The Role Of D1/dDA1 And D5/DAMB Dopamine Receptors In Ethanol Induced Behavioral Disinhibition

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THE ROLE OF D1/dDA1 AND D5/DAMB DOPAMINE RECEPTORS IN ETHANOL INDUCED BEHAVIORAL DISINHIBITION

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THE ROLE OF D1/dDA1 AND D5/DAMB DOPAMINE RECEPTORS IN ETHANOL INDUCED BEHAVIORAL DISINHIBITION

by

IVAN MERCADO, BS

THESIS

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Abstract

Alcohol drinking causes changes in behaviors by acting on various neurotransmitters (e.g. GABA, acetyl choline and glutamate) (Kumar et al., 2009), neuromodulators (e.g. dopamine, and serotonin) (Banerjee, 2014) and ion channels (e.g. Ca\(^{2+}\) and K channels) (Harris and Hood, 1980). Behaviors associated with alcohol consumption include loss of motor coordination, impaired decision-making, altered circadian rhythm, and disinhibited behaviors (NIH-NIAAA, Bethesda, MD). Behavioral disinhibition is defined as having poor judgment and acting in a manner that one would not usually behave (Greenspan and Ferveur, 2000) and commonly caused by substance abuse such as alcohol consumption and cocaine use. Excessive alcohol intake can also have detrimental impacts such as car accidents leading to death, violent attacks, and unsafe sexual behaviors. Furthermore, alcohol consumption affects the sleep cycle (Sharma et al., 2017), which could aggravate behavioral disinhibition. This research aims to identify the mechanism by which ethanol induces behavioral disinhibition. Drosophila melanogaster was used as a model organism for this study. Drosophila and mammals have similar behavioral responses to ethanol (Kong et al., 2010). Wild-type flies exhibit disinhibited inter-male courtship under the influence of ethanol and the level of disinhibited courtship increases with recurring ethanol exposure (Lee et al., 2008), which represents behavioral sensitization (Berger et al., 2004). Behavioral sensitization is an enhanced response to stimulus after multiple exposures and underlies drug addiction (Walker, 1999). Dopamine is a major neuromodulator in both flies and mammals and plays roles in reward, attention, learning, memory, motivation, sleep, and ethanol-induced disinhibition Oishi and Lazarus, 2017; van Gaalen et al., 2006). Additionally, dopamine is involved in the development of drug dependence
and addiction (Volkow et al., 2009). To understand the mechanism by which dopamine mediates ethanol-induced behavioral disinhibition and sensitization, this study focused on the D1-family receptors dDA1 and DAMB, the insect homologs of mammalian D1 and D5 dopamine receptors, respectively. The major finding of this study is the essential role of DAMB receptor in alcohol-induced behavioral disinhibition. This may provide insight into new intervention strategies for alcohol abuse and addiction.
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Chapter 1: Introduction

Alcohol consumption is known to have beneficial health effects at moderate levels (NIH-NIAAA, Bethesda MD, USDA, Washington, D.C), however many people consume it as a way to detach themselves from physical and emotional pain and problems. According to the 2015 National Survey on Drug Use and Health (NSDUH), 15.1 million adults (ages 18 and older) and 623,000 adolescents (ages 12-17) had alcohol use disorders (NIH-NIAAA, Bethesda MD, SAMHSA, Rockville, MD). High alcohol consumption leads to impaired judgement creating a negative impact in society. Over 85,000 people die from alcohol-related deaths annually (Mokdad et al., 2004), making it the fourth leading preventable cause of death in the United States.

Alcohol interacts with a diverse number of signaling molecules and effector cells (Crews and Nixon, 2009; Moore et al., 1998). Behaviors such as motor coordination, tolerance, sensitization, sleep and inhibition are altered when alcohol is being consumed. Improper amounts of sleep can alter behaviors by inducing tiredness, reduced cognitive throughput, and poor decision-making (Alhola and Polo-Kantola, 2007; Banks and Dinges, 2007). Behavioral disinhibition can be associated to risky behaviors, violent temperament and exasperation, leading to detrimental effects such as, but not limited to, accidents caused by drunk drivers and sexual assaults. Moreover, the mechanism by which alcohol affects sleeping behaviors remains unknown. This research aims to elucidate the neural mechanisms underlying ethanol-induced disinhibition and sleeping behaviors. Drosophila melanogaster will be used as a model organism to study the neural mechanisms underlying ethanol-disinhibited behaviors. The
hypothesis for this study is that D1 family dopamine receptors, dDA1 and DAMB, are important for ethanol induced behavioral disinhibition.

1.1. Behavioral Disinhibition

Doing something you would not do in your regular state of mind is a simple explanation of behavioral disinhibition (i.e. dancing on top of a table while singing karaoke, or becoming best friends to a foe of yours). Behavioral disinhibition is represented by a loss of restraint behaviors acting upon emotions, perceptual and cognitive functions, and motor skills (Grafman et al., 2002, Greenspan and Ferveur, 2000). Impulsivity, aggression, misconduct, risk taking behaviors, and hyper-sexuality are common signs and symptoms that are observed during behavioral disinhibition (Grafman et al., 2002, Iacono et al., 2015). Moreover, substance abuse, such as alcohol and other drugs, lead to these disinhibited behaviors (Grafman et al., 2002, Bartholow and Wood 2000). Though behavioral disinhibition can be observed in various ways, alcohol consumption is one of the highest drug of abuse leading to aggression and risky behaviors (Kaun et al., 2011). In this study, a distinct behavioral disinhibition phenomenon was observed in Drosophila while under the influence of alcohol.

1.2. Alcohol Induced Behaviors

Unlike any other drugs of abuse, ethanol is a non-specific drug. Its metabolites can interfere with gene transcription, therefore altering gene expression patterns (NIAAA, Bethesda MD). In addition, distinct behavioral responses can be affected by alcohol consumption. Alcohol has rewarding effects, which contribute to the development of abuse and addiction (Kaun et al.,
Acute ethanol effects are biphasic (Devineni and Heberlein, 2012; Kaun et al., 2011), acting as a stimulant and a depressant. As a stimulant, ethanol consumption enhances mood, disinhibits behaviors and increases locomotor activity. However, as a depressant it induces a lack of motor coordination and leads to sedation (Kong et al., 2010). When these behaviors are combined, they can lead to violence, assault, euphoria, and risky sexual practices (Moore et al., 1998). Heavy alcohol drinkers often express heightened pleasure feelings while being dominant and assertive towards other people, which can be attributed to recurrent alcohol intake. On the other hand, chronic alcohol consumption affects brain structures such as hippocampus and amygdala, involved in cognitive functions (Crews and Nixon, 2009; Pereira et al., 2016) which underlie dependence and addiction. Alcohol can also mediate abnormal sleep patterns. It modifies the circadian rhythm by increasing the amounts of sleep (Sharma et al., 2017). Alcohol effects are mediated by adaptive changes in brain functions that can be observed by the development of tolerance and sensitization (Berger et al., 2004). A simple animal model will allow us to better understand the underlying mechanism by which ethanol induces disinhibited behaviors.

1.3. Drosophila model

*Drosophila melanogaster*, commonly known as the fruit fly, is a widely used model in alcohol-associated research (Berger et al., 2004; Kaun et al., 2011; Lee et al., 2008; Moore et al., 1998). It allows for easy genetic manipulation and identification of cellular and molecular components in behavior manifestations (McClung and Hirsh, 1998). Its large genetic resources, tools and databases facilitate research to understand neurobiological sources of distinct behaviors, such as ethanol-induced disinhibition (Lee et al., 2008). Fruit flies have a relatively
short life cycle, which facilitates the process of generating a large number of offspring in a short time period. Additionally, the fly brain has architecturally simpler brain regions yet homologous structures compared to those in mammals (Moore et al., 1998). Furthermore, many complex behaviors are also conserved between flies and mammals (He et al., 2013; Hendricks et al., 2000; Lee et al., 2008; McClung and Hirsh, 1998). Hence, the use of Drosophila for scientific studies is relevant and can be applied to the study of mammals.

1.4. DROSOPHILA AND ALCOHOL

It is not rare for flies to reproduce in ambiences containing low levels of ethanol. In fact, flies eat, mate and lay eggs in fermented fruit containing ethanol concentrations up to 5% (Ashburner, 1998; Azanchi et al., 2013). Flies and mammals share similar behavioral responses and common molecular pathways to different ethanol doses. At low doses fruit flies show increased locomotor activity (Devineni and Heberlein, 2012) and disinhibited behaviors such as altered sexual activity (Lee et al., 2008). At high doses, ethanol contributes to a lack of motor coordination, and the development of tolerance to its sedative effects (Berger et al., 2004; Devineni and Heberlein, 2012; Kong et al., 2010). Drosophila’s predisposition to ethanol intake is highly associated with increased sensitivity to stimulant effects, consistent to the observations in humans. Furthermore, sexual dimorphism to ethanol consumption can also be seen in both fruit flies and humans (Ceylan-Isik et al., 2010; Devineni and Heberlein, 2012; Grant et al., 2004). Male fruit flies show higher tolerance development to acute ethanol exposures and greater resistance to ethanol sedation compared to female flies (Devineni and Heberlein, 2012). In humans, women are also more strongly affected by acute ethanol intake compared to men (Ceylan-Isik et al., 2010; Devineni and Heberlein, 2012). Additionally, fruit flies and mammals
share molecular pathways regulating ethanol-induced behaviors (Dominguez et al., 2016; Moore et al., 1998). For example, cAMP pathways have been demonstrated to be involved in the sedative effect of ethanol (Dominguez et al., 2016; Rodan and Rothenfluh, 2010). Thus, *Drosophila* is a good model system to study ethanol-associated behaviors since dDA1 and DAMB regulate the levels of cAMP.

### 1.5. Dopamine and *Drosophila*

Dopamine is a major neurotransmitter involved in reward-motivated behaviors (Soderpalm et al., 2009; Wise, 2000). In both mammals and *Drosophila*, tyrosine hydroxylase (TH) synthesizes dopamine from amino acid tyrosine. It has been shown that flies with mutation in TH have undetectable dopamine levels in the CNS and show deficits in learning and memory (Schwaerzel et al., 2003). Dopaminergic neurons project to various brain structures of the fly such as the mushroom bodies, homologs to mammal’s hippocampus. (Han et al., 1996; Kim et al., 2007). The mushroom bodies (Illustration 1) are important structures in the fly brain that mediate multiple behaviors including sleep (Sitaraman et al., 2015), learning and memory (Kim et al., 2007), and startled-induced locomotor activity (Riemensperger et al., 2013). Thus, dopamine is essential for adaptive behavioral changes in *Drosophila* and mammals. Dopamine D1-type, D2-type, and transporter are found in both *Drosophila* and mammals (Illustration 2). D1-family receptors (dDA1/D1, DAMB/D5, and DopEcR/GPR30 - *Drosophilalmammals, respectively) enhance cAMP levels (Han et al., 1996; Kim et al., 2007) and D2-family receptors (dD2R/D2 - *Drosophilalmammals, respectively) inhibit the increase of cAMP upon binding of dopamine (Hearn et al., 2002). In *Drosophila*, ethanol-induced behaviors can be mapped to
neural sites such as the mushroom bodies. The receptors involved in these neural sites, however, need to be identified.

1.6. Dopamine and Alcohol

Alcohol, like all other drugs of abuse, activates the brain’s reward pathway (Soderpalm et al., 2009; Wise, 2000). Increased dopamine levels are observed in mammals when alcohol is consumed (Soderpalm et al., 2009). Studies on mice demonstrate that at high ethanol concentrations dopamine is released from the striatum (Cohen et al., 1997). Moreover, low doses of ethanol produce a dose-dependent increase of dopaminergic neurons’ firing in the ventral tegmental area (Gessa et al., 1985), supporting the relationship of dopamine with the rewarding effects of ethanol. Silencing dopamine outputs from the mushroom bodies in fruit flies have been shown to inhibit ethanol reward memory (Kaun et al., 2011). Thus, the mammalian mesolimbic system and the Drosophila mushroom bodies play roles in drug addiction. Dopamine is able to interact with the two classes of dopamine receptors (D1-like and D2-like). Antagonists of dopamine D1 and D2 receptors block mice to self-administer drugs (Cohen et al., 1997). Also administration of D1 receptor antagonist results in the suppression of stimulant effects in ethanol self-administering rats (Cohen et al., 1997). Overall, the dopaminergic system is involved in modulating the reward pathway that drugs, including ethanol, target. Nonetheless, specific dopamine receptors involved in ethanol-induced disinhibited behaviors remain to be elucidated.
1.7. Dopamine and disinhibition

In both mammals and fruit flies, dopamine plays a fundamental role in behavioral responses towards drugs. In fact, altered levels of extracellular dopamine are seen with the usage of various drugs of abuse such as cocaine and methamphetamines. Altered dopamine transmission can be seen in the mesolimbic system of mice with drug-induced changes (Bocklisch et al., 2013). Dopamine regulates motor coordination as well as ethanol-induced behaviors (Bainton et al., 2000). Enhanced dopamine activity leads to abnormal inter-male courtship activity in fruit flies (Lee et al., 2008; Liu et al., 2008). Although dopamine is involved in distinct behavioral responses the specific neural pathways remain unclear.

1.8. Sleep, Alcohol and Dopamine

Sleep is a key physiological function for good health and well-being tending the body’s needs. Sleep deprivation, however, has been shown to affect various activities such as learning and memory, attention, creativity, decision-making, problem solving, and emotion and behavior control (NIH-NHLBI, Bethesda MD). Dopamine plays a role in regulating wakefulness by activating the ventral tegmental area, nucleus accumbens and several forebrain structures in various animal models (Dzirasa et al., 2006; Monti and Jantos, 2008). Drosophila share many behavioral similarities to those in mammals including sleep-wake behaviors (Hendricks et al., 2000; Shaw et al., 2000). Like mammals, flies have a circadian rhythm with peak points of activity and periods of inactivity considered as sleep (Hendricks et al., 2000). Alcohol has a sedative effect on both humans and flies (Lee et al., 2008), disturbing the circadian clock. This study aimed to clarify the mechanism by which alcohol interacts with dopamine receptors that mediate sleep-wake behaviors using the fruit fly as our animal model.
Illustration 1: Mushroom bodies
Illustration 2: *Drosophila*/Mammalian dopamine receptors
Chapter 2: Materials and Methods

2.1. Drosophila Strains and Culture

All flies were maintained in a standard cornmeal/agar medium. Flies were raised at 25° C with ~50% humidity under light/dark cycles (12 h: 12 h). One to two day-old flies were collected under carbon dioxide (sedative) and housed in vials (33 male flies per vial for Flypub Assay, and 10-15 male/female flies per vial for sleep studies) containing food. Vials were kept in an incubator for two additional days to clear potential carbon dioxide residues. Three to five day-old males were used for all behavioral tests.

2.2. Genetic Tools

The GAL4/UAS binary system is commonly used for targeted gene expression. The extensive genetic resources allow driving the expression of certain genes in a temporal or spatial manner. GAL4 is a yeast transcriptional activator that binds to a complementary upstream activating sequence (UAS) for gene expression (Feany, 2000) (Illustration 3). A wide array of GAL4 and UAS lines are available upon request via stock centers (i.e. Bloomington, and Vienna stock centers).

2.3. Immunohistochemistry

For dDA1 immunoreactivity, 4 to 5 day-old fly-brains were dissected in phosphate-buffered saline (PBS). Dissected brains were then fixed with 2% Periodate-lysine-paraformaldehyde (PLP) for 20 min each, and then rinsed three times in 1X-PBHT w/ Triton X-
100 over 30 min. Brains were then grouped together and solubilized in 1% Triton X-100 in PBHT for 1 hr. Thereafter, brains were blocked in 5% normal goat serum (NGS) for 2 hr and then incubated with anti-dDA1 antibody (1:1000 diluted in 5% NGS) overnight (12-18 hr). Brains were then washed in 1X-PBHT 4 times over 1 hr and kept in 1X-PBHT overnight. Samples were then incubated for 2 hrs with Alexa 488 conjugated goat anti-mouse IgG (1:1000 diluted in 5% NGS) (Molecular Probes; Invitrogen) and then washed in 1X-PBHT 4 times over 1 hr. Brains were then mounted with Vectashield® (Vector Laboratories) on a slide and imaged using a confocal microscope (Zeiss LSM 700). All steps were performed at room temperature.

For DAMB immunoreactivity, to 5 day-old fly brains were dissected in PBS. Dissected brains were then fixed with 2% PLP for 3 hrs, rinsed three times with 1X-PBHT w/ Triton X-100 over 30 min. Brains were then blocked in 5% NGS for 3 hrs and then incubated with anti-DAMB antibody (1:1000 diluted in 5% NGS) for 4-6 days. Brains were then washed in 1X-PBHT 4 times over 1 hr and kept in 1X PBHT for 4-6 days. Samples were then incubated for 3 days with Alexa 488 conjugated goat anti-rabbit IgG (1:1000 diluted in 5% NGS), washed in 1X-PBHT 4 times over 1 hr and left in 1X-PBHT for 5 additional days. On the final day, brains were mounted with Vectashield® (Vector Laboratories) on a slide for imaging using confocal microscope (Zeiss LSM 700). All steps were performed at 4°C.

2.4. FLYPUB ASSAY

Plastic chambers with clear tops and an open hole at the bottom (Flypub; Illustration 4) were used to measure acute and chronic effects to ethanol (Lee et al., 2008). The clear top allows for the recording of behavior, and the open hole at the bottom allows for the administration of ethanol vapor. Thirty-three 4 to 5 day-old male flies were gently transferred into the pub and
allowed 10 min of acclimation prior to ethanol exposure. A small petri dish containing a cotton pad with 1 ml of 95% ethanol was inserted into the bottom opening of the pub. The pub was videotaped for the first 15 min of ethanol exposure. The dish containing the cotton pad with ethanol was removed after all flies became sedated (~25-30 min) and were gently transferred to their corresponding food vial. Pubs were individually analyzed for inter-male courtship. All flies were exposed to ethanol vapor once a day for six consecutive days.

2.5. DROSOPHILA ACTIVITY MONITOR SYSTEM

After eclosion, flies were maintained in a 12 hr:12 hr light-dark cycle with ~50% humidity in their food vial for 2 days. 3-4 day old flies were then transferred into glass tubes (65mm x 5mm) containing 5% sucrose based food. Tubes were then placed into the Drosophila Activity Monitor (DAM) System (Trikinetics, Waltham, MA) and were allowed one day of acclimation. Locomotor activity data was collected every 5 min for 3 consecutive days.

2.6. DATA ANALYSIS

Enhanced courtship are changes in behavior that were seen in flies exposed to ethanol. Videos recorded from the Flypub Assay were used to score courtship activity. The maximum number of males engaged in courtship activity was scored for every 30 seconds (1 block). Thereafter, the average of the 10 highest consecutive blocks was calculated to obtain a percentage of courtship for each pub. For sleep assay, locomotor activity data was extracted from the DAM system, processed and analyzed by Sleepy and Circadian Analysis MATLAB Program.
(SCAMP) (Trikinetics, Waltham, MA). SCAMP quantified and averaged the total amounts of sleep per day and night, respectively.

All statistical analyses were performed using Minitab16 software. Normal distribution test were conducted for all individual data sets. ANOVA and Student’s t-test was used for normal distributed data, and Kruskal-Wallis and Mann-Whitney test was used for nonparametric data.
Illustration 3: GAL4/UAS Binary System
Illustration 4: Flypub
Chapter 3: The role of dopamine D1 (dDA1) receptor in ethanol-induced courtship disinhibition

3.1 Characterization of dDA1 Receptor

To characterize the role of D1 receptor in ethanol-induced disinhibition, flies deficient in dDA1 (dumb²) were tested and compared to wild-type control (Canton-S). All flies were exposed to ethanol for six consecutive days and measured the number of males courting other males to calculate the percentage of flies engaged in courtship. Male flies normally court females and rarely other males. Upon ethanol exposure, wild-type Canton-S male flies actively court other males in a similar manner to that of females. Previous studies in the lab demonstrated that the flies with non-functional dopamine D1 receptor showed higher levels of courtship disinhibition compared to wild-type flies (data not shown). My results were not consistent with the previous data since no significant difference was seen on dumb² when compared with Canton-S (p > 0.05; Figure 1).

To see if knocking down dDA1 receptor in specific structures of the fly brain, RNA interference (RNAi) techniques were combined with the GAL4/UAS system. RNAi targets and breaks down mRNA affecting the levels of protein expression. For this study, UAS-PBDP (empty promoter-GAL4) was used as genetic control to match the number of transgenes in each fly line tested. A dDA1RNAi was expressed in multiple areas of the fly brain including pan-neurons (ELAV-GAL4) and the mushroom bodies (MB247-GAL4, 238y-GAL4, 30Y-GAL4) (Table 1). When dDA1RNAi got expressed pan-neuronally there was no significant difference compared to control (p > 0.05; Figure 2). However, one of the three mushroom bodies GAL4 lines (238y-GAL4) showed a significant reduction of courtship disinhibition starting at the
second exposure (E2) and continuing throughout the sixth (E6) \( (p < 0.05; \text{Figure 2}) \). To prove that dDA1RNAi>238y exhibited a true phenotype caused by the RNAi targeting dDA1 mRNA, additional assays were conducted. Unfortunately, 238y-GAL4/+ by itself exhibited decreased levels of courtship disinhibition in all ethanol exposures compared to PBDP-GAL4 \( (p < 0.05; \text{Figure 3}) \). This demonstrated that 238y-GAL4 insertion is sensitive to the effects of ethanol. This data concluded that dDA1 is not important for ethanol-induced behavioral disinhibition.

### 3.2. Dopamine D1 Over Expression

Dopamine is a well-known molecule involved in the “good feeling” effect, which typically leads to drug dependence and addiction. Multiple drugs of abuse, including alcohol, increase the levels of dopamine in the brain (Cohen et al., 1997). To determine if dDA1 activation leads to behavioral sensitization flies were subjected to ethanol exposure. The first set of experiments were performed by over-expressing dDA1 in the white genetic background utilizing the following GAL4 lines: MB247, OK107, 30Y, and ELAV (Table 1). Flies exhibited low levels of courtship disinhibition with all GAL4 drivers when dDA1 was over-expressed. Notably, MB247 showed the highest sensitivity to ethanol effects compared to the all other mushroom body drivers \( (p < 0.0001; \text{Figure 4}) \). The expression pattern of MB247-GAL4 is not only in the mushroom bodies but in the glial neurons as well (Aso et al., 2009). To determine if over-expression of dDA1 receptors in glial neurons contributed to elevated levels of sensitization, a specific GAL4 (repo-GAL4) driver was used for dDA1 over-expression. Figure 5 shows that over-expression of dDA1 in glial cells had no difference in behavioral sensitization compared to our control flies \( (p > 0.05) \). Hence, dDA1 in the mushroom bodies but not in glia is involved in behavioral sensitization.
Since over-expression of dDA1 in the white genetic background led to the apparent importance of the mushroom bodies in behavioral sensitization, the assay was repeated by placing UAS-dDA1 fly line in the Canton-S genetic background. Similarly to the white genetic background data, cantonized flies show sensitivity to ethanol compared to transgenic control (Figure 6). As stated in Figure 3, 238Y-GAL4 is not a good driver to test as it shows behavioral desensitization on its own which is why it was not used in this set of experiments. dDA1 overexpression driven by 30Y led to significantly decreased levels of courtship disinhibition or sensitization when compared to control in the second and sixth exposure (E2: \( p < 0.001 \), E6: \( p < 0.0001 \)). These finding provide additional evidence demonstrating the importance of the mushroom body dDA1 in ethanol induced disinhibition.

To confirm that dDA1 was in fact over-expressed, immunoreactivity using receptor specific antibodies was performed. Figure 7A demonstrates the localization of dDA1 receptor in the mushroom body in Canton-S flies. Immunoreactivity in dumb\(^2\) mutant flies showed no presence of dDA1 receptor (Figure 7B), demonstrating the specificity of the antibody used in this study. Figure 7C displays a qualitative representation of dDA1 immunoreactivity in the brain with over-expressed dDA1 in the mushroom body driven by MB247-GAL4. This confirms that dDA1 is in fact expressed in the mushroom bodies as previously reported (Kim, et al 2007) and that we were able to over-express the receptor.
Figure 1. dDA1 receptor mutant *dumb*<sup>2</sup> shows no difference in courtship disinhibition.

Mutant flies exposed to ethanol scored for inter-male courtship and had same levels of disinhibition compared to Canton-S. (E1: $p > 0.05$ by Mann-Whitney Test; E2: $p > 0.05$ by Two-Sample T-Test; E6: $p > 0.05$ by Two-Sample T-Test; n = 5-6)
Figure 2. dDA1 knockdown.

The only statistical difference in induced courtship disinhibition was in 238Y-GAL4 driving dDA1RNAi. (E1: F_{4,25} = 2.16, p > 0.05; E2: F_{4,25} = 4.85, * p < 0.01; E6: F_{4,25} = 9.96, ** p < 0.001; all by ANOVA; n = 6)
Figure 3. 238Y-GAL4 is sensitive to the effects of ethanol.

Empty promoter (PBDP/+) heterozygous used as transgenic control to 238Y-GAL4 heterozygous. (E1: *, $p < 0.05$ by Mann-Whitney Tests; E2 and E6: ***, $p < 0.0001$ by Two sample T-test, n = 5-6)
Figure 4: dDA1 over-expression in the white genetic background.

Over-expression of dDA1 in mushroom body lobes (α, β, γ lobes) using MB247-GAL4 have the highest sensitization levels to ethanol. (E1: **, p < 0.001; ***, p < 0.0001, by Mann-Whitney Test; E2: F_{4,76} = 73.10, *** p > 0.0001; E6: p > 0.05; all by Mann-Whitney; n = 8-19)
Figure 5: dDA1 over-expression in repo-GAL4 has no difference in inter-male courtship.

Over-expression of dDA1 in glia cells do not show differences in behavioral disinhibition compared to control. (E1: $p > 0.05$; E2: $p > 0.05$; E6: $p > 0.05$; all by Mann-Whitney; n = 11-17)
Figure 6: dDA1 over-expression in the Canton-S genetic background.

Over-expression of the dDA1 in mushroom body lobes (α/α', β/β', and γ) using 30Y-GAL4 show behavioral desensitization to ethanol effects. (E1: \( p > 0.05 \) by Mann-Whitney Test; E2: \( F_{4,25} = 4.72, \, ** \, p < 0.001 \); E6: \( F_{5,63} = 9.29, \, ** \, p < 0.005, \, *** \, p < 0.0001 \); by ANOVA; n=8-14)
Figure 7: dDA1 Immunohistochemistry

(A) Canton-S immunoreactivity seen in all mushroom body (MB) (α/α’, β/β’, and γ) neurons. (B) A lack of receptor expression in dumb² mutant flies. (C) Ectopic dDA1 receptor over-expression in the mushroom bodies. Scale bar, 50 µm.
Chapter 4: The role of dopamine D5 (DAMB) receptor in ethanol-induced courtship disinhibition

4.1 CHARACTERIZATION OF DAMB RECEPTOR

To characterize the role of DAMB receptor in ethanol-induced disinhibition, the flies deficient in DAMB (damb) were tested and compared to the wild-type Canton-S using the Flypub assay as described above. Previous studies performed in the lab demonstrated that the flies with non-functional dopamine D5 receptor showed higher levels of courtship disinhibition compared to Canton-S flies (data not shown). When replicating the experiments using damb mutant flies they did not exhibit enhanced levels of behavioral disinhibition when compared to Canton-S. Due to these findings, damb flies were crossed with the deficiency lines that contain deletion of damb (Df(3R)BSC500 and Df(3R)BSC547, referred as Df(a) and Df(b) respectively) in order to replicate the phenotype. Trans-heterozygous damb/Df(a) and damb/Df(b) showed enhanced disinhibited courtship ($p < 0.05$; Figure 8), this corroborated that D5 receptor mutation induced higher courtship disinhibition with recurrent ethanol exposure compared to Canton-S. In addition, Figure 9 shows that having a heterozygous mutation in DAMB induced the same response to ethanol ($p < 0.0001$), hence damb having a dominant phenotype.

Next, RNA interference (RNAi) techniques were combined with the GAL4/UAS system to knock down DAMB receptor. This study used PBDP-GAL4 as a transgenic control. DAMB$^{RNAi}$ was expressed using the same GAL4 drivers as in the dDA1 studies (Table 1). Like the dDA1 receptor experiments, the only significant difference was the DAMB knockdown driven by 238y-GAL4 ($p < 0.05$; Figure 10), which is due to the 238y insertion as opposed to DAMB knockdown (Figure 3).
4.2. Dopamine D5 Over Expression

As aforementioned, substance abuse increases the levels of dopamine in the brain. To determine if D5 receptors are involved in ethanol-induced behavioral disinhibition and sensitization, flies over-expressing DAMB were tested in the Flypub assay. DAMB was over-expressed in all neurons using ELAV-GAL4 as well as in the mushroom body neurons using MB247, 30Y, and OK107. These experiments were performed in the Canton-S genetic background. Figure 11 demonstrates that there is no difference between the flies with DAMB over-expression and the control \((p > 0.05)\), indicating that over-expressed DAMB does not cause changes in behavioral disinhibition or sensitization.

Over-expression of DAMB was confirmed by immunohistochemical analysis using receptor specific antibodies. Figure 12A shows DAMB expression pattern in the wild-type Canton-S brain. This confirms that DAMB is in fact expressed in the mushroom bodies as previously reported (Han et al 1996). damb mutants were also tested to confirm the absence of D5 receptors (Figure 12B). Moreover, Figure 12C shows a qualitative representation of DAMB over-expressivity in the mushroom bodies. These data confirm that DAMB was indeed over-expressed when tested in ethanol induced behavioral sensitization.

4.3 DAMB Behavioral Rescue

To identify the brain structure where DAMB is involved in ethanol-induced behavioral disinhibition, DAMB expression was restored by multiple GAL4 drivers in damb mutant flies. In the first data set damb mutants did not show the disinhibition phenotype as noted above. In ELAV-GAL4/UAS-DAMB;damb, courtship levels were not significantly different from that of Canton-S \((p < 0.05;\) Figure 12A). In the second set of experiments the previously used DAMB
deficiency lines (Df(a) and Df(b)) were used as control after observing that damb mutant flies did not show the phenotype. Similarly to the first set of experiments, ELAV-GAL4/UAS-DAMB;damb showed rescued behavior when compared to Canton-S, damb/Df(a) and damb/Df(b). However, in this set of experiments (Figure 12B), reinstating DAMB in mushroom bodies (MB247-GAL4) rescued the disinhibited behavior phenotype, which was not seen in the first set of experiments (p < 0.05, p < 0.001). The next set of experiments focused on confirming that restored DAMB in all neurons recue the damb’s disinhibition phenotype. The damb mutants with UAS-DAMB or ELAV-GAL4 alone showed enhanced disinhibition but the damb mutants with restored DAMB in the all neurons displayed comparable disinhibition to Canton-S (p < 0.05, Figure 12C). The last set of experiments (Figure 12D) were performed to determine if DAMB in the mushroom bodies plays a role in disinhibited courtship since pervious results of MB247-GAL4 rescue experiments (Figure 12A and 12B) were inconsistent. In this set, DAMB was reinstated in the subset of the mushroom bodies, α’β’ (c305a-GAL4) and γ (np1131-GAL4) neurons. When compared to UAS-DAMB/+;damb control, there was no significant difference in disinhibited courtship (p < 0.05). The GAL4 drivers used in this study did not include all mushroom body subsets. Additional studies need to be performed to establish the regions of the mushroom bodies where DAMB may be important for ethanol induced courtship disinhibition.
Figure 8: DAMB receptor mutant *damb* shows ethanol induced courtship disinhibition

*damb* mutants crossed with DAMB deficiency lines (*Df(3R)BSC500* and *Df(3R)BSC547*, referred as Df(a) and Df(b) respectively) show enhanced levels of courtship disinhibition. (E1: $F_{3,15} = 1.18$, $p > 0.05$; E3: $F_{3,15} = 7.37$, **$p < 0.001$; E6: $F_{3,15} = 9.38$, ***$p < 0.0001$; all by ANOVA; n = 3-6)
Figure 9: *damb* is a dominant phenotype mutation

One copy of *damb* mutation is enough for ethanol-induced courtship disinhibition phenotype. (E1: $F_{2.37} = 8.55$, *, $p < 0.05$, *** $p < 0.0001$ by ANOVA; E2: ** $p < 0.001$, *** $p < 0.0001$ by Mann-Whitney Test; E6: *** $p < 0.0001$ by Mann-Whitney Test; n=6
(*, $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$ by Mann-Whitney Test; n = 6-12)
Figure 10: DAMB knockdown.

The only statistical difference in induced courtship disinhibition was in 238Y-GAL4 driving DAMB\textsuperscript{RNAi} (E1: $F_{4,25} = 1.43$, $p > 0.05$; E2: $F_{4,25} = 4.72$, **$p < 0.001$; E6: $F_{4,25} = 8.33$, **$p < 0.005$; all by ANOVA; n=6)
Figure 11: DAMB over-expression in the Canton-S genetic background.

Over-expression of the DAMB receptor in pan-neurons and in mushroom body lobes (α/α’, β/β’, and γ) do not show difference in ethanol-induced courtship disinhibition (E1: $p > 0.05$ by Mann-Whitney Test, n = 5-19; E2: $F_{4,61} = 0.22, p > 0.05$; E6: $F_{4,56} = 1.00, p > 0.05$; by ANOVA; n = 5-19)
Figure 12: Expression of DAMB receptor on *damb* mutant background

(A-D) Reinstated expression of DAMB receptor pan-neuronally rescued courtship disinhibition phenotype. Reinstated expression in mushroom bodies remains inconclusive (A) (E1: $F_{3,29} = 2.68, p > 0.05$ by ANOVA, n = 6; E2: * $p < 0.05$, **, $p < 0.001$ by Mann-Whitney, n = 6; E6: $F_{3,29} = 17.16, * p < 0.05$, **, $p < 0.001$ by ANOVA, n = 6) (B) (E1: $F_{5,33} = 2.26, p > 0.05$; E2: $F_{5,32} = 7.49, * p < 0.05$, **, $p < 0.001$; E6: $F_{5,33} = 7.45, *** , p < 0.0001$; all by ANOVA; n=6-8) (C) (E1: $F_{3,20} = 10.28, * p < 0.05$, **, $p < 0.001$; E2: $F_{3,20} = 3.86, * p < 0.05$; E6: $F_{3,20} = 33.96$, *** , $p < 0.0001$; all by ANOVA; n=6) (D) (E1: $F_{3,20} = 5.89, * p < 0.05$, **, $p < 0.001$; E2: $F_{3,19} = 14.17$, ** $p < 0.001$; E6: $F_{3,19} = 6.96, * p < 0.05$, **, $p < 0.001$; all by ANOVA; n=6)
Figure 13: DAMB Immunohistochemistry

(A) Canton-S immunoreactivity seen in all mushroom body (α/α’, β/β’, and γ) neurons. (B) A lack of receptor expression in damb mutant flies. (C) Ectopic DAMB receptor over-expression in the mushroom bodies. Scale bar, 50 µm.
Chapter 5: Sleep behaviors

5.1 Sleep baseline studies

To investigate the effect of ethanol exposure on sleep, I set up the *Drosophila* Activity Monitor (DAM) System to measure sleep (Shaw, et al., 2000). Research on sleep usually focuses on testing either males or females and does not address sex difference. To identify whether males and females have same sleep, *Canton-S* males as well as females were examined in the DAM system. Flies were individually placed in glass tubes to monitor activity (infrared beam break) and their activity profiles were analyzed every 5 min using the SCAMP software (Trikinetics, Waltham, MA). Additional factors tested in this study include age and mating status, which likely affect sleep but have not been investigated. Figure 14 shows total amounts of sleep per day or night of virgin or naïve flies at different ages. As shown in Figure 14A, the naïve male flies at all ages tested (4, 12, 24 and 36 day-old) had equal amounts of sleep throughout daytime ($p > 0.05$) and nighttime ($p > 0.05$). In contrast, virgin females had decreased amounts of sleep with aging during daytime, but not nighttime, ($R^2 = 0.3528$). As shown in Figure 15, the mated males and females did not show changes in the total amount of sleep during daytime or nighttime with aging. Nonetheless, the mated females slept less during daytime compared to the virgin females (Figure 14B and 15B).

5.2 *Drosophila* sleeping behaviors with alcohol intake

Alcohol intake leads to multiple behavioral changes including disinhibition, aggression, loss of motor coordination and altered sleep pattern. To test the effect of ethanol exposure on
sleep in flies, 4 to 5 day-old flies were exposed to ethanol at 1 hr before nighttime (8:00 p.m.) for six consecutive days in a Flypub. Sleep time was measured, for three consecutive days following the last ethanol exposure, in the DAM system. Figure 16 shows the average amounts of sleep per day or night on the naïve males and females after chronic ethanol exposure. There was no difference between chronic ethanol and control ($p > 0.05$) in the daytime or nighttime sleep for both males and females. In the females with chronic ethanol, there were sight increases in day and night sleep time, although not statistical significant, ($p > 0.05$; Figure 16B). This suggest that females may exhibit a stronger response to chronic ethanol consumption compared to males.
Figure 14: Day/night average total sleep on naïve/virgin flies at different age groups

Sleep time differed only on females different age points. (A) Male total daytime sleep ($p > 0.05$ by Mann-Whitney Test, $n = 18-24$) and night sleep time ($p > 0.05$ by Mann-Whitney Test, $n = 18-24$) (B) Female total daytime sleep time ($R^2 = 0.3528$, $F_{3,88} = 17.53$) and nighttime sleep time ($F_{3,88} = 1.84$, $p > 0.05$ by ANOVA, $n=20-24$)
Figure 15: Day/night average total sleep on mated flies at different age groups

No difference in sleep time in males or female flies at different age point. (A) Male total daytime sleep ($F_{3.55} = 3.94$, $p > 0.05$ by ANOVA; $n = 12-16$) and nighttime sleep time ($F_{3.55} = 1.42$, $p > 0.05$ by ANOVA; $n = 12-16$) (B) Female total daytime sleep ($F_{3.51} = 0.40$, $p > 0.05$ by ANOVA; $n = 7-16$) and nighttime sleep time ($F_{3.51} = 2.07$, $p > 0.05$ by ANOVA; $n = 7-16$)
Figure 16: No EtOH Exposure (0x) vs. Chronic EtOH Exposure (6x)

No difference in sleep time in either naïve males or virgin female flies when exposed to ethanol. (A) Male average total day/night sleep time (ns, $p > 0.05$ by Two-sample student T-test; n = 8-11) (B) Female average total day/night sleep time (ns, $p > 0.05$ by Two-sample student T-test; n = 8-12)
Chapter 6: Discussion

6.1 dDA1 and DAMB in ethanol induced disinhibition

Ethanol is a non-specific drug of use that targets and affects multiple organs including the liver, heart, and brain. Additionally, ethanol mediates multiple behaviors including loss of motor skills, cognition, abnormal sleeping patterns, learning and memory, aggression, and loss of response inhibition. Dopamine has been associated with ethanol induced behavioral disinhibition, yet the mediation of this behavior is still to be elucidated. This study has given insight and demonstrated how D5 dopamine receptor, part of the D1-family like receptors, plays a role in ethanol-induced disinhibition.

D1 receptor mutant was first tested to determine if it played a role in ethanol-induced disinhibition. Studies by a previous lab member demonstrated that non-functional D1 receptor enhanced the levels of courtship activity; however, results could not be replicated. Possible suppresser buildup due to the nature of the mutation would be an explanation of why the behavior was not seen, or perhaps flies were not carefully managed. Previous D5 receptor data was replicated. damb mutant flies were crossed with deficiency lines which then increased behavioral disinhibition as seen with previous results. Having non-functional D5 receptor increased the levels of inhibition; hence, playing role in ethanol-induced courtship disinhibition. Additionally, it was shown that lack of one copy of the damb gene leads to behavioral disinhibition, making it a dominant phenotype.

To further the studies of non-functional receptors (dDA1 and DAMB) in ethanol-induced courtship disinhibition in a tissue specific manner, RNAi technique was utilized. RNAi knocked down dDA1 and DAMB, respectively, in a site specific manner using various GAL4 drivers.
Tests using RNAi were inconclusive. The RNAi lines used (dDA1RNAi and DAMBRNAi) did not worked properly since there was no difference in inhibition with any of the GAL4 lines tested. RNAi might have not decreased the level of protein expression, nevertheless, additional tests using functional RNAi lines need to be conducted to demonstrate specific sites where the receptors could play a role in ethanol-induced behavioral disinhibition.

Since damb mutants show increased disinhibited behaviors, genetic manipulations were used to rescue the phenotype. Functional DAMB was expressed pan-neuronally under damb genetic background (Illustration 5). Expression of DAMB successfully rescued the mutant phenotype. This demonstrated that the phenotype was due to the lack of the functional receptor and not due to the mutation itself. Based on data presented, ethanol mediates dopamine D5 receptor for inhibition control in the nervous system. Additional studies were performed to determine brain sites where ethanol could potentially mediate courtship-disinhibited behaviors. Multiple mushroom body GAL4 drivers were used to rescue the phenotype. Additional GAL4 drivers need to be tested to cover all sites of the mushroom bodies and to determine if they play a role in ethanol-induced behavioral disinhibition.

Dopamine plays a role in multiple neurological disorders such as schizophrenia and attention deficit hypertension disorder (ADHD) as well as in drug addiction were the levels of dopamine are higher than normal. dDA1 and DAMB, respectively, were over-expressed to see if activation of these receptors led to distinct behavioral disinhibition, since it is a common characteristic seen in people with drug addiction. No difference was seen when DAMB was over-expressed in either the mushroom bodies or pan-neuronally, however when dDA1 was over-expressed in different GAL4 lines flies became more sensitive to the effects of ethanol. The results were consistent when tested in different genetic background. Specifically, the
mushroom bodies seem to play a role in sensitization when dDA1 was over-expressed. This could potentially give insight on how ethanol interacts with specific receptors to promote sensitivity to ethanol’s effects.

6.2 Sleep

Sleep is an important component for synaptic (McGaugh, 2000; Vyazovskiy et al., 2017), memory retention and consolidation (Boyce et al., 2017; Patrick and Gilbert, 1896). However, these brain functions can be altered with improper sleep. Drosophila has been widely used as an animal model to observe and study sleep patterns and behaviors as it shares homologous behaviors to those found in mammals (Hendricks et al., 2000; Shaw et al., 2000). Sleep baselines studies were performed to view the differences of total sleep comparing males and females of different ages. Furthermore, flies were exposed to ethanol for six consecutive days to mimic binge drinking and were analyzed to measure their amounts of sleep.

The first part of this study suggests that age affects the amounts of sleep virgin females get but has no effect on naïve males. This could be due to the fact that female flies have the necessity to mate in order to maintain the species. Since Drosophila has a short life span, older virgin flies need to mate to continue reproducing, hence flies spend more time looking for a partner rather than sleeping. Additionally, naïve males have similar amounts of sleep during the daytime and nighttime, yet virgin flies sleep less during the day compared to the night, and the difference increases as these flies get older. Once again, showing that virgin flies rather be awake during the day to perhaps look for a suitable mate. In mated flies age has no effect on the amounts of sleep on either females or males, both sleep the same amount of time disregarding age. Nevertheless, mated female flies have decreased amounts of sleep during the day compared
to the nighttime. A possible explanation could be the fact that mated female flies rather stay awake longer during the day for continuous egg laying to maintain the species.

The second part of this study was to determine whether ethanol affects the amounts of sleep. Naïve male flies did not show a difference in amounts of sleep when chronically exposed (6x) compared to flies that were not exposed. Additionally, the amount of sleep was constant during both day and nighttime. However, virgin females, although not significantly different, showed increased levels of sleep when chronically exposed to ethanol. Females are typically more sensitive to the effects of alcohol, which could explain the high amounts of sleep with recurrent ethanol exposures on females yet not in males. The number of animals tested needs to be increased. Furthermore, this study needs to be continued using mated flies to determine if that has any effect on sleep time.

This study has given preliminary data to better understand the effects of age in sleep time, and how chronic exposure to ethanol affects this behavior. To understand how ethanol mediates sleep time through the dopaminergic system, flies deficient or non-functional receptors would need to be tested.

6.3 Conclusion

Alcohol abuse and addiction is a problem affecting society worldwide. Alcohol is able to mediate multiple behaviors such as motor coordination, learning and memory, cognition, sleep, and disinhibited behaviors. Excessive alcohol consumption, however, can lead to detrimental effects such as accidents caused by drunk drivers, aggression, risky sexual behaviors, and death. Dopamine, a major neuromodulator, acts upon alcohol consumption. The relationship between alcohol and dopamine remains unclear. This research aimed to identify the neural mechanism
underlying ethanol-associated disinhibition. Using *Drosophila* as a model organism allowed us to understand the principles by which ethanol affects dopamine receptors and mediates disinhibited behaviors. This study demonstrates how dopamine D1 and D5 receptors play a role in ethanol-induced disinhibition. Additionally, the phenotype seen with non-functional D5 receptor was rescued by reinstating the receptor in all neurons. Although experiments on D1 receptors were not replicated, results on over-expression correlated to previous finding demonstrating that having an abnormal amounts of dDA1 receptor lead to disinhibited behaviors. Data on sleep provided useful information on how age and alcohol affect amount of sleep. Further studies need to be performed to determine how ethanol consumption mediates sleeping behaviors.

I hope that this study will help better understand how alcohol mediate behaviors by acting upon dopamine receptors. This way, dopamine receptors could be a potential target for effective treatments for alcohol abuse and addiction.
Illustration 5: Reinstation of DAMB receptor
## Appendix

### Table 1: Fly lines used

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References


Sharma, R., Sahota, P., and Thakkar, M.M. Lesion of the basal forebrain cholinergic neurons attenuates sleepiness and adenosine after alcohol consumption. Journal of Neurochemistry, n/a-n/a.


Vita

Ivan Mercado was born and raised in El Paso, Texas. He graduated top ten percent from San Elizario High school. He continued on pursuing a Bachelor’s degree in the field of biology at the University of Texas at El Paso (UTEP) graduating class of 2014. During his undergraduate career, Ivan was involved in multiple organizations including Medical Professions Organization (MPO), and the American Society of Microbiology (ASM) where he partook in volunteer activities. Additionally, Ivan served UTEP as a member of the universities marching band (Marching Miner Regiment) for the years of 2011 and 2013. During his undergraduate career, Ivan was given the opportunity to enter Dr. Kyung-An Han laboratory research team. Doing so, Ivan became a Research Initiative for Scientific Enhancement (RISE) scholar. While being a RISE scholar, he attended the 2013 Society for Neuroscience annual conference at San Diego, CA. where he was author of a poster being presented. After graduating with a BS in Biology, Ivan was accepted as a Master’s student at UTEP. During his career as graduate student, Ivan became a NIAA Diversity Supplement Awardee. He attended the 2015 Research Society on Alcoholism annual meeting at San Antonio, Texas and the 2014/2015 Society for Neuroscience annual conferences at Washington D.C/Chicago, Ill, respectively. In all conferences attended he gave poster presentations. In addition to his work as a research graduate assistant from 2014-2017, Ivan became a teaching assistant for Fall 2016.

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