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Role of Dynorphin/Kappa Opioid Receptor Activity Within the Extended Amygdala in Binge Alcohol Drinking.

By

Harold Lewis Haun

Department of Neuroscience

A dissertation submitted to the faculty of the Medical University of South Carolina in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biomedical Science in the College of Graduate Studies

June 25, 2020

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Chairman, Howard C. Becker

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Thomas C. Jhou

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Thomas L. Kash

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Patrick J. Mulholland

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John J. Woodward

ABSTRACT

HAROLD LEWIS HAUN. Role of Dynorphin/Kappa Opioid Receptor Activity Within the Extended Amygdala in Binge Alcohol Drinking (Under the direction of HOWARD C. BECKER).

Alcohol Use Disorder (AUD) is a significant national and global public health problem. Of concern, binge drinking is the most common pattern of excessive alcohol consumption and serves as a risk factor for the development of AUD. Recent studies have implicated the dynorphin/kappa opioid receptor (DYN/KOR) neuropeptide system in this pattern of drinking but the precise circuitry mediating these effects are poorly understood. The central amygdala (CeA) and bed nucleus of the stria terminalis (BNST) are two interconnected structures within the extended amygdala macrostructure that are rich in DYN/KOR and thought to contribute to binge drinking behavior. In the present studies, we demonstrate that KOR in the BNST contribute to excessive drinking by showing that site-specific delivery of a KOR antagonist decreased, while an agonist increased, binge-like alcohol consumption. Furthermore, we show that high levels of drinking induced by systemic administration of a KOR agonist were reversed by selective KOR blockade within the BNST. These findings suggest that KOR in the BNST promote binge drinking behavior, however, the endogenous dynorphinergic circuitry underlying this effect remains unknown. The CeA is a likely candidate in that it is involved in excessive drinking and sends dense dynorphinergic projections to the BNST (CeA-BNST^{DYN}). In support of this hypothesis, we demonstrate that neuronal activity is increased within the CeA during a binge drinking session and that chemogenetic inhibition of the CeA-BNST^{DYN} pathway selectively decreased

binge-like alcohol consumption. Collectively, these studies suggest that the CeA-BNST^{DYN} circuit contributes to binge-like alcohol consumption and KORs in the BNST likely mediate this effect. These studies provide valuable insight into neuronal circuitry underlying a key aspect of AUD and point to the DYN/KOR system as a potential therapeutic target for the treatment of excessive drinking.

DEDICATION

This dissertation is dedicated in loving memory of my mother, who passed away in late 2018. She instilled in me an unwavering positive outlook on life and this mentality has kept a smile on my face throughout the various ups and down of my graduate studies.

To my father, who equally helped shape my work ethic and never allowed me to cut corners. You always said, “A job worth doing is worth doing right.” You are certainly correct and I hope this dissertation embodies these words.

To my wife and son, I am forever thankful for your unyielding patience, love, and support. This has been a long journey and I am happy to have shared it with you.

Finally, this dissertation would not have been possible without the unwavering support of Dr. Howard C. Becker. The skills you have taught me over the course of my graduate studies will be critical for my advancement in the field and I could not have chosen a better mentor to help lay this foundation. I cannot thank you enough for the opportunities you have provided and hope that I have sufficiently expressed my gratitude along the way. It is truly amazing what you have done for me and I am forever grateful.

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CHAPTER 1: Background and Significance

INTRODUCTION

Alcohol Use Disorder

The earliest reported consumption of alcohol by humans dates to 7000 BC and alcohol use continues to be a stable fixture in most societies across the globe (McGovern et al., 2004; WHO, 2018). In fact, Roughly 55% of the global adult population has consumed alcohol and 20% of adults have engaged in heavy episodic drinking placing alcohol as the most widely used drug of abuse next to tobacco (GBD, 2018; Peacock et al., 2018; SAMHSA, 2018). In the United States, however, the vast majority (86%) of the population has reported drinking alcohol within their lifetime and 26% of adults have reported heavy drinking (SAMHSA, 2018). Alcohol drinking to excess presents a multitude of adverse societal and health effects creating a significant socioeconomic burden (WHO, 2018). Further, excessive drinking is associated with 88,000 deaths per year in the U.S. and 3.3 million deaths worldwide, propelling alcohol abuse into the spotlight as a serious epidemiological concern (Rehm and Imtiaz, 2016; Rehm et al., 2003; WHO, 2018). While alcohol abuse is a broad term, the *Diagnostic and Statistical Manual of Mental Disorders, 5th Edition* (DSM-V) systematically defines a diagnosis of Alcohol Use Disorder (AUD) based on 11 criteria that encompasses both alcohol dependence and abuse (Dawson et al., 2005; Grant et al., 2015; Hasin, 2012). AUD is characterized as behavior consisting of compulsive, uncontrolled bouts of heavy episodic drinking accompanied by a negative emotional state and craving during periods of abstinence, which contributes to chronic relapse (Grant et al.,

2015; Hasin, 2012). A diagnosis of AUD is not uncommon given a 30% life-time prevalence in the U.S. (Grant et al., 2015). Given the adverse health effects of alcohol abuse and high occurrence of AUD, it is not surprising that alcohol drinking is a significant contributor to the global burden of disease. In fact, alcohol-related disease is the third leading cause of preventable death behind tobacco use and poor diet and exercise, and alcohol-related mortality has doubled in the last 20 years (Mokdad et al., 2004; SAMHSA, 2018; White et al., 2020). Alcohol abuse taxes the US economy a staggering $250 billion annually generated largely by a loss of workplace productivity, healthcare expenses associated with treating alcohol-related injury and disease, and criminal justice expenses (Sacks et al., 2015; SAMHSA, 2016; WHO, 2018). Therefore, better understanding of the mechanisms that contribute to and promote heavy alcohol consumption is of the utmost importance to develop treatment strategies aimed at attenuating harmful alcohol drinking.

The exact cause of uncontrolled drinking and relapse in patients diagnosed with an AUD arises from the diverse and complex interaction between genetic, epigenetic, and environmental factors that influence the neurobiological basis of behavior (Koob and Volkow, 2010, 2016). Presently, viable treatment strategies for patients suffering from an AUD include behavioral interventions and pharmacotherapies, which are modestly effective given the 60% relapse rates in treatment-seeking individuals within the first year (Dawson et al., 2005; Grant et al., 2015; SAMHSA, 2016). Brief interventions, cognitive behavioral therapy, and motivational-enhancement therapy are the most common forms of psychosocial

treatments for AUD (Miller and Wilbourne, 2002). These include behavioral and cognitive interventions aimed at addressing the maladaptive behavioral patterns that promote compulsive alcohol consumption and contribute to relapse (Coates et al., 2018; McHugh et al., 2010; Miller and Wilbourne, 2002). Because chronic alcohol consumption promotes maladaptive changes within the brain that drive uncontrolled drinking and amplify negative affect during withdrawal, it is not surprising that treatment outcomes are greatly improved when psychosocial interventions are combined with pharmacotherapy (Anton et al., 2006; Koob, 2013; Koob and Volkow, 2010). Presently, pharmacological treatment is limited to three clinically approved drugs by the U.S. Food and Drug Administration (Heilig and Egli, 2006; Swift and Aston, 2015). These include naltrexone, disulfiram, and acamprosate yet the efficacy of these drugs alone is modest and clinical utilization low (Akbar et al., 2018; Heilig and Egli, 2006). This is, in part, due to each compound having specific clinical utility based on distinct neurochemical and peripheral systems involved in alcohol's effects. For example, naltrexone is an opioid antagonist that attenuates the reinforcing properties of alcohol thereby reducing the drive to drink (Anton et al., 2004). On the other hand, disulfiram decreases alcohol consumption by amplifying the negative reinforcing properties of alcohol by blocking the metabolism of acetaldehyde, a metabolite of alcohol, that is toxic and causes nausea, tachycardia, and flushing (Banys, 1988; Brewer, 1984; Lester et al., 1952). Acamprosate, albeit a biologically inactive molecule, has anti-relapse properties through the actions of calcium salt acting as a calcium therapy (Heilig, 2014; Spanagel et al., 2014). While medication-assisted therapy is

an effective treatment strategy, less than 15% of individuals meeting the criteria for AUD actually receive formal treatment due to the high cost of healthcare services and fear of social exclusion (SAMHSA, 2018). Social exclusion and stigma are due, in part, to a lack of lay knowledge concerning the neuroadaptations that occur in patients with AUD and dependence that justify a diagnosable medical disorder (Glass et al., 2014; Schomerus et al., 2011; Weine et al., 2016). Thus, a major component of the NIAAA is to disseminate scientific findings to the public in regards to the neuroadaptive changes that accompany chronic alcohol consumption. More specifically, recent attention has focused on binge drinking as a particularly risky behavior that is associated with the development of AUD (Llerena et al., 2015; McCarty et al., 2004)

BINGE DRINKING

Habitual heavy drinking is a behavioral pattern acquired over time and is accompanied by neuroadaptations that further promote uncontrolled drinking behavior despite negative consequences. A behavioral pattern consisting of repeated bouts of heavy episodic drinking is a hallmark of AUD and defined as 5 or more instances of binge drinking within a month. The NIAAA further defines binge drinking as 5 or more units of alcohol consumed within a 2-hour period for men, or 4 units within a 2-hour period for women (Alcoholism, 2004; SAMHSA, 2018). Binge drinking is a common occurrence within the U.S. population in that 1 in 6 persons displays this pattern of drinking within a 30-day period and, among individuals with a history of excessive drinking, roughly 90% report patterns of

binge drinking (Kanny et al., 2013). Furthermore, this pattern of excessive drinking has the greatest potential for harm because of the cognitive impairments and organ damage caused by high levels of alcohol circulating the body (Centers for Disease and Prevention, 2012; Jennison, 2004). Rapid consumption of alcohol in this fashion results in a blood alcohol concentration (BAC) in excess of the 0.08 g/dL (80 mg/dL) legal limit of intoxication (Alcoholism, 2004; Courtney and Polich, 2009; Kanny et al., 2013). Total alcohol metabolism under basal conditions is roughly 0.015 g per hour per 100 mL, or the equivalent of 1 standard drink per hour (Cederbaum, 2012; Holford, 1987). Alcohol is metabolized at a steady state and the rapid consumption occurring during a binge quickly overwhelms metabolic processes leading to exponential increases in BACs (Holford, 1987). Generally, 2-3 unit doses of alcohol consumed in an hour result in a BAC below 0.08 g/dL and is accompanied by a feeling relaxation and well-being (Hughes et. al., 2001). Consumption of 4-5 standard drinks, however, elevates BACs into a binge range between 0.08-0.09 g/dL. This level of intoxication is accompanied by reduced judgement, impairments in memory and reasoning, and impairments in motor coordination and reflex time. As alcohol consumption continues, BACs ranging from 0.10-0.25 g/dL result in gross impairments in motor coordination, slurring of speech, blurred vision, dysphoria, and nausea. Symptoms of alcohol poisoning begin to present when BACs in excess of 0.25 mg/dL are reached, which include confusion, severe nausea, vomiting, seizures, hypothermia, irregular breathing, and loss of consciousness. In cases of severe acute intoxication, coma and respiratory arrest can lead to death when BACs supersede 0.40 g/dL. The rapid

elevation of BAC achieved during a binge is potentially harmful because alcohol is a central nervous system (CNS) depressant and the subjective effects of alcohol are not felt until roughly 10-30 minutes after ingestion (Holford, 1987; Tagawa et al., 2000). Thus, a high volume of alcohol consumed during a 10-30 minute period can lead to an elevation in BAC to a damaging and potentially lethal level before an individual experiences negative feedback and slows subsequent drinking.

Risk for Injury

The high BAC achieved during a binge is associated with cognitive impairments that negatively affect decision making and increase the users risk for self-harm and harm to others (Jennison, 2004). For example, BACs superseding 0.08 g/dL are associated with deficits in motor coordination, reduced ability to visually track moving objects, and impaired decision-making that collectively increase the risk of harm to the individual and others (Balodis et al., 2009; Courtney and Polich, 2009; Kanny et al., 2013). Acute intoxication results in deficits in motor coordination that are highly correlated with accidental injury and 42% of hospitalizations involve intoxication or alcohol-related disease (Gerke et al., 1997; Tagawa et al., 2000). In fact, binge drinking increases the risk for accidental injury and the level of alcohol intoxication is associated with emergency room visit severity in a dose-dependent manner (Borges et al., 1998; Cherpitel et al., 2004; Gmel et al., 2007). Injury most often presents as a fracture, abrasion, or concussion resulting from falls (Charalambous, 2002). Binge drinking is thought to result in injury in moderate drinkers because the high level of intoxication achieved

during an isolated binge is a foreign experience (Gruenewald and Nephew, 1994; Treno et al., 1997). However, habitual heavy episodic drinking is associated with repeated injury resulting in chronic hospitalization or emergency room visits (D'Onofrio and Degutis, 2002; Ponzer et al., 1999). Repeated episodes of binge drinking and injury are also associated with a diagnosis of AUD (Gmel et al., 2007; Mancino et al., 1996). Thus, it is likely that the high BAC achieved during a binge is predictive of a generalized increased risk for accidental injury.

Accidental injuries are common in individuals with BACs above 0.08 g/dL due to the cumulative effect of deficits in psychomotor coordination and impaired decision making (Grant et al., 2000). Acute alcohol intoxication decreases an individuals perceived probability of negative outcomes and consequences of their actions (Fromme et al., 1997). Furthermore, alcohol is often referred to as a social lubricant because it decreases inhibition and increases self-confidence that can lead to an overestimation of one's own abilities (Tiplady et al., 2004). Alcohol also increases impulsive behaviors in a dose-related manner and increased impulsivity is associated with increased propensity for risk taking (Kovacs et al., 2017; Lyvers et al., 2015; Upton et al., 2011). It is therefore not surprising that individuals are more likely to engage in dangerous behaviors that they would otherwise not take part in whilst sober. For example, individuals are more likely to engage in risky sexual behavior while intoxicated that increases the risk for spread of sexually transmitted disease, unwanted pregnancy, and/or sexual abuse (Hines and Straus, 2007; Naimi et al., 2003). Because intoxication during a binge drinking episode results in reduced cognitive control over emotional reactivity, incidences

of domestic violence and violent crime are also higher among binge drinkers (Brewer and Swahn, 2005; Llerena et al., 2015). Finally, because acute alcohol intoxication negatively affects the ability to track moving objects, impairs cognitive processes, and delays motor responsiveness, it is of no surprise that high BAC achieved during a binge negatively affects an individual's ability to operate a motor vehicle (Brumback et al., 2007; Christoforou et al., 2013). Cognitive impairments associated with intoxication significantly contribute to mortality rates in intoxicated drivers (Marczinski and Fillmore, 2009; Wundersitz and Raftery, 2017). In fact, 28% of all traffic related deaths involve alcohol-impaired drivers and, of self-reports within the US, there are roughly 115 million instances of alcohol-impaired driving annually (Mancino et al., 1996; National Highway Traffic Safety, 2010; Wundersitz and Raftery, 2017). The collective ensemble of cognitive processes affected by binge drinking contributes to but one of multiple facets of the negative effects of alcohol.

Alcohol Toxicity

The rapid consumption of alcohol during a binge exerts an extreme stress on metabolic pathways that work to process the large bolus of alcohol entering the system (Cederbaum, 1980; Llerena et al., 2015). The liver is of particular importance because it is the principal site of first pass metabolism and the most susceptible to life threatening complications due to excessive drinking (Badawy, 1978; Zakhari, 2006). Prolonged stress on this metabolic pathway leads to high levels of alcohol circulating in the bloodstream as well as metabolites that are toxic

to organs and tissue (Holford, 1987). After oral consumption, alcohol enters the body and is readily absorbed in the intestines and passes through the portal vein to the liver (Badawy, 1978). Primary hepatic alcohol metabolism involves alcohol dehydrogenase (ADH) that converts alcohol to acetaldehyde through oxidative metabolism (Edenberg, 2007). Acetaldehyde is highly toxic and buildup produces nausea, headache, and vomiting. Acetaldehyde is metabolized through oxidation by mitochondrial aldehyde dehydrogenase 2 (ALDH2) into acetate in an irreversible reaction (Cederbaum, 2012; Holford, 1987). Acetate is then further metabolized in the liver or excreted to the periphery and metabolized. This occurs as mitochondrial acetyl-CoA synthetase (acetyl-CoA synthetase 2 in cytosol) reduces acetate to acetyl-CoA, an essential molecule in the tricarboxylic acid (TCA) cycle. This primary process of alcohol metabolism accounts for roughly 90% of alcohol metabolism, but when high concentrations of alcohol enter the system, the remaining 10% of alcohol is metabolized through microsomal oxidation (Cederbaum, 2012; Zakhari, 2006). This also occurs in the liver and involves CYP2E1, a cytochrome P450 enzyme that converts alcohol to acetaldehyde (Zakhari, 2006). An important byproduct of this reaction is the generation of reactive oxygen species (ROS) that can cause damage to DNA and mitochondria at high levels (Zakhari, 2006). The majority of acetaldehyde is converted by ALDH into acetate, but it can also be converted by CYP2E1 into acetate generating ROS in the process (Yun et al., 2014). The rapid consumption of alcohol during a binge exerts a taxing force on the liver because of the zero-order kinetics of alcohol metabolism. The prolonged stress on these metabolic pathways leads to high

levels of alcohol circulating in the bloodstream as well as acetaldehyde, acetate, NADH, acetyl-CoA, and ROS (Zakhari, 2006). Heavy drinkers have higher levels of acetaldehyde within the liver that promotes oxidative stress and lipid peroxidation (Barry, 1988; Setshedi et al., 2010). Increased NADH and acetyl-CoA production causes dysregulation of metabolic pathways including glycolysis, TCA cycle, and gluconeogenesis (Badawy, 1978; Cederbaum, 1980; Zakhari, 2006). Dysregulation of these pathways directly contributes to alcoholic liver disease. For example, fatty acid oxidation is suppressed in the liver during alcohol metabolism and, as a result of prolonged heavy episodic drinking, contributes to fatty liver disease (Lieber, 1975; Rasineni and Casey, 2012). Acetaldehyde and ROS generated during chronic heavy drinking directly damage the liver as well (Barry, 1988; Setshedi et al., 2010). Alcohol-induced damage to the liver most commonly presents as fatty liver disease and cirrhosis of the liver, and together are the leading causes of death in heavy drinkers (Lieber, 1975; Llerena et al., 2015; SAMHSA, 2018).

Apart from direct effects on tissues involved in metabolism, the large bolus of alcohol entering the body during a binge can cause damage to multiple tissues and organs, especially with chronic use. For example alcohol causes a proinflammatory response in the tongue and gums, and chronic inflammation is a common risk factor for oral cancer (Ogden, 2005). After oral intake, alcohol travels down the esophagus damaging the esophageal mucosa that precipitates and exacerbates the symptoms of gastroesophageal reflux disease (Pan et al., 2019). Once reaching the gastrointestinal (GI) tract, alcohol suppresses prostaglandin

function further promoting inflammation and erosion of the lining within the large intestine (Punchard et al., 1994). Furthermore, alcohol decreases digestive enzyme levels within the small intestine resulting in poor nutrient extraction from food, which creates a metabolic imbalance and disrupts general health (Bode and Bode, 1997). Damage to the gut, however, is not limited to direct tissue damage. The gut microbiome balance shifts toward dysbiosis with heavy alcohol consumption that is associated with a number of different pathologies including obesity, diabetes, irritable bowel syndrome, and celiac disease (Purohit et al., 2008). Finally, excessive alcohol consumption has long been associated with pancreatitis and chronic binge drinking is a risk factor for pancreatic cancer (Gupta et al., 2010; Juliusson et al., 2018). In fact, heavy episodic drinking is associated with 70% of cases of chronic pancreatitis (Dufour and Adamson, 2003). Therefore, alcohol drinking disrupts various systems within the body that negatively affect general health and binge drinking significantly contributes to mortality associated with alcohol-induced disease.

Risk Factor for AUD

Social drinking is common worldwide and individuals at risk for developing AUD are more likely to binge drink in social situations than those at low risk (Gowin et al., 2017; Peacock et al., 2018). This is of importance because binge drinking is the most common pattern of excessive alcohol consumption regardless of age and sex (Kanny et al., 2013). In fact, roughly 50% of individuals that consumed alcohol in the past year did so in a binge fashion and instances of binge drinking continue

to rise within the US, as do corresponding emergency room visits, alcohol-related mortality, and diagnoses of AUD (Castle et al., 2016; Hingson et al., 2017; White et al., 2020). Even more, the intensity and duration of binge drinking serves as a strong predictor for the development of AUD (Gowin et al., 2017). Furthermore, the subjective response to alcohol is predictive of AUD in that binge drinkers report increased stimulation and craving for alcohol after a single beverage whereas non drinkers report a sedative effect (Holdstock et al., 2000). The predictive nature of the response to alcohol and patterns of binge drinking has been documented in humans, non-human primates, and rodents suggesting an evolutionarily conserved mechanism of addiction pathology (Baker et al., 2017; Hingson et al., 2017; Zhou et al., 2017). Engagement in binge drinking may predispose an individual to AUD because BACs in excess of 0.08 g/dL are associated with a host of genetic and neuroadaptive changes that promote excessive drinking (Ferguson et al., 2019; Mulligan et al., 2011). Thus, study of the neurobiological mechanisms underlying binge drinking is critical to understanding the trajectory of AUD.

NEUROBIOLOGICAL BASIS OF AUD AND BINGE DRINKING

Alcohol is consumed, in part, because of the feelings of relaxation and euphoria experienced during intoxication that contributes to a positive affective state and gives the drug reinforcing properties. At the most basic level, alcohol influences mood and behavior through a pharmacological interaction with neurotransmitter systems that modulate inhibition and excitation within the brain. Alcohol is a nonspecific drug that is considered to be a CNS depressant because

it potentiates neural inhibition and attenuates excitatory processes. For example, the $GABA_A$ receptor is a ligand-gated ion channel that is activated by gamma-aminobutyric acid (GABA), the major inhibitory neurotransmitter system in the CNS (Allan et al., 1987). Activation of $GABA_A$ receptors results in inward chloride ion flux that has a net effect of hyperpolarization of the cell (Mihic and Harris, 1997). Alcohol and other sedatives such as benzodiazepines and barbiturates are considered negative allosteric modulators because they have a synergistic effect with endogenous GABA interactions at the receptor that facilitate, and potentiate, $GABA_A$-mediated neuronal inhibition (Mihic et al., 1997). Alcohol suppresses excitatory activity in the CNS through its action as an antagonist at the N-methyl D-aspartate (NMDA) receptor (Lovinger et al., 1989). NMDA receptors are ligand-gated ion channels that modulate inward flux of sodium and calcium and outward flux of potassium, thereby resulting in depolarization of a neuron (Loftis and Janowsky, 2003; Yamakura and Shimoji, 1999). Activation of NMDA receptors occurs after binding of both glutamate, the major excitatory neurotransmitter, and glycine. In fact, alcohol inhibits synaptic functions of both ionotropic NMDA and AMPA glutamate receptors, which is somewhat unique in the adult brain (Gonzales and Jaworski, 1997; Lovinger et al., 1990; White et al., 1990). Thus, modulation of ionotropic glutamate and GABA receptors by alcohol functions to directly affect intracellular ionic balance and subsequent neuronal activity. However, alcohol also exerts its effects on the CNS through interactive effects of neuromodulatory monoamine systems. Briefly, alcohol increases dopamine, norepinephrine, and serotonin release that promotes the rewarding properties of the drug, enhances

arousal, and affects mood (Lovinger, 1997; Zahr and Pfefferbaum, 2017). Lastly, alcohol interacts with a number of different neuropeptide systems that similarly contribute to the reinforcing properties of alcohol and promote alcohol consumption, which will be discussed in detail later. Because the acute effects of alcohol enhance inhibition and blunt excitation through GABA and glutamate respectively, compensatory neuroadaptations occur after chronic alcohol abuse that result in rebound excitation during periods absence of drug that contribute to withdrawal syndrome. For example, internalization of $GABA_A$ and upregulation of NMDA receptors results in decreased inhibitory tone and hyperexcitability within brainstem structures that contributes to generalized seizure activity (Banerjee, 2014; Rogawski, 2005).

Human neuroimaging of patients with AUD and animal studies that approximate AUD have revealed that discrete neurocircuitry contributes to behaviors involved in the cycle of addiction, which are more precisely defined as 1) binge consumption and intoxication, 2) withdrawal and negative affect, and 3) preoccupation and craving (Koob and Volkow, 2010). Different neuroanatomical substrates are involved in the behaviors observed in each stage of this cycle that collectively contribute to the manifestation of AUD. Preclinical models of excessive alcohol drinking and dependence have provided further insight into brain regions and neurochemical systems that are affected by chronic alcohol intake/exposure. For example, alcohol consumption to excess during a bout of binge drinking involves signaling within the mesolimbic dopamine system that reinforces the rewarding properties of alcohol and neuroadaptations within the dorsal striatum

promotes habitual binge drinking (Di Chiara, 1997; Nutt et al., 2015; Wang et al., 2015). The negative affective state experienced during withdrawal involves activity within the extended amygdala and hypothalamus that promotes an internal stress response (Koob, 2009; Stephens and Wand, 2012). Finally, craving and preoccupation with alcohol consumption during periods of abstinence is associated with activity within the prefrontal cortex and hippocampus that contribute to relapse (Sinha and O'Malley, 1999). The dominant theory of the neurobiology of addiction proposes that a homeostatic balance is necessary within the brain for normal processing (Koob and Volkow, 2010). Alcohol disrupts this process by driving a positive affective state whilst intoxicated. But, with every action there is an equal and opposite reaction. Therefore, a negative affective state is experienced during withdrawal consistent with the opponent process theory. As a result of chronic binge drinking, the homeostatic balance skews towards a net negative state that presents during withdrawal as the emergence of persistent negative affect, drives craving, and promotes chronic relapse. Thus, engagement in repeated binge drinking is an effort to restore baseline homeostatic balance and regain an allosteric set-point. The discrete neurocircuitry underlying the stages of the addiction cycle as they relate to this shift in allostasis are described in greater detail below.

Binge Drinking and Reward

As we have previously described, binge drinking is a destructive pattern of behavior that involves the rapid consumption of alcohol over a short amount of time. Binge drinking and intoxication is a key component of the conceptual framework underlying the neurobiological basis for AUD (Koob and Volkow, 2010). Alcohol, like all drugs of abuse, is consumed because it has positive reinforcing properties that promote excessive alcohol drinking. Operant conditioning paradigms are used to study reward-driven behavior in rodents and early experiments revealed that animals will lever press for intracranial self-stimulation (ICSS) of the medial forebrain bundle. Mice and rats will establish lever press behavior to receive ICSS, natural rewards, or drugs of abuse, which suggests a common neuronal mechanism involved in reward. In fact, responding for ICSS is decreased when subjects are pretreated with alcohol, demonstrating the innate rewarding properties of the drug (Negus and Miller, 2014). The medial forebrain bundle encompasses fibers of passage that originate in the ventral tegmental area (VTA) and terminate in the basal forebrain. The VTA is a midbrain structure involved in reward-related behaviors and is comprised of largely GABAergic neurons that express the neuromodulator, dopamine (DA). Systemic challenge or microinjection of alcohol directly into the VTA increases firing of DA neurons through an excitatory mechanism (Brodie et al., 1999; Gessa et al., 1985). Activation of neurons within the VTA results in release of DA in downstream structures in the ventral striatum involved in reward, such as the nucleus accumbens (NAc) (Kohl et al., 1998; Yim et al., 1998). Alcohol also facilitates

endogenous opioid release within the VTA resulting in activation of Mu-opioid receptor (MOR) locally on GABAergic terminals arising from the rostromedial tegmental nucleus (RMTg) (Font et al., 2013; Jhou et al., 2009; Matsui and Williams, 2011). Activation MOR on these terminals results in disinhibition of VTA projections to the NAc and increased phasic DA release that contributes to reward salience (Jhou et al., 2009). In fact, alcohol and all drugs of abuse increase extracellular DA release in the NAc and suggests a common mechanism involved in the reinforcing properties of these substances (Di Chiara, 1997; Di Chiara and Imperato, 1988).

The mesolimbic DA system is not only responsive to the pharmacological effects of alcohol, but also drives alcohol drinking and seeking behavior. For example, alcohol consumption and anticipation of access to alcohol results in DA release within the NAc and blockade of DA receptors therein decreases voluntary intake (Hodge et al., 1997; Rassnick et al., 1992; Weiss et al., 1993). Furthermore, cues associated with alcohol are sufficient to induce activation of the VTA and NAc. Selective optogenetic activation of the VTA-NAc circuit promotes reinstatement of alcohol seeking, an effect that is dependent upon DA signaling in the NAc (Bass et al., 2013; Budygin et al., 2020; Juarez et al., 2017). Thus, projections from the VTA to the NAc play a prominent role in reward salience that promotes goal driven behavior, such as alcohol seeking and binge drinking. The NAc is further subdivided into the NAc core and NAc shell. Both the core and shell receive DA input from the VTA but each play a nuanced role in motivated behaviors. More specifically, the NAc core is involved in general reward processes such as alcohol

drinking and seeking behavior in response to discrete cues (Chaudhri et al., 2010). The NAc shell plays a more prominent role in the balance of stimulus-outcome and response-outcome processing (Corbit et al., 2001). Finally, D1- and D2-expressing neurons in the NAc core project to the ventral pallidum (VP) via the direct “go” and indirect “no-go” pathways, respectively, that are involved in various reward behaviors, such as drug seeking and contribute to the reinforcing properties of alcohol (Heinsbroek et al., 2020; Kupchik and Kalivas, 2013; Melendez et al., 2004). D2-expressing neurons in the striatum are of interest because patients with AUD show decreased D2 availability compared to controls (Volkow et al., 2002). Furthermore, individuals with high levels of D2 expression are less likely to develop an AUD even if they are predisposed through familial alcoholism (Volkow et al., 2006). Therefore, DA signaling within VTA-NAc-VP circuitry plays an important role in the rewarding properties of alcohol that promote drinking behavior. Interestingly, pharmacological inactivation of the VTA or NAc blocks the expression of conditioned place preference (CPP) for alcohol but lesion of DA terminals in the NAc does not affect voluntary alcohol consumption (Bechtholt and Cunningham, 2005; Rassnick et al., 1993), suggesting that the mesolimbic DA systems works in concert with other regions and systems to provide the cumulative rewarding properties of alcohol that promote binge drinking.

Habitual alcohol consumption is a key component to the addiction cycle and AUD. The dorsal striatum (DS) is of relevance because it plays a prominent role in assigning reward value to a behavior, and compulsive, habitual drug-seeking (Belin and Everitt, 2008; Malvaez and Wassum, 2018). Generally speaking the DS

is a part of the basal ganglia that is involved in the initiation and control of motor behaviors. The DS can be subdivided into the dorsal medial striatum (DMS) and the dorsal lateral striatum (DLS), which are analogues of the human caudate and putamen, respectively. The DMS is involved in goal-directed behavior and inactivation of the DMS disrupts lever-press responding for alcohol only when responding is sensitive to outcome value, indicative of action/outcome associations (Corbit et al., 2012; Fanelli et al., 2013). Devaluation studies revealed that selective inactivation of the DLS only decreased inflexible lever-press responding for alcohol, suggesting that the DLS is more so associated with habit-like behavior (Corbit et al., 2012; Fanelli et al., 2013). This DMS/DLS dichotomy in responding for alcohol is in agreement with the general theory that behavioral acquisition and skill learning is mediated by associative corticostriatal activity involving the DMS. As behaviors are repeated and actions refined, behavioral responses become habitual, driven by a shift involving engagement of sensorimotor circuitry involving the DLS (Kupferschmidt et al., 2017). Indeed, the DS is a key component of the basal ganglia and DA signaling therein is involved in the initiation and continuation of motor behavior involved in alcohol seeking and consumption (Everitt and Robbins, 2013). Unlike the NAc, dopaminergic signaling in the DS arises from projections originating in the substantia nigra pars compacts (SNc), a midbrain DA-containing structure that innervates both the DMS and DLS. SNc inputs to the DS are classically associated with the direct and indirect pathways that drive thalamic inputs to the motor cortex through respective D1- and D2-expressing striatal neurons. Acute alcohol treatment increases extracellular DA

release within the striatum that has important consequences for behaviors that involve the DS (Gonzales et al., 2004; Sulzer, 2011). For example, D1 antagonist microinjection into the DMS decreases alcohol drinking and D2 antagonist in DLS decreases habitual responding for alcohol (Corbit et al., 2012; Wang et al., 2015). Interestingly, nonspecific D1/D2 antagonist into DLS decreases PR-breakpoint for alcohol suggesting a role for DA signaling in the DLS in the motivation to work for alcohol (Spoelder et al., 2017). Thus, the DMS is likely recruited after the engagement of mesolimbic reward circuity to promote and facilitate drug seeking behavior and, over time, the DLS is promotes more habitual patterns of drinking that contribute to compulsive alcohol consumption (Barker and Taylor, 2014).

The amygdala is a structure located within the rostral pole of the temporal lobe that is classically associated with emotional behavior. Recent studies, however, suggest that subregions of the amygdala contribute to the reinforcing properties of alcohol that promote binge drinking. For example, lesion of the amygdala blocks the acquisition and expression of alcohol-induced CPP (Gremel and Cunningham, 2008). Further studies revealed that selective lesion of the central amygdala (CeA) attenuates voluntary alcohol consumption (Moller et al., 1997). The CeA receives direct dopaminergic input from the VTA and SNc and, similar to the striatum, extracellular DA is increased within the CeA after acute alcohol challenge (Hasue and Shammah-Lagnado, 2002; Yoshimoto et al., 2000). Furthermore, blockade of D1 receptors in the CeA attenuates alcohol drinking and seeking behavior (Yoshimoto et al., 2000). Interestingly, activation of D2 expressing neurons in the CeA promotes impulsive behavior that is involved in

chronic binge drinking (Kim et al., 2018). The CeA sends monosynaptic projections to the VTA and SNc and this circuitry is involved in appetitive learning (Lee et al., 2010). Within the CeA, bath application of alcohol decreases glutamate release through inhibition of N-type calcium channels and *in-vivo*, nonselective blockade of NMDA/AMPA receptors in the CeA blocks the expression of CPP (Zhu et al., 2007). Finally, the CeA is also the most sensitive site of $GABA_A$ antagonists' effects on alcohol consumption and a history of chronic intermittent ethanol (CIE) exposure increases GABAergic tone and responsiveness to alcohol in the CeA (Gilpin et al., 2015; Hyytia and Koob, 1995; Roberto et al., 2012). Together, these studies suggest involvement of the CeA in the rewarding properties of alcohol and processes involved in the motivation to drink excessively.

Therefore, alcohol is consumed because of the rewarding properties of the drug that arise from the collective activity of VTA-NAc-VP circuitry in addition to activity in the CeA. Neuroadaptations within the aforementioned brain regions as well as the DLS occur over time and contribute to the transition to habitual, compulsive, uncontrolled bouts of binge drinking that are a hallmark of AUD.

Withdrawal and Negative Affect

Chronic excessive drinking leads to neuroadaptations that promote withdrawal symptomology in the absence of alcohol. This is not to be confused with what is commonly referred to as a "hangover" that manifests as nausea, vomiting, and headache due to dehydration that is experienced after acute binge intoxication (Swift and Davidson, 1998). In fact, this acute negative experience has

strong aversive properties that are associated with stimuli related to alcohol and act as a positive punishment, which decreases the likelihood of further drinking. Alcohol consumption, however, can alleviate this symptomology and promote further drinking through negative reinforcement (Koob, 2013). Over time, repeated instances of binge drinking can result in neuroadaptations that contribute to withdrawal syndrome, which is characterized by physical symptomology, such as increased heart rate, sweating, tremor, seizure activity, and increased pain sensitivity combined with a negative emotional state that is experienced when access to alcohol is prevented (Becker and Hale, 1993; Jesse et al., 2017; Koob and Le Moal, 2008).

Chronic binge drinking results in neuroadaptive changes that promote tolerance, dependence, and the manifestation of withdrawal syndrome that is associated with an imbalance of excitatory and inhibitory neurotransmitter systems. More specifically, alcohol is a $GABA_A$ PAM and NMDA antagonist, and chronic alcohol exposure results in a rebound effect of decreased inhibition due to $GABA_A$ hypofunction and NMDA-mediated hyperexcitability during withdrawal (Davies, 2003; Gonzales and Jaworski, 1997). Cessation of alcohol after chronic consumption results in CNS autonomic hyperexcitability that contributes to generalized tonic-clonic seizure activity (Becker and Hale, 1993; Rogawski, 2005). More specifically, withdrawal-related seizure activity occurs most commonly within 48 hours of alcohol cessation and is related to a decrease in seizure threshold (Brathen et al., 1999; Victor and Brausch, 1967). Seizure activity originates in the brainstem during withdrawal due to decreased $GABA_A$-mediated gating of

neuronal activity (Rogawski, 2005). Thus, drugs that restore activity of the $GABA_A$ receptor, such as benzodiazepines and alcohol, act as anticonvulsants and normalize seizure threshold during withdrawal (Mayo-Smith, 1997). Glutamatergic hyperexcitability also occurs that contributes to seizure activity through an upregulation of NMDA receptors and NMDA antagonists block withdrawal-related seizures (Grant et al., 1990; Kalluri et al., 1998). Cognitive processes are also greatly affected during acute withdrawal, and in the most severe cases, symptoms of alcohol withdrawal delirium, or Delirium Tremens (DTs) present as extreme confusion, hallucinations, autonomic hyperactivity, and cardiovascular arrhythmia (Grover and Ghosh, 2018; Schuckit, 2014). Dysregulation of monoamine systems contribute to confusion, hallucinations, and extreme shifts in mood. DA in particular plays an important role in major alcohol withdrawal whole body tremor. For example, heavy episodic drinking decreases DA signaling in basal ganglia and promotes cerebellar degeneration that presents as resting tremor during withdrawal (Deik et al., 2012; Yokota et al., 2006).

As previously discussed, alcohol increases DA release within reward circuitry and promotes the positive reinforcing effects of the drug. Chronic alcohol consumption, however, drives a maladaptive shift towards a hypodopaminergic state within the NAc that contributes to a reward-deficit (Edwards and Koob, 2010; Karkhanis et al., 2015). For example, under basal conditions acute alcohol increases endogenous opioid and DA release that is associated with positive affect (Yim et al., 1998). As drinking escalates over time, MOR-related signaling and subsequent DA release in response to alcohol is attenuated, necessitating greater

levels of alcohol consumption to achieve a positive state (Koob and Volkow, 2016). This rationale is supported by imaging studies that show reduced methylphenidate-induced DA release within the striatum of patients with AUD (Volkow et al., 2013). Furthermore, chronic alcohol treatment increases ICSS reward threshold during withdrawal suggesting an increased hedonic drive to drink during withdrawal (Chester et al., 2006; Schulteis et al., 1995). Thus, heavy episodic drinking emerges as a maladaptive behavioral process to relieve the reward-deficit. The homeostatic set point shifts towards a net negative as signaling of the pro-affective MOR decreases and signaling in systems involved in dysphoria, aversion, and stress are increased.

Withdrawal is generally accepted to be a stressful state and is associated with increased circulation of stress-responsive hormones and neuropeptides. The heightened stress/anxiety state resulting from repeated activation of the reward system contributes to negative hedonic value that has been conceptualized as an anti-reward system (Koob, 2013; Koob and Le Moal, 2008). For example, the hypothalamic-pituitary-adrenal (HPA) axis regulates the endogenous stress response and is severely impacted by chronic alcohol consumption (Blaine et al., 2016). The paraventricular nucleus of the hypothalamus (PVN) produces corticotrophin release factor (CRF) that is secreted in response to input from structures that detect and are responsive to stress-associated stimuli (Turnbull and Rivier, 1997). CRF-expressing neurons within the PVN project to various structures within the CNS and also send axonal projections through the infundibular stalk that release CRF into the median eminence. CRF travels through

the hypophyseal portal system and drives the release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary gland. ACTH then moves freely through the body and stimulates the secretion of cortisol/corticosterone (CORT) and glucocorticoids from the adrenal cortex. Feedback from CRF, ACTH, and CORT regulates the stress response and disrupted in patients with AUD (Stephens and Wand, 2012). Indeed, a moderate dose of alcohol reflecting BACs below 0.08 g/dL blunt HPA axis responsiveness to experimenter-delivered CORT, supporting the anxiolytic properties of alcohol (Waltman et al., 1993). However, higher BACs achieved during a binge increase CRF, ACTH, and CORT release, as well as norepinephrine, and promotes positive feedback at the level of the PVN (Borg et al., 1981; Wright, 1978). Chronic alcohol consumption further disrupts HPA axis function by dynamically altering the response to alcohol, abstinence, and stress. For example, HPA axis activity is increased during chronic consumption and withdrawal, but is suppressed in response to stressors (Adinoff et al., 1998; Becker, 2012). Increased circulating stress hormone levels during withdrawal contribute to the shift toward negative allostasis that is relieved by binge drinking, consistent with the tension-reduction hypothesis (Becker, 2012; Cappell and Herman, 1972; Koob, 2013). However, repeated instances of binge drinking to alleviate withdrawal symptoms facilitate neuroadaptations that exacerbate the dysregulation of HPA axis reactivity and the stress response that perpetuates the addiction cycle.

The extended amygdala macrostructure is considered to be a key node in the interface of stress and arousal systems with limbic drivers of hedonic behavior

affected during withdrawal (Koob and Le Moal, 2008). The extended amygdala is comprised of the CeA, bed nucleus of the stria terminalis (BNST), substantia innominata, and NAc shell (Alheid and Heimer, 1988). The CeA and BNST in particular modulate the stress response through projections to the PVN and lesion of either structure disrupts HPA axis activation (Crestani et al., 2013; Xu et al., 1999). C-Fos immunoreactivity is increased within these structures during acute withdrawal and this heightened neuronal activity is thought to promote stress and anxiety-like behavior (Kimbrough et al., 2020; Smith et al., 2019). Interestingly, activity and connectivity between the CeA and BNST is observed during withdrawal that may contribute to negative affective states (Hu et al., 2018; Pedersen et al., 2020). In fact, inhibition of a select populations of projections between the CeA and BNST decreased alcohol intake and attenuated anxiety-like behavior in alcohol-dependent rats (de Guglielmo et al., 2019). Further, the CeA and BNST are rich in CRF and glucocorticoid receptors that are activated during withdrawal and contribute to excessive drinking (Kash et al., 2015; Pomrenze et al., 2019a; Silberman and Winder, 2013). Indeed, CRF and glucocorticoid antagonists have shown promise in attenuating excessive drinking and withdrawal-related stress and anxiety in preclinical models of AUD (Funk et al., 2007; Shaham and de Wit, 2016; Stephens and Wand, 2012). More specifically, microinjection of a CRF1 antagonist into the CeA decreased dependent-like escalation in alcohol intake and reduced anxiety-like behavior during protracted withdrawal (Baldwin et al., 1991; Funk et al., 2006). CRF-expressing neurons within the BNST are also affected by chronic alcohol exposure and modulate activity in the VTA through

CRF1 receptors therein (Rinker et al., 2017; Silberman et al., 2013). Within the BNST, dysregulation of excitatory/inhibitory balance by alcohol-induced alteration of monoamine and peptide release mediate withdrawal-induced anxiety and are thought to also promote excessive drinking (Kash, 2012; Kash et al., 2015; Pleil et al., 2016).

Koob et al., have described the negative affective state experienced during withdrawal that drives negative reinforcement and promotes alcohol drinking as the "dark side of addiction", embodied by dysphoria, anxiety, and irritability (Koob, 2013; Schulteis and Koob, 1994). The dark side emerges over time as functioning of anti-reward systems increase concurrent with frequent bouts of heavy episodic drinking. Thus, the chronic relapsing nature of AUD is evident as individuals seek to restore a homeostatic set point through alcohol consumption to alleviate withdrawal symptomology and persistent negative affect. However, since reward systems are desensitized over time, alcohol drinking to excess in repeated/chronic binges emerge as an effort to offset the negative allosteric load experienced during the reward deficit.

Opponent Process Theory and Craving

Individuals diagnosed with AUD report that craving, and an inability to regulate the behavioral response to craving, significantly contributes to relapse that perpetuates the chronic relapsing nature of the disorder (Breese et al., 2011; Fox et al., 2007). Therefore, understanding the neurobiological mechanisms that drive craving is essential to break the cycle of addiction and prevent relapse. Craving presents as an intense, urgent, and abnormal desire to consume alcohol that

arises from the complex interplay between neuroadaptations that occur as a result of prolonged alcohol abuse and environmental influences that trigger craving and relapse (Seo and Sinha, 2014; Sinha, 2013). As previously described, chronic excessive alcohol consumption results in neuroadaptations that create a maladaptive imbalance in positive and negative affect. The resulting increase in allosteric load leaves individuals with AUD susceptible to relapse because of increased reward salience, persistent negative affect, and deficits in the top-down control of impulsive behavior and emotional processing (Koob, 2013). It is not surprising that neurocircuitry involved in reward (striatum), behavioral inhibition (PFC), and emotional control (limbic system/amygdala) are affected by chronic alcohol abuse and drive craving (Koob and Volkow, 2016). Thus, allosteric pressure and resultant dysregulation of cortico-striatal-limbic circuitry is a likely culprit contributing to craving in patients with AUD.

We have previously discussed the role of the NAc within the context of reward and provided evidence demonstrating that alcohol dependence drives a hypodopaminergic state during withdrawal. Concurrent neuroadaptations within reward circuitry shift during the transition to dependence to assign greater salience to alcohol and alcohol-related stimuli compared to natural rewards. Robinson and Berridge describe this process as incentive sensitization that is consistently observed across drugs of abuse (Robinson and Berridge, 1993). Therefore, craving for alcohol emerges in place of natural rewards as reward circuits are sensitized over time resulting in a homeostatic shift toward compulsive drinking. This is particularly evident in the sensitized response to cues associated with

alcohol. For example, increased activity is observed in the ventral and dorsal striatum in response to alcohol-related cues in subjects diagnosed with AUD and activity within these regions is predictive of the likelihood of relapse (Seo et al., 2011). Preclinical models have shown that inactivation of the NAc attenuated reinstatement of alcohol-seeking behavior suggesting a causal role for this structure in what is considered the rodent approximation of craving and relapse (Chaudhri et al., 2010).

Beyond mesolimbic DA, alcohol-predictive cues trigger the intense urge to seek alcohol and this craving is related to altered glutamatergic tone in the NAc. In fact, increased glutamate within the NAc of humans is observed during withdrawal and strongly predicts the intensity of craving (Bauer et al., 2013). In rodents, extracellular glutamate is increased within the NAc of alcohol-dependent mice and pharmacological attenuation of glutamatergic tone normalized alcohol intake to a moderate level (Griffin et al., 2014). Similarly, glutamate is released within the NAc in response to alcohol-associated cues and triggers reinstatement of alcohol-seeking in a mGlur2-dependent manner (Gass et al., 2011; Meinhardt et al., 2013). Thus, glutamate overflow within the NAc resulting from chronic alcohol consumption is thought to promote craving, preoccupation, and obsessive thoughts that drive relapse (Kalivas, 2009).

The mPFC is classically associated with cognitive processing and top-down control over limbic structures, such as the NAc and amygdala, that drive hedonic behavior (Kouneiher et al., 2009). Neuroimaging studies have revealed that chronic alcohol abuse is associated with decreased gray matter volume within the

mPFC that manifests as deficits in higher order functioning such as decision making, impulse control, and emotional regulation (Bechara, 2005; Goldstein and Volkow, 2011; Yang et al., 2016). Even though there is decreased volume within the medial PFC (mPFC) and subdivisions therein, increased activity within these structures as a function of AUD have been associated with craving (Sinha, 2013). The orbitofrontal cortex (OFC) and insular cortex (IC) in particular are associated with incentive motivation and behavioral inhibition (Arana et al., 2003; Naqvi et al., 2014). Activity within the OFC is increased in response to alcohol-associated cues and correlates with craving (Myrick et al., 2004). In fact, heightened OFC activity predicts the likelihood of relapse during protracted abstinence (Reinhard et al., 2015). Similarly, alcohol-associated cues result in increased activity within the IC and this increase is more pronounced in alcohol-dependent individuals (Schacht et al., 2013). The heightened responsiveness of the IC is also predictive of the severity of AUD symptomology (Claus et al., 2011). Another region of the PFC, the dorsolateral prefrontal cortex (dlPFC), has been extensively studied in the context of craving and alcohol seeking behavior. The dlPFC is highly reactive to cues associated with alcohol and this activity is strongly correlated with craving (Park et al., 2007). Furthermore, modulation of neuroplasticity within the dlPFC using noninvasive techniques such as repeated transmagnetic stimulation (rTMS) has shown promise in reducing craving and relapse in treatment-seeking patients with AUD (Zhang et al., 2019). In rodents, the dlPFC is analogous to the prelimbic cortex (PL). The PL exerts excitatory control over the VTA through a glutamatergic projection involved in incentive salience (Carr and Sesack, 2000). The PL also

sends a direct monosynaptic projection to the NAc core and promotes drug seeking behavior (McGlinchey et al., 2016). Indeed, cues associated with alcohol increase c-Fos expression within the PL and selective inhibition or ablation of the PL-NAc core pathways is sufficient to block cue-induced reinstatement of alcohol seeking (Keistler et al., 2015; Keistler et al., 2017).

During early withdrawal, uncontrolled craving is associated with dysregulation of the HPA axis that presents as a negative emotional state and promotes compulsive drinking (Koob, 2013). An inability to control emotional behavior in response to stress is associated with relapse and increased drinking (Claus et al., 2011). The ventromedial prefrontal cortex (vmPFC) has been implicated in the top-down control over emotional reactivity and dysregulated activity has been observed in abstinent patients with AUD (Seo et al., 2013). More specifically, basal activity is increased within the vmPFC and the level of resting state activity is positively correlated with craving scores and predicts relapse (Seo et al., 2013). In healthy subjects, vmPFC activity is increased in response to a stressor but this effect is not observed after chronic alcohol abuse suggesting a blunting in stress-responsivity. The vmPFC projects to the amygdala and decreased coupling of these structures is associated with negative affect (Urry et al., 2006). As a function of chronic alcohol consumption or a familial history of alcoholism, amygdala volume is decreased and the reduced size predicts craving and relapse (Hill et al., 2013; Wrase et al., 2008; Zhang et al., 2013). However, cues associated with alcohol illicit a robust increase in activity within the amygdala that is associated with relapse (Schact et al., 2013). Further study of PFC-

amygdala connectivity in rodent models of AUD and relapse support these observations, although the neuroanatomical terminology for these structures differs slightly. For example, the human vmPFC is analogous to the rodents infralimbic cortex (IL) and sends glutamatergic projections to the BLA. Studies of conditioned fear and extinction learning have demonstrated that activity of IL-BLA projections is necessary for extinction learning and the expression of extinction behavior, which is sensitive to alcohol abuse (Bloodgood et al., 2018; Do-Monte et al., 2015; Holmes et al., 2012). Indeed, CIE exposure reduces activity within the IL and impairs extinction learning, reflective of a dysregulation in the top-down control of emotional behavior (Scarlata et al., 2019). The BLA, however, is not only involved in the regulation/expression of fear, but is highly reactive to cues associated with alcohol and a key node in reinstatement circuitry (Gass et al., 2011; Sinclair et al., 2012). In fact, ablation of BLA projections to the NAc attenuates cue-induced reinstatement of alcohol-seeking (Keistler et al., 2017). The BLA also sends projections to the hippocampus, which is involved in contextual associations with alcohol that trigger relapse, and inhibition of BLA projections to the ventral hippocampus decreases excessive drinking (Ewin et al., 2019). Thus, cortico-striatal-limbic circuity drives craving in response to cues associated with alcohol.

Dysregulation of the HPA axis and the endogenous response to stress is a major component of craving and not only facilitates excessive drinking but sensitizes the response to cues associated with alcohol that drive relapse (Koob, 2013; Koob and Le Moal, 2008). We have previously described that CRF, ACTH,

and CORT levels are elevated at rest as a function of dependence that contributes to craving and further drinking to alleviate the hyper aroused state, consistent with the tension reduction hypothesis (Sinha, 2001). Stress reactivity, however, is also altered as a function of dependence that drives craving. Rodent models have been useful in demonstrating this relationship in that rats show increased response to a mild stressor during withdrawal that promotes alcohol-seeking behavior (Zorilla et al., 2001; Breese et al., 2005; Le at al., 1998). Repeated cycles of CIE exposure results in increased voluntary alcohol consumption in mice, and the addition of chronic FSS further elevates drinking beyond that of CIE alone (Anderson et al., 2016; Lopez et al., 2016a). This suggests that a history of dependence sensitizes stress-related circuitry that selectively promotes excessive alcohol consumption, an effect not seen in non-dependent subjects. Furthermore, a history of alcohol dependence blunts the HPA axis response to stress that may present behaviorally as a maladaptive coping response (Adinoff et al., 1998; Becker, 2012). The extended amygdala and specifically the CeA and BNST are highly enriched in CRF-expressing neurons and adaptations within these structures has been suggested to play a role in the altered stress response that contributes to craving (Merlo Pich et al., 1995; Olive et al., 2002).

In summary, the loss of control over craving is associated with compulsive alcohol drinking, especially when individuals are faced with situations, cues, or environments associated with alcohol, and in the face of stressful events. Indeed, circuits that are involved in emotional responses and cognitive control over behavior become dysregulated and contribute to craving and relapse. Thus,

cortico-striatal-limbic circuitry is a likely culprit driving craving and relapse because these regions are involved in regulation of behavioral control, reward, and emotion. Koob and Volkow propose a framework underlying craving where neuroadaptations within the NAc occur first that promote incentive salience. This is followed by alterations within the PFC resulting in a lack of top-down control of behavior. Finally, a gain of function in limbic structures, specifically the extended amygdala that modulate stress, push the system to promote alcohol drinking over natural rewards.

Summary

The development of AUD and dependence arises from the complex interplay between neuroadaptations within multiple brain regions that occur as a result of repeated binge intoxication. Intoxication achieved during a binge activates reward circuitry and reinforces further alcohol consumption. As binge drinking continues, reward pathways are sensitized in response to stimuli associated with alcohol and drinking behavior that dysregulates reward circuitry. Furthermore, with repeated instances of binge drinking, withdrawal sequalae emerge from a gain of function in stress-related systems that presents as a persistent negative emotional state. Thus, chronic binge drinking and subsequent withdrawal create an allosteric load that shifts the act of alcohol consumption from positive to negative reinforcement. During abstinence, the negative emotional state experienced during withdrawal creates a strong drive to consume alcohol. Relapse presents as an effort to relive this state and Koob et al, 2013 suggested classifying AUD as a

stress surfeit disorder for this reason. The extended amygdala structure is a shared neuroanatomic substrate of binge drinking, withdrawal, and craving and the CeA and BNST in particular have garnered much interest as a druggable target for the treatment of AUD. More specifically, the CeA and BNST are home to a plethora of neuropeptides that are involved in the addiction cycle and pharmaceutical compounds targeting these systems have shown promise in decreasing drinking behavior, relieving withdrawal-related negative affect, and curbing craving/relapse. Of particular interest is the kappa opioid receptor, which is expressed within the extended amygdala and implicated in each stage of the addiction cycle. We will expand upon the function of this receptor and its endogenous ligand in relation to AUD in the following section.

DYNORPHIN/KAPPA OPIOID RECEPTOR SYSTEM

Neuropeptide systems play a critical role in the modulation of binge drinking behavior, withdrawal, and craving that comprise the addiction cycle. Dynorphin (DYN) is one such neuropeptide that is involved in the various aspects of the addiction cycle and has emerged as a potential therapeutic target for the treatment of AUD. Dynorphins (DYN) are a class of endogenous opioid peptides classically associated with stress, dysphoria, pain, and aversion and are the primary ligand to the kappa opioid receptor (KOR) (Bruchas et al., 2010; Chavkin et al., 1982; Mucha et al., 1985; Pfeiffer et al., 1986). DYN arises from a single precursor protein, preprodynorphin, which is enzymatically processed within the soma by proprotein convertase-2 into prodynorphin (PDYN) and further processed to

functionally active DYN peptides A and B (together referred to as “Big Dynorphin”) and neo-endorphin (Chavkin et al., 1982; Massotte and Kieffer, 1998). Like other opioid peptides, DYN is stored in dense-core vesicles where it is released from axon terminals or dendritic processes. The rate of DYN metabolism varies by brain region but occurs through proteolysis resulting in leu-enkephalin production (Sandin et al., 1997). Leu-enkephalin is an active metabolite with high affinity at the delta-opioid receptor, moderate affinity at the mu-opioid receptor, and very low affinity for KOR (Sandin et al., 1997). Upon release, DYNs cross the synaptic cleft and bind to KORs that are expressed primarily on presynaptic terminals. The KOR is a seven transmembrane G-protein coupled receptor encoded by the OPRK1 gene (Chavkin, 2013). KORs exert a largely inhibitory effect on neuronal activity through Gi/o intracellular signaling. For example, upon DYN binding to KOR, GDP is displaced allowing for GTP association with the Gi/o subunit. GTP-bound Gi/o and beta/gamma subunits then dissociate from the c-terminus of KOR and have distinct signaling pathways. The Gi/o complex acts to inhibit adenylyl cyclase activity that, in turn, decreases cAMP production (Law et al., 2000). The GTP-bound Gi/o subunits further exerts a hyperpolarizing effect on tonic neuronal activity by facilitating G-protein gated inward rectifying potassium channels (Kir3) (Henry et al, 1995). Finally, the beta-gamma subunit promotes hyperpolarization by blocking N-type voltage-gated calcium channels (Tallent et al., 1994). After G-protein dissociation, the c-terminus is phosphorylated by G-protein receptor kinase 2 (GRK2) at serine 369 that acts to functionally desensitize the receptor (McLaughlin et al., 2003b). Interestingly, KOR can be desensitized after ligand

binding and GRK2 phosphorylation at Ser-369, yet remain in the membrane without internalization (McLaughlin et al., 2004; McLaughlin et al., 2003b). Prolonged agonist activity at the receptor, however, results in internalization via beta-arrestin recruitment at the sites of phosphorylation (McLaughlin et al., 2004). Dephosphorylation occurs via protein phosphatase 1 and 2a allowing for receptor recycling to the membrane (Appleyard et al., 1997).

Location and Function

KORs are widely expressed throughout both the peripheral nervous system (PNS) and central nervous system (CNS). Within the PNS, KOR are expressed on primary sensory afferent neurons, within the gastrointestinal tract, and heart, and compounds with selective KOR agonist properties have been considered as potential treatment strategies for maladies of these regions (Galligan and Sternini, 2017; Snyder et al., 2018; Sobanski et al., 2014). For example, KORs are expressed throughout primary afferent neurons on the cell body, dorsal root ganglion, and terminals within the spinal cord and, like mu- and delta-opioid receptors, activation of KOR within peripheral nerves has analgesic properties (Ji et al., 1995; Snyder et al., 2018). In fact, peripherally restricted KOR agonists attenuate the behavioral response to thermal allodynia, sciatic nerve injury, and hind-paw inflammation suggesting involvement of KOR in various pain models (Berg et al., 2011; Jamshidi et al., 2015). KOR agonists have spinal anesthetic properties and modulate the peripheral response to pain through reduced excitatory neurotransmission from sensory input to the CNS. Opioid receptors are

widely expressed in the gastrointestinal tract and morphine, through agonist action at the mu-opioid receptor, has strong analgesic properties, but reduces intra-gastric contractions that cause constipation (Pasternak and Pan, 2013). KOR are also expressed within the gastrointestinal tract and modulate acetylcholine release at the neuromuscular junction within the colon (Chamouard et al., 1993; Riviere, 2004). The use of KOR agonists to treat chronic pain is viable alternative to classic opiates because they do not cause gastrointestinal distress and there is little concern for dependence or abuse (Riviere, 2004). Of concern for drug development, KOR are expressed on myocardial cells within the heart and have anti- and pro- arrhythmic effects depending on dosage (Coles et al., 2003; Mousa et al., 2010). However, KOR agonists reduce the size of myocardial infarction and show promise for the prevention of tissue damage caused by heart attack (Peart et al., 2004). Finally, KOR agonists have also been considered for drug development given the anti-pruritic, anti-inflammatory, and anti-emetic properties of peripheral KOR activation (Phan et al., 2012; Liang, 2016; Porreca et al., 2009).

Within the CNS, KOR are expressed in brain regions implicated in the experience of consciousness, mood/affect, and motivational behavior, such as the claustrum, frontal cortex, mesolimbic dopamine system, substantia nigra, hypothalamus, and the extended amygdala network (Bruchas et al., 2010; Chavkin, 2013; DePaoli et al., 1994; Simonin et al., 1995). Interestingly, KOR agonists have a profound effect on human consciousness and use of plants containing naturally occurring compounds with KOR agonist properties, such as *Salvia divinorum*, have been used in religious ceremonies or for medicinal

purposes by shamans in South America and Mexico for many years (Valdes et al., 1983). More specifically, salvinorin A is a selective KOR agonist found within the leaves of the *Salvia divinorum* (salvia) plant and is considered to be one of the most potent naturally occurring hallucinogenic drugs (Chavkin et al., 2004; Roth et al., 2002). Inhalation of vapor derived from extracts of dried salvia leaves or ingestion of plant material has extreme hallucinogenic and dissociative properties (Valdes, 1994). Individuals report profound changes in all sensory modalities, out of body experiences, and psychotomimetic effects during clinical experimentation with salvinorin A (Pfeiffer et al., 1986; Siebert, 1994; Valdes, 1994). In support of DYN/KOR-mediated psychotomimetic effects, DYN levels are increased in the CSF of patients with schizophrenia and KOR antagonists attenuate hallucinations in these subjects (Gunne et al., 1977; Heikkila et al., 1990). Interestingly, documentation of experiences with salvinorin A have played an important role in unraveling one of the greatest unanswered questions in neuroscience, the "problem of consciousness". In the last article written before his death, Francis Crick postulated that the claustrum, a structure rich in KOR and located between the insular cortex and striatum, may act as a "grand conductor of consciousness" (Crick and Koch, 2003). The claustrum is highly interconnected with cortical structures and is involved in the integration of sensory modalities and the experience of "self". Thus, it is not surprising that activation of KOR by salvinorin A results in vivid hallucinations, disrupted perception of self and, what many refer to as ego death or ego dissolution (Stiefel et al., 2014). Even though the psychotropic effects of acute salvia intoxication resemble that of psychedelic

compounds such as psilocybin, lysergic acid diethylamide, or dimethyltryptamine, salvia is not classified as a psychedelic because it does not have agonist properties at the 5-HT2A receptor (Roth et al., 2002). Even more interesting, salvinorin A decreases release of DA in the NAc setting this recreational drug apart from drugs of abuse (Zhang et al., 2005). Indeed, the use of salvia has gained popularity but the pattern of usage does not reflect abuse potential due to the dysphoric properties of the drug (SAHMSA, 2012). These unique properties have led many to be interested in the development of KOR agonists for various treatments due to the lack of abuse potential.

Aversion, Stress, and Anxiety

Within the CNS, however, KOR antagonists are more commonly investigated as treatment strategies for psychiatric disorders involving negative affective states, such as those of anxiety, depression, and addiction. This is because KOR agonists, while novel in their analgesic properties, promote dysphoric states in humans and rodents (Pfeiffer et al., 1986; Roth et al., 2002). More specifically, rodents display a variety of behaviors associated with dysphoria in response to KOR agonists such as aversion, a stress response, and anxiety-like behavior, all of which are critical components of alcohol withdrawal (Bals-Kubik et al., 1993; Lalanne et al., 2014; Shippenberg et al., 2001). For example, early studies revealed that repeated treatment with a KOR agonist produced conditioned place aversion (CPA) to a drug-paired environment, suggesting that KOR activation has aversive properties (Shippenberg and Herz, 1986). Further studies

revealed a dynamic relationship between DYN/KOR and DA signaling in the NAc that mediate aversion (Di Chiara and Imperato, 1988; Maisonneuve et al., 1994). KOR are found on presynaptic terminals of NAc-projecting dopaminergic neurons within the VTA (Margolis et al., 2003; Svingos et al., 1999). During reward-related behavior or exposure to drugs of abuse, DA is released from these VTA terminals in the NAc. Upon release, DA binds D1 receptors on medium spiny neurons (MSNs) that express DYN. D1 MSNs within the medial shell send long range projections to the VTA and release GABA and DYN onto dopaminergic cells therein and inhibit downstream release (Yang et al., 2018). More prominently, Gs-mediated intracellular signaling after D1 MSN activation drives local release of DYN that activates KOR on VTA terminals and decreases DA release in the NAc (Al-hassani). DYN and KOR agonists alike decrease extracellular DA release within the NAc through Gi-mediated signaling on presynaptic VTA terminals (Margolis et al., 2003; Spanagel et al., 1992). Intracellular KOR signaling also enhances activity of the DA transporter (DAT) that increases clearance of DA from the extracellular space (Thompson et al., 2000). Thus, DYN/KOR activation in the NAc acts as a negative feedback mechanism gating mesolimbic DA activity. In the context of aversion, selective delivery of a KOR agonist into the NAc decreases local DA release and is sufficient to drive CPA (Bals-Kubik et al., 1993; Donzanti et al., 1992). A series of elegant studies from the Bruchas lab revealed that photostimulation of DYN-containing neurons within the ventral NAc shell results in the display of real-time place aversion and decreased responding for a natural reward, both of which are reversed by KOR antagonist challenge (Al-Hasani et al.,

2015). Furthermore, genetic deletion of KOR from VTA-DA terminals in the NAc blocks CPA learning and can be restored by selective KOR knock-in (Chefer et al., 2013). These data suggest that DYN-containing neurons within the NAc contribute to aversive-like behavior in a KOR-dependent fashion, likely though the modulation of local DA release. However, KOR in the NAc also modulate CPP and, more specifically, photoactivation of dorsal NAc shell DYN-containing neurons has rewarding properties suggesting that topographical organization of KOR expression may differentially regulate opposing behaviors (Al-Hasani et al., 2015; Castro and Berridge, 2014).

It is also important to note that aversive behavior is also observed after microinjection of a KOR agonist into the PFC and VTA, and there is strong evidence suggesting that the aversive properties of KOR activation in the VTA is due to disrupted DA release within the PFC (Chavkin, 2013; Knoll and Carlezon, 2010; Tejeda et al., 2013). In fact, KOR agonist microinjection into the VTA induces CPA by decreasing activity in projections to the PFC, but projections to the NAc are not affected (Bals-Kubrick et al., 1993). This finding was bolstered by the Shippenberg lab who demonstrated that KOR agonists decrease DA overflow within the PFC and this effect can be blocked by genetic deletion of KOR on DAT-expressing neurons, suggesting KOR regulation of DA release within the PFC (Tejeda et al., 2013). Furthermore, systemic KOR agonist challenge resulted in CPA, as expected, but was blocked by selective KOR antagonist microinjection in the PFC. Decreased DA tone within the PFC and NAc have important clinical implications because positive valence disorders, such as depression, addiction,

some aspects of schizophrenia, and ADHD involve disruption to DA signaling in the PFC and NAc that mediates cognitive symptomology and reward deficits (Braver et al., 1999; Brennan and Arnsten, 2008; Ford et al., 2006).

We have thus far described involvement of the DYN/KOR system in cognition and the balance of reward/aversion. Activity of DYN/KOR is also involved in the dynamic processes that mediate the physiological response to stress as well as behaviors involved in the stress response. Preclinical models of stress, such as the forced swim stress (FSS) task or fear conditioning have revealed an endogenous release of DYN in response to stress exposure (Land et al., 2008; McLaughlin et al., 2003a; Nabeshima et al., 1992). Release of DYN within the CNS has a number of effects that promote an internal stress response, which is not surprising given the expression pattern of DYN/KOR within the hypothalamus. Most notably, endogenous KOR activity in the hypothalamus triggers activation of the HPA axis and release of CRF, ACTH, and corticosterone (Drolet et al., 2001; Nikolarakis et al., 1987). Similarly, systemic administration of a KOR agonist increases c-Fos expression within the PVN and is accompanied by increased serum corticosterone levels indicative of pharmacological activation of the HPA axis (Buckingham and Cooper, 1986; Pechnick, 1993). Although these studies are correlative in nature, they suggest involvement of KOR in the physiological response to acute stress. More causal studies have been conducted in relation to DYN/KOR involvement in the behavioral response to repeated stress exposure. For example, KOR antagonists or global *Pdyn*-knockout blocks the emergence of pro-depressive behavior as a result of repeated foot-shock stress (Land et al.,

2008). Furthermore, associative aversive learning involving an odor or context paired with a foot-shock is blocked by systemic KOR antagonist challenge or *Pdyn*-knockout (Land et al., 2008). Similarly, repeated exposure to FSS results in increased immobility indicative of a pro-depressive state and is blocked by systemic KOR antagonist treatment (Mague et al., 2003; McGlaughlin et al., 2003). Increased immobility resulting from chronic FSS can be recapitulated in naïve animals when challenged with a KOR agonist (McGlaughlin et al., 2006). Involvement of KOR in response to the other ethologically relevant stressors, such social defeat stress, has also been demonstrated (Donahue et al., 2015). As with foot-shock or FSS, treatment with a KOR antagonist or *Pdyn*-knockout decreased the display of submissive posture, heightened nociception, and analgesia observed after social defeat stress (McGlaughlin et al., 2003).

Foot-shock, FSS, and social defeat stress provoke DYN release and indicate activity of KOR in the behavioral response to stress (Knoll et al., 2010). CRF is also released in response to these stressors and it appears that CRF can stimulate release of DYN in the hypothalamus, suggesting a dynamic interaction between the DYN and CRF systems involved in the stress response (Almeida et al., 1986). For example, systemic challenge with CRF increases KOR phosphorylation, indicative of CRF activity provoking DYN release and KOR activation (Land et al, 2008; Bruchas et al., 2010). From a behavioral perspective, administration of CRF (ICV) promotes aversive learning and the expression of CPA is blocked by pharmacological blockade of KOR or *Pdyn*-knockout (Land et al.,

2008). These data suggest a reciprocal interaction between the CRF and DYN/KOR systems as both a cause and causality involved in the stress response.

The DYN/KOR system has also been strongly implicated in anxiety-like behavior but a clear role has been difficult to ascertain due to similar behavioral outcomes in response to KOR agonists and antagonist. This discrepancy arises from studies demonstrating that acute systemic treatment with a KOR agonist or antagonist both promote an anxiolytic phenotype (Knoll et al., 2007; Kuzmin et al., 2006; Narita et al., 2006). Further studies have revealed a more prominent role for DYN/KOR activation in promoting anxiety-like behavior. For example, global *Pdyn*-knockout attenuates the startle response and presents as an anxiolytic phenotype within the open field, elevated plus maze, and light/dark box tests (Bilkei-Gorzo et al., 2008; Wittmann et al., 2009). Anxiogenic behavior is restored within knockout mice, and potentiated in wild-type mice, by systemic challenge with a KOR agonist, suggesting that endogenous DYN/KOR activity promotes the expression of anxiety-like behavior (Wittmann et al., 2009). Furthermore, KOR antagonists increase open arm entries in the elevated plus maze and decrease the fear-potentiated startle response indicative of anxiolytic properties of the drug (Knoll et al., 2007).

The extended amygdala is rich in DYN/KOR and recent studies suggest that connectivity between the CeA the BNST is involved in the expression of anxiety-like behavior (Alheid, 2003; Fallon and Leslie, 1986; Mansour et al., 1994; Marchant et al., 2007; Poulin et al., 2009; Sim-Selley et al., 1999). For example, projection neurons in the CeA express and co-express various neuropeptides,

such as DYN, somatostatin (SST), and CRF. Optogenetic stimulation of CeA projections to the BNST (CeA-BNST) produces an anxiogenic phenotype in an open field task (Ahrens et al., 2018). Neurons within this circuit largely express *Pdyn* mRNA (along with *SST*) and the anxiogenic response resulting from circuit-level activation can be blocked by KOR antagonist administration into the BNST. Interestingly, microinjection of the long-lasting KOR antagonist nor-BNI into the BNST alone does not affect anxiety-like behavior in the open field task suggesting that strong stimuli that provoke DYN release within the BNST are involved in the expression of anxiety, opposed to tonic dynorphinergic tone. In contrast, DYN-containing neurons within the BNST release DYN locally and may promote anxiety (Crowley et al., 2016). The mechanism by which KOR in the BNST modulates anxiety is not completely clear, but it is likely through affecting excitatory/inhibitory balance through the regulation of glutamate/GABA release in select circuits. For example, activation of KOR in the BNST decreases presynaptic release of GABA through an ERK-dependent signaling mechanism from projections originating in the CeA (Li et al., 2012). This is in stark contrast to p38-MAPK signaling that mediates the dysphoric component of KOR activation in other structures (Bruchas et al., 2007). Interestingly, activation of BLA projections to the BNST results in anxiolytic behavior presumably through glutamate release downstream in the BNST, and optogenetic stimulation of DYN-containing neurons within the BNST decreases glutamate release by activation of KOR on BLA terminals (Crowley et al., 2016). The behavioral consequence of KOR modulation of glutamatergic and GABAergic terminals may depend on the topographical input to sub-compartments

in the BNST that modulate anxiety. More specifically, the oval, anterodorsal, and ventral BNST are involved in anxiety and direct GABAergic input onto these population may be anxiolytic (Jennings et al., 2013; Kash and Winder, 2006; Kim et al., 2013; Lebow and Chen, 2016; Lovinger and Kash, 2015). However, there are numerous GABAergic interneurons within the BNST and KOR antagonists may restore glutamate release onto these subpopulations driving feed-forward inhibition of anxiogenic populations. Thus, KOR antagonists may promote anxiolytic behavior directly, or indirectly through local circuits, by inhibition of anxiogenic hotspots in the BNST.

Given the complex interplay with CRF and DYN in the stress response, it is not surprising that both are synergistically involved in anxiety. This interaction was clearly demonstrated by the Chavkin lab who showed that central administration of CRF provokes an anxiogenic phenotype in the elevated plus maze that is blocked by nor-BNI treatment within the BLA (Bruchas et al., 2009). Because KOR agonists decrease LTP and glutamate release in the BLA, it is tempting to speculate that intra-BLA nor-BNI disinhibits the BLA-BNST circuit thereby promoting anxiolytic behavior, but this has not been directly tested to the best of our knowledge (Huge et al., 2009; Crowley et al., 2016). CRF and DYN largely co-localize within the nearby CeA and compensatory mechanisms have been observed in CRF expression after genetic alteration of DYN (Pomrenze et al., 2015; Wittmann et al., 2009). For example, global *Pdyn*-knockout or nor-BNI treatment decreased *Crf* mRNA expression in the CeA (and PVN), which is associated with decreased expression of anxiety-like behavior (Wittmann et al.,

2009). This is interesting because it demonstrates an influence of DYN/KOR activity on CRF expression, whereas CRF1 activation also promotes DYN release in other regions (Bruchas et al., 2009). Finally, direct infusion of CRF into the CeA is anxiogenic and enhances excitability of CeA neurons that project to the BNST (Asok et al., 2018). Indeed, anxiogenic neurons within the CeA-BNST circuit are known to express DYN and CRF, and expression of anxiety-like behavior is dependent upon KOR and the CRF1 receptor activity in the BNST (Ahrens et al., 2018; Pomrenze et al., 2019). Thus, DYN and CRF play complimentary roles within the extended amygdala that contribute to anxiety-like behavior.

DYN/KOR IN AUD AND DEPENDENCE

Clinical Perspective

Given the role of DYN/KOR in aversion, stress, and anxiety, it is not surprising that this system has gained much attention as a druggable target for the treatment of indices of negative effect in individuals with AUD. Indeed, examination of polymorphisms in the genes coding for DYN and KOR have revealed a strong association with AUD and dependence. More specifically, a single nucleotide polymorphism (SNP) in the *PDYN* gene has been identified in patients with AUD and is associated with drinking severity (Preuss et al., 2013; Williams et al., 2007; Xuei et al., 2006). In fact, *PDYN* SNPs are associated with negative craving and predict the likelihood of drinking severity in response to stress (Karpyak et al., 2013; Preuss et al., 2013). SNPs in the *Oprk1* gene coding for KOR are similarly found in individuals with AUD and are predictive of the severity of symptoms

assessed by AUDIT (Edenberg et al., 2008; Park et al., 2020). Furthermore, polymorphisms in *PDYN* and *Oprk1* are associated with increased impulsivity, anxiety, and negative craving that predicts drinking severity (Edenberg et al., 2008; Park et al., 2020; Votinov et al., 2014; Xuei et al., 2006; Xuei et al., 2007). A more casual role for KOR in AUD has been identified in clinical studies through the use of the non-selective opioid antagonist naltrexone. Naltrexone is primarily thought to reduce alcohol intake with greater efficacy in individuals with a select polymorphism in the gene coding for the mu-opioid receptor (MOR), although there is some evidence suggesting this effect may be mediated by the KOR (Anton, 2008; Oroszi et al., 2009). For example, alcohol-dependent individuals show less KOR bioavailability in the amygdala, insular cortex, frontal cortex, and striatum, all regions implicated in excessive drinking, withdrawal, and craving (Vijay et al., 2018). Furthermore, naltrexone was found to decrease craving and alcohol intake in nontreatment-seeking heavy drinkers and this response was associated with KOR availability prior to treatment (de Laat et al., 2019). Similarly, nalmefene is a MOR antagonist and partial KOR agonist that has been shown to reduce the frequency and intensity of drinking in alcohol-dependent subjects (Mann et al., 2013; van den Brink et al., 2013). Therefore, the development and application of selective KOR antagonists for the treatment of AUD has shown promise, and preclinical studies have been successful in demonstration the underlying processes that mediate these effects.

Preclinical Models of AUD

For roughly 30 years, the relationship between DYN/KOR activity and alcohol consumption has been studied in preclinical models of AUD. Because KOR are located within brain regions that are involved in reward and aversion, much work has been done to determine the contribution of KOR to alcohol-induced CPP and CPA. However, a clear role for KOR within these behaviors has been difficult to ascertain due to mixed results. For example, systemic administration of the KOR agonist, U50,488, 10-min before alcohol conditioning blocks CPP learning in DBA/2 mice (Logrip et al., 2009). However, U50,488 treatment 90-min before alcohol pairings potentiates CPP in C57BL/6J mice, suggesting that the anxiolytic properties of alcohol relieved the aversive state driven by U50,488 (Sperling et al., 2010). However, a provocateur of KOR signaling, be it endogenous DYN release in response to stress or exogenous administration of a KOR agonist, may be necessary to uncover KOR involvement in CPP. For example, FSS drives endogenous release of DYN and repeated FSS prior to conditioning potentiates CPP expression, an effect that is blocked by nor-BNI (McLaughlin et al., 2003a; Sperling et al., 2010). Interestingly, nor-BNI alone has no effect on CPP learning or expression, but does potentiate state-dependent CPP expression (Nguyen et al., 2012). These data are indicative of a facilitation of reward-pathway signaling during alcohol challenge, stress, or KOR antagonist administration that potentiates the rewarding properties of alcohol. Few studies, however, have explored the relationship between CTA and the DYN/KOR system. Nor-BNI does not affect alcohol-induced CTA during conditioning or testing but does attenuate CTA in

stressed rats suggesting potentiation of the aversive properties of alcohol by stress in a KOR-dependent manner (Anderson et al., 2013; Roma et al., 2008). Thus, further studies are necessary to determine a clear role for KOR in mediating reward/aversion related to alcohol.

In the context of voluntary home-cage alcohol drinking, systemic administration of a KOR antagonist generally reduces intake that has been observed across multiple limited-access drinking paradigms ranging from 30-min to 24-hours of access (Anderson and Becker, 2017; Karkhanis et al., 2017). More specifically, nor-BNI reduced alcohol drinking during a 3-hour 2-bottle choice session in Rhesus monkeys and produced a reduction of intake in male C57BL/6J mice during 18- and 24-hour access to alcohol (Logrip et al., 2008; Williams and Woods, 1998; Zhou et al., 2017). However, the effect of a KOR antagonist on home-cage drinking is not consistent given that, under similar experimental conditions, either no effect or an increase in drinking has been observed. For example, nor-BNI reduced alcohol intake in female Long Evans rats during a 30-min drinking session but increased drinking in males, suggesting possible sex differences in responsiveness to KOR antagonists (Morales et al., 2014). Nor-BNI also increased drinking in a subset of male Lewis rats that were high drinkers, but had no effect in females (Lindholm et al., 2001; Mitchell et al., 2005). Effects of KOR agonists on voluntary consumption have also been inconsistent across species and drinking paradigms. More specifically, chronic administration of enadoline increased alcohol intake in Wistar rats as does U50,488 treatment in C57BL/6J mice during continuous access drinking (Holter et al., 2000; Sperling et

al., 2010). Similarly, U50,488 increased alcohol intake during 1- and 2-hour limited access drinking sessions in C57BL/J5 mice (Anderson et al., 2016; Rose et al., 2016). However, others have shown that U50,488 decreased intake in rats in both 2- and 24-hour access drinking paradigms (Lindholm et al., 2001; Sandi et al., 1990). Thus, differences in species, strain, time-course of drug delivery, and experimental design likely account for the inconsistency of the results in these studies.

Although results have been mixed in the context of home cage drinking, a clear role for KOR has been demonstrated in models of operant self-administration in rats. Systemic administration of a KOR antagonist decreased operant self-administration, blocked spontaneous recovery, and lowered progressive ratio breakpoints in Wistar and P-rats, respectively (Deehan et al., 2012; Rorick-Kehn et al., 2014; Schank et al., 2012; Walker et al., 2011). Furthermore, KOR antagonists block alcohol-seeking behavior in response to cues associated with alcohol or a pharmacological stressor, such as yohimbine (Schank et al., 2012; Funk et al., 2014). In fact, systemic challenge with U50,488 acts like a pharmacological stressor and is sufficient to induce reinstatement behavior in rats under extinction conditions, and this effect is blocked by nor-BNI (Funk et al., 2014; Hayes and Stewart, 1985). Studies involving mice, however, are mainly conducted in the context of home-cage drinking whereas the vast majority of studies involving reinstatement involve rats. This is particularly true in the context of models of alcohol dependence where rats typically self-administer alcohol opposed to mice

that are commonly presented alcohol in limited-access home cage drinking paradigms.

Recently, focus has shifted toward probing DYN/KOR involvement dependence and negative affect associated with withdrawal that drives excessive drinking, aka the "dark side of addiction" (Koob, 2013; Schulteis and Koob, 1994). Rodent models of alcohol dependence, such as the chronic intermittent ethanol (CIE) exposure paradigm, produce a phenotypic escalation of voluntary alcohol consumption, withdrawal-related sequalae, and genetic adaptions that are seen in humans thus serving as a robust platform for the study of AUD (Becker and Lopez, 2004; Lopez and Becker, 2005; Melendez et al., 2012; Osterndorff-Kahanek et al., 2015). The CIE model involves repeated cycles of alcohol vapor exposure and withdrawal that mimics repeated bouts of binge drinking and withdrawal observed in humans. In a groundbreaking study, Walker and Koob compared the effects of naltrexone, nalmefene, and the selective KOR antagonist nor-BNI on alcohol self-administration in CIE-exposed Wistar rats (Walker and Koob, 2008). Interestingly, naltrexone and nalmefene had a general effect on decreasing responding for alcohol, but nor-BNI selectively decreased self-administration in dependent rats without affecting moderate responding and intake in non-dependent subjects (Walker and Koob, 2008). These data suggest that the DYN/KOR system in particular is sensitive to a history of dependence/CIE exposure and further studies have supported this notion. For example, systemic challenge with nor-BNI decreased high levels of alcohol intake and attenuated anxiety-like behavior during withdrawal from CIE exposure (Walker et al., 2011). The anxiolytic properties of

KOR antagonists have been demonstrated in measures of withdrawal-induced anxiety, such as marble burying, elevated plus maze, and ultrasonic vocalizations (Rose et al., 2016; Berger et al., 2013; Schank et al., 2012). The extended amygdala is a likely culprit in mediating these effects because this macrostructure is rich in DYN/KOR and involved in excessive alcohol consumption (Al-Hasani et al., 2015; Bloodgood et al., 2020; Marchant et al., 2007). In fact, site-specific delivery of nor-BNI into the NAc, CeA, or BNST attenuates dependent-like drinking in CIE-exposed subjects without affecting drinking in non-dependent controls (Erikson et al., 2018; Kissler et al., 2014; Nealey et al., 2011). We will describe the role of KOR within the NAc, CeA, and BNST below in further detail.

We have previously described the role of DA within the NAc in relation to the motivation to drink excessively and it is theorized that KOR antagonists may be effective in restoring dopaminergic homeostasis in the NAc under extreme conditions such as alcohol dependence. For example, a history of alcohol dependence achieved through CIE exposure results in increased expression of *Pdyn* mRNA within the NAc and selective microinjection of nor-BNI decreased dependent-like drinking (Karkhanis et al., 2015; Nealey et al., 2011). Alcohol is known to increase extracellular DA in the NAC but this response is inverted in CIE-exposed mice in that DA levels decrease in response to acute alcohol challenge (Karkhanis et al., 2016). Furthermore, DA release within the NAc is decreased in dependent mice through enhanced functionality of KOR on VTA terminals (Karkhanis et al., 2015). The hypodopaminergic state observed during withdrawal is specific to alcohol-dependent mice and reflective of increased sensitivity of KOR

to agonists. The functional consequence of the so-called CIE-induced "super-sensitivity" to KOR agonists is a hypodopaminergic state that promotes dysphoria, anxiety, and drug-seeking, which can be blocked by nor-BNI (Rose et al., 2016). This effect has been observed in rodent models as well as in non-human primates suggesting a conserved mechanism across species in the development of AUD-like phenotypes (Siciliano et al., 2015; Siciliano et al., 2016). In support of the hypothesis that KOR-mediated suppression of DA activity drives negative affective states, microinjection of a KOR agonist into the NAc promotes anhedonia-like behavior that is blocked by a KOR antagonist (Muschamp et al., 2011). It is reasonable to suspect then that KOR antagonists would, in theory, block KOR activity on VTA terminals and disinhibit DA release in the NAc, reflecting rewarding properties and a potential for abuse. Indeed, KOR antagonists increased extracellular DA and KOR knockout potentiated evoked-DA release in the NAc, but ICSS reward threshold is not affected (Karkhanis et al., 2016; Todtenkopf et al., 2004; Zapata and Shippenberg, 2006). Thus, it is theorized that DYN/KOR modulation of phasic DA release in the NAc is activity dependent and that KOR antagonists may be effective in restoring homeostasis under extreme conditions, such as alcohol dependence. It is also important to note that increased *Pdyn* mRNA has been observed within the NAc shell of seizure-prone mice during withdrawal from chronic alcohol (Beadles-Bohling and Wiren, 2005). Nor-BNI increased seizure activity in these mice during early withdrawal, although administration was via systemic injection and not localized to the NAc (Beadles-Bohling and Wiren, 2006). Furthermore, systemic U50,488 increased seizure

threshold suggesting opposing KOR involvement in seizure activity and withdrawal related-negative affect. Future studies with targeted treatment will be of importance.

Less is known about DYN/KOR function within the CeA and BNST in the context of alcohol dependence but the existing data support a similar role in excessive drinking and withdrawal-related negative affect. For example, *Pdyn* expression and DYN-A immunoreactivity are increased within the CeA after chronic alcohol treatment and KOR therein display increased GTPyS signaling indicative of super-sensitivity, similar to the that observed in the NAc (D'Addario et al., 2013; Kissler and Walker, 2016). Kissler and collogues further demonstrated that microinjection of nor-BNI into the CeA selectively decreased drinking in dependent rats suggesting that the increase in KOR sensitivity contributes to excessive drinking (Kissler et al., 2014). However, physical signs of withdrawal were not affected by nor-BNI thus KOR within the CeA appear to play a predominant role in drinking behavior that is dissociable from negative affect. The exact mechanism driving the decrease in drinking is not entirely clearly, but could be through modulation of GABA release onto select populations within the CeA. For example, output neurons of the centromedial amygdala (CeM) are known to promote alcohol drinking and seeking behavior (Gilpin et al., 2015; Roberto et al., 2012). Acute alcohol increases GABA release within the CeM and CIE exposure not only increases basal extracellular GABA, but potentates GABA release in response to alcohol (Roberto et al., 2003; Roberto et al., 2004). Interestingly, KOR regulate tonic inhibition within the CeM through modulation of GABA release from

presynaptic terminals (Kang-Park et al., 2013). The KOR agonist, U69593 reduces IPSCs indicative of KOR regulation of tonic GABAergic tone. Acute alcohol increases evoked IPSCs within the CeM from nearby KOR-expressing neurons within the centrolateral amygdala (CeL) and this effect is potentiated by nor-BNI or by genetic deletion of KOR (Kang-Park et al., 2013). GABA within the CeA is also associated with the reinforcing properties of alcohol as both $GABA_A$ antagonists and nor-BNI decrease self-administration (Hyytia and Koob, 1995; Kissler and Walker, 2016). The discrepancy between increased GABAergic tone resulting from CIE-induced dependence and increased GABA release in response to KOR antagonists is not fully understood, but may be reflective of topography of KOR-expressing populations within the CeA. For example, KOR-expressing neurons within the CeL may functionally gate output neurons of the CeM such that nor-BNI restores tonic inhibitory control over this output population resulting in decreased drinking. Thus, future studies mapping expression patterns of KOR within the CeA in relation to output neurons that are known to promote excessive drinking will be of importance.

Much like the CeA, the BNST is highly enriched in DYN/KOR and sensitive to the effects of chronic alcohol. For example, a history of CIE exposure increased alcohol self-administration and upregulated *Oprk1* expression within the BNST (Erikson et al., 2018). Interestingly, *Pdyn* expression was not affected by moderate alcohol self-administration nor CIE exposure. This observed increase in *Oprk1* contributes to excessive drinking because site-specific microinjection of nor-BNI into the BNST decreased alcohol self-administration in dependent rats without

affecting moderate responding in non-dependent control groups (Erikson et al., 2018). In contrast to the CeA, KOR blockade in the BNST decreased ultrasonic vocalizations during withdrawal suggesting a reduction in negative affect (Erikson et al., 2018; Kissler et al., 2014). Thus, KOR within the BNST likely modulate maladaptive excessive drinking behavior by relieving withdrawal-related sequala in the negative affective domain, but the endogenous source of DYN mediating this effect is unknown.

Extended Amygdala and Binge Drinking

We have thus far discussed DYN/KOR involvement within the extended amygdala in relation to excessive drinking as a function of alcohol dependence. Binge drinking, however, is unique in that drinking is excessive yet infrequent, and occurs prior to development of dependence or AUD. Therefore, neuroadaptive processes involved in early binge drinking likely change over time during the transition to dependence that promote frequent, uncontrolled binge drinking. The amygdala has been implicated in binge drinking because it is a critical hub in reward circuitry involved in emotional reactivity and appetitive behavior (Gilpin et al., 2015; Koob, 2003; Roberto et al., 2012; Stephens and Duka, 2008). A history of binge drinking produces alterations in amygdala activity that present as poor mood states, heightened anxiety, altered stress-responsiveness, and increased impulsivity (Stephens and Duka, 2008; Stephens et al., 2005; Townshend and Duka, 2005). Furthermore, human functional magnetic resonance imaging (fMRI) studies show that increased activity at rest in the amygdala is associated with

heightened anxiety and impulsive decision-making in binge drinkers (Xiao et al., 2013). Anxiety and impulsivity have a positive relationship with binge drinking and are associated with aberrant connectivity between the amygdala and the prefrontal cortex (PFC), which reflects deficits in networks mediating top-down control of excessive drinking (Balodis et al., 2009; Stautz and Cooper, 2013). In fact, decreased coupling of amygdala and PFC connectivity predicts long-term alcohol intake as a function of drinking during adolescence, suggesting neuroadaptations within amygdala-circuitry that contribute to the trajectory of AUD (Peters et al., 2017). In contrast, acute alcohol intoxication to binge levels is anxiolytic and dampens amygdala activity, blunting emotional processing and threat responsiveness (Gilman et al., 2008; Gorka et al., 2013). Risky decision-making is a hallmark of binge intoxication and is associated with a dysregulation of emotional processing and decreased inhibitory control over behavior. Therefore, the amygdala plays a critical role in the motivation to consume alcohol excessively and is affected by a history of binge drinking. Given the role of DYN/KOR within the amygdala in preclinical models of AUD, it is likely that this system is recruited to promote binge drinking behavior as well. However, examination of DYN/KOR within the amygdala and projection sites in humans is currently not possible given the limitations of current imaging technology.

An animal models that approximate binge drinking, the Drinking in the Dark (DID) paradigm, has been widely used in the field and proven to be an effective platform for pharmacological and circuit-level interrogation of brain systems that drive binge drinking (Crabbe et al., 2017; Rhodes et al., 2005; Sprow and Thiele,

2012). An important aspect of the DID model is that it captures excessive drinking behavior prior to the development of dependence when neuroadaptations are likely to occur that promote the progression to compulsive, inflexible drinking (Ferguson et al., 2019). The DID model capitalizes on natural rhythms in rodent behavior such that alcohol bottles are present 3 hours into the dark cycle, coinciding with a period of high alcohol consumption. Thus, mice drink more alcohol during this time period when presented with a single bottle of 20% alcohol (vol/vol) compared to any other period in the circadian cycle (Rhodes et al., 2005). Furthermore, BACs achieved after 2 or 4 hours of drinking exceed the 0.08 g/dL legal limit of intoxication and effectively models binge drinking behavior in humans. Indeed, a history of binge drinking results in lasting neuroadaptations that are associated with increased intake over time in mice, rats, non-human primates, and humans suggesting a conserved mechanism across species (Baker et al., 2017; Hingson et al., 2017; Zhou et al., 2017). Further, a growing body of literature has demonstrated involvement of the extended amygdala and neuropeptide systems therein in excessive alcohol drinking in the DID model (Anderson et al., 2014; King et al., 2017; Pleil et al., 2015; Rinker et al., 2017).

There has been considerable interest in the contribution of the DYN/KOR system to excessive drinking and negative affect produced by alcohol dependence, but recent studies suggest a general role in the regulation of binge drinking. For example, we have shown that systemic administration of the KOR agonist U50,488 increased binge-like alcohol consumption in male mice (Anderson et al., 2018). In contrast, systemic administration of the short acting

KOR antagonist LY2459989 decreased binge drinking. These findings compliment those that demonstrate efficacy of KOR antagonists to decrease dependent-like drinking, and are reflective of a general reduction of excessive drinking independent of a history of dependence. Furthermore, these findings are consistent with others that have shown that KOR agonists and antagonists bidirectionally modulate voluntary alcohol consumption, and the extended amygdala is a likely candidate in mediating these effects. The CeA is enriched in dynorphinergic neurons that may promote binge drinking behavior through activation of KOR locally or in downstream structures, such as the BNST (Marchant et al., 2007; Bloodgood et al., 2020). Indeed, a history of binge drinking increased excitability in DYN-containing neurons within the CeA of male mice suggesting a neuroadaptation in endogenous dynorphinergic circuitry underlying excessive drinking (Bloodgood et al., 2020). This rationale is supported by studies demonstrating that chemogenetic inhibition of DYN-containing neurons within the CeA, or genetic deletion of DYN in the CeA, decreased binge-like drinking in male and female mice (Anderson et al., 2018; Bloodgood et al., 2020). These studies demonstrate a clear role for DYN within the extended amygdala, but the downstream KORs mediating these effects are not known. Local release of DYN within the CeA is a possible explanation in that microinjection of nor-BNI or genetic deletion of KOR therein attenuated binge drinking in male mice (Anderson et al., 2018; Bloodgood et al., 2020). However, KOR knockout in the CeA of female mice does not affect binge-like alcohol consumption alluding to possible KOR activity in a downstream structure (Bloodgood et al., 2020). Interestingly, systemic U50,488

increased c-Fos immunoreactivity within the CeA and the BNST, but expression in the BNST strongly correlated with reinstatement of alcohol-seeking (Le et al., 2018). Therefore, KOR in the BNST may more directly contribute to binge drinking. Indeed, the CeA sends dense dynorphinergic projections to the BNST (CeA-BNST^{DYN}) and the observed increase in c-Fos immunoreactivity within the CeA and BNST during bouts of binge drinking may be reflective of circuit level activation (Anderson et al., 2018; Ahrens et al., 2018; Le et al., 2018). A considerable gap exists in the literature in relation to KOR activity in the BNST and CeA-BNST connectivity in the context of binge drinking. Studies addressing this open question are critical to understanding the mechanisms that promote binge drinking prior to the development of dependence.

Summary

Binge drinking is the most common form of excessive drinking and presents a multitude of adverse effects on health and behavior, propelling binge patterns of drinking into the spotlight as a serious epidemiological concern (Sacks et al., 2015; SAMHSA, 2018; WHO, 2018). This pattern of intake is associated with increased engagement in risky behavior, poor health, and increased risk of developing alcohol dependence and/or an AUD (Centers for Disease and Prevention, 2012; Jennison, 2004). Binge drinking is a critical component in the cycle of addiction and understanding the mechanisms that promote binge drinking is important to understanding the trajectory of neuroadaptations that contribute to the development of an AUD. Among several neurochemical and neuropeptide

systems implicated in binge drinking, recent findings suggest a prominent role for the DYN/KOR system. This present dissertation uses a multifaceted approach involving pharmacology, immunohistological assessment of neuronal activity, and chemogenetics to interrogate the DYN/KOR neuropeptide system within extended amygdala circuity as it relates to excessive binge-like alcohol consumption in male and female mice. More specifically, we aim to determine the effect of KOR agonist/antagonist microinjection in the BNST on binge drinking behavior. We then examine endogenous activity of CeA projections to the BNST during drinking and functionally probe the role of the CeA-BNST^{DYN} circuit as it relates to binge-like alcohol consumption. Results from these studies provide important information regarding this neuropeptide system as a potential target for the development of therapeutics in treating individuals that engage in dangerous, excessive levels of alcohol drinking and provide valuable insight into the trajectory of AUD.

CHAPTER 2: Kappa Opioid Receptors in the Bed Nucleus of the Stria Terminalis Regulate Binge-Like Alcohol Consumption in Male and Female Mice.

INTRODUCTION

As described in Chapter 1, a growing body of literature has focused on the dynorphin/kappa opioid receptor (DYN/KOR) system in mediating excessive drinking (Anderson and Becker, 2017; Koob and Le Moal, 2008; Walker and Koob, 2008). Activation of the DYN/KOR system following chronic alcohol exposure has been associated with behaviors reflective of a negative affective state experienced during alcohol withdrawal, and this has been suggested to increase relapse vulnerability as well as promote excessive levels of drinking (Karkhanis et al., 2017; Sirohi et al., 2012). In fact, KOR antagonists have garnered much interest as a potential therapeutic intervention for the treatment of AUD (Karkhanis et al., 2017). In support of this idea, studies have shown that systemic administration of the KOR antagonist, nor-BNI, attenuates dependence-related escalation of alcohol consumption and alleviates withdrawal symptomology in rats (Walker et al., 2011). These effects appear to be mediated by blockade of KORs in the extended amygdala as direct injection of nor-BNI into the central nucleus of the amygdala (CeA), bed nucleus of the stria terminalis (BNST), or nucleus accumbens shell (NAc shell) reduced elevated drinking and anxiety-like behavior in alcohol dependent animals (Erikson et al., 2018; Kissler et al., 2014; Rose et al., 2016).

While these findings strongly implicate a role for the DYN/KOR system within the extended amygdala in alcohol dependence, recent findings suggest a more general role for KORs in the regulation of alcohol consumption in non-

dependent animals. For example, mice lacking prodynorphin or KORs show decreased alcohol drinking and preference (Blednov et al., 2006; Kovacs et al., 2005; Van't Veer et al., 2016). However, few studies have probed the role of KORs within the context of binge drinking. The Drinking-in-the-Dark (DID) paradigm models binge drinking in rodents by producing high levels of alcohol consumption within a relatively short period of time such that subjects reliably achieve BACs above the 80 mg/dL threshold of intoxication (Rhodes et al., 2005; Thiele et al., 2014; Thiele and Navarro, 2014). We previously demonstrated that systemic administration of a KOR agonist increased, while a KOR antagonist decreased, binge-like alcohol consumption in male C57BL/6J mice (Anderson et al., 2018a). Since the BNST is rich in KORs and sensitive to alcohol (Burnham and Thiele, 2017; Poulin et al., 2009), the present study examined whether manipulation of KORs in the BNST influence binge-like alcohol consumption using the DID model.

MATERIALS AND METHODS

Subjects

Male and female C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) ranging from 10-12 weeks old were singly housed and tested in a temperature and humidity controlled AAALAC approved facility on a reverse 12-hr light/dark cycle with food and water continuously available. For all experiments, mice were treated in accordance with both the NIH Guide for the Care and Use of Laboratory Animals (National Research Council, 2011) and the Institutional Animal Care and Use Committee at MUSC.

Surgical Procedures

Mice were anaesthetized with isoflurane and bilateral guide cannula (Plastics One, Inc.) were positioned above the BNST (AP: +0.8, ML: +/- 0.75, DV: -3.6) and secured to the skull with a light-cured resin system (Haun et al., 2018). Once inserted, microinjector tips extended 1 mm beyond the guide to target the BNST for microinjection. After surgery, all mice were given 2 weeks to recover prior to the start of experiments.

Alcohol Binge Drinking Procedure

After recovery from surgery, mice were habituated to the microinjection procedure by removing and then replacing dummy guides from the microinjector tract 30 minutes prior to drinking. Similarly, for studies involving systemic administration of drug, mice were habituated to the injection procedure by administering daily intraperitoneal (ip.) injections of vehicle for several days before alcohol access and then at 30 minutes prior to drinking sessions. Mice were trained to drink alcohol in their home cage in the limited access "Drinking-in-the-Dark" (DID) procedure, as previously described (Anderson et al., 2018a). A single bottle of alcohol (20% v/v) was presented 3 hours into the dark cycle in place of the water bottle. Access to alcohol was for 2 hours on 3 consecutive days, and then extended for 4 hours on the 4th day. Alcohol intake was determined for each 2-hour session and then for the 0-2 and 2-4 hour time periods during the final (4th day) 4-hour drinking session. An identical procedure was used for assessing sucrose (0.5% w/v) consumption. All mice were given one 4-day cycle of binge drinking, 3 days rest in the home cage, followed by a second 4-day binge cycle. For all experiments,

drug challenges occurred during the second binge cycle, prior to the 4-hour access (test) session. Average drinking across the first 3 days of the second binge cycle were used to separate subjects evenly into drug treatment groups. Immediately after the 4-hour test drinking session, blood samples were collected, plasma extracted, and blood alcohol concentrations determined using an AM1 Alcohol Analyzer (Analox Instruments, Stourbridge, UK). Separate, experimentally-naïve groups of mice were used in each experiment to ensure that a history of prior alcohol or sucrose consumption did not influence drinking or locomotor activity.

Locomotor Activity Test

Activity chambers (ENV-510; Med Associates) were used to assess locomotor activity as previously described (May et al., 2015). Briefly, the open field arenas measured 27.5 cm wide × 27.5 cm long × 20.5 cm deep. Mice were placed into the locomotor activity apparatus 16-hours after microinjection and distance traveled (cm) was measured in 1 min bins for 10 minutes. Additionally, cumulative time spent in the center (10 cm) of the open field arenas was collected to assess possible pharmacological effects on anxiety-like behavior.

Drugs

The KOR antagonist nor-Binaltorphimine dihydrochloride (nor-BNI; 2.5 µg/side, Tocris) and the KOR agonist U50,488 (0, 0.1, 0.2 µg/side) was dissolved in 1xPBS for microinjection. The KOR agonist U50,488 (5 mg/kg; Tocris) was dissolved in 0.9% saline. Doses of nor-BNI and U50,488 were based on previous studies (Anderson et al., 2018a).

Microinjection Procedures

Microinjections of nor-BNI were administered 16 hours before Day 4 of drinking during the second binge cycle in a between-subjects design. Vehicle or nor-BNI (2.5 μg/side) was delivered bilaterally into the BNST at 0.25 μL/min for 2-min, followed by a 2-min diffusion period before microinjector removal (Anderson et al., 2018a; Griffin et al., 2014; Haun et al., 2018). Obdurators were replaced and mice returned to their home cage where they remained undisturbed until the following day. Microinjections of U50,488 (0, 0.1, 0.2 μg/side) occurred 30 min prior to drinking on Day 4 of the second, third, and fourth binge cycles in a counterbalanced, within-subjects design. For systemic administration, saline or U50,488 (5 mg/kg) was administered via intraperitoneal (ip) injection (10 ml/kg) 30-min prior to the 4-hour test binge session.

Histology

At the conclusion of all experiments, mice were euthanized with urethane and transcardially perfused with 10 mL saline followed by 10 mL of paraformaldehyde (PFA; 4%). Brains were extracted, post fixed in 4% PFA for 24 hours and cryoprotected in sucrose (30% wt/vol) until sectioning. Tissue was sliced in serial 40 μM sections and mounted on Permafrost slides. The tissue was then dehydrated in alcohol and stained with Cresyl Violet for histological verification of microinjector placement within the BNST, as previously described (Haun et al., 2018). Only mice with verified bilateral placements in the BNST in reference to a mouse stereotaxic atlas were included in the final analyses (Franklin and Paxinos, 2008).

Statistical Analysis

The primary dependent variables were alcohol intake (g/kg), BEC (mg/dL), sucrose intake (mL/kg), and distance traveled (cm). All data were analyzed by ANOVA, with Time as a repeated factor as necessary. Significant factor interactions were further evaluated using the Student-Newman–Keuls (SNK) for post-hoc comparisons. Alpha was set to $p < 0.05$ for all analyses.

RESULTS

Effect of KOR Antagonist Microinjection Into the BNST on Binge-Like Alcohol Consumption.

Male (N= 17) and female (N= 17) mice were split evenly into drug treatment groups based on alcohol intake averaged across the three preceding 2-hour limited-access drinking sessions. During these three sessions, males consumed an average of 2.01 ± 0.11 g/kg and females 3.01 ± 0.10 g/kg alcohol, consistent with other reports indicating female mice consume more alcohol than males (Finn et al., 2005; Rhodes et al., 2005; Sneddon et al., 2019).

Alcohol intake during the test drinking session for the vehicle and KOR antagonist (nor-BNI) treatment groups are summarized in **Figure 2.1**. As shown in **Figure 2.1A**, females consumed more alcohol than males during the 0-2 and 2-4 hour time periods following vehicle treatment. Further, nor-BNI strongly reduced alcohol consumption in both male and female mice. Although ANOVA did not indicate a significant 3-way interaction (Sex x Drug x Time; F= 0.43), there was a significant Sex x Drug interaction [$F(1,30)= 6.53$, $p< 0.025$], as well as main effects of Sex [$F(1,30)= 19.06$, $p< 0.001$] and Drug [$F(1,30)= 67.50$, $p< 0.001$]. Subsequent post-hoc analyses showed that nor-BNI significantly reduced alcohol intake in both male in female mice during both time periods (* $p< 0.001$). Also, vehicle-treated females consumed significantly more alcohol than vehicle-treated males (^ $p< 0.001$). Lastly, both sexes generally consumed more alcohol during the first 2-hour time period compared to the second 2-hour time period that was supported by a main effect of Time [$F(1,30)= 10.34$, $p< 0.01$].

Analysis of alcohol intake during the entire 4-hour test session showed that intra-BNST injection of nor-BNI significantly reduced alcohol consumption (**Figure 2.1B)**. This was supported by a significant Sex x Drug interaction [$F(1,30) = 6.53$, $p < 0.025$], as well as main effects of Drug [$F(1,30) = 67.50$, $p < 0.001$] and Sex [$F(1,30) = 19.06$, $p < 0.001$]. Post-hoc analyses showed that nor-BNI decreased alcohol intake in both sexes compared to vehicle (* $p < 0.001$), and female mice consumed significantly more alcohol than males following vehicle treatment (^ $p < 0.001$).

At the conclusion of the 4-hour test drinking session, blood samples were collected and blood alcohol concentrations (BACs) were assessed. Consistent with the alcohol intake data, BACs were significantly lower in both sexes after nor-BNI treatment and, under vehicle conditions, the higher alcohol intake in females resulted in significantly higher BACs compared to males (**Figure 2.1C**). This was supported by 2-way ANOVA that revealed a Sex x Drug interaction [$F(1,30) = 5.54$, $p < 0.025$] as well as main effects of Drug [$F(1,30) = 49.64$, $p < 0.001$] and Sex [$F(1,30) = 7.15$, $p < 0.02$]. Post hoc analyses revealed significantly reduced BACs in male and female mice as a result of nor-BNI treatment compared to vehicle (* $p < 0.001$) and higher average BACs in females compared to males receiving vehicle (^ $p < 0.001$). Finally, a schematic representation of microinjector guide placements within the BNST is represented in **Figure 2.1D**.

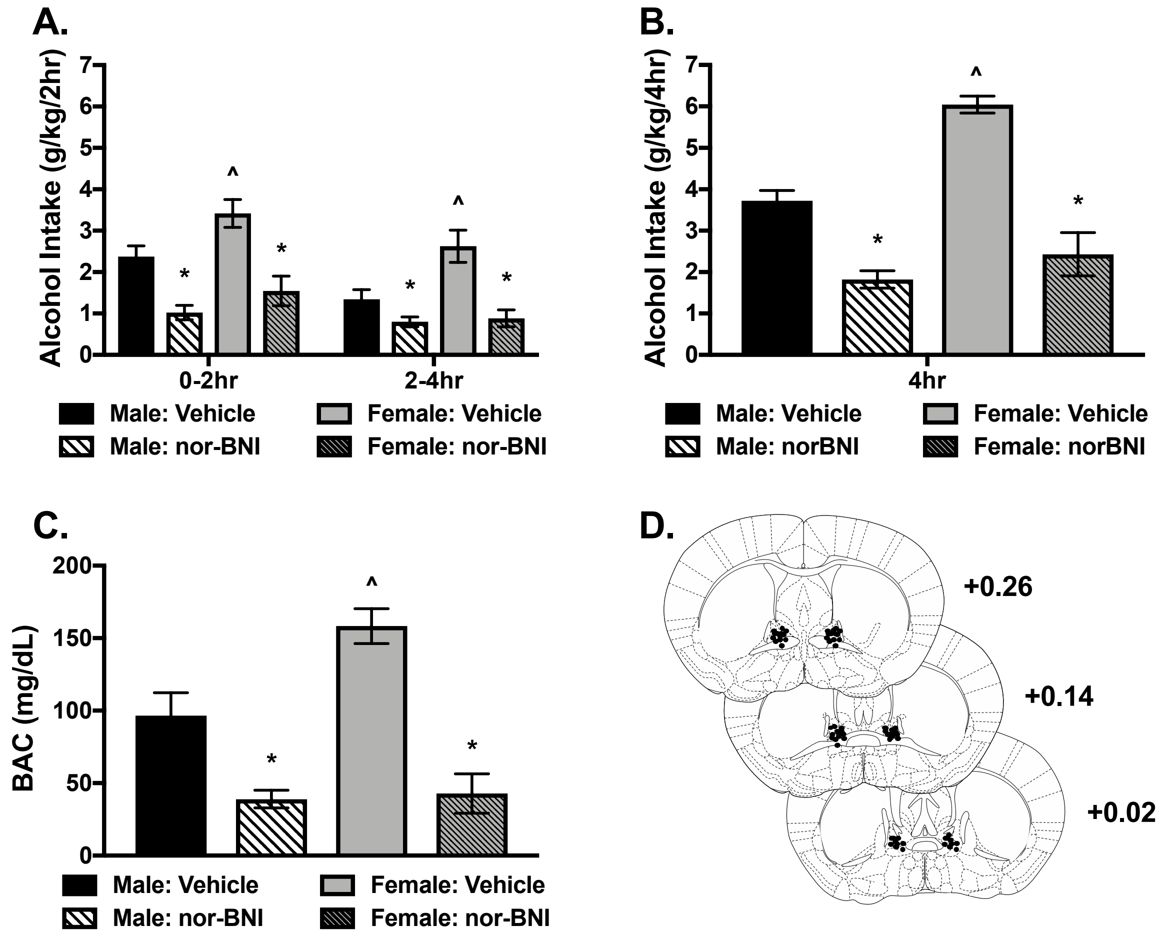


Figure 2.1: KOR antagonist infusion into the BNST reduced binge-like alcohol consumption. Male and female mice received a bilateral microinjection of vehicle or the KOR antagonist, nor-BNI (2.5 μg/side), 16 hours before a binge drinking session. **A)** Alcohol intake (g/kg) during the consecutive 2-hour time periods (0-2 and 2-4 hour) in the 4-hour access session. Females consumed more alcohol than males receiving vehicle across both timepoints (^ $p< 0.001$). Nor-BNI significantly reduced alcohol intake in males and females (*ps<0.001) relative to vehicle but drinking did not differ between sexes treated with nor-BNI at either time point. **B)** Cumulative alcohol intake across the 4-hour session. When treated with vehicle, females consumed more alcohol than males (^ $p< 0.001$). Nor-BNI reduced alcohol intake in both males and females compared to their respective vehicle (* ps< 0.001). However, no difference was observed between males and females receiving nor-BNI. **C)** Blood alcohol concentration (BAC; mg/dL) was assessed at the end of the 4-hour session. Vehicle treated females had greater BACs than males receiving vehicle (^ $p< 0.001$). BACs were significantly reduced in both male and females compared to their respective vehicle groups (* ps< 0.001) but no difference was observed between males and females treated with nor-BNI. **D)** Representative microinjector tip placements within the BNST.

Effect of KOR Antagonist Microinjection Into the BNST on Binge-Like Sucrose Consumption.

Male (N= 16) and female (N= 16) mice were split into treatment groups based on average intake across the preceding three days of 2-hour sucrose drinking. Males consumed an average of 36.94 ± 1.97 mL/kg and females consumed 47.85 ± 2.22 mL/kg during these three days prior to drug challenge. During the test session, female mice generally consumed more sucrose than males and nor-BNI treatment produced a modest reduction in intake in both sexes (**Figure 2.2A**). ANOVA indicated main effects of Drug [$F(1,28) = 9.29$, * $p < 0.005$] and Sex [$F(1,28) = 10.47$, ^ $p < 0.01$], but the Dug x Sex and Drug x Sex x Time interaction terms did not achieve statistical significance ($Fs < 2.2$). Likewise, analysis of sucrose intake during the entire 4-hour test session indicated that nor-BNI reduced sucrose consumption in both sexes and females generally consumed more sucrose than males (**Figure 2.2B**). This was supported by significant main effects of Drug [$F(1,28) = 9.29$, * $p < 0.01$] and Sex [$F(1,28) = 10.47$, ^ $p < 0.01$]. BNST microinjector placements for mice in this study are represented in **Figure 2.2C**.

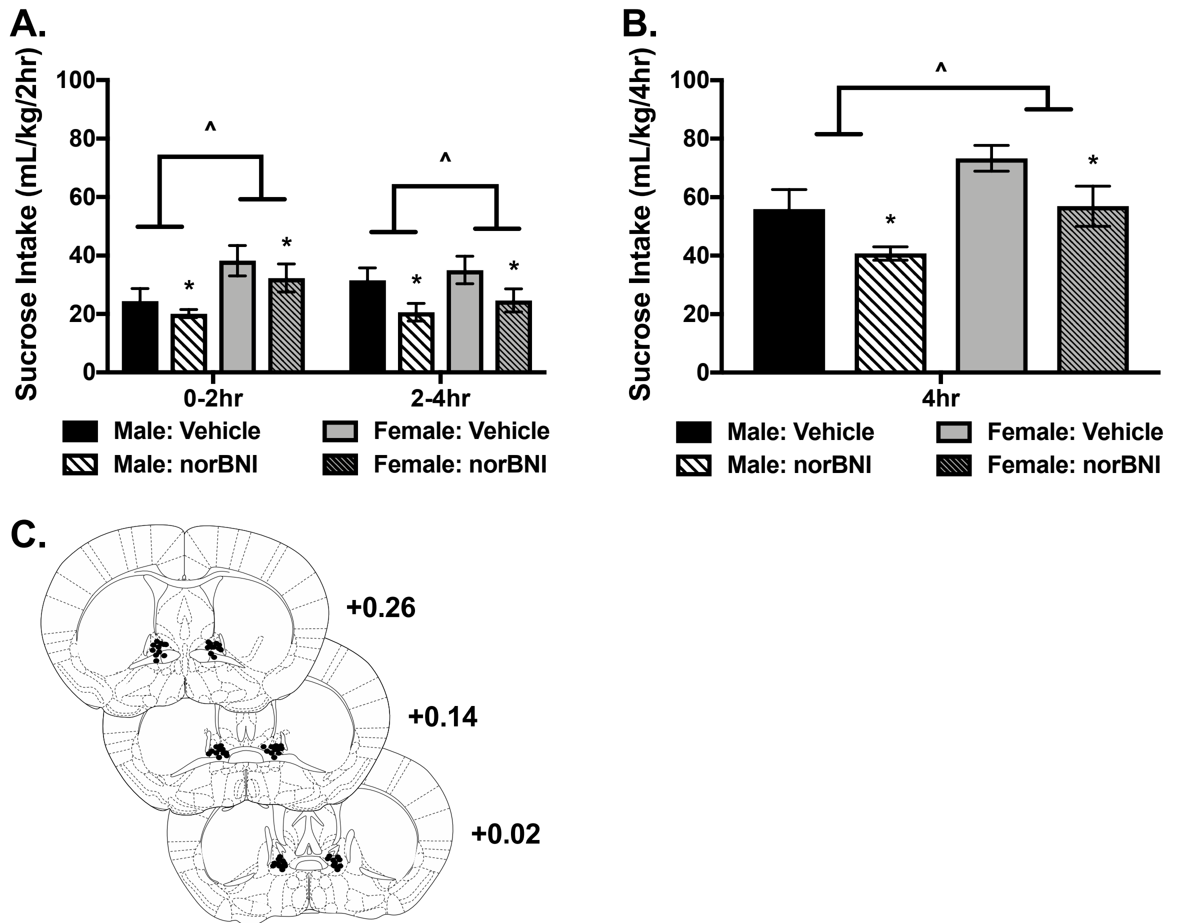


Figure 2.2: KOR antagonist infusion into the BNST reduced binge-like sucrose consumption. **A)** Sucrose intake (mL/kg) during the consecutive 2-hour time periods (0-2 and 2-4 hour) in the 4-hour access session. Females consumed more sucrose than males (^ $p < 0.01$) and infusion of nor-BNI into the BNST resulted in a general suppression of sucrose intake (* $p = 0.005$). **B)** Cumulative sucrose intake across the 4-hour session. Sucrose intake was greater in females (^ $p < 0.01$) while intake was lower in mice treated with nor-BNI compared to vehicle across sexes (* $p = 0.005$). **C)** Representative microinjector tip placements within the BNST.

Comparison of nor-BNI's effects in the BNST on alcohol and sucrose consumption.

Because direct administration of nor-BNI into the BNST decreased consumption of both alcohol (**Figure 2.1**) and sucrose (**Figure 2.2**), the effect size was compared by expressing data from these 2 experiments as the percent change from vehicle (mL/kg). As can be seen in **Figure 2.3**, intra-BNST nor-BNI injection reduced alcohol consumption to a greater extent than sucrose. This was supported by ANOVA that indicated a significant Solution x Drug interaction [$F(1,58)= 8.99$, $p< 0.005$] as well as a main effect of Drug [$F(1,58)= 61.45$, $p< 0.001$] and Solution [$F(1,58)= 8.50$, $p< 0.005$]. There was no main effect of Sex or interactions with this variable. Post-hoc analyses showed that the percent decrease in intake after nor-BNI was significantly greater for alcohol compared to sucrose (# $p< 0.001$). This suggests that the overall suppressive effect of local KOR antagonist treatment in the BNST is more pronounced for alcohol compared to sucrose.

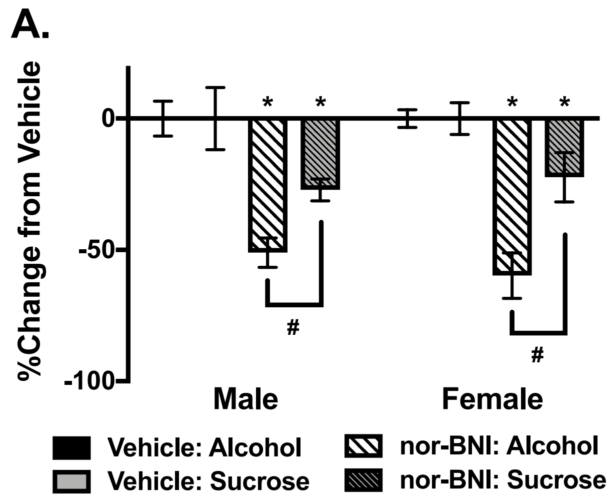


Figure 2.3: Suppressed intake after KOR antagonist infusion into the BNST is greater for alcohol compared to sucrose. **A)** Using data collected in Experiments 1 and 2, a percent change from vehicle calculation was made based on 4-hour intake of alcohol or sucrose for each sex. Nor-BNI microinjection into the BNST resulted in a significant decrease compared to vehicle for intake of alcohol and sucrose (* ps< 0.001). However, the percent change from vehicle after nor-BNI treatment was significantly greater in mice consuming alcohol compared to those drinking sucrose (# ps< 0.001).

Examination of Non-Specific Locomotor Effects of KOR Antagonist Microinjection in the BNST.

A separate cohort of male (N= 17) and female (N= 16) mice were used to determine if the decrease in alcohol and sucrose consumption following intra-BNST nor-BNI microinjection resulted from non-specific sedative effects by examining locomotor activity in an open-field arena. While females were generally more active than males [$F(1,29) = 31.40$, $p < 0.001$], nor-BNI infused into the BNST 16 hours prior to testing did not affect distance traveled in either sex (**Figure 2.4A**). This was supported by the fact that ANOVA did not reveal a main effect of Drug ($F = 0.03$) or an interaction of Drug with Sex or Time variables. This suggests that the suppressive effect of nor-BNI on drinking behavior is not likely related to a general sedative-like effect. Further, analysis of time spent in the center of the open field indicated no significant main effects of Sex ($F = 0.53$) or Drug ($F = 0.02$). This suggests that KOR blockade within the BNST did not affect anxiety-like behavior under these behavioral testing conditions (**Figure 2.4B**). Microinjector placements within the BNST for this study are represented in **Figure 2.4C**.

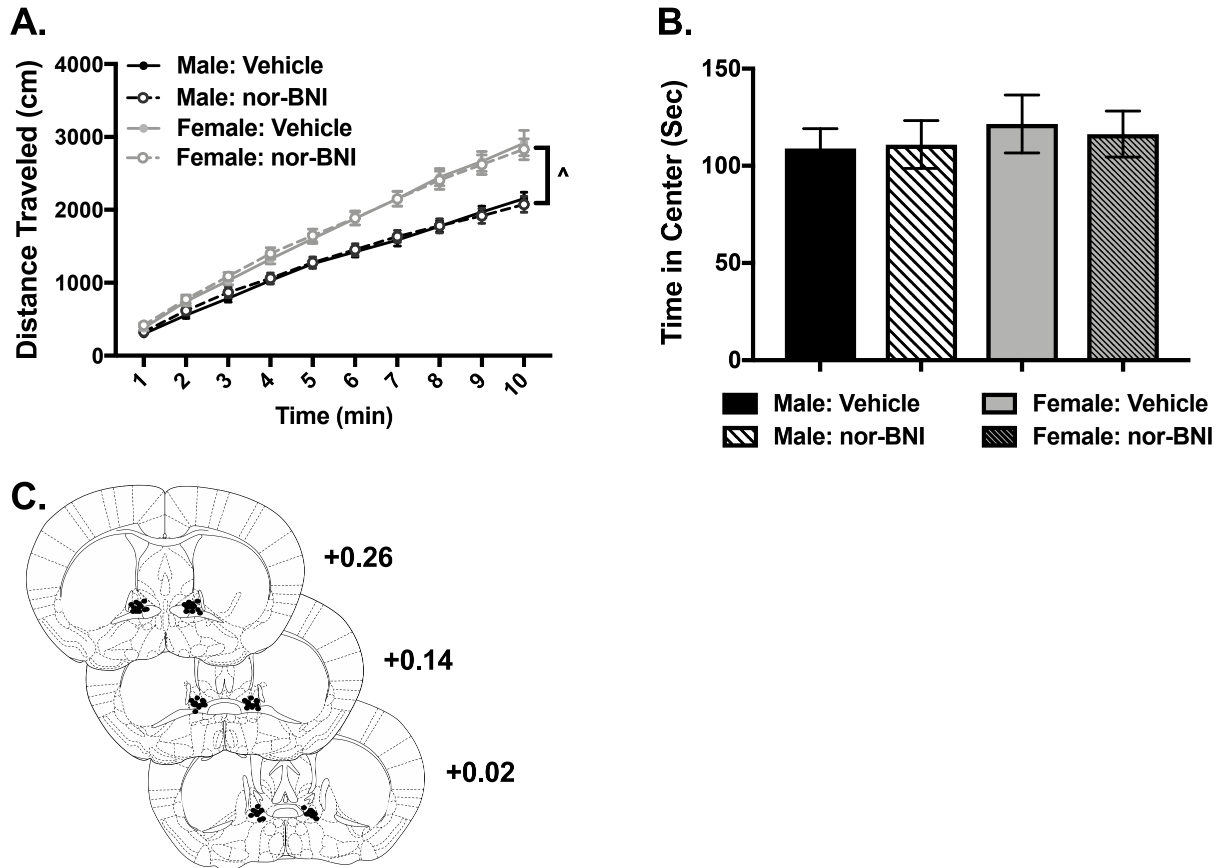


Figure 2.4: KOR antagonist infusion into the BNST does not affect locomotor activity. **A)** Cumulative distance traveled (cm) after vehicle or nor-BNI microinjection into the BNST 16 hours before open field testing. Females generally traveled a greater distance than males (^ $p < 0.001$) but nor-BNI did not affect locomotor activity. **B)** Representative microinjector tip placements in the BNST.

Effect of KOR Agonist Microinjection Into the BNST on Binge-Like Alcohol Consumption.

Male (N= 10) and female (N= 10) mice were split evenly into drug treatment groups based on alcohol intake averaged across the three preceding 2-hour limited-access drinking sessions. Male mice consumed an average of 2.12 +/- 0.2 g/kg and females 2.72 +/- 0.28 g/kg alcohol during these three days. Two female mice were excluded from testing due to microinjector head-cap dislocation.

Alcohol intake during the test drinking sessions after microinjection of the KOR agonist U50,488 (0, 0.1, 0.2 μg/side) is shown in **Figure 2.5**. Because neither a main effect of Sex [$F(1,32)= 0.39$, $p= 0.54$) nor a factor interaction was observed during the 4-hour binge drinking test sessions, data were averaged across males and females. Microinjection of U50,488 resulted in an increase in alcohol intake (**Figure 2.5A**). Repeated-measures ANOVA revealed a main effect of Drug [$F(2,64)= 3.61$, $p< 0.05$) and a Drug x Time interaction neared significance [$F(2,64)= 2.48$, $p= 0.09$). As can be seen, the increase in alcohol intake was likely driven by drinking during the first 0-2 hr epoch of the test given the equivalent levels of intake during the latter 2-4 hr timepoint. A 2-way ANOVA of the 0-2 hr timepoint was conducted to further evaluate the dose dependency of U50,488. Analysis revealed a main effect of Drug [$F(2,32)= 7.57$, $p< 0.005$] and post-hoc analysis showed that alcohol intake was greater than vehicle for both the low dose (0.01 μg/side; $p< 0.05$) and high dose (0.02 μg/side; $p< 0.001$) of U50,488. Similarly, analysis of alcohol drinking during the entirety of the 4-hour drinking session indicated that U50,488 resulted in a modest increase alcohol drinking compared to vehicle supported by a near significant main effect of Drug [$F(2,32)=$

3.18, p= 0.054] (**Figure 2.5B**). Microinjector placements within the BNST for this study are represented in **Figure 2.5C**.

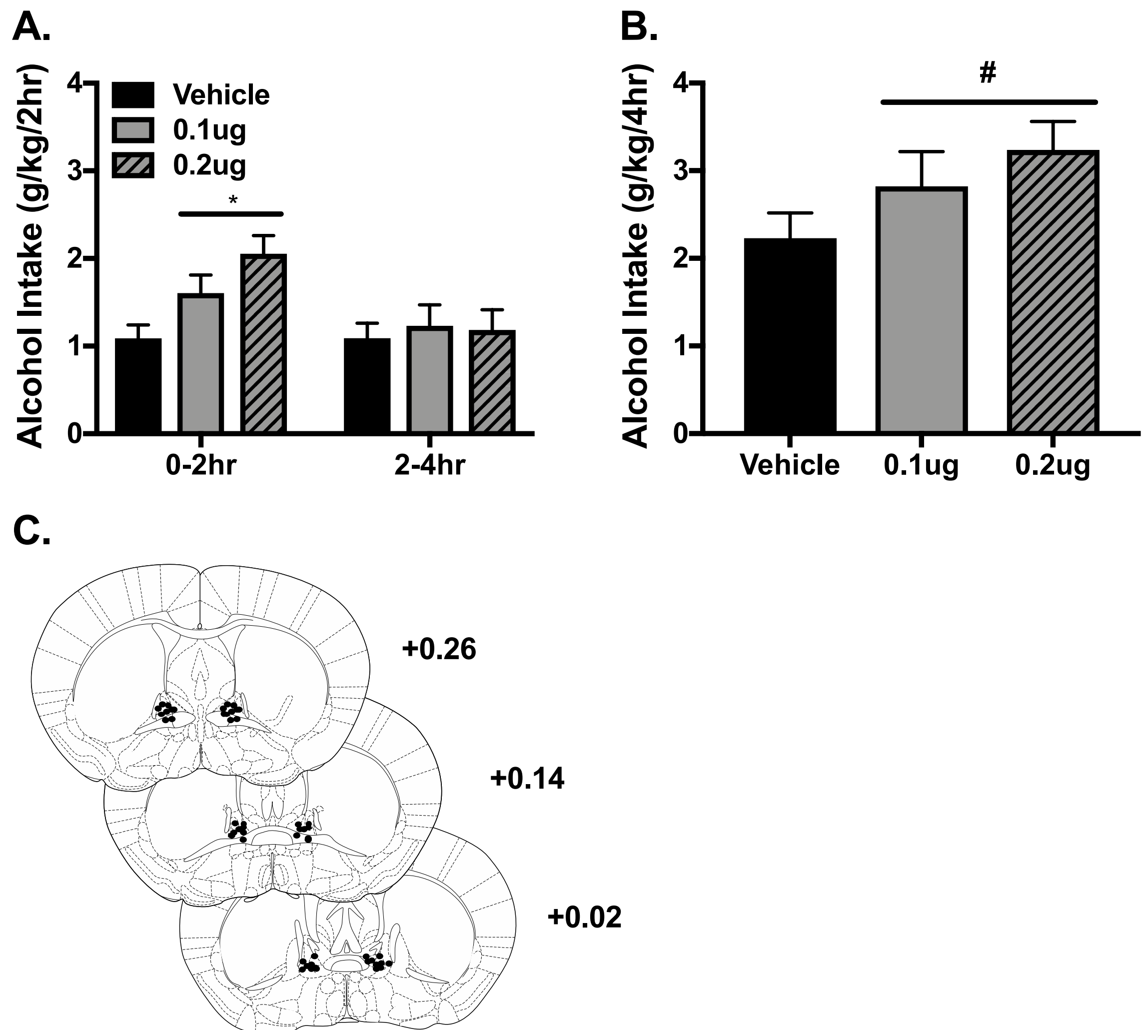


Figure 2.5: KOR agonist infusion into the BNST increased binge-like alcohol consumption. Male and female mice received a bilateral microinjection of vehicle or the KOR agonist, U50,488 (0, 0.1, 0.2 µg/side), 30-min before binge drinking sessions in a within-subjects design. Data are averaged across males and females due to a nonsignificant main effect of Sex. **A)** Alcohol intake (g/kg) during the consecutive 2-hour time periods (0-2 and 2-4 hour) in the 4-hour access session. BNST infusion of U50,488 increased alcohol intake compared to vehicle at the 0-2 hr timepoint (*ps< 0.05). **B)** Cumulative alcohol intake across the 4-hour session. U50,488 microinjection into the BNST modestly increased alcohol intake independent of dose supported by a near significant main effect of Drug (#p= 0.054). **C)** Representative microinjector tip placements within the BNST.

Effect of KOR Agonist Microinjection Into the BNST on Binge-Like Sucrose Consumption.

Male (N= 10) and female (N= 10) mice were split evenly into drug treatment groups based on sucrose intake averaged across the three preceding 2-hour limited-access drinking sessions. Male mice consumed an average of 24.4 +/- 2.5 mL/kg and females 33.8 +/- 6.0 mL/kg sucrose during these three days.

Sucrose intake during the test drinking sessions after microinjection of the KOR agonist U50,488 (0, 0.1, 0.2 µg/side) is shown in **Figure 2.6**. Because neither a main effect of Sex [F(1,38)= 2.90, p= 0.1) nor a factor interaction was observed during the binge drinking test sessions, data were averaged across males and females. Microinjection of U50,488 in the BNST did not affect sucrose intake across the 0-2 hr and 2-4 hr timepoints in the binge drinking session [F(2,76)= 257.61, p= 0.26] (**Figure 2.6A**). Similarly, U50,488 had no effect on sucrose drinking cumulatively during the 4 hr drinking session (**Figure 2.6B**). Microinjector placements within the BNST for this study are represented in **Figure 2.6C**.

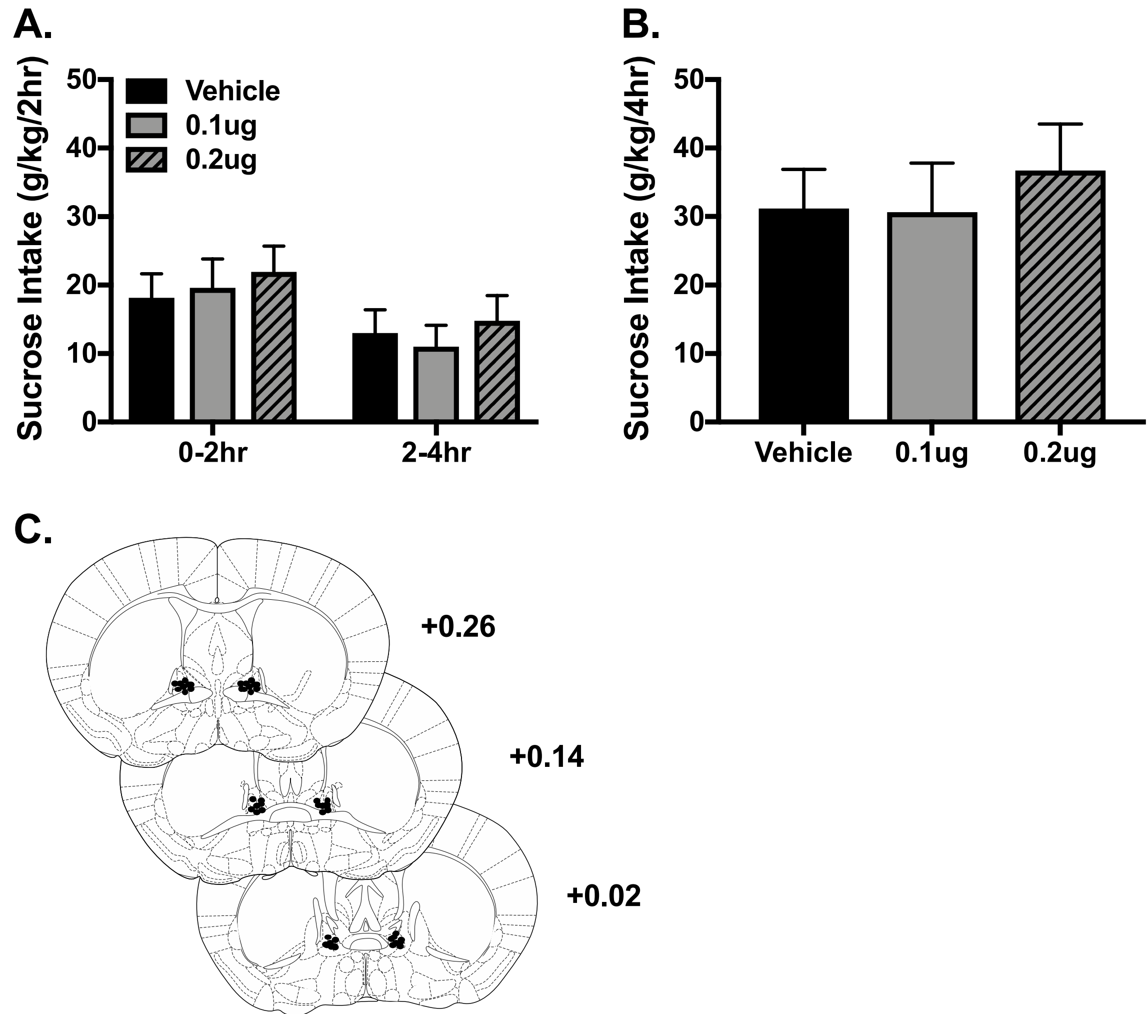


Figure 2.6: KOR agonist infusion into the BNST did not affect binge-like sucrose consumption. **A)** Sucrose intake (mL/kg) during the consecutive 2-hour time periods (0-2 and 2-4 hour) in the 4-hour access session. Compared to Vehicle, U50,488 did not influence sucrose drinking across the binge session. **B)** Cumulative sucrose intake across the 4-hour session. No effect of U50,488 was observed. **C)** Representative microinjector tip placements within the BNST.

Microinjection Into the BNST 30-min Prior to Testing Negatively Affects Behavior.

For studies in **Figure 2.1** and **Figure 2.2**, vehicle or nor-BNI were administered 16-hr prior to testing and drinking during the first 2 hours of the binge session after vehicle treatment was similar to the average of intake over the 3 days preceding testing. However, drinking appeared lower after vehicle microinjection into the BNST 30-min prior to testing relative to drinking on the prior 3 days. To further explore this disruption to drinking, data during the first 2 hours of drinking from studies involving U50,488 microinjection into the BNST are shown in **Figure 2.7A** and include average intake across the three 2-hr limited access sessions prior to the binge session. Alcohol intake was significantly impacted by microinjection of vehicle into the BNST, supported by a main effect of Microinjection [$F(3,48) = 10.03$, $p < 0.001$]. Further post hoc analysis showed that alcohol drinking after vehicle or U50,488 (0.1 μg/side) was significantly less than drinking after mock microinjection handling on Days 1-3 ($ps < 0.05$). No effect of Sex was observed so data are presented as male and female group averages. Similar to alcohol drinking, sucrose drinking is presented in **Figure 2.7B** that was disrupted by microinjection into the BNST, evidenced by a main effect of Microinjection [$F(3,54) = 3.07$, $p = 0.03$). Post hoc analysis revealed that sucrose drinking decreased after microinjection of U50,488 (0, 0.1, 0.2 μg/side) compared to handling on days 1-3. ($ps < 0.05$).

To determine if the microinjection procedure was resulting in sedation or impaired locomotor activity, mice were then tested in an open field task after receiving either Handling, or a microinjection of Vehicle or U50,488 (0.2 μg/side;

Figure 2.7C). ANOVA revealed a main effect of Microinjection and further post hoc analysis showed that cumulative locomotor activity for mice receiving a microinjection into the BNST was significantly less that those handled under mock microinjections conditions 30-min prior to testing.

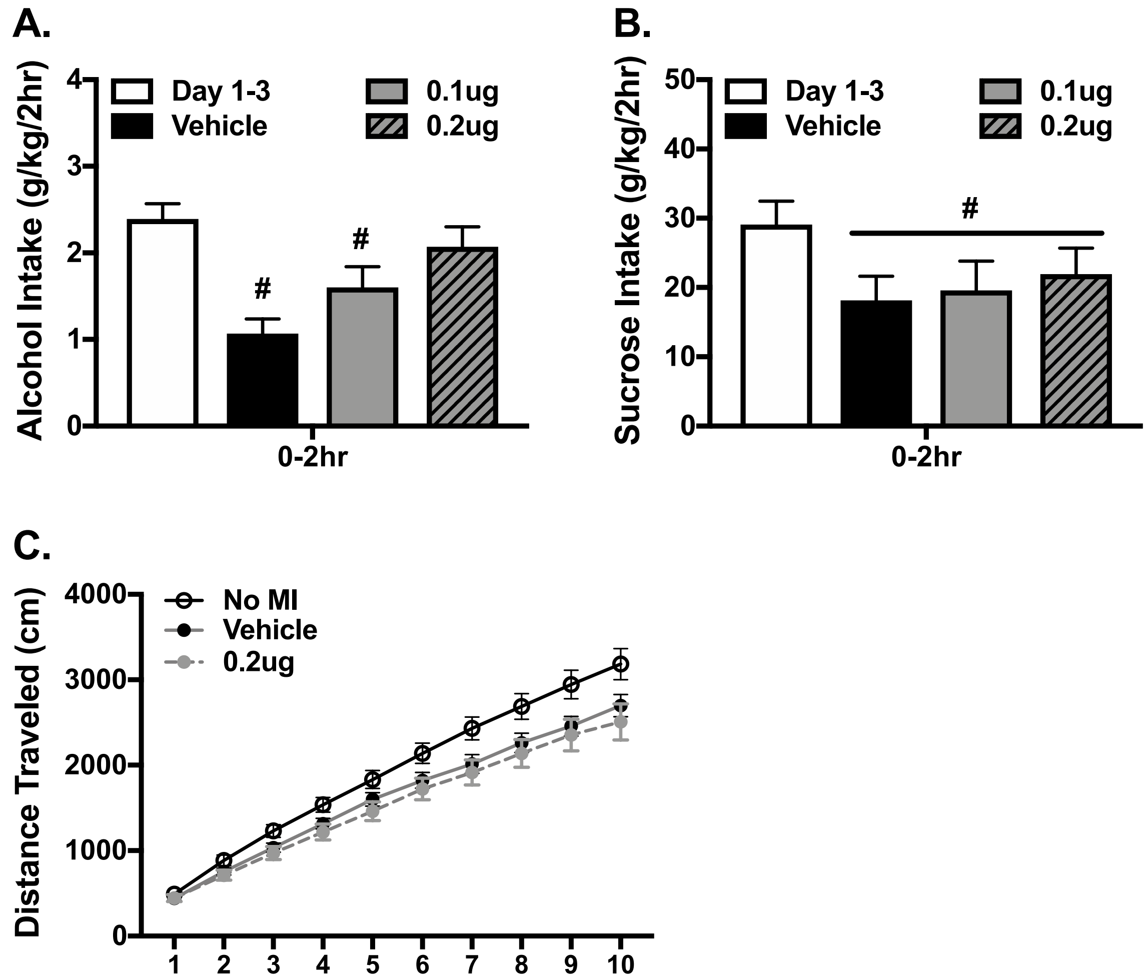


Figure 2.7: Microinjection into the BNST 30-min prior to testing disrupted drinking and locomotor activity. **A)** Microinjection of Vehicle or 0.1 μg/side of U50,488 into the BNST resulted in significantly lower drinking than the previous Day 1-3 involving handling. **B)** Similarly, sucrose intake was negatively affected by microinjection of Vehicle, and both doses of U50,488 compared to the previous three days of handling only. **C)** Compared to handling, mice receiving a microinjection of Vehicle or U50,488 (0.2 μg/side) displayed significantly decreased locomotor activity in an open field task.

Effect of Systemic KOR Agonist Challenge on Binge-Like Alcohol Consumption After Microinjection of a KOR Antagonist Into the BNST.

We previously showed that systemic administration of the KOR agonist, U50,488, increased binge-like alcohol consumption in male mice (Anderson et al., 2019). Experiment 5 was conducted to determine whether KOR within the BNST contribute to U50,488's ability to increase alcohol intake in male and female mice.

Male (N= 35) and female (N= 34) mice were separated into drug treatment groups based on average intake across the three 2-hour drinking sessions preceding testing on Day 4. During these sessions, males consumed an average of 2.42 ± 0.08 g/kg and females consumed 2.93 ± 0.12g/kg alcohol. Initial analysis indicated a trend but no significant main effect of Sex [F(1,60)= 3.20, p= 0.08]. Since Sex did not interact with the other factors, data for the 4-hour test binge session were averaged across males and females. Consistent with our previous report, systemic administration of U50,488 robustly increased binge-like alcohol consumption (Anderson et al., 2018a). Further, microinjection of nor-BNI into the BNST decreased alcohol intake and blocked the ability of U50,488 to increase alcohol consumption in this binge-drinking model (**Figure 2.8A**). ANOVA indicated a Systemic Treatment x Microinjection x Time interaction [F(1,60)= 24.52, p< 0.001] and subsequent post hoc analysis showed that U50,488 robustly increased alcohol intake during the first 2 hours of the test binge session relative to vehicle (* p< 0.001). In contrast, alcohol consumption was lower in mice treated with U50,488 compared to vehicle (* p< 0.001) during the latter half (2-4 hour) of the binge session, an effect likely due to a ceiling effect reached in the first 2 hours of drinking. As observed in Experiment 1, nor-BNI microinjection into the BNST

significantly decreased alcohol intake compared to vehicle during both time periods of the binge session (* $p < 0.001$). Further, blocking KOR in the BNST via direct injection of nor-BNI completely blocked increased drinking following systemic administration of the KOR agonist U50,488 during the first 2 hours of the session (+ $p < 0.001$).

Cumulative alcohol intake during the entire 4-hour test session is presented in **Figure 2.8B**, also collapsed across Sex. Analysis revealed a significant Systemic Treatment x Microinjection interaction [$F(1,60) = 7.33$, $p = 0.009$]. Post hoc tests indicated that systemic administration of U50,488 increased alcohol intake compared to vehicle (* $p < 0.01$), intra-BNST nor-BNI treatment alone reduced alcohol intake (* $p < 0.001$), and blocking KOR in the BNST blocked the ability of systemic U50,488 to elevate drinking (+ $p < 0.001$). Analysis of BACs determined immediately following the test binge-drinking session indicated a significant Systemic Treatment x Microinjection interaction [$F(1,60) = 16.46$, $p < 0.001$]. Resultant BACs mirrored cumulative drinking data in that BACs were elevated in mice treated with U50,488, reduced in mice that received nor-BNI in the BNST alone, and lower than U50,488 or vehicle levels in mice that received both systemic U50,488 and intra-BNST injection of nor-BNI ($ps < 0.001$) (**Figure 2.8C**). Representative microinjector placements are depicted in **Figure 2.8D**.

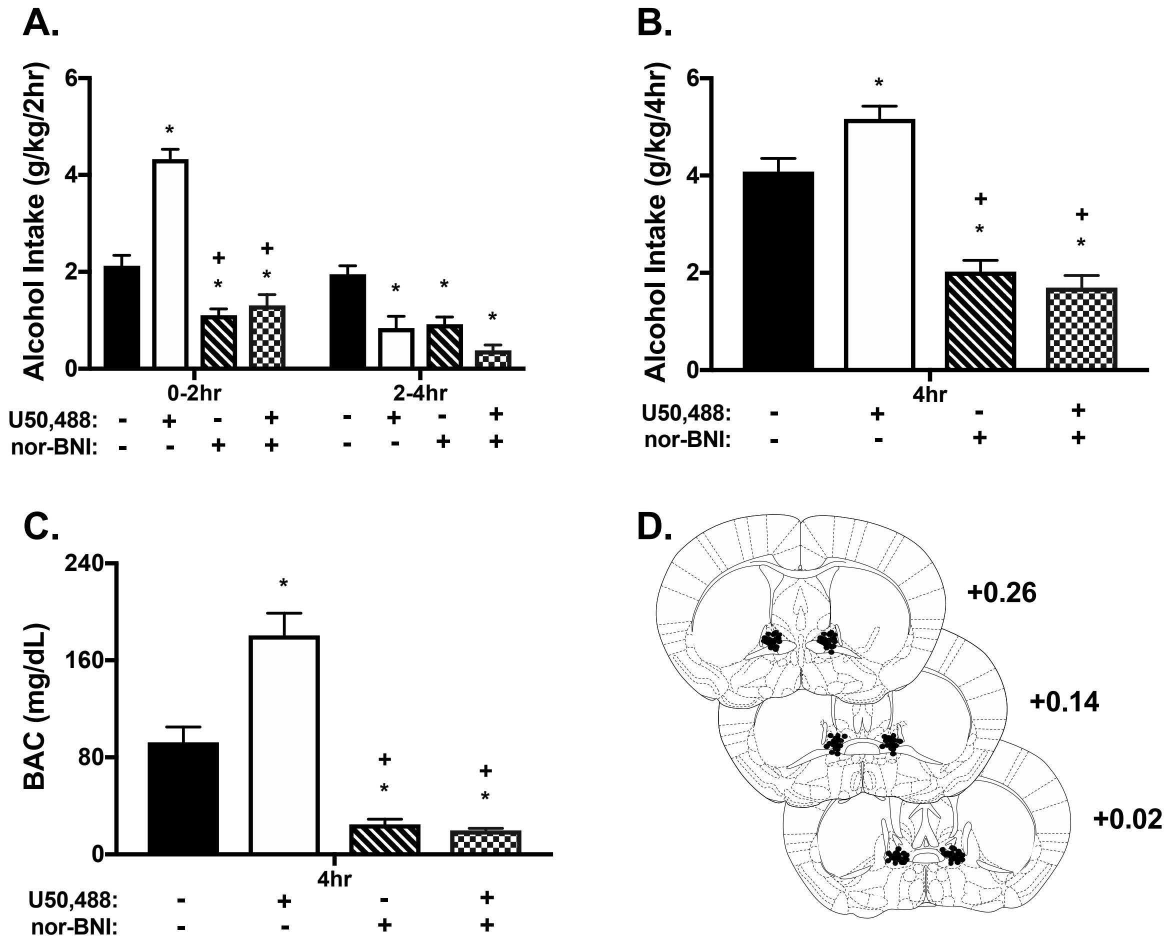


Figure 2.8: KOR antagonist infusion into the BNST blocked elevated alcohol consumption after systemic administration of a KOR agonist. Nor-BNI was microinjected into the BNST 16 hour prior to the 4-hour drinking session and the KOR agonist, U50,488 (5 mg/kg; ip.) was administered 30 min prior to drinking. Alcohol drinking data are collapsed across sex given a nonsignificant main effect (p= 0.079). **A)** Systemic U50,488 treatment resulted in a significant increase in alcohol intake during the first 2 hours of drinking compared to vehicle (*p<0.001). However, a ceiling effect was likely reached given that intake was lower in the 2-4 hour period (* p< 0.001). Microinjection of nor-BNI into the BNST resulted in significantly lower alcohol consumption compared to vehicle (* p< 0.001) and U50,488 (+ p< 0.001) during the first 2 hours. In mice receiving nor-BNI infusion into the BNST combined with systemic U50,488, alcohol intake was reduced compared to vehicle (* p< 0.001) and U50,488 alone (+ p< 0.001) during the first 2 hours. Drinking was also lower in the drug combination group than vehicle during the final 2 hours of testing (*p<0.001). **B)** A similar pattern of drinking was observed for cumulative intake across the 4-hour session. U50,488 (ip.) resulted in a significant increase in alcohol intake (* p< 0.01) while nor-BNI microinjection in the BNST decreased intake compared to vehicle (*<0.001). Intake was significantly less than both vehicle and U50,488 in mice receiving the combined U50,488 and

nor-BNI (* $p < 0.001$; + $p < 0.001$). **C)** BAC was assessed at the end of the 4-hour drinking session. Systemic U50,488 resulted in significantly greater BACs compared to vehicle while mice treated with nor-BNI microinjection into the BNST or combined nor-BNI and U50,488 (ip.) had lower BACs relative to vehicle (* $ps < 0.001$). Furthermore, mice receiving nor-BNI in the BNST or nor-BNI and U50,488 had lower BACs compared to U50,488 alone (+$ps < 0.001$). **D)** Representative microinjector tip placements within the BNST.

DISCUSSION

Results from these studies show that KORs in the BNST play a role in regulating binge-like alcohol drinking in male and female mice. Direct administration of the KOR antagonist nor-BNI into the BNST significantly reduced, while microinjection of the KOR agonist U50,488 increased alcohol consumption in the DID model. Further, intra-BNST nor-BNI injection completely blocked the ability of systemically administered U50,488 to increase binge-like alcohol consumption in the model. While nor-BNI injection into the BNST reduced sucrose consumption, the magnitude nor-BNI's effect on sucrose intake was smaller than the suppression of alcohol intake. Infusion of U50,488 into the BNST had no effect on sucrose drinking. Collectively, these data are consistent with our earlier report showing that systemic administration of KOR a agonist or antagonist bi-directionally alter alcohol consumption in the DID model (Anderson et al., 2018a) and extend those findings to show that KOR signaling in the BNST is significantly involved in regulating binge-like alcohol consumption.

These findings agree with several reports suggesting that KOR antagonists attenuate excessive alcohol consumption in several rodent models, and the effects are mediated by action within the extended amygdala (Anderson et al., 2018b; Crowley and Kash, 2015; Koob, 2013). For example, nor-BNI was shown to decrease binge-like alcohol intake when administered systemically, as well as via microinjection into the CeA, or as reported here, within the BNST (Anderson et al., 2018a). The CeA is of particular relevance because it expresses dynorphinergic (DYN+) neurons that send dense projections to the BNST (Ahrens et al., 2018; Al-

Hasani et al., 2015; Mansour et al., 1994; Marchant et al., 2007; Normandeau et al., 2018). Chemogenetic inhibition of DYN+ neurons in the CeA decreased binge alcohol consumption and, thus, it is possible that reduced activity of KORs within the BNST may mediate this effect (Anderson et al., 2018a). Since there is a high degree of co-expression of DYN and other neuropeptides in the CeA (Kim et al., 2017; McCullough et al., 2018; Pomrenze et al., 2019a), it is possible that targeted chemogenetic inhibition of the CeA-BNST pathway may produce its effects by also altering release of other peptides. For example, DYN is co-expressed with CRF within the CeA and inactivation of the CeA-BNST^{Crf} pathway decreased dependence-related drinking and anxiety-like behavior (de Guglielmo et al., 2019; Marchant et al., 2007; Pomrenze et al., 2019b). Further, increased anxiety-like behavior induced by CeA-BNST excitation is dependent upon KOR signaling within the BNST, suggesting involvement of the CeA-BNST^{DYN} circuit (Ahrens et al., 2018). It is likely that CeA-BNST^{DYN} projections are similarly recruited during binge drinking and influence KOR actions in the BNST. Future studies will need to selectively target the CeA-BNST^{DYN} pathway using the DID model to more directly address this issue. Nevertheless, while it is difficult to rule out the involvement of other neuropeptide systems, results from the present study support the notion that KOR signaling in the BNST contributes to regulating alcohol drinking in the DID binge model.

While KOR blockade within the BNST decreased alcohol consumption in a robust manner (55% reduction), nor-BNI injection into the BNST also produced a modest decrease (25% reduction) in sucrose consumption under the same binge-

drinking conditions. Interestingly, systemic administration of nor-BNI did not affect sucrose intake in the DID model, suggesting that KORs in the BNST may play a more prominent role in general consummatory behavior (Anderson et al., 2018a). The DYN/KOR system is known to influence feeding behavior, including consumption of natural (palatable) rewards (Karkhanis et al., 2017; Nogueiras et al., 2012). However, the mechanisms and site of action for these effects are not entirely clear. For example, neither systemic nor intracerebroventricular (ICV) administration of nor-BNI altered sucrose or saccharine intake (Lopez et al., 2011). In another study, ICV infusion of nor-BNI reduced feeding behavior, but targeted blockade of KORs in the BNST did not alter responding for palatable food pellets (Le et al., 2018; Lopez et al., 2011). Thus, it is unclear to what extent pharmacological antagonism of KORs produce general reductions in food intake. Results from the present study suggest that alcohol intake is more sensitive to KOR antagonism, but since only a single dose of nor-BNI was evaluated, future studies are needed to determine whether an alcohol-selective effect reflects a shift to the left in the dose-response function. Finally, since KOR antagonists do not appear to influence water intake (Lindholm et al., 2001; Zhou and Kreek, 2019), reduced binge-drinking in this study is not likely due to a general effect on fluid consumption.

It is possible that nor-BNI influences the rewarding properties of alcohol and sucrose that is reflected in reduced consumption of these two solutions. For example, nor-BNI (ip.) administration prior to conditioning potentiated state-dependent conditioned place preference (CPP) when mice were challenged with

2 g/kg alcohol (Nguyen et al., 2012). Similarly, nor-BNI potentiated CPP expression to a lower dose of alcohol (0.8 g/kg) only in mice with a history of forced swim stress prior to conditioning (Sperling et al., 2010). However, nor-BNI does not affect CPP expression during testing in the absence of alcohol under non-stressed conditions (Nguyen et al., 2012; Sperling et al., 2010). These studies suggest recruitment of DYN/KOR under conditions involving a high dose of alcohol or under stressful conditions that affect the reinforcing properties of alcohol. KOR antagonists similarly have no effect on the expression of alcohol-induced conditioned taste aversion (CTA) when given before conditioning or during testing (Anderson et al., 2013; Roma et al., 2008). KOR specifically within the BNST have received less attention in relation to CPP or CTA. Therefore, it is difficult to determine whether nor-BNI infused into the BNST in the present studies is affecting the reinforcing properties of alcohol.

Heightened anxiety is thought to promote excessive drinking and KOR antagonists have been shown to exert anxiolytic effects (Koob, 2013; Van't Veer and Carlezon, 2013). More specifically, systemic administration of nor-BNI decreases anxiety-like behavior in open field (OF) and elevated plus-maze (EPM) tests (Knoll et al., 2007; Wittmann et al., 2009). Given the expression of KOR within the BNST, a structure known to be involved in anxiety (Kash et al., 2015), nor-BNI administration in the BNST was expected to result in an anxiolytic phenotype in the present studies. However, we observed no difference in the amount of time spent in the center portion of the open field arena between vehicle and nor-BNI treated mice. This is consistent with another report that showed a similar dose of

nor-BNI (2 μg/side) microinjected into the BNST of male mice had no effect on center time in the OF nor in time spent in the open arm of an EPM (Ahrens et al., 2018). However, nor-BNI administration into the BNST attenuates ultrasonic vocalizations, physiological withdrawal scores, and alcohol intake in rats with a history of alcohol dependence (Erikson et al., 2018). Different measures of anxiety such as novelty suppressed feeding (Carr and Lucki, 2010), fear-potentiated startle (Knoll et al., 2007), and forced swim stress (Mague et al., 2003) are also sensitive to KOR antagonists. Thus, it is likely that KOR in the BNST mediate anxiety-like behavior, but they may be selectively recruited under specific testing conditions and/or the state of the subjects.

The present studies found, in agreement with others, that female mice generally consume more alcohol than males, resulting in significantly greater BACs (Finn et al., 2005; Rhodes et al., 2005; Sneddon et al., 2019). Females also exhibited greater sucrose intake and higher levels of locomotor activity upon first introduction to an open-field arena, consistent with other reports (Archer, 1975; Crabbe et al., 1999; Kaur et al., 2012; Tucker et al., 2016). Interestingly, no studies have directly explored sex differences in the effect of KOR antagonists on binge-drinking. We observed a proportionately larger suppression of alcohol intake resulting from nor-BNI microinjection in the BNST for female mice compared to males. This finding points to potential sex differences in the ability of KOR antagonists to modulate alcohol intake and merits further exploration including more thorough testing of nor-BNI dosing. While sex differences were observed in alcohol consumption in Experiment 1, no significant differences were observed in

Experiment 5. In this case, the observation of females consuming more alcohol than males neared significance (p=0.08), with females consuming roughly 0.5 g/kg more than males. Sex differences in alcohol intake are not consistently observed in different models of excessive alcohol consumption and, in this case, may be due to differences in experimental design between Experiment 1 and 5 (Hilderbrand and Lasek, 2018). Mice in Experiment 1 received a single microinjection into the BNST prior to drinking while mice in Experiment 5 received a microinjection combined with an ip. injection 30-min prior to the test drinking session. The more robust handling procedure may have resulted in slightly lower intake, obscuring potential sex differences in alcohol intake.

In contrast to KOR antagonists, sex differences in response to KOR agonists have been reported but vary by brain region. For example, male guinea pigs exhibit greater U50,488-induced GTPγS activity in the cortex, claustrum, periaqueductal gray, and substantia nigra while females show greater GTPγS activity in the dentate gyrus and hypothalamus (Wang et al., 2011). Females are also less sensitive than males to U50,488-induced responding for intracranial self-stimulation suggesting sex differences in the ability of KOR to mediate motivated behaviors, such as binge-like alcohol consumption (Russell et al., 2014). The BNST is a sexually dimorphic region of particular interest in relation to KOR activation given that after U50,488 administration, expression of the immediate early gene c-Fos is greater in females than males, and sex differences in KOR mRNA expression have been reported (Conway et al., 2019; Russell et al., 2014). In the context of binge drinking, however, our studies indicate no sex differences

in the ability of U50,488 to enhance alcohol drinking to a high level, nor in the ability of nor-BNI to counter U50,488's effects within the BNST. The effect of KOR agonists to increase intake appears to be selective to alcohol under binge conditions given that the same dose of U50,488 (5 mg/kg; ip.) does not affect sucrose intake (Anderson et al., 2018a). Others have similarly reported no change in sucrose consumption after challenge with the KOR agonist, Mesyl Salvinorin B (Zhou et al., 2017). To more selectively probe the role of KOR activation, U50,488 was microinjected into the BNST resulting in increased alcohol intake in both male and female mice. These data build upon the previous findings of systemic administration of U50,488 and pinpoint the BNST as a likely site mediating increased alcohol intake. Interestingly, the increase in intake after U50,488 microinjection was not observed in a separate cohort of mice drinking sucrose suggesting that KOR activation in the BNST selectively promotes alcohol drinking and does not affect consummatory behavior involving a natural reward.

While the present studies indicate that KOR activity within the BNST bidirectionally modulates alcohol drinking in the binge model, direct microinjection into the BNST in close proximity to behavioral testing negatively affected consumption of both alcohol and sucrose. This finding was supported by analyzing drinking levels during the 3 days of mock microinjection handling compared to the analogous first 2 hours of drinking during the 4-hour test session. The disruption to drinking may be due to impaired locomotor activity given than mice receiving a microinjection were less mobile in an open field task compared to mice being handled prior to testing. However, microinjection of vehicle into the BNST 16-hours

prior to testing did not affect alcohol or sucrose drinking given that drinking during the first 2 hours of the 4-hour test day was comparable to drinking the prior 3 days during handling. Previous studies have utilized the same procedure for microinjections into the CeA, mPFC, or NAc prior to testing without any significant disruptions to drinking (Anderson et al., 2018a; Griffin et al., 2014; Haun et al., 2018). Therefore, it is difficult to determine the exact cause of the disruption to behavior after microinjection into the BNST immediately preceding testing. Microinjection into the BNST of rats within the context of alcohol drinking is more common and negative effects on behavior are not commonly observed (Erikson et al., 2018; Le et al., 2018). Of note in mice, microinjection of a neuropeptide-Y receptor agonist into the BNST 2-hours prior to drinking in the DID model decreased intake while drinking was relatively unaffected by vehicle (Pleil et al., 2015). The microinjection procedure utilized by Pleil et al., 2015 involved a much longer pretreatment time (2 hours) and a slower infusion rate of 0.100 uL/min. This is an important consideration given that our studies involving 16-hour pretreatment of vehicle did not influence drinking. A similar procedure with increased pretreatment time and smaller injection volume will be of consideration for future studies when possible. None the less, a clear effect of intra-BNST U50,488 to enhance alcohol drinking was observed in the present studies supporting the contribution of KOR within this structure to alcohol drinking.

Taken together, these findings point to a role for KOR within the BNST in regulating excessive alcohol drinking. However, it remains unclear as to how DYN/KOR activity influences drinking in the context of non-dependent binge-like

alcohol consumption when the majority of studies suggest efficacy of KOR antagonists only under conditions of alcohol dependence. Interestingly, many neuropeptide systems associated with dependence-related drinking also affect binge-like drinking in non-dependent subjects. For example, antagonists targeting the CRF1 receptor (Funk et al., 2007), orexin1 receptor (Lopez et al., 2016b), or KOR (Walker and Koob, 2008) all attenuate dependence-related drinking without affecting more moderate levels of consumption in non-dependent animals. And yet, these systems are also engaged in and promote binge-like drinking prior to the development of dependence. Selective antagonists targeting the CRF1 receptor (Lowery-Gionta et al., 2012), orexin1 receptor (Olney et al., 2015), or KOR (Anderson et al., 2018a) decrease binge-drinking, effectively normalizing intake to a moderate level. Since all of these neuropeptide systems are responsive to stress, they may become engaged under drinking-in-the-dark conditions that engenders high levels of alcohol intake, that presents as a potent stressful event (Anderson et al., 2018b; Koob, 2013; Rivier, 1996; Stephens and Wand, 2012). In the context of binge drinking, the high level of alcohol intake achieved results in a rapid elevation in BAC and may recruit the DYN/KOR system. For example, systemic administration of alcohol at doses generating BACs only in excess of 80 mg/dL results in increased extracellular DYN release within the nucleus accumbens (Marinelli et al., 2006). This increase in DYN occurs during the acute effects of alcohol intoxication suggesting a dynamic response of DYN to alcohol beyond the contribution of DYN/KOR signaling to dysphoria experienced during withdrawal (Chavkin and Koob, 2016). Therefore, KOR antagonists may modulate the stress

response to alcohol or anxiety associated with binge drinking in non-dependent mice resulting in a normalization of intake to more moderate levels. This is supported by the fact that nor-BNI has no effect on moderate alcohol consumption in non-dependent subjects when delivered by systemic injection (Walker et al., 2011) or into extended amygdala structures (Erikson et al., 2018; Kissler et al., 2014).

Thus, the DYN/KOR system, along with other stress-related peptides contribute to excessive alcohol consumption, and are further recruited as bouts of binge drinking and intoxication increase in frequency, leading to escalated and uncontrolled drinking observed in dependence. This provides a potential prophylactic window during binge drinking to selectively target these systems early in the trajectory of the addiction cycle to normalize intake such that regular bouts of binging become less frequent, thereby reducing the likelihood of escalation of intake over time. In conclusion, the present series of studies demonstrate that targeted KOR antagonist treatment within the BNST attenuates binge-like alcohol consumption and normalizes KOR agonist-potentiated drinking in both male and female mice. Future studies aimed at targeting the precise dynorphinergic circuity within the extended amygdala that contributes to activity at KOR within the BNST will shed further light on the role the DYN/KOR system plays in mediating excessive binge-like alcohol drinking.

CHAPTER 3: Increased Neuronal Activity Within the Central Amygdala is Associated With Binge Drinking.

INTRODUCTION

In Chapter 2, we demonstrated that KOR activity within the BNST modulates binge-like alcohol consumption in male and female mice. The precise BNST afferent projection sites that contribute to binge drinking, however, are poorly understood. The CeA is a likely candidate in that it is involved in various alcohol-related behaviors and sends dense peptide-rich, projections to the BNST (Ahrens et al., 2018; de Guglielmo et al., 2019; Koob, 2003, 2009; Li et al., 2012; Waraczynski, 2006). Preclinical models have demonstrated a role for the CeA in the motivational effects of alcohol and withdrawal-related sequalae that promote excessive drinking (Gilpin et al., 2015; Koob, 2009; Sharko et al., 2016). Further, a growing body of literature has focused on the CeA within the context of binge drinking. For example, gene expression is up-regulated in the CeA of rats with a history of binge-like drinking (McBride et al., 2010). The immediate early gene (IEG), C-Fos, is often used as a proxy for neuronal activity and increased c-Fos expression has been observed in the CeA after a bout of binge drinking (Burnham and Thiele, 2017; McReynolds et al., 2018). In fact, there is a distinct population of neurons within the CeA that is recruited prior to bouts of binge drinking that promote excessive drinking behavior (George et al., 2012). Ensemble recruitment in the CeA is also critical to network remodeling that contributes to excessive drinking as a function of dependence (de Guglielmo et al., 2016; George et al., 2012; Kimbrough et al., 2020). Of note, projections from the CeA to the BNST mediate high levels of alcohol consumption in dependent rats and the CeA-BNST

circuit is thought to be involved in the motivation to drink excessively during a binge (de Guglielmo et al., 2019).

While the CeA is involved in alcohol consumption, it is also responsive to the pharmacological effects of alcohol. For example, human imaging studies have reported decreased activity within the amygdala and BNST after acute alcohol challenge (Hur et al., 2018). The dampening of extended amygdala activity is thought to account for, in part, the anxiolytic properties of alcohol (Koob, 2013). In fact, emotionally charged cues typically evoke a strong BOLD response in the amygdala, but this response is blunted when alcohol is on-board (Gilman et al., 2008; Sripada et al., 2011). Preclinical studies measuring c-Fos activity in the CeA after acute alcohol challenge also support the pharmacological action of alcohol (Hitzemann and Hitzemann, 1997; Ryabinin et al., 1997). Relevant to binge drinking, acute administration of alcohol in doses generating blood alcohol concentrations (BACs) in excess of 80 mg/dL resulted in increased c-Fos expression in the CeA (Hansson et al., 2008; Hitzemann and Hitzemann, 1997; McBride, 2002). This effect was not observed with lower doses of alcohol that result in lower BACs (Ryabinin et al., 1997). Similarly, moderate levels of alcohol drinking do not alter c-Fos activity suggesting that the high BAC achieved during a binge is sufficient to induce c-Fos expression within the CeA (Burnham and Thiele, 2017; Hitzemann and Hitzemann, 1997; McBride, 2002). Little is known, however, about activity within projections from the CeA to the BNST during binge drinking and or in response to pharmacologically relevant BACs.

The drinking-in-the-dark (DID) model has been widely used in the field and proven to be effective in serving as a platform for pharmacological and circuit-level interrogation of brain systems that drive binge drinking (Rhodes et al., 2005; Sprow and Thiele, 2012; Thiele and Navarro, 2014). Few studies, however, have examined c-Fos expression as a function of binge drinking in the DID paradigm. The only study to date characterized c-Fos expression in C57BL/6J male mice across multiple brain regions relative to water drinking controls, with tissue collected 30 min after a 2 hour drinking session (Burnham and Thiele, 2017). This study found that c-Fos expression was elevated within the CeA and BNST of binge drinking male mice. It is unclear, however, if activity within the CeA and BNST are related or at what time these populations are recruited during a 4 hour drinking session. Therefore, the present study was designed to address this gap in the literature by determining c-Fos activity within projections from the CeA to the BNST during multiple timepoints of a binge-drinking session.

MATERIALS AND METHODS

Subjects

Male and female (N= 30/sex) C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) at 10 weeks of age were singly housed and tested in a temperature and humidity controlled AAALAC approved facility on a reverse 12-hr light/dark cycle with food and water continuously available. For all experiments, mice were treated in accordance with both the NIH Guide for the Care and Use of Laboratory

Animals (National Research Council, 2011) and the Institutional Animal Care and Use Committee at MUSC.

Surgical Procedures

Originally, this study was designed to determine activity within projections from the CeA to BNST in the context of binge drinking. This, mice were anaesthetized with isoflurane and 0.15 uL of red Retrobeads (Lumafluor) were bilaterally infused into the BNST (AP: + 0.22, ML: +/- 0.75, DV: -4.6) using a 0.5 uL Hamilton Neuros syringe at a flow rate of 0.05 uL/min for 5 min. The syringe was then left in place during a 10 min diffusion period and retracted over 5 min (Haun et al., 2018). The volume of Retrobead infusion was based on a pilot study but retrograde labeling in neurons within the CeA of experimental animals was not consistently measurable. Therefore, co-labeling of c-Fos and Retrobeads within CeA projections to the BNST was not quantified and is not described further.

Alcohol Binge Drinking Procedure

After surgery, all mice were given 2 weeks to recover prior to the start of experiments and were trained to drink alcohol in their home cage in the limited access “Drinking-in-the-Dark” (DID) procedure, as previously described (Anderson et al., 2018a; Haun et al., 2020; Rhodes et al., 2005). A single bottle of alcohol (20% v/v) was presented 3 hours into the dark cycle in place of the water bottle. Access to alcohol was for 2 hours on 3 consecutive days and then extended to 4 hours on the 4th day. Alcohol intake was determined for each 2-hour session and then for the 2- and 4-hour time periods during the final (4th day) drinking session. All mice were given one 4-day cycle of binge drinking, 3 days rest in the home

cage, followed by a second 4-day binge cycle. On the final 4-hour binge drinking session, mice were perfused and blood samples collected at the 2-hour and 4-hour timepoints (**Figure 3.1**). These time points were chosen because peak c-Fos expression is detected 90 min after experimental manipulation, but activity can be detected between 30 and 120 min post manipulation (Barros et al., 2015; Cullinan et al., 1995; Hope et al., 1994; Kovacs, 1998). Therefore, tissue collected at the 2-hour timepoint is reflective of neuronal activity occurring roughly 30 min into the drinking session. Similarly, c-Fos expression for tissue collected at the 4-hour timepoint coincides with activity 150 min into the binge session. Plasma was extracted from blood samples and blood alcohol concentrations (BAC) determined using an AM1 Alcohol Analyzer (Analox Instruments, Stourbridge, UK). Water drinking control mice were housed under the same conditions as alcohol drinking subjects and handled equally (weekly cage changes and body weight assessments). These mice remained alcohol naïve for the duration of experimentation and were sacrificed at the same 2- and 4-hr timepoints, were perfused, and their tissue extracted in an identical manner to alcohol drinking test subjects. However, BAC was not assessed in this group.

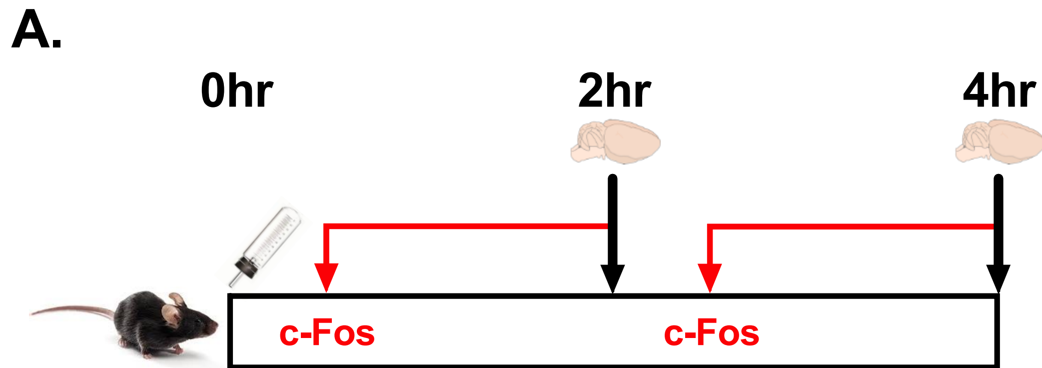


Figure 3.1: Procedure overview. **A)** Mice consumed alcohol across a 4 hr limited-access drinking session. Tissue was collected at the 2 hr and 4 hr timepoints for blood alcohol concentration (BAC) and c-Fos assessment. Tissue collected at the 2 hr timepoint indicates c-Fos expression after 30 min of drinking and the 4 hr timepoint reflects activity after 150 min of drinking.

Immunohistochemistry

Mice were euthanized with urethane and transcardially perfused with 10 mL saline followed by 10 mL of paraformaldehyde (PFA; 4%). Brains were extracted, post fixed in 4% PFA for 24 hours and cryoprotected in sucrose (30% wt/vol) until sectioning. Tissue was sliced in serial 40 μM sections and processed for immunohistochemical staining as previously described (Smith et al., 2019). Free-floating sections were rinsed (1-min washes, repeated 6 times) in 1x phosphate buffered saline (PBS) then blocked in 0.3% H2O2 and 0.3% triton-X 100 (PBST) for 1 hr. Tissue was then rinsed and incubated overnight in rabbit anti-c-Fos primary antibody (1:8000; Synaptic Systems) in 5% normal goat serum and PBST at room temperature. The following morning, tissue was rinsed and incubated for 1 hr in biotinylated goat anti-rabbit secondary antibody (1:1000; Jackson Immuno Research). After washing, tissue was incubated for 1 hr in ABC (1:1000; Vector Elite Kit, Vector Labs) and rinsed. Finally, immunolabeling was visualized after a 20 min incubation 3,3' diaminobenzidine (DAB; 0.025%), nickel ammonium sulfate (0.05%), and H202 (0.015%) in PBST for 20 min. The tissue was then rinsed and free-floating sections were mounted onto Permafrost slides, and coverslipped with Permount mounting medium.

Images were collected under 10X magnification on an EVOS-FL microscope (AMF-4300, ThermoFisher) and c-Fos immunoreactivity was assessed with ImageJ imaging software. A representative image of the CeA was collected from the left and right hemisphere of two sections corresponding to AP: -1.46 and AP: -1.58 (Franklin and Paxinos, 2008). ImageJ software was used to

generate a single standard region of interest (ROI) encompassing the CeA and c-Fos immunoreactive nuclei were counted. C-Fos counts were averaged across hemisphere and serial sections to generate a single mean for each subject.

Statistical Analysis

The primary dependent variables were alcohol intake (g/kg), BAC (mg/dL), and c-Fos+ nuclei. Alcohol intake (g/kg) and BAC (mg/dL) were analyzed by 2-way ANOVA to assess main effects of Sex and Time (2 hr, 4 hr). Pearson's correlation was used to determine the relationship between alcohol intake and BAC. Linear regression analysis was then used to determine the correlation between alcohol intake and BAC, accounting for Sex and Time as between-subjects factors. C-Fos data were analyzed by 3-way ANOVA with Sex, Solution (Water, Alcohol), and Time as between-subjects factors. Pearson's correlation was used to determine the general relationship between alcohol intake (or BAC) and c-Fos expression in the CeA. Linear regression analysis was then used to determine the combined effect of the predictors on alcohol intake (or BAC) and c-Fos expression, accounting for Sex and Time as between-subjects factors. Significant factor interactions were further evaluated using the Student-Newman–Keuls (SNK) for post-hoc comparisons. Alpha was set to $p < 0.05$ for all analyses.

RESULTS

Drinking-in-the-Dark Paradigm Engenders Excessive Drinking and Elevated BAC.

Male (N= 20) and female (N= 20) mice trained in the drinking-in-the-dark (DID) paradigm consumed alcohol during a final 4-hr binge drinking session and tissue was collected for c-Fos analysis at the 2-hr and 4-hr timepoints (**Figure 3.1**). Additional groups of alcohol naïve, water drinking male and female mice (N= 10/sex) were sacrificed at the same timepoints. Prior to analysis, three male and three female mice were excluded due to technical errors.

Alcohol intake during the test drinking session for the 2-hr and 4-hr drinking groups are summarized in **Figure 3.2A**. ANOVA revealed a main effect of Sex [$F(1,33)= 4.75$, $p< .05$] and Time [$F(1,33)= 76.08$, $p< .001$] suggesting that females generally consumed more alcohol than males, and intake was greater in mice drinking for 4 hr compared to 2 hr regardless of sex. BAC was assessed immediately after drinking for 2 hr and 4 hr (**Figure 3.2B**). As expected, BAC was greater after 4 hr of drinking compared to 2 hr [$F(1,33)= 13.4$, $p< .001$), but no sex differences were observed, despite females drinking more than males (def discuss this in the discussion). Pearson's correlation analysis showed a significant positive relationship between alcohol intake and BAC under these binge drinking conditions collapsing across Sex and Time [$R^2= .692$, $p< .001$; **Figure 3.2C]**. . Multivariate linear regression analysis further revealed that Sex and Time together explained a significant amount variance in BAC relating to alcohol intake [$F(3,33)= 22.51$, $p< .001$, $R^2= .69$]. Linear regression analysis investigating the individual contributions of these variables to BAC showed that alcohol intake [Beta= 1.16, $t(33)= 5.80$, p<

.001] and Time [Beta= 0.41, t(33)= 2.12, $p<.05$], but not Sex [Beta= 0.05, t(33)= 0.41, $p> 0.6$] predicted BAC in response to alcohol drinking. Further analysis of 2-hr and 4-hr epochs revealed a strong positive correlation between alcohol intake and BAC at 2-hr [R^2=.48, $p<.005$] and 4 hr [R^2=.64, $p<.001$], collapsed across sex (**Figure 3.2D**).

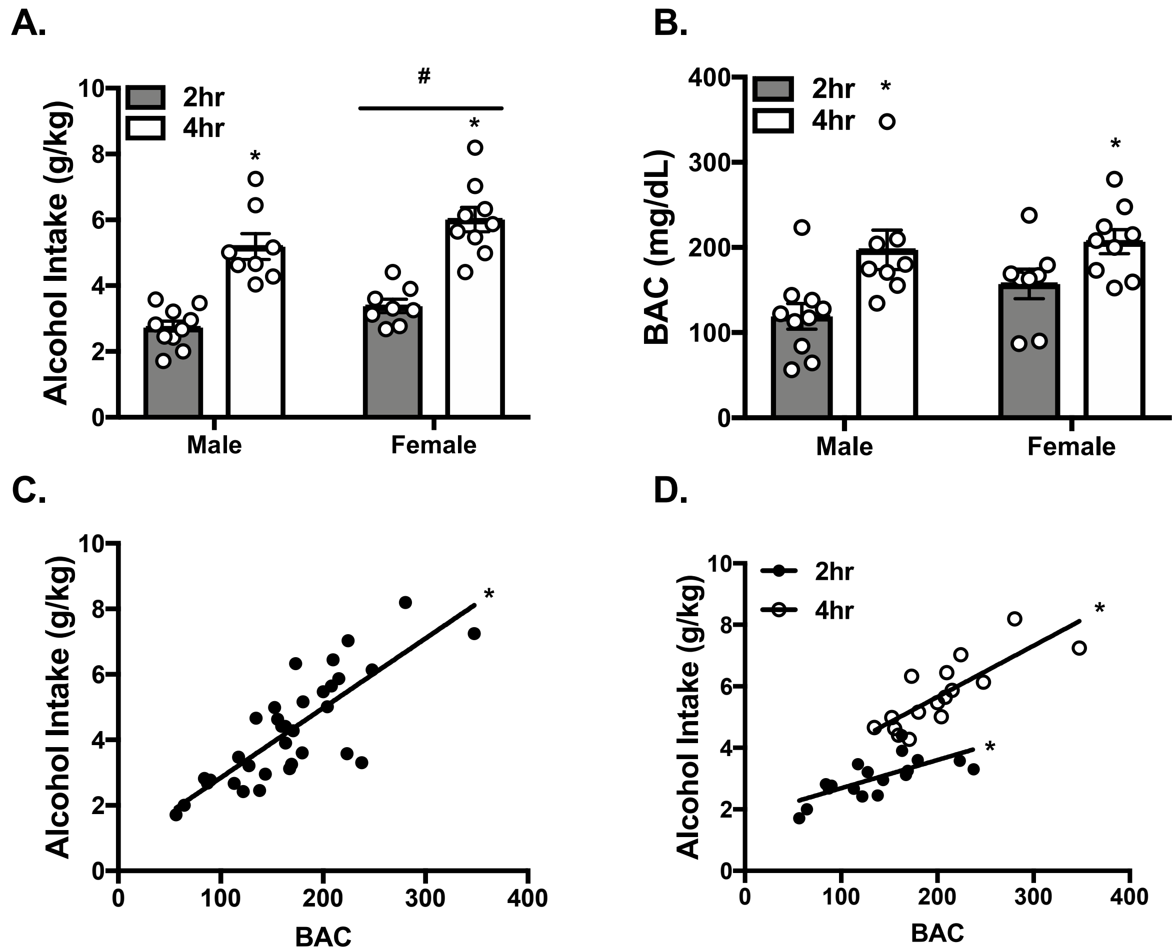


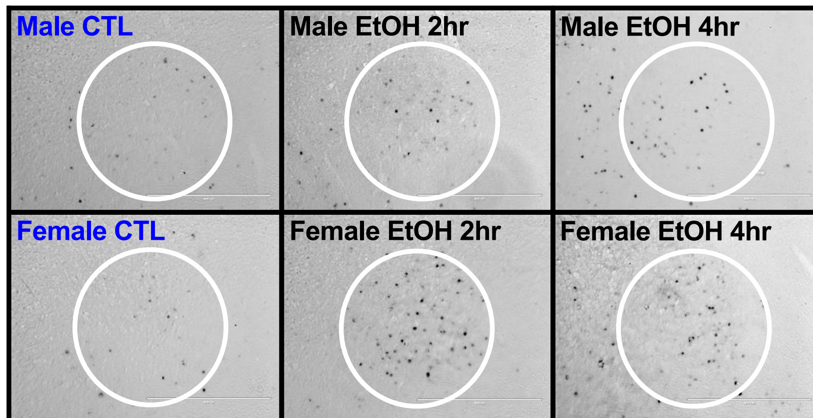
Figure 3.2: Alcohol drinking in the DID paradigm resulted in high levels of intake and elevated BAC. **A)** Cumulative alcohol intake across the final 2 and 4-hour session. Alcohol intake was greater in mice drinking for 4 hr compared to 2 hr (*p< .001) and females generally consumed more alcohol than males (# p< .05). **B)** Blood alcohol concentration (BAC; mg/dL) was assessed at the end of the final 2 or 4-hour session. BACs were higher after 4 hr of drinking compared to 2 hr of drinking in both males and females (*p< .001) **C)** Alcohol intake strongly predicts BAC independent of time and sex (*p<.001). **D)** Alcohol intake and BAC were significantly correlated after 2 hr and 4 hr of drinking (*ps< .005).

Effect of Binge Drinking on C-Fos Expression Within the CeA.

Representative images of c-Fos immunoreactivity within the CeA of water drinking alcohol-naïve control mice (CTL), and those consuming alcohol (EtOH) for 2 or 4 hours is shown in **Figure 3.3A**. For CTL mice, data were analyzed from a total of 10 males (2 hr: N= 5; 4 hr: N= 5) and 10 females (2 hr: N= 5; 4 hr: N= 5). For alcohol drinking mice, data were analyzed for 17 males (2 hr: N= 10; 4 hr: N= 7) and 17 females (2 hr: N= 8; 4 hr: N= 9). Three-way ANOVA revealed a significant main effect of Solution [$F(1,46)= 121.97$, $p< 0.001$] indicating that c-Fos expression was elevated in mice consuming alcohol (**Figure 3.3B)**. Furthermore, c-Fos expression was overall greater in females supported by a main effect of Sex [$F(1,46)= 10.6$, $p< 0.005$]. Finally, a main effect of Time [$F(1,46)= 5.52$, $p< 0.025$] indicated greater c-Fos activity after 4 hours of drinking compared to 2 hours. There was a significant factor interaction between Sex and Time [$F(1,46)= 4.39$, $p< 0.05$) and post hoc analysis revealed that c-Fos expression in females consuming alcohol was greater at 2 hr compared to males at 2 hr ($p< 0.05$), but expression was equivalent at 4 hr across sex. For males, c-Fos activity was greater after 2 and 4 hr of alcohol drinking compared to CTL at each timepoint (ps< 0.005). C-Fos expression was also greater after 4 hr of alcohol drinking compared to 2 hr ($p< 0.001$). No differences in c-Fos expression were observed between CTL mice as a function of time in males ($p= 0.56$). For female mice, c-Fos expression was elevated after 2 and 4 hr of alcohol drinking compared to CTL at each timepoint ($p< 0.001$). However, there was no difference in expression in c-Fos levels of

alcohol drinking mice after 2 and 4 hr (p= 0.67), nor in CTL mice at each timepoint (p= 0.93).

A.



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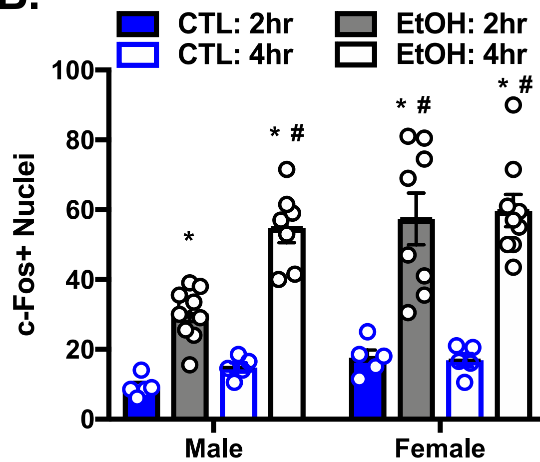


Figure 3.3: C-Fos expression in the CeA is elevated after binge-like alcohol consumption. **A.)** Representative images of c-Fos immunoreactivity within the CeA of water-drinking control mice (CTL) and alcohol-drinking mice (EtOH) after 2 or 4 hr of drinking. **B)** Mean c-Fos+ nuclei. C-Fos expression within the CeA was increased in male and female mice relative to CTL at both 2 hr and 4 hr (*ps< 0.001). In males, c-Fos activity was greater after 4 hr of drinking compared to 2 hr (#p< 0.001). C-Fos activity was also greater in female alcohol-drinking mice at 2 and 4 hr compared to male alcohol-drinking mice at 2 hr (#ps< 0.05). No differences in c-Fos expression were observed in male or female CTL groups.

Relationship Between Alcohol Intake and C-Fos Expression in the CeA.

Pearson's correlation was used to determine if a relationship exists between alcohol intake and CeA c-Fos expression (**Figure 3.4A**). Pearson's correlation revealved a strong possitive association between alcohol intake (g/kg) and c-Fos expression [r= 0.46, $p< 0.01$]. Multivariate regression analysis was then conducted to determine if Sex and Time together predicted CeA c-Fos expression in alcohol drinking mice. It was found that Sex and Time explained a significant amount variance in the levels of CeA c-Fos expression [$F(3,33)= 6.30$, $p< 0.001$, $R^2= 0.39$]. However, analyzing these factors separately, Sex strongly predicted the CeA c-Fos response to alcohol drinking [Beta= 0.45, $t(33)= 2.89$, $p< 0.01$], and not Time [Beta= 0.31, $t(33)= 1.57$, $p> 0.2$]. Given that sex predicted c-Fos expression, data were analyzed separeately for males and females. Among male mice (**Figure 3.4B**), there was a strong positive correlation between alcohol intake and c-Fos expression after 2 hr of drinking [$R^2= 0.641$, $p< 0.01$] and not after 4 hr of drinking in males ($p> 0.4$). There was no association between alcohol intake and c-Fos expression in female mice at either the 2 or 4 hr timepoints (ps> 0.6; **Figure 3.4C**).

Because BACs above 80 mg/dL have a pharmacolocal effect on c-Fos expression in the CeA (Hansson et al., 2008; Hitzemann and Hitzemann, 1997), data were analyzed to determine the relationship between BAC and c-Fos activity (**Figure 3.4D**). Pearson's correlation revealed that BAC strongly predicted CeA c-Fos expression [r= 0.46, $p< 0.01$], independent of Sex and Time. Controlling for Sex and Time, multivariate linear regression analysis revealed that Sex and Time were associated with c-Fos expression and BAC [$F(3,33)= 7.05$, $p< 0.001$, $R^2=$

0.41], but Sex strongly predicted the c-Fos response to BAC [Beta= 0.42, t(33)= 2.91, $p < 0.01$]. Thus, separate regression analysis were conducted for males and females. Similar to alcohol drinking, BAC strongly predicted c-Fos expression in males at the 2 hr timepoint ($R^2 = 0.47$, $p < 0.05$) but not at 4 hr ($p > 0.7$) (**Figure 3.4E**). Finally, BAC did not predict c-Fos expression in female mice at either 2 hr or 4 hr (ps> 0.2) (**Figure 3.4F**).

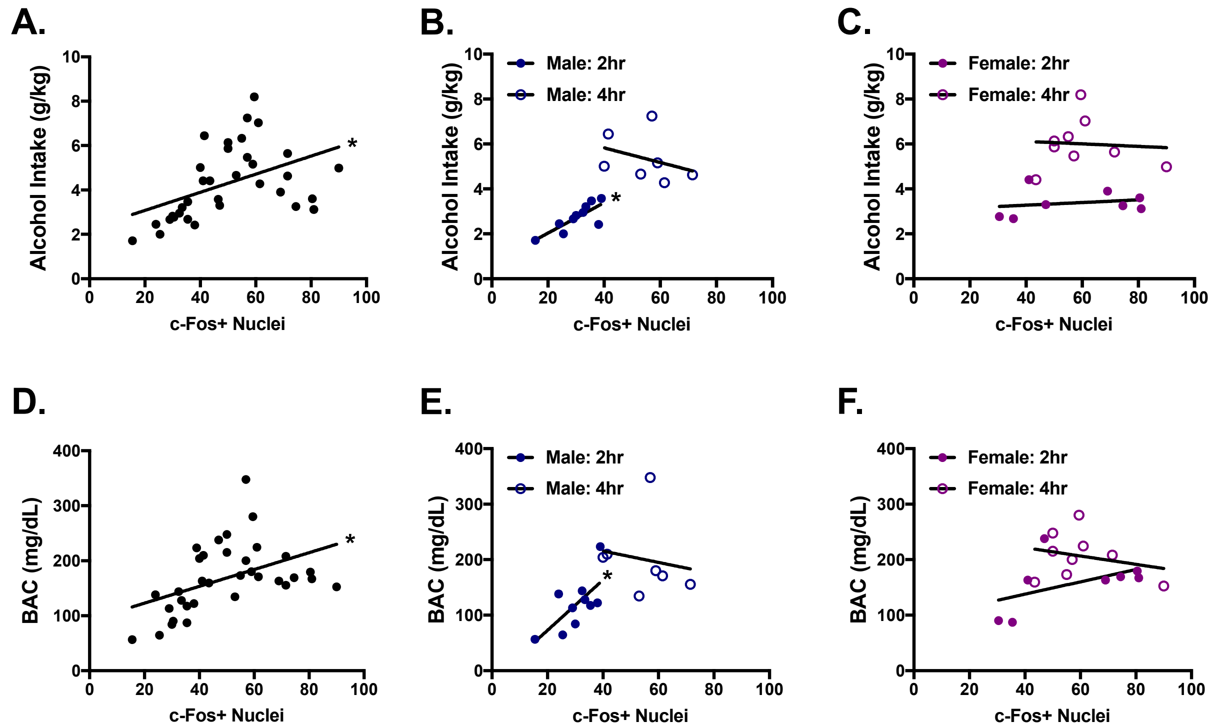


Figure 3.4: Alcohol drinking and BAC correlate with c-Fos expression in the CeA. **A)** Alcohol drinking strongly predicted c-Fos+ neurons within the CeA ($p < 0.01$). **B)** The correlation between alcohol drinking and c-Fos expression was significant after 2 hr of drinking in male mice ($p < 0.01$) but not at the 4 hr timepoint. **C)** Although c-Fos expression was increased in the CeA of female mice consuming alcohol, the level of intake did not predict with c-Fos expression. **D)** BAC strongly predicted c-Fos expression in the CeA overall ($p < 0.01$). **E)** BAC was associated with c-Fos expression in male mice at 2 hr ($p < 0.05$) but not at 4 hr. **F)** BAC did not predict c-Fos levels in the CeA of female mice at either timepoint.

DISCUSSION

The present study adds to the growing body of literature demonstrating that alcohol drinking in the DID paradigm effectively models binge-like drinking behavior in mice by reliably producing clinically relevant BACs. Further, these findings extend previous work by assessing sex differences in CeA neuronal activity during binge drinking. We found that females consumed significantly more alcohol than males and that drinking in this paradigm produced BACs in excess of 80 mg/dL in both sexes, consistent with the literature (Finn et al., 2018; Rhodes et al., 2007; Sneddon et al., 2019). Unfortunately, sufficient Retrobead immunoreactivity was not detected within CeA projections to the BNST precluding observations of circuit level activity. However, we found that c-Fos expression was elevated within the CeA of both male and female mice relative to alcohol naïve controls and that the amount of alcohol consumed and subsequent BAC predicted the level of c-Fos expression. When accounting for sex, c-Fos activity was generally greater in female mice but the correlation between alcohol intake and c-Fos expression was driven largely by males after 2 hours of drinking, suggesting sex differences in CeA activity during binge drinking behavior in this model.

Activity of select populations within the CeA is associated reward saliency that promotes appetitive behaviors, including alcohol consumption (Kim et al., 2017; Koob, 2009; Koob and Volkow, 2016; Mahler and Berridge, 2009). For example, the CeA is activated in response to the acquisition and maintenance of consummatory behavior as well as emotionally salient stimuli like alcohol and reward-related cues (Knapska et al., 2007). Optogenetic interrogation further

revealed that neurons within the CeA promote positive reinforcement and that activity of these neurons, assessed via calcium imaging, coincides with consummatory behavior (Douglass et al., 2017). Likewise, reinstatement of lever-press behavior in the context of cue-induced reinstatement of alcohol seeking predicts c-Fos expression in the CeA (Knapska et al., 2007; Radwanska et al., 2008; Walker et al., 2017). In the context of voluntary consumption, increased c-Fos expression has been observed in the CeA of male mice in the DID paradigm after 2 hours of drinking (Burnham and Thiele, 2017). Consistent with these reports, our results indicate that c-Fos activity was elevated relative to water drinking mice after 2 and 4 hours of alcohol drinking that supports involvement of the CeA in binge drinking behavior. We also found that the amount of alcohol consumed generally predicted c-Fos immunoreactivity within the CeA. Few studies, however, have directly manipulated the CeA specifically in the context of binge drinking to determine a causal role of this region in binge drinking behavior. Pharmacological interrogation of CeA peptide systems has revealed that microinjection of KOR or Crf1R antagonists attenuate binge drinking in male mice (Anderson et al., 2018a; Lowery-Gionta et al., 2012). Direct manipulation of neuronal activity within the CeA using chemogenetic inhibition of DYN-containing neurons or selective knockdown of DYN/KOR also decreased binge drinking (Anderson et al., 2018a; Bloodgood et al., 2020). Together, these findings implicate the CeA as having a causal role in the expression of excessive binge-like alcohol consumption.

While the CeA is involved in motivated behaviors, there is also sufficient evidence demonstrating that this structure is sensitive to the pharmacological effects of alcohol. For example, dose-response studies indicate that acute systemic administration of a high dose of alcohol is sufficient to induce c-Fos expression in the CeA (Hitzemann and Hitzemann, 1997). This elevation in CeA activity is not observed with lower doses of alcohol that result in BACs below 80 mg/dL, suggesting that a threshold is needed for c-Fos induction (Chang et al., 1995; Ryabinin et al., 1997; Ryabinin and Wang, 1998). Importantly, these studies delivered alcohol independent of drinking behavior, suggesting that these responses were purely due to the pharmacological effects of alcohol. The temporal dynamics of the distribution of alcohol after oral self-administration follows a similar pattern to systemic treatment, albeit strongly related to the intensity and duration of drinking behavior (Griffin et al., 2009b; Robinson et al., 2000). For example, alcohol can be detected in brain dialysate samples 30 min after bouts of drinking (Griffin et al., 2009a; Griffin et al., 2007; Robinson et al., 2000). In the present study, tissue was collected 2 hours after the onset of drinking, reflecting c-Fos expression during the first 30 min of drinking session. This is a period when bout frequency and duration are the highest during a 4 hour session, meaning that BACs will rapidly rise thereafter from the large bolus of alcohol flooding the system (Wilcox et al., 2014). Therefore, activity at the 2 hour timepoint reflects the initiation of binge drinking behavior during the first 30 min of the session prior to the circulation of alcohol throughout the brain at a relevant BAC. Because we observed BACs in excess of 80 mg/dL after 2 and 4-hours of drinking, it is reasonable to

suspect then that BACs remained elevated during the final 2 hours of drinking, and that tissue collected during this time reflects a period of binge-level intoxication. C-Fos expression at this time was elevated in mice consuming alcohol indicative of a pharmacological effect of alcohol. The temporal dynamics of c-Fos induction limit interpretation of our results thus it is unclear if c-Fos activity at the 4 hour timepoint reflects activity related to alcohol drinking or a pharmacological effect of alcohol. Future studies can disentangle the drinking behavior and effect of alcohol by utilizing genetically encoded calcium indicators to assess activity within the CeA with high temporal specificity during a binge drinking session.

Studies of sex differences in alcohol intake across various limited-access drinking paradigms generally show that female mice consume more alcohol than males (Almeida et al., 1998; Crabbe et al., 2009; Jury et al., 2017). We and others have observed this phenotypic difference specially in the context of binge drinking in the DID paradigm (Crabbe et al., 2009; Finn et al., 2018; Haun et al., 2020; Sneddon et al., 2019). Here, we replicate this finding and show that females consumed more alcohol than males. The difference in intake was marginal in that subsequent BACs after 2 or 4-hours of drinking were not different in males and females. Studies of the neurobiological mechanisms underlying sex differences in binge-like alcohol consumption, however, remain limited. Here, we show that c-Fos activity was elevated in mice drinking alcohol and also greater overall in the CeA of female mice. The general elevated c-Fos levels in females may be reflective of greater consummatory behavior under DID conditions. For example, we and other have shown that females readily consume more sucrose than males

in the DID model (Crabbe et al., 1999; Haun et al., 2020; Kaur et al., 2012). The idea that the appetitive, consummatory behavior involved in binge drinking is associated with activity in the CeA is supported by our finding that alcohol intake predicted levels of c-Fos expression. However, this effect was largely driven by male mice and thus, the association between binge drinking and activity of the CeA in females remains unclear.

The CeA sends dense projections to the BNST and this pathway is thought to contribute to various alcohol-related behaviors (de Guglielmo et al., 2019; Li et al., 2012; Pomrenze et al., 2019a). For example, acute alcohol treatment results in disinhibition of CeA projections to the BNST and it is hypothesized that activation of the CeA-BNST circuit promotes alcohol drinking (Herman et al., 2013). To date, one study has directly manipulated this circuit in the context of alcohol drinking that demonstrated that optogenetic silencing of CeA-BNST^{Crf} neurons attenuates excessive drinking as a function of alcohol dependence (de Guglielmo et al., 2019). Of note, both Crf- and SST-containing neurons within the CeA-BNST circuit promote anxiety-like behavior, the latter of which is mediated by KOR in the BNST (Ahrens et al., 2018; Pomrenze et al., 2019b). Within the context of binge drinking, antagonists targeting the Crf1 receptor and KOR attenuate binge drinking (Haun et al., 2020; Lowery-Gionta et al., 2012). Therefore, it is likely that activity of Crf- and DYN-containing neurons within the CeA-BNST circuit contribute to binge drinking. A major aim of the present study was to determine c-Fos activity within the CeA-BNST circuit during binge-like alcohol consumption. The present findings were limited by inconsistent labeling of the CeA-BNST circuit but do support a

general role for the CeA in binge drinking behavior. Because KOR within the BNST modulate binge drinking behavior (Haun et al., 2020), it is reasonable to suspect involvement of DYN-containing neurons within the CeA-BNST circuit given that the CeA is activated during periods of binge drinking and that dynorphinergic neurons within the CeA promote binge drinking behavior (Anderson et al., 2018a; Bloodgood et al., 2020). Thus, these data support the further exploration of DYN-containing neurons within the CeA-BNST circuit in the context of binge drinking.

CHAPTER 4: Chemogenetic Inhibition of the CeA-BNST^{DYN} Circuit Attenuates Binge-Like Alcohol Consumption.

INTRODUCTION

We have previously shown that KOR within the BNST contribute to binge-like alcohol consumption and it is hypothesized that dynorphinergic projections from the CeA to the BNST mediate this effect. Neuroimaging of the CeA or BNST in subjects with AUD or those with a history of binge drinking is limited, but there is evidence to suggest that these structures are involved in affective disorders that have a high co-occurrence with AUD. For example, high resting state connectivity has been observed between the CeA and BNST and synchronized activity is associated with the behavioral response to emotionally salient negative stimuli (Pedersen et al., 2019; Pedersen et al., 2020). Functional activity within these regions is also associated with anxiety in non-human primates (Fox et al., 2018; Oler et al., 2012; Torrisi et al., 2015). Activity within the extended amygdala is thought to promote maladaptive behaviors that alleviate stress and anxiety, such as alcohol consumption (Koob, 2003; Koob and Volkow, 2016). For example, amygdala activity during withdrawal is positively associated with AUDIT score and is a strong predictor of AUD (Fede et al., 2019; Hu et al., 2018; Peters et al., 2017). Conversely, acute alcohol dampens amygdala and BNST reactivity in response to stressors including emotionally charged images in humans (Gilman et al., 2008; Hur et al., 2018; Sripada et al., 2011). Increased activity within the extended amygdala observed during withdrawal reflects a shift in allosteric load that is thought to contribute to relapse, and the subsequent binge consumption of alcohol transiently quells withdrawal-related sequalae (Koob, 2013; Koob and Le Moal,

2008). The DYN/KOR system contributes to stress and anxiety experienced during withdrawal that drives excessive drinking (Koob, 2003; Koob and Le Moal, 2008). For example, a polymorphism in the genes coding for DYN and KOR, *Pdyn* and *Oprk1* respectively, have been reported in patients with AUD and are associated with increased anxiety, impulsivity, and drinking severity (Edenberg et al., 2008; Park et al., 2020; Votinov et al., 2014; Xuei et al., 2006; Xuei et al., 2007). Current human imaging techniques, however, are limited by resolution of subcortical structures making observation of DYN/KOR activity specifically within the CeA and BNST of humans difficult at this time.

Studies involving various preclinical models of AUD have demonstrated a similar recruitment of the DYN/KOR system in the promotion excessive drinking and relapse-like behavior. For example, Pdyn/DYN expression is elevated within the extended amygdala in rats with a history of alcohol dependence achieved through chronic intermittent ethanol (CIE) exposure (Erikson et al., 2018; Kissler et al., 2014). High levels of alcohol intake achieved after CIE can be attenuated by systemic delivery of a KOR antagonist supporting a more causal role for KOR in excessive drinking (Walker and Koob, 2008; Walker et al., 2011). Further probing of the extended amygdala revealed that site-specific delivery of a KOR antagonist into the CeA, BNST, or NAc similarly decreased dependent-like alcohol consumption (Erikson et al., 2018; Kissler et al., 2014; Nealey et al., 2011). It is likely that circuity promoting high levels of alcohol intake in models of dependence may also contribute to binge-drinking as this pattern of drinking is positively correlated with the emergence of dependence across species (Addolorato et al.,

2018; Baker et al., 2017; Kroenke et al., 2014). In fact, recent studies support a more general role for KOR in excessive drinking beyond the context of dependence. For example, systemic delivery of a KOR antagonist attenuates binge-like alcohol drinking in mice (Anderson et al., 2018a; Haun et al., 2020). Direct delivery of a KOR antagonist into the CeA or BNST decreased binge-like drinking, similar to the effect observed in subjects with a history of dependence (Anderson et al., 2018a; Erikson et al., 2018; Haun et al., 2020; Kissler et al., 2014). Finally, systemic KOR agonist treatment increased binge drinking, an effect that is blocked by KOR antagonist delivery into the BNST suggesting that KOR in the BNST are actively engaged to promote alcohol drinking behavior (Haun et al., 2020). However, the endogenous circuity that provides dynorphinergic input to the BNST involved in drinking behavior is unclear.

The CeA sends dense projections to the BNST and the CeA-BNST circuit contributes to various alcohol-related behaviors, such as anxiety and fear (Asok et al., 2018; Gilpin et al., 2014; Le et al., 2018; Roberto et al., 2012). A dense population of neurons within the lateral subdivision of the CeA expresses DYN, among other neuropeptides, and sends direct projections to the BNST (CeA-BNST^{DYN}) (Ahrens et al., 2018; Li et al., 2012; Marchant et al., 2007). No studies to date have directly investigated a functional role of CeA-BNST^{DYN} circuitry, however, chemogenetic inhibition of DYN-containing neurons within the CeA (CeA^{DYN}) reduced binge-like alcohol consumption (Anderson et al., 2018). This study provided the first evidence of DYN-containing neurons within the CeA contributing to binge drinking. Further studies revealed that genetic deletion of

DYN from the CeA attenuated binge-like alcohol consumption, directly pointing to DYN as a driver of excessive drinking (Bloodgood et al., 2020). Therefore, chemogenetic inhibition and deletion of DYN from CeA^{DYN} point to this population as a key hub for binge drinking, but the downstream site of these projections has yet to be determined. Manipulation of other CeA-BNST projections provide valuable insight given the high levels of peptide co-expression within the CeA. For example, DYN is largely co-expressed with CRF in the CeA and optogenetic silencing of the CeA-BNST^{Crf} circuit attenuated dependence-related drinking and decreases fear and anxiety-like behavior in rats (Asok et al., 2018; de Guglielmo et al., 2019; Pomrenze et al., 2019b). Somatostatin (SST) colocalizes with DYN to a large extent within the CeA and activation of CeA-BNST^{SST} projections increased anxiety like behavior in mice, an effect that is dependent on KOR in the BNST providing evidence for functional CeA-BNST^{DYN} circuitry (Ahrens et al., 2018). Because the CeA-BNST^{DYN} pathway has not been directly manipulated in the context of binge drinking, the present studies were designed to use a chemogenetic approach to inhibit CeA-BNST^{DYN} in the context of binge drinking in male and female mice.

MATERIALS AND METHODS

Subjects

Male and female Pdyn-IRES-Cre mice ranging from 10-12 weeks old were from an in-house colony as previously described (Anderson et al., 2018a; Krashes et al., 2014). All animals were singly housed and tested in a temperature and humidity controlled AAALAC approved facility on a reverse 12-hr light/dark cycle with food and water continuously available. For all experiments, mice were treated in accordance with both the NIH Guide for the Care and Use of Laboratory Animals (National Research Council, 2011) and the Institutional Animal Care and Use Committee at MUSC.

Surgical Procedures

Mice were anaesthetized with isoflurane and 0.25 uL of AAVrg-hSyn-DIO-hM4Di-mCherry or AAVrg-eF1a-eGFP was bilaterally infused into the BNST (AP: + 0.22, ML: +/- 0.75, DV: -4.6) using a 0.5 uL Hamilton Neuros syringe at a flow rate of 0.05 uL/min for 5 min. The syringe was then left in place during a 10 min diffusion period and retracted over 5 min as previously described (Haun et al., 2018). Bilateral guide cannula (Plastics One, Inc.) were then positioned above the CeA (AP: -1.2, ML: +/- 3.0, DV: -3.6) and secured to the skull with a light-cured resin system (Haun et al., 2018). Once inserted, microinjector tips extended 1 mm beyond the guide to target the CeA for microinjection. After surgery, all mice were given 4 weeks to recover for adequate viral expression prior to the start of experiments.

Alcohol Binge Drinking Procedure

Mice were trained to drink alcohol in their home cage in the limited access "Drinking-in-the-Dark" (DID) procedure, as previously described (Anderson et al., 2018a; Haun et al., 2020). Prior to drinking, mice were habituated to the microinjection procedure by removing and then replacing dummy guides from the microinjector tract 30 minutes prior to drinking. A single bottle of alcohol (20% v/v) was then presented 3 hours into the dark cycle in place of the water bottle. Access to alcohol was for 2 hours on 3 consecutive days, and then extended for 4 hours on the 4th day. Alcohol intake was determined for each 2-hour session and then for the 0-2 and 2-4 hour time periods during the final (4th day) 4-hour drinking session. An identical procedure was used for assessing sucrose (0.5% w/v) consumption. All mice were given one 4-day baseline cycle of binge drinking followed by 3 days rest in the home cage. This 4-day binge drinking cycle followed by 3 days of rest was repeated for 2 more cycles involving experimental testing. For all experiments, microinjection challenge occurred 30-min before the 4-hour binge session during the second and third binge cycle. Average drinking across the first 3 days of the second binge cycle were used to separate subjects evenly into drug treatment groups in a balanced within-subjects design. Immediately after the 4-hour test drinking session, blood samples were collected, plasma extracted, and blood alcohol concentrations determined using an AM1 Alcohol Analyzer (Analox Instruments, Stourbridge, UK).

After the three 4-day binge cycles of alcohol drinking, mice were given 4-weeks rest with food and water provided *ad-libitum* prior to sucrose drinking. Sucrose (0.5% v/v) drinking was carried out in an identical fashion to alcohol

drinking. This included one 4-day baseline week of sucrose binge drinking followed by 3 days of rest. This 4-day binge cycle was repeated for 2 more test weeks with microinjections occurring 30-min prior to testing on day 4 of the cycle.

Microinjection Procedures

The DREADD agonist clozapine-*N*-oxide (CNO; 1 mM/side, Tocris) was dissolved in 1xPBS for microinjection. This dose was chosen given strong Gi-induced neuronal quiescence in various brain regions and documented usage for circuit level manipulation in the DID model (Mahler and Aston-Jones, 2018; Mahler et al., 2014; Rinker et al., 2017). Microinjections were administered 30-min before Day 4 of drinking during the second and third binge cycle in a within-subjects design. Vehicle or CNO (1 mM/side) was delivered bilaterally into the CeA at a flow rate of 0.25 µL/min for 2-min, followed by a 2-min diffusion period before microinjector removal (Anderson et al., 2018a; Griffin et al., 2014; Haun et al., 2018). Obdurators were replaced and mice returned to their home cage where they remained undisturbed until testing.

Histology

At the conclusion of all experiments, mice were euthanized with urethane and transcardially perfused with 10 mL saline followed by 10 mL of paraformaldehyde (PFA; 4%). Brains were extracted, post fixed in 4% PFA for 24 hours and cryoprotected in sucrose (30% wt/vol) until sectioning. Tissue was sliced in serial 40 µM sections and processed for verification of viral expression as previously described (Anderson et al., 2018a). Briefly, tissue was washed in 1xPBS and incubated in rat anti-mCherry (1:1000; Invitrogen) overnight at room

temperature. The following day, tissue was washed and incubated in AlexaFluor goat anti-rat 555 (1:1000; ThermoFisher) for 2 hr. Tissue was then mounted on permafrost slides with Prolong Diamond with Dapi for imaging. Tissue from mice receiving AAVrg-eF1a-DIO-eGFP was perfused, sectioned, washed, and mounted onto slides for imaging. IHC was not necessary given the strong endogenous eGFP signal. Images were collected under 4x, 10x, and 20x magnification on an EVOS-FL microscope (AMF-4300, ThermoFisher). Only mice with viral expression and bilateral guide placements in the CeA in reference to a mouse stereotaxic atlas were included in the final analyses (Franklin and Paxinos, 2008).

Statistical Analysis

The primary dependent variables were alcohol intake (g/kg), blood alcohol concentration (BAC; mg/dL), and sucrose intake (mL/kg). All data were analyzed by ANOVA, with Sex and Virus (hM4Di; eGPF) as between-subjects factors and Drug (Vehicle; CNO) and Time (0-2; 2-4 hr) as a repeated factors as necessary. Significant factor interactions were further evaluated using the Student-Newman–Keuls (SNK) for post-hoc comparisons. Alpha was set to $p < 0.05$ for all analyses.

RESULTS

Dynorphin-Containing Neurons in the CeA Project to the BNST.

We have previously demonstrated that both CeA^{DYN} neurons and KOR within the BNST contribute to excessive alcohol drinking in the DID model (Anderson et al., 2018a; Haun et al., 2020). Therefore, direct connectivity between CeA-BNST^{DYN} circuitry may mediate binge-like alcohol consumption. To directly test this, Pdyn-IRES-Cre mice were used to selectively target the CeA-BNST^{DYN} circuit for chemogenetic silencing during binge drinking sessions. Commonly used viral strategies for circuit level chemogenetic manipulation utilize an anterograde-expressing viral vector that allows for receptor expression in the downstream terminal fields where CNO is directly applied for selective circuit activation/inhibition (Armbruster et al., 2007; Lee et al., 2014; Mahler et al., 2014; Roth, 2016; Urban and Roth, 2015). However, an alternative approach was necessary to target CeA^{DYN} projections terminating in the BNST given the disruption to behavior previously observed when microinjecting vehicle into the BNST 30-min prior to drinking (**Figure 2.7**). Therefore, the AAVrg serotype was utilized that allows for efficient retrograde expression restricted to a select cell population when utilizing a Cre-driver line (Tervo et al., 2016). A double-floxed Gi-coupled DREADD (or a control vector) under the AAVrg serotype was applied to the BNST and CNO was targeted directly into the CeA to selectively influence hM4Di receptors on cell bodies in CeA-BNST^{DYN} circuit for chemogenetic inhibition.

Mice received bilateral infusions (0.25 uL/side) of AAVrg-hSyn-DIO-hM4Di-mCherry (hM4Di) or AAVrg-eF1a-DIO-eGFP (eGFP) into the BNST and guide cannulae were positioned above the CeA to selectively target cell bodies in CeA-BNST^{DYN} circuit with vehicle or CNO (**Figure 4.1A**). Cre-dependent expression of hM4Di selectively within CeA^{DYN} of Pdyn-IRES-Cre mice has been previously validated (Anderson et al., 2018a). Functionality has also been confirmed by electrophysical testing that showed that bath application of CNO resulted in significant hyperpolarization indicative of hM4Di-mediated inhibition (Anderson et al., 2018a).

Expression of the mCherry (or eGFP) fluorescent tag was observed in terminals within the dorsal and ventral subdivisions of the BNST (**Figure 4.1B**). Expression of mCherry (or eGFP) was observed within the central lateral amygdala (CeL) consistent with viral tracing studies demonstrating that neuronal populations expressing DYN in the CeL send dense projections to the BNST (**Figure 4.1C**) (Ahrens et al., 2018; Li et al., 2012). Microinjector tip placements within the CeA are represented with black circles. Additional images showing bilateral DYN+ terminal expression in the BNST and expression in upstream DYN+ populations including the CeA, BMA, and PVN are shown in **Supplemental Figure 1A**. Representative images of AAVrg-eF1a-DIO-eGFP expression in the CeA is shown in **Supplemental Figure 1B.**

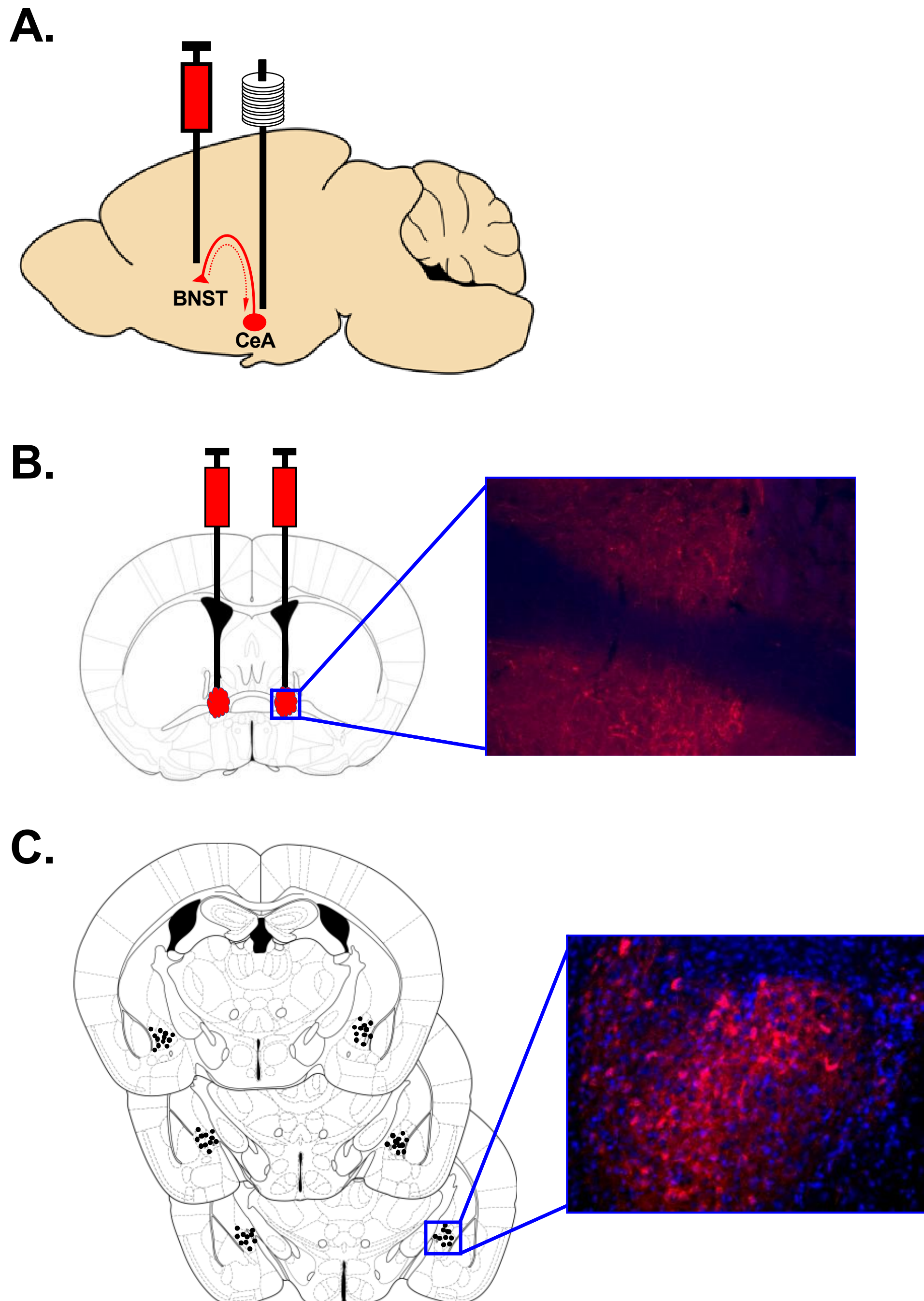


Figure 4.1: Viral strategy for targeting CeA-BNST^{DYN} circuit. **A)** Sagittal section depicting AAVrg-hSyn-DIO-hM4Di-mCherry or AAVrg-eF1a-DIO-eGFP infusion into the BNST of Pdyn-IRES-Cre and guide cannula positioning above the CeA. **B)** Stereotaxic delivery of AAVrg-hSyn-DIO-hM4Di-mCherry into the BNST resulted in expression of mCherry within DYN+ afferent terminals. Red= mCherry; Blue= Dapi **C)** AAVrg uptake by Cre+ DYN-containing terminals in the BNST results in expression within cell bodies within the CeA. Black circles represent microinjector tip placements within the CeA targeting CeA-BNST^{DYN} neurons. Red= mCherry; Blue= Dapi.

Effect of CeA-BNST^{DYN} Inhibition on Binge-Like Alcohol Consumption.

Male and female mice expressing AAVrg-hSyn-DIO-hM4Di-mCherry (hM4Di) or AAVrg-eF1a-DIO-eGFP (eGFP) were split into treatment groups based on average intake across the preceding three days of 2-hour alcohol drinking. Drinking data from 17 males (hM4Di= 9; eGFP= 8) and 16 females (hM4Di= 9; eGFP= 7) were included in the final analysis after viral expression and microinjector guide placement were confirmed. No differences in alcohol consumption were observed during 4-hour testing based on Sex [$F(1,29)= 3.21$, $p= 0.08$] with males consuming an average of 2.19 ± 0.12 g/kg and females 2.50 ± 0.12 g/kg. Therefore, drinking data were collapsed across Sex for further analysis.

During the test session, silencing of the CeA-BNST^{DYN} circuit resulted in decreased alcohol consumption across both 0-2 and 2-4 hour timepoints (**Figure 4.2A**). 4-way ANOVA (Sex x Virus x Time with Drug as a repeated factor) revealed a main effect of Drug [$F(1,58)= 5.26$, $p< 0.05$] and a Drug x Virus interaction [$F(1,58)= 10.99$, $p< 0.01$). There were no significant main effects of Sex, Virus, or Time (Fs< 2.49). Post hoc analysis indicated significantly lower alcohol consumption during the 0-2 and 2-4 hour timepoints in mice expressing hM4Di within the CeA-BNST^{DYN} circuit after CNO microinjection compared to vehicle (ps< 0.01). Furthermore, intake in the hM4Di group after CNO challenge was lower than both vehicle and CNO treatment in mice expressing the eGFP control virus at both timepoints (ps< 0.01). Drinking after CNO challenge in mice expressing eGFP was no different than mice receiving vehicle.

Likewise, analysis of alcohol intake during the entire 4-hour test session indicated that CNO reduced alcohol consumption compared to vehicle after silencing of the CeA-BNST^{DYN} circuit (**Figure 4.2B**). This was supported by a significant main effect of Drug [$F(1,29)= 6.60$, $p< 0.05$] and Drug x Virus interaction [$F(1,29)= 13.79$, $p< 0.001$). Post hoc analysis further supported overall decreased alcohol intake in mice expressing hM4Di after CNO compared to vehicle ($p< 0.001$) and compared to eGFP-expressing mice receiving vehicle ($p< 0.01$) or CNO ($p< 0.005$). Compared to vehicle, no effect of CNO was observed in eGFP-expressing mice. Lastly, resultant BACs were significantly lower after silencing of the CeA-BNST^{DYN} circuit (**Figure 4.2C**). ANOVA revealed a main effect of Drug [$F(1,31)= 6.86$, $p< 0.01$] and Drug x Virus interaction [$F(1,31)= 12.99$, $p< 0.001$). Further post hoc analysis determined that, compared to vehicle, hM4Di-expressing mice receiving CNO had significantly lower BACs ($p< 0.005$). This BAC was also lower than eGFP-expressing mice treated with vehicle or CNO ($ps< 0.05$).

For reference, alcohol drinking data from mice excluded from the above analysis due to lack of viral expression within the CeA is shown in **Supplemental Figure 2A**. 4-way ANOVA (Sex x Virus x Time with Drug as a repeated factor) revealed no significant main effects nor factor interactions across the 0-2 and 2-4 hour timepoints ($Fs< 1.73$). Similarly, analysis of alcohol intake during the entire 4-hour test session was similar across groups receiving vehicle microinjection, compared to control ($Fs< 0.15$).

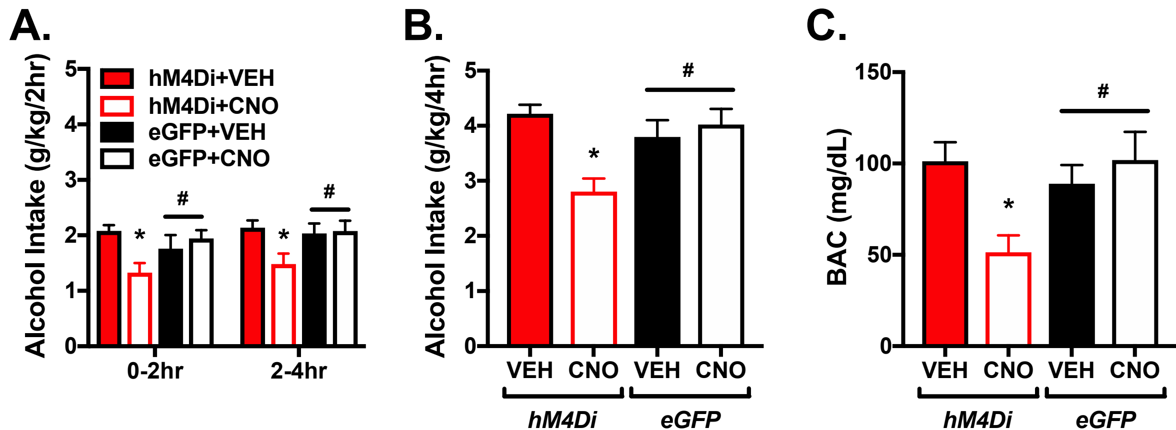


Figure 4.2: Chemogenetic inhibition of CeA-BNST^{DYN} reduces binge-like alcohol consumption. **A)** Alcohol intake (g/kg) during the consecutive 2-hour time periods (0-2 and 2-4 hour) in the 4-hour access session. Microinjection of CNO to silence the CeA-BNST^{DYN} circuit in hM4Di-expressing mice resulted in a significant decrease in alcohol intake across both time points compared to vehicle (VEH; *ps <0.005) and compared to eGFP-expressing mice receiving VEH or CNO (#ps< 0.005). No differences in drinking were observed after CNO microinjection in eGFP expressing mice. **B)** Cumulative alcohol intake across the 4-hour session. Cumulative alcohol intake across the entire 4-hour session was lower in hM4Di-expressing mice after CNO compared to VEH (*p <0.005) and compared to eGFP-expressing mice receiving VEH or CNO (#ps< 0.005). **C)** Blood alcohol concentration (BAC) after binge drinking. CeA-BNST^{DYN} silencing with CNO resulted in significantly lower BACs in hM4Di-expressing mice compared to VEH (*p< 0.005). BACs were significantly lower than eGFP-expressing mice receiving VEH and CNO (#p< 0.005). No differences in BAC were observed compared to VEH after CNO microinjection in eGFP-expressing mice.

Effect of CeA-BNST^{DYN} inhibition on binge-like sucrose consumption.

While silencing of CeA^{DYN} does not affect binge-like sucrose drinking, targeted blockade of KOR within the BNST modestly reduced sucrose drinking in the DID model (Anderson et al., 2018a; Haun et al., 2020). To determine the effect of chemogenetic CeA-BNST^{DYN} inhibition on sucrose intake, drinking was assessed in 17 males (hM4Di= 9; eGFP= 8) and 16 females (hM4Di= 9; eGFP= 7) after a 4-week cessation from alcohol drinking. Males consumed an average of 32.47 ± 4.82 mL/kg and females 44.10 ± 2.88 mL/kg during 4-hour drinking and a main effect Sex neared significance [$F(1,29)= 3.155$, $p= 0.09$]. Thus, drinking data were collapsed across Sex for further analysis.

Silencing of the CeA-BNST^{DYN} circuit had no effect on sucrose intake during the 0-2 and 2-4 hour portions of the test session (**Figure 4.3A**). 4-way ANOVA (Sex x Virus x Time with Drug as a repeated factor) indicated no significant main effect of Drug [$F(1,58)= 0.124$, $p= 0.73$] suggesting that CNO had no effect on sucrose intake in both hM4Di and eGFP-expressing mice. A main effect of Time [$F(1,58)= 4.26$, $p< 0.05$] and Time x Sex intereaction [$F(1,58)= 31.84$; $p< 0.001$) were observed and further post hoc analysis indicated that females consumed more sucrose during the 2-4 hour epoch compared to males ($p< 0.01$). Cumulative drinking across the entire 4 hour session was not affected by microinjection of vehicle or CNO into the CeA of both hM4Di- and eGFP-expressing mice (**Figure 4.3B**). While a main effect of Sex neared significance [$F(1,29)= 3.16$, $p= 0.09$], no effect of Virus or factor interaction was observed ($Fs< 0.90$).

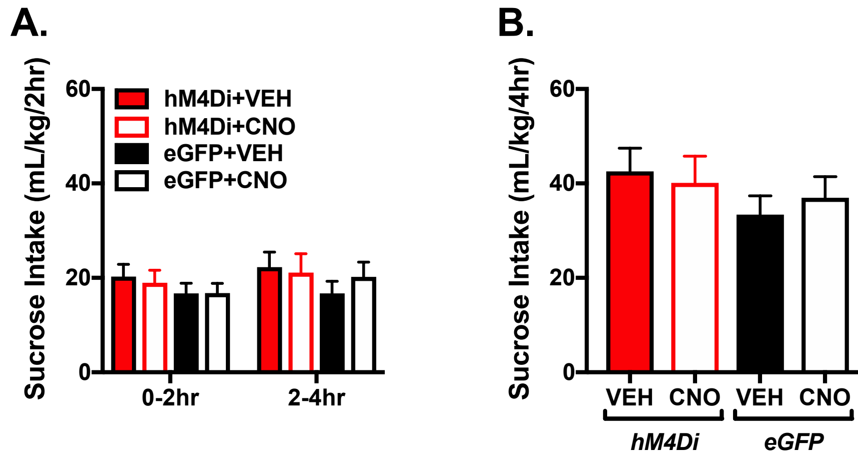


Figure 4.3: Inhibition of CeA-BNST^{DYN} does not affect binge-like sucrose consumption. **A)** Sucrose intake (mL/kg) during the consecutive 2-hour time periods (0-2 and 2-4 hour) in the 4-hour access session. Microinjection of CNO into the CeA to silence the CeA-BNST^{DYN} circuit in hM4Di-expressing mice did not affect sucrose consumption across the binge session. CNO had no effect in eGFP-expressing controls. **B)** Similarly, cumulative sucrose intake across the 4-hour session was not affected by CNO in both hM4Di and eGFP-expressing groups.

DISCUSSION

The extended amygdala has long been implicated in excessive alcohol drinking and we have previously demonstrated recruitment of neurons within the CeA during periods of binge drinking. Furthermore, recent studies have supported a role for CeA^{DYN} and implicated KOR within the CeA and BNST specifically in the context of binge-like drinking behavior (Anderson et al., 2018a; Bloodgood et al., 2020; Haun et al., 2020). Given the strong connectivity between the CeA and BNST, it was hypothesized that dynorphinergic projections from the CeA to the BNST (CeA-BNST^{DYN}) contribute to binge drinking. Here we report that inhibition of the CeA-BNST^{DYN} circuit using a novel chemogenetic strategy significantly attenuated binge-like alcohol consumption in male and female mice. This effect was not observed in mice harboring a control vector nor in those that received viral surgery but failed to exhibit hM4Di expression within the CeA-BNST^{DYN} circuit. Furthermore, the reduction in drinking behavior was specific to alcohol because CeA-BNST^{DYN} inhibition had no effect on sucrose drinking under similar conditions. Taken together, these data suggest that the CeA-BNST^{DYN} circuit promotes excessive alcohol consumption within the DID model of binge drinking.

To the best of our knowledge, this is first use of an AAVrg strategy involving the microinjection of CNO in a target neuronal population for circuit level manipulation. This strategy was implemented as an alternative to direct CNO application at CeA^{DYN} terminal fields in the BNST because vehicle microinjection into the BNST disrupted both drinking behavior and locomotor activity in previous experiments. The limited use of this AAVrg strategy is likely due to the potential for

activation of collateralized axonal inputs at the site of CNO administration. For example, Cre-dependent AAVrg infusion in the BNST resulted in expression within multiple DYN-expressing BNST afferent sites, including the CeA, BMA, and PVN (**Appendix Figure 1A**). The BMA sends peptide-rich projections to both the CeA and BNST and there is evidence of collateralization between these regions (Bienkowski and Rinaman, 2013; Poulin et al., 2006). Because KOR antagonist delivery into the CeA decreased binge drinking behavior, it is possible that collateral BMA^{DYN} projections terminating in the BNST and CeA could be affected by CNO microinjection and influence drinking (Anderson et al., 2018a). While we acknowledge this as a possibility, it is unlikely because dual retrograde tracing studies show minimal collateralization (~6%) within CeA- and BNST-projecting neurons of the BMA (Bienkowski and Rinaman, 2013). Similarly, the BNST is also rich in DYN-containing neurons and projections from the BNST to the CeA have been described (Crowley et al., 2016; Gungor et al., 2015; Kash et al., 2015). However, we only observed terminal expression in the BNST after Cre-dependent viral infusion suggesting that the virus was not expressed locally within soma in the BNST. Thus, under the current experimental conditions, it is possible but unlikely that the effects observed were due to collateral activation of DYN-expressing terminals within the CeA. An optogenetic approach for CeA-BNST^{DYN} terminal inhibition is a viable option for future studies in that this strategy has been effectively utilized in mice to target the CeA-BNST circuit with cell type specificity (Ahrens et al., 2018; Li et al., 2012).

While these data demonstrate a causal role for the CeA-BNST^{DYN} circuit in binge-like alcohol consumption, further studies will be of importance to clarify the exact role of DYN within this pathway. Combined chemogenetic hM3Dq-mediated activation and pharmacological blockade of target receptors in the downstream terminal field is a common strategy to determine the contribution of a select peptide in a circuit. For example, microinjection of a CRF1 receptor antagonist into the BNST blocked the expression anxiety-like behavior after activation of the CeA-BNST^{Crf} pathway (Pomrenze et al., 2019b). Similarly, optogenetic activation of CeA-BNST^{SST} is sufficient to induce an anxiogenic phenotype and expression of anxiety-like behavior is blocked by nor-BNI administration in the BNST (Ahrens et al., 2018). The use of the AAVrg serotype in the present study involved microinjection of CNO into the CeA to target cell bodies within the CeA-BNST^{DYN} pathway. Therefore, pharmacological challenge within the BNST after circuit level manipulation was not feasible under these methodological constraints. Therefore, the exact role of DYN within the CeA-BNST circuit is unclear under these experimental conditions because DYN-containing neurons within the CeA also express GABA, CRF, NTS, and SST, among other neuropeptides (Ahrens et al., 2018; Marchant et al., 2007; Pomrenze et al., 2019a; Sanford et al., 2017; Torruella-Suarez et al., 2020). To address these concerns, we propose an intersectional viral approach for future studies to selectively knock down DYN expression within the CeA-BNST pathway. More specifically, this approach involves the use of 2 viral vectors. The first, AAVrg-Cre, can be infused in the BNST such that the transgene Cre would be expressed in BNST afferents, including

neurons within the CeA that terminate in the BNST. Secondly, a double-floxed Pdyn-shRNA infused into the CeA would then target neurons within the CeA-BNST circuit for pathway selective DYN-knockdown. While the present study demonstrated a role for bulk CeA-BNST^{DYN} circuit activity in binge drinking behavior, implementation of the strategy described above will better isolate the contribution of DYN, and DYN alone, within the circuit to drinking.

In contrast with our previous studies, no differences in alcohol intake were observed between male and female mice consuming alcohol in the DID model. Females generally consume more alcohol than males across various limited access drinking procedures, but this effect is not consistently observed (Finn et al., 2005; Hilderbrand and Lasek, 2018; Sneddon et al., 2019). It is likely that different experimental handling procedures across our studies accounts for this variation. In the present study, mice received a microinjection 30-min prior to a binge drinking session that involved robust handling in close temporal proximity to drinking behavior. Similarly, we did not observe sex differences in alcohol intake when drug was administered into the BNST 30-min prior to drinking (**Figure 2.5**). In contrast, females consumed more alcohol than males when microinjections occurred 16-hr prior to drinking or when mice did not receive handling prior to drinking (**Figure 2.1; Figure 3.2**). Thus, the robust handling involved in the microinjection procedure in close proximity to drinking may have resulted in slightly lower alcohol intake, masking potential sex differences. Different experimental handling procures in future studies for CeA-BNST^{DYN} manipulation may uncover differential reactivity to circuit level manipulation. It is also important to note that sex differences in c-Fos

immunoreactivity within the BNST after U50,488 administration and in *KOR* mRNA expression have been reported suggesting that CeA-BNST^{DYN} activation may reveal sex differences in drinking behavior (Conway et al., 2019; Russell et al., 2014). In the context of binge drinking, however, our studies indicate no sex differences in the ability of U50,488 to enhance alcohol drinking to a high level, nor in the ability of nor-BNI to counter U50,488's effects within the BNST. Furthermore, inhibition of the CeA-BNST^{DYN} circuit did not differentially affect alcohol consumption in male and female mice but we cannot rule out the possibility of sex differences given our experimental design.

An important finding of the present study was alcohol, but not sucrose drinking was attenuated by CeA-BNST^{DYN} inhibition suggesting pathway specific recruitment involved in motivated behavior exclusive to alcohol. This is in general agreement with our studies showing that systemic administration a KOR antagonist decreased binge-like alcohol consumption but did not affect sucrose intake (Anderson et al., 2018a). Interestingly, site-specific KOR blockade in the BNST decreased alcohol drinking but modestly reduced sucrose consumption. These data seem to suggest that KOR in the BNST play a role in general consummatory behaviors but selective inhibition of the CeA-BNST^{DYN} circuit did not influence sucrose consumption. This is a potentially important finding because the CeA is associated with reward saliency, involved in the maintenance of consummatory behavior, and it would be feasible for projections to the BNST to be involved in a motivated behavior such as sucrose drinking (Douglass et al., 2017; Kim et al., 2017; Knapska et al., 2006; Mahler and Berridge, 2009). The

DYN/KOR system is also known to influence feeding behavior, including consumption of natural rewards like sucrose (Karkhanis et al., 2017; Nogueiras et al., 2012). Manipulation of CeA^{DYN} in the context of sucrose drinking or general consummatory behavior has not been directly tested, but manipulation of other populations within the CeA that colocalize with DYN, such as nociceptin and neurotensin, provide valuable insight into the role of select CeA projections (Hardaway et al., 2019; Normandeau et al., 2018; Serafin et al., 2019). For example, chemogenetic inhibition of nociceptin-containing neurons in the CeA decreased sucrose intake supporting a role for peptidergic neurons within the CeA in natural reward behavior (Hardaway et al., 2019). However, ablation of neurotensin-expressing neurons in the CeA decreased alcohol intake but not sucrose consumption, consistent with our findings with CeA-BNST^{DYN} inhibition (Torruella-Suarez et al., 2020). Thus, KOR activity influenced by CeA-BNST^{DYN} projections may act on discreet populations of neurons within the BNST that are involved in alcohol drinking behavior opposed to circuitry involved in the general consumption of palatable rewards.

It is important to note that DYN has both anterograde and retrograde release properties (Gupta and Gintzler, 2003; Lindholm et al., 2007; Margolis et al., 2003). The present chemogenetic strategy for circuit level inhibition involved CNO delivery to cell bodies within the CeA. Thus, local KOR activity in the CeA could have been affected because retrograde release of DYN has been described in the extended amygdala (Crowley et al., 2016; Gilpin et al., 2014; Kang-Park et al., 2013; Lindholm et al., 2007). Because microinjection of a KOR antagonist or

genetic KOR deletion within the CeA decreased binge-like alcohol consumption, we cannot rule out the possibility that the effect on drinking observed in the present study was due to decreased retrograde or terminal release of DYN within the CeA after CeA-BNST^{DYN} inhibition (Anderson et al., 2018a; Bloodgood et al., 2020). This does not negate involvement of the circuit itself, but does raise the question as to the site of DYN-mediated effects on drinking behavior. In support of local KOR engagement in the CeA, $GABA_A$ receptors are expressed on presynaptic terminals and microinjection of a $GABA_A$ antagonist into the CeA decreases drinking in non-dependent rats suggesting a similar presynaptic mechanism as KOR (Hyytia and Koob, 1995). In fact, KOR antagonist application to the CeA increased local inhibition via increased presynaptic release of GABA and this mechanism is sensitive to the effects of alcohol (Gilpin et al., 2014; Kang-Park et al., 2013). However, an inherent discrepancy exists in that KOR antagonists increase GABA release but GABA activity is also enhanced within the CeA of alcohol-dependent rats and mice that contributes to excessive drinking (Kang-Park et al., 2013; Gilpin et al., 2014). Therefore, it is counterintuitive that KOR-antagonist treatment dually increases presynaptic GABA release and also decreases alcohol intake since increased GABA is associated with increased drinking. It is important to note that study of alcohol's effects on CeA functionality, be it chronic or acute, has primarily focused on the medial subdivision (CeM) of the CeA, which is the main source of behavioral output in the amygdala. Interestingly, KOR-expressing neurons within the CeA are primarily found within the lateral subdivision (CeL) and neurons within the CeL impinge directly on output

neurons within the CeM and functionally gate behavioral output through a monosynaptic GABAergic connection (Bloodgood et al., 2020; Herman et al., 2013). Therefore, KOR antagonists may facilitate GABA release onto CeM output neurons resulting in decreased drinking behavior (Kang-Park et al., 2013). Similarly, within the CeL, DYN or KOR agonist activity at presynaptic GABAergic terminals would have the net effect of CeL disinhibition that may influence the activity of other peptide rich populations within the CeL that drive drinking behavior (De Guglielmo et al., 2019). More specifically, CRF-expressing neurons in the CeL promote excessive drinking in the context of alcohol dependence and there is extensive literature supporting interactions between the DYN and CRF systems (Bruchas et al., 2010; de Guglielmo et al., 2019; Pomrenze et al., 2019a). Therefore, KOR antagonists may drive increased GABA release onto CeL^{Crf} neurons and thereby decrease alcohol intake, a possibility that has not been tested to the best of our knowledge. We suspect that CeA-BNST^{DYN} inhibition may influence local release of DYN in the CeA and attenuate drinking similar to the effect of intra-CeA nor-BNI through the scenario outlined above. Further study of the topography of KOR expression within the microcircuitry of the CeL and CeM may be the key to unlocking the discrepancy between KOR-mediated regulation of GABAergic activity and the effects of alcohol on GABA release in the CeA.

Concerning KOR activity in the BNST, a series of elegant studies from the Kash lab supports a role for presynaptic KOR modulation of GABA release similar to that described above within the CeA. More specifically, bath application of the KOR agonist, U69593, or DYN results in decreased presynaptic release of GABA

within the BNST (Li et al., 2012). Because the BNST is rich in KOR-expressing nuclei, it is possible that KOR modulation of GABA release is derived from local interneurons or from presynaptic terminals of BNST afferents. Sorting of neuronal populations based on cellular properties revealed that the effect of U69593 on BNST activity was specific to KOR-expressing afferents because activity in interneurons was not affected. Therefore, we posit that the decrease in drinking behavior observed after CeA-BNST^{DYN} inhibition or nor-BNI microinjection into the BNST is due to enhanced presynaptic GABA release. Interestingly, KOR-mediated suppression of GABAergic release in the BNST has been observed in BNST-projecting neurons of the CeA (Li et al., 2012). This suggests the existence of two parallel circuits within CeA-BNST circuitry because there is minimal co-expression of DYN and KOR within the CeA (Bloodgood et al., 2020). The functional role of these dissociable CeA-BNST^{DYN} and CeA-BNST^{KOR} pathways has not been explored. In support of this hypothesis, there is evidence for recruitment of presynaptic KOR on discrete populations within the BNST based on the source of input. For example, activation of glutamatergic BNST-projecting neurons of the BLA promotes the expression of an anxiolytic phenotype (Crowley et al., 2016). However, activation of DYN-containing neurons within the BNST attenuates glutamate release from the BLA and not from the PFC, suggesting pathway specific KOR modulation of neurotransmitter release relevant to anxiety and drinking behavior (Crowley et al., 2016). Furthermore, glutamatergic projections from the mPFC that terminate in the BNST promote activity of local DYN-containing neurons that drive anxiety-like behavior and excessive drinking (Hwa et

al., 2019). This suggests that activity of DYN/KOR within the BNST can be derived from different sources of input that act on discrete circuity. However, in-vivo evidence of endogenous DYN release within the BNST has been limited to stimulated release of local DYN-containing neurons (Crowley et al., 2016). Retrograde release of DYN has been observed in the NAc, CeA, and BNST and action at presynaptic KOR in these regions decreases GABA release (Crowley et al., 2016; Gilpin et al., 2014; Lindholm et al., 2007). There is also evidence for anterograde release of DYN directly onto presynaptic terminals or dendrites/cell bodies expressing KOR (Gupta and Gintzler, 2003; Margolis et al., 2003). Therefore, it is possible that CeA-BNST^{DYN} directly influences activity of CeA-BNST^{KOR} through an axoaxonic interaction. Future studies utilizing genetically encoded peptide sensors will allow for the detection of DYN/KOR activity within these select circuits.

Within the BNST, projections to downstream structures involved in alcohol drinking, such as the VTA, are likely affected as a result of KOR activation/inactivation or CeA-BNST^{DYN} inhibition. For example, the BNST sends long-range GABAergic and glutamatergic projections to the VTA, placing this pathway in direct relevance to addiction circuitry (Kudo et al., 2012; Dedic et al., 2018; Jennings et al., 2013). In fact, this pathway is involved in excessive drinking because chemogenetic inhibition of CRF-expressing neurons in the BNST-VTA circuit decreased alcohol intake in the DID model (Companion and Thiele, 2018; Rinker et al., 2017). Therefore, it is possible that endogenous activity of CeA-BNST^{DYN} results in KOR-mediated inhibition of GABAergic presynaptic terminals

in the BNST that disinhibit the BNST-VTA^{Crf} circuit thereby promote alcohol drinking behavior. Likewise, inhibition of CeA-BNST^{DYN} would restore inhibition over BNST-VTA^{Crf} and attenuate binge drinking. This model, however, does not account for the inhibitory influence of GABA release from CeA-BNST^{DYN} in the BNST. However, GABAergic activity could have an inhibitory effect on local interneurons and influence activity of output populations such as BNST-VTA^{Crf}. There is evidence in support for local microcircuit activity in the BNST and these discrete populations are dysregulated by a history of alcohol dependence (Pati et al., 2020). More specifically, excitability is increased in local CRF-expressing neurons within the BNST during withdrawal from chronic alcohol and this is thought to affect activity in non-CRF neurons that project to the VTA or LH. Furthermore, these findings suggest that local BNST circuits are sensitive to alcohol and contribute to decreased activity in otherwise anxiolytic circuitry, thereby facilitating the expression of negative affective behaviors that promote excessive drinking. Because alcohol use disorder is conceptualized as stress surfeit disorder, it is possible that early binge drinking sensitizes these stress-related circuits and facilitates an escalation of intake over time (Koob, 2013). Therefore, future studies will be of importance to determine the precise circuitry and directionality of DYN release within the extended amygdala that contributes to excessive alcohol drinking behavior.

CHAPTER 5: Conclusion and Final Thoughts

DYN/KOR and Binge Drinking

The goal of this dissertation was to use a multifaceted approach involving pharmacology, histological assessment of neuronal activity, and chemogenetic tools to interrogate the dynorphin/kappa opioid (DYN/KOR) neuropeptide system within extended amygdala circuity as it relates to excessive binge-like alcohol consumption. Here, we have shown that activity of KOR within the bed nucleus of the stria terminalis (BNST) bidirectionally modulates binge-like alcohol consumption in the drinking-in-the-dark (DID) model. More specifically, microinjection of a KOR antagonist into the BNST decreased excessive drinking and blocked the ability of systemic KOR activation to enhance alcohol intake. Furthermore, microinjection of a KOR agonist selectively into the BNST increased alcohol consumption and together, these data demonstrate that KOR activity within the BNST is both necessary and sufficient to promote binge drinking behavior. Because the central amygdala (CeA) sends dynorphinergic projections to the BNST and the CeA-BNST circuit has been implicated in excessive drinking behavior, a study was conducted to assess neuronal activity within this pathway during a binge drinking session. This study revealed that c-Fos immunoreactivity, serving as a proxy for neuronal activity, was increased within the CeA of mice consuming alcohol compared to those drinking water. The level of alcohol consumption was positively correlated with c-Fos expression in the CeA, an effect that was largely driven by male mice. While determination of activity within CeA projections to the BNST was not quantified due to methodological constraints, a

more rigorous study was conducted to determine a causal role for the CeA-BNST pathway in binge drinking behavior. More specifically, we demonstrated that chemogenetic inhibition of the CeA-BNST^{DYN} circuit decreased binge-like alcohol consumption, an effect that was selective to alcohol because sucrose drinking was not affected. Taken together, these data support the overarching hypothesis that DYN/KOR activity within the extended amygdala contributes to excessive drinking and that the effect observed after CeA-BNST^{DYN} inhibition is likely mediated by KOR in the BNST.

While this dissertation work outlines the role KOR in the BNST and implicates the CeA-BNST^{DYN} circuit in mediating binge drinking, the exact role of DYN within endogenous CeA-BNST circuitry still remains unclear. DYN-containing neurons within the CeA are GABAergic and also co-express a number of peptides involved in drinking behavior, such as CRF, NTS, and SST. Chemogenetic strategies confer cell-type and pathway specificity, but influence bulk neurotransmitter and peptide release within the targeted population. Thus, it is possible that the release of GABA or a neuropeptide such as CRF was affected by CeA-BNST^{DYN} inhibition and may influence drinking behavior. To address this concern, future studies will employ pathway-specific shRNA-mediated knockdown of DYN within CeA-BNST projections. This approach will deplete DYN expression within the circuit but leave other systems intact. Utilization of this strategy will build upon the present findings and more clearly define the role of DYN within CeA-BNST circuitry.

One important limitation of this dissertation work is that microinjection into the BNST in close proximity to behavioral testing resulted in a disruption to drinking behavior. This methodological constraint limits interpretation of potential sex differences in response to U50,488 microinjection into the BNST on alcohol drinking. However, U50,488 did elevate alcohol consumption as hypothesized albeit a modest increase. This limitation also directed the use of a retroviral strategy to target the CeA-BNST^{DYN} circuit for chemogenetic studies. The most commonly used chemogenetic strategy to target select circuitry involves the use of an anterograde vector harboring a DREADD and CNO delivery into the downstream terminal field. In the present studies, a retro-viral approach was used that involved microinjection of CNO into the CeA. While this strategy allowed for effect circuit-level inhibition,

Finally, studies aimed at determining neuronal activity within the CeA-BNST circuit during binge drinking were inconclusive due to poor expression of the retro-tracer in the CeA. Within the CeA, c-Fos expression was elevated in mice consuming alcohol relative to water drinking control and supports a general involvement of this region in binge drinking. However, the temporal dynamics of c-Fos induction limit interpretation of our results. Future studies utilizing genetically encoded calcium indicators will build upon the present findings by measuring neuronal activity within DYN-containing neurons with high temporal specificity. For example, targeted viral expression using a Cre-dependent genetically-encoded fluorescent calcium biosensor in PDYN-IRES-Cre mice will allow for selective

measurement of real-time activity within the CeA-BNST^{DYN} pathway that can be time-locked to licks at an alcohol bottle.

Clinical Perspectives

AUD arises from the diverse and complex interaction between genetic, epigenetic, and environmental factors that influence the neurobiological basis of behavior (Koob and Volkow, 2010, 2016). The DYN/KOR systems accounts for but one of the plethora of variables that contribute to AUD and targeting this system alone is unlikely to restore proper functioning in all systems that together contribute to excessive drinking, negative affect, and craving that embody AUD. Thus, KOR antagonists are not a likely “all-inclusive” cure for AUD, but may be better suited as an adjunct therapy to treat persistent negative affect, promote stress resilience, and thereby reduce the drive to drink excessively.

The widely accepted theoretical framework of the addiction cycle involves chronic binge drinking that precipitates the emergence of withdrawal syndrome and persistent negative affect that, in turn, drive craving, relapse, and further binge drinking. Indeed, KOR antagonists attenuate excessive alcohol drinking, withdrawal-related anxiety, and negative affective behaviors in preclinical models of alcohol dependence through action within the extended amygdala. The present dissertation builds upon these findings and indicates that KORs within the extended amygdala also modulate excessive binge-like alcohol consumption prior to the development of alcohol dependence. Together, these findings suggest that the DYN/KOR system is a shared neurobiological mechanism involved in initial

binge drinking and excessive drinking in the context of dependence. It is likely that the emergence of withdrawal syndrome and negative affect, which are absent in initial sporadic episodes of binge drinking, promote increased frequency and intensity of subsequent drinking through an increase in function of the DYN/KOR system.

Increased dynorphinergic tone is hypothesized to promote excessive drinking and a *PDYN* SNP is associated with drinking severity in patients with AUD (Preuss et al., 2013; Williams et al., 2007; Xuei et al., 2006). Furthermore, a SNP in the gene coding for KOR, *Oprk1*, is positively associated with diagnoses of AUD as well as severity of alcohol drinking, withdrawal symptomology, impulsivity, and craving (Park et al., 2020). However, further imaging studies are needed to determine exactly how SNPs in *PDYN* and *Oprk1* affect expression and signaling of DYN/KOR throughout the brain, and specifically within the extended amygdala. It also remains to be seen how KOR antagonists may influence dysregulated limbic network activity as a function of AUD. None the less, KOR antagonists may serve as a viable option to treat patients with a genetic vulnerability to AUD.

Clinical trials involving selective KOR antagonists are currently underway although initial trials raised concern over drug safety. The long-lasting receptor inactivating antagonist, JDTic, has entered into Phase 2 clinical trials but have yield mixed success for the treatment of drug abuse, anxiety disorders, and treatment resistant depression. More specifically, studies were aborted due to off-target cardiovascular effects in a subset of patients. More conventional antagonists that compete at the receptor, unlike JDTic that inactivates c-Jun Kinase signaling,

serve as a viable alternative because they are well tolerated and have shown promise in clinical trials for the treatment of anxiety and depression (Schattauer et al., 2017; Lowe et al., 2014). For example, Opra Kappa (formerly LY2456302 or CERC-501) is a conventional KOR antagonist that has recently completed clinical trials in persons with cocaine dependence, nicotine use disorder, and in healthy controls (Jones et al., 2020; Reed et al., 2017). While the drug failed to attenuate craving or measures of depression, the KOR antagonist was well tolerated and holds promise for further studies.

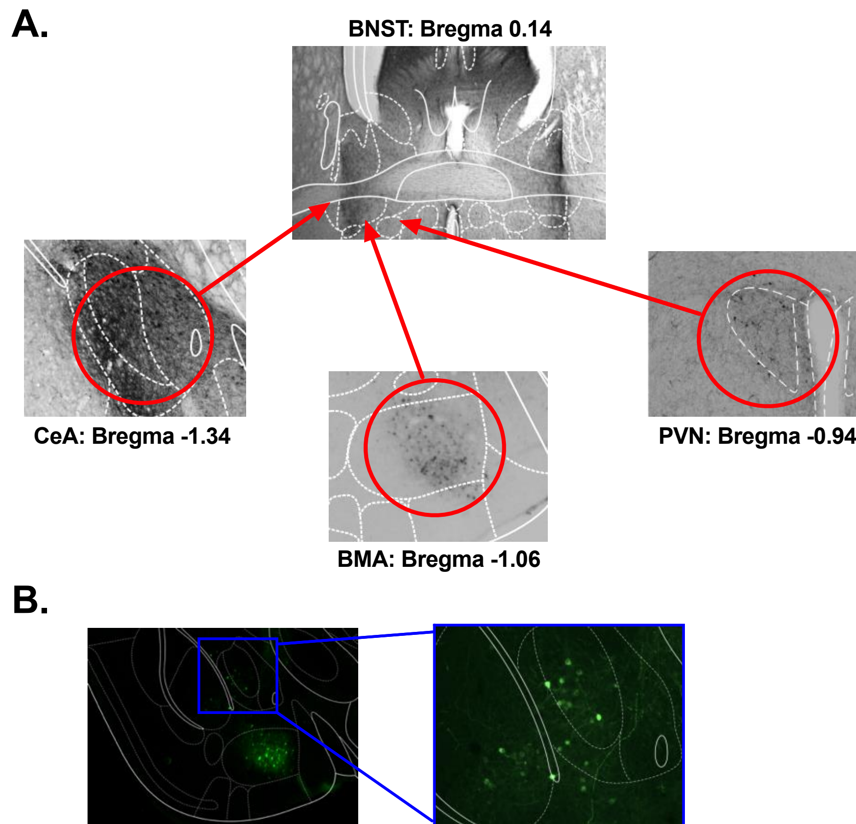
Lastly, de Laat and colleges provide compelling evidence in support of KOR pharmacotherapies by demonstrating that the non-selective opioid antagonist naltrexone reduces alcohol intake and attenuates craving in patients diagnosed with AUD through activity at KOR (de Laat et al., 2018). In fact, an ongoing clinical trial is utilizing combined naltrexone and buprenorphine to treat subjects with AUD and comorbid PTSD. While buprenorphine is a KOR antagonist and partial MOR agonist, combined treatment with naltrexone allows for MOR blockade and more selective KOR antagonist activity. Combined pharmacotherapies offer a viable alternative to costly drug development while achieving KOR selectivity.

Final Thoughts

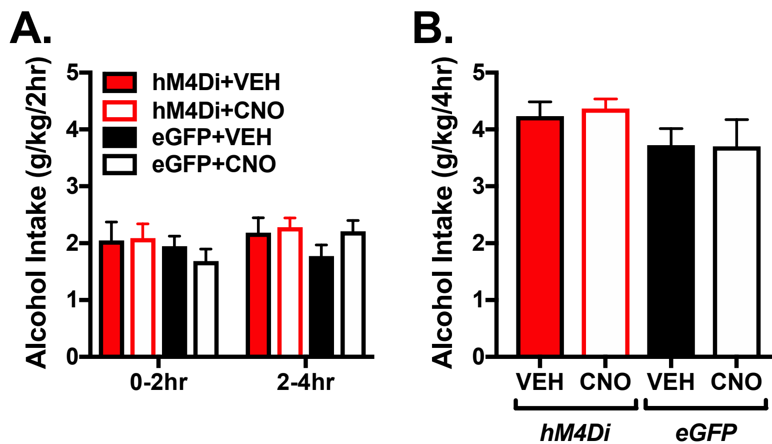
Presently, a comprehensive understanding of the neurobiological underpinnings of AUD is far from complete and, although great advances have been made in the last decade, the translation of preclinical findings into new therapeutic interventions that attenuate excessive alcohol consumption remain

limited. Thus, there is a pressing need for the development of novel compounds to treat individuals suffering from an AUD and alleviate the enormous health and financial burden caused by excessive drinking. There is currently ample evidence indicating that neuropeptide systems contribute to excessive alcohol drinking and AUD. This dissertation adds to the growing body of preclinical literature supporting a role for the DYN/KOR system in excessive alcohol consumption and supports further study of this system as a promising druggable target for the treatment of AUD.

SUPPLEMENTAL FIGURES



Supplemental Figure 1: Pattern of cre-dependent AAVrg expression after infusion into the BNST of Pdyn-IRES-Cre mice. **A)** Expression of mCherry was enhanced by colorimetric staining with DAB to better visualize terminal expression in the BNST relative to landmarks that can be difficult to see with fluorescent staining. Bilateral terminal expression can be seen within the BNST at the site of viral infusion. Expression can be seen in DYN-containing neurons within the CeA, BMA, and PVN. **B)** Infusion of the control vector, AAVrg-eF1a-DIO-eGFP, resulted in eGFP expression within the CeA.



Supplemental Figure 2: Alcohol drinking in mice that received surgery to target the CeA-BNST^{DYN} circuit but lacked viral expression. **A)** Alcohol drinking across the 0-2 and 2-4 hr timepoints was similar after VEH or CNO microinjection into the CeA. **B)** Similarly, cumulative alcohol intake at 4 hours was similar after VEH or CNO treatment suggesting that hM4Di expression within the CeA-BNST^{DYN} circuit is necessary for CNO to affect drinking behavior.

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