

Fall 12-15-2013

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Alzheimer's Disease and Circadian Rhythms in a *Drosophila* Model

2013

By

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Biology

Submitted in Partial Fulfillment of the
Requirements for the Degree of Bachelor of Science

In the Honors Program at
Coastal Carolina University

December 2013

Abstract

Sleep disorders are commonly reported in Alzheimer's disease, a debilitating, age-related neurodegenerative disorder that affects neurons in the brain. Recently, several studies have suggested a role for sleep abnormalities and the internal "body clock" known as the circadian system, in the disease onset and progression. Since most of the data has been collected from mammals with complex neural circuitry, the molecular and cellular mechanisms that integrate the two neural networks are still limited. This study examined the relationship between circadian rhythmicity and Alzheimer's Disease presentation using a *Drosophila* model. Flies were crossed using the GAL4-UAS system to display Alzheimer's disease neuropathology. Flies were then subjected to (3) weekly negative geotaxis assays to measure fly motility and circadian rhythmicity was observed using a locomotor assay. Results from the study suggest that circadian arrhythmicity may contribute to advanced neurodegeneration as flies with arrhythmic circadian cycles were associated with lower motility in the geotaxis assay. While the cause-and-effect relationship is not entirely conclusive, this study can serve as a basis for future experiments that seek to expand our knowledge of the disease neuropathology and related molecular/cellular mechanisms.

Introduction

Neurodegenerative diseases (ND), such as Parkinson's Disease (PD), Huntington's Disease (HD), and Amyotrophic Lateral Sclerosis (ALS) affect millions of people around the world. They are serious, debilitating illnesses that are characterized by a loss of neuronal structure and function (Cannon and Greenamyre, 2011). The neurons are highly specialized cells of the nervous system that are responsible for receiving and processing sensory information, as well as controlling higher cognitive thinking and behavior. These postmitotic cells are not actively dividing and so lost cells are never replaced. Neurodegeneration affects several everyday processes including balance, movement, memory, talking,

breathing and heart functioning – overtime, as the neurons deteriorate and these processes weaken, the eventual result is death.

One of the most common and lethal ND is Alzheimer’s Disease (AD); it affects an estimated 5.2 million Americans (Cannon and Greenamyre, 2011). Approximately one in every three senior dies with Alzheimer’s or a similar form of dementia, leaving it the sixth-leading cause of death (Gaugler et al., 2013). Like many other ND, Alzheimer’s is highly associated with aging and onset typically occurs around age 65; as age increases, the risk for developing the disease increases significantly (Gaugler et al., 2013). There are cases of early-onset Alzheimer’s, but they only account for about 4% of all cases. This form of the disease has genetic origin and is inherited, thus giving it the name familial Alzheimer’s disease (Gaugler et al., 2013). The prognosis for AD is often poor and there is no effective cure.

Overtime, neurons in the brain deteriorate, causing severely diminished cognitive abilities. Early signs of AD are decreased memory, apathy, and depression while later symptoms include impaired judgment, disorientation, confusion, behavior changes and difficulty speaking, swallowing, and walking. Once AD sets in, mean life expectancy is around seven years; fewer than 3% survive 14 years after diagnosis (Cannon and Greenamyre, 2011). In future decades, we will likely see an influx of AD cases as a result of longer life spans and the aging Baby Boomer generation (Gaugler et al 2013). Therefore it is imperative that we broaden our understanding of the disease progression and the related pathological mechanisms so that effective therapeutics might be developed

Genetic mutations play a key role in familial Alzheimer’s disease and most likely in late-onset AD, although the causes for this form are not as well understood. Researchers have identified four AD gene mutations that can be assigned to one of two categories - they can either be deterministic genes, meaning they directly cause a disease if inherited, or risk genes, which increase the likelihood of developing a disease, but do not guarantee that it will happen (“Risk Factors” 2013). Three of the

mutations are in autosomal dominant, deterministic genes that code for amyloid precursor protein (APP), presenilin-1 (PS-1), and presenilin-2 (PS-2); these mutations lead to the accumulation of an insoluble protein, β -amyloid-42 ($A\beta_{42}$), which is a hallmark of AD neuropathology (Williamson et al., 2009). The fourth gene on the other hand is considered a susceptibility factor or risk gene and contributes to late onset AD; it codes for apolipoprotein E (APOE), which is involved in central nervous system lipid homeostasis (Coogan and Greenamyre, 2011). There are three variants of this gene: $\epsilon 2$, $\epsilon 3$, and $\epsilon 4$ with allele $\epsilon 4$ having the highest risk factor (Williamson et al., 2009). Since the discovery of APOE in 1993, researchers have examined hundreds of genes of interest, but the results from these studies are not entirely conclusive.

Another key area of research focuses on environmental and lifestyle influences in AD pathogenesis (Cannon and Greenamyre 2011). Decades ago, much of the work was centered on environmental metals after aluminum was reported in amyloid plaques; although this relation has since been discarded (Cannon and Greenamyre, 2011). More recent studies suggest that alterations to microvasculature are central to AD pathogenesis. Experimental evidence has found that reduced microvascular density, increased fragmented vessels, changes in vessel diameter, and capillary basement membrane thickening, affect the blood brain barrier (BBB) and blood- cerebral spinal fluid (CSF), which increases the risk for $A\beta$ accumulation (Cannon and Greenamyre, 2011). Studies have also examined various environmental toxins and their relation to reactive oxygen species (ROS) and mitochondrial dysfunction in AD. The results suggest that oxidative stress may be a factor in disease presentation (Cannon and Greenamyre, 2011). The cause-and-effect relationships between these environmental neurotoxicants and AD are not definitive and so the mechanisms behind AD pathogenesis have remained under considerable debate.

Interestingly, as the disease pathways and biochemical functions are defined, it seems that there is evidence to support a link between certain lifestyle factors and AD including diet, exercise, mental health activity, social engagement, and overall well-being (National Institute on Aging, 2013). Included in this well-being is the role of sleep and functional circadian rhythms, which if altered or insufficient, may contribute to neurodegeneration. The circadian system is the internal “body clock” of an organism that is responsible for regulating the ~24 hour sleep-wake cycle. This central time keeping system is controlled by the suprachiasmatic nucleus (SCN) of the hypothalamus, although secondary structures, such as the bed nucleus of the stria terminalis (BNST), cingulate cortex, and pineal gland, also seem to play a role (Coogan et al., 2013). The SCN is composed of a neurochemically and functionally heterogeneous assembly of neurons that are maintained by a panel of clock genes and other hormones (Coogan et al., 2013). The clock genes are regulated by transcriptional feedback/feedforward loops that translate into physiological and behavioral circadian rhythms (Coogan et al., 2013). Such physiological effects include melatonin secretion, and changes in the biological clock, core body temperature, and sleep itself (Weldemichael and Grossberg, 2010).

Maintenance of normal circadian rhythms is crucial to an organism’s overall well-being. Desynchronisation can have adverse consequences in sleep and subsequently, on other aspects of health including metabolism dysfunction, cognitive impairment, cardiovascular abnormalities, gastrointestinal and genitourinary dysfunctions (Zhu and Lee, 2012). Occasional desynchronisation can occur as a result of shiftwork, air travel through time zones, substances and medication, and more severe disturbance is displayed in neuropsychiatric disorders and ND (Thome et al., 2011). Day-time napping, night-time insomnia, and restlessness are often observed in AD (Weldemichael and Grossberg, 2010). These individuals often display decreased amplitude of sleep as well as a fragmented sleep-wake cycle, suggesting abnormalities in the circadian system (Coogan et al., 2013).

It is fairly well documented that even in healthy people, circadian rhythms deteriorate with age leading to a reduction in sleep quality and impairment in cognitive performance (Weldemichael and Grossberg, 2010). These effects are exaggerated in patients with AD and seem to be associated with disease severity (Thome et al., 2011). Furthermore, evidence suggests that the pattern of desynchronisation is dependent upon the type of neurodegeneration (Harper et al., 2001). Thome et al., have reviewed several studies that had similar findings which led them to support the hypothesis that disturbed circadian rhythmicity in patients with AD is mediated by pathological CNS changes (2011). Experiments investigating this hypothesis have largely involved postmortem or neuropathology studies on humans or animal studies in mice, rats and hamsters (Coogan et al., 2013). However, due to the complexity of the central nervous system in mammals, our understanding of the molecular and cellular mechanisms that integrate circadian rhythms and AD is extremely limited.

In this study, the fruit fly, *Drosophila melanogaster* is used as a model to observe circadian rhythms and neurodegeneration in AD. The *Drosophila* model is a great alternative to mammal models for it can easily be genetically manipulated, it has a simple neural circuitry, and a shorter lifespan for longitudinal observation of neurodegeneration. Knowledge of molecular oscillation of clock genes in *Drosophila* has already led to the discovery of mammalian homologs and their functioning in the mammalian circadian system (Hardin, 2011). Examining these genes and sleep-wake patterns in the fruit fly will help increase our understanding of the cause-and-effect relationship to neurodegeneration so that potential therapeutics may be created. To do so, this study will have three parts: (1) Grow fly lines and cross flies to mimic AD presentation; (2) Verify the AD model; and (3) Observe patterns of the sleep-wake cycle in F₁ (AD presenting) flies compared to the controls. By analyzing the circadian rhythms from the locomotor assay with the decline in motility as determined by the geotaxis climbing assays, this study seeks to correlate circadian arrhythmicity as a preface to AD onset and progression.

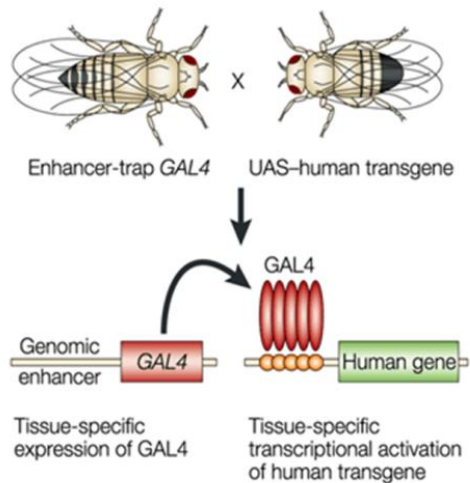
Materials and Methods

Fly Lines and Experimental Strains

Four fly lines (stock number 458, 33770, 33771, and 33772) were purchased from FlyBase and shipped from Bloomington *Drosophila* Stock Center at Indiana University. The four fly lines were grown in separate flasks with JAZZ-Mix *Drosophila* food (Fisher Scientific, Pittsburgh, PA) - 33771 and 33772 flies did not survive. Once the other flies had emerged, virgins were collected and crossed (3 females: 2 males) to form three experimental strains. 458 parents produced one control strain and 33770 parents produced a second control strain. The third strain served as the AD model which was reproduced using the GAL4-UAS system. This system, developed by Brand and Perrimon in 1993, allows for *in vivo* transcription of specific genes in the *Drosophila* model using two components known as the driver and the responder. One fly line carries the driver, which is the GAL4 gene from the yeast *Saccharomyces cerevisiae*, while a separate fly line carries the responder, a specific gene of interest, as well as an Upstream Activation Sequence (UAS). When the two fly lines are crossed, progeny obtain both the driver and responder – the GAL4 gene product (an enhancer protein) can then bind to the UAS to induce transcription of downstream genes, and the gene of interest will be expressed (see Figure 1).

In this study, the gene of interest is the autosomal dominant, deterministic gene that codes for human beta-amyloid protein ($A\beta_{42}$); incorporation of this gene in *Drosophila* has been reported to produce amyloid plaques in the cerebral cortex, a hallmark of AD neuropathology. 458 flies carry the GAL4 driver on the X chromosome while 33770 flies express the $A\beta_{42}$ fragment of APP under the control of UAS on chromosome 2. Thus to reproduce the AD neuropathology model, 458 virgin females had to be crossed with 33770 males. The three strains, 458, 33770, and 458 x 33770 were monitored for approximately two weeks, until enough flies had emerged for experimentation.

Figure 1 The bipartite Gal4-UAS system in *Drosophila*.



Virgin females carrying the GAL4 driver were mated with 33770 males carrying the UAS-Abeta42 responder; progeny express the human gene of interest ($A\beta_4$) and AD phenotype. Taken from Miratul and Feany, 2002.

Verification of AD Model

Movement disorder is a hallmark of ND, and decreased motility often becomes noticeable in the late stages of AD. The presence of AD can be verified based on this characteristic by subjecting flies to three (3) weekly negative geotaxis climbing assays as described by Le Bourg and Lints (1992). Before beginning experimentation, the flies were separated by gender into 18 vials so that all three strains had three female vials (labeled 1-3) and three male vials (4-6) with about 10 flies in each. Assays were performed each Monday morning starting around 9:00.

Fly motility was measured by recording the number of flies capable of climbing up 8 cm in 10 seconds, immediately after being tapped down to the bottom of the tube. Each vial was tested 10 times with 60 seconds of rest in between each trial; all 458 vials were tested first followed by 33770 vials and then 458 x 33770 vials. Decline in percent flies capable of crossing the 8cm line indicates the onset and degree of neurodegeneration.

Observation of Sleep-Wake Patterns

Sleep and circadian rhythmicity was measured using a locomotor assay. Prior to recording, flies were entrained to the 12 hour light (10:00 to 22:00): 12 hour dark (22:00 to 10:00) cycle at room temperature (~22°C), on the Jazz-mix diet. Then, 15 males and 15 females were collected from each of the three strains and placed individually in small glass tubes with sucrose-agar media. The tubes were placed in a locomotor monitor (Trikinetics, Waltham, MA) under constant darkness (DD) for two weeks at 25°C, to observe endogenous clock activity without environmental factors such as light or sound. 458 flies were placed in monitor 1 (males: 1-15, females 16-30); 33770 flies were placed in monitor 2 and F₁ (458 x 33770) flies in monitor 3 in the same order.

Locomotor activity was measured by recording the number of times the infrared beam was interrupted due to the movement of a fly. The total number of movements was summed at the end of each 30-minute interval, and shown as a single bar on each line. The height of the bar is proportional to the number of movements recorded during that period. All of the data was collected using DAMSystem Collection software (Trikinetics) and analyzed using Clock-Lab software (Actimetrics, Wilmette, IL).

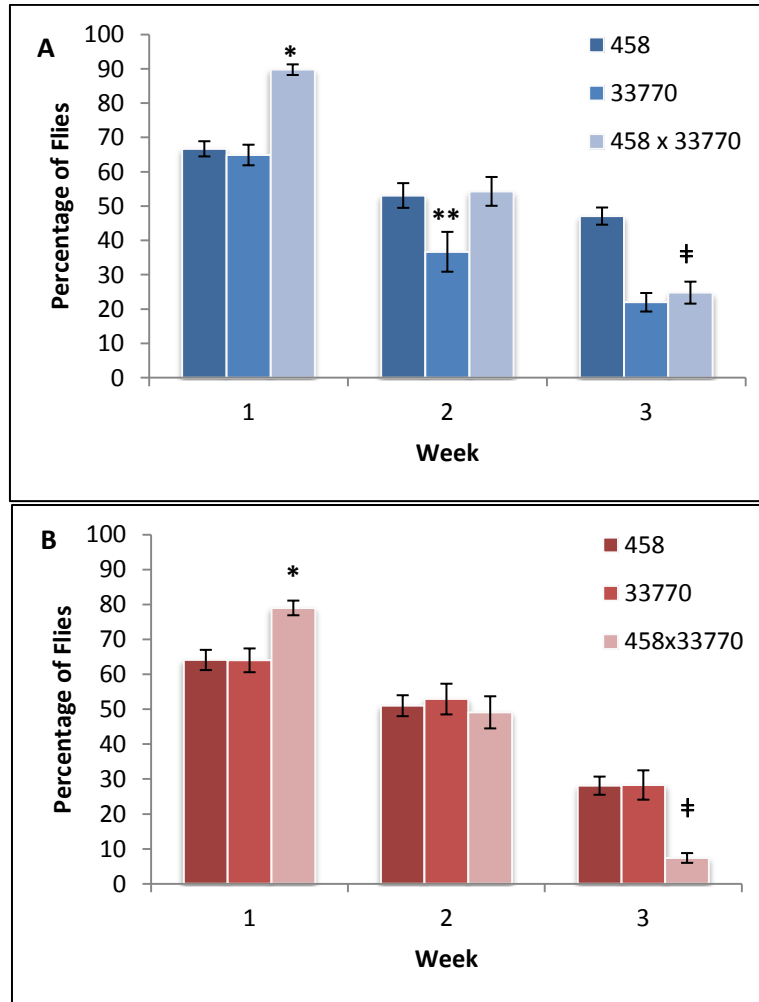
Results

Geotaxis Climbing Assay

In week one, both male and female 458 x 33770 flies (F₁ generation) displayed hyperactivity compared to the controls 458 and 33770. 89.75% of F₁ males were capable of crossing the 8 cm line while only 66.7% of 458 males and 64.9% of 33770 males were capable; females displayed similar numbers with 79.0% of F₁ females compared to 64.0% for both 458 and 33770 females. From week one to week two, the percentage of flies dropped significantly for all strains except for the 33770 females. The percentage of females capable of crossing the line was similar between all three strains (458: 51%,

33770: 52.9%, F_1 : 49.1%), but the males exhibited a different pattern. 458 and F_1 males had similar percentages with 53.1% and 54.3% respectively, but 33770 had a much lower percentage of 36.7%. From week two to week three, percentages of flies dropped significantly for all flies except for 458 males. There were significant differences between F_1 females (7.4%) and the control females (458: 28.1% and 33770: 28.3%). There was also a significant difference between F_1 males (24.8%) and 458 males (47.1%), but not between F_1 and 33770 males (22.0%). The results of the three weekly geotaxis assays for males and females are shown below in Figure 2.

Figure 2 (A) Geotaxis climbing assay for males over a period of three weeks. (B) Geotaxis climbing assay for females.



Results refer to the percentage of flies capable of crossing the 8 cm line in 10 seconds.

* F_1 flies (458 x 33770) had significantly higher percentages ($p < 0.05$) than 458 and 33770 controls

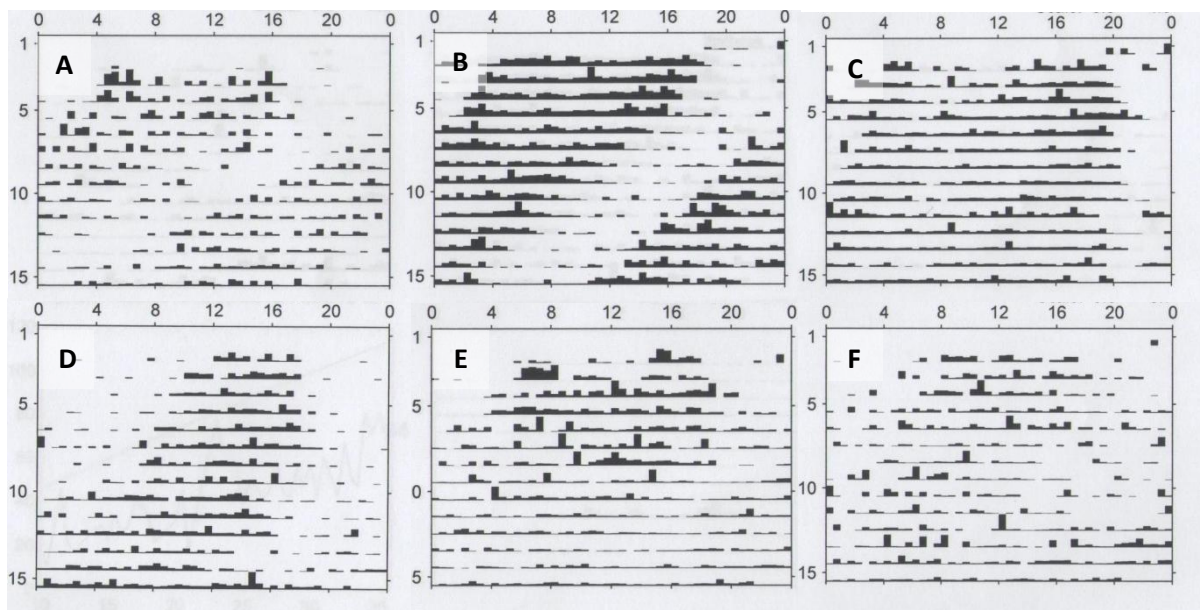
**33770 males had a significantly lower percentage ($p < 0.05$) than F_1 males and 458 males

\ddagger F₁ flies (458 x 33770) had significantly lower percentages ($p < 0.05$) than the controls (F₁ males significantly lower than 458; F₁ females significantly lower than both 458 and 33770)

Circadian Rhythm Data

Circadian rhythmicity differed between males and females; however, in all strains, males were more rhythmic than females. 458 males were 40.0% rhythmic ($n=15$) and 458 females were 28.6% rhythmic ($n= 14$). 33770 males were 86.7% rhythmic ($n=14$) and 33770 females were only 7.1% rhythmic ($n=14$). For 458 x 33770 flies, F₁ males were 75.0% rhythmic ($n=12$) and females were 20.0% rhythmic ($n=10$). 458 and 33770 males seemed to display a short mutant phenotype, meaning they had a shorter period between activity bouts. The average cycle for 458 males was 22 hours instead of 24; thus, with each successive day, locomotor activity gradually shifts to the left, as shown on the actogram in Figure 3A. 33770 males were mostly rhythmic with a 23 hour cycle; F₁ flies were also fairly rhythmic with a 23 to 24 hour cycle. Actograms for a rhythmic individual fly of each strain and gender are shown in Figure 3.

Figure 3 Individual actograms recording locomotor activity for a male and female fly in each strain.



(A) 458 males, **(B)** 33770 males, **(C)** F₁ males, **(D)** 458 females, **(E)** 33770 females, and **(F)** F₁ females. The x-axis indicates the 24 hour timeline on top of each actogram and the y-axis represents successive days of recording as described in the Methods. Each actogram is set at a scale around 3.0. Male strains display more rhythmicity than females, and 458 and 33770

males display short-mutant rhythmicity patterns.

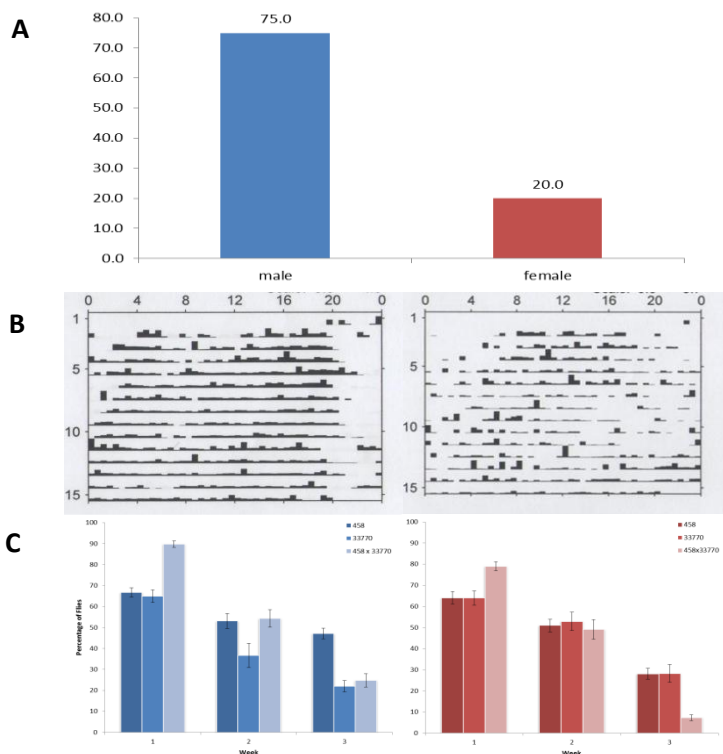
Discussion

The results from the geotaxis climbing assay indicate that the F_1 , 458 x 33770, flies exhibited decreased motility, thus confirming the AD model. Looking back at Figure 1, it is clear that all of the strains decreased in percentage of flies capable of crossing the 8 cm line in 10 seconds; however, both male and female flies from the F_1 generation decreased by much larger margins when compared to the controls. From week one to week two, F_1 males dropped 35.5%, 33770 males dropped 28.2% and 458 flies dropped 13.6%. F_1 females dropped 29.9%, 33770 dropped 11.1% and 458 dropped 13.1%. From week two to week three, F_1 males dropped 29.5%, while the controls 33770 and 458 only dropped 14.7% and 6.0%, respectively. Once again, females displayed a similar trend with F_1 females dropping an entire 41.7%; 33770 females dropped 24.6% and 458 females dropped 22.9%.

The climbing assay results for female flies were more consistent than the male flies with what we originally expected to see. F_1 flies displayed hyperactivity in the first week, but then leveled off with the controls in the second week. By the third week, AD has progressed resulting in a severe loss of motility. The male strains exhibited this trend to some degree: F_1 males were hyperactive and then leveled off with the 458 control before dropping significantly in week three. The results from the 33770 males on the other hand do not support the original hypothesis. Instead of declining gradually, like the 458 control, 33770 flies dropped quickly and by a fairly large amount, much like the pattern displayed by the AD model strain. This result may be due to an unhealthy fly line or an unknown mutation that caused the obvious decline in motility. 33770 flies only carried the UAS-Abeta42 responder, and so they should not have developed AD neuropathology like the F_1 flies. Although these flies dropped significantly from week one to week two, their circadian rhythms remained the most rhythmic of the three strains. This suggests that the decline in the percentage of 33770 flies was caused by alternate factors which may have skewed the results.

In examining the sleep-wake cycles of each of the three strains, it is most important to note that the circadian cycles of all of the males were much more rhythmic compared to those of the females. Figure 4 shows the male and female circadian rhythmicity from the AD strain (458 x 33770) along with the related actograms and geotaxis climbing assay. By analyzing the three experimental parts together, it seems evident that arrhythmicity played a role in advancing neurodegeneration, especially in the F₁ females. The actogram of the female fly is much more arrhythmic compared to the actogram of the male which shows relatively consistent periods between activity and rest. The disturbance in circadian rhythms resulted in an overall 20% rhythmicity for F₁ females which subsequently led to a lower percentage of flies capable of crossing the 8 cm line in the geotaxis assays. While the results are not entirely conclusive, it can be inferred that interrupted and arrhythmic circadian rhythms may contribute to advanced neurodegeneration in AD.

Figure 4 Comprehensive experimental data of males and females from the AD model strain, 458 x 33770.



Data representing the males is on the left and data representing the females is on the right. **(A)** Circadian Rhythmicity of F_1 flies. Males ($n=12$) were 75.0% rhythmic and females ($n=10$) were 20.0% rhythmic; percentages were calculated from the circadian recordings for the locomotor assay. **(B)** Actograms represent locomotor activity of an individual F_1 male and F_1 female. **(C)** Comparison of fly motility for all fly strains over a period of three weeks, as recorded from the geotaxis climbing assays. By week three, F_1 males and females were significantly lower than the controls (458 and 33770), indicating AD progression. Taken together, the results indicate a positive correlation between circadian arrhythmicity and decreased motility due to neurodegeneration.

While the data suggests that circadian rhythms and AD are associated with one another, a true cause-and-effect relationship cannot be determined at this time. The experiment from this study needs to be repeated with a larger sample size to limit the affect dead or unhealthy flies had on the results. Also, it would be of considerable interest to repeat the experiment only crossing 458 females with 33771 flies or 33772 flies. Along with the Abeta42 fragment of APP, these lines express human MAPT (tau) under the control of UAS. The tau protein has been identified in neurofibrillary tangles, another hallmark of AD; inclusion of this gene could enhance the AD model and produce different effects that alter circadian rhythms. Postmortem and neuropathology studies of humans have found neuronal loss and neurofibrillary tangles in the SCN more often than amyloid plaques (Coogan et al., 2013), so using 337701 or 33772 fly lines may reproduce a more accurate AD model in the fruit fly. Should a cause-and-effect relationship be identified through these studies, there is great potential to develop novel therapeutics to improve AD prognosis. Already, researchers have proposed phototherapy and melatonin treatments to reduce circadian and sleep abnormality, thus hoping to improve AD manifestation. The *Drosophila* model is a great alternative to complex mammalian systems to further our understanding of the causes and characteristics of AD and all of the other ND.

Acknowledgements

The author gives a special thanks to Dr. Fang-Ju Lin for advisement in designing this study, assistance in analysis and presentation, and for opening the doors to new ideas of scientific knowledge and discovery; also to Dr. Karen Aguirre for supporting this project throughout its entirety; and finally to the Coastal

Carolina University Honors Program for providing the opportunity for academic enrichment and personal development.

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