2015). Research has shown distinct differences in the circadian phase and the amplitude of the rhythms for lymphocyte subsets in older adults (Mazzocchi *et al.*, 2011). Disrupted circadian regulation of the neuro-immune system with aging could increase disease susceptibility and affect longevity.

Circadian desynchronization or arrhythmicity accelerates aging

In humans, with the exception of a few notable familial disorders such as advanced familial sleep phase syndrome (Jones *et al.*, 2013), most individuals with circadian disorders are due to environmental perturbations rather than genetic disorders. The number of individuals affected by circadian disorders, in particular internal circadian desynchronization, has skyrocketed over the past few decades for a number of reasons. The proliferation of smartphone and personal electronic use, due to technological advances and increased affordability, has contributed to increased artificial light exposure at night weakening circadian stability (Cajochen *et al.*, 2011; Oh *et al.*, 2015). Longer work hours during the work week (Alterman *et al.*, 2013) and a shift in social schedules and sleep patterns on weekends cause circadian desynchronization from the biweekly phase shifts, a condition referred to as social jet-lag (Wittmann & Werfel, 2006). Social jetlag is associated with

many adverse health impacts including metabolic and cardiac diseases (Roenneberg *et al.*, 2012; Rutters *et al.*, 2014; Wong *et al.*, 2015). Shiftwork also has contributed to the number of individuals suffering from circadian disorders with between 15–30% of the adult population performing shiftwork in developed countries (Drake *et al.*, 2004a; Drake *et al.*, 2004b; Alterman *et al.*, 2013; Di Milia *et al.*, 2013).

As circadian disruption and desynchronization exacerbate numerous health conditions from metabolic diseases, cancers to neuropsychiatric disorders (Khan *et al.*, 2002; Scheer *et al.*, 2009; Arble *et al.*, 2010; Evans & Davidson, 2013; Golombek *et al.*, 2013; Albrecht, 2017; James *et al.*, 2017; Khan *et al.*, 2018), it is important to understand the interactions and impacts that circadian dysfunction or desynchronization has on the aging process. Research in hamsters has shown that circadian disorganization resulting from a mismatch between the light-dark cycle and the free-running period results in severe cardiac pathologies and kidney disease (Martino *et al.*, 2008). It should be noted that recent research in a mouse model of circadian desynchronization did not find a link between desynchronization and glucose intolerance or obesity suggesting that additional factors are needed to observe metabolic consequences of circadian desynchronization (van der Vinne *et al.*, 2018). As this study was performed in young animals, it is possible that the adverse health concerns associated with circadian desynchronization in humans may be aggravated in middle age and older individuals.

In animal models, experimental paradigms employing phase shifts of the light-dark cycle to simulate acute and chronic jet lag are commonly used for studying circadian desynchronization. Experimental protocols in which the light-dark cycle is changed on a weekly basis also can be employed to mimic rotating shiftwork or social jetlag conditions. Research in *Drosophila* has directly addressed the interactions of circadian desynchronization with age-related impairments using a chronic jet-lag model in which flies with a short circadian period of 16 hours were maintained on a 24 hour cycle (12:12 LD cycles; Vaccaro *et al.*, 2016). Under these conditions, the short period flies that were constantly experiencing phase shifts exhibited accelerated declines in age related motor impairments as well as decreased longevity compared to arrhythmic *per⁰¹* flies and wild-type CS flies (Vaccaro *et al.*, 2016). However, when the short period mutants were housed with 16 hour cycles (8:8 LD schedule) the increased rate of age-related declines in motor function were mitigated while wild-type flies displayed a greater rate of decline in age-related decreases in motor function suggesting that constant phase shifts and circadian desynchronization accelerate age-related phenotypes (Vaccaro *et al.*, 2016). Circadian disruption also negatively affects longevity in mammalian models. In one of the initial studies investigating the link between circadian function and longevity, researchers found that when mutant hamsters with a short circadian period of ~ 20 hours were maintained on a 24 hour LD cycle that was outside of the range of entrainment, these animals had significantly reduced lifespans suggesting that circadian desynchronization affected longevity (Hurd & Ralph, 1998). Similarly, aged mice placed on rotating LD schedules simulating chronic jet lag exhibit significantly higher mortality compared to younger mice on these schedules (Davidson *et al.*, 2006). It is possible that reduced tolerance to stressors and perturbations of cellular function seen with aging make these populations more sensitive to the adverse effects of desynchronization. Circadian desynchronization using a chronic jet

lag model increases cellular aging in diurnal grass rats resulting in telomere shortening (Grosbellet *et al.*, 2015). The metabolic profiles of the desynchronized young rats were intermediate between control young and old rats, suggesting increased oxidative stress and impaired glucose tolerance due to the desynchronization (Grosbellet *et al.*, 2015). Mammalian studies have identified correlations between disruption of core oscillator function and acceleration of aging phenotypes (Kondratov *et al.*, 2006; Antoch *et al.*, 2008; Vinogradova *et al.*, 2009; Ali *et al.*, 2015). For example, BMAL1 and CLOCK knockout mice have disrupted redox homeostasis, accelerated aging and deficits in cognition (Kondratov *et al.*, 2006; Antoch *et al.*, 2008; Ali *et al.*, 2015). Similarly rats housed in constant light exhibit accelerated metabolic pathologies and tumorigenesis and significantly shortened lifespan (Vinogradova *et al.*, 2009). In human studies, longer participation in shiftwork increases the degree of adverse health impacts and in men cognitive impairments such as cognitive efficiency and immediate recall (Rouch *et al.*, 2005; Wirtz & Nachreiner, 2012).

Reinforcement of the Circadian System Mitigates Age-Related Pathologies

In addition to the challenges and daily perturbations of the circadian system that people face in a 24/7 society, many individuals also experience weak entrainment of the circadian system due to the urban nature of society with limited outdoor time and increased artificial light at night (Espiritu *et al.*, 1994; Diffey, 2011; Matz *et al.*, 2014; Smolensky *et al.*, 2015; Falchi *et al.*, 2016; Lunn *et al.*, 2017). Poor functioning of the circadian system accelerates aging and aging weakens circadian function, such that these reciprocal interactions aggravate age-induced pathologies. Human longevity has significantly increased worldwide (Vaupel, 2010) with recent research suggesting that the average length of human lifespan will continue to increase significantly (Brown *et al.*, 2017; de Beer *et al.*, 2017; Lenart & Vaupel, 2017; Barbi *et al.*, 2018). Consequently, it is imperative to develop new strategies for healthy aging to minimize susceptibility to age-related pathologies and increase the manageability of chronic conditions. The bidirectional interactions of the circadian clock with aging suggests that strengthening of the circadian system may mitigate age-related declines and pathologies. Indeed, there have been intriguing studies from *Drosophila* to humans that strongly suggest that reinforcement of the circadian clock through stronger entrainment paradigms or the use of multiple zeitgebers provides health benefits in aging. Table 1 outlines genetic and environmental manipulations of the circadian clock that have been used in *Drosophila* to address age-related problems while Table 2 presents key studies from mammalian models and humans that employ circadian interventions to mitigate age-related declines.

In *Drosophila*, several studies have analyzed the impact of overexpression of core clock genes on longevity and age-related pathologies. Researchers found that overexpression of the *timeless* gene throughout the body significantly increased longevity (Katewa *et al.*, 2016). With tissue specific gene expression to manipulate individual tissue peripheral oscillators, it was found that no single tissue overexpression of *tim* increased longevity to the extent achieved by whole body *tim* overexpression suggesting that aging and longevity is influenced by the integration of multiple peripheral oscillators (Katewa *et al.*, 2016). Reinforcement of the circadian oscillator through overexpression of *cry* in central and

peripheral clock cells of older flies results in robust circadian oscillations of *per*, *tim*, *Pdp1e* and *vri* mRNA similar to those observed in younger flies (Rakshit & Giebultowicz, 2013). In addition to strengthening the molecular oscillator, *cry* overexpression suppressed the age-related locomotor activity phenotypes when *cry* was expressed throughout central and peripheral oscillators and decreased the age-related vulnerability to oxidative stress in older flies (Rakshit & Giebultowicz, 2013). Interestingly, *cry* overexpression in central brain oscillators alone was insufficient to rescue either the age-dependent motor deficits, the increase in longevity or the age-induced susceptibility to oxidative-stress (Rakshit & Giebultowicz, 2013). These studies from *Drosophila* emphasize the critical role of peripheral oscillators for the resilience to age-induced pathologies, although it remains to be determined whether it is the function of multiple peripheral oscillators that is crucial in healthy aging and/or the synchronization between oscillators.

Genetic strategies to enhance the output signaling activity of specific circadian neuron groups have also been employed to counter age-induced declines. In the *Drosophila* central brain circadian system, the small ventral lateral neurons provide signaling for the morning oscillator (Grima *et al.*, 2004; Stoleru *et al.*, 2004) or potentially act as the main oscillator (Rieger *et al.*, 2006), while the large ventral lateral neurons are generally thought to provide an arousal function as part of the circadian circuit (Parisky *et al.*, 2008; Shang *et al.*, 2008; Sheeba *et al.*, 2008; Potdar & Sheeba, 2012). When the activity of the ventral lateral neurons was manipulated in aging flies via input dopaminergic signaling into these neurons, age-dependent declines in neural structural plasticity were rescued as was activity dependent increases in sleep (Donlea *et al.*, 2014a). With the exception of the 5th small ventral lateral neuron, all of the small and large ventral lateral neurons express PDF (Renn *et al.*, 1999; Kaneko & Hall, 2000). Overexpression of PDF in these neurons rescued the age-related deficits in locomotor activity rhythms and significantly increased TIM levels in all seven groups of circadian neurons in aged flies, although only small increases were observed in PER protein levels (Umezaki *et al.*, 2012). These studies suggest that age-dependent connectivity issues between neuronal groups in the circadian circuit and the wake-sleep/arousal circuits can also be targeted to mitigate age-related declines in behavior and neural plasticity.

Strengthening circadian entrainment through the coupling of zeitgebers enhances circadian function in both flies and mammals and has been used to mitigate age-related phenotypes. In *Drosophila*, temperature entrainment in sync with the light dark cycle significantly increases behavioral rhythmicity in older flies by consolidating activity to the daytime period and reducing activity during the early night resulting in longer and more consolidated sleep bouts (Luo *et al.*, 2012). Given the numerous consequences of sleep disorders and mistimed sleep on health and cognitive function, circadian reinforcement presents a potential option to increase sleep quality and regulate the timing of sleep in older individuals to mitigate adverse health impacts. In humans, the use of bright light therapy in the morning has been used for decades to strengthen circadian entrainment for seasonal affective disorder and other mood disorders (Pail *et al.*, 2011; Oldham & Ciraulo, 2014). Circadian genes and the circadian clock also have been implicated as a neuroprotective buffer against age-induced brain pathologies (Musiek *et al.*, 2013; Musiek & Holtzman, 2016; Musiek *et al.*, 2018). Weak or fragmented circadian rhythm may be a predictive biomarker for Alzheimer's

disease and increase susceptibility to age-related neurodegeneration (Musiek *et al.*, 2013; Musiek & Holtzman, 2016; Musiek *et al.*, 2018). Recently the potential for bright light therapy with age-related neurodegenerative disorders to reinforce circadian rhythms has also been tested. Studies in individuals suffering from Alzheimer's and Parkinson's disease found bright light therapy to be effective in reducing the severity of some disease symptoms. Short exposures of bright light appear to improve sleep wake rhythms as well as partially rescue the deficits in performance on cognitive and pathology tests (Yamadera *et al.*, 2000; Skjerve *et al.*, 2004; Videnovic *et al.*, 2017).

The circadian system and metabolism are tightly intertwined as are the connections between metabolism and longevity. Time-restricted feeding paradigms in which food delivery is limited to the active period strengthens entrainment and synchronization of circadian oscillators. In *Drosophila*, seven days of time-restricted feeding in middle-aged flies (35 days old) improved sleep-wake activity cycles (Gill *et al.*, 2015). When middle-aged flies were switched to time restricted feeding for two weeks, age-related declines in cardiac function were significantly attenuated compared to age-matched controls (Gill *et al.*, 2015). Moreover, the suppression of cardiac aging was associated with temporal gene expression and was dependent upon a functional circadian clock (Gill *et al.*, 2015). Time-restricted feeding, a paradigm that entrains the liver circadian clock in mammals, synchronized with activity has been used in numerous rodent studies to minimize chronic metabolic conditions often associated with aging (Manoogian & Panda, 2017). Time-restricted feeding has also been used in a mouse model of Huntington's disease to restore molecular circadian rhythms and behavioral activity rhythms (Morton *et al.*, 2005; Maywood *et al.*, 2010). An interactive approach in which people used their smartphones to monitor eating patterns and then shift the timing of food consumption to avoid late night eating was found to improve sleep patterns and metabolism (Gill & Panda, 2015). These studies suggest that circadian reinforcement with time-restricted feeding may be a feasible option to mitigate age-related declines in circadian function and manage chronic conditions. Time restricted feeding has also been suggested as a possible therapeutic option with Alzheimer's disease (Kent, 2014).

One of the earliest studies linking caloric restriction to increased longevity was published in 1947 (Ball *et al.*, 1947) with more than 1500 papers published since then investigating the parameters and mechanisms through which dietary restriction affects longevity demonstrating the phylogenetic conservation of the advantages of caloric restrictions. Recent research in *Drosophila* and mammals suggests that caloric restriction enhances the core circadian oscillator function in aged animals. Young flies subject to caloric restriction displayed enhanced circadian rhythms with increased peak expression for *tim*, *per*, *Pdp1e*, *vri*, and *Clk* mRNAs as well as higher amplitude PER and TIM protein rhythms (Katewa *et al.*, 2016). Although the oscillations were dampened with aging, middle-aged flies (33 days old) on dietary restriction exhibited significantly higher oscillations of all the clock genes compared to middle-aged flies on an *ad libitum* diet (Katewa *et al.*, 2016). A functional circadian oscillator was found necessary for the benefit of dietary restriction on lifespan as flies with functional circadian clocks showed significantly greater lifespan extension compared to flies with mutations in *per* and *tim* or flies rendered arrhythmic due to constant light conditions (Katewa *et al.*, 2016). It should be noted that the necessity of a circadian clock for longevity benefits of dietary restriction may depend upon environmental

factors or microbiota as other researchers have found that dietary restriction increases lifespan even in the absence of a functional circadian clock (Ulgherait *et al.*, 2016). In mammalian studies, mice exposed to caloric restriction were found to self-impose a temporal restriction of feeding consolidating their feeding patterns (Patel *et al.*, 2016b; Acosta-Rodríguez *et al.*, 2017), although differences in gene expression between caloric restriction and time-restricted feeding can be observed (Patel *et al.*, 2016b). Caloric restriction significantly increases the rhythm amplitude and peak mRNA expression levels for several core clock genes including *Bmal1*, *per1* and *per2* (Patel *et al.*, 2016a). Similar to what has been observed in *Drosophila* for core clock mutants, the efficacy of caloric restriction is also impaired in *Bmal1* knockout mice (Patel *et al.*, 2016a). Thus, caloric restrictions appear to enhance the robustness of the circadian system and this reinforcement may provide an explanation, at least in part, for how dietary restriction increases longevity. Several recent reviews provide more detailed information and comparisons of timed feeding and caloric restriction on metabolism and longevity (Froy & Miskin, 2007; Taormina & Mirisola, 2014; Longo & Panda, 2016; Kapahi *et al.*, 2017; Manoogian & Panda, 2017; Giebultowicz, 2018).

The timing of activity and exercise has been suggested as another mechanism through which the circadian system may be potentially strengthened to mitigate age-related declines, as exercise has been shown to increase the rate of resynchronization after light-induced phase-shifts in young female mice (Castillo *et al.*, 2011). However, the efficacy of exercise to strengthen circadian rhythms under normal physiological conditions associated with aging is unclear. In *Drosophila*, forced daily morning exercise, using a 180° vial rotation protocol that takes advantage of the flies' natural negative geotaxic behavior, had no effect on age-related locomotor activity declines or longevity (Rakshit *et al.*, 2013). In aged mice, voluntary exercise with a running wheel increased the proportion of mice exhibiting rhythmic locomotor activity reflecting a strengthening of the circadian system (Leise *et al.*, 2013). Voluntary exercise also ameliorates some age-related impairments associated with phase-shifts of the light-dark cycle by increasing the rate of resynchronization between activity rhythms and the new light-dark cycle as well as the resynchronization of peripheral oscillators, although aged mice still take longer to phase-shift than young mice (Leise *et al.*, 2013). In addition to the health benefits of an active lifestyle, daily exercise on a fixed schedule may promote circadian function to sustain healthy aging. Overall, the research described above highlights the promise of therapeutic options targeted to support the circadian system as viable options for the management of age-related pathologies and chronic conditions.

Future Perspective

In the past two decades, advances in circadian research have underscored the importance of circadian function for health and the consequences of circadian dysfunction in disease. With age, circadian function declines in multiple ways impacting molecular, physiological and behavioral rhythms as well as increasing disease risk and exacerbating neuropsychiatric disorders. However, we still do not understand how aging targets the circadian system at the molecular level. More research is necessary to characterize the impact of aging on the molecular rhythms of clock-controlled target genes in individual tissues to identify

molecules and nodes for potential manipulation. We do not yet have a clear understanding of how aging interferes with neural circuit level communication between circadian pacemakers or at the systems level with synchronization between central and peripheral oscillators. On the reciprocal side, research defining how circadian decline contributes to cellular aging and increases disease risk and neurodegeneration is also in its early stages. Given the reciprocity between the circadian clock and healthy aging, answering these questions appears critical for improving public health.

Despite the importance of understanding the bidirectional interactions between the circadian system and aging, there are challenges for performing age-related studies including the expense and time required for studies using aged rodents or mammalian models. *Drosophila* as a model system, with its neurogenetic tools and ease of molecular analysis, should provide a tremendous asset in future research of circadian and aging interactions. The low cost of animal husbandry and relatively short life-span allow experimenters to conduct complex genetic experiments in a fraction of the time compared to similar experiments in vertebrate models providing a foundation for rapid translation into rodent models. The next few years will undoubtedly bring significant advances in understanding the interactions between the circadian system and healthy aging with exciting possibilities including the development of future therapies using circadian reinforcement as a potential treatment for mitigating age-related physiological vulnerabilities.

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Abbreviations:

Bmal1

Brain and Muscle ARNT-like protein 1

dClk or *mClk*

Drosophila clock or mammalian *clock*

dCry or *mCry* (1 or 2)

Drosophila cryptochrome or mammalian *cryptochrome* (1 or 2)

dCyc

Drosophila cycle

Gcl

Glutamate cysteine ligase

LD

Light-dark

dPer, *mPer1*, *mPer2*

Drosophila period gene, mammalian *period* 1 or 2

PDF

Pigment dispersing factor

SCN

Suprachiasmatic nucleus

tim*timeless* gene**VIP**

Vasopressin intestinal peptide

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Table 1:Approaches for Reinforcement of the Circadian System in Aging *Drosophila*

Genetic rescue of circadian function				
Gene	Problem	Manipulation	Outcome	Reference
<i>pdf</i>	Aging related reductions in circadian rhythmicity	Overexpression of <i>pdf</i> in clock neurons	Increased rhythmicity, restoration of TIM levels in older flies	Umezaki et al., 2012
<i>per</i>	Increased neurodegeneration in <i>per⁰¹</i> flies	Rescue of <i>per</i>	Increased resistance to hypoxia and oxidative stress, reduction in neuronal degeneration	Krishnan et al., 2008
<i>cry</i>	Reduced <i>cry</i> mRNA expression and protein levels with aging	Overexpression of <i>cry</i> in all clock cells in the body	Increased resistance to oxidative stress. Increased rhythmicity and lifespan	Rakshit et al., 2013
<i>tim</i>	Age-related problems with metabolism	Overexpression of <i>tim</i> in peripheral tissues	Increased lifespan, metabolism and resistance to pathogens on a protein rich diet	Katewa et al., 2016
Environmental reinforcement of circadian clock				
Paradigm	Problem	Manipulation	Outcome	Reference
Time restricted feeding	Reduced sleep in older flies and deterioration of cardiac function	Food restricted to 12 h/day during light cycle	Improved sleep/activity rhythms and decreased cardiac aging	Gill et al, 2015
Dietary Restriction	Reduced molecular rhythms of clock genes with aging	Ten days feeding with dietary restriction media	Increased molecular oscillations of core clock genes; extended lifespan	Katewa et al., 2016
Temperature entrainment	Disrupted sleep-wake rhythms with aging	Coupled entrainment of light and thermal cycles	Improved sleep and locomotor activity rhythms	Luo et al., 2012

Table 2:

Enhancement of Circadian Function in Aging Mammals and Humans

Genetic rescue of circadian function				
Gene	Problem	Manipulation	Outcome	Reference
<i>Bmal1</i>	Decreased longevity in mutant mice	Restoration of <i>Bmal1</i> in brain	Improved survival in 75% of mice	McDearmon et al. 2006
		Restoration of <i>Bmal1</i> in muscles	Increased longevity in 100% of mice	
Environmental reinforcement of circadian clock				
Paradigm	Problem	Manipulation	Outcome	Reference
Time restricted feeding	Disrupted entrainment to light	Restricted feeding for 2 h during the light cycle for 14 days	Aged rats entrained to feeding rhythms	Walcott and Tate, 1996
	Disrupted circadian rhythms in metabolism in Huntington's disease	Restricted feeding for 5 hr/day for 12 days in R6/2 Huntington's disease mouse model	Restoration of behavioral rhythms and hepatic circadian gene expression	Maywood et al., 2010
Activity dependent reinforcement	Disrupted circadian rhythms in peripheral tissues	Voluntary exercise in VIP mutant mice	Improved heart rate, body temperature and locomotion rhythms	Schroeder et al., 2012
	Age related fragmentation of activity rhythms and slow resetting of the clock during phase shifting	Voluntary exercise over a 2-month period.	Stronger circadian rhythms in locomotor activity, increased rate of internal synchronization following phase shifting and increased amplitude of <i>Per2</i> in SCN	Leise et al., 2013
Bright light therapy	Sleep disturbance and reduced performance on cognitive tests in Alzheimer's dementia patients	Bright light exposure for 2 h during early-mid day for 4 weeks	Improved circadian rhythms and performance on cognitive tests	Yamadera et al., 2000
	Poor performance on the Alzheimer's rating scale	Bright light exposure for 45 min every morning for 4 weeks	Increased performance	Skjerve et al., 2004
	Disturbed sleep/wake rhythms in PD patients	Light exposure 2 times daily in 1-hr intervals for 14 days	Improved sleep/wake rhythms, reduced daytime sleepiness and increased daytime activity	Videnovic et al., 2017