

Alcohol-Induced Plasticity within Dopaminergic Memory Circuits in *Drosophila*

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B.S. in Biomedical Sciences, Central Michigan University, Mt. Pleasant, MI. 2018.

A dissertation submitted in partial fulfillment of the requirements for the degree of
- Doctor of Philosophy -
in the Department of Neuroscience from
Brown University

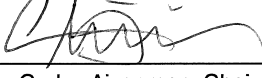
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This dissertation by Tariq M. Brown is accepted in its present form by
the Department of Neuroscience as satisfying
the dissertation requirement for the degree of Doctor of Philosophy.

Date 3/14/25 
Dr. Karla Kaun, Advisor

Recommended to the Graduate Council

Date 3/14/25 
Dr. Carlos Aizenman, Chair

Date 3/14/25 
Dr. Kate O'Connor-Giles, Reader

Date 3/14/25 
Dr. Leslie Griffith, External Reader

Approved by the Graduate Council

Date _____
Thomas A. Lewis, Ph.D., Dean of the Graduate School

CURRICULUM VITAE

EDUCATION

Brown University

Graduation: TBD

Degree: Doctorate of Philosophy

Program: Neuroscience

Central Michigan University – Honors College

Graduation: May 2018

Degree: Bachelor of Science

Major: Biomedical Sciences

SCIENTIFIC INTERESTS

I investigate the role of alternative splicing in memory circuits in response to alcohol exposure. Using *Drosophila melanogaster*, I aim to understand how the Dopamine D2 receptor's alternative splicing influences neural dynamics and animal behavior.

RESEARCH EXPERIENCE

Neurogenetics and Behavior Laboratory– Ph.D. Student August 2019-Present

Advisor: Karla Kaun, Ph.D.

Affiliation: Brown University

Investigating the role of alcohol-induced alternative splicing in memory formation in *Drosophila melanogaster*.

Neocortical Dynamics Laboratory– Rotation Student

May 2019-August 2019

Advisor: Chris Moore, Ph.D.

Affiliation: Brown University

Successfully implemented the use of a photoswitchable Bioluminescent-Optogenetic molecule *in vivo* in mouse model.

Neural Circuits Laboratory – Rotation Student

September 2018 – January 2019

Advisor: Carlos Aizenman, Ph.D.

Affiliation: Brown University

Looked at the impacts of valproic acid on visual circuitry development in *Xenopus laevis*

Biological-Tools Laboratory – Research Assistant

May 2018-August 2018

Advisor: Nathan Shaner, Ph.D.

Affiliation: Scintillon Institute, San Diego, CA

Characterized previously undiscovered fluorescent protein (FP) homologs from *Aequorea victoria* and a related *Aequorea* species. Took advantage of directed evolution and other molecular biological techniques to optimize bioluminescent and fluorescent proteins.

Bioluminescent Optogenetics Laboratory- Research Assistant

May 2015-May 2018

Advisor: Ute Hochgeschwender, M.D.

Department: CMU Neuroscience

Assisted Dr. Ute Hochgeschwender in ongoing research concerning bioluminescent optogenetics. Used cloning techniques and electrophysiological examination develop and analyze multiple optogenetic constructs termed “luminopsins.” These luminopsins are

inserted into neurons and are bimodally activated by addition of coelenterazine or via laser light stimulation to regulate cell activity. This novel technology allows for practical and easy interrogation of neuronal function.

PEER-REVIEWED PAPERS

Petrucelli, E., **Brown, T.**, Ledru, N., & Kaun, K. R. (2020). Alcohol causes lasting differential transcription in *Drosophila* mushroom body neurons. *Genetics*, <https://doi.org/10.1534/genetics.120.303101>

Zenchak, J. R., Palmateer, B., Dorka, N., **Brown, T. M.**, Wagner, L. M., Medendorp, W. E., ... Hochgeschwender, U. (2018). Bioluminescence-driven optogenetic activation of transplanted neural precursor cells improve motor deficits in a Parkinson's disease mouse model. *Journal of Neuroscience Research*, 10.1002/jnr.24237.

REVIEWS

Brown, T., Hernandez, J., Kaun, K. R. (2023) Reward Circuits in *Drosophila*. Oxford Encyclopedia of Neuroscience.

ORAL PRESENTATIONS

Brown T., Kaun K, Scaplen K, (2024, May) Neural Dynamics of Acquisition of Alcohol Memory in *Drosophila* Memory Circuits. Oral presentation at Albert Einstein's Einstein Dialogues in Graduate Education, Bronx, New York.

Brown T., Kaun K, (2023, May) Alternative Splicing of Dop2R in Memory Circuits. Oral presentation at International Behavior and Neural Genetics Society's Genes, Brain and Behavior 2023, Galway Ireland.

Brown T., Kaun K, (2021, May) Alternative Splicing in Memory Circuits. Oral presentation at International Behavior and Neural Genetics Society's Genes, Brain and Behavior 2021, Virtual.

Brown T., Kaun K, (2020, November) Alternative Splicing in Memory Circuits. Oral presentation at Brown University Neuroscience Department In-House, Providence, RI.

Brown T., Kaun K, (2020, July) Alternative Splicing in Memory Circuits. Oral presentation at Brown University NEUR 10 Course, Providence, RI.

Brown T., Kaun K, (2020, May) Alternative Splicing in Memory Circuits. Oral presentation at International Behaviour and Neurogenetics Society Meeting 2020, Woods Hole, MA.
(Invited)

Brown, T., Hochgeschwender, U, (2017, September). *Genetically Encoded Light Sources for Noninvasive Optogenetics*. Oral presentation at the Annual Ronald E. McNair Scholar Symposium, Mt. Pleasant, MI.

POSTER PRESENTATIONS

Brown T., Kaun, K. Scaplen K., Neural Dynamics of Intoxication in *Drosophila* Memory Circuits (2024, September). Poster presentation at Neurofly, Birmingham ,England.

Brown T., Kaun, K. Scaplen K., Neural Dynamics of Intoxication in *Drosophila* Memory Circuits (2024, September). Poster presentation at 54th Society for Neuroscience Annual Conference, Chicago ,IL.

Brown T., Hernandez J., Petruccelli E., Scaplen K., Gratz S., O'Connor-Giles, K., Kaun K., Alcohol-induced Alternative Splicing in *Drosophila* Memory Circuits (2023, June). Poster presentation at 4th Mushroom Body Meeting, Gottingen, Germany.

Brown T., Hernandez J., Petruccelli E., Scaplen K., Gratz S., O'Connor-Giles, K., Kaun K, Alcohol-induced Alternative Splicing in *Drosophila* Memory Circuits (2022, Nov). Poster presentation at 51st Society for Neuroscience Annual Conference, San Diego, CA.

Brown T., Kaun K, Alcohol-induced Alternative Splicing in *Drosophila* Memory Circuits (2022, May). Poster presentation at International Behaviour and Neurogenetics Society Meeting 2022, Memphis TN.

Brown T., Petruccelli E., Waterman A., Nuñez K., O'Connor-Giles K., Kaun K, (2021, November) Alternative Splicing in Memory Circuits. Poster presentation at 50th Society for Neuroscience Annual Conference, held virtually.

Brown T., Petruccelli E., Waterman A., Nuñez K., O'Connor-Giles K, Kaun K, Alternative Splicing in Memory Circuits (2021, Sept). Poster presentation at Brown University's Neuroscience Graduate Program Annual Retreat, Providence, RI.

Brown T., Petruccelli E., Waterman A., Nuñez K., O'Connor-Giles K, Kaun K, Alternative Splicing in Memory Circuits (2020, Sept) . Poster presentation at Brown University's Neuroscience Graduate Program Annual Retreat, Providence, RI.

Brown, T., Lambert, G., Pal, A., Prakash, M., Shaner, N., Hochgeschwender, U., (2018, April). *Genetically Encoded Light Sources for Noninvasive Optogenetics*. Poster presentation at Annual Capitol Scholars Exhibition in Lansing, Michigan.

Brown, T., Lambert, G., Pal, A., Prakash, M., Shaner, N., Hochgeschwender, U., (2018, April). *Genetically Encoded Light Sources for Noninvasive Optogenetics*. Poster presentation at Student Research and Creative Endeavors Exhibition in Mt. Pleasant, Michigan.

Brown, T., Lambert, G., Pal, A., Prakash, M., Shaner, N., Hochgeschwender, U., (2017, November). *Genetically Encoded Light Sources for Noninvasive Optogenetics*. Poster presentation at 47th Society for Neuroscience Annual Conference Scholars in Washington, DC.

Brown, T., Lambert, G., Pal, A., Prakash, M., Shaner, N., Hochgeschwender, U., (2017, October). *Genetically Encoded Light Sources for Noninvasive Optogenetics*. Poster presentation at Annual Biomedical Research Conference for minority Students Scholars Exhibition in Pheonix, Arizona.

Brown, T., Hochgeschwender, U., (2017, April). *Genetically Encoded Light Sources for Noninvasive Optogenetics*. Poster presentation at Student Research and Creative Endeavors Exhibition in Mt. Pleasant, Michigan.

Brown, T., Hochgeschwender, U., (2017, September). *Genetically Encoded Light Sources for Noninvasive Optogenetics*. Presentation at Emerging Researcher's National in Washington, DC.

PROFESSIONAL DEVELOPMENT

NIDA Virtual Early Career Workshop Fall 2022 – NIDA hosted workshop focused on providing young researchers with the knowledge and the tools necessary for becoming a successful grant writer. Held virtually, November 21st, 2022.

Cold Spring Harbor Laboratories *Drosophila* Neurobiology Course – 3-week course focused on various aspects of *Drosophila* neurobiology. Cold Spring Harbor, New York, July 1st – July 21st 2022.

Cellular Mechanisms of Brain-Body Interactions – 2-day Janelia-sponsored workshop focused on 4D Cellular Physiology and other novel techniques to look at intact tissues. February 25th-26th, 2021.

Applying and Winning Graduate STEM Fellowships – Initiative to Maximize Student Diversity’s workshop focused on improving applications for STEM fellowships. Held September 21st, 2020.

Carney Methods Meetups – Carney Institute workshop focused on calcium imaging data collection and analysis. Hosted by Drs. Alex Fleischmann & Christopher Deister. June 2, 2020.

NSGP Ethics & Skills Workshop – Research ethics workshop focused on the publication process. Hosted by Dr. Kate O’Connor Giles. Nov. 15, 2019.

TEACHING EXPERIENCE

NEUR10 – Guest lecture for introductory neuroscience course for incoming undergraduate students.

NEUR1650: Neuroanatomy – Teaching Assistant for class led by Dr. David Berson. Led lab sections including teaching students fluorescent microscopy, immunohistochemical analysis, and sheep brain dissections. Fall 2019.

Sheridan Center – Completed *Sheridan Center Teaching Seminar* certificate program offered through Brown University’s Sheridan Center to gain the skills necessary to be an effective teacher. Fall 2019.

HONORS & AWARDS

Ruth L. Kirschstein National Research Service Award (NRSA) – NRSA from the National Institute of Alcohol Abuse and Alcoholism. Fellowship will support research into alcohol-induced alternative splicing in memory circuits. Received February 2022.

Carney Graduate Award in Brain Science – Awarded to graduate students who have demonstrated scientific merit, productivity, contributions to diversity, and contributions to the Carney Institute community. Awarded August, 2021, for a year of fully funded research and \$3000 of travel funds.

Brown University Neuroscience Graduate Program Poster Award – Awarded by Brown University Faculty to student poster presenters during annual retreat. Provides \$300 of travel funds.

Central Michigan University's President Award - Awarded for outstanding research conducted at Central Michigan University. Presented by President George Ross with \$500 monetary award May, 2018.

ABRCMS Neuroscience Presentation Award & Travel Award - Received \$300 prize for Annual Biomedical Research Conference for Minority Students poster presentation. Awarded \$500 in travel funds based on academics, background, and various essays.

Centralis Scholar – Central Michigan University's most prestigious scholarship. Awards full tuition and housing expenses for four years. Awarded Spring, 2014.

McNair Scholar – Awarded a prestigious scholarship to participate in research over the summer of 2017. Conducted research at Central Michigan University under Dr. Ute Hochgeschwender studying noninvasive optogenetics. Received training in diversity, graduate school preparation, and team building.

Benjamin A. Gilman Scholar - The U.S. Department of State's grant program that enables students of limited financial means to study or intern abroad, in order to gain skills critical to our national security and economic competitiveness. Awarded \$3000 Spring 2016.

Freeman-Asia Scholar – Grant providing students with limited financial means to participate in study abroad in eastern Asia. Awarded \$3000 Spring 2016.

Dean's List – Fall 2014, Spring 2015, Fall 2015, Spring 2016, Fall 2016, Spring 2017

RELATED COURSEWORK

Cellular and Molecular Neuroscience II <i>Professor:</i> <i>Dr. Gilad Barnea</i>	Jan. 2019-May 2019
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Statistics using Python <i>Professor: Dr. David Sheinberg</i>	Jan. 2019-May 2019
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Systems Neuroscience <i>Professor: Dr. Theresa Desrochers</i>	Sept. 2018-Dec. 2018
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Cellular and Molecular Neuroscience <i>Professor: Dr. Anne Hart</i>	Sept. 2018-Dec. 2018
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ORGANIZATIONS AND COMMUNITY ENGAGEMENTS

Brain Wave Rhode Island	Feb. 2024
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Talked to Rhode Island undergraduates and early-career professionals about paths to graduate school and provided resources for engaging the Rhode Island community concerning neuroscience.

PREP Peer Mentor	Sep. 2021 – May 2023
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Mentored students participating in the National Institutes of Health-funded Postbaccalaureate Research Education Program. Provided assistance with graduate school applications, as well as research and career advice.

Brown University Neuroscience Department DIAP Student Rep. June 2020 – August 2021 Elected by the department to address equity and inclusion issues facing the department.

Frontier’s School Division Science Week Aide April 2020
Gave a virtual lab tour to middle school and elementary students from the Frontier’s School District in Winnipeg, MB.

Graduate Student Council Social Chair Jan. 2020 – Jan. 2021
Elected by the council to organize GSC social events such as formals and game nights open to the graduate student population. Also serving as liaison for graduate students to Brown administration.

Brain Week Rhode Island Organizer Sept. 2019 - March 2020
Cure Alliance sponsored organization dedicated to raising awareness about the brain and disorders that affect the brain in Rhode Island.

Brown University Aikido President April 2019 – Aug. 2020
Graduate Student President of the Brown University Aikido Club. Organize practice and seminars dedicated to the martial art of peace.

Graduate Student Council Finance Committee Sept. 2019 – Jan. 2021
Member of the GSC committee responsible for allocating funding to graduate student events, organizations and other affairs.

Brown University Neuroscience Department Social Chair May 2019 – June 2020
Elected by the department to organize department-wide social events.

Brown University Neuroscience Department Representative May 2019 - May 2020
Elected by the Neuroscience department to represent the department at Graduate Student Council meetings.

Brown University Conversation Partner Sept. 2018 – March 2020
Helping visiting scholars advance their English.

Samuel M. Nabrit Black Graduate Student Association. Sept. 2018-Present
Student organization committed to the personal, professional, and academic enrichment of the graduate student community at Brown University.

MENTORSHIP EXPERIENCES

PAST MENTEES

Raffee Wright

Aug. 2020 - May 2021

Currently a Ph.D. candidate at University of Edenborough.

Rohan Freedman

Sept. 2021-May 2023

Currently a research assistant at Brown University School of Public Health.

Imaad Said

Sept. 2022-May 2024

Enrolling at University of Madison Wisconsin as a medical student.

CURRENT MENTEES

Brian Lee

July 2023-May 2024

Currently completing undergraduate studies at Brown University.

PROFESSIONAL MEMBERSHIPS

Society for Neuroscience

November 2017 - Present

International Behavioural And Neural Genetics Society

March 2020 – Present

Genetics Society of America

April 2020 – Present

PERSONAL INTERESTS

I believe a strong community is necessary for meaningful progress. As such, I have always been a very active community member, doing my best to aid those who need it the most. I have a long history of volunteering at community centers and assisting at mutual aids. I am also a strong proponent for diversity, equity, and inclusion, so I am constantly working to achieve better outcomes for those from disadvantaged backgrounds, especially in STEM. On my own time, I enjoy cooking, learning languages, trivia, and learning martial arts such as Sanshou Kickboxing, Brazilian Jiu-Jitsu, and Krav Maga.

PREFACE

*“Conquer all mysteries by rule and line,
Empty the haunted air, and gnomed mine –
Unweave a rainbow,”* –John Keats, *Lamia*, 1820

The flexibility of circuits is integral for learning and adaptive behavior. However, there are stimuli like substances of abuse that are able to corrupt these circuits and increase their rigidity, generating repetitive behavioral loops. These same stimuli are also able to invade cellular machinery and bias the assembly of genetic products to further solidify maladaptive behavior cycles. The mechanisms underlying how substances of abuse co-opt neural circuitry to generate long lasting, and inflexible memories and behavioral patterns is still poorly understood.

This dissertation addresses the impact of alcohol on genetic, circuit, and behavioral levels to better characterize the impacts of alcohol on reward response. In chapter 1, I provide background for *Drosophila* reward circuitry and set the stage for the work of the next two chapters. In chapter 2, I look at the plasticity that occurs during the association of a cue with intoxicating doses of ethanol within dopaminergic circuits. In chapter 3, I explore the connection between alcohol and the coopting of cellular machinery to generate differential alternative splicing of memory relevant genes and tie those changes to behavior. In chapter 4, I discuss how this work contributes to our knowledge of alcohol memory formation and the contributions of alternative splicing to behavior.

Acknowledgements

I would first like to thank my mentor, Dr. Karla Kaun. It has been a wonder 6 years under your guidance. I look at the difference between the scientist I was then, and the scientist I am now, and the difference is tremendous. The way I conduct my science, think about experiments, convey my contributions to neuroscience whether that be through written or visual means are all skills I was able to carefully hone with your mentorship. I am constantly floored by your ingenuity and intuition. Your “opportunity vigilance” is unparalleled, and I appreciate you always thinking of opportunities for my professional development. I hope to one day to have the scientific vision you have and seamlessly weave together complex ideas into a cohesive research direction.

I’d next like to thank my committee members Kate O’Connor-Giles and Carlos Aizenman. You two have provided so much mentorship and support during my entire PhD career. I have always felt comfortable going to either of you for questions regarding science or career advice. I hope to continue to rely on the both of you as I move to the next stage of my career.

I would like to thank the entire Kaun lab both past and present, with a special thanks to J Amanda Waterman, John Hernandez, Kristin Scaplen, Kavin Nuñez, Lewis Sherer, Rebecca Orasmas, Nelson Le, Imaad Said, Rohan Freedman, & Raffee Wright. All of you have had such a major impact on my scientific career, in addition to being such great mentors, colleagues and friends.

I would like to thank Reza Azanchi, if it wasn’t for your infinite wisdom and supportive presence in lab, I don’t think I would have made it nearly as far as I have. I truly

appreciate your commitment to fostering a positive work environment, and willingness to solve problems big and small.

I would like to thank my cohort, and the greater Neuroscience community at Brown. You all have been extremely important from the very moment I stepped on Brown's campus.

I would like to thank Adrian Thompson, Jess Sevetson, Rajan Thakur, and Amanda and Scott Gratz. You all have been tremendous influences on my scientific journey, and I appreciate your continued support throughout the years.

I would also thank Valerie Estela, and the many others who advocated for me to be here after my initial rejection from Brown University. Your voices have forever changed my life for the better. On that same note, I would like to thank Guillaume Pagnier for ensuring I had wonderful interview experience at Brown and showing me the all the best parts of Brown (the people).

I would also like to thank Carol Viveiros, Rosanna Cabral, Jennifer Blackwell, Courtney Hamann, Patricia Manceiri, Kathleen Gerlock, and the many other members of the administration aspect of the department that keep the department running.

Next, I would first like to thank my parents and their undying support. They are the reason I strive to accomplish all my goals. My mom is the reason I have the passions I do, getting me interested in medicine, and by extension, general science. My mom also developed and nourished my love for cooking (It was between the culinary arts and cooking when I entered college and neuroscience won by a hair) I always thought she had superpowers when she would work full-time as a respiratory therapist and still find

time to cater on the side. And through the years I am even more convinced of her superpowers, being one of the strongest individuals I know. My dad, whose philosophical insights are guiding beacons within my life. I continually strive to emulate your intellect, creativity, and undying love for community. I would be nothing without your early insights into metacognition, religion, and music (Rest in Power, Prince!). I truly hope that I can make both of you proud.

Next, I would like to thank my siblings, Marcus, Alex, Andres, Maya, Franky, and Breanna. I have always said that my personality is an amalgamation of all your peculiarities, for better or worse. I know I can always rely on you all for anything, regardless of the circumstances. Whenever my future is uncertain, I know that it will be happy one regardless, because I have you all in my life. The bond we share is eternal, and I cannot thank you enough to be the souls I feel most connected to on this earth.

I would also like to thank my grandparents Iretha, Dewey, John, and Brenda. I love all of you from the bottom of my heart, and your love has kept me going through all of the years.

I would also like to thank Jim Fingleton, Jennfier Gass, Anne Gass, Sam Fingleton and the greater Fingleton family. From the first time I met all of you, I already felt like I was a part of the family. Thank you for support.

I would like to thank all my friends throughout the years. Naming each one of you would be longer than my thesis, but you know who you are. Enough said. But seriously, I appreciate all the wonderful times we have had together, I wouldn't have maintained my sanity without you all bringing a smile to my face day-in and day-out.

And finally, I would like to thank my beautiful partner, Erin Fingleton. Your infinite kindness, empathy and understanding constant reminder of how I want to be when I interact with the world. Your intelligence is boundless, it seems like you have thought deeply about so many topics and can hold a conversation about practically anything. It makes me wonder why you aren't a world leader. I would love to be a first gentleman one day. Thank you for putting up with the most chaotic side of me that never rears its head in public.

My journey has been an extremely fortunate one. I am forever indebted to the multitude of people who have helped propel me to my current position. They say it takes a village, but I'm pretty sure it was decent-sized metropolis that has gotten Tariq Brown to be Tariq Brown. And to all the inhabitants of that metropolis, and I hope I can bring a smile to each one of your faces.

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CHAPTER 1
Reward Circuits in Drosophila

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1.1 ABSTRACT

The ability to sense and respond to a rewarding stimulus is a key advantage for animals in their natural environment. The circuits that mediate these responses are complex, and it has been difficult to identify the fundamental principles of reward structure and function. However, the well-characterized brain anatomy and sophisticated neurogenetic tools in *Drosophila melanogaster* make the fly an ideal model to understand the mechanisms through which reward is encoded. *Drosophila* find food, water, intoxicating substances, and social acts rewarding. Basic monoaminergic neurotransmitters, including dopamine (DA), serotonin (5-HT), and octopamine (OA), play a central role in encoding these rewards. DA is central to sensing, encoding, responding, and predicting reward, whereas 5-HT and OA carry information about the environment that influences DA circuit activity. In contrast, slower-acting neuromodulators such as hormones and neuropeptides play a key role in both encoding the pleasurable stimulus and modulating how the internal environment of the fly influences reward sensation and seeking. Recurring circuit motifs for reward signaling identified in *Drosophila* suggest that these key principles will help elucidate understanding of how reward circuits function in all animals.

1.2 INTRODUCTION

1.2.1 - Introduction

Appetitive stimuli induce positive sensations that motivate animals to perform tasks, and these sensations are termed as “rewarding.” These experiences are fundamental to an animal’s ability to obtain and assess food and water, reproduce, and ensure fitness of its progeny. Perhaps unsurprisingly, reward circuits are strikingly similar in anatomy and function across species from flies to primates (Dvořáček & Kodrík, 2021; Landayan & Wolf, 2015; Scaplen & Kaun, 2016). It has traditionally been difficult to understand the structure and function of reward circuits at the level of individual cells. However, the relative simplicity, detailed anatomy, and genetic accessibility of the *Drosophila melanogaster* brain make the fly a powerful model to understand how reward circuits function (Honda, 2022; Hulse et al., 2021; Li et al., 2020; Scheffer et al., 2020; Takemura et al., 2017; Venken et al., 2011). The ability to investigate the structure and function of circuits in both larval and adult stages also provides a cross-developmental opportunity to extract general principles underlying the function of reward circuits

In *Drosophila*, rewards include macronutrients like sugar, fat, protein, and water; social rewards like the act of copulation; and intoxicating substances such as alcohol (Dus et al., 2011; Fougéron et al., 2011; Kaun et al., 2011; Lin et al., 2014; Shohat-Ophir et al., 2012; Tempel et al., 1983; Toshima & Tanimura, 2012). These rewards and their cues are perceived through sensory structures in the *Drosophila* brain such as the optic lobe (vision), subesophageal zone (taste), antennal lobe (smell), and the antennal

mechanosensory motor center (audition). Sensory information is then sent through divergent and convergent connections to central brain structures to dictate behavior. Artificial activation of reward-associated regions, including primary sensory brain regions and their direct outputs, can be inherently rewarding to *Drosophila* (Lyutova et al., 2019; Nuwal et al., 2012; Schneider et al., 2012; Seidenbecher et al., 2020; Thoener et al., 2022; Wen et al., 2005). For example, *Drosophila* will optogenetically self-stimulate sugarsensing neurons (Deere & Devineni, 2022). Because reward behavior manifests in a variety of ways, a wide variety of reward contexts are discussed. Internal state is intrinsically linked to reward response, as it changes the motivational response for reward. For example, thirsty flies find water rewarding, and hungry flies find food rewarding. This makes it difficult to parse out circuits that are responsible for inducing a particular internal state and encoding reward. Here, *Drosophila* circuits that directly encode reward behavior are reviewed within the appropriate context in which reward is perceived. Neural circuits and neurotransmitters underlying various rewards often overlap and share similar structural circuit motifs. The circuit function, however, may differ according to the internal state, reward stimulus, environmental context, and the appropriate output response. This review focuses on circuits that reside downstream of the primary sensory pathways and elicit a behavioral response in which a fly purposefully and/or repeatedly engages with a stimulus response.

1.3 – Rewarding Stimuli

Most reward circuits in *Drosophila* have been characterized in the context of food reward. Food consumption behavior is broken down into three components: initiation,

ingestion, and cessation. Food consumption initiation involves sensing food odors and nutrients within food (Aryal et al., 2022; Liman et al., 2014), whereas metabolic nutrient sensing occurs during the ingestion process (Yapici et al., 2016). Food consumption cessation occurs once an animal experiences satiety (Huetteroth et al., 2015).

Experiencing both the taste and metabolic properties of food is perceived as rewarding to flies (Burke & Waddell, 2011; Huetteroth et al., 2015; May et al., 2020; Yamagata et al., 2015). *Drosophila* food typically contains a mixture of macronutrients, including carbohydrates, protein, and fats, and micronutrients, including vitamins and minerals. *Drosophila* consume mixtures of carbohydrates like sucrose, fructose, and glucose in rotting fruits (Burke & Waddell, 2011; Lebreton et al., 2014; Lüersen et al., 2019), as well as proteins in the yeasts that grow on fermenting fruit (Becher et al., 2012).

Nutritive sugars are sensed through two distinct mechanisms: (a) taste sensing via peripheral taste receptor cells that detect the palatability of sugar and (b) and taste-independent behavioral responses to the nutritional value of sugar (Clyne et al., 2000; Dus et al., 2011; Thorne et al., 2004; Wang et al., 2013). Sucrose is the most extensively studied reward in *Drosophila*, resulting in mechanistic knowledge of how circuits function during sugar reward encoding, how this alters sugar ingestion, and how olfactory cues associated with sugar help the fly navigate to a source of nutritious sugar. Less is understood about the rewarding properties of proteins, although a fly's ability to sense and consume appropriate proteins directly contributes to their survival and fitness (Liman et al., 2014), and glucose consumption alters protein consumption (Lebreton et al., 2014). For this review, circuits for mixed media and individual macronutrients overlap in the context of food reward. Water, essential for maintaining proper osmolarity

of cells within living organisms, is rewarding to thirsty flies. In order to seek water, flies detect humidity gradients using humidity-sensing and dry-sensing receptors (Enjin et al., 2016; Ji & Zhu, 2015). Thirsty *Drosophila* are able to seek out sources of humidity through hygrosensation (Sayeed & Benzer, 1996), and upon encountering water, thirsty flies will extend their proboscis and ingest water rapidly using their pharynx (Enjin et al., 2016). Because thirsty flies find water rewarding, they actively seek out cues previously associated with water reward (Lin et al., 2014). Intoxicating rewards such as alcohol induce an alternative internal state that is perceived as rewarding to *Drosophila*. Moderate doses of ethanol increase motor activity, whereas high concentrations of ethanol reduce postural control and induce sedation (Corl et al., 2005; Dzitoyeva et al., 2003; Moore et al., 1998; Rothenfluh et al., 2006; Wolf et al., 2002). Ethanol consumption evaluated by measuring proboscis extension (Scheiner et al., 2014), fluorometry (Peru Y Colón de Portugal et al., 2014), oligonucleotide tagging (A. Park et al., 2018), or capillary feeding demonstrates *Drosophila* preferentially consume alcohol (Adel & Griffith, 2021; Devineni & Heberlein, 2009; Ja et al., 2007). Flies remember pharmacologically stimulating doses of ethanol as rewarding and will walk over a 120-V electric shock to attain a cue previously associated with ethanol (Kaun et al., 2011). Social behavior is also rewarding to flies in the context of courtship and copulation. In order for sexual engagement to occur in *Drosophila*, adult males court adult females in an intricate series of behaviors that involve auditory and visual sensory cues demonstrated to either a receptive or a dismissive female (Pavlou & Goodwin, 2013). If courtship is successful, the pair will copulate, which is the rewarding outcome of courtship behavior (Shohat-Ophir et al., 2012; Zer-Krispil et al., 2018). In contrast,

repetitively suppressing copulation in male flies results in alternative reward seeking (Shohat-Ophir et al., 2012). These rewards are all encoded within layered neuropeptidergic and monoaminergic signaling within central brain structures, and these circuits function distinctly and cohesively to promote reward behavior, depending on context.

1.4 Neurotransmitters

1.4.1 – Dopamine

Dopamine (DA) signaling is central to reward processing in *Drosophila* as DA neurons are activated by hunger, encode valence, and are required for cue-induced appetitive memories (Y.-C. Kim et al., 2007; W.-P. Lee et al., 2021; Pitman et al., 2011; Figure 1A). There are approximately 280 DA neurons in the *Drosophila* central brain (Budnik & White, 1987; Mao & Davis, 2009; Nässel & Elekes, 1992), half of which innervate the mushroom body (MB), a well-studied structure important for learning and memory (Mao & Davis, 2009; Nässel & Elekes, 1992). Two main types of DA neurons innervate the MB: 12 protocerebral posterior medial lateral (PPL1) neurons innervate the vertical (α , α' , γ_1 and γ_2) lobes, and ~100 protocerebral anterior medial (PAM) neurons innervate the horizontal (β , β' , γ and α_1) lobes. Together, these DA neurons innervate the parallel axons of intrinsic MB neurons, called Kenyon cells (KCs), and the dendrites of cholinergic, glutamatergic, or γ -aminobutyric acid (GABAergic) mushroom body output neurons (MBONs), which divide the MB axons into distinct compartments (Figure 1B; Aso, Hattori, et al., 2014; Stahl et al., 2022). DA neurons provide information about reward to both the KCs and MBONs to bias the fly toward engaging in behavior that leads to a rewarding outcome (Aso, Hattori, et al., 2014; Aso & Rubin, 2016; Aso,

Sitaraman, et al., 2014; Huetteroth et al., 2015; Ichinose et al., 2021; Yamagata et al., 2015). The mechanisms by which DA neurons encode reward within the MB depends on the type and timing of reward, the internal state of the fly, and which behavior is being executed to attain the reward. Accumulating data suggest that the two main DA neuron types that innervate the MB provide information about opposing valences, where PAMs largely convey information about reward. PAM neurons that innervate the $\alpha 1$, $\gamma 4$, $\gamma 5$, and $\beta'2$ compartments of the MB are activated by water, sucrose, and alcohol (Cohn et al., 2015; Li et al., 2020; C. Liu et al., 2012; Otto et al., 2020; Scaplen et al., 2020; Yamagata et al., 2015; Figure 1B). This compartmentalization makes it possible for the flies to encode multiple forms of reward memories such as the short-term memory for sugar taste simultaneously with the long-term memory for sugar calories (Burke & Waddell, 2011; Chouhan & Sehgal, 2022; Huetteroth et al., 2015; Pardo-Garcia et al., 2023; Yamagata et al., 2015; Y. Zhang et al., 2015). The number of compartments involved appears to scale with the intensity of the reward. For example, alcohol indiscriminately activates all PAMs (Scaplen et al., 2020), whereas sucrose activates select compartments (Harris et al., 2015; C. Liu et al., 2012). Some MB compartments encode multiple reward types, whereas others assess value or are specific to long-lasting cue-induced memories of reward (Huetteroth et al., 2015; Lin et al., 2014; Scaplen et al., 2020; Yamagata et al., 2015). An example of this is nicely highlighted with the $\beta'2$ compartment, which plays a key role in food reward but is also implicated in alcohol and water reward. $\beta'2$ DA neuron activity controls feeding rate for sucrose and subsequent satiation (May et al., 2020), whereas signaling from the neighboring $\gamma 5$ PAM neurons is required for positive reinforcement of the nutritious quality of food/sucrose

(Huetteroth et al., 2015; Yamagata et al., 2015). These neurons both innervate MBON- $\gamma 5\beta'2a$, and optogenetic activation of this MBON is sufficient to induce sucrose memory formation (Otto et al., 2020). This process occurs through the interplay of neurons that encode both aversive and appetitive responses since the aversive GABAergic $\gamma 1pedc$ -MBON induces an appetitive response through feedforward inhibition to the $\gamma 5\beta'2a$ and $\beta'2mp$ MBONs (Owald et al., 2015; Perisse et al., 2016). Plasticity within this compartment is dependent on the previous food experience of the fly. Flies maintained on a high-sucrose diet show reduced activity of PAM- $\beta'2$ DA neurons in response to sugar (May et al., 2020). This increases activity of $\gamma 5\beta'2a$ and $\beta'2mp$ MBONs in response to the odor paired with sucrose, causing an inability to form an associative sucrose memory (PardoGarcia et al., 2023). Subsequent activation of these PAM- $\beta'2$ DA neurons reduces overeating in flies on high-sucrose diets (May et al., 2020). This suggests that $\beta'2$ DA neurons encode the appetitive properties of sugar and receive information about satiety to control sugar seeking and feeding rate. The $\beta'2$ MB compartment also plays a key role in alcohol response, which elicits a unique internal state. Activity in the $\beta'2a$ PAM DA neurons and the corresponding compartmental MBON- $\beta 2\beta'2a$ neurons is required for cue-induced alcohol seeking (Scaplen et al., 2020). In contrast, inhibiting in the neighboring MBON- $\beta'2mp$ overnight during memory consolidation enhances alcohol memory, a response dependent on the inhibitory dopamine 2-like receptor (Dop2R; Scaplen et al., 2020). $\beta'2a$ PAM DA neurons likely inhibit MBON- $\beta'2mp$ during sleep to promote memory consolidation, and that activity of the $\beta'2a$ compartment during cue presentation is required to express reward memory. This suggests that alcohol acts on a natural reward memory circuit but alters dynamics

within that circuit to induce long-lasting memories for alcohol reward. Internal state significantly influences DA-dependent reward encoding in the MB. Hunger enhances activity of PAM DA neurons projecting to the $\alpha 1$, $\beta 1$, $\beta 2$, and $\gamma 4$ MB compartments in response to an odor (Siju et al., 2020) and recruits involvement of the PAM DA neurons in food occupancy (Landayan et al., 2018). Hunger also promotes yeast food seeking by recruiting the $\gamma 1 \text{pedc} > \alpha \beta$ MBON, which inhibits the $\beta 2$ MBON and $\alpha 1$ MBON (Tsao et al., 2018), and the protocerebral posterior medial (PPM) DA neurons in protein seeking in adult females (Q. Liu et al., 2017). Likewise, DA input into the $\alpha 1$, $\beta 1$, $\beta 2$, and $\gamma 5$ MB compartments inhibits the proboscis extension reflex to sucrose reward in hungry flies (Chia & Scott, 2020). Together, this suggests that hunger may act through DA MB circuits to both enhance reward perception and amplify behavioral responses to reward. PAM DA neurons also play a key role in water reward response. The $\beta 1$, $\beta 2$, and $\gamma 4$ DA PAMs are all active in response to water consumption in both water-sated and water-deprived flies, with $\beta 1$ having the most robust response in thirsty flies and $\beta 2$ having a consistently high response regardless of state (Lin et al., 2014; Shyu et al., 2017). $\gamma 4$ DA neurons are necessary for water memory following associations between an olfactory cue and a water reward (Lin et al., 2014; Shyu et al., 2017). Inhibition of signaling from the MBON- $\gamma 3 \beta 1$ GABAergic neuron does not directly affect water consumption but disrupts water long-term memory (W.-P. Lee et al., 2021). Flies lacking dopamine 1-like receptors (Dop1R1) also have decreased water consumption and decreased proboscis extension reflex to water reward (Lau et al., 2017). This demonstrates that water reward, like food or alcohol reward, is largely encoded through DA neurons that innervate several adjacent horizontal MB compartments that act in

concert to elicit an appropriate behavioral response. The vertical MB compartments also play a key role in memory for sugar, water, and alcohol reward. Interestingly, these compartments are more associated with 24-hour memory as opposed to immediate or 3-hour memory. The $\alpha 1$ PAM DA neuron is activated upon sucrose exposure and provides stimulatory input via the $\alpha 1$ KCs and the $\alpha 1$ MBON (Ichinose et al., 2015; Yamagata et al., 2015). During appetitive memory expression, the $\alpha 1$ MBON forms a feed-forward loop with $\alpha 1$ PAM DA neurons (Ichinose et al., 2015). Silencing the $\gamma 1$ pedc MBON and PPL1 $\gamma 1$ pedc DA neurons in hungry flies suppresses expression of appetitive memory formation. In contrast, satiated flies require activity of $\gamma 2a'1$ MBON and the PPL1- $\gamma 2a'1$ DA neurons for appetitive memory expression (Chouhan et al., 2021). Activity of the $\alpha 2\alpha'2$ PPL1 DA neurons and $\alpha'2$ MBONs is required for odor cue-induced alcohol seeking 24 hours after formation of alcohol–odor memories (Scaplen et al., 2020). Finally, the cholinergic $\alpha 3$ MBONs are required for odor-induced reward seeking 24 hours after an odor–sugar association (Ichinose et al., 2015; Plaçais & Preat, 2013). Similarly, inhibition of signaling from cholinergic MBON- $\alpha'2$, or MBON- $\alpha'1$, MBON- $\alpha'3m$, and MBON- $\alpha 3ap$, does not alter water consumption directly but does disrupt preference for a water-paired odor during the first 4-hour period after training (W.-P. Lee et al., 2021). Although it is clear there are many connections between the horizontal and vertical MB compartments (Li et al., 2020; Scheffer et al., 2020), how these compartments interact in the context of reward is largely unknown. Thus, it appears that a complex interplay of DA-MBON compartments along KC axons within the MB encodes information about reward, memory, and internal state and is required for appropriate behavioral responses. Although the mechanisms through which multiple

rewards are encoded within PAM MB compartments seem complex, the relative simplicity of its anatomy allows for extraction of key principles of reward encoding. Study of the β^2 MB compartment in particular demonstrates that (a) multiple rewards activate this compartment through DA neurons, (b) odor cues encoded within KCs alter activity of the glutamatergic MBON as a consequence of concurrent DA activation, (c) activation of the glutamatergic MBON elicits learned reward seeking, (d) reward memory encoding within this compartment is dependent on activity of interconnected MB compartments, and (e) both internal state and previous experience alter dynamics within this compartment. Other compartments appear to be dependent on the type or timing of reward, or they are recruited as a response to a shift in internal state. DA neurons outside the MB also contribute to reward response. Activity of DA neurons projecting to the fan-shaped body is required for ethanol consumption preference (Shamsideen et al., 2019). DA neurons that project to the ellipsoid body, another central complex structure, and their postsynaptic GABAergic R2/R4 ring ellipsoid body neurons contribute to both ethanol-induced hyperactivity and tolerance (Kong et al., 2010; Scaplen et al., 2019; Urizar et al., 2007), which are behavioral components of alcohol reward memory formation (Berger et al., 2008; Nunez et al., 2018). Interestingly, these circuits overlap with those required for the ethologically advantageous ethanol oviposition preference (Azanchi et al., 2013). Mating is also rewarding to male flies (Shohat-Ophir et al., 2012; Zer-Krispil et al., 2018), and DA signaling is required for sex drive in both male and female *Drosophila*. Depleting DA via tyrosine hydroxylase inhibitor 3-iodo-tyrosine reduces the likelihood to copulate and increases the time to copulation in females (Neckameyer, 1998). Activation of protocerebral anterior lateral,

protocerebral posterior lateral (PPL) 2ab and 2c, PPM 2, and 3 DA neurons drives courtship behavior and increases duration of copulation in male flies (Zhang et al., 2016). These DA neurons synapse on downstream circuitry acting through dopamine 1-like receptor 2 (Dop1R2) expressing P1 interneurons that directly contribute to male sex drive (Kimura et al., 2008; Zhang et al., 2016). Activated P1 neurons will generate courtship behavior in males and, upon successful courting, result in copulation (Kimura et al., 2008). The role of DA receptors in appetitive response is less well understood, in part because cell type– specific manipulation of these receptors in the context of reward behavior is a fairly recent approach. Ablation of Dop1R1 leads to deficits in odor–sugar memory in adults but does not affect sucrose consumption (Y.-C. Kim et al., 2007), suggesting these behaviors are mediated by different DA receptors. More recent evidence suggests that Dop1R1 can act in parallel to Dop1R2 in the formation and updating of memories, as well as shaping behavioral responses (Cohn et al., 2015; Handler et al., 2019). Dop2R plays a key role in increased sucrose sensitivity and consumption in *Drosophila* raised on diets lacking sugar (Ganguly et al., 2021). As a general rule, DA signaling plays a role in arousal, learning, and memory, as well as valence encoding, for a wide-variety of reward modalities. Some DA neurons encode multiple types of reward, whereas others are reward specific, and the amount of DA neuron involvement scales with reward intensity. Modularity of DA circuits within the MB plays a pivotal role in encoding different types of reward and eliciting appropriate behavioral responses. Finally, behavioral responses are shaped by the excitatory and inhibitory properties of DA and how this is altered by experience, internal state, and environmental context.

1.4.2 – Octopamine

In parallel with dopamine (DA) signaling, acquisition of the olfactory cues associated with sucrose consumption also requires octopamine (OA) signaling (Figure 1C). Mutations in tyramine- β -hydroxylase (T β h), the enzyme required for OA synthesis, or the OA receptor in mushroom bodies (OAMBs) reduces the ability of flies to form a positive odor–sucrose association (Y.-C. Kim et al., 2013; Schwaerzel et al., 2003) and decreases ethanol preference (Scheiner et al., 2014). Moreover, *Drosophila* will optogenetically self-stimulate OA neurons (Schneider et al., 2012). These OA neurons simultaneously act on Kenyon cells (KCs) and protocerebral anterior medial DA neurons through OAMBs in KCs and OA receptor Octopamine Beta-2 Receptor (OctB2R) in γ 1pedc mushroom body (MB) DA neurons to signal positive reinforcement for sucrose consumption (Burke et al., 2012). OA also plays a key role in how the internal state influences reward response. For example, hunger significantly suppresses the activity of OA neurons (LeDue et al., 2016). In addition, food and sex-deprived flies will choose to participate in feeding behavior over courtship behavior, and this behavior is caused by a decrease in excitatory tyramine, a metabolite of OA, acting on P1 neurons to activate courtship and decreased inhibitory tyramine acting on Posterior Lateral Protocerebrum (PLP) neurons that activate feeding behavior (Cheriyamkunnel et al., 2021). Positive reinforcement from OA-releasing neurons is necessary and sufficient for ethanol attraction, with a very specific subset of OA neurons being required for manifestation of ethanol preference (i.e., G3a/AL2 and VMI–VMIII; Schneider et al., 2012). When *Drosophila* lack the rate-limiting enzyme for producing OA, they are unable to form ethanol preference, but this lack of ethanol preference is reversed if flies are prefed

ethanol food at least 3.5 hours prior to testing (Claßen & Scholz, 2018). Together, these data suggest OA is required and necessary for innate ethanol attraction, but ethanol exposure itself could alter non-OA circuits to induce ethanol preference. As a general rule, OA seems to play a role in general state setting that contributes to the direction in which other neurotransmitter systems influence reward behavior.

1.4.3 – Serotonin

Serotonin (5-HT) also appears to play a key role in reward in the *Drosophila* brain (Figure 1D). Although evidence suggesting 5-HT directly encodes a reward response is lacking, current evidence points to its role in mediating how the internal state influences reward. Activation of a subset of 5-HT neurons induces starvation-like feeding in sated flies, suggesting that activation of these neurons elicits a hunger response, thereby increasing motivational response for sucrose (Albin et al., 2015). Similarly, serotonergic ellipsoid body R4 neurons increase food consumption when artificially activated (J.-Y. Park et al., 2016). In the subesophageal zone (SEZ), 5-HT neurons that encode stimuli with opposing valences dictate balanced sucrose consumption through feedback signals from enteric neurons (Yao & Scott, 2022). Two different populations of 5-HT neurons are required for sucrose memory: neurons that project outside of the mushroom body (Albin et al., 2015) and the Dorsal Paired Medial (DPM) neurons that innervate Kenyon cell axons (Krashes & Waddell, 2008; Sitaraman et al., 2012). The DPM neurons also promote sleep (Haynes et al., 2015), which is required to learn an appetitively reinforced operant task in *Drosophila* (Wiggin et al., 2021). Finally, pharmacologically increasing 5-HT signaling decreases ethanol odor attraction, whereas prolonged signaling from the 5-HT neurons provides greater attraction to

higher concentrations of ethanol (Xu et al., 2016). This may be because restriction of the 5-HT transporters to different neuronal compartments differentially alters ethanol attraction (Kasture et al., 2019). Thus, 5-HT generally functions to modulate sucrose behavior and ethanol response, which could reflect its role in both natural and drug reward encoding. However, most evidence suggests that like OA, 5-HT modulates state setting, which contributes to how other neurotransmitter systems influence reward behavior.

1.4 – Neuropeptides and Hormones

Similar to mammals, stress hormones and peptides modulate reward behavior in *Drosophila* (Figure 1E). *Drosophila* will optogenetically self-activate neurons expressing neuropeptide F (NPF), the *Drosophila* ortholog to neuropeptide Y (Shao et al., 2017; Wen et al., 2005). Activation of NPF neurons enhances preference for an odor associated with sucrose (Krashes et al., 2009), whereas knockdown of NPF receptors in shock-encoding dopamine (DA) neurons reduces preference for an odor associated with sucrose (Aso, Hattori, et al., 2014; Aso et al., 2012; Krashes et al., 2009). NPF signaling is also critical for the increase in ethanol intake that occurs in males as a consequence of sexual rejection, and this occurs in concert with corazonin (Crz) signaling. During and after copulation, Crz neurons are activated, ultimately resulting in ejaculation and cessation of sexual behavior (Tayler et al., 2012). Flies will optogenetically self-activate Crz neurons and approach odors associated with optogenetic activation of these neurons (Zer-Krispil et al., 2018). Successful mating or activation of Crz neurons ultimately generates an increase in NPF levels in the brains of males (Zer-Krispil et al., 2018), which decreases the likelihood of engaging in reward-

seeking behavior (Shohat-Ophir et al., 2012). Thus, optogenetic activation of Crz neurons in males is rewarding and gates release of NPF, which reduces ethanol self-administration in males by inducing a hedonic-like state (Shohat-Ophir et al., 2012). Together, these data suggest that monoaminergic signaling influences alcohol reward and preference, and peptidergic signaling appears to modulate alcohol attraction due to complex social interactions. Crz-releasing neurons also contribute to food reward. RNA interference-mediated Crz receptor knockdown in adipocytes and salivary glands, as well as Crz production knockdown, significantly reduces food intake in flies (Kubrak et al., 2016). Similarly, gain of function of short NPF (sNPF) increases food consumption, whereas the loss of function of sNPF suppresses food consumption (K. S. Lee et al., 2004). Knockdown of sNPF in the mushroom body and sNPF receptors on protocerebral anterior medial (PAM) neurons reduces the efficacy of odor-sugar memory encoding (Knapek et al., 2013; Lyutova et al., 2019). Hormones in *Drosophila* also play a key role in sugar reward. Neurons releasing diuretic hormone 44 (Dh44), orthologous to the mammalian stress hormone corticotropin releasing factor (Cannell et al., 2016; Lovejoy & Jahan, 2006), are key sensors for promoting food consumption (Yang et al., 2018). Dh44, released from neurons in the central brain, is necessary and sufficient for the detection and consumption of nutritive sugar (Dus et al., 2015). Dh44 binds to the Dh44 receptor in R1 neurons and R2 cells in the gut, which are required for behavioral responses to the nutritional value of sugar (Dus et al., 2015). Allatostatin A (AstA) neurons function in response to hunger and thirst. Activating AstA neurons in food-deprived animals suppresses feeding, and this effect is reversed by activating NPF neurons (Hergarden et al., 2012). Additionally, AstA neurons act on the allatostatin A

receptor 1 (DAR1) in PAM- γ 3 DA neurons to suppress their activity after sucrose consumption. γ 3 PAM inactivation mediates reward during sucrose consumption and associative learning (Yamagata et al., 2016). AstA also enhances water seeking while simultaneously suppressing feeding via AstA receptor 2 in NPF neurons (Landayan et al., 2021). The neuropeptides drosulfakinin (Dsk) and tachykinin contribute to reward indirectly. Activating or silencing Dsk neurons (Agrawal et al., 2020; Wu et al., 2020) elicits social isolation-induced fighting behavior phenotypes in *Drosophila* (Y.-K. Kim et al., 2018). Activation of tachykinin-releasing neurons is sufficient to induce same-sex aggression in males (Asahina et al., 2014). Since winning or losing a fight influences subsequent risk taking, social behavior, and reward behavior (Y.-K. Kim et al., 2018; Trannoy et al., 2016), activity from both Dsk and tachykinin neurons could disrupt reward perception. As a rule, hormone, peptidergic, and neurotransmitter circuits function together to directly modulate reward behavior, although the exact mechanisms through which this occurs remains to be understood.

1.6 – Larval Reward Circuits

One of the advantages of studying *Drosophila* is the unique ability to compare circuit frameworks within the same animal across different life stages. Despite having 10-fold fewer neurons than adults, larvae appear to have remarkable similarities in reward circuit form and function. Emerging research suggests a rich network of feeding circuits in larvae, making larvae an ideal model to study the mechanisms of food reward. Much of the larval feeding circuitry has been mapped with high resolution and is organized into a series of parallel pathways that connect sensory to motor neurons with interneuron signaling to the mushroom body (MB; Miroschnikow et al., 2018, 2020). In

larvae, as in adults, a complex interplay of neuropeptidergic and dopaminergic circuits mediates the behavioral response to food (Figure 2). Feeding initiation is regulated by insulin signaling in the MB (Zhao & Campos, 2012), whereas subsets of neurons expressing cyclic guanosine monophosphate (cGMP)-dependent protein kinase regulate the amount of food consumed and triglyceride levels in larvae, suggesting they play a role in feeding cessation (Allen et al., 2018). Adipokinetic hormone (AKH)-releasing cells release AKH and receive modulatory input from midgut neuropeptide F (NPF) and Allatostatin-C (AstC) neurons to alter hunger and satiation (Nelson et al., 2021). Similarly, cholinergic neurons that also release the neuropeptide hugin suppress feeding behavior by increasing searching behavior (Melcher & Pankratz, 2005; Schlegel et al., 2016). Interestingly, these neurons project to distinct parts of the larval brain (Figure 2), although it is unclear how these circuits function together to guide food consumption in larvae. As with adults, the dopamine (DA)-NPF circuit also plays a role in reward response in larvae. Neurons expressing NPF contribute to gustatory stimulation by sugar (Shen & Cai, 2001), contribute to odor-induced appetitive arousal (Pu et al., 2018), and interact with DA neurons to gate appetitive response (Wang et al., 2013). The Dopamine Neuron (DAN) k1 DA neurons (DANK1) directly contribute to fructose seeking and consumption (Saumweber et al., 2018). Similarly, ablation of dopamine 1 receptor 1 (Dop1R1) reduces odor-sugar memory of larvae but does not affect sucrose consumption (Honjo & Furukubo-Tokunaga, 2009; Selcho et al., 2009). Larval DA neuron activation is sufficient to induce reward memory (Thoener et al., 2022), suggesting this induces a pleasurable interoceptive state. DA neurons may be mediated by octopamine (OA) neurons (Eichler et al., 2017), as OA is required to

rescue reduced preference for consuming sucrose in larval *Drosophila* exposed to artificial repeated failure in reward pursuit (Fei et al., 2018). Larval *Drosophila* will optogenetically self-stimulate Kenyon cells (KCs; Lyutova et al., 2019) and sugar-sensing gustatory neurons (Nuwal et al., 2012; Seidenbecher et al., 2020). The γ -aminobutyric acid (GABAergic) anterior paired lateral neuron, which broadly innervates KC axons and contributes to odor discrimination (Amin et al., 2020; Prisco et al., 2021), is also critical for appetitive memory in larvae (Amin et al., 2020; Sabandal et al., 2020; Saumweber et al., 2018). *Drosophila* larvae display diversity in sensing and subsequent preference for amino acids (Kudow et al., 2017), which requires glutamatergic input to larval taste neurons (Croset et al., 2016). DA neurons contribute to *Drosophila* larvae by rejecting food that lacks essential amino acids (Bjordan et al., 2014). DL1 DA neurons in larvae are necessary and sufficient for the expression of preference for amino acids (Bjordan et al., 2014). When *Drosophila* larvae are reared in a protein-deficient diet, they reduce food seeking and consumption, which is dependent on OA neurons in the subesophageal zone (Bjordan et al., 2014; Fei et al., 2018). Together, this suggests that glutamate, OA, and DA function together to shape amino acid, and thus protein, preference. 5-HT also conveys information on the nutritional value of protein. Of the five known 5-HT receptors, serotonin 2A (5-HT_{2A}) receptors are the main mechanism by which *Drosophila* establish the value of dietary protein (Ro et al., 2016), and 5-HT_{2A} receptors are necessary for normal larval feeding (Gasque et al., 2013). Together, the above circuits function in larvae to sense and consume amino acids and proteins required for proper development. Despite larvae being a different developmental stage, with large differences in morphology and behavior, their reward circuits look remarkably

similar to those in adults. Peptidergic, hormone, OA, and 5-HT neurons signal information about arousal and internal state to DA neurons, which modulate valence and drive responses in cue-encoding neurons and premotor neurons required for eliciting reward behaviors.

1.7 Discussion

As understanding of the structure and function of *Drosophila* reward circuits develops, it is increasingly clear why *Drosophila* is an effective model for comprehending key basic principles underlying reward circuit function. In both larvae and adult *Drosophila*, slow, long-acting neuropeptidergic neurons connect to neurons that release faster-acting neurotransmitters that influence premotor and motor neurons to control directionality (goal orientation). Reward circuits thus act in concert to integrate internal state and reward sensation to produce reward behavior, much like a five-piece band works together to produce music for an audience (Figure 3). Octopaminergic or serotonergic state-setting neurons (Figure 3B1) act as percussive instruments (Figure 3A1), setting the tempo for the rest of the players. Slow-acting peptidergic neurons (Figure 3B2) work together with these state-setting neurons to layer rhythm, similar to the slow tones of a bass guitar (Figure 3A2). Bass guitars can alter the perception of music by providing notes that act as a bridge between treble (guitar) and percussion, similar to how neuropeptide F (NPF) can induce wakefulness that alters reward perception. Dopaminergic neurons (Figure 3B3) provide valence in the same way the lead guitar (Figure 3A3) drives the direction of the music. Cue-encoding Kenyon cells (KCs; Figure 3B4) sparsely activate in response to sensory information in the same way that a

synthesizer (Figure 3A4) sporadically punctuates the music. Sequential activation of KC compartments helps encode the memory the way keys on a piano/ synthesizer are activated to produce a melody (Owald & Waddell, 2015). Finally, the mushroom body output neuron (MBONs; Figure 3B5) are the star of the show as they guide the behavior in the same way the vocalist of the band communicates to the audience an overall message of the music through lyrics (Figure 3A5). Together, the percussion, bass, guitar, synthesizer, and vocalist act in concert to produce a song in a similar way that octopamine (OA), NPF, dopamine (DA), acetylcholine, and fast-acting neurotransmitter circuits directly produce reward behavior. Although this metaphor is based on mushroom body (MB) circuits, other circuits in the *Drosophila* brain encompass the described overlapping motifs for responses to sugar, water, and alcohol reward, suggesting it is a generalized model (Ichinose et al., 2015; Lin et al., 2014; Scaplen et al., 2020; Shyu et al., 2017; N. Yamagata et al., 2015). This pattern of connectivity is repeated across different types of reward in *Drosophila* (Figure 4). Remarkably, this also appears to be functionally similar across development, across different structures within the same brain, and across species. This suggests that key principles extracted from how reward circuits are structured and function in flies can enhance knowledge of the function of reward circuits and brain systems pivotal for reward behavior in mammals (Borst & Helmstaedter, 2015; Hunter et al., 2021; Kaiser, 2015; Scaplen & Kaun, 2016).

Some of the key principles that can already be extracted include the following:

- Rewards are encoded through the actions of monoaminergic, peptidergic, and hormone signaling within the brain.
- Reward response is dependent on the internal state of the animal.

- Reward encoding is dependent on timing and intensity of reward.
- A compartmentalized code within reward structures defines reward response.
- There are common circuits for all forms of reward.
- Unique circuits for different rewards are dependent on the context in which the reward was received.

A noticeable gap in understanding includes identifying which neurons directly contribute to appetitive internal states and which elicit the appropriate appetitive response. This is further complicated by how prior experiences can influence internal state and reward experience. Sleep is an excellent example of this. Hungry flies sleep less than satiated flies, and hunger-induced sleep deprivation during memory consolidation reduces sucrose-associated odor cue seeking (Chouhan et al., 2021). This effect is dependent on the activity of the $\gamma 2\alpha'1$ DA neuron–MBON circuit (Chouhan et al., 2021). DA neurons also likely contribute reward prediction error (RPE). Although direct data demonstrating this RPE are still lacking, the anatomy and function of the DA–MB circuit suggest it would be an ideal model to identify the detailed circuit mechanisms underlying this phenomenon (i.e., Adel & Griffith, 2021; Bennett et al., 2021). Future research using synthetic manipulation of neurons will help elucidate this and further interrogate how reward-encoding and reward-expressing circuits function distinctly and collaboratively to modulate appetitive behavior. Ultimately, understanding how experience and internal state alters neuromodulation and neural dynamics is key to understanding the mechanisms underlying motivational behavior for reward seeking and consumption.

1.8. CHAPTER 1 REFERENCES

- Adel, M., & Griffith, L. C. (2021). [The role of dopamine in associative learning in *Drosophila*: An updated unified model](#). *Neuroscience Bulletin*, 37(6), 831–852.
- Agrawal, P., Kao, D., Chung, P., & Looger, L. L. (2020). [The neuropeptide Drosulfakinin regulates social isolation-induced aggression in *Drosophila*](#). *Journal of Experimental Biology*, 223(2), jeb207407.
- Albin, S. D., Kaun, K. R., Knapp, J.-M., Chung, P., Heberlein, U., & Simpson, J. H. (2015). [A subset of serotonergic neurons evokes hunger in adult *Drosophila*](#). *Current Biology*, 25(18), 2435–2440.
- Allen, A. M., Anreiter, I., Vesterberg, A., Douglas, S. J., & Sokolowski, M. B. (2018). [Pleiotropy of the *Drosophila melanogaster* foraging gene on larval feeding-related traits](#). *Journal of Neurogenetics*, 32(3), 256–266.
- Amin, H., Apostolopoulou, A. A., Suárez-Grimalt, R., Vrontou, E., & Lin, A. C. (2020). [Localized inhibition in the *Drosophila* mushroom body](#). *eLife*, 9, e56954.
- Aryal, B., Dhakal, S., Shrestha, B., & Lee, Y. (2022). [Molecular and neuronal mechanisms for amino acid taste perception in the *Drosophila* labellum](#). *Current Biology*, 32(6), 1376–1386.e1374.
- Asahina, K., Watanabe, K., Duistermars, Brian J., Hoopfer, E., González, Carlos R., Eyjólfsson, Eyvindr A., Perona, P., & Anderson, David J. (2014). Tachykinin-expressing neurons control male-specific aggressive arousal in *Drosophila*. *Cell*, 156(1), 221–235.

Aso, Y., Hattori, D., Yu, Y., Johnston, R. M., Iyer, N. A., Ngo, T.-T. B., Dionne, H., Abbott, L. F., Axel, R., Tanimoto, H., & Rubin, G. M. (2014). [The neuronal architecture of the mushroom body provides a logic for associative learning](#). *eLife*, 3, e04577.

Aso, Y., Herb, A., Ogueta, M., Siwanowicz, I., Templier, T., Friedrich, A. B., Ito, K., Scholz, H., & Tanimoto, H. (2012). [Three dopamine pathways induce aversive odor memories with different stability](#). *PLoS Genetics*, 8(7), e1002768.

Aso, Y., & Rubin, G. M. (2016). [Dopaminergic neurons write and update memories with cell-type-specific rules](#). *eLife*, 5, e16135.

Aso, Y., Sitaraman, D., Ichinose, T., Kaun, K. R., Vogt, K., Belliart-Guérin, G., Plaçais, P.-Y., Robie, A. A., Yamagata, N., Schnaitmann, C., Rowell, W. J., Johnston, R. M., Ngo, T.-T. B., Chen, N., Korff, W., Nitabach, M. N., Heberlein, U., Preat, T., Branson, K. M., . . . Rubin, G. M. (2014). [Mushroom body output neurons encode valence and guide memory-based action selection in *Drosophila*](#). *eLife*, 3, e04580.

Azanchi, R., Kaun, K. R., & Heberlein, U. (2013). [Competing dopamine neurons drive oviposition choice for ethanol in *Drosophila*](#). *Proceedings of the National Academy of Sciences*, 110(52), 21153–21158.

Becher, P. G., Flick, G., Rozpędowska, E., Schmidt, A., Hagman, A., Lebreton, S., Larsson, M. C., Hansson, B. S., Piškur, J., Witzgall, P., & Bengtsson, M. (2012). [Yeast, not fruit volatiles mediate *Drosophila melanogaster* attraction, oviposition and development](#). *Functional Ecology*, 26(4), 822–828.

- Bennett, J. E. M., Philippides, A., & Nowotny, T. (2021). [Learning with reinforcement prediction errors in a model of the Drosophila mushroom body](#). *Nature Communications*, 12(1), 2569.
- Berger, K. H., Kong, E. C., Dubnau, J., Tully, T., Moore, M. S., & Heberlein, U. (2008). [Ethanol sensitivity and tolerance in long-term memory mutants of *Drosophila melanogaster*](#). *Alcoholism, Clinical and Experimental Research*, 32(5), 895–908.
- Bjordal, M., Arquier, N., Kniazeff, J., Pin, J. P., & Léopold, P. (2014). [Sensing of amino acids in a dopaminergic circuitry promotes rejection of an incomplete diet in *Drosophila*](#). *Cell*, 156(3), 510–521.
- Borst, A., & Helmstaedter, M. (2015). [Common circuit design in fly and mammalian motion vision](#). *Nature Neuroscience*, 18(8), 1067–1076.
- Boto, T., Stahl, A., Zhang, X., Louis, T., & Tomchik, S. M. (2019). [Independent contributions of discrete dopaminergic circuits to cellular plasticity, memory strength, and valence in *Drosophila*](#). *Cell Reports*, 27(7), 2014–2021.e2012.
- Budnik, V., & White, K. (1987). Genetic dissection of dopamine and serotonin synthesis in the nervous system of *Drosophila melanogaster*. *Journal of Neurogenetics*, 4(6), 309–314.
- Burke, C. J., Huetteroth, W., Oswald, D., Perisse, E., Krashes, M. J., Das, G., Gohl, D., Silies, M., Certel, S., & Waddell, S. (2012). [Layered reward signalling through octopamine and dopamine in *Drosophila*](#). *Nature*, 492(7429), 433–437.

- Burke, C. J., & Waddell, S. (2011). [Remembering nutrient quality of sugar in *Drosophila*](#). *Current Biology*, 21(9), 746–750.
- Cannell, E., Dornan, A. J., Halberg, K. A., Terhzaz, S., Dow, J. A. T., & Davies, S. A. (2016). [The corticotropin-releasing factor-like diuretic hormone 44 \(DH44\) and kinin neuropeptides modulate desiccation and starvation tolerance in *Drosophila melanogaster*](#). *Peptides*, 80, 96–107.
- Cheriyamkunnel, S. J., Rose, S., Jacob, P. F., Blackburn, L. A., Glasgow, S., Moorse, J., Winstanley, M., Moynihan, P. J., Waddell, S., & Rezaval, C. (2021). [A neuronal mechanism controlling the choice between feeding and sexual behaviors in *Drosophila*](#). *Current Biology*, 31(19), 4231–4245.e4234.
- Chia, J., & Scott, K. (2020). [Activation of specific mushroom body output neurons inhibits proboscis extension and sucrose consumption](#). *PLoS ONE*, 15(1), e0223034.
- Chouhan, N. S., Griffith, L. C., Haynes, P., & Sehgal, A. (2021). [Availability of food determines the need for sleep in memory consolidation](#). *Nature*, 589(7843), 582–585.
- Chouhan, N. S., & Sehgal, A. (2022). [Consolidation of sleep-dependent appetitive memory is mediated by a sweet-sensing circuit](#). *The Journal of Neuroscience*, 42(18), 3856–3867.
- Claßen, G., & Scholz, H. (2018). [Octopamine shifts the behavioral response from indecision to approach or aversion in *Drosophila melanogaster*](#). *Frontiers in Behavioral Neuroscience*, 12, 131.

- Clyne, P. J., Warr, C. G., & Carlson, J. R. (2000). [Candidate taste receptors in *Drosophila*](#). *Science*, 287(5459), 1830–1834.
- Cohn, R., Morante, I., & Ruta, V. (2015). [Coordinated and compartmentalized neuromodulation shapes sensory processing in *Drosophila*](#). *Cell*, 163(7), 1742–1755.
- Corl, A. B., Rodan, A. R., & Heberlein, U. (2005). [Insulin signaling in the nervous system regulates ethanol intoxication in *Drosophila melanogaster*](#). *Nature Neuroscience*, 8(1), 18–19.
- Croset, V., Schleyer, M., Arguello, J. R., Gerber, B., & Benton, R. (2016). [A molecular and neuronal basis for amino acid sensing in the *Drosophila* larva](#). *Scientific Reports*, 6(1), 34871.
- Deere, J. U., & Devineni, A. V. (2022). [Taste cues elicit prolonged modulation of feeding behavior in *Drosophila*](#). *iScience*, 25(10), 105159.
- Devineni, A. V., & Heberlein, U. (2009). [Preferential ethanol consumption in *Drosophila* models features of addiction](#). *Current Biology*, 19(24), 2126–2132.
- Dus, M., Lai, J. S.-Y., Gunapala, K. M., Min, S., Tayler, T. D., Hergarden, A. C., Geraud, E., Joseph, C. M., & Suh, G. S. B. (2015). [Nutrient sensor in the brain directs the action of the brain–gut axis in *Drosophila*](#). *Neuron*, 87(1), 139–151.
- Dus, M., Min, S., Keene, A. C., Lee, G. Y., & Suh, G. S. (2011). [Taste-independent detection of the caloric content of sugar in *Drosophila*](#). *Proceedings of the National Academy of Sciences of the United States of America*, 108(28), 11644–11649.

- Dvořáček, J., & Kodrík, D. (2021). [Drosophila reward system—A summary of current knowledge](#). *Neuroscience & Biobehavioral Reviews*, 123, 301–319.
- Dzitoyeva, S., Dimitrijevic, N., & Manev, H. (2003). [γ-Aminobutyric acid B receptor 1 mediates behavior-impairing actions of alcohol in Drosophila: Adult RNA interference and pharmacological evidence](#). *Proceedings of the National Academy of Sciences*, 100(9), 5485–5490.
- Eichler, K., Li, F., Litwin-Kumar, A., Park, Y., Andrade, I., Schneider-Mizell, C. M., Saumweber, T., Huser, A., Eschbach, C., Gerber, B., Fetter, R. D., Truman, J. W., Priebe, C. E., Abbott, L. F., Thum, A. S., Zlatic, M., & Cardona, A. (2017). [The complete connectome of a learning and memory centre in an insect brain](#). *Nature*, 548(7666), 175–182.
- Enjin, A., Zaharieva, E. E., Frank, D. D., Mansourian, S., Suh, G. S., Gallio, M., & Stensmyr, M. C. (2016). [Humidity sensing in Drosophila](#). *Current Biology*, 26(10), 1352–1358.
- Fei, Y., Zhu, D., Sun, Y., Gong, C., Huang, S., & Gong, Z. (2018). [Repeated failure in reward pursuit alters innate Drosophila larval behaviors](#). *Neuroscience Bulletin*, 34(6), 901–911.
- Fougeron, A.-S., Farine, J.-P., Flaven-Pouchon, J., Everaerts, C., & Ferveur, J.-F. (2011). [Fatty-acid preference changes during development in Drosophila melanogaster](#). *PLoS ONE*, 6(10), e26899.

- Ganguly, A., Dey, M., Scott, C., Duong, V.-K., & Arun Dahanukar, A. (2021). [Dietary macronutrient imbalances lead to compensatory changes in peripheral taste via independent signaling pathways](#). *The Journal of Neuroscience*, 41(50), 10222.
- Gasque, G., Conway, S., Huang, J., Rao, Y., & Vosshall, L. B. (2013). [Small molecule drug screening in *Drosophila* identifies the 5HT2A receptor as a feeding modulation target](#). *Scientific Reports*, 3(1), srep02120.
- Handler, A., Graham, T. G. W., Cohn, R., Morantte, I., Siliciano, A. F., Zeng, J., Li, Y., & Ruta, V. (2019). [Distinct dopamine receptor pathways underlie the temporal sensitivity of associative learning](#). *Cell*, 178(1), 60–75.e19.
- Harris, D. T., Kallman, B. R., Mullaney, B. C., & Scott, K. (2015). [Representations of taste modality in the *Drosophila* brain](#). *Neuron*, 86(6), 1449–1460.
- Haynes, P. R., Christmann, B. L., & Griffith, L. C. (2015). [A single pair of neurons links sleep to memory consolidation in *Drosophila melanogaster*](#). *eLife*, 4, e03868.
- Hergarden, A. C., Tayler, T. D., & Anderson, D. J. (2012). [Allatostatin-A neurons inhibit feeding behavior in adult *Drosophila*](#). *Proceedings of the National Academy of Sciences*, 109(10), 3967–3972.
- Honda, T. (2022). [Optogenetic and thermogenetic manipulation of defined neural circuits and behaviors in *Drosophila*](#). *Learning & Memory*, 29(4), 100–109.
- Honjo, K., & Furukubo-Tokunaga, K. (2009). [Distinctive neuronal networks and biochemical pathways for appetitive and aversive memory in *Drosophila* larvae](#). *The Journal of Neuroscience*, 29(3), 852–862.

- Huetteroth, W., Perisse, E., Lin, S., Klappenbach, M., Burke, C., & Waddell, S. (2015). [Sweet taste and nutrient value subdivide rewarding dopaminergic neurons in *Drosophila*](#). *Current Biology*, 25(6), 751–758.
- Hulse, B. K., Haberkern, H., Franconville, R., Turner-Evans, D. B., Takemura, S.-y., Wolff, T., Noorman, M., Dreher, M., Dan, C., Parekh, R., Hermundstad, A. M., Rubin, G. M., & Jayaraman, V. (2021). [A connectome of the *Drosophila* central complex reveals network motifs suitable for flexible navigation and context-dependent action selection](#). *eLife*, 10, e66039.
- Hunter, I., Coulson, B., Zarin, A. A., & Baines, R. A. (2021). [The *Drosophila* larval locomotor circuit provides a model to understand neural circuit development and function](#). *Frontiers in Neural Circuits*, 15, 684969.
- Ichinose, T., Aso, Y., Yamagata, N., Abe, A., Rubin, G. M., & Tanimoto, H. (2015). [Reward signal in a recurrent circuit drives appetitive long-term memory formation](#). *eLife*, 4, e10719.
- Ichinose, T., Kanno, M., Wu, H., Yamagata, N., Sun, H., Abe, A., & Tanimoto, H. (2021). [Mushroom body output differentiates memory processes and distinct memory-guided behaviors](#). *Current Biology*, 31(6), 1294–1302.e1294.
- Ja, W. W., Carvalho, G. B., Mak, E. M., de la Rosa, N. N., Fang, A. Y., Liong, J. C., Brummel, T., & Benzer, S. (2007). [Prandiology of *Drosophila* and the CAFE assay](#). *Proceedings of the National Academy of Sciences of the United States of America*, 104(20), 8253–8256.

- Ji, F., & Zhu, Y. (2015). [A novel assay reveals hygrotactic behavior in *Drosophila*](#). *PLoS ONE*, 10(3), e0119162.
- Kaiser, M. (2015). [Neuroanatomy: Connectome connects fly and mammalian brain networks](#). *Current Biology*, 25(10), R416–R418.
- Kasture, A. S., Bartel, D., Steinkellner, T., Sucic, S., Hummel, T., & Freissmuth, M. (2019). [Distinct contribution of axonal and somatodendritic serotonin transporters in *Drosophila* olfaction](#). *Neuropharmacology*, 161, 107564.
- Kaun, K. R., Azanchi, R., Maung, Z., Hirsh, J., & Heberlein, U. (2011). [A *Drosophila* model for alcohol reward](#). *Nature Neuroscience*, 14(5), 612–619.
- Kim, Y.-C., Lee, H.-G., & Han, K.-A. (2007). [D1 dopamine receptor dDA1 is required in the mushroom body neurons for aversive and appetitive learning in *Drosophila*](#). *The Journal of Neuroscience*, 27(29), 7640.
- Kim, Y.-C., Lee, H.-G., Lim, J., & Han, K.-A. (2013). [Appetitive learning requires the alpha1-like octopamine receptor OAMB in the *Drosophila* mushroom body neurons](#). *The Journal of Neuroscience*, 33(4), 1672.
- Kim, Y.-K., Saver, M., Simon, J., Kent, C. F., Shao, L., Eddison, M., Agrawal, P., Texada, M., Truman, J. W., & Heberlein, U. (2018). [Repetitive aggressive encounters generate a long-lasting internal state in *Drosophila melanogaster* males](#). *Proceedings of the National Academy of Sciences*, 115(5), 1099–1104.

Kimura, K.-i., Hachiya, T., Koganezawa, M., Tazawa, T., & Yamamoto, D. (2008).

[Fruitless and doublesex coordinate to generate male-specific neurons that can initiate courtship.](#) *Neuron*, 59(5), 759–769.

Knapek, S., Kahsai, L., Winther, A. M., Tanimoto, H., & Nässel, D. R. (2013). [Short neuropeptide F acts as a functional neuromodulator for olfactory memory in Kenyon cells of *Drosophila* mushroom bodies.](#) *The Journal of Neuroscience*, 33(12), 5340–5345.

Kong, E. C., Woo, K., Li, H., Lebestky, T., Mayer, N., Sniffen, M. R., Heberlein, U., Bainton, R. J., Hirsh, J., & Wolf, F. W. (2010). [A pair of dopamine neurons target the D1-like dopamine receptor DopR in the central complex to promote ethanol-stimulated locomotion in *Drosophila*.](#) *PLoS ONE*, 5(4), e9954.

Krashes, M. J., DasGupta, S., Vreede, A., White, B., Armstrong, J. D., & Waddell, S. (2009). [A neural circuit mechanism integrating motivational state with memory expression in *Drosophila*.](#) *Cell*, 139(2), 416–427.

Krashes, M. J., & Waddell, S. (2008). [Rapid consolidation to a radish and protein synthesis-dependent long-term memory after single-session appetitive olfactory conditioning in *Drosophila*.](#) *The Journal of Neuroscience*, 28(12), 3103.

Kubrak, O. I., Lushchak, O. V., Zandawala, M., & Nässel, D. R. (2016). [Systemic corazonin signalling modulates stress responses and metabolism in *Drosophila*.](#) *Open Biology*, 6(11), 160152.

- Kudow, N., Miura, D., Schleyer, M., Toshima, N., Gerber, B., & Tanimura, T. (2017). [Preference for and learning of amino acids in larval *Drosophila*](#). *Biology Open*, 6(3), 365–369.
- Landayan, D., Feldman, D. S., & Wolf, F. W. (2018). [Satiation state-dependent dopaminergic control of foraging in *Drosophila*](#). *Scientific Reports*, 8(1), 5777.
- Landayan, D., Wang, B. P., Zhou, J., & Wolf, F. W. (2021). [Thirst interneurons that promote water seeking and limit feeding behavior in *Drosophila*](#). *eLife*, 10, e66286.
- Landayan, D., & Wolf, F. W. (2015). [Shared neurocircuitry underlying feeding and drugs of abuse in *Drosophila*](#). *Biomedical Journal*, 38(6), 496–509.
- Lau, M.-T., Lin, Y. Q., Kisling, S., Cotterell, J., Wilson, Y. A., Wang, Q.-P., Khuong, T. M., Bakhshi, N., Cole, T. A., Oyston, L. J., Cole, A. R., & Neely, G. G. (2017). [A simple high throughput assay to evaluate water consumption in the fruit fly](#). *Scientific Reports*, 7(1), 16786.
- Lebreton, S., Witzgall, P., Olsson, M., & Becher, P. G. (2014). [Dietary glucose regulates yeast consumption in adult *Drosophila* males](#). *Frontiers in Physiology*, 5.
- LeDue, E. E., Mann, K., Koch, E., Chu, B., Dakin, R., & Gordon, M. D. (2016). [Starvation-induced depotentiation of bitter taste in *Drosophila*](#). *Current Biology*, 26(21), 2854–2861.
- Lee, K. S., You, K. H., Choo, J. K., Han, Y. M., & Yu, K. (2004). [Drosophila short neuropeptide F regulates food intake and body size](#). *The Journal of Biological Chemistry*, 279(49), 50781–50789.

- Lee, W.-P., Chiang, M.-H., Chang, L.-Y., Shyu, W.-H., Chiu, T.-H., Fu, T.-F., Wu, T., & Wu, C.-L. (2021). [Serotonin signals modulate mushroom body output neurons for sustaining water-reward long-term memory in *Drosophila*](#). *Frontiers in Cell and Developmental Biology*, 9, 755574.
- Li, F., Lindsey, J. W., Marin, E. C., Otto, N., Dreher, M., Dempsey, G., Stark, I., Bates, A. S., Pleijzier, M. W., Schlegel, P., Nern, A., Takemura, S.-y., Eckstein, N., Yang, T., Francis, A., Braun, A., Parekh, R., Costa, M., Scheffer, L. K., . . . Rubin, G. M. (2020). [The connectome of the adult *Drosophila* mushroom body provides insights into function](#). *eLife*, 9, e62576.
- Liman, E. R., Zhang, Y. V., & Montell, C. (2014). [Peripheral coding of taste](#). *Neuron*, 81(5), 984–1000.
- Lin, S., Oswald, D., Chandra, V., Talbot, C., Huetteroth, W., & Waddell, S. (2014). [Neural correlates of water reward in thirsty *Drosophila*](#). *Nature Neuroscience*, 17(11), 1536–1542.
- Liu, C., Plaçais, P.-Y., Yamagata, N., Pfeiffer, B. D., Aso, Y., Friedrich, A. B., Siwanowicz, I., Rubin, G. M., Preat, T., & Tanimoto, H. (2012). [A subset of dopamine neurons signals reward for odour memory in *Drosophila*](#). *Nature*, 488(7412), 512–516.
- Baldoni, D., Liu, Q., Tabuchi, M., Liu, S., Kodama, L., Horiuchi, W., Daniels, J., Chiu, L., & Baldoni, D., Wu, M. N. (2017). [Branch-specific plasticity of a bifunctional dopamine circuit encodes protein hunger](#). *Science*, 356(6337), 534–539.

- Lovejoy, D. A., & Jahan, S. (2006). [Phylogeny of the corticotropin-releasing factor family of peptides in the metazoa](#). *General and Comparative Endocrinology*, 146(1), 1–8.
- Lüersen, K., Röder, T., & Rimbach, G. (2019). [Drosophila melanogaster in nutrition research—The importance of standardizing experimental diets](#). *Genes & Nutrition*, 14(1), 3.
- Lyutova, R., Selcho, M., Pfeuffer, M., Segebarth, D., Habenstein, J., Rohwedder, A., Frantzmann, F., Wegener, C., Thum, A. S., & Pauls, D. (2019). [Reward signaling in a recurrent circuit of dopaminergic neurons and peptidergic Kenyon cells](#). *Nature Communications*, 10(1), 3097.
- Mao, Z., & Davis, R. L. (2009). [Eight different types of dopaminergic neurons innervate the Drosophila mushroom body neurophil: Anatomical and physiological heterogeneity](#). *Frontiers in Neural Circuits*, 3, 5.
- May, C. E., Rosander, J., Gottfried, J., Dennis, E., & Dus, M. (2020). [Dietary sugar inhibits satiation by decreasing the central processing of sweet taste](#). *eLife*, 9, e54530.
- Melcher, C., & Pankratz, M. J. (2005). [Candidate gustatory interneurons modulating feeding behavior in the Drosophila brain](#). *PLoS Biology*, 3(9), e305.
- Miroschnikow, A., Schlegel, P., & Pankratz, M. J. (2020). [Making feeding decisions in the Drosophila nervous system](#). *Current Biology*, 30(14), R831–R840.

Miroschnikow, A., Schlegel, P., Schoofs, A., Hueckesfeld, S., Li, F., Schneider-Mizell, C. M., Fetter, R. D., Truman, J. W., Cardona, A., & Pankratz, M. J. (2018).

[Convergence of monosynaptic and polysynaptic sensory paths onto common motor outputs in a *Drosophila* feeding connectome.](#) *eLife*, 7.

Moore, M. S., DeZazzo, J., Luk, A. Y., Tully, T., Singh, C. M., & Heberlein, U. (1998).

[Ethanol intoxication in *Drosophila*: Genetic and pharmacological evidence for regulation by the cAMP signaling pathway.](#) *Cell*, 93(6), 997–1007.

Nässel, D. R., & Elekes, K. (1992). [Aminergic neurons in the brain of blowflies and *Drosophila*: Dopamine- and tyrosine hydroxylase-immunoreactive neurons and their relationship with putative histaminergic neurons.](#) *Cell and Tissue Research*, 267(1), 147–167.

Neckameyer, W. S. (1998). [Dopamine modulates female sexual receptivity in *Drosophila melanogaster*.](#) *Journal of Neurogenetics*, 12(2), 101–114.

Nelson, J. M., Saunders, C. J., & Johnson, E. C. (2021). [The intrinsic nutrient sensing adipokinetic hormone producing cells function in modulation of metabolism, activity, and stress.](#) *International Journal of Molecular Sciences*, 22(14), 7515.

Nunez, K. M., Azanchi, R., & Kaun, K. R. (2018). [Cue-induced ethanol seeking in *Drosophila melanogaster* is dose-dependent.](#) *Frontiers in Physiology*, 9, 438.

Nuwal, N., Stock, P., Hiemeyer, J., Schmid, B., Fiala, A., & Buchner, E. (2012). [Avoidance of heat and attraction to optogenetically induced sugar sensation as operant behavior in adult *Drosophila*.](#) *Journal of Neurogenetics*, 26(3–4), 298–305.

Otto, N., Pleijzier, M. W., Morgan, I. C., Edmondson-Stait, A. J., Heinz, K. J., Stark, I., Dempsey, G., Ito, M., Kapoor, I., Hsu, J., Schlegel, P. M., Bates, A. S., Feng, L., Costa, M., Ito, K., Bock, D. D., Rubin, G. M., Jefferis, G., & Waddell, S. (2020). [Input connectivity reveals additional heterogeneity of dopaminergic reinforcement in *Drosophila*](#). *Current Biology*, 30(16), 3200–3211.e3208.

Owald, D., Felsenberg, J., Talbot, C. B., Das, G., Perisse, E., Huetteroth, W., & Waddell, S. (2015). [Activity of defined mushroom body output neurons underlies learned olfactory behavior in *Drosophila*](#). *Neuron*, 86(2), 417–427.

Owald, D., & Waddell, S. (2015). [Olfactory learning skews mushroom body output pathways to steer behavioral choice in *Drosophila*](#). *Current Opinion in Neurobiology*, 35, 178–184.

Pardo-Garcia, T. R., Gu, K., Woerner, R. K. R., & Dus, M. (2023). [Food memory circuits regulate eating and energy balance](#). *Current Biology*, 33(2), 215–227.e213.

Park, A., Tran, T., & Atkinson, N. S. (2018). [Monitoring food preference in *Drosophila* by oligonucleotide tagging](#). *Proceedings of the National Academy of Sciences*, 115(36), 9020–9025.

Park, J.-Y., Dus, M., Kim, S., Abu, F., Kanai, M. I., Rudy, B., & Suh, G. S. B. (2016). [*Drosophila* SLC5A11 mediates hunger by regulating K⁺ channel activity](#). *Current Biology*, 26(15), 1965–1974.

Pavlou, H. J., & Goodwin, S. F. (2013). [Courtship behavior in *Drosophila melanogaster*: Towards a “courtship connectome.”](#) *Current Opinion in Neurobiology*, 23(1), 76–83.

- Perisse, E., Oswald, D., Barnstedt, O., Talbot, C. B., Huetteroth, W., & Waddell, S. (2016). [Aversive learning and appetitive motivation toggle feed-forward inhibition in the *Drosophila* mushroom body](#). *Neuron*, 90(5), 1086–1099.
- Peru Y Colón de Portugal, R. L., Ojelade, S. A., Penninti, P. S., Dove, R. J., Nye, M. J., Acevedo, S. F., Lopez, A., Rodan, A. R., & Rothenfluh, A. (2014). [Long-lasting, experience-dependent alcohol preference in *Drosophila*](#). *Addiction Biology*, 19(3), 392–401.
- Pitman, J. L., Huetteroth, W., Burke, C. J., Krashes, M. J., Lai, S.-L., Lee, T., & Waddell, S. (2011). [A pair of inhibitory neurons are required to sustain labile memory in the *Drosophila* mushroom body](#). *Current Biology*, 21(10), 855–861.
- Plaçais, P. Y., & Preat, T. (2013). [To favor survival under food shortage, the brain disables costly memory](#). *Science*, 339(6118), 440–442.
- Prisco, L., Deimel, S. H., Yelisseyeva, H., Fiala, A., & Tavosanis, G. (2021). [The anterior paired lateral neuron normalizes odour-evoked activity in the *Drosophila* mushroom body calyx](#). *eLife*, 10, e74172.
- Pu, Y., Zhang, Y., Zhang, Y., & Shen, P. (2018). [Two *Drosophila* neuropeptide Y-like neurons define a reward module for transforming appetitive odor representations to motivation](#). *Scientific Reports*, 8(1), 11658.
- Ro, J., Pak, G., Malec, P. A., Lyu, Y., Allison, D. B., Kennedy, R. T., & Pletcher, S. D. (2016). [Serotonin signaling mediates protein valuation and aging](#). *eLife*, 5, e16843.

- Rothenfluh, A., Threlkeld, R. J., Bainton, R. J., Tsai, L. T. Y., Lasek, A. W., & Heberlein, U. (2006). [Distinct behavioral responses to ethanol are regulated by alternate RhoGAP18B isoforms](#). *Cell*, 127(1), 199–211.
- Sabandal, J. M., Sabandal, P. R., Kim, Y.-C., & Han, K.-A. (2020). [Concerted actions of octopamine and dopamine receptors drive olfactory learning](#). *The Journal of Neuroscience*, 40(21), 4240.
- Saumweber, T., Rohwedder, A., Schleyer, M., Eichler, K., Chen, Y. C., Aso, Y., Cardona, A., Eschbach, C., Kobler, O., Voigt, A., Durairaja, A., Mancini, N., Zlatic, M., Truman, J. W., Thum, A. S., & Gerber, B. (2018). [Functional architecture of reward learning in mushroom body extrinsic neurons of larval *Drosophila*](#). *Nature Communications*, 9(1), 1104.
- Sayed, O., & Benzer, S. (1996). [Behavioral genetics of thermosensation and hygrosensation in *Drosophila*](#). *Proceedings of the National Academy of Sciences of the United States of America*, 93(12), 6079–6084.
- Scaplen, K. M., & Kaun, K. R. (2016). [Reward from bugs to bipeds: A comparative approach to understanding how reward circuits function](#). *Journal of Neurogenetics*, 30(2), 133–148.
- Scaplen, K. M., Mei, N. J., Bounds, H. A., Song, S. L., Azanchi, R., & Kaun, K. R. (2019). [Automated real-time quantification of group locomotor activity in *Drosophila melanogaster*](#). *Scientific Reports*, 9(1), 4427.

Scaplen, K. M., Talay, M., Nunez, K. M., Salamon, S., Waterman, A. G., Gang, S., Song, S. L., Barnea, G., & Kaun, K. R. (2020). [Circuits that encode and guide alcohol-associated preference](#). *eLife*, 9, e48730.

Scheffer, L. K., Xu, C. S., Januszewski, M., Lu, Z., Takemura, S.-y., Hayworth, K. J., Huang, G. B., Shinomiya, K., Maitlin-Shepard, J., Berg, S., Clements, J., Hubbard, P. M., Katz, W. T., Umayam, L., Zhao, T., Ackerman, D., Blakely, T., Bogovic, J., Dolafi, T., . . . Plaza, S. M. (2020). [A connectome and analysis of the adult *Drosophila* central brain](#). *eLife*, 9, e57443.

Scheiner, R., Steinbach, A., Claßen, G., Strudthoff, N., & Scholz, H. (2014). [Octopamine indirectly affects proboscis extension response habituation in *Drosophila melanogaster* by controlling sucrose responsiveness](#). *Journal of Insect Physiology*, 69, 107–117.

Schlegel, P., Texada, M. J., Miroshnikow, A., Schoofs, A., Hückesfeld, S., Peters, M., Schneider-Mizell, C. M., Lacin, H., Li, F., Fetter, R. D., Truman, J. W., Cardona, A., & Pankratz, M. J. (2016). [Synaptic transmission parallels neuromodulation in a central food-intake circuit](#). *eLife*, 5, e16799.

Schneider, A., Ruppert, M., Hendrich, O., Giang, T., Ogueta, M., Hampel, S., Vollbach, M., Büschges, A., & Scholz, H. (2012). [Neuronal basis of innate olfactory attraction to ethanol in *Drosophila*](#). *PLoS ONE*, 7(12), e52007.

Schwaerzel, M., Monastirioti, M., Scholz, H., Friggi-Grelín, F., Birman, S., & Heisenberg, M. (2003). [Dopamine and octopamine differentiate between aversive](#)

[and appetitive olfactory memories in *Drosophila*](#). *The Journal of Neuroscience*, 23(33), 10495.

Seidenbecher, S. E., Sanders, J. I., von Philipsborn, A. C., & Kvitsiani, D. (2020). [Reward foraging task and model-based analysis reveal how fruit flies learn value of available options](#). *PLoS ONE*, 15(10), e0239616.

Selcho, M., Pauls, D., Han, K. A., Stocker, R. F., & Thum, A. S. (2009). [The role of dopamine in *Drosophila* larval classical olfactory conditioning](#). *PLoS ONE*, 4(6), e5897.

Shamsideen, A. O., Andrew, R. B., Collin, B. M., Eve Privman, C., Yoshinori, A., Danielle, W., Roberto, U. C., Rueben, A. G., Gerald, M. R., Venton, B. J., Aylin, R. R., & Adrian, R. (2019). [Dopaminergic learning and arousal circuits mediate opposing effects on alcohol consumption in *Drosophila*](#). *bioRxiv*, 624833.

Shao, L., Saver, M., Chung, P., Ren, Q., Lee, T., Kent, C. F., & Heberlein, U. (2017). [Dissection of the *Drosophila* neuropeptide F circuit using a high-throughput two-choice assay](#). *Proceedings of the National Academy of Sciences of the United States of America*, 114(38), E8091–E8099.

Shen, P., & Cai, H. N. (2001). [Drosophila neuropeptide F mediates integration of chemosensory stimulation and conditioning of the nervous system by food](#). *Journal of Neurobiology*, 47(1), 16–25.

Shohat-Ophir, G., Kaun, K. R., Azanchi, R., Mohammed, H., & Heberlein, U. (2012). [Sexual deprivation increases ethanol intake in *Drosophila*](#). *Science*, 335(6074), 1351–1355.

Shyu, W. H., Chiu, T. H., Chiang, M. H., Cheng, Y. C., Tsai, Y. L., Fu, T. F., Wu, T., & Wu, C. L. (2017). [Neural circuits for long-term water-reward memory processing in thirsty *Drosophila*](#). *Nature Communications*, 8, 15230.

Siju, K. P., Štih, V., Aimon, S., Gjorgjieva, J., Portugues, R., & Grunwald Kadow, I. C. (2020). [Valence and state-dependent population coding in dopaminergic neurons in the fly mushroom body](#). *Current Biology*, 30(11), 2104–2115.e2104.

Sitaraman, D., LaFerriere, H., Birman, S., & Zars, T. (2012). [Serotonin is critical for rewarded olfactory short-term memory in *Drosophila*](#). *Journal of Neurogenetics*, 26(2), 238–244.

Stahl, A., Noyes, N. C., Boto, T., Botero, V., Broyles, C. N., Jing, M., Zeng, J., King, L. B., Li, Y., Davis, R. L., & Tomchik, S. M. (2022). [Associative learning drives longitudinally graded presynaptic plasticity of neurotransmitter release along axonal compartments](#). *eLife*, 11, e76712.

Takemura, S.-y., Aso, Y., Hige, T., Wong, A., Lu, Z., Xu, C. S., Rivlin, P. K., Hess, H., Zhao, T., Parag, T., Berg, S., Huang, G., Katz, W., Olbris, D. J., Plaza, S., Umayam, L., Aniceto, R., Chang, L.-A., Lauchie, S., . . . Scheffer, L. K. (2017). [A connectome of a learning and memory center in the adult *Drosophila* brain](#). *eLife*, 6, e26975.

Tayler, T. D., Pacheco, D. A., Hergarden, A. C., Murthy, M., & Anderson, D. J. (2012). [A neuropeptide circuit that coordinates sperm transfer and copulation duration in *Drosophila*](#). *Proceedings of the National Academy of Sciences of the United States of America*, 109(50), 20697–20702.

- Tempel, B. L., Bonini, N., Dawson, D. R., & Quinn, W. G. (1983). [Reward learning in normal and mutant *Drosophila*](#). *Proceedings of the National Academy of Sciences of the United States of America*, 80(5), 1482–1486.
- Thoener, J., Weiglein, A., Gerber, B., & Schleyer, M. (2022). [Optogenetically induced reward and “frustration” memory in larval *Drosophila melanogaster*](#). *The Journal of Experimental Biology*, 225(16), jeb244565.
- Thorne, N., Chromey, C., Bray, S., & Amrein, H. (2004). [Taste perception and coding in *Drosophila*](#). *Current Biology*, 14(12), 1065–1079.
- Toshima, N., & Tanimura, T. (2012). [Taste preference for amino acids is dependent on internal nutritional state in *Drosophila melanogaster*](#). *Journal of Experimental Biology*, 215(16), 2827–2832.
- Trannoy, S., Penn, J., Lucey, K., Popovic, D., & Kravitz, E. A. (2016). [Short and long-lasting behavioral consequences of agonistic encounters between male *Drosophila melanogaster*](#). *Proceedings of the National Academy of Sciences of the United States of America*, 113(17), 4818–4823.
- Tsao, C.-H., Chen, C.-C., Lin, C.-H., Yang, H.-Y., & Lin, S. (2018). [Drosophila mushroom bodies integrate hunger and satiety signals to control innate food-seeking behavior](#). *eLife*, 7, e35264.
- Urizar, N. L., Yang, Z., Edenberg, H. J., & Davis, R. L. (2007). [Drosophila Homer is required in a small set of neurons including the ellipsoid body for normal ethanol sensitivity and tolerance](#). *The Journal of Neuroscience*, 27(17), 4541.

- Venken, K. J., Simpson, J. H., & Bellen, H. J. (2011). [Genetic manipulation of genes and cells in the nervous system of the fruit fly](#). *Neuron*, 72(2), 202–230.
- Wang, Y., Pu, Y., & Shen, P. (2013). [Neuropeptide-gated perception of appetitive olfactory inputs in *Drosophila* larvae](#). *Cell Reports*, 3(3), 820–830.
- Wen, T., Parrish, C. A., Xu, D., Wu, Q., & Shen, P. (2005). [Drosophila neuropeptide F and its receptor, NPFR1, define a signaling pathway that acutely modulates alcohol sensitivity](#). *Proceedings of the National Academy of Sciences of the United States of America*, 102(6), 2141–2146.
- Wiggin, T. D., Hsiao, Y., Liu, J. B., Huber, R., & Griffith, L. C. (2021). [Rest is required to learn an appetitively-reinforced operant task in *Drosophila*](#). *Frontiers in Behavioral Neuroscience*, 15, 681593.
- Wolf, F. W., Rodan, A. R., Tsai, L. T., & Heberlein, U. (2002). [High-resolution analysis of ethanol-induced locomotor stimulation in *Drosophila*](#). *The Journal of Neuroscience*, 22(24), 11035–11044.
- Wu, F., Deng, B., Xiao, N., Wang, T., Li, Y., Wang, R., Shi, K., Luo, D.-G., Rao, Y., & Zhou, C. (2020). [A neuropeptide regulates fighting behavior in *Drosophila melanogaster*](#). *eLife*, 9, e54229.
- Xu, L., He, J., Kaiser, A., Gräber, N., Schläger, L., Ritze, Y., & Scholz, H. (2016). [A single pair of serotonergic neurons counteracts serotonergic inhibition of ethanol attraction in *Drosophila*](#). *PLoS ONE*, 11(12), e0167518.

- Yamagata, N., Hiroi, M., Kondo, S., Abe, A., & Tanimoto, H. (2016). [Suppression of dopamine neurons mediates reward](#). *PLoS Biology*, 14(12), e1002586.
- Yamagata, N., Ichinose, T., Aso, Y., Plaçais, P. Y., Friedrich, A. B., Sima, R. J., Preat, T., Rubin, G. M., & Tanimoto, H. (2015). [Distinct dopamine neurons mediate reward signals for short- and long-term memories](#). *Proceedings of the National Academy of Sciences of the United States of America*, 112(2), 578–583.
- Yang, Z., Huang, R., Fu, X., Wang, G., Qi, W., Mao, D., Shi, Z., Shen, W. L., & Wang, L. (2018). [A post-ingestive amino acid sensor promotes food consumption in *Drosophila*](#). *Cell Research*, 28(10), 1013–1025.
- Yao, Z., & Scott, K. (2022). [Serotonergic neurons translate taste detection into internal nutrient regulation](#). *Neuron*, 110(6), 1036–1050.e1037.
- Yapici, N., Cohn, R., Schusterreiter, C., Ruta, V., & Vosshall, L. B. (2016). [A taste circuit that regulates ingestion by integrating food and hunger signals](#). *Cell*, 165(3), 715–729.
- Zer-Krispil, S., Zak, H., Shao, L., Ben-Shaanan, S., Tordjman, L., Bentzur, A., Shmueli, A., & Shohat-Ophir, G. (2018). [Ejaculation induced by the activation of Crz neurons is rewarding to *Drosophila* males](#). *Current Biology*, 28(9), 1445–1452.e1443.
- Zhang, S. X., Rogulja, D., & Crickmore, M. A. (2016). [Dopaminergic circuitry underlying mating drive](#). *Neuron*, 91(1), 168–181.

Zhang, Y., Liu, G., Yan, J., Zhang, Y., Li, B., & Cai, D. (2015). [Metabolic learning and memory formation by the brain influence systemic metabolic homeostasis](#). *Nature Communications*, 6, 6704.

Zhao, X. L., & Campos, A. R. (2012). [Insulin signalling in mushroom body neurons regulates feeding behaviour in *Drosophila* larvae](#). *Journal of Experimental Biology*, 215(15), 2696–2702.

1.9: CHAPTER 1 FIGURES

Figure 1.1

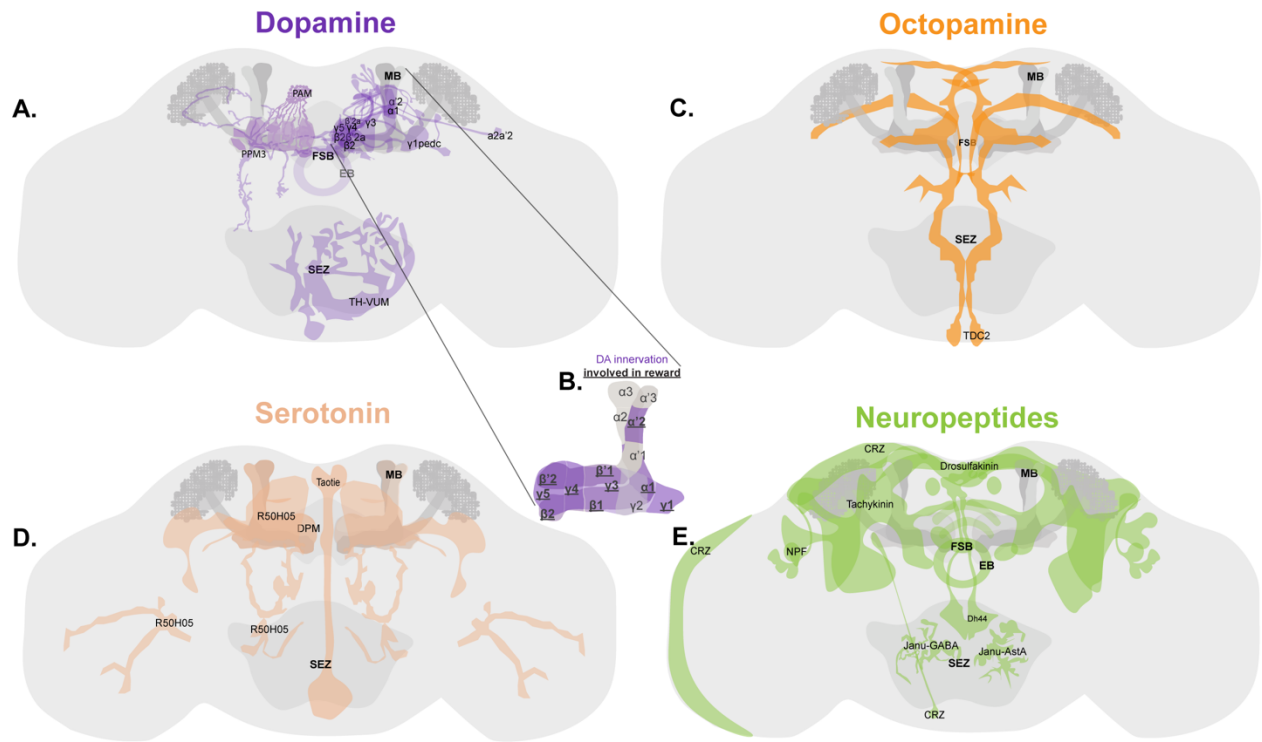


Figure 1. Reward circuits in adult *Drosophila*. Circuits identified in behavioral responses to a variety of rewards in *Drosophila* include (A) dopaminergic circuits; (B) dopamine mushroom body (MB) neurons implicated in reward synapse onto distinct MB compartments as follows: $\alpha 1$ – $\alpha 3$, $\alpha' 1$ – $\alpha' 3$, $\beta 1$ and $\beta 2$, $\beta' 1$ and $\beta' 2$, and $\gamma 1$ – $\gamma 5$; (C) octopaminergic circuits, (D) serotonergic circuits, and (E) neuropeptidergic circuits.

Figure 1.2

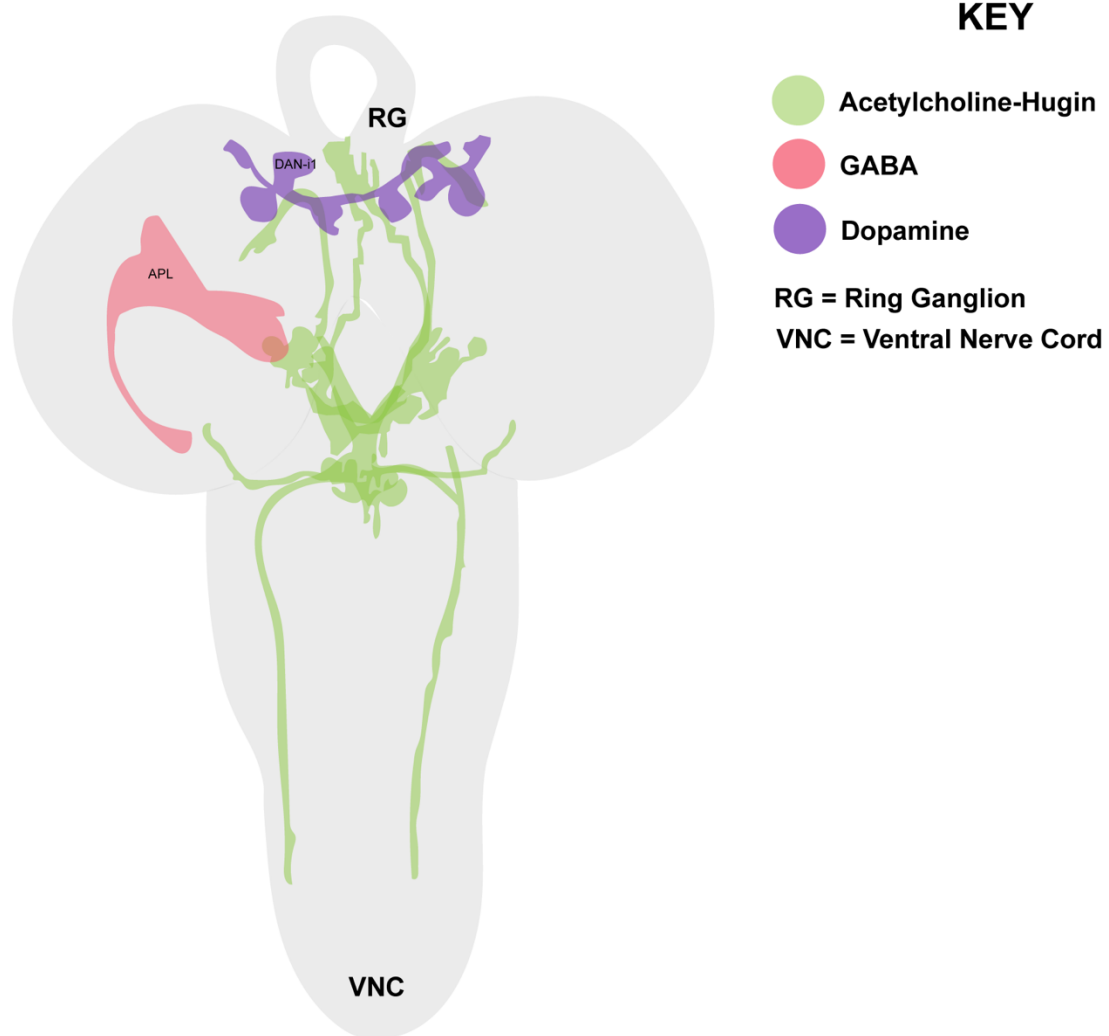


Figure 2. Reward circuits in larval *Drosophila*. Acetylcholine-releasing neurons as well as GABA and DAergic neurons contribute to the expression of reward behavior. Acetylcholine-expressing Hugin neurons differ in their projection to protocerebrum (PC), pharynx (PH), and two populations that project to the ring gland (RG).

Figure 1.3

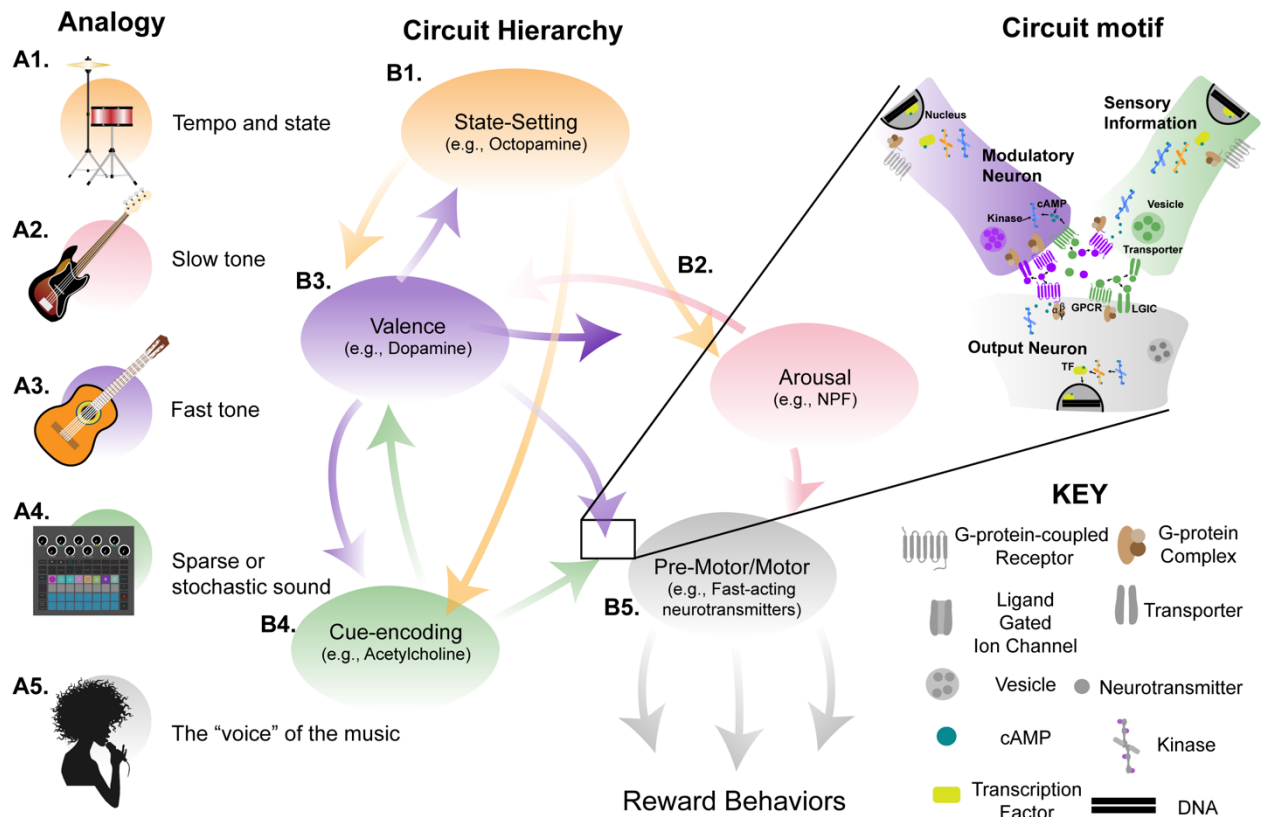


Figure 3. General model for reward signaling using a metaphor of a five-member band. Band components such as percussion (A1), bass (A2), guitar (A3), synthesizers (A4), and vocalists (A5) function similarly to state-setting (B1), arousal-eliciting (B2), valence-encoding (B3), cue-encoding (B4), and premotor/motor (B5) neurons to produce music or reward-related behaviors, respectively. Our inset circuit motif demonstrates the synapse between modulatory, sensory, and output neurons that contribute to reward behavior expression.

Figure 1.4

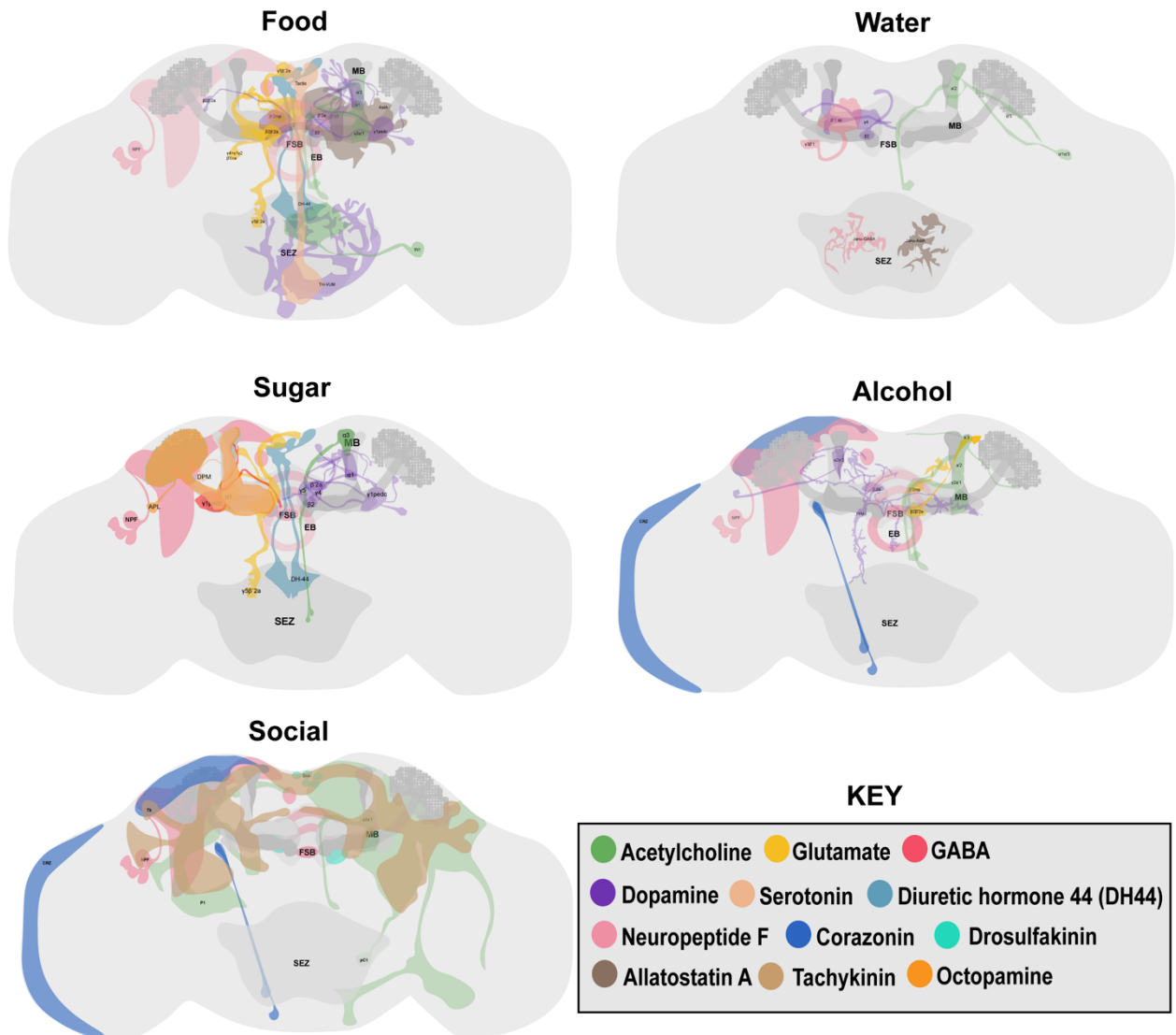


Figure 1.4 Circuits for different rewards in *Drosophila*. Illustrations of neurons that are directly involved in (A) food, (B) water, (C) sugar, (D) alcohol, and (E) social reward in the adult *Drosophila* central brain (gray). Neuron types are identified by color (see key), and coverage is based on peer-reviewed publications referenced in the article or from the Janelia FlyLight Split-Gal4 Driver Collection.

CHAPTER 2:

Dopamine circuit dynamics underlying acquisition of alcohol memory

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Contributions:

- Tariq Brown performed all imaging experiments, generated all figures, and composed text.
- Karla Kaun provided feedback on experimental design, data analysis, and visualization, and helped draft and revise the manuscript
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2.1 ABSTRACT

Alcohol-associated cues can induce cravings, but the neural mechanisms through which ambient sensory cues become associated with the pharmacological properties of alcohol aren't well understood. Dopaminergic reinforcement is a crucial part of encoding reward memories across multiple brain structures. To clarify how dopamine dynamics change across reward-related learning, we investigate changes in dopamine signaling across multiple-spaced cue-ethanol associations in the *Drosophila* mushroom body. We found that alcohol in the absence of a cue does not increase dopaminergic activity across sessions. However, dopamine and post-synaptic cholinergic cue-encoding neurons potentiate with each alcohol-cue association. This potentiation occurs as a consequence of both increased neuronal activity, and recruitment of additional neurons with subsequent sessions. These findings demonstrate that changes in dopamine over multiple-spaced sessions modulate cue-encoding neurons to generate lasting memories linked to alcohol experiences.

2.2 SIGNIFICANCE

Investigating the neural dynamics that occur during alcohol-cue memory formation is critical for understanding how cue associations can drive maladaptive behaviors. We found that reward-encoding dopamine neurons and cue-encoding cholinergic neurons increase their activity over trials in response to repeated cue-ethanol presentations, but not in response to repeated exposure to alcohol alone or a complex odor. These results fill a previous gap in the field of understanding how cue-alcohol associations impact reward circuits differently than alcohol or cue-alone.

2.3 INTRODUCTION

The ability to associate a reward with a cue is essential for an animal's survival, and dopamine has an established role in this process (Krause, 2015; Morand-Ferron, 2017). As cues become associated with a water, food, or sucrose reward, dopaminergic neural activity gradually shifts from signaling the actual reward to responding to the predictive cue associated with that reward (Amo et al., 2022; Cohen et al., 2012; Hsu et al., 2020; Schultz et al., 1997; Stuber et al., 2008). This phenomenon is evident across organisms, and within mammals, across several brain systems (Day et al., 2007; Lee et al., 2014; Lefner et al., 2022; Sias et al., 2024). Psychostimulants like alcohol, cocaine, and methamphetamines co-opt these neural mechanisms, leading to the formation of persistent cue-associated memories linked to the euphoric properties of intoxication (Bijoch et al., 2023; Di Chiara et al., 2004; Imperato & Di Chiara, 1986; Kufahl et al., 2005; Leyton et al., 2002; Mandeville et al., 2011; Volkow et al., 2004). Numerous studies have investigated the neurophysiological changes induced by alcohol exposure. Together they show that alcohol increases the activity of dopaminergic neurons (Brodie, 2002; Deehan et al., 2016; Ericson et al., 1998; Imperato & Di Chiara, 1986; Juarez et al., 2017; Koyama et al., 2007; Y. Liu, Montgomery, et al., 2020; Myrick et al., 2004; Sharma et al., 2024; Siciliano et al., 2016; Stuber et al., 2008; Weiss et al., 1996), and repeated exposure is reported to increase the resting membrane potential of dopaminergic neurons thereby increasing their excitability (Hopf et al., 2007; Mulholland et al., 2009; Tateno & Robinson, 2011).

Understanding how alcohol-associated cues modify an animal's internal representation of alcohol's rewarding properties is crucial to understanding the

mechanisms underlying intense cravings. Cues can trigger powerful cravings for alcohol (Jones et al., 2013; Pina & Williams, 2016), which may progressively evolve into problematic drinking patterns (Koob & Volkow, 2016), leading to harmful consequences for those afflicted (Evans-Polce et al., 2024; Patrick & Terry-McElrath, 2021; Varghese & Dakhode, 2022). However, little is known about how the addition of a cue might modulate alcohol's impact on dopaminergic circuitry dynamics. Further how these cues are encoded to form memories powerful enough to elicit maladaptive behavioral responses remains unclear. Understanding how dopaminergic circuits are responding to cue-ethanol pairings is critical for understanding problematic substance use.

Drosophila melanogaster offer a powerful model for investigating how neural dynamics within memory circuits change across multiple cue-alcohol pairings because of its well-characterized circuitry, expanding toolkit for circuitry dissection, cost-effectiveness, and short lifespan which enables faster experimental timelines—advantages not available in mammalian models (Aso, Hattori, et al., 2014; Fiala & Kaun, 2024; Hernandez et al., 2023; Margulies et al., 2005; Scaplen & Kaun, 2016). Indeed, *Drosophila* has a long history of being used as a model to study the neurological effects of alcohol from a molecular, genetic, and behavioral standpoint (Devineni & Heberlein, 2009; Heberlein, 2000; Scholz et al., 2000; Wolf & Heberlein, 2003; Xu et al., 2012), and more recent associative conditioning protocols provide an opportunity to investigate the development of alcohol-associative preference (Kaun et al., 2011; Nunez et al., 2018).

In *Drosophila*, activation of the cholinergic mushroom bodies (MB) and dopaminergic reward-encoding Protocerebral Anterior Medial (PAM) neurons are

required for the formation of odor-alcohol associative memories (Kaun et al., 2011; Scaplen et al., 2020). Adding a pharmacologically relevant dose of alcohol to an odor cue increases PAM dopamine neural activity throughout 10 minutes of presentation, whereas presentation of alcohol alone, or odors alone results in consistent neural activity throughout the presentation (Scaplen et al., 2020). However, it remains unclear how PAM dopamine neural activity changes in response to multiple-spaced pairing sessions, which is required for long-lasting memory.

Here, we implement three different paradigms to investigate the effects of cue-alcohol associations on MB and PAM neural dynamics (Figure 2.1D). To understand how alcohol in the presence of cues shapes the activity of reward circuitry, we used an associative paradigm (Figure 2.1D). We used a complex odor paradigm to discriminate the activity of PAM and MB neurons during association between two odors without alcohol (Figure 2.1D). Finally, we used a non-associative paradigm to dissociate the changes in PAM and MB dynamics in response to the presentation of alcohol and the presentation of a cue in the absence of an association (Figure 2.1D).

We show that dopamine neural activity, measured at the MB axon terminals, gradually increases across multiple sessions of alcohol-odor pairings. Similarly, MB axon terminals increase their activity and recruit additional MB neurons across multiple sessions of alcohol-odor pairing. This phenomenon was only evident when odors were paired with pharmacologically relevant doses of alcohol, and not when alcohol alone was presented to the fly, or when two different odors were presented simultaneously. Together, these data suggest that during learning, cues that predict alcohol increase the representation of alcohol's rewarding properties by increasing dopaminergic activity.

Additionally, the recruitment of additional cue-responding neurons potentially strengthens the association between cues and intoxicating doses of alcohol. These results further our understanding of how cue-ethanol pairings shape neural dynamics within memory circuitry to generate long-lasting memories.

2.4 RESULTS

2.4.1 - Sequential odor-ethanol pairings increase the activity of dopaminergic PAM neurons

Previous work from our lab showed that dopaminergic PAM neurons are required for the formation of cue-ethanol memory, and increase their activity when ethanol is added to an odor stimulus (Scaplen et al., 2020). We hypothesized that compared to an unpaired odor, alcohol-odor pairings would increase the activity of the PAM neurons, and this response would be enhanced across training sessions. To test this hypothesis, we exposed flies expressing GCaMP7b under the control of the PAM neuron driver line R58E02 to three separate sessions of 10 minutes of an odor alone, followed by 10 minutes of a different odor paired with psychoactive doses of ethanol (Figure 2.2A, B). Each session was separated by a 50-minute intersession interval. Calcium activity was simultaneously recorded at the axon terminals and cell bodies.

In the first training session (Figure 2.2C), the unpaired odor (Odor A) initially increased the activity of PAM neurons (Figure 2.2Di; Baseline vs first 15 seconds of Odor A $W = 78$, $p < 0.001$). However, continued odor exposure over 10 minutes inhibited the activity of PAM neurons (Figure 2.2Ei, odor alone 1st minute vs last minute $W = 5$, $p = 0.0195$). On the other hand, the ethanol-odor pairing did not increase activity upon initial

exposure (Figure 2.2Di, last 15 seconds of odor alone vs first 15 seconds of ethanol-odor pairing $W = 19$, $p = 0.583$). Continued ethanol-odor exposure inhibited the activity of PAM neurons (Figure 2Eii; Session 1 alcohol-odor pairing 1st minute vs Last minute $W = 0$, $p = 0.00195$).

During the second training session (Figure 2F), the unpaired odor again caused an initial increase in activity (Figure 2.2Gi, last 15 seconds of baseline vs first 15 seconds of odor alone $W = 103$, $p = <0.001$). Following the initial response, unpaired odor presentation once again elicited a decrease in activity over the 10 minutes (Figure 2.2Hi; odor alone first minute vs last minute $W = 65$, $p = 0.042$). Ethanol-odor pairing generated an increase in activity but was not statistically significant (Figure 2.2Gi; last 15 seconds of odor alone vs first 15 seconds of ethanol-odor pairing $W = 59$, $p = 0.068$), followed by a reduction in calcium activity over 10 minutes (Figure 2.2Hii; Session 2 ethanol-odor pairing First-minute min vs Last minute $W = 21$, $p = 0.049$).

Finally, during the third training session (Figure 2.2I), the unpaired odor once again causes an initial increase in activity (Figure 2.2Ji; last 15 seconds of baseline vs first 15 seconds of odor alone $W = 85$, $p = 0.005$), however, this activity remained constant during the 10 minutes of presentation as opposed to the first and third sessions (Figure 2.2Ki; first-minute odor alone vs. last-minute odor alone $W = 23$, $p = 0.502$). Ethanol-odor pairing also generated an initial increase in activity (Figure 2.2Ji; last 15 seconds odor alone vs first 15 seconds of ethanol-odor pairing $W = 63$, $p = 0.049$), that was once again followed by a decrease in activity over 10 minutes (Figure 2.2Kii, first minute vs. last-minute $W = 71$, $p = 0.0245$). Similar to the second session, the response to ethanol

paired with odor was not significantly different than the response to the unpaired odor (Figure 2.2Kiii; $W = 34$, $p = 0.204$).

A Friedman test was conducted to determine whether activity significantly changed across sessions within the odor-alone condition. The results show a significant difference (Figure 2.2Li, χ^2 (df) = 8.71, $p = 0.013$; Session 1 vs Session 2 posthoc Dunn $p = 0.042$, Session 1 vs Session 3 posthoc Dunn p -value = 0.024). Similarly, alcohol-odor pairing potentiates the activity of PAM axon terminals over the three sessions (Figure 2.2Lii; χ^2 (df) = 7.8, $p = 0.018$, Session 1 vs Session 3 Nemenyi posthoc = 0.022).

We hypothesized that PAM potentiation of activity across sessions was due to a gradual recruitment of PAM neurons over subsequent sessions as opposed to an increase in activity of the same PAM neurons. To test this, we extracted signals from single cells and tracked them through multiple recordings. Cells that were not overlapped with other cells were selected for analysis. We observed heterogeneity in patterns of activity in response to both odor and ethanol paired with an odor (Figure 2.2Ni-iii). To categorize cellular activity profiles, we segregated them into three groups. Cells that had at least a 10% increase in activity across a session were considered “responders”, cells that had their activity decreased by 10% were considered “suppressed”, and activity profiles that did not increase or decrease by 10% were considered “non-responders” (see Methods).

During session 1 (Figure 2.2Ni), most of the recorded cells responded with an increase in activity to the presentation of odor alone (Figure 2.2Oi, 55 responders, 1 suppressed, 22 non-responders). Most cells also responded to ethanol paired with odor, although

there were more suppressed cells in this condition (Figure 2.2Oi; 65 responders, 9 suppressed, 14 non-responders). A Fisher's exact test was conducted to examine the distribution of response profiles between odor and odor paired with ethanol. The results revealed a significant difference (Figure 2.2Oi; $p = 0.003$).

During session 2 (Figure 2.2Nii), presentation of odor alone and ethanol paired with odor again resulted in most of the recorded cells increasing their activity (Figure 2.2Oii; 51 responders for odor alone, 47 responders for ethanol paired with odor), with a not significant increase in suppressed cells for ethanol paired with an odor compared to odor alone (Figure 2.2Oii; 14 suppressed cells for odor alone, 23 suppressed cells for ethanol paired with odor; Fisher's exact test p -value = 0.213).

In the final session (Figure 2.2Niii), most of the recorded cells were still responding with an increase in activity to odor alone (Figure 2.2Oiii; 65 responders) and odors paired with ethanol (Figure 2.2Oiii; 62 responders). Once more, ethanol paired with odor had a higher number of suppressed cells (Figure 2.2Oiii; 17 suppressed) compared to odor alone (Figure 2.2Oiii; 9 suppressed cells). Despite these differences, a Fisher's exact test revealed that the overall response distributions were not statistically significantly different (Figure 2.2Oiii; $p = 0.171$).

To gain a better understanding of how the numbers of responders, suppressed and non-responders evolve, we aimed to examine any potential changes within each condition across the sessions. We compared the distribution of responders, suppressed, and non-responders within session 1 to session 3 for odor alone. A Fisher's exact test revealed significant differences between the two distributions (Figure 2.2Oi and iii; $p = 0.013$).

Specifically, session 3 contained more suppressed cells than session 1. Conversely, we

observed no differences between the distribution of cellular responses for ethanol paired with odor and a Fisher's exact test reported no significant differences (Figure 2.2O*i* and *iii*; $p = 0.063$).

To measure whether cells were recruited, we isolated cells that could reliably be tracked over all three sessions (supplemental Figure 2.S1A) and categorized these cells as recruited (10% increase or more from session 1 to session 3), inhibited (10% decrease or more from session 1 to session 3), or consistent (see methods). In contrast to our hypothesis, we did not observe greater recruitment of PAM cell bodies in response to alcohol-odor pairing compared to odor alone (Figure 2.2P; 16 recruited, 15 inhibited, 3 consistent for odor A, 17 recruited, 14 inhibited, 3 consistent for ethanol paired with odor). Of the 17 cells that were categorized as recruited for ethanol paired with odor, 13 of them were responders during the initial exposure. These data suggest that the potentiation across sessions seen at PAM axons is due to active cells becoming more active.

Collectively these results suggest the ethanol-odor pairing increases the activity of dopaminergic axon terminals across sessions. Interestingly, rather than the recruitment of dopaminergic cells to respond to odors paired with ethanol, we saw that this increase in the axon terminals is likely due to initially active dopamine neurons increasing their activity during training in response to the ethanol-odor pairing.

2.4.2 - Odor-ethanol pairing potentiates axon terminals of MB neurons and recruits MB neurons

The changes observed within the axon terminals of PAM neurons during associative conditioning could have functional consequences for the postsynaptic MB neurons (Figure 2.3B). We hypothesized that alcohol paired with odor would also increase calcium dynamics within MB neurons when compared to the presentation of odor alone and generate potentiation of the axon terminals across multiple-spaced sessions.

During the first session (Figure 2/3C), calcium dynamics measured at the MB axon terminals increased in response to the unpaired odor (Figure 2.3Di; baseline vs first 15 seconds of odor alone $W = 87$, $p = <0.001$). Ethanol-odor pairing also elicited an increase in response initially (Figure 2Di; last 15 seconds of odor alone vs. first 15 seconds of ethanol-odor pairing $W = 91$, $p = <0.001$), however, this was followed by a decrease throughout the 10 minutes of presentation (Figure 2.3Ei, first minute vs last minute $W = 7$, $p = 0.0093$).

During the second session (Figure 2.3F), again we observed that the unpaired odor elicited an initial increase in activity (Figure 2.3Gi; baseline vs. first 15 seconds odor alone $W = 91$, $p = <0.001$) that was consistent throughout the 10 minutes of exposure (Figure 3Hi). Ethanol-odor pairing also resulted in an initial increase (Figure 2.3Gi; last 15 seconds of odor alone vs first 15 seconds of ethanol-odor pairing $W = 91$, $p = <0.001$), but was again followed by a decrease in activity during exposure (Figure 2.3Hii, ethanol-odor pairing first minute vs last minute $W = 60$, $p = 0.016$). However, when comparing the first minute of activity for both the unpaired odor and the ethanol-odor pairing, we find that the ethanol-odor pairing increased activity more than the

unpaired odor (Figure 3Hi and ii, odor alone first minute vs ethanol-odor first minute $W = 70$, $p = 0.012$). The increase observed during the first minute is not maintained when comparing the average activity across the ten minutes (Figure 2.3H, odor alone 10-minute average vs ethanol-odor pairing 10-minute average $W = 51$, $p = 0.38$).

During the final session (Figure 2.3I), the unpaired odor once again caused an increase in activity (Figure 2.3Ki; baseline vs. first 15 seconds odor alone $W = 91$, $p < 0.001$) that remained constant throughout the 10 minutes (Figure 2.3Ki). Ethanol-odor pairing also generated an increase in initial activity (Figure 3Ji; last 15 seconds of odor alone vs first 15 seconds of ethanol-odor pairing $W = 90$, $p < 0.001$), however, this did not significantly decrease across the 10 minutes, unlike sessions 1 and 2 (Figure 2.3Kii, Ethanol-odor pairing first minute vs last minute $W = 18$, $p = 0.10$). Additionally, overall activity throughout the 10 minutes was higher when comparing average activity during the 10 minutes of the pairing to the unpaired odor (Figure 2.3Kiii, odor alone 10-minute average vs Ethanol-odor pairing 10-minute average $W = 68$, $p = 0.021$)

Using a Friedman test to evaluate calcium dynamics across sessions within the same stimulus condition, we observe that axon terminal calcium dynamics remain consistent in response to Odor A (Figure 2.3Li, Friedman $\chi^2 = 3.600$, $p = 0.165$), but with the ethanol-odor pairing there was a significant increase in activity when comparing session 1 to session 3 (Figure 2.3Lii; $\chi^2 = 8.4$, $p = 0.015$, Session 1 vs Session 3 posthoc Nemenyi $p = 0.0185$) suggesting that alcohol-odor pairing is potentiating the activity of MB neurons during cue-ethanol learning. Thus, we provide evidence that sequential ethanol-odor pairings enhanced the activity of MB axon terminals compared to odor

alone, as seen in session 3. Furthermore, the activity in response to odor-alcohol pairing increases throughout the three sessions.

To gain a detailed understanding of which MB cells were active, we analyzed the calcium dynamics of MB cell bodies. It is possible that the increase in calcium dynamics in response to odors paired with intoxicating doses of alcohol observed in the MB axon terminals was a result of an increase in the number of cue-responsive MB neurons. Thus, we hypothesized that alcohol-associative training would increase calcium activity within cell bodies and expected to see a large increase in the number of cells responding to the paired condition.

In session 1 (Figure 2.3Ni, $n = 125$ cells), there was a significant difference in the distribution of cell activity profiles for odor alone compared to alcohol-odor pairing (Figure 2.3Oi, $N=125$; $\chi^2, p = 0.015$). With odor alone, 50% of cells were identified as responders whereas with alcohol-odor pairing only 33% of cells were identified as responders (Figure 2.3Oi). Additionally, only 26% of the cells were suppressed by odor presentation alone, but 40% were suppressed during the odor-alcohol pairing.

During session 2 (Figure 2.3Nii, $n = 161$ cells), the activity profiles dramatically shifted with both odor alone and odor-alcohol pairing generating mostly responders (Figure 3Oii; Session 2 odor alone responders = 88%, Session 2 ethanol-odor pairing responders = 96%). However, a Fisher's exact test revealed that the number of non-responders within odor alone was greater than the alcohol-odor pairing (Session 2 odor alone non-responders = 8.6%, odor-alcohol pair non-responders = 2.5%, $p = 0.0281$).

During session 3 (Figure 2.3Niii; $n = 140$ cells), the percentage of responders did not change during odor-alcohol pairing whereas, within the odor-alone condition, a significant number of cells stopped responding. (Figure 2.3Oiii; Session 3 odor alone responders = 81%, odor-alcohol responders = 97%, $p < 0.0001$).

Comparing the distributions of responses for session 1 to session 3, we observed that odor alone had a large increase in the number of responsive cells from session 1 to session 3. A Fisher's exact test revealed a statistically significant distribution between the two time points (Figure 2.3Ni and Niii; $p < 0.0001$). Similarly, ethanol-odor pairing also caused a large increase in the number of responding cells compared to session 1 (Figure 2.3P; $p < 0.0001$).

When single cells were tracked across the three sessions (Supplemental Figure 2.1B), we observed that the paired condition recruits more cells when compared to the presentation of odor alone (Figure 2.3P; $p = 0.0013$, $n = 43$ cells). These data provide support for our hypothesis that alcohol-odor pairing is uniquely recruiting cells to respond to cues. The increase in activity observed at the axon terminals during learning is likely due to the recruitment of more cue-encoding neurons during spaced, sequential exposures to ethanol-odor pairings.

2.4.3 - Compartment-Specific Responses to Ethanol-Odor Pairing

Mushroom body axons can be segregated into compartments based on the innervation patterns of dopaminergic neurons and extrinsic Mushroom Body Output Neurons (MBON; Aso et al., 2014; Tanaka et al., 2008). The balance of activity across these compartments dictates approach and avoidance behaviors and is important for

associative learning. (Aso, Sitaraman, et al., 2014; Cohn et al., 2015; Hattori et al., 2017; Oswald & Waddell, 2015; Scaplen et al., 2020; Tanaka et al., 2008; Wang et al., 2023; Yamagata et al., 2015). We previously demonstrated that the encoding of alcohol memory requires the activity of the entire dopaminergic population encompassed in the R58E02 driver line (Scaplen et al., 2020). Thus, for this study, we investigated compartments of the horizontal lobe where the majority of the *R58E02* dopaminergic axon terminals innervate. We outlined compartments to best match the innervation patterns represented in Aso et al., 2014a, and measured responses occurring within the third session of the associative paradigm. We focused on compartments innervated by the PAM neurons ($\gamma 3\beta^1$, $\gamma 4$, $\gamma 5\beta^2a$). Because of the established role of the β^2a compartment in the expression of alcohol memory (Scaplen et al., 2020) we hypothesized that this region would respond greater to ethanol-odor pairings as compared to odor alone.

When measuring PAM $\gamma 3\beta^1$ axon responses during the third session (Figures 2.4Ci and ii), we observed a consistent response to odor alone (Figure 2.4Di), however, there was a decrease in activity during the 10 minutes of exposure for ethanol-paired with odor (Figure 2.4Dii; ethanol-odor first minute vs last minute $W = 54$, $p = 0.034$).

However, overall activity during the 10 minutes of exposure was similar for odor alone and the ethanol-odor pairing (Figure 2.4Diii). This pattern was also observed within the $\gamma 3\beta^1$ region of the MB axon terminals (Figure 2.4Eii). Odor-alone responses remained stable (Figure 2.4Fi), but ethanol-odor pairing caused a decrease in activity throughout the 10 minutes of exposure (Figure 2.4Fii; ethanol-odor first minute vs last $W = 48$, $p = 0.032$).

There were similar responses to both odor alone and ethanol-odor pairing during the third session for the $\gamma 4$ region of the PAM axon terminals (Figure 2.4Gi and Figure 2.4Gii). There were no observed changes throughout the ten minutes in response to odor alone or ethanol-odor pairings (Figure 2.4Hi and 2.4Hii). Additionally, there were no differences in 10-minute average activity between odor alone and ethanol-odor pairing (Figure 2.4Hiii). Odor alone generated constant activity levels within the $\gamma 4$ of the MB axon terminals (Figure 2.4Iii, 2.4Ji), but ethanol-odor pairing caused a decrease over the 10 minutes of exposure (Figure 2.4Jii; ethanol-odor first minute vs last minute $W = 50$, $p = 0.024$). Average activity remained similar between odor alone and ethanol-odor pairing (Figure 2.4Jiii).

Within the $\gamma 5\beta'2a$ region of the PAM axon terminals, we observed similar responses to odor alone and ethanol odor pairing, with no changes in levels of activity during exposure (Figure 2.4Ki – 4Liii). However, at the $\gamma 5\beta'2a$ region of the MB axon terminals (Figure 2.4Mi, and 2.4Mii) we observed decreases in activity during the 10 minutes of exposure for odor alone (Figure 2.4Ni; odor alone first minute vs last minute $W = 46$, $p = 0.042$) and ethanol odor pairing (Figure 2.4Nii; ethanol-odor first minute vs last minute $W = 50$, $p = 0.024$). The overall responses were similar between the odor alone and ethanol odor pairing (Figure 2.4Niii). Data suggest compartment-specific responses to odor alone and ethanol odor pairing conditions, however, perplexingly, none of the compartments demonstrated an increase in response to ethanol-odor pairing compared to odor alone in the third session.

2.4.4 - A non-associative paradigm does not generate increased calcium dynamics within dopaminergic axon terminals or recruit dopaminergic neurons

Previous work demonstrates that the presentation of intoxicating doses of alcohol unpaired from odor cues is not sufficient to generate odor-induced alcohol memory (Kaun et al., 2011). We hypothesized that alcohol in the absence of an odor cue would not be sufficient to generate potentiation of PAM axon terminals with repeated exposure. To test this, we used a non-associative paradigm (Figure 2.5A, Kaun et al., 2011), while recording *R58E02>GCaMP7b* fluorescent signals (Figure 2.5B). Like the associative paradigm, we exposed the flies to 10 minutes of each of the stimuli (Figure 2.5A). A ten-minute gap of air was presented between odor B and ethanol to ensure no association was made between the odors and ethanol.

During session 1, we observed that the PAM neurons respond to the presentation of both odors as well as ethanol (Figure 2.5C). Presentation of the first and second odors increased activity (Figure 2.5Di; baseline vs odor a first 15 seconds $W = 54$, $p = 0.004$, last 15 seconds of odor a vs first 15 seconds of odor B $W = 41$, $p = 0.037$) that was sustained throughout the 10 minutes of presentation (Figure 2.5Ei, 2.5Eii). Ethanol alone also generated an increase initially (Figure 2.5Di; pre-ethanol baseline vs. first 15 seconds of ethanol $W = 47$, $p = 0.014$), but was followed by a decrease in activity throughout the ten minutes of presentation (Figure 2.5Eiii; Session 1 $W = 16$, $p = 0.015$). Strikingly, overall activity between the three stimuli (odor 1, odor 2, and ethanol) did not differ (Figure 2.5Eiv; Friedman $\chi^2 = 0.800$, $p = 0.710$).

During session 2, the presentation of the first odor increased activity (Figure 2.5Gi; baseline vs first 15 seconds of odor A $W = 55$, $p = 0.002$). This increase in activity was

stable throughout the ten minutes of stimulation (Figure 2.5Hi). Presentation of the second odor on the other hand generated an increase in activity (Figure 5Gi; last 15 seconds of odor A vs first 15 seconds of odor b $W = 41$, $p = 0.037$) that gradually decreased throughout the ten minutes of presentation (Figure 2.5Hii; Odor B 1st minute vs last minute $W = 49$, $p = 0.010$). Ethanol alone again caused an increase in activity (Figure 5Gi; last 15 seconds of pre-ethanol vs first 15 seconds of ethanol $W = 53$, $p = 0.004$) that gradually decreased to below baseline levels of activity (Figure 2.5G). There was a significant decrease when comparing activity during the first minute to the last minute (Figure 2.5Hiii; Session 2 $W = 49$, $p = 0.009$). Overall activity levels between the conditions did not differ (Figure 2.5Hiv; Friedman $\chi^2 = 0.200$, $p = 0.973$).

For the final session, the first odor produced an increase in activity (Figure 2.5Ji; baseline vs. first 15 seconds odor A $W = 55$, $p = 0.0002$) that remained constant throughout the ten minutes of exposure (Figure 2.5Ki), whereas the second odor caused an increase in activity that was not statistically significant (Figure 2.5Ji; last 15 seconds odor A vs first 15 seconds odor B $W = 46$, $p = 0.064$) and slightly decreased throughout the 10 minutes (Figure 2.5Kii; Odor B first minute vs last minute $W = 45$, $p = 0.195$). Again, ethanol caused an increase in activity (Figure 2.5Ji; last 15 seconds pre-ethanol exposure vs. first 15 seconds ethanol $W = 54$, $p = 0.004$) that was followed by a return to baseline levels of activity. The decrease from ethanol exposure's first minute to the last minute was significant (Figure 2.5Kiii; alcohol alone Session 3 $W = 5$, $p = 0.019$). As with sessions 1 and 2, there was no difference in overall activity between the different stimuli (Figure 2.5Kiv; Friedman $\chi^2 = 0.800$, $p = 0.710$).

Looking across sessions we see that both odors alone and alcohol alone were insufficient to drive changes in activity across sessions (Figure 2.5Li-5Liii; odor A Friedman $\chi^2 = 1.400$, $p = 0.601$; odor B Friedman $\chi^2 = 1.400$, $p = 0.601$; odor A Friedman $\chi^2 = 0.800$, $p = 0.710$).

Altogether, data demonstrate that alcohol in the absence of an odor cue does not increase the activity of PAM axon terminals relative to the presentation of odor alone within each of the three sessions. Additionally, both alcohol alone and odor alone did not drive increases in activity across sessions as observed with the associative conditioning.

We hypothesized that we would not observe the recruitment of PAM neurons during the non-associative paradigm based on the lack of changes observed across sessions for the axon terminal.

During the first session (Figure 2.5Ni), both the first odor and the second odor had similar numbers of cells in each category (Figure 2.5Oi; 32 responders, 27 suppressed, 28 non-responders for odor A, 24 responders, 39 suppressed, 24 non-responders for odor B). Conversely, most of the cells were suppressed by alcohol alone (Figure 2.5Oi; 28 responders, 53 suppressed, 6 non-responders). When comparing the distributions, a Fisher's exact test revealed a significant difference between conditions (Figure 2.5Oi; $p = <0.0001$).

During the second session (Figure 2.5Nii), the cellular responses to the first odor were mostly divided into responders and non-responders (Figure 2.5Oii; 37 responders, 12 suppressed, 31 non-responders for odor A). The second odor generated an even

distribution between responders, suppressed, and non-responding cells (Figure 2.5Oii; 27 responders, 28 suppressed, 25 non-responders for odor B). Once again, ethanol had many inhibited cells relative to responding and non-responding cells (Figure 2.5Oii; 27 responders, 34 suppressed, 19 non-responders for ethanol alone). Similar to session 1, a Fisher's exact test revealed that these distributions were also significantly different (session 2 $p = 0.002$).

In the final session (Figure 2.5Niii; $n = 73$), we observed that most cells were not responding to the first odor (Figure 2.5Oi; 20 responders, 7 suppressed, 46 non-responders). The second odor cellular responses were predominantly responders and suppressed cells (Figure 2.5Oiii; 34 responders, 26 suppressed, 13 non-responders). Suppression of cell body activity was most frequently observed during the final exposure to ethanol alone (Figure 2.5Oiii; 60 responders, 15 suppressed, 68 non-responders). A Fisher's exact test revealed that the distribution of cellular activity was again significantly different between the three conditions (Figure 2.5Oiii; $p = <0.0001$).

From session 1 to session 3, a Fisher's exact test revealed a significant increase in suppressed cells for the first odor (Figure 2.5Oi and 2.5Oiii; $p < 0.0001$). The second odor also significantly increased the number of suppressed cells when compared to the first odor, resulting in significantly different distributions of responses (Figure 2.5Oi and 2.5Oiii, $p = 0.0404$). Similarly, ethanol exposure increased the number of suppressed cells in session 3 as compared to session 1, resulting in significant differences between the populations during the two different time points as revealed by a Fisher's exact test (Figure 2.5Oi and 2.5Oiii; $p = 0.0390$).

Looking at the subset of cells that were tracked across all three sessions (supplemental Figure 1E), we observed that for the first odor, inhibited cells comprised the largest portion of the distribution (Figure 2.5P; 5 recruited, 14 inhibited, 10 consistent). For the second odor, consistent cells were the most numerous (Figure 2.5P; 7 recruited, 5 inhibited, 17 suppressed). Similarly, ethanol alone was unable to recruit many cells to respond to ethanol (Figure 2.5P; 5 recruited, 7 inhibited, 10 consistent). This data suggests that alcohol in the absence of an odor cue is not sufficient to drive changes in calcium dynamics within the cell bodies of the PAM neurons.

2.4.5 - A non-associative paradigm does not generate increased calcium dynamics within MB axon terminals or recruit neurons

Like the PAM axon terminals, associative training generated an increase in calcium dynamics at the MB axon terminals across sessions. Based on our findings within the PAM axon terminals, we hypothesized that alcohol in the absence of an odor cue would not be sufficient to drive dynamic changes within MB axon terminals. Using *MB010B>GCaMP7b* flies, we observed MB calcium dynamics during non-associative training (Figure 2.6A). Both odors (Figure 2.6Dii; baseline vs odor a first 15 seconds $W = 54$, $p = 0.004$, last 15 seconds of odor a vs first 15 seconds of odor B $W = 52$, $p = 0.0186$), as well as ethanol alone increased MB activity (Figure 2.6Di, last 15 seconds of pre-ethanol baseline vs. first 15 seconds of ethanol $W = 64$, $p = 0.002$), with no significant differences between the first minute and last minute of activity (Figure 2.6Ei-2.6Eiii). We observed that the overall activity was consistent between the odors and ethanol alone (Figure 2.6Eiv; Friedman chi-square statistic = 1.273, $p = 0.629$).

Again, in session 2, both odor A and ethanol alone generated an increase in activity (Figure 2.6Gi; baseline vs odor a first 15 seconds $W = 66$, $p = 0.001$, last 15 seconds of pre-ethanol baseline vs. first 15 seconds of ethanol $W = 66$, $p = 0.001$) that was stable throughout the 10 minutes of exposure (Figure 2.6Hi and 2.6Hiii). Odor B, on the other hand, was not able to generate a significant increase from prior levels of activity (last 15 seconds of odor a vs first 15 seconds of odor B $W = 38$, $p = 0.101$,) The overall activity between the conditioned remained similar as well (Figure 2.6Hiv; Friedman $\chi^2 = 0.181$, p -value = 0.9761).

During the final session, both odors alone generated an increase in response (Figure 2.6Ji; baseline vs odor A first 15 seconds $W = 64$, $p = 0.002$, last 15 seconds of odor A vs first 15 seconds of odor B $W = 46$, $p = 0.042$) as in sessions 1 and 2, however this increase in activity was not sustained throughout the 10 minutes of exposure. When comparing the first minute to the last minute of activity for the first odor and the second odor we observed a decrease during the final minute of exposure (Figure 2.6Ki and 2.6Kii; odor a $W = 3$, $p = 0.005$; odor B $W = 10$, $p = 0.042$). Alcohol alone did not experience a significant decrease from the initial increase in activity (Figure 2.6Ji; last 15 seconds of pre-ethanol exposure vs. first 15 seconds of ethanol $W = 66$, $p = 0.001$) when comparing it to the final minute of exposure (Figure 2.6Kiii; $W = 15$, $p = 0.123$). (Figure 2.6Kiv; Friedman $\chi^2 = 1.400$, $p = 0.601$).

Similar to the responses recorded at the dopamine neuron axon terminals, odor alone was also unable to increase the activity of the MB axon terminals across the three-spaced sessions (Figure 2.6Li and 2.6Lii; Odor A Friedman $\chi^2 = 0.181$, $p = 0.976$; Odor B Friedman $\chi^2 = 0.181$, $p = 0.976$). Similarly, alcohol in the absence of an odor cue did

not cause potentiation of MB axon terminals with repeated exposure (Figure 2.6Loii; Friedman $\chi^2 = 0.545$, $p = 0.844$). As predicted, repeated exposure to alcohol in the absence of an odor cue did not cause an increase in activity relative to odor alone, nor did it increase activity in response to alcohol alone across the spaced sessions.

Because we did not observe changes within calcium dynamics in response to alcohol alone, we hypothesized that alcohol alone would not be sufficient to drive increased responsivity and recruitment of MB cells. During the first exposure (Figure 2.6Ni; $n = 122$), most of the cells respond to the presentation of the first odor (Figure 2.6Oi, 69 responders, 2 suppressed, 51 non-responders for odor A), the second odor (Figure 2.6P, 77 responders, 6 suppressed, 39 non-responders for odor B), and ethanol alone (Figure 2.6P, 87 responders, 9 suppressed, 26 non-responders for ethanol alone).

Despite most of the cells being responders, a Fisher's exact test revealed that the distributions of cellular responses were significantly different during session 1 (Figure 2.6Ni; $p = 0.005$).

For the second session (Figure 2.6Nii; $n = 119$), cellular activity in response to the first odor was comprised of mostly non-responsive cells (Figure 2.6Oii; 33 responders, 5 suppressed, 81 non-responders), whereas with the second odor, there was a more even split between responsive and non-responsive cells (Figure 2.6Oii; 49 responders, 14 suppressed, 56 non-responders). Ethanol had slightly more suppressed cells compared to both odors but was still mostly comprised of responsive and non-responsive cells (Figure 2.6Oii; 49 responders, 21 suppressed, 49 non-responders). A Fisher's exact test revealed that the distribution of cellular activity between the three stimuli was different (Figure 2.6P; $p < 0.0001$)

In the last session (2.6Niii; $n = 144$), the first odor still did not generate an increase in activity from a majority of the cells (Figure 2.6Oiii; 59 responders, 2 suppressed, 82 non-responders). For the second odor, most of the cellular responses were either responders or non-responders as observed in session 2 (Figure 5Oiii; 62 responders, 9 suppressed, 72 non-responders). Interestingly, ethanol alone had the a higher number of suppressed cells, but most of the responses were split between responders and non-responders (Figure 2.6Oiii; 60 responders, 15 suppressed, 68 non-responders). Again, a Fisher's exact test revealed that the distribution of the cellular responses was different between the stimuli ($p = 0.016$).

In the associative paradigm, we saw recruitment of MB neurons across sessions, however, the same phenomenon was not evident when tracking subsets of cells during the presentation of alcohol alone (Supplementary Figure 2.S1F). For both odors, most of the cells were inhibited (Figure 2.6P; 5 recruited, 38 inhibited, 4 consistent for odor A, 8 recruited, 34 inhibited, 5 consistent). Interestingly, alcohol also caused inhibition of most of the tracked cells (Figure 2.6P; 5 recruited, 36 inhibited, 6 consistent). We observed no significant difference between the distribution of cellular responses for the three conditions (odor 1, odor 2, ethanol, Figure 2.6P; $p = 0.845$). This increase in inhibited cells is likely due to responsive cells becoming non-responsive. Overall, alcohol presentation in the absence of an odor cue does not recruit cue-encoding neurons. Like with PAM neurons, we did not observe potentiation of MB axon terminal dynamics or recruitment of MB neurons when flies are exposed to alcohol in the absence of an odor cue. This further underscores that associations between odor and

alcohol are driving distinct dynamics at both the axon terminals and cell bodies, ultimately contributing to the formation of long-lasting, appetitive memories.

2.4.6 - Complex odor presentation does not potentiate dopamine axon terminal responses or recruit dopaminergic neurons

To investigate whether the increase in activity during the associative paradigm within the dopaminergic and MB neurons is a general memory process, we presented the *R58E02>GCaMP7b* flies with three spaced sessions of 10 minutes of odor alone (Odor A), followed by 10 minutes of two different odor presented simultaneously, termed a complex odor (Odor B and Odor C; Figure 2.7A). This methodology allowed us to delineate the effects of ethanol-odor pairing from general associative learning mechanisms within dopaminergic circuits. We hypothesized that a complex odor would not be sufficient to drive increased activity within dopaminergic axon terminals relative to odor alone, and would not be able to drive the recruitment of dopaminergic neurons. During session 1 (Figure 2.7C), we observed that the presentation of odor alone was able to increase the activity of the dopaminergic axon terminals (Figure 2.7Di; baseline vs first 15 seconds of odor alone $W = 47$, $p = 0.014$), that remained constant throughout the 10 minutes (Figure 2.7Ei). Likewise, the complex odor elicited a consistent increase in activity (Figure 2.7Di; last 15 seconds Odor A vs. first 15 seconds Odor B + Odor C $W = 52$, $p = 0.010$) throughout the 10 minutes (Figure 2.7Eii). Additionally, the activity during exposure to the complex odor did not differ from the activity during odor alone (Figure 2.7Eiii).

During session 2 (Figure 2.7F), both odor alone and the complex odor again generated a consistent increase in activity (Figure 2.7Gi; baseline vs first 15 seconds of Odor A, $W = 55$, $p = 0.002$, last 15 seconds of Odor A vs first 15 seconds of Odor B + Odor C $W = 39$, $p = 0.049$) across the 10 minutes of exposure (Figure 2.7Hi and 2.7Hii). There were no observed differences between the activity during odor alone and the activity during exposure to the complex odor (Figure 2.7Hiii).

During session 3 (Figure 2.7I), again odor alone elicits an increase in activity (Figure 2.7Ji; baseline vs first 15 seconds of Odor A, $W = 50$, $p = 0.019$) that remains consistent throughout the 10 minutes of presentation (Figure 2.7Li). In contrast, the complex odor initially caused an increase in activity compared to the end of Odor A (Figure 2.7Ji; last 15 seconds of Odor A vs first 15 seconds of Odor B + Odor C, $W = 49$, $p = 0.027$) that remained below baseline throughout the 10 minutes of exposure (Figure 2.7J and 2.7Ki). Activity in response to complex odor did not differ significantly from odor alone (Figure 2.7Kiii). Additionally, there was no significant difference in activity across sessions in response to odor A or the complex odor (Figure 2.7Li and 2.7Lii; Odor A, $\chi^2 = 0.200$, $p = 0.974$, Complex Odor, Friedman $\chi^2 = 0.600$, $p = 0.830$). These data demonstrate that, in contrast to what was observed with ethanol-odor pairings, there is no change occurring within the axon terminals of the dopaminergic neurons in response to repeated, spaced exposure to a complex odor. Taken together, this suggests that the dopamine neural dynamic changes in response to ethanol-odor pairing are distinct from non-associative experiences and a general memory process.

We hypothesized that the lack of change observed at the axon terminals of the dopaminergic neurons in response to repeated, spaced exposure to a complex odor

would be mirrored by similar levels of cell body activity and cell body recruitment in response to odor alone and complex odor.

During session 1 (Figure 2.7Ni; $n = 30$), most of the cells are responding to both odor alone (24 cells out of 30) and the complex odor (29 cells out of 30), and a Fisher's exact test revealed no significant difference in the distribution of the responders, suppressed cells, and non-responders (Figure 2.7Oi; session 1 $p = 0.102$).

Our observations for the second session (Figure 2.7Nii; $n = 40$), showed most of the cells respond to both odor alone (Figure 2.7Oii; 28 responders, 1 suppressed, 11 non-responders) and the complex odor (Figure 2.7Oii; 28 responders, 3 suppressed, 9 non-responders). A Fisher's exact test revealed no significant difference in the distribution of cellular activity (Figure 2.7Oii; $p = 0.681$).

During session 3 (2.7Niii; $n = 29$), there was an increase in the number of suppressed cells for both the odor alone (Figure 2.7Oiii, P; 7 suppressed cells of 29) and complex odor conditions (Figure 2.7Oiii; 8 suppressed cells of 29). However, many of the cells were still responding (Figure 2.7Oiii; odor A responding cells: 15 of 29; complex odor responding cells: 15 of 29). The increase in suppression of cellular responses was common to both stimuli. A Fisher's exact test revealed no differences in the distribution of cellular activity between responding, suppressed, and non-responding cells (Figure 2.7Oiii; $p = 0.886$).

When comparing the ratio of responders, suppressed, and non-responders from session 1 to session 2 for odor alone and the ethanol-odor pairing, a Fisher's exact test revealed that the distribution of responses was significantly different (Figure 2.7Oi and

2.7Oiii; $p = 0.023$). Notably, there were more suppressed cells in the odor-alone condition. Likewise, the increase in the number of suppressed cells relative to non-responders and responders was also obvious between session 3 and session 1. A Fisher's exact test revealed that the distribution of responses in session 3 and 1 were significantly different (Figure 2.7Oi and 2.7Oiii; $p < 0.0001$)

When comparing activity from session 3 to session 1 in a subset of cells tracked across all three sessions (Supplemental Figure 2.S1C), we find that most of the cells were inhibited by repeated, spaced exposure to odor alone (Figure 2.7P; 6 recruited, 10 inhibited 3 consistent) and the complex odor (Figure 2.7P; 5 recruited, 12 inhibited, 2 consistent). A Fisher's exact test revealed that the distributions between odor alone and complex odor were not significantly different (Figure 2.7P; $p = 0.816$).

These data collectively suggest that complex odor, representative of arbitrary associations, is not sufficient to cause potentiation of dopaminergic axon terminals or recruit dopaminergic neurons to respond to the complex odor.

2.4.7 -Complex odor Presentation does not potentiate mushroom body axon terminal responses or recruit mushroom body neurons

Like the dopaminergic axon terminals, MB axon terminals also showed an increase in activity in response to repeated exposure to ethanol-odor pairings. The dopaminergic axon terminals and the cell bodies did not undergo increases in activity in response to complex odor presentation. We sought to determine whether MB axon terminals, which serve as a locus for associative learning, responded similarly. We hypothesized that like

the PAM neurons, the MB would not increase its activity in response to repeated, spaced complex odor exposure.

During session 1 (Figure 2.8C), we observed that the presentation of odor alone induced an increase in the activity of the MB axon terminals (Figure 7Di; baseline vs first 15 seconds of odor A $W = 49$, $p = 0.001$), that remained constant throughout the 10 minutes (Figure 2.8Ei; first minute vs last minute Odor A $W = 7$, $p = 0.769$). Immediate presentation of the complex odor did not change activity compared to the final seconds of odor alone exposure (Figure 2.8Di; last 15 seconds of Odor A vs first 15 seconds of Odor B + Odor C, $W = 13$, $p = 0.556$) that was sustained for the 10 minutes of exposure (Figure 2.8Eii; first minute vs last minute Odor B + Odor C, $W = 13$, $p = 0.695$). The activity observed during both stimuli did not differ from one another (Figure 2.8Eii, $W = 7$, $p = 0.770$).

During session 2 (Figure 2.8F), odor alone again increased activity (Figure 2.8Gi; baseline vs first 15 seconds of Odor A, $W = 53$, $p = 0.004$) that is sustained throughout the 10 minutes of exposure (Figure 7Hi; first minute Odor A vs last minute Odor A $W = 19$, $p = 0.375$). The second exposure to the complex odor exposure initially resulted in similar levels of activity compared to odor alone (Figure 2.8Gi; last 15 seconds of Odor A vs first 15 seconds of Odor B + Odor C, $W = 13$, $p = 0.556$). Interestingly, the complex odor generates a decrease in activity from the first minute to the last (Figure 2.8Eii; First minute vs Last Minute $W = 55$, $p = 0.002$). Additionally, when comparing the average activity during odor alone to the average activity with exposure to the complex odor, we also observed a decrease during exposure to the complex odor (Figure 2.8Eiii; odor alone vs complex odor, $W = 53$, $p = 0.027$).

During session 3 (Figure 2.8I), odor alone once again generated an increase in activity (Figure 2.8Ji; baseline vs first 15 seconds of Odor A, $W = 55$, $p = 0.002$), however, this significantly decreased throughout the 10 minutes of exposure (Figure 2.8Ki, first minute vs last minute $W = 39$, $p = 0.048$). The complex odor initially generated activity levels similar to the odor alone (Figure 2.8Ji; last 15 seconds of Odor A vs first 15 seconds of Odor B + Odor C $W = 43$, $p = 0.131$), which dropped below baseline levels of activity (Figure 7I). Despite this, no differences were observed between the first minute of complex odor exposure and the last minute (Figure 2.8Kii, first minute vs last minute $W = 15$, $p = 0.234$). Again, we observed that the average activity in response to complex odor was significantly lower than activity during odor alone (Figure 2.8Kiii, odor alone vs complex odor $W = 8$, $p = 0.048$). When looking across sessions, however, we observed no changes in average activity for odor alone (Figure 2.8Li; Friedman $\chi^2 = 0.800$, $p = 0.7103$) or the complex odor (Figure 2.8Lii; Friedman $\chi^2 = 0.600$, $p = 0.830$).

We hypothesized that there would be no increase in activity at the axon terminals in response to the complex odor. However, we observed an unexpected decrease when comparing odor alone to the complex odor during sessions 2 and 3. This is in stark contrast to the potentiation observed during the presentation of repeated ethanol-odor pairings and further supports our hypothesis that arbitrary associations are not sufficient to cause plasticity within MB circuitry.

Once again, we looked to the cell bodies of the MB to better understand the dynamics occurring at the axon terminals during complex odor presentation. We observed that complex odor presentation resulted in decreased activity during sessions 2 and 3 of exposure when compared to odor alone. We hypothesized that this decrease in activity

may be the result of an increase in the number of cells suppressed by the complex odor, or an increase in the number of nonresponding cells across the sessions.

During session 1 (Figure 2.8Ni, $n=52$), there is heterogeneity in cell body responses to both odor alone and complex odor. A plurality of the cells were responders for odor alone (Figure 2.8Oi; 23 responders, 13 suppressed, 16 non-responders), however for complex odor, a majority were suppressed (Figure 2.8Oi; 17 responders, 23 suppressed, 12 non-responders). However, the distribution of cellular activities was overall similar during session 1 between odor alone and complex odor as determined by a Fisher's Exam Test (Figure 2.8Oi; $p = 0.125$).

During session 2 (Figure 2.8Nii; $n = 74$), we observed that many of the cells are responding to odor alone (Figure 2.8Oii; 45 responders, 8, suppressed, 21 non-responders). Similarly, the complex odor also caused most of the recorded cells to respond (Figure 2.8Oii; 45 responders, 12, suppressed, 17 non-responders). Again, the distributions remained consistent between odor alone and the complex odor ($p = 0.575$).

During session 3 (Figure 2.8Niii; $n= 89$), most of the recorded cells did not respond to odor alone (Figure 2.8Oiii; 32 responders, 6 suppressed, 51 non-responders). The complex odor also exhibited an increase in non-responders during session 3 (Figure 2.8Oiii; 36 responders, 16 suppressed, 37 non-responders). However, when comparing the distributions we observed that there was a significant difference between the two conditions as determined by a Fisher's exact test (Figure 2.8Oiii; $p = 0.0313$).

Both the odor alone and the complex odor conditions experienced a large shift from session 1 to session 3, with a majority of the cells becoming nonresponsive as

determined by Fisher's exact tests (Figure 2.8Oi and 2.8Oiii; odor alone session 3 vs session 1, $p = 0.001$; complex odor session 3 vs session 1, $p = 0.003$).

When measuring the change in activity from session 1 to session 3 within a subset of tracked cells (Supplementary Figure 2.S1D), we observed no large-scale recruitment of neurons to either odor alone or the complex odor (Figure 2.8P; 8 recruited, 6 inhibited, 5 consistent for odor alone; 6 recruited, 3 inhibited, 10 consistent for complex odor), and the distribution of recruited, inhibited, and consistent cells were not significantly different between the two conditions (Figure 2.8P; $p = 0.2914$).

2.5. DISCUSSION

We set out to understand how cue-ethanol memories influence the activity of dopaminergic circuits. We implemented functional imaging within dopaminergic neurons and their post-synaptic partners to investigate ethanol-odor associations. Here we provide evidence that repeated, spaced exposures to intoxicating doses of alcohol in the presence of an odor cue potentiates the activity of dopaminergic neurons and post-synaptic cue-encoding neurons. Potentiation was not observed when flies were exposed to ethanol alone, or when two stimuli presented simultaneously, suggesting that alcohol in the presence of an odor cue is uniquely acting upon dopaminergic circuits. This work provides novel insight into the dynamic changes in the activity of dopaminergic circuits during the association of cues with intoxicating doses of alcohol, as opposed to looking after repeated exposure to alcohol alone or in the presence of a cue (Y. Liu, Jean-Richard-Dit-Bressel, et al., 2020; Schreiner et al., 2023).

We observe that multiple sessions of spaced alcohol-odor pairing increased the activity of PAM dopamine axon terminals. However, this increase in activity at the axon terminals was not accompanied by increased activity at the PAM cell bodies. When looking at post-synaptic MB axon terminals, we observed that alcohol-odor memory formation increased activity across the three-spaced sessions. MB neurons were also recruited to respond to the odor-alcohol pairing at higher rates when compared to the odor alone. We propose that the stability of alcohol-cue memories is caused by the increased recruitment of many cue-encoding neurons to respond to ethanol-associated cues (Figure 2.9). These effects were not seen when flies were presented with alcohol in the absence of an odor cue (non-associative paradigm) or an arbitrary association of odors (complex odor paradigm). Taken together, we conclude that changes in dopaminergic neurons during alcohol-cue learning correspond to increased activity and recruitment of cue-encoding neurons. This likely results in strong cravings and maladaptive behavior in response to alcohol-associated cues that are characteristic of AUD.

Interestingly, we observed that alcohol-odor pairings generate a decrease in dopamine neuron activity as measured by GCaMP during the initial association. This is in contrast to what we observed during the presentation of alcohol alone in the non-associative paradigm, and what previous literature has observed in mice (Juarez et al., 2017), rats (Gessa et al., 1985), and humans (Boileau et al., 2003; H.-S. Kim et al., 2014; Oberlin et al., 2015). The hypoactivity observed during session 1 could be reflective of the aversive nature of the intoxicating dose of ethanol, decreasing activity at reward-encoding PAMs. However, this decrease in dopamine neuron activity is absent

during the last presentation of odors paired with alcohol, where the activity elicited in session 3 is significantly greater than the activity present in session 1. This increase in the response to alcohol-associated cues is consistent with studies demonstrating increased dopamine neuron activity in mice (Lei et al., 2024, p. 202), rats (Katner et al., 1996; Weiss et al., 1993), and humans (Filbey et al., 2008; Kareken et al., 2004; Myrick et al., 2004), following formation of alcohol-cue memory.

We observed that alcohol-cue pairings during learning increased the activity of postsynaptic MB axon terminals during the later stage of acquisition (Session 3) when compared to odor alone. This is in agreement with previous literature that has found increased activity within cue-encoding medial prefrontal cortex neurons in response to cues associated with alcohol (Filbey et al., 2008; Myrick et al., 2004). Here we extend the field's understanding, by demonstrating that increases in activity from cue-encoding neurons are the result of increased recruitment during the learning process, similar to what has been observed during sucrose-cue associations in mice (Ottenheimer et al., 2023). In contrast when odor was paired with another odor, as well as when ethanol was presented alone in the absence of an odor cue, we observed the MB cell bodies shifted towards being non-responsive or suppressed and a large-scale inhibition of cells when comparing their third-session activity to the first-session activity suggesting that the recruitment of neurons during learning is unique to alcohol associations.

In the context of the dopamine neurons, there were more subtle changes occurring during all three of the paradigms. Unlike the MB cell bodies, we did not observe any major shifts in cell body dynamics. This is especially surprising in the

context of the associative paradigm, given the necessity of the entire PAM neuron population during the acquisition of alcohol memory (Scaplen et al., 2020).

Our recordings at the cell bodies illustrated that alcohol alone is not able to recruit the cue-encoding MB neurons at the same rate as alcohol in the presence of an odor cue. These findings may provide insight into the classification of alcohol as a depressant (Eckardt et al., 1998) or a stimulant (Eckardt et al., 1998). Alcohol may elicit disparate behavioral responses depending on context, stimulating in the presence of environmental cues, or depressing in the absence of them, as seen with our results.

The PAM axon terminal compartmentalized data suggests that the *y5B'2a* compartment is a central locus for encoding ethanol-odor memories. However, when investigating this compartment within the MB axon terminals, we were unable to replicate the increase in activity across sessions. There may be additional dopaminergic signaling that occurs during sleep that modifies the plasticity of the compartment, as suggested by Scaplen et al., 2020.

Perplexingly, overall dopamine release onto the MB from the PAM dopamine neurons remained consistent in response to the alcohol-odor pairings (as measured by fluorescent dopamine sensor, dLight, Supplementary Figure 3), despite their increased calcium dynamics from the *R58E02>GCaMP7b* recordings. There may be a need for higher activity levels within PAM neurons to generate and release the same amount of dopamine. It is also possible that PAMs, known to co-transmit nitric oxide (Aso et al., 2019) and glutamate (Aso et al., 2019; Buck et al., 2021), are releasing another neurotransmitter. Nitric oxide can change the valence of a reinforcer (insert citation) Thus, perhaps nitric oxide plays a role in changing the innately aversive concentrations

of alcohol to a stimulus that can drive approach. Glutamate could also increase the activity of the mushroom bodies by binding glutamate receptors on the axon terminals, which could lead to more active MBs during alcohol-odor pairings. Lastly, it is also possible that dopamine signaling could be affecting other modulatory neurons like the anterior paired lateral (APL) or dorsal paired median (DPM) neurons, both of which modulate the mushroom body.

Dopamine released from PAM neurons could be acting on a multitude of dopamine receptors in postsynaptic neurons. G_{α_s} -engaging Dopamine-1-like receptors (Dop1R1), are critical for olfactory memory formation (Y.-C. Kim et al., 2007), and could be a critical molecule for signaling. G_{α_i} -interacting Dopamine-2-like receptors (Dop2R) expressed on the APL have been implicated in shaping olfactory responses (Okray et al., 2023; Zhou et al., 2019) and could suppress GABAergic inhibition of the MBs during alcohol-odor conditioning, leading to increased activity with repeated exposures. Furthermore, Dop2R within the mushroom body is critical for the formation of alcohol memory (Petrucelli et al., 2018). Additionally, PAM neurons are likely releasing dopamine onto negative-valence, mushroom body output neurons (MBONs) which may be the site of increased dopamine release.

Dopamine released by the PAM neurons onto the MB could also be acting on G_{α_s} Dopamine-1-like receptors on the axon terminals of the MB, potentiating their activity due to coincident activity-associated calcium increases and dopaminergic modulation (Tomchik & Davis, 2009). G_{α_i} D2-receptors, which have also been shown to be important for alcohol-odor memory formation (Petrucelli et al., 2018, Scaplen et al., 2020) may also be depressing the connections between the MBON dendrites and

horizontal lobe Kenyon cell compartments, weakening avoidance behaviors in response to an odor associated with alcohol.

Another means by which activity within MB circuitry could be changing is through the molecular mechanisms underlying alcohol tolerance. The impacts of alcohol on large conductance calcium channels (BK) are important for organisms to develop rapid alcohol tolerance (Bettinger & Davies, 2014; Dopico et al., 2014; Scaplen & Petrucci, 2021; Treistman & Martin, 2009). The modification in the clustering and activity of these channels may drive changes in underlying circuit dynamics. Alcohol also interacts with the presynaptic SNARE complex protein, Dunc13 (Munc18 in mammals), and may alter synaptic transmission through a Dunc13-mediated pathway (Das et al., 2013).

It is still unclear how neural dynamics change in the context of alcohol-associated memory retrieval. Future studies should investigate how internal representations of alcohol's rewarding properties are changing throughout learning and retrieval. Ideally, these studies would be conducted all within the context of the imaging environment, recording neural signatures during training, and 24 hours later recording the responses to odor cues. There are previously identified alcohol memory retrieval microcircuits, α^2 PPL1 $\rightarrow$ α^2 MBON and PAM- β^2 a innervating MBON- β^2 mp and MBON- β^2 2a, (Scaplen et al., 2020) that are likely the loci for the largest change in plasticity. Using this approach, it is possible to get an even more detailed understanding of how odor cues induce craving by analyzing single neurons known to contribute to cued approach. Repeating the methodology of this study in the context of chronic exposure to alcohol over multiple days could also provide insights into how chronic alcohol use further co-opts reward circuitry to lead to drug dependence.

In summary, we have uncovered how dopaminergic circuits are uniquely impacted by alcohol-odor associations in comparison to alcohol and complex odor presentations. We found that repeated, spaced exposure to odors paired with intoxicating doses of ethanol increases the activity of multiple neural substrates important for encoding reward. Additionally, odor-alcohol associations are potentially stabilized by the recruitment of multiple cue-encoding neurons which can drive stronger behavioral responses when cues associated with alcohol are encountered. Using this approach, we have further established *Drosophila* as a model for understanding substance use disorders by leveraging its expansive genetic toolkit and tractability to investigate how alcohol shapes reward circuitry in the presence of a cue. Elucidating how these associations co-opt reward circuits further our understanding of how alcohol-associated cues drive strong cravings and seeking for alcohol. This knowledge will help us develop better therapeutics for treating AUD and other substance use disorders.

2.6. MATERIALS AND METHODS

2.6.1 - Key Resources Table

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Genetic reagent (<i>D. melanogaster</i>)	<i>UAS-GCaMP7b</i>	Dana et al., 2019	FLYB: FBal0061578 RRID: BDSC_9313	
Genetic reagent (<i>D. melanogaster</i>)	<i>MB010B-Gal4</i>	Courtesy of Yoshinori Aso Aso et al., 2014	FLYB: FBst0009313	
Genetic reagent (<i>D. melanogaster</i>)	<i>UAS-dLight</i>	Courtesy of Vivek Jayaraman		
Genetic reagent (<i>D. melanogaster</i>)	<i>R58E02-Gal4</i>	Heberlein Lab Liu et al., 2012		
Chemical	Iso-amyl alcohol	Fisher Scientific	Cat#A393	(1:36)
Chemical	Ethyl-acetate	Fisher Science	Cat#S25305	(1:36)
Chemical	Iso-amyl acetate	Fisher Science	Cat#150662500	(1:36)
Chemical	Mineral oil	Fisher Science	Cat#BP2629	N/A
Chemical	CaCl ₂	Sigma	Cat#C1016	2 mM

Chemical	KCl	Sigma	Cat#P9541	5 mM
Chemical	HEPES	Sigma	Cat#H0887	5 mM
Chemical	MgCl ₂	New England BioLabs	Cat#B9021S	8.2 mM
Chemical	NaCl	Sigma	Cat#S9888	108 mM
Chemical	NaHCO ₃	Boston BioProduct	Cat#BB-2612	4 mM
Chemical	NaH ₂ PO ₄	Sigma	Cat#S5011	1 mM
Chemical	Ribose	Sigma	R9629	15 mM
Chemical	UV-cured adhesive	Loctite	Cat# AA 3106	N/A

2.6.2 -Fly Husbandry

All *Drosophila melanogaster* lines were raised on standard cornmeal-agar media with tegosept anti-fungal agent and maintained at 24C humidity-controlled chambers under 14/10 hr light/dark cycles on standard cornmeal-agar media with tegosept anti-fungal agent. If flies were CO₂ anesthetized, they were given 2 days of recovery before use for two-photon imaging.

2.6.3 - Preparation of flies for 2-Photon Calcium Imaging

To generate flies for imaging, virgin females carrying UAS-GCaMP7B (Chromosome III) (Dana et al., 2019), were crossed to MB010B-Gal4 (Bräcker et al., 2013) for imaging the mushroom body, R58E02-Gal4 (C. Liu et al., 2012, p. 2) for imaging the Protocerebral Anterior Medial dopaminergic neurons Flies (4-10 days old) were briefly cold

anesthetized (up to 15 minutes in a plastic vial) and mounted on custom-built stages using Loctite ultraviolet-cured glue. Flies then had the cuticle above the region of interest removed using a tungsten wire (Roboz Surgical Instruments Tungsten Dissecting Needle, 125 mm, Ultra Fine) and forceps #5. The imaging area was covered in a chilled, adult-hemolymph-like solution (formula here, osmolality ~7.1). Flies had trachea and fat bodies above the imaging area removed or pushed to the edges of the region of interest to provide a clear imaging area. Flies were then placed under a Scientifica 2-photon Microscope on a levitating polystyrene ball, allowing them to walk. (flow rate supporting the ball ~65ml/min). Z-stack volumes (ranging from 100um to 150um) were acquired using SciScan software at 1.08Hz/30.94 frames per second with resonant scanning. The field of view was 512 x 512 and saved as 16-bit raw files. Flies were presented with stimulus at flow rates from 5-7.5 ml/min. Recordings were performed at 18.5°C room temperature and 59% humidity.

2.6.4 -Imaging Paradigm for Odor-Ethanol Memory Acquisition

Flies were exposed to an odor-alcohol associative memory paradigm (Kaun et al., 2011, Nunez et al., 2018) while under the scope. Flies received 2 mins of airflow at 5ml/min, followed by 5ml/min of Odor A for 10 minutes. Flies were then exposed for 10 minutes to 2.5ml/min of Odor B, and 5 ml/min of 50% ethanol before air again for an additional minute before the end of the recording. Recording sessions were spaced by 50 minutes.

2.6.5 - Imaging Paradigm for Non-associative Odor-Ethanol Memory Acquisition

Flies were exposed to 2 mins of air, followed by 5ml/min of Odor A for 10 minutes. Flies were then exposed for 10 minutes to 5ml/min of odor B. 10 minutes of air flow was given before presenting flies with 5 ml/min of Ethanol to prevent any associations from occurring between odors and ethanol. Flies were then exposed to air again for an additional minute before the end of the recording. Flies were then given 30 minutes to fully metabolize ethanol before recording the subsequent session.

2.6.6 - Imaging paradigm for Mixed odor presentation

Flies were exposed to 2 mins of air, followed by 5ml/min of Odor A (Isoamyl Acetate) for 10 minutes. Flies were then exposed for 10 minutes to 3.75ml/min of Odor B (Isoamyl Alcohol) and Odor C (Ethyl Acetate). Flies were then exposed to air again for an additional minute before the end of the recording. Flies were then given 50 minutes to rest before the subsequent session.

2.6.7 - Imaging Data Analysis Pipeline

16-bit raw files were converted to hyperstacks, then to max-intensity projections, and subsequently saved as tiffs. If tiffs required motion correction, Caiman and NoRMCore MATLAB scripts were used to perform motion correction. Tiffs were then manually segmented using the image-analysis GUI developed by Dr. Chris Deister (available here: https://github.com/cdeister/imageAnalysis_gui). Raw fluorescence traces from the GUI were then imported into custom Python Scripts as CSVs (available here:) to perform baseline normalization and for data visualization. The baseline fluorescence was defined for each stimulus-response as the mean fluorescence from 30 s before

stimulus presentation. Baseline normalization was conducted as follows $F - \text{Baseline}_F / \text{Baseline}_F$.

2.6.8 - Single Cell Analysis

Using max-intensity projections, individual cellular-shaped ROIs were selected. ROIs were preferentially selected based on the amount of movement, and lack of overlap with neighboring cells. The maximal number of identifiable cells was measured from each video. If possible, single cells were tracked across the same fly based on anatomical similarity.

Cells were categorized based on the following equations:

$$\text{Responder} = (\text{Session}_{\mu_{t1-t2}} - \text{Session}_{\text{baseline}}) > 0.1 \text{ (Delestro et al., 2020)}$$

$$\text{Suppressed} = (\text{Session}_{\mu_{t1-t2}} - \text{Session}_{\text{baseline}}) < -0.1$$

$$\text{Non-responder} = 0.1 > \text{Session}_{\mu} > -0.1$$

$$\text{Recruited} = (\text{Session } 3_{\mu_{t1-t2}} - \text{Session } 1_{\mu_{t1-t2}}) > 0.1$$

$$\text{Inhibited} = (\text{Session } 3_{\mu_{t1-t2}} - \text{Session } 1_{\mu_{t1-t2}}) < -0.1$$

$$\text{Consistent} = 0.1 > \text{Session } 3_{\mu_{t1-t2}} - \text{Session } 1_{\mu_{t1-t2}} > -0.1$$

2.6.8 - Odor delivery system

Teflon tubing was positioned directly in front of the fly. Tubing received input from up to 4 separate tubes that could carry odor. These tubes were connected to vials that carried

3ml of odorant or 15ml of 50% ethanol. Odorants were diluted in mineral oil. Pure ethanol was diluted to 50% using distilled water. The odor was delivered by manually turning stopcocks at appropriate times.

2.8 CHAPTER 2 REFERENCES

- Amo, R., Matias, S., Yamanaka, A., Tanaka, K. F., Uchida, N., & Watabe-Uchida, M. (2022). A gradual temporal shift of dopamine responses mirrors the progression of temporal difference error in machine learning. *Nature Neuroscience*, 25(8), 1082–1092. <https://doi.org/10.1038/s41593-022-01109-2>
- Aso, Y., Hattori, D., Yu, Y., Johnston, R. M., Iyer, N. A., Ngo, T.-T. B., Dionne, H., Abbott, L. F., Axel, R., Tanimoto, H., & Rubin, G. M. (2014). The neuronal architecture of the mushroom body provides a logic for associative learning. *eLife*, 3, e04577. <https://doi.org/10.7554/eLife.04577>
- Aso, Y., Ray, R. P., Long, X., Bushey, D., Cichewicz, K., Ngo, T.-T., Sharp, B., Christoforou, C., Hu, A., Lemire, A. L., Tillberg, P., Hirsh, J., Litwin-Kumar, A., & Rubin, G. M. (2019). Nitric oxide acts as a cotransmitter in a subset of dopaminergic neurons to diversify memory dynamics. *eLife*, 8, e49257. <https://doi.org/10.7554/eLife.49257>
- Aso, Y., Sitaraman, D., Ichinose, T., Kaun, K. R., Vogt, K., Belliart-Guérin, G., Plaçais, P.-Y., Robie, A. A., Yamagata, N., Schnaitmann, C., Rowell, W. J., Johnston, R. M., Ngo, T.-T. B., Chen, N., Korff, W., Nitabach, M. N., Heberlein, U., Preat, T., Branson, K. M., ... Rubin, G. M. (2014). Mushroom body output neurons encode valence and guide memory-based action selection in *Drosophila*. *eLife*, 3, e04580. <https://doi.org/10.7554/eLife.04580>
- Bettinger, J. C., & Davies, A. G. (2014). The role of the BK channel in ethanol response behaviors: Evidence from model organism and human studies. *Frontiers in Physiology*, 5. <https://doi.org/10.3389/fphys.2014.00346>

- Bijoch, Ł., Klos, J., Pawłowska, M., Wiśniewska, J., Legutko, D., Szachowicz, U., Kaczmarek, L., & Beroun, A. (2023). Whole-brain tracking of cocaine and sugar rewards processing. *Translational Psychiatry*, 13(1), 1–14. <https://doi.org/10.1038/s41398-023-02318-4>
- Boileau, I., Assaad, J., Pihl, R. O., Benkelfat, C., Leyton, M., Diksic, M., Tremblay, R. E., & Dagher, A. (2003). Alcohol promotes dopamine release in the human nucleus accumbens. *Synapse*, 49(4), 226–231. <https://doi.org/10.1002/syn.10226>
- Bräcker, L. B., Siju, K. P., Varela, N., Aso, Y., Zhang, M., Hein, I., Vasconcelos, M. L., & Grunwald Kadow, I. C. (2013). Essential role of the mushroom body in context-dependent CO₂ avoidance in *Drosophila*. *Current Biology: CB*, 23(13), 1228–1234. <https://doi.org/10.1016/j.cub.2013.05.029>
- Brodie, M. S. (2002). Increased Ethanol Excitation of Dopaminergic Neurons of the Ventral Tegmental Area After Chronic Ethanol Treatment. *Alcoholism: Clinical and Experimental Research*, 26(7), 1024–1030. <https://doi.org/10.1111/j.1530-0277.2002.tb02637.x>
- Buck, S. A., Steinkellner, T., Aslanoglou, D., Villeneuve, M., Bhatte, S. H., Childers, V. C., Rubin, S. A., De Miranda, B. R., O’Leary, E. I., Neureiter, E. G., Fogle, K. J., Palladino, M. J., Logan, R. W., Glausier, J. R., Fish, K. N., Lewis, D. A., Greenamyre, J. T., McCabe, B. D., Cheetham, C. E. J., ... Freyberg, Z. (2021). Vesicular glutamate transporter modulates sex differences in dopamine neuron vulnerability to age-related neurodegeneration. *Aging Cell*, 20(5), e13365. <https://doi.org/10.1111/accel.13365>

- Cohen, J. Y., Haesler, S., Vong, L., Lowell, B. B., & Uchida, N. (2012). Neuron-type-specific signals for reward and punishment in the ventral tegmental area. *Nature*, 482(7383), 85–88. <https://doi.org/10.1038/nature10754>
- Cohn, R., Morantte, I., & Ruta, V. (2015). Coordinated and Compartmentalized Neuromodulation Shapes Sensory Processing in *Drosophila*. *Cell*, 163(7), 1742–1755. <https://doi.org/10.1016/j.cell.2015.11.019>
- Dana, H., Sun, Y., Mohar, B., Hulse, B. K., Kerlin, A. M., Hasseman, J. P., Tsegaye, G., Tsang, A., Wong, A., Patel, R., Macklin, J. J., Chen, Y., Konnerth, A., Jayaraman, V., Looger, L. L., Schreiter, E. R., Svoboda, K., & Kim, D. S. (2019). High-performance calcium sensors for imaging activity in neuronal populations and microcompartments. *Nature Methods*, 16(7), 649–657. <https://doi.org/10.1038/s41592-019-0435-6>
- Das, J., Xu, S., Pany, S., Guillory, A., Shah, V., & Roman, G. W. (2013). The presynaptic Munc13-1 binds alcohol and modulates alcohol self-administration in *Drosophila*. *Journal of Neurochemistry*, 126(6), 715–726. <https://doi.org/10.1111/jnc.12315>
- Day, J. J., Roitman, M. F., Wightman, R. M., & Carelli, R. M. (2007). Associative learning mediates dynamic shifts in dopamine signaling in the nucleus accumbens. *Nature Neuroscience*, 10(8), 1020–1028. <https://doi.org/10.1038/nn1923>
- Deehan, G. A., Knight, C. P., Waeiss, R. A., Engleman, E. A., Toalston, J. E., McBride, W. J., Hauser, S. R., & Rodd, Z. A. (2016). Peripheral Administration of Ethanol Results in a Correlated Increase in Dopamine and Serotonin Within the Posterior Ventral Tegmental Area. *Alcohol and Alcoholism (Oxford, Oxfordshire)*, 51(5), 535–540. <https://doi.org/10.1093/alcalc/agw037>

- Devineni, A. V., & Heberlein, U. (2009). Preferential ethanol consumption in *Drosophila* models features of addiction. *Current Biology: CB*, 19(24), 2126–2132.
<https://doi.org/10.1016/j.cub.2009.10.070>
- Di Chiara, G., Bassareo, V., Fenu, S., De Luca, M. A., Spina, L., Cadoni, C., Acquas, E., Carboni, E., Valentini, V., & Lecca, D. (2004). Dopamine and drug addiction: The nucleus accumbens shell connection. *Neuropharmacology*, 47, 227–241.
<https://doi.org/10.1016/j.neuropharm.2004.06.032>
- Dopico, A. M., Bukiya, A. N., & Martin, G. E. (2014). Ethanol modulation of mammalian BK channels in excitable tissues: Molecular targets and their possible contribution to alcohol-induced altered behavior. *Frontiers in Physiology*, 5.
<https://doi.org/10.3389/fphys.2014.00466>
- Eckardt, M. J., File, S. E., Gessa, G. L., Grant, K. A., Guerri, C., Hoffman, P. L., Kalant, H., Koob, G. F., Li, T., & Tabakoff, B. (1998). Effects of Moderate Alcohol Consumption on the Central Nervous System*. *Alcoholism: Clinical and Experimental Research*, 22(5), 998–1040. <https://doi.org/10.1111/j.1530-0277.1998.tb03695.x>
- Ericson, M., Blomqvist, O., Engel, J. A., & Söderpalm, B. (1998). Voluntary ethanol intake in the rat and the associated accumbal dopamine overflow are blocked by ventral tegmental mecamylamine. *European Journal of Pharmacology*, 358(3), 189–196.
[https://doi.org/10.1016/s0014-2999\(98\)00602-5](https://doi.org/10.1016/s0014-2999(98)00602-5)
- Evans-Polce, R. J., Maggs, J. L., Lanza, S. T., & Patrick, M. E. (2024). Negative consequences associated with daily alcohol use as a nonlinear function of number of drinks in a

daily diary study. *Drug and Alcohol Dependence*, 256, 111089.

<https://doi.org/10.1016/j.drugalcdep.2024.111089>

Fiala, A., & Kaun, K. R. (2024). What do the mushroom bodies do for the insect brain?

Twenty-five years of progress. *Learning & Memory*, 31(5), a053827.

<https://doi.org/10.1101/lm.053827.123>

Filbey, F. M., Claus, E., Audette, A. R., Niculescu, M., Banich, M. T., Tanabe, J., Du, Y. P., &

Hutchison, K. E. (2008). Exposure to the taste of alcohol elicits activation of the mesocorticolimbic neurocircuitry. *Neuropsychopharmacology: Official Publication of the American College of Neuropsychopharmacology*, 33(6), 1391–1401.

<https://doi.org/10.1038/sj.npp.1301513>

Gessa, G. L., Muntoni, F., Collu, M., Vargiu, L., & Mereu, G. (1985). Low doses of ethanol

activate dopaminergic neurons in the ventral tegmental area. *Brain Research*, 348(1), 201–203. [https://doi.org/10.1016/0006-8993\(85\)90381-6](https://doi.org/10.1016/0006-8993(85)90381-6)

Hattori, D., Aso, Y., Swartz, K. J., Rubin, G. M., Abbott, L. F., & Axel, R. (2017).

Representations of Novelty and Familiarity in a Mushroom Body Compartment. *Cell*, 169(5), 956-969.e17. <https://doi.org/10.1016/j.cell.2017.04.028>

Heberlein, U. (2000). Genetics of alcohol-induced behaviors in *Drosophila*. *Alcohol*

Research & Health: The Journal of the National Institute on Alcohol Abuse and Alcoholism, 24(3), 185–188.

Hernandez, J. S., Brown, T. M., & Kaun, K. R. (2023). *Drosophila* Reward Circuits. In *Oxford*

Research Encyclopedia of Neuroscience.

<https://doi.org/10.1093/acrefore/9780190264086.013.495>

Hopf, F. W., Martin, M., Chen, B. T., Bowers, M. S., Mohamedi, M. M., & Bonci, A. (2007).

Withdrawal from intermittent ethanol exposure increases probability of burst firing in VTA neurons in vitro. *Journal of Neurophysiology*, 98(4), 2297–2310.

<https://doi.org/10.1152/jn.00824.2007>

Hsu, T. M., Bazzino, P., Hurh, S. J., Konanur, V. R., Roitman, J. D., & Roitman, M. F. (2020).

Thirst recruits phasic dopamine signaling through subfornical organ neurons.

Proceedings of the National Academy of Sciences of the United States of America, 117(48), 30744–30754. <https://doi.org/10.1073/pnas.2009233117>

Imperato, A., & Di Chiara, G. (1986). Preferential stimulation of dopamine release in the

nucleus accumbens of freely moving rats by ethanol. *The Journal of Pharmacology and Experimental Therapeutics*, 239(1), 219–228. [https://doi.org/10.1016/S0022-3565\(25\)23929-5](https://doi.org/10.1016/S0022-3565(25)23929-5)

Jones, A., Rose, A. K., Cole, J., & Field, M. (2013). Effects of Alcohol Cues on Craving and Ad

Libitum Alcohol Consumption in Social Drinkers: The Role of Disinhibition. *Journal of Experimental Psychopathology*, 4(3), 239–249.

<https://doi.org/10.5127/jep.031912>

Juarez, B., Morel, C., Ku, S. M., Liu, Y., Zhang, H., Montgomery, S., Gregoire, H., Ribeiro, E.,

Crumiller, M., Roman-Ortiz, C., Walsh, J. J., Jackson, K., Croote, D. E., Zhu, Y., Zhang, S., Vendruscolo, L. F., Edwards, S., Roberts, A., Hodes, G. E., ... Han, M.-H. (2017).

Midbrain circuit regulation of individual alcohol drinking behaviors in mice. *Nature Communications*, 8(1), 2220. <https://doi.org/10.1038/s41467-017-02365-8>

- Kareken, D. A., Claus, E. D., Sabri, M., Dzemidzic, M., Kosobud, A. E. K., Radnovich, A. J., Hector, D., Ramchandani, V. A., O'Connor, S. J., Lowe, M., & Li, T. (2004). Alcohol-Related Olfactory Cues Activate the Nucleus Accumbens and Ventral Tegmental Area in High-Risk Drinkers: Preliminary Findings. *Alcoholism: Clinical and Experimental Research*, 28(4), 550–557.
<https://doi.org/10.1097/01.ALC.0000122764.60626.AF>
- Katner, S. N., Kerr, T. M., & Weiss, F. (1996). Ethanol anticipation enhances dopamine efflux in the nucleus accumbens of alcohol-preferring (P) but not Wistar rats. *Behavioural Pharmacology*, 7(7), 669–674.
- Kaun, K. R., Azanchi, R., Maung, Z., Hirsh, J., & Heberlein, U. (2011). A Drosophila model for alcohol reward. *Nature Neuroscience*, 14(5), 612–619.
<https://doi.org/10.1038/nn.2805>
- Kim, H.-S., Kim, D., Kim, R. G., Kim, J.-M., Chung, E., Neto, P. R., Lee, M.-C., & Kim, H.-I. (2014). A Rat Model of Photothrombotic Capsular Infarct with a Marked Motor Deficit: A Behavioral, Histologic, and microPET Study. *Journal of Cerebral Blood Flow & Metabolism*, 34(4), 683–689. <https://doi.org/10.1038/jcbfm.2014.2>
- Kim, Y.-C., Lee, H.-G., & Han, K.-A. (2007). D1 dopamine receptor dDA1 is required in the mushroom body neurons for aversive and appetitive learning in Drosophila. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 27(29), 7640–7647. <https://doi.org/10.1523/JNEUROSCI.1167-07.2007>

- Koob, G. F., & Volkow, N. D. (2016). Neurobiology of addiction: A neurocircuitry analysis. *The Lancet. Psychiatry*, 3(8), 760–773. [https://doi.org/10.1016/S2215-0366\(16\)00104-8](https://doi.org/10.1016/S2215-0366(16)00104-8)
- Koyama, S., Brodie, M. S., & Appel, S. B. (2007). Ethanol inhibition of m-current and ethanol-induced direct excitation of ventral tegmental area dopamine neurons. *Journal of Neurophysiology*, 97(3), 1977–1985. <https://doi.org/10.1152/jn.00270.2006>
- Krause, M. A. (2015). Evolutionary perspectives on learning: Conceptual and methodological issues in the study of adaptive specializations. *Animal Cognition*, 18(4), 807–820. <https://doi.org/10.1007/s10071-015-0854-4>
- Kufahl, P. R., Li, Z., Risinger, R. C., Rainey, C. J., Wu, G., Bloom, A. S., & Li, S.-J. (2005). Neural responses to acute cocaine administration in the human brain detected by fMRI. *NeuroImage*, 28(4), 904–914. <https://doi.org/10.1016/j.neuroimage.2005.06.039>
- Lee, C., Mayfield, R. D., & Harris, R. A. (2014). Altered Gamma-Aminobutyric Acid Type B Receptor Subunit 1 Splicing In Alcoholics. *Biological Psychiatry*, 75(10), 765–773. <https://doi.org/10.1016/j.biopsych.2013.08.028>
- Lefner, M. J., Dejeux, M. I., & Wanat, M. J. (2022). Sex Differences in Behavioral Responding and Dopamine Release during Pavlovian Learning. *eNeuro*, 9(2), ENEURO.0050-22.2022. <https://doi.org/10.1523/ENEURO.0050-22.2022>
- Lei, J., Zhang, P., Li, T., Cui, C., Li, M., Yang, X., Peng, X., Ren, K., Yang, J., Shi, Y., Luo, G., Yao, Y., & Tian, B. (2024). Alternating bilateral sensory stimulation alleviates alcohol-

- induced conditioned place preference via a superior colliculus-VTA circuit. *Cell Reports*, 43(7), 114383. <https://doi.org/10.1016/j.celrep.2024.114383>
- Leyton, M., Boileau, I., Benkelfat, C., Diksic, M., Baker, G., & Dagher, A. (2002). Amphetamine-Induced Increases in Extracellular Dopamine, Drug Wanting, and Novelty Seeking: A PET/[11C]Raclopride Study in Healthy Men. *Neuropsychopharmacology*, 27(6), 1027–1035. [https://doi.org/10.1016/S0893-133X\(02\)00366-4](https://doi.org/10.1016/S0893-133X(02)00366-4)
- Liu, C., Plaçais, P.-Y., Yamagata, N., Pfeiffer, B. D., Aso, Y., Friedrich, A. B., Siwanowicz, I., Rubin, G. M., Preat, T., & Tanimoto, H. (2012). A subset of dopamine neurons signals reward for odour memory in *Drosophila*. *Nature*, 488(7412), 512–516. <https://doi.org/10.1038/nature11304>
- Liu, Y., Jean-Richard-Dit-Bressel, P., Yau, J. O.-Y., Willing, A., Prasad, A. A., Power, J. M., Killcross, S., Clifford, C. W. G., & McNally, G. P. (2020). The Mesolimbic Dopamine Activity Signatures of Relapse to Alcohol-Seeking. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 40(33), 6409–6427. <https://doi.org/10.1523/JNEUROSCI.0724-20.2020>
- Liu, Y., Montgomery, S. E., Juarez, B., Morel, C., Zhang, S., Kong, Y., Calipari, E. S., Nestler, E. J., Zhang, L., & Han, M.-H. (2020). Different adaptations of dopamine release in Nucleus Accumbens shell and core of individual alcohol drinking groups of mice. *Neuropharmacology*, 175, 108176. <https://doi.org/10.1016/j.neuropharm.2020.108176>

- Mandeville, J. B., Choi, J.-K., Jarraya, B., Rosen, B. R., Jenkins, B. G., & Vanduffel, W. (2011). fMRI of Cocaine Self-Administration in Macaques Reveals Functional Inhibition of Basal Ganglia. *Neuropsychopharmacology*, 36(6), 1187–1198.
<https://doi.org/10.1038/npp.2011.1>
- Margulies, C., Tully, T., & Dubnau, J. (2005). Deconstructing Memory in *Drosophila*. *Current Biology: CB*, 15(17), R700–R713. <https://doi.org/10.1016/j.cub.2005.08.024>
- Morand-Ferron, J. (2017). Why learn? The adaptive value of associative learning in wild populations. *Current Opinion in Behavioral Sciences*, 16, 73–79.
<https://doi.org/10.1016/j.cobeha.2017.03.008>
- Mulholland, P. J., Hopf, F. W., Bukiya, A. N., Martin, G. E., Liu, J., Dopico, A. M., Bonci, A., Treistman, S. N., & Chandler, L. J. (2009). Sizing up Ethanol-Induced Plasticity: The Role of Small and Large Conductance Calcium-Activated Potassium Channels. *Alcoholism, Clinical and Experimental Research*, 33(7), 1125–1135.
<https://doi.org/10.1111/j.1530-0277.2009.00936.x>
- Myrick, H., Anton, R. F., Li, X., Henderson, S., Drobos, D., Voronin, K., & George, M. S. (2004). Differential Brain Activity in Alcoholics and Social Drinkers to Alcohol Cues: Relationship to Craving. *Neuropsychopharmacology*, 29(2), 393–402.
<https://doi.org/10.1038/sj.npp.1300295>
- Nunez, K. M., Azanchi, R., & Kaun, K. R. (2018). Cue-Induced Ethanol Seeking in *Drosophila melanogaster* Is Dose-Dependent. *Frontiers in Physiology*, 9, 438.
<https://doi.org/10.3389/fphys.2018.00438>

- Oberlin, B. G., Dziedzic, M., Tran, S. M., Soeurt, C. M., O'Connor, S. J., Yoder, K. K., & Kareken, D. A. (2015). Beer self-administration provokes lateralized nucleus accumbens dopamine release in male heavy drinkers. *Psychopharmacology*, 232(5), 861–870. <https://doi.org/10.1007/s00213-014-3720-1>
- Okray, Z., Jacob, P. F., Stern, C., Desmond, K., Otto, N., Talbot, C. B., Vargas-Gutierrez, P., & Waddell, S. (2023). Multisensory learning binds neurons into a cross-modal memory engram. *Nature*, 617(7962), 777–784. <https://doi.org/10.1038/s41586-023-06013-8>
- Ottenheimer, D. J., Hjort, M. M., Bowen, A. J., Steinmetz, N. A., & Stuber, G. D. (2023). A stable, distributed code for cue value in mouse cortex during reward learning. <https://doi.org/10.7554/eLife.84604.1>
- Owald, D., & Waddell, S. (2015). Olfactory learning skews mushroom body output pathways to steer behavioral choice in *Drosophila*. *Current Opinion in Neurobiology*, 35, 178–184. <https://doi.org/10.1016/j.conb.2015.10.002>
- Patrick, M. E., & Terry-McElrath, Y. M. (2021). Drinking Motives and Drinking Consequences across Days: Differences and Similarities between Moderate, Binge, and High-Intensity Drinking. *Alcoholism: Clinical and Experimental Research*, 45(5), 1078–1090. <https://doi.org/10.1111/acer.14591>
- Petrucelli, E., Feyder, M., Ledru, N., Jaques, Y., Anderson, E., & Kaun, K. R. (2018). Alcohol Activates Scabrous-Notch to Influence Associated Memories. *Neuron*, 100(5), 1209–1223.e4. <https://doi.org/10.1016/j.neuron.2018.10.005>

- Pina, M. M., & Williams, A. R. (2016). Alcohol Cues, Craving, and Relapse: Insights from Animal Models. In *Recent Advances in Drug Addiction Research and Clinical Applications*. IntechOpen. <https://doi.org/10.5772/63105>
- Scaplen, K. M., & Kaun, K. R. (2016). Reward from bugs to bipeds: A comparative approach to understanding how reward circuits function. *Journal of Neurogenetics*, 30(2), 133–148. <https://doi.org/10.1080/01677063.2016.1180385>
- Scaplen, K. M., & Petruccelli, E. (2021). Receptors and Channels Associated with Alcohol Use: Contributions from *Drosophila*. *Neuroscience Insights*, 16, 26331055211007441. <https://doi.org/10.1177/26331055211007441>
- Scaplen, K. M., Talay, M., Nunez, K. M., Salamon, S., Waterman, A. G., Gang, S., Song, S. L., Barnea, G., & Kaun, K. R. (2020). Circuits that encode and guide alcohol-associated preference. *eLife*, 9, e48730. <https://doi.org/10.7554/eLife.48730>
- Scholz, H., Ramond, J., Singh, C. M., & Heberlein, U. (2000). Functional ethanol tolerance in *Drosophila*. *Neuron*, 28(1), 261–271. [https://doi.org/10.1016/s0896-6273\(00\)00101-x](https://doi.org/10.1016/s0896-6273(00)00101-x)
- Schreiner, D. C., Wright, A., Baltz, E. T., Wang, T., Cazares, C., & Gremel, C. M. (2023). Chronic alcohol exposure alters action control via hyperactive premotor corticostriatal activity. *Cell Reports*, 42(7), 112675. <https://doi.org/10.1016/j.celrep.2023.112675>
- Schultz, W., Dayan, P., & Montague, P. R. (1997). A Neural Substrate of Prediction and Reward. *Science*, 275(5306), 1593–1599. <https://doi.org/10.1126/science.275.5306.1593>

- Sharma, R., Chischolm, A., Parikh, M., Kempuraj, D., & Thakkar, M. (2024). Cholinergic Interneurons in the Accumbal Shell Region Regulate Binge Alcohol Self-Administration in Mice: An In Vivo Calcium Imaging Study. *Brain Sciences*, 14(5), 484. <https://doi.org/10.3390/brainsci14050484>
- Sias, A. C., Jafar, Y., Goodpaster, C. M., Ramírez-Armenta, K., Wrenn, T. M., Griffin, N. K., Patel, K., Lamparelli, A. C., Sharpe, M. J., & Wassum, K. M. (2024). Dopamine projections to the basolateral amygdala drive the encoding of identity-specific reward memories. *Nature Neuroscience*, 27(4), 728–736. <https://doi.org/10.1038/s41593-024-01586-7>
- Siciliano, C. A., Calipari, E. S., Yorgason, J. T., Lovinger, D. M., Mateo, Y., Jimenez, V. A., Helms, C. M., Grant, K. A., & Jones, S. R. (2016). Increased presynaptic regulation of dopamine neurotransmission in the nucleus accumbens core following chronic ethanol self-administration in female macaques. *Psychopharmacology*, 233(8), 1435–1443. <https://doi.org/10.1007/s00213-016-4239-4>
- Stuber, G. D., Klanker, M., de Ridder, B., Bowers, M. S., Joosten, R. N., Feenstra, M. G., & Bonci, A. (2008). Reward-predictive cues enhance excitatory synaptic strength onto midbrain dopamine neurons. *Science (New York, N.Y.)*, 321(5896), 1690–1692. <https://doi.org/10.1126/science.1160873>
- Tanaka, N. K., Tanimoto, H., & Ito, K. (2008). Neuronal assemblies of the *Drosophila* mushroom body. *Journal of Comparative Neurology*, 508(5), 711–755. <https://doi.org/10.1002/cne.21692>

- Tateno, T., & Robinson, H. P. C. (2011). The mechanism of ethanol action on midbrain dopaminergic neuron firing: A dynamic-clamp study of the role of I_h and GABAergic synaptic integration. *Journal of Neurophysiology*, 106(4), 1901–1922.
<https://doi.org/10.1152/jn.00162.2011>
- Tomchik, S. M., & Davis, R. L. (2009). Dynamics of learning-related cAMP signaling and stimulus integration in the *Drosophila* olfactory pathway. *Neuron*, 64(4), 510–521.
<https://doi.org/10.1016/j.neuron.2009.09.029>
- Treistman, S. N., & Martin, G. E. (2009). BK Channels: Mediators and models for alcohol tolerance. *Trends in Neurosciences*, 32(12), 629–637.
<https://doi.org/10.1016/j.tins.2009.08.001>
- Varghese, J., & Dakhode, S. (2022). Effects of Alcohol Consumption on Various Systems of the Human Body: A Systematic Review. *Cureus*, 14(10), e30057.
<https://doi.org/10.7759/cureus.30057>
- Volkow, N. D., Fowler, J. S., Wang, G.-J., & Swanson, J. M. (2004). Dopamine in drug abuse and addiction: Results from imaging studies and treatment implications. *Molecular Psychiatry*, 9(6), 557–569. <https://doi.org/10.1038/sj.mp.4001507>
- Wang, C.-M., Wu, C.-Y., Lin, C.-E., Hsu, M.-C., Lin, J.-C., Huang, C.-C., Lien, T.-Y., Lin, H.-K., Chang, T.-W., & Chiang, H.-C. (2023). Forgotten memory storage and retrieval in *Drosophila*. *Nature Communications*, 14(1), 7153. <https://doi.org/10.1038/s41467-023-42753-x>
- Weiss, F., Lorang, M. T., Bloom, F. E., & Koob, G. F. (1993). Oral alcohol self-administration stimulates dopamine release in the rat nucleus accumbens: Genetic and

- motivational determinants. *The Journal of Pharmacology and Experimental Therapeutics*, 267(1), 250–258.
- Weiss, F., Parsons, L. H., Schulteis, G., Hyytiä, P., Lorang, M. T., Bloom, F. E., & Koob, G. F. (1996). Ethanol self-administration restores withdrawal-associated deficiencies in accumbal dopamine and 5-hydroxytryptamine release in dependent rats. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 16(10), 3474–3485. <https://doi.org/10.1523/JNEUROSCI.16-10-03474.1996>
- Wolf, F. W., & Heberlein, U. (2003). Invertebrate models of drug abuse. *Journal of Neurobiology*, 54(1), 161–178. <https://doi.org/10.1002/neu.10166>
- Xu, S., Chan, T., Shah, V., Zhang, S., Pletcher, S. D., & Roman, G. (2012). The propensity for consuming ethanol in *Drosophila* requires rutabaga adenylyl cyclase expression within mushroom body neurons. *Genes, Brain, and Behavior*, 11(6), 727–739. <https://doi.org/10.1111/j.1601-183X.2012.00810.x>
- Yamagata, N., Ichinose, T., Aso, Y., Plaçais, P.-Y., Friedrich, A. B., Sima, R. J., Preat, T., Rubin, G. M., & Tanimoto, H. (2015). Distinct dopamine neurons mediate reward signals for short- and long-term memories. *Proceedings of the National Academy of Sciences*, 112(2), 578–583. <https://doi.org/10.1073/pnas.1421930112>
- Zhou, M., Chen, N., Tian, J., Zeng, J., Zhang, Y., Zhang, X., Guo, J., Sun, J., Li, Y., Guo, A., & Li, Y. (2019). Suppression of GABAergic neurons through D2-like receptor secures efficient conditioning in *Drosophila* aversive olfactory learning. *Proceedings of the National Academy of Sciences*, 116(11), 5118–5125. <https://doi.org/10.1073/pnas.1812342116>

2.9: CHAPTER 2 FIGURES

Figure 2.1

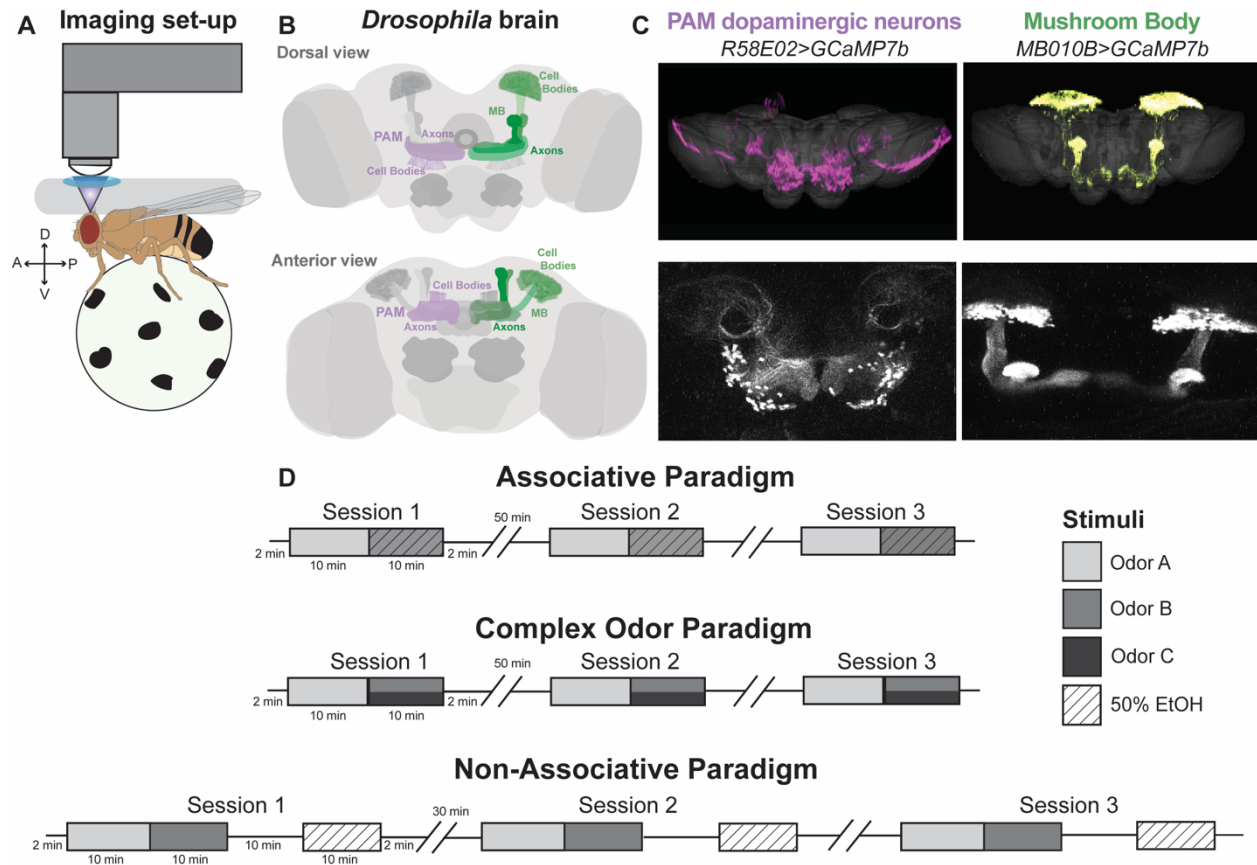


Figure 2.1: Paradigms investigating the impact of alcohol-odor associations on *Drosophila* memory circuitry.

(A) Representation of in vivo imaging of neural circuits under a 2-photon. (B) Dorsal (Top) and Anterior (Bottom) view schematics of PAM and MB neural population in the *Drosophila* brain. (C) Virtual Fly Brain depictions of R58E02 and MB010B Gal4 expression patterns (Top). Representative images of *R58E02>GCaMP7b* and *MB010B>GCaMP7b* (Bottom). D. Different paradigms implemented to investigate the impact of cues-alcohol pairing on neural dynamics

Figure 2.2

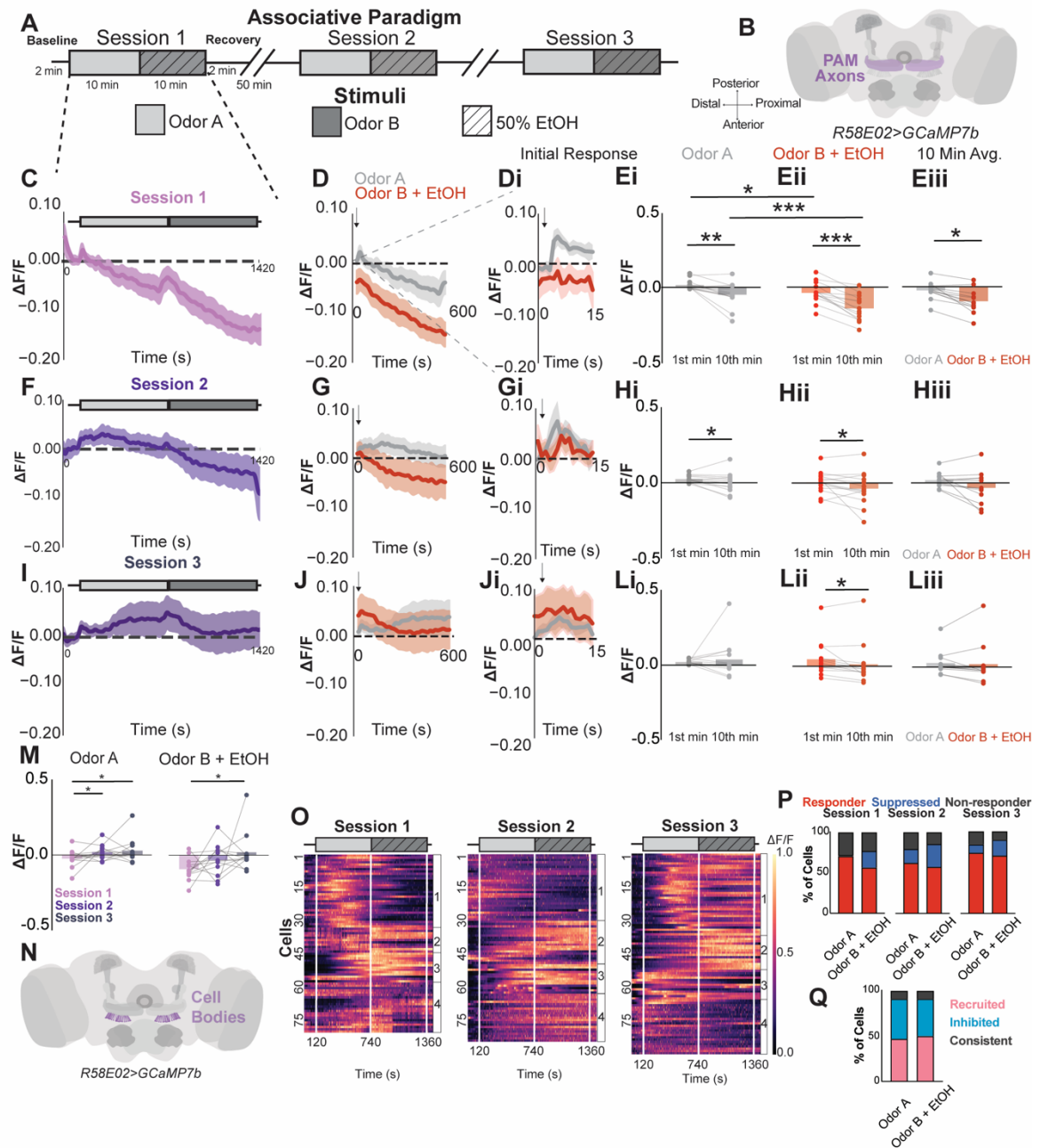


Figure 2.2: Acquisition of alcohol memory increases dopaminergic axon terminal activity.

(A) Representative schematic of the associative paradigm. (B) Schematic of the *Drosophila* brain with the PAM axon terminals highlighted in purple. (C) PAM axon

terminal activity during the first session of the associative paradigm ($n = 14$ flies). Odor alone causes an increase in activity, however odor paired with ethanol decreases activity during the stimulation period. (D) Overlay of odor-alone response (grey) with ethanol paired with odor response (red) for the first session. The arrow marks the onset of the stimulus. (Di) Zoom in on the first 15 seconds of stimulation for both odor alone and ethanol paired with odor for the first session. (Ei) Bar plot comparing the first minute of odor alone to the last-minute during session 1. (Eii) Comparison between the first minute of exposure to ethanol paired with odor to the last minute of exposure for the first session. (Eiii) Average activity during the entire 10-minute stimulation period for odor alone and ethanol paired with odor. (F) PAM axon terminal activity during the second session of the associative paradigm. Pairing ethanol with odor still decreases the activity of the PAM axon terminals. (G) Overlay of odor alone response with ethanol paired with odor response for the second session. (Gi) Zoom in on the first 15 seconds of stimulation for both odor alone and ethanol paired with odor for the second session. (Hi) Bar plot comparing the first minute of odor alone to the last-minute during session 2. (Hii) Comparison between the first minute of exposure to ethanol paired with an odor to the last minute of exposure for the second session. (Hiii) Average activity during the entire 10-minute stimulation period for odor alone and ethanol paired with odor for the second session. (I) PAM axon terminal activity during the third session of the associative paradigm. Pairing ethanol with odor still decreases the activity of the PAM axon terminals. (J) Overlay of odor alone response with ethanol paired with odor response for the third session. (Ji) Zoom in on the first 15 seconds of stimulation for both odor alone and ethanol paired with odor for the third session. (Ki) Bar plot comparing the first

minute of odor alone to the last minute during session 3. (Kii) Comparison between the first minute of exposure to ethanol paired with an odor to the last minute of exposure for the third session. (Kiii) Average activity during the entire 10-minute stimulation period for odor alone and ethanol paired with odor for the second session (right). (Li) Bar plot comparing the response to odor alone across the three sessions (left, pink = Session 1, purple = Session 2, dark purple = Session 3). (Lii) Bar plot comparing the activity of the axon terminals across the three sessions to odor paired with ethanol. (M) Schematic of *Drosophila* brain highlighting the cell bodies of the PAM neurons. Heatmaps of the cell bodies responses during session 1 (Ni), session 2 (Nii), and session 3 (Niii). Distribution of within-session cellular responses to odor alone and odor paired with ethanol for sessions 1 (Oi), 2 (Oii), and 3 (Oiii). Responders (increased their activity by 10% from baseline) are red, suppressed (decreased their activity by 10% or more from baseline) are blue, and non-responders ($-10\% < \text{activity} < 10\%$) are grey. (P) Categorization of cells based on whether they increased their activity by 10% from session 1 to session 3 (Recruited, pink), decreased their activity by 10% or more from session 1 to session 3 (Inhibited, light blue), or remained consistent across the sessions (Consistent, gray).

Figure 2.3

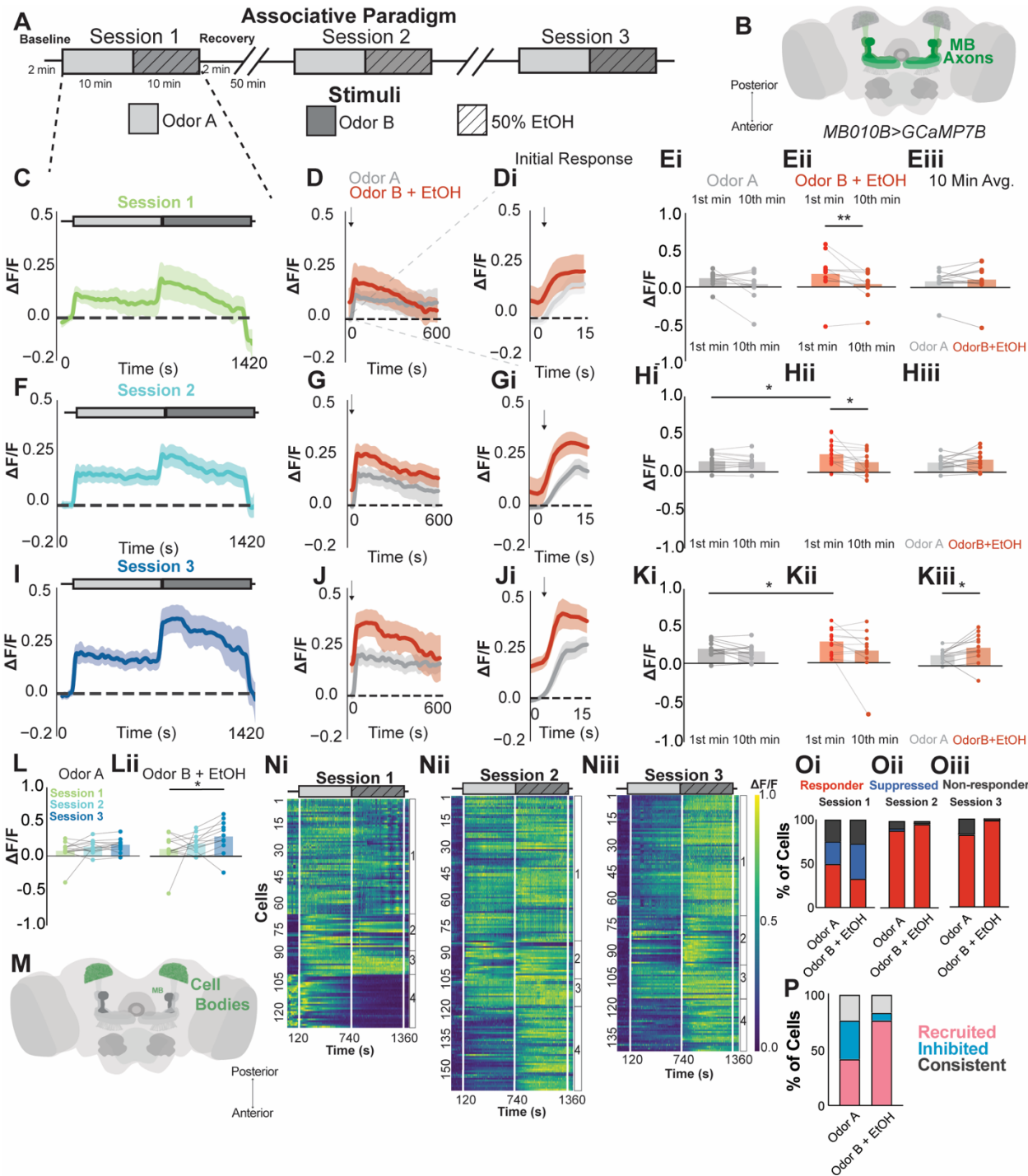


Figure 2.3: Acquisition of alcohol memory increases MB axon terminal activity and recruits MB neurons.

(A) Representative schematic of the associative paradigm. (B) Schematic of the *Drosophila* brain with the MB axon terminals highlighted in green. (C) MB axon terminal activity during the first session of the associative paradigm. (D) Overlay of odor-alone response with ethanol paired with odor response for the first session. The arrow marks the onset of the stimulus. (Di) Zoom in on the first 15 seconds of stimulation for both odor alone and ethanol paired with odor for the first session. (Ei) Bar plot comparing the first minute of odor alone to the last minute during session 1. (Eii) Comparison between the first minute of exposure to ethanol paired with an odor to the last minute of exposure for the first session. (Eiii) Average activity during the entire 10-minute stimulation period for odor alone and ethanol paired with odor. (F) MB axon terminal activity during the second session of the associative paradigm. Pairing ethanol with odor still decreases the activity of the PAM axon terminals. (G) Overlay of odor alone response with ethanol paired with odor response for the second session. (Gi) Zoom in on the first 15 seconds of stimulation for both odor alone and ethanol paired with odor for the second session. (Hi) Bar plot comparing the first minute of exposure to odor alone to the last minute during session 2. (Hii) Comparison between the first minute of exposure to ethanol paired with an odor to the last minute of exposure for the second session. (Hiii) Average activity during the entire 10-minute stimulation period for odor alone and ethanol paired with odor for the second session (right). (I) MB axon terminal activity during the third session of the associative paradigm. Pairing ethanol with odor still decreases the activity of the PAM axon terminals. (J) Overlay of odor alone response with ethanol paired with odor response for the third session. (Ji) Zoom in on the first 15 seconds of stimulation for both odor alone and ethanol paired with odor for the third session. (Ki)

Bar plot comparing the first minute of odor alone to the last-minute during session 3.

(Kii) Comparison between the first minute of exposure to ethanol paired with an odor to

the last minute of exposure for the third session. (Kiii) Average activity during the entire

10-minute stimulation period for odor alone and ethanol paired with odor for the second

session (right). (Li) Bar plot comparing the response to odor alone across the three

sessions (left, pink = Session 1, purple = Session 2, dark purple = Session 3). (Lii) Bar

plot comparing the activity of the axon terminals across the three sessions to odor

paired with ethanol. (M) Schematic of *Drosophila* brain highlighting the cell bodies of the

MB neurons. Heatmaps of the cell bodies responses during session 1 (Ni), session 2

(Nii), and session 3 (Niii). Distribution of within-session cellular responses to odor alone

and odor paired with ethanol for sessions 1 (Oi), 2 (Oii), and 3 (Oiii). (Q) Categorization

of cells based on whether they increased their activity by 10% from session 1 to session

3 (Recruited, pink), decreased their activity by 10% or more from session 1 to session 3

(Inhibited, light blue), or remained consistent across the sessions (Consistent, gray).

Figure 2.4

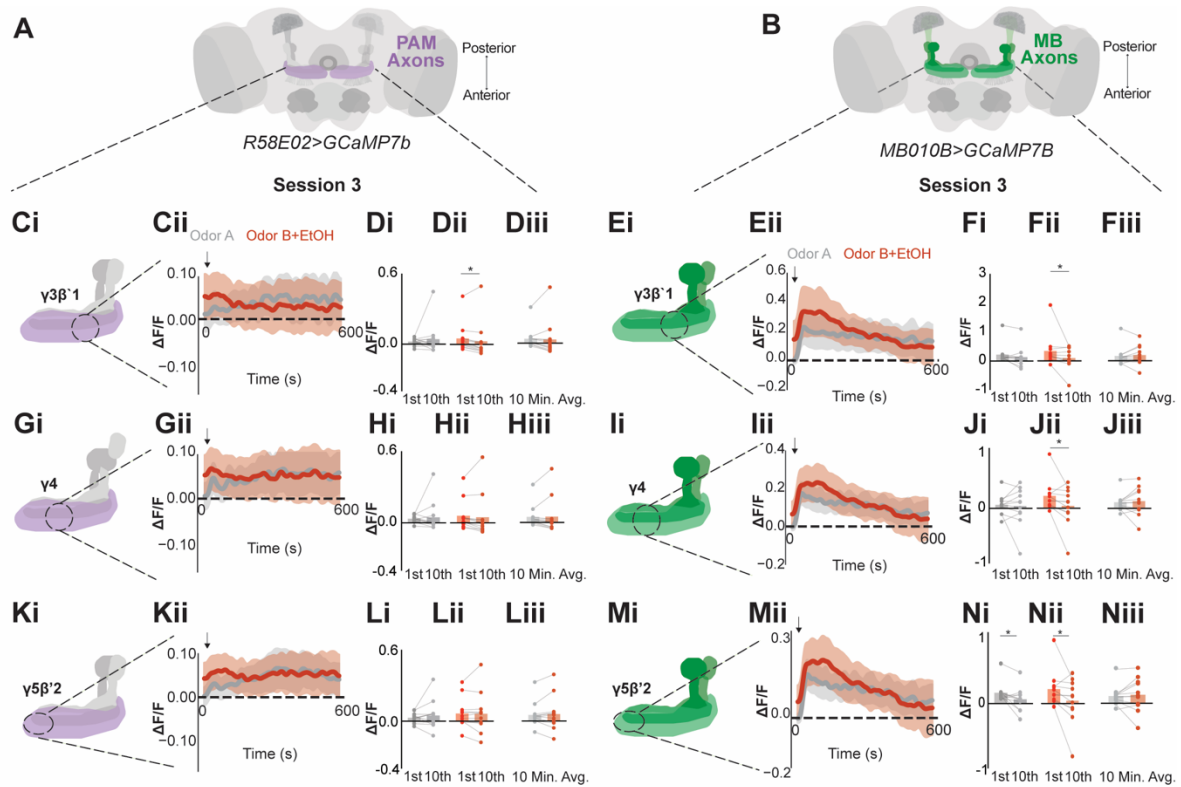


Figure 2.4: Compartmentalized responses to the acquisition of alcohol memory.

(A) Schematic of the *Drosophila* brain with the PAM axon terminals highlighted in purple. (B) Schematic of the *Drosophila* brain with MB axon terminals highlighted in green. (Ci) $\gamma 3\beta'1$ compartment highlighted within the axon terminals of the PAM neurons (*R58E02>GCaMP7b*). (Cii) (C) Odor alone response (gray) overlaid with odor paired with ethanol (red) for $\gamma 3\beta'1$ during the third session ($n = 12$). Bar plots showing the first and last-minute responses for odor alone (Di, gray) and odor paired with ethanol (middle, red). Bar plots quantifying the first and last-minute response for odor alone (Di) and the pairing (Dii). Overall activity during session 3 quantified in a bar plot (Diii). (Ei) $\gamma 3\beta'1$ compartment highlighted within the axon terminals of the MB neurons

(*MB010B>GCaMP7b*). (Eii) Odor alone response (gray) overlaid with odor paired with ethanol (red) for y3B`1 during the third session (n = 11). Bar plots showing the first and last-minute responses for odor alone (Fi, gray) and odor paired with ethanol (Fii, red). Bar plot comparison of overall activity during each stimuli's 10-minute presentation (Fiii).

(Gi) y4 compartment highlighted within the axon terminals of the PAM neurons

(*R58E02>GCaMP7b*). (Gii) (C) Odor alone response (gray) overlaid with odor paired with ethanol (red) for y4 during the third session (n = 12). Bar plots showing the first and last-minute responses for odor alone (Hi, gray) and odor paired with ethanol (Hii, red). Bar plot comparison of overall activity during each stimuli's 10-minute presentation (Hiii).

(Ii) y4 compartment highlighted within the axon terminals of the MB neurons

(*MB010B>GCaMP7b*). (Iii) (C) Odor alone response (gray) overlaid with odor paired with ethanol (red) for y4 during the third session (n = 11). Bar plots showing the first and last-minute responses for odor alone (Ji, gray) and odor paired with ethanol (Jii, red). Bar plot comparison of overall activity during each stimuli's 10-minute presentation (Jiii).

(Ki) y5 compartment highlighted within the axon terminals of the PAM neurons

(*R58E02>GCaMP7b*). (Kii) (C) Odor alone response (gray) overlaid with odor paired with ethanol (red) for y5 during the third session (n = 12). Bar plots showing the first and last-minute responses for odor alone (Li, gray) and odor paired with ethanol (Lii, red). Bar plot comparison of overall activity during each stimuli's 10-minute presentation (Liii).

(Mi) y5 compartment highlighted within the axon terminals of the MB neurons

(*MB010B>GCaMP7b*). (Mii) (C) Odor alone response (gray) overlaid with odor paired with ethanol (red) for y5 during the third session (n = 11). Bar plots showing the first and

last-minute responses for odor alone (Ni, gray) and odor paired with ethanol (Nii, red).

Bar plot comparison of overall activity during each stimuli's 10-minute presentation (Niii).

Figure 2.5

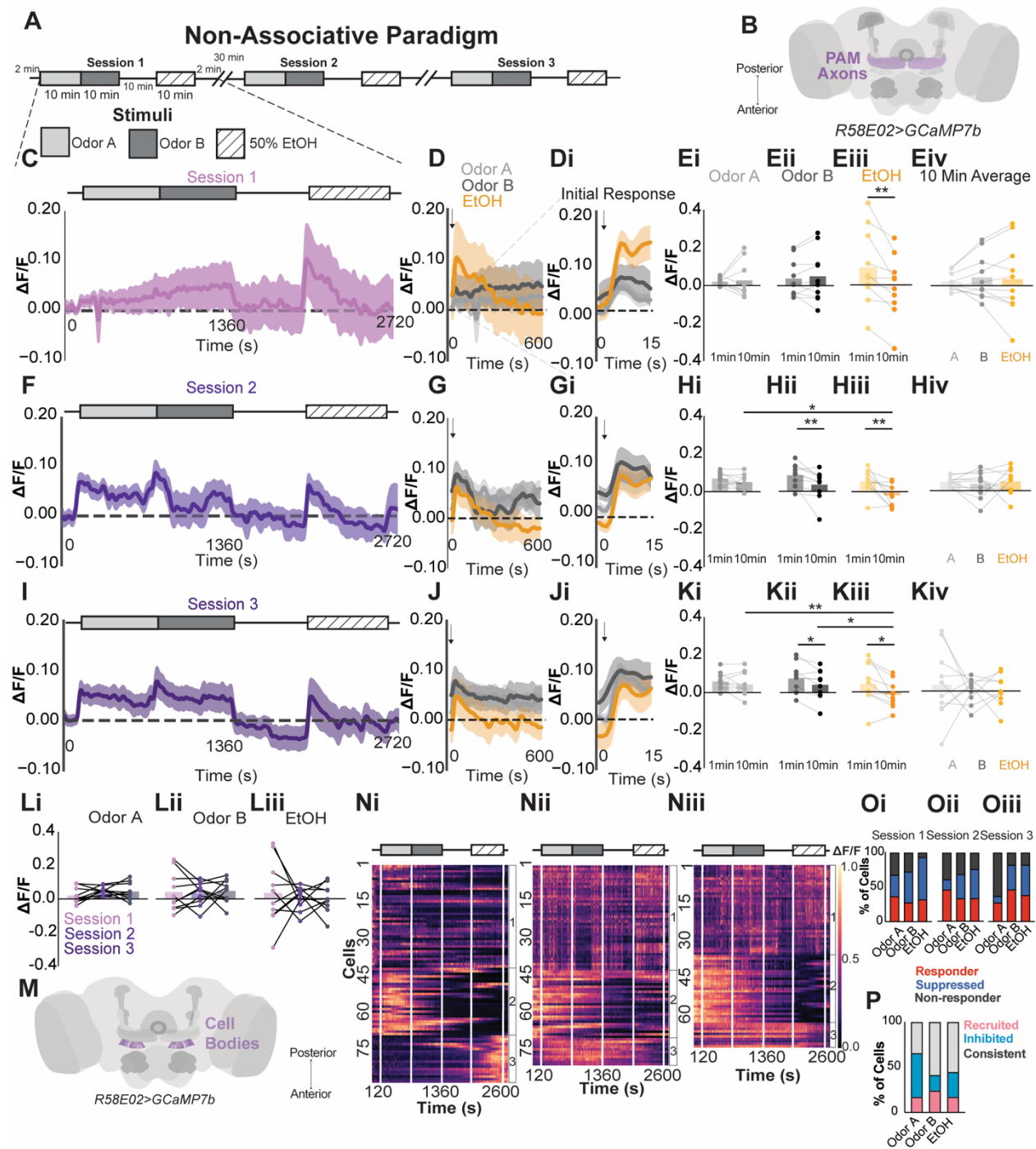


Figure 2.5: Alcohol unpaired from odors does not potentiate dopaminergic axon terminals.

(A) Representative schematic of the non-associative paradigm. (B) Schematic of the *Drosophila* brain with the PAM axon terminals highlighted in purple. (C) PAM axon terminal activity during the first session of the associative paradigm (n = 10). (D) Overlay of odor A response with odor B (gray) response (dark gray) and ethanol alone (yellow) for the first session. The arrow marks the onset of the stimulus. (Di) Zoom in on the first 15 seconds of stimulation for odors and alcohol. Bar plot comparison between the first minute of exposure to Odor A (Ei), Odor B (Eii), and alcohol alone (Eii) to the last minute of exposure for the first session. (Eiv) Average activity during the entire 10-minute stimulation period for odors and alcohol alone. (F) PAM axon terminal activity during the second session of the non-associative paradigm. (G) Overlay of odor alone response with ethanol paired with odor response for the second session. (Gi) Zoom in on the first 15 seconds of stimulation for each stimulus during the second session. Bar plot comparison between the first minute of exposure to Odor A (Hi), Odor B (Hii), and alcohol alone (Hiii) to the last minute of exposure for the second session. (Hiv) Average activity during the entire 10-minute stimulation period for each stimulus during the second session. (I) PAM axon terminal activity during the third session of the non-associative paradigm. (J) Overlay of odors and alcohol response for the third session. (Ji) Zoom in on the first 15 seconds of stimulation for each stimulus during the third session. Bar plot comparison between the first minute of exposure to Odor A (Ki) and odor B (Kii) and alcohol alone (Kiii) to the last minute of exposure for the second session (Kiv). Average activity during the entire 10-minute stimulation period for odor alone and ethanol paired with odor for the second session. (Li) Bar plot comparing the response to odor A across the three sessions (pink = Session 1, purple = Session 2,

dark purple = Session 3). (Lii) Bar plot comparing the activity of the axon terminals across the three sessions to odor B. (Liii) Bar plot comparing the activity of axon terminals across three sessions to ethanol. (M) Schematic of *Drosophila* brain highlighting the cell bodies of the PAM neurons. Heatmaps of the cell bodies responses during session 1 (Ni), session 2 (Nii), and session 3 (Niii). (P) Distribution of within-session cellular responses to both odors and the ethanol odor for sessions 1 (Oi), 2 (Oii), and 3 (Oiii). (P) Categorization of cells based on whether they increased their activity by 10% from session 1 to session 3 (Recruited, pink), decreased their activity by 10% or more from session 1 to session 3 (Inhibited, light blue), or remained consistent across the sessions (Consistent, gray).

A Non-Associative Paradigm

B

Posterior
Anterior
MB Axons
MB010B>GCaMP7B

C

Session 1
ΔF/F
Time (s)
1360 2720

D

Odor A
Odor B
EtOH
ΔF/F
Time (s)
0 600

E

Initial Response
ΔF/F
Time (s)
0 15

F

Session 2
ΔF/F
Time (s)
1360 2720

G

ΔF/F
Time (s)
0 600

H

ΔF/F
Time (s)
0 15

I

Session 3
ΔF/F
Time (s)
1360 2720

J

ΔF/F
Time (s)
0 600

K

ΔF/F
Time (s)
0 15

L

Odor A
Odor B
EtOH
ΔF/F
Time (s)
0 15

M

Posterior
Anterior
Cell Bodies
MB

N

Session 1
Cells
Time (s)
120 1360 2600

O

Session 2
Cells
Time (s)
120 1360 2600

P

Session 3
Cells
Time (s)
120 1360 2600

Q

Recruited
Inhibited
Consistent
% of Cells
Session 1 Session 2 Session 3

R

Recruited
Inhibited
Consistent
% of Cells
Session 1 Session 2 Session 3

(A) Representative schematic of the non-associative paradigm. (B) Schematic of the *Drosophila* brain with the MB axon terminals highlighted in green. (C) MB axon terminal activity during the first session of the associative paradigm (n = 11). (D) Overlay of odor A response with odor B (gray) response (dark gray) and ethanol alone (yellow) for the first session. The arrow marks the onset of the stimulus. (Di) Zoom in on the first 15 seconds of stimulation for odors and alcohol. Bar plot comparison between the first minute of exposure to Odor A (Ei), Odor B (Eii), and alcohol alone (Eii) to the last minute of exposure for the first session (Eiv). Average activity during the entire 10-minute stimulation period for odors and alcohol alone. (F) MB axon terminal activity during the second session of the non-associative paradigm. (G) Overlay of odor alone response with ethanol paired with odor response for the second session. (Gi) Zoom in on the first 15 seconds of stimulation for each stimulus during the second session. Bar plot comparison between the first minute of exposure to Odor A (Hi), Odor B (Hii), and alcohol alone (Hiii) to the last minute of exposure for the second session. (Hiv) Average activity during the entire 10-minute stimulation period for each stimulus during the second session. (I) MB axon terminal activity during the third session of the non-associative paradigm. (J) Overlay of odors and alcohol response for the third session. (Ji) Zoom in on the first 15 seconds of stimulation for each stimulus during the third session. Bar plot comparison between the first minute of exposure to Odor A (Ki), Odor B (Kii), and alcohol alone's (Kiii) to the last minute of exposure for the second session. (Kiv) Average activity during the entire 10-minute stimulation period for odor alone and ethanol paired with odor for the second session. (Li) Bar plot comparing the response to odor A across the three sessions (green = Session 1, light blue = Session 2, dark blue =

Session 3). (Lii) Bar plot comparing the activity of the axon terminals across the three sessions to odor B. (Liii) Bar plot comparing the activity of axon terminals across three sessions to ethanol. (M) Cartoon of *Drosophila* brain highlighting the cell bodies of the MB neurons. Heatmaps of the cell bodies responses during session 1 (Ni), session 2 (Nii), and session 3 (Niii). (P) Distribution of within-session cellular responses to both odors and the ethanol odor for sessions 1 (Oi), 2 (Oii), and 3 (Oiii). (P) Categorization of cells based on whether they increased their activity by 10% from session 1 to session 3 (Recruited, pink), decreased their activity by 10% or more from session 1 to session 3 (Inhibited, light blue), or remained consistent across the sessions (Consistent, gray).

Figure 2.7

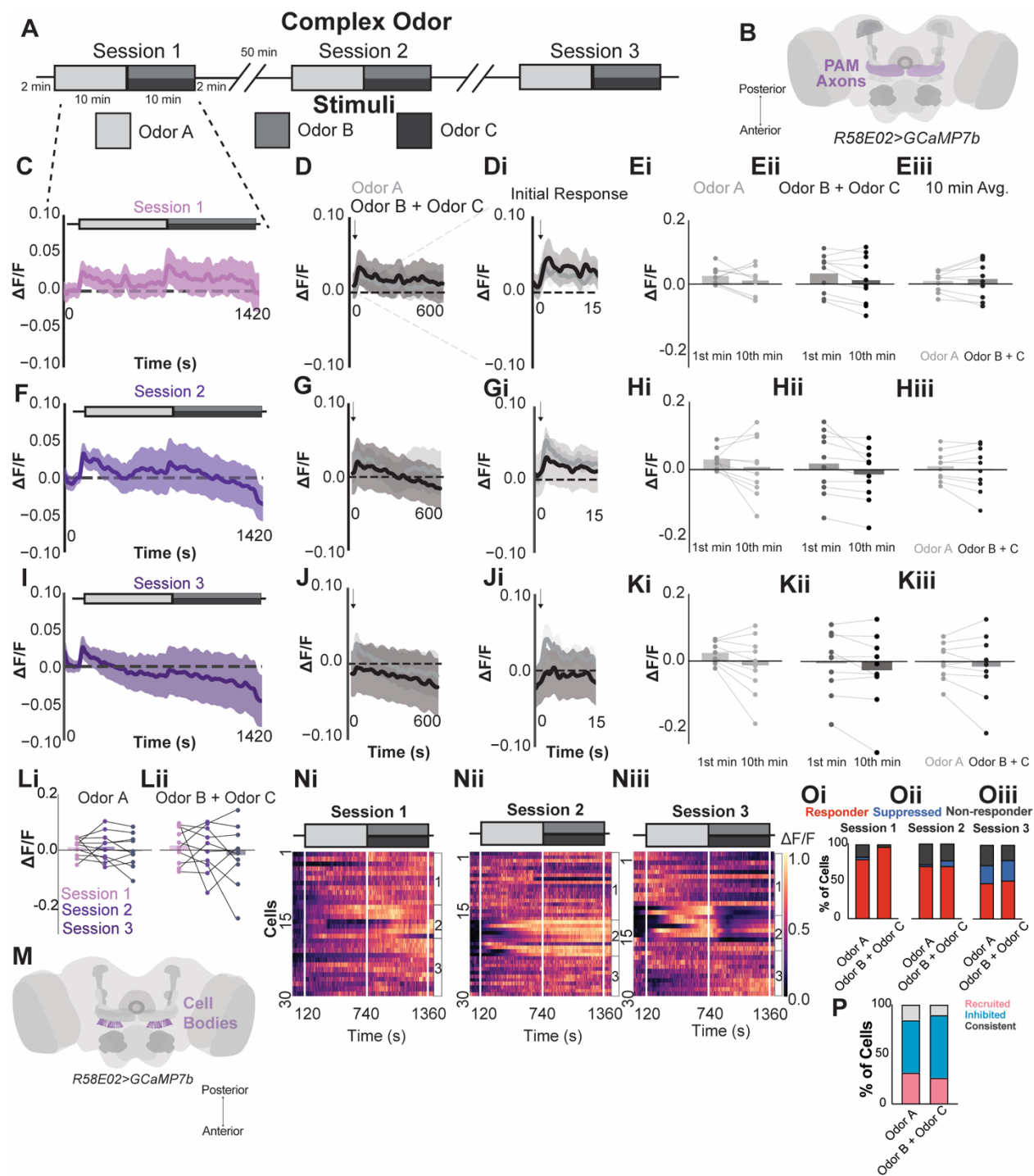


Figure 2.7: Complex odor presentation does not generate plasticity within dopaminergic axon terminals and does not recruit neurons.

(A) Representative schematic of the complex odor paradigm. Flies are exposed to 10 minutes of odor alone, followed by 10 minutes of two odors presented simultaneously. This is repeated for a total of 3 spaced sessions. (B) Schematic of the *Drosophila* brain with the PAM axon terminals highlighted in purple. (C) PAM axon terminal activity during the first session of the complex odor paradigm. Both the odor alone and the complex odor increase the activity of the dopaminergic axon terminals. (D) Overlay of odor-alone response (gray) with complex odor response (black) for the first session. The arrow marks the onset of the stimulus. (Di) Zoom in on the first 15 seconds of stimulation for both odor alone and ethanol paired with odor for the first session. (Ei) Bar plot comparing the first minute of odor alone to the last minute during session 1. (Eii) Comparison between complex odor first minute of exposure to the last minute of exposure for the first session. (Eiii) Average activity during the entire 10-minute stimulation period for odor alone and complex odor. (F) PAM axon terminal activity during the second session of the complex odor paradigm. (G) Overlay of odor alone response with complex odor response for the second session. (Gi) Zoom in on the first 15 seconds of stimulation for both odor alone and ethanol paired with odor for the second session. (Hi) Bar plot comparing the first minute of odor to the last minute during session 2. (Hii) Comparison between the first minute of exposure to the complex odor to the last minute of exposure for the second session. (Hiii) Average activity during the entire 10-minute stimulation period for odor alone and complex odor for the second session (right). (I) PAM axon terminal activity during the third session of the complex

odor paradigm. (J) Overlay of odor alone response with complex odor response for the second session. (Ji) Zoom in on the first 15 seconds of stimulation for both odor alone and the complex odor for the second session. (Ki) Bar plot comparing the first minute of exposure to the odor alone to the last minute during session 2. (Kii) Comparison between the first minute of exposure to the complex odor to the last minute of exposure for the second session. (Kiii) Average activity during the entire 10-minute stimulation period for odor alone and complex odor for the second session. (Li) Bar plot comparing the response to odor alone across the three sessions (left, pink = Session 1, purple = Session 2, dark purple = Session 3). (Lii) Bar plot comparing the activity of the axon terminals across the three sessions to odor paired with ethanol. (M) Schematic of *Drosophila* brain highlighting the cell bodies of the MB neurons. Heatmaps of the cell bodies responses during session 1 (Ni), session 2 (Nii), and session 3 (Niii). Distribution of within-session cellular responses to odor alone and complex odor for sessions 1 (Oi), 2 (Oii), and 3 (Oiii). (P) Categorization of cells based on whether they increased their activity by 10% from session 1 to session 3 (Recruited, pink), decreased their activity by 10% or more from session 1 to session 3 (Inhibited, light blue), or remained consistent across the sessions (Consistent, gray).

Figure 2.8

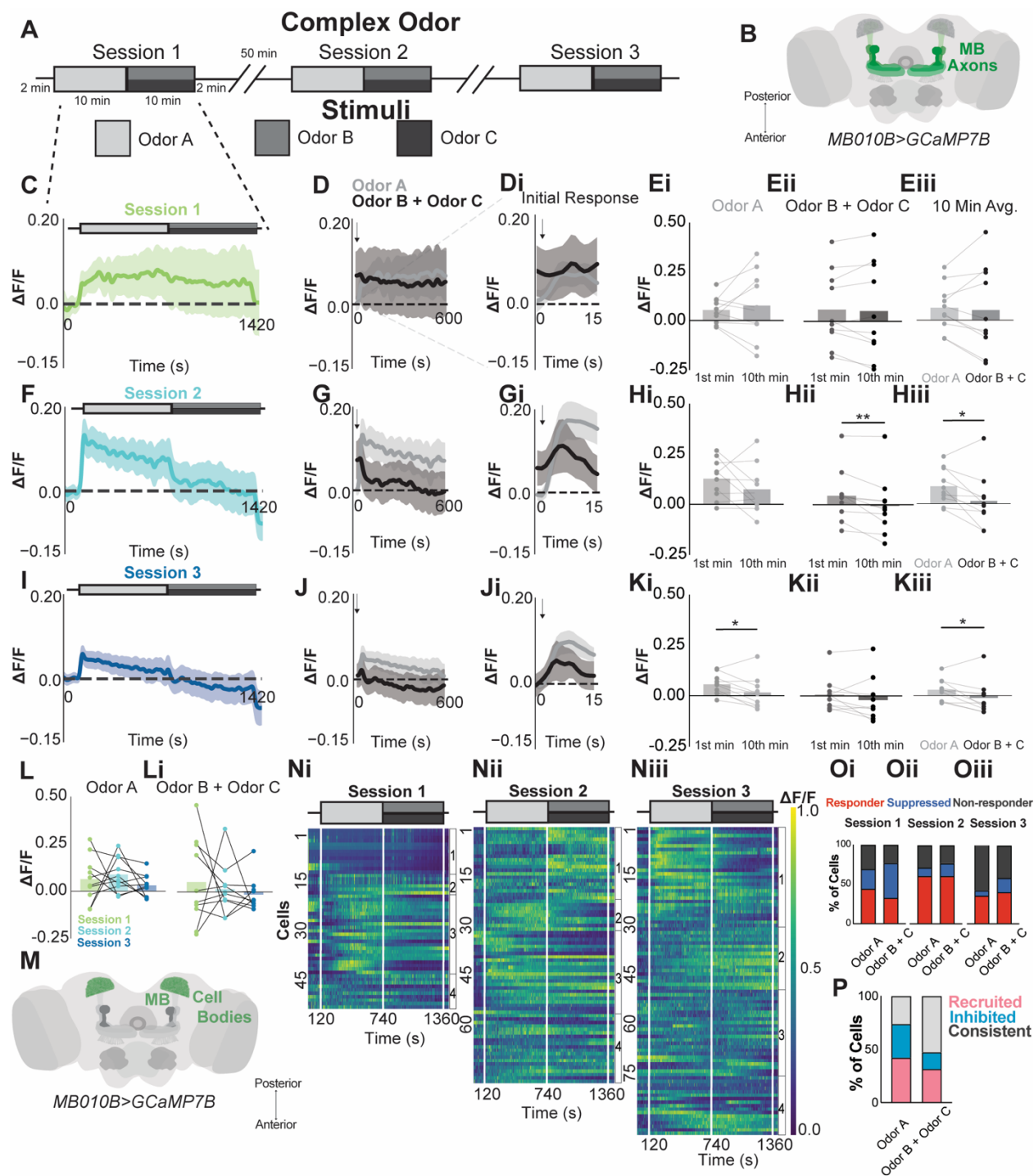


Figure 2.8: Complex odor presentation suppresses MB axon terminal activity and does not recruit cells.

(A) Representative schematic of the complex odor paradigm. (B) Schematic of the *Drosophila* brain with the MB axon terminals highlighted in green. (C) MB axon terminal activity during the first session of the associative paradigm. (D) Overlay of odor-alone response with ethanol paired with odor response for the first session. The arrow marks the onset of the stimulus. (Di) Zoom in on the first 15 seconds of stimulation for both odor alone and complex odor for the first session. (Ei) Bar plot comparing the first minute of exposure to the odor alone to the last minute during session 1. (Eii) Comparison between the first minute of exposure to the complex odor to the last minute of exposure for the first session (middle). (Eiii) Average activity during the entire 10-minute stimulation period for odor alone and complex odor (right). (F) MB axon terminal activity during the second session of the associative paradigm. (G) Overlay of odor alone response with ethanol paired with odor response for the second session. (Gi) Zoom in on the first 15 seconds of stimulation for both odor alone and complex odor for the second session. (Hi) Bar plot comparing the first minute of odor alone to the last minute during session 2. (Hii) Comparison between the first minute of exposure to ethanol paired with an odor to the last minute of exposure for the second session. (Hiii) Average activity during the entire 10-minute stimulation period for odor alone and ethanol paired with odor for the second session. (I) MB axon terminal activity during the third session of the associative paradigm. (J) Overlay of odor alone response with complex odor response for the second session. (Ji) Zoom in on the first 15 seconds of stimulation for both odor alone and ethanol paired with odor for the second session. (Ki) Bar plot comparing the first minute of odor alone to the last minute during session 2. (Kii) Comparison between the first minute of exposure to the complex odor to the last

minute of exposure for the second session. (Kiii) Average activity during the entire 10-minute stimulation period for odor alone and complex odor for the second session (right). (Li) Bar plot comparing the response to odor alone across the three sessions (left, pink = Session 1, purple = Session 2, dark purple = Session 3). (Lii) Bar plot comparing the activity of the axon terminals across the three sessions to complex odor. (M) Schematic of *Drosophila* brain highlighting the cell bodies of the MB neurons. Heatmaps of the cell bodies responses during session 1 (Ni), session 2 (Nii), and session 3 (Niii). Distribution of within-session cellular responses to odor alone and the complex odor for sessions 1 (Oi), 2 (Oii), and 3 (Oiii). (P) Categorization of cells based on whether they increased their activity by 10% from session 1 to session 3 (Recruited, pink), decreased their activity by 10% or more from session 1 to session 3 (Inhibited, light blue), or remained consistent across the sessions (Consistent, gray).

Figure 2.9

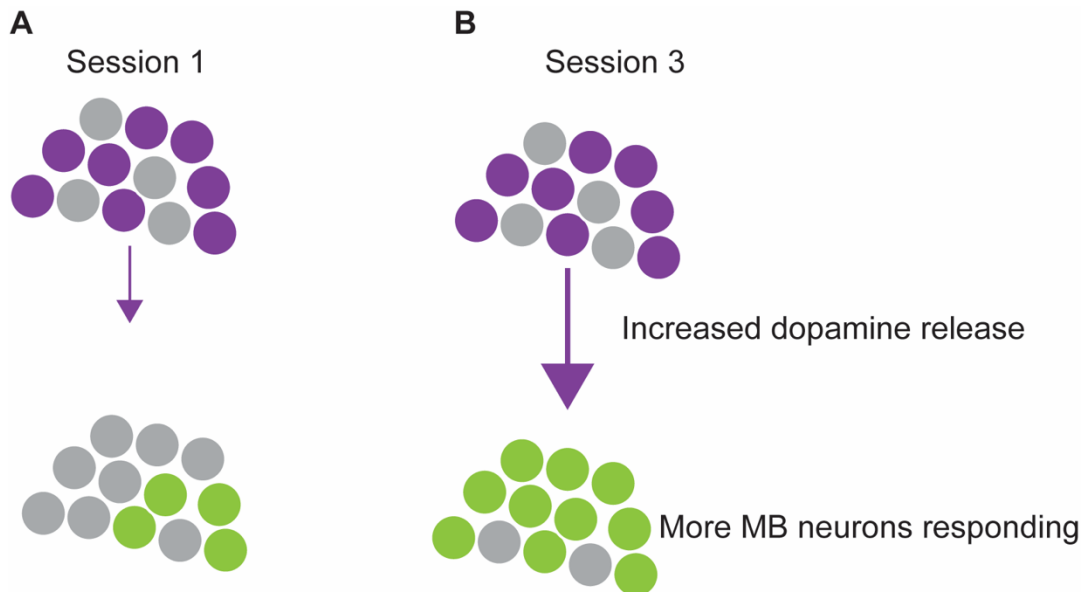
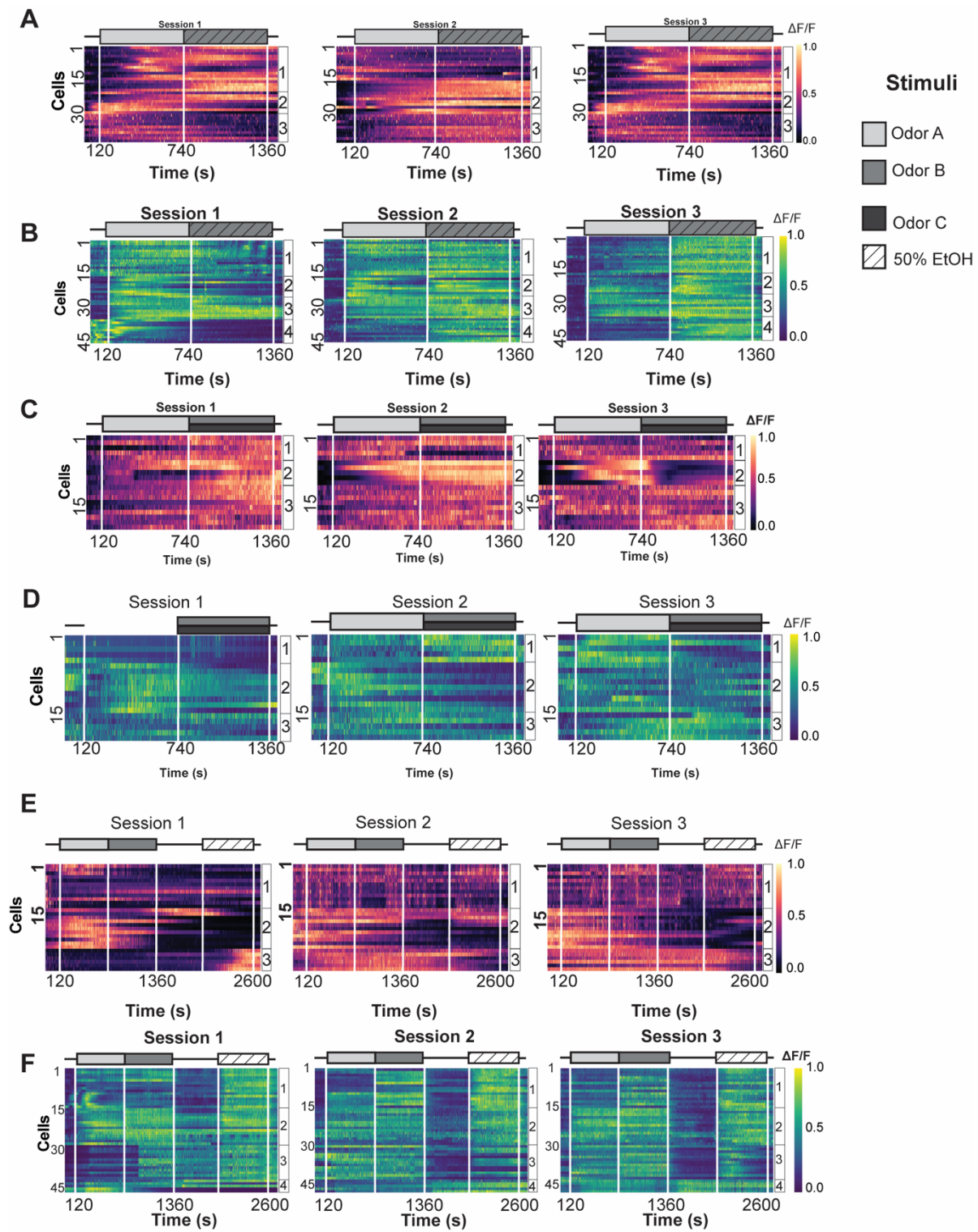


Figure 2.9: Dopamine neurons drive the recruitment of MB neurons during the acquisition of alcohol memory.

(A) Representation of PAM (purple) and MB (green) dynamics during the first session of the associative paradigm. Initially, there is low dopaminergic drive (purple arrow) that corresponds to a smaller number of MB neurons responding to odors paired with alcohol. (B) Representation of PAM and MB dynamics during the final session of ethanol-odor pairings. By the third session, there is increased activity at the axon terminals of the dopamine neurons, likely driving an increased number of MB neurons to respond to odors paired with ethanol.

2.10: CHAPTER 2 SUPPLEMENTAL FIGURES

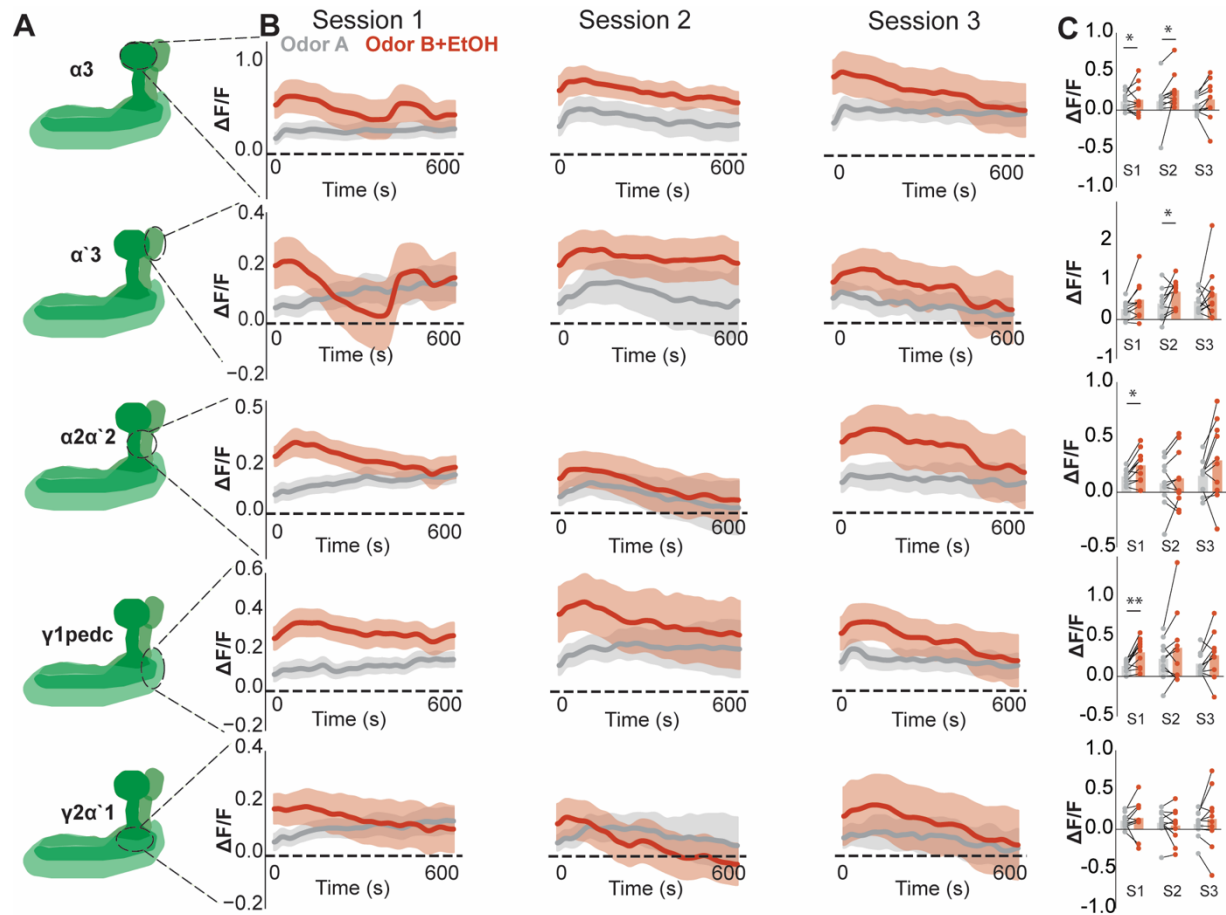
Supplementary Figure 1



Supplemental Figure 2.S1: Subset of tracked cell bodies for each experimental paradigm

Heatmap for cells tracked across all three sessions during: R58E02>GCaMP7b associative paradigm (A), MB010B>GCaMP7b associative paradigm (B), R58E02>GCaMP7b complex odor paradigm (C), MB010B>GCaMP7b complex odor paradigm (D), R58E02>GCaMP7b non-associative paradigm (E), MB010B>GCaMP7b non-associative paradigm (F).

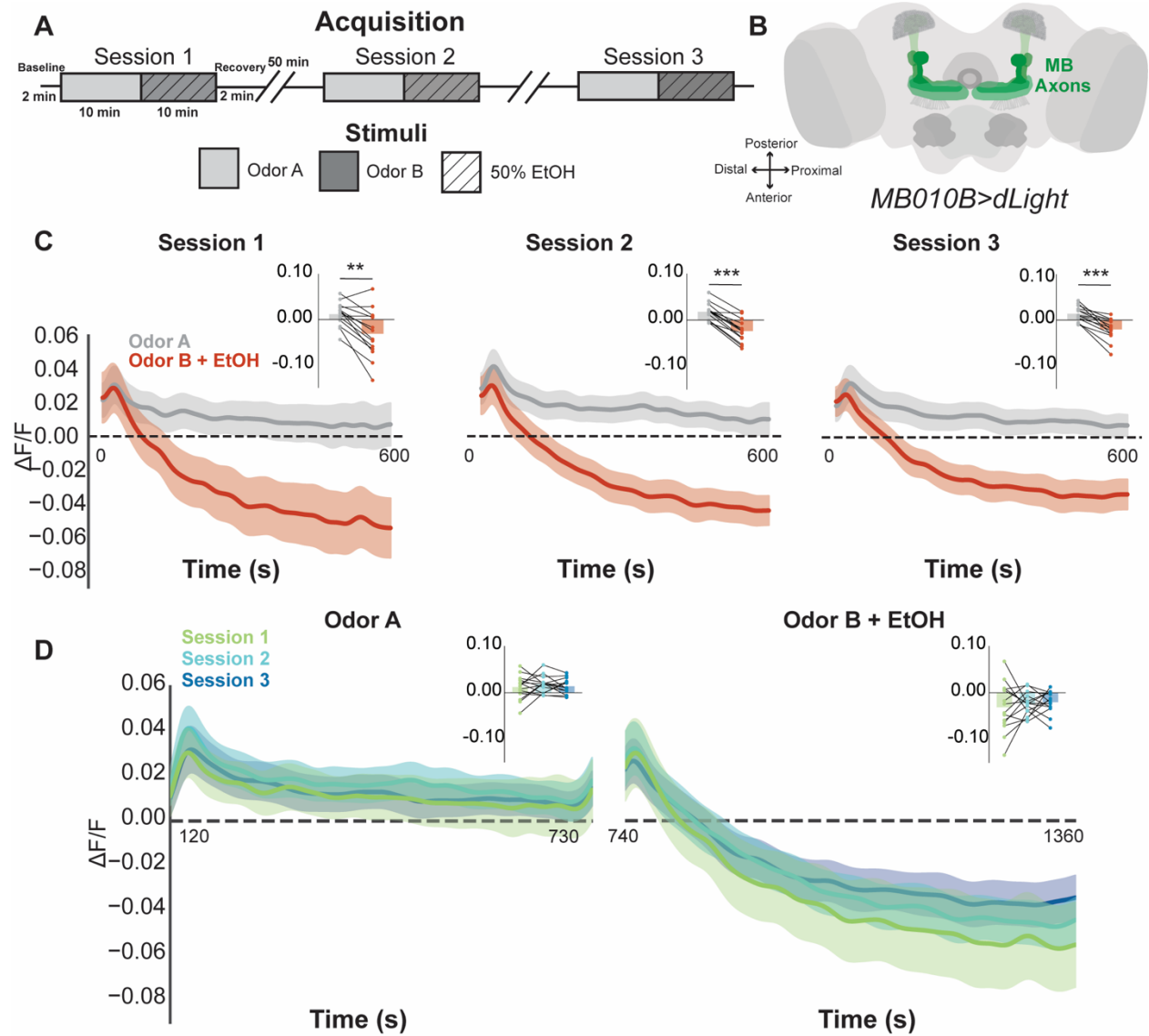
Supplementary Figure 2.S2



Supplemental Figure 2.S2: Vertical lobe compartments during the associative paradigm.

(A) Schematic representations of the MB axon compartments being recorded. (B) Responses to odor alone (grey) and odor paired with ethanol (red) for session 1 (left), session 2 (middle), and session 3 (right) for each compartment. (C) Quantification of responses to each of the stimuli for each compartment across the three sessions.

Supplementary Figure 2.S3



Supplementary Figure 2.S3: Dopamine release onto the mushroom bodies remains consistent throughout the associative paradigm.

(A) Representation of the associative paradigm. (B) Schematic depicting expression of dLight under control of the *MB010B* split gal4. (C) *MB010B>dLight* signal during Sessions 1 (left), 2 (middle), and 3 (right), of the associative paradigm (n = 14). The grey line and shaded overlay represent the average signal and SEM during odor alone.

The red line and shaded overlay represent the average response and SEM to odor paired with ethanol. (D). Overlay of all three sessions with session 1 represented as green, session 2 as light blue, and session 3 as dark blue.

Supplemental Figure 2.S4: Compartments of the MB during dLight acquisition recordings.

(A) Schematic representations of the MB axon compartments being recorded. (B) Responses to odor alone (grey) and odor paired with ethanol (red) for session 1 (left), session 2 (middle), and session 3 (right) for each compartment. (C) Quantification of responses to each of the stimuli for each compartment across the three sessions.

CHAPTER 3

Alcohol Causes Lasting Differential Transcription in *Drosophila* Mushroom Body Neurons

This chapter is adapted from a peer-reviewed publication.

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Author contributions

E.P. designed the research, wrote the manuscript, performed INTACT nuclear extraction, and completed revisions for the manuscript.

T.B. performed immunohistochemistry and acquired brain images.

A.W. performed quantitative PCR experiments.

N.L. performed INTACT nuclear extraction and developed the pipeline to align RNA-seq reads.

K.R.K. designed the research, wrote the manuscript, acquired alcohol memory data and associated data analysis, and completed revision of the manuscript.

3.1. ABSTRACT

Repeated alcohol experiences can produce long-lasting memories for sensory cues associated with intoxication. These memories can problematically trigger relapse in individuals recovering from alcohol use disorder (AUD). The molecular mechanisms by which ethanol changes memories to become long-lasting and inflexible remain unclear. New methods to analyze gene expression within precise neuronal cell types can provide further insight toward AUD prevention and treatment. Here, we used genetic tools in *Drosophila melanogaster* to investigate the lasting consequences of ethanol on transcription in memory-encoding neurons. *Drosophila* rely on mushroom body (MB) neurons to make associative memories, including memories of ethanol-associated sensory cues. Differential expression analyses revealed that distinct transcripts, but not genes, in the MB were associated with experiencing ethanol alone compared to forming a memory of an odor cue associated with ethanol. Adult MB-specific knockdown of spliceosome-associated proteins demonstrated the necessity of RNA-processing in ethanol memory formation. These findings highlight the dynamic, context-specific regulation of transcription in cue-encoding neurons, and the lasting effect of ethanol on transcript usage during memory formation.

3.2 INTRODUCTION

Alcohol use disorder (AUD) affects millions of individuals and constitutes one of the most serious public health problems in the world today (Kendler et al. 2016; Grant et al. 2017; Cheng et al. 2018). This chronic, relapsing brain disorder can affect individuals for extremely long periods of time regardless of alcohol abstinence. Relapse is often triggered by exposure to cues that predict alcohol availability, as they evoke memories of the drug's effects to result in cravings (Jasinska et al. 2014; Courtney et al. 2016; Groefsema et al. 2016; Valyear et al. 2017; Clemens and Holmes 2018; Logge et al. 2019). The complexity of alcohol's molecular actions and our limited understanding of how these actions change distinct neurons in the brain's reward memory circuitry has prevented our understanding of the mechanisms underlying these maladaptive memories.

Unlike the majority of other abused drugs, ethanol does not act on a particular molecular target, but instead affects a variety of molecules (Nestler 2013; Trudell et al. 2014). Many of the molecules implicated in ethanol-induced behaviors have broad roles in regulating diverse processes such as cell signaling, transcription, and neuronal plasticity (Ron and Barak 2016; Erickson et al. 2019). Furthermore, these roles are often dependent on both cell type and developmental stage. This complexity, as well as the diversity of experimental approaches in AUD research, often obscures ethanol's context-specific molecular effects. However, recent advances in cell-type-specific isolation and sequencing technology can reveal the precise consequences of ethanol exposure on gene expression.

The genetic tools available in the fruit fly *Drosophila melanogaster* provide the ability to define where, when, and how alcohol may be acting in the nervous system, including within memory circuitry. *Drosophila* demonstrate ethanol-induced hyperactivity, tolerance (Wolf et al. 2002), and consummatory preference (Devineni and Heberlein 2009). They also remember and prefer the experience of intoxication, and this process requires mushroom body (MB) neuron activity (Kaun et al. 2011; Nunez et al. 2018). The MB integrates both sensory odor information from olfactory projection neurons and valence (aversive/rewarding) information from dopamine neurons. Downstream MB output neurons then drive avoidance of, or approach toward, an odor cue (Aso et al. 2014).

Long-term memory formation in many species requires both de novo transcription and protein synthesis (Bailey et al. 1996; Alberini and Kandel 2014; Sweatt 2016). Although long-term memory formation requires transcriptional changes (Alberini and Kandel 2014; Uchida and Shumyatsky 2018), it remains unclear how repeated ethanol experiences influence these processes. Similarly, whether the presentation of ethanol alone or ethanol paired with an odor induces the same transcriptional events is unknown. We hypothesized that ethanol exposures, alone or paired with an odor, would produce lasting transcriptomic changes within memory-associated MB neurons, thus altering memory formation.

Recent RNA-sequencing (RNA-seq) analysis in *Drosophila* (hereafter “flies”) has systematically identified the transcriptomic profiles of various cell types within MB circuitry (Shih et al. 2019), and examined gene expression changes in the context of associative memory formation (Crocker et al. 2016; Widmer et al. 2018). Here, we used

nuclear, MB-specific RNA-seq to better understand transcriptional changes after the formation of odor-cue-induced ethanol memory. We found that ethanol exposures caused lasting expression changes at the transcript level, but not gene level, and that altered transcript usage was distinct between ethanol alone and ethanol cue memory groups. This suggests that differences in RNA-processing provide a distinct molecular landscape for encoding behavioral experiences.

3.3 RESULTS

3.3.1 RNA-seq library characterization

We generated trans-heterozygous flies that express the nuclear-tagged GFP “INTACT” transgene (5xUAS-unc84-GFP) (Henry et al. 2012) in all MB neurons (MB010B-Gal4). Expression of INTACT was MB-specific (Supplemental Material, Figure 3.S1, A and B) and did not disrupt odor-cue-induced ethanol memory (Figure 3.S1C). MB-INTACT flies were subjected to one of four treatment paradigms—air, EtOH, odors, or trained—and flash frozen the following day (Figure 3.1A). MB nuclei were isolated and RNA-sequenced to a depth of ~30 million reads per sample (Figure S3.2). Nearly all libraries showed >80% uniquely mapped reads and ~15 million fragments per kilobase of transcript per million mapped reads (FPKM) per sample.

Across treatment conditions there was a similar frequency distribution of mean expression of the fly’s 17560 annotated genes (Figure S3.1D). Within the entire data set, 7984 genes (45%) showed little to no expression [arbitrarily defined as $\log_2(\text{FPKM} + 1) < 2$], whereas 9576 genes (55%) had robustly detected levels of gene expression

[$\log_2(\text{FPKM} + 1) \geq 2$]. Thus, adult MB nuclei were actively transcribing roughly 55% of annotated genes in the genome at the time of isolation.

The BDGP6 Dm6 annotation of the fly transcriptome is comprised of 17560 genes, with 34741 corresponding transcript isoforms, where 10010 genes are uniquely represented by a single transcript isoform. According to the current information (flybase.org), the Dm6 contains roughly 85% protein-coding isoforms, 2% noncoding RNAs, 1.5% microRNAs, 0.9% transfer RNAs, 0.8% small nucleolar RNAs, and 9.7% other/nonannotated biotype sequences. When pooling all expression counts across treatments, our data were comprised of 92% protein-coding genes/isoforms, 0.9% noncoding RNAs, 0.06% microRNAs, 0.06% transfer RNAs, 0.05% small nucleolar RNAs, and 6.3% other/nonannotated sequences (Figure S3.1E). The characterization of our RNA-seq libraries shows that adult MB neurons are actively transcribing detectable protein-coding genes to allow for interesting differential expression analyses.

3.3.2 Expression of a priori and novel gene lists across treatments

In light of the cell-type-specific RNA-seq and exposure conditions assayed (Figure 3.1A), we expected to see prominent expression of genes previously associated with MB function and or previously implicated ethanol-related behaviors. Using the “Vocabularies” search function on FlyBase (flybase.org), a list of genes was acquired representing “adult mushroom body” (FBbt:00003684) and ethanol-associated genes termed “behavioral response to ethanol” (FBbt:0048149). As expected, many of the MB-associated genes showed robust mean expression across our treatment libraries

(Figure 3.1A, Figure 3.S3A). Interestingly, many ethanol-associated genes were also detectable (Figure 3.S3B).

We next determined the highest 20 expressed genes detected across treatments and found the “INTACTlabel” (manually added to the Dm6 transcriptome) was highly expressed across the libraries, demonstrating the robust nature of the GAL4/UAS binary expression system and further validating the specificity of the MB isolation technique. Most of the highly transcribed genes were RNA-associated genes (Figure 3.S3Ci), which we posit to be a mix of transcripts from rough endoplasmic reticulum that was pulled down during nuclear isolation, as well as newly transcribed or nuclear-localized RNAs. We further curated the highest 20 mean expressed genes by excluding RNA-associated genes (Figure 3.1B). Together, these descriptive analyses indicate that the libraries represent comparable MB data sets in which lasting experience-dependent molecular effects could be explored.

3.3.3 Experience-dependent differential gene expression

To uncover experience-dependent effects on transcription, we examined genes that showed the greatest variance in expression (log-transformed counts per million means) across repeated air, ethanol, odors, and paired ethanol-odor (trained) treatments. Among the 100 most variable genes, many ribosomal proteins and previously uncharacterized (unnamed CG#) genes were identified (Figure 3.S3D). Also included were Esterase-6 (Est-6), Cytochrome P450-4g1 (Cyp4g1), la costa (lcs), Mucin related 18B (Mur18B), alpha and beta Trypsin (α Try, β Try), yippee interacting protein

7 (yip7), fab1 kinase (fab1), Troponin C (TpnC73F and TpnC47D), Odorant binding protein 51a (Obp51a), obstructor-B (obst-B), inaF-B (inaF-B), Autophagy-related 10 (Atg-10), short spindle 2 (ssp2), neurotransmitter transporter-like (Ntl), Multiple inositol polyphosphate phosphatase 2 (Mipp2), and a number of Jonah peptidase transcripts (Figure 3.1C). Excluding RNA-associated genes further revealed Maltase Ai (Mal-A1), Sarcolamban B (ScIB), Cuticular protein 49Ab (Cpr49Ab), and Coiled-coil domain containing 56 (Ccdc56) (Figure 3.1C).

To determine statistically significant differential expression between treatment conditions, we used “Ballgown” (Perteira et al. 2016), an R Bioconductor package. No genes or transcripts were removed from analyses and the adjusted P-value (a.k.a. q-value) was set to the standard false discovery rate < 0.05 . All pairwise condition contrasts were examined at both the gene and transcript level. Only two genes were found to be differentially expressed 24 hr after the various treatments, the actin filament nucleating protein cappuccino (capu) (air vs. odors) (Figure 3.1D) and the nuclear DNA-binding protein pre-mod(mdg4)-G (odors vs. EtOH) (Figure 3.1E).

3.3.4 Intersectional analysis of differential transcript expression

In contrast to gene-level analysis, there were more statistically significant differences found at the transcript level, despite this data being subjected to more repeated measure corrections (Figure S4A). This implicates altered transcriptional start/stop site or RNA-processing events that influence transcript diversity. In light of this finding,

subsequent analyses focused on the differential expression of transcripts between treatments.

Using air treatment as a control, we compared the top 200 most significantly differentially expressed (lowest P-values) transcripts from air vs. odors (Figure 3.2Ai), air vs. EtOH (Figure 3.2Aii), and air vs. trained (Figure 3.2Aiii). There was surprisingly little intersectional overlap in specific transcripts between condition comparisons, including between air vs. EtOH and air vs. trained (Figure 3.2B). This indicated that experiencing odors, ethanol intoxication, or making ethanol-odor memories produced distinct, molecularly separable changes in the lasting transcriptional state of MB nuclei. Using hypergeometric statistics, the number of overlapping transcripts was found to be statistically greater than would be expected by chance alone (Figure S4B). The extent of intersection was visualized with an upgraded Venn diagram plot generated by an R package called “UpSetR” (Figure 3.2B, Figure 3.S4B). Overlapping top P-value isoforms and their predicted human orthologs between condition comparisons are listed in Figure 3.2C.

We furthered compared the top 200 transcripts with the largest fold changes in expression (Figure S5). This approach demonstrated that specific transcripts have greater dynamic range in expression in response to experiencing odors, ethanol, or ethanol-odor training. Interestingly, there were more overlapping transcripts between pairwise comparisons than observed in the top P-value transcripts intersection analysis (Figure S5C), suggesting that particular transcripts in the MB neurons are more plastic than others. Hypergeometric statistics again showed that the number of overlapping

transcripts was statistically greater than would be expected by chance alone (Figure S4C). A subset of the overlapping top fold-changing transcripts between treatment conditions are listed in Figure S5D.

3.3.5 Enrichment of genes associated with differential transcripts

To determine which types of transcripts are enriched in the top 200 P-value comparisons, a DAVID Gene Ontology (GO) analysis was performed on the associated gene IDs for each treatment: 167 air, 175 odor, 175, ethanol, and 188 trained genes. Using default DAVID settings, the top three GO annotations were identified (Figure S6). Compared to air controls, repeated ethanol exposures produced statistically significant GO terms: “Alternative splicing” (P-value = 0.0002), “Phosphoprotein” (P-value = 0.041), and “Coiled Coil” (P-value = 0.047). Interestingly, the response to trained treatment also produced “Alternative splicing” (P-value = 0.062) as a high-scoring enrichment category. It is important to note that the “Alternative splicing” GO term does not refer to spliceosome genes, but rather to genes that are “known to be alternatively spliced.” The enrichment analysis results further support the idea that there are experience-dependent changes in transcript usage, but not overall gene-level changes. Optimally, future ontology databases will include transcript-specific functional categorization.

3.3.6 - Candidate transcripts underlying ethanol cue memories

Motivated by our interest in identifying lasting gene expression changes associated with formation of ethanol-cue-induced memories, we focused on the comparisons between odor vs. ethanol, ethanol vs. trained, and odor vs. trained flies (Figure 3.3).

Seven transcripts (six genes) were found to be differentially expressed in response to ethanol treatment as compared to odors-only controls (Figure 3.3A). One transcript was downregulated (CG17982-RA) and six were upregulated {translational regulator Dodeca-satellite-binding protein 1 (Dp1-RH), transcription factor elbow B (elB-RD), transcription factor modifier of mdg4 [pre-mod(mdg4)-G-RA], hypoxia-induced protein CG11825-RA, eukaryotic translation release factor 3 (eRF3), and a gene of unknown function CG17982-RB}.

Four transcripts (three genes, all of unknown function) were found to be differentially expressed in response to trained treatment as compared to ethanol alone (Figure 3.3B). One transcript was downregulated (CG10809-RB) and three were upregulated (CG11970-RA, CG13055-RB, and CG10809-RA).

Eight transcripts (seven genes) were found to be differentially expressed in response to trained treatment as compared to odors-only controls (Figure 3.3C, Figure 3.S7). Three of these transcripts were downregulated: the Sterol Regulatory Element Binding Protein SREBP (HLH106-RC), the Drosophila homolog of dynamin shibire (shi-RP), and a predicted Ras guanine-nucleotide exchange factor CG9098-RA. Four transcripts were upregulated: [a retinoic acid-like protein CG3558-RB, the same predicted Ras guanine-nucleotide exchange factor CG9098-RB, a Coat Protein Complex II factor Secretory 31 (Sec31-RA), and a transmembrane heparin sulfate proteoglycan Syndecan (Sdc-RK)], as well as a transcription factor that functions in the JAK/STAT pathway, Signal-transducer and activator of transcription protein at 92E (Stat92E-RH). Using the DRSC Integrative Ortholog Prediction Tool (DIOPT) (Hu et al. 2011), we identified, when

possible, the human genes corresponding to the implicated fly genes (Figure 3.3Aiii, Figure 3.3Biii, Figure 3.3Ciii).

3.3.7 Molecular interactions with candidate ethanol-cue-induced memory genes

To visualize and probe deeper into the known functions of differentially expressed transcripts, we used the open source Cytoscape platform (Shannon et al. 2003) and MIST database (Hu et al. 2018) to identify proteins that interact with odor vs. trained transcripts of interest (Figure 3.4). By overlaying our data set information including expression level and fold change onto this network, we provided a foundation for prospective hypothesis-driven bioinformatic inquiries and experiments. Notably, Stat92E had the largest known network, including a number of proteins associated with nucleosome remodeling (HDAC1, brm, mor), transcriptional regulation (Taf1, Ada2b, kay, Jra, CG13510), and splicing (Cdc5, CG7564, tsu) (Figure 3.4).

Considering our finding of differential transcript usage, we were intrigued to follow up on the possible requirement of spliceosomal proteins such as cell division cycle 5-like protein (Cdc5). A GFP-tagged version of Cdc5 was expectantly observed within nuclei throughout the adult central brain (Figure 3.5A, i and ii), including within MB neurons (Figure 3.5A, iii and iv). To test the necessity of Cdc5 in ethanol-cue-induced memory, Cdc5-RNAi was expressed during the adult stage using a pan-MB-specific Gal4 driver (R19B03-Gal4) (Figure S9A). Flies with adult Cdc5 knockdown had significant impairment in ethanol-cue-induced memory as compared to genetics controls (Figure 3.5B). To determine whether Cdc5 could affect individual Stat92E transcript

expression, we performed whole-head quantitative RT-PCR with primers specific to each alternative first exon in the Stat92E transcripts, where Exon1 includes Stat92E-RH and Exon1a includes Stat92E-RI (Henriksen et al. 2002) (Figure 3.5C, Figure 3.S8A). Decreasing expression of Cdc5 in all adult neurons significantly altered the ratio of Exon1 to 1a (Stat92E-RH to -RI) transcripts (Figure 3.5D, Figure 3.S8B).

3.3.8 - Knockdown of splice factors impairs ethanol-cue-induced memory

In light of differential transcript usage, GO analysis, and Cdc5 requirement, we assessed the detectable expression of FlyBase's "spliceosome complex" gene list (GO:0005681) (Figure 3.6A). We predicted that decreasing expression of highly expressed splice factors in the MB neurons would affect alcohol associative memories. We choose candidate targets RNA export and export binding protein 1 (Ref1), Protein on ecdysone puffs (Pep), RNA helicase Rm62 (also referred to as p68), and CG7971, which is orthologous to serine/arginine repetitive matrix 2 (SRRM2) (Figure 3.6B). Adult MB-specific knockdown of each of these targets impaired ethanol-cue-induced memory without affecting odor sensitivity (Figure 3.6C, Figures 3.S9 and 3.S10).

3.4 DISCUSSION

The nuclear, cell-type-specific nature of our data provides a unique analysis of genes actively transcribed in memory-encoding neurons 24 hr after experience with ethanol or ethanol cue associative memory formation. The RNA-seq analysis highlighted the differential expression of particular transcripts. This work supports the essential role of RNA processing in the lasting transcriptional changes encoding ethanol-cue-induced memory.

3.4.1 - Confirmation of known genes implicated with memory

Two of the genes implicated in this work have been previously implicated in memory and or ethanol-related behaviors. *shi*, the fly homolog of Dynamin (van der Bliek and Meyerowitz 1991), plays a role in endocytosis, which is critical for synaptic transmission in MB neurons during memory formation (McGuire et al. 2001; Kasuya et al. 2009) and in ethanol tolerance (Krishnan et al. 2012). We have also previously shown that expression of a temperature-sensitive form of *shi* in MB neurons disrupts ethanol cue memory at higher temperatures (Kaun et al. 2011). Stat92E, a transcription factor in the JAK/STAT signaling pathway, plays a role in long-term memory in *Drosophila* (Copf et al. 2011), and we have recently shown that reducing Stat92E in MB neurons similarly reduces ethanol cue memory (Petruccelli et al. 2018). Other targets identified in this work have been implicated in long-term memory in *Drosophila* (Figure 3.S11). Similarly, a recent study showing the effects of acute response to alcohol shows some overlap in transcriptomic targets (Figure 3.S11) (Signor and Nuzhdin 2018).

3.4.2 - Context-specific transcript expression

An intriguing pattern that emerged from the data is that administration of repeated mild doses of ethanol, odors alone, or odors paired with ethanol all produced significantly altered transcript isoforms. Remarkably, the particular transcripts identified were specific to the behavioral paradigm the animals experienced. Even altered transcripts associated with the presentation of ethanol alone were very different from those when ethanol was presented concomitantly with an odor. This suggests that transcript expression is specific to the type of behavioral context flies experienced.

3.4.3- Alternative splicing associated with behavior in MB neurons

Alternative transcripts can be created through the use of alternative transcriptional start sites or alternative splicing. Alternative splicing of pre-mRNA transcribed from a finite genetic sequence enhances proteomic diversity. It is estimated that 95% of human genes undergo alternative splicing (Pan et al. 2008) and disruptions in the splicing status quo can lead to various human disease (Cieply and Carstens 2015). The most common cause of abnormal splicing is due to a mutation in the core splicing consensus sequences (Cieply and Carstens 2015). Our data, however, are derived from animals with the same genetic backgrounds and environmental conditions. This suggests that mutagenesis is not the cause of the potential transcript usage differences we observed.

Our data did not demonstrate differential expression in spliceosome factors with behavior condition, but indirectly implicated spliceosomal machinery. Specifically, known protein-protein interactions were revealed between Stat92E and spliceosome complex associated proteins, including Cdc5, CG7564, and tsu (Guruharsha et al. 2011) (Figure 3.4). The lack of differentially expressed splicing factors with treatment condition does not, however, rule out the possibility that spliceosome-associated proteins were acutely affected during treatment exposure. Consequently, our data may represent the aftermath of these, or post-translational response, effects.

A caveat to our data is that because we did not a priori expect to see splicing differences, our sequencing data were not performed with long-read sequencing techniques. Nor were statistical considerations, such as k-means clustering of transcripts or weighting exon-exon junction reads, used to compare splicing across

genes. However, our data do show that reducing gene expression of five known spliceosome associated proteins in the adult MB disrupts formation of ethanol-cue-induced memory. Thus, we believe that the alternative transcript usage in our data, is in part, due to splicing that occurs during memory formation.

3.4.4 - Splicing associated with memory formation

Splicing of particular targets in response to memory formation is not without precedent. For example, the splicing of the Orb2A transcript is required for both associative appetitive and courtship suppression memory in *Drosophila* (Gill et al. 2017). There is also evidence that this phenomenon may be conserved across species, since contextual fear conditioning induces differential splicing in the hippocampus of mice (Poplawski et al. 2016). It is, therefore, conceivable that while splicing may occur during all forms of synaptic plasticity, the isoform targets may be specific to the type of memory formed. The broad implication of this prediction is that the molecular engram of memory within relevant cells uses transcriptional diversity to provide the cellular plasticity associated with the type of memory being formed. This cellular encoding is bolstered by the diversity of epigenetic changes caused by neuronal activity associated with memory formation. For example, activity-induced histone modifications caused a late-onset shift in Neurexin-1 splicing reduced the stability of memories (Ding et al. 2017). Our data suggest that alcohol can affect transcriptional events, and thus shape how context is encoded during formation of memories.

3.4.5 - Alcohol regulates splicing in cue-encoding neurons

Several recent studies have shown that alternative splicing is correlated to chronic alcohol consumption. Chronic self-administration of alcohol in cynomolgus macaques (*Macaca fascicularis*) is associated with alternative splicing of AMPA subunits in the prefrontal cortex (Acosta et al. 2012). Broader transcriptomic analysis demonstrated overrepresentation of genes associated with splicing across brain regions in chronic ethanol self-administrating rhesus macaques (*Macaca mulatta*) (Iancu et al. 2018). Similar alcohol-induced effects on splicing extend to humans (Farris and Mayfield 2014). Postmortem analysis of the brains of patients with AUD showed novel splicing in GABAB1 that decreased expression of the GABA binding site (Lee et al. 2014). Similarly, alcohol-induced splicing events also occurred in the developing human cortex in utero, potentially resulting in devastating neurodevelopmental consequences (Kawasawa et al. 2017).

Our data suggest that mild repeated alcohol exposures have lasting consequences on the transcript isoforms expressed in *Drosophila*. Importantly, altered isoform transcription occurred in a diversity of genes, including transcription factors like Stat92E, which could have long-lasting and broad effects on transcription within memory circuits (Copf et al. 2011). As alcohol alone did not induce expression of alternative transcripts of Stat92E, this suggests that splicing of this gene might occur due to memory formation. Since our analysis was restricted to neurons necessary for memories associated with punishment or reward, it is possible that even small effects could have widespread consequences for subsequent memory formation and vulnerability to dependence on drugs of abuse. Uncovering whether this is a broader phenomenon will

require identifying lasting transcriptional events that occur in the same cell types across a diversity of memory and drug exposure paradigms.

3.4.6 - Diverse splicing mechanisms across addictions

Alternative splicing has been recently implicated in cocaine addiction (Cates et al. 2018). Transcription factor E2F3a regulates cocaine-induced alternative splicing in the mouse nucleus accumbens, and E2F3b mediates cocaine responses in the prefrontal cortex (Cates et al. 2019). *Drosophila* E2F1 was expressed in our MB nuclei data set (Figure 1Cii), although it was not alternatively spliced in response to alcohol or alcohol-cue training (see transcript count data in Gene Expression Omnibus). We speculate that cocaine utilizes the E2F transcription factor family more than alcohol does to drive drug-cue memory formation and retention. Future investigations will likely identify both conserved drug-specific and convergent molecular mechanisms that influence transcriptional activity in reward circuitry.

3.5. METHODS AND MATERIALS

3.5.1 - Fly husbandry

Flies were raised on cornmeal agar food at 24°, 70% humidity, and with a 14:10 light:dark cycle. All flies used in this study were male. Male 4- to 5-day-old flies were isolated under CO₂, given 1 day to recover, and then conditioned with specified paradigms detailed below. The fly lines used in this study include two pan-MB specific drivers, a MB010B split Gal4 line [#68293; Bloomington *Drosophila* Stock Center (BDSC)] (Aso et al. 2014) and R19B03 Gal4 line (#49830; BDSC) (Jenett et

al. 2012), tubP-Gal80ts-7 (#7018; BDSC) (McGuire et al. 2004) recombined with R19B03-Gal4, a 5xUAS-unc84-2xGFP “INTACT” line (Henry et al. 2012), UAS-Cdc5-RNAi (#57425; BDSC) (Perkins et al. 2015), UAS-Rm62-RNAi (#34829; BDSC) (Perkins et al. 2015), UAS-Ref1-RNAi (#34626; BDSC) (Perkins et al. 2015), UAS-Pep-RNAi (#32944; BDSC) (Perkins et al. 2015), UAS-CG7971-RNAi (#52936; BDSC) (Perkins et al. 2015), GFP-RNAi (9330; BDSC), yw (Janelia Research Campus), and TRiP control (#36303; BDSC) (Perkins et al. 2015). elavC155-Gal4 (#458; BDSC) and Act5C-Gal4 (#25374; BDSC) were used to test efficacy of the RNA interference (RNAi) lines. Unless otherwise specified, experimental flies were heterozygous for transgenes. Control heterozygote crosses were performed with consideration to the respective genetic background of the trans-heterozygous Gal4 > UAS flies.

3.5.2 - Odor-cue-induced ethanol memory

Odor-cue-induced ethanol memory was performed as published previously (Kaun et al. 2011; Nunez et al. 2018; Petruccelli et al. 2018). One minor difference for the RNA-seq data were the use of 50 flies per 14 ml training vial for the isolation of nuclei tagged in specific cell types (INTACT) experiment, whereas memory experiments were performed with 30 flies per vial. Humidified ethanol vapor (90:60 EtOH:air) for 10 min causes a moderate 13.8 ± 3 mM (0.01 g/dl) internal body ethanol concentration. Ethanol, odors (1:36 odorant:mineral oil with ethyl acetate or isoamyl alcohol), or both were delivered to flies in perforated vials for 10 min, followed by a 50 or 60 min rest before second and third trainings were performed (Figure 3.1A). Vapor treatments were delivered to flies on 1% agar and supplemented with yeast pellets overnight before being tested or killed the next day.

Memory testing was performed in a Y-Maze where flies were given a choice between the paired and unpaired odor. Conditioned preference index was calculated by counting the number of flies that moved toward the paired odor, subtracting the flies that moved toward the unpaired odor, and dividing that number by the total number of flies. The average conditioned preference index for pair of reciprocal tests was used as biological n = 1 for each genotype. Odor controls were performed similarly, but with preference indices corresponding to the number of flies facing a choice of either an odor (ethyl acetate or isoamyl alcohol) or no odor.

3.5.3 - Isolation of MB nuclei (INTACT procedure)

INTACT was adapted from the method described in Pankova and Borst (2016) and pioneered in flies by Henry et al. (2012). The INTACT method of extracting nuclear RNA for sequencing provides several advantages compared to a general RNA-seq approach. First, the ability to use flash-frozen tissue, in contrast to FACS or dissected tissue samples, allows for an accurate examination of the current transcriptional profile of genetically targeted cells. Second, nuclear RNA from neurons contributes to the integrity of the active transcriptome snapshot by minimizing contamination from messenger RNA (mRNA) stored in the cytoplasm, along dendrites, and within axons, while allowing for the detection of experience-dependent differential expression (Lacar et al. 2016). Finally, given evidence that mRNA handling in subcellular compartments has been implicated in the formation and memory storage (Bramham and Wells 2007; Richter 2010; Shigeoka et al. 2016; Nakahata and Yasuda 2018; Biever et al. 2019), this approach provides a robust profile of the stable postexposure transcriptome unencumbered by the diversity of whole-cell RNA.

For each biological replicate used for INTACT RNA-seq, flies were pooled such that 2000 fly heads were collected via liquid nitrogen freezing and sieve-separated 24 hr after treatment conditioning. Flies were treated with one of four exposure paradigms (Figure 3.1A). Biological replicates were performed for air (n = 3), EtOH (n = 3), odors (n = 4), and trained (n = 4) conditions. Odors and trained conditions were performed with reciprocal odor groups (n = 2 ethyl acetate→isoamyl alcohol; n = 2 isoamyl alcohol→ethyl acetate).

Frozen heads were homogenized in a Kontes glass homogenizer (D9938-1SET; Sigma, St. Louis, MO) with 10 ml of Dounce buffer (10 mM β -glycerophosphate, 2 mM MgCl₂, 0.5% IGEPAL buffer) and homogenized for ~2 min with the loose A pestle. Homogenate was passed through a 190- μ m nylon net filter (CMN-0185-C; Small Parts). The filter was subsequently rinsed with 2 ml of the same buffer before discarding. Homogenate was subsequently further homogenized gently ~6–7 times with the tight B pestle, passed through a 20- μ m filter (F020N-12-C; Small Parts), brought up to 50 ml by adding sucrose buffer (10 mM β -glycerophosphate, 2 mM MgCl₂, 25 mM KCl, 250 mM sucrose). Next, 300 μ l of Dynabead A magnetic beads (10001D; Thermo Fisher) were preincubated with rabbit α -GFP antibody (G10362; Thermo), and incubated with the homogenate for 30 min at 4° with gentle agitation. Beads were then captured with a magnet for 15 min and washed five times with 600 μ l of sucrose buffer for 5 min at 4° with gentle agitation. Finally, RNA was extracted from bead-bound nuclei using TRIzol (Ambion, Life Technologies), resuspended in RNA-ase free water and DNA-ase treated according to manufacturer's instructions (Ambion DNA-Free Kit).

3.5.4 - RNA-seq

RNA libraries were polyA-enriched to identify mRNA transcripts ready for nuclear export and sequenced using a Hi-Seq 4000 (Illumina) machine at a depth of ~30 million single-end 1 × 50 bp reads by GENEWIZ (South Plainfield, NJ). Read quality was assessed using FastQC 0.11.5 (Babraham Bioinformatics). Adapters were removed and trimmed using Trimmomatic 0.36 (Bolger et al. 2014). The “new tuxedo suite” was used to further process reads (Pertea et al. 2016). Reads were aligned with HISAT2 2.0.5 (Pertea et al. 2016) to the Ensembl BDGP6_transcriptome reference (Dm6), modified to include the 5xUAS-unc-84-2xGFP sequence present in our flies. Next, samtools 1.3.1 (Li et al. 2009) and Stringtie 1.3.3 (Pertea et al. 2016) were used to sort, merge, and quantify transcripts. Lastly, assembly and analysis of Ballgown objects (Pertea et al. 2016) and data visualization with ggplot2 was performed in RStudio 1.0.136 (Wickham 2009). No samples were discarded, and default settings were used for the pipeline unless otherwise noted; command line code as follows:

#Trimmomatic:

```
TruSeq3-SE.fa:2:30:10:8:true LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15  
MINLEN:36
```

#HISAT2:

```
hisat2 -q -x <reference index> -U <input fastq file> -S <output sam file> -p 50
```

#Samtools:

```
samtools view -Su alns.sam | samtools sort -o alns.sorted -@ 4
```

#StringTie:

```
stringtie <input.sorted.bam> -b <path to Ballgown tables> -o <output.gtf> -G  
<reference.gtf>
```

3.5.5 - Immunohistochemistry

Fly brains were dissected in 1 × PBS solution and fixed in 2% paraformaldehyde S2 media overnight at 4°. Brains were continuously rocked on a nutator throughout the remainder of the protocol. Brains were washed four times for 15 min with PBS with 0.5% Triton X-100 (PBT), blocked at room temperature for 1 hr in PBT with 5% goat serum (PBT-GS). Primary antibodies (1:400 or 1:20) in PBT-GS were incubated overnight at 4°. Brains were then washed four times for 15 min with PBT, and secondary antibodies (1:500) in PBT-GS were incubated at room temperature for 2 hr. Again, brains were washed four times for 15 min with PBT, then mounted in DAPI Fluoromount-G (SouthernBiotech) or ProLong Diamond Antifade Mountant (Invitrogen, Carlsbad, CA).

3.5.6 - Imaging

Images were obtained using a Nikon A1R Multiphoton Upright Confocal Microscope using NIS-Elements Software. Nonsaturating laser power and gain were determined for each channel and held constant throughout an experiment. We used 2-μm Z-sections with a ×20 air objective, and 0.5-μm Z-sections with a ×60 oil objective. Max stack images vary in depth shown. Z-series are available upon request. FIJI was used to apply contrast and brightness settings for visualization purposes.

3.5.7 - Quantitative PCR

Total RNA was extracted from ~90 heads or 30 bodies using TRIzol (Ambion, Life Technologies). RNA samples were treated with DNase (Ambion DNA-Free Kit), and equal amounts of RNA (1 µg) were reverse-transcribed into complementary DNA (Applied Biosystems). Biological and technical replicates were then analyzed with SYBR Green Real-Time PCR (ABI PRISM 7700 Sequence Detection System; Bio-Rad) and performed using the following PCR conditions: 39 cycles (30 s at 95°, 30 s at 60°, 30 s at 72°). The Ct threshold was manually adjusted to 0.6 across all samples and targets. Then, each of the target genes was normalized to rp49 expression for the comparative Δ Ct method analysis following subsequent comparison to the control genotype to assess fold enrichment ($\Delta\Delta$ CT method). Primers, listed below, were all tested for efficiency before using for experiments.

Stat92E-Exon1a_for TGC GCA ACC AGT TGA ATT CTT

Stat92E-Exon1a_rev CATTACACACACGACGCAGT

Stat92E-Exon1_for GGTAGTCGCGTTCGCAAAAA

Stat92E-Exon1_rev GCAGGTGTTGGGGGAAAAAC

Pep_for CGTCGTTCGGATCGGAG

Pep_rev GTCGAGAAGCGATGGTGG

Rm62_for GCGAGATCGTCATTGCCACAC

Rm62_rev GCGGAACTCACGGAGC

Ref1_for CGTCGGTAACCTGGACTAC

Ref1_rev CACGGCCACGTCTGAG

Rp49_for CATAACAGGCCCAAGATCGTGAAG

Rp49_rev CACGTTGTGCACCAGGAACTTC

3.5.8 - Statistical analysis

For RNA-seq, libraries were statistically analyzed in R to determine differential expression between treatment conditions. Raw P-values are shown, as well as corrected P-values (false discovery rate < 0.05). The freely available Ballgown package (Pertea et al. 2016) afforded generalized linear modeling statistics for differential expression analysis and data visualization tools. Libraries were normalized with Ballgown package default – sum of each library's log expression measurements below the 75th percentile (Frazee et al. 2015). For intersectional analyses, hypergeometric statistics were performed on pairwise comparisons and considered different from chance at P-value < 0.05.

Memory and quantitative PCR experiment statistical analyses were performed using JMP Pro 14. Levene's test was used to determine homogeneity of variance; Shapiro–Wilk goodness-of-fit was used to test normality. Data that did not violate assumptions of homogeneity of variance or normality were tested using an ANOVA test followed by Student's t-test post hoc comparisons. Data that violated assumptions of normality and homogeneity of variance were tested with a rank-sums Wilcoxon test followed by Wilcoxon post hoc analysis for each pair. For all figures, the bar represents mean, error bars represent SE of the mean, and significance defined by *P < 0.05.

3.5.9 - Data availability

Raw sequencing files and count data presented in this work are freely available on NCBI Gene Expression Omnibus via accession number GSE108525. Supplemental material available at figshare: <https://doi.org/10.25386/genetics.11929458>.

3.6. CHAPTER 3 REFERENCES

1. Acosta G., Freidman D. P., Grant K. A., and Hemby S. E., 2012. Alternative splicing of AMPA subunits in prefrontal cortical fields of cynomolgus monkeys following chronic ethanol self-administration. *Front. Psychiatry* 2: 72 10.3389/fpsyt.2011.00072
2. Alberini C. M., and Kandel E. R., 2014. The regulation of transcription in memory consolidation. *Cold Spring Harb. Perspect. Biol.* 7: a021741 10.1101/cshperspect.a021741
3. Aso Y., Hattori D., Yu Y., Johnston R. M., Iyer N. A. et al. , 2014. The neuronal architecture of the mushroom body provides a logic for associative learning. *eLife* 3: e04577 10.7554/eLife.04577
4. Bailey C. H., Bartsch D., and Kandel E. R., 1996. Toward a molecular definition of long-term memory storage. *Proc. Natl. Acad. Sci. USA* 93: 13445–13452. 10.1073/pnas.93.24.13445
5. Berson A., Goodman L. D., Sartoris A. N., Otte C. G., Aykit J. A. et al. , 2019. *Drosophila* Ref1/ALYREF regulates transcription and toxicity associated with ALS/FTD disease etiologies. *Acta Neuropathol. Commun.* 7: 65 10.1186/s40478-019-0710-x
6. Biever A., Donlin-Asp P. G., and Schuman E. M., 2019. Local translation in neuronal processes. *Curr. Opin. Neurobiol.* 57: 141–148. 10.1016/j.conb.2019.02.008
7. Bolger A. M., Lohse M., and Usadel B., 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30: 2114–2120. 10.1093/bioinformatics/btu170

8. Bramham C. R., and Wells D. G., 2007. Dendritic mRNA: transport, translation and function. *Nat. Rev. Neurosci.* 8: 776–789. 10.1038/nrn2150
9. E. A., Lardner C. K., Purushothaman I., Pena C. J. et al. , 2018. Transcription factor E2F3a in nucleus accumbens affects cocaine action via transcription and alternative splicing. *Biol. Psychiatry* 84: 167–179. 10.1016/j.biopsych.2017.11.027
10. Cates H. M., Bagot R. C., Heller E. A., Purushothaman I., Lardner C. K. et al. , 2019. A novel role for E2F3b in regulating cocaine action in the prefrontal cortex. *Neuropsychopharmacology* 44: 776–784. 10.1038/s41386-018-0296-1
11. Cheng H. G., Kaakarli H., Breslau J., and Anthony J. C., 2018. Assessing changes in alcohol use and alcohol use disorder prevalence in the United States: evidence from national surveys from 2002 through 2014. *JAMA Psychiatry* 75: 211–213. 10.1001/jamapsychiatry.2017.4008
12. Cieply B., and Carstens R. P., 2015. Functional roles of alternative splicing factors in human disease. *Wiley Interdiscip. Rev. RNA* 6: 311–326. 10.1002/wrna.1276
13. Clemens K. J., and Holmes N. M., 2018. An extended history of drug self-administration results in multiple sources of control over drug seeking behavior. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 87: 48–55. 10.1016/j.pnpbp.2017.11.011
14. Copf T., Goguel V., Lampin-Saint-Amaux A., Scaplehorn N., and Preat T., 2011. Cytokine signaling through the JAK/STAT pathway is required for long-term memory in *Drosophila*. *Proc. Natl. Acad. Sci. USA* 108: 8059–8064. 10.1073/pnas.1012919108

15. Courtney K. E., Schacht J. P., Hutchison K., Roche D. J., and Ray L. A., 2016.
Neural substrates of cue reactivity: association with treatment outcomes and relapse.
Addict. Biol. 21: 3–22. 10.1111/adb.12314
16. Crocker A., Guan X. J., Murphy C. T., and Murthy M., 2016. Cell-type-specific transcriptome analysis in the *Drosophila* mushroom body reveals memory-related changes in gene expression. *Cell Rep.* 15: 1580–1596. 10.1016/j.celrep.2016.04.046
17. Devineni A. V., and Heberlein U., 2009. Preferential ethanol consumption in *Drosophila* models features of addiction. *Curr. Biol.* 19: 2126–2132.
10.1016/j.cub.2009.10.070
18. Ding X., Liu S., Tian M., Zhang W., Zhu T. et al. , 2017. Activity-induced histone modifications govern Neurexin-1 mRNA splicing and memory preservation. *Nat. Neurosci.* 20: 690–699. 10.1038/nn.4536
19. Erickson E. K., Grantham E. K., Warden A. S., and Harris R. A., 2019.
Neuroimmune signaling in alcohol use disorder. *Pharmacol. Biochem. Behav.* 177: 34–60. 10.1016/j.pbb.2018.12.007
20. Farris S. P., and Mayfield R. D., 2014. RNA-Seq reveals novel transcriptional reorganization in human alcoholic brain. *Int. Rev. Neurobiol.* 116: 275–300.
10.1016/B978-0-12-801105-8.00011-4
21. Frazee A. C., Pertea G., Jaffe A. E., Langmead B., Salzberg S. L. et al. , 2015.
Ballgown bridges the gap between transcriptome assembly and expression analysis.
Nat. Biotechnol. 33: 243–246. 10.1038/nbt.3172

22. Gill J., Park Y., McGinnis J. P., Perez-Sanchez C., Blanchette M. et al. , 2017.
Regulated intron removal integrates motivational state and experience. *Cell* 169: 836–848.e15. 10.1016/j.cell.2017.05.006
23. Grant B. F., Chou S. P., Saha T. D., Pickering R. P., Kerridge B. T. et al. , 2017.
Prevalence of 12-month alcohol use, high-risk drinking, and DSM-IV alcohol use disorder in the United States, 2001–2002 to 2012–2013: results from the national epidemiologic survey on alcohol and related conditions. *JAMA Psychiatry* 74: 911–923. 10.1001/jamapsychiatry.2017.2161
24. Groefsema M., Engels R., and Luijten M., 2016. The role of social stimuli content in neuroimaging studies investigating alcohol cue-reactivity. *Addict. Behav.* 58: 123–128. 10.1016/j.addbeh.2016.02.033
25. Guruharsha K. G., Rual J. F., Zhai B., Mintseris J., Vaidya P. et al. , 2011. A protein complex network of *Drosophila melanogaster*. *Cell* 147: 690–703. 10.1016/j.cell.2011.08.047
26. Henriksen M. A., Betz A., Fuccillo M. V., and Darnell J. E. Jr, 2002. Negative regulation of STAT92E by an N-terminally truncated STAT protein derived from an alternative promoter site. *Genes Dev.* 16: 2379–2389 (erratum: *Genes Dev.* 16: 2729). 10.1101/gad.1020702
27. Henry G. L., Davis F. P., Picard S., and Eddy S. R., 2012. Cell type-specific genomics of *Drosophila* neurons. *Nucleic Acids Res.* 40: 9691–9704. 10.1093/nar/gks671

28. Herold N., Will C. L., Wolf E., Kastner B., Urlaub H. et al. , 2009. Conservation of the protein composition and electron microscopy structure of *Drosophila melanogaster* and human spliceosomal complexes. *Mol. Cell. Biol.* 29: 281–301.
10.1128/MCB.01415-08
29. Hu Y., Flockhart I., Vinayagam A., Bergwitz C., Berger B. et al. , 2011. An integrative approach to ortholog prediction for disease-focused and other functional studies. *BMC Bioinformatics* 12: 357 10.1186/1471-2105-12-357
30. Hu Y., Vinayagam A., Nand A., Comjean A., Chung V. et al. , 2018. Molecular Interaction Search Tool (MIST): an integrated resource for mining gene and protein interaction data. *Nucleic Acids Res.* 46: D567–D574. 10.1093/nar/gkx1116
31. Iancu O. D., Colville A., Walter N. A. R., Darakjian P., Oberbeck D. L. et al. , 2018. On the relationships in rhesus macaques between chronic ethanol consumption and the brain transcriptome. *Addict. Biol.* 23: 196–205. 10.1111/adb.12501
32. Jasinska A. J., Stein E. A., Kaiser J., Naumer M. J., and Yalachkov Y., 2014. Factors modulating neural reactivity to drug cues in addiction: a survey of human neuroimaging studies. *Neurosci. Biobehav. Rev.* 38: 1–16.
10.1016/j.neubiorev.2013.10.013
33. Jenett A., Rubin G. M., Ngo T. T., Shepherd D., Murphy C. et al. , 2012. A GAL4-driver line resource for *Drosophila* neurobiology. *Cell Rep.* 2: 991–1001.
10.1016/j.celrep.2012.09.011

34. Kasuya J., Ishimoto H., and Kitamoto T., 2009. Neuronal mechanisms of learning and memory revealed by spatial and temporal suppression of neurotransmission using shibire, a temperature-sensitive dynamin mutant gene in *Drosophila melanogaster*. *Front. Mol. Neurosci.* 2: 11 10.3389/neuro.02.011.2009
35. Kaun K. R., Azanchi R., Maung Z., Hirsh J., and Heberlein U., 2011. A *Drosophila* model for alcohol reward. *Nat. Neurosci.* 14: 612–619. 10.1038/nn.2805
36. Kawasaki Y. I., Mohammad S., Son A. I., Morizono H., Basha A. et al. , 2017. Genome-wide profiling of differentially spliced mRNAs in human fetal cortical tissue exposed to alcohol. *Alcohol* 62: 1–9. 10.1016/j.alcohol.2017.05.001
37. Kendler K. S., Ohlsson H., Sundquist J., and Sundquist K., 2016. Alcohol use disorder and mortality across the lifespan: a longitudinal cohort and Co-relative analysis. *JAMA Psychiatry* 73: 575–581. 10.1001/jamapsychiatry.2016.0360
38. Krishnan H. R., Al-Hasan Y. M., Pohl J. B., Ghezzi A., and Atkinson N. S., 2012. A role for dynamin in triggering ethanol tolerance. *Alcohol. Clin. Exp. Res.* 36: 24–34. 10.1111/j.1530-0277.2011.01587.x
39. Lacar B., Linker S. B., Jaeger B. N., Krishnaswami S. R., Barron J. J. et al. , 2016. Nuclear RNA-seq of single neurons reveals molecular signatures of activation. *Nat. Commun.* 7: 11022 [corrigenda: *Nat. Commun.* 8: 15047 (2017)]. 10.1038/ncomms11022

40. Lee C., Mayfield R. D., and Harris R. A., 2014. Altered gamma-aminobutyric acid type B receptor subunit 1 splicing in alcoholics. *Biol. Psychiatry* 75: 765–773.
10.1016/j.biopsych.2013.08.028
41. Li H., Handsaker B., Wysoker A., Fennell T., Ruan J. et al. , 2009. The sequence alignment/map format and SAMtools. *Bioinformatics* 25: 2078–2079.
10.1093/bioinformatics/btp352
42. Logge W. B., Morley K. C., Haber P. S., and Baillie A. J., 2019. Executive functioning moderates responses to appetitive cues: a study in severe alcohol use disorder and alcoholic liver disease. *Alcohol Alcohol.* 54: 38–46.
10.1093/alcalc/agy083
43. McGuire S. E., Le P. T., and Davis R. L., 2001. The role of Drosophila mushroom body signaling in olfactory memory. *Science* 293: 1330–1333.
10.1126/science.1062622
44. McGuire S. E., Mao Z., and Davis R. L., 2004. Spatiotemporal gene expression targeting with the TARGET and gene-switch systems in Drosophila. *Sci. STKE* 2004: pl6 10.1126/stke.2202004pl6
45. Nakahata Y., and Yasuda R., 2018. Plasticity of spine structure: local signaling, translation and cytoskeletal reorganization. *Front. Synaptic Neurosci.* 10: 29
10.3389/fnsyn.2018.00029
46. Nestler E. J., 2013. Cellular basis of memory for addiction. *Dialogues Clin. Neurosci.* 15: 431–443.

47. Nunez K. M., Azanchi R., and Kaun K. R., 2018. Cue-induced ethanol seeking in *Drosophila melanogaster* is dose-dependent. *Front. Physiol.* 9: 438
10.3389/fphys.2018.00438
48. Pankova K., and Borst A., 2016. RNA-seq transcriptome analysis of direction-selective T4/T5 neurons in *Drosophila*. *PLoS One* 11: e0163986
10.1371/journal.pone.0163986
49. Pan Q., Shai O., Lee L. J., Frey B. J., and Blencowe B. J., 2008. Deep surveying of alternative splicing complexity in the human transcriptome by high-throughput sequencing. *Nat. Genet.* 40: 1413–1415 [corrigenda: *Nat. Genet.* 41: 762 (2009)].
10.1038/ng.259
50. Perkins L. A., Holderbaum L., Tao R., Hu Y., Sopko R. et al. , 2015. The transgenic RNAi project at harvard medical school: resources and validation. *Genetics* 201: 843–852. 10.1534/genetics.115.180208
51. Pertea M., Kim D., Pertea G. M., Leek J. T., and Salzberg S. L., 2016. Transcript-level expression analysis of RNA-seq experiments with HISAT, StringTie and Ballgown. *Nat. Protoc.* 11: 1650–1667. 10.1038/nprot.2016.095
52. Petruccelli E., Feyder M., Ledru N., Jaques Y., Anderson E. et al. , 2018. Alcohol activates scabrous-notch to influence associated memories. *Neuron* 100: 1209–1223.e2. 10.1016/j.neuron.2018.10.005

53. Poplawski S. G., Peixoto L., Porcari G. S., Wimmer M. E., McNally A. G. et al. , 2016. Contextual fear conditioning induces differential alternative splicing. *Neurobiol Learn Mem* 134 Pt B: 221–235. 10.1016/j.nlm.2016.07.018
54. Richter J. D., 2010. Translational control of synaptic plasticity. *Biochem. Soc. Trans.* 38: 1527–1530. 10.1042/BST0381527
55. Ron D., and Barak S., 2016. Molecular mechanisms underlying alcohol-drinking behaviours. *Nat. Rev. Neurosci.* 17: 576–591. 10.1038/nrn.2016.85
56. Shannon P., Markiel A., Ozier O., Baliga N. S., Wang J. T. et al. , 2003. Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res.* 13: 2498–2504. 10.1101/gr.1239303
57. Shigeoka T., Jung H., Jung J., Turner-Bridger B., Ohk J. et al. , 2016. Dynamic axonal translation in developing and mature visual circuits. *Cell* 166: 181–192. 10.1016/j.cell.2016.05.029
58. Shih M. M., Davis F. P., Henry G. L., and Dubnau J., 2019. Nuclear transcriptomes of the seven neuronal cell types that constitute the *Drosophila* mushroom bodies. *G3 (Bethesda)* 9: 81–94. 10.1534/g3.118.200726
59. Signor S., and Nuzhdin S., 2018. Dynamic changes in gene expression and alternative splicing mediate the response to acute alcohol exposure in *Drosophila melanogaster*. *Heredity* 121: 342–360. 10.1038/s41437-018-0136-4
60. Sweatt J. D., 2016. Neural plasticity and behavior - sixty years of conceptual advances. *J. Neurochem.* 139: 179–199. 10.1111/jnc.13580

61. Trudell J. R., Messing R. O., Mayfield J., and Harris R. A., 2014. Alcohol dependence: molecular and behavioral evidence. *Trends Pharmacol. Sci.* 35: 317–323. 10.1016/j.tips.2014.04.009
62. Uchida S., and Shumyatsky G. P., 2018. Synaptically localized transcriptional regulators in memory formation. *Neuroscience* 370: 4–13. 10.1016/j.neuroscience.2017.07.023
63. Valyear M. D., Villaruel F. R., and Chaudhri N., 2017. Alcohol-seeking and relapse: a focus on incentive salience and contextual conditioning. *Behav. Processes* 141: 26–32. 10.1016/j.beproc.2017.04.019
64. van der Bliek A. M., and Meyerowitz E. M., 1991. Dynamin-like protein encoded by the *Drosophila shibire* gene associated with vesicular traffic. *Nature* 351: 411–414. 10.1038/351411a0
65. Wickham H., 2009. *Ggplot2: Elegant Graphics for Data Analysis*, Springer, New York: 10.1007/978-0-387-98141-3
66. Widmer Y. F., Bilican A., Bruggmann R., and Sprecher S. G., 2018. Regulators of long-term memory revealed by mushroom body-specific gene expression profiling in *Drosophila melanogaster*. *Genetics* 209: 1167–1181. 10.1534/genetics.118.301106
67. Wolf F. W., Rodan A. R., Tsai L. T., and Heberlein U., 2002. High-resolution analysis of ethanol-induced locomotor stimulation in *Drosophila*. *J. Neurosci.* 22: 11035–11044. 10.1523/JNEUROSCI.22-24-11035.2002

3.7: CHAPTER 3 FIGURES

Figure 3.1

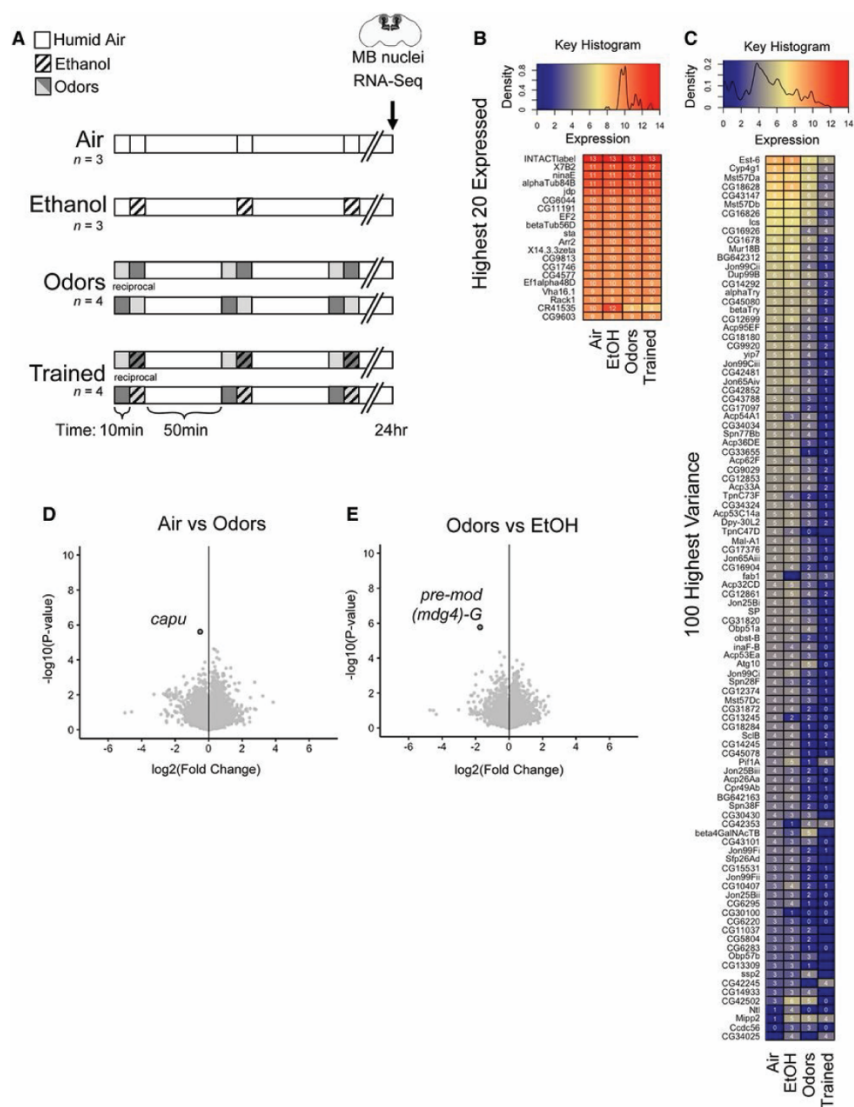


Figure 3.1: Exposure paradigms and differentially expressed genes in MB nuclei.

(A) Paradigm depicting three spaced 10 min exposures of “Air”, “Ethanol”, “Odors”, or “Trained” (odor + ethanol) and flies killed 24 hr later. RNA for each biological replicate (n) was extracted from mushroom body nuclei isolated from ~2000 male heads [Air: n = 3, Ethanol: n = 3, Odors: n = 4 (two reciprocals of opposite odor order), Trained: n = 4 (two reciprocals of opposite odor order)]. (B and C) Volcano plots showing fold change

of gene expression [$\log_2(\text{fc}+1)$] compared to the inverse of statistical significance [$-\log_{10}(\text{P-value})$] (dark outline, FDR < 0.05). Both (B) Air vs. Odors and (C) Odors vs. EtOH had one statistically significant differentially expressed gene. (D and E) Density histogram and normalized FPKM expression (blue, yellow, and red color representing relative levels) of (D) the 100 most variable genes—the squared deviation from the mean—and (E) the 20 highest expressed genes postfiltering of RNA-associated genes. FDR, false discovery rate.

Figure 3.2

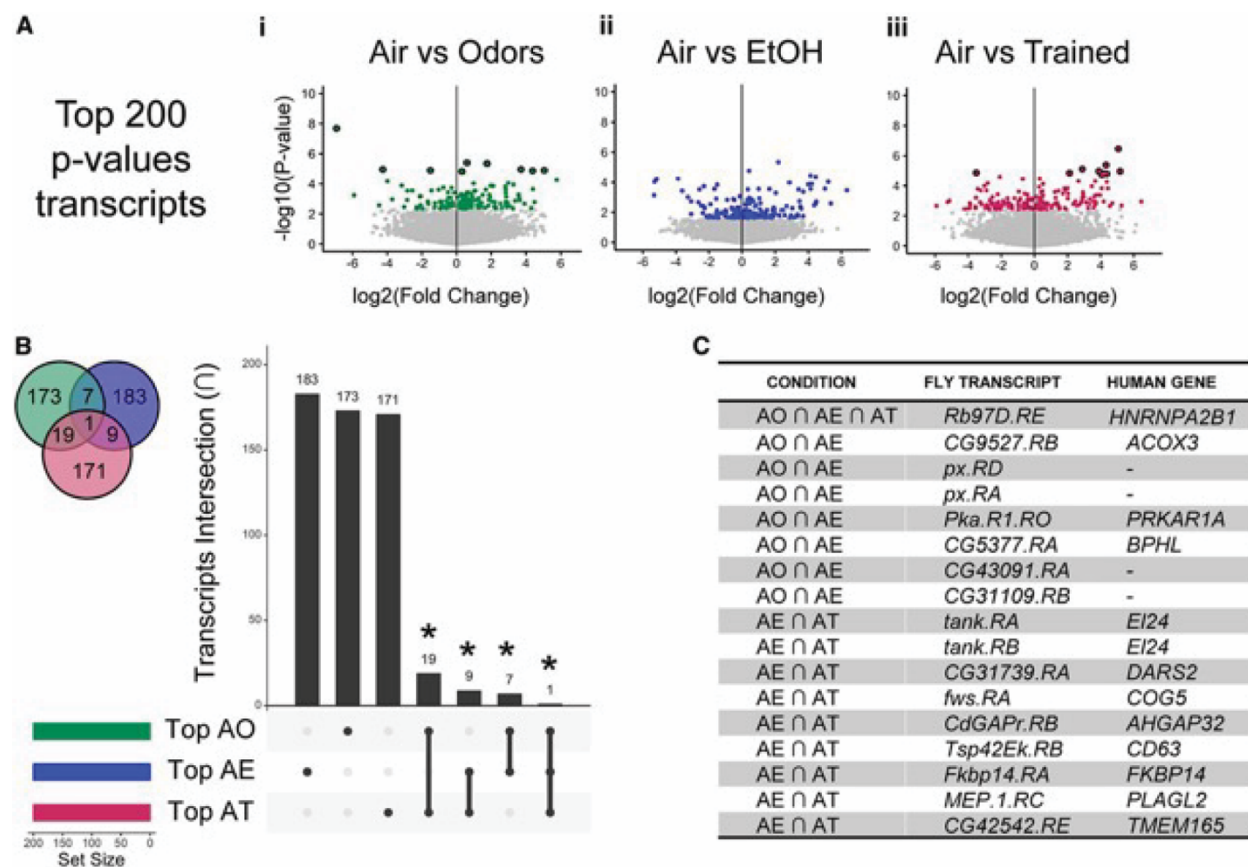


Figure 3.2: Comparison of differential transcripts in response to odor, ethanol, or trained (odor-ethanol) treatment.

(A) Volcano plots showing fold change of transcript expression [$\log_2(fc+1)$] compared to the inverse of statistical significance [$-\log_{10}(P\text{-value})$] (dark outline, FDR < 0.05). Plots for (i) Odors, (ii) EtOH, and (iii) Trained (ethanol-odor pairing) compared to Air (colors depict the top 200 P-value transcripts). (B) An upgraded Venn Diagram plot generated by an R package called “UpSetR” demonstrating the intersection in top 200 P-value transcripts (abbreviated by first letter in treatment). (C) A few of the transcripts from intersectional analysis are listed, along with the corresponding highest DIOPT-scored

human genes. Hypergeometric statistics, $*P < 0.05$ (Figure S4C). FDR, false discovery rate.

Figure 3.3

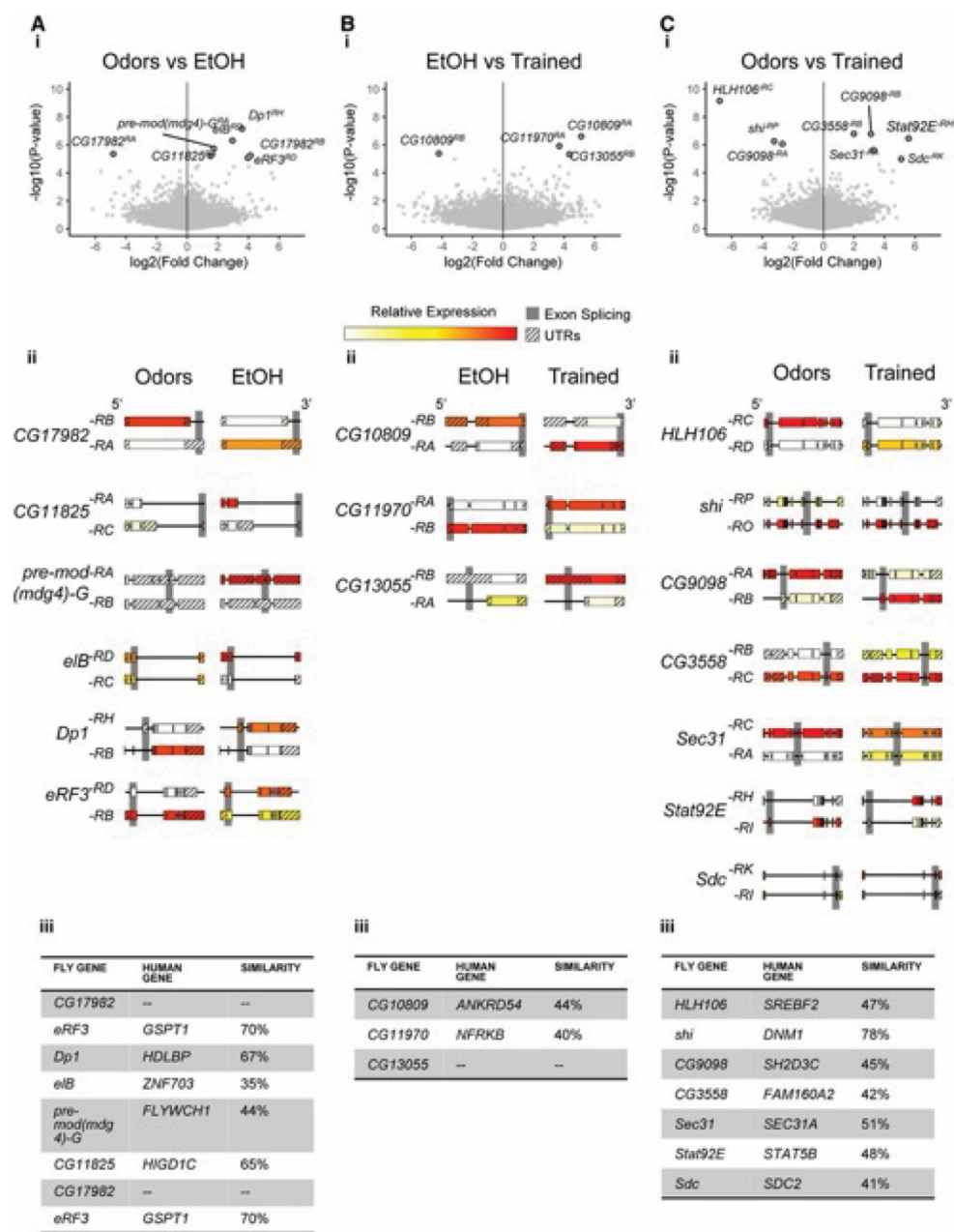


Figure 3.3: Differential transcripts in particular pairwise treatment comparisons.

(Ai–Ci) Volcano plots depicting (A) how EtOH differentially affects transcript expression compared to odors, (B) how Training (ethanol-odor pairing) differentially affects transcript expression compared to EtOH alone, and (C) how Training (ethanol-odor

pairing) differentially affects transcript expression compared to Odors alone (dark outline, FDR < 0.05). (Aii–Cii) Significantly altered transcripts are depicted alongside the most highly expressed isoform of the same gene, gray shaded regions denote sequence differences between isoforms. (Aiii–Ciii) Transcripts are listed, along with the corresponding highest DIOPT-scored human genes. FDR, false discovery rate.

Figure 3.4

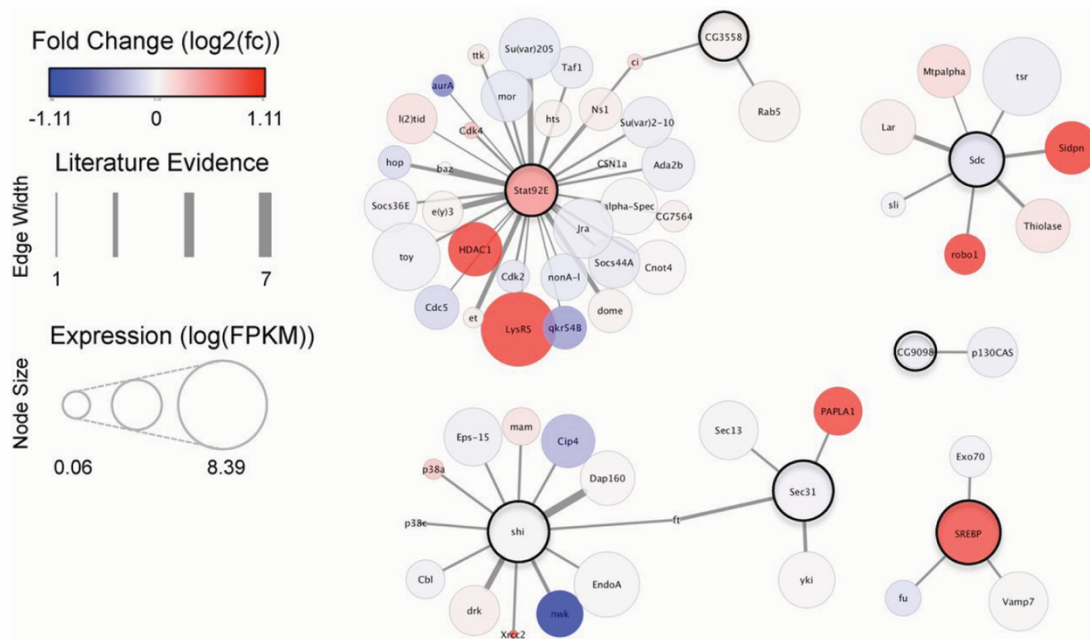


Figure 3.4. Protein interaction network analysis of alternatively expressed transcripts.

Protein interaction network of proteins associated with “Odor vs. Trained” significant transcripts. Each node represents a protein, dark outlines denote proteins associated with significant “Odor vs. Trained” transcripts. Attributes of the nodes include fold change [$\log_2(fc+1)$] as color, expression level as size [$\log_2(FKPM+1)$] and edge thickness between nodes represents the extent of protein-protein interaction evidence from the MIST database.

Figure 3.5

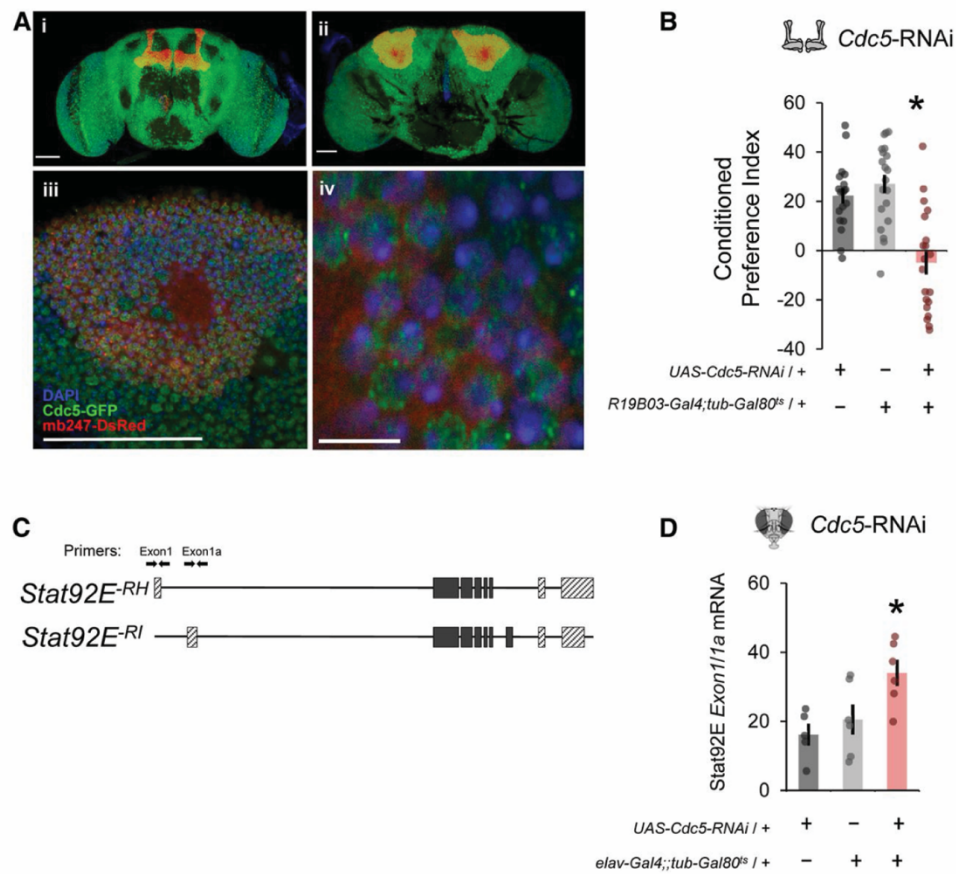


Figure 3.5: Spliceosome complex gene *Cdc5* is required for cue-induced ethanol memory in adult MB neurons.

(A) A *Cdc5*-GFP fusion shows that *Cdc5* is expressed diffusely in the (i) anterior and (ii) posterior adult central brain, including (iii and iv) within MB nuclei (red). Bar in i, ii, and iii is 50 μ M, Bar in iv is 5 μ M). (B) Expression of *Cdc5*-RNAi in adult MB neurons significantly reduced conditioned preference for odor-cue-induced ethanol memory [$F(2,56) = 21$, $*P < 0.0001$]. Expression pattern of R19B03-Gal4 is shown in Figure S9A, and efficacy of the RNAi in Figure S10. (C) Primers designed to measure expression of

transcripts that include the Stat92E–RH (Exon 1) or –RI (Exon 1a) isoforms (Henriksen et al. 2002), see Figure S8A for depiction of all Stat92E transcripts. (D) Decreasing expression of Cdc5 in adult neurons significantly alters the ratio of Exon1/1a transcripts in whole-head tissue [$F(2,14) = 5.7$, $*P = 0.02$].

Figure 3.6

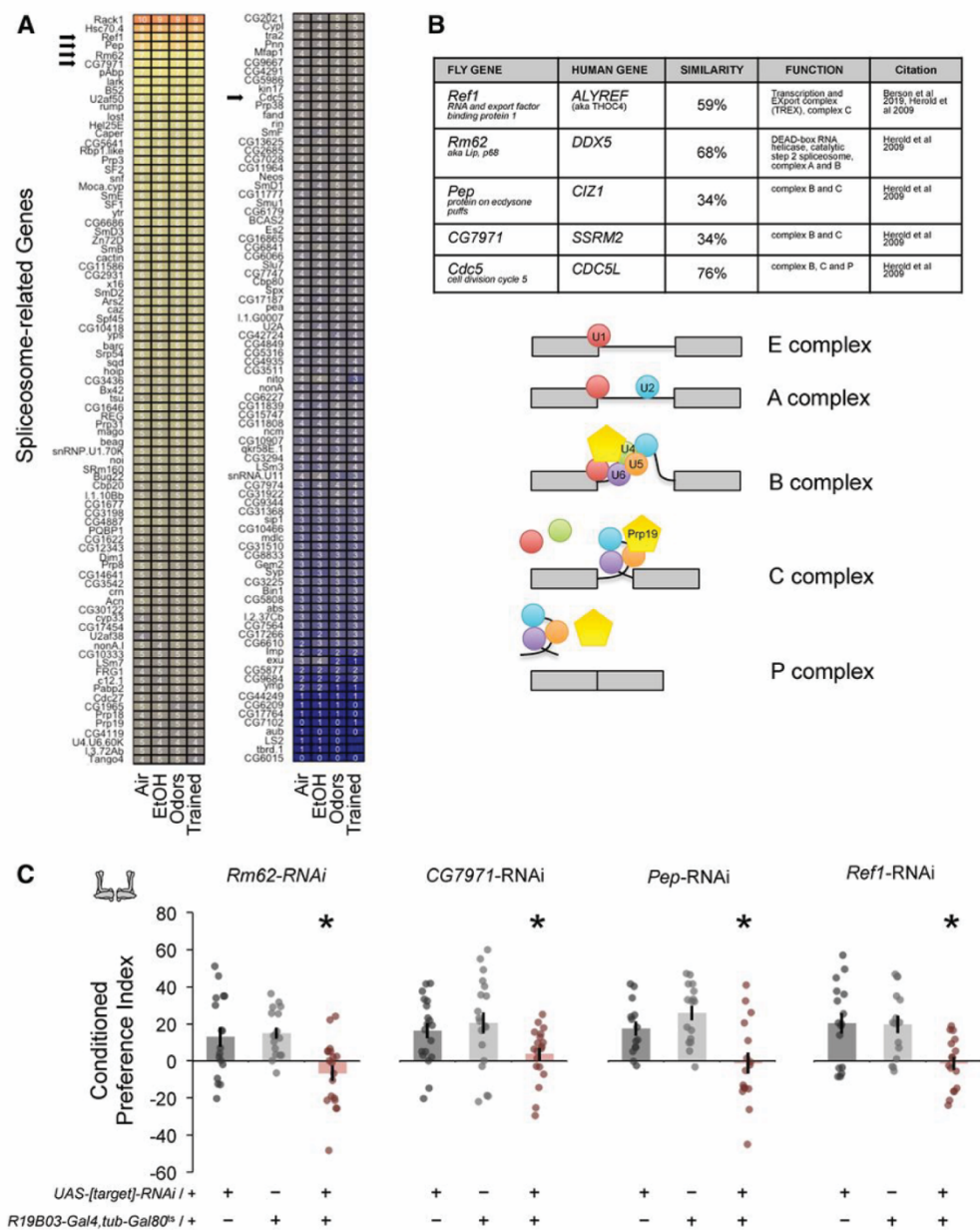


Figure 3.6: Knockdown of splice-associated targets in MB neurons reduces odor-cue-induced ethanol memory.

(A) Density histogram and normalized FPKM expression (blue, yellow, and red color representing relative levels) of genes associated with the spliceosome; arrows mark selected candidate targets for testing. (B) Table listing four top-expressed spliceosome-associated genes and *Cdc5*, along with their corresponding highest DIOPT-scored human genes (Herold et al. 2009; Berson et al. 2019), and schematic demonstrating the complexes associated with these genes during splicing. (C) Expression of RNAi targeting *Rm62* [$F(2,49) = 5.72$, $P = 0.02$], *CG7971* [$F(2,55) = 3.75$, $P = 0.03$], *Ref1* [$F(2,44) = 6.96$, $P = 0.002$], or *Pep* [$F(2,43) = 9.30$, $P = 0.0004$] in adult MB neurons significantly reduced conditioned preference for odor-cue-induced ethanol memory.

3.8: CHAPTER 3 SUPPLEMENTARY FIGURES

Figure 3.S1

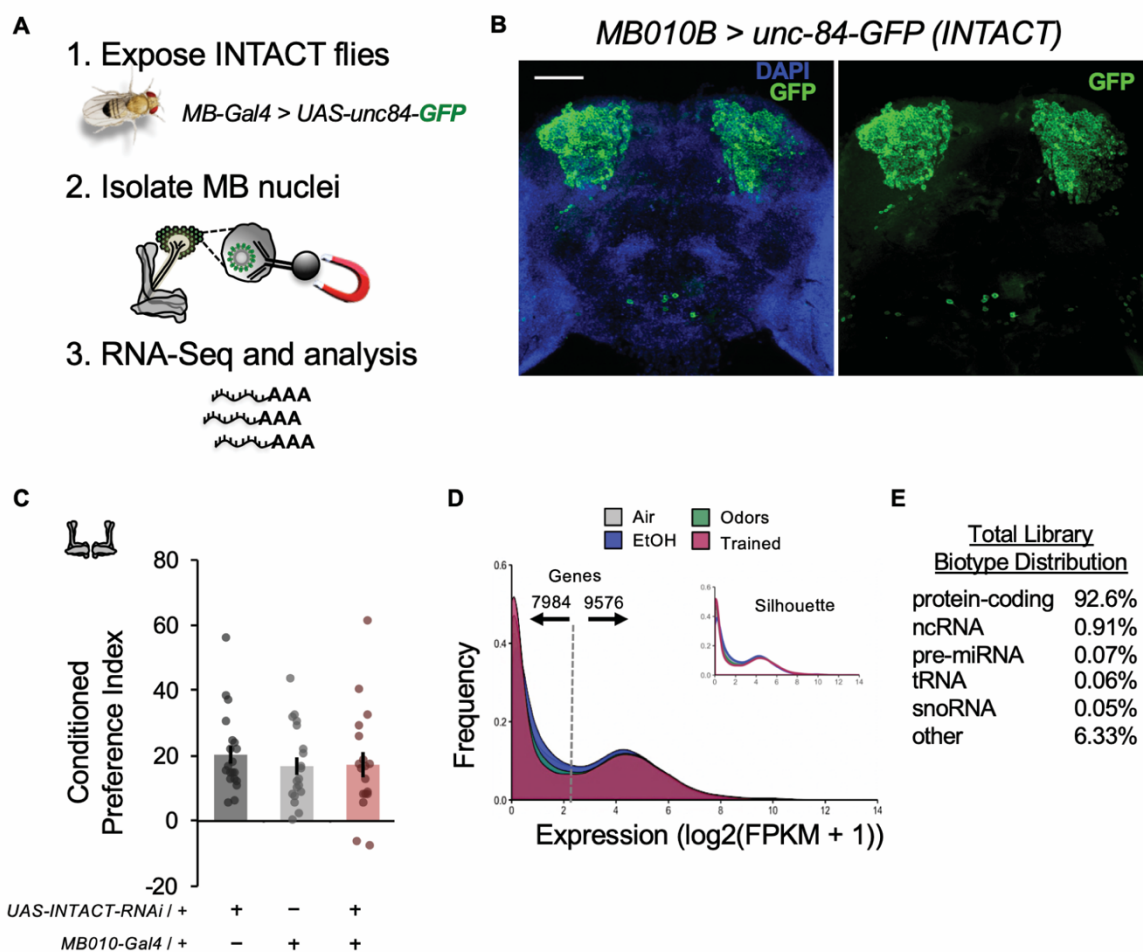


Figure 3.S1: MB INTACT paradigm and initial analysis.

(A) RNA-seq was performed on MB nuclei of flies expressing the INTACT transgene (MB010B-Gal4 > UAS-unc-84-GFP). Each fly brain contains ~4000 MB nuclei. (B) A representative immunohistochemical staining of the restricted INTACT (unc-84-GFP) tag expressed in MB010B-splitGal4 neurons in a 3-5-day-old adult male brain (DAPI staining DNA, Blue; anti-GFP staining GFP, Green). Confocal max stack image taken at 20x resolution (Scale bar 50 μ m). (C) Expression of the INTACT transgene (unc84-GFP)

in MB neurons did not interfere with odor cue-induced ethanol memory $F(2,56)=71.36$, $p=0.68$). (D) Histogram of the mean gene expression in fragments per kilobase (FPKM) across treatment libraries. Inset silhouette lacking fill color for increased visibility between treatments. (E) Percent distribution of biotypes according to current annotated Flybase 'feature' types across treatment libraries.

Figure 3.S2

	Total Sequences	Uniquely Mapped	Exonic / Intronic / Intergenic	Transcript Counts (FPKM)
Air	33844558	85.87%	97% / 1.5% / 0.7%	15784305
	37211293	87.31%	97% / 1.9% / 0.7%	15262082
	46395515	76.84%	97% / 1.8% / 0.8%	17714446
EtOH	65772590	85.91%	93% / 4.1% / 2.5%	12129006
	25243988	87.39%	97% / 1.6% / 0.9%	14165946
	29887470	83.14%	97% / 1.7% / 0.9%	15497176
Odors	30059906	89.08%	97% / 1.4% / 0.8%	15415177
	25082770	90.57%	97% / 1.4% / 0.8%	14377060
	41694314	89.92%	97% / 1.4% / 0.8%	21702932
	29669861	89.92%	97% / 1.6% / 0.9%	15614864
Trained	27938257	89.86%	97% / 1.4% / 0.8%	16104880
	24496938	89.84%	97% / 1.7% / 1%	14242031
	29179965	90.72%	97% / 1.4% / 0.8%	15455975
	33911395	89.6%	97% / 1.5% / 0.8%	17410191
AVERAGE	34313487	87.5%	96.7% / 1.7% / 0.94%	15776862

Figure 3.S2: QualiMap (BAM QC) analysis of the RNA-seq libraries.

The total sequences, uniquely mapped reads %, genomic biotype, and normalized transcript counts (FPKM) are displayed for all 14 libraries.

Figure 3.S3

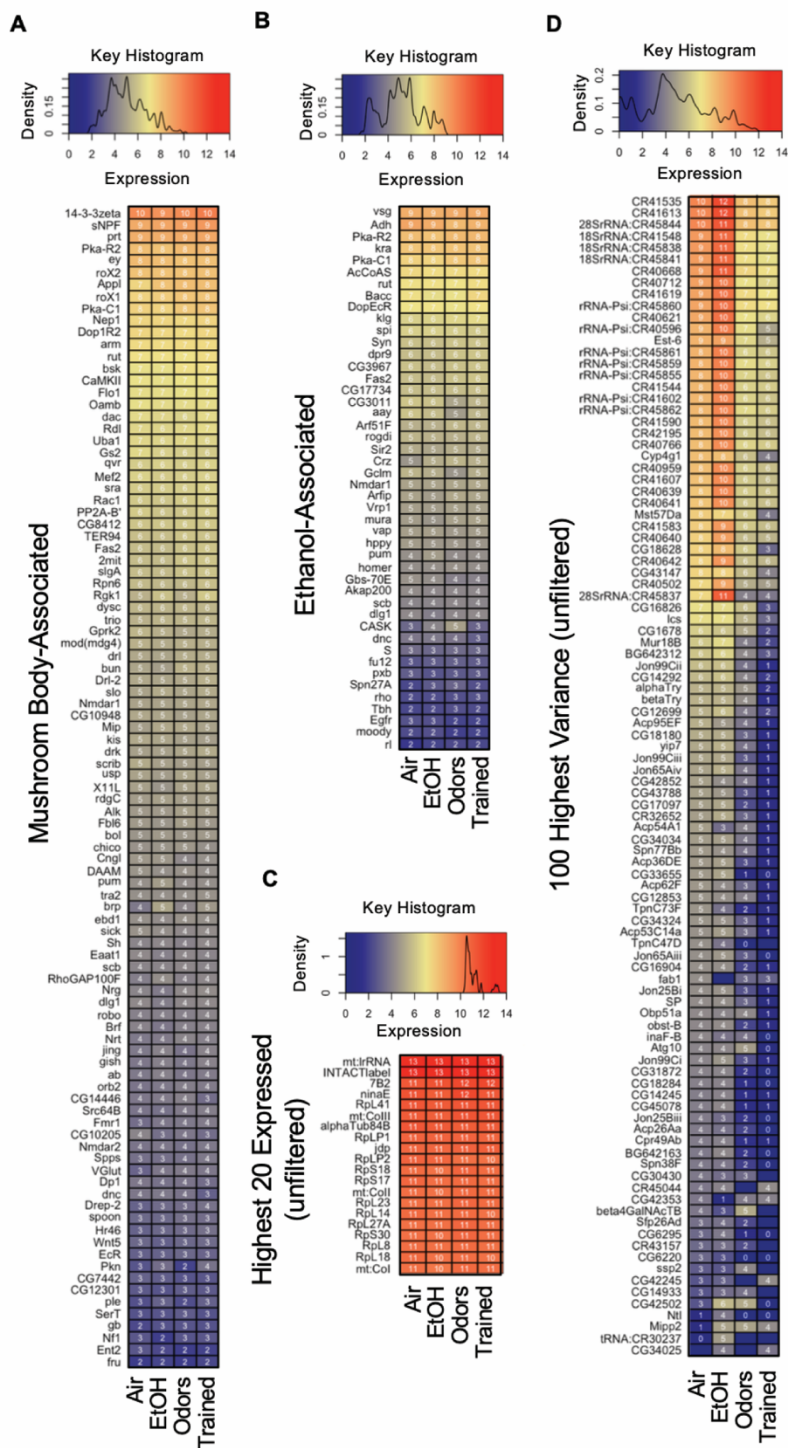


Figure 3.S3: Expression of genes-of-interest, highest expressed, and most variable genes across behavioral conditions

(A-D) Density histogram and normalized FPKM expression (blue, yellow, and red color representing relative levels) of (A) MB-associated, (B) ethanol- associated, (C) the highest 20 genes expressed genes without filtering, and (D) 100 most variable genes without filtering. All gene expression represented as condition means ($\log_2(\text{FPKM}+1)$) and ordered by mean expression.

Figure 3.S4

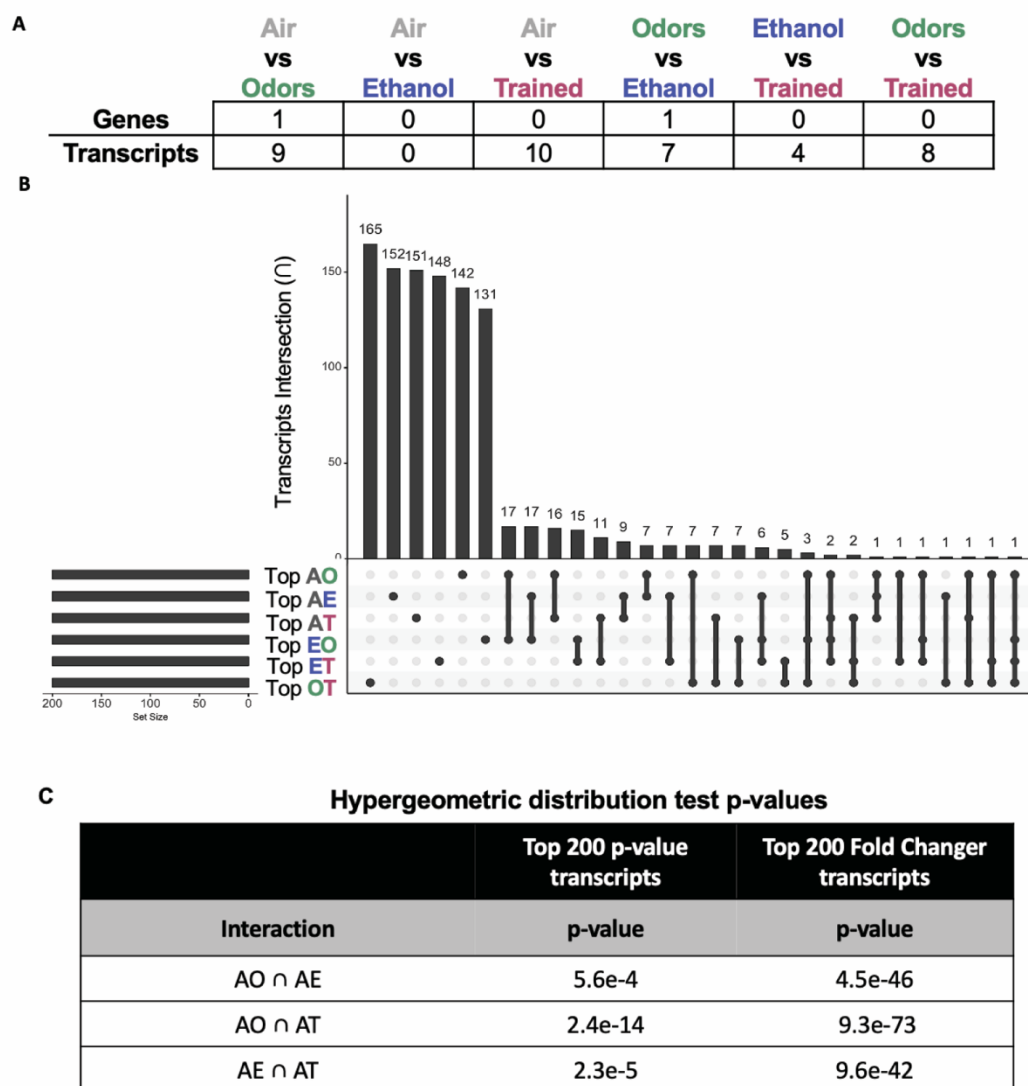


Figure 3.S4: Comparison of top differentially expressed transcripts across treatments, as compared to 'Air'

(A) The number of genes or transcripts reaching statistical significance at FDR < 0.05 between all pairwise comparisons: 'Air vs Odors', 'Air vs EtOH', 'Air vs Trained', 'Odors vs EtOH', 'EtOH vs Trained', and 'Odors vs Trained'. Colors depict treatment condition.

(B) An upgraded Venn Diagram plot generated by an R package called 'UpSetR'

demonstrating the intersection between top 200 p-value transcripts (abbreviated by first letter in treatment). (C) The phyper function in R was used to determine hypergeometric p-values. The 'universe' test number was 34,741 transcripts from the current Dm6 transcriptome. Values are as follows: phyper(q, m, n, k, lower.tail=FALSE) ... eg. phyper(overlap#, 200, (34741-200), 400, lower.tail=FALSE).

Figure 3.S5

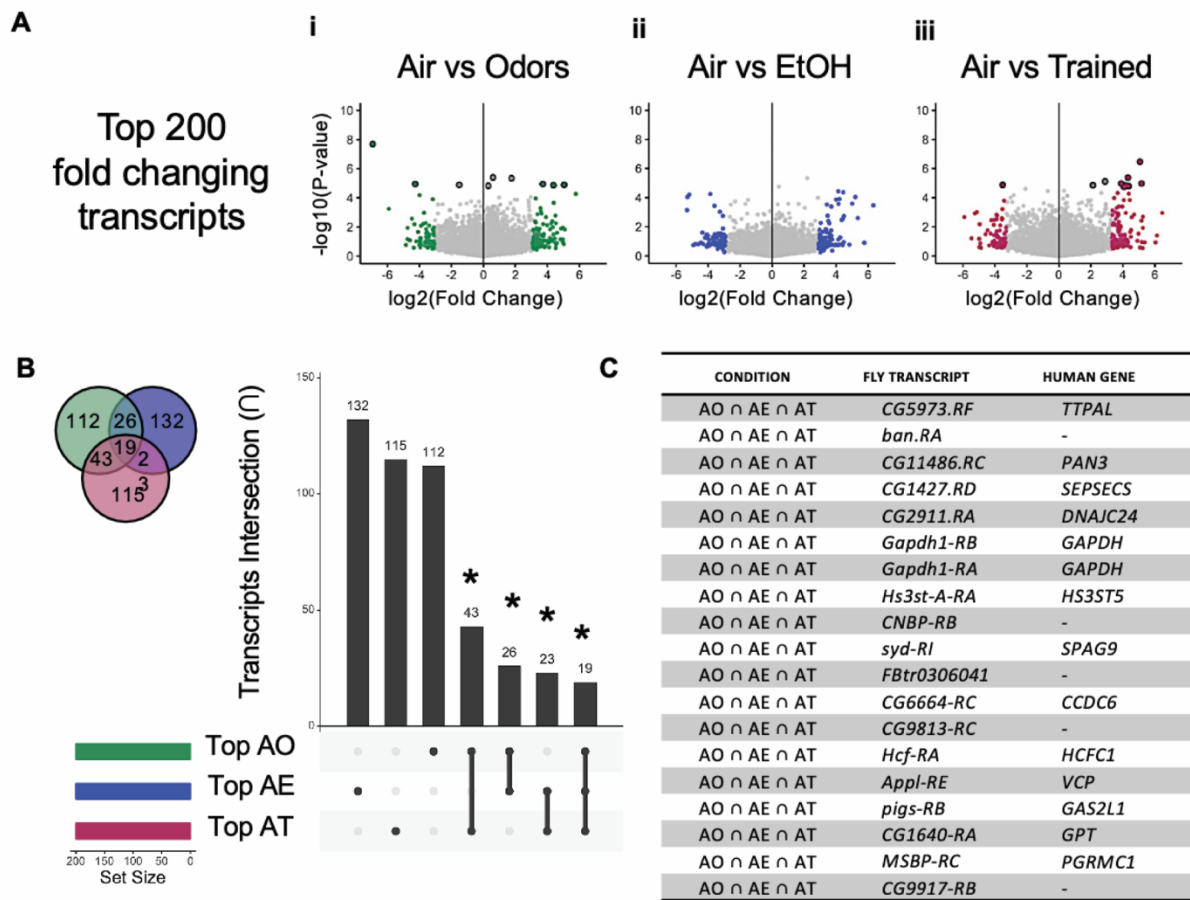


Figure 3.S5: Comparison of top 200 folding-changing transcripts in response to odor, ethanol or trained (odor- ethanol) treatment.

(A) Volcano plots showing fold change of transcript expression ($\log_2(\text{fc}+1)$) compared to the inverse of statistical significance ($-\log_{10}(\text{p-value})$) (dark outline, FDR < 0.05). Plots for (i) Odors, (ii) EtOH and (iii) Trained (ethanol-odor pairing) compared to Air (colors depict the top 200 fold changing transcripts). (B) An upgraded Venn Diagram plot generated by an R package called 'UpSetR' demonstrating the intersection in top 200 fold changing transcripts (abbreviated by first letter in treatment). (C) The 19 transcripts

found to be similar across all pairwise intersectional analyses are listed, along with the corresponding highest DIOPT-scored human genes.

Figure 3.S6

	DAVID GO terms	Adjusted p-value
Air vs Odor	phototransduction	0.86
	NADH dehydrogenase (ubiquinone) activity	0.91
	protein binding	0.92
Air vs Ethanol	Alternative splicing	0.0002
	Phosphoprotein	0.041
	Coiled Coil	0.047
Air vs Trained	Alternative splicing	0.062
	Developmental protein	0.2
	Phosphoprotein	0.25

Figure 3.S6: DAVID analysis of gene ontology (GO) of top 200 p-value genes.

Enrichment of current GO terms within the top 200 p-value genes found between Air vs Odor, Air vs Ethanol, and Air vs Trained pairwise comparisons (Bolted adjusted p-value < 0.05).

Figure 3.S7

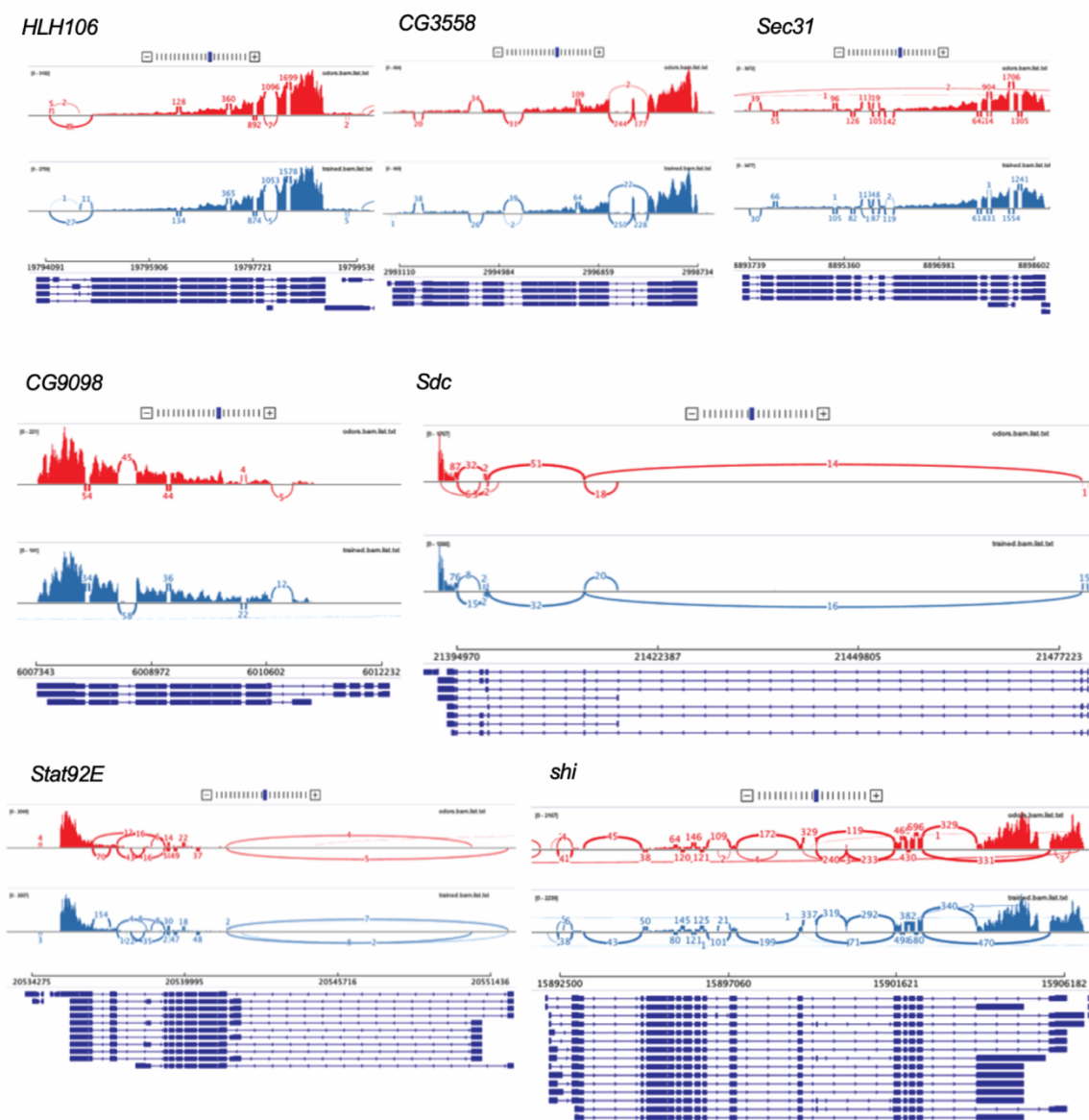


Figure 3.S7: Sashimi plots (IGV, Broad Institute) for alternatively expressed transcripts.

Sashimi plots help to visualize splice junctions across merged 'Odors' and 'Trained' samples for differentially expressed transcripts.

Figure 3.S8

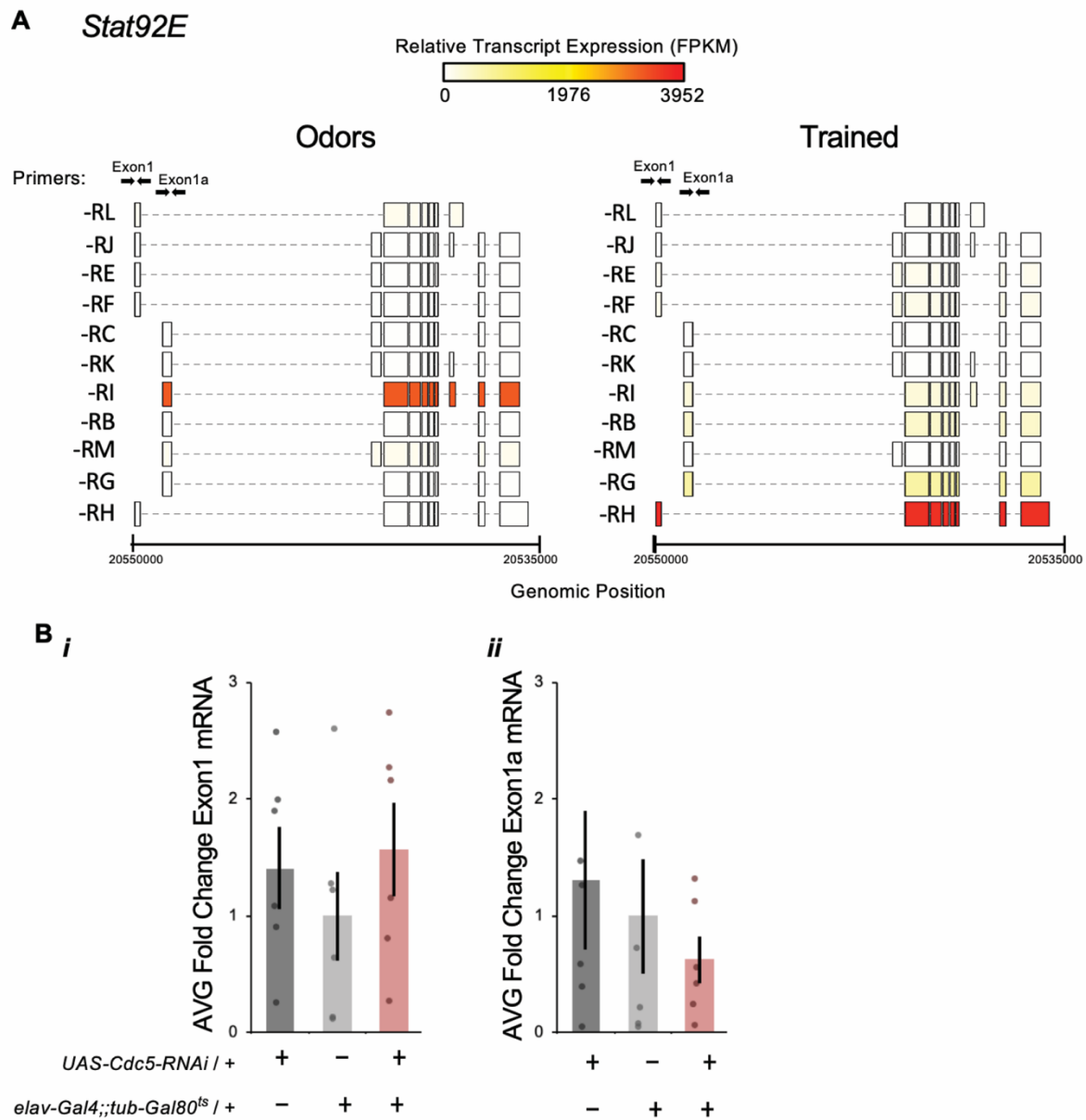


Figure 3.S8: Decreasing expression of *Cdc5* in adult neurons affects *Stat92E* expression.

(A) All transcripts of Stat92E expressed in MB neurons of flies treated with odors, or odor-ethanol pairings. Stat92E-RI was most highly expressed in MB nuclei of control flies whereas Stat92E-RH was more highly expressed in trained flies. (B) Average fold change of Stat92E Exon1 transcripts (including Stat92E-RH) and Exon 1a transcripts (including Stat92E-RI). Data for both graphs are normalized to *elav-Gal4;;tub-Gal80ts/+*. Decreasing Cdc5 in adult neurons did not significantly affect Stat-92E Exon 1 ($F(2,16)=2.56$, $p=0.45$) or Exon 1a ($F(2,16)=0.99$, $p=0.40$) transcript expression alone.

Figure 3.S9

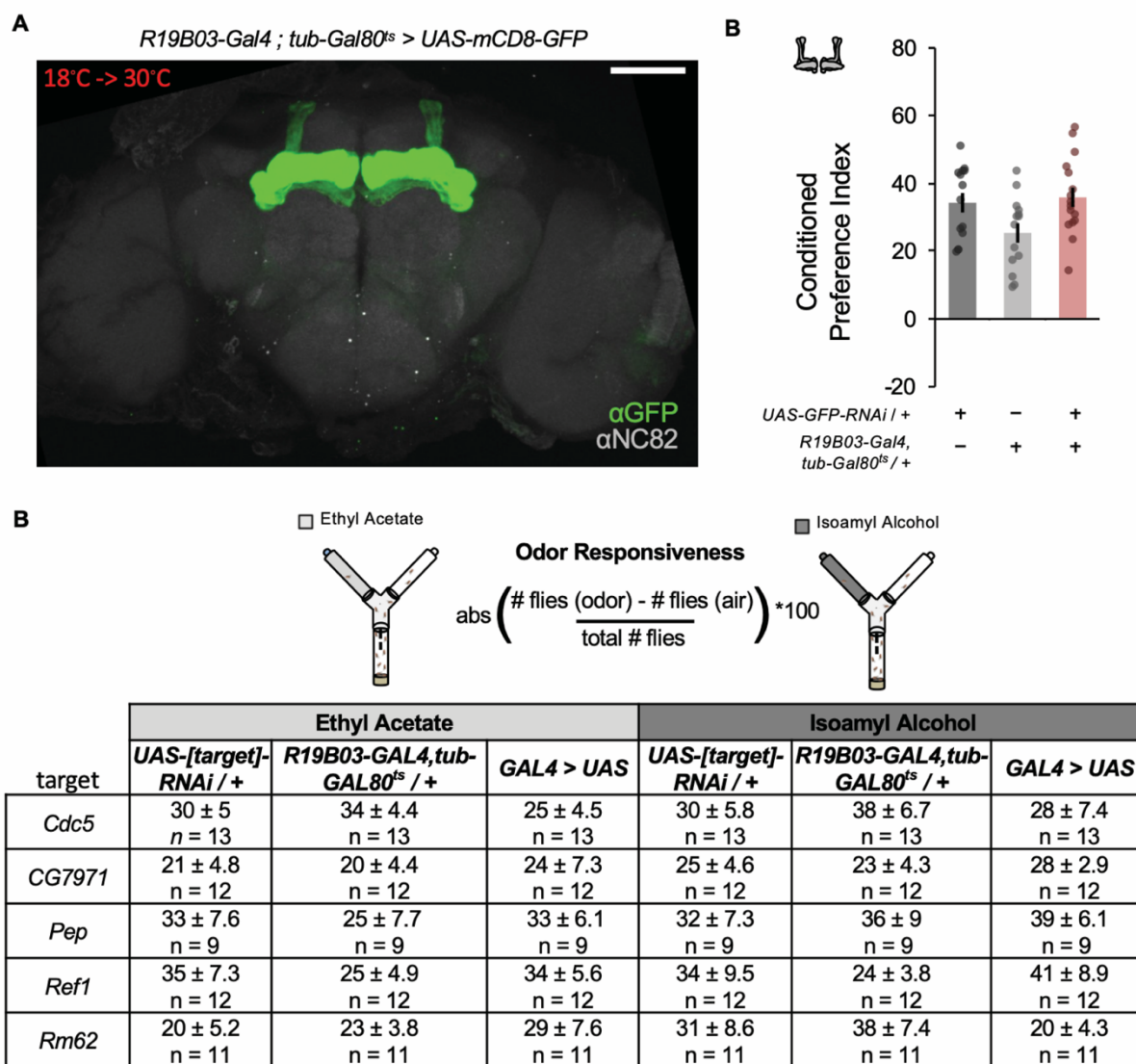


Figure 3.S9: Knockdown of splice-associated targets in adult MB neurons does not affect odor responses.

(A) R19B03-Gal4 is expressed in all MB neurons when using a UAS-mcd8-GFP reporter. Temporal expression was achieved by rearing flies in 18°C, then permitting expression by shifting one-day-old flies to 30°C for 3 days. (Scale bar 50μM) (B)

Expressing GFP-RNAi in all MB cells using R19B03-Gal4 did not reduce ethanol-odor memory ($F(2,41)=4.45$, $p=0.02$ with Student's t-test UAS/Gal4 vs Gal4,G80/+ $p=0.007$, and UAS/Gal4 vs UAS/+ $p=0.54$). (C) Odor responses as defined by preference between an odor or no odor in a Y-maze test to 1:36 ethyl acetate in mineral oil or 1:36 isoamyl alcohol in mineral oil were not significantly affected by expressing RNAi's for Cdc5, CG7971, Pep, Ref1 or Rm62 in adult MB neurons, as compared to their respective genetic controls.

Figure 3.S10

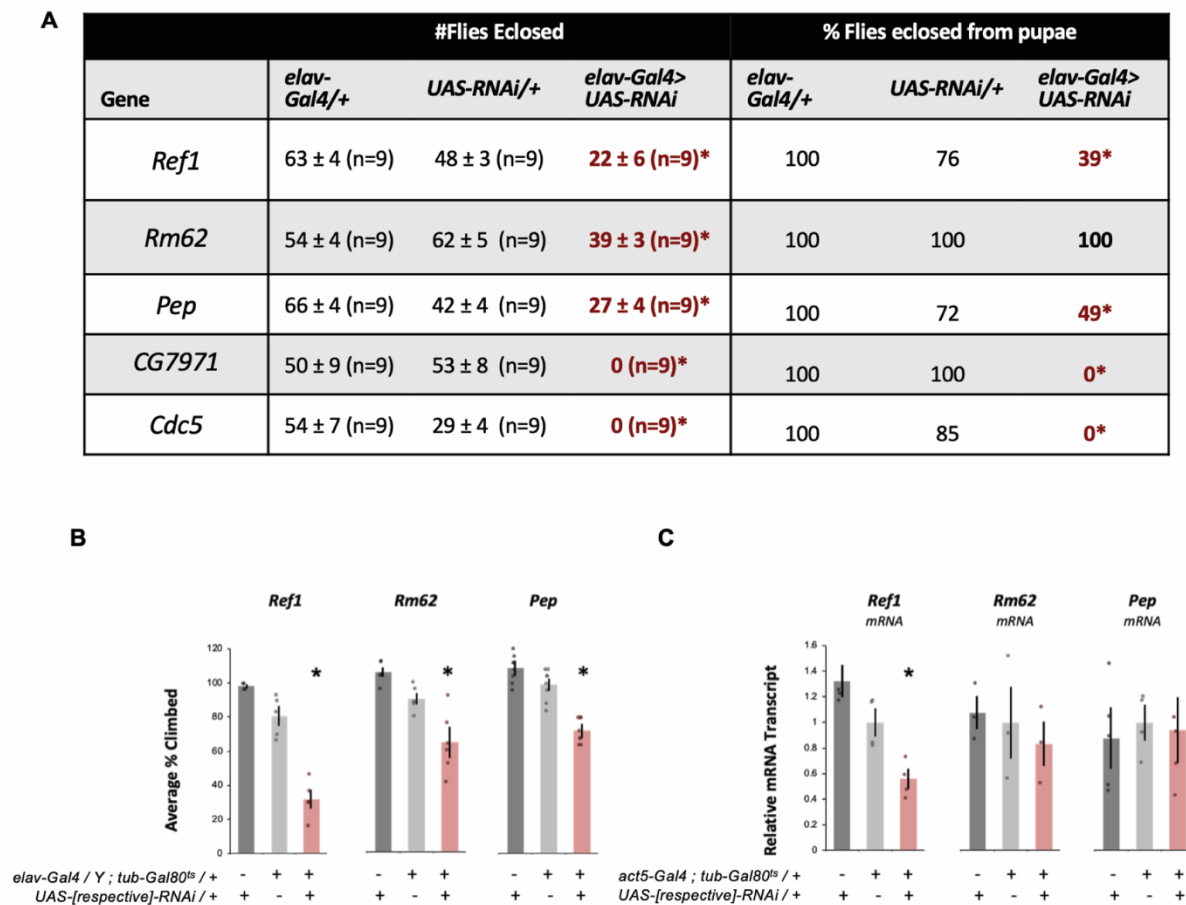


Figure 3.S10: Efficacy of RNAis of spliceosome associated genes.

(A) Expressing RNAi for Ref1, Pep, CG7971 or Cdc5 in all neurons throughout the life of the flies significantly affected the number of non-balanced flies eclosed, resulting in decreased survival to adulthood. Expressing RNAi for Rm62 significantly decreased the number of non-balanced flies eclosed. Wilcoxon rank sum tests were performed for experimental lines vs respective heterozygous controls. $p < 0.05$ are depicted in a red color with an *. (B) Expressing RNAi for Ref1 ($p < 0.001$), Rm62 ($p < 0.001$) or Pep ($p < 0.001$) in all neurons using *elav-Gal4* significantly reduced the ability to climb to a 5

cm mark in a plastic vial within 10 seconds after tap down. (C) Expressing RNAi in all adult cells using actin5c-Gal4 significantly reduced expression of Ref1 in adult fly heads ($p < 0.001$).

Figure 3.S11

Publication and study details	Gene lists
Petrucelli et al, 2019 (this study) Time: 24hr after training Tissue: Mushroom body nuclei Condition: appetitive alcohol LTM, 3x 10min (sequential odors) interval spaced 60min Technique: INTACT Analysis: RNA-seq (HISAT2, StringTie, Ballgown)	Genes associated with top 200 transcripts 'Odors vs Trained': Aats-trp, aay, Act5C, Adgf-E, Amnionless, Anx89, asparagine-synthetase, Atg8b, aurA, 3B54, beg, ben, Cad96Cb, CBP, Cdk1, Cdk8, CG10170, CG10428, CG10512, CG10839, CG11131, CG11148, CG11425, CG12116, CG12746, CG12909, CG13484, CG13563, CG13607, CG13708, CG13872, CG14075, CG14275, CG14285, CG14435, CG14621, CG14688, CG14717, CG14879, CG14926, CG15093, CG1513, CG15925, CG1673, CG16756, CG16926, CG16953, CG17570, CG17612, CG17816, CG17821, CG18130, CG2316, CG2911, CG3040, CG30430, CG31419, CG31431, CG3176, CG31787, CG31882, CG32533, CG33098, CG33111, CG33120, CG33795, CG3402, CG34126, CG34166, CG34376, CG34417, CG3505, CG3558, CG42394, CG42502, CG42540, CG42559, CG42577, CG42724, CG42752, CG43093, CG43172, CG43347, CG43630, CG4462, CG4662, CG4842, CG4962, CG5174, CG5446, CG5953, CG5958, CG6067, CG6770, CG8216, CG8508, CG8726, CG9098 , CG9149, CG9263, CG9300, CG9663, CG9723, CG9777, CG9784, CG9975, CR32205, CR43653, CR43887, CR44265, CR44301, CR44886, CR45113, CR45170, CR45172, CR45884, Crk, crol, cu, Cyp12c1, Cyp4e3, cype, Cyt-b5-r, dbo, Desat2, DhpD, Dip-B, dor, endos, endos, fabp, fit, fus, Glut3, Gpdh, gus, HLH106 , HmgZ, HPS4, Hr39, Hr4, hubl, Idh, intr, jnj, l(2)k09913, LKR, mav, mir-4963, mod, mod(modg4), mRp5188, mtsh, NaCP60E, net, Not1, nsl1, Obp57b, odd, Or85f, Pask, Pitsre, pix, Rb97D, Reps, Reps, Rm62, Rpl1, Rpl3, Sdc , Sec31 , Sh, shi, sif, SkpA, snoRNA:Me285-A2634c, snoRNA:Me285-C3420b, Sodh-2, Stat92E , su(r), Syt1, tau, TFAM, Tgi, Thor, TM45F, TotA, Trpm, UK114, viaf, Xport *bold FDR < 0.05
Crocker et al, 2016 Time: 30min after training Tissue: Kenyon Cells, male only? Condition: aversive shock olfactory LTM, 8x 1min interval spaced 10min Technique: Single Cell RNA-seq Analysis: TopHat2, HTseq-count, DESeq2	no differentially expressed changes found in KCs, likely due to pooling
Widmer et al, 2018 Time: T2 (12-24hr) and T3 (24-48hr) after training Tissue: Heads Condition: appetitive sucrose olfactory LTM, starve 19-21hr then 2min (sequential odors spaced 1min) Technique: Dam-Pol II Analysis: Bowtie2, damidseq_pipeline with FDR via 50,000 simulations	T2: Acox57D-p, Acp54A1, Adam, Adk2, Asciz, ATPsynC, Cdk7, CG10344, CG10562, CG10581, CG10584, CG11069, CG11137, CG11597, CG11668, CG11693, CG11777, CG11852, CG12118, CG13026, CG13031, CG13082, CG13239, CG13640, CG13731, CG13748, CG14096, CG14147, CG14572, CG14646, CG14661, CG14968, CG15098, CG15515, CG15545, CG15546, CG16884, CG17068, CG17224, CG17337, CG17549, CG17919, CG18067, CG2604, CG30101, CG30280, CG31626, CG31717, CG31812, CG32170, CG32579, CG32650, CG3301, CG33170, CG33946, CG34010, CG3552, CG3566, CG3700, CG42269, CG42568, CG42615, CG43367, CG4407, CG44286, CG45676, CG5390, CG5397, CG5867, CG5885, CG5934, CG6272, CG6429, CG6908, CG7457, CG7872, CG7896, CG8317, CG8319, CG8525, CG8788, CG9372, CG9521, CG9928, CHKov2, CIAPIN1, Cpr47Ea, Cpr49Aa, Cpr49Ah, Cpr64Aa, Cpr64Ad, Cpr78Cb, CR31144, CR43196, CR44107, CR44168, CR44264, CR44383, CR44468, CR44469, CR44621, CR44756, CR44888, CR44893, CR45150, CR45350, CR45501, CR45541, CR45682, CR45827, CR45828, CR45925, CR46108, CREG, Cyp6a15Psi, Cyp6d5, Cyp6g1, Cyp9f2, Dip-B , Dnal-60, E(spl)mbeta-HLH, Eflbeta, Fmo-2, FucT6, Gapdh1, GstE8, GstO1, GstO2, GstO2, GstZ1, Hsf, Hsp70Bb, Lcp65Aa, Lsm10, mir-282, Mmp1, mRp525, Myb, ND-816.6, Nxt1, Obp56d, obst-A, olf186-M, Osl19, PCB, Pcd, PGRP-LF, pinta, Probeta3, Prx2540-1, Ptr, rempa, Rpl15, Rps15Ab, scu, snmRNA:838, snoRNA:Psi185-301, Syx8, tal-1A, tal-2A, tal-3A, tal-AA, Taldo, tRNA:CR32289, tRNA:H:48F, Ugt35b, vajk-1, ver, wal, ylp2, ylp2 T3: Acox57D-p, Acp54A1, Adk2, B-H1, Cdk7, CG10257, CG10344, CG10602, CG10754, CG11137, CG11191, CG11668, CG11852, CG12118, CG13082, CG13299, CG13731, CG13751, CG13928, CG14096, CG14572, CG14968, CG15515, CG15545, CG17337, CG17549, CG30280, CG32039, CG32170, CG3226, CG32579, CG32650, CG32806, CG32815, CG33170, CG33946, CG3566, CG3939, CG42239, CG44286, CG5397, CG5867, CG6272, CG6429, CG7457, CG7872, CG8500, CG8788, CG9319, CG9521, CG9928, CG9960, CR43700, CR44371, CR44420, CR45008, CR45172 , CR45350, CR45554, CR45911, CR45919, CR45925, CR46108, CREG, Cyp6a15Psi, Efl1alpha100E, Faf2, Flo1, Fmo-2, Gapdh1, GstO2, Hsp70Bb, jdp, mei-5332, mir-282, mRpL27, mRpL35, mRp525, ND-816.6, Nup54, Obp56d, olf186-M, pinta, pncr015:3L, Prx2540-1, Pu, put, rempa, scu, Snapin, snoRNA:lola-a, snoRNA:Psi185-301, Syx8, Taldo, Tes, TRAM, tRNA:CR32287, tRNA:CR32289, tRNA:H:48F, Vps4, ylp2 *bold similar in this study
Pacifico et al, 2018 Time: 5-, 20-, 30-, and 40-day-old Tissue: Sex- and age-matched brains Condition: Age-related cognitive decline, aversive shock olfactory STM, 1min (sequential odors spaced 30sec) Technique: RNA-seq Analysis: STAR, HTSeq-count, DESeq2 with default filtering and WGCNA	Male, likelihood ratio test (LRT) q-value < 0.05 (1076 genes identified): too many to list here Similar in this study: aay, aurA, ben, CG11425, CG13607, CG14879, CG1673, CG42394, CG5174, CG5446, CG5953, CG9098, cype, Gpdh, Rpl1, Sodh-2, Thor, TM45F, TotA *bold similar in this study
Signor and Nuzhdin, 2018 Time: 10, 20, 30min post exposure Tissue: Male and female heads, heterozygous inbred lines Condition: Ethanol vapor exposure from food Technique: RNA-seq Analysis: Exon delineation, a priori gene list filtering and abundance cutoffs	Genes associated with exonic P_Treatment < 0.05 (1941 unique genes of 2402 identified): too many to list here Similar in this study: CG12116, CG13607, CG17816, CG2911, CG34166, CG3505, CG42540, CG9098, Crk, dor, endos, fus, jnj, l(2)k09913, mod(modg4), NaCP60E, odd, Pitsire, Rpl1, Sec31, su(r), Trpm *bold similar in this study
Other important studies with different methodologies: Kong et al, 2010 (microarray); Morozova et al, 2009 (microarray); Morozova et al, 2015 (QTL analysis).	

Figure 3.S11: RNA-seq comparison to previous studies.

The gene lists from previous studies (in no particular order), most relevant to this study.
Study details are provided to bear in mind contextual relevance.

Chapter 4:

Alcohol-Induced Alternative Splicing of *Dopamine-2-receptors* in *Drosophila* Memory Circuits

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Contributions:

- Tariq Brown assisted in generating Dop2R splice mutants and Dop2R tagged with T-STEP flies, performed crosses and dissections for Fig 3.2, performed all analyses, generated all figures, and composed text.
- John Hernandez performed OPEN BARR experiments.
- Ramsha Gul performed FLIC experiments.
- Samuel Stea performed Tandem two-choice experiments.
- Emily Petruccelli performed the RNA-seq experiments in Figure 1.

- Scott Gratz conceptualized the CRISPR modifications in order to generate Dop2R-splice mutants.
- Imaad Said assisted in elucidating the endogenous expression of Dop2R.
- Amanda Waterman conceptualized and helped generate Dop2R T-STEP flies.
- Kavin Nunez conceptualized and helped generate Dop2R T-STEP flies.
- James Kentro provided valuable insight into possible neuropil expressing Dop2R.
- Kate O'Connor-Giles conceptualized the CRISPR modifications to generate Dop2R splice mutants and provided necessary *Drosophila* reagents for generation of Dop2R splice mutants.
- Kristin Scaplen performed Y-Maze experiments and FLIC experiments
- Karla Kaun provided valuable feedback on experimental design, structure, figures, and writing.

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4.1 ABSTRACT

Sensory memories for the feeling of being intoxicated can problematically trigger relapse in individuals recovering from alcohol use disorder (AUD). However, the molecular mechanisms underlying these memories have yet to be fully understood. Dopamine-2 receptor (D2R) is thought to be an important factor in determining susceptibility to AUD. Splicing of D2R from the long isoform (D2RL) to the short isoform (D2RS) changes subcellular localization, altering the properties of dopaminergic signaling and potentially conveying increased risk for AUD. In *Drosophila*, Dopamine-2-Receptor (*Dop2R*) isoform expression changes with alcohol exposure in memory-encoding neurons in *Drosophila melanogaster*. To understand the functional consequences of this alcohol-induced splicing, we generated mutant *Drosophila* that have CRISPR modifications that only permit the expression of the naive or trained isoform of DOP2R. We observed that *Dop2R* splicing affected the way flies consumed alcohol and sucrose, without impacting memory for these rewards. These findings demonstrate that alcohol-induced splicing directly alters reward consumption.

4.2 SIGNIFICANCE

Alcohol-induced alternative splicing is conserved across species from flies to mice to humans. However, the functional consequences of this alternative splicing are not well understood. We found that alcohol-induced alternative splicing of the Dopamine-2-Receptor in *Drosophila* impacts both alcohol and natural reward consumption. These results suggest that Dop2R splicing plays an important role in dictating reward response.

4.3 INTRODUCTION

Alcohol is the most abused drug in the United States (World Health Organization, 2018). The pharmacological properties of alcohol are anxiolytic, and induce euphoric feelings (Baum-Baicker, 1985; Gilman et al., 2008; Sayette et al., 2012). However, there are negative consequences associated with its consumption at both the molecular and behavioral level (Evans-Polce et al., 2024; Patrick & Terry-McElrath, 2021; Varghese & Dakhode, 2022). The increased willingness to endure the negative consequences association with alcohol consumption can result in the development of alcohol use disorder (AUD (Koob & Volkow, 2016)). AUD is a substantial burden on health systems, having an economical toll of ~\$250 billion dollars within the United States annually (Sacks et al., 2015). According to the 2023 National Survey on Drug Use and Health, 28.9 million people ages 12 and older were had AUD in 2022-2023 (SAMSHA, 2024). While current therapeutics are effective for a subset of the population (Celik et al., 2024; Mattle et al., 2022), they are not substantial long-term solutions (Fuller et al., 1986; Lyons et al., 2017). The molecular mechanisms that change the function of neural circuits, and thus behavior, require further investigation to develop better pharmaceutical interventions.

An under-explored possible contributor to the development of AUD is the alternative splicing that occurs within neural circuits following ethanol exposure (Donadoni et al., 2019; Downing et al., 2012; Farris et al., 2015; Huggett et al., 2023; Iancu et al., 2018; Petruccelli et al., 2018; Signor & Nuzhdin, 2018). Alternative splicing is a fundamental biological mechanism that allows our cells to generate multiple forms of proteins from a single genetic template, and occurs within approximately 95% of genes (Gilbert, 1978;

Marasco & Kornblihtt, 2023; Pan et al., 2008). This is an essential process that expands genetic diversity and allows cells to flexibly adapt their proteins to better suit the environment they reside within (Liu et al., 2022; Wright et al., 2022). AUD causes global changes in splicing in the human brain (Van Booven et al., 2021). In recent years, alternative splicing has also been associated with alcohol use in the brains of non-human primates (Iancu et al., 2018), rats (Hardy et al., 1999; Petrie et al., 2001), mice (Wolfe et al., 2019), chicks (Al-Shaer et al., 2019), and flies (Petrucelli et al., 2018, 2020; Signor & Nuzhdin, 2018). The conservation of this process across such a diversity of species suggests it is a fundamental mechanism through which alcohol affects the molecular composition and function of cells.

Alcohol-induced alternative splicing has been observed in immune-related genes (Li et al., 2023; Petrucelli et al., 2018) and plasticity-associated genes (Li et al., 2023; Petrucelli et al., 2018) including dopamine receptors (Oomizu et al., 2003), GABA receptors (Edenberg et al., 2004; Lee et al., 2014). Of these genes, Dopamine-2 receptors (D2R) have a conserved role in learning and memory across species (Puig et al., 2014), and D2R is a therapeutic target for multiple neuropsychiatric diseases (Creese et al., 1976; Jiménez-Urbietá et al., 2015; Lawford et al., 1995; Zhang et al., 2020). Thus, D2R is an interesting candidate to better understand the contributions of alcohol-associated splicing to changes in behavior and the development of AUD.

Along with its conserved role in memory, the splicing of D2R is observed across species, including flies (Petrucelli et al., 2018), mice (Monsma et al., 1989), rats (Giros et al., 1989), and humans (Dal Toso et al., 1989). D2R splicing manifests as the removal of a 29-amino acid insert in the third intracellular loop of the protein (Dal Toso et al.,

1989; Giros et al., 1989; Monsma et al., 1989; Žuk et al., 2020). *In vitro* and *in vivo* studies have demonstrated that the third intracellular loop, and serines within the 29-amino acid stretch are instrumental to the function of D2R, determining the strength of protein-protein interactions (Fernández-Dueñas et al., 2012; Guiramand et al., 1995; Senogles et al., 2004) and ability to signal through β arrestin (Mann et al., 2021). D2R-Long is predominantly expressed on dendrites on a subset of neurons within the striatum (Hernandez-Lopez et al., 2000; Khan et al., 1998). Conversely, D2R-short is mainly expressed on the presynaptic sites at the axon terminals dopaminergic neurons (Anzalone et al., 2012; Khan et al., 1998; Lindgren et al., 2003; Usiello et al., 2000). There are many single nucleotide polymorphisms (SNPs) associated with AUD that bias the splicing of D2R towards the short isoform, and overall decreases in the expression of D2 receptors (Bertolino et al., 2009; Eisenstein et al., 2016; Konishi et al., 2004; Smith et al., 2017). This decrease in D2 receptors can lead to excitatory D1-receptors on MSNs being preferentially stimulated by upstream dopaminergic circuits, causing increased approach to reward (Walle et al., 2024). These changes in the form and density of D2 receptors need to be further investigated to understand how alcohol-induced splicing of D2 receptors is changing the properties of reward circuitry and behavioral responses to natural and alcohol reward.

While previous studies have been able remove splicing of certain isoforms of genes implicated in AUD (Bulwa et al., 2011; Radl et al., 2018; Wang et al., 2000), studying the impact of splicing using mammalian models has been difficult due the lack resolution to make precise splicing alterations and manipulate and record from precise circuits.

Drosophila melanogaster has been an effective model for understanding the

development of AUD for decades (Cummins-Beebee et al., 2023; Kaun et al., 2011; Nunez et al., 2018; Singh & Heberlein, 2000). *Drosophila*'s behavioral responses to intoxication often mirror that of humans, and they develop lasting preferences for intoxicating doses of ethanol (Devineni & Heberlein, 2013; Kaun et al., 2011; Singh & Heberlein, 2000).

We have previously shown that splicing machinery is required for the formation of alcohol memory in *Drosophila melanogaster* (Petruccelli et al., 2020), and following formation of odor-alcohol memory, aberrant splicing occurs in multiple genes with functions involved in synaptic plasticity, regulation of transcription, and G-protein coupled receptors (GPCR) like *Drosophila Dopamine-2-like receptor (Dop2R)*, the *Drosophila* ortholog of D2R (Hearn et al., 2002; Petruccelli et al., 2018).

Dop2R is broadly expressed in memory relevant neuropil, such as the mushroom body (Figure 4.S2). Dop2R is expressed within the axon terminals, calyx, and cell bodies of the mushroom body (Figure 4.S2). Previous work has demonstrated the necessity of Dop2R in the mushroom body for odor-alcohol memory formation (Petruccelli et al., 2020; Scaplen et al., 2020). Odor-alcohol memory formation results in the omission of a single serine (S658) within the third intracellular loop of Dop2R that changes *Dop2R transcript-H (Dop2R-RH)*, to *Dop2R transcript-I (Dop2R-RI)* (Figure 3.1A)

To better understand the functional consequences of this alcohol-induced alternative splicing of Dop2R, we generated CRISPR-Cas9 splice mutants that have forced expression of Dop2R-RH or Dop2R-RI by modifying the splice donor and acceptor sites preceding exon 8 (Figure 4.1D and 4.1E). With this technique we restricted expression within mutants to just Dop2R transcripts B, G, and H (herby referred to as

Dop2R-RH) or mutants that have forced expression of Dop2R transcript-I (hereby referred to as Dop2R-RI). Because the only difference between these two isoforms is the presence of a serine-658 within the protein structure, we also generated mutants that have a phosphoinactive alanine (Dop2R-S658A), or a phosphomimetic aspartic acid (Dop2R-S658D), which will provide insight into whether the serine is driving functional differences between the isoforms. In this work, we use Dop2R splice mutants to investigate the relationship between alternative splicing and reward-associated behaviors involving reward consumption, choice, and motivation.

4.4 RESULTS

4.4.1 - Alternative splicing of Dop2R increases alcohol and sucrose consumption.

Previous work has shown that decreased D2R-long expression in mice leads to increased alcohol and sugar consumption (Bulwa et al., 2011). Since *Drosophila* Dop2R enhances sugar taste response following a reduction in sugar intake (Ganguly et al., 2021) and is required for formation of ethanol memory (Petrucelli et al., 2018), we hypothesized that expression of specific Dop2R isoforms would change sucrose and alcohol consumption. Because lack of the long isoform within mice increased alcohol and sucrose consumption, we expected the Dop2R-RH-deficient Dop2R-RI flies would exhibit greater consumption of both sucrose and ethanol-containing food compared to Dop2R-RH and Dop2R-RI. We further hypothesized that Dop2R-RI mutants would exhibit higher preference for both sucrose-containing food and ethanol-containing food using the food-liquid interaction counter (FLIC) assay (Figure 3.2A (Ro et al., 2014)) which provides real-time monitoring of consumption. Flies were allowed to consume a food in the form of 5% sucrose in one well or 15% ethanol in 5% sucrose in another well

for up to 5 days. We measured the number of times the fly contacted the food port (Approaches), the number of times they extend their proboscis to feed (Licks), the time they spend at the food ports (Duration), and how frequently they return to the food port (Latency).

Over the 5 days of the flies were monitored within the assay, behavior trends were consistent within each strain (Figure 4.S4), so we report data on day 1 below, and days 2-5 in the supplement.

We observed that control, Dop2R-RH and Dop2R-RI flies approached both the sucrose port to consume food (Figure 4.2Bi), however, Dop2R-RI flies approached the sucrose containing food port almost twice as many times on average compared to Dop2R-RH and control flies (Figure 4.2Bi). There were no differences observed in the number of approaches between control and Dop2R-RH flies. Control and Dop2R-RH flies also licked a similar amount from the sucrose containing food port (Figure 4.2Bii), whereas Dop2R-RI licked at significantly higher amount from the sucrose food (Figure 4.2Bii). Interestingly, there were no differences between the genotypes in the amount of time per feeding bout (Figure 4.2Biii). Control flies took longer to return to the sucrose-containing food port when compared to the RH flies. From this data, we can conclude that Dop2R-RI flies are engaging more with sucrose compared to Dop2R-RH or control flies. Stats are reported in Table 4.S1.

A similar trend emerges with ethanol-containing food. On the first day, Dop2RI flies approached the ethanol containing food port more than the control and Dop2R-RH flies (Figure 4.2Ci). Also, Dop2R-RI flies lick more from the ethanol-containing food port when compared to both RH and control flies, which did not differ from one another.

Feeding bout duration is consistent across genotypes (Figure 4.2Ciii), and Dop2R-RI flies have a significant decrease in the latency between approaches when compared to both control and Dop2R-RH flies. Thus, we conclude that the Dop2R-RI flies are engaging more with ethanol compared to Dop2R-RH or control flies. Stats are reported in Table 4.S1.

When investigating the minute-to-minute fluctuations for engagement with the sucrose-containing food and ethanol-containing food ports (Figure 4.2Di-4.2Div, 4.2Ei- 4.2Eiv), we observed that all genotypes predominantly fed between zeitgeber time 6 and zeitgeber time 10 (Figure 4.2Dii and 4.2Eii; 200 to 500 minutes, lights on at 9:00AM). For both sucrose and ethanol, we observed that the Dop2R-RI flies engaged with the port during this period more than control and Dop2R-RH flies. Stats are reported in Figure 4.S20.

4.4.2 - *Alternative splicing of Dop2R does not influence alcohol-cue response*

Dop2R is critical for the encoding of odor-alcohol memory (Petruccelli et al., 2018). Additionally, our FLIC recordings have provided evidence that the Dop2R-RI isoform increases alcohol engagement. This increased drive for alcohol could influence preference for cues associated with alcohol. We hypothesized that the change from transcript-H to transcript-I is a driving factor for approach to alcohol-associated odor cues. More specifically, we hypothesized that the *Dop2R-RH*-expressing mutants would exhibit lower preference for cues associated with alcohol after Pavlovian conditioning compared to *Dop2R-RI*-expressing mutants. To test approach toward cues associated with alcohol, we conditioned flies to associate intoxicating doses of ethanol with odor

cues (Figure 4.3A (Kaun et al. 2011, Nunez et al., 2018). We subsequently tested examined cue-memory in the well-established memory assay, Y-maze (Figure 4.3B; (Kaun et al., 2011; Mohandasan et al., 2022; Nunez et al., 2018)).

When cue-memory was assessed using the Y-maze, Control flies exhibited expected levels of memory within this assay (Figure 4.4C). Unexpectedly though, both Dop2R-RH and Dop2R-RI exhibited normal levels of memory (Figure 4.4C; Table 4.S3 for stats). These results provide evidence that Dop2R mutants, regardless of their splicing profile, can learn to associate an odor-cue with intoxicating doses of ethanol.

Though Y-maze may lack the sensitivity to thoroughly distinguish between the mutant phenotypes. As such, we have implemented a novel two-choice assay to better understand choice dynamics (Tandem two-choice assay; Figure 4.3E). Given that the Dop2R-RI isoform also increased approach towards sucrose as measured by the FLIC assay, Dop2R-RI might also be influencing associative learning with natural reward. We hypothesized that Dop2R-RI mutants would have higher preference for cues associated with sucrose compared to Dop2R-RH flies (but not controls). We trained flies to associate an odor with sucrose (Figure 4.3D). by exposing flies to 15 seconds of air, followed by 2 minutes of simultaneous presentation of both the paired odor and the unpaired odor. Flies were allowed to freely move to the side of the arena containing the paired or unpaired odor. Flies were then given 15 seconds of air after the stimulation period.

We observed that the control flies approached the odor paired with sucrose after odor onset (Figure 4.3Fi). Likewise, Dop2R-RH and Dop2R-RI flies also approached the odor paired with sucrose (Figure 4.3Fii, and Figure 4.3Fiii). We found that there were no

differences between the genotypes when looking at overall cue memory (Figure 4.3G; Table 4.S4). However, Dop2R-RI flies appear to be less active when expressing the memory. Dop2R-RI flies had less transitions (Figure 4.3H), and distance moved in both paired and unpaired regions (Figure 4.3J) compared to Control and Dop2R-RH flies (Table 4.S4 for statistical comparisons). Although overall cue memory remained consistent between genotypes, using the Tandem two-choice assay we were able to identify behavioral metrics that could potentially be indicative of how Dop2R-RI flies engage differently with reward cues compared to Dop2R-RH and controls.

4.4.3- Alternative splicing of Dop2R has mixed effects on operant alcohol response

Previous studies have shown that D2R expression levels impact self-administration of alcohol in both rats and mice (Phillips et al., 1998; Thanos et al., 2001). However, these studies have lacked the ability to look at splice-specific alterations within the D2 receptor and their connection to alcohol self-administration. Using the Operant Assay for Behavioral Reinforcement Response (OPEN BARR; Figure 4.4A & 4.4B), we investigated how Dop2R splicing affected operant self-administration for alcohol. In this assay, flies obtain volatilized ethanol when in a demarcated region of self-administration (ROSA, Hernandez et al., unpublished data). Otherwise, flies can spend their time in a non-marked portion of the arena and receive humidified air (Region of Non-self-Administration, RONSA).

Within a typical wild-type population, some flies engage at less frequently in administering ethanol (low responders) and some escalate their alcohol preference over three days (high responders (Hernandez et al., unpublished data)). We hypothesized that if Dop2R-RI is connected to increased approach to alcohol, then Dop2R-RI

expressing flies should exhibit the highest occupation within ROSA compared to control (only on Day 1) and Dop2R-RH flies.

As observed within wildtype populations, control flies showed a variety of response profiles (Figure 4.4C). By Day 3, the control fly population exhibited various self-administration patterns (Figure 4.4C). These flies could be segregated into quartiles based on their self-administration on Day 3. Of particular interest are the high engagers (Quartile 1; Figure 4.4C). Distribution of responses and the quartile breakdown for Dop2R-RH and Dop2R-RI flies can be found in Supplemental Figure 4.S8.

When comparing ROSA occupation on the first day between genotypes (Figure 4.4Di), we observed that control flies self-administered vaporized ethanol more compared to both Dop2R-RH and Dop2R-RI flies (Table 4.S5). There were no differences observed between the splice mutants (Figure 4.4D; See Supplemental Table 4.S5). The increased time spent within ROSA for the control flies appears to be concentrated within the first 5 minutes of the 15-minute recording period (Figure 4G). The average bout duration within ROSA on the first day were also similar between groups, with RH and RI flies spending a non-significantly less amount of time within ROSA (Figure 4.4Ei, Supplemental Table 4.S5).

By Day 3, Control flies exhibited greater occupation of ROSA (Figure 4.4Di) paired with longer bout durations compared to Dop2R-RH flies, but not Dop2R-RI flies (Figure 4.4E; Supplemental Table 4.S5).

The distance moved within the ROSA could also be an important factor in determining motivation, as moderate doses of ethanol have been shown to increase fly locomotion

(Scaplen et al., 2019). We hypothesized that Dop2R-RI flies should also have a higher amount of distance traversed within the ROSA compared to control (only on Day 1) and Dop2R-RH flies. However, we did not observe any differences between the genotypes (Figure 4.4Fi and 4.4Fii).

By the third day, Control flies were only self-administering more than Dop2R-RH flies (Figure 4.4Dii, Table 4.S5). The mutant flies self-administered at similar levels (Figure 4.4Dii; Table 4.S5). Unexpectedly, we found that control flies had higher rates of self-administration compared to Dop2R-RH and Dop2R-RI. We were not able to observe differences in ROSA occupation between Dop2R-RH and Dop2R-RI flies.

4.4.4 – Highest engaging Dop2R-RI increase their occupation of ROSA in Open BARR

At the population level, Dop2R-RH and Dop2R-RI behave similarly in response to intoxicating doses of ethanol vapor. Due to the subtlety of the mutation however, there could be potentially interesting interactions occurring within high and low responders exclusively. We hypothesized that high-engaging Dop2R-RI flies would have exhibit longer durations of ROSA occupation compared to control and Dop2R-RH flies. With this approach, we found that only the highest engaging (Figure 4.S8; Quartile 1) Dop2-RI flies increased their total time in ROSA over the three days of self-administration (Figure 4.S11A). On day 3, both Dop2R-RI and control had longer bout durations compared to Dop2R-RH (Figure 4.S11; Table 4.S6), but Dop2R-RH and Dop2R-RI flies were not significantly different from each other. Movement in ROSA remained similar for all three days when comparing all three strains (Figure 4.S11; Table 4.S6).

Taken all together we see that alternative splicing has a variety of effects on alcohol engagement strategies in the OPEN BARR assay. High-engaging Dop2R-RI flies displayed increases in total time spent in ROSA. This is coupled by increased bout durations compared to Dop2R-RH on the final day of self-administration. This is in contrast with high-engaging Dop2R-RH that did not alter their behavior within the operant assay.

4.5. DISCUSSION

Understanding the functional consequences of alternative splicing on behavior is a rapidly developing field within neuroscience (Carvalho & Lasek, 2024; Nik & Bowman, 2019; Petruccelli et al., 2020; Xu et al., 2021). We demonstrate that alcohol-induced alternative splicing of *Drosophila* Dop2R influences reward engagement. We observed that Dop2R-RI flies, which have an isoform lacking Ser658 in the 3rd intracellular loop, increase consumption of both natural and alcohol rewards.

While previous studies have shown that splicing of D2R is connected to alcohol consumption (Van Booven et al., 2021), our findings provide direct evidence to suggest that this splicing is causative in increased engagement with alcohol across multiple contexts. Additionally, we observed that the shift from Dop2R-RH to Dop2R-RI may also be driving increased approach to general reward, since Dop2R-RI flies had increased approach and consumption of both sucrose and alcohol. The increased sucrose engagement with Dop2R-RI flies may also be connected to a Dop2R-mediated mechanism that enables increased sucrose sensitivity following sucrose deprivation (Ganguly et al., 2021).

These phenotypes did not manifest as difference in overall preference in choice assays following Pavlovian conditioning for alcohol. This contradicted our hypothesis that Dop2R-RI flies would have higher levels of approach to alcohol-associated odor cues when compared to Dop2R-RH. It is possible that the presence of a natural reward is necessary to resolve the impacts of Dop2R splicing on behavior. However, since a previous study demonstrated that knockdown of Dop2R did not alter the rewarding properties of sucrose as determined by olfactory-sucrose conditioning (Sun et al., 2020,), it could be that Dop2R splicing plays a role in consumption behavior as opposed to sensing the rewarding properties of reward. However, the Sun et al., 2020 study implemented an assay that is not capable of extracting multiple behavioral metrics to better characterize preference.

This contrasts with apparatus like FLIC, Tandem two-choice assay, and OPEN BARR that allow for real time monitoring of reward-motivated behavior in addition to providing the rewarding stimuli. These results highlight the importance of implementing behavioral assessments that can extract multidimensional behavioral data. Using higher resolution data, we were able to pull out differences in how Dop2R splice mutants were engaging with sucrose-associated cues (Figure 4.3), despite similarities in overall preference. The observed difference in overall movement between Dop2R-RI and Dop2R-RH could be indicative of different strategies used to engage with reward and reward-associated cues. Dop2R-RH constantly transitioned between the unpaired and paired odor, whereas Dop2R-RI remained more stationary when expressing memory for a sucrose cue.

Utilizing T-STEP, we were able to expand the number of known circuits that express Dop2R (Figure 4.S2) and provide a foundation for Dop2R is shaping other behaviors. Dop2R's expression within the fan-shaped body (Figure 4.S2D) could be influencing behaviors like navigation (Matheson et al., 2022), and nociception (Hu et al., 2018). And the expression within the optic lobe may be important in visual perception, possibly aiding circuit formation during development (Tian et al., 2015). Dop2R and its splicing events may be fundamental to shaping circuit properties across a wide range of brain structures and possibly influencing behavior through multiple neural substrates.

Future studies will also have the capacity to look at how the presence of Dop2R splice isoforms is directly changing neural dynamics using functional imaging. GCaMP sensors under the control of gal4 drivers could be expressed within the background of the mutants to observe how the different isoforms of Dop2R are changing the activity profiles of reward circuitry like the mushroom body and protocerebral anterior medial dopamine neurons. It is likely that the RI isoform which more closely resembles D2R-short, is not able to signal as effectively as the Dop2R-RH due to the lack of the phosphorylation events at the 658th amino-acid residue. This could result in increased activity within neural circuits that express Dop2R-RI.

In summary, our investigation into the alcohol-induced alternative splicing of Dop2R provides insight into how different isoforms of Dop2R might be shaping reward response. Our approach will serve as a model for future studies investigating the role of splicing in connection to neuropsychiatric disease.

4.6 MATERIALS AND METHODS

4.6.1 List of Reagents

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Genetic reagent (<i>D. melanogaster</i>)	<i>Dop2R-Control</i>	This Paper	N/A	Chromosome I
Genetic reagent (<i>D. melanogaster</i>)	<i>Dop2R-RH</i>	This Paper	N/A	Chromosome I
Genetic reagent (<i>D. melanogaster</i>)	<i>Dop2R-RI</i>	This Paper	N/A	Chromosome I
Genetic reagent (<i>D. melanogaster</i>)	<i>Dop2R-S658D</i>	This Paper	N/A	Chromosome I
Genetic reagent (<i>D. melanogaster</i>)	<i>Dop2R-S658A</i>	This Paper	N/A	Chromosome I

Genetic reagent (<i>D. melanogaster</i>)	Dop2R T-STEP	This Paper	N/A	Chromosome I
Genetic reagent (<i>D. melanogaster</i>)	nSyb-Gal4	Courtesy of Kate O'Connor-Giles	BDSC#51635	Chromosome III
Chemical	Iso-amyl alcohol	Fisher Scientific	Cat#A393	(1:36)
Chemical	Ethyl-acetate	Fisher Science	Cat#S25305	(1:36)
Chemical	Mineral oil	Fisher Science	Cat#BP2629	N/A
Chemical	Triton-X	Sigma-Aldrich	Cat#X-100	0.3%
Chemical	10x PBS Buffer	Thermo-Fisher	Cat#AM9624	N/A
Chemical	Normal Goat Serum	Southern Biotech	Cat#0060-01	(1:20)

Chemical	Citifluor Antifadent Mountant Solution	Agar Scientific	Cat#AGR132 0	~20uL
Chemical	16% PFA	Electron Microcopy	Cat#15710	1 mM
Chemical	Poly-L-Lysine Hydrobromide	Sigma Alrich	Cat#P1524- 25MG	N/A
Antibody	α -GFP (Rabbit polyclonal)	Thermo- Fisher	Cat#G10362	(1:200)
Antibody	DSHB	Life Tech	nc82	(1:20)

4.6.2 - Generation of *Dop2R*-Splice Mutants

Dop2R-splice mutants were generated using CRISPR-Cas9. Splice acceptor sites preceding and within exon 8 of the *Dop2R* gene were modified to generate the *Dop2R*-RH and *Dop2R*-RI mutants (Figure 4.1). Phosphomimetic and phosphoinactive mutants were generated by modifying the RH splice acceptor site and modifying the codon encoding the the 658th reisdue to generate either a aspartic acid or alanine, respectively. w¹¹¹⁸ Embryos were injected with custom plasmids and guide RNAs using RainbowGene Inc. Plasmids that contained DsRed under the expression of the *eyeless* promoter were inserted into the target locus to allow tracking of flies positive for uptake

of the plasmid. DsRed Positive flies were then backcrossed into w^{1118} flies to generate stable lines. DsRed was then removed using flies containing PBaC Transposase (Crossing Schematic in Figure 4.S21). Once DsRed was excised, flies were molecularly characterized using custom primers to confirm presence of the specific mutations. Sequencing was conducted using Eurofins Genomic SimpleSeq kits.

4.6.3 - Generation of T-STEP lines

The T-STEP cassette (Kohl et al., 2015) was inserted into the Dop2R locus of w^{1118} embryos CRISPR Cas9 (RainbowGene Inc.). DsRed under expression of the *eyeless* promoter within the T-STEP cassette allowed for tracking of the insertion throughout crosses. Custom plasmids and guide RNAs were designed to target the c-terminus to capture all isoforms (Figure 4.S2). DsRed-containing flies were used for all crosses to tissue-specific Gal4s.

4.6.4 - Immunohistochemistry

Flies were cold anesthetized on ice. Brains were dissected in chilled 1% PBS. Brains are then placed in 2% PFA or 4% PFA. For experiments with 2% PFA, brains were left overnight at 4C nutating. For experiments with 4% PFA, brains were left on ice for up to 2 hours. Brains were washed in 0.5% Phosphate buffer solution with Triton-X (PBT) for 15 minutes, 4 times (exchanging for new washing buffer after each 15-minute interval). Brains were then placed in blocking solution (PBT with 5% Normal Goat Serum) nutating for 1 hour at room temperature before the addition of antibodies. Brains were left for 2 days in the solution containing primary antibodies before washing again using the protocol described above. Brains were then placed in fresh blocking solution with

the addition of secondary antibodies overnight. Brains were washed and mounted between 1.5mm thick coverslips that were dipped in Poly-L-Lysine to arrange brains in preferred orientations. Citifluor mounting solution is then applied to the edges between the two coverslips to allow capillary action to pull the mounting solution over the brains. The slides are then sealed together using nail polish and left to cure for 24 hours before imaging. Z-stack confocal images were acquired using 20x or 60x oil objectives on either a Nikon Yokogawa CSUW1 Spinning Disk Confocal microscope, or an Olympus FV3000 confocal microscope.

4.6.5 - Fly Husbandry

All *Drosophila melanogaster* lines were raised on standard cornmeal-agar media with tegosept anti-fungal agent and maintained at 24°C in humidity-controlled chambers under 14/10 hr light/dark cycles on standard cornmeal-agar media with tegosept anti-fungal agent.

4.6.6 - FlyGRAM

The FlyGrAM (Fly Group Activity Monitor) was used for real-time monitoring and quantification of *Drosophila* group locomotion. 2-3 day-old male flies were collected using brief CO₂ exposure, 2-3 days before experiment were performed and kept at 24°C at 70% humidity under a 14:10 Light:Dark Cycle. During the day of experiments 10 flies were placed in each of the four wells of the FlyGRAM apparatus. Genotypes were rotated through the four arenas during experimentation to control for any arena-specific effects. This protocol uses the standard 20-minute timeline in which flies receive air for 5 minutes, ethanol vapor for 10 minutes at 70mL flow rate with 40mL flow rate of air,

followed by 5 minutes of just air. Experiments were conducted in the dark at 21-22°C. Data was processed and visualized using a custom python script.

4.6.7. - Odor cue-induced ethanol memory

Flies were collected 2 days prior to experimentation using brief CO₂ exposure. 4-5 day old flies were trained using the Pavlovian paradigm described in Kaun et al., 2011 and Nunez et al., 2018, briefly summarized here: Experiments were performed using 30 flies in perforated conical culture tubes with plastic mesh lids containing 1ml of agar. Flies were exposed to 10 minutes of an unpaired odor, followed by 10 minutes of a paired odor with 66% ethanol. Each training session is spaced by 50 minutes to allow flies to fully metabolize the ethanol. The odors used were isoamyl alcohol (1:36) and isoamyl acetate (1:36), and flies were reciprocally changed to account for any inherent odor preferences. Flies were then provided a yeast food source and allowed to rest in a 24°C incubator at 70% humidity.

4.6.8 - Y-Maze

Memory retrieval was conducted in a vertical Y-Maze, where flies were given the choice between the unpaired and paired odors being streamed (at ~10ml/min) into the maze through 2 separate arms. Testing occurred 20-24 hours following training. Testing was conducted under red light illumination at 23°C-24°C with 70% humidity. Flies were allowed to climb the maze and decide between the two arms. Preference Index was calculated as total number of flies in the paired odor, subtracting the number of flies in the unpaired odor, then dividing by the total number of flies that climbed. Conditioned preference index was calculated by averaging across reciprocally trained groups of flies

4.6.9 - Open-BARR

For the Operant Behavioral Apparatus for Reinforcement Response (OPEN-BARR), Virgin female and virgin male flies kept in food vials containing standard cornmeal-agar food supplemented with the antifungal agent tegosept. Vials were maintained at 24°C with 70% humidity, and kept on a 14:10 light:dark cycle. First-generation flies were collected following a brief CO₂ exposure and grouped in separate vials containing 15 flies each (housing females and males separately) 3 days before experimentation. Flies used in experiments were 3-4 days old at the start of each experiment. Flies were kept on food prior to experiments. Experiments were conducted starting at zeitgeber time 0-2 (when the lights in the incubator turned on). On the experimental day, individual flies were removed from group-housed containers by gentle aspiration and placed in each of the 6 chambers for their first 15-minute self-administration session. The chambers were then placed within the OPEN-BARR apparatus box, which was kept at 24°C with 70% humidity. Before tracking, flies were allowed to acclimate to the arena for two minutes. When flies reach 4 days old, they were placed into individual arenas that have half of the arena marked with yellow tape on the bottom. Air flow was maintained via a 3-way solenoid valve (ASCO valves, St. Louis, MO) at 200 standard cubic centimeters per minute (sscm) and measured using a digital flow monitor (Alicat Scientific, Tucson, AZ) before experimentation. Once the flies entered the region demarcated by yellow tape, also called the Region of self-administration (ROSA), they receive vaporized ethanol (75%) controlled by a solenoid system. Ethanol self-administration of individual flies was recorded for 15 minutes. Flies are then individually housed, and their ethanol self-administration is recorded for 2 more days. Once flies finished their 15-minute self-

administration session they were individually placed in food vials and maintained at 24°C with 70% humidity. Overall time spent self-administering, locomotion, and other metrics are then characterized using custom scripts in MATLAB as well as Python.

4.6.10 Fly Liquid-Food Interaction Counter

For two-choice FLIC assays, male flies were collected after brief CO₂ exposure. Flies were kept in food vials containing standard cornmeal-agar food supplemented with the antifungal agent tegosept. 6, male flies aged 3-5 days were placed in 6 individual chambers within a *Drosophila* feeding monitors (Ro et al., 2014) able to detect engagement with liquid food sources. Flies had access to either a well containing 5% sucrose liquid food, or a well containing 15% ethanol + 5% sucrose liquid food. Flies must stand on a conductive metal surface and extend their proboscis to access food. Upon contact, flies complete a voltage divider circuit, resulting in voltage changes measured by an external microcontroller board that then relays these signals to a data-processing Master Control Unit before storing data in FLIC software. Flies' engagement with the liquid food was measured over 5 consecutive days at room temperature (20-21°C) at 50%-60% humidity. Flies that did not engage with food for longer than a 24-hour period were excluded from analyses. Behavior was analyzed using publicly available R (Ro et al., 2014) scripts.

4.6.11 - Statistical Analyses

Statistical analyses were performed on GraphPad Prism 10 Version 10.4.0 (GraphPad Software) or Python. Y-Maze data was analyzed using nonparametric Kruskal-Wallis

statistical testing. Open BARR data was analyzed using the nonparametric Kruskal–Wallis test followed by Dunn's multiple-comparisons test for within-day genotype comparisons, and Two-way ANOVAs with post-hoc Tukey to look at Open Barr behavioral metrics over time. FLIC data was analyzed using Kruskal-Wallis tests followed by Dunn's multiple comparison for within-day genotype comparisons. The significance level of statistical tests was set to 0.05. For detailed statistics see Supplemental Tables. Asterisks indicate statistically significant differences, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

4.7 CHAPTER 4 REFERENCES

- Al-Shaer, A. E., Flentke, G. R., Berres, M. E., Garic, A., & Smith, S. M. (2019). Exon level machine learning analyses elucidate novel candidate miRNA targets in an avian model of fetal alcohol spectrum disorder. *PLoS Computational Biology*, 15(4), e1006937. <https://doi.org/10.1371/journal.pcbi.1006937>
- Anzalone, A., Lizardi-Ortiz, J. E., Ramos, M., Mei, C. D., Hopf, F. W., Iaccarino, C., Halbout, B., Jacobsen, J., Kinoshita, C., Welter, M., Caron, M. G., Bonci, A., Sulzer, D., & Borrelli, E. (2012). Dual Control of Dopamine Synthesis and Release by Presynaptic and Postsynaptic Dopamine D2 Receptors. *Journal of Neuroscience*, 32(26), 9023–9034. <https://doi.org/10.1523/JNEUROSCI.0918-12.2012>
- Baum-Baicker, C. (1985). The psychological benefits of moderate alcohol consumption: A review of the literature. *Drug and Alcohol Dependence*, 15(4), 305–322. [https://doi.org/10.1016/0376-8716\(85\)90008-0](https://doi.org/10.1016/0376-8716(85)90008-0)
- Bertolino, A., Fazio, L., Caforio, G., Blasi, G., Rampino, A., Romano, R., Di Giorgio, A., Taurisano, P., Papp, A., Pinsonneault, J., Wang, D., Nardini, M., Popolizio, T., & Sadee, W. (2009). Functional variants of the dopamine receptor D2 gene modulate prefronto-striatal phenotypes in schizophrenia. *Brain: A Journal of Neurology*, 132(Pt 2), 417–425. <https://doi.org/10.1093/brain/awn248>
- Bulwa, Z. B., Sharlin, J. A., Clark, P. J., Bhattacharya, T. K., Kilby, C. N., Wang, Y., & Rhodes, J. S. (2011). Increased consumption of ethanol and sugar water in mice lacking the dopamine D2 long receptor. *Alcohol (Fayetteville, N.Y.)*, 45(7), 631–639. <https://doi.org/10.1016/j.alcohol.2011.06.004>

- Carvalho, L., & Lasek, A. W. (2024). It is not just about transcription: Involvement of brain RNA splicing in substance use disorders. *Journal of Neural Transmission*, 131(5), 495–503. <https://doi.org/10.1007/s00702-024-02740-y>
- Celik, M., Gold, M. S., & Fuehrlein, B. (2024). A Narrative Review of Current and Emerging Trends in the Treatment of Alcohol Use Disorder. *Brain Sciences*, 14(3), 294. <https://doi.org/10.3390/brainsci14030294>
- Creese, I., Burt, D. R., & Snyder, S. H. (1976). Dopamine Receptor Binding Predicts Clinical and Pharmacological Potencies of Antischizophrenic Drugs. *Science*, 192(4238), 481–483. <https://doi.org/10.1126/science.3854>
- Cummins-Beebee, P. N., Chvilicek, M. M., & Rothenfluh, A. (2023). The Stage-Based Model of Addiction-Using *Drosophila* to Investigate Alcohol and Psychostimulant Responses. *International Journal of Molecular Sciences*, 24(13), 10909. <https://doi.org/10.3390/ijms241310909>
- Dal Toso, R., Sommer, B., Ewert, M., Herb, A., Pritchett, D. B., Bach, A., Shivers, B. D., & Seeburg, P. H. (1989). The dopamine D2 receptor: Two molecular forms generated by alternative splicing. *The EMBO Journal*, 8(13), 4025–4034. <https://doi.org/10.1002/j.1460-2075.1989.tb08585.x>
- Devineni, A. V., & Heberlein, U. (2013). The evolution of *Drosophila melanogaster* as a model for alcohol research. *Annual Review of Neuroscience*, 36, 121–138. <https://doi.org/10.1146/annurev-neuro-062012-170256>
- Donadoni, M., Cicalese, S., Sarkar, D. K., Chang, S. L., & Sariyer, I. K. (2019). Alcohol exposure alters pre-mRNA splicing of antiapoptotic Mcl-1L isoform and induces

- apoptosis in neural progenitors and immature neurons. *Cell Death & Disease*, 10(6), 1–16. <https://doi.org/10.1038/s41419-019-1673-3>
- Downing, C., Flink, S., Florez-McClure, M. L., Johnson, T. E., Tabakoff, B., & Kechris, K. J. (2012). Gene expression changes in C57BL/6J and DBA/2J mice following prenatal alcohol exposure. *Alcoholism, Clinical and Experimental Research*, 36(9), 1519–1529. <https://doi.org/10.1111/j.1530-0277.2012.01757.x>
- Edenberg, H. J., Dick, D. M., Xuei, X., Tian, H., Almasy, L., Bauer, L. O., Crowe, R. R., Goate, A., Hesselbrock, V., Jones, K., Kwon, J., Li, T.-K., Nurnberger, J. I., O'Connor, S. J., Reich, T., Rice, J., Schuckit, M. A., Porjesz, B., Foroud, T., & Begleiter, H. (2004). Variations in GABRA2, encoding the alpha 2 subunit of the GABA(A) receptor, are associated with alcohol dependence and with brain oscillations. *American Journal of Human Genetics*, 74(4), 705–714. <https://doi.org/10.1086/383283>
- Eisenstein, S. A., Bogdan, R., Love-Gregory, L., Corral-Frías, N. S., Koller, J. M., Black, K. J., Moerlein, S. M., Perlmutter, J. S., Barch, D. M., & Hershey, T. (2016). Prediction of striatal D2 receptor binding by DRD2/ANKK1 TaqIA allele status. *Synapse*, 70(10), 418–431. <https://doi.org/10.1002/syn.21916>
- Evans-Polce, R. J., Maggs, J. L., Lanza, S. T., & Patrick, M. E. (2024). Negative consequences associated with daily alcohol use as a nonlinear function of number of drinks in a daily diary study. *Drug and Alcohol Dependence*, 256, 111089. <https://doi.org/10.1016/j.drugalcdep.2024.111089>

- Farris, S. P., Arasappan, D., Hunicke-Smith, S., Harris, R. A., & Mayfield, R. D. (2015). Transcriptome organization for chronic alcohol abuse in human brain. *Molecular Psychiatry*, 20(11), 1438–1447. <https://doi.org/10.1038/mp.2014.159>
- Fernández-Dueñas, V., Gómez-Soler, M., Jacobson, K. A., Kumar, S. T., Fuxe, K., Borroto-Escuela, D. O., & Ciruela, F. (2012). Molecular determinants of A2AR-D2R allosterism: Role of the intracellular loop 3 of the D2R. *Journal of Neurochemistry*, 123(3), 373–384. <https://doi.org/10.1111/j.1471-4159.2012.07956.x>
- Fuller, R. K., Branchey, L., Brightwell, D. R., Derman, R. M., Emrick, C. D., Iber, F. L., James, K. E., Lacoursiere, R. B., Lee, K. K., & Lowenstam, I. (1986). Disulfiram treatment of alcoholism. A Veterans Administration cooperative study. *JAMA*, 256(11), 1449–1455.
- Ganguly, A., Dey, M., Scott, C., Duong, V.-K., & Dahanukar, A. A. (2021). Dietary Macronutrient Imbalances Lead to Compensatory Changes in Peripheral Taste via Independent Signaling Pathways. *Journal of Neuroscience*, 41(50), 10222–10246. <https://doi.org/10.1523/JNEUROSCI.2154-20.2021>
- Gilbert, W. (1978). Why genes in pieces? *Nature*, 271(5645), 501–501. <https://doi.org/10.1038/271501a0>
- Gilman, J. M., Ramchandani, V. A., Davis, M. B., Bjork, J. M., & Hommer, D. W. (2008). Why we like to drink: A functional magnetic resonance imaging study of the rewarding and anxiolytic effects of alcohol. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 28(18), 4583–4591. <https://doi.org/10.1523/JNEUROSCI.0086-08.2008>

- Giros, B., Sokoloff, P., Martres, M. P., Riou, J. F., Emorine, L. J., & Schwartz, J. C. (1989). Alternative splicing directs the expression of two D2 dopamine receptor isoforms. *Nature*, 342(6252), 923–926. <https://doi.org/10.1038/342923a0>
- Guiramand, J., Montmayeur, J. P., Ceraline, J., Bhatia, M., & Borrelli, E. (1995). Alternative splicing of the dopamine D2 receptor directs specificity of coupling to G-proteins. *The Journal of Biological Chemistry*, 270(13), 7354–7358. <https://doi.org/10.1074/jbc.270.13.7354>
- Hardy, P. A., Chen, W., & Wilce, P. A. (1999). Chronic ethanol exposure and withdrawal influence NMDA receptor subunit and splice variant mRNA expression in the rat cerebral cortex. *Brain Research*, 819(1–2), 33–39. [https://doi.org/10.1016/s0006-8993\(98\)01340-7](https://doi.org/10.1016/s0006-8993(98)01340-7)
- Hearn, M. G., Ren, Y., McBride, E. W., Reveillaud, I., Beinborn, M., & Kopin, A. S. (2002). A Drosophila dopamine 2-like receptor: Molecular characterization and identification of multiple alternatively spliced variants. *Proceedings of the National Academy of Sciences of the United States of America*, 99(22), 14554–14559. <https://doi.org/10.1073/pnas.202498299>
- Henry, G. L., Davis, F. P., Picard, S., & Eddy, S. R. (2012). Cell type-specific genomics of Drosophila neurons. *Nucleic Acids Research*, 40(19), 9691–9704. <https://doi.org/10.1093/nar/gks671>
- Hernandez-Lopez, S., Tkatch, T., Perez-Garci, E., Galarraga, E., Bargas, J., Hamm, H., & Surmeier, D. J. (2000). D2 dopamine receptors in striatal medium spiny neurons reduce L-type Ca²⁺ currents and excitability via a novel PLC[β]1-IP3-calcineurin-signaling cascade. *The Journal of Neuroscience: The Official Journal*

of the Society for Neuroscience, 20(24), 8987–8995.

<https://doi.org/10.1523/JNEUROSCI.20-24-08987.2000>

Hu, W., Peng, Y., Sun, J., Zhang, F., Zhang, X., Wang, L., Li, Q., & Zhong, Y. (2018).

Fan-Shaped Body Neurons in the *Drosophila* Brain Regulate Both Innate and Conditioned Nociceptive Avoidance. *Cell Reports*, 24(6), 1573–1584.

<https://doi.org/10.1016/j.celrep.2018.07.028>

Huggett, S. B., Ikeda, A. S., Yuan, Q., Benca-Bachman, C. E., & Palmer, R. H. C.

(2023). Genome- and transcriptome-wide splicing associations with alcohol use disorder. *Scientific Reports*, 13(1), 3950. <https://doi.org/10.1038/s41598-023-30926-z>

Iancu, O. D., Colville, A., Walter, N. A. R., Darakjian, P., Oberbeck, D. L., Daunais, J. B.,

Zheng, C. L., Searles, R. P., McWeeney, S. K., Grant, K. A., & Hitzemann, R.

(2018). On the relationships in rhesus macaques between chronic ethanol consumption and the brain transcriptome. *Addiction Biology*, 23(1), 196–205.

<https://doi.org/10.1111/adb.12501>

Jiménez-Urbieto, H., Gago, B., de la Riva, P., Delgado-Alvarado, M., Marin, C., &

Rodríguez-Oroz, M. C. (2015). Dyskinesias and impulse control disorders in Parkinson's disease: From pathogenesis to potential therapeutic approaches.

Neuroscience & Biobehavioral Reviews, 56, 294–314.

<https://doi.org/10.1016/j.neubiorev.2015.07.010>

Jumper, J., Evans, R., Pritzel, A., Green, T., Figurnov, M., Ronneberger, O.,

Tunyasuvunakool, K., Bates, R., Žídek, A., Potapenko, A., Bridgland, A., Meyer,

C., Kohl, S. A. A., Ballard, A. J., Cowie, A., Romera-Paredes, B., Nikolov, S., Jain,

- R., Adler, J., ... Hassabis, D. (2021). Highly accurate protein structure prediction with AlphaFold. *Nature*, 596(7873), 583–589. <https://doi.org/10.1038/s41586-021-03819-2>
- Kaun, K. R., Azanchi, R., Maung, Z., Hirsh, J., & Heberlein, U. (2011). A *Drosophila* model for alcohol reward. *Nature Neuroscience*, 14(5), 612–619. <https://doi.org/10.1038/nn.2805>
- Khan, Z. U., Mrzljak, L., Gutierrez, A., de la Calle, A., & Goldman-Rakic, P. S. (1998). Prominence of the dopamine D2 short isoform in dopaminergic pathways. *Proceedings of the National Academy of Sciences of the United States of America*, 95(13), 7731–7736. <https://doi.org/10.1073/pnas.95.13.7731>
- Konishi, T., Calvillo, M., Leng, A.-S., Lin, K.-M., & Wan, Y.-J. Y. (2004). Polymorphisms of the dopamine D2 receptor, serotonin transporter, and GABA(A) receptor beta(3) subunit genes and alcoholism in Mexican-Americans. *Alcohol (Fayetteville, N.Y.)*, 32(1), 45–52. <https://doi.org/10.1016/j.alcohol.2003.11.002>
- Koob, G. F., & Volkow, N. D. (2016). Neurobiology of addiction: A neurocircuitry analysis. *The Lancet. Psychiatry*, 3(8), 760–773. [https://doi.org/10.1016/S2215-0366\(16\)00104-8](https://doi.org/10.1016/S2215-0366(16)00104-8)
- Lawford, B. R., Young, R. M., Rowell, J. A., Qualichefski, J., Fletcher, B. H., Syndulko, K., Ritchie, T., & Noble, E. P. (1995). Bromocriptine in the treatment of alcoholics with the D2 dopamine receptor A1 allele. *Nature Medicine*, 1(4), 337–341. <https://doi.org/10.1038/nm0495-337>

- Lee, C., Mayfield, R. D., & Harris, R. A. (2014). Altered Gamma-Aminobutyric Acid Type B Receptor Subunit 1 Splicing In Alcoholics. *Biological Psychiatry*, 75(10), 765–773. <https://doi.org/10.1016/j.biopsych.2013.08.028>
- Li, R., Reiter, J. L., Chen, A. B., Chen, S. X., Foroud, T., Edenberg, H. J., Lai, D., & Liu, Y. (2023). RNA alternative splicing impacts the risk for alcohol use disorder. *Molecular Psychiatry*, 28(7), 2922–2933. <https://doi.org/10.1038/s41380-023-02111-1>
- Lindgren, N., Usiello, A., Goiny, M., Haycock, J., Erbs, E., Greengard, P., Hökfelt, T., Borrelli, E., & Fisone, G. (2003). Distinct roles of dopamine D2L and D2S receptor isoforms in the regulation of protein phosphorylation at presynaptic and postsynaptic sites. *Proceedings of the National Academy of Sciences*, 100(7), 4305–4309. <https://doi.org/10.1073/pnas.0730708100>
- Liu, Q., Fang, L., & Wu, C. (2022). Alternative Splicing and Isoforms: From Mechanisms to Diseases. *Genes*, 13(3), 401. <https://doi.org/10.3390/genes13030401>
- Lyons, P. G., Snyder, A., Sokol, S., Edelson, D. P., Mokhlesi, B., & Churpek, M. M. (2017). Association Between Opioid and Benzodiazepine Use and Clinical Deterioration in Ward Patients. *Journal of Hospital Medicine*, 12(6), 428–434. <https://doi.org/10.12788/jhm.2749>
- Mann, A., Keen, A. C., Mark, H., Dasgupta, P., Javitch, J. A., Canals, M., Schulz, S., & Robert Lane, J. (2021). New phosphosite-specific antibodies to unravel the role of GRK phosphorylation in dopamine D2 receptor regulation and signaling. *Scientific Reports*, 11(1), 8288. <https://doi.org/10.1038/s41598-021-87417-2>

- Marasco, L. E., & Kornblihtt, A. R. (2023). The physiology of alternative splicing. *Nature Reviews Molecular Cell Biology*, 24(4), 242–254. <https://doi.org/10.1038/s41580-022-00545-z>
- Matheson, A. M. M., Lanz, A. J., Medina, A. M., Licata, A. M., Currier, T. A., Syed, M. H., & Nagel, K. I. (2022). A neural circuit for wind-guided olfactory navigation. *Nature Communications*, 13(1), 4613. <https://doi.org/10.1038/s41467-022-32247-7>
- Mattle, A. G., McGrath, P., Sanu, A., Kunadharaju, R., Kersten, B., Zammit, K., & Mammen, M. J. (2022). Gabapentin to treat acute alcohol withdrawal in hospitalized patients: A systematic review and meta-analysis. *Drug and Alcohol Dependence*, 241, 109671. <https://doi.org/10.1016/j.drugalcdep.2022.109671>
- Monsma, F. J., McVittie, L. D., Gerfen, C. R., Mahan, L. C., & Sibley, D. R. (1989). Multiple D2 dopamine receptors produced by alternative RNA splicing. *Nature*, 342(6252), 926–929. <https://doi.org/10.1038/342926a0>
- Nik, S., & Bowman, T. V. (2019). Splicing and neurodegeneration: Insights and mechanisms. *WIREs RNA*, 10(4), e1532. <https://doi.org/10.1002/wrna.1532>
- Nunez, K. M., Azanchi, R., & Kaun, K. R. (2018). Cue-Induced Ethanol Seeking in *Drosophila melanogaster* Is Dose-Dependent. *Frontiers in Physiology*, 9, 438. <https://doi.org/10.3389/fphys.2018.00438>
- Oomizu, S., Boyadjieva, N., & Sarkar, D. K. (2003). Ethanol and estradiol modulate alternative splicing of dopamine D2 receptor messenger RNA and abolish the inhibitory action of bromocriptine on prolactin release from the pituitary gland. *Alcoholism, Clinical and Experimental Research*, 27(6), 975–980. <https://doi.org/10.1097/01.ALC.0000071743.57855.BE>

- Pan, Q., Shai, O., Lee, L. J., Frey, B. J., & Blencowe, B. J. (2008). Deep surveying of alternative splicing complexity in the human transcriptome by high-throughput sequencing. *Nature Genetics*, 40(12), 1413–1415. <https://doi.org/10.1038/ng.259>
- Pankova, K., & Borst, A. (2016). RNA-Seq Transcriptome Analysis of Direction-Selective T4/T5 Neurons in *Drosophila*. *PloS One*, 11(9), e0163986. <https://doi.org/10.1371/journal.pone.0163986>
- Patrick, M. E., & Terry-McElrath, Y. M. (2021). Drinking Motives and Drinking Consequences across Days: Differences and Similarities between Moderate, Binge, and High-Intensity Drinking. *Alcoholism: Clinical and Experimental Research*, 45(5), 1078–1090. <https://doi.org/10.1111/acer.14591>
- Petrie, J., Sapp, D. W., Tyndale, R. F., Park, M. K., Fanselow, M., & Olsen, R. W. (2001). Altered GABA_A Receptor Subunit and Splice Variant Expression in Rats Treated With Chronic Intermittent Ethanol. *Alcoholism: Clinical and Experimental Research*, 25(6), 819–828. <https://doi.org/10.1111/j.1530-0277.2001.tb02285.x>
- Petrucelli, E., Brown, T., Waterman, A., Ledru, N., & Kaun, K. R. (2020). Alcohol Causes Lasting Differential Transcription in *Drosophila* Mushroom Body Neurons. *Genetics*, 215(1), 103–116. <https://doi.org/10.1534/genetics.120.303101>
- Petrucelli, E., Feyder, M., Ledru, N., Jaques, Y., Anderson, E., & Kaun, K. R. (2018). Alcohol Activates Scabrous-Notch to Influence Associated Memories. *Neuron*, 100(5), 1209–1223.e4. <https://doi.org/10.1016/j.neuron.2018.10.005>
- Phillips, T. J., Brown, K. J., Burkhart-Kasch, S., Wenger, C. D., Kelly, M. A., Rubinstein, M., Grandy, D. K., & Low, M. J. (1998). Alcohol preference and sensitivity are

- markedly reduced in mice lacking dopamine D2 receptors. *Nature Neuroscience*, 1(7), 610–615. <https://doi.org/10.1038/2843>
- Puig, M. V., Rose, J., Schmidt, R., & Freund, N. (2014). Dopamine modulation of learning and memory in the prefrontal cortex: Insights from studies in primates, rodents, and birds. *Frontiers in Neural Circuits*, 8, 93. <https://doi.org/10.3389/fncir.2014.00093>
- Radl, D., Chiacchiaretta, M., Lewis, R. G., Bami-Cherrier, K., Arcuri, L., & Borrelli, E. (2018). Differential regulation of striatal motor behavior and related cellular responses by dopamine D2L and D2S isoforms. *Proceedings of the National Academy of Sciences*, 115(1), 198–203. <https://doi.org/10.1073/pnas.1717194115>
- Ro, J., Harvanek, Z. M., & Pletcher, S. D. (2014). FLIC: High-throughput, continuous analysis of feeding behaviors in *Drosophila*. *PloS One*, 9(6), e101107. <https://doi.org/10.1371/journal.pone.0101107>
- Sacks, J. J., Gonzales, K. R., Bouchery, E. E., Tomedi, L. E., & Brewer, R. D. (2015). 2010 National and State Costs of Excessive Alcohol Consumption. *American Journal of Preventive Medicine*, 49(5), e73–e79. <https://doi.org/10.1016/j.amepre.2015.05.031>
- SAMSHA. (n.d.). 2023 NSDUH Detailed Tables | CBHSQ Data. Retrieved February 28, 2025, from <https://www.samhsa.gov/data/report/2023-nsduh-detailed-tables>
- Sayette, M. A., Creswell, K. G., Dimoff, J. D., Fairbairn, C. E., Cohn, J. F., Heckman, B. W., Kirchner, T. R., Levine, J. M., & Moreland, R. L. (2012). Alcohol and group formation: A multimodal investigation of the effects of alcohol on emotion and

- social bonding. *Psychological Science*, 23(8), 869–878.
<https://doi.org/10.1177/0956797611435134>
- Scaplen, K. M., Talay, M., Nunez, K. M., Salamon, S., Waterman, A. G., Gang, S., Song, S. L., Barnea, G., & Kaun, K. R. (2020). Circuits that encode and guide alcohol-associated preference. *eLife*, 9, e48730. <https://doi.org/10.7554/eLife.48730>
- Senogles, S. E., Heimert, T. L., Odife, E. R., & Quasney, M. W. (2004). A Region of the Third Intracellular Loop of the Short Form of the D2 Dopamine Receptor Dictates Gi Coupling Specificity*. *Journal of Biological Chemistry*, 279(3), 1601–1606.
<https://doi.org/10.1074/jbc.M309792200>
- Signor, S., & Nuzhdin, S. (2018). Dynamic changes in gene expression and alternative splicing mediate the response to acute alcohol exposure in *Drosophila melanogaster*. *Heredity*, 121(4), 342–360. <https://doi.org/10.1038/s41437-018-0136-4>
- Singh, C. M., & Heberlein, U. (2000). Genetic control of acute ethanol-induced behaviors in *Drosophila*. *Alcoholism, Clinical and Experimental Research*, 24(8), 1127–1136.
- Smith, C. T., Dang, L. C., Buckholtz, J. W., Tetreault, A. M., Cowan, R. L., Kessler, R. M., & Zald, D. H. (2017). The impact of common dopamine D2 receptor gene polymorphisms on D2/3 receptor availability: C957T as a key determinant in putamen and ventral striatum. *Translational Psychiatry*, 7(4), e1091.
<https://doi.org/10.1038/tp.2017.45>
- Sun, H., Nishioka, T., Hiramatsu, S., Kondo, S., Amano, M., Kaibuchi, K., Ichinose, T., & Tanimoto, H. (2020). Dopamine Receptor Dop1R2 Stabilizes Appetitive Olfactory

- Memory through the Raf/MAPK Pathway in *Drosophila*. *The Journal of Neuroscience*, 40(14), 2935–2942. <https://doi.org/10.1523/JNEUROSCI.1572-19.2020>
- Thanos, P. K., Volkow, N. D., Freimuth, P., Umegaki, H., Ikari, H., Roth, G., Ingram, D. K., & Hitzemann, R. (2001). Overexpression of dopamine D2 receptors reduces alcohol self-administration. *Journal of Neurochemistry*, 78(5), 1094–1103. <https://doi.org/10.1046/j.1471-4159.2001.00492.x>
- Tian, N., Xu, H., & Wang, P. (2015). Dopamine D2 receptors preferentially regulate the development of light responses of the inner retina. *The European Journal of Neuroscience*, 41(1), 17–30. <https://doi.org/10.1111/ejn.12783>
- Usiello, A., Baik, J.-H., Rougé-Pont, F., Picetti, R., Dierich, A., LeMeur, M., Piazza, P. V., & Borrelli, E. (2000). Distinct functions of the two isoforms of dopamine D2 receptors. *Nature*, 408(6809), 199–203. <https://doi.org/10.1038/35041572>
- Van Booven, D., Mengying Li, Sunil Rao, J., Blokhin, I. O., Dayne Mayfield, R., Barbier, E., Heilig, M., & Wahlestedt, C. (2021). Alcohol use disorder causes global changes in splicing in the human brain. *Translational Psychiatry*, 11(1), 1–9. <https://doi.org/10.1038/s41398-020-01163-z>
- Varghese, J., & Dakhode, S. (2022). Effects of Alcohol Consumption on Various Systems of the Human Body: A Systematic Review. *Cureus*, 14(10), e30057. <https://doi.org/10.7759/cureus.30057>
- Walle, R., Petitbon, A., Fois, G. R., Varin, C., Montalban, E., Hardt, L., Contini, A., Angelo, M. F., Potier, M., Ortole, R., Oummadi, A., De Smedt-Peyrusse, V., Adan, R. A., Giros, B., Chaouloff, F., Ferreira, G., de Kerchove d'Exaerde, A., Ducrocq,

- F., Georges, F., & Trifilieff, P. (2024). Nucleus accumbens D1- and D2-expressing neurons control the balance between feeding and activity-mediated energy expenditure. *Nature Communications*, 15(1), 2543.
<https://doi.org/10.1038/s41467-024-46874-9>
- Wang, Y., Xu, R., Sasaoka, T., Tonegawa, S., Kung, M.-P., & Sankoorikal, E.-B. (2000). Dopamine D2 Long Receptor-Deficient Mice Display Alterations in Striatum-Dependent Functions. *Journal of Neuroscience*, 20(22), 8305–8314.
<https://doi.org/10.1523/JNEUROSCI.20-22-08305.2000>
- Wolfe, S. A., Farris, S. P., Mayfield, J. E., Heaney, C. F., Erickson, E. K., Harris, R. A., Mayfield, R. D., & Raab-Graham, K. F. (2019). Ethanol and a rapid-acting antidepressant produce overlapping changes in exon expression in the synaptic transcriptome. *Neuropharmacology*, 146, 289–299.
<https://doi.org/10.1016/j.neuropharm.2018.11.007>
- World Health Organization. (2018). *Global status report on alcohol and health 2018*.
World Health Organization. <https://iris.who.int/handle/10665/274603>
- Wright, C. J., Smith, C. W. J., & Jiggins, C. D. (2022). Alternative splicing as a source of phenotypic diversity. *Nature Reviews Genetics*, 23(11), 697–710.
<https://doi.org/10.1038/s41576-022-00514-4>
- Xu, S.-J., Lombroso, S. I., Fischer, D. K., Carpenter, M. D., Marchione, D. M., Hamilton, P. J., Lim, C. J., Neve, R. L., Garcia, B. A., Wimmer, M. E., Pierce, R. C., & Heller, E. A. (2021). Chromatin-mediated alternative splicing regulates cocaine-reward behavior. *Neuron*, 109(18), 2943-2966.e8.
<https://doi.org/10.1016/j.neuron.2021.08.008>

- Zhang, C., Li, Q., Meng, L., & Ren, Y. (2020). Design of novel dopamine D2 and serotonin 5-HT_{2A} receptors dual antagonists toward schizophrenia: An integrated study with QSAR, molecular docking, virtual screening and molecular dynamics simulations. *Journal of Biomolecular Structure & Dynamics*, 38(3), 860–885.
<https://doi.org/10.1080/07391102.2019.1590244>
- Żuk, J., Bartuzi, D., Matosiuk, D., & Kaczor, A. A. (2020). Preferential Coupling of Dopamine D_{2S} and D_{2L} Receptor Isoforms with Gi1 and Gi2 Proteins—In Silico Study. *International Journal of Molecular Sciences*, 21(2), 436.
<https://doi.org/10.3390/ijms21020436>

4.8: CHAPTER 4 FIGURES

Figure 4.1

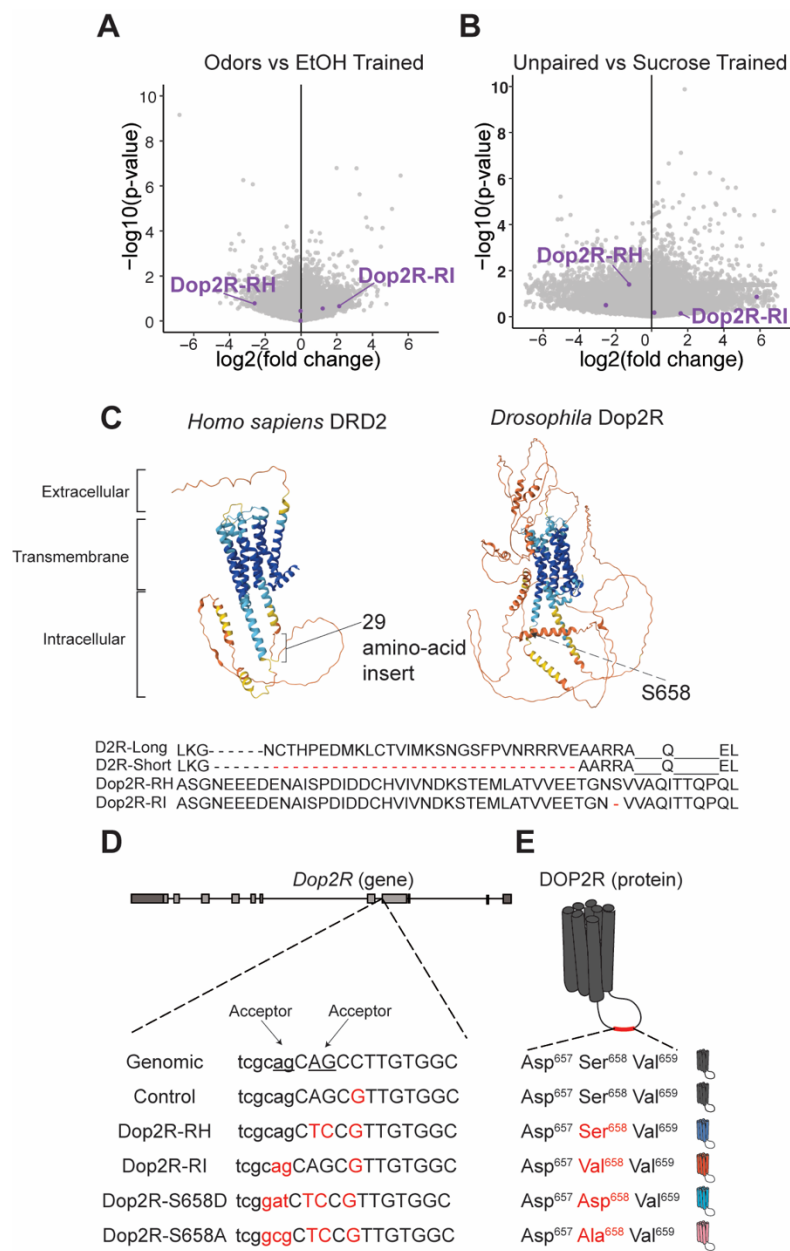


Figure 4.1: Dynamic splicing of *Dop2R* and generation of *Dop2R* splice mutants.

Volcano plots showing RNA-sequencing conducted against *Drosophila* mushroom body (MB) nuclei using the intact approach (Henry et al., 2012; Pankova & Borst, 2016;

Petrucelli et al., 2018, 2020). Volcano plots compare the expression levels of *Dop2R* transcripts following exposure to just odors or just ethanol (A), odors compared to flies repeatedly exposed to odors paired with intoxicating doses of ethanol, (B) flies who had odors paired with sucrose compared to odors unpaired with sucrose. (C) DOP2R protein structure prediction rendered by AlphaFold (Jumper et al., 2021), and comparison of splicing events within mammalian D2 receptor and *Drosophila* Dop2R. Red hyphens indicate regions omitted during alternative splicing. (D) Dop2R gene structure and representation of CRISPR-Cas9 mediated mutations in the Dop2R sequence preceding exon 8 to force isolation of specific isoforms. (E) Cartoon depiction of DOP2R with resultant amino acid sequences following CRISPR modifications.

Figure 4.2

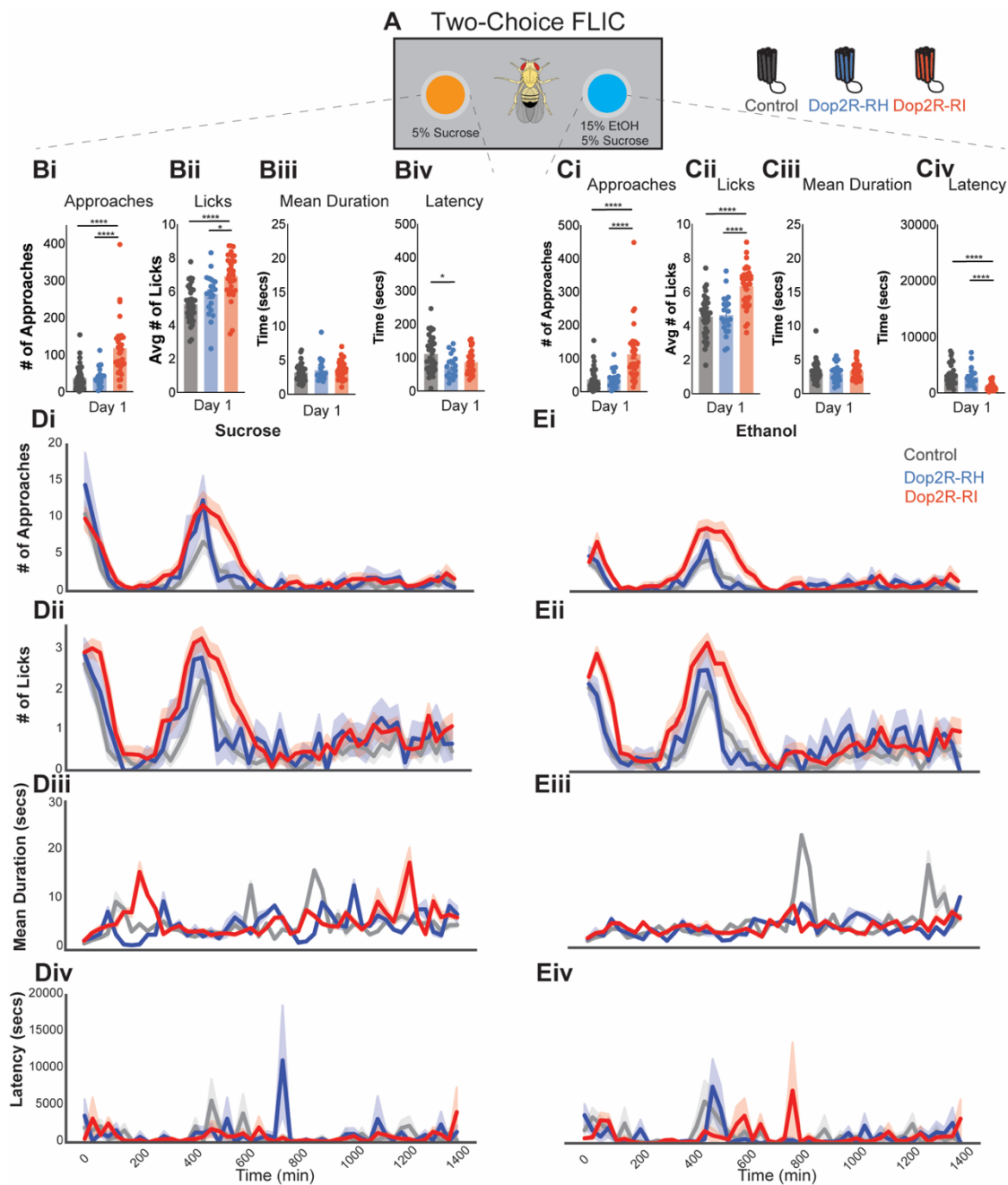


Figure 4.2: Dop2R-Ri increases both natural and drug reward consumption

(A) Cartoon depicting the two-choice Fly liquid-food interaction counter (FLIC) where flies can either consume 5% sucrose or 15% ethanol in 5% sucrose. Barplots

representing the average with swarmplot overlay for approaches (Bi), Licks (Bii), mean duration (Biii), and latency to return to sucrose (Biv). Barplots representing the average with swarmplot overlay for approaches (Ci), Licks (Cii), mean duration (Ciii), and latency to return to ethanol-containing food (Civ). Lineplots representing the average with SEM shaded overlay for approaches (Di), Licks (Dii), mean duration (Diii), and latency to return to sucrose (Div) over a 24 hour period. Lineplots representing the average with shaded SEM overlay for approaches (Di), Licks (Dii), mean duration (Diii), and latency to return to ethanolc(Div) over a 24 hour period. Gray is control (n =40), blue is Dop2R-RH (n=22), and red is Dop2R-RI (n =40). Lineplots representing the average with SEM overlay for approaches (Di), Licks (Dii), mean duration (Diii), and latency to return to sucrose (Div) over a 24 hour period. Stats reported in Supplemental Table 4.S1 and Supplemental Figure 4.S20.

Figure 4.3

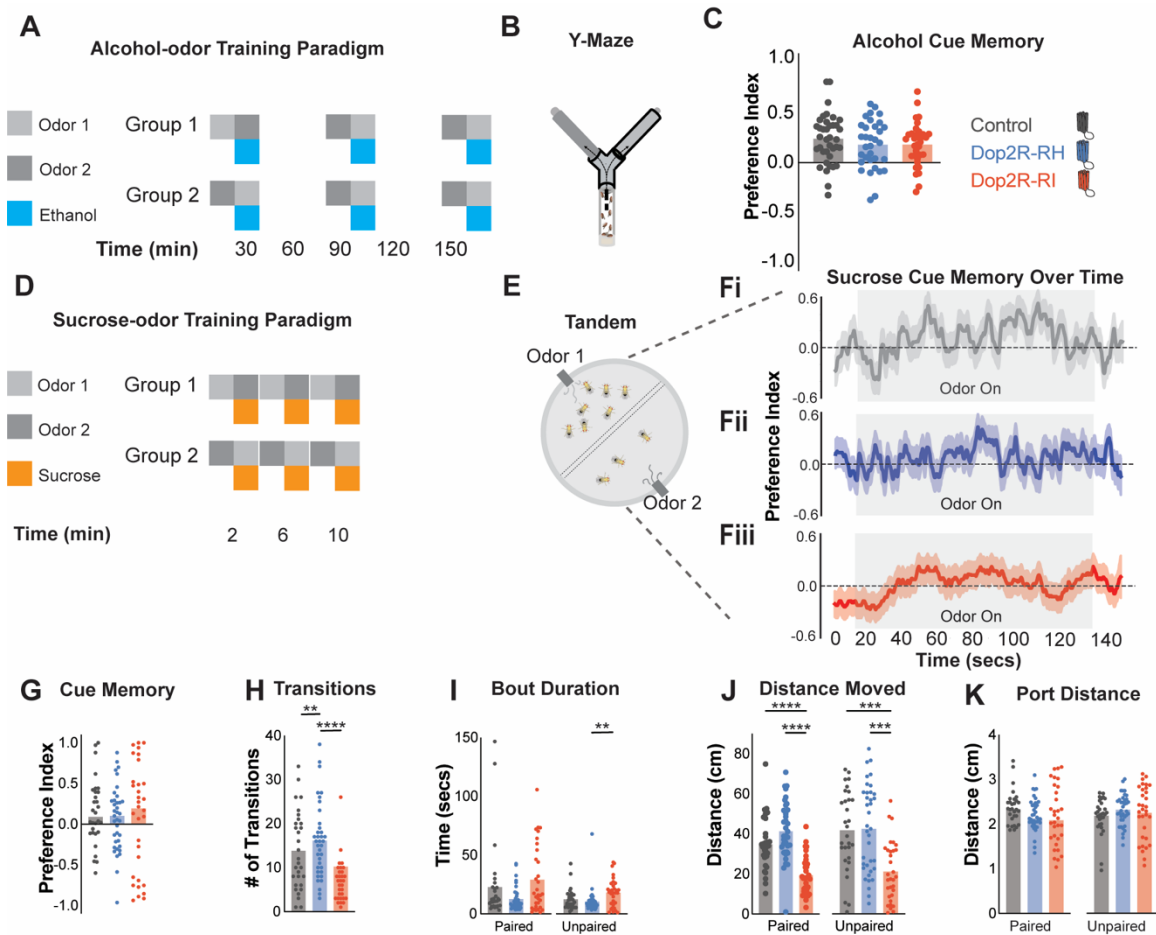


Figure 4.3: Pavlovian conditioning of *Dop2R* mutants for alcohol and sucrose cues.

(A) Paradigm used to condition flies to associate intoxicating doses of ethanol with neutral odors. (B) Depiction of the vertical y-maze. (C) Y-maze preference indices for control (gray, n= 33), *Dop2R-RH* (blue, n =33), and *Dop2R-RI* (red, n =33) flies following alcohol-odor training paradigm. (D) Pavlovian conditioning of *Drosophila* for immediate sucrose memory. (E). Depiction of Tandem two-choice assay where flies can move freely between two regions that are filled with odors. Preference index over time for the 2-minute odor stimulation period for Control (Fi, n = 40), *Dop2R-RH* (Fii, n = 22), and

Dop2R-RI (Fiii, n = 40) flies. (G) Overall sucrose cue memory from the tandem two-choice assay (Supplemental Table 4.S3 for stats). (H) Total number of transitions during the odor stimulation period. (I) Average amount of time spent in the paired (left) and unpaired (right) odor regions. (J) Average distance moved in the paired (left), and unpaired (right) regions. (K) Average distance from the paired (left) and unpaired (right) odor ports. Bar plots represent the mean, and the scatterplot overlay represent individual flies' metric value. Stats reported in Supplemental Table 4.S5.

Figure 4.4

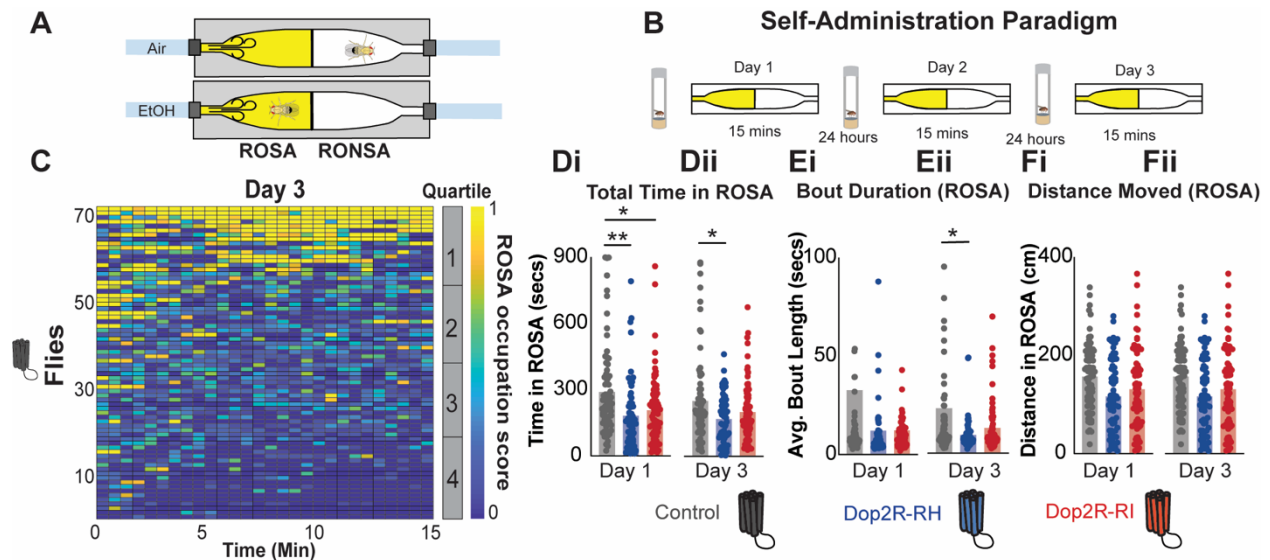


Figure 4.4: Behavioral metrics for control, Dop2R-RH, and Dop2R-RI within Open BARR.

(A) Representation of the Open BARR apparatus. Flies are exposed to air when in the Region of Non-Self Administration (RONSA, white), but can be exposed to 75% ethanol when they enter the Region of Self-Administration (ROSA, yellow). (B) Paradigm used to assess alcohol motivation within flies. Flies are placed within an Open BARR arena and allowed to move freely within the arena for 15 minutes for three days consecutive days. (C) Heatmap of self-administration for control flies on the third day of self-administration. Grey bar to the right of the heatmap labels the quartile breakdown for flies. Quartile 1 are high engaging flies, while quartile 4 are the low engaging flies. Total time within ROSA for each of the three genotypes on Day 1 (Di) and Day 3 (Dii) Average bout duration for Day 1 (Ei) and Day 3 (Eii). Total distance moved within ROSA

on Day 1 (Fi), and Day 3 (Fii). (Control = Gray, n =73, Dop2R-RH = Blue, n = 69, Dop2R-RI = Red, n =71).

4.9: CHAPTER 4 SUPPLEMENTAL FIGURES

Figure 4.S1

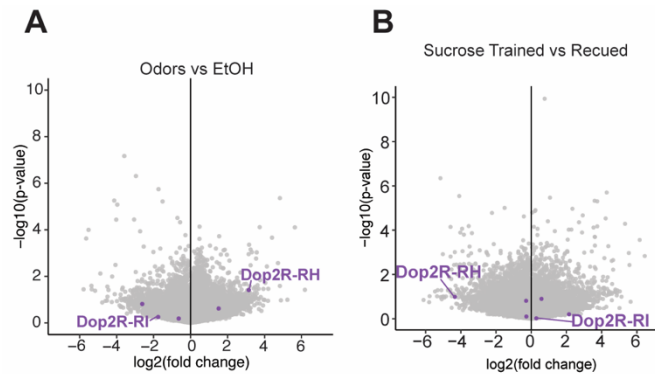


Figure 4.S1: Alternative splicing of *Do2R* following ethanol exposure and sucrose cues.

(A) Volcano plot comparing sequencing data from flies exposed to odors vs ethanol alone. Highlighted in purple are *Dop2R* isoforms, with *Dop2R-RH* and *Dop2R-RI* clearly labeled. Unlike Odors paired with ethanol compared to odors alone (Figure 4.1A), *Dop2R-RH* is upregulated, and *Dop2R-RI* is downregulated. (B) Volcano plot comparing sequencing data from flies that have been sucrose trained compared to flies that have been sucrose-trained and re-exposed to the sucrose-predicting cues. *Dop2R-RH* is downregulated compared to *Dop2R-RI*, which doesn't change much between the two conditions.

Figure 4.S2

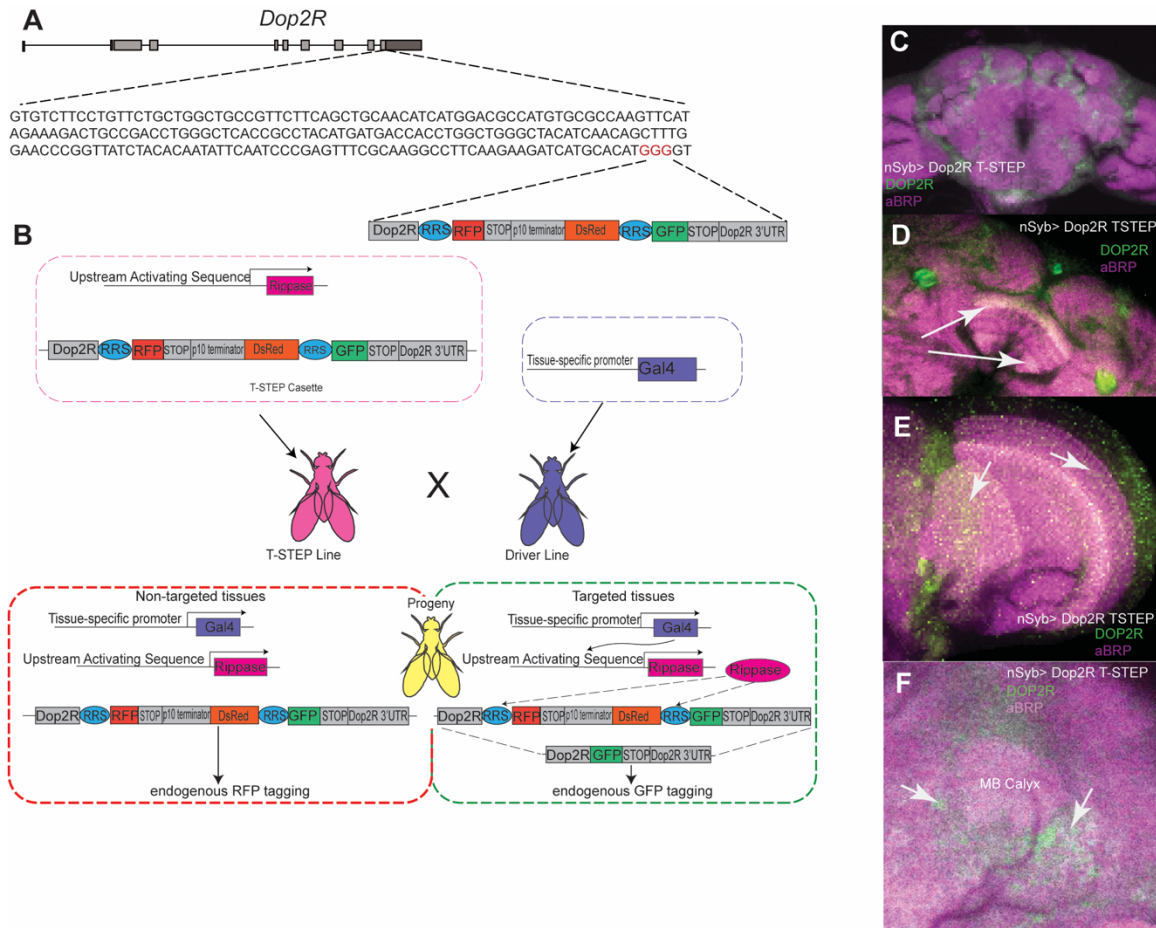


Figure 4.S2: Resolving subcellular localization of DOP2R using an intersectoral genetics approach.

(A) Representation of the insertion site for the T-STEP cassette to allow for endogenous tagging of the DOP2R protein (See methods). (B) Representation of the genetics that allow for endogenous tagging of DOP2R in a tissue specific manner. (C) Pan-neuronal expression of DOP2R using *nSyb-Gal4*. (D) Midbrain sagittal slice revealing expression of Dop2R in multiple layers of the Fan-Shaped Body (arrows). (E). Max-intensity projection of *nSyb>Dop2R T-STEP* in the optic lobe. Arrows show expression in the

medulla (right) and lobula (left). (F) Saggital slice of the posterior portion of *nSyb>Dop2R T-STEP* highlighting the expression around the mushroom body calyx and within Kenyon cell bodies.

Figure 4.S3

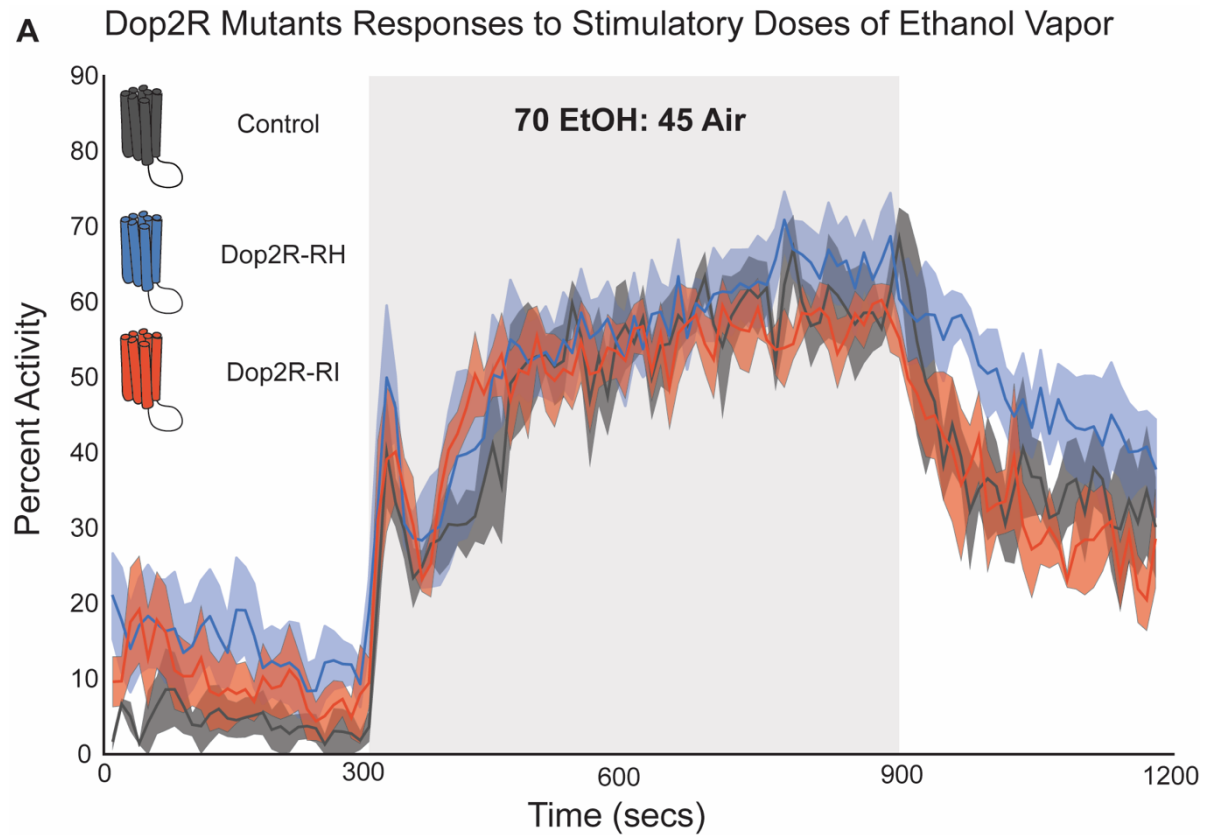


Figure 4.S3: Alcohol-induced locomotion in *Dop2R* splice mutants.

(A) Splice mutant locomotor responses to stimulatory doses of ethanol vapor. Control is gray (n = 8 runs of 10 flies), Dop2R-RH (n = 8 runs of 10 flies) is blue, and Dop2R-RI (n = 8 runs of 10 flies) is red.

Figure 4.S4

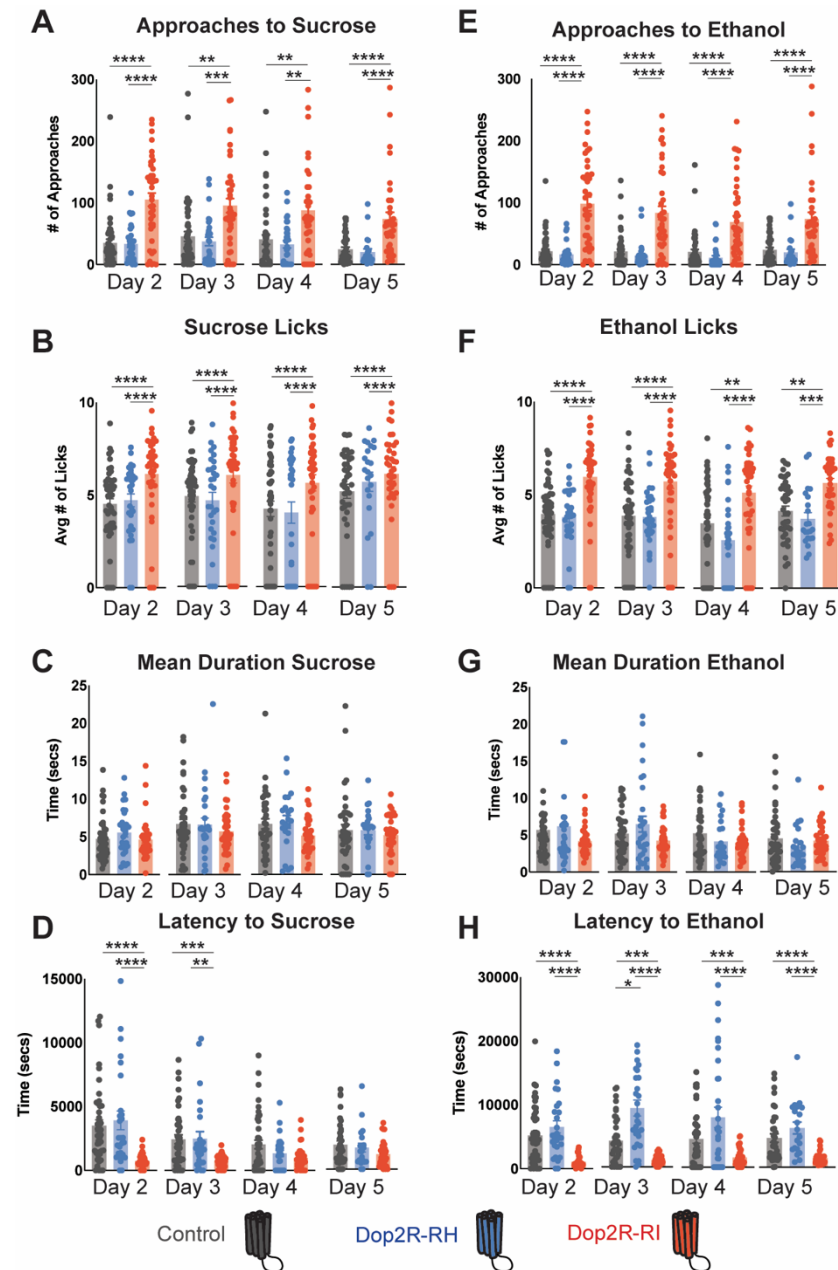


Figure 4.S4: FLIC metrics for days 2-5 for Control, Dop2R-RH, and Dop2R-RI flies.

(A) Average approaches to sucrose-containing food port on Days 2 through 5. (B) Average number of licks per contact for a sucrose-containing food port on days 2

through 5. (C) Average duration spent feeding at a sucrose-containing food port on days 2 through 5. (D) Average latency to return to a sucrose containing food port on Days 2 through 5. (E) Average approaches to sucrose-containing food port on Days 2 through 5. (F) Average number of licks per contact for a ethanol-containing food port on days 2 through 5. (G) Average duration spent feeding at an ethanol-containing food port on Days 2 through 5. (H) Average latency to return to an ethanol-containing food port on Days 2 through 5. Stats reported in Supplemental Table 4.S2.

Figure 4.S5

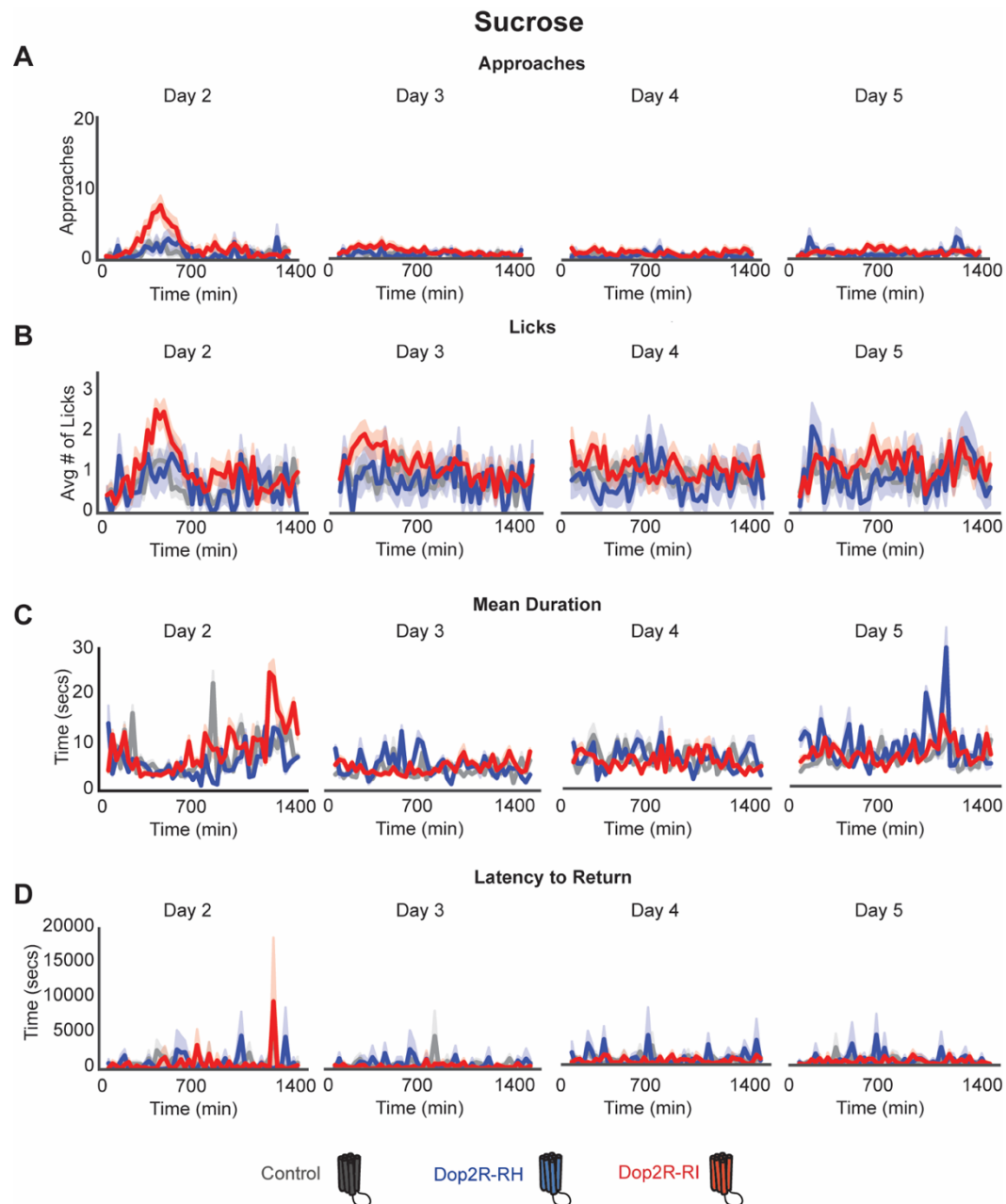


Figure 4.S5: FLIC Engagement with Sucrose over time across days 2-5.

(A) Average number of sucrose approaches over a 24-hour period on days 2 through 5.

(B) Average number of sucrose licks over a 24-hour period on days 2 through 5. (C)

Mean duration of sucrose approaches over a 24-hour period on days 2 through 5. (D)

Average latency to return to a sucrose-containing food source over a 24-hour period on days 2 through 5. Stats reported in Supplemental figure 4.S19.

Figure 4.S6

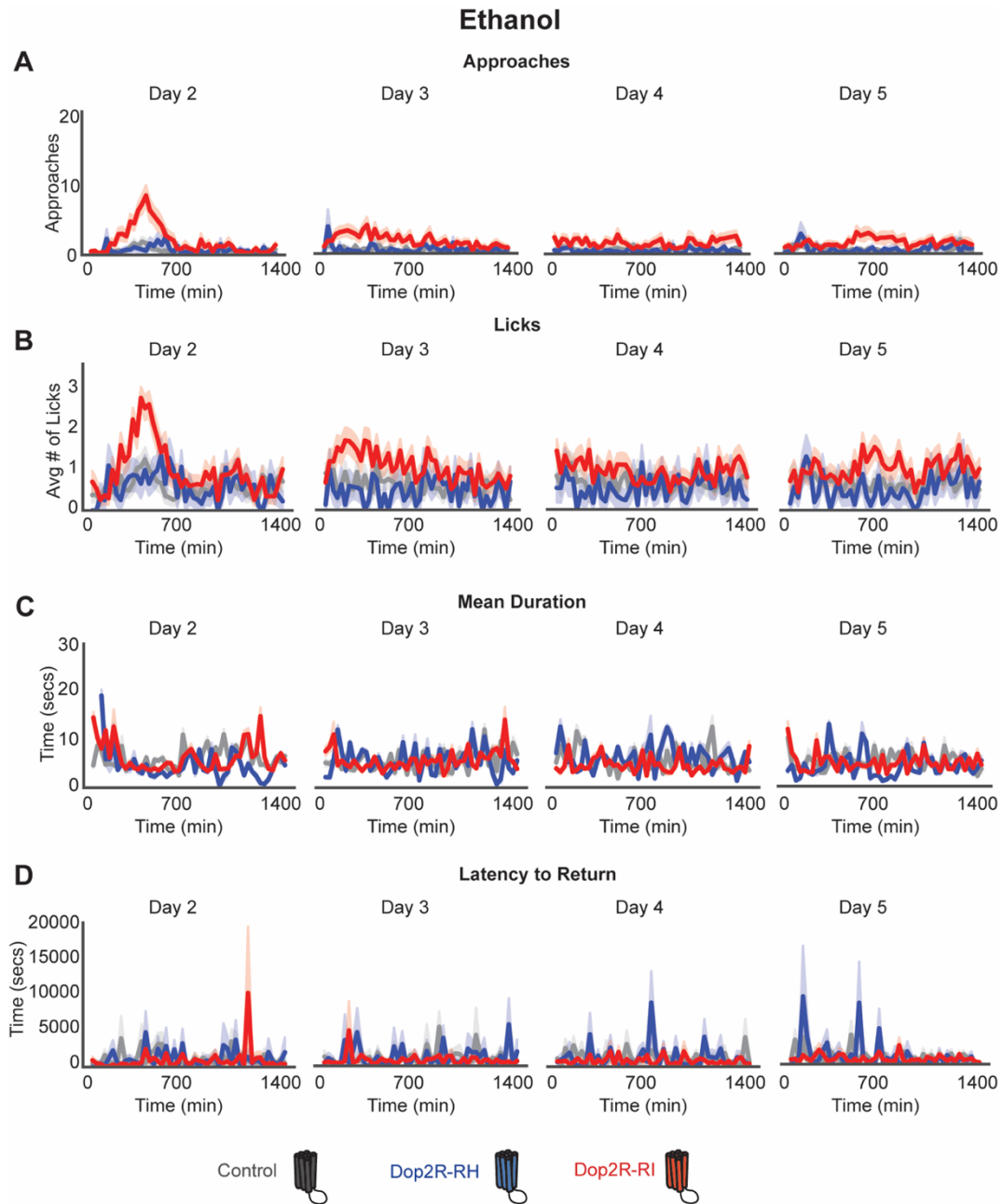


Figure 4.S6: FLIC Engagement with ethanol in sucrose over time across days 2-5.

(A) Average number of ethanol approaches over a 24-hour period on days 2 through 5.

(B) Average number of ethanol licks over a 24-hour period on days 2 through 5. (C)

Mean duration of ethanol approaches over a 24-hour period on days 2 through 5. (D)

Average latency to return to an ethanol-containing food source over a 24-hour period on days 2 through 5. Stats reported in Supplemental figure 4.S20.

Figure 4.S7

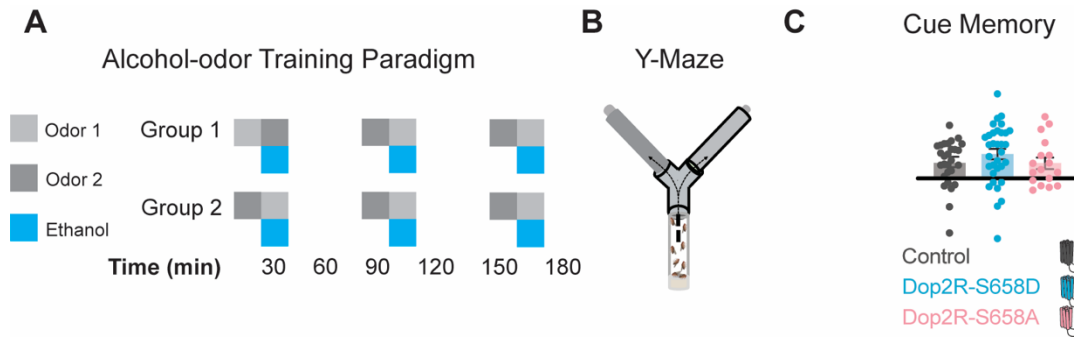


Figure 4.S7: Alcohol-cue memory assessment via Y-Maze for Control, Dop2R-S658D, and Dop2R-S658A flies.

(A) Alcohol-odor training paradigm schematic. (B) Depiction of Y-Maze. (C) Alcohol-cue memory 24 hours after training for control and phosphomutant flies. Control is gray (n = 28), Dop2R-S658D is blue (n = 33), and Dop2R-S658A is pink (n = 17). Stats reported in supplemental table 4.S3.

Figure 4.S8

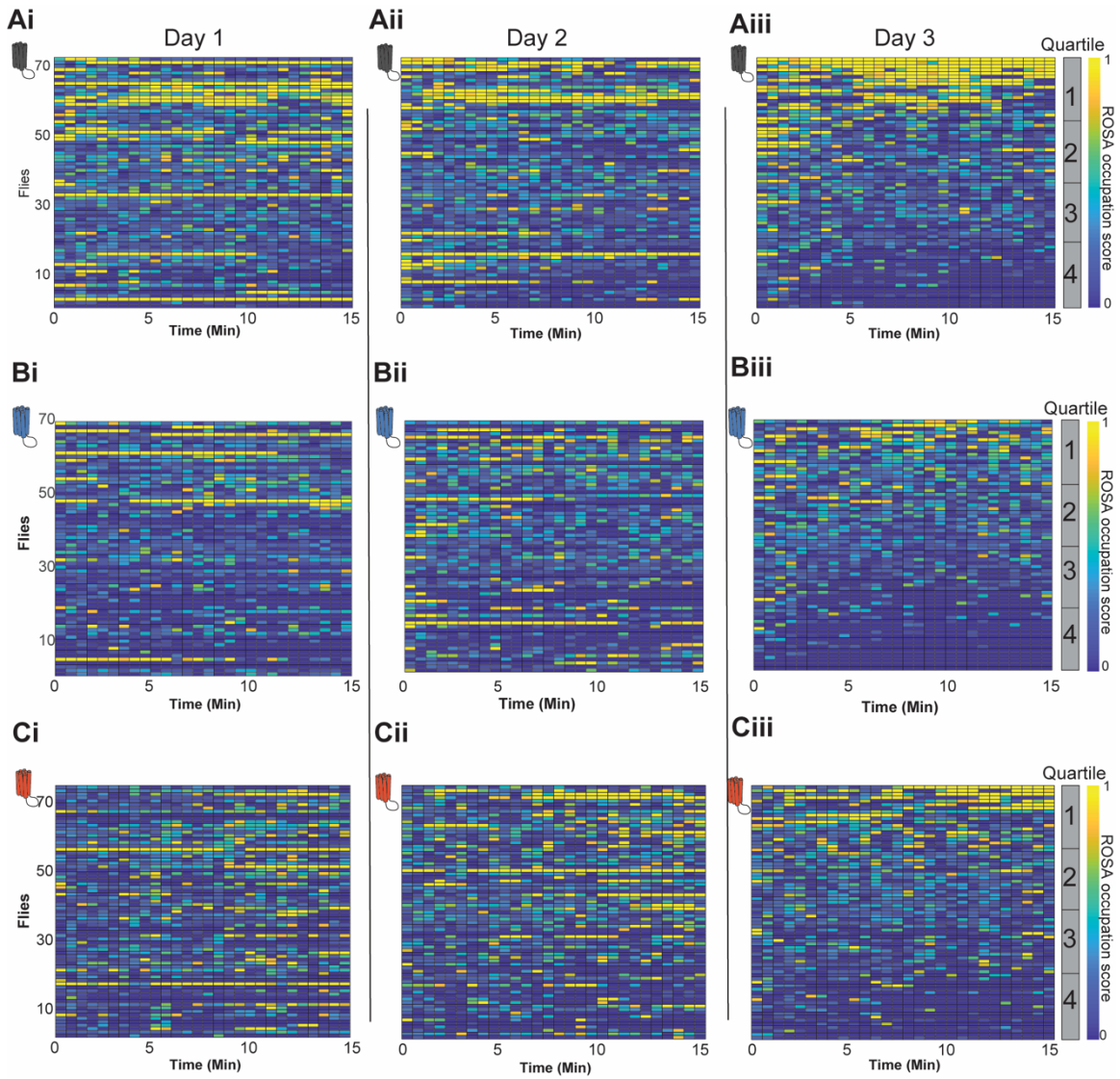


Figure 4.S8: Open BARR Heatmaps for Control, Dop2R-RH, and Dop2R-RI.

Heatmaps for Control flies on day 1 (Ai), Day 2 (Aii), and Day 3 (Aiii). Heatmaps for Dop2R-RH flies on day 1 (Bi), Day 2 (Bii), and Day 3 (Biii). Heatmaps for Dop2R-RI flies on day 1 (Ci), Day 2 (Cii), and Day 3 (Ciii).

Figure 4.S9

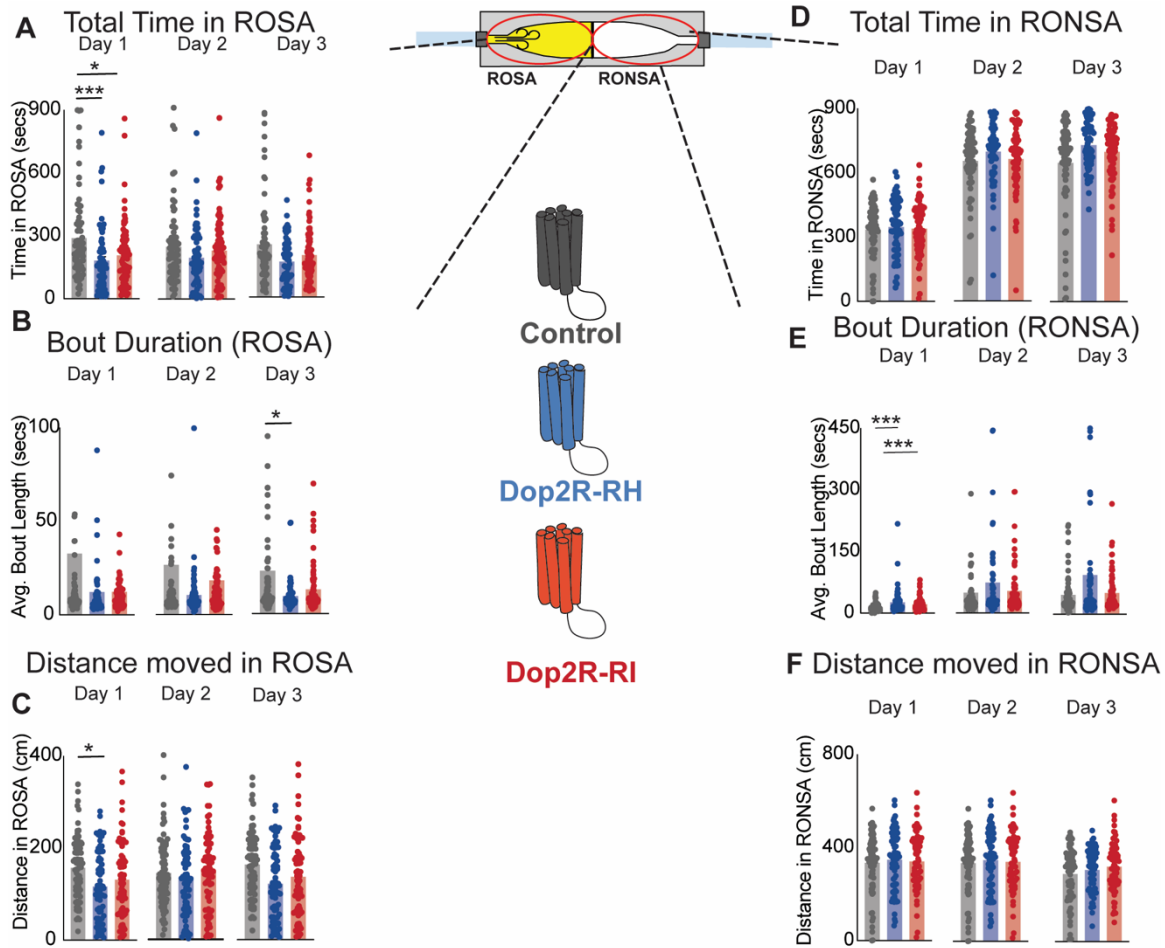


Figure 4.S9 Open BARR Behavioral Metrics for Control, Dop2R-RH, and Dop2R-RI

(A) Total time spent in ROSA by each genotype for each of the three days. (B) Average bout duration in ROSA for each genotype on each of the three days. (C) Total distance moved in ROSA by each of the genotypes on each of the three days. (D) Total time spent in RONSA by each of the genotypes on each of the three days. (E) Average bout duration in RONSA for each the genotypes on each of the three days. (F) Total distance moved in RONSA by each genotype on each of the three days. Bar plots represent the

mean, and the scatterplot overlay represent individual flies' metric value. Control is gray, Dop2R-RH is blue, and Dop2R-RI is red.

Figure 4.S10

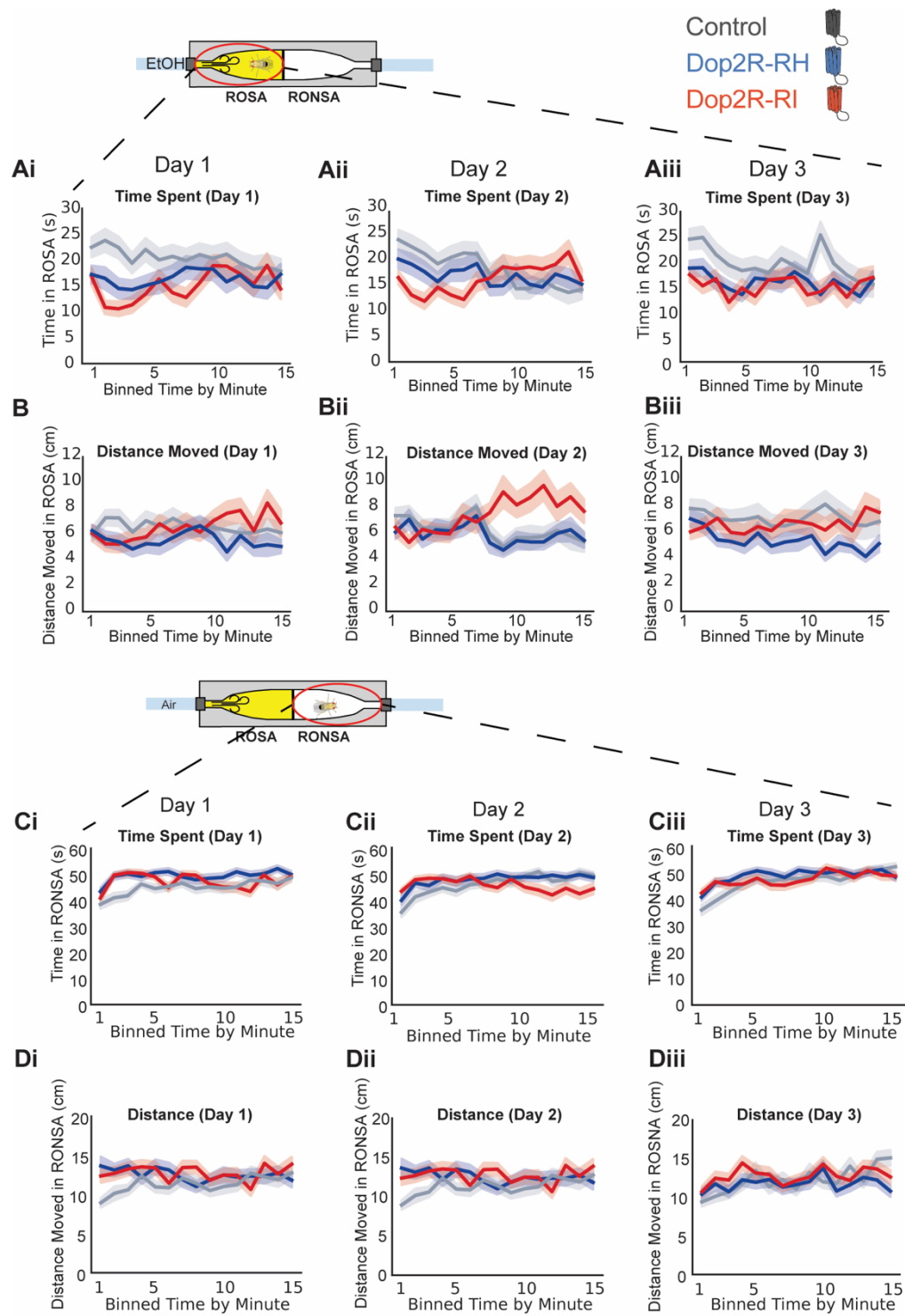


Figure 4.S10: Behavioral metrics in ROSA and RONSA over time for Control, RH, RI

Average time spent in ROSA over time for Day 1 (Ai), Day 2 (Aii), and Day 3 (Aiii).

Average distance moved in ROSA over time for Day 1 (Bi), Day 2 (Bii), and Day 3 (Biii).

Average time spent in RONSA over time for Day 1 (Ci), Day 2 (Cii), and Day 3 (Ciii).

Average distance moved in RONSA over time for Day 1 (Di), Day 2 (Dii), and Day 3 (Diii). Bold line represents mean, and shaded line represents SEM. Control is gray, Dop2R-RH is blue, and Dop2R-RI is red.

Figure 4.S11

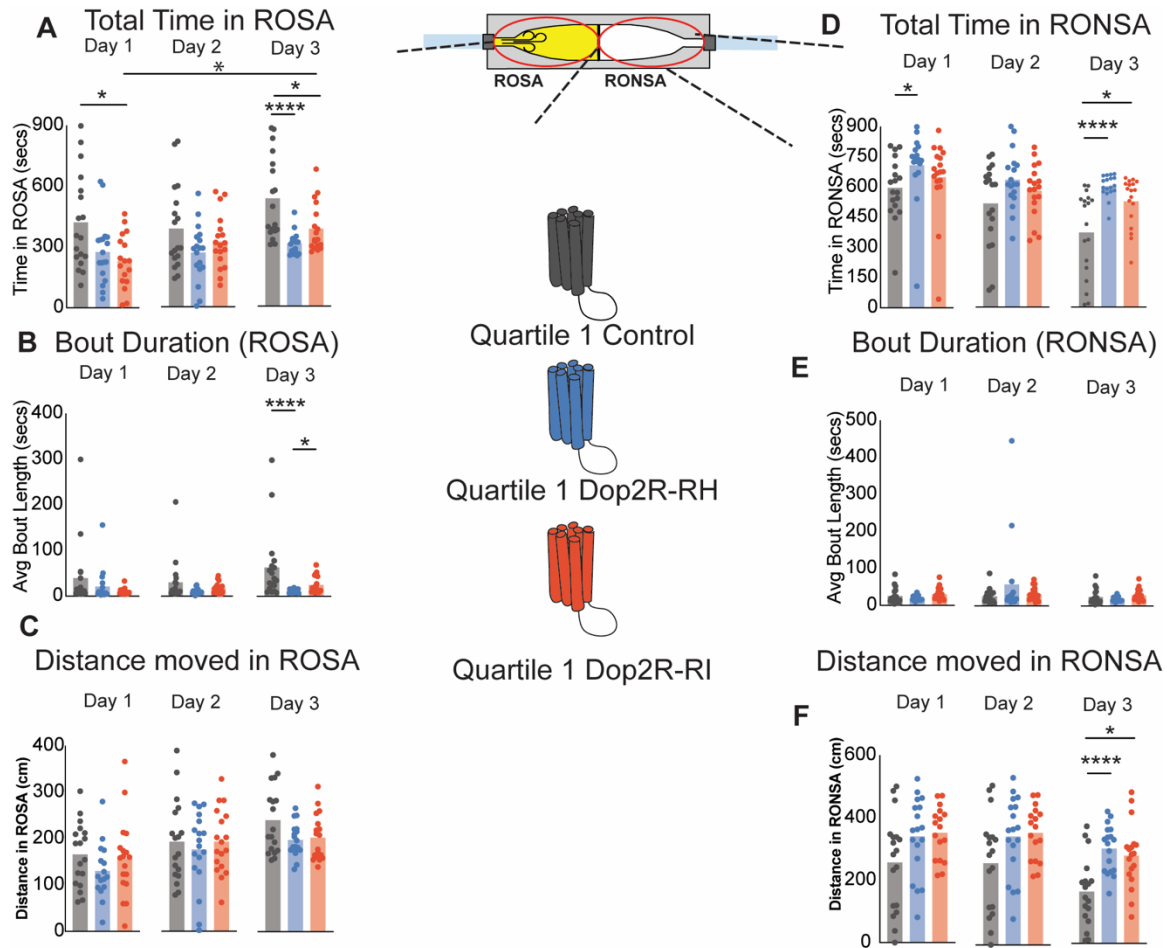


Figure 4.S11: High engaging fly metrics within ROSA and RONSA.

(A) Total time spent in ROSA by each genotype for each of the three days. (B) Average bout duration in ROSA for each genotype on each of the three days. (C) Total distance moved in ROSA by each of the genotypes on each of the three days. (D) Total time spent in RONSA by each of the genotypes on each of the three days. (E) Average bout duration in RONSA for each the genotypes on each of the three days. (F) Total distance moved in RONSA by each genotype on each of the three days. Bar plots represent the

mean, and the scatterplot overlay represent individual flies' metric value. Control is gray (n = 18), Dop2R-RH is blue (n =18), and Dop2R-RI is red (n = 18).

Figure 4.S12

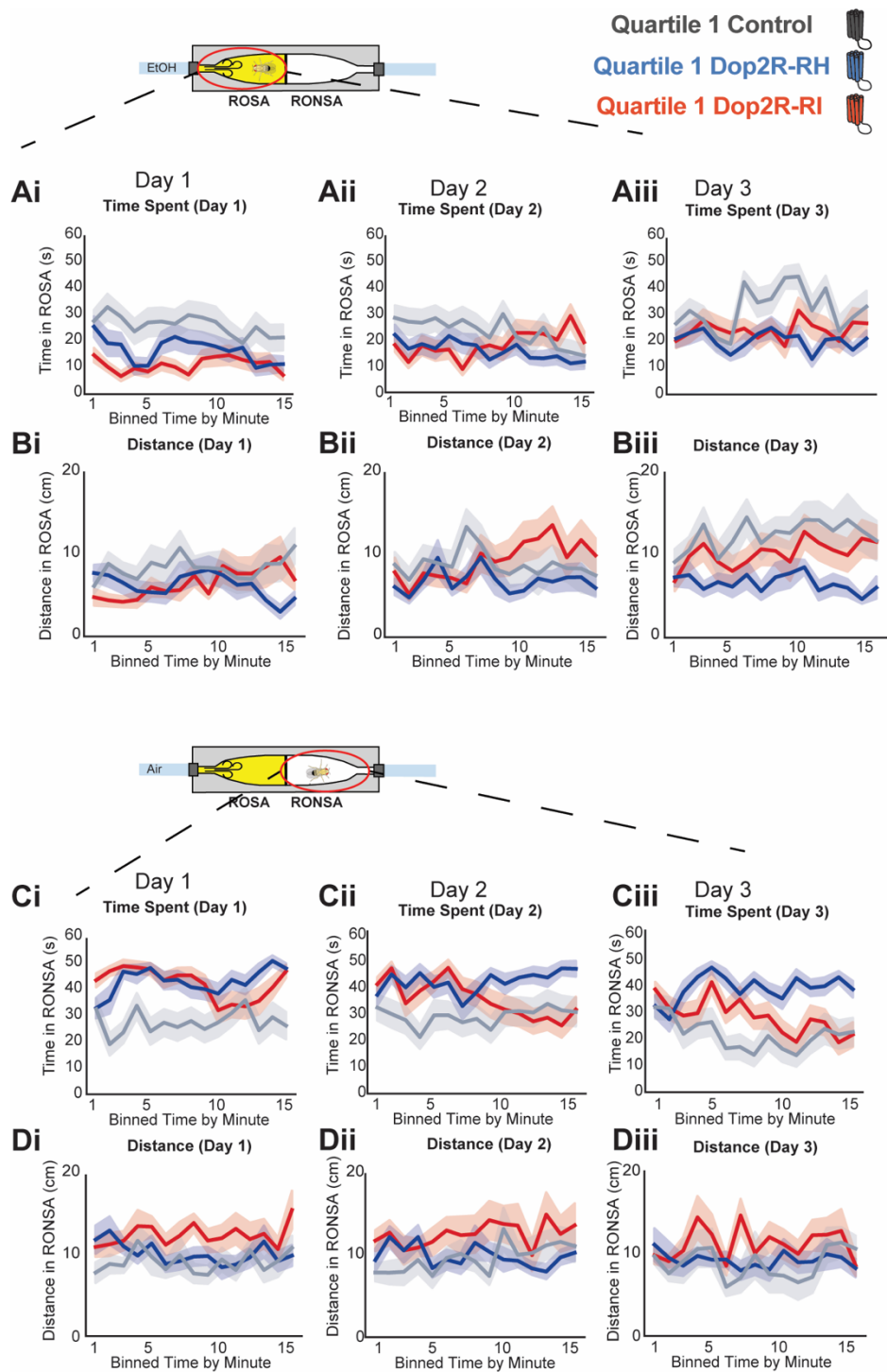


Figure 4.S12 Open BARR metrics overtime for high engaging Control, Dop2R-RH, and Dop2R-RI flies.

Time in ROSA per minute for high engaging control and flies on Day 1 (Ai), Day 2 (Aii), and Day 3 (Aiii). Average distance moved in ROSA over 15-minute bins on Day 1 (Bi), Day 2 (Bii), and Day 3 (Biii). Time spent in RONSA over the 15 minutes on Day 1 (Ci), Day 2 (Cii), and Day 3 (Ciii). Average distance moved for high engaging flies within RONSA on days 1(Di), 2 (Dii), and 3 (Diii).

Figure 4.S13

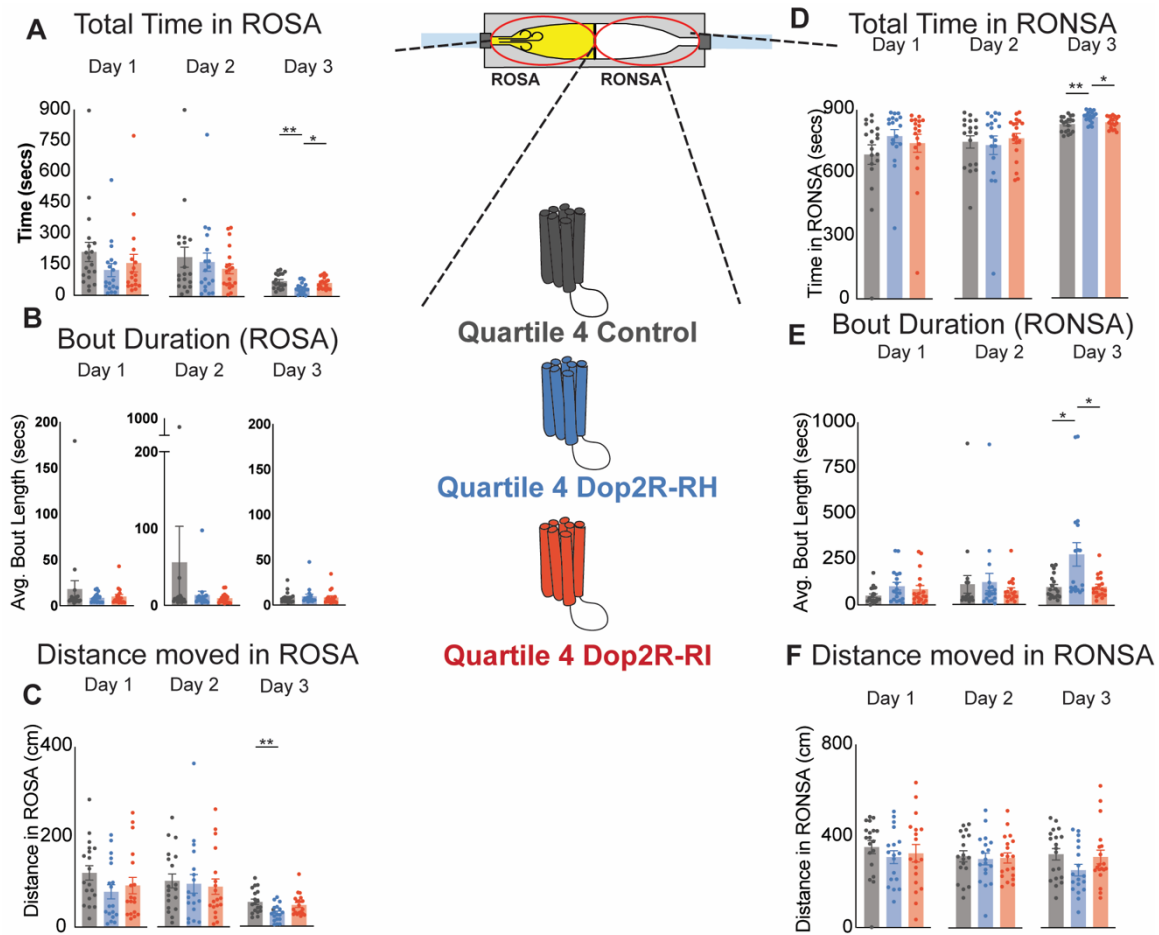


Figure 4.S13: Open BARR metrics for low engaging flies

(A) Total time spent in ROSA by each genotype for each of the three days. (B) Average bout duration in ROSA for each genotype on each of the three days. (C) Total distance moved in ROSA by each of the genotypes on each of the three days. (D) Total time spent in RONSA by each of the genotypes on each of the three days. (E) Average bout duration in RONSA for each the genotypes on each of the three days. (F) Total distance moved in RONSA by each genotype on each of the three days. Bar plots represent the

mean, and the scatterplot overlay represent individual flies' metric value. Control is gray (n = 18), Dop2R-RH is blue (n = 18), and Dop2R-RI is red (n = 18).

Figure 4.S14

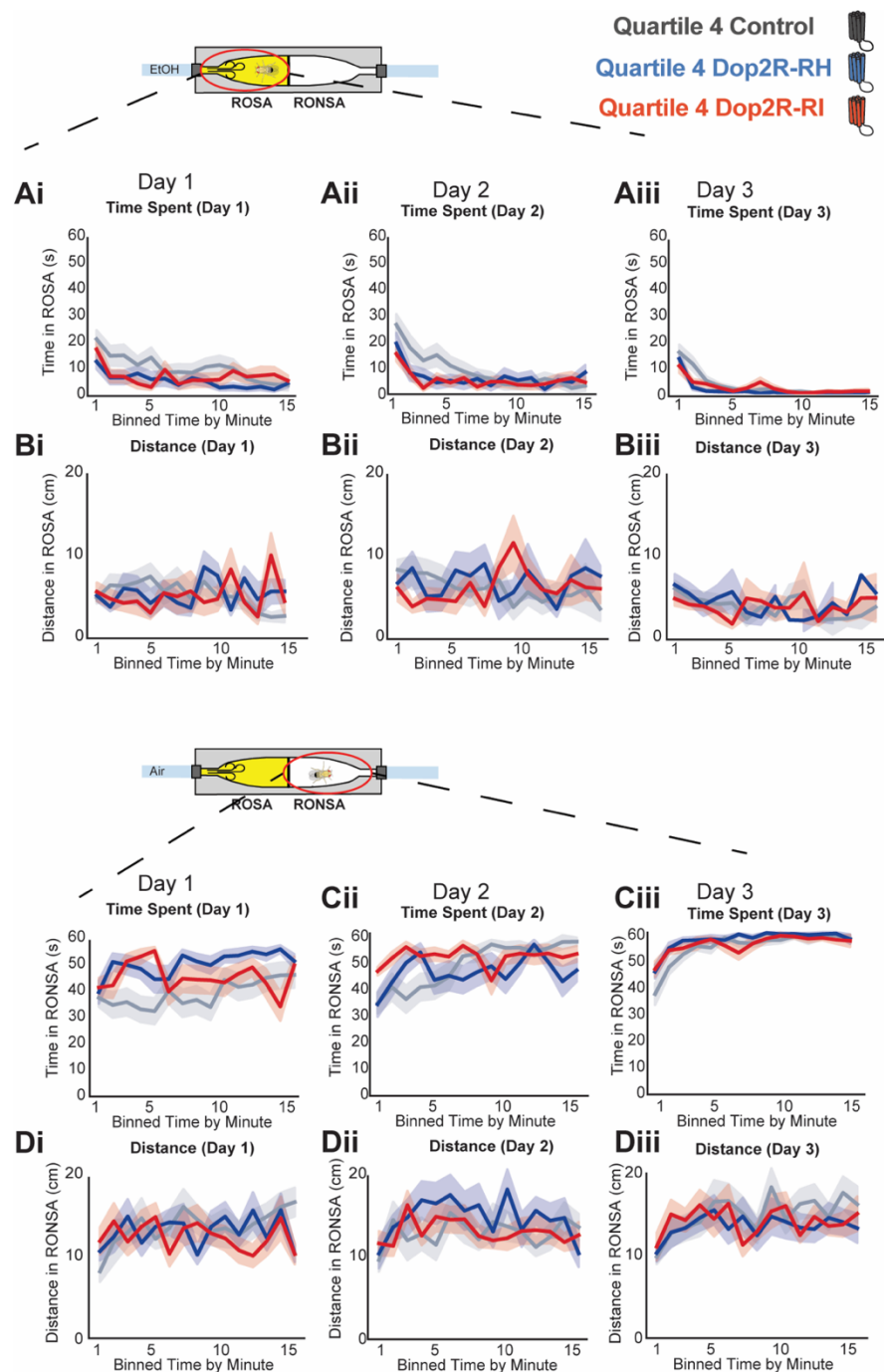


Figure 4.S14 Open BARR behavioral metrics over time for low engaging flies

Behavioral metrics for low engaging (quartile 4 flies)

Time in ROSA per minute for high engaging control and flies on Day 1 (Ai), Day 2 (Aii), and Day 3 (Aiii). Average distance moved in ROSA over 15-minute bins on Day 1 (Bi), Day 2 (Bii), and Day 3 (Biii). Time spent in RONSA over the 15 minutes on Day 1 (Ci), Day 2 (Cii), and Day 3 (Ciii). Average distance moved for high engaging flies within RONSA on days 1 (Di), 2 (Dii), and 3 (Diii).

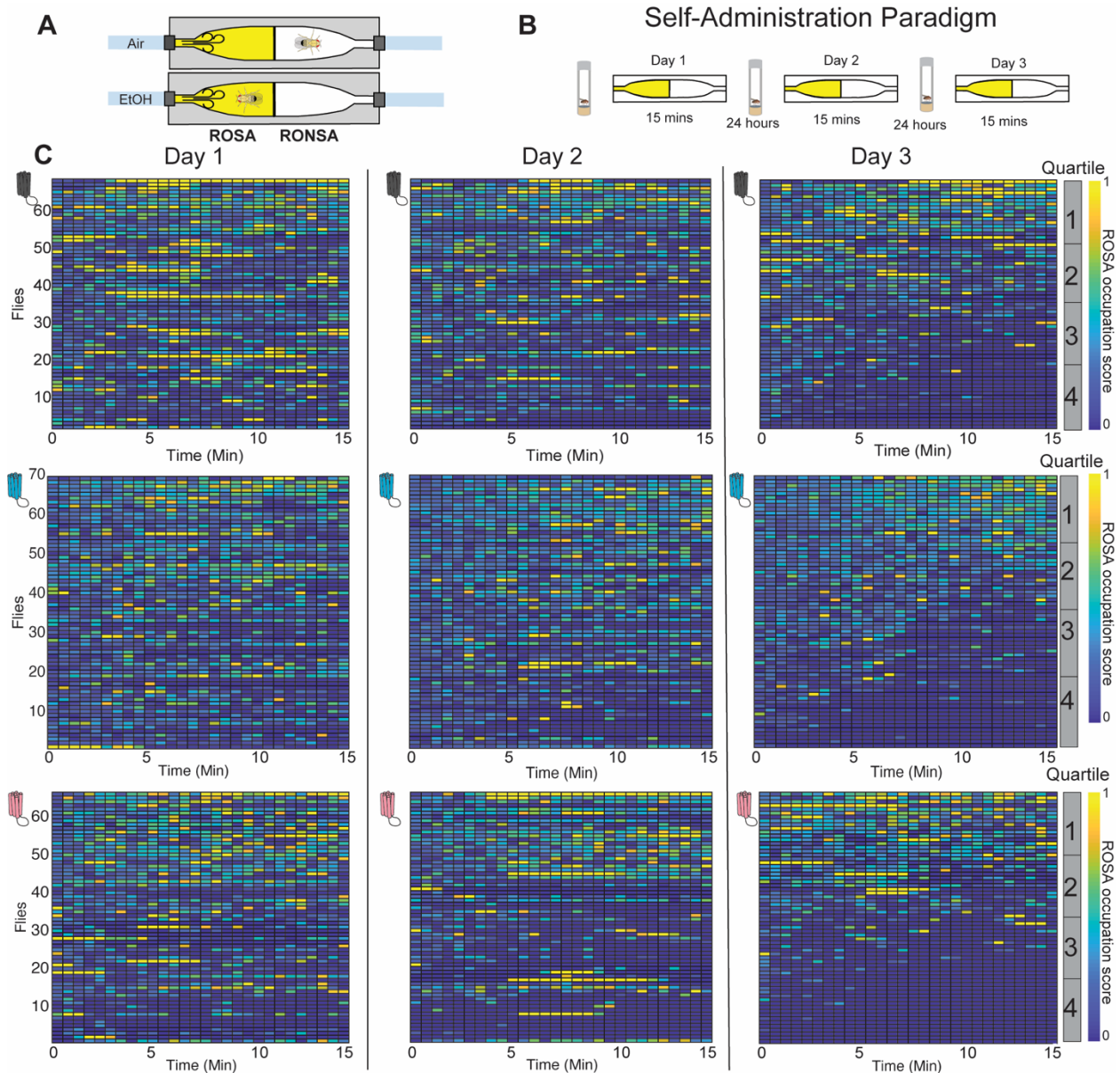


Figure 4.S15: Heatmaps showing alcohol self-administration for Control, *Dop2R-S658D*, and *Dop2R-S658A* flies.

(A) Depiction of the OPEN BARR assay. (B) Flies are grouped house before being placed in OPEN BARR and allowed to self-administer ethanol for 15 mins. Flies are then individually housed and allowed to self-administer again on days 2 and 3. (C) (Left) Day 1 self-administration for control (1st row), *Dop2R-S658D* (middle row), and *Dop2R-*

S658A flies (bottom row). (Middle Column) Day 2 self-administration of ethanol vapor for control (1st row), Dop2R-RH (middle row), and Dop2R-RI (bottom row). (Right) Day 3 self-administration of ethanol vapor for control (1st row), Dop2R-RH (middle row), and Dop2R-RI (bottom row). Flies are the row across heatmaps within genotype (e.g. Fly 3 in Day 1 heatmap is the same as Fly 3 in Day 3 heatmap within the same row) Numbered bars at the end of 3rd columns heatmaps represent the quartile the fly is placed in. Time is binned into 30-second bins. Control n = 73, Dop2R-RH n = 70, Dop2R-RI n = 75.

Figure 4.S16

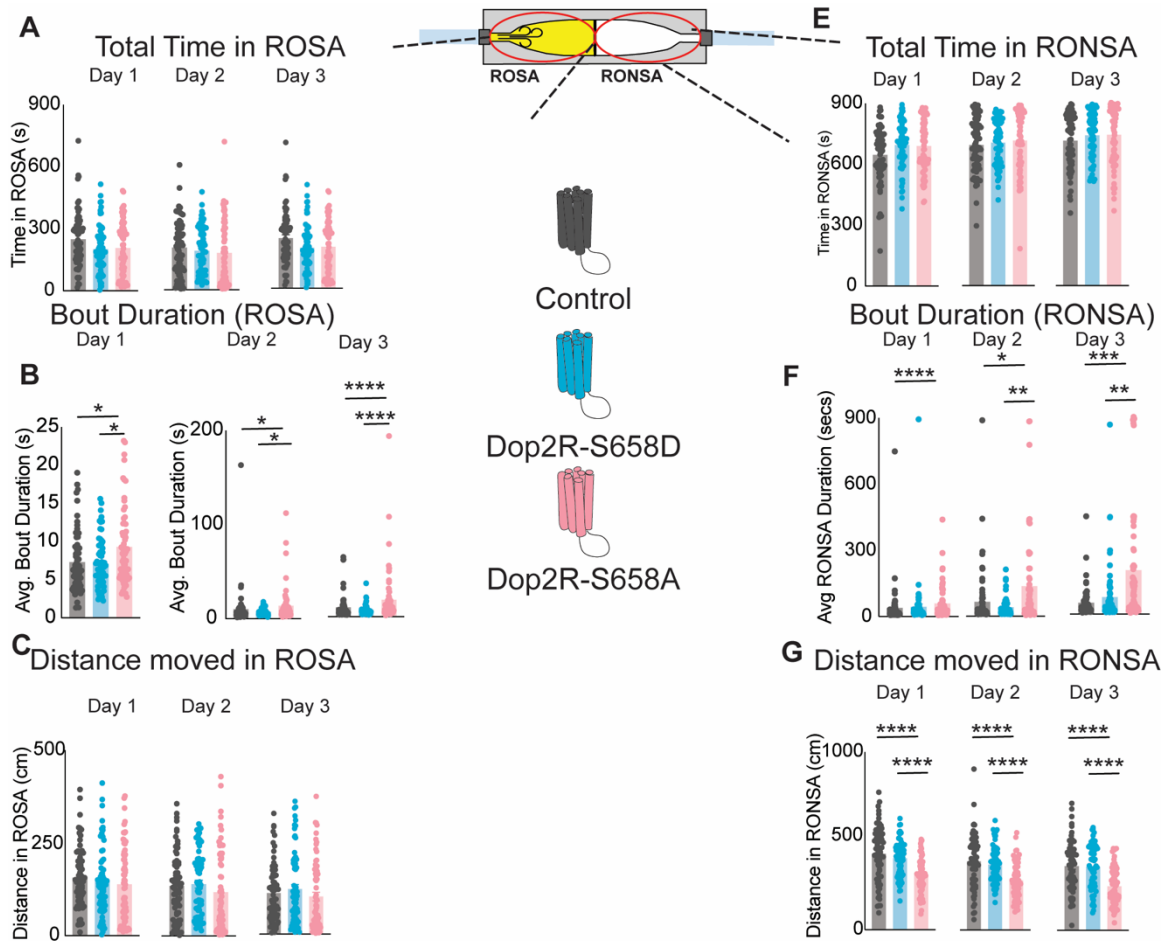


Figure 4.S16: Behavioral metrics for Control, Dop2R-S658D, and Dop2R-S658A.

(A) Total time spent in ROSA by each genotype for each of the three days. (B) Average bout duration in ROSA for each genotype on each of the three days. (C) Total distance moved in ROSA by each of the genotypes on each of the three days. (D) Total time spent in RONSA by each of the genotypes on each of the three days. (E) Average bout duration in RONSA for each the genotypes on each of the three days. (F) Total distance moved in RONSA by each genotype on each of the three days. Bar plots represent the

mean, and the scatterplot overlay represent individual flies' metric value. Control is gray, Dop2R-S658D is light blue, and Dop2R-S658A is pink.

Figure 4.S16

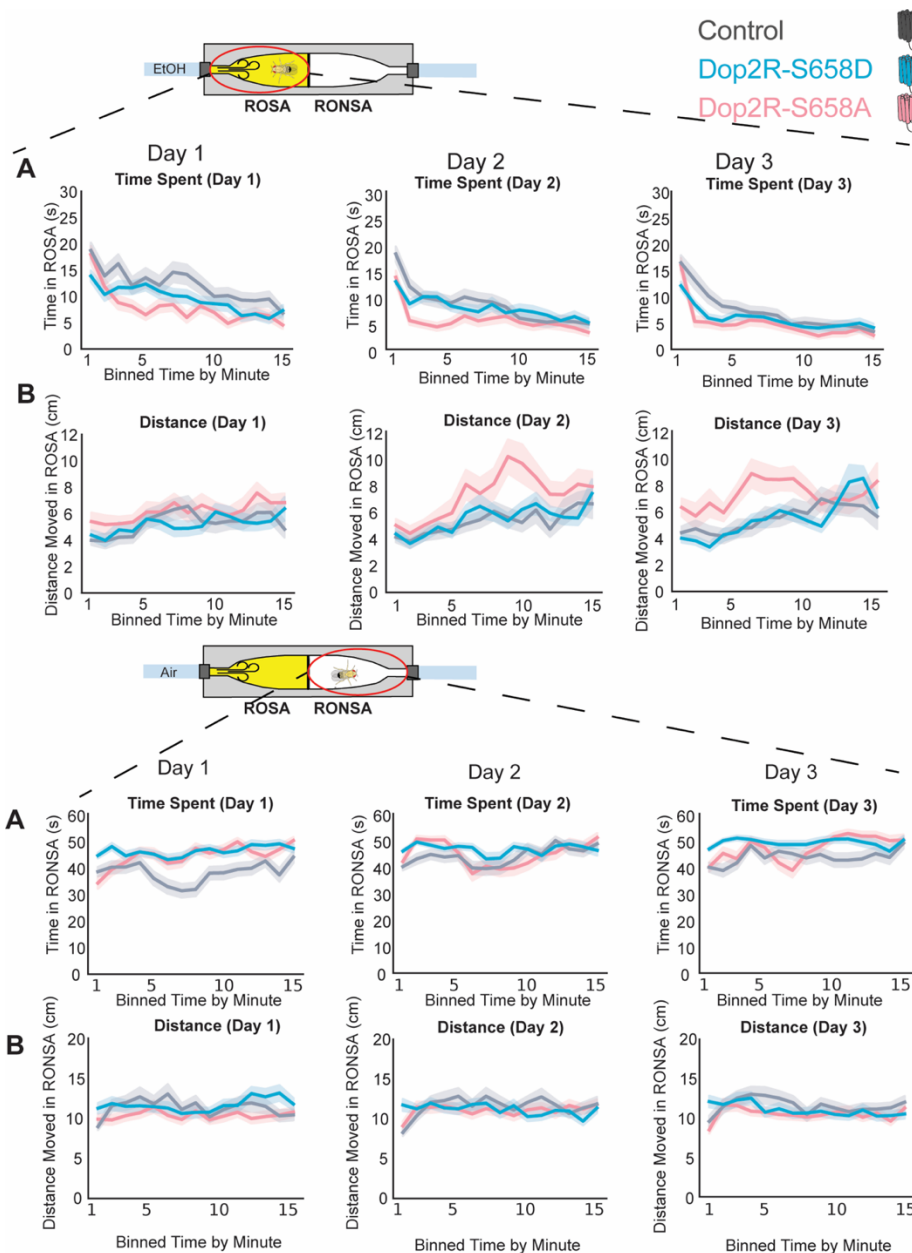


Figure 4.S17: Behavioral metrics over time in ROSA for Control, Dop2R-S658D, and Dop2R-S658A.

(A) Average time spent in ROSA over time for Day 1 (Left), Day 2 (Middle), and Day 3 (Right). (B) Average distance moved in ROSA over time for Day 1 (Left), Day 2

(Middle), and Day 3 (Right). (C) Average Time Spent in RONSA over time for Day 1 (Left), Day 2 (Middle), and Day 3 (Right). (D) Average distanced moved in RONSA over time for Day 1 (Left), Day 2 (Middle), and Day 3 (Right). Bold line represents mean, and shaded line represents SEM. Control is gray, Dop2R-S658D is light blue, and Dop2R-S658A is pink.

Figure 4.S18

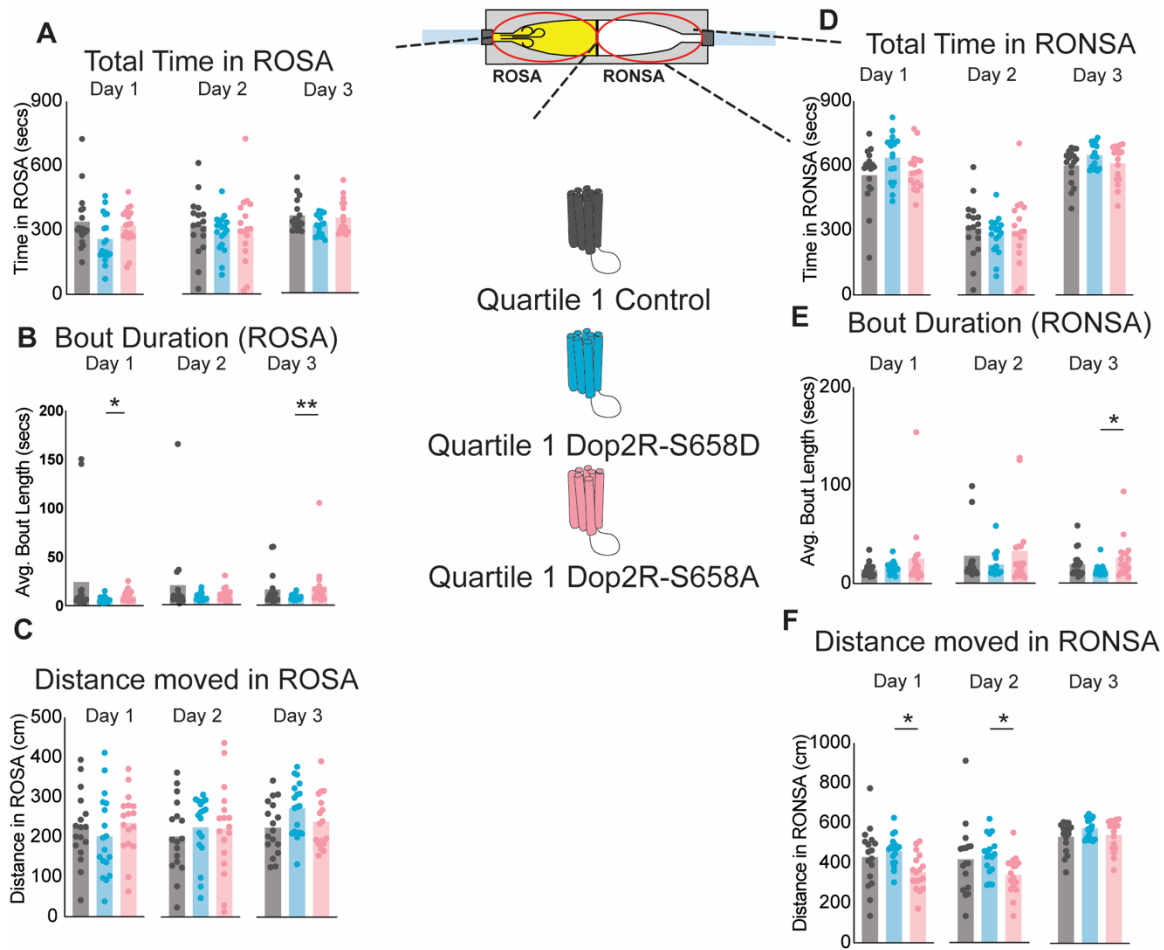


Figure 4.S18 Behavioral metrics for Quartile 1 (High Engaging) Control, Dop2R-S658D, and Dop2R-S658A flies

(A) Total time spent in ROSA by each genotype for each of the three days. (B) Average bout duration in ROSA for each genotype on each of the three days. (C) Total distance moved in ROSA by each of the genotypes on each of the three days. (D) Total time spent in RONSA by each of the genotypes on each of the three days. (E) Average bout duration in RONSA for each the genotypes on each of the three days. (F) Total distance moved in RONSA by each genotype on each of the three days. Bar plots represent the

mean, and the scatterplot overlay represent individual flies' metric value. Control is gray (n = 17), Dop2R-S658D (n = 18) is light blue, and Dop2R-S658A (n = 17) is pink.

Figure 4.S19

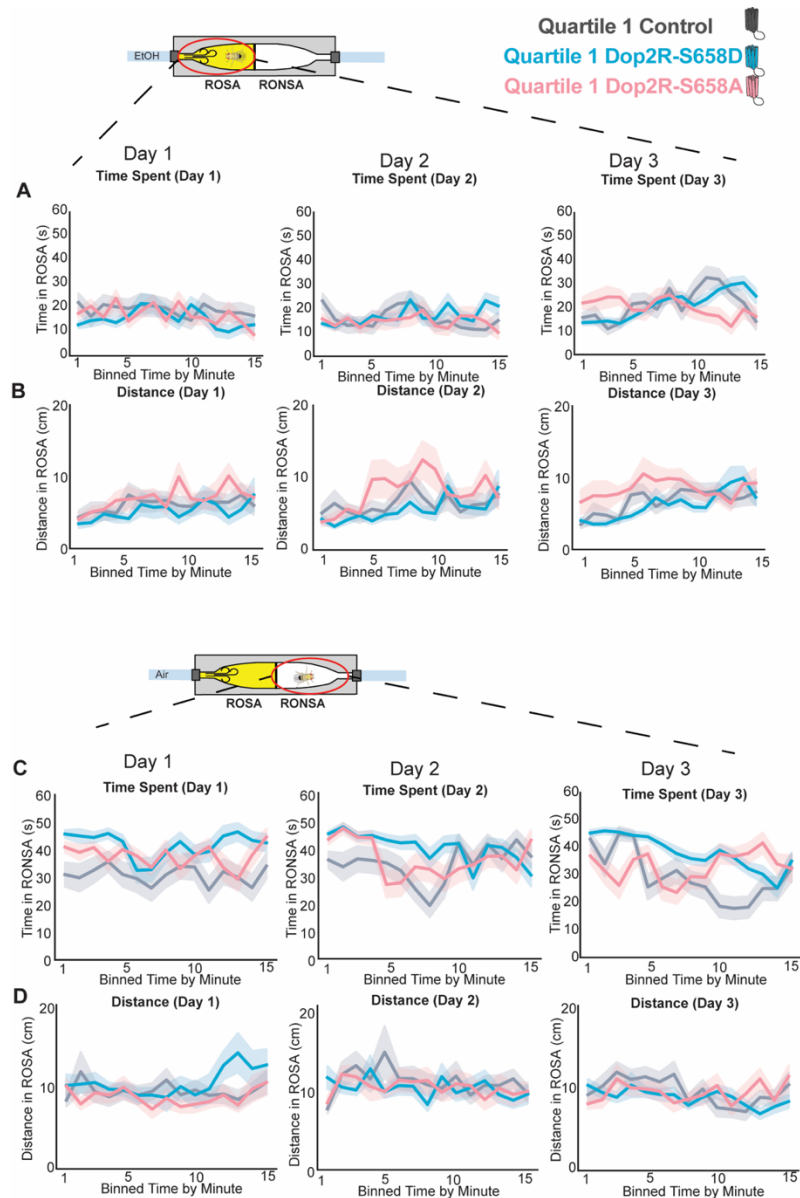


Figure 4.S19 Open BARR metrics overtime for high engaging control, Dop2R-S658D, and Dop2R-S658A flies.

(A) Time in ROSA per minute for high engaging control and phosphomutant flies on Day 1 (left), Day 2 (middle), and Day 3 (right). (B) Average distance moved in ROSA over

15-minute bins on Day 1 (left), Day 2 (middle), and Day 3 (right). (C) Time spent in RONSA over the 15 minutes on Day 1 (left), Day 2 (middle), and Day 3 (right). (D) Average distance moved for high engaging flies within RONSA on days 1(left), 2 (middle), and 3 (right).

Figure 4.S20

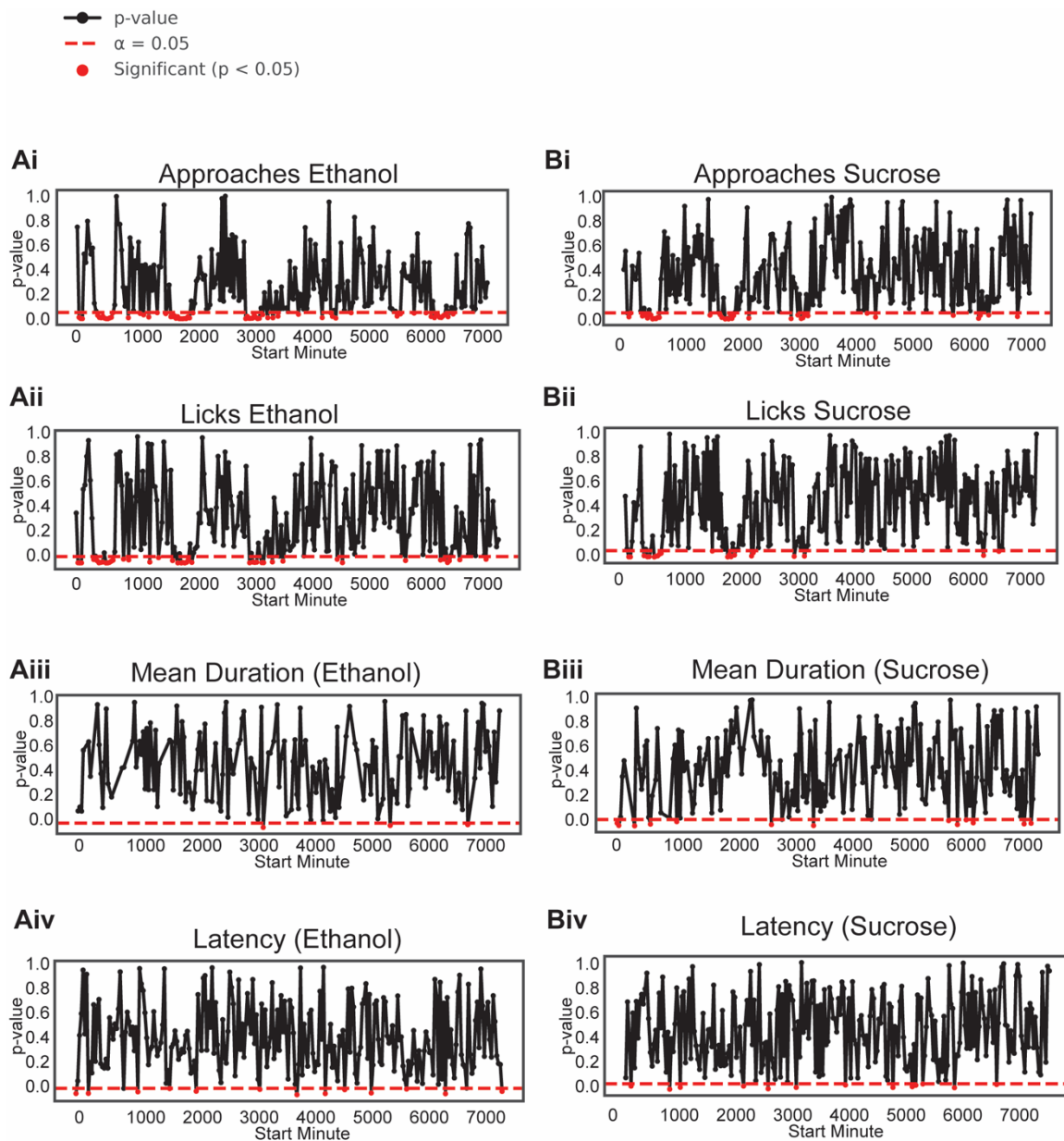


Figure 4.S19 – FLIC Metrics Overtime Significance

P-value over the 5 days of observations for FLIC. Red dashed line indicates the significance level cut-off. Data is binned into 60-minute bins.

Figure 4.S21

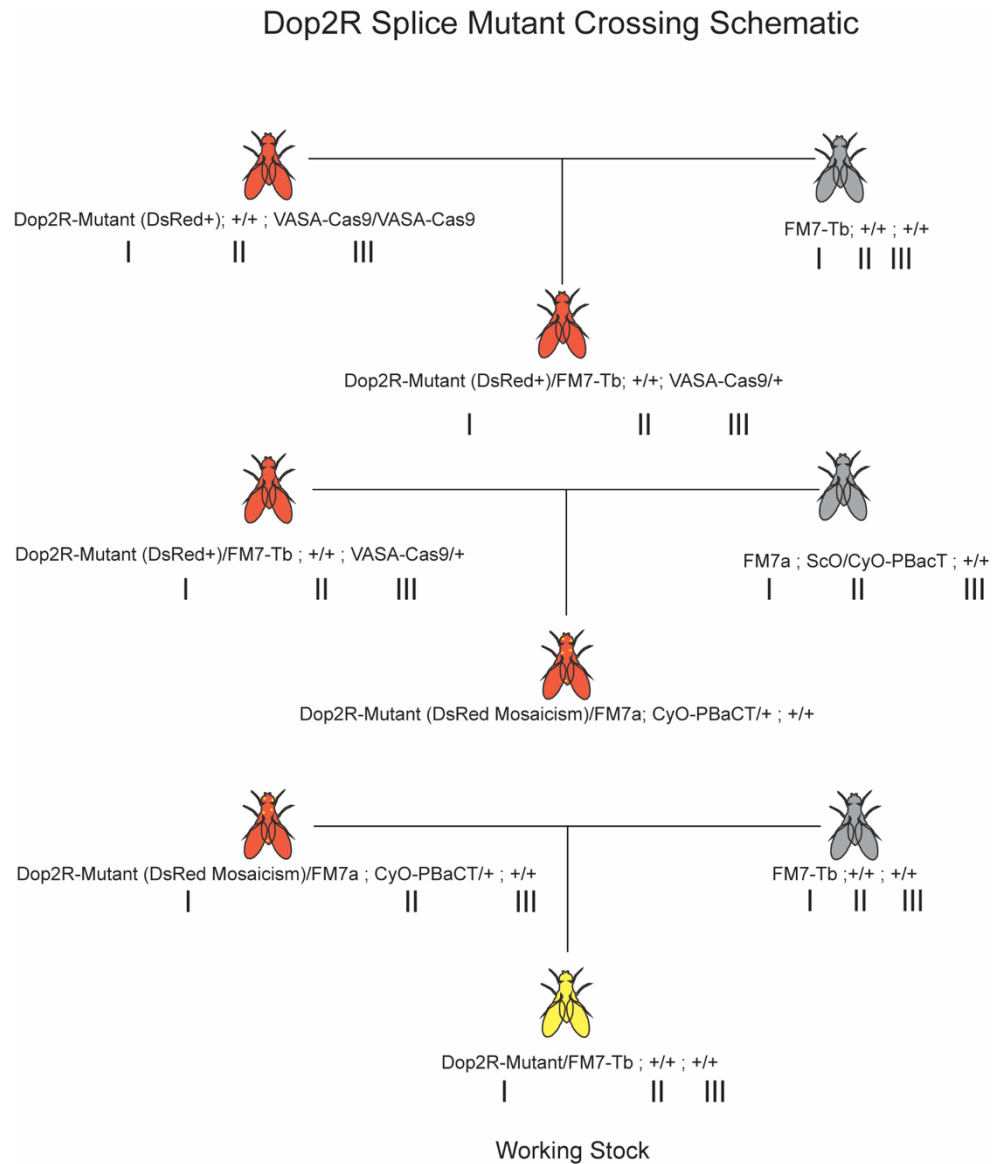


Figure 4.S20 Removal of DsRed from Dop2R-CRISPR mutants.

DsRed under control of the *eyeless* promoter was removed from Dop2R-mutants using PBaC Transposase.

Table 4.S1

Figure 4.8Ei	FLIC	Control vs RH vs RI Licks for Sucrose DAY 1	Kruskal-Wallis	<0.0001	Control vs RH	Dunn	0.1574
					Control vs RI		<0.0001
					RH vs RI		0.0218
Figure 4.2Bii	FLIC	Control vs RH vs RI Events for Sucrose DAY 1	Kruskal-Wallis	<0.0001	Control vs RH	Dunn	>0.9999
					Control vs RI		<0.0001
					RH vs RI		<0.0001
Figure 4.2Biii	FLIC	Control vs RH vs RI Mean Duration for Sucrose DAY 1	Kruskal-Wallis	0.1646			
Figure 4.2Biv	FLIC	Control vs RH vs RI Latency for Sucrose DAY 1	Kruskal-Wallis	0.0119			0.0129
							0.1594
							0.7113
Figure 4.2Ci	FLIC	Control vs RH vs RI Approaches for EtOH DAY 1	Kruskal-Wallis	<0.0001	Control vs RH	Dunn	>0.9999
					Control vs RI		<0.0001
					RH vs RI		<0.0001
Figure 4.2Cii	FLIC	Control vs RH vs RI Licks for EtOH DAY 1	Kruskal-Wallis	<0.0001	Control vs RH	Dunn	>0.9999
					Control vs RI		<0.0001
					RH vs RI		<0.0001
Figure 4.2Ciii	FLIC	Control vs RH vs RI Mean Duration for EtOH DAY 1	Kruskal-Wallis	0.5645			
Figure 4.2Civ	FLIC	Control vs RH vs RI Latency for EtOH DAY 1	Kruskal-Wallis	<0.0001	Control vs RH	Dunn	>0.9999
					Control vs RI		<0.0001
					RH vs RI		<0.0001

Table 4.S1: Stats for Day 1 overall metrics for FLIC assay.

Table 4.S2

Figure	Experiment Type	Comparison Description	Statistical Test	p-value	Post-Hoc Comparison	Statistical Test	P-Value
Figure 4.28a	FLIC	Control vs RH vs RI Approaches for Sucrose DAY 2	Kruskal-Wallis	<0.0001	Control vs RH Control vs RI	Dunn	>0.9999 <0.0001
Figure 4.28b	FLIC	Control vs RH vs RI Licks for Sucrose DAY 2	Kruskal-Wallis	<0.0001	RH vs RI Control vs RH Control vs RI	Dunn	>0.9999 <0.0001 0.0029
Figure 4.28c	FLIC	Control vs RH vs RI Mean Duration for Sucrose DAY 2	Kruskal-Wallis	0.2642			
Figure 4.28d	FLIC	Control vs RH vs RI Latency for Sucrose DAY 2	Kruskal-Wallis	<0.0001	Control vs RH Control vs RI	Dunn	>0.9999 <0.0001
Figure 4.29a	FLIC	Control vs RH vs RI Approaches for ETOH DAY 2	Kruskal-Wallis	<0.0001	RH vs RI Control vs RH Control vs RI	Dunn	<0.0001 >0.9999 <0.0001
Figure 4.29b	FLIC	Control vs RH vs RI Licks for ETOH DAY 2	Kruskal-Wallis	<0.0001	RH vs RI Control vs RH Control vs RI	Dunn	<0.0001 >0.9999 <0.0001
Figure 4.29c	FLIC	Control vs RH vs RI Mean Duration for ETOH DAY 2	Kruskal-Wallis	0.8323			
Figure 4.29d	FLIC	Control vs RH vs RI Latency for ETOH DAY 2	Kruskal-Wallis	<0.0001	Control vs RH Control vs RI	Dunn	>0.9999 <0.0001 <0.0001
Figure 4.30a	FLIC	Control vs RH vs RI Licks for Sucrose DAY 3	Kruskal-Wallis	0.0026	Control vs RH Control vs RI	Dunn	>0.9999 0.0074
Figure 4.30b	FLIC	Control vs RH vs RI Approaches for Sucrose DAY 3	Kruskal-Wallis	<0.0001	RH vs RI Control vs RH Control vs RI	Dunn	0.0105 >0.9999 0.0012
Figure 4.30c	FLIC	Control vs RH vs RI Mean Duration for Sucrose DAY 3	Kruskal-Wallis	0.1646			0.0007
Figure 4.30d	FLIC	Control vs RH vs RI Latency for Sucrose DAY 3	Kruskal-Wallis	0.0002			
Figure 4.31a	FLIC	Control vs RH vs RI Approaches for ETOH DAY 3	Kruskal-Wallis	<0.0001	Control vs RH Control vs RI	Dunn	>0.9999 0.0005
Figure 4.31b	FLIC	Control vs RH vs RI Licks for ETOH DAY 3	Kruskal-Wallis	<0.0001	RH vs RI Control vs RH Control vs RI	Dunn	>0.9999 <0.0001 <0.0001
Figure 4.31c	FLIC	Control vs RH vs RI Mean Duration for ETOH DAY 3	Kruskal-Wallis	0.5645			>0.9999 0.0004
Figure 4.31d	FLIC	Control vs RH vs RI Latency for ETOH DAY 3	Kruskal-Wallis	<0.0001	Control vs RH Control vs RI	Dunn	<0.0001 0.012
Figure 4.32a	FLIC	Control vs RH vs RI Licks for Sucrose DAY 4	Kruskal-Wallis	0.0309	RH vs RI Control vs RH Control vs RI	Dunn	0.0001 0.0461 0.1108
Figure 4.32b	FLIC	Control vs RH vs RI Approaches for Sucrose DAY 4	Kruskal-Wallis	0.0007	Control vs RH Control vs RI	Dunn	>0.9999 0.0021
Figure 4.32c	FLIC	Control vs RH vs RI Mean Duration for Sucrose DAY 4	Kruskal-Wallis	0.1646			0.0043
Figure 4.32d	FLIC	Control vs RH vs RI Latency for Sucrose DAY 4	Kruskal-Wallis	0.311			
Figure 4.33a	FLIC	Control vs RH vs RI Approaches for ETOH DAY 4	Kruskal-Wallis	<0.0001	Control vs RH Control vs RI	Dunn	0.3031 <0.0001
Figure 4.33b	FLIC	Control vs RH vs RI Licks for ETOH DAY 4	Kruskal-Wallis	0.0189	RH vs RI Control vs RH Control vs RI	Dunn	<0.0001 >0.9999 0.0611
Figure 4.33c	FLIC	Control vs RH vs RI Mean Duration for ETOH DAY 4	Kruskal-Wallis	0.5645			0.0325
Figure 4.33d	FLIC	Control vs RH vs RI Latency for ETOH DAY 4	Kruskal-Wallis	<0.0001	Control vs RH Control vs RI	Dunn	>0.9999 <0.0001 <0.0001
Figure 4.34a	FLIC	Control vs RH vs RI Licks for Sucrose DAY 5	Kruskal-Wallis	0.185			
Figure 4.34b	FLIC	Control vs RH vs RI Approaches for Sucrose DAY 5	Kruskal-Wallis	<0.0001	Control vs RH Control vs RI	Dunn	>0.9999 <0.0001 <0.0001
Figure 4.34c	FLIC	Control vs RH vs RI Mean Duration for Sucrose DAY 5	Kruskal-Wallis	0.1646			
Figure 4.34d	FLIC	Control vs RH vs RI Latency for Sucrose DAY 5	Kruskal-Wallis	0.0119			0.0129 0.1594 0.7113
Figure 4.35a	FLIC	Control vs RH vs RI Approaches for ETOH DAY 5	Kruskal-Wallis	<0.0001	Control vs RH Control vs RI	Dunn	>0.9999 <0.0001 <0.0001
Figure 4.35b	FLIC	Control vs RH vs RI Licks for ETOH DAY 5	Kruskal-Wallis	<0.0001	RH vs RI Control vs RH Control vs RI	Dunn	>0.9999 <0.0001 <0.0001
Figure 4.35c	FLIC	Control vs RH vs RI Mean Duration for ETOH DAY 5	Kruskal-Wallis	0.5645			0.4755 <0.0001 <0.0001
Figure 4.35d	FLIC	Control vs RH vs RI Latency for ETOH DAY 5	Kruskal-Wallis	0.2099			

Table 4.S2: FLIC Stats for days 2 through days 5

Table 4.S3

Figure	Experiment Type	Comparison Description	Statistical Test	p-value	Post-Hoc Comparison	Statistical Test	P-Value
Figure 4.3	Y-Maze Preference Index	Control vs RH vs RI	Kruskal-Wallis	0.525			
Figure 4.S7		Control vs S658D vs S658A		0.231			

Table 4.S3: Stats for Alcohol-Cur Memory (Y-maze)

Table 4.S4

Figure	Experiment Type	Comparison Description	Statistical Test	p-value	Post-Hoc Comparison	Statistical Test	P-Value
Figure 4.3	Tandem (Sucrose)	Preference Index	Kruskal-Wallis	0.8171			
Figure 4.3	Tandem (Sucrose)	Transitions	Kruskal-Wallis	<0.0001	Control vs RH Control vs RI RH VS RI	Dunn	0.6822 0.006 <0.0001
Figure 4.3	Tandem (Sucrose)	Bout Duration (Paired)	Kruskal-Wallis	0.1066	Control vs RH Control vs RI RH VS RI		
Figure 4.3	Tandem (Sucrose)	Bout Duration (Unpaired)	Kruskal-Wallis	0.0067	Control vs RH Control vs RI RH VS RI	Dunn	0.795 0.1684 0.005
Figure 4.3	Tandem (Sucrose)	Distance Moved (Paired)	Kruskal-Wallis	<0.0001	Control vs RH Control vs RI RH VS RI	Dunn	0.245 <0.0001 <0.0001
Figure 4.3	Tandem (Sucrose)	Distance Moved (Unpaired)	Kruskal-Wallis	<0.0001	Control vs RH Control vs RI RH VS RI	Dunn	>0.9999 0.0003 0.0002
Figure 4.3	Tandem (Sucrose)	Port Distance (Paired)	Kruskal-Wallis	0.2041	Control vs RH Control vs RI RH VS RI		
Figure 4.3	Tandem (Sucrose)	Port Distance (Unpaired)	Kruskal-Wallis	0.4955	Control vs RH Control vs RI RH VS RI		

Table 4.S4: Stats table for Tandem-Two Choice Sucrose Memory

Table 4.S5

Figure 4.4	Open Bar	Control vs RH vs RI Total Duration (ROSA) D1	Kruskal-Wallis	0.0005	Control vs RH Control vs RI RH VS RI	Dunn's	0.004 0.031 0.5912
Figure 4.S8	Open Bar	Control vs RH vs RI Total Duration (ROSA) D2	Kruskal-Wallis	0.1616			
Figure 4.S8	Open Bar	Control vs RH vs RI Total Duration (ROSA) D3	Kruskal-Wallis	0.0774			
Figure 4.4	Open Bar	Control vs RH vs RI Average Duration (ROSA) D1	Kruskal-Wallis	0.0753			
Figure 4.S8	Open Bar	Control vs RH vs RI Average Duration (ROSA) D2	Kruskal-Wallis	0.1739			
Figure 4.4	Open Bar	Control vs RH vs RI Average Duration (ROSA) D3	Kruskal-Wallis	0.197			0.0163 0.8569 0.2449
Figure 4.4	Open Bar	Control vs RH vs RI Total Distance (ROSA) D1	Kruskal-Wallis	0.0063	Control vs RH Control vs RI RH VS RI	Dunn's	0.0063 0.0809 >0.9999
Figure 4.S8	Open Bar	Control vs RH vs RI Total Distance (ROSA) D2	Kruskal-Wallis	0.2654	Control vs RH Control vs RI RH VS RI		
Figure 4.4	Open Bar	Control vs RH vs RI Total Distance (ROSA) D3	Kruskal-Wallis	0.2163	Control vs RH RH VS RI Control vs RH		

Table 4.S5: Stats for Open BARR part 1.

Table 4.S6:

Figure 4.S8	Open Bar	Control vs RH vs RI Total Duration (RONSA) D1	Kruskal-Wallis	0.8452	Control vs RI RH VS RI Control vs RH Control vs RI		
Figure 4.S8	Open Bar	Control vs RH vs RI Total Duration (RONSA) D2	Kruskal-Wallis	0.1872	RH VS RI Control vs RH Control vs RI		
Figure 4.S8	Open Bar	Control vs RH vs RI Total Duration (RONSA) D3	Kruskal-Wallis	0.0811	RH VS RI Control vs RH Control vs RI		
Figure 4.S8	Open Bar	Control vs RH vs RI Bout Duration (RONSA) D1	Kruskal-Wallis	0.0001	Control vs RI RH VS RI Control vs RI RH VS RI	Dunn's	0.0007 0.0007 >0.9999
Figure 4.S8	Open Bar	Control vs RH vs RI Bout Duration (RONSA) D2	Kruskal-Wallis	0.1093	Control vs RH Control vs RI RH VS RI		
Figure 4.S8	Open Bar	Control vs RH vs RI Bout Duration (RONSA) D3	Kruskal-Wallis	0.6667	Control vs RH Control vs RI RH VS RI		
Figure 4.S8	Open Bar	Control vs RH vs RI Total Distance (RONSA) D1	Kruskal-Wallis	0.0001	Control vs RH Control vs RI RH VS RI		
Figure 4.S8	Open Bar	Control vs RH vs RI Total Distance (RONSA) D2	Kruskal-Wallis	0.0398	RH VS RI Control vs RH Control vs RI	Dunn's	0.2217 0.0423 >0.9999
Figure 4.S8	Open Bar	Control vs RH vs RI Total Distance (RONSA) D3	Kruskal-Wallis	0.282	Control vs RH Control vs RI RH VS RI		
Figure 4.S8	Open Bar	Quartile 1 Control vs RH vs RI Total Duration (ROSA) D1	Kruskal-Wallis	0.0364	Control vs RH Control vs RI RH VS RI	Dunn's	0.1857 0.0414 >0.9999
Figure 4.S10	Open Bar	Quartile 1 Control vs RH vs RI Total Duration (ROSA) D2	Kruskal-Wallis	0.359	Control vs RH Control vs RI RH VS RI		
Figure 4.S10	Open Bar	Quartile 1 Control vs RH vs RI Total Duration (ROSA) D3	Kruskal-Wallis	0.0001	Control vs RI RH VS RI	Dunn's	0.0004 0.031 0.5912
Figure 4.S10	Open Bar	Quartile 1 Control vs RH vs RI Average Duration (ROSA) D1	Kruskal-Wallis	0.1764			
Figure 4.S10	Open Bar	Quartile 1 Control vs RH vs RI Average Duration (ROSA) D2	Kruskal-Wallis	0.0661			
Figure 4.S10	Open Bar	Quartile 1 Control vs RH vs RI Average Duration (ROSA) D3	Kruskal-Wallis	0.0001	Control vs RH Control vs RI RH VS RI	Dunn's	<0.0001 0.3735 0.01
Figure 4.S10	Open Bar	Quartile 1 Control vs RH vs RI Total Distance (ROSA) D1	Kruskal-Wallis	0.2536			
Figure 4.S10	Open Bar	Quartile 1 Control vs RH vs RI Total Distance (ROSA) D2	Kruskal-Wallis	0.9675			
Figure 4.S10	Open Bar	Quartile 1 Control vs RH vs RI Total Distance (ROSA) D3	Kruskal-Wallis	0.2275			
Figure 4.S10	Open Bar	Quartile 1 Control vs RH vs RI Total Duration (RONSA) D1	Kruskal-Wallis	0.022		Dunn's	0.0174 0.5217 0.4676
Figure 4.S10	Open Bar	Quartile 1 Control vs RH vs RI Total Duration (RONSA) D2	Kruskal-Wallis	0.3542			
Figure 4.S10	Open Bar	Quartile 1 Control vs RH vs RI Total Duration (RONSA) D3	Kruskal-Wallis	0.0001		Dunn's	<0.0001 0.0361 0.2414
Figure 4.S10	Open Bar	Quartile 1 Control vs RH vs RI Bout Duration (RONSA) D1	Kruskal-Wallis	0.056			
Figure 4.S10	Open Bar	Quartile 1 Control vs RH vs RI Bout Duration (RONSA) D2	Kruskal-Wallis	0.3121			
Figure 4.S10	Open Bar	Quartile 1 Control vs RH vs RI Bout Duration (RONSA) D3	Kruskal-Wallis	0.0434		Dunn's	>0.9999 0.15 0.0593
Figure 4.S10	Open Bar	Quartile 1 Control vs RH vs RI Total Distance (RONSA) D1	Kruskal-Wallis	0.1178			
Figure 4.S10	Open Bar	Quartile 1 Control vs RH vs RI Total Distance (RONSA) D2	Kruskal-Wallis	0.3313			
Figure 4.S10	Open Bar	Quartile 1 Control vs RH vs RI Total Distance (RONSA) D3	Kruskal-Wallis	0.006		Dunn's	0.0007 0.0176 0.006

Table 4.S6: Stats for Open BARR part 2.

Table 4.S7

	Metric	Source	SS	DF	MS	F	p-unc	p-GG-corr	ng2	eps	sphericity	W-spher	p-spher
	time_in_ROSA	odor	114.0556	2	57.0277998	2.21895119	0.11324042	0.12244299	0.02364066	0.84175538	FALSE	0.81200641	0.00238355
	time_in_ROSA	Error	3032.64011	118	25.70034								
	time_in_RONSA	odor	2131.63094	2	1065.81547	3.05298749	0.05096472	0.05798854	0.03787681	0.87914225	FALSE	0.86252765	0.01372201
	time_in_RONSA	Error	41194.4779	118	349.105745								
	distance_in_ROSA	odor	38248.46	2	19124.23	2.45012365	0.0906621		0.02689134	0.92388715			
	distance_in_ROSA	Error	921038.878	118	7805.41422								
	distance_in_RONSA	odor	289322.988	2	144661.494	2.89191309	0.05941118		0.03529102	0.91877569			
	distance_in_RONSA	Error	5902686.48	118	50022.7667								
	latency_to_reenter_ROSA	odor	1666.61257	2	833.306285	2.29439655	0.10530356		0.02536882	0.94981885			
	latency_to_reenter_ROSA	Error	42856.6464	118	363.191919								
	latency_to_reenter_RONSA	odor	28623.909	2	14311.9545	13.659949	4.61E-06	2.44E-05	0.14339299	0.81988099	FALSE	0.78031079	0.00075121
	latency_to_reenter_RONSA	Error	123632.279	118	1047.73118								
	depth_in_ROSA	odor	2451.21114	2	1225.60557	2.74365551	0.06844169	0.07457948	0.03214309	0.899099	FALSE	0.88777543	0.03168039
	depth_in_ROSA	Error	52711.2303	118	446.705341								
	depth_in_RONSA	odor	2387.92351	2	1193.96176	2.81116365	0.06416828		0.02616585	0.99428996			
	depth_in_RONSA	Error	50117.1417	118	424.72154								
Repeated Measures ANOVA	Metric	group1	group2	meandiff	p-adj	lower	upper	reject					
	latency_to_reenter_RONSA	Dop2R-Cont	Dop2R-RH					FALSE					
	latency_to_reenter_RONSA	Dop2R-Cont	Dop2R-RI					FALSE					
Tukey	latency_to_reenter_RONSA	Dop2R-RH	Dop2R-RI					FALSE					

Table 4.S7 Repeated Measures Anova for Open BARR Metrics Overtime.

Chapter 5

Discussion and Future Directions

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Contributions:

Tariq Brown performed calcium imaging experiments in Figure 4.1, generated Figure 4.1 and composed text.

5.1. ABSTRACT

Alcohol drives plasticity within circuits by influencing circuit dynamics and changing molecular profiles. In this work, I have elucidated the how cues are changing the internal representation of alcohol, and hopefully by extension, other substances of abuse (Chapter 2). We found that repeated exposure to an alcohol-cue pairing generates increased activity within the axon terminals of dopamine neurons, and cue-encoding cells post-synaptic from those dopamine neurons. Additionally, I found that the memory is potentially stabilized and strengthened by the increased number of cue-encoding neurons responding to cue-alcohol pairings. Additionally, I have also been able to illustrate the importance of neuropsychiatric-disease-associated alternative splicing by uncovering behavioral consequences associated with alcohol-induced alternative splicing (Chapter 3). We found that an isoform expressed after alcohol-memory formation drives not only increased motivation for alcohol in multiple contexts but appears to generalize to increased motivation for naturally rewarding substances as well. The methodology implemented in this study will serve as an archetype for future work trying to understand neuropsychiatric diseases by analyzing genes, circuits, and behavior.

5.2. – THE EXPANSION OF DROSOPHILA'S UTILITY WITHIN MULTIPLE FIELDS

This dissertation provides two clear methodologies for studying how a substance of abuse like alcohol is changing plasticity within reward circuitry. Firstly, we implemented the functional imaging to directly observe the acquisition of alcohol memory within dopaminergic circuits. Secondly, we used CRISPR-Cas9 technology to identify how the omission of a single amino acid shifts reward responses across contexts. This dissertation is another testament to the power of the *Drosophila* model and serves as an additional in not only our understanding of alcohol memory formation, but also an example of alternative splicing is behaviorally relevant.

5.3. HETEROGENEOUS DOPAMINERGIC POPULATIONS

One of the barriers to measuring alcohol's effect of neural circuits was the imprecision of tools used in mammalian models. Many studies lack the resolution to properly differentiate subpopulations within large neural populations like the ventral tegmental area (VTA) (Juarez & Han, 2016; Lammel et al., 2014; Nelson et al., 2018; Robinson & Carelli, 2008; You et al., 2018) This inability to home in on subpopulations of neurons via precise genetic manipulations and recordings makes it hard to develop conclusions about their functionality in reward response. We showed that we were able to narrow in on contributions of a well-characterized population of dopamine neurons to the formation of alcohol memory. This is thanks to the expansive genetic toolkit available within the *Drosophila* model. The previously observed dual role for VTA neurons in aversion and reward may also be investigated using *Drosophila*. We might be able to

identify a role for both in response to alcohol observed within mammals may also be elucidated using *Drosophila*. We have also expressed GCaMP7b in a separate subset of dopamine neurons thought to be important for aversive memory formation (Figure 5.1). Like the PAM neurons, the Protocerebral Posterior Later-1 (PPL1) neurons are responsive to both odors and odors paired with intoxicating doses of ethanol ($n = 8$). Unlike the PAM neurons, PPL1 neurons do not change their responses to odor paired alcohol relative to odor or increase their activity in response to odors paired with alcohol. In addition, we can also observe dynamics within single cells using the (Fig A.C.1D and 1E). The resolution at which *Drosophila* researchers can address questions surrounding the involvement of specific circuits has can be a transformative element in our understanding of the development of substance use disorders.

In this work, we were able to observe changes in dopamine release using dLight within the context of cue-alcohol learning. We observed that alcohol-cue pairing generated the largest change in dopamine dynamics within the a3 compartment of the mushroom body. This is interesting, as previous studies have found that the a3 compartment is critical for expression of long-term appetitive memory (Plaçais et al., 2013). Other microcircuits had been identified as being necessary for the retrieval of alcohol memory (Scaplen et al., 2020), and we hypothesized that we would see unique dopamine dynamics within those compartments. It is possible that the dopamine dynamics are occurring outside of the learning context, potentially during consolidation or sleep (as proposed in Scaplen et al., 2020).

5.4. LOCALIZATION OF D2 AND BEHAVIOR

One of the gaps in our understanding between the connection of D2 receptor availability and propensity to consume alcohol. There are many contradicting findings across species, some work stating that decreased D2 receptor availability has no connection with alcohol craving (Gleich et al., 2021; Jangard et al., 2023), while other work has been able to connect low D2 levels to increases in preference for alcohol and increased responsiveness to alcohol-associated cues (Bocarsly et al., 2019; Heinz et al., 2004). We used an intersectional genetics approach (T-STEP) to identify subcellular localization within specific neural populations (Koles et al., 2016). A clear next step for would to be to identify how subcellular localization of receptors are changing with alcohol consumption or alcohol memory formation to see how D2 receptor availability or localization is connected to preference. Although with the current tool, all isoforms are tagged with the reporter, it may be possible to still infer the localization of different isoforms based on exposure to reward-associated cues. One could condition flies to form an alcohol memory and compare Dop2R expression to flies that have received non-associative conditioning, where the odor cue was presented unpaired from ethanol. We know that following alcohol-memory formation Dop2R-RH is decreased, and Dop2R-RI is increased (Figure 4.1A; (Petrucelli et al., 2018)). Changes in Dop2R-localization between the two conditions may provide evidence for differential isoform localization. Additionally, using tools like this, we can also look concurrently at the distribution of both D1-like receptors and D2-like receptors.

Previous studies have shown that rewarding substances like sucrose, and substances of abuse like ethanol and meth increase the expression of D1-like receptors in the

mushroom body without affecting Dop2R expression (Kanno et al., 2021) Recent results have also found bidirectional modifications of dopamine receptor levels depending on internal state within memory relevant circuitry (Hiramatsu et al., 2025). However, they have not been able to quantify the ratio between the two dopamine receptors within the same animal and connect it to behavior.

Additionally, using T-STEP we found expression of Dop2R across a broad range of neural structures including the fan-shaped body and the optic lobe (Figure 4.S2). Splicing of Dop2R may also be impacting spatial information processing through alterations in the visual system (Souza et al., 2018), or by changing circuit dynamics within the navigation-relevant fan-shaped body (Matheson et al., 2022). Though it is important to note that our findings only resolved splicing of Dop2R following alcohol-odor memory formation in the mushroom body.

5.5 ALCOHOL'S ROLE AS DEPRESSED, OR STIMULANT?

Alcohol is classified as a depressant though has multiple effects associated with stimulants (Hendler et al., 2013). We observed alcohol acting in a stimulatory fashion when presented alongside an odor cue (Chapter 2 Figures 2.2, 2.5, 2.7), however we observed more depressant qualities when alcohol was presented alone (Chapter 2, Figure 2.13F). Alcohol alone was not sufficient to generate increased activity across repeated exposure in cue-encoding neurons or in dopaminergic neurons. This is in contrast to alcohol paired with a cue generating strong potentiation within the axon terminals of the MB, and more mild increases with the dopamine neurons. It is possible

that context is important for dictating the pharmacological effects of alcohol. These findings would help delineate the seeming paradoxical behavioral impacts of alcohol.

5.6. COMPLEX ODOR SUPPRESSION OF MB ACTIVITY

The suppression of activity within MB was an unexpected consequence during presentation of a complex odor. Possible mechanisms underlying this depression is the lateral inhibition coming from MB neurons onto each other acting through nAChRB3 receptors. Or Over activation of Kenyon cells by two food relevant odors causes increased release of GABA release from the anterior paired lateral neuron (APL) to normalize odor-evoked activity (Prisco et al., 2021)

5.7. INVOLVEMENT OF APL/DPM

The GABAergic APL neuron and serotonin and GABA releasing dorsal paired medial neuron (Lee et al., 2011) densely innervate the mushroom body and are electrically coupled (Wu et al., 2011). APL neurons express Dop2R, and the increased release of dopamine during alcohol-odor pairing causes decreased APL activity, and subsequently less GABAergic inhibition of the MB. A possible source for the increased strength of the memory is enabled by serotonin increasing the associative window between the rewarding properties of alcohol and the odor stimulus allowing for more MB neurons to become responsive to alcohol (Zeng et al., 2023).

5.8. BEHAVIORALLY RELEVANT SUBTLE CHANGES IN SPLICING

There have been many studies that have made connections between D2 receptors and AUD, whether that be through genome-wide association studies, splicing within the D2 receptor following alcohol consumption (Oomizu et al., 2003; Petruccelli et al., 2018), or D2 receptor availability changing alcohol sensitivity and influencing alcohol cravings (Bocarsly et al., 2019; Heinz et al., 2004). The mechanism behind which D2 receptors were modifying alcohol response, and likely reward behavior in general (Bulwa et al., 2011), remained poorly elucidated. Here we found a direct connection between splice isoform, and increased reward motivation in multiple contexts. Additionally, we were able to provide evidence that the isoforms functionality is likely dependent on a serine within the 3rd intracellular loop. This is a novel finding not just in the context of reward, but also broader implications to the role alternative splicing may be playing in determining behavior. The removal of a single serine from Dop2R was sufficient to drive a massive shift in behavior. The level of impact given the seemingly subtle nature of the splicing brings into question how subtle changes in other genes may be generating large shifts in behavior.

A clear next set of experiments would be to merge the *Drosophila* reagents used in Chapter 3, with the techniques of Chapter 2 to look directly at how splicing is changing the internal representation of alcohol and other rewards. This can be accomplished by crossing GCaMP7b and Gal4 reagents into the background of the Dop2R splice mutants. This would allow for us to confirm whether Dop2R-RI's impact on behavior is a result of decreased signaling, resulting in less inhibition of reward-relevant circuitry. Additionally, if the Dop2R-S658A mutants phenocopy the decreased inhibition, we

would have more solid evidence to conclude that the serine is a functionally relevant for Dop2R's functionality. These findings would be among the first connecting splice isoforms to both neural physiology and behavioral consequences.

The strategy of making splice specific mutations, observing behavioral differences, and uncovering the underlying neural dynamics, can be implemented to identify the behavioral relevance of other genes significantly alternatively spliced following alcohol odor training. This includes proteins like *Stat92E* and *shibire* (Petrucelli et al., 2018, Petrucelli et al., 2020). *Stat92E* is also a memory relevant transcription (Copf et al., 2011), and *shibire* is a dynamin-like protein that is important for synaptic transmission (van der Bliek & Meyerowitz, 1991). The diversity of types of proteins alcohol odor memory formation is impacting

5.9. CAS13 AS A TOOL TO HELP RESOLVE THE BEHAVIORAL CONSEQUENCES OF ALTERNATIVE SPLICING

Due to the nature of the splicing event within *Drosophila* Dop2R, we were unable to develop tools with high enough resolution to tag the specific isoforms. However, there are newer tools that might be able to provide the resolution to not only tag the specific isoforms, but also modify them solely at the mRNA level (Kelner et al., 2019), and not the genomic level like CRISPR. This would allow for high spatial and temporal resolution for the modification of RNAs. We could potentially force switches in Dop2R splice isoform expression, something difficult to achieve using traditional approaches. This approach would unlock refined experiments looking at the evolution of behavior

because of the splicing event within individuals. Within the same a single fly, we could gauge initial reward response and see if we can alter it using Cas-13-mediated, time-locked, ablation/knockdown of specific isoforms. This could even apply to broader neuropsychiatric diseases that have aberrant splicing associated with them. We could screen for potential antisense oligonucleotides by using Cas13 to knockdown isoforms in disease models. This approach could help make large advancements in treatment of splicing defects in diseases like Alzheimer's, Parkinson's and Huntington's disease. (Dredge et al., 2001, Glatt et al., 2011, Nikom and Zheng).

The results from both chapter 2 and chapter 3 converge to elucidate the role dopaminergic signaling in formation and of alcohol memory, and its enduring properties. Alcohol is recruiting more cells to respond to cues associated with alcohol, and within those cells, memory-relevant genes like Dop2R are being alternatively spliced. We know this splicing pattern is not observed when flies are presented with vs odors (Figure 3.1A and 3.1B). We know that there is increased activity within *Drosophila* mushroom bodies during odor paired with intoxicating doses of ethanol. Is it possible that the splicing is not driven by ethanol, but rather activity? Perhaps the compartment specific increase in the release dopamine during odor-alcohol may be influencing MB activity, and subsequent activity dependent splicing events.

5.10 CONCLUSION

The co-opting of memory circuitry during the formation of drug-associative memories is a complex process. Understanding this phenomenon has been slowed by conflicting results within mammalian systems on the impact of alcohol, and the lack of tools to adequately access circuit dynamics. This work has made great strides in understanding

the signaling dynamics between dopaminergic neurons and cue encoding neurons during the formation of alcohol memory. Dopaminergic neurons increase their activity in response to repeated alcohol-odor cue pairings, potentially driving increased plasticity within downstream memory relevant circuitry. We also provide evidence that cue-encoding neurons are recruited to generate long-lasting, intense memories. Additionally, we have produced a major finding within the splicing field by connecting the removal of a single amino acid in a memory-relevant protein to dramatic shifts in behavior. Together these results elucidate the consequences of plasticity that occurs within memory circuits following alcohol memory formation and advance the fields understanding of context-dependent representation of reward, and behaviorally relevant splicing events.

5.11 Chapter References

- Bocarsly, M. E., da Silva E Silva, D., Kolb, V., Luderman, K. D., Shashikiran, S., Rubinstein, M., Sibley, D. R., Dobbs, L. K., & Alvarez, V. A. (2019). A Mechanism Linking Two Known Vulnerability Factors for Alcohol Abuse: Heightened Alcohol Stimulation and Low Striatal Dopamine D2 Receptors. *Cell Reports*, 29(5), 1147-1163.e5.
<https://doi.org/10.1016/j.celrep.2019.09.059>
- Bulwa, Z. B., Sharlin, J. A., Clark, P. J., Bhattacharya, T. K., Kilby, C. N., Wang, Y., & Rhodes, J. S. (2011). Increased consumption of ethanol and sugar water in mice lacking the dopamine D2 long receptor. *Alcohol (Fayetteville, N.Y.)*, 45(7), 631–639.
<https://doi.org/10.1016/j.alcohol.2011.06.004>
- Copf, T., Goguel, V., Lampin-Saint-Amaux, A., Scaplehorn, N., & Preat, T. (2011). Cytokine signaling through the JAK/STAT pathway is required for long-term memory in *Drosophila*. *Proceedings of the National Academy of Sciences of the United States of America*, 108(19), 8059–8064. <https://doi.org/10.1073/pnas.1012919108>
- Gleich, T., Spitta, G., Butler, O., Zacharias, K., Aydin, S., Sebold, M., Garbusow, M., Rapp, M., Schubert, F., Buchert, R., Heinz, A., & Gallinat, J. (2021). Dopamine D2/3 receptor availability in alcohol use disorder and individuals at high risk: Towards a dimensional approach. *Addiction Biology*, 26(2), e12915.
<https://doi.org/10.1111/adb.12915>
- Heinz, A., Siessmeier, T., Wrase, J., Hermann, D., Klein, S., Grüsser, S. M., Flor, H., Braus, D. F., Buchholz, H. G., Gründer, G., Schreckenberger, M., Smolka, M. N., Rösch, F., Mann, K., & Bartenstein, P. (2004). Correlation between dopamine D(2) receptors in

- the ventral striatum and central processing of alcohol cues and craving. *The American Journal of Psychiatry*, 161(10), 1783–1789.
<https://doi.org/10.1176/appi.ajp.161.10.1783>
- Hendler, R. A., Ramchandani, V. A., Gilman, J., & Hommer, D. W. (2013). Stimulant and sedative effects of alcohol. *Current Topics in Behavioral Neurosciences*, 13, 489–509. https://doi.org/10.1007/7854_2011_135
- Hiramatsu, S., Saito, K., Kondo, S., Katow, H., Yamagata, N., Wu, C.-F., & Tanimoto, H. (2025). Synaptic enrichment and dynamic regulation of the two opposing dopamine receptors within the same neurons. *eLife*, 13, RP98358.
<https://doi.org/10.7554/eLife.98358.3>
- Jangard, S., Jayaram-Lindström, N., Isacson, N. H., Matheson, G. J., Plavén-Sigra, P., Franck, J., Borg, J., Farde, L., & Cervenka, S. (2023). Striatal dopamine D2 receptor availability as a predictor of subsequent alcohol use in social drinkers. *Addiction (Abingdon, England)*, 118(6), 1053–1061. <https://doi.org/10.1111/add.16144>
- Juarez, B., & Han, M.-H. (2016). Diversity of Dopaminergic Neural Circuits in Response to Drug Exposure. *Neuropsychopharmacology*, 41(10), 2424–2446.
<https://doi.org/10.1038/npp.2016.32>
- Kanno, M., Hiramatsu, S., Kondo, S., Tanimoto, H., & Ichinose, T. (2021). Voluntary intake of psychoactive substances is regulated by the dopamine receptor Dop1R1 in *Drosophila*. *Scientific Reports*, 11(1), 3432. <https://doi.org/10.1038/s41598-021-82813-0>

- Koles, K., Yeh, A. R., & Rodal, A. A. (2016). Tissue-specific tagging of endogenous loci in *Drosophila melanogaster*. *Biology Open*, 5(1), 83–89.
<https://doi.org/10.1242/bio.016089>
- Lammel, S., Lim, B. K., & Malenka, R. C. (2014). Reward and aversion in a heterogeneous midbrain dopamine system. *Neuropharmacology*, 76, 351–359.
<https://doi.org/10.1016/j.neuropharm.2013.03.019>
- Lee, P.-T., Lin, H.-W., Chang, Y.-H., Fu, T.-F., Dubnau, J., Hirsh, J., Lee, T., & Chiang, A.-S. (2011). Serotonin-mushroom body circuit modulating the formation of anesthesia-resistant memory in *Drosophila*. *Proceedings of the National Academy of Sciences of the United States of America*, 108(33), 13794–13799.
<https://doi.org/10.1073/pnas.1019483108>
- Matheson, A. M. M., Lanz, A. J., Medina, A. M., Licata, A. M., Currier, T. A., Syed, M. H., & Nagel, K. I. (2022). A neural circuit for wind-guided olfactory navigation. *Nature Communications*, 13(1), 4613. <https://doi.org/10.1038/s41467-022-32247-7>
- Nelson, A. C., Williams, S. B., Pistorius, S. S., Park, H. J., Woodward, T. J., Payne, A. J., Obray, J. D., Shin, S. I., Mabey, J. K., & Steffensen, S. C. (2018). Ventral Tegmental Area GABA Neurons Are Resistant to GABA(A) Receptor-Mediated Inhibition During Ethanol Withdrawal. *Frontiers in Neuroscience*, 12.
<https://doi.org/10.3389/fnins.2018.00131>
- Oomizu, S., Boyadjieva, N., & Sarkar, D. K. (2003). Ethanol and estradiol modulate alternative splicing of dopamine D2 receptor messenger RNA and abolish the inhibitory action of bromocriptine on prolactin release from the pituitary gland.

- Alcoholism, Clinical and Experimental Research*, 27(6), 975–980.
- <https://doi.org/10.1097/01.ALC.0000071743.57855.BE>
- Petrucelli, E., Feyder, M., Ledru, N., Jaques, Y., Anderson, E., & Kaun, K. R. (2018). Alcohol Activates Scabrous-Notch to Influence Associated Memories. *Neuron*, 100(5), 1209–1223.e4. <https://doi.org/10.1016/j.neuron.2018.10.005>
- Plačais, P.-Y., Trannoy, S., Friedrich, A. B., Tanimoto, H., & Preat, T. (2013). Two pairs of mushroom body efferent neurons are required for appetitive long-term memory retrieval in *Drosophila*. *Cell Reports*, 5(3), 769–780.
- <https://doi.org/10.1016/j.celrep.2013.09.032>
- Prisco, L., Deimel, S. H., Yeliseyeva, H., Fiala, A., & Tavosanis, G. (2021). The anterior paired lateral neuron normalizes odour-evoked activity in the *Drosophila* mushroom body calyx. *eLife*, 10, e74172. <https://doi.org/10.7554/eLife.74172>
- Robinson, D. L., & Carelli, R. M. (2008). Distinct subsets of nucleus accumbens neurons encode operant responding for ethanol versus water. *The European Journal of Neuroscience*, 28(9), 1887–1894. <https://doi.org/10.1111/j.1460-9568.2008.06464.x>
- Scaplen, K. M., Talay, M., Nunez, K. M., Salamon, S., Waterman, A. G., Gang, S., Song, S. L., Barnea, G., & Kaun, K. R. (2020). Circuits that encode and guide alcohol-associated preference. *eLife*, 9, e48730. <https://doi.org/10.7554/eLife.48730>
- Souza, B. O. F., Abou Rjeili, M., Quintana, C., Beaulieu, J. M., & Casanova, C. (2018). Spatial Frequency Selectivity Is Impaired in Dopamine D2 Receptor Knockout Mice. *Frontiers in Integrative Neuroscience*, 11. <https://doi.org/10.3389/fnint.2017.00041>

- van der Bliek, A. M., & Meyerowitz, E. M. (1991). Dynamin-like protein encoded by the *Drosophila shibire* gene associated with vesicular traffic. *Nature*, 351(6325), 411–414. <https://doi.org/10.1038/351411a0>
- Wu, C.-L., Shih, M.-F. M., Lai, J. S.-Y., Yang, H.-T., Turner, G. C., Chen, L., & Chiang, A.-S. (2011). Heterotypic Gap Junctions between Two Neurons in the *Drosophila* Brain Are Critical for Memory. *Current Biology*, 21(10), 848–854. <https://doi.org/10.1016/j.cub.2011.02.041>
- You, C., Vandegrift, B., & Brodie, M. S. (2018). Ethanol actions on the ventral tegmental area: Novel potential targets on reward pathway neurons. *Psychopharmacology*, 235(6), 1711–1726. <https://doi.org/10.1007/s00213-018-4875-y>
- Zeng, J., Li, X., Zhang, R., Lv, M., Wang, Y., Tan, K., Xia, X., Wan, J., Jing, M., Zhang, X., Li, Y., Yang, Y., Wang, L., Chu, J., Li, Y., & Li, Y. (2023). Local 5-HT signaling bi-directionally regulates the coincidence time window for associative learning. *Neuron*, 111(7), 1118-1135.e5. <https://doi.org/10.1016/j.neuron.2022.12.034>

Chapter 5.12 : CHAPTER 5 FIGURES

Figure 5.1

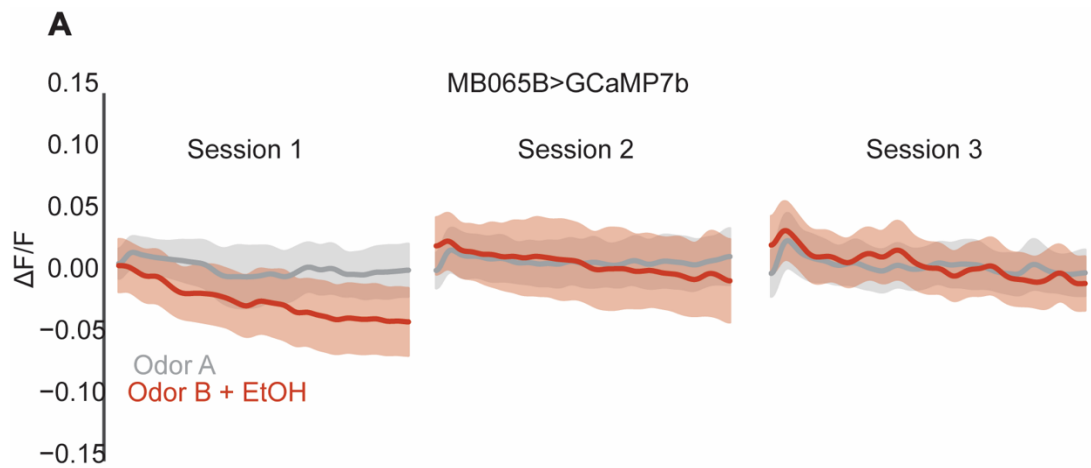


Figure 5.1 – Acquisition of odor-alcohol odor memory in PPL1 neurons

MB065B was used to drive GCaMP7b in PPL1 neurons. (Left) Session 1 responses to odor alone (gray) and odor paired with ethanol (red). (Middle) Session 2 responses to odor alone and odor paired with ethanol. (Right) Session 3 responses to odor alone and odor paired with ethanol. Bold line represents mean and shaded line represents SEM. $n = 8$.

Appendix A:

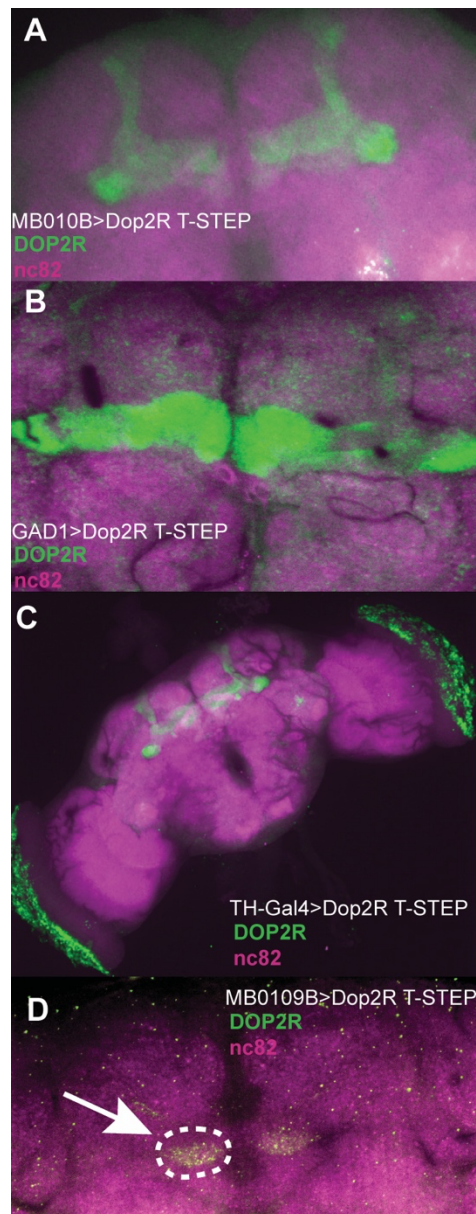
Tissue-specific Tagging of *Drosophila* D2-like receptor

A.A.1 RESULTS

Previous studies have shown that *Drosophila Dopamine-2-like receptor* is broadly expressed across the *Drosophila* central nervous system using an intersectional genetics approach. Here, we implement a different intersectional genetics approach (Koles et al., 2016) to uncover subcellular localization of DOP2R. Using a MB010B split-gal4 (Bräcker et al., 2013), we were able to recapitulate previous findings of DOP2R within the mushroom bodies. We next wanted to know whether Dop2R might be acting as an autoreceptor in adult *Drosophila* dopaminergic neurons as suggested. Previous studies have investigated the role of Dop2R in suppressing the activity of the GABAergic anterior paired lateral neuron (APL) during olfactory learning (Zhou et al., 2019), but were unable to uncover the subcellular localization of Dop2R within the APL. We hypothesized that Dop2R would be broadly expressed throughout the APL neuron. We used *GAD1-Gal4* to drive expression T-STEP-tagged DOP2R. We uncovered expression of Dop2R throughout a putatively GABAergic structure that densely innervates the mushroom bodies (Fig A.B.1B). This anatomical structure is highly consistent with that of the APL neuron (Prisco et al., 2021). Though, APL neuron has highly interconnected innervation patterns with the electrically coupled dorsal paired medial neuron (DPM; (Wu et al., 2011)) which also expresses GAD1 and releases GABA (Aso et al., 2019; Haynes et al., 2015). As such, it is difficult to determine the exact identity of the putative GABAergic neuron(s) innervating the mushroom body. Previous work done in mice, rats, and guinea pigs have found that mammalian Dopamine-2 receptor functions as an autoreceptor for ventral tegmental area dopaminergic neurons (Anzalone et al., 2012; Courtney et al., 2012; Ford, 2014). Work

in *Drosophila* larva has also suggested that Dop2R acts as an autoreceptor (Vickrey & Venton, 2011). Using TH-Gal4, we were able to confirm expression within the axon terminals of the dopaminergic neurons innervating the mushroom body. Results from our lab has also implicated the importance of Dop2R within the and PAM- β^2 a>MBON- β^2 mp and MBON- β^2 β^2 a microcircuit for the retrieval of alcohol-cue-induced memory (Scaplen et al., 2020). We hypothesized that Dop2R would be expressed in PAM- β^2 a. We observed punctate expression of Dop2R specifically within the axon terminals of PAM- β^2 a (Fig A.B.1D.)

A.A.2 APPENIX A FIGURE



A.A.2 Figure Subcellular localization of Dop2R using an intersectional genetics approach

(A) Expression of endogenously tagged Dop2R using mushroom body split gal4 driver *MB010B*. (B) expression of endogenous Dop2R within GABAergic neurons using *GAD1-gal4*. (C) Expression of Dop2R within dopaminergic populations using *TH-gal4*. (D) Expression of Dop2R within the MB109B split gal4, revealing subcellular localization to the axon terminals (outlined) of the PAM-β`2a.

A.A.2 REFERENCES

- Anzalone, A., Lizardi-Ortiz, J. E., Ramos, M., Mei, C. D., Hopf, F. W., Iaccharino, C., Halbout, B., Jacobsen, J., Kinoshita, C., Welter, M., Caron, M. G., Bonci, A., Sulzer, D., & Borrelli, E. (2012). Dual Control of Dopamine Synthesis and Release by Presynaptic and Postsynaptic Dopamine D2 Receptors. *Journal of Neuroscience*, 32(26), 9023–9034. <https://doi.org/10.1523/JNEUROSCI.0918-12.2012>
- Aso, Y., Ray, R. P., Long, X., Bushey, D., Cichewicz, K., Ngo, T.-T., Sharp, B., Christoforou, C., Hu, A., Lemire, A. L., Tillberg, P., Hirsh, J., Litwin-Kumar, A., & Rubin, G. M. (2019). Nitric oxide acts as a cotransmitter in a subset of dopaminergic neurons to diversify memory dynamics. *eLife*, 8, e49257. <https://doi.org/10.7554/eLife.49257>
- Bräcker, L. B., Siju, K. P., Varela, N., Aso, Y., Zhang, M., Hein, I., Vasconcelos, M. L., & Grunwald Kadow, I. C. (2013). Essential role of the mushroom body in context-dependent CO₂ avoidance in *Drosophila*. *Current Biology: CB*, 23(13), 1228–1234. <https://doi.org/10.1016/j.cub.2013.05.029>
- Courtney, N. A., Mamaligas, A. A., & Ford, C. P. (2012). Species differences in somatodendritic dopamine transmission determine D2-autoreceptor-mediated inhibition of ventral tegmental area neuron firing. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 32(39), 13520–13528. <https://doi.org/10.1523/JNEUROSCI.2745-12.2012>

- Ford, C. P. (2014). The role of D2-autoreceptors in regulating dopamine neuron activity and transmission. *Neuroscience*, 282, 13–22.
<https://doi.org/10.1016/j.neuroscience.2014.01.025>
- Haynes, P. R., Christmann, B. L., & Griffith, L. C. (2015). A single pair of neurons links sleep to memory consolidation in *Drosophila melanogaster*. *eLife*, 4, e03868.
<https://doi.org/10.7554/eLife.03868>
- Koles, K., Yeh, A. R., & Rodal, A. A. (2016). Tissue-specific tagging of endogenous loci in *Drosophila melanogaster*. *Biology Open*, 5(1), 83–89.
<https://doi.org/10.1242/bio.016089>
- Prisco, L., Deimel, S. H., Yeliseyeva, H., Fiala, A., & Tavosanis, G. (2021). The anterior paired lateral neuron normalizes odour-evoked activity in the *Drosophila* mushroom body calyx. *eLife*, 10, e74172. <https://doi.org/10.7554/eLife.74172>
- Scaplen, K. M., Talay, M., Nunez, K. M., Salamon, S., Waterman, A. G., Gang, S., Song, S. L., Barnea, G., & Kaun, K. R. (2020). Circuits that encode and guide alcohol-associated preference. *eLife*, 9, e48730. <https://doi.org/10.7554/eLife.48730>
- Vickrey, T. L., & Venton, B. J. (2011). *Drosophila* Dopamine2-like receptors function as autoreceptors. *ACS Chemical Neuroscience*, 2(12), 723–729.
<https://doi.org/10.1021/cn200057k>
- Wu, C.-L., Shih, M.-F. M., Lai, J. S.-Y., Yang, H.-T., Turner, G. C., Chen, L., & Chiang, A.-S. (2011). Heterotypic Gap Junctions between Two Neurons in the *Drosophila* Brain Are Critical for Memory. *Current Biology*, 21(10), 848–854.
<https://doi.org/10.1016/j.cub.2011.02.041>

Zhou, M., Chen, N., Tian, J., Zeng, J., Zhang, Y., Zhang, X., Guo, J., Sun, J., Li, Y., Guo, A., & Li, Y. (2019). Suppression of GABAergic neurons through D2-like receptor secures efficient conditioning in *Drosophila* aversive olfactory learning. *Proceedings of the National Academy of Sciences*, 116(11), 5118–5125.
<https://doi.org/10.1073/pnas.1812342116>

Appendix B

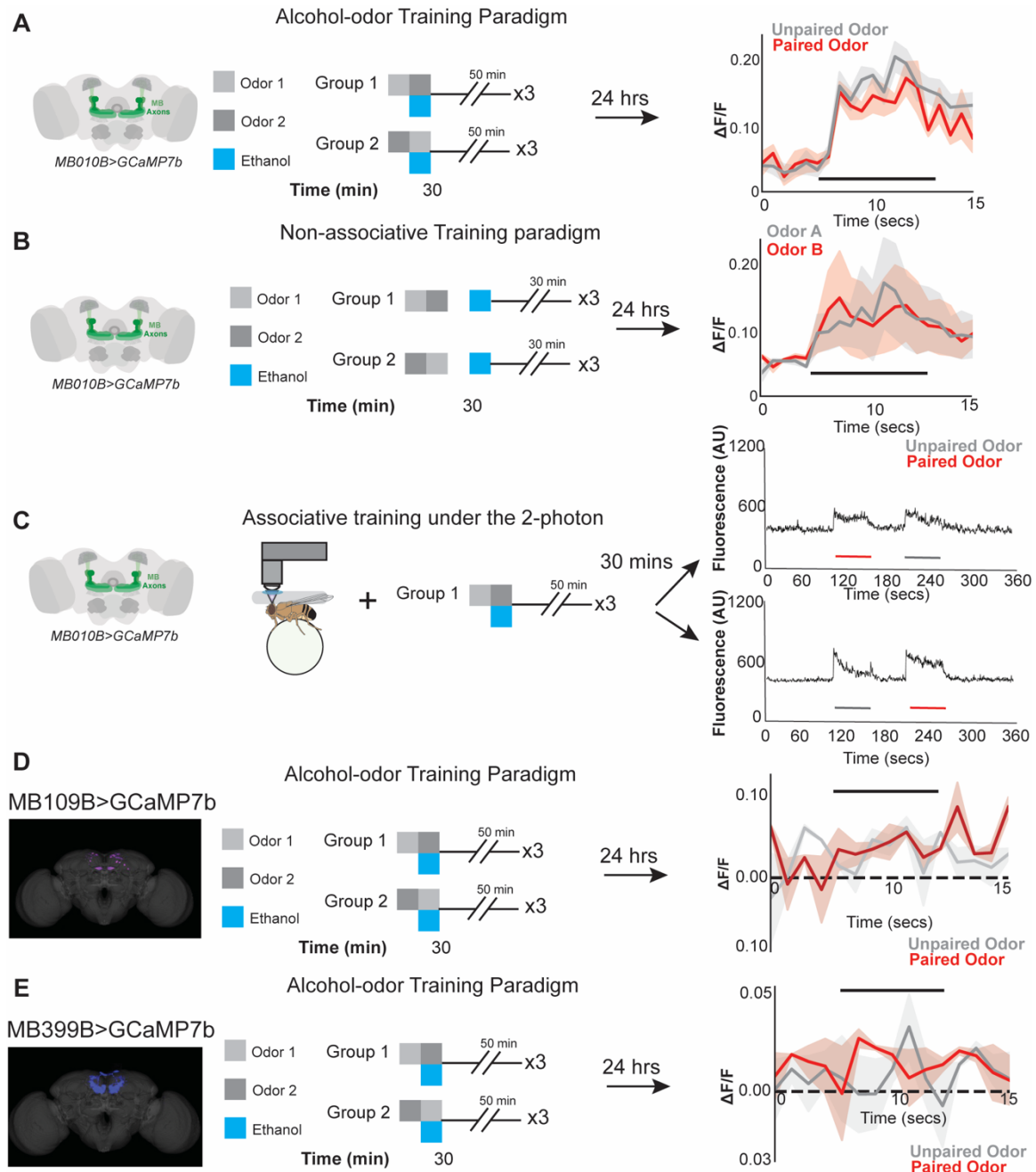
Retrieval of alcohol memory in Mushroom Body circuitry

A.B.1 RESULTS

The work presented in this dissertation's second chapter uncovered the changes in activity of reward-encoding circuitry during the acquisition of alcohol memory. We found that dopaminergic neurons and cue-encoding neurons increase their activity in response to odor paired with pharmacologically relevant doses of vaporized alcohol. However, whether this plasticity is maintained during cue-induced alcohol memory is yet to be seen within *Drosophila*. Previous literature has shown that alcohol-associated cues generate increased c-fos reactivity within rat medial prefrontal cortex (Pfarr et al., 2015), and that rat ventral tegmental area neurons increase their activity during acquisition and reinstatement of reward response after extinction (Liu et al., 2020), but the expression of alcohol memory has yet to be investigated in reward-encoding dopaminergic neurons, and post-synaptic cue-encoding neurons. Work from our lab has identified microcircuits important for the retrieval of alcohol memory (Scaplen et al., 2020). We sought to understand the neural dynamics underlying the expression of alcohol memory within relevant circuits. Using the split Gal4 *MB010B*, we expressed GCaMP7b in the *Drosophila* mushroom bodies, which are known to be important for alcohol memory retrieval (Kaun et al., 2011; Petruccelli et al., 2018). We trained MB010B>GCaMP7b expressing flies using an established odor-alcohol conditioning protocol in a behavior box (Kaun et al., 2011; Nunez et al., 2018), and recorded from mushroom body neurons 24 hours later (A.C.1A). We presented the flies with 2 mins of air, followed by 5 seconds of either the paired or unpaired odor, then 30 seconds of air followed by the other odor. Unexpectedly, we observed no change in calcium dynamics between the paired odor and unpaired odor (Figure A.C.1A, Wilcoxon p-value = 0.354, n

= 16). We also observed that following non-associative training, there were no changes in calcium dynamics between the paired and unpaired odor. (Figure A.C.1B, Wilcoxon p-value = 0.74, n = 8). The failure resolve differences between the two stimuli may be due to the context in which the cues are being presented under the scope being too different from the context of the behavioral training boxes. We next sought to observe retrieval of aversive alcohol memory immediately following associative training under the scope (Figure A.C.1C, n = 2). Though the sample size is small, there is some evidence to suggest that the paired odor may be causing sustained responses within the MB axon terminals not observed with the unpaired odor. These should make for interesting follow-up experiments. We also wanted to see if calcium dynamics may be restricted to either downstream or upstream populations from the Kenyon cells, so we analyzed calcium dynamics of retrieval in a retrieval relevant microcircuit, PAM-B`2a (MB109B), MBON-B2B`2a (MB399B) (Scaplen et al., 2020). Again, we foundk no differences in calcium dynamics between the paired and unpaired odor. Potentially due to context being important for the retrieval of alcohol-odor memories.

A.B.2. FIGURE



A.B.2 Figure – Calcium dynamics of retrieval.

(A) (Left) Cartoon depicting a dorsal view of the *Drosophila* brain. Green are the axon terminals of the mushroom body. (Middle) Alcohol associative training paradigm used

prior to functional imaging. (Right) *MB010B>GCaMP7b* calcium responses to the unpaired and paired odor (n = 16). (B). (Left) Cartoon depicting a dorsal view of the *Drosophila* brain. Green are the axon terminals of the mushroom body. (Middle) Non-associative training paradigm used prior to functional imaging. (Right) Sham trained flies response to odors A and B (n = 8). (C) (Left) Cartoon depicting a dorsal view of the *Drosophila* brain. Green are the axon terminals of the mushroom body. (Middle) Alcohol associative training paradigm during functional imaging, and prior to retrieval of aversive memory 30 minutes later. (Right) Sample traces from 2 different flies following odor-associative training under the scope. Red underline represents presentation of paired odor, and gray underline represents presentation of the unpaired odor. (D) (Left) Virtual Fly Brain representation of *MB109B* split Gal4's expression pattern. (Middle) Alcohol associative training paradigm used prior to exposure to odors under the scope. (Right) *MB109B>GCaMP7b* axon terminal responses to paired and unpaired odors (n = 8). (E) (Left) Virtual Fly Brain representation of *MB399B* split Gal4's expression pattern. (Middle) Alcohol associative training paradigm used prior to exposure to odors under the scope. (Right) *MB399B>GCaMP7b* axon terminal responses to paired and unpaired odors (n = 8). Bold lines represent mean and shaded line represents SEM.

A.B.3 REFERENCES

- Kaun, K. R., Azanchi, R., Maung, Z., Hirsh, J., & Heberlein, U. (2011). A *Drosophila* model for alcohol reward. *Nature Neuroscience*, 14(5), 612–619.
<https://doi.org/10.1038/nn.2805>
- Liu, Y., Montgomery, S. E., Juarez, B., Morel, C., Zhang, S., Kong, Y., Calipari, E. S., Nestler, E. J., Zhang, L., & Han, M.-H. (2020). Different adaptations of dopamine release in Nucleus Accumbens shell and core of individual alcohol drinking groups of mice. *Neuropharmacology*, 175, 108176.
<https://doi.org/10.1016/j.neuropharm.2020.108176>
- Petrucelli, E., Feyder, M., Ledru, N., Jaques, Y., Anderson, E., & Kaun, K. R. (2018). Alcohol Activates Scabrous-Notch to Influence Associated Memories. *Neuron*, 100(5), 1209-1223.e4. <https://doi.org/10.1016/j.neuron.2018.10.005>
- Pfarr, S., Meinhardt, M. W., Klee, M. L., Hansson, A. C., Vengeliene, V., Schönig, K., Bartsch, D., Hope, B. T., Spanagel, R., & Sommer, W. H. (2015). Losing Control: Excessive Alcohol Seeking after Selective Inactivation of Cue-Responsive Neurons in the Infralimbic Cortex. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 35(30), 10750–10761.
<https://doi.org/10.1523/JNEUROSCI.0684-15.2015>
- Scaplen, K. M., Talay, M., Nunez, K. M., Salamon, S., Waterman, A. G., Gang, S., Song, S. L., Barnea, G., & Kaun, K. R. (2020). Circuits that encode and guide alcohol-associated preference. *eLife*, 9, e48730. <https://doi.org/10.7554/eLife.48730>