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# Examining the Role of the Drosophila Melanogaster Unc13 Protein in Open Field Activity Using RNAi

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# EXAMINING THE ROLE OF THE *DROSOPHILA MELANOGASTER* UNC13 PROTEIN IN OPEN FIELD ACTIVITY USING RNAi

By

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DEDICATION

This thesis is dedicated to everyone who guided and encouraged me throughout my college years on my journey to medical school. I could not have done it without the support of my family and my friends.

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ABSTRACT

ISLAM ORABI: Examining the Role of the *Drosophila Melanogaster* Unc13 Protein in Open Field Activity Using RNAi

Unc13 are proteins in the presynaptic neurons essential in controlling synaptic vesicle fusion and synaptic transmission. Recently, the reduction of *Drosophila melanogaster* Unc13 proteins (Dunc13), were found to result in a resistance to alcohol, highly reminiscent of tolerance formation. I investigated whether genetically reducing *Dunc13* activity in *Drosophila* brain regions leads to different forms of behavioral plasticity using an open field activity paradigm. In my approach, a Dunc13 RNAi transgene was expressed in the *Drosophila* brain within the mushroom body, the ellipsoid body, and in all neurons. The activities of the flies were examined in the open field paradigm to measure the locomotor and exploratory behavior. In my experiments, I did not find a significant effect of Dunc13 RNAi expression in any of the tested areas. I conclude by describing the effect of the reduction of *Dunc13* activity in the neurons on the behavior of the fly and proposing methods of improvement.

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# Chapter One: Introduction and Background

## Introduction

Unc13 plays a central role in synaptic vesicle docking and priming, assembly and release, and in coupling calcium entry to fusion (Dittman, 2019). The composition and function of the active zone are known to impact many forms of presynaptic plasticity. The active zone provides the platform for rapid fusion of neurotransmitter-filled synaptic vesicles after $Ca^{2+}$ influx by $Ca^{2+}$ channels (Sigrist & Schmitz, 2011). Recently, the Roman lab has identified the inhibition of *Drosophila melanogaster* Unc13 (Dunc13) majorly influenced alcohol tolerance formation (Xu et al., 2018). This suggests that alcohol reduces the activity of Dunc13 leading to homeostatic changes in presynaptic activity to compensate for the loss in Dunc13. Preliminary data suggest that a genetic reduction of Dunc13 activity in small subsets of the *Drosophila* brain is sufficient for a tolerance like-state in alcohol naïve animals to develop (Shrestha and Roman, unpublished data). Furthermore, a reduction in Dunc13 activity may lead to changes in other forms of behavioral plasticity observed in the open field arena exploration (Shrestha and Roman, unpublished data). The overall goal of this project is to determine whether a genetic reduction of Dunc13 activity in *Drosophila* brain regions implicated in alcohol tolerance leads to different forms of behavioral plasticity seen in an open field exploration.

Active zone

Synapses are intercellular junctions between a presynaptic neuron and a postsynaptic neuron (Südhof, 2012). Information in the form of an action potential arrives at a presynaptic terminal and is transmitted to the postsynaptic neuron via a chemical neurotransmitter. Neurotransmitters are packaged into synaptic vesicles in the presynaptic terminal (Südhof, 2012). When an action potential opens presynaptic voltage-gated $Ca^{2+}$ channels, $Ca^{2+}$ enters the compartment and triggers neurotransmitter release in which synaptic vesicles are exocytosed into the synaptic cleft. The active zone is a small section in the presynaptic plasma membrane containing electron-dense material and where synaptic vesicle exocytosis is restricted (Südhof, 2012). Consequently, the active zone is located at the edge between the presynaptic terminal and the synaptic cleft. The active zone functions to transform a presynaptic action potential signal into a released neurotransmitter signal (Südhof, 2012).

Synapses act as computational devices where they transmit and transform action potential encoded information (Südhof, 2012). The majority of synaptic computation of information occurs in the active zone. Active zones perform four functions in neurotransmitter release. First, they dock and prime synaptic vesicles. Then, recruit voltage-gated $Ca^{2+}$ channels to the presynaptic membrane allowing rapid synchronous excitation and release coupling. Next, the active zone contributes to the location of pre- and postsynaptic specializations opposite to each other, organized by transsynaptic cell adhesion molecules. Finally, active zones mediate presynaptic plasticity either by directly

responding to second messengers, such as $Ca^{2+}$ or diacylglycerol, or indirectly recruiting other responsible proteins for plasticity (Südhof, 2012).

Unc13 proteins

Sequencing of early branching animals that lack neurons and muscles such as the sponge *Amphimedon queenslandica* and closely related unicellular organisms to choanoflagellate *Monosiga brevicollis*, disclose that Unc13 homolog was present before the emergence of neurons and true synapses (Burkhardt & Sprecher, 2017; Varoqueaux & Fasshauer, 2017). For example, both long and short isoforms of the 1100 residue core functional unit of Unc13 exist in choanoflagellates despite the lack of neurons, muscle, and synapses in them. Based on the conservation of Unc13 in metazoan and the presence of vital presynaptic proteins such as Synaptotagmin 1 and complexin in sponges, it is probable that a favorably regulated form of calcium-triggered membrane fusion existed before the development of neurons and synapses (Burkhardt et al., 2011; Dittman, 2019; Smith et al., 2014; Yang et al., 2015).

Unc13 proteins are highly conserved synapse-specific proteins crucial in regulating neurotransmitter release through the direct modulation of the SNARE exocytosis complex in flies, mammals, and worms (Aravamudan & Broadie, 2003). Most synapses rely on the Unc13 proteins to regulate synaptic vesicles fusion and synaptic transmission (Dittman, 2019). The Unc13 proteins have C1 and C2 domains that bind to diacylglycerol (DAG) and $Ca^{2+}$, respectively. Additionally, *Drosophila* Unc13 (Dunc13) contains a nonconsensus calmodulin-binding domain. Unc13 proteins interact with t-

SNARE Syntaxin and other key synaptic proteins (Figure 1) (Aravamudan & Broadie, 2003).

SNARE proteins on the plasma membrane, Syntaxin 1 and SNAP25, which comprise the t-SNARE complex, bind to VAMP2 on the synaptic vesicle membrane preparing to fuse upon local $Ca^{2+}$ elevation. Synaptotagmin 1 and complexin help prevent premature synaptic vesicle fusion such that Synaptotagmin 1 promotes vesicle fusion only when calcium levels in the cell are high. The Unc13 and Sec1/Munc18 protein families are essential for synaptic vesicle fusion since the loss of either protein eliminates spontaneous and calcium-triggered synaptic transmission (Dittman, 2019). Sec1/Munc18 family proteins assist in SNARE assembly; spontaneous assembly of SNARE complexes in the absence of Sec1/Munc18 proteins is inefficient (Baker et al., 2015). In the nematode *Caenorhabditis elegans*, Unc13 initiates the open conformation of Syntaxin, preparing vesicles and allowing the core complex to mediate fusion (Dittman, 2019). The abundance of Unc13 at the synaptic terminal determines the synaptic transmission efficacy, and the location of the Unc13 synaptic transmission is modulated by multiple signaling pathways, including local recruitment by diacylglycerol (DAG) (Aravamudan & Broadie, 2003).

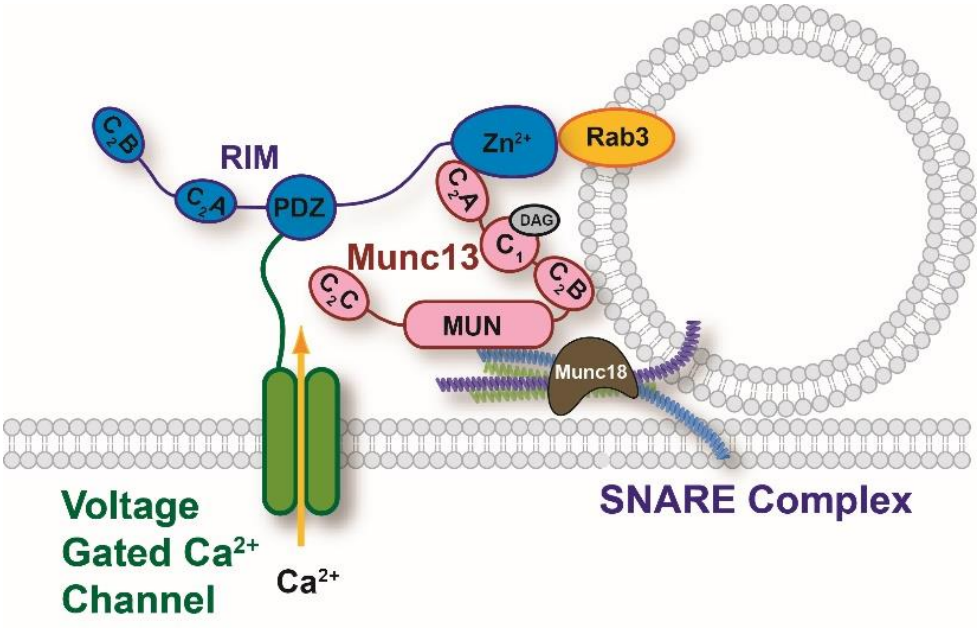



**Figure 1. Munc13-1 and its interactions with other presynaptic active zone proteins.** Drawing showing Munc13 interacting with SNARE proteins creating a SNARE complex for membrane vesicle fusion with neuron membrane to occur when $Ca^{2+}$ levels are high enough. Munc13 consists of C1, C2, MUN, and calmodulin-binding domains. C1 and C2 domains bind to DAG and $Ca^{2+}$. Munc13 and Sec1/Munc18 are essential for synaptic vesicle fusion (Dittman, 2019).

*Drosophila melanogaster* neuroanatomy

Positioned in the center of the insect brain, the central complex is a neuropil of adult insect brains, and its structure is similar across species from diverse habitats (Franconville et al., 2018; Hanesch et al., 1989). Moreover, the central complex encompasses additional neuropils that straddle the midline of the protocerebrum in the brain (Wolff et al., 2015). The central complex serves as an integration center for motor, sensory, learning, and memory activities in insects, including but not limited to coordinating locomotor behavior (flight and walking) and memory in flies (Ilius et al., 2007; Pan et al., 2009; Strauss & Heisenberg, 1993; Wolff et al., 2015). It has been shown in wild-type flies that walking and flight cause an increase in central complex neuropil activity and that different neuropil regions are activated by each behavior (Kim et al., 2019; Renn et al., 1999). Flies display a variety of visual pattern and position-independent behaviors such as stripe fixation, short-term memory, pattern and place learning, all of which require the central complex (Seelig & Jayaraman, 2013). When the central complex structures are disrupted by genetic mutations, abnormal walking behavior and flight behavior are exhibited (Strauss & Heisenberg, 1993). However, only specific components of the behavior are disrupted in each case. For instance, the basic leg coordination of walking may be normal, but the speed, activity, and turning are affected (Strauss et al., 1992; Strauss & Heisenberg, 1993). There are four interconnected substructures in the central complex, the protocerebral bridge, the fan-shaped body, the

ellipsoid body and the paired noduli and, three accessory neuropils (also known as the lateral complex), the gall, the lateral accessory lobes (also known as the ventral body) and the bulbs (commonly referred to as the lateral triangles) (Franconville et al., 2018; Pan et al., 2009; Renn et al., 1999; Wolff et al., 2015). Studies have implicated the protocerebral bridge and the fan-shaped body in the maintenance of the locomotor activity (Martin et al., 2001; Martin et al., 1999). In addition to the central complex, the mushroom body, another structure of the insect central brain, plays a role in memory and olfactory learning (Erber et al., 1980; Heisenberg et al., 1985).

The protocerebral bridge lies between two mushroom body calyces at the most posterior of the brain (Lin et al., 2013). The paired noduli lie rostral to the protocerebral bridge (Wolff et al., 2015). The fan-shaped body, the largest of the central complex neuropils, is posterior to the ellipsoid body. The ellipsoid body is partially embedded in the fan-shaped body and its ventral half is the most anterior neuropil of the central complex (Wolff et al., 2015). The lateral accessory lobes and the bulbs are predominately formed by the processess of extrinsic neurons with approximately 30 different neuronal types categorized as either large-field or small-field neurons (Hanesch et al., 1989). Large-field neurons arborize within one or more tangential layers of a single central complex subunit and provide connectivity to one or more accessory structures or non-central complex areas. Small-field neurons divide the protocerebral bridge and the fan-shaped body as columnar elements, and the ellipsoid body into radial elements. Furthermore, small-field neurons interconnect elements of different substructures and several project to the accessory areas or other brain regions (Renn et al., 1999). Typically, the large-field type connects one central complex structure to areas surrounding the brain

extrinsic to the central complex while the small-field type connect domains within the central complex (Martín-Peña et al., 2014). The central complex, a large repertoire of neurotransmitters, receptors and peptides, display a high degree of intrinsic, topographic order and contributes with many functionally diverse and widely situated brain centers (Martín-Peña et al., 2014; Renn et al., 1999).

The ellipsoid body is shaped like a donut and is partitioned along its radius into rings (Figure 2) (Wolff et al., 2015). A study by Struass and Heisenberg showed that a defect in the ellipsoid body slows the walking behavior in the fly and suppresses fast phototaxis (Strauss & Heisenberg, 1993). Moreover, the ellipsoid body is essential for visual place learning such that severing certain circuits within the ellipsoid body impairs visual place memory (Ofstad et al., 2011). A subset of large field neurons in the ellipsoid body play a part in long-term memory consolidation (Wu et al., 2007) and another group of neurons are necessary for spatial orientation memory (Neuser et al., 2008; Pan et al., 2009). Additionally, the ellipsoid body is involved in the process of olfactory aversive learning and memory (Zhang et al., 2013). The ellipsoid body is implicated in the regulation of alcohol-induced behaviors such that certain genes expressed in the ellipsoid body mediates the rescue of alcohol sensitivity and rapid tolerance (Kang et al., 2020; Urizar et al., 2007).

Another integrative brain center, the mushroom body, plays an essential role in associative learning of olfactory information and short-term memory (Figure 2) (McGuire et al., 2001; Pascual & Préat, 2001; Zhang et al., 2013). In *Drosophila*, mushroom bodies consist of approximately 2500 neurons per brain hemisphere in which the cell bodies of these neurons positioned in the dorsal posterior region of the brain extend their axons to

give rise to lobes (McGuire et al., 2001). These lobes contain the intrinsic mushroom body axons and other processes that synapse with mushroom body neurons. Genes involved in olfactory learning and memory show enriched expression in the mushroom bodies (Heisenberg, 2003). Synaptic transmission from the mushroom bodies is required for the retrieval of short-term memories (Dubnau et al., 2001; McGuire et al., 2001). The mushroom bodies have also been implicated in several other behaviors and in spatial learning (Besson & Martin, 2005; Joiner et al., 2006; Zars, 2000). For example, there is a probable role for the mushroom bodies in the spatial learning during the exploration of a novel arena (de la Flor and Roman, unpublished data). Expressions of certain genes in the the mushroom body promotes alcohol tolerance indicating that mushroom body is implicated in alcohol tolerance and sensitivity (Adhikari et al., 2019; Engel et al., 2016).

In this study, I will investigate how the reduction of Dunc13 expression in the ellipsoid and mushroom bodies of *Drosophila* by RNAi, affects behavioral changes in the open field arena (further explained in the next section).

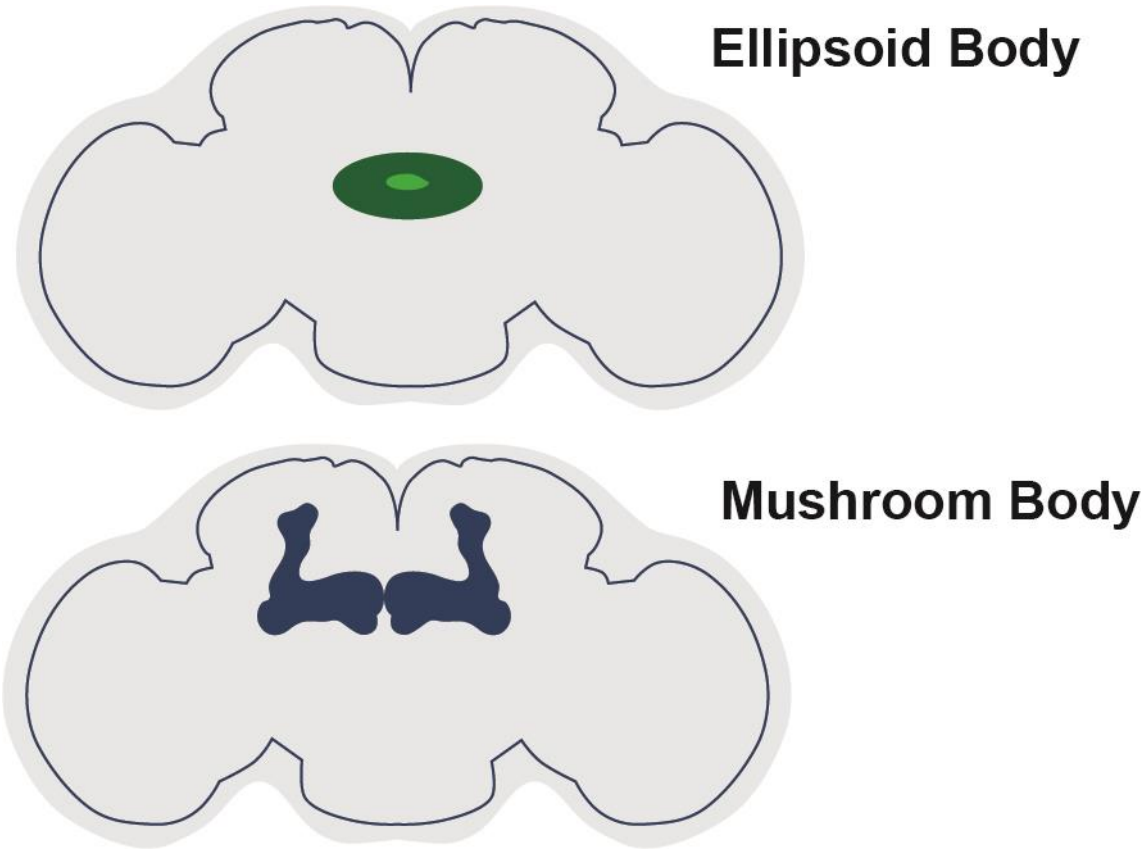



**Figure 2. Visual representation of the location of the ellipsoid and mushroom bodies in *Drosophila* brain.** The ellipsoid body is located in the central complex while the mushroom body is positioned in the dorsal posterior of the brain (McGuire et al., 2001; Renn et al., 1999).

Gal4/UAS pathway

Yeast Gal4 protein and its target upstream activating sequence (UAS) is the most widely used system for generating spatially restricted transgene expression in *Drosophila* (Roman, 2004). Gal4 is a transcriptional activation protein that will bind to the UAS promoter sequence to activate transcription. The Gal4-UAS system utilizes a specific cloned promoter or enhancer to direct the expression of Gal4. Consequently, the expression of the gene of interest that has been cloned downstream of a UAS promoter will be specifically activated in the cell where Gal4 is expressed. The Gal4/UAS system may be used in mosaic expression of transgenes for cell-specific rescue of mutant phenotypes, synaptic activity, genetic perturbation of cellular signaling pathways and many other experimental applications (Roman, 2004). The *elav*-Gal4 is a widely used driver line for neuron-specific ectopic gene expression (Berger et al., 2007) such that *elav*-Gal4 expresses the gene in all neurons (Luo et al., 1994). Embryonic lethal abnormal visual gene or "elav" is vital for the development and maintenance of the nervous system (Antic & Keene, 1997).

Chimeric versions of the Gal4 protein have been developed in which activity of the transcriptional activator depends on the presence of steroid hormones such as mifepristone or chemically related compounds (McGuire et al., 2004). The tissue-specific promoters and the activation by the systemic application of the ligand express Gal4 proteins provides for spatial and temporal regulation of the expression of UAS transgenes (Roman et al., 2001). Gene-Switch is a Gal4-progesterone receptor chimeric protein used

in *Drosophila* (Roman & Davis, 2002; Roman et al., 2001). In the absence of the anti-progestin RU486, the Gene-Switch is in the "off" state. When RU486 is present, the Gene-Switch molecule changes to an active conformation in which it can bind to a UAS sequence and activate transcription of a transgene (Roman et al., 2001). Gene-Switch drivers have been generated that allow for temporally controlled gene expression in the *Drosophila* mushroom body neurons (Mao et al., 2004).

*Dunc13*RNAi gene of interest

RNA interference (RNAi) is a technique in which a double-stranded RNA is expressed to inhibit the expression of a target gene (McGuire et al., 2004). The double-stranded RNA will produce short interfering RNAs (siRNAs) that will guide the RNA-induced silencing complex to degrade mRNAs with sequence identity to the siRNA. In my experiments, I used a UAS- transgene that generated a double-stranded RNA with sequence identity to the *Dunc13* gene. This UAS-*Dunc13*RNAi transgene would reduce the *Dunc13* mRNA present in cells where it is expressed (Xu et al., 2018).

Open Field Test

Characterizing the locomotor behavior of *Drosophila* is essential to any study of the phenotyope-genotype interaction and the complex behaviors that are all based on measures that depend upon some observable movement of the flies (Valente et al., 2007). These observable movements may include the average speed of flies in some environments (Strauss & Heisenberg, 1993; Valente et al., 2007) or a simple line-crossing assay (Kume et al., 2005; Valente et al., 2007). Generally, locomotor activity

refers to regular facets of spatial and temporal patterns in animal trajectories (Soibam et al., 2014).

Establishing a quantitative description of a behavior by taking into consideration the trajectory that the fly takes during a given behavioral test can be analzyed to elucidate the neurological and biochemical processes involved as the behavior unfolds over time (Valente et al., 2007). A study of trajectory is most frequently accomplished in an open field arena (Valente et al., 2007). An Open Field Test typically involves a circular arena, a thin transparent plastic ceiling to be placed over the arena to prevent the fly from escaping and a camera hovering over the arena and attached to a personal computer to track the movements of the fly (Liu et al., 2007; Valente et al., 2007).

The Open Field Test is commonly used to measure the quantity and quality of exploratory behavior and general activity (Gould et al., 2009). Furthermore, the test is also used to assess the sedative, toxic or stimulant effects of compounds (Gould et al., 2009). More recently, the Open Field Test is utilized as a valid assay for determining fly locomotor behavior (Liu et al., 2007; Valente et al., 2007). In an open field arena, *Drosophila* display a non-linear activity (Soibam et al., 2013) and significant preference for the arena's edge due to centrophobicity and arousal gained by contact with walls (Besson & Martin, 2005; Götz & Biesinger, 1985; Soibam et al., 2012). When a fly is initally introduced to an arena, the fly exhibits high exploration activity induced by the novelty of the arena (Liu et al., 2007; Soibam et al., 2013). Over time, the decay in activity of the fly is attributed to the habituation to the arena's novelty through visual learning (Liu et al., 2007; Soibam et al., 2013).

The experiments conducted in this study will apply the Open Field Test on genetically modified flies to quantitatively analyze the neurological and biochemical processes involved in the behavior of the fly.

The purpose behind investigating the reduction of *Dunc13* activity in the ellipsoid and mushroom bodies is that genetically reducing *Dunc13* activity mimics the initial effects of intoxicating concentrations of alcohol which produces synaptic depression (Xu et al., 2018). The reduction of *Dunc13* activity in the mushroom body affects the naive senstivity to alcohol, suggesting a role for Dunc13 related plasticity in alcohol tolerance formation (Shrestha and Roman, unpublished data). However, we do not know if other functions are affected by the reduction of *Dunc13* activity. Hence, my experiment's objectives are to examine whether other mushroom body and ellipsoid body functions were impaired when *Dunc13* activity was reduced in the mushroom and ellipsoid bodies.

I hypothesized that when *Dunc13* activity is reduced by the expression of the *Dunc13*RNAi transgene in the ellipsoid body, the fly's locomotor activity will be reduced due to the role of the ellipsoid body in regulating complex motor patterns. Moreover, I predicted that the reduction of *Dunc13* activity in the mushroom body either through constitutive or inducible expression will impact the fly’s spatial orientation and exploratory behavior since the mushroom body is involved with spatial learning. This prediction was to be addressed with the following aims:

Aim 1: Determine if a genetic reduction of *Dunc13* activity in the ellipsoid body leads to a change in exploratory behavior.

Aim 2: Determine if a pan-neural genetic reduction of *Dunc13* activity leads to a change in exploratory behavior.

Aim 3: Determine if a constitutive or inducible reduction of *Dunc13* activity in the mushroom body leads to a change in exploratory behavior.

## Chapter Two: Materials and Methods

### Methods

#### Fly stocks and genetics

All flies were maintained on standard *Drosophila* food except MBGeneSwitch/*Dunc13*RNAi, MBGeneSwitch/+ and *Dunc13*RNAi/+ heterozygous flies that were treated with RU486 to induce gene of interest expression. RU486 (mifepristone) is used to induce gene expression using the "Gene-Switch" in *Drosophila* (Roman et al., 2001).

The c819 Gal4 driver (RRID:BDSC_30849) was used to direct the expression of the Dunc13 RNAi transgene in the ellipsoid bodies and was a gift of P. Taghert (Washington University St. Louis) (Renn et al., 1999). The pan-neural *elav*-Gal4 line (FBst0008760, RRID: BDSC_8760) was obtained from the Bloomington Drosophila Stock Center. The MB-Gene Switch line (RRID:BDSC_81013) was as described (Mao et al., 2004). The mushroom body P247 Gal4 line driver (RRID:BDSC_50742) was a gift of Ronald Davis (Scripps Research Inst., Jupiter FLA). The UAS-Dunc13 RNAi line used in my experiments was the *Dunc13*$^{JF02440}$ transgene (referred to hereafter as Dunc13 RNAi; RRID: BDSC_29548) was obtained from the Bloomington Drosophila Stock Center. I also used the P{UAS-Dcr-2.D}10 transgene (RRID:BDSC_24651), referred to as UAS-*dcr2*, to increase the efficacy of the *Dunc13*$^{JF02440}$ transgene.

For all the RNAi experiments, crosses were set to generate an experimental genotype containing both the Gal4 line and the UAS-*Dunc13* RNAi (e.g., Gal4/UAS-

*Dunc13*RNAi), a Gal4 control genotype containing one copy of the Gal4 line (Gal4/+), and a UAS control genotype containing one copy of the UAS-*Dunc13* transgene (*Dunc13*RNAi/+).

For the ellipsoid body experiment, the c819/+ Gal4 controls were generated by crossing 10 virgin females of wild type Canton-S (CS) with 10 males of c819 and selecting male progeny. The *Dunc13*RNAi/+ controls were generated by crossing 10 wild type Canton-S virgin females with 10 $w^+$; *Dunc13*RNAi males and selecting male progeny. The experimental genotype was generated by crossing 10 virgin $w^+$; c819 females with 10 $w^+$; *Dunc13*RNAi males and selecting male progeny.

For the pan-neural experiment, the *elav*-Gal4/+ controls were generated by crossing 10 virgin females of wild type Canton-S with 10 males of *elav*-Gal4 and selecting male progeny. The UAS-*Dunc13*RNAi, UAS-*dcr2*/+ controls were generated by crossing 10 wild type Canton-S virgin females with 10 $w^+$; UAS-*Dunc13*RNAi, UAS-*dcr2* males and selecting male progeny. The experimental genotype was generated by crossing 10 virgin $w^+$; *elav*-Gal4 females with 10 $w^+$; UAS-*Dunc13*RNAi, UAS-*dcr2* males and selecting male progeny.

For the inducible mushroom body experiment, the MBGeneswitch/+ Gal4 controls were generated by crossing 20 virgin females of wild type Canton-S with 20 males of MBGeneswitch and selecting male progeny. The *Dunc13*RNAi/+ controls were generated by crossing 20 wild type Canton-S virgin females with 20 $w^+$; *Dunc13*RNAi males and selecting male progeny. The experimental genotype was generated by crossing 20 virgin $w^+$; MBGeneSwitch females with 20 $w^+$; *Dunc13*RNAi males and selecting

male progeny. Flies of each genotype were either treated with RU486 or treated with standard 2% sucrose food.

For the constitutive mushroom body experiment, the P247-Gal4/+ controls were generated by crossing 10 virgin females of wild type Canton-S with 10 males of P247-Gal4 and selecting male progeny. The UAS-*Dunc13*RNAi, UAS-*dcr2*/+ controls were generated by crossing 10 wild type Canton-S virgin females with 10 $w^+$; UAS-*Dunc13*RNAi, UAS-*dcr2* males and selecting male progeny. The experimental genotype was generated by crossing 10 virgin $w^+$; P247-Gal4 females with 10 $w^+$; UAS-*Dunc13*RNAi, UAS-*dcr2* males and selecting male progeny. In all experiments, 3-5 days old male flies were used. Flies were maintained at 25° C on a 12/12h light/dark cycle.

Exploration assay using Buridan Tracker

Nine fly arenas were set in 25° C room temperature under the same light intensity. Each arena consisted of a square-shaped posterboard tray. PVC pipes were used to attach a Logitech camera directly facing down into the arena. In the tray, an 8.2 cm diameter arena was taped in place and petri dish lid was used to enclose the arena. To safely transfer flies into and away from the arena, a small, short plastic tubing with an inside net was constructed to suck the fly into the tubing where the net catches it and softly blow the fly out of the tubing at its designated destination. The Buridan Tracker program was used to record the movement of each fly from the camera for 10 minutes (Colomb et al., 2012). A standard trial involved the transferal of nine flies with a single genotype by the plastic tubing from the vial to the petri dishes and running Buridan Tracker simultaneously for 10 minutes. An n of 50 trials was used for each genotype.

Bioinformatics

The raw files collected from Buridan Tracker were converted to MS Excel extension files using Extension Changer program, then translated using the Distance per Minute Macro. In Microsoft Excel, the average distance per minute for the genotypes were calculated and a graph was constructed using the average to compare the traveled distance (in cm) in each minute across the genotypes. Tables were constructed to organize and sum the total average distance for each genotype.

Statistical analysis

The total distance a fly traveled in each trial were calculated for the genotypes and analyzed using the statistical analysis software SigmaPlot. Initially, the Shapiro-Wilk test was run on test normality. If the data were normally distributed, an ANOVA test would be run. If the data failed the Shapiro-Wilk normality test, then a Kruskal-Wallis test was performed, followed by a pair-wise comparison using Dunn's Method.

## Chapter Three: Results

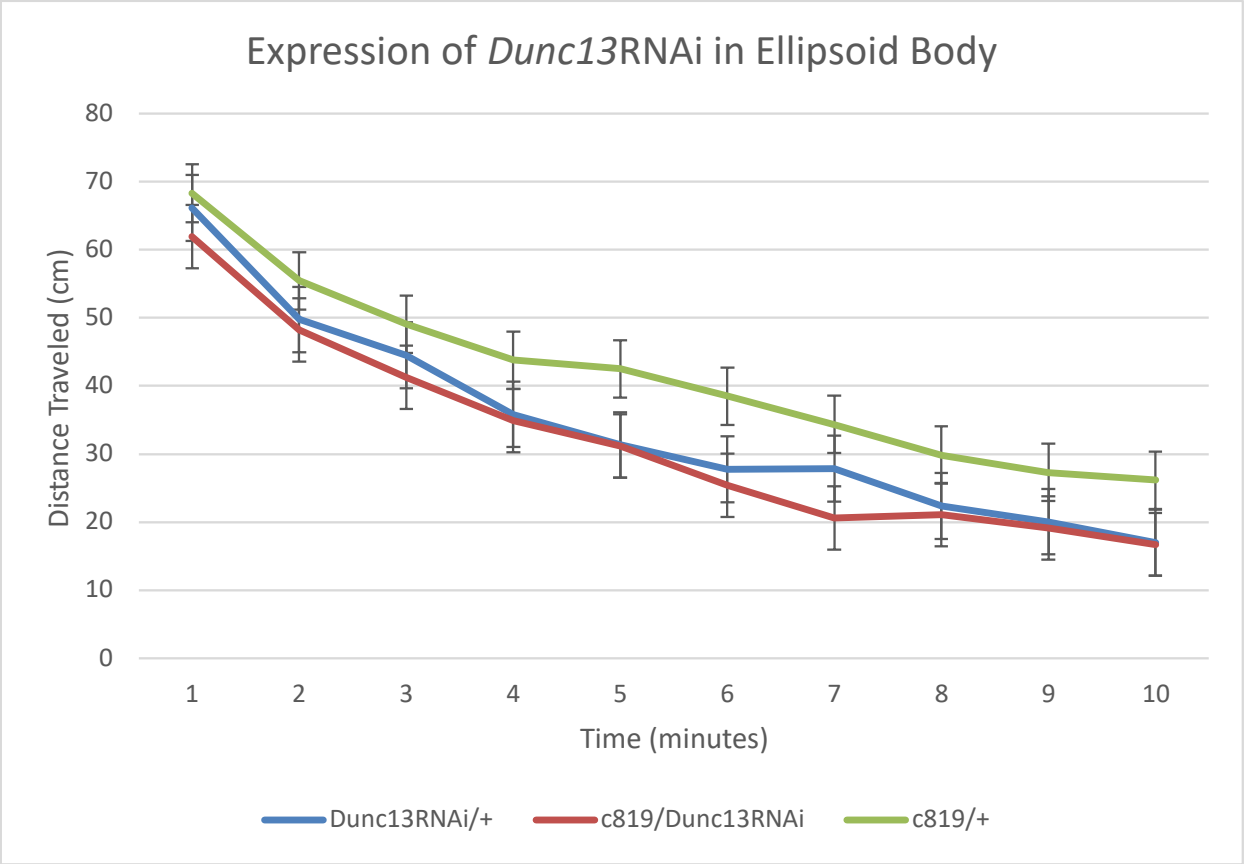
### Results

#### Inducing *Dunc13*RNAi expression in the ellipsoid body did not significantly impact locomotor activity in the open field

To investigate the significance of the ellipsoid body in the locomotor activity of the fly, I examined the distance traveled per minute in flies heterozygous for *Dunc13* loss-of-function in the ellipsoid body against the genotypes c819/+ and *Dunc13*RNAi/+. Locomotor activity was determined as the distance in centimeters a fly traveled each minute for 10 minutes, that is, the longer distance the fly traveled per minute, the more functional the locomotor behavior of the fly is. There was a significant effect of genotype in this experiment (Kruskal-Wallis, $H = 23.280$, $p < 0.001$). There was a significant difference between c819/+ and c819/*Dunc13*RNAi (Dunn's Method, $Q = 4.691$, $p < 0.001$) and, between c819/+ and *Dunc13*RNAi/+ (Dunn's Method, $Q = 3.362$, $p < 0.005$), but no significant difference between *Dunc13*RNAi/+ and c819/*Dunc13*RNAi (Dunn's Method, $Q = 1.366$, $p = 0.516$). The c819/+ heterozygotes have the highest locomotor activity in the fly with a total distance of 415 cm traveled over 10 minutes followed by *Dunc13*RNAi/+ genotype with a total distance of 343 cm traveled in 10 minutes and lastly, c819/*Dunc13*RNAi genotype with a total distance of 321 cm traveled throughout 10 minutes (Figure 3). The difference in distance presented between *Dunc13*RNAi/+ and c819/*Dunc13*RNAi genotypes is insignificant. These data suggest

that the effect of RNAi expression in the ellipsoid bodies did not affect locomotor activity.

A



B

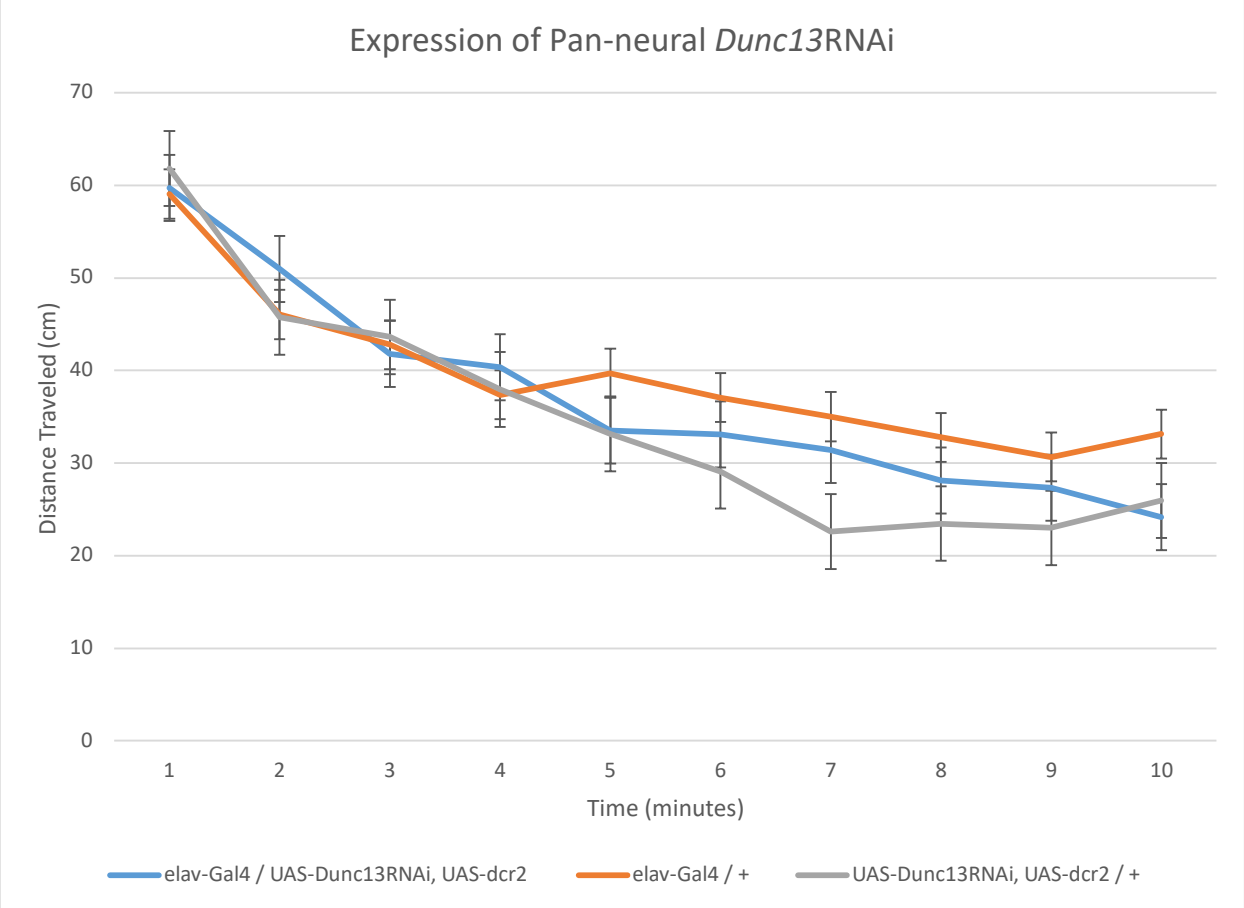
| Genotype | Minute (1-10) | Average distance (cm) | Genotype | Minute (1-10) | Average distance (cm) | Genotype | Minute (1-10) | Average distance (cm) |
|---|---|---|---|---|---|---|---|---|
| c819/+ | 1 | 68.30 | *Dunc13*RNAi/+ | 1 | 66.18 | c819/*Dunc13*RNAi | 1 | 61.93 |
| | 2 | 55.45 | | 2 | 49.76 | | 2 | 48.24 |
| | 3 | 49.07 | | 3 | 44.49 | | 3 | 41.25 |
| | 4 | 43.80 | | 4 | 35.83 | | 4 | 34.95 |
| | 5 | 42.51 | | 5 | 31.35 | | 5 | 31.21 |
| | 6 | 38.49 | | 6 | 27.74 | | 6 | 25.39 |
| | 7 | 34.34 | | 7 | 27.84 | | 7 | 20.62 |
| | 8 | 29.84 | | 8 | 22.37 | | 8 | 21.12 |
| | 9 | 27.29 | | 9 | 20.06 | | 9 | 19.12 |
| | 10 | 26.15 | | 10 | 16.99 | | 10 | 16.74 |
| | Total | 415.24 | | Total | 342.61 | | Total | 320.57 |

**Figure 3. c819/*Dunc13*RNAi expression in the ellipsoid body did not lead to a reduced locomotor activity.** (A) The distance traveled by flies were measured in centimeters per minute for 10 minutes. On the graph, the blue colored curve represents *Dunc13*RNAi/+ genotype, the red colored curve represents c819/*Dunc13*RNAi genotype and the green colored curve represents c819/+ genotype. The c819/+ genotype curve line is the topmost, followed by *Dunc13*RNAi/+ and then c819/Dunc13RNAi genotypes. (B) The distance per minute flies traveled in each trial and the sum are shown. There were significant differences between c819/+ flies and both the c819/*Dunc13*RNAi and *Dunc13*RNAi/+ genotypes (Dunn's Method, $p < 0.005$), but no significant difference between *Dunc13*RNAi/+ and c819/*Dunc13*RNAi was found (Dunn's Method, $p = 0.516$).

Inducing *Dunc13*RNAi expression pan-neurally does not significantly impact exploration in the open field arena

To measure the impact of reducing *Dunc13* activity globally in the nervous system, I used *elav*-Gal4 to drive the UAS-*Dunc13*RNAi transgene. The distance traveled per minute over 10 minutes in the open field arena were recorded for flies with the following genotypes: 1) *elav*-Gal4/UAS-*Dunc13*RNAi, UAS-*dcr2* (Experimental), 2) *elav*-Gal4/+ (Gal4 control) and 3) UAS-*Dunc13*RNAi, UAS-*dcr2*/+ (UAS control). There were no significant genotype effects within this experiment (Kruskal-Wallis, H = 0.157, $p$ = 0.924) (Figure 4). These data indicate that the expression of the *Dunc13*RNAi driven by the *elav*-Gal4 transgene had no detectable effect on locomotor activity.

A



B.

| Genotype | Minute (1-10) | Average distance (cm) | Genotype | Minute (1-10) | Average distance (cm) | Genotype | Minute (1-10) | Average distance (cm) |
|---|---|---|---|---|---|---|---|---|
| *elav*-Gal4/+ | 1 | 59.05 | *elav*-Gal4/UAS-*Dunc13*RNAi, UAS-*dcr2* | 1 | 59.74 | UAS-*Dunc13*RNAi, UAS-*dcr2*/+ | 1 | 61.82 |
| | 2 | 46.05 | | 2 | 50.98 | | 2 | 45.73 |
| | 3 | 42.79 | | 3 | 41.79 | | 3 | 43.63 |
| | 4 | 37.37 | | 4 | 40.34 | | 4 | 37.97 |
| | 5 | 39.72 | | 5 | 33.52 | | 5 | 33.17 |
| | 6 | 37.07 | | 6 | 33.08 | | 6 | 29.10 |
| | 7 | 35.01 | | 7 | 31.43 | | 7 | 22.61 |
| | 8 | 32.77 | | 8 | 28.14 | | 8 | 23.46 |
| | 9 | 30.65 | | 9 | 27.36 | | 9 | 23.00 |
| | 10 | 33.13 | | 10 | 24.18 | | 10 | 25.97 |
| | Total | 393.61 | | Total | 370.56 | | Total | 346.46 |

**Figure 4. Pan-neural expression of *Dunc13*RNAi did not significantly affect activity in the open field arena.** (A) Each minute, the distance traveled by a fly with a genotype was measured over 10 minutes. The blue-colored curve represents *elav*-Gal4/UAS-*Dunc13*RNAi, UAS-*dcr2*, the orange colored curve represents *elav*-Gal4/+ and the gray colored curve represents UAS-*Dunc13*RNAi, UAS-*dcr2*/+. *elav*-Gal4/+ curve is the highest curve followed by the *elav*-Gal4/UAS-*Dunc13*RNAi, UAS-*dcr2* curve with UAS-*Dunc13*RNAi, UAS-*dcr2*/+ curve being the lowest. (B) The mean distance per minute flies traveled and the sum total distances are shown. There was no significant difference between the three genotypes in total distance traveled (Kruskal-Wallis, $p > 0.05$).

Inducing *Dunc13*RNAi expression in the mushroom body neurons did not significantly affect activity in the open field

*Dunc13*RNAi was induced post-developmentally in the mushroom bodies using the MB-GeneSwitch driver (Figure 5). The activity of MB-GeneSwitch is induced by feeding the flies 500 μM RU486 mixed in 2% sucrose feeding solution. Control groups were fed vehicle (2% sucrose). The two-way ANOVA indicated that there was not a significant effect of treatment nor genotype on activity in this experiment ($F = 1.45$; $p > 0.05$ and $F = 2.02$; $p > 0.05$ ). These data indicate that the *Dunc13*RNAi induced post-developmentally in the mushroom bodies by the MB-GeneSwitch driver does not alter locomotor activity.

A.

B.

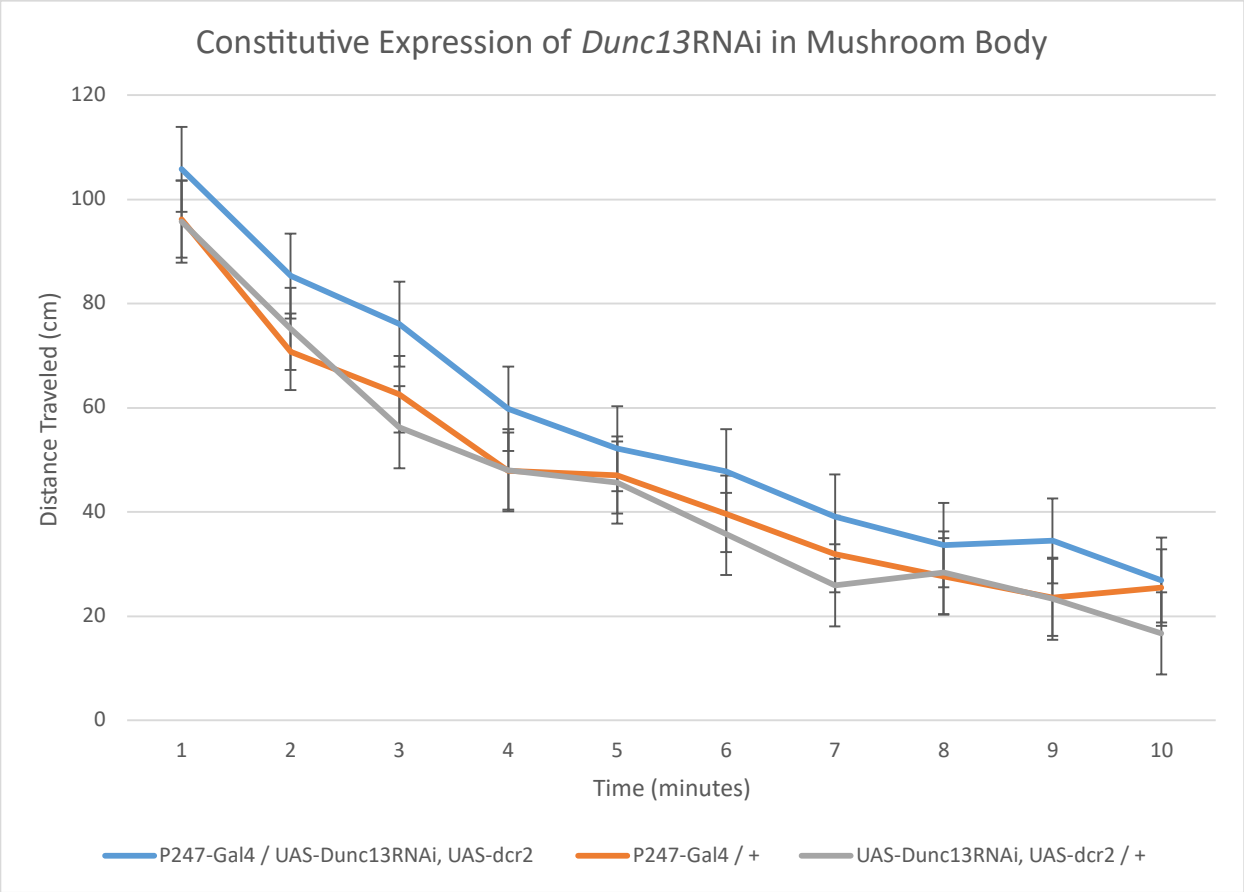
| Genotype and Treatment | Minute (1-10) | Average distance (cm) | Genotype and Treatment | Minute (1-10) | Average distance (cm) |
|---|---|---|---|---|---|
| MBGeneSwitch/*Dunc13*RNAi with 2% sucrose | 1 | 57.91 | MBGeneSwitch/*Dunc13*RNAi with RU486 | 1 | 59.82 |
| | 2 | 47.06 | | 2 | 46.98 |
| | 3 | 45.14 | | 3 | 42.78 |
| | 4 | 41.62 | | 4 | 41.63 |
| | 5 | 33.16 | | 5 | 33.59 |
| | 6 | 31.72 | | 6 | 30.48 |
| | 7 | 25.23 | | 7 | 26.91 |
| | 8 | 30.57 | | 8 | 24.67 |
| | 9 | 24.36 | | 9 | 24.02 |
| | 10 | 23.55 | | 10 | 22.53 |
| | Total | 360.32 | | Total | 353.41 |

**Figure 5. Treating MBGeneSwitch/*Dunc13*RNAi with RU486 did not lead to a significant reduction in locomotor behavior in fly.** (A) Over the course of 10 minutes, the distance the fly traveled each minute was recorded for 2% sucrose- and RU486-treated flies of the indicated genotypes. The aqua colored curve represents MBGeneSwitch/*Dunc13*RNAi treated with 2% sucrose, the red colored curve represents MBGeneSwitch/*Dunc13*RNAi treated with RU486, the colors purple, turquoise, green and orange curves represent MBGeneSwitch/+ treated with RU486, *Dunc13*RNAi/+ treated with 2% sucrose, MBGeneswitch/+ treated with 2% sucrose and *Dunc13*RNAi/+ treated with RU486, respectively. MBGeneSwitch/*Dunc13*RNAi treated with 2% sucrose curve is higher than MBGeneSwitch/*Dunc13*RNAi treated with RU486 curve. (B) The mean distance per minute that the experimental genotype traveled are shown. There were no significant effects of genotype nor treatment in this experiment (two-way ANOVA, $F = 1.45$; $p > 0.05$ and $F = 2.02$; $p > 0.05$).

*Dunc13*RNAi constitutively expressed in the mushroom body did not significantly affect activity in the open field

*Dunc13*RNAi constitutively expressed in the mushroom body was investigated by recording the distance the fly traveled each minute throughout the course of 10 minutes and compared with the control genotypes P247-Gal4/+ and UAS-*Dunc13*RNAi, UAS-*dcr2*/+. There was no significant difference between the three genotypes (ANOVA, $F = 2.523$, $p = 0.084$) (Figure 6).

A.



B.

| Genotype | Minute (1-10) | Average distance (cm) | Genotype | Minute (1-10) | Average distance (cm) | Genotype | Minute (1-10) | Average distance (cm) |
|---|---|---|---|---|---|---|---|---|
| P247-Gal4/UAS-*Dunc13*RNAi, UAS-*dcr2* | 1 | 105.75 | P247-Gal4/+ | 1 | 96.18 | UAS-*Dunc13*RNAi, UAS-*dcr2*/+ | 1 | 95.72 |
| | 2 | 85.26 | | 2 | 70.72 | | 2 | 75.12 |
| | 3 | 76.05 | | 3 | 62.57 | | 3 | 56.29 |
| | 4 | 59.81 | | 4 | 47.88 | | 4 | 48.03 |
| | 5 | 52.14 | | 5 | 47.08 | | 5 | 45.69 |
| | 6 | 47.76 | | 6 | 39.64 | | 6 | 35.83 |
| | 7 | 39.10 | | 7 | 31.95 | | 7 | 25.92 |
| | 8 | 33.63 | | 8 | 27.66 | | 8 | 28.34 |
| | 9 | 34.45 | | 9 | 23.62 | | 9 | 23.39 |
| | 10 | 26.94 | | 10 | 25.48 | | 10 | 16.70 |
| | Total | 560.89 | | Total | 472.78 | | Total | 451.03 |

**Figure 6. *Dunc13*RNAi constitutively expressed in the mushroom body did not lead to the reduction of locomotor behavior in fly.** (A) The distance for flies with P247-Gal4/UAS-*Dunc13*RNAi, UAS-*dcr2*, P247-Gal4/+ and UAS-*Dunc13*RNAi, UAS-*dcr2*/+ genotypes were measured each minute over 10 minutes. The blue colored curve represents P247-Gal4/UAS-*Dunc13*RNAi, UAS-*dcr2*, the orange-colored curve represents P247-Gal4/+ and the gray colored curve represents UAS-*Dunc13*RNAi, UAS-*dcr2*/+. P247-Gal4/UAS-*Dunc13*RNAi, UAS-*dcr2* curve is the highest curve followed by P247-Gal4/+ curve and lastly, UAS-*Dunc13*RNAi, UAS-*dcr2*/+ curve being the lowest. (B) The distance per minute flies traveled in each trial and the sum are shown. There was no significant difference between the three genotypes (ANOVA, $F = 2.52$; $p > 0.05$).

## Chapter Four: Discussion and Conclusion

### Discussion

Genetically reducing Dunc13 activity mimics the effects of intoxicating concentrations of alcohol on tolerance formation (Xu et al., 2018). Preliminary data suggest that inhibition of Dunc13 proteins in the mushroom body is sufficient to produce alcohol resistance (Shrestha and Roman, unpublished data). These data may suggest a role for Dunc13 activity in the mushroom bodies in alcohol tolerance formation. However, it is unclear if changes in Dunc13 activity and the associated changes in presynaptic plasticity have additional roles in the mushroom bodies. Consequently, this study was intended to investigate the role that *Dunc13*, a *Drosophila* ortholog of *Unc13*, plays in the mushroom body, and the ellipsoid body in a general form of behavioral plasticity – activity responses in an open field arena.

We used an Open Field Test to examine the locomotor activity of the fly by measuring their trajectory over the course of 10 minutes. In this work, we demonstrated that *Dunc13*RNAi expression in the ellipsoid body did not significantly affect the locomotor activity in the open field. Furthermore, we revealed that expressing *Dunc13*RNAi pan-neurally did not show a significant impact in the fly's exploration in the open field arena. Lastly, the constitutive and inducible expression of *Dunc13*RNAi in the mushroom body did not show a meaningful impact on the locomotor activity of the fly in the arena. These results indicate that our attempt to reduce *Dunc13* activity in the

ellipsoid, mushroom body, and throughout all neurons did not significantly influence the exploratory and locomotor behavior of the fly.

c819/*Dunc13*RNAi heterozygotes did not have a significant effect on locomotor behavior in fly

The expression of the *Dunc13*RNAi transgene, in which RNAi should induce a reduction in *Dunc13* activity within the ellipsoid body reduces the fly's behavioral walking activity. This prediction, however, was not the case. c819/*Dunc13R*NAi heterozygotes did not demonstrate a significant difference in locomotor behavior from a background control genotype (Figure 3). Hence, the *Dunc13*RNAi expression did not sufficiently reduce *Dunc13* activity enough to observe an effect, as would be expected from a complete loss of ellipsoid body activity (Strauss & Heisenberg, 1993). Dunc13 proteins are vital components in regulating synaptic vesicle fusion and neurotransmitter release. In theory, there is a correlation between the abundance of Dunc13 in the ellipsoid body and the locomotor activity of the fly such that without Dunc13, the synaptic transmission efficacy is reduced (Aravamudan & Broadie, 2003), altering the overall behavior of the fly. As such, the inhibition of a fully functioning ellipsoid body by reducing the Dunc13 activity affects the central complex resulting in the modification of the fly's locomotor and exploratory behavior. Specifically, the decline of the fly's walking behavior.

*Dunc13*RNA/+ and c819/+ heterozygotes were used to compare c819/*Dunc13*RNAi heterozygote with. *Dunc13*RNAi/+ and c819/+ do not express *Dunc13*RNAi transgene because *Dunc13*RNAi/+ heterozygote is missing the Gal4

component, and c819/+ is missing the UAS component from the Gal4/UAS system. Surprisingly, the *Dunc13*RNAi/+ heterozygote exhibited a reduced locomotor behavior compared to c819/+ despite both genotypes not expressing the *Dunc13*RNAi responsible for the inhibition of Dunc13 activity. Since both the UAS RNAi and the c819 Gal4 transgenes are insertions into the genome, it is possible that either one may disrupt a gene that plays a role in locomotor behavior. It is also possible that in c819, which expresses Gal4 in the ellipsoid bodies, this heterologous transcription factor may have a dominant role in neural activity by modulating transactional profiles (Gill & Ptashne, 1988). The significant difference in activity between the background control genotypes makes it challenging to assess the role of *Dunc13*RNAi expression and may warrant further investigation.

Pan-neural induction of *Dunc13*RNAi gene did not show a significant effect on locomotor behavior in fly

When the *Dunc13*RNAi activity was expressed throughout the neurons of the fly, the locomotor and exploratory behaviors of the fly was not affected (Figure 4). Unlike my previous experiment with the c819 Gal4 line, the *elav*-Gal4/+ control did not result in a significant difference in activity in the Open Field Test. This difference might suggest that negative effects of Gal4 expression in ellipsoid body neurons might not exist. Still, there are also likely significant differences in the level of Gal4 expression between these two Gal4 drivers, which could still account for a domain effect of c819 on behavioral activity in the open field arena.

An inducible and constitutive expression of *Dunc13*RNAi in the mushroom body did not lead to a significant change in the locomotor behavior

When *Dunc13*RNAi was induced in the mushroom bodies of the MBGeneSwitch/*Dunc13*RNAi flies with RU486, the locomotor behavior was not significantly altered (Figure 5). The Gene-Switch system (Roman et al., 2001), a Gal4-progesterone receptor chimera in *Drosophila*, was used to induce the Gene-Switch to an active conformation in the presence of the hormone RU486.

Similarly, when *Dunc13*RNAi was constitutively reduced in the mushroom body of the P247-Gal4/UAS-*Dunc13*RNAi, UAS-*dcr2* heterozygote, the effect on the locomotor behavior was not signifcant (Figure 6), indicating that neither an inducible nor a constitutive expression of *Dunc13*RNAi in the mushroom body affected the fly's exploratory behavior. The results suggest there were no significant effects on the locomotor behavior of the fly when expressing the *Dunc13*RNAi transgene in the mushroom and ellipsoid bodies. Since the mushroom bodies have been implicated in spatial learning and regulation of locomotor activity, a loss of Dunc13 might have been expected to generate a change in open field activity (Besson & Martin, 2005; Joiner et al., 2006; Zars, 2000). The absence of an effect of *Dunc13*RNAi expression in the mushroom bodies may suggest that Dunc13-related plasticity in these neurons does not impact open field behavior. The lack of an effect on activity may also be because the expression of the *Dunc13*RNAi transgene is not reducing the expression of Dunc13 in these neurons. This *Dunc13*RNAi has been previously shown to have an effect on ethanol sensitivity when expressed pan-neurally (Xu, et al. 2018).

Summary

Dunc13 is crucial in regulating neurotransmitter release through the direct modulation of the SNARE exocytosis complex (Aravamudan & Broadie, 2003). Moreover, most synapses rely on Dunc13 proteins to regulate synaptic vesicle fusion and neurotransmitter release in which the C1 and C2 domains of Dunc13 bind to DAG and $Ca^{2+}$ (Aravamudan & Broadie, 2003; Dittman, 2019). A reduction in the activity of *Dunc13* lowers the synaptic transmission efficacy (Aravamudan & Broadie, 2003). By reducing Dunc13 activity in the ellipsoid body neurons, the synaptic vesicle fusion and neurotransmitter release should be hindered, causing an interruption in the activity of the ellipsoid body and thus the central complex. The result of this hindrance is expected to be a decrease in the ellipsoid body's function, a reduction in the locomotor and exploratory behaviors of the fly (Martin et al., 1999; Strauss et al., 1992; Strauss & Heisenberg, 1993). On the contrary, reducing the activity of Dunc13 in the mushroom body should primarily impact memory and spatial learning. The mushroom body, a distinct brain structure and another integrative brain center, has a necessary role in associative learning of olfactory information, short-term memory and spatial learning (Besson & Martin, 2005; Joiner et al., 2006; Martín-Peña et al., 2014; McGuire et al., 2001; Zars, 2000). In this experiment, we did not observe a significant effect in the expression of *Dunc13*RNAi transgene in the ellipsoid, mushroom bodies and throughout all neurons in the locomotor and exploratory behavior of the fly. This may be because the *Dunc13*RNAi simply did not function at all or the expression of the transgene was insufficient to cause an impact in the neural activity sufficiently disrupting the behavior of the fly on the open field arena.

Future study

A major question that remains to be explored pertains to the outcome in the locomotor and exploratory behavior of the fly when *Dunc13* activity is completely reduced in the ellipsoid body. Further, it would be of interest to examine flies with a complete reduction in *Dunc13* activity in the mushroom body and assess their locomotor behavior in an open field arena to see how much the mushroom body is associated with the fly's walking behavior. We know that reducing *Dunc13* activity mimics the initial effects of intoxicating concentrations of alcohol (Xu et al., 2018). However, by completely reducing *Dunc13* activity in the ellipsoid and mushroom bodies, will the fly become more tolerant and resistant to alcohol? A question remains to be answered. Finally, it would be of interest to establish alternative methods for reducing *Dunc13* activity and to verify that the reduction occurred.

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