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THE GENETIC AND NON-GENETIC FACTORS FOR IMPULSIVITY

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Doctoral Program in Biological Sciences

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Erick Benjamin Saldes

2023

Dedication

I want to dedicate this entire journey to my father Juan Manuel Saldes Canales who always told me to never to give up, and work as hard as I could to achieve my goals. To my stepfather who gave me all the encouragement to continue to pursue this degree. You may not be able to be here with me at this moment, but I know you are proud of me.

THE GENETIC AND NON-GENETIC FACTORS FOR IMPULSIVITY

by

Erick Benjamin Saldes, B.S.

DISSERTATION

Presented to the Faculty of the Graduate School of

The University of Texas at El Paso

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Abstract

Inhibitory control is an executive function suppressing inappropriate actions or thoughts and inhibitory control dysfunction leads to impulsivity. Impulsivity is associated with wide-ranging brain disorders including attention deficit hyperactivity disorder, autism spectrum disorder, substance use disorder, and dementia. The genetic and non-genetic factors contributing to impulsivity are poorly understood. This dissertation aims to begin closing this knowledge gap by identifying the genetic and non-genetic factors for impulsivity and the underlying mechanism in the model organism *Drosophila melanogaster*. For this task, we developed a new Go/No-Go test, which measures the fly's ability to suppress movements in situations that can endanger its safety or survival such as strong wind and predatory sound. When flies were unable to maintain movement suppression, they displayed impulsive flying behavior, which is inappropriate in a small chamber. In this dissertation work, I identified 3 major factors important for impulsivity. First, I identified nighttime caffeine as a key non-genetic factor for impulsivity. In the study, I also observed sex differences where caffeine-fed females displayed more severe impulsivity than caffeine-fed males. Furthermore, I identified dopamine signaling as a key cellular mechanism for caffeine-induced impulsivity. The second impulsivity factor I identified is the Shaker/KCNA voltage-gated potassium channel complex Shaker, Hyperkinetic, and quiver/sleepless. I further clarified the mushroom body lobe neurons as the neural site where the Shaker complex play a role in impulsivity. Lastly, through a functional genetic screen, I identified the novel genetic factor *Kekkon5* that encodes a synaptic cell adhesion molecule. *Kekkon5* caused impulsivity upon interaction with hyper

dopamine. This dissertation is the first to uncover the aforementioned genetic and non-genetic factors as key contributors to impulsivity. This study may provide insights into how these factors contribute to impulsivity-related brain disorders and may lead to the development of preventive and therapeutic interventions.

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Chapter 1: Introduction

1.1 Inhibitory Control

Executive function is a collection of high-order cognitive processes like working memory, cognitive flexibility, attention, and inhibitory control (Bari & Robbins, 2013; Miyake et al., 2000)Logue (Diamond, 2013; Logue & Gould, 2014). These cognitive processes are required for the successful completion of goal-directed behaviors (Bredemeier & Miller, 2015; Dorman et al., 2022; Duff & Sulla, 2015; Jones et al., 2018). Inhibitory control is a core executive function responsible for suppressing inappropriate actions and thoughts (Best & Miller, 2010; Diamond, 2013). For example, when someone is dieting, inhibitory control is represented by the ability to restrain themselves from eating unhealthy snacks. Inhibitory control dysfunction leads to impulsivity which is associated with a wide range of brain disorders such as attention deficit hyperactivity disorder (ADHD), substance use disorder, autism spectrum disorder, and dementia (Daban et al., 2006; Schoenbaum, Roesch, & Stalnaker, 2006; Willcutt, Doyle, Nigg, Faraone, & Pennington, 2005). Our knowledge on how genetic and non-genetic factors affect inhibitory control remains unclear. Inhibitory control has been studied in rats, mice, primates, and human subjects, where experimental manipulations are limited due to the complex organization of the nervous system, ethical issues, and time-consuming. Understanding the contributions of genetic and non-genetic factors to impulsivity is crucial to develop preventive and therapeutic measures.

1.2 Neural structures and neurotransmitters important for inhibitory control in mammals

The mammalian prefrontal and orbital frontal cortex are linked to regulating inhibitory control (Boehme et al., 2017; Mansouri et al., 2017). Eagle et al. (Eagle et al., 2008) demonstrated that the orbital frontal cortex is responsible for the response inhibition of rats. As for attention, lesions in the medial prefrontal cortex of the rat brain result in impairments as measured by the 5-choice serial reaction time task (Maddux & Holland, 2011). Using the

same assay, Chudasama et al. (Chudasama et al., 2003) showed that lesions in the orbital frontal cortex also affect response inhibition but not attention.

The prefrontal cortex receives input from dopaminergic, serotonergic, cholinergic, and norepinephrine neurons (Bloem, Poorthuis, & Mansvelder, 2014; Fan et al., 2022; Lambe, Fillman, Webster, & Shannon Weickert, 2011; Ott & Nieder, 2019) Overexpression of the dopamine D1 receptor in the prefrontal cortex of adult rats results in impulsive choices as measure by the delay discount task (Sonntag et al., 2014).

Norepinephrine plays a role in attention and response inhibition. Lesions in the medial prefrontal cortex of rats that dampen norepinephrine activity result in attention deficits in the 5-choice serial reaction time task (Milstein, Lehmann, Theobald, Dalley, & Robbins, 2007). Newman et al. (Newman, Darling, & McGaughy, 2008), showed that the attention deficits caused by lesions to the noradrenergic afferents in the prefrontal cortex in rats were improved by treatment with the norepinephrine reuptake inhibitor atomoxetine. Navarra et al. (Navarra et al., 2008) showed that treatment with atomoxetine, a norepinephrine reuptake inhibitor, improved attention and reduced impulsivity in rats tested in the 5-choice series reaction test.

The medial prefrontal and orbital frontal cortexes receive and send projections of serotonin neurons to the dorsal raphe (Enge, Fleischhauer, Lesch, Reif, & Strobel, 2011). Serotonergic activity is linked to response inhibition (Dalley & Roiser, 2012). Homberg et al. (Homberg et al., 2007) showed that increased serotonin levels in rats with no functional serotonin transporter improved in the 5-choice serial reaction time test.

The cholinergic neurons innervate the medial prefrontal cortex and the orbital frontal cortex. This system has two classes of receptors, muscarinic and nicotinic. Muscarinic receptors are known to play a role in attention since injecting a muscarinic antagonist in the rat brain results in impaired reversal learning in rats (Chen, Baxter, & Rodefer, 2004). Rhodes et al. (Rhodes, Hawk, Ashare, Schlienz, & Mahoney, 2012), showed that adult smokers given an α4β2 partial nicotinic acetylcholine receptor improved attention as measured by the stop-

signal test Nicotine also improved the inhibitory control of non-smokers as measured by the Stroop test (Wignall & de Wit, 2011).

1.3 Experimental approaches to study inhibitory control in mammals

The anatomy of the prefrontal cortex, the cognitive processes regulated in this area and experimental tests are similar between humans and animal models. An example is the 5-choice serial reaction time test used to assess attention in human, which works for nonhuman primates and rodents (Fizet, Cassel, Kelche, & Meunier, 2016). There are two categories of impulsivity. The first group measures impulsive action known as motor impulsivity, while the second measures impulsive choice or decision-making (Bari & Robbins, 2013; Logue & Gould, 2014). There are three primary assessments for measuring motor impulsivity. These include the stop-signal reaction time task, the continuous performance test, and the Go/No-Go test (Logue & Gould, 2014; Verbruggen, Best, Bowditch, Stevens, & McLaren, 2014).

The stop-signal reaction time test requires individuals to respond rapidly upon the presentation of a go signal. Afterward, a stop signal is presented at different times after the Go signal is displayed (Eagle et al., 2008). The Go/No-Go test also uses two cues, one for the Go response and the other for when the response should be inhibited (No-Go) (Verbruggen & Logan, 2008).

The continuous performance test measures attention while incorporating aspects of motor impulsivity. During this test, participants memorize a sequence of five digits and respond when the numbers match the stimulus. Errors are defined as the subject responding to sequences that do not match the memorized sequence. These errors are considered premature responses and are used to measure motor impulsivity. In rodent studies, the animals are trained to react to a displayed light in one of five apertures, and they must wait for a five-second inter-trial interval before selecting the aperture. When a premature response occurs, the experiment reset (Carli, Robbins, Evenden, & Everitt, 1983).

Impulsivity can also be measured using cognitive tests that involve choice and preference. One of the most commonly used tests is the delay-discounting paradigm. It assesses how much a subject's reward value decreases when delayed by observing how often the subject chooses a smaller but more immediate reward over a larger one that will be delivered later. Choosing the immediate but smaller reward represents an impulsive choice due to the inability to wait for the larger reward and opting to get a smaller reward in the long run (Regier, Claxton, Zlebnik, & Carroll, 2014).

There is still a lot of research to be done on the different genetic and non-genetic factors that impact inhibitory control. Studies on inhibitory control are conducted on a range of animal models including primates, rats, and mice. However, genetic approaches have limitations due to functional compensation and the lengthy life cycles of mammalian models, making them time-consuming. Here we adopt the Drosophila melanogaster model system that can be easily manipulated and is less complex, thus allowing us to focus on understanding of the basic cell biology of how genetic and non-genetic factors affect inhibitory control.

1.4 Drosophila melanogaster as a model organism

Drosophila melanogaster has been a model organism since the 1900s and provides many advantages for studying non-genetic and genetic factors important for brain disorders. First, Drosophila has a simple nervous system (~100 000 neurons in the brain) yet displays diverse behavioral plasticity. Second, it has a short life cycle allowing for rapid generation of flies to perform multiple independent studies. Third, the fully sequenced genome and advanced genetics allowed the production of a comprehensive collection of genetic and transgenic lines, many of which are orthologs of human disease-related genes. Fourth, various genetic tools allow us to manipulate genes' spatial or temporal expression or neuronal activity, visualize synaptic plasticity, and clarify molecular interactions and structural or functional changes in

the brain. Fifth, the molecules necessary for synaptic transmission, plasticity, and behavioral control are conserved between flies and mammals.

Diverse genetic approaches have been used to study behavior. For example, Gal4/UAS system allows for the expression of genes under study in a temporally and spatially restricted manner. GAL4 is a yeast transcriptional activator that binds the upstream activating sequence (UAS) for downstream gene expression (Duffy, 2002). In addition, the Gal4/UAS system is used for RNA interference to knock down a molecule of interest to investigate its role in cellular mechanisms and behavior (Hales, Korey, Larracuente, & Roberts, 2015).

1.5 Inhibitory Control in *Drosophila melanogaster*

Recent work in our lab has identified the role of dopamine in the regulation of inhibitory control in Drosophila. For example, the dopamine transporter mutant *fumin* (*fmn*) displays dysfunctional inhibitory control leading to motor impulsivity, which indicates that elevated dopamine signaling contributes to impulsivity. Dopamine's role in impulsivity was further demonstrated by ectopic activation of a specific cluster of dopaminergic neurons (Sabandal et al., manuscript under revision). In addition, a non-genetic factor that influenced impulsivity was social context. For example, with more flies in a chamber, fmn mutants displayed more severe impulsivity. When tested alone, fmn flies do not show impulsivity (Sabandal manuscript under revision). More recently our studies show aging as another non-genetic factor contributing to impulsivity. We found acetylcholine to play a role in aging-sensitive impulsivity. The aging-sensitive impulsivity is diminished by reducing acetylcholine breakdown and is increased by reducing the acetylcholine biosynthesis (P. R. Sabandal, Saldes, & Han, 2022).

1.6 Caffeine

Caffeinated drinks stimulate the central nervous system (CNS), increasing alertness. One example of highly caffeinated beverages is energy drinks. These drinks are intended to keep people awake at work and during leisure activities. Caffeine blocks the binding of adenosine to its receptor (Bridgette E. Garrett, 1994; S. Ferre, 2016; Sergi Ferre et al., 2008; Garrett & Holtzman, 1994; Horrigan, Kelly, & Connor, 2006). By blocking the binding of adenosine to its receptor, caffeine indirectly affects the release of neurotransmitters such as norepinephrine, serotonin, glutamate, acetylcholine, gamma-aminobutyric acid (GABA), and dopamine (Sebastiao & Ribeiro, 2009). Solinas et al. (Solinas et al., 2002) study in rats showed that caffeine administration in the nucleus accumbens resulted in elevated levels of GABA and dopamine and increased motor activity. Garrett and Holtzman's study in rats found that the increased locomotor activity resulting from caffeine administration was through enhanced D1 and D2 receptor activity (Garrett & Holtzman, 1994). Similarly, Cauli and Morelli's study also showed that subchronic caffeine administration potentiated the effects of both D1 and D2 receptor agonists in rats lead to elevated locomotor activity (Cauli & Morelli, 2002). Caffeine also inhibits cyclic nucleotide phosphodiesterase (Astrid Nehlig, 1992).

1.7 Sleep

Sleep is defined by specific behavioral criteria where animals adopt a specific posture and limit their movement as much as possible. The arousal threshold of the animals is elevated, but strong stimuli can interrupt this state (Helfrich-Forster, 2018). Sleep and wakefulness are in a circadian cycle: the need to sleep accumulates during the wakefulness period and only reduces after the appropriate amount of sleep is obtained. Whenever sleep is disturbed and the necessary amount of sleep is not met and it needs to be compensated during the next sleep opportunity, which is known as the sleep rebound (Helfrich-Forster, 2018).

Sleep is a vital and ubiquitous state that is evolutionarily conserved and plays a significant role in executive brain functions such as attention, decision-making, learning, and memory (Bringmann, 2018; Ly, Pack, & Naidoo, 2018). Mammals and flies share several similarities, such as age-dependent changes in sleep amount and patterns, susceptibility to drugs that induce or suppress sleep like antihistamines and caffeine, and molecular markers of sleep (Kume, Kume, Park, Hirsh, & Jackson, 2005; Lin et al., 2010). Sleep deficit impacts health and the economy. For example, sleep deficits can impair cognition, alertness, metabolism, and immune function, increase the risk of accidents, and in extreme cases increase mortality (Bringmann, 2018; Kayser, Mainwaring, Yue, & Sehgal, 2015).

Sleep is affected by environment's illumination. A recent study showed that a slight change in light could significantly affect sleep patterns in healthy human subjects and, more importantly, aggravate the symptoms of the patients suffering from Alzheimer's disease (AD) (M. Kim et al., 2018). This is a serious problem because some places have constant light illumination, which adversely affects people in the shift work system (M. Kim et al., 2018). Sleep fragmentation happens when an individual consistently wakes up afte falling asleep. Sleep deficit affects fly behavior as well. Previous studies have demonstrated that acute mechanical sleep disruption suppresses aggression in flies (Kayser et al., 2015).

1.8 Shaker voltage-gated potassium channel

Shaker, *Hyperkinetic* and *quiver/sleepless* mutants have reduced sleep (Chiara Cirelli, 2005; Daniel Bushey, 2007; M. Wu, Robinson, & Joiner, 2014). The Shaker gene codes for the alpha subunit of the Shaker voltage-gated potassium channel complex (Schwarz, Tempel, Papazian, Jan, & Jan, 1988). The Sh mutant with the most severe sleep phenotype is *Shaker mininsleep* (Sh^{mns}), which has decreased duration but a normal number of sleep episodes, nonetheless are viable and fertile (Chiara Cirelli, 2005).

The *Hyperkinetic* (Hk) gene codes for the β subunit that binds to the α-subunit, which regulates Sh activity by increasing the amplitude and altering voltage dependence and kinetics

for activation and inactivation (Chouinard, Wilson, Schlimgen, & Ganetzky, 1995). Mutation in the *Hk* gene results in a short sleep phenotype (Daniel Bushey, 2007). Contrary to the lack of changes in locomotor activity seen in the *Sh*^{mns}, *Hk* mutants show increased locomotor activity (Daniel Bushey, 2007).

The *quiver/sleepless* (*qvr/sss*) codes for Ly6/neurotoxin protein linked to the plasma membrane by a glycosylphosphatidyl-inositol (GPI) anchor (M. N. Wu et al., 2010). Shaker protein level and activity are reduced in *qvr/sss* mutants. Mutations in *qvr/sss* severely affect daytime and nighttime sleep (Koh (Koh et al., 2008). Unlike *Sh*, this phenotype is attributed to the reduced duration and number of sleep bouts (Koh et al., 2008). Like *Sh*, locomotor activity is unaffected in *qvr/sss* (Koh et al., 2008). Contrary to Hyperkinetic, which is a cytosolic protein, quiver/sleepless is anchored to the extracellular membrane, activity and expression (Koh et al., 2008; M. N. Wu et al., 2010).

The voltage-gated potassium channel is important in mammals as in Drosophila. While Drosophila only has one *Shaker* gene, the human genome has 8 genes that code for the Sh alpha subunit of potassium channels (Douglas et al., 2007). One of these genes is the *Kcna2* which codes for the Kv1.2 and *Kcna2* knockout mice show less slow wave sleep and an increased number of waking episodes than wild-type mice (Douglas et al., 2007).

1.9 Overall Significance

Impulsivity is associated with many brain disorders including ADHD, autism spectrum disorder, substance use disorder, and dementia (Bari & Robbins, 2013; Logue & Gould, 2014); however, the underlying mechanisms remain poorly understood. Several genetic factors affecting inhibitory control have been identified (Bowirrat et al., 2012). For example, the ADHD children with the dopamine transporter 1 variable number of tandem repeat polymorphism have severe response inhibition deficits when tested in a Go/No-Go test (Braet et al., 2011). Non-genetic factors are also associated with dysfunctional inhibitory control

((Magnuson, Kang, Dalton, & McNeil, 2022). For example, sleep deficits are associated with dysfunctional inhibitory control. Sleep disruption in healthy individuals significantly decreases response accuracy in the Go/No-Go test (Demos et al., 2016). However, whether the same neurobiological mechanism regulates inhibitory control and sleep remains unclear.

Here I investigated how genetic and non-genetic factors contribute to inhibitory control dysfunction, specifically motor impulsivity in *Drosophila*. In Chapter 2 of this dissertation, I clarify the effects of caffeine and aberrant sleep on impulsivity. I further uncover the mechanism by which caffeine induces impulsivity. In Chapter 3, I identify the role of the Shaker voltage-gated potassium channel for impulsivity as well as the neuronal site where the Shaker complex plays a role in impulsivity. In Chapter 4, I identify the *Kekkon5* gene that encodes a synaptic cell adhesion molecule as a novel enhancer of dopamine signaling for impulsivity. All these findings are novel and will provide insight into the mechanism by which genetic and non-genetic factors contribute to impulsivity associated with brain disorders and may lead to the development of preventive and therapeutic interventions.

Chapter 2: Nighttime caffeine intake augments motor impulsivity

2.1 Introduction

Caffeine is the most consumed psychostimulant worldwide. Approximately 85% of adults in the United States consume caffeine (Fulgoni, Keast, & Lieberman, 2015). For example, shift workers, medical professionals, and military personnel routinely use caffeine to stay awake and improve performance during nighttime (Franke et al., 2015; Irwin, Khalesi, Desbrow, & McCartney, 2020; Lieberman, Tharion, Shukitt-Hale, Speckman, & Tulley, 2002; Muehlbach & Walsh, 1995; Ogeil et al., 2018; Walsh et al., 1990). Caffeine consumption during nighttime improves cognitive performance in the logical reasoning test and productivity in the simulated assembly line test (Bonnet & Arand, 1994) (Muehlbach & Walsh, 1995). Caffeine is known to improve reaction times and attention in individuals who have been sleep-deprived (Huffmyer et al., 2020; Kamimori et al., 2015; Killgore, Kamimori, & Balkin, 2014; Lieberman et al., 2002; Wingelaar-Jagt, Bottenheft, Riedel, & Ramaekers, 2023). In surgeons, caffeine consumption after sleep disruption restored laparoscopic psychomotor skills to rested baseline levels (Aggarwal, Mishra, Crochet, Sirimanna, & Darzi, 2011). Repantis et al., (Repantis, Bovy, Ohla, Kuhn, & Dresler, 2021) showed that reaction time improvement could also be seen in subjects without sleep loss as measured by the sustained attention task. Furthermore, caffeine has been shown to affect memory positively. Memory retention was improved in Wistar rats when given caffeine after training in the Morris water maze (Angelucci, Cesário, Hiroi, Rosalen, & Cunha, 2002). Alheider et al. (Alhaider, Aleisa, Tran, Alzoubi, & Alkadhi, 2010) showed that chronic caffeine treatment prevented the memory decline resulting from sleep disruption in Wistar rats as measured by the radial arm water maze task. Also, caffeine treatment improved short- and long-term memory in rats (Assis et al., 2018; Sallaberry et al., 2013).

Caffeine also has detrimental effects, such as nausea, jitteriness, trembling, and anxiety (Daly & Fredholm, 1998; Parry, Iqbal, Harrap, Oeppen, & Brennan, 2023). Caffeine

consumption's negative effects on motor control can increase errors in fine hand movement activities. Jacobson et al. found that naïve caffeine drinkers display reduced hand steadiness (via steadiness tester), decreased dexterity (tweezer dexterity tester), and increased errors (Steadiness tester and tweezer dexterity tester) after consuming caffeine one hour before testing (Jacobson, Winter-Roberts, & Gemmell, 1991). These effects were also seen using the steadiness and maze coordination tests (Bovim, Naess, Helle, & Sand, 1995). Similarly, microsurgeons that ingested caffeine showed steadiness deficit or "shakiness" when doing the free-handed steadiness test (Arnold, Springer, Engel, & Helveston, 1993). There is also an increase in completion time and hand movements necessary to complete the laparoscopic skills test (Quan, Alaraimi, Elbakbak, Bouhelal, & Patel, 2015). First-year surgeons were less effective after caffeine consumption based on the spatial accuracy test showing increased errors (Bykanov et al., 2021). Caffeine is also associated with gamblers' impulsive responses and irrational and risky choices as measured by the Barratt questionnaire, a computerized gambling task, and Cambridge gamble task (Grant & Chamberlain, 2018). Anderson et al. (Anderson, Hagerdorn, Gunstad, & Spitznagel, 2018) also demonstrated that individuals with sleep deficits show more commission errors in the standard continuous performance test after consuming caffeine.

Caffeine has both positive and negative effects on behavior. However, the mechanism of how caffeine intake affects cognitive function is unclear. Here we investigate the effect of nighttime caffeine intake on inhibitory control by evaluating the inhibitory control of *Drosophila* after nighttime caffeine feeding. *Drosophila* has been used as a major tool for understanding the mechanisms of how caffeine affects sleep (R. Andretic, Y.-C. Kim, F. S. Jones, K.-A. Han, & R. J. Greenspan, 2008; Nall et al., 2016; M. N. Wu et al., 2009). Here we show that nighttime caffeine intake results in the loss of inhibitory control, which we define as impulsivity in flies. We also identify dopamine as the key neurotransmitter in caffeine's effect on impulsivity by showing that this phenomenon requires dopamine signaling via the dopamine receptors in the mushroom bodies.

2.2 Materials and Methods

Fly strains and culture

The wild-type strain used in this study is *Canton-S* (*CS*). The dopamine transporter mutant *fmn* was obtained from Dr. Jackson (Tufts University, Boston, MA) and crossed with *CS* to generate the heterozygous dopamine transporter *fmn/+*. The dopamine receptor mutants used in this study are *dumb*^{1} with an inversion mutation in the dDA1/Dop1R1 (D1 dopamine receptor) gene, *dumb*^{2} with the PBac{WH} inserted in the first intron and *dumb*^{4} with the Minos Mi{MIC} inserted in the first intron (Y. C. Kim, Lee, & Han, 2007; Lim et al., 2018). All mutants are in the *CS* background. The following fly strains were obtained from the Bloomington Drosophila Stock Center (BDSC; Bloomington, IN): w1118; PBac{WH}plef01945/TM6B, Tb1 (*pale-1*) (BDSC #18492), y1 w*; Mi{MIC}pleMI02717/TM3, Sb1 Ser1 (*pale-2*) (BDSC #36034), *OK107-GAL4* (BDSC #854), *c739-GAL4* (BDSC #7362), and *c305a-GAL4* (BDSC #30829); *NP1131-GAL4* and *NP3061-GAL4* from Dr. Dubnau (Stony Book University School of Medicine, Stony Brook, NY, USA); *MB247-GAL4* from Dr. Waddell (The University of Oxford, Oxford, UK). I generated all the different heterozygous mutants by collecting virgin flies from the *CS* three times a day (8 am, 12 pm, and 6 pm). Accumulating 25-30 virgin flies per cross that I needed, using 20-25 males of *fmn* or *ple* flies and placing them in a bottle. Crosses were made reciprocally. Once the crosses were made, I waited 7-10 days to empty the bottles. Flies were raised on a standard cornmeal/sucrose/yeast/agar medium at 25° C with 50 % relative humidity under a 12 h light/12 h dark cycle. Female and male flies were collected under carbon dioxide within two days after eclosion and used separately when 3 to 4 days old. During collection, the occasional flies with damaged wings were discarded as they could not display flying behaviors, and the flies with good wings were housed in same-sex groups of 13-15 per vial (representing n=1). The vials were placed for two days in the light-

, temperature-, and humidity-controlled incubator before caffeine feeding and behavioral assays. Both males and females were examined separately.

Caffeine feeding

Caffeine-containing food was made as follows: 200 ml of water was placed in a 600 ml beaker and heated on a hot plate. 1 ml of ddH_2O in a 50 ml falcon tub was placed inside the heated water to warm it, and then the appropriate amount of caffeine (C0750, Sigma-Aldrich St. Louis, MO; 0, 10, 50, 75, or 100 mg for 0, 1, 5, 7.5 or 10 mg/ml, respectively) was added. The tube was vortexed until caffeine was completely dissolved, 1 ml of water with green food dye was added (dye was used to confirm flies' feeding of caffeine-containing food), and 8 ml of melted food was added to make the total 10 ml of food solution, the tube was vortexed to mix all ingredients thoroughly, and then 1 ml of the caffeine- and dye-containing food was aliquoted into individual fly food vials. After the food was solidified, a group of flies (about 13 males or females, which represents n = 1) were transferred to the caffeine-containing food vial about 30 min before the dark cycle began, kept in the food during the dark cycle, and subjected to behavioral tests after the dark cycle ended.

Sleep Disruption

Two different methods were used to disrupt flies' sleep. Mechanical sleep deprivation (MSD) was carried out using a vortexer (TriKinetics Inc., Waltham, MA) to shake the vials containing flies randomly for 2 s within every 20 s block during the dark cycle (Kayser et al., 2015). Light-induced sleep disruption (LSD) was done by randomly turning on lights once an hour for 30 min during the dark cycle (Williams et al., 2016). Flies were subjected to the Go/No-Go test immediately after sleep disruption.

Go/No-Go test

Flies were transferred into a rectangular plexiglass chamber (60 mm L X 60 mm W X 15 mm H) connected to filtered air, let to explore the chamber for 1 min, and then exposed to the 10 L/min airflow for 10 min. The chamber was video recorded to monitor fly movements before and after airflow. Videos were analyzed manually to score the number of flying events or using the Viewer3 tracking software (BiObserve Technologies, Bonn, Germany) that allows tracking and measuring the average speed of individual flies' movements in mm/sec per fly. Raw data were exported to Excel (Microsoft, Redmond, WA), and the number of movements exceeding 60 mm/sec that we defined as loss of inhibition events (LIE;(P. R. Sabandal et al., 2022)) was scored per fly per min. The highest number of LIE per fly per min was used to compare control and experimental groups. When the LIE number per fly per min is low, all LIE numbers during the entire 10 min were used for comparison. All behavioral experiments were performed blind to the experimenter. The control and experimental groups were tested in the same experimental session. Multiple sets of flies obtained from independent seeding or crosses were used for behavioral experiments.

Caffeine analysis

The flies were anaesthetized using CO_2, sorted (by sex and caffeine concentration), and collected into 1.5 microcentrifuge tubes. The flies were then frozen and stored at -80°C freezer. To measure caffeine in the flies, we used a commercially available Caffeine ELISA kit (Catalog no. 515575; ABRAXIS, Warminster, PA, USA). For each sample, 5 whole flies were transferred into an ice-cold KONTES Micro Tissue Grinder (ThermoFisher Scientific, Waltham, MA), 50 μl of the Sample Diluent was added to each sample (thus, 1 whole fly per 10 μl) and then homogenized for 30 to 45 s. The samples were transferred to fresh tubes, centrifuged at 14,000 rpm for 10 min at 4°C, and placed on ice until the Caffeine ELISA assay. Caffeine quantification was conducted per the manufacturer instructions (Catalog no. 515575; ABRAXIS, Warminster,

PA, USA). Three independent sets of samples obtained from independent broods and feedings were used for caffeine quantification.

Statistical analysis

All statistical analyses were performed using the Minitab software (Minitab, State College, PA) or JMP (SAS, Cary, NC). Raw data were analyzed using the Anderson–Darling goodness-of-fit test for distribution and are reported as mean + SEM. Normally distributed data were analyzed by either a two-tailed Student's *t*-test for two groups or by ANOVA followed by *post hoc* Tukey's multiple-comparison for three or more groups or Dunnett's test to compare experimental groups with a control group. Non-normally distributed data were analyzed by Kruskal-Wallis and post hoc Mann-Whitney tests. Significant difference among the groups under comparison was determined using an α level of 0.05 in all analyses. All raw data files are available on request.

2.3 Results

Caffeine feeding at night causes inhibitory control deficit

To investigate the effect of nighttime caffeine consumption on behavioral control, independent groups of *Canton-S* (*CS*) female and male flies were fed with the food containing different amounts of caffeine (1, 5, 7.5, and 10 mg/ml), and then we measured the caffeine content per fly using a caffeine ELISA kit. As the caffeine dose increased, the amount of caffeine within the females and males also increased (Figure 1; female: ANOVA, $F_{4,14} = 443.97$, ***, $p < 0.0001$, $n = 3$; male: ANOVA, $F_{4,14} = 226.31$; ***, $p < 0.0001$, $n = 3$). Notably, the caffeine content in the females was consistently higher compared to males, especially in the 5, 7.5, and 10 mg/mL concentrations (Figure 1). These results indicate that the caffeine content in flies tends to be sexually dimorphic, where the caffeine quantity was larger in females than in

males. We hypothesize that caffeine's effect on impulsivity would follow the same pattern. After measuring the caffeine content, we then subjected the flies to the Go/No-Go test that measures the fly' capacity to suppress movements under the condition (e.g., the presence of strong wind or a predator) for movements to jeopardize wellbeing or survival ((P. R. Sabandal et al., 2022), unpublished data). Briefly, flies placed in a novel chamber actively move around but stop moving when strong airflow is introduced and maintain movement suppression till the airflow is turned off. The flies that lose inhibitory control exhibit impulsive flying behavior (movements exceeding 60 mm/sec), which we quantify as a readout of inhibitory control deficit (LIE, (P. R. Sabandal et al., 2022)). In the absence of caffeine feeding, both female and male flies showed strong movement suppression with sporadic flying (Figure 1, 0 mg/ml). Upon caffeine feeding, however, flies displayed increased LIEs in a dose-dependent manner to a greater extent in females (Figure 2A: Kruskal Wallis test, $p = 0.0001$, $n = 30\text{-}32$) than in males (Figure 2B: Kruskal Wallis test, $p = 0.0118$, $n = 30\text{-}32$). This result indicates that nighttime caffeine consumption causes impaired inhibitory control and aligns with the measured caffeine content.

We asked whether the observed caffeine-induced LIEs are related to hyperactivity. To address it, we measured the walking speeds of the *CS* female and male flies fed with or without caffeine in the absence of airflow. *CS* females did not show any change in walking speeds after caffeine intake (Figure 2C: Student *t*-test, $p = 0.1407$, $n = 30\text{-}32$), while *CS* males showed rather decreased walking speeds (Figure 2D: Student *t*-test, $p = 0.0001$; $n = 30\text{-}32$). This indicates that the caffeine-induced impulsive flying is not due to hyperactivity. Caffeine promotes wakefulness, altering sleep (R. Andretic, Y. C. Kim, F. S. Jones, K. A. Han, & R. J. Greenspan, 2008; Nall et al., 2016; M. N. Wu et al., 2009). We investigated whether sleep deficits contribute to impulsivity. For the task, *CS* females and males were subjected to mechanical sleep disruption (Kayser et al., 2015) or light sleep disruption (LSD; (Williams et al., 2016)) during the dark phase and then examined in the Go/No-Go test. Mechanical sleep disruption caused a small yet significant increase in LIEs in females (Figure 2E MSD: Mann-Whitney, p

= 0.0036, *n* = 15-17) but not to the level seen with caffeine feeding. There was no increase in LIEs in the *CS* females subjected to light sleep disruption and the *CS* males subjected to either mechanical or light sleep disruption (Figures 1E and 1F: Mann-Whitney, $p > 0.05$; *n* = 14-17). These results indicate that the impaired inhibitory control induced by nighttime caffeine intake is not due to sleep loss per se.

Dopamine mediates the caffeine's effect on inhibitory control

The wake-promoting effect of caffeine is mediated by dopamine signaling (Rozi Andretic et al., 2008) (Nall et al., 2016). To explore whether the caffeine's effect on inhibitory control involves dopamine signaling, we examined the flies with the heterozygous mutation (2 independent alleles) in tyrosine hydroxylase, the rate-limiting enzyme for dopamine biosynthesis (*ple*/+) (Silva, Hidalgo, & Campusano, 2020). Similar to *CS*, both alleles of *ple*/+ females and males showed strong movement suppression in the absence of caffeine (Figure 3A, Kruskal Wallis test, $p = 0.64$, *n* = 5-6; Figure 3B, Kruskal Wallis test, $p = 0.32$, $n = 6$). Upon caffeine feeding, both alleles of *ple/+* females and males also displayed strong movement suppression, unlike the control *CS* (Figure 3A, ANOVA, $p = 0.0005$, $n = 6$; Figure 3B, ANOVA, $p = 0.0002$, $n = 6$) indicating that the reduced dopamine biosynthesis fully suppressed the caffeine-induced impulsive flying. As a complementary approach, we tested the flies with the heterozygous mutation in dopamine transporter (*fmn/+*), which have the elevated extracellular dopamine level (Makos, Han, Heien, & Ewing, 2010), and found that the *fmn/+* females and males exhibited significantly augmented LIEs upon caffeine feeding (Figure 3C, Student *t*-test, $p = 0.0008$, *n* = 8-9; Figure 3D, Student *t*-test, $p = 0.0023$, *n* = 8-9). In the absence of caffeine feeding, the fmn/+ females and males exhibited negligible LIEs similar to *CS* (Figure 3C, Mann-Whitney, $p = 0.21$, *n* = 8-9; Figure 3D, Mann-Whitney, $p = 0.36$, *n* = 8-9). These data together demonstrate that dopamine signaling is required for the caffeine effect on impulsivity.

Caffeine-induced inhibitory control deficit is mediated by the dDA1 receptor

We have previously shown that the D1 dopamine receptor dDA1/Dop1R1 is a major receptor mediating caffeine's wake-promoting effect (R. Andretic et al., 2008). To investigate whether dDA1 is also important for the caffeine-induced inhibitory control deficit, we examined three independent dDA1 mutant alleles: *dumb*[1], *dumb*[2], and *dumb*[4] (Y. C. Kim et al., 2007; Lim et al., 2018). Upon caffeine feeding, all three *dumb* alleles showed strong movement suppression similar to the *ple/+* flies shown in Figure 2 but unlike the control *CS* (Figure 4A and 4B, ANOVA, $p < 0.0001$, $n = 6\text{-}14$). These show that the dDA1 receptor plays a major role in caffeine-induced impulsivity in flies.

To identify the neural site where dDA1 mediates inhibitory control deficit, as we previously have done in our study of learning and memory (Lim et al., 2018), we took advantage of the UAS inserted in the *dumb*[2] allele to express endogenous dDA1 in the mushroom body neurons using the well-established GAL4 drivers (Y. C. Kim et al., 2007; Qin et al., 2012). Since dDA1 is highly expressed in a majority of mushroom body neurons (Y. C. Kim, Lee, Seong, & Han, 2003) and the dDA1 in the mushroom body neurons is important for the caffeine's wake-promoting effect (Rozi Andretic et al., 2008) we focused on the mushroom body neurons in this study. The mushroom body neurons have three substructures: γ-lobes, α/β-lobes, and α'/β'-lobes. We observed full reinstatement of the caffeine-induced inhibitory control deficit when dDA1 was restored in the γ-lobes (via NP1131-GAL4) or the α/β-lobes (via NP3061 GAL4), but not in the α'/β'-lobes (via c305a GAL4), in the *dumb*[2] mutant background (Figure 4C; $p < 0.0001$ by ANOVA, $n = 6\text{-}8$). However, we did not observe any significant reinstatement when dDA1 was restored in the γ- and α/β-lobes (via MB247 GAL4) or all mushroom body neurons (via OK107 GAL4). In the absence of caffeine, all fly lines under study did not show significant LIEs (Figure 4D, $p = 0.098$, $n = 6\text{-}8$) thus GAL4-driven dDA1 reinstatement per se was insufficient to induce loss of inhibitory control. Taken together, these

results suggest that the dDA1 in the NP1131 and NP3061 GAL4-expressing neurons may be sufficient, but the dDA1 in the MB247 or OK107-expressing neurons may not be sufficient to mediate caffeine-induced inhibitory control deficit.

2.4 Discussion

This study focused on clarifying the effects of nighttime caffeine consumption on inhibitory control. We first measured the caffeine content in flies after overnight feeding, and we found that caffeine content in flies increased in a dose-dependent manner. In line with the caffeine content data, our results show that nighttime caffeine intake affects the inhibitory control of female and male flies in a somewhat dose-dependent manner, where inhibitory control declines as the caffeine concentration increases. We also show that nighttime caffeine intake does not affect baseline locomotor activity and that sleep deficit alone is insufficient to cause dysfunctional inhibitory control. Lastly, we show the role of dopamine signaling by identifying the D1 dopamine receptor dDA1/Dop1R1 as responsible for caffeine’s effect on inhibitory control.

We found that nighttime caffeine intake induced impulsivity in flies. Human studies have found mixed results to the effects of caffeine on inhibitory control. Some studies have shown that acute caffeine intake can improve inhibitory control as measured by the Go-No/Go test, mainly by decreasing omission errors and increasing reaction times (Barry, De Blasio, & Cave, 2014; Barry, Fogarty, & De Blasio, 2020). Pasman et al. (Pasman, Boessen, Donner, Clabbers, & Boorsma, 2017) reported similar increases in reaction time while also showing a decrease in correct responses via the Go-No/Go test. Tieges et al. (Tieges, Snel, Kok, & Richard Ridderinkhof, 2009) also investigated the effects of caffeine on inhibitory control where they found that processing speed was increased (via stop task), but the effects on inhibitory control were negligible as measured by the flanker test. Thomas et al. (Thomas, Rothschild, Earnest, & Blaisdell, 2019) investigated caffeine's effects on esports players and found that a single dose

of an energy drink containing 150 mg of caffeine did not affect the player's inhibitory control. Kahathuduwa et al. (Kahathuduwa et al., 2020) found that caffeine improved inhibitory control on the Go/No-Go task. However, in the same study, inhibitory control was decreased based on the stop signal reaction task in children with ADHD. The mixed results seen in these studies can also be attributed to the highly diverse age of the human subjects, the different caffeine concentrations used, the differences in sample size, and the diverse forms of caffeine delivery. Our approach allows us to avoid some of the variables encountered in the aforementioned studies and helps us better study the complexity of how caffeine affects inhibitory control.

Caffeine exerts pleiotropic effects in flies and mammals; for example, caffeine has been shown to reduce sleep in both flies and mammals. In humans, daytime caffeine consumption reduces melatonin, promoting sleep at night (O'Callaghan, Muurlink, & Reid, 2018). Another example is that adolescents who consume high concentrations of caffeine are more tired in the morning, suggesting lower sleep quality (Orbeta, Overpeck, Ramcharran, Kogan, & Ledsky, 2006). Caffeine treatment reduces rest levels in flies (Rozi Andretic et al., 2008; Joan C. Hendricks, 2000; Kayser et al., 2015; M. N. Wu et al., 2009). It is also known to increase locomotor activity. Rat studies have shown that treatment with caffeine increases locomotor activity (Cauli & Morelli, 2002; Daly & Fredholm, 1998; Garrett & Holtzman, 1994). Our study systematically investigates the possible contributions of sleep loss and locomotor activity to caffeine-induced impulsivity in flies. We found that the baseline activity of female groups was unaffected by nighttime caffeine feeding, while the males showed decreased baseline activity. These findings partially align with Suh et al. and Koh et al.'s daytime activity data, where they only see increased activity during nighttime (Ko et al., 2017) (Suh, Shin, Han, Woo, & Hong, 2017). Another study has shown that flies do not exhibit changes in their locomotor activity after 24 hours of caffeine intake (Kmetova, Maronek, Borbelyova, Hodosy, & Celec, 2021). We clarify that sleep loss was not responsible for the dysfunctional inhibitory control seen after caffeine feeding by disrupted flies' sleep using two different methods. Our study found that

after reducing flies' sleep using mechanical or light exposure, the changes in inhibitory control were not at the level seen in caffeine feeding. Suggesting that sleep perturbation alone is not sufficient to impact inhibitory control.

Caffeine increases the activity of a subset of dopaminergic neurons in the fly brain, which promotes wakefulness (Nall et al., 2016). We also found that caffeine affects flies' inhibitory control via the dopamine system. In our study, reducing dopamine levels suppressed the effects of caffeine on inhibitory control, while enhanced dopamine levels amplified the dysfunctional inhibitory control caused by caffeine. These results go in line with human studies that show elevated levels of dopamine affect inhibitory control. Balachandran et al. show that amphetamines, which increase synaptic dopamine levels, decrease accuracy on the 5-choice series reaction time task (Balachandran et al., 2018). Cheng et al. also show that injection of amphetamine disrupts the rat's performance in differential reinforcement of low-rate tasks which require proper impulse control. In addition, amphetamine and methylphenidate treatment increase premature responses by rats tested in the stop signal reaction task (Maguire & France, 2019). Lastly, a study on children with ADHD shows the detrimental effect of caffeine on inhibitory control based on the stop signal reaction task (Kahathuduwa et al., 2020). These results suggest that dopamine signaling plays a key role in caffeine's effects on inhibitory control.

Our study found that the dysfunctional inhibitory control resulting from the high concentration of caffeine feeding is regulated via the dDA1 receptor. Our result aligns with recent studies in rats where D1 receptor antagonist treatment after amphetamine injection improves inhibitory control as measured by the differential reinforcement of low-rate tasks (Balachandran et al., 2018; Cheng & Liao, 2017). Self et al. showed that in rats, caffeine acts similarly to D1 receptor agonists, where it does not induce cocaine-seeking behavior at dosages that induce locomotor activity. In contrast, pretreatment for cocaine-seeking behavior enhances the priming behavior (Self, Barnhart, Lehman, & Nestler, 1996). These results

highlight the complexity of how caffeine affects both inhibitory control and locomotor activity.

We identified the neural site where the dDA1 receptor plays a role in the caffeine-induced loss of inhibitory control. In *Drosophila,* the mushroom bodies are a critical neural site that regulates multiple complex behaviors such as sleep, locomotor activity, learning, and memory (Guven-Ozkan & Davis, 2014; Heisenberg, 1994; Y. C. Kim et al., 2007; J.-R. Martin, R. Ernst, & M. Heisenberg, 1998; Pitman, McGill, Keegan, & Allada, 2006). Our previous study also shows that cholinergic transmission in the mushroom bodies is responsible for the aging-related loss of inhibitory control (P. R. Sabandal et al., 2022). We found that dDA1 receptor activity in the NP1131- or NP0361-GAL4 expressing neurons that include the γ or α/β lobe neurons, respectively, is sufficient for the caffeine-induced loss of inhibitory control the caffeine-induced loss of inhibitory control. Caffeine activates dopaminergic PAM neurons, which project to the mushroom bodies, and the signal is then conveyed from the mushroom bodies to the mushroom body output neurons. It is possible that dDA1 activity in the mushroom body alone is not enough to regulate caffeine-induced locomotor activity. It may require dDA1 activity in the mushroom body output neuron or both the mushroom bodies and mushroom body output neurons together. We found a similar mechanism in Sabandal et al. (P. R. Sabandal et al., 2022) where the ACh mushroom body neuron MBON-γ2α'1 was necessary for the loss of inhibitory control.

Our study found that females displayed a higher loss of inhibitory control than males. Nunez et al. used male and female spontaneously hypertensive rats and showed that caffeine treatment from childhood to adolescence increases locomotor activity in spontaneously hypertensive female rats (Nunes, Pochmann, Almeida, Marques, & Porciuncula, 2018). Leffa et al. only focused on male spontaneously hypertensive rats and showed that acute doses of caffeine increase delay choice in rats, while chronic treatment results in more impulsive choices (Leffa et al., 2019). Pandolfo et al. showed that chronic caffeine treatment of spontaneously hypertensive male rats did not affect locomotor activity but positively affected

memory (Pandolfo, Machado, Kofalvi, Takahashi, & Cunha, 2013). Hughes and Hancock compared 3 different strains of rats showed that acute caffeine treatment increased rearing behavior in Wistar female rats and increased risk anxiety in both male and female Long-Evans rats (Hughes & Hancock, 2016). A follow-up study using PVG/c female rats showed they are less affected by chronic caffeine treatment, showing less anxiety behavior and more locomotor activity (Hughes & Hancock, 2017). A study of adult human subjects showed that with low to moderate doses of caffeine, female adults are three times more likely to report more adverse effects like anxiety (Domaszewski, 2023). The responses to caffeine are varied depending on sex thus is important for more studies to report any sexual dimorphism observed.

Studies on caffeine can be susceptible to expectancy or a placebo effect. In human studies, the results might be affected by a subject's previous experience with caffeine (Shabir, Hooton, Tallis, & M, 2018; Wicht, De Pretto, Mouthon, & Spierer, 2022). Experiments investigating this matter show that individuals who previously experienced positive effects from caffeine consumption report feeling the same positive effect even when only suggested they consume caffeine. The same is true for individuals who experience negative effects, they report adverse effects when suggested they consumed caffeine (Shabir et al., 2018). The use of *Drosophila* in this study avoids that effect and allows us to focus on the neurobiology of caffeine.

Unpublished data from our lab (Sabandal manuscript under review) and our study on aged flies have shown that the γ-mushroom body neurons are important for inhibitory control. Here we also show the role of mushroom body neurons in inhibitory control but now in the context of a psychostimulant like caffeine which affects the dopamine system. This highlights the importance of further research into how different neurotransmitters play a role in regulating inhibitory control.

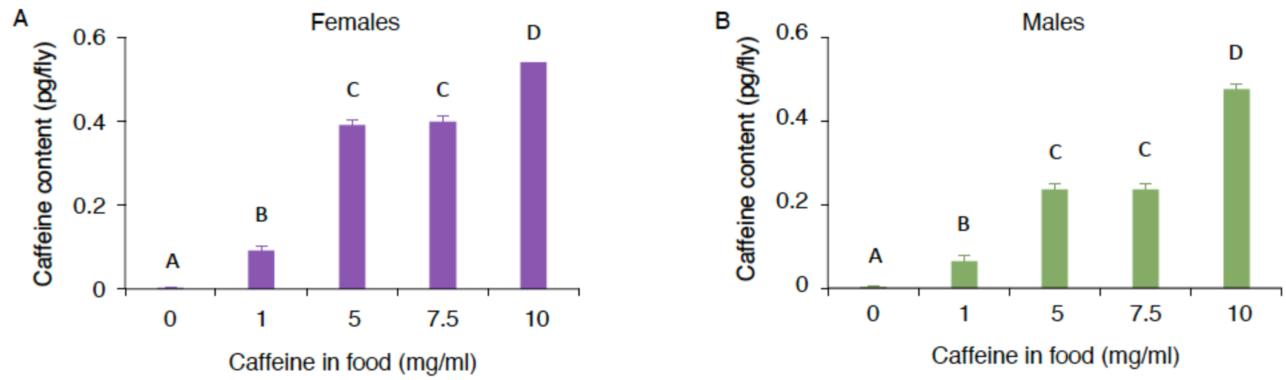

Figure 2.1: Caffeine content in the male and female *Canton-S* (*CS*) flies.

A-B. In both sexes, the caffeine quantity per fly increases in a dose-dependent manner. ANOVA with *post hoc* Tukey multiple-comparison of five caffeine concentrations in food for each sex: different letters denote statistically significant differences ($p > 0.05$; $n = 3$).

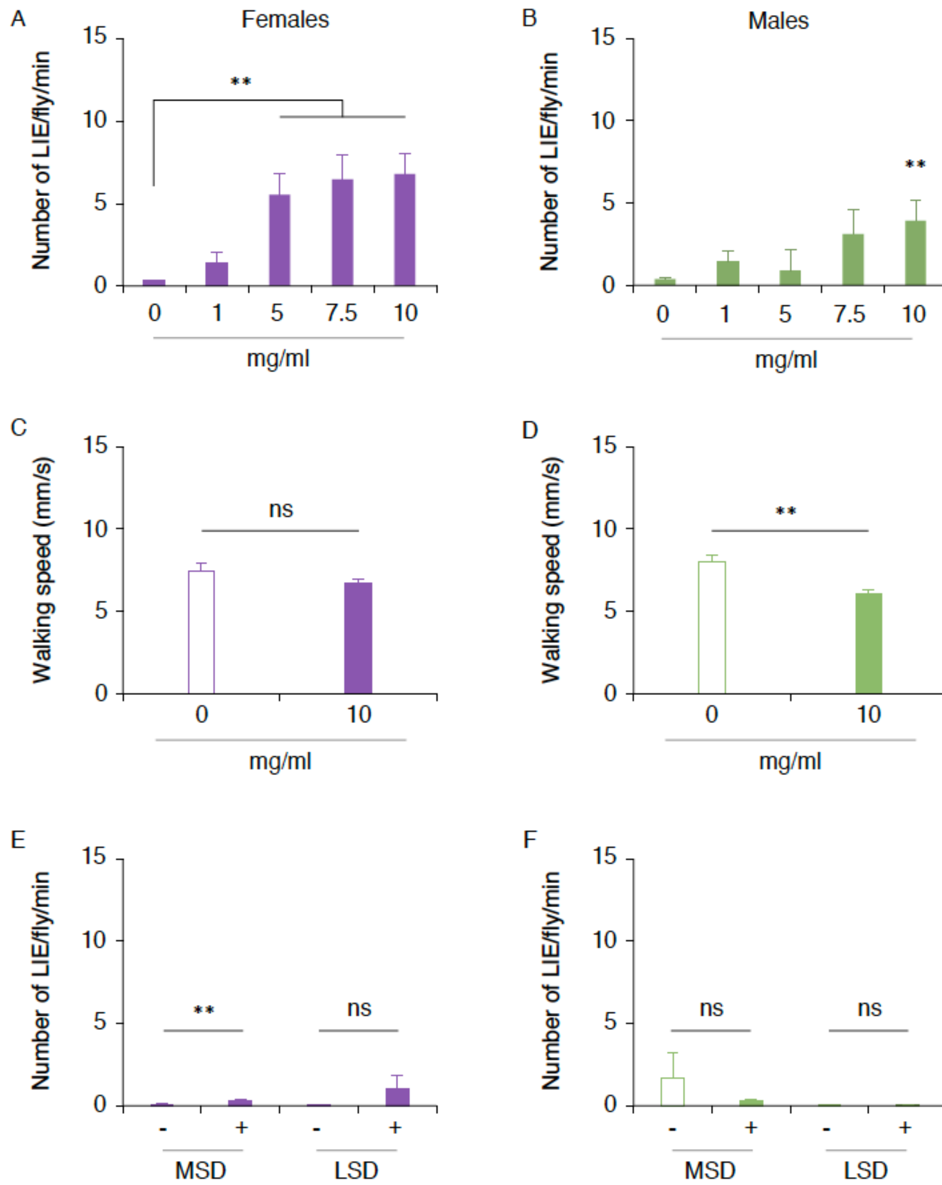

Figure 2.2: Caffeine's effect on inhibitory control in flies.

Caffeine feeding causes inhibitory control dysfunction in a dose-dependent manner in both females (A) and males (B). Kruskal Wallis followed by Dunn test as CS as a control: ns, $p > 0.05$; **, $p < 0.005$; ***; $n = 30\text{-}32$. **C-D.** The baseline activity of females (C: Student *t*-test: ns, $p > 0.05$; $n = 30\text{-}32$) and male (Student *t*-test: ns, $p > 0.05$; **, $p < 0.005$; $n = 30\text{-}32$). **E-F.** Effects

of mechanical sleep disruption (MSD) and light-induced sleep disruption (LSD) in females (E: Mann-Whitney: ns, $p > 0.05$; **, $p < 0.005$; $n = 14\text{-}17$) and males (F: Mann-Whitney: ns, $p > 0.05$; $n = 14\text{-}17$).

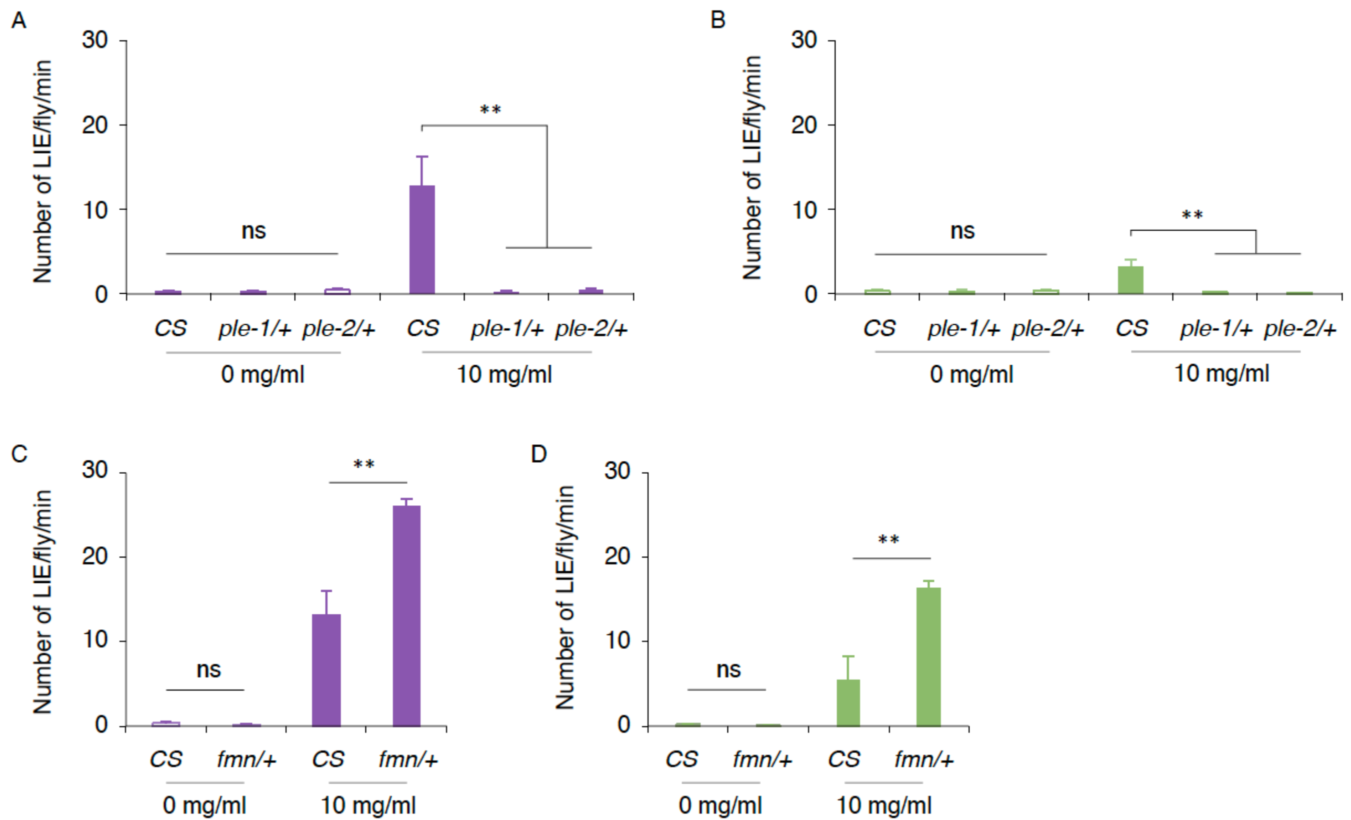

Figure 2.3: Elevated dopamine signaling aggravated caffeine-induced impulsivity

Without caffeine feeding, the *ple*/+ showed normal inhibitory control similar to *CS* in both females (A) and males (B). When fed with 10 mg/ml of caffeine, both female and male *ple/+* showed normal inhibitory control unlike *CS* (A: ANOVA with *post hoc* Dunnett using *CS* as a control: ns, $p > 0.05$; **, $p < 0.001$; $n = 5\text{-}6$. B: ANOVA with *post hoc* Dunnett using *CS* as a control: ns, $p > 0.05$; ** $p < 0.005$; $n = 6\text{-}7$). **C-D.** Unfed *fmn*/+ flies showed normal inhibitory control similar to *CS* in both females (C: Mann-Whitney; ns, $p > 0.05$) and males (D: Mann-Whitney; ns, $p > 0.05$). When fed with 10 mg/ml of caffeine, both female and male *fmn/+* flies showed higher LIEs than *CS* (Student *t*-test; **, $p < 0.005$; $n = 8\text{-}9$).

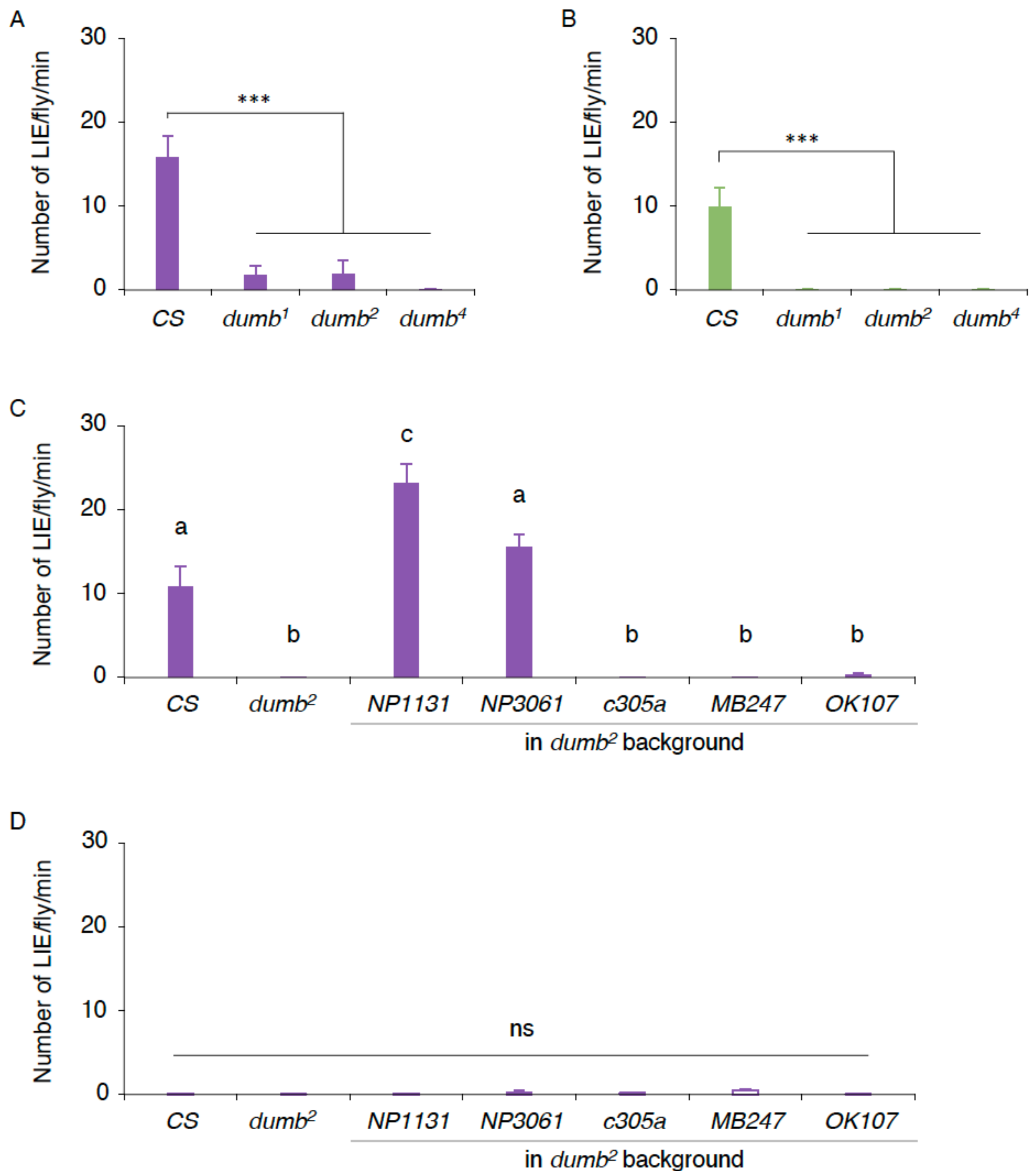

Figure 2.4: The dDA1 receptor mediates caffeine-induced impulsivity

Unlike CS, the caffeine-fed *dumb*^{1}, *dumb*^{2}, and *dumb*^{4}, three independent mutant alleles of dopamine D1 receptor dDA1, exhibited negligible LIEs in females (A: ANOVA with *post hoc*

Dunnett using *CS* as a control: ***, $p < 0.0001$; $n = 9\text{-}14$) and males (B: ANOVA with *post hoc* Dunnett using *CS* as a control: ***, $p < 0.\ 0.0001$; $n = 6\text{-}14$). **C**. Re-expression of dDA1 in the γ (*NP1131;dumb*^{2}) or α/β (*NP3061;dumb*^{2}) neurons in the caffeine-fed *dumb*^{2} females caused high levels of caffeine-induced LIE, whereas dDA1 re-expression in the α'/β' (*c305a;dumb*^{2}) or γ and α/β together (*MB247*;*dumb*^{2}) or in all MB (*dumb*^{2}*;OK107/+*) neurons did not have any effect. (ANOVA: $p < 0.0001$; $n = 6\text{-}8$; different letters denote statistically significant difference). **D.** dDA1 re-expression in the γ (*NP1131;dumb*^{2}), α/β (*NP3061;dumb*^{2}), α'/β' (*c305a;dumb*^{2}) or γ and α/β (*MB247*;*dumb*^{2}) and in all MB (*dumb*^{2}*;OK107/+*) neurons in the absence of caffeine feeding did not cause loss of inhibitory control (Kruskal-Wallis test: ns, $p > 0.05$;$n = 6\text{-}8$).

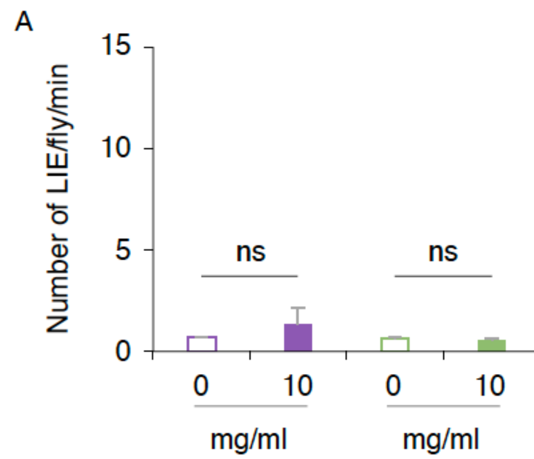

Figure S2.1: Daytime caffeine feeding does not cause impulsivity.

Female (purple shade) and male (green shade) flies showed normal inhibitory control when fed with caffeine for 4 h during daytime (Kruskal-Wallis test: ns, $p > 0.05$; n = 13-14).

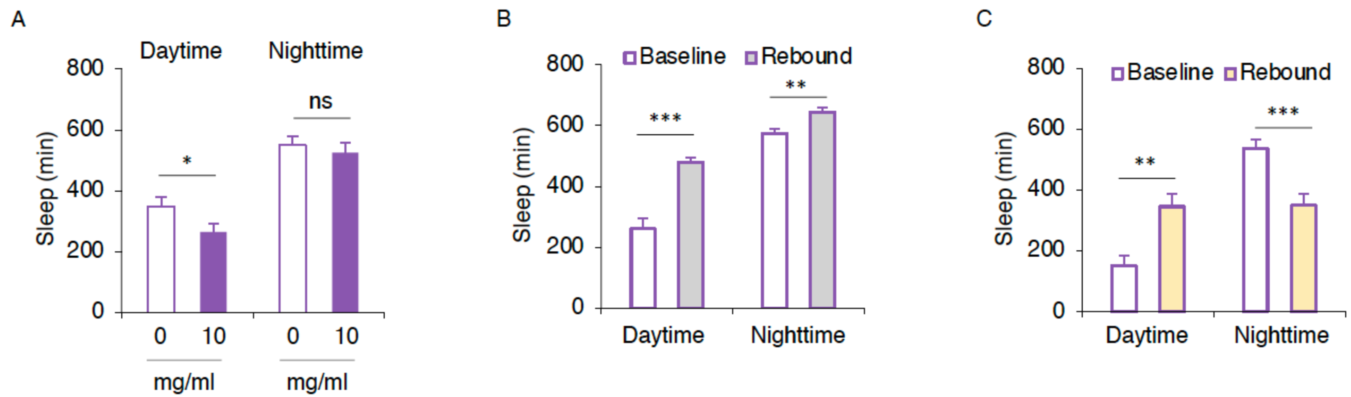

Figure S2.2: Effects of nighttime caffeine feeding, mechanical stimulation-induced sleep disruption (MSD), and light-induced sleep disruptions (LSD) on sleep on the subsequent day

A. Sleep measurement of the nighttime-caffeine-fed flies showed a decrease in daytime sleep but not nighttime sleep, (Student *t*-test: ns, $p > 0.05$; *, $p < 0.05$; $n = 13$-14). **B.** Flies show sleep rebound after sleep disruption via bright light (Student *t*-test: **, $p < 0.005$; ***, $p < 0.0001$; $n = 21$). **C.** Flies show sleep rebound after mechanical sleep disruption (Student *t*-test: **, $p < 0.005$; ***, $p < 0.0001$; $n = 18$).

Chapter 3: The Shaker voltage-gated potassium plays a role in inhibitory control

3.1 Introduction

Paragraphs with the style *Heading 3,h3* applied can be extracted to appear in the table of contents as level 1 sub sections under the chapters. Potassium channels are the most diverse and large group of ion channels; their function is essential for controlling neuronal excitability (Schwarz et al., 1988). Potassium channels are divided into 4 families: two-pore, inward rectifier, calcium-activated, and voltage-gated (Allen, Weckhuysen, Gorman, King, & Lerche, 2020). The voltage-gated potassium channel (Kv) family has 12 sub-families (Kohling & Wolfart, 2016). Voltage-gated potassium channels are activated in response to membrane depolarization for repolarization and hyperpolarization (Bahring, Barghaan, Westermeier, & Wollberg, 2012). One member of the aforementioned family is the Shaker (Sh) complex composed of the alpha subunit Sh and auxiliary proteins such as the beta subunit Hyperkinetic (Hk) and Quiver/Sleepless (Qvr/Sss). The tetrameric Sh channel regulates the kinetics of action potentials and the quantity of neurotransmitters released (Schwarz et al., 1988). Hyperkinetic β-subunits bind to the α-subunits to increase the current amplitude and change the kinetics of inactivation and activation of the Sh channel (Chouinard et al., 1995). Qvr/Sss is a glycosylphosphatidylinositol-anchored Ly6 protein (Koh et al., 2008) (M. N. Wu et al., 2010). Qvr/sss binds to Sh and enhances its activity (M. N. Wu et al., 2010).

Prior studies have demonstrated the roles of the Sh channel complex in various brain functions. For example, mutations in both alpha and beta subunits as well as qvr/sss cause sleep defits (Chiara Cirelli, 2005; Daniel Bushey, 2007; Koh et al., 2008; M. Wu et al., 2014; M. N. Wu et al., 2010). The *Shaker* mutant flies have also been shown to have an increased aggression (Davis, Thomas, Liu, Campbell, & Dierick, 2018). The *Hyperkinetic* mutants are known to have impaired memory (Daniel Bushey, 2007). In human subjects, genetic variants in these channels are linked to ataxia, epileptic encephalopathies, ADHD and autism spectrum disorder (Allen et al., 2020; Boland, Price, & Jackson, 1999). The mechanism by which Sh dysfunction

affects cognitive function is not fully understood. In this study, we show that the Sh, Hk and qvr/sss mutants display impulsivity. We also identify the mushroom body neurons as the major neural site where Sh is important for inhibitory control and the neural site where Sh regulates sleep is distinct.

3.2 Materials and Methods

Fly strains and culture

The wild-type strain used in this study is *Canton-S* (*CS*). The dopamine transporter mutant *fmn* (Kume et al., 2005) was obtained from Dr. Jackson (Tufts University, Boston, MA). All mutants were placed in the *CS* background. The following fly strains were obtained from the Bloomington Drosophila Stock Center (BDSC; Bloomington, IN): Sh^5(BDSC# 111), Sh^{mns}(BDSC# 24149), Hk^1(BDSC# 3562), Hk^2 (BDSC# 55), TRiP.HMC03576}attP40 (Sh^{RNAi}) (BDSC# 53347), HMJ21916}attP40 (qvr^{RNAi}) (BDSC# 58061), c739-GAL4 (BDSC #7362), and c305a-GAL4 (BDSC #30829). P{GD15937}v47805 (Hk^{RNAi}) (VDRC #47805) is obtained from the Vienna *Drosophila* Resource Center (VDRC; Vienna, AT); NP1131-GAL4 from Dr. Dubnau (Stony Book University School of Medicine, Stony Brook, NY, USA); and MB247-GAL4 from Dr. Waddell (The University of Oxford, Oxford, UK. Flies were raised on a standard cornmeal/sucrose/yeast/agar medium at 25° C with 50 % relative humidity under a 12 h light/12 h dark cycle. Flies were collected under carbon dioxide within two days after eclosion. During collection, we occasionally found flies with damaged wings, which were discarded as they could not display flying behaviors, and the flies with good wings were housed in groups of 13 per vial (representing n = 1). The vials were placed for two days in the light-, temperature-, and humidity-controlled incubator before behavioral assays. Only females were used in experiments because *Shaker* and *Hyperkinetic* loci are in the x chromosome.

Go/No-Go test

Single or group flies were transferred into a rectangular plexiglass chamber (60 mm L X 60 mm W X 15 mm H) connected to filtered air, let to explore the chamber for 1 min, and then exposed to the 10 L/min airflow for 10 min. The chamber was video recorded to monitor fly movements before and after airflow. Videos were analyzed manually to score the number of flying events or using the Viewer3 tracking software (BiObserve Technologies, Bonn, Germany) that allows tracking and measuring the average speed of individual flies' movements in mm/sec per fly. Raw data were exported to Excel (Microsoft, Redmond, WA), and the number of movements exceeding 60 mm/sec that we defined as loss of inhibition events {LIE;(P. R. Sabandal et al., 2022)} was scored per fly per min. The highest number of LIE per fly per min was used to compare control and experimental groups. When the LIE number per fly per min is low, all LIE numbers during the entire 10 min were used for comparison. All behavioral experiments were performed blind to the experimenter. The control and experimental groups were tested in the same experimental session. Multiple sets of flies obtained from independent seeding or crosses were used for behavioral experiments.

Sleep assay

For sleep experiments, flies were gently loaded via aspiration into small glass tubes (5 mm diameter × 65 mm length) containing standard cornmeal/sucrose/yeast/agar medium and caped with a small piece of cotton on the other. The tubes were then loaded into DAM2 boards and placed in a 25 °C with 50% relative humidity incubator with a 12 h light/12 h dark cycle. The activity of each fly was monitored using the Drosophila Activity Monitoring (DAM) system (Trikinetics, Waltham, MA) and analyzed in 1 min bins. Sleep was identified as 5 min of consolidated inactivity (Hendricks et al., 2000; Shaw et al., 2000). Data were processed using the Sleep and Circadian Analysis MATLAB Program (SCAMP) (Donelson et al., 2012).

3.3 Results

Shaker channel dysfunction results in impulsivity

To investigate whether the Sh voltage-gated potassium channel plays a role in inhibitory control, we tested *Sh*^{mns} and *Sh*^{5} (Chiara Cirelli, 2005) for the Sh alpha subunit in a Go/No-Go test. This test measures the fly's ability to suppress its movements in situations that can endanger its safety or survival, such as strong winds or predators (P. R. Sabandal et al., 2022). Flies are placed in a chamber where they freely move around but stop when a strong airflow is introduced. Flies display impulsive flying behavior {movements above 60 mm/sec = LIE, (P. R. Sabandal et al., 2022)} when they are unable to initiate or maintain movement suppression. The wild-type *Canton-S* (*CS*) flies displayed strong movement suppression in the presence of strong airflow. However, both *Sh*^{mns} and *Sh*^{5} were unable to maintain movement suppression and displayed a significant number of impulsive flying behavior (Figure 1A. ANOVA, $p < 0.0001$, $n = 8\text{-}12$) with *Sh*^{mns} mutant flies showing more impulsive flying than *Sh*^{5}. We next asked whether Hk β-subunit deficiency also results in impulsivity and found that two independent *Hk* mutants *Hk*^{1} and *Hk*^{2} exhibited significantly increased LIEs compared to *CS*, with *Hk*^{1} mutant flies having the more severe phenotype (Figure 1B: ANOVA, $p < 0.0001$; $n = 8\text{-}10$). These results suggest a role for the Sh complex in inhibitory control.

Unpublished data from the lab show that social settings can alter impulsivity, where the flies tested in a group showed more severe impulsivity than tested alone (Sabandal et al. manuscript under revision). To test if social environment affects the *Sh* and *Hk* mutant flies' impulsivity, we tested the *CS*, *Sh* and *Hk* in a single-fly setting. The flies with the strong alleles of Sh (Figure 1C) and Hk (Figure 1D), *Sh*^{mns} and *Hk*^{1} (Gramates et al., 2022), showed significant LIEs while the flies with week alleles, *Sh*^{5} and *Hk*^{2} displayed normal inhibition similar to *CS* (Figure 1C: Mann-Whitney: $p < 0.0001$, $n = 22\text{-}24$). The LIEs of both *Sh*^{mns} and *Hk*^{1} in a single-fly-setting were lower than those in a group setting (Figure 1A and 1B). These results suggest

that deficits in Sh function leads to impulsivity in a single-fly setting, but social environment enhances impulsivity.

To investigate a possible interaction between the subunits for inhibitory control, we tested individual and double heterozygous flies for Sh^{mns} and Hk^{1} in a Go/NoGo test. while $Sh^{mns}/+$ displayed high LIEs, $Hk^{1}/+$ exhibited normal movement suppression similar to *CS* (Figure 1E; $p < 0.0001$ by ANOVA, $n = 8$-10). Interestingly, the double heterozygous Sh^{mns}/Hk^{1} flies displayed LIEs similar to that of $Sh^{mns}/+$. These results suggest that Sh^{mns} is dominant, Hk^{1} is recessive and $Sh^{mns}/+$ and $Hk^{1}/+$ do not interact for impulsivity.

Interaction between the Shaker channel and dopamine for inhibitory control

Previous studies in the lab have shown that the dopamine transporter mutant *fmn* display impulsivity (Sabandal manuscript in revision). We asked if Sh or Hk interact with fmn for impulsivity. To address this question, we generated double heterozygous Sh^{mns} or Hk^{1} and *fmn*. The double heterozygous $Sh^{mns}/+$:*fmn*/+ flies showed higher LIEs than $Sh^{mns}/+$ while *fmn/+* flies showed normal inhibition, suggesting synergistic interaction of $Sh^{mns}/+$ and *fmn/+* (Figure 2A; $p < 0.0001$ by ANOVA, $n = 11$-17). The double heterozygous $Hk^{1}/+$;*fmn*/+, however, showed movement suppression similar to *CS*, *fmn/+* and $Hk^{1}/+$ (Figure 2C; $p > 0.05$ by ANOVA, $n = 11$-19), suggesting no interaction of *fmn/+* and $Hk^{1}/+$ for impulsivity. When the double homozygous flies were examined, Sh^{mns};*fmn* flies displayed the level of LIE similar to that of *Sh* but lower than that of *fmn* flies, indicating Sh^{mns} is epistatic to *fmn* (Figure 2B; $p < 0.0001$ by ANOVA, $n = 10$-18). The double homozygous Hk^{1};*fmn* flies, on the other hand, showed more LIEs than *fmn* or Hk^{1} (Figure 2C; $p < 0.0001$ by ANOVA, $n = 14$-17), suggesting additive interaction of Hk^{1} and *fmn.*

Mushroom bodies are the neural site for Shaker's role in inhibitory control

Sh is highly expressed in the mushroom bodies (Rogero & Tejedor, 1995; M. Wu et al., 2014; M. N. Wu et al., 2010) and our previous work has shown that the mushroom bodies (MB) neurons are important for inhibitory control (Sabandal et al., manuscript under revision)(P. R. Sabandal et al., 2022). Hence, we hypothesized that the MB neurons could be the neural site important for the Sh's role in impulsivity. To address this, we used the GAL4/UAS system to perform conditional knockdown of *Sh* and its auxiliary proteins *Hk* and Qvr/Sss using RNA interference (RNAi). The MB has three sub-structures, γ, α/β and α'/β' neurons (Tanaka, Tanimoto, & Ito, 2008). Sh, Hk or Qvr/Sss knockdown (KD) in individual lobe neurons (γ via np1131-GAL4; α/β via c739-GAL4 or α'/β' via c305-GAL4) or the γ and α/β neurons together (via MB247-GAL4) resulted in the significant increase in LIE (Figures 3A, 3B and 3C; ANOVA, $p < 0.0001$; $n = 6 - 30$), suggesting that Sh, Hk and Qvr/Sss in all MB subsets are important for inhibitory control.

Sh is expressed in additional brain areas other than MB (Rogero & Tejedor, 1995). To idenfiy whether Sh, Hk and Qvr/Sss in other brain area are involved in inhibitory control, we used the pan-neuronal driver elav-GAL4 for KD of Sh, Hk, and Qvr/Sss, and found that the LIE levels of pan-neuronal Sh, Hk, and Qvr/Sss KD are similar to those of the MB Sh, Hk, and Qvr/Sss KD (Figure 3A, 3B and 3C). This suggests that the MB may be the major functional site of Sh, Hk and Qvr for inhibitory control.

Sh, Hk and *qvr* mutants have the reduced sleep phenotype (Chiara Cirelli, 2005; Daniel Bushey, 2007). While the functional sites of Sh, Hk and Qvr/Sss for sleep are unknown, the MB is known to regulate sleep in flies (Joiner, Crocker, White, & Sehgal, 2006). We asked if the MB is also responsible for the sleep deficits of *Sh*, *Hk* and *qvr* mutants. When the nighttime sleep of the MB Sh, Hk and Qvr KD flies were compared with the control UAS-RNAi lines, Sh, Hk and Qvr KD in the α/β neurons but not in other MB subsets reduced sleep (Figure 4A, 4B, and 4C; ANOVA, $p < 0.0001$; $n = 11\text{-}16$). As for daytime sleep, Sh and Qvr KD in the α/β neurons as well as Sh KD in α'/β' neurons induced sleep reduction (Figure 4D, 4E and 4F;

ANOVA, $p < 0.0001$; $n = 11\text{-}16$) This suggests that the neural sites that Sh, Hk and Qvr regulate inhibitory control and sleep are distinct.

3.4 Discussion

We investigated the role of Sh in inhibitory control. We found that changes in neuronal excitability due to mutations in the Sh complex result in impulsivity. We also show that the social environment plays a role in impulsivity in that group environments lead to increased impulsivity. We found no interaction between the subunits for inhibitory control and the *Sh*^{mns} to be dominant, while *Hk*^{1} is recessive for impulsivity. Lastly, we identify that activity of the Sh$_{V}$ complex (Sh, Hk, and qvr/sss) in all MBs is important for inhibitory control, while only activity in α/β MB neurons is for sleep.

Our results show that mutations in *Shaker* result in loss of inhibitory control, with *Sh*^{mns} showing more impulsivity than *Sh*^{5}. *Shaker* codes for the α-subunit of the Shaker voltage-gated potassium channel. Each subunit is made of six transmembrane domains S1-S6. The transmembrane domains also separate into two regions where S1 to S4 form the voltage sensing region and S5 to S6 shape the pore region (Chiara Cirelli, 2005; Davis et al., 2018). Studies have shown that mutations in *Shaker* produced a severe sleep phenotype and increased aggression (Chiara Cirelli, 2005; Davis et al., 2018). *Sh*^{mns} is the most severe of the two alleles tested in sleep, while *Sh*^{5} is more susceptible to modifiers that affect the short-sleep phenotype (Chiara Cirelli, 2005). Other findings also show that *Sh*^{mns} has a learning deficit phenotype (Daniel Bushey, 2007). In the study of aggression, *Sh*^{mns} show an elevated percentage of fighting frequency. However, *Sh*^{5} has no aggression phenotype (Davis et al., 2018). Our studies also show a more severe impulsivity phenotype in the *Sh*^{mns}. The difference in the phenotype level can result from *Sh*^{mns} point mutation being a change from Threonine (polar uncharged) to Isoleucine (Hydrophobic), impacting the voltage-sensing region of the channel. In contrast, the *Sh*^{5} point mutation is a Phenylalanine (Hydrophobic) to Isoleucine (Hydrophobic) at the

pore region. Our studies also show that the loss of inhibitory control phenotype in *Sh*^{mns} is dominant, contrary to the sleep and shaking phenotypes which are recessive (Chiara Cirelli, 2005).

Similar to the *Shaker*, we found that mutations in *Hyperkinetic results* in impulsivity. *Hyperkinetic* codes for the β-subunit of the Shv channel. Hk subunits bind to the Shaker subunit, modulating the channel's kinetics (Chouinard et al., 1995) (Wang & Wu, 1996). Studies using *Drosophila* neuromuscular junction have shown that *Hk* mutations affect neurotransmitter release in the same fashion that mutations in *Sh* (Stern & Ganetzky, 1989). *Hk*^{1} has been previously characterized as having sleep deficits and memory impairments, while the *Hk*^{2} mutant allele shows impulsivity, it lacks the short sleep and memory phenotypes (Daniel Bushey, 2007). *Hk*^{1} shows a more severe phenotype than *Hk*^{2} in the jumping behavior (Kaplan & Trout, 1974). Similar to our study on impulsivity, the jumping behavior phenotype is recessive (Kaplan & Trout, 1974).

Our study found no interaction between *Sh*^{mns} and *Hk*^{1} for impulsivity, contrary to studies in the neuromuscular junction where it was also shown that *Sh* phenotype is epistatic to *Hk,* suggesting that the effects on neurotransmitter release at the neuromuscular junction are a consequence of *Hk* acting on the Sh (Stern & Ganetzky, 1989). The same is true for the sleep phenotype, where *Hk/Sh* mutants do not show a more severe sleep deficit than *Hk* and *Sh* mutants alone (Daniel Bushey, 2007).

Previous studies in the lab have shown that the dopamine system plays a role in flies' inhibitory control. Elevated dopamine levels in the dopamine transporter mutant *fmn* lead to impulsivity in homozygous flies, but heterozygous flies show strong movement suppression (Sabandal in review). Also, similar to the Sh mutants tested here, *fmn* flies have sleep deficits (Kume et al., 2005). We decided to investigate whether Sh interacted with the dopamine system for impulsivity. Our results show that *Sh* and *fmn* double homozygous mutants show lower impulsivity levels than *fmn* homozygous, while the double heterozygous show increased levels of impulsivity compared to *Sh*^{mns}/+. These results suggest a synergistic interaction

between Sh and the dopamine transporter for inhibitory control. Interestingly, the *Hk*^{1} mutant allele only showed an increase in phenotype in the double homozygous background. These results suggest that *Hk*^{1} and *fmn* have and additive interaction for impulsivity. Preliminary studies exploring Sh's downstream effect on the dopamine system show that PKA-catalytic subunit mutant in *Sh*^{mns};*fmn* background decreases the impulsivity phenotype. Suggesting that the Sh potassium affects inhibitory control by disrupting the downstream dopamine pathway.

We identify the neural site where Sh activity is important for inhibitory control. The MBs are a major neural site where complex behaviors like sleep, locomotor activity, learning, and memory are regulated (Guven-Ozkan & Davis, 2014; Heisenberg, 1994; Y. C. Kim et al., 2007). Unpublished data from our lab has also shown them to be important for impulsivity in flies (Sabandal in review). Our study shows that the knockdown of the Sh, Hk, and qvr/sss subunits in the MBs results in impulsivity. We demonstrate that sleep and inhibitory control are regulated via different MB lobes. MBs are known to promote sleep since ablation or inactivation of these neurons reduces flies' sleep (Pitman et al., 2006). However, increasing excitability in the MB neurons also results in decreased sleep (Joiner et al., 2006). This aligns with our finding that altered neuronal excitability due to knockdown of Sh, HK and qvr/sss at α/β MB neurons affects sleep. This finding becomes more relevant since a recent study showed that the knockdown of Sh and HK in the dorsal fan shape body, which is the main brain structure associated with sleep regulation, does not result in sleep loss (De, Wu, Lambatan, Hua, & Joiner, 2023). Altogether, these findings suggest that the increase in neuronal excitability resulting from Shv channel mutations is responsible for the loss of sleep and the inhibitory control deficits.

Sh homolog is the potassium voltage-gated channel subfamily A member 3 (KCNA3), mainly linked to autoimmune diseases like multiple sclerosis (Lioudyno et al., 2021). One study has found that a link between the reduction in KCNA3 expression and function contributes to the lower neuronal number and brain size seen in Down Syndrome (Lu et al., 2012). Hk homolog is the potassium voltage-gated channel subfamily A regulatory beta subunit 1

(KCNAB1). This subunit has been associated with synaptic facilitation in the hippocampus (I. H. Cho et al., 2020). Another study found via a genetic screen that KCNAB1 is a susceptibility gene for lateral temporal epilepsy (Busolin et al., 2011). Lastly, like the deficit in learning and memory observed in flies, mice with mutated KCNAB1 showed impaired hippocampal-dependent learning (Giese et al., 1998). With the limited information about the role of Shaker/KCNA, this study furthers our understanding of how this voltage-gated potassium channel contributes to impulsivity.

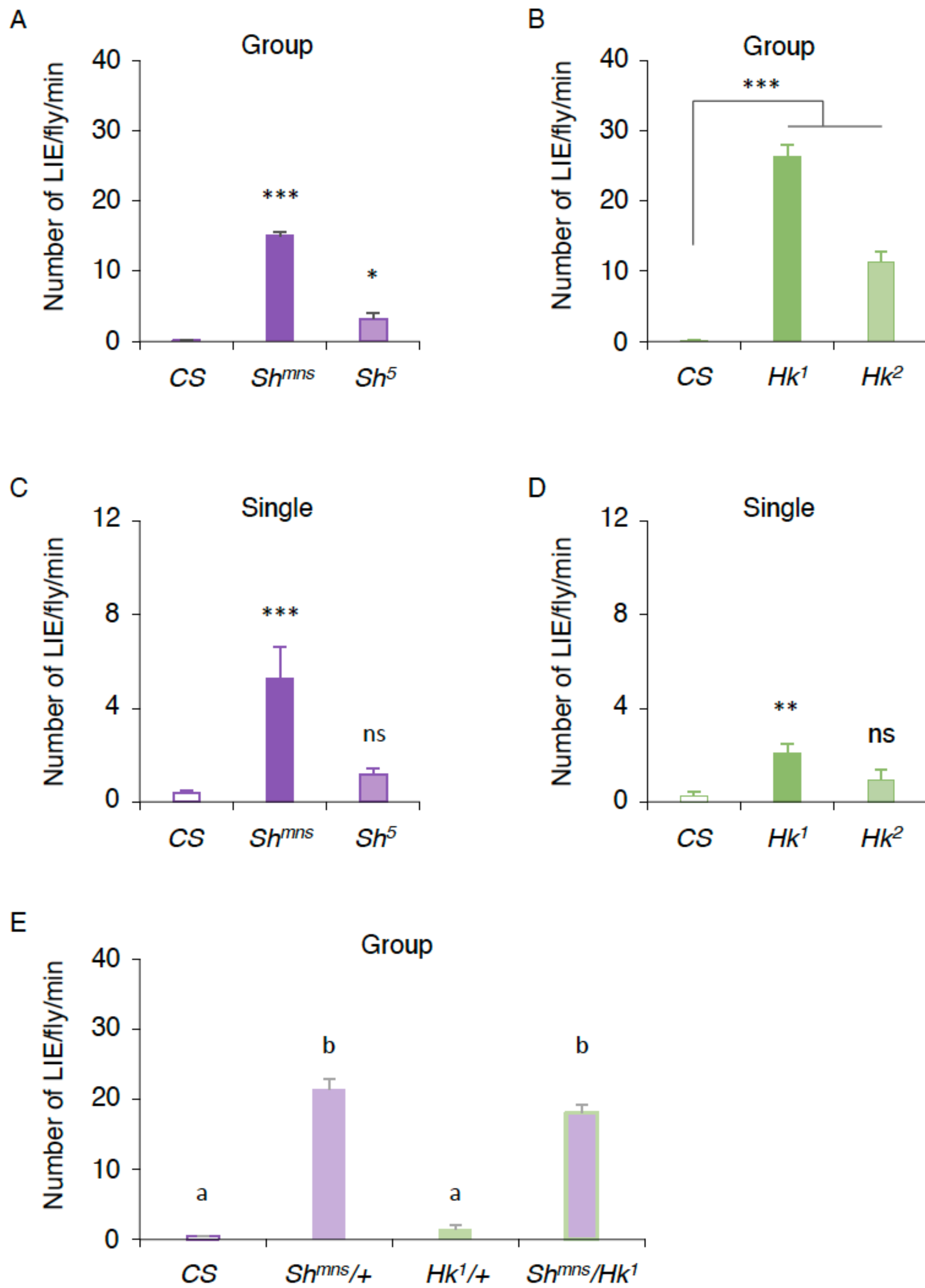

Figure 3.1: Shaker channel mutants showed impulsivity.

A. The mutants for the α-subunit of the voltage-gated potassium channel Shaker, *Sh*^{mns}, and *Sh*^{5} showed higher LIEs compared to *CS* when a group of flies were tested together in a

Go/NoGo test (ANOVA with *post hoc* Dunnett using *CS* as a control: *, $p < 0.05$; ***, $p < 0.0001$; $n = 8\text{-}12$). **B.** The mutants for the β-subunit of the voltage-gated potassium channel Shaker, Hk^{1}, and Hk^{2} showed higher LIEs compared to *CS* when a group of flies were tested together in a Go/NoGo test (ANOVA with *post hoc* Dunnett using *CS* as a control: ns, $p > 0.05$; ***, $p < 0.0001$; $n = 8\text{-}10$). **C.** The mutant for the α-subunit of the voltage-gated potassium channel Shaker, Sh^{mns} showed higher LIEs compared to Sh^{5} mutant and *CS* when single flies were tested in a Go/NoGo test (Mann-Whitney: ns, $p > 0.05$; ***, $p < 0.0001$; $n = 22\text{-}24$). **D.** The mutant for the β-subunit of the voltage-gated potassium channel Shaker, Hk^{1} showed higher LIEs compared to Hk^{2} mutant and *CS* when single flies were tested in a Go/NoGo test (Mann-Whitney: ns, $p > 0.05$; **, $p = 0.001$ $n = 22\text{-}24$). **E.** The Sh^{mns}/+ flies displayed higher LIEs than the Hk^{1}/+ and *CS*. The transheterozygous Sh^{mns}/ Hk^{1} showed comparable levels of LIEs to those seen in both Sh^{mns}/+ (ANOVA with *post hoc* Tukey: $p < 0.0001$; $n = 8\text{-}10$; different letters denote statistically significant difference).

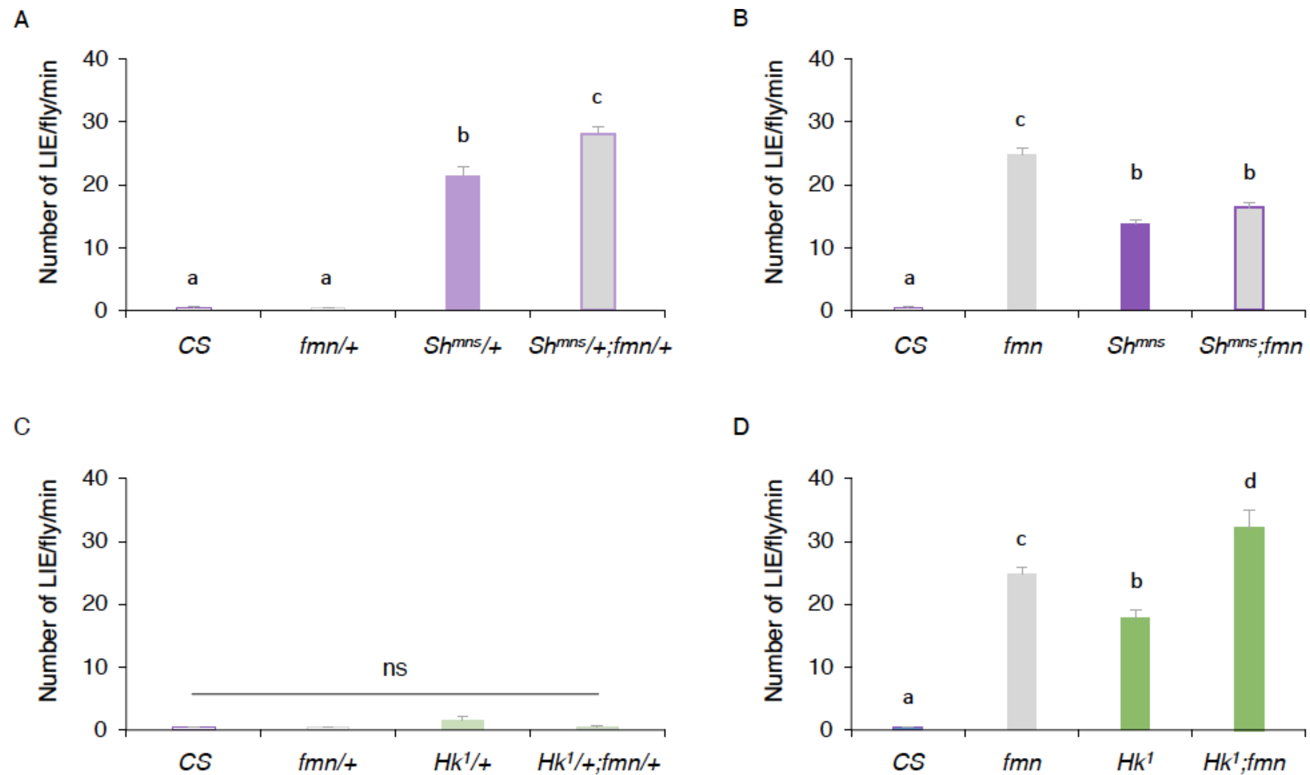

Figure 3.2: Interaction of *Sh* and *Hk* with the dopamine system.

A. *Sh*^{mns}/+;*fmn*/+ showed significantly more LIE than *Sh*^{mns}/+ while *CS* and *fmn*/+ displayed negligible LIE (ANOVA with *post hoc* Tukey: $p < 0.0001$; $n = 11\text{-}18$; different letters denote statistically significant difference). **B.** The double homozygous mutants *Sh*^{mns};*fmn* showed comparable LIE to *Sh*^{mns}, but less LIE than *fmn* (ANOVA with *post hoc* Tukey: $p < 0.0001$; $n = 11\text{-}18$; different letters denote statistically significant difference). **C.** All genotypes under study that include *CS, fmn/+, Hk*^{1}*/+,* and *Hk*^{1}*/+;fmn/+* showed strong movement suppression (Kruskal-Wallis, *ns*, $p > 0.05$; $n = 11\text{-}19$). **D.** The double homozygous *Hk*^{1}*;fmn* mutant displayed higher levels of LIEs compared to both homozygous *fmn* and *Hk*^{1} flies (ANOVA with *post hoc* Tukey: $p < 0.0001$; $n = 14\text{-}17$; different letters denote statistically significant difference).

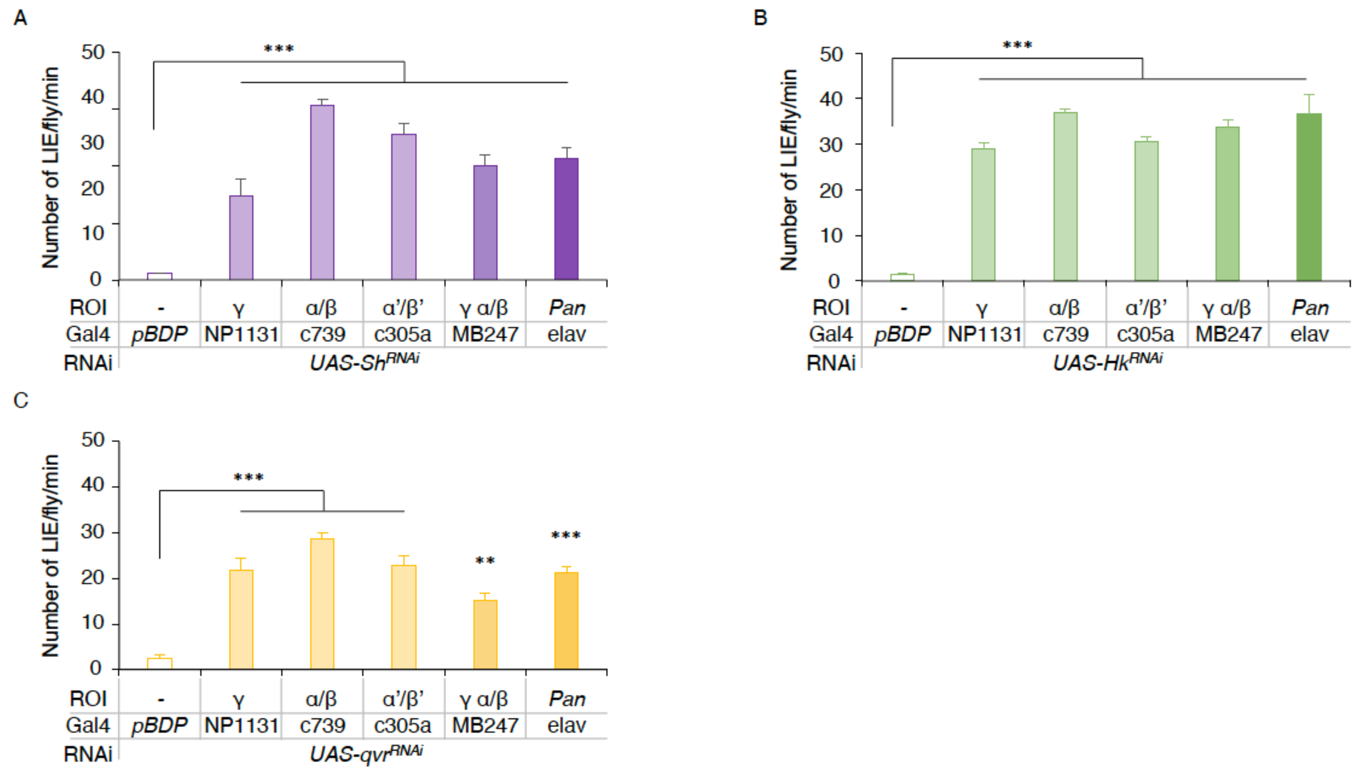

Figure 3.3: All mushroom body subsets are important for *Sh*, *Hk* and *qvr* in impulsivity.

A. *Sh* KD in the individual MB subsets (γ via NP1131-GAL4, α/β via c739-GAL4 or α'/β' via c305a-GAL4), the γ and α/β together (MB247-GAL4) or all neurons (elav-GAL4) resulted in elevated LIE (ANOVA with *post hoc* Dunnett using *pBDP-Gal4* as a control: ***, $p < 0.0001$; $n = 9\text{-}13$). **B.** *Hk* KD in the individual MB subsets (γ via NP1131-GAL4, α/β via c739-GAL4 or α'/β' via c305a-GAL4), the γ and α/β together (MB247-GAL4) or all neurons (elav-GAL4) resulted in elevated LIE (ANOVA with *post hoc* Dunnett using *pBDP-Gal4* as a control: ***, $p < 0.0001$; $n = 6\text{-}17$).**C.** *qvr* KD in the individual MB subsets (γ via NP1131-GAL4, α/β via c739-GAL4 or α'/β' via c305a-GAL4), the γ and α/β together (MB247-GAL4) or all neurons (elav-GAL4) resulted in elevated LIE (ANOVA with *post hoc* Dunnett using *pBDP-Gal4* as a control: **, $p < 0.005$; ***, $p < 0.0001$; $n = 9\text{-}30$).

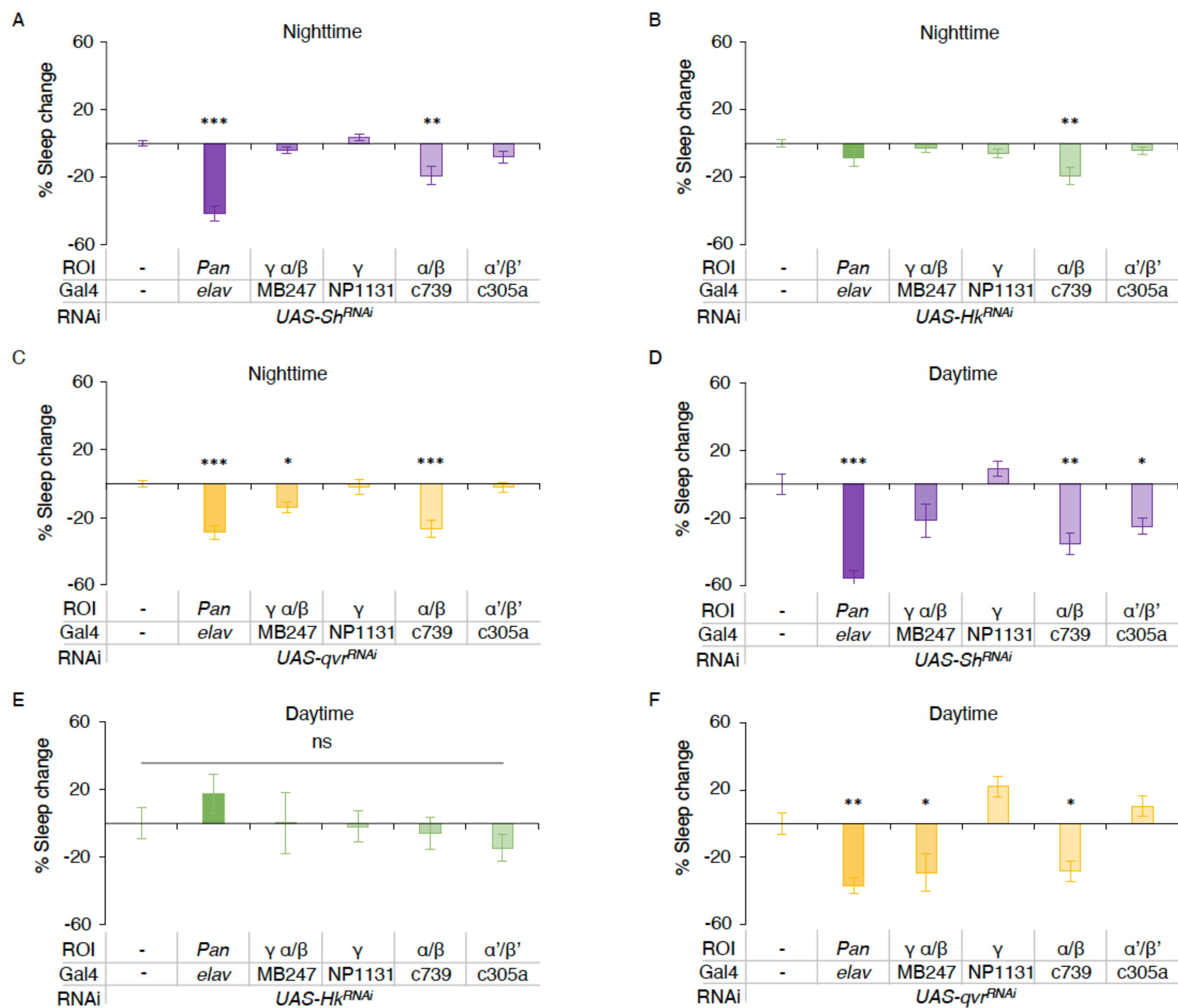

Figure 3.4: Distinct mushroom bodies are important for *Sh*, *Hk* and *qvr* in sleep.

A. *Sh* KD in the α/β (via c739-GAL4) or in all neurons (elav-GAL4) resulted in nighttime sleep deficits (ANOVA with *post hoc* Dunnett using Sh^{RNAi}/+ as a control: **, $p < 0.005$; ***, $p < 0.0001$; $n = 13$-16). **B.** *Hk* KD in the α/β (via c739-GAL4) resulted in nighttime sleep deficits (ANOVA with *post hoc* Dunnett using Hk^{RNAi}/+ as a control: **, $p < 0.005$; $n = 14$-16). **C.** *qvr* KD in α/β (via c739-GAL4), the γ and α/β together (MB247-GAL4) or all neurons (elav-GAL4) resulted in a reduction in nighttime sleep (ANOVA with *post hoc* Dunnett using qvr^{RNAi}/+ as a control: *, $p < 0.05$; ***, $p < 0.0001$; n = 11-16). **D.** *Sh* KD in the individual MB subsets (α/β via c739-

GAL4 or α'/β' via c305a-GAL4) or all neurons (elav-GAL4) resulted in daytime sleep deficits (ANOVA with *post hoc* Dunnett using Sh^{RNAi}/+ as a control: *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0001$; $n = 13$-16). **E.** *Hk* KD in the individual MB subsets subsets (γ via NP1131-GAL4, α/β via c739-GAL4 or α'/β' via c305a-GAL4), the γ and α/β together (MB247-GAL4)or all neurons (elav-GAL4) did not affect daytime sleep (ANOVA with *post hoc* Dunnett using Hk^{RNAi}/+ as a control: ns, $p > 0.05$; $n = 14$-16). **F.** *qvr* KD in the the α/β (via c739-GAL4) the γ and α/β together (MB247-GAL4) or all neurons (elav-GAL4) resulted in daytime sleep deficits (ANOVA with *post hoc* Dunnett using qvr^{RNAi}/+ as a control: *, $p < 0.05$; **, $p < 0.005$; $n = 11$-16).

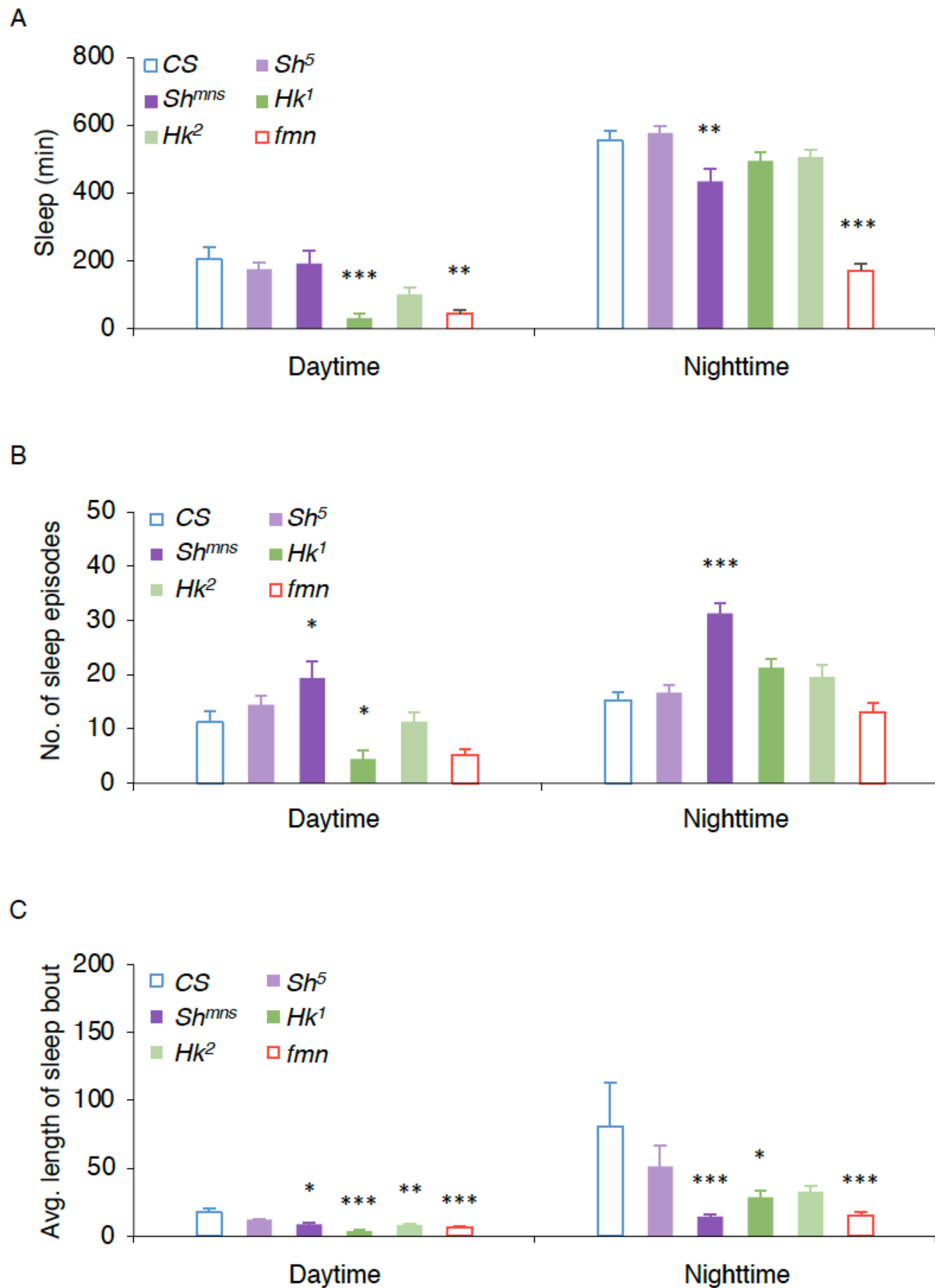

Figure S3.1: Sleep analysis of both *Sh* and *Hk* alleles.

A. *Hk*^{1} shows significantly reduced daytime sleep, while *Sh*^{mns} shows reduced nighttime sleep both *Sh*^{5} and *Hk*^{2} did not show any changes in sleep (Daytime: Mann-Whitney or Student *t*-

test: ns, $p > 0.05$; **, $p < 0.005$; ***, $p < 0.0001$, $n = 17$-22) (Nighttime: ANOVA with *post hoc* Dunnett using *CS* as a control: ns, $p > 0.05$; **, $p < 0.005$; ***, $p < 0.0001$; $n = 17$-22). Sh^{mns} has significantly more sleep episodes in both daytime and nighttime, consistent with the short sleep phenotype. Hk^{1} shows a low number of sleep episodes only during the daytime. Sh^{5} and Hk^{2} do not show changes in the number of sleep episodes (ANOVA with *post hoc* Dunnett using *CS* as a control: ns, $p > 0.05$; **, $p < 0.005$; ***, $p < 0.0001$; $n = 17$-22). **C.** During the daytime, all but Sh^{5} showed reduced average length of sleep bout (Mann-Whitney: ns, $p > 0.05$; **, $p < 0.005$; ***, $p < 0.0001$, $n = 17$-22). During the nighttime, only Sh^{mns} and Hk^{1} showed a reduction in the average length of sleep bout (Mann-Whitney: ns, $p > 0.05$; *, $p < 0.05$; ***, $p < 0.0001$, $n = 17$-22).

Chapter 4: *Kekkon5* interacts with the dopamine system for inhibitory control

4.1 Introduction

Inhibitory control is the ability to suppress inappropriate actions or thoughts and its dysfunction is associated with many brain disorders such as ADHD, autism spectrum disorder, substance use disorder, Parkinson's disease and Alzheimer's disease and related dementia (Bari & Robbins, 2013; Logue & Gould, 2014). Nonetheless, our knowledge of the genetic factors contributing to inhibitory control dysfunction remains unclear. Inhibitory control has been studied previously utilizing diverse animal models such as primates, rats, and mice (Bari & Robbins, 2013). However, genetic approaches are limited due to functional compensation and are time-consuming because of the length of their life cycles. By taking an advantage of the *Deficiency* (*Df*) library that covers 98.3% of the euchromatic genes (Bloomington Drosophila Stock Center) in *Drosophila melanogaster* we performed an unbiased genetic screen to identify novel genetic factors for inhibitory control deficit. Each *Df* line has deletion of a relatively large genome segment thus allows us to screen multiple genes at once. Here we report the identification of a novel genetic factor for impulsivity from the screen of the X chromosome *Df* kit.

4.2 Materials and Methods

Fly strains and culture

The wild-type strain used in this study is *Canton-S* (*CS*). The dopamine transporter mutant *fmn* was obtained from Dr. Jackson (Tufts University, Boston, MA). The following fly strains were obtained from the Bloomington Drosophila Stock Center (BDSC; Bloomington, IN): *Df(1)Exel7468* (BDSC #7768), $nAChR\alpha7^{1}$ (BDSC #24880), $nAChR\alpha7^{EY10801}$ (BDSC #20216), $nAChR\alpha7^{MI12545}$ (BDSC #59317), $kekkon5^{MI11781}$(BDSC #56624), *Naa-15-16* (BDSC #16782), *pacman*(BDSC #33263), $Pfrx^{MI12503}$ (BDSC #59429) and $Pfrx^{EP1150}$ (BDSC #11457). Flies were

raised on a standard cornmeal/sucrose/yeast/agar medium at 25° C with 50 % relative humidity under a 12 h light/12 h dark cycle. Flies were collected under carbon dioxide within two days after eclosion and were housed in a group of 13-15 per vial (representing $n = 1$). The vials were placed for two days in the light-, temperature-, and humidity-controlled incubator before behavioral assays.

Go/No-Go test

Flies were placed in a rectangular plexiglass chamber (60 mm L X 60 mm W X 15 mm H) connected to filtered air and allow to move around for 1 min. The 10 L/min airflow was delivered to the chamber for 10 min and the chamber was video recorded to monitor fly movements before and after airflow (P. R. Sabandal et al., 2022). Videos were analyzed manually to score the number of flying events or using the Viewer3 tracking software (BiObserve Technologies, Bonn, Germany) that allows tracking and measuring the average speed of individual flies in mm/sec. Raw data were exported to Excel (Microsoft, Redmond, WA), and the number of movements exceeding 60 mm/sec that represent impulsive flying behavior {loss of inhibition event (LIE)} was scored per fly per min. To quantitively compare different genotypes, we analyzed LIE numbers in 1 min intervals for the entire 10 min of the No-Go phase and used the 1 min bin with the highest LIE count. All behavioral experiments were performed blind to the experimenter. The control and experimental groups were tested in the same experimental session randomly. Multiple independent sets of flies were obtained from independent seedings and were used for behavioral experiments. Only females were used in the experiments since *Kekkon 5* gene is on the X chromosome.

Statistical analysis

All statistical analyses were performed using the Minitab software (Minitab, State College, PA) or JMP (SAS, Cary, NC). Raw data were analyzed using the Anderson–Darling

goodness-of-fit test for distribution and are reported as mean + SEM. Normally distributed data were analyzed by either a two-tailed Student's *t*-test for two groups or by ANOVA followed by *post hoc* Tukey's multiple-comparison for three or more groups or Dunnett's test to compare experimental groups with a control group. Non-normally distributed data were analyzed by Kruskal-Wallis and post hoc Mann-Whitney tests. Significant difference among the groups under comparison was determined using an α level of 0.05 in all analyses. All raw data files are available on request.

4.3 Results

We previously found that the *fumin* (*fmn*) flies with homozygous mutation in dopamine transport exhibit impulsive flying behavior when subjected to the Go/NoGo test (Sabandal et al., manuscript under revision). To identify the genetic loci interacting with *fmn*, we performed the functional genetic screen of the X chromosome *Df* lines using the Go/NoGo test and found *Df(1)Exel7468* (Figure 1A) as a positive hit. Specifically, the heterozygous *fmn* (*fmn/+*) flies showed normal inhibitory control; however, addition of *Df(1)Exel7468/+* led to high LIEs (Figure 1B). *Df(1)Exel7468* has deletion of a large genome segment containing 20 genes (Figure 1A). To identify the gene(s) interacting with *fmn/+*, we selected five genes within *Df(1)Exel7468* that are known to be important for nervous system functions: *nAChRalpha7* coding for nicotinic acetylcholine receptor α7 subunit, *kekkon 5* (*kek5*) coding for synaptic cell adhesion molecule, *Naa15-16* coding for N(α)-acetyltransferase 15/16, pacman (*pcm*) coding for exoribonuclease, and *Pfrx* coding for 6-phosphofructo-2-kinase. Upon testing their heterozygous mutants in the *fmn/+* background, we found only *kek5/+;fmn/+* showing significantly elevated LIEs (Figure 1B. ANOVA with *post hoc* Dunnett using *CS* as a control: $p < 0.0001$; $n = 8\text{-}18$). We next tested *kek5* without *fmn*/+ to clarify if it truly interacted with *fmn* to cause dysfunctional inhibitory control or if mutated *kek5* alone was sufficient for elevated LIEs. The results showed normal inhibitory control of the *kek5/+* mutant (Figure 1C.

$p > 0.05$, $n = 4$-9). This indicates that *kek5* is a novel genetic factor that interacts with dopamine signaling for impulsivity.

4.4 Discussion

Impulsivity is associated with wide-ranging brain disorders including ADHD, autism spectrum disorders, substance use disorder and dementia but its etiology is largely unknown. Through an unbiased genetic screen, we have identified *kek5* as a novel gene that interacts with dopamine signaling to regulate inhibitory control. *Kek5* is a member of synaptic cell adhesion molecule family with leucine-rich repeat and immunoglobulin-like domain (Musacchio & Perrimon, 1996), is involved in receptor tyrosine kinase signaling (Ulian-Benitez et al., 2017), and regulates the Bone Morphogenic Protein signaling pathway for wing development (Evans, Haridas, & Duffy, 2009). Aside from this finding there is no information on the role of *kek5*. This study thus uncovers a novel function for Kek5 in inhibitory control and serves as a baseline for future studies on the relevant mechanism.

kek5 has several human homologs that include CHAD, LRIT2, LRRC24, LRTM1 and SLITRK (SLIT and NTRK-like protein) family (SLITRK 2-6) (Gramates et al., 2022).Of particular interest is SLITRK 2 whose mutations are linked to X-linked intellectual developmental disorder-111 (www.omim.org). The SLITRK 2 knockout mice are hyperactive in novel environments (Katayama et al., 2022) and the hyperactivity phenotype is recapped in the mice with conditional Slitrk2 knockout in dopamine neurons (Salesse et al., 2020). At the cellular level, SLITRK 2 interacts with PSD 95 and Shank3 for excitatory synapse formation and transmission in midbrain dopamine neurons and the CA1 hippocampal neurons (Han et al., 2019). Thus, it seems tantalizing to postulate that SLITRK 2 is a mammalian ortholog of the fly kek5. Our study identifying Kek5 as an enhancer of dopamine signaling sets the stage to uncover the inhibitory control mechanism and also to provide insight into the mechanism

underlying dysfunctional inhibitory control present in ADHD, autism spectrum disorder, addiction and dementia.

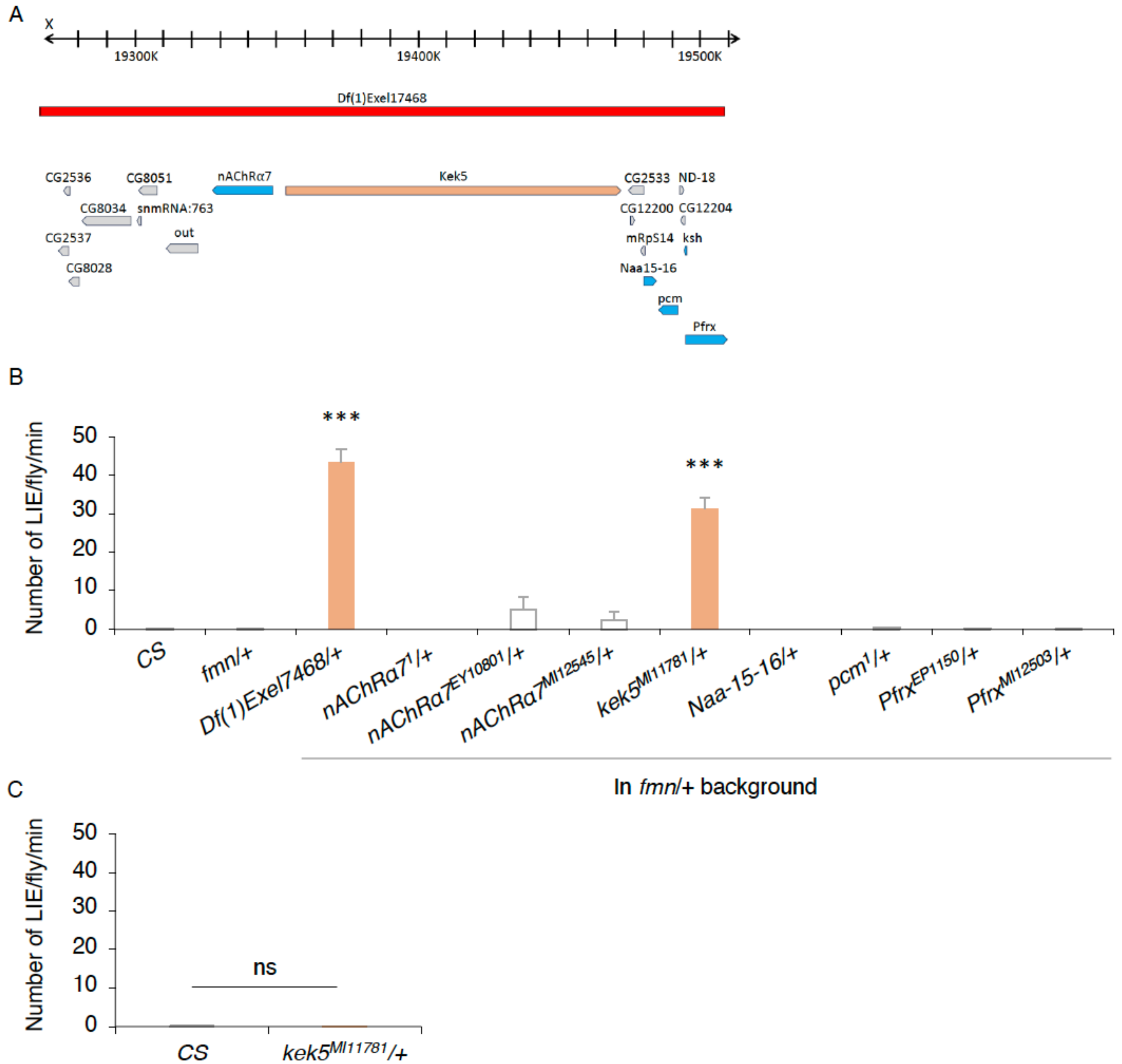

Figure 4.1: The novel inhibitory control gene *kek5*.

A. Genomic region of *Df(1)Exel7468* and the genes deleted in *Df(1)Exel7468* are noted (adapted form flybase.org). **B.** Interaction of five candidate genes with *fmn/+* in a Go/NoGo test. $kek5^{MI11781}$, but not other mutants, displayed loss of inhibitory control in the *fmn/+* genetic

background (ANOVA with *post hoc* Dunnett using *CS* as a control: ***, $p < 0.0001$; $n = 8$-18).
C. *kek5/+* without *fmn/+* did not show loss of inhibitory control. (Mann-Whitney: ns, $p > 0.05$; $n = 4$-9).

Chapter 5: Acetylcholine deficit causes dysfunctional inhibitory control in an aging-dependent manner

The following chapter presents a research manuscript written, submitted, and published in Scientific Reports on December 03, 2022. This manuscript's work describes the effects of aging on the inhibitory control of *Drosophila melanogaster* and how acetylcholine 's decline with aging contributes to this dysfunctional inhibitory control. Inhibitory control is an executive function that terminates inappropriate actions in a specific social setting. This executive function is crucial for the completion of goal-oriented behaviors. Dysfunctional inhibitory control can be observed in aging and neurodegenerative disorders. Notably, ACh neurotransmission is also essential in the regulation of executive functions. In addition, ACh signaling weakens with aging, but its role in inhibitory control deficits related to aging and neurodegenerative disorders remains unclear.

We first established the inhibitory control decline with aging in wild-type control flies to close this knowledge gap using our developed Go/No-Go test. Our results show an increase in *Drosophila's* loss of inhibitory control in an age-dependent manner. Next, we investigated how ACh neurotransmission plays a role in inhibitory control. Here we found that elevated levels of ACh improved the inhibitory control decline via testing an acetylcholinesterase mutant shown to have high levels of ACh in the brain. We also identify the mushroom bodies as the neural site where ACh is the major contributor to the regulation of inhibitory control. Lastly, we also highlight the mushroom body output neuron responsible for maintaining movement suppression. With this study, we demonstrated for the first time the major role ACh signaling plays in regulating an essential executive function, one being inhibitory control.

Author Contributions

The following manuscript discusses the findings and observations of the collaborative efforts of multiple individuals: Paul Rafael Sabandal (P.R.S.), Erick Benjamin Saldes (E.B.S.),

and Kyung-An Han (K.A.H.). PRS performed and analyzed all Go/No-Go tests, ELISA experiments and wrote the manuscript. EBS performed and analyzed the immunohistochemical experiments and contributed to writing the manuscript. PRS and EBS collaborated to generate schematic representations of the acetylcholine neurons. KAH and PRS synthesized the hypothesis, performed data management and analysis, and wrote the manuscript.

Acetylcholine deficit causes dysfunctional inhibitory control in an aging-dependent manner

Abbreviated Title: acetylcholine in inhibitory control

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5.1 Abstract

Inhibitory control is a key executive function that limits unnecessary thoughts and actions, enabling an organism to appropriately execute goal-driven behaviors. The efficiency of this inhibitory capacity declines with normal aging or in neurodegenerative dementias similar to memory or other cognitive functions. Acetylcholine signaling is crucial for executive function and also diminishes with aging. Acetylcholine's contribution to the aging- or dementia-related decline in inhibitory control, however, remains elusive. We addressed this in *Drosophila* using a Go/No-Go task that measures inhibition capacity. Here, we report that inhibition capacity declines with aging in wild-type flies, which is mitigated by lessening acetylcholine breakdown and augmented by reducing acetylcholine biosynthesis. We identified the mushroom body (MB) γ neurons as a chief neural site for acetylcholine's contribution to the aging-associated inhibitory control deficit. In addition, we found that the MB output neurons MBON-γ2α'1 having dendrites at the MB γ2 and α'1 lobes and axons projecting to the superior medial protocerebrum and the crepine is critical for sustained movement suppression per se. This study reveals, for the first time, the central role of acetylcholine in the aging-associated loss of inhibitory control and provides a framework for further mechanistic studies.

5.2 Introduction

Acetylcholine is important for executive function in human subjects and rodents. In healthy non-smokers, for example, acute nicotine administration enhances attention when tested in the spatial attentional resource allocation and rapid visual information processing tasks (Hahn et al., 2020). Consistently, the nicotinic acetylcholine receptor (nAChR) antagonist mecamylamine administration in rats and the nAChR beta2 knockout in mice impair attention performance in the 5-choice serial reaction time task (5-CSRTT) (Balachandran et al., 2018; Guillem et al., 2011), substantiating a crucial role of acetylcholine

in attention. The role of acetylcholine in inhibitory control, however, seems rather complex. In healthy non-smokers, acute nicotine administration improves behavioral inhibition in the stop signal task (Logemann, Bocker, Deschamps, Kemner, & Kenemans, 2014). Nicotine treatment in rats, on the other hand, impairs inhibitory action and choice when tested in the 5- or 3-CSRTT, go/no-go task and systemic delayed reward task(Balachandran et al., 2018; Kolokotroni, Rodgers, & Harrison, 2011; Tsutsui-Kimura et al., 2010). The inhibitory effect of nicotine is blocked by mecamylamine pretreatment (Kolokotroni et al., 2011) or the infusion of the alpha4beta2 nAChR antagonist dihydro-beta-erythroidine (DHbetaE) in the infralimbic cortex(Tsutsui-Kimura et al., 2010). Moreover, the DHbetaE infusion(Tsutsui-Kimura et al., 2010) or systemic mecamylamine administration (Balachandran et al., 2018) without nicotine administration is sufficient to suppress impulsive action, suggesting pro-impulsive function of endogenous acetylcholine in rats, which is not in line with the findings in human subjects. It seems of great value to study an additional model organism for deeper understanding of the mechanism by which acetylcholine regulates inhibitory response control.

Acetylcholine neurotransmission declines with normal aging and the decline is highly augmented in Alzheimer's disease and related dementias such as frontotemporal dementia and Lewy body dementia(Ballinger, Ananth, Talmage, & Role, 2016; Barrett, Cloud, Shah, & Holloway, 2020; Murley & Rowe, 2018; Noufi, Khoury, Jeyakumar, & Grossberg, 2019; Schliebs & Arendt, 2011). Executive dysfunctions, in particular impaired inhibitory control, represent prominent symptoms of early-stage Alzheimer's disease, frontotemporal dementia and other dementias(Collette et al., 2007; Crawford & Higham, 2016; Crawford et al., 2005; Johns et al., 2009; Martyr, Boycheva, & Kudlicka, 2017); however, there is no information on the role of acetylcholine in inhibitory control dysfunction associated with normal aging or dementias. To address this knowledge gap, we developed a simple behavioral paradigm to study a fundamental form of inhibitory response control in *Drosophila melanogaster*. Here, we show that acetylcholine significantly contributes to the aging-dependent deficit in inhibitory control and its major neural site is the mushroom body neurons.

5.3 Materials and methods

Fly strains and culture

Paragraphs with the style *Heading 5,h5* applied can be extracted to appear in the table of contents as level 3 sub headings. The wild-type strain used in this study is *Canton-S* (*CS*). The following fly strains were obtained from the Bloomington Drosophila Stock Center (BDSC; Bloomington, IN): *Ace^{c00215}* (BDSC #10026), *ChAT^{MI08244}* (BDSC #55439), *ChAT^{MI04508}* (BDSC #37817), *OK107-GAL4* (BDSC #854), *MB010B-GAL4* (BDSC #68293), *201y-GAL4* (BDSC #4440), *MB009B-GAL4* (BDSC #68292), *c739-GAL4* (BDSC #7362), *MB008B-GAL4* (BDSC #68291), *c305a-GAL4* (BDSC #30829), *MB005B-GAL4* (BDSC #68306), *MB077B-GAL4* (BDSC #68283), *MB051B-GAL4* (BDSC #68275), *MB018B-GAL4* (BDSC #68296), *MB082C-GAL4* (BDSC #68286), *MB543B-GAL4* (BDSC #68335), *MB027B-GAL4* (BDSC #68301), *MB549C-GAL4* (BDSC #68373), *MB542B-GAL4* (BDSC #68372), *GH146-GAL4* (BDSC #30026) and *UAS-ChAT^{RNAi}* (BDSC #25856); *pBDP-GAL4* from David Anderson (California Institute of Technology, Pasadena, CA); *NP225-GAL4* from Andreas Thum (Universität Leipzig, Leipzig, Germany); *MB247-GAL4* from Scott Waddell (University of Oxford, Oxford, UK); and *NP1131-GAL4* from Josh Dubnau (Stony Brook University School of Medicine, Stony Brook, NY).

Flies were raised on a standard cornmeal/sucrose/yeast/agar medium at 25° C with 50 % relative humidity under a 12 h light/12 h dark cycle. Flies were collected under carbon dioxide within two days after eclosion and were housed in mixed sex groups. For aging, all fly strains were transferred to fresh food every 2-3 d until the ages of 4 d, 2 wk and 4 wk. These ages were selected to examine flies at young (4 d), mid (2 wk) and early old (4 wk) ages. The 4-wk old allows capturing the brain and behavioral changes associated with early-stage dementia and potentially distinguishing them from those occurring during normal aging(Tonoki & Davis, 2012). Prior to behavioral tests, flies were sorted into a group of 13 per food vial (representing n = 1) and were then left to rest for 2 d in the light-, temperature-, and

humidity- controlled incubator before behavioral or immunohistochemical analyses. Both males and females were examined separately but there was no sex difference thus combined data are presented

Behavioral analysis

Flies were gently transferred into a rectangular plexiglass chamber (60 mm L X 60 mm W X 15 mm H) connected to filtered air and acclimated to the chamber for 10 min. The 10 L/min airflow was delivered to the chamber for 10 min. The chamber was video recorded to monitor fly movements before and after airflow. Videos were analyzed manually to score the number of flying events or using the Viewer3 tracking software (BiObserve Technologies, Bonn, Germany) that allows tracking and measuring the average speed of individual flies in mm/sec per fly. Raw data were transported to Excel (Microsoft, Redmond, WA) and the number of the movements exceeding 60 mm/sec (flying event) that we defined as loss of inhibition events (LIE) was scored per fly per min. To quantitively compare different conditions (e.g., age or genotype), we analyzed LIE numbers in 1 min bins for the entire 10 min of the No-Go phase and compared the 1 min bin having the highest LIE counts. All behavioral experiments were performed blind to the experimenter. The control and experimental groups were tested in the same experimental session in a randomized manner. Multiple independent sets of flies obtained from independent crosses were used for behavioral experiments.

Immunohistochemical analysis

Immunostaining was performed as previously described(Lim et al., 2018; Yasuyama & Salvaterra, 1999) with several modifications. Fly brains were dissected in ice-cold 1X phosphate buffered saline (1X PBS), fixed in 4% paraformaldehyde in 1X PBS for 3 h at 4°C. The fixed brains were washed with 1X PBS once for 10 min, washed twice with 1X PBHT (20

mM PO_4, 0.5 M NaCl, 0.2 % Triton X-100, pH 7.4) for 10 min each, solubilized with 1% Triton X-100 in 1X PBHT for 1 h at room temperature, blocked with 5% normal goat serum (NGS) in 1X PBHT for 16-18 h at 4° C and incubated with the 1X PBHT containing 1:1000 anti-ChAT antibody (ChAT 4B1(Takagawa & Salvaterra, 1996); DSHB, Iowa City, IA) and 5% NGS for 48 h at 4° C. After two 10 min washes at room temperature and a 16-24 h wash at 4° C with 1X PBHT, the brains were incubated with the Alexa Flour 488 conjugated goat anti-mouse IgG (A-11001; Invitrogen/Life Technologies, Eugene, OR) for 48 h at 4° C followed by three 20 min washes with 1X PBHT and three 10 min washes with 0.12M Tris-HCl, pH 7.2. The brains were then mounted in the Vectashield mounting medium (H-1000; Vector Laboratories, Burlingame, CA) for imaging with the 40X oil immersion objective in the LSM700 confocal microscope (Zeiss, Thornwood, NY). Pixel resolution was set at 2048 x 2048. Optical sections were made at 1 μm thickness and 50 sections were stacked for the representative images in Figure 2B. ChAT immunoreactivity in the superior medial protocerebrum was quantified using the ImageJ (NIH, Bethesda, MD). The fluorescent signals were converted to grayscale and the fluorescence intensity per pixel in the SMP was calculated by $\{\text{Integrated density}_{\text{Total}}/\text{Area}_{\text{Total}}\}$ per brain. The fold change of the fluorescent intensities in the *ChAT/+* mutant brains from those in the control *Canton-S* brains was used for data presentation.

Acetylcholine analysis

To measure acetylcholine, we utilized a commercially available Choline/Acetylcholine assay kit (catalog # ab65345, Abcam, Cambridge, United Kingdom). Briefly, 50 frozen fly heads per condition (genotype and age) were homogenized 50 μl of the Choline Assay Buffer (1 fly head per 1 μl) on ice with the KONTES Micro Tissue Grinder (Thermo Fisher Scientific, Waltham, MA) for 30 to 45 s. The homogenates were transferred to fresh tubes, centrifuged at 14,000 rpm for 10 min at 4°C, and subjected to the Choline/Acetylcholine assay per the

manufacture's instruction. Multiple independent sets of fly heads (e.g., 4 to 8 sets) obtained from independent crosses were used for acetylcholine quantification.

Statistical analysis

All statistical analyses were performed using the Minitab software (Minitab, State College, PA) or JMP (SAS, Cary, NC). Raw data were analyzed using the Anderson–Darling goodness-of-fit test for distribution and are reported as mean + SEM. Normally distributed data were analyzed by either two-tailed Student's *t* test for two groups or by ANOVA followed by *post hoc* Tukey's multiple-comparison for three or more groups or Dunnett's test to compare experimental groups with a control group. Significant difference among the groups under comparison was determined using an α level of 0.05 in all analyses. All raw data files are available on request.

5.4 Results

Inhibitory control diminishes with age in *Drosophila*

Inhibitory control confers the ability to suppress inappropriate actions or thoughts and is a key executive function supporting flexible and goal-directed behaviors. This capacity declines with aging in human subjects, for example when tested in the go/no-go (GNG)(Rey-Mermet & Gade, 2018). A GNG task measures action restraint, requiring subjects to withhold a motor response to a "no-go" signal (Chikazoe et al., 2009). To assess whether inhibitory control declines with aging in *Drosophila* similar to humans, we developed a *Drosophila* version of a GNG test, in which a group of 13 flies placed in a chamber were video-tracked to quantify their movements. Without salient stimuli, the wild-type *Canton-S* (*CS*) flies freely move around. Upon introduction of strong airflow, flies halt movements presumably to keep their behavior under control (Figure 1A)(Tsubouchi et al., 2017; Yorozu et al., 2009). The fly's

ability to suppress ongoing activity (walking) inappropriate in a given context (strong "wind"), and we postulate that it represents a fundamental form of inhibitory response control. We tested the female and male *CS* flies at three different ages 4 d (young), 2 wk (mid), and 4 wk (early old). Regardless sex and age, the *CS* flies exhibited robust movement suppression under strong airflow with infrequent instances of flying behavior, which is an impulsive act resulting from loss of movement inhibition (Figure 1A). To identify whether the frequency of the loss of inhibition events (LIE) changes with aging, we counted the movement with the speed over 60 mm/sec, consisting of flying. Notably, older *CS* flies exhibited more LIE (Figure 1B: one way ANOVA; $R^2 = 0.704$, $F_{2,33} = 39.24$, $p < 0.0001$; $n = 12$) at shorter latency for LIE (Figure 1C: one way ANOVA; $R^2 = 0.372$, $F_{2,33} = 9.77$, $p = 0.0005$), indicating that aging diminishes inhibitory capacity. We did not observe any difference in LIE between females and males at all ages thus the data obtained from both sexes are combined and presented in Figures 1B and 1C.

It is known in human subjects and rodents that acetylcholine neurotransmission modestly declines with aging(Ballinger et al., 2016; Schliebs & Arendt, 2011) but this has not been demonstrated in flies. To test whether diminished acetylcholine neurotransmission is responsible for the aging-associated increase in LIE, we examined the $Ace^{c00215}/+$ flies with the heterozygous mutation in acetylcholinesterase (Ace; EC 3.1.1.7) that breaks down acetylcholine and is a target for the first line medication to treat Alzheimer's disease. The Ace^{c00215} allele is a severe hypomorph or null since it has a piggyBac insertion in the 5' non-coding region of the gene and homozygous lethal(Larkin et al., 2021). We found that the heterozygous *Ace* mutation rescued fully at 2 wk and partly at 4 wk the movement suppression deficit (Figure 1B: two-way ANOVA, $F_{5,66} = 37.98$, $p < 0.0001$; genotype effect, $p < 0.0001$; age effect, $p < 0.0001$; genotype x age, $p = 0.0009$) as well as the LIE latency at 4 wk (Figure 1C: two-way ANOVA, $F_{5,66} = 5.236$, $p = 0.0004$; genotype effect, $p = 0.0112$; age effect, $p = 0.0007$; genotype x age, $p = 0.2021$). Consistently, the $Ace^{c00215}/+$ flies showed higher acetylcholine levels compared to *CS* at all ages tested and both genotypes have declining acetylcholine levels

with aging (Figure 1D: two-way ANOVA, $F_{5,18} = 36.93$, $p < 0.0001$; genotype effect, $p < 0.0001$; age effect, $p < 0.0001$; genotype x age, $p = 0.116$). This finding supports that diminished acetylcholine neurotransmission accounts for inhibitory control decline in normal aging, and the GNG test is well suited to study aging-related changes in inhibitory control in flies.

Choline acetyltransferase (ChAT) deficit augments inhibitory control impairments with aging

To directly address the role of acetylcholine in inhibitory control, we used the flies defective in ChAT. ChAT (EC 2.3.1.6) is the biosynthetic enzyme for acetylcholine, which aids in the transfer of an acetyl group in Acetyl-CoA to choline, and *Drosophila* has a single gene for ChAT as in humans(Larkin et al., 2021). We examined two mutant alleles $ChAT^{MI04508}$ and $ChAT^{MI08244}$ containing the gene trap MiMIC vector(Venken et al., 2011) (Figure 2A). The homozygous $ChAT^{MI04508}$ or $ChAT^{MI08244}$ mutants die during development thus we investigated their heterozygous mutants. The ChAT immunoreactivity was widespread in the wild-type *CS* brain and its level was significantly reduced in both mutants at the age of 4 to 5 d old (Figure 2B, representative images; 2C, immunoreactivity quantifications; ANOVA, $F_{2,26} = 15.96$, $p < 0.0001$, $n = 8 - 11$). When subjected to the GNG test, both heterozygous mutants showed dysfunctional movement suppression in an aging-dependent manner (Figures 2D and 2E: the representative movement traces per chamber containing 13 flies of the $ChAT^{MI04508}/+$ or $ChAT^{MI08244}/+$ genotypes at the ages of 4 d, 2 wk or 4 wk). Specifically, the heterozygous *ChAT* mutants at all ages showed robust movement suppression when strong air was introduced (time 0 on the X-axis in Figures 2D and 2E) similar to *CS*; however, they exhibited highly augmented LIEs as they got older (Figure 2F: $F_{2,33} = 65.28$, $p < 0.0001$, $n = 12$ for $ChAT^{MI04508}/+$; $F_{2,33} = 61.56$, $p < 0.0001$, $n = 12$ for $ChAT^{MI08244}/+$). At the age of 4 d, there was no difference in LIEs of the $ChAT^{MI04508}/+$ and $ChAT^{MI08244}/+$ mutants compared to that of *CS* (Figure 2G 4 d: $F_{2,33} = 0.1377$, $p = 0.87$, $n = 12$); however, there was significant increases in the LIEs of the $ChAT^{MI04508}/+$ and

ChAT^{MI08244}*/+* flies at the ages of 2 wk ($F_{2,33} = 65.35$, $p < 0.0001$, $n = 12$) and 4 wk ($F_{2,33} = 41.3$, $p < 0.0001$, $n = 12$) compared to those of *CS*. Also, the LIE onsets of *ChAT*^{MI04508}*/+* and *ChAT*^{MI08244}*/+* flies were significantly earlier than CS at 2 wk (Figure 2H; $F_{2,33} = 26.996$, $p < 0.0001$, $n = 12$) and 4 wk ($F_{2,33} = 32.365$, $p < 0.0001$, $n = 12$) but not at 4 d ($F_{2,33} = 0.0346$, $p = 0.97$, $n = 12$). Together, these data indicate that the flies with reduced ChAT expression have augmented sensitivity to aging in movement suppression deficit, indicating the critical role of acetylcholine in the aging-dependent loss of inhibitory control.

Ace deficiency rescues anomalous inhibitory control of the ChAT mutants

We reasoned that dampened cholinergic transmission causes the dysfunctional inhibitory control of the ChAT mutant. Should it be the case, we should be able to rescue the phenotype by reducing the amount of the acetylcholine degradation enzyme Ace. We tested this notion by examining the *ChAT/+* mutant flies carrying the heterozygous *Ace* mutation(W. Kim et al., 2011) (i.e. *ChAT*^{MI04508}/*Ace*^{c00215}) along with the flies with the heterozygous mutation only in *ChAT* or *Ace* and *CS* as controls. When subjected to the GNG test, there was no differences in LIEs of all genotypes at the age of 4 d as expected (Figure 3A: ANOVA; $F_{3,44} = 0.1621$, $p = 0.92$, $n = 12$). Notably, the double heterozygous *ChAT*^{MI04508}*/Ace*^{c00215} mutants exhibited the LIEs comparable to those of *CS* and *Ace*^{c00215}/+ but significantly different from that of *ChAT*^{MI04508}/+ at the ages of 2 wk (Figure 3A: ANOVA; $F_{3,44} = 89.13$, $p < 0.0001$, $n = 12$) and 4 wk ($F_{3,44} = 87.43$, $p < 0.0001$, $n = 12$). Likewise, the double heterozygous *ChAT*^{MI04508}*/Ace*^{c00215} mutants exhibited the latency to the first LIE comparable to those of *CS* and *Ace*^{c00215}/+ but significantly delayed than that of *ChAT*^{MI04508}/+ at the age of 2 wk (Figure 3B: ANOVA; $F_{3,44} = 23.07$, $p < 0.0001$, $n = 12$). At the age of 4 wk, the *ChAT*^{MI04508}*/Ace*^{c00215} mutants showed the latency comparable to that of *Ace*^{c00215}/+ but significantly delayed than those of *ChAT*^{MI04508}/+ as well as *CS* ($F_{3,44} = 49.22$, $p < 0.0001$, $n = 12$). Together, the heterozygous *Ace*^{c00215} mutation fully suppressed the *ChAT*^{MI04508}*/+* mutant's LIE and latency phenotypes. In

line with these results, the acetylcholine levels in the double heterozygous *ChAT*^{MI04508}*/Ace*^{c00215} were indistinguishable from those of *CS* at all ages and they were significantly higher than those of *ChAT*^{MI04508}*/+* (Figure 3C: ANOVA with *post hoc* Tukey multiple comparison of the genotypes in each age. For 4 d: $F_{3,28} = 62.71$, $p < 0.0001$; For 2 wk: $F_{3,12} = 63.26$, $p < 0.0001$; For 4 wk: $F_{3,12} = 89.62$, $p < 0.0001$; ns, $p > 0.05$; **, $p < 0.005$; ***, $p < 0.0001$; n = 4-8). This indicates that the acetylcholine deficiency is responsible for the aging-associated deficit in inhibitory control.

The cholinergic neurons contributing to the aging-associated deficit in inhibitory control

Acetylcholine neurons consist of approximately 58% of the central brain(Croset, Treiber, & Waddell, 2018) and their axons project to most brain areas (Figure 2B). The mushroom body (MB) neurons in the central brain are known to modulate locomotor behaviors(J. R. Martin, R. Ernst, & M. Heisenberg, 1998; Sun et al., 2018) and their three substructures, namely α/β, α'/β' and γ neurons, are cholinergic(Croset et al., 2018). We asked whether the MB ChAT neurons contribute to the aging-sensitive LIE by using the RNA interference (RNAi)-mediated ChAT knockdown (KD). When ChAT was knocked down in all MB neurons (via the OK107- or MB010B-GAL4 driver), two MB substructures γ and α/β (via MB247- or 201y-GAL4) or γ (via NP1131- or MB009B-GAL4) but not in α/β (via c739- or MB008B-GAL4) or α'/β' (via c305a- or MB005B-GAL4) neurons led to the aging-dependent increase in LIEs (Figure 4A, two-way ANOVA: $F_{32,231} = 79.83$, $p < 0.0001$; genotype effect, $p < 0.0001$; age effect, $p < 0.0001$; genotype x age, $p < 0.0001$; $n = 8$). These data indicate that the MB γ cholinergic neurotransmission is important for the aging-related LIEs.

Eight types of the mushroom body output neurons (MBON) are known to be cholinergic and their dendrites are mapped to the γ2α'1, α2sc, α2p3p, α3, α'1, α'2, α'3ap and α'3m lobes(Aso et al., 2014). ChAT KD in the MBON-γ2α'1 (via MB077B- or MB051B-GAL4) but not in other cholinergic MBONs, namely MBON-α'2 (via MB018B-GAL4), MBON-α3 (via

MB082C-GAL4), MBON-α’1 (via MB543B-GAL4), MBON-α’3ap and -α’3m (via MB027B-GAL4), MBON-α2sc (via MB549C-GAL4), and MBON-α2p3p (via MB542B-GAL4), resulted in significant LIEs in all ages (4 d, 2 wk and 4 wk) tested (Figure 4B, two-way ANOVA: $F_{26,189} = 39.01$, $p < 0.0001$; genotype effect, $p < 0.0001$; age effect, $p = 0.448$; genotype x age, $p = 1$; $n = 8$). Thus, the MBON-γ2α’1 cholinergic transmission is critical for inhibitory control. The MBN receive input from the projection neurons (PN) and the PN are cholinergic(Takagawa & Salvaterra, 1996). We found that ChAT KD in the PN (via NP225- or GH146-GAL4) had no effect on movement inhibition at all ages under test (Figure 4C, two-way ANOVA: $F_{8,63} = 22.33$, $p < 0.0001$; genotype effect, $p = 0.94$; age effect, $p = 0.0001$; genotype x age, $p = 1$; $n = 8$). This suggests that the PN cholinergic transmission is dispensable for inhibitory control.

5.5 Discussion

Inhibition or inhibitory control is a fundamental brain function and is critical for fitness and survival. Poor inhibition is associated with many brain disorders including attention deficit hyperactivity disorder, autism spectrum disorder, addiction and neurodegenerative disorders such as Alzheimer’s disease and frontotemporal dementia(Bari & Robbins, 2013; Logue & Gould, 2014; Migliaccio et al., 2020; Opwonya et al., 2022; Rochat et al., 2008). Mechanistic studies of inhibition have been done on neurodevelopmental disorders and addiction whereas limited studies have been reported on neurodegenerative dementias. In the report, we show that inhibition capacity declines with aging in wild-type flies, which is halted by the mutation in the acetylcholine breakdown enzyme Ace and augmented by the decreased acetylcholine biosynthesis in the γ MB neurons.

Motor behaviors have been studied in *Drosophila* and they typically entail startle responses to olfactory, visual or mechanosensory stimuli in which increases in locomotor activity or movements represent the expected behavioral outcome(Campbell & Nash, 1998; W. Cho, Heberlein, & Wolf, 2004; Erlenmeyer-Kimling & Hirsch, 1961; Rogers et al., 2012). In

the startle-induced negative-geotaxis/climbing assay, for example, flies exhibit rapid climbing movements when tapped down. Older flies in this assay exhibit slower or less negative-geotaxis movements than young flies(Rhodenizer, Martin, Bhandari, Pletcher, & Grotewiel, 2008; Riemensperger et al., 2013). The GNG test used in our study measures movement suppression (no or decreased movements) in the presence of strong airflow or predator sound (unpublished data), and we show that aging causes poor suppression represented by hyperkinetic or flying behavior. Reduced or lack of motor activity of aged flies in the startle-induced negative-geotaxis assay could be confounded by general slowness of behavior related to weakened muscle or other physiological changes. The GNG test is beneficial to uncover aging-related changes in neural control of movements, in particular those leading to impulsive acts.

The genetic mutation in the rate-limiting enzyme for acetylcholine biosynthesis ChAT in mice causes lethality at birth(Misgeld et al., 2002) but all mutant mice defective in the individual subunits of nAChR and muscarinic acetylcholine receptor (mAChR) except for α3 are viable to adulthood(Zhang, 2006). The mice lacking the α7 nAChR subunit or the mice with the conditional knockout of M4 mAChR in the D1 receptor expressing neurons(Hoyle, Genn, Fernandes, & Stolerman, 2006; Klawonn et al., 2018) show significant increases in impulsive acts, supporting our finding on the acetylcholine's role in inhibitory control. The genetic knockout studies are very insightful however no human diseases identified to date involves total absence of acetylcholine production nor signaling. It is conceivable that acetylcholine signaling via α7 nAChR and M4 mAChR may decline with aging, which in turn contributes to aging-associated inhibitory control dysfunction. The role of acetylcholine signaling in aging is unknown in *Drosophila*. The only related studies are those demonstrating either reduced or overexpressed vesicular acetylcholine transporter dramatically reduces lifespan of flies, suggesting that acetylcholine is important for longevity(Showell, Martinez, Gondolfo, Boppana, & Lawal, 2020; White et al., 2020). Our approach to employ the

heterozygous ChAT mutant offers a useful strategy to identify the molecular and cellular players crucial for inhibitory control that is sensitive to aging, a key risk factor for dementia.

The MB is a key neural site processing olfactory behaviors as well as non-olfactory brain functions(Boto, Stahl, & Tomchik, 2020; Donlea, 2017; Modi, Shuai, & Turner, 2020; J. M. Sabandal, Sabandal, Kim, & Han, 2020). Our study points to the cholinergic transmission in the γ MB being responsible for aging-related loss of inhibitory control. We have previously shown that the γ neurons, upon receiving dopaminergic and octopaminergic inputs, form appetitive and aversive memory, modulate courtship motivation and develop behavioral sensitization to the disinhibition effect of ethanol(Aranda, Hinojos, Sabandal, Evans, & Han, 2017; Y. C. Kim et al., 2007; Y. C. Kim, Lee, Lim, & Han, 2013; Lim et al., 2018). The γ independently with α'/β' neurons are also shown to modulate startle-induced negative geotaxis or climbing behavior in which ectopic activation of their activities inhibits the locomotor reactivity to startle but ectopic inhibition of their synaptic outputs has no effect(Sun et al., 2018). Since the ChAT RNAi used in this study would dampen cholinergic output from the γ neurons, the neural controls for startle-induced negative geotaxis and movement suppression in the GNG task are likely independent. The study by Martin et al.(J. R. Martin et al., 1998) shows that the MB ablation or inhibition of γ and/or α/β synaptic output causes basal hyperactivity. While it seems possible that higher basal activity could be associated with more frequent loss of movement suppression, we do not see significant difference in basal activities of the flies with and without ChAT KD at 4 weeks (data not shown). Thus, the neural control of the stimulus-induced movement suppression is distinct from that of basal activity. Together, our study is the first identifying progressive loss of acetylcholine and inhibitory control with aging in which γ cholinergic neurotransmission plays a key role.

The MB axons convey information to 21 types of MBONs among which 8 types are cholinergic(Aso et al., 2014). Our study uncovers only 1 type of the cholinergic MBONs – MBON-γ2α'1 (2 neurons per hemisphere) receiving inputs from the MB γ2 and α'1 lobes and sending outputs to the crepine and the superior medial protocerebrum – contributing to

movement suppression, which is further supported by our unpublished study demonstrating that ectopic inhibition of MBON-γ2α’1 via Shi^{ts} leads to loss of inhibitory control. MBON-γ2α’1 activity is shown to be crucial for various aspects of appetitive and aversive memory(Aso et al., 2014; Berry, Phan, & Davis, 2018; Chouhan, Griffith, Haynes, & Sehgal, 2021; Felsenberg, Barnstedt, Cognigni, Lin, & Waddell, 2017; Ichinose et al., 2021; Yamazaki et al., 2018), courtship memory(Montague & Baker, 2016), sleep promotion(Aso et al., 2014; Lei, Henderson, & Keleman, 2022; Sitaraman et al., 2015), fat accumulation(Al-Anzi & Zinn, 2018), food seeking(Tsao, Chen, Lin, Yang, & Lin, 2018) and attraction/positive valence(Aso et al., 2014). Regarding stimulus-induced motor behaviors, ectopic activation of MBON-γ2α’1 causes inhibition of proboscis extension response to sucrose but ectopic inhibition has no effect(Chia & Scott, 2020). The MBONs important for startle-induced negative geotaxis include the cholinergic MBON-α2sc and α’3(Sun et al., 2018) but they are not important for movement suppression in the present study. Together, our study reveals a novel role for MBON-γ2α’1 in sustained inhibitory control per se. It remains to be determined whether this function requires the input from the γ MB lobes or dopamine/octopamine neurons or both, and how this MBON with only 2 neurons in each hemisphere accommodates so many functions.

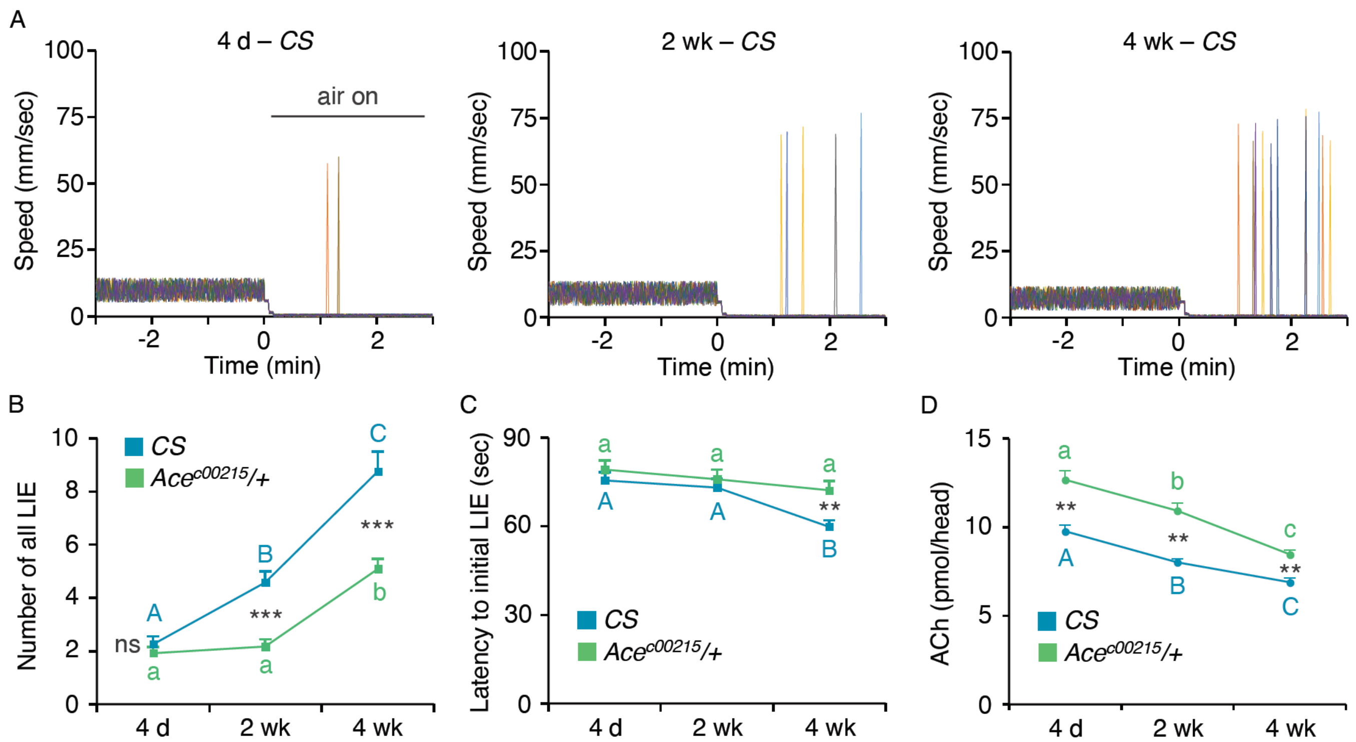

Figure 5.1: Aging diminishes inhibitory control in flies.

A. Representative movement traces of a group of 13 *CS* flies subjected to the GNG test in three chambers – one with the 4 d old, the other with the 2 wk old and the last with the 4 wk old. Each panel illustrates individual fly traces, which are denoted by the different colored lines. The timepoint when strong airflow was introduced is marked as 0 in the X axis. The *CS* flies of all three ages exhibited robust inhibitory control under airflow with infrequent instances of flying behavior representing LIE (movement > 60 mm/sec). The sporadic LIEs increased with aging. **B.** Quantitative representation of the aging-dependent increases in LIE and rescue by *Ace* deficiency. All LIEs displayed by 13 flies in a chamber for the entire 10 min under airflow during GNG test were manually counted revealing more frequent LIE in older *CS* flies (blue line). The aging-dependent increases in the LIE were alleviated by the heterozygous *acetylcholinesterase* (*Ace*) mutation (green line; $Ace^{c00215}/+$) fully at the 2 wk old and partly at

the 4 wk old. ANOVA with *post hoc* Tukey multiple comparison of three ages in each genotype: different letters (A, B and C for *CS*; a and b for $Ace^{c00215}/+$) denote statistically significant differences ($p > 0.05$; $n = 12$). Student *t*-test between two genotypes ($Ace^{c00215}/+$ and *CS*) in each age: ns, $p > 0.05$; ***, $p < 0.0001$; $n = 12$. **C.** Latency to initial LIE. ANOVA with *post hoc* Tukey multiple comparison of three ages in each genotype: different letters (A and B for *CS*) denote statistically significant differences ($p < 0.0001$; $n = 12$). When the age-matching *CS* and $Ace^{c00215}/+$ were compared by Student *t*-test, there is no difference in the LIE latency at 4 d or 2 wk (p > 0.05, $n = 12$; not noted in the graph) but there is significant difference at 4 wk (**, $p < 0.005$, $n = 12$). The heterozygous *Ace* mutation fully reversed the aging-related premature LIE latency. **D.** Acetylcholine levels in the *CS* and $Ace^{c00215}/+$ heads. The acetylcholine levels were significantly higher in $Ace^{c00215}/+$ than *CS* at all ages and dampened with aging in both genotypes. ANOVA with *post hoc* Tukey multiple comparison of three ages in each genotype: different letters (A, B and C for *CS*; a, b and c for $Ace^{c00215}/+$) denote statistically significant differences ($p < 0.05$; $n = 4$). Student *t*-test between two genotypes ($Ace^{c00215}/+$ and *CS*) in each age: **, $p < 0.005$; $n = 4$.

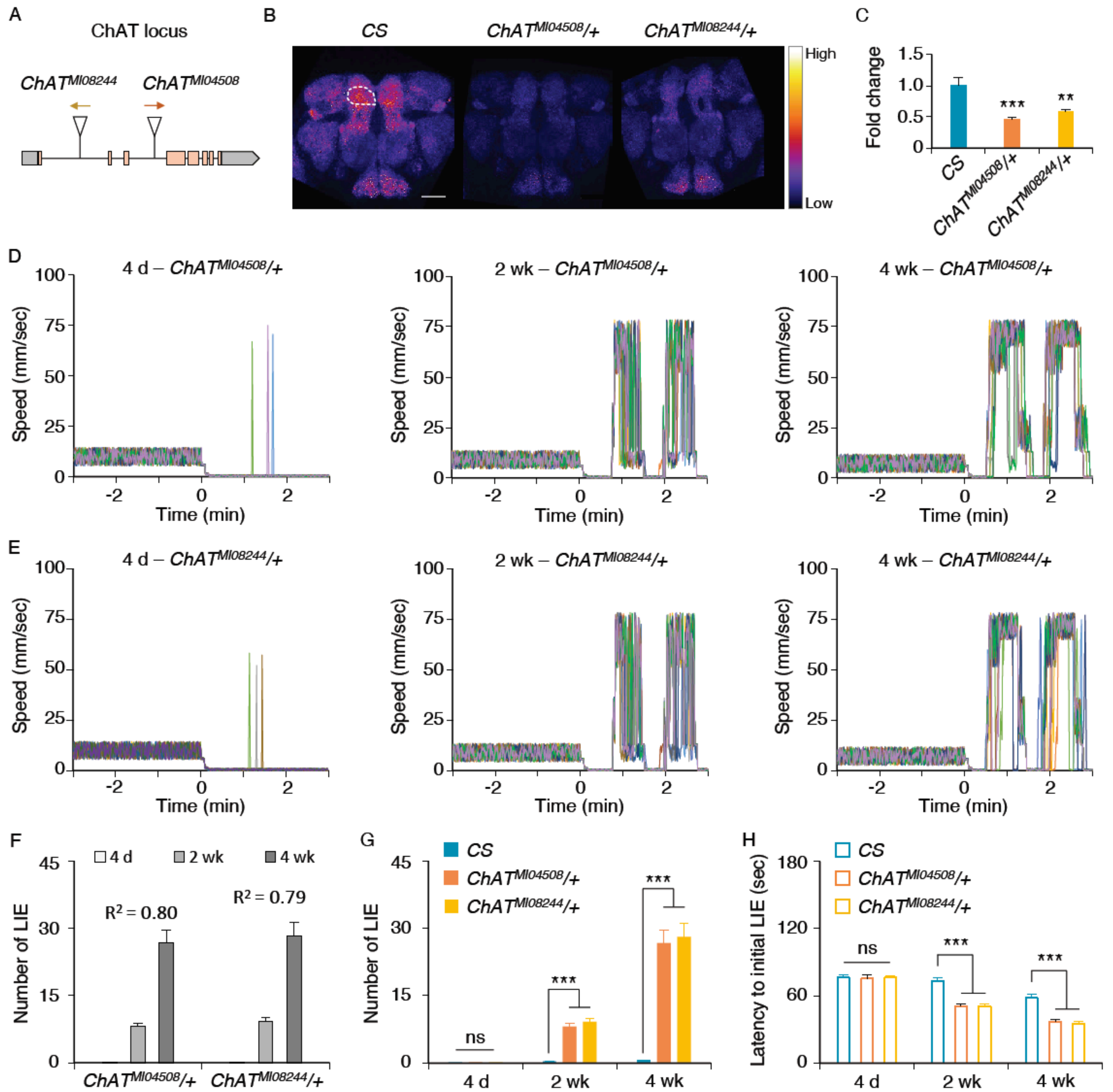

Figure 5.2: ChAT deficit amplifies the impaired inhibitory control with aging

A. The scheme depicts the locations of the transposon MiMIC insertions in the *ChAT* locus: *ChAT*^{MI04508} (the orange arrow denoting direction of the splice acceptor in the transposon) and *ChAT*^{MI08244} (yellow arrow denoting direction of the splice acceptor). The coding regions are

noted in orange boxes, noncoding regions in gray boxes, and introns in gray lines (not to scale). **B.** ChAT immunoreactivity in the brains of *CS, ChAT*^{MI04508}/+ and *ChAT*^{MI08244}/+. Signal intensity is converted into a fire scale. Quantification of ChAT immunoreactivity was done in the superior medial protocerebrum (SMP) in both hemispheres, one of which is demarcated with a white circle. Both heterozygous *ChAT* mutants had less ChAT immunoreactivity compared to *CS*. Scale bar, 50 μm. **C**. Quantified ChAT immunoreactivity comparison. ChAT immunoreactivity levels were normalized with the average ChAT immunoreactivity level of *CS*. The heterozygous ChAT mutants showed significantly reduced ChAT immunoreactivity in the SMP compared to *CS* (ANOVA with *post hoc* Dunnett test using *CS* as a control: **, $p = 0.0006$; ***, $p < 0.0001$; $n = 8\text{-}11$). **D-E.** Each graph depicts the representative movement traces of 13 flies in a chamber: *ChAT*^{MI04508}/+ (**D**) and *ChAT*^{MI08244}/+ (**E**) across different ages (4 d, 2 wk, and 4 wk). **F-G.** Comparison of the number of LIEs per fly per min for *ChAT*^{MI04508}/+, *ChAT*^{MI08244}/+ and *CS* at three different ages. The older heterozygous *ChAT* mutants exhibited higher LIEs (**F**: ANOVA R^2 values are noted in the graph; $n = 12$), which are significantly more than those of *CS* at the ages of 2 wk and 4 wk but not at the age of 4 d (**G:** ANOVA with *post hoc* Dunnett test using *CS* as a control: ns, $p > 0.05$; ***, $p < 0.0001$; $n = 12$). **H.** Latency to initial LIE. ANOVA with *post hoc* Dunnett using *CS* as a control: ns, $p > 0.05$; ***, $p < 0.0001$; $n = 12$). The heterozygous *ChAT* mutation caused significantly earlier onset of LIE at the ages of 2 wk and 4 wk.

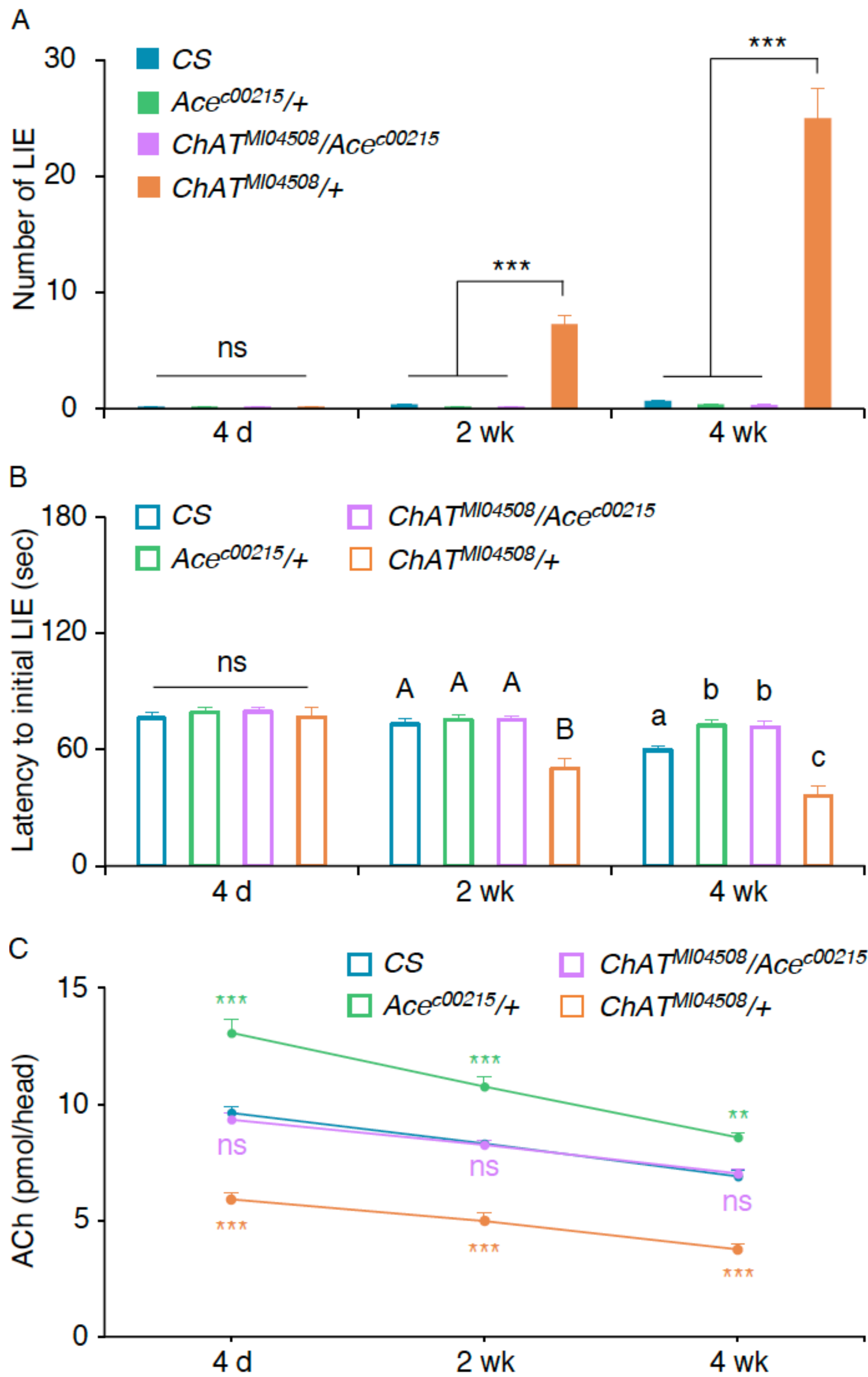

Figure 5.3: The heterozygous *Ace* mutation fully suppresses the *ChAT/+*'s inhibitory control deficits

A. The double heterozygous *ChAT* ^{MI04508}/*Ace* ^{c00215} (light purple) mutants displayed robust movement restraint with rarely detectable LIEs similar to *CS* (blue), *Ace*^{c00215}/+ (light green) and *ChAT*^{MI04508}/+ (orange) at the age of 4 d (one way ANOVA with *post hoc* Tukey: ns, $p > 0.05$; $n = 12$). At the ages of 2 wk and 4 wk, the *ChAT*^{MI04508}/*Ace*^{c00215} mutants displayed a small increase in LIE similar to *CS* and *Ace*^{c00215}/+ but the level was significantly different from that of *ChAT*^{MI04508}/+ (ANOVA with post hoc Tukey: ***, $p < 0.0001$; $n = 12$). **B.** The latency to the initial LIE was comparable in all genotypes under study at the age of 4 d (ANOVA with *post hoc* Tukey: ns, $p > 0.05$; $n = 12$). The double heterozygous *ChAT*^{MI04508}/*Ace*^{c00215} exhibited the significantly delayed latency compared to that of *ChAT*^{MI04508}/+ at the ages of 2 wk and 4 wk (ANOVA with post hoc Tukey: different letters denote statistically significant difference, $p < 0.01$, $n = 12$). **C.** The acetylcholine levels in the double heterozygous *ChAT*^{MI04508}/*Ace*^{c00215} were comparable to *CS*, but significantly lower than *Ace*^{c00215}/+ and significantly higher than *ChAT* ^{MI04508}/+ at all ages. ANOVA with *post hoc* Tukey multiple comparison of the genotypes within an age: ns, $p > 0.05$; **, $p < 0.005$; ***, $p < 0.0001$; $n = 4\text{-}8$.

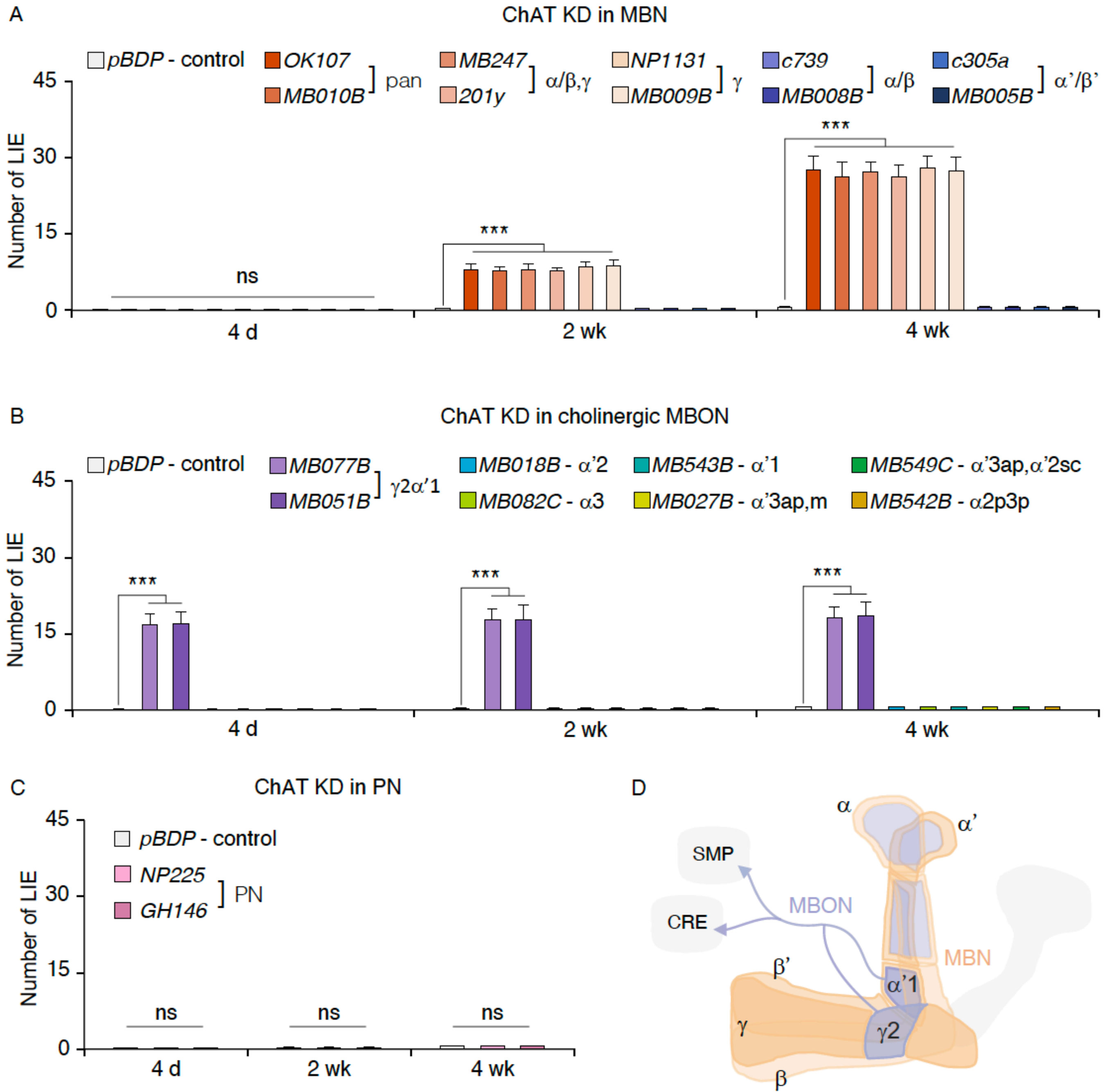

Figure 5.4: The cholinergic neurons important for inhibitory control.

A-C. To identify the cholinergic neurons involved in the aging-related LIEs, we knocked down ChAT by expressing *UAS-ChAT RNAi* in MBNs, MBONs or PNs. The promoter-less *pDBP*-GAL4 driver served as a control. **A.** ChAT KD in either all-MBNs (via OK107-GAL4 or

MB010B-GAL4), MBN α/β and γ (via MB247-GAL4 or 201y-GAL4), or MBN γ (via NP1131-GAL4 or MB009B-GAL4), but not in MBN α/β (via c739-GAL4 or MB008B-GAL4) or MBN α'/β' (via c305a-GAL4 or MB005B-GAL4), led to the aging-dependent increase in LIEs (ANOVA with *post hoc* Dunnett test using *pDBP*-GAL4 as a control: ***, $p < 0.0001$; $n = 8$). **B.** ChAT KD in the MBON-$\gamma 2\alpha'1$ (via MB077B or MB051B-GAL4) increased LIEs at all ages tested (***, $p < 0.0001$; $n = 8$). ChAT KD in other cholinergic MBONs (the GAL4 drivers used for KD and their dendritic sites are noted in the top panel) did not affect the flies' movement inhibition ($n = 8$). **C.** ChAT KD in the PNs (via NP225-GAL4 or GH146-GAL4) did not affect flies' inhibitory control (ns, $p > 0.05$; $n = 8$). **D**. Scheme of the cholinergic neurons important for inhibitory control that include the mushroom body neurons (MBNs; orange) and several mushroom body output neurons (MBONs; purple) as well as the PNs (not shown). The PNs have dendrites in the antenna lobe and project axons to the MBN calyx. The MBNs include γ, α/β and α'/β' among which ChAT in γ (dark orange) is important for the aging-related LIEs. The cholinergic MBON-$\gamma 2\alpha'1$ (dark purple), whose axons project to the superior medial protocerebrum (SMP) and the crepine (CRE), is important for inhibitory control.

Chapter 6: Flypub To Study Ethanol-Induced Behavioral Disinhibition and Sensitization

The following chapter presents a research manuscript written, submitted, and published in JoVE on May 18, 2020. This manuscript describes and details the laboratory's protocol for studying alcohol-induced behavioral disinhibition and sensitization. Behavioral disinhibition is a significant consequence of ethanol consumption, and it affects motor, social, sexual, and cognitive functions up to the point of being dangerous. Sensitization, however, is the increased response to repeated exposure to a drug. This escalated response is an adaptive change that contributes to the development of addiction. Alcohol use disorder (AUD) is a constant burden in our society, costing several millions of dollars to those suffering from it and their families. To develop an efficient countermeasure, we need to understand how the main ingredient in alcoholic beverages, ethanol, causes changes to the nervous system. While the study of ethanol's induced behavioral disinhibition has been studied in several models, behavioral sensitization still is highly unrepresented. To this end, the laboratory developed the Flypub assay, where behavioral disinhibition and sensitization can be studied simultaneously using the *Drosophila melanogaster* animal model.

In the report, we detail the list of materials necessary and have step-by-step instructions on how to build and set up each Flypub chamber. We provide information on the criteria for the experiment's care and collection of the flies. We also include a detailed explanation of quantifying and analyzing both behavioral outputs and how to process and analyze the data collected properly. In the end, we also include a section on the possible issue an experimenter might encounter and how to solve them.

Author Contributions

The following manuscript discusses the findings and observations of the collaborative efforts of multiple individuals: Nataly M. Delgado (N.M.D.), Carmen Mariana Sierra (C.M.S),

Abraham Arzola (A.A.), Erick Benjamin Saldes (E.B.S.), Kyung-An Han (K.A.H.) and Paul Rafael Sabandal (P.R.S.). PRS, NMD and CMS performed, analyzed all experiments, and wrote the manuscript. AA and EBS created the pictures detailing the instructions for building the Flypub chambers and contributed to the writing of the manuscript. EBS generated the section with the statistical analysis and contributed to writing the manuscript. KAH and PRS synthesized the hypothesis, performed data management and analysis, and wrote the manuscript.

Title

Flypub To Study Ethanol Induced Behavioral Disinhibition and Sensitization

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KEYWORDS:

Ethanol, *Drosophila melanogaster*, Fruit fly, Behavioral sensitization, Disinhibition, Courtship, Octopamine, Alcohol use disorder

SUMMARY:

The Flypub assay measures the behaviors that the fruit fly *Drosophila melanogaster* displays under the influence of ethanol. The assay can be readily mastered by experimenters at all levels and applied to various vaporized stimuli, facilitating substance abuse and addiction studies.

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DISCLOSURES:

The authors have nothing to disclose.

6.1 Abstract

Alcohol use disorder (AUD) remains a serious problem in our society. To develop effective interventions for addiction, it is important to understand the underlying neurobiological mechanisms, for which diverse experimental approaches and model systems are needed. The main ingredient of alcoholic beverages is ethanol, which causes adaptive changes in the central nervous system and behavior upon chronic intake. Behavioral sensitization (i.e., escalated responses) in particular represents a key adaptive change underlying addiction. Most ethanol-induced behavioral sensitization studies in animal models have been conducted on the locomotor activating effect of ethanol. A prominent effect of ethanol is behavioral disinhibition. Behavioral sensitization to the disinhibition effect of ethanol, however, is underrepresented. To address this issue, we developed the Flypub assay that allows measuring the escalated increase in disinhibited courtship activities upon recurring ethanol exposure in *Drosophila melanogaster*. Here, we report the step-by-step Flypub assay including assembly of ethanol exposure chambers, setup of the assay station, criteria for fly care and collection, ethanol delivery, quantification of disinhibited courtship activities, data processing and statistical analysis. Also provided are how to troubleshoot critical steps, overcome limitations and expand its utility to assess additional ethanol-induced behaviors. The Flypub assay in combination with powerful genetic tools in *Drosophila melanogaster* will facilitate the task of discovering the mechanism underlying ethanol-induced behavioral sensitization.

6.2 Introduction

Alcohol is one of the most readily available and widely consumed drugs in the world. It has high potential for misuse and addiction; however, the mechanism underlying this process remains incompletely understood. Ethanol induces disinhibition, euphoria, cognitive impairment, hyperactivity, loss of motor control and sedation in worms(Scholz, 2019), fruit

flies(Devineni & Heberlein, 2013; Park, Ghezzi, Wijesekera, & Atkinson, 2017; Scholz, 2019), mice(Kippin, 2014), rats(Bell et al., 2017) and humans(Nona, Hendershot, & Le, 2018), indicating common neurobiological components mediating ethanol's effects from invertebrates to mammals including humans. Chronic ethanol intake causes neural adaptations and behavioral modifications that underlie AUD. One of the adaptations is behavioral sensitization defined as the augmented response with repeated experiences of ethanol(Camarini & Pautassi, 2016; Masur & Boerngen, 1980; Robinson & Berridge, 1993) or other addictive substances(Short & Shuster, 1976; Shuster, Yu, & Bates, 1977; Vanderschuren & Pierce, 2010).

Over the decades, the studies on ethanol-induced behavioral sensitization (EIBS) have focused on the locomotor-stimulating effect, which is used as a proxy for a euphoric response(Camarini & Pautassi, 2016; Kong et al., 2010; Masur & Boerngen, 1980; Robinson & Berridge, 1993). For example, rats or mice upon repeated (every 24, 48 or 72 h) ethanol administration display the augmented locomotor activity as measured by walking speed(Broadbent & Harless, 1999; Camarini, Andreatini, & Monteiro, 1995; Camarini & Hodge, 2004; Hoshaw & Lewis, 2001; Kawakami, Quadros, Takahashi, & Suchecki, 2007; Lessov & Phillips, 1998; Masur & Boerngen, 1980; Melon & Boehm, 2011; Pastor & Aragon, 2006). Similarly, the fruit flies subjected to the second exposure to ethanol vapor 4 h after the first exposure exhibit the enhanced locomotor response as measured by walking speed as well(Scholz, Ramond, Singh, & Heberlein, 2000). While no information is available on the mechanism underlying EIBS to the locomotor-stimulating effect in fruit flies, the studies in rats and mice have uncovered the molecular and signaling components (for example, the dopamine, glutamate and GABA systems) as well as neural substrates and circuit (for example, the ventral tegmental area, nucleus accumbens, amygdala and prefrontal cortex) that play major roles for EIBS(Camarini & Pautassi, 2016; Cofresi, Bartholow, & Piasecki, 2019; Nona et al., 2018).

Disinhibition is a major effect of ethanol and leads to manifestation of behaviors that are typically restricted. The disinhibiting effect is exerted on motor, emotional, social, sexual and cognitive functions, which may lead to inappropriate sexual behavior, verbal or physical aggression and impulsive acts in humans and animal models(Heinz, Beck, Meyer-Lindenberg, Sterzer, & Heinz, 2011; Marinkovic, Halgren, Klopp, & Maltzman, 2000; Miczek, DeBold, Hwa, Newman, & de Almeida, 2015; Prause, Staley, & Finn, 2011; Stoner, George, Peters, & Norris, 2007; Topper, Aguilar, Topper, Elbel, & Pierce-Shimomura, 2014). Ethanol-induced disinhibition has been investigated in animal models for mechanistic studies and they include motor impulsivity and aggression in rodents and monkeys as well as foraging disinhibition in worms(Camarini & Pautassi, 2016; Heinz et al., 2011; Miczek et al., 2015; Nona et al., 2018; Schwandt, Higley, Suomi, Heilig, & Barr, 2008; Topper et al., 2014). We have demonstrated that fruit flies show disinhibited sexual behavior under the influence of ethanol(Lee, Kim, Dunning, & Han, 2008). Specifically, wild-type males flies rarely court other males without ethanol(Lee et al., 2008) and when they do, courtees actively reject courting males. Under the influence of ethanol, however, male flies show more courtship toward other males and courtees exhibit less rejection, resulting in overall enhanced intermale courtship. Notably, flies develop behavioral sensitization to the disinhibition effect upon recurring ethanol exposure, which serves as a unique system to study EIBS(Aranda et al., 2017; Lee et al., 2008).

In this report, we describe how to set up, perform, troubleshoot and analyze the Flypub assay and data to study ethanol-induced disinhibition and sensitization in the fruit fly *Drosophila melanogaster*. To provide its utility and effectiveness, we tested the wild-type *Canton-S* (*CS*; control fly strain) along with the flies deficient in tyramine β hydroxylase (TβH) that synthesizes octopamine (OA). OA is a major neuromodulator in invertebrates(Gallo, Accordi, Chimenti, Civinini, & Crivellato, 2016; Roeder, 1999) and plays a key role in the development of ethanol tolerance in flies(Scholz et al., 2000). We report here for the first time that OA is important for EIBS.

6.3 Protocol:

NOTE: The protocol section details the preparatory, Flypub assay and analysis steps that include (1) assembly of the chamber, (2) fly care and collection, (3) assay station setup, (4) ethanol exposure, (5) courtship scoring and data analysis, and (6) statistical analysis. The key steps for conducting the Flypub assay and analysis is depicted in a workflow (**Figure 1**).

1. Assembly of the chamber (Figure 2)

1.1. Cut off the bottom portion of the round *Drosophila* bottle at the 25 mL mark using a razor blade. 1.2. Make a hole, 5 mm in diameter, at the 50 mL mark of the bottle using a hot soldering iron. NOTE: This is the access point where the flies will be transferred into the chamber. 1.3. Cut a mesh sheet into a circle, 54 mm in diameter, to fit in the *Drosophila* bottle at the 75 mL mark. 1.4. Secure the mesh at the 75 mL mark of the bottle using hot glue. 1.5. Cut the polycarbonate plastic sheet into a circle, 70 mm in diameter. 1.6. Attach the polycarbonate plastic sheet to the bottle at the 25 mL mark (bottom open area made in step 1.1) using hot glue. 1.7. Pressure down using weights to ensure that the polycarbonate round is firmly attached to the bottom. 1.8. Wash the pubs with ethanol to remove any odors and profusely rinse them multiple times under running distilled water. Shake the pubs vigorously to remove excess water. 1.9. Dry the pubs by laying them down horizontally on paper towels at room temperature.

Fly care and collection

2.1. Maintain the flies on a standard cornmeal/agar/sugar/yeast food medium (https://bdsc.indiana.edu/information/recipes/harvardfood.html). 2.2. Collect one- to two-days old male flies into a group of 33, which represent one data point, under carbon dioxide (CO_2) anesthesia. Make sure to select the flies with intact morphology and put them in a food vial to recover. NOTE: Two more or three less flies per group are tolerable. Behaviors can be

sensitive to experimental settings thus a total fly number per pub may need to be adjusted with a control fly line. NOTE: Make sure that the food vial is laid down on the side so the anesthetized flies do not get stuck to the food. 2.3. Keep the flies in the 25 °C incubator with at least 50% relative humidity and a 12 h light / 12 h dark cycle for 2 days prior to ethanol exposure. NOTE: CO_2 clearance is critical to eliminate any CO_2-induced physiological or behavioral effects that may alter ethanol-induced responses. 2.4. Use codes to blind fly genotypes or treatment conditions to the experimenters conducting ethanol exposure and scoring courtship behaviors. NOTE: Blind tests help eliminate experimental bias.

Assay station set up (Figure 3A)

3.1. Set up a copy stand with an attached center arm on a bench top in a well-ventilated room. NOTE: The copy stand is not mandatory. Any staging device that provides a level platform is sufficient. 3.2. Clamp the two lateral arms to the stand, with each arm approximately 18 cm out from the center of the stand. 3.3. Place a fluorescent light on each arm of the stand and one in the middle. 3.4. Attach the video recorder to the center arm, approximately 38 cm above the center of the base. This will record the pubs from a top view. 3.5. Cover the base of the stand with white paper, which helps visualize dark-colored flies to create contrast. 3.6. During the day of exposure, turn on the fluorescent lights and the computer connected to the video camera that is attached to the copy stand (**Figure 3A**). NOTE: The light intensity 2100-2200 lux provides good quality of recorded behaviors for scoring. However, ambient lighting conditions in the laboratory are sufficient to observe ethanol-induced courtship activities. 3.7. Prepare the items to be used for ethanol exposure depicted in **Figure 3B**. 3.8. Gather six clean, assembled pubs for a set of experiments and label them with the code 1 through 6. NOTE: Make sure to place the randomized codes on fly genotypes or treatment conditions.

Ethanol exposure (Figure 3)

4.1. Gently transfer a group of 33 males into a flypub chamber through the hole at the 50 mL mark using a small funnel. NOTE: To minimize mechanical stress to the flies, place a mouse pad or any cushioning material under the pub during transfer. 4.2. Cover the hole with a tape. NOTE: The tape is used to close the hole, preventing flies from escaping out of the pub. 4.3. Align the pubs on the stage from 1 to 6. 4.4. Acclimate the flies to the chamber for 10 min (**Figure 3D**). 4.5. Adjust the camera settings including the focus, zoom and brightness, and record the last 5 min of acclimation to measure a basal courtship level. NOTE: To eliminate glare generated by light reflection from a pub, place lab wipes (typically 4 layers or less than 1 mm thickness) at the bottom of the pub to adjust the angle. 4.6. Prepare cotton pads for ethanol delivery by cutting a pad into four equal quadrants with clean scissors and then trim the corners to make it fit into a Petri dish during acclimation (**Figure 3C**). NOTE: Do not use bare hands to handle the cotton pads. Use forceps to handle the cotton pads to avoid any potential transfer of odors. 4.7. Add a cotton pad into each Petri dish. 4.8. Add 1 mL of 95% ethanol to each cotton pad, make sure for ethanol solution to be evenly distributed on the entire area of the pad. 4.9. Cover with double-layered lab wipes to avoid fast ethanol evaporation. 4.10. Place the small Petri dish containing the ethanol-soaked cotton pad and the double-layered lab wipes through the bottom opening of the pub after acclimation. 4.11. Align the pubs on the stage, begin recording and simultaneously start a timer. 4.12. Record the pubs containing flies during ethanol exposure until the flies stop courting or moving due to sedation. 4.13. Remove the Petri dish containing ethanol from each pub with a spatula when over 90% of the flies are sedated. 4.13. Gently transfer flies back to their assigned vials through the hole at the 50 mL mark in the pub. NOTE: Place a funnel on top of food vials to aid in transfer. Make sure to place sedated flies on the side of the food vials to prevent them from getting stuck in the food. 4.14. Clean the pubs with ethanol to remove any odors and profusely rinse them multiple times under running distilled water. Shake the pubs vigorously to remove excess water. 4.15. Dry the pubs by laying them down horizontally on paper towels at room temperature. 4.16. Keep

the flies in the 25 °C incubator with at least 50% relative humidity and a 12 h light / 12 h dark cycle. 4.17. Repeat the steps 4.1-4.16 every 24 h for six consecutive days and make sure to conduct the ethanol exposure at the same time of the day to avoid any circadian effects. NOTE: Change food vials every 2 – 3 days to maintain healthy flies.

Courtship scoring and data analysis (Figure 4-6)

5.1. Open the recorded videos using a media player (e.g., VLC) and zoom in the video to clearly observe flies to score (**Figure 4A**). 5.2. Attach the time code to the video (**Figure 4B**). 5.3. Count the number of males engaged in courtship activities including following, unilateral wing extension, courtship chain, courtship circle, abdominal bending and mounting for every 10 sec time block(Lee et al., 2008) (**Figure 5**). 5.4. Enter the number of males displaying courtship for every 10 s time block into a worksheet (**Figure 6A**). 5.5. Use the maximal number of courting males in the three consecutive 10 s time blocks as a representative data point (**Figure 6B**). 5.6. Calculate the average of 10 consecutive data points having the highest value (**Figure 6C**) and this represents the percentage of intermale courtship per pub (**Figure 6A**).

6. Statistical analysis (Supplemental Figure 1)

6.1. Open statistical analysis software (e.g., Minitab 17) and add courtship data in the worksheet. NOTE: Any statistical analysis software can be used. 6.2. To determine the distribution of the data (either normal or non-normal distribution), go to the **Stat** tab, select **Basic Statistics**, and click on the **Normality Test** option (**Supplemental Figure 1Ai**). 6.3. In **Variable**, select individual columns (each column representing a data set of a genotype or treatment under study), choose the **Anderson-Darling** test, and click **OK** (**Supplemental Figure 1Aii**). NOTE: The Normality Probability Plot will show the calculated P-Value: if the P-Value is greater than 0.05, then the data are normally distributed. If the P-Value is less than 0.05, the data are non-normally distributed (**Supplemental Figure 1Aiii**). 6.4. For comparison

of multiple groups, stack the columns to compare by clicking the **Data** tab, select **Stack**, and then **Columns** (**Supplemental Figure 1Bi**). 6.5. In the **Stack Columns** window, select the data columns to be stacked, select the stacking done either in **New worksheet** or **Column of current worksheet** with the next column designated for denoting subscript (e.g., data group identity; **Supplemental Figure 1Bii-1Biii**). 6.6. Click the **Stat** tab, select the **ANOVA** test, select the **General Linear Model** and then click the **Fit General Linear Model** (**Supplemental Figure 1Ci**). 6.7. In the **General Linear Model** window, select the columns to be compared in the **Responses** box, select the column with subscript in the **Factors** box and click **OK**, which leads to the statistical analysis results (**Supplemental Figure 1Cii-1Ciii**). 6.8. For comparison of two groups with normally distributed data, click the **Stat** tab, select the **Basic Statistics**, and select the 2-**Sample t-test** (**Supplemental Figure 1Di**). 6.9. In the **2-Sample t for the Mean** window, select **Each sample is in its own column**, from a dropdown box, select the two groups to compare in the **Sample 1** and **Sample 2** boxes and then click **OK**, which leads to the statistical analysis results (**Supplemental Figure 1Dii-1Diii**). 6.10. For comparison of two groups with non-normally distributed data, go to the **Stat** tab, select **Nonparametrics** and click **Mann-Whitney** (**Supplemental Figure 1Ei**). 6.11. In the **Mann-Whitney** window, select the two groups to compare in the **First Sample** and **Second Sample** boxes and then click **OK**, which leads to the statistical analysis results (**Supplemental Figure 1Eii-1Eiii**). 6.12. For comparison of three or more groups of non-normally distributed data, go to the **Stat** tab, select **Nonparametrics**, and then click the **Kruskal-Wallis test** (**Supplemental Figure 1Fi**). 6.13. In the **Kruskal-Wallis** window, select the columns to be compared in the **Response** box, select the column with subscript in the **Factor** box and click **OK**, which leads to the statistical analysis results (**Supplemental Figure 1Fii-1Fiii**).

6.3 REPRESENTATIVE RESULTS:

This section demonstrates the results of a representative Flypub experiment. *Drosophila* males rarely court other males(Curcillo & Tompkins, 1987; Spieth, 1974). During the first ethanol exposure, the wild type *Canton-S* (*CS*) males exhibited a small but insignificant increase in the disinhibited intermale courtship(Lee et al., 2008) (**Figure 7A**). However, *CS* males showed the escalated increases in the disinhibited courtship activity in subsequent ethanol exposures (ANOVA GLM, *CS*: R^2=0.83, $F_{(5,66)}$ =65.21, p < 0.0001; n = 12; **Figure 7A**), which indicates behavioral sensitization to the disinhibition effect of ethanol. We have previously shown that this type of EIBS requires dopamine and the dopamine receptor DopEcR in the mushroom neurons(Aranda et al., 2017; Lee et al., 2008).

To identify whether additional neuromodulators are involved in EIBS, we investigated the role of OA by testing the flies (*tβh; nM18 null allele*)(Certel, Savella, Schlegel, & Kravitz, 2007; Monastirioti, Linn, & White, 1996) lacking tyramine β hydroxylase, the rate-limiting enzyme in the OA biosynthesis, thus deficient in OA. The *tβh* males in the *CS* genetic background (a kind gift from Dr. Andreas Thum, University of Leipzig , Germany) displayed the sensitized disinhibited courtship response upon daily ethanol exposures (ANOVA GLM, *tβh*: R^2=0.67, $F_{(5,66)}$ =27.60, p < 0.0001; n = 12; **Figure 7B**) but at the reduced level compared to *CS (*ANOVA GLM, interaction effect: F=2.50, p <0.034). Upon post hoc analysis, *tβh* males exhibited lower levels of intermale courtship at each exposure that is most evident during the fourth through sixth ethanol exposures when compared to *CS* (Two-sample t-test: p < 0.002 in EXP4, p < 0.004 in EXP5, p < 0.021 in EXP6; n = 12; **Figure 7C**). Together, these results indicate that OA may play a role in EIBS to the disinhibition effect of ethanol. More importantly, these data sets clearly demonstrate the utility and effectiveness of the Flypub assay in studying ethanol-induced disinhibition and sensitization.

6.4 DISCUSSION:

In this report, we have described the setup and detailed protocol of the Flypub assay; a novel method to measure how recurring ethanol exposure triggers disinhibited courtship and behavioral sensitization. Although the Flypub assay is relatively straightforward, several steps require care and attention to ensure reliable results. Firstly, the flies for testing must be fully pigmented (i.e., fully developed adult flies), healthy and intact. Deformities or damage especially in their wings or legs can affect the male's ability to court. Secondly, the fly age is important and must be matched among control and experimental groups (optimal age: 3-5 days old at ethanol exposure 1). Two weeks and older wild-type male flies tend to display the elevated levels of disinhibited courtship(Lee et al., 2008). Thus, proper age-matching of flies under study is essential to avoid variable results. Thirdly, the fly number per pub is vital (optimal: 33 per pub). The lower or higher fly numbers per pub can greatly skew courtship scores (data not shown). Fourthly, the Flypub chambers need to have identical volumes as illustrated in **Figure 2B**. This ensures that flies receive ethanol vapor synchronously and the elicited behaviors are consistent. Fifthly, clear video recording and precise courtship scoring are essential. This protocol heavily depends on behavioral observations, so meticulous observance to the standardized courtship scoring protocol is fundamental to minimize inconsistent results. Lastly, it is highly recommended that both ethanol exposure and the courtship scoring steps be performed blindly, where an experimenter is unaware of fly genotypes or experimental treatments, thereby preventing experimental bias.

The Flypub assay has multiple advantages. Firstly, multiple groups of flies can be tested and compared simultaneously. Secondly, it is inexpensive, simple to setup and easy to learn, making it highly amenable to experimenters at all levels including elementary through high school, undergraduate and graduate students, postdocs and faculty as well as teaching laboratories with limited space and budgets. Thirdly, it can be utilized to measure additional behaviors such as disinhibited courtship of female flies and the sedative effect of ethanol or other sedatives to assess initial sensitivity and tolerance development and maintenance(Aranda

et al., 2017; Lee et al., 2008). Together, the Flypub is a versatile method to study diverse features of AUD.

The major limitation of the Flypub assay is the rigorous and laborious courtship scoring regimen. The courtship behavior under the influence of ethanol is highly dynamic in a manner that the courtship duration ranges from less than one second to many minutes and the flies engaged in courtship are rather frequently changing. The scoring regime presented here was developed to incorporate this dynamic nature and to provide consistent scores on individual ethanol exposures for a given genotype(Aranda et al., 2017; Lee et al., 2008). As noted in the protocol, the courtship activity is manually scored, which is time consuming. Several automated scoring programs have been developed to facilitate unbiased high-throughput behavioral screening and all of which rely on individual flies' movements and locations(Branson, Robie, Bender, Perona, & Dickinson, 2009; Dankert, Wang, Hoopfer, Anderson, & Perona, 2009; Keleman, Kruttner, Alenius, & Dickson, 2007; Kido & Ito, 2002; Reza et al., 2013; Schneider & Levine, 2014; Winbush et al., 2012). We also attempted to develop a computer software to automatically count the courtship activity but was unable to obtain consistent and reliable outcomes. This could be due to the fact that behavioral scoring includes multiple courtship steps (i.e., following, unilateral wing extension, abdominal bending and mounting)(Curcillo & Tompkins, 1987; Greenspan & Ferveur, 2000; Spieth, 1974) of multiple flies at once. Even with this limitation, an experimenter with adequate training should be able to quantify the ethanol-induced courtship behaviors with consistency and accuracy. Nonetheless, it would be of great help and importance to adopt machine learning or other advanced algorithms as a follow-up.

Similar to rodent models, the studies on ethanol in the fly model have largely focused on the ethanol's locomotor stimulating and sedative effects. The Flypub assay however, measures disinhibited courtship, a type of cognitive disinhibition which is novel(Aranda et al., 2017; Lee et al., 2008). Therefore, the Flypub can aid to elucidate the molecular players, cellular pathways and neural circuits as well as the risk factors (e.g., age, sleep, diet or social

environment) critical for behavioral disinhibition and sensitization. We have previously demonstrated that dopamine signaling is required for EIBS, which is in line with the findings in rodent models and human subjects(Camarini & Pautassi, 2016; Lee et al., 2008; Nona et al., 2018). Also as a proof of concept, we examined the *tβh* mutant lacking OA (the invertebrate counterpart of norepinephrine) and found that OA is also important for behavioral sensitization to the ethanol's disinhibition effect although its contribution is relatively small compared to that of dopamine(Lee et al., 2008). This finding is in contrast to the observation by Scholz(Scholz, 2005) that the *tβh* mutant flies exhibit no obvious impairments in sensitization to the ethanol's locomotor activating effect(Scholz, 2005). This suggests distinct molecular, cellular and neural pathways mediating behavioral sensitization to the disinhibition versus locomotor activation. Follow-up studies should further collaborate this tantalizing notion.

In summary, the Flypub is a low-cost, multifaceted and effective method to investigate the behavioral responses to ethanol, particularly disinhibition and behavioral sensitization, that may help advance our understanding of AUD and provide insight into effective interventions for this chronic disorder.

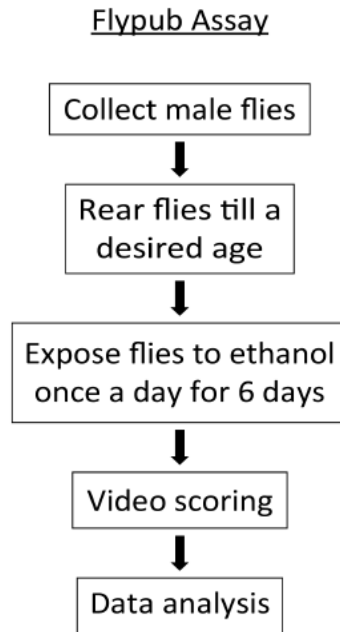

Figure 6.1: Flypub assay workflow.

A workflow diagram highlighting the key steps for conducting

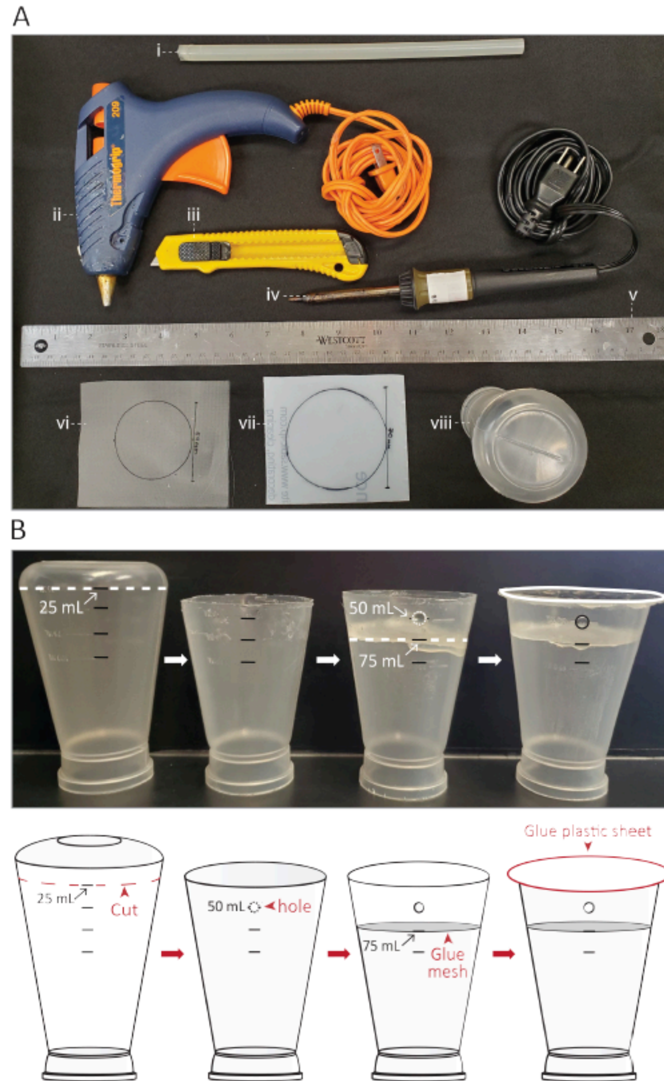

Figure 6.2: Flypub chamber materials and assembly.

(**A**) Materials required to build a Flypub chamber include (**i**) hot glue gun glue stick, (**ii**) hot glue gun, (**iii**) razor blade, (**iv**) soldering iron, (**v**) ruler, (**vi**) mesh, (**vii**) polycarbonate plastic sheet, and (**viii**) round-bottom *Drosophila* bottle. (**B**) Schematic representation of the Flypub chamber assembly.

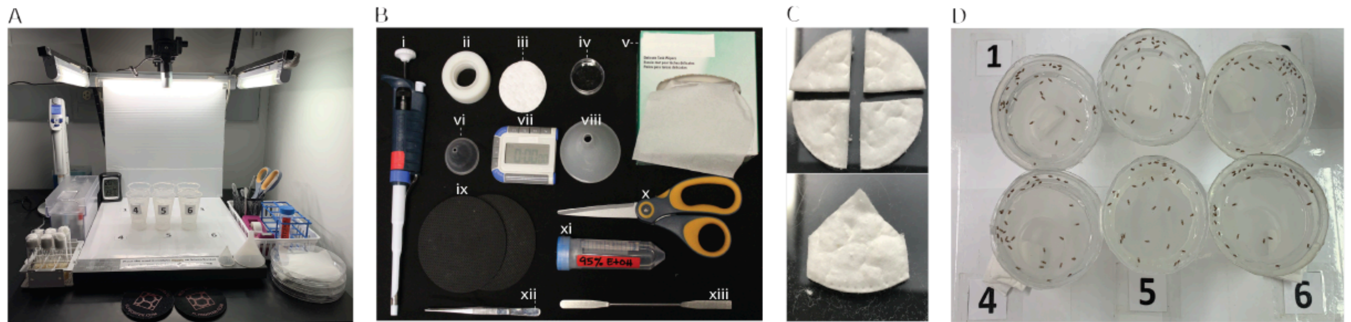

Figure 6.3: Ethanol exposure.

(**A**) A fully assembled Flypub station. (**B**) Materials required for ethanol exposure include (**i**) P1000 micropipette, (**ii**) tape, (**iii**) cotton pad, (**iv**) Petri dish, (**v**) lab wipes, (**vi**) small funnel, (**vii**) timer, (**viii**) mid-size funnel, (**ix**) mouse pad, (**x**) scissors, (**xi**) 95% ethanol, (**xii**) forceps and (**xiii**) spatula. (**C**) Steps on how to cut cotton pads. (**D**) Top view image of the pubs aligned on the stage.

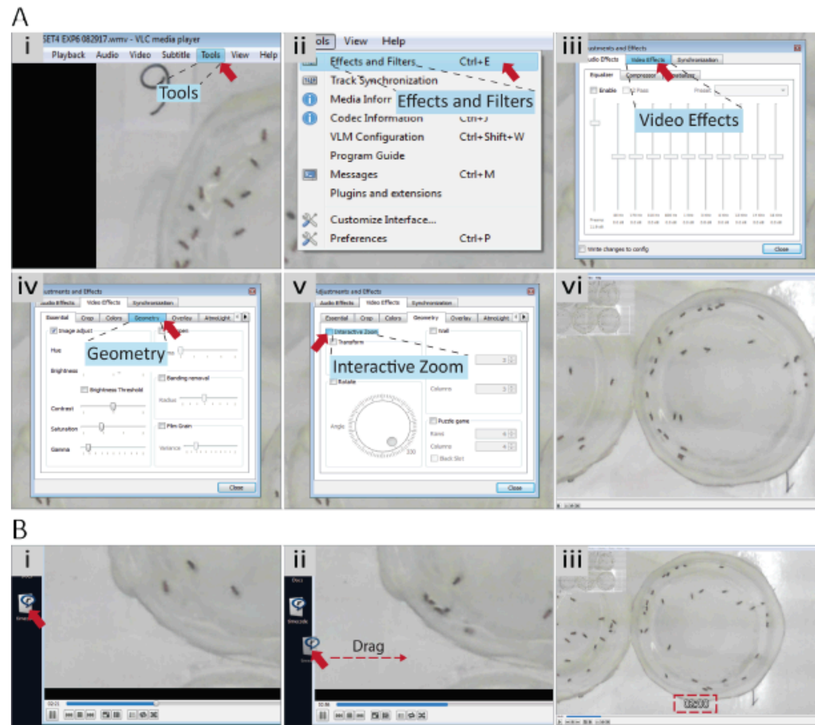

Figure 6.4: Video setup for behavioral scoring.

Shown is the step-by-step guide on (**A**) how to zoom in on the video and (**B**) how to insert the timecode file into the VLC media player for behavioral scoring.

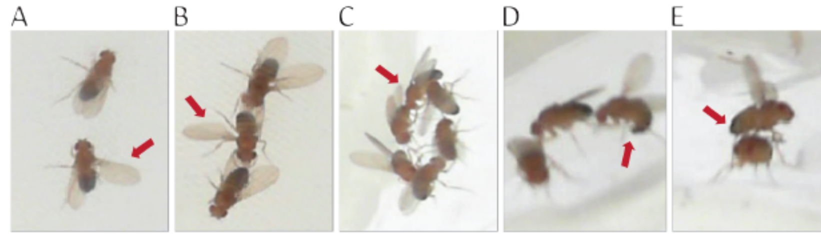

Figure 6.5: Male courtship behaviors.

Representative images illustrate the *Drosophila* male courtship behaviors including following and unilateral wing extension for (**A**) courtship song, (**B**) courtship chain, (**C**) courtship circle (**D**) abdominal bending and (**E**) mounting that are used for behavioral scoring.

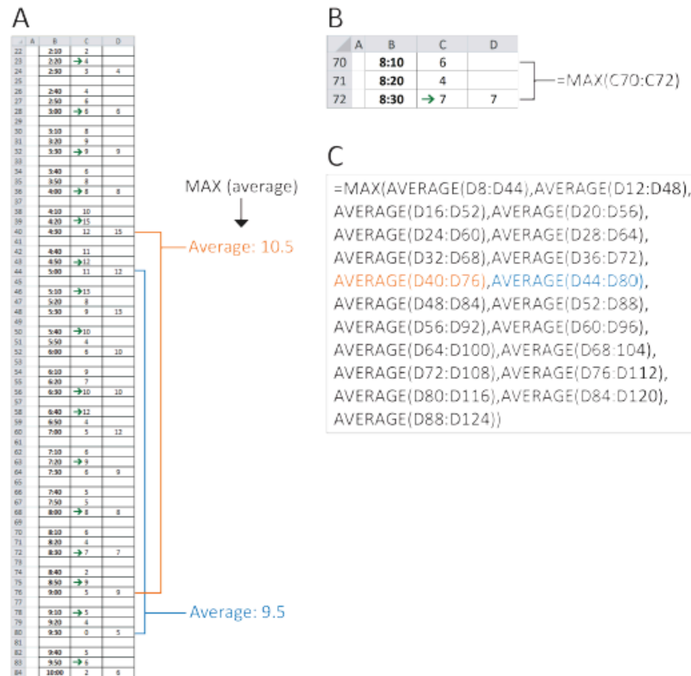

Figure 6.6: Data input and analysis.

(**A**) The number of males engaged in courtship in every 10 s time block is transcribed into a worksheet. The highest number of courting males of three consecutive 10 s time blocks (green arrow) is used as a representative data point. The average of 10 consecutive data points (blue or orange bracket) having the maximal value represent the percentage of inter-male courtship per pub {orange bracket; MAX (average), black arrow}. (**B,C**) The worksheet formulas used to calculate the maximal representative data point and the maximal average of 10 consecutive representative data points per pub.

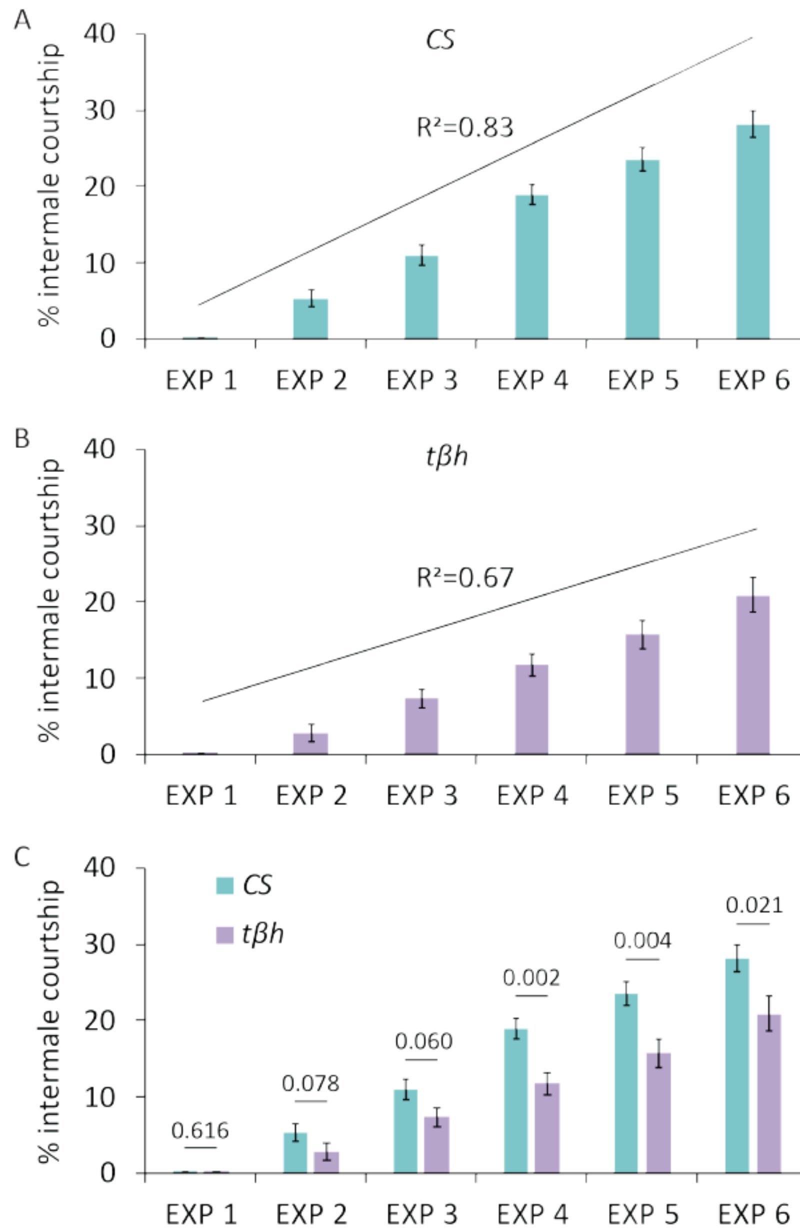

Figure 6.7: Fly Ethanol induced behavioral disinhibition and sensitization in CS and tβh..

A,B) The *CS* and *tβh* males displayed sensitized courtship disinhibition with repeated ethanol exposures (ANOVA GLM, *CS:* R^2=0.83, $F_{(5,66)}$=65.21, p < 0.0001; *tβh:* R^2=0.67, $F_{(5,66)}$=27.60, p < 0.0001; n = 12). (**C**) The *tβh* males showed less disinhibited courtship compared to *CS* (n=12). The p values of the post hoc analyses are shown above the line. The intermale courtship

activity was analyzed from the videos generated for each ethanol exposure. All data are reported as means ± standard error of the mean.

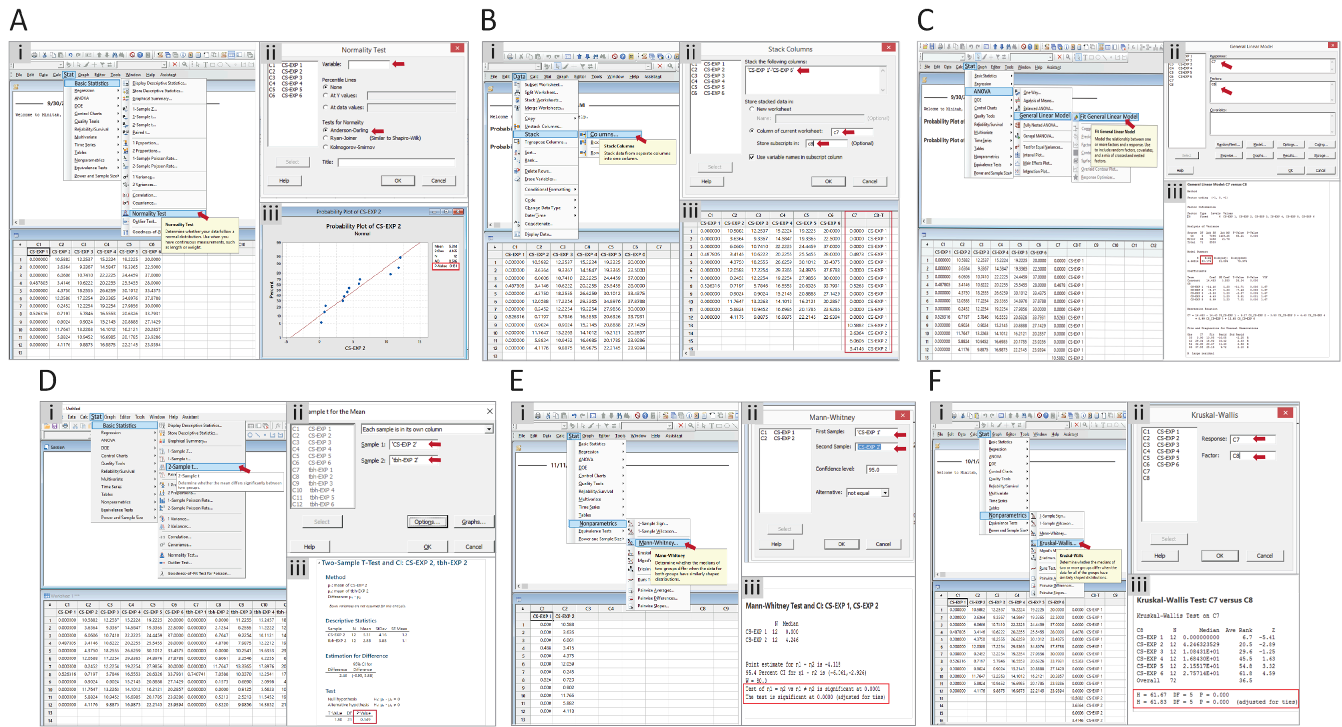

Figure S6.1: Statistical analysis.

The steps in the Minitab 17 software on how to perform the (**A**) Normality test, (**B**) stacking the data, (**C**) General linear model ANOVA test, (**D**) Two-sample t-test, (**E**) Mann-Whitney test and (**F**) Kruskal-Wallis test.

Chapter 7: Final Discussion

This dissertation work begins closing the gap in our understanding of how genetic and non-genetic factors affect inhibitory control. Using the newly designed Go/No-Go test, we have identified three major factors crucial for inhibitory control. The first nighttime caffeine as a non-genetic factor affecting inhibitory control. Secondly, we identified the Shaker/KCNA voltage-gated potassium channel complex (Shaker, Hyperkinetic, and Quiver/Sleepless) which codes for a synaptic cell adhesion molecule. We have found *kek5* to interacts with hyper dopamine to cause impulsivity. Altogether, the findings in this dissertation provides new insights into how these factors contribute to impulsivity, which may lead to new preventive and therapeutic approaches for impulsivity-related brain disorders.

With the increase popularity of caffeinated energy drinks, it is important to understand the effects of caffeine in cognitive functions. Studies focusing on caffeine and inhibitory control have produced mixed results. Some studies show caffeine positively affects caffeine by improving inhibitory control as measured by the Go/No-Go test (Barry et al., 2014). In contrast, others show that it negatively affects inhibitory control by increasing the errors in the stop-signal reaction task (Kahathuduwa et al., 2020). The variability in the outcomes of these studies can be attributed to the difference in sample size, the dose used, and even the caffeine delivery. Another factor that contributes that can possibly contribute to the mixed results observed in caffeine studies in human subjects is their previous experience with caffeine. Positive experiences or negative experiences can lead to unintentional bias at the moment of testing (Shabir et al., 2018). The use of *Drosophila* in this study allows to avoid this variable and helps us focus on the mechanism by which caffeine affects inhibitory control.

Our study is the first one to measure the quantity of caffeine in flies after caffeine feeding. We used an ELISA assay to measure the caffeine content in flies after nighttime caffeine feeding (Figure 1.1). The results from this experiment directly correlate to the result we observed in our behavioral experiments where we see a dose-dependent increase in

impulsivity in flies. Another important finding is the sex differences observed in both caffeine content and impulsivity. We found females to have more caffeine content and display more severe impulsivity (Figure 2.1 and Figure 2.2 A B). This goes in line with a Caretta et al. (Mucignat-Caretta, 1998) who showed similar results in humans, where females are more susceptible to the effects of caffeinated energy drinks. These results stress the importance of evaluating each gender independently to identify any differences in their behavioral processes.

Caffeine reduces the sleep of both humans and flies (Grant & Chamberlain, 2018; Joan C. Hendricks, 2000; Lieberman et al., 2002). We also know that sleep disruption in humans has been linked to poor performance in inhibitory control (Demos et al., 2016) and that individuals with ADHD develop sleep disorders (van der Voet, Harich, Franke, & Schenck, 2016). In this study we demonstrate that disruption of sleep (strong shaking and bright light exposure during the nighttime) alone is not enough to induce significant levels of impulsivity (Figure 2.2 EF).

Our studies also found that the effects of caffeine on impulsivity involved dopamine signaling (Figure 2.3). Similar to previous reports that found the dDA1 receptor is important for caffeine effects on sleep (R. Andretic et al., 2008), our study found the dDA1 receptor to play a role in impulsivity (Figure 2.4 AB). We also found the γ or α/β MB neurons to be the neural site where the dDA1 receptor is necessary for the caffeine-induce impulsivity (Figure 2.4 B). Studies in the lab have previously identified the MB γ lobe neurons as the major neural site where dopamine signaling is important for inhibitory control (Sabandal et al. in revision). However, the contribution of α/β lobe neurons was less pronounced in that study than what we see here. While the dDA1 activity in the γ and α/β is necessary for the caffeine-induced impulsivity upon re-expression in all Kenyon cells we saw no effect of caffeine in inhibitory control (Figure 2.4 B). However, we observed rescue of the caffeine-induce impulsivity using the Gal4 drivers NP1131 (γ lobe neurons) and NP0361 (α/β lobe neurons). These results suggest that dDA1 in the γ and α/β the neurons are necessary but not sufficient for caffeine-induced impulsivity. Furthermore, it suggests that the additional neurons labeled by the Gal4 drivers

NP1131 and NP0361 play a role in caffeine-induced impulsivity. Thus, future experiments will require more specific MB drivers to answer this question.

Previous studies in the lab have shown the MB to be the major site for impulsivity in flies (Sabandal et al. in revision,(P. R. Sabandal et al., 2022) our findings align with those studies. This also explains why the sleep disruptions via both mechanical and light did not induce impulsivity since mechanical sleep disruption is known to affect the ellipsoid body neurons (Shafer & Keene, 2021), which according to previous experiments, do not play a role in the inhibitory control of *Drosophila*. While MB neurons are involved in the responses to light signals, their contribution is not as big as the ones from other central brain structures like the ellipsoid body, fan-shaped body, and helicon cells. In addition, light processing has a complex signaling pathway that does not involve the MB lobes and inhibits dopaminergic sleep-promoting neurons (Agrawal et al., 2017; Mazzotta, Damulewicz, & Cusumano, 2020; Shafer & Keene, 2021).

The results from this study identify caffeine as a non-genetic factor contributing to impulsivity. We found sex differences in the effects of caffeine on impulsivity, where females show more severe impulsivity than males, underscoring the importance of evaluating each gender independently. We found that caffeine-induced impulsivity involves dopamine-D1 receptor signaling in the MB neurons.

One of the limitations of this study is the caffeine delivery method, which is done by mixing caffeine into regular fly food. This influences the amount of caffeinated food each fly consumes, resulting in heterogenous caffeine content and effects. One way to address this is by delivering caffeinated food in glass capillary feeding tubes, allowing us to measure caffeinated food intake precisely (Ja et al., 2007). Another way to address this would be by volatilizing caffeine, which would minimize individual differences in caffeine intake and effects. Another factor we did not consider in this study was the mating status of the flies, where the caloric and nutrient demand tends to be altered after mating. To address this, future studies need to compare the virgin versus the mated flies' caffeinated food consumption.

Regarding the dopamine-related mechanism, we need to investigate the possible contribution(s) of other dopamine receptors like D2 dopamine receptors, which play a role in caffeine-induced hyperactivity in rats (Garrett & Holtzman, 1994). This can be addressed by testing individual D2 receptor mutants. Furthermore, future studies will address the role of neurons other than the MB Kenyon cells (i.e., MB output neurons) in nighttime caffeine-induced impulsivity. This will continue to enhance our understanding of how caffeine affects inhibitory control.

We clarified the role of the Shaker voltage-gated potassium complex for impulsivity. Studies show that mutations in the Shaker voltage-gated potassium complex can affect behaviors like aggression, sleep, learning and memory (Chiara Cirelli, 2005; Daniel Bushey, 2007; Davis et al., 2018). Here we showed that mutation to Sh also leads to impulsivity (Figure 3.1 AB). Unpublished data from the lab showed that social environment plays a role in impulsivity. This study also finds a role for social environment in impulsivity. While we observe slight levels of impulsivity in the flies with the strongest alleles for *Sh* and *Hk*, when tested in a single-fly setting, they are not to the level observed in a group (Figure 3.1). These results suggest that the social environment enhances impulsivity.

In our efforts to investigate the interaction between the Sh and Hk subunits, we found that the Sh^{mns} is dominant and the Hk^{1} to be recessive for impulsivity. These results align with the "shaking" phenotype in Sh^{mns}, which is also dominant however, both sleep and learning phenotypes are recessive (Chiara Cirelli, 2005; Daniel Bushey, 2007). Our study found no interaction between the $Sh^{mns}/+$ and $Hk^{1}/+$ for impulsivity. One limitation of our Sh and Hk interaction study is that we did not test double homozygous interaction, which requires the generation of a recombinant fly. Future studies using a double knockdown of both Sh and Hk will address this limitation. Also, the interaction studies between the Sh-Qvr and Hk-Qvr are currently being conducted to investigate a possible interaction for impulsivity.

More importantly, when we investigated if Sh^{mns} or Hk^{1} interact with dopamine transporter mutant *fmn* we found that the double heterozygous mutant results in a synergistic

interaction, since the elevated PKA probably results in more phosphorylation of Sh channels thus elongating depolarization resulting in enhanced neurotransmission. However, the double homozygous interaction results suggest *Sh^mn^* to be epistatic to *fmn*. Additional studies need to be done to clarify the interaction between *Sh^mns^* and hyper-dopamine signaling. When we tested *Hk^1^* we found a possible additive interaction between *Hk^1^* and *fmn.* These results suggest that Hk might interact with an additional channel such as ether-a-go-go (Wilson, Wang, Chouinard, Griffith, & Ganetzky, 1998) and hyper dopamine for impulsivity. These results suggest that contrary to it role in sleep Hk might not have a major impact on Sh for impulsivity. To further confirm these result,s experiments using a PKC mutant, which has reduced PKA activity, are currently underway.

Our study also found the MB bodies to be the neural site where the Sh channel complex (Sh, Hk, and Qvr/Sss) plays a role in sleep and impulsivity. Our results show that Sh, Hk, and Qvr/Sss in all MB neurons are important for inhibitory control. While we see a significant increase of LIEs in all KD experiments, KD in α/β appears to show more than the others suggesting that either Sh, Hk, and Qvr/Sss play a more significant role in at α/β or the Gal4 driver we used might be causing more expression of RNAi. We also found the α/β lobe to be the neural site where Sh, Hk, and Qvr/Sss regulate sleep in drosophila. This becomes more relevant since a recent study found that KD of Sh and Hk in the dorsal fan-shaped, which is the main brain structure associated with sleep regulation, does not result in sleep loss (De et al., 2023). Altogether, these studies provide new insights into how Sh, Hk, and Qvr/Sss regulate sleep and impulsivity. Additional knockdown experiments are being planned to clarify these results.

The is very limited information on the voltage-gated potassium channels and their role in human disease. The Sh, homolog is the potassium voltage-gated channel subfamily A member 3 (KCNA3), winked to autoimmune diseases like multiple sclerosis (Lioudyno et al., 2021). It has also been shown that the reduced in expression of linked KCNA3 contributes to the lower neuronal number and brain size seen in Down Syndrome (Lu et al., 2012). In the

case of Hk homolog the potassium voltage-gated channel subfamily A regulatory beta subunit 1 (KCNAB1). Has been associated with synaptic facilitation in the hippocampus and it was found to be a susceptibility gene for lateral temporal epilepsy (Busolin et al., 2011; I. H. Cho et al., 2020). Lastly, like the deficit in learning and memory observed in flies, this is also seen in mice with mutated KCNAB1 which showed impaired hippocampal-dependent learning (Giese et al., 1998). The findings in this dissertation advance our understanding on the mechanism by which Sh affect behavior.

In our genetic screen study, we found a novel gene that interact with hyper dopamine for impulsivity. This gene is the *Kekkon5* (*Kek5*) which codes for a synaptic cell adhesion molecule. There is very limited information on the role of *Kek5*. Our studies show a novel function for this gene and serves as a starting point to uncovering the mechanism by which it interacts with dopamine signaling for impulsivity. Future studies in our lab will focus on investigating the neural site for *Kek5* for impulsivity. In addition to looking for additional interacting molecules that can for part of the network by which *Kek5* plays a role in impulsivity.

All the major findings in this dissertation work provide novel insight into the mechanisms by which genetic and nongenetic factor contribute to impulsivity. In addition, this finding may lead to the development of new preventive and therapeutic interventions for impulsivity related brain disorders.

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Vita

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Erick was also actively involved in extracurricular activities during his time at UTEP, serving as a representative for the Biological Sciences Graduate Student Assembly and helping found the Society for Neuroscience chapter at UTEP.

His doctoral research has been focused on investigating the role of genetic and non-genetic factors in impulsivity. Erick has demonstrated his proficiency in presenting his research multiple times at the Society of Neuroscience and mentoring other students and peers in laboratory and classroom settings.

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