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# BEHAVIORAL PHARMACOLOGY OF ALCOHOL AND LEGAL PSYCHOSTIMULANTS

by

**Meridith Tracy Doyle**

**A Dissertation**

*Submitted to the Faculty of Purdue University*

*In Partial Fulfillment of the Requirements for the degree of*

**Doctor of Philosophy**

Department of Medicinal Chemistry and Molecular Pharmacology

West Lafayette, Indiana

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*To my parents,*

*for reminding me, early and often,*

*that education is everything.*

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# LIST OF COMMON ABBREVIATIONS

| | |
|---|---|
| A1R | Adenosine receptor A1 |
| A2AR | Adenosine receptor 2A |
| AMPA | α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid |
| AUC | Area under the curve |
| AUD | Alcohol use disorder |
| Barr1 | β-arrestin 1 |
| Barr1 | β-arrestin 2 |
| cAMP | Cyclic adenosine monophosphate |
| CNO | clozapine-N-oxide |
| CNS | Central nervous system |
| CPP | Conditioned place preference |
| D1R | Dopamine receptor D1 |
| D2R | Dopamine receptor D2 |
| DAT | Dopamine transporter |
| DID | Drinking-in-the-dark |
| DMSO | Dimethyl sulfoxide |
| DOR | Delta-opioid receptor |
| DPDPE | [D-Pen$^{2,5}$]Enkephalin |
| DREADD | Designer Receptors Exclusively Activated by Designer Drugs |
| EPH | Ethylphenidate |
| GABA | γ-aminobutyric acid |
| GFP | Green fluorescent protein |
| GPCR | G protein-coupled receptor |
| $hM_4Di$ | Human muscarinic $M_4$ inhibitory DREADD |
| HET | Heterozygous |
| i.p. | Intraperitoneal |
| KO | Knockout |
| KOR | Kappa-opioid receptor |
| LTD | Long-term depression |

| | |
|---|---|
| LTP | Long-term potentiation |
| MOR | Mu-opioid receptor |
| MPH | Methylphenidate |
| MSN | Medium spiny neuron |
| NET | Norephinephrine transporter |
| NMDA | N-Methyl-D-aspartic acid |
| o.g. | Oral gavage |
| SERT | Serotonin transporter |
| WT | Wild-type |
| VTA | Ventral tegmental area |

# ABSTRACT

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Substance abuse, including alcohol and psychostimulant abuse, is a widespread and dangerous public health issue. In the United States, 8-10% of people 12 years of age or older (accounting for 20-22 million persons) are addicted to alcohol or other drugs, and the results of substance abuse are costly at both the individual and society level. Despite the large financial burden of substance abuse to society, efficacious psychosocial and pharmacologic treatment options are lacking. For example, in the pharmacologic treatment of alcohol use disorders (AUD), only three drugs have been approved by the Food and Drug Administration, and each have their own limitations that restrict efficacy and recovery outcomes. Here, the behavioral pharmacology of alcohol and psychostimulants is investigated using a variety of *in vitro* and *in vivo* techniques to better develop treatment options for AUD and to further our basic understanding of adolescent psychostimulant use. Overall, these studies provide significant progress towards the development of novel, functionally selective delta-opioid therapeutics for alcohol use disorder and also help elucidate the potential aversive behavioral outcomes of adolescent psychostimulant use.

# CHAPTER 1. INTRODUCTION

Drug abuse, both of legal and illegal substances, remains an important health issue, as noted by the significant $740 billion total annual cost of substance abuse in the United States [1]. Despite this, pharmacologic treatment options are limited for treating addiction to the majority of drugs of abuse. Furthermore, for substance use disorders with medications available, compliance remains an ongoing issue (especially in individuals with comorbid mental health issues) [2]. Here, I present my findings investigating novel treatments for alcohol use disorders and drug-associated behaviors of repeated legal psychostimulant use in adolescence.

## 1.1 Alcohol use disorder (AUD)

### 1.1.1 Etiology and prevalence

Alcohol use disorder (AUD) is defined as a chronic relapsing brain disease characterized by compulsive alcohol use, loss of control of alcohol intake, and negative emotional state when not using alcohol [3]. Diagnosis of AUD requires the presence of at least 2 out of 11 symptoms associated with AUD behaviors [4], and diagnosis of AUD can be classified as mild, moderate, or severe depending on the number of alcohol-related behaviors present. In 2017, approximately 20.2 million adults aged 18 or older were diagnosed with a substance use disorder. Of all individuals diagnosed with a substance use disorder (including AUD and illicit drug use disorder), a 2013 study found that 4 out of 5 these adults were diagnosed with AUD, as compared with 3 out of 10 diagnosed with an illicit drug use disorder [5]. Moreover, AUD has a 29.1% lifetime prevalence [6] and only a 35-40% long-term remission rate [7]. Previously, AUD prevalence in men was higher than prevalence in women, although recent reports suggest that this gender gap is decreasing as the number of women diagnosed with AUD is increasing [8, 9]. The magnitude of the number of individuals diagnosed with AUD is reflected by the cost of AUD to society; the total cost of excessive drinking was estimated to be $223.5 billion in 2006 (with the majority of cost attributed to binge drinking) [10]. Yet, the high cost at both an individual and societal level does not correlate with a large number of treatment options available for patients - for pharmacological intervention, only three medication options exist and each are associated with limited efficacy [11].

### 1.1.2 Current treatment options

In the United States, AUD is commonly treated with psychosocial therapy (including motivational enhancement therapy, cognitive-behavioral therapy, brief interventions, and Alcoholics Anonymous) while pharmacotherapy is less available [11]. Psychosocial therapies have proven effective in patients with alcohol use disorder and are essential components of a comprehensive treatment program as they promote behavioral changes necessary for remission [12]. Importantly, all aforementioned psychosocial methods are most efficacious in treating alcoholism (reducing frequency and intensity of drinking) when used in combination with pharmacotherapy [12, 13]. For pharmacotherapy, three FDA approved medications are available for AUD treatment: disulfiram, acramprosate, and naltrexone (as an oral or extended release injectable formulation).

In 1951, disulfiram (Antabuse®) became the first FDA approved drug for the treatment of AUD [14]. In contrast to acamprosate and naltrexone – whose mechanism of action involve modulating neurotransmitter systems – disulfiram enhances the negative and punishing effects of alcohol consumption by inhibiting the enzyme acetaldehyde dehydrogenase. As a result, alcohol metabolism is altered and acetaldehyde (a neurotoxic chemical responsible for disulfiram-associated neuropathy [15, 16]) levels increase in the blood, leading to the negative symptoms associated with alcohol intake such as skin flushing, tachycardia, and vomiting [17, 18]. Yet, patient adherence is required for disulfiram to be effective [19], and typically this is low [20]. Additionally, conflicting reports exist on the efficacy of disulfiram (as compared with placebo) in treating AUD patients. A 1986 study involving 605 men found that there were no significant differences in total abstinence, time to first drink, employment, and social stability between those assigned to placebo, 1 mg disulfiram, or 250 mg disulfiram [21, 22].

Despite its FDA approval in 2004, the exact molecular mechanism of action of acamprosate (Campral®) has yet to be elucidated. Studies have shown that acamprosate acts by inhibiting the metabotropic glutamate receptor 5 (mGluR5) which thereby decreases glutamatergic hyperactivity observed upon chronic alcohol use [23]. Furthermore, acamprosate binds to sites on NMDA receptors which may also help regulate the imbalance between upregulated excitatory glutamate systems and downregulated inhibitory GABA systems observed in AUD [24, 25]. Additional research has shown that the calcium ion of the calcium salt formulation of acamprosate (calcium-bis (N-acetylhomotaurinate)) may indeed be the active moiety of acamprosate responsible for altering drinking behavior, as shown by the lack of efficacy of sodium salt formulations of

acamprosate to reduce alcohol consumption in rodent drinking models [26]. Overall, the efficacy of acamprosate in reducing alcohol intake in AUD patients is controversial. A 2006 wide-scale study investigating the efficacy of acamprosate found that acamprosate was ineffective in reducing drinking compared with placebo [13].

In the same study, the medication naltrexone (ReVia®, Vivitrol®) was found to be most efficacious in treating AUD, particularly when administered in combination with medical management (defined as intervention sessions focusing on medication adherence and abstinence in a primary care setting) [13]. Naltrexone, a nonspecific opioid antagonist, was approved for AUD treatment by the FDA in 2006 and decreases alcohol drinking by inhibiting the rewarding effects of alcohol intake. Upon alcohol ingestion, the endogenous opioid ligand endorphin is released and acts as an agonist at mu- and delta-opioid receptors on GABAergic neurons in reward areas of the brain (including - but not limited to - the ventral tegmental area, VTA), thus resulting in neuronal disinhibition and increased dopamine release onto the nucleus accumbens, amygdala, and forebrain [17]. As an antagonist, naltrexone blocks endogenous endorphin activity and consequently decreases the rewarding effects of alcohol (and also reduces alcohol craving). Patients prescribed naltrexone report decreases in the number of heavy drinking days, increases in abstinent days, and a delay in relapse to heavy drinking [13, 27, 28]. However, despite these positive behavioral outcomes, patients prescribed naltrexone exhibit poor adherence [2, 29-31], which is sometimes combated through the use of a sustained-release naltrexone injectable rather than oral administration.

#### 1.1.3 Brain regions involved

Unlike many drugs of abuse, alcohol has no principal receptor target or cellular mechanism, but instead can interact with many cellular targets and functions. This promiscuity has generated a large body of literature on the effects of alcohol on the body, thus resulting in a variety of theories and mechanism. It is accepted that alcohol activates the reward system of the brain similarly to many drugs of abuse [32] through its actions as a positive allosteric modulator for $GABA_A$ [33] and by acute inhibition of NMDA-activated ion currents [34]. This increased GABAergic activity increases neuronal inhibition, specifically in neuronal pathways regulating drug reward, thus leading to an overall increase in dopamine release in the mesolimbic reward pathway [35, 36]. Increased dopamine release in the mesolimbic reward pathway is associated with the initial,

hedonistic effects of alcohol [37, 38]. Conversely, chronic alcohol use is not associated with increased dopaminergic activity in the mesolimbic reward pathway, yet consumption continues, leading to physiological and psychological tolerance [37, 39-41]. This increased alcohol intake despite a decrease from the initial rewarding effects of alcohol is characteristic of alcohol-dependent individuals [40], and can be associated with changes in neuronal plasticity in both the dorsal and ventral striatal regions of the brain [42-47], which is more finely detailed in Chapters 3 and 4.

## 1.2 Legal stimulant use and abuse

### 1.2.1 Etiology and prevalence

Psychostimulants are a drug class that include legal psychoactive agents (such as caffeine, nicotine), non-scheduled, novel psychoactive drugs, and illegal drugs (such as methamphetamine and cocaine) that stimulate the central nervous system. Caffeine is the most widely used psychoactive substance in the world [48, 49], with approximately 85% of adult in the United States consuming 180 mg caffeine daily (the caffeine content of up to 2 cups of coffee) [50, 51]. The DSM-5 acknowledges both caffeine use disorder and caffeine withdrawal disorder [4, 52]. Caffeine withdrawal disorder is defined as headaches, difficulty concentration, fatigue, nausea, flu-like symptoms, and mood changes upon cessation of caffeine intake [49, 53]. For caffeine use disorder, this diagnosis requires the following three criteria met: 1) persistent desire or unsuccessful effort to control caffeine use, 2) “use despite harm,” and 3) withdrawal upon caffeine intake cessation [54, 55]. Overall, the vast majority of caffeine users do not abuse caffeine [50, 51], although recently concerns over the use of caffeine simultaneously with other psychoactive substances, such as alcohol, have led to the removal of pre-mixed alcohol-energy drinks from United States markets [49]. A number of human [56-58] and animal models [59, 60] have attempted to address these potential issues associated with caffeine-mixed alcohol intake.

A variety of novel, unscheduled (and thus legal) psychoactive substance have emerged since 2008, including novel synthetic cathinones (often referred to as “bath salts”) [61] and methylphenidate-based designer drugs [62, 63]. Cathinones will not be the focus of this dissertation, but for comprehensive review on this class of psychostimulants, see German et al [64]. In Chapter 8, I will focus on a specific derivative of the psychostimulant methylphenidate (MPH,

Ritalin®, commonly prescribed for attention-deficit/hyperactivity disorder and narcolepsy), known as ethylphenidate (EPH), which has recently gained popularity as an alternative to MPH for cognitive enhancement, increased alertness, and reward (Figure 1-1) [62, 65].

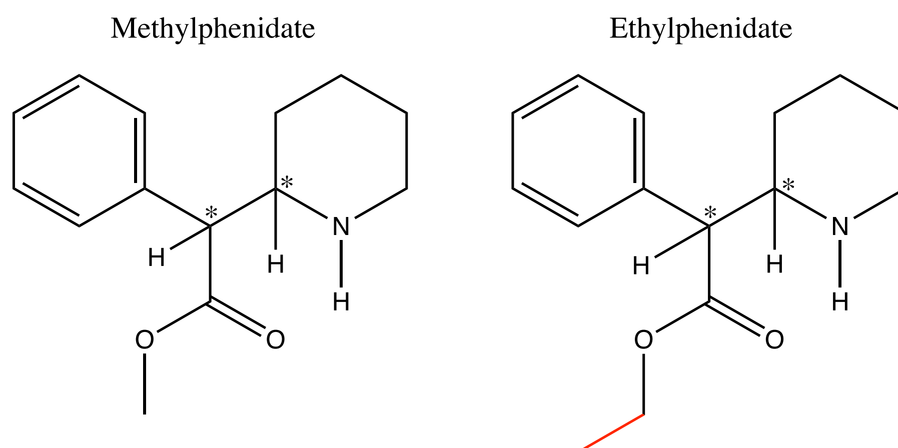



Figure 1-1. Structural similarity between methylphenidate and ethylphenidate.
Structural differences in alkyl group shown in red between methylphenidate (left) and ethylphenidate (right), stereocenters shown by asterisks, *.

### 1.2.2 Current treatment options

No treatment options are available for caffeine withdrawal [52], in part because symptoms are not highly debilitating and generally dissipate within 2-9 days following abstinence. Additionally, over-the-counter, non-steroid anti-inflammatory drugs can provide temporary pain relief associated with headaches during this time. Yet, with the exception of nicotine, for most other psychostimulants (such as cocaine), overdose and withdrawal treatment options are limited. This presents an issue for treating overdose toxicity in patients who have consumed novel psychoactive substances, as the uncertainty of the exact pharmacology of these substances adds an additional barrier to treatment care. Instead, patient stabilization and supportive care is administered [66].

### 1.2.3 Brain regions involved

Psychostimulants, such as caffeine and novel methylphenidate-based derivatives, exert their rewarding and stimulating behavioral effects by increasing dopamine signaling through either antagonism of striatal adenosine receptors [67-69] or blockade of the dopamine transporter (DAT) [70, 71], leading to increased dopamine activity in brain regions associated with reward and locomotor activity [32]. While the outcomes on dopaminergic signaling are similar for both

caffeine and methylphenidate-based drugs, the exact mechanisms in doing so are pharmacologically distinct (Figure 1-2).

Caffeine is a small methylxanthine alkaloid that exhibits its pharmacological actions primarily through antagonism of adenosine $A_1$ and $A_{2A}$ receptors (more discussion on adenosine receptors in Section 1.4.3) [72]. Expression of the $A_{2A}$ receptor is required for caffeine's positive effects on wakefulness, as $A_{2A}$ receptor knockout animals do not display hyperlocomotion upon caffeine administration [73]. Moreover, adenosine $A_{2A}$ receptors may engage in protein-protein interactions to form so-called heterodimers with postsynaptic dopamine $D_2$ receptors [74-76], where antagonism of $A_{2A}$ receptor by caffeine may alter striatal dopamine $D_2$ receptor expression or enhance $D_2$ receptor affinity for dopamine [77]. As for methylphenidate-based novel psychoactive substances, pharmacological activity is primarily restricted to monoamine transporters rather than receptors [62]. For example, racemic novel psychoactive substance ethylphenidate exhibits greater selectivity than cocaine for DAT relative to the norepinephrine transporter (NET) and significantly increases locomotor activity as compared with vehicle [62, 78]. The rewarding effects of a number of these methylphenidate-based substances have yet to be determined, although the higher DAT and NET inhibition affinity compared with serotonin transporter (SERT) would suggest increased abuse liability [79], as the potency of DAT and NET inhibition is correlated with psychotropic effective doses of psychostimulants in humans [80]. Furthermore, these methylphenidate-based novel psychostimulants have been associated with prolonged mental health effects [81] and death [82].

## 1.3 Use of animal models to study drug behaviors

We can infer certain behavioral qualities a drug may possess based upon its *in vitro* pharmacology; however, *in vivo* behavioral pharmacology allows us to understand how acute and chronic drug use truly influences behavior and how drug behavior may change depending on the age or sex of the subject. Animal models, such as rodent or non-human primate studies, allow us to study the effects of drug administration in a controlled environment with experimenter-set manipulations, such as exposure timeline, route/location of administration, and age or sex of subjects during drug testing. As the majority of human clinical trials are only conducted in adults, the ability to work with different developmental ages in animal models provides important insights into how drug behaviors may vary across life stages. Importantly, a number of these unique developmental

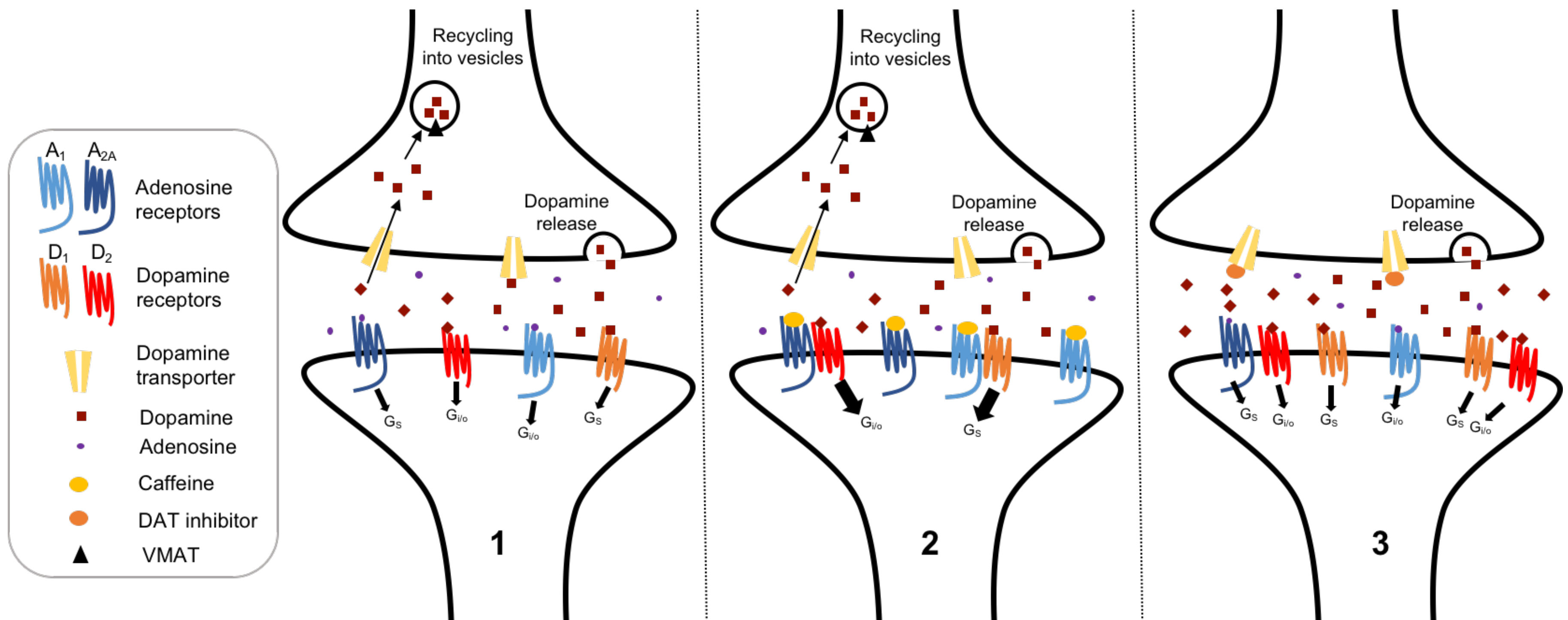



Figure 1-2. Post-synaptic signaling upon caffeine exposure or DAT inhibition.

Dopamine is released into the synaptic cleft through monoamine vesicle fusion with the presynaptic membrane, where dopamine molecules can then activate postsynaptic dopamine receptors ($D_1$, orange, $D_2$, red) or be recycled back through the presynaptic dopamine transporter (DAT) for re-packing in vesicles through vesicular monoamine transporters (VMAT2, specifically). Endogenous adenosine signaling at adenosine receptors is shown ($A_1$, light blue, $A_{2A}$, dark blue) (1). In panel (2), caffeine acts as a nonspecific adenosine receptor antagonist, inhibiting $A_1$ and $A_{2A}$ signaling and leading to the formation of adenosine-dopamine heterodimers which may potentially increase the potency of dopamine for dopamine receptors, leading to increased dopamine receptor signaling. In panel (3), DAT inhibitors such as ethylphenidate prevent the recycling of dopamine back into the presynaptic synapse, thereby increasing extracellular dopamine and resulting in an increase and/or sustained postsynaptic dopamine receptor signaling. Presynaptically expressed dopamine and adenosine receptors omitted for visual clarity.

behaviors are in fact conserved across species [83]. Another benefit of animal model testing is the ability to genetically modify animals, which allows researchers to perform more precise mechanistic studies on specific proteins, receptors, or circuitry which may contribute to drug-related behaviors.

### 1.3.1 Common animal models in drug abuse research

In utilizing animal models, behavior models must offer validity. Three different types of validity exist for animal models: predictive validity (ability to use animal model to identify new therapies for disease found in humans), face validity (similarity in outward appearance/phenotyope between an animal model and the modeled disease in humans), or construct validity (theoretical rationale of disease mechanism) [84]. While all three types of validity are desirable for a single model of behavior, this is not always feasible. Frequently, multiple complimentary animal models are required to model different aspects of a single mental disorder.

For drugs of abuse, the acute locomotor effects of a drug are commonly measured to classify a drug as either stimulating or sedative [85, 86]. Furthermore, locomotor activity can be measured following repeated drug administration, where increased locomotor activity upon each drug administration is termed "sensitization." This sensitization can be indicative of a drug's abuse liability as the appearance of locomotor sensitivity suggests alterations of neuronal networks have occurred [87, 88]. To measure the rewarding or aversive properties of a drug, conditioned place preference (CPP), a Pavlovian conditioning technique where an animal associates an unconditioned stimulus (drug) with a distinct environment (positive conditioned stimulus) [89], is frequently used. Following drug conditioning, an animal that spends more time in the drug-paired compartment after conditioning compared with before conditioning is interpreted to have found the drug rewarding. Conversely, an animal that spends more time in the drug-unpaired compartment following training is interpreted to have found the drug aversive. One significant criticism of CPP is that the animal does not administer the drug, but rather, the experimenter administers the drug to the animal involuntarily. Thus, behavioral assays for animal voluntary reward consumption (such as the two-bottle choice, drinking-in-the-dark model [90]) or animal self-administration protocols can measure volitional drug-taking in rodents [91]. These voluntary drug-taking assays may offer more face and predictive validity to human drug-taking behavior,

although limitations of interspecies differences in taste (intake studies) and metabolism (self-administration) can be problematic.

Regardless of protocol, significant limitations do exist in animal models and models can fail when complex behaviors, such as drug abuse, are oversimplified through experimental manipulation. For example, animals undergoing behavioral observation may be subjected to isolated housing, which can heavily alter drug intake behavior as this single housing removes the social aspect of drug addiction [92]. Additionally, recorded behaviors may be confounded by multiple physiological and/or mental states (for example, anxiety-like behavior being interpreted in the presence of hyperlocomotion [85]).

### 1.3.2 Neuronal plasticity in adolescence and adulthood

Adolescence is a time of unique neurochemical development, as highlighted by increased synaptic pruning [93, 94], alterations in receptor expression [95, 96], and increased susceptibility to drug abuse [97-99]. While adolescence is not generally classified as a "sensitive period" of development (as compared with early development, which is marked by a number of sensitive periods to behaviors, i.e. language development [97]), increased autonomy during adolescence allows individuals in this age group to actively choose and engage with different environmental stimuli. Behaviorally, this is a time marked by increased impulsivity and risk-taking [100] where experimentation with alcohol and other drugs is frequently observed [101] because of increased time spent with peers rather than family members [102]. These adolescent behaviors observed in humans are also observed in adolescent animals [103], where rodents experience increased social interaction and increased risk-taking during this age [104-106].

In humans, alcohol is the most widely consumed substance of abuse during adolescence [107]. Heavy adolescent alcohol use has been implicated in deficits in attention and information processing, memory, spatial functioning, and executive functioning [108] together with decreased volume in the hippocampus, prefrontal cortex, and cerebellum [109, 110]. Similarly, in rodent models, studies have shown that early-adolescent rats consume more 10% ethanol during adolescence than in adulthood [111] and exhibit inhibition of hippocampal neurogenesis following adolescent alcohol exposure [112]. In mice, deficits in reversal learning - as well loss of cholinergic neurons and forebrain structure - are observed following adolescent alcohol exposure [113]. Aside from alcohol alone, many adolescents also report consuming alcohol with psychostimulants, such

as caffeine or methylphenidate, to maintain alertness while counteracting alcohol-induced sedation [114, 115]. A survey of undergraduate students in 2002 found that approximately 20% of students that used prescription stimulants consumed them with alcohol for recreational purposes, and a large number of students taking stimulants non-medically had binged on alcohol in the previous two weeks [115, 116].

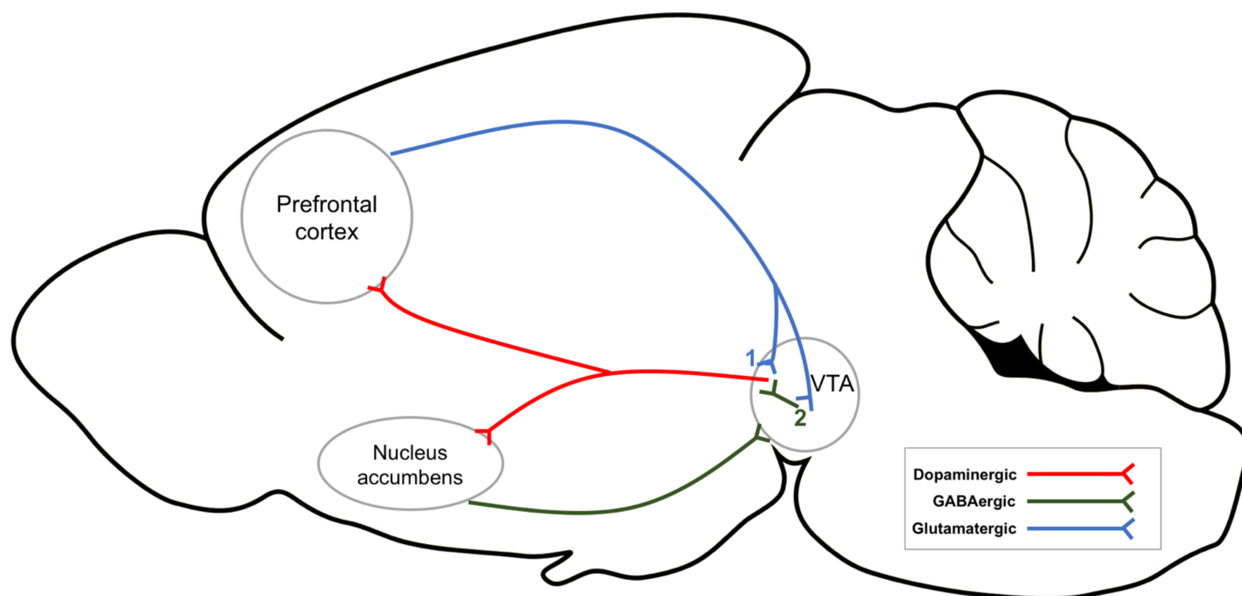



Figure 1-3. Neurocircuitry activated by drugs of abuse.

As described in Section 1.3.2, increased LTP at (1) or decreased LTP at (2) can result in increased dopamine release onto the nucleus accumbens. Note: brain regions not drawn to scale, graphic does not encompass many or all projections reported in these regions.

In contrast to adolescence, adulthood is characterized as a period of more stable neuronal adaptation, although synaptic neuroplasticity and hippocampal neurogenesis are still active during this time and readily influence drug-related behavior [117, 118]. Regardless of age, repeated use of drugs that alter neuronal activity can lead to long-term changes in brain circuitry responsible for regulating behavioral responding and learning [119, 120]. Following the rules of Hebbian plasticity [121], drug-evoked synaptic plasticity is observed in the mesocorticolimibic dopamine system following repeated increases in dopamine concentrations in the ventral tegmental area (VTA) and its projecting regions, the nucleus accumbens and prefrontal cortex [122-124] upon acute or repeated drug exposure (a mechanism shared by the majority of drugs of abuse). In both the VTA and nucleus accumbens, alterations in evoked long-term potentiation (LTP) or long-term depression (LTD) of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor

currents can be observed following drug exposure, although exact magnitude and direction of changes in LTP/LTD are influenced by drug administration strategy [125] and/or duration of drug exposure [126, 127]. In general, an increase in LTP at excitatory glutamatergic VTA afferents onto dopaminergic cells upon drug exposure results in enhanced dopamine release into the nucleus accumbens, which in turn inhibits GABAergic projections from the nucleus accumbens to the VTA, resulting in disinhibition and further dopamine release [128]. Opioids, such as morphine, inhibit LTP at inhibitory GABAergic VTA afferents, thus resulting in a similar mechanism downstream with respect to dopamine release onto the nucleus accumbens [129] (Figure 1-3). Additional alterations in LTP and LTD on projections from the cortex, amygdala, and hippocampus to the nucleus accumbens are observed upon repeated drug exposure [130, 131]. Importantly, these alterations in drug-evoked plasticity are not unique to mesocorticolimbic nuclei, as further described in Chapter 3 on the dorsal striatum's role in alcohol abuse.

### 1.3.3 Considerations of sex

Until recently, the majority of rodent studies were conducted in male animals in an attempt to reduce total animal use, decrease research cost, and remove potential variability in behaviors that may be associated with hormonal fluctuations [132]. However, a number of studies in both human and nonhuman subjects suggest that drug reward-related behaviors are not identical between males and females [133-136]. Women tend to escalate their rate of drug consumption more quickly than men for drugs such as alcohol, opioids, and cocaine [137, 138]. In rodents, female routinely consume more alcohol than males [139-141], and female rodents are more sensitive to psychostimulants-induced behaviors compared with males [142, 143]. A large number of studies investigating sex differences involve ovariectomized animals, as compared with intact females, which greatly disrupts the ability to draw general conclusions on differences in drug behaviors between free cycling male and females, although it appears that in general females are more susceptive to escalated intake because of sexually dimorphic brain development/receptor expression differences rather than hormonal fluctuations [144].

### 1.3.4 Genetic manipulations as mechanistic tools

Genetic animal models have been used successfully to establish the role of different molecular mechanisms associated with drug-related behaviors by selectively altering expression of proteins,

receptors, or kinases. Types of genetic manipulation include complete (embryonic or conditional) gene knockout, gene mutation, or gene knock-in [145-147]. For example, in drug abuse research, knockout studies for dopamine transporters [148] and receptors [149-152] have helped identified the role of dopamine in locomotor and reward-like behaviors upon cocaine or amphetamine administration. One significant drawback of genetic models is that the models are only as strong as the genetic manipulation's contribution to the behavioral phenotype [153], and potential physiological compensation resulting from genetic manipulation (especially in complete gene knockout studies) cannot be distinguished from a single gene knockout effect unless a conditional knockout strategy is utilized [145].

## 1.4 Role of G protein-coupled receptors in drug abuse

G protein-coupled receptors (GPCRs) are metabotropic, seven transmembrane receptors which many drugs of abuse engage either directly or indirectly [154]. Because of their ubiquitous expression in the nervous system and ability to "fine-tune" ionotropic signaling (or influence neuronal firing on their own), therapies targeting GPCRs comprise 33% of all neuronal-based drugs [155] and 34% of all drugs approved by the United States Food and Drug Administration (FDA) [156]. GPCRs are expressed presynaptically near axon terminals of neurons to influence presynaptic function (such as neurotransmitter release) or postsynaptically on dendrites, where they can bind and become activated by endogenously released neurotransmitters [157]. GPCRs coupled to heterotrimeric G proteins, comprised of α, β, and γ subunits, and activation upon agonist binding to the receptor allows the dissociation of this heterotrimeric G protein from the receptor through the exchange of GDP for GTP [158, 159]. Upon this guanine nucleotide exchange, the Gα subunit separates from the Gβγ dimer, and these two separate portions of the original heterotrimeric G protein can distinctly activate a variety of signal transduction pathways [159, 160]. Gα subunits are commonly classified based on their downstream signaling function as $G_s$, $G_{i/o}$, $G_{q/11}$, or $G_{12/13,}$ [161]. For $G_s$- or $G_{i/o}$-coupled receptors, activity of these GPCRs either increases or decreases intracellular cycle adenosine monophosphate (cAMP) concentrations through either stimulation ($G_s$-coupled) or inhibition ($G_{i/o}$-coupled) of the enzyme adenylyl cyclase (AC), respectively, and the resulting AC-cAMP-PKA signaling pathway [159, 162-164]. For $G_q$-coupled receptors (see review for more discussion, [165]), activation of $G_q$-coupled leads to increases in intracellular calcium which can result in increased neuronal firing [166]. $G_{12/13}$ coupled

receptors (see reviews for more discussion, [167, 168]) activate RhoGEF signaling, an important regulator of cellular proliferation, movement, and morphology.

Following constitutive or continued ligand activation, neuronal G protein signaling can be "arrested" or desensitized by proteins known as arrestins [169, 170]. Arrestins, including non-visual β-arrestin 1 and β-arrestin 2, are proteins recruited to GPCRs upon receptor phosphorylation by G protein-coupled receptor kinases (GRKs) [171-173]. The recruitment of arrestin to a GPCR is thought to occlude the heterotrimer G protein from re-associating with the receptor (although recent biochemical studies suggest GPCR-G protein-arrestin supercomplexes are possible [174]) and trigger receptor internalization. Internalized GPCRs can then be recycled back to the plasma membrane, degraded within lysosomes, or potentially engage in arrestin-dependent signaling cascades [170, 175-178]. The implications of β-arrestin and GPCR signaling on neuronal physiology and behavior are further investigated in Section 1.4.4 with respect to drug behavior.

Activation of presynaptic and postsynaptic $G_s$-and $G_q$-coupled receptors typically excites synaptic function, while activation of $G_{i/o}$-coupled receptors can lead to neuronal inhibition [157]. This metabotropic influence on synaptic excitation and inhibition has led to the development of Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) as chemogenetic tools to alter neuronal activity [179-181]. DREADDs are GPCRs engineered to be selectively activated by designer drugs (which are otherwise inert ligands to endogenous receptors), such as the drug clozapine-N-oxide (CNO). By expressing DREADDs under viral promoters, DREADDs can selectively activate or inhibit specific neuronal subtypes or circuits through their metabotropic activity. As such, DREADD technology has led to the elucidation of neuronal circuits involved in feeding [182], drug sensitization and seeking [183, 184], and anxiety-like behaviors [185], although limitations in interpretation of behavioral DREADD studies do exist because of the potential back-transformation of CNO to clozapine, which is an agonist of endogenous muscarinic receptors expressed in the CNS [179, 186, 187].

### 1.4.1 Dopamine receptors

The dopamine receptor family includes two major branches: D1-like ($D_1$ and $D_5$) and D2-like ($D_{2long}$, $D_{2short}$, $D_3$, $D_4$) receptors [188-190]. The D1-like family of dopamine receptors are $G_s$-coupled receptors, which upon activation can increase neuronal firing and are highly enriched (primarily $D_1$ rather than $D_5$) on D1-medium spiny neurons (MSNs) in the direct striatonigral

pathway [188, 191]. On the other hand, activation of D2-like receptors can lead to neuronal inhibition or silencing through coupling to $G_{i/o}$, and these receptors (primarily $D_{2long}$ and $D_{2short}$, expression of $D_3$ and $D_4$ is limited) are located on D2-medium spiny neurons which form a major component of the indirect striatopallidal pathway [191, 192]. Furthermore, two distinct $D_2$ receptor isoforms exist as the result of alternative splicing of the $D_2$ gene (*Drd2*), and it is believed that $D_{2short}$ acts as an autoreceptor presynaptically in dopaminergic cell bodies while $D_{2long}$ is primarily a postsynaptic receptor [193].

Expression and activity of D1-like and D2-like receptors has been heavily implicated in drug reward, as observed in genetic knockout animal studies [194]. Historically, increased dopamine signaling has been strongly associated with reward reinforcement, motivation, and learning behaviors - all behaviors highly associated with the development of drug abuse and addiction [195]. As such, it is no surprise that drugs of abuse increase extracellular dopamine concentrations in reward centers of the brain, such as the nucleus accumbens [122, 196-198]. This increase in dopaminergic activity upon drug-taking is thought to not only be rewarding, but also to process the drug experience as a salient stimulus, which may cause increased future drug-taking through conditioned learning processes [198, 199].

### 1.4.2 Opioid receptors

The opioid receptor family contains three major receptor subclasses, mu-, kappa-, and delta-opioid receptors [200]. Additionally, the nociceptin receptor (NOP) is commonly discussed among mu-, kappa-, and delta- because of its high sequence homology with these receptors, although NOP peptide nociceptin has little affinity for classical opioid receptors and classical opioid peptides (enkephalins, dynorphin) exhibit limited affinity for NOP [201]. Activation of opioid receptors is most commonly associated with physiological responses to pain (not discussed in detail in this dissertation, see [202] for a review on opioids' role in nociception) as well as drug reward. Opioid receptors play an important role in natural and drug reward processes because of indirect increases in endogenous opioid activity upon reward intake (as observed in alcohol, described in detail in Chapters 2-4) or because of direct binding of drugs of abuse to these receptors (as observed by morphine) [203].

All members of opioid family are $G_{i/o}$-coupled receptors which decrease intracellular levels of cAMP upon activation through $G\alpha_{i/o}$ inhibition of adenylyl cyclase. Furthermore, heterotrimeric

G protein dissociation into Gα and Gβγ upon ligand binding can lead to neuronal hyperpolarization/inhibition by Gβγ activation of G protein-coupled inwardly-rectifying potassium channels (GIRKs) [204, 205]. Opioid receptors are ubiquitously expressed in the brain, with heavy enrichment in the cortex, limbic system, and brain stem [200]. Despite the similarities in intracellular function and gross anatomical expression of the three major opioid subtypes, at the neuronal level, synaptic localization of each receptor subclass varies. For example, in the striatum (a brain region of strong interest for this dissertation), mu-opioid receptors are expressed on both D1- and D2-MSNs in striatal regions while delta-opioid receptors are thought to only be expressed on D2-MSNs [47, 206-208]. This differential neuronal expression and synaptic localization may explain the subtleties between the different behavioral roles of each opioid receptor subtype in specific drug-related behaviors. For example, global knockout of mu- or kappa- opioid receptors decreases alcohol intake [209, 210], while global knockout of delta-opioid receptors increases alcohol [211]. In Chapters 3 and 4, I will describe localization of GPCRs in the dorsal striatum and their roles in alcohol-related behaviors in more detail.

### 1.4.3 Adenosine receptors

The family of adenosine receptors includes adenosine $A_1$, $A_{2A}$, $A_{2B}$, and $A_3$ receptors, where $A_1$ and $A_3$ are $G_{i/o}$-coupled, $A_{2A}$ is $G_s$-coupled, and $A_{2B}$ is both $G_s$- and $G_q$-coupled [157]. $A_1$ and $A_{2A}$ receptors are highly expressed in the brain [212, 213], while $A_{2B}$ and $A_3$ are more highly expressed in peripheral tissue [213, 214]. As expected by their $G_{i/o}$-coupling, activation of $A_1$ receptors inhibits synaptic transmission [215] and $A_1$ receptors are highly expressed both pre- and postsynaptically in the hippocampus [216]. Conversely, activation of $A_{2A}$ receptors facilitates synaptic transmission [217], and $A_{2A}$ is more heavily expressed postsynaptically in the striatum with lower expression presynaptically in the hippocampus, suggesting complementary expression levels of $A_1$ and $A_{2A}$ exist between the striatum and the hippocampus [218]. $A_1$ expression is localized to D1-MSNs [219] while $A_{2A}$ localize to D2-MSNs [218], allowing for both anatomical and functional segregation of these two major adenosine receptor subtypes [218, 220]. Animal studies employing genetic knockout of $A_1$ and/or $A_{2A}$ have helped elucidate the roles of these receptors in behaviors related to cognition, locomotion, sleep/wake cycles, emotion, and anxiety [221, 222]. Because of these roles, therapies targeting adenosine receptors, such as adenosine $A_{2A}$

receptor antagonists for Parkinson's disease [222] or agonists for decreased reward intake [223, 224] have been investigated.

### 1.4.4 Implications of GPCR signaling bias, or functional selectivity, in physiology and behavior

In the past decade, the identification of GPCR functional selectivity has unveiled a new frontier in GPCR pharmacology. Functional selectivity, also known as signaling bias, is defined as the ability of a ligand to induce a unique, ligand-specific receptor conformation which can distinctively activate downstream signaling pathways, such as those associated with G protein-dependent signaling or β-arrestin [225-228]. Therapeutically, ligand signaling bias is relevant as a number of ligands exhibiting signaling bias for either G protein or β-arrestin signaling decrease side effects associated with otherwise potent, unbiased ligands [229]. For example, TRV130 (oliceridine), a G protein-biased mu-opioid receptor agonist, [230, 231], provides a faster onset of action and decreased respiratory depression compared with typical in-patient analgesic, morphine. Another mu-opioid G protein-biased ligand, PZM21, displays a similar therapeutic profile (analgesia without significant respiratory depression) [232], further suggesting that β-arrestin recruitment is to be avoided in mu-opioid-based analgesic development. The therapeutic relevancy of G protein signaling bias is not unique to the mu-opioid receptor; in Chapters 2 and 4, the role of signaling bias of delta-opioid ligand will be discussed with respect to alterations in alcohol intake. Importantly, G protein bias is not always preferred in drug discovery; in the development of new antipsychotics, β-arrestin 2 recruitment of dopamine D2 receptor agonists is associated with decreased psychotic-like behavior while also preventing catalepsy in animal models of schizophrenia [233, 234].

## 1.5 Scope of dissertation

To further investigate the topics introduced here, Chapter 2 will focus on the characterization and use of orally-active, non-opium-derived opioid alkaloids of the plant *Mitragyna speciosa* as functionally selective, delta-opioid chemical scaffolds to be used in the development of novel treatments of alcohol use disorder. Chapter 3 offers a broad review on how $G_{i/o}$-coupled receptors expressed in the dorsal striatum, a brain region heavily implicated in goal-directed and habitual action, may play an important role in alcohol-related behavior. In Chapter 4, the role of $G_{i/o}$-

coupled receptors in the dorsal striatum are investigated through the use of DREADD technology and by targeting endogenous delta-opioid receptors in this region. Importantly, the findings in Chapter 4 suggest that dorsal striatal $G_{i/o}$-protein signaling decreases alcohol intake, and β-arrestin 2 expression is required for the delta-opioid agonist and β-arrestin 2 super-recruiter, SNC80, to increase alcohol intake. As β-arrestin 2 is frequently the focus of drug-related behavioral pharmacology, Chapter 5 characterizes β-arrestin 1's role in baseline locomotor, anxiety-like, reward intake, and alcohol-related behaviors while also assessing sex as a biological variable.

Switching the emphasis away from alcohol use disorder, the later Chapters 6-8 focus on the consequences of repeated use of psychostimulants, such as caffeine or the novel psychoactive substance ethylphenidate, in adolescence. Chapter 6 uncovers the (lack of) correlation between adolescent caffeine intake and adult alcohol intake, while Chapter 7 describes the unique behavioral and neurochemical outcomes associated with repeated adolescent caffeine-mixed alcohol exposure. In Chapter 8, the cognitive- and reward-related behaviors associated with repeated ethylphenidate in adolescence are described. Finally, in Chapter 9, the potential future directions of this dissertation's topics are discussed, as well as some preliminary findings on the role of β-arrestin isoforms in delta-opioid agonist-induced seizure behavior.

# CHAPTER 2. PRECLINICAL CHARACTERIZATION OF MITRAGYNA SPECIOSA ALKALOIDS TO DECREASE ALCOHOL INTAKE

*Mitragyna speciosa* (kratom) is an alkaloid containing plant that has recently gained traction in opioid-dependent individuals to mitigate effects of opioid withdrawal. Interestingly, a number of alkaloids present in kratom target opioid receptors but interact in a manner which does not recruit β-arrestin. It has been suggested that mu-opioid receptor (MOR) targeting drugs that preferentially interact with G-proteins over β-arrestin 2 show beneficial characteristics, including reduced respiratory depression with increased onset of analgesic action. Similarly, we have previously demonstrated that delta-opioid receptor (DOR) targeting drugs lacking β-arrestin 2 recruitment are able to decrease voluntary alcohol consumption in C57BL/6 mice; therefore, we hypothesized that kratom-derived alkaloids may decrease alcohol consumption in mice. To test our hypothesis, we first characterized kratom extract and kratom-derived alkaloids for their $G_{i/o}$-protein signaling activity and β-arrestin 2 recruitment *in vitro* at DOR, where negligible β-arrestin 2 recruitment was observed for all. The effect of intraperitoneal administration of each alkaloid on 10% alcohol intake was modeled in male C57BL/6 mice using a two-bottle choice, limited access procedure, where all alkaloids decreased voluntary alcohol intake (with the alkaloid 7-hydroxymitragynine as the most potent). This decrease in alcohol intake by 7-hydroxymitragynine was attenuated in DOR knockout mice. Only the alkaloid 7-hydroxymitragynine increased locomotor activity upon administration; other alkaloids decreased locomotion at doses efficacious in reducing alcohol intake. Furthermore, kratom extract, mitragynine, and 7-hydroxymitragynine did not induce acute conditioned place preference (in contrast to mu-opioid agonist, morphine). Our results indicate that kratom alkaloids have the potential to reduce alcohol consumption based on their ability to activate G protein signaling at DOR in a biased signaling manner, without acute abuse potential. Thus, we propose that kratom alkaloids may provide a useful chemical template to develop novel pharmacological interventions for the treatment of alcohol use disorder.

## 2.1 Introduction

Use of the psychoactive plant *Mitragyna speciosa* (commonly known as kratom, krathrom, or ketum) has risen dramatically across North America and Europe throughout recent years [235].

Historically, kratom has been used in its native Southeast Asian region to relieve pain, diarrhea, cough, intestinal distress, and/or stimulation, and overuse is not accepted in society [236]. Currently, the rationale for kratom use varies depending on the region and user. In the West, kratom is used as self-medication from opioid withdrawal, management of chronic pain, relief from mood disorders, or recreationally [235, 237, 238]. With increasing use rates and reports of adverse effects following consumption [239], reservations on the safety of kratom has increased scrutiny of its current legal status in the United States [240, 241] (although no overdoses have been attributed to kratom use alone to date [242]).

The kratom plant contains several orally-active, indole alkaloids including mitragynine, speciogynine, paynantheine, and 7-hydroxymitragynine [243] which may differentially contribute to the reported positive effects of kratom use [244]. The use of a kratom as self-treatment for opioid withdrawal and chronic pain management logically derives from the activity of some of kratom's alkaloids at the mu-opioid receptor (MOR) [245-247], and depending on the alkaloid, activity is also observed at delta-opioid receptors (DOR) and to a lesser extent at kappa-opioid receptors (KOR) [248]. For example, kratom alkaloid 7-hydroxymitragynine is 4-5x more potent in antinociceptive assays and causes less constipation in rodents than morphine [248-251], and displays *in vitro* receptor binding affinities ($K_i$) for MOR, DOR, and KOR of 37±4 nM, 91±8 nM, and 132±7 nM respectively [248]. At MOR, the signaling bias (or functional selectivity) - a term defined by the propensity of a ligand to engage one signaling pathway over another [252-254] - of 7-hydroxymitragynine for inhibition of adenylyl cyclase-stimulated cAMP production versus β-arrestin 2 recruitment suggests that 7-hydroxymitragynine is bias towards inhibition of cAMP [248, 255]. Importantly, this bias profile has been associated with an increased therapeutic window for MOR agonists [229] and has led to the development of biased MOR agonists such as TRV130 (oliceridine, which is currently in Phase III clinical trials) and PZM21 [230, 232, 256].

In addition to pain management, kratom use has been used as self-medication to alleviate symptoms of alcohol withdrawal [257-260]. This is important as the therapeutic relevancy of signaling bias is not unique to MOR agonists. DOR agonists biased towards $G_{i/o}$-protein pathways decrease voluntary alcohol intake in C57Bl/6 male mice, while DOR agonists biased with β-arrestin 2 recruitment bias increase voluntary alcohol intake in rodents [261-263]. The *in vitro* bias of kratom alkaloids has yet to be determined at DOR and KOR; therefore, for this study we hypothesized that this DOR therapeutic bias profile ($G_{i/o}$-protein pathway activation > β-arrestin 2

recruitment) may be present for the orally active alkaloid kratom species, as the binding affinity of alkaloids such as 7-hydroxymitragynine at DOR and its known MOR bias suggests that a subset of kratom alkaloids may also be bias toward G-protein pathways (versus β-arrestin 2 recruitment) at DOR [248]. In addition to characterizing DOR bias, elucidating KOR bias of kratom alkaloids is necessary as β-arrestin 2 activity upon stress-induced KOR activation is responsible for increased dysphoria [264]. Furthermore, the biased KOR agonist noribogaine lacks significant β-arrestin 2 recruitment and does not induce dysphoria [265].

Here, we assessed the *in vitro* signaling bias of kratom extract and four major kratom alkaloids (mitragynine, speciogynine, paynantheine, 7-hydroxymitragynine) as well as a kratom extract at DOR prior to *in vivo* characterization for alcohol-related behaviors, locomotor effects, and acute reward. We observed that all tested kratom alkaloids and kratom extract exhibit negligible β-arrestin 2 recruitment but are capable of inhibiting forskolin-stimulated cAMP production at DOR, indicating cAMP bias at this receptor. All alkaloids were capable of reducing voluntary alcohol intake in wild-type male C57Bl/6, although only 7-hydroxymitragynine lacked hypolocomotor at the lowest relevant dose necessary to decrease alcohol intake. The ability of 7-hydroxymitragynine to decrease alcohol intake was dependent on DOR expression, as observed by the lack of efficacy in 7-hydroxymitragynine to reduce alcohol intake in DOR knockout C57Bl/6 mice. Additionally, kratom extract, mitragynine, and 7-hydroxymitragynine were not acutely rewarding at doses effective in reducing voluntary alcohol intake. Overall, *Mitragyna speciosa*-derived opioid alkaloids provide a novel chemical scaffold for DOR functional selectivity and further implicate DOR signaling bias as a relevant tool for altering alcohol intake in rodents.

## 2.2 Materials and methods

### 2.2.1 Drugs and chemicals

Kratom powder (MoonKratom, Austin, TX USA) was purchased from Amazon.com (Seattle, WA, USA) and alkaloids were extracted as described previously by Orio et al. [266]. In brief (and shown in Figure 1-1), extract from leaves was performed in 95% ethanol (EtOH) at 50 °C for four hours followed by vacuum filtration and solvent removal. Crude extract was then re-suspended in water (pH=3, hydrochloric acid) and washed with hexanes to remove plant material. Solution pH was adjusted to 9 with ammonia and solution as extracted with dichloromethane (DCM) prior to drying

over sodium sulfate, where a final solid crystalline light brown extract was obtained after vacuum solvent removal. Extract composition was evaluated on a 6550-QTOF (Aligent, Santa Clara, CA, USA) (scan 105-100 amu) using a Zorbax Extend-C18 column (Aligent) held at 30 °C and a 0.3 mL/minute flow rate. TRV130, speciogynine, paynantheine, 7-hydroxymitragynine, and mitragynine were synthetically derived and provided by the lab of Dr. Sushruta Majumdar (Memorial Sloan Kettering Cancer Center, New York NY). Morphine sulfate pentahydrate, leu-enkephalin, hydrochloric acid, sodium sulfate, dichloromethane, ammonia, hexanes, and ethyl alcohol (pure) were purchased from Sigma-Aldrich (St. Louis, MO USA). For animal drinking assays, pure ethyl alcohol was diluted to 10% alcohol in reverse osmosis water.

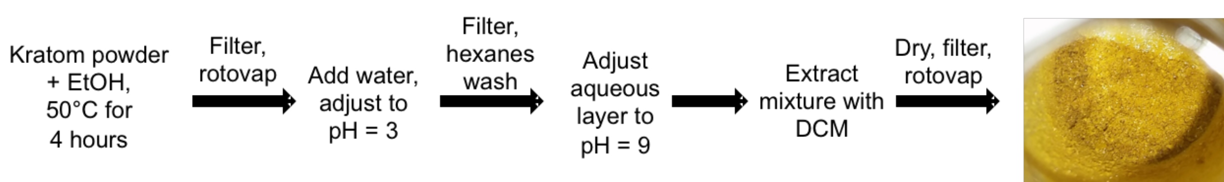



Figure 2-1. Extract workflow of kratom alkaloids from kratom leaf powder results in 0.94% yield.

### 2.2.2 Cellular assays

cAMP inhibition and β-arrestin 2 recruitment assays were performed as previously described [261]. In brief, for cAMP inhibition assays HEK293 (Life Technologies, Grand Island, NY, USA) cells (15,000 cells/well, 7.5 µl) transiently expressing FLAG-mDOR and pGloSensor22F-cAMP plasmids (Promega, Madison, WI, USA) were incubated with Glosensor reagent (Promega, 7.5 µl, 2% final concentration) for 90 minutes at 37°C/5% $CO_2$. Cells were stimulated with 5 µl agonist 20 minutes prior to 30 µM forskolin (5 µl) stimulation for an additional 15 minutes. For β-arrestin 2 recruitment assays, CHO-hDOR PathHunter β-arrestin 2 cells (DiscoverX, Fremont, CA, USA) were plated (2500 cells/well, 10 µl) prior to stimulation with 2.5 µl agonist for 90 minutes at 37°C/5%$CO_2$, after which cells were incubated with 6 µl cell assay buffer for 60 minutes at room temperature as per the manufacturer's protocol. Luminescence and fluorescence for each of the assays was measured using a FlexStation3 plate reader (Molecular Devices, Sunnyvale, CA, USA).

### 2.2.3 Animal husbandry

Male C7Bl/6, wild-type adult (age 6 weeks) mice were purchased from Envigo (Indianapolis, IN USA) and habituated for one week to the animal facility prior to behavioral testing. For DOR knockout mice stain information, see van Rijn et al. [267]. Food was provided ad libitum; water

was provided ad libitum unless specified for ethanol consumption experiments. Throughout the experiments, animals were housed at ambient temperature of (21°C) in a room maintained on a reversed 12L:12D cycle (lights off at 10.00, lights on at 22.00) in Purdue University's animal facility, as accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. All animal procedures were pre-approved by Purdue University's Institutional Animal Care and Use Committee and conducted in accordance with National Institutes of Health Guide for the Care and Use of Laboratory Animals.

### 2.2.4 Voluntary alcohol consumption

Mice were trained to voluntarily consume alcohol in a limited access (4 hours/day), 2-bottle choice (water vs. 10% ethanol), drinking-in-the-dark (DID) paradigm during their active phase (dark light cycle) until the alcohol intake was stable as previously described [267, 268]. Mice were trained for three weeks during which the mice initially increased their alcohol intake prior to reaching steady state consumption. After the completion of the third week of training, injections were administered every Friday prior to the drinking session. Drug effect on alcohol intake was measured as a change in Friday total drinking minus average alcohol intake between Tuesday-Thursday (g/kg). Bottle weights were measured directly before and after the 4-hour access period to the second decimal point to determine fluid intake during this access period and weights of bottles were corrected for any spillage.

### 2.2.5 Locomotor activity

Square locomotor boxes from Med Associates (L 27.3 cm x W 27.3 cm x H 20.3 cm, St. Albans VT, USA) were used to monitor locomotor activity. For all locomotor studies, animals were moved to the testing room for 60 minutes prior to testing. A 90-minute baseline habituation session to the boxes was conducted prior to drug administration to reduce novelty locomotor differences. The following day, locomotor activity was monitored for a total of 90 minutes after drug injection. All testing was conducted during the dark light/active phase.

### 2.2.6 Conditioned place preference

An acute conditioned place preference (CPP) as described previously [269, 270] was performed with two modifications: 1) 40-minute conditioning sessions rather than 30 minutes and 2) a two-chamber apparatus rather than a three-chamber design. To determine initial compartment bias, a

vehicle injection was administered intraperitoneally immediately prior to the pre-conditioning session to create an unbiased, counterbalanced approach for drug-pairing (half of the animals received drug on the pre-test preferred side, while half received drug on the pre-test non-preferred side). Animals exhibiting >70% preference for one of the two chambers were removed from further testing. Four conditioning sessions (two to vehicle on the non-drug-paired side and two to drug on the drug-paired side) were conducted with two sessions a day, four hours apart. On the post-conditioning testing day, a vehicle injection was administered directly before placing the animals in the testing apparatus to determine post-conditioning preference. For all sessions, animals were habituated to the testing room 60 minutes before sessions and all behavior was conducted during the dark light/active phase.

### 2.2.7 Statistical analysis

All data are presented as means ± standard error of the mean, and analysis was performed using GraphPad Prism 7 software (GraphPad Software, La Jolla, CA). For *in vitro* assays, nonlinear regression using a dose-response to either inhibition (cAMP) or stimulation (β-arrestin 2 recruitment) was conducted to determine pIC50 or pEC50, respectively. Significant changes in average alcohol intake was determined by one-way, repeated measures ANOVA with Bonferroni multiple comparisons. For CPP, two-way, repeated measures ANOVA with Bonferroni multiple comparisons was used to determine significant differences in time spent on the drug-paired side pre- versus post-conditioning. One-way ANOVA with Bonferroni multiple comparisons determined significance for locomotor studies.

Table 2-1. Alkaloid composition of alcohol-based kratom extraction (*analysis performed by Robert Cassell, Van Rijn lab).*

| Alkaloid | % of total |
|---|---|
| Mitragynine | 44.87 |
| Speciociliatine | 15.51 |
| Paynantheine | 14.08 |
| Isopaynantheine | 14.08 |
| Speciogynine | 8.94 |
| 7-hydroxymitragynine | 2.85 |
| Corynantheidine | 2.12 |

## 2.3 Results

### 2.3.1 Kratom extract alkaloid composition

Our alcohol-based kratom extract technique contained 7 known alkaloid species, included mitragynine (44.9%), speciociliatine (15.5%), paynantheine (14.1%), isopaynantheine (14.1%), speciogynine (8.9%), 7-hydroxymitragynine (2.9%), and corynantheidine (2.1%) (Table 2-1), as evaluated by LC/MS.

### 2.3.2 *In vitro* characterization of kratom extract and alkaloids at DOR

All kratom alkaloids and kratom extract inhibited forskolin-stimulated cAMP in HEK293 cells transiently transfected with mouse DOR (Figure 2-2A, Table 2-2), although with decreased potency and efficacy compared with reference endogenous ligand, leu-enkephalin (Leu-enk.). None of the kratom alkaloids nor kratom extract exhibited full agonist activity for cAMP inhibition. For β-arrestin 2 recruitment, negligible recruitment was observed for all kratom

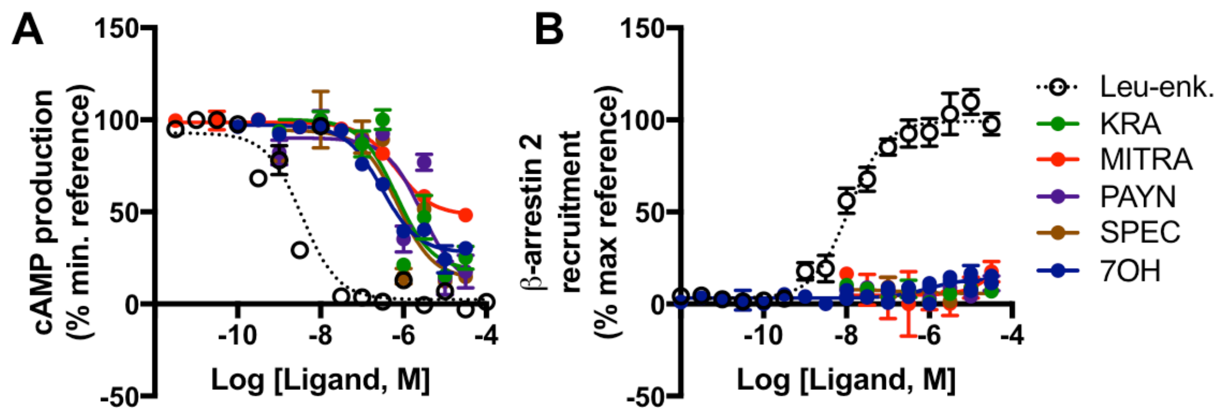



Figure 2-2. At DOR, kratom alkaloids and kratom extract inhibit forkolin-stimulated cAMP production but do not recruit β-arrestin 2 recruitment.

Kratom extract (KRA) and kratom alkaloids (mitragynine, MITRA, paynantheine, PAYN, speciogynine, SPEC, 7-hydroxymitragynine, 7OH) inhibited forskolin-stimulated cAMP production in HEK293 cells expressing DOR although with decreased potency and efficacy compared with endogenous ligand, leu-enkephalin (Leu-enk.) (A). For β-arrestin 2 recruitment, as measured in CHO PathHunter cells expressing DOR and β-arrestin 2-galactosidase, kratom alkaloids and kratom extract induced negligible β-arrestin 2 recruitment to DOR as compared with leu-enkephalin (B). Data are represented as mean ± SEM. *Experiments performed by Robert Cassell and Anna Gutridge (Van Rijn lab).*

Table 2-2. $G_{i/o}$ cAMP inhibition and β-arrestin 2 recruitment by kratom alkaloids at DOR.

Potency and maximal inhibition (cAMP) or recruitment (β-arrestin 2) as normalized to reference ligand, leu-enkephalin (leu-enk.), response. For pIC50 and pEC50, the 95% confidence interval is shown in parentheses. N/A = not converged, no estimate available.

| **Ligand** | **cAMP inhibition** | | **β-arrestin 2 recruitment** | |
|---|---|---|---|---|
| | **pIC50 (M)** | **$E_{max}$ (%inhibition leu-enk.)** | **pEC50 (M)** | **$E_{max}$ (%recruitment leu-enk.)** |
| Leu-enkephalin (Leu-enk.) | 8.4 (8.0-8.9) | 97.6 | 7.9 (7.8-8.1) | 99.3 |
| Kratom extract (KRA) | 6.2 (5.7-6.6) | 81.8 | N/A | N/A |
| Mitragynine (MITRA) | 6.2 (5.9-6.4) | 51.8 | N/A | N/A |
| Paynantheine (PAYN) | 5.6 (45.0-6.2) | 90.7 | N/A | N/A |
| Speciogynine (SPEC) | 6.1 (5.6-6.6) | 86.5 | N/A | 5.8 |
| 7-hydroxymitragynine (7OH) | 6.5 (6.4-6.7) | 72.3 | 6.0 (5.5-6.6) | 13.8 |

alkaloids and kratom extract as compared with leu-enkephalin in CHO PathHunter cells expressing human DOR and β-arrestin 2-galactosidase (Figure 2-2B, Table 2-2).

### 2.3.3 Significant decrease in voluntary 10% alcohol intake upon kratom alkaloid administration

Following four weeks of exposure to the two-bottle choice between water and 10% alcohol, drinking-in-the-dark protocol, animals were injected with kratom alkaloids directly prior to Friday drinking sessions. 30 mg/kg kratom extract administration (KRA, i.p.) significantly deceased alcohol intake (Figure 2-3A, $F_{2,16}$=15.0, $p$=0.0002: 0 vs. 30, $p$=0.0002, 10 vs. 30, $p$=0.0041), as well as administration of 30 mg/kg and 100 mg/kg mitragynine (MITRA, Figure 2-3B, $F_{3,24}$=5.15, $p$=0.0068: 0 vs. 30, $p$=0.045, 0 vs. 100, $p$=0.02) compared with average alcohol intake change after vehicle (0.9% saline) injection. Speciogynine decreased alcohol intake at the highest 30 mg/kg dose (SPEC, Figure 2-3C, $F_{2,18}$=12.5, $p$=0.0004: 0 vs. 30, $p$<0.0001, 10 vs. 30, $p$=0.0008), while paynantheine decreased voluntary intake at both 10 mg/kg and 30 mg/kg (PAYN, Figure 2-3D, $F_{2,18}$=12.5: $p$=0.0004, 0 vs. 10, $p$=0.013, 0 vs. 30, $p$=0.0003). For 7-hydroxymitragyine, the most potent of the alkaloids, a decrease in alcohol intake was observed at both 3 and 10 mg/kg with a dose-response in effectiveness (7OH, Figure 2-3E, $F_{3,24}$=10.4, $p$=0.0001: 0 vs. 3, $p$=0.03, 0 vs. 10, $p$=0.02, 1 vs. 10, $p$=0.032).

### 2.3.4 Varying locomotor effects of kratom alkaloids

The locomotor effects of the lowest, most efficacious dose of the alkaloids to reduce alcohol intake was determined in alcohol-exposed animals to see if these decreases in alcohol intake were the result of drug-induced hypolocomotion. At the lowest, most efficacious doses, 30 mg/kg kratom extract (KRA), 30 mg/kg mitragynine (MITRA), 10 mg/kg paynantheine (PAYN), and 30 mg/kg speciogynine (SPEC) all significantly decreased locomotion compared with vehicle saline 0.9% control (Figure 2-4A,B; $F_{5,72}$=31.9, $p$<0.0001; compared with VEH: $p$<0.0001 for MITRA, PAYN, SPEC, $p$<0.05 for KRA). The lowest dose of 7-hydroxymitragynine (7OH), 3 mg/kg, significantly increased ambulation compared with vehicle control ($p$<0.0001). No significant differences in total 3 mg/kg 7-hydroxymitragynine-induced locomotion were observed between wild-type alcohol-exposed and wild-type alcohol naïve-mice (*graph omitted from dissertation text*, unpaired student's t-test, t(14)=1.42, p=0.18), suggesting that previous alcohol exposure was not responsible for this increased ambulatory response.

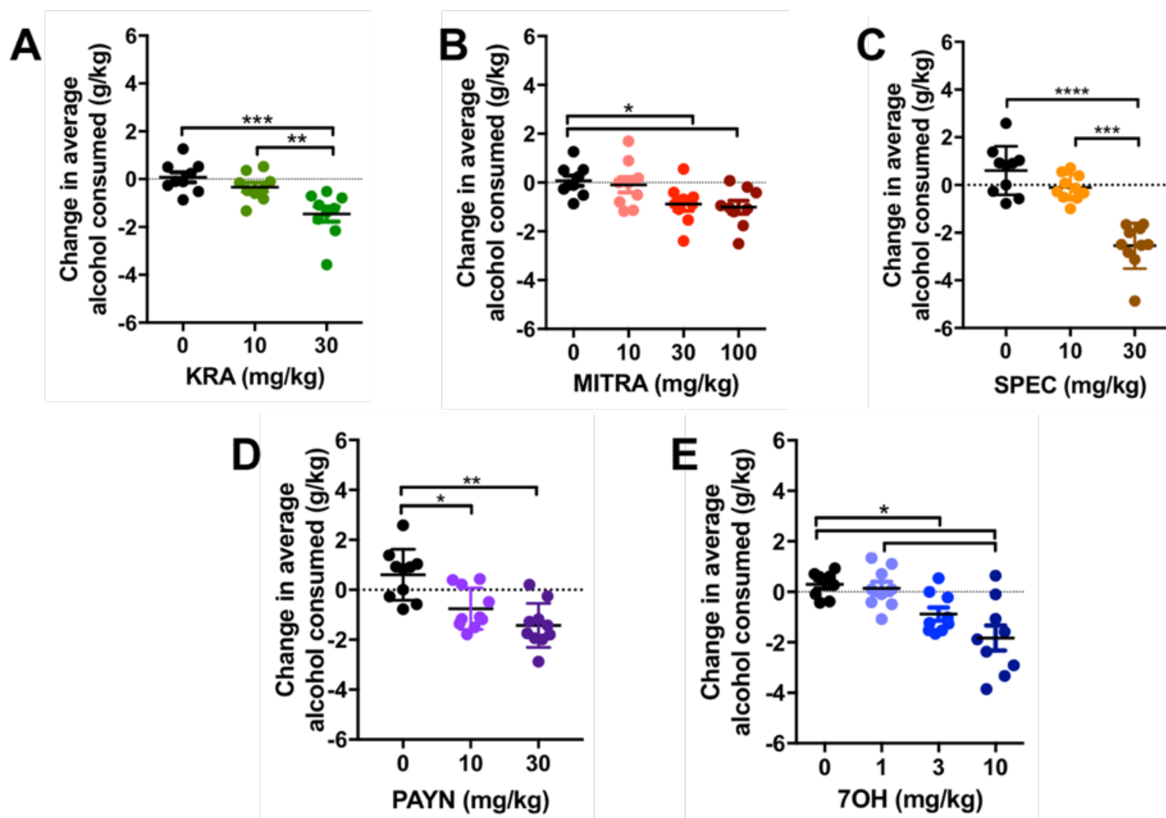



Figure 2-3. Decreased 10% alcohol intake upon kratom alkaloid administration.

Following three weeks of exposure to a two-bottle choice (10% alcohol vs. water), limited access, drinking-in-the-dark protocol, male C57Bl/6 adult wild-type mice were injected with kratom alkaloids (i.p.) to address changes in volitional alcohol consumption. Kratom extract (KRA, A), mitragynine (MITRA, B), speciogynine (SPEC, C), paynantheine (PAYN, D), and 7-hydroxymitragynine (7OH, E) all decreased alcohol intake. Significance by repeated measures, multiple comparisons (Bonferroni) 1-way ANOVA, *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$, ****, $p<0.0001$; data are represented as mean ± SEM.

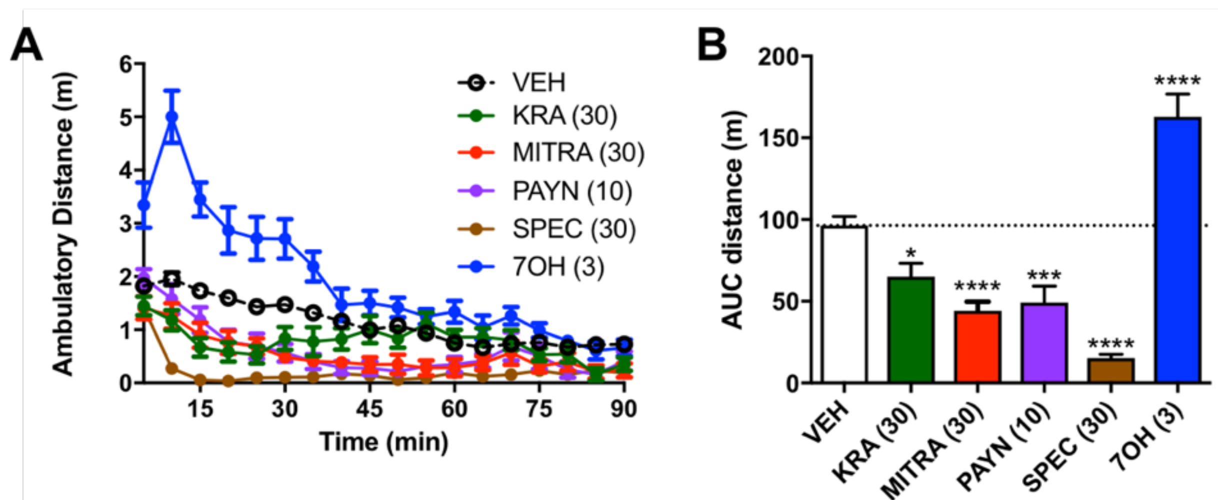



Figure 2-4. Locomotor effects of kratom alkaloids at lowest, most efficacious dose to decrease 10% alcohol intake.

One day after a 90 minute habituation session to the locomotor boxes, ambulation of 90 minutes (A) and area under the curve (B) following kratom alkaloid administration (i.p.) was measured for 30 mg/kg kratom extract (KRA (30)), 30 mg/kg mitragynine (MITRA (30)), 10 mg/kg paynantheine (PAYN (10)), 30 mg/kg speciogynine (SPEC (30)), and 3 mg/kg 7-hydroxymitragynine (7OH (3)) in alcohol-exposed C57Bl/6 wild-type male mice. Significance by 1-way ANOVA with multiple comparisons to VEH ambulation, *, $p<0.05$, ***, $p<0.001$, ****, $p<0.0001$Data are represented as mean ± SEM.

### 2.3.5 Decrease in alcohol intake for 7-hydroxymitragynine is DOR-dependent and DOR G protein bias ligand-dependent

To further support our *in vitro* evidence that 7-hydroxymitragynine is pharmacologically active at DOR and the overarching hypothesis that biased signaling at DOR is responsible for the observed decrease in alcohol intake upon 7-hydroxymitragynine administration (rather than activity at MOR), the two-bottle choice, drinking-in-the-dark protocol was repeated in DOR knockout mice where no changes in average alcohol intake were observed at both 3 ($p>0.99$) and 10 mg/kg ($p=0.402$) 7-hydroxymitragynine compared with vehicle saline 0.9% (Figure 2-5A, $F_{2,20}=1.77$, $p=0.20$). Additionally, known biased MOR agonist, TRV130, did not decrease voluntary alcohol intake (Figure 2-5B, $F_{2,16}=1.51$, $p=0.25$) at 1 or 3 mg/kg ($p=0.32$, $p=0.69$, respectively) compared with vehicle saline 0.9% in wild-type mice, at antinociceptive doses of TRV130 [256], suggesting that potential biased signaling at MOR by 7-hydroxymitragynine does not contribute to the decrease in alcohol intake, and decreases in alcohol intake upon biased opioid ligand activation may be specific to DOR.

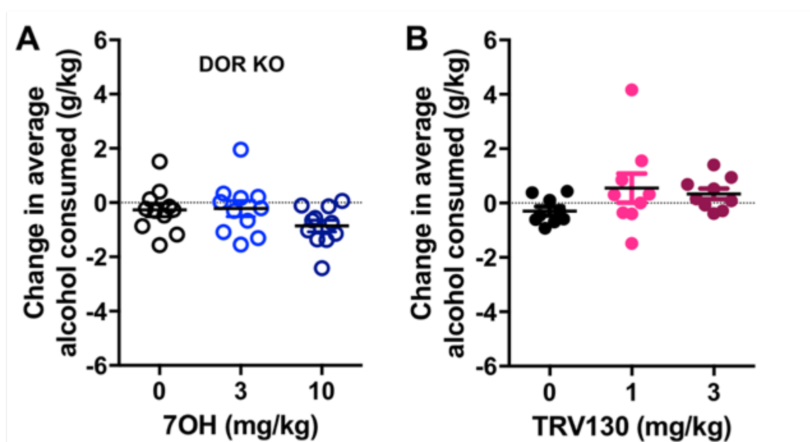



Figure 2-5. Specificity of decreased 10% alcohol intake to DOR receptor bias activity.

Following three weeks of exposure to a two-bottle choice (10% alcohol vs. water), limited access, drinking-in-the-dark protocol, male C57Bl/6 adult DOR knockout (DOR KO) mice exhibited no change in alcohol intake following 3 or 10 mg/kg 7-hydroxymitragynine (i.p.) administration (A). For TRV130, 1 and 3 mg/kg of G protein, mu-opioid agonist TRV130 (i.p.) did not significantly decrease alcohol intake in C57Bl/6 wild-type male mice exposed to the same drinking protocol previously described. Significance by one-way, repeated measures ANOVA; data are represented as mean ± SEM.

### 2.3.6 Lack of acute conditioned place preference for kratom extract, mitragynine, and 7-hydroxymitragynine

As observed by the opioid activity of kratom extract, mitragynine, and 7-hydroxymitragynine *in vitro* and previous reports of conditioned place preference to these alkaloids in rodents [271-273], the acute rewarding activity of these compounds was determined compared with 6 mg/kg morphine sulfate. 6 mg/kg morphine sulfate was rewarding acutely as observed by significantly (MS (6), $p$=0.0032) increased time spent on the drug-paired side before vs. after conditioning (Figure 2-6; effect of drug: $F_{4,31}$=0.82, $p$=0.53; effect of conditioning: $F_{1,31}$=9.63, p=0.0041; effect of drug x conditioning: $F_{4,31}$=2.11, p=0.103; effect of matching $F_{31,31}$=1.89, $p$=0.041). Surprisingly, 30 mg/kg kratom extract (KRA (30), $p$>0.99), 30 mg/kg mitragynine (MITRA (30), $p$>0.99), and 3 and 10 mg/kg 7-hydroxymitragynine (7OH (3), $p$>0.99; 7OH (10), $p$>0.99) were on average not rewarding in the acute CPP protocol in alcohol-naïve mice; however, the drugs were rewarding for some mice, while aversive to others (increased preference for the vehicle paired side).

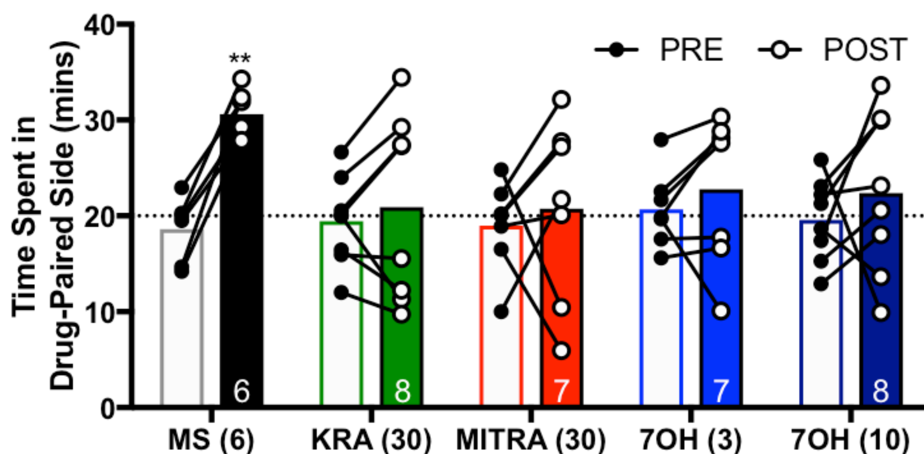



Figure 2-6. No acute conditioned place preference observed for kratom extract, mitragynine, or 7-hydroxymitragynine.

Pre- and post-conditioning time spent on the drug paired side in a model of acute conditioned place preference to intraperitoneal administration of 6 mg/kg morphine sulfate (MS (6)), 30 mg/kg kratom extract (KRA (30)), 30 mg/kg mitragynine (MITRA (30)), 3 mg/kg 7-hydroxymitragynine (7OH (3)), 10 mg/kg 7-hydroxymitragynine (7OH (10)) in C57Bl/6 wild-type male mice. Significance by repeated measures, 1-way ANOVA, **, *p*<0.01; data are represented as individual animals, bars represent mean per group.

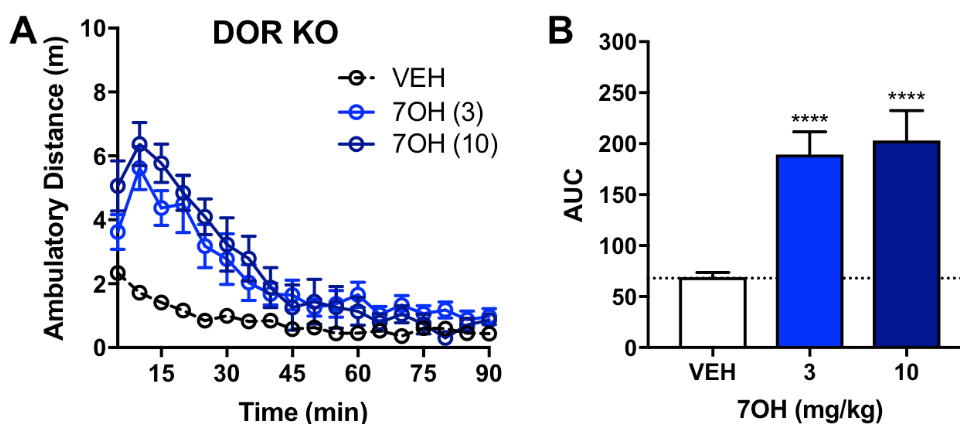



Figure 2-7. Hyperlocomotion following 7-hydroxymitragynine administration observed in DOR KO mice.

One day after a 90 minute habituation session to the locomotor boxes, ambulation of 90 minutes (A) and area under the curve (B) was measured following 3 or 10 mg/kg 7-hydroxymitragynine in alcohol-naïve C57Bl/6 DOR knockout (DOR KO) male mice, where significant hyperlocomotion was observed compared with vehicle saline (0.9%). Significance by 1-way ANOVA with multiple comparisons to VEH ambulation, ***, *p*<0.001, ****, *p*<0.0001; data are represented as mean ± SEM.

### 2.3.7 Hyperlocomotion at 3 and 10 mg/kg 7-hydroxymitragynine observed in DOR KO mice

With no decrease in voluntary alcohol intake in DOR KO mice, the locomotor stimulating effects of 7-hydroxymitragynine were determined at both 3 mg/kg (the lowest effective dose in wild-type animals tested) and 10 mg/kg (the highest, but still effective, dose tested in wild-type animals). A significant increase in total locomotor activity was observed compared with vehicle injection in alcohol-naïve DOR KO mice (Figure 2-7A,B; $F_{2,25}=24.3$, $p<0.0001$, multiple comparisons: VEH vs. 3 $p<0.0001$, VEH vs. 10 $p<0.0001$) at both 3 mg/kg and 10 mg/kg 7-hydroxymitragynine administration, i.p.

## 2.4 Discussion

Use of *Mitragyna speciosa* (kratom) has gained attention as potential self-medication or substitute for heroin and prescription-opioid dependence [245-247], where the mechanism of action for kratom is hypothesized to stem from a strong signaling bias of active kratom compounds for $G_{i/o}$-protein signaling compared with β-arrestin 2 recruitment. Here, we characterized DOR $G_{i/o}$-protein activity versus β-arrestin 2 recruitment *in vitro* for *Mitragyna speciosa*-derived alkaloids including crude kratom extract, mitragynine, speciogynine, paynantheine, and 7-hydroxymitragynine. Furthermore, we determined the *in vivo* effects of alkaloid administration on alcohol consumption, locomotion, and acute reward to investigate whether kratom and/or kratom alkaloids would be efficacious to reduce alcohol use. Of these alkaloids, 7-hydroxymitragynine displayed the most promising *in vitro* and *in vivo* profile: limited β-arrestin 2 recruitment was observed at DOR and low doses (3 mg/kg) of 7-hydroxymitragynine decreased voluntary alcohol intake in C57Bl/6 male mice without inducing hypolocomotion or acute reward. While kratom alkaloids such as 7-hydroxymitragynine may be rewarding with more continued exposure models of CPP (potentially because of significant MOR activity) [271-273], these kratom alkaloids still provide both beneficial structural and pharmacological insights into how biased signaling at DOR may be important in the modulation of voluntary alcohol intake. Additionally, these alkaloids provide novel chemical scaffolds for further biased DOR agonist drug development.

Previous opioid signaling bias/functional selectivity research has strongly focused on understanding the role of signaling bias at the mu-opioid receptor (with respect to analgesia versus respiratory depression) [230, 232, 256]. Similarly, kratom-based research has emphasized the

antinociceptive properties of 7-hydroxymitragynine and kratom-derived alkaloid synthetic derivatives, such as mitragynine pseudoindoxyls [248-251]. In doing so, limited β-arrestin 2 recruitment was observed at MOR for both mitragynine and 7-hydroxymitragynine compared with $G_{i/o}$-protein activity [248]. Our results suggest that this functional selectivity is present at DOR as well for kratom extract, mitragynine, speciogynine, paynantheine, and 7-hydroxymitragynine (Figure 2-2), where inhibition of forskolin-stimulated cAMP is observed but all alkaloids lacked detectable β-arrestin 2 recruitment. Importantly, in our hands, alkaloids such as 7-hydroxymitragynine act as partial agonists at DOR rather than antagonists as previously reported [248]. This discrepancy is possibly the result of the use of different cellular assays (GTPyS versus inhibition of adenylyl cyclase cAMP production) to measure $G_{i/o}$-protein activation. The cAMP assay may amplify signals such that a strong partial agonist becomes a full agonist [274], but on the same token, an antagonist in the GTPγS assay may turn out to be a weak partial agonist functionally.

For alcohol use disorder, targeting DOR has received considerable attention because of its potential role as drug target for not only alcohol abuse [275], but also mood disorders (which frequently are co-morbid) [276]. Previously, the rationale behind the ability of different DOR agonists to differentially affect voluntary alcohol intake was poorly understood [261, 262, 277]. Through the consideration of DOR biased signaling, we now understand that DOR agonists which strongly recruit β-arrestin 2 increase voluntary alcohol intake, while DOR agonists which weakly recruit β-arrestin 2 decrease alcohol intake [261]. As the kratom alkaloids presented here display this bias profile towards G protein signaling *in vitro*, it was unsurprising that administration of all alkaloids decreased voluntary alcohol intake (Figure 2-3). 7-hydroxymitragynine was the most potent at decreasing alcohol intake (Figure 2-3), followed by paynantheine and finally mitragynine and kratom extract. Speciogynine was capable of decreasing alcohol intake at 30 mg/kg, although it is likely that the large decrease in alcohol intake observed at this dose was likely the result of hypolocomotion upon speciogynine administration (Figure 2-4). For 7-hydroxymitragynine, this decrease in alcohol intake was at least in part driven by DOR (versus MOR, where it higher displays affinity [248]), as DOR knockout mice exhibited no significant change in alcohol intake for 7-hydroxymitragynine compared with vehicle (Figure 2-5A). Interestingly, administration of the MOR functionally selective G protein agonist, TRV130 (at doses known to induce analgesia), did not alter alcohol use. This result suggests that changes in alcohol behavior by G protein-biased

opioid agonists are primarily related to biased ligand activity at DOR as opposed to MOR (Figure 2-4B).

Opioid agonists have a major propensity for development of physical and psychological dependence, and preferably, kratom users would not simply replace one drug of abuse (alcohol) with another (opioid) when using kratom as a self-medication for alcohol or opioid withdrawal [257, 258]. While no acute reward was observed for our main alkaloids of interest (kratom extract, mitragynine, and 7-hydroxymitragynine (Figure 2-6)), reward has been previously reported in rodent conditioned place preference for mitragynine and 7-hydroxymitragynine using non-acute conditioning models [271, 278]. It is possible that the acute locomotor effects of the kratom compounds or use of a two-chamber (versus three-chamber) apparatus decreased the expression of acute CPP overall [89]. Interestingly, in our protocol, the majority of the mice preferred the drug-paired chamber for each alkaloid, while the minority found the drug-paired side aversive, suggesting potential non-opioid receptor activity at $\alpha_2$ adrenergic or 5-$HT_{2A}$ receptors [279]. Further studies are necessary to understand how 7-hydroxmitragynine administration alters alcohol reward, as known G protein bias DOR agonist TAN-67 decreases alcohol intake yet non-significantly increases alcohol conditioned place preference in C57Bl/6 mice [280], suggesting that treatment methods developed under our hypothesis may decrease total alcohol consumption but not support abstinence.

In our study, the majority of kratom alkaloids decreased locomotor activity following acute exposure, except for 7-hydroxymitragynine (Figure 2-4). Similarly, a previous study by Yusoff et al. observed that rats acutely exposed to 30 mg/kg (i.p.) mitragynine reduced their total locomotor distance [273], although lower doses of 1 mg/kg were stimulatory. For 7-hydroxymitragynine, locomotor stimulation was previously observed at 2 mg/kg (s.c.) in ddY-strain mice [249], in line with our increase in total locomotion in both wild-type and DOR knockout C57Bl/6 mice at 3 mg/kg 7-hydroxymitragynine. This increase in locomotion upon administration may be the result of kratom alkaloid activity at MOR (as observed with lower doses of morphine administration, [281]) or on the noradrenergic or serotonergic systems, as activity of mitragynine has been observed at $\alpha_2$ adrenergic or 5-$HT_{2A}$ receptors in the central nervous system in studies investigating the analgesic properties of mitragynine [279].

In conclusion, of the kratom alkaloids investigated here, 7-hydroxymitragynine provides the most promising pharmacologic and behavioral profile for future drug development efforts

targeting functional selectivity at the DOR receptor, as acute administration 7-hydroxymitragynine was devoid of hypolocomotion and reward at a dose efficacious in reducing voluntary alcohol intake. Potential polypharmacology of all studied kratom alkaloids herein at additional GPCRs requires consideration, as exemplified by the non-DOR-selective locomotor effects of 7-hydroxymitragynine (as observed by hyperlocomotion in both wild-type and DOR knockout mice) and the aversion observed in a small number of mice to kratom extract, mitragynine, and 7-hydroxymitragyine in acute CPP testing. As such, our findings provide additional support for efforts to develop DOR selective G protein-biased agonists, either through the use of 7-hydroxymitragynine as starting scaffold or by structure-based drug design [232], for alcohol use disorder treatment.

# CHAPTER 3. GI’N TONIC: DORSAL STRIATAL $G_{I/O}$ SIGNALING FOR TREATMENT OF ALCOHOL USE DISORDER

Ethylalcohol (alcohol) is a small molecule with a big impact on our central nervous system. In 2016, 16.9% of the adult US population reported binge drinking, with 15 million estimated to suffer from alcohol use disorder (AUD). It is troubling that few medications exist to treat AUD, and this is reflected in the massive $250 billion annual cost alcohol places on the United States. Alcohol directly interacts with $GABA_A$ channels through a positive allosteric mechanism; yet, as a small molecule, alcohol perturbs many other CNS receptor systems. This promiscuity explains why, for example, the opioid receptor antagonist naltrexone is one of the three approved medications for AUD treatment. The mesolimbic and nigrostriatal pathways are two conserved midbrain projections heavily implicated in drug use disorders, including AUD. In contrast to the mesolimbic reward pathway which is important in the initial stages of drug use, hyperactivity of the nigrostriatal pathway (specifically in the dorsal striatum) is associated with the later stages of drug and alcohol use. Reducing dorsal striatal hyperactivity may be a potential therapeutic option to treat AUD patients. Interestingly, over 50% of the G protein-coupled receptors (GPCRs) expressed in the dorsal striatum are Gi-coupled. In this review article, we describe the role of the dorsal striatum in AUD, detail the pharmacology and function of striatal $G_{i/o}$-coupled GPCRs, and present preclinical and clinical findings of drugs acting at these GPCRs in behavioral models of alcohol use. It is our impression that agonists of dorsal striatally-expressed $G_{i/o}$-coupled GPCRs hold great promise for novel AUD treatments.

## 3.1 Significance of finding new treatment options for alcohol use disorder

Alcohol is a legal psychotropic drug that is widely abused across the world. Results from the 2016 National Survey on Drug Use and Health indicate that in the United States alone, 15.1 million adults reportedly suffer from alcohol use disorder (AUD). A recent 2017 study indicates that alcohol use in the US is on the rise [9], which is in juxtaposition to the general downward trend of nicotine use (another major legal drug of abuse) [282]. Alcohol abuse is not only harmful to the user; it also affects the family of the user and produces collateral damage to society at an annual cost surpassing $250 billion in the USA [283]. Given the magnitude of AUD as a societal health

issue, surprisingly few pharmacological treatment options exist for those seeking help. Only three distinct drugs have been approved by the Food and Drug Administration (FDA) to treat AUD, but few patients actively receive or adhere to these medications [284, 285].

One potential reason for the difficulty in identifying effective pharmacological interventions relates to the physical nature of the drug itself. Alcohol, or more accurately ethylalcohol or ethanol, is a very small molecule that can perturb lipid membranes, readily cross the blood brain barrier, and interact with a variety of different proteins [286, 287]. The primary target of alcohol is the $GABA_A$ channel, which alcohol modulates in a positive allosteric manner to increase the potency of the inhibitory function of this channel when bound to its endogenous agonist, GABA [288-290]. As alcohol increases GABAergic activity, this would suggest that blocking $GABA_A$ channel activity may be a therapeutic option for AUD. However, because GABA is the most abundant inhibitory neurotransmitter in the central nervous system (and thus provides a counterbalance to excitatory systems, such as the glutamate system, which become hyperactive upon chronic alcohol use), $GABA_A$ antagonism leads to a general stimulant effect. As a serious side effect, this resulting stimulant effect may cause seizures, thus explaining why $GABA_A$ antagonists are not regularly prescribed for alcohol use disorder treatment in a clinical setting [291]. A different treatment approach for AUD therapeutics is to not focus on the activity of a single drug target (such as $GABA_A$), but instead to manipulate activity of specific brain areas and/or circuits perturbed by prolonged heavy alcohol use. In this review, we focus on the dorsal striatum and G protein-coupled receptors (GPCRs) - specifically those that signal via inhibitory $G\alpha_{i/o}$ proteins (shortened to $G_{i/o}$ throughout this review) - that exhibit enriched expression in this brain region. In some cases, these receptor targets are already considered novel targets for possible AUD treatment.

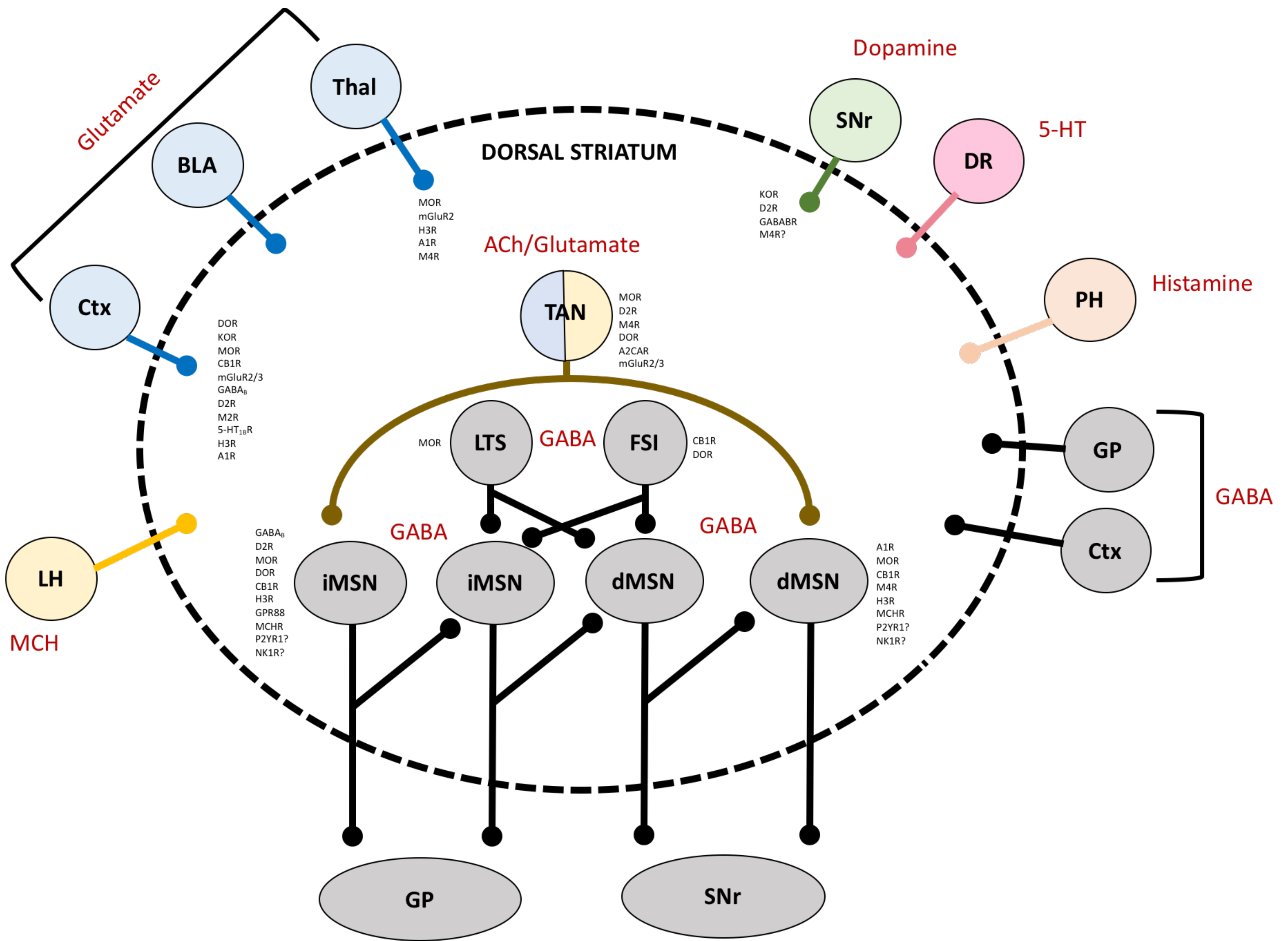
## 3.2 Neurophysiology of the dorsal striatum

The human caudate and putamen roughly correspond to the dorsomedial and the dorsolateral striatal subdivisions of the rodent dorsal striatum, respectively, and the ventral portion of the striatum is more commonly referred to as the nucleus accumbens (NAcc). The striatum contains two separate populations of GABAergic medium spiny projection neurons (MSNs) that are commonly distinguished by their expression of either dopamine $D_1$ receptors ($D_1R$) or dopamine $D_2$ receptors ($D_2R$) (Figure 3-1). $D_1R$-type MSNs are further characterized by expressing dynorphin and substance P, whereas $D_2R$-type MSNs characteristically express enkephalin and the

Figure 3-1. Neuroanatomy of the dorsal striatum.

The dorsal striatum receives melanin containing projections from the lateral hypothalamus (LH), glutamatergic projections from the cortex (Ctx), basolateral amygdala (BLA), thalamus (Thal), dopaminergic projections from the substantia nigra (SNr), serotonergic projections from the dorsal raphe (DR), histaminergic projections from the posterior hypothalamus (PH), and GABAergic projections from the globus pallidus (GP) and cortex (Ctx). Within the dorsal striatum, tonically active cholinergic or glutamatergic interneurons (TANs) project onto GABAergic medium spiny neurons (MSN) in the direct (dMSN) or indirect (iMSN) pathway, which then project to the substantia nigra (SNr) or globus pallidus (GP), respectively. Additionally, GABAergic low threshold spiking (LTS) and fast spiking (FSI) interneurons project to dMSNs and iMSNs. Within the MSN population, iMSNs form collaterals onto other iMSNs or dMSNs, and dMSNs onto other dMSNs, although no dMSN to iMSN collaterals have been established to date. $G_{i/o}$-coupled GPCRs expressed either pre- or post-synaptically are listed adjacent to their neuronal projections and/or neuron and the listed receptors may not be exhaustive.

*Figure provided by Dr. Brady Atwood (Indiana University-Purdue University-Indianapolis).*


Glutamate
Thal
BLA
Ctx
MOR
mGluR2
H3R
A1R
M4R
DORSAL STRIATUM
Dopamine
SNr
5-HT
DR
KOR
D2R
GABABR
M4R?
ACh/Glutamate
TAN
MOR
D2R
M4R
DOR
A2CAR
mGluR2/3
PH
Histamine
DOR
KOR
MOR
CB1R
mGluR2/3
$GABA_B$
D2R
M2R
$5\text{-}HT_{1B}R$
H3R
A1R
MOR
LTS
GABA
FSI
CB1R
DOR
GP
GABA
Ctx
LH
MCH
$GABA_B$
D2R
MOR
DOR
CB1R
H3R
GPR88
MCHR
P2YR1?
NK1R?
GABA
iMSN
iMSN
dMSN
GABA
dMSN
A1R
MOR
CB1R
M4R
H3R
MCHR
P2YR1?
NK1R?
GP
SNr

adenosine $A_{2A}$ receptor [292]. The striatum is rather homogenous in that ~95% of all striatal neurons are MSNs, with the remainder of neurons consisting of tonically active cholinergic neurons (TANs), somatostain-expressing low threshold spiking (LTS) interneurons, and parvalbumin-expressing fast-spiking GABAergic interneurons (FSIs) (Figure 3-1) [293, 294]. The dorsal striatum receives (*i*) serotonergic inputs from the raphe nucleus, (*ii*) dopaminergic inputs from the substantia nigra, (*iii*) histaminergic inputs from the tuberomamillary nucleus of the posterior hypothalamus, (*iv*) glutamatergic inputs from the cortex, thalamus, and basolateral amygdala, (*v*) GABAergic inputs from cortex and globus pallidus, and (*vi*) melanin concentrating hormone containing inputs from the lateral hypothalamus (Figure 3-1) [292, 295-302]. In the direct pathway of the basal ganglia, $D_1$R-MSNs project to the globus pallidus internus (GPi) and substantia nigra pars reticulata (SNr), whereas the $D_2$R-MSNs project to neurons within the globus pallidus externus (GPe), which in turn project to subthalamic nucleus and then the GPi and SNr in the indirect pathway. Activation of GABAergic $D_1$R-MSNs results in inhibition of GPi and SNr GABAergic neurons, ultimately leading to a disinhibition of the thalamic neurons and concomitant activation of the motor cortex neurons. Activation of the GABAergic $D_2$R-MSNs inhibits GPe GABAergic neurons, ultimately leading to disinhibition of GPi and SNr neurons and thereby strengthening their inhibitory control of the thalamic neurons [292, 303].

### 3.3 Role of dorsal striatum in alcohol consumption and transition to alcohol use disorder

The transition from casual, non-dependent alcohol use to alcohol dependence involves increased habitual and/or compulsive alcohol intake despite a decrease in the hedonistic effects (or reinforced devaluation) of alcohol [37, 304]. The initial rewarding effects of alcohol require engagement of the ventral striatum [37, 39-41], which becomes less engaged upon habitual or compulsive alcohol intake, as observed in heavy drinking human subjects compared to light social drinkers when measured by cue-induced fMRI responses [40]. With increased dorsal striatal engagement upon alcohol cue presentation in heavy drinking individuals [40], it is suggested that a change in drug behavior from "wanting" (associated with the ventral striatum) to a "must do" (associated with the dorsal striatum) begins, thus leading to increased habitual or compulsive drug-seeking and taking [39, 40].

Evidence of this shift is apparent by changes in neuronal plasticity in the dorsal striatum, as observed by increased neuronal hyperexcitability by altered glutamatergic and GABAergic

transmission upon alcohol exposure in rodents [305-307]. Additionally, subregion-specific plasticity changes are observed within the dorsal striatum to mediate alcohol behavioral reinforcement, where activity of the dorsomedial striatum (DMS) is associated with goal-directed behaviors and the dorsolateral striatum (DLS) is associated with habit formation [42, 43, 308-310]. It is thought that a response control shift from the DMS to the DLS is responsible for observed alcohol devaluation insensitivity in alcoholics [46, 311]. A study in rats observed that initial alcohol seeking was attenuated upon inactivation of the DMS (with no effect upon inactivation of the DLS); yet, upon longer exposure to operant alcohol training, animals became insensitive to alcohol devaluation, and inactivation of the DLS re-sensitized the animals to devaluation [46]. Alcohol exposure can also decrease inhibition of DLS MSNs in the dorsal striatum, leading to increased dorsal striatal MSN excitation output [312, 313] which may be further associated with maladaptive alcohol-related behaviors.

### 3.4 Role of striatal expressed GPCRs on alcohol use.

Based off mRNA copy number, approximately 55-60% of striatal GPCRs are $G_{i/o}$-coupled receptors, which by definition as $G_{i/o}$-coupled receptors inhibit adenylyl cyclase upon activation (Table 3-1, [314, 315]). Under the hypothesis that habitual alcohol use increases neuronal hyperactivity in the dorsal striatum, it is logical that agonists of $G_{i/o}$-coupled receptors may alter neurotransmitter release or neuronal excitability depending on their expression location, as increased $G_{i/o}$ protein activity leads to decreased levels of cAMP and associated downstream signaling pathways [316]. To investigate this hypothesis, we review relevant studies on $G_{i/o}$-coupled GPCRs expressed in the dorsal striatum.

#### 3.4.1 Opioid receptors

Alcohol use can increase the release of endogenous opioids in limbic reward areas of the brain [317-320], and this increase in endogenous opioid concentration upon alcohol intake is rewarding and involves increases in both endogenous enkephalins and β-endorphins levels [321]. Furthermore, selective infusion of non-endogenous opioid agonists in these regions can increase alcohol intake [262, 322-325], thereby suggesting that opioid antagonists may decrease alcohol consumption. Indeed, of the three FDA-approved AUD treatments, the relatively non-selective opioid receptor antagonist naltrexone (slightly preferring mu-opioid receptors (MOR) over kappa-

and delta-opioid receptors (KOR, DOR) [326]) has shown the most pronounced effects in reducing alcohol use in patients compared with other AUD therapeutics [327]. Including the nociceptin receptor, all four known opioids receptors are $G_{i/o}$-coupled and exhibit wide-spread CNS expression particularly in pain and reward centers [328-331].

In the dorsal striatum, MORs are located on both $D_1R$-type and $D_2R$-type MSNs, LTS interneurons, TANs, and glutamatergic inputs from thalamus and cortex [206, 208, 332-336](*Munoz et al., in revision)*. DORs are located presynaptically on $D_2R$-type MSNs and FSIs but may also be expressed postsynaptically on multiple cell types including TANs [206, 207, 337-339]. DOR are presynaptically expressed on corticostriatal projections into the dorsal striatum, but not thalamic projections, whereas MORs inhibit glutamate release from thalamostriatal and a subpopulation of corticostriatal projections [208](*Munoz et al., in revision).* DOR knockout (KO) mice exhibit enhanced alcohol place preference and self-administration compared to wild-type mice [211, 267], suggesting a protective effect of DOR expression on the rewarding effects of alcohol. Yet, modulation of alcohol intake by DOR selective compounds is complex; in the dorsal striatum, the DOR agonist SNC80 increases alcohol intake and the DOR antagonist naltrindole decreases intake [262]. However, in rats, the DOR agonist DPDPE injected into the ventral tegmental area (VTA) decreases alcohol intake [263].

One factor that may contribute to the different effects of DOR alcohol intake is that alcohol exposure can change DOR expression and/or signaling efficacy [340]. In mouse striatum, 7% alcohol exposure did not change DOR mRNA or DOR binding, but increased the efficacy of the DOR agonist DADLE to inhibit forskolin-induced cAMP production [341], thus suggesting an increase in $G_{i/o}$ protein coupling. Conversely, in other brain regions, alcohol exposure has been reported to decouple $G_{i/o}$ protein binding from DORs, as evident from reduced DOR agonist-induced GTPγS binding in rats after continued alcohol exposure [342]. In rats, it was also reported that DOR $G_{i/o}$ protein coupling decreases with age, but that alcohol exposure maintains DOR GTPγS signaling [262]. No significant changes in DOR binding were observed in the dorsal striatum between alcohol-preferring AA (alko, alcohol) and alcohol avoiding ANA (alko, non-alcohol) rats [343, 344], but alcohol-preferring sP (Sardinian preferring) rats showed significant lower DORs in the dorsal striatum than non-preferring sNP rats. This lower DOR binding in non-preferring sNP rats was partly reversed following alcohol use [345]. A different study looking at the effect of a single alcohol injection on DOR expression in the dorsal striatum observed changes

in [$^3$H]DPDPE binding, suggestive of increased DOR expression in the medial and anterior dorsal striatum, but not of decreased DOR expression in posterior dorsal striatum [346]. As mentioned previously, another factor in the role of DORs on alcohol consumption relates to downstream signaling of DOR agonists. Modulation of alcohol consumption by DOR agonists is tightly correlated with the agonist's ability to efficaciously recruit the scaffolding protein β-arrestin 2 compared to canonical $G_{i/o}$ protein pathway activation [347]. For example, infusion of the strong β-arrestin 2 recruiting DOR agonist SNC80 into the dorsal striatum of wild-type mice increases alcohol intake, but the same agonist decreases alcohol consumption when infused in β-arrestin 2 KO mice (*Robins et al., in revision*).

KORs have gained traction as target to treat cocaine use disorder [348], primarily based on KOR presynaptic expression on dopamine terminals in the NAcc and dorsal striatum [349-353], but also because of KORs role in the negative affect-like states induced by some drugs of abuse [354, 355]. Human studies have found that single nucleotide polymorphisms (SNPs) in the KOR and *predyn* genes are associated with increased risk for alcohol dependence [356, 357]. Additionally, alcohol exposure and/or withdrawal alters KOR and pDyn mRNA expression in humans [358] and animals [359, 360]. KOR KO, as well as preprodynorphin KO, mice self-administer less alcohol [209, 361], and KOR antagonists tend to decrease alcohol operant self-administration in rodents and reduce reinstatement of alcohol-seeking induced by the KOR agonist, U50,488 [362-364]. However, in voluntary, 2-bottle choice alcohol drinking protocols, U50,488 reduces alcohol intake [365] and preprodynorphin KO mice display increased alcohol use [366]. Similarly, the KOR antagonist NorBNI increases volitional alcohol intake in rats [367]. These contrasting results suggest that operant self-administration and volitional consumption may involve different brain regions (or, at least, different KOR-directed mechanisms). A number of studies investigating KOR modulation of alcohol use have primarily focused on the NAcc and central amygdala [368-370], where acute alcohol exposure leads to increased dopamine release in the NAcc which can be enhanced by blocking or disrupting KOR [371]. This increased dopamine release in the NAcc can conversely be attenuated by KOR agonists [372]. Chronic alcohol exposure reduces dopamine release in response to acute alcohol exposure because of increased KOR activity by dynorphins [373]. The increased sensitivity of the KOR system follow alcohol exposure is exemplified by the enhanced ability of U50,488 to inhibit dopamine release in alcohol exposed mice [370] and alcohol-drinking macaques [353, 374]. Long-term alcohol drinking in

macaques also exhibit increased KOR system sensitivity in the caudate nucleus; although, unlike in the NAcc, this increase sensitivity is not correlated with the lifetime amount of alcohol consumed [374]. Few studies have investigated the role of KORs in the dorsal striatum in alcohol behavior; however, it appears that KORs mimic DORs in this region, as observed by how alcohol exposure can increase transcription of preprodynorphin and activation of KORs by dynorphin in the dorsal striatum contributes to decreased alcohol intake [375].

MOR agonists such as morphine, oxycodone, and fentanyl are well known to be rewarding, and persistent use of MOR agonists can lead to the development of physiological and psychological dependence. However, MORs play not only an integral role in the development of opioid use disorders, but also to AUD [376-378], and the FDA-approved AUD treatment naltrexone preferentially blocks MORs. As such, it is not surprising that most alcohol studies investigating MOR have focused on antagonists and find that MOR agonists decrease alcohol use [379-382]. The rewarding effects mediated by MOR may stem from the large presence of MORs in the VTA on GABAergic interneurons that disinhibit the dopamine neurons projecting to the NAcc [383]. Alcohol self-administration does not alter MOR agonist DAMGO-induced GTPγS in most brain regions, including the striatal regions [384]. Local blockade of MORs in striatopallidal MSNs increases voluntary alcohol intake, while activation with DAMGO reduces alcohol intake [385]. However, a recent study using conditional MOR knockout mice demonstrated that deletion of MOR expression in GABAergic forebrain neurons - but not midbrain neurons - reduces alcohol consumption and conditioned place preference to the same degree as in the full MOR knockout [386]. The loss of MOR in these mice was most abundant in the striatum, thereby implicating the significant importance of these receptors in alcohol reward. The same mice were used to demonstrate that these forebrain MORs also control responding for heroin and palatable foods, further suggesting these receptors in general reward function [387].

### 3.4.2 Dopamine receptors

Dopamine receptors are classified into two families: $D_1R$-like ($D_1R$ and $D_5R$) or $D_2R$-like ($D_{2S}R$, $D_{2L}R$, $D_3R$, and $D_4R$), where $D_1$-like receptors couple to $G_s$-proteins leading to adenylyl cyclase-stimulated cAMP production and $D_2$-like receptors couple to $G_{i/o}$-proteins to inhibit cAMP production [316]. Alcoholism has been consistently associated with decreased dopamine release in the striatum [388, 389], and in humans, decreased striatal $D_2R/D_3R$ availability was observed in

detoxified alcoholics compared to controls [389-392]. Alcohol use has additionally been associated with decreased activity in the prefrontal brain regions necessary for executive functions such as salience attribution, inhibitory control, and planning. This reduction in $D_2$-like receptor activity may correlate with reports of increased vulnerability and impulsivity [393, 394] and increased craving and relapse in alcoholics [395], overall suggesting that increased inhibitory $D_2$-like receptor activity or expression in the dorsal striatum may prevent or reduce these alcohol-related behaviors. Indeed, other human studies suggest that increased $D_2R/D_3R$ expression in the striatum is protective against excessive alcohol intake [396].

Acute alcohol self-administration increases phasic dopamine concentration in both the DLS and DMS striatum in rats, but with more robust increases in the DLS [397]. In contrast, tissue levels of dopamine and the dopamine metabolite 3,4-dihydroxyphenylacetic acid, as well as tyrosine hydroxylase protein levels, are significantly decreased in the dorsal striatum of rats chronically exposed to alcohol compared to controls [398]. $D_1R$ KO mice drink and prefer alcohol less than wild-type or heterozygous C57Bl/6 mice [399]; while, somewhat surprisingly, global $D_2R$ KO mice also exhibit reductions in alcohol consumption, place preference, and increased alcohol aversion [400], which counters the aforementioned human effects of $D_2Rs$.

Recently, specific targeting of $D_1R$-MSNs versus $D_2R$-MSNs in the dorsal striatum has allowed us to better understand the individual contributions of these MSN subtypes on alcohol behaviors. The $D_1R$ antagonist SCH23390 significantly reduced dorsal striatal basal firing rates, thus decreasing neuronal activation of “anticipatory” cues which initiate alcohol seeking in rats who previously self-administered alcohol [401]. In the DLS region of the dorsal striatum, infusion of a $D_2R$ antagonist restored sensitivity to alcohol devaluation in extended alcohol-trained animals which previously demonstrated habitual and devaluation insensitive alcohol-seeking behavior [402], suggesting that $D_2R$ activity in the DLS is necessary for alcohol-seeking behavior, although this effect may be part of the general role of dopamine in motivated behavior rather than alcohol reinforcement [403, 404]. In the DMS, blockade of $D_1R$ activity by local antagonist administration attenuated alcohol consumption, while no changes in alcohol intake were observed upon blockade of $D_2R$ activity [307]. 8 weeks of intermittent, 2-bottle choice alcohol consumption increased glutamatergic transmission in $D_1R$-MSNs and GABAergic transmission in $D_2R$-MSNs [305]. Chemogenetic excitation of $D_1R$-MSNs and chemogenetic inhibition of $D_2R$-MSNs (to respectively mimic the alterations in input) increased alcohol consumption. Collectively these data

suggest that DMS dopamine signaling in $D_2$R-MSNs may contribute to "no-go" signals that reduce or prevent alcohol intake and dopamine signaling in $D_1$R-MSNs may allow a "go" signal for alcohol intake, leading to increased alcohol consumption

The partial $D_2$R agonist aripiprazole [405] can lower alcohol use in humans, specifically in subjects with lower impulse control [406], and can reduce craving and alcohol-free maintenance in detoxified alcohol-dependent individuals [407]. The partial agonist activity of aripiprazole may be key to its efficacy as full $D_2$R agonists, such as bromocriptine, increased sweetened alcohol solution intake in rats [408] and administration of full agonists can decrease $D_1$R and $D_2$R densities in the striatum of C57BL/6J mice [409]. The effect of signal-biased $D_2$R agonists on alcohol intake remains uninvestigated, although functional selectively has been observed at $D_2$R [234, 410, 411], and $D_2$R $G_{i/o}$ protein biased agonists may prevent receptor degradation compared to β-arrestin biased agonists thus providing therapeutic benefit.

The other $D_2$-like receptors, the $D_3$R and $D_4$R exhibit significantly lower expression in the dorsal striatum. $D_4$R knockout in male mice reduces mean alcohol intake, although this effect was specific to male mice, not females [135]. $D_3$R KO does not alter alcohol reward or intake in C57Bl/6 mice [412], although $D_3$R expression in the striatum is reportedly higher in rats after voluntary alcohol intake [413]; however, human studies suggest that this alcohol-induced increase in $D_3$R density is highest in the NAcc compared to the dorsal striatum [414]. Administration of the $D_3$R antagonist SB-277011-A and the partial agonist BP 897 reduces alcohol drinking reinstatement in an alcohol deprivation effect model [413, 415]. Importantly, the role of $D_3$R in alcohol dependence may be complicated by $D_3$R's autoreceptor control of the synthesis and release of dopamine [416, 417].

### 3.4.3 Serotonin 5-HT receptors

M any serotonergic receptors subtypes are thought to be expressed in the dorsal striatum, including the $G_{i/o}$-coupled 5-$HT_{1B}$ and 5-$HT_{1D}$, the $G_q$-coupled 5-$HT_{2A}$ and 5-$HT_{2C}$, and the $G_s$-coupled 5-$HT_6$, and 5-$HT_7$ receptor subtypes [418, 419]. Most binding studies in both rat and human reveal little-to-no evidence of 5-$HT_{1A}$R protein in this region [418]; instead, 5$HT_{1A}$Rs are primarily expressed in the dorsal raphe and hippocampus [420]. Still, in the dorsal raphe, 5-$HT_{1A}$R autoreceptors can reduce serotonin levels in the striatum, which is even more pronounced after voluntary alcohol consumption in C57Bl/6 mice [421]. Withdrawal from chronic ethanol increases

the sensitivity of presynaptic 5-$HT_{1A}$Rs which modulate synthesis of serotonin and dopamine in rat striatum [422]. Alcohol exposure has been shown to increase serotonin concentrations in the corpus striatum [423], yet other studies have shown reductions in serotonin levels in the striatum of alcohol-exposed rats [424] and human alcoholics [425], which is in line with data showing that serotonin depletion results in increased ethanol consumption in animals and humans [426-429]. Drugs which raise the central serotonin tone, such as selective serotonin reuptake inhibitors (SSRIs), decrease spontaneous alcohol intake in rodents [430-432].

In contrast to 5-$HT_{1A}$Rs, there is a dense distribution of 5-$HT_{1B}$Rs in the striatum. [433-438]. 5-$HT_{1B}$Rs regulate dorsal striatal and NAcc glutamate transmission [439, 440]. Interestingly, a significant increase in 5-$HT_{1B}$R mRNA levels are observed in the striatum of alcohol-exposed rats (2 weeks, 9% alcohol, liquid diet). However, heavy alcohol gastric feeding (12 g/kg/day) for 4 days shows no change in either 5-$HT_{1B}$R or 5-$HT_2$R binding [441]. Similarly, no change in 5-$HT_{1B}$R binding in dorsal striatum is observed between alcohol-preferring P versus non-preferring NP rats [442]. Similar to the DOR KO mice, 5$HT_{1B}$R KO mice consume more alcohol in a voluntary, 2-bottle choice [443]. Still, 5-$HT_{1B}$R modulation of alcohol intake is not unidirectional. For example, increased 5-$HT_{1B}$R expression through viral overexpression in the NAcc leads to augmented voluntary alcohol intake [444, 445], which may be related to findings that local infusion of the selective 5-$HT_{1B}$R agonist CP-93,129 facilitates striatal dopamine release *in vivo* and increases dopamine release in the NAcc [446, 447]. Alcohol increases dopamine release in VTA and NAcc [448], and this is blocked by VTA infusion of a 5-$HT_{1B}$R antagonist but enhanced by a 5-$HT_{1B}$R agonist [449]. This is in line with human findings of increased 5-$HT_{1B}$R binding potential values in the ventral striatum of AUD patients compared to healthy individuals [450].

While 5-$HT_{1A}$ and 5-$HT_{1B}$ receptors facilitate dopamine release, 5-$HT_2$Rs mediate inhibition of dopamine efflux [451]. Alcohol enhances 5-$HT_{2A}$R binding in the corpus striatum [423] as well as 5-$HT_{2C}$R mRNA, but not receptor protein expression [452]. Thus far, the 5-$HT_{1D}$R appears to play little role in the pathophysiology of alcohol addiction [453]. Systemic administration of an agonist for the Gs-coupled [454] 5-$HT_6$R increase dopamine release in the NAcc [455] and antagonists of 5-$HT_6$R reduce alcohol-seeking [456] and voluntary alcohol consumption in rats [457]. Overexpression of 5-$HT_6$R in the dorsomedial striatum (DMS) prevents mice from acquiring a reward-based instrumental learning task [458]. The finding that blocking the 5-$HT_6$R, which is $G_s$-coupled, results in decreased alcohol intake is in agreement with the

increased alcohol intake phenotype of the $G_{i/o}$-coupled $5HT_{1B}R$ KO mice, and in agreement with the general consensus is that $G_{i/o}$-coupled 5-HT receptors have a protective role, whereas $G_s$-coupled 5-HT receptors negatively impact alcohol use [459]. Few studies have investigated the $G_s$-coupled 5-$HT_7R$ in relation to alcohol use disorders [460]. Alcohol exposure has been shown to increase 5-$HT_7R$ mRNA in several brain regions, including the dorsal striatum; however, antagonism of 5-$HT_7R$ does not reduce or alter voluntary alcohol consumption in C57BL/6 mice [452]. Overall, it appears that alcohol preference is inversely correlated with serotonin levels [461] i.e. low serotonin correlates with higher alcohol intake, and the dorsal striatum plays an important role in this behavior as it is a major recipient of serotonergic projections from the dorsal raphe nucleus.

#### 3.4.4 Histamine $H_3$ receptors.

Of the four histamine receptors, the histamine $H_3$ receptor is most abundantly expressed in the central nervous system, including the dorsal striatum [462]. The $H_3R$ is a known autoreceptor controlling release of histamine from histaminergic neurons that originate in the tuberomamillary nucleus of the posterior hypothalamus and project to many brain regions, including the striatum [463, 464]. Radioligand binding suggests the $G_q$-coupled receptor $H_1$ and the $G_s$-coupled $H_2$ receptors may be expressed in the striatum [465], causing activation of cholinergic interneurons [466] and MSNs [467]. However, the predominantly expressed histamine receptor is the $H_3R$, which modulates release of histamine and other neurotransmitters, such as GABA and glutamate, via its presynaptic action, but may also be expressed postsynaptically, including in the striatum where the $H_3R$ are expressed on $D_1R$- and $D_2R$-expressing MSNs with activation leading to decreased GABA release [467-471]. The balance of $H_2R$ and $H_3R$ activation in MSNs seems tilted towards $H_3Rs$ given histamine reduces GABA release from MSN collaterals within dorsal striatum [467]. The majority of studies investigating the histamine system in alcohol behaviors have focused on the role of $H_3Rs$, where studies have found that $H_3R$ KO mice drink less alcohol than wild-type mice in a drink-in-the-dark model of volitional consumption [472]. In line with this observation, $H_3R$ antagonists, such as ciproxifan, DL77, and ST1283 reduce alcohol intake and alcohol place preference, whereas the agonist immepip increased alcohol use [472-474]. It is currently difficult to speculate by what mechanism $G_{i/o}$-coupled $H_3Rs$ increases alcohol

consumption given its post- and pre-synaptic expression pattern on both $D_1R$-type and $D_2R$-type MSNs.

### 3.4.5 Melanin-concentrating hormone MCHR1 receptors

The melanin-concentrating hormone receptor (MCHR1, previously SLC-1 and GPR24), is a $G_{i/o}$-coupled receptor (although it may promiscuously couple to $G_q$-proteins as well [475]) that is activated by melanin-concentrating hormone, a hypothalamic peptide. Lateral hypothalamic neurons projecting to the striatum can release MCH which can act on MCHRs that are expressed on striatal MSNs [476]. The ability of MCHR1 to impact dopamine function in the striatum is evident from findings that MCHR1 KO mice show several adaptations in their dopamine systems - including decreased $D_2R$ expression, increased dopamine transporter expression, and altered locomotor activity induced by psychostimulants [477]. Activation of MCHR1 can increase dopamine levels that appear to encode both reward and nutritional values [478]. While MCHR1 is expressed in the dorsal striatum, previous investigations have focused on MCHR1 in the NAcc. Rats previously exposed to intermittent alcohol exhibit a decrease in NAcc MCHR1 mRNA during abstinence [479]. Similar to the $H_3R$ function, it has been reported that the MCHR1 antagonist GW803430 suppresses alcohol self-administration [479, 480]. It is unclear if there is a common mechanism behind the observation that both the hypothalamic histamine and melanin systems appear to promote alcohol use.

### 3.4.6 GPR88

GPR88 is an orphan $G_{i/o}$-coupled receptor [481] with strong expression in GABAergic forebrain neurons, including $D_1R$-type and $D_2R$-type type MSNs in the dorsal striatum [482, 483]. In GPR88 KO mice, these MSNs display augmented glutamatergic excitation and reduced GABAergic inhibition [484], and it has been reported that GPR88 KO mice demonstration enhanced alcohol intake and preference over water as well as increased alcohol place preference compared to wild-type mice [485]. This would suggest that GPR88 plays a protective role in alcohol use and that small molecule GPR88 agonists [481, 486] could serve as novel therapeutics for AUD. Furthermore, GPR88 KO mice show increased $G_{i/o}$ coupling to MORs and DORs in striatum, and inhibition of DORs in these mice partially restores multiple behavioral phenotypes, including striatal-based behaviors such as motor coordination [487]. Certain anxiety-like behaviors

(light/dark box, elevated plus maze) are lost in $D_2$R-MSN-specific GRP88 KO mice [488]. $D_2$R-MSN-specific GRP88 KO mice also display significantly altered responses to dopamine receptor agonists [489]. It is conceivable that alcohol-induced behavioral impairments could also be targets of GPR88 small molecule agonists alone or in concert with DOR ligands.

### 3.4.7 $\alpha_2$-adrenergic receptors.

$\alpha_2$-ARs likely reside primarily on MSNs [490], and the $G_{i/o}$-coupled $\alpha_{2C}$ adrenergic receptor ($\alpha_{2C}$-AR) is abundantly expressed in the striatum [491], where it is thought to increase GABA release when activated [492]. The $\alpha_2$-ARs are known to play an important role in pain transmission [493, 494], but are also recognized for their therapeutic role in alcohol and opioid withdrawal [495, 496] as well as stress-induced reinstatement of alcohol drinking [497-500]. The non-isoform-specific $\alpha_2$-AR agonist guanfacine reduces alcohol intake, seeking, and reinstatement in rats [497]; yet, despite its striatal expression, no studies have investigated the specific role of $\alpha_{2C}$-AR activity on addictive behavior. The lack of $\alpha_{2C}$-AR research may be because of the lack of many commercially available subtype selective agonists. (R)-(+)-m-nitrobiphenyline [501] and fadolmidine [502] appear to be the only subtype selective $\alpha_{2C}$-AR receptor agonists; however, it is unclear if these drugs are brain penetrable and/or have cardiovascular side effects. To investigate the role of $\alpha_{2C}$-ARs in modulation of alcohol behaviors, these agonists could be microinfused into the dorsal striatum of alcohol drinking animals to measure changes in alcohol consumption following $\alpha_{2C}$-AR activation. Future work will therefore need to resolve the possible role of $\alpha_2$-ARs, such as $\alpha_{2C}$-AR, in alcohol-related behaviors.

### 3.4.8 Muscarinic receptors

The muscarinic $M_4$ receptors ($M_4$R) is another $G_{i/o}$-coupled receptor [503] which is heavily expressed in the striatum, both postsynaptically on primarily $D_1$R-type MSNs [504, 505] as well as presynaptically on cholinergic interneurons and thalamic inputs [506-508]. This localization of $M_4$Rs allow $M_4$R activity to directly inhibit MSNs as well as reduce dopamine release from nigrostriatal projections by inhibiting acetylcholine release, thus preventing activation of presynaptic nicotinic acetylcholine receptors on the nigrostriatal terminals. The striatal function of $M_4$Rs on the dopaminergic system is reflected in the antipsychotic properties of $M_1$R/$M_4$R agonists. In relation to alcohol use, it has been shown that $M_4$R KO mice display elevated alcohol

consumption [509]. Producing muscarinic receptor subtype selective agonists is not trivial, with many muscarinic $M_4R$ drugs also binding $M_1R$, a $G_q$-coupled receptors that is also expressed in the striatum but has functions that are distinct from $M_4Rs$ on behaviors such as place reversal learning [510]. In the striatum, $M_1R$ antagonists prevent alcohol-induced hyperpolarization of MSNs [511]. The difficulty in developing selective muscarinic agonists has led to increased efforts to identify of $M_4R$ selective positive allosteric modulators [512, 513], where positive allosteric modulation of $M_4R$ has been shown to successfully reduce amphetamine- and cocaine-induced dopamine release in the NAcc and dorsal striatum [512, 514], suggesting the therapeutic value of $M_4R$ positive allosteric modulators in altering drug-induced dopaminergic responses. The $G_{i/o}$-coupled $M_2$ receptor does not appear to play a major role in regulating acetylcholine release in striatum [508] and, because of the overlapping pharmacologies of $M_2R$ and $M_4R$, many effects attributed to $M_2R$ signaling in striatum may result from $M_4R$ instead.

#### 3.4.9 $GABA_B$ receptors

Alcohol's ability to act as a positive allosteric modulator of $GABA_A$ channels is well-known. However, the $G_{i/o}$-coupled $GABA_B$ receptors [515] also play a role in alcohol-related behaviors. Baclofen, particularly R-baclofen [516-518], a $GABA_BR$ agonist has been used off-label as treatment option for AUD [519], although clinical results have not been convincing [520] despite positive results in preclinical studies [521]. Sodium oxybate (gamma hydroxybutyrate), an endogenous precursor of GABA, is another $GABA_BR$ agonist that has been used clinically for treatment of AUD [522]. Side effects associated with $GABA_BR$ agonists have led researchers to investigate positive allosteric modulation of $GABA_BR$ as an alternative preclinical approach to reduce alcohol intake [523], and it is thought that activating the inhibitory $GABA_BR$ may counter the enhanced glutamatergic action observed during alcohol withdrawal [524].

$GABA_BRs$ are expressed in midbrain projections to the striatum [525-531]. Interestingly, alcohol-preferring sP rats show lower potency for baclofen-induced GTPγS activation compared to non-alcohol preferring rats. However, alcohol exposure in sP rats normalizes the baclofen response [532], and similarly, alcohol increases baclofen-induced activity of G protein inwardly rectifying potassium (GIRK) channels mediated by post-synaptic $GABA_BR$ located on VTA and substantia nigra neurons projecting to the striatum [533]. Microinjection of baclofen into the VTA reduces binge-like consumption of alcohol in mice [534]. It has been difficult to study $GABA_BR$

KO mice as they show viability issues [535, 536]; therefore, while the $GABA_BR$ is a $G_{i/o}$-coupled receptor with expression in the dorsal striatum and a protective role in alcohol use, there currently is not enough mechanistic data available to link striatal $GABA_BRs$ to this behavior.

### 3.4.10 Metabotropic glutamate receptors

The excitatory neurotransmitter glutamate acts both at ionotropic (NMDA, AMPA, kainate) and metabotropic glutamate receptors (mGluRs). The glutamate system plays an important role in the progression of AUD, particularly in association with aspects of negative effect and relapse behavior during abstinence [524]. It has been suggested that adaptations to glutamatergic circuits in the corticostriatal reward network, including increased glutamatergic tone, are important in development of drug use disorders, including AUD [537-540]. Based on these reports, antagonism of the glutamate system would be expected to reduce alcohol use [541]. For example, acamprosate is one of three FDA-approved drugs for the treatment of AUD, and while the pharmacology of acamprosate is unclear, it's mechanism of action is thought to involve modulation of glutamatergic NMDA receptors (potentially as a partial agonist) [542].

There are eight different mGluRs which are commonly organized into three groups. Group I mGluRs include mGluR1 and mGluR5, are mostly expressed postsynaptically, and couple through $G_q$ proteins [543, 544]. On the other hand, Group II (mGluR2/3) and Group III (mGluR4/6/7/8) all signal through $G_{i/o}$ proteins and are largely - but not exclusively - located presynaptically [543, 544]. Glutamatergic projections from the thalamus and cortex synapse in the dorsal striatum [298, 545-547], and cholinergic interneurons within the striatum co-release glutamate [548]. mGluR2/3s inhibit glutamate release from cortical inputs [549-551] and thalamic inputs are inhibited by mGluR2 activation, consistent with high mGluR2 mRNA expression in thalamus [550, 552]. In addition, activation of presynaptic mGluR2 on thalamic inputs reduces dopamine release through an indirect action on cholinergic interneurons [550] and activation of mGluR2/3 reduces NMDA evoked acetylcholine release [553]. The mGluR2/3 agonist LY379268 reduces alcohol self-administration and seeking in rats [554, 555], whereas the mGluR2/3 antagonist, LY341495, increases alcohol consumption in Wistar rats [556]. Infusion of LY379268 into NAcc core reduces alcohol-seeking behavior [555]. Interestingly, the mGluR2 positive allosteric modulators, BINA and AZD8529, produce only moderate reductions in alcohol-seeking behavior in Wistar rats [555, 557], but AZD8529 significantly blocks cue-induced alcohol-seeking

behavior [557]. Alcohol-preferring P rats carry a genetic mutation introducing a stop codon into the mGluR2 gene, (Grm2 *407), thus disrupting mGluR2 expression and Group II mGluR-mediated inhibition of glutamatergic transmission in dorsolateral striatum. These findings are recapitulated in mice with a genetic disruption of mGluR2 that display increased alcohol intake [556]. It should be noted that that other genes encoding proteins involved in synaptic transmission differ between P rats and the selected line of non-preferring rats. AZD8529 was ineffective in P rats, supporting its specificity for mGluR2 [557]. Further work is required to unravel the exact mechanism of striatal $mGlu_{2/3}$ receptors in relation to alcohol intake behaviors.

The Group III mGluRs, including mGluR4 and mGluR7, are localized and inhibit neurotransmitter release at glutamatergic and GABAergic synapses in dorsal striatum [551, 558-565]. At glutamatergic synapses, mGluR4 is exclusively expressed on cortical inputs [566]. Knockout of mGluR7 expression globally or within nucleus accumbens results in increased alcohol drinking behavior [567, 568] and increased alcohol conditioned place preference [567]. The mGluR7 antagonist MMPIP increases alcohol consumption and preference, whereas the allosteric agonist AMN082 decreases alcohol consumption and preference [569]. AMN082 decreases operant self-administration of alcohol and sucrose [570]. Inbred strains of mice that have lower mGluR7 expression consume more alcohol than those with higher expression, although these genetic differences were interestingly limited to hippocampus and certain regions of cortex [568, 571]. mGluR4 null mice display reduced alcohol-stimulated motor responses compared to wild-type mice, but did not show differences in alcohol consumption, preference, withdrawal or impaired righting reflex [572]. Knockout or inhibition of mGlu5R, a Gq-coupled receptor, reduced alcohol self-administration [554, 573] as well as the discriminative stimulus of alcohol, but this effect seems to originate from the NAcc and not the dorsal striatum [574].

### 3.4.11 Cannabinoid $CB_1$ receptors

Cannabinoid receptors have increasingly been recognized as potential therapeutic target for a wide variety of diseases and disorders, including AUD [575, 576]. Two cannabinoid receptors have been identified ($CB_1$ and $CB_2$), both of which are $G_{i/o}$-coupled, but show distinct distribution patterns [577]. $CB_1$ receptors are ubiquitously expressed throughout the CNS, including heavy expression in the striatum, cerebellum, hippocampus and cortex and globus pallidus [577, 578]. $CB_1$ receptors are found on cortical inputs to dorsal striatum, on both classes of MSNs as well as

FSIs [579-582]. $CB_1Rs$ might be expressed postsynaptically at thalamostriatal synapses [582] and $CB_1R$ activation regulates glutamate, GABA, acetylcholine, and dopamine release in striatum [579, 583-586]. The role of $CB_1Rs$ in alcohol use is evident from reports finding that $CB_1R$ KO mice consume and self-administer less alcohol than wild-type mice and show reduced alcohol place preference [587-591], whereas mice lacking the gene for fatty acid amide hydrolase (FAAH), the enzyme responsible for breaking down the endogenous cannabinoid anandamide, show enhanced alcohol intake [592]. In line with these findings, $CB_1R$ antagonists also decrease alcohol use in animals [593-595] and agonists increase alcohol consumption [596, 597]. Induction of $CB_1R$ plasticity at corticostriatal synapses onto direct pathway MSNs reduces ethanol self-administration [598].

In low alcohol-preferring mice, such as the DBA strain, a $CB_1R$ agonist will actually increase alcohol intake [599] and $CB_1R$ receptor expression is generally lower in alcohol-preferring C57BL/6 mice than in DBA mice [600]. Compared to Wistar rats, alcohol-preferring fawn-hooded rats display lower $CB_1R$-stimulated GTPγS in various brain regions, including the dorsal striatum [601]. However, another study comparing naïve alcohol-preferring sP rats with sNP rats found higher levels of $CB_1R$ protein and mRNA in the striatum and other regions [602]. Alcohol consumption in these animals was associated with increased concentration of endocannabinoids in the striatum and resulted in a reduction in striatal $CB_1R$-mediated $G_{i/o}$ protein coupling. Alcohol withdrawal reduced these effects [602]. Rimonabant, a $CB_1R$ receptor antagonist, decreases alcohol intake, in line with the $CB_1R$ KO data, and also corrects the $CB_1R$-mediated $G_{i/o}$ protein activation [602]. Acute and chronic alcohol exposure causes a significant reduction in $CB_1R$ mRNA and protein expression in the dorsal striatum, as well as other regions [317, 603], which is in line with the alcohol-induced reduction in $CB_1R$ function [604-607]. Although other studies have observed enhanced $CB_1R$ activity in the dorsal striatum of alcoholics, [608], in human subjects, patients with a history of alcohol dependence show a reduction of $CB_1R$ receptor binding during abstinence [609]. The wide distribution pattern of the cannabinoid system and the extra-vesicular release of endogenous cannabinoids make it difficult to determine a precise role of striatal $CB_1Rs$ in alcohol behavior using the available data.

### 3.4.12 Purinergic receptors.

Purinergic receptors are defined by their response to ATP, ADP or adenosine and can be either metabotropic (adenosine $A_{1-3}$ and P2Y receptors) or ionotropic (P2X receptors) [610-612]. Adenosine-mediated glutamate signaling in the striatum regulates sensitivity to ethanol intoxication and intake [613]. Adenosine receptors, especially $A_1$ and $A_{2A}$ receptors are well known to be expressed alongside dopamine receptors in striatal MSNs. Specifically, the $G_{i/o}$-coupled $A_1R$ is commonly found in $D_1R$-type MSNs, and local infusion of the $A_1R$ selective agonist, N6-cyclohexyladenosine into the striatum decreases striatal cAMP in alcohol-exposed (but not alcohol-naïve) animals via $G_{i/o}$ protein activation [614]. $A_1Rs$ are also expressed presynaptically on cortical and thalamic inputs to dorsal striatum where they inhibit glutamate release [615]. $A_1R$ antagonism reduces ethanol consumption and blood ethanol levels in a mouse model of binge drinking, but interestingly, had no effect on sucrose consumption [616]. While $A_1R$ KO mice exist [617], to our knowledge they have not been investigated in alcohol intake studies thus far.

Adenosine receptors have been extensively reported to engage in heteromeric protein-protein interactions forming $A_{2A}R$-$D_2R$ heterodimers on excitatory striatopallidal neurons and $A_1R$-$D_1R$ heterodimer on striatonigral and striato-entopeduncular inhibitory GABA pathways [618]. The $G_s$-coupled $A_{2A}$ receptor is primarily expressed in $D_2R$-type MSNs [619-622]. One study found that $D_1R$+$A_{2A}R$ knockout mice exhibited a larger reduction in ethanol consumption compared to $D_1R$-deficient mice [623]. Specifically, in the DMS, a reduction in $A_{2A}R$ functionality has been associated with increased alcohol consumption in mice because of non-alcohol-specific increases in goal-oriented behavior [624, 625]. Consequently, $A_{2A}R$ knockout mice demonstrate augmented alcohol intake over wild-type mice [624], and $A_{2A}R$ antagonists similarly increase alcohol consumption in rats, where conversely an $A_{2A}R$ agonist such as CGS 21680 reduces alcohol self-administration [626-628]. However, in a mouse model of binge drinking, an $A_{2A}R$ antagonist had no effect on alcohol consumption, but did produce locomotor stimulation [616]. As stated earlier, the striatum is an important component of the direct and indirect pathways of movement. As such several studies have investigated the role of adenosine receptors in ethanol-induced motor incoordination [625, 629-632], and adenosine receptors have been implicated in modifying alcohol-withdrawal associated behaviors such as anxiety-like behavior, tremors, and seizures [618]. Mice lacking $A_{2A}R$ display reduced sensitivity to the hypnotic effects of alcohol

and reduced alcohol-withdrawal seizures [624, 633]. Overall, these studies further suggest that adenosine signaling, both at $A_1R$ and $A_{2A}R$, may provide important fine-tuning of striatal synapses in alcohol-related behaviors.

The ionotropic P2X receptors, particularly $P2X_4R$, is expressed in the striatum and has been linked with alcohol use in mice [634-636]. In rat striatum, the $G_{i/o}$-coupled $P2Y_{12}R$ is expressed on oligodendrocytes [637, 638] [639]; nonetheless, little is known about the role of striatal P2Y receptors and their ability to modulate alcohol behaviors

### 3.5 Impact of modulation of striatal G-protein signaling on alcohol use

GPCRs do not function in a vacuum but instead rely on a machinery of accessory proteins for signal transduction as well as signal termination (Figure 3-2). $G_{i/o}$-coupled receptors inhibit cAMP production by adenylyl cyclases, and alcohol-dependent patients and those in abstinence display decreased AC activity [640]. Numerous isoforms of adenylyl cyclase exist, but the dorsal striatum is highly enriched in adenylyl cyclase type 5 (AC5) [641-643]. AC5 has a preference to be activated by 'Gs-like' olfactory G proteins [644, 645], and exhibits high expression in the dorsal striatum (striosomes) [646, 647]. AC5 function is particularly linked to two $G_s$-coupled receptors abundantly expressed in the striatum: $D_1$ and $A_{2A}$ receptors [648]. AC5 KO mice demonstrate increased alcohol intake [649], an observation in agreement with decreased AC activity in alcohol-dependent patients [640]. However, AC5 KO also exhibit peculiar feeding habits [650], therefore alterations in olfaction may be a potential factor in the increased behavioral responding of AC5 KO mice to alcohol. Because of the role of the dorsal striatum in locomotor activity, the striatal expression of AC5 may also be associated with the observation that AC5 KO mice are less sensitive to the hypolocomotive effects of alcohol [649]. The alcohol phenotype of AC5 KO mice resembles that observed for mice with a disruption in the RII-regulatory subunit of protein kinase A (PKA), the enzyme activated by AC-produced cAMP [651]. Striatal AC1 may also play a role in behavioral responses to ethanol. AC1 is a calcium/calmodulin-sensitive isoform of AC, but it is also targeted by G proteins [652] and it is more highly expressed in dorsal striatum than in ventral striatum [653]. Mice treated with the AC1 inhibitor NB001 or AC1 KO mice display locomotor responses that are equivalent to those of wild-type or untreated mice in response to acute alcohol, but fail to develop locomotor sensitization [654]. These effects were linked to differences in dorsal striatal NMDAR signaling. Human alcoholic brains show increased levels of AC1 [655], thus

GPCR-mediated regulation of AC1 - in conjunction with calcium/calmodulin signaling - may be a critical determinant in behavioral responding to alcohol.

Under normal circumstances, cAMP is inactivated by phosphodiesterases (PDEs) to form 5-AMP. Because of the important roles of $G_{i/o}$- and $G_s$-coupled receptors in alcohol use, several studies have investigated the role of particular PDE isoforms on alcohol consumption in mice [656]. Inhibition of PDE isoforms which are enriched in striatal neurons (particularly PDE4 and PDE10a) [657, 658] leads to an increase in intracellular cAMP and results in decreased alcohol behaviors in mice [659-664]. These observed changes in cAMP and alcohol-related behaviors agrees with the AC5 KO alcoholic phenotype. These observations would argue that it is $G_s$ (and not $G_{i/o}$) signaling

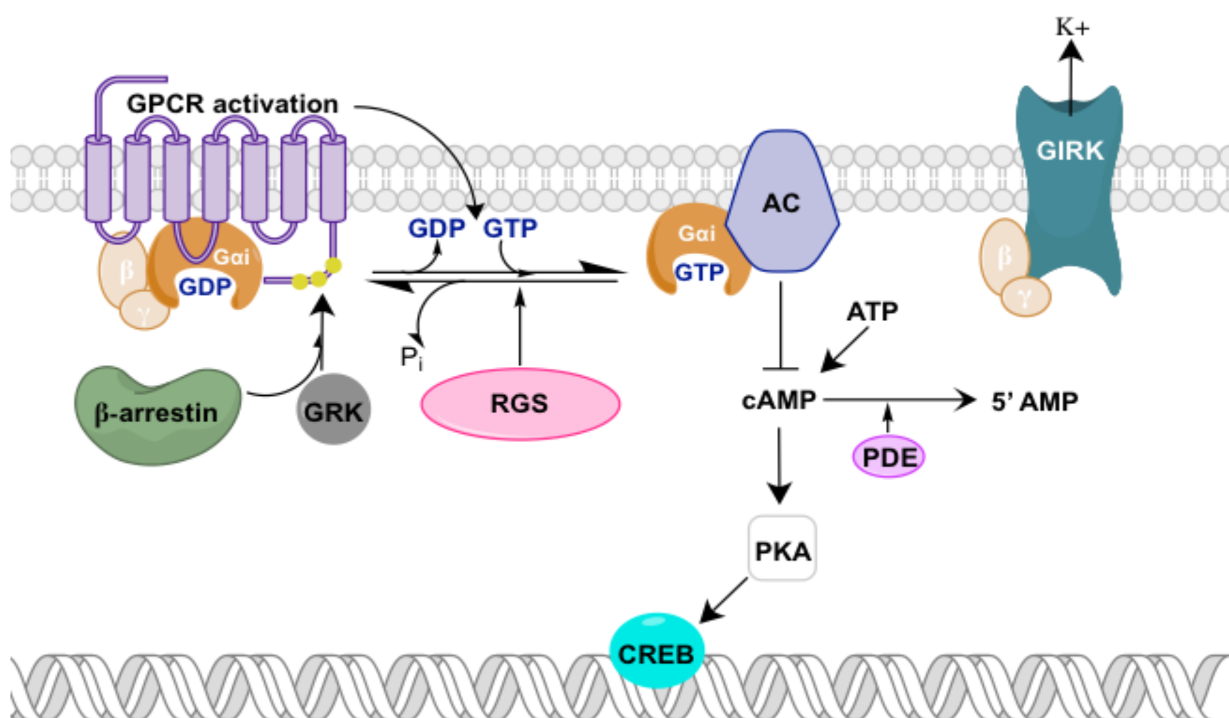



Figure 3-2. Modulation of GPCR signaling.

Following activation, the $G_{i/o}$-protein dissociates from the receptor to bind to adenylyl cyclase (AC) and inhibit the production of cAMP. cAMP will otherwise activate protein kinase A (PKA) which can activate transcription factors such as CREB. cAMP is dephosphorylated to from 5'AMP, a process can be blocked by phosphodiesterase (PDE). The Gβγ subunits of the heterotrimeric G-protein can activate G-protein inwardly rectifying potassium channels (GIRK). Regulator of G-protein signaling (RGS) will hydrolyze the GTP activated $G_{i/o}$-protein to attenuate $G_{i/o}$-signaling. After receptor activation G-protein receptor kinases (GRK) can phosphorylate the receptor, which will increase the receptors affinity for binding β-arrestin and promote receptor desensitization and internalization.

that is protective for alcohol use, although one aspect of $G_{i/o}$-coupled receptor pharmacology is that chronic activation can lead to so-called cAMP superactivation or heterologous sensitization of adenylyl cyclase [204, 665, 666]. This phenomenon has been demonstrated for several striatal $G_{i/o}$-coupled receptors, such as the $D_2R$, DOR, and $M_4R$, and the impact of AC sensitization and cAMP superactivation on alcohol-related behaviors has yet to be investigated.

Besides inhibition of adenylyl cyclase, activation of $G_{i/o}$-coupled receptors can also lead to activation of G protein inwardly rectifying potassium channels (GIRKs or Kir3) via the dissociation of Gβγ subunit from the original heterotrimeric G protein [667, 668]. Activation of GIRKs induces a hyperpolarization and decreases neuronal excitability via increased potassium efflux [669]. GIRKs in the mesolimbic system play a role in drug addiction ([669, 670] and can be directly activated by alcohol [671, 672]. Interestingly, GIRK3 KO mice display increased alcohol intake [673], suggesting a potential protective role of GIRK3 expression in alcohol behavior. Considering that $G_{i/o}$ proteins activate GIRK3, this would extrapolate to activation of $G_{i/o}$ protein-coupled receptors having a propensity to be protective for alcohol use through subsequent increases in Gβγ-activation of GIRKs.

For GPCR signal termination, activated G proteins can be deactivated by regulators of G protein signaling (RGS) proteins which promote the hydrolysis of GTP in the active Gα-protein [674]. As such, inhibition of RGS proteins potentiates and/or prolongs GPCR signaling. Several RGS isoforms show high expression in the dorsal striatum, particularly RGS2, RGS4, RGS9-2, and RGS20 (or RGSZ1) [674, 675]. Human studies have shown that SNPs in RGS4 and RGS17 are associated with increased risk of alcohol dependence [676]. RGS6 KO mice consume less alcohol and exhibit lower alcohol conditioned place preference than wild-type mice, suggesting that decreases in RGS activity may be protective to increased alcohol consumption and reward. The RGS6 KO mice also display significantly lower dopamine content in the striatum, although this phenotype could partially be reversed using either $D_2R$ or $GABA_BR$ antagonists [677]. Interestingly, RGS proteins do not only interfere with Gα-protein modulation of AC but can also modulate GIRK activity [678, 679], which may be additionally protective for alcohol-related behaviors as stated previously.

Following receptor activation, GPCRs are phosphorylated by G protein receptor kinases (GRKs). This phosphorylation causes GPCR signaling desensitization by increasing the receptors' affinity for β-arrestin proteins, where recruitment of β-arrestin contributes to receptor

internalization [680-682]. GRKs are widely expressed in the CNS and extensively studied in relation to opioid tolerance [683-685], and psychostimulant-induced hyperlocomotion is altered when disrupting GRK2 in striatal neuronal populations [686]. However, few studies have investigated GRK isoforms in relation to alcohol use or drug self-administration. Chronic alcohol exposure has been reported to increase the interaction of GRK2 with MORs in the hippocampus [687], but decrease GRK2 expression and MOR endocytosis in the VTA [688]. Additional studies are necessary to understand how GRK activity is altered upon alcohol exposure.

Over the last two decades, it has become apparent that besides their role in GPCR desensitization and trafficking, β-arrestin proteins can also scaffold with signaling proteins, such as MAP kinases and take part in the signal transduction cascade independent of G proteins [689]. Moreover, these signaling pathways may be distinct from those engaged by G proteins, leading to unique behavioral effects that can be therapeutic or adverse in nature [690]. Increased efforts are ongoing to develop drugs targeting GPCRs that are biased towards either the G protein or β-arrestin pathway as a way to produce medications that have fewer side effects [691]. There are two β-arrestin isoforms (1 and 2) both of which are ubiquitously expressed in the CNS [692]. β-arrestin 1 KO female mice have a tendency to consume more alcohol (*Robins et al., accepted*), suggesting a protective role for this isoform. In contrast, it has been reported that alcohol preferring AA rats have increased amount of β-arrestin 2 mRNA in the dorsal striatum compared to ANA rats. Additionally, β-arrestin 2 KO mice drink less alcohol [693]. A follow up study found that alcohol causes dopamine release at lower concentrations of alcohol in β-arrestin 2 KO mice than wild-type mice, and that β-arrestin 2 KO mice exhibit alcohol CPP at lower alcohol doses than wild-type mice [694]. For DOR agonists, β-arrestin 2 recruitment is tightly correlated with increased voluntary alcohol intake in mice, and specifically DOR agonists that strongly recruit β-arrestin 2 to DORs increase alcohol intake. In contrast, $G_{i/o}$-biased DOR agonists decrease alcohol consumption [347]. This behavior can be pinpointed to DOR activity in the striatum; injecting a β-arrestin biased DOR agonist into the dorsal striatum of β-arrestin 2 KO mice reduces alcohol intake, in sharp contrast with the same agonist's effect in wild-type mice, where it increases alcohol drinking (*Robins et al., under review*).

### 3.6 How best to selective target striatal GPCRs to treat AUD?

The dorsal striatum has a well-established role during the later stages of alcohol dependence, as characterized by the observed hyperactivity of this region in alcohol-exposed subjects [40, 306]. With a large number of striatal GPCRs being $G_{i/o}$-coupled, there is a hypothetical rationale to target these specific receptors to reduce habitual alcohol use. Purely based on observed alcoholic phenotypes in KO mice, it can be argued that expression and/or activity at several of these GPCRs (DOR, 5-$HT_{1B}$R, $M_4$R, $mGlu_2$R, GPR88) is protective against escalated alcohol use, with some of these protective effects directly linked to actions of these GPCR in the dorsal striatum.

A counter argument can be made that several other $G_{i/o}$-coupled GPCR KO mice show decreased alcohol intake. However, some of those receptors have wide distribution patterns and modulate alcohol behavior in multiple ways. For example, specific activation of the DOR and KOR in the striatum reduces alcohol behavior [375, 385]. While, $D_2$R KO mice drink less than wild-type, the $D_{2L}$R-isoform specific KO mice drink more than wild-type (although an increase in sugar water consumption was also observed in $D_{2L}$R KO female mice suggesting that this KO effect may not be specific to alcohol) [695]. It is therefore important to acknowledge that targeting striatal $G_{i/o}$-coupled receptors with ubiquitous CNS expression may not be efficacious in treating excessive alcohol consumption observed in AUD. Targeting GPCRs that are highly enriched in the dorsal striatum compared to other brain regions, such as GPR88, may provide a valuable target for reducing habitual alcohol drinking in alcohol-dependent patients. Furthermore, it will be interesting to find out how GPR88 agonists modulate alcohol intake in β-arrestin 2 KO mice.

GPCRs are great druggable therapeutic targets as their ligand binding pocket is located on the extracellular/cytosolic site. Still, brain and cell penetrable drugs can be produced to target non-GPCR downstream effectors of GPCR signaling, such as PDE10a or RGS9-2, which are also highly expressed in the dorsal striatum. In conclusion, striatal $G_{i/o}$-coupled receptors are an intriguing therapeutic target for AUD; however, many aspects on how and where these GPCRs operate within the dorsal striatum is not known. We hope this review will motivate researchers to engage in more mechanistic studies aimed at addressing questions pertaining to the synaptic location, the anatomical location, and downstream signaling pathways of striatal $G_{i/o}$-coupled receptors for their potential role in modulating alcohol use.

Table 3-1. Overview of the most prominently expressed GPCRs in the dorsal striatum, preferred G-protein, and alcohol-related phenotype in respective KO mice.

Out of thirty-two GPCRs, eighteen are coupled to $G_{i/o}$-proteins, 8 to $G_q$-proteins and 6 to $G_s$-proteins. *Signaling preference for the orphan receptors comes from references [314, 315, 481, 696-702].

| Striatal receptors | Preferred G protein | Knockout effect on alcohol use |
|---|---|---|
| **Opioid receptors** | | |
| MOR | $G_{i/o}$ | Drink less |
| KOR | $G_{i/o}$ | Drink less |
| DOR | $G_{i/o}$ | Drink more |
| **Dopamine receptors** | | |
| $D_1R$ | $G_s$ | Drink less |
| $D_2R$ | $G_{i/o}$ | Drink less |
| $D_{2L}R$ | $G_{i/o}$ | Drink more |
| **Purinergic receptors** | | |
| $A_1R$ | $G_{i/o}$ | TBD |
| $A_{2A}R$ | $G_s$ | Drink more |
| $P2Y_{12}R$ | $G_{i/o}$ | TBD |
| **Cannabinoid receptors** | | |
| $CB_1R$ | $G_{i/o}$ | Drink less |
| **Histamine receptors** | | |
| $H_3R$ | $G_{i/o}$ | Drink less |
| **Muscarinic receptors** | | |
| $M_1R$ | $G_q$ | TBD |
| $M_2R$ | $G_{i/o}$ | TBD |
| $M_4R$ | $G_{i/o}$ | Drink more |
| **Neurokinin receptors** | | |
| $NK_1R$ | $G_q$ | Drink less |
| **Orphan receptors*** | | |
| GPR6 | $G_s$ | TBD |
| GPR52 | $G_s$ | TBD |
| GPR88 | $G_{i/o}$ | Drink more |
| GPR83 | $G_q$ | TBD |
| GPR12 | Gs | TBD |
| GPR26 | $G_{i/o}$ and $G_s$ | TBD |
| GPR139 | $G_q$ | TBD |
| GPR27 | $G_{i/o}$ | TBD |
| GPR103 | $G_q$ | TBD |
| **Metabotropic glutamate receptors** | | |
| mGlu5R | $G_q$ | Drink less |
| mGluR2 | $G_{i/o}$ | Drink more |

Table 3-1 continued

| | | |
|---|---|---|
| mGluR3 | $G_{i/o}$ | TBD |
| mGluR4 | $G_{i/o}$ | No change |
| mGluR7 | $G_{i/o}$ | Drink more |
| **Serotonin receptors** | | |
| 5-$HT_{1B}$R | $G_{i/o}$ | Drink more |
| 5-$HT_{1D}$R | $G_{i/o}$ | TBD |
| 5-$HT_{2A}$R | $G_q$ | TBD |
| 5-$HT_{2C}$R | $G_q$ | TBD |
| 5-$HT_6$R | $G_s$ | TBD |
| 5-$HT_7$R | $G_{i/o}$ | TBD |
| **GABA receptors** | | |
| $GABA_B$R | $G_{i/o}$ | TBD |
| **Melanin concentrating hormone receptors** | | |
| MCHR1R | $G_{i/o}$ | TBD |
| **Adrenergic receptors** | | |
| $\alpha_{2c}$R | $G_{i/o}$ | TBD |
| **Auxiliary proteins** | | |
| AC5 | $G_s$ | Drink more |
| GIRK3 | $G_{i/o}$ | Drink more |
| RGS6 | $G_{i/o}$ | Drink less |
| β-arrestin 1 | NA | Drink more |
| β-arrestin 2 | NA | Drink less |

# CHAPTER 4. CRITICAL ROLE OF $G_{I/O}$-PROTEIN ACTIVITY IN THE DORSAL STRIATUM IN THE REDUCTION OF VOLUNTARY ALCOHOL INTAKE

The transition from non-dependent alcohol use to alcohol dependence involves increased activity of the dorsal striatum. Interestingly, the dorsal striatum expresses a large number of inhibitory G-protein coupled receptors (GPCRs), which when activated may inhibit alcohol-induced increased neuronal hyperactivity and decrease alcohol consumption. Here, we explore the hypothesis that dorsal striatal $G_{i/o}$-protein activation is sufficient to reduce voluntary alcohol intake. Using a voluntary, limited access, 2-bottle choice, drink-in-the-dark model of alcohol (10%) consumption, we validated the importance of $G_{i/o}$-signaling in this region by locally expressing neuron-specific, adeno-associated-virus encoded $G_{i/o}$-coupled muscarinic $M_4$ DREADDs (Designer Receptors Exclusively Activated by Designer Drug) and observed a decrease in alcohol intake upon DREADD activation. We validated our findings by activating $G_{i/o}$-coupled delta-opioid receptors, which are natively expressed in the dorsal striatum, using either a G protein-biased agonist or a β-arrestin-biased agonist. Local infusion of TAN-67, an *in vitro*-determined $G_{i/o}$ protein-biased delta-opioid receptor agonist, decreased voluntary alcohol intake in wild-type and β-arrestin 2 KO mice. SNC80, a β-arrestin 2-biased delta-opioid receptor agonist, increased alcohol intake in wild-type mice; however, SNC80 decreased alcohol intake in β-arrestin 2 KO mice, thus resulting in a behavioral outcome generally observed for $G_{i/o}$-biased agonists and suggesting that β-arrestin recruitment is required for SNC80-increased alcohol intake. Overall, these results suggest that activation $G_{i/o}$-coupled GPCRs expressed in the dorsal striatum, such as the delta-opioid receptor, by G-protein-biased agonists may be a potential strategy to decrease voluntary alcohol consumption and β-arrestin recruitment is to be avoided.

## 4.1 Introduction

Alcoholism and alcohol abuse is a widespread health issue, placing a large burden at both the individual and societal level. Yet, pharmacological treatment options are still limited. Currently, only three drugs have been approved by the Food and Drug Administration for the treatment of alcohol use disorders (AUD) and each come with their own limitations in therapeutic efficacy

[703]; therefore, it is imperative to identify novel targets for more effective drug development, with hopes of increasing the number of treatment options and compliance for AUD management.

One potential AUD treatment approach is to increase inhibition of the dorsal striatum, a brain region with observed increasing activation upon alcohol tasting in heavy alcohol drinking human subjects [41]. In contrast to the ventral striatum, which is implicated in reward-associated learning and behavior, the dorsal striatum is heavily involved in the transition to compulsive drug or alcohol seeking and taking [37, 40, 41, 604]. In rats, habitual alcohol self-administration increases habit-like responding with decreased sensitivity to alcohol devaluation [46]. This shift towards habit-like responding, as well as reports of increased hyperexcitability and altered glutamatergic and GABAergic transmission in the dorsomedial striatum upon alcohol exposure [44, 306, 307], suggests molecular alterations in this brain region lead to behavioral reinforcement of alcohol intake resulting in habitual excessive alcohol intake [37, 44, 306]. We hypothesized that one conceivable strategy to inhibit this alcohol-induced neuronal excitability is by activation of metabotropic, inhibitory $G_{i/o}$ protein signaling pathways via G-protein coupled receptors (GPCRs) expressed on neurons in this region.

Interestingly, a large number of GPCRs expressed in the dorsal striatum couple to inhibitory G proteins ($G_{i/o}$) [314, 315], thereby providing an ideal target for inhibiting this hyperexcitability observed in the dorsal striatum following persistent alcohol use. To investigate our hypothesis, $G_{i/o}$ coupled-Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) can provide powerful tools [181, 704] to increase $G_{i/o}$-signaling in a specific brain region, such as the dorsal striatum, on an experimenter's predetermined time point to determine the role of dorsal striatum in modulating alcohol consumption. In addition to artificially increasing $G_{i/o}$-signaling using viral DREADD strategies, agonists have been developed to preferentially activate $G_{i/o}$ protein signal pathways over the competing β-arrestin recruitment and signaling pathways for receptors endogenously expressed in the dorsal striatum, with recent advances in opioid receptor pharmacology being a prime example [230, 232, 705]. For this study, the delta-opioid receptor (DOR), a $G_{i/o}$ coupled GPCR with strong expression in the dorsal striatum [200], provided us with a powerful tool to investigate our hypothesis that $G_{i/o}$-signaling in the dorsal striatum can reduce alcohol use. Delta-opioid receptors are thought to play a protective role in alcohol use disorders, as DOR knockout (KO) mice display increased alcohol consumption and preference compared with wild-type, kappa-, or mu-opioid receptor knockout mice, suggesting

that DOR expression prevents escalated alcohol intake compared to other opioid receptor subtypes [211]. Moreover, DORs are heavily expressed in the dorsal striatum presynaptically on corticostriatal glutamatergic inputs [336] and both pre- and postsynaptically on interneurons within this brain region (with enrichment on $D_2$ receptor expressing-MSN compared with $D_1$ receptor expressing-MSNs) [206, 207, 706], and direct activation [208] or indirect activation via alcohol-induced release of endogenous enkephalins [47] in the dorsal striatum induces long-term depression.

The importance of the activation of dorsal striatal DORs in the modulation of alcohol intake was first evident in a report by Nielsen et al., where infusion of the DOR selective agonist SNC80 into the dorsal striatum increased alcohol intake in rats while the DOR antagonist naltrindole reduced intake [262]. This finding that DOR agonist SNC80 increased alcohol intake was somewhat surprising as DOR expression was previously mentioned to be protective against increased alcohol intake [211]. Yet, our recent work investigating a panel of DOR agonists suggests that SNC80 prefers to recruit β-arrestin 2 protein through a mechanism called biased signaling (as termed functional selectivity) [707, 708], where we have additionally correlated *in vitro* β-arrestin biased with *in vivo* increased alcohol intake [347]. In our same study investigating the behavioral effects of DOR biased signaling, we also observed that DOR agonists that weakly recruit β-arrestin, particularly TAN-67 (and thus are G protein-biased), decreased alcohol intake in mice in a limited-access, drinking-in-the-dark protocol to 10% alcohol [347].

Therefore, here we hypothesized that activation of $G_{i/o}$-signaling in the dorsal striatum would be beneficial in reducing alcohol intake, whereas β-arrestin recruitment will lead to enhanced alcohol use. To begin to investigate this hypothesis, we first utilized $hM_4Di$ DREADD technology [181] to identify the broad role of $G_{i/o}$ coupled receptor activation in the dorsal striatal on voluntary alcohol intake in C57Bl/6 male mice using a two-bottle choice, limited-access drinking-in-the-dark protocol. Additionally, we selectively infused our previously identified differentially biased DOR agonists in wild-type and β-arrestin 2 knockout (KO) mice to more specifically investigate the effect of increased dorsal striatal DOR $G_{i/o}$-protein signaling (versus β-arrestin recruitment) on voluntary alcohol intake.

## 4.2 Materials and methods

### 4.2.1 Drugs and chemicals

SNC80 and SB205607 (TAN-67), were purchased from Tocris, R&D systems (Minneapolis, MN, USA); naltrindole hydrochloride, forskolin, 200 proof ethyl alcohol, leu-enkephalin, sodium chloride, DMSO, cocaine hydrochloride, and clozapine-N-oxide (CNO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). For dorsal striatal infusion studies, TAN-67 and SNC80 were diluted in 0.9% saline to a concentration of 10 μM; for cellular assays, drugs were dissolved in water. Cocaine was dissolved in 0.9% saline for an administered dose of 15 mg/kg and CNO was dissolved in 100% DMSO and diluted to a concentration of 0.2 mg/mL in saline (final DMSO concentration of 0.5% and administered dose of 2 mg/kg). Both cocaine and CNO were injected intraperitoneally (i.p.) during experimentation. Non-Cre dependent AAV8-hSyn-hM$_4$Di-mCherry ($7.4\times10^{12}$ vg/ml) virus and AAV8-hSyn-EGFP ($3.9\times10^{12}$ vg/ml) virus were obtained from the University of North Carolina Vector Core. Both viruses were chosen as they specifically express in neurons through use of the synapsin promotor. A 100 mg/kg ketamine (Henry Schein, Dublin, OH, USA):10 mg/kg xylazine (Sigma Aldrich, St. Louis MO, USA) cocktail was administered to induce anesthesia for cannulation surgeries and prior to transcardial perfusion. All systemic drugs were injected at a volume of 10 ml/kg.

### 4.2.2 Cell culture and biased signaling assays

Competition binding assays were performed using the Tag-lite assay according to the manufacturer's protocol (Cis-Bio, Bedford, MA, USA). In short, Tb-labeled HEK293-SNAP-hDOR cells/well (4000 cells/well) were plated in 10 μl Tag-lite medium into low-volume 384 well plates in the presence of 5 μl 8 nM fluorescent naltrexone (final concentration) and 5 μl of an increasing concentration of TAN-67, leu-enkephalin, or SNC80 and incubated at room temperature for 3 hours. cAMP inhibition and β-arrestin 2 recruitment assays were performed as previously described [347]. In brief, for cAMP inhibition assays HEK293 (Life Technologies, Grand Island, NY, USA) cells (15,000 cells/well, 7.5 μl) transiently expressing FLAG-mDOR [267, 709], SNAP-rDOR, or SNAP-hDOR (Cis-Bio), and pGloSensor22F-cAMP plasmids (Promega, Madison, WI, USA) were incubated with Glosensor reagent (Promega, 7.5 μl, 2% final concentration) for 90 minutes at 37°C/5% $CO_2$. Cells were stimulated with 5 μl DOR agonist 20 minutes prior to 30 μM forskolin (5 μl) stimulation for an additional 15 minutes. For β-arrestin 2

recruitment assays, CHO-hDOR PathHunter β-arrestin 2 cells (DiscoverX, Fremont, CA, USA) were plated (2500 cells/well, 10 µl) prior to stimulation with 2.5 µl DOR agonists for 90 minutes at 37°C/5%$CO_2$, after which cells were incubated with 6 µl cell assay buffer for 60 minutes at room temperature as per the manufacturer's protocol. Luminescence and fluorescence for each of the assays was measured using a FlexStation3 plate reader (Molecular Devices, Sunnyvale, CA, USA).

#### 4.2.3 SNAP-rDOR construction

Rat DOR cDNA was amplified from the pUC17-rDOR plasmid (Versaclone cDNA NP_036749, R&D systems) using the following forward [5'-CTTCGATATCTTGGAGCCGGTGCCTTCTG-3'] and a standard M13 reverse primer using the Pfu Ultra II Hotstart PCR Mastermix (Agilent, Santa Clara, CA, USA) according to the manufacturer's protocol. The amplified rDOR PCR product and the pSNAP-hDOR plasmid (Cis-Bio) were restricted using EcoRV and XhoI restriction enzymes (New England BioLabs, Ipswich, MA, USA) and the rDOR construct was exchanged with the hDOR gene followed by ligation with T4 DNA Ligase (New England BioLabs) and transformation into NEB5α competent cells (New Englang BioLabs). The SNAP-rDOR was fully sequenced to ensure correct orientation and absence of point mutations introduced during amplification.

#### 4.2.4 Animals

37 male C57BL/6 mice (age 6 weeks) were purchased from Harlan and habituated for to the facility one week prior to surgery. For β-arrestin 2 KO animals, animals were bred in house and 16 animals were selected for surgery (for complete details on strain origin see [347]). Throughout the experiment, animals were kept in at ambient temperature of (21°C) in a room maintained on a reversed 12L:12D cycle (lights off at 10.00, lights on at 22.00) in Purdue University's animal facility, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. This study was carried out in accordance with the recommendations of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The protocol (#1305000864) was approved by the Purdue University Institutional Animal Care and Use Committee.

#### 4.2.5 Surgical cannulation

Directly prior to surgery, mice were anesthetized with ketamine/xylazine (i.p.). A Kopf model 1900 stereotaxic alignment system (David Kopf Instruments, Tujunga, CA, USA) was used to drill two holes using Kyocera #69 drill bits at the following coordinates from bregma: AP = +1 mm, ML = +/-1.5mm, DV: -3.25 mm [710, 711]. For experiments involving drug infusion, an additional two holes were drilled using Kyocera #60 drill bits at the following coordinates from bregma: AP=-2.4 mm, ML= +/- 1.6 mm and 1 mm screws were positioned to ensure head-cap stability. A bilateral 22-gauge guide cannula (cut 1.5 mm below pedestal, PlasticsOne, Roanoke, VA, USA) was attached to the skull using Geristore dental cement (DenMat, Lompoc, CA, USA). In total, two animals did not wake up from surgery and eight animals were euthanized after their cannulas came off post-operation or throughout alcohol training and/or experimentation.

#### 4.2.6 Viral injection

After cannulation surgery, animals were single housed in double grommet cages to allow recovery and individual measurement of fluid intake. One week post-surgery, mice were anesthetized as previously described and injected bilaterally with 450 nl of virus using a Harvard Apparatus infusion pump at a speed of 50 nl/min via internal cannula with 0.5 mm projection (PlasticsOne). The internal cannula was left in place for an additional five minutes to allow viral dispersion and prevent backflow of the viral solution into the injection syringe. All biohazard work was approved by the Institutional Biohazard Committee at Purdue University (#13-013-16).

#### 4.2.7 Voluntary alcohol intake

One week post-surgery and/or one week post-viral injection, mice were exposed to a limited access (4 hours/day), 2-bottle choice (water vs. 10% ethanol), drinking-in-the-dark (DID) protocol during their active cycle (dark phase) until the alcohol intake was stable as previously described [267]. This model has previously shown that TAN-67 administration prior to the four-hour session decreases alcohol intake with a correlated decrease in blood ethanol concentration (with no TAN-67 effects on alcohol metabolism) [267]. Mice were trained for three weeks during which the mice initially increased their alcohol intake prior to reaching steady state consumption. Bottle weights were measured directly before and after the 4-hour access period to the second decimal point to

determine fluid intake during this access period. Weights of bottles were corrected for any spillage with fluid bottles placed on empty cages.

#### 4.2.8 Drug infusion or injection

After three weeks of exposure to the drinking model described above, alcohol and water intake on the day of infusion (Friday) was compared with the average alcohol intake over the preceding three days (Tuesday-Thursday) to determine if either drug injection or infusion altered voluntary alcohol intake in the following manipulations. For experiments involving viral expression, the AAV injected mice were injected with i.p. saline (with 0.5% DMSO) for vehicle measurements in week 4 and 2 mg/kg CNO (i.p.) the following week (week 5). The dose of CNO of 2 mg/kg was utilized as it has previously been shown to be effective in mice in activating expressed DREADDs [712, 713]. Also, this relatively low dose limits high concentrations of clozapine caused by metabolism of CNO [187]. For experiments involving direct drug infusion into the dorsal striatum, animals received a 150 nL bilateral infusion of saline into the dorsal striatum on Friday of the fourth week of alcohol exposure. In weeks 5 and 6, animals received either a 150 nL infusion of 10 μM TAN-67 and SNC80, respectively, thereby allowing for a within subjects' analysis. The order of the drug infusions was chosen to mitigate potential DOR internalization and/or degradation as SNC80 is a high internalizing agonist *in vitro* and *in vivo* [714, 715]. Doses of TAN-67 and SNC80 were determined based on previous studies of SNC80 infusions in rats [262] and *in vivo* specificity of TAN-67 and SNC80 for the DOR over MOR or KOR had been previously established using KO animals [267, 340]. Importantly, no seizure behavior was observed up SNC80 infusion [716] following any dorsal striatal infusions.

#### 4.2.9 Locomotor activity

Square locomotor boxes from Med Associates (L 27.3 cm x W 27.3 cm x H 20.3 cm, St. Albans VT, USA) were used to monitor locomotor activity during the active/dark phase as previously described [347]. For AAV experiments, animals were placed in the locomotor box 15 minutes prior to CNO (2 mg/kg, i.p.) injection to allow baseline locomotor activity scoring. After 15 minutes, all animals were injected with CNO and then placed back into the box for an additional 60 minutes of testing to measure the total distance traveled in 60 minutes following drug injection. For intra-dorsal striatal infusion of SNC80, animals received either 10 μM SNC80 or vehicle

(saline 0.9%) infusion and were placed immediately in the boxes for 90 minutes; locomotor data was analyzed 30 minutes after drug infusion as this is when drinking experiments began in the previously described alcohol intake studies.

#### 4.2.10 Cannula location and immunohistochemical analysis

For animals undergoing drug infusions, animals were sacrificed via transcardial perfusion within one week following their final drinking session. During analysis, it was determined that the cannula of one mouse from these experiments was not positioned properly and this animal was removed from analysis (placement was too ventral). Cannulation location and viral expression was verified with confocal microscopy (Nikon A1, Nikon, Melville, NY, USA) with an area of capture of 1.69 $mm^2$. The experimenter preforming analysis was blind to the experimental conditions; all images were evaluated in greyscale to prevent unintentional bias.

#### 4.2.11 Cocaine-induced c-Fos activation in DREADD-expressing animals

For viral expression studies, both groups of mice were injected with 2 mg/kg CNO (i.p.) during the dark/active phase for each animal. Twenty minutes later, animals were injected with 15 mg/kg cocaine (i.p.) to induce expression of immediate-early gene c-Fos. Brains were collected 90 minutes following cocaine exposure via transcardial perfusion. Extracted brain samples embedded and frozen in Tissue-Tek® O.C.T. compound (VWR, Radnor, PA, USA) in tissue molds (VWR) and sliced into 50 µm coronal sections via cryostat (Leica Microsystems Inc., Buffalo Grove, IL). Immunohistochemical staining was conducted using primary rabbit anti-c-Fos antibody (sc-52, Santa Cruz Biotechnology, Dallas, TX), diluted 1:1000. Control-GFP animal brains were applied Alexa-Fluor 594 goat anti-rabbit antibody (A-11012, Life Technologies, Grand Island, NY, USA) diluted 1:1000. $hM_4Di$-mCherry animal brains were applied Alexa-Fluor 488 goat anti-rabbit (A-11008, Life Technologies, Grand Island, NY, USA) diluted 1:1000. Brain slices were mounted onto microscope slides (Fisher Scientific, Hampton NH, USA) for confocal microscopy with an area of the capture of 0.40 $mm^2$. Images were processed using ImageJ software (NIH, Bethesda MD, USA) for the number of c-Fos positive cells in the dorsal striatum surrounding the viral injection site in infected cell populations. The experimenter preforming analysis was blind to the experimental conditions; all images were evaluated in greyscale to prevent unintentional bias.

### 4.2.12 Statistical analysis

All data are presented as means ± standard error of the mean and was performed using GraphPad Prism7 software (GraphPad Software, La Jolla, CA). Differences between control-GFP and $hM_4Di$-mCherry animals were analyzed by student two-tailed t-test for differences in baseline water intake, alcohol intake, alcohol preference, locomotion after CNO injection, and c-Fos expression in the dorsal striatum. Differences in alcohol intake after saline injection and CNO injection were evaluated by repeated measures, multiple comparisons (Bonferroni) 2-way ANOVA. For in vitro assays, nonlinear regression using a dose-response to either inhibition (binding, cAMP) or stimulation (β-arrestin 2 recruitment) was conducted to determine pIC50 or pEC50, respectively. In direct dorsal striatal drug infusion experiments, differences in voluntary alcohol intake, water intake, and alcohol preference were analyzed by repeated measures, multiple comparisons (Tukey) 2-way ANOVA. The Grubb's outlier test (alpha = 0.05) was used to identify potential outliers throughout this study. Statistical analysis was conducted in guidance with and approved by Purdue University's Department of Statistics.

## 4.3 Results

### 4.3.1 Activation of a $G_{i/o}$-coupled DREADD in the dorsal striatum decreases alcohol intake

Cannula placement was verified post-mortem (n=10-11) through immunohistochemical analysis of viral expression (Figure 4-1A). Viral infusions of control-GFP (green fluorescent protein) or $hM_4Di$-mCherry in the dorsal striatum did not alter baseline alcohol intake, water intake, or alcohol preference when comparing the two groups (Figure 4-1B, $t(20)=0.81$, $p=0.32$; Figure 4-C, $t(20)=0.60$, $p=0.42$; graph omitted from text, $t(20)=1.01$, $p=0.55$). Vehicle injection (0.5% DMSO, i.p.) did not affect alcohol intake for GFP-control or $hM_4Di$ expressing animals in alcohol intake (Figure 4.1D, see Table 4-1 for full statistical analysis for experimental group), water intake (*graph omitted from text*), or alcohol preference in control-GFP or $hM_4Di$-mCherry mice (*graph omitted from text*). Unlike saline injection, CNO injection (2 mg/kg, i.p.) significantly reduced alcohol intake in $hM_4Di$ expressing mice compared with control, as evaluated by 2-way ANOVA (Figure 4-E, effect of drug x virus: $p=0.03$), where Bonferroni post-test analysis revealed that CNO significantly reduced alcohol intake only in $hM_4Di$ expressing animals and not control-GFP expressing mice ($p<0.002$). No significant change in water intake was observed after CNO

injection in the testing period for in either group of animals (*graph omitted from text,* Table 4-1). CNO injection did not alter alcohol preference in control-GFP or $hM_4Di$-mCherry mice (*graph omitted from text,* Table 4-1).

Both viruses properly expressed in the dorsal striatum (Figure 4-2A). Differences in visualization of the control-GFP and $hM_4Di$-mCherry expression may potentially result from differences in viral load and protein expression or inherent differences in quantum yield and extinction coefficients between GFP and mCherry [717]. Considering that the striatum is part of the basal ganglia that controls movement [46, 718], we determined whether CNO activation of dorsal striatal $hM_4Di$ altered locomotor activity where we observed that CNO did not alter locomotor activity between control-GFP and $hM_4Di$-mCherry expressing mice in a 60-minute locomotor period after injection (Figure 4-2B, $t(19)=0.78$, $p=0.45$). To confirm the inhibitory functionality of $hM_4Di$ expression, we determined if CNO activation of $hM_4Di$ could prevent cocaine-induced c-Fos expression [182, 719], an acceptable approach previously used in other studies to validate functionality of inhibitory DREADDs [182, 719-721]. We observed that activation of striatal $hM_4Di$ with CNO (2 mg/kg, i.p.) prior to a cocaine challenge (15 mg/kg, i.p.) significantly inhibited c-Fos activation in animals expressing $hM_4Di$ versus GFP controls (control were also administered CNO prior to cocaine challenge) (Figure 4-2C,D; $t(13)=2.78$, $p<0.02$), suggesting that activation of $hM_4Di$ via CNO before cocaine challenge inhibited cAMP pathway activity by $G_{i/o}$-coupled inhibition. The variability in c-Fos expression in control-GFP may be a result of intrinsic differences in response to psychostimulants between animals, which has been commonly observed in C57Bl/6 mice [722].

### 4.3.2 In vitro characterization of β-arrestin 2 biased DOR agonist, SNC80

We have previously established that systemic activation of the $G_{i/o}$-coupled DOR with TAN-67, an agonist that only weakly recruits β-arrestin-2 to hDOR (Figure 4-3A), reduces voluntary intake in mice, but that SNC80, a hDOR agonist that strongly recruits β-arrestin-2 (Figure 4-3A) increases alcohol intake [347]. However, we previously had not determined if a difference in receptor binding was observed between TAN-67 and SNC80 at hDOR to potentially explain differences in ligand bias. Using a SNAP-tag HTRF® (Cis-Bio) approach we found that hDOR, TAN-67 exhibited a pKi = 7.7 ± 0.1 and SNC80 a pKi = 7.2 ± 0.2, with pKi =5.8 ± 0.1 for leu-enkephalin (Figure 3B),

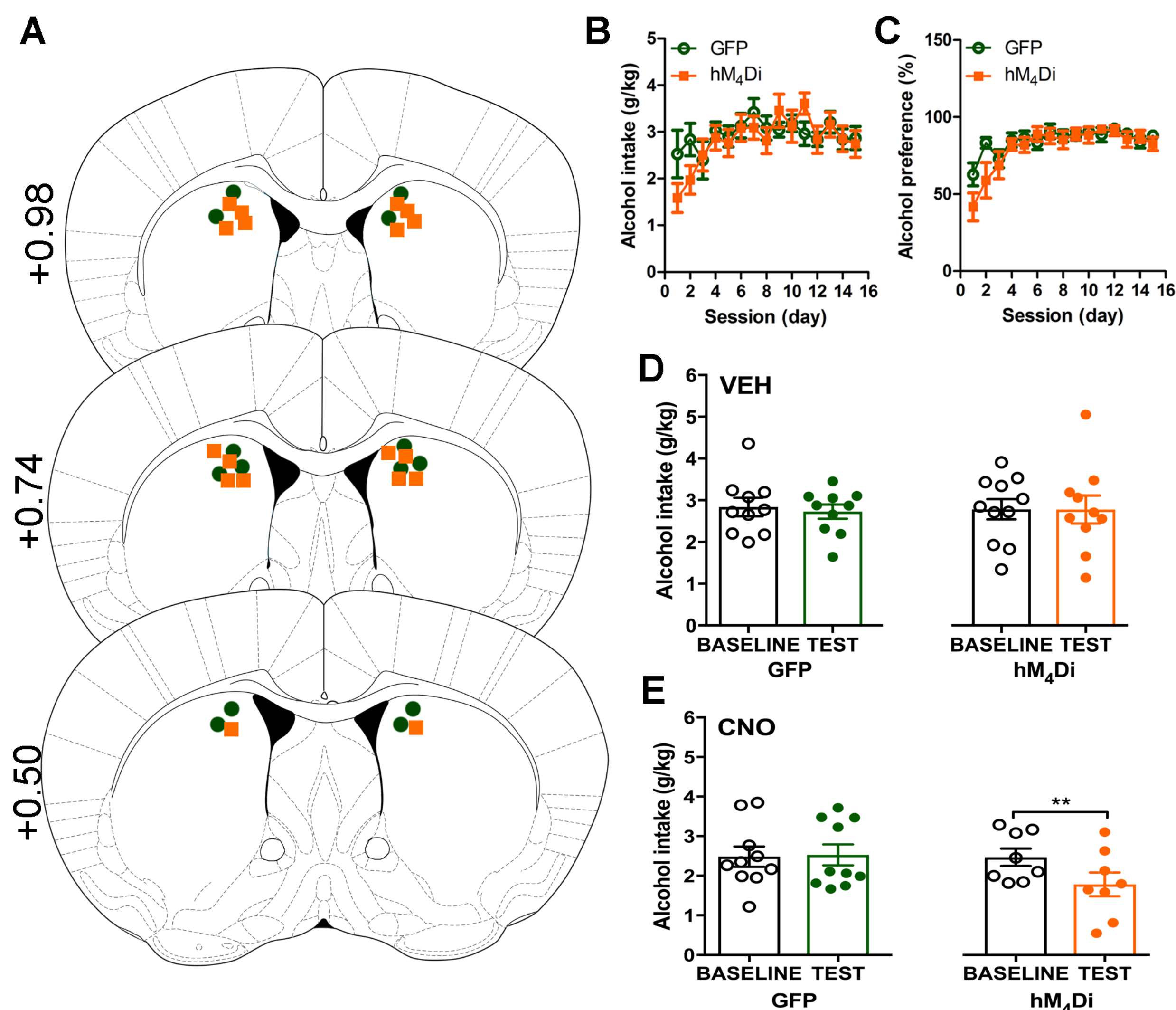



Figure 4-1. Activation of virally expressed hM4Di in the dorsal striatum decreases alcohol intake in mice.

Cannula placement was verified for all animals included in behavioral analysis (**A**). C57BL/6 mice (n=10-11) injected in the dorsal striatum with either GFP or hM4Di were trained to consume alcohol in a two-bottle, DID protocol. Both groups of animals displayed a similar increase in alcohol intake (**B**) and preference (**C**). Vehicle injection (saline 0.9%, i.p.) did not change alcohol intake (**D**). Systemic CNO injection (2 mg/kg i.p.) significantly decreased alcohol intake in mice expressing hM4Di, but not GFP, in the dorsal striatum (**E**). Significance by unpaired, student's t-test for AUC or 2-way ANOVA with Bonferroni post-test for matching, **, $p<0.01$.

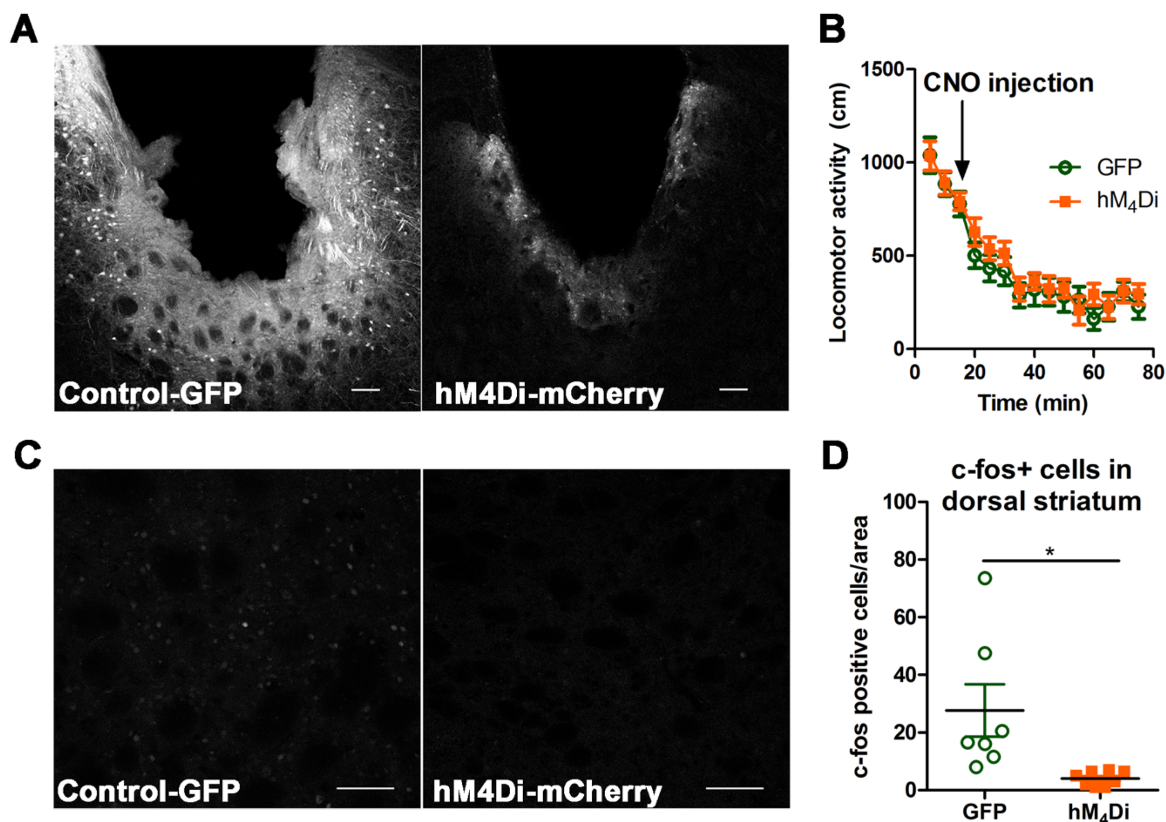



Figure 4-2. Verification of viral expression and functionality of control-GFP or $hM_4Di$-mCherry in the dorsal striatum.

Viral expression verification via confocal microscopy of control-GFP (left) and $hM_4Di$-mCherry (right) in the dorsal striatum (scale bar = 100 μm) (A). C57BL/6 mice (n=10-11 per group) expressing GFP or $hM_4Di$ in the dorsal striatum did not display significant CNO (2 mg/kg, i.p.) induced locomotor activity in the 60-minute session after CNO injection (B). Immunohistochemical representation of c-Fos activation in the dorsal striatum in animals expressing control-GFP (left) and $hM_4Di$-mCherry (right) in the dorsal striatum (scale bar = 100 μm) (C). Decreased c-Fos expression in dorsal striatum after cocaine challenge (15 mg/kg, i.p.) in C57BL/6 mice (n=7-8) expressing $hM_4Di$-mCherry versus control-GFP observed confocal microscopy (D). Significance by unpaired two-tailed t-test, *, $p<0.05$.

Table 4-1. Analysis of alcohol-related behaviors in control-GFP versus $hM_4Di$-mCherry DREADD expressing mice.

(*, student's t-test; #, two-way, repeated measures (Bonferroni) ANOVA).

| | df | Alcohol intake | Water intake | Alcohol preference |
|---|---|---|---|---|
| **Baseline*** (student's t-test) | 20 | t=0.812 p=0.32 | t=0.603 p=0.42 | t=1.01 p=0.55 |
| **Vehicle#** | | | | |
| Drug | 1,19 | F=0.18 p=0.86 | F=0.52 p=0.48 | F=3.82 p=0.07 |
| Virus | | F=0.04 p=0.85 | F=0.00 p=0.96 | F=0.26 p=0.42 |
| Drug x virus | | F=0.00 p=0.99 | F=0.26 p=0.61 | F=2.85 p=0.11 |
| **CNO#** | | | | |
| Drug | 1,19 | F=4.00 p=0.06 | F=1.20 p=0.29 | F=0.42 p=0.52 |
| Virus | | F=1.26 p=0.28 | F=2.37 p=0.14 | F=0.90 p=0.35 |
| Drug x virus | | F=5.17 **p=0.03 Control vs. DREADD p<0.002** | F=0.28 p=0.60 | F=1.01 p=0.33 |

suggesting that the only clear difference between TAN-67 and SNC80 is β-arrestin-2 recruitment efficacy. The surprisingly low affinity observed for leu-enkephalin may be an artifact of the fluorescent binding assay that relies on a large N-terminal SNAP-tag that may potential interfere with the binding of relatively large peptide ligand, such as leu-enkephalin, but not small molecules.

Expanding from our previous study, we determined the equiactive bias factors for TAN-67 and SNC80 at hDOR using leu-enkephalin as a reference ligand [723] for β-arrestin-2 recruitment compared with $G_{i/o}$-stimulated cAMP inhibition (a more positive bias factor = indicative of bias towards β-arrestin-2, more negative bias factor = indicative of bias towards cAMP activity). TAN-67 displayed a bias factor of -1.4 (cAMP biased) versus a +0.85 bias factor for SNC80 (β-arrestin-2-biased) (Figure 4-3C). To estimate what concentration of SNC80 to infuse *in vivo*, we relied on the Nielsen et al. reported findings in rat [262]. Our in vitro assays suggest minimal differences in cAMP inhibition between human hDOR (Figure 4-3D), rat rDOR (Figure 4-3E) and mDOR (Figure 4-3F) for SNC80 ($pIC_{50} = 7.8 \pm 0.3$, n=3, $pIC_{50} = 8.4 \pm 0.1$, n=5, $pIC_{50} = 8.4 \pm 0.4$, n=3, respectively) and leu-enkephalin ($pIC_{50} = 8.7 \pm 0.2$, n=5, $pIC_{50} = 8.9 \pm 0.2$, n=5, $pIC_{50} = 8.3 \pm 0.1$, n=6, respectively). Because the affinity and efficacy of TAN-67 is very comparably to SNC80 (Figure 4-3A and 4-3B), we decided to infuse 10 nM TAN-67 and SNC80 into the mouse dorsal striatum to investigate the role of $G_{i/o}$-signaling versus β-arrestin-2 recruitment in the modulation of alcohol use.

### 4.3.3 Differential modulation of alcohol intake following dorsal striatal DOR activation by $G_{i/o}$-biased versus β-arrestin 2-biased DOR agonists

Cannula terminus location and patency were validated via trypan blue dye infusion into the dorsal striatum upon experimental completion (Figure 4-4A). Wild-type male animals (n=9-10) were successfully trained to consume alcohol using a limited-access, two-bottle-choice (water vs 10% alcohol), drinking-in-the-dark (DID) protocol as shown by increased daily alcohol intake and preference (Figure 4-4B,C) compared with water intake (*graph omitted from text*). For intra-striatal infusions, a significant drug ($p$=0.03, see Table 4-2 for full statistical analysis for experimental group) and drug x test session ($p$<0.0001) was observed, with no effect of test session alone, where Tukey multiple comparisons test revealed that 10 μM of TAN-67 significantly decreased voluntary alcohol intake ($p$=0.04) while 10 μM SNC80 significantly increased alcohol intake ($p$=0.0005). Importantly, vehicle (saline 0.9%) infusion did not affect alcohol intake (Figure 4-4D**).** No changes

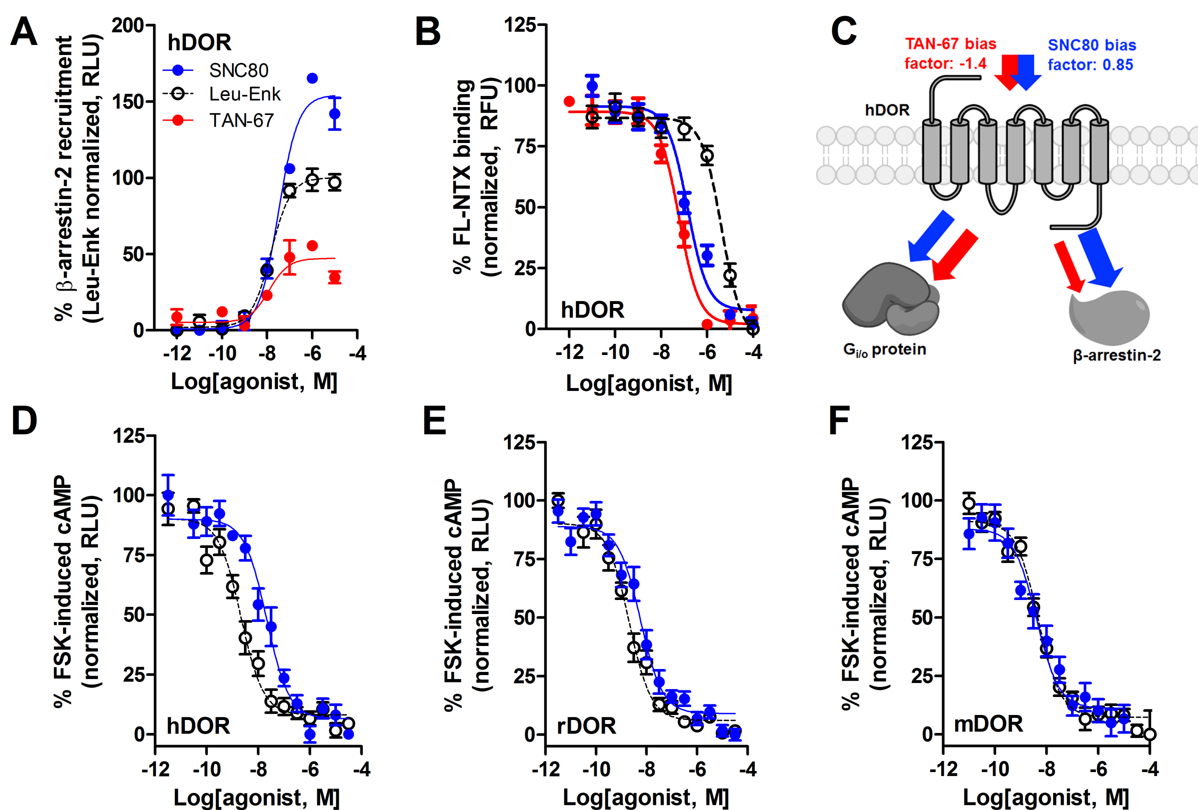



Figure 4-3. SNC80 is a β-arrestin 2 biased agonist with comparable potency across species in heterologous cell systems.

At the hDOR, SNC80 acts as a β-arrestin 2 super-agonist compared with the endogenous agonist leu-enkephalin and the weak β-arrestin-2 recruiter TAN-67 (A). SNC80 and TAN-67 bind to hDOR with similar affinity (B). Schematic representation of the observed ligand bias of TAN-67 and SNC80 at hDOR, with calculated bias factor (C). SNC80 and Leu-enkephalin have similar potency to inhibit forskolin-induced cAMP production at hDOR (D), rDOR (E) and mDOR (F). A representative summation is shown (n≥3). *Experiments performed by Dr. Richard van Rijn and Kendall Mores (Van Rijn lab).*

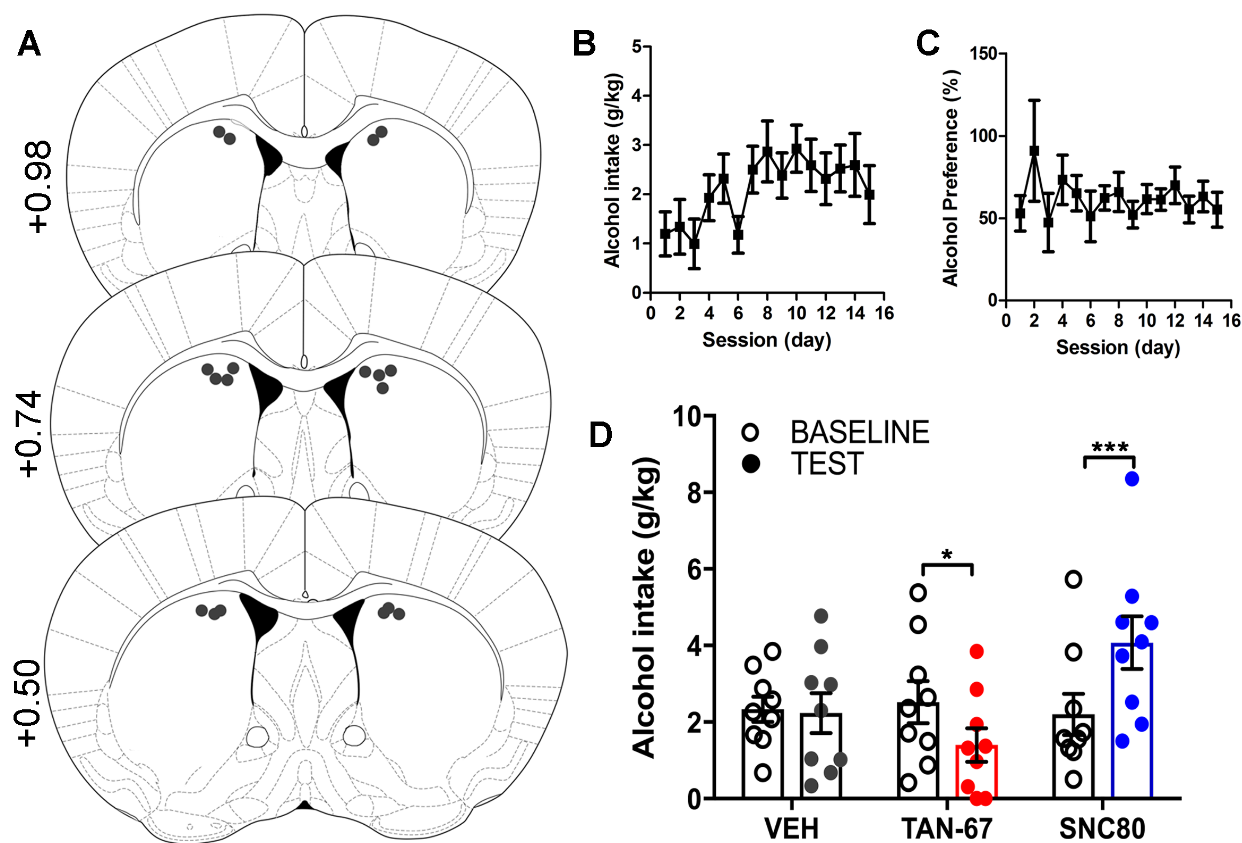



Figure 4-4. Dorsal striatal infusion of $G_{i/o}$ protein-biased DOR agonist TAN-67 decreases voluntary alcohol intake, while β-arrestin 2-biased DOR agonist SNC80 increases alcohol intake in wild-type mice.

Cannula placement was verified for all animals included in behavioral analysis (A). C57BL/6 male, wild-type mice (n=9-10) were trained to consume 10% alcohol over the course of three weeks, during which they increased their alcohol intake (B) and alcohol preference (C). Vehicle saline (0.9%) infusion did not change alcohol intake while TAN-67 (10 μM) significantly decreased alcohol intake and SNC80 (10 μM) significantly increased alcohol intake (D). Significance by repeated measures, multiple comparisons (Tukey) 2-way ANOVA, *, $p<0.05$, ***, $p<0.001$.

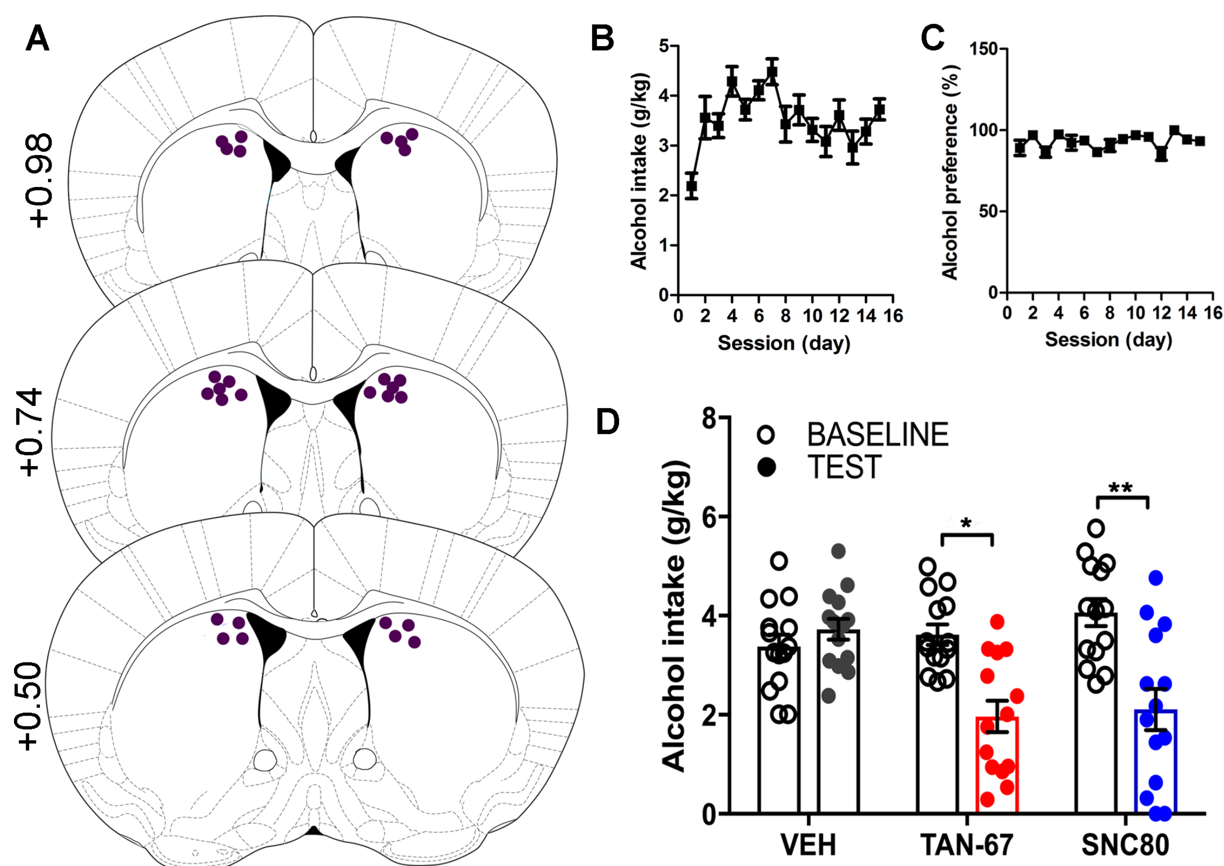



Figure 4-5. Genetic KO of β-arrestin 2 reveals critical role of $G_{i/o}$-signaling in reducing alcohol intake via dorsal striatal DOR activation.

Cannula placement was verified for all animals included in behavioral analysis (A). C57BL/6 male, β-arrestin 2 KO mice (n=12) were trained to consume 10% alcohol over the course of three weeks, during which they increased their alcohol intake (B) and alcohol preference (C). Vehicle saline (0.9%) infusion did not change alcohol intake, but both TAN-67 and SNC80 (10 µM) significantly decreased alcohol intake (D). Significance by repeated measures, multiple comparisons (Tukey) by 2-way ANOVA, *, $p<0.05$, **, $p<0.01$.

Table 4-2. Analysis of alcohol-related behaviors in wild-type mice upon biased DOR agonist infusion in the dorsal striatum.

| Two-way ANOVA | df | Drug injection, alcohol intake | Drug injection, water intake | Drug injection, alcohol preference |
|---|---|---|---|---|
| | | | | |
| Drug | 2.16 | F=4.38<br>p=0.03 | F=0.63<br>p=0.56 | F=1.40<br>p=0.28 |
| Test session | 1,8 | F=0.39<br>p=0.55 | F=2.11<br>p=0.18 | F=0.33<br>p=0.58 |
| Drug x test session | 2,16 | F=20.22<br>P<0.0001 | F=0.49<br>p=0.62 | F=1.39<br>p=0.28 |
| Multiple comparisons (Tukey) | | VEH p>0.99<br>**TAN-67 p=0.042**<br>**SNC80 p=0.0005** | VEH p>0.96<br>TAN-67 p>0.99<br>SNC80 p=0.54 | VEH p>0.97<br>TAN-67 p=0.85<br>SNC80 p=0.88 |

Table 4-3. Analysis of alcohol-related behaviors in β-arrestin 2 knockout mice upon biased DOR agonist infusion in the dorsal striatum.

| Two-way ANOVA | df | Drug injection, alcohol intake | Drug injection, water intake | Drug injection, alcohol preference |
|---|---|---|---|---|
| | | | | |
| Drug | 2,26 | F=7.31<br>p=0.003 | F=1.07<br>p=0.36 | F=3.11<br>p=0.062 |
| Test session | 1,13 | F=25.28<br>p=0.0002 | F=0.93<br>p=0.35 | F=7.19<br>p=0.019 |
| Drug x test session | 2,26 | F=7.92<br>p=0.0021 | F=2.46<br>p=0.11 | F=4.03<br>p=0.03 |
| Multiple comparisons (Tukey) | | VEH p>0.97<br>**TAN-67 p=0.011**<br>**SNC80 p=0.0021** | VEH p>0.99<br>TAN-67 p=0.95<br>SNC80 p=0.28 | VEH p>0.99<br>TAN-67 p>0.99<br>**SNC80 p=0.018** |

in water intake or alcohol preference were noted during these drug infusion testing sessions (*graphs omitted from text*, Table 4-2).

#### 4.3.4 Genetic knockout of β-arrestin 2 provides additional support for the critical role of DOR mediated $G_{i/o}$-coupling in the dorsal striatum in decreasing alcohol intake

β-arrestin 2 KO male C57Bl/6 mice (n=12) were surgically implanted with a bilateral cannula into the dorsal striatum prior to alcohol training and cannula terminus location and patency were validated via trypan blue dye infusion upon experimental completion (Figure 4-5A). KO animals were successfully trained to consume alcohol using a limited-access, two-bottle-choice (water vs 10% alcohol), DID protocol (Figure 4-5B,C) compared with water intake (*graph omitted from text*). A significant effect of drug ($p$=0.003, see Table 4-3 for full statistical analysis), test session ($p$=0.002), and drug x test session ($p$=0.0021) was identified for intra-dorsal striatal infusions, where multiple comparisons test found no effect of vehicle (saline 0.9%, p=0.968) on alcohol intake. 10 μM of TAN-67 significantly decreased voluntary alcohol intake ($p$=0.0113) and 10 μM SNC80 also significantly decreased alcohol intake ($p$=0.0021) (Figure 4-5D). This decrease was in contrast with that observed in wild-type animals and is the first report of SNC80's ability to decrease voluntary alcohol intake, further suggesting that β-arrestin 2 functionality is key for SNC80-increased voluntary alcohol intake. No changes in water intake were noted during testing periods (*graph omitted from text,* Table 4-3) but a decrease in alcohol preference was noted for SNC80 infusion ($p$=0.0018, *graph omitted from text,* Table 4-3). We have previously observed hyperlocomotion upon systemic SNC80 administration in both wild-type and β-arrestin-2 KO mice with increased alcohol intake or no change in alcohol intake, respectively [347, 724]. While we did not visually observe typical SNC80-induced hyperlocomotive behavior upon infusion in WT mice, we did question whether the decrease in alcohol intake upon dorsal striatal SNC80 infusion in the β-arrestin-2 KO was the result of changes in locomotion. However, SNC80 (10 μM) infusion into the dorsal striatum of β-arrestin-2 KO animals did not cause hyperlocomotion compared with vehicle infusion (*graph omitted from thesis text,* paired two-tailed student's t-test: $t(6)=1.68$, $p$=0.14).

## 4.4 Discussion

Through both chemogenetic and pharmacologic activation of $G_{i/o}$ protein signaling, we observed that activation of $G_{i/o}$ protein coupled receptors in the dorsal striatum significantly decreases alcohol intake in male C57BL/6 mice by either inhibitory DREADD activation or activation of endogenously expressed DORs using a G protein-biased agonist. We specifically targeted the dorsal striatum as it plays an important role in modulating habitual alcohol use [37, 41, 44, 306], has strong DOR expression [207], and, crucially, is a region where DOR agonist SNC80 has been shown to increase alcohol intake in rats [262]. Here, activation of virally expressed $G_{i/o}$-coupled DREADDs in the dorsal striatum was capable of decreasing alcohol intake while no effect was observed in control-GFP animals upon CNO administration (Figure 4-1). For activation of endogenous dorsal striatal DORs, our findings that local dorsal striatal infusion of TAN-67 decreased alcohol intake and SNC80 increased alcohol use (Figure 4-4) agreed with our systemic findings [347] and also confirmed the previously observed alcohol intake increase following local dorsal striatal infusion of SNC80 in rats [262]. Furthermore, through the use of β-arrestin 2 KO mice, we were able to shift the direction of alcohol intake modulation by SNC80 from significantly increasing intake to significantly decreasing consumption when β-arrestin 2 signaling pathways is not present (Figure 4-5). This was expected as TAN-67 and SNC80 displayed similar binding and G-protein pathway efficacy at DOR *in vitro,* suggesting that the removal of potential β-arrestin 2 recruitment would allow the agonists to behave similarly (Figure 4-3). This shift is in agreement with our hypothesis that DOR mediated $G_{i/o}$-signaling is a potential strategy to reduce alcohol use, whereas DOR-mediated β-arrestin signaling is to be avoided.

To validate the role of the dorsal striatum in alcohol consumption, we virally expressed a $G_{i/o}$-coupled DREADD (hM4Di) to artificially activate $G_{i/o}$ protein signaling pathways in this region to determine how increased $G_{i/o}$ protein activity altered alcohol intake. In the present study, activation by the $hM_4Di$ DREADD ligand CNO decreased alcohol intake of animals expressing $hM_4Di$ in the dorsal striatum and had no effect on control-GFP animals (Figure 4-1). Despite the dorsal striatum's role in motor skills and directed movements, our viral DREADD construct was expressed under a human synapsin promotor which specifically targets neurons [725]. We did not characterize if the $hM_4Di$ preferentially expressed in a subset of dorsal striatum neurons, but given that the majority of the dorsal striatum consists of MSNs, activation of virally expressed striatal $hM_4Di$ receptors in our experimental design likely inhibited both the $D_1R$-MSNs and D2R-MSNs

of the direct and indirect pathways, respectively. This net inhibition may be responsible for the observed no net change in locomotor activity and a modest - albeit significant - decrease in alcohol intake [37, 46, 187, 307, 718]. Despite recent concerns on the use of DREADD technology and CNO's conversion to clozapine *in vivo*, the low dose of 2 mg/kg was specific in its behavioral effects on the $hM_4Di$-expressing mice compared with control GFP-expressing animals in drinking behavior [187], thus ruling out the potential issue that decreased consumption resulted from clozapine-N-oxide (or clozapine following CNO conversion) activating endogenous muscarinic $M_4$ receptors, which are also highly expressed in the striatum [726]. Additionally, as previously mentioned, no differences in locomotor activity were observed upon CNO administration in either control or DREADD-expressing mice, suggesting that the observed decrease in consumption did not result from sedation and lack of preferential tropism of DREADD expression on D1- versus D2-MSNs (Figure 4-2). While our DREADD strategy was successful in confirming that inhibition of dorsal striatum by increased $G_{i/o}$-protein signaling can decrease alcohol consumption, CNO is known to be an unbiased ligand for DREADD receptors [727, 728]. Therefore, we next continued with an approach where we could more selectively activate $G_{i/o}$ protein signaling over β-arrestin pathways.

Our finding that activation of $G_{i/o}$-signaling in the dorsal striatum reduces alcohol intake would suggest a role for adenylyl cyclase and cAMP in this behavior. Recently, reductions in cAMP levels in the dorsal striatum by adenylyl cyclase type 1 (AC1) inhibition and AC1-knockout have been associated with decreased ethanol-induced locomotor sensitization [654]. Furthermore, blockade of dorsal striatal $G_s$-coupled dopamine $D_1$ receptors (but not blockade of $G_{i/o}$-coupled dopamine $D_2$) attenuates alcohol consumption [307], suggesting indeed that inhibition of cAMP production in the dorsal striatum may contribute to reduced alcohol use. In the dorsal striatum, alcohol can induce long-term depression (LTD) of fast spiking interneuron-medium spiny neuron synapses via a mechanism involving DORs, as the LTD was blocked by a DOR antagonist and the effect was mimicked when using the DOR agonist DPDPE [47]. Moreover, the effects of DPDPE can also be blocked by activating adenylyl cyclases with forskolin [47]. In our hands, we find that DPDPE is relatively unbiased and thus also efficiently recruits β-arrestin [347]. This may be relevant as it has been shown that long-term depression may also rely on functional β-arrestin 2 expression: activation of hippocampal metabotropic glutamatergic receptors attenuated LTD in β-arrestin 2 KO animals [729, 730] and, upon metabotropic glutamate receptor activation, β-arrestin

2 scaffolding proteins increase the synaptic strength of hippocampal neurons [731]. Currently, no studies have investigated the role of β-arrestin 2 in alcohol and DOR-mediated LTD in the dorsal striatum, nor have studies investigated if contributions of cAMP and β-arrestin to LTD change in alcohol-exposed or alcohol-dependent animals.

The observation that β-arrestin 2 activation in the dorsal striatum increases alcohol intake in mice is in agreement with reported elevated expression levels of β-arrestin 2 gene (*Arrb2*) and β-arrestin 2 protein levels in the striatum of ethanol-preferring alko alcohol (AA) rats in comparison to alko non-alcohol (ANA) rat counterparts as well as decreased voluntary alcohol intake in β-arrestin 2 KO [693]. Despite these connections of β-arrestin expression and voluntary alcohol intake, conflicting results exist on how alcohol intake is altered in β-arrestin 2 KO animals. Li et al. [732] observed that their β-arrestin 2 KO mice displayed increased voluntary alcohol consumption compared to wild-type mice, in line with behavior by our β-arrestin 2 KO mice which also showed slightly higher alcohol intake than wild-type mice (Figure 4-5, [347]). One potential explanation is that the Bjork et al. study used alcohol solutions that contained saccharin [347, 693, 732]. Importantly, as a number of these aforementioned studies (including ours presented here) utilize global β-arrestin 2 KO animal models, we are limited in our interpretation on how global β-arrestin 2 expression affects general alcohol behavior because of potential compensatory expression of the β-arrestin 1 isoform particularly because isoform-selective differences in behavior have been observed [733]. The effect of β-arrestin expression on alcohol intake is noteworthy as altered levels of β-arrestin 2 have been observed as a result of acute and/or chronic morphine exposure in rats [734], elevated glucocorticoid activity *in vitro* [735], during inflammation *in vivo* in synoviocytes, and after cerebral hypoxia/ischemia [736]. It is possible that alcohol intake and preference by subjects in these situations is enhanced, and that effectiveness of therapeutic drugs may be altered in these subjects i.e. an unbiased drug may become β-arrestin-biased and increase alcohol use.

The dorsal striatum contains a large variety of $G_{i/o}$-coupled GPCRs besides DORs, including the muscarinic $M_4$ and serotonin 5-$HT_{1B}$ receptors [314, 315]. In line with our current findings, all three $G_{i/o}$-coupled receptors the respective knockout animals (DOR KO, $M_4$R KO, and 5-$HT_{1B}$ KO mice) consume more alcohol compared with wild-type littermates [211, 443, 509]. Here, our findings indicate that activation of dorsal striatal $G_{i/o}$-coupled receptors, either via endogenous DORs or by virally expressed DREADDs, is sufficient to decrease voluntary alcohol

intake in C57Bl/6 male mice. As β-arrestin 2 recruitment is associated with rapid internalization of DORs *in vitro* and *in vivo* (where DORs are degraded upon internalization [737]) [714, 715], we hypothesize that β-arrestin 2 recruitment to delta-opioid receptors by SNC80 can lead to rapid desensitization of endogenously expressed DORs, resulting in increased alcohol consumption similar to that observed in DOR KO mice [211]. Additionally, SNC80-induced β-arrestin 2 recruitment may lead to β-arrestin-dependent signaling events [170], such as increased phosphorylation of ERK [738, 739]. Previously, we discovered that agonists of the $G_{i/o}$-coupled DOR can either decrease or increase alcohol intake in mice [267, 277, 280], and closer examination of the pharmacology of the DOR agonists revealed that agonists that strongly recruit β-arrestin 2 increased alcohol intake, whereas agonists that were $G_{i/o}$ protein-biased decreased alcohol intake in mice [347], suggesting that $G_{i/o}$ protein-biased ligands may be a therapeutic option in treating AUD. Combined with our current results, these studies suggest a potentially broad role for striatal $G_{i/o}$-coupled signaling decreases alcohol intake, which could be accomplished via $G_{i/o}$ protein-biased ligands that activate $G_{i/o}$-coupled receptors robustly expressed in the dorsal striatum, such as the DOR. Therefore, the development of $G_{i/o}$ protein-biased DOR agonists or agonists for other striatal $G_{i/o}$-coupled receptors, such as the $M_4$, 5-$HT_{1B}$, dopamine $D_2$ [740], kappa-opioid [741], and/or GPR88 receptor [482], could present a novel strategy to treat AUD by decreasing excessive alcohol consumption.

# CHAPTER 5. BEHAVIORAL ALTERATIONS IN β-ARRESTIN 1 KNOCKOUT MICE IN ANXIETY-LIKE AND ALCOHOL-RELATED BEHAVIORS

As accepted for publication as:

**Robins MT, Chiang T, Berry JN, Ko MJ, Ha JE, van Rijn RM (2018). Behavioral characterization of β-arrestin 1 knockout C57Bl/6 mice in anxiety- and alcohol-related behaviors. Frontiers in Behavioral Neuroscience. doi: 10.3389/fnbeh.2018.00054**

β-arrestin 1 and 2 are highly expressed proteins involved in the desensitization of G protein-coupled receptor signaling which also regulate a variety of intracellular signaling pathways. Gene knockout studies suggest that the two isoforms are not homologous in their effects on baseline and drug-induced behavior; yet, the role of β-arrestin 1 in the central nervous system has been less investigated compared to β-arrestin 2. Here, we investigate how global β-arrestin 1 knockout affects anxiety-like and alcohol-related behaviors in male and female C57BL/6 mice. We observed increased baseline locomotor activity in β-arrestin 1 knockout animals compared with wild-type or heterozygous mice with a sex effect. Knockout male mice were less anxious in a light/dark transition test, although this effect may have been confounded by increased locomotor activity. No differences in sucrose intake were observed between genotypes or sexes. Female β-arrestin 1 knockout mice consumed more 10% alcohol than heterozygous females in a limited four-hour access, two-bottle choice, drinking-in-the-dark model. In a 20% alcohol binge-like access model, female knockout animals consumed significantly more alcohol than heterozygous and wild-type females. A significant sex-effect was observed in both alcohol consumption models, with female mice consuming greater amounts of alcohol than males relative to body weight. Increased sensitivity to latency to loss of righting reflex was observed in β-arrestin 1 knockout mice although no differences were observed in duration of loss of righting reflex. Overall, our efforts suggest that β-arrestin 1 may be protective against increased alcohol consumption in females and hyperactivity in both sexes.

## 5.1 Introduction

Arrestins are a family of auxiliary proteins associated with G protein-coupled receptors (GPCRs) that consists of four members: visual arrestin (arrestin 1), cone arrestin (arrestin 4), β-arrestin 1 (arrestin 2) and β-arrestin 2 (arrestin 3) [742]. The non-visual β-arrestin subtypes 1 and 2 are both highly expressed in the central nervous system [743] and are heavily implicated in negative regulation of GPCR signaling; β-arrestins can uncouple the G-protein from receptors to "arrest" signaling and assist in receptor internalization [169, 744-747], but interestingly have also been found capable of initiating signal transduction at GPCRs in a G-protein-independent manner [689, 745, 748, 749].

The critical importance of β-arrestins on neurophysiology was first demonstrated through studies using β-arrestin 2 knockout animals in which mice lacking β-arrestin 2 failed to develop antinociceptive tolerance and sensitization to chronic morphine, thereby implicating β-arrestin 2 in opioid tolerance [750, 751]. Since the first reports of differing drug responses between wild-type and β-arrestin 2 knockout animals in 1999, both pharmaceutical companies and academic laboratories have been eager to develop molecules that either preferably recruit and signal via β-arrestins over G proteins [234, 752, 753] or, conversely, that are G protein-biased [230, 232, 261, 705, 754] in order to treat neurophysiological disorders while avoiding adverse side effects. In these endeavors, however, little attention is given to the different β-arrestin isoforms, β-arrestin 1 and 2. While β-arrestin 1 or β-arrestin 2 global knockout mice are viable and healthy, each display altered physiology and behavior compared with wild-type mice [693, 733]. In contrast, double knockout mice are embryonically lethal, indicating a degree of functional compensation and overlap within the isoforms [755]. Still, despite the high 78% sequence homology between the β-arrestin 1 and 2 [692], differential expression and functionality of the two isoforms has been observed in various CNS disorders [756] and following drug exposure [734].

In general, compared to β-arrestin 2, fewer studies have investigated behavioral responses in β-arrestin 1 knockout mice, although the role of β-arrestin 1 has been investigated in non-CNS contexts such as cancer [757, 758], autoimmunity [759], and cardiology [760]. Recently, a study by Zurkovsky et al., found that β-arrestin 2 knockout mice exhibit reduced amphetamine-induced locomotion, while β-arrestin 1 knockout mice exhibit increased locomotor responsiveness to amphetamine [733]. This divergent modulation of amphetamine-induced locomotion further

suggests that the β-arrestin isoforms may differentially contribute to drug-related behaviors. Additionally, most studies that have used β-arrestin knockout mice in the past have solely used male mice; therefore, we decided to characterize both male and female wild-type, heterozygous and homozygous β-arrestin 1 knockout C57BL/6 mice in animal models of reward intake and anxiety-like behavior. Here, we observed β-arrestin 1-specific changes (with sex specific effects) in behaviors, such as increased locomotor activity, increased alcohol intake, and faster alcohol-induced sedation.

## 5.2 Materials and methods

### 5.2.1 Drugs and chemicals

200 proof ethyl alcohol and sucrose were purchased from Sigma-Aldrich (St. Louis, MO, USA).

### 5.2.2 Animal husbandry

β-arrestin 1 knockout C57BL/6 mice were obtained from the laboratory of Dr. Amynah Pradhan at the University of Illinois-Chicago, but originated in the laboratory of Dr. Robert Lefkowitz (Duke University)[714, 761]. The original knockout strain was created by crossing a 129/Sv strain with a black Swiss strain [762], but was then backcrossed to C57BL/6 mice for more than 10 generations. The β-arrestin 1 (-/-) KO C57BL/6 mice were obtained via β-arrestin 1 (-/-) x β-arrestin 1 (-/-) breeding pairs. The β-arrestin 1 (+/-) heterozygous KO were obtained via β-arrestin 1 (-/-) x wild-type C57BL/6 mice breeding pairs.

Food was provided ad libitum; water was provided ad libitum unless specified for ethyl alcohol and sucrose consumption experiments. Throughout the experiment, animals were housed in Plexiglas® cages in ventilated racks at ambient temperature of (21°C) in a room maintained on a reversed 12L:12D cycle (lights off at 10.00, lights on at 22.00) in Purdue University's animal facility, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. This study was carried out in accordance with the recommendations of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The protocol was approved by the Purdue University Institutional Animal Care and Use Committee (#1305000864).

In designing the behavior assays, two separate experimental batteries were created to efficiently run the experiments while reducing/refining the number of mice necessary. All

behavioral experiments were performed during the dark/active phase of the mice. Mice in the first group were tested for locomotor activity/open field (2 days total) three days prior to alcohol intake studies, which took 5 weeks total (3 weeks 10%, followed by 2 weeks 20%). Mice assigned to the second group underwent light/dark transition testing (1 day) three days prior to sucrose intake assays (10 days total). Five days later loss of righting reflex (1 day) was assessed in this second group. All mice were approximately 55 days post-natal at the beginning of testing.

### 5.2.3 Western blot analysis of β-arrestin 1 expression

Mice were euthanized for rapid brain dissection by $CO_2$ asphyxiation and brain samples were stored on dry ice following organ harvest. For sample preparation, 30 mg of cerebellar tissue was lysed using 200 μL RIPA buffer and 1x protease inhibitors (ThermoFisher, Waltham, MA USA). Samples were then homogenized using a manual hand-homogenizer and sonicated prior to centrifugation at 12,000 rpm for 20 minutes at 4°C, where the supernatant was kept for further analysis and pellet was discarded. Protein concentration was determined by Bradford protein determination assay (Bio-Rad Hercules, CA, USA), and 10 mg protein/20 μL sample were loaded into a gradient gel (Life Technologies, Carlsbad, CA USA). Following gel transfer to a nitrocellulose membrane (ThermoFisher) and blocking (Li-Cor blocking buffer, Lincoln, NE USA), the membrane was stained using mouse anti-α-tubulin (SC-5286, lot #63117, Santa Cruz Biotechnology, Dallas, TX, USA) and rabbit anti-β-arrestin 1 XP® (D723W, 300365, lot #1, Cell Signaling, Danvers, MA, USA) primary antibodies, both at a 1:1000 dilution in blocking buffer with 0.2% Tween for 1 hour at room temperature. Secondary antibodies IRdye 680 LT goat anti-mouse (925-68020, lot #C60824-02 Li-Cor) and IRdye 800 CW goat anti-rabbit (925-32211, lot #C61103-06, Li-Cor) were applied 1:5000 in blocking buffer for 1 hour at room temperature. The membrane was then imaged using Li-Cor Odyssey CLx, and relative β-arrestin 1 protein concentration was determined by normalized intensity to loading α-tubulin (loading control) as well as average normalized β-arrestin 1 protein concentration of wild-type mice (genotype control) using ImageJ software (NIH, Bethesda, MA USA).

### 5.2.4 Open field locomotion

Square locomotor boxes from Med Associates (L 27.3 cm x W 27.3 cm x H 20.3 cm, St. Albans VT, USA) were used to monitor locomotor activity. For all locomotor studies, animals were moved

to the testing room for 60 minutes prior to testing. A 60-minute habituation session to the boxes was conducted 1 day prior to testing, to avoid measuring locomotor activity driven by novelty and/or anxiety. During this habituation session for the open field test, anxiety-like behavior was measured. For open field anxiety testing, the time spent in the center of the area (12x12 cm) during the first five minutes of the testing session and the total number of crosses into the center area was recorded. The following day, locomotor activity was monitored for a total of 60 minutes for analysis. All testing was conducted during the dark/active light phase.

#### 5.2.5 Light/dark box

Animals were habituated to the experimental room >30 minutes prior to testing. Testing was conducted without a habituation session to the boxes and a 1/3 area dark insert was placed in the locomotor boxes, leaving the remaining 2/3 of the area lit as described previously [763]. Two LED lights were inserted above the light portion of the testing chamber where the lux of the light region ranged from 390-540 lumens and dark chamber lux ranged from 0-12 lumens. For testing, animals were placed in the light portion of the chamber and testing began upon animal entry. Analysis of the 10 minute sessions utilized MedAssociates software to measure total locomotion per area, total time spent in area, total crosses between areas, and latency to enter the dark area [763-765]. All testing was conducted during the dark/active light phase.

#### 5.2.6 Sucrose consumption

Mice were individually housed in double-grommet ventilated Plexiglas® cages to monitor individual fluid consumption. Animals were offered increasing concentrations of sucrose in water (0.25-4%) in two day increments for a total of 10 days where the total amount of sucrose liquid was recorded daily [60, 766]. Sucrose was offered in a two-bottle (sucrose vs. reverse osmosis water), limited-access (4 hour) choice two hours into the dark/active phase, where the location of each bottle was alternated daily (right vs. left grommet) to prevent habit formation. After each drinking session, bottles were removed and weighed for total volume water and sucrose consumed during the four-hour session. Water intake during the 20-hour single access to water was measured for changes in general fluid intake. Fluid spillage during these sessions was corrected for using control bottles located on empty cages.

### 5.2.7 Alcohol consumption

Animals were evaluated for voluntary alcohol consumption over the course of five weeks as previously described [267] and throughout the testing weeks, mice were individually housed in double-grommet ventilated Plexiglas® cages for individual fluid consumption. Moderate alcohol intake was tested in the first 3 weeks followed by binge-like consumption in the final 2 weeks. During the first three weeks of alcohol testing, animals were offered a two-bottle, limited-access choice between 10% ethyl alcohol and reverse osmosis water for four hours a day, two hours into the dark/active phase, where the location of each bottle was alternated daily (right vs. left grommet) to prevent habit formation. Bottles were removed and weighed prior to and after each drinking session; these measurements were recorded Monday-Friday. but only values from Tuesday-Friday were used in analysis as drinking varied on Mondays following two days of forced abstinence. Total daily and weekly average alcohol consumption was recorded for analysis for sex, genotype, and genotype+sex effect.

To measure binge-like-alcohol intake, we used a behavioral model described by Rhodes et al. where in weeks 4-5 [268] the animals had access to a single bottle of 20% ethyl alcohol for two-hours a day, Monday-Thursday. On Fridays, the duration of the drinking session increased to four hours. As mentioned for previous fluid consumption experiments, the location of the single 20% alcohol bottle was alternated daily (right vs. left grommet) to prevent habit formation. In these sessions, total alcohol consumed during the two weeks of 20% alcohol access was analyzed for sex, genotype, and genotype+sex effect.

### 5.2.8 Loss of righting reflex

Alcohol-naïve animals were moved to a testing room during the dark/active phase and allowed one hour for acclimation prior to injection with 3.8 g/kg 20% ethyl alcohol (i.p.). For analysis of the sedative hypnotic effects of acute alcohol intoxication, loss of righting reflex was defined as the inability of a mouse to right itself after being placed on its back three times within 30 seconds [767]. Latency to LORR (time from injection to immobile) and duration of LORR (time from immobile to regaining LORR) was recorded for sex, genotype, and genotype+sex effect.

### 5.2.9 Statistical analysis

All data are presented as means ± standard error of the mean (SEM) using GraphPad Prism 7 software (GraphPad Software, La Jolla, CA). Statistical analysis for the effect of genotype, sex, and sex+genotype interaction was analyzed by two-way ANOVA with either Sidek (genotype differences between the sexes) or Tukey (for within sex genotype differences) post-tests for multiple comparisons.

## 5.3 Results

### 5.3.1 Western blot confirmation of β-arrestin 1 expression in different genotypes and lower body weight in β-arrestin 1 knockout mice.

To confirm that the heterozygous and homozygous β-arrestin 1 knockout mice indeed exhibited altered β-arrestin 1 expression we measured β-arrestin 1 protein levels from cerebellum in these animals (as high β-arrestin 1 mRNA levels had been measured in male C57Bl/6 previously, Experiment #70305395, Allen Brain Atlas) and compared with β-arrestin 1 levels in wild-type β-arrestin 1 mice, which we normalized as a control (Figure 5-1A,B). We observed a significant decrease in both heterozygous and knockout mice β-arrestin 1 expression as determined by one-way, multiple comparisons ANOVA ($F_{2,7}$ =32, $p$=0.0002, WT vs. HET: $p$=0.037, WT vs. KO: $p$=0.0001). Negligible β-arrestin 1 expression observed in knockout animals, observed staining at this protein weight is likely non-selective antibody staining of β-arrestin 2, which is also 51 kDa.

Age matched β-arrestin 1 knockout mice of both sexes exhibited decreased body weight compared with wild-type mice over the course of five weeks (Figure 5-1C, D). In male mice, a significant effect of genotype ($F_{2,19}$=20.8, $p$<0.0001), time ($F_{4,76}$=25.2, $p$<0.0001) and interaction ($F_{8,76}$=6.39, $p$<0.0001) was found, where multiple comparisons revealed a significant difference between KO vs. WT ($p$<0.0001) and vs. HET ($p$=0.0002). In females, a similar decrease in body weight was observed in β-arrestin 1 knockout mice with a significant effect of genotype ($F_{2,21}$=12.2, $p$=0.0003)

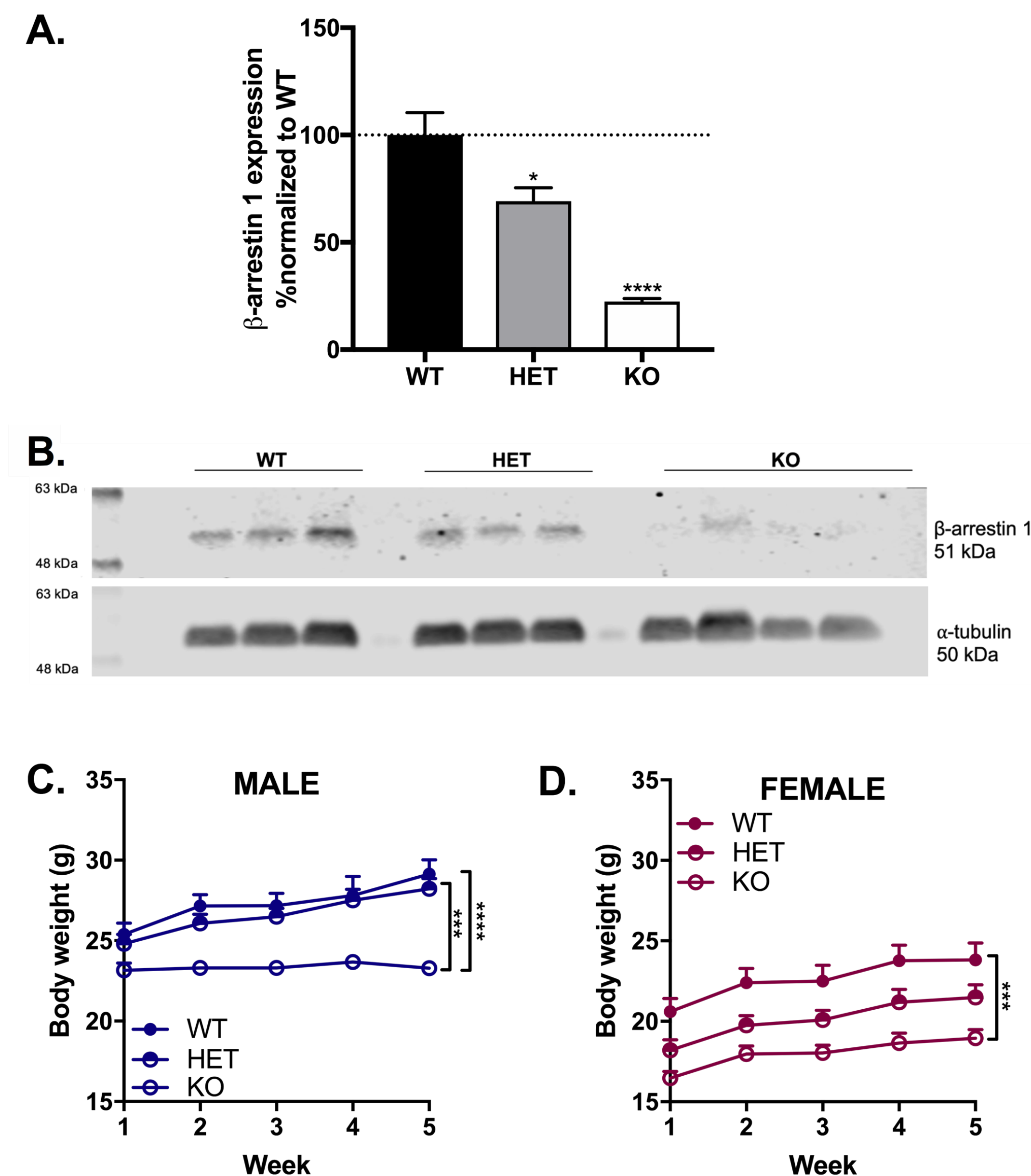



Figure 5-1. Decreased β-arrestin 1 expression in heterozygous mice and negligible β-arrestin 1 expression in knockout mice; decreased body weight in β-arrestin 1 KO mice.

Compared to wild-type, decreased β-arrestin 1 expression was observed via Western blot of cerebellar protein extracts in heterozygous and knockout β-arrestin 1 mice (A), as normalized by α-tubulin expression and adjusted for background (B). A decrease in body weight was observed in β-arrestin 1 knockout male (C) and female (D) mice. Significance by one-way ANOVA (β-arrestin 1 Western blot) or two-way ANOVA (body weight over time) with multiple comparisons (Tukey), $p<0.05$, *, $p<0.01$, **, $p<0.001$, ***, $p<0.0001$, ****; data represented as mean ± SEM.

and time ($F_{4,84}$=25.2, *p*<0.0001). No interaction ($F_{8,84}$=1.04, *p*<0.0001) effect was observed, although multiple comparisons revealed a significant difference in body weight between KO vs. WT (*p*=0.0002).

### 5.3.2 Increased locomotor activity in β-arrestin 1 knockout animals with no significant differences in open field anxiety behavior compared with wild-type or heterozygous.

For the 60 minute locomotor session, a significant effect of genotype ($F_{2,46}$=29.4, p<0.0001) and sex ($F_{1.46}$=5.54, *p*=0.0229) was found, with no significant interaction effect $F_{2,46}$=0.810, *p*=0.451) (Figure 5-2A). Within males, a significant increase in locomotion was observed between KO and both WT (*p*=0.0022) and HET (*p*=0.0003) with no significant difference in total ambulation between WT and HET (*p*=0.866). In females, KO mice ambulated significant more than WT (*p*<0.0001) and HET (*p*<0.0001) with no significant difference in ambulation between WT and HET (*p*=0.703). For time spent in the center of the open field box in the first 5 minutes of the first habituation session, a significant effect of sex ($F_{1,46}$=5.39, *p*=0.0248) was observed as male spent more time in the center than females, although no significant effect of genotype ($F_{2,46}$=0.706, *p*=0.499) or interaction ($F_{2,46}$=1.96, *p*=0.153) were noted (Figure 5-2B). Within genotypes, male WT mice ambulated significantly more than female WT mice (p=0.033). For number of entries into the center quadrant during the first five minutes, a similar sex effect was found ($F_{1,46}$=5.36, *p*=0.0251) as males entered the center more than females, but no effect of genotype ($F_{2,46}$=0.070, *p*=0.932) or interaction ($F_{2,46}$=0.121, *p*=0.886) (Figure 5-2C) was observed.

### 5.3.3 β-arrestin 1 male knockout animals spend more time in light with higher ambulation in light/dark transition test.

No sex ($F_{1,49}$=0.00470, *p*=0.946) nor interaction of sex+genotype effect ($F_{2,49}$=1.37, *p*=0.264) was detected for time spent in the light compartment during the 10 minute light/dark transition test, although a genotype effect ($F_{2,49}$=11.7, *p*<0.0001) was observed (Figure 5-3A). Multiple comparisons test revealed that male β-arrestin 1 KO spent more time in the light (anxiogenic) compartment compared with male WT (*p*=0.0011) or male HET (*p*=0.0028) mice. For total distance traveled in the light compartment, a significant effect of genotype ($F_{2,49}$=15.5, *p*<0.0001)

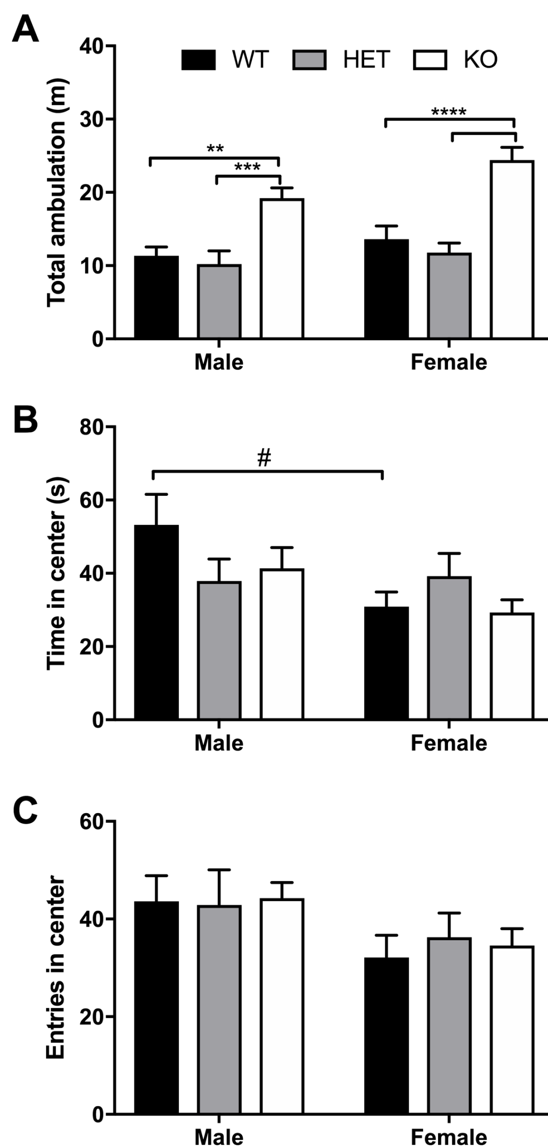



Figure 5-2. Increased locomotor activity in β-arrestin 1 knockout animals but no genotype effect on anxiety-like behavior

Locomotor activity was assessed for wild-type (WT), heterozygous (HET), and β-arrestin 1 knockout (KO) male and female C57BL/6 mice in a 60-minute session. An overall sex effect in total locomotion were observed (n=27 males, n=25 females), and KO male mice (n=10) displaying higher ambulation than WT (n=8) or HET (n=9) males (**A**). This genotype effect was also observed between female KO mice (n=9) and WT (n=8) and HET (n=7) females (**A**). Male mice spent more time in the center of the arena than females during the first five minutes of testing (**B**), although no genotype effect was observed. Similarly, males entered the center area of the arena more times than females, although again no genotype effect was noted (**C**). Significance by two-way ANOVA with multiple comparisons (Tukey within sex, Sidek between genotype), $p<0.01$, **, $p<0.001$, ***, $p<0.0001$, ****; #, $p<0.05$ data represented as mean ± SEM.

was observed with no effect of sex ($F_{1,49}$=0.0632, $p$=0.803) nor interaction ($F_{2,49}$=2.06, $p$=0.138) (Figure 5-3B). Multiple comparisons revealed that this genotype effect was prevalent between male β-arrestin 1 KO and WT ($p$=0.0002) and HET ($p$=0.0002), with higher ambulation in the light in male KO mice. This significant effect of genotype was not observed in distance traveled in the dark compartment, as no effect of genotype ($F_{2,49}$=0.709, $p$=0.497), sex ($F_{1,49}$=01.54, $p$=0.221), or interaction ($F_{2,49}$=0.249, $p$=0.781) was observed (Figure 5-3C). For number of crosses between the two compartments, no sex ($F_{1,49}$=3.87x10$^{-6}$, p=0.998), genotype ($F_{2,49}$=2.18, $p$=0.125), or interaction effect ($F_{2,49}$=0.154, $p$=0.857) was noted (Figure 5-3D). A similar lack of sex ($F_{2,49}$=1.53, $p$=0.223), genotype ($F_{1,49}$=0.425, $p$=0.656), or interaction effect ($F_{2,49}$=0.116, $p$=0.891) was observed for latency to enter the dark compartment (Figure 5-3E). Overall, these results suggest that the increased locomotor activity observed in male β-arrestin 1 KO accounted for the increased in time spent in the light rather than a difference in anxiety-like behaviors

### 5.3.4 No differences in sucrose preference between β-arrestin 1 genotypes.

No effect of sex ($F_{1,53}$=0.501, $p$=0.482), genotype ($F_{2,53}$=0.2943, $p$=0.396), or interaction ($F_{2,53}$=0.140, $p$=0.870) were observed for total sucrose consumption within β-arrestin 1 (Figure 5-4), suggesting no alterations in natural reward intake behavior. To assess if differences in general fluid intake were present, we measured total water intake during the 20-hour access period to water alone. No significant difference in 20-hour water intake was noted by volume alone (sex: $F_{1,53}$=0.0194, $p$=0.890; genotype: $F_{2,53}$=0.157, $p$=0.855; interaction: $F_{2,53}$=2.52, $p$=0.0897, *graph omitted from thesis text*) or by volume consumed per body weight for genotype ($F_{2,53}$=2.00, $p$=0.146) or interaction ($F_{2,53}$=1.52, $p$=0.227), although female mice consumed more water per body weight than males (sex: $F_{1,53}$=16.4, $p$=0.0002) (*graph omitted from thesis text*).

### 5.3.5 Ethanol consumption higher in female animals in general, and β-arrestin 1 expression prevents increased binge consumption in females.

An overall sex effect was observed between male and female animals for average alcohol intake in the limited-access, 10% two-bottle choice protocol ($F_{1,51}$=46.45, $p$<0.0001), as well as a genotype ($F_{2,51}$=4.52, $p$=0.0157) and interaction effect ($F_{2,51}$=4.11, $p$=0.0221). Within male animals, no observed differences in average alcohol intake were present, yet HET female mice consumed less 10% alcohol ($p$=0.0025) on average than KO female animals (Figure 5-5). Across

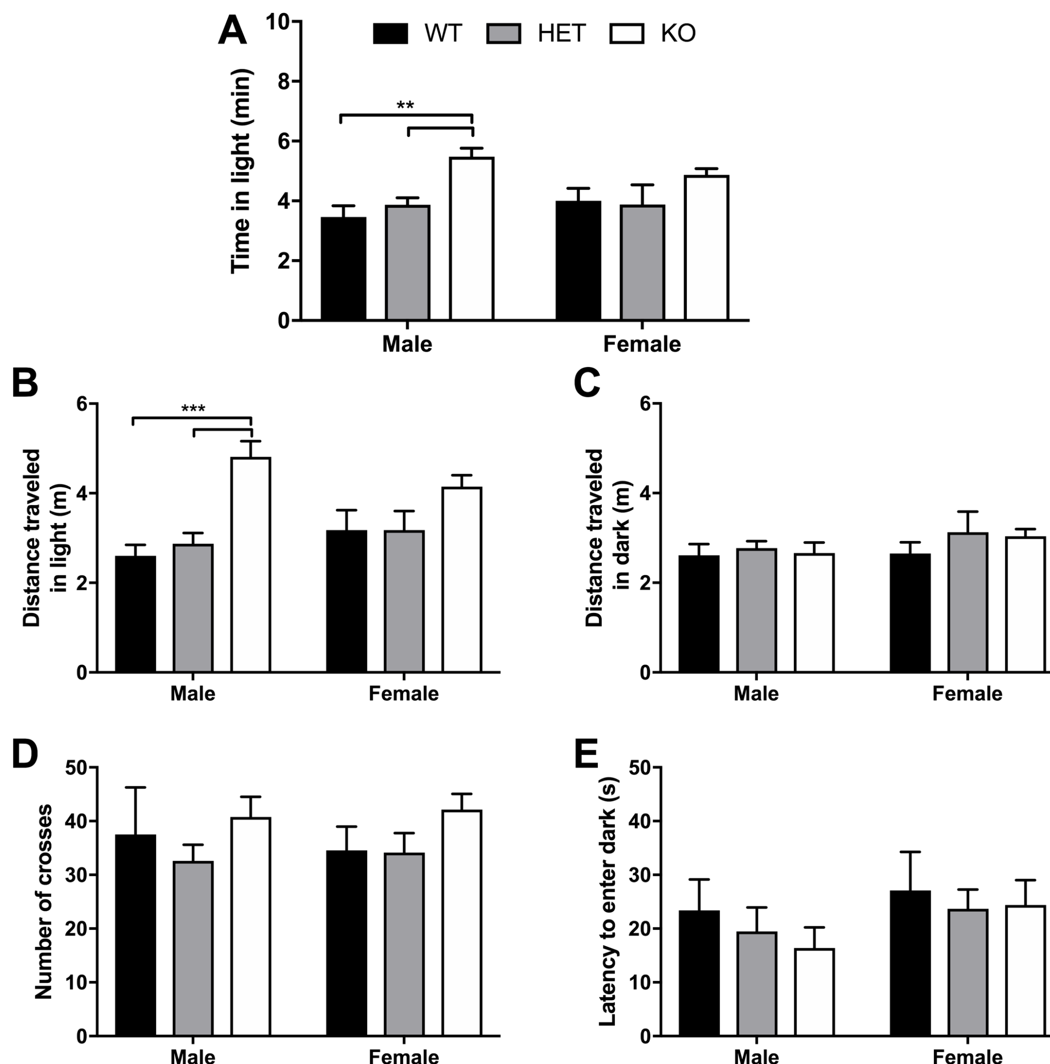



Figure 5-3. Light/dark transition test for anxiety-like behavior reveals male β-arrestin 1 knockout animals spend more time in anxiogenic compartment with higher locomotion.

Light/dark transition was performed for wild-type (WT), heterozygous (HET), and β-arrestin 1 knockout (KO) male and female C57BL/6 mice in a 10-minute session. No sex effect was observed for time spent in the light (anxiogenic) compartment (n=26 males, n=29 females) but a significant genotype effect was observed between KO male mice (n=10) and WT (n=6) and HET male mice (n=10) (**A**). No genotype effect was observed between female β-arrestin 1 WT (n=7), HET (n=7), or KO mice (n=15) (**A**). Total ambulation in the light (anxiogenic) compartment was significant increased in β-arrestin 1 KO males compared with male WT or HET mice, with no genotype effect observed in females (**B**). No sex or genotype effects were observed for distance traveled in the dark (anxiolytic) compartment (**C**). No sex or genotype differences were observed for number of crosses between the light and dark compartments (**C**) or latency to enter the dark (anxiolytic) compartment (**D**). Significance by two-way ANOVA with multiple comparisons (Tukey within sex, Sidek between genotype), $p<0.01$, **, $p<0.001$, ***; data represented as mean ± SEM.

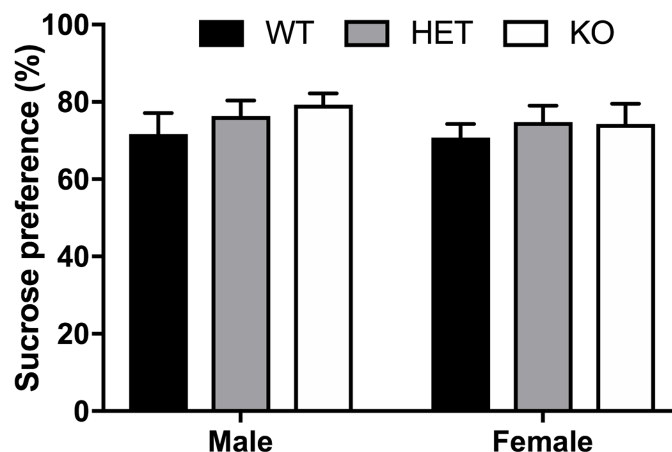



Figure 5-4. No sex or β-arrestin 1 genotype effect on preference of natural reward, sucrose.

A two-bottle, limited access procedure of increasing concentrations of sucrose (0.25-4%) for a total of 10 days revealed no sex (n=26 males, n=24 females), genotype, or interaction effect on average sucrose preference. Significance by two-way ANOVA with multiple comparisons (Tukey within sex, Sidek between genotype); data represented as mean ± SEM.

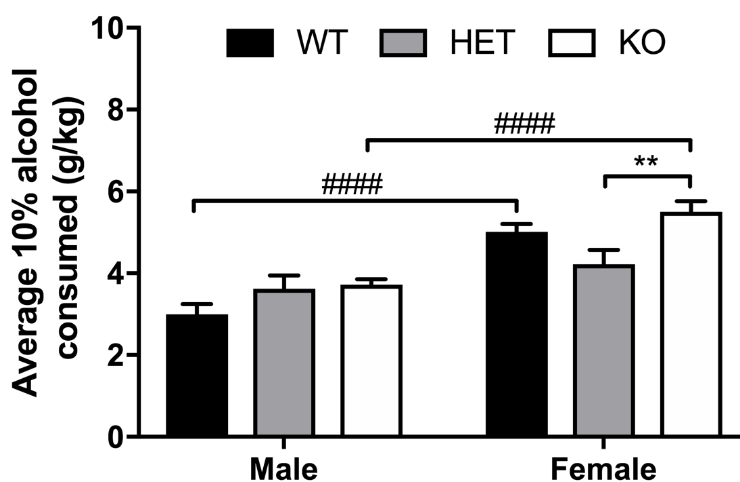



Figure 5-5. Large sex effect observed for average 10% alcohol, with β-arrestin 1 knockout females consuming more alcohol than heterozygous females.

Average daily 10% alcohol consumption was significantly higher in female (n=29) mice compared with male mice (n=28). No overall genotype effect was observed between male animals (n=7 WT, n=10 HET, n=11 KO) although KO female mice consumed significantly more alcohol than HET females (n=9 WT, n=10 HET, n=11 KO). When comparing the two sexes within the same genotype, female WT or KO consumed more alcohol that male WT or KO, respectively. Significance by two-way ANOVA with multiple comparisons (Tukey within sex, Sidek between genotype), $p < 0.01$, **; $p < 0.0001$, ####; data represented as mean ± SEM.

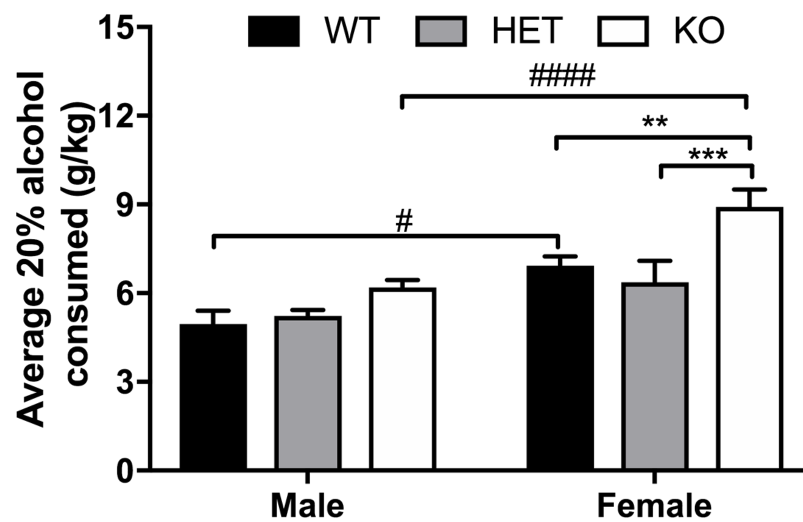



Figure 5-6. Overall sex effect on binge 20% intake, binge 20% alcohol intake is higher in female β-arrestin 1 knockout animals.

Average binge intake during the 4-hour session was significantly higher in females (n=27) compared with male mice (n=25) as observed by an overall sex effect. In males, KO males (n=11) did not drink significantly more than WT (n=7) or HET (n=7). Female KO mice (n=11) consumed more alcohol than WT (n=9) and HET (n=7) females. When comparing the two sexes within the same genotype, female WT or KO mice also consumed more alcohol than male WT or KO mice, respectively. Significance by two-way ANOVA with multiple comparisons (Tukey within sex, Sidek between genotype), $p<0.01$, **, $p<0.001$, ***; $p<0.05$, #; $p<0.00001$, ####; data represented as mean ± SEM

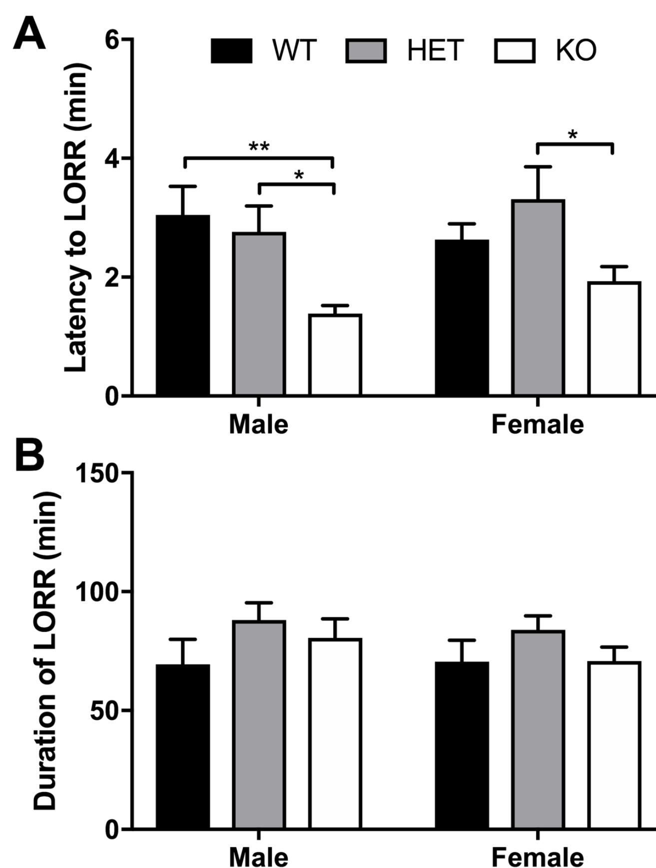



Figure 5-7. Decreased latency to loss of righting reflex (LORR) observed in β-arrestin 1 knockout mice but duration of LORR not affected by β-arrestin 1 expression.

Following 3.8 g/kg 20% ethanol administration (intraperitoneal), no sex effect was observed for latency to LORR (n=26 males, n=25 females), although a genotype effect was observed in both male (n=7 WT, n=8 HET, n=10 KO) and female mice (n=8 WT, n=8 HET, n=8 KO), with decreased latency was observed in mice lacking β-arrestin 1 expression (**A**). Duration of LORR was not affect by sex or β-arrestin 1 genotype alone, nor were differences observed by genotype+sex (**B**). Significance by two-way ANOVA with multiple comparisons (Tukey within sex, Sidek between genotype), $p < 0.05$, *; $p < 0.01$, **; data represented as mean ± SEM.

genotypes, female KO mice consumed more alcohol than male KO ($p<0.0001$). Female WT mice also consumed more alcohol than male WT ($p<0.0001$). In the binge 20% alcohol consumption model, a significant sex effect was again observed ($F_{1,46}=25.9$, $p<0.0001$). An overall genotype effect ($F_{2,46}=9.83$, $p<0.0003$) was observed although no interaction effect was noted ($F_{2,46}=1.50$, $p<0.233$). Within male animals, no significant genotype effect was observed by multiple comparisons, although within females, β-arrestin 1 KO mice consumed more alcohol than WT ($p=0.0056$) and HET ($p=0.0009$) (Figure 5-6). Furthermore, within genotypes, a significant increase in alcohol intake was observed between female β-arrestin 1 KO compared with male β-arrestin 1 knockout ($p<0.0001$) and female WT compared to male WT ($p=0.0168$).

### 5.3.6 Decreased latency to loss of righting reflex in β-arrestin 1 knockout mice but no alterations in duration of LORR.

No sex ($F_{1,44}=0.596$, $p=0.448$) or interaction ($F_{2,44}=1.10$, $p=0.342$) effect was observed for latency to LORR, although a significant genotype effect ($F_{2,42}=9,07$, $p=0.0005$) was noted (Figure 5-7A). Multiple comparisons revealed that male β-arrestin 1 KO mice exhibited decreased latency to LORR as compared with male WT ($p=0.0008$) and HET ($p=0.0177$). In female mice, multiple comparisons revealed that female β-arrestin 1 KO mice had decreased latency to LORR as compared with female HET ($p=0.0249$) but not WT ($p=0.367$). For duration of LORR, no sex ($F_{1,44}=0.439$, $p=0.511$), genotype ($F_{2,44}=2.08$, $p=0.137$), or interaction ($F_{2,44}=0.238$, $p=0.790$) effect was observed (Figure 5-7B).

## 5.4 Discussion

Using a global knockout strategy, we explored the role of β-arrestin 1 in relation to basal anxiety-like behavior and behavior associated with consumption of rewarding substances. A potential pitfall when utilizing a global, congenic knockout strategy is that mice may display altered cellular or behavioral responses resulting from compensatory adaptations to cope with the absence of β-arrestin 1 expression. However, currently no conditional β-arrestin 1 isoform knockouts have been produced to overcome this.

Here we observed increased baseline locomotion in both male and female β-arrestin 1 knockout mice and decreased trait anxiety-like behavior in male β-arrestin 1 knockout mice. In our animals, we observed that male mice without β-arrestin 1 expression exhibit decreased trait

anxiety, as measured by increased time spent in the more anxiogenic light compartment in the light/dark test (Figure 5-3), although this increased time spent in the light compartment may be the result of increased locomotor activity in general (Figure 5-2,3). However, one study has reported that anxious mice display reductions in β-arrestin 1 protein levels in blood plasma [769]. For alcohol consumption, a large sex effect was observed with female animals consuming more alcohol than male animals in both limited-access and binge alcohol models, as observed by other studies [139-141]. A recent study by Mittal et al. observed that β-arrestin 1 expression is essential for regulating reward-motivated behaviors associated with cocaine self-administration and natural food reward through a mechanism associated with altered glutamatergic function, as β-arrestin 1 KO mice exhibited deficits in both types of reward responding [770]. This observed deficit in natural reward intake may be reflected in the decreased body weights observed in our β-arrestin 1 KO mice compared with wild-type (Figure 5-1). However, we did not observe differences for sucrose preference (Figure 5-4), in contrast with the previously observed decrease in natural food reward in these mice [770]. The discrepancies in the observed behavior of β-arrestin 1 knockout mice may be the result of differences related to the type of learning involved in operant self-administration (Pavlovian) versus volitional intake assays (non-Pavlovian). Surprisingly, in our voluntary alcohol consumption assays, we did observe that female knockout β-arrestin 1 mice consumed more alcohol (Figure 5-5,6) than wild-type (for binge) or heterozygous (for limited access and binge) females. These results are suggestive of a potential protective role of β-arrestin 1 expression in increased voluntary alcohol intake or protective genetic compensation upon β-arrestin 1 knockout in females. These alterations in alcohol consumption are not believed to be the result of differences in alcohol metabolism, as no differences in the time it takes for animals to regain consciousness following alcohol sedation in a loss of duration of righting reflex assay were apparent by genotype or sex, although β-arrestin 1 knockout mice were quicker to sedate to alcohol compared with wild-type (in male mice only) and heterozygous (in both sexes), which may indicate increased $GABA_A$ receptor function in the KO animals [771].

We can speculate on what is driving the ability of β-arrestin 1 to modulate certain behaviors; β-arrestin 1 has been implicated in signaling events requiring translocation to the nucleus, such as increased Bcl2, P27, arachidonic acid, and ROCK/LIMK signaling [759, 772, 773]. The effects of β-arrestin 1 expression in neuronal cell survival following a variety of neurological insults remains

to be more fully investigated, although increased β-arrestin 1 expression has been correlated with decreased negative neurological behavioral effects following ischemic insult [756]. β-arrestin isoform selective signaling has been previously observed at the metabotropic glutamate receptor 7 (mGlu7) *in vitro*, where β-arrestin 1 signaling increased ERK1/2 and inhibited JNK, while β-arrestin 2 performed the opposite [774], although this reciprocal regulation may be cell-type and receptor specific [761], as *in vivo* β-arrestin 2 disruption decreased murine hippocampal metabotropic glutamate receptor 5 (mGlu5)-associated ERK activation [729].

Besides differences in receptor-mediated downstream signaling, varied responses to drug exposure and disease have been observed between β-arrestin 1 and 2 using *in vivo* and *in vitro* models. For example, amphetamine-induced hyperlocomotion is initially enhanced in male β-arrestin 1 KO mice while β-arrestin 2 KO mice are hyposensitive to amphetamine and display poor locomotor sensitization [733, 775]. Also, expression of β-arrestin 2, but not β-arrestin 1, is required for morphine-induced hyperlocomotion [776], where interestingly expression of β-arrestin 2 increases morphine, but not cocaine, reward [777]. Our behavioral results suggest that selectively activating β-arrestin 1 recruitment and/or signaling may result in decreased alcohol intake in females and prevent locomotor hyperactivity. We have previously observed that β-arrestin 2 recruitment at the δ-opioid receptor is associated with increased alcohol intake [261]. Similarly, β-arrestin 2 KO mice have been reported to consume less alcohol and mice bred to prefer alcohol display increased β-arrestin 2 expression [693]. In the opioid field, there has been a push to develop G-protein biased drugs that do not recruit β-arrestin [230, 232, 705, 754]; however, our data suggests that sometimes it may be beneficial to selectively recruit β-arrestin 1, i.e. selectively avoid β–arrestin 2 recruitment. A few recent studies suggest that it may be possible to identify drugs that preferentially recruit a specific β-arrestin isoform upon receptor binding. Agonist-selective recruitment of β-arrestin isoforms has been observed at the δ-opioid receptor, where high-internalizing agonists recruit β-arrestin 1 to the receptor while low-internalizing agonists preferentially recruit β-arrestin 2 [714]. Additionally, reports of the δ-opioid agonist etorphine suggest that this alkaloid agonist – compared with peptide agonists such as [D-Pen2,D-Pen5]enkephalin (DPDPE) and deltorphin I – promotes δ-opioid receptor endocytosis in a β-arrestin 1-dependent manner which is not observed upon DPDPE or deltorphin I activation,

although β-arrestin 2 was not expressed in this study and therefore β-arrestin 2-dependent effects were not discussed [778].

In summary, here we provide additional support that β-arrestin 1 may have similar, as well as unique, roles in behavior compared with β-arrestin 2, as described in β-arrestin 2 studies previously conducted. Our study also revealed instances of β-arrestin 1-associated sex differences, such as those described here for alcohol intake, suggesting that it is important to consider both sex and β-arrestin isoforms when studying biased signaling at G protein-coupled receptors. Our studies warrant continued investigation into the mechanisms that underlie these observed behavioral differences in β-arrestin 1 KO mice and potential underlying differences in downstream signaling cascades between the two β-arrestin isoforms. There is an increased interest in developing β-arrestin-biased agonists for specific indications [234, 760, 779-781]; ours and future studies could promote the idea of developing of β-arrestin-isoform-biased agonists.

# CHAPTER 6. ADOLESCENT CAFFEINE EXPOSURE DOES NOT INFLUENCE ADULT ALCOHOL INTAKE

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The rise in marketing and mass consumption of energy drink products by adolescents poses a largely unknown risk on adolescent development and drug reward. Yet, with increasing reports of acute health issues present in young adults who ingest large quantities of energy drinks alone or in combination with alcohol, the need to elucidate these potential risks is pressing. Energy drinks contain high levels of caffeine and sucrose; therefore, exposure to energy drinks may lead to changes in drug-related behaviors since caffeine and sucrose consumption activates similar brain pathways engaged by substances of abuse. With a recent study observing that adolescent caffeine consumption increased cocaine sensitivity, we sought to investigate how prolonged energy drink exposure in adolescence alters alcohol use and preference in adulthood. To do so, we utilized three different energy-drink exposure paradigms and two strains of male mice (C57BL/6 and BALB/c) to monitor the effect of caffeine exposure via energy drinks in adolescence on adult alcohol intake. These paradigms included two models of volitional consumption of energy drinks or energy drink-like substances and one model of forced consumption of sucrose solutions with different caffeine concentrations. Following adolescent exposure to these solutions, alcohol intake was monitored in a limited-access, two-bottle choice between water and increasing concentrations of alcohol during adulthood. In none of the three models or two strains of mice did we observe that adolescent 'energy drink' consumption or exposure was correlated with changes in adult alcohol intake or preference. While our current preclinical results suggest that exposure to large amounts of caffeine does not alter future alcohol intake, differences in caffeine metabolism between mice and humans need to be considered before translating these results to humans.

## 6.1 Introduction

Consumption of highly caffeinated energy drinks has increased rapidly over the last decade. Between the years of 2002 to 2006, sales of energy drinks grew at a rate of 55% annually with sales in the United States reaching $5.4 billion in 2006 [782, 783]. The consumption of energy drinks is particularly prevalent in adolescents and young adults, with reports of more than 30% of this population consuming these drinks on a regular basis [784]. Adolescents and young adults are selectively targeted by energy drink manufacturers and marketing, thus increasing the desire and probability of consumption in this age group [783, 785, 786]. Importantly, the current generation of energy drinks contains much higher caffeine concentrations (ranging from 9–30 mg/oz, [783] than standard caffeinated sodas such as cola which generally contain roughly 3 mg/oz caffeine [783].

Several reports have indicated that caffeine can induce behavioral effects commonly associated with drugs of abuse, such as increased self-administration, reward, withdrawal, and tolerance [786, 787]. Adolescent caffeine consumption produces cross-sensitized responses to methylphenidate [788], increased self-administration of nicotine [789], increased cocaine sensitivity [786, 790], and increased self-administration of alcohol after caffeine exposure [791]. This is not unexpected as caffeine induces dopamine release in brain regions that process behavioral reinforcement in ways similar to those of drugs of abuse [792-794]. As the adolescent brain is still under development, adolescent exposure to highly caffeinated energy drinks could potentially influence short- and long-term behaviors, specifically relating to drug reward and consumption. Brain structures such as the prefrontal cortex, which is important for the rewarding value of taste [795] and decision making/reward-guided learning [796], are still developing throughout adolescence [83, 100, 797]. As a result of this continued development, adolescents are known to demonstrate less impulse control than adults [100, 798] and exhibit increased susceptibility to drugs of abuse [83, 790, 799].

A relatively small number of human studies have reported correlations between energy drink consumption and negative alcohol outcomes [800-805]. However, interpretation and applicability of these studies are heavily limited to user self-reports or acute in-laboratory behavioral tests, which limit the evaluation of objective long-term consequences of energy drink exposure in adolescence on adult alcohol consumption. To better understand the consequences and potential risks of adolescent consumption of caffeinated energy drinks, rodent animal models

provide a convenient way to conduct developmental studies in a much shorter time period as adolescence in rodents takes weeks versus years in humans [806].

In order to study the impact of prolonged adolescent exposure to energy drinks on adult alcohol intake in a well-controlled manner, we developed three different rodent models for energy drink consumption or exposure during adolescence. We used C57BL/6 mice that readily consume large quantities of alcohol or sucrose solutions and BALB/c mice, who consume lower levels of alcohol [140, 807]. Adolescent male mice were exposed to 'energy drinks' (actual energy drinks or caffeinated sucrose solutions mimicking energy drink concentrations) either voluntarily using a continuous-access or limited-access two-bottle choice paradigm or involuntary by oral gavage of the caffeinated sucrose solutions. Directly following adolescent 'energy drink' exposure, alcohol intake and preference was measured in the young adult mice. No correlations between adolescent caffeinated energy drink exposure and adult alcohol consumption were observed in any of our exposure paradigms, concluding that adolescent exposure to caffeinated energy drinks in male C57BL/6 or BALB/c mice does not affect adult alcohol consumption. Reconciling our negative results to those observed in human correlations between energy drink use and alcohol intake requires further exploration.

## 6.2 Materials and methods

### 6.2.1 Animals

Male C57BL/6 and BALB/c wild-type inbred mice were purchased from Harlan (Indianapolis, IN, USA). For Experiments 1 and 2, animals were single-housed in double grommet, ventilated Plexiglas® cages throughout testing. In Experiments 3 and 4, animals were group-housed throughout adolescence and moved to single housing in double grommet, ventilated Plexiglas® cages for adult alcohol intake testing. All mice were housed in a 12-h reverse dark-light cycle to allow energy drink exposure and alcohol intake studies to be conducted during each animal's active light cycle. The temperature of the housing room was maintained at 21 °C; food and water were provided *ad libitum* throughout all experiments.

Mice were 4 weeks old, 30-days postnatal (P30) when shipped and allowed to acclimate for 7–10 days prior to the experiment initiation. Experiments started when mice were approximately 40 days old and could be described as being in mid-adolescence [83, 808]. On

Fridays, cages were changed and the animals were weighed. All procedures were approved by the Institutional Animal Care and Committee and performed in an Association for Assessment and Accreditation of Laboratory Animal Care (AALAC) -certified facility in accordance with the National Institutes for Health Guide for Care and Use of Laboratory Animals.

### 6.2.2 Drugs and solutions

The alcohol solutions were prepared by diluting 200 proof ethanol (Goldshield, Hayward, CA, USA) in reverse-osmosis filtered water to produce 1%, 3%, 6%, 12% and 20% (vol/vol) ethanol solutions. Red Bull® (Santa Monica, CA, USA), Monster® (Corona, CA, USA), and NOS® (Coca-Cola Company, Atlanta, GA, USA) were decarbonated and filter-sterilized prior to consumption. Sucrose (Fisher Scientific, Pittsburgh, PA, USA) + caffeine (Sigma-Aldrich, St. Louis, MO, USA) and sucrose + quinine (Sigma-Aldrich) solutions were prepared in reverse-osmosis filtered water and filter-sterilized.

### 6.2.3 Experiment 1: Voluntary continuous access to energy drinks in adolescence in C57BL/6 male mice.

To model the effects of adolescent energy drink consumption on future alcohol consumption in mice, we provided groups of adolescent C57BL/6 mice (n = 6 per group) with continuous access (24 h/day) to a two-bottle choice consisting of water and one of three different energy drinks (Red Bull®, Monster®, or NOS®) for 10 consecutive days. The water group was exposed to two bottles of water to control for the two-bottle access option. Of the three energy drinks, NOS® has the highest caffeine concentration (550 mg/L versus 326 mg/L for Red Bull® and Monster®). To control for additional ingredients present in energy drinks such as vitamins, taurine, ginseng, and guarana, an additional two groups of mice (n = 6 per group) had continuous access to either water and a sucrose + caffeine solution containing an amount of caffeine that was equivalent to the amount present in Red Bull® and Monster® solutions (326 mg/L caffeine, n = 6 per group). The Red Bull®, Monster®, and NOS® energy drink solutions contained 115–120 g/L of sugar, predominantly made up of sucrose (although Western Red Bull® also contains glucose). Therefore, we used 120 g/L sucrose for our control solution with the exception of the water control solution. The weights of the bottles were measured to the nearest 0.1 g and replaced afterward.

At the end of the 10-day period, the energy drink solutions were exchanged for alcohol solutions. Mice were presented with water and solutions of increasing alcohol concentration (3%,

6%, 12%, and 20%) in a two-bottle choice, limited-access paradigm for a 4-h period (11:00 AM to 3:00 PM) for 4 days each for 16 consecutive days (see Figure 6-1 for timeline). The weight of each bottle was measured to the nearest 0.1 g at the end of the alcohol access and bottle location was reversed every day to prevent habit formation. Throughout, combined alcohol intake per alcohol concentration is denoted as 'total intake', whereas combined intake of alcohol during the entire 4-week period is denoted as 'cumulative intake'.

### 6.2.4 Experiment 2: Voluntary limited access to energy drinks in adolescence in C57Bl/6 male mice

A two-bottle limited-access (4 h/day, 5 days/week) paradigm was performed during which adolescent C57BL/6 male mice (n = 6 per group) received water or a choice between water and one of five test solutions for 20 days (5 days a week for 4 weeks, water solutions only on weekends). The water control group received two bottles of water for control during the two-bottle choice paradigm. Based on the results obtained in Experiment 1, we decided to test only NOS® and Red Bull®, since we found no clear difference between Red Bull® and Monster®. Caffeinated sucrose solutions with sucrose concentrations (120 g/L) and caffeine concentrations that matched that of Red Bull® (326 mg/L) and NOS® (550 mg/L) were also tested. An additional control of sucrose solutions laced with quinine was used to mimic the bitter taste caused by the presence of caffeine. For Red Bull®, we used 1 mM quinine and for NOS® we used 1.75 mM quinine. The quinine concentrations were based on the concentration at which mice drank the same amount of sucrose as they drank of the caffeinated sucrose solution used as the Red Bull® control or NOS® control (data not shown). The bottles were distributed to the cages at 11:00 AM and removed at 3:00 PM (5 days/week) during the active cycle (dark cycle) of each mouse. The weights of the bottles were measured to the nearest 0.1 g. Bottle locations (between water and energy drink solution) were altered between days to prevent habit formation.

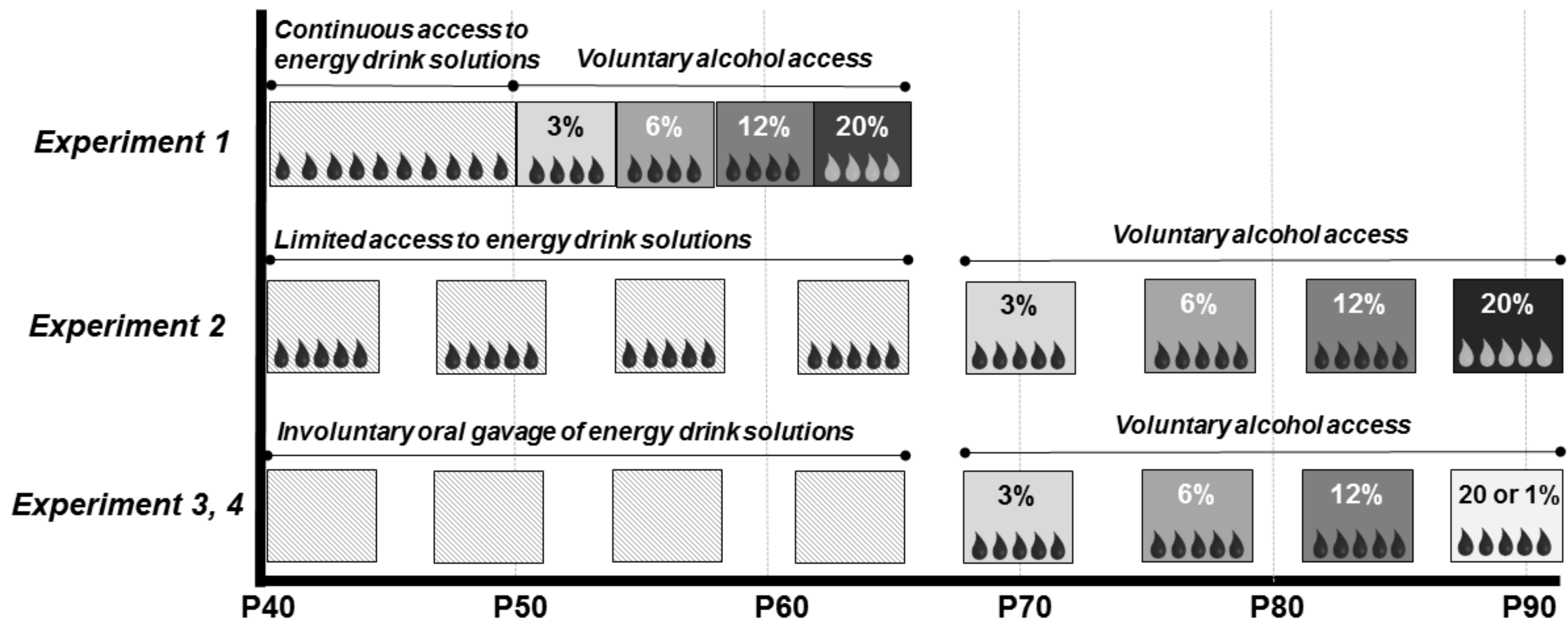


Figure 6-1. Timelines for energy drink exposure and alcohol access in Experiment 1, 2, 3, and 4.

Male C57BL/6 mice (Experiments 1, 2, 3) or male BALB/c mice (Experiment 4) were exposed to energy drinks or energy drink-like solutions in adolescence to assess subsequent alterations in voluntary adult alcohol intake. Water drops indicate two-bottle, drinking-in-the-dark access to solutions; syringes represent oral gavage administration. In Experiment 3, male C57BL/6 mice were exposed to 20% alcohol solutions during the final week, whereas in Experiment 4, male BALB/c mice were exposed to 1% alcohol solutions during that week.

At the end of the 4-week period, the energy drink solutions were removed and adult alcohol intake was monitored. Mice were presented with water and solutions of increasing alcohol concentration (3%, 6%, 12%, and 20%) in a two-bottle choice paradigm for a 4-h period (11:00 AM to 3:00 PM) for 5 days each for 4 weeks (see Figure 6-1 for timeline). The weight of each bottle was recorded to the nearest 0.1 g at the end of the alcohol access. The location of the bottles was reversed daily to limit habit formation.

### 6.2.5 Experiment 3: Involuntary exposure to caffeinated energy drinks in adolescence via oral gavage in C57BL/6 male mice

Adolescent C57BL/6 male mice were exposed to test solutions with constant 120 g/L sucrose and increasing concentrations of caffeine (15, 50, 75, 100, and 150 mg/kg caffeine, n = 3–4 per group). An additional group of animals received the sucrose solution alone with no caffeine. Solutions were administered via oral gavage, once daily, 5 days a week for 4 weeks starting at postnatal day 40. Despite the high concentrations of caffeine administered, no ill behavioral effects were observed upon administration of caffeinated sucrose solutions. After 4 weeks of exposure to the sucrose + caffeine solutions, mice were exposed to alcohol as described in Experiment 2 in a limited-access, two-bottle choice paradigm (see Figure 6-1 for timeline).

### 6.2.6 Experiment 4: Involuntary exposure to caffeinated energy drinks in adolescence via oral gavage in BALB/c male mice

We repeated the experimental setup described in Experiment 3 in adolescent male BALB/c mice with the exception that during the fourth week of adult alcohol exposure a 1% alcohol solution was used instead of 20% alcohol solutions, because the BALB/c mice drank very little 6% and 12% alcohol solutions during the prior weeks, and at a low preference (see Figure 6-1 for timeline).

### 6.2.7 Statistical analysis

To compare cumulative energy drink intake versus total alcohol intake, a one-way ANOVA was performed and statistical differences were obtained by performing Tukey’s post hoc analysis. A two-way ANOVA was performed to determine if differences existed between adolescent energy drink or caffeine exposures and the adult intake of alcohol at different concentrations or cumulative alcohol intake. A Bonferroni post hoc analysis was performed when significant

differences were observed. Correlations between energy drink and alcohol consumptions were fitted by linear regression and Pearson's r correlation coefficient (r) values were determined using GraphPad Prism5 (GraphPad Software, San Diego, CA).

## 6.3 Results

### 6.3.1 Experiment 1: Voluntary continuous accesses to energy drink solutions in male C57BL/6 mice.

Adolescent mice did not show an escalation of energy drink intake over a period of 10 days (Figure 6-2A). One-way ANOVA analysis revealed a significant difference in cumulative sucrose intake (Figure 6-2B) between the different types of energy drinks ($F[3,20] = 9.509$, $p < 0.001$). In particular, *post hoc* analysis revealed that mice drank significantly more of the sucrose + caffeine solution than the authentic energy drink solutions as measured by cumulative sucrose consumption. Mice consumed the least amount of cumulative sucrose when given NOS®, the energy drink solution with the highest caffeine concentration (Figure 6-2B). Because NOS® contains more caffeine than Red Bull® and Monster®, we used a one-way ANOVA to identify that caffeine intake by adolescent mice was significantly different between the four types of energy drinks ($F[3,20] = 3.6$, $p = 0.032$). This significant difference was primarily driven by a small but significant difference in caffeine intake between adolescent mice exposed to Red Bull® or the caffeinated sucrose solution (Figure 6-2C). After 10 days of continuous access to energy drink solutions, mice received limited access (4 h/day) to a two-bottle choice of water and an alcohol solution of increasing concentrations (4 days per concentration). Two-way ANOVA analysis revealed no significant difference in alcohol consumption between groups of mice exposed to different types of energy drinks or water ($F[4,25] = 0.75$, $p = 0.5647$). There was also no significant interaction between adolescent treatment and the consumption of alcohol at different ethanol percentages ($F[12,75] = 1.02$, $p = 0.442$), further highlighting the lack of significance between treatment groups (Figure 6-2D). No significant difference ($F[4,25] = 0.75$, $p = 0.56$) was observed in the cumulative alcohol intake over the entire period of alcohol access between treatment groups (Figure 6-2E). In none of the mice exposed to caffeinated energy drinks did adolescent intake of caffeine correlate with future alcohol intake (Figure 6-2F, Table 6-1).

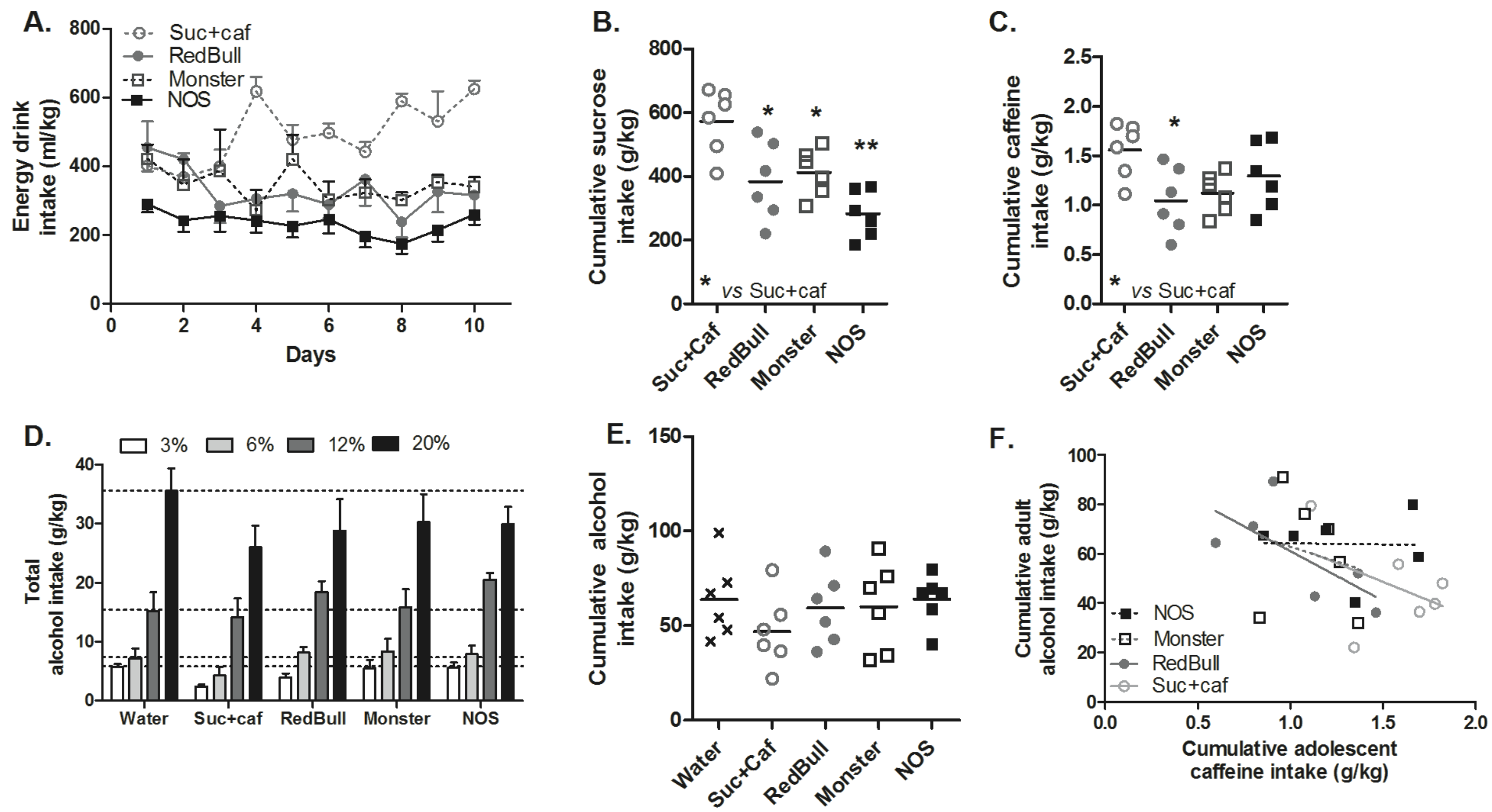


Figure 6-2. Continuous access to caffeinated energy drink solutions does not alter future alcohol intake in male C57BL/6 mice.

(A) Daily intake of sucrose + caffeine (326 mg/L), Red Bull®, Monster®, or NOS® solutions available 24 h/day to adolescent male C57BL/7 mice (n = 6 per group) for 10 consecutive days. (B–C) Cumulative intake of sucrose and caffeine from energy drink solutions during adolescence. Total intake of alcohol solutions with increasing concentrations (D) and cumulative intake of all alcohol solutions (E) by C57BL/6 male mice previously exposed to water, sucrose + caffeine, Red Bull®, Monster®, or NOS® solutions. (F) Correlation between cumulative energy drink intake and cumulative alcohol intake. Statistical significance was assessed by one-way or two-way ANOVA (time and treatment or treatment and alcohol percentage) followed by Tukey's Multiple Comparison Test, *$p < 0.05$; **$p < 0.01$. Correlation coefficients are depicted in Table 6-1. Dotted lines in panel D indicate intake of 3%, 6%, 12%, and 20% alcohol solutions by water-exposed mice. Data are represented as mean ± SEM.

### 6.3.2 Experiment 2: Voluntary limited access to energy drink solutions in male C57BL/6 mice

Adolescent mice generally drank equal amounts of energy drinks, with equal caffeine concentrations, on the first day of energy drink access, but stratification was observed over the course of the 20-day exposure period (Figure 6-3A). Adolescent mice significantly differed ($F[4,25] = 26.55$, $p < 0.0001$) in cumulative sucrose consumption (Figure 6-3B). While all solutions contained the same amount of sucrose, the energy drinks differed in their caffeine content. One-way ANOVA analysis revealed no significant difference ($F[3,20] = 1.182$, $p = 0.34$) in cumulative caffeine intake between treatment groups (Figure 6-3C). Similar to the results obtained in Experiment 1, mice exposed to sucrose or caffeinated energy drink solutions did not consume significantly more or less alcohol ($F[5,30] = 0.55$; $p = 0.7402$) at any of the different ethanol concentrations than mice exposed to water during adolescence (Figure 6-3D). No interaction was observed between the consumption of different percentage alcoholic solutions and adolescent treatment ($F[15,90] = 0.50$, $p = 0.9331$). Similarly, there was no significant difference ($F[5,30] = 0.55$, $p = 0.74$) in cumulative alcohol consumption between treatment groups (Figure 6-3E). In none of the mice exposed to caffeinated energy drinks did adolescent intake of these solutions correlate with their future alcohol intake (Figure 6-3F, Table 6-1).

### 6.3.3 Experiment 3 and 4: Involuntary energy drink exposure in male C57BL/6 and BALB/c mice

To better correlate adolescent caffeine exposure with adult alcohol consumption, mice were administered differing concentrations of caffeine via oral gavage while sucrose concentrations (120 g/L) were held constant. In C57BL/6 male mice, exposure to different concentrations of caffeine in adolescence did not significantly affect adult alcohol consumption ($F[5,15] = 1.82$, $p = 0.1777$), nor did we observe a significant interaction between the consumption of different percentages of alcohol solutions and treatment groups ($F[15,39] = 1.74$, $p = 0.82$) (Figure 6-4A). In these animals, cumulative adult alcohol consumption was not correlated with adolescent caffeine exposure ($r = -0.04$, $p = 0.88$, Figure 6-4B, Table 6-1) or any alcohol solution (Table 6-2). Similar to the higher alcohol-preferring C57BL/6 mice, in the moderate alcohol-preferring BALB/c male mice, exposure to different concentrations of caffeine in adolescence did not affect adult alcohol consumption at each tested alcohol concentration (Figure 6-4C, $F[15,39] = 0.73$, $p = 0.74$) nor cumulative alcohol consumption during adulthood ($r = 0.36$, $p = 0.13$, Figure 6-4E).

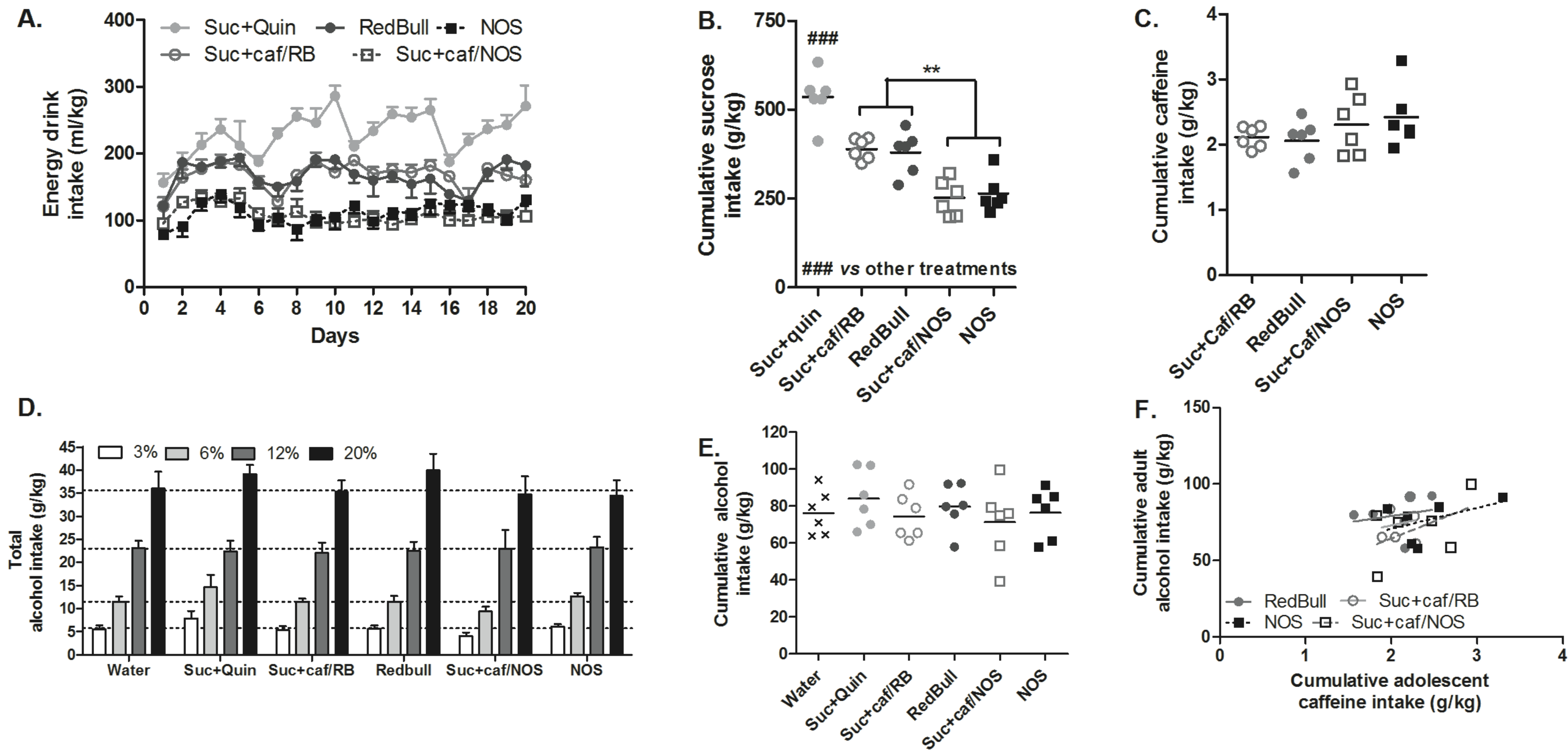


Figure 6-3. Limited access to caffeinated energy drink solutions does not alter future alcohol intake in male C57BL/6 mice.

(A) Daily intake of sucrose + quinine, sucrose + caffeine/Red Bull® (RB) (326 mg/L), Red Bull®, sucrose + caffeine/NOS® (550 mg/L), or NOS® solutions available 4 h/day by adolescent male C57BL/6 mice (n = 6 per group) for 20 days (5 days/week) (A). (B–C) Cumulative intake sucrose and caffeine from energy drink solutions during adolescence. Total intake of alcohol solutions with increasing concentrations (D) and cumulative intake of all alcohol solutions (E) by C57BL/6 male mice previously exposed to water, sucrose + quinine, sucrose + caffeine/Red Bull®, Red Bull®, sucrose + caffeine/NOS®, or NOS® solutions. (F) Correlation between cumulative energy drink intake and cumulative alcohol intake. Statistical significance was assessed by one-way or two-way ANOVA (time and treatment or treatment and alcohol percentage) followed by Tukey's Multiple Comparison Test, $**p < 0.01$, $***p < 0.0005$. Correlation coefficients are depicted in Table 6-1. Dotted lines in panel D indicate intake of 3%, 6%, 12%, and 20% alcohol solutions by water-exposed male mice. Data are represented as mean ± SEM.

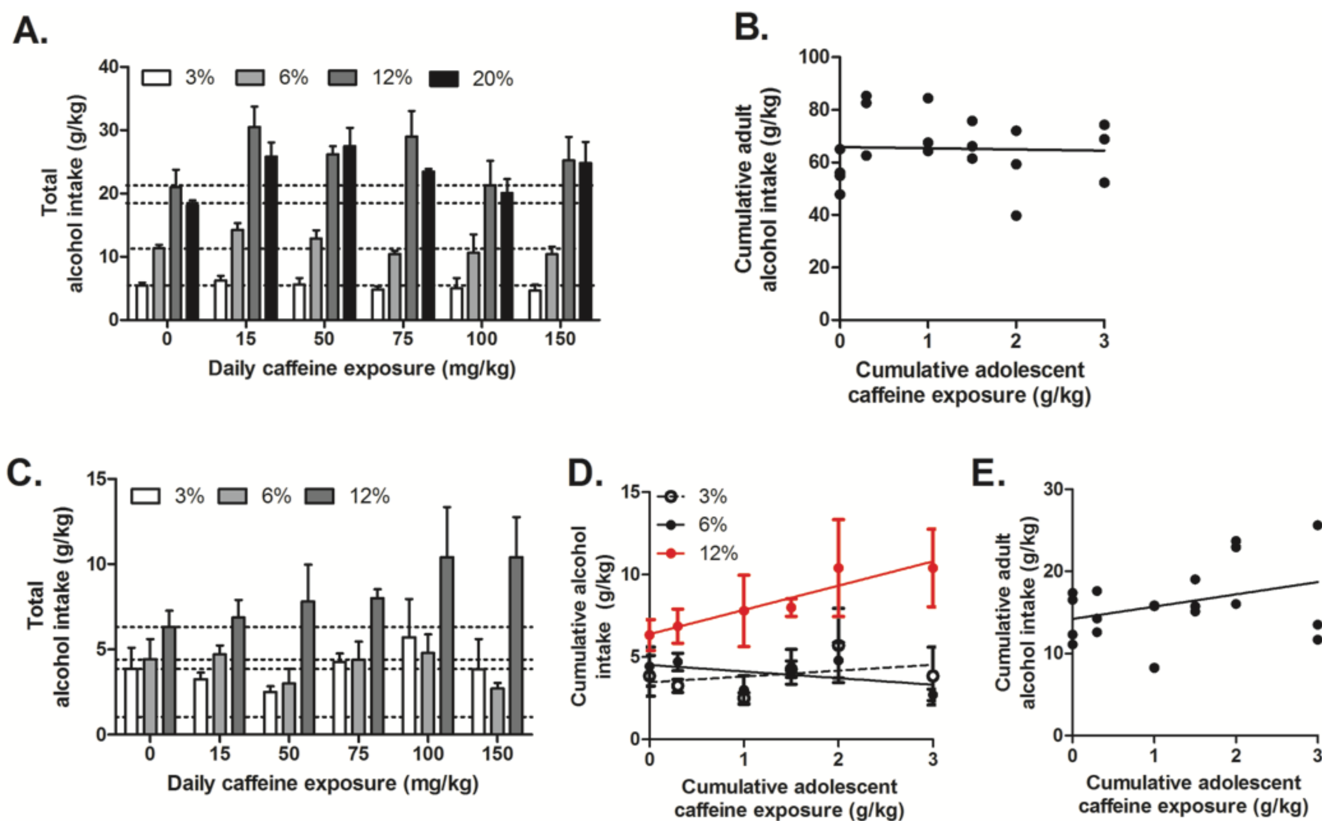


Figure 6-4. Involuntary exposure to caffeinated energy drink solutions does not alter future alcohol intake in male C57BL/6 nor BALB/c mice.

(A) Total intake of alcohol solutions with increasing concentrations by C57BL/6 mice and (C) BALB/c mice previously exposed throughout adolescence by oral gavage to sucrose solutions with increasing concentrations of caffeine (n = 3 per caffeine concentration per group, n = 4 for sucrose-only group). (B) Correlation between cumulative alcohol consumption and cumulative adolescent caffeine exposure in C57BL/6 and (E) BALB/c mice. (D) Correlation between cumulative alcohol intake and cumulative adolescent caffeine intake in BALB/c mice for 3%, 6%, and 12% cumulative alcohol intake. Statistical significance was assessed by one-way or two-way ANOVA (treatment and alcohol percentage) followed by Tukey's Multiple Comparison Test. Correlation coefficients are depicted in Table 6-1 and 6-2. Dotted line in panels A and C indicate intake of 3%, 6%, 12%, and 20% by mice exposed to sucrose solutions without caffeine. Data are represented as mean ± SEM.

No correlations were observed at 3% or 6% solutions, but a correlation was observed at 12% alcohol for BALB/c mice (Figure 6-4D, Table 6-2).

### 6.3.4 Over a broad range of caffeine concentrations, no correlation was observed between adolescent caffeine exposure and adult alcohol consumption in male C57BL/6 mice.

It is plausible that no obvious correlation is observed within the narrow range of caffeine intake in Experiment 1 (0.6–1.8 g/kg, Figure 6-5A – red data points] and Experiment 2 (1.6–3.3 g/kg, Figure 6-5A – blue data points), but that a correlation becomes apparent when analyzing the data of these experiments together, thereby broadening our range of caffeine intake and also strengthening our statistical power over the range obtained in experiment (0–3 g/kg, Figure 6-5A – green data points). Because mice in Experiment 1 were exposed to alcohol only for 4 days per alcohol concentration, compared to 5 days for mice in Experiments 2 and 3, we calculated average daily alcohol consumption to enable side-by-side comparison of the data. We still observed no correlation between adolescent caffeine exposure and voluntary adult alcohol consumption (Figure 6-5B, Table 6-1).

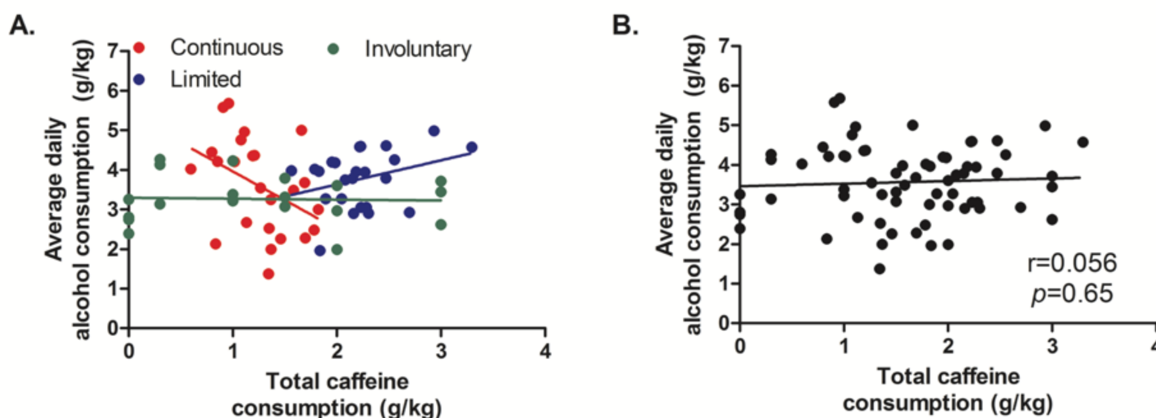


Figure 6-5. Over a broad range of caffeine concentrations, adolescent caffeinated energy drink exposure does not correlate with adult alcohol consumption in male C57BL/6 mice.

Correlations between total caffeine exposure in adolescence between average daily alcohol consumption in adulthood in each of the three paradigms (continuous access, limited access, involuntary access) (A) Combined results from all three drinking paradigms further reveal lack of correlation between caffeine consumption and adult alcohol consumption with each route of administration of adolescent caffeine exposure. (B) Lack of correlation is independent of route of administration of caffeine during adolescence. Correlation coefficients are shown in Table 6-1; data are represented as mean ± SEM.

Table 6-1. Correlation between adolescent caffeine consumption and adult alcohol intake overall in C57BL/6 mice.

| **Continuous, voluntary** | **Pearson coefficient, r** | ***p*** |
|---|---|---|
| Suc+caf | -0.43 | 0.39 |
| RedBull | -0.69 | 0.13 |
| Monster | -0.20 | 0.70 |
| NOS | -0.024 | 0.97 |
| ALL | -0.41 | 0.046 |
| **Limited, voluntary** | **Pearson coefficient, r** | ***p*** |
| Suc+Quin | 0.32 | 0.53 |
| RedBull | 0.22 | 0.68 |
| Suc+caf/RB | 0.17 | 0.75 |
| NOS | 0.47 | 0.35 |
| Suc+caf/NOS | 0.50 | 0.31 |
| ALL | 0.32 | 0.12 |
| **Involuntary** | **Pearson coefficient, r** | ***p*** |
| Suc+caf | -0.038 | 0.88 |
| **ALL GROUPED** | **0.057** | **0.65** |

Table 6-2. Correlation between adolescent caffeine exposure and individual alcohol solutions in BALB/c mice.

| **Involuntary** | **C57BL/6** | | **BALB/c** | |
|---|---|---|---|---|
| Alcohol | **Pearson coefficient, r** | ***p*** | **Pearson coefficient, r** | ***p*** |
| 3% | -0.80 | 0.055 | 0.40 | 0.44 |
| 6% | -0.64 | 0.17 | -0.50 | 0.32 |
| 12% | -0.093 | 0.86 | 0.94 | 0.005 |
| 20% | 0.16 | 0.76 | N.D. | N.D. |

## 6.4 Discussion

Based on previous studies in mice and self-reports from human subjects suggesting that adolescent caffeine exposure can induce adult sensitization to drugs of abuse in adulthood [790, 801], we assessed whether energy drink consumption during adolescence increased alcohol consumption. Adolescent male C57BL/6 mice were exposed to caffeinated energy drinks using voluntary continuous-access and limited-access drinking paradigms or involuntary oral gavage administration before monitoring adult alcohol intake. We additionally monitored adult alcohol intake in BALB/c male mice after involuntary exposure to caffeine via oral gavage in adolescence to assess cross-strain variability and specificity. In none of the three exposure paradigms or mouse strains did adolescent intake of caffeinated sucrose solutions correlate with a change in voluntary adult alcohol consumption in adulthood. We also did not observe a correlation between adolescent caffeinated sucrose intake and alcohol preference in any of the paradigms (data not shown). Our results suggest that exposure to caffeinated energy drinks in adolescence does not influence adult alcohol consumption in either C57BL/6 or BALB/c male mice.

Our rationale for using a continuous-access model was to mimic a human situation in which an adolescent can consume an energy drink at any time of the day. In contrast, the use of a limited-access paradigm was chosen to mimic a 'party setting' in which mice would only have access for a short period of the day (e.g., only at a bar or club). As it has been shown that rodents will consume larger volumes of alcohol in a limited-access model compared to a continuous paradigm, thus inducing a more binge-like consumption of the energy drinks [809], we hypothesized that perhaps such a binge-like pattern of energy drink intake would affect future alcohol intake more prominently than continuous 24-h access. Mice exposed to energy drink solutions in a limited-access paradigm drank the same amount of energy drink (Red Bull® = $3.2 \pm 0.5$ mL/g, NOS® = $2.2 \pm 0.4$ mL/g) in 80 h (4 weeks × 5 days × 4 h) as mice exposed to the same solution (Red Bull® = $3.2 \pm 1.0$ mL/g, NOS® = $2.4 \pm 0.6$ mL/g) in a continuous-access paradigm in 240 h (10 days × 24 h). These results suggest that mice indeed consumed energy drinks in a more binge-like manner when exposure occurs in a limited-access paradigm.

Mice consumed lower volumes of sucrose solutions with higher concentrations of caffeine, potentially due to the bitterness of the solution (Figure 6-2). To make the control sucrose solution equally bitter to the caffeinated sucrose solution, we added quinine to the sucrose solutions to control for caffeine's bitterness. While intake of the quinine sucrose solution initially was equal to

the caffeinated sucrose solutions, over the duration of the experiment mice consumed less of the caffeinated sucrose solutions compared to the equivalent quinine-sucrose solution, suggesting that caffeine may have an inhibitory effect that extends beyond the bitter taste on the consumption of the sucrose solution [810]. Nevertheless, while the total intake of energy drinks with the highest caffeine concentration was lower than those with intermediate caffeine concentrations, mice consumed roughly the same total amount of caffeine (Red Bull® 1.0 ± 0.2 mg/kg versus NOS® 1.2 ± 0.2 mg/kg, $p = 0.16$, Figure 6-3). It was therefore less surprising that we did not observe a difference in alcohol consumption between the mice exposed to sucrose solutions with medium or high caffeine concentrations. Still, humans may be more adaptive to ingesting bitter solutions in their diet than mice, which is an area where our mouse model may fail to properly relate with human consumption preferences [811]. In our mouse models, we did not find any difference in consumption of Red Bull® or NOS® and their respective control solutions containing only the equivalent doses of sucrose and caffeine, suggesting that additives such as taurine, ginseng, and vitamins did not influence the intake of these solutions, which is in agreement with a lack of effects in humans [57, 812-814].

To overcome the limitation in our studies that mice voluntarily consuming energy drinks did not display a wide range of caffeine intake, we exposed mice to sucrose solutions with increasing caffeine concentrations via oral gavage. We chose the 15 mg/kg dose of caffeine because it has frequently been used to study hyperlocomotion in adolescent and adult rodents [815], with a maximum dose of 150 mg/kg to give us a 10-fold window and to span the concentrations reached during voluntary consumption in Experiments 1 and 2. C57BL/6 mice readily consume alcohol and can drink as much as 8 g/kg in a 4-h drinking-in-the-dark session [90, 268, 277]. With the exception of the 20% alcohol solution, our mice were not drinking at their maximum limit. However, to provide additional validity to our negative results and to determine if these results were comparable across mouse strains, we subjected BALB/c mice, a low-to-moderate alcohol-preferring strain [140, 816], to the same experimental setup to establish that the lack of increase in adult alcohol consumption in C57BL/6 animals was not an artifact of this particular strain's strong alcohol preference. Both C57BL/6 and BALB/c mice are known to consume equally large amounts of sucrose, suggesting drinking differences between strains are otherwise similar [817]. Regardless of the amount of caffeine given to the adolescent mice, we found no overall correlation between adolescent caffeine exposure and adult alcohol exposure (Figure 6-4).

Overall, adolescent caffeine exposure did not correlate with cumulative adult alcohol consumption in any of the behavioral paradigms in C57BL/6 male mice (Figure 6-5) or at any alcohol concentration tested (Table 6-2). However, upon further investigation we observed a significant correlation between caffeine exposure and 12% alcohol intake in the BALB/c animals (Table 6-2, Figure 6-4). The significance of this result, however, should not be overestimated as the BALB/c mice on average drank only 0.2 mL of 12% alcohol per 4-h session (daily spillage was ~0.1 mL per session), and became less interested in consuming alcohol with each increasing concentration (Figure 6-6A, B). This was further apparent from the lack of interest of BALB/c mice to consume a 1% alcohol solution following the 12% solution (Figure 6-6C).

Environmental cues, social settings, and learning associated with drug use may further promote consumption of alcohol caused by consumption of caffeinated energy drinks [818], yet our results suggest that exposure to energy drinks alone cannot account for the potential changes in adult alcohol consumption in mice. One limitation of our studies is the use of non-facility bred animals. Animals were shipped to our animal facility during a vulnerable developmental phase (postnatal day 30, 4-weeks-old), and this process may have induced significant stress on the mice, resulting in altered adult alcohol consumption. Increased stress in early developmental stages has been associated with alterations in adult alcohol consumption [819-821]. However, because all of

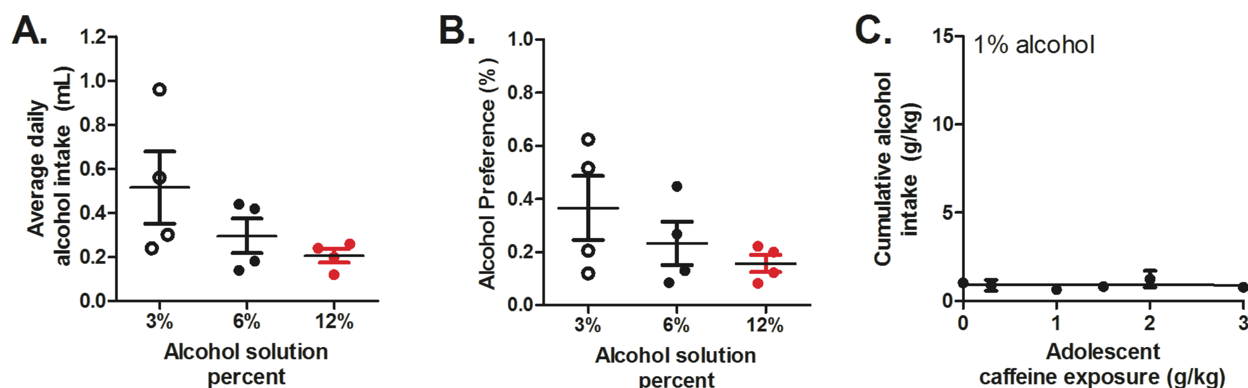


Figure 6-6. BALB/c mice loss of training observed during alcohol intake testing.

(A) Average daily intake by volume and (B) preference in male BALB/c mice (n = 4) exposed to 0 mg/kg caffeine + 120 g/L sucrose via oral gavage during adolescence. (C) Following exposure to 12% alcohol, BALB/c mice were highly unmotivated to consume even a more palatable 1% alcohol solution. Data are represented as mean ± SEM.

our animals were shipped at the same age in each experiment, changes resulting from this stress inducement are similar across all experiments. Another potential confounding factor in our animal studies is that mice metabolize caffeine 2–4 times faster than humans do [822, 823]. These metabolic differences between humans and rodents may result in differences in the ability of prolonged caffeine consumption to influence drug reward or consumption that has been reported in humans.

While our studies found no correlation between adolescent caffeine consumption and voluntary adult alcohol intake, the effects of caffeine on future alcohol preference and intake may be further modified when caffeine is co-consumed with alcohol in adolescence. Co-consumption of caffeine with alcohol has been associated with self-reports of increased alcohol intake [785], driving under the influence of alcohol [824], unwanted sexual encounters [782], and increased aggression [825], signifying that co-consumption poses a significant risk to public health. Additionally, consumption of caffeine with alcohol has been reported to give rise to a "wide-awake-drunk" behavior characterized by an increasingly stimulated state despite the depressive drug effects observed by alcohol consumption alone. This behavior has been validated using animal models, as shown by Fritz and colleagues, where co-ingestion of caffeine with alcohol increased animal locomotor stimulation and decreased alcohol-associated ataxia [826], suggesting that mouse models of energy drink and alcohol intake are translatable to humans. While Fritz and colleagues did not observe an increase in voluntary, binge alcohol consumption or resulting blood ethanol concentrations (BEC) between animals offered caffeine with alcohol and alcohol alone (in agreement with our study), the unique behavioral responses observed in animal studies and human reports suggest that co-consumption of caffeine with alcohol may result in unique behavioral effects not observed with caffeine or alcohol consumption alone.

In summarizing the results obtained in our four experiments, we are confident in stating that chronic exposure to caffeinated sucrose solutions such as energy drinks does not produce long-lasting effects in male C57BL/6 or BALB/c mice that promote future alcohol consumption. It remains to be investigated whether this also holds true for other drugs of abuse, in particular, stimulant drugs such as cocaine. The establishment of a direct association between energy drinks and alcohol abuse remains controversial [827]. In human studies, Arria and colleagues found correlations between energy drink consumption and alcohol consumption in college students, reporting that students who were heavy energy drink users displayed increased alcohol

consumption [800]. Additionally, O'Brien and colleagues found that the consumption of energy drinks containing alcohol was linked to an increased number of drinking days per week and an increased number of drinks consumed on those days [785]. Future studies are necessary to better understand the potential effects of caffeine and alcohol on the adolescent brain and long-term behavioral plasticity, especially in context of other drugs of abuse, such as stimulants.

# CHAPTER 7. CAFFEINE-MIXED ALCOHOL: MIXING HIGHS AND LOWS

As published as:

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The number of highly caffeinated products has increased dramatically in the past few years. Among these products, highly caffeinated energy drinks are the most heavily advertised and purchased, which has resulted in increased incidences of co-consumption of energy drinks with alcohol. Despite the growing number of adolescents and young adults reporting caffeine-mixed alcohol use, knowledge of the potential consequences associated with co-consumption has been limited to survey-based results and in-laboratory human behavioral testing. Here, we investigate the effect of repeated adolescent (post-natal days P35-61) exposure to caffeine-mixed alcohol in C57BL/6 mice on common drug-related behaviors such as locomotor sensitivity, drug reward and cross-sensitivity, and natural reward. To determine changes in neurological activity resulting from adolescent exposure, we monitored changes in expression of the transcription factor ΔFosB in the dopaminergic reward pathway as a sign of long-term increases in neuronal activity. Repeated adolescent exposure to caffeine-mixed alcohol exposure induced significant locomotor sensitization, desensitized cocaine conditioned place preference, decreased cocaine locomotor cross-sensitivity, and increased natural reward consumption. We also observed increased accumulation of ΔFosB in the nucleus accumbens following repeated adolescent caffeine-mixed alcohol exposure compared to alcohol or caffeine alone. Using our exposure model, we found that repeated exposure to caffeine-mixed alcohol during adolescence causes unique behavioral and neurochemical effects not observed in mice exposed to caffeine or alcohol alone. Based on similar findings for different substances of abuse, it is possible that repeated exposure to caffeine-mixed alcohol during adolescence could potentially alter or escalate future substance abuse as means to compensate for these behavioral and neurochemical alterations.

## 7.1 Introduction

Over the last decade, numerous products containing high levels of caffeine have emerged [783, 801]. These products include energy drinks, powdered caffeine, caffeine pills, buccal caffeine pouches, caffeinated peanut butter, and caffeine vaporizer sticks. These highly caffeinated products are disproportionally targeted to adolescents and young adults [828]. Of these products, the most widely used are highly caffeinated energy drinks, which come in a variety of different volumes (from 1.7 oz energy shots to 20 oz. cans) and caffeine concentrations (9-170 mg/oz.) [783, 785, 812]. Sales of energy drinks grew 60% from 2008 to 2013, illustrating the increased popularity and consumption of these beverages. Yet, increased accessibility of highly caffeinated products has coincided with increased reports of emergency departments visits because of energy drink consumption [829], highlighting the potential harms of exposure to highly caffeinated solutions to adolescents.

While the consumption of large quantities of caffeine itself is problematic [783, 830], added health risks arise when caffeine is consumed with alcohol. It has been reported that 23% to 47% of adolescents and young adult alcohol users consume alcohol-mixed energy drinks [803, 831]. Surveys of college-aged students suggest this population consumes large amounts of caffeine-mixed alcohol to fulfill hedonistic motives, such as increased pleasure from intoxication and increasing the intensity and/or nature of intoxication [832, 833]. However, serious – and sometimes fatal – consequences can occur when mixing caffeine with alcohol [782, 834, 835]. While it is clear that consumption of caffeine-mixed alcohol solutions by adolescents and young adults carries a significant acute health risk, the long-term consequences of repeated exposures to caffeine-mixed alcohol are not yet well understood.

The lack of information on the potential long-term risks is particularly concerning given that adolescents, who are the predominant consumers of caffeine-mixed alcohol, are known to be more susceptible to changes in behavioral and neuronal adaptations from exposure to psychostimulants and drugs of abuse than adults [790, 836, 837]. Increased responses to cocaine-induced locomotor stimulation and reward have been observed in adolescent mice exposed to caffeine but not in animals exposed to caffeine in adulthood [790], suggesting chronic exposure outcomes in adolescence are not synonymous with exposures outcomes in adulthood. Legal and ethical issues surrounding alcohol use in minors heavily limits caffeine-mixed alcohol studies in human to self-reported survey-based results or in-laboratory performance tasks [838, 839]; yet,

animal studies provide a viable option for studying the effects of caffeine-mixed alcohol on adolescent behavior in a controlled setting [826]. Importantly, results observed in previous animal studies correlate with reported effects in adolescents and young adults [790, 826, 840, 841]. Here we developed an animal model using adolescent mice to mimic exposure to caffeine-mixed alcohol as reported by college-aged adults [829, 832, 833].

Both caffeine and alcohol are known to increase dopamine release in dopaminergic reward pathways, specifically through their actions involving adenosine and dopamine receptors in the dorsal striatum and nucleus accumbens [842, 843]. We hypothesized that repeated consumption of caffeine-mixed alcohol causes stronger activation of the dopaminergic reward pathway than caffeine or alcohol alone and could be on par with the levels of dopamine released by commonly abused psychostimulants, such as cocaine, leading to unique behavioral and pharmacological adaptations. To evaluate how chronic adolescent exposure to caffeine-mixed alcohol alters drug-related behaviors, we exposed C57BL/6 mice to caffeine-mixed alcohol throughout adolescence and monitored changes in locomotor sensitivity, ΔFosB accumulation, cocaine preference, cocaine sensitivity, and natural reward to saccharin. We observed unique behavioral and neurochemical effects of repeated caffeine-mixed alcohol exposure in adolescent mice that may indicate that these animals will experience future events involving caffeine-mixed alcohol, natural rewards, or cocaine and/or other psychostimulants differently than animals not exposed to caffeine-mixed alcohol in adolescence.

## 7.2 Materials and methods

### 7.2.1 Animals

A dolescent (approximately postnatal day 28 [P28]) male and female C57BL/6 mice were obtained from Harlan Inc. (Indianapolis IN, USA) and allowed to acclimate for one week to handling and drug administration before behavioral testing began at postnatal day 35 [83, 808]. Unless specified otherwise, mice were grouped housed in single grommet ventilated Plexiglas® cages at ambient temperature (21°C) in a room maintained on a reversed 12L:12D cycle (lights off at 10.00, lights on at 22.00) in animal facilities, accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. Food and water were provided ad libitum and mice were not deprived of food or water at any time. All animal procedures were pre-approved by Institutional Animal

Care and Use Committees of Purdue University and the University of California San Francisco and conducted in accordance with National Institutes of Health Guide for the Care and Use of Laboratory Animals.

### 7.2.2 Drugs and solutions

Caffeine, ethyl alcohol (200 proof), cocaine hydrochloride, and saccharin were obtained from Sigma Aldrich (St. Louis MO, USA). Caffeine (15 mg/kg), alcohol (1.5 g/kg), and caffeine (15 mg/kg) mixed alcohol (1.5 g/kg) solutions were administered via intraperitoneal injection (i.p., diluted in 0.9% saline) or oral gavage (o.g., dissolved in reverse osmosis water). Cocaine (1.5-30 mg/kg, diluted in 0.9% saline) was administered intraperitoneally (i.p.). For transcardial perfusion, a ketamine (Henry Schein Animal Health, Dublin OH, USA) and xylazine (Sigma Aldrich) cocktail of 100:10 mg/kg solution was administered (10 mg/mL i.p.) to induce anesthesia. Phosphate-buffered saline (PBS), 16% paraformaldehyde ampules (Electron Microscopy Sciences, Hatfield PA, USA), and heparin (10 units/mL) (Sigma) were utilized during perfusion. Saccharin solutions were prepared in reverse osmosis water to concentrations of 0.25 mM, 0.5 mM, 1.0 mM, and 2.0 mM.

### 7.2.3 Locomotor sensitization via intraperitoneal exposure

Adolescent male and female C57BL/6 mice (n=9-11 per group) were administered saline (0.9%), caffeine (15 mg/kg), alcohol (1.5 g/kg), or caffeine-mixed alcohol (15 mg/kg caffeine, 1.5 g/kg alcohol) by intraperitoneal injection for either five days a week for two weeks (male only animals, Figure 7-1A) or four weeks (male and female animals, Figure 7-1B). Locomotor activity was measured for 60 minutes in locomotor activity boxes (L 27.3 cm x W 27.3 cm x H 20.3 cm, Med Associates, St Albans City VT, USA) immediately following drug administration on the days depicted in Fig 1A-B. Behavioral testing was conducted during the light cycle for each mouse. Mice were habituated to the behavioral testing room one-hour prior to acclimate to fan noise. To reduce the effect of novelty on locomotor activity, mice were habituated to the locomotor boxes the day before the first experiment.

### 7.2.4 Locomotor sensitization via oral gavage exposure

A dolescent male C57BL/6 mice (n=6 per group) were administered water, caffeine (15 mg/kg), alcohol (1.5 g/kg), or caffeine-mixed alcohol (15 mg/kg caffeine, 1.5 g/kg alcohol) by oral gavage

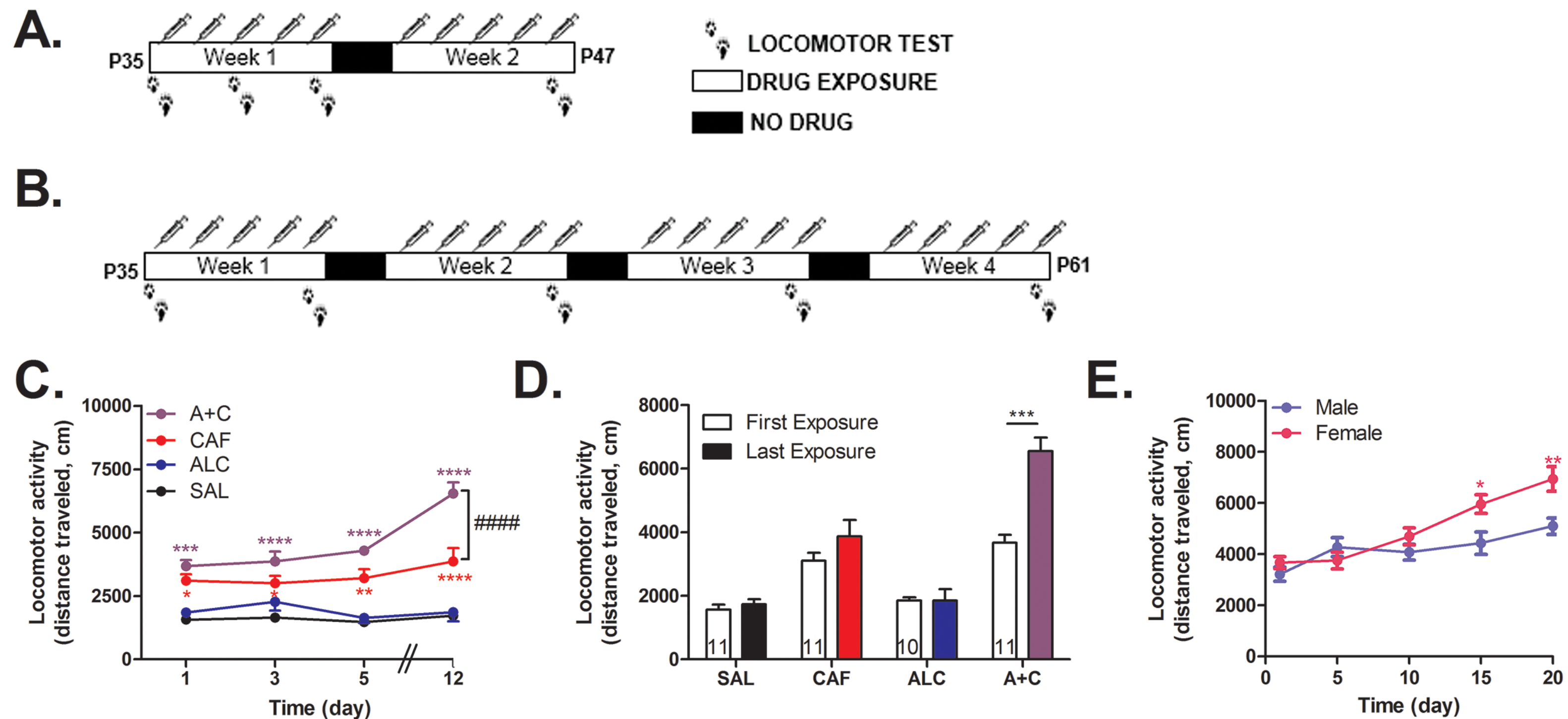


Figure 7-1. Repeated caffeine-mixed alcohol exposure by intraperitoneal injection during adolescence sensitizes locomotor response with sex specific differences.

Adolescent C57BL/6 mice were repeatedly exposed to saline (SAL), 1.5 g/kg alcohol (ALC), 15 mg/kg caffeine (CAF), or caffeine-mixed alcohol (A+C) daily via intraperitoneal injection (n = 9–11 per group) for two weeks (male only, A) of four weeks (male and female, B). Locomotor activity was measured for 60 minutes directly following injection. Total distance traveled per session increased in animals exposed to caffeine-mixed alcohol over the exposure time for adolescent male mice (C). Adolescent male mice exposed to caffeine-mixed alcohol exhibited acute hyperlocomotion and significant locomotor sensitization between first and last exposure session measure in locomotor boxes over two weeks (D). Adolescent female animals sensitized more quickly and robustly than male mice (E) for animals exposed to caffeine-mixed alcohol over four weeks. Statistical significance was assessed by two-way, repeated measures ANOVA (time and treatment) followed by Bonferroni's Multiple Comparison Test, *, $p<0.05$; **, $p<0.01$, ***, $p<0.0005$, ****, $p<0.0001$, ####, $p<0.0001$; data represented as mean ± SEM.

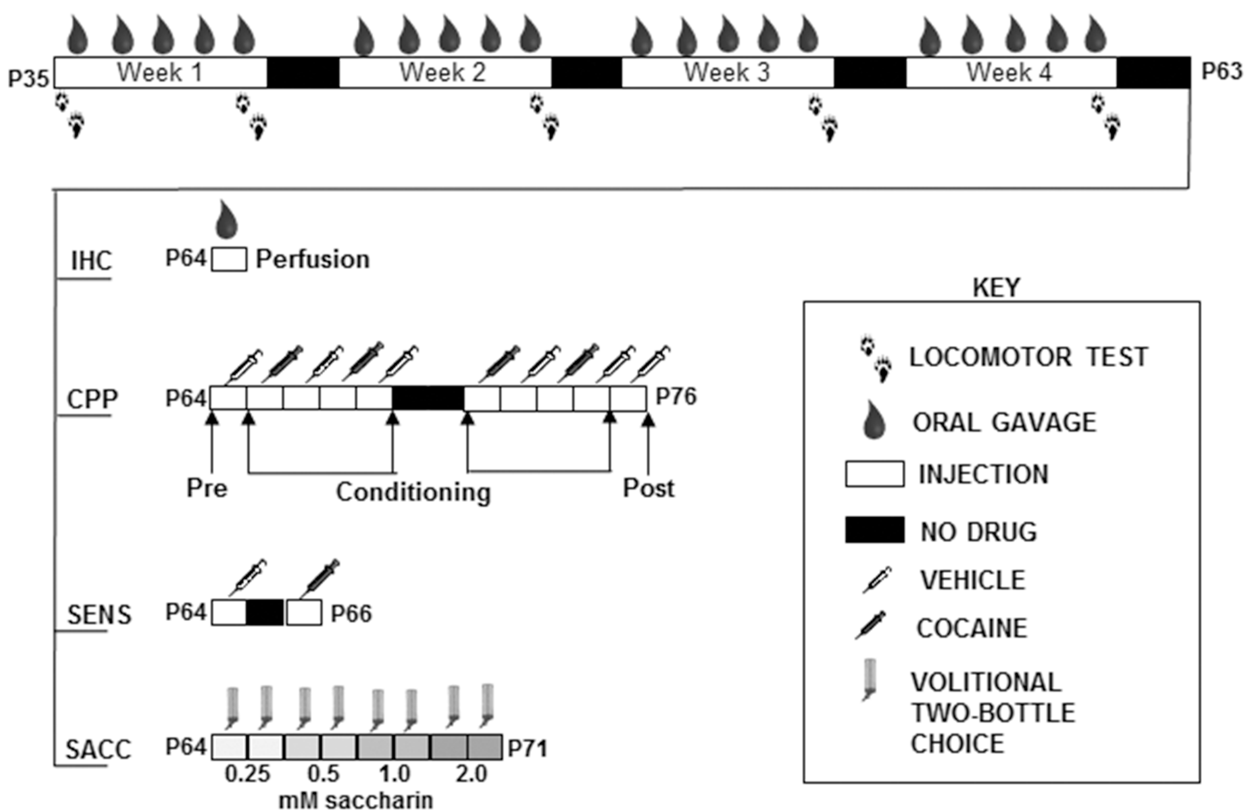


Figure 7-2. Timeline for adolescent drug exposure via oral gavage for experiments characterizing the effects of caffeine-mixed alcohol on drug related behaviors.

Male adolescent C57BL/6 mice were repeatedly exposed to exposed to water (H2O), 1.5 g/kg alcohol (ALC), 15 mg/kg caffeine (CAF) or caffeine-mixed alcohol (A+C), exposure by daily oral gavage (n = 6 per group) for 4 weeks for locomotor monitoring as depicted by the arrows. At the end of four weeks, animals were either perfused after one more drug administration ("IHC") or subjected to behavioral tasks. Animals under "CPP" were subjected to cocaine conditioned place preference for cross-sensitization to cocaine reward. Animals in "SENS" were monitored for cocaine locomotor cross-sensitization. Natural reward consumption of saccharin was measured in "SACC" through four-hour limited-access, two-bottle choice between concentrations of saccharin (0.25, 0.5, 1.0, and 2.0 mM saccharin) and water for two days at each saccharin concentration.

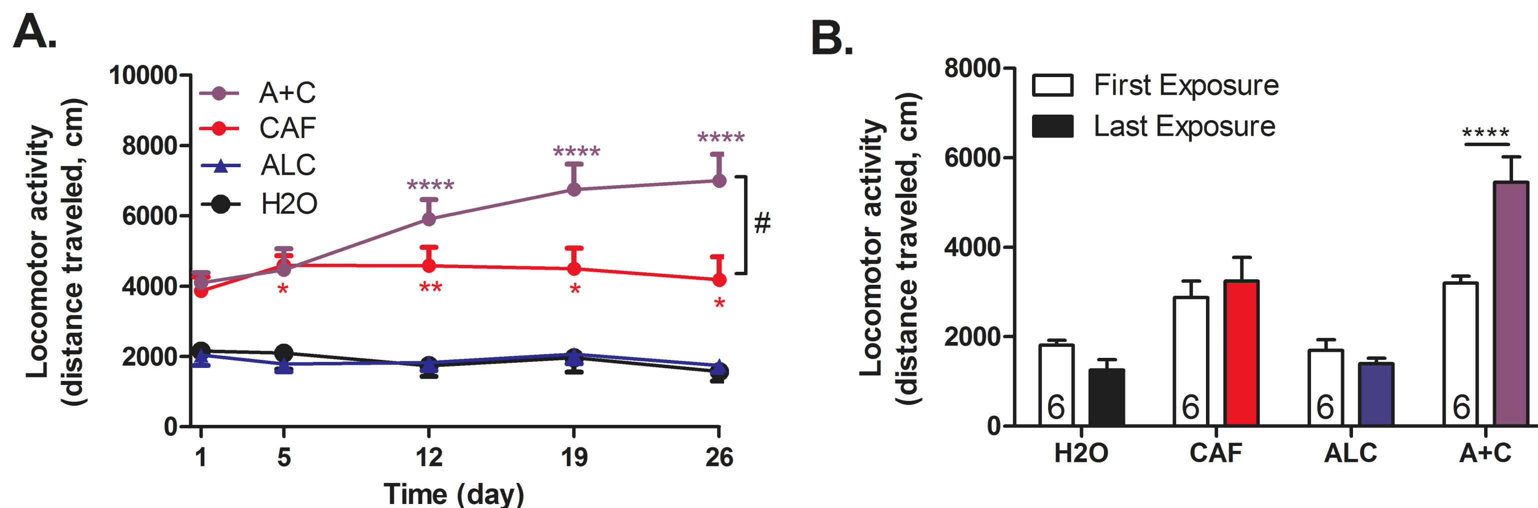


Figure 7-3. Repeated caffeine-mixed alcohol exposure by oral gavage during adolescence sensitizes locomotor responses.

Adolescent C57BL/6 mice were exposed to water (H2O), 1.5 g/kg alcohol (ALC), 15 mg/kg caffeine (CAF) or caffeine-mixed alcohol (A+C), exposure by daily oral gavage (n = 6 per group) for 4 weeks (Figure 7-2). Locomotor activity was measured for 60 minutes directly following injection. Mice exposed to caffeine-mixed alcohol showed acute hyperlocomotion and significant locomotor sensitization over the course of four weeks (A). Differences in first and last exposure demonstrate the increase in locomotor activity over the locomotor testing sessions (B). Statistical significance was assessed by two-way, repeated measures ANOVA (time and treatment) followed by Bonferroni's Multiple Comparison Test, *, $p<0.05$; **, $p<0.01$, ***, $p<0.0005$, ****, $p<0.0001$, #, $p<0.05$; data represented as mean ± SEM.

for five days a week for four weeks (Figure 7-2). Locomotor activity was measured for 60 minutes in the locomotor activity boxes immediately following drug administration on the days depicted in Figure 7-2. Behavioral testing was conducted during the active/dark cycle for each mouse. Mice were habituated to the behavioral testing room one-hour prior to acclimate to fan noise. To reduce the effect of novelty on locomotor activity, mice were habituated to the locomotor boxes the day before the first experiment.

#### 7.2.5 ΔFosB expression levels changes in dorsal striatum and nucleus accumbens.

Adolescent male C57BL/6 mice (n=6 per group) were administered water, caffeine, alcohol, or caffeine-mixed alcohol via oral gavage or cocaine (15 mg/kg, i.p.), five days a week for four weeks during the animal's dark/active cycle (Figure 7-2). Three days after the four week period of adolescent exposure, animals were once more exposed to their respective treatment and brains were collected 30 minutes later via transcardial perfusion as previously described by Engle et al, 2013 [844] (Figure 7-2 "IHC"). Brains were fixed in a 4% paraformaldehyde solution for 24 hours before transfer into 30% sterile sucrose (Sigma) for one week for cryoprotection. Brains were embedded and frozen in Tissue-Tek® O.C.T. compound (VWR, Radnor PA, USA) in tissue molds (VWR) and 50 µm coronal sections were prepared using a cryostat (Leica Microsystems Inc., Buffalo Grove IL, USA). Staining was conducted on free-floating slices for ΔFosB positive cells using primary goat anti-ΔFosB antibody (sc-48-G, Santa Cruz Biotechnology, Dallas TX, USA), diluted 1:1000 and secondary Alexa-Fluor 594 donkey anti-goat antibody (A-11058, Life Technologies, Grand Island NY, USA), diluted 1:1000. Slices were mounted with VectaShield (Vector Laboratories, Burlingame CA, USA) mounting media on microscope slides (Fischer Scientific, Hampton NH, USA), fitted with coverglass (Fischer Scientific), and sealed with nail polish.

Images were acquired via confocal microscopy (Nikon A1) at 20x magnification using an oil immersion objective. Gain and exposure were standardized to slices from a water-treated animal for proper control throughout image capture. For each animal, two images were collected, one image from the left hemisphere and one from the right hemisphere for the brain region of interest. Images were processed using ImageJ software (National Institutes of Health) for the number of ΔFosB positive cells in the dorsal striatum and shell of the nucleus accumbens per image. Positive cells were identified as areas with a specific intensity and area compared to

background, as identified through Image J analysis. The total area of analysis for each images = 403072 $um^2$.

### 7.2.6 Conditioned place preference to cocaine.

Adolescent male C57BL/6 mice (n=8-12 per group) were administered water, caffeine, alcohol, or caffeine-mixed alcohol via oral gavage, five days a week for four weeks as previously described (Figure 7-2). The following week, mice were conditioned to cocaine in a conditioned place preference paradigm (CPP, Figure 7-2 "CPP") [89]. On day 1, mice were injected i.p. with saline and placed in a two-chamber conditioned place preference box (ENV-3013-2, Med Associates) to establish baseline preference the two chambers. Testing chambers contained unique tactile (wired mesh versus metal rod flooring) and visual (horizontal or vertical black and white striped wallpaper) cues for contextual usage to differentiate between the two chambers. Over the following eight conditioning days, mice received daily i.p. injection alternatively with saline or cocaine (1.5, 5, 15, or 30 mg/kg) and were confined for 30 minutes to either a cocaine-paired side or saline-paired side of the box in an unbiased approach. On the final day, saline was administered and the mice were placed in the CPP box in order to freely move between the two boxes for preference testing for 30 minutes (Figure 7-2). Preference was calculated as the difference in time spent in the cocaine-paired side between the pre- and post-conditioning tests. Mice that spent 70% of time in one side on the pre-conditioning day were excluded from the test. All conditioning was conducted during the dark/active cycle for each mouse.

### 7.2.7 Cocaine cross sensitization

Adolescent male C57BL/6 mice (n=7-8 per group) were administered water, caffeine (15 mg/kg), alcohol (1.5 g/kg), or caffeine-mixed alcohol (15 mg/kg caffeine, 1.5 g/kg alcohol) by oral gavage for five days a week for four weeks (Figure 7-2). Locomotor activity was measured for 60 minutes in the locomotor activity boxes on the first and final day of drug administration. Locomotor activity was measured as described previously for 60 minutes following habituation to the testing room during the animals' dark/active cycle. Three days after final drug administration, animals were injected with 0.9% saline (i.p.) and placed in the locomotor boxes for baseline locomotor activity for 60 minutes. Two days after this baseline measurement (total of 5 days since last drug treatment),

animals were injected with 15 mg/kg cocaine (i.p.) and placed in the locomotor boxes for 60 minutes for total locomotor activity measurement (Figure 7-2 “SENS”).

### 7.2.8 Natural reward to saccharin.

Natural reward was monitored through preference of sweet solution (saccharin) versus water in a four-hour, two bottle choice, drinking-in-the-dark paradigm [90] following adolescent exposure to drug solutions. Male adolescent C57BL/6 mice (n=6-8 per group) were exposed to water or caffeine-mixed alcohol via oral gavage as described previously for four weeks in adolescence, shown in Figure 7-2. Upon final drug administration during the fourth week, animals were moved into single housing, double grommet cages for fluid consumption monitoring and to allow one weekend of acclimation to new cages. Three days after, saccharin solutions (0.25, 0.5, 1.0, 2.0 mM in reverse osmosis water) were prepared in 50 mL Falcon tubes, fitted with sippers, and distributed to the animals alongside a water control bottle during a four-hour, drinking-in-the-dark period to monitor saccharin consumption preference and volume (Fig 2 “SACC”) [209, 766]. Bottles were added two hours into the dark cycle and removed four hours later, allowing behavioral testing during the animals’ active cycle. Weights of the bottles were measured to 0.1 gram. Each concentration was offered to the animals for two consecutive days before moving to the next concentration for total of eight days of drinking. The location of the water and saccharin bottles was reversed between days to prevent habit formation.

### 7.2.9 Statistical analysis

All data are presented as means ± standard error of the mean. The analysis of pharmacological drug effects over time was performed using one-way or two-way ANOVA for drug effect and time, followed by a Bonferroni post-hoc test to determine statistically significant differences between groups using GraphPad Prism5 software (GraphPad Software, La Jolla, CA, USA). Student’s unpaired t-test was used for analyzing less than two groups using GraphPad Prism5.

## 7.3 Results

### 7.3.1 Repeated adolescent caffeine-mixed alcohol exposure induces significant locomotor sensitization

We observed that adolescent mice exposed to caffeine-mixed alcohol or caffeine alone by i.p. injection (Figure 7-1A) displayed significant locomotor activity compared to water or alcohol alone as determined by two-way, repeated measures ANOVA (treatment: $F_{3, 158} = 85$, $p<0.0001$, time: $F_{4, 158} = 7.74$, $p<0.0001$), where we also observed a statistically significant interaction effect (interaction time x treatment: $F_{12, 158} = 3.22$, $p<0.0004$, Figure 7-1C). Comparison of locomotor activity after the first injection versus the last injection revealed that only caffeine-mixed alcohol exposure caused statistically significant locomotor sensitization (two-way, repeated measures ANOVA for time: $F_{1, 67} = 16.70$, $p<0.0001$, treatment: $F_{3, 67} = 48.50$, $p<0.0001$, interaction time x treatment: $F_{3, 67} = 8.03$, $p<0.0001$, Figure 7-1D). Female animals sensitized more quickly and robustly than male animals, although this difference was only apparent three weeks into testing (Figure 7-1B,E) as shown by two-way, repeated measures ANOVA for sex: $F_{1, 17} = 5.51$, $p<0.0313$, time: $F_{4, 68} = 23.15$, $p<0.0001$, and interaction time x sex: $F_{4, 68} = 4.96$ $p<0.0014$.

In order to increase the physiological relevance of the animal model while maintaining the ability to administer controlled amounts, we changed the exposure route from i.p. to oral gavage (Figure 7-2 and 7-3). We found that caffeine and caffeine-mixed alcohol significantly increased locomotor activity over four weeks of exposure (treatment: $F_{4, 133} = 66.64$, $p<0.0001$, time: $F_{4, 133} = 0.67$, $p<0.6117$, time x treatment $F_{16, 133} = 2.13$, $p=0.01$, Figure 7-3A). In this model, we again observed that only adolescent mice exposed to caffeine-mixed alcohol showed significant locomotor sensitization versus caffeine alone between first and last drug exposure (two-way, repeated measures ANOVA for time: $F_{3, 38} = 3.63$, $p=0.06$, treatment: $F_{3, 38} = 35.18$, $p<0.0001$, interaction time x treatment: $F_{3, 38} = 7.82$, $p<0.0003$ Figure 7-3B), although four weeks of exposure were necessary for these effects to be significantly different from the locomotor activity induced by caffeine alone.

### 7.3.2 Animals exposed to caffeine-mixed alcohol in adolescence exhibit significant ΔFosB expression in nucleus accumbens

The locomotor sensitization we observed in adolescent mice exposed to caffeine-mixed alcohol resembled the locomotor sensitization commonly observed upon chronic cocaine exposure [845].

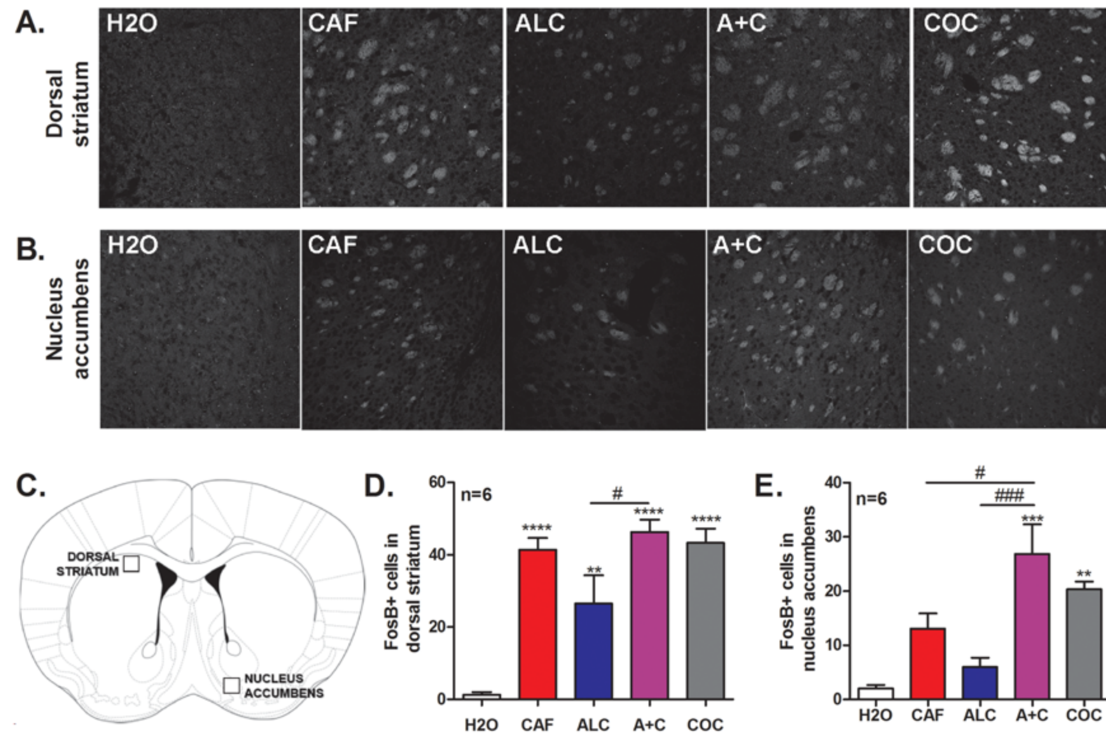


Figure 7-4. Adolescent exposure to caffeine-mixed alcohol during adolescence significantly increases ΔFosB expression levels in the nucleus accumbens similar to cocaine.

Adolescent C57BL/6 mice (n = 6 per group) were repeatedly exposed by oral gavage to water (H2O), 15/mg/kg caffeine (CAF), 1.5 g/kg alcohol (ALC), caffeine-mixed alcohol (A+C), or 15 mg/kg cocaine (i.p., COC) for four weeks in adolescence, as shown in Figure 7-2. Three days after the final locomotor session, animals were exposed once more to their respective treatment. Brains were removed 30 minutes after exposure to last treatment via transcardial perfusion. Coronal brain slices were immunohistochemically stained for ΔFosB expression in the dorsal striatum (A, D) and nucleus accumbens (B, E), as indicated in C. All treatments increased ΔFosB accumulation in the dorsal striatum compared to water controls (A, D). Increases in ΔFosB accumulation were observed in the nucleus accumbens in animals exposed to caffeine-mixed alcohol compared to alcohol or caffeine alone (B, E). Quantification was achieved by counting the number of ΔFosB for each treatment using ImageJ software. Statistical significance was determined by one-way ANOVA followed by Bonferroni's Multiple Comparison Test, *, $p<0.05$; **, $p<0.01$, ***, $p<0.0005$, #, $p<0.05$, ###, $p<0.0005$; data represented as mean ± SEM.

Chronic cocaine exposure is known to induce long-term increases in ΔFosB expression in the mesocortical and nigrostriatal dopaminergic pathways [846], thus we examined whether changes in ΔFosB expression occurred in the dorsal striatum and nucleus accumbens as a result of drug exposure (Figure 7-2). The shell of the nucleus accumbens was chosen (compared to nucleus accumbens core) as dopamine concentrations are known to preferentially increase in the shell following exposure to drugs of abuse [847]. One-way ANOVA analysis of these data was statistically significant for both dorsal striatum ($F_{4, 29} = 17.43$, $p<0.0001$, Figure 7-4A,C,D) and nucleus accumbens ($F_{4, 28} = 10.73$, $p<0.0001$, Figure 7-4B,C,E) indicating that treatment in general affected ΔFosB expression. Post-hoc analysis with Bonferroni's multiple comparison test revealed that mice exposed to cocaine, caffeine, alcohol, or caffeine-mixed alcohol exhibited a significant increase in the number of ΔFosB positive cells in the dorsal striatum compared to water controls. Interestingly, mice exposed to caffeine-mixed alcohol or cocaine during adolescence, but not alcohol or caffeine alone, exhibited increased ΔFosB expression in the nucleus accumbens versus water controls.

### 7.3.3 Adolescent caffeine-mixed alcohol desensitizes cocaine conditioned place preference

Considering the similarities between caffeine-mixed alcohol and cocaine with regard to locomotor sensitization, ΔFosB expression, and previous reports of caffeine induced sensitization of cocaine place preference [790, 845], we next tested whether adolescent mice exposed to caffeine-mixed alcohol would show altered sensitivity to the rewarding properties of cocaine [845, 846]. Mice were exposed to daily oral gavage injections of water, caffeine (15 mg/kg), alcohol (1.5 g/kg) or caffeine-mixed alcohol for four weeks during adolescence. Three days after final drug exposure, animals were subjected to cocaine conditioned place preference (Figure 7-2). Dose of 1.5, 5, 15, and 30 mg/kg were used to test preference exposed to caffeine-mixed alcohol in adolescence in separate cohorts of animals. Whereas animals exposed to water exhibited the strongest cocaine place preference to a dose of 15 mg/kg (Figure 7-5A) in accordance with that previously reported Hnasko et al., 2007 [848], caffeine-mixed alcohol exposed mice only showed significant place preference at 30 mg/kg of cocaine (two-way, repeated measures ANOVA for time: $F_{1,13} = 13.47$, $p=0.0023$, treatment: $F_{1,13} = 0.90$, $p=0.3600$, interaction time x treatment: $F_{1,13} = 2.14$, $p=0.1668$, S1C Fig). No cocaine conditioned place preference was observed at 1.5 mg/kg for animals exposed to caffeine-mixed alcohol (graph omitted from text, see supplemental in [59]) and no conditioning

was observed in caffeine-mixed alcohol or water animals at 5 mg/kg cocaine (two-way, repeated measures ANOVA for time: $F_{1,16} = 4.36$, $p = 0.053$, treatment: $F_{1,16} = 0.04$, $p = 0.8402$, interaction time x treatment: $F_{1,16} = 1.54$, $p = 0.2320$, graph omitted from text, see supplemental in [59]). Cocaine induced place preference at a dose of 15 mg/kg cocaine across all treatment groups except caffeine-mixed alcohol exposed animals, indicating that only caffeine-mixed alcohol exposed mice displayed desensitized place preference (two-way, repeated measures ANOVA for time: $F_{1,29} = 28.17$, $p<0.0001$, treatment: $F_{3,29} = 0.70$, $p<0.5600$, interaction time x treatment: $F_{3,29} = 0.72$, $p<0.5501$, Figure 7-5B).

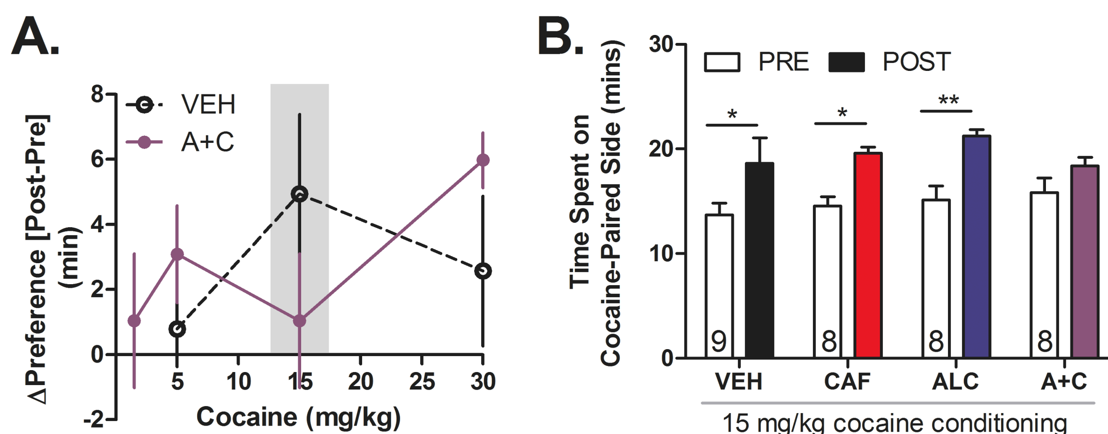


Figure 7-5. Adolescent exposure to caffeine-mixed alcohol desensitizes cocaine conditioned place preference in early adulthood.

Pre-conditioning and post-conditioning time spent on cocaine-paired side for mice treated with water (H2O), 15 mg/kg caffeine (CAF), 1.5 g/kg alcohol (ALC), or caffeine-mixed alcohol (A+C), o.g., for four weeks prior to cocaine conditioning (n = 8–12). Cocaine conditioned began three days after final adolescent drug administration. Cocaine was administered at 1.5, 5, 15, or 30 mg/kg, i.p. doses Cocaine preference, is depicted as the difference in time spent on the cocaine-paired side [change in preference = post-test (minutes)–pre-test (minutes)], (n = 8–12 per group) (A). Animals exposed to water, caffeine, or alcohol alone exhibited conditioned place preference to 15 mg/kg cocaine conditioning (n = 8–11 per group) (B), while this response was attenuated in animals exposed to caffeine-mixed alcohol. Open bars depict pre-conditioning measurement, closed bars depict post-conditioning measurement. Significance by two-way, repeated measures ANOVA with Bonferroni's Multiple Comparisons Test, *, p<0.05; **, p<0.01; data represented as mean ± SEM.

We observed no difference in cocaine induced hyperlocomotion between water and caffeine-mixed alcohol exposed animals upon their first cocaine exposure during conditioning at any of the tested cocaine conditioning doses (graph omitted from text, see supplemental in [59]). Additionally, there were no differences in 15 mg/kg cocaine induced locomotor activity during first conditioning session to cocaine between adolescent treatment groups (graph omitted from text, see supplemental in [59]), suggesting that the attenuation in place preference observed in animals exposed to caffeine-mixed alcohol was not a result of alterations in locomotor response to cocaine. Adolescent exposure to caffeine-mixed alcohol also did not impact general locomotor activity during the pre-conditioning test day compared to water controls, although Bonferroni's post-hoc analysis did show that caffeine exposed mice had significantly more locomotor activity than animals exposed to alcohol in adolescence (one-way ANOVA $F_{3,29} = 4.976$, $p$=0.004, graph omitted from text, see supplemental in [59]).

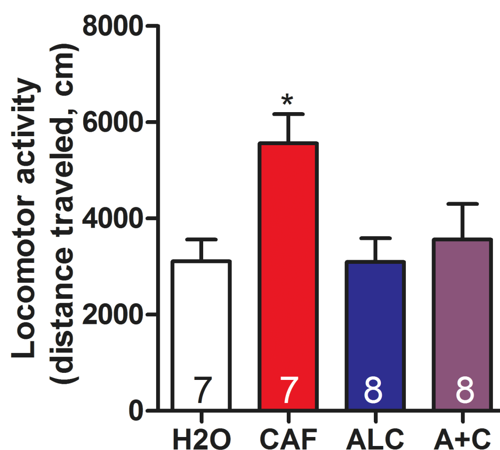


Figure 7-6. Exposure to caffeine-mixed alcohol attenuates caffeine-induced cocaine locomotor cross-sensitivity.

Adolescent male animals exposed to water (H2O), 15 mg/kg caffeine (CAF), 1.5 g/kg alcohol (ALC), or caffeine-mixed alcohol (15 mg/kg caffeine, 1.5 g/kg alcohol, A+C) were challenged to 15 mg/kg cocaine in adulthood (n = 7–8 per group). Animals repeatedly exposed to caffeine alone exhibited increased cocaine locomotor cross-sensitization than animals exposed to water, alcohol, or caffeine-mixed alcohol. Significance by one-way ANOVA, *, p<0.05; data represented as mean ± SEM.

### 7.3.4 Repeated exposure to caffeine-mixed alcohol attenuates the sensitizing effects of caffeine alone to cocaine locomotor cross-sensitization

To investigate the effects of caffeine-mixed alcohol on cocaine locomotor cross-sensitivity, animals were exposed to 15 mg/kg cocaine after adolescent treatment (Figure 7-2). Exposure to caffeine alone increased both baseline (graph omitted from text, see supplemental in [59]) and cocaine-induced increases in ambulation after adolescent treatment (Figure 7-6), while exposure to water, alcohol, or caffeine-mixed alcohol did not (one-way ANOVA for baseline: $F_{3,29} = 5.556$, $p=0.0044$, cocaine: $F_{3,29} = 3.723$, $p=0.0237$).

### 7.3.5 Caffeine-mixed alcohol exposure increases natural reward consumption and preference

We next investigated if exposure to caffeine-mixed alcohol during adolescence altered natural reward consumption and preference [766]. To prevent satiation, saccharin solutions were chosen because of saccharin's lack of caloric value compared to sucrose, which could inhibit drinking during the four-hour access period. Animals exposed to caffeine-mixed alcohol (15 mg/kg caffeine, 1.5 g/kg alcohol) during adolescence increased saccharin solution preference compared to animals exposed to water as observed by two-way, repeated measures ANOVA for adolescent treatment: $F_{1,12} = 5.95$, $p=0.031$, saccharin concentration: $F_{3,36} = 3.59$, $p=0.023$ (Figure 7-7A). Two-way, repeated measures ANOVA revealed significant differences in saccharin consumption as well, with Bonferroni post-hoc analysis indicating that animals exposed to caffeine-mixed alcohol consumed significantly larger quantities of 2 mM saccharin (Figure 7-7B, treatment: $F_{1,12} = 7.62$, $p=0.017$, saccharin concentration: $F_{3,36} = 16.13$, $p<0.0001$). Analysis of cumulative saccharin intake revealed the same significant effect as (area under the curve shown in Figure 7-7C) as analyzed by student's t-test.

## 7.4 Discussion

To study the effect of adolescent caffeine-mixed alcohol exposure on drug-related behaviors, we developed a mouse model that enabled us to observe several unique features resulting from repeated adolescent exposure to caffeine-mixed alcohol compared to caffeine or alcohol alone. We exposed animals to caffeine-mixed alcohol (15 mg/kg caffeine, 1.5 g/kg alcohol) by intraperitoneal and oral gavage administrations throughout the span of mouse adolescence [post-natal days 30-60] [808, 849]. The alcohol dose of 1.5 g/kg was chosen as it is high enough to induce intoxication

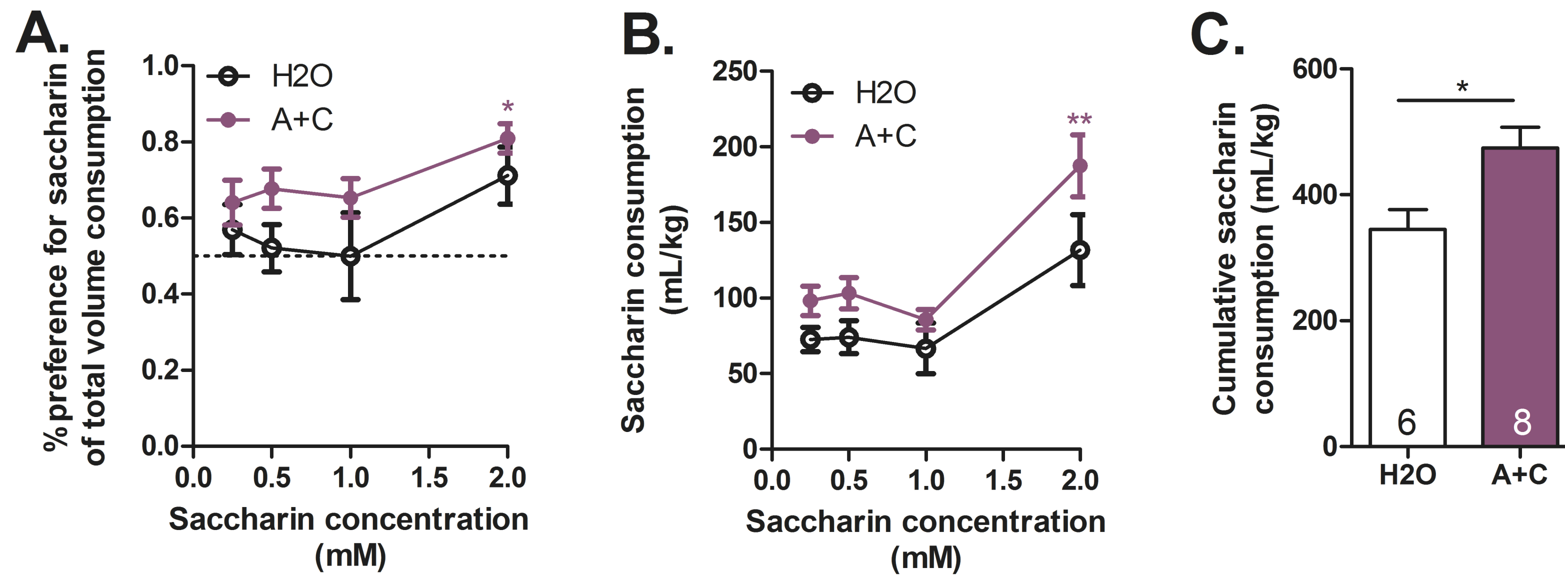


Figure 7-7. Caffeine-mixed alcohol exposure in adolescence increases natural reward consumption and preference.

Adolescent exposure to caffeine-mixed alcohol (15 mg/kg caffeine, 1.5 g/kg alcohol, A+C) or water (H2O) altered natural reward consumption and preference in adulthood (n = 6–8 per group). An increase in saccharin preference was observed throughout testing between animals exposed to caffeine-mixed alcohol versus water controls (A). Animals exposed to caffeine-mixed alcohol consumed significantly more 2.0 mM saccharin solution (B) with a greater saccharin solution consumption overall compared to water controls (C). Significance by two-way ANOVA, *, $p<0.05$; **, $p<0.01$ or unpaired t-test, *, $p<0.05$; data represented as mean ± SEM.

without inducing severe locomotor impairment and C57BL/6 mice routinely reach BEC of >0.08 mg/ml [850]. The caffeine dose chosen provided clear stimulation in C57BL/6 mice as evident from increased locomotor activity (Figures 7-1 and 7-3). The exposure model utilized mirrors patterns and levels of repeated binge consumption of caffeine-mixed alcohol self-reported by adolescents and young adults [833]. Our results, obtained by monitoring changes in locomotor activity, ΔFosB accumulation, and natural and drug reward sensitivity, support the emerging idea that repeated exposure to caffeine-mixed alcohol poses a risk to adolescent behavioral and neurological development.

Previous studies have shown that the addition of alcohol (1.75-3.25 g/kg) to caffeine (15 mg/kg) can acutely enhance the locomotor effects induced by caffeine alone [840] and locomotor sensitization observed upon repeated exposures of caffeine-mixed alcohol (15 mg/kg + 4 g/kg) [841]. In our animal model using the same dose of caffeine (15 mg/kg), we observed significant locomotor sensitization at lower doses of alcohol (1.5 g/kg) than previously observed (4 g/kg) [841]. This effect was retained for both intraperitoneal and oral gavage drug administration, the latter method being a more relevant route of administration for proper comparison to human consumption and metabolism (Figures 7-1-3). The increased locomotor activity upon adolescent exposure to caffeine-mixed alcohol was in accordance with previous data [826, 840, 841] showing that mixing caffeine with alcohol may diminish the sedative properties of alcohol through caffeine's stimulant properties, giving rise to a "wide-awake drunk" behavioral state [826]. Additionally, we observed sex differences in response to repeated caffeine-mixed alcohol exposure, with female adolescent animals sensitizing more quickly and robustly than male mice (Figure 7-1). Our results suggest that repeated caffeine-mixed alcohol consumption in females may be more problematic than repeated exposures in male, aged-matched counterparts. The similarity in increased locomotor sensitivity observed with caffeine-mixed alcohol in females is in accordance with that observed for other psychostimulants [142, 851], such as cocaine, suggesting that activation of similar brain regions may occur upon repeated administration of caffeine-mixed alcohol and common psychostimulants.

Neurons in the nucleus accumbens and dorsal striatum that are exposed for a prolonged period to high concentrations of dopamine, e.g. by repeated cocaine exposure, are known to increase expression of ΔFosB, a transcription factor that accumulates upon chronic drug exposure [845, 846, 852]. Both caffeine and alcohol increase dopamine levels in the mesolimbic and

nigrostriatal dopamine systems by affecting firing of dopaminergic neurons [792, 793, 842], and increased dopaminergic tone in the dorsal striatum has been correlated with enhanced locomotor activity [853]. Therefore, we hypothesized that if caffeine-mixed alcohol-induced-locomotor sensitization was attributed to additive or synergistic striatal release of dopamine, we would observe increased ΔFosB expression in the dorsal striatum and/or nucleus accumbens. We observed that adolescent exposure to caffeine, caffeine-mixed alcohol, and cocaine significantly increased ΔFosB expression in the dorsal striatum compared to water controls (Figure 7-4). More importantly, significant increases in ΔFosB expression were observed in the nucleus accumbens in animals exposed to caffeine-mixed alcohol or cocaine, but not mice exposed to alcohol or caffeine alone (Figure 7-4), supporting our hypothesis that caffeine-mixed alcohol can induce stronger dopamine release than caffeine or alcohol exposure alone. Previous studies have observed statistically significant alcohol-induced ΔFosB expression in the nucleus accumbens, but this increase was observed at concentrations much higher than used in our experiment (8-12 g/kg vs 1.5 g/kg) [852], further suggesting that the combination of caffeine-mixed alcohol induces ΔFosB accumulation at lower levels of alcohol intoxication than those previously reported. Our observation that only mice exposed to caffeine-mixed alcohol and not caffeine alone display increased accumbal ΔFosB expression and heightened locomotor sensitization is in agreement with reports showing that cocaine directly injected in the nucleus accumbens induces locomotor sensitization [854-856], emphasizing the role of increased dopamine levels in the nucleus accumbens and increased locomotor stimulation.

The nucleus accumbens is heavily involved in reward-associated learning and behaviors, specifically to drugs of abuse, while the dorsal striatum is involved in decision-making, habitual action, and response control [37]. Enhanced ΔFosB expression in the nucleus accumbens has been previously correlated with increased locomotor sensitization, increased cocaine reward [845], as well as increased place preference to other non-stimulant drugs of abuse, such as morphine [857]. As our mice exposed to repeated caffeine-mixed alcohol exhibited locomotor sensitization and increased accumbal ΔFosB expression, we hypothesized that these mice would also exhibit enhanced cocaine conditioned place preference compared to animals exposed to caffeine or alcohol alone [845, 846].

Against our initial hypothesis, animals exposed to caffeine-mixed alcohol exhibited attenuated cocaine place preference to 15 mg/kg cocaine. Instead, caffeine-mixed alcohol exposed

animals exhibited "equi-rewarding" effects to 30 mg/kg cocaine when compared to 15 mg/kg cocaine reward for animals exposed to water, caffeine, or alcohol alone (Figure 7-5). One possible interpretation of our data is that repeated exposure to caffeine-mixed alcohol caused stronger dopamine release than exposure to caffeine or alcohol alone and potentially desensitized subsequent cocaine reward responses. This hypothesis is in line with our observation that cocaine place preference was only desensitized in mice exposed to caffeine-mixed alcohol, but not caffeine or alcohol.

If exposure to repeated caffeine-mixed alcohol during adolescence caused desensitization of the dopamine system in young adults, we hypothesized that mice repeatedly exposed to caffeine-mixed alcohol would show limited cocaine cross-sensitization, which has been reported for previous exposure to alcohol and caffeine [790, 858]. Indeed, whereas adolescent exposure to 15 mg/kg caffeine sensitized locomotor responses to cocaine as previously reported [790], mice exposed to caffeine-mixed alcohol or 1.5 g/kg alcohol did no show cocaine locomotor cross-sensitization (for alcohol alone, this effect may result from the lower cocaine and alcohol doses utilized compared to those previously reported by Itzhak and Martin, 1999) (Figure 7-6). Several studies have shown that a decrease in drug reward found in conditioned place preference can be associated with increased self-administration, because animals need to administer more of the drug to obtain the same reward or stimulatory effect [280, 859-863]. Thus, our results could indicate that mice exposed to repeated caffeine-mixed alcohol during adolescence may be at greater risk for future abuse of rewarding substances. To test this hypothesis, we investigated how repeated exposure to caffeine-mixed alcohol in adolescence would alter intake of a natural reward (saccharin) in adulthood [209, 766, 864, 865]. In support of our hypothesis, we found that mice exposed to caffeine-mixed alcohol increased voluntary saccharin consumption and preference compared to water control mice (Figure 7-7). Importantly, animals exposed to caffeine-mixed alcohol did not display an anhedonic response, as animals continued to consume saccharin at levels higher than control animals suggesting that caffeine-mixed alcohol exposure did not decrease reward-seeking motivation.

It is important to note that our adolescent locomotor and ΔFosB measurements were conducted in animals directly after drug administration, while the rest of our behavioral data was collected from animals at a minimum of three days after final adolescent drug exposure. Abstinence and possible withdrawal from the adolescent drug treatments may explain why the

increase in locomotor sensitization and ΔFosB expression did not correlate with increased drug reward and sensitivity [845, 846, 866]. Yet, with attenuated cocaine preference, decreased cocaine locomotor sensitivity, and increased natural reward consumption in adulthood, our results continue to suggest that repeated caffeine-mixed alcohol in adolescence alters reward and response threshold to psychostimulants and natural rewards via desensitization of dopamine reward pathways. Previously, animals characterized as low quinpirole (selective dopamine $D_2$ receptor agonist) responders exhibited decreased cocaine conditioned place preference compared to animals with high quinpirole response, potentially as a result of decreased dopamine $D_2$ receptor levels in the brain [722]. Dopamine $D_2$ receptors are known to undergo receptor downregulation and degradation upon stimulation [867, 868]. Thus it is possible that repeated exposure to caffeine-mixed alcohol increased dopamine release by such extent (i.e. more so than alcohol or caffeine alone can accomplish) that it caused desensitized/downregulation of $D_2$ receptors [867, 869, 870]. We hypothesize that additional measurements on drug self-administration to drugs of abuse, such as cocaine or other psychostimulants, would observe an escalation in drug administration resulting from this desensitized response and reward threshold alteration.

The persistent marketing of highly caffeinated products will increase the likelihood of adolescent exposure to highly caffeinated alcoholic beverages, thus understanding the developmental risks of caffeine-mixed alcohol consumption on adolescent behavior and drug reward is vital. Here, we developed a physiologically relevant animal model to investigate the effects of repeated caffeine-mixed alcohol exposure during adolescence for alterations in drug-related behaviors and neuronal activation of the dopamine reward systems. From our model, we observed that repeated adolescent exposure to caffeine-mixed alcohol induced locomotor sensitization, increased expression of transcription factors related with chronic neuronal activation, and altered cocaine conditioned place preference. A desensitized response to cocaine preference and locomotor cross-sensitization was observed in animals exposed to caffeine-mixed alcohol, suggesting a desensitized dopamine reward system, which was supported by increased natural reward consumption and preference to saccharin solutions. Our data provides *in vivo* evidence that highlights several potential health risks associated with repeated exposure to caffeine-mixed alcohol. How these results compare with future drug taking events is currently unknown, but our results suggest that repeated caffeine-mixed alcohol consumption may lead to increased reward thresholds for natural and drug-related rewards, leading to an escalation in reward consumption to

reach that threshold. Combined with human data reporting the dangers of acute adolescent consumption of caffeine-mixed alcohol [813, 829, 833], our results should open up a dialogue about the potential safety risks and marketing strategies of highly caffeinated products to adolescents and young adults.

# CHAPTER 8. PHARMACOLOGICAL CHARACTERIZATION OF THE NOVEL PSYCHOACTIVE SUBSTANCE ETHYLPHENIDATE IN MALE AND FEMALE MICE

A rising trend is the misuse of prescription psychostimulants, such as amphetamine (Adderall®) and methylphenidate (MPH, Ritalin®) as nootropic drugs, i.e. drugs that enhance focus and cognition. As the designation of these medications as Schedule II drugs provides a hurdle for drug users to obtain them, a market for the development of novel psychoactive substances (NPSs) that are not (yet) controlled by the DEA has developed. Logically, much less is known about the potential therapeutic - as well as adverse effects - of these nootropic NPSs with regards to their influence on cognitive- or addiction-related behaviors. Here, we characterized the behavioral outcomes associated with repeated use of the NPS ethylphenidate (EPH), a close analog of MPH, in adolescent male and female C57Bl/6 mice. EPH displays a higher preference for the dopamine transporter (DAT) than the norepinephrine transporter (NET) compared to MPH. Given the important role of dopamine in decision making and reward learning, we hypothesized that because of the higher preference of EPH for DAT, EPH exposure in adolescent mice would be rewarding and alter cognition. Repeated exposure to 15 mg/kg decreased spatial cognitive performance as assessed by the Barnes maze spatial learning task. For drug-sensitization, we found that acute EPH exposure induced hyperlocomotion at a high dose (15 mg/kg, i.p.), but not a low dose (5 mg/kg, i.p.) of EPH, and no locomotor sensitization was observed upon repeated exposure to either dose. Interestingly, both male and female mice exhibited significant conditioned place preference at both low (5 mg/kg) and high (15 mg/kg) doses of EPH, suggesting that even non-stimulating doses of EPH are rewarding. In both males and females, repeated EPH exposure increased levels of ΔFosB in the dorsal striatum, nucleus accumbens, and prefrontal cortex. Overall, our results suggest that EPH use in adolescence is rewarding and decreases spatial performance, with no overall sex differences observed in EPH locomotor sensitivity, reward, or cognition.

## 8.1 Introduction

Over the past decade, a significant increase in the number of novel psychoactive substances (NPS) - also known as “legal highs” - has emerged. These substances, which are either novel in origin or novel in their use as a consumed chemical [871], can alter the mental and behavioral performance

of the user, although the risks of acute or repeated use are typically unknown [872]. Indeed, some of these unregulated research chemicals may be toxic upon consumption or lead to significant modification of mental status after intake [82, 873-875].

Many of the NPS that have emerged, such as synthetic cathinones, are psychostimulants [62]. One novel psychostimulant of recent interest is ethylphenidate (EPH), which is similar in structure and function to the ADHD medication methylphenidate (MPH, Ritalin®) and initially rose in prominence in Europe in the past decade under the name of "diet coke" or "nopaine" [876, 877]. In humans, self-reports of EPH consumption are associated with increased socialness, euphoria, cognitive enhancement, as well as bodily agitation, insomnia, anxiety, and compulsive redosing [875, 878]. Administration strategies of EPH vary and include nasal insufflation, oral or anal administration, and intramuscular or intravenous injection [875, 878]. In cases where EPH is injected [879], EPH use has been associated with weight loss, irritability, and paranoia and potential long-term mental health alterations, although no controlled studies have formally investigated the association between EPH use and cognition or reward [81]. As a result of the increased reports of EPH use and toxicity [82], EPH became illegal to manufacture, sell, or import in many European countries such as the United Kingdom, Germany, Austria, Denmark, Poland, and Sweden starting in 2012. EPH remains uncontrolled in the Netherlands, and the United States in 2018, EPH is not explicitly controlled but may be considered illegal as it is an analog of MPH, a Schedule II substance [880].

While many NPSs lack any pharmacological data, some information is known of EPH, partly from *in vitro* and in *in vivo* pharmacology studies on EPH and through what is known about the pharmacologic and physiologic effects of EPH's structurally similar chemical cousin, MPH [62, 78, 881]. EPH's mechanism of action is similar to that of MPH [78, 881], and interestingly, small concentrations of EPH form upon co-consumption of large quantities of alcohol and MPH through a mechanism known as hepatic transesterification [78, 882]. In HEK293 cells expressing human dopamine transporter (DAT), racemic (±)-EPH displays increased DAT inhibition (95±18 nM) compared to cocaine (289±38 nM). This increased DAT inhibition is primarily driven by (+)-EPH, with DAT inhibition at 26±6 nM, versus (-)-EPH with 1730±180 nM DAT inhibition [881]. Negligible binding and inhibition is observed at the serotonin transporter (SERT) for (±)-EPH, while similar norepinephrine transporter (NET) inhibition and binding is detected between cocaine and (±)-EPH [881]. Overall, (±)-EPH displays a higher preference for DAT versus NET in terms

of inhibition and binding when compared with (±)-MPH or cocaine. This profile may correlate with reports of euphoria in human subjects upon EPH ingestion [875], as increased DAT preference compared with NET or SERT is correlated with psychotropic effective doses of psychostimulants in humans [80].

The stimulatory effects of EPH administration have been previously investigated using rodent behavioral models, where hyperlocomotion was observed following 5 and 10 mg/kg (±)-EPH exposure in C57Bl/6 mice. Yet, the rewarding and cognitive effects of repeated EPH exposure have yet to be elucidated, and the increased DAT preference of EPH suggests that EPH exposure may be rewarding and alter cognition similarly to MPH or cocaine [881, 883]. Here, we present the first *in vivo* reports of the reward and cognitive profile of repeated (±)-EPH exposure in C57Bl/6 male and female adolescent mice. Overall, our results suggest that EPH is rewarding even at non-stimulatory doses and may impair cognitive recall in both male and female adolescent mice, with neurochemical correlates in increased ΔFosB expression.

## 8.2 Materials and methods

### 8.2.1 Drugs and chemicals

(±)-threo-ethylphenidate hydrochloride (ethylphenidate, EPH) was purchased from Cayman Chemical (Ann Arbor, MI USA). Ketamine was purchased from Henry Schein Animal Health (Dublin OH, USA) and xylazine, heparin (10 units/mL), and isopropyl alcohol were purchased from Sigma Aldrich (St. Louis, MO, USA). Paraformaldehyde ampules were obtained from (Electron Microscopy Sciences, Hatfield PA, USA).

### 8.2.2 Animal husbandry

Male and female C57Bl/6, wild-type adolescent (post-natal day 35) mice were purchased from Taconic Biosciences, Inc. (Cambridge, IN USA) and habituated for one week to the animal facility prior to behavioral testing. Food and water was provided ad libitum. Throughout the experiment, animals were kept in at ambient temperature of (21°C) in a room maintained on a 12L:12D cycle (lights on at 9.00, lights off at 21.00) in Purdue University's animal facility, as accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. All animal procedures were pre-approved by Purdue University's Institutional Animal Care and Use Committee and

conducted in accordance with National Institutes of Health Guide for the Care and Use of Laboratory Animals.

### 8.2.3 Barnes maze task for spatial learning

The Barnes maze task for spatial learning was initially conducted in drug-naïve male and female adolescent mice as described previously [884] over the course of 6 days in a light room with bright light and geometric shapes taped to the walls for spatial cueing. On day 1, mice were habituated to the maze's escape route over the course of three sessions. On days 2-5, four trials a day, at least 15 minutes apart in time, were conducted to assess latency to enter the escape route. Each trial ended once the mouse entered the escape route or after 3 minutes (if the animal did not enter the escape route before the 3 minute session was finished, the animal was guided to the escape route and placed inside). Following each trial, the animal was left in the escape route for 1 minute. On day 6, a probe trial was conducted for 90 seconds, where the escape route was covered, and the animal was free to explore the entire maze.

Following the initial Barnes maze training, animals were exposed to a once daily injection of 15 mg/kg EPH (i.p.) for 12 consecutive days. Three days after the final EPH injection, Barnes maze re-testing began as described previously (days 1-6 described above). Total time to enter escape route per trial and total errors per trial per recorded manually by an unbiased experimenter. All testing was conducted during the animals' light cycle, and animals were habituated to the testing room for at least 1 hour prior to testing and conditioning sessions. The maze was cleaned with 70% isopropyl alcohol between animal trials to prevent lingering scent trials.

### 8.2.4 Acute and repeated exposure locomotor activity

On day 1 (first day of drug exposure), animals were weighed and injected with vehicle (saline, 0.9%), 5, or 15 mg/kg EPH i.p. directly prior to a 60-minute locomotor session in square locomotor boxes from Med Associates (L 27.3 cm x W 27.3 cm x H 20.3 cm, St. Albans VT, USA). Locomotor testing was conducted on days 1, 3, 5, 8, and 12 following drug exposures; on days without locomotor testing, animals were injected with vehicle or EPH and placed back in their home cage. All testing and drug administration was conducted during the animals' light cycle. For locomotor testing, animals were habituated to the testing room for at least 1 hour prior to testing sessions.

### 8.2.5 Conditioned place preference

An unbiased conditioned place preference protocol was performed as previously described [59, 89]. In brief, adolescent male and female animals were placed in a two-chamber apparatus for 30 minutes following a vehicle injection (0.9% saline, i.p.) to establish initial bias. Animals exhibiting an initial bias for one compartment >70% were excluded from further testing. Over the following conditioning days, one conditioning session (30 minutes) was performed per day by confining the animal to either drug- (5 or 15 mg/kg EPH) or vehicle-paired side for a total of 8 conditioning sessions. On the final day, animals were placed in the two-chamber apparatus following a vehicle injection to freely explore both compartments where preference of the two chambers was assessed over 30 minutes. All testing was conducted during the animals' light cycle, and animals were habituated to the testing room for at least 1 hour prior to testing and conditioning sessions.

### 8.2.6 Immunohistochemistry

Male and female adolescent mice were exposed to daily vehicle, 5 mg/kg, or 15 mg/kg EPH (i.p.) injections for 12 consecutive days. Three days following the final drug administration, animals were transcardially perfused as previously described [59]. Brains were fixed in a 4% paraformaldehyde solution for 24 hours before transfer into 30% sterile sucrose (Sigma) for at least one week for cryoprotection. The sucrose solution was changed once during this time. Brains were embedded and frozen in Tissue-Tek® O.C.T. compound (VWR, Radnor PA, USA) in tissue molds (VWR) and 50 μm coronal sections were prepared using a cryostat (Leica Microsystems Inc., Buffalo Grove IL, USA). Staining was conducted on free-floating slices for ΔFosB positive cells using primary goat anti-ΔFosB antibody (sc-48-G, Santa Cruz Biotechnology, Dallas TX, USA), diluted 1:1000 and secondary Alexa-Fluor 594 donkey anti-goat antibody (A-11058, Life Technologies, Grand Island NY, USA), diluted 1:1000. Slices were mounted with VectaShield (Vector Laboratories, Burlingame CA, USA) mounting media on microscope slides (Fischer Scientific, Hampton NH, USA), fitted with coverglass (Fischer Scientific), and sealed with nail polish.

Images were acquired via confocal microscopy (Nikon A1) at 20x magnification using an oil immersion objective. Gain and exposure were standardized to slices from a vehicle-treated animal for proper control throughout image capture. For each animal, two images were collected, one image from the left hemisphere and one from the right hemisphere for the brain region of

interest. Images were processed using ImageJ software (National Institutes of Health) for the number of ΔFosB positive cells in the dorsal striatum, shell of the nucleus accumbens, or prefrontal cortex (prelimbic cortex) per image. Positive cells were identified as areas with a specific intensity and area compared to background, as identified through ImageJ analysis. The total area of analysis for each images = 403072 $um^2$.

### 8.2.7 Statistical analysis

All data are presented as means ± standard error of the mean and analysis was performed using GraphPad Prism 7 software (GraphPad Software, La Jolla, CA). Two-way, repeated measures ANOVA with Bonferroni multiple comparisons test was used for locomotor differences between first and last drug exposure and conditioned place preference studies. Two-way ANOVA with Tukey (dose effect within sex) or Sidek (sex effect across same dose) multiple comparisons test were utilized to assess drug, sex, or interaction effect for Barnes maze performance, locomotor differences at first drug exposure, last drug exposure, difference in time spent on the EPH-paired side in CPP, and ΔFosB accumulation.

## 8.3 Results

### 8.3.1 Deficit in spatial Barnes maze task following EPH exposure, although no within group significance

To study how EPH exposure may impact learning and memory, we chose to use the Barnes maze task as it is capable of assessing spatial learning without inducing significant anxiety [885], as compared with the Morris water maze [886]. Following repeated exposure to either 0.9% saline (VEH) or 15 mg/kg EPH, animals (n=6 per group) were trained on the Barnes maze to locate an escape route within 3 minutes over the course of four days of training (with 4 sessions per day). A significant overall effect of drug exposure ($F_{1,19}=5.07$, $p=0.0363$) exposure was found, with no sex ($F_{1,19}=2.96$, $p=0.102$) or interaction effect ($F_{1,19}=0.0204$, $p=0.888$) on latency to enter the escape route following EPH exposure (Figure 8-1A,B). No significant differences in latency to enter the escape route were observed by Tukey multiple comparisons test for drug effect between drug-exposed and drug-naïve animals within the same sex, likely because of the limited number of animals tested.

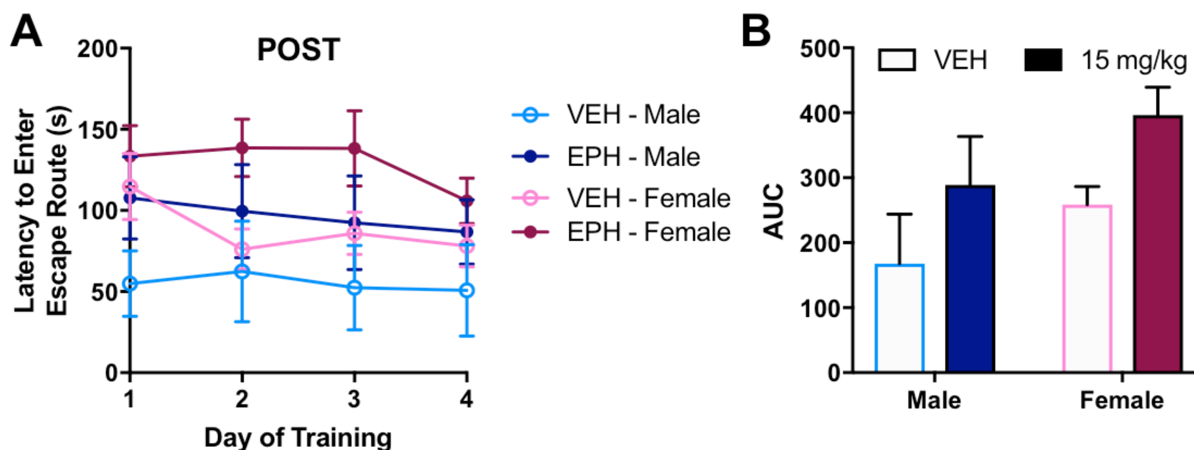


Figure 8-1. Overall significant drug effect of EPH to increase latency to enter escape route, no within sex significance.

Adolescent male and female C57Bl/6 mice were trained on the Barnes maze for one week prior to 12 consecutive days of vehicle (VEH) or 15 mg/kg EPH exposure (i.p.). Following this drug exposure, a second Barnes maze training week was conducted (POST) where a drug effect was observed (A, B), although no significance was observed within sex in mice exposed to vehicle compared with 15 mg/kg EPH. Significance by two-way ANOVA with Tukey (dose) or Sidek (sex) multiple comparisons; data represented as mean ± SEM.

### 8.3.2 Repeated EPH exposure does not induce locomotor sensitization

As locomotor sensitization is a sign of synaptic plasticity and has been correlated with increased DAT activity [887], we assessed locomotor activity following both acute and repeated exposure to EPH at a low (5 mg/kg) or high (15 mg/kg) dose. No locomotor sensitization was observed in adolescent male or female mice (n=5-6 per group) at 5 or 15 mg/kg EPH, as observed by no increase in total ambulatory distance between day 1 (first drug exposure) and day 12 (last drug exposure) (Figure 8-2A). No significant effect of drug dose x exposure date ($F_{2,28}$=0.71, $p$=0.62) or effect of exposure date ($F_{28,1}$=0.09, $p$=0.77) was found, while a significant effect of drug dose ($F_{5,28}$=48.0, $p$<0.0001) was observed by two-way ANOVA. Additionally, no effect of matching $F_{28,28}$=1.03, $p$=0.47) was determined.

No sex ($F_{1,28}$=0.178, $p$=0.676) or interaction ($F_{2,28}$=3.15, $p$=0.0581) effect was observed for total ambulation following first drug exposure, although a significant effect of dose was noted ($F_{2,28}$=64.0, $p$<0.0001). In male and female mice, significantly higher ambulation was observed between VEH and 15 mg/kg EPH ($p$<0.0001) and 5 and 15 mg/kg EPH ($p$<0.0001) (Figure 8-2B).

Similarly, for repeated exposure, no sex ($F_{1,28}$=2.08, $p$=0.160) or interaction ($F_{2,28}$=0.135, $p$=0.875) effect was observed, although a significant effect of dose was found ($F_{2,28}$=55.6, $p$<0.0001). Again, in both male and female mice, significantly higher ambulation was observed between VEH and 15 mg/kg EPH ($p$<0.0001) and 5 and 15 mg/kg EPH ($p$<0.0001) (Figure 8-2C) at this last exposure.

### 8.3.3 EPH conditioned place preference observed at non-stimulatory doses

DAT expression is known to play a role in the reward conditioning effects of psychostimulants such as cocaine [888]; therefore, we investigated how male and female adolescent mice (n=5-10) conditioned to either a non-locomotor stimulatory 5 or stimulatory 15 mg/kg dose of EPH. As evident by the increased time spent on the drug-paired side following 8 sessions of conditioning (Figure 8-3A), both the non-stimulatory (5 mg/kg) and stimulatory (15 mg/kg) dose of EPH increased time spent on the EPH-paired side, suggesting reward present at both doses of EPH. A significant effect of conditioning ($F_{27,1}$=88.9, $p$<0.0001) was observed, while no effect of drug dose ($F_{3,27}$=0.580, $p$=0.63) or drug dose x conditioning ($F_{3,27}$=1.14, $p$=0.35) was noted by 2-way ANOVA, with a significant effect of matching ($F_{27,27}$=3.17, $p$=0.0019). For adolescent male mice exposed to 5 mg/kg or 15 mg/kg EPH, a significant increase in time spent on the EPH-paired side was observed ($p$<0.0001, $p$=0.0001, respectively). This increased in time spent on the EPH-paired side was also observed in female adolescent mice at 5 mg/kg and 15 mg/kg as well ($p$=0.0218, $p$<0.0001, respectively). No significant effect of sex ($F_{1,23}$=0.0656, $p$=0.800), drug dose ($F_{1,23}$=2.06, $p$=0.165), or interaction ($F_{1,23}$=1.56, $p$=0.224) effect was observed for difference in time spent on the EPH-paired side after - before conditioning (Figure 8-3B).

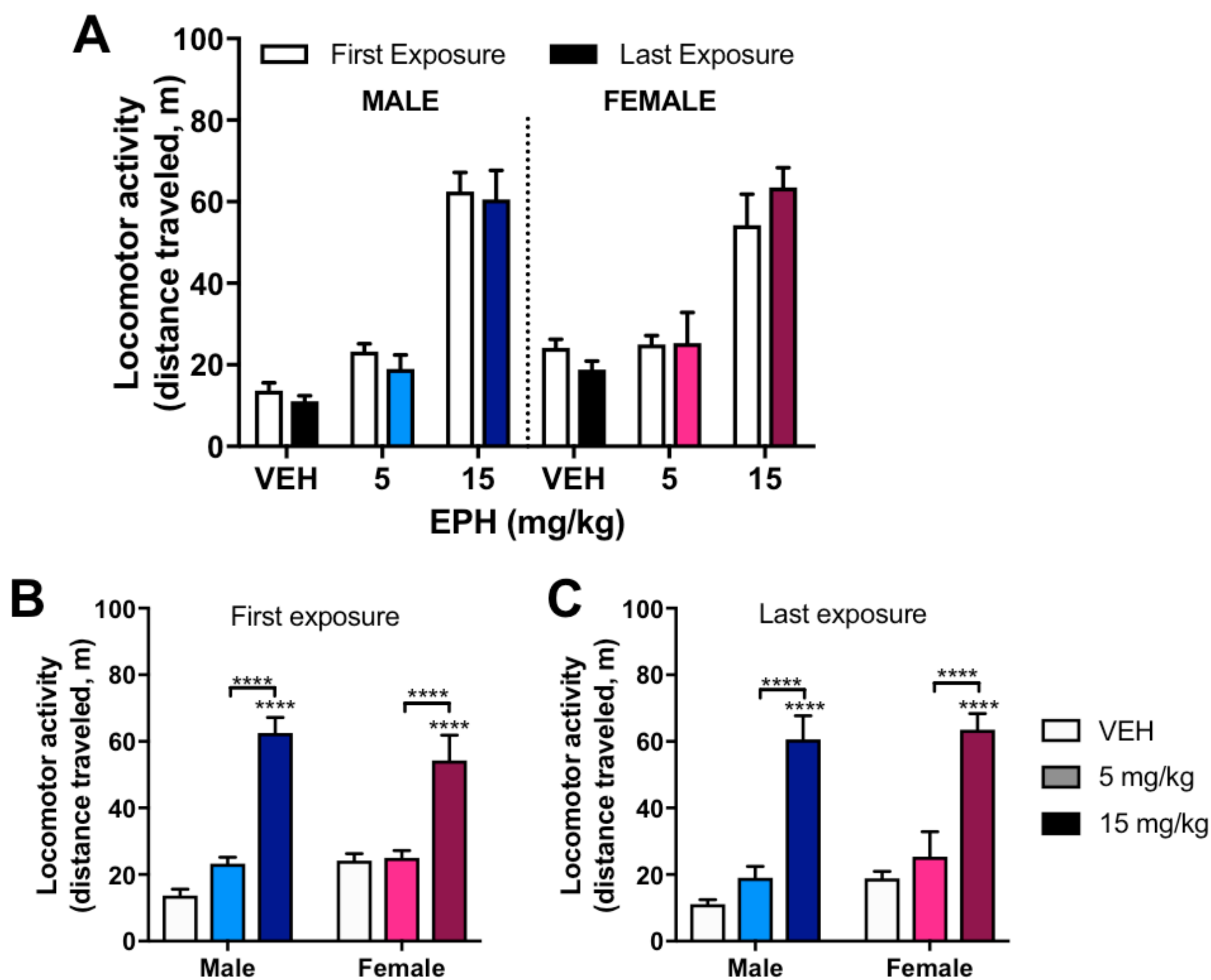


Figure 8-2. No locomotor sensitivity observed at 5 or 15 mg/kg EPH but increased locomotion at 15 mg/kg EPH upon acute and repeated exposure.

No locomotor sensitivity observed between the first or last exposure to EPH at 5 or 15 mg/kg (A) in adolescent male or female C57Bl/6 mice. Increase locomotion observed at 15 mg/kg in male (B) and female (C) mice compared with vehicle or 5 mg/kg EPH. Significance by two-way ANOVA with Tukey (dose) or Sidek (sex) multiple comparisons, ****, $p<0.0001$; data represented as mean ± SEM.

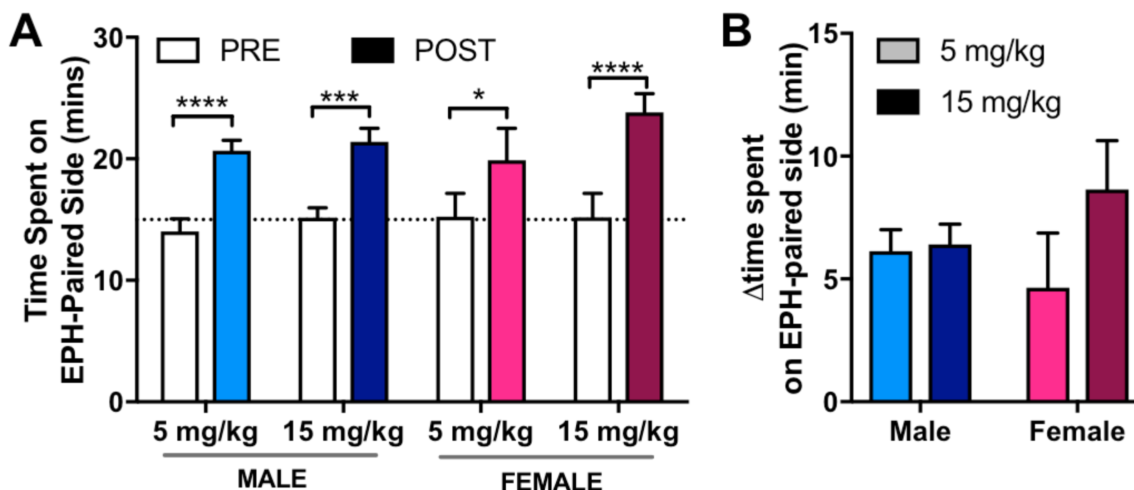


Figure 8-3. EPH conditioning observed at both non-stimulatory and stimulatory doses.

Male and female adolescent C57Bl/6 mice spent more time on the EPH-paired compartment in a two-chamber, conditioned place preference protocol following 8 total conditioning sessions (A). No significant change in time spent on the EPH-paired side by dose or sex (B). Significance by two-way ANOVA with Tukey (dose) or Sidek (sex) multiple comparisons, *, $p<0.05$; ***, $p<0.0005$; ****, $p<0.0001$; data represented as mean ± SEM.

### 8.3.4 EPH dose-dependently increases ΔFosB expression in striatal and cortical areas

ΔFosB, a neuronal marker heavily implicated in drug addiction [845, 846, 852], has been shown to increase upon repeated exposure to drugs of abuse [846], thus we exposed male and female adolescent mice (n=8-9) to vehicle, 5, or 15 mg/kg EPH (i.p.) once daily for 12 consecutive days to measure changes in ΔFosB in the brain. Three days after the final exposure, mice were sacrificed, and brains were extracted. A significant increase in ΔFosB accumulation was observed in the prefrontal cortex (Figure 8-4A), dorsal striatum (Figure 8-4B), and nucleus accumbens (Figure 8-4C) in animals exposed to EPH. In the prefrontal cortex, a significant effect of drug dose ($F_{2,43}=74.4$, $p<0.0001$) was observed, with no sex ($F_{1,43}=0.418$, $p=0.521$) or interaction ($F_{2,43}=0.205$, $p=0.816$) effect, and multiple comparisons reveled that both 5 and 15 mg/kg significant increased ΔFosB staining in both male and female mice ($p<0.0001$) as compared with vehicle (Figure 8-4A). In the dorsal striatum, a significant effect of drug dose ($F_{2,43}=37.8$, $p<0.0001$) was observed, with no sex ($F_{1,43}=2.71$, $p=0.107$) or interaction ($F_{2,43}=1.63$, $p=0.207$) effect. Multiple comparisons revealed that 15 mg/kg EPH significant increased ΔFosB as compared with vehicle ($p<0.00001$) in both male and female mice. In males, a significant increase was observed

between 5 and 15 mg/kg ($p$=0.0005), and in females, a significant increase was noted between vehicle and 5 mg/kg ($p$=0.0006). For the nucleus accumbens, a significant effect of drug dose ($F_{2,43}$=32.9, $p$<0.0001) was observed, with no sex ($F_{1,43}$=3.49, $p$=0.0686) or interaction ($F_{2,43}$=0.453, $p$=0.639) effect, where multiple comparisons revealed that 15 mg/kg EPH significant increased ΔFosB as compared with vehicle ($p$<0.00001) in both male and female mice. In the nucleus accumbens, a significant increase in ΔFosB in male and female mice exposed to 5 mg/kg compared with vehicle ($p$=0.0265, $p$=0.0004, respectively) was found.

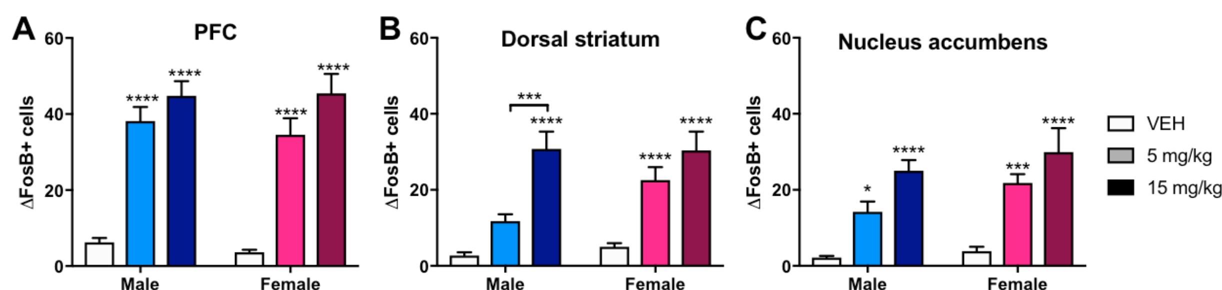


Figure 8-4. Increased ΔFosB expression in striatal and cortical areas following repeated 5 and 15 mg/kg EPH exposure.

Repeated EPH exposure (i.p.) significantly increased ΔFosB expression in the prefrontal cortex (A), dorsal striatum (B), and nucleus accumbens (C) in both male and female adolescent mice. Dose-dependent increases in ΔFosB expression is observed in the dorsal striatum and nucleus accumbens, but not in the prefrontal cortex. Significance by two-way ANOVA with Tukey (dose) or Sidek (sex) multiple comparisons, *, $p$<0.05; ***, $p$<0.0005; ****, $p$<0.0001; data represented as mean ± SEM.

## 8.4 Discussion

Here, we assessed the behavioral and neurochemical impact of repeated adolescent EPH exposure in both male and female C57Bl/6 mice. As adolescent reports of NPS use (and drug experimentation in general) are prevalent [101, 889], we evaluated drug responses in this specific age group. We observed that repeated exposure to 15 mg/kg EPH decreased spatial cognitive performance as assessed by the Barnes maze task. EPH increased locomotor activity at 15 mg/kg, but not 5 mg/kg, and did not induce locomotor sensitization upon repeated exposure. Reward to EPH (as measured by conditioned place preference) was observed at both the non-locomotor stimulatory dose of 5 mg/kg and the stimulatory dose of 15 mg/kg EPH. Repeated EPH exposure dose-dependently correlated with increased ΔFosB expression in the dorsal and ventral striatum,

while in the prefrontal cortex, both 5 and 15 mg/kg EPH significantly increased ΔFosB similarly with no difference in dose. Overall, our results suggest that EPH is indeed rewarding and stimulating as human reports would suggest [875, 878], although it causes spatial cognitive deficits at doses which cause hyperactivity. Importantly, no sex differences were observed between male and female animals throughout our testing. This was surprising as female rodents typically exhibit increased sensitivity to psychostimulants such as MPH [890], cocaine [891], modafinil [892], and amphetamine [142] in behaviors associated with reward and drug self-administration.

Human self-reports suggest that EPH is consumed for its perceived cognitive enhancing effects [875, 878], and this is unsurprising given similar reports of misuse of ADHD medications such as amphetamine and MPH [893]. The cognitive benefits of MPH have been observed across species, as rodents have shown increased performance on spatial tasks upon MPH exposure [883]. Thus, we may predict that similar psychostimulants, such as EPH, may equally increase spatial cognition. However, here we found that repeated exposure to 15 mg/kg EPH increased latency to the escape route in our Barnes maze task (Figure 8-1), suggestive of a decrease in spatial cognitive performance rather than an increase. It is possible that the tested 15 mg/kg did not provide optimal arousal for increasing spatial task performance, as this dose also induces hyperactivity (Figure 8-2). The necessity of optimal arousal by psychostimulants to enhance cognition is illustrated by differences in 10 mg/kg MPH to enhance performance in spatial tasks (such as the Morris water maze) but to decrease performance in fear conditioning tasks [883, 894]. Thus, testing the effects of repeated exposure to a non-stimulatory dose of EPH, such as 5 mg/kg, may indeed increase Barnes maze performance.

In adolescent mice, EPH was stimulatory (Figure 8-2) at a 15 mg/kg dose but not at 5 mg/kg. The tested doses of EPH chosen were based on those used for MPH in C57Bl/6 male mice [894, 895], where 10 mg/kg MPH is stimulatory and displays locomotor sensitization after seven days of exposure [894], while a 1 mg/kg dose does not induce hyperlocomotion or locomotor sensitivity. Interestingly, we observed no locomotor sensitization to either 5 or 15 mg/kg EPH despite the 12 days of exposure (Figure 8-2), although both doses were found to be rewarding in our conditioned place preference paradigm (Figure 8-3). In the study by Carmack et al. investigating the locomotor and rewarding properties of MPH, both a non-stimulatory 1 mg/kg and 10 mg/kg stimulatory MPH dose were rewarding as measured by conditioned place preference [894], similar to our results for both the non-stimulatory 5 mg/kg EPH and 15 mg/kg EPH (Figure

8-3,4). Overall, these results suggest that the locomotor and reward profile of EPH at 5 and 15 mg/kg is similar to that observed at 1 and 10 mg/kg MPH despite the differences in DAT versus NET preference observed by *in vitro* transporter binding [78, 881].

Repeated exposure to EPH increased ΔFosB expression in both male and female adolescent mice in cortical and striatal regions of the brain (Figure 8-4), and this increase in the striatum is similar to the increase in fosB (a non-truncated splice variant of ΔFosB [846]) immunoreactivity observed in adolescent male rats exposed to 2 or 10 mg/kg MPH for 14 days [896]. As increased ΔFosB expression is associated with increased sensitivity to the behavioral effects of certain drugs of abuse [846], both our results for EPH and previous results for MPH would suggest that these drugs may have an abusive profile and/or alter future drug seeking behaviors. Increases in ΔFosB expression observed in our study are presumed to be the result of increased dopamine levels in brain regions following drug exposure [846] as the result of DAT inhibition by EPH [896]. Interestingly, a study by Cummins et al. found that repeated exposure to 5 mg/kg MPH in adolescence decreases DAT expression in the nucleus accumbens and striatum compared with vehicle control [897, 898], suggesting that repeated drug exposure downregulates one of the molecular targets (DAT) of both MPH and EPH and importantly, decreased DAT expression would presumably increase extracellular dopamine levels by preventing dopamine reuptake. As this decrease in transporter expression upon MPH exposure in adolescent and adult rodents appears to be unique to DAT (as compared with NET or SERT) [898, 899], future studies may evaluate if EPH has a similar effect on DAT expression in the regions where increased ΔFosB expression was observed.

In summary, we characterized the novel psychoactive substance EPH in relation to its self-reported effects in humans [875, 878] on drug sensitization, reward, and cognition following repeated exposure in adolescent male and female C57Bl/6 mice. With findings of decreased cognitive performance, significant reward, and increased ΔFosB expression following prolonged, repeated EPH exposure, our animal models provide evidence that EPH is indeed stimulating and rewarding, and thus may have an abusive profile. As the current legal status of EPH in the United States is not explicitly clear, these determined behaviors in male and female adolescent mice suggest that EPH's effects on behavior are similar to its similar chemical analog, MPH, a Schedule II substance. However, EPH use may have stronger negative aspects in terms of learning, but this will require further comparative studies with MPH to properly address these concerns.

# CHAPTER 9. FUTURE DIRECTIONS

## 9.1 Understanding to what degree the rewarding properties of kratom alkaloids influence their ability to modulate alcohol use

In the evaluation of *Mitragyna speciosa* (kratom) alkaloids on alcohol-related behaviors in Chapter 2, I established that all studied alkaloids were capable of reducing voluntary 10% alcohol intake (Figure 2-3). I also characterized the acutely rewarding properties of kratom extract, mitragynine, and 7-hydroxymitragynine, and observed no significant acute reward compared (while mu-opioid agonist morphine sulfate was found rewarding in this acute CPP protocol) (Figure 2-6). However, a potential caveat of this conclusion is that the conditioned preference was measured in alcohol-naïve male C57Bl/6 mice and not in alcohol-exposed animals. As opioid receptors are known to be dynamically expressed in response to drug exposure (discussed further in Section 9.3) [900-902], it is possible that the tested kratom alkaloids would display different conditioned preference in alcohol-exposed mice compared with alcohol-naïve as a result of altered opioid receptor expression in response to alcohol exposure [903]. Activation of DOR by DOR agonist TAN-67 (which has a similar bias profile as the kratom alkaloids and is sometimes referred to as a DOR1-type agonist [267, 904]) does not induce reward in non-acute conditioned place preference models [267]. Similarly, intra-VTA injection of the DOR1 agonist DPDPE does not produce CPP in alcohol-naïve or alcohol-exposed rats [905]. Therefore, I do not expect alcohol-induced changes in DOR expression to contribute to potential differences in alkaloid reward between alcohol-exposed and alcohol-naïve mice. However, it has been shown that morphine reward is increased in alcohol-exposed animals [906, 907], suggesting alcohol-exposure may alter MOR expression and functionality. Furthermore, if increased kappa-opioid receptor expression occurs in alcohol-exposed mice, increased alkaloid aversion may be observed [248, 908-910], thus it is important to understand how alcohol exposure impacts KOR/MOR expression and the resulting rewarding properties of kratom alkaloids.

To address this, the acute conditioned place preference experiment outlined in Chapter 2 could be repeated in alcohol-exposed C57Bl/6 male mice following exposure to the 10% alcohol in a two-bottle choice, limited access protocol for a minimum four weeks. Under the hypothesis that expression of opioid peptides and receptors is altered in response to alcohol exposure and in alcohol-preferring rodent strains [903, 911], I would expect an increase in time spent on drug-

paired side for kratom alkaloids which exhibit high affinity for MOR (such as 7-hydroxymitragynine) if alcohol exposure indeed increases MOR expression in the mesocorticolimbic regions [248, 903].

A second question that remains is how kratom alkaloid use alters the rewarding properties of alcohol (rather than intake, as measured in Chapter 2), as human self-reports suggest users may consume kratom as a method of self-medication to prevent escalated alcohol intake [257, 258]. One strategy to address this question would be to test how kratom alkaloid administration influences the expression, extinction, and reinstatement of alcohol conditioned place preference [89]. This experiment would be performed using kratom alkaloid doses efficacious in decreasing alcohol intake outlined in Chapter 2. Interestingly, DOR G protein-biased ligands (our desired DOR therapeutic profile), such as TAN-67, can increase (albeit not significantly) expression of alcohol place preference but decrease volitional alcohol intake [277, 280], suggesting that increased reward to alcohol in fact decreases consumption. For alcohol-CPP extinction, inhibition of the opioid system by pan-opioid antagonist naloxone [912] prior to extinction sessions decreases the expression of alcohol-CPP in DBA/2 mice compared with vehicle saline [913]. This decrease is thought to occur because blockade of opioid receptor signaling by naloxone interferes with a conditioned motivated response requiring constitutive opioid system activity following alcohol exposure, and this opioid-influenced motivated response is partially responsible for the alcohol-seeking behavior observed during CPP expression [913]. Thus, activation of the opioid system by kratom alkaloids may in fact increase alcohol-CPP expression and prevent extinction, as activation of the opioid system by kratom alkaloids would maintain this conditioned motivated response to alcohol-seeking behavior [913].

## 9.2 Comprehending the potential of behavioral polypharmacology of kratom alkaloids

As shown by a number of previous studies [248, 255, 279], *Mitragyna speciosa* compounds may exhibit activity at a number of opioid, noradrenergic, or serotonin receptors (although data collected from a National Institute of Mental Health Psychoactive Drug Screening Program suggests that at least one alkaloid, 7-hydroxymitragynine, is rather opioid selective and does not bind to serotonin receptors/transporter, adrenergic receptors, dopamine receptors/transporter, muscarinic receptors, norepinephrine transporter, or sigma receptors with binding affinity of $K_i >$ 10 uM [255]). The locomotor effects of 7-hydroxymitragynine observed in wild-type mice may be

explained solely through its actions at the different opioid receptors. Given that this hyperactivity upon 7-hydroxymitragynine administration is still present in DOR knockout mice (Figure 2-4, 2-7), the increase in locomotion may stem from MOR activity and would agree with reports of increased locomotor responses upon morphine administration [281]. The polypharmacology of other kratom alkaloids, such as mitragynine, speciogynine, and paynantheine, has yet to be elucidated through any broad-panel CNS receptor screening techniques, although the strategy described in Kruegel et al. (2016) could provide a viable method to do so [255]. Additionally, results from this drug screening technique would help guide future behavioral pharmacology efforts. For example, using global knockout mice or subcutaneous administration of receptor antagonists for identified receptors of interest could be utilized to address how a specific behavior is the result of kratom alkaloid's actions on said receptor of interest. Once the polypharmacology of kratom alkaloids are elucidated, medicinal chemistry efforts and/or structural-activity relationships of these alkaloids with the opioid receptor of interest (such as DOR, for our interests) could lead efforts to develop opioid therapeutics with increased selectivity for a specific receptor subtype.

## 9.3 DOR receptor expression as a protective marker for alcohol use disorder

DOR expression and/or constitutive DOR $G_{i/o}$ protein signaling appears to be protective against alcohol intake, as supported by the increase in alcohol intake observed in the initial characterization of alcohol-related behaviors in DOR knockout mice [211]. Our previous study investigating the correlation between DOR agonists' β-arrestin recruitment and increased alcohol intake further supports this hypothesis [347], as β-arrestin recruitment to DOR by the high-internalizing, β-arrestin 2 superrecruiter agonist SNC80 increased alcohol intake and leads to rapid *in vivo* DOR internalization [176, 715]. As such, exposure to SNC80 prior to alcohol drinking sessions may cause a transient "DOR knockout," thus leading to increased alcohol intake. The importance of DOR expression in alcohol-related behaviors further supported by a study by Margolis et al. [263], where DOR expression in the VTA was protective against increased alcohol intake in rats, as observed by a decrease in alcohol intake in both high and low drinking rats upon intra-VTA microinjection with the DOR agonist DPDPE. Conversely, intra-VTA microinjection of TIPP-ψ, a DOR antagonist, increased alcohol consumption in chronically drinking rats.

Mechanistically, DPDPE inhibition of both evoked and spontaneous $GABA_A$ signaling in the VTA of rats exposed to alcohol was negatively correlated with alcohol consumption [263].

However, the observation of increased DOR receptor expression in alcohol-preferring C57Bl/6 mice in limbic regions of the brain compared with alcohol-aversive DBA/2 mice [911] appears to be at odds with the original hypothesis that DOR expression is protective (as hypothesized from the original DOR KO studies where DOR KO mouse consume more alcohol [211]). In contrast to the study by Margolis et al. [263], a study investigating the role of DORs in the central nucleus of the amygdala (CeA), where DOR functionality was measured by DPDPE inhibition of glutamatergic synaptic currents in CeA neurons, only observed an emergence of functional DORs in rats exposed to alcohol conditioned place preference (CPP), but not in those exposed to saline [914]. This study would suggest that alcohol exposure increases DOR functionality, and thus DOR expression/function is not protective against alcohol-related behaviors. However, as increased opioid activity in the CeA may increase GABA-mediated inhibition of downstream CeA target regions [915], this increased DOR activity may indeed decrease anxiety-like behavior and thus, be reinforcing [916]. Also importantly, these animals were not exposed to alcohol voluntarily but rather by the experimenter (as is necessary in CPP), thus the interpretation to how this increase in DOR function and/or expression following alcohol exposure may correlated with alcohol intake as observed by Margolis et al. in the VTA is limited [263]. Furthermore, a study in human subjects observed a positive correlation between caudate DOR expression (as measured by positron emission (PET) scan of binding of [$^{11}$C]methylnaltrindole (MeNTL), a DOR specific antagonist [917]) and recent alcohol intake (average drinks per drinking day) in alcohol-dependent subjects [918]. This increase in DOR expression observed in humans following recent alcohol intake is in agreement with the observed increase in DOR expression in the CeA following alcohol exposure [914]. Interestingly, no significant differences in DOR expression were noted between healthy control subjects and alcohol-dependent subjects [918], although alcohol-dependent controls were at least 5 days abstinent at the time of imaging and DOR expression rapidly fluctuates in response to drug exposure [900, 901] or drug removal [902].

As the exact role of DOR expression in alcohol-related behaviors is not clear, it would be valuable to address the following questions: 1) Can DOR expression serve as a predictive marker for alcohol intake? 2) Does DOR expression correlate with duration or severity of alcohol use? 3)

Does DOR expression decrease during abstinence/alcohol withdrawal? and 4) Does DOR expression predict the treatment efficacy when using an opioid drug with DOR affinity? However, current technological limitations exist for DOR visualization *in vitro* or in animal models *in* and *ex vivo*. As reviewed in detail by Gendron et al. [919], these issues include poor antibody specificity, altered basal receptor expression/trafficking, and fluorescent protein-tagged receptor oligomerization, thus limiting the interpretation of results using fluorescently tagged DORs. Therefore, I propose using a PET imaging approach to assess *in vivo* DOR dynamics in response to alcohol-related behaviors in mice using [$^{11}$C]methylnaltrindole (MeNTL), as this PET ligand displays specificity for DOR in both rodents and humans [920]. Although antagonist-based PET ligands are preferred as they bind receptors in both high and low affinity states (as compared with agonist-based PET ligands [921]), a G protein-biased DOR agonist PET ligand (perhaps using the TAN-67 scaffold) could be developed to image high affinity DOR receptor differences in the aforementioned proposed experiments and prevent receptor internalization (as compared with developing a DOR agonist PET ligand based upon SNC80, which heavily recruits β-arrestin and induces rapid receptor internalization [176, 715]).

## 9.4 Investigating neuronal- and regional-specific roles of $G_{i/o}$ signaling in the dorsal striatum

In Chapter 4, I expressed the inhibitory $hM_4Di$ DREADD in the dorsal striatum under a synapsin promotor to monitor how neuronal inhibition of the dorsal striatum by increased $G_{i/o}$-coupled receptor activity influences alcohol intake, where a decrease in alcohol intake was observed. While the synapsin promotor is highly specific to neurons [922], this promoter does not allow us to differentiate between DREADD $G_{i/o}$-coupled receptor activity on striatal D1-MSNs versus D2-MSNs. As the activity of dorsomedial striatal D2-MSNs is thought to be part of the "no-go" pathway of rewarding behavior and excessive alcohol intake suppresses D2-MSN activity [44, 307], an increase in $G_{i/o}$-protein activity solely in D2-MSNs may be enough to decrease voluntary alcohol intake by reinstating this "brake." This could be accomplished by viral expression of $hM_4Di$ specifically on D2-MSNs (under a *Drd2* promotor or through a Cre-recombinase *Drd2*-Cre strategy) and repeating the DREADD experiment outlined in Chapter 4. As DOR receptors are thought to be solely expressed on D2-MSNs in the striatum [206], our dorsomedial microinfusion experiments performed previously to activate endogenously expressed DOR are still valid and in agreement with this hypothesis. Importantly, in addition to controlling for non-DREADD CNO

effects on alcohol intake behavior, locomotor changes in response to specific D2-MSN inhibitory DREADD activation would be imperative because of the role of D2-MSN activity in the indirect pathway with respect to movement (and increased activity may be observed following inhibition of the indirect pathway).

While the dorsal striatum as a region is in general implicated in procedural learning [923-925], the dorsolateral striatum sub-region is heavily associated with habitual behavior (behavioral actions that persist despite reward devaluation) [308] and the dorsomedial striatum with goal-directed learning [42]. In our study described in Chapter 4, cannula terminals for microinfusion and viral expression were largely localized to the dorsomedial striatum. Chronic alcohol exposure may preferentially activate the dorsolateral striatum versus the dorsomedial striatum, as observed in increased glutamatergic transmission [312] and decreased GABAergic transmission [312, 313] in animals exposed to chronic intermittent alcohol. As such, an increase in dorsolateral activation upon chronic alcohol intake was likely not completely inhibited in our study by $G_{i/o}$-coupled DOR or DREADD activation. Instead, our study likely largely inhibited the dorsomedial striatum, leading to a decrease in goal-directed behavior (alcohol intake upon bottle presentation [46]). Notably, we were not able to assess changes in alcohol devaluation using our two-bottle choice, drinking-in-the-dark protocol; thus, we cannot interpret our observed decrease in alcohol intake as a change in “habitual” alcohol intake, which is desensitized to reward devaluation [46], and therefore cannot differentiate between DMS or DLS effects on behavior.

Two different follow-up experiments investigating DMS versus DLS can be proposed: repeat the experiments in Chapter 4 but with terminals placed in either DMS (Group 1-DMS) or DLS (Group 2-DLS), and in animals exposed to repeated alcohol for an extended period of time, perform 1) Experiment 1: measure changes in alcohol intake in a two-bottle choice with increasing concentrations of quinine to devaluate alcohol [926], or 2) Experiment 2: measure changes in alcohol self-administration using an operant training technique [46]. Increased $G_{i/o}$-coupled receptor activity in Group 2-DLS should attenuate alcohol-seeking in both Experiment 1 and 2, while increased $G_{i/o}$-coupled receptor activity in Group 1-DMS may have no affect (if alcohol intake is indeed habitual in both experiments, as the DLS would still be overactive in this group). Additionally, the aforementioned experiments investigating inhibition of the specific influence of the direct (D1-MSNs) or indirect (D2-MSNs) pathways by $G_{i/o}$-coupled receptors on alcohol intake could be repeated within the dorsomedial and the dorsolateral striatum regions as well.

## 9.5 Limitations on “the perfect biased ligand” for Class A GPCRs

Does the perfect biased ligand exist? It may depend on the pathway of interest. A recent study by Grundmann et al. found upon Class A GPCR activation in HEK293 cells devoid of G protein expression (through CRISPR/Cas9 deletion of $G_{s/q/12}$ and pertussis toxin-inactivation of $G_{i/o}$), β-arrestin was still recruited, but activation of previously attributed β-arrestin-dependent, G protein-independent signaling pathways, such as phosphorylation for ERK1/2 MAP kinases, was not detected [927]. As the first study to completely eliminate G protein activity (as compared with previous studies using siRNA knockdown of G protein expression [928-930]), this study suggests that cellular responses previously defined as G protein-independent, β-arrestin dependent signaling (including - but not limited to - activation of tyrosine kinases, MAP kinases, and E3 ubiquitin ligases [170]) may need to be reassessed in this new “zero functional G” protein cell line. One competing hypothesis to β-arrestin-dependent, G protein-independent signaling may be that cellular responses associated with β-arrestin-dependent signaling are indeed functions of an internalized GPCR-G protein-β-arrestin supercomplex [174], where G protein signaling continues despite GPCR internalization. As the supercomplex formation and internalization would be delayed compared to "rapid onset” GPCR G protein-dependent signaling at the membrane, this may explain the slow onset, sustained duration of signaling previously associated with β-arrestin-dependent signaling events [170].

While our studies in Chapters 2 and 4 outlined the desire to develop G protein-biased DOR drugs devoid of β-arrestin recruitment, the precise mechanism by which β-arrestin recruitment to DOR leads to increased alcohol intake is unknown. One simple hypothesis, as mentioned in Section 9.3, is that β-arrestin recruitment to DOR by agonists such as SNC80 rapidly internalizes the receptor from the membrane [176, 715], thus leading to an increase in alcohol intake similar to that observed in DOR KO mice [211, 347]. Another more mechanistic hypothesis is that the strong β-arrestin recruitment of SNC80 leads to not only receptor internalization, but also increased β-arrestin-dependent signaling events (where the outcomes of these signaling events are responsible for increased alcohol intake). Yet, the study by Grundmann et al. (and an additional study by O’Hayre et al.) proposes that signaling events previously deemed β-arrestin-dependent require G protein signaling [927, 931], thus suggesting that SNC80 must have a different $G_{i/o}$-protein signaling profile than TAN-67 (a DOR agonist with weak β-arrestin recruitment and decreases alcohol intake). However, TAN-67 and SNC80 exhibit similar potency and efficacy for

$G_{i/o}$-protein signaling (as measured by $G_{i/o}$-protein inhibition of forskolin-stimulated cAMP, Figure 4-3), thus the differences in $G_{i/o}$-protein signaling alone cannot explain the differences in alcohol intake. Instead, it is possible that the formation and signaling of GPCR-G protein-β-arrestin supercomplexes may explain the differences in the behavioral responses to SNC80 and TAN-67, as SNC80 would exhibit increased supercomplex formation and/or signaling because of its ability to robustly induce β-arrestin 2 recruitment to DOR [347, 715] compared with TAN-67 *in vitro*.

## 9.6 Role of β-arrestin isoforms in DOR agonist-induced seizure behavior

One major limitation of DOR therapeutics is the potential for convulsions/seizure at high concentrations [716, 932, 933]. This undesirable effect of DOR agonist administration has been commonly observed with a subset of DOR agonists, including BW373U86, SNC80, and SNC162 [347, 932, 934]. Interestingly, *in vitro* characterization of these specific DOR ligands indicates that these agonists are strong recruiters of β-arrestin 2 [347, 932, 934] and DOR agonists which do not heavily recruit β-arrestin 2, such as KNT-127 [347], do not produce convulsions in rodents [935]. As GPCRs such as DOR can recruit β-arrestin 2 in addition to β-arrestin 1 (a β-arrestin less well studied) [714], I sought to identify if these isoforms differentially contributed to DOR seizure behavior. The DOR agonist SNC80 was chosen to induce seizure behavior as it readily produces seizures in rodents and can recruit β-arrestin 1 as well as β-arrestin 2 to DOR *in vitro* and *in vivo* [347, 714]. I hypothesized that β-arrestin 1 expression may be protective during SNC80-induced seizures because of β-arrestin 1's reported ability to induce Bcl-2 activity (and Bcl-2 activity is anti-apoptotic, therefore increasing Bcl-2 activity may attenuate seizure-induced cell death) [759, 773, 936-938].

Using β-arrestin 1 KO (βarr1 KO) and β-arrestin 2 KO (βarr2 KO) mice, I observed higher Modified Racine Scale scores [939] for seizure behavior in β-arrestin 1 KO mice compared with wild-type (WT) or β-arrestin 2 KO mice following 20 mg/kg SNC80 intraperitoneal administration (Figure 9-1). This increase in Modified Racine Scale score in β-arrestin 1 KO mice suggests that lack of β-arrestin 1 expression reduces seizure threshold to SNC80-induced seizures. As observed in previous studies, wild-type mice exhibited convulsive behavior at 20 mg/kg [347, 932, 934], and a lower Modified Racine Scale score was observed in β-arrestin 2 knockout mice, potentially suggesting that global knockout of β-arrestin 2 may further decrease SNC80-induced seizure potential. Our observed results are in agreement with a recent study by Dripps et al., where a

similar increase in seizure score was observed upon SNC80 administration in β-arrestin 1 KO mice [940].

Upon global β-arrestin single isoform knockout, it is expected that the remaining β-arrestin isoform will compensate for the function of the knocked-out isoform based on high sequence homology and the embryonic lethal nature of double β-arrestin 1+2 knockout [692, 755]. However, it is also conceivable that upon a single β-arrestin isoform knockout, the expression of the other isoform is increased to help compensate for the loss. Further studies are necessary to quantify the expression of β-arrestin 1 and 2 in wild-type and knockout mice, as altered seizure threshold in β-arrestin 1 KO mice may be explained in two ways: 1) the result of β-arrestin 1 compensatory expression being protective against SNC80-induced seizures in wild-type and β-arrestin 2 KO mice (as observed by the small - albeit not significant - decrease in highest Modified Racine scale score between wild-type and β-arrestin 2 KO mice), or 2) a compensatory increase in β-arrestin 2 in β-arrestin 1 KO mice, which may be harmful and lower seizure threshold. Genetic strategies, such as overexpression of β-arrestin 1 by viral vector or knockin, would address if β-arrestin 1 is indeed protective to SNC80-induced seizure activity.

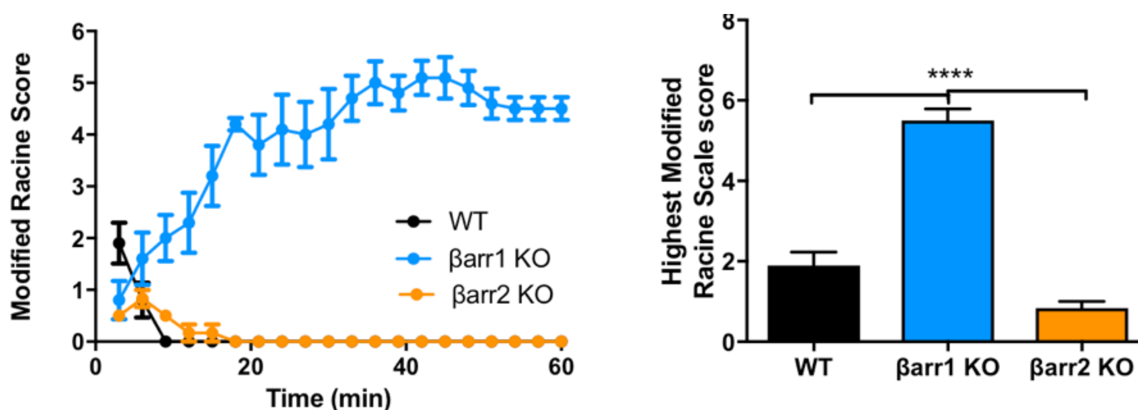


Figure 9-1. Decrease in seizure threshold in β-arrestin 1 knockout mice.

β-arrestin 1 male C57Bl/6 knockout mice (n=6) exhibited increase seizure behavior (higher Modified Racine Score) for a longer duration of time compared with β-arrestin 2 male knockout C57Bl/6 mice (n=3) or wild-type male C57Bl/6 mice (n=4) (A,B).

## 9.7 Using conditional genetic manipulations to better understand mechanistic details

The majority of the genetic techniques described in this dissertation utilize global knockout of the protein of interest, such as DOR or β-arrestin 1/2 (see Chapters 4, 5, Section 9.6). While this strategy may be successful in broadly generalizing the effect of a single receptor/protein knockout, alterations in genetic robustness may occur when the gene of interest is knocked out but 1) overlapping expression domains exist with a gene which has similar functions or 2) knockout of the gene of interest results in upregulation of other similar functional genes [941]. This is particularly concerning in β-arrestin 1 or 2 global knockout animals, as both arrestin isoforms are exhibit recruitment to GPCRs such as DOR [714], are highly expressed in the nervous system [743], exhibit functional compensation (as observed by double knockout as embryonic lethal [755]), and share 78% sequence homology [692]. The major difference between β-arrestin 1 and 2 appears to be their cellular localization, as β-arrestin 2 is located in the cytosol as the result of a nuclear export sequence located on the C-terminus compared to the cytosolic and nuclear localization of β-arrestin 1 [942]. This difference in localization may explain the differences in downstream β-arrestin-dependent signaling between the two isoforms [749, 772, 773, 775].

To better understand the contribution of β-arrestin 1 and 2 to drug-related behavioral responses, conditioned, cell-type specific (with or without induction by agents such as tetracycline) knockout strategies [943] could be utilized to repeat our studies performed in Chapters 4 and 5. For our studies characterizing how β-arrestin 1 knockout influences anxiety- and reward-related behaviors in Chapter 5, an inducible (tetracycline cre/loxP system) knockout strategy to selectively knockout β-arrestin 1 (*Arrb1*) expression in adulthood (a few days prior to behavioral testing) would address issues of developmental compensatory expression of complementary β-arrestin 2 expression [944]. As for Chapter 4, to further validate that the differences in drinking observed with SNC80 upon dorsal striatal microinfusion between wild-type and β-arrestin 2 knockout mice were the result of DOR β-arrestin 2 recruitment, a strain created by crossing *Oprd1*-Cre with floxed *Arrb2*$^{flx/flx}$ (β-arrestin 2) mice [945] could be generated for specific knockdown of β-arrestin 2 expression in DOR expressing neurons (note: the *Oprd1*-Cre strain does not exist and is hypothetical). Results in Chapter 4 should be similar in this hypothetical strain of mice if the SNC80 alterations in drinking are indeed the result of the loss of β-arrestin 2 recruitment specifically to DOR. This may additionally address if compensatory arrestin isoform expression

explains why both β-arrestin 1 knockout and 2 knockout animals have been reported to drink more than wild-type (Chapter 5, [347, 732]). Importantly, without an inducible knockout system, this aforementioned strategy to test DOR neuron expressing-specific, β-arrestin 2 activity would still be limited by genetic knockout present in embryonic development [943] and could be developed into an inducible knockout strategy.

## 9.8 Adolescent and adult social influences on behavioral pharmacology

Despite humans reports of energy drink consumption correlating with negative alcohol outcomes, I observed no correlations between adolescent caffeine intake and adult alcohol intake in Chapter 6 [800-805]. Importantly, I was limited to monitoring changes in adult alcohol intake in singly housed animals, thus removing any potential influence of social behavior on drinking [818]. A study by Logue et al. observed that postnatal 28-30 day old, adolescent male and female C57Bl/6 mice (the strain used for the majority of our study in Chapter 6) consumed more 5% v/v alcohol and spent more time drinking when alcohol behavior was measured in social triads compared with isolated, individual age-matched controls [946]. This peer effect appeared to be greatest in adolescent male mice [946], and no peer effect was observed in adulthood (P84-86) [946]. In a separate study measuring the peer effect on sweetened alcohol intake across multiple mice strains with varying sociability, the opposite effect was observed with increased sweetened alcohol intake in isolated P34-54 aged mice compared to group-housed, same-sex pairs regardless of genetic strain [947]. This is further supported with a report that social and environmental enrichment decreases alcohol preference in age P40 C57Bl/6 mice, although this effect was also observed for sucrose preference, suggesting that enrichment decreases general reward intake behaviors [948].

As our animals in Chapter 6 had entered adulthood by the start of the alcohol intake experiments (Figure 6-1), these abovementioned studies would suggest that social housing would either have no effect [946] or potentially decrease [947, 948] alcohol intake based upon the factors of age and social environment. Yet, as our animals had been exposed to caffeine throughout adolescence (and thus not drug-naïve like those animals used in these social factor studies [946-948]), differences in alcohol intake and preference may be observed as a factor of social environment + previous caffeine exposure.

## 9.9 Age- and sex-dependent outcomes of caffeine and caffeine-mixed alcohol use

In addition to the social aspects of drug behavior mentioned above, the influence of age and sex on drug-related behaviors were only moderately addressed in Chapters 5 through 8. In Chapters 6 and 7, the effect of caffeine or caffeine-mixed alcohol administration on future drug-related behaviors (adult alcohol intake or locomotion, cocaine conditioned place preference, and natural reward, respectively) were tested only in adolescent animals with no adult aged group to compare with for age-specific effects. For caffeine exposure alone, caffeine exposure in adolescence results in higher locomotor activity [949] and increased cocaine reward sensitivity compared with adults in rats [790], suggesting that some of the sensitivity to caffeine-mixed alcohol observed in Chapter 7 may be unique to adolescence. Future studies repeating these behavioral assays and neurochemical experiments outlined in Chapters 7 in C57Bl/6 adults would help elucidate if the observed alterations in locomotor activity, cocaine conditioned place preference, ΔFosB, and saccharin reward upon caffeine-mixed alcohol exposure are distinct to adolescence. As no association between adolescent caffeine use and adult alcohol intake was observed in Chapter 6, these studies in Chapter 6 would be less crucial to repeat in adults alone.

As for the investigation of sex as a biological variable that may influence caffeine or caffeine-mixed alcohol behavior, sex differences have been observed in response to psychostimulants such as cocaine and D-amphetamine with female mice exhibiting enhanced psychostimulant-induced behavior [142, 143]. This increased response to psychostimulants in females begs the question that female mice may respond differently to caffeine or caffeine-mixed alcohol than male mice. In Chapter 7, I indeed observed that female adolescent animals displayed increased locomotor sensitivity to caffeine-mixed alcohol exposure (intraperitoneal) compared with adolescent male C57Bl/6 mice. This increased locomotion in female adolescent mice upon repeated caffeine-mixed alcohol exposure was not compared to female mice exposed to caffeine or alcohol alone, thus I am unable to conclude that this locomotor sensitivity is unique to caffeine-mixed alcohol (as observed in males) compared with caffeine or alcohol alone in females. Thus, the experiments outlined in Chapter 7 could be repeated in female adolescent C57Bl/6 to best address this limitation. Importantly, in humans, male adolescents report increased energy levels, increased positive subjective effects, and improved athletic performance following caffeine intake compared with female adolescents [950, 951], suggesting that the male sex may be more sensitive to the rewarding effects of caffeine.

### 9.10 Ethylphenidate: cognitive alterations and treatment for attention deficit hyperactivity disorder?

In Chapter 8, a drug effect on Barnes maze performance was reported where repeated exposure to 15 mg/kg ethylphenidate in adolescent male and female C57Bl/6 mice increased latency to finish the Barnes maze task. As the Barnes maze testing was conducted following repeated drug administration (and not concurrent with EPH administration), the observed decrease in spatial performance in Chapter 8 appears to be the result of long-term changes in spatial cognition following EPH exposure (as animals were drug-free during Barnes maze testing). In future studies, it would be interesting to test how EPH exposure alters Barnes maze performance when EPH is given directly prior to the Barnes maze training trials. As 15 mg/kg EPH induces hyperlocomotion, I would expect a decrease in performance (measured as an increase in latency to enter the escape route) as the stimulating dose of 15 mg/kg would likely be above the optimal arousal threshold for spatial learning [883, 894]. However, at 5 mg/kg EPH (which does not cause hyperlocomotion), increases in spatial learning may be observed, similar to those observed for optimal doses of methylphenidate in spatial learning tasks such as the Morris water maze [883, 894].

In our study investigating the behavioral effects of repeated ethylphenidate in adolescence in male and female C57Bl/6 mice, I utilized wild-type mice as I was most interested in how repeated ethylphenidate (EPH) exposure alters behavior in the "general" population for reward-related behaviors and cognitive enhancement. A number of EPH users report ingesting EPH for the positive cognitive benefits [875, 878], and importantly, the chemical cousin of EPH, methylphenidate (MPH), is used as treatment in individuals with attention deficit hyperactivity disorder (ADHD) [952]. The use patterns of EPH, the therapeutic use of MPH, and their chemical and pharmacologic similarity between EPH and MPH suggest that EPH may also offer positive cognitive enhancement in ADHD individuals by increasing alertness. Therefore, the potential cognitive enhancing properties of EPH could be measured in animal models of ADHD to assess if anecdotal claims of increased cognitive performed following EPH ingestion are valid [875, 878]. However, animal models for ADHD lack significant treatment validity, in addition to lacking face, construct, and predictive validity (which is somewhat expected as the exact pathology of ADHD is unknown and may not be homogeneous across individuals) [953]. Common models of ADHD include spontaneously hypertensive rats (SHR) [954], dopamine depleted animals (using neurotoxin 6-hydroxydopmaine, 6-OHDA) [955], or dopamine transporter knockout mice (DAT

KO) [956], where MPH's efficacy varies depending on the task and the model [953, 957, 958] and in some cases, can exacerbate symptoms associated with ADHD (such as increased impulsivity [958]). Additionally, MPH's positive effects on concentration do not appear to be specific to individuals with ADHD, as observed by enhanced performance in the CPT model in children [959] and adults without ADHD following MPH administration [960, 961]. Despite this, assessing if the observed alterations in locomotor, reward, and cognitive properties of EPH in Chapter 8 still exist in a model of ADHD (such as the recently reported knockin dopamine $D_4$ polymorphic variant in C57Bl/6 background mice [962]) can be performed. Blunted extracellular dopamine release and locomotor activity were observed in response to methamphetamine in this transgenic strain, although no changes in cocaine ambulation between this ADHD model strain and wild-type were found [962], suggesting that EPH responses may not be altered because of the similarity in mechanism of action between EPH and cocaine on dopamine reuptake inhibition [881].

## 9.11 General conclusions

Overall, the research performed in this dissertation provides several significant findings to help guide future drug development strategies for delta-opioid therapeutics for alcohol use disorder and to evaluate the risks associated with adolescent psychostimulant exposure. In addition to the more rigorous examination of the role of signaling bias at the delta-opioid receptor for alcohol-related behaviors, the investigation into the role of β-arrestin 1 versus 2 isoform expression in DOR-agonist seizure behavior greatly assists our understanding of a previous limitation of delta-opioid drug development. Other studies, including my studies on kratom alkaloids, adolescent ethylphenidate use, and energy drinks with or without alcohol, may also help drive health policy and inform the public to make more informed choices about their use of (currently) legal substance of abuse.

Of course, with every investigation, we reveal new directions to explore, particularly as new techniques arrive that allow us to perform behavioral pharmacology in more detailed/controlled manner (as outlined in throughout Chapter 9). For alcohol abuse, efforts to develop novel DOR therapeutics require further investigation into how these drugs may alter alcohol behaviors in self-administration and/or alcohol devaluation paradigms to best mimic human behaviors for those diagnosed with alcohol use disorder. Furthermore, for alcohol and legal

psychostimulants, age, sex, and social aspects of use must be considered to increase the validity of these animal models in order to properly model the complexity of drug use.

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# VITA

Meridith Tracy Robins was born September 28th, 1990 to John and Karen Robins in Flint, Michigan. Upon graduation from Carmel High school in 2009, she attended the University of Wisconsin-Madison to study Genetics before transferring to Purdue University in 2011. At Purdue, she graduated with a degree in Chemistry (as approved by the American Chemical Society) with a Biochemistry emphasis and a Biology minor in 2013. During her time as an undergraduate, her largest research project involved the discovery of macrophage-targeted radioimaging agents to predict early response to therapy in autoimmune and inflammatory diseases (under the guidance of Dr. Phillip Low). Upon graduation, Meridith returned to Purdue University to pursue her PhD in the Department of Medicinal Chemistry and Molecular Pharmacology, where she joined Dr. Richard van Rijn's lab to study the behavioral pharmacology of alcohol and psychostimulants. Here, her research was awarded supported through the Charles C. Chappelle Fellowship, the Medicinal Chemistry and Molecular Pharmacology Endowment, and the Bilsland Dissertation Fellowship. In 2017, she was awarded the Enoch Gordis Research Recognition Award from the Research Society on Alcoholism for her research efforts, and the Kienly Excellence in Teaching Award and Teaching Academy Graduate Teaching Award from Purdue University for her teaching efforts. After finishing her PhD, Meridith will join the lab of Dr. Andrey Ryabinin at Oregon Health and Sciences University in Portland, Oregon as a postdoctoral researcher studying the neurocircuitry and social aspects of alcohol abuse and drug addiction.

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**Robins MT**, Atwood BK, van Rijn RM. Gi'n tonic – Modulation of alcohol intake by inhibitory $G_{i/o}$ protein-coupled receptors in the dorsal striatum (*in final preparation*).

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