The Effects of the Immunity Factor *Relish* on Aging Phenotypes in *Drosophila melanogaster*

by
Nandita Kumar

A THESIS

submitted to

Oregon State University

University Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Microbiology
(Honors Scholar)

Presented March 4, 2016
Commencement June 2016

AN ABSTRACT OF THE THESIS OF

Nandita Kumar for the degree of Honors Baccalaureate of Science in Microbiology presented on March 4, 2016. Title: The Effects of the Immunity Factor *Relish* on Aging Phenotypes in *Drosophila melanogaster* .

Abstract approved: _____

Jadwiga Giebultowicz

Aging is a complex biological phenomenon that alters many different physiological processes. Although many age-related phenotypes, such as neurodegeneration, weakening circadian rhythms, and inflammaging, are well-studied, the links between them are not. To elucidate these links we created an inflammation-like state in *Drosophila melanogaster* by overexpressing *Relish (Rel)*, an NFκB transcription factor in the *Imd* innate immunity pathway. Then we observed how aging parameters such as lifespan, locomotor activity rhythms, and neurodegeneration were affected. We found that systemic *Rel* overexpression causes significant lifespan shortening and increased neurodegeneration. By overexpressing *Rel* specifically the gut, fat body, head fat body, or neurons, we found that these tissues were not responsible for the lifespan shortening that was observed with systemic *Rel* overexpression. While increased Rel shortens lifespan and increase neurodegeneration, it has no detrimental effects on rhythms of locomotor activity. We conclude that age-related increase in *Rel* expression in wild-type flies may be linked with accelerated aging and that *Rel* overexpression could serve as possible model to study mechanisms of inflammaging in *Drosophila*. Further experiments are needed to understand why *Rel* overexpression causes lifespan shortening and neurodegeneration, especially to investigate the possible role of the glia and genes downstream of *Rel*.

Key Words: aging, circadian rhythms, chronic inflammation, *Drosophila melanogster*, *Relish*, neurodegeneration, inflammaging, innate immunity

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

Nandita Kumar, Author

Acknowledgments

First and foremost, I would like to thank my thesis mentor, Dr. Jaga Giebultowicz. Jaga saw my enthusiasm for science when she interviewed me two and a half years ago, and gave me my first real job. She has been a mentor to me in so many ways, and has challenged me to reach my full potential. Working with Jaga has easily been the most educational experience in my college career, and she has taught me everything I know about professionalism and research. I am forever grateful to Jaga for giving me this opportunity.

Next, I would like to express my utmost gratitude to Eileen Chow. From the very first day that I started working it the lab, Eileen was so welcoming and answered every question I had with patience. Eileen is someone that I know I can count on for help inside and outside the lab. She has been a wonderful teacher, and made it a priority to explain the scientific reasoning behind everything she taught me. Eileen is a wonderful mentor and a caring friend: someone I will miss very much when I graduate.

I would like to thank Tara Bonar, my thesis twin. She and I started our research at roughly the same time, and she has always been a shoulder I can lean on. We have been a support system to each other throughout every step of this process. I would also like to thank all of my lab-mates, Barbara Gvakharia, Dani Long, Rachel Kuintzle, and Braden Mattison. They have all given so much support over the years. I would also like to thank Lizzy Sunderhaus and Marlène Cassar in Dr. Doris Kretzschmar lab at OHSU who have been very gracious in performing the neurodegeneration experiments included in this thesis.

Special thanks also go to Dr. Malcolm Lowry and Dr. Euan Allen for serving on my defense committee and taking the time to review my thesis.

Finally I would like to thank URSA, the DeLoach Work Scholarship, the LIFE Scholars Program, and the Honors Experience Scholarship for funding this experience and research. I am so grateful to all the donors who recognize the importance of undergraduate research.

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1. Introduction

1.1 The Multifaceted Role of Circadian Rhythms

Circadian rhythms are endogenous oscillations of biological processes with a circa 24 hour period. Circadian rhythms are controlled by the circadian clock, which is the molecular mechanism within organisms that is responsible for producing and regulating circadian rhythms. Circadian rhythms are known to exist in both evolutionarily simple life-forms such as bacteria, as well as complex mammalian organisms. These endogenous rhythms keep organisms in tune with light-dark cycles of 24 hours that naturally occur on Earth. Strong circadian rhythms have been shown to be important in many different biological functions such as sleep, metabolism, and cognition (Akerstedt, 2003, Delezie et al., 2011).The weakening or genetic disruption of circadian rhythms has been linked to impairments of key biological functions. For example, shift workers, whose circadian rhythms are disrupted, have a greater risk of cardiovascular disease, obesity, and diabetes (Maury et al., 2010). Since impaired circadian rhythms are correlated with many diseases, understanding their multifaceted role of the circadian system in biological processes is very important.

1.2 A Macroscopic View of Circadian Rhythms

To be considered a circadian rhythm, a biological process must be an endogenous, but entrainable rhythm with a circa 24 hour period. Therefore, all circadian rhythms have three main components, an input pathway that transmits environmental cues, a circadian oscillator that can respond to cues, and output pathways that respond to changes dictated by the circadian oscillator (Yu et al., 2006). The most important environmental cue is light because most circadian oscillators are entrained by light. Output pathways often

affect important physiological functions such metabolism, hormone secretion, and body temperature (Meyer-Bernstein et al., 2001). Behavioral functions such as locomotor activity, feeding, and sleep are affected by output pathways of circadian oscillators.

On an organismal level, the central clock in mammals is located in suprachiasmatic nucleus (SCN), which is a cluster of around 10,000 neurons located in the hypothalamus (Williams et al., 2001). These neurons have the ability to synchronize their rhythms, and can be entrained to light entering to retina. Many peripheral tissues such as the liver and testis also maintain their own circadian clocks that are synchronized by the central clock (Okamura, 2004). Similar to mammals, *Drosophila* have a central clock that is located in a network of lateral and dorsal neurons, and peripheral clocks in tissues such as the male reproductive system, fat body, and gut (Meyer-Bernstein et al., 2001, Giebultowicz, 2001). Unlike in mammals, peripheral clocks in *Drosophila* are directly entrained by light (Ivanchenko et al., 2001).

On a cellular level, both mammalian and *Drosophila* cells maintain their own cell-autonomous circadian clocks using interlocking molecular pathways. How synchrony is achieved between the SCN and peripheral clocks in individual cells is not well understood (Okamura, 2004). However, the molecular mechanism used by cells to maintain their clocks is well understood. We will first discuss the circadian molecular mechanism in *Drosophila*, and then compare it to the mammalian circadian mechanism.

1.3 The Molecular Circadian Clock Mechanism in Drosophila melanogaster

The circadian clock in the fruit fly is controlled by a molecular feedback loop. In a molecular pathway, a gene is expressed when a transcription factor binds DNA in the gene's promoter region. Then, the gene is transcribed into mRNA by RNA polymerase

and transported outside of the nucleus where it is translated into protein. In the clock molecular mechanism of *Drosophila melanogaster*, the clock genes *Clock* (*Clk*) and *Cycle (cyc*) encode the transcription factors CLK and CYC. CLK and CYC heterodimerize in the nucleus and promote the expression of two other clock genes *period* (*per*) and *timeless* (*tim*) by binding to DNA sequences called the E-boxes in the promoter regions of these genes. The *per* and *tim* genes encode the proteins PER and TIM respectively. PER and TIM have been shown to act as regulators, and affect the expression of other clock-controlled genes. Very importantly, they are involved in creating a negative feedback loop to inhibit the transcriptional activity of the CLK/CYC heterodimer, thereby stopping the production of their own mRNA. This inhibitory process occurs when PER and TIM heterodimerize, localize within the nucleus, and bind to CLK/CYC preventing their transcriptional activity (Hardin, 2011)(Figure 1).

Although circadian rhythms are endogenous, the expression of clock genes is entrained to light/dark cycles via an important clock-associated gene called *cryptochrome* (*cry*). When protein CRY is activated by sunlight, it binds to TIM causing its breakdown, thereby stopping the PER/TIM heterodimer from forming. Since the PER/TIM heterodimer no longer inhibits the CLK/CYC heterodimer, *per* and *tim* expression is activated again, thus restarting the molecular clock mechanism (Figure 1). Since *cry* is activated by sunlight, its expression is greatest in the morning hours and PER/TIM levels are greatest in the evening hours (Hardin, 2011). These changes in clock gene expression levels allow the organism to align with the 24 hour light/dark cycles present on Earth (Allada et al., 2010). In summary, the *Drosophila* circadian molecular mechanism

consists of a negative feedback loop that is directly affected by light. We will now consider how the *Drosophila* molecular clock compares to the mammalian clock.

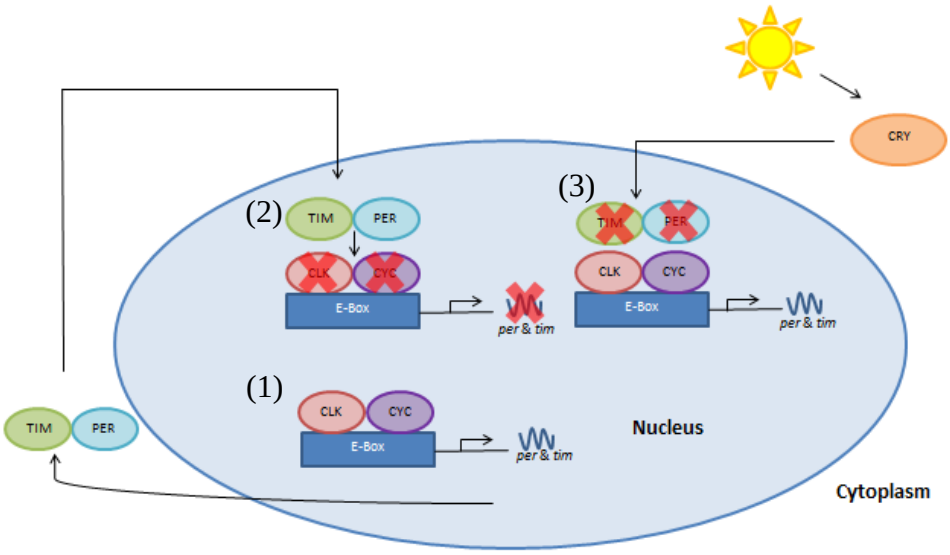

Figure 1: The Circadian Clock Mechanism in *Drosophila melanogaster*. (1) The heterodimer of clock (CLK) and cycle (CYC) proteins promotes the expression of *period* (*per*) and *timeless* (*tim*) genes. (2) Period (PER) and timeless (TIM) proteins heterodimerize and inhibit the transcriptional activity of the CLK/CYC heterodimer, creating a negative feedback loop.(3) The cryptochrome (CRY) protein is activated by sunlight and degrades TIM allowing the CLK/CYC heterodimer to resume transcription of *per* and *tim* expression. *Cryptochrome* therefore aligns endogenous circadian rhythms with light/dark cycles.

1.4 The Circadian Clock mechanism is Conserved between insects and humans

Although mammals are vastly more complex than *Drosophila*, their circadian molecular pathways are evolutionarily conserved to a large extent. We have already discussed that the circadian organization in mammals and *Drosophila* is quite similar at the organismal level. The molecular circadian mechanisms of mammals and *Drosophila* are also very similar since both use interlocking feedback loops to created oscillatory rhythms (Hardin et al., 2013). Like *Drosophila*, the mammalian mechanism is also controlled through the transcription and translation of clock genes. The mammalian mechanism contains many orthologs of *Drosophila* clock genes (Hardin et al., 2013).

Notably, BMAL-1 is the ortholog *of the cycle* gene, and the mPER/mCry heterodimer is the ortholog of the PER/TIM heterodimer in *Drosophila*. Many of the *Drosophila* clock genes have more than one functional equivalent in the mammalian mechanism.

One large difference between the mammalian and *Drosophila* circadian molecular mechanisms is the way they are affected by light. The *Drosophila* circadian pathway uses the light-dependent degradation of TIM, which cause repression of the mechanism during light hours. However in mammals, light causes *mPer1* (*period* homolog) expression to increase, which causes the mechanism to be activated during light hours. Therefore light causes *mPer1* activation in mammals and *per* repression in *Drosophila* which is a major difference in the mechanisms (Hardin et al., 2013).

Although the mammalian circadian system is slightly more complex, the core clock mechanism and organismal structure in *Drosophila* are very similar. This allows us to use *Drosophila* as model to investigate circadian rhythms and their functions.

1.5 Weakening Circadian Rhythms and Premature Aging

Studying circadian rhythms is very important because many physiological functions are affected by output pathways of circadian oscillators. Therefore, weak circadian rhythms are detrimental to healthspan. Particularly, the circadian clock is known to weaken with age, and weakening circadian rhythms lead to premature aging in mammals (Kolker et al., 2003). Mice without BMAL1 (*Cycle* homolog) expression have been shown to have shorter lifespans and other age-related symptoms (Kondratov et al., 2006). Similar findings have been reported in *Drosophila* as well. Specifically, the negative feedback of the clock mechanisms has been shown to be altered by age (Krishnan et al., 2012). In response to these alterations, behavioral rhythms in

Drosophila were also shown to decrease when the clock genes are expressed at lower levels (Krishnan et al., 2012). Additionally flies with mutated clock mechanisms were shown to have slower climbing times and other behavioral impairments with age (Krishnan et al., 2009). Taken together, these findings suggest that circadian rhythms naturally weaken with age and weakened circadian rhythms leads to accelerated aging. Understanding the links between circadian rhythms and aging could help to uncover the mechanisms of age-related diseases.

1.6 Inflammaging: An Age-Related Phenomenon

As discussed above, the weakening of circadian rhythms is an age-related phenomenon. Another phenomenon detrimental to aging is inflammaging, the increase in chronic inflammation with age. Inflammation is a complex immune response that is mounted to combat pathogens and repair damaged tissues. Therefore innate immunity is very important to maintaining health. However, inflammatory responses are known to occur and reoccur even when the organism is not presented with pathogenic threat, known as chronic inflammation. Chronic inflammation is detrimental to health because healthy tissues are often damaged when excessive inflammatory responses occur. Since chronic inflammation is detrimental to health, inflammaging is detrimental to healthy aging. The role of inflammation in aging is a well-studied phenomenon in (Franceschi et al., 2014). For example, increase in Il-6, a cytokine biomarker of inflammation is correlated with many aging phenotypes (Franceschi et al., 2014)This increase in chronic inflammation with age is thought to be a contributing factor to many age-related diseases in mammals such as atherosclerosis, obesity and type 2 diabetes (Salminen et al., 2012). While correlative links between chronic inflammation, and aging are well established in

mammals, the mechanisms of these phenomena are not well understood. Since weakening of circadian rhythms and elevated chronic inflammation are both age-related phenomena, it is important to understand the links between them.

Innate immunity and circadian rhythm mechanisms are well studied in *Drosophila* and are greatly conserved will mammals. Therefore *Drosophila* could be used as model organism to elucidate the age-related links between circadian and the immune system. To this end, we will now explore the *Drosophila* immune system to better understand how a chronic inflammation-like state may occur in fruit flies.

1.6 Innate Immunity Pathways in *Drosophila*

Mammalian immune systems combat pathogens using two types of immunity: innate and adaptive immunity. Innate immunity is the first line of defense used by the immune system to combat broad range of pathogens. Adaptive immunity is the secondary response used to target specific antigens by creating immunological memory of an antigen following initial exposure. Like all invertebrates, the *Drosophila* immune system only uses innate immunity for pathogen defense. Innate immunity is considered a culprit of inflammaging, so the lack of adaptive immunity in the *Drosophila* makes it a simple model to study this phenomenon (Franceschi et al., 2014)

In *Drosophila*, there are two main pathways that are used for innate immune response, the Toll pathway and the Imd pathway (Fig.2). The Toll pathway is activated by the presence of fungi or Gram-positive bacteria, whereas the Imd pathway is activated by Gram-negative bacteria. Both of these pathways lead to the production of anti-microbial peptides (AMPs), which are effector molecules that attack the pathogen. The exact mechanism by which the attack is mounted is not well understood, but AMPs are

known to puncture the membranes of pathogen and cause cell lysis. Different AMP genes are transcribed by different pathways. For example *Drosomycin* transcription is associated with the Toll pathway, whereas *Diptericin* transcription is associated with the Imd pathway. Some AMPs are transcribed through both pathways (Williams et al., 1997).

Expression of AMPs is stimulated by a cascade of transcription factors. An important transcription factor in the Imd pathway is *Relish* (*Rel*). This transcription factor is activated when the membrane receptor PGRP-LC is stimulated by a pathogen. This triggers the IMD protein to bind with Fadd, which then triggers the binding of the caspase DREDD. DREDD then cleaves off a region in the N-terminus of REL, causing the activation of REL. REL then enters the nucleus and induced the transcription of AMP genes to combat the pathogen. (Meister et al., 2004)

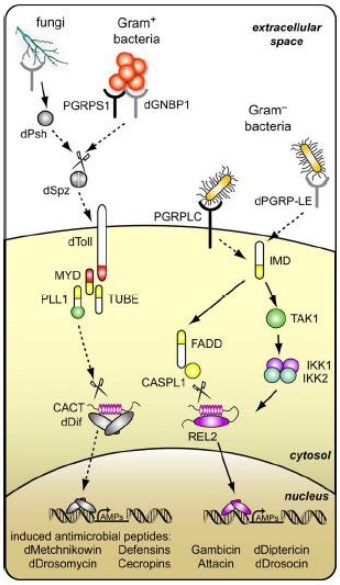

Figure 2: Toll and Imd Pathways in *Drosophila melanogaster*. *Drosophila* possesses two major innate immunity pathways called Toll and Imd. The Toll pathway is shown on the left where Gram-positive and fungi stimulate the Toll receptor which eventually causes the NFκB transcription factor Dorsal-related immune factor (*Dif*) to be activated. The activated *Dif* leads to the transcription of many antimicrobial peptides. The Imd pathway is shown on the right where Gram-negative bacteria stimulate the PGRP-LC membrane receptor. This stimulation eventually leads to the activation of the *Relish* transcription factor. Relish then induces the expression of antimicrobial peptides (after Meister et al., 2004).

The innate immunity pathways are conserved between *Drosophila* and humans. The Toll pathway is a homologue of the Toll pathway in humans, and was actually discovered first in *Drosophila melanogaster* (Brennan et al., 2004). One difference between the human Toll pathway and the *Drosophila* Toll pathway is that in humans microbial antigens are directly presented to Toll-like receptors, whereas *Drosophila* uses the endogenous ligand called Spätzle. The Imd pathway is thought to be similar to the tumor necrosis factor (TNF) receptor pathways in humans. *Relish* is a homologue of NFκB in humans, which is important in the production of cytokines. Five NFκB proteins exist in humans that all have highly conserved *Relish* homology domains (Courtois et al., 2006). Because *Drosophila* and human innate pathways are highly conserved, *Drosophila* may be a good model for chronic inflammation research.

1.7 The Effect of Inflammation on Neurodegeneration

Innate immunity in *Drosophila* has been well studied in the context of pathogenic defense. How innate immunity pathways in *Drosophila* relate to inflammaging is not well understood. However, chronic inflammation in mammals and *Drosophila* is known to play a role in neurodegeneration, another age-related phenotype. Neurodegeneration in mammals plays a large role in neurodegenerative diseases, such as Alzheimer's disease (Hung et al., 2010). The role of inflammation on neurodegeneration has been shown in a few previous studies (Amor et al., 2014) (Gonzalez-Scarano et al., 1999). In mammals, innate immune responses are reported to be involved in both repair and damage processes linked with neurodegenerative disease. Innate immune response can aid repair because it

can clear apoptotic cells and lead to tissue homeostasis. However, damage processes are also linked with immune response because prolonged immune response can damage healthy tissue: exacerbating neurodegeneration (Amor et al., 2014). Particularly in the case of Alzheimer's disease, immune cells in the central nervous system called microglia are thought to have a role in neurodegeneration, although their exact effects are not known. (Gonzalez-Scarano et al., 1999). Because of its importance to human health, we would like to explore the effects of inflammaging on neurodegeneration in *Drosophila*.

Like mammals, innate immunity pathways in *Drosophila* have also been linked to neurodegeneration. Specifically, *Relish* has recently be shown to contribute to neurodegeneration in a study showing that neurodegeneration was dependent on *Relish*, and that overexpression of AMPs in the brain alone can result in neurodegeneration (Cao et al., 2013). Another study about ataxia-telangiectasia (AT), a condition that impairs motor function due to neurodegeneration was conducted using *Drosophila*. AT was modelled in *Drosophila* by reducing the expression of an ATM kinase which is responsible for cell cycling, DNA repair, and programmed cell death in the brain. In this experiment, it was shown that the reduced expression of this kinase cause the increased expression of innate immunity genes (Petersen et al., 2012). This shows that the induction of neurodegeneration in the brain activates innate immune response. Taken together,

these studies show a possible link between innate immunity response and neurodegeneration in *Drosophila*.

1.8 The Link between Circadian Rhythms and Neurodegeneration

As discussed above, chronic inflammation plays a role in neurodegeneration. Another age-related phenotype linked to neurodegeneration is weakening circadian rhythms. In mammals, mutation in BMAL1 (CYC homolog) have not only been linked to accelerated aging, but also to increased levels of neurodegeneration (Musiek et al., 2013). Similar links between neurodegeneration and circadian rhythms have been observed in *Drosophila*. In a recent study, a *Drosophila* model of Alzheimer's disease that causes aggregates of amyloid peptides in the brain, like those found in Alzheimer's disease, was used to investigate any possible links to the circadian clock. This study found that clock mutants did not show greater levels of neurodegeneration, but *Drosophila* with the AD model showed high levels of neurodegeneration and greatly impaired circadian rhythms with age (Long et al., 2014). Another study showed that *Drosophila* mutants prone to greater neurodegeneration had shorter lifespans, reduced climbing ability, and greater neurodegeneration when a clock mutation was introduced (Krishnan et al., 2012). Taken together, these studies show that mutations in the clock mechanism not only accelerate aging, but also increase levels of neurodegeneration. Although the circadian clock seems to have a role in neurodegeneration, the mechanism of this interaction has yet to be deduced.

1.9. Inflammation, Circadian Rhythms, and Neurodegeneration: An Aging Trifecta?

Preliminary research and recent studies have shown that aging is linked to inflammation, weakening circadian rhythms, and neurodegeneration in mammals and

Drosophila. Chronic inflammation is known to play a role in neurodegeneration, and neurodegeneration and weakening circadian rhythms have been linked. However, the relationship between these three age-related phenotypes has yet to be explored. Links between chronic inflammation and weakening circadian rhythms are especially unclear. Additionally, there is currently no *Drosophila* model for chronic inflammation since innate immunity has mostly been studied in the context of pathogen defense. Therefore, the primary goal of this project is to explore *Drosophila* as a model for inflammaging, and assess the effects of age-related parameters on this model. Secondarily we used this model to explore possible links between chronic inflammation and weakening circadian rhythms and other aging parameters.

We used the NFκB transcription factor *Relish* to model inflammaging in *Drosophila*. As discussed above *Relish* has been shown to be involved in age-related phenotypes, especially neurodegeneration. The expression of *Relish* has also been shown to increase with age using RNA-seq data collected in collaboration with Dr. David Hendrix (Rachael Kuintzle, personal communication) (Fig.3).The increase was confirmed by qRT-PCR (Eileen Chow, personal communication). Due to these indicators that *Relish* may play an important role in aging, we proposed that altering *Relish* expression may mimic inflammaging in *Drosophila*.

In the project, we altered *Relish* expression in *Drosophila* to create an inflammation-like state, and observe how this affects healthspan, circadian rhythms and neurodegeneration. Understanding the possible role of *Rel* in the age-related phenotypes will give a better understanding of how different aging processes are related. Since many

age-related diseases are affected by these phenotypes, understanding their relationships may help us to better understand why these diseases occur.

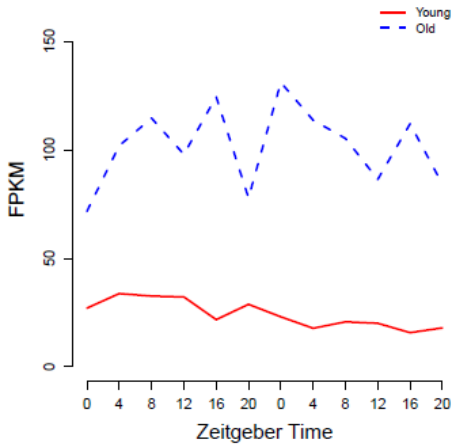

Figure 3. RNA-seq analysis of *Relish* expression around the clock. RNA-seq shows that *Rel* expression increase ~4 fold between young and old flies. Data contributed by Rachael Kuintzle and David Hendrix,

2. Materials and Methods

2.1 Fly Rearing and strains

In all experiments *white* (w^{1118}) genotype was used as the control wild-type strain. The UAS-Gal4 system was used for genetic crosses to altered *Relish* (*Rel*) expression. To increase *Rel* mRNA, we used UAS-*Rel* and to decrease *Rel*, we used a *Rel*-RNAi responder strain (Hedengren et al., 1999). In the experiments described in Section 3.1, the *tim-GAL4* driver was used, which is active in all central and peripheral clock cells (Kaneko et al., 2000). *tim-Gal4* was crossed with *UAS-Rel* to obtain *tim>Rel* flies or with *UAS-Rel-RNAi* to obtain *tim>Rel-RNAi*. Controls were *tim/+* (*tim-Gal4* crossed with *w*), *+/Rel* (*UAS-Rel* crossed with *w*), and *+/Rel-RNAi* (*UAS-Rel-RNAi* crossed with *w*), where + indicates *w* strain. Both driver and responder flies were backcrossed to the *w* stock for 8 generations to equalize their genetic background.

For the experiments performed in Section 3.2 the RU-486 gene switch system was used to over-express *Rel* in specific tissues only in adult flies. UAS-*Rel* flies were crossed to the following RU-inducible driver lines: *elav-GS* targeting neurons, *S32-GS* targeting head fat body, *S106-GS* targeting head and abdominal fat body, and *5966-GS* targeting gut cells. All fly lines were obtained from the Bloomington Drosophila Stock Center.

All flies used in the experiments from Section 3.1 were raised on a standard diet containing 1% agar, 6.25% cornmeal, 6.25% molasses, and 3.5% Red Star yeast at 2 1C. Experimental flies in Section 3.2 over-expressing Rel using RU-486 gene switch were fed the standard diet with a 50µM concentration of RU-486 in ethanol. Control flies were fed standard diet with same volume of ethanol as was used for the experimental flies. Flies were entrained to 12-hour light: dark (LD, 12:12) cycles (with an average light

intensity of ~1500 lx). The time at which the lights were switched on is denoted as Zeitgeber Time (ZT) 0 and the time they were switched off is denoted as ZT 12. Flies of a given genotype were mated, separated by sex 2-3 days after emerging, and transferred either to 8 oz. round bottom polypropylene bottles (Genesee Scientific, San Diego, CA) or wide 28.5mm diameter plastic vials, each containing 50 flies, or to narrow 25mm diameter plastic vials, each containing 25 flies, depending on the experiment. Both male and female flies were used for all experiments except locomotor activity and gene expression studies in Section 3.2.1 data where only males were used.

2.2 Longevity test

To determine differences in lifespan between crosses, the number of dead flies was recorded each time flies were transferred onto new diet (3 times a week). For each longevity experiment, ~200 males and females of a given genotype were used to monitor mortality until no individuals remained alive.

2.3 Quantitative Real-Time PCR

To verify altered *Rel* expression, *tim>Rel*, *tim>Rel-RNAi*, and *tim>+*, young males and females were collected and frozen. To verify *Rel* over-expression in specific tissues, *elav>Rel*, *FB>Rel*, and *tim>+* males were fed RU or EtOH , until 30-day-old and then collected and frozen. Fly heads and bodies were separated using metal sieves frozen with liquid nitrogen. Total RNA was extracted from 50 heads or bodies, which were homogenized using a Kontes handheld motor in Trizol (Life Technologies, Grand Island, NY) followed by ethanol precipitation. Samples were treated with DNAse (Takara, Mountain View, CA). DNAse was deactivated by phenol/chloroform extraction, and samples were purified with sodium acetate. RNA concentration was measured using

a Nanodrop spectrophotometer. Synthesis of cDNA was achieved with the iScript cDNA synthesis kit (BioRad, Hercules, CA) according to the manufacturer's protocol. Quantitative real-time PCR was performed with Power SYBR Green Master Mix (Life Technologies) on an Applied Biosystems Step-One Plus machine. Data from before backcrossing were normalized to the reference gene *rp49* and data from after backcrossing were normalized to *Dcp2*. All data were analyzed using the standard $2^{-\Delta\Delta CT}$ method. The following primers were obtained from IDT Technology (Coralville, IA): Relish forward: 5' GGCCATTCGACAGAACAAGT 3'; *Relish* reverse: 5' TGCCATGTGGAGTGCATTAT 3'; *Dcp2* forward: 5' CCAAGGGCAAGATCAATGAG 3'; *Dcp2* reverse: 5' GCATCGATTAGGTCCGTGAT 3' *rp49* forward: 5' GCCCAGCATACAGGCCCAAG 3' *rp49* reverse: 5' AAGCGGCGACGCACTCTGTT 3' . All primers used in this study had efficiency > 96%.

2.4 Locomotor Activity Measurements.

Flies were entrained in LD 12:12 at 25°C. Locomotor activity of 5- and 30-35-day-old males was recorded for 3 days in LD 12:12, followed by 7 days in constant darkness (DD) using the Trikinetics locomotor activity monitor (Waltham, MA). Actograms and periodograms (measuring average period in DD) were generated for individual flies using ClockLab software (Actimetrics; Coulbourn Instruments, Whitehall, PA). For a quantitative measure of circadian rhythmicity, fast Fourier transform (FFT) analysis was performed using ClockLab software. Flies with FFT values <.04 at a period of 24h were classified as arrhythmic. Flies with FFT values above .04 and a single peak in the

periodogram were classified as rhythmic and were included in the calculation used to determine the percent of rhythmic flies.

2.5 Neurodegeneration Measurements.

Neurodegeneration experiments were performed by Lizzy Sunderhaus in collaboration with the Dr. Kretzschmar Lab (Oregon Institute of Occupational Health Sciences, Oregon Health and Science University). Heads of ~50 day old flies were embedded in paraffin using methods described in (Kretzschmar et al., 1997). Next, the embedded heads were cut in 7mm 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). 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 the average vacuolized area was calculated as described in (Bettencourt Da Cruz et al., 2008).

2.6 Statistical analysis of data

All survival curves were generated using Kaplan-Meier survival analysis. Data comparing the genotypes and feeding regimes were analyzed using a Gehan-Breslow Wilcoxon Test to determine if there was a significant difference in lifespan among treatments compared to the control. All statistical analysis and graphs for this data were generated using GraphPad Prism 6 (San Diego, USA). In Section 3.1, an unpaired t-test with Welch's correction was used to determine significance between day 5 and day 35 FFT for locomotor activity. In Section 3.2, a two-way ANOVA test was used to

determine significance between day 5 and day 30 FFT for locomotor activity. Neurodegeneration was analyzed using unpaired t-test with Welch's Correction.

3. Results

3.1 Effects of Altered *Rel* Expression on Aging Parameters

The effects of NF-kB transcription factor *Relish* (*Rel*) on aging have not been studied previously; therefore, we genetically manipulated the expression of *Rel* in the fruit flies to determine its effects on different parameters of aging. We over-expressed *Rel* to simulate an inflammation-like state in all clock cells of the body by crossing *tim-Gal4* driver line with *UAS-Rel* responder line to obtain *tim-Gal4/ UAS-Rel* (*tim>Rel*) progeny. By measuring mRNA levels using qRT-PCR, we confirmed that *Rel* expression was increased strongly in *tim>Rel* flies compared to controls (Figure 4A). To study the effects of reduced *Rel* expression, we crossed the *tim-Gal4* driver line with *UAS-Rel-RNAi* responder line to obtain the *tim>Rel-RNAi* progeny. The *Rel*-RNAi construct reduces the translation of *Rel* by targeting and degrading *Rel* mRNA. We first confirmed that *Rel* expression was decreased in the progeny of this cross to less than 40% of controls by measuring mRNA levels using qRT-PCR (Figure 4B). We crossed driver *tim-Gal4* and both responders to *white* (*w* denoted as *+) to* obtain *tim/+, +/ Rel*, and +/*Rel*-RNA as our controls with unaltered *Rel* expression. These data confirmed that *Rel* was strongly over-expressed in *tim>Rel* flies and substantially reduced in *tim>Rel-RNAi* flies as compared to controls (Fig. 4). Because some of our initial experiments were performed before *UAS-Rel* and *UAS-Rel-RNAi* were backcrossed to *w*, we tested *Rel* expression in both scenarios and obtained similar data (Fig 4)

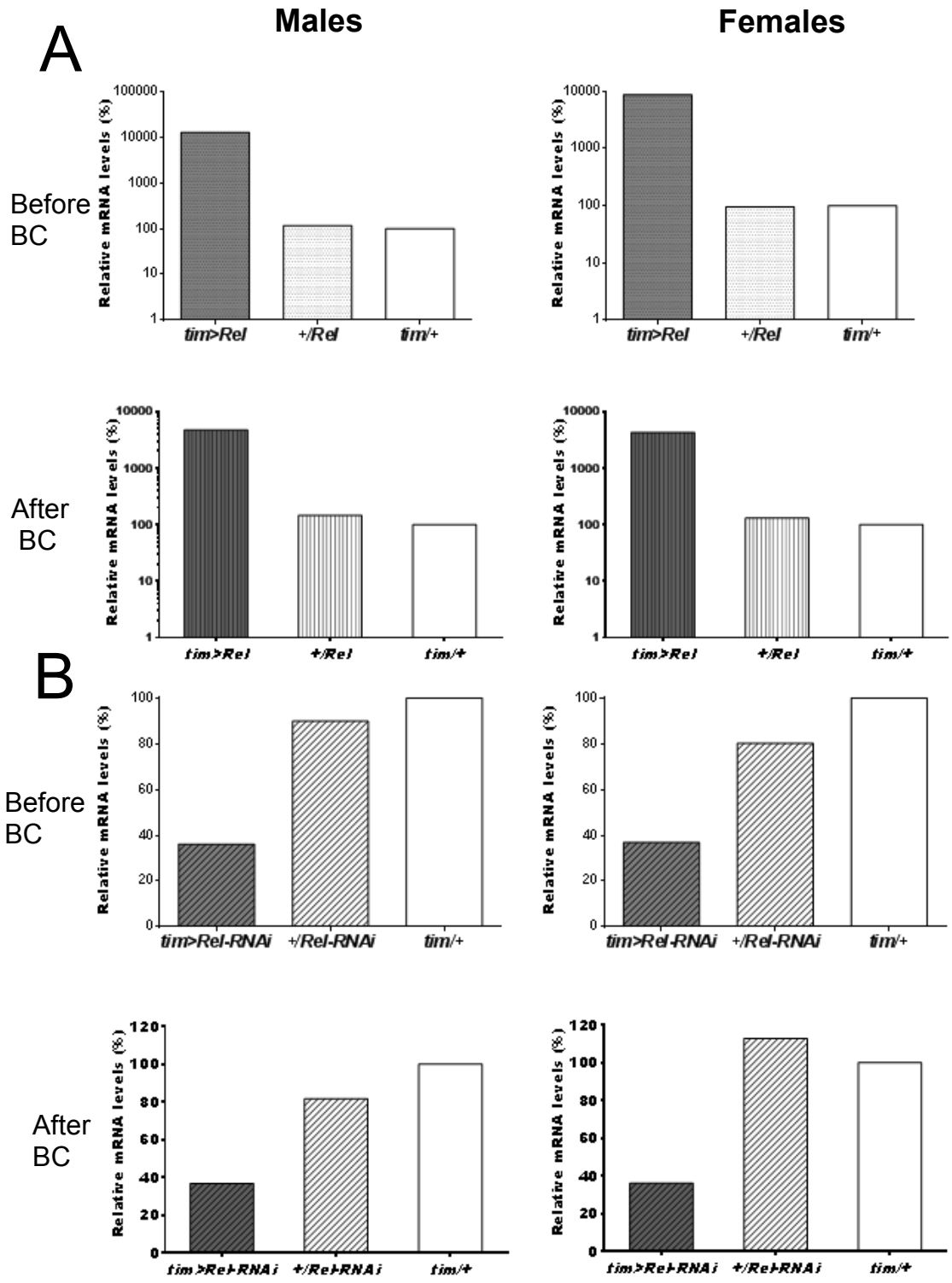

Figure 4. Verification of the effects of *Rel* manipulation. mRNA levels of *Rel* were measured using qRT-PCR in young males and females. *Rel* was strongly over-expressed in *tim>Rel* as compared to *+/Rel* and tim/+ controls (A). *Rel* was greatly inhibited by *tim>Rel-RNAi* as compared to *+/Rel-RNAi* and *tim/+* controls (B). BC denotes backcrossing. Expression analysis was done using the standard $2^{-\Delta\Delta CT}$ method. Data were normalized to *Rel* expression in *tim/+* control set as 100%.

3.1.1 The Effects of *Rel* on Longevity: *Rel* overexpression reduces Lifespan.

The first parameter that we used to measure the effects of *Rel* on aging was longevity. We measured the lifespan of *tim>Rel*, *tim>Rel-RNAi*, and *tim/+* males and females (Fig. 5). Statistical analysis of mortality curves using the Gehan-Breslow-Wilcoxon test showed that *tim> Rel* flies had significantly shorter lifespan than *tim/+* flies in both males and females (Table 1, p-value <0.0001). In contrast, the lifespan of *tim>Rel-RNAi* flies was modestly but significantly longer than in *tim/+* flies (p-value 0.0188) indicating the inhibiting *Rel* expression may promote longevity (Table 1). To verify these results, we performed a second experiment, which included additional controls, namely *white* flies crossed to *UAS-Rel* (*+/ Rel*) or to *Rel-RNAi (+/ Rel-RNAi*) (Figure 5B). We confirmed that flies over-expressing *Rel* lived significantly shorter (p<0.0001) than both control lines in males and females (Table 1). Reducing levels of *Rel* mRNA significantly extended lifespan of females compared to both controls, and males compared to one control but not the other (Table 1). Overall, these experiments strongly suggest that *Rel* over-expression significantly shortens lifespan in both sexes while inhibiting *Rel* expression may slightly lengthen lifespan.

Table 1. Statistical Comparison of Lifespan Differences due to altered *Rel* Expression

Exp	Genotype	Male lifespan	p-value	Female lifespan	p-value
1	*tim>Rel* vs. *tim/+*	shortened	<0.0001	shortened	<0.0001
	tim>Rel-RNAi vs.*tim/+*	lengthened	0.0465	no change	0.3976
2	*tim/+* vs. *tim>Rel*	shortened	<0.0001	shortened	<0.0001
	+/Rel vs. *tim>Rel*	shortened	<0.0001	shortened	0.0036
	tim/+ vs. *tim>Rel-RNAi*	lengthened	<0.0001	lengthened	0.0237
	+/Rel-RNAi vs. *tim>Rel-RNAi*	no change	0.0906	lengthened	0.0134

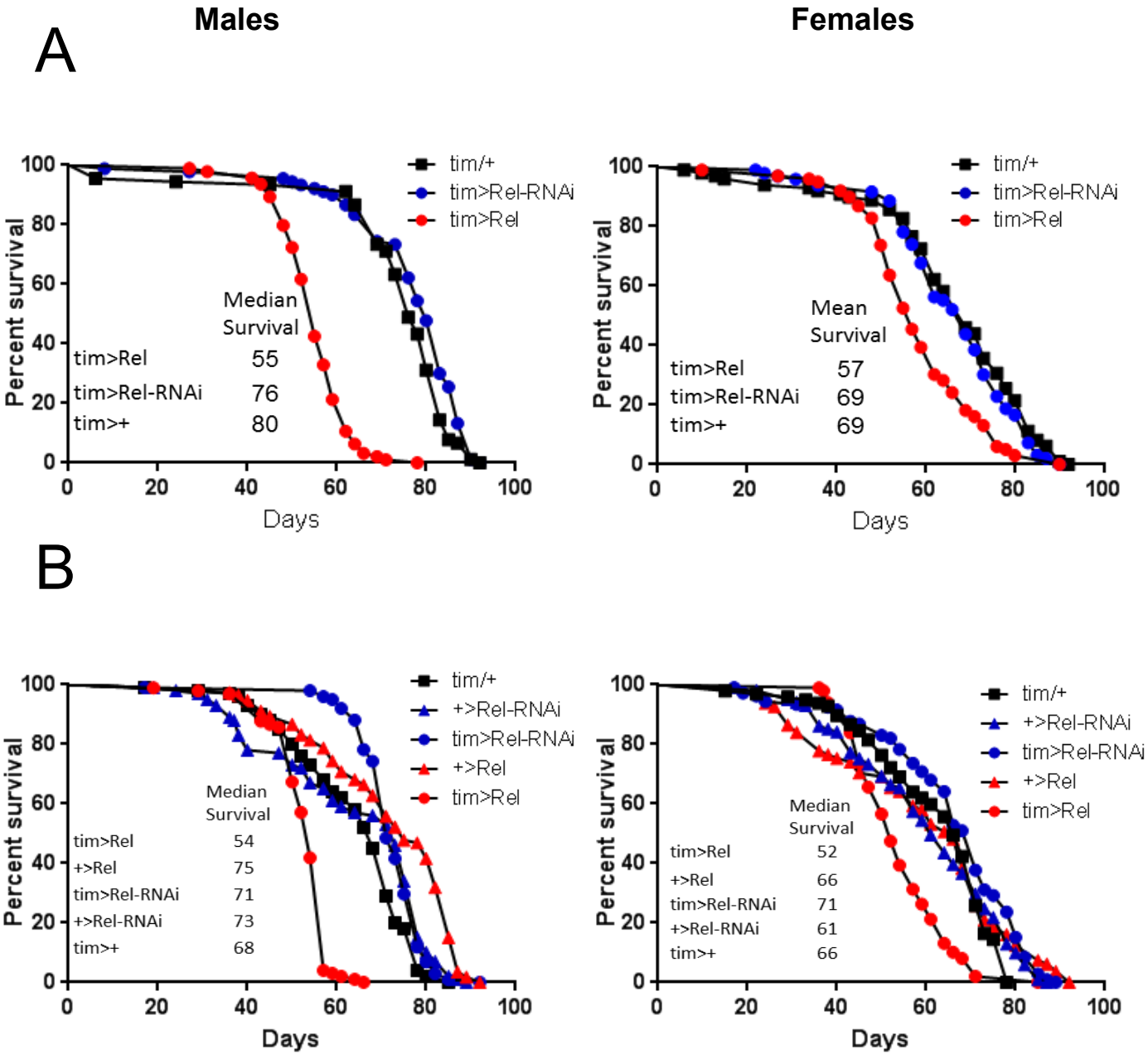

Figure 5. Lifespan of flies with manipulated Rel Expression. (A) The first experiment assessing longevity where *Rel* is over-expressed (*tim>Rel*) or inhibited (*tim>Rel-RNAi*) relative to control (*tim/+*) flies. The lifespan of *tim>Rel* is significantly shorter that *tim>Rel-RNAi* or *tim/+*. (B) The second experiment on longevity confirmed the findings of the first experiment. *+/Rel* and *+/Rel-RNAi* were added as controls and as expected showed lifespan similar to *tim/+*. Median survival of each genotype is indicated below graphs. For statistical analysis see Table 1 .

3.1.2 The Effects of Altered *Rel* Expression on Locomotor Activity: The Clock remains Intact

Since *Rel* over-expression was detrimental to longevity, we next investigated what factors may contribute to this phenotype. Given that circadian rhythms weaken with age and weak circadian rhythms may accelerate aging (Kolker et al., 2003), we hypothesized that *Rel* over-expression may disrupt circadian rhythms. To test if *Rel* over-expression affected circadian rhythms, we monitored the locomotor activity of flies whose *Rel* expression was elevated. We used males from the same cohort of flies used for the longevity experiments, and measured locomotor activity at day 5 (D5) and day 35 (D35) to see if circadian rhythms weakened with age in a *Rel*-dependent way. Flies were entrained for 3 days in LD and then transferred to DD to assess free-running locomotor rhythms which reflect the function of the central clock.

Analysis of data from two combined bio-repeats of locomotor activity showed that over-expressing *Rel* was not detrimental to circadian rhythms. The average period for all crosses was very close to 24hr, indicating that the central clock mechanism was intact. As expected, the *tim/*+control did experience deteriorating rhythms since the percent rhythmic flies and average FFT values were lower at D35 than D5. The *+/Rel* control also showed a decrease in % rhythmic flies and a slight, non-significant increase in FFT values between D5 and D35. Interestingly, the *tim>Rel* flies showed slight strengthening in rhythms with age since the percent of rhythmic flies increased and average FFT significantly increased between D5 and D35 (Table 2, p-value:0.0407). Taken together, these results indicate that deteriorations of central clock appear not to be responsible for effects of *Rel* over-expression on lifespan; in fact *Rel* over-expression seemed to slightly strengthen locomotor activity rhythms.

Table 2. Comparison of Locomotor Activity due to altered *Rel* Expression

Genotype	Age	N	% Rhythmic flies	Average FFT (Sig?[1]) (p-value)	Average Period[2]
tim>Rel	5	26	96%	0.053	23.81
	35	19	100%	0.078* (0.0407)	24.43
tim/+	5	25	100%	0.058	23.72
	35	39	74%	0.044 ns (0.0706)	24.36
+/Rel	5	13	100%	0.049	23.22
	35	20	80%	0.055 ns (0.5333)	23.65

[1] significance between D5 and D35 FTT using unpaired t-test with Welch's correction,
[2]free running period in DD

3.1.3 The Effects of Manipulating *Rel* Expression on Neurodegeneration

Since circadian rhythms were unaffected by manipulating levels of *Rel* expression, the question of why *Rel* overexpression shortens lifespan remained open. Previous research has shown that increased expression of pro-inflammatory factors such as *Rel* may play a role in neurodegeneration (Cao et al., 2013). We hypothesized that *Rel* over-expression could cause neurodegeneration which may be linked to shortened lifespan. The involvement of neurodegeneration in shortening lifespan is further supported by the fact that neurodegeneration is an age-related phenotype (Hung et al., 2010).

To test whether neurodegeneration may be a cause for the shortened lifespan that we observed in *tim>Rel* flies, we measured neurodegeneration in the brains of old *tim>Rel, tim>Rel-RNAi* and *tim/+* flies in collaboration with the Kretzschmar Lab at Oregon Health and Science University. These measurements showed that *tim>Rel* flies did exhibit significantly more neurodegeneration in the form of average vacoulized area in the brain than *tim/+* (p-value=0.0015) (Figure 6). *tim>Rel-RNAi* flies did not show a

significant difference in neurodegeneration when compared to the *tim/+* control (Fig. 6). Since we observed significantly increased levels of neurodegeneration in flies over-expressing *Rel*, we concluded that elevated activity of this gene is associated with neurodegeneration which may lead to lifespan shortening.

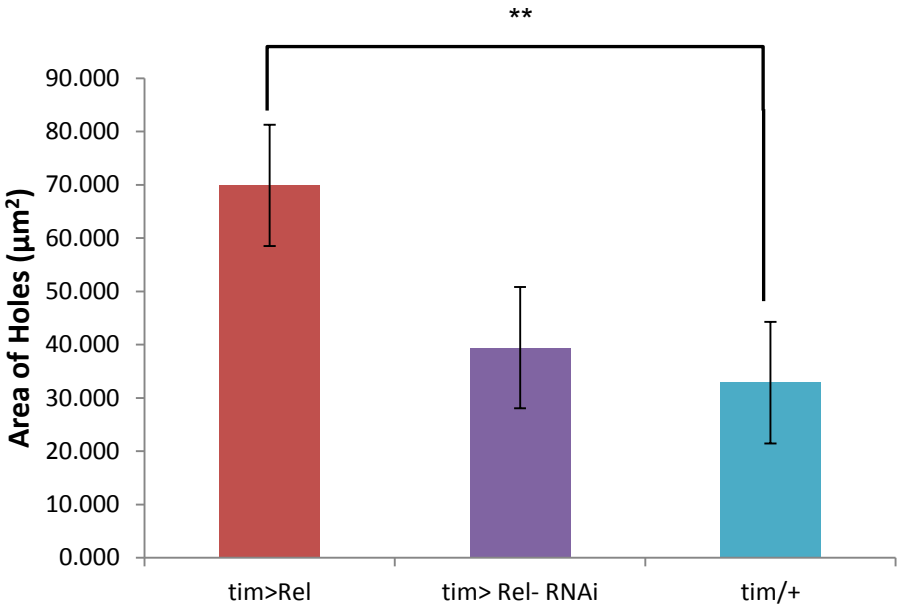

Figure 6. Neurodegeneration in flies with manipulated *Rel* expression. Neurodegeneration was measured in ~50 day-old flies as average area of holes (µm²). Analysis based on X-Y brain hemispheres showed significantly higher average levels of neurodegeneration in *tim>Rel* flies than *tim>+* flies (p=0.0015). *tim>Rel-RNAi* flies showed levels of neurodegeneration not significantly different from the *tim/+* control. Statistical analysis was done using unpaired t-test with Welch-s correction and error bars indicate SEM.

3.2 Effects of Altering *Rel* Expression in Specific Tissues on Aging Parameters

Experiments described in Sec. 3.1 showed that *Rel* over-expression significantly shortened lifespan and increased neurodegeneration. Because *tim* is expressed in multiple tissues, it could not be determined whether and how *Rel* over-expression in specific tissues contributes to lifespan shortening. To address this question, we examined how overexpressing *Rel* in specific tissues affects aging parameters. Since we showed a correlation between *Rel* overexpression and neurodegeneration, we used the *elav* driver line to obtain the *elav>Rel* cross that overexpresses *Rel* in neurons. We also overexpressed *Rel* in fatbody and head fatbody tissues using the *FB>Rel* and *h-FB>Rel* crosses respectively since *Rel* is endogenously expressed in the fatbody. In addition, we overexpressed *Rel* in the gut using the *gut>Rel* cross since previous studies have suggested that overexpression of inflammation-related genes in the gut is detrimental to lifespan (Rera et al., 2012). To over-express *Rel* in adults only, we used the RU-486 gene-switch system (see Methods). Using this system, *Rel* was only overexpressed when diet with RU-486/ethanol solution (RU) was consumed. This was done to eliminate any extraneous effects of *Rel* overexpression during development. Therefore we fed control flies diet with the vehicle, ethanol (EtOH). We also confirmed *Rel* over-expression in *elav>Rel* and *FB>Rel* flies that were fed RU diet compared to flies fed EtOH (Fig. 7A &7B). Using qRT-PCR, we verified that *Rel* expression was not altered in flies without the Gene-switch construct that were fed RU or EtOH diet (Fig. 7C).

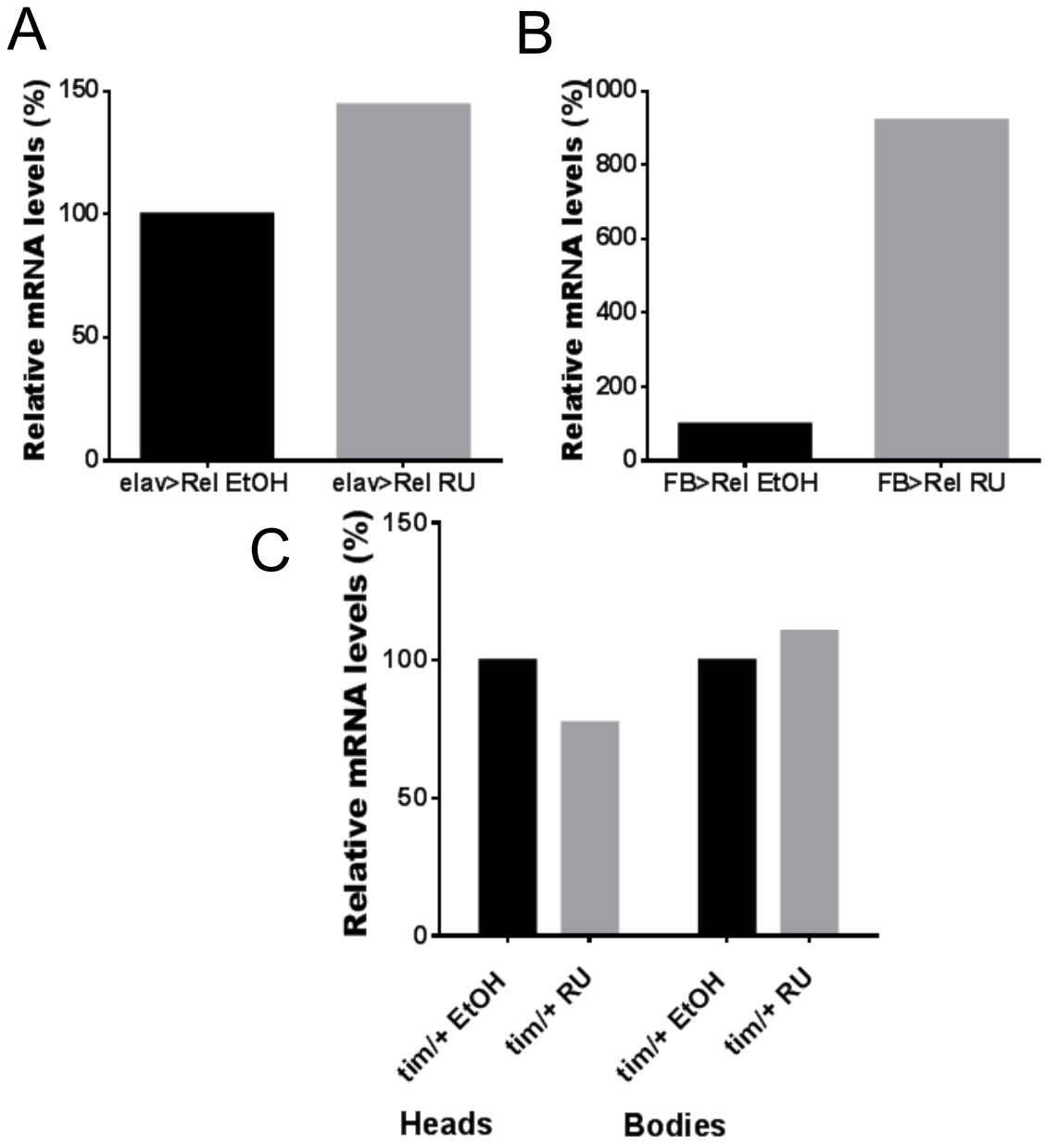

Figure 7. mRNA levels of *Rel* verify *Rel* over-expression in specific tissues. mRNA levels of *Rel* were measured using qRT-PCR in 30-day-old males. qRT-PCR was done using only fly heads or bodies depending on the location of the tissue. (A) *Rel* was over-expressed by about 50% in heads by e*lav>Rel* RU when normalized to *elav>Rel* EtOH *Rel* expression. (B) *Rel* was overexpressed by about 850% in bodies by *FB>Rel* RU when normalized to *FB>Rel* EtOH *Rel* expression. (C) mRNA levels of *Rel* in head and bodies were compared between *tim>+* RU and EtOH flies to determine if RU diet alone without a gene-switch driver line affects *Rel* expression. The mRNA levels of *Rel* were not affected by simply feeding RU diet without a gene-switch driver. Since *Rel* expression in *tim/+* RU and *tim/+* EtOH was very similar, this indicates that EtOH diet is a good control. Expression analysis was done using the standard $2^{-\Delta\Delta CT}$ method.

3.2.1 Effects of Altering *Rel* Expression in Specific Tissues on Longevity

The main purpose of creating crosses that specifically overexpress *Rel* in certain tissues was to determine if *Rel* overexpression in a specific tissue may be responsible for shortening of the lifespan observed in *tim>Rel* flies with multi-tissue overexpression of *Rel* (Fig. 5). Therefore, we measured the longevity of *elav>Rel*, *FB>Rel*, *h-FB>Rel*, and *gut>Rel* flies using flies that were fed either RU or EtOH in diet. We used *tim/+* as a control to ensure that RU and EtOH diet did not inherently affect lifespan. We found that over-expressing *Rel* in specific tissues did not significantly shorten lifespan (Fig. 8). Interestingly, *elav>Rel* flies on RU showed slight lengthening in lifespan that was significant in females compared to the EtOH fed control (p-value=0.023). We also observed slight but significant lengthening in lifespan in *FB>Rel* males on RU compared to the EtOH fed control (p-value=0.026). As expected, the *tim/+* control showed that RU and EtOH diet did not significantly affect lifespan, which validated our results. From these results, we concluded that overexpression of *Rel* in the fat body and the gut has no effect on longevity (with the exception of *FB>Rel* males), and that overexpression of *Rel* in neurons may slightly lengthen lifespan in females.

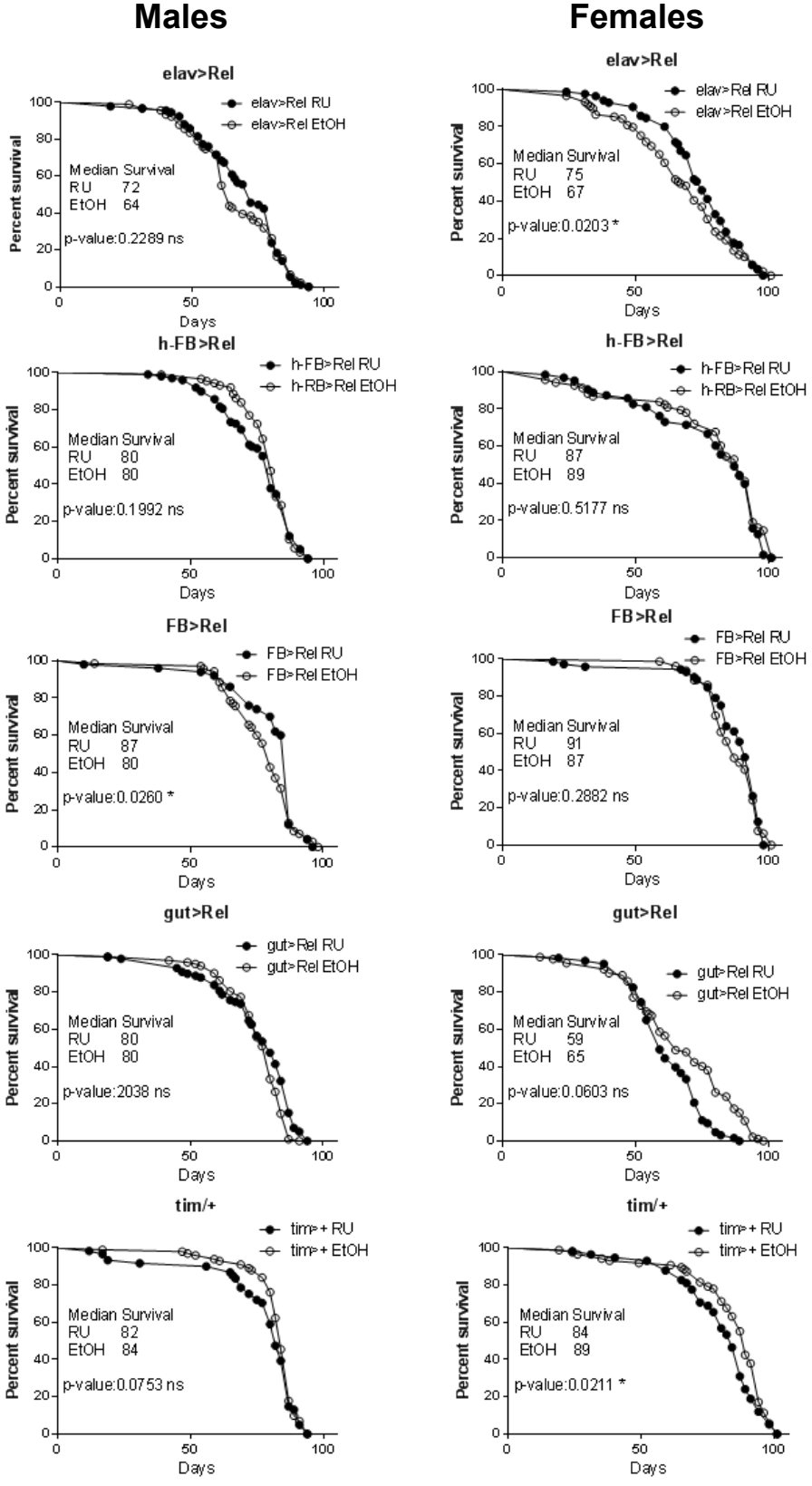

Figure 8. Lifespan of flies with *Rel* over-expression in specific tissues. Lifespan of *elav>Rel*, *FB>Rel*, *h-FB>Rel*, *gut>Rel*, and *tim/+* males and females maintained on RU and EtOH. Median survival and p-value calculated using Gehan-Breslow-Wilcoxon test are indicated within each graph.

3.2.2 Effects of Altering *Rel* Expression in Specific Tissues on Locomotor Activity

To determine how circadian rhythms were affected by the overexpression of *Rel* in specific tissues, we measured locomotor activity in *elav>Rel*, *FB>Rel*, *h-FB>Rel*, *gut>Rel*, and *tim/+* males. Data were collected at day 5 and day 30 in both RU and EtOH fed flies (Table 3). From the analysis of the strength of the rhythm (FFT) using two-way ANOVA, we determined that *elav>Rel* RU flies showed significantly stronger rhythms and a higher percentage of rhythmic flies at day 30 than their EtOH counterparts (Table 3). The *FB>Rel*, *h-FB>Rel*, and *gut>Rel* flies did not show statistical differences in locomotor activity. However *gut>Rel* RU flies gained rhythmicity with age and *gut>Rel* EtOH flies lost rhythmicity with age. This suggests that Rel overexpression in the gut may affect circadian rhythms, although lifespan is not affected.

Table 3. Comparison of Locomotor Activity due to altered *Rel* Expression in Specific Tissues

Genotype	Age	Average % Rhythmicity (N)		Average FFT	
		EtOH	RU	EtOH	RU
elav>Rel	**5**	100% (13)	88% (8)	0.072	0.065
	30	82% (28)	100% (29)	0.079	0.102*
h-FB>Rel	**5**	100% (13)	90% (10)	0.08	0.078
	30	100% (28)	96% (24)	0.119	0.133
FB>Rel	**5**	100% (13)	81% (11)	0.118	0.078
	30	97% (29)	96% (25)	0.095	0.085
gut>Rel	**5**	77% (13)	50% (12)	0.117	0.029
	30	55% (31)	82% (22)	0.037	0.052
tim/+	**5**	100% (13)	100% (8)	0.059	0.073
	30	100% (29)	90% (20)	0.063	0.075

*indicates significance compared to EtOH D30 flies using two-way ANOVA

4. Discussion

The goal of this project was to test whether increased levels of *Rel* expression, which is a major transcription factor regulating innate immunity, could create an inflammation-like state in *Drosophila*. We determined that *Rel* overexpression causes significant lifespan shortening and significant increases in neurodegeneration in aging flies, suggesting that these flies may be used as a model for inflammation in short-lived organisms. We also showed that *Rel* overexpression does not impair locomotor activity rhythms but actually slightly strengthened these rhythms, suggesting that the central clock is not weakened in these flies.

The primary finding of this project was that *Rel* is an important gene that affects longevity. We determined that *Rel* overexpression dramatically shortens lifespan, while reducing *Rel* expression caused a small but significant extension in lifespan. These results are consistent with the previous finding showing that a mutation in the gene *dnr1*, a *Rel* repressor, leads to shorter lifespan (Cao et al., 2013). Since mutating a *Rel* repressor and *Rel* overexpression have similar effects, these studies cumulatively show the detrimental effects of increased *Rel* activity for longevity. To determine the cause of lifespan shortening due to systemic *Rel* overexpression, we overexpressed *Rel* in specific tissues: neurons, fat body, head-fat body, and the gut. We measure lifespan in these crosses and found no significant shortening in contrast to flies with systemic *Rel* overexpression. In fact, *Rel* overexpression in neurons causes slight but significant lengthening in lifespan. These results indicate that either *Rel* overexpression in multiple tissues is need to cause lifespan shortening, or *Rel* overexpression in another tissue may be responsible for shorter lifespan observed in *tim>Rel*. The slight lifespan lengthening by *Rel*

overexpression in neurons is consistent with studies that show that NFκB, mammalian *Rel* homolog, is important for neuronal cell survival (Camandola et al., 2007). Studies also show that NFκB expression in neurons is needed to induced TNF-α, which has a neuroprotective, anti-apoptotic role in neurons (Cheng et al., 1994, Barger et al., 1995). However, other studies reported that TNF-α expressed in both neurons and glia is pro-apoptotic. Specifically NFκB activation in glia is thought to cause neuronal pathologies (Camandola et al., 2007). These findings suggest that *Rel* overexpression in glia may cause inflammation-like state in the brain, which causes shortening of lifespan. Because an RU-inducible glia driver was not available during this project, the role of glial overexpression of *Rel* on longevity remains to be tested in the future.

In addition to shortening lifespan, we showed that systemic overexpression of *Rel* caused significant neurodegeneration in brains of aging flies. Our data are consistent with many reports showing that increased activity of innate immune system is linked to neurodegeneration (Amor et al., 2014). Results of our study also corroborate the findings of Cao et. al, since they found that *dnr1* mutants exhibited high levels of age-dependent neurodegeneration. Taken together, these data suggest the importance of *Rel* in mediating neurodegenerative processes. From these results combined with the fact that *Rel* expression naturally increase with aging (Fig. 3), we can conjecture that endogenous *Rel* expression may lead to neurodegeneration during aging in wild-type flies.

The nature of links between innate immunity and neurodegeneration is not well understood and appears to be complex and tissue dependent. For example, as discussed above NFκB activation in neurons is thought to be neuroprotective, but in glia is thought to be detrimental. The possible importance of glia in causing neuroinflammation and

therefore lifespan is underscored by our finding that systemic *Rel* overexpression causes neurodegeneration and shortens lifespan, but only overexpressing *Rel* in neurons actually causes slight increases in lifespan. Therefore overexpressing *Rel* in glial cells will be an important future experiment to better understand the effects of *Rel* overexpression on both lifespan shortening and neurodegeneration since these phenomena appear to be linked. Additionally the effect of *Rel* overexpression on these aging parameters may be due to genes that are activated by *Rel*, since this family of transcription factor regulates many genes involved in inflammation, immunity, cell proliferation, differentiation, and survival (Oeckinghaus et al., 2009). Therefore investigating the effects of genes regulated by *Rel* on neurodegeneration may help to explain our results.

Another important finding in this project is that in contrast to our hypothesis, *Rel* overexpression does not weaken locomotor activity rhythms. In fact, locomotor activity rhythms were slightly strengthened with age when *Rel* was overexpressed systemically via *tim-Gal4* driver. Measuring locomotor activity rhythms in flies overexpressing *Rel* in specific tissues further supported this finding since locomotor activity rhythms improved with age when *Rel* was overexpressed in neurons and the gut. Many previous studies have explored the effects of impaired circadian rhythms on immunity, and have established the importance of strong circadian rhythms for robust immune response (Cermakian et al., 2014) but reciprocal links are not understood. Therefore, exploring the effects of elevated immune response on circadian rhythms during aging is a novel aspect of this project. The fact that *Rel* overexpression did not impair locomotor rhythms is important to better understand how different aging parameters may or may not be related. Measuring locomotor activity rhythms in flies overexpressing *Rel* in specific tissues

showed that improvement of locomotor activity rhythm during aging was observed in flies with systemic or neuronal *Rel* overexpression. This would be consistent with reports that *Rel* plays protective role in neurons (Camandola et al., 2007).

Another goal of this study was to assess if altering *Rel* expression could be used to simulate inflammaging in *Drosophila*. This is very important since no such model currently exists in a short-lived model organism. We tested how different aging parameters are affected by altered *Rel* expression, and found that overexpression of *Rel* causes lifespan shortening and increases neurodegeneration but did not affect circadian rhythms. Altering *Rel* expression may be a promising model for inflammaging because it does cause age-related phenotypes. This model will be especially useful in deducing links between longevity and neurodegeneration. However further research needs to be done to determine downstream effects of overexpressing *Rel* to determine the mechanisms behind these age-related phenotypes. In summary, altered *Rel* expression may be a promising model for inflammaging research in *Drosophila*, especially in studying neurodegeneration and longevity.

5. References Cited

Akerstedt, T. (2003). "Shift work and disturbed sleep/wakefulness." Occup Med (Lond) **53**(2): 89-94.

Allada, R. and B. Y. Chung (2010). "Circadian organization of behavior and physiology in *Drosophila*." Annual Review of Physiology **72**: 605-624.

Amor, S., L. A. Peferoen, D. Y. Vogel, M. Breur, P. van der Valk, D. Baker and J. M. van Noort (2014). "Inflammation in neurodegenerative diseases--an update." Immunology **142**(2): 151-166.

Barger, S. W., D. Horster, K. Furukawa, Y. Goodman, J. Krieglstein and M. P. Mattson (1995). "Tumor necrosis factors alpha and beta protect neurons against amyloid beta-peptide toxicity: evidence for involvement of a kappa B-binding factor and attenuation of peroxide and Ca2+ accumulation." Proc Natl Acad Sci U S A **92**(20): 9328-9332.

Bettencourt Da Cruz, A., J. Wentzell and D. Kretzschmar (2008). "Swiss Cheese, a protein involved in progressive neurodegeneration, acts as a noncanonical regulatory subunit for PKA-C3." J Neurosci **28**(43): 10885-10892.

Brennan, C. A. and K. V. Anderson (2004). "Drosophila: the genetics of innate immune recognition and response." Annu Rev Immunol **22**: 457-483.

Camandola, S. and M. P. Mattson (2007). "NF-kappa B as a therapeutic target in neurodegenerative diseases." Expert Opin Ther Targets **11**(2): 123-132.

Cao, Y., S. Chtarbanova, A. J. Petersen and B. Ganetzky (2013). "Dnr1 mutations cause neurodegeneration in Drosophila by activating the innate immune response in the brain." Proc Natl Acad Sci U S A **110**(19): E1752-1760.

Cermakian, N., S. Westfall and S. Kiessling (2014). "Circadian clocks and inflammation: reciprocal regulation and shared mediators." Arch Immunol Ther Exp (Warsz) **62**(4): 303-318.

Cheng, B., S. Christakos and M. P. Mattson (1994). "Tumor necrosis factors protect neurons against metabolic-excitotoxic insults and promote maintenance of calcium homeostasis." Neuron **12**(1): 139-153.

Courtois, G. and T. D. Gilmore (2006). "Mutations in the NF-kappaB signaling pathway: implications for human disease." Oncogene **25**(51): 6831-6843.

Delezie, J. and E. Challet (2011). "Interactions between metabolism and circadian clocks: reciprocal disturbances." Ann N Y Acad Sci **1243**: 30-46.

Franceschi, C. and J. Campisi (2014). "Chronic inflammation (inflammaging) and its potential contribution to age-associated diseases." J Gerontol A Biol Sci Med Sci **69 Suppl 1**: S4-9.

Giebultowicz, J. M. (2001). "Peripheral clocks and their role in circadian timing: insights from insects." Phil. Trans. R. Soc. B **356**: 1791-1799.

Gonzalez-Scarano, F. and G. Baltuch (1999). "Microglia as mediators of inflammatory and degenerative diseases." Annu Rev Neurosci **22**: 219-240.

Hardin, P. E. (2011). "Molecular genetic analysis of circadian timekeeping in Drosophila." Adv Genet **74**: 141-173.

Hardin, P. E. and S. Panda (2013). "Circadian timekeeping and output mechanisms in animals." Curr Opin Neurobiol **23**(5): 724-731.

Hedengren, M., B. Asling, M. S. Dushay, I. Ando, S. Ekengren, M. Wihlborg and D. Hultmark (1999). "Relish, a central factor in the control of humoral but not cellular immunity in Drosophila." Mol Cell **4**(5): 827-837.

Hung, C. W., Y. C. Chen, W. L. Hsieh, S. H. Chiou and C. L. Kao (2010). "Ageing and neurodegenerative diseases." Ageing Res Rev **9 Suppl 1**: S36-46.

Ivanchenko, M., R. Stanewsky and J. M. Giebultowicz (2001). "Circadian photoreception in *Drosophila*: functions of cryptochrome in peripheral and central clocks." J. Biol. Rhythms **16**: 205-215.

Kaneko, M. and J. C. Hall (2000). "Neuroanatomy of cells expressing clock genes in *Drosophila*: transgenic manipulation of the period and timeless genes to mark the perikarya of circadian pacemaker neurons and their projections." J Comp Neurol **422**(1): 66-94.

Kolker, D. E., H. Fukuyama, D. S. Huang, J. S. Takahashi, T. H. Horton and F. W. Turek (2003). "Aging alters circadian and light-induced expression of clock genes in golden hamsters." J Biol Rhythms **18**(2): 159-169.

Kondratov, R. V., R. K. Shamanna, A. A. Kondratova, V. Y. Gorbacheva and M. P. Antoch (2006). "Dual role of the CLOCK/BMAL1 circadian complex in transcriptional regulation." Faseb J **20**(3): 530-532.

Kretzschmar, D., G. Hasan, S. Sharma, M. Heisenberg and S. Benzer (1997). "The swiss cheese mutant causes glial hyperwrapping and brain degeneration in Drosophila." J Neurosci **17**(19): 7425-7432.

Krishnan, N., D. Kretzschmar, K. Rakshit, E. Chow and J. Giebultowicz (2009). "The circadian clock gene *period* extends healthspan in aging *Drosophila melanogaster*." Aging **1**(11): 937-948.

Krishnan, N., K. Rakshit, E. S. Chow, J. S. Wentzell, D. Kretzschmar and J. M. Giebultowicz (2012). "Loss of circadian clock accelerates aging in neurodegeneration-prone mutants." Neurobiol Dis **45**(3): 1129-1135.

Long, D. M., M. R. Blake, S. Dutta, S. D. Holbrook, J. Kotwica-Rolinska, D. Kretzschmar and J. M. Giebultowicz (2014). "Relationships between the circadian system and Alzheimer's disease-like symptoms in Drosophila." PLoS One **9**(8): e106068.

Maury, E., K. M. Ramsey and J. Bass (2010). "Circadian rhythms and metabolic syndrome: from experimental genetics to human disease." Circ Res **106**(3): 447-462.

Meister, S., A. C. Koutsos and G. K. Christophides (2004). "The Plasmodium parasite--a 'new' challenge for insect innate immunity." Int J Parasitol **34**(13-14): 1473-1482.

Meyer-Bernstein, E. L. and A. Sehgal (2001). "Molecular regulation of circadian rhythms in Drosophila and mammals." Neuroscientist **7**(6): 496-505.

Musiek, E. S., M. M. Lim, G. Yang, A. Q. Bauer, L. Qi, Y. Lee, J. H. Roh, X. Ortiz-Gonzalez, J. T. Dearborn, J. P. Culver, E. D. Herzog, J. B. Hogenesch, D. F. Wozniak, K. Dikranian, B. I. Giasson, D. R. Weaver, D. M. Holtzman and G. A. Fitzgerald (2013). "Circadian clock proteins regulate neuronal redox homeostasis and neurodegeneration." J Clin Invest **123**(12): 5389-5400.

Oeckinghaus, A. and S. Ghosh (2009). "The NF-kappaB family of transcription factors and its regulation." Cold Spring Harb Perspect Biol **1**(4): a000034.

Okamura, H. (2004). "Clock genes in cell clocks: roles, actions, and mysteries." J Biol Rhythms **19**(5): 388-399.

Petersen, A. J., S. A. Rimkus and D. A. Wassarman (2012). "ATM kinase inhibition in glial cells activates the innate immune response and causes neurodegeneration in Drosophila." Proc Natl Acad Sci U S A **109**(11): E656-664.

Rera, M., R. I. Clark and D. W. Walker (2012). "Intestinal barrier dysfunction links metabolic and inflammatory markers of aging to death in Drosophila." Proc Natl Acad Sci U S A **109**(52): 21528-21533.

Salminen, A. and K. Kaarniranta (2012). "AMP-activated protein kinase (AMPK) controls the aging process via an integrated signaling network." Ageing Res Rev **11**(2): 230-241.

Williams, J. A. and A. Sehgal (2001). "Molecular components of the circadian system in Drosophila." Annu Rev Physiol **63**: 729-755.

Williams, M. J., A. Rodriguez, D. A. Kimbrell and E. D. Eldon (1997). "The 18-wheeler mutation reveals complex antibacterial gene regulation in Drosophila host defense." EMBO J **16**(20): 6120-6130.

Yu, W. and P. E. Hardin (2006). "Circadian oscillators of Drosophila and mammals." J Cell Sci **119**(Pt 23): 4793-4795.