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Cover Photograph: Drosophila courtship behavior. Photo and art by Andres Castillo and Kimberly Weaver.

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Loss of Courtship Suppression Memory in a *Drosophila melanogaster* Model of Alzheimer's Disease

Eric Robles[1], Johannes Berlandi[2], Chris Ellis[1], Tianyi Wu[1], Astrid Jeibmann[2], and Fang Ju Lin[1,*]

Abstract - Alzheimer's disease (AD) is the most prevalent and lethal neurodegenerative disease. Memory loss and motor dysfunction are accompanied by pathological hallmarks like neurofibrillary tangles or amyloid plaques. In this study, courtship suppression assay was used to assess learning and memory of a transgenic *Drosophila melanogaster* (the fruit fly) line expressing human Amyloid beta 42 (Aβ42). At young age (4–6 days old), both parental control and AD flies displayed lower courtship indices during training after being rejected by previously mated females. However, in the subsequent testing phase, young AD flies showed compromised recall memory, unlike that of parental controls. Neither control nor AD flies at 16–18 days old showed significant learning or recall memory. AD flies also exhibited age-related motor defects and presented amyloid plaques in brain sections. Interestingly, older AD flies displayed persistent chasing throughout the one-hour training period, and they attempted copulation at higher frequency than the untrained AD controls. Thus, transgenic AD flies displayed early onset of memory deficit, and aggressive courtship behavior as they aged.

Introduction

Alzheimer's disease (AD) is the sixth-leading cause of death in the US, with an estimated 5.3 million Americans currently suffering from the disease. Approximately one in every three seniors dies with AD or another form of dementia. As a chronic neurodegenerative disease, AD is characterized by declining memory and cognitive abilities over decades. AD pathological hallmarks include accumulation of neurofibrillary tangles containing the TAU protein, loss of synapses and neurons in neocortex, hippocampus and cerebrovasculature, decreased axonal transport, and the presence of extracellular amyloid plaques composed of the beta-amyloid (Aβ) proteins (Goguel et al. 2011, Rogers et al. 2012). In AD, both Aβ and TAU proteins are found to be misfolded and aggregated (Folwell et al. 2009). In particular, Aβ accumulation is derived from proteolytic cleavage of amyloid precursor protein (APP) by β- and γ-secretases (reviewed by Chen et al. 2017). The murine model has been extensively studied, as they carry mouse APP and β-secretases (BACE-1) that are human homologs. With transgenic constructs using known human genes involved in AD, the animals produced plaques and tangles, as well as displayed behavioral symptoms. Unfortunately, almost all drugs that were effective in alleviating symptoms in those transgenic animals failed in human clinical trials (reviewed by Mckean et al. 2021). While large animals are ideal for human disease models, the cost to maintain and their long lifespan make the research challenging.

Drosophila melanogaster, commonly known as the fruit fly, is an excellent alternative model for many neurodegenerative diseases such as AD, Parkinson's, and

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Huntington's diseases (reviewed by Hirth 2010, Jeon et al. 2020). In addition to their fast reproduction rate and ease of gene manipulation, the *Drosophila* genome contains three important homologs for humans: APP-like 1 (APPL-1), APP-like 2 (APPL-2), and a γ-secretase (Luo et al. 1990, Torroja et al. 1999). Despite the fact that human Aβ sequences are not conserved in the *Drosophila* APPL protein, expression of human Aβ in flies did result in amyloid plaques and behavioral deficits similar to both AD patients and the mouse model (Goguel et al. 2011, Iijima et al. 2004). It has been shown that olfactory learning and memory were reduced when Aβ40 and Aβ42 were induced in *Drosophila* neurons (Iijima et al. 2004), using the yeast *GAL4-UAS* system (Brand and Perrimon 1993). In addition, Ling's group (2009) found exogenous *Aβ42* expression in flies induced extensive damage and death of neurons through progressive injury of the autophagic-lysosomal degradation pathway. These findings recapitulate one of the underlying mechanisms proposed for human AD (Kasanin et al. 2022).

There are two commonly used approaches to assess cognitive function in *Drosophila*: 1) olfactory conditioning, in which a volatile odor is paired with electric shock to test aversive memory (Akalal et al. 2006, Beck et al. 2000, Kim et al. 2013, Tully and Quinn 1985), and 2) courtship suppression assay, to investigate conditioned response (McBride et al. 1999, Mhatre et al. 2014, Siegel and Hall 1979). The latter assay tests the fly's ability to modify its courtship behavior after learning from prior sexual experiences: naïve males that experience an unreceptive female for a period of time will eventually reduce male courtship behavior, even towards virgin females. To date, there are different fly transgenics that express human AD-related proteins: full-length APP in combination with β-secretase, Aβ42 fragment, or TAU construct. Such flies have different lifespans and learning and memory defects. Most courtship suppression assay utilized 4-5 days old flies to assess memory function. To model the memory loss after aging humans, Mhatre's group (2014) reared the transgenic *elav*; *App*/*BACE* flies at a lower temperature to prolong their lifespan and mimicking mild progression of disease. The recall memory loss did not occur until they were 80 days old, whereas learning during training remained intact throughout their lifespan (100 days). McBride's group (2010) also compared the learning and memory in 5 days old and 30-45 days old mutant flies with reduced level of presenilin (γ-secretase) in brain tissue. Presenilin is involved in formation of Aβ fragments as well as in mediating inflammatory response (reviewed by Saura 2010). The authors found that immediate recall and short-term memory were intact in 5 days old mutants, but impaired in 30 days old mutants. While there is general agreement that transgenics or mutants had altered learning and memory, it is difficult to consolidate findings due to the facts that various strains, conditions and age groups were tested. Here we used a transgenic line from Konsolaki's group (Finelli et al. 2004) using *elav-Gal4* driver to express human Aβ42 throughout the central nervous system (*elav-Gal4>UAS-Aβ42*$^{H29.3}$; hereafter referred as AD flies) to investigate their memory function of two age groups: young (4–6 days old) and old (16–18 days old). As neurodegeneration in humans impacts both cognitive and motor function which are pivotal to the fly courtship behavior, we chose a moderate age group that was two weeks older than conventional 4–5 days old flies for courtship suppression assay, but younger than other published age group (e.g. 30–45 days) to circumvent the confounding factor of muscle weakness during courtship. We first characterized the motor dysfunction and AD molecular markers to confirm that the flies retain transgene as reported previously (Finelli et al. 2004, Iijima et al. 2004). Then courtship suppression behaviors in age groups were examined to correlate age, neurodegeneration, and cognitive defects.

Materials and Methods

Drosophila stocks and genetic crosses

Aβ42-expressing flies (*UAS- Aβ42$^{H29.3}$/CyO*) were a generous gift from M. Konsolaki (2013). *Elav^{c155}Gal4* strain was from Bloomington *Drosophila* Stock Center (Bloomington, IN). All flies were maintained at 23ºC in a 12:12 light:dark cycle. Flies were fed with JAZZ-Mix *Drosophila* food, consisting of a mixture of sugar, corn meal, yeast, and agar recipe (Fisher Scientific, Pittsburgh, PA), or as described previously (Ruland et al. 2018). Transgenic AD flies were generated by crossing pan-neuronally expressing *elav-Gal4* line to *UAS- Aβ42$^{H29.3}$* strain.

Negative geotaxis assay

Male flies were separated into groups immediately after eclosion, placed in plastic vials containing *Drosophila* standard food, and kept at 25°C in an incubator. The climbing assays were conducted weekly at the same time of the day (10 am–12 pm) as long as enough animals were available. One day before the experiment, flies were separated into groups of five animals each. Before the climbing assay, individual groups were transferred into a 15 ml falcon tube without using anesthesia. After one minute of habituation, flies were gently tapped down to the bottom of the tube and animals attaining a 9 cm-high threshold within 15 seconds were counted. The procedure was repeated five times to obtain mean values for each single group. To exclude an effect of lighting conditions, the assay was carried out under red light.

Immunohistochemistry

Fly heads from 10- and 15- day old were fixed in 4% paraformaldehyde and embedded in paraffin. Five µm thin paraffin sections were deparaffinized, rehydrated and washed in distilled water. For antigen retrieval, slides were pretreated with formic acid. Anti-*β* amyloid (M872, mouse monoclonal, 1:100, DAKO, Glostrup, Denmark) was used. After washes in PBT, the slides were incubated with a biotinylated goat anti-rabbit secondary antibody (E0432; 1:500 dilution; DAKO) for 45 minutes at room temperature after incubation with the ABC kit (SK6100; Vectastain avidin-biotin complex-horseradish peroxidase (ABC-HRP; Vector Laboratories, Burlingame, CA, USA) for 45 minutes after washing in PBT. The signal was developed using a 3,3-diaminobenzidine (DAB) substrate kit (SK4100; Vector Laboratories), and the sections were counterstained with hematoxylin. For negative controls, sections were stained as described above using only the secondary antibody.

Courtship suppression assay

For courtship behavioral training, methodology was adapted from McBride et al. (1999) with the following modifications. Naïve males were collected between 0 to 6h after eclosion (Day 1) and transferred to food vials (5 males per vial). Virgin females were collected with 10 females per vial. All flies were maintained at 23ºC in a 12:12 light:dark cycle. All behavioral tests were conducted in a separate room maintained at 23ºC and under 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 spent performing courtship behaviors divided by the total observed time (10 minutes) for unmated males. If successful copulation occurred between 2-10 minutes, observation was stopped and CI was calculated

using the time period leading up to copulation instead of entire 10 minutes (McBride et al. 1999). Males that mated within the first two minutes of observation were excluded from data.

Two age groups of naïve males were tested: young (4–6 days after eclosion) and old (16–18 days after eclosion) for both parental control (*elav-Gal4*) and for AD flies. Virgin female *elav-Gal4* flies were collected and kept in normal food vials in groups of 10. Trainer (mated) females were obtained by mating 4-day old *elav-GAL4* virgin females with naïve *elav-GAL4* males on the day before training and testing. Only successfully mated female trainers were recovered and kept individually. The next day, each naïve male was transferred by gently aspirating to an empty well in a 4-well plate (ThermoFisher Scientific, Waltham, MA) and allowed to acclimate for 1 minute. Next, a mated *elav-GAL4* female trainer was added to the well with the naïve male, and the training lasted for 60 minutes. For sham control, naïve males were transferred to 4-well plates, without any female for 60 minutes. The amount of time the males exhibited courtship behavior during training was assessed during the first and the last 10 minutes. To test their immediate recall memory after 60 minutes of training, both trained males and sham control males were transferred individually 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. In addition to calculating CI, frequency of attempts to copulate in the 10 minutes of testing was also recorded. All observers were blind as to the fly's genotype or experimental status during courtship behavior analysis.

Statistical analysis

Data were analyzed with a two-tailed Student's t-test or Mann-Whitney-U test (Fig. 1 only). Statistical significance was set at the 95% confidence level. p-values ≤ 0.05 are marked *; p-values < 0.01, **; and p-values < 0.001, ***.

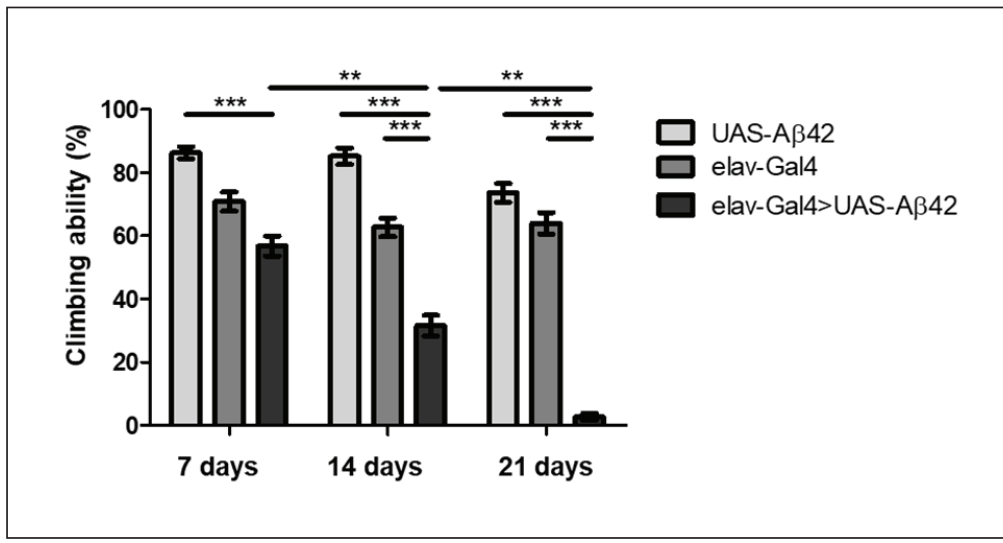

Figure 1. Decreased locomotor performance of AD flies over time. The locomotor performance of flies with pan-neuronally expressing Aβ42 under the control of elav-Gal4 driver (darkest grey) was compared to climbing behavior of parental strains, respectively carrying the UAS-Aβ42 construct (lightest grey) or the Gal4 driver (intermediate grey) only. Each group represents the average of up to 10 replicates including 5 animals each. Horizontal bars indicate significant differences observed. **p < 0.01; *** p < 0.001. Vertical bars represent Standard Error of the Mean (SEM).

Results

Pan-neuronal expression of Aβ42 leads to locomotor defects

A negative geotaxis assay was performed with AD flies and two parental control flies: *elav-Gal4* and *UAS-Aβ42*. A decline in locomotor activity was observed (Fig. 1), due to progressive neurodegeneration of the AD flies (Fig. 2 and Iijima et al. 2004). Mann-Whitney-U test was performed for statistical analysis. The AD flies progressively lost their climbing ability over the course of the three-week testing period. On average, between the first and second week, the climbing ability in AD flies decreased from 56.8% to 31.6% ($p = 0.0056$). Between the second to third week the climbing ability decreased again for another 2.8% ($p = 0.0059$). In contrast, parental control groups (*UAS-Aβ* or *elav-Gal4*) showed no significant decrease in three weeks. Furthermore, the climbing ability in AD flies was reduced when compared to that of parental control: after one week (AD vs. *UAS-Aβ42*: $p = 0.0005$); after two weeks (AD vs. *elav-Gal4*: $p = 0.0006$; AD vs. *UAS-Aβ42*: $p = 0.0002$); and after three weeks (AD vs. *elav-Gal4*: $p = 0.0003$; AD vs. *UAS-Aβ42*: $p = 0.0001$).

Aβ42 expression leads to amyloid deposits in the adult fly brain

Presence of human amyloid protein in day-10 and day-15 fly heads were verified using immunohistochemistry. Iijima's group has previously reported that amyloid load was detectable on day 3 and a severe built up on day 48 staining (Finelli et al. 2004, Iijima et al. 2004). Our result established additional time points that correspond to our old age group in subsequent courtship suppression assay between day 10 and day 15 and showed an age-dependent increase in quantity of extracellular deposits of *Aβ42*. Iijima's group also

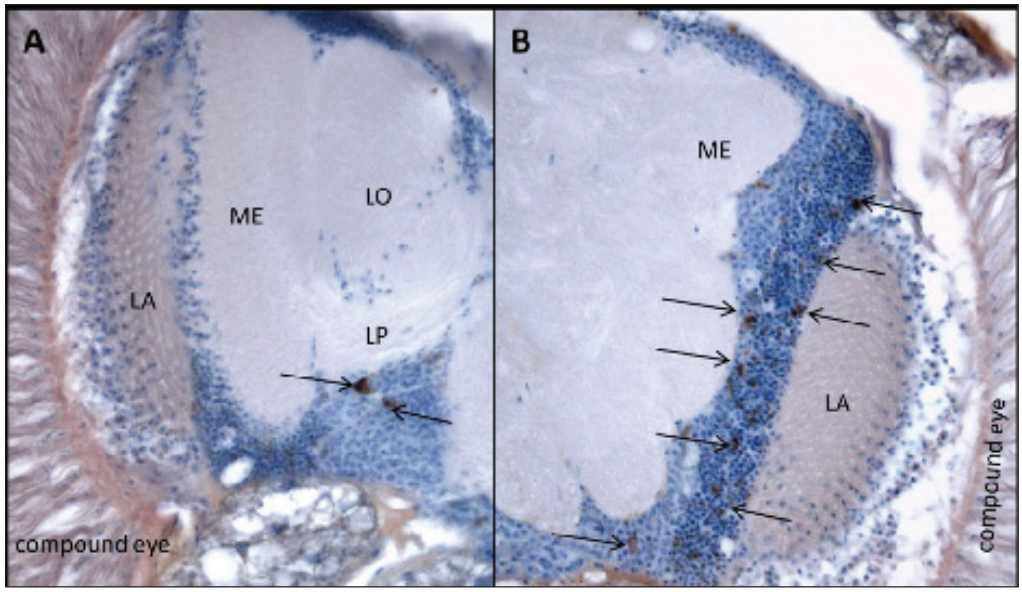

Figure 2. Extracellular Aβ42 aggregates in paraffin sections of 10- and 15-days old *Drosophila melanogaster* brains. Paraffin sections of 10- and 15- days old flies were stained using the 6F/3D α-Aβ42 antibody to highlight amyloid-beta (brown) and nuclear stain DAB K5001 (blue). Arrows mark several positively stained aggregations. Aβ42 accumulations in 10 days old adult brain (A) and in 15 days old adult brain (B). LA: lamina, ME: medulla, LO: lobula, LP: lobula plate.

reported the diffusible *Aβ42* in the Kenyon cells and neuropil, but no amyloid fibril. Using a different antibody, accumulation of *Aβ42* in our AD flies were detected between lamina and medulla, and in the ventral nuclei region to lobula plate (Fig. 2).

Young AD flies displayed short-term memory deficit despite an ability to learn

Courtship suppression assays were performed by testing young (4–6 days old) *elav-Gal4* and AD flies. The fraction of time that a male tester spent on courtship during a ten-minute window was expressed as the courtship index (CI). As the flies experience rejection with the mated trainer female, we adapted the definition of learning during training (LDT) by Joiner's and McBride's groups as more than 40% decrease in courtship index (CI) between first- and last- ten minutes of one-hour training periods (Joiner and Griffin 1997, McBride 2010). At 4–6 days of age, both *elav-Gal4* and AD flies showed a significant LDT with training (87% reduction, $p = 0.0002$; and 74% reduction, $p = 0.019$, respectively; Figure 3A). These data suggest that both young *elav-Gal4* and AD flies were able to respond to sensory signals and modify behavior accordingly. In the testing phase, trained *elav-GAL4* males had a significantly lower courtship index when compared to the age- and genotype-matched, untrained males (*i.e.*, sham control; $p = 0.0015$, Fig. 3B), an indicative of intact immediate recall memory. In contrast, young AD flies already exhibited deficits in short-term, recall memory (trained vs. sham: $p = 0.35$). Lastly, comparing Figures 3B and 3D, we found that CI of sham control from old *elav-Gal4* is significantly lower than that of young sham control ($p = 0.00027$), whereas sham control of AD showed no difference between the two age groups ($p = 0.847$).

Loss of LDT and immediate recall memory in aged groups

When comparing courtship behaviors in older (16–18 days old) *elav-Gal4* and AD flies, neither group showed significant LDT (32% reduction, $p = 0.55$; and 3% reduction; $p = 0.95$, respectively; Fig. 3C). Within the *elav-Gal4* groups, total CIs of first 10 minutes were similar between young (Fig. 3A) and old (Fig. 3C); but the CI of last ten minutes in older group went up, making the LDT insignificant. This phenomenon was more pronounced in the last 10 minutes of old AD flies, in comparison to that of young AD group. It suggests that disinhibition occurs with aging, and enhanced even more by Aβ transgene, like the behaviors reported in some AD and dementia patients (Eshmawey 2021, Yu et al. 2019). Upon closer examination, we noted that some older AD flies were persistent in courtship behaviors throughout the entire hour of training, and not just the last ten minutes, clearly ignoring the rejection from the previously mated female trainer. In the subsequent testing of immediate recall, neither trained *elav-GAL4* nor AD flies showed any differences in CI when compared to their respective age-matched sham males ($p = 0.625$ and 0.077, respectively, Fig. 3D). Despite only two weeks older, courtship behaviors at the age appear to be more complexed than the younger group, possibly due to the sexual maturation, prolonged social interaction with other male flies in the same vial, and in the case of AD, disruption of learning and memory with presence of Aβ (more in discussion).

Older AD flies exhibited higher frequency of copulation attempts after training

Intrigued by the unusual hyperactive courtship behavior in some older AD flies during training, we re-analyzed a subset of courtship behavior and focused on copulation attempts during 10 minutes of testing (Fig. 4). We found a trend toward fewer copulation attempts in trained young *elav-Gal4* (2.19 attempts) and AD flies (1.81 attempts), when compared to their untrained sham control (2.67 and 3.45 attempts, respectively), although such difference was not statistically significant ($p = 0.35$ for *elav-Gal4*; and $p = 0.12$ for AD). Similarly, the

average frequency in the trained older *elav-Gal4* group was lower than that of sham (1.30 vs. 1.69), also not significant (p = 0.28). Finally, the frequency of attempts by older, trained AD flies was significantly higher than that of sham control (1.95 vs. 1.12; p = 0.044). While number of attempts seemed low in a 10-minute window, the total CI was much higher in sham group than in trained flies (Fig. 3D), making the ratio of copulation attempts: total CI much higher in trained group (5.82) than sham (2.55). As copulation attempts resemble a more aggressive courtship behavior, this shift is likely triggered by rejection during training.

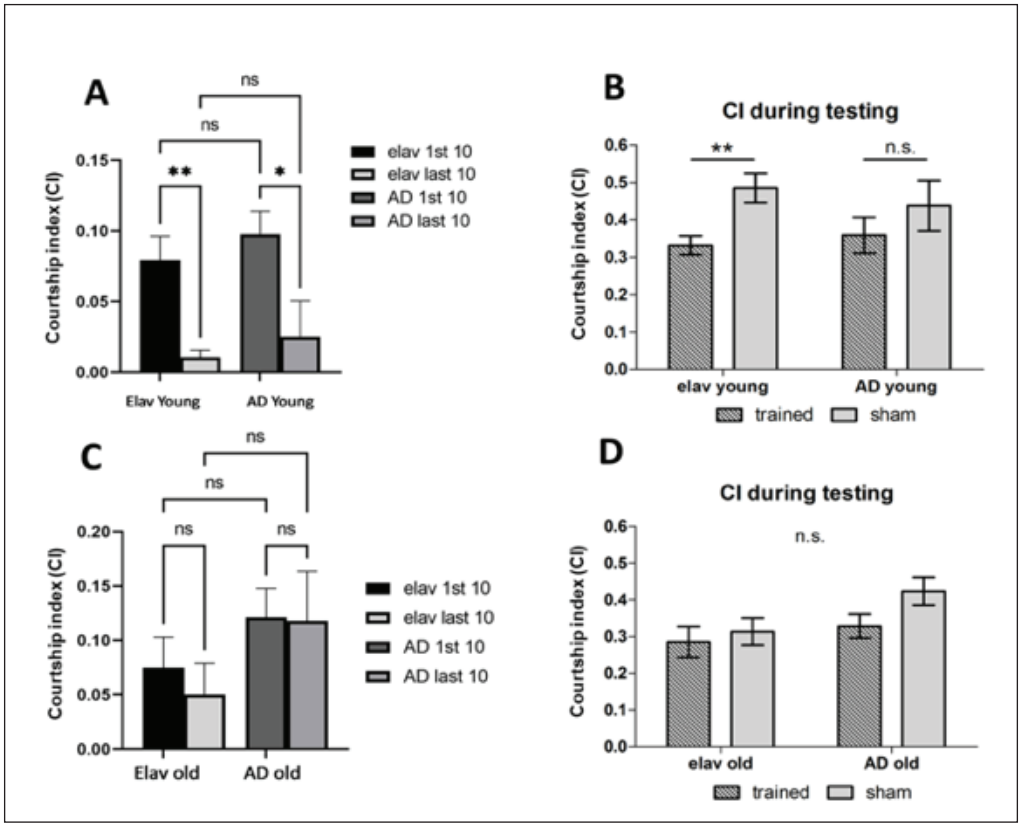

Figure 3. Training and testing in young (4-6 days old) *elav-Gal4* and AD flies (**A-B**), and in old (16-18 days old) *elav-Gal4* and AD flies (**C-D**). **A**: average courtship index between the first 10 min and last 10 min during training phase. In young *elav-GAL4* (n = 63), a significant difference (p = 0.000158) between the first 10 (0.079 ± 0.016) and last 10 min of training (0.01 ± 0.004), suggesting the efficacy of training. In young AD males (n = 27), similar training efficacy (p = 0.019) was observed between first 10 (0.097 ± 0.015) and last 10 min (0.025 ± 0.0024). **B**: comparison of average CI in young *elav-GAL4* between trained (0.332 ± 0.025; n = 63) and sham control (0.485 ± 0.039; n = 35) during testing. A significant difference was observed for *elav-GAL4* group (p= 0.00158). No significant difference (0 = 0.35) in AD flies between trained (0.359 ± 0.048; n = 26) and sham control (0.438 ± 0.067; n = 14). **C**: no significant differences were observed between first 10 min (0.074 ± 0.027) and last 10 min (0.05 ± 0.028) of training in old *elav-GAL4* group (p = 0.546; n = 27). Also, no significant difference (p = 0.949; n = 29) was observed for old AD flies between first 10 min (0.121 ± 0.026) and last 10 min (0.117 ± 0.044). **D**: no significant difference was observed between trained (0.285 ± 0.042; n = 27) and sham control (0.313 ± 0.037; n = 32) in old *elav-GAL4* (p = 0.625). No significant difference (p = 0.077) between trained (0.328 ± 0.032; n = 28) and sham (0.423 ± 0.038; n = 18). Horizontal bars or brackets indicate significant differences observed. *p < 0.05; ** p < 0.001. Error bars represent Standard Error of the Mean (SEM).

Discussion

We investigated the effect of amyloid beta plaques on *Drosophila* learning and memory using the yeast *Gal4*/*UAS* system to drive the expression of human amyloid beta fragment (aa 1-42) in flies. We observed amyloid deposits in brain regions of 10- and 15- day old flies (Fig. 2) as well as 25–55% declined locomotor function (Fig. 1). Courtship suppression assays showed that young AD flies displayed a recall memory deficit, while maintaining the ability to learn during training. Furthermore, no significant difference in total courtship index (CI) in older flies was observed between trained and sham control (Fig. 3C-D). Nevertheless, copulation attempts were significantly higher in the trained AD group, in comparison to that of the sham control group (Fig. 4).

For the purpose of *Drosophila* courtship behaviors, two major groups of molecules involved in olfactory and gustatory signals are considered here: a) non-volatile cuticular hydrocarbons (CHCs) produced in females as sex pheromone to attract male flies (Ferveur 2005), and b) anti-aphrodisiacs such as cis-vaccenyl acetate (cVA) and (Z)-7-Tricosene produced in males. Male flies prefer young over old females because of their CHCs differ as part of the age-related sexual maturity (Hu et al. 2014). In addition, in *Drosophila ananassae*, older males had better courting and reproductive success than young males (Prathibha et al. 2011). The anti-aphrodisiacs are deposited on females during mating to make her less

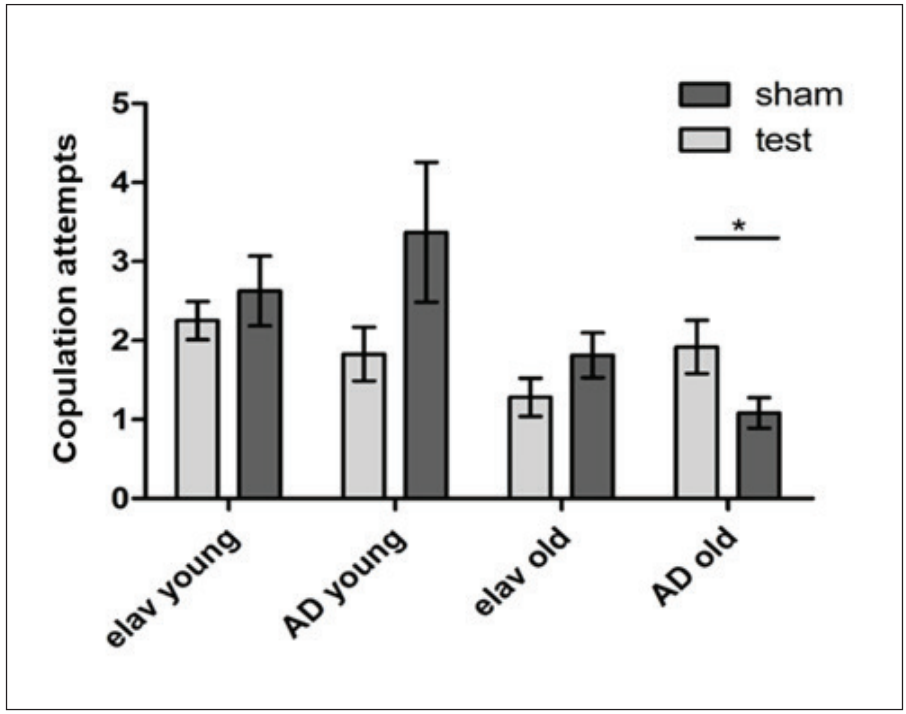

Figure 4. Copulation attempts frequency (counts /10 min) in two age groups during testing. Sample size for each group: *elav-Gal4* young trained (n = 63) vs. sham (n = 34); AD young trained (n = 25) vs. sham (n = 13); *elav-Gal4* old trained (n = 26) vs. sham (n = 31); and AD old trained (n = 27) vs. sham (n = 17). * P = 0.044. Horizontal bars indicate significant differences observed. *p < 0.05. Vertical bars represent Standard Error of the Mean (SEM).

attractive to other males (Chin et al. 2014). Z-7-Tricosene is also the sex pheromone that inhibits male-male courtship (Lacaille et al. 2007), which is detected by gustatory receptor, *Gr32a* (Moon et al. 2009). It has been shown that both odorant receptors (e.g. *Or67d* or *Or65a;*) and *Gr32a* attribute to the reduced male courtship toward mated females. Chemosensory interaction is transmitted to the brain and subsequently behavioral output, either engagement or rejection, is determined. Mutations in those genes abolished the discrimination between virgin and mated females (Laturney and Billeter 2016, Miyamoto and Amrein 2008). Furthermore, Hu's group reported that preference of young females was eliminated when human APP was ectopically expressed in another gustatory receptor, *Gr33a* (Hu et al. 2014). It is possible that Aβ42 targeted the chemosensory system in our AD flies and reduced their sensitivity to distinguish between virgin and mated females; and more importantly, disrupted the neuronal function that is involved in learning and memory inputs for decision-making process.

In our study, sham control flies served as a baseline for the courtship index in each age group within the same genotype, as their first and only encounter with virgin females took place during the 10 minutes of testing. Prathibha's group (2011) have previously described that individually housed older males had higher CI compared to the young ones. However, in our group-housing condition we observed a significantly reduced CI in sham control of old *elav-Gal4* (Fig. 3D), compared to that of young ones (Fig. 3B). In addition, no difference of CI was observed between two age groups of AD sham control (Fig. 3B &D). It is conceivable that interaction among group-housed males, either in short term (4–6 days) or long term (16-18 days), includes sending inhibitory signals through anti-aphrodisiacs and/or physically rejecting each other. It is possible that old *elav-Gal4* flies in the sham control group learned from this experience, which made them hesitant to initiate courtship towards other flies including virgin females during the 10- minute testing, compared to the young *elav-Gal4* flies. This experience-induced modification did not occur with either group of AD flies. In comparison, Iijima's group (2004) reported olfactory associative learning started to decline in AD flies on day 6–7, and progressively worsen on day 14–15 while their olfactory acuity remained the same as that of *elav-Gal4*. Our result in sham control group of AD flies agreed with theirs on the lack of learning, despite different methods being used.

CIs observed during the first 10 minutes of training were much lower than CIs during testing, suggesting the inhibitory signals (e.g. anti-aphrodisiacs) on mated females were effective and perceived by naïve males. The mixture of female CHCs, the anti-aphrodisiacs, and the experience of prior social interaction all play into the decision of naïve males in training. However, in the last 10 minutes of training, both old *elav-Gal4* and AD flies continued their courtship activities, with only 2% difference of CIs between the first and the last 10 minutes for old AD flies. Unlike the sham control, males in the last 10 min of training had been continuously exposed to competing positive (CHCs) and negative (anti-aphrodisiacs) signals, which, paired with their learning during social interaction to other males, compelled them to ignore the rejection from mated females. For AD flies, even the 3 days old already showed some amyloid in brain (Iijima et al. 2004), so while the inhibitory signals were present during training, young AD managed to respond to it, but failed to retain the memory immediately after. Old AD flies also responded to the presence of anti-aphrodisiacs, but disinhibition occurred quickly during training and continued into testing, most likely due to more amyloid deposit in the brain that impaired the relay and decision-making processes. Additionally, although olfactory acuity to two organic compounds (OCT and MCH) appeared intact in 14–15 days old AD flies (Iijima et al. 2004), amyloid impact on olfactory/gustatory receptors should also be considered.

Not only was disinhibition more pronounced in older AD, the one-hour training with mated females also induced shift in the type of courtship to more aggressive copulation attempts (Fig. 4). Aggression and inappropriate sexual behavior are noted as symptoms found in some AD patients: 28% aggression in AD (Yu et al. 2019) and 25% in patients with dementia (Eshmawey 2021). Because both trained and sham control groups are siblings with same genetic makeup and age, the observed behavioral differences are likely triggered by stimuli (i.e. rejection) in that one hour. For future study, proteomic analyses or whole genome RNA sequencing from those trained AD fly heads may shed light on signal relay that leads to their aggressiveness, and to identify potential target for treating AD patients.

Most reports on learning and memory using courtship suppression assay focused on young (4–5 days old) males that were individually housed. Our study tested on a different age group (16–18 days old) that were older and group housed. Based on our results, we hypothesized that old *elav-Gal4* learned from prolonged male-male interaction which reflects on their subsequent encounters with either virgin or mated females. Furthermore, while young AD males could still modify their courtship behaviors towards mated trainers, the inhibition disappeared quickly during testing and showed no difference in CI from that of sham control. The group housing became a conditioning itself, prior to training and testing. In our previous study, we showed that social interaction in AD flies was crucial to their survival: an iron response protein 1B was upregulated in the individually housed flies that had shorter lifespans and more severe locomotor phenotypes (Ruland et al. 2017). By studying older AD flies in different social settings, we hope to further investigate the significance of age and environmental factors and their corresponding signal pathways underlying symptoms of Alzheimer's Disease.

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