DEVELOPING AND CHARACTERIZING A TDP-43 DROSOPHILA MODEL OF FRONTOTEMPORAL DEMENTIA

By

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Abstract:

Frontotemporal Dementia (FTD) is a progressive neurodegenerative disease that is characterized by intraneuronal protein aggregates. In 45% of FTD cases, these intracellular inclusions contain TAR-DNA binding protein (TDP-43). Though many animal models of FTD exist, none have been generated in *Drosophila melanogaster* through TDP-43 overexpression that recapitulate learning and memory deficits found in late stages of the disease. In an attempt to develop such a model, we overexpressed TDP-43 and disease-associated mutant variants in the mushroom bodies of the fly. Their learning and memory was tested using a courtship assay that measures conditioned decreases in male courtship behavior after experience with an unreceptive female. Subsequent courtship behavior with a receptive female was also measured. TDP-43 expressing flies did not show significant differences from controls in their courtship behavior with the unreceptive female, but all genotypes and ages showed a reduction in courtship when paired with the receptive female. Differences in courtship behavior between flies that subsequently mated with the receptive female and those that did not were also found. This suggests that genetic background of the transgenic flies and the behavior of the receptive female have profound effects on the courtship behavior of male *Drosophila.*

Introduction:

Frontotemporal Dementia (FTD) is a neurodegenerative disease that is the second leading cause of dementia outside of Alzheimer's Disease (AD) and is the primary cause of dementia in 45-60 year olds (Ji et al, 2017). It affects between 50,000 to 60,000 people in the United States. FTD was first characterized by Arnold Pick in 1892, and was often referred to as Pick's Disease—a diagnosis that now specifically refers to forms of FTD that have intracellular inclusions called Pick bodies (Olszewska et al, 2016). FTD can manifest itself in two forms; a familial, inherited form and a sporadic form. The familial cases account for 20-50 % of all FTD cases and the remaining are sporadic with no family history. Interestingly, mutations in FTD-associated genes are present in many sporadic cases, supporting the concept that FTD has a genetic component.

FTD can be broken up into several clinical subtypes. The first is behavioral variant FTD (bvFTD), in which patients show personality changes and marked increases in apathy. Other FTD patients exhibit severe decreases in language skills. Patients who exhibit primary progressive aphasia can also be further broken down into subtypes: semantic variant and non-fluent/agrammatic variant. Semantic variant dementia patients have trouble understanding or speaking sentences whereas non-fluent/agrammatic aphasia patients have labored speaking that is agrammatical and have difficulty understanding complex syntax (Olszewska et al, 2016). When the disease has progressed far enough, FTD patients show impairments to the ability to learn and remember information. Finally, many FTD patients exhibit motor deficits, which is in alignment with the idea that FTD is a spectrum disorder with amyotrophic lateral sclerosis (ALS), a neurodegenerative disease of motor neurons. The two diseases exhibit significant clinical, pathological, and

mechanistic overlap supporting the idea that these two phenotypes are bookends of a spectrum with many patients showing various degrees of motor deficits and cognitive decline.

A significant finding supporting the idea of an FTD/ALS spectrum was the discovery of the chromosome 9 open reading frame 71 (C9orf72) mutations that accounts for 25% of familial cases of FTD and 30-50% of familial cases of ALS. It also accounts for a smaller percentage of sporadic cases of each (Ji et al, 2017). Additionally, TAR DNA-binding protein (TDP-43) pathology is a common feature of both diseases. TDP-43 cytoplasmic inclusions are found in 97% of all ALS cases and 45% of FTD cases, making TDP-43 the most common protein found in aggregates between both diseases.

TDP-43 is a nuclear RNA binding protein involved in RNA splicing, processing, and transport. In both FTD and ALS, it is found mislocalized and aggregated in the cytoplasm. There are several theories as to why TDP-43 aggregates are toxic to cells and they include defects in protein homeostasis and RNA metabolism. Protein homeostasis theories include loss of function (LOF) or gain of function (GOF) roles of TDP-43. TDP-43 aggregates can also sequester mRNAs, leading to defects in RNA processing as well.

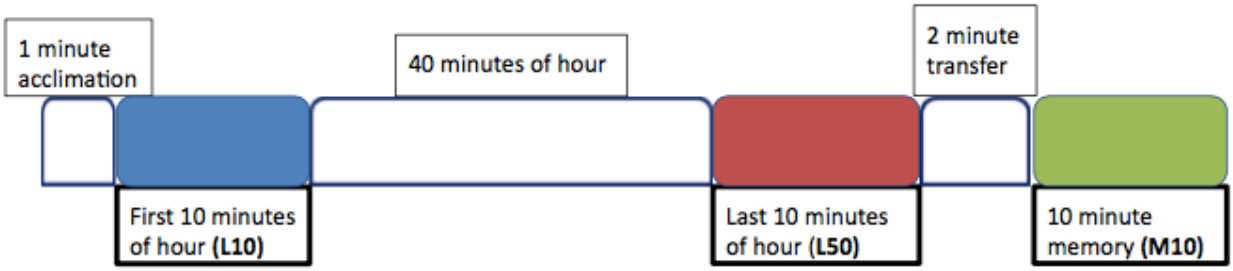
There are a few *Drosophila melanogaster* models of FTD, but none that recapitulate cognitive behavioral symptoms of the disease in this model organism. The overall goal of this project was to develop and characterize a model of FTD in *Drosophila* through the overexpression of human TDP-43 and mutant variants in the mushroom bodies of the fly. The mushroom body is a learning and memory structure in the fly brain analogous to the hippocampus in mammals. Developing a *Drosophila* model of FTD and characterizing it through a behavioral learning and memory is a novel approach to modeling the disease.

Although other studies have shown the toxicity of TDP-43 in the Drosophila mushroom body (Li *et al, 2010*), there have not been studies examining the effects on behaviors such as learning and memory. Similarly, the courtship assay has been used in the literature to characterize other disease models such as Fragile X syndrome (McBride et al, 2005) and Alzheimer's Disease (Mhatre et al, 2014).

The literature shows that the courtship assay can be used to measure learning and memory in *Drosophila*. Its basis is in the decreased male courtship behavior over time in response to an unreceptive female. It was shown that if a male fly was paired with an unreceptive female in a courtship chamber, he would court her at first, but over time his behavior would decrease as he learned the female is unreceptive. Further, his courtship behavior would remain suppressed when placed with a new female who was receptive.

Male *Drosophila* demonstrate a very stereotyped progression of behaviors when courting females, allowing for quantifiable measurement of behavior. First, males will orient themselves toward the female and will follow her around the chamber. He will then engage in wing-song, where he spreads one wing away from his body and vibrates it to create a "song". The frequency of this wing-song is highly species-specific. The male will also mechanically stimulate the female through tapping on her abdomen and will also lick her vaginal plate with his proboscis. If the female allows mating, the male will then mount and copulate with her. These behaviors can be easily seen on a video-recording of the flies. By summing the total amount of time courtship behavior was observed during a given time period, a simple proportion of courtship behavior is calculated—called the courtship index. We measured three courtship indices corresponding to specific time points in the experiment for each fly. The L10 period is the first ten minutes with the unreceptive

female, L50 is the last ten minutes of the hour with the unreceptive female, and M10 is the ten minutes with the new receptive female (Siegal and Hall, 1979; Mhatre et al, 2014).



This assay was chosen as a way to measure the learning and memory ability for flies that were overexpressing TDP-43 wild-type and disease-associated mutant variants in the mushroom bodies. Due to the late age of onset for many neurodegenerative diseases, FTD included, multiple ages of flies was tested: 5 days, 10 days, and 15 days post-eclosion. This was to test if the flies exhibit age-dependent decline in learning and memory.

In order to target the expression TDP-43 wild-type and mutant variants to the mushroom bodies, the GAL4-UAS system was used (as previously described for motor neurons by the Zarnescu lab; Estes et al, 2011; Estes et al, 2013). GAL4 is a transcription factor derived from yeast that binds to the upstream activating sequence (UAS). Transgenic flies expressing GAL4 under a spatial or temporal specific promoter can be crossed to transgenic flies expressing the UAS element upstream of the gene of interest. (Duffy, 2002) This results in spatial or temporal expression of the gene of interest.

Results:

Testing Courtship Assay with Wild-Type Flies

Five-day-old wild-type OregonR flies were tested in the courtship assay to establish a baseline (Figure 1). A paired t-test did not show a significant reduction in courtship

behavior for L10 to M10 (p=0.078). However, the mean for L10 values was only 0.51—much lower than L10 values seen in the literature which vary from 0.6 to 0.8. This means that a significant reduction may not have been observed in M10 because of a floor effect set by L10. The proportion of flies that were discarded for low courtship behavior in L10 (30%), was also higher than normal, suggesting that OregonR flies demonstrate lower courtship indices as a whole. Therefore, due to the trend for a decrease in courtship behavior from L10 to M10, transgenic flies were tested and analyzed.

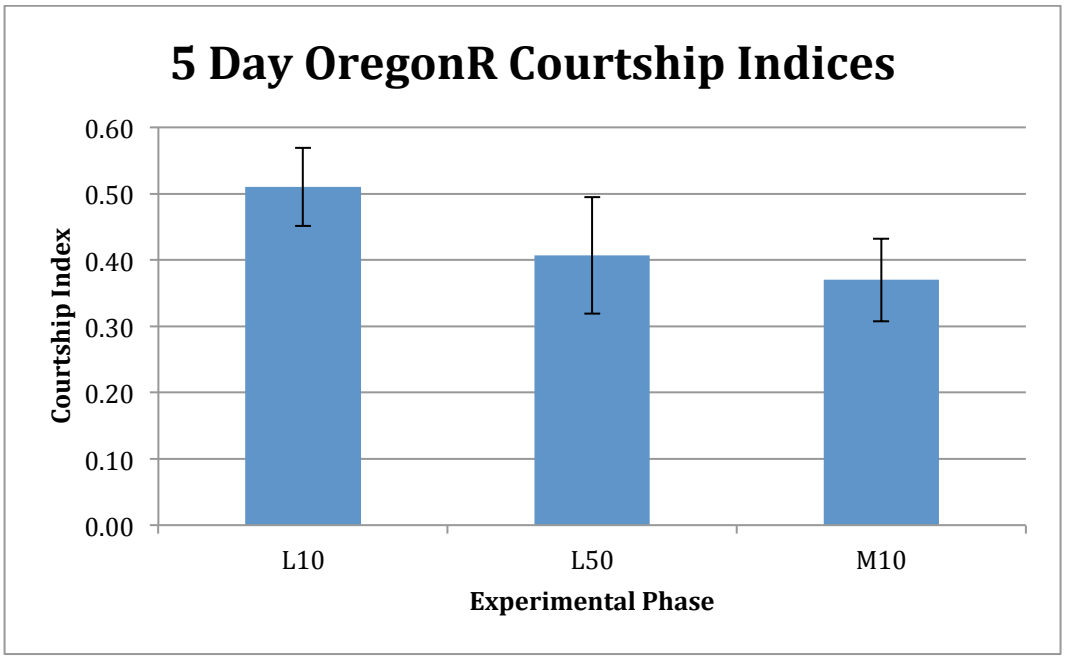


Figure 1. Mean courtship indices for 5-day-old wild-type OregonR male flies. n=16. A total of 23 males were tested and 7 were discarded for having a courtship index less than 0.1 in the learning phase. Error bars represent standard error of the mean.

Optimizing the Mushroom Body GAL4 Driver

The flies tested next were transgenic 201Y GAL4 driver flies crossed to UAS-TDP-43 or mutant variants. The 201Y driver had fairly good localization to the mushroom body as visualized by larval dissections (Figure 2), but the driver also expresses in the larval musculature. This resulted in early lethality of the progeny in their pupal cases and a very

low number of naïve males being collected for courtship experiments. We then decided to switch to another mushroom body driver, OK107, to overcome the pupal lethality associated with 201Y. However, this driver had less specificity than 201Y to the mushroom bodies and expressed in other brain regions (Figure 2). While running these flies in courtship experiments, steps were taken to reduce as much variability as possible. All courtship flies were housed in a new incubator, humidity was controlled, and the courtship assay was standardized. (Courtship data not shown)

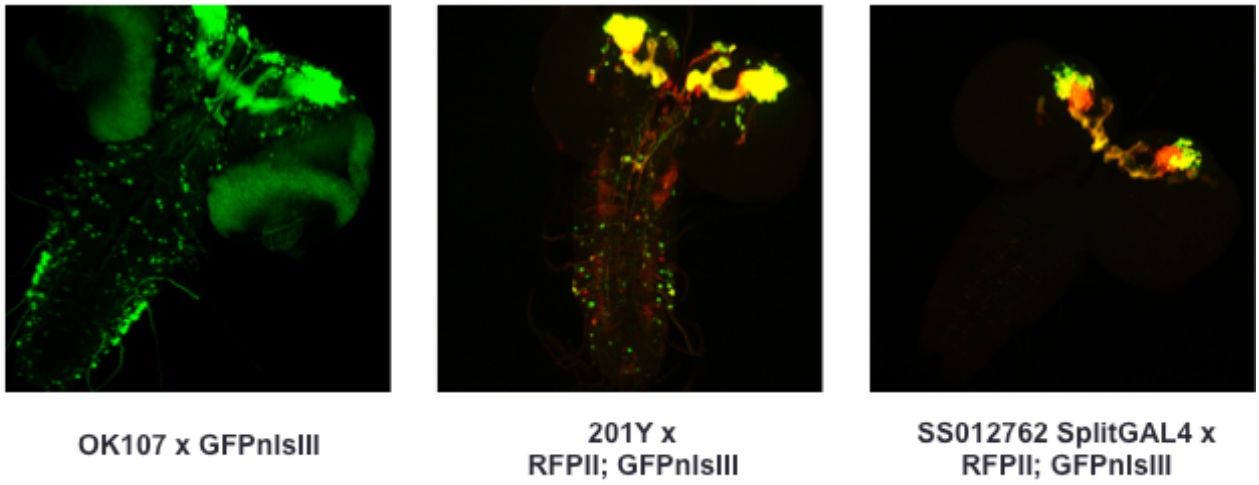


Figure 2. Third instar larval dissections showing spatial expression of the GAL4 drivers used in the courtship experiments. (Images provided by Dr. Robert Kraft)

The next step was to use a split GAL4 driver, SS01276, where two different halves of the GAL4 protein are expressed under two different tissue specific promoters that have overlap in the mushroom bodies. This results in much higher tissue specificity localization of the GAL4 protein to the mushroom bodies (Figure 2).

Courtship Assay with Transgenic Flies

After switching to the new SS01276 driver, the first time point to be tested was 5-day-old naïve male flies. The SS01276 driver was crossed to UAS flies driving expression of human TDP-43 wild-type and two mutant variants; G298S9 and A315T6. To control for

genetic background, the SS01276 flies were crossed to w^{1118} males as controls. Five days is when males flies are at their peak fecundity, therefore, we predicted this time point would show robust differences between controls and TDP-43 wild-type or mutant variant-expressing flies if the TDP-43 overexpression had profound effects on learning and memory.

A two-factor repeated measures ANOVA was performed to analyze the effects of genotype and time, which corresponds to the L10, L50, and M10 time points of the experiment, on courtship indices. There was no significant difference between genotypes, but there was a significant effect of time on courtship indices (Figure 3. $p<0.0001$). This indicated a significant decrease in courtship behavior over the course of the experiment.

We then decided to test flies at older time points to investigate the possibility of an age-dependent decline in courtship behavior. The next two time-points to be tested were of the same genotypes as above at 10 days post-eclosion and 15 days post-eclosion. The same two factor repeated measures ANOVA was performed for each time point and only time was a significant factor (Figure 3, $p<0.0001$).

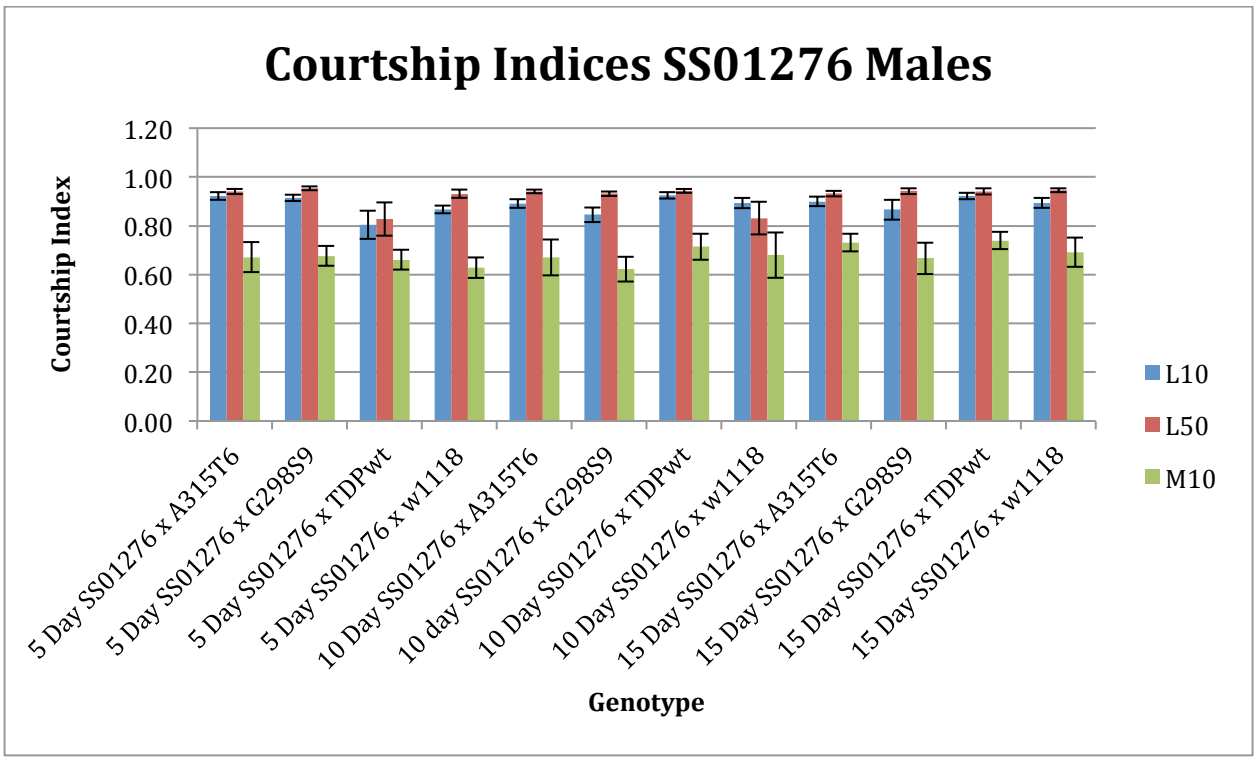


Figure 3. Mean courtship indices for 5-day, 10-day, and 15-day old SS01276 male flies. n≥15 for every age and genotype. Time, as represented by the L10, L50, and M10 time points was significant for all ages and genotypes. $p<0.0001$. A total of 203 males were tested and 9 were discarded for mating with the unreceptive female during the learning period. Error bars represent standard error of the mean.

After analysis of each individual age group, we decided to normalize all courtship data for each fly. Calculating the M10 value for an individual fly as a proportion of its own L10 value and subtracting this from 1.0 created a value for the proportional change in its courtship behavior. This accounts for differences in absolute courtship behavior that is a result of individual variation to give a more standardized value for each fly. A two factor non-repeated measures ANOVA was then performed to analyze differences across genotype and age groups, but no significant differences were found for the difference between L10 and M10 after normalization (Figure 4). Therefore, the proportional reduction in courtship behavior from L10 to M10 was not significantly different between genotypes or age groups.

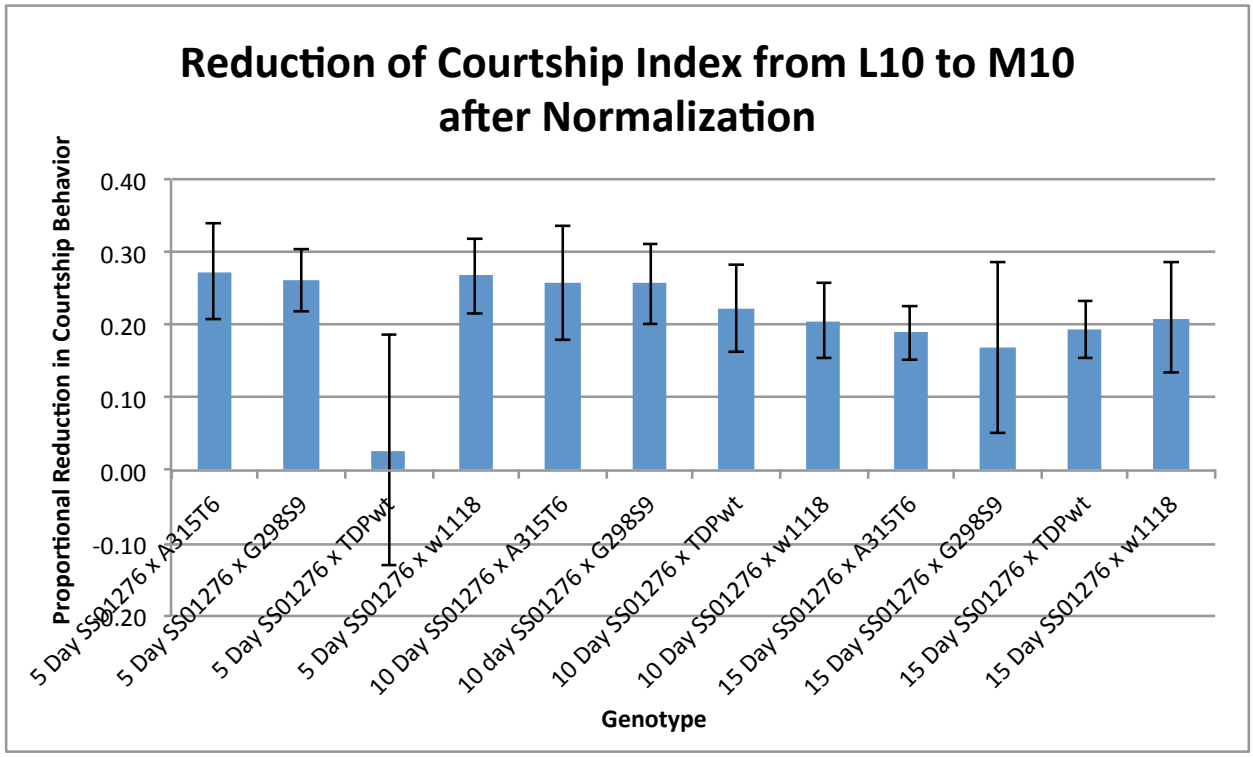


Figure 4: Mean reduction in courtship behavior from L10 to M10 for SS01276 males. Reduction in courtship behavior was calculated by normalizing each individual fly's M10 value to its L10 value and then subtracting this normalized value from 1.0. n≥15 for every age and genotype combination. Error bars represent standard error of the mean.

Mating Behavior in M10

The reduction in courtship behavior from L10 to M10 needed to be investigated further because courtship behavior between L10 and L50 was either not decreased or even increased for some ages and genotypes. A majority of males also mated with the receptive female in M10 across all groups (Table 1), but there was no significant difference in the rate of mating in M10 compared to SS01276 x w^{1118} controls. This led to the hypothesis that the receptive females in M10 were having a bigger impact on the male's courtship behavior compared to the hypothesis that courtship reduction was indicative of learning and memory. Two new pieces of information were then collected from every male: the amount

of time it took for them to start courting in M10 and the amount of time it took for them to mate.

First, the courtship reduction from L10 to M10 after normalization between flies that mated and those that did not was compared, but no difference was found. We then decided to analyze only the non-mater flies to investigate possible differences in age and genotype on their courtship indices (Figure 5). A two factor non-repeated measures ANOVA was performed, but no differences found. However, the n for each group was very low after eliminating flies that mated in M10.

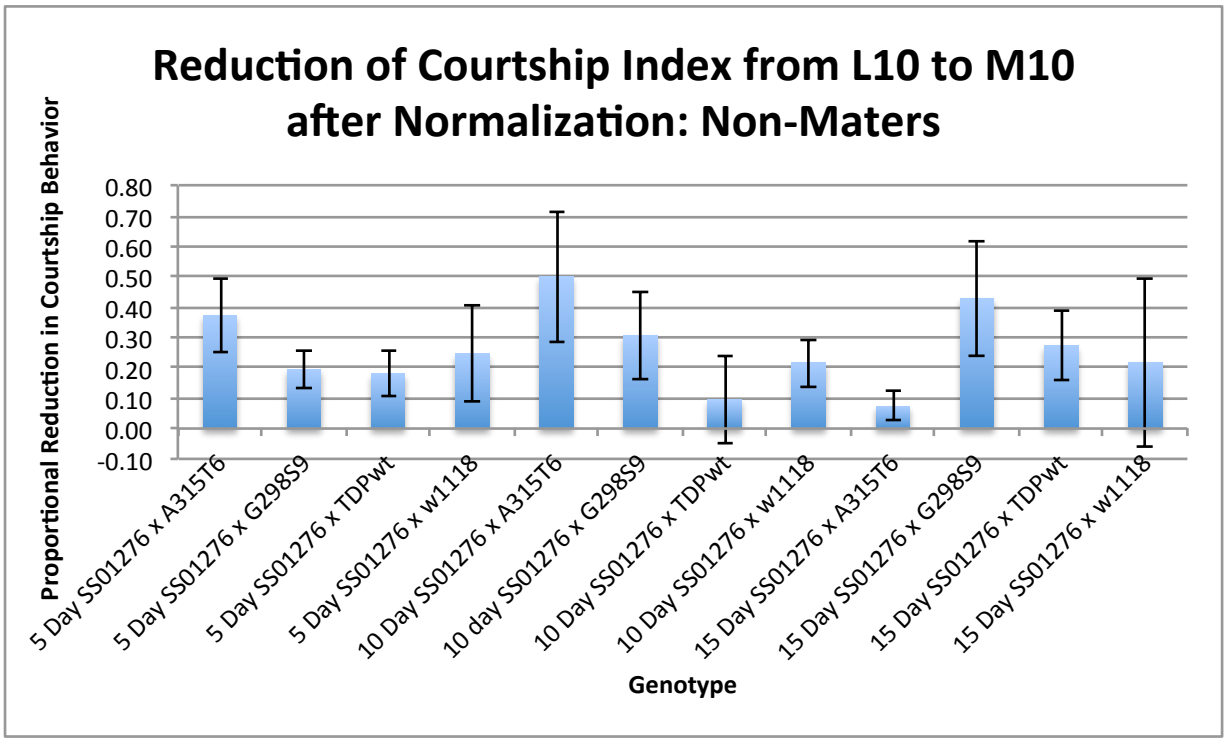


Figure 5. Mean reduction in courtship behavior from L10 to M10 for SS01276 males that did not mate in M10. Reduction in courtship behavior was calculated by normalizing each individual fly's M10 value to its L10 value and then subtracting this normalized value from 1.0. n is between 3 and 7 for every age and genotype combination. Error bars represent standard error of the mean.

When comparing means across all age groups and genotypes, in order to examine only the effect of mating, there was a significant difference between the time it took to

begin courting for flies that ultimately mated in M10 and those that did not (Figure 6. Unpaired t-test, p=0.01, df=22). When then comparing the M10 CI across all groups between flies that mated and those that did not, there was also a significant difference (Figure 6. Unpaired t-test, p<0.05, df=22). However, there was no correlation within flies that mated between how long it took them to court and how long it took them to ultimately mate with the female.

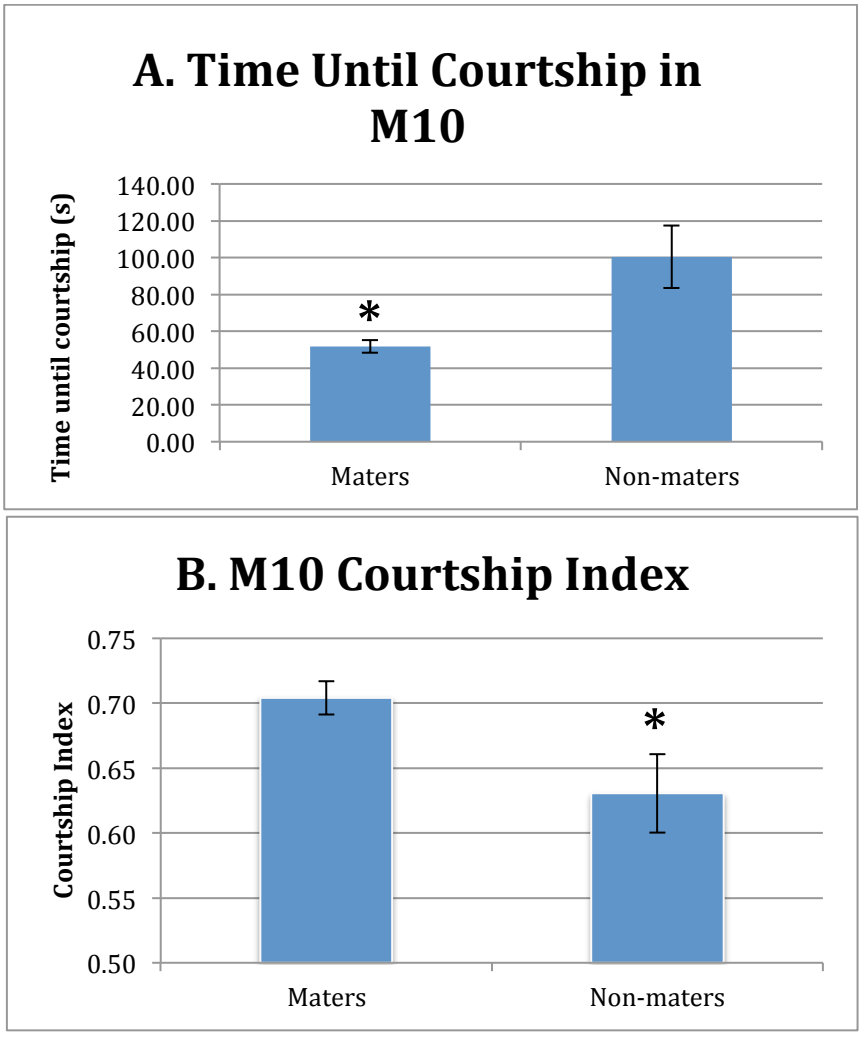


Figure 6. A) Average time until courtship for males that mated with the receptive female in M10 compared to males that did not mate in M10. Males that mated had a significantly faster time to begin courtship behavior. Unpaired t-test, p=0.01, df=22. **B) Average M10 courtship index for males that mated with the receptive female in M10 compared to males that did not mate in M10.** Males that mated in M10 had a significantly higher M10 value than non-maters. Unpaired t-test, p<0.05, df=22. Error bars represent standard error of the mean.

Age and Genotype	Percent Mated in M10
5 Day SS01276 x A315T6	61%
5 Day SS01276 x G298S9	84%
5 Day SS01276 x TDPwt	74%
5 Day SS01276 x w^{1118}	70%
10 Day SS01276 x A315T6	69%
10 day SS01276 x G298S9	65%
10 Day SS01276 x TDPwt	81%
10 Day SS01276 x w^{1118}	60%
15 Day SS01276 x A315T6	63%
15 Day SS01276 x G298S9	67%
15 Day SS01276 x TDPwt	75%
15 Day SS01276 x w^{1118}	75%

Table 1. The percentage of male flies that mated with the receptive female in the M10 period.

Examining Mushroom Body Morphology

Dissections were taken of SS01276 x TDPwt 15-day-old adult flies showed no disruption of mushroom body morphology (Figure 7).

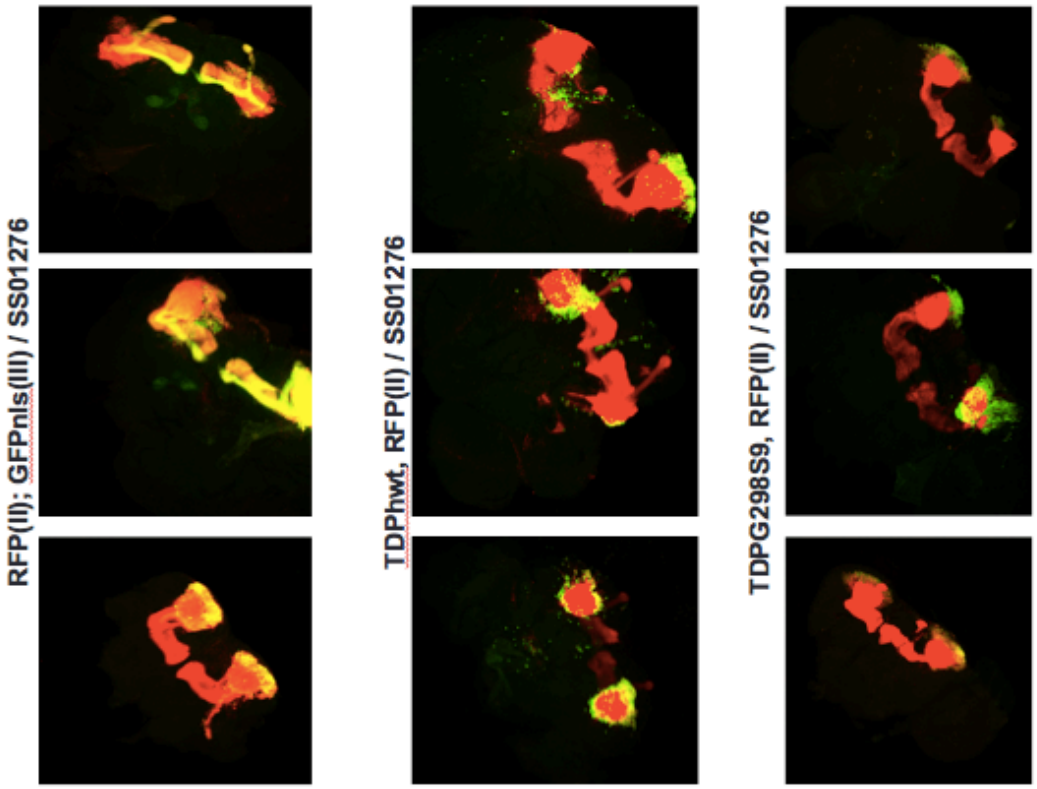


Figure 7. Dissections of 15-day-old SS01276 adult flies. Mushroom bodies are tagged with RFP to outline morphology. (Images courtesy of Elise Muñoz)

Discussion

FTD is a devastating disease that affects thousands of Americans every year. While many animal models are utilized to study neurodegenerative disease, few have utilized *Drosophila* as a model system. Taking a behavioral approach to modeling FTD based on TDP-43 overexpression in *Drosophila* has not been done. Our goal was to develop a model of FTD based on TDP-43 overexpression in the mushroom bodies of the fly using the GAL4-UAS system.

Initial courtship data taken from the 201Y and OK107 to TDP-43 flies was ultimately inconclusive because of the spatial specificity problems associated with each GAL4 driver. The 201Y driver caused expression in larval musculature, which caused some level of pupal lethality. This made it difficult to collect the number of adult males required for testing in the courtship experiments. 'Escaper' adult flies that were collected from these crosses may have also had a reduced susceptibility to the TDP-43 overexpression that could have confounded the courtship experiments. The OK107 driver resolved the pupal lethality issues associated with 201Y, but had much less specificity to the mushroom body drivers.

A split GAL4 driver, SS01276, which solved the mushroom body specificity issue, was used for the rest of the experiments. When first comparing 5-day-old OregonR males to 5-day-old SS01276 males, the differences in courtship behavior were stark. The OregonR males had a trend in decreasing courtship index from L10 to L50 to M10 and the mean for L10 was 0.51. In contrast, the means for L10 values for SS01276 control males were 0.8 or higher. Furthermore, the reduction from L10 to L50 was either only slightly reduced or even increased compared to L10 values. However, there was a significant reduction from L10 to M10 for all genotypes. When just examining the differences between L10 and L50,

there was no evidence of learning across all SS01276 genotypes in the 5-day-old males. Two more ages were then tested of the same genotypes: 10-day-old and 15-day-old males. The same results were seen in these two age groups. For all ages and genotypes, L10 started at a high courtship index, had either a slight decrease or even an increase in L50 compared to L10, and then had a significant decrease in courtship index to M10.

Given there was no difference between TDP-43 wild-type/mutant-expressing flies and controls, we had to consider the possibility of other genetic factors influencing courtship behavior. SS01276 x w^{1118} flies were chosen as controls to control for the genetic background of our transgenic flies, since they are generated in a w^{1118} background. However, the *white* gene in *Drosophila* has now been implicated in erratic courtship behavior. Loss of the *white* gene can cause an increase in male sexual arousal—males will court females vigorously and will also court other males (Krstic et al, 2013). Additionally, an intact *white* gene has been associated with mating success and loss of the gene severely impacts mating success (Xiao et al, 2016). Given these results, using SS01276 crossed to w^{1118} is likely to have had a profound impact on their courtship behavior. This also creates a problem of being unable to distinguish the effects of TDP-43 expression when attempting to control for genetic background. This problem could be addressed in the future by altering the genetic background of the transgenic flies so that controls are not generated from w^{1118} flies.

Despite the high courtship indices in L10 and L50, a significant difference between L10 and M10 needed to be further investigated. When examining these two different parameters, the main variable changed is the type of female that the male is placed with. L10 represents the courtship behavior in the first ten minutes spent with an *unreceptive*

female and the M10 period represents the courtship behavior in the ten minutes spent with a *receptive* female. We hypothesized that the receptive female is giving cues that govern male courtship behavior. To examine these differences, we separated males based on whether they mated with the receptive female in M10 or not, since the female ultimately determines whether mating occurs. We also took two new measures for all males: the amount of time it took them before initiating courtship in M10 and the amount of time it took to successfully mate (non-successful males were given a value of 600 seconds if they did not mate, which corresponds to the ten minute M10 period). On average, the males that mated in M10 initiated their courtship earlier than non-maters and they had a higher courtship index for M10. This suggests that the receptive female gave cues that decreased the latency and increased total proportion of male courtship. The assay could potentially be modified so that males are presented with another *unreceptive* female in M10 to eliminate possible effects of receptive female cues that stimulate male courtship behavior.

Furthermore, mushroom body morphology was normal in 15-day-old flies. This shows that TDP-43 wild-type or mutant expression had no repercussions on mushroom body development, at least when using the specific SS01276 driver. It is possible that overexpression would show synapse loss, perhaps in an age-dependent manner, which is not detectable in these images.

Ultimately, it is difficult to draw conclusions about the learning and memory ability of these TDP-43 expressing flies due to possibly confounding variables of genetic background and receptive female behavior.

Future Directions/Current Experiments:

Current experiments ongoing are testing the same SS01276 genotypes as reported above in 20-day-old flies to continue studying age-dependent effects. Since all flies tested above showed comparable reductions in courtship behavior, it is possible that further aging is required to detect deficits in learning and memory. Experiments that will begin once the 20-day-old males are finished testing are "sham" males. These males are placed in the courtship chamber for an hour *without* an unreceptive female. After an hour, they are transferred by mouth pipet to a chamber containing a receptive female in order to see the behavior that occurs independent of any "learning" period. Finally, an ongoing experiment is a lifespan study to see if there are significant differences in survival of TDP-43 wild-type or mutant variants as compared to SS01276 x w^{1118} controls.

Methods:

Courtship Assay

A 5-day-old OregonR female who had been mated the day before was transferred by mouth pipet into a courtship chamber (Aktogen). The experimental male was then also transferred into the courtship chamber by mouth pipet. They were given a one-minute acclimation period in the chamber, but were not allowed to interact because of separation by a divider. After one minute, the camera recording was started and the divider pulled back to allow the male and female to interact. Two chambers, and thus two males, were recorded at a time. After one hour, the divider was closed and the male and unreceptive female were separated. The male was transferred by mouth pipet to a new chamber containing a 4-day-old virgin female. The male was kept separate from the receptive female until two minutes after the learning period and the divider was once again removed

beneath the camera, which was still recording. The chamber was recorded for an additional ten minutes to constitute the memory test.

The courtship chambers are made of clear acrylic and were placed on a white sheet of paper on a piece of glass held up three centimeters above a light box (Apollo). All videos were recorded with a Sony DSR-SR47 handycam with a Carl Zeiss optical lens. The courtship experiments were run in a separate room to minimize noise or other disruptions. After starting the video recoding, the experimenter would leave the room and allow the experiment to proceed for the hour without disruption. The experimenter would only return at the end of the hour to transfer the male to a new courtship chamber and once again would leave.

Environmental variables were controlled. Lights were kept off in the room except for the light from the light box that illuminated the experiment. Temperature was kept at 21-23 ^{o}C and humidity was between 30-50%.

Collecting Experimental Males

Experimental males were collected from crosses that were kept in an incubator at 22 ^{o}C. Males were collected 0-6 hours post-eclosion and placed individually in unyeasted vials. These experimental males were also kept in a 22 ^{o}C incubator and flipped once a week into new food vials.

Collecting Females

Both the receptive and unreceptive females were collected 0-6 hours post-eclosion and kept in yeasted vials at room temperature. Females used in the learning test were mated the day before in order to make them unreceptive to the males. This was done by separating the females and males with CO_2 and placing four age-matched OregonR males

into a single vial with a single female. The cotton plug in the vial was pushed to leave a centimeter space to promote mating. The vials were laid horizontally and monitored for mating behavior. Once mating was observed, it was timed to ensure it lasted at least ten minutes. Once the male and female had completed mating, they were separated with CO_2 and the females placed back in the vial for the experiment the next day. Receptive females were kept in vials together at room temperature and used in experiments when they were 4-days-old.

Measuring Courtship Indices

Courtship indices were calculated by measuring the total courtship behavior within the L10, L50, and M10 periods and divided by the total amount of time, which was usually ten minutes. Many males mated with the receptive female during the M10 period. In these cases, the courtship behavior was measured up until the point of mating and divided by only the time until mating occurred. Two individuals, who had previously matched analysis on the same videos for consistency, performed all courtship analysis. All videos were analyzed using iMovie ’11 version 9.0 (Apple).

Male Disqualification

Males were only disqualified from results under two circumstances. The first was if they mated with the unreceptive female during the learning period. The second was if upon analysis, the courtship index for the L10 period was below 0.1. Such a low index during the first learning period would create an effect where further decreases in courtship behavior would not be detected. Data below does not include flies that were not successfully transferred to have an M10 recording.

Age	Genotype	Total Tested	Eliminated	Percent Eliminated
5 Day	SS01276 x A315T6	20	2	10.0%
5 Day	SS01276 x G298S9	20	1	5.0%
5 Day	SS01276 x TDPwt	20	1	5.0%
5 Day	SS01276 x w1118	20	0	0.0%
10 Day	SS01276 x A315T6	17	1	5.9%
10 Day	SS01276 x G298S9	17	0	0.0%
10 Day	SS01276 x TDPwt	17	1	5.9%
10 Day	SS01276 x w1118	17	2	11.8%
15 Day	SS01276 x A315T6	16	0	0.0%
15 Day	SS01276 x G298S9	15	0	0.0%
15 Day	SS01276 x TDPwt	17	1	5.9%
15 Day	SS01276 x w1118	16	0	0.0%

Statistical Analyses

All statistical analyses were performed in Graphpad Prism (Version 7.0). Two factor repeated measures ANOVAs were performed for each age group of flies with time (courtship phase) and genotype being the independent variable and included the raw L10, L50, and M10 values for each individual fly. Two factor ANOVAs to analyze the reduction in courtship between L10 and M10 were performed on the L10 to M10 difference mean, standard deviation, and n of groups with genotype and age being the independent variables.

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