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Eric Robles
Coastal Carolina University

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Learning and Memory in a *Drosophila melanogaster* model of Alzheimer's Disease

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BY

Eric Robles

Department of Biology

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ABSTRACT

Human Alzheimer's disease (AD) is the most prevalent and lethal neurodegenerative disease; it involves the accumulation of neurofibrillary tangles, loss of synapses and neurons in specific areas of the brain, and the presence of extracellular amyloid plaques, particularly Amyloid beta-42 (A β -42). In this study, two *Drosophila* transgenic fly lines carrying either elav-GAL4 driver or UAS-A β -42 transgene, were crossed to generate AD flies that expressed low levels of human A β -42. Male AD flies (experimental) and elav-GAL4 flies (as parental control without A β -42) were tested for learning and short-term memory using the courtship suppression assay (Siegal and Hall, 1979). The courtship suppression assay includes "training" and "testing" periods, where male flies rejected by a previously mated female during training will retain the memory and therefore exhibit less courtship behaviors in testing period. First, a single virgin male was assigned to one of three training conditions: paired with a previously mated (trainer) female (experimental condition), a virgin female, or no female (sham control). After one hour of training, all males were transferred and paired with virgin females for ten minutes. Independent raters reviewed the training and testing videos and calculated courtship indices (CI) reflecting the amount of time males engaged in characteristic courtship behaviors (ex. orientation, wing vibration, tapping). Both 4-6 days-old elav-GAL4 and AD males exhibited less courtship behaviors towards mated females, suggesting the efficacy of training. In addition, trained elav-GAL4 males had a lower average CI than the sham control in testing, indicating their short term memory is intact. However, the average testing CI for 4-6 days-old trained AD males was similar compared to their sham control group. Our results showed that four-to-five days-old AD males already exhibited deficits in short-term memory.

INTRODUCTION

Neurodegeneration can cause a wide variety of neurological disorders, such as Parkinson's disease (PD) and Alzheimer's disease (AD). These are dementing cognitive disorders that are characterized by a loss of structure or function of neurons, leading to severe mental and physical effects (Mershin et al., 2004). Neurons are highly specialized cell types responsible for processing and transmitting cellular signals. A loss of structure or function of neurons can thus be devastating. Neurons are also classified as post-mitotic, meaning they do not undergo cell division after fetal development is complete. Therefore, lost neurons and their associated function are typically not replaced. Consequently, neurodegeneration can lead to loss of synapses and neurons in specific areas of the brain affecting everyday processes such as memory, movement, speech, or balance—gradually, as the neurons deteriorate, vital bodily functions are lost, resulting in death.

Alzheimer's disease is the most common and lethal neurodegenerative disease accounting for the majority of cases of dementia in the elderly, with an estimated 5.3 million Americans with the disease. It is the sixth-leading cause of death in the US, with approximately one in every three seniors dying with AD or another dementia (Alzheimer's Association). This chronic neurodegenerative disease starts off slowly, characterized by impairments in memory and cognitive abilities, and gets worse over time. There are two classes of the disease: early-onset (EOAD) and late-onset (LOAD). EOAD manifests before 60 years of age and LOAD occurs most often in those over 65 years of age (Mhatre et al., 2014). EOAD has been found to have a genetic origin and thus inherited, giving it the name familial AD, but only makes up about 5% of all cases. About 95% of people with AD were reported to have LOAD, but similar neuropathology is seen with both classes of AD (Mhatre et al., 2014). Age is the greatest risk

factor for AD, similar to other common, chronic, lethal conditions like cancer and cardiovascular disease (Rogers et al., 2012). The disease itself is not a normal part of aging, as brains of elderly, *nondemented* people show substantial hallmark indications of AD (Goguel et al., 2011). The number of Americans with AD will increase as the size of the population over 65 years of age continues to rise as a result of longer life spans and the aging Baby Boomer generation. At the current rate, AD prevalence is projected to increase to 13.8 million people in the USA by 2050, excluding any medical breakthroughs to prevent or slow the disease (Alzheimer's Association). Therefore, it is crucial for further research and understanding of how the disease progresses and the underlying mechanisms contributing to the pathogenesis of AD so effective treatments can be developed to slow or stop the neuronal malfunction and resulting death.

The pathological hallmarks of the disease are the accumulation of neurofibrillary tangles, loss of synapses and neurons in specific areas of the brain, decreased axonal transport, and the presence of extracellular amyloid plaques (Goguel et al., 2011; Rogers et al., 2012). These plaques are mainly composed of amyloid beta ($A\beta$) peptides and intraneuronal neurofibrillary tangles (NFTs) comprised mostly of aggregated TAU, an insoluble fibrillar microtubule binding protein (Mershin et al., 2004; Rogers et al., 2012). There are two $A\beta$ peptides that make up amyloid plaques: Amyloid beta with 40 and 42 amino acids in length. The $A\beta$ peptides result from the cleavage of amyloid precursor protein (APP) by presenilins PS1 and PS2 (Rogers et al., 2012). There is still no conclusive correlation between the presence of amyloid plaques and neuronal loss as the cause of AD, due to a lack of definitive pathogenic pathway linking them together (Ling et al., 2009). Although no definitive pathway, in AD, both proteins ($A\beta$ and TAU) are found to be misfolded and aggregated. Aging could play an important role in increasing vulnerability to protein toxicity, as the duration and extent of exposure can induce neuronal

dysfunction and reduce protein turnover leading to an accumulation (Sofola et al., 2014; Rogers et al., 2012). A study conducted by Ling et al. has found that autophagic vesicles become accumulated and increasingly dysfunctional with age and A β 42 expression (2009). Normally, autophagy, a lysosome-mediated process, is responsible for the turnover of long-lived proteins and organelles. Expression of A β 42 induced an age-dependent injury to this autophagic-lysosomal degradation pathway which led to extensive damage and death of neurons (Ling et al., 2009). The mechanisms by which A β or TAU proteins contributes to the clinical progression of AD is not entirely clear, but there has been strong support for the amyloid cascade hypothesis. This is due to mutations found in EOAD, affecting the amyloid precursor protein (APP) from which both A β peptides are derived from. This causes an accumulation of A β peptides, specifically a greater increase in A β 42 peptide. This peptide has a greater tendency to aggregate and be more neurotoxic than A β 40, thus, it is associated more with the development of AD (Ling et al., 2009). The precursor of A β peptides plays a crucial role in the early stages of the disease and thus serves as an important molecule in the pathogenic cascade leading to AD (Rogers et al., 2012).

Many diseases are researched *in vivo* due to a better understanding of the overall effects a disease has on a living organism. The development of an *in vivo* disease model for AD has increased over the last 30 years, thanks to a better understanding of the disease. An important and influential model organism for understanding the mechanisms of many neurodegenerative diseases, such as AD, is the invertebrate model organism *Drosophila melanogaster* (fruit fly). *D. melanogaster* is a prime candidate because it is inexpensive, has its complete genome sequenced, has a short life span, short generation time, and has a large number of genetic tools that enable gene expression to be restricted to specific areas of the brain (Xu et al., 2014). Most importantly,

the genome of *D. melanogaster* contains homologs of approximately 70% of human disease-related genes (Goguel et al., 2011). One of them includes homology among APP, such as mammalian APL P1/2 and *Drosophila* Beta-Amyloid Precursor Protein-Like (APPL). Homology among these two members of the APP family allows them to functionally substitute for each other (Torroja et al., 1999). A β sequences are not conserved in the *Drosophila* APPL, but expression of human A β results in similar features observed in the mouse model, such as behavioral deficits and neurodegeneration (Goguel et al., 2011; Iijima et al., 2004). This finding further proves that *D. melanogaster* serves as a reliable model for the pathogenesis of AD.

In order to study the effects human A β , or other disease-related proteins, there has to be a way to introduce the genes into *D. melanogaster*. Developing a system to target gene expression has always been an area of increased study because it is a useful tool for analyzing genes of interest. A landmark study by A.H. Brand and N. Perrimon was able to accomplish this. Brand and Perrimon developed a system, called the GAL4/UAS system, which allows for genes to be turned on in any cell type at any time in development, even incorporate and drive genes from other species (1993). This allows for the analysis of genes and the proteins that they may code for. The system works by two parts: the GAL4 gene (the activator/driver) and the Upstream Activation Sequence (UAS). The GAL4 is a transcriptional activator derived from yeast, and can activate transcription in flies but only from promoters that have GAL4 binding sites. In order for the GAL4-dependent target gene to be activated, it has to be subcloned behind a tandem array of five optimized GAL4 binding sites, the UAS. Both work together to have an effect on cells. With just the GAL4 gene, there is no effect on *Drosophila* cells, but with UAS, there is an effect and the gene (responder) next to the UAS is transcribed. This system can be used to generate dominant phenotypes for use in genetic screening, such as expressing A β 42 to model

neurodegenerative diseases like AD (Brand and Perrimon, 1993). By using *Drosophila* and utilizing the GAL4/UAS system, there have been hundreds of fruit fly GAL4 lines developed over the years.

Using the GAL4-UAS system, researchers have been able to study the molecular basis of AD pathogenesis and other diseases. With AD, researchers can examine the effects of A β in the *Drosophila* brain to analyze the possible roles it has in causing learning defects, memory decline, and neurodegeneration (Iijima et al., 2004). Significant research has been focused on the *Drosophila* mushroom bodies (MBs), which are paired neuropil structures in the central brain (Akmal et al., 2006). It has been suggested that the MBs serve different functional roles and are important for associative learning and memory, since genes associated with them have elevated levels of expression in the MB neurons (Akmal et al., 2006). It has also been found that MBs are centers for courtship behavior, as well as a role in circadian clock, and generation, regulation, and coordination of motor patterns (Martin et al., 1998). Therefore, *D. melanogaster* serves as a powerful model for studying mechanisms related to learning, memory, and motor patterns, all of which have an important implication in AD. One of the most widely used methods to study learning and memory is olfactory conditioning. This type of associative conditioning tests the fly's ability to avoid an odor (conditioned stimulus CS+) associated with an electric shock punishment (unconditioned stimulus US) in opposition to a second odor (the CS-) without a shock, to test aversive memory (Beck et al., 2000; Kim et al., 2013).

Another method to study learning and memory without the use of electric shocks is the conditioned courtship behavior assay. This behavior assay tests the fly's ability to alter its courtship behavior after learning from prior sexual experiences (Siegel and Hall, 1979). Most studies in the past on behavioral plasticity have focused on male courtship choices between

virgin and non-virgin females. The role of aging and neurodegeneration can also be implemented in this type of assay to examine the males' choice behavior based on previous learning and the resulting memory (Hu et al., 2014). The courtship behaviors elicited by male *Drosophila* flies are done in a pattern. Examples of courtship behaviors include orientation of the male fly towards the female, tapping the female with his foreleg, extension and vibration of his wing to produce a "courtship song," extending his proboscis to lick the female, and copulation attempts (Moehring and Mackay, 2004; Siegel and Hall, 1979). These activities are performed by the male naturally, which means that their actions are independent of conditioning. Researchers, such as Siegel and Hall (1979), Mhatre et al. (2014), and McBride et al. (1999) use experience-dependent courtship conditioning to modify the male courtship behavior by allowing naive males to experience an unreceptive female for a period of time. Exposure to an unreceptive female will eventually reduce male courtship behavior, even towards virgin females. McBride et al. point out that males experiencing virgin females *did not* show a depressed courtship activity towards other virgin females. This means that the conditioned courtship suppression with unreceptive females and subsequent virgin females was a result of experience with an unreceptive female and resulting change in behavior (1999). This type of courtship conditioning can be used to assess the roles age and neurodegeneration have on learning and memory. Maintenance of normal mating ability is crucial to the organism's reproductive fitness (Goguel et al., 2011). Therefore, disruptions in this behavior can be used to study cognitive impairments and memory decline.

The studies reviewed here highlight the importance of the animal model *Drosophila melanogaster*, and the work behind developing an effective disease model for AD and other neurodegenerative disease. The ability to model AD *in vivo* allows researchers to study the effects the disease has as well as the mechanisms of the disease. The *Drosophila* model has

allowed researchers to further investigate age-related behavioral defects. This can help to provide essential information on how the nervous system changes with age, affecting other key systems such as motor control or memory recall/retention/formation, similar to the pathology seen in AD patients. *Drosophila* model also has a set of genetic tools that can be used to manipulate its genome for use in investigating mechanisms related to behavior and age. This has been taken advantage of by the landmark study from Brand and Perrimon (1993) on the development of a system to target gene expression, the GAL4-UAS system. Consequently, many researchers have taken advantage of this model to enhance our understanding of aging and its influence on age-related behavioral declines (ex. activity and coordination, learning, and memory) by coming up with different, influential, and reproducible behavioral and learning assays.

Siegel and Hall's 1979 experiment on courtship conditioning has been used by many researchers throughout the years to study learning and memory. Researchers can use this behavioral assay to help answer the question as to how neurodegeneration and age affect memory. In respect to flies, is courtship behavior completely eliminated by neurodegeneration? Does age play a major factor in memory decline, and if it does, is it exacerbated with both age and neurodegeneration? Is *Drosophila* a good model for examining these questions? This study will hopefully aid in answering some of these questions by using the fruit fly as a model of AD. It will be conducted in multiple parts, including rearing the flies to express A β 42, conducting the courtship suppression assay, and observing any changes in the characteristic patterns of courtship behavior in relation to age. This will help in enhancing our research question which is whether there is a relationship between neurodegeneration, age, and memory loss. A decline in memory associated with the proposed AD-causing protein can help to correlate it with neurodegeneration, similar to the one seen in the progression to AD in humans.

METHODOLOGY

Courtship behavior has been widely used to investigate activity and coordination, as well as learning and memory. It remains to be useful for the investigation of age-dependent decreases in cognitive ability due to A β . *Drosophila melanogaster* courtship behavior, as stated earlier, involves the exchange of various sensory stimuli in the courtship pattern between males and females. These characteristic motor behaviors are crucial because behavior relies on coordination of the nervous system and musculature. Any defects in these behaviors can help to investigate how age and neurodegenerative diseases affect these two key organ systems. As stated previously, the fruit fly will be used as a model to observe the effects the proposed AD-causing protein, A β , and age has on experience-dependent courtship behaviors. A courtship-suppression assay on *Drosophila* flies will be conducted in the lab. Protocols from Siegel and Hall (1979) will be used as a starting point for the assay, with some slight modifications. Special concerns include developing an effective schedule to keep the experiment going and keeping the stock alive. We should also take into consideration developing a way to record the activity for review at a later date. Also, making sure that the evaluator is blind to the flies being tested, so that there is no bias in describing changes in courtship behavior. The data obtained will be evaluated according to past protocols on how to analyze courtship assays. This study and others alike are vital for investigating mechanisms that influence neurodegeneration-related behavioral and memory declines. Unlike other experiments on mammals, which often involve postmortem studies, studies on *Drosophila* can occur in real time. The nervous system is less complex because it is smaller and more understood. This knowledge can be applied to real world neurodegenerative disease, such as AD, to help in developing potential therapeutics.

***Drosophila* Stocks and Genetic Crosses**

Wild-type flies used were elav-Gal4 purchased from Bloomington Drosophila Stock Center at Indiana University (stock number 458). All flies were maintained at 23°C in a 12:12 light:dark cycle. Flies were grown with JAZZ-Mix Drosophila food, consisting of a mixture of sugar, corn meal, yeast, and agar recipe (Fisher Scientific, Pittsburgh, PA). Experimental strain used was transgenic fly line carrying human A β 42 under yeast UAS promoter control (Novartis International), a generous gift from Jeibmann's lab.

All crosses were carried out at 23°C 12:12 light:dark cycle on a JAZZ-Mix diet (Fisher Scientific, Pittsburgh, PA). 458 (elav-GAL4) flies carry the GAL4 driver on the X chromosome while the UAS-A β 42 express the A β 42 fragment of APP under the control of UAS on chromosome 2. To cross, elav-GAL4 virgin females were crossed with UAS-A β 42 virgin males. After approximately two weeks, experimental male flies were collected from the first generation and transferred to smaller food vials, with only five males per vial.

Behavioral Testing

For courtship behavioral training, methodology was adapted from McBride et al. (1999) with slight modifications. Virgin males were collected between 0 to 6h after eclosion (Day 1) and transferred to food vials (5 males per vial). All flies were maintained at 23°C in a 12:12 light:dark cycle. All behavioral tests were conducted in a separate room maintained at 23°C and under a constant dim lighting. All behavior was digitally recorded using a Sony Handycam with Carl Zeiss optics. The total time a male performed courtship behaviors (ex. orientation, following, wing extension and vibration, attempted copulation, tapping) were measured using a

stopwatch and scored. The courtship index (CI) was calculated as the total time males performing courtship behavior divided by the total time observed.

Virgin female *elav-Gal4* flies were collected and kept in normal food vials in groups of 10. Trainer (mated) females used for training were obtained by mating *elav-GAL4* virgin females with virgin *elav-GAL4* males on Day 4, and keeping the mated females in individual food vials. On Day 5, individual virgin males were transferred by gently aspirating to an empty well in a 4-well plate (Thermo Scientific, catalog # 144444) and allowed to acclimate for 1 minute. A microscope slide was used to cover the open wells. Then, a previously mated *elav-GAL4* female trainer was added to the well by sliding the microscope slide and the males were trained for 60 minutes. The amount of time the males exhibited courtship behavior (ex. orientation, following, wing extension and vibration, attempted copulation, tapping) was assessed during the first 10 minutes and the last 10 minutes of the training phase. A CI was calculated for both time the first 10 minutes and the last 10 minutes. After 60 minutes, male flies were transferred within 2 minutes without anesthesia to a new, clean well that already contained a virgin *elav-GAL4* female. Courtship behaviors were recorded for 10 minutes. A group of sham controls (virgin males) of the same genotype were transferred to a separate well without any female for 60 minutes, then virgin *elav-GAL4* females were added to the wells for 10 minutes during testing. All observers were blind as to the fly's experimental status during courtship behavior analysis.

Statistical Analysis

To determine the significance between different measures, a two-tailed Student's t-test was performed. Significance was determined at the 95% confidence level.

RESULTS

During the 60 minutes of training, 4-6 days-old elav-GAL4 and AD flies showed a significant drop in courtship behavior in the last 10 minutes of training when compared with the first 10 minutes (Figure 1). Although not significantly different, this trend was also observed in 16-17 days-old elav-GAL4 males. This was not the case with 16-17 days-old AD males. These data suggest that both elav-GAL4 and AD flies were able to modify behavior at 4-6 days-old in response to successful interpretation of sensory stimuli.

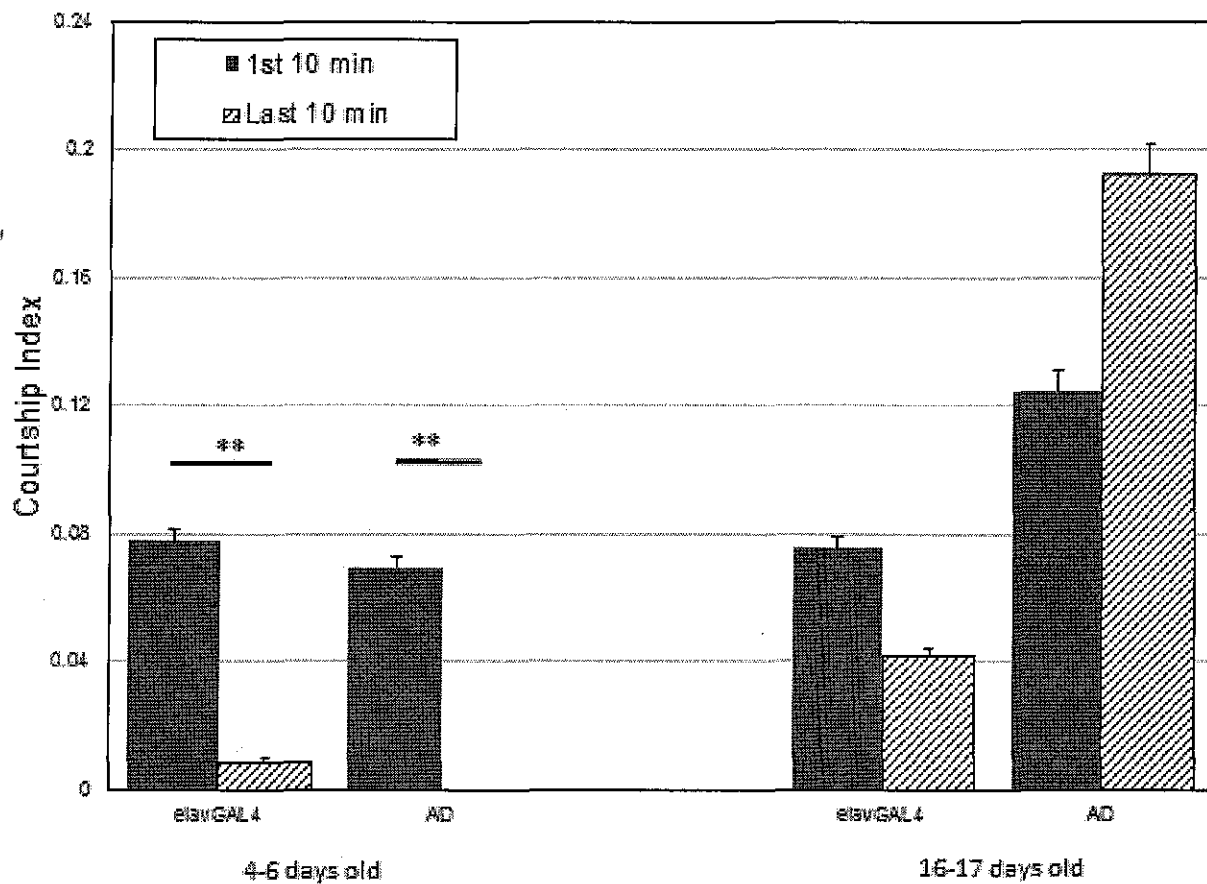


Figure 1: Average courtship index between the first 10 min and last 10 min during training phase. 4-6 days-old elav-GAL4 males show a significant difference between the first 10 and last 10 minutes of training, suggesting the efficacy of training. A significant difference was also observed for 4-6 days-old AD males. No significant differences were observed between any groups at 16-17 days-old, or when comparing the first 10 minutes at different ages of the same genotype, or when comparing the first 10 minutes between different genotypes, within the same age. Horizontal bars indicate significant differences observed. A double asterisk indicates $p < 0.001$ when comparing to 1st 10 minutes. Error bars

represent 95% confidence interval. N=60, N=17 for 4-6 days old elav-GAL4 and AD, respectively. N=5, N=13 for 16-17 days old elav-GAL4 and AD, respectively.

During the 10 minutes of testing, trained 4-6 days-old elav-GAL4 males showed a significantly lowered courtship index when compared to age-matched sham males (Figure 2). However, such a decrease in courtship behavior was not observed in flies expressing A β 42 in the same age group. AD flies 4-6 days-old showed no significant difference between age-matched untrained males (Figure 2). Trained 16-17 days-old elav-GAL4 or AD showed no significant difference in courtship index compared to their respective age-matched sham males (Figure 2).

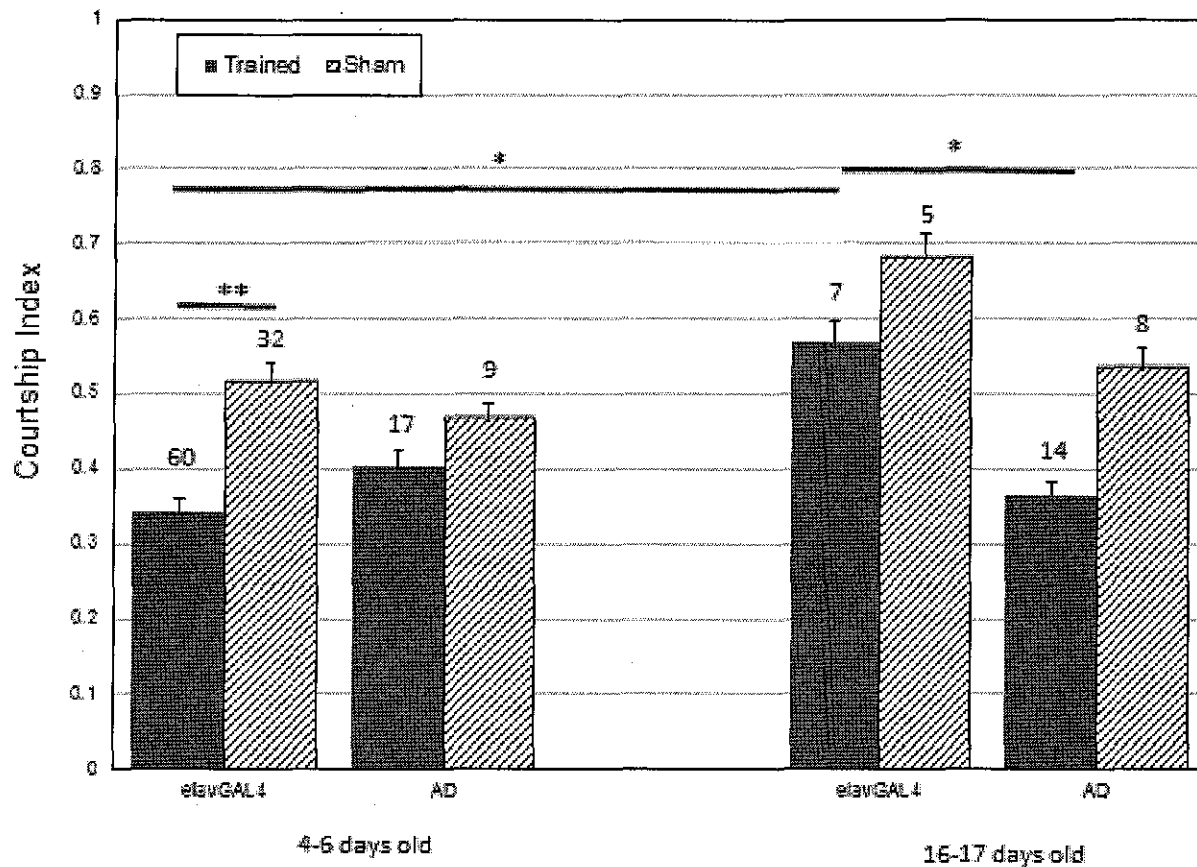


Figure 2: Average courtship index between trained and sham males during testing phase. 4-6 days-old elav-GAL4 males show a significant difference between trained and sham males, indicative of immediate recall of memory. No significant difference was observed between 4-6 days-old AD trained and sham males. When comparing between different ages of same genotype, a significant difference was seen between 4-6 days-old elav-GAL4 and 16-17 days-old elav-GAL4 trained males. When comparing between different genotypes, within the same age group, a significant difference was observed between 16-17 days-old elav-GAL4 and AD trained males. Horizontal bars indicate

significant differences observed. A single asterisk indicates $p < 0.05$ and a double asterisk indicates $p < 0.001$. Error bars represent 95% confidence interval. Sample size (N) indicated above each bar.

During testing, trained 4-6 days-old AD males showed a significantly higher courtship latency compared to the sham males of the same genotype (Figure 3). A significant difference between 4-6 days-old elav-GAL4 and AD sham males was also observed and a significant difference between 16-17 days-old elav-GAL4 and AD trained males. A significant difference in the courtship latency between 4-6 days old and 16-17 days old trained elav-GAL4 males was also observed (Figure 3).

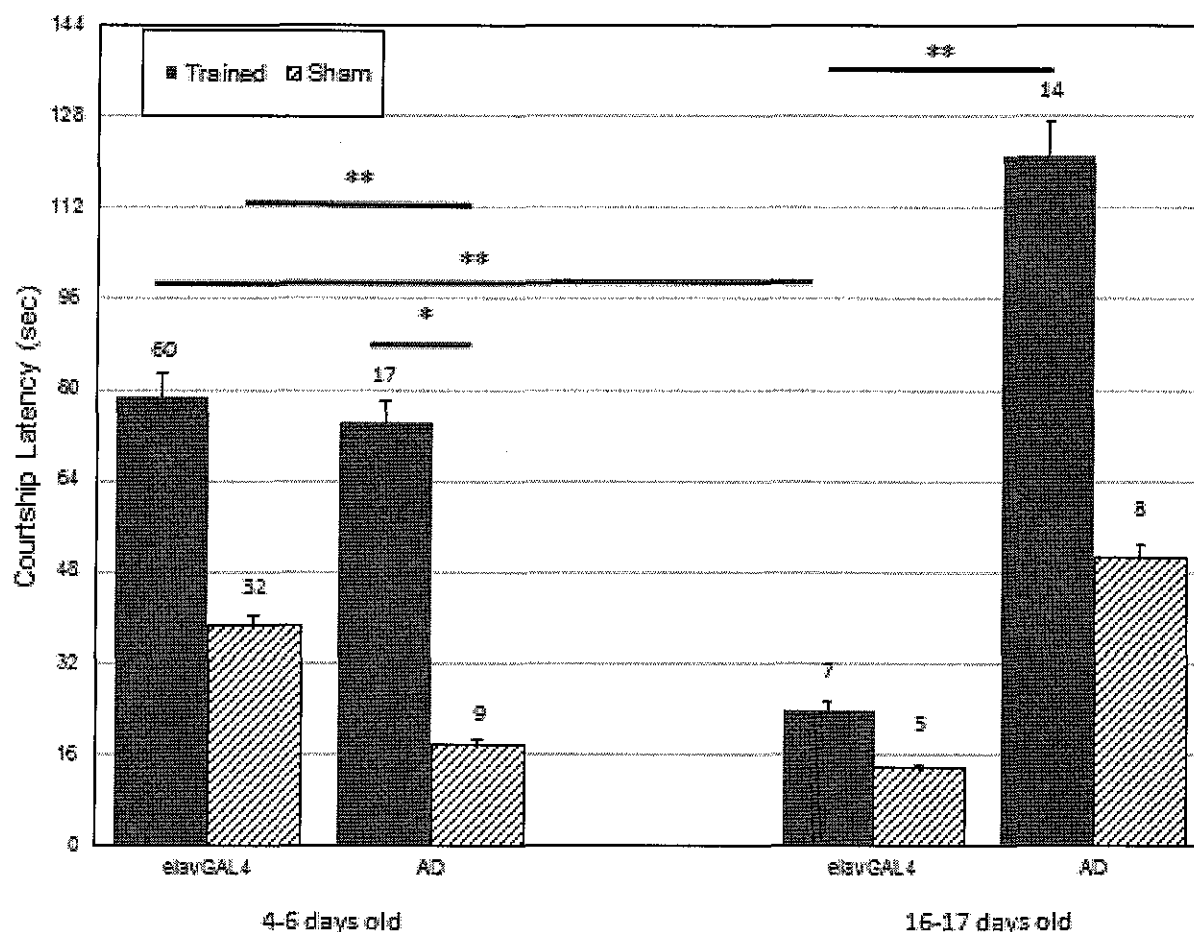


Figure 3: Average courtship latency between trained and sham males during testing phase. Significant differences were observed between 4-6 days-old AD and its sham and

between 4-6 days-old sham males of elav-GAL4 and AD. Significant differences were also observed between 16-17 days-old trained males of elav-GAL4 and AD and between 4-6 days-old elav-GAL4 and 16-17 days-old elav-GAL4 trained males. Horizontal bars indicate significant differences observed. A single asterisk indicated $p < 0.05$ and a double asterisk indicates $p < 0.001$. Error bars represent 95% confidence interval. Sample size (N) indicated above each bar.

DISCUSSION

With an increase in average life expectancy, there is an expected increase in the prevalence of dementia and AD (Alzheimer's Association, 2014). Developing a treatment that would simply delay onset of AD could lead to tremendous reductions in burdens felt by families and medical fields. In this study, we have tried to develop a model of AD that stresses the most important risk factor for developing AD: age. To develop a fly AD model, we used the GAL4/UAS system developed by Brand and Perrimon (1993) to express A β 42 to the nervous system of *Drosophila*. Virgin red-eyed, straight-winged males from the F1 generation were used for the experiments. These males carried the tissue-specific driver (GAL4) that then binds to the UAS to induce transcription of the gene of interest (A β 42).

The courtship conditioning behavior assay was used to test deficits in learning and memory in *Drosophila*. In this associative conditioning procedure, male flies exposed to mated females during the training phase will have their courtship inhibited, and when exposed to a virgin female during the testing phase, will exhibit suppressed courtship behavior (Siegal and Hall, 1979). Learning and memory formation are important during experience-driven behavioral changes, as it directly impacts cognitive ability (Xu et al., 2014). Results from this assay will aid in determining whether age and neurodegeneration have an impact on memory, similar to what has been seen with human AD patients. To determine the effects on learning, virgin male flies were placed in a courtship chamber with a previously mated (trainer) elav-GAL4 female for 60

minutes. Courtship behavior was assessed during the first 10 minutes compared with the last 10 minutes. Our results for 4-6 days old wild-type *elav-GAL4* flies show a significant reduction in courtship behavior in the last 10 minutes compared with the first 10 minutes, indicative of behavior modification and appropriate learning (Figure 1). The rapid initial decrease in courtship behavior probably reflects simple interruption of initial courtship by females' repelling movements. Our results for 4-6 days old male AD flies also show a significant decrease in courtship behavior in the last 10 minutes compared with the first 10 minutes. This indicates that our flies expressing $A\beta 42$ are capable of interpreting sensory stimuli and able to alter their behavior, and thus $A\beta 42$ does not affect the fly's ability to learn.

During training, no significant decrease in courtship behavior for both *elav-GAL4* and AD flies at 16-17 days old was observed. Although there was no significant difference, a similar pattern of decreased courtship behavior was observed for 16-17 days-old *elav-GAL4* males during the last 10 minutes compared to the first 10 minutes (Figure 1). A larger sample size would aid in determining whether or not there is a significant difference, but current results indicate a slight decrease in courtship behavior, similar to 4-6 days-old *elav-GAL4* males. The same cannot be said for 16-17 days-old AD flies. Although not significant, AD flies at this age show a higher courtship index in the last 10 minutes compared to the first 10 minutes (Figure 1). This result could be due to development of age-dependent neuronal dysfunction, with older males unable to effectively perceive and interpret sensory stimuli to alter their behavior appropriately (learn).

There exists several phases of memory in *Drosophila*, with short-term memory retained out to 1 hour post training. To test this phase of memory, we assayed *elav-GAL4* and AD flies for their recall memory by transferring trained male flies to clean mating chambers containing a

virgin elav-GAL4 female within 2 minutes and scoring their courtship behavior for 10 minutes. Trained 4-6 days-old elav-GAL4 males show a significant decrease in courtship activity when compared with sham control males of the same age and genotype (Figure 2), indicating a change in behavior that is consistent with normal recall memory of training. Though no significant difference was observed with 4-6 days-old AD males, a similar pattern of trained males having lower CIs than their sham controls was observed (Figure 2). Because these flies are capable of interpreting sensory stimuli and alter their behavior (Figure 1), their inability to significantly suppress courtship behavior during the testing phase could indicate defects in short-term memory recall at this young age. As mentioned previously, a significant decrease in courtship behavior for 4-6 days-old AD males during the last 10 minutes compared to the first 10 minutes was observed. This indicates that A β 42 does not affect the fly's ability to learn during training, but it does have an effect on recall memory during testing. This outcome could also be explained by a low population size for the AD sham controls. Further experiments at this age range would have to be conducted for definitive differences to be seen.

In this study, we were especially interested in developing an aged model of AD in flies to determine the role age and A β 42 has on memory. The testing phase of this assay therefore would help to provide us with possible answers to this question. No significant decrease in courtship behavior for both elav-GAL4 and AD flies at 16-17 days old was observed, when compared to their respective sham controls (Figure 2). Interestingly, AD males in this age group as a whole exhibited average CIs comparable to 4-6 days-old elav-GAL4 males. The data *suggests* that AD flies at this age group are capable of successfully altering their behavior during training and suppressing courtship behavior during testing. This is not the case with AD males at 16-17 days-old, as evident in Figure 1. Overall, these flies had a higher CI during the last 10 minutes

compared to the first 10 minutes of training, indicative of impaired learning, despite the rejection from mated female trainers. Upon further analysis, 16-17 days-old AD males exhibited significantly prolonged courtship latency during testing compared to their sham controls, which directly affected the CI calculation (Figure 3). With AD males at this age group taking on average two minutes before starting any courtship behavior, this resulted in less time for the males to be analyzed for courtship behavior. The results observed for elav-GAL4 males at this age during testing were consistent with the results seen for the same flies during training. Although not significant in both cases, an expected pattern of decreased courtship behavior in the last 10 minutes compared to the first 10 minutes during training was observed, as well as suppressed courtship behavior during testing when compared to their respective age-matched sham controls. Nonetheless, elav-GAL4 males in this age group showed more persistent courtship behaviors during training (Figure 1) and testing (Figure 2) compared to the younger elav-GAL4 flies, despite the rejection from mated female trainers during training.

A possible explanation could be that older males in general are more sexually mature, and may ignore any cues imposed by trainer females during training. Therefore, when exposed to a virgin female, 16-17 days-old elav-GAL4 males are no longer subdued by any previous rejection cues. Analysis of courtship latencies, especially for the older elav-GAL4 flies supports this hypothesis. As seen in Figure 3, these older flies exhibited a significantly lower courtship latency when compared to younger elav-GAL4 flies (Figure 3). In *Drosophila*, male mating ability is a critical component of reproductive fitness, and therefore results obtained for the older elav-GAL4 flies could be an indication of the fly's drive to pass on their genetic code.

Our study aimed to further understand the impact age and neurodegeneration have on short-term memory. As seen with many AD patients, a progressive decline in memory, along

with other histological changes, is what characterizes this neurodegenerative disorder. Through this study, we were able to utilize *Drosophila melanogaster* as a model for AD to study the effects A β 42 and age had on memory. Through the courtship suppression assay, we were able to determine that courtship behavior was not completely eliminated by neurodegeneration. Flies were still able to perform courtship activity at both age groups assayed. As with previous results discussed herein, the experiments from this study needs to be repeated with a larger sample size to obtain clearer differences. *Drosophila* served as an excellent model for examining these relationships, although they lack the complexity of humans and the many issues that often accompany neurodegenerative disorders (ex. infections, heart attacks, accidents). Even so, this model can still be useful for assessing and developing interventions that could help to decelerate and better understand the progression of AD. Further studies with this experimental setup could be used for drug screening and looking at the effects it has on AD pathogenesis. It would be beneficial in any future experiments to look at both progressive neuronal or muscular dysfunctions determined by age-dependent memory defects or muscular defects, as these are often observed with human AD patients.

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