EXAMINING SIMULTANEOUS ALCOHOL AND Δ9-TETRAHYDROCANNABINOL SELF-ADMINISTRATION ON BEHAVIORAL FLEXIBILITY AND DORSAL STRIATAL CB_1 EXPRESSION IN CHAP MICE

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Lauren A. Millie

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THE PURDUE UNIVERSITY GRADUATE SCHOOL
STATEMENT OF COMMITTEE APPROVAL

Dr. Nicholas Grahame, Chair
Department of Psychology

Dr. Stephan Boehm
Department of Psychology

Dr. Marian Logrip
Department of Psychology

Dr. Ken Mackie
Department of Psychology

Approved by:
Dr. Nicholas Grahame

To my family and friends, thank you for your never-ending support and encouragement, I wouldn't be who I am today without you.

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ABSTRACT

Although marijuana and alcohol are two of the most commonly used drugs in the United States, relatively little is understood about how these drugs interact to effect drug use, cognitive behaviors, and neurophysiological changes. Specific drug use patterns such as simultaneous use may produce differential effects for consumption and other behaviors in addition to unique neurobiological changes compared to singular drug use. In order to better understand the effects of simultaneous alcohol and marijuana (SAM) use, we used the selectively bred crossed High Alcohol Preferring mice to examine consummatory, cognitive, and neurobiological changes following chronic alcohol and THC self-administration. We hypothesized that SAM mice would consume more drug than animals exposed to either substance alone. We used an operant behavioral flexibility paradigm to assess cognitive impairments believing that drug-exposed animals would show deficits relative to Control animals, with SAM mice being the most impaired of all drug conditions. Finally, we assessed CB_1 receptor changes in the dorsal striatum, as this region is critical for behavioral flexibility (Bissonette & Powell, 2012; Ragozzino, 2007), CB_1 receptors are the primary target of THC and these receptors are involved in numerous alcohol related behaviors (Maldonado et al., 2006; Pava & Woodward, 2012). Contrary to our hypothesis, SAM animals did not consume higher levels of drug compared to mice exposed to only THC or alcohol. Interestingly, female THC consumption was robust when THC was consumed alone but was reduced when simultaneous access to alcohol was available. Surprisingly, although we speculated that drug-exposed mice would be impaired compared to Control animals, and that SAM animals would likely be more compromised than THC and alcohol for Reversal Learning and Attentional Set-Shifting respectively, behavioral flexibility deficits were absent in our paradigm. Finally, alterations to dorsal striatal CB_1 receptor expression were observed following a Short Abstinence period. Despite an absence of cognitive behavioral effects, this research contributes to furthering our understanding of co-drug use for consummatory and neurobiological changes, both of which are critically necessary given the evolving landscape surrounding simultaneous alcohol and recreational marijuana use.

CHAPTER 1 INTRODUCTION

1.1 An Introduction to Simultaneous Alcohol and Marijuana Use

Marijuana is frequently cited as the most commonly used illicit substance of the 21st century (Feeney & Kampman, K. M., 2016; Tzilos, Reddy, Caviness, Anderson & Stein, 2014). However, the legalization of recreational marijuana in the United States has been rapidly increasing over the past decade. With a growing number of states removing the *illicit* distinction and even more states recognizing some form of medicinal use, it is important to consider how co-drug use may change as a result of increased legalization. Easier access to marijuana may facilitate simultaneous alcohol use, as legalization generates opportunities to combine these substances by removing barriers to marijuana possession (Guttmannova et al., 2018; Lynskey et al., 2003).

There is some evidence to suggest that marijuana legalization that results in greater availability and lowered costs may reduce harmful alcohol use, as these substances can produce similar intoxicating effects (Wen & Cummings, 2015). For instance, at low doses, the major psychoactive component of marijuana, Δ9-Tetrahydrocannabinol (THC), can generate feelings of euphoria, whereas high doses can produce drowsiness and sedation (Basavarajappa & Hungund, 1999; Hollister & Gillespie, 1970), mirroring dose-related effects of alcohol. Thus, a substitution effect, wherein marijuana is used in place of alcohol to achieve the same desired effects, may occur (Guttmannova, Lee, Kilmer, Fleming, Rhew, Kosterman & Larimer, 2018; Reiman, 2009). However, rising permissive attitudes towards recreational marijuana use may promote complementary use, with individuals using both substances rather than solely alcohol (Kilmer, Caulkins, Pacula, MacCoun, & Reuter, 2010; White et al., 2019). Moreover, when considering the reported pleasurable effects produced by simultaneous alcohol and marijuana (SAM) use, complementary use may be more likely to occur, rather than a substitution effect (Wen et al., 2015). Using alcohol and marijuana to produce overlapping effects and subsequently enhancing positive properties of both substances has been described and popularized in recent years by the term "cross-fading" (Patrick & Lee, 2018). Therefore, changing drug laws raise public health concerns not only for marijuana use but also for alcohol use, as alcohol already is used regularly in the United States (Guttmannova et al., 2018).

Studying the temporal pattern of drug ingestion allows researchers to distinguish between simultaneous use (i.e., the intake of multiple drugs at the same time) and sequential use (i.e., one drug is ingested on one occasion, and a different drug is ingested on a separate occasion, such as the following day). New research has found a willingness of SAM users to spend more money on alcohol compared to sequential users, indicating behavioral economic disparities between these different user populations (Ramirez et al., 2019). The impaired judgment resulting from either substance may lead to higher-than-intended use for both alcohol and marijuana (Guttmannova et al., 2018). When temporal patterns have been examined, SAM users report drinking more alcohol when also using marijuana compared to drinking-only days; these individuals also report the highest alcohol use levels in terms of quantity and frequency compared to alcohol-only users (Lee et al., 2020; Subbaraman & Kerr, 2015). Participants also consumed more marijuana when simultaneously drinking alcohol, compared to marijuana-only days (White et al., 2019). Thus, simultaneous use in humans has been associated with greater consumption of both substances, compared to using either substance alone.

In addition to producing similar subjective intoxicating effects in humans, THC and alcohol can generate parallel behavioral effects in animals. Both substances show dose-dependent depressant effects including hypo-locomotion, hypothermia, and ataxia (Pava & Woodward, 2012). Preclinical research has established symmetrical cross-tolerance for multiple behavioral assays in rats: initial administration of alcohol or THC produced behavioral impairments in a one-way avoidance task and a rotarod task, but subsequent tests using the opposite drug showed tolerance and unimpaired performance (Newman, Lutz, Gould, & Domino, 1972; Siemens & Doyle, 1979; Sprague & Craigmill, 1976). These effects were not attributable to altered pharmacokinetic processes, considering that, regardless of the initial treatment with either THC or alcohol, metabolism (assessed via blood disappearance curves) of the subsequent drug was not increased (Siemens & Doyle, 1979). Although marijuana is comprised of many different natural components, examining THC in isolation is a necessary first step into understanding the complex effects exogenous cannabinoids may have on a variety of behavioral and neurobiological targets.

1.2 CB_1 Receptor Involvement in Addiction

While the endocannabinoid system is the primary site of action for both the psychological and pharmacological responses to the THC present in marijuana, growing evidence demonstrates that endocannabinoid signaling is involved in the rewarding and addictive properties of many drugs of abuse including alcohol, nicotine, opiates, and psychostimulants (Maldonado, Valverde, & Berrendero, 2006; Onaivi, 2008; Serrano & Parsons, 2011). The endocannabinoid system consists of three primary components: the endogenous ligands (endocannabinoids: anandamide, AEA; 2- arachidonoyl-glycerol, 2-AG); the proteins that are responsible for AEA (N-acylphosphatidylethanolamide-phospholipase D, NAPE-PLD) and 2-AG (diacylglycerol lipase, DAGL) synthesis and degradation (fatty acid amide hydrolase, FAAH, and monoacylglycerol lipase, MAGL, respectively) of these endocannabinoids; and the cannabinoid receptors (cannabinoid receptor subtype 1, CB_1; subtype 2 CB_2) (Maldonado et al., 2006; Basavarajappa, 2007; Henderson-Redmond, Guindon, & Morgan, 2016). CB_2 receptors are predominantly expressed in the immune cells in the periphery but have recently been identified in the central nervous system (Onaivi, Ishiguro & Liu, 2017). However, more research is needed to examine whether CB_2 receptors are as abundant in the central nervous system as the CB_1 subtype, as well as how this discovery impacts addiction research involving the endocannabinoid system.

Alcohol alters CB_1 receptor activity in the brain by changing both CB_1 receptor expression and the synthesis of the endogenous cannabinoids, producing differential effects depending on the brain region examined, the type of alcohol administration used (i.e., acute versus chronic), and the point at which these factors are assessed (e.g., intoxication, withdrawal, relapse) (for a comprehensive review see Zlebnick & Cheer, 2016). Studies focused on the effects of chronic alcohol administration on the endocannabinoid system are highly variable in their methodology, and consequently, as with all drug research, subsequent observations depended on the length of alcohol administration, presence or absence of withdrawal periods, and brain regions examined. However, decreased expression in CB_1 receptors following chronic alcohol is consistently reported throughout numerous studies. Chronic alcohol exposure can lead to increased levels of AEA in several brain structures, such as the prefrontal cortex and the nucleus accumbens with a corresponding downregulation of CB_1 receptors (Basavarajappa, Cooper, & Hungund, 1998; Pava & Woodward, 2012); although, reduced FAAH expression has also been reported (Pava & Woodward, 2012). These are transient alterations following short

term inhalation (i.e., 72 hours) and can return to basal levels in as little as 24 hours (Basavarajappa et al., 1998; Pava & Woodward, 2012). Although chronic alcohol exposure also reduces CB_1 receptors in the dorsal striatum, a decrease rather than an increase, in AEA is reported for this region. Mitrirattanakul and colleagues (2007) also report downregulation of hippocampal CB_1 receptors following chronic intermittent ethanol (CIE) vapor and short-term withdrawal (i.e., 2 days); however, after an extended period of abstinence (i.e., 40 days) they observed an upregulation of CB_1 receptors and AEA.

Evidence suggests that alcohol and cannabinoids activate similar reward pathways and that CB_1 receptors regulate the reinforcing properties of alcohol (Hungund & Basavarajappa, 2000; Maldonado et al., 2006). This is demonstrated by the acute administration of CB_1 receptor agonists increasing voluntary alcohol consumption in both rats and mice (Colombo et al., 2002; Linsenbardt & Boehm, 2009; Wang, Liu, Harvey-White, Zimmer, & Kunos, 2003). CB_1 receptor knockout mice exhibit low alcohol preference and intake, lower alcohol-induced conditioned place preference, and do not display the suppressive effects of CB_1 receptor antagonists on drinking behaviors observed in wild type mice and other rodents (Houchi et al., 2005; Hungund, Szakall, Adam, Basavarajappa, & Vadasz, 2003; Thanos, Dimitrakakis, Rice, Gifford, & Volkow, 2005).

Downregulation of CB_1 receptors in multiple brain regions has been observed in humans with alcohol use disorder (AUD) (Henderson-Redmond et al., 2016). Interestingly, C57BL/6 mice, that readily consume alcohol during drinking in the dark paradigms, display lower CB_1 receptor densities compared to DBA/2 mice that avoid alcohol consumption in drinking paradigms (Hungund, Baslingappa & Basavarajappa, 2000). However, CB_1 receptors may regulate alcohol drinking behaviors but not the subjective effects of alcohol as DBA/2 mice have shown conditioned place preference following an alcohol injection (Grisel et al., 2014). CB_1 receptor-deficient mice demonstrate normal alcohol tolerance and preference but fail to show alcohol withdrawal symptoms (Racz et al., 2003). Additionally, the selectively bred High Alcohol Preferring (HAP3) mice have lower levels of dorsal striatal CB_1 expression compared to their Low Alcohol Preferring counterparts (LAP; Millie, Boehm, & Grahame, 2020), and other HAP replicates are less affected by alcohol withdrawal than their LAP counterparts (Lopez, Grahame & Becker, 2011). Together, these studies implicate a modulatory role of the endocannabinoid system, specifically via CB_1 receptors, in alcohol preference, in strains with

low withdrawal susceptibility. Low withdrawal susceptibility and other genetic phenotypes such as a low-level response (LR) to alcohol that may initially act as protective factors against alcohol use also are present in individuals at risk of developing AUDs, and LR has been associated with higher alcohol intake (Schukit, 2002). Another phenotype that could be associated with a vulnerability for the development of alcoholism may be a preference towards specific drug use patterns such as simultaneous use.

1.3 Using Selectively Bred Mice as a Model of Familial Alcoholism

Individuals genetically predisposed to developing substance use disorders may be at an elevated risk for problems as a result of simultaneous use. Genetic factors may underlie an individual's susceptibility towards simultaneous use patterns and exacerbate adverse outcomes of combined cannabis and alcohol use (Subbaraman & Kerr, 2015), evidenced by polymorphisms of the gene that encodes the CB_1 receptor that have been associated with problematic drug use in humans (Serrano & Parsons, 2011), and differences between alcohol-preferring and non-alcohol preferring mice (Hungund & Basavarajappa, 2000; Millie et al., 2020). Selectively bred alcohol-preferring lines could help to clarify whether genetic susceptibility for AUDs is a core factor for use driven by a preference for combining the effects of alcohol and THC and could help assess the behavioral consequences of these combined substances in subjects with a genetic predisposition to drink.

One such genotype is the HAP selectively bred line, generated from a Hs/Ibg progenitor strain and chosen for high alcohol preference and intake on a 24-hour two-bottle choice (2BC) task where they were given continuous access to 10% alcohol and water (Grahame, Li & Lumeng, 1999). Mice were crossed from the HAP1 and HAP2 lines to produce the crossed HAP (cHAP) line that consistently consumes enough alcohol to reach or surpass blood alcohol levels of 250 mg/dl during 2BC (Matson & Grahame, 2013). These levels of alcohol drinking make the cHAPs an ideal model for alcohol research as they freely consume enough alcohol to make contact with the BALs observed in patients with alcohol use disorder (Mello & Mendelson, 1970). Additionally, this level of alcohol consumption produces BALs similar to those targeted in many CIE vapor paradigms, allowing for the potential comparison of different routes of alcohol administration while controlling for similar alcohol exposure.

1.4 THC Administration in Animal Models

As with alcohol, there are a number of paradigms that can be implemented to assess the effects of THC on cognitive, neurobiological, and physiological changes in animal models, but few offer face validity to human THC consumption. Specifically, models utilizing injections lack both the voluntary nature and route of administration associated with human THC consumption. Injections are also accompanied by unavoidable stressors (e.g., restraint stress) that produce acute stress reactions like increases in plasma corticosterone (Meijer et al., 2006). Other administration routes such as intravenous (I.V.) administration may allow for animals to self-administer, but as with injections, I.V. paradigms are likely to have numerous stressors, stemming from the surgery, that are unrelated to the target drug. Vapor inhalation paradigms have also been explored; however, placebo-exposed mice demonstrated locomotor inhibitory, hypothermic, and cataleptic effects which could interfere with performance on behavioral tasks (Lichtman et al., 2001). Fortunately, recent paradigms have confirmed that animals will freely consume THC when made available in an edible form (Nelson et al., 2018; Smoker, Mackie, Lapish & Boehm, 2019). Edible models offer the opportunity to examine voluntary THC consumption, as 2BC enables researchers to examine voluntary alcohol consumption, without stressors associated with injections, surgeries, restraint, food or water deprivation. Utilizing paradigms which minimize additional stressors is important when examining alterations stemming from drug administration, as stress effects might confound changes to the endocannabinoid system (Morena et al., 2016). Furthermore, edible models align with trends of human edible-THC intake that have accompanied marijuana legalization.

1.5 Behavioral Inflexibility in Addiction

Clinical researchers have observed increased cognitive dysfunction in individuals that were simultaneous users compared to sequential users of alcohol and marijuana. The combined use of these drugs is associated with worse performance for a variety of executive functions, including complex attention, memory, processing speed, and visuospatial functioning (Jacobus et al., 2015). Behavioral flexibility is another process of executive function impaired following drug use, and drug-induced deficits may, in turn, contribute to persistent drug-taking behaviors.

Behavioral flexibility can be described as an operation of executive functioning that involves multiple complex processes and enables an organism to use multifaceted information to inhibit an undesirable response. It requires both cortical and distinct subcortical regions to accurately distinguish between strategies and rules based on multifaceted environmental stimuli (Floresco & Jentsch, 2011; Ragozzino, 2007). Behavioral inflexibility has been hypothesized to drive addiction due to the inability of drug-dependent individuals to change their drug-taking behavior when it becomes problematic (Millie et al., 2020; Seip-Cammack & Shapiro, 2014). Innate deficits in behavioral flexibility, drug-induced behavioral inflexibility, or some combination of these factors may, therefore, contribute to addiction.

Tasks designed to evaluate behavioral flexibility begin with the formation of a rule that acts as the initial discrimination sometimes referred to as the Attentional Set. The relevant and irrelevant stimuli necessary for the initial discrimination and all the following discriminations are determined based on the type of paradigm being used. The relevant stimuli and corresponding discriminations are made based on either intradimensional (IDS) or extradimensional (EDS) shifts (Bissonette & Powell, 2012; Floresco et al., 2006; Keeler & Robbins, 2011). For example, in an operant task, the Attentional Set could require an animal to use an egocentric discrimination (i.e., the relevant dimension is spatial), always requiring the animal to select the left lever to obtain the reward. During this Attentional Set, the animal would be required to simultaneously ignore an additional stimulus such as a visual cue (i.e., the irrelevant dimension is visual). The first discrimination (i.e., the IDS or reversal) would require the animal to select the right lever instead of left to receive the reward. For the EDS required in Attentional Set-Shifting, the previously irrelevant dimension becomes the relevant dimension. In an operant chamber, the lever stimulus light (i.e., visual cue) would become the relevant dimension, indicating the correct lever choice for the subsequent reward. Several different paradigms have been developed to assess Reversal Learning and Attentional Set-Shifting, including digging, maze, and operant tasks. Regardless of the paradigm used, behavioral flexibility in animals can be measured by tasks that require subjects to learn a series of changing rules, where cues within the same dimension indicating a correct choice are switched (Reversal Learning); alternatively, once a set criterion is met, cues from one dimension may become irrelevant, and a correct choice becomes determined via another dimension (Attentional Set-Shifting).

Impaired behavioral flexibility has been observed in clinical studies following chronic singular drug abuse for a variety of substances, including alcohol and marijuana (Bolla, Brown, Eldreth, Tate, & Cadet, 2002; Fernández-Serrano, Pérez-García, Schmidt Río-Valle, & Verdejo-García, 2010; Pope, Gruber, Hudson, Huestis, & Yurgelun-Todd, 2001; Ratti, Bo, Giardini, & Soragna, 2002; Verdejo-García, Bechara, Recknor, & Pérez-García, 2006). The Wisconsin Card Sorting Task (WCST), a clinical measure of behavioral flexibility analogous to animal Attentional Set-Shifting tasks, has been used to examine the impairments of individuals with AUD. Consistently, individuals with AUD have been found to commit more errors compared to nonalcoholic controls (Ratti et al., 2002; Oscar-Berman, Kirkley, Gansler & Couture, 2004). Additional studies utilizing the WCST have been conducted to characterize behavioral flexibility in individuals that may be at risk for developing AUD. Research indicates that both adolescents with a family history of alcoholism (Corral, Holguín, & Cadaveira, 2003) and adults from high-density alcoholic families (Gierski et al., 2013) have innate deficits in behavioral flexibility as measured by the WCST. When evaluating the impact of heavy marijuana use on behavioral flexibility, there are conflicting findings on whether neurocognitive deficits improve over time. Some research has shown that heavy marijuana use is associated with persistent impairments measured by the WCST after 28-day abstinence (Bolla et al., 2002); however, other research failed to detect a significant effect of heavy cannabis use, also following 28-day abstinence, on the same task (Pope, Gruber, Hudson, Huestis & Yurgelun-Todd, 2001). Therefore, it is unclear whether deficits associated with marijuana use are transient or what factors may contribute to deficits that persist over time.

Animal models have demonstrated that alcohol typically interferes with Attentional Set-Shifting (Gass et al., 2014; Hu, Morris, Carrasco & Kroener, 2015; Kroener et al., 2012; Trantham-Davidson et al., 2014), although there is some evidence that it can also interfere with Reversal Learning (Fernandez, Lew, Vedder & Savage, 2017). Synthetic cannabinoid agonists, as well as THC, primarily impair Reversal Learning (Egerton, Brett & Pratt, 2005; Gomes, Guimarães & Grace, 2015). Unexpectedly, some evidence suggests that modulation of the Cannabinoid receptor subtype 1 (CB_1) by cannabinoid agonists promotes Attentional Set-Shifting, although this phenomenon is likely due to a disruption in the consolidation of Reversal Learning memories (Hill et al., 2006). Thus, from a behavioral standpoint, CB_1 receptor activation through co-administration of THC and alcohol would likely disrupt Reversal Learning

and Attentional Set-Shifting more than either substance alone, although to what extent currently remains unclear.

Several factors may affect the outcome of the combination of these substances specifically on animal models of behavioral flexibility. For instance, alcohol and CB_1 agonists may produce more profound impairment on only Reversal Learning or Attentional Set-Shifting rather than overall impairment, as the combination of these drugs may induce recruitment of additional brain regions to perform cognitive tasks through complex signaling alterations (Eldreth, Matochik, Cadet, & Bolla, 2004; Kanayama, Rogowska, Pope, Gruber, & Yurgelun-Todd, 2004; Ramaekers et al., 2011). Alternatively, although somewhat unlikely, it also is possible that one drug may ameliorate the effects of the other drug, resulting in either a total absence of deficits for either task or no increased impairment compared to singular drug administration. A recent study examining oral and subcutaneous administration of THC and simultaneous alcohol access in adolescent male rats found altered consummatory behaviors for sucrose, chow, and alcohol but did not observe any negative effects on spatial learning or behavioral flexibility using the Barnes maze task (Nelson et al., 2018). However, the relatively low blood alcohol concentrations achieved in that study (i.e., BACs 25.4 ± 11.6 mg/dL) may indicate a minimum drug threshold is necessary to produce behavioral deficits. Research targeting pharmacologically relevant levels of both THC and alcohol is needed to assess the impact simultaneous intoxication can have on behavioral flexibility measures and the corresponding brain regions that regulate this executive function.

1.6 The Dorsal Striatum is a Critical Brain Region for Behavioral Flexibility and Addiction

Direct and indirect dopaminergic signaling from the dorsal striatum participates in feedforward and feedback signaling cascades that contribute to the basal ganglia circuitry's role in numerous behaviors, including action selection (Gerfen & Surmeier, 2011). There is a modulatory role for the dorsal striatum in Reversal Learning and Attentional Set-Shifting tasks which utilize action selection (Bissonette & Powell, 2012; Ragozzino, 2007), in addition to containing some of the highest levels of CB_1 receptors in the brain (Pattij, Wiskerke, & Schoffelmeer, 2008; Zlebnick & Cheer, 2016). Seminal research examining motor activity demonstrated that neuronal interactions between CB_1 receptors and dopaminergic systems

contribute to striatal signaling, independent from drug administration (Giuffrida et al., 1999). However, the endocannabinoid system has a diverse role in drug addiction as synaptic plasticity altered by endocannabinoid signaling can subsequently produce a variety of complex downstream effects on behavior.

The endocannabinoid system is directly involved in the primary rewarding effects of many drugs of abuse through the release of endocannabinoids that act as retrograde messengers to inhibit the release of monoamines, GABA, and glutamate. Endocannabinoid and monoamine release, in turn, has a modulatory effect on the reward circuitry through shared cellular mechanisms of mesolimbic dopamine neurotransmission (Maldonado et al., 2006; Onaivi, 2008). Inhibition of CB_1 receptors in regions such as the NAc reduces the rewarding properties of drugs such as alcohol and heroin (Caillé, Alvarez-Jaimes, Polis, Stouffer, & Parsons, 2007). Additionally, drug-induced stimulation of CB_1 receptors in the dorsal and ventral striatum results in altered short-term and long-term synaptic plasticity integral to the adaptive learning of reward-motivated behaviors (Zlebnik & Cheer, 2016). Although the NAc is frequently studied, comparatively little is known about the effects of specific drugs, such as alcohol and THC, in the dorsal striatum. Potentially the complexity of dorsal striatal circuitry and signaling has limited research relative to the NAc.

The dorsal striatum has been established as a part of the brain's reward circuitry crucial for behavioral flexibility (Bissonette & Powell, 2012; Ragozzino, 2007). Moreover, this area may also be the primary site responsible for changes observed in Reversal Learning and Attentional Set-Shifting tasks following drug administration. Considering neuronal interactions between endocannabinoid and dopaminergic systems within the dorsal striatum, we hypothesize that drug-induced CB_1 receptor expression in this brain region may contribute to maladaptive changes in behavioral flexibility. The extent to which alcohol, THC, and the combination of these drugs alter CB_1 receptor expression in the dorsal striatum and, furthermore, how this expression changes with the timing of drug exposure is currently unclear.

1.7 Conclusions

Given the increased legalization of marijuana and the heightened likelihood of the simultaneous use of alcohol and marijuana (SAM), more research is needed to elucidate how

these drugs interact and subsequently affect both behavior and the brain. Animal models offer an opportunity to explore potential effects of co-use on behaviors ranging from basic consumption patterns to complex cognitive processes, such as behavioral flexibility. These models may provide useful information for human populations, considering the escalating simultaneous use of these two commonly used substances. To date, minimal animal research has explored the combined effects of THC and alcohol on executive functions, and more research is needed to examine the neurobiological changes that may occur as a result of chronic simultaneous drug consumption, especially in understudied regions such as the dorsal striatum.

CHAPTER 2 SPECIFIC AIMS

2.1 Aim 1: Examine the effects of chronic simultaneous self-administration of oral THC and alcohol on drug consumption.

This aim sought to understand if voluntary simultaneous use, in a preclinical model of familial alcoholism, affected levels of drug consumption compared to the use of a single drug, and whether this pattern of polysubstance use was associated with deficits in measures of behavioral flexibility. We hypothesized that simultaneous use would increase the consumption of THC and alcohol compared to the consumption of either drug alone.

2.2 Aim 2: Examine the effects of different abstinence periods following chronic simultaneous self-administration of oral THC and alcohol on behavioral flexibility using operant reversal learning and attentional set-shifting tasks.

This aim evaluated the self-administration of alcohol and THC, alone and in combination, for potential behavioral impairments following either a Short or Prolonged Abstinence period. We hypothesized that THC administration would impair Reversal Learning and alcohol administration would impair Attentional Set-Shifting following a Short Abstinence period relative to drug-naïve Controls. Additionally, SAM use would produce deficits for both Reversal Learning and Attentional Set-Shifting relative to Controls after both a Short and Prolonged Abstinence period.

2.3 Aim 3: Assess potential time-dependent changes following simultaneous THC and alcohol self-administration on CB_1 receptor protein expression in the dorsal striatum.

Alterations in the dorsal striatum, a crucial area involved in behavioral flexibility, following THC and alcohol administration have not been fully characterized. By examining various abstinence periods (No Withdrawal, Short Abstinence, and Prolonged Abstinence) following the self-administration of drugs in mice, this experiment will assess whether alcohol, THC, or self-administration of both substances results in sustained or transient alterations in a region important for behavioral flexibility and other addiction-related behaviors. We predicted that dorsal striatal levels of CB_1 receptors would be reduced following two weeks of drug administration compared to Control animals, with a return to basal levels after a brief abstinence

period. We also hypothesized that after extended abstinence, there would be an upregulation of dorsal striatal CB_1 receptors in drug-exposed animals, relative to both Controls and to the other abstinence periods.

CHAPTER 3 MATERIALS AND METHODS

3.1 General Design (Aims 1-3)

Animals initially went through the behavioral flexibility paradigm detailed below (Figure 1) and subsequently went through an identical drug access paradigm but were placed into one of three abstinence groups: Short Abstinence, Prolonged Abstinence, or No Abstinence. Along with the No Abstinence cohort, an additional group of cHAPs and one group of C57BL/6J (B6) mice received no behavioral training or exposure to the drug consumption paradigm to function as a complete control and alternate strain comparison for western blot analysis. Table 1 provides details on the experimental group sizes for each cohort by strain and sex.

Table 1. Experimental Subjects

Cohort	Strain	Sex	Control (n=42)	EtOH (n=42)	THC (n=48)	SAM (n=48)	No Behavior (n=23)	Total (n=203)
1. Short Abstinence	cHAP	Female	6	6	8	8		28
		Male	8	8	8	8		32
2. Prolonged Abstinence	cHAP	Female	7	7	8	8		30
		Male	7	7	8	8		30
3. No Abstinence	cHAP	Female	7	7	8	8	7	37
		Male	7	7	8	8	5	35
	B6	Female					6	6
		Male					5	5

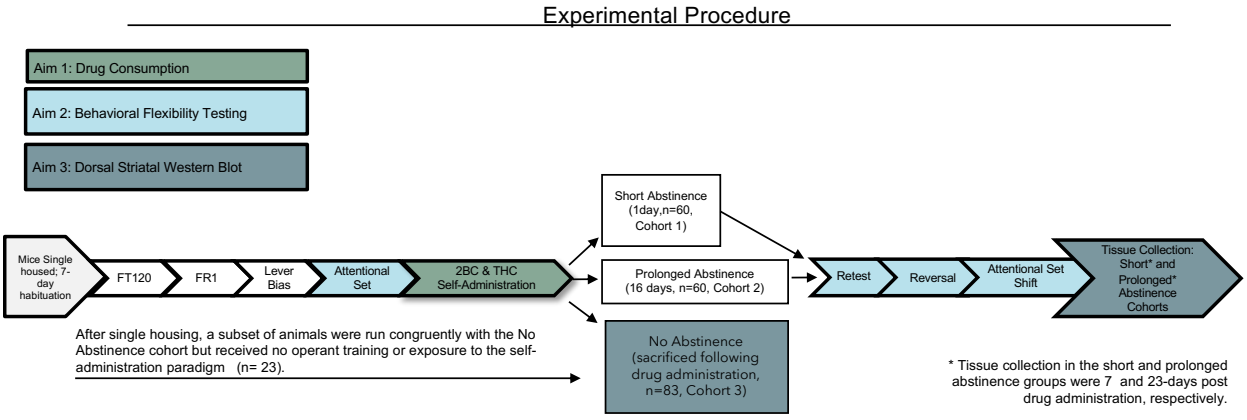


Figure 1. Experimental Schematic depicting the sequence of operant procedures, drug administration, and tissue collection. Color coding indicates the focus of the individual dissertation aims and is separated as they will be discussed in the results section.

3.1.1 Subjects

All experiments were performed following the Institutional Animal Care and Use Committee (IACUC) of IUPUI and NIH Guide for the Care and Use of Laboratory Animals. All mice were born in the IUPUI Animal Care Facilities from maintained colonies and were between 56-73 days old at the start of behavioral testing. Male and female cHAP mice were from the 43rd selection generation. For each experiment, cHAP mice were initially counterbalanced by Family and Sex and counterbalanced by attentional set performance prior to drug condition assignment. All mice were singly housed in standard Plexiglas cages lined with pine bedding on standard 12-hour reverse light cycle (lights off at 0700).

3.1.2 Drug Administration

Mice were divided into one of four drug exposure groups: Control (water and control dough), Alcohol only (EtOH and control dough), THC only (water and THC dough), and SAM (EtOH and THC dough) and were run in three cohorts. Drug self-administration commenced on the day following the completion of the attentional set for all animals. Animals were weighed weekly to calculate g/kg/day EtOH consumption and dough preparation. A two-bottle choice (2BC) paradigm was used to administer EtOH for fourteen days. The 2BC procedure consisted of two tubes, one 50 mL and one 25 mL, both containing tap water (Water only) or one 50 mL tube of 10% EtOH and one 25 mL tube of tap water (EtOH). Alcohol concentration was set to 10% as this matches the concentration used for the selective breeding of this line. Bottles were read each before dough administration and bottle sides were switched daily to avoid the establishment of a side preference.

On the first two days of 2BC, control dough was given to all animals. On the third day of 2BC, animals in the THC and SAM groups received 1mg/kg THC dough, while all others continued to receive control dough. THC was procured from the National Institute on Drug Abuse (Bethesda, MD) dissolved in 95% ethanol at a concentration of 100 mg/ ml. The concentration of THC was increased daily for four days (3mg, 5mg, 7mg) until a 10mg/kg dose was achieved and was given for the remainder of the drug consumption period (i.e., 8 days at 10mg/kg). The utilization of escalating THC concentrations is common in edible THC paradigms and the concentrations here are similar to those used in published edible paradigms (Nelson et

al., 2019; Smoker et al., 2019). A pilot study to evaluating whether cHAPs would voluntarily consume THC in addition to EtOH demonstrated consumption of both substances up to 5mg doses, and 10mg doses in the absence of EtOH supporting the escalation chosen here.

3.1.3 Dough

THC and control doughs were made based on the recipe found in Smoker et al. (2019). The dough was comprised of flour, sugar, salt, glycerol (3 g:0.4 g:0.1 g:2 mL ratio), and given in 5 mg dough per 1 g mouse body weight portions. THC was suspended in 95% alcohol at 100 mg/mL. For each mouse, the control dough contained an equivalent amount of alcohol as the THC dough. THC doses ranged from 1 to 10 mg/kg, resulting in alcohol doses between 0.0075 g/kg and 0.075 g/kg, per serving with corresponding amounts of alcohol in the control dough based on THC dough provided on the same day. This amount of alcohol is below the metabolic threshold of pharmacologically relevant alcohol consumption in cHAPs (Matson & Grahame, 2013). For dough consumption periods, mice were transferred to a clean, empty cage and allowed to consume dough with access to water but not food for 60 minutes. Mice remained single-housed during dough access. Following access, animals were returned to their home cage, and bottles were read. The amount of uneaten dough was recorded following each period of dough access.

3.1.4 Apparatus

Ten operant boxes were used (Med-Associates, St. Albans, VT). A retractable sipper with a 10-mL graduated sipper tube was used to provide and measure the saccharin solution reward and two retractable levers were in use at various points during training and testing. The lever stimulus light, located above each retractable lever, was used during all testing phases but had different functions depending on the Reversal Learning and Attentional Set-Shifting tasks, described below. During all operant procedures, Cellsorb bedding was placed under wire-grid flooring and changed bi-weekly. A 0.1% w/v saccharin solution was used as the reward in the operant chambers based on Grahame lab operant procedures and to maintain consistency with published works (Millie et al., 2020).

3.2 Behavioral Flexibility Operant Procedures (Aim 2)

Animals were water restricted ~22 hours a day for all behavioral testing periods. Following operant sessions, water bottles were placed on the home cage for ~2 hours. Animals having met criteria for the set-shift ceased water deprivation.

3.2.1 Pre-training

Animals were trained to lever press using fixed time 120s (FT120) and fixed-ratio 1 (FR1) schedules in 30-minute sessions for a 0.1% saccharin solution reward. The criteria to move to the next stage of training was a minimum of 20 lever presses and 0.2mL of reward consumption per session. For the FR1 training, only one lever was present at a time, left and right levers were counterbalanced across animals. After meeting the criteria for the first lever, animals were training on the opposite lever.

3.2.2 Lever Bias Test

A 5-minute session in which both levers were active measured responses to determine if a lever bias was present. The correct egocentric lever for the attentional set was assigned opposite any lever bias. Animals were considered to have a lever bias if they responded for more than 50% of their total lever presses on a single lever. If no lever bias was present, correct egocentric levers were randomly assigned and counterbalanced across animals.

3.2.3 Attentional Set

Animals were required to always choose either the left or right lever regardless of the illumination of the lever stimulus light (which randomly alternated between levers ~50% of the time) in 30-minute sessions. Following a correct lever press, the levers retracted, the lever light was turned off and the reward became available (2 seconds of access to 0.1% saccharin reward) followed by a 4-second intertrial interval. The session terminated after 30 minutes or immediately following correct responding on 8 of the most recent 10 trials.

3.2.4 Retest

Mice completed one session of the Attentional Set to assess retention of the initial discrimination, either the day after drug access ended (Short Abstinence cohort) or 16 days after drug access ended (Prolonged Abstinence cohort). The Retest of the Attentional Set was completed before mice moved on to the Reversal Learning task.

3.2.4 Reversal Learning Task (IDS)

Following the retention Retest of the Attentional Set, animals were tested on an egocentric reversal (IDS), wherein the previously incorrect lever became the correct lever. The session terminated after 30 minutes or when animals respond correctly on eight consecutive trials, whichever came first. Animals not meeting the criterion of 8 consecutive correct trials on the first session were administered additional sessions until this criterion was met.

3.2.5 Attentional Set-Shift (EDS)

Following a successful reversal, the shift to visual cue responding (EDS) occurred wherein animals were required to always choose the active lever indicated by the illumination of the previously irrelevant lever light stimulus. Like the Reversal Learning sessions, shift sessions were 30 minutes long but would terminate early if animals met criterion by responding correctly on 10 consecutive trials. Animals continued in sessions until the criterion was met or the testing period (5 days) ended. Animals who did not meet the criteria (n=1) were excluded from the analysis.

3.3 Brain Extraction and Western Blot (Aim 3)

The Short Abstinence Cohort was sacrificed 7 days following drug administration, and the Prolonged Abstinence Cohort was sacrificed 23 days after cessation of drug self-administration, at which point both groups had completed the Reversal Learning and Attentional Set-Shift tests. Within these groups, only the final day of drug consumption was held constant to the time of euthanasia. Animals completed the Reversal Learning and Attentional Set-Shift

procedures within 6 days, however testing ceased upon successful task completion, creating a 4-day range between the last set-shift and euthanasia. The No Abstinence cohort was sacrificed on the same day as their final drug exposure.

Brains were collected via rapid extraction following cervical dislocation for all animals. Western blot procedures followed the method used in Kasten, Zhang, and Boehm (2017). A single 2-mm coronal slice was taken from each brain and bilateral tissue punches of the dorsal striatum were extracted. Tissue was snap-frozen using liquid nitrogen and stored in a -80°C freezer until sample preparation. Samples were homogenized in radioimmunoprecipitation assay (RIPA) buffer, containing: 25 mM Tris HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, with protease inhibitor (1mL of RIPA buffer containing 100 µl of 10X Protease Inhibitor and 10 µL of .1M phenylmethylsulphonyl fluoride; Thermo Fisher), for ~30 seconds until the tissue thoroughly homogenized using a Pellet pestles cordless motor. Samples were then centrifuged for 15 minutes (20,000 X g, 4°C) and the resulting supernatant was separated from the pellet and placed into a new tube. Protein concentration was calculated using the Bio-Rad Protein Assay kit (Hercules, California). Samples were denatured using 20 ug of sample protein with 5 ul of 4X Loading Dye and adding 5% of 1 M DTT. The final volume was adjusted to 20 ul with RIPA buffer and denatured at 95°C for 5 minutes. Bio-Rad 18-well Midi protein gels (4%-20% criterion TGX precast) were used for electrophoresis at 120V in a 1X Tris/Glycine/SDS buffer (Bio-Rad) with Standard Plus2 added to the first well, experimental samples to wells 2-16, and the standard sample to well 18. Gels were transferred to Bio-Rad Midi format 0.2 um nitrocellulose single application membranes using the Trans-Blot Turbo System for 7 minutes at 2.5A/25V. Membranes were then blocked using 5% nonfat milk in tris-buffered saline (TBS).

Following blocking, the primary antibody (Anti-Cannabinoid Receptor 1, Rabbit polyclonal to Cannabinoid Receptor 1; Abcam) was added to the blocking buffer (5% nonfat milk in TBS-T (TBS with 0.1% Tween-20)), at 1:1000 dilution and incubated at 4°C overnight on a rocking plate. After incubation the membrane was washed with TBS-T 3X, 10 min each, at ~45rpm before addition of the secondary antibody (IRDye 800 CW Goat anti-Rabbit IgG H+L [heavy and light chains]; LI-COR) in a 1:5000 dilution. Following the application of the secondary antibody, membranes were washed identically, and membranes were scanned using Odyssey CLx imager and Image Studio software (LI-COR, Lincoln, NE, USA). Following the

same procedure used for the primary and secondary antibodies, the protein loading control, β-actin (mouse monoclonal antibody, LI-COR) and its secondary (IRDye 680RD Donkey anti-Mouse IgG (H+L), LI-COR) were then applied to the membranes and subsequently scanned for the β-actin signal. Target protein quantification for each mouse was calculated as a ratio of the signal strength of CB_1 expression to the signal strength of β-actin expression and normalized to the standard sample to enable analysis across membranes.

3.4 Statistical Analysis

Data were analyzed and graphed using SPSS (SPSS, Version 26, Chicago, IL) and GraphPad Prism (GraphPad Prism, v. 8.0, La Jolla, CA). Significance was set at $p < 0.05$, followed by post hoc analyses when appropriate. ANOVAs were structured as Drug Group (Control, EtOH, THC, SAM) x Sex (Female, Male). In the absence of a significant interaction, the data were collapsed across Sex.

3.4.1 Drug Self-Administration (Aim 1)

Previous research has assessed daily EtOH intake and corresponding BACs for cHAPs (Matson & Grahame, 2013); therefore, an ANOVA was used to assess average EtOH intake across the 2-week self-administration period. This average indicates whether mice are consistently reaching pharmacologically relevant EtOH during 2BC. We choose to assess total THC consumption, instead of a daily average, as we have not previously assessed pharmacologic responses to single or repeated THC consumption in cHAPs. THC consumption was not normally distributed and therefore nonparametric tests were used to examine whether total THC consumption differed by Drug Group or Sex for total THC consumed during the self-administration period. Repeated Measures ANOVAs (RMANOVA), Day (day of drug administration) x Drug Group split by Sex (e.g., Female EtOH), were used to examine daily drug intake for EtOH and THC.

3.4.2 Behavioral Flexibility Testing (Aim 2)

Considering the Short and Prolonged Abstinence periods were run as separate cohorts, they are treated as separate experiments for the behavioral flexibility analyses. In both cohorts, performance on the Attentional Set was used to counterbalance animals into the four possible drug conditions to ensure there were no baseline behavioral differences prior to drug administration. RMANOVAs with Drug Group and Sex as the between-subject factors were run to examine within-subject differences between the Attentional Set and the retention Retest.

For the Reversal Learning task, two error types were calculated. A perseverative error was scored whenever the mouse chose the lever which was correct under the initial discrimination. Based on Floresco et al., (2008)'s operant procedure, all trials were divided into blocks of eight. After making fewer than five perseverative errors in a block of trials, all the following perseverative errors were considered regressive errors. Regressive errors indicate that the animal is following an alternative strategy at least 50% of the time. As with Reversal Learning, for the Attentional Set-Shift, all attempted trials in which a perseverative error could have been made were assessed in blocks of eight trials and transitioned to regressive errors when an alternative strategy was being used ~50% of the time. Additionally, never-reinforced errors were scored when a mouse selected the lever opposite the correct response when the visual-cue stimulus was active above the same assigned egocentric lever from the Reversal Learning task. ANOVAs assessing Reversal Learning and Attentional Set-Shifting were followed by planned pairwise comparisons based on *a priori* hypotheses.

3.4.3 Dorsal Striatal Western Blot Analysis (Aim 3)

Analysis of CB_1 receptor protein expression was performed with nonparametric tests due to non-normal distributions. The western blot analysis was performed by calculating a ratio of CB_1 signal to the β-actin signal and normalized to an identical gel standard (sample from a single, whole-brain male HAP3) to normalize blotting differences across membranes. Each gel contained samples from male and female animals from all drug conditions within an abstinence period (4 gels per abstinence period, 12 total gels). Results are graphed using the normalized CB_1 receptor expression in arbitrary units. Regressions assessing whether drug consumption corresponded to CB_1 receptor expression were also conducted.

CHAPTER 4 RESULTS

4.1 Attrition

One female SAM mouse in the Short Abstinence cohort died from hypothermia-related issues after the home cage flooded. Animals in the EtOH drug conditions consuming a daily average of less than a pharmacologically relevant amount of EtOH (i.e. <12g/kg/day; Matson & Grahame, 2013) were excluded from analysis (EtOH n=2; SAM n=3, 1 female).

4.2 Drug Consumption (Aim 1)

A small pilot study demonstrated that cHAP mice would repeatedly consume THC and alcohol but prior to this study, large-scale THC consumption with and without simultaneous alcohol access had not been examined in this strain. Although the majority of THC and SAM mice consumed large quantities of THC, there was a wide range in the total amount of drug consumed over the two-week drug administration period (Figure 2). This range implies that, similarly to EtOH, mice titrate to preferred levels of THC intake, although the range of THC consumption may be more variable than EtOH consumption typically is within this selectively bred line.

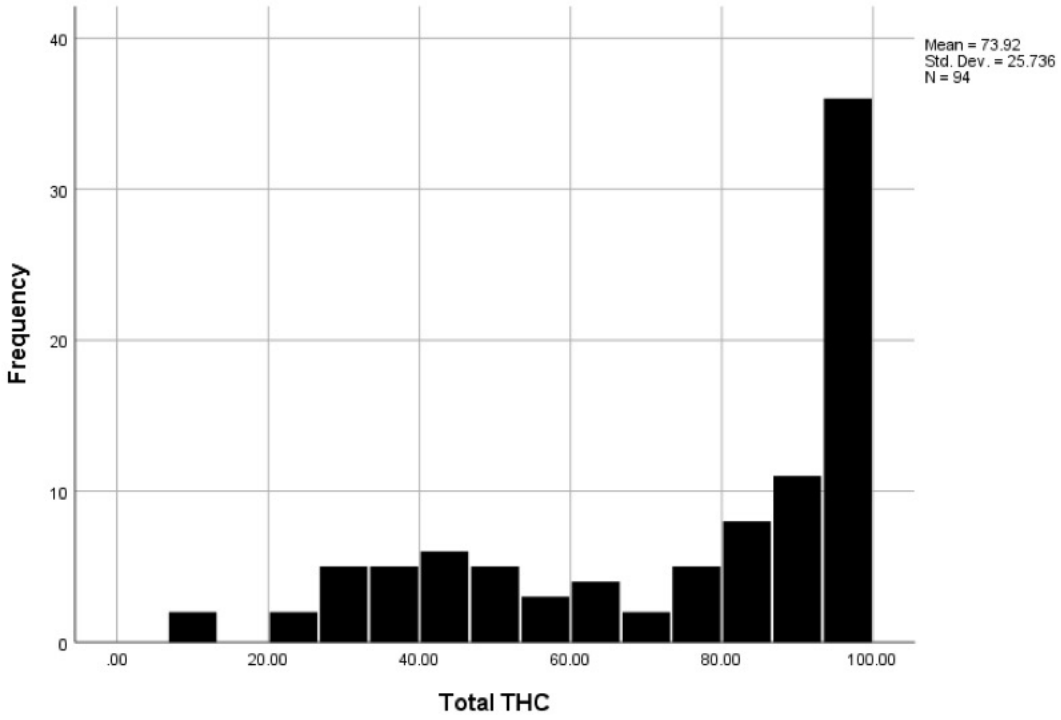


Figure 2. Histogram of Total THC intake for all SAM and THC animals collapsed by group, across the two-week drug administration period in which animals could consume a maximum of 96mg/kg of THC.

A RMANOVA of weights found no difference within-subjects from baseline to the secondary weight assessment (F[1,108]=.861, p=.355) and no Day*Group (F[3,108]=.179, p=.910), or a Day*Group*Sex (F[3,108]=.104, p=.958) interactions. There was a Weight*Sex interaction (F[1,108]=6.001, p=.001) with females (M= 20.171) weighing less than males (M = 22.378). Between-subjects there was a main effect of Group (F[3,108]=3.357, p=.022) and Sex (F[1,108]=55.275, p<.0001) but no Group*Sex interaction (F[3,108]=.1.672, p=.177). Bonferroni post hoc correction found that Control animals (M= 21.910) weighed slightly more than EtOH (M= 20.766; F[3,108]=3.357, p=.030) and SAM (M= 20.865; F[3,108]=3.357, p=.034) mice.

4.2.1 THC Consumption is Modulated by Sex and Simultaneous Alcohol Access

Aim 1 examined whether simultaneous drug consumption, in mice with a predisposition to drink, would affect levels of drug consumption compared to the intake of a single substance.

We examined total THC and average EtOH consumption, across the entire two-week self-administration period. Contrary to our hypothesis, simultaneous use did not increase the consumption of THC compared to THC alone. Due to the skewed distribution of total THC intake (Figure 2), a nonparametric Mann-Whitney U test was used to look for Sex and Group differences for total THC consumption. There was a main effect of Sex (n = 92, U = 637.5, p=.001) with females (M= 82.74, SEM =3.21) consuming more total THC than males (M= 67.41, SEM =3.72) (Figure 3A). The nonparametric post hoc comparisons found that this effect is largely driven by female THC animals, as female THC mice consume more THC (M= 92.44, SEM =1.99) than SAM (M= 73.05, SEM =5.46) female mice (n = 46, U = 128.0, p=.001; Figure 3A). Over the eight days of 10mg/kg THC access, THC females consumed an average of 20mg/kg more THC than SAM females. This difference in consumption indicates that simultaneous access to alcohol increases variability and reduces THC self-administration at this dose in female mice. Interestingly, male THC mice (M= 64.74, SEM =5.26) were the lowest THC consumers, although they do not statistically differ from SAM males (M= 70.33, SEM =5.23) for total THC intake (n = 46, U = 307.0, p=.949; Figure 3A). These findings signal that THC consumed alone is more preferable to female than male cHAPs and simultaneous access to alcohol in females reduces THC consumption. Furthermore, this is a sex-specific phenomenon as male SAM animals did not differ from either THC males or SAM females for total THC consumption.

Daily drug intake for both THC and EtOH was assessed using RMANOVAs to understand how SAM use may have altered day to day drug consumption. Sphericity was not assumed for RMANOVAs and Geisser-Greenhouse corrections were applied for these analyses. For THC intake, there was a main effect of Day (F[11,91]=73.882, p<.0001), Sex (F[3,91]=8.748, p=.004), and a Day*Sex*Group interaction (F[11,91]=2.48, p<.048; Figure 3B). Differences in daily intake are present following the higher (10mg/kg) doses of THC. Tukey's multiple comparisons for the RMANOVA showed the most consistent differences in THC consumption are between Female and Male THC-only mice (Table 2). This is not surprising as female THC mice consumed more THC than any other group, while male THC mice consumed the lowest amount of THC of any group.

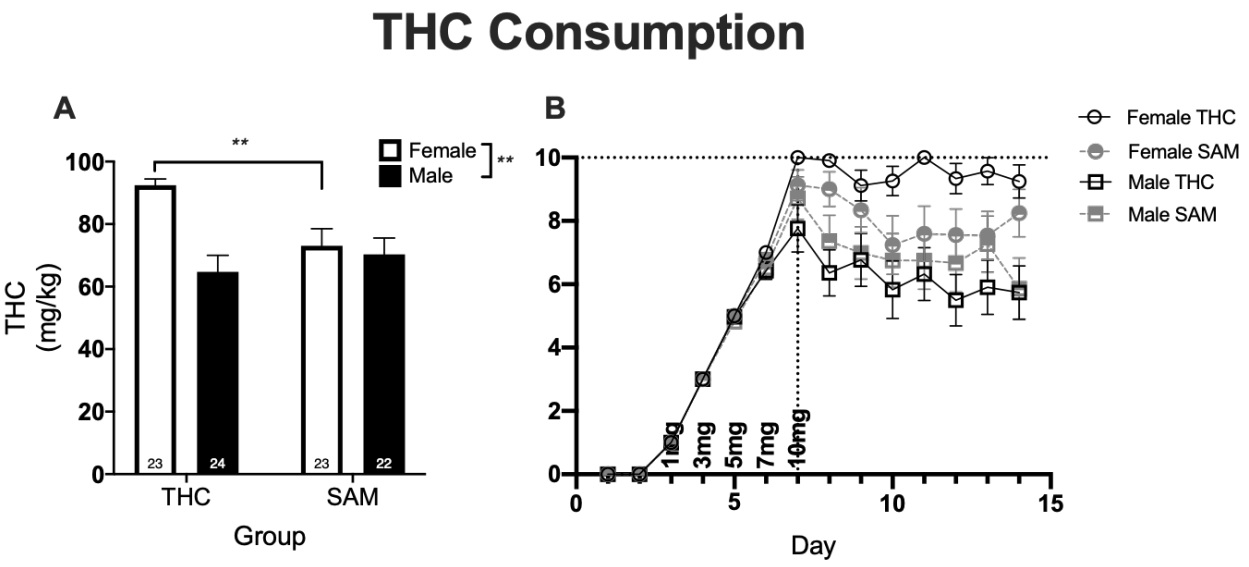


Figure 3. THC consumption across the 2-week self-administration period, y-axis for both panels is THC mg/kg consumption. A: Total THC intake across self-administration. Animals could consume up to 96mg/kg THC. Female mice consume more THC (*M*= 82.74, *SEM* =3.21) than males (*M*= 67.41, *SEM* =3.72; *n* = 92, *U* = 637.5, *p*=.001); *n's* are reported within bars. Female THC only mice consume more THC (*M*= 92.44, *SEM* =1.99) than female SAM (*M*= 73.05, *SEM* =5.46) mice (*n* = 46, *U* = 128.0, *p*=.001). Male THC (*M*= 64.74, *SEM* =5.26) and male SAM (*M*= 70.33, *SEM* =5.23) mice do not differ from one another for total consumption (*n* = 46, *U* = 307.0, *p*=.949). B: Daily intake of THC. Mice consumed increasing concentrations of THC across the self-administration period. The dotted line indicates that following day 7, allotted daily THC was 10mg/kg doses. RMANOVA showed a main effect of Day (*F* [13,87] =183.1, *p*<.0001) and a main effect of Drug Group (*F* [3,87] =6.927, *p*=.0003). There was also a Day*Group interaction (*F* [39,87] =2.78, *p*<.0001) with female THC animals consistently consuming more THC than all other groups. Table 2 reports the signification results from the Tukey's multiple comparison post hoc test for Group*Day interactions. *p*<.01**

Table 2. Tukey's multiple comparisons for RMANOVA of Daily THC Consumption for Group (split by Sex).

Group	Day	95.00% CI	Adjusted P- Value
Female THC vs. Male THC	7	0.1872 to 4.294	0.0290*
	8	1.509 to 5.579	0.0004***
	10	0.6792 to 6.196	0.0098**
	11	1.374 to 5.982	0.0011**
	12	1.304 to 6.376	0.0013**
	13	1.078 to 6.254	0.0029**
	14	0.8348 to 6.174	0.0059**
Female THC vs. Male SAM	8	0.2440 to 4.846	0.0266*
	11	0.7493 to 5.759	0.0080**
	14	0.3905 to 6.377	0.0218*
Female SAM vs. Male THC	8	0.1987 to 5.086	0.0296*

$p<.05$*, $p<.01$**, $p<.001$***

4.2.2 SAM Males Reduce EtOH Intake Following Higher Concentrations of Edible THC

Like THC, contrary to our hypothesis, SAM use did not increase alcohol consumption relative to mice exposed to only EtOH. Unlike total THC, average EtOH consumption did not differ between Drug Groups (F [1,84] =3.186, p=.078) or by Sex (F [1,84] =3.518, p=.064). Nor was there a Group*Sex interaction (F [1,84] =.004, p=.949; Figure 5A). However, even though average EtOH did not differ, there is a pattern of reduced alcohol intake for male SAM mice following increasing concentrations of edible THC (Table 3) during several days of the 2-week

self-administration. Due to the main effect of Sex (F [1, 83] =5.338, p=.02; Figure 4) in the RMANOVA for daily EtOH intake, Drug Group was not collapsed across Sex (Figure 5B).

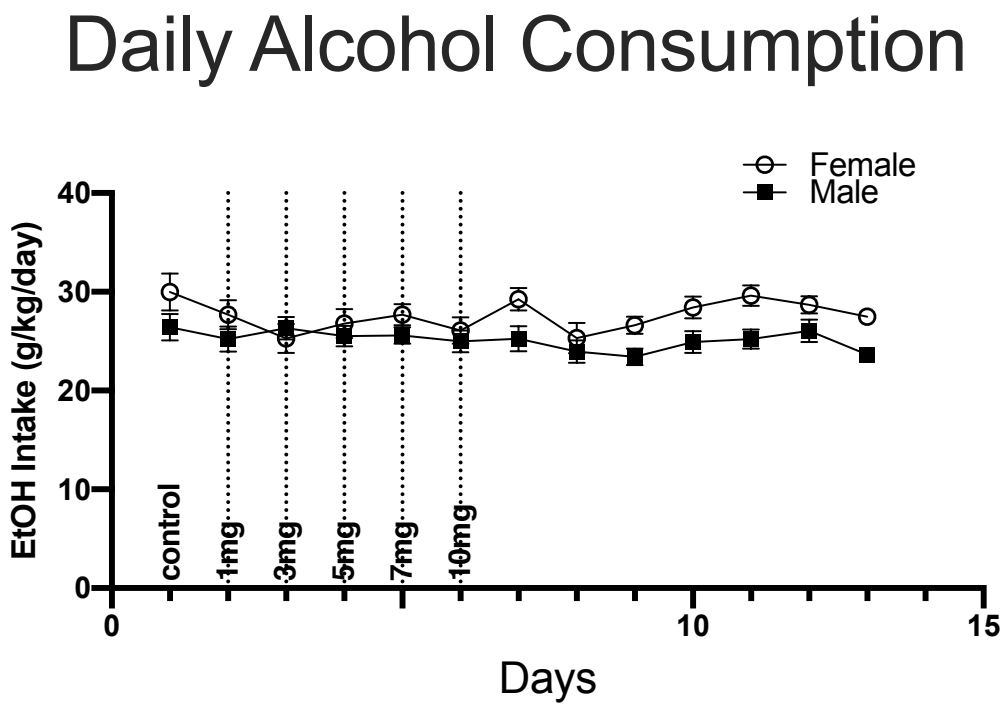


Figure 4. Alcohol consumption across the two-week self-administration period. Females consumed more EtOH than males during several days of 2BC (F [1, 83] =5.338, p=.02).

Subsequently, there was a main effect of Day (F[12,81]=2.373, p=.017), a main effect of Drug Group split by Sex (F[3,87]=6.927, p=.0003), and a Day*Group interaction (F[39,87]=2.78, p<.0001; Figure 5B). These results suggest that male SAM mice are more sensitive to alcohol consumption than their female counterparts, although alcohol intake remains high even on days in which it is reduced. Given these findings and those of the daily THC intake, it seems that male SAM animals may titrate their alcohol intake in response to SAM use, whereas SAM females titrate their THC intake in reaction to SAM use.

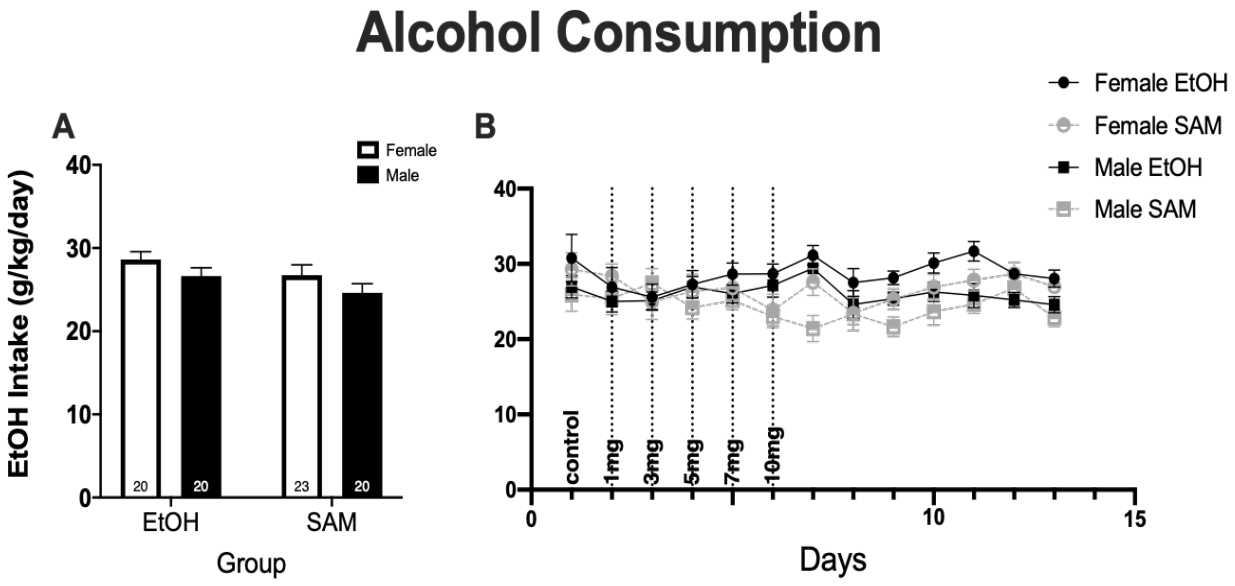


Figure 5. Alcohol consumption across the two-week self-administration period. A: Average EtOH consumption did not differ between Groups (F[1,84]=3.186, p=.078) or by Sex (F[1,84]=3.518 p=.064). There was no Group*Sex (F[1,84]=.004, p=.949); n's are reported within bars. B: Daily intake of EtOH, dotted lines designate increasing THC concentrations for SAM animals, the first day of control dough is not shown as this was also the first-day mice were exposed to EtOH. After day 7, SAM animals were continually exposed to 10mg/kg THC dough balls. The RMANOVA showed a main effect of Day (F[12,81]=2.399, p=.016) and a main effect of Group (F[3,81]=2.869, p=.041). Male SAM mice most frequently consumed less EtOH than other groups on multiple days during 2BC. Table 3 reports the signification results from the Tukey's multiple comparison post hoc test for Group*Day interactions.

Table 3. Tukey's multiple comparisons for RMANOVA of Daily EtOH Consumption by Group (split by Sex).

Group	Day	95.00% CI	Adjusted P- Value
Female EtOH vs. Male SAM	6	0.3958 to 10.93	0.0309*
	7	3.901 to 15.54	0.0004***
	9	2.219 to 10.78	0.0013**
	10	.5241 to 12.41	0.0284*
	11	2.188 to 11.84	0.0020**
	13	0.9024 to 9.536	0.0124*
Female EtOH vs. Male EtOH	11	0.2510 to 11.47	.0377*
Female SAM vs. Male SAM	13	0.6574 to 7.668	.0148*
Male EtOH vs. Male SAM	7	1.899 to 14.09	0.0059**

$p<.05$*, $p<.01$**, $p<.001$***

4.3 Behavioral Results (Aim 2)

4.3.1 Retention of an Egocentric Response Strategy is Unimpaired following Drug Consumption

Aim 2 examined potential behavioral impairments following either a Short or Prolonged Abstinence period after self-administration of alcohol and THC alone, or in combination. As expected, an ANOVA confirmed that there were no differences for the initial discrimination (i.e., Attentional Set) variables between Drug Group, Sex, or any Group*Sex interactions for either the Short (*F's*[3,56] <2.1540, *p's* > .05) or Prolonged Abstinence period cohorts (*F's*[3,58]

<1.789, *p's* > .05). This is a result of the core Attentional Set variables (e.g. Trials to Criterion) being used to assign animals to the various experimental groups (i.e., Control, EtOH, THC, SAM) to ensure there were no baseline behavioral differences among groups prior to drug self-administration.

A RMANOVA comparing within-subject performance for the Attentional Set to the retention Retest showed maintenance of the initial behavioral strategy (i.e., egocentric lever selection) across all Drug Groups. There were no between-subject effects of Group (F[3,49]=.152, p=.928), Sex (F[1,49]=.340, p=.563), or Group*Sex interactions (F[3,49]=.631, p=.610) for Attentional Set to retention Retest variables in the Short Abstinence cohort. Within-subject effects showed preservation of the Attentional Set demonstrated by a decrease in the number of trials need to reach criteria during the Retest (Figure 6, Table 4). The self-administration period did not interfere with recall of the initial discrimination acquired two weeks before, for any Drug Group.

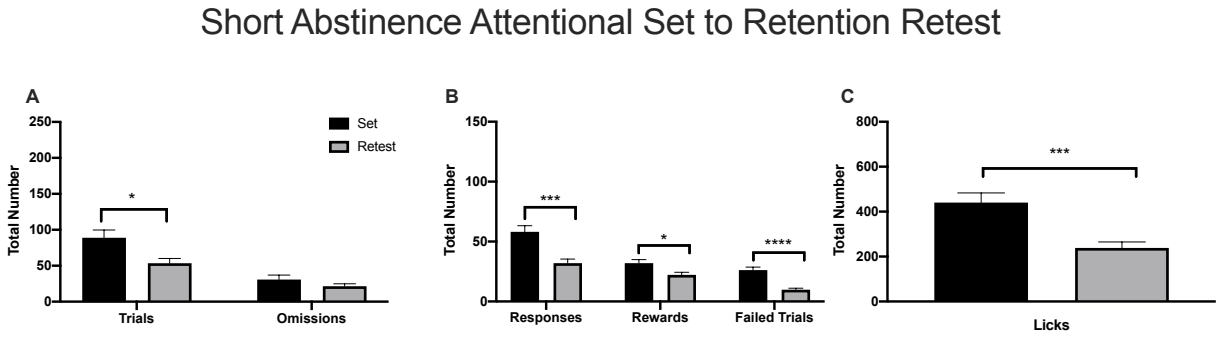


Figure 6. RMANOVA for Attentional Set to retention Retest variables. Within-subject comparisons confirmed maintenance of the initial discrimination as nearly all variables were significantly reduced upon Retest (Table 4). A: The total number of Trials to reach criteria for the Attentional Set (black) summed across sessions and the number of Trials needed to reach criteria in a single session Retest of the initial discrimination (grey). B: The total number of Responses (i.e., completed trials), Rewards, and Failed Trials, to reach criteria for the Attentional Set summed across sessions or in a single session Retest of the initial discrimination. C: Number of Licks was reduced in the Retest, corresponding to the reduction in rewards. N= 57, p<.05*, p<.01**, p<.001***

Similarly, there were no between-subject effects of Group (F[3,49]=.152, p=.928), Sex (F[1,49]=.340, p=.563), or Group*Sex interactions (F[3,49]=.631, p=.610) for Attentional Set to

the retention Retest variables in the Prolonged Abstinence cohort. Again, as with the Short Abstinence cohort, within-subject effects showed improved performance indicating that mice retained the initial discrimination (Figure 7, Table 4). Repeated drug consumption, combined with extended abstinence between the acquisition of the initial discrimination and the Retest of this discrimination, did not interfere with the retention of the ego-centric rule required to meet criteria.

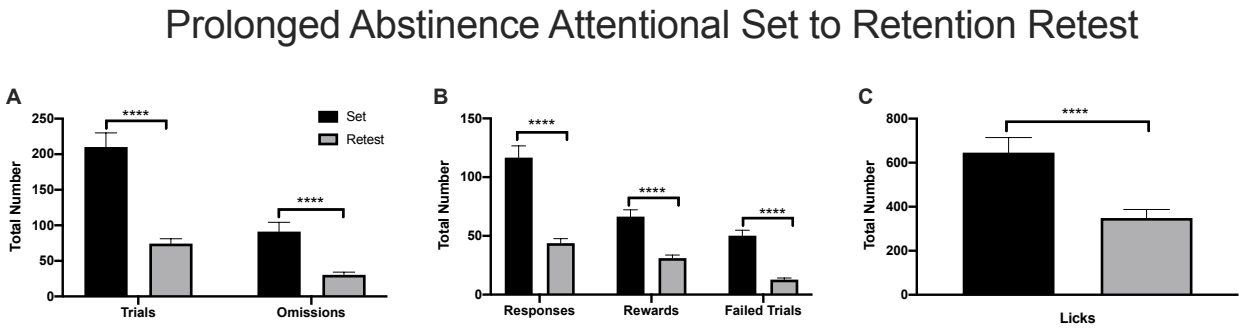


Figure 7. RMANOVA for Attentional Set to retention Retest variables. Within-subject comparisons confirmed maintenance of the initial discrimination as nearly all variables were significantly reduced upon Retest (Table 4). A: Total number of Trials to reach criteria for the Attentional Set (black) summed across sessions and the number of trials needed to reach criteria in a single session Retest of the initial discrimination (grey). B: Total number of Responses (i.e., completed trials), Rewards, and Failed Trials, to reach criteria for the Attentional Set summed across sessions or in a single session Retest of the initial discrimination. C: Number of Licks was reduced in the Retest, corresponding to the reduction in rewards. N= 59, p<.05*, p<.01**, p<.001***, p<.0001****

Table 4. Within-Subject Contrasts for the Attentional Set to Retest used to evaluate retention of the initial discrimination.

Cohort	Variable	F	Sig.
Short Abstinence (n=57)	Rewards	5.926	0.019*
	Failed Trials	29.582	0.0001****
	Omissions	1.581	0.215
	Responses	15.341	0.0001***
	Trials	6.694	0.013*
	Licks	15.190	0.0001***
	Intake	9.126	0.004**
Prolonged Abstinence (n=59)	Rewards	25.845	0.0001****
	Failed Trials	57.163	0.0001****
	Omissions	22.672	0.0001****
	Responses	39.511	0.0001****
	Trials	38.021	0.0001****
	Licks	14.559	0.0004***
	Intake	23.352	0.0001****

$p<.05$*, $p<.01$**, $p<.001$***, $p<.0001$****

Although it initially appears as though animals in the Prolonged Abstinence cohort demonstrated stronger retention of the egocentric discrimination than the Short Abstinence cohort due to the substantial decrease in Trials to criterion and other variables, Retest variable numbers appear similar across cohorts. This strong reduction is likely the result of the Prolonged Abstinence cohort requiring more Trials to reach criteria during the initial Attentional Set phase of the behavioral flexibility paradigm. The average number of sessions to complete the Attentional Set in the Short Abstinence cohort was M= 1.08, whereas the average for the Prolonged Abstinence cohort was M= 1.633. Collectively, these results indicate that any impairment, or conversely improvement, observed in the Reversal Learning or Attentional Set-Shifting procedures would not be due to a deficit in the ability of the animals to perform the initial Attentional Set.

4.3.2 Reversal Learning and Attentional Set Shifting is Unaffected following Drug Self-Administration

We hypothesized that Reversal Learning would be impaired following THC and SAM administration, however, ANOVA's failed to show significant differences between drug conditions for Reversal Learning variables for either the Short Abstinence (Figure 8, Table 5) or Prolonged Abstinence cohorts (Figure 9, Table 5). ANOVA confirmed that there were no differences by Sex, or any Group*Sex interactions for either the Short ($F's$[3,56] <2.199, $p's$ > .05) or Prolonged Abstinence period cohorts ($F's$[3,58] <1.61, $p's$ > .05). Results indicate there were no Reversal Learning deficits induced by drug self-administration in our paradigm.

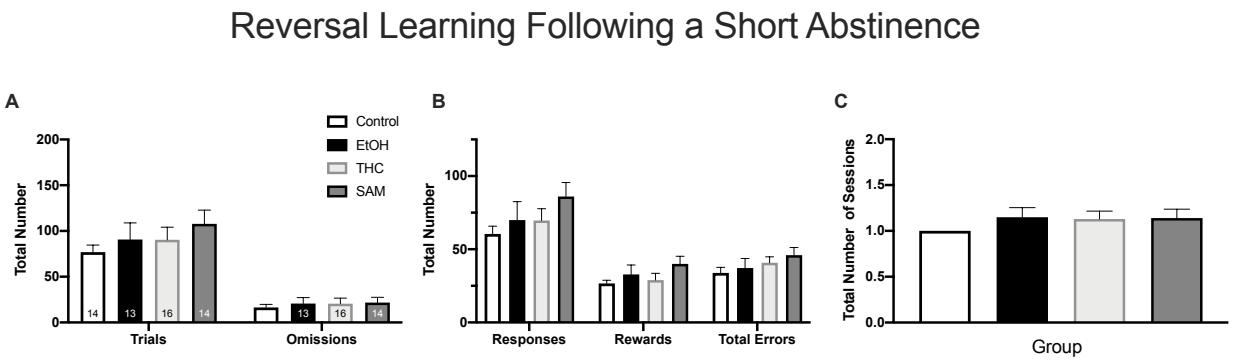


Figure 8. A: The total number of Trials and Omissions scored in reaching Reversal Learning Criteria (i.e., 8 consecutive correct responses); Control n= 14, EtOH n=13, THC n=16, SAM n=14. B: Total number of Responses (i.e., completed trials), Rewards, and Total Errors, to reach criteria. C: Number of Sessions required to reach criteria.

Table 5. ANOVA Results for the Reversal Learning Variables

Cohort	Variable	F	Sig.
Short Abstinence	Trials	0.799	0.500
	Omissions	0.175	0.913
	Responses	1.341	0.271
	Rewards	1.475	0.232
	Total Errors	1.102	0.357
	Perseverative Errors	1.602	0.200
	Regressive Errors	0.538	0.658
	Reversal Sessions	0.726	0.541
Prolonged Abstinence	Trials	1.084	0.364
	Omissions	1.151	0.337
	Responses	0.634	0.596
	Rewards	0.902	0.447
	Total Errors	0.709	0.551
	Perseverative Errors	0.356	0.785
	Regressive Errors	0.167	0.918
	Reversal Sessions	2.830	0.047

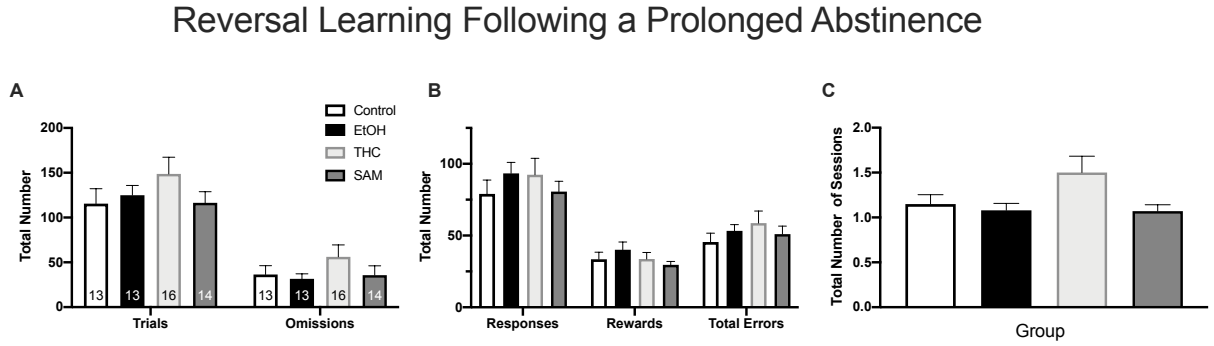


Figure 9. A: Total number of trials and omissions scored in reaching Reversal Learning Criteria (i.e., 8 consecutive correct responses); Control n= 13, EtOH n=13, THC n=16, SAM n=14. B: Total number of Responses (i.e., completed trials), Rewards, and Total Errors, to reach criteria. C: Number of Sessions required to reach criteria.

We also hypothesized that alcohol and SAM self-administration would impair performance on an operant Attentional Set-Shifting procedure. Similarly, to Reversal Learning, ANOVAs did not indicate significant deficits stemming from drug consumption for the Attentional Set-Shift in either the Short Abstinence (Figure 10, Table 6) or Prolonged Abstinence cohorts (Figure 11, Table 6). Although we speculated that drug-exposed mice would be impaired compared to Control animals, and that SAM animals would likely be more compromised than THC and alcohol for Reversal Learning and Attentional Set-Shifting respectively, no behavioral flexibility impairments were observed in our paradigm.

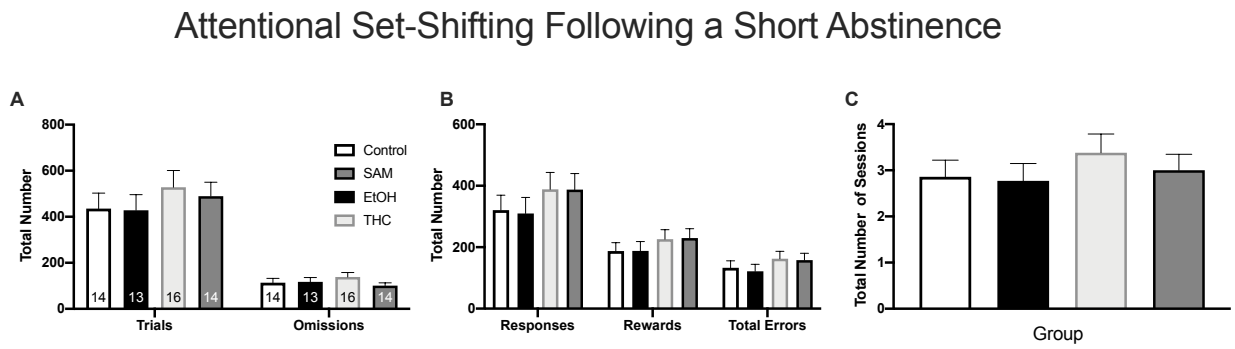


Figure 10. A: The total number of Trials and Omissions scored in reaching Attentional Set-Shifting Criteria (i.e., 10 consecutive correct responses); Control n= 14, EtOH n=13, THC n=16, SAM n=14. B: Total number of Responses (i.e., completed trials), Rewards, and Total Errors, to reach criteria. C: Number of Sessions required to reach criteria.

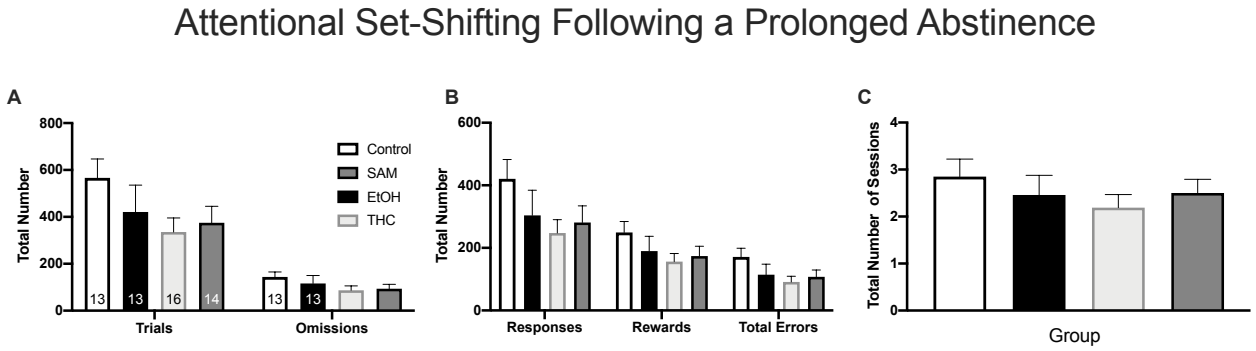


Figure 11. A: The total number of Trials and Omissions scored in reaching Attentional Set-Shifting Criteria (i.e., 10 consecutive correct responses); Control n= 13, EtOH n=13, THC n=16, SAM n=14. B: Total number of Responses (i.e., completed trials), Rewards, and Total Errors, to reach criteria. C: Number of Sessions required to reach criteria.

Table 6. ANOVA Results for Attentional Set-Shifting Variables

Cohort	Variable	F	Sig.
Short Abstinence	Trials	0.497	0.686
	Omissions	0.822	0.488
	Responses	0.645	0.590
	Rewards	0.603	0.616
	Total Errors	0.708	0.552
	Perseverative Errors	1.505	0.224
	Regressive Errors	0.170	0.916
	Never Reinforced Errors	0.450	0.718
	Shift Sessions	0.526	0.666
Prolonged Abstinence	Trials	1.514	0.222
	Omissions	1.205	0.317
	Responses	1.574	0.207
	Rewards	1.332	0.274
	Total Errors	1.889	0.143
	Perseverative Errors	1.089	0.362
	Regressive Errors	0.918	0.439
	Never Reinforced Errors	1.568	0.208
	Shift Sessions	0.653	0.585

Considering the *a priori* hypothesis that drug conditions would be different from Controls for individual parts of the behavioral flexibility testing we also performed planed pairwise comparison analyses. We postulated that SAM use would produce deficits for both Reversal Learning and Attentional Set-Shifting relative to Controls after both a Short and Prolonged Abstinence period. Although the pairwise comparison indicated that SAM mice required more

Responses (M= 86.00; t (26) = -2.320, p=.031) during the Reversal Learning Task in the Short Abstinence cohort compared to Control animals Responses (M= 60.50) to reach criteria, there were no differences for error subtypes (Table 7) or Total Errors t (26) = -1.901, p=.068. No additional differences were found between SAM animals relative to Controls for Reversal Learning in the Prolonged Abstinence cohort, nor were there any deficits for Attentional Set-Shifting in either cohort. Although we also theorized that THC administration would impair Reversal Learning, and alcohol administration would produce deficits for Attentional Set-Shifting, following a Short Abstinence period, no impairments were observed for these drug conditions in the pairwise comparisons

Table 7. Attentional Set-Shifting Error Subtypes Means and SEMs.

Cohort	Group	Variable	Mean	SEM
Short Abstinence	Control	Perseverative	29.14	9.00
		Regressive	60.21	12.28
		Never Reinforced	43.64	8.40
	EtOH	Perseverative	41.54	12.87
		Regressive	40.38	12.50
		Never Reinforced	39.85	9.80
	THC	Perseverative	59.69	13.26
		Regressive	53.38	11.57
		Never Reinforced	49.31	11.11
	SAM	Perseverative	58.57	12.00
		Regressive	54.57	12.10
		Never Reinforced	44.79	8.00
Prolonged Abstinence	Control	Perseverative	74.43	23.11
		Regressive	25.64	4.37
		Never Reinforced	34.07	7.11
	EtOH	Perseverative	33.79	13.43
		Regressive	17.86	3.80
		Never Reinforced	28.57	8.31
	THC	Perseverative	56.31	14.92
		Regressive	19.13	5.22
		Never Reinforced	18.63	4.40
	SAM	Perseverative	40.47	13.34
		Regressive	33.80	9.11
		Never Reinforced	26.87	7.20

4.4 Western Blots (Aim 3)

To assess potential time-dependent changes following simultaneous THC and alcohol self-administration for CB_1 receptor expression in the dorsal striatum, we ran western blot analyses. We hypothesized that expression would be reduced following two weeks of drug administration compared to Control animals, with a return to basal levels following a brief abstinence. We also predicted that after an extended abstinence there would be an upregulation of expression in drug-exposed animals compared to Control mice, as well as the other abstinence conditions. CB_1 receptor expression was analyzed using nonparametric tests, and corrected for multiple comparisons when appropriate, as protein expression did not follow a normal distribution for the No Abstinence cohort, Drug Groups, or Sex (Table 8). There was no effect of Sex across cohorts with the Independent-Samples Mann-Whitney U test (U=4687, p=.833) nor was there an effect within cohorts (U's >359, p's>.575), subsequent analyses are collapse across sex.

Table 8. Shapiro-Wilk Tests of Normality for Normalized CB1

Variables		Statistic	df	Sig.
Cohort	Short Abstinence	0.970	55	0.189
	Prolonged Abstinence	0.968	59	0.128
	No Abstinence	0.787	78	0.000****
Group	Control	0.922	41	0.008**
	EtOH	0.900	37	0.003**
	THC	0.886	47	0.000***
	SAM	0.896	44	0.001***
	No Behavior Control	0.972	12	0.928
	B6	0.928	11	0.388
Sex	Female	0.877	98	0.000****
	Male	0.912	94	0.000****

p<.05*, p<.01**, p<.001***, p<.0001****

Figure 12 is a representative image of the western blots showing CB_1 receptor expression (upper green band; predicted molecular weight of 53 kDa) and β-actin expression (lower red band; predicted molecular weight of 45 kDa).

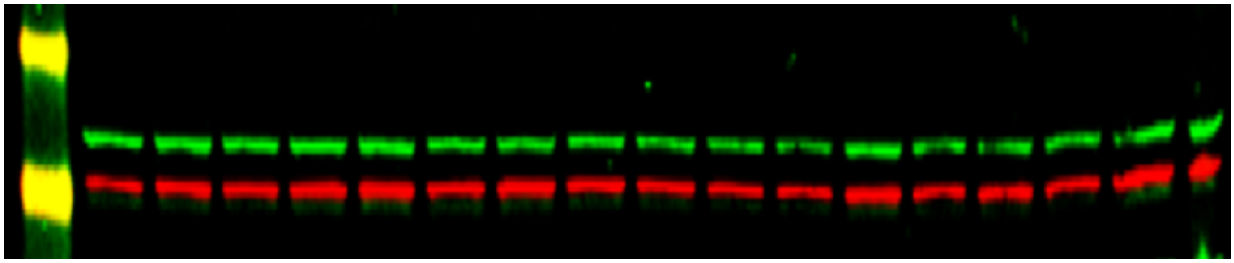


Figure 12. Representative fluorescent image of the western blots with the sample ladder (left, yellow) CB_1 (green bands) and β-actin (red bands) expression.

4.4.1 Short Abstinence Promotes Dorsal Striatal CB1 Expression

Contrary to our hypothesis, CB_1 protein expression was not elevated following a Prolonged Abstinence period relative to the Short and No Abstinence cohorts. Instead, an Independent-Samples Kruskal-Wallis test indicated that the Short Abstinence cohort had elevated CB_1 protein expression compared to the No Abstinence ($p<.0001$) and Prolonged Abstinence ($p<.0001$) cohorts (Figure 13). A simple linear regression analysis found that Total THC consumption predicted CB_1 expression ($b = .317$) and accounts for a proportion of the variance in CB_1 expression ($R^2 = .100$, $F\ (1,54) = 5.904$, $p=.019$) only within the Short Abstinence cohort. Alcohol consumption was not found to account for any variance in CB_1 expression in any cohort (Table 8).

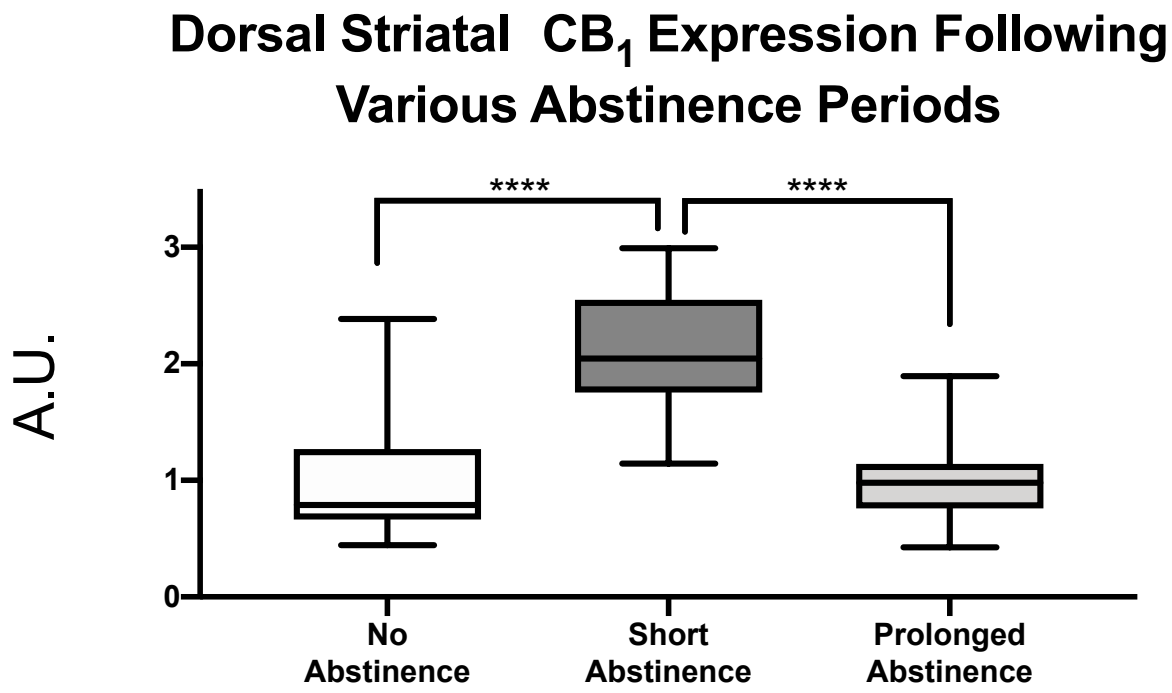


Figure 13. Normalized CB_1 expression is graphed in arbitrary units (A.U.). CB_1 expression was elevated following a Short Abstinence period compared to both No Abstinence and Prolonged Abstinence conditions. The No Abstinence and Prolonged Abstinence conditions did not differ from one another. n=192, $p<.0001$****

Table 9. Linear Regression Results of Drug Consumption Predicting Dorsal Striatal CB_1 Expression

Cohort	Predictor	Standardized Beta	R^2	F	Sig.
No Abstinence	THC	-0.209	0.044	3.487	0.066
	EtOH	-0.199	0.040	2.731	0.103
Short Abstinence	THC	0.317	0.100	5.904	0.019*
	EtOH	-.0240	0.057	3.225	0.078
Prolonged Abstinence	THC	-0.209	0.002	0.094	0.761
	EtOH	-0.110	0.012	0.697	0.407

*$p<.05$

4.4.2 Dorsal Striatal CB_1 Expression is Not Altered Immediately Following 2 Weeks of Drug Self-Administration

Within the No Abstinence cohort, we predicted reduced expression of dorsal striatal CB_1 receptors stemming from drug consumption; however, we observed no differences between drug conditions and Control mice with an Independent-Samples Kruskal-Wallis test (p=.950; Figure 14).

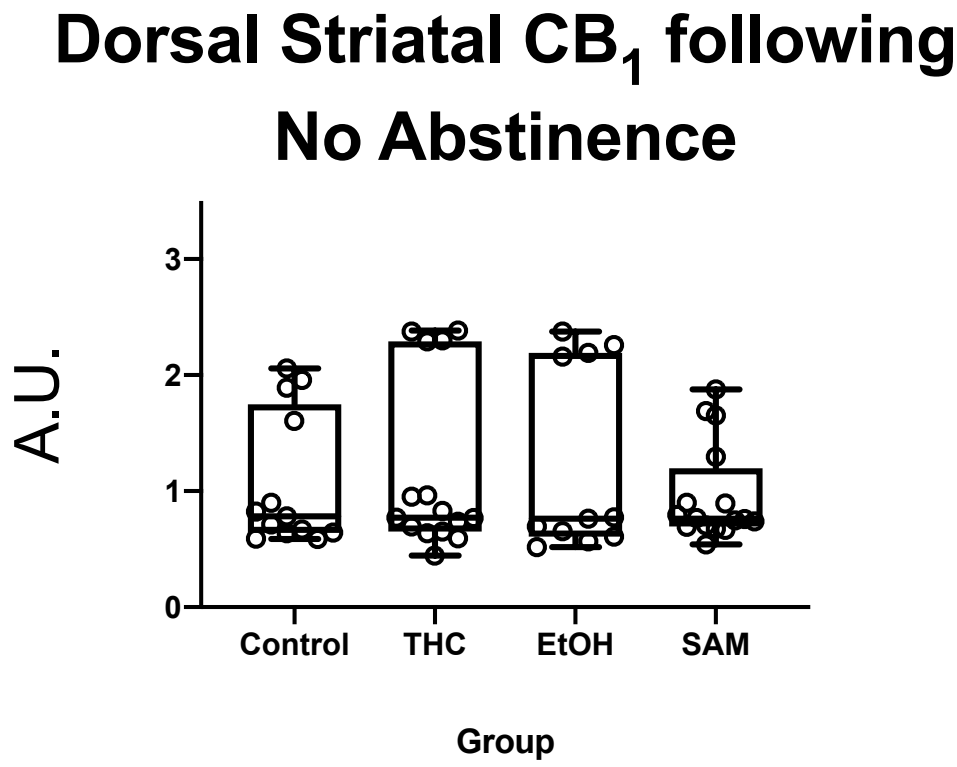


Figure 14. Normalized CB_1 expression is graphed in arbitrary units (A.U.). Drug consumption did not alter CB_1 expression compared to Control animals when assessed immediately following the drug-exposure paradigm, n=55.

4.4.3 Dorsal Striatal CB_1 Expression is Elevated Following THC Self-Administration and a Short Abstinence

Although we predicted that, following drug self-administration and a Short Abstinence period, CB_1 expression in the dorsal striatum would return to basal levels, instead we found an overall increase compared to the other abstinence periods, as well as selective increase based on the type of drug consumed. An Independent-Samples Kruskal-Wallis test indicated that CB_1 protein expression for THC animals was elevated within the Short Abstinence cohort compared

to EtOH mice (p=.01 Bonferroni corrected; Figure 15). Although initial assessment indicated the THC group was also higher than Control animals (p=.032), this difference was not significant following Bonferroni correction for multiple comparisons (p=.192). Interestingly, alcohol self-administration before a brief abstinence period may block the upregulation resulting from the Reversal Learning and Attentional Set-Shifting tasks or THC administration, as the EtOH group had the lowest mean of normalized CB_1 receptor expression. Potentially the lack of effects observed between the SAM group and other conditions may be explained by inverse effects of alcohol and THC on CB_1 receptor expression in the dorsal striatum.

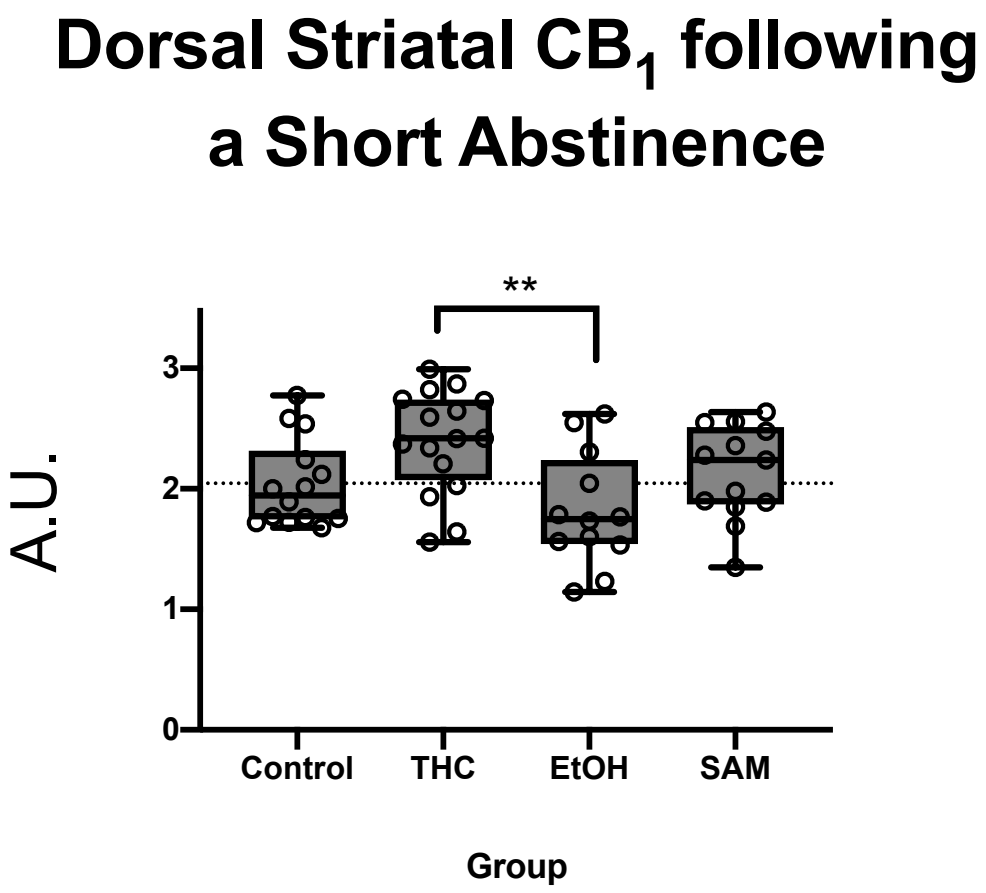


Figure 15. Normalized CB_1 expression is graphed in arbitrary units (A.U.), the dotted line represents the grand median. CB_1 expression was elevated following THC consumption compared to animals that consumed EtOH (p=.01). p<.01**

4.4.4 Dorsal Striatal CB_1 Expression is Not Upregulated Following Prolonged Abstinence

We hypothesized that following an extended abstinence period CB_1 receptor expression would be upregulated in animals with the previous drug-exposure. However, we failed to see a drug-induced difference compared to Controls at this timepoint (Independent-Samples Kruskal-Wallis test p=.300; Figure 16).

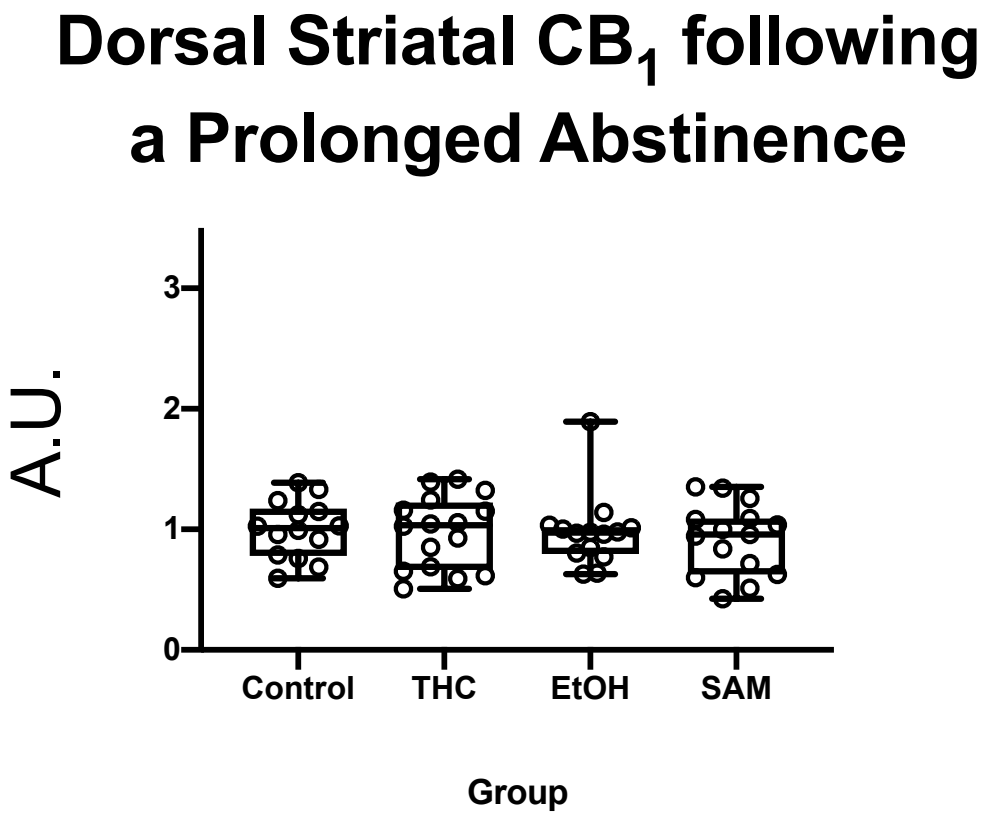


Figure 16. Normalized CB_1 expression is graphed in arbitrary units (A.U.). CB_1 receptor expression was not upregulated in Drug Groups compared to Controls following a Prolonged Abstinence.

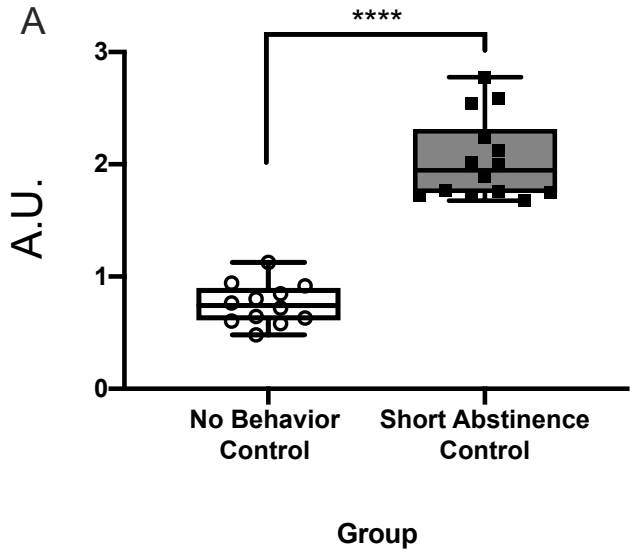
A comparison of Control cHAPs that underwent behavioral training and testing in the shortest succession of these cohorts to the No Behavioral Control cHAPs with no operant training indicates that behavioral flexibility paradigms alone may upregulate CB_1 receptor expression in the dorsal striatum under certain conditions ($U = 0$, p=<.0001; Figure 17). This finding could explain why THC and SAM groups did not differ from Controls in the Short Abstinence cohort for CB_1 receptor expression.

Additionally, B6 mice show elevated levels of dorsal striatal CB_1 expression compared to cHAPs, who were not exposed to either the drug consumption paradigm or any operant training and behavioral testing (U =17, p =.0017, Figure 17). This shows that the cHAPs innately have lower levels of CB_1 expression than a commonly used strain of alcohol-consuming mice, in a region important for many alcohol-related behaviors.

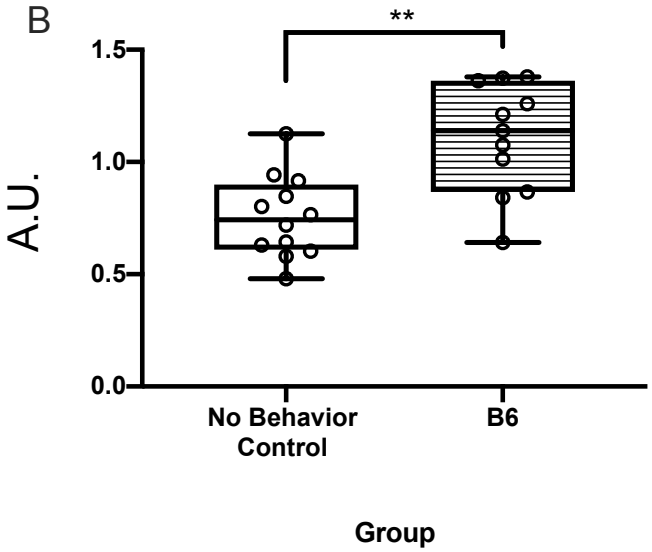
In addition to the nonparametric tests, we used a log10 transformation in an attempt to normalize data, however this transformation failed to normalize the No Abstinence cohort. Subsequently we retained nonparametric analysis for consistency, but Group x Sex ANOVAs for the Short Abstinence and Prolonged Abstinence confirmed the reported nonparametric findings. As stated, there was a main effect of group due to the difference between EtOH and THC in the Short Abstinence cohort (F [3,54] =4.402, p=.006), but no effect of Sex (F [1,54] =.038, p=.847), or Group* Sex interaction (F [3,54] =.636, p=.596). Nor was there any effect of Group (F [3,58] =.258, p=.855) or Sex (F [1,58] =.023, p=.881) or Group*Sex interaction (F [3,58] =1.280, p=.291) in the Prolonged Abstinence cohort.

Figure 17. (Displayed on the following page) Normalized CB_1 expression is graphed in arbitrary units (A.U.). A: Mice in the Short Abstinence cohort have an upregulation of CB_1 levels compared to behaviorally naïve Controls. B: cHAP mice have lower levels of dorsal striatal CB_1 compared to B6 mice. $^{**}p<.01$, $^{****}p<.0001$

Control cHAPs
Dorsal Striatal CB_1 Expression



No Behavior
Dorsal Striatal CB_1 Expression



CHAPTER 5 DISCUSSION

5.1 General Discussion

In this study, drug consumption was altered by simultaneous access to multiple drugs, and differential effects were modulated by sex. Female cHAP mice consistently consumed more THC than their male counterparts. Furthermore, alcohol self-administration reduced THC consumption in female mice, but simultaneous access to THC and alcohol did not alter alcohol intake. Males SAM animals may be more sensitive to alcohol than female SAM mice following high doses of THC, as they reduced alcohol consumption compared to other Drug Groups on several days of the 2-week self-administration period. Behavioral flexibility was largely unaffected following drug self-administration, although a minor impairment was found in SAM animals compared to Controls for Reversal Learning following a pairwise comparison. Dorsal striatal CB_1 expression is upregulated following a Short Abstinence period compared to No abstinence and Prolonged Abstinence assessments. Within the Short Abstinence cohort, THC appears to drive an increase in CB_1 expression relative to alcohol administration. Interestingly, behavioral flexibility tasks may increase CB_1 levels but alcohol administration could block this effect. Comparatively, this selectively bred line has lower basal dorsal striatal CB_1 expression than B6 mice.

5.2 Simultaneous Use Alters Drug Consumption Relative to Intake of a Singular Drug

Using 2BC and an edible model of THC administration, this study found that cHAPs readily consume THC and will simultaneously self-administer THC and alcohol in large quantities. Although we speculated that a genetic predisposition to drink alcohol could be a predictive factor for individuals who prefer the simultaneous use of marijuana and alcohol, in this animal model, SAM use did not result in an escalation of alcohol or THC intake. There are several considerations as to why the proposed drug escalation, such as that reported in human populations, was absent in the present study.

Notably, it is possible that in animals, the combination of THC and alcohol does not produce a synergistic effect that lends itself to elevated drug use. Alternatively, although this

model was termed “simultaneous” SAM animals received edible THC separately from alcohol due to experimental constraints. Therefore, although mice returned to alcohol consumption within an hour of THC consumption, possibly both substances need to be present at the same time and not just in overlapping exposures for SAM escalation to occur. However, as SAM animals continued to consume alcohol after THC administration, it is also possible that an increase in substance use may center around THC administration but is not robust enough to produce a cumulative effect. A more in-depth study examining alcohol consumption periodically following THC administration could elucidate this possibility.

Unexpectedly, THC consumption was reduced for female mice with simultaneous access to alcohol. Although we originally discussed the idea of a substitution effect reducing alcohol intake, our model indicates that this effect may also be applicable in terms of reducing high levels of marijuana consumption. Furthermore, there may be divergent substitution effects between the sexes considering the decrease in THC consumption for female SAM animals and reduced alcohol intake for male SAM animals following higher THC concentrations.

Similar to alcohol, many animals consuming THC showed day-to-day fluctuations over the twelve days of dough administration. It appears that animals titrate their use to achieve preferred levels of intoxication. Another potential explanation for a difference between these findings and those presents in the clinical literature surrounding heightened drug consumption is that simultaneous oral drug use is affecting the mouse metabolism of the individual drugs, such that lower levels of these drugs are needed to produce sustained effects. This, however, may be unlikely considering Siemens & Doyle (1979) found no modification of alcohol disappearance following 10.1mg/kg THC administered intragastrically by gavage and alcohol elimination is unaltered following THC injections in rats (Sprague & Craigmill, 1976).

Access to alcohol reduced female consumption of THC but, conversely, THC access did not alter alcohol intake. Although it was proposed that SAM use would increase alcohol consumption relative to alcohol alone, another consideration for why the hypothesized effect was not observed is that alcohol consumption in these animals may be approaching a ceiling effect; as cHAPs were consistently consuming over 25 g/kg of alcohol per day. This intake is largely occurring over the twelve hours of the dark portion of the light/dark cycle. Therefore, it may be difficult for animals to surpass these already high levels of sustained alcohol intake. The clinical studies citing increases in alcohol consumption during SAM use have been conducted in more

generalized populations. Individuals in these studies were likely not consuming alcohol in the quantity or frequency that individuals with AUD do, which may explain the increases in alcohol consumption during SAM use. Research exploring individuals with a predisposition towards AUD, as modeled by the cHAPs, as well as diagnosed AUD subjects are necessary to better understand how SAM use may alter drug consumption in different populations.

5.3 Behavioral Flexibility is Largely Unaffected Following Drug Self-Administration

As we expected, SAM animals required more Responses to reach criteria compared to Control mice for the Reversal Learning task, following a Short Abstinence period. However, this deficit compared to Controls was only present following a pairwise comparison. Additionally it is minor impairment as there was no significant effect of SAM drug consumption on the number or type of errors committed. We failed to observe the theorized deficit that SAM animals would show greater impairment for both Reversal Learning and Attentional Set-Shifting following a Prolonged Abstinence period. Opposing our predictions, SAM animals also were not more impaired than animals consuming either alcohol or THC alone for any measure of behavioral flexibility. Previous research discovering deficits in Reversal Learning has predominately focused on acute systemic THC or CB_1 agonists administration (Egerton, Brett, & Pratt, 2005; Gomes, Guimarães, & Grace, 2015; Hill et al., 2006) whereas repeated edible THC has failed to produce behavioral flexibility impairments (Nelson et al., 2018). Considering our lack of findings, in conjunction with those of Nelson and colleagues (2018), it is possible that either repeated edible administration of THC does not produce behavioral flexibility impairments, or that THC must be acutely administered to impair responding in rodents. Another possibility as to why we failed to observe the hypothesized effects of THC on Reversal Learning could be that the 10 mg/kg dose used for the majority of self-administration is not high enough to produce cognitive impairments. Although notably, hypo-locomotor effects have been observed at a dose of 5 mg/kg THC in C57BL/6J (B6) mice bred (Smoker et al., 2018) indicating these doses are adequate to produce some behavioral changes in another mouse strain.

A combination of drug and stress effects from systemic injections also may contribute to the divergent findings between other studies and those using an edible model of THC administration. It is also possible that only certain measures of behavioral flexibility such as

cross maze paradigms are capable of identifying deficiencies following THC. Although the operant procedure employed in our study utilizes the same egocentric discriminations used in cross maze studies, the encumbrance of physical distance mice must travel in maze studies is likely much higher than what is required for operant testing.

Contrary to our hypothesis there were also no observed deficits following alcohol administration for Attentional Set-Shifting. Although this finding conflicts with most of the literature that finds impairments in Attentional Set-Shifting following alcohol administration (Gass et al., 2014; Hu, et al., 2015, Kroener et al., 2012; Trantham-Davidson et al., 2014), it bolsters our previous findings that 2BC does not produce deficits in Attentional Set-Shifting, although that study used the inverse Attentional Set and Shift than what was implemented in this paradigm (Millie et al., 2020). Here, even though we utilized what can be considered a more difficult shift, we again observed no effect of 2BC. These findings highlight the possibility that other paradigms of alcohol administration (e.g., CIE vapor) may produce deficits due to a combination of stress, withdrawal cycles, and drug administration, rather than solely as a result of drug effects (Heilig, Egli, Crabbe & Becker, 2010; Maldonado-Devincci et., 2016). Alternatively, although alcohol consumption in two weeks of 2BC in cHAPs meets or surpasses levels achieved in CIE vapor paradigms used to assess behavioral flexibility, the selectively bred lines may be in some ways less susceptible to negative consequences resulting from these high intakes (Houck, Carron, Millie & Grahame, 2019). Despite reaching comparable amounts of alcohol-exposure as other studies after 2 weeks of 2BC, cHAPs may require an extended alcohol history in order to be susceptible to the negative consequences of alcohol self-administration on behavioral flexibility.

However, it is also important to consider that the operant behavioral flexibility paradigm we utilized does not produce a sufficient enough challenge to identify possible deficits in behavioral flexibility within mice. Future experiments could incorporate several methodological changes to increase the difficulties of these tasks such as including the lever stimulus lights with training to reduce salience of this cue in subsequent testing, as well as increasing the burden of responding by not allowing for omissions between correct responses.

5.4 Dorsal Striatal CB_1 Receptors

Based on the literature that demonstrates chronic alcohol reduces CB_1 receptors in the NAc (Pava & Woodward, 2012), it was predicted that dorsal striatal levels of CB_1 receptors would be reduced immediately following drug consumption compared to Control animals and would return to basal levels after a brief abstinence period. Withdrawal has been associated with an increase in endocannabinoid release and concomitant reduction in receptor expression, which results in the upregulation of CB_1 receptors to compensate for elevated endocannabinoid levels (Pava & Woodward, 2012). Therefore, it was also hypothesized that following a Prolonged Abstinence there would be an upregulation of dorsal striatal CB_1 receptors in drug-exposed animals, relative to Controls. As discussed in the methods, the normalization procedure enables us to evaluate samples across gels. However, between abstinence period conclusions should be interpreted with caution as all cohorts were not represented on every gel although there were no differences in β-actin across gels. Contrary to our hypothesis, there was no observed downregulation immediately following repeated drug consumption in animals that went through no withdrawal or abstinence period. One consideration as to why we did not find a reduction in receptor levels immediately following drug consumption, as expected, is the already low basal level of dorsal striatal CB_1 receptors. Previously, we found that HAP3's have lower levels of dorsal striatal CB_1 receptors than their low alcohol-preferring counterparts, and here we observed a reduction in expression for cHAPs relative to B6 mice (Figure 17). Consequently, a floor effect may be obscuring a significant suppression of these receptors.

There was also no upregulation observed following Prolonged Abstinence compared to Control mice or the other abstinence periods. However, this abstinence period was potentially too short to capture the hypothesized adaptation and may account for the lack of observed neurobiological changes, as the upregulation in rat brains observed by Mitrirattanakul and colleagues (2007) occurred after 40 days. Instead, there was an increase in CB_1 receptor expression in the mice following a Short Abstinence period (i.e., 7 days since their last drug exposure) compared to the No Abstinence and Prolonged Abstinence cohorts. While we postulate that the Prolonged Abstinence period used here may have been too short to capture an upregulation observed in other studies following an extended abstinence, alternatively the upregulation was captured, but unexpectedly occurred in the Short Abstinence cohort. Potentially

these animals may have more rapid upregulation compared to other rodents and subsequently return to basal levels as abstinence continues.

Surprisingly, animals who received operant training, water-only 2BC, control dough, and went through Reversal Learning and Attentional Set-Shifting tasks expressed higher levels of CB_1 receptors in the dorsal striatum compared to animals with no exposure to those experimental procedures (Figure 17). This was only true for Control animals who went through the shortest version of this experimental paradigm, as the Prolonged Abstinence Controls did not differ from the No Behavior Control group. It is possible that water deprivation could have contributed to this difference, as the Short Abstinence animals were exposed to two sequences of water deprivation whereas the No Behavior Control group-maintained ad lib water access throughout the experiment. This suggests that Reversal Learning and Attentional Set-Shifting that closely follows operant training promotes the upregulation of CB_1 receptors in the dorsal striatum.

Alcohol may block upregulation as alcohol animals had the lowest levels of expression in this cohort. Alcohol in the Short Abstinence cohort had reduced expression compared to THC animals, whereas SAM mice had lower expression than THC mice but higher than alcohol animals although not statistically divergent (Figure 15). Instead, it is also possible that THC paired with behavior alone may be responsible for the changes in receptor expression in the dorsal striatum observed here. Therefore, the reduction of CB_1 receptor expression in SAM animals compared to THC may be due to a decrease in THC consumption, a behavioral effect of simultaneous alcohol consumption, rather than a neurobiological effect of alcohol.

Naïve cHAPs have lower levels of dorsal striatal CB_1 receptors compared to B6 mice (Figure 17). Considering the numerous ways that CB_1 receptors can alter addiction-related behaviors (Onaivi, 2008; Serrano & Parsons, 2011) this finding reinforces the theory that reduced CB_1 receptor expression in cHAPs may in part account for the extremely high alcohol intake seen in this selectively bred line. This research highlights the need to evaluate not only different drug consumption patterns but various time points including different abstinence periods to fully characterize neurobiological and behavioral changes following chronic drug consumption. Future research should consider the time at which particular behaviors are assessed based on the type of drug administered as certain changes may be difficult to encapsulate.

Increases in dorsal striatal CB_1 receptor expression in cHAPs following a Short Abstinence period did not correspond to our measures of behavioral flexibility as we failed to

observe fundamental changes in Reversal Learning and Attentional Set-Shifting. Likewise, considering there was a lack of neurobiological and behavioral changes observed for both the No Abstinence and the Prolonged Abstinence cohorts we cannot draw definitive conclusions as to the involvement of CB_1 receptors in regulating behavioral flexibility assessed with an operant paradigm. Although we believed that dorsal striatal CB_1 receptors might have a vital role in regulating behavioral flexibility, the low levels present in cHAPs are sufficient to perform Reversal Learning and Attentional Set-Shifting tasks. These results indicate a minimal role for these receptors in these behaviors, although we cannot rule out compensatory mechanisms that may support behavioral flexibility in the presence of low receptor levels. However, the increase in CB_1 receptor expression observed in Control animals does indicate that CB_1 receptors can change in response to operant training and testing.

5.5 Limitations and Future Directions

One limitation of this research is the route of administration of THC. Although utilizing a self-administration paradigm with edible THC provides a translationally valid paradigm to human THC consumption and avoids potential confounding effects such as injection stress, edible models are still relatively novel. Thus, we do not fully understand the effects of continuous consumption and potential discrepancies of edible versus injected THC in the body for rodents, and how these and other potentially yet unknown factors surrounding chronic edible consumption may affect behavior. These caveats are especially important to consider as a diverse number of factors may affect behavioral paradigms that occur over several sessions or days.

Although selectively bred lines enable researchers to model the potential influence of genetics on alcohol use and addiction-related behaviors for individuals with a family history of alcoholism, they are not designed to model the general population. As such, it can be difficult to make predictions based on the literature. Drawing conclusions based on comparative research in other less-specialized mouse strains is also challenging. Although any animal model chosen innately has its limitations, through the inclusion of different strains we may begin to be able to make more direct comparisons across research paradigms. For that reason, B6 mice, a commonly used alcohol drinking strain, were included with the primary cHAP population for a basal assessment of dorsal striatal CB_1 receptor expression. The differences observed here may

contribute to the alcohol consumption disparities that have been reported between these strains (Matson & Grahame, 2013). An effort to continually compare selectively bred lines to frequently used strains would enable broader conclusions to be drawn for a variety of paradigms, especially those incorporating drug self-administration.

Overall, we failed to see cognitive effects corresponding to drug self-administration. Future studies might examine acute testing of edible THC to better understand whether THC must be actively modulating neurotransmission to produce deficits, or whether edible THC fails to produce operant behavioral flexibility deficits altogether. Evaluating edible THC effects on other paradigms of Reversal Learning such as a cross maze would also elucidate whether there is a true lack of effects and confirm these discrepancies with the majority existing literature. Additionally, higher concentrations of THC should be explored if animals are willing to freely consume increased doses; although notably, the other edible models discussed had issues with reduced consumption of 10 mg/kg doses.

Even though the length of the Prolonged Abstinence was chosen to approach timepoints assessed in the human literature (i.e., 28 days), task retention within our animals has not been systematically assessed and longer abstinence periods than the one used here may be useful for exploring behavioral and neurobiological changes. It is possible that other alterations in CB_1 receptor levels do occur with this drug-consumption model but at different times than assessed here. Distinguishing between the dorsomedial and dorsolateral striatum due to the distinct roles these regions have in behavioral flexibility, habit learning, and drug-related behaviors (Bissonette & Powell, 2012; Ragozzino, 2007; Malvaez & Wassum, 2018; Maldonado et al., 2006; Onaivi, 2008) may also prove to be informative for studying THC and SAM drug administration. This distinction would help to generate further understanding of the dorsal striatum, as the role of the dorsal striatum in drug-addiction still lags behind what is known for the ventral striatum (Lipton et al., 2019).

5.6 Implications and Conclusions

Our research indicates that tasks designed to assess behavioral flexibility can contribute to neurobiological alterations such as an increase in CB_1 receptor expression in the dorsal striatum through training and testing alone. Changes CB_1 expression in this study were not separated by

dorsomedial and dorsolateral striatum, instead, this structure was assessed as a whole. However, the alterations observed in the Short Abstinence cohort were not necessarily unilateral changes in both the dorsomedial and dorsolateral striatum. Especially as the dorsomedial striatum has been linked to Reversal Learning (Ragozzino, 2007) and the dorsolateral striatum is prominent in habitual behaviors (Malvaez & Wassum, 2018), which conceptually are similar to behavioral flexibility. Seeing as these regions likely differentially contribute to behavioral flexibility tasks in addition to habitual behaviors, alterations in one may be responsible for the generalized increase observed in the dorsal striatum. Consequently, the absence of effects within both the Prolonged and No Abstinence periods was due to the lack of the distinction made between dorsomedial and dorsolateral striatum. The examination of the dorsal striatum as a whole may have occluded inverse regulation in these areas. Evaluations distinguishing between these regions may illuminate behavioral and neurobiological differences that this experiment failed to observe. Studying multiple strains for basal levels of CB_1 receptors in the whole brain and specific regions of interest could also promote a greater understanding of the differences that are observed between animals that freely consume drugs and those that do not.

Thus far edible models have not produced deficits in Reversal Learning and therefore depart from the findings within the published literature. There may be a minimum threshold of THC necessary to assess deficits in complex behavioral assays that are above concentrations used here. This may explain why Nelson et al. (2018) failed to see any behavioral effects as they also used 10 mg/kg doses of THC as well as only male rats with low alcohol intakes compared to those achieved in this study. Some researchers have observed impaired behavioral performance for only female rodents following THC administration; however, more pronounced THC effects have been found for male mice when assessing general activity (Smoker et al., 2019) as opposed to task-specific deficits (Siemens & Doyle, 1979). The disparity in THC consumption between female and male animals underscores the importance of the inclusion of female animals in research, especially for drug investigations. The conflicting findings for THC's effect on behavioral assays within the literature implicate sex-specific effects may depend on the paradigm in which THC is being assessed.

Home cage drinking is one behavioral procedure that shows consistent sex differences. The stress from social isolation may contribute to home cage drinking differences between the sexes and an increase in drug consumption may ameliorate that stress in certain strains. Female

mice in the THC group consumed more THC than either THC males or SAM animals of either sex. The anxiolytic effects of THC may mitigate the stress from social isolation, which is more prominent in female rodents (Senst, Baimoukhametova, Sterley, & Bains, 2016). THC's ability to alleviate stress could be especially important in light of the different user populations that have been identified in the clinical literature (White et al., 2019), and given the high levels of comorbid anxiety and substance use disorders found in women (Brandy & Randall, 1999).

Animal models are crucial to developing theories surrounding the effects that exogenous cannabinoids can have on the complex endocannabinoid system and subsequently other brain systems as a result of downstream effects. Furthermore, animal models offer an opportunity to explore how the use of multiple drugs can affect everything from receptor expression to plasticity in specific regions such as the dorsal striatum, as well as in pathways like the mesolimbic dopamine system. Although there are challenges to evaluating co-use, selectively bred lines offer an opportunity to examine how self-administration of these drugs may change behavior without the introduction of additional unintended variables such as stress effects from injections or forced consumption (e.g., CIE vapor or gavage). Considering the evolving landscape surrounding simultaneous alcohol and recreational marijuana use, there is a critical need for research that furthers our understanding of not only THC but SAM effects on the brain and, by extension, behavior.

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