CURRICULUM VITAE

CATHERINE (CASSIE) STAPF

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DEGREE AND DATE TO BE CONFERRED: PH.D. IN NEUROSCIENCE, FALL 2024

EDUCATION

PhD University of Maryland, Baltimore — August 2019-October 2024
Program in Neuroscience
PhD Candidate, GPA: 4.0

BS University of Scranton, Neuroscience — 2015-2019
GPA: 3.95, *Summa Cum Laude*
Second Major in Philosophy, Minor in Chemistry

RESEARCH EXPERIENCE

University of Maryland, Baltimore, Baltimore, MD — 2020-2024
Dissertation, **Advisor**: Donna Calu
Dissertation Committee: Joseph Cheer, Melanie Pina, Brian Mathur, Marco Venniro

University of Scranton, Scranton, PA — 2016-2019
Research Volunteer, Lab of Patrick Orr
Summer Research Intern, Lab of Kate Stumpo — 2018

Drexel University, Philadelphia, PA — 2017
Summer Undergraduate Research Fellowship, Lab of Andreia Mortensen

RESEARCH PUBLICATIONS

Bacharach, S.S, Martin, D.A, **Stapf, C.A**, Sun, F., Li, Y., Cheer, J.F., Calu, D.J. (2023) Decreased ventral tegmental area CB1R signaling reduces sign-tracking and shifts cue-outcome dynamics in rat nucleus accumbens. J Neuro 43 (25) 4684-4696; https://doi.org/10.1523/JNEUROSCI.1486-22.2023

Ruiz-Whalen DM, Aichele CP, Dyson ER, Gallen KC, Stark JV, Saunders JA, Simonet JC, Ventresca EM, Fuentes IM, Marmol N, Moise E, Neubert BC, Riggs DJ, Self AM, Alexander JI, Boamah E, Browne AJ, Correa I, Foster MJ… **Stapf CA**, Tondapu T, Tsiobikas C, Habas R, O'Reilly AM. (2023) Gaining Wings to FLY: Using Drosophila Oogenesis as an Entry Point for Citizen Scientists in Laboratory Research. Methods Mol Biol. 2626:399-444. https://doi.org/10.1007/978-1-0716-2970-3_22

Choi, E.Y., Franco, D., **Stapf, C.A.**, McGlincy, M., Chow, A., Cover, K.C., Chandra, R., Lobo, M.K. (2023) Inducible CRISPR Epigenome Systems Mimic Cocaine Induced Bidirectional Regulation of Nab2 and Egr3. J Neuro 43 (13) 2242-2259; https://doi.org/10.1523/JNEUROSCI.1802-22.2022

Stapf, C.A., Keefer, S.E., McInerney, J.M., Cheer, J.F., Calu, D.J. (2024) Dorsomedial Striatum CB1R signaling is required for Pavlovian outcome devaluation in male Long Evans rats and reduces inhibitory synaptic transmission in both sexes. bioRxiv https://www.biorxiv.org/content/10.1101/2024.05.01.592059v2

ABSTRACTS

Stapf,C.A., Walter,K.N., Orr, P.T (2017). Acute restraint stress impairs object recognition memory in female mice. *Society for Neuroscience Abstracts*. Program #424.15.

Milewski, T.M., Patel,S., **Stapf,C.A.**,Wierbowski,S.N., Orr, P.T. (2017) Acute exposure to acetaminophen impairs object recognition memory in mice. *Society for Neuroscience Abstracts*, Program #424.09.

Stapf,C.A., Wislotsky,A.A., Orr,P.T. (2019) Acute acetaminophen exposure impairs object recognition memory in female mice. *Society for Neuroscience Abstracts*, Program #603.25

Bacharach, S.Z., Martin, D.A., **Stapf, C.A.**, Calu, D.J. (2021) The role of VTA cannabinoid receptor-1 in modulating dopamine and sign-tracking rats. *Pavlovian Society Conference Symposium*

Stapf C.A., Calu, D.J. (2022). Sex Differences in Endocannabinoid Regulation of Behavioral Flexibility. *Gordon Research Conference: Neurobiology of Addiction*

Stapf C.A., Keefer, S.E., McInerney, J., Calu, D.J. (2023) Cannabinoid-1-receptor signaling promotes sensitivity to devaluation in male, but not female, sign-tracking rats. *Eastern Psychological Association Annual Meeting*

Stapf C.A., Keefer, S.E., McInerney, J., Calu, D.J. (2023) Sex Differences in Endocannabinoid Regulation of Behavioral Flexibility. *International Behavioral Neuroscience Society Annual Meeting*

Stapf, C.A., Keefer, S.E., McInerney, J., Calu, D.J. (2023) Cannabinoid receptor-1 signaling promotes sensitivity to devaluation in male, but not female, sign-tracking rats. *Society for Neuroscience Abstracts,* Program #290.17

Stapf, C.S., Keefer, S.E., McInerney, J.M., Cheer, J.F., Calu, D.J. (2024) Sex-specific effects of dorsomedial striatal cannabinoid receptor-1 signaling on Pavlovian Outcome Devaluation. *Society for Neuroscience Abstracts.* Program #183.25

PRESENTATIONS AND INVITED LECTURES

Career Seminar, "Career Pathways in Neuroscience" Chemistry and Biology clubs speaker series at Morgan State University. October 2021

Departmental Seminar, "Sex Differences in Endocannabinoid Regulation of Behavioral Flexibility," Second Monday Series in UMB Dept. of Anatomy and Neurobiology, February 2022.

Inter-Institutional Seminar, "Sex Differences in Endocannabinoid Regulation of Behavioral Flexibility," Baltimore Brain Series at National Institute of Drug Abuse, October 2022.

Visitor Seminar, "Cannabinoid receptor-1 signaling promotes sensitivity to devaluation in male, but not female, sign-tracking rats," Rutgers University Neuro-RECEPTR Symposium, April 2023

Career Seminar, "My Path to Neuroscience" Research and Mentoring Program speaker series at University of Maryland, Baltimore, Department of Physical Therapy and Rehabilitation Science, July 2023

Departmental Seminar, "Sex specific effects of cannabinoid receptor-1 signaling on behavioral flexibility." Second Monday Series in UMB Dept. of Neurobiology, May 2024.

GRANTS AND FUNDING

Active 1/13/2023-1/12/2025. PI: 100% effort

"Sex Differences in Endocannabinoid Regulation of Behavioral Flexibility" F31 DA057817, National Research Service Award

Completed. 1/1/2023-5/20/2023.

"Neuroscience Outreach Volunteer Association: Brewing Biology Event" Research !America Civic Engagement Microgrant. Total funds: $1,800.00

OUTREACH AND SERVICE

Neuroscience Outreach Volunteer Association

Member, Baltimore, MD, 2019-2021

Coordinated psychiatric hospital visits. Presented at Morgan State University. Contributed to Brain Awareness Week.

Co-President, Baltimore, MD, 2022

Acquired funding from Research!America. Led all events throughout the year, approx. one event per month with events happening on UMB campus, Maryland Science Center, local elementary schools, and local coffee shops to better reach Baltimore community.

President, Baltimore, MD 2023-present

Acquired funding from UM-Medicine Institute for Neuroscience Discovery for our annual Brewing Biology public forum event series. Lead all meetings and events throughout the year, approx. one event per month with majority of events aimed at neuroscience education of school-aged children. Managed internal and external communications to notify about upcoming events. Created social media postings about group efforts. Collaborated with UMB campus groups and Baltimore City organizations in planning events.

Graduate Program in Life Sciences Tutor

Group Session Tutor, Baltimore, MD, Fall 2021

Mechanisms of Biomedical Sciences Core Course

1-on-1 Tutor, Baltimore, MD, Fall 2020

Mechanisms of Biomedical Sciences Core Course

Mid-Atlantic Neuroscience Diversity Scholars (MINDS) Program

Lab mentor, Baltimore, MD, Summers 2021 and 2022

Trained undergraduate students in lab techniques such as animal handling, histology, surgery, and research project management.

UMB Program in Neuroscience Recruitment
Student Host, Baltimore, MD, 2021-2024
Acted as primary contact for multiple prospective students before and after recruitment weekends. Presented lab tours during virtual recruitment weekends.

UMB Program in Neuroscience IDEAS Committee
Speaker Host, Baltimore, MD, 2023
Selected and invited Dr. Kale Edmiston to speak as part of our Inclusion, Diversity, Equity, Antiracism in Science Committee Seminar Series and acted as the host on the day of the seminar.
Student Panel Participant, Baltimore, MD, 2024
Represented the Program in Neuroscience student body as a student leader to ask questions during the panel, "Looking to the Future: Building a Diverse and Belonging Community Together."

UMB Career Advisory Group
Industry Group Member, Baltimore, MD, 2024
Provided input on career development programs for trainees (graduate students and postdoctoral fellows) aimed at career readiness for individuals interested in industry positions.

Skype A Scientist
Scientist Volunteer, Virtual, 2024
Met virtually with a high school classroom from Argentina to answer their questions about the brain and research in neuroscience.

TEACHING EXPERIENCE

University of Scranton, Scranton, PA Aug 2016 to Dec 2016
Teaching Assistant, Department of Biology
- General Biology Lab, an undergraduate course with less than 20 students
- Setup lab demos and assisted with running experiments, assisted in proctoring and grading exams

University of Scranton, Scranton, PA Aug 2017 to Dec 2018
Teaching Assistant, Department of Biology, Neuroscience Program
- Behavioral Neuroscience Lab, an undergraduate course with 15 students, assisted on two sections of the course across two Fall semesters
- Setup lab demos, assisted in running the labs, graded weekly assignments, designed and graded neuroanatomy exams

Goucher University, Towson, MD Aug 2022 to Dec 2022
Teaching Assistant, Department of Psychology
- Teaching Fellowship run through the Collaborative Teaching Fellows Program between Johns Hopkins University, University of Maryland, Baltimore and regional undergraduate universities.

- Intro to Psychology, an undergraduate course with 25 students, lectured on the topics of the neurobiology of addiction and psychological disorders.

PROFESSIONAL TRAINING

CIRTL Teaching Workshop, University of Maryland, Baltimore, April 13, 2022
An Introduction to Evidence-Based Undergraduate Teaching
Description: Completed a 6-week course with focuses on the principles of learning, learning objectives, assessment, active learning and inclusive teaching.

CIRTL Network Course Completion, Center for Integration of Research, Teaching, and Learning Massive Online Open Course, March 28, 2022
Advancing Learning Through Evidence-Based Teaching, completed with distinction.
Description: Completed a 6-week online course with a focus on teaching techniques of peer instruction, inquiry-based labs, cooperative learning, problem-based learning, diversity in the classroom, and the flipped classroom.

HONORS AND AWARDS

Royal Experience Internship Program, University of Scranton	2018
Neuroscience Research Award, University of Scranton	2018
ORSP Research as High Impact Award, University of Scranton	2018
Nu Rho Psi Honor Society, University of Scranton	2018
Alpha Sigma Nu Honor Society, University of Scranton	2018
Excellence in Neuroscience Major Award, University of Scranton	2019
GPILS Educational Enrichment Scholarship, University of Maryland, Baltimore	2019
Travel Award, Gordon Research Conference: Neurobiology of Addiction	2022
2nd Quarter Graduate Student Association Travel Fellowship	2023
International Brain Bee Leadership Award	2024
Trainee Professional Development Award – Society for Neuroscience	2024

PROFESSIONAL AFFILIATIONS

Society for Neuroscience, 2017-Present
Association for Women in Science, 2022-Present
Eastern Psychological Association, 2023-Present
International Behavioral Neuroscience Society, 2023-Present
American Association for the Advancement of Science, 2024-Present

Abstract

Title of Dissertation: Examining Behavioral Flexibility in Pavlovian processes through the lens of Endocannabinoid Regulation and Sex Differences.

Catherine A. Stapf, Doctor of Philosophy, 2024

Dissertation directed by: Donna J. Calu, PhD, Associate Professor, Department of Neurobiology.

Behavioral flexibility is an adaptive process where one alters behavior due to a change in the internal or external environment. Prior research in the field shows that both the dorsolateral striatum (DLS) and dorsomedial striatum (DMS) contribute to the regulation of behavioral flexibility in instrumental conditioning. I begin in Chapter 1 by presenting background literature on behavioral flexibility, comparing instrumental and Pavlovian paradigms, and considering sex differences in either context. In Chapter 2, I focus on how male and female Long Evans rats differ in the expression of a Pavlovian conditioned response and sensitivity to outcome devaluation and how DMS cannabinoid-1 receptors (CB1Rs) mediate these processes. I find that females show more lever-directed behavior and are insensitive to outcome devaluation. I also find that DMS CB1Rs are required for the devaluation sensitivity of males. In Chapter 3, I use slice electrophysiology to assess whether DMS inhibitory tone at baseline differs between males and females and how CB1R activation affects inhibitory synaptic transmission. I find that cells recorded from male rats show reduced frequency of inhibitory events and that CB1R activation reduces inhibitory tone in both males and females. In Chapter 4, I investigate the relationship between social reward and sign-tracking. I find that sign-tracking behavior persists under extinction and in the presence of an alternative reward choice. Collectively, this research establishes a role for DMS CB1R in Pavlovian outcome devaluation and supports previously established sex differences in behavioral flexibility.

Examining Behavioral Flexibility in Pavlovian processes through the lens of Endocannabinoid Regulation and Sex Differences.

by
Catherine Stapf

Dissertation submitted to the faculty of the Graduate School of the
University of Maryland, Baltimore in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2024

Dedication

To Michael.

I'm grateful to have a family who has always inspired me to be curious and one where we share in each other's successes and challenges.

Thank you for paving the way for me.
I love you.

Acknowledgements

I first want to thank my advisor, Donna Calu, for her commitment to my training and to the training of many graduate students during my time at UMB. It is rare to find a mentor who cares just as much about your personal life as they do about your professional development or experiment timelines. With Donna, I found an incredible mentor who helped me stay balanced throughout grad school and who was a wonderful role model as a woman in science. Thank you.

I want to thank the other members of my committee: Joe Cheer, Brian Mathur, Melanie Pina, and Marco Venniro. Each of you has contributed to my training as a behavioral pharmacologist and slice electrophysiologist and I'm lucky to have gotten a chance to learn from you. Without this help I would have been lost, especially in the last year. I also want to thank each of the coordinators of the Program in Neuroscience that helped me stay on track – Georgia Rogers, Renee Cockerham, and Jenn McFarland.

I am grateful to each member of the Calu lab for teaching me so many things. I could not have become an independent researcher without your help. Sara, thank you for being my bench mate and commiserating with me on so many things from non-lab life stresses to experiment failures. Thank you to Sam and Utsav for teaching me the many techniques of the Calu lab and for being the grad student models I follow at UMB. Utsav, your laid-back demeanor and cleverness showed me it's okay when things don't go as planned and that one of the best skills of a PhD is resourcefulness. Sam, your dedication to your research inspired me to be persistent in the face of confusing results. Thank you, Dave, for reminding me of the value of a healthy dose of skepticism. Thank you for the many volunteers that have helped me in running my experiments throughout the years. Scientific exploration is and will always be a team effort.

Thank you to the Department of Neurobiology for being a great and welcoming place to conduct neuroscience research. I know that I'll always find a smile and well wishes walking around the halls here. I especially want to thank the members of the Lobo lab who adopted me as a lab mate once Calu lab numbers dwindled. Thank you for the lunch conversations and for the education in pop culture.

One of my greatest joys while being at UMB was being the president of the Neuroscience Outreach and Volunteer Association for two years. As with any community group, all the events I organized would not have been possible without the many volunteers who worked with me. Thank you to everyone who helped found Brewing Biology and who helped plan, host, and clean up our many other events, especially my fellow officers and committee members: Garrett Crutcher-Bunce, Anna Maximova, Christie Dionisos, Kali Engel, Ali Siclair, Jessica Cornell, and Loryn Johnson.

There are many things that have led me to fall in love with Baltimore. I have found a great sense of belonging from my time as part of Charm City Sings, which started as the Patterson Park Community Chorus. Thank you to all members, especially my other altos. Thank you to BARCS, the shelter where I got my dog and one of the best nonprofits in Baltimore. Thank you to my St. Ignatius church community, for challenging and supporting me. Thank you, Baltimore, for being the "Greatest City in America."

I could not have made it through grad school without my many different support networks. Thank you to the people who I met through UMB: Megan, Cali, Sara, Utsav, Sam, Jack, Daniela, Emily. Thank you to the Scranton gang: Emily, Laura, Sarah, Meg, Serge, Nick, Joey, Rob, Anna, Krista, Jake, Bri, and Christie. Thank you to the high school crew, I'll see you on Thanksgiving Eve: Maureen, Peggy, Nordy and many others.

I am incredibly grateful for the support of my extended family. The large Cassidy clan has always reminded me of the importance of being present and finding reasons to laugh. It has been a great privilege to see all the things my cousins have achieved as we have grown into adults. Thank you to the House family that welcomed me with open arms. For my siblings and in-laws: Kevin, Michael, Elizabeth, Laura, and Bill, thank you for always asking how things were going, how I was doing, and how much longer I'd be in grad school – haha, not actually that last one. Thank you for being a huge support, welcoming me when I was able to stop by, and facetiming me so I could still be part of the best family moments. Thank you to my nephews: Brayden, Owen, and Griffin for being my reminders that the world is full of new things to learn and explore; thank you for sharing your wonder with me.

For those who know me well, they also know how close I am to my mom, Rita. I have many things from her for which to be grateful. She was my first PI – she taught me to be independent and helped me to think critically. Without my mom, I probably would never have become a scientist. I would not have turned out as balanced as I am now - with a love of family and community, an appreciation for good music and good times, and an understanding of how all success in life has come because of the support you've received from others. Mom, I made it – thank you.

Thank you to my little family – Tom and Millie. Millie, bark bark bark woof (she'll know what that means). Tom, I'm forever grateful Millie decided that you were a good one. Thank you for reminding me to be a goofball from time to time and letting me know when Unusual Memes came out during the week so we could take a moment to sit down and watch it. My life wouldn't be as interesting without you. You have always reminded me to prioritize my passions and have supported me in pursuing those. Thank you for making me Dr. House this October. I promise to support you in pursuing your passions in return.

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List of Abbreviations

aCSF	Artificial Cerebrospinal Fluid
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
A-O	Action-outcome association
CB1R	Cannabinoid Receptor-1
CS	Conditioned Stimulus
CTA	Conditioned Taste Aversion
DS	Dorsal Striatum
DLS	Dorsolateral Striatum
DMS	Dorsomedial Striatum
DMSO	Dimethyl Sulfoxide
eCB	Endocannabinoid
FSI	Fast-Spiking Interneuron
GT	Goal-Tracker
IEI	Inter-Event Interval
INT	Intermediate Responder
MSN	Medium Spiny Neuron
NMDAR	N-methyl-D-Aspartate Receptors
NMDG	N-methyl-D-Glucamine
PavCA	Pavlovian Conditioned Approach index
PLA	Pavlovian Lever AutoShaping
sIPSC	Spontaneous Inhibitory Post-Synaptic Current
S-R	Stimulus-response association
ST	Sign-Tracker
SUD	Substance Use Disorder
US	Unconditioned Stimulus
WIN	WIN 55,212-2

Chapter 1: General Introduction

1.1. Behavioral Flexibility

Changing circumstances and unpredictable outcomes in everyday life require the ability to adapt behavior to fit new contexts and succeed. Behavioral flexibility is the ability to alter behavior due to a change in the internal or external environment (Brown & Tait, 2010). Multiple contexts require flexible responding to ensure the survival of a species. It is adaptive to stop searching for food that elicits illness and is no longer rewarding due to toxicity or being spoiled. It is adaptive to vary exploration strategies in the search for reward due to a change in location or presence of multiple reward types. In some cases, it is more adaptive to respond inflexibly and persist in the pursuit of a reward. In the laboratory, we model conditions where a previously rewarding outcome is devalued, or multiple rewarding outcomes are present to study the neurobiology of flexible versus inflexible responding.

Animal models of behavioral flexibility often involve training an animal under instrumental, or operant, conditioning procedures where the animal must respond to earn a reward. The value of this reward can be altered by devaluation procedures. One method to achieve devaluation is satiety-induced outcome devaluation where we provide free access to the high value reward, typically sugar pellets, so the animal is no longer in a hungry state. An alternative method of devaluation is conditioned taste aversion (CTA) or illness-induced devaluation where we induce an illness in the animal following consumption of the high value reward. (Colwill & Rescorla, 1985; Adams & Dickinson, 1981; Adams, 1982; Garcia & Koelling, 1967). Animals who persist in reward seeking following these manipulations are considered 'habit-driven' while animals who reduce

their responding are deemed 'goal-directed.' Researchers in the field of behavioral flexibility suggest learning in instrumental conditioning involves two different associations – action-outcome associations (A-O) and stimulus-response associations (S-R; Yin & Knowlton, 2006). A-O associations underlie goal-directed behavior, where consequences guide actions. In contrast, S-R associations underlie habitual where presentation of a stimulus in in the environment elicits a given response regardless of the outcome. The schedule of reinforcement during training may also bias behavior towards goal-directed responding or habitual responding. Random ratio schedules of reinforcement, where the number of lever presses required to earn a reward varies, biases behavior towards goal-directed responding, while random interval schedules, where the trial time between reward delivery varies, biases behavior towards habitual responding (Adams, 1982; Adams & Dickinson, 1981; Balleine & O'Doherty, 2010; Colwill & Rescorla, 1985; Dickinson, 1985; Gremel & Costa, 2013). Prior work in this field also establishes a bias for more goal-directed behavior following limited training and this bias shifts to habit-driven behavior with more training experience (Dickinson & Balleine, 1994; Dickinson et al., 1995; Balleine et al., 2003; Graybiel, 2008). From the instrumental literature, it is clear how critical it is to study behavioral flexibility with particular focus on the training parameters and test conditions.

In contrast, the length of training does not impact the devaluation sensitivity of Pavlovian associations, (Holland, 1998). Pavlovian, or classical, conditioning typically involves the presentation of a visual and/or auditory cue (conditioned stimulus, CS+) followed by reward delivery (unconditioned stimulus, US) without requiring a behavioral response (Pavlov (1927), 2010). Researchers also assess Pavlovian devaluation

sensitivity through satiety-induced devaluation or CTA (Hatfield et al., 1996; Holland & Rescorla, 1975; Pickens et al., 2003). Importantly, there can be individual differences in the sensitivity to Pavlovian outcome devaluation, which predicts differences in the preferred conditioned responding in Pavlovian Lever AutoShaping (Nasser et al., 2015).

1.1.1 Behavioral Flexibility of the Sign-Tracking Model

Pavlovian Lever AutoShaping (PLA) is a task that reveals significant individual differences in reward learning. In PLA, a lever extends at the start of a trial and acts as a food predictive cue. The lever then retracts at the end of the 10s trial and is followed by the delivery of two food pellets, regardless of the behavioral response of the animal. This task reveals distinct behavioral phenotypes in which a portion of rodents will emerge as sign-trackers (STs), who will press on and attend to the lever cue, and another portion will emerge as goal-trackers (GTs), who will focus more on the foodcup where the reward is presented after the cue period (Flagel et al., 2007, 2009; Meyer et al., 2012; Pitchers et al., 2015). Learning theories behind these distinct behavioral phenotypes suggest that the lever takes on incentive value in STs and becomes an incentive stimulus that bias attention towards it and invigorates reward-seeking behaviors (Robinson & Flagel, 2009; Saunders & Robinson, 2012).

GT and ST rats respond differently to satiety-induced devaluation or CTA. Following limited training in PLA, GTs are sensitive to devaluation while STs are not (Keefer et al., 2020; Morrison et al., 2015; Nasser et al., 2015; Patitucci et al., 2016). Following the theories of learning in instrumental literature, these findings suggest that S-R associations guide ST behavior. Surprisingly, both GTs and STs are sensitive to Pavlovian outcome devaluation after extended training (Keefer et al., 2020). The emergence of flexible

responding in STs suggests that different mechanisms mediate Pavlovian outcome devaluation. In this dissertation, I explore how satiety-induced outcome devaluation affects responding in a Pavlovian paradigm, PLA, after extended training and explore a potential neurobiological mechanism for mediating Pavlovian flexibility.

1.2. Sex and Training Differences in Behavioral Flexibility

Original studies of outcome devaluation in rodents included only males, a notable shortcoming of these prior findings. (Adams, 1982; Adams & Dickinson, 1981; Dickinson et al., 1983). Numerous studies that include both males and females reveal distinct sex-specific behavioral strategies such as active fear response in females and passive fear response in males (Gruene et al., 2015; Kokras & Dalla, 2017). Benefits of studying both sexes in preclinical studies includes revealing distinct molecular signaling pathways (Tabatadze et al., 2015), uncovering factors which protect against disease (Golden & Voskuhl, 2017), and understanding divergent behavioral strategies that vary due to sex, as described above. Recently, the National Institute of Health released new requirements to include both sexes and consider sex as a biological factor in all preclinical studies (Miller et al., 2017). The inclusion of females in studies focusing on behavioral flexibility reveals that sex is an important influence on the expression of flexible or inflexible responding depending on the context. Many studies suggest that females are less flexible and do not show sensitivity to outcome devaluation under conditions in which males are sensitive. Females express habit-driven behaviors at an earlier timepoint in training than their male counterparts and are less sensitive to outcome devaluation (H. Schoenberg et al., 2018). Extending this training in an instrumental paradigm (360 presentations of reward) results in

habit-driven, devaluation-insensitive behavior in both males and females (Dickinson, 1985; Thrailkill & Bouton, 2017). In another study there was no difference in behavioral flexibility following outcome devaluation or in other measures of flexibility such as reversal learning or risk-based decision making (Westbrook et al., 2018). In this dissertation, I include both males and females to examine sex differences in responding following Pavlovian outcome devaluation.

1.1.2 Sex Differences in Sign-tracking Model

Studies using both sexes suggest females may be less sensitive to satiety-induced outcome devaluation (Keefer et al., 2020; Kochli et al., 2020; Keefer et al., 2022). In fact, female rats are more likely to be classified as STs and show more lever-directed behavior than males, suggesting they may be less sensitive to devaluation (King et al., 2020). Recent studies that focus on sex differences in tracking and behavioral flexibility show that females are less sensitive to satiety-induced devaluation (Sood & Richard, 2023) but this is not the case for CTA (Bien & Smith, 2023). However, neither of these studies focused on the interaction between tracking group and sex nor did they probe for a neurobiological basis for the sex differences. In this dissertation, I aimed to better understand the interaction between tracking group and sex in Pavlovian outcome devaluation and provide one possible mechanistic difference between males and females.

1.3. Social Reward and Alternative Reward Choice

As described above, behavior can change following devaluation of a specific reward. Another facet of behavioral flexibility is decision-making between different rewards.

Recent experiments in the field of Substance Use Disorder (SUD) research focus on how the presentation of an alternative reward such as food and/or social interaction can alter choice when presented alongside a drug reward (Beckmann & Chow, 2015; Chow & Beckmann, 2021; Lenoir et al., 2013; Venniro et al., 2018; Venniro & Shaham, 2020). Social interaction is a strong reinforcer that outcompetes drug reward choice (Venniro et al., 2017, 2018; Venniro & Shaham, 2020). In these studies, voluntary abstinence due to social choice over drug is protective against relapse which suggests positive social interaction disrupts addiction-related behavioral patterns.

Sign-tracking predicts escalation of drug use and a higher risk to relapse for cocaine and amphetamines (Kucinski et al., 2018; Versaggi et al., 2016). Thus, ST is considered an "addiction-vulnerable" phenotype. Similarities in the reward learning behaviors and neurobiology behind sign-tracking correlate with the patterns that characterize substance use disorder (Tomie et al., 2008). As an example, STs display enhanced Nucleus Accumbens dopamine release in response to reward-predictive lever cue (Flagel et al., 2007, 2011). Enhanced Nucleus Accumbens dopamine release follows drug self-administration and contributes to the development of SUD (Di Chiara, 1999). Social interaction protects against negative outcomes related to SUDs such as escalation of drug use and relapse. It is possible that social interaction reduces sign-tracking and alters the stimulus-reward associations established between the lever and cue, similar to how social interaction disrupts SUD related behaviors. I aimed to determine whether social interaction outcompetes the motivation to sign-track and Chapter 4 of this dissertation combines PLA with the operant social interaction task to ask this question.

1.4. Neurobiology of Behavioral Flexibility

Many brain regions are involved in the regulation of behavioral flexibility, most notably the dorsal striatum (Amaya & Smith, 2018; Balleine & O'Doherty, 2010; G. L. Gerdeman et al., 2003; Malvaez & Wassum, 2018). The DS can be subdivided into two regions, the dorsomedial striatum (DMS) and the dorsolateral striatum (DLS). Activity of these striatal subregions depends on the training experience of the individual. Following limited training in an instrumental paradigm, the DMS shows high neuronal activity and after extended training, this activity shifts to the DLS (Averbeck & Costa, 2017; Gremel & Costa, 2013; Yin et al., 2004, 2005, 2009). A recent study looking at the spike activity of neurons in DMS and DLS shows that the activity of the two regions is similar after extended training with peaks of DMS activity at the start and end of behavioral sequences (Vandaele et al., 2019). The DMS receives inputs from limbic and associative cortices and lesions of the DMS result in inflexible responding; whereas the DLS receives inputs from motor cortices and lesions of the DLS result in flexible responding (Allichon et al., 2021; Guo et al., 2015). Because this dissertation focuses on Pavlovian paradigms and the associations made between the cues and outcomes, I focus my experiments on the region of the DS where associative information is stored. Thus, the experiments found in chapters 2 and 3 focus on the neurobiology and physiology of the DMS.

1.1.3 Dorsomedial Striatum Circuitry

The DMS is a critical area in mediating the associations necessary to support flexible responding. The area receives connections from associative cortices such as the orbitofrontal cortex and cingulate cortex, from the thalamus, and from the amygdala

(Guo et al., 2015). These projections into the area are glutamatergic and they project onto local interneurons or onto medium spiny neurons (MSNs). MSNs receive dopaminergic projections from the substantia nigra pars compacta (Allichon et al., 2021). MSNs are the main class of neurons projecting from the DMS. These GABAergic cells make up approximately 90-95% of the neurons within the DMS and can be subdivided into two distinct populations, direct or dopamine receptor-1 containing (D1), and indirect, or dopamine-receptor 2 containing (D2; (Graveland & Difiglia, 1985)). These DMS projection neurons differ in their downstream connections where D1 neurons project directly to substantia nigra pars reticulata, while D2 neurons project first to the globus pallidus (Kawaguchi, 1997; Lobo et al., 2006). These MSNs also project locally and are a source of lateral inhibition in the DMS (Burke et al., 2017; Lalchandani et al., 2013; Tunstall et al., 2002). In addition to these projection neurons, the DMS also expresses numerous interneurons that shape its local physiology, including parvalbumin positive, fast-spiking (FSI) interneurons, somatostatin-positive (SOM) interneurons, calretinin-positive (CR) interneurons and tyrosine hydroxylase-positive (TH) interneurons. These types altogether make up the DMS GABAergic interneurons which compose 3-4% of the neurons in the striatum (Do et al., 2013; Kawaguchi et al., 1995). FSIs project to the soma of MSNs and thus have a strong influence on the physiology of these neurons (Kawaguchi, 1993). FSIs receives cortical input which drives FSI synchrony (Gittis et al., 2010; McKeon et al., 2022). The remaining interneuron types are cholinergic and play an important role in mediating dopamine release (Cachope et al., 2012). In addition to the excitatory and inhibitory neurotransmitters of glutamate and GABA, many neuromodulators such as acetylcholine and endocannabinoids work in the DMS to alter the region's activity. In

Chapter 3 of this dissertation, I record inhibitory inputs from neurons in the DMS which are most likely to be MSNs due to their high abundance in the DMS relative to other cell types.

1.5. Endocannabinoids and Behavioral Flexibility

Endocannabinoids (eCBs) are important neuromodulators that are found in high density throughout the brain and the main eCBS are anandamide (AEA) and 2-arachidonoylglycerol (2-AG; (G. L. Gerdeman et al., 2002; Lovinger & Mathur, 2012)). These compounds are produced in post-synaptic cells and signal through retrograde transport to activate cannabinoid receptors located on the presynaptic cell. Cannabinoid receptor-1 (CB1R) is the main eCB receptor type located in the brain (Castillo et al., 2012). Activation of this receptor results in a reduction of the release probability of neurotransmitters (G. L. Gerdeman et al., 2002).

eCBs are critical in the regulation of behavioral flexibility. One study in humans showed that increased 2-AG in blood correlated with worse cognitive flexibility performance while increased AEA correlated with better performance (Fagundo et al., 2013). Rodent studies which systemically activate CB1Rs and studies with more site-directed manipulations, like overexpression of CB1Rs in the prefrontal cortex, show that CB1Rs contribute to reduced cognitive flexibility (Hilário et al., 2007; Klugmann et al., 2011). Due to the high density of CB1Rs throughout the brain (Liu et al., 2020), eCBs can elicit very different effects depending on the region of the brain where they act.

1.1.4 Endocannabinoids in the Dorsal Striatum

CB1R are located on glutamatergic projections into the DMS and locally on MSNs and FSIs (G. L. Gerdeman et al., 2002; G. Gerdeman & Lovinger, 2001; Lovinger & Mathur, 2012a; Mathur et al., 2013a; Y. W. Wu et al., 2015). Signaling through these receptors is important for the expression of inflexible or habit-driven behaviors in instrumental paradigms. Chronic stimulation of CB1Rs will result in habitual or compulsive responding that can be maladaptive (Gremel et al., 2016; Hilário et al., 2007; Nazzaro et al., 2012). In addition to the expression of CB1Rs, both 2-AG and AEA are released in the DMS (Ade & Lovinger, 2007; Xu et al., 2020; Augustin et al., 2023) and act to signal at CB1Rs.

1.6. Chapter Objectives

Prior research into behavioral flexibility focuses heavily on the mechanism of instrumental outcome devaluation in males. Through the experiments presented in this dissertation, I hope to better understand how sex influences flexibility of Pavlovian behaviors and to determine the contribution of eCB in the DMs on sex differences in Pavlovian behaviors.

In Chapter 2, I provide evidence for the necessity of DMS CB1R for devaluation sensitivity in male rats, which runs counter to established theories of eCB regulation of behavioral flexibility as described above in Section 1.5. I suggest a possible mechanistic framework for the neurobiological regulation of Pavlovian flexibility. I also uncover sex differences in the acquisition of a Pavlovian conditioned response and in Pavlovian outcome devaluation after extended training.

In Chapter 3, I use slice electrophysiology to explore DMS physiology and the influence of CB1R signaling. I provide evidence that suggests there are sex differences in DMS inhibitory synaptic transmission at baseline. I also replicate a well-established role for DMS CB1Rs: activation of CB1Rs reduces the frequency of inhibitory synaptic events regardless of sex.

In Chapter 4, I make use of a novel operant social interaction model to probe the social sensitivity of sign-tracking rats and begin comparing between males and females. Overall, these experiments are underpowered to evaluate sex differences but suggest that sign-tracking is largely unresponsive to social alternatives under the conditions tested. These experiments also provide a case to explore the social sensitivity between tracking groups.

In the final chapter, I provide a general discussion of the main findings of this dissertation and present reasoning for sex differences in behavioral flexibility as well as the likely role of eCBs in supporting flexibility in Pavlovian behaviors.

Chapter 2: Sex differences in Pavlovian Outcome Devaluation and the role of CB1Rs in mediating devaluation sensitivity.

2.1. Introduction

Impairments in behavioral flexibility occur across a range of mental health disorders including substance use disorder, schizophrenia, obsessive-compulsive disorder, and depression (Geramita et al., 2020; Jordan & Andersen, 2017; Kalivas & Volkow, 2005; Listunova et al., 2018; Simmler & Ozawa, 2019; Thoma et al., 2007). Preclinical studies suggest that sex and individual differences influence behavioral control when environmental conditions change from what is expected (Amaya et al., 2020; Bien & Smith, 2023; Keefer et al., 2020; Morrison et al., 2015; Nasser et al., 2015). Specifically, the pre-clinical sign-tracking model suggests more rigid response strategies in females compared to males (Kochli et al., 2020; Pitchers et al., 2015). Understanding the neurobiological underpinnings of individual and sex differences in behavioral flexibility may help to identify novel therapeutic targets for disorders of behavioral control.

Instrumental conditioning procedures in rats identified dorsal striatal regulation of behavioral flexibility, which involves dorsomedial and dorsolateral striatal (DMS, DLS) subdivisions. After limited training in an instrumental task in which an action leads to a food outcome, the DMS is critical for decreasing actions when the associated food outcome is devalued (Peak et al., 2019; Yin et al., 2004, 2005). After extended training, actions become insensitive to outcome devaluation, and this rigid responding requires DLS activity (Amaya & Smith, 2018; Dickinson et al., 1995; Gremel & Costa, 2013; Yin et al., 2004). The shift from goal-directed to habitual behavior that occurs with instrumental experience is mediated by a shift from DMS to DLS control. An instrumental study in mice established

that CB1Rs on glutamatergic synapses projecting from the orbitofrontal cortex (OFC) to the dorsal striatum (DS) gate behavioral flexibility in outcome devaluation (Gremel et al., 2016). In mice, CB1R deletion in the OFC-DS projection promotes devaluation sensitivity even during schedules of reinforcement that ordinarily drive rigid responding (Gremel et al., 2016). While these studies are not DS-subregion specific, they suggest that CB1R-mediated inhibition of OFC synaptic inputs to the outcome devaluation sensitive DMS may shift behavior towards rigid, devaluation-insensitive actions. We hypothesize that DMS CB1R signaling also biases behavior towards devaluation-insensitive behavior in a Pavlovian task. Here we use intracranial delivery of a CB1R inverse agonist, rimonabant, to determine the role of DMS CB1R in mediating Pavlovian devaluation sensitivity in male and female rats.

While far less is known about the role of the DS in driving behavioral flexibility in Pavlovian outcome devaluation, Pavlovian procedures are ideal for identifying individual and sex differences in behavioral flexibility. The sign-tracking model has uncovered considerable individual, sex and, experience-dependent differences in Pavlovian devaluation sensitivity (Flagel et al., 2009; Keefer et al., 2020; Kochli et al., 2020; Madayag et al., 2017; Pitchers et al., 2015).

After limited training (<10 sessions) in Pavlovian lever autoshaping (PLA), in which an insertable lever cue predicts a food outcome, goal-tracking rats (GT) show sensitivity to outcome devaluation while sign-tracking (STs) rats do not (Keefer et al., 2020; Morrison et al., 2015; Nasser et al., 2015; Patitucci et al., 2016). After extended training (>10 session), both GT and ST rats show sensitivity to satiety-induced outcome devaluation (Keefer et al., 2020), an effect established in male rats. Female rats show increased levels of sign-

tracking, or lever-directed approach during PLA compared to males (Hammerslag & Gulley, 2014; Keefer et al., 2022; King et al., 2020; Kochli et al., 2020; Madayag et al., 2017; Pitchers et al., 2015) suggesting they may be less sensitive to outcome devaluation even after extended training. Here we evaluate sex differences in behavioral flexibility using the sign-tracking model and determine whether DMS CB1R signaling contributes to individual and sex differences in devaluation sensitivity after extended Pavlovian training.

2.2. Materials and Methods

Subjects. We used 68 Long Evans rats (33 Male, 35 Female; run as 5 cohorts) in the age range of 7-9 weeks old at the start of training for this study. All rats were double-housed upon arrival and then single-housed 24-48 hours therafter. We maintained rats on a reverse 12hr:12hr light-dark cycle (lights off at 1000). We performed all behavioral procedures during the dark phase of the light cycle. All rats had *ad libitum* access to standard laboratory chow and water before we food deprived them to maintain 90% of their baseline weight. We surgerized one cohort prior to any behavioral training and testing and surgerized the remaining cohorts after three days of training. There were no pre- or post-surgery differences in behavior between groups. We performed all procedures in accordance with the "Guide for the Care and Use of Laboratory Animals" (8th edition, 2011, US National Research Council) and with approval by the University of Maryland, School of Medicine Institutional Animal Care and use Committee (IACUC).

Apparatus. We conduct behavioral experiments in identical operant chambers (25 X 27 X 30 cm; Med Associates) located in a separate room from the animal colony. An individual sound-attenuating cubicle with a ventilation fan surrounds each chamber. One

wall contains a red house light and the opposing wall contains a food cup with photobeam detectors that rests 2 cm above the grid floor. A programmed pellet dispenser attached to the foodcup dispensed 45 mg food pellets (catalog #1811155; Test Diet Purified rodent Tablet [5TUL]; protein 20.6%, fat 12.7%, carbohydrate 66.7%). We installed one retractable lever at 6cm above the grid floor on either side of the foodcup and we counterbalanced the lever side between subjects.

Surgical Procedures. After three days of PLA training, we gave *ad libitum* access to food before we performed intracranial cannula placement surgery. We anesthetized 8-week-old rats with isoflurane (VetOne, Boise, ID, USA; 5% induction, 2-3% maintenance) then administered the pre-operative analgesic carprofen (5mg/kg, s.c.) and lidocaine (10mg/mL subdermal at incision site). We placed rats in a stereotaxic frame (Model 900, David Kopf Instruments, Tujunga, CA, USA) over a heating pad to maintain stable body temperature throughout surgery.

We implanted guide cannula (23G; PlasticsOne INC, Roanoke, VA, USA) bilaterally at an 8-degree angle, 1mm above the injection site in the DMS (coordinates from bregma in mm: -0.24 AP, ± 2.6 ML and -4.5 DV). We determined distance from bregma using the Paxinos and Watson rat brain atlas (Paxinos & Watson, 2006). Cannula were secured to the skull with jeweler's screws and dental cement. At the end of surgery, we inserted dummy cannula into the guide cannula, which we only removed during infusion habituation and infusion test procedures. We moved rats to a recovery cage, placed over a heating pad, and administered carprofen analgesic at 24 hr, 48 hr and 72 hr post-surgery. We gave animals 1 week of recovery before resuming behavioral procedures.

Pavlovian Lever Autoshaping Training. Prior to training, we exposed all rats to the food pellets in their home cage to reduce novelty to the food. Then we trained them in daily Pavlovian lever autoshaping (PLA) sessions which lasted ~ 26 minutes and included 25 trials of non-contingent lever presentations (conditioned stimulus; CS) and occurred on a VI 60 s schedule (50-70s). At the start of the session, the houselight turned on and remained on for the duration of the session. Each trial consisted of a 10 s lever presentation and retraction of the lever was followed immediately by delivery of two 45 mg food pellets into the foodcup. At the end of the session, we returned rats to their cage and colony room. We trained rats in PLA first for 5 days to determine their tracking group, then continued training through 12 days following PLA testing.

Pavlovian Lever Autoshaping Testing. We tested the effects of blocking DMS CB1R during reinforced PLA. We infused rimonabant (SR141716; 1 µg/µl or 2 µg/µl; dissolved in 1:1:18 ethanol: emulphor: saline solution) or vehicle bilaterally into DMS at a rate of 0.5 µl/min over the span of 1 minute. We left the infusion cannula in place for an additional minute before slowly removing them and replacing the dummy cannula. We waited 10 min after infusion before placing rats into the behavioral chamber and running the PLA test. We infused all rats with vehicle, low (1 µg/µl) or high (2 µg/µl) dose of rimonabant across three days and we counterbalanced the dose across days.

Satiety-Induced Outcome Devaluation Testing. After the 12th training session, we gave rats two sessions of satiety-induced outcome devaluation. Rats had one hour of *ad libitum* access to 30 g of either their homecage chow (valued condition) or food pellets used during PLA training (devalued condition) in a ceramic ramekin. Within 15 min of the end of the satiation hour, we infused rimonabant into the DMS as described in the previous

section. We waited 10 min after the infusion before placing rats into the behavioral chamber and running the PLA test. Tests consisted of 10 non-rewarded lever presentations on VI 60s schedule (50-70s). Immediately after each test, we gave rats a 30 min food choice test in their homecage which included 10 g of homecage chow and 10 g of food pellets in separate ceramic ramekins to confirm satiety was specific to the outcome they had been fed before the test session. We retrained rats on 25 reinforced trials on a separate day between devaluation probe tests.

Measurements. For training and devaluation probe tests, we recorded the number and duration of foodcup and lever contacts, the latency to contact, and the probability during the 10 s CS (lever) period. On trials with no contacts, a latency of 10s was recorded. To determine tracking group, we used a Pavlovian Conditioned Approach (PavCA) analysis (Meyer et al., 2012) which quantifies behavior along a continuum where +1.00 indicates behavior is primarily lever directed (sign-tracking) and -1.00 indicates behavior is primarily foodcup directed (goal-tracking). PavCA scores are the average of three separate scores: the preference score (lever contacts minus foodcup contacts divided by the sum of these measures), the latency score (time to contact foodcup minus the time to contact lever divided by 10 s (duration of the cue)) and the probability score (probability to make a lever contact minus the probability to make a foodcup contact across the session). We use the PavCA score from the 5th day of training to determine an individual's tracking group as follows: STs have a PavCA sore +0.33 to +1.00, GTs have a PavCA score -1.00 to -0.33, intermediates (INT) have scores ranging from -0.32 to +0.32. Rats in GT and INT groups were combined into a single GT/INT group as in our prior studies (Keefer et al., 2020). On

day 6, we were unable to record latency data for 6 rats and only retained lever and foodcup contacts for these rats. Preference score was used in place of PavCA for rats on this day.

For devaluation probe tests, we also report total approach (the sum of food cup and lever contacts during the 10 s CS period) and individual contact measurements. We recorded consumption on the test days and calculated the amount of pellet or chow consumed in grams during the satiety hour and during the 30 min choice test.

Histology. At the end of experiments, we deeply anesthetized rats with isoflurane and transcardially perfused 100ml of 0.1M sodium phosphate buffer (PBS), followed by 200ml of 4% paraformaldehyde (PFA) in PBS. We removed brains and post-fixed them in 4% PFA over night before we transferred them to 30% sucrose in PBS for 48-72 hr at 4 °C. We rapidly froze brains in dry ice before storing them in -20 °C until slicing. We sliced brains with the Leica Microsystems 1850 cryostat to collect 40 µm coronal sections in three series through the cannula placements in the DMS. We mounted sections onto gel-coated slides and then stained with cresyl violet before coverslipping with Permount. We examined placements under a light microscope for confirmation of cannula placement in the DMS (Fig. 2D). We excluded 11 rats due to cannula placements being outside the region of interest.

Experimental Design and Statistical Analysis. We analyzed behavioral data using SPSS 29.0 statistical software (IBM) with mixed-design repeated measures analysis of variance (ANOVA). Significant main effects and interactions ($p<0.05$) were followed by post-hoc repeated-measures ANOVA or Bonferroni paired t-tests. Analyses included between subject factors of Tracking (ST, GT/INT) Sex (male, female) and Treatment

(vehicle, rimonabant) and within-subject factors of Session (1-12), Outcome Value (Valued, Devalued), or Outcome (Nonsated, Sated).

2.3. Results

Acquisition of Pavlovian Lever Autoshaping. We trained rats for 12 days in PLA before outcome devaluation testing. We used the Pavlovian Conditioned Approach Index (PavCA) on the 5th session of training to determine tracking groups (Fig. 2.1A). Consistent with our prior studies (Bacharach et al., 2018; Keefer et al., 2020) showing that GT and INT rats shift away from food-cup approach and towards lever approach with extended training, we observe a main effect of Session for PavCA Index, $F(11,583)=106.292$, $p<0.001$, and a Session x Tracking (ST, GT/INT) interaction, $F(11,583)=13.909$, $p<0.001$. Next, we examined whether there were sex differences in the acquisition and expression of PLA (Fig. 2.1B).

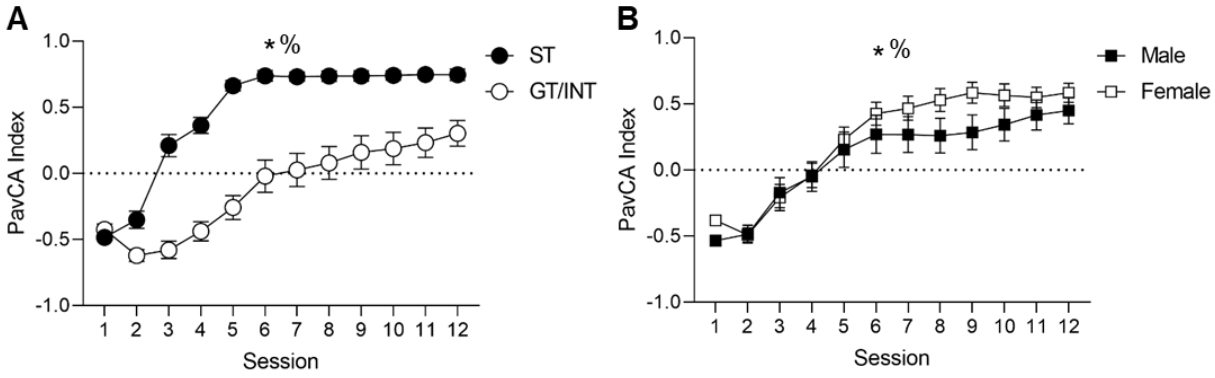

Figure 2.1: Pavlovian Conditioned Approach during training differs by Tracking and Sex. ***A,*** PavCA Index mean ± SEM for ST and GT/INT (collapsed on sex) that acquire individual differences in conditioned responding in PLA task. *Main effect of Session. %Significant Session X Tracking interaction. ***B,*** PavCA Index mean ± SEM for Male and Female rats (collapsed on tracking) that acquire conditioned responding in a PLA task. *Main effect of Session. %Significant Session X Sex interaction.

We used a mixed design repeated measures ANOVA with between-subject factors of Sex (Male, Female) and within subject factor of Session (1-12) and found a Session x Sex interaction for PavCA Index, F(11,605)=1.823, p=0.047). While males and females have similar approach during initial acquisition, female rats showed more sign-tracking, via a higher PavCa Index, than males with extended training (day 8, t(55)=-1.754, p=0.043; day 9,t(55)=-2.007, p=0.025).

We observe a significant Sex x Tracking x Session interaction for PavCA (Table 2.1; F(1,53)=3.501, p<0.001). When male and female acquisition is also split by tracking, we observe in the GT/INT group that there is a significant main effect of Sex (Fig. 2.2b; F(1,27)=4.236, p=0.049) and we see that the shift towards sign-tracking is driven by female GT/INT rats. In contrast, ST acquisition is very stable after day 5 and there are no differences by sex in this group (*Fs*<0.489*, ps*>0.491*).* A closer look into lever contacts and food cup contacts alone also reveal interesting sex differences.

Effect	Degrees of Freedom	PavCA Index		Lever						Foodcup					
				Contact		Latency		Probability		Contact		Latency		Probability	
		F	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Session	(11, 583)	**110.46**	**<0.001**	**46.953**	**<0.001**	**67.952**	**<0.001**	**77.062**	**<0.001**	**16.093**	**<0.001**	**29.551**	**<0.001**	**34.997**	**<0.001**
Tracking Group	(1,53)	**49.293**	**<0.001**	**15.401**	**<0.001**	**19.568**	**<0.001**	**26.117**	**<0.001**	**41.543**	**<0.001**	**33.349**	**<0.001**	**41.326**	**<0.001**
Sex	(1,53)	3.69	0.06	0.103	0.750	3.980	0.052	3.491	0.068	**8.264**	**0.006**	2.399	0.128	1.341	0.253
Session X Tracking	(11,583)	**14.917**	**<0.001**	**4.248**	**<0.001**	**7.701**	**<0.001**	**8.109**	**<0.001**	**8.322**	**<0.001**	**11.170**	**<0.001**	**10.442**	**<0.001**
Session X Sex	(11,583)	**2.284**	**0.01**	1.330	0.203	**2.168**	**0.015**	1.636	0.085	**1.997**	**0.027**	0.904	0.536	0.633	0.801
Tracking Group X Sex	(11, 583)	3.484	0.067	0.925	0.340	1.214	0.276	1.365	0.248	**9.268**	**0.004**	3.061	0.087	**4.139**	**0.047**
Session X Sex X Tracking	(11, 583)	**3.501**	**<0.001**	1.652	0.081	**2.140**	**0.016**	**3.193**	**<0.001**	**2.429**	**0.006**	1.213	0.275	1.763	0.058

Table 2.1: *Repeated Measures analysis of variance (ANOVA) for PLA across all tracking groups during training (session 1-12).*

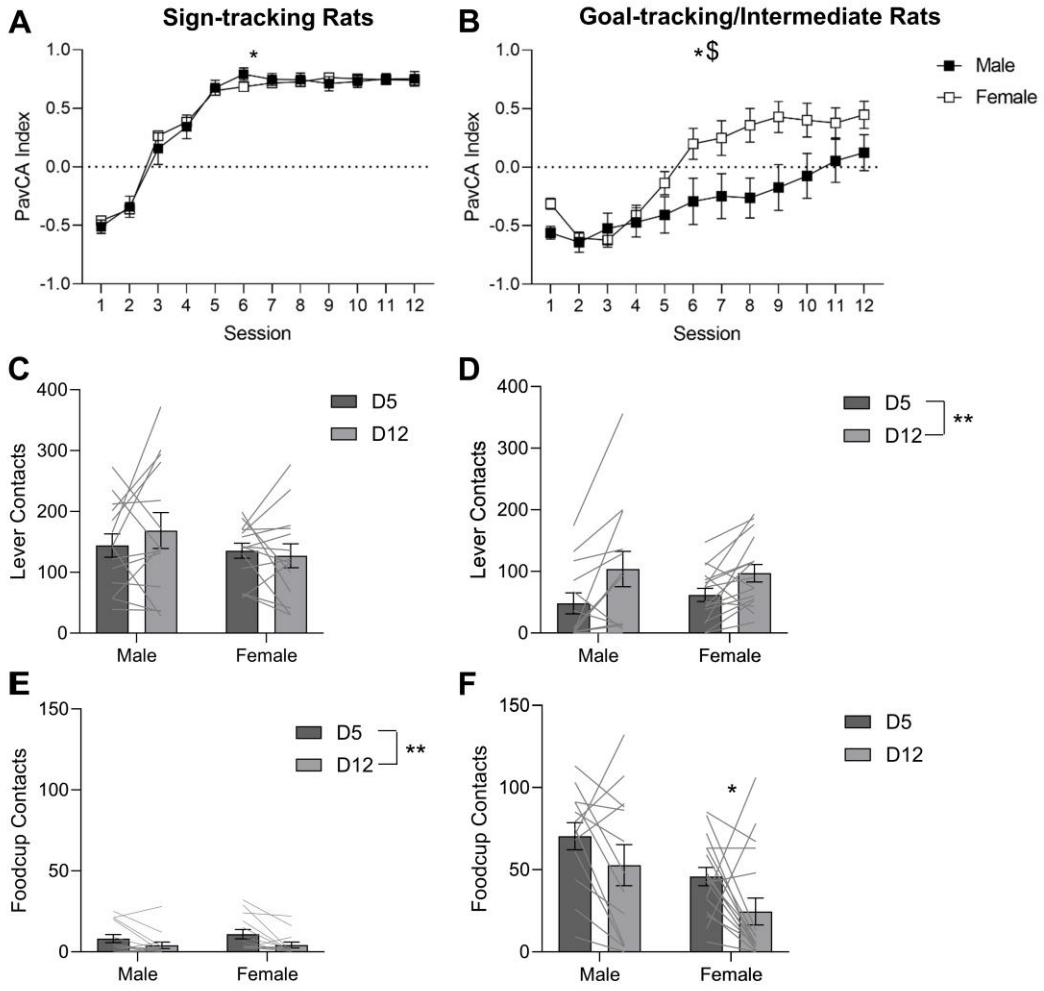

Figure 2.2: Pavlovian Conditioned Approach shifts in GT/INT rats toward lever directed behaviors which is driven by female GT/INT rats. We observed a significant 3-way Tracking X Sex X Session interaction on PavCA Index. Data are represented as per day mean ± SEM. ***A,*** PavCA Index for Male and Female ST rats ***B,*** PavCA Index for Male and Female GT/INT rats. *Main effect of Session. $Main effect of Sex. Day 5 and Day 12 lever contact (***C,D***) and food cup contact (***E,F***) data are represented as within-subject individual data (lines) and group data (bars; mean ± SEM). ***C,*** We did not observe any significant differences in ST rats. ***D,*** We observed a significant main effect of Session GT/INT rats. ***E,*** We observed a significant difference only in Session for ST rats ***F,*** We observed a significant interaction with Sex where only female GT/INTs reduced food cup contacts due to Session. *$p<0.05$ **$p<0.025$

Analysis for foodcup contacts between session 5, when we categorize tracking phenotype, and session 12, the final PLA session before outcome devaluation, reveals that there is a significant main effect of Session ($F=10.597$, $p=0.002$), Sex ($F=4.888$, $p=0.031$) and Tracking ($F=6.179$, $p=0.016$) as well as a significant Sex x Tracking interaction ($F(1,53)=6.179$, $p=0.016$). When we split analysis further by tracking groups, we find that in ST rats, both males and females reduce their food cup contacts by session 12 with no interaction of Sex (Fig 2.2e, Session; $F(1,26)=10.432$, $p=0.003$, Session x Sex; $F=0.585$, $p=0.451$). However, in GT/INT rats, we observe a significant main effect of Sex for food cup contacts (Fig. 2.2f; $F(1,27)=6.136$, $p=0.02$). We find that female GT/INTs significantly reduce their food cup contacts by session 12 (Session; $F(1,15)=4.554$, $p=0.05$) while male GT/INTs do not (Session; $Fs<2.776$, $ps>0.122$). Analysis of lever contacts reveals significant main effects of Session ($F(1,53)=7.573$, $p=0.008$) and Tracking ($F(1,53)=15.325$, $p<0.001$) but no interaction with Sex ($Fs<0.707$, $ps>0.404$). We ran a similar analysis to the above for lever contacts and split by tracking group and sex. We find that there are no differences between session 5 and session 12 for lever contacts of STs (Fig. 2.2c, $Fs<1.112$, $ps<0.301$) while in GT/INTs, both males and females increase the amount of lever pressing in session 12 (Fig. 2.2d, Session; $F=14.881$, $p<0.001$). Regardless of these differences in acquisition, there were no sex differences in responding on the last day of training (PavCa Index; $t(55)=-1.099$, $p=0.277$). Thus, we observe no sex differences in rats' overall autoshaping behavior on the day prior to testing in outcome devaluation.

Effects of intra-DMS blockade of CB1R signaling on Pavlovian Approach during outcome devaluation. We tested rats using within-subject satiety-induced outcome devaluation. To determine the effects of intra-DMS rimonabant injections on devaluation sensitivity of Pavlovian approach, we analyzed total approach which is the sum of lever and foodcup contacts. First, to examine treatment effects on devaluation sensitivity across all rats, we compared responding during the valued (chow sated) versus devalued (pellet sated) conditions. Figure 2.3A shows the performance of all rats that received either intra-DMS vehicle or rimonabant infusions during the outcome devaluation probe test.

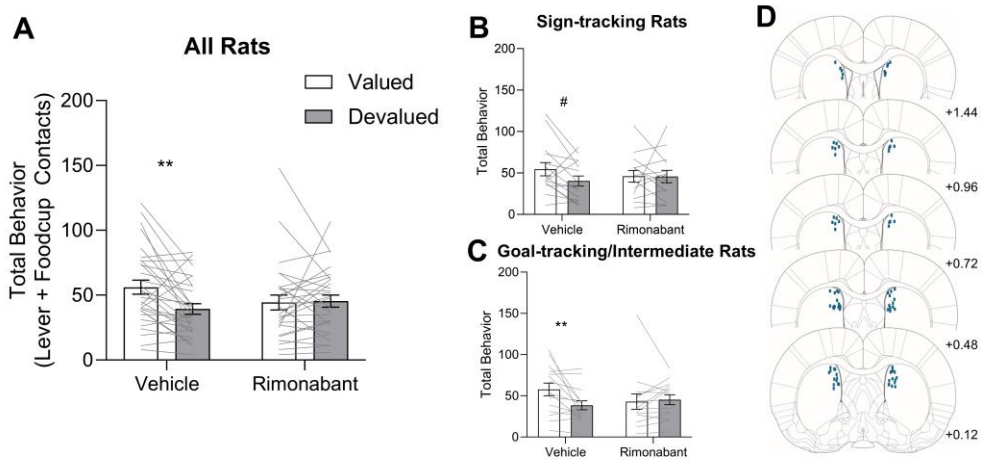

Figure 2.3: Intra-DMS Rimonabant prevents sensitivity to Pavlovian Outcome devaluation, regardless of Tracking. Data are represented as within-subject individual data (lines) and group data (bars; mean ± SEM). Rats received intra-DMS injections of either vehicle (left) or rimonabant (right) 10 minutes prior to probe test. ***A,*** Total behavior (sum of lever and food cup contacts) in outcome devaluation across all rats. We observed a main effect of Outcome Value and a significant Outcome Value X Treatment interaction. ***B,*** In ST rats, we did not observe any significant main effects or interactions, when collapsed across sex. ***C,*** In GT/INT rats**,** we observed a significant Outcome Value X Treatment interaction. ***D,*** Coronal sections (in mm) depicting the location of DMS injector tips for rimonabant infusion. #p=0.051, **p<0.025

We found a main effect of Outcome Value ($F(1,49)=5.558$, $p=0.022$) and an Outcome Value x Treatment interaction ($F(1,49)=6.663$, $p=0.013$), indicating that intra-DMS rimonabant blocked Pavlovian devaluation sensitivity across all rats (Table 2.2). Under vehicle conditions, rats decreased total approach when sated on the training pellet (devalued state) compared to when they were sated on the homecage chow (valued state).

In contrast, with intra-DMS rimonabant, rats showed a similar amount of Pavlovian approach in the valued and devalued state. These results suggest a divergent mechanism for eCBs in mediating Pavlovian behavioral flexibility where CB1R promote flexibility, in contrast to prior studies employing instrumental outcome devaluation (Gremel et al., 2016; Hilário et al., 2007; Navarro et al., 2001).

		Total Behavior		Lever Contacts		Food cup Pokes	
Effect	**Degrees of Freedom**	***F***	***p***	***F***	***p***	***F***	***p***
Outcome Value	(1,56)	**5.558**	**0.022**	**3.902**	**0.054**	2.530	0.118
Treatment	(1,56)	0.276	0.602	0.312	0.579	0.060	0.808
Tracking	(1,56)	0.004	0.949	0.542	0.465	**5.814**	**0.020**
Sex	(1,56)	1.989	0.165	0.735	0.395	**14.500**	**0.000**
Outcome Value X Treatment	(1,55)	**6.663**	**0.013**	**4.442**	**0.040**	**4.091**	**0.049**
Outcome Value X Tracking	(1,55)	0.093	0.762	0.000	0.988	2.054	0.158
Outcome Value X Sex	(1,55)	2.088	0.155	1.937	0.170	0.003	0.958
Outcome Value X Treatment X Tracking	(1,53)	0.566	0.455	0.153	0.697	2.982	0.091
Outcome Value X Treatment X Sex	(1,53)	2.316	0.134	0.761	0.387	**9.466**	**0.003**
Outcome Value X Tracking X Sex	(1,53)	0.572	0.453	0.202	0.655	2.103	0.153
Outcome Value X Treatment X Tracking X Sex	(1,49)	**4.545**	**0.038**	**5.334**	**0.025**	1.502	0.226

Table 2.2: *Repeated Measures analysis of variance (ANOVA) for Pavlovian outcome devaluation across Outcome Value (Valued, Devalued) for total behavior, lever contacts, and food cup pokes.*

Considering the established individual differences in devaluation sensitivity during PLA, we also added Tracking and Sex as factors in this analysis. We observed an Outcome Value x Treatment x Tracking x Sex interaction ($F(1,49)=4.545$, $p=0.038$) which points to differences in the effects of treatment on devaluation sensitivity due to Sex and/or Tracking. In ST rats, we did not observe any significant main effects or interactions, when collapsed across Sex. (Fig. 2.3B).

In GT/INT rats, we observed a significant Outcome Value x Treatment interaction (Fig. 2.3C, $F(1,27)=5.377$, $p=0.028$) when data were collapsed across Sex. When collapsed across Tracking, in male rats we observed a main effect of Outcome Value and an Outcome Value x Treatment interaction (Fig. 2.4A, Value: $F(1,25)=6.084$, $p=0.021$; Value X Treatment: $F(1,25)=6.440$, $p=0.018$), while in female rats, we did not observe any significant main effects or interactions (Fig. 2.4B). We confirm that under vehicle conditions, male rats are sensitive to outcome devaluation ($t(13)=4.670$, $p<0.001$), while female rats are not. Altogether, these results point to sex differences in the effects of treatment on devaluation sensitivity within Tracking groups.

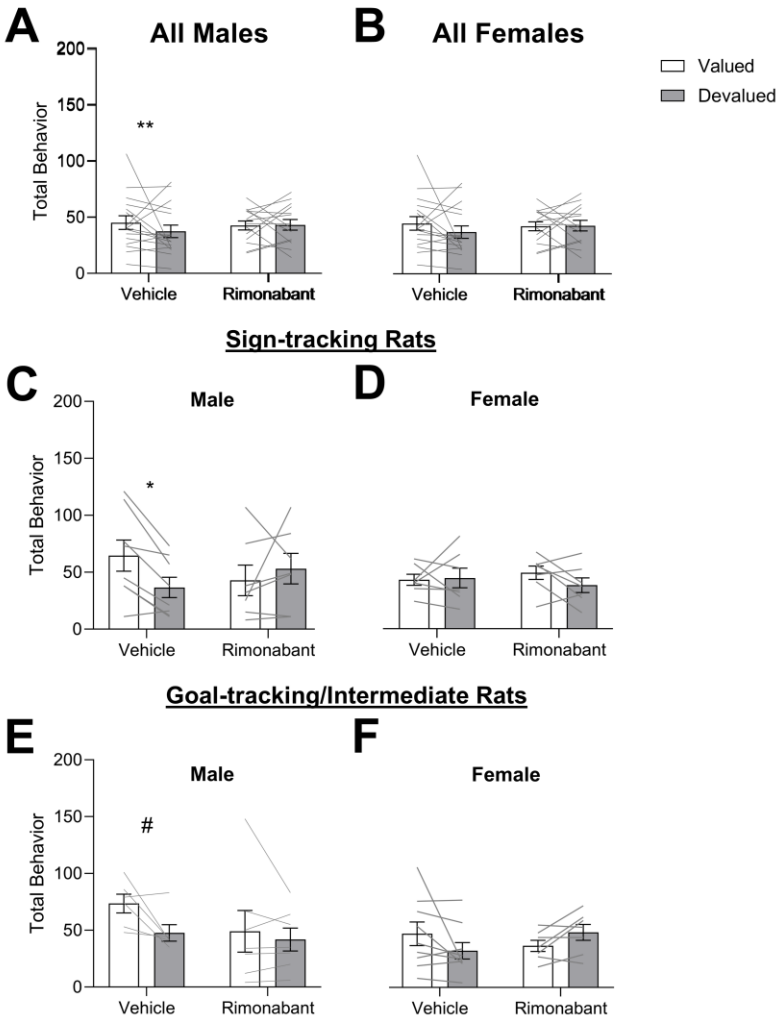

Figure 2.4: Male, but not female, rats are sensitive to Pavlovian Outcome Devaluation, and this sensitivity is blocked by intra-DMS Rimonabant regardless of Tracking type. Data are represented as within-subject individual data (lines) and group data (bars; mean ± SEM). Rats received intra-DMS injections of either vehicle (left) or rimonabant (right) 10 minutes prior to probe test. ***A,*** In Male rats, we observed a significant main effect of Outcome Value and a significant Outcome Value X Treatment interaction. ***B,*** In Female rats, we did not observe any significant main effects or interactions. ***C, D,*** In ST rats, we observe a significant Outcome Value X Treatment X Sex interaction on total behavior. ***C,*** In Male ST rats we observed a significant Outcome Value X Treatment interaction. ***D,*** In ST Female rats there were no main effects or interactions. We then performed a parallel analysis in our GT/INT rats. ***E, F*** In GT/INT rats, we observe an Outcome Value X Treatment interaction, but no interaction with Sex. #p=0.067 *p<0.05 **p<0.025

In a prior study using male rats, it was established that initially devaluation insensitive ST rats become devaluation sensitive after extended training (Keefer et al., 2020). The present study replicates this finding and shows that under vehicle conditions, male ST rats are sensitive to outcome devaluation (Fig. 2.4C, Bonferroni post-hoc; t(13)=2.679, p=0.037). Here we use both sexes and identify an Outcome Value x Treatment x Sex interaction in ST rats (F(1,24)=6.210, p=0.020), suggesting potential sex differences in devaluation sensitivity and/or effects of CB1R signaling inhibition. First, we confirmed the Outcome Value x Treatment interaction that was observed overall (Fig. 2.4A) is also observed in male ST rats (Fig. 2.4C, F(1,12)=5.063, p=0.044). Post-hoc analyses confirmed that intra-DMS rimonabant injections impaired devaluation sensitivity in male rats with similar levels of Pavlovian approach for valued and devalued conditions (t(13)=0.9205, p=0.7482). We found similar trends for male ST rats in lever contacts (the dominant response of ST rats) during outcome devaluation (Fig. 2.5A), in which there was a significant Outcome Value X Treatment interaction (F(1,13)=4.810, p=0.047) but post hoc tests did not reach significance even for the vehicle condition (t<2.484, p>0.0548). As expected, we observed no significant effects when analyzing male ST foodcup contacts (Fig. 2.6A). In contrast to males, female ST rats showed similar levels of responding in all probe tests and intra-DMS rimonabant had no effects (Total Behavior, Fig. 2.4D, *Fs*<1.236, *ps*>0.288; Lever, Fig. 2.5B; Foodcup, Fig. 2.6B).

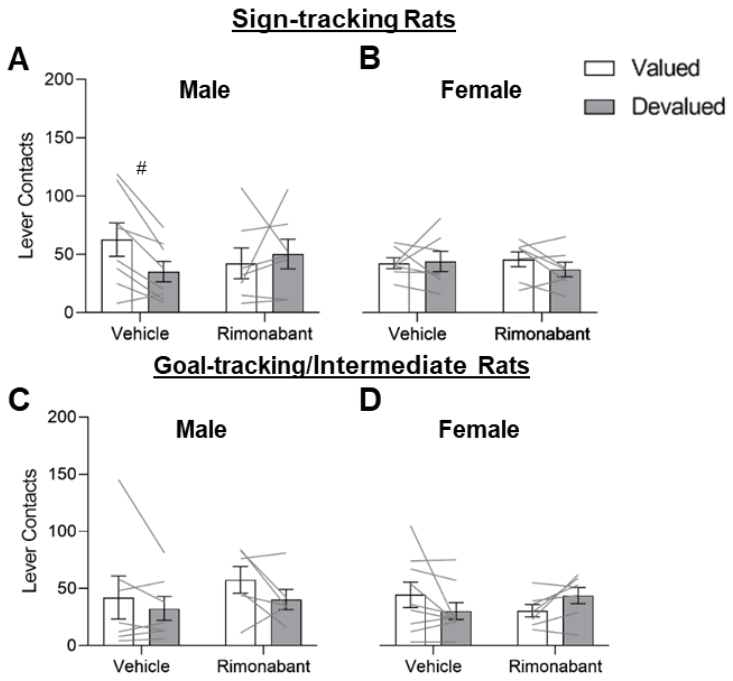

Figure 2.5: Male ST rats are sensitive to Pavlovian Outcome Devaluation for lever responding, which is blocked by intra-DMS Rimonabant. Data are represented as within-subject individual data (lines) and group data (bars; mean ± SEM). Rats received intra-DMS injections of either vehicle (left) or rimonabant (right) 10 minutes prior to probe test. We observed a significant Outcome Value X Treatment X Tracking X Sex interaction for lever presses. ***A, B,*** In ST rats, we observe a significant Outcome Value X Treatment X Sex interaction on lever presses. ***A,*** In Male ST rats we observed a marginal Outcome Value X Treatment interaction (p=0.073). ***B,*** In ST Female rats there were no main effects or interactions. We then performed a parallel analysis in our GT/INT rats. ***C, D*** In GT/INT rats, we did not observe any significant main effects or interactions. #p=0.054

Consistent with prior studies, male GT/INT rats were sensitive to outcome devaluation after extended training (main effect of Outcome Value (Fig. 2.4E; $F(1,11)=5.203$, $p=0.043$). In contrast to the ST group, we observed no significant main effects or interactions with Sex in GT/INT group. Despite this, we performed parallel analyses and found a marginal devaluation effect under vehicle condition in male GT/INT rats ($t(11)=2.425$, $p=0.0675$).

For GT/INT the dominant response is food cup contacts, and for this measure there was a significant Outcome Value X Treatment interaction (Fig. 2.6C; F(1,11)=7.279, p=0.0207). Post hoc analysis revealed that under vehicle conditions, male GT/INT rats were sensitive to outcome devaluation (t(11)=2.872, p=0.0304) which was not the case with intra-DMS rimonabant (t(11)=0.8692, p=0.8066). We observed no significant differences when analyzing lever contacts alone (Fig. 2.5C).

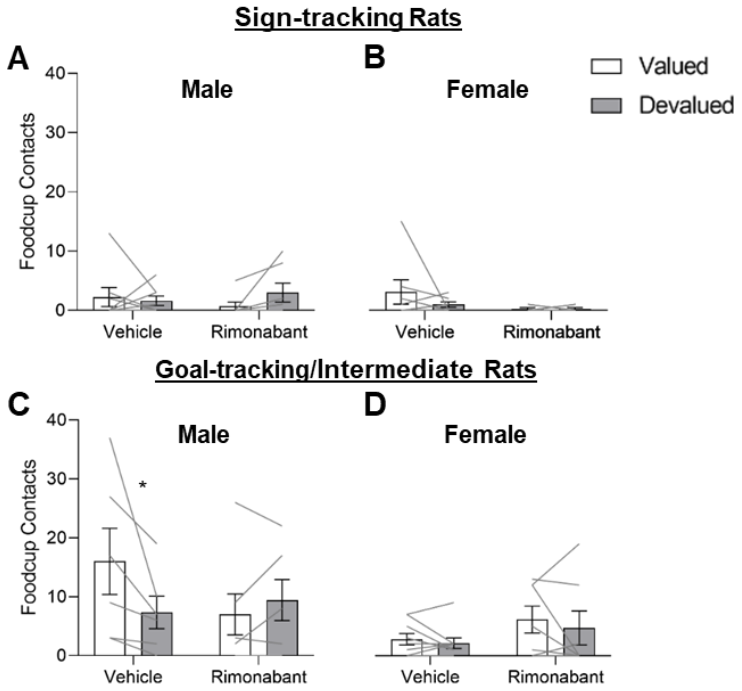

Figure 2.6: Male GT/INT rats are sensitive to Pavlovian Outcome Devaluation for food cup responding, which is blocked by intra-DMS Rimonabant. Data are represented as within-subject individual data (lines) and group data (bars; mean ± SEM). Rats received intra-DMS injections of either vehicle (left) or rimonabant (right) 10 minutes prior to probe test. For all rats, we observed a significant Outcome Value X Treatment interaction and main effects of Sex and Tracking. ***A, B,*** In ST rats, we did not observe any significant main effects or interactions. We then performed a parallel analysis in our GT/INT rats. ***C, D*** In GT/INT rats, we observe an Outcome Value X Treatment interaction, and a main effect of Sex. ***C,*** In male GT/INT rats, we observed a significant Outcome Value X Treatment interaction. ***D,*** In female GT/INTs, we did not observe any significant interactions or main effects. *p<0.05

Female GT/INT rats showed a significant Outcome Value X Treatment interaction for total behavior (Fig. 2.4F; $F(1,14)=5.100$, $p=0.040$) that was driven by opposite patterns of behavior for the two treatments, however differences between value conditions did not reach significance for either treatment (vehicle, valued vs. devalued, $t(14)=1.907$, $p=0.1545$, rimonabant, valued vs. devalued ($t(14)=1.329$, $p=0.410$). We found a similar Outcome Value X Treatment interaction when looking at female GT/INT lever contacts alone (Fig. 2.5D; $F(1,14)=4.953$, $p=0.043$) but no significant interactions for food cup contacts (Fig. 2.6D); however, none of the post hoc analysis for these measures reached significance in female GT/INT rats. Altogether, these results point to sex differences in Pavlovian outcome devaluation sensitivity and to treatment effects on Pavlovian devaluation sensitivity in male rats. Male rats are sensitive to devaluation after extended training, while female rats are not. The effects of intra-DMS CB1R blockade on devaluation sensitivity in male rats are consistent across tracking groups but are response specific. In male ST rats this sensitivity is driven by lever contacts, while in male GT/INTs, this sensitivity is driven by food cup contacts. Additionally, we find a significant difference in the number of foodcup pokes made between males and females. Males made significantly more foodcup pokes than females in both the vehicle and rimonabant groups (Main effect of Sex; $F(1,25) = 5.198$, $p=0.031$). This suggests that male GT/INTs display more goal-tracking behavior during devaluation tests than females that are show more sign-tracking during tests as we have shown previously (Keefer et al., 2022; Kochli et al., 2020).

These effects of DMS CB1R signaling inhibition were specific to the satiety-specific outcome devaluation test. We found no difference in responding between vehicle and rimonabant groups during a non-sated, non-reinforced lever autoshaping test of the same

duration (10 trials, Fig. 2.7A; Sex x Treatment x Response (lever, foodcup), Fs<0.479, ps>0.493). This suggests that sex-specific intra-DMS rimonabant treatment effects on Pavlovian approach emerge only after outcome-specific satiety.

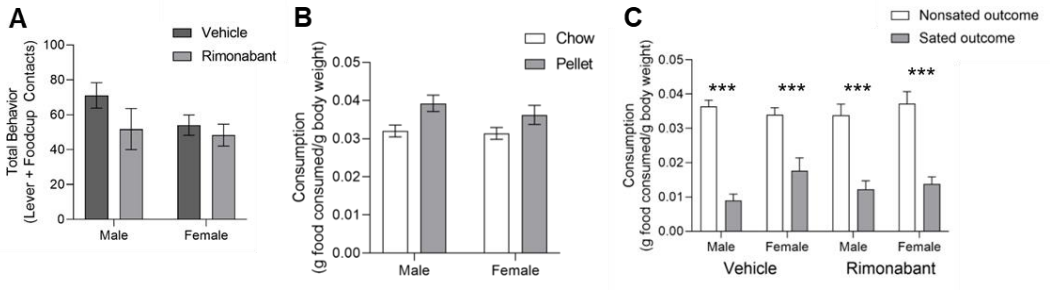

Figure 2.7: Consumption and response in a non-sated test do not change due to Sex or Treatment. Data are represented as group data (bars; mean ± SEM) ***A,*** In a post-session choice test, we gave rats 30 minutes of access to both of the outcomes and they consumed less of the outcome they were stated on, regardless of Sex or Treatment. ***B,*** Prior to devaluation probe tests, rats ate a similar amount of chow to pellets overall. ***C,*** In a non-sated extinction test identical in duration to the sated tests, we observed no effects of CB1R inhibition on total behavior for male or female rats. ***p<0.001

The observed effects are also not due to differences in consumption between male and female rats during the 1-hour satiation period. We normalized the amount (g) of food consumed during the either satiation period or post-probe choice test to the average body weight of each rat across both days of outcome devaluation tests to account for the difference in body weight between male and female rats of the same strain and age (Council, 1995; Lenglos et al., 2013). We found no difference in the amount of food consumed during the satiation period prior to the probe test (Fig. 2.7B; Fs<1.153, ps>0.288). To confirm devaluation of the sated food, we gave rats a post-probe choice test between the chow and training pellets (Fig. 2.7C) immediately after the end of the outcome devaluation probe test. Rats consumed less of the food that they were sated on and more of the alternative food, as verified by a main effect of Outcome ($F(1,45)=8.134$, $p<0.007$) and

this did not differ by sex or treatment (*Fs*<1.790, *ps*>0.187). These data also serve as an important control for rimonabant effects on consumption as prior studies report that rimonabant reduces consummatory behaviors (Thornton-Jones et al., 2005). We find no significant effects of intra-DMS infused rimonabant at the dose tested (1µg/µL). The observed effects of blockade of CB1R signaling following outcome devaluation were not evident during non-sated, reinforced PLA sessions. We tested the effect of intra-DMS rimonabant infusion on a subset of rats (n=12) during non-sated, reinforced PLA sessions and found no significant difference across sex or tracking between vehicle, low (1µg/µl) or high dose (2µg/µl) of rimonabant on PavCA (Fig. 2.8A; *Fs*<2.062, *ps*>0.16), lever presses (Fig. 2.8B; *Fs*<1.972, *ps*>0.198) or on foodcup contacts (Fig. 2.8C; *Fs*<1.078, *ps*>0.329). Prior research reports that rimonabant reduces locomotion (Tallett et al., 2007). Responses were not significantly impacted by intra-DMS infused rimonabant, suggesting no reduction in overall locomotion. We did observe a significant main effect of Sex for lever contacts ($F(1,10)=5.395$, $p=0.043$), which is in line with acquisition data, during which females showed more sign-tracking early in training. Overall, rimonabant inhibition of DMS CB1R signaling did not impact conditioned approach under reinforced conditions.

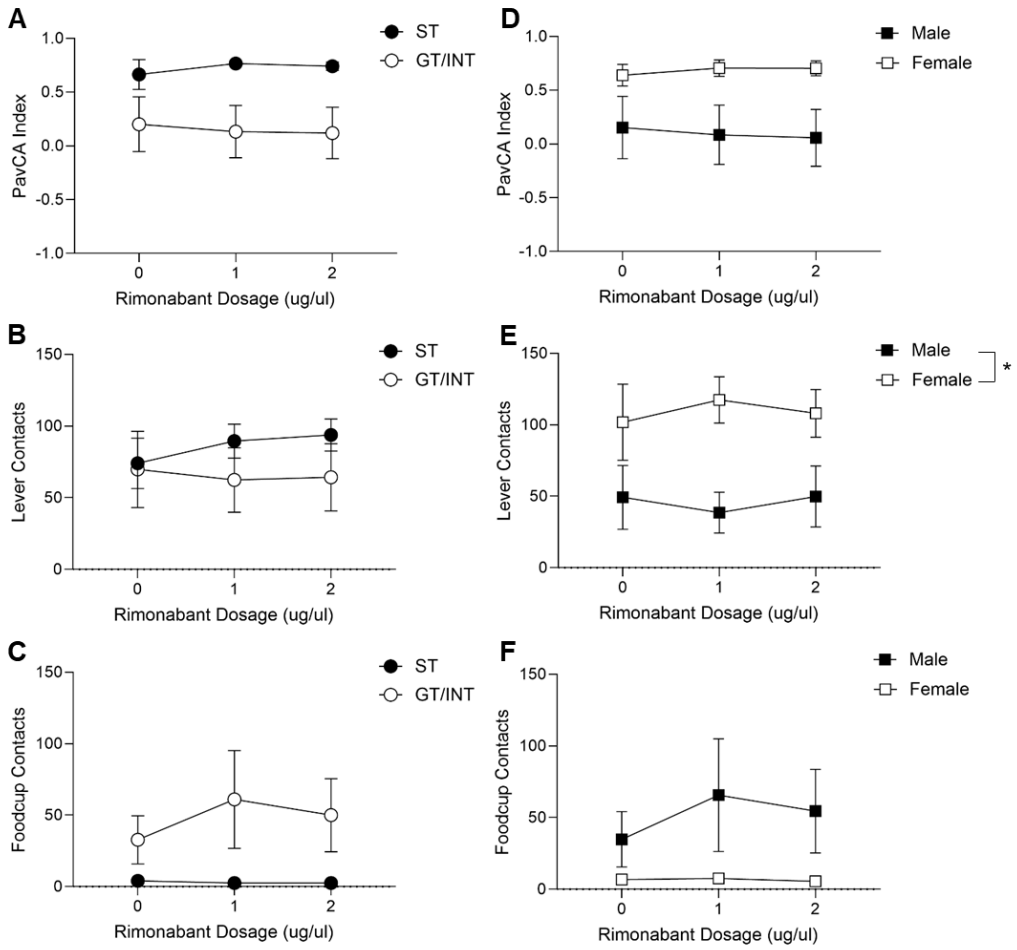

Figure 2.8: Intra-DMS inhibition of CB1Rs by Rimonabant does not alter behavior in reinforced PLA sessions. Data are represented as per day group averages; mean ± SEM. All rats received intracranial infusion of vehicle or Rimonabant (1µg/µl, 2 µg/µl) 10 min prior to the start of the PLA session. ***A,B,C,*** We observed no significant interactions with Treatment for Tracking or Sex on PavCA Index, lever, or foodcup contacts. We observed a significant main effect of Sex on lever contacts. *p<0.05

2.4. Discussion

In the current study, we examined the influence of sex on the acquisition of Pavlovian conditioned approach and tested the effects of blocking DMS CB1R signaling on Pavlovian satiety-specific outcome devaluation. Consistent with previous studies (Keefer et al., 2022; Kochli et al., 2020; Pitchers et al., 2015), we found that female rats showed more lever-

directed behaviors than males during extended training which is driven by female GT/INT rats, but this difference diminished before testing in outcome devaluation. Under vehicle conditions, we replicated previous findings that male ST rats show devaluation sensitivity after extended training in PLA (Keefer et al., 2020) but found that female ST rats persist in being insensitive to devaluation after extended training. Similarly, female GT/INT rats also show insensitivity to devaluation in this study. These results echo the findings of other studies that indicate females are less sensitive to instrumental and Pavlovian devaluation and thus, are prone to habit-driven responding (Quinn et al., 2007; H. L. Schoenberg et al., 2019; Sood & Richard, 2023). Further analyses into either lever or foodcup contacts alone revealed that male rats were sensitive to devaluation for their preferred response of their tracking group. ST male rats reduced lever contacts when the outcome is devalued (Fig. 2.5A), while GT/INT male rats reduce foodcup contacts (Fig. 2.6C) as has been shown previously in studies that examine Pavlovian outcome devaluation after extended training (Keefer et al., 2020, 2022). Surprisingly, we found that DMS CB1R signaling promotes devaluation sensitivity in male rats, without affecting the behavior of female rats.

At first, we predicted that DS CB1R signaling would promote rigid, or habitual, behaviors as has been shown for instrumental outcome devaluation (Gremel et al., 2016). However, our study suggests that CB1Rs in DMS promote behavioral flexibility in male rats, running counter to this established understanding. There are several factors that contribute to the divergence of results including species differences, the use of Pavlovian versus instrumental devaluation procedures and the subregion-specific effects of experimental manipulations. This prior study trained CB1R flox mice in both random-ratio (RR) and random interval (RI) schedules of instrumental reinforcement and generated an

OFC-DS specific CB1R knockout. Competing action-outcome and stimulus-response associations mediate instrumental devaluation, and studies show that goal-directed behaviors shift to habit with extended training or with RR schedules of reinforcement (Adams, 1982; Adams & Dickinson, 1981; Gremel et al., 2016). This is not the case with Pavlovian behaviors that are sensitive to devaluation even after extended training (P. Holland, 1998; Keefer et al., 2020), suggesting stimulus-outcome associations support adaptive reward seeking despite overtraining. Thus, differences in Pavlovian and instrumental processes may, in part, underline divergent findings in male rodents between studies. Never-the-less, the present study suggests that Pavlovian behaviors are insensitive to devaluation in females after extended training, suggesting that female rats rely on stimulus-response associations to drive behavior. This is in line with other studies where females more readily acquire a Pavlovian approach and remain insensitive to devaluation (Hammerslag & Gulley, 2014; Keefer et al., 2022; Kochli et al., 2020; Madayag et al., 2017; Pitchers et al., 2015; Stringfield et al., 2019).

Another possibility is methodological differences in the way CB1R were manipulated between studies. CB1R deletion in the OFC-DS projection promoted "goal-directed" devaluation sensitivity even during schedules of reinforcement that ordinarily drive "habitual" devaluation insensitivity (Gremel et al., 2016). Our current study inhibits CB1R signaling indiscriminately- likely affecting both inhibitory and excitatory synaptic transmission- rather than specifically on glutamatergic OFC afferents to the DS, as in the prior study. Additionally, our use of rimonabant, a CB1R inverse agonist, will block binding of eCBs and inhibit the activity of constitutively active CB1Rs. Prior work has shown that systemic activation of CB1Rs promotes rigid responding (Hilário et al., 2007;

Nazzaro et al., 2012) and while both DLS and DMS express CB1Rs (Fusco et al., 2004; Hohmann & Herkenham, 2000; Van Waes et al., 2012), more of the CB1R work in the DS has focused on the DLS. The DLS does express CB1R more densely than DMS, thus, it is possible that off-target effects impacted DLS function, an area with high CB1R density (Hohmann & Herkenham, 2000; Fusco et al., 2004; Van Waes et al., 2012) and this could confound our results. However, we think this is unlikely given the volume of the rimonabant solution injected (0.5 µL per hemisphere). The current targeting of DMS, as compared to DLS, may in part explain why our results diverge from previous observations that dorsal striatal CB1Rs support rigid responding via inhibition of glutamatergic inputs and our findings fit within the context of the DMS' role of biasing behavior towards "goal-directed" responding (Yin et al., 2005; Corbit & Janak, 2010; Gremel & Costa, 2013; Li et al., 2022).

These prior studies established that the DMS supports flexible, goal-directed conditioned responses. Reducing the activity of the DMS through lesion or pharmacological inhibition impairs flexible responding in a variety of tasks (Corbit & Janak, 2010; Gremel & Costa, 2013; Li et al., 2022; Ragozzino et al., 2002; Yin et al., 2005). To be interpreted in this conceptual framework, the present results suggest that CB1R signaling promotes DMS activation and that inhibiting CB1Rs signaling decreases DMS output to impair Pavlovian devaluation sensitivity. A primary prediction stemming from this interpretation is that DMS CB1R signaling at GABAergic inputs to MSNs reduces inhibitory tone in the area, allowing for DMS activation to promote flexible responding in Pavlovian devaluation. While the current study does not identify the cell-type affected by DMS CB1R manipulations, parvalbumin-expressing fast-spiking interneurons

(FSIs) are a prime candidate mechanism to explain the observed results here with intra-DMS CB1R antagonism during Pavlovian devaluation. While FSIs make up only 1-5% of striatal neurons (Kawaguchi et al., 1995), each cell is estimated to project onto ~100 MSNs and receive glutamatergic cortical projections, forming a strong feedforward inhibitory circuit (Gittis et al., 2010; Koós & Tepper, 1999; Plenz, 2003; Tepper & Bolam, 2004). Direct manipulation of DLS PV-FSIs shows that their activity is critical to supporting habitual responding (O'Hare et al., 2017; Patton et al., 2020) but less is known about DMS PV-FSIs and their contribution to habitual or goal-directed responding. CB1Rs are expressed on striatal PV-FSIs and mediate a form of inhibitory LTD that disinhibits MSNs, a mechanism that is associated with striatal regulation of behavioral flexibility (DePoy et al., 2013; Mathur et al., 2013). CB1Rs are also expressed on cortical inputs that target MSNs (G. L. Gerdeman et al., 2002; G. Gerdeman & Lovinger, 2001; Lovinger et al., 2022; Lovinger & Mathur, 2012; Y. W. Wu et al., 2015), but it has not yet been established in the DMS whether cortical projections targeting FSIs also contain CB1Rs. CB1R signaling at cortical-striatal FSI synapses would be expected to reduce inhibitory tone and increase DMS MSN activation, a similar result to CB1R signaling at FSI-MSN synapses. Thus, these two hypotheses must be tested further to discover the mechanism of DMS CB1R regulation of Pavlovian devaluation sensitivity. Direct manipulation of either cortical-FSI or FSI-MSN circuitry through optogenetics or chemogenetics would uncover a role for one or both circuits in Pavlovian outcome devaluation. However, completing these studies in rats may prove difficult due to the limited availability of specific strains of transgenic rats. A transition to transgenic mouse models may be necessary to ask circuit specific questions.

Overall, the current studies show that DMS CB1Rs promote sensitivity to Pavlovian devaluation for male rats, regardless of tracking. More work is needed to identify the cell-type specific population of DMS CB1Rs that support flexible responding. Additionally, it is possible that CB1Rs would be necessary for the devaluation sensitivity of females in cases where they respond flexibly at baseline. Thus, future studies should manipulate DMS eCBs under conditions in which males and females respond similarly to determine if CB1Rs play a sex-specific role in mediating behavioral flexibility. Alternative methods of outcome devaluation such as illness-induced outcome devaluation, result in similar patterns of flexibility in males and females (Bien & Smith, 2023). I discuss this possibility more in-depth in the final chapter of this dissertation.

Chapter 3: Sex Differences in Recordings of Spontaneous Inhibitory Currents in the Dorsomedial Striatum.

3.1. Introduction

Behavioral flexibility is an adaptive mechanism by which a subject alters a previously learned behavior due to a change in internal state, external environment, or previously learned contingencies (Audet & Lefebvre, 2017; Brown & Tait, 2010; Lea et al., 2020). Research shows that the dorsal striatum (DS) is critical in regulating the flexibility of behavior (Belin et al., 2009; Malvaez & Wassum, 2018; Yin et al., 2005). The DS is subdivided into two regions, medial (DMS) and lateral (DLS) and many studies have focused on these regions to better understand how their activity maps onto the expression of behavioral flexibility.

Lesion studies show that the DLS is critical for the expression of inflexible, or habitual, behavioral strategies while the DMS is critical for flexible, or goal-directed, behavior (Amaya & Smith, 2018; Dickinson et al., 1995; Peak et al., 2019; Yin et al., 2004, 2005). Additional in vivo electrophysiology studies reveal that activity of the DMS is high after limited training and that with extended training, the DMS activity attenuates and the activity of the DLS then increases (Gremel & Costa, 2013). Within the DS, multiple cell-types mediate the activity of either subregion. Approximately 90% of cells across the DS are medium spiny neurons (MSNs), the main type of projection neurons arising from the DS (Graveland & Difiglia, 1985). Remaining local neuron types are the Parvalbumin positive fast-spiking interneurons (FSIs), acetylcholine interneurons, somatostatin/neuropeptide Y containing-neurons, tyrosine hydroxylase positive interneurons, and calretinin-containing interneurons.

One of the most abundant receptor types in the DS is the Cannabinoid receptor-1 (CB1R) which is a G-protein coupled receptor that is expressed presynaptically on glutamatergic inputs into the DS and locally on MSNs and FSIs (G. L. Gerdeman et al., 2002; G. Gerdeman & Lovinger, 2001; Lovinger & Mathur, 2012a; Mathur et al., 2013a; Y. W. Wu et al., 2015). Signaling through these receptors is important for the expression of inflexible or habit-driven behaviors in instrumental paradigms and chronic stimulation of these receptors will result in habitual or compulsive responding that can be maladaptive (Gremel et al., 2016; Hilário et al., 2007; Nazzaro et al., 2012). However, our work in the previous chapter has shown that CB1Rs in the DMS are necessary for the expression of flexibility in PLA (Figure 2.2). Since inactivation of the DMS can lead to inflexible responding (Ragozzino et al., 2002; Yin et al., 2005), we theorize that CB1Rs located on inhibitory synapses from MSNs or FSIs in the DMS act to reduce inhibition, leading to higher activity of the DMS and allowing for flexible responding.

Sex differences in DS-related behaviors and electrophysiology have been shown in prior research, especially in the case of dopamine release and signaling (Becker & Beer, 1986; Becker & Ramirez, 1981; Di Paolo et al., 1986; Walker et al., 2006; Zachry et al., 2021). However, there is limited research into the influence of sex on endocannabinoid (eCB) mechanisms in the DS. Studies focusing on other areas of the brain such as the Prefrontal Cortex and Hippocampus have uncovered sex differences in basal physiology (Borsoi et al., 2019; Tang et al., 2005; Wyrofsky et al., 2018). Thus, this study includes both male and female Long Evans rats to investigate potential sex differences in eCB effects on DS physiology.

We set out to record spontaneous inhibitory postsynaptic currents (sIPSCs) in the DMS to better understand sex differences through the lens of whole-cell patch clamp electrophysiology and to assess how CB1R signaling may impact inhibitory transmission in the DMS. We focus on only the DMS as a follow-up to our behavioral pharmacology findings where CB1Rs in the DMS were necessary for the flexible responding of male rats. We recorded sIPSCs from cells in the DMS before and after application of a CB1R agonist and compared all measurements between males and females. We found no sex differences in the effect of CB1R activation on sIPSCs but uncover sex differences under baseline conditions.

3.2. Materials and Methods

Subjects. We used 24 Long Evans rats (13 Male, 11 Female) in the age range of 9-15 weeks old at the time of slice electrophysiology recording. All rats were double housed upon arrival. We maintained rats on a reverse 12hr:12hr light-dark cycle (lights off at 1000). All rats had *ad libitum* access to standard laboratory chow and water before we food deprived them 24 hours prior to slice electrophysiology recording. We performed all procedures in accordance with the "Guide for the Care and Use of Laboratory Animals" (8th edition, 2011, US National Research Council) and with approval by the University of Maryland, School of Medicine Institutional Animal Care and use Committee (IACUC).

Brain Slice Preparation for Slice Electrophysiology. We anesthetized rats with isoflurane then perfused with chilled N-Methyl-D-Glucamine (NMDG)-modified artificial cerebrospinal fluid (NMDG-aCSF; in mM; 92 NMDG, 20 HEPES, 25 Glucose, 30 $NaHCO_3$, 1.3 NaH_2PO_4, 2.5 KCl, 5 Sodium Abscorbate, 3 Sodium Pyruvate, 2 Thiourea,

10 MgSO4, 0.5 CaCl2) that had been bubbled with carbogen (95% oxygen, 5% carbon dioxide). Coronal sections of the DMS (350 μm) were cut in chilled, carbogen-bubbled NMDG-aCSF, using a Leica VT 1200 vibratome. Slices were incubated in carbogen-bubbled NMDG-aCSF at 40ºC for 5-8 minutes and then transferred to room temperature, carbogen-bubbled HEPES-buffered holding solution (in mM; 92 NaCl, 20 HEPES, 25 Glucose, 30 NaHCO3, 1.3 NaH2PO4, 2.5 KCl, 5 Sodium Abscorbate, 3 Sodium Pyruvate, 2 Thiourea, 1 MgSO4, 2 CaCl2). We waited 1 hour before collecting the first recordings. Sections remained in the holding solution until electrophysiological recordings were performed.

Recordings and Bath Application of Drug. We visualized cells in the DMS using an Olympus BX50 light microscope. We recorded sIPSCs from neurons using borosilicate, fire-polished glass pipettes with resistance in the 3-5 MΩ range. We pulled pipettes with a Narishige PC-100 pipette puller and filled them with a CsCl-based internal solution (in mM; 150 CsCl, 10 HEPES, 2 MgCl2*H2O, 0.3 Na-GTP, 3 Mg-ATP, 0.2 BAPTA). We recorded from hemisected slices that were constantly perfused with 37°C, carbogen-bubbled aCSF (in mM; 126 NaCl, 25 NaHCO3, 11 Glucose, 1.2 MgCl2*H2O, 1.4 NaH2PO4, 2.5 KCl, 2.4 CaCl2) containing blockers of AMPAR (DNQX, 20μM) and NMDAR (AP5, 50μM). We perfused the recording chamber with a basic Longer Pump BT100-2J peristaltic pump. We also recorded from slices submerged in a bath containing a vehicle of DMSO (0.065%) and 2-hydroxypropyl-beta-cyclodextrin (0.006%). We clamped cells at -60 mV using a Molecular Devices Multiclamp 700B amplifier and digitized recordings with a Molecular Devices Axon Digidata 1550B digitizer. We used Molecular Devices Clampex 10.7 software for data acquisition. We excluded recordings when holding

current was <-200 pA, series resistance was >40 MΩ, or series resistance changed >20% throughout the course of the experiment.

Measurements. We processed sIPSC traces using the template search function in Molecular Devices Clampfit 10.7 software to determine event peak amplitude and event peak start time. We report these measurements in each experiment: Amplitude, calculated as the peak amplitude of an event and averaged across each recording; Frequency, calculated as the number of events per recording divided by the duration of the recording in seconds; Inter-Event Interval (IEI), calculated as the inverse of the time (in seconds) between the peak of an event and the peak of the event prior and represented through a cumulative frequency distribution.

Statistical Analysis. Data are represented as mean ± standard error. We performed repeated measures two-way ANOVA, student's t-test, Mann-Whitney and Wilcoxon matched pairs analyses as appropriate using either SPSS 29.0 (IBM software) or Prism (GraphPad software). Analysis included within-subject variable of Bath (pre-DMSO, post-DMSO; pre-WIN, post-WIN) and between-subject variable of Sex (Male, Female). We removed two data points, one from each sex, based on results from Grubb's Test for Outliers.

3.3. Results

Baseline spontaneous IPSC recordings in DMS cells between male and female Long Evans rats. Based on the sex differences in behavioral flexibility, we predicted that there may be differences in inhibitory synaptic transmission in the DMS where male rats may show reduced inhibitory currents. We recorded sIPSCs from cells in the DMS in males

and females (Figure 3.1; n=18 cells total, 1-2 cells per rat; males, n=5 rats; females, n=6 rats). We examined the mean amplitude (absolute value in pA), the mean frequency (in Hz), or total events across the duration of the recording, and the cumulative frequency distribution for inter- event interval (IEI), or the time between event peaks from 5-min recordings. We found that there is no difference in the mean amplitude of DMS sIPSCs between males and females when slices are perfused with aCSF (Figure 3.1b, t= -1.226, p=0.239). However, we did see a difference in both the frequency and IEI.

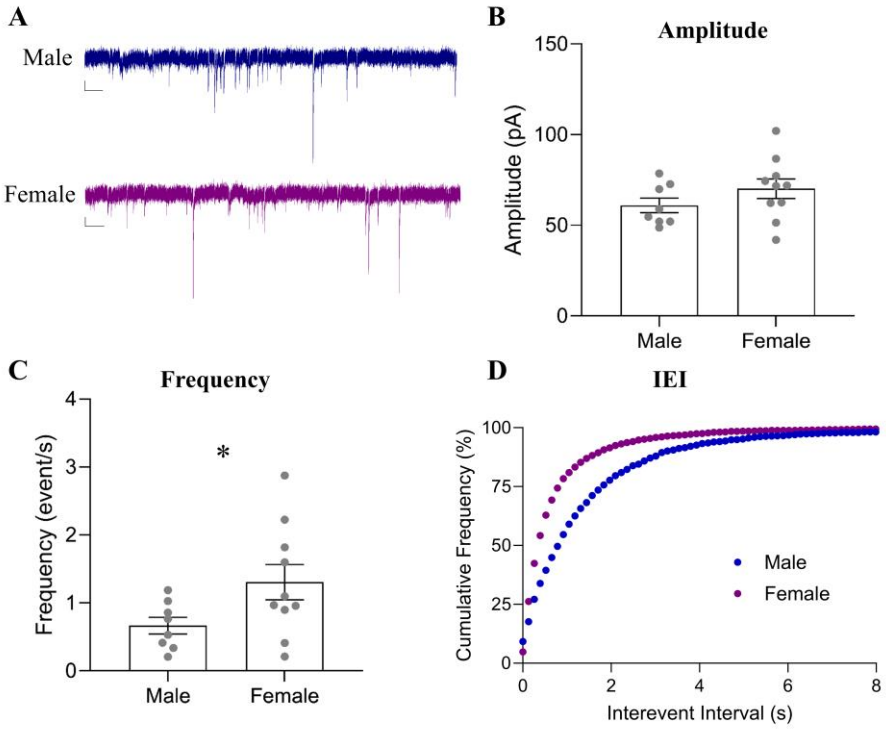

Figure 3.1: DMS cells show reduced sIPSC frequency in males as compared to females. ***A,*** Representative sIPSC traces from DMS cells in aCSF bath of male (blue, n=8 cells) and female (purple, n=10 cells) Long Evans Rats. Scale bars: 20 pA and 1 sec. Data are presented as mean ± SEM. ***B,*** Mean Amplitude ***C,*** Mean Frequency ***D,*** Cumulative Frequency Distribution of IEI for all events. *p<0.05

Male rats show a sIPSC lower frequency as compared to females (Figure 3.1c, t= -2.561, p=0.022) and a right shifted IEI curve, suggesting a larger IEI in males (Figure 3.1d, Kolmogorov-Smirnov test, D=0.2498, p<0.0001, Hedge's g = 0.426). This difference in frequency and IEI of sIPSCs suggests that male rats show less inhibitory synaptic transmission onto cells in the DMS than females.

Comparison of inhibitory synaptic transmission with normal aCSF and aCSF containing DMSO. We used a subset of animals (N=9 cells total, 1-2 cells per rat; males, n=3 rats; females, n=6 rats) to determine if there were any changes in sIPSC frequency and amplitude when DMSO (0.065%) and 2-hydroxypropyl-beta-cyclodextrin (0.006%) were included in the bath, as these were necessary to dissolve WIN 55,212-2 in future experiments. We recorded sIPSCs from DMS cells for 5 min then switched to a bath containing the DMSO and beta-cyclodextrin vehicle. After a 10-minute wash-on period, we then recorded from DMS cells for 5 min (Figure 3.2a). We found no difference in mean amplitude or frequency of sIPSCs (Figure 3.2b-c, Fs< 4.519, ps>0.071). When analyzing the cumulative distributions of IEI pre- and post-DMSO application and between Sex, we did find significant differences between the curves (Fig. 3.2d, Kruskal Wallis, H =231.1, p<0.0001).

Post hoc analyses revealed that there were no differences due to DMSO (Dunn's comparison, ps>0.0844) but that there was a significant right shift in the distribution curve for males as compared to females under both baseline and DMSO conditions (aCSF, p<0.001, Hedge's g = 0.583; DMSO, p<0.001, Hedge's g = 0.428); this finding mimics what was previously seen that males showed a larger IEI under aCSF conditions (Fig. 3.1).

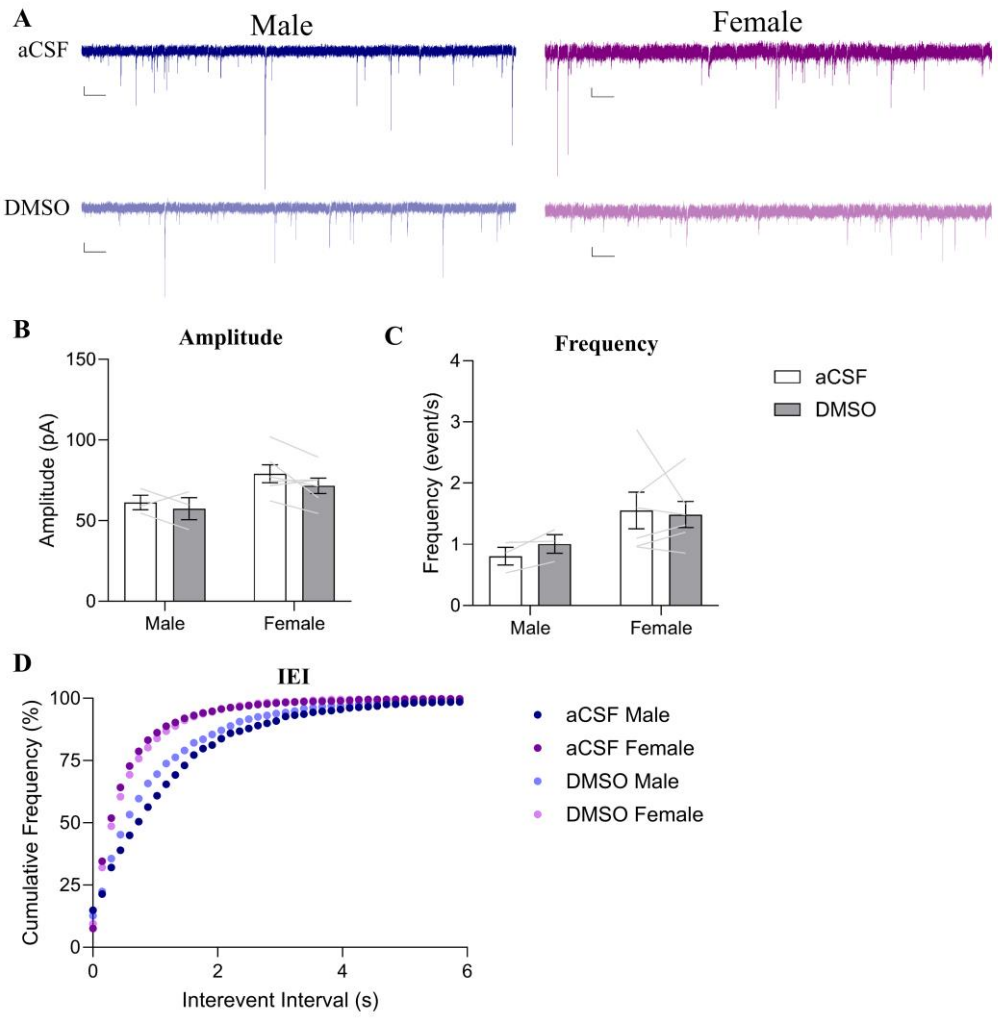

Figure 3.2: DMSO bath application does not alter inhibitory synaptic transmission as compared to baseline. ***A,*** Representative sIPSC traces from DMS cells pre- (darker shades) and post-DMSO (lighter shades) bath application for male (blue; n=3 cells) and female (purple; n=6 cells) Long Evans Rats. Scale bars: 20 pA and 1 sec. Data are presented as mean ± SEM. ***B,*** Mean Amplitude ***C,*** Mean Frequency ***D,*** Cumulative Frequency Distribution of IEI for all events.

We then analyzed recordings from different DMS neurons bath-treated with DMSO only to examine if there were any sex differences in sIPSC measurements (Fig. 3.3a; N=25 cells total, 1-3 cells per rat; males n=10 rats; females, n=8 rats). We found that there is no difference in sIPSC mean amplitude in DMS neurons of males and females in the presence of DMSO (Figure 3.3b, t= -0.446, p=0.660). We also found no difference in sIPSC frequency between males and females (F3.3c; t=0.913, p=0.371).

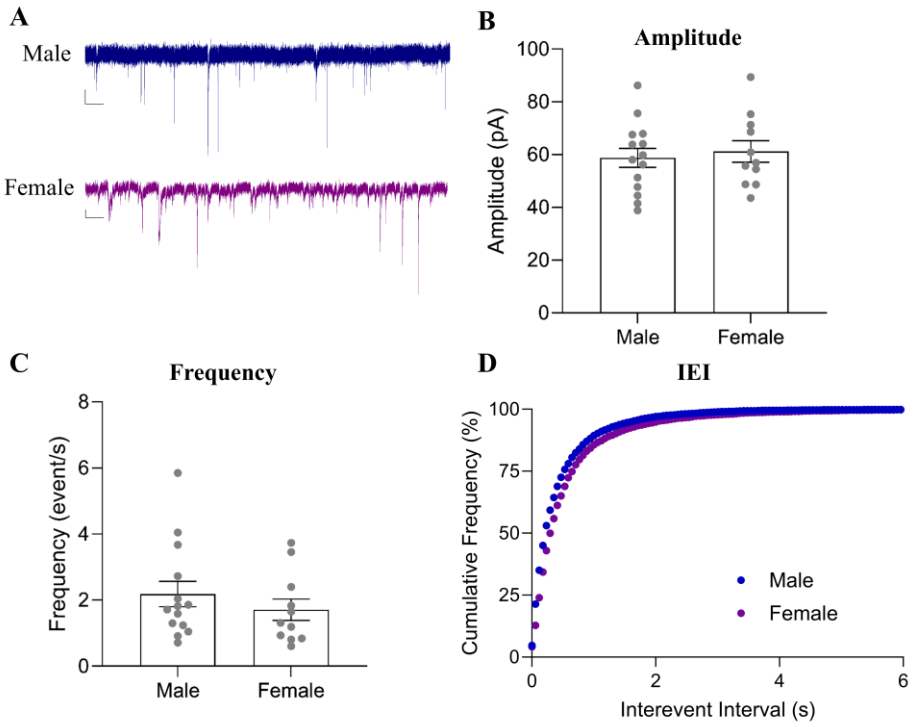

Figure 3.3: Male and female rats show no difference in sIPSC frequency under vehicle (DMSO) bath application but do differ in IEI. ***A,*** Representative sIPSC traces from DMS cells during DMSO bath application in male (blue, n=14 cells) and female (purple, n=11 cells) Long Evans Rats. Scale bars: 20 pA and 1 sec. Data are presented as mean ± SEM. ***B***, Mean Amplitude ***C,*** Mean Frequency ***D,*** Cumulative Frequency Distribution of IEI for all events.

There were significant differences in the cumulative distribution curves of the IEI between males and females. Analysis shows that there is a rightward shift for females as compared to males (Kolmogorov-Smirnov, $D=0.1160$, $p<0.001$, Hedge's $g = 0.180$) suggesting a larger IEI for females overall. However, the difference is miniscule and possibly negligible as the effect size was small.

Comparison of inhibitory synaptic transmission in male and female rats between DMSO and WIN 55, 212-2 bath applications. We hypothesized that activation of CB1R would reduce inhibitory synaptic transmission in male rats and we included females to investigate sex differences in the effect of CB1R manipulation on sIPSCs in the DMS. We recorded sIPSCs from DMS cells for 5 mins at baseline and following a 10-minute bath application of a CB1R agonist, WIN 55,212-2 (WIN; 10μM, Fig. 3.4a; N=21 cells total, 1-2 cells per rat; males n=8 rats; females, n=7 rats). We found that there were no differences in sIPSC mean amplitude due to WIN or Sex (Fig. 3.4b and 3.5b, $Fs<1.182$, $ps>0.290$). We found a main effect of WIN on sIPSC frequency ($F(1,19)=6.306$, $p=0.021$) but no main effect of or interaction with Sex ($Fs<0.825$, $ps>0.375$). We found that the application of WIN shifted IEI cumulative distribution curves to the right (Kruskal Wallis, $H=1359$, $p<0.001$).

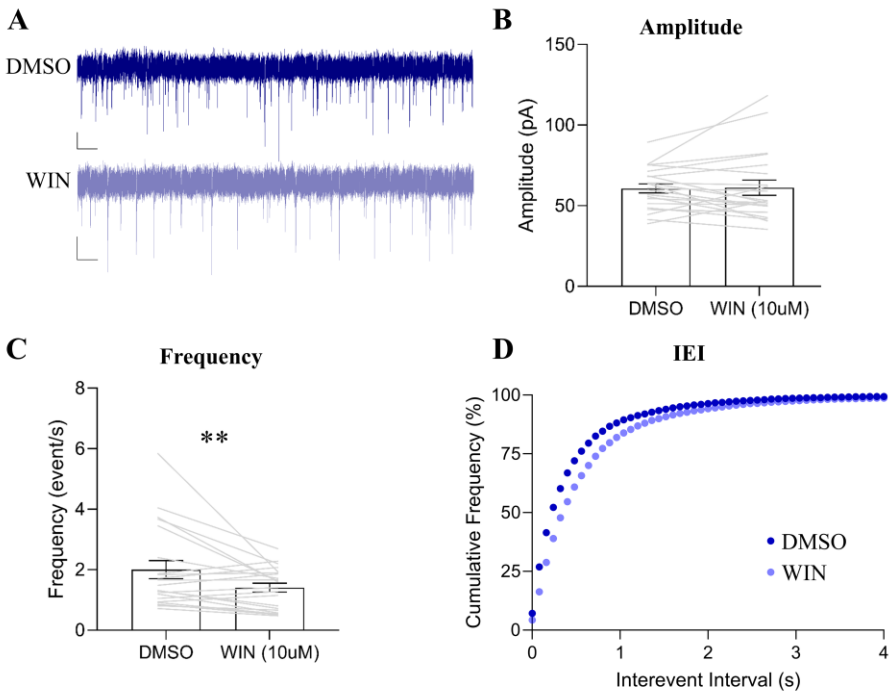

Figure 3.4: Activation of CB1Rs by WIN reduces frequency of inhibitory synaptic events. ***A,*** Representative sIPSC traces from DMS neurons pre- (blue) and post-WIN (light blue) bath application (n=21 cells). Scale bars: 20 pA and 1 sec. Data are presented as mean ± SEM. ***B,*** Mean Amplitude ***C,*** Mean Frequency ***D,*** Cumulative Frequency Distribution of IEI for all events. **p<0.025

Post hoc comparisons revealed a difference between males and females under DMSO conditions, as females showed a rightward shift in the distribution curve (Dunn's comparison; p<0.001, Hedge's g = 0.126); however, the effect size for this finding was small. We also show that WIN impacted both male and female rats (Fig. 3.5; DMSO vs. WIN, male, p<0.0001, Hedges' g = 0.2085; female, p<0.0001, Hedges' g = 0.2291). This rightward shift suggests that WIN increases IEI regardless of sex. Bath application of WIN reduced sIPSC frequency and increased IEI across all rats, suggesting that presynaptically expressed CB1Rs suppress release of GABA in the DMS.

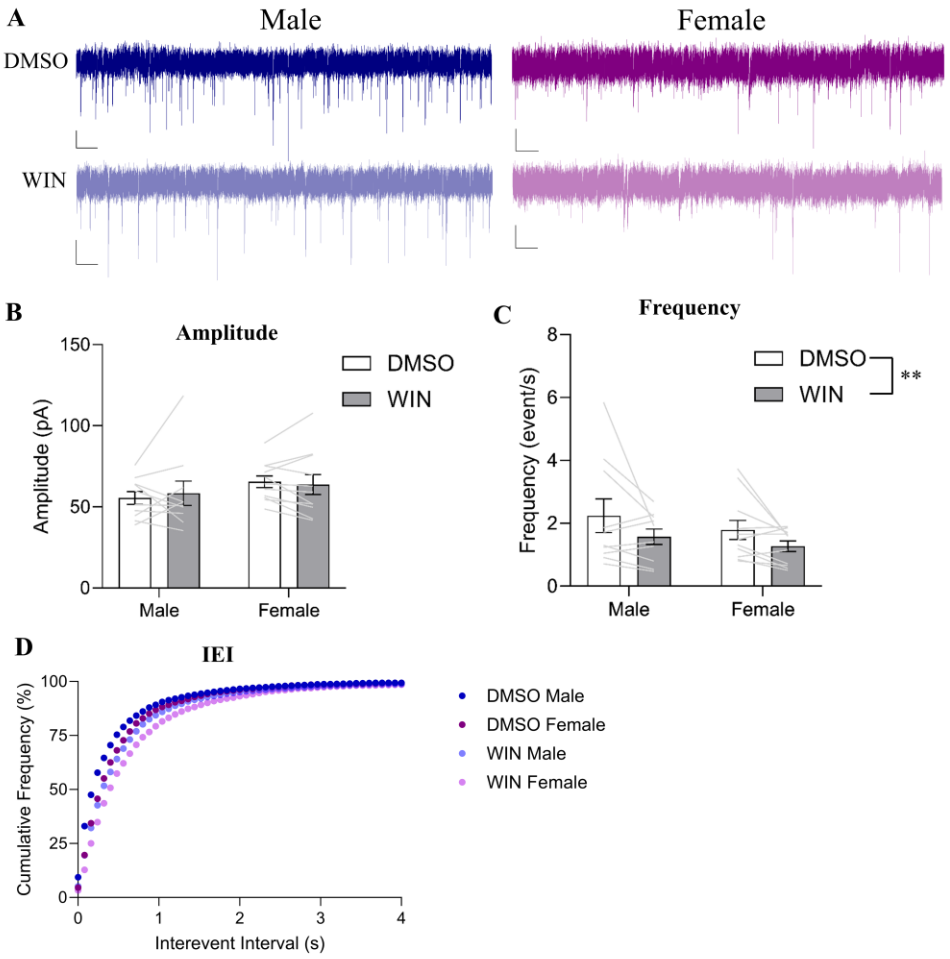

Figure 3.5: Activation of CB1Rs by WIN reduces sIPSC frequency regardless of Sex. A, Representative sIPSC traces from DMS cells pre- (darker shades) and post-WIN (lighter shades) bath application for male (blue; n=10 cells) and female (purple; n=11 cells) Long Evans Rats. Scale bars: 20 pA and 1 sec. Data are presented as mean ± SEM. B, Mean Amplitude C, Mean Frequency D, Cumulative Frequency Distribution of IEI for all events. **p<0.025

3.4. Discussion

These slice electrophysiology studies reveal that there are sex differences in baseline inhibitory synaptic transmission in the DMS. We find that male rats show a lower frequency of inhibitory synaptic events at baseline as compared to females. We also show that bath application of DMSO (vehicle) alone does not alter inhibitory synaptic

transmission but note that there may be sex differences in this effect. Bath application of DMSO following aCSF recordings in experiment 3.2 suggests males have a larger IEI (Fig. 3.2d) while experiment 3.3, where slices begin in DMSO, suggests that females have a marginally larger IEI (Fig. 3.3d) as compared to their opposite sex counterparts, though this difference is miniscule. These divergent findings require further exploration to determine the basal sex differences under DMSO bath. Finally, we find that CB1R activation reduces the frequency of inhibitory synaptic events in both male and female rats. Ultimately this data serves to establish sex differences in inhibitory synaptic transmission in the DMS, but additional work must be done to define more precisely how the male and female DMS differs in terms of its electrophysiological properties, inputs, and the influence of neuromodulators likes eCBs.

Within the above framework of striatal contributions to goal-directed and habitual control of behavior, lower levels of DMS inhibitory transmission (as seen in males; Fig. 3.1) would promote flexibility and higher levels of inhibitory transmission (as seen in females) would prevent the expression of outcome devaluation, consistent with our devaluation findings in male and female rats, respectively. While we did not confirm the identity of the cells we recorded from, approximately 90% of cells across the DS are medium spiny neurons (MSNs), the main type of projection neurons arising from the striatum (Graveland & Difiglia, 1985). Due to their abundance, we are likely to be recording from MSNs in the DMS. Multiple studies have shown that intact female rats and males treated with estradiol have increased striatal MSN excitability (Tansey et al., 2008; Dorris et al., 2015; Cao et al., 2018; Proaño et al., 2018) and estradiol decreases GABA release (Schultz et al., 2009). However, these studies are not specific to the DMS.

Additionally, some studies have shown lower numbers of GABAergic neurons in males compared to females (Ovtscharoff et al., 1992), which may explain reduced inhibitory synaptic transmission in males. However, there are many types of GABAergic cells in the DMS. GABAergic MSNs project locally to other MSNs (Wilson & Groves, 1980; Somogyi et al., 1981; Graveland & Difiglia, 1985; Tunstall et al., 2002; Czubayko & Plenz, 2002; Burke et al., 2017). There are also multiple GABAergic interneuron types, predominately Parvalbumin positive fast-spiking interneurons (FSIs) and somatostatin interneurons (SOM). In fact, a study focusing on sex differences in the number of interneurons shows that FSIs and cholinergic interneurons in the DS are more dense in males than females while SOM interneurons are less dense in the DS than the ventral striatum but display no sex differences (Van Zandt et al., 2024). Thus, further work must be done to isolate inhibitory synaptic transmission from these different sources and better understand baseline sex differences in the DMS with cell-type specificity. Further investigations should assess individual inhibitory inputs to DMS MSN such as local interneurons or collateral MSNs through the combination of optogenetic stimulation and electrophysiology recordings as has been done in other studies (Mathur et al., 2013).

We also uncover a possible effect of DMSO on sIPSCs. Our second experiment suggests that DMSO does not alter sIPSCs after changing from an aCSF bath to one containing 0.06% DMSO for a subset of DMS neuron recordings and that males continue to show a greater IEI than females (Fig. 3.2). However, the following analysis, including only cells recorded under DMSO bath conditions, and in the final analysis, including only cells under DMSO as vehicle conditions, prior to WIN application, show an opposite finding – that females have a larger IEI as compared to males (Fig. 3.3 and Fig. 3.5).

Previous studies have shown that DMSO may alter electrophysiological recordings by increasing excitatory post synaptic potentials at concentrations lower than what was used in this study (Tsvyetlynska et al., 2005) but this effect should not be present in our studies as we blocked excitatory currents via DNQX and AP-5. Both findings are statistically significant for IEI between sexes but have a small effect size which may be due to the sensitivity of running statistical analyses on data with many data points such as cumulative distribution. Additionally, we did not find a significant effect of DMSO bath application on sIPSC frequency between sexes which may point to a false positive in the difference between IEI of males and females, as frequency and IEI are inversely related. In the aCSF versus DMSO experiment, the total number of cells in males was very low (n=3) and the best practice would be to replicate this experiment with a greater number of cells from males and females to compare inhibitory transmission under aCSF and DMSO bath conditions with an increasing concentration of DMSO to determine the impact of DMSO-containing aCSF on inhibitory transmission between sexes.

We show that CB1R activation reduces sIPSC frequency regardless of sex. However, this finding should be interpreted with caution, as we only tested a single high concentration (10, bath applied) of the CB1R agonist, WIN 55,212-2. Other studies using much lower doses (1 μM) have reported sex differences in other brain regions (Tabatadze et al., 2015; Ferraro et al., 2020). Both males and females express CB1Rs in the DS and males express CB1Rs more densely in the striatum and other brain regions than females (Laurikainen et al., 2019; Liu et al., 2020). Thus, it is possible that a lower concentration of WIN may be more sensitive at uncovering CB1R-based sex differences due to the greater abundance of these receptors in males. Another caveat of these electrophysiological

findings is that rats we recorded from did not have any behavioral training. It is possible that behavioral experience alters DMS inhibitory tone or changes DMS activity, as has been shown in other studies examining DMS activity after extended training or under different schedules of reinforcement (Fanelli et al., 2013; Gremel & Costa, 2013; Vandaele et al., 2019).

CB1Rs are located on multiple cell types in the DS so further work must been done to identify the cell-type that supports Pavlovian flexibility in male rats. One notable possibility is the parvalbumin positive FSIs. CB1Rs are expressed on striatal PV-FSIs and mediate a form of inhibitory LTD that disinhibits MSNs, a mechanism that is associated with striatal regulation of behavioral flexibility (DePoy et al., 2013b; Mathur et al., 2013). CB1Rs are also expressed on cortical inputs that target MSNs or MSNs themselves (G. Gerdeman & Lovinger, 2001; G. L. Gerdeman et al., 2002; Y. W. Wu et al., 2015; Lovinger & Mathur, 2012b; Lovinger et al., 2022), but it has not yet been established whether cortical projections targeting PV-FSIs also contain CB1Rs. CB1R signaling at cortical-striatal FSI synapses would be expected to reduce inhibitory tone and increase DMS MSN activation, a similar result to CB1R signaling at FSI-MSN or MSN-MSN synapses. Direct manipulation of DLS FSIs shows that their activity is critical to supporting habitual responding (O'Hare et al., 2017; Patton et al., 2020) but much less is known about DMS FSIs and their contribution to habitual or goal-directed responding. Thus, these hypotheses must be tested further to discover the mechanism of CB1R regulation of DMS inhibitory synaptic transmission and the influence of MSN or FSI CB1R regulation on Pavlovian outcome devaluation. Either cell type can be targeted using Cre-dependent viral approaches

to manipulate either FSIs or MSNs independently (Ciriachi et al., 2019; Vormstein-Schneider et al., 2020).

Overall, we show that there are baseline sex differences in DMS inhibitory transmission and that CB1Rs reduce the release of GABA in both sexes. Future studies should expand these experiments to test sIPSC changes under multiple concentrations of WIN and other experiments of antagonizing CB1Rs with the inverse agonist, rimonabant, as we use in the prior chapter. Additionally, there should be follow-ups to test the effect of CB1R manipulation in animals that have undergone extended training in PLA to model DMS electrophysiology after learning.

Chapter 4: Sign-tracking is a Rigid Behavior which persists under Extinction and in the Presence of an Alternative Choice.

4.1. Introduction

Neuropsychiatric conditions involving disorders of behavioral control often have an impaired social interaction component such as in schizophrenia, substance use disorder (SUD), and depression where there is a decrease in the motivation to seek out social interaction (Allsop et al., 2014; Kennedy & Adolphs, 2012; Christie, 2021). Preclinical models of these conditions have ignored this impairment in social motivation in the past but recent development of the operant social interaction paradigms allows us to study social motivation in the context of neuropsychiatric conditions and symptom expression and the connections between social interaction and symptom expression (Venniro & Shaham, 2020; Venniro et al., 2021; Chow et al., 2022).

Preclinical experiments that employ the operant social interaction task show that social reward is a strong reinforcer and can out-compete other alternative rewards when available (Venniro & Shaham, 2020; Chow et al., 2022). In fact, the operant social self-administration model established that rats voluntarily abstain from drug use and instead choose to work for social interaction, which results in protective effects against relapse for cocaine in cue-induced reinstatement paradigms (Venniro et al., 2018, 2019, 2021). SUD develops overtime after chronic and escalating drug use and in human populations, only a small percentage of people who use illicit drugs will develop a SUD (Anthony et al., 1994; Jordan & Andersen, 2017). In both humans and animals, social stress and social isolation promote drug use and increase the risk for developing a SUD (Miczek et al., 2008; Bardo et al., 2013; Buckner et al., 2013; Nader & Banks, 2014). Still other studies show that social

support or being active with regular social groups prevents the development of a SUD or lessens drug use (Sigfúsdóttir et al., 2009; Sloboda et al., 2012; Yang et al., 2020). While prior studies using the operant social interaction task show that social interaction can improve SUD progression and prevent relapse, minimal work aims to study the protective effects of social interaction on the expression of symptoms related to neuropsychiatric conditions like SUD.

Pavlovian Lever AutoShaping (PLA) reveals distinct behavioral phenotypes: sign-trackers (STs), who attend to the 10 second lever cue, and goal-trackers (GTs), who attend to the food cup where food is delivered following the cue. Further research shows that STs are more impulsive, prone to habit-like devaluation insensitivity after early training, and display cue engagement even at the expense of receiving the reward suggesting compulsive-like behavior (Lovic et al., 2011; Swintosky et al., 2021; Morrison et al., 2015; Nasser et al., 2015; Chang & Smith, 2016). Additionally, the tracking model reveals that STs are more susceptible to relapse for cocaine and methamphetamine as compared to GTs (Saunders & Robinson, 2010; Everett et al., 2020). For this reason, sign-tracking is considered an addiction-vulnerable phenotype. Studies in humans also reveal that sign-tracking, or a high level of reward-paired cue engagement, correlates strongly with both addiction-related and obsessive-compulsive behaviors (Albertella et al., 2019, 2021; Colaizzi et al., 2023; Cope et al., 2023; Garofalo & di Pellegrino, 2015). Few studies have combined the operant social interaction task with PLA to test how social interaction connects to tracking phenotype and vice versa. One study shows that rats do sign-track to a social reward, and that STs show a higher motivation for social interaction, especially novel social interactions, as compared to GTs (Fitzpatrick & Morrow, 2020). However, this study

does not incorporate operant reinforcement to test if STs or GTs will work to earn a social reward, and it is possible that the motivation to earn a social reward differs due to tracking phenotype. Social reward strongly reinforces behavior and animals earn and choose social reward over other strong reinforcers such as food and drug (Venniro et al., 2018; Venniro & Shaham, 2020). Thus, it is possible that social reward will outcompete with another strong reinforcer, the ST lever.

In the following experiments, we aim to test whether rats will choose to respond for social reward instead of responding on the sign-tracking lever, an addiction-related behavior. We establish the operant social interaction task in ST rats and lever-preferring intermediates (INTs) and test how they respond when presented with both the possibility to earn social reward and interact with the ST lever. We also explore whether there are any sex differences in social reinforcement or choice in STs. In addition to the tracking-specific differences in reward learning, there are also sex differences in the acquisition of a Pavlovian conditioned response and the expression of impulsivity, devaluation sensitivity, and addiction-related behaviors (Pitchers et al., 2015; King et al., 2020; Burton & Fletcher, 2012; Hammerslag & Gulley, 2014; Barker et al., 2017; Barker & Taylor, 2019). Thus, it is critical to consider both sexes using models related to behavioral control such as PLA and choice tasks.

We find that sign-tracking is persistent under extinction conditions and that male and female rats may differ in their sensitivity to extinction. We also find that there are no sex differences in the acquisition of operant social reinforcement. Despite matching cue period and reward delivery timing for the social and sign-tracking responses, rats persisted in sign-tracking even when a social alternative was present.

4.2. Materials and Methods

Subjects. We used 16 Long Evans rats (8 Male, 8 Female) that were 8 weeks old at the start of training. All rats were double-housed upon arrival at the animal facilities and single-housed 48 hours after their arrival. We maintained rats on a reverse 12hr:12hr light-dark cycle (lights off 0900). We performed all behavioral procedures during the dark phase of the light cycle. All rats had *ad libitum* access to standard laboratory chow and water before we food deprived them to maintain 90% of their baseline weight. We performed all procedures in accordance with the "Guide for the Care and Use of Laboratory Animals" (8th edition, 2011, US National Research Council) and with approval by the University of Maryland, School of Medicine Institutional Animal Care and use Committee (IACUC).

Apparatus. We performed all behavioral procedures in a room that is separate from the animal colony. We conduct behavioral experiments in identical operant chambers (25 X 27 X 30 cm; Med Associates) with an attached social chamber (25 X 18 X 18 cm) that was separated with a guillotine door. During PLA, one wall contained a red house light and a closed guillotine door, and the opposite wall contained a food cup with photobeam detectors that rests 2 cm above the grid floor. A programmed pellet dispenser attached to the foodcup dispensed 45 mg food pellets (catalog #1811155; Test Diet Purified rodent Tablet [5TUL]; protein 20.6%, fat 12.7%, carbohydrate 66.7%). We installed one retractable lever at 6cm above the grid floor on either side of the foodcup and we counterbalanced the lever side between subjects. During social reinforcement, we replaced the foodcup and retractable lever with plain walls and included a lighted nose poke on the wall that contained the guillotine door. We placed a perforated barrier between the main

chamber and social chamber so that when the guillotine doors were raised, animals could interact via sniffing and pawing but not leave their assigned chambers. During Social-PLA Hybrid tests, one wall contained the red house light, guillotine door, and lighted nose poke (social response) while the other wall contained the foodcup port and retractable lever (sign-tracking response).

Pavlovian Lever Autoshaping Training and Extinction Test. Prior to training, we exposed all rats to the food pellets in their home cage to reduce novelty to the food. Then we trained them in daily PLA sessions which lasted ~ 26 minutes and included 25 trials of non-contingent lever presentations (conditioned stimulus; CS) and occurred on a VI 60 s schedule (50-70s). At the start of the session, the house light turned on and remained on for the duration of the session. Each trial consisted of a 10 s lever presentation and retraction of the lever followed immediately by delivery of two 45 mg food pellets into the foodcup. At the end of the session, we returned the rats to their cages and colony room. We trained rats in PLA for 5 days to determine their tracking group and selected the Sign-trackers (STs; n=6; 2 Males, 4 Females) and lever-biased Intermediates (INTs; n=2; both male) to continue training in social reinforcement and further behavioral tests following the PLA extinction test (Fig. 4.1A). Remaining animals were used either as social partners for the remainder of the experiments (n= 4; 1 GT and 3 INT) or trained in food reinforcement (n= 3; all ST) and one rat was not used beyond the extinction test. Only data from the STs and INTs (experimental groups) are represented in graphs. We performed an extinction test the day following the last PLA training day to establish a baseline for sign-tracking prior to social operant training. The extinction test consisted of 10 non-rewarded lever presentations

on a VI 60s schedule (50s-70s). Rats were then retrained in a reinforced session of PLA following the extinction test.

Social Reinforcement and Social Reinforcement Shaping. We trained the ST and INT rats daily on a fixed-ratio 1 schedule with one session per day. All sessions contained 60 trials with a 60s inter trial interval. The light in the nose poke zone was illuminated at the start of each trial and remained on until either the end of the cue period or after a nose poke. To match the social operant and sign-tracking response parameters, the length of the cue period was changed throughout the training period as follows: session 1-3, 60s cue; sessions 4-5, 40s cue; sessions 6-10, 20s cue; sessions 11-13, 10s cue. This was done to shape behavior and reduce the latency to nose poke for social reward. To match the delivery of reward between social interaction and hybrid tests, sessions 4-5 had a 5s delay before the guillotine door raised and sessions 6-8 had a 10s delay before the guillotine door raised. Successful nose pokes resulted in the nose poke light turning off and the guillotine door being raised. We allowed the resident rat to interact with the social partner through the perforated barrier for 60s, at which point the guillotine door closed.

Social Reinforcement-PLA Hybrid Testing. Hybrid tests followed the session structure as described above in the PLA training. Each trial started when the sign-tracking lever extended and the social operant nose poke light illuminated. Rats could engage with both responses in non-mutually exclusive trials of lever autoshaping. Successful nose pokes for social reward resulted in raising of the guillotine door at the end of the 10s cue period and resident rats had 20s of interaction time with partner rats before the door would close. Food pellets were delivered at the end of the 10s cue after lever retraction regardless of whether responses were made or not on the lever or food cup port on the PLA side of the

chamber. We designed hybrid tests to keep the Pavlovian and operant reward contingencies consistent with training, thus two pellets were delivered and the end of the 10s cue period and only one response was necessary to earn a social reward.

Measurements. For PLA training and the extinction test, we recorded the number and duration of foodcup and lever contacts, the latency to contact, and the probability during the 10 s CS (lever) period. On trials with no contacts, a latency of 10s was recorded. To determine tracking group, we used a Pavlovian Conditioned Approach index (PavCA;(Meyer et al., 2012) which quantifies behavior along a continuum where +1.00 indicates behavior is primarily lever directed (sign-tracking) and -1.00 indicates behavior is primarily foodcup directed (goal-tracking). PavCA scores are the average of three separate scores: the preference score (lever contacts minus foodcup contacts divided by the sum of these measures), the latency score (time to contact foodcup minus the time to contact lever divided by 10 s (duration of the cue)) and the probability score (probability to make a lever contact minus the probability to make a foodcup contact across the session). We use the PavCA score from the 5th day of training to determine an individual's tracking group as follows: STs have a PavCA sore +0.33 to +1.00, GTs have a PavCA score -1.00 to -0.33, INTs have scores ranging from -0.32 to +0.32.

For social reinforcement, we recorded the number of social rewards earned in each session, the total number of nose pokes across the whole session and the nose poke latency for each trial. During hybrid tests we also calculated the Response Probability, which is defined as the difference of the lever probability (probability to press the lever on a given trial) and the nose poke probability (probability to nose poke for social reward on a given trial) divided by the sum of the lever and nose poke probabilities. A response probability

closer to +1.0 suggests the subject was more likely to engage with the PLA lever on any given trial than the social nose poke while a score closer to -1.0 suggests the subject was more likely to engage with the social nose poke.

Experimental Design and Statistical Analysis. We analyzed data using SPSS 29.0 statistical software (IBM) with mixed-design repeated measures analysis of variance (ANOVA). Significant main and interaction effects ($p<0.05$) were followed by post-hoc repeated measures ANOVA or Bonferroni comparisons. Analyses included between subject factors of Tracking (ST, INT) or Sex (Male, Female) and within-subject factors of Session (PLA 1-5, Extinction; Social Reinforcement 1-13) or Test (Hybrid 1, Hybrid 2).

4.3. Results

Acquisition of Pavlovian lever AutoShaping and testing under extinction conditions. We trained rats in PLA and determined their tracking phenotype based on their day 5 Pavlovian Conditioning Approach index (PavCA; Fig. 4.1B). We found that both ST and INT rats showed more lever directed behavior over time, shown by a main effect of Session on PavCA ($F(4,13)= 33.822$, $p<0.001$). These groups were significantly different from each other with an observed main effect of Tracking ($F(1,13)=64.055$, $p=<0.001$). We found that in extinction tests, which are non-reinforced and contain only 10 trials, behavior remained stable in both groups, meaning that their lever-biased behavior is persistent and stable ($Fs<2.999$, $ps>0.107$). In addition to PavCA, we also examined Probability and Preference scores during training and extinction (Fig. 4.1c and Fig. 4.1d).

We saw similar trends in both scores where there was a significant main effect of Session (Probability, $F(4,13)=31.467$, $p<0.001$; Preference, $F(4,13)=28.408$, $p<0.001$)) and a main effect of Tracking (Probability, $F(1,13)=75.032$, $p<0.001$; Preference, $F(1,13)=38.801$, $p=0<0.001$)) for the training sessions. We found no significant differences in ST or INT behavior between D5 PLA and extinction tests for Probability scores (*Fs*<0.35, *ps*>0.564).

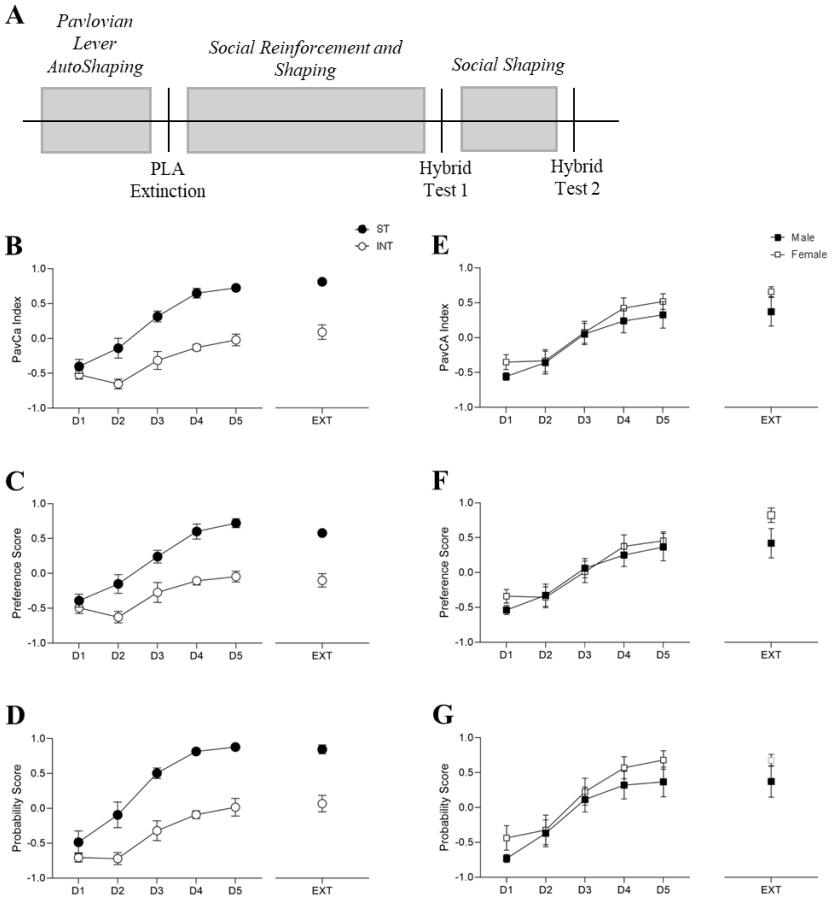

Figure 4.1: Sign-tracking is a rigid behavior that persists under extinction conditions. ***A,*** Experimental timeline for PLA training and testing followed by Social Reinforcement Shaping and Social/PLA Hybrid Tests. ***B,E,*** PavCA Split between Sex and Tracking. ***C,F,*** Preference Score split between Sex and Tracking. ***D,G,*** Probability Score split between Sex and Tracking. Data are group means ± SEM. % Main effect of Session (1-5).

However, we did observe that both ST and INT reduced their preference scores in the extinction tests as compared to D5 PLA shown by a main effect of Session ($F(1,13)=9.47$, $p=0.009$). As we show in Chapter 2, there can be substantial differences in acquisition of PLA behaviors due to sex, so we analyzed PLA training and extinction tests split by sex. We found that both males and females developed more lever-directed behavior over time, seen by a significant main effect of session on PavCA ($F(4,13)=30.475$, $p<0.001$) but there were no significant differences in training due to sex on any measure ($Fs<0.775$, $ps>0.315$). We also see no differences due to sex between training and extinction for either PavCA or Probability Scores ($Fs<1.887$, $ps>0.193$). We do see sex differences in responding between training and the extinction tests for Preference Score only, where we observe a significant Session x Sex interaction ($F(1,13)=8.615$, $p=0.012$). We find that females increase their lever-bias in extinction tests as compared to training (Bonferroni comparisons, $t(7)=5.944$, $p=0.0086$) while males remain stable in their behavioral preference in extinction ($t(6)=0.5995$, $p>0.999$). This suggests females are more habit-driven which we discuss more in the final chapter of the discussion.

Acquisition and Social Reinforcement and Shaping of Responding in Social Reinforcement. Following the PLA extinction test, we trained STs and the most lever-biased INTs (n=8 total; Male, n=4; Female; n=4) in Social Reinforcement on a fixed ratio 1 response requirement.

We did not analyze social training, or any following tests based on Tracking due to the small number of INTs (n=2) which did not allow properly powered comparisons between tracking groups. Sex was better powered with even groups between males and females. Comparisons between males and females shows that both groups earn a similar number of social rewards through training which reduces as the cue period reduces and a delay is introduced, shown by a main effect of session (Fig. 4.2B, $F(12,72)=11.138$, $p<0.001$) and no interaction with sex ($Fs<0.61$, $ps<0.464$).

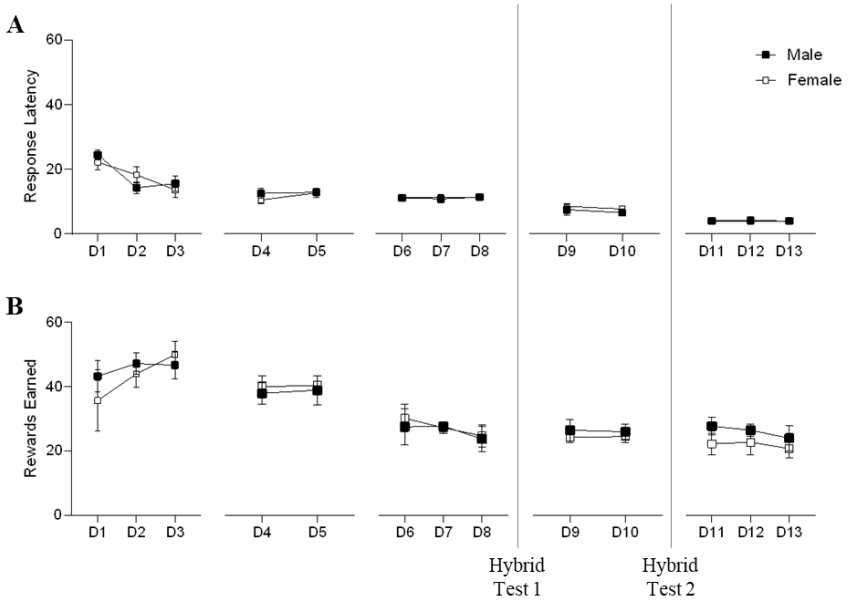

Figure 4.2: Social Reinforcement responses occur with shorter latency when the cue period is reduced. Social Reinforcement was 60 trials with 60s cue for sessions 1-3 then changed for the following sessions as stated: 4-5, 40s cue and 5s delay; 6-8, 20s cue and 10s delay; 9-10, 20s cue and no delay; 11-13, 10s cue and no delay. ***A,*** Latency to nose poke for social reward split by Sex. ***B,*** Rewards earned during 60-trial training session split by Sex. Data are group means ± SEM.

However, as our group sizes are small (n=4 male, n=4 female), results based on data split by sex should be considered lightly. Similarly, we also see a significant reduction in the response latency to nose poke for social reward across all rats (Fig. 4.2A, $F(12,72)=40.847$, $p<0.001$).

By the last day of training, both males and females are responding within 10 seconds (males, average = 4.02s ± 0.16; females, average = 3.85s ± 0.25).

Responding in Social Reinforcement-PLA Hybrid Tests. The final behavioral tests we conducted were nonexclusive choice tests between responding for social reward and on the PLA cue lever. Hybrid tests differed on the timing of their tests during social reinforcement training. We conducted Hybrid test 2 after the social reinforcement sessions where latency to respond for social reward occurred within 10 seconds, which is the cue period presented in the hybrid tests.

Thus, we expected to see some differences between Hybrid tests 1 and 2 since only in Hybrid 2 were the parameters matched for social response and sign-tracking. We found no difference in the amount of lever contacts across 25 trials between the last PLA session and either Social-PLA hybrid tests (Fig. 4.3A, $Fs < 1.389$, $ps > 0.284$). However, we did see a significant increase in the latency to sign-track, shown by a main effect of Session (Fig. 4.3C, $F=4.408$, $p=0.037$). Post hoc comparisons reveal no significant differences between the last PLA session and either hybrid day but there is a trending increase in lever latency between the hybrid tests ($t(7)=3.607$, $p=0.085$).

Analysis of the engagement on the social reinforcement side of the chamber shows no significant difference between the hybrid tests (Fig. 4.4A, $Fs < 3.916$, $ps > 0.105$) but there is a trending increase in the number of social rewards earned during hybrid test 2 as compared to the first test ($t(7)=-1.749$, $p=0.062$).

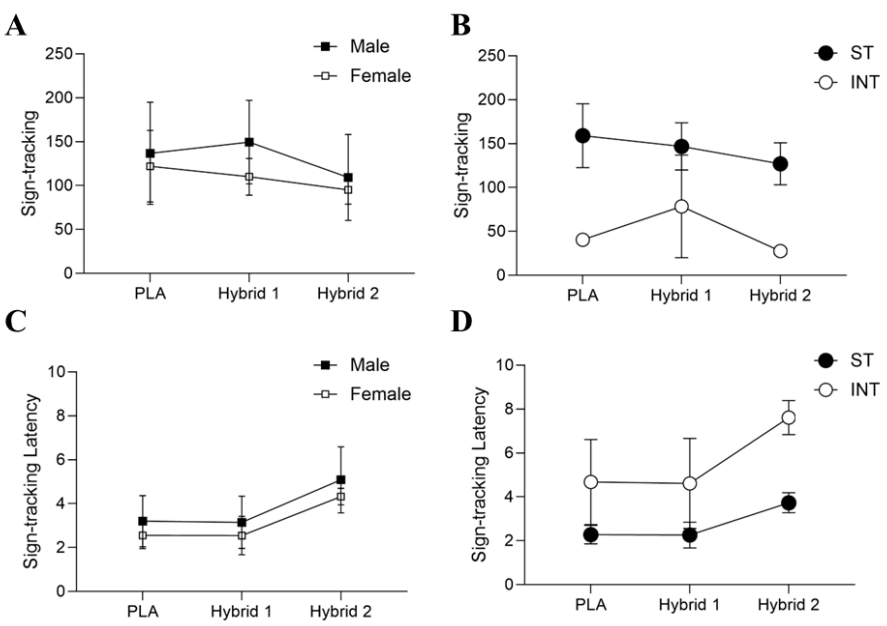

Figure 4.3: Sign-tracking for a lever cue persists even with the availability of social reward. ***A, B,*** Lever Presses made during the final day of PLA training and during both 25 Trial Hybrid Tests and split by Sex or Tracking. ***C, D,*** Latency to press on the lever cue during the final day of PLA and during both tests split by Sex and Tracking. Data are group means ± SEM.

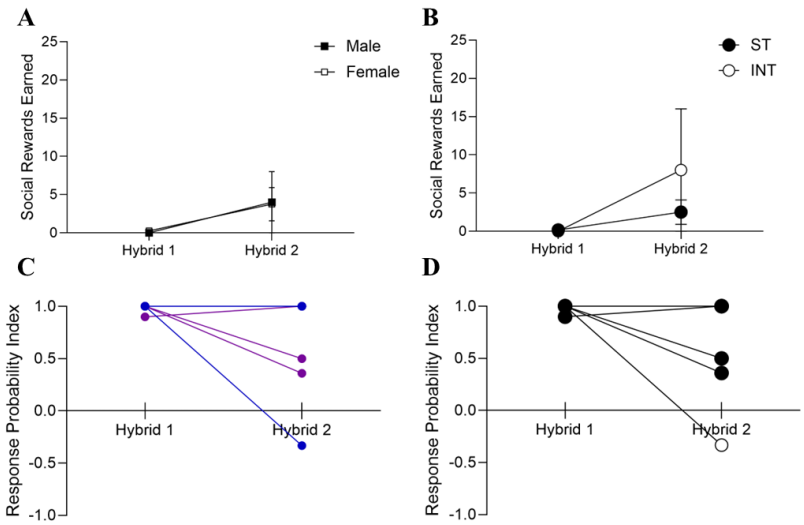

Figure 4.4: Probability of choosing Social Reward increases after rats have been shaped in social reinforcement to respond within a cue period that matches PLA sessions. ***A, B,*** Social rewards earned during 25 Trial Hybrid Tests split by Sex and Tracking. ***C,D***, Response Probability for Hybrid Tests split by Sex (male, blue; female, purple) and Tracking (ST, black dot; INT, white dot). Data are individual values for Response Probability.

Likewise, when we compare the Response Probability Index which is an index comparing the probability of engaging with the PLA lever with the probability of engaging with the social nose poke (index closer to +1.0 means rats display more sign-tracking, closer to -1.0 means rats display more social nose poke responses, we find no significant differences due to session or sex (Fig. 4.4C, individual data, $Fs<3.809$, $ps>0.108$). Only a subset of animals alters their behavior (n= 4), all other animals (n=4) sign-track the entire time which results in a +1.0 Response Probability Index on both tests and overlapping individual data points in Fig. 4.4C and D.

There is a trend where response probability decreases in hybrid test 2, suggesting the subset of subjects that alter their behavior are more likely to engage with the social nose poke in the final hybrid test ($t(7) = 1.681$, $p=0.068$).

4.4. Discussion

In these experiments we trained rats in both PLA and operant social interaction to investigate the relationships between sign-tracking, social motivation, and sex differences. Our experiments reveal that female ST rats are less sensitive to Pavlovian extinction (Fig. 4.1) and persist in the expression of a lever-directed responses in PLA. Procedurally, we successfully matched Pavlovian and operant contingencies, reducing the latency to respond on a social nose poke by reducing the cue period in an operant social interaction task (Fig. 4.2). In our hybrid tasks, we found that rats maintained a high level of sign-tracking even when an alternative is present (Fig. 4.3). We also show that latency to sign-track increased between Hybrid 1 and Hybrid 2. While there are trends suggesting that rats altered their pattern of responding in the final hybrid session, in which more social rewards were earned (Fig. 4.4), further research is needed to test whether social reinforcement can modify rigid sign-tracking patterns of behavior.

Sign-tracking rats persist in lever responding under multiple conditions. We found in this study that sign-tracking persists under extinction conditions where the reward is withheld (Fig. 4.1B) and this replicates an established finding where ST is resistant to extinction (Ahrens et al., 2016; Fitzpatrick et al., 2019). Researchers attribute this phenomenon to the transfer of incentive salience to the reward-predictive cue in ST rats (Flagel et al., 2009; Morrow et al., 2011; Beckmann & Chow, 2015; Pitchers et al., 2015). In ST rats, Pavlovian conditioning results in the transfer of incentive-motivational value from natural food reward, the US, to the paired cue, the CS. Additional tests such as conditioned reinforcement and Pavlovian-Instrumental transfer show that the lever itself

becomes a potent reinforcer in STs (Villaruel & Chaudhri, 2016; Bacharach et al., 2018). We show that female rats are less sensitive to extinction than males (Fig. 4.1F) which suggests they may be more likely to attribute incentive salience to the lever cue and this aligns with other studies that show females are more likely to sign-track and more prone to addiction-related behaviors (Anker & Carroll, 2011; Barker & Taylor, 2019; King et al., 2020; Stapf et al., 2024). While ST is a persistent behavioral phenotype, some studies have shown that environmental enrichment reduces the likelihood to sign-track and thus attenuate the attribution of incentive salience to reward-paired cues (Lomanowska et al., 2011; Beckmann & Bardo, 2012).

Social reward is a potent reinforcer that outcompetes alternative rewards such as food and intravenous drugs in operant tasks (Venniro & Shaham, 2020). Alterations to the task such as increasing the FR, introducing a delay, or increasing the dosage of a drug reward can reduce the reinforcing effects of social reward and change the response bias in a choice task (Venniro et al., 2021; Chow et al., 2022). In this study, we aimed to shape responses for a social reward to occur within 10 seconds and attempted to introduce a delay to mimic the presentation of rewards in the Social-PLA hybrid tasks. We were successful in reducing the response latency by reducing the cue period (Fig. 4.2a). However, this is confounded by the introduction of a reward delay as we altered both parameters simultaneously during training. We found minimal sex differences in social reinforcement, mainly that males reduce their response latency more readily than females (Fig. 4.2A, D1-D3) which may be non-specific to social reinforcement. However, this study is underpowered to conclude findings based on sex differences but suggest sex differences in operant social interaction should be explored. Prior studies do not show a consensus on whether there are sex

differences in the acquisition of social reinforcement where some studies show no difference due to sex (Venniro & Shaham, 2020; Chow et al., 2022) and others show females show greater social motivation (Raymond et al., 2024). These divergent findings may be due to factors such as strain, housing conditions, and partner rat sex. In this study, we use single-house Long Evans rats and the prior study from Chow et al. which found no sex differences also utilized Long Evans rats.

In our Social-PLA hybrid tasks, we found changes in behavior only during the final hybrid tests, after response latency in social reinforcement fell below 10s. However, these changes were minimal. We found a significant increase in lever latency in the final hybrid test which could suggest that rats took longer to respond on the lever or that they were not responding on the lever during some trials. A look into the trial-by-trial data reveals that in trials in which rats responded for social reward, there were no PLA lever responses. We did not collect time-stamped behavioral data which would enhance the resolution of the current analysis. In future studies, time-stamped data should be collected to analyze nose poke behaviors during trial periods to better understand task engagement. One difference between the two tasks was the presentation of a lever predictive cue for PLA and a nose poke and light cue for social reward. These differences in the cue modality could partially explain the high preference for PLA in our hybrid tests. Prior studies show that a reward-paired lever elicits stronger motivational drive than a nose poke response, shown by increased responding in progressive ratio tests (Haluk & Wickman, 2010; Beasley et al., 2024). It is possible that training social reinforcement using a lever response may create better conditions to compare social and PLA responses in our hybrid tasks.

This study is the first to examine how operant social interaction experience alters sign-tracking, thus we focused on STs and lever-biased INTs. Unfortunately, these studies were underpowered to compare tracking differences in social reinforcement and our hybrid tests. Due to the significant differences between STs and GTs in responding for other reward types (Villaruel & Chaudhri, 2016; King et al., 2020; Raymond et al., 2024), it is possible that there are tracking-specific differences in social motivation revealed by this operant training. Future studies should better optimize the PLA versus social reinforcement test conditions to better compare lever or social preference between sex and tracking groups in rats.

Chapter 5: General Discussion

5.1. Summary of Findings

In this dissertation, I investigated sex differences in tracking behaviors, Pavlovian outcome devaluation and the effects of social choice on sign-tracking. Additionally, I explored the role of DMS CB1Rs in regulation of Pavlovian outcome devaluation and DMS inhibitory synaptic transmission. These findings expand our understanding of sex differences in learning under Pavlovian conditioning and how eCBs mediate sensitivity to outcome value changes and DMS inhibitory tone. Using a series of experiments, I tested whether sex differences in PLA tracking behaviors extend to Pavlovian outcome devaluation sensitivity and whether DMS mediate devaluation sensitivity. In addition, I tested whether there are basal sex differences in DMS inhibitory postsynaptic transmission and whether CB1R agonism impacts inhibitory postsynaptic events. Finally, I explored whether operant social interaction protects against the expression of an addiction-related phenotype, sign-tracking. Below are key findings from each research chapter:

Chapter 2: I replicated prior studies by showing that female rats display more lever-directed behavior during training in PLA. Female rats, especially in the GT/INT group, show more lever-directed behavior than males. However, there is no difference in behavior on the final day of training before outcome devaluation testing. Thus, differences in responding after outcome devaluation are due to differences in updating outcome value and not in the baseline conditioned response. I show that female rats are less flexible than males and that DMS CB1Rs mediate the flexibility of males. Male rats reduce their responding to cues when sated on the reward pellets prior to a non-reinforced test, regardless of tracking phenotype. In contrast, females do not alter responding to cues whether they are sated on

chow or pellets. Additionally, infusion of the CB1R inverse agonist, Rimonabant, prior to the devaluation test prevents the flexibility of male rats, without affecting responding on fully reinforced or brief non-sated test PLA sessions.

Chapter 3: Because our behavioral pharmacology results suggested DMS CB1R-dependent flexibility in male rats, I hypothesized that DMS is more active in males than females and that CB1R activation results in greater activation of the DMS. I demonstrated that DMS inhibitory synaptic transmission differs at baseline between males and females. Males showed lower spontaneous inhibitory postsynaptic current (sIPSC) frequency and higher sIPSC interevent interval. There is no difference in sIPSC amplitude due to sex. Additionally, CB1R activation reduces the frequency of inhibitory synaptic events in the DMS, regardless of sex. Bath application of the CB1R agonist, WIN 55-212,2, reduced sIPSC frequency and increased sIPSC interevent interval of inhibitory events in male and female rats.

Chapter 4: Because sign-tracking is a classically rigid behavior, I explored how social reinforcement, a potent alternative reinforcer, affected sign-tracking in male and female rats. I explored sex differences in reward choice between sign-tracking and social interaction and consider the interaction between sign-tracking and social motivation. Consistent with earlier findings (Chapter 2, King et al., 2020), female rats show enhanced sign-tracking under extinction conditions where males do not alter their tracking behavior. I show that male and female rats prone to sign-tracking learn to respond in the operant social interaction task at similar rates and that both groups reduce their latency to respond as the cue period is shortened. I find that sign-tracking outcompetes the choice for social reward

in a non-exclusive hybrid choice task. Both male and female rats are more likely to respond on the sign-tracking lever than the social nose poke.

Overall, these results emphasize the rigidity of sign-tracking, which is more strongly expressed in females, impervious to social alternative, and opposed by DMS CB1R signaling in male rats. These findings raise questions about the specific population of DMS neurons responsible for devaluation sensitivity in PLA in addition to the exact mechanism of eCB signaling which differs between males and females. In this discussion, I present what is currently known about sex differences in incentive learning processes and value updating. I then describe the key players in DMS neurobiological regulation of behavioral flexibility and present a model for how eCBs act during Pavlovian outcome devaluation. I discuss sign-tracking as an addiction vulnerable phenotype and explore the connection between sign-tracking and social motivation. Finally, I present the limitations of the above experiments and propose future studies which build upon these findings.

5.2. Sex differences in Pavlovian conditioned responding and sensitivity to outcome devaluation

Several studies reveal sex differences in acquisition of a Pavlovian conditioned approach and show that females display enhanced responding compared to males (Dalla & Shors, 2009; Hammerslag & Gulley, 2014; Lefner et al., 2022). This is true for PLA where a lever extends for the 10 second cue period then retracts and is followed by reward delivery. Female rats display more lever-directed behavior measured through more lever contacts, higher probability to contact the lever, and reduced latency to press the lever (Pitchers et al., 2015; Madayag et al., 2017). Additionally, in studies that categorize

subjects based on their tracking phenotype, females are more likely to be STs (Stringfield et al., 2019; King et al., 2020). These findings suggest that female rats show enhanced incentive learning of Pavlovian lever cues as compared to males. Incentive learning refers to the process where behavior is driven by current physiological state and previously learned reward-cue associations (Hyman, 2005; Berridge & Robinson, 2016). In PLA, sign-tracking occurs due to the incentive salience of the reward transferring to the conditioned cue so that the cue itself reinforces and invigorates behaviors (Cardinal et al., 2002; Robinson & Flagel, 2009).

In Chapter 2, I showed that females shift to display more sign-tracking at an earlier time point than males and this difference is driven by female GT/INTs (Fig. 2.1, Fig. 2.2). Female GT/INTs show more sign-tracking due to a change in the response ratio due to a simultaneous increase in lever contacts and decrease in foodcup contacts, while male GT/INTs only increase lever contacts (Fig. 2.2). This suggests that female rats show stronger encoding of cue-reward associations where the conditioned cue becomes a reinforcer of behavior. In fact, Pitchers et al., 2015 tested female and male rats in conditioned reinforcement, a task which tests the reinforcing properties of a previously learned conditioned stimulus (CS). In this task, animals must respond to earn presentation of the previously learned CS. Pitchers et al. showed that female rats made more active nose poke responses for delivery of the lever-CS than male rats which suggests that the lever reinforces behavior in females more readily than in males. Additionally, this study also tested estrous cycle during training in PLA and showed that estrous cycle phase did not affect responding in PLA because female rats showed similar PavCA Index regardless of estrous cycle phase. Our data also suggests enhanced reinforcing properties of the lever-cue

for female rats though we did not test this directly via conditioned reinforcement. Prior work in the lab shows that female rats are more sensitive to rimonabant attenuation of conditioned reinforcement, though this was a systemic injection and more work should be done to extend the findings to DMS CB1Rs (Bacharach et al., 2018). We do not expect that the estrous cycle would impact our findings due to the previous findings of Pitcher et al. where there is no effect of estrous cycle on conditioned responding. We also do not observe increased variability in our female group compared to males. In the field of sex differences, experts suggest that the estrous cycle phase of the animal should be considered when there is a clear case that the variability in females is greater and could be explained by an additional variable, such as reproductive stage (McCarthy et al., 2012). Thus, our findings that show sex differences in the Pavlovian conditioned approach may be explained by existing neurobiological differences between males and females.

Another facet of incentive learning is the dependence on the current physiological state of the subject to motivate responding (Hyman, 2005). In many studies, animals are food-restricted to enhance the motivational value of the food reward cue. I food restrict animals throughout the above studies to maintain 90% of their baseline body weight. Satiety-induced outcome devaluation causes a change in the satiety state of the animal to induce a motivational change (Hetherington & Rolls, 1996). Research into the motivational drive for food reward reveals that, when compared with males, female rats show a higher demand for palatable food and escalate their food intake under binge-eating paradigms (Babbs et al., 2011; Freeman et al., 2021). These findings suggest that the motivational value for palatable food is higher in females and can elicit stronger reinforcement of behavior. The current findings where females are insensitive to devaluation brings up an

important question: Does satiation reduce the palatability of the pellets and/or change the incentive motivational value of the reward and cue-reward associations? Results of the consumption choice tests in Chapter 2 do show that females reduced consumption of the sated outcome as compared to the non-sated outcome (Fig. 2.7). Based on these results, the satiation paradigm was able to satiate female rats enough to reduce the palatability of the reward pellets.

It is possible that we observe an inability to update the cue-outcome association between the lever and food pellet for female rats rather than a lack of satiety. Future studies should examine whether females are less sensitive to changes in outcome value as compared to males which may be due to differences in the encoding or retrieval of the value changes. In Wassum et al., 2009, researchers separate the reward value encoding and retrieval components through a paradigm of induced mild satiety (2-hour food restriction) and hunger (23-hour food restriction). In this paradigm, all rats were trained under mild satiation then put through a revaluation period where half of the rats were presented with the same food reward while mildly sated and the other half while hungry. Hungry rats updated the value of the food reward through 'incentive learning' and when tested in an instrumental seeking task showed an escalation of behavior as compared to the rats who only experienced the food reward while mildly sated. These manipulations test behavior after value inflation (hungry condition) rather than a value deflation as we use in outcome devaluation (sated condition). However, this study design does reveal that there are distinct neurobiological mechanisms that underlie reward value encoding and retrieval (Malvaez et al., 2019; Wassum et al., 2009). Female rats may show impairments in the retrieval of reward value if tested under this paradigm. Additionally, our timepoint of rimonabant

injection occurs prior to the behavioral test and after satiety, thus we are testing whether DMS CB1Rs mediate the retrieval of reward value and not encoding the value change. Future studies could manipulate CB1Rs to examine whether their role is also necessary during the encoding of the new outcome value, i.e. inject rimonabant prior to the satiation period.

In this dissertation, I used satiety-induced outcome devaluation to test the flexibility of a Pavlovian conditioned response. However, researchers often use CTA as an additional method to test sensitivity to changes in reward value. Two recent studies observed Pavlovian and tracking behaviors following different devaluation protocols and found either no sex difference (Bien & Smith, 2023) or that males were sensitive to devaluation while females were insensitive (Sood & Richard, 2023). In Bien & Smith, researchers induced devaluation through CTA and found both males and females were sensitive to reward devaluation showing reduced sign-tracking in a reacquisition Pavlovian session. Other studies show that females respond flexibly following CTA and reduce responding (Chambers & Sengstake, 1976; Dacanay et al., 1984; Dalla & Shors, 2009; Rinker et al., 2008). However, females are quicker to extinguish their aversion when given multiple exposures to the cue-reward pairings (Dalla & Shors, 2009). These studies again point to the idea that females are sensitive to the changes in palatability of food rewards but may show impairments in the revaluation of previously learned cue-reward associations, or in other words, impaired incentive learning.

One notable difference between satiety-induced and CTA devaluation procedures is the internal state of the animal during the behavioral test. For CTA, animals are not tested while sick and there are no pairings between the illness and the cue, only pairings between

the illness and the food reward itself. Thus, devaluation sensitivity following CTA requires inference from prior illness-outcome associations that responding to the cue should decrease. However, when CTA occurs in the behavioral chamber, no inference is necessary since the illness is then paired with the context. This results in devaluation sensitivity of STs in studies who use this CTA within the behavioral context (Bien & Smith, 2023; Cleland & Davey, 1982; Derman et al., 2018). Under satiety-induced devaluation, animals are tested in the sated state and no inference is necessary for devaluation sensitivity. It is possible that CTA devaluation procedures where the illness is paired with the outcome outside of the behavioral chamber would also show sex differences in devaluation sensitivity.

Both Bien & Smith and Sood & Richard test Pavlovian outcome devaluation test devaluation sensitivity after an extended amount of Pavlovian conditioning, as we do in the experiment found in Chapter 2. The length of training is important in shaping the stimulus-reward associations that guide responding in Pavlovian outcome devaluation (P. Holland, 1998). In fact, Female rats are prone to developing devaluation insensitive, or habitual, responding at earlier time points than males following instrumental conditioning (H. Schoenberg et al., 2018). However, prior work in the Calu lab reveals that extended Pavlovian training results in an emergence of flexible responding in male ST rats (Keefer et al., 2020). Female rats are more likely to sign-track (King et al., 2020). Thus, future studies should consider testing female rats after a limited amount of Pavlovian training to determine whether they would show flexible responding early on and if their flexibility changes with extended training as in the male ST rats.

5.3. Endocannabinoid regulation of Pavlovian outcome devaluation and DMS physiology

In this dissertation, I focus on the impact of CB1R manipulation on Pavlovian outcome devaluation and DMS inhibitory currents. Several studies show that systemic CB1R activation promotes habits (Hilário et al., 2007; Nazzaro et al., 2012) and that striatal eCBs regulate flexibility (Bilbao et al., 2020; G. L. Gerdeman et al., 2002; Gremel et al., 2016; Renteria et al., 2021; M. Wu et al., 2022). Glutamatergic projections into the striatum and local inhibitory synapses both express CB1Rs; orbitofrontal cortex (OFC) , fast-spiking interneurons, and medium spiny neurons are key players in eCB regulation of DMS function (Gremel et al., 2016; Mathur et al., 2013). Prior work reveals that *habit-driven* responding requires OFC-DS CB1Rs (Gremel et al., 2016). These receptors function to reduce the signaling of OFC terminals in DMS. Since the OFC is critical for value encoding, this renders the animal less sensitive to changes in outcome value. Counter to this established mechanism, the study in Chapter 2 reveals that CB1Rs are critical for the expression of *goal-directed* responding of male rats in Pavlovian outcome devaluation. This finding suggests that CB1Rs function to enhance the activation of the DMS to support goal-directed responding. Thus, we hypothesize that CB1Rs located on inhibitory synapses of either FSIs or MSNs are the key player in regulating Pavlovian outcome devaluation.

Our follow-up study in Chapter 3 focuses on inhibitory synaptic transmission in the DMS. We confirm that DMS CB1Rs reduce the frequency of inhibitory synaptic events in both males and females. However, this study does not identify the source of the inhibitory currents and future work must be done to identify whether FSI or MSN inhibition exerts more control over Pavlovian behavioral flexibility.

5.3.1. Potential mechanisms for DMS eCB regulation of behavioral Flexibility

We reveal sex differences in flexibility between male and female rats where male rats are flexible and female rats are not. Additionally, male rats require CB1R activation to express flexible responding. Multiple explanations may explain this difference: (1) Males express more CB1Rs in the DMS or show enhanced function of CB1R signaling, (2) Males release more eCBs during outcome devaluation or (3) Males and females show cell-type specific patterns of CB1Rs.

Our work using slice electrophysiology reveals that both males and females are sensitive to CB1R pharmacological activation at the single dose tested in the DMS and suggests that differences are unlikely between sexes in CB1R signaling. However, this is not a direct exploration of CB1R expression or function. Follow-up studies should directly address these possibilities by using immunohistochemistry or RNAscope to assess CB1R expression and GTP-γ-S binding assays to assess CB1R signaling. Additionally, both FSIs and MSNs express CB1Rs (Mathur et al., 2013) and are a source of inhibition in the DMS that differ in their connection to other DMS neurons. FSIs synapse onto MSN somas while MSNs synapse onto distal dendrites of other MSNs, thus the FSIs elicit strong lateral inhibition of MSN activity while MSN-MSN connections do not elicit inhibition of MSNs to the same degree but rather are weaker (Burke et al., 2017; Koos et al., 2004). A higher expression of CB1R in FSIs instead of MSNs would result in CB1R signaling in males eliciting stronger disinhibition of MSNs due to their action at axo-somatic synapses of FSI-MSN connections. Thus, CB1Rs at FSI-MSN synapses achieve stronger control over the total output of DMS activity and present as a critical focus for future experiments.

Endocannabinoid (eCB) synthesis from the post-synaptic cell remains unexplored in the context of Pavlovian outcome devaluation. Based on our behavioral pharmacology and slice electrophysiology findings, we can conclude that behavioral flexibility of males results from CB1R activation but that both males and females are sensitive to CB1R manipulations. This suggests that the eCB system upstream of CB1R may differ between males and females. Both anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are present in the DMS (Ade & Lovinger, 2007; Maccarrone et al., 2008). A recent study looking at both excitatory and inhibitory transmission following deletion of DAG lipase, the enzyme necessary for 2-AG synthesis reveals that 2-AG impairment results in changes only in excitatory transmission (Shonesy et al., 2018). Thus, it is possible that AEA may be at play in the mechanism proposed in this dissertation due to the current hypothesis that inhibitory DMS inhibitory transmission drives Pavlovian behavioral flexibility differences between males and females.

Prior research has uncovered sex differences in other neurotransmitter/neuroendocrine systems within the striatum (Meitzen et al., 2018; Zachry et al., 2021). These systems may be upstream of eCB release and CB1R and may more directly drive sex differences in behavioral flexibility. One notable example is the difference in amphetamine-induced dopamine release within the striatum resulting in higher extracellular dopamine in female rats as compared to males, but this dopamine release also fluctuates in the striatum based on female estrous cycle phase (Xiao & Becker, 1994). In times of higher dopamine release for females (proestrus/estrus), expression of D2 receptors decreases resulting in reduced autoregulation of the dopamine system in the Nucleus Accumbens (Calipari et al., 2017). D2 receptor activation promotes the release of eCBs from the post-synaptic cell (Pan et al.,

2008; Yin & Lovinger, 2006). This increase in dopamine release but reduction in D2 receptors may underly the heightened incentive salience in females and the lack of eCB-mediated flexibility in females if this is occurring within the DMS.

5.4. Persistence of Sign-tracking in the face of alternative reward choice

In Chapter 4, we find that sign-tracking resists change under extinction and in the presence of an alternative response, the social nose poke. We were able to find a marginal change in sign-tracking once response times were matched and occurred within the desired cue period (10 seconds). While I am tempted to make the conclusion that sign-tracking is a stronger reinforcer than social reward, I conclude that more testing must be done to make this conclusion. These two paradigms, PLA and operant social interaction, are not well matched since one response is Pavlovian while the other is instrumental. Additionally, social reward can be a weak reinforcer when parameters vary like shortening social interaction access duration and introducing a delay (Chow et al., 2022). Choice procedures such as the Hybrid tests in Chapter 4 are sensitive to changing parameters in increased price and reinforcement frequency (Beckmann et al., 2019; Thomsen et al., 2013; Woolverton & Rowlett, 1998). In our training of social reinforcement, we introduced a delay during sessions 4-8 which may have weakened the associations between the action (nose poke) and the outcome (social interaction). We also have a shorter access duration for social interaction during the testing (20s) as we do during training (60s). We present different response operandi for either sign-tracking (lever) or social interaction (nose poke). Prior studies show that a reward-paired lever elicits stronger motivational drive than a nose poke response, shown by increased responding in progressive ratio tests (Haluk &

Wickman, 2010; Beasley et al., 2024). It is possible that training social reinforcement using a lever response may create better conditions to compare social and PLA responses in our hybrid tasks. Future studies should design choice paradigms with particular focus on response type, reinforcement schedule, and access duration to the reward.

While Chapter 4 suggests that ST is a persistent behavioral phenotype, some studies have shown that environmental enrichment reduces the likelihood to sign-track and thus attenuate the attribution of incentive salience to reward-paired cues (Lomanowska et al., 2011; Beckmann & Bardo, 2012). Voluntary abstinence due to social reward choice protects against incubation of drug craving (Venniro et al., 2021). Under our test conditions, we found that social interaction did not significantly alter sign-tracking under choice procedures. While we tested sign-tracking under extinction conditions just after PLA, we did not test again following operant social interaction training. We found that females increased sign-tracking while males did not at during the early extinction test. It is possible that additional extinction tests where the reward is not available and are similar to drug relapse tests would reveal that sign-tracking reduces following social interaction. Future studies should include additional extinction tests to observe how sign-tracking changes following social interaction training.

5.5. Limitations

A few alterations to study design would improve interpretations of and conclusions from the studies presented in this dissertation. In Chapters 2 and 3, I used only one dose of rimonabant for outcome devaluation and only one dose of WIN for slice electrophysiology recordings. We found in Chapter 2 that Rimonabant did not impact the conditioned

response of rats regardless of dose (1µg/µL and 2µg/µL). We went forward to test rimonabant infusions prior to outcome devaluation with the lower dose because there was no impact on reinforced non-sated behavioral responses. Our lab previously used the higher dose (2µg/µL) for intracranial infusions of rimonabant to the VTA, another region that densely expresses CB1Rs, and observed significant changes to reinforced PLA (Bacharach et al., 2023). A higher dose of rimonabant could reveal that CB1Rs may regulate reinforced, nonsated PLA behaviors. Slice electrophysiology studies showed that 10 µM WIN reduced inhibitory current frequency in both males and females, which is a high concentration for bath application. Other studies use much lower doses (1 µM) and have seen sex differences in other brain regions (Tabatadze et al., 2015; Ferraro et al., 2020). Both males and females express CB1Rs in the DS and males express CB1Rs more densely in the striatum and other brain regions than females (Laurikainen et al., 2019; Liu et al., 2020). Thus, it is possible that application of this high concentration (10 µM) of WIN masks sex differences in the sensitivity CB1R agonism. Regardless of whether the dosages were too low or too high, future studies should include multiple doses to better understand the degree to which CB1R manipulation impacts behavior or physiology in males and females.

The difference in CB1R manipulations also confounds any holistic conclusions made from the presented studies. In Chapter 2, we use rimonabant, an inverse agonist, to block CB1R signaling during behavior, while in the next chapter, we use WIN, a CB1R agonist, to activate CB1Rs during slice electrophysiology recordings. Both drugs bind to the same location on the receptor but exert different effects: WIN promotes activation of any CB1R and rimonabant promotes inactivation of constitutively active CB1Rs. However, their

functions are not direct opposites due to the difference in affinities for the CB1R (Rinaldi-Carmona et al., 1994). Rimonabant shows a higher affinity for CB1Rs than WIN and will exert measurable receptor effects at lower doses. For the behavioral pharmacology experiments, we expected DMS neurons would release eCBs during outcome devaluation procedures as other studies have shown that activation of DMS neurons causes the release of both AEA and 2-AG (Ade & Lovinger, 2007; Maccarrone et al., 2008). In this case, rimonabant was the best choice because we wanted to block activation of receptors as well as inactivate any constitutively active CB1Rs. When transitioning to slice electrophysiology in the DMS, we did not expect release of eCBs while we recorded spontaneous synaptic events without stimulation of the neurons. We did not expect eCB release because release occurs in an activity-dependent manner and requires depolarization of the post-synaptic cell (Di Marzo et al., 1994; Di et al., 2005; Hashimotodani et al., 2007). Thus, we aimed to study the effect of CB1R manipulation on DMS physiology and used WIN to activate CB1R without requiring the presence of eCBs. The inclusion of stimulation procedures would improve future slice electrophysiology studies as researchers could incorporate rimonabant as a pharmacological blockade and investigate the role of eCBs like 2-AG and AEA.

Another consideration for future studies is the consideration of the DMS and DLS as regions working in parallel to gate action strategies. In this dissertation, we focused on the DMS to manipulate the flexibility of rats after extended training in PLA and to explore eCBs in a less studied region. However, many studies show that the processing between the DMS and DLS is not binary, where one region is active and the other is not, but rather the output of behavioral flexibility results from the balance between the activity of both regions

(Bonnavion et al., 2019; Gremel & Costa, 2013; Thorn et al., 2010; Yin et al., 2009). Gremel & Costa show similar encoding of action between the DLS and DMS through *in vivo* electrophysiology behavior but goal-directed or habitual responding correlates to an increase in activity of the DMS and DLS, respectively. These dynamic activity patterns allow a subject to readily change between goal-directed and habitual action strategies as they see fit. Future studies manipulating the DMS would benefit from including an experimental group that targets the DLS to understand how neurobiological mechanisms may differ between the two areas within the same behavior.

Similarly to the issue above, confirmation of the exact target area for experimental manipulations is important to make specific conclusions about striatal subregions. We observe sex differences in inhibitory synaptic transmission under aCSF bath conditions in Chapter 3. A major caveat of this finding is that we do not find this difference under DMSO-containing aCSF. Alongside the switch to DMSO studies, I also aimed to record more lateral neurons but from those that still located in the DMS. I recorded from neurons along the medial edge of the DMS by the ventricle during early tests like the aCSF experiments. Unfortunately, we did not collect slices after recording to confirm the exact location of neurons. Future studies should include an approach such as biocytin-filling cells to systematically assess the anatomical location and cell-type identity of recorded neurons as has been used to identify D1 or D2 cells (Kawaguchi et al., 1990).

Finally, experiments in Chapters 3 and 4 did not include findings related to tracking phenotype due to lack of behavioral training (Chapter 3) or a lack of statistical power to compare groups (Chapter 4). Future studies should extend the findings here to include tracking phenotype characterization due to the significant individual differences in both

neurobiology and behavior between tracking groups (Flagel & Robinson, 2017; Keefer et al., 2022; Kochli et al., 2020).

5.6. Final Remarks

In this dissertation, I explored behavioral flexibility in Pavlovian paradigms following outcome devaluation and in the presence of an alternative reward. These studies reveal individual differences in the ability to adapt behavior to new environmental contexts and suggest that males and females differ in their tendency to respond flexibly or inflexibly. In addition to these conclusions, I find that DMS eCBs regulate the flexibility of Pavlovian behaviors which may be due to their action in reducing the activity of the DMS. Constantly changing environmental contexts in day-to-day life requires that behavior be goal-directed under some conditions and habitual in others. Thus, activity-dependent modulation of DS circuits through eCBs are critical to this dynamic role of flexibility. This dissertation contributes to the field of behavioral flexibility research by expanding upon previously revealed sex differences and countering the established role of dorsal striatal endocannabinoids.

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