The Role of Dopamine in Operant Learning and Memory

Jennifer Leigh Johnson
University of Texas at El Paso, jjohnson2@miners.utep.edu

Follow this and additional works at: https://digitalcommons.utep.edu/open_etd

Part of the Behavioral Neurobiology Commons, and the Social and Behavioral Sciences Commons

Recommended Citation
Johnson, Jennifer Leigh, "The Role of Dopamine in Operant Learning and Memory" (2011). Open Access Theses & Dissertations. 2322.
https://digitalcommons.utep.edu/open_etd/2322
THE ROLE OF DOPAMINE IN OPERANT LEARNING AND MEMORY

JENNIFER JOHNSON

Department of Biological Sciences

APPROVED:

_________________________________________________________________
Kyung-An Han, Ph.D., Chair

_________________________________________________________________
Renato Aguilera, Ph.D.

_________________________________________________________________
Manuel Miranda, Ph.D.

_________________________________________________________________
Christina Sobin, Ph.D.

_________________________________________________________________
Benjamin C. Flores, Ph.D.
Acting Dean of the Graduate School
ACKNOWLEDGEMENTS

First and foremost I would like to express my sincere gratitude to my advisor, Dr. Kyung-An Han. Her guidance throughout my graduate studies has greatly influenced my development as a research scientist and I will always be grateful for her wisdom, support and encouragement. Special thanks to my thesis committee members Drs. Christina Sobin, Manuel Miranda, and Renato Aguilera for their comments, suggestions, and most importantly, for their valuable time.

I am indebted to members and extended members of the lab: Junghwa Lim, Dr. Youngcho Kim, Paul Sabandal, Erick Saldes, Paula Villareal, Josh Frederick, and Anais Martinez for their cherished friendship, support, and collaboration. I wish you all the best of luck in your future endeavors.

Last, but not least of importance, a special thank you goes to my mother, Judy Johnson, for the tremendous amount of love, support, and encouragement. I would not have been able to accomplish what I did without her by my side.
ABSTRACT

Dopamine is a neurotransmitter that regulates many physiological processes including reward, motivation, movement, learning and memory, and reinforcement. The cognitive processes in which one associates a specific action or behavior with a positive or negative consequence is referred to as operant learning, and is a robust occurrence in everyday life. It is also believed to play a significant role in the development of drug addiction and has been shown that increased levels of dopamine are associated with the intake of addictive drugs such as cocaine and alcohol; however, the underlying cellular mechanisms of this learned behavior are not well understood. By using *Drosophila melanogaster* as a model system, we can genetically manipulate and dissect the mechanisms underlying operant learning and memory. Because these complex biological processes are evolutionarily conserved, the data obtained here can be related to the behavioral plasticity observed in vertebrates. In a conditioned courtship assay, a male fly’s courtship behavior is influenced by persistent rejection of a mated female. The male fly learns to associate unsuccessful courtship and copulation with the female fly and displays a generalized aversion (via courtship suppression) toward a virgin female. The goal of this study is to elucidate dopamine’s roles in this operant conditioning. For this task, we have tested flies lacking D1 receptor dDA1 (*dumb*), D2 receptor dDR2 (*dd2r*), or dopamine transporter DAT (*fmn*, *fmnZuker*). Once we identify important components mediating operant learning and memory, their functional sites (brain structures) and underlying cellular mechanisms will be clarified. Knowledge obtained in this study should shed light onto the complex and multifaceted pathways in which learning through association occurs.
## TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>iii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>iv</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>vii</td>
</tr>
<tr>
<td>CHAPTER</td>
<td></td>
</tr>
<tr>
<td>1. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>2. MATERIALS AND METHODS</td>
<td>7</td>
</tr>
<tr>
<td>2.1 Drosophila strains and husbandry</td>
<td>7</td>
</tr>
<tr>
<td>2.2 Basal Courtship</td>
<td>9</td>
</tr>
<tr>
<td>2.3 Conditioned Courtship</td>
<td>9</td>
</tr>
<tr>
<td>2.4 Pharmacological administration of methamphetamine</td>
<td>11</td>
</tr>
<tr>
<td>2.5 Immunohistochemical staining of dopaminergic cells in the adult fly brain</td>
<td>11</td>
</tr>
<tr>
<td>3. RESULTS</td>
<td>13</td>
</tr>
<tr>
<td>3.1 Basal Courtship</td>
<td>13</td>
</tr>
<tr>
<td>3.2 Conditioned Courtship</td>
<td>17</td>
</tr>
<tr>
<td>3.3 Characterization of Basal Courtship in Methamphetamine-fed Wild-Type Flies</td>
<td>30</td>
</tr>
<tr>
<td>3.4 Immunohistochemical staining of dopaminergic cells in the adult fly brain</td>
<td>34</td>
</tr>
<tr>
<td>4. DISCUSSION</td>
<td>39</td>
</tr>
</tbody>
</table>
LIST OF REFERENCES ................................................................................................................43

APPENDIX: FMN AND FMN/FMNZUKER OBSERVATIONS ................................................48

CURRICULUM VITA ......................................................................................................................58
LIST OF FIGURES

Figure 1 Characterization of basal courtship behavior in dd2r males ......................15
Figure 2 Characterization of basal courtship behavior in dumb$^1$ and dumb$^2$ males .................................................................16
Figure 3 Learning Indices for dd2r, dumb$^1$, and dumb$^2$ males...............................19
Figure 4 Courtship latency with mated female during 1 hr training period ...............20
Figure 5 Memory Indices for dd2r, dumb$^1$, and dumb$^2$ males tested 3 min after Training ........................................................................22
Figure 6 Pair-wise comparison of courtship levels between mock and trained dd2r, dumb$^1$, and dumb$^2$ males during 3 min memory testing ......................23
Figure 7 Memory Indices for dd2r, dumb$^1$, and dumb$^2$ males tested 1 hr after Training ........................................................................26
Figure 8 Pair-wise comparison of courtship levels between mock and trained dd2r, dumb$^1$, and dumb$^2$ males during 1 hr memory testing .........................27
Figure 9 Memory Indices for dd2r, dumb$^1$, and dumb$^2$ males tested 3 hr after training ........................................................................28
Figure 10 Pair-wise comparison of courtship levels between mock and trained dd2r, dumb$^1$, and dumb$^2$ males during 3 hr memory testing .........................29
Figure 11 Characterization of basal courtship behavior in methamphetamine-fed wild-type males .................................................................32
Figure 12 Characterization of courtship latency in methamphetamine-fed wild-type Males .............................................................................33
Figure 13 Schematic of TH-GAL4 expressing neurons in the Drosophila brain ...........37
Figure 14 Canton-S and fmn$^{Zuker}$ anti-TH immunostaining ....................................38
Figure A1 Characterization of basal courtship behavior in fmn and fmn/fmn$^{Zuker}$ Males .............................................................................49
Figure A2 Learning Indices for fmn and fmn/fmn$^{Zuker}$ males ..................................50
Figure A3 Courtship latency with mated female during 1 hr training period .............52
Figure A4 Memory Indices for $f_{mn}$ and $f_{mn}/f_{mn}^{\text{Zuker}}$ males tested 3 min after training…………………………………………………………………………………………………..53

Figure A5 Pair-wise comparison of courtship levels between mock and trained $f_{mn}$ and $f_{mn}/f_{mn}^{\text{Zuker}}$ males during 3 min memory testing………………………………54

Figure A6 Memory Indices for $f_{mn}$ and $f_{mn}/f_{mn}^{\text{Zuker}}$ males tested 1 hr after training…………………………………………………………………………………………………..56

Figure A7 Pair-wise comparison of courtship levels between mock and trained $f_{mn}$ and $f_{mn}/f_{mn}^{\text{Zuker}}$ males during 1 hr memory testing………………………………57
CHAPTER 1. INTRODUCTION

Defining the mechanisms that mediate experience-dependent behavioral plasticity has become a challenging undertaking in neuroscience. Communication between neurons, driven by neurotransmission, greatly impacts the development of the neural circuit leading to an appropriate learned response. Dopamine (DA), is known to play a neuromodulatory role in many brain functions in both vertebrates and invertebrates including arousal, attention, motivation, locomotion, learning and memory. Dysregulation of the DA system has been associated with a number of neurological and psychiatric disorders including Parkinson’s disease, depression, drug addiction, attention deficit disorder, and schizophrenia. In Drosophila melanogaster there are approximately 600 DA neurons whose cell bodies are grouped into fifteen clusters distributed throughout the brain [8-9]. Compared to the mammalian brain, the fly brain is architecturally simple; however, DA in the fly performs functions similar to those in mammals. Therefore, the fly system could be helpful in understanding the mechanisms by which DA mediates diverse functions. As in mammals, in the fly DA is synthesized from tyrosine by the rate-limiting enzyme tyrosine hydroxylase (TH) [8,10]. Mutant flies deficient in neuronal TH have greatly reduced DA levels in the brain and show a significant impairment in learning and memory when tested in aversive olfactory conditioning [11]. The aversive olfactory conditioning paradigm elicits a passively learned behavior and tests the fly’s ability to associate an aversive electric shock (unconditioned stimulus, US) with a neutral odor (conditioned stimulus, CS) so that upon future presentation of the associated odor (CS+) in the absence of shock, the fly will exhibit avoidance [10]. Blocking synaptic output from DA neurons or altering DA
neurotransmission by inhibiting dDAT has been shown to cause disruption in memory acquisition and memory retention in the same olfactory conditioning paradigm [12-13]. These findings suggest that DA signaling is necessary for behavioral plasticity in the fly and also validates the use of Drosophila as a model for understanding the mechanisms of DA functions.

Additional model systems have been used to investigate the roles of biogenic amines in associative learning and memory and further support a conserved role of DA in behavioral plasticity. Similar to olfactory conditioning in flies, honeybees can learn to extend their sting in response to odorants that were previously paired with electric shocks. Pharmacological administration of DA receptor antagonists, fluphenazine and flupentixol, causes memory impairment and bees are unable to discriminate the reinforced from the non-reinforced odorant [14]. These results have also been observed in aversive olfactory conditioning using crickets, suggesting that intact synaptic transmission from DA neurons is crucial for learning and memory [15]. Research examining behavioral plasticity in invertebrates has largely focused on classical conditioning.

The current study will utilize another form of learning known as operant conditioning, which requires the organism to associate a voluntary action with a positive or negative consequence. Based on the association made, the frequency of a given behavior will be altered. In Drosophila, operant learning and memory can be modeled in a conditioned courtship paradigm. In this process, male courtship behavior is modified by experience with a previously mated, unreceptive female [16]. Although the sensory inputs for the CS and US are not well defined, the US likely represents an aversive
pheromone, cis-vaccenyl acetate (cVA), transferred to the female fly during copulation along with physical and/or psychological rejection [17] while the CS may involve visual stimuli and/or attractive pheromones produced normally by mature female flies [16]. The behavioral outcome results in a generalized avoidance towards all female flies [17].

The precise mechanisms that underlie learning and memory in courtship conditioning are largely unknown, however, studies have identified several neural system components that contribute to memory acquisition and consolidation. Using a GAL4/UAS expression system, Joiner and Griffith [18-20] demonstrated that Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) is an important signaling molecule in learning and memory. CaMKII is required during training in the antennal lobe and lateral protocerebrum whereas short-term memory requires CaMKII activity in the MB, lateral protocerebrum, and central complex. This suggests that the initial transient sites of memory formation in conditioned courtship are sensory neuropils outside of the MB and that inputs from both the visual system and antennal lobe may play a role in this early phase of suppression [18-20]. McBride and colleagues [21] further show that ablation of the MBs with hydroxyurea does not impair immediate memory recall; however, short-term memory is significantly impaired 1 hr following training. Taken together, these results provide evidence for two phases of memory; an early, MB-independent component and a longer-lasting phase where memory formation takes place in the MB.

A critical question remains: how does DA signaling fit into the model above? The sensory pathways that are activated upon presentation of the US are believed to stimulate monoaminergic modulatory neurons [12]. In *Aplysia*, short- and long-term
memories can be induced in operant reward conditioning when presentation of the US is replaced by electrical stimulation of DA-containing nerve processes [22]. DA’s role as a learning signal is conserved across species. When monkeys learn to associate a reward with a predictable CS, DA neurons in the substantia nigra pars compacta (SNRc) and ventral tegmental area (VTA) engage in burst firing at the moment the CS is presented [23-24]. This suggests that when behavior results in a positive consequence, the associated DA release will strengthen the neural connections that are necessary to carry out that behavior and will allow for the increase in frequency of that behavior.

In the fly, DA may mediate both the US during classical olfactory conditioning [12] and the coincidental reinforcement during operant conditioning. Schroll and colleagues [25] support this idea by demonstrating that Drosophila larvae expressing a light-activated cation channel channelrhodopsin 2 in DA neurons were classically conditioned in the absence of US by directly stimulating DA neurons with light. A likely site for this DA-mediated reinforcement in the fly is in the MB; eight different types of DA neurons have been shown to innervate the MB neuropil [26]. The MB is composed of approximately 2,500 neurons known as Kenyon cells. The cell bodies form the calyx that represent the site of sensory input from several different sensory pathways including olfaction. Kenyon cells project their axons away from the calyx where they bifurcate to form the vertical and medial output lobes. Specific DA fibers innervate the dendritic arborizations in the calyx and distinct areas of the lobes, possibly modulating output of the MB neurons. These fibers respond differently to electric shock and odor stimuli, suggesting that the DA circuitry mediating learning and memory in the fly are more complex than originally thought [26].
Several genes expressed in the MB have been implicated in regulating short-term memory during aversive classical conditioning. The first two mutants reported in the literature that were found defective in learning and memory, *dunce* (*dnc*) and *rutabaga* (*rut*) are involved in the cyclic AMP cascade. The *dunce* mutant encodes a cAMP phosphodiesterase, which breaks down cAMP and regulates the duration of cAMP signaling. The *rutabaga* mutant codes for a type I Ca$^{2+}$/calmodulin-stimulated adenyl cyclase and functions by elevating levels of cAMP in response to both Ca$^{2+}$/calmodulin and Gs proteins [7]. Immunohistochemistry demonstrated that both genes were found preferentially expressed in the MB [24-25]. Contributing to the essential role that the MB plays in behavioral plasticity, experiments have shown that selective expression of *rut* cDNA in the MBs of *rut* mutant flies is sufficient to rescue the associated memory defect. *Rutabaga* may then function as a molecular coincidence detector during the convergence of the CS and US at the MBs [7]. Additionally, mutations in the PKA catalytic subunit and a PKA anchoring protein, both important mediators of signaling in the cAMP pathway, lead to a significant impairment in memory performance, suggesting that the cAMP/PKA signaling pathway is likely to contribute to olfactory memory formation [29-30]. In this process, DA represents the reinforcing property of the aversive US [31], serving as a ligand responsible for stimulating G protein-coupled receptors and thus, the cAMP cascade in the MB.

In the fly, three DA receptors have been characterized. dDA1 and DAMB (DA receptor in MBs) are classified as D1-like receptors increase levels of cAMP while dD2R, a D2-like receptor, inhibits the increase of cAMP [1-4, 32]. Immunohistochemical analysis shows that dDA1 and DAMB exhibit distinct expression patterns in both the MB.
and central complex [6]. Kim et al. demonstrated that the dDA1 receptor is specifically required in the MBs for acquisition of memory in both appetitive and aversive olfactory classical conditioning [33]. It remains unclear as to where dD2R is expressed in Drosophila; however, current development of a dD2R antibody should help to correlate behavioral phenotypes observed in this study with functional localization. Similar to classical conditioning, it is conceivable that DA receptors in the MBs become activated once DA is released in response to the US, and the downstream signaling events lead to formation of memory in operant conditioning. It is necessary to examine whether D1 and D2 receptors control memory formation or consolidation by differentially regulating cAMP signaling in specific MB areas. The findings may provide insight into which receptors are activated in the MBs, which areas in the MBs these events occur, and what downstream signaling cascades are important for aversive operant learning and memory.

Up until now, research has focused on defining the intracellular molecules involved in signal transduction pathways mediating learning and memory in Drosophila. Little is known however, regarding the influences of monoamine neurotransmission or the function of specific receptors that activate the signaling mechanisms. With the availability of dDA1, dD2R, and DA transporter (dDAT) mutants, we are now able to investigate, for the first time, the functions of these proteins in learning and memory using an operant conditioning paradigm.
CHAPTER 2. MATERIALS AND METHODS

2.1 Drosophila strains and husbandry

The control used for all experiments in this study was the wild-type fly line Canton-S. Two different dDAT mutants were used in this study. One mutant, *fumin* (*fmn*), was first described by Kume and colleagues [34] in a sleep behavior study and has an autosomal recessive mutation resulting in a truncated form of dDAT. This is due to a *roo* transposon inserted in intron 6 that prevents the appropriate splicing event to occur and causes premature termination of the dDAT protein [34]. Makos and colleagues [35] have shown using fast-scan cyclic voltammetry coupled with carbon-fiber microelectrodes that the truncated dDAT in *fmn* likely represents a non- or less-functional transporter. The second dDAT mutant, *fumin*-*Zuker* (*fmn*-*Zuker*), was identified in a forward genetic screen for sleep mutants [36]. The Zuker collection has more than 12,000 lines carrying ethylmethane sulfonate (EMS)-induced mutations [37]. One of the short sleep lines, *fmn*-*Zuker*, has a single nucleotide mutation in the *dat* gene causing a change of glycine to glutamine at amino acid 108 in dDAT. This location falls into a putative junction of the 1st intracellular loop and the 3rd transmembrane domain and is highly conserved among fly, mouse, and human DATs [36]. The parental line for *fmn*-*Zuker* contains genetic markers, *cinnabar brown* (*cn bw*) [37], to demarcate the mutated dDAT, thus, the *cn bw* line will be included as a control to account for any genetic background effects on behavioral phenotype. The use of two DAT alleles will help identify behavioral phenotypes that specifically result from a nonfunctional DA transporter. Unfortunately, a contamination issue with my dopamine transporter mutant stock, *fmn*, was discovered towards the completion of this project. Therefore, the
associated results are not reproducible. All data obtained in this study involving the dopamine transporter mutants, \textit{fmn} and \textit{fmn/fmn}\textsuperscript{zuker} can be found in the appendix. The experiments will need to be repeated using a clean stock of \textit{fmn} flies to determine if the phenotypes observed here are, in fact, due to a mutation in the dopamine transporter.

Flies containing mutations in DA receptors D1 and D2 were also used in this study to assess whether abnormal receptor function affects performance in the conditioned courtship paradigm. Two different dDA1 mutants, \textit{In(3LR)234} and \textit{f02676} represent hypomorphic alleles and contain lesions in the regulatory sequence coding for tissue-specific expression of dDA1. \textit{In(3LR)234}, designated \textit{dumb}\textsuperscript{1}, is an inversion line with two break points at 67D and 88A-88B (chromosomal location where dDA1 gene resides). \textit{f02676}, designated \textit{dumb}\textsuperscript{2}, contains the transposable element piggyBac inserted at the first intron in the dDA1 locus [33]. The dD2R mutant, f06521 designated \textit{dd2r}, is also hypomorphic (Han lab, unpublished data) and contains a piggyBac insertion at the second intron of the \textit{dd2r} gene. Controls for behavioral experiments include wild-type \textit{Canton-S} and heterozygous alleles for all mutants investigated in this study.

All flies were reared on standard cornmeal-yeast medium under a 12:12 light:dark cycle at 25°C and 60% relative humidity.
2.2 Basal Courtship

Experimental males were collected after eclosion and housed individually in small transfer vials containing food for a period of five days. Each male was then transferred by aspiration to a single-pair mating chamber (8 mm in diameter, 3 mm deep) containing a 5-day old wild-type virgin female. A wet filter paper was placed at the bottom of each chamber in order to maintain humidity. Flies were videotaped for 1 hr and the percentage of time spent courting during the first 10 min of pairing was calculated. Additional characteristics of basal courtship were examined including courtship latency, copulation latency, and copulation duration. Data obtained was tested for significance using the nonparametric Kruskal-Wallis test followed by the Mann-Whitney post-hoc analysis. Minitab 15 software was used for statistical analysis.

2.3 Conditioned Courtship

To test DA’s role in this behavioral plasticity, the conditioned courtship protocol established by Siegel and Hall was used [16]. Female flies were mated 24 hr in advance by housing an individual virgin female with 3-4 wild type males in a small vial containing food. To ensure that the female had been copulated with, only those females that had laid a significant number of eggs were used for training. A wet filter paper was placed at the bottom of each chamber in order to maintain humidity. The 1 hr training period was videotaped for scoring and the percentage of time spent courting during the first and last 10 min of pairing was calculated. This percentage represents the courtship index (CI). Test males that copulated with the trainer female or who had initial CIs of <10% were not used in analysis. For immediate memory recall (3 min delay), the trained male fly
was transferred by aspiration to a new mating chamber housing a decapitated 5-day old wild-type virgin female and videotaped for 10 min. For 1 hr and 3 hr memory recall, trained male flies were first transferred individually into transfer vials containing food and kept in a 22°C incubator for the allotted 1 or 3 hr delay period before being paired in a mating chamber with a decapitated 5-day old wild type virgin female. To decapitate, the virgin female was mildly anesthetized with carbon dioxide and the head was removed using 7mm blade scissors. The body was quickly transferred to a wet filter paper and observed for movement. Decapitated virgin females remain stationary; however they display normal grooming behavior. They are used during testing to prevent female responses from influencing male’s courtship behavior. This ensures that the male’s courtship suppression is a specific result of experience with a mated female. As a control, sham or mock tests were performed in which experimental males were housed alone in the mating chamber during the 1 hr training period and then paired with a decapitated virgin after the respective delay periods. All experiments were performed in an environmental chamber that maintained temperature at 25°C and 70% humidity. Courtship conditioning has two measurable behavioral outputs: the acquisition or learning component represented by the change of courtship levels during the training period with the mated female and the memory component represented by the subsequent suppression of courtship towards a decapitated tester virgin. A Learning Index (LI) is calculated by dividing the CI for the final 10 min period (Ci) of training by that of the initial 10 min period (Ci) and then multiplying by 100. Memory retention is measured as a percent reduction (PI) in courtship activity with and without conditioning as defined by the following equation:
Memory Index (MI) = \[100 \times 1-(\text{CI}_{\text{test}}/\text{mCI}_{\text{sham}})\]

where \(\text{CI}_{\text{test}}\) refers to the CI of the first 10 min during testing and \(\text{mCI}_{\text{sham}}\) refers to the mean of sham CIs. To test for statistical significance, all CIs were subjected to the nonparametric Kruskal-Wallis test followed by the Mann-Whitney post-hoc analysis using Minitab 15 software.

### 2.4 Pharmacological administration of methamphetamine

Four-day old wild type males collected after eclosion and housed individually were transferred 24 hr before testing, to small vials with food containing 0, 1, or 5 mg/ml methamphetamine and 0.25 mg/ml of ascorbic acid to prevent drug oxidation. Green food coloring was added to the food to monitor drug intake before testing. Males were kept under dark conditions during the 24 hr drug feeding period to further prevent oxidation of the drug and moved to light 3 hr prior to the experiment. Basal courtship was examined in drug-treated male flies to assess whether methamphetamine affects courtship and copulation.

### 2.5 Immunohistochemical staining of dopaminergic cells in the adult fly brain

Immunostaining was performed by a standard lab protocol. Briefly, whole brains were dissected from 5-day old males in phosphate buffered saline (PBS) with careful forcep manipulation, washed briefly with PBS to remove tissue debris, and fixed in 4% paraformaldehyde containing lysine for 3 hr at 4°C. Following fixation, the brains were washed three times, for approximately 20 min each in PBHT solution [0.02 M NaPO₄, 0.5M NaCl, 0.2% Triton X-100 (pH 7.4)] and transferred to a 5% normal goat serum (NGS) blocking solution. After blocking for 2 hr at room temperature, the brains were
incubated overnight at 4°C in primary antibody consisting of a 1:1000 dilution of mouse anti-tyrosine hydroxylase (anti-TH) in 5% NGS and PBHT. The primary antibody was removed the next day and the brains were washed three times in PBHT. In order to efficiently remove background from staining, the brains were left at 4°C in PBHT for three days before the secondary antibody is applied. On the fourth day, the brains were transferred to a 1:1000 dilution of goat anti-mouse IgG conjugated with Alexa 568 in PBHT and incubated for 2 hr at room temperature. Following three washes in PBHT, the brains were mounted with VectaShield on a standard glass microscope slide. Imaging was performed using a Zeiss LSM 700 Confocal microscope and 2 µM optical sections were obtained for each brain. Anti-TH stained DA cell bodies in $\text{fmr}^\text{Zuker}$ mutant brains were manually counted and compared to the numbers obtained for wild-type Canton-S brains.
CHAPTER 3. RESULTS

3.1 Basal Courtship

Upon initial presentation of a female, a naïve male begins a stereotyped courtship ritual after detection of attractive mature female pheromones [38-39]. In order to investigate whether the potential phenotypes displayed during conditioned courtship are due to a sensory deficit, basal courtship levels were measured for males paired with a virgin female.

Surprisingly, dd2r males exhibited very low levels of courtship compared to wild-type males during the first 10 min (Fig. 1A) (p< 0.0001; n=33; Kruskal-Wallis, post-hoc Mann-Whitney) and also displayed a significant delay in courtship and copulation initiation (p< 0.0001, p= 0.001 respectively; n=33, n=14; Kruskal-Wallis, post-hoc Mann-Whitney) (Figs. 1B and 1C). The dd2r mutants also exhibited a significant decrease in copulation duration, about 17.5 min, as compared to wild-type (p= 0.04; n=14; Kruskal-Wallis, post-hoc Mann-Whitney) (Fig. 1D). Despite reduced courtship levels in these flies, they display a normal courtship ritual and copulate successfully (the number of males that copulated in this assay is low; however, healthy homozygous stocks are maintained in the laboratory). It is possible that the dd2r male’s reduced courtship level may be the result of decreased motivational state, and/or a higher threshold for arousal. Further experiments will need to be conducted to delineate the role of the Drosophila DA D2 receptor to clarify these possibilities.

The D1 DA receptor mutant dumb² males display significantly higher levels of courtship within the first 10 min. of pairing with a virgin female compared to wild-type CS
males and $dumb^1$ males (p= 0.005, p= 0.0001 respectively; n=33; Kruskal-Wallis, post-hoc Mann-Whitney). (Fig. 2A) Interestingly, $dumb^2$ males are also significantly impaired in maintaining a copulation duration (p< 0.0001; n=31; Kruskal-Wallis, post-hoc Mann-Whitney). (Fig. 2D) Taken together, both $dumb^1$ and $dumb^2$ males are hypomorphic alleles of the D1 receptor, however $dumb^2$ males display a more severe phenotype in basal courtship behavior. Nevertheless, both alleles show a normal courtship ritual and copulation, indicating that D1 receptor mutants are not impaired in sensory processing required for courtship.
Figure 1. Characterization of basal courtship behavior in *dd2r* males.
Figure 2. Characterization of basal courtship behavior in $dumb^1$ and $dumb^2$ males.
3.2 Conditioned Courtship

Females that have recently been mated are usually unwilling to mate a second time and will reject a male fly’s advances by kicking and/or running away. In addition, mated females also release a pheromone, cis-vaccenyl acetate (cVA), which is transferred by the male during copulation and is perceived as aversive to the male fly. Exposure to cVA produces a strong inhibitory drive onto the lateral horn which suppresses the male’s subsequent impulse to court even when paired with virgin female flies [17]. The effects of this conditioning can last up to 2-3 hr [16]. This generalized aversion is an example of operant learning and memory where males modify their courtship behavior based on prior courtship experience. Males of all genotypes were tested in this paradigm and two behavioral components, acquisition and memory, were measured. The LIs were calculated to evaluate the level of courtship suppression by males during the 1 hr training period. The MIs were calculated to quantitatively compare the percent reduction in courtship behavior for 3 min, 1 hr, and 3 hr memory recall among males of all genotypes.

Learning Index

D2 DA receptor mutant dd2r males display a significantly higher LI compared to wild-type males suggesting that these males may be defective in acquisition and are unable to successfully suppress their courtship towards a mated female (p= 0.1; n= 38; Kruskal-Wallis, post-hoc Mann-Whitney) (Fig. 3B). It is also possible that since basal courtship levels for dd2r mutants are already low, there may not be a substantial
amount of interaction with the mated female during the first 10 min of training to allow for a significant reduction in courtship by the end of the training period.

The D1 DA receptor mutant $dumb^1$ males exhibit a significant lower LI compared to wild-type males and to the other D1 DA receptor allele, $dumb^2$, indicating that these mutants are able to successfully reduce their courtship towards a mated female during the 1 hr training period ($p=0.003$, $p=0.05$ respectively; $n=23-57$; Kruskal-Wallis, post-hoc Mann-Whitney) (Fig. 3B). Similar to basal courtship, these results also support the idea that the two D1 DA receptor alleles, though both hypomorphic, may possess different phenotypes.

Figure 4 demonstrates the amount of time it took for males of all genotypes to initiate courtship with a mated female during the first 10 min of the training period. D1 DA receptor mutants’ $dumb^1$ and $dumb^2$ males both took significantly longer to initiate courtship with a mated female compared to wild-type males ($p=0.001$, $p=0.1$ respectively; $n=61-77$; Kruskal-Wallis, post-hoc Mann-Whitney) (Fig. 5B). Comparing both alleles, the $dumb^1$ males display a more severe phenotype, taking longer to initiate courtship than $dumb^2$ males ($p=0.0006$; $n=61-77$; Kruskal-Wallis, post-hoc Mann-Whitney) (Fig. 5B). Despite higher courtship latency, $dumb^1$ males have a significantly lower LI than wild-type males (Fig. 3) suggesting that the time spent courting the mated female during the first 10 min of training was sufficient to produce a significant reduction in courtship during the last 10 min of training.
Figure 3. Learning Indices for $dd2r$, $dumb^1$, and $dumb^2$ males.
Figure 4. Courtship latency with mated female during 1 hr training period.
Memory Index

3 Min Memory

The \textit{dd2r}, \textit{dumb}^1, and \textit{dumb}^2 males all exhibited a percent reduction in courtship similar to wild-type, suggesting that they are able to successfully form an association and recall it 3 min later (Figs. 5A and 5B). The mock or sham group consists of males that are kept in isolation and are not paired with a mated female during the 1 hr training period. These male flies do not form an association between rejection and the female fly and therefore, should exhibit high levels of courtship towards a decapitated virgin female during the testing phase. Males that have been trained with a mated female show reduced courtship levels towards a decapitated virgin female during testing. Figure 6 presents a pair-wise comparison between the mock and trained males tested with a decapitated virgin female 3 min after training. Trained males from all genotypes, except \textit{dd2r}, show a significant reduction in courtship compared to their respective mock group suggesting that the courtship suppression observed during 3 min memory testing is most likely due to the interactions with a mated female during training. A significant reduction in courtship during testing could not be obtained for trained \textit{dd2r} males possibly because of already low courtship levels exhibited by the mock group and a low number of experimental males used for testing (n= 16).
Figure 5. Memory Indices for dd2r, dumb¹, and dumb² males tested 3 min after training.
**Figure 6.** Pair-wise comparison of courtship levels between mock and trained *dd2r*, *dumb*¹, and *dumb*² males during 3 min memory testing.
1 Hr Memory

All mutants were tested 1 hr following the training period. Increasing the amount of time between training and testing requires the ability of the male fly to effectively store the formed association in short term memory and be able to retrieve this information 1 hr later when presented with a decapitated virgin female. However, increasing the delay period also increases the possibility that the association may be forgotten. Surprisingly, both dd2r and dumb\textsuperscript{1} males performed significantly better than Canton-S in 1 hr memory recall (p = 0.008, p = 0.001 respectively; n = 18, n = 12 respectively; Kruskal-Wallis, post-hoc Mann-Whitney) and dumb\textsuperscript{1} males scored significantly higher than dumb\textsuperscript{2} males (p = 0.02; n = 12; Kruskal-Wallis, post-hoc Mann-Whitney) (Fig. 7). This suggests that dd2r and dumb\textsuperscript{1} males are able to effectively recall the memory up to 1 hr following training and/or may possess a slower memory decay rate for courtship conditioning.

Figure 8 examines the pair-wise comparison between mock and trained males tested with a decapitated virgin 1 hr after the training period. Trained wild-type males exhibited a significant reduction in courtship compared to their respective mock group (p = 0.001; n = 25; Kruskal-Wallis, post-hoc Mann-Whitney). In addition, dumb\textsuperscript{1} males also perform significantly different from mock males (p = 0.01; n = 12; Kruskal-Wallis, post-hoc Mann-Whitney). The results suggest that those genotypes that failed to show a significant reduction in performance from mock males may not have been able to properly retrieve the memory 1 hr following training and exhibited courtship levels comparable to males that were never trained with a mated female. The 1 hr delay period may also be a point at which extinction of the memory becomes apparent.
### 3 hr Memory

In the original courtship conditioning paper published by Siegel and Hall, results showed that wild-type Canton-S males were unable to retain the association made during 1 hr training for longer than a 3 hr period. The data presented in Figure 9 reflect this same finding with a percent reduction in courtship close to 0 for wild-type males during 3 hr memory recall. Surprisingly, $dumb^1$ and $dumb^2$ males performed significantly better than wild-type males suggesting that they are still able to recall the memory 3 hr later ($p=0.004$, $p=0.02$ respectively; $n=4$ $n=17$ respectively; Kruskal-Wallis, post-hoc Mann-Whitney). This also raises the possibility that the memory extinction rate for these mutants is considerably slower. However, the pair-wise comparison in Figure 10 demonstrates that statistical significance could not be achieved for any genotype when comparing mock and trained males. This could be a result of the high variability in courtship levels for $dd2r$, $dumb^1$, and $dumb^2$ males or a result of the already low courtship levels for mock males. In addition, the number of $dumb^1$ males used for testing was extremely low ($n=4$) and will need to be increased in future experiments to determine if the results obtained here are a true representation of the mutants' phenotype.
Figure 7. Memory Indices for $dd2r$, $dumb^1$, and $dumb^2$ males tested 1 hr after training.
Figure 8. Pair-wise comparison of courtship levels between mock and trained $dd^{2r}$, $dumb^1$, and $dumb^2$ males during 1 hr memory testing.
Figure 9. Memory Indices for *dd2r*, *dumb*¹, and *dumb*² males tested 3 hr after training.
Figure 10. Pair-wise comparison of courtship levels between mock and trained *dd2r*, *dumb*<sup>1</sup>, and *dumb*<sup>2</sup> males during 3 hr memory testing.
3.3 Characterization of Basal Courtship in Methamphetamine-fed Wild-Type Flies

The stimulant drug methamphetamine binds to the DA transporter and inhibits DA reuptake [42-43]. *In vivo* studies in *Drosophila* have shown that methamphetamine-treated wild-type flies exhibit an approximate 30% increase in extracellular DA concentration in the brain. In addition, *fmn* flies did not exhibit a change in DA reuptake following treatment with methamphetamine, suggesting that this stimulant effectively blocks normal DA transporter function in *Drosophila* [35]. In this study, wild-type *Canton-S* males were fed 0, 1, or 5 mg/mL of methamphetamine for a 24 hr period and courtship levels with a decapitated virgin female were then measured. This was performed to help elucidate whether the behavioral phenotypes of dDAT mutants (*fmn* and *fmn\textsuperscript{Zuker}* ) was due to an enhanced level of extracellular DA at the time of courtship testing or adaptive changes associated with dDAT deficiency during development.

Wild-type males fed for 24 hr with 1 or 5 mg/mL of methamphetamine displayed decreased courtship compared to males that were fed only 0.25 mg/mL of ascorbic acid. Statistical significance was obtained with males fed the highest concentration of methamphetamine (p= 0.03; n= 24; Kruskal-Wallis, post-hoc Mann-Whitney) (Fig. 11). However, males from all three feeding groups initiated courtship at similar times and significant differences were unable to be obtained between methamphetamine-fed flies and the control (Fig. 12). It is possible that the 24 hr feeding period was too long and may have caused long-term adaptive changes in the male fly, reducing courtship levels. In addition, courtship levels may have already been too high in the 0 mg/mL control group to observe a significant difference in the methamphetamine-fed experimental groups. Future experiments should investigate shorter feeding times (ex. 4 hr feeding)
to determine if the phenotype observed in this study is a true representative of the effects methamphetamine has on courtship behavior.
Figure 11. Characterization of basal courtship behavior in methamphetamine-fed wild-type males.
Figure 12. Characterization of courtship latency in methamphetamine-fed wild-type males.
3.4 Immunohistochemical staining of dopaminergic cells in the adult fly brain

DAT is the primary means for clearance of DA from the synaptic cleft. DA transporter deficiency or inhibition leads to enhanced levels of extracellular DA, which could lead to adaptive changes in DA synthesis or ultimately lead to the generation of highly damaging reactive oxygen species. Long-term exposure to these neurotoxic molecules is likely associated with decreases in DA concentrations, decreases in tyrosine hydroxylase activity, and nerve-terminal degeneration. These effects are observed with administration of high doses of methamphetamine, a known inhibitor of DAT [44-47]. To assess whether changes in DA neuron properties can be observed in the adult brain of the DA transporter mutant, *fmnZuker*, immunohistochemical staining was performed using an antibody against tyrosine hydroxylase. Tyrosine hydroxylase is the rate-limiting enzyme in DA synthesis and is localized specifically to DA neurons. Therefore, an antibody generated against this enzyme should restrict expression to DA neurons.

Table 1 lists the number of anti-tyrosine hydroxylase positive neurons per hemisphere in wild-type and *fmnZuker* males, categorized into 13 different classes. Every class is bilaterally symmetrical in each hemisphere except for PPM1, which is positioned along the midline (Fig. 13). Noticeable differences in cell body number between the two genotypes can be observed in the PPM2 and PPL2ab clusters (Fig. 14). PPM2 mainly projects to the ventral medial protocerebrum and the subesophageal ganglion. PPL2ab projects to the calyx of the mushroom body as well as the lateral
horn, or pre-motor center. A decrease in cell body number in both neuropil, as observed in $f_{mn}^{Zuker}$ males, could potentially affect learning and memory in conditioned courtship. The subesophageal ganglion and mushroom body calyx are responsible for sensory input processing. Changes in the dopaminergic projections to these structures may hinder the male fly’s ability to effectively process aversive information, including both physical rejection and pheromones. Also, the motor control necessary to suppress courtship during testing may also be disrupted if a smaller number of dopaminergic processes are projecting to the lateral horn. It is important to note that neuronal cell bodies were manually counted and only those stained cell bodies that were visible above the threshold set by background staining were included. It is possible that there may have been additional neurons that were not included in the count because their staining was unable to be distinguished from background staining. Further investigation should focus on whether differences can be observed in the number and intensity of actual dopaminergic processes projecting to important structures in the fly brain implicated in learning and memory.
Table 1. Number of anti-TH positive neurons per hemisphere in the male fly brain. N= 5

<table>
<thead>
<tr>
<th>DA neuron cluster</th>
<th>CS</th>
<th>fmn$^{Zuker}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAM</td>
<td>35.3 ± 9.33</td>
<td>45.2 ± 8.28</td>
</tr>
<tr>
<td>PAL</td>
<td>4.2 ± 0.46</td>
<td>4.3 ± 0.47</td>
</tr>
<tr>
<td>PPM1</td>
<td>0.8 ± 0.19</td>
<td>1 ± 0</td>
</tr>
<tr>
<td>* PPM2</td>
<td>7.3 ± 0.47</td>
<td>5.9 ± 0.58</td>
</tr>
<tr>
<td>PPM3</td>
<td>6.1 ± 0.77</td>
<td>6 ± 0.76</td>
</tr>
<tr>
<td>PPL1</td>
<td>10.9 ± 0.39</td>
<td>11.5 ± 0.32</td>
</tr>
<tr>
<td>* PPL2ab</td>
<td>6 ± 0.37</td>
<td>4.7 ± 0.76</td>
</tr>
<tr>
<td>PPL2c</td>
<td>1.6 ± 0.23</td>
<td>1.5 ± 0.32</td>
</tr>
<tr>
<td>PPD</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>PPM4</td>
<td>0.2 ± 0.2</td>
<td>0.2 ± 0.2</td>
</tr>
<tr>
<td>PPL3</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>PPL4</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>PPL5</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
</tbody>
</table>
Figure 14. Canton-S (top) and fmrZuker (bottom) anti-TH immunostaining demonstrating noticeable cell body differences in PPM2 (blue circle) and PPL2ab (green circles) classes. Cell bodies were manually counted and only those that were visible above the background threshold were included. N= 5
CHAPTER 4. DISCUSSION

The major objective of this research was to understand the mechanism by which DA modulates learning and memory. This was carried out using *Drosophila melanogaster* as a model system and focused on several proteins involved in DA signaling including the D1 and D2 receptors as well as the dopamine transporter. DA neurotransmission has already been shown to be involved in mediating aversive stimuli in classical learning and memory, however, little is known regarding how this monoamine influences aversive stimuli in operant learning and memory. Using dDA1, dD2R, and DA transporter mutants in a conditioned courtship paradigm, this study has provided evidence suggesting that DA signaling plays a role in more than one form of associative learning.

Characterization of DA receptor mutants in basal courtship and courtship conditioning behavior

D2 receptor mutants, *dd2r*, exhibit a very distinct phenotype. These males do not readily court a virgin female fly as demonstrated by their significantly lower basal courtship level compared to wild-type males. In addition, they take significantly longer to initiate courtship, longer to initiate copulation, and spend a significantly shorter time copulating. This factor makes it extremely difficult to examine operant learning and memory in these mutants using the conditioned courtship paradigm because a minimum amount of courtship must be reached during training in order to form an association. Therefore, very few *dd2r* males meet this requirement, the experimental number for memory testing is low, variability is high, and statistical significance is hard to obtain.
It appears that there may be an underlying explanation for this behavior that is beyond the scope of this study; however, one hypothesis is that dd2r mutants may have a motivational impairment. As mentioned previously, dopamine has come to be identified with motivational as well as motor function. Though it is not clear which receptor dopamine acts through to regulate locomotion, dd2r males do not exhibit locomotor behavior different from wild-type males; strongly suggesting that these mutants lack the motivation to court a female fly. Further investigation will need to confirm whether motivation is a factor that affects dd2r males' ability to court and if so, a different operant learning and memory paradigm may need to be used to study behavioral plasticity in these flies.

In Drosophila, several other paradigms have been developed to model operant conditioning. The heat box conditions flies to avoid one half of a chamber that is associated with an aversive increase in temperature. In addition to the heat box paradigm, flies can be trained to discriminate different shapes and colors in the flight simulator. Flies learn to avoid specific landmarks (shapes, colors, etc.) that have been paired with either heat or an aversive odor. To date, dopamine has not been implicated in place memory or visual learning and memory; however, several components involved in dopamine signaling including adenylyl cyclase and cAMP phosphodiesterase have been shown to be critical for memory formation in these paradigms [48]. It would be interesting to investigate if the dopamine receptor mutants tested in this study also display distinct phenotypes in these paradigms.

Two alleles, dumb1 and dumb2, were used to investigate D1 receptor's role in basal courtship and learning and memory. Based on the results presented, it becomes
evident that they may possess distinctive phenotypes. D1 receptor mutant \( \text{dumb}^2 \) exhibit significantly higher courtship levels toward a virgin female compared to wild-type and spent significantly less time copulating while basal courtship behavior for \( \text{dumb}^1 \) males did not differ significantly from wild-type. However, the Learning Index for \( \text{dumb}^1 \) males’ was significantly better than \( \text{dumb}^2 \) males. Overall, \( \text{dumb}^1 \) males appeared to perform significantly better than wild-type males during memory testing (with the exception of 3 min STM in which there was no statistical significance) suggesting these mutants may have an enhanced ability to retain the association made during courtship conditioning. This is an interesting finding because both \( \text{dumb}^1 \) and \( \text{dumb}^2 \) alleles were previously found to be impaired in olfactory conditioning, possibly inferring that a separate mechanism exists for classical and operant learning and memory. Nonetheless, the experimental number of males used for memory testing was extremely low due to the inability to obtain males that met the minimum level of courtship during the first 10 min of training. Similar to \( \text{dd}2r \) males, this may be a result of a motivational impairment and further investigation is needed to confirm this.

It is important to note in Figure A6 that wild-type \( \text{Canton-S} \) males displayed very little courtship suppression during 1hr memory testing. Though it is not common for wild-type males to forget after only 1 hr, it is possible that exposure to extraneous sensory information from the environment during the delay period may have interfered with memory recall. Conversely, in Figure 7, \( \text{Canton-S} \) males exhibited a higher performance index than the \( \text{Canton-S} \) males in Figure A6 suggesting that additional sensory input from the environment did not have as great an effect on memory recall in this set of experiments. Future experiments will need to be conducted in which the males are
housed in a lit incubator kept at room temperature during the delay period with minimal environmental interference.

In summary, dopaminergic signaling is important for associative learning and memory in *Drosophila melanogaster*. This study suggests DA neurotransmission may play a significant role in a specific type of learning and memory known as operant learning and memory. Though many intracellular components have previously been identified as necessary for courtship conditioning, these results provide the first line of evidence that dDA1 and dDA2 receptor function may be involved in initiating the signal transduction events that are known to occur in operant learning and memory.
LIST OF REFERENCES


APPENDIX: *FMN* AND *FMN/FMN*\(^{ZUKER}\) OBSERVATIONS

**Basal Courtship**

Interestingly, *fmn/fmn\(^{Zuker}\)* transheterozygous mutants spent more time courting during the first 10 min compared to wild-type CS and heterozygous controls (Fig. A1A). In addition, *fmn/fmn\(^{Zuker}\)* mutants spent less time copulating, approximately 15 min, compared to wild-type and heterozygous controls. The *fmn* homozygous mutants also displayed a decrease in copulation duration compared to controls (Fig. A1D).

**Learning Index**

Both *fmn* homozygous and *fmn/fmn\(^{Zuker}\)* transheterozygous males did not perform significantly different from wild-type males however, their LIs were different from their heterozygous controls (Fig. A2).
Figure A1. Characterization of basal courtship behavior in \textit{fmn} and \textit{fmn/fmn} \textit{Zuker} males.
Figure A2. Learning Indices for *fmn* and *fmn/fmnZuker* males.
Conditioned Courtship

3 min memory

Figure A3 demonstrates the amount of time it took for males of all genotypes to initiate courtship with a mated female during the first 10 min of the training period. Interestingly, \textit{fmn} and \textit{fmn/fmn} \textit{Zuker} males initiate courtship faster than wild-type and heterozygous control males (Fig. A3). This finding correlates well with the LIs of these mutants from Figure A2 and supports the idea that \textit{fmn} and \textit{fmn/fmn} \textit{Zuker} males spend more time interacting with the mated female during the first 10 min of training.

The transheterozygous mutant \textit{fmn/fmn} \textit{Zuker} displayed a defect in 3 min memory compared to wild type and the heterozygous controls (Fig. A4A). Surprisingly, \textit{fmn} homozygous mutants do not show a memory impairment compared to wild-type (Fig. A4B). Both of these mutants have abnormal DA transporter function; however, \textit{fmn/fmn} \textit{Zuker} transheterozygous mutants displayed a more severe phenotype.

Figure A5 presents a pair-wise comparison between the mock and trained males tested with a decapitated virgin female 3 min after training. Trained \textit{fmn} and \textit{fmn/fmn} \textit{Zuker} males showed a reduction in courtship compared to their respective mock group suggesting that the courtship suppression observed during 3 min memory testing is most likely due to the interactions with a mated female during training.
Figure A3. Courtship latency with mated female during 1 hr training period.
Figure A4. Memory Indices for *fmn* and *fmn/fmn<sup>Zuker</sup>* males tested 3 min after training.
Figure A5. Pair-wise comparison of courtship levels between mock and trained *fmn* and *fmn/fmn<sup>Zuker</sup>* males during 3 min memory testing.
1 hr memory

In Figure A6, *fmn* homozygous males were the only ones to display higher performance in 1 hr memory recall compared to wild-type males.

Figure A7 examines the pair-wise comparison between mock and trained males tested with a decapitated virgin 1 hr after the training period. Only *fmn* and two heterozygous controls, *fmn/+* and *cnbw/+*, showed a reduction in courtship between their respective mock and trained males. As mentioned above, wild-type *Canton-S* males did not exhibit a percent reduction in courtship during 1 hr memory testing (Fig. A6) and Figure A7 demonstrates that compared to the mock group, trained *Canton-S* males also did not perform differently.
Figure A6. Memory Indices for *fmn* and *fmn/fmnZuker* males tested 1 hr after training.
Figure A7. Pair-wise comparison of courtship levels between mock and trained $f_{mn}$ and $f_{mn}/f_{mn}^{Zuker}$ males during 1 hr memory testing.
CURRICULUM VITA

Jennifer Johnson was born in El Paso, Texas. The only daughter of Judy Johnson, she graduated valedictorian of Burges High School, El Paso, TX, in the spring of 2005 and entered The University of Texas at El Paso in the fall with the Presidential Leadership Scholarship. While pursuing a bachelor’s degree in microbiology, she worked as a General Chemistry Peer Leader during the 2007-2008 school year where she taught several chemistry workshop courses. She was also a participant in the Howard Hughes Medical Institute Undergraduate Research Grant in 2008 and 2009 and worked under the guidance of Dr. Marc Cox, focusing on prostate cancer research. She received her bachelor’s of science degree from the University of Texas at El Paso in the spring of 2009 and in the fall of that same year, she entered the Graduate School at The University of Texas at El Paso.

Permanent Address: 1430 Miracle Way #8
El Paso, TX 79925