Serotonergic Modulation of Novelty Habituation During Exploration in *Drosophila*

A Dissertation Presented to

the Faculty of the Department of Biology & Biochemistry

University of Houston

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

By

Miguel Angel de la Flor, Jr

August 2019

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For my parents Cleo and Miguel

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I am indebted to my parents Cleo and Miguel for always encouraging my curiosity in the natural world from a young age, for supporting my desire to explore and for instilling in me a deep appreciation of education.

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Abstract

In a natural environment, animals must attend to and process countless streams of stimuli. Novel stimuli, or a change in the environment, may signal an opportunity for food, mates or indicate danger. A novel stimulus may elicit an approach response motivating an animal to inspect and learn about the new environmental feature. Locomotor exploration allows an animal to gain information about the features in the environment. However, animals can attend to only one stimulus at a time. Once the aim of exploring has been achieved, the exploratory behaviors triggered by novelty should cease to allow the animal to attend to other tasks. Novelty habituation is the process whereby an animal gradually decreases behaviors elicited by novelty, as the unfamiliar transitions to familiar. Herein, this study demonstrates that the decrease in locomotor activity *Drosophila* display in the open-filed arena is habituation to the novelty presented by the arena. In addition, experiments presented here show that serotonin signaling modulates locomotor activity, that the 5-HT1A receptor may be required in α/β and γ neurons of the mushroom bodies for locomotor modulation and that activation of the Dorsal Paired Medial neurons and possibly the Posterior Lateral Protocerebrum neurons is sufficient to decrease locomotor activity in the open-field arena. These data suggest a putative serotonergic circuit that modulates locomotor exploration in response to plasticity in the mushroom bodies as novelty transitions to familiarity.

Table of Contents

List of Figures

List of Tables

Chapter 1: Introduction

After watching *Drosophila melanogaster*, the common fruit fly, for a few moments, it becomes apparent that flies display a collection of interesting behaviors. If observed long enough, there is little doubt that flies are making decisions about what to do, and when and where to do it. Somehow, with a brain of roughly 150,000 neurons flies detect and process a battery of incoming sensory-rich information about their environment and execute the appropriate behaviors based on their biological needs. How do flies “know” what to attend to in an environment filled with competing sensory signals? This is an important question not just for flies, but for all animals, including humans.

Habituation is an active learning process that filters out inconsequential, repeated stimuli freeing up precious neural resources to attend to tasks that are more important. However, habituation is not just a filter, it is a dynamic, active learning process that allows an animal to transition from attending to one task to another. In other words, once the aim of a task has been reached, habituation is part of the mechanism that promotes the reduction of the behaviors involved the first task and allows the transition to another. Herein, novelty habituation is the process whereby a novel stimulus transitions to familiarity after exploration and learning. At a behavioral level, the focus of this study is the attenuation of locomotor exploration as a component of novelty habituation, and at a neural circuit level, the focus is the role of serotonin (5-HT) in the attenuation of locomotor exploration.

1.1 **Learning and Memory**

Flies can learn. Flies form, consolidate and retrieve memories and modify their behavior based on experience. For decades, the focus of learning and memory research in flies has been on associative learning models, specifically olfactory associative learning (Roman & Davis, 2001; Waddell & Quinn, 2001). Flies are an excellent model for this type of learning as much of their central nervous system is dedicated to the processing of olfactory cues.

Associative and non-associative learning can be divided into classical (Pavlovian) conditioning and operant (instrumental) learning. Classical conditioning is the temporal pairing of a conditioned (neutral) stimulus to an unconditioned stimulus (e.g., sight of food). The unconditioned stimulus in this case would produce reflexive salivation, the unconditioned response. After a period of training, the conditioned stimulus alone elicits the unconditioned response (Pearce & Bouton, 2001).Operant conditioning, on the other hand, is a process where learning is achieved through the presentation of a reward or punishment stimulus based on the organism's actions. Over time, the organism associates a behavior with an outcome (Pearce & Bouton, 2001).

Non-associative learning is the modification of a behavioral response to the presentation of a single repeated stimulus (Rankin et al., 2009; Thompson & Spencer, 1966). One type of non-associative learning is habituation. In this type of learning, the behavioral response to the repeated presentation of a stimulus decreases. Sensitization occurs when the behavioral response to a repeated

stimulus increases. There is debate as to whether habituation and sensitization are strictly non-associative modes of learning. For instance, a study in *C. elagans* showed that habituation to a tap stimulus was facilitated if training and testing occurred in the same chemosensory context However, another study in mice reported that habituation of the acoustic-startle response is not context specific (Pilz, Arnold, Rischawy, & Plappert, 2014). Both associative and non-associative learning share much of the same molecular machinery, it may be the case that rather than being discrete forms of learning, associative learning are part of a spectrum.

The memories formed through associative and non-associative learning can be separated into short-term memory (STM) and long-term memory (LTM). STM is a transient form of memory dependent on cAMP/PKA signaling modulated by monoamines like serotonin (5-HT), but not dependent on protein synthesis. LTM is also cAMP/PKA signaling dependent, but involves CREB-mediated protein synthesis and remodeling of synapses (Kandel, Dudai, & Mayford, 2014; Margulies, Tully, & Dubnau, 2005). In associative learning, there are intermediate forms of memory, middle-term memory (MTM) and anesthesia-resistant memory (ARM). MTM has been observed in various model organisms, while ARM is a phenomenon described in flies dependent on the product of the *radish* gene (Margulies et al., 2005). These intermediate forms of learning have not been described in non-associative learning paradigms. The focus of this study is likely a short-term form of habituation involved in the behavioral modulation to novelty.

1.2 **Habituation, Novelty, Exploration and 5-HT Signaling**

Novelty habituation begins with the detection of a novel stimulus, which may elicit exploratory behavioral responses that allow organisms to learn about the novel feature, facilitating the transition from the unfamiliar to familiar. Familiarity in turn, may attenuate some of the behavioral responses initially triggered by novelty, like exploration. More clearly, once the aims of the behavioral responses to novelty have been attained, habituation allows organisms to end those behaviors and attend to others. In this section, the behavioral components of the novelty habituation model proposed here are described.

As mentioned, habituation is a form of learning that results in the decrement of a behavioral response to a repeated, inconsequential stimulus (Rankin et al., 2009; Thompson & Spencer, 1966). Here a comparator (the brain of the organism) analyzes repeated sensory stimuli for relevance or importance, if the stimuli are not important then the behavioral responses elicited by the repeated stimulus are decreased or habituated (Figure 1.1).

While habituation may share certain mechanistic features with other forms of learning, it has distinct behavioral characteristics that distinguish it from decrement responses like sensory adaptation or motor fatigue (Groves & Thompson, 1970; Rankin et al., 2009; Thompson & Spencer, 1966). See table in the appendix for a complete list of all habituation characteristics. A key characteristic of habituation is dishabituation, the recovery of the habituated

response when another stimulus is introduced (Rankin et al., 2009). Dishabituation is an important characteristic because it eliminates sensory adaptation and motor fatigue as a causal factor in the behavioral decrease as the organism readily recovers the habituated response. For this reason, dishabituation is often used to detect habituation within behavioral paradigms. In this work, dishabituation was detected in the open-field assay while *Drosophila* (flies) explore, a topic which will be discussed in more detail in a subsequent section.

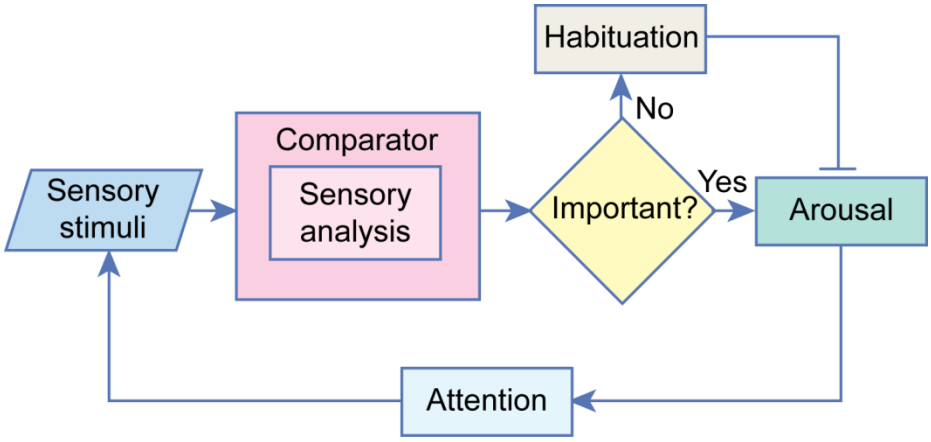

Figure 1. 1 **Habituation**. In this habituation model, repeated sensory stimuli are actively analyzed for importance by a comparator (the brain in an organism). If the stimuli are not consequential, behavioral responses to the stimulus decrease over time (Flowchart after Sokolov, 1963).

While there is not a list of characteristics for novelty, there are behavioral criteria that indicate novelty. At its most basic novelty is a mismatch between an animal's internal representations of its environment based on recent sensory inputs and new sensory inputs, in other words, a change detected in the environment. However, the mismatch must exceed a threshold to trigger a behavioral response that may motivate approach behaviors, but not exceed the

threshold such that it elicits a startle response and prompt avoidance (Figure 1.2) (Gati & Ben-Shakhar, 1990; Snyder, Blank, & Marsolek, 2008; Sokolov, 1963).

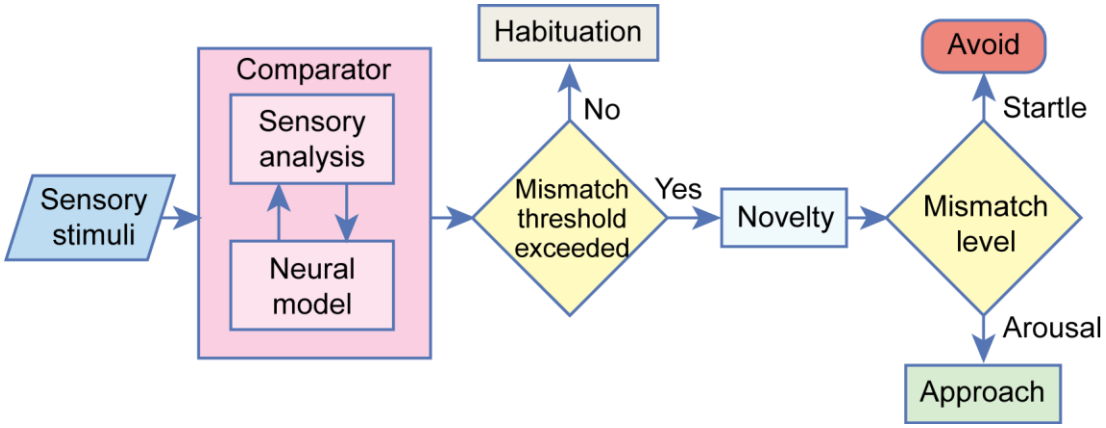

Figure 1. 2 **Novelty.** Novelty is a mismatch between an existing neural model of the environment and newly incoming sensory stimuli, in other words, a detected change in the environment. However, the mismatch must exceed a threshold before novelty is detected, otherwise all mismatches would trigger behavioral responses. Moreover, large mismatch levels may elicit a startle response and avoidance, whereas a smaller mismatch level may promote arousal and approach. (Flowchart after Sokolov, 1963, Gati, 1990 and Snyder, 2008).

The detection of novelty may trigger arousal and increased attention, which may prompt an orienting response to focus attention. Novelty may also produce an intrinsic anxiety-like state due to a lack of information about the novel stimulus and motivate *specific* exploration (Figure 1.3) (Berlyne, 1966; van Swinderen & Andretic, 2003). Specific exploration may be accomplished through locomotor exploration where an organism ambulates to inspect and learn about the novel features of its environment (Berlyne, 1966). Over time however, locomotor exploratory behaviors may wane as familiarity increases. This decrease in exploratory behaviors may suggest habituation to novelty. How the

transition from novelty to familiarity attenuates behavioral responses to novelty is not well understood.

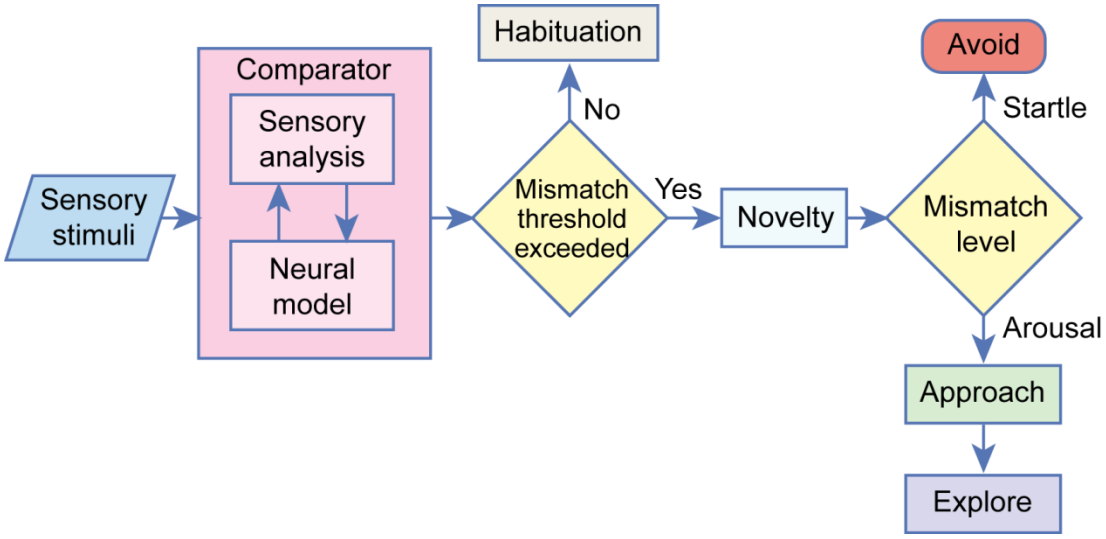

Figure 1. 3 **Specific exploration.** Specific exploration is motivated by a lack of information caused by novelty. Specific exploration may be undertaken through locomotor activity, ambulating to investigate and learn about the novel features of the environment (Flowchart after Sokolov, 1963, Berlyne, 1966, Gati, 1990 and Snyder, 2008).

In flies, dopamine (DA) signaling has been implicated in arousal (Andretic, van Swinderen, & Greenspan, 2005; Kume, 2007; Q. Liu, Liu, Kodama, Driscoll, & Wu, 2012). The work on serotonin (5-HT) signaling in flies is sparse compared to DA. Nonetheless, a comprehensive study of 5-HT signaling in flies showed that 5-HT signaling suppresses general activity, feeding and courtship (Pooryasin & Fiala, 2015). Pooryasin proposed a general, bimodal modulatory model in flies in which the DA system initiates behaviors and the 5-HT system induces behavioral quiescence. However, the Pooryasin study does not rule out the effects of other neuromodulators nor does it rule out other roles for DA and 5-HT. In addition to the Pooryasin work, 5-HT signaling has been shown to modulate

sleep, locomotion, aggression, learning and memory, place memory among other physiological processes and behaviors in flies (Dierick & Greenspan, 2007; Keene, Krashes, Leung, Bernard, & Waddell, 2006; Majeed et al., 2016; Sitaraman et al., 2008; Yuan, Joiner, & Sehgal, 2006).

Based on the literature, a central hypothesis in the work presented here was that at the neural circuit level 5-HT signaling may suppress locomotor exploration as flies habituate to novelty (Figure 1.4). In addition, because the mushroom bodies (MB) in the fly brain have been implicated in sensory processing, learning and memory, the processing of novelty to familiarity and the regulation of behaviors like locomotion and different forms of habituation, it was further hypothesized that 5-HT signaling to the mushroom bodies may play a key role in components of novelty habituation while flies explore (Figure 1.4) (Acevedo, Froudarakis, Kanellopoulos, & Skoulakis, 2007; J. R. Martin, Ernst, & Heisenberg, 1998; Pitman et al., 2011; Silva, Goles, Varas, & Campusano, 2014; Sun et al., 2018).

Under the behavioral models of habituation and novelty presented here, novelty habituation may be generally defined as the decrease in the behavioral responses elicited by novelty as a result of the transition of the novel stimulus from the unfamiliar to the familiar. This definition is congruent with fly behaviors observed in the open-field arena. Flies introduced to a novel open-filed arena are motivated to explore by the novelty the arena presents. The exploratory

behaviors initially triggered by novelty decrease once novelty is satiated through exploration.

Based on the data presented in chapter 3, it is proposed that feedback signaling from the mushroom bodies (MB) to the Dorsal Paired Medial (DPM) neurons in response to novelty satiety, activates serotonergic (5-HT) signaling from the DPM neurons to subsets of MB neurons. This 5-HT signaling activates the 5-HT1A receptor which initiates an inhibitory signaling cascade which may quiesce certain behaviors, like locomotor exploration.

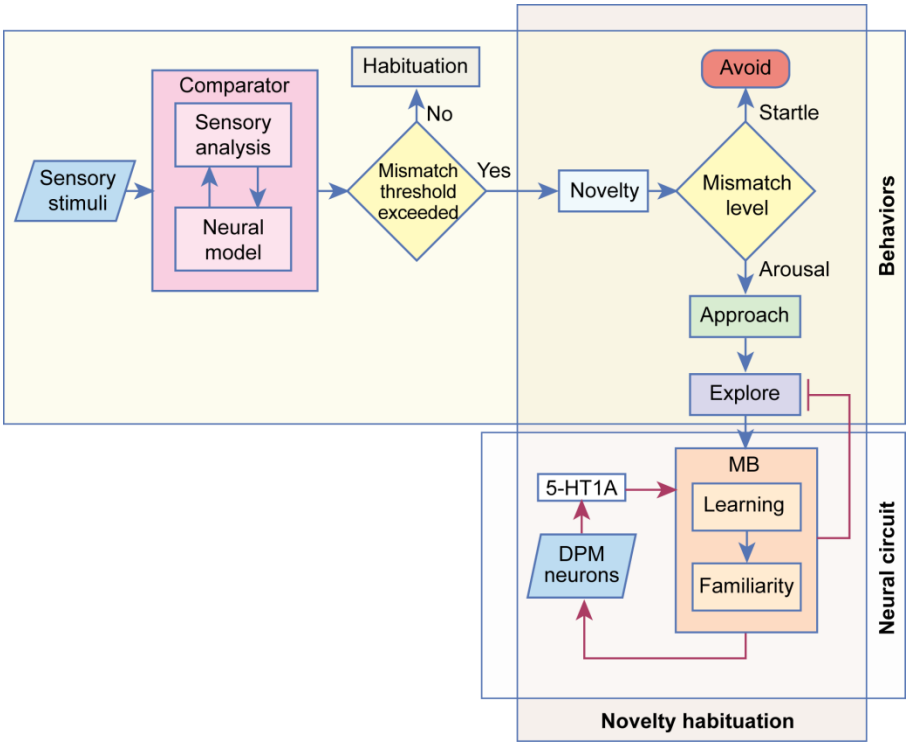

Figure 1. 4 **Model of novelty habituation.** Habituation is a dynamic process that allows organisms to transition from attending to one task to another. Once the aim of a particular set of behaviors is accomplished, habituation is part of the mechanisms that stops the actions involved in reaching that aim. This allows organisms to move from one behavior to another. Here it is proposed that 5-HT plays a role in suppressing locomotor exploration activity once flies have satiated the novelty.

Habituation has been the focus of intense research for many decades because it was realized early that habituation is a necessary first step to downstream cognitive processes (Stein, 1966). In fact, many human mental disorders like Autism spectrum disorders and attention deficit disorders exhibit habituation defects (McDiarmid, Bernardos, & Rankin, 2017; Schmid, Wilson, & Rankin, 2014). Habituation has too often been labeled a simple, non-associative form learning and memory, when in fact habituation is a dynamic form of neural plasticity sharing the same molecular machinery as other forms of learning, featuring associative learning components and is involved in the regulation of behaviors at different levels (Ardiel, Yu, Giles, & Rankin, 2017; Rankin, 2000; Rankin et al., 2009; Schmid et al., 2014). It is within these contexts that novelty habituation is presented as the decrease of behaviors elicited to investigate the novel object or feature due to increasing familiarity. The neural substrates that signal or represent satiety to novelty, for example the MBs in flies, may drive the decrease in investigatory behaviors (e.g., arousal, orienting, exploration) through inhibitory feedback loops (Figure 1.4). This contrasts with habituation of a startle response or proboscis extension response for example, where repeated exposure to a visual or chemical stimulus attenuates a reflex response (Engel & Wu, 2009). In novelty habituation, it is the transition of the unfamiliar to the familiar, a learning process that involves multiple sensory modalities, investigatory behaviors, and associative learning like place learning, that drives a behavioral decrease.

1.3 **Drosophila as a Model Organism**

Drosophila melanogaster has been used as model organism in biology for over 100 years going back to Thomas Hunt Morgan's experiments with the *white* gene. Since then flies have been used extensively to investigate many basic biological processes like genetics, development, behavior, and neurobiology (Jennings, 2011; Wangler, Yamamoto, & Bellen, 2015). In recent years, flies have also become a common model for human diseases from cancer to metabolic disorders and neurodegenerative diseases (Wangler et al., 2015). Flies are ubiquitous in basic science research and biomedical research because despite their humble stature and small brain size, about 40% of fly genes have mammalian homologs. In highly conserved genes, the similarity can be up to 90%. Moreover, flies, display a rich-repertoire of complex behaviors providing the perfect platform in which to dissect the genetic basis of behaviors (Pandey & Nichols, 2011). A prominent genetic tool for these studies is the Gal4/UAS system. This system is central to many of the experiments in this work.

1.3.1 The Gal4/UAS System

The *Drosophila* Gal4/UAS expression system has made it possible to visualize and manipulate neurons with high levels of temporal and spatial control. Briefly, in a specific fly line, the yeast GAL4 transcription factor is expressed under the control of an endogenous fly enhancer or promoter element, limiting expression of the GAL4 to a specific group of cells. In a separate fly line the upstream activation sequence (UAS) is expressed upstream of a gene of

interest. When the lines are crossed, the progeny express both transgenes, the GAL4 binds the UAS element recruits RNA polymerase and the gene of interest is expressed only in the cells where GAL4 is expressed (Brand & Perrimon, 1993) (Figure 1.5).

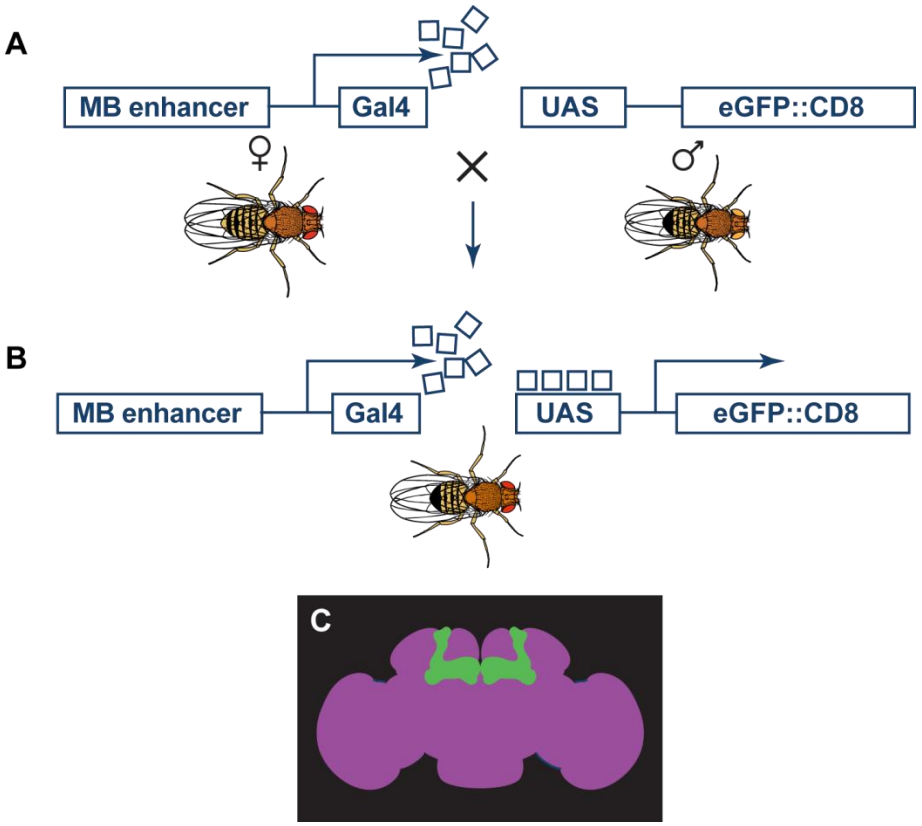

***Figure 1. 5* Gal4/UAS system.** The Gal4/UAS system allows the expression of genes in a tissue specific manner and temporal control. (A) A fly line expressing the Gal4 under control of an enhancer for a specific fly tissue, mushroom bodies in this case, is crossed with a fly line expressing the UAS upstream of a reporter. (B) In the progeny, the Gal4 transcription factor, binds the UAS and promotes transcription of the reporter gene. (C) The mushroom bodies are labeled.

In this study, the GAL4/UAS system is used in three main ways. First, to visualize neurons, a GAL4 line that targets the neurons of interest are crossed to a *UAS-mCD8::eGFP* (T. Lee, Lee, & Luo, 1999) reporter that labels the neuron soma and neurites. Second, to dissect neural circuits (circuit breaking), specific

neurons or populations of neurons are activated or inhibited by driving expression of a neural effector. For example, expression of the temperature-sensitive cation channel *UAS-TrpA1* (Hamada et al., 2008) depolarizes neurons at the permissive temperature. This method permits loss of function or gain of function experiments to determine sufficiency and necessity for the neurons targeted by the Gal4, in a particular behavior. Finally, the Gal4/UAS system is used here to express the 5-HT1A receptor in the mushroom bodies and ellipsoid body in genetic rescue and overexpression experiments.

1.3.2 Anatomical Nomenclature

The fly central brain is a bilateral structure composed of approximately 150,000 neurons organized into about 33 distinct neuropil structures contained in three main regions; the protocerebrum, which contains the optic lobes, mushroom bodies, central complex and other neuropil, the deutocerebrum, made up mainly of the antenna lobes, and the tritocerebrum composed of the suboesophageal structures (Ito et al., 2014; Yu et al., 2013). The neuropil in these regions are described by their anatomical locations and relationships to other structures. About 90 5-HT secreting neurons have been identified in the fly brain (Pooryasin & Fiala, 2015). 5-HT secreting neurons, and other neuron types, are named by their anatomical locations or relationships. For example, the somas of the PLP neurons are located in the posterior lateral protocerebrum (Figure 1.6). The name APL, for the anterior paired lateral neurons, describes their number and location. Though efforts are underway to standardize the

anatomical nomenclature of the insect brain, there remain discrepancies. The nomenclature used in the neuron screen in Section 3.2.6 of this report is shown in Table 1, after Pooryasin 2015.

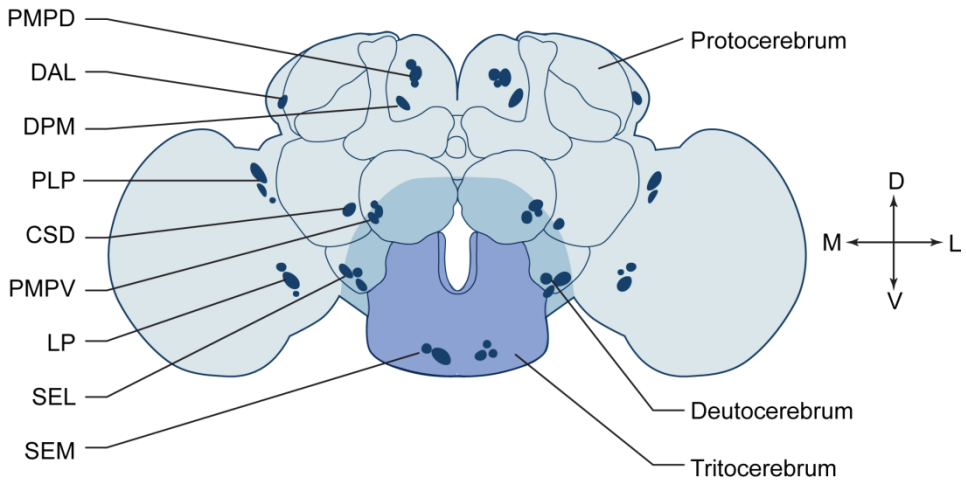

Figure 1. 6 **Anatomical nomenclature and location of neurons.** The insect brain is divided into three regions, the protocerebrum, deuterocerebrum, and the tritocerebrum. The somas of the neurons assayed in this study are named after their anatomical and locations and relationships. The schematic shows a collapsed AP plane as some neurons are in the anterior or posterior of the fly brain.

Table 1. 1 Neuron name and location

Neuron cluster/name	Brain region/location
PMPD	Posterior medial protocerebrum, dorsal
PMPV	Posterior medial protocerebrum, ventral
PLP	Posterior lateral protocerebrum
LP	Lateral protocerebrum
DAL	Dorsal anterior lateral (protocerebrum)
CSD	Contralaterally projecting, serotonin-immunoreactive deutocerebral (protocerebrum)
DPM	Dorsal paired medial (protocerebrum)
SEL	Subesophageal ganglion, lateral
SEM	Subesophageal ganglion, medial

1.3.3 Mushroom Bodies

The mushroom bodies (MB) are prominent paired bilateral neuropil in the central brain. Each MB is composed of about 2000-2500 intrinsic neurons called Kenyon cells (KC). The somas of the KC are grouped in the dorsolateral, posterior protocerebrum of the fly brain. The KC dendrites form the calyx of each MB and are the main region of inputs to these neurons (Ito et al., 2014; Tanaka, Tanimoto, & Ito, 2008). Each MB calyx is composed of a main calyx, dorsal and ventral accessory calices. The axons of the KC project anteriorly forming the MB peduncles as they course forward and at the anterior protocerebrum terminate to form the MB lobes (Aso et al., 2009) (Figure 1.7A and C). The lobes of the MBs are organized into functional regions composed of the axons of the KC. The γ lobe is made of KC that medially send axons. The α/β and α'/β' lobes are comprised of KC axons with medial and dorsal bifurcations (Figure 1.7C). The lobes may be further subdivided into distinct populations of neurons; α/β surface, core and posterior and α'/β' anterior, middle, posterior (Figure 1.7D) (Tanaka et al., 2008). In addition to the KC, the dorsal paired medial neurons (DPM) are a focus of this study. The DMP neurons, considered MB intrinsic neurons, heavily innervate the MB lobes and anterior peduncles, and are involved in the modulation of many behaviors (Haynes, Christmann, & Griffith, 2015; Tanaka et al., 2008; Waddell, Armstrong, Kitamoto, Kaiser, & Quinn, 2000; Wu, Fu, Chou, & Yeh, 2015).

The fly MBs have been a focus of research for decades because these interesting structures are involved in the processing and regulation of many physiological functions and behaviors. The MBs process multi-modal sensory information, from gustatory to visual and mechanosensory inputs, but most prominent is the processing of olfactory information from the antenna lobes (Vogt et al., 2014). The MBs also regulate behaviors such as feeding, sleep and locomotion (Joiner, Crocker, White, & Sehgal, 2006; Mabuchi et al., 2016; Tsao, Chen, Lin, Yang, & Lin, 2018). However, the MBs are best studied for their role in learning and memory, mainly in associative olfactory learning and memory, but the MBs are also required for visual learning and memory (R. L. Davis, 2011; Roman & Davis, 2001; Vogt et al., 2014). Interestingly, the MBs are also required for habituation and are involved in the processing of novel stimuli (Acevedo et al., 2007; Hattori et al., 2017).

The fly MBs play an important role in this study, because they are involved in key behaviors central to novelty habituation. At the behavioral level, they are involved in learning and memory, processing novel stimuli and regulating locomotion. In addition, at the cell and molecular level the KC of MBs express the 5-HT1A receptor and form circuits with key 5-HT secreting neurons like the DPM neurons (Tanaka et al., 2008). Given that 5-HT signaling is a known modulator of habituation and activity, it was hypothesized that the 5-HT circuits that the MB forms may be involved in novelty habituation in flies.

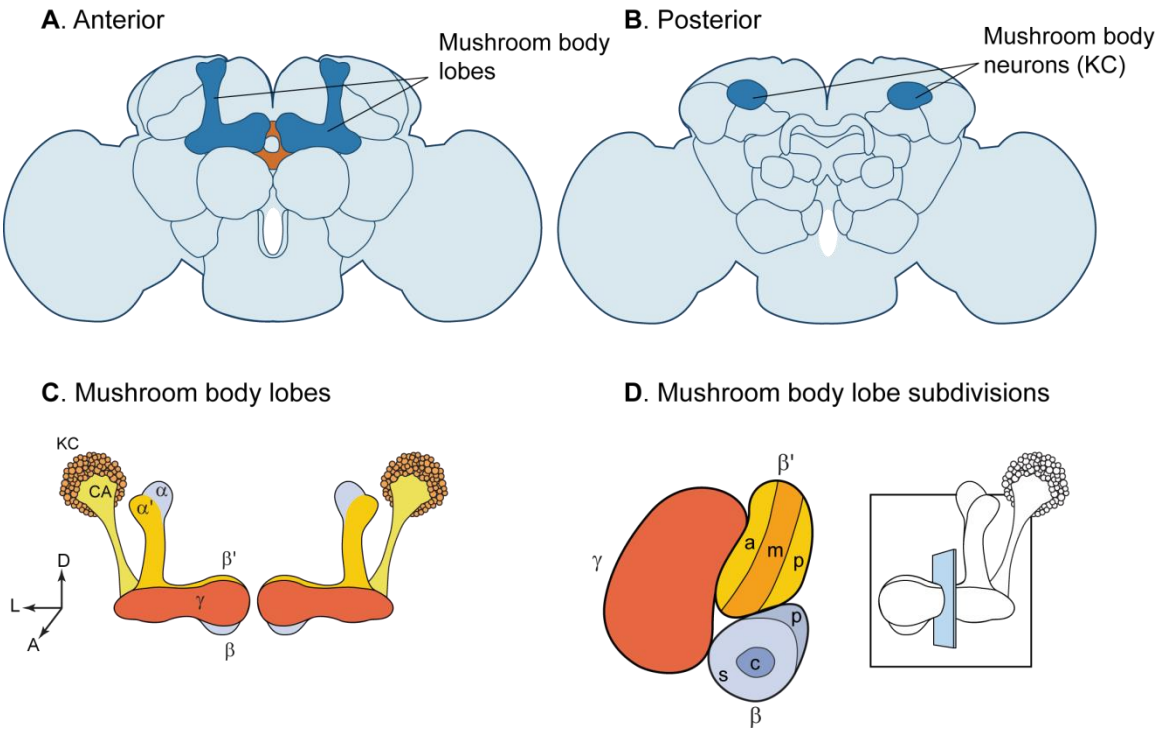

***Figure 1. 7* Anatomy of the mushroom bodies.** (A) The mushroom body (MB) lobes are the forward axonal projections of the Kenyon Cells (KC). (B) The KC somas are situated in the posterior dorsal region of the protocerebrum. (C) The MB lobes are made up of distinct groups of neurons, α/β, α'/β' and γ. (D)Each MB lobe is further subdivided into anatomical regions. The α/β is made up surface (s), core (c), and posterior (p) neurons and the α'/β' is made up of anterior (a), middle (m) and posterior (p) neurons (Based on Tanaka, 2008). Fasdfasd

1.3.4 Ellipsoid Body

The ellipsoid body (EB) of the central complex (CC) is an easily distinguished neuropil of the protocerebrum. The EB is intricately interconnected with the other structures of the CC like the fan shaped body and other brain regions (Hanesch, 1989) (Figure 1.8A). The curious anatomical shape of the EB comes from the concentric, laminar layering of R1 through R4 neuron axons forming connections in distinct rings of glomeruli (Figure 1.8C) (Martín-Peña et

al., 2014). The EB is reported to be involved in locomotion and flight coordination, visual place learning and visual cognition (Ilius, Wolf, & Heisenberg, 1994; Ofstad, Zuker, & Reiser, 2011; Pan et al., 2009).

The EB is of interest to this study because like the MBs, the EB regulates behaviors relevant to novelty habituation. Notably, the EB is involved in visual place learning, suggesting a role for the EB in the processing of spatial information and the formation of place memories, which may contribute to the satiety of novelty. A model of the interactions between the MB and EB posits that the MBs regulate locomotion and the CC (of which the EB is a part) initiates and coordinates locomotion (J. R. Martin et al., 1998; Strauss, 2002). Specifically, the R1-R4 neurons of the EB have been shown to be necessary for locomotion (Martín-Peña et al., 2014). At the cell and molecular level, the work reported here and other studies show that the EB expresses the 5-HT1A receptor, implicating 5-HT signaling in the modulation of these behaviors (Alekseyenko & Kravitz, 2014).

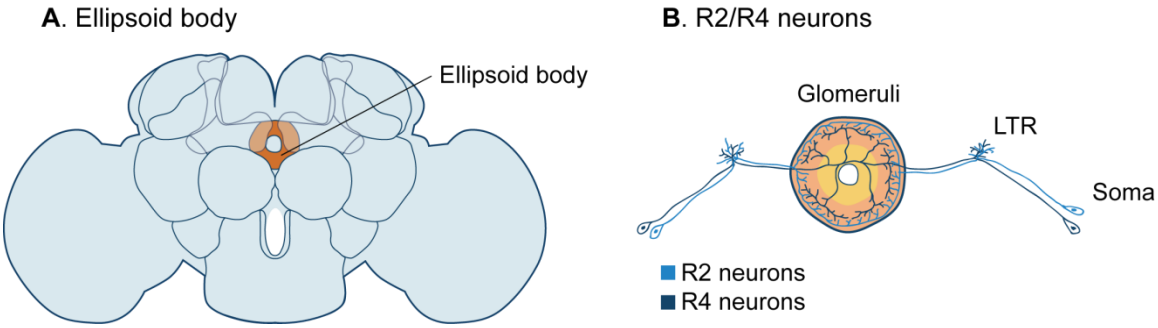

Figure 1. 8 **Anatomy of the ellipsoid body.** (A) The ellipsoid body EB is located in the central complex in the protocerebrum. (B) Simplified schematic of the EB. The shape of the EB comes from the concentric, laminar glomeruli of the EB neuron axons. The lateral triangle (LTR) are the dendrites of the EB neurons and main area of inputs. (based on Xie, 2017).

1.4 **Summary and Aims**

Flies (and humans) can attend to only one stimulus at a time. In a natural environment, flies are exposed to a continuous stream of competing stimuli. Some environmental stimuli may arise from novel objects or features of the environment. These novel features may elicit a behavioral response, such as arousal and orienting, followed by approach and locomotor exploration. However, once the fly becomes familiar with the formerly novel object or feature, the series of behaviors initiated by novelty must stop so that the fly can attend to other tasks. The internal states signaling satiety to novelty has to be integrated and processed such that the behavioral output can be changed. In the assay used here, attenuation of locomotor exploration occurs once learning has taken place. When flies are introduced to a novel open-field arena they display increased levels of locomotor activity indicative of locomotor exploration that decreases over time.

In flies, the mushroom bodies (MB) are higher order processing structures that are known to integrate internal states, sensory stimuli, and experience to regulate behavioral outputs. Moreover, the MBs are required for some forms of habituation and are involved in the neural processing underlying the transition from novelty to familiarity. Serotonin (5-HT) signaling has been shown to modulate many behaviors and in flies is reported to induce behavioral quiescence. Further, the MBs express 5-HT receptors and form synapses with 5-HT secreting neurons. These findings suggest a system where the MBs may play

a role in the habituation to novelty, possibly by integrating internal and external cues and by promoting the execution of several behavioral outputs. For instance, through a known feedback loop the MBs interact with the dorsal paired medial neurons (DPM) which in turn modulate MB outputs through 5-HT signaling. The aim of this work was to apply pharmacological, transgenic and behavioral approaches to investigate the characteristics of habituation in the open-field arena, characterize the role of 5-HT signaling in novelty habituation while flies explore, and identify and describe 5-HT circuits involved in modulation of novelty habituation.

The aims of this work are:

1. To establish that the decrease in locomotor activity observed in the open-field arena while flies explore is habituation using the known characteristics of habituation.
2. To characterize the modulatory role of 5-HT signaling while flies explore a novel open-filed arena using pharmacological approaches.
3. To identify the 5-HT secreting neurons involved in the modulation of novelty habituation using circuit-breaking techniques.
4. To investigate the role of the mushroom bodies in novelty habituation.

Chapter 2: Materials and Methods

2.1 **Animal Stocks**

All *Drosophila melanogaster* (fly) lines were housed and reared at room temperature 22 °C or at 18 °C and fed a standard yeast-cornmeal agar food. Flies used in behavioral assays were raised on standard food at 25 °C, 60% relative humidity in 12 h light/dark cycles. Crosses with the temperature sensitive cation channel *UAS-TrpA1* were raised at room temperature ~22 °C. Crosses with the red-shifted light activated cation channel *UAS-CsChR* were raised in dark conditions after third instar larval stage to reduce effects of *UAS-CsChR* low levels of activity in white light. All transgenic and mutant lines were outcrossed into the Roman Canton-S (CS) genetic background for six generations and balanced. Most stocks were obtained from the Bloomington Drosophila Stock Center (BDSC, Bloomington, Indiana). Others were generous gifts as listed in Table 2.1. Male flies to be used in behavioral experiments were sorted and collected at 1-3 days old (except where noted in results) under brief CO_2 anesthesia and placed in fresh food vials and allowed to recover from anesthesia for 24-36 hours prior to experimentation. Flies used in behavioral experiments were between 3-7 days old.

Predators were raised at room temperature in individual culture containers and fed flies for several weeks. A week prior to use in experiments predators and non-predator controls were habituated to being handled and to the cage in the open-field arena by being placed in the cage for about four h for five days to

reduce attempts to escape. Predators were starved for one day prior to experiments.

Table 2. 1 Animal Stocks

Name	Description	Source
Mutants		
w^+; 5-HT1A^{MB09978}; +	5-HT1A receptor mutant	BDSC 27820
w^+; 5-HT1A ^{MB09812}; +	5-HT1A receptor mutant	BDSC 27807
w^+; 5-HT1B^{MB09812}; +	5-HT1B receptor mutant	BDSC 24240
w^+; +: orco^2	Anosmic mutant	Gift from Leslie Vosshall
w^+, norpA^7; +; +	Blind mutant	BDSC 5685
w^{1118}	ABC-like transporter mutant	NA
Gal4 drivers		
w^+; +; Trh-D2	Tryptophan hydroxylase	Gift from Dr. Dierick
w^+; +; Trh-Kartic	Tryptophan hydroxylase	Gift from Dr. Dierick
w^-; +; R67B05	All & LP	BDSC 46576
w^-; +; R7011A	PMPV	BDSC 46630
w^-; +; R65DO3	SEL	BDSC 48233
w^-; +; R23E12	PMPD, LP	BDSC 49034
w^-; +; R35C08	SEL	BDSC 49900
w^-; +; VT64246	DPM	VDRC 204311
w^+; NP2721; +	DPM	Hayes 2015
w^+; C316; +	DPM	BDSC 30830
w^+; +; P247	MB	BDSC 50742
w^+; C739; +	MB	BDSC 7362
w^+; 238Y; +	MB	BDSC 81009
w^+; +; C819	EB R2/R4M	BDSC 30849
w^+; +; 5.30	EB R2/R4M	NA
w^+; +; R37DO4, R51B02	CSD (MB465C)	Roy 2007
w^-, G0338; +; +	DAL	Chen 2012
w^-; +; G0431	DAL	Chen 2012
UAS responders		
w^+; +; UAS-CsChR	Optogenetic activator	Klapoetke 2014
w^+; UAS-TrpA1; +	Thermogenetic activator	BDSC 26263
w^+; +; UAS-Kir 2.1	Constitutive inhibitor	BDSC 6595
w^+; UAS-TNT-LC; +	Constitutive inhibitor	BDSC 28838

Table 2.1 continued

w-; +; UAS-mCD8::eGFP	eGFP reporter	Lee, 2001
w^+; +; UAS-5HT1A	5-HT1A receptor cDNA	BDSC 27631
w^+; +; UAS-5HT1B	5-HT1B receptor cDNA	BDSC 27635
w^+; +; UAS-SERT	5-HT reuptake transporter	BDSC 24464
Predators and controls		
Pantropical jumping spider	*Plexippus paykulli*	Captured at UH
Twin-flagged jumping spider	*Anasaitis canosa*	Captured at UH
Texas unicorn mantis	*Phyllovates chlorophaena*	Gift from Dayne Jordan
Milkweed bug	*Oncopeltus fasciatus*	Carolina Biological 143810

2.2 **Pharmacology**

For experiments where 5-HT signaling was manipulated, flies were fed 2% sucrose water solution (vehicle) with or without drug treatment. The following drugs were used at the concentrations specified in the results section: Hydroxy-L-tryptophan (5-HTP) (H9772 Sigma, Santa Cruz, CA), Way100365 (15599 Cayman Chemical, Ann Arbor, MI), αMethyl-DL-tryptophan (αMTP) (M8377 Sigma, Santa Cruz, CA). Drugs were delivered in standard fly food vials with a half a Kimtech Kimwipe tissue (1/2 of 4.4 x 8.4" tissue) tamped down to the bottom. 1 ml of the proper solution is then added to the tissue. 1 to 3 day old flies were anesthetized with CO_2, about 15-20 collected, and placed in the prepared vials. Flies were incubated in the solution for 36-40 h. 1 hour prior to activity experiments, flies are transferred by careful flipping to either vials with fresh, standard food or clean, empty vials to allow them to clean off any residues from the solutions. For optogenetic experiments with *UAS-CsChR* flies were fed 100

μM of all-trans retinal (R2500-100MG Sigma, Santa Cruz, CA) in 50 ml of standard fly food (0.04 mM concentration) for 72 h in food vials covered in aluminum foil to reduce light exposure.

2.3 **Behavioral Assays**

2.3.1 Open-field Arenas

Clear Plexiglas arenas 8.2 cm in diameter by 0.7 cm high were used to assay locomotor activity (University of Houston Physics machine shop, Houston, TX). Arenas were covered with clear polystyrene plastic lids to prevent flies or other predators from escaping during experiments (Figure 1C).

An array of 16 identical behavioral set-ups was used to produce a high-throughput behavioral assay system. All behavioral set-ups were networked to a central server where raw X, Y tracking data for each fly from each behavioral set-up was collected, parsed and organized using proprietary Excel scripts prior to statistical analyses.

Arenas with predator cages were machined to hold centrally located predator cages fabricated from 5 cm diameter by 0.07 cm high circular Nylon Nitex mesh strips (FS57-103 Genesse Scientific, San Diego, CA). Arenas with predator cages and 1.2 cm by 1.5 cm alcoves were custom built by the University of Houston Physics machine shop

Automated arena shaking in habituation experiments was achieved by mounting behavioral setups on a standard 13 cm, 120 V, 75 W speaker secured

to a heavy base. A succession of five 0.2 second, 50 Hz tones were played at designated time intervals (see results section) under computer control to shake the arena in a controlled and repeatable manner.

2.3.2 Environmental Controls

Ambient lighting at each arena was maintained at approximately 900 lux by overlapping arrays of full-spectrum, compact white fluorescent lights (100W, 5000K) positioned about 1 meter above the arenas and by 3 white LED lights housed in wide-angle lens modules (MSS-4W50 Samsung) inside the arena box (Figure 1C). All experiments were carried out at 22-23°C in approximately 35-45% humidity. Arenas were surrounded on all sides by a 10 cm tall white Foam Core board box to prevent environmental cues from interfering with fly behavior.

2.3.3 Animal Tracking

Video capture of fly centroids was done with Logitech C920 HD Pro cameras or Panasonic WC-BP334 CCTV cameras with Navitar 7000 zoom lenses (described in Lui 2007) at 30 frames per s (Figure 2.1C). The primary software used to track X, Y coordinates and time stamps for each fly was BuriTrack (Colomb 2011). Ethovision XT 5.1 or 8.5 (Noldus, Leesburg, VA) software was also used. Flies were tracked for 10 min (or longer where noted in the results).

2.3.4 Optogenetics and Thermogenetics

Optogenetic experiments with the red-shifted Channelrhodopsin *UAS-CsChR* neural effector were carried out under the full-spectrum fluorescent lights as noted above and augmented with 625 nm wavelength red LED lights with wide-angle lenses (MSS-4R50 Samsung) placed 15 cm above the arena. Temperature control inside the open-field arena for thermogenetic experiments with the temperature sensitive cation channel *UAS-TrpA1* neural effector was achieved with 7 cm diameter 12V polyimide flexible heaters (Jaye Industries, Guandong, China) placed 3 cm under each arena. Dual stage 12V digital temperature controllers (Inkbird Technologies, Shenzhen, China) were used to monitor and maintain constant temperatures inside the arena (Figure 2.1 A-B).

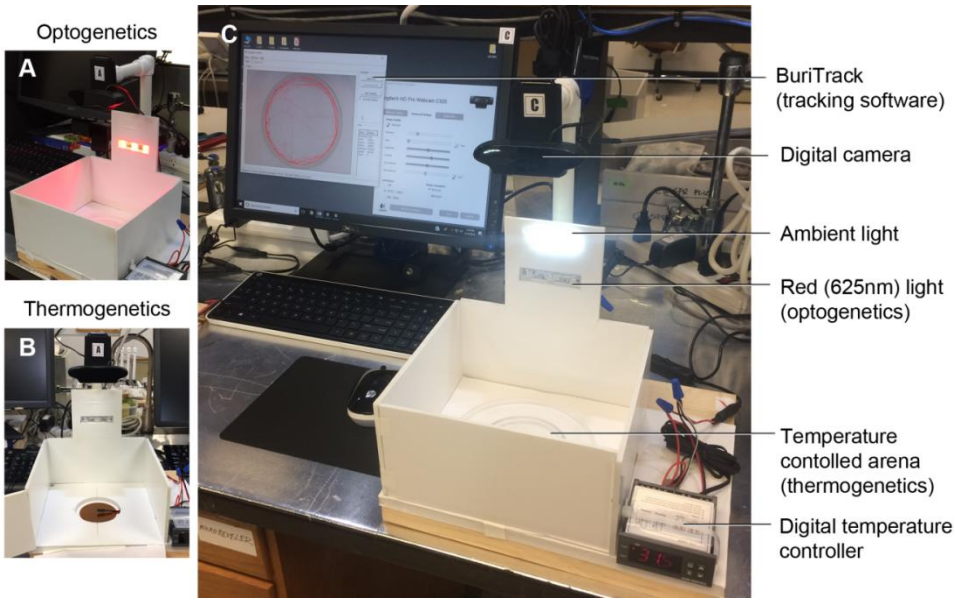

Figure 2. 1 **Behavioral assay.** An array of 16 custom-built behavioral set-ups was created to produce a high-throughput tracking system (one shown). (A) a 625 nm LED was used for optogenetic experiments. (B) Thermogenetic experiments were done with a heater managed by digital temperature controller. (C) Each behavioral set-up was enclosed to reduce environmental artifacts.

2.4 **Tissue Dissection and Immunohistochemistry**

2.4.1 Brain Dissection

Flies were anesthetized on ice prior to dissection. Whole brains were dissected in PBS and fixed in 4% paraformaldehyde for 30 minutes at room temperature. Brains were then washed in fresh PBS-T (PBS with 0.3% Triton-X) 3 times for 20 minutes each wash on a rocker at room temperature. After washing, brains were placed in blocking solution (PBS-T with 5% goat serum) for 1 hour at room temperature or overnight at 4 °C on a rocker. Prior to application of antibodies the brains were washed in PBS-T 3 times for 20 min each wash on a rocker at room temperature.

2.4.2 Double Staining Protocol and Confocal Microscopy

Primary antibodies used were Chicken pAb to GFP 1:200 dilution (13970 Abcam Cambridge, MA) and nc82 1:200 dilution (DSHB, Iowa City, IA). Dissected brains were incubated with primary antibodies diluted in blocking solution for two nights at 4 °C with gentle rocking. Prior to application of secondary antibodies, brains are washed in fresh PBS-T 3 times for 20 min each time on a rocker at room temperature. The secondary antibodies used were 1:500 dilution Alexa Fluor® 594 Donkey Anti-Mouse IgG Antibody (R37115, ThermoFischer Scientific, Waltham, MA) and 1:500 dilution Alexa Fluor® 488 Goat Anti-Chicken IgG (A-11039, ThermoFischer Scientific, Waltham, MA). Brains were incubated with secondary antibodies for two nights at 4 °C with gentle rocking. After staining with secondary antibodies, brains were washed in

fresh PBS-T 3 times for 20 min each time on a rocker at room temperature. Brains were then placed in Vectashield® Antifade Mounting Media (H-1000, Vector Labs, Burlingame, CA) and mounted on standard microscope slides. Confocal microscopy was carried out on a Leica SP8 microscope using LAS-X 2.0.0.14332 software for Leica DM6000B-CFS.

2.5 **Statistics**

Parametric data: Analysis of variance (ANOVA) was used to establish overall significance. One-factor or two-factor ANOVA was used where appropriate. Post hoc analysis for multiple comparisons was carried out with Tukey (HSD) and Bonferroni correction. Dunnett's test was used for comparisons to controls where appropriate. Student's T-tests were used for comparisons between two groups as required. P values < 0.05 were considered statistically significant. All statistical calculations were done using XLSTAT (Addinsoft, NY, NY) running on Microsoft Excel 2010. All ±error bars are standard error of the mean (SEM).

Non-parametric data: Comparisons between two groups were carried out using the Mann-Whitney two-tailed test. The Kruskal-Wallis test was used for comparisons of more than two groups, followed by Dunn's two-tailed test with Bonferonni corrections for multiple comparisons. These statistics were calculated using XLSTAT (Addinsoft, NY, NY).

Chapter 3: Results

3.1 **Habituation in the Open-field Assay**

3.1.1 Habituation to Novelty

Wild type CS flies introduced to a novel circular, open-field arena engage in a burst of locomotion in the initial activity phase, which gradually decreases over time through an intermediate activity phase concluding in a spontaneous activity phase with little or no locomotion (L. Liu, Davis, & Roman, 2007). In an open-field arena, flies will orient away from the arena center, quickly walk to the arena wall or boundary, and continue to walk proximally to the wall in a persistent direction, rarely deviating, stopping or reversing (Soibam, Goldfeder, et al., 2012). This locomotor activity is consistent with *specific exploration*, a class of exploratory behaviors that are motivated by an animal's drive to satiate a lack of information about a novel environment or object (Berlyne, 1966).

When exploring flies primarily employ vision, olfaction, and graviception to navigate, ambulate and learn about their environment (de la Flor et al., 2017; Gaudry, Nagel, & Wilson, 2012; L. Liu et al., 2007; Robie, Straw, & Dickinson, 2010). As flies visit discreet areas of a novel environment, they form place memories, reported to be processed by neurons of the ellipsoid body, a structure possibly analogous in function to that of vertebrate hippocampal place cells (Ofstad et al., 2011). However, over time locomotor exploratory behaviors progressively decrease. This may be explained by satiation of the initial drive to explore as flies become familiar with their environment. The gradual decrease in locomotor behaviors may represent habituation, a form of non-associative

learning, to the novelty presented by the arena. Habituation is the decrease in the behavioral response to a repeated stimulus (Rankin, 2009, characteristic 1), Nonetheless, changes in locomotor activity levels may be unrelated to habituation. To investigate if the decrease in locomotor activity may *be* habituation, experiments to elicit several of the known characteristics of habituation were performed.

Dishabituation, where the presentation of a different stimulus results in recovery of the habituated response, is an important characteristic of habituation (Rankin, 2009, characteristic 8). Demonstration of dishabituation in the open-field would support the hypothesis that the decrease in locomotor activity observed may be habituation. Wild type CS flies were introduced to the open-field arena and allowed to explore, by one to two min, a significant decrease in locomotor activity is observed (Figure 3.1A). At three min, a different stimulus is presented by mechanically shaking the arena using an automated system. (Figure 3.1A red asterisk at three min.). Flies display recovery of the habituated response to the arena's novelty by an increase in locomotor activity, but not to the level of the first-minute suggesting dishabituation, followed by a subsequent habituation bout in minutes four through six.

To eliminate a startle response or other confounding variables as a possible cause of the increased locomotor activity at four min, the arena is shaken again with the same intensity at six, nine and 12 min (Figure 3.1A red asterisks). This produced dishabituation/habituation responses that decreased

over time, culminating with no response at 13 min. The locomotor activity at one, four, 10 and 13 min are significantly different (Figure 3.1A, $p < .0001$). These data show that the habituation response to the arena's novelty may be partially recovered by introducing a different but salient stimulus, in other words, the habituation response is dishabituated.

Interestingly, the data also suggest habituation to dishabituation (Rankin, 2009, characteristic 9) where there is a decrease in the behavioral response to the dishabituation stimulus. The total distance means after each dishabituation response steadily decreases indicating habituation to dishabituation (Figure 3.1B, $p < .0001$). Together, these data indicate dishabituation and habituation to dishabituation in the open field assay, which are important characteristics of habituation.

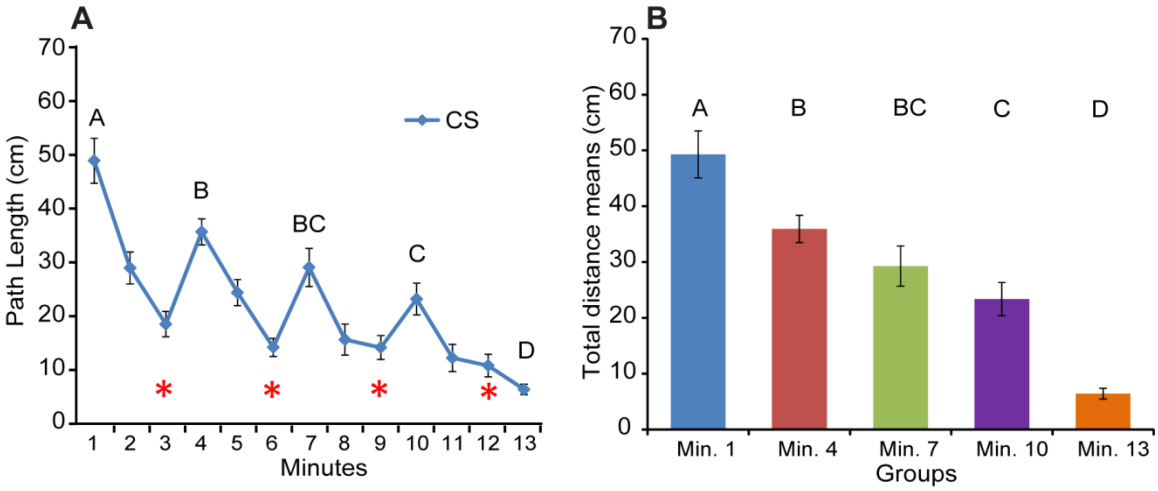

Figure 3. 1 **Flies display characteristics of habituation in the open-field assay.** (A-B) In the first-minute, flies display robust locomotor activity, which decreases rapidly by minute three suggesting habituation to the arena's novelty. Increased mean activity at four, seven, 10 and 13 min after arena shaking indicate dishabituation. Decreasing total distance means after subsequent shaking suggests habituation to dishabituation ($F_{4, 179} = 26.96$, $p<.0001$, N=36, error bars ±SEM).

Another important characteristic of habituation is that a strong stimulus may result in reduced or no habituation (Rankin, 2009, characteristic 5). Habituation is the decrease in the behavioral response to a repeated stimulus that *is not* important (Rankin, 2009, characteristic 1), allowing the organism to attend to other tasks. However, if the stimulus *is* important, for instance, the presence of a threat, that stimulus may yield no habituation.

To test for this characteristic in the open-field arena, CS flies were allowed to explore in the presence of or not in the presence of a caged, but visible, pantropical jumping spider (*Plexippus paykulli*), a natural predator that presents a threat to flies (described in de la Flor et al., 2017). In addition, flies were divided into a group fed 2% sucrose and water solution (vehicle) or a group fed vehicle plus 40 mM 5-hydroxytryptophan (5-HTP) for 36 h. 40 mM 5-HTP promotes serotonin (5-HT) signaling. Increased 5-HT signaling, in turn induces quiescence.

In the no spider condition, flies fed vehicle displayed typical locomotor exploration behaviors and habituation rates, but flies fed 40 mM 5-HTP displayed significantly decrease locomotor exploration (Figure 3.2A, $p < .001$). The decreased locomotor exploration may indicate increased habituation. In contrast, flies fed the same solutions, but in the presence of a spider showed no differences in locomotor exploration between treatments (Figure 3.2B $p > .05$). The increased habituation rate seen in Figure 3.2A produced by 5-HTP treatment is significantly reduced by the presence of a predatory threat.

First-minute distance means are significantly different between the vehicle or 5-HTP treatments in the no spider condition, but not in the presence of a spider (Figure 3.2C, $p < .001$ and NS, respectively). These same differences are observed in the total distances covered as well (Figure 3.2D, $p <. 001$ and NS). These data show that the presence of a predatory threat significantly increases locomotor exploration in the 5-HTP condition, eliminating the decreased locomotor exploration, indicative of faster habituation produced by 5-HTP in the no spider condition. These data suggest a reduction of habituation by a strong a stimulus, which is a characteristic of habituation.

Taken together, the results from these experiments demonstrate habituation, dishabituation, habituation to dishabituation and the reduction of habituation by a strong stimulus in the open field assay. Thus, the decrease in locomotor activity observed in the open-field assay is likely habituation.

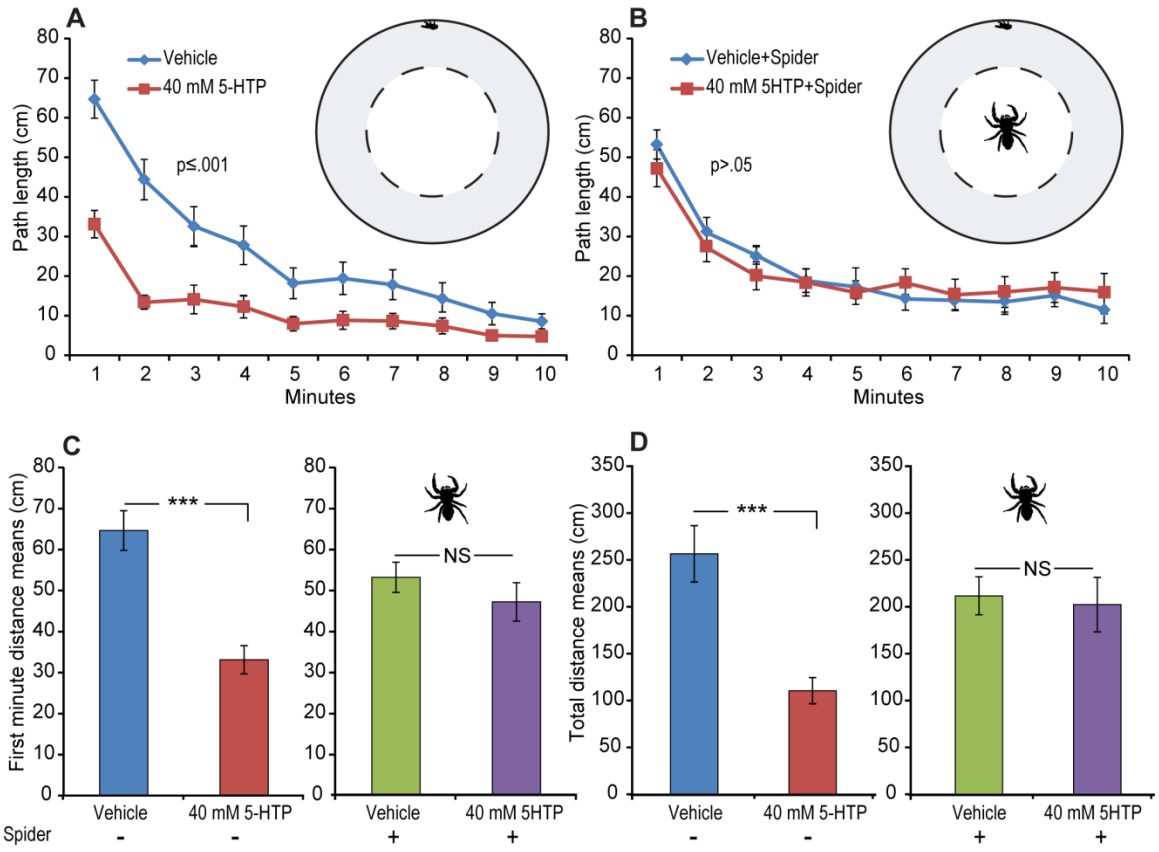

Figure 3. 2 **The presence of a predator reduces habituation.** (A) In the no spider condition, flies fed vehicle display normal exploration and habituation rates. Flies fed 40 mM 5-HTP show a significant decrease in exploration and increase in habituation. (B) The presence of a spider abolishes the increased habituation caused by 5-HTP suggesting that the spider stimulus is sufficient to reduce habituation. (C-D) First-minute distance means and total distance means are significantly different between treatments in the no spider condition, but not in the presence of a spider. (T-tests, ***p ≤ .001,. NS = not significant, N>24, error bars ±SEM).

3.2 **5-HT Modulates Habituation During Exploration**

3.2.1 Pharmacological Manipulation of 5-HT Signaling

To investigate the possible modulation of exploratory activity by 5-HT signaling, pharmacological experiments to globally increase or decrease 5-HT signaling levels in the fly brain were performed. 5-hydroxytryptophan (5-HTP) is

the product of the enzyme tryptophan hydroxylase (Trh) and its substrate L-tryptophan, components of the first enzymatic step in a two-step 5-HT synthesis pathway (Coleman & Neckameyer, 2005). Administering 5-HTP increases 5-HT signaling and has been shown to modulate many behaviors in different species. In mice, 5-HTP treatment decreases wakefulness and increases non-REM sleep (Morrow, Vikraman, Imeri, & Opp, 2008). While in rats 5-HTP reduces food intake (Moon, Choi, Yoo, Lee, & Jahng, 2010). In flies, 5-HTP reduces anxiety-like state (Ries, Hermanns, Poeck, & Strauss, 2017) and increases aggression (S. M. Davis, Thomas, Liu, Campbell, & Dierick, 2018; Dierick & Greenspan, 2007).

CS flies were fed either 2% sucrose water solution (vehicle), 10 mM, 20 mM or 40 mM concentrations of 5-HTP in vehicle for 36 hours. First-minute distance means between vehicle and 10 mM and 20 mM 5-HTP were not significantly different (Figure 3.3A, $p > .05$), though vehicle and 40 mM 5-HTP were different ($p < .0001$). In the total distance means flies fed 10 mM and 20 mM 5-HTP concentrations were not different from each other but were significantly different from the vehicle ($p > .0001$). Flies fed 40 mM 5-HTP displayed a more pronounced decrease in locomotor activity compared to vehicle (Figure 3.3B, $p < .0001$), and when compared to the 10 and 20 mM concentrations of 5-HTP ($p > .001$). Path length means curves are of a similar slope but with increasing habituation as the concentration of 5-HTP increases (Figure 3.03C). This work suggests that globally increasing 5-HT signaling with 5-HTP significantly decreases locomotor activity and increases habituation in a dose-dependent manner.

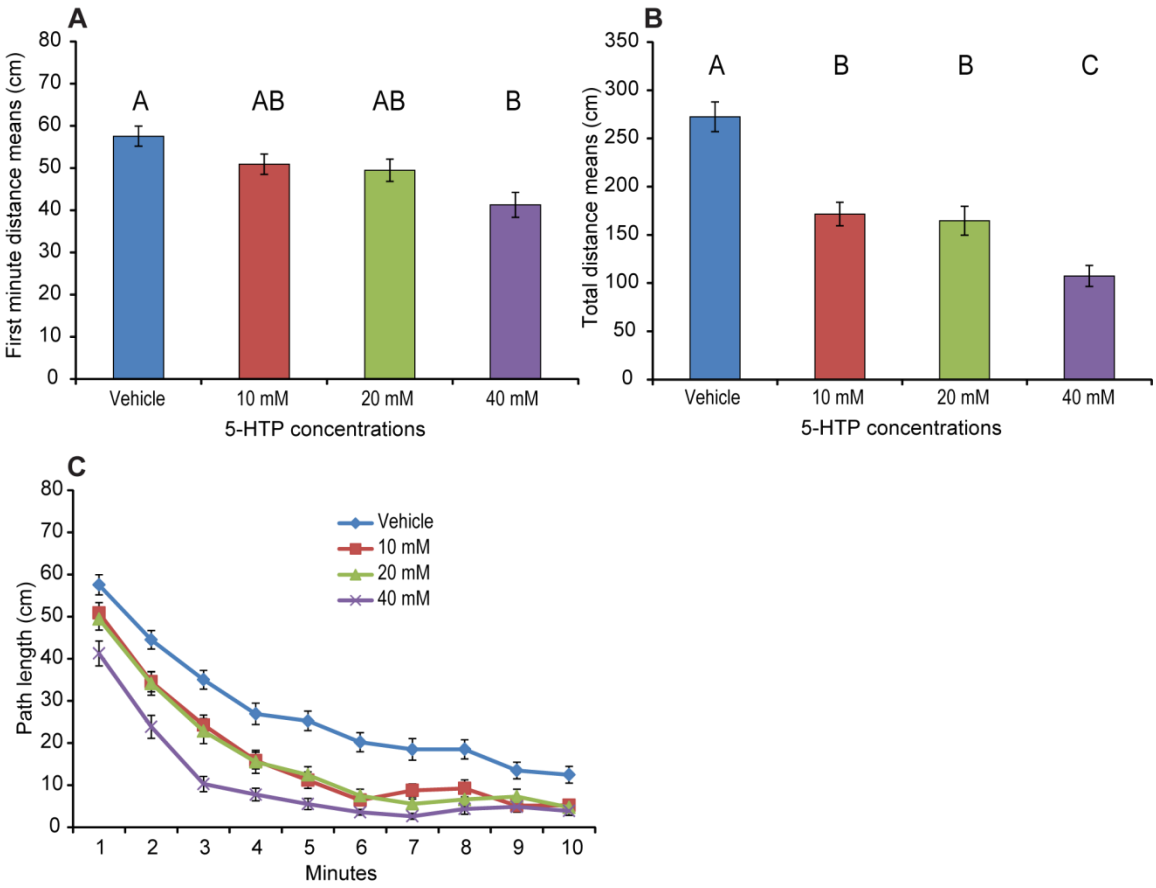

Figure 3. 3 **Increase of 5-HT signaling with 5-HTP increases habituation.** (A) First-minute distance means between vehicle and 10 mM and 20 mM 5-HTP are not different but 40 mM 5-HTP is significantly different, compared to vehicle ($F_{3, 155} = 6.60$, $p < .001$). (B)Total distance means between vehicle and all 5-HTP concentrations are significantly different ($F_{3, 155} = 25.92$, $p < .0001$), increasing 5-HTP concentrations decreases total distance covered. (C) Path length means were significantly reduced by all concentrations of 5-HTP. (All groups N>32, groups with the same letter are not different according to Tukey (HSD), Bonferroni correction and Dunnett test, error bars ±SEM).

To investigate if the effects of 5-HTP on locomotor activity are reversible and to examine the possibility that high concentrations of 5-HTP may be toxic three groups of CS flies were tested after administering different vehicle, food, and 40 mM 5-HTP regimens. The first group was fed 2% sucrose solution (vehicle) for 36 hours, then normal fly food for 36 h and then switched back to the vehicle for the last 36 h (Figure 3.4A blue line). The second group had a similar

feeding regimen except that the *first* 36 hours were on 40 mM 5-HTP (Figure 3.4A green line) and in the third group the 40 mM 5-HTP feeding occurred in the *last* 36 hours.

When tested in the arena at 108 hours there was no difference in path length means between the first two groups (Figure 3.4A, blue and green, $p > .05$), but the third group that was fed 40 mM 5-HTP in the last 36 hours displayed a significant locomotor activity decrease (Figure 3.4A red, $p<.001$). The total distance covered by the group on the vehicle/food/vehicle regimen and the group on 40 mM 5-HTP/ food/vehicle regimen showed no difference ($p < .05$), but the group that was on 40 mM 5-HTP in the last 36 hours was remarkably different ($p < .0001$). These results suggest that the effects of 5-HTP are reversible and that high concentrations of 5-HTP do not have lasting adverse effects.

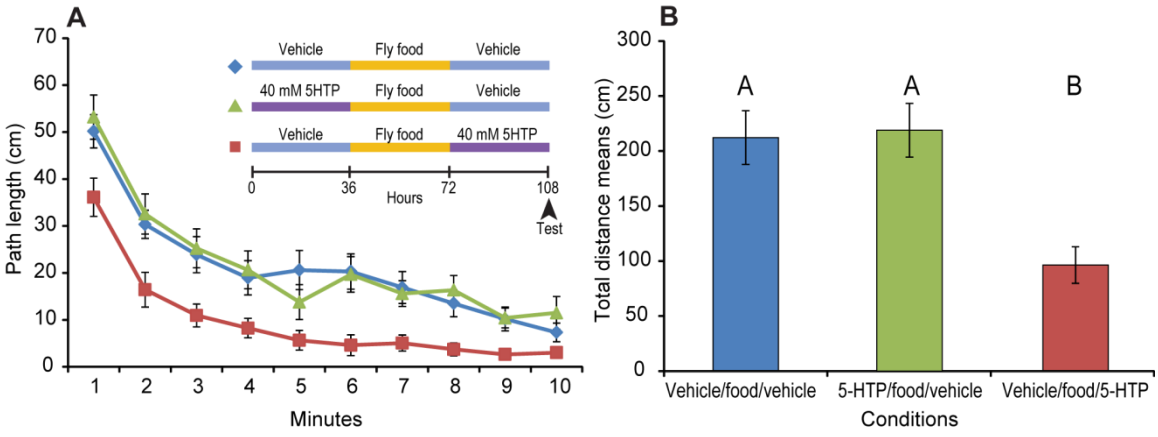

Figure 3. 4 **The effect of 5-HTP on locomotor activity is reversible and has no toxic effects.** (A-B) Flies fed 40 mM 5-HTP and allowed to recover for 72 hours display similar habituation rates to flies not fed 5-HTP. Flies on 40 mM 5-HTP at the time of testing were significantly different. ($F_{2, 56} = 9.67$, $p<.0001$, N=19 all groups, groups with the same letter are not different according to Tukey (HSD) , Bonferroni correction and Dunnett test, error bars ±SEM).

α-methyl-tryptophan (αMTP), an analog of L-tryptophan, is a competitive inhibitor of 5-HT synthesis. αMTP inhibits the first enzymatic reaction in the 5-HT synthesis pathway that produces 5-HTP, decreasing 5-HT signaling. Here flies were either fed vehicle, 40 mM 5-HTP, 50 mM αMTP, or 40 mM 5-HTP plus 50 mM αMTP for 36 hours. In the first-minute distance means, vehicle, 50 mM αMTP and 40 mM 5-HTP & 50 mM αMTP groups were not different (Figure 3.5A, $p > .05$), though 40 mM 5-HTP alone decreased locomotor activity compared to vehicle (Figure 3.5A $p < .001$). The total distance means reveal the opposing effects of manipulating 5-HT signaling on locomotor activity, where 40 mM 5-HTP decreased and 50 mM αMTP increased activity compared to vehicle (Figure 3.5B, $p < .0001$).

However, when feeding flies both 5-HTP *and* αMTP, 5-HTP rescued locomotor activity (Figure 3.5B, $p > .05$ vehicle compared to 5-HTP & αMTP). This suggests that 5-HTP feeding bypassed αMTP inhibition of the first step in the 5-HT synthesis pathway allowing 5-HT signaling to modulate locomotor activity (Figure 3.5D). Path length curves show that manipulation of 5-HT signaling with 5-HTP or αMTP increases and decreases habituation respectively, whereas administering 40 mM 5-HTP rescues habituation when fed with 50 mM αMTP (Figure 3.05C). These data indicate that 5-HT signaling modulates habituation during locomotor exploration.

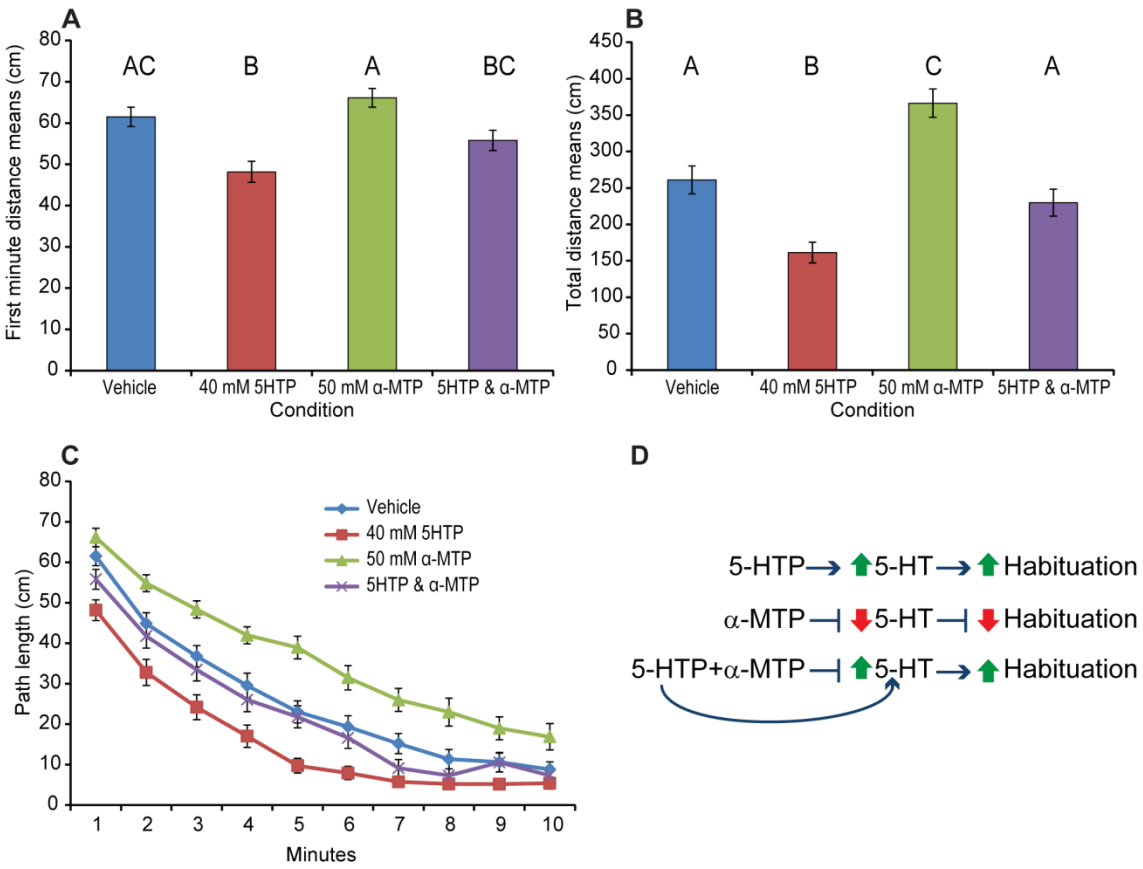

Figure 3. 5 **5-HTP rescues αMTP habituation decrease.** (A-B) 40 mM 5-HTP significantly decreased whereas 50 mM αMTP significantly increased locomotor activity ($F_{3, 127} = 10.32$, $p < .0001$). (C) Path length means show that pharmacological manipulation of 5-HT signaling modulates habituation and that 5-HTP rescues normal habituation in the 5-HTP & αMTP condition. (N>32, groups with the same letter are not different according to Tukey (HSD), Bonferroni correction and Dunnett test, error bars ±SEM).

3.2.2 Transgenic Manipulation of 5-HT Signaling

The fly brain is composed of about 150,000 neurons of which fewer than 100 have been identified as serotonergic (Pooryasin & Fiala, 2015). The somas of 5-HT secreting neurons are stereotypically located in the midbrain as contralateral pairs or clusters and their dendrites and axons extensively innervate the fly brain in an ipsilateral and/or contralateral manner.

To assess which 5-HT secreting neurons may be involved in modulating habituation during exploration a top-down approach using the Gal4/UAS system (Figure 2.x) was used to activate and inhibit populations of 5-HT secreting neurons. The Gal4 lines used are Trh-Kartic (referred to as Trh-K) and Trh-D2, kindly provided by Dr. Herman Dierick. Expression of the Gal4 transcription factor in these lines is controlled by promoter/enhancer sequences of the neural Tryptophan hydroxylase (Trh) gene expressed by neurons that synthesize 5-HT, ostensibly limiting expression of activating and inhibiting neural effectors to populations of 5-HT neurons. Two different Trh Gal4 lines were employed to detect possible confounding artifacts from either Gal4 line.

Both Trh-Gal4 lines have similar expression patterns, driving UAS-mCD8::eGFP expression in the PMPD, PMPV, PLP, LP, SEM and SEL 5-HT neuron clusters (Pooryasin & Fiala, 2015). 5-HT secreting neurons that may also be included are the Dorsal Anterior Lateral neurons (DAL) and the contralaterally projecting, serotonin-immunoreactive deutocerebral neurons (CSD), the somas for Dorsal Paired Medial neurons (DPM) were not located (Figure 3.6). These Gal4 lines include overlapping populations of Trh-positive, 5-HT secreting neurons but may also include 5-HT negative neurons. The effect of activating or inhibiting these cells along with 5-HT secreting cells is unknown.

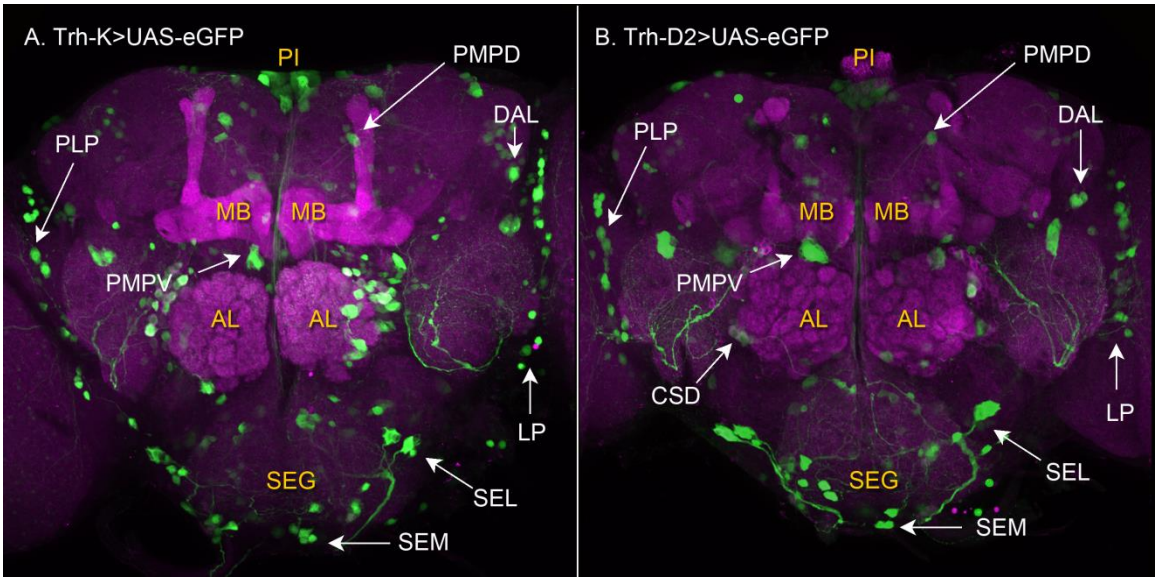

***Figure 3. 6* Expression patterns of Trh-K and Trh-D2 Gal4 lines.** Both Gal4 lines target the PMPD, PMPV, PLP, LP, SEM, and SEL 5-HT neuron clusters. (B) The soma for one of the CSD neurons is visible. DAL neurons may also be included in these Gal4 lines. (Anterior view of the fly midbrain. MB=mushroom bodies, AL=antenna lobes, PI=pars intercerebralis, SEG=subesophageal ganglia).

Conditional thermogenetic and optogenetic approaches were employed to activate 5-HT secreting neurons. Both Trh-Gal4 lines were used to drive expression of the temperature sensitive cation channel UAS-TrpA1 (Hamada et al., 2008). The permissive and restrictive temperatures of UAS-TrpA1 are ~23 °C and ~29 °C respectively. Both Trh-Gal4s were also used to drive expression of the light-sensitive, red-shifted (~620 nm) channelrhodopsin UAS-CsChR (Klapoetke et al., 2014) in 5-HT neurons. UAS-CsChR is a robust neural effector having some level of activity in white light alone, either with or without the photosensitive pigment all-trans retinal (ATR). However, the presence of ~620 nm red light and ATR increase the ability of the neural effector to depolarize neurons.

To inhibit 5-HT secreting neurons both Trh-Gal4 lines drove expression of UAS-Kir 2.1 (Baines, Uhler, Thompson, Sweeney, & Bate, 2001). UAS-Kir 2.1 is a human inward rectifying potassium channel that hyperpolarizes neurons effectively reducing their ability to fire an action potential thereby inhibiting neurotransmitter release.

Thermogenetic activation at 29 °C of Trh-positive neurons covered by Trh-K Gal4 significantly increased activity. In the first-minute and total distance means comparisons between temperature conditions showed no differences in the controls (Figure 3.9A-B, $p > .05$), but the locomotor activity difference between experimental groups was significant (Figure 3.9A-B, $p < .0001$). Within the 23 °C condition in both the first-minute distance means and total distance means there were no differences (Figure 3.9A-B, $p > .05$), however, within the 29 °C condition the experimental groups were significantly different from the controls (Figure 3.9A-B, $p < .0001$).

Optogenetic activation of Trh-positive neurons targeted by Trh-K Gal4 also decreased locomotor activity. The optogenetic neural effector UAS-CsChR has some level of constitutive activity in white light and in the -ATR condition. As a result, when large populations of Trh-positive neurons are manipulated, as in this experiment, there is often no difference between ±ATR conditions in all groups. However, *within* -ATR or +ATR conditions the differences are frequently significant.

In the first-minute distance means and total distance means there were no differences between ±ATR conditions in the control and experimental groups (Figure 3.9C-D, $p > .05$). Within the -ATR or the +ATR conditions, there were no differences in controls, but the experimental groups displayed significantly decreased locomotor activity (Figure 3.7C-D, $p < .0001$).

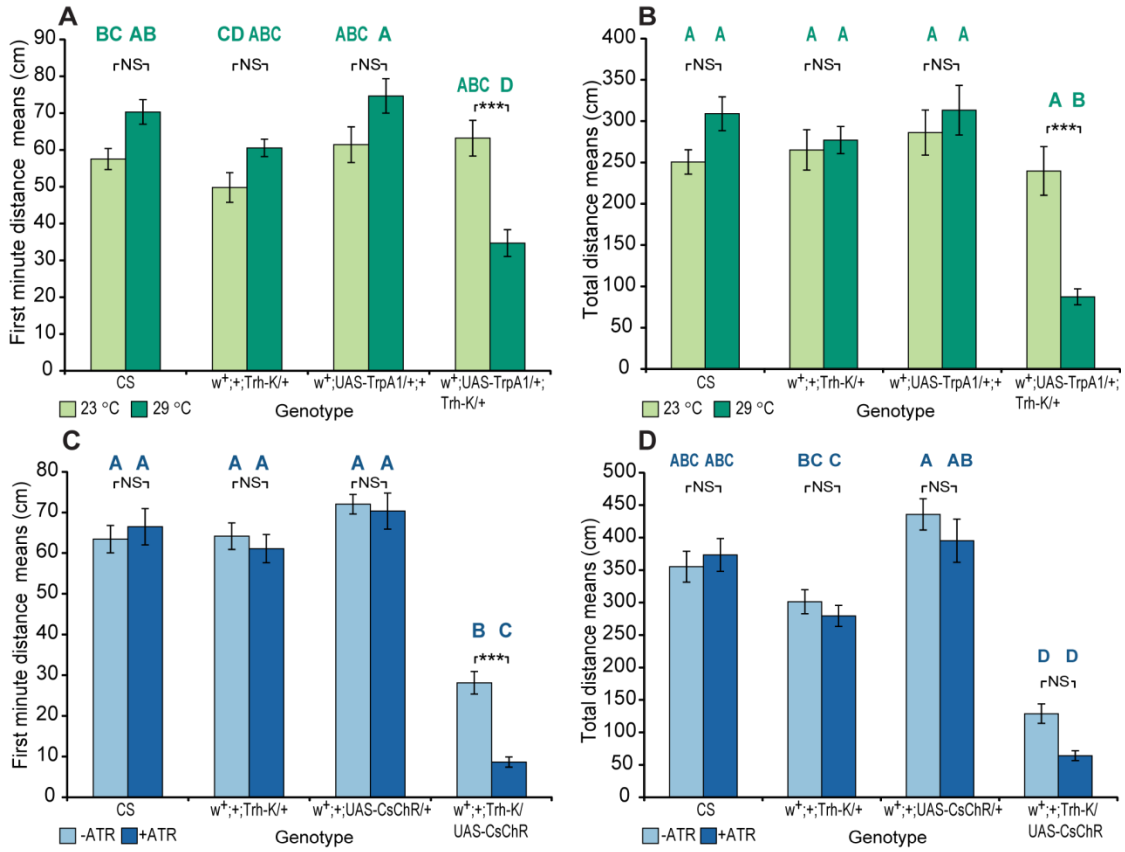

Figure 3. 7* Activation of neurons targeted by Trh-K Gal4 increases habituation.** (A and B) Thermogenetic activation: Trh-K>UAS-TrpA1 at 29 °C significantly decreases activity compared to 23 °C in first-minute and total distance means (A, $F_{7, 352} = 8.76$, $p < .0001$; B, $F_{7, 348} = 8.54$, $p < .0001$). (C and D) Optogenetic activation: Trh-K>UAS-CsChR +ATR significantly decreases activity compared to controls. (C, $F_{7, 204} = 39.70$, $p < .0001$; D, $F_{7, 204} = 32.42$, $p < .0001$) ($p \leq .001$, **$p \leq .01$, NS $p > .05$, groups with the same letter are not different according to Tukey (HSD) , Bonferroni correction, error bars ±SEM).

Inhibition of Trh-positive neurons covered by Trh-K by hyperpolarization with UAS-Kir 2.1 increased locomotor activity in the first-minute distance means (Figure 3.8A $p < .001$) and total distance means (Figure 3.8B, $p < .001$). In the total distance means the Gal4 control did not reach significance when compared to the experimental group due to behavioral variability. Path length means over time show the Trh-K>UAS-Kir 2.1 experimental group beginning at a lower activity level in the first-minute and remains low compared to controls indicating faster habituation (Figure 38C, $p < .001$).

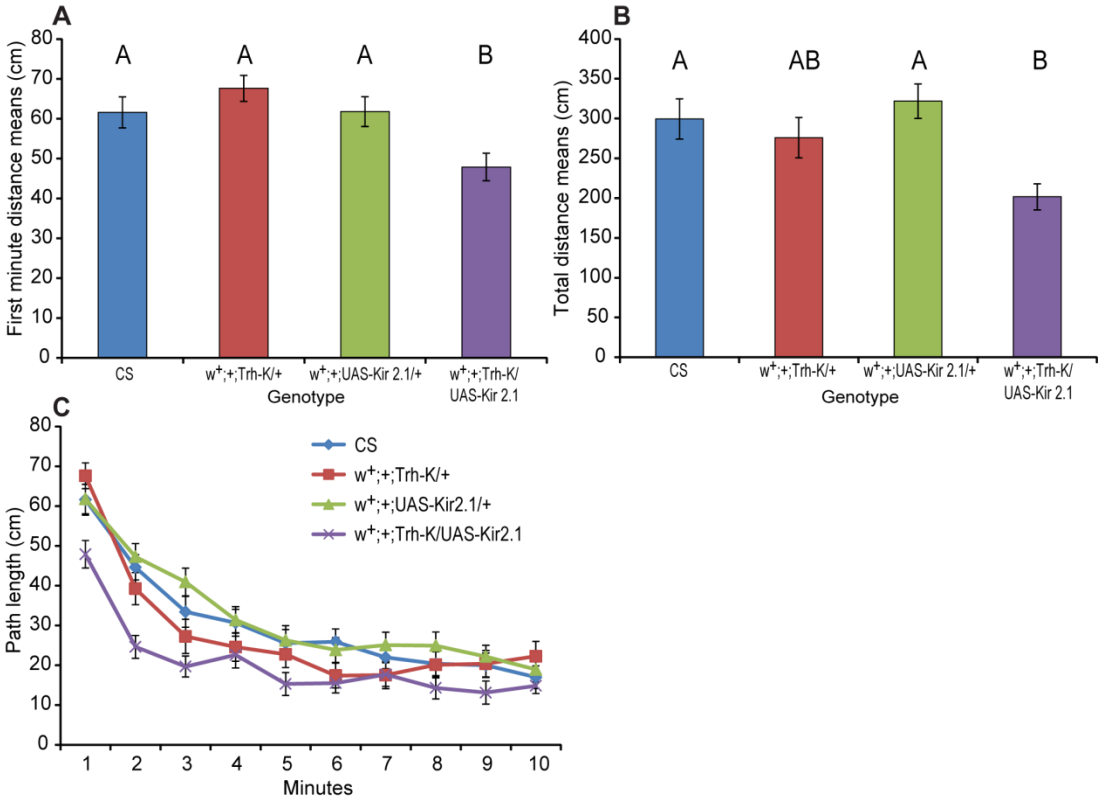

Figure 3. 8 **Inhibition of neurons targeted by Trh-K Gal4 increases habituation.** Trh-K>UAS-Kir 2.1 significantly increases habituation in the (A) first-minute means ($F_{3, 117} = 5.45$, $p < .002$) and (B) total distance means ($F_{7, 117} = 5.80$, $p < .001$). (C) Path length means over time in the experimental groups display a shallower habituation curve. ($N \geq 32$, groups with the same letter are not different according to Tukey(HSD), Bonferroni correction and Dunnett test, error bars ±SEM.)

The experiments were repeated with the Trh-D2 Gal4, which targets overlapping but different groups of neurons. Thermogenetic activation at 29C of neurons covered by Trh-D2 Gal4 also decreased locomotor activity means. The first-minute distance and total distance means revealed similar results (Figure 3.9A-B). In the first-minute distance and total distance means comparisons between 23 °C and 29 °C conditions were not different between controls ($p > .05$), but there were significant differences between the experimental groups ($p < .0001$) showing decreased locomotor activity when the neurons covered by Trh-D2 were depolarized. Within group analysis of the 23 °C condition showed no differences among controls or experimental groups ($p > .05$), but in the 29 °C permissive condition the experimental groups displayed decreased locomotor activity means and were significantly different than controls ($p < .0001$).

Optogenetic activation with Trh-D2>UAS-CsChR also showed decreased distance means. Trh-D2 optogenetic activation produced consistent results in the first-minute distance and total distance means (Figure 3.9 C-D). There were no statistically significant differences between ±ATR controls or between ±ATR experimental groups in the first-minute distance and total distance means ($p > .05$). However, within-group analyses, -ATR or +ATR, in the first-minute distance and total distance means, showed significant differences in distance means between controls and experimental groups ($p < .0001$).

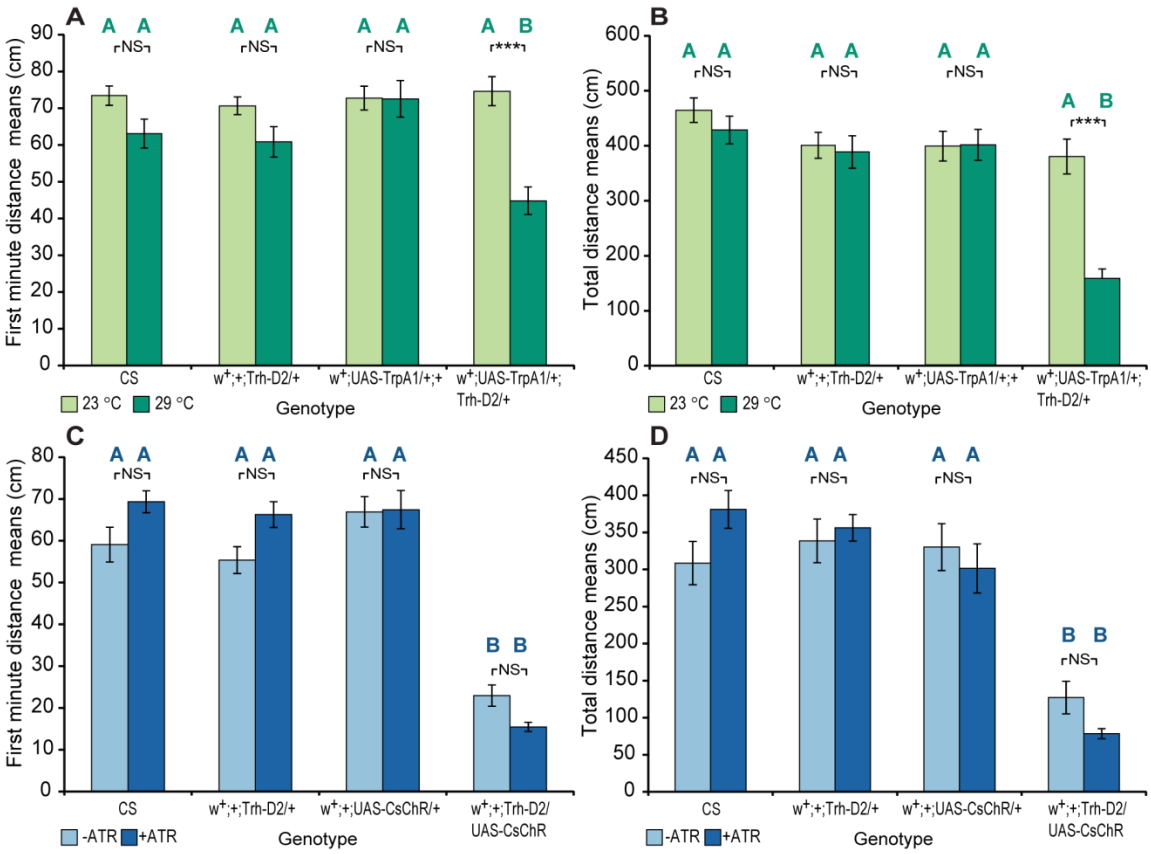

***Figure 3. 9* Activation of neurons targeted by Trh-D2 Gal4 increases habituation.** (A and B) Thermogenetic activation: Trh-D2>UAS-TrpA1 at 29 °C significantly increases habituation compared to controls at 23C in first-minute and total distance means (A, $F_{7, 302} = 7.83$, $p < .0001$; B, $F_{7, 299} = 18.33$ $p < .0001$). (C and D) Optogenetic activation: Trh-D2>UAS-CsChR +ATR significantly increases habituation compared to -ATR controls. (C, $F_{7, 241} = 42.46$, $p < .0001$; D, $F_{7, 241} = 18.63$ $p < .0001$). ($N \geq 30$, ***$p \leq .001$, **$p \leq .01$, NS $p > .05$, groups with the same letter are not different according to Tukey (HSD), Bonferroni correction, error bars ±SEM).

Inhibiting neurons with Trh-D2>UAS-Kir 2.1 decreased distance means in the first-minute distance (Figure 3.10A, $p < .0001$) and in the total distance (Figure 3.10B, $p < .0001$) when compared to controls. In the first-minute distance means the Gal4 control did not reach significance when compared to the experimental group. Path length means curves over time show a remarkable difference between controls and experimental groups, where the controls are

tightly grouped and the experimental group habituates faster and has consistently lower activity (Figure 3.10C, $p < .0001$).

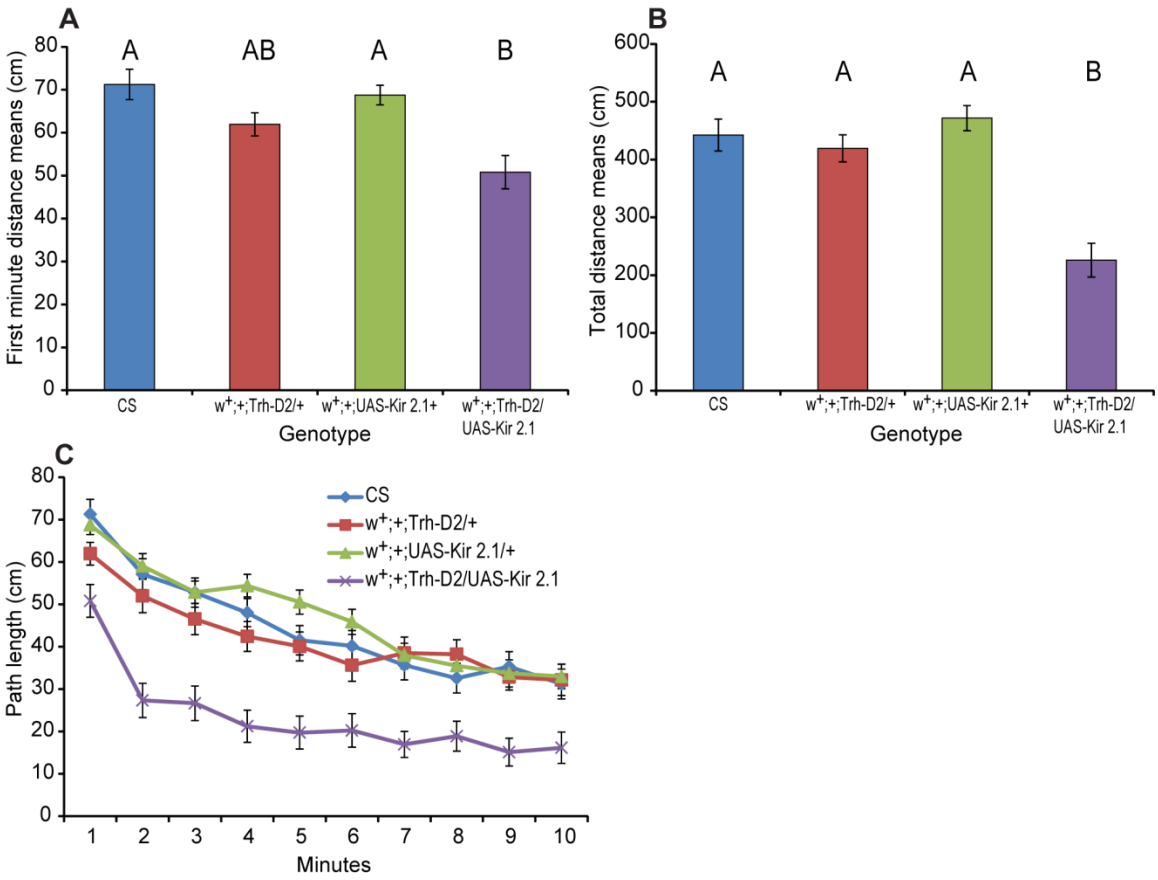

Figure 3. 10 **Inhibition of neurons targeted by Trh-D2 Gal4 increases habituation.** Trh-D2>UAS-Kir 2.1 increases habituation in (A) the first-minute distance means ($F_{3, 159} = 8.29$, $p < .0001$) and (B) total distance means (($F_{3, 159} = 17.44$, $p < .0001$). (C) Controls display similar habituation curves but the experimental habituates faster and has lower activity consistently. (N>32, groups with the same letter are not different according to Tukey (HSD), Bonferroni correction and Dunnett test, error bars ±SEM).

Overall Trh-K and Trh-D2 Gal4s produced consistent and comparable results indicating that activation or inhibition of the neurons covered by these two Gal4 drivers modulates locomotor activity. Pharmacological and transgenic promotion of 5-HT signaling decrease activity possibly indicative of increased habituation. However, transgenic inhibition of 5-HT neurons also decreased

locomotor activity, which contrasts with pharmacological inhibition experiments where inhibiting 5-HT signaling increased activity (Figure 3.5). This may be in part due to developmental compensation through up or down gene regulation as both the UAS-CsChR and UAS-Kir 2.1 neural effectors are active through development.

3.2.3 Transgenic Manipulation of the Dorsal Paired Medial Neurons

Pharmacological or transgenic manipulation to promote or inhibit 5-HT signaling may affect large populations of neurons and could have off-target effects. These global approaches may simultaneously activate 5-HT inhibitory and stimulatory pathways, promote the release of multiple neurotransmitters, or target non-5-HT secreting neurons. To reduce possible confounding effects a screen targeting specific 5-HT neurons or smaller subsets of 5-HT neurons was carried out.

The initial neurons tested were the Dorsal Paired Medial (DPM) neurons. Anatomically the DPMs are two large neurons located on each side of the fly brain which heavily innervate all lobes and the anterior portion of the peduncles of the mushroom bodies in an ipsilateral manner, suggesting a comprehensive circuit with mushroom body (MB) neurons (Figure 3.11F) (Waddell et al., 2000). The DPM neurons reportedly release 5-HT, GABA and a neuropeptide produced by the *amnesiac* gene (Dubnau & Chiang, 2013; Waddell et al., 2000). Functionally, the DPM neurons modulate many behaviors including sleep (Haynes et al., 2015), egg laying (Wu et al., 2015), and long term memory

consolidation and retrieval (Margulies et al., 2005; Pitman et al., 2011; Waddell et al., 2000). To investigate whether the DPM neurons are involved in habituation during exploration, the Gal4 drivers VT64246, C316, and NP2721 were used separately to drive expression of the neural effectors *UAS-CsChR* to activate and *UAS-Kir 2.1* to inhibit the DPM neurons.

First, the DPMs were manipulated with the VT64246 Gal4, which targets the DPMs and little else in the fly brain (Figure 3.11F), but is not considered a strong driver (Haynes et al., 2015). Optogenetic depolarization of the DPM neurons with VT64246>UAS-CsChR decreased locomotor activity. No significant differences are detected between ±ATR control or experimental groups in either the first-minute distance or total distance means. Nonetheless, analyses comparing controls and experimental groups within -ATR or +ATR conditions show a significant decrease in locomotor activity in experimental groups (Figure 3.11 A and B, $p < .0001$), in both first-minute distance and total distance means. However, inhibiting the DPMs with VT64246>UAS-Kir 2.1 did not affect habituation, possibly because the VT64246 is not a strong driver (Figure 3.11C-E, all $p > .05$).

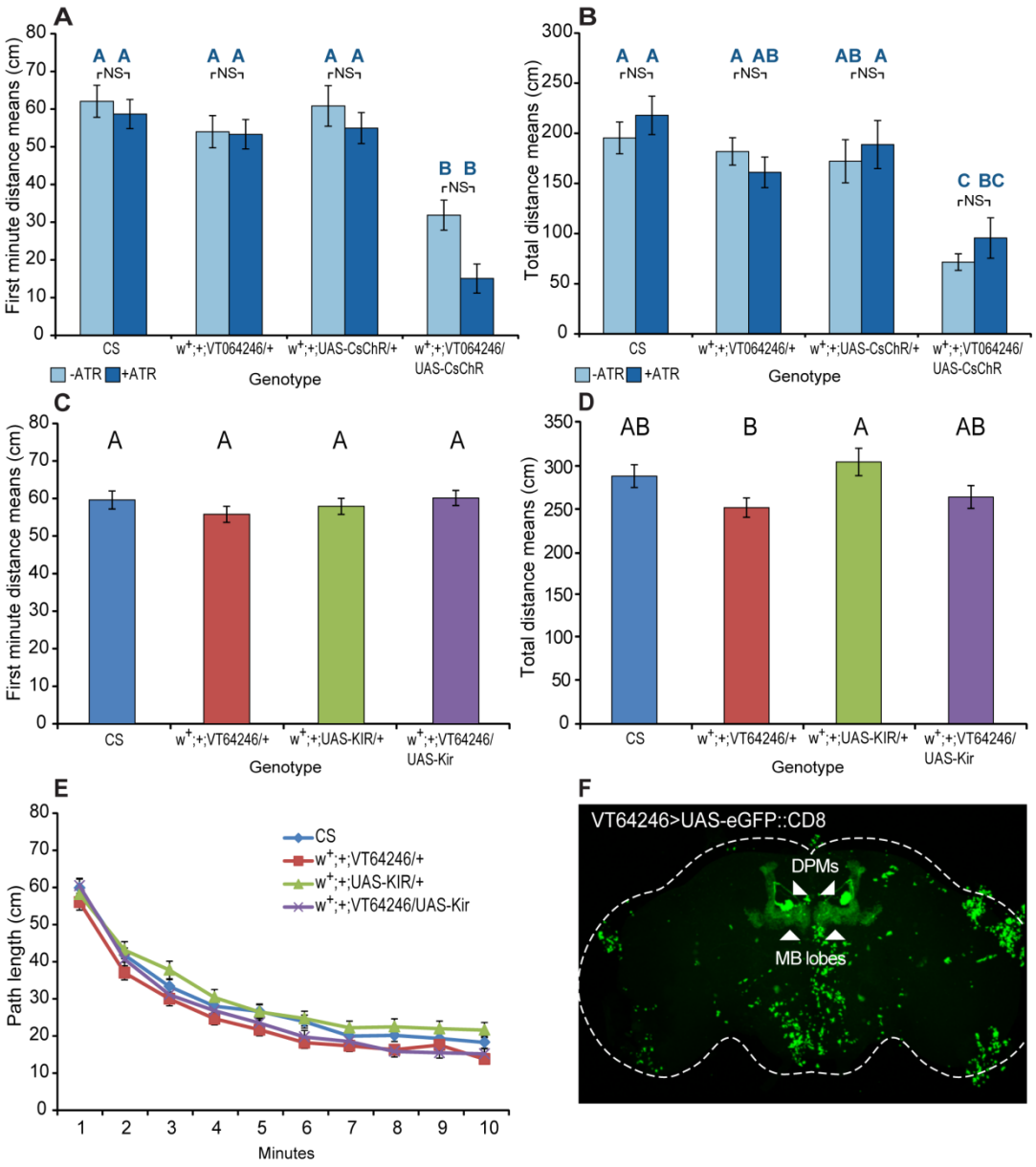

Figure 3. 11 **Activation of the DPM neurons with VT64246 increases habituation but inhibition has no effect.** (A-B) Activating the DPM neurons with VT64246>UAS-CsChR significantly decreased activity in first-minute distance ($F_{7, 248} = 15.43$, $p < .0001$) and total distance means ($F_{7, 248} = 7.11$, $p < .0001$) in both conditions indicative of habituation. (C-D) Inhibiting the DPM neurons with VT64246>UAS-Kir 2.1 did not affect activity. First-minute distance means ($F_{3,424} = 0.79$, $P = .498$) and total distance means ($F_{3,424} = 2.93$, $P = .032$). (E) Path length means show no differences in habituation rates between controls and experimental group. (F) VT64246>UAS-mCD8::eGFP labels the somas and axons of the DPMs. The dashed line indicates the border of the brain. (NS $p > .05$, groups with the same letter are not different according to Tukey (HSD) , Bonferroni correction, error bars ±SEM).

Next, the DPM neurons were manipulated using the C316 Gal4 driving the same neural effectors. The C316 Gal4 is reported to be a strong DPM driver (Haynes et al., 2015). GFP expression pattern by C316 labels the DPMs and also labels cells in the SOG and OL (Figure 3.12F). In the first-minute distance means activating the DPMs revealed no significant differences between ±ATR conditions or within -ATR or +ATR groups (Figure 3.12A $p > .05$). Similarly, in the total distance means there were no differences between ±ATR conditions but in contrast within the -ATR or +ATR conditions the experimental groups showed significantly decreased locomotor activity (Figure 3.12B $p < .0001$).

Unlike the VT64246 Gal4 experiment, inhibiting the DPMs with C316>UAS-Kir 2.1 showed decreased activity in the experimental groups similar to the Trh-Gal4 experiments (Figures 3.8 and 3.10). While the first-minute distance means indicate a trend towards decreased locomotor activity, there were no significant differences between groups (Figure 3.12C $p > .05$). On the other hand, in the total distance means there was a difference in activity levels between the controls and experimental groups (Figure 3.12D $p < .0001$). Path length curves are similar to the Trh-D>UAS-Kir 2.1 experiment, showing clustering of the controls and faster habituation and consistently decreased locomotor activity in the experimental group. (Figure 3.12E $p < .0001$)

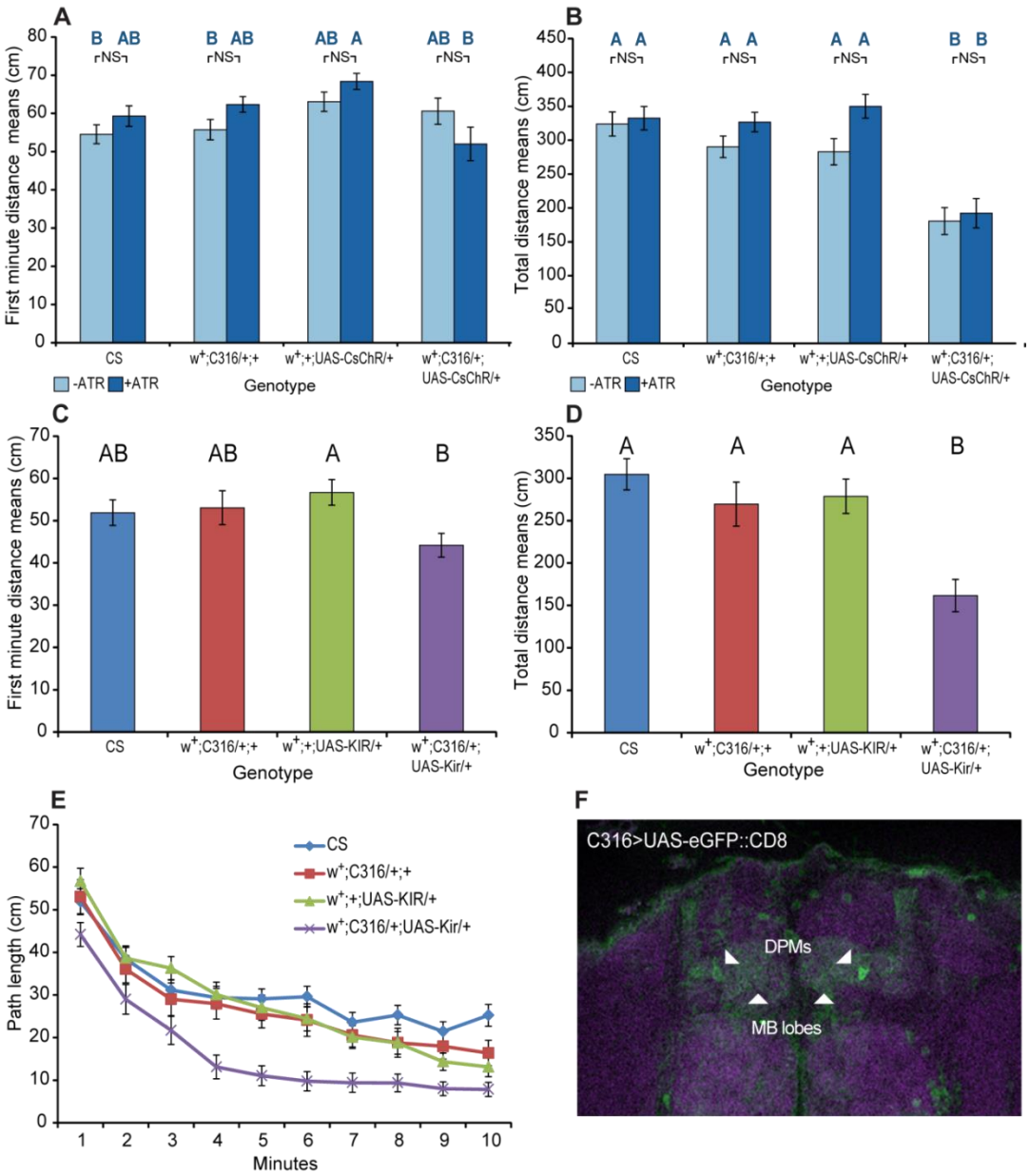

Figure 3. 12 **Activation or inhibition of the DPM neurons with C316 increases habituation.** Activating the DPM neurons with C316>UAS-CsChR showed no consistent differences in (A) the first-minute distance means ($F_{7, 478}$ = 3.65, p < .001), but (B) the total distance means showed significantly decreased activity suggesting modulation of habituation ($F_{7, 477}$ = 11.19, p < .0001). Inhibiting the DPM neurons with C316>UAS-Kir 2.1 showed no differences in (C) the first-minute distance means ($F_{7, 198}$ = 2.49, p > .05) while the (D) total distance means showed significant decreased locomotor activity ($F_{7, 198}$ = 8.31, p > .0001). (E) Path length means show significant differences in habituation rates between controls and experimental group. (F) C316>UAS-mCD8::eGFP labels the somas of the DPMs. (NS p >.05, groups with the same letter are not different according to Tukey (HSD), Bonferroni correction, error bars ±SEM).

The DPM neurons were also manipulated with the NP2721 Gal4 driving UAS-CsChR to activate, however in this case the neural effector UAS-TNT-LC was used to inhibit synaptic activity. Like the C316 Gal4, the NP2721 Gal4 is a robust driver (Haynes et al., 2015). GPF expression by NP2721 Gal4 labels the DPMs as well as the AL, SOG, and PI (Figure 3.13F).

Comparisons between ±ATR conditions in the first-minute distance means show differences between the Gal4 controls (Figure 3.13A, $p = .002$) possibly due to behavioral variability, but no differences between the other two controls ($p > .05$). Moreover, ±ATR comparisons between experimental groups showed decreased locomotor activity ($p < .0001$) when DPMs are depolarized. Within the -ATR condition there were no differences between all groups ($p > .05$), but in contrast in the +ATR condition, there was a significant decrease in locomotor activity in the experimental group compared to controls ($p < .0001$).

In the total distance means there were no differences between ±ATR control groups (Figure 3.13B, $p > .05$) however, there was a significant decrease in activity in the +ATR experimental group when compared to the -ATR experimental group (Figure 3.13B, $p < .001$). Within the -ATR condition there were no differences ($p > .05$), but once again there was a significant difference locomotor activity between controls and the experimental group in the +ATR condition ($p < .0001$) when the DPMs are depolarized.

Though the UAS-TNT-LC neural effector is used in this experiment to inhibit the DPMs the results are similar to the C316 Gal4 experiment where UAS-

Kir 2.1 was used to inhibit (Figure 3.12 C-E). The first-minute distance means show decreased locomotor activity when the experimental group is compared to CS and Gal4 controls (Figure 3.12C $p < .0001$) but not when compared to the UAS control. In contrast, in the total distance means there was a clear difference between all controls and the experimental group ($p < .0001$). These data, much like the Trh-Gal4 experiments suggest that inhibiting 5-HT signaling from the DPMs modulate locomotor activity.

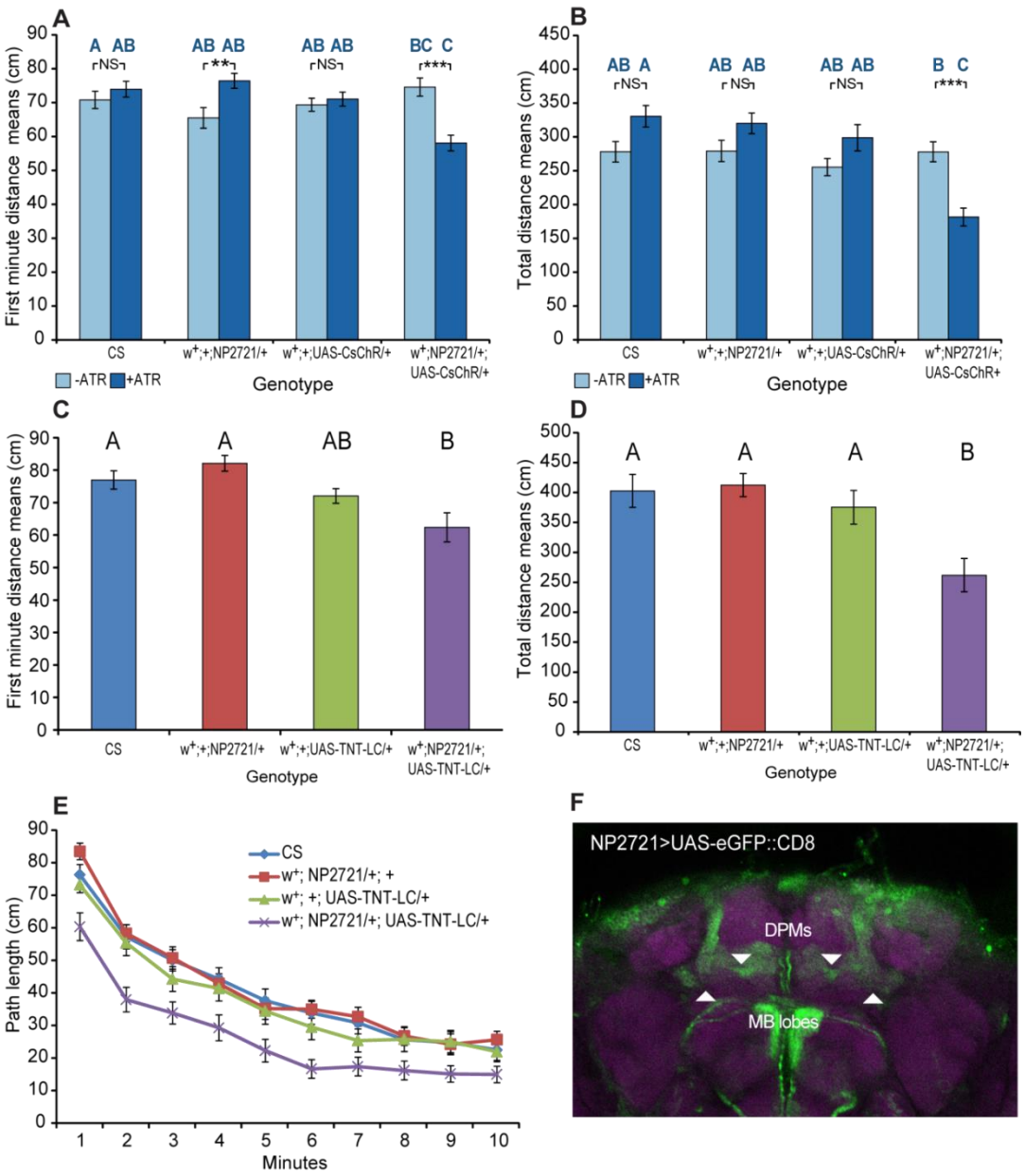

Figure 3. 13 **Activation or inhibition of the DPM neurons with NP2721 increases habituation.** Activating the DPM neurons with NP2721>UAS-CsChR showed decreased activity in (A) the first-minute distance means ($F_{7, 679} = 6.18$, $p < .0001$) and (B) total distance means ($F_{7, 678} = 9.27$, $p < .0001$) suggesting increased habituation. Inhibiting the DPM neurons with NP2721>UAS-TNT-LC also decreased activity in the (C) first-minute distance means ($F_{7, 160} = 6.78$, $p < .0001$) and (C) total distance means ($F_{7, 161} = 6.04$, $p < .001$). (E) Path length means over time suggest an increase in habituation in the experimental group when compared to controls ($p < .0001$). (F) NP2721UAS-mCD8::eGFP labels the somas of the DPMs and SOG. (***$p \leq .001$, **$p \leq .01$, NS $p > .05$, Groups with the same letter are not different according to Tukey (HSD), Bonferroni correction and Dunnett test, error bars ±SEM).

The DPM neurons are reported to release 5-HT, GABA and a neuropeptide produced by the *amnesiac* gene (AMN). Both 5-HT and GABA are generally inhibitory while AMN has a role in memory consolidation (Haynes et al., 2015; Pitman et al., 2011; Waddell et al., 2000). When the DPMs are transgenically depolarized they may co-release neurotransmitters. To determine if 5-HT may be responsible for the locomotor activity phenotypes observed in the previous pharmacological and transgenic experiments, 5-HT signaling but not GABA or AMN signaling, was reduced from the DPM neurons. The Drosophila Serotonin Transporter (dSerT) is expressed by 5-HT secreting neurons. dSerT regulates 5-HT signaling by 5-HT reuptake into the presynaptic neuron from the synaptic cleft (Demchyshyn et al., 1994; Giang, Ritze, Rauchfuss, Ogueta, & Scholz, 2011). Here VT64246 Gal4 is used to *drive UAS-SERT* to overexpress SerT in the DPM neurons. In addition, the experiment is run in vehicle or 40 mM 5-HTP conditions.

In the first-minute distance means there are no significant differences within or between groups (Figure 3.14A, $p > .05$). In contrast, in the total distance means there are significant differences between ±5-HTP controls (Figure 3.14B, $p < .001$) in response to 5-HTP treatment, but not in the experimental group ($p > .05$). Further, within the vehicle condition, the experimental group shows reduced activity compared to controls ($p < .0001$), however, within the 5-HTP condition all control groups respond to 5-HTP treatment, but not the experimental group ($p > .05$).

The distance means in Figure 3.14B are recapitulated, but are more readily clear in the path length line plots (Figure 3.14C-D). Within the vehicle condition there was a remarkable decrease in locomotor activity in the experimental group where SerT is overexpressed ostensibly by reducing 5-HT signaling, but not GABA or AMN (Figure 3.14C $p > 0.0001$). These results agree with results from the DPM inhibition experiments (Figure12E and 13E).

However, within the 40 mM 5-HTP condition, the controls respond to 5-HTP treatment with decreased locomotor activity, but the experimental group, where SerT is overexpressed, is not different from the controls (Figure 3.14D, $p > .05$). This may be due to the 40 mM 5-HTP treatment increasing 5-HT signaling, bypassing the reducing effects of 5-HT reuptake by overexpressed SERT, rescuing normal activity.

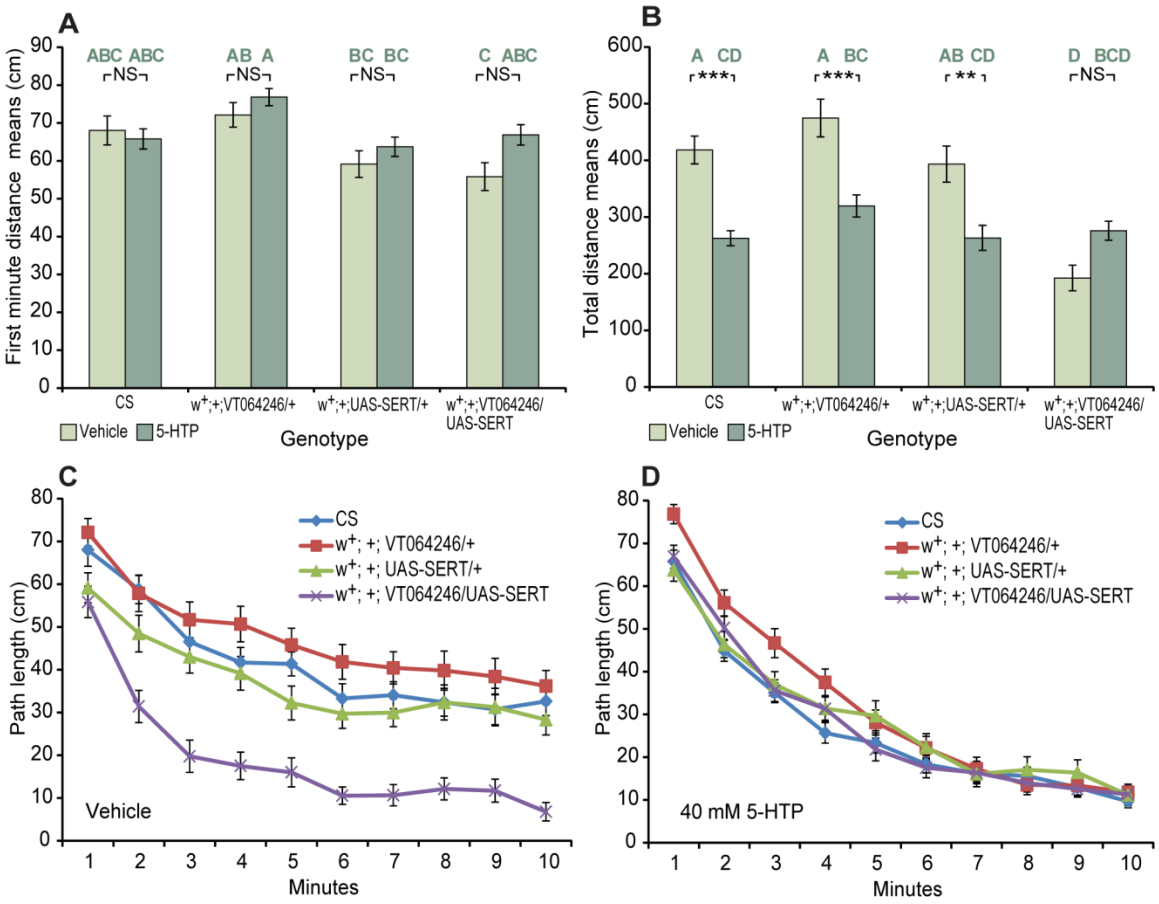

Figure 3. 14 **Overexpression of SERT in the DPM neurons increases habituation.** (A) There are no significant within or between-group differences in the first-minute distance means ($F_{7, 346}$ + 4.72, p < .0001) (B) Expression of UAS-SERT decreases locomotor activity. Administering 40 mM 5-HTP rescues habituation ($F_{7, 346}$ + 15.82, p < .0001). (C) Overexpression of SERT increases habituation in the vehicle condition. (D) 40 mM 5-HTP rescues normal habituation rates. (***p ≤ .001, **p ≤ .01, NS p > .05 groups with the same letter are not different according to Tukey (HSD) , Bonferroni correction, error bars ±SEM).

3.2.4 Transgenic Manipulation of the DAL Neurons

The Dorsal Anterior Lateral neurons (DAL) are a pair of large 5-HT secreting neurons involved in the formation and retrieval of LTM (Xia 2005, Chen 2012, Dubnau 2013). In the adult fly brain, the somas of the DAL neurons are located in the dorsal anterolateral protocerebrum. Like the DPM neurons, there is

one DAL neuron per brain hemisphere. Dendrites from the DALs extend to the superior dorsofrontal protocerebrum and axonal processes extend to several brain regions including domains of the MB calices that contain α/β neuron dendrites, suggesting a circuit between the DAL neurons and MB cells (Figure 3.15F). In these experiments, the DAL G0338-Gal4 is used to drive neural effectors. G0338-Gal4 is an insertion on the X chromosome, therefore females were assayed instead of males.

Optogenetic activation of the DAL neurons with DAL(G0338) Gal4 driving *UAS-CsChR* showed no between or within group differences in first-minute distance and total distance means (Figure 15A-B, $p > .05$). Inhibition of the DAL neurons with DAL(G0338) Gal4 driving a UAS-Kir 2.1 also revealed no between or within group differences in first-minute distance and total distance means (Figure 3.15C-D, $p > .05$). Path length means show no differences in habituation rates (Figure 3.15E, $p > .05$). These results suggest that the DAL neurons may not be involved in modulating habituation.

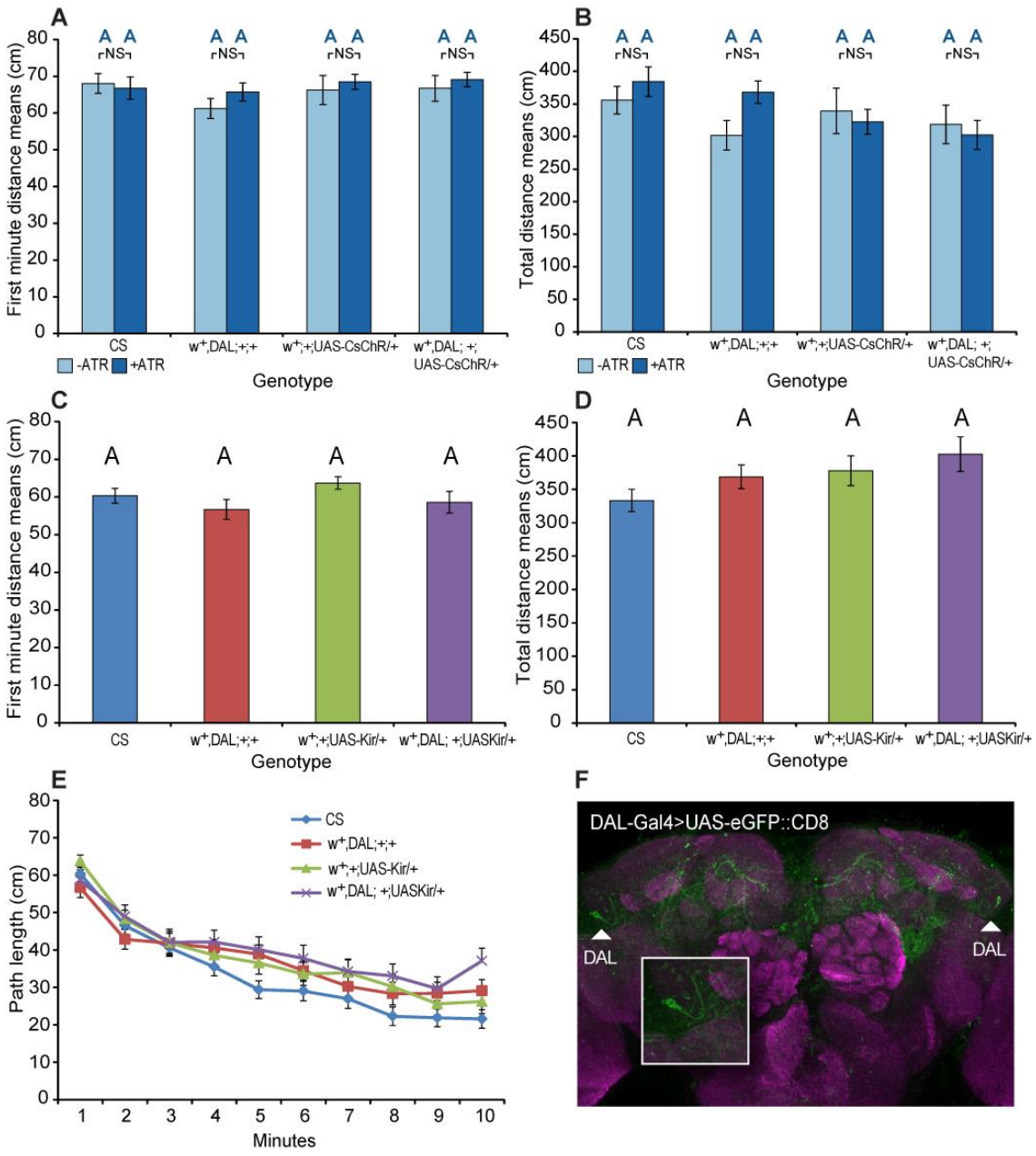

Figure 3. 15 **Activating or inhibiting the DAL neurons does not affect habituation.** (A-B) Activating DAL neurons did not affect habituation in the first-minute means ($F_{7,266}$ = 0.8, p > .05) or total distances means ($F_{7,266}$ = 1.77, p > .09). (C-D) Inhibiting the DAL neurons did not affect habituation ($F_{3,186}$ = 1.61, p > .18). (E) Path length means show no significant differences. (F) Expression of GFP reporter with G0338 Gal4 labels the somas of the DALs and their neurites. (NS p > .05, groups with the same letter are not different according to Tukey (HSD), Bonferroni correction error bars ±SEM).

3.2.5 Transgenic Manipulation of the CSD Neurons

First characterized in the sphinx moth (*Manduca sexta*) (Kent, 1987) the contralaterally projecting, serotonin-immunoreactive deutocerebral neurons (CSD) are a pair of 5-HT secreting neurons found in many insects including *Drosophila* (Dacks, Christensen, & Hildebrand, 2006; Hill, Iwano, Gatellier, & Kanzaki, 2002; Roy et al., 2007; Tsuji, Aonuma, Yokohari, & Nishikawa, 2007). In the adult fly brain, the CSD neurons display an intricate morphology innervating various brain regions in an ipsilateral and contralateral manner. The somas of each CSD neuron are located in the deutocerebrum lateral to the AL and project ipsilateral to the AL glomeruli, the MB calyx (CA), the superior medial protocerebrum (SMPR) and the lateral horn (LH), then contralateral to the same structures (Figure 3.16F).

In flies, the CSD neurons are involved in the modulation of sensitivity to odorants by regulating the activity of neurons in the AL, MB and LH (Dacks et al., 2006; Roy et al., 2007). Findings by (Xu et al., 2016) suggest that the CSD neurons also modulate the activity of 5-HT neurons in the IP and LP1 regions of the protocerebrum. These 5-HT neurons are thought to suppress innate attraction to odorants like ethanol. Sufficient inputs to the CSDs disinhibit these neurons allowing the animal to approach certain olfactory stimuli.

The 5-HT neurons in the LP1 cluster are intrinsic to the ventrolateral protocerebrum (VLPR), a region of the fly brain involved in multimodal sensory integration and regulation of internal states like aggression and hunger

(Alekseyenko & Kravitz, 2014; Xu et al., 2016). These findings suggest that the CSD neurons form circuits with MB neurons and other brain structures and may modulate approach/avoidance behaviors. To determine if the CSD neurons are involved in modulating activity in the open-field, the split-Gal4 MB465C was employed to drive expression of the neural effectors UAS-CsChR and UAS-Kir 2.1 to activate and inhibit these neurons respectively.

Optogenetic activation of the CSD neurons with MB465C>UAS-CsChR showed no between or within group differences in the first-minute distance means or total distance means (Figure 3.16A-B, all $p > .05$). Inhibition of the CSD with MB465C>UAS-Kir 2.1 showed no differences in first-minute distance and total distance means (Figure 3.16C-D, all $p > .05$). Path length means show no differences in habituation rates (Figure 3.15E, $p > .05$). Similar to the DAL neuron results these results suggest that the CSD neurons may not be involved in modulating habituation.

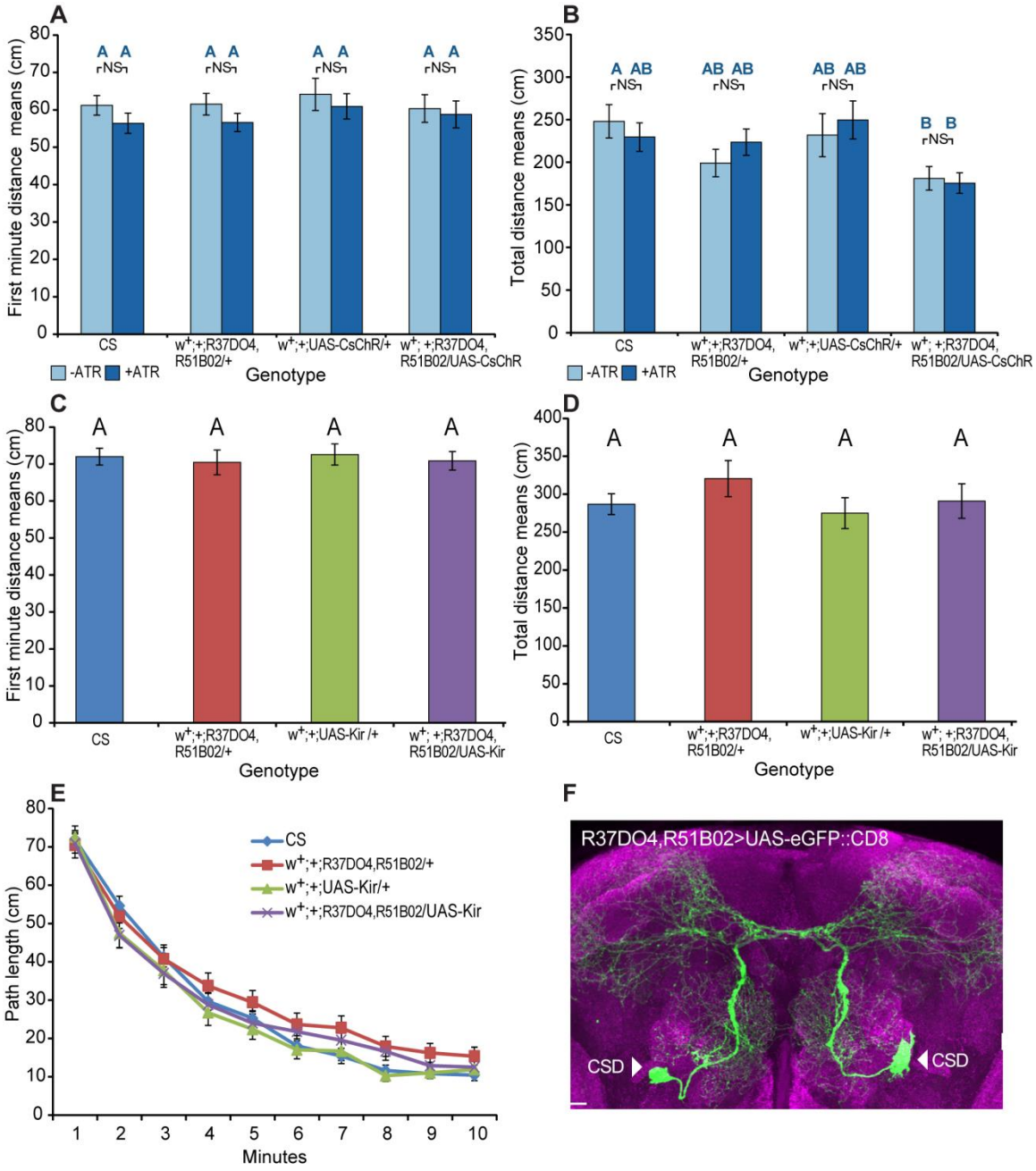

Figure 3. 16 **Activating or inhibiting the CSD neurons does not affect habituation.** (A-B) Activating or CSD neurons did not affect habituation in the first-minute means ($F_{7,414} = 0.52$, $p > .07$) or total distances means ($F_{7,414} = 3.459$, $p < .001$). (C-D) Inhibiting the CSD neurons did not affect habituation ($F_{3,250} = 0.12$, $p > .94$). (E) Path length means show no significant differences. (F) Expression of GFP reporter with MB456C Gal4 labels the somas of the CDS neurons and their neurites (image licensed under CC, Coates 2017). (NS $p > .05$, groups with the same letter are not different according to Tukey (HSD), Bonferroni correction error bars ±SEM).

3.2.6 Preliminary Transgenic Manipulation of 5-HT Neuron Clusters

5-HT positive neurons are located throughout the fly brain (Pooryasin & Fiala, 2015). Unlike the DPM, DAL or CSD neurons, which are large, paired neurons, with distinct neurites, the somas of many 5-HT positive neurons are clustered in discrete brain regions. Thermogenetic activation of these 5-HT positive neurons with UAS-TrpA1 promotes a general behavioral quiescence, specifically reducing feeding, courtship and locomotor behaviors (Pooryasin & Fiala, 2015). That activating these 5-HT positive neurons reduces feeding and courtship are relevant to this study because these are goal-directed behaviors, so it may be that specific 5-HT positive neurons in these clusters may reduce or stop the behavior after a goal is attained or drive is satiated. Similarly, after exploring a novel environment flies habituate to the novelty and reduce locomotor activity, this reduction in locomotor activity may be modulatory actions by some of these 5-HT positive neurons.

To investigate if the 5-HT positive neurons in these clusters are involved in habituation while flies explore, the Gal4 drivers R23E12, R35C08, R65D03, R67B05 and R7A011 were used in a pilot study to drive neural effectors to activate or inhibit the neurons targeted by these Gal4 drivers. These five Gal4 drivers include populations of 5-HT positive neurons in the PMVP, PMPD, PLP, LP, SEL and SEM but also include populations of non-5-HT positive neurons (Figure 3.18 A-G). Table 3.1 outlines where each Gal4 drives expression in the fly brain. Green plus signs are regions reported by Pooryasin 2015 and the red

plus signs are based on publically available immunohistochemistry images by Janelia Farms showing expression patterns of these Gal4 drivers. R67B05 Gal4 covers most brain regions shown, the remaining four Gal4 drivers target specific subsets of the same brain regions.

Table 3. 1 Neurons covered by Gal4s based on (+) Pooyarsin et al, 2015 reports and (+) FlyLight IHC imaging.

	Brain region									
Gal4	PMPV	PMPD	SEM	SEL	PLP	LP	MB	EB	OL	FB
R23E12		++			+	+				+
R35C08				++			+		+	
R65D03			++	++	++			+	+	
R67B05	++	+	++	++	+		+		+	
R7A011	++								+	

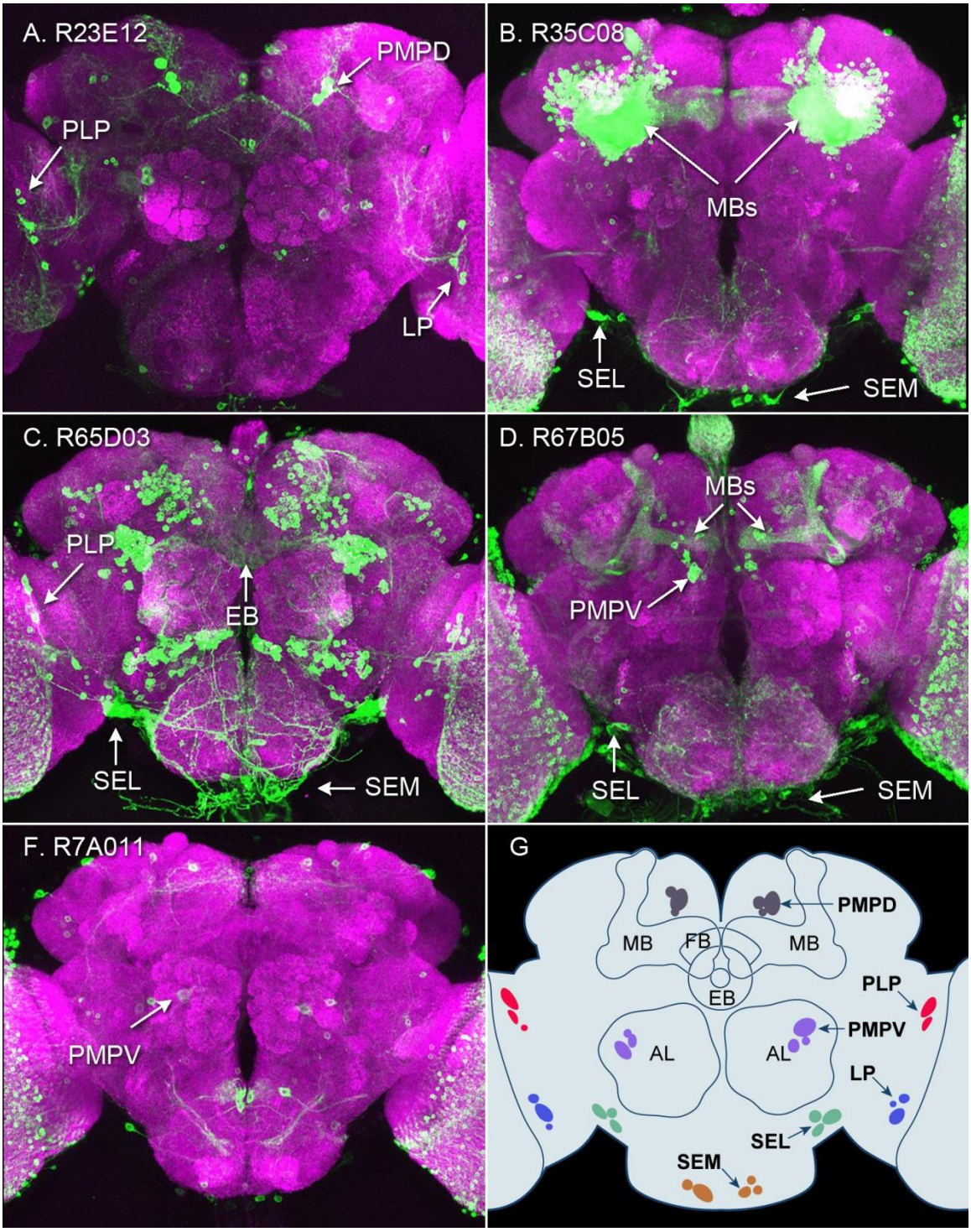

Figure 3. 17 **Expression patterns of Janelia Farms FlyLight Gal4 drivers.** (A-G) All images are Z-stacks. Gal4s include the 5-HT neuron clusters of interest as well as other neuron types. (F) Anatomical schematic showing all 5-HT neuron clusters. (All confocal images from Janelia Farm).

To activate the neurons covered by these Gal4 drivers the neural effector UAS-NaChBac was used. UAS-NaChBac is a bacterial Na^+ channel that excites neurons facilitating depolarization be leaking Na^+. To inhibit the same neurons, the UAS-Kir 2.1 neural effector was employed, UAS-Kir 2.1 is a K^+ inward rectifier that hyperpolarizes neurons making it more difficult to depolarize. Both neural effectors are constitutive.

First-minute distance means show locomotor activity differences only between activation and inhibition of neurons covered by R35C08 Gal4 and R67B05 Gal4 drivers (Figure 3.18A, $p < .0001$). However, the differences are opposite, whereas activating R35C08 Gal4 increases locomotor activity, activating R67B05 Gal4 decreases locomotor activity. The same opposing differences are seen when inhibition with neural inhibition using these GAL4 drivers. R35C08 Gal4 covers only the SEL brain region, a subset of that covered by R67B05 Gal4 (Table 3.1). The opposing differences could be explained by the activation of non-5-HT neurons in the SEL or other brain regions or possibly by activation of stimulatory or inhibitory 5-HT neurons simultaneously.

In the total distance means the activity differences with R67B05 persist and there are significant similar activity differences with R65D03 Gal4 (Figure 3.18B, $p < .0001$). R65D03 Gal4 covers neurons in the SEM, SEL and PLP regions, a subset of that covered by R67B05 Gal4 (Table 3.1). The total distance means suggest that activating or inhibiting neurons covered by R67B05 Gal4 and R65D03 Gal4 is sufficient and necessary to modulate activity. Of the subsets

shared by these GAL4 drivers neurons in the SEL show, no activity modulation in the total distance means. GAL4 drivers for only the SEM were not assayed in this study. The PLP brain region covered by both R67B05 Gal4 and R65D03 Gal4 is interesting because 5-HT neurons in the PLP arborize to the ventrolateral protocerebrum, a region known to integrate multisensory information that may modulate approach/avoidance behaviors. In addition, the PLP neurons may form circuits with the mushroom body peduncles (Alekseyenko & Kravitz, 2014).

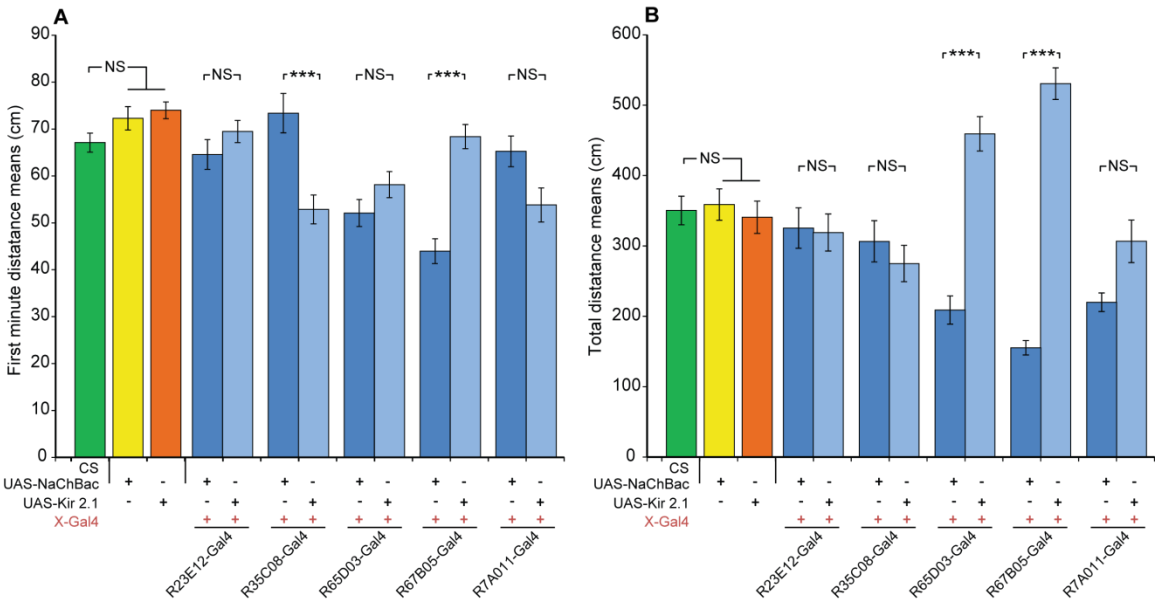

Figure 3. 18 **The PLP neurons may play a role in habituation modulation.** (A) R35CO8 Gal4 and R67B05 Gal4 display opposing modulatory effects on habituation when activated or inhibited ($F_{12,835} = 13.47$, $p < .0001$). (B) R65D03 Gal4 and R67B05 Gal4s modulate habituation when activated or inhibited ($F_{12,682} = 20.64$, $p < .0001$). (***$p \leq .0001$, NS $p > .05$, error bars ±SEM).

3.3 **Postsynaptic Component Screen**

Results from pharmacological and transgenic manipulation of 5-HT signaling suggest that 5-HT modulates habituation. Pharmacological activation of 5-HT signaling with 5-HTP decreased locomotor activity during exploration and

pharmacological inhibition with αMTP increased locomotor activity during exploration. However, transgenic activation or inhibition of Trh-positive neurons or specific 5-HT secreting neurons decreased locomotor activity, suggesting a physiological 5-HT signaling threshold that is flanked by the levels produced by the neural effectors.

5-HT modulates behaviors by binding to G-protein coupled receptors (GPCRs) or ligand-gated ion channels. Drosophila express five 5-HT GPCRs, 5-HT1A, 5-HT1B, 5-HT2A, 5-HT2B, and 5-HT7. All Drosophila GPCRs share substantial structural homology and function with vertebrate GPCRs in the same classes (Hauser, Cazzamali, Williamson, Blenau, & Grimmelikhuijzen, 2006; Saudou, Boschert, Amlaiky, Plassat, & Hen, 1992). The 5-HT1A receptor is well characterized as preferentially Gαi/o coupled, activation of which reduces intracellular cAMP levels through inhibition of adenylyl cyclase type I resulting in a hyperpolarization/inhibitory effect (Blenau & Thamm, 2011; Rojas & Fiedler, 2016). Signaling through 5-HT1A has presynaptic autoregulatory as well as postsynaptic modulatory functions (Hidalgo et al., 2017). In flies, the 5-HT1A receptor has been implicated in the regulation of sleep, feeding, aggression, locomotion, and is required for learning and memory (Albin et al., 2015; Johnson, Becnel, & Nichols, 2009, 2011; Silva et al., 2014; Yuan et al., 2006).

3.3.1 5-HT Receptor Mutants Display Habituation Defects

The 5-HT receptor mutants *5-HT1A*^{MB09978} and *5-HT1A*^{MB09812} were assayed to investigate a possible role for the 5-HT1A receptor in habituation. The

Drosophila 5-HT1A receptor is located on the right arm of the second chromosome spanning 54,803 nucleotides at sequence location 19067155-19121958. The *5-HT1A*^{MB09978} mutant has a Minos transposable element Mi(ET1) at the in the first intron and the *5-HT1A*^{MB09821} mutant has a Minos transposable element Mi(ET1) at the eighth intron (Figure 3.19). Stocks used in these experiments were homozygous for the mutation and were hypomorphs.

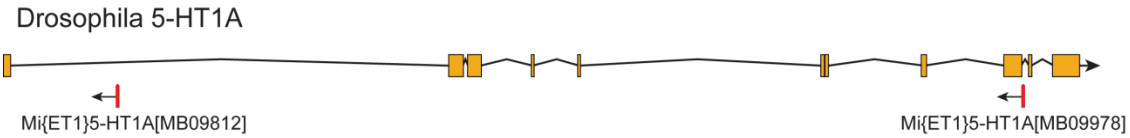

Figure 3. 19 **5-HT1A mutant.** Schematic of the Drosophila 5-HT1A gene showing the location of intronic transposon insertions MBO9812 and MB09978 (based on FlyBase GBrowse data).

Compared to CS controls the w^{+}; *5-HT1A*^{MB09978};+ and w^{+}; *5-HT1A*^{MB09812}; + mutants display increased locomotor activity suggesting habituation deficits. In the first-minute distance means comparisons between ±5-HTP groups show significant differences in the CS controls (Figure 3.20A $p < .001$), but not in the 5-HT1A mutants which show resistance to increased 5-HT signaling ($p > .05$). Within the vehicle condition, there are no differences among groups ($p > .05$), but within the 5-HTP condition, the CS control displays reduced locomotor activity ($p < .001$), but not the mutants, again indicating resistance to increased 5-HT signaling.

In the total distance means ±5-HTP comparisons show differences between the CS controls in response to 5-HTP treatment (Figure 3.20B, $p < .0001$) but not among the mutants ($p > .05$). Within the vehicle condition, the

mutant phenotype of increased locomotor activity compared to CS controls is present ($p < .0001$). Within the 5-HTP condition, the CS control responds to 5-HTP treatment but not the mutants indicating resistance to increased 5-HT signaling ($p < .0001$). In the vehicle condition, path length means show that mutants have increased activity or shallower habituation curves compared CS controls (Figure 3.20C, $p < .0001$). Within the 40 mM 5-HTP condition, the CS controls respond to 5-HTP but not the mutants.

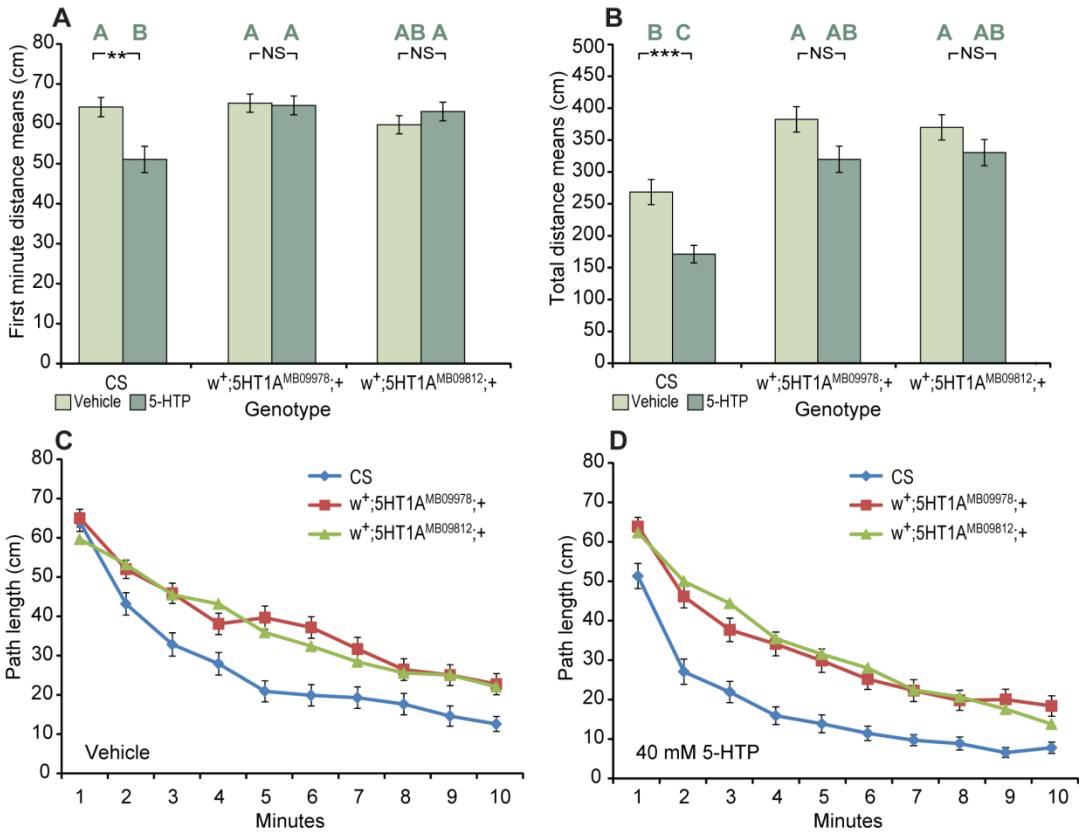

***Figure 3. 20* 5-HT1A receptor mutants display habituation defects.** (A) CS controls respond to increased 5-HT signaling, but not 5-HT1A receptor mutants ($F_{5,342} = 4.37$, $p < .001$). (B) The mutant phenotype is apparent in the total distance means ($F_{5,342} = 16.52$, $p < .0001$) (C) 5-HT1A receptor mutants display increased locomotor activity suggesting a habituation deficit, (D) and do not respond to increased 5-HT signaling. (**$p \leq .001$, ***$p \leq .0001$, NS $p > .05$, groups with the same letter are not different according to Tukey (HSD), Bonferroni correction, error bars ±SEM).

3.3.2 Antagonizing the 5-HT1A Receptor Reduces Habituation

WAY 100635 is a potent antagonist of the 5-HT1A receptor and agonist of the D4 receptor (Chemel, Roth, Armbruster, Watts, & Nichols, 2006; Fletcher et al., 1996; Marona-Lewicka, Chemel, & Nichols, 2009). In flies, WAY100635 is a selective 5-HT1A receptor antagonist as flies are not known to express the D4 receptor. CS flies fed 3 mM or 6 mM WAY100635 in the vehicle (36 h) display increased locomotor activity compared to vehicle only. In the first-minute distance means there are no statistical differences between conditions, though 6 mM WAY100635 shows a trend towards increased habituation (Figure 21A, $p > .05$).

However, in the total distance means flies fed 3 mM or 6 mM WAY100635 displayed significantly increased activity when compared to vehicle (Figure 21B, $p < .0001$). The habituation curves of the WAY100635 fed flies were similar to but more extreme than the 5-HT1A receptor mutant curves. Though not statistically different, it is interesting that the 3 mM dose of WAY100635 increases activity more when compared to the 6 mM dose. There may be a threshold where too little 5-HT signaling begins to decrease locomotor activity. Together the 5-HT1A mutant and 5-HT1A WAY100635 antagonist data indicate that the Drosophila 5-HT1A receptor may play a role in habituation.

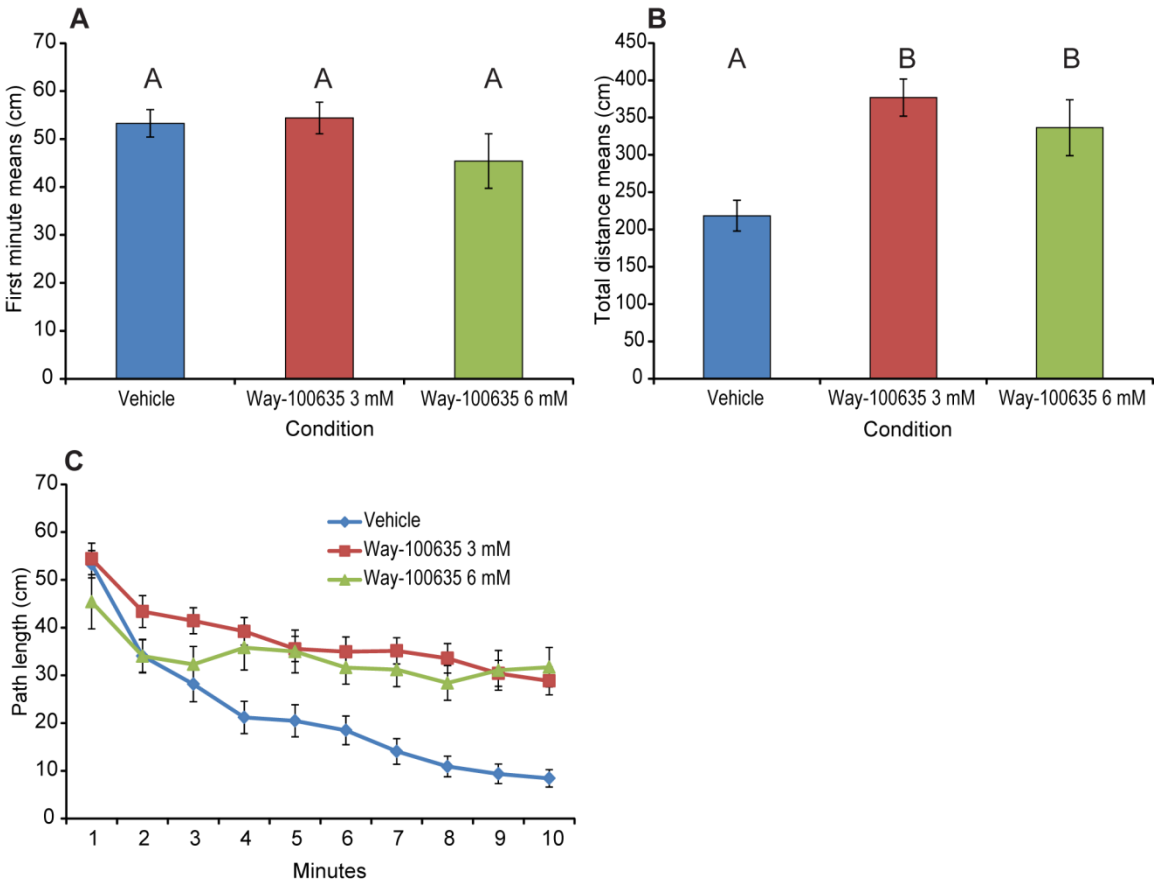

Figure 3. 21 **Antagonizing the 5-HT1A receptors with WAY100635 affects habituation.** (A) There are no differences between groups in the first-minute distance means ($F_{2,71} = 1.45$, $p > .05$). (B) In the total distance means WAY100635 treated groups display decreased habituation when compared to CS controls ($F_{2,71} = 10.66$, $p < .0001$). (C) WAY100635 treated groups have shallower rates of habituation when compared to controls. (Groups with the same letter are not different according to Tukey (HSD), Bonferroni correction and Dunnett test, error bars ±SEM).

3.3.3 The 5-HT1A Receptor is Expressed in the MBs

Promotion or activation of 5-HT signaling is sufficient to modulate activity in the open field arena. 5-HT1A receptor mutants display increased locomotor activity suggesting decreased habituation and antagonizing the 5-HT1A receptor mimics the habituation deficits seen in the mutants. Together, the work thus far

shows that 5-HT signaling through the 5-HT1A receptor may modulate habituation.

The 5-HT1A receptor is expressed in the peripheral and central nervous system of the fly. In the fly brain, the 5-HT1A receptor is expressed primarily in the OL, EB, and MBs (Saudou et al., 1992) (Blenau & Thamm, 2011). Expression of the 5-HT1A receptor in the MBs has been implicated in learning and memory, and the modulation of behaviors, such as aggression, olfaction, sleep, hunger, and mood (Albin et al., 2015; Johnson et al., 2009; Ogren et al., 2008; Ries et al., 2017; Tierney, 2001; Yuan et al., 2006).

To visualize the expression pattern of the 5-HT1A receptor in the fly brain a Gal4 under control of *5-HT1A* endogenous regulatory elements was employed using the *5-HT1A-T2-Gal4*^{MiMIC1468} transposon insertion to drive the UAS-mCD8::eGFP reporter responder. From posterior to anterior Figure 3.22: MB intrinsic neurons (Kenyon cells) somas (A), calices composed of dendritic arbors (B), peduncles, axonal projections (C) and all MB lobes (D) were clearly labeled by the GFP reporter, indicating expression of the 5-HT1A receptor in these structures

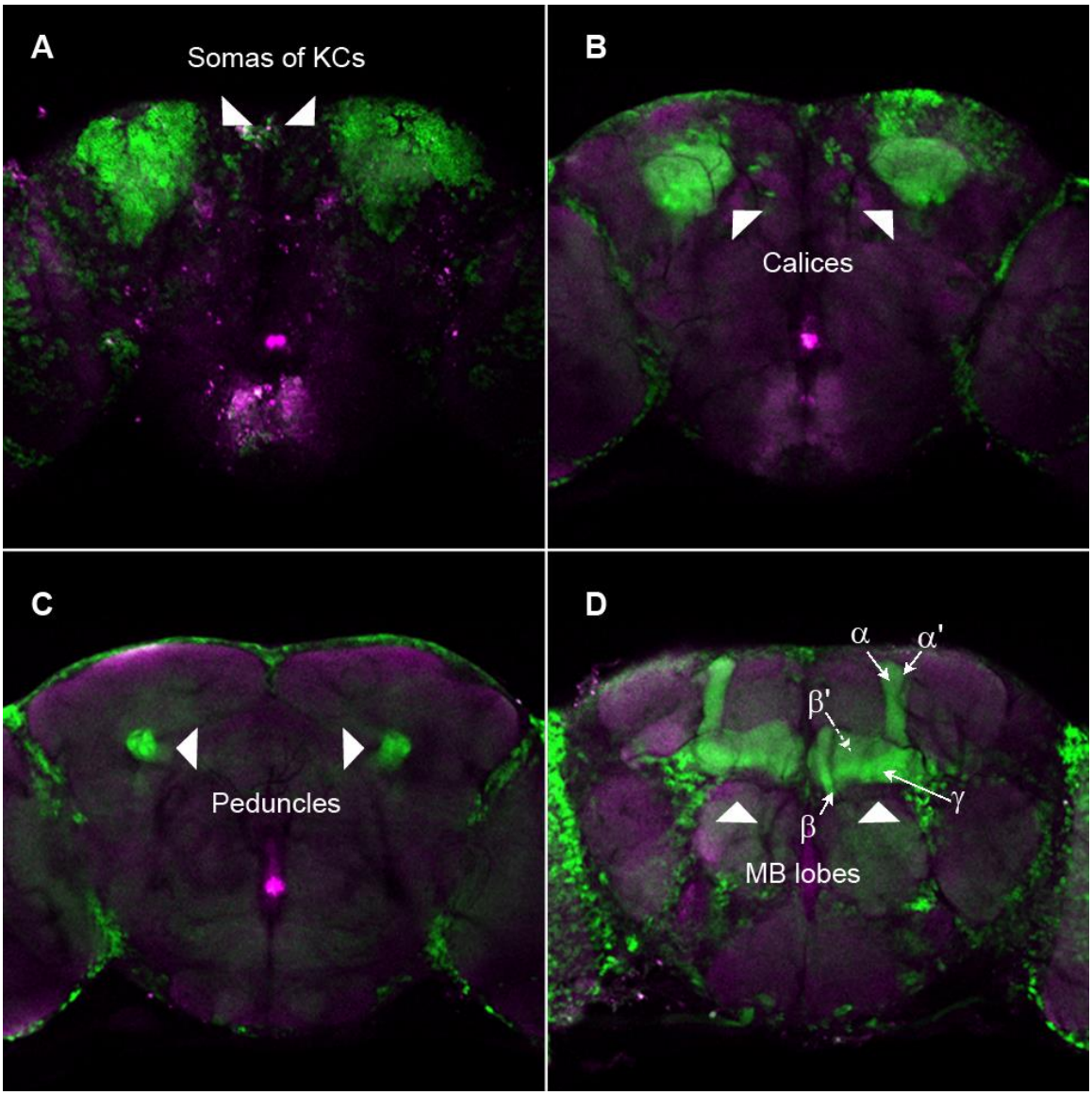

Figure 3. 22 **5-HT1A-T2-G4^{MiMIC1468}>UAS-mCD8::eGFP expression pattern.** Posterior to anterior: (A) Somas of MB intrinsic neurons (Kenyon cells), (B) calices (dendritic arbors) of MB intrinsic neurons, (C) peduncles (forward axonal projections of MB intrinsic neurons), (D) terminal bifurcations of axons into the α/β, α'/β' and γ lobes are all labeled with GFP suggesting expression of the 5-HT1A receptor in these structures.

To determine if the 5-HT1A receptor is required in the MBs for modulation of locomotor activity, a series of genetic rescue experiments were carried out where *5-HT1A* cDNA was expressed in the *5-HT1A^{MB09978}* mutant background.

The GAL4 drivers used to drive expression of *UAS-5-HT1A* were: Embryonic Lethal, Abnormal Vision (ELAV) Gal4, which drives pan-neural expression in postmitotic neurons in the central nervous system of the adult fly (Berger, Renner, Luer, & Technau, 2007).; 238Y-Gal4 which covers all of the MB intrinsic neurons, including α/β, α'/β', γ, C739 Gal4 drives expression primarily in the α/β MB intrinsic neurons; P247 Gal4 drives expression primarily in the α/β and γ MB intrinsic neurons (Figure 3.23A-C) (Aso et al., 2009).

In the following rescue experiments, CS flies are used as wild type controls as they display typical locomotor activity levels. Flies expressing the Gal4 and UAS transgenes are also homozygous for the *5-HT1A*^{MB09978} mutation and display the mutant phenotype of increased activity. The experimental rescue crosses between the Gal4 and UAS stocks are also *5-HT1A*^{MB09978} homozygous.

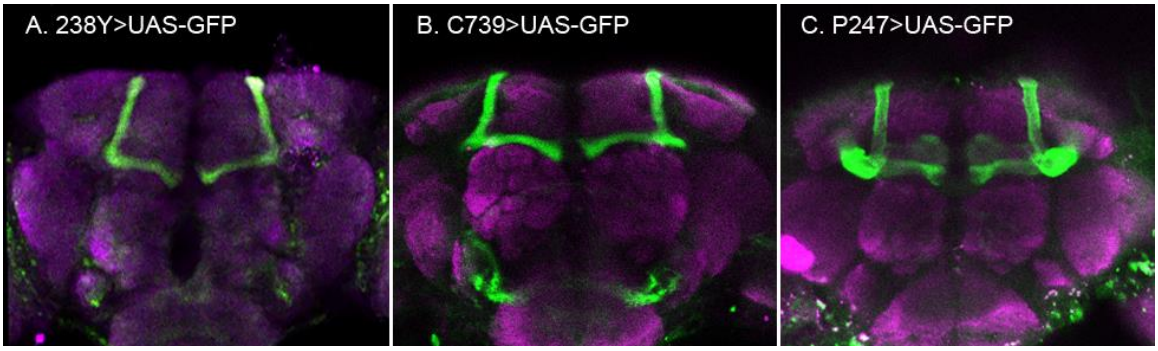

Figure 3. 23 **Expression patterns of MB Gal4s.** (A) 238Y Gal4 drives expression all MB intrinsic neurons, but mainly in α/β neurons. (B) C739 Gal4 drives expression in α/β neurons only. (C) P247 Gal4 drives expression in α/β and γ' MB intrinsic neurons.

3.3.4 The 5-HT1A Receptor is sufficient in the MBs for Habituation

3.3.4.1 Partial Rescue of Habituation by Pan-neural Expression of 5-HT1A cDNA.

The ELAV Gal4 was used as a preliminary rescue attempt because it drives expression in all CNS neurons, including MB intrinsic neurons. *UAS-5-HT1A* expression under control of the ELAV Gal4 in the 5-HT1A mutant background partially rescues the mutant phenotype. In the first-minute distance means there is no difference between the Gal4 and UAS controls and the experimental rescue group (Figure 24A, $p > .05$), though all three groups are significantly different from the CS control (Figure 32A, $p < .001$) possibly due to the mutant phenotype.

Much like the first-minute distance means, in the total distance means the Gal4 and UAS controls are not significantly different from the experimental group (Figure 32B, $p > .05$). However, unlike the first-minute distance means, in the total distance means there is also no difference between the CS control and the experimental rescue group ($p > .05$), and both Gal4 and UAS controls are significantly different from the CS control ($p < .001$) suggesting a partial rescue of locomotor activity.

The partial rescue can be seen in the faster habituation rate and in early spontaneous activity of the experimental rescue group (Figure 24C, purple line). The experimental rescue group begins at about the same locomotor activity means as the genetic controls (red and green) in the first-minute, but habituates

faster reaching almost the same means of the CS control (blue) by two minutes and keeps similar levels of spontaneous activity (Figure 24C).

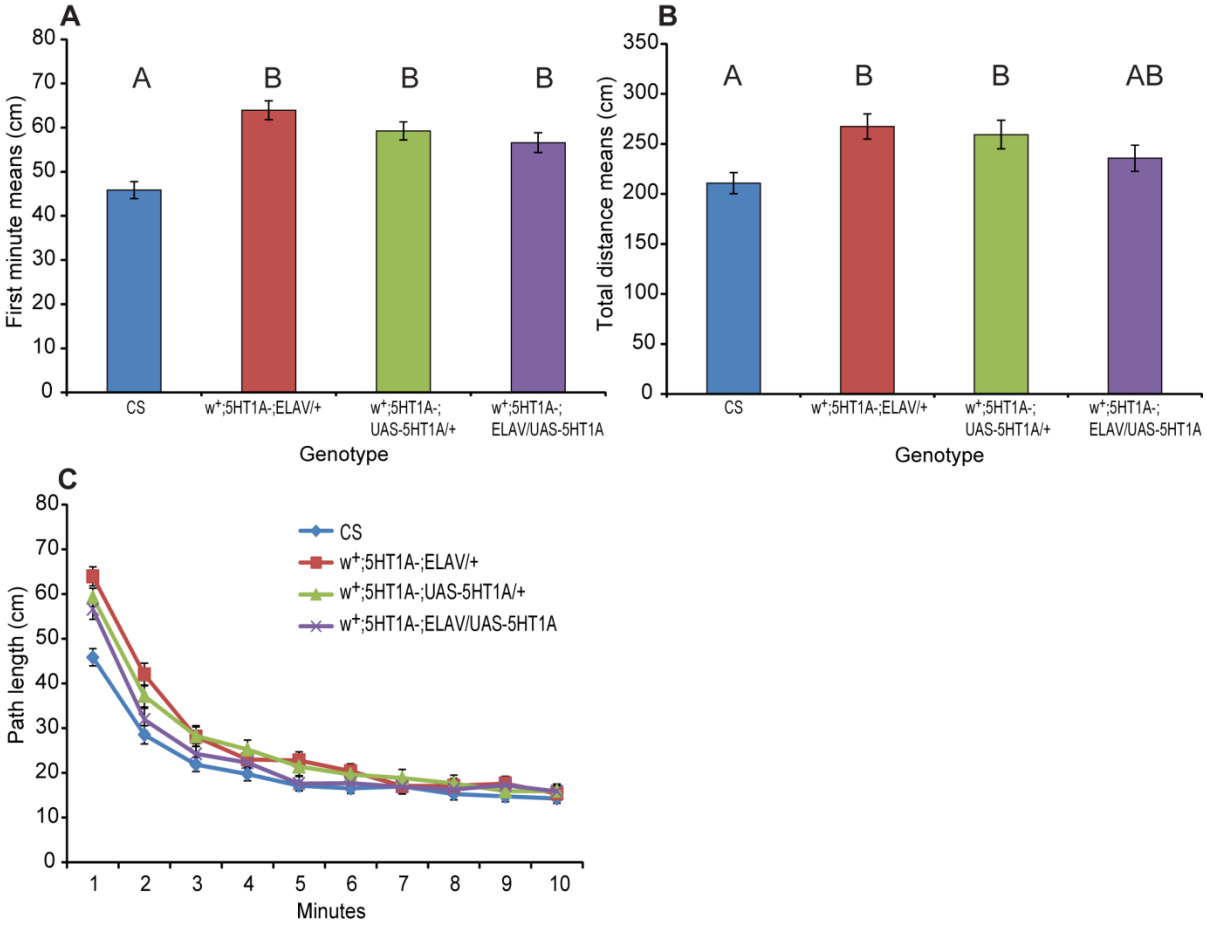

Figure 3. 24 **Partial rescue of habituation by pan-neural expression of 5-HT1A cDNA.** (A)In the first-minute distance means Gal4, UAS, and experimental groups are not different, but are all are different from the CS control ($F_{3, 234}$ = 13.869, $p < .0001$). (B) In the total distance means the Gal4, UAS, and experimental rescue group are not different, however, the experimental rescue group is also not different from the CS controls suggesting a partial rescue ($F_{3, 234}$ = 4.11, $p < .01$). (All groups with the same letter are not different according to Tukey (HSD), Bonferroni correction, and Dunnett test, error bars ±SEM).

3.3.4.2 Partial Rescue of Habituation by Expression of 5-HT1A cDNA in the MBs with 238Y Gal4

In the first-minute distance means there are no locomotor activity differences between any groups (Figure 25A, $p > 0.05$). The differences are more

apparent in the total distance means where the Gal4 and UAS controls are significantly different from the CS controls (Figure 25B, $p < .0001$), due to the mutant phenotype of increased locomotor activity. However, the experimental rescue group failed to reach statistical significance when compared Gal4 ($p = .026$) and UAS ($p = .015$) control. Nonetheless, there is also no difference between the CS controls and experimental group ($p > .05$)) suggesting a strong partial rescue.

The partial rescue is evident in the habituation curves seen in Figure 25C. While all groups begin about the same activity level (60-64 cm), the Gal4 and UAS controls (red and green) have shallower habituation rates typical of the mutants. The experimental and CS groups habituate at about the same rate, but the experimental group has higher levels of spontaneous activity which produces a partial rescue instead of a full rescue (Figure 25C).

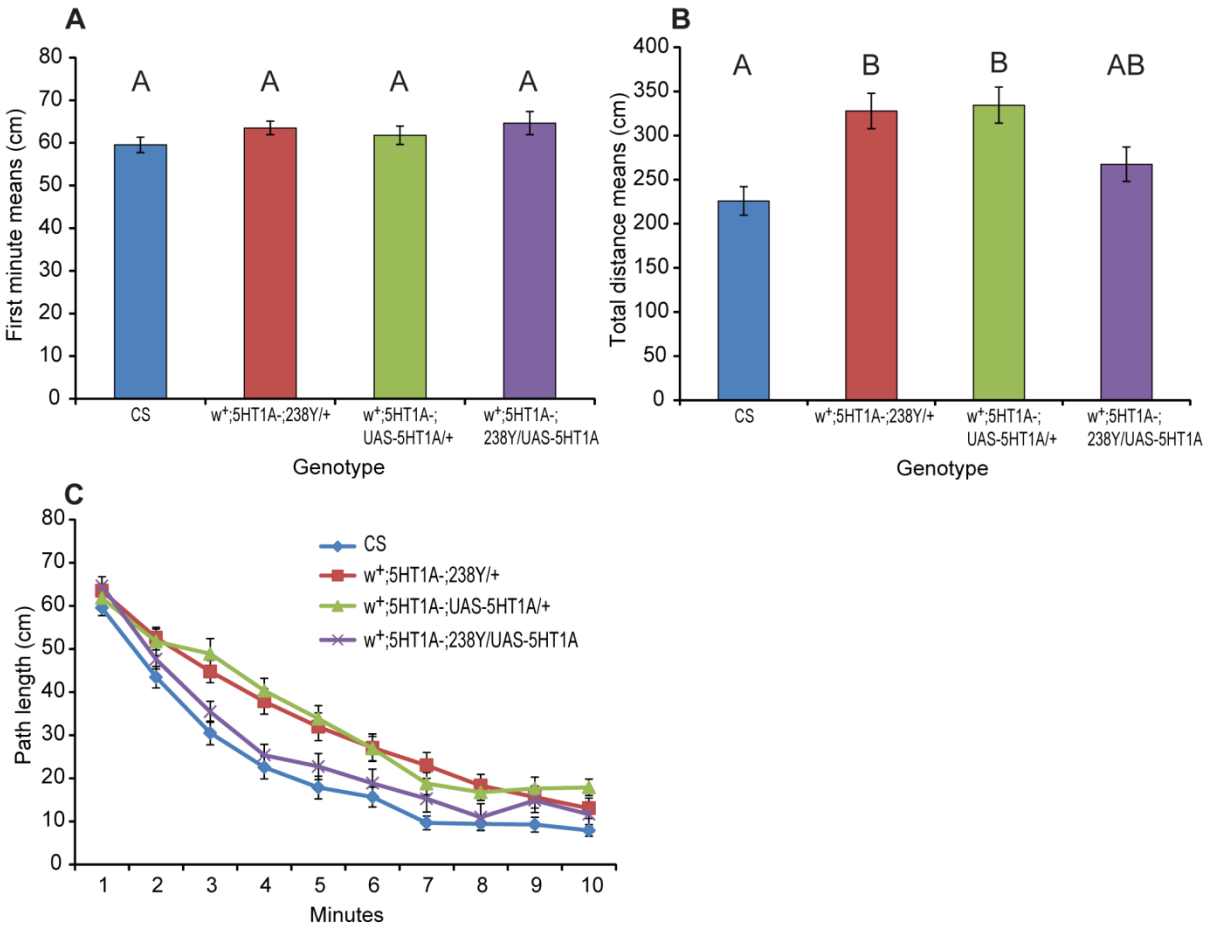

Figure 3. 25 **Partial rescue of habituation by 238Y-Gal4 driving expression of 5-HT1A cDNA.** (A) In the first-minute distance means all groups are the same. ($F_{3,165} = 1.12$, $p > .05$). (B) The total distance means shows a strong partial rescue ($F_{3,165} = 7.33$, $p < .0001$). (All groups with the same letter are not different according to Tukey (HSD), Bonferroni correction, and Dunnett test, error bars ±SEM).

3.3.4.3 Full Rescue of Habituation by Expression of *5-HT1A* cDNA in the MBs with C739 Gal4

C739 Gal4 driving *UAS-5-HT1A* produces a full rescue of normal activity in the first-minute means and total distance means. In the first-minute distance means the Gal4 and UAS controls display a clear mutant phenotype when compared to the CS control (Figure 3.26A, $p < .0001$) and the experimental

rescue group ($p < .0001$), but the CS control and experimental group are not different ($p > .05$) indicating a full rescue.

The total distance means mirror the first-minute distance means in that the CS and experimental rescue groups are not different (Figure 26B, $p > .05$) indicating rescue of locomotor activity. In addition, the CS and experimental groups are significantly different from the Gal4 control ($p < .001$) due to the mutant phenotype. However, the UAS control is not significantly different from the CS or experimental rescue group failing to reach statistical significance, nonetheless, there is a strong trend towards the mutant phenotype.

The Path length means show a clear rescue, as both the CS and experimental rescue group start at the same level and mirror each other through the 10-minute assay (Figure 3.26C). The Gal4 and UAS controls display the decreased habituation and increased activity typical of the mutant, though the UAS activity decreases to the of the CS controls in the spontaneous activity phase (Figure 3.26C).

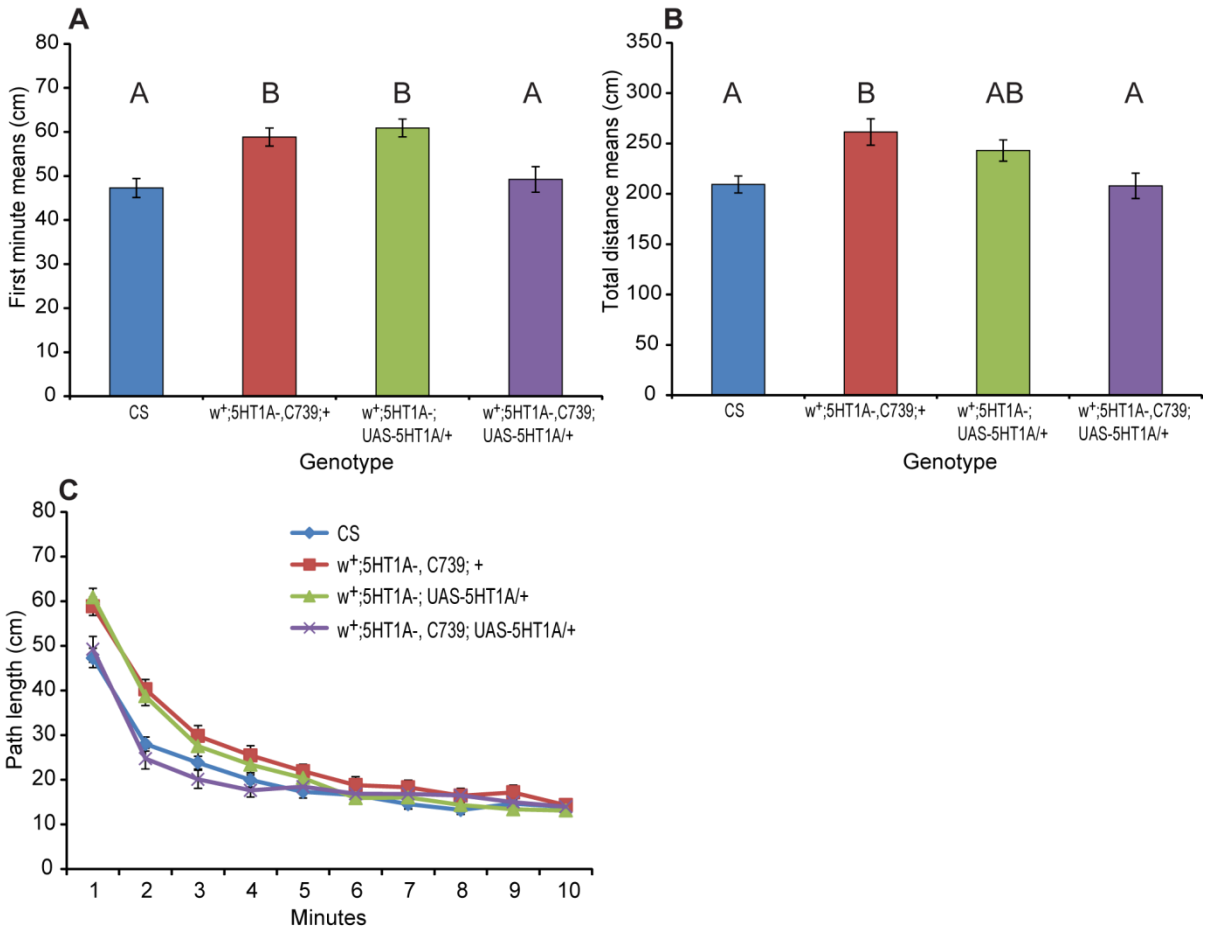

Figure 3. 26 **Rescue of habituation by C739-Gal4 driving expression of 5-HT1A cDNA.** Expression of 5-HT1A cDNA in the α/β lobes of the MBs with C739 Gal4 rescues normal habituation in first-minute means ($F_{3,203} = 8.84$, $p > .0001$). and total distance means ($F_{3,203} = 5.42$, $p > .001$). (C) The CS and experimental rescue group display similar habituation rates, which are dissimilar from the Gal4 and UAS controls. (N≥49, all groups with the same letter are not different according to Tukey (HSD), Bonferroni correction, and Dunnett test, error bars ±SEM).

3.3.4.4 Full Rescue of Habituation by Expression of 5-HT1A cDNA in the MBs with P247 Gal4

There are no substantive differences between groups in the first-minute distance means that would indicate a rescue (Figure 27A, $p > .05$). Nonetheless, in the total distance means the Gal4 and UAS controls display the mutant phenotype when compared to the CS controls (Figure 27A, $p < .0001$) and there

is no difference between the CS and experimental groups ($p > .05$) suggesting a strong rescue of locomotor activity levels.

Similar to the 238Y Gal4 partial rescue the habituation curves unmistakably show the mutant phenotype with shallower habituation rates in the Gal4 and UAS controls (Figure 27C). Even though the CS control starts at a higher activity mean the habituation rate is comparable to that of the experimental rescue group (Figure 27C).

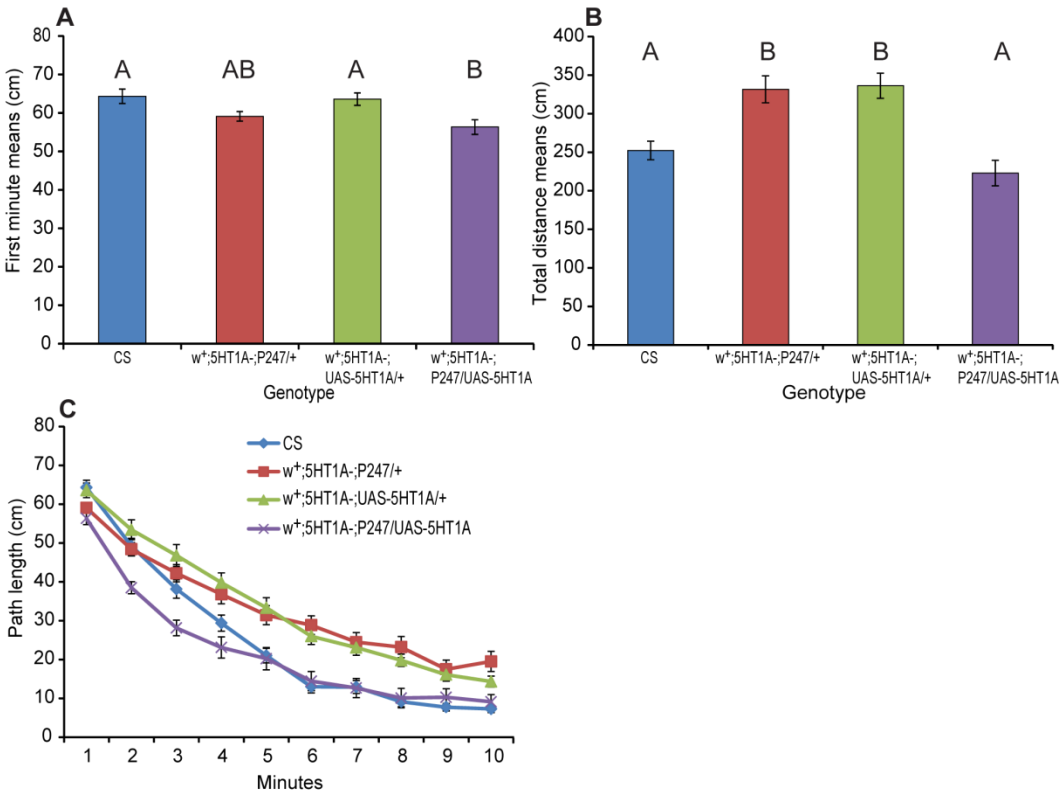

Figure 3. 27 **Rescue of habituation by P247-Gal4 driving expression of 5-HT1A cDNA.** Expression of 5-HT1A cDNA in the α/β and γ lobes of the MBs with P247 Gal4 shows no rescue in the (A) first-minute distance means ($F_{3,235} = 5.04$, $p < .002$). (B) Total distance means indicate a rescue habituation in the (B) total distance means ($F_{3,235} = 12.80$, $p < .0001$). (C) The habituation curves of the Gal4 and UAS are typical the mutant phenotype, while the CS and experimental display similar habituation curves. (All groups with the same letter are not different according to Tukey (HSD), Bonferroni correction, and Dunnett test, error bars ±SEM).

3.3.4.5 Overexpression of 5-HT1A cDNA in the MBs with 238Y Gal4 has No Phenotype.

In the first-minute distance means there are no consistent differences between groups as the CS control is the same as all other groups (Figure 28A, $p > .05$). Similarly, all groups in the total distance means are the same (Figure 28B, $p > .05$). All groups display similar habituation rates and slopes (Figure 28C).

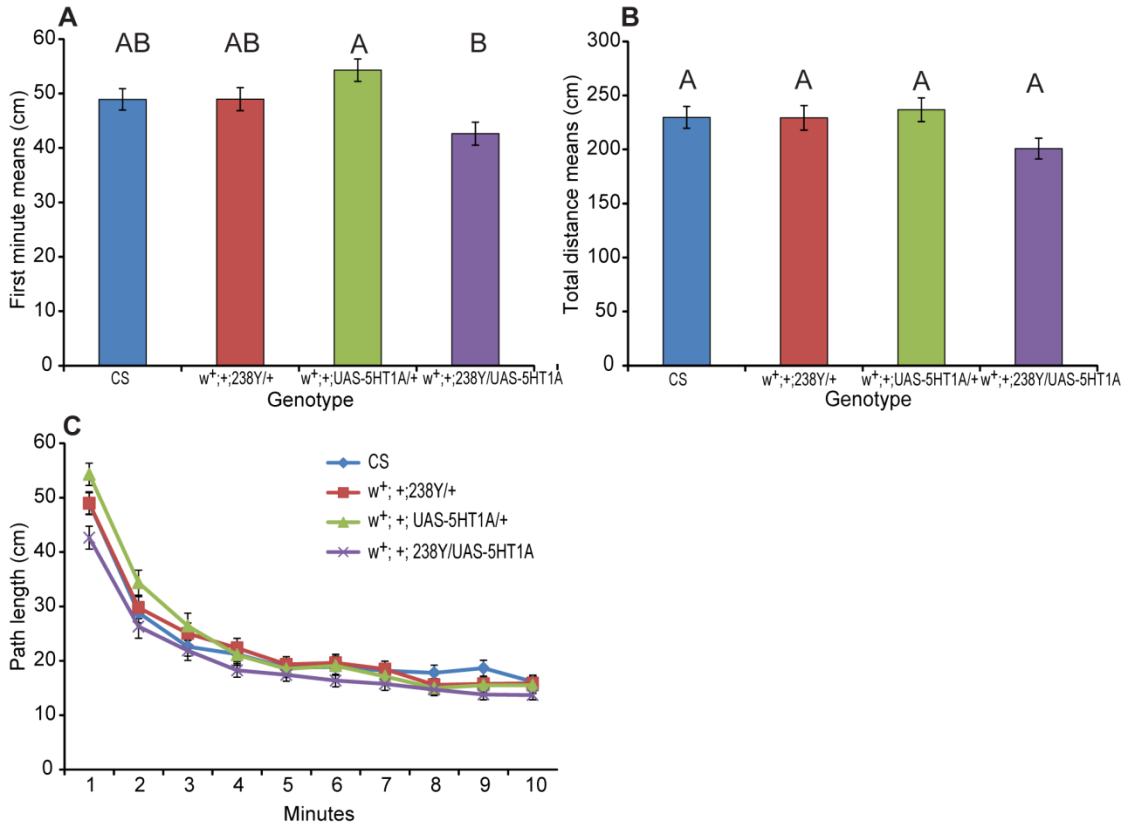

Figure 3. 28 **Overexpression of the 5-HT1A cDNA with 238Y Gal4 does not have a phenotype.** Overexpression of 5-HT1A cDNA in the α/β, α'/β' and γ lobes of the MBs with 238y Gal4 does not produce any differences between groups in the first-minute distance means ($F_{3,254} = 5.13$, $p < .002$). (B) Total distance means ($F_{3,254} = 2.30$, $p > .05$). (All groups with the same letter are not different according to Tukey(HSD) , Bonferroni correction, and Dunnett test, error bars ±SEM).

3.3.4.6 Overexpression of 5-HT1A cDNA in the MBs with C739 Gal4 has no Phenotype.

Overexpressing the 5-HT1A receptor with C739 Gal4 produces similar results as overexpression with 238Y Gal4. Both Gal4s cover α/β MB intrinsic neurons, but 238Y Gal4 targets additional MB intrinsic neurons. There are no consistent differences between groups in the first-minute distance means (Figure 29A, $p > .05$). All groups in the total distance means are the same (Figure 29B, $p > 0.5$). All groups display similar habituation rates (Figure 29C).

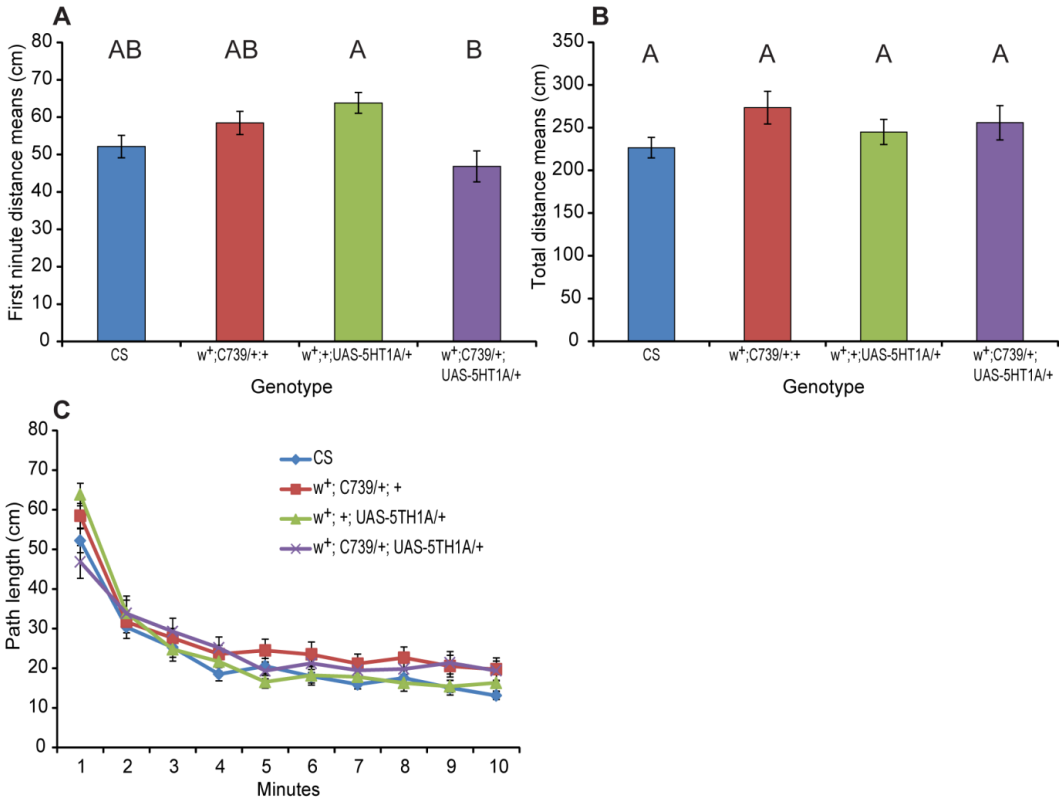

Figure 3. 29 **Overexpression of the 5-HT1A cDNA with C739 Gal4 does not have a phenotype.** Overexpression of 5-HT1A cDNA in the α/β lobes of the MBs with C739 Gal4 does not produce any differences between groups in the first-minute distance means ($F_{3,119} = 4.84$, $p < .003$) and total distance means. ($F_{3,119} = 1.36$, $p > .05$). (All groups with the same letter are not different according to Tukey (HSD), Bonferroni correction, and Dunnett test, error bars ±SEM).

3.3.4.7 Overexpression of 5-HT1A cDNA in the MBs with P247 Gal4 has no Phenotype.

Overexpression of the 5-HT1A receptor in the MBs with P247 Gal4 does not produce a discernable phenotype. P247 Gal4 targets the α/β and γ MB intrinsic neurons. In the first-minute and total distance means there are no consistent differences between controls and the experimental group. (Figure 28A-B, $p > .05$). Path length curves are not different (Figure 28C).

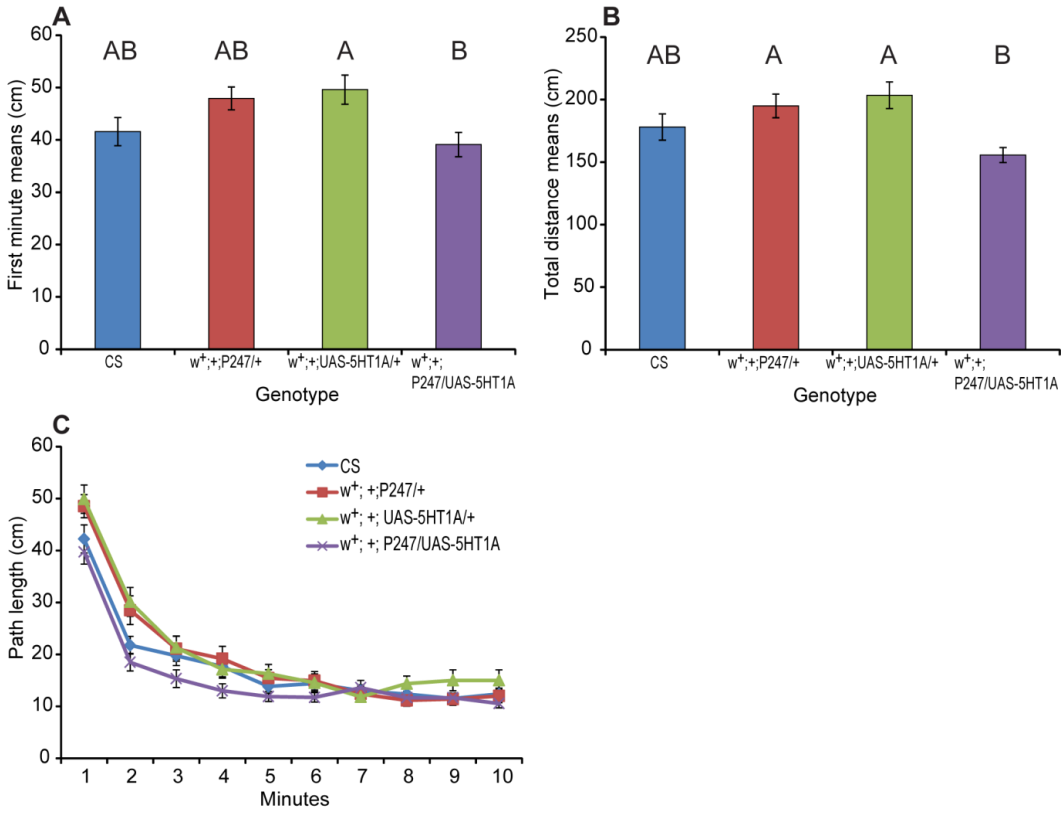

Figure 3. 30 **Overexpression of the 5-HT1A cDNA with P247 Gal4 does not have a phenotype.** Overexpression of 5-HT1A cDNA in the α/β and γ lobes of the MBs with P247 Gal4 does not produce any differences between groups in (A) the first-minute distance means ($F_{3,142} = 3.80$, $p < .01$). or (B) total distance means ($F_{3,119} = 5.05$, $p < .01$). (All groups with the same letter are not different according to Tukey (HSD), Bonferroni correction, and Dunnett test, error bars ±SEM).

3.3.5 The 5-HT1A Receptors are Expressed in the Ellipsoid Body

The ellipsoid body (EB) of the central complex is a prominent neuropil in the fly midbrain involved in locomotor regulation, flight coordination, visual place learning and visual pattern learning (Ilius et al., 1994; J. P. Martin, Guo, Mu, Harley, & Ritzmann, 2015; Ofstad et al., 2011; Pan et al., 2009). The EB is formed by the concentric, laminar layering of EB neuron axons forming connections in distinct glomeruli rings (R1 through R4 rings) (Martín-Peña et al., 2014).

EB neurons express different types of 5-HT receptors including the 5-HT1A receptor (Martín-Peña et al., 2014; Sitaraman et al., 2008). The *5-HT1A-T2-Gal4*^{MiMIC1140} and *5-HT1A-T2-Gal4*^{MiMIC1468} insertions in the *5-HT1A* locus were used to drive the UAS-mCD8::eGFP responder. The R2-R4 EB ring neurons are labeled (Figure 3.31A and B) indicating expression of the 5-HT1A receptor in these structures.

To determine if the 5-HT1A receptor is required in the EB for habituation, genetic rescue experiments were carried out where *5-HT1A* cDNA was expressed in the *5-HT1A*^{MB09978} mutant background in the EB R2 and R4 ring neurons using 5.30 Gal4 and C819 Gal4 (Figure 3.31C and D).

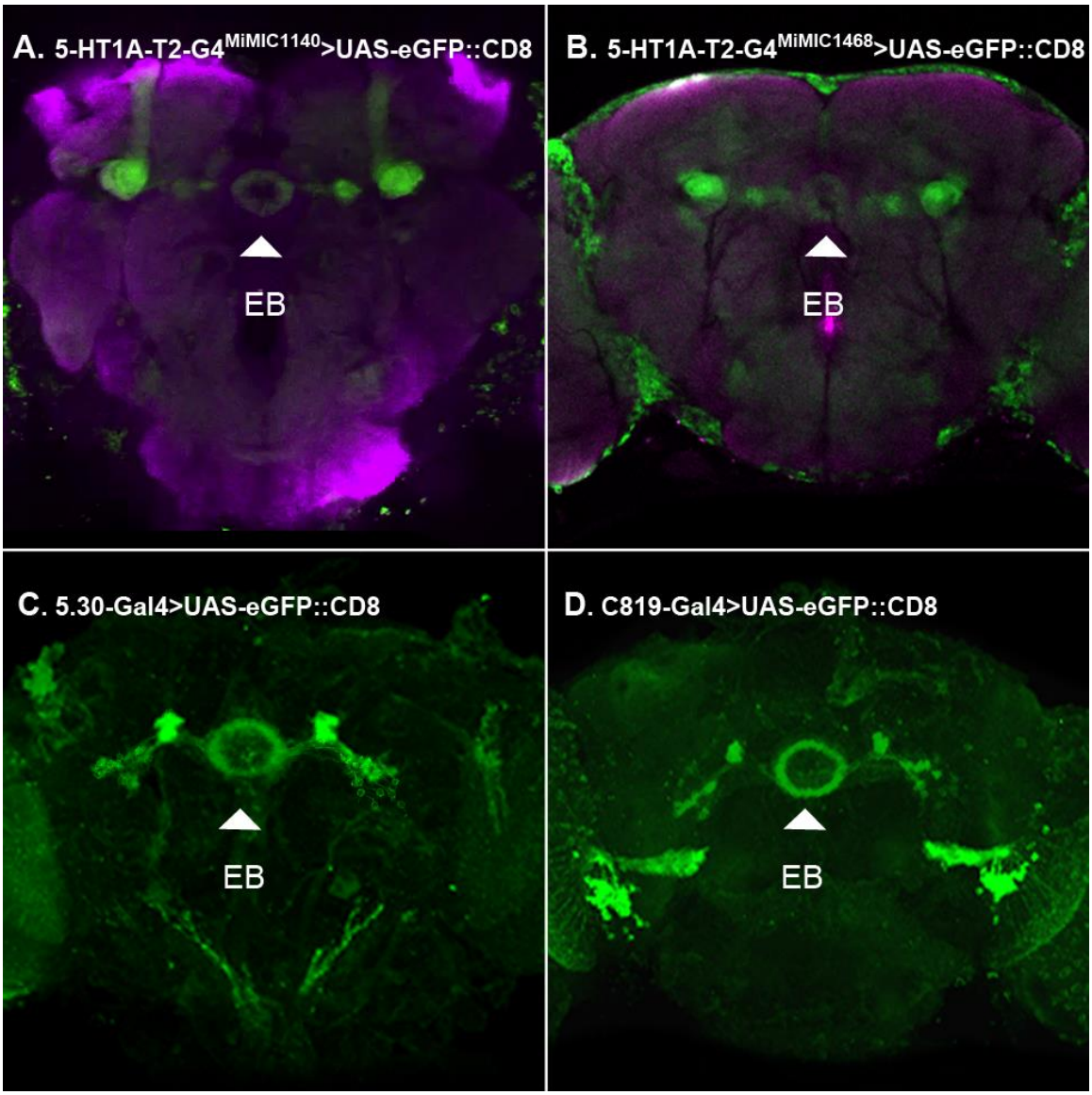

Figure 3. 31 **5-HT1A receptor is expressed in the EB.** (A-B) The EB R2 and R4 ring neurons are labeled with GFP suggesting expression of the 5-HT1A receptor in these structures. (C-D) The Gal4 drivers 5.30 and C819 were used to drive expression of the 5-HT1A cDNA in the mutant background to attempt a rescue.

3.3.5.1 Expression of 5-HT1A cDNA in the EB R2/R4 Neurons with C819 Gal4 does not Rescue Habituation

The first-minute distance means and total distance means produced similar results. In both measures, the 5-HT1A mutant phenotype is present in the

Gal4 and UAS controls when compared to the CS group (Figure 3.32A $p < .01$), but the experimental rescue group is not different from the Gal4 and UAS controls ($p > .05$). In the path length means the Gal4 and UAS controls and experimental are grouped together when compared to the CS control indicating no rescue of locomotor activity.

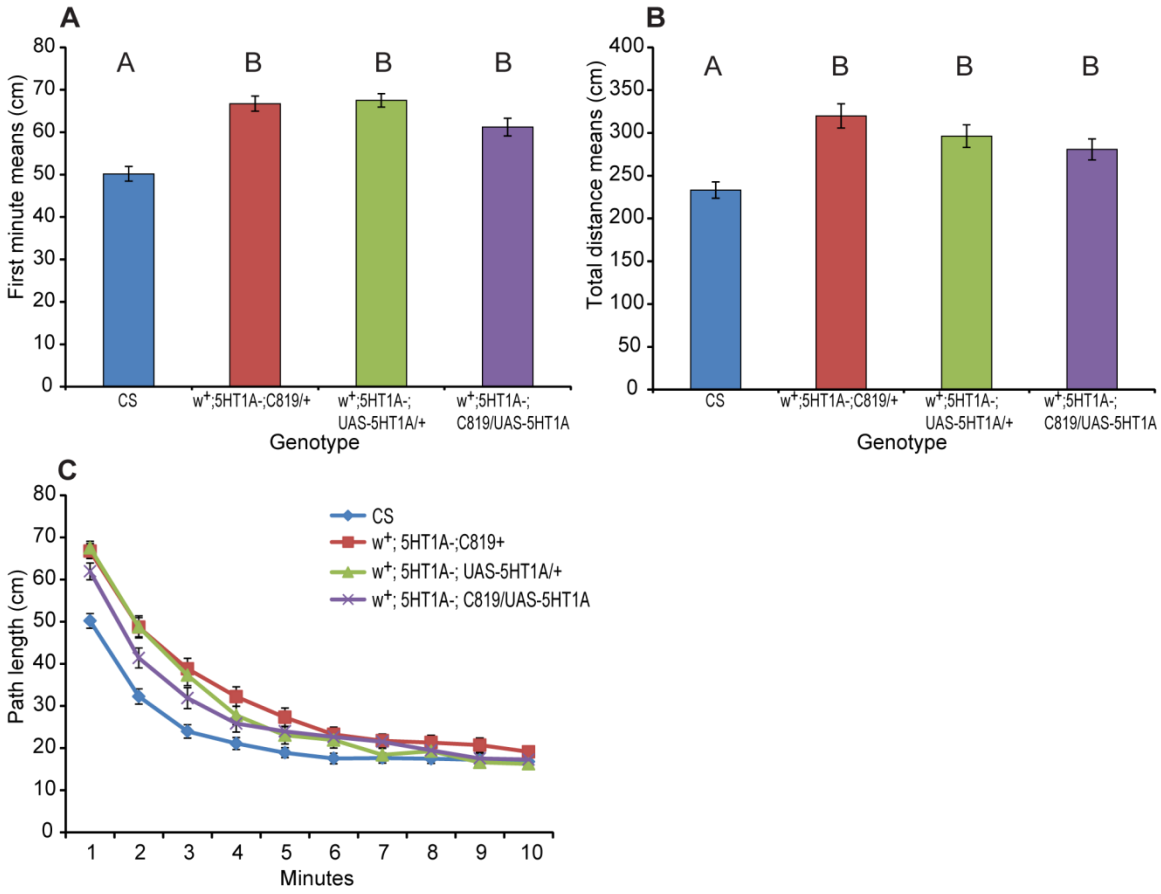

Figure 3. 32 **Expression of the 5-HT1A cDNA in the EB with C819 Gal4 does not rescue habituation.** (A) First-minute distance means ($F_{3,239} = 20.142$, $p < .0001$) and total distance means ($F_{3,239} = 20.142$, $p < .0001$) show no rescue as the genetic controls are not different from the experimental group. (C) The mutant phenotype is present in the curves of the Gal4 and UAS controls and experimental group indicating no rescue. (All groups with the same letter are not different according to Tukey (HSD) , Bonferroni correction, and Dunnett test, error bars ±SEM).

3.3.5.2 Expression of 5-HT1A cDNA in the EB R2/ R4 neurons with 5.30 Gal4 Rescues Habituation in the First-minute Distance Means but not in the Total Distance Means

The first-minute distance means in this experiment indicate a rescue of habituation in the initial phase (Figure 3.33A $p < .0001$), as the CS and experimental groups begin at the same place and have nearly identical curves and the genetic controls show the mutant phenotype (Figure 33C). However, while the total distance means suggest a rescue similar to that in the first-minute distance means, the differences do not reach statistical significance (Figure 3.33B, $p > .05$).

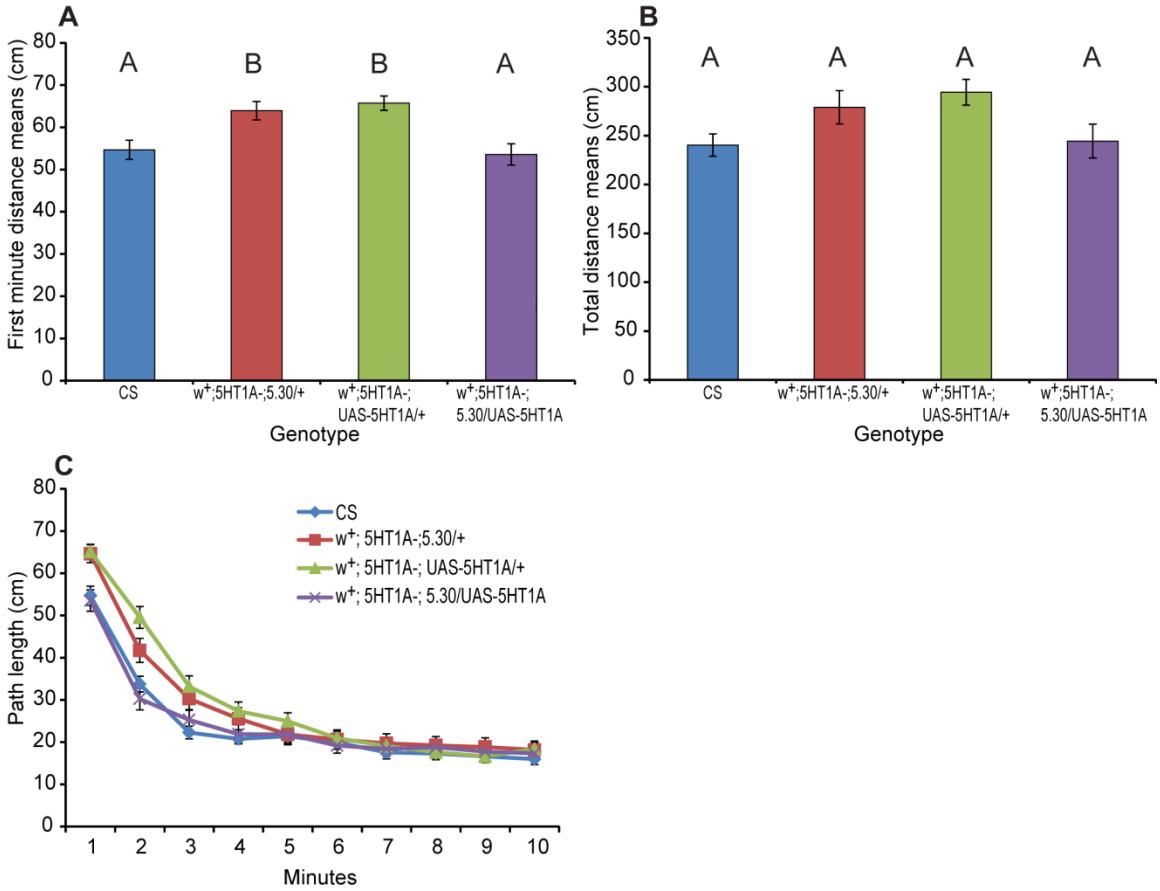

Figure 3. 33 **Expression of the 5-HT1A cDNA in the EB with 5.30Gal4 rescues early habituation** (A) First-minute distance means suggest a rescue of habituation in the initial phase ($F_{3,205} = 8.11$, $p < .0001$) (B) Total distance means also indicate a rescue but does not reach significance ($F_{3,205} = 3.19$, $p < .025$). (C) The CS and experimental group's curves are nearly identical. (All groups with the same letter are not different according to Tukey(HSD), Bonferroni correction, and Dunnett test, error bars ±SEM).

3.3.5.3 Overexpression of 5-HT1A cDNA in the EB R2 and R4 Neurons with C819 Gal4 has no Phenotype

Both first-minute distance means and total distance means produced very similar results. In both measures the experimental groups show a non-significant trend towards decreased locomotor activity, suggesting a possible phenotype of 5-HT1A overexpression (Figure 3.34A-B, $p < .05$). The difference between the

UAS control and other groups is likely due to behavioral variability. Figure 3.34C shows all curves generally grouped over the entire 10 minutes. However, from minute 1 to minute 4 the experimental group appears to habituate faster.

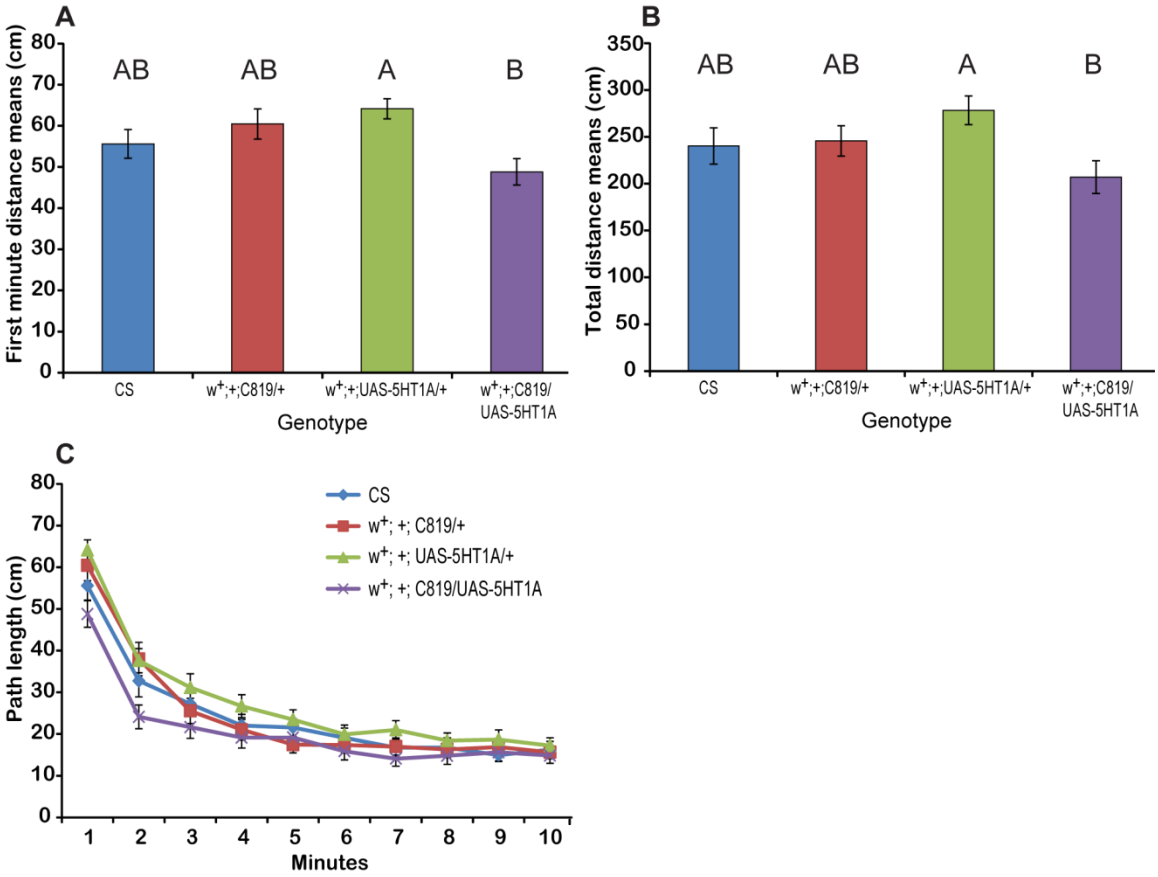

Figure 3. 34 **Overexpression of the 5-HT1A cDNA in the EB with C819 Gal4 does not have a phenotype.** (A) First-minute distance means ($F_{3,123} = 4.37$, $p < .001$) and total distance means ($F_{3,123} = 3.02$, $p < .05$) do not show any difference between groups. (C) All groups in the distance means show similar rates of habituation. (All groups with the same letter are not different according to Tukey(HSD), Bonferroni correction, and Dunnett test, error bars ±SEM).

3.3.5.4 Overexpression of 5-HT1A cDNA in the EB R2 and R4 Neurons with 5.30 Gal4 has no Phenotype

Similar to the C819 Gal4 overexpression experiment there are no differences between groups in the first-minute distance means and total distance means. In this case, the similarities between groups are more pronounced (Figure 3.34A $p > .05$). Figure 3.34C shows all curves tightly grouped, displaying similar habituation rates.

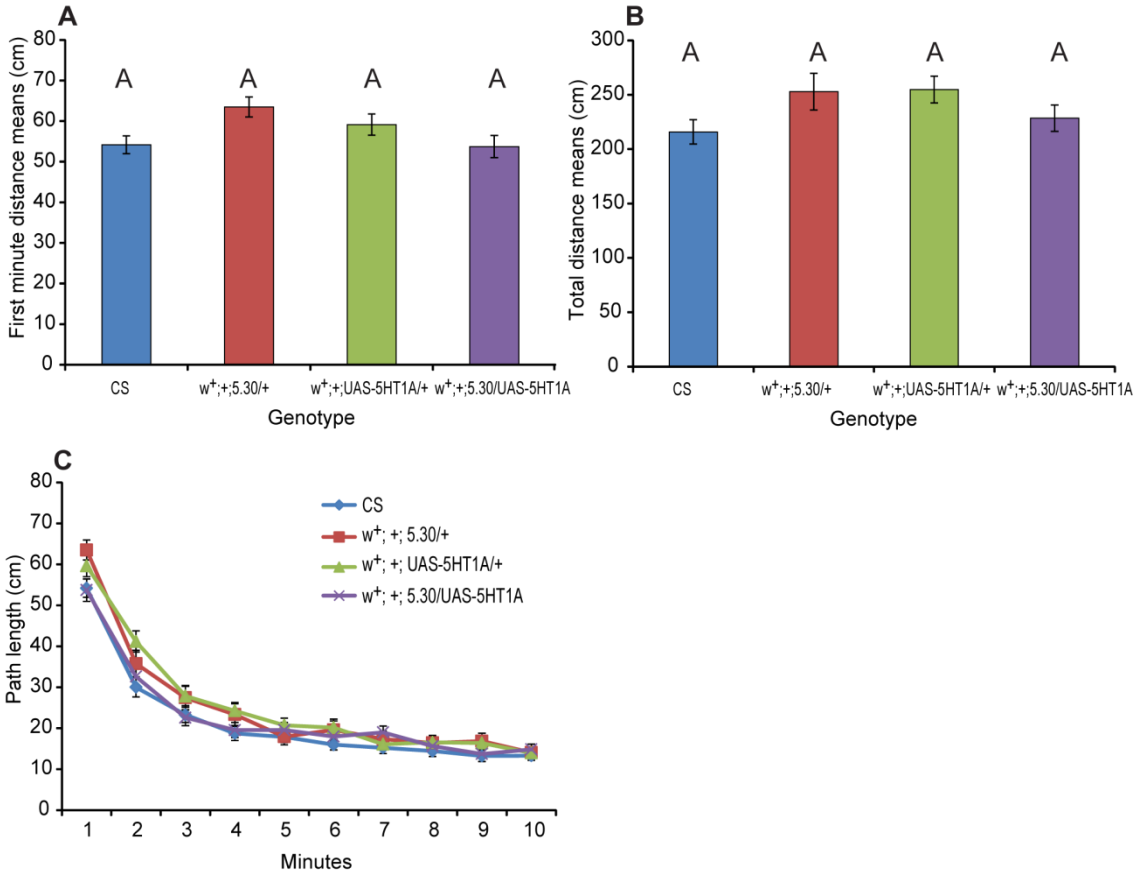

***Figure 3. 35* Overexpression of the 5-HT1A cDNA in the EB with 5.30 Gal4 does not have a phenotype.** (A) First-minute distance means ($F_{3,212} = 2.94$, $p = .034$) and total distance means ($F_{3,211} = 2.03$, $p > .05$) do not show any difference between groups. (C) All groups in the distance means show similar rates of habituation. (All groups with the same letter are not different according to Tukey (HSD), Bonferroni correction, and Dunnett test, error bars ±SEM).

3.3.6 Visual Detection of Predators Increases Exploration in *Drosophila*

Prey animals that successfully detect and avoid predators have an adaptive advantage. The behaviors in which prey animals may engage to avoid predation include combinations of fleeing, freezing or fighting back (Cooper, Goldenberg, & Arndt, 2010; Eilam, 2005; McNaughton & Corr, 2004; Stankowich & Blumstein, 2005). Fleeing behaviors may involve locomotor exploration to search for shelter or escape (L. Liu et al., 2007; Soibam, Shah, Gunaratne, & Roman, 2013). Interestingly, many anti-predator behaviors are innate, unlearned responses conferring upon the naïve prey animal the ability to recognize and avoid predators (Adamo, Kovalko, & Mosher, 2013). In contrast, responses to predatory threats may be altered through experience. For example, prey animals may habituate to the presence of predators, thereby reducing metabolically costly and unnecessary defensive behaviors (Herberholz & Marquart, 2012). Defensive behaviors may depend on predator type, predator behavior and predator proximity. To understand better how prey behave in the presence of predatory threat, the following investigates how fly behavior is affected by the presence of a predator. Wild type CS flies used in the following experiments were reared for thousands of generations in laboratory conditions in the absence of predators.

In the following experiments standard 8.2 cm diameter arenas were machined to hold a 4.5 cm diameter centrally located cage fabricated out of Nylon Nitex mesh. The cages allowed predators and flies to detect each other through most sensory cues like vision and olfaction. The area outside the cage

was digitally separated into outer and inner zones (Figure 3.36A). Avoidance was measured by tracking the fly's positional preferences with and without a predator present in the cage. Three predators were assayed, Pantropical jumping spider (*Plexippus paykulli*), Twin-flagged jumping spider (*Anasaitis canosa*) and the Texas unicorn mantis (*Phyllovates chlorophaena*). The Milkweed bug (*Oncopeltus fasciatus)* was used as a non-predator control.

In the presence of a Pantropical jumping spider flies spent less time in the middle zone, adjacent to the cage when compared to an empty cage (Figure 36B, $p = .008$). When the smaller Twin-flagged jumping spider occupied the cage, flies spent about the same time in in the middle zone when compared to a vacant cage ($p = .419$). The Texas unicorn mantis produced similar positional preference as the Pantropical jumping spider with flies spending less time in the middle zone in the presence of the mantis compared to an empty cage ($p = .021$). The control Milkweed bug elicited no significantly different positional preferences from flies ($p = .808$). These results demonstrate that naïve flies detect and avoid the Pantropical jumping spider and Texas unicorn mantis when compared to an empty cage, but not the Twin-flagged jumping spider or control Milkweed bug. That naïve flies that have been raised in laboratory conditions in the absence of predators for decades can still detect and avoid is an important finding and supports existing work in other organisms.

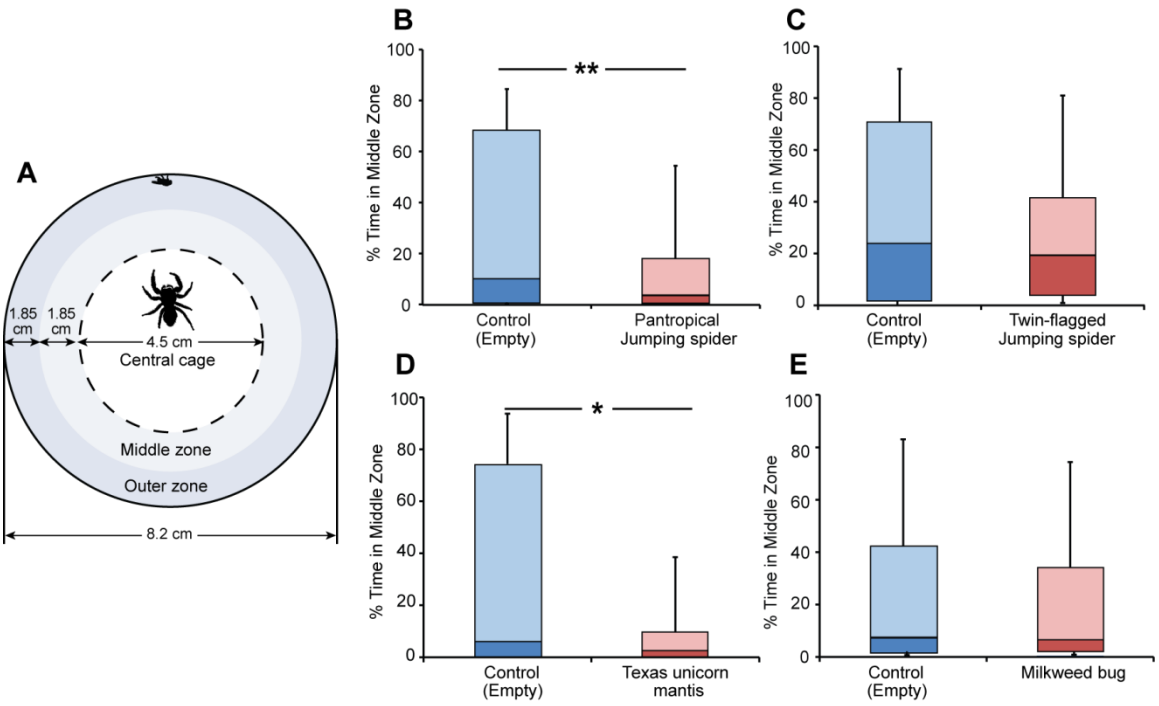

Figure 3. 36 **Drosophila avoid predators.** (A) Schematic of arena set-up. A Nylon Nitex central cage housed predators. The outer arena was digitally separated into an outer and middle zone. Avoidance was measured by the amount of time spent in the middle zone. (B) CS flies spent less time in the middle zone in the presence of a Pantropical jumping spider (U=6413.5, N=126, p = .008), and the (D) Texas unicorn mantis (U=5389, N=95, p = .021), but not in the presence of the (C) Twin-flagged jumping spider (U = 8671.5, N=128, p = .419). (E) Milkweed bug controls did not affect positional preference (U = 5825, N=107, p = .808). (** p < .01. Box plot midline represents the median, upper box 3rd quartile and lower box 2nd quartile, whiskers are 90% confidence levels).

Previous work in our laboratory has shown that flies explore and loiter in outward-projecting alcoves built into the wall of circular arenas (Soibam, Mann, et al., 2012). This behavior may represent a drive to locate sheltered areas in the environment. As a result, it was hypothesized that in the presence of a predator, flies may prefer to be in the alcove to increase distance from the threat posed by the predator. In this experiment an arena with an outward-projecting 1.2 cm x 1.5 cm alcove built into the outer wall was used (Figure 3.37A). Flies introduced into

the arena in the presence of a caged Pantropical jumping spider spent significantly more time in the alcove when compared to an empty cage ($p = .001$) indicating that wild type flies detect and avoid predators. However, flies exposed to the other two predators, the Twin-flagged jumping spider and Texas unicorn mantis did not show statistically different positional differences in the presence of the predator or empty cage ($p > .05$). These data show that naïve flies detect and avoid predators, and that flies innately seek to maximize distance between themselves and predators. These findings offer support to an existing body of work that demonstrates that many prey species exhibit innate anti-predator behaviors (Fendt, 2006; Hawkins, Magurran, & Armstrong, 2004; Kindermann, Siemers, & Fendt, 2009; Veen, Richardson, Blaakmeer, & Komdeur, 2000; Zheng, Kashimori, Hoshino, Fujita, & Kambara, 2005).

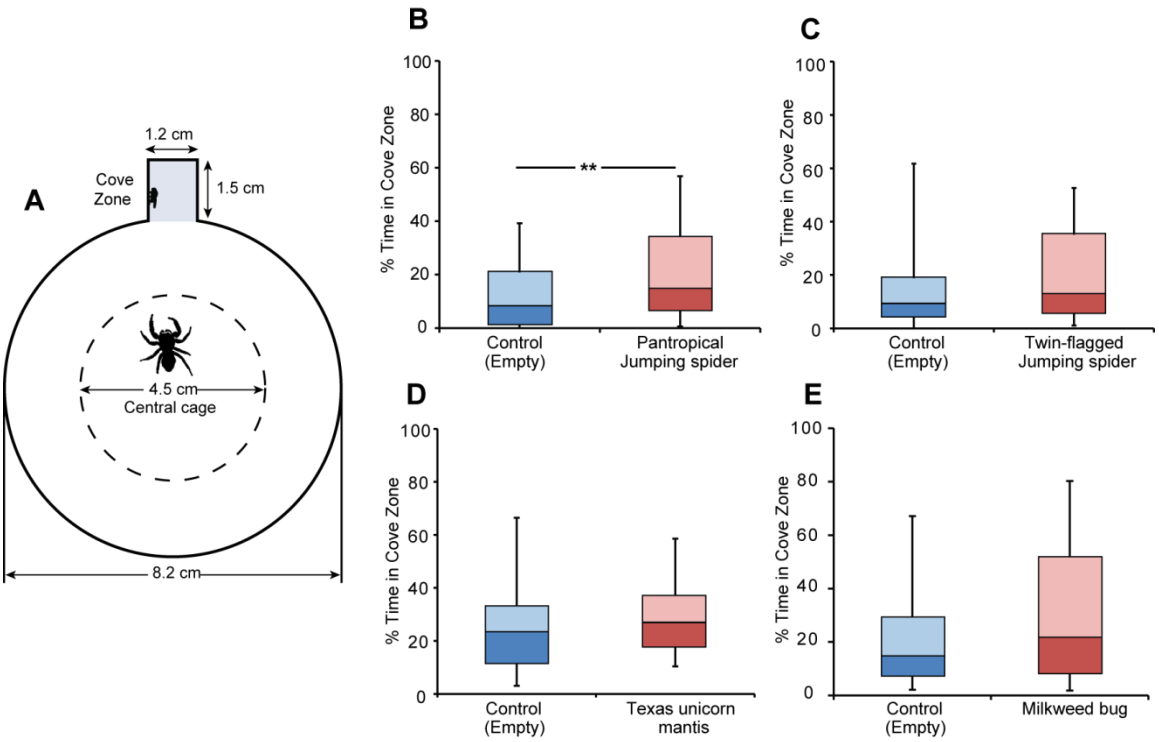

Figure 3. 37 **Drosophila predator avoidance in a circular arena with alcove.** (A) Schematic of arena with alcove. The alcove was set up as a digital zone to track position. Avoidance was measured by the amount of time spent in the alcove. (B) CS flies spent more time in the alcove in the presence of a Pantropical jumping spider compared to an empty cage (U=5635, N=95, p = .0001). But the (C) Twin-flagged jumping spider (U = 5088, N=95, p = .129) and (D) Texas unicorn mantis (U=4076, N=96, p = .167, did not affect positional preference. (E) Milkweed bug controls did not affect positional preference (U = 4325, N=86, p = .076). (** p < .01. Box plot midline represents the median, upper box 3rd quartile and lower box 2nd quartile, whiskers are 90% confidence levels).

To this point, the predator work suggests that standard laboratory strains of wild type CS flies that have been reared for thousands of generations in the absence of predatory stimuli can still detect and avoid predators, indicating an innate response to predator threats that does not require experience. Which sensory modalities flies used to detect caged predators in the arena was investigated next using one olfaction mutant and two vision mutants. *orco*^{2} mutants are anosmic (smell blind) due a loss of function mutation in the odorant

receptor co-receptor (orco) locus (Engels, Johnson-Schlitz, Eggleston, & Sved, 1990; Libert et al., 2007). *norpA*^{7} mutants have a phospholipase Cβ null mutation that disrupts visual signaling leaving these mutants physiologically blind (Harris & Stark, 1977). *w*^{1118} mutants have a loss of function mutation that disrupts an ABC-like transporter necessary for the production of eye pigments leaving these animals phototactic, but with low visual acuity. Wild type CS flies were used a controls. All strains were assayed in the arena shown in Figure 3.36A either with or without a Pantropical jumping spider, since this predator reliably elicited robust avoidance responses.

Comparisons between all strains revealed significant differences in avoidance of the Pantropical jumping spider (Figure 3.38, $p < .0001$). Consistent with previous experiments CS flies spent less time in the middle zone in the presence of a jumping spider compared to a vacant cage ($p < .0001$). *orco*^{2} mutants also spent less time next to the cage in the middle zone when the jumping spider was present compared to an empty cate ($p < .0001$), but not the vision mutants *norpA*^{7} ($p = .584$) or *w*^{1118} ($p = .957$). Overall, flies with intact vision, CS and *orco*^{2} mutants, detected and avoided the caged jumping spider, but not the two vision mutants. These results indicate that olfaction is not required to detect predators and that flies primarily use vision to detect and avoid predators.

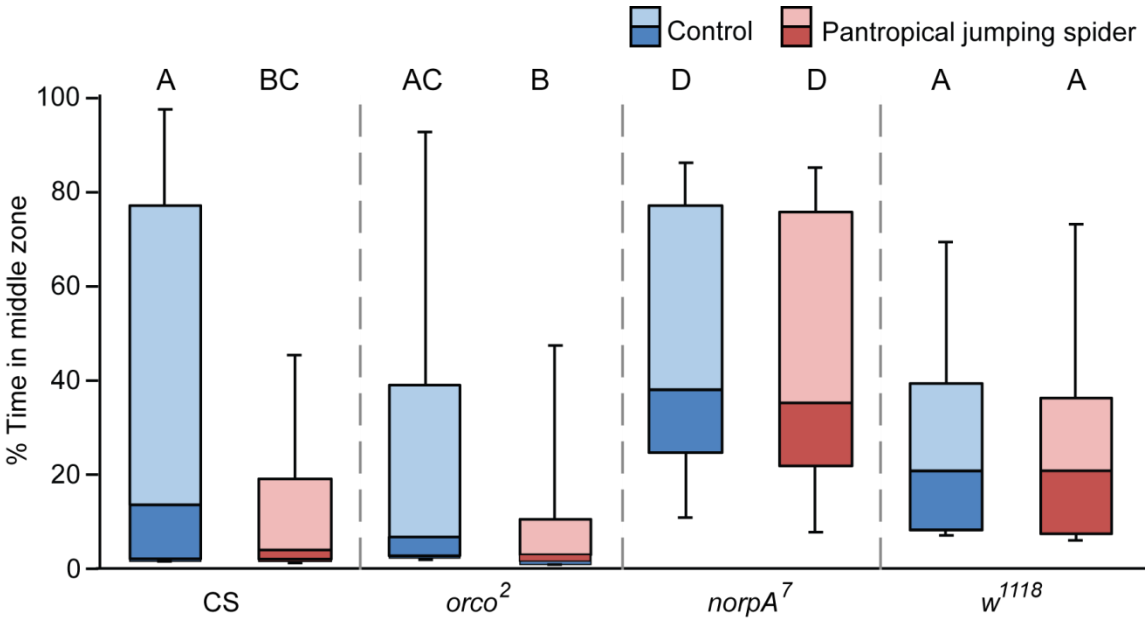

Figure 3. 38 **Flies with impaired vision do not detect predators.** There were significant differences between CS, olfaction and vision mutants in the median time spent in the middle zone with a caged jumping spider and empty cage ($H7=219.61$, $N=126$, $p < .0001$). (Box plot midline represents the median, upper box 3rd quartile and lower box 2nd quartile, whiskers are 90% confidence levels).

Visual cues from a live predator were sufficient to prompt anti-predator responses from wild type CS flies. To test this observation further the live Pantropical jumping spiders were replaced with mock plastic spiders. The mock spiders had a general spider shape, black, but about 15% larger than the Pantropical jumping spider. The mock spiders lacked most other natural physical characteristics such as odors and behavioral cues. Much like with the Pantropical jumping spider wild type CS flies detected and avoided the mock spider spending significantly less time in the middle zone when the mock spider was present compared to a unoccupied cage, suggesting that the mock spider had sufficient predator cues to elicit a response (Figure 3.39A, $U = 5793.5$, $N = 96$, $p = 0.002$).

Among flies' repertoire of defensive behaviors is their ability to attend to and almost instantly move to avoid looming targets, whether flies are standing still, walking or in flight (Muijres, Elzinga, Melis, & Dickinson, 2014). Flies visually detect and move to avoid looming threats (Gotz, 1980), thus it was hypothesized that a moving mock spider may be perceived as looming threat and elicit a defensive response. Mock spiders were made to move by adding a small magnet bar to the underside of the abdomen and then placing the entire arena set up on top of a stir plate offset from the plate center to produce slow asymmetrical lunging movements. A large black stir bar was used as a non-spider control. Significant differences in the time spent in the middle zone were found between all groups (Figure 3.39B, $H_3 = 33.62$, $p < .0001$). Wild type CS flies spend significantly less time in the middle zone when the mock spider was moving compared to a still mock spider (Figure 3.39B, W = 5.498, N=32, p = .0001). The moving stir bar also decreased the amount of time CS flies spent in the middle zone compared to a still bar (Figure 3.39B, W = 4.140, N=32, p = .018).

norpA^7 blind mutants did not detect caged predators in the previous experiments (Figure 3.38), so it was hypothesized that these mutants may also not respond to looming stimulus posed by the moving mock spider. Results with wild type CS flies as controls and *norpA*^{7} blind mutants show that there were significant differences between groups in time spent in the middle zone (Figure 3.39C, $H_3 = 48.278$, N= 32, $p < .001$). Like the previous experiment wild type CS controls significantly spent less time in the middle zone (Figure 3.39C, W = 7.140, N= 32, $p < .0001$), however, the blind *norpA*^{7} mutants did not respond to

the presence or movement of the mock spider or any other possible stimuli (Figure 3.39C, W = 1.595, N= 32, p = .672). Taken together these findings suggest that flies detect and avoid a mock predator. Further, that a looming mock predator also elicits anti-predator responses. These novel findings in flies are consistent with many studies that show that vertebrate and invertebrate prey animals respond to the general shape or movements of mock predators, suggesting that the ability to detect predatory cues is a highly conserved behavior (Adamo et al., 2013; Helfman, 1989; Luca & Gerlai, 2012a, 2012b; Magurran, 1986; Raderschall, Magrath, & Hemmi, 2011; Tinbergen, 1939).

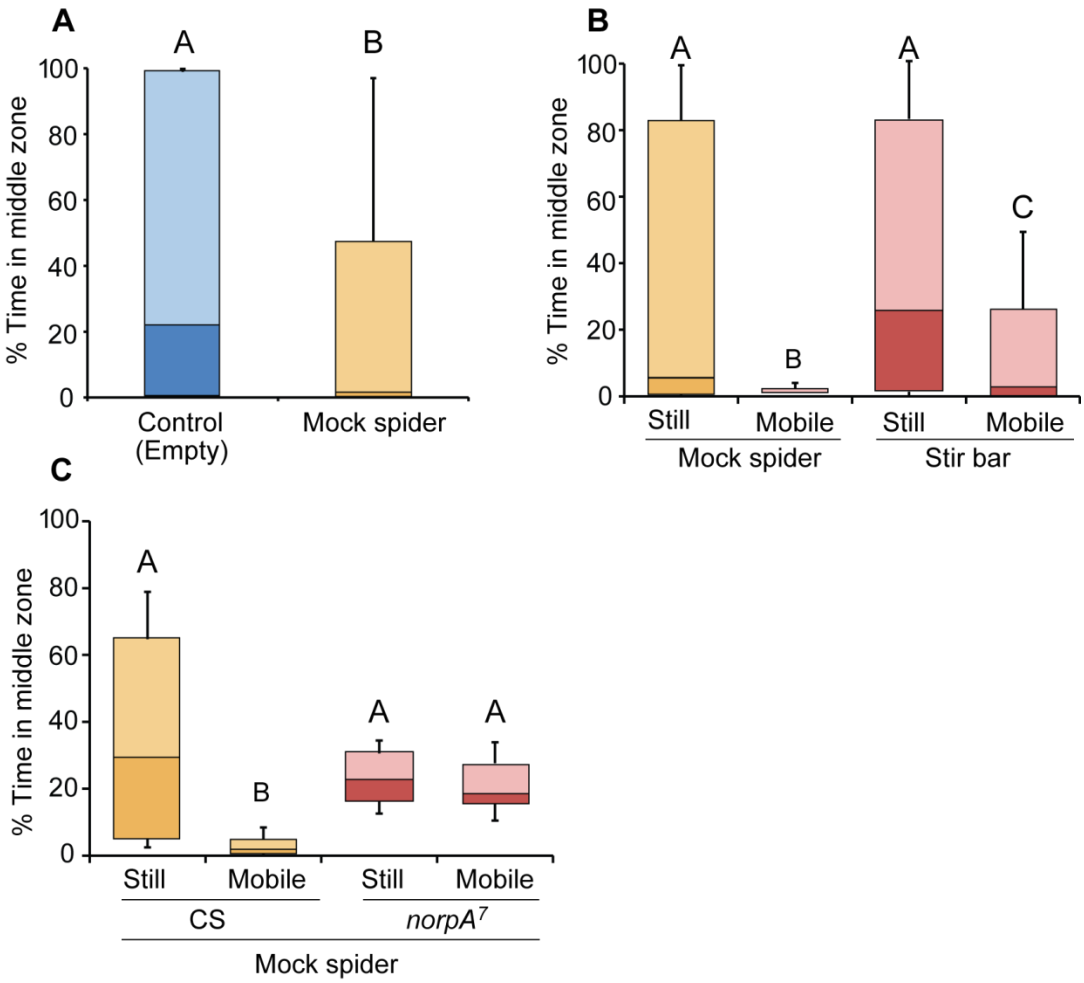

***Figure 3. 39* Vision is required to avoid the mock spider.** (A) CS flies detected and avoided the mock spider by reducing the amount of time spent in the middle zone next to the cage. (B) Movement of a mock spider or control stir bar reduced time spent in the middle zone, suggesting a response to a looming threat. (C) Blind norpA^{7} mutants did not detect or avoid the still or moving mock spider suggesting that vision alone is required to detect the mock spider and is sufficient to illicit a defensive response. (Box plot midline represents the median, upper box 3rd quartile and lower box 2nd quartile, whiskers are 90% confidence levels).

Prey animals may engage in fleeing or freezing behaviors when predators are detected. Predators that are near may motivate a prey animal to explore for escape routes, while freezing behaviors may reduce detection by distant predators (Cooper et al., 2010; Eilam, 2005; McNaughton & Corr, 2004;

Stankowich & Blumstein, 2005). To determine if flies engage in fleeing and/or freezing behaviors, locomotor activity was measured using coverage. Wild type CS flies engage in directionally persistent locomotor activity when introduced into a novel open-field arena, but within a few minutes this locomotor activity decreases to spontaneous activity levels. The initial directional persistence in locomotion represents movement driven by the novelty of the arena and the decrease in locomotion is habituation to the novelty stimulus. In other words, as flies explore, they learn about the arena by visiting and often revisiting patches of the arena boundary, relying heavily on vision to gather information. As a result, the increase in familiarity attenuates the novelty drive.

To quantify coverage, the entire arena boundary is digitally discretized into patches equal to the noise threshold of tracking to a distance 1 cm from the arena wall. Coverage (C) is defined as the minimal number of times that a fly visits each patch of the arena boundary. If C=1 then a fly has visited all patches once, if C=1.75 then all patches have been visited once and three-quarters have been revisited. In the coverage model P++ describes the probability of persistent forward motion, while P+0 is the probability of stopping and P+- is the probability of reserving trajectory (Soibam et al., 2013).

In the presence of Pantropical Jumping spider, wild type CS flies displayed significantly increased locomotor activity in the first two minutes when compared to an empty cage (Figure 40A, $F_{1, 205} = 4.56$, $p = .034$). There was no difference in P++ or directional persistence (Figure 40B, $F_{1, 205} = 2.85$, $p = .092$).

These data suggest that the presence of a predator increases exploratory activity in the first two minutes.

In Figure 3.39A the presence of a mock spider elicted a strong avoidance response compared to an empty cage. Thus it was hypothesized that the mock predator may also increase exploration similar to a natural predator. However, results show that the mock spider did not increase exploratory behaviors (Figure 40C, $F_{1, 238} = 0.079$, $p = .779$) and no differences in P++ were detected (Figure 40D, $F_{1, 238} = 0.035$, $p = .851$). Nonetheless, a moving mock predator signifcantly increased exploration (Figure 40E, $F_{1, 149} = 3.98$, $p = .047$), albeit later in the habituation curve when coverage value was 4-7. Continuing the trend, no differences were detected between still and moving mock spiders in P++ (Figure 40F, $F_{1, 149} = 1.33$, $p = .251$). In summary, these data suggest that presence of a Pantropical jumping spider and moving mock spider increase activity as a function of coverage, but directional persistence P++ is not affected. Further, no freezing behaviors were detected as flies displayed robust locomotor exploratory behaviors.

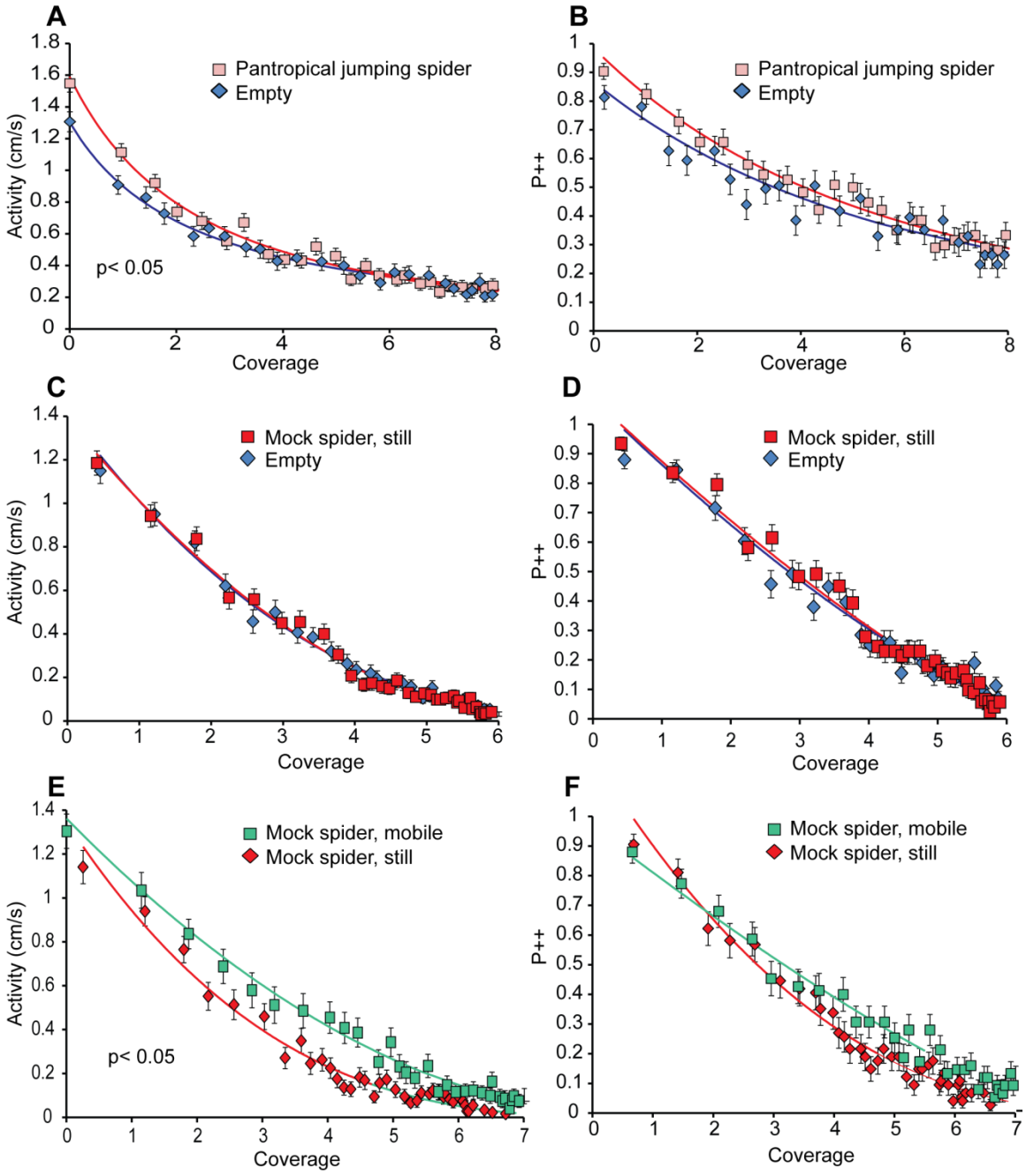

Figure 3. 40 **Drosophila increase locomotor activity in the presence of a Pantropical jumping spider and mock spider.** (A) Wild type CS displayed increased activity in the early phase of exploration when a spider was present compared to an empty cage. (B) P++ or directional persistence was not affected by the presence of a spider. (C) No activity differences or (D) P++ differences were detected between a still mock spider and empty cage. (E) A moving mock spider significantly increased activity but (F) P++ was unchanged. (Regression curves fit to $y=a*(1+x/b^2$, ±error bars are SEM).

Chapter 4: Discussion

4.1 **Results in Brief**

Important characteristics of habituation were detected in the open-field assay: habituation, dishabituation, habituation to dishabituation and reduction or abatement of habituation to a strong stimulus, demonstrating for the first time, that the decrease in locomotor activity while flies explore an open-field arena is habituation (Figures 3.01-3.02). This important finding establishes a new paradigm in which the neural substrates of novelty habituation may be investigated.

Using pharmacological and transgenic approaches, a novel role for 5-HT signaling in the modulation of locomotor exploration in novelty habituation was demonstrated (Figures 3.03-3.10). Moreover, results suggest that locomotor modulation by 5-HT signaling may involve a DPM-MB and possibly PLP-MB neural circuits, and that the 5-HT1A receptor is sufficient in the α/β and γ lobes of the MBs for novelty habituation (Figures 3.11-3.35)

Findings from a related study revealed that laboratory fly strains that have not encountered predators in thousands of generations detected and avoided predators, suggesting that these are innate responses to predatory cues. Further work showed that flies visually detect predators. Additional investigations demonstrated that flies increase exploration in response to live or mock predators, gradually habituate to the presence of the live predator but not a looming mock predator (Figures 36-40).

4.2 **Novelty Habituation is observed in the Open-field Arena**

4.2.1 Habituation

Animals display a wide range of experience-dependent behavioral modifications in response to stimulus changes in their environments. Habituation is a highly conserved, dynamic form of behavioral plasticity observed across taxa through which animals learn to ignore a repeated or inconsequential sensory stimulus, freeing up limited neural resources to attend to tasks that are more salient (Groves & Thompson, 1970; Rankin, 2009). As a result, habituation is a necessary first step or building block for different forms of cognition. In keeping with this role, many human mental disorders such as ADHD, autism spectrum disorder and schizophrenia have habituation deficits (McDiarmid et al., 2017).

Historically what constitutes habituation and what is not habituation has been a point of debate. Different experimental approaches such as (Thompson & Spencer, 1966) with prepared animal spinal cords or (Sokolov, 1963) behavioral observations in animals produced different models of habituation. Further, habituation has been contested as a distinct phenomenon, apart from sensory fatigue or motor fatigue and possibly *not* learning. Based on their work Groves and Thompson proposed in 1970 key characteristics that separated habituation from other behaviors and forms of learning. Over a half-century later in 2009, Rankin clarified and updated the descriptions of habituation and added a new habituation characteristic. Today researchers ostensibly have a habituation

“Rosetta stone” with which to identify the phenomenon in different contexts. Using behavioral and pharmacological approaches the evidence presented here demonstrates that the decrease in locomotor activity observed while flies explore in the open-field arena is habituation to the novelty presented by the arena.

4.2.2 Novelty

Much like habituation novelty has been described using different experimental approaches. Unfortunately, there has been a propensity to view the differing models as competitors rather than building on one another. In Sokolov’s comparator model of the orienting reflex, novelty is described as a mismatch between an animal’s current “neuronal model” based on recent sensory inputs and comparisons to newly incoming inputs (Sokolov, 1963). So in this model a behavioral response indicating novelty like the orienting reflex is elicited only when a mismatch between old and new sensory stimuli is detected.

A proposed feature-matching approach incorporates a significance threshold that the mismatch must reach to elicit a behavioral response indicating detection of a novel stimulus (Gati & Ben-Shakhar, 1990). Further, an optimal level model proposed that a novel stimulus has to be within an ideal range to elicit an approach or avoidance response. In this model, smaller mismatches or discrepancies are thought to produce an optimal level of arousal resulting in an approach response. Whereas, large mismatches produce high levels of arousal or a startle response, resulting in avoidance (Snyder et al., 2008).

While there may be limitations with these models, together they provide a coherent behavioral description of novelty. Consistent with this model, wild type CS flies moved from their holding vials into an open-filed arena will engage in righting and orienting behaviors (unpublished personal observation), and quickly walk towards the arena boundary where they begin to explore in a directionally persistent manner, indicating the detection of a novel stimulus. After a few minutes, locomotor exploration begins to decrease as flies habituate to the novelty presented by the arena.

4.2.3 Characteristics of Habituation Detected in the Open-field arena

Results reported here demonstrate that wild type CS flies engage in exploration in the form of locomotor activity when introduced to a novel open-field arena and that over time that locomotor activity decreases to spontaneous activity levels as familiarity increases. These data and previous reports from our laboratory suggest that the decrease in locomotor activity may represent habituation to the novelty of the arena (L. Liu et al., 2007; Soibam, Goldfeder, et al., 2012; Soibam, Mann, et al., 2012; Soibam et al., 2013).

Nonetheless, as mentioned before the decrease in locomotor activity may be explained by factors other than habituation to novelty, such as sensory or motor fatigue or unexplained transient changes in behavior. To establish habituation in an experimental context, researchers often looked for dishabituation, a chief characteristic of habituation (Engel & Wu, 2009). Dishabituation is the recovery of the habituated response when another strong

stimulus is presented. For example, (Cho, Heberlein, & Wolf, 2004) reports finding dishabituation in a study on habituation to an odorant-induced startle response. Following that lead, a series of key experiments were performed where a second novel stimulus was presented by briefly shaking the arena mechanically under computer control. Initial results showed that one shaking event produced what appeared to be dishabituation (data not shown). Next, the arena was shaken an additional three times at specific intervals with the same intensity. The data clearly showed that dishabituation or a partial recovery of the habituated response had occurred. In addition, the data also revealed habituation to dishabituation, where the second novel stimulus is also habituated (Rankin, 2009).

Our laboratory has published work with predators and flies, where among other findings habituation to a natural predator was observed, but not a looming mock predator (de la Flor et al., 2017). Other work in our laboratory has shown that administering 5-HTP to flies increases habituation in a dose dependent manner and is reversible. These findings made it possible to investigate another characteristic of habituation, where an intense stimulus may produce no detectable habituation (Rankin et al., 2009). An experiment where flies fed either vehicle (no 5-HTP) or 40 mM 5-HTP in the presence of spider showed that the usual increase of habituation by 5-HTP was reduced. In other words, the presence of a live spider, an intense stimulus, eliminated the increase in habituation produced by 5-HTP feeding. This is a strong indicator characteristic 5 of habituation.

The work presented here detected four of the ten characteristics: (1) habituation, (8) dishabituation, (9) habituation to dishabituation and (5) reduction of habituation in the presence of a strong stimulus. See table in the appendix for a complete list of all habituation characteristics. It is important to note that not all 10 characteristics are necessary to establish habituation, as several may be specific to certain stimuli or conditions. For example, work with the gill withdrawal reflex in *Aplysia* found six habituation characteristics (Pinsker, Kandel, Castellucci, & Kupfermann, 1970). Because habituation is a dynamic behavior that is still being understood, there may be challenges to the findings reported here. For example, under Thompson and Spencer's 1966 habituation characteristics the definition of dishabituation fits the data reported here. However, Rankin 2009 extends the definition to include that the increase in response (recovered response) must be to the original stimulus and not the dishabituating stimulus. In the open-field arena the original stimulus is the novelty of the arena. It is difficult to determine if flies are responding to the original stimulus or the second stimulus. However, the fact that flies also habituate to the second stimulus suggests that the phenomenon observed is habituation. Despite possible issues, taken together these findings demonstrate for the first time in flies that the decrease in locomotor activity while exploring an open-field arena is habituation. This opens up a new research paradigm in which the genetic toolkits available in flies can be used to dissect the genetic mechanism of novelty habituation.

4.3 **Manipulation of 5-HT Signaling Modulates Novelty Habituation**

Biogenic amines are key modulators of behaviors in vertebrates and invertebrates. The genes involved in the synthesis of biogenic amines and the mechanisms of action of these ancient neuromodulators are highly conserved across taxa (Vleugels, Verlinden, & Vanden Broeck, 2015). In flies, dopamine is involved in development, the regulation of locomotion, courtship, learning and memory, addiction, attention and reward (Andretic et al., 2005; Q. Liu et al., 2012; Niens et al., 2017; Owald, Lin, & Waddell, 2015; Riemensperger et al., 2013; Schwaerzel et al., 2003; Yamamoto & Seto, 2014). Often neuromodulators have synergistic but differential roles within a behavioral paradigm, for example, in olfactory memory conditioning octopamine is required for appetitive conditioning while dopamine is required form aversive conditioning (Matsumoto, Matsumoto, Wakuda, Ichihara, & Mizunami, 2015; Schwaerzel et al., 2003). And whereas 5-HT is implicated in behavioral quiescence dopamine is associated with arousal (Kume, 2007; Pooryasin & Fiala, 2015). 5-HT signaling has been shown to be a key modulator of many complex behaviors in flies including feeding, courtship, aggression, learning and memory, and sleep, (Becnel, Johnson, Luo, Nässel, & Nichols, 2011; Dierick & Greenspan, 2007; Neckameyer, 2010; Sitaraman et al., 2008; Yuan et al., 2006).

4.3.1 Pharmacological Manipulation of 5-HT Signaling

Results from this study show that pharmacological increases in 5-HT signaling with 5-HTP feeding decreased locomotor activity in a dose dependent manner where increasing concentrations of 5-HTP further decreased locomotor activity. In addition, the quiescent effects of 5-HTP on locomotor activity were entirely reversible, again suggesting that 5-HT signaling modulates locomotor activity while flies explore. In stark contrast, pharmacological inhibition of 5-HT signaling with 50 mM αMTP increased locomotor activity. However, when administering 5-HTP and αMTP together, 5-HTP rescued locomotor activity. These data are consistent with work in *Drosophila* larvae that show that 5-HT signaling modulates locomotor activity (Majeed et al., 2016; Silva et al., 2014).

4.3.2 Transgenic Manipulation of 5-HT Signaling

Here the Gal4 drivers Trh-D2-Gal4 and Trh-Kartic-Gal4 were used to drive expression of optogenetic *UAS-CsChR* and temperature sensitive *UAS-TrpA1* neural activators in large populations of Trh-positive neurons. Ostensibly, Trh-positive neurons produce and secrete 5-HT. The results show that conditional optogenetic or thermogenic activation of Trh-positive neurons was sufficient to decrease locomotor activity in the open-field arena. These findings are consistent with pharmacological activation of 5-HT signaling reported here and with results from Pooryasin (2015), that activation of Trh-positive neurons with different Trh-Gal4 drivers decreases locomotor activity.

Interestingly, transgenic inhibition Trh-positive neurons also decreased locomotor activity in stark contrast to the results from pharmacological inhibition of 5-HT signaling with αMTP. Work with the conditional temperature sensitive inhibitory neural effector *UAS-Shibire* did not produce reliable results. Consequently, the constitutive inhibitory neural effectors *UAS-KIR 2.1* or *UAS-TNT-LC* were used to inhibit Trh-positive neurons with Trh-D2-Gal4 and Trh-Kartic-Gal4. However, the constitutive nature of these neural effectors may have contributed to confounding artifacts because their inhibitory effects are expressed during development. This may result in compensation through up-regulation of 5-HT signaling thereby confusing the inhibition results.

In addition, the Trh-Gal4 drivers used here may drive expression in populations of non-5HT secreting neurons (unpublished), which could produce off target effects. Finally, activation of large populations of Trh-positive neurons may simultaneously activate inhibitory and stimulatory 5-HT signaling pathways. Despite these limitations, overall the data show that pharmacological and transgenic activation of 5-HT signaling is sufficient to decrease locomotor activity while flies explore. Coupled with findings that demonstrate that the decrease in locomotor activity is habituation, these data strongly suggest that 5-HT signaling is playing a role in novelty habituation to decrease locomotor exploration while flies explore.

4.4 **Activation of DPM Neurons is Sufficient for Novelty Habituation**

4.4.1 DPM, CSD and DAL Neuron Screen

5-HT secreting neurons throughout the fly brain have been implicated in the modulation of different behaviors, often the same neurons have been reported to modulate multiple behaviors. In the current study, the 5-HT secreting neurons DPM, CSD, DAL were screened for possible involvement in novelty habituation while flies explore. These neurons were selected for this screen because they are reported to be presynaptic to the Kenyon cells or the intrinsic neurons of the mushroom bodies (MB) (Dacks et al., 2006; Dubnau & Chiang, 2013; Roy et al., 2007; Waddell et al., 2000). This is key because the MBs are involved in processes relevant to habituation, such as non-associative and associative learning and memory processing and the regulation of locomotion (Mabuchi et al., 2016; J. R. Martin et al., 1998; Xiong, Lv, Gong, & Liu, 2010). Furthermore, other studies show that the MBs process the transition from novelty to familiarity (Hattori et al., 2017; Zhang & Roman, 2012). The MBs have also been shown to integrate multi-modal sensory stimuli with internal states like hunger, sleep, courtship, arousal, relaying outputs through mushroom body output neurons to other structures like the ellipsoid body allowing animals to make decisions based on experience (Aso et al., 2014; Perisse et al., 2013; Tsao et al., 2018).

Results in this study show that optogenetic activation of the DPM neurons but not the CSD or DAL neurons was sufficient to decrease locomotor activity while flies explore. In addition, inhibition of the DPM neurons but not the CSD or DAL neurons also decreased locomotor activity. These novel findings suggest that signaling from the DPM neurons to the MBs may modulate novelty habituation while flies explore. These data are congruent with reports that the DPM-MB circuit modulates behaviors. For example, egg laying behaviors in females involve the DPM neurons (Wu et al., 2015).

That inhibiting the DPM neurons produces the same phenotype as activating them is an interesting observation that may shed light on the physiological function of the DPM-MB circuit. The DPM neurons were inhibited using three different Gal4 drivers, but only two of the three, C316 and NP2721 driving *UAS-KIR 2.*1 or *UAS-TNT-LC* respectively, decreased locomotor activity. The VT64246 Gal4 driver, a weaker driver than C316 or NP2721 Gal4s, produced no detectable phenotype in repeated experiments.

This suggests a physiological model in which the DPM neurons may signal to the MB intrinsic neurons at a tonic, basal rate possibly stabilizing the MB intrinsic neurons. This model is consistent with the extremely dense anatomical innervation of the MB lobes and anterior peduncles by the DPM neurons. Signaling from the DPM neurons to the MB intrinsic neurons may transition into phasic firing upon sufficient inputs to the DPM neurons, possibly via a feedback loop from the MB intrinsic neurons to the DPM neurons (Keene et

al., 2006; Wu et al., 2015). Neuronal signaling shifting from basal firing to phasic firing is common in vertebrates and invertebrates and is found in the regulation of rhythmic behaviors, sensory processing and memory consolidation (Radulescu, 2010; Stopfer, 2014). If the model proposed here is correct then inhibiting the DPM neurons, in other words, reducing or removing the basal signaling rate to the MB intrinsic neurons may sufficiently destabilize the MB intrinsic neurons to dysregulate locomotion.

Another important issue to consider is that the DPM neurons are reported to secrete 5-HT, GABA and the peptide product of the *amnesiac* gene (AMN) (P. T. Lee et al., 2011; Waddell et al., 2000). Though there is some controversy as to whether the DPM neurons secrete 5-HT at all (unpublished). Assuming that the DPM neurons secrete all three neurotransmitters, it may be challenging to determine which is acting when the DPM neurons are artificially activated. Furthermore, inhibition of the DPM neurons may interfere with all three signaling pathways.

In an attempt to address this issue 5-HT signaling from the DPM neurons, but not GABA or AMN, was reduced by overexpressing the *Drosophila* plasma membrane serotonin transporter (dSerT) using the Gal4/UAS system in ±5-HTP conditions. Like the vertebrate orthologue, in flies dSerT is expressed in 5-HT neurons to tune 5-HT signaling by regulating the amount of 5-HT available at the synaptic cleft (Giang et al., 2011). Thus, overexpressing dSerT reduces 5-HT signaling by 5-HT reuptake into the DPM neurons. Results show that in the

vehicle condition (no 5-HTP) overexpression of dSerT in the DPM neurons phenocopied the decreased locomotor activity of transgenic inhibition of the DPM neurons, which supports the model that 5-HT signaling, but not GABA or AMN, from the DPM neurons to the MBs may be involved in the modulation of locomotor activity. In the 5-HTP condition, 5-HTP reduces locomotor activity in the controls as expected, but not in the experimental group, suggesting that 5-HT reuptake by dSerT is robust and offsetting increased 5-HT signaling by 5-HTP.

4.4.2 PMPV, PMPD, SEL, SEM, PLP and LP Screen

A pilot study using five different Gal4 drivers that cover 5-HT secreting and non-5-HT secreting neurons in the PMPV, PMPD, SEL, SEM, PLP and LP brain regions revealed that activation of the neurons targeted by R67B05-Gal4 and R65D03-Gal4 with *UAS-NaChBac* significantly decreased locomotor activity. Conversely, inhibition of the same population of neurons with *UAS-KIR 2.1* increased locomotor activity. The results from these preliminary experiments identified the neurons in the SEM and PLP brain region as sufficient and necessary in the regulation of locomotor activity while flies explore. Of particular interest are the 5-HT secreting neurons clustered in the PLP region, which are reported to synapse directly (or indirectly) with the peduncles of the MBs (Alekseyenko & Kravitz, 2014). Further studies may establish a novel role for the PLP neurons in the modulation of locomotion and possibly novelty habituation. However, the SEM neurons, which are also covered by the R65D03-Gal4, have not been ruled at this time as having some role in these behaviors. In addition, it

is important to note that these Gal4 drivers also cover many neurons that do not secrete 5-HT, which may produce off target effects. Experiments to address both of these issues are planned.

4.5 **5-HT1A Receptor Function is Sufficient for Novelty Habituation**

Based on the findings reported so far a plausible model emerges in which 5-HT signaling from the DPM neurons and possibly the PLP neurons to the MBs may modulate novelty habituation. However, flies express five 5-HT receptors, 5-HT1A, 5-HT1B, 5-HT2A, 5-HT2B and 5-HT7, all of which are GPCRs (Gasque, Conway, Huang, Rao, & Vosshall, 2013; Johnson et al., 2009; Ofstad et al., 2011). Which 5-HT receptor and where in the fly brain transduces 5-HT signaling to effect novelty habituation is an important question. The likely scenario is that more than one receptor is involved, as 5-HT receptors are known to differentially modulate behaviors and crosstalk between 5-HT receptor types and other receptor types (e.g., dopamine) has also been described in the literature (Majeed et al., 2016; Ries et al., 2017; Rojas & Fiedler, 2016).

Confocal images in this study, reveal that the 5-HT1A receptor is expressed in the MBs and other brain structures like the ellipsoid body and optic lobes. This finding confirms the expression pattern of the 5-HT1A receptor reported by (Gnerer, Venken, & Dierick, 2015). Furthermore, in vertebrates and invertebrates postsynaptic 5-HT1A receptors are preferentially Gαi/o coupled, thus, activation of these receptors hyperpolarizes neurons decreasing firing rates

by inhibiting adenylyl cyclase (Bickmeyer, Heine, Manzke, & Richter, 2002; Blenau & Thamm, 2011). In addition, 5-HT signaling through the 5-HT1A receptor has been shown to regulate sleep, locomotion, learning and memory, aggression and anxiety-like states among other behaviors (Johnson et al., 2009, 2011; Ries et al., 2017; Silva et al., 2014; Yuan et al., 2006). Taken together, these findings suggest that the 5-HT1A receptor is a good candidate to investigate the transduction of 5-HT signaling in novelty habituation.

Experiments with *5-HT1A*^{MBO9978} and *5-TH1A*^{MBO9812} receptor mutants show that these mutants have increased levels of locomotor activity in the open-field assay. Further, unlike wild type CS controls, these mutants do not respond to pharmacologically increased levels of 5-HT signaling with 40 mM 5-HTP, suggesting a disruption of 5-HT signaling through the 5-HT1A receptor. In addition, antagonizing the 5-HT1A receptor in wild type CS flies with WAY100365 (selective for the 5-HT1A receptor in flies) phenocopied the increased locomotor activity levels of the mutants. These novel results confirm that in the adult fly brain 5-HT signaling through the 5-HT1A receptor is necessary for the modulation of locomotor activity and adds to a growing body of evidence that the 5-HT1A receptor plays a key role in the modulation of important behaviors.

4.6 **Expression of the 5-HT1A Receptor in the MBs but not the EBs is Sufficient for Novelty Habituation**

4.6.1 The 5-HT1A Receptor is Sufficient in the α/β MB Neurons for Novelty Habituation

The MBs have been extensively studied in the regulation many of physiological and behavioral functions. The MBs are bilateral neuropil consisting of about 2500 intrinsic neurons each which are generally subdivided into α/β, α'/β' and γ neurons comprising the lobes of the MBs. The α/β, α'/β' and γ neurons can be further subdivided into α/β, surface, core, and posterior layers, α'/β' anterior, middle and posterior layers, and γ dorsal layer (Aso et al., 2009; Tanaka et al., 2008). Neurons in these subsets have been characterized as having differential roles within a behavior modality. For example, studies in olfactory associative learning and memory have characterized differential roles for specific subsets of MB intrinsic neurons in memory acquisition and stabilization (α'/β'), storage (γ) and retrieval (α/β) (Akalal, Yu, & Davis, 2010; Huang, Wang, Xie, Wang, & Zhong, 2013; Krashes, Keene, Leung, Armstrong, & Waddell, 2007; Wang, Mamiya, Chiang, & Zhong, 2008). The rescue data presented here shows that 5-HT signaling transduced through the 5-HT1A receptor to subsets of neurons in the α/β/y lobes, but not α'/β' lobes modulates locomotion and may play a role in novelty habituation.

Using three different Gal4 drivers to express cDNA of the 5-HT1A receptor in the MBs, in the 5-HT1A mutant background, partially or fully rescued novelty habituation. Specifically, experiments with 238Y-Gal4 and C739-Gal4 produced strong partial rescues of novelty habituation, while P247-Gal4 produced a full rescue.

All three Gal4 drivers target overlapping, but distinct populations of MB intrinsic neurons. The 238Y-Gal4 targets all MB intrinsic neurons, but drives stronger expression in the α/β surface, core and posterior neurons. On the other hand, P247-Gal4 drives strong expression in α/β surface and posterior neurons, but not core neurons, and includes γ lobe neurons, while C739-Gal4 is limited to α/β surface, core, and posterior neurons (Aso et al., 2009). Combined, the reported expression patterns and the rescue data presented here suggest that expression of the 5-HT1A receptor in the α/β surface and possibly posterior neurons may be required for novelty habituation but not in the α/β core neurons. Also expression of the 5-HT1A receptor does not seem to be required in the α'/β' neurons for novelty habituation as these neurons are not targeted by P247-Gal4 or C739-Gal4 and weakly covered by 238Y-Gal4, though α'/β' have not been explicitly excluded. Together, these data corroborate findings that the MBs are involved in the regulation of locomotion (Martín-Peña et al., 2014; Silva et al., 2014; Sun et al., 2018) and that that the MB α/β lobes may be involved in the regulation of habituation (Acevedo et al., 2007). In addition, these findings

establish a novel role for expression of the 5-HT1A receptor in the MB α/β surface and posterior neuron in the regulation of novelty habituation.

However, while the Gal4/UAS system makes it possible to express genes under temporal and spatial control it is difficult to titrate the level of gene expression. Results from Gal4/UAS rescue experiments could be confounded by ectopic expression, overexpression or underexpression of genes that could produce neopmorphic phenotypes. To address this issue the 5-HT1A receptor was overexpressed in the MBs using the same Gal4 drivers, 238Y-Gal4, C730-Gal4 and P247-Gal4. The results show no detectable phenotype, indicating that expression of the 5-HT1A receptor in the mutant background using the Gal4/UAS system is not producing neomorphic phenotypes, supporting the genetic rescue results.

4.6.2 5-TH1A Receptor may not be required in the EB for Novelty Habituation

In flies, the central complex (CC) is a centrally located, prominent group of unpaired, densely interconnected neuropil composed of the fan shaped body, protocerebral bridge, noduli and ellipsoid body (EB) (Hanesch, 1989). The CC is reported to process and integrate visual, acoustic, and olfactory inputs, with internal states (e.g. hunger) and memory to initiate and coordinate locomotion and flight (Ilius et al., 1994; Martín-Peña et al., 2014; Strausfeld & Hirth, 2013). Thus a prevailing model of the regulation of locomotion in flies is that the MBs modulate locomotion and the central complex (CC) initiates and coordinates

locomotion (J. R. Martin et al., 1998; Strauss, 2002). Specifically, the R1-R4 neurons of the EB have been shown to be necessary for locomotion (Martín-Peña et al., 2014). Further, immunostaining experiments in this study show that the EB expresses the 5-HT1A receptor, corroborating reports in the literature of expression of this receptor in the EB (Alekseyenko & Kravitz, 2014).

However, expressing cDNA of the 5-HT1A receptor in the 5-HT1A mutant background using C819-Gal4 and 5.30-Gal4, which target EB R2/R4 neurons, did not show clear rescues of the mutant phenotype. Moreover, overexpressing the 5-HT1A receptor produced no detectable phenotypes. Nonetheless, experiments with 5.30-Gal4 showed a rescue in the first-minute distance means but not the total distance means suggesting a possible role for 5-HT signaling through the 5-HT1A receptor to the EB R2/R4 neurons in the initiation of exploration but not habituation. Along the same lines, experiments with C819-Gal4 showed a trend towards a rescue but did not reach statistical significance. These results suggest that the 5-HT signaling in the EB may be involved in the regulation of locomotion. Much like the MB overexpression experiments, overexpressing 5-HT1A receptor in the EB R2/R4 neurons produced no phenotype indicating that results from the genetic rescue experiments were not due to neomorphic effects.

4.7 **Drosophila Increase Locomotor Exploration after Visually Detecting Predators**

Using a combination of behavioral and genetic approaches the data here demonstrates that laboratory strains of wild type CS flies that have not been exposed to predators for thousands of generations detected and avoided live and mock predators in a circular open-field arena. This finding suggests not only that these behaviors are innate but also highly conserve. In addition, results show that flies use vision as the primary sensory modality to detect predators and that the presence of predators increases exploration.

When exposed to a caged Pantropical jumping spider or a Texas unicorn mantis, naïve wild type CS flies spend less time in the middle zone (the zone adjacent to the caged predator) compared to a control empty cage or a caged non-predator Milkweed bug. However, flies did not avoid the middle zone when a Twin-flagged jumping spider was caged, compared to an empty cage. In addition, wild type CS flies also avoided the middle zone in the presence of a mock spider in contrast to an empty cage, even though the mock spider lacked many natural predatory cues. Seemingly, the mock spider's general "spider" silhouette may have been sufficient to motivate flies to move away from the cage. Further, in a circular open-field arena with a small, outward cove zone, flies spent more time in the cove zone when a Pantropical jumping spider was caged, but not the Twin-flagged jumping spider or Texas praying mantis, compared to an empty cage or Milkweed bug control.

That flies, as prey animals, detect and avoid predatory threats is unremarkable. However, that naïve laboratory wild type strains that have not been exposed to predators for decades *still* detect and avoid a predator suggests that these responses are highly conserved, innate defensive behaviors. This is a significant finding because while there is a large body of work on innate defensive behaviors in vertebrates going back to Lorenz and Tinbergen's famous 1937 goose/hawk experiments, there is a paucity of research of these behaviors in invertebrates (Albecker & Vance-Chalcraft, 2015; Amo, 2011; Epp, 2008; Fendt, 2006; Hawkins et al., 2004; Kindermann et al., 2009; Veen et al., 2000; Zheng et al., 2005). Interestingly, the retention of innate defensive behaviors in captive animals is not always the case. A 2003 study showed that wild-caught field mice bred in captivity for generations were less likely to engage in anti-predator behaviors compared to mice not bred in captivity indicating that in some cases these behaviors are lost (Elsbeth McPhee, 2004). Given *Drosophila's* extensive tractability as a model organism, these findings create a unique opportunity for future research to dissect the neurogenetic basis of innate behaviors in general and innate defensive behaviors specifically.

However, in the circular arena flies failed to detect the Twin-flagged jumping spider and in the circular arena with cove flies did not detect the Twin-flagged jumping spider as well or the Texas unicorn mantis. The Twin-flagged jumping spider's small stature may explain fly's inability to detect this predator behind the Nitex mesh. It is conceivable that in certain circumstances the position of the predator inside the central cage in relation to the position and angle of the

fly in the outer arena may have made it difficult for the fly to detect certain predator behaviors. For instance, as an ambush predator the Texas unicorn mantis remains relatively still compared to the Pantropical jumping spider, which moves to track and hunt its prey thereby creating a moving, looming target. Despite of these limitations, after repeated experiments of over several months the data confirm that laboratory strains of flies that have not been exposed to predators for decades maintain the ability of to recognize and avoid predators.

Prey animals use combinations of sensory modalities to detect predators. To determine which senses flies use to detect predators the blind *norpA*^{7}, visually impaired *w*^{1118} and the smell-blind *orco*^{2} mutants were assayed in the presence of a Pantropical jumping spider. The blind and visually impaired mutants did not detect or avoid the caged Pantropical jumping spider. However, the smell-blind and wild type CS flies detected and avoided the caged spider. Along the same lines, the *norpA*^{7} blind mutant flies spent about the same in the middle zone with either a still or moving mock spider, indicating a failure to detect the mock predator, when compared to wild type CS controls.

These data demonstrate that vision is the primary sense used by flies to detect predators. This is unsurprising because flying insects of the order Diptera have excellent vision, but *Drosophila melanogaster* specifically is also known for its powerful sense of smell. In fact, much of the sensory processing by the mushroom bodies is dedicated to olfaction. Therefore, it is somewhat surprising that visually impaired flies that had intact olfaction failed to avoid the caged

predators, given that olfactory cues usually play some role in predator detection in many prey animals. However, this discrepancy could be a limitation of the artificial conditions of the assay. Also, w^{1118} mutants are visually impaired because of a mutation in an ABC-like transporter that interrupts the production of red eye pigments. However, the mutation may also be at the root of behavioral problems (unpublished data), which may confound these findings. Nonetheless, the data from the blind *norpA*^{7} mutant, which are healthy otherwise, and the smell-blind *orco*^{2} mutants demonstrate that flies detect predators using vision.

When faced with a predatory threat animals may choose from a repertoire of escape behaviors. If available, prey may seek shelter like running into a burrow or hiding under brush. However, in the absence of shelter, freezing to decrease detection or fleeing to put distance between the predator and prey are common. Often prey will alternate between freezing and fleeing behaviors in response to cues from the predator or type of predator (Eilam, 2005). The coverage data presented here show that in general flies do not freeze, but instead increased locomotor activity in the presence of a Pantropical jumping spider, compared to an empty cage. However, it is also possible that as flying animals, freezing serves little purpose as an anti-predator response and within the constraints of the arena, fleeing was the only option. No statistical differences in directional persistence (P++) were detected in either condition. Given that wild type CS flies naturally move to the arena wall and explore in a directionally persistent manner (Soibam et al., 2013) this assay may be limited in detecting variations in this behavior caused by the predator.

In addition, coverage data revealed that overall locomotor activity and directional persistence (P++) were not different between mock spider and no mock spider conditions. Nevertheless, when the mock spider was moving flies significantly increased locomotor activity and showed decreased habituation, suggesting that the moving spider presented a stimulus that could not be habituated. Nonetheless, in conditions with jumping spider, after an initial period of robust locomotor exploration, activity declined to spontaneous levels indicating that flies can habituate to the presence of a natural predator. This finding aligns reports that prey animals may eventually habituate to the presence of a predator and attend to other tasks (Dacier, Maia, Agustinho, & Barros, 2006).

4.8 **Summary**

Habituation is a basic form of behavioral plasticity that allows animals to ignore unimportant stimuli and focus on relevant features of their environment. When flies are introduced into a novel open-field arena, they engage in locomotor activity to explore the arena boundary. Within in a few minutes locomotor activity gradually decreases to low levels of spontaneous activity. Key experiments in this study identified four important characteristics of habituation while flies explore; (1) habituation, (8) dishabituation, (9) habituation to dishabituation and (5) abolishment of habituation, demonstrating that the decrease in locomotor activity during exploration is habituation to the novelty presented by the arena. Decades of research have revealed many of the cellular and molecular mechanisms of habituation, however, there is still much that is

unknown. These findings establish a novel paradigm for investigations into novelty habituation, that in combination with the genetic toolkits available in flies, and the high throughput system developed for this study, make it possible to screen for neural substrates of this behavior.

5-HT signaling is a known modulator of important behaviors in vertebrates and invertebrates. Data presented here show that pharmacological and transgenic increases in 5-HT signaling are sufficient to reduce locomotor activity, indicating that 5-HTsignaling modulates locomotor activity while flies explore. In addition, a systematic screen of 5-HT secreting neurons shows that signaling from the DPM neurons, possibly the PLP neurons, was sufficient to suppress locomotor activity after flies have explored the arena for a few minutes. These findings agree with a model proposed by Pooryasin 2015 in which specific populations of 5-HT secreting neurons in combination with other modulatory systems, like the dopaminergic system, function to initiate and terminate behaviors.

Work with 5-HT1A receptor mutants revealed increased levels of locomotor activity and decreased habituation rates. The results with mutants were recapitulated in wild type CS flies by pharmacologically antagonizing the 5-HT1A receptor. Being preferentially Gαi/o coupled the 5-HT1A receptor's role is inhibitory, generally suppressing neuronal activity. 5-HT1A receptor mutants being hyperactive, lines up with the inhibitory role of the 5-HT1A receptor. This suggests that disruption of 5-HT signaling through the 5-HT1A receptor

dysregulates locomotion. However, the complexities of neuromodulation are such that it is unlikely that signaling through the 5-HT1A receptor alone modulates locomotion as different subtypes of 5-HT receptors in different parts of the fly brain likely orchestrate this behavior.

The mushroom bodies (MB) in the fly brain are second order processing structures responsible for the integration of sensory inputs with internal states and stored memories to produce outputs that regulate behaviors. The intrinsic cells of the MBs are divided into specific neuron types that make up the MB lobes and the lobes can be further dissected to distinct layers of neurons. Results from genetic rescue experiments reported here show that expression of the 5-TH1A receptor is sufficient in the α/β surface neurons, possibly the α/β posterior neurons and the γ neurons, but not the α'/β' neurons for the modulation of locomotor activity involved in novelty habituation. These results align with several reports that the α/β neurons are involved in the regulation of important behaviors, more specifically, these findings confirm reports by Acevedo 2007 that α/β neurons are required for habituation.

In summary, these findings posit a model in which the open-field arena serves as a novel stimulus that motivates flies to explore the new environment. Sensory inputs to the mushroom bodies indicating novelty are integrated with an anxiety-like state from a lack of information about the arena, which drives exploration. As the fly visits and revisits patches of the arena, it forms place memories and learns about the arena. Internal cues indicating satiety to novelty

(familiarity) are also processed by the mushroom bodies, which may signal the DPM neurons via a feedback loop. In turn, the DPM neurons signal through the 5-HT1A receptor to the mushroom bodies to suppress locomotion. From a behavioral perspective, the initial response to the novelty stimulus posed by the arena is reduced, in other words habituated. At a circuit and molecular level 5-HT signaling from the DPM neurons is recruited to modulate locomotion in response to familiarity, in essence the mechanics of habituation.

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Appendix

Appendix Table 1. 1 Habituation characteristics from Rankin, et al, 2009.

Number	Description
1	Repeated application of a stimulus results in a progressive decrease in some parameter of a response to an asymptotic level. This change may include decreases in frequency and/or magnitude of the response. In many cases, the decrement is exponential, but it may also be linear; in addition, a response may show facilitation prior to decrementing because of (or presumably derived from) a simultaneous process of sensitization.
2	If the stimulus is withheld after response decrement, the response recovers at least partially over the observation time (‘‘spontaneous recovery”).
3	After multiple series of stimulus repetitions and spontaneous recoveries, the response decrement becomes successively more rapid and/or more pronounced (this phenomenon can be called potentiation of habituation).
4	Other things being equal, more frequent stimulation results in more rapid and/or more pronounced response decrement, and more rapid spontaneous recovery (if the decrement has reached asymptotic levels).
5	Within a stimulus modality, the less intense the stimulus, the more rapid and/or more pronounced the behavioral response decrement. Very intense stimuli may yield no significant observable response decrement.
6	The effects of repeated stimulation may continue to accumulate even after the response has reached an asymptotic level (which may or may not be zero, or no response). This effect of stimulation beyond asymptotic levels can alter subsequent behavior, for example, by delaying the onset of spontaneous recovery.
7	Within the same stimulus modality, the response decrement shows some stimulus specificity. To test for stimulus specificity/stimulus generalization, a second, novel stimulus is presented and a comparison is made between the changes in the responses to the habituated stimulus and the novel stimulus.
8	Presentation of a different stimulus results in an increase of the decremented response to the original stimulus. This phenomenon is termed ‘‘dishabituation.” It is important to note that the proper test for dishabituation is an increase in response to the original stimulus and not an increase in response to the dishabituating stimulus.
9	Upon repeated application of the dishabituating stimulus, the amount of dishabituation produced decreases (this phenomenon can be called habituation of dishabituation).
10	Some stimulus repetition protocols may result in properties of the response decrement (e.g., more rapid rehabituation than baseline, smaller initial responses than baseline, smaller mean responses than baseline, less frequent responses than baseline) that last hours, days or weeks. This persistence of aspects of habituation is termed long-term habituation.