EXAMINATION OF CORTICAL/THALAMIC-STRIATAL CIRCUITRY IN MODULATING SENSITIVITY TO ALCOHOL AND RELAPSE

Anel A. Jaramillo

A dissertation submitted to the faculty at the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Neurobiology Curriculum in the School of Medicine

Chapel Hill
2017

Approved by:

Joyce Besheer

Thomas L. Kash

Darin J. Knapp

A. Leslie Morrow

Scott E. Parnell

ABSTRACT

Anel A. Jaramillo: Examination of Cortical/Thalamic-Striatal Circuitry In Modulating Sensitivity To Alcohol And Relapse
(Under the direction of the Joyce Besheer)

All drugs of abuse produce unique interoceptive/subjective (i.e., discriminative stimulus) effects that can impact drug-taking, seeking, and relapse in both clinical and pre-clinical studies. However, the neural circuitry modulating the interoceptive effects of alcohol has yet to be established. The nucleus accumbens core (AcbC), a region known to modulate alcohol-related behaviors, also plays a central role in modulating the discriminative stimulus effects of alcohol. Thus, by investigating the insular cortex (IC) and rhomboid thalamic nucleus (Rh), two brain regions with projections to the AcbC, the experiments in this dissertation sought to investigate the circuitry underlying alcohol-induced interoceptive states and how those internal cues can modulate alcohol-seeking and relapse-like drinking. The IC is implicated in processing interoceptive cues and responding to alcohol-related cues, although its functional role in modulating alcohol-induced interoceptive effects has not been investigated to date. The Rh is proposed to modulate inhibition, behavior flexibility, and motivation, but the role of Rh in modulating any drug-related behaviors has yet to be determined. Utilizing an alcohol discrimination task, pharmacological inhibition of the IC or Rh produced partial alcohol-like effects. Furthermore chemogenetic silencing of the IC or Rh and specific silencing of the IC or Rh outgoing projections to the AcbC potentiated the interoceptive effects of alcohol. Interestingly, in a model of moderate alcohol self-administration, chemogenetic silencing of all IC and Rh outgoing projections did not affect maintenance or reinstatement of alcohol self-administration or the alcohol loading dose effect. However, chemogenetic silencing of IC to AcbC projections decreased alcohol self-administration and increased sensitivity to an alcohol

loading dose (i.e., satiation), resulting in attenuated maintenance and reinstatement of alcohol self-administration. Interestingly chemogenetic silencing of the IC outgoing projections and specific IC to AcbC projections did not affect ongoing sucrose self-administration, but did affect relapse-like behavior. Overall, results from the studies within the present dissertation provide a novel role for the insular/thalamic-striatal circuit in modulating sensitivity to alcohol and implicate the insular-striatal circuit in modulating the alcohol-reinforced behavior, while demonstrating the complex role of interoceptive effects in modulating on alcohol-related behaviors.

Para mis padres, gracias por todo.

Gracias por pensar que avia algo mejor atraves de esos cerros. Gracias por ser generosos al pensar que su familia merecia algo mejor. Algo que talvez ni se imaginavan, pero sabian que valia la pena intentar. Gracias por demonstrarme que el mundo bendice al que trabaja duro y con buenas intenciones. Gracias por el amor y el apoyo que siempre me han brindado. Yo se que tal vez nunca entiendan mi trabajo pero deseo que sepan que en mis momentos mas duros yo siempre supe que esto nunca compararia con en valor que tuvieron ustedes desde el dia que decidieron irse “al norte”, y que sepan que al fin del dia todas las madrugadas en la pollera y en state farm valieron la pena.

To las chicas, thank you for supporting your nerd in pink heels, for keeping me sane and allowing me to vent nonstop. Thank you for the silly calls and the many adventures we wont ever tell our kids about. Most of all, thank you for being guerrilleras in this fight with me.

“sueno …..para encontrar un regalito”

To my BA, thank you for the early morning texts and the fire snaps. Thank you for somehow convincing me I had time and money to join you all on our BA adventures. Thank you for being with me from the start, for influencing me with your strength, intelligence, wit, and confidence. But overall thank you for believing in me, for watching me grow and finding a way to still maintain our friendships across the nation.

ACKNOWLEDGEMENTS

The work presented in this dissertation would not have been possible without the amazing support that I have been blessed with. First and foremost, thank you to my mentor Dr. Joyce Besheer for giving me the opportunity to learn and gain confidence as a scientist. It has been an honor to work under your direction. Thank you for your encouragement, patience, and for believing in me.

Thank you to my former and current lab mates who have contributed immensely to this work. You all have provided a great working environment and it has been a pleasure doing science with you all. Thank you to Kristen Fisher and Dr. Reginald Cannady for getting me started in the lab and for assisting me in the preliminary findings for this work. To Suzanne Frisbee, Dr. Patrick Randall, Spencer Stewart, Brayden Fortino, Verda Agan, Kalynn Van Voorhiees, and Viren Makhijani thank you for all of your hard work and for helping me make this into a reality.

To my committee, thank you for your support, advice, and encouragement. You have each contributed to my success and to my growth as a scientist. Thank you for your willingness to help and for making this project the best it could be. To Dr. Zoe McElligott, thank you for your support and help with the electrophysiological recordings. To Dr. Michael Chua, thank you for your support with the imaging portions of this project.

Anel was supported by an NSF Graduate Research Fellowship (DGE-1144081) and then by an NIH National Research Service Award (F31-AA024973). This work was also supported in part by NIH AA019682.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS AND SYMBOLS

AcbC: Nucleus accumbens core

AUD: Alcohol use disorder

DREADDs: Designer Receptors Exclusively Activated by Designer Drugs

FG: Fluoro-Gold

Fixed Ratio: FR

$GABA_A$: [gamma]-aminobutyric acid type A

$GABA_B$: [gamma]-aminobutyric acid type B

IC: Insular cortex

IG: Intragastric

IP: Intraperitoneal

IHC: Immunohistochemistry

IR: Immunoreactivity

mPFC: Medial prefrontal cortex

NMDA: n-methyl-D-aspartate

Rh: Rhomboid thalamic nucleus

RM ANOVA: repeated measures analysis of variance

CHAPTER 1: INTRODUCTION

AN OVERVIEW OF ALCOHOL USE

Reports of alcohol consumption and production date back as early as 7000 B.C. Today, alcohol continues to be a staple at social gatherings, celebrations, sporting events, and religious ceremonies across the world. Furthermore, alcohol use is not limited to special or social occasions, with people often consuming alcoholic beverages during mealtimes or for relaxation. According to the 2015 National Survey of Drug Use and Health, over 56% of Americans report drinking alcohol at least once within the past month (SAMHSA, 2014), demonstrating the integral role of alcohol in everyday life. Unfortunately despite the thousands of years of alcohol consumption among humans, our understanding of the role of alcohol in our society and on our health continues to perplex clinicians and investigators alike.

For most consumers of alcohol, the occasional indulgence produces little to no long-term consequences. However, to a select population (e.g., high risk individuals) maladaptive drinking patterns and alcohol misuse makes them susceptible to develop an alcohol use disorder (AUD; as defined by DSM-V), with approximately 1 in 6 individuals developing an AUD in their lifetime (SAMHSA, 2014). Although alcohol can directly produce health-related effects (e.g., cirrhosis, cancer, and injuries) amongst individuals with AUD(s), the negative effects of alcohol have a profound impact on all members of society. Approximately 5.9% of deaths across the world are alcohol-related (WHO, 2004). Furthermore alcohol-related deaths are the 4th leading preventable cause of death in the US (Mokdad *et al*, 2004), thus demonstrating an important need for intervention of the deadly consequences of alcohol.

Years of clinical and preclinical research have helped to highlight the complexity of alcohol and the role of alcohol on behavior. The release of DSM-V broadens the criteria for AUDs while allowing the classification of mild, moderate and severe diagnosis. This acknowledges the diverse efficacy of treatments related to the severity and history of the disease. Thus further understanding the neurological adaptations underlying AUDs will assist in the development of new treatments to alleviate the symptoms or consequences of AUDs. While understanding the neurological adaptations that can contribute to AUDs is important, the basic understanding of alcohol actions can in turn elucidate the complex role of alcohol. To this end, studying alcohol abuse under non-dependent conditions not only assists investigators and clinicians in understanding the neural circuitry and behavioral mechanisms that modulate alcohol-related behaviors but it can also assist in developing treatment to prevent the development of AUDs.

INTEROCEPTIVE EFFECTS OF ALCOHOL & BEHAVIOR

One of the many factors modulating drug use is the subjective/interoceptive effects produced by drugs of abuse. All drugs of abuse produce unique internal/discriminative stimulus effects. For example, following consumption of alcohol, the alcohol effects are often described as producing "euphoria, light-headedness, calmness, or sedation". The ability for individuals to perceive these stimuli and the general state of the body is termed interoception (Craig, 2002). Although varying definitions exist, most commonly interoception is defined as the integration of visceral sensations, emotions, and learned associations resulting in a subjective representation of the body state (Ceunen *et al*, 2016). Thus, interoception encompasses receiving, processing, and integrating body-relevant signals that can be internal and external. As such, the stimuli commonly associated with or resulting in an interoceptive state, are proposed to affect behavior, particularly decision-making processes needed to reach an ideal homeostatic state (Damasio, 2003; Paulus *et al*, 2009). Given the ability of drugs of abuse to produce unique interoceptive effects, it is no surprise that interoceptive processing is proposed to contribute to and affect drug-use (Kostowski and Bienkowski, 1999).

Under experimental settings, clinical studies have demonstrated that alcohol produces distinguishable discriminative stimulus effects, which can be pharmacologically characterized and further investigated (i.e., effects on behavior) (Preston and Bigelow, 1991; Stolerman *et al*, 2011). As such humans can be trained to discriminate the interoceptive effects of alcohol vs placebo, and to discriminate breath alcohol levels (Duka *et al*, 1998; Kamien *et al*, 1993). Furthermore these studies have masked the taste of alcohol and accomplished appropriate discrimination of experimenter or self-administered alcohol vs placebo, demonstrating that discrimination is independent of taste or route of administration (Duka *et al*, 1998; Kamien *et al*, 1993). In addition to being able to discriminate the interoceptive effects of alcohol, studies have also demonstrated that the interoceptive effects produced by an acute alcohol loading dose (i.e., priming), in social and individuals with AUDs, results in self-reports of craving and increased motivation to drink (e.g., (de Wit and Chutuape, 1993; Fernie *et al*, 2012; Rose and Grunsell, 2008; Stockwell *et al*, 1982). It is the self-reported craving or "desire for previously experienced effects of a psychoactive substance" (Koob and Volkow, 2010) that is proposed to drive reinstatement of alcohol-drinking in previously abstinent individuals. Such that following a period of prolonged abstinence, cravings and relapse are triggered by acute re-exposure to alcohol, external alcohol-associated stimuli, or certain stressors (Bossert *et al*, 2013; O'Brien *et al*, 1998). These studies demonstrate the continued prominent role of the interoceptive effects of alcohol to drive behavior. However, due to the experimental design in studies investigating the effect of a loading dose (use of placebo with alcohol taste), the role of anticipation/expectancy of interoceptive states (i.e., due to alcohol-associated external cues) must also be considered (Christiansen *et al*, 2017). Nonetheless other studies utilizing the proper controls do demonstrate a specific role for the pharmacological induced-interoceptive effects of alcohol (Christiansen *et al*, 2017). Interestingly despite having a long history with alcohol, subjects with AUD are capable of also discriminating the interoceptive effects of alcohol (Kamien *et al*, 1993; Kostowski and Bienkowski, 1999), indirectly demonstrating the role of interoceptive effects despite expected tolerance (Kostowski and Bienkowski, 1999). Furthermore, only one study to date has

demonstrated the ability of alcohol discrimination to directly modulate alcohol drinking in individuals with AUD (lovibond). As such, following acquisition of the interoceptive effects of self-administered alcohol (Kamien *et al*, 1993), individuals with AUDs were trained to successfully stop drinking after reaching the alcohol training BAL. This resulted in decreased alcohol intake and drinking episodes in individuals with AUD despite the loss of control often attributed to relapse drinking.

Despite the well-established role of interoceptive alcohol effects as potent modulators of alcohol-related behaviors (Naqvi and Bechara, 2010; Paulus and Stewart, 2014; Verdejo-Garcia *et al*, 2012), the neural circuity modulating the interoceptive effects of alcohol has been understudied. Therefore, understanding the neurobiological mechanisms underlying alcohol-induced interoceptive states and how they relate to drinking and relapse is critical. As such given the complexity of neurobiological processes implicated in modulating addition, preclinical models of alcohol-use are extremely valuable and necessary.

MEASURING ALCOHOL-RELATED BEHAVIORS IN RODENTS

Interoceptive Effects of Drugs and Alcohol

Given the vast amount of literature detailing the rodent neurobiology and the variety of techniques available, rodents are very practical for studying various aspects of drug-related behavior (i.e., drug-seeking, intake). Animal models of drug discrimination are viewed as analogous to measuring subjective effects and also provide an indirect measure of abuse potential of a drug (solinas). Similar to clinical studies (Preston and Bigelow, 1991), rodents can be trained to discriminate the discriminative stimulus effects of a drug by utilizing a two choice procedure (e.g., operant, Pavlovian) producing a contingent reinforcer (e.g., food). The studies usually consist of two phases: a training phase and a testing phase. During the discrimination training phase, the experimenter administers the drug or vehicle (e.g., intragastric gavage, intraperitoneal) injection on separate occasions. Following extensive training with the experimenter-administered drug, the animal

learns to associate the discriminative stimulus effects of the drug with a behavioral response (i.e., lever selection in the operant method or goal-tracking in the Pavlovian method). During the testing phase appropriate identification of the drug is measured. Through this way preclinical drug discrimination procedures have paralleled the clinical studies demonstrating proper discrimination of the same drugs of abuse, including alcohol (Colpaert, 1999; Kostowski and Bienkowski, 1999; Stolerman, 1992). Utilizing alcohol discrimination models, studies have demonstrated that the interoceptive effects of alcohol are dose and time dependent and can be attributed to brain alcohol content (Grant and Colombo, 1993b; Quertemont *et al*, 2003; Schechter, 1989). Furthermore, drug discrimination procedures have been used to identify several receptor systems that modulate the discriminative stimulus effects of alcohol ($GABA_A$, NMDA, serotonin, opioid, mGluRs (Besheer *et al*, 2009; Grant, 1999; Kostowski and Bienkowski, 1999) with the early stimuli being more stimulating and then becoming sedating (Grant and Colombo, 1993b; Schechter, 1989).

Alcohol Self-administration and Relapse

Rodent models of self-administration utilizing operant conditioning can be utilized to investigate the complex processes modulating voluntary drug-taking. The self-administration model provides the most direct measure of the reinforcing effects of the drug (Solinas *et al*, 2006), as the drug acts as a reinforcer that the rodent must respond for. Traditionally, in operant alcohol self-administration procedures, alcohol is contingent on a conditioned response (e.g., lever). The response requirement or schedule of reinforcement can be manipulated and provides an index of the reinforcing properties of alcohol. Among humans (dependent and nondependent), alcohol consumption often occurs in interspersed episodes with periods of abstinence. Furthermore, AUDs are specifically characterized by periods of abstinence and subsequent relapse episodes (McLellan *et al*, 2000). It is widely accepted that relapse episodes are triggered, in part, by alcohol-associated cues (e.g., internal/interoceptive cues, contextual cues) (Koob and Volkow, 2010; Verdejo-Garcia *et al*, 2012). Animal studies commonly model this behavior through periods of abstinence or by

extinguishing the previously reinforcer-associated behavior. As such, utilizing an operant alcohol self-administration procedure, relapse-like behavior is commonly examined following extinction of drug-reinforced behavior, and the ability of conditioned stimuli (e.g., acute loading dose of the drug or drug associated cue) to reinstate drug-seeking is measured (e.g., lever responding; Bossert *et al*, 2013). As such, the present study will utilize an operant alcohol self-administration paradigm to model alcohol intake, and to investigate the effects of internal/interoceptive cues by alcohol pre-exposure (i.e., alcohol loading dose) on subsequent alcohol self-administration and relapse-like behaviors.

INTEROCEPTIVE EFFECTS OF AN ALCOHOL LOADING DOSE ON ALCOHOL DRINKING AND RELAPSE

Numerous preclinical studies have replicated the clinical findings demonstrating that a low alcohol dose can prime alcohol-related behaviors, including craving, relapse, and additional or increased alcohol intake (Bigelow *et al*, 1977; de Wit and Chutuape, 1993; Gass and Olive, 2007; Hodgson *et al*, 1979; Kirk and de Wit, 2000; Le *et al*, 1998; Vosler *et al*, 2001). Conversely, pretreatment with a high alcohol dose (i.e., loading dose) can decrease alcohol self-administration, alcohol-seeking, and relapse-like drinking, likely related to processes such as satiation or devaluation (Czachowski *et al*, 2006; Randall *et al*, 2015; Samson *et al*, 2003). Other studies utilizing similar moderate alcohol loading doses to induce devaluation, also demonstrate decreased alcohol intake under both experimenter-administered and self-administered preload conditions (Czachowski *et al*, 2006; Samson *et al*, 2002; Samson *et al*, 2003), specific to the alcohol reinforcer (Samson *et al*, 2002; Samson *et al*, 2003). Additionally, devaluation of alcohol reinforcement through the use of alcohol paired with lithium chloride to induce malaise, results in decreased alcohol consumption (Samson *et al*, 2004), indicating that postingestive interoceptive effects and internal cues associated with alcohol directly contribute to alcohol-related behaviors. Together, these studies demonstrate that titration of

self-administration and relapse-like drinking is sensitive to the interoceptive effects produced by pretreatment with a loading dose of alcohol (i.e., alcohol pre-exposure.

CIRCUITRY

The discriminative stimulus effects of alcohol are at least partially regulated centrally and are not limited to peripheral sensations, as site-specific and ventricular intracranial injections generalize to alcohol (Hodge, 1994), confirming the brain as a site of pharmacology action for alcohol. Furthermore, various region specific receptors have been demonstrated to functionally regulate the interoceptive effects of alcohol (e.g., amygdala, mPFC; (Hodge and Cox, 1998; Jaramillo *et al*, 2016). The present study will focus on investigating circuitry modulating the interoceptive effects of alcohol within the following three regions.

Nucleus Accumbens Core

The ventral striatum, commonly implicated in modulating motivational behavioral output has been extensively studied and proposed to encompass the nucleus accumbens shell and core (AcbC). It is well-documented that the AcbC modulates self-administration and reinstatement; (Besheer *et al*, 2010; Chaudhri *et al*, 2008; Chaudhri *et al*, 2010; Gass *et al*, 2011; Griffin *et al*, 2014; Rassnick *et al*, 1992a; Rassnick *et al*, 1992b). Furthermore, the existing literature heavily implicates the nucleus accumbens core (AcbC; and possible projections to the AcbC) as a central region in modulating sensitivity to the interoceptive effects of alcohol (Besheer *et al*, 2003; Besheer *et al*, 2010; Hodge and Alken, 1996; Hodge and Cox, 1998). One of the goals of the present work was to focus on upstream regions to the AcbC, as general inhibition in the AcbC has been shown to modulate sensitivity to alcohol (Besheer *et al*, 2003; Hodge and Alken, 1996; Hodge and Cox, 1998; Hodge *et al*, 2001b). As such, the present dissertation is focused on the insular cortex and the rhomboid nucleus and their projections to the AcbC.

Insular Cortex

One of the regions of interest in the present work is the anterior IC, due to its projections to the AcbC (IC→AcbC; McGeorge and Faull, 1989; Wright and Groenewegen, 1996) and its role in integrating internal and external stimuli into interoceptive states to drive motivated behavior (Craig, 2009; Paulus and Stewart, 2014). Clinical-imaging studies demonstrate increased IC activity in response to the interoceptive effects and cue-induced urges for various drugs of abuse (i.e., cigarettes, cocaine, heroin), including alcohol (*see*: (Naqvi and Bechara, 2009). Interestingly, damage to the IC in cigarette smokers has been shown to result in the cessation of smoking and the abolishment of "the urge to smoke" (Naqvi *et al*, 2007), further supporting a role for the IC in processing internal drug cues (Kusumoto-Yoshida *et al*, 2015). Despite the vast clinical literature, no preclinical study has investigated the functional role of the IC in modulating the discriminative stimulus effects of alcohol or any other drug of abuse. However, one of the few studies investigating the role of the IC in alcohol-related behaviors determined that inactivation of IC→AcbC projections decreased aversion-resistant alcohol consumption in rats, implicating an important role within the IC→AcbC circuit (Seif *et al*, 2013).

Rhomboid Thalamic Nuclei

An additional focus of this application is on the rhomboid (Rh) thalamic ventral midline nuclei (commonly grouped with the reuniens due to their close proximity; Cassel *et al*, 2013). Rh modulate spatial learning and memory consolidation (Cholvin *et al*, 2013; Hembrook *et al*, 2012; Loureiro *et al*, 2012). However, recent preclinical studies implicate a larger role for the Rh in modulating behavioral inhibition and motivation (Cassel *et al*, 2013; Cholvin *et al*, 2013; Prasad *et al*, 2013). For example using a 5-choice reaction time task, lesions to the Rh increased impulsive behavior in the presence of a conditioned stimulus, with varying stimulus durations (Prasad *et al*, 2013), thus, implicating a role for the Rh in modulating cue-induced behavior, particularly under conditions that require behavioral flexibility (Prasad *et al*, 2013). Interestingly, Rh lesions also

resulted in decreased number of omitted responses and decreased latency to obtain reward, suggesting a role for Rh in motivation (Prasad *et al*, 2013). Additionally, neuronal activation of Rh in response to various antipsychotic drugs suggests a role in the drug-modulated circuitry (Cohen *et al*, 1998). The recent literature along with the Rh central anatomical location and extensive connections with the cortex and limbic regions, including projections to the AcbC (Vertes *et al*, 2006), suggest that Rh integrates various inputs to affect psychological, affective, and cognitive functions required to induce behavioral flexibility in a changing environment (Cassel *et al*, 2013; Cholvin *et al*, 2013; Prasad *et al*, 2013). Interestingly, although these behavioral processes are often associated with drug self-administration and relapse-like behavior no study to our knowledge has investigated the functional role of Rh in alcohol- or drug-related behaviors.

RATIONALE

Despite the well-established role of interoceptive drug states as potent modulators of drug-related behaviors (Naqvi and Bechara, 2010; Paulus and Stewart, 2014; Verdejo-Garcia *et al*, 2012), the neural circuity modulating these states remains understudied. Thus, understanding the neurobiological mechanisms underlying drug-induced interoceptive states and how they relate to alcohol-seeking and relapse-like drinking is critical for both the preclinical and clinical drug abuse fields. Therefore, a goal of the present work is to investigate the circuitry modulating the discriminative stimulus effects of alcohol (**Aim 1**). Given the well-documented central role of the AcbC in regulating the discriminative stimulus effects of alcohol (Besheer *et al*, 2003; Besheer *et al*, 2009; Hodge and Alken, 1996; Hodge and Cox, 1998; Hodge *et al*, 2001b), we aim to broaden our understanding of the AcbC-related brain circuitry by investigating two potential brain regions, with projections to the AcbC (IC and Rh), as modulators of the discriminative stimulus effects of alcohol. In humans, the IC has been implicated in modulating interoceptive states and responds to drug/alcohol-related cues, although its functional role in the preclinical alcohol field has not been fully established (Paulus and Stewart, 2014). The Rh is proposed to modulate motivation and

behavioral inhibition, thus implicating a possible role for the Rh in modulating drug-related behaviors.

Additionally, a major challenge in the alcohol field is to better understand how interoceptive states can serve as internal cues to modulate relapse-like behavior. Pretreatment with a low alcohol dose can prime alcohol-related behaviors, including craving, relapse, and additional or increased alcohol consumption (Bigelow *et al*, 1977; de Wit and Chutuape, 1993; Gass and Olive, 2007; Hodgson *et al*, 1979; Kirk and de Wit, 2000; Le *et al*, 1998; Vosler *et al*, 2001). Conversely, pretreatment with a high alcohol dose (i.e., to induce satiation) decreases alcohol self-administration, alcohol-seeking, and relapse-like drinking (Fig 7B; Czachowski *et al*, 2006; Randall *et al*, 2015; Samson *et al*, 2003). Together, this demonstrates that titration of self-administration and relapse-like drinking is sensitive to the interoceptive effects produced by pretreatment with a loading dose of alcohol (i.e., alcohol pre-exposure). Thus, another goal of the work described in this dissertation is to examine the neural circuity modulating sensitivity to the effects of an alcohol loading dose on relapse-like behavior (**Aim 2**). Thus, by utilizing an alcohol loading dose strategy we aim to elucidate the roles of the IC and Rh in modulating the interoceptive effects produced by an alcohol loading dose and their roles in modulating relapse-like behaviors.

Aim 1: Investigate the role of the IC and Rh in modulating the discriminative stimulus effects of alcohol.

The functional role of IC and Rh in modulating the interoceptive effects of alcohol has never been investigated. Therefore in **Chapter 2** we utilize male Long Evans rats, trained on operant alcohol discrimination, to examine a functional role of the IC and Rh in modulating sensitivity to the discriminative stimulus effects of alcohol by pharmacologically inactivating the IC or Rh by $GABA_A$ and $GABA_B$ agonists, musimol+baclofen, prior to a discrimination test. Additionally, to confirm and expand our understanding of the role of IC and Rh on the interoceptive effects of alcohol, in **Chapter 3** we chemogenetically silence the IC or Rh by utilizing inhibitory Designer Receptors Exclusively

Activated by Designer Drugs (DREADDs) in rats trained on operant or Pavlovian alcohol discrimination. Utilizing DREADDs, which are activated by the inert ligand clozapine-n-oxide (CNO), allowed us to also examine the role of the IC→ and Rh→AcbC projections through site specific intra-cranial infusion of CNO. Thus, male Long-Evans trained to discriminate alcohol were pre-treated with CNO prior to a discrimination test. We hypothesize that silencing IC→AcbC and Rh→AcbC (independently), will increase sensitivity to the discriminative stimulus effects of alcohol.

Aim 2: Investigate the role of the IC and Rh in modulating the effects of a preload dose of alcohol on relapse-like behaviors.

A major challenge in the alcohol field is to understand how interoceptive states can modulate alcohol-related behaviors. Thus, to investigate the role of IC and Rh in modulating sensitivity to the interoceptive effects of a loading dose of alcohol on ongoing alcohol self-administration and relapse-like behaviors, male Long-Evans rats were trained to self-administer alcohol. Utilizing the similar chemogenetic technique as in the previous aim, in **Chapter 4** we chemogenetically silence the IC, IC→AcbC and Rh prior to pretreatment with a loading dose of alcohol to investigate the IC and Rh role in modulating on going alcohol self-administration following pretreatment with an alcohol loading dose (i.e., to induce satiation). Next in **Chapter 5** to investigate the role of IC, IC→AcbC, and Rh in modulating relapse-like behavior (i.e., alcohol-seeking/reinstatement) following a loading dose of alcohol, the male Long Evants rats trained to self-administer alcohol from Chapter 4 underwent extinction of alcohol-reinforced behavior and then prior to a alcohol-seeking/reinstatement test we chemogenetically silenced the IC, IC→AcbC or Rh prior to pretreatment with a loading dose of alcohol. We hypothesize that inactivation will potentiate the effects of an alcohol loading dose (i.e., further decrease alcohol-seeking and relapse-like drinking.

These studies seek to broaden our understanding of the IC, Rh and AcbC-related circuitry in modulating sensitivity to the discriminative stimulus effects of alcohol and in modulating sensitivity

to the effects of an alcohol loading dose on relapse-like behaviors. The present findings have potential to provide novel information on the functional roles of the IC, Rh, and their projections to the AcbC given that behavior is examined under control conditions and following alcohol. Further, by conducting these experiments in parallel, this innovative approach has the ability to elucidate the role of regions not previously implicated or understudied in the preclinical alcohol field while elucidating the neurobiology modulating the interoceptive effects of alcohol and their behavioral effects, thus informing both the clinical and preclinical drug-abuse field.

CHAPTER 2: MODULATION OF SENSITIVITY TO ALCOHOL BY CORTICAL AND THALAMIC BRAIN REGIONS[1]

INTRODUCTION

Despite the well-known deleterious effects of alcohol, its consumption among the general population remains high, with approximately 2 billion people worldwide consuming alcohol (WHO, 2004) and 57% of Americans consuming at least one alcoholic beverage within the past month (SAMHSA, 2014). Thus, understanding the neurobiological mechanisms that modulate sensitivity to alcohol, especially the subjective/interoceptive (discriminative stimulus) effects of alcohol, is important given that interoceptive drug cues can impact drug-related behaviors from onset of drug use and throughout dependence (Bevins and Besheer, 2014; Koob and Volkow, 2010; Paulus and Stewart, 2014; Verdejo-Garcia *et al*, 2012).

Drug discrimination procedures are commonly used to assess the interoceptive/discriminative stimulus effects of drugs of abuse in animal models (Solinas *et al*, 2006) and these procedures have identified several receptor systems that modulate the interoceptive effects of alcohol ([gamma]-aminobutyric acid type A [$GABA_A$], N-methyl-D-aspartate [NMDA], serotonin, metabotropic glutamate, opioid; (Besheer *et al*, 2010; Besheer and Hodge, 2005; Grant and Barrett, 1991; Grant and Colombo, 1993a; Grant *et al*, 1997; Helms *et al*, 2009; Hodge and Cox, 1998; Jaramillo *et al*, 2015; Kostowski and Bienkowski, 1999; Maurel *et al*, 1998; Platt and Bano, 2011; Shelton and

[1] This chapter has been previously published (Jaramillo AA. et al., (2016). Modulation of sensitivity to alcohol by cortical and thalamic brain regions. *European Journal of Neuroscience, 44,* 8: 2569-2580). It has been included with permission from Wiley, and with additional editing by the author.

Grant, 2002; Vivian *et al*, 2002). Additionally, the existing literature heavily implicates the nucleus accumbens core (AcbC; and possible projections to the AcbC) as a central region in modulating sensitivity to the interoceptive effects of alcohol (Besheer *et al*, 2003; Besheer *et al*, 2010; Hodge and Alken, 1996; Hodge and Cox, 1998).

The goal of the present work was to broaden understanding of potential AcbC-related neural circuitry modulating the interoceptive effects of alcohol by identifying brain regions with projections to the AcbC and whether these regions may regulate sensitivity to alcohol. Thus, in behaviorally naïve male Long-Evans rats, projections to the AcbC were identified using a neuronal retrograde tracer. Second, neuronal response to alcohol was examined in alcohol discrimination-trained rats based on the selected brain regions that were identified to have projections to the AcbC. Lastly, to determine the functional role of these brain regions in modulating sensitivity to alcohol pharmacological inactivation was used (intra-brain regional administration of $GABA_A$+$GABA_B$ agonists - muscimol+baclofen; (Chaudhri *et al*, 2013; Lasseter *et al*, 2011; Willcocks and McNally, 2013). The present retrograde tracing study identified and led to the focus of two regions of interest with projections to the AcbC, the anterior insular cortex (IC) and the rhomboid thalamic nucleus (Rh). These regions were selected for the following reasons. **1)** The IC is proposed to integrate internal and external stimuli into interoceptive states to drive motivated behavior, which has extensive implications for drug addiction (Craig, 2009; Paulus and Stewart, 2014) and various preclinical studies have determined a functional role for the IC in modulating self-administration of several drugs of abuse (Di Pietro *et al*, 2008; Hollander *et al*, 2008; Pushparaj and Le Foll, 2015). Thus, we hypothesized that the IC is involved in modulating sensitivity to alcohol and that pharmacological inactivation would disrupt expression of the discriminative stimulus effect of alcohol. **2)** The Rh is implicated in modulating behavioral inhibition and motivation (Cassel *et al*, 2013; Cholvin *et al*, 2013; Prasad *et al*, 2016; Prasad *et al*, 2013), and has been proposed to integrate and modulate arousal and attention (Cassel *et al*, 2013), all of which are key behavioral components in drug use and may have implications for modulating sensitivity to the interoceptive effects of alcohol. Accordingly, we

hypothesized, that similar to the IC, pharmacological inactivation of the Rh would disrupt expression of the discriminative stimulus effects of alcohol.

MATERIALS AND METHODS

Animals

This study used single-housed male Long-Evans rats (Harlan Sprague–Dawley, Indianapolis, IN). All rats were weighed and handled daily for at least 1 week before the start of training. Food intake was restricted to maintain body weight (325–340 g) for all experiments. Water was available ad libitum in the home cage unless noted. The colony room was maintained on a 12-h light/dark cycle and experiments were conducted during the light cycle. Animals were under continuous care and monitoring by veterinary staff from the Division of Laboratory Animal Medicine at UNC-Chapel Hill. All procedures were conducted in accordance with the NIH Guide to Care and Use of Laboratory Animals and institutional guidelines.

Apparatus

All behavioral experiments occurred in chambers (Med Associates, Georgia, VT) measuring 31 × 32 × 24 cm. The right wall of the chamber contained a liquid dipper receptacle, two retractable response levers, and stimulus lights (mounted above each lever). Lever press responses activated a dipper mechanism that presented 0.1 mL of a 10% (w/v) sucrose solution for 4 seconds. All chambers were equipped with infrared beams that divided the chamber into 4 parallel zones to measure general locomotor data during the sessions. Each chamber was located in a sound-attenuating cubicle equipped with an exhaust fan that provided both ventilation and masking of external sounds. Additionally, chambers were interfaced (Med Associates) to a computer programmed to control sessions and record lever responses and locomotor data.

Discrimination training

Daily training sessions (Monday-Friday) were identical to those previously described (Besheer *et al*, 2015; Jaramillo *et al*, 2015; Randall *et al*, 2015). Briefly, following administration of water or alcohol (1 g/kg) by intragastric gavage (IG), rats were placed in the chambers for a 10-min timeout period. Next, both levers were introduced into the chamber and the house light was illuminated signaling commencement of the 15-min session. During an alcohol session, completion of a fixed ratio 10 (FR10) on the alcohol-appropriate lever (e.g., left lever) resulted in sucrose delivery. Alternatively, during a water session, completion of an FR10 on the water-appropriate lever (e.g., right lever) resulted in the delivery of sucrose reinforcer. During both alcohol and water sessions, responding on the inappropriate lever was recorded but had no programmed consequence. Alcohol- and water-associated levers were counterbalanced across animals and training days varied on a double alternation schedule (alcohol, alcohol, water, water,..). Testing began once the following criteria were met: the percentage of appropriate lever responses before the first reinforcer, and during the entire session was >80% for at least 8 out of the 10 consecutive days.

Discrimination Testing

Test sessions began following a 10-min delay and were similar to training sessions except they were 2-min in duration. Additionally, an FR10 on either lever resulted in sucrose delivery, thus sucrose reinforcement was delivered independent of lever-appropriate responding so as not to bias lever selection and to allow for the analysis of the effects of treatments on overall response rates (internal measure of nonspecific motor effects). Prior to the start of testing in all rats, a cumulative alcohol curve (0.1, 0.3, 1.0, and 1.7 g/kg) was generated to confirm discriminative stimulus control by alcohol (Schechter, 1997) as described in detail (Besheer *et al*, 2012c; Besheer *et al*, 2014). Briefly, rats initially received 0.1 g/kg alcohol and were placed in the chamber for the test session (i.e., 10-

min pre-session delay and 2 min test session). At the conclusion of the session, rats received a subsequent alcohol administration of 0.2 g/kg and immediately began another test session. This procedure was repeated with two subsequent administrations of 0.7 g/kg alcohol, thus administration of alcohol was additive to produce the stated dose range (0.1, 0.3, 1.0, and 1.7 g/kg). Once discriminative stimulus control by alcohol was confirmed experimental testing began. In Experiment 3, testing was interspersed with training sessions and only occurred when accuracy criteria was met during 3 of 4 previous training sessions. No more than two test sessions were conducted per week.

Cannulae Implantation Surgery and Microinjection Procedures, and Verification

Site-specific microinjections were delivered by a microinfusion pump (Harvard Apparatus, MA) through 1.0 µl Hamilton syringes connected to 33-gauge injectors (Plastics One, VA). For Experiment 1, anesthetized rats received a unilateral microinjection of FG into the AcbC (AP +1.7, ML +1.5, DV -6.8 from skull) at a volume of 0.5 µl across 8-min. The injector remained in place for an additional 4-min to allow for diffusion. For Experiment 3, anesthetized rats received implantation of 26-gauge guide cannulae (Plastics One, Roanoke, VA) aimed to terminate 2 mm above the anterior IC (bilateral coordinates: AP +3.2, ML ±4.0 mm, DV -4.0 mm) and Rh (unilateral coordinates: AP -2.3, ML -1.7 mm (15º angle), DV−5.2 mm). Coordinates were based on (Paxinos and Watson, 2007). Muscimol+baclofen microinjections were delivered through injectors extending 2 mm below the guide cannulae at a volume of 0.5 µl/side across 1 min. The injector(s) remained in place for an additional 2-min after the infusion to allow for diffusion. Additional microinjection procedures are described in detail in (Besheer *et al*, 2014; Cannady *et al*, 2011). At the end of Experiment 3, brain tissue was stained with cresyl violet to verify cannulae placement. Only data from rats with cannulae/injector tracts determined to be in the target brain regions were used in the analyses. For bilateral cannulae (IC), both cannulae had to be in the target region. As such, for the IC, three rats had a confirmed cannula on one side (depicted as solid circles on **Figure 2.3A**), but the cannula for the

opposite side was outside of the target region or we were unable to visibly confirm the injector tract and thus, were considered misses (depicted as solid triangles on **Figure 2.3A**). Data from these rats and others with cannulae determined to be out of the other target brain regions were combined and analyzed to serve as anatomical controls.

Immunohistochemistry Procedure and Quantification

To obtain brain tissue for Experiment 2.2, rats were deeply anesthetized with pentobarbital and perfused with 0.1 M PBS, followed by 4% paraformaldehyde, 4°C; pH=7.4. The brains were removed from the skull and placed in the same fixative solution for approximately 24 h. Next, they were transferred to 30% (w/v) sucrose in a 0.1 M PBS solution, and subsequently sliced on a freezing microtome into 40 μm coronal sections. Tissue was then stored in cryoprotectant (−20°C) until immunohistochemistry (IHC) processing. IHC staining and quantification procedures were similar to those we have previously described (Besheer *et al*, 2012a; Besheer *et al*, 2014; Cannady *et al*, 2011). Free-floating coronal sections were incubated in rabbit anti-Fluorogold antibody (1:8,000; Millipore) for 24 h or rabbit anti-c-Fos antibody (1:20,000; Millipore) for 48 h at 4 °C with agitation. The brain regions examined were the anterior insular cortex (IC; +2.8 to +1.9 mm), the nucleus accumbens core (AcbC; AP -2.3 to -1.3) and rhomboid thalamic nucleus (Rh; AP -1.8 to -3.2 mm), according to (Paxinos and Watson, 2007). Images were acquired utilizing Olympus CX41 light microscope (Olympus America) and analyzed utilizing Image-Pro Premier image analysis software (Media Cybernetics, MD). IR data (c-Fos positive pixels/mm^2) were acquired from a minimum of three sections/brain region/animal, and the data were averaged to obtain a single value per subject.

Experimental procedures

Experiment 2.1: Confirmation of incoming AcbC projections utilizing a neuronal retrograde tracer

To confirm afferent neuronal projections to the AcbC, a region known to modulate the discriminative stimulus effects of alcohol, and to determine anatomical coordinates for those brain sites of interest for the discrimination studies (i.e., the c-Fos analyses and the inactivation studies, Experiments 2.2 and 2.3, respectively), behaviorally naïve rats (n=6) received a unilateral microinjection of the neuronal retrograde tracer Fluoro-Gold (2%; FG) aimed at the AcbC. One week following injection, allowing time for recovery and diffusion of the tracer, brain tissue was collected and analyzed for FG expression using IHC.

Experiment 2.2: Alcohol-induced neuronal activation in IC and Rh in discrimination-trained rats

After identifying the regions of interest with projections to the AcbC (i.e., IC, and Rh), we sought to investigate whether those regions and the nucleus accumbens would show changes in neuronal activity following alcohol in rats whose behavior was under the discriminative control of alcohol. As such, discrimination-trained rats were administered water or alcohol (1 g/kg, IG; n=4-5/group) and underwent a standard 2-min discrimination test session. 90-min after the end of the test, rats were sacrificed and brain tissue was collected and processed for c-Fos IR. c-Fos IR in the nucleus accumbens (core and shell), IC, and Rh was then analyzed.

Experiment 2.3: Examination of the functional role of IC and Rh on the discriminative stimulus effects of alcohol, through pharmacological inactivation

Discrimination-trained rats were implanted with bilateral cannulae aimed at the IC and a unilateral cannula aimed at the Rh (n=11). Dual cannulae implantation was conducted to minimize the number of animals required for this study. Cannulae implantation coordinates were based on FG expression from Experiment 2.1 and previous work (Besheer *et al*, 2010; Cholvin *et al*, 2013; Cosme

et al, 2015; Kesner and Gilbert, 2007). To determine the functional role of each brain region in modulating the discriminative stimulus effects of alcohol, each region was independently inactivated with a muscimol+baclofen cocktail infusion prior to a discrimination test session. Testing was interspersed between both regions. On test days, rats received vehicle or microinjection of muscimol+baclofen, 15-min prior to receiving water or the alcohol training dose (1 g/kg, IG). Rats were then placed in the chamber for a 2-min test session (following the 10 min time out period).

Drugs

Alcohol (95% w/v) was diluted in distilled water to a concentration of 20% (v/v) and administered IG, with volumes varied by weight to obtain the desired dose. Fluoro-Gold (FG; Fluorochrome, LLC, Denver, Colorado) was dissolved in 0.9% saline (w/v)/2% (v/v) FG per manufacturer instructions (Schmued and Fallon, 1986). Muscimol and baclofen (R&D systems, Minneapolis, Minnesota) were dissolved in sterile 0.9% saline to produce a cocktail of 0.1mM muscimol + 1mM baclofen, and the doses were chosen based on previous work and our own pilot studies (Chaudhri *et al*, 2013; Lasseter *et al*, 2011).

Data Analysis

For the discrimination experiments, response accuracy was expressed as the percentage of alcohol-appropriate lever responses upon delivery of the first reinforcer. Complete expression of the discriminative stimulus effects of alcohol (i.e., full substitution) was defined as ≥80% alcohol-appropriate responding and partial substitution was defined as >40% and <80% alcohol-appropriate responses (Besheer *et al*, 2015; Solinas *et al*, 2006). If an animal did not complete an FR10 during these test sessions, data from that animal were not included in the response accuracy analysis, but were included in the response rate analysis. Response rate (responses/min) and general locomotor rate (beam breaks/min) were analyzed for the entire session and served as an index of motor activity.

Group differences in discrimination behavior and c-Fos IR for Experiment 2.2 were determined by *t*-test. In Experiments 2.2 and 2.3, one or two-way repeated measures analysis of variance (RM ANOVA) were used to analyze response accuracy, response rate, and locomotor rate data. Tukey *post hoc* analyses were used to explore significant interactions. Significance was declared at $p \leq 0.05$. Injector tip placements are shown in **Figures 2.3A** and **2.4A** and only animals with accurate bilateral cannulae placements (IC) or unilateral placement (Rh) were included in the analyses. Data from the rats with inaccurate cannulae placements were analyzed separately and served as anatomical controls.

RESULTS

Experiment 2.1: Confirmation of incoming AcbC projections utilizing a neuronal retrograde tracer

Injection of FG, a neuronal retrograde tracer, in the AcbC (**Fig. 2.1A**) resulted in dense FG IR in the IC (**Fig. 2.1B**) and Rh (**Fig. 2.1C**). FG IR was also found in other regions (e.g., mPFC[2], amygdala, hippocampus, etc.); however, the focus of the present study was on the IC and Rh.

Experiment 2.2: Alcohol-induced neuronal activation in IC and Rh in discrimination-trained rats

Alcohol stimulus control was confirmed by testing a cumulative alcohol dose response curve. Alcohol-appropriate responding increased with the alcohol dose as confirmed by the one-way RM ANOVA [$F(3,30)=54.639$, $p<0.001$], with higher alcohol-appropriate responding at the training dose (1 g/kg) and the highest dose (1.7 g/kg) relative to the lowest dose (0.1 g/kg; $p<0.001$; **Table 2.1**). No effects on response rate were observed (**Table 2.1**). However, a significant decrease in locomotor rate

[2] Originally one of the areas of focus in (Jaramillo AA. et al., (2016). Modulation of sensitivity to alcohol by cortical and thalamic brain regions. European Journal of Neuroscience, 44, 8: 2569-2580). It has been omitted by the author.

[F(3,10)=9.70, p<0.001] was observed for all the alcohol doses relative to the lowest dose (0.1 g/kg; p<0.002; **Table 2.1**). Discrimination accuracy performance on the final test showed a significant increase in responding on the alcohol-appropriate lever following the alcohol training dose (1 g/kg; t=4.46, p=0.002; **Fig. 2.2A**). There were no significant differences in response rate (**Fig. 2.2B**) or locomotor rate (beam breaks– Water: 272.10±21.84; Alcohol 271.92±31.52), suggesting that any group differences in c-Fos expression is likely not related to a change in response output or general motor behavior. IHC analysis of the brain tissue demonstrated a decrease in c-Fos IR following alcohol (1 g/kg) in the AcbC (t=2.36, p=0.04; but not shell, **Fig. 2.2C**), the IC (**Fig, 2.2D**; t=2.61, p<0.03), and the Rh (**Fig. 2.2E**; t=2.25, p=0.05).

Experiment 2.3: Examination of the functional role of IC and Rh on the discriminative stimulus effects of alcohol, through pharmacological inactivation

Confirmation of stimulus control

Alcohol stimulus control was confirmed for the cannulated IC/Rh group with a cumulative alcohol curve as shown in **Table 2.1**. One-way RM ANOVA showed an increase in alcohol-appropriate lever responding the IC/Rh group [F(3,30)=29.20, p<0.001], at the training dose (1 g/kg) and the highest dose (1.7 g/kg) relative to the lowest dose (0.1 g/kg; p<0.001). In the IC/Rh group [F(3,30)=3.81, p=0.02] a significant reduction was observed at the highest dose (1.7 g/kg) relative to the lowest dose (0.1 g/kg; p<0.03). Additionally, locomotor rate was significantly decreased [F(3,30)=32.33, p<0.001] at all doses (0.3, 1.0, and 1.7 g/kg) relative to the lowest dose (0.1 g/kg; $p\leq$0.001).

Pharmacological inactivation of the insular cortex

The two-way RM ANOVA on alcohol-appropriate responding following IC inactivation (**Fig. 2.3A**), showed a significant main effect of alcohol dose [F(1,6)=19.81, p=0.004] and

muscimol+baclofen treatment [F(1,6)=7.38, p<0.04], and a significant interaction ([F(1,6)=5.95, p=0.05]; **Fig. 2.3B**). IC inactivation prior to water administration induced increased alcohol-appropriate responding (p=0.004), resulting in partial substitution for the 1 g/kg alcohol training dose. IC inactivation prior to the alcohol-training dose (1 g/kg) did not affect discrimination performance, again as behavior was likely at a ceiling effect. One rat did not complete an FR10 following IC inactivation and thus was not included in the response accuracy measure, but was included in the response rate analysis. Two-way RM ANOVA of response rate as shown in **Figure 2.3C** showed a significant main effect of muscimol+baclofen treatment [F(1,7)=10.18, p<0.015], with lower response rates following inactivation relative to vehicle and there was a trend for an interaction (p<0.07). Muscimol+baclofen treatment significantly affected locomotor rate [F(1,7)=34.84, p<0.001; **Fig. 2.3D**] and a significant interaction between alcohol dose and treatment was also observed [F(1,7)=6.62, p<0.04], with significantly decreased locomotor rate compared to vehicle following water (p=0.002) and alcohol (p<0.001).

Pharmacological inactivation of the rhomboid thalamic nucleus

The two-way RM ANOVA of Rh inactivation (**Fig. 2.4A**) on alcohol-appropriate responding showed a main effect of alcohol dose [F(1,3)=185.63, p<0.001] and a significant alcohol dose by muscimol+baclofen treatment interaction [F(1,3)=28.39, p=0.01]. Interestingly, Rh inactivation prior to Water resulted in a significant increase in alcohol-appropriate responding relative to Water under vehicle conditions (p<0.05), resulting in partial substitution for the training dose. However, Rh inactivation prior to administration of the alcohol-training dose (1 g/kg) did not affect discrimination performance. One rat did not complete an FR10 following Rh inactivation and thus was not included in the response accuracy measure, but was included in the response rate analysis. There was a significant main effect of muscimol+baclofen treatment on response rate [F(1,4)=23.26, p=0.009], but

no significant main effect of alcohol or interaction (**Fig. 2.4B-C**). Additionally, Rh inactivation produced no effect on locomotor rate (**Fig. 2.4D**).

Pharmacological inactivation of anatomical controls/misses

Following verification of cannulae implantation, data from animals considered to be outside the target regions (n=10), as depicted by triangles in each of the figures (**Fig. 2.3A and 2.4A**), were considered misses and not included in the analyses of that brain region. As such, the data from this group of animals were combined to serve as anatomical controls. Discrimination performance was analyzed with a two-way RM ANOVA which demonstrated a significant main effect of alcohol dose (**Fig. 2.5A**; [$F(1,9)=65.29$, $p<0.001$]) with a significant increase in alcohol-appropriate lever responding following alcohol (1 g/kg) relative to water, as would be expected. No significant main effect of muscimol+baclofen treatment was observed. Two-way RM ANOVA of response rate demonstrated a significant main effect of muscimol+baclofen treatment (**Fig. 2.5B**; [$F(1,9)=21.34$, $p<0.001$]), with a decreased response rates following inactivation relative to vehicle. There was no main effect of alcohol dose or interaction. Additionally, two-way RM ANOVA also showed a significant main effect of muscimol+baclofen treatment on locomotor rate (**Fig. 2.5C**; [$F(1,9)=5.80$, $p<0.04$]), with significantly less locomotor activity following muscimol+baclofen relative to vehicle condition.

DISCUSSION

The findings from the present work demonstrate that the IC and Rh are targets of alcohol (1 g/kg), as measured by c-Fos IR in rats trained to discriminate alcohol (1 g/kg) from water, suggesting that these brain regions may be recruited in modulating sensitivity to alcohol. Indeed, we confirm the functional involvement of these regions as temporary pharmacological inactivation of the IC or Rh

partially substitutes for the discriminative stimulus effects of a moderate alcohol dose (1 g/kg). While the data patterns in the IC and Rh are contrary to our original hypotheses, the findings from the present work identify the functional role of the IC and Rh in modulating sensitivity to alcohol, which is an important and novel contribution to the literature.

Neuronal response as measured by c-Fos expression has been widely used to determine the brain regional site of action of alcohol (*see*: (Vilpoux *et al*, 2009). A previous study utilizing a higher alcohol dose (1.5 g/kg, IP) found an increase in c-Fos IR in the IC, both in alcohol-naïve and -experienced rats, an effect not seen with a lower alcohol dose (0.5 g/kg; (Ryabinin *et al*, 1997). In the present work, decreases in c-Fos IR within the AcbC, IC, and the Rh were observed following alcohol in discrimination-trained animals, suggesting that these regions may be recruited when the animal is using the alcohol interoceptive cue to guide behavior. The animals were tested following a discrimination session as we sought to examine the brain response in conjunction with the discrimination behavior; therefore, it would be interesting to determine whether a similar pattern of c-Fos response would occur if the rats were sacrificed without undergoing the behavioral session on the final session, as it is possible that basal levels of c-Fos IR are elevated, in general, as a consequence of engaging in the behavior. Additionally, the alcohol-induced decrease in c-Fos IR was observed in the AcbC, but not the nucleus accumbens shell. This data pattern is consistent with the observed decrease in the AcbC projection regions (IC and Rh) as confirmed by the FG retrograde tracer study. Analysis of FG positive cells that co-express c-Fos would allow for determination of whether the alcohol-induced decreases in neuronal activity are specific to projection neurons from the IC or Rh to the AcbC. This strategy was not implemented in the present work as the FG retrograde tracer study (Experiment 2.1) was conducted in naïve rats in order to identify projection regions to the AcbC and not in the discrimination-trained rats that were used for the c-Fos analyses (Experiment 2.2), but will be an interesting future direction. Importantly, in the present study, the alcohol-induced decrease in c-Fos IR in these brain regions is likely not due to differences in motor output (i.e., lever responding), as response rates were similar between the groups that received water or alcohol on the test (**Fig.**

2.2B). Given that only one alcohol training dose (1 g/kg) was examined it will be interesting for future work to broaden the range of alcohol training doses, as these studies may identify dose-related effects on these anatomical sites of action of alcohol.

In general, as reflected in the alcohol discrimination literature, pharmacological manipulations that result in CNS inhibition (e.g., $GABA_A$ agonists, NMDA antagonist) tend to have "alcohol-like" effects (Grant and Colombo, 1993a; Hiltunen and Jarbe, 1989; Hodge and Alken, 1996; Hodge and Cox, 1998; Hodge *et al*, 2001b). Thus, while utilization of a muscimol+baclofen cocktail is commonly used as a tool by which to "temporarily inactivate" a specific brain region, and was used for that purpose in the present work, this pharmacological strategy also allows for a mechanistic interpretation. That is, while co-activation of $GABA_A$ and $GABA_B$ receptors (i.e., muscimol+baclofen cocktail infusions) in the IC and Rh intrinsically "inactivate" the brain regions, we are also able to conclude that these receptors in these brain regions contribute, in part, to the discriminative stimulus effects of alcohol, as partial substitution (Rh and IC) for alcohol was observed. Therefore, the present results mechanistically implicate the importance of $GABA_A$ and $GABA_B$ receptors and indicate that activating these receptors is critical for the expression of the discriminative stimulus effects of alcohol. Although, pharmacological inactivation of the Rh resulted in a decrease in response rate, responding on the alcohol-appropriate lever was not altered following the training dose of alcohol (e.g., appropriate accuracy performance). Additionally, pharmacological inactivation of the IC did not alter response rates, confirming that changes in discrimination performance were not due to nonspecific changes in motor output, or motivation to respond for the sucrose reinforcer. This latter point suggests that there was also no change in sucrose palatability, which is important given that the IC (albeit further posterior IC than that targeted in the present work) has been implicated in food-seeking and taste processing (Carleton *et al*, 2010; Kusumoto-Yoshida *et al*, 2015).

Pharmacological manipulation in the IC and Rh resulted in partial substitution for the discriminative stimulus effects of alcohol (1 g/kg). Even though full substitution was not observed,

these findings implicate, in part, the functional importance of the IC and Rh and activation of $GABA_A$ and $GABA_B$ receptors within these brain regions in modulating sensitivity to alcohol. These findings are highly novel given that, to date, these brain regions have not been previously examined in terms of modulating sensitivity to the interoceptive effects of alcohol in an animal model. Further, it is possible that $GABA_A$ and $GABA_B$ activation in the IC and Rh may potentiate the effects of low alcohol doses (e.g., 0.3 or 0.5 g/kg), resulting in full substitution. Unfortunately, this was not tested in the present study, but will be important for future work to determine. Moreover, these findings also suggest that co-activation of $GABA_A$ and $GABA_B$ receptors only constitute a partial target site of action in the IC and Rh as other receptor systems are likely also recruited in modulating interoceptive sensitivity to alcohol.

Many studies suggest a motivational network involving the IC and the AcbC as both have been implicated in regulating motivationally relevant events (Clithero *et al*, 2011; Damasio, 1996), which is highly relevant for drug-related stimuli. Therefore, it is not surprising that in human imaging studies the IC responds to alcohol-related cues in individuals with alcohol-use disorders (Filbey *et al*, 2008) and among at-risk individuals (Ihssen *et al*, 2011; Ray *et al*, 2010), an effect absent in social drinkers (George *et al*, 2001; Myrick *et al*, 2004; Tapert *et al*, 2004). Further pre-clinical data also implicates the role of the IC in modulating compulsive alcohol drinking, in which optogenetic inactivation of IC projections to the AcbC decreased aversion-resistant alcohol intake (Seif *et al*, 2013). Taken together, the current findings lend further support for the importance of the IC in modulating sensitivity to alcohol.

Interestingly, there is relatively little literature on the functional role of the Rh, especially in relation to drug and alcohol-related behaviors. The Rh receives dense projections from the brainstem and shares reciprocal projections with the cortices (Ohtake and Yamada, 1989; Vertes, 2002; Vertes *et al*, 2006); *see*: (Cassel *et al*, 2013; Vertes *et al*, 2015). Historically, the Rh is studied with the reuniens ventral thalamic nucleus, as together they form the ventral midline nuclei (Cassel *et al*, 2013). Inactivation and lesions to the Rh implicate their role in modulating behavioral flexibility

(Cholvin *et al*, 2013; Prasad *et al*, 2016; Prasad *et al*, 2013). Additionally lesions to the Rh increase accuracy, decrease number of omitted responses and latency to obtain reward during behavioral tasks, suggesting a role for Rh in motivation and executive control (Prasad *et al*, 2016; Prasad *et al*, 2013). The presence of these known connections along with the current findings, suggest that Rh integrate cognitive and arousal processes to induce behavioral flexibility in a changing environment (Cassel *et al*, 2013). The majority of those studies attribute the Rh with the role of modulating working-memory particularly with reference to spatial context (Cholvin *et al*, 2013; Hallock *et al*, 2013; Hembrook and Mair, 2011; Layfield *et al*, 2015; Prasad *et al*, 2016). Therefore, it is possible that pharmacological inactivation of these regions may induce memory impairments. Indeed, a memory impairment in a two-lever discrimination task, would be reflected by 50% responding on either lever. While this was the behavioral pattern observed following inactivation under the water condition (i.e., ~50% alcohol-appropriate responding), alcohol-appropriate responding under the alcohol condition was unaffected by inactivation (i.e., similar to the control condition). Therefore, this accurate discrimination performance would argue against a memory impairment (**Fig. 2.4B**). To date the role of the Rh in drug-related behaviors has been understudied, however there is growing interest in this midline thalamic nucleus especially given its projections to limbic structures such as the mPFC, hippocampus, nucleus accumbens and its role in cognitive function (*see*: (Vertes *et al*, 2015). The present findings implicating the Rh in modulating sensitivity to alcohol suggest the importance of future work to examine the role of this brain region in modulating other alcohol- and drug-related behaviors. However, it is important to consider the small sample size in the Rh inactivation studies, which was the consequence of several inaccurate cannula placements primarily due to the location and the small target area. Therefore, it will be important for future work to replicate this finding.

One of the goals of the present work was to focus on upstream regions to the AcbC, as general inhibition in the AcbC has been shown to modulate sensitivity to alcohol (Besheer *et al*, 2003; Hodge and Alken, 1996; Hodge and Cox, 1998; Hodge *et al*, 2001b). It is important to consider that infusion of muscimol+baclofen into these regions inactivates all of the regions' outgoing projections.

Thus, the partial and full substitution of alcohol obtained through pharmacological inactivation may not be specific to inactivation of the outgoing AcbC projections but rather of a widespread regional effect. In addition to projecting to the AcbC, the IC and Rh all share reciprocal projections (Ohtake and Yamada, 1989; Sesack *et al*, 1989; Vertes *et al*, 2006). Thus, the present findings may be an indirect result of communication within these regions and may explain the partial vs. full substitution of "alcohol-like" effects. Further, while the FG study led to the focus on the IC and Rh as being AcbC-projecting regions, which is consistent with other findings (Ding *et al*, 2001; Vertes *et al*, 2006; Wright and Groenewegen, 1996), it is important to consider that FG diffusion into the proximal shell or caudate nucleus may have occurred. Therefore, it will be important for future studies to isolate the specific neural circuitry modulating sensitivity to alcohol, and whether projections from the IC and Rh to the AcbC are functionally involved.

The present findings provide evidence that $GABA_A$+$GABA_B$ receptor systems in the IC and Rh functionally modulate, in part, the interoceptive effects of alcohol. Studies also utilizing muscimol+baclofen infusions in the IC demonstrate decreased alcohol self-administration (Pushparaj and Le Foll, 2015). Thus, it is possible that the decrease in alcohol self-administration and seeking (Pushparaj and Le Foll, 2015; Willcocks and McNally, 2013) may be related to "alcohol-like" effects induced by the pharmacological inactivation. In conclusion, the current results have identified novel brain regional involvement in modulation of the discriminative stimulus effects of alcohol.

	Cumulative Alcohol Dose (g/kg, IG)				Cumulative Alcohol Dose (g/kg, IG)				Cumulative Alcohol Dose (g/kg, IG)			
	0.1	**0.3**	**1.0**	**1.7**	**0.1**	**0.3**	**1.0**	**1.7**	**0.1**	**0.3**	**1.0**	**1.7**
Exp 2	9.1±3.9	17.6±8.9	86.7±8.9*	98.4±1.1*	54.7±3.7	48.0±3.4	59.0±3.5	48.1±3.9	17.7±1.4	13.2±0.7*	12.5±0.8*	12.7±1.2
Exp 3	11.4±2.4	34.9±10.6	77.5±8.8*	92.5±4.5*	55.4±4.2	53.6±6.2	46.5±4.3	40.6±5.9*	21.5±2.2	15.4±1.5*	10.2±1.2*	9.1±0.8

Table 2.1- Performance during the initial cumulative alcohol discrimination test to confirm discriminative control (mean ± S.E.M.).

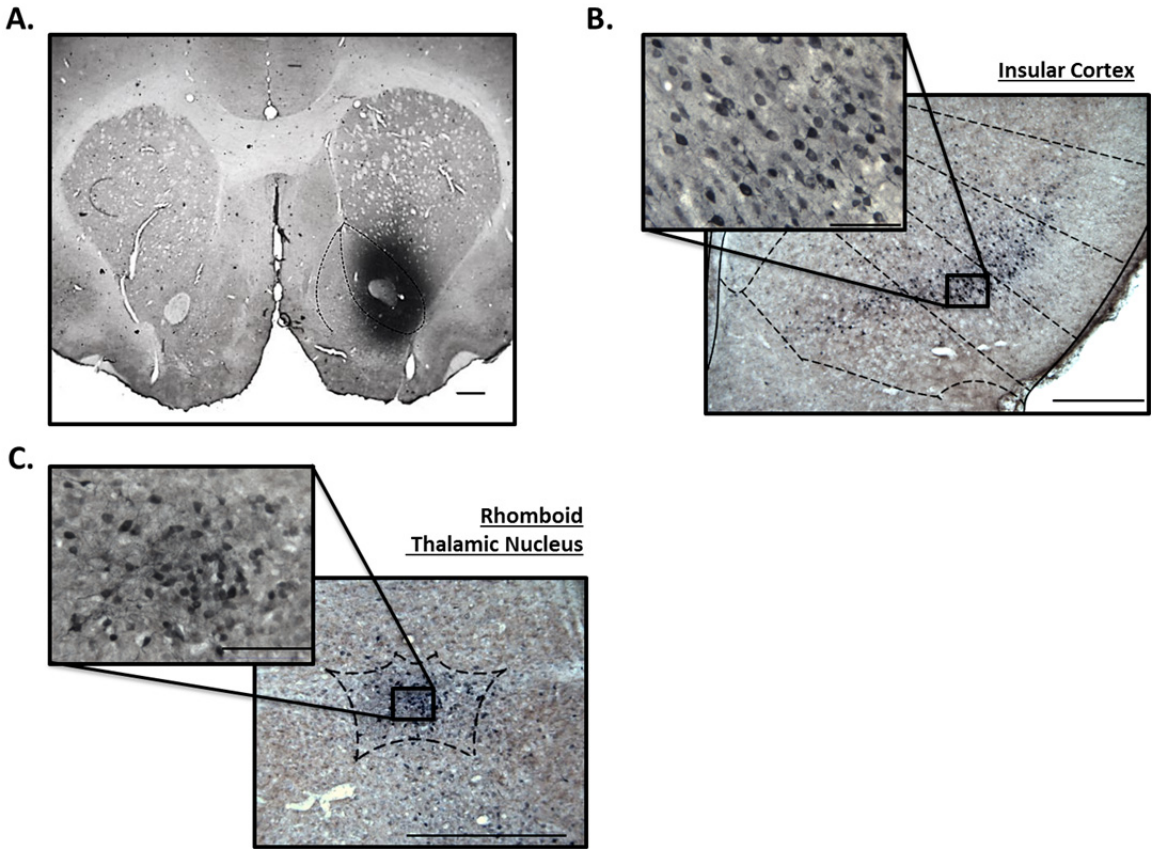

Figure 2.1- FG immunoreactivity identifies incoming neuronal projections to the nucleus accumbens core.

Representative photomicrograph to show **(A)** unilateral FG infusion into the nucleus accumbens core (1.25X) and FG expression in the **(B)** insular cortex (5X), and **(C)** rhomboid thalamic nucleus (10X). Photomicrograph insets in panels B and C represent FG-positive cells within the regions (B =32X, C=40X). Scale bars represent 250 μm in pictographs, insets represent 50 μm.

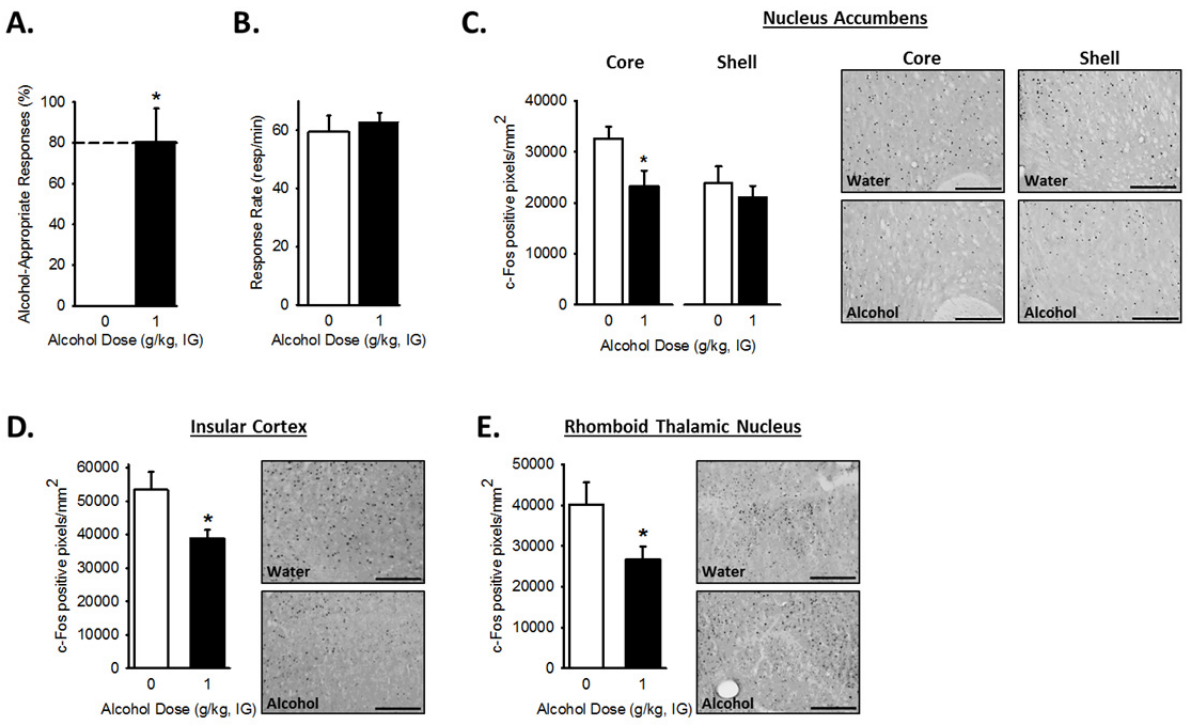

Figure 2.2- Decreased brain regional neuronal activity in response to the training dose of alcohol

(A) Increased alcohol-appropriate responses following the training dose of alcohol (1 g/kg) with no effect on **(B)** response rate on the terminal test prior to sacrifice. c-Fos IR, following the discrimination test, shows a significant decrease in c-Fos-positive cells in response to the training dose of alcohol (1 g/kg) in the **(C)** nucleus accumbens core, but not shell, **(D)** insular cortex **(E)** and rhomboid thalamic nucleus. Representative photomicrographs (20X) to show c-Fos positive cells for each brain region. Scale bars represent 250 µm. Dashed line (>80%) represents full expression of the discriminative stimulus effects of alcohol.* $p<0.05$, significant difference from water (i.e., 0 g/kg; *t*-test; n=4-5/ group). Values on graphs represent mean ± SEM.

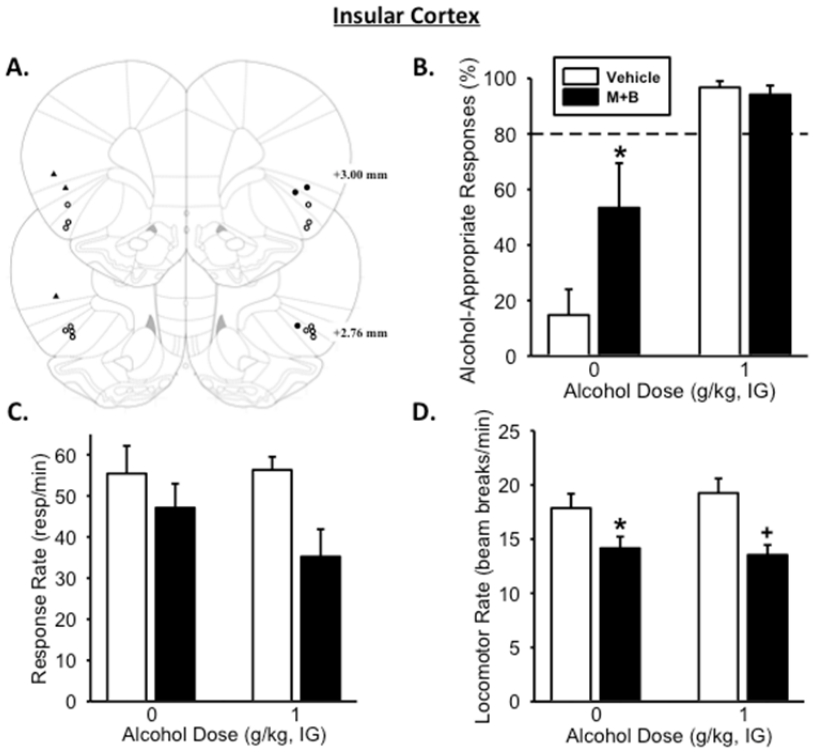

Figure 2.3- Pharmacological inactivation of the insular cortex partially substitutes for the discriminative stimulus effects of the alcohol training dose

(A) Insular cortex bilateral injector tip placements from individual discrimination-trained rats with accurate placements (depicted as open circles) and inaccurate placements (depicted as solid triangles/circles). **(B)** Pharmacological inactivation of the insular cortex, through bilateral infusion of muscimol+baclofen (M+B), significantly increased mean (±SEM) percentage of alcohol-appropriate responses following Water (IG). However, IC inactivation had no effect on alcohol-appropriate responses following the training dose of alcohol (1 g/kg, IG). **(C)** M+B infusion did significantly decrease response rate relative to vehicle. **(D)** Locomotor rate was significantly decreased with M+B infusion following Water and 1 g/kg (IG). Dashed line (>80%) represents full expression of the discriminative stimulus effects of alcohol. * significant difference from vehicle in the Water condition (i.e., 0 g/kg; Tukey, $p<0.05$; n=7). Values on graphs represent mean ± SEM.

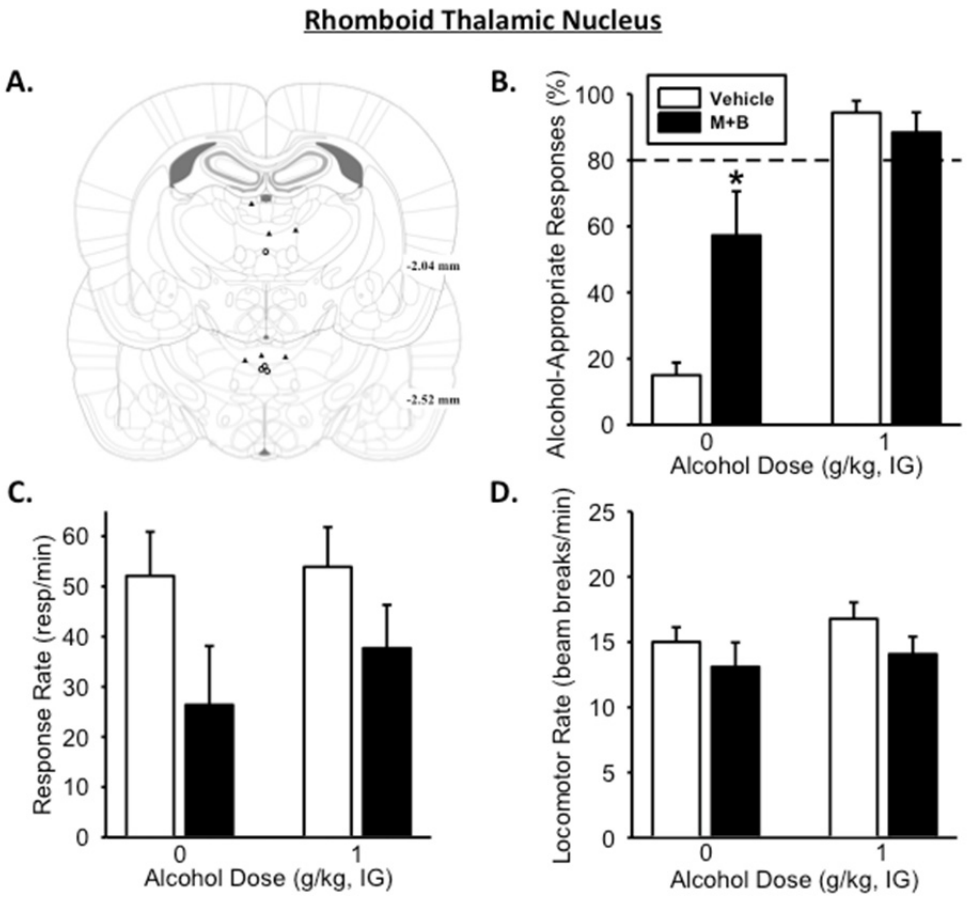

Figure 2.4- Pharmacological inactivation of the rhomboid thalamic nucleus partially substitutes for the discriminative stimulus effects of the alcohol training dose

(A) Rhomboid thalamic nucleus unilateral injector tip placements from individual discrimination-trained rats with accurate placements. **(B)** Temporary inactivation of the rhomboid thalamic nucleus, through unilateral infusion of muscimol+baclofen (M+B), increased mean (±SEM) percentage of alcohol-appropriate responses following Water (IG) but had no effect following the training dose of alcohol (1 g/kg, IG). **(C)** Response rate was significantly decreased with M+B infusion relative to vehicle. **(D)** However there was no effect on locomotor rate. Dashed line (>80%) represents full expression of the discriminative stimulus effects of alcohol. *significant difference from vehicle in the Water condition (i.e., 0 g/kg; Tukey, $p \leq 0.05$; n=4). Values on graphs represent mean ± SEM.

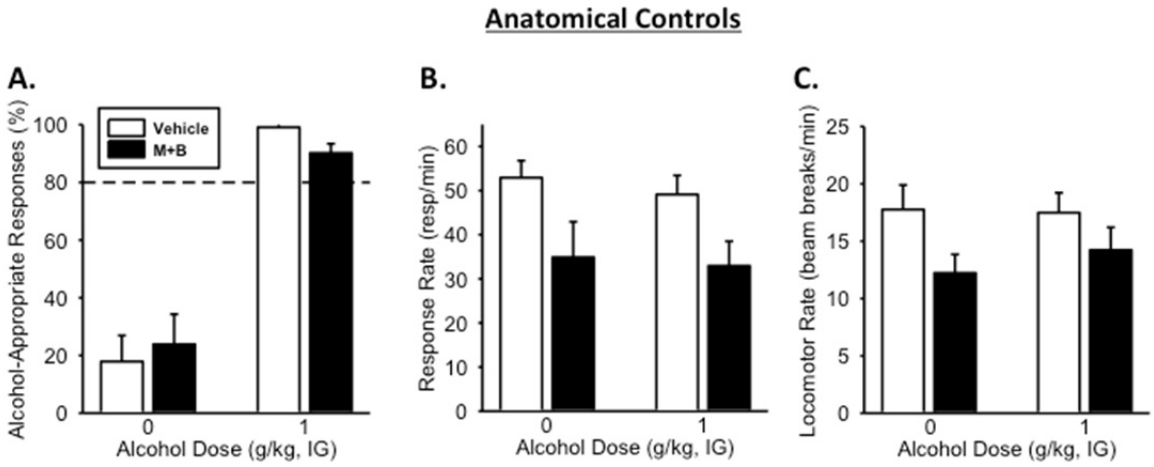

Figure 2.5- Pharmacological inactivation of anatomical controls/misses produced no effects on the discriminative stimulus effects of the alcohol training dose

(A) Alcohol significantly increased the mean (±SEM) percentage of responding on the alcohol-appropriate lever relative to Water. However, infusion of muscimol+baclofen (M+B) had no effect alcohol-appropriate responses following Water or alcohol (1g/kg, IG). **(B)** Response rate and **(C)** locomotor rate were significantly lowered with M+B infusion, relative to vehicle. Dashed line (>80%) represents full expression of the discriminative stimulus effects of alcohol. (Tukey, $p<0.05$; n=10) Values on graphs represent mean ± SEM.

CHAPTER 3: FUNCTIONAL ROLE FOR CORTICAL/THALAMIC-STRIATAL CIRCUIT IN MODULATING INTEROCEPTIVE EFFECTS OF ALCOHOL[3]

INTRODUCTION

The IC receives somatosensory and viscerosensory information from the thalamus and somatosensory cortices in the granular IC (Craig, 2009). This information spreads vertically and is integrated through a columnar organization relayed to the agranular/anterior IC, a region highly interconnected with limbic structures (Craig, 2009; Gu *et al*, 2013; Maffei *et al*, 2012). As such the IC is positioned to be a central hub for interoceptive processing within the central nervous system (Gu *et al*, 2013; Naqvi and Bechara, 2010). Such that, a proposed function of the IC is to project relevant information on interoception to influence decision-making processes and drive motivated behavior through efferent limbic projections, which is highly relevant to drug-related behaviors (Naqvi and Bechara, 2010; Paulus and Stewart, 2014). The suggested role of the IC in regulating drug-related interoceptive effects is based on findings from the clinical literature (Droutman *et al*, 2015; Paulus and Stewart, 2014). Interestingly, in the preclinical field, only recently has a functional role for the IC been identified in modulating the discriminative stimulus/interoceptive effects of a drug of abuse (Jaramillo *et al*, 2016). That is, pharmacological inactivation of the IC in rats results in partial substitution for the discriminative stimulus effects of alcohol (i.e., produces some "alcohol-like" interoceptive effects). Of specific interest to the present study are projections from the IC to the

[3]This chapter is currently under review (Jaramillo AA. et al., (2016). Functional role for suppression of the insular-striatal circuit in modulating interoceptive effects of alcohol. *Neuropsychopharmacology,* It has been included with additional editing by the author to include the Rh as an additional focus.

nucleus accumbens core (IC→AcbC; (Jaramillo *et al*, 2016; Wright and Groenewegen, 1996), a limbic region also implicated in modulating interoceptive sensitivity to alcohol (Besheer *et al*, 2003; Besheer *et al*, 2009; Hodge and Cox, 1998; Hodge *et al*, 2001b; Jaramillo *et al*, 2016). In preclinical work, IC→AcbC projections have been shown to functionally regulate compulsive alcohol drinking (Seif *et al*, 2013) and imaging studies demonstrate strong functional connectivity between the IC and AcbC (Cauda *et al*, 2011) particularly in response to reward processing (Cho *et al*, 2013; Clithero *et al*, 2011). Therefore, understanding the neurobiological circuitry modulating the interoceptive effects of drugs and the potential role of the IC is beneficial for the addiction field.

Traditionally, drug discrimination methods have been used to investigate the discriminative stimulus/interoceptive effects of drugs. Thus, a goal of the present work was to test the functional role of the IC and IC→AcbC circuit in modulating the interoceptive effects of alcohol in rats by implementing a chemogenetic strategy (i.e., hM4Di Designer Receptors Exclusively Activated by Designer Drugs [DREADDs]). Moreover, we utilized two alcohol discrimination-training methods (i.e., operant and Pavlovian). Under both training conditions, behavior (i.e., lever selection in the operant method or goal-tracking in the Pavlovian method) is under the control of the alcohol (1 g/kg) drug state. Using two methods allows us to confirm functional involvement, and also to determine whether there is differential involvement of the IC→AcbC circuit related to the behavioral output (e.g., lever responding, goal-tracking), particularly in reference to interoceptive effects of alcohol and reward associations.

Additionally, we investigate the rhomboid thalamic nucleus, as in Chapter 2 we also demonstrate that pharmacological inactivation of the Rh produces partial “alcohol-like” effects. Furthermore similar to the IC, the Rh also sends projections to the AcbC, receives dense projections from the brainstem and is also highly interconnected with the cortices and limbic regions (Cassel *et al*, 2013; Ohtake and Yamada, 1989; Vertes, 2002; Vertes *et al*, 2006; Vertes *et al*, 2015). However, in addition to its proposed role in modulating the interoceptive effects of alcohol, the Rh is also proposed to modulate cue-induced behavior, particularly under conditions that require behavioral

flexibility (Prasad *et al*, 2013), as lesions to the Rh increased impulsive behavior in the presence of a conditioned stimulus, with varying stimulus durations (Prasad *et al*, 2013). The presence of these known connections along with the past findings, suggest that Rh integrates cognitive and arousal processes to induce behavioral flexibility in a changing environment (Cassel *et al*, 2013). However, only one study has implicated Rh as a site of alcohol action (Jaramillo *et al*, 2016). Therefore the present study also investigates the role of Rh and Rh→AcbC circuit in modulating the interoceptive effects of alcohol in rats by utilizing a chemogenetic strategy (i.e., DREADDs) and the two-lever operant alcohol discrimination method. Additionally, given the proposed role of the Rh in modulating cue-induced behavioral flexibility, we utilize a testing paradigm, which tests the discriminative stimulus of self-administered alcohol (Besheer *et al*, 2015; Besheer *et al*, 2006; Hodge *et al*, 2001a).

Based on our previous findings with the IC and Rh (Chapter 2) and the known functional importance of the AcbC in modulating sensitivity to alcohol (Besheer *et al*, 2009; Hodge and Cox, 1998; Jaramillo *et al*, 2016), we hypothesized that chemogenetic silencing of the IC, Rh, IC→AcbC, and Rh→AcbC projections would potentiate the interoceptive effects of experimenter-administered alcohol, thus functionally demonstrating the role of the IC, Rh and insular/thalamic-striatal circuit in modulating behavior under the stimulus control of alcohol.

MATERIALS AND METHODS

Animals

Male Long-Evans rats (Harlan Sprague–Dawley, Indianapolis, IN) were individually housed in a vivarium maintained on a 12-h light/dark cycle (experiments conducted during the light cycle). Water was available ad libitum in the home cage and food intake was restricted to maintain body weight (325–340 g). Animals were under continuous care and monitoring by veterinary staff from the Division of Laboratory Animal Medicine at UNC-Chapel Hill. All procedures were conducted in accordance with the NIH Guide to Care and Use of Laboratory Animals and institutional guidelines.

Alcohol Discrimination Training and Testing Procedures

Rats were trained to discriminate alcohol (1 g/kg, IG) vs. water using operant (two-lever) or Pavlovian alcohol discrimination procedures as we describe in (Jaramillo *et al*, 2016; Randall *et al*, 2016) and Chapter 2. Prior to any testing, alcohol stimulus control was confirmed by a cumulative alcohol dose-response curve (**Table 3.1**).

Operant Alcohol Discrimination

During the alcohol and water training sessions (i.e., 10 min after alcohol or water IG, respectively), completion of a fixed ratio 10 (FR10) on the appropriate (i.e., correct) lever resulted in the delivery of sucrose reinforcer (i.e., 0.1 ml of 10% sucrose (w/v) solution; **Fig. 3.2a**). Testing began when the following accuracy criteria were met: the percentage of appropriate lever responses before the first reinforcer and during the entire session were >80% for at least 8 out of 10 consecutive days.

Pavlovian Alcohol Discrimination

During alcohol training sessions (10 min following alcohol, IG), each light presentation was followed by a sucrose presentation (i.e., 0.1 ml of 26% sucrose [w/v]). During water training sessions (10 min following water, IG), light presentations did not result in sucrose presentations (**Fig. S2b**). Head entries into the sucrose receptacle during the light presentation and the 15 sec preceding the light presentation were measured and a discrimination score was calculated. Testing began when the following accuracy criterion was met: the mean of the first discrimination score from the preceding two alcohol sessions had to be ≥150% of the mean of the first discrimination score from the preceding two water sessions (Randall, 2016 #236; adapted from (Besheer *et al*, 2012b; Palmatier *et al*, 2005).

Tracer and Viral Vectors

Fluoro-Gold (0.4 µl [FG]; Fluorochrome, LLC, CO) was dissolved in 0.9% saline (w/v)/2% (v/v) FG, per manufacturer instructions and as we previously report (Jaramillo *et al*, 2016). hM4Di-DREADDs (AAV8-hSyn-DIO-hM4Di-mCherry; UNC Vector Core, NC) or Control-mCherrys (AAV8-hSyn-DIO-mCherry; UNC Vector Core, NC), previously described by (Krashes *et al*, 2011; Roth, 2016), were combined with Cre recombinase (AAV8-CMV-Cre-GFP; Vector Biolabs, PA) in a ratio of 7:3 (v/v) and infused 2 µl/side in the IC or 1 µl into the Rh.

Microinjection Procedures for Viral Vectors, Tract Tracer, and Drug Infusions

Site-specific microinjections were delivered by a microinfusion pump (Harvard Apparatus, MA) through 1.0 µl Hamilton syringes (Hamilton Robotic, NV) connected to 33-gauge injectors (Plastics One, VA) as described in (Besheer *et al*, 2014; Jaramillo *et al*, 2016). For Experiment 3.1, anesthetized rats received a unilateral microinjection of FG into the AcbC at a volume of 0.05 µl across 8-min as we describe in (Jaramillo *et al*, 2016). The injector remained in place for an additional 4-min to allow for diffusion. For Experiment 3.2-3.5, anesthetized rats received bilateral microinjection of viral vectors into the IC at a volume of 2.0 µl across 10-min or into the Rh at a volume of 1.0 µl across 5-min. The injector remained in place for an additional 10- or 5- min, respectively, to allow for diffusion. CNO microinjections were delivered in Experiment 3.3-3.5 through injectors extending 2 mm below the previously implanted (aimed to terminate 2 mm above the AcbC) 26-gauge guide (Plastics One, VA) at a volume of 0.5 µl/side across 1 min. The injectors remained in place for an additional 2-min after the infusion to allow for diffusion.

Electrophysiological Validation of DREADD-Based Silencing

Slice Preparation

Rats with intra-IC or intra-Rh hM4Di-DREADDs were anesthetized, rapidly decapitated and brains were quickly removed into a modified ice-cold aCSF solution containing (in mM): 75 sucrose; 87 NaCl; 2.5 KCl; 1.25 NaH_2PO_4 ; 25 $NaHCO_3$; 0.5 CaCl; 7 MgCl for 1-2 min. Coronal sections (300 μM), prepared by a vibatome (Leica, Germany), were placed in a holding chamber and allowed to recover for at least 30 min before being placed in the recording chamber and superfused with bicarbonate-buffered solution saturated with 95% O_2 and 5% CO_2 and containing (in mM): 119 NaCl, 2.5 KCl, 1.0 NaH_2PO_4, 1.3 $MgCl_2$, 2.5 $CaCl_2$, 26.2 $NaHCO_3$, and 11 glucose (at 32-34°C).

Patch Clamp Electrophysiology

Cells were visualized with an upright microscope using infrared differential interference contrast (IR-DIC) illumination and fluorescent LED (550nM) and whole-cell current clamp recordings of IC or Rh neurons were made with a Multiclamp 700B amplifier (Axon Instruments, CA). Patch electrodes (3-5 MΩ) were backfilled with a potassium gluconate internal solution containing (in mM): 135 K Gluc, 5 NaCl, 2 $MgCl_2$-$6H_20$, 10 HEPES, 0.6 EGTA, 4 Na_2ATP, 0.4 Na_2GTP. Access resistance (< 40 MΩ, 20% threshold for change) was monitored online with a 5 mV hyperpolarizing step (50 ms). Resting membrane potential and rheobase were measured before and after 10 μM CNO bath application.

Experimental Procedures

Experiment 3.1: Examination of alcohol-induced neuronal activity in projections from the IC and the Rh to the AcbC.

Naïve rats received a unilateral microinjection of the neuronal retrograde tracer Fluoro-Gold (FG) aimed at the AcbC (AP +1.7, ML +1.5, DV -6.8 from skull; **Fig**. **3.1a**). One week following injection, allowing time for recovery and diffusion of the tracer, rats were habituated to daily water IG for 5 days. On the test day, rats received alcohol (0 or 1 g/kg, IG; n=11/group) and were sacrificed 90 min later. Coronal brain sections were processed for co-localization of c-Fos and FG fluorescence to determine alcohol-induced neuronal activation in IC→AcbC projections (**Fig. 3.1b-g**) or Rh→AcbC projections (**Fig. 3.1h-k**).

Experiment 3.2: Validation of DREADDs.

Naïve rats received bilateral microinjection of hM4Di-DREADDs in the IC (n =10; AP +3.2, ML ±4.0, DV -6.0 from skull; **Fig. 3.2a-e**) or Rh (n=4; AP -2.3, ML ± 1.7 , DV -7.2 with a 5° angle from skull; **Fig. 3.2f-g**). Seven weeks later, allowing time for expression of DREADDs, brain tissue was collected to confirm hM4Di-mCherry neuronal expression in the IC (**Fig**. **3.2a-b** and the Rh (**Fig. 3.f-g**) or to validate functional activity by *ex vivo* slice electrophysiological recordings in the IC (**Fig. 3.1c-e**) and the Rh, following bath application of clozapine-N-oxide (CNO; **Fig. 3.1c-e**). Additionally, tissue was analyzed for immunofluorescence colocalization of hM4Di-DREADDs and NeuN or GFAP (i.e., neuronal and glial markers, respectively) in the IC (**Fig. 3.3a-b**) and Rh (**Fig. 3.3c-d**).

Experiment 3.3: Examination of the functional role of the IC and IC→AcbC on the alcohol drug state utilizing operant alcohol discrimination procedure.

IC silencing. Discrimination-trained rats received bilateral infusion of hM4Di-DREADDs into the IC (n=11). Following 1 week of recovery, training continued until stable discrimination was established (>6 weeks). To determine a functional role of the IC in modulating the discriminative stimulus effects of alcohol, rats received CNO (0, 1 mg/kg, intraperitoneal [IP]) and 45 min later an alcohol dose (0.3, 0.5, 1.0 g/kg, IG), after which rats were placed in the chambers for a test session (**Fig. 3.4a**). Another group of discrimination-trained rats that did not receive surgery were used as CNO-injected behavioral controls (n=7).

IC→AcbC silencing. To determine the role of IC→AcbC projections, another group of discrimination-trained rats were infused with hM4Di-DREADDs (n=6) or Control-mCherry (n=6) in the IC and implanted with bilateral AcbC cannulae. Following acquisition of stable discrimination behavior (>6 weeks), rats received intra-AcbC infusion of CNO (0 or 9 μM/side) 5 min prior to alcohol (0.3, 0.5, 1.0 g/kg, IG) and then underwent a test session (**Fig. 3.5a**).

Experiment 3.4. Examination of the functional role of the IC on the alcohol drug state utilizing a Pavlovian alcohol discrimination procedure.

IC silencing. Rats trained in the Pavlovian alcohol discrimination procedure received bilateral infusion of hM4Di-DREADDs (n=18) or Control-mCherrys (n=6) into the IC. Following recovery and continued training (>6 weeks), testing began. Rats received CNO (0, 1 mg/kg, IP) and 45 min later an alcohol dose (0, 0.1, 1.0 g/kg, IG) after which rats were placed in the chambers for a test session (**Fig. 3.6a**).

IC→AcbC silencing. Following systemic CNO testing, the same hM4Di-DREADD rats and Control-mCherry rats were implanted with bilateral AcbC cannulae. Rats received pretreatment of CNO (0 or 0.3 μM/side) 5 min prior to alcohol (0, 0.1, 1.0 g/kg, IG) and then underwent a test session (**Fig. 3.7a**).

Experiment 3.5. Examination of the functional role of the Rh→ and Rh→AcbC on the alcohol drug state utilizing operant alcohol discrimination procedure.

Rh silencing. Discrimination-trained rats received unilateral infusion of the hM4Di-DREADDs into the Rh (n=12). Following 1 week of recovery, training continued until stable discrimination was established (>6 weeks). To determine a functional role of the Rh in modulating the discriminative stimulus effects of alcohol, rats received CNO (0.5 mg/kg, IP) and 45 min later an alcohol dose (0, 0.3, 1.0 g/kg, IG), after which rats placed in the chambers for a test session (**Fig. 3.8a**)

Rh→AcbC silencing. To determine the role of the Rh→AcbC projections, another group of discrimination-trained rats were infused with hM4Di-DREADDs and implanted with bilateral AcbC cannulae (n=11). Following acquisition of stable discrimination behavior (>6 weeks), rats received intra-AcbC infusion of CNO (0 or 9 μM/side) 5 min prior to alcohol (0.3, 0.5, 1.0 g/kg, IG) and then underwent a test session (**Fig. 9a**).

Experiment 3.6. Examination of the functional role of the Rh→ and Rh→AcbC on the discriminative stimulus effect of self-administered alcohol

To determine whether Rh or Rh→AcbC silencing would alter the discriminative stimulus effects of self-administered alcohol, rats expressing hM4Di-DREADD in the Rh and implanted with AcbC cannulae from Experiment 3.5 were tested on a discrimination/self-administration (Discrim/SA) test session as conducted in (Besheer *et al*, 2015; Besheer *et al*, 2006; Hodge *et al*, 2001a). These test sessions differed in duration (30 min) and reinforcer (sweetened alcohol solution; 10%, w/v sucrose+10%, v/v alcohol) from the standard test sessions; however, as in the standard test session behavior was free to vary between the two levers since completion of an FR10 on either lever resulted in reinforcer presentation. Briefly, in these sessions, following water (IG) administration, rats begin the session responding predominantly on the water-appropriate lever; as the session continues

and rats have consumed significant amounts of the sweetened alcohol reinforcer, responding shifts to the alcohol-appropriate lever, indicating that the interoceptive effects of the consumed alcohol are detected by the animal (i.e., behavior under discriminative stimulus control of alcohol). For testing, rats received CNO (0 or 3 mg/kg, IP) 45 min before water (IG), or intra-AcbC infusion of CNO (0 or 9 μM/side) 5 min before water (IG), after which rats were placed in the chambers for a Discrim/SA test session (**Fig**. **3.10a**)

Tissue Preparation, Immunohistochemistry Procedure, and Analysis

Tissue collection, immunohistochemistry (IHC) staining and quantification procedures were similar as previously described in (Jaramillo *et al*, 2016). The brain regions examined were the AcbC (AP -2.3 to -1.3), IC (+2.8 to +1.9 mm), and Rh (-1.8 to -3.2 mm) according to (Paxinos and Watson, 2007). Free-floating coronal sections (40 μm) were incubated in rabbit anti-c-Fos antibody (1:3,000; Millipore, MA; Experiment 1) for 48 h or rabbit anti-DSRed (1:2,500; Clontech, CA; Experiment 2-4) for 24 h at 4 °C. For confirmation of neuronal DREADD expression in Experiment 2, sections were incubated with mouse anti-DSRed (1:2,500; Clontech, CA;) and rabbit anti-GFAP (1:1000; Acbcam, MA) or rabbit anti-NeuN (1:500, Millipore, MA) for 24 h at 4°C. Sections were then incubated at RT in appropriate fluorescent conjugated secondary antibody (goat anti-rabbit 594; goat anti-rabbit 647 and goat anti-mouse 568; Life Technologies, MA). For Experiment 3.1, only rats with FG injection site determined to be within the AcbC were analyzed for FG and c-Fos positive cells in the IC or Rh. Images were taken on Nikon 80i Upright microscope (Nikon Instruments, NY) and analyzed utilizing Image-Pro Premier image analysis software (Media Cybernetics, MD). Immunoreactivity (IR) data (positive cells/frame [i.e., 89,741.78 μm^2]) were acquired from a minimum of three IC and Rh sections/animal, and the data were averaged to obtain a single value per subject. In Experiment 2-5 viral expression was confirmed by IHC (individual expression represented as 20% opacity [**Fig**. 3.2-3.5]). Nikon 80i Upright microscope and Olympus FV1000 MPE SIM Laser Scanning Confocal Microscope (Olympus America, PA) were used to acquire representative

photomicrographs of hM4Di-mCherry expression. For Experiment 3.3-3.5 cannulae placements were confirmed by Nissl staining (injector placements represented by circles in Fig. 3.3b and 3.5b). Only rats with accurate injections and placements were included in the analyses and data presentation.

Drugs

Alcohol (95% w/v) was diluted in distilled water to a concentration of 20% (v/v) and administered IG, with volumes varied by weight to obtain the desired dose. For systemic administration CNO, injected at a volume of 1 ml/kg (NIDA Drug Supply Program, NC or Enzo Life Sciences, NY), was dissolved in 1% dimethyl sulfoxide in water (v/v) or 0.9% saline (w/v), respectively, or in aCSF for intracranial administration. The CNO doses were chosen based on previous work (Krashes *et al*, 2011; Roth, 2016; Stachniak *et al*, 2014) and pilot studies from our lab.

Data Analysis

Group differences in IR for Experiment 1 were determined by *t*-test. For Experiment 2, *t*-tests, were used to analyze the effects of CNO on modulation of synaptic transmission. For Experiment 3 (i.e., operant drug discrimination), one- or two-way repeated measures analysis of variance (RM ANOVA; within subject design) were used to analyze response accuracy and response rate. Response accuracy was expressed as the percentage of alcohol-appropriate lever responses upon delivery of the first reinforcer. If during the test session an animal did not complete an FR10, data from that animal were not included in the response accuracy analysis, but were included in the response rate analysis. Partial expression of the discriminative stimulus effects of alcohol (i.e., partial substitution) was defined as >40% and <80% alcohol-appropriate responses. Complete expression of the discriminative stimulus effects of alcohol (i.e., full substitution) was defined as ≥80% alcohol-appropriate responding (Jaramillo *et al*, 2016; Solinas *et al*, 2006). Response rate (responses/min) was analyzed for the entire session and served as an index of motor activity. For Experiment 3.5 (i.e.,

Pavlovian discrimination), one- or two-way RM ANOVA was used to analyze discrimination score and locomotor rate. The discrimination score was calculated by subtracting the number of head entries that occurred in the 15 s before light onset (i.e., pre-CS) from the head entries that occurred during the 15-s light CS (Besheer *et al*, 2012b; Palmatier *et al*, 2005; Randall *et al*, 2016).To confirm that during the test the alcohol training dose (1 g/kg) resulted in similar discrimination performance as the training sessions, a paired samples *t*-test was used to compare the discrimination score from the training dose (under the vehicle condition) to the training session baseline. Full substitution for the alcohol training dose (1 g/kg) was determined when the discrimination score did not differ from the vehicle+alcohol training dose condition (Randall *et al*, 2016). Tukey *post hoc* analyses were used to explore significant interactions. Data represented as means ± S.E.M. and significance was declared at $p \leq 0.05$. Figures were assembled using Photoshop (Adobe, CA).

RESULTS

Experiment 3.1: Alcohol-induced neuronal activation in afferent projections to the AcbC.

Individual unilateral FG expression is represented in **Figure 3.1a**. A total of 9 rats showed FG outside the boundaries of the AcbC (e.g., inaccurate injection) or had inefficient FG infusions (e.g., clogged injector) and thus were excluded from the analysis. FG injected into the AcbC (**Fig. 3.1a**) resulted in FG expression in the IC that was similar in the water and alcohol injected groups (**Fig. 3.1c**). Analysis of c-Fos IR within the IC (**Fig. 3.1b**) revealed an alcohol-induced increase in c-Fos in the IC ($t=2.52$, $p<0.03$; **Fig. 1d**). Examination of co-localization of FG and c-Fos IR showed an alcohol-induced increase in cells with co-localized c-Fos+FG ($t=2.80$, $p<0.02$; **Fig. 3.1e-g**), indicating neuronal activation in response to alcohol in IC→AcbC projections. FG injected into the AcbC (**Fig. 3.1a**) also resulted in FG auto-fluorescence in the Rh (**Fig. 3.1i**). Alcohol induced no

change on c-Fos IR (**Fig. 3.1j**) or co-localized FG and c-Fos IR (**Fig. 3.1k**), indicating no neuronal response to alcohol in Rh→AcbC projections.

Experiment 3.2: Validation of DREADDs.

Intra-insula.

Representative hM4Di-mCherry expression is shown in IC (**Fig. 3.1h-i**), 7 weeks following bilateral intra-IC viral vector infusion (hSyn-DIO-hM4Di-mCherry+Cre). To test the efficacy of the hM4Di-DREADDs, using whole-cell current clamp recordings (**Fig. 3.2c-e**), resting membrane potential (RMP; representative trace in **Fig. 3.2d-e**) and rheobase were measured before and after 10 μM CNO bath application. Following CNO, a significant hyperpolarization (pre-CNO: -62.7 + 1.6 mV [**Fig. 3.2d**]; post-CNO -66.3 + 2.1 mV [**Fig. 3.2e**]; delta: -3.5 + 1.3 mV ([F(1,11)=2.84, $p<0.03$]; **Fig**. 1j]), and a significant change in rheobase (pre-CNO: 119.4 + 45.4 nA; post-CNO: 215.1 + 72.0 nA; delta: 104.7 + 36.0 nA; [F(1,11)=2.70, $p\leq0.03$]) were found, thereby confirming the functional activity of the hM4Di-DREADDs in the IC. Additionally hM4Di-mCherry expression was colocalized to NeuN, a neuronal cell marker, and not to the glia marker, GFAP. Thus, indicating DREADD expression only within neurons and not glia.

Intra-rhomboid.

Representative hM4Di-mCherry expression is shown in Rh (Fig. 3.Xh-i) and in the AcbC (Fig. 3.X), 7 weeks following bilateral intra-Rh viral vector infusion (hSyn-DIO-hM4Di-mCherry+Cre). To test the efficacy of the hM4Di-DREADDs, using whole-cell current clamp recordings (**Fig**. 3.1j-l), rheobase were measured before and after 10 μM CNO bath application. Following CNO, a significant a significant change in rheobase ([F(1,2)=4.96, $p\leq0.04$; not shown] was found, thereby confirming the functional activity of the hM4Di-DREADDs in the Rh. Additionally

hM4Di-mCherry expression was colocalized to NeuN, a neuronal cell marker, and not to the glia marker, GFAP. Thus, indicating DREADD expression only within neurons and not glia.

Experiment 3.3-3.6: Confirmation of alcohol stimulus control prior to testing.

Operant Discrimination.

One-way RM ANOVA demonstrated an increase in alcohol-appropriate responding for intra-insular hM4Di-DREADD [$F(3,29)=78.71$, $p<0.001$; **Table 3.1**], intra-rhomboid hM4Di-DREADD [$F(3,13)=139.0$, $p<0.001$; **Table 3.1**] and Control [$F(3,26)=46.7$, $p<0.001$; **Table 3.1**] groups, at the training dose and the highest dose (1.0 and 1.7) relative to the lowest dose (0.1 g/kg; $p<0.001$), demonstrating alcohol stimulus control. In the intra-insular hM4Di-DREADD group, response rate increased with alcohol dose [$F(3,29)=78.71$, $p<0.001$; **Table 3.1**], at the highest alcohol dose relative to the lowest dose (0.1 g/kg; $p<0.05$). No change in response rate was observed for the intra-rhomboid or Control groups.

Pavlovian Discrimination.

One-way RM ANOVA demonstrated a significant effect of alcohol dose on discrimination score for both intra-insular hM4Di-DREADD [$F(3,30)=4.80$, $p=0.008$; **Table 3.1**] and Control-mCherry [$F(3,21)=7.65$, $p=0.004$; **Table 3.1**] groups. An increase in discrimination score was observed at the training dose (1 g/kg; $p\leq0.007$) and the highest dose (1.7 g/kg; $p\leq0.05$) relative to the lowest dose (0.1 g/kg) for both intra-insular hM4Di-DREADD and Control-mCherry groups, demonstrating appropriate alcohol stimulus control. No change in locomotor rate was observed for hM4Di-DREADD and Control-mCherry groups.

Experiment 3.3: Examination of the functional role of the IC and IC→AcbC on the alcohol drug state utilizing operant alcohol discrimination procedure.

IC silencing.

Bilateral hM4Di-mCherry expression is represented in **Figure 3.4b**. Four rats died prior to completion of testing and 1 rat had an inefficient hM4Di-DREADD infusion (i.e., no hM4Di-mCherry expression). These rats are not shown in **Figure 3.4** and are not included in any analyses. Baseline discrimination performance (i.e., 2 sessions prior to initiation of testing) is shown to the left of the x-axis break (**Fig. 3.4c and e**) as a visual reference (i.e., not included in the overall analyses). In rats expressing hM4Di-DREADDs in the IC, CNO treatment significantly increased alcohol-appropriate responding, as the two-way RM ANOVA showed a significant main effect of CNO treatment [$F(1,5)=6.56$, $p=0.05$; **Fig. 3.4c**], indicating potentiation of the discriminative stimulus effects of alcohol. There was also a significant main effect of alcohol dose [$F(2,5)=32.06$, $p<0.001$; **Fig. 3.4c**], with an increase in alcohol-appropriate responding as the alcohol dose increased, demonstrating appropriate discriminative stimulus control. Following CNO, partial substitution (>40%) for the alcohol training dose was observed at the lowest alcohol dose (0.1 g/kg) tested. No change in response rate was observed (**Fig. 2d**).

In the CNO-Control group, the two-way RM ANOVA showed a significant main effect of alcohol dose on alcohol-appropriate responses [$F(2,6)=111.51$, $p<0.001$; **Fig. 3.4e**], confirming discriminative stimulus control, and no effect of CNO treatment. CNO also did not affect response rate (**Fig. 3.4f**).

IC→AcbC silencing.

For the hM4Di-DREADD group, bilateral AcbC injector tip placements (red circles) and bilateral hM4Di-mCherry expression are represented in **Figure 3.5b** and **Figure 3.5c**, respectively. One rat died prior to completion of testing and is not shown in **Figure 3** nor included in the analyses.

Baseline discrimination performance is shown to the left of the x-axis break (**Fig. 3d and 3g**) as a visual reference (i.e., not included in overall analyses). In rats expressing hM4Di-DREADDs in the IC, intra-AcbC CNO treatment significantly increased alcohol-appropriate responding, as the two-way RM ANOVA showed a significant main effect of CNO treatment [$F(1,4)=7.88$, $p<0.05$; **Fig. 3.5d**], indicating potentiation of the discriminative stimulus effects of alcohol following IC→AcbC silencing. There was also a significant main effect of alcohol dose [$F(2,4)=21.69$, $p<0.001$; **Fig. 3.5d**], with an increase in alcohol-appropriate responding as the alcohol dose increased, demonstrating appropriate discriminative stimulus control. Additionally, the two-way RM ANOVA revealed a significant interaction between alcohol dose and CNO treatment [$F(2,8)=8.98$, $p=0.009$; **Fig. 3.5d**]. That is with increasing alcohol dose, alcohol-appropriate responses differed significantly between CNO and vehicle conditions. Indeed, following intra-AcbC CNO, partial substitution (>40%) for the alcohol training dose was observed at the lower alcohol doses (0.3 and 0.5 g/kg; $p\leq0.004$) tested. No effect on response rate was observed (**Fig. 3.5e**).

In the Control-mCherry group, bilateral AcbC injector placements (blue circles) and bilateral Control-mCherry expression are represented in **Figure 3.5b** and **Figure 3.5f**, respectively. Three rats died prior to completion of testing and are not shown in **Figure 3** nor included in the analyses. Two-way RM ANOVA showed a significant main effect of alcohol dose [$F(2,2)=6.72$, $p\leq0.05$; **Fig. 3.5g**], confirming discriminative stimulus control, and no effect on intra-AcbC CNO treatment (**Fig. 3.5g**). CNO also did not affect response rate (**Fig. 3h**).

Experiment 3.4. Examination of the functional role of the IC on the alcohol drug state utilizing a Pavlovian alcohol discrimination procedure.

IC silencing.

Bilateral hM4Di-mCherry expression is represented in **Figure 3.6b**. Eight rats died prior to completion of testing and four rats had inefficient hM4Di-DREADD infusions (i.e., no hM4Di-

mCherry expression or unilateral expression likely due to a clogged injector), and are not shown nor included in **Figure 3.6b and d**. However, the 2 rats showing no hM4Di-DREADDs were included in the Control-mCherry group analyses (**Fig. 3.6 f-g**). Baseline discrimination performance (i.e., 2 sessions prior to initiation of testing) is shown to the left of the x-axis break (**Fig. 3.6c and 3.6f**) as a visual reference (i.e., not included in the overall analyses). In rats expressing hM4Di-DREADDs in the IC, CNO treatment significantly increased the discrimination score (i.e., head entries during the 15-s light CS minus head entries 15 s before light onset), as the two-way RM ANOVA showed a significant main effect of CNO treatment [$F(1,5)=18.23$, $p=0.008$; **Fig. 3.6c**], indicating potentiation of the interoceptive effects of alcohol. There was also a significant main effect of alcohol dose [$F(2,5)=33.25$, $p=0.006$; **Fig. 3.6c**], with an increase in the discrimination score as the alcohol dose increased, demonstrating appropriate alcohol stimulus control. Following CNO, full substitution for the alcohol training dose at water and the lowest alcohol dose (0.1 g/kg) was observed. Analysis of locomotor activity revealed a main effect of alcohol dose [$F(2,5)=4.80$, $p<0.04$; **Fig. 3.6d**], as locomotor activity increased with increasing alcohol dose.

Bilateral Control-mCherry expression is represented in **Figure 3.6e** and two-way RM ANOVA showed a significant main effect of alcohol dose on the discrimination score [$F(2,7)=13.08$, $p<0.001$; **Fig. 3.6f**], confirming appropriate alcohol stimulus control and no effect on CNO treatment (**Fig. 3.6f**). Analysis of locomotor activity revealed a trend ($p<0.056$) but no main effect of alcohol dose (**Fig. 3.6g**).

IC→AcbC silencing.

For the hM4Di-DREADD group, bilateral AcbC injector placements (red circles) and bilateral hM4Di-mCherry expression are represented in **Figure 3.7b** and **Figure 3.7c**, respectively. Baseline discrimination performance is shown to the left of the x-axis break (**Fig. 3.7d** and **3.7g**) as a visual reference (i.e., not included in overall analyses). In rats expressing hM4Di-DREADDs in the IC, intra-AcbC CNO treatment significantly increased the discrimination score, as the two-way RM

ANOVA demonstrated a significant main effect of CNO treatment [$F(1,5)=16.36$, $p=0.01$; **Fig. 3.7d**]. There was also a main effect of alcohol dose [$F(2,5)=8.01$, $p=0.008$; **Fig. 3.7d**], with an increase in the discrimination score as the alcohol dose increased, demonstrating appropriate alcohol stimulus control. Following intra-AcbC CNO, full substitution for the alcohol training dose at water and the lowest alcohol dose (0.1 g/kg) was observed. No effect on locomotor rate was observed (**Fig. 3.7e**).

In the Control-mCherry group, bilateral AcbC injector placements (blue circles) and bilateral Control-mCherry expression are represented in **Figure 3.7b** and **Figure 3.7f**, respectively. One rat died prior to completion of testing and is not shown in Figure 5 and is not included in the analyses. Two-way RM ANOVA showed a significant main effect of alcohol dose [$F(2,6)=27.53$, $p<0.001$; **Fig. 3.7g**], confirming appropriate alcohol stimulus control, and no effect on intra-AcbC CNO treatment (**Fig. 3.7g**). CNO also did not affect locomotor rate (**Fig. 3.7h**).

Experiment 3.5: Examination of the functional role of the Rh and Rh→AcbC on the alcohol drug state utilizing operant alcohol discrimination procedure.

Rh silencing.

hM4Di-mCherry expression is represented in **Figure 3.8b**. Two rats died prior to completion of testing and two rats had inaccurate placements or inefficient hM4Di-DREADD infusions (i.e., no hM4Di-mCherry expression), and are not shown nor included in **Figure 3.8**. Baseline discrimination performance (i.e., 2 sessions prior to initiation of testing) is shown to the left of the x-axis break (**Fig. 3.8c**) as a visual reference (i.e., not included in the overall analyses). In rats expressing hM4Di-DREADDs in the Rh, CNO treatment significantly increased alcohol-appropriate responding, as the two-way RM ANOVA showed a significant main effect of CNO treatment [$F(1,7)=16.56$, $p=0.005$; **Fig. 3.8c**], indicating potentiation of the discriminative stimulus effects of alcohol. There was also a significant main effect of alcohol dose [$F(2,7)=78.00$, $p<0.001$; **Fig. 3.8c**], with an increase in alcohol-appropriate responding as the alcohol dose increased, demonstrating appropriate

discriminative stimulus control. Additionally, the two-way RM ANOVA revealed a significant interaction between alcohol dose and CNO treatment [$F(2,7)=6.29$, $p=0.01$; **Fig. 3.8c**]. That is with increasing alcohol dose, alcohol-appropriate responses differed significantly between CNO and vehicle conditions. Indeed, following intra-AcbC CNO, partial substitution (>40%) for the alcohol training dose was observed at the lower alcohol dose (0.3 g/kg; $p\leq0.001$) tested. No effect on response rate was observed (**Fig. 3.8e**).

Rh→AcbC silencing.

For the hM4Di-DREADD group, bilateral AcbC injector tip placements and hM4Di-mCherry expression are represented in **Figure 3.9b** and **Figure 3.9c**, respectively. Four rats died prior to completion of testing and to completion of testing and two rats had an inefficient hM4Di-DREADD infusion (i.e., no hM4Di-mCherry expression), and are not shown or included in **Figure 3.9**. Baseline discrimination performance is shown to the left of the x-axis break (**Fig. 3.9d**) as a visual reference (i.e., not included in overall analyses). In rats expressing hM4Di-DREADDs in the Rh, intra-AcbC CNO treatment did not significantly affect alcohol-appropriate responding (albeit a trend, p=0.065). There was a significant main effect of alcohol dose [$F(2,4)=7.66$, $p<0.01$; **Fig. 3.9d**], with an increase in alcohol-appropriate responding as the alcohol dose increased, demonstrating appropriate discriminative stimulus control. However, the two-way RM ANOVA revealed a significant interaction between alcohol dose and CNO treatment [$F(2,8)=8.44$, $p=0.01$; **Fig. 3.9d**]. That is with increasing alcohol dose, alcohol-appropriate responses differed significantly between CNO and vehicle conditions. Indeed, following intra-AcbC CNO, partial substitution (>40%) for the alcohol training dose was observed at the lower alcohol doses (0.3 and 0.5 g/kg; $p\leq0.004$) tested.. No effect on response rate was observed (**Fig. 3.9e**).

Experiment 3.6. Examination of the functional role of the Rh→ and Rh→AcbC on the discriminative stimulus effect of self-administered alcohol

Addition of alcohol (10%, v/v) to the sucrose (10%, w/v) reinforcer resulted in an increase in alcohol-appropriate responding across the session (**Fig. 3.10b**), indicating that behavior was under discriminative stimulus control of the consumed alcohol. Under vehicle conditions, almost full substitution for the alcohol training dose was observed 20 min into the session. In contrast, following CNO (3 mg/kg [IP] and 9μM [intra-AcbC]) almost full substitution was observed 10 min into the session. This was confirmed by a significant main effect of CNO [$F(2,30)=7.20$, $p\leq0.04$], significant main effect of time [$F(5,30)= 29.69$, $p < 0.001$], and a significant interaction [$F(10,30) = 2.71$, $p\leq0.02$]. CNO (3 mg/kg and 9μM) significantly increased alcohol-appropriate responses during the first 10 and 15 min ($p < 0.05$) indicating potentiated sensitivity to alcohol early in the session (**Fig. 3.10b**). Importantly, CNO did not affect response rate (**Fig. 3.10a** *inset*) alcohol intake (g/kg; **Fig. 3.10c**). Alcohol intake (g/kg) increased across time [$F(5,30) = 203.83$, $p \leq0.001$]. Thus, the potentiation of the discriminative stimulus effects of alcohol within the first 10-15 min of the session following CNO (**Fig. 3.10b**), was directly related to increased sensitivity to the discriminative stimulus effects of the consumed alcohol and not to differences in the alcohol dose consumed. These findings support and extend the findings in Experiment 3.5 with experimenter-administered alcohol, to show that silencing the Rh and Rh→AcbC, by CNO also potentiates sensitivity to the interoceptive effects of consumed/self-administered alcohol.

DISCUSSION

The present findings demonstrate the IC and specifically its projections to the AcbC are important for the expression of the interoceptive effects of alcohol. Here we show IC and IC→AcbC projections respond to alcohol, as measured by c-Fos IR suggesting this insular-striatal circuit is a

target for alcohol action. Indeed, by chemogenetically silencing the IC or IC→AcbC projections, in rats trained on two alcohol discrimination tasks (i.e., the two-lever operant or Pavlovian task), we show increased sensitivity to the interoceptive effects of alcohol. Additionally, the present findings demonstrate that chemogenetic silencing of Rh or Rh→AcbC projections also increase sensitivity to the discriminative stimulus effects of experimenter and self-administered alcohol, in rats trained on two-lever operant alcohol discrimination task. Thus these findings further implicate a central role for the insula/thalamic-striatal circuitry in modulating the discriminative stimulus effects of alcohol.

In humans, brain imaging studies show IC activity in response to alcohol and alcohol-related cues (Droutman *et al*, 2015; Jasinska *et al*, 2014; Naqvi and Bechara, 2010). Alternatively, preclinical studies have analyzed c-Fos expression patterns to measure IC response to alcohol (Jaramillo *et al*, 2016; Ryabinin *et al*, 1997). Previous work has shown an increase in c-Fos IR following alcohol (1.5 g/kg, IP) in alcohol-experienced and alcohol-naïve rats (Ryabinin *et al*, 1997). Consistent with those findings, here we show an increase in c-Fos expression following alcohol (1 g/kg, IG) in alcohol-naïve rats. Additionally, by utilizing the FG retrograde tracer infused into the AcbC (Experiment 3.1), we found an alcohol-induced increase in c-Fos expression within IC→AcbC projections. These results demonstrate alcohol-induced molecular activity within this insular-striatal circuit and implicate the IC→AcbC circuit as a site of action for alcohol. Our previous work identified recruitment of the IC and Rh in rats performing an operant alcohol discrimination, as an alcohol-induced decrease in c-Fos IR was observed in the IC and Rh following a discrimination test session (Jaramillo *et al*, 2016). While this finding is in contrast to the alcohol-induced increase in the IC and lack of effect in the Rh of the present work, it is important to consider that the previous work examined c-Fos response following ongoing discrimination behavior. Further, in that study, confirmation of IC and Rh involvement was determined, as pharmacological inactivation ($GABA_A$ + $GABA_B$ agonist cocktail) of the IC or Rh partially substituted for the interoceptive effects of alcohol (1 g/kg, IG; Jaramillo *et al*, 2016). Here, we confirm and extend those findings by demonstrating potentiated sensitivity to the interoceptive effects of low alcohol doses following chemogenetic

silencing of the IC, Rh, or specific silencing of IC /Rh projections to the AcbC, in rats trained on operant alcohol discrimination. Furthermore, chemogenetic silencing of IC or IC→AcbC projections fully substituted for the interoceptive effects of alcohol, in the Pavlovian alcohol discrimination procedure. Additionally despite the proposed role of the Rh in modulating behavioral flexibility, silencing of the Rh or Rh→AcbC did not disrupt behavioral flexibility under the control of the discriminative stimulus effects of alcohol, rather confirming the potentiated sensitivity to alcohol, even with the small sample size. Thus, for the first time we demonstrate a functional role for IC→AcbC and Rh→AcbC circuit in modulating the discriminative stimulus effects of a drug of abuse.

Drug discrimination procedures have been used to identify several receptor systems that modulate the discriminative stimulus effects of alcohol ($GABA_A$, NMDA, serotonin, opioid, mGluRs (Besheer *et al*, 2009; Grant, 1999; Kostowski and Bienkowski, 1999). Therefore, while the present study identified two striatal circuits that are, in part, recruited in the expression of the interoceptive effects of alcohol, it will be important for future work to identify the receptor mechanism(s) within both IC→ and Rh→AcbC circuit. Based on the extensive alcohol discrimination literature, pharmacological manipulations that inhibit the CNS (e.g., GABA agonists, NMDA antagonists) tend to produce "alcohol-like" effects (Grant, 1999; Kostowski and Bienkowski, 1999) and these effects likely involve inhibition in the AcbC (Besheer *et al*, 2009; Hodge and Cox, 1998; Jaramillo *et al*, 2016). Given the presumed glutamatergic projections from the IC→AcbC (Leong *et al*, 2016; Seif *et al*, 2013), and Rh→AcbC (Ohtake and Yamada, 1989)we posit that chemogenetic silencing of these projections following activation of the $G_{i/o}$ DREADDs likely results in inhibition of the AcbC. Consistent with this suggestion are previous findings showing that GABAergic activation in the AcbC produces alcohol-like effects (Besheer *et al*, 2003; Hodge and Cox, 1998; Hodge *et al*, 2001b). However, given that silencing of both IC→ or Rh→AcbC produce alcohol-like effects, it is also possible that general silencing of glutamatergic afferents to the AcbC produce alcohol-like effects. Thus, future experiments would need to directly test the specific role of IC and Rh afferents to the

AcbC. Furthermore as evident from IC and Rh silencing (following systemic CNO injection), other outgoing projections likely contribute to the increase in sensitivity to alcohol and this will be important for future work to investigate (e.g., projections to hippocampus, mPFC). Furthermore, given the current findings demonstrating increased sensitivity to alcohol following Rh and Rh→AcbC silencing, despite the absence of alcohol-induced effects on c-Fos IR within the Rh→AcbC, the shared reciprocal projections between the IC and Rh are of particular interest (Mufson and Mesulam, 1984; Vertes *et al*, 2006). Therefore, together with the c-Fos data, our findings suggest that alcohol has effects on this IC→AcbC and Rh→AcbC circuit and these circuits, in part, are important for the expression of the interoceptive effects of alcohol.

Although increased sensitivity to alcohol following silencing of the IC and IC→AcbC projections was observed under both discrimination training conditions, full substitution for alcohol was only observed in the Pavlovian-trained group. That is, under the Pavlovian discrimination training conditions, silencing of the IC and IC→AcbC projections potentiated the effects of the low alcohol dose (0.1 g/kg) resulting in full substitution for the 1 g/kg training dose an effect that was also observed in the absence of alcohol (i.e., following water, IG). In contrast, under the operant discrimination training conditions, silencing of the IC resulted in partial substitution for the alcohol training at the 0.1 g/kg dose (approximately 40% alcohol-appropriate responses), and silencing of the IC→AcbC projections resulted in partial substitution at the 0.3 and 0.5 g/kg alcohol doses (approximately 60% and 75% alcohol-appropriate responding, respectively). These data suggest that the Pavlovian discrimination procedure may be a more sensitive tool for detection of low drug doses than the operant procedures, which is consistent with previous suggestions (Besheer *et al*, 2012b; Palmatier *et al*, 2005; Randall *et al*, 2016), and is likely related to the different response costs and distinct behavioral outputs (i.e., lever response, goal-tracking) of the procedures (see (Besheer *et al*, 2012b; Palmatier *et al*, 2005). This was also a reason why a lower intra-AcbC CNO dose was used in the Pavlovian-trained group, as pilot studies showed general decreases in behavior at higher doses. Moreover, by definition the two discrimination-training procedures are inherently different and thus,

alcohol and CNO doses were selected in accordance with each procedure. However, parallel examination of the discrimination behavior in both procedures allows for a point of comparison and identification of potential overlap in mechanism/neural circuitry. To this end, these data suggest overlap, but also, the possibility of the recruitment of different IC projections and/or neuromodulator systems by the different procedures.

Previous work has demonstrated involvement of the IC in taste processing (albeit gustatory cortex (granular; (Maffei *et al*, 2012), consummatory feeding, and the processing of anticipatory food cues (Kusumoto-Yoshida *et al*, 2015). Therefore, consideration of these appetitive and cognitive processes is important for the interpretation of the outcome of the present studies. In the operant discrimination procedure, silencing of the IC or IC→AcbC projections did not alter response rates and locomotor activity was unchanged in the Pavlovian discrimination group, confirming that changes in discrimination performance were not due to nonspecific changes in motor output, or motivation to respond for the sucrose reinforcer. This latter point also suggests that there was likely no change in sucrose palatability. However, it is also important to note that for the tests in both discrimination procedures, the primary dependent measure of alcohol sensitivity (alcohol-appropriate responses or discrimination score) is determined prior to sucrose delivery, which makes an explanation based on altered taste or consummatory behavior less likely. That is for the operant discrimination, alcohol-appropriate responses are calculated upon completion of the first FR10 and for the Pavlovian procedure the discrimination score is calculated for the single light presentation (prior to sucrose presentation). Next, consideration of the known role of the IC in processing external cues associated with food or drugs (Cosme *et al*, 2015; Li *et al*, 2013; Ma *et al*, 2014; Wu *et al*, 2014) but see (Kusumoto-Yoshida *et al*, 2015; Wu *et al*, 2016) is important. For example, optogenetic silencing of the IC decreases cue (tone and light)-triggered food seeking (i.e., goal-tracking) behavior (Kusumoto-Yoshida *et al*, 2015), and pharmacological inhibition of the IC decreases drug reinstatement or drug self-administration (Cosme *et al*, 2015; Droutman *et al*, 2015; Hamlin *et al*, 2007; Kufahl *et al*, 2009; Kutlu *et al*, 2013; Pushparaj and Le Foll, 2015; Wu *et al*, 2014). Therefore,

the behavioral findings (i.e., after chemogenetic silencing of the IC/Rh or the IC/Rh→AcbC projections) of a specific increase in alcohol-appropriate responding and lack of change in response rate (in the operant task), and an increase in goal-tracking following the light cue with no change in locomotor activity (IC silencing in the Pavlovian task), are likely not related to a deficit in cue processing and are specific to the drug state.

Next, it is possible that IC/Rh or IC/Rh→AcbC silencing impaired decision-making processes, as a role for the Rh in modulating working-memory particularly with reference to spatial context (Cholvin *et al*, 2013; Hallock *et al*, 2013; Hembrook and Mair, 2011; Layfield *et al*, 2015; Prasad *et al*, 2016) has been implicated. Furthermore, the IC has been implicated in altered decision-making in methamphetamine-treated rats (Mizoguchi *et al*, 2015), and reduced IC activity is observed during cognitive control tasks in drug-dependent populations (Paulus and Stewart, 2014). Therefore, it is possible that pharmacological inactivation of these regions may induce memory impairments. Indeed, in the two-lever operant discrimination task, 50% responding on either lever may suggest such impairment. However, discrimination performance following chemogenetic silencing was either below or above this level. Additionally, in the Pavlovian procedure, discrimination scores were at levels comparable to the training condition, again making an argument of impaired decision making less tenable. However, (Seif *et al*, 2013) demonstrated increased NMDAR function in the IC→AcbC circuit attributed to compulsive drinking, demonstrating the circuit as a site of alcohol action after extensive alcohol history. Additionally, imaging studies show GABAergic concentration within the IC is correlated with neural response to interoceptive stimuli (Wiebking *et al*, 2014) and dysregulation in body awareness is often found in drug-dependent individuals (Paulus and Stewart, 2014). Therefore, it will be relevant for future discrimination studies to investigate the role of IC→AcbC in modulating the interoceptive effects of higher alcohol doses (i.e., >1.0 g/kg), as it is possible that this circuit may be recruited to a greater (or lesser) degree at higher alcohol doses and following a history of exposure to higher alcohol doses.

An important feature of the present work is the incorporation of the CNO-treated controls. That is, the lack of modulatory control of discrimination behavior by the non-DREADD injected controls (i.e., CNO-Controls) and CNO in the Control-mCherrys (i.e., intra-IC) throughout the studies allows us to conclude that the CNO doses used (systemically and intra-AcbC) do not have alcohol-like effects and do not potentiate the effects of alcohol. These doses also did not affect general response rate or locomotor behavior. These are important findings as CNO, a major metabolite of the anti-psychotic drug clozapine, may have biological effects (perhaps due to retroconversion to clozapine or N-desmethylclozapine) which are likely to depend on animal, strain, and CNO dose (Chang *et al*, 1998; Jann *et al*, 1994; MacLaren *et al*, 2016). For example, in Long Evans rats 30 min following administration of CNO (5 mg/kg, IP), clozapine levels were detected in the plasma, albeit at a tenth of the CNO levels at that time point and diminished across the 360 min of the assessment (MacLaren *et al*, 2016). Interestingly, clozapine can serve as a discriminative stimulus and selectively guide behavior as demonstrated by drug discrimination experiments (Goudie *et al*, 1998; Prus *et al*, 2016). Further, to our knowledge overlap between clozapine and alcohol discriminative stimulus effects have not been demonstrated. Additionally, clozapine has been shown to decrease alcohol-stimulated activity (Thrasher *et al*, 1999), an effect absent in our CNO-manipulations (Fig. 4d). Thus, the inclusion of these CNO-only control groups and the absence of behavioral effects within the context of this study are highly relevant.

Together these data identify a role for the IC/Rh→AcbC circuit in modulating behavior driven by an alcohol drug state, which to date has been unstudied. Here, we demonstrate the ability to change behavior that is under the control of an alcohol-interoceptive state through chemogenetic silencing of IC/Rh→AcbC circuit. That is, silencing of the insular-striatal circuit potentiates and produces alcohol-like effects. While silencing the thalamic-striatal circuit potentiates sensitivity to the interoceptive effects of self-administered alcohol. Together with the previous data, these findings inform us of the complex IC and Rh structure while providing evidence of the critical nature of striatal circuitry in underlying drug states and interoceptive sensitivity. Furthermore, the

insular/thalamic-striatal circuit may account for modulation of internal drug states and thus directly affect drug-induced behavioral changes (i.e., drug-seeking and -taking).

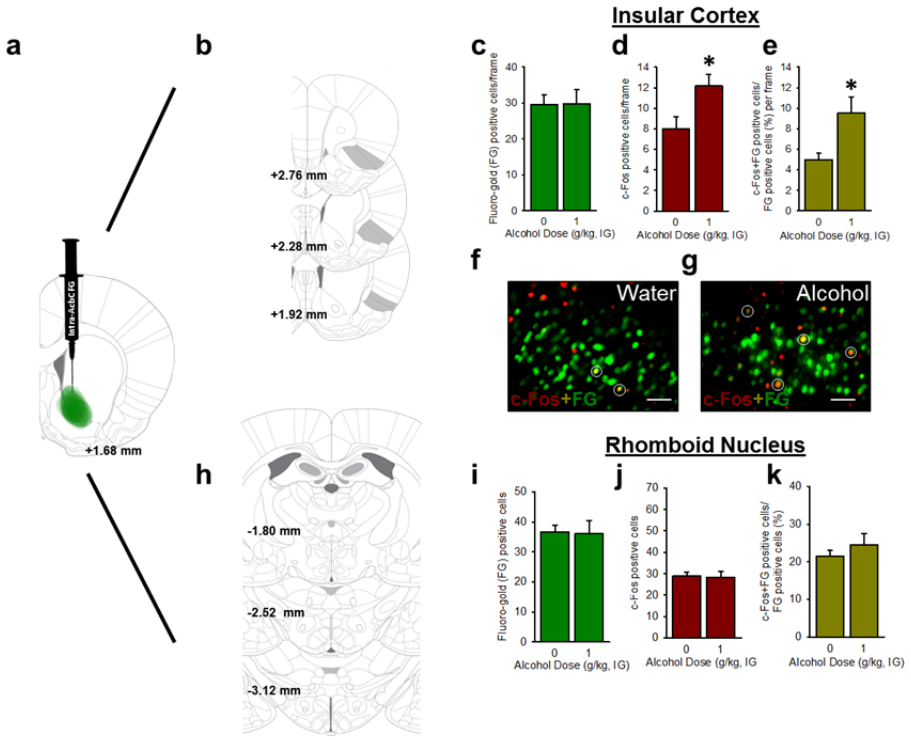

Figure 3.1- Co-expression of FG and c-Fos to identify neuronal response to alcohol in IC and Rh afferent projections to the AcbC.

(**a**) Representative diagram demonstrating individual FG expression following intra-AcbC infusion of FG in behaviorally naïve rats, with (**b**) insets representing the region of analysis, the insular cortex (IC) and rhomboid nucleus (Rh; **h**). (**c**) No difference in mean (± S.E.M) number of FG positive cells in the IC following water or alcohol (1 g/kg) administration (IG). **(d)** Increased number of c-Fos positive cells and (**e**) FG+c-Fos positive cells in IC following alcohol. (**f-g**) Photomicrographs (20X; 100 µm scale bar) of c-Fos (red) and FG (green) co-localization (yellow, marked by white circles) in the IC after (**f**) water and (**g**) alcohol (n=6-7/group). (**j**) No difference in mean (± S.E.M) number of FG positive cells, (**k**) c-Fos positive cells, (**j**) and FG+c-Fos positive cells in the Rh following water or alcohol (1 g/kg) administration (IG).

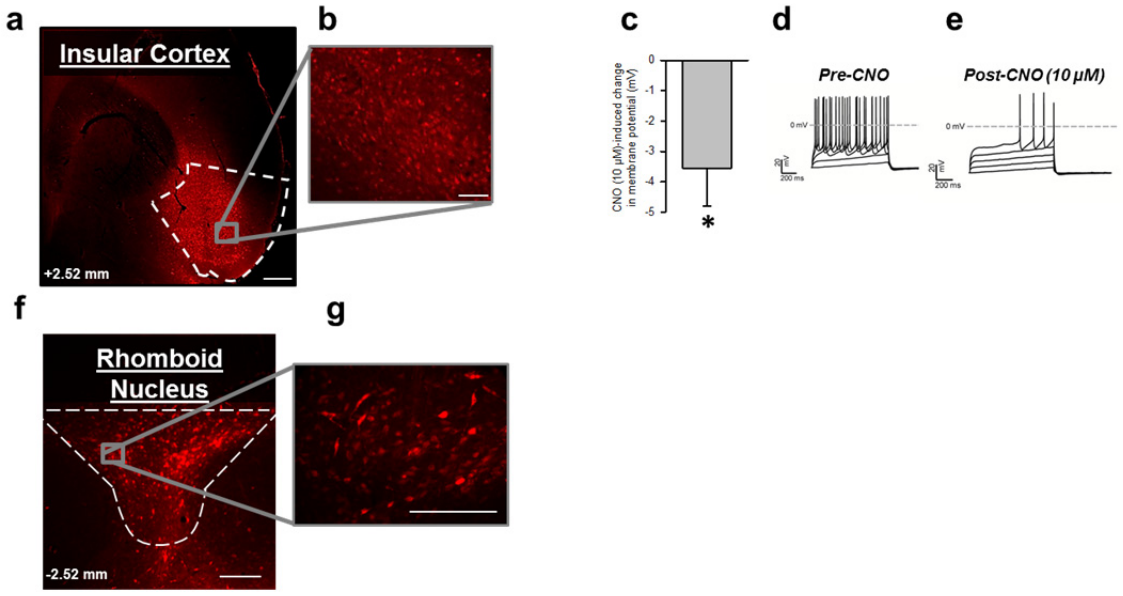

Figure 3.2- hM4Di-DREADDs validation for chemogenetic silencing of IC.

(**a**) Representative image of m-Cherry immunofluorescence in IC (2X, 2 mm scale bar) and (**b**) IC neurons (10X, 100 µm scale bar) following stereotaxic injection of AAV-hSyn-DIO-hM4Di-mCherry+Cre into IC. (**c**) Decreased membrane potential in IC neurons (n=8 neurons from 6 rats), (**d**) demonstrated by representative traces of neuronal firing and (**e**) neuronal silencing following bath application of clozapine-n-oxide (CNO; 10 µM). (**f**) Representative image of m-Cherry immunofluorescence in Rh (10X, 100 µm scale bar) and (**g**) Rh neurons (100X, 100 µm scale bar) following stereotaxic injection of AAV-hSyn-DIO-hM4Di-mCherry+Cre into Rh. *Significant difference from 0, (*t*-test, $p<0.05$). Values on graphs represent mean ± S.E.M.

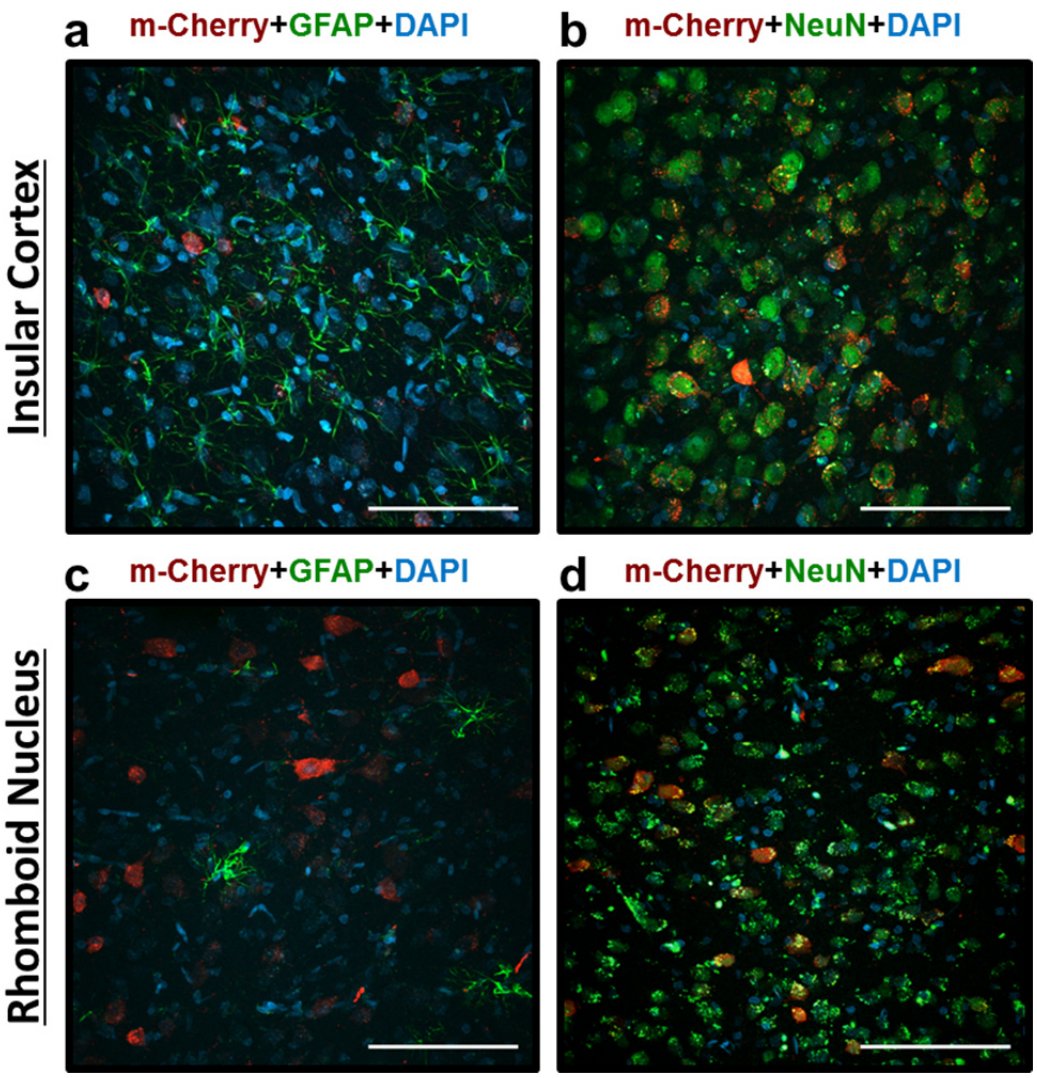

Figure 3.3- Validation of hM4Di-DREADDs expression in neurons.

Representative images of m-Cherry immunofluorescence in IC following stereotaxic injection of AAV-hSyn-DIO-hM4Di-mCherry+Cre into the IC, (**a**) demonstrating no colocalization with the glial marker GFAP but (**b**) dense colocalization with the neuronal marker NeuN. Representative images of m-Cherry immunofluorescence in Rh following stereotaxic injection of AAV-hSyn-DIO-hM4Di-mCherry+Cre into the Rh, (**a**) demonstrating no colocalization with the glial marker GFAP but (**b**) dense colocalization with the neuronal marker NeuN. (40X, 100 μm scale bar)

	Alcohol-appropriate Responses (%) Cumulative Alcohol Dose (g/kg, IG)				Response Rate (resp/min) Cumulative Alcohol Dose (g/kg, IG)			
Operant Discrim.	**0.1**	**0.3**	**1.0**	**1.7**	**0.1**	**0.3**	**1.0**	**1.7**
Intra-Rhomboid								
hM4Di	8.6±3.3	13.6±6.9	93.7±2.2*	96.6±1.6*	46.6±4.1	47.4±3.8	45.5±4.4	42.0±4.7
Intra-Insula								
hM4Di	5.3±2.5	17.7±8.7	90.1±6.6*	99.1±0.9*	51.7±6.1	51.9±7.3	46.9±4.2	37.1±6.2*
CNO-Controls	10.7±3.1	4.8±2.1	73.0±12.7*	97.8±2.0*	50.6±6.2	45.4±5.7	48.0±5.1	41.5±6.1

	Discrimination Score (head entries during CS-PreCS) Cumulative Alcohol Dose (g/kg, IG)				Locomotor Rate (beam breaks/min) Cumulative Alcohol Dose (g/kg, IG)			
Pavlovian Discrim.	**0.1**	**0.3**	**1.0**	**1.7**	**0.1**	**0.3**	**1.0**	**1.7**
Intra-Insula								
hM4D(G_i)	1.3±0.7	2.6±0.8	4.9±0.9*	3.9 ±0.8*	50.1±5.5	60.3±4.9	66.6±13.2	55.3±15.3
Controls	1.5±0.4	2.7±0.4	6.4±1.0*	5.3±1.2*	50.5±6.2	46.5±5.4	43.9±7.1	48.7±7.5

*Significant difference from lowest alcohol dose (0.1 g/kg; $p<0.05$).

Table 3.1- Performance during the initial cumulative alcohol discrimination test to confirm discrimination control (mean ± S.E.M.).

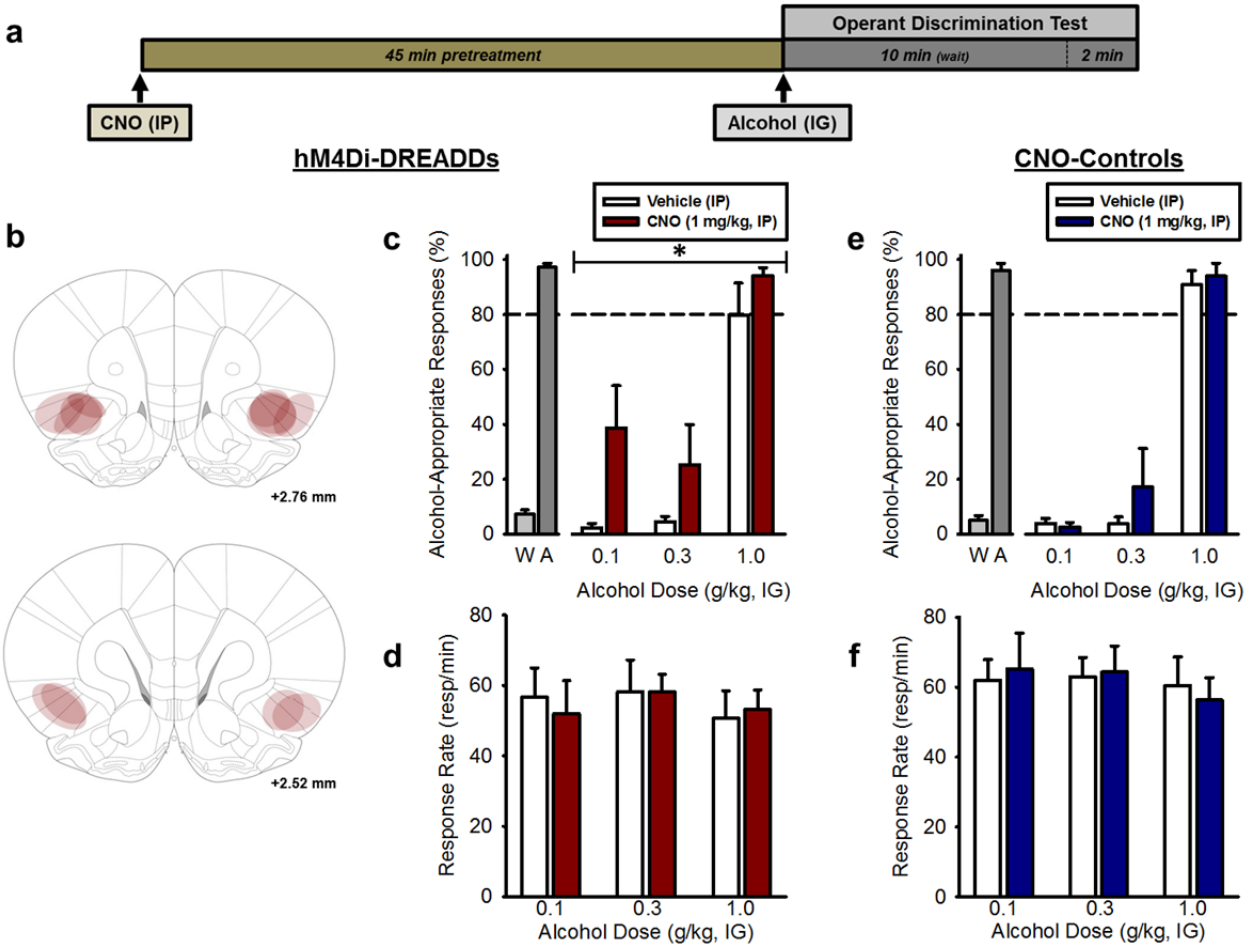

Figure 3.4- Chemogenetic silencing of IC increases sensitivity to the interoceptive effects of alcohol in an operant alcohol discrimination task.

(**a**) Schematic diagram of test session. (**b**) Bilateral intra-IC hM4Di-mCherry expression from individual discrimination-trained rats. (**c**) Silencing of IC, by CNO, increased the percentage of alcohol-appropriate responses in hM4Di-DREADD group (n=6). (**d**) CNO did not affect response rate (responses/min). (**e**) CNO did not affect alcohol-appropriate responses or (**f**) response rate in CNO-Control group (n=7). Dashed line (>80%) represents full expression of the discriminative stimulus effects of alcohol. Baseline discrimination performance on Water and Alcohol (1.0 g/kg, IG) training days shown to the left of x-axis break. *Significant main effect of CNO dose (two-way RM ANOVA, $p \leq 0.05$). Values on graphs represent mean ± S.E.M.

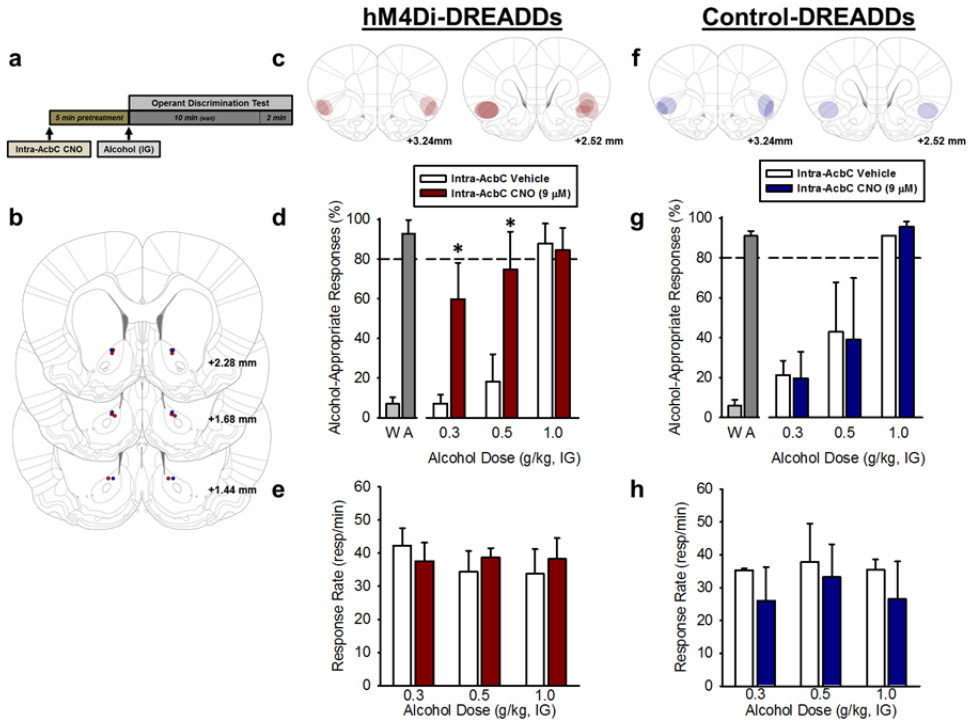

Figure 3.5- Chemogenetic silencing of IC→AcbC projections increases sensitivity to low alcohol doses in an operant alcohol discrimination task.

(**a**) Schematic diagram of test. (**b**) bilateral AcbC injector tip placements from individual discrimination-trained rats in hM4Di-DREADD (depicted as red circles) or Control-mCherry (depicted as blue circles) groups. (**c**) Bilateral intra-IC hM4Di-mCherry expression from individual discrimination-trained rats. (**d**) Infusion of CNO into AcbC increased percentage of alcohol-appropriate responses following 0.3 and 0.5 g/kg alcohol in the hM4Di-DREADD group (n=5). (**e**) Response rate (responses/min) was unaffected by CNO or alcohol dose. **(f)** Bilateral Control-mCherry expression from individual discrimination-trained rats (n=3). (**g**) Intra-AcbC infusion of CNO did not affect alcohol-appropriate responses or (**h**) response rate in the Control-mCherry group. Dashed line (>80%) represents full expression of the discriminative stimulus effects of alcohol. Baseline discrimination performance on Water and Alcohol (1.0 g/kg, IG) training days shown to the left of x-axis break. *Significant difference from Vehicle (Tukey, $p<0.05$). Values on graphs represent mean ± S.E.M.

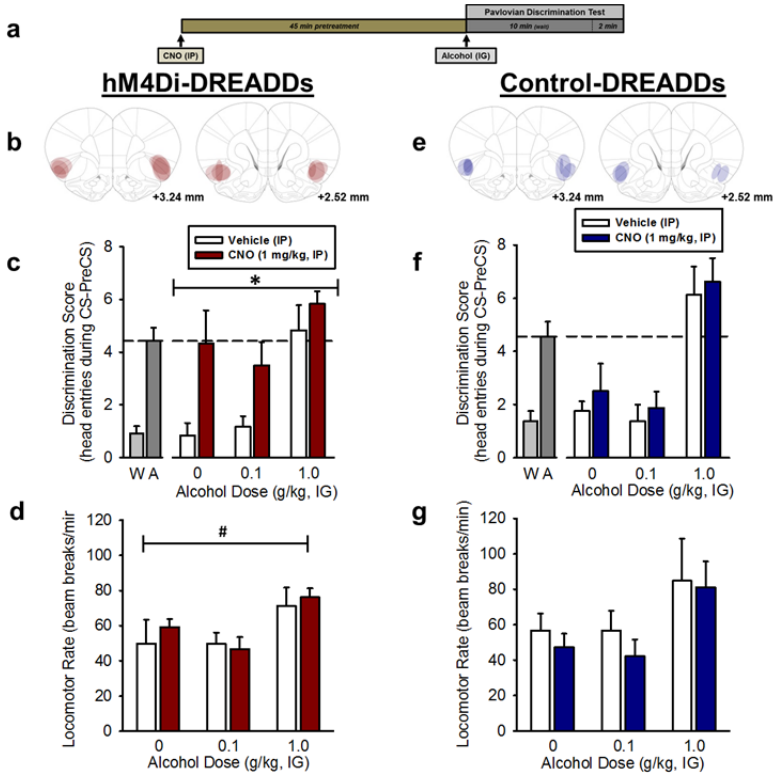

Figure 3.6- Chemogenetic silencing of IC substitutes for the interoceptive effects of alcohol in a Pavlovian alcohol discrimination task.

(**a**) Schematic diagram of test. (**b**) Bilateral intra-IC hM4Di-mCherry expression from individual discrimination-trained rats. (**c**) Silencing of IC, by CNO, increased the discrimination score (head entries into the liquid receptacle during the 15-s light CS minus head entries 15 s before light onset) in the hM4Di-DREADD group (n=6). (**d**) Locomotor rate (beam breaks/min) was unaffected. (**e**) Bilateral Control-mCherry expression from individual discrimination-trained rats. CNO did not affect (**f**) discrimination score or (**g**) locomotor rate in Control-mCherry group (n=8). Baseline discrimination performance on Water and Alcohol (1.0 g/kg, IG) training days shown to the left of x-axis break. Dashed line represents full expression of the interoceptive effects of alcohol. *Significant main effect of CNO, #Significant main effect of alcohol (two-way RM ANOVA, $p<0.05$). Values on graphs represent mean ± S.E.M.

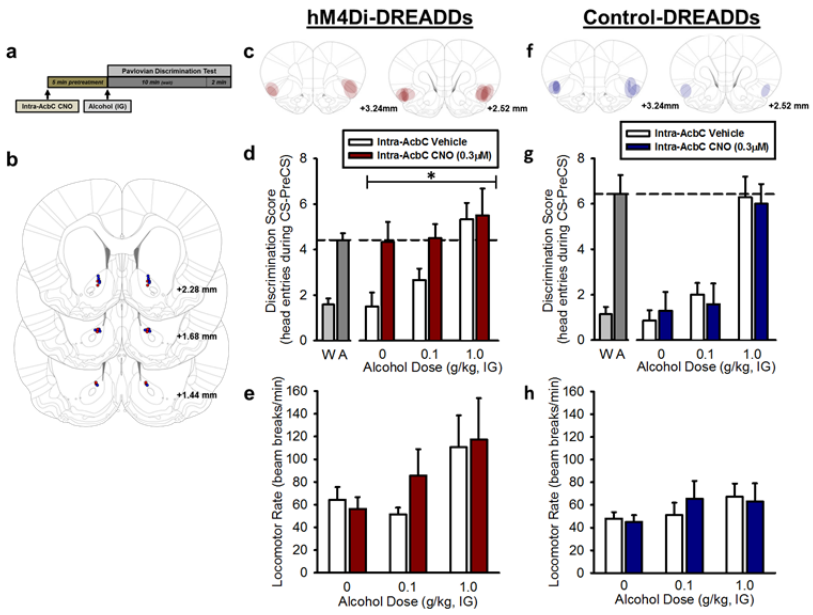

Figure 3.7- Chemogenetic silencing of IC→AcbC projections substitutes for the interoceptive stimulus effects of alcohol in a Pavlovian alcohol discrimination task.

(**a**) Schematic diagram of test. (**b**) Bilateral AcbC injector tip placements from individual discrimination-trained rats in hM4Di-DREADD (depicted as red circles) or Control-mCherry (depicted as blue circles) groups. (**c**) Bilateral intra-IC hM4Di-mCherry expression from individual discrimination-trained rats. (**d**) Infusion of CNO into AcbC increased the discrimination score (head entries into the liquid receptacle during the 15-s light CS minus head entries 15 s before light onset) in the hM4Di-DREADD group (n=6). (**e**) Locomotor rate (beam breaks/min) was unaffected. (**f**) Bilateral Control-mCherry expression from individual discrimination-trained rats. Intra-AcbC CNO did not affect (**g**) discrimination score or (**h**) locomotor rate in the Control-mCherry group (n=7). Baseline discrimination performance on Water and Alcohol (1.0 g/kg, IG) training days shown to the left of x-axis break. Dashed line represents full expression of the interoceptive effects of alcohol. *Significant main effect of CNO (two-way RM ANOVA, $p<0.05$). Values on graphs represent mean ± S.E.M.

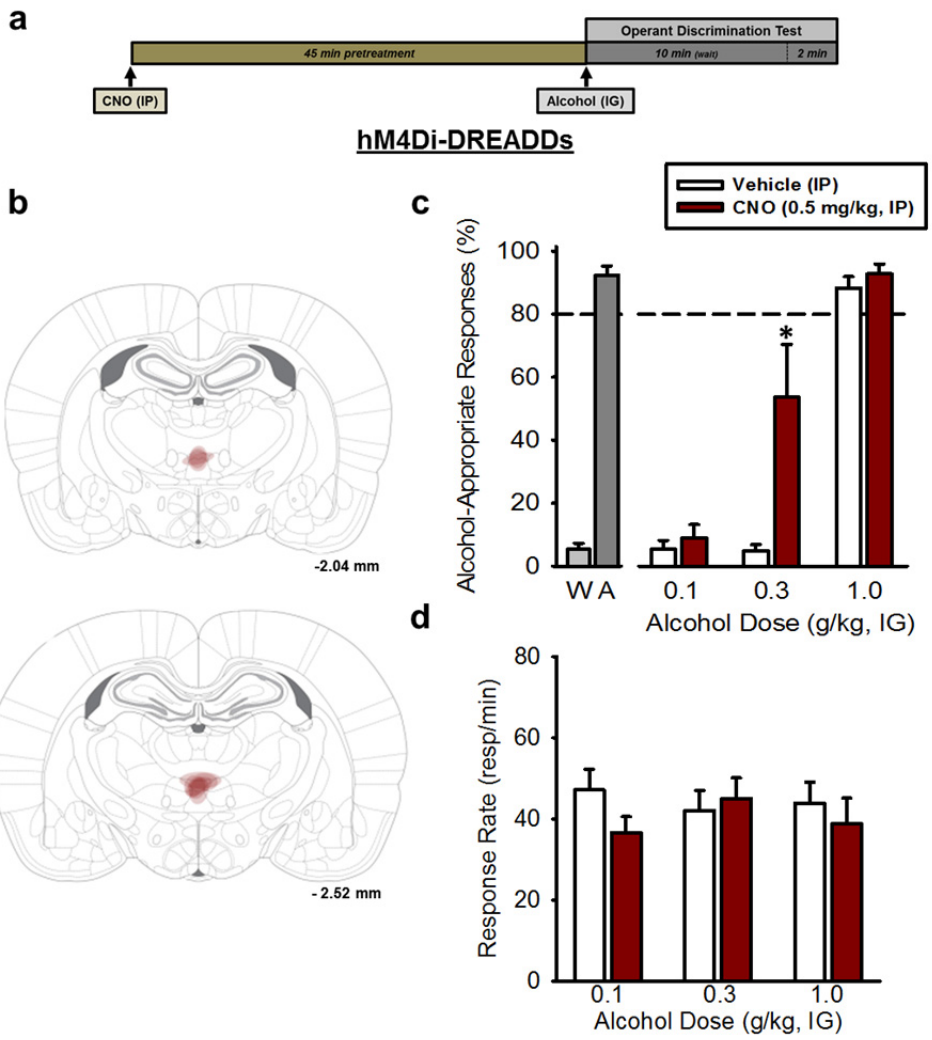

Figure 3.8- Chemogenetic silencing of Rh increases sensitivity to the interoceptive effects of a low alcohol dose in an operant alcohol discrimination task.

(**a**) Schematic diagram of test session. (**b**) Intra-Rh hM4Di-mCherry expression from individual discrimination-trained rats. (**c**) Silencing of Rh, by CNO, increased the percentage of alcohol-appropriate responses in hM4Di-DREADD group (n=8). (**d**) CNO did not affect response rate (responses/min). Dashed line (>80%) represents full expression of the discriminative stimulus effects of alcohol. Baseline discrimination performance on Water and Alcohol (1.0 g/kg, IG) training days shown to the left of x-axis break. * Significant difference from Vehicle (Tukey, $p<0.05$). Values on graphs represent mean ± S.E.M.

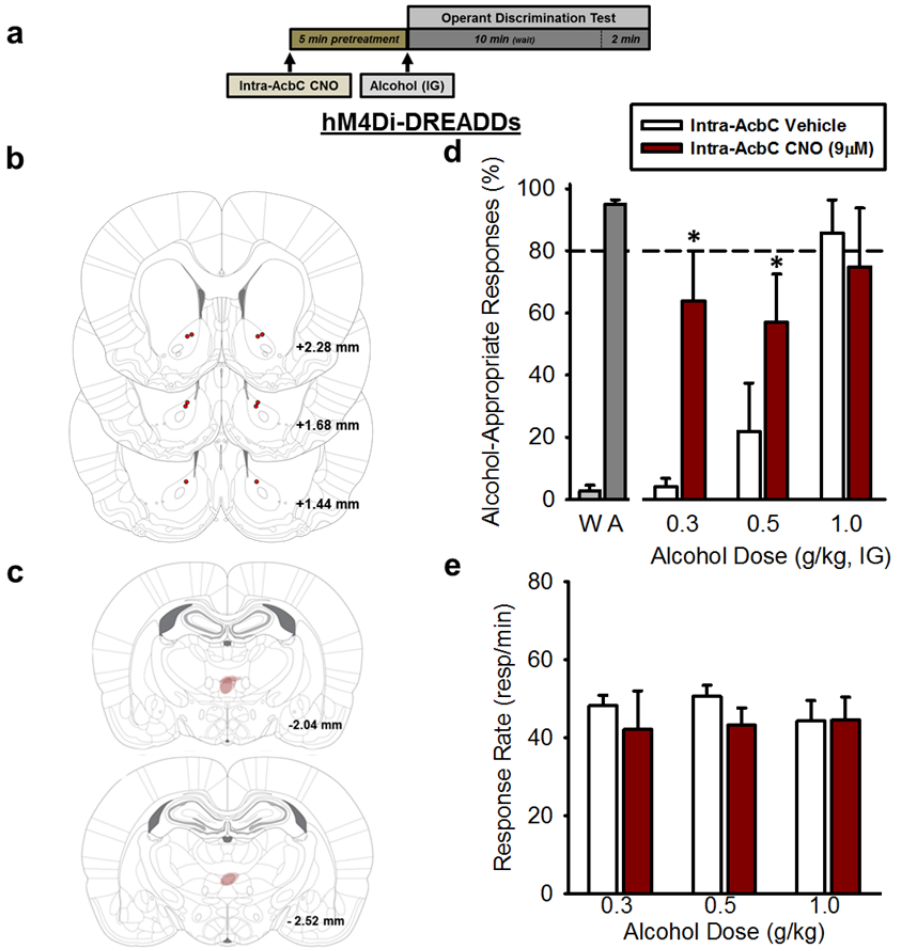

Figure 3.9- Chemogenetic silencing of Rh→AcbC projections increases sensitivity to a low alcohol dose in an operant alcohol discrimination task.

(**a**) Schematic diagram of test. (**b**) Bilateral AcbC injector tip placements (depicted as circles) and (**c**) intra-Rh hM4Di-mCherry expression from individual discrimination-trained rats (n=5) (**d**) Infusion of CNO into AcbC significantly increased percentage of alcohol-appropriate responding at 0.3 and 0.5 alcohol doses. (**e**) Response rate (responses/min) was unaffected by CNO or alcohol dose. (**f**) Dashed line (>80%) represents full expression of the discriminative stimulus effects of alcohol. Baseline discrimination performance on Water and Alcohol (1.0 g/kg, IG) training days shown to the left of x-axis break. *Significant difference from vehicle (Tukey, $p<0.05$). Values on graphs represent mean ± S.E.M.

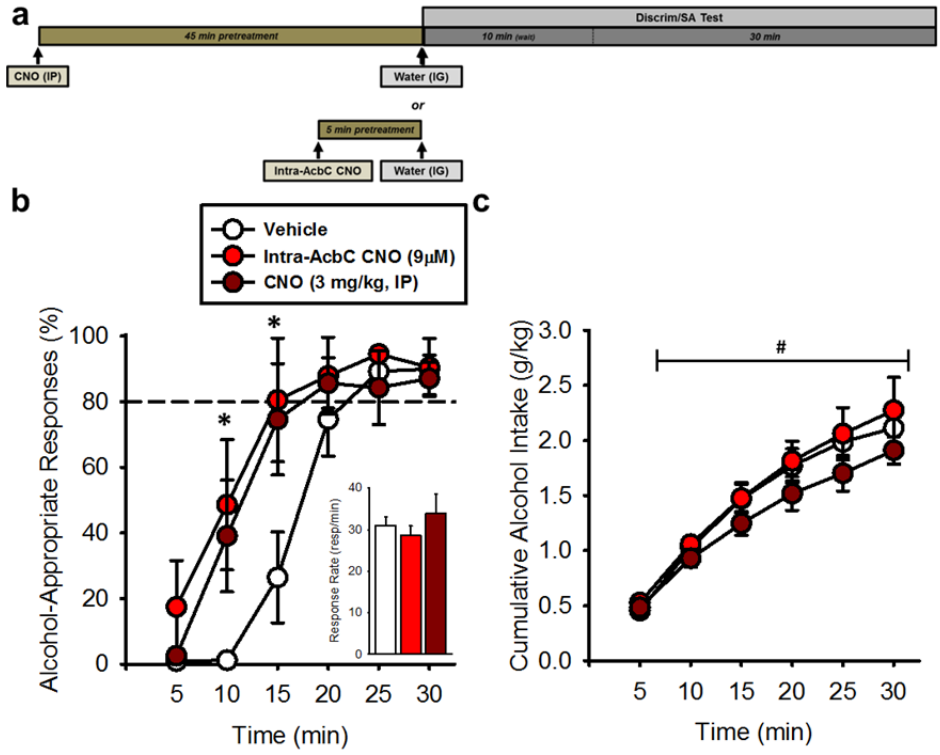

Figure 3.9- Chemogenetic silencing of Rh or Rh→AcbC projections increases sensitivity to the interoceptive effects of self-administered alcohol.

(**a**) Schematic diagram of test. (**b**) Alcohol-appropriate responses and (**c**) cumulative alcohol intake in 10-min intervals for the sweetened alcohol (10% w/v sucrose/10% v/v alcohol) test session (i.e., sweetened alcohol reinforcer). Silencing of Rh or Rh→AcbC, by systemic CNO or intra-AcbC CNO respectively, significantly increased alcohol-appropriate responding at 10 and 15 min, and cumulative sucrose intake increased across time. Inset demonstrates response rates (responses/min) were unaffected by CNO. Dashed line (>80%) represents full expression of the discriminative stimulus effects of alcohol. *Significant difference from vehicle (Tukey, $p<0.05$; n=4). #Significant main effect of time (two-way RM ANOVA, $p<0.05$).Values on graphs represent mean ± S.E.M.

CHAPTER 4: FUNCTIONAL ROLE FOR CORTICAL/THALAMIC-STRIATAL CIRCUIT IN MODULATING THE EFFECTS OF AN ALCOHOL LOADING DOSE ON SELF-ADMINISTRATION

INTRODUCTION

Previous work has shown that pharmacological inhibition and chemogenetic silencing of the IC or Rh produces "alcohol-like" effects and increases sensitivity to the discriminative stimulus effects of alcohol (Chapters 2 and 3). Additionally both the IC and Rh send projections to the nucleus accumbens core (AcbC; Vertes *et al*, 2006), a limbic region proposed to play a central role in modulating the interoceptive effects of alcohol (Besheer *et al*, 2003; Besheer *et al*, 2009; Hodge and Cox, 1998; Hodge *et al*, 2001b; Jaramillo *et al*, 2016) and alcohol self-administration (Chaudhri *et al*, 2008; Chaudhri *et al*, 2010). Furthermore, specific chemogenetic inactivation of the IC→AcbC or Rh→AcbC increases sensitivity to alcohol, thus implicating a role for the insular/thalamic-striatal circuitry in modulating the discriminative stimulus effects of alcohol and possibly alcohol-related behaviors. The IC is proposed to integrate internal and external stimuli into interoceptive states to drive motivated behavior (Craig, 2009; Paulus and Stewart, 2014), which is highly relevant to drug-use (Naqvi and Bechara, 2010; Paulus and Stewart, 2014). Therefore not surprisingly, preclinical studies have confirmed the functional role for the IC in modulating drug self-administration, drug-reinstatement, and drug-seeking (Droutman *et al*, 2015). Brain imaging studies in humans consistently show IC activity in response to alcohol and alcohol-related cues (Droutman *et al*, 2015; Jasinska *et al*, 2014; Naqvi and Bechara, 2010), however the role of the IC in modulating alcohol-related behaviors in preclinical models remains largely unstudied. One preclinical study demonstrated that pharmacological inhibition of the IC decreases alcohol self-administration

(Pushparaj and Le Foll, 2015), and another showed that optogenetic silencing of IC→AcbC decreased compulsive alcohol drinking (Seif *et al*, 2013). Another focus of the present work is the Rh, a region implicated in modulating behavioral flexibility and motivation (Prasad *et al*, 2013). The Rh is known to regulate affective, and cognitive functions required to drive behavioral flexibility, and this is likely related due to the central anatomical location of the region and the extensive connections with the cortex and limbic regions (Cassel *et al*, 2013; Cholvin *et al*, 2013; Prasad *et al*, 2013). Although prior studies have demonstrated neuronal response in the Rh to antipsychotic drugs and alcohol, (Cohen *et al*, 1998) Jaramillo), the functional role of the Rh in modulating drug self-administration has to date not been investigated.

The goal of the present work was to test the functional role of the IC, Rh, and the efferent projections to the AcbC in modulating the effects of an alcohol preload on maintenance of ongoing operant alcohol self-administration. As such, male Long Evans rats were trained to self-administer alcohol and a chemogenetic strategy (i.e., hM4Di Designer Receptors Exclusively Activated by Designer Drugs [DREADDs]) was implemented to silence the IC, Rh or IC/Rh →AcbC projections to test the functional role of the these regions in regulating self-administration following alcohol preload doses. Given that chemogenetic silencing of these regions and projections increases sensitivity to alcohol as shown in Chapter 3(Jaramillo *et al*, 2016), we hypothesized that chemogenetic silencing of these regions and the projections to the AcbC, would increase sensitivity to the alcohol preload dose resulting in decreased responding for alcohol. Given the role of interoceptive effects as potent modulators of drug-related behaviors, understanding the circuitry of these internal cues and their functional role in modulating self-administration will be important to better understand the neural mechanisms driving drug taking and seeking.

METHODS

Animals

Male Long Evans rats (Harlan Sprague–Dawley, Indianapolis, IN) were double housed and then individually housed, following cannulae implantation surgery, in ventilated cages. Water and food were available ad libitum in the home cage. The colony room was maintained on a 12-h light/dark cycle, with lights on at 07:00. All experiments were conducted during the light cycle. Animals were under continuous care and monitoring by veterinary staff from the Division of Laboratory Animal Medicine (DLAM) at UNC-Chapel Hill. All procedures were conducted in accordance with the NIH Guide to Care and Use of Laboratory Animals and institutional guidelines. As described below, several strategies (within subject testing; animals also used in Chapter 5 experiments) were taken throughout this work to reduce the number of animals needed for the conduct of the studies.

Self-Administration Training

Rats were trained using the same two lever (i.e., active lever and inactive lever) behavioral chambers and procedures as previously described in (Besheer *et al*, 2015; Randall *et al*, 2015). Self-administration sessions (30 min) took place 5 days/week (M−F) with active lever responses on a fixed ratio 2 (FR2) schedule of reinforcement such that every second response on the lever resulted in delivery of alcohol (0.1 ml) into a liquid receptacle. Responses on the inactive lever were recorded, but produced no programmed consequences. Locomotor activity was measured during the self-administration sessions by infrared photobeams that divided the behavioral chamber into 4 parallel zones. A sucrose fading procedure was used in which alcohol was gradually added to a 10% (w/v) sucrose solution. The exact order of exposure was as follows: 10% sucrose (w/v)/2% (v/v) alcohol (10S/2A), 10S/5A, 10S/10A, 5S/10A, 5S/15A, 2S/15A. There were one or two sessions at each concentration. Following sucrose fading, sweetened alcohol (2S/15A) continued as the reinforcer for

the remainder of the study. Based on our previous findings using similar self-administration procedures, we typically observe moderate daily alcohol intake ranging from 0.5 to 0.8 g/kg (Randall et al., 2015 and Besheer et al., 2013) and corresponding to approximately 40 mg/dl when blood alcohol concentration is measured immediately after the 30 min session (Besheer et al., 2013). Sucrose self-administration trained rats did not receive alcohol and were faded to 0.8% (w/v) sucrose. The exact order of sucrose fading was as follows: 10S, 5S, 2S, 1S, 0.5S, 0.3S, 0.8S, with one or two sessions at each concentration. The final sucrose concentration was 0.8% (w/v) sucrose because this concentration produced similar lever responding as compared to 2S/15A alcohol-trained animals and this would allow for similar reinforcement history. Testing was only conducted following stable self-administration behavior, (i.e., defined as no change greater than 15% in the total number of responses during the session prior to testing). For all tests, preload and CNO doses were experienced in a random order.

Viral Vectors

hM4Di-DREADDs (AAV8-hSyn-DIO-hM4Di-mCherry; UNC Vector Core, NC) or Control-mCherrys (AAV8-hSyn-DIO-mCherry; UNC Vector Core, NC) previously described by (Krashes *et al*, 2011; Roth, 2016) were combined with Cre recombinase (AAV8-CMV-Cre-GFP; Vector Biolabs, PA) in a ratio of 7:3 (v/v) and bilaterally infused into the IC (2 µl/side; AP +3.2, ML ±4.0, DV -6.0 from skull) or unilaterally in the Rh (1 µl; AP -2.3, ML ± 1.7 , DV -7.2 with a 5° angle from skull).

Experimental Procedures

Experiment 4.1: Examination of the functional role of IC and Rh on an alcohol-preload prior to alcohol self-administration, through chemogenetic silencing

IC-silencing. Rats (n=24) trained to self-administer alcohol received bilateral infusions of hM4Di-DREADDs in the IC. Following 1 week of recovery and stable self-administration rats were tested to determine a functional role of the IC in modulating the effects of an alcohol loading dose on alcohol self-administration. Rats received CNO (0, 1 mg/kg, intraperitoneal [IP]), 35 min prior to an alcohol loading dose (0, 0.5, 1.0 g/kg, IG). Then 10 min later commenced the self-administration session.

Rh-silencing. Rats trained to self-administer alcohol received a unilateral infusion of hM4Di-DREADDs in the Rh (n=12). Following 1 week of recovery, training continued until stable self-administration was established. To determine a functional role of the Rh in modulating alcohol self-administration, rats received CNO (0, 1 mg/kg, IP), 35 min prior to an alcohol loading dose (0, 0.5, 1.0 g/kg, IG). Then 10 min later commenced the self-administration session.

Experiment 4.2: Examination of the functional role of IC→AcbC on an alcohol-preload prior to alcohol self-administration

To determine a role for the IC→AcbC projections, additional groups trained to self-administer alcohol were infused with hM4Di-DREADDs (n=11) or Control-mCherryS (n=12) in the IC. After 1 week of recovery and stable self-administration behavior, rats were implanted with bilateral AcbC cannulae. Following 1 week of recovery and reacquisition of self-administration behavior, rats received intra-AcbC infusion of CNO (0 or 3 μM/side) 5 min prior to an alcohol loading dose (0, 0.5, 1.0 g/kg, IG). Then 10 min later commenced a self-administration session.

Experiment 4.3: Examination of the functional role of IC→AcbC on sucrose self-administration

IC-silencing. Rats received bilateral infusions of hM4Di-DREADDs (n=11) in the IC. Following 1 week of recovery, rats were trained to self-administer sucrose until stable self-administration was established. To determine a functional role of the IC in modulating sucrose self-administration, rats received CNO (0, 1 mg/kg, IP), 45 min prior to a sucrose self-administration session.

Intra-AcbC silencing. Following systemic CNO testing, rats were implanted with bilateral AcbC cannulae (n=11) to determine a role for the IC→AcbC projections in modulating sucrose self-administration. Following 1 week recovery and reacquisition of sucrose self-administration behavior, rats received intra-AcbC infusion of CNO (0 or 3 µM/side) 5 min prior to a sucrose self-administration session.

Microinjection Procedures for Viral Vectors, Tract Tracer, and Drug Infusions

Site-specific microinjections were delivered by a microinfusion pump (Harvard Apparatus, MA) through 1.0 µl Hamilton syringes (Hamilton Robotic, NV) connected to 33-gauge injectors (Plastics One, VA) as described in (Besheer *et al*, 2014; Jaramillo *et al*, 2016). For Experiment 4.1-4.3, anesthetized rats received bilateral microinjection of viral constructs into the IC at a volume of 2.0 µl across 10-min. The injector remained in place for an additional 10 min to allow for diffusion. For Experiment 4.2-4.3, anesthetized rats received unilateral microinjection of viral constructs into the Rh at a volume of 1.0 µl across 5-min. The injector remained in place for an additional 5 min to allow for diffusion. CNO microinjections were delivered in Experiment 4.2-4.3 through injectors extending 2 mm below the previously implanted (aimed to terminate 2 mm above the AcbC; AP +1.7, ML +1.5, DV -6.8 from skull), 26-gauge guide (Plastics One, VA) at a volume of 0.5 µl/side across 1 min. The injectors remained in place for an additional 2-min after the infusion to allow for diffusion.

Tissue Preparation for Viral Vector and Cannulae Confirmation

Tissue collection, immunofluorescent and Nissl staining were similar as previously described in Chapter 3. The brain regions examined were the IC (+2.8 to +1.9 mm; Experirment 4.1-4.3) and Rh (-1.92 to -2.76 mm; Experiment 4.1), according to (Paxinos and Watson, 2007). Free-floating coronal sections (40 μm) were incubated in rabbit anti-DSRed (1:2,500; Clontech, CA) for 24 h at 4 °C. Sections were then incubated at RT in fluorescent conjugated secondary antibody (goat anti-rabbit 594; Life Technologies, MA). hM4Di-mCherry expression was confirmed by immunofluorescence (individual expression represented as 20% opacity [Fig. 4.1-4.4]) using a Nikon 80i Upright microscope (Nikon Instruments, NY). For Experiment 2/3 cannulae placements were confirmed by Nissl staining (injector placements represented by circles in Fig. 4.3 and 4.4). Only rats with accurate injections and cannulae placements were included in the analyses and data presentation.

Drugs

Alcohol (95% w/v) was diluted in distilled water to a concentration of 20% (v/v) and administered IG, with volumes varied by weight to obtain the desired dose. For systemic administration CNO, injected at a volume of 1 ml/kg (NIDA Drug Supply Program, NC), was dissolved in 1% dimethyl sulfoxide in water (v/v), or in aCSF for intracranial administration. The CNO doses were chosen based on previous work (Krashes *et al*, 2011; Roth, 2016; Stachniak *et al*, 2014) and pilot studies from our lab.

Data Analysis

For all experiments, doses were assigned in a repeated measures design with each rat receiving all treatments in a randomized order, with at least one baseline self-administration session between testing days. Alcohol intake (g/kg) was approximated based on body weight and number of reinforcements delivered. For all studies, one- or two-way RM ANOVA was used to analyze data as

appropriate. Post hoc analysis (Tukey) was used to determine differences between specific treatment conditions. Statistical significance was declared at $P \leq 0.05$.

RESULTS

Experiment 4.1: Examination of the functional role of IC and Rh on an alcohol-preload prior to alcohol self-administration, through chemogenetic silencing

IC- silencing.

hM4Di-mCherry expression is represented in **Figure 4.1b** (n=13). Eleven rats had inaccurate cannulae placements or inefficient hM4Di-DREADD infusions (i.e., no hM4Di-mCherry expression likely due to a clogged injector), and are not included in analyses or in **Figure 4.1b-4.1e**. The two-way RM ANOVA of alcohol-reinforced responding (**Fig. 4.1d**) demonstrated a significant main effect of alcohol loading dose [$F(2,24)=17.75$, $p<0.001$], with decreased alcohol responses at both the 0.5 and 1.0 (g/kg) alcohol loading dose relative to the water loading dose ($p<0.05$). Similarly, two-way RM ANOVA of total alcohol intake (includes experimenter-administered alcohol loading dose, shown in black; [**Fig. 4.1d**]) demonstrated a significant main effect of alcohol loading dose [$F(2,24)=19.35$, $p<0.001$], with increased total intake at 0.5 and 1.0 (g/kg) alcohol loading dose relative to the water loading dose ($p<0.05$). Therefore, indicating that self-administration behavior was under the control of the alcohol loading dose. There was no effect of CNO treatment demonstrating that silencing the IC did not disrupt the loading dose-induced decrease on self-administration behavior. Two-way RM ANOVA demonstrated no effects on locomotor activity (**Fig. 4.1e**) or inactive lever responding (**Table 4.1**).

Rh- silencing.

hM4Di-mCherry expression is represented in **Figure 4.2b** (n=9). One rat died prior to completion of testing and for two rats hM4Di-DREADD infusions could not be verified (i.e., experimenter error), and are not included in any analyses and not shown in **Figure 4.2b-4.2e**. The two-way RM ANOVA of alcohol-reinforced responding (**Fig. 4.2c**) demonstrated a significant main effect of alcohol loading dose [$F(2,16)=7.83$, $p=0.004$], with significantly less responding at the highest alcohol loading dose (1 g/kg) relative to the water loading dose. Similarly, two-way RM ANOVA of total alcohol intake (includes experimenter administered alcohol loading dose shown in black in **Fig. 4.2d**) demonstrated a significant main effect of alcohol loading dose [$F(2,16)=13.26$, $p<0.001$] with decreased alcohol intake at the 0.5 and 1.0 (g/kg) alcohol loading dose relative to the water loading dose ($p<0.05$). Therefore, indicating that self-administration behavior was under the control of the alcohol loading dose. There was no effect of CNO treatment, demonstrating that silencing the Rh did not disrupt the loading dose-induced decrease on self-administration behavior. Two-way RM ANOVA demonstrated no effect on locomotor activity (**Fig. 4.2e**) or inactive lever responding (**Table 4.1**).

Experiment 4.2: Examination of the functional role of IC→AcbC on an alcohol-preload prior to alcohol self-administration

hM4Di-mCherry expression and bilateral AcbC injector placements (red circles) are represented in **Figure 4.3b** (n=7). Two rats died prior to completion of testing and one rat had inefficient hM4Di-DREADD infusions (i.e., no hM4Di-mCherry expression), are not included in analyses or in **Figure 4.3b-f**. Two-way RM ANOVA of alcohol-reinforced responding (**Fig. 4.3c**) showed a significant main effect of alcohol loading dose [$F(2,12)=6.38$, $p\leq0.01$], with significantly less responding at the highest alcohol loading dose (1 g/kg) relative to the water loading dose ($p<0.05$), indicating that self-administration behavior was under the control of the alcohol loading dose. There was also a main effect of CNO [$F(1,12)=10.35$, $p<0.02$], with significantly less

responding following IC→AcbC silencing. There was no significant two-way interaction. However, based on our *a priori* hypothesis that silencing IC→AcbC projections would potentiate sensitivity to the alcohol loading dose, we conducted planned comparisons to examine the effect of CNO at each loading alcohol dose. Indeed, CNO significantly decreased responding at the water loading dose ($t=4.45$, $p=0.004$) and at the 0.5 (g/kg; $t=2.848$, $p\leq0.03$) alcohol loading dose relative to vehicle. Similarly, two-way RM ANOVA of alcohol intake (**Fig. 4.3d**) showed a significant main effect of alcohol loading dose [$F(2,12)=27.46$, $p<0.001$] with significantly more alcohol intake at both 0.5 and 1.0 (g/kg) relative to the water loading dose ($p<0.05$). Additionally, there was a significant main effect of CNO [$F(1,12)=7.75$, $p\leq0.03$] with planned comparisons demonstrating significantly less intake following CNO treatment at the water loading dose ($t=4.87$, $p=0.003$) and at the 0.5 (g/kg) alcohol loading dose ($t=3.04$, $p<0.02$). Thus, intra-AcbC CNO decreased self-administration following a water loading dose and potentiated the effect of the 0.5 (g/kg) alcohol loading dose. There was no effect on locomotor activity (**Fig. 4.2e**) or inactive lever responding (**Table 4.1**).

Control-mCherry expression and bilateral AcbC injector placements (blue circles) are represented in **Figure 4.3f** (n=8). Two rats died prior to completion of testing and 3 rats had inaccurate placements or inefficient Control-mCherry infusions (i.e., no Control-mCherry expression), and are not included in any analyses and not shown in **Figure 4f-i**. Two-way RM ANOVA of alcohol-reinforced responding (**Fig. 4.3g**) showed a main effect of alcohol loading dose [$F(2,14)=5.893$, $p\leq0.01$], with significantly less responding at the highest alcohol loading dose (1 g/kg) relative to the water loading dose ($p<0.05$), indicating that self-administration behavior was under the control of the alcohol loading dose. Similarly, two-way RM ANOVA of total alcohol intake (includes experimenter-administered alcohol loading dose, shown in black; [**Fig. 4.3i**]) demonstrated a significant main effect of alcohol loading dose [$F(2,14)=64.66$, $p<0.001$], with increased total intake at 0.5 and 1.0 (g/kg) alcohol loading dose relative to the water loading dose ($p<0.05$). Therefore, indicating that self-administration behavior was under the control of the alcohol loading dose. There was no effect of CNO treatment demonstrating no off target effect by CNO as there was not effect on

the loading dose-induced decrease on self-administration behavior. Two-way RM ANOVA demonstrated no effects on locomotor activity (**Fig. 4.2e**) or inactive lever responding (**Table 4.1**).

Experiment 4.3: Examination of the functional role of IC and IC→AcbC on alcohol or sucrose self-administration.

IC-silencing

hM4Di-mCherry expression is represented in **Figure 4.4**. Five rats had inaccurate placements or inefficient hM4Di-DREADD infusions (i.e., no hM4Di-mCherry expression), are not included in analyses or in **Figure 4.4b-d**. Two-way RM ANOVA of sucrose-reinforced responding (**Fig. 4.4d**) and locomotor activity (**Fig. 4.4e**) or inactive lever responding (**Table 4.1**) did not show a significant main effect of CNO treatment.

Intra-AcbC silencing.

Bilateral AcbC injector placements (red circles) are represented in **Figure 4.4b**, respectively. Two-way RM ANOVA of lever responses (sucrose [**Fig. 4.4g**] and inactive [**Table 4.1**]), or locomotor activity (**Fig 4.4h**) demonstrated no effect by intra-AcbC CNO.

DISCUSSION

The present findings demonstrate that IC projections to the AcbC modulate ongoing alcohol intake, in a reinforcer-specific manner, implicating an insular-striatal role on alcohol-related behaviors. Here we reliably demonstrate titration of alcohol drinking (i.e., decreased alcohol self-administration) following a loading dose of alcohol, in rats trained on an operant self-administration paradigm. Contrary to our hypothesis, silencing the IC or Rh outgoing projections (in general) did not modulate ongoing alcohol self-administration or self-administration following the alcohol loading dose, as alcohol self-administration was unaffected following systemic CNO treatment. However,

following specific chemogenetic silencing of IC→AcbC projections, we demonstrate decreased alcohol self-administration, and increased sensitivity to a moderate (0.5 g/kg) alcohol loading dose, implicating a role for insular-striatal circuitry, in modulating the effects of alcohol pre-exposure on maintenance of ongoing operant alcohol self-administration.

An important aspect of this study was to assess the effect of an alcohol pretreatment (i.e., loading dose) on ongoing self-administration. Previously, work from our lab demonstrated a decrease in alcohol self-administration following an experimenter administered (1 g/kg) alcohol loading dose (Randall *et al*, 2015). Here we confirm and expand our findings by reliably demonstrating a decrease in alcohol self-administration following a 0.5 and 1.0 (g/kg) loading dose of alcohol (Fig 4.3). Other studies utilizing similar moderate alcohol loading doses also demonstrate decreased alcohol intake under both experimenter-administered and self-administered preload conditions (Czachowski *et al*, 2006; Samson *et al*, 2002; Samson *et al*, 2003), implicating the postingestive interoceptive effects of an alcohol loading dose, regardless of route of administration. Although a study has attributed a loading dose-induced decrease in self-administration to gastric distention (Czachowski *et al*, 2006); albeit lower alcohol dose and higher volume), others have attributed the effects to reinforcer-specific pharmacological processes, as the effects are specific to alcohol loading doses and not sucrose or water (Samson *et al*, 2002; Samson *et al*, 2003). Additionally, utilizing sham ingestion (open gastric fistula) to minimize gastric absorption of alcohol, (Rowland and Barnett, 1992) demonstrates acquisition of increased alcohol intake in rats, interpreted as an attempt to titrate consumption in the absence of the postingestive pharmacological effects of alcohol. Additionally, devaluation of alcohol reinforcement through the use of alcohol paired with lithium chloride to induce malaise, results in decreased alcohol consumption (Samson *et al*, 2004). Together, these studies demonstrate that postingestive interoceptive effects and internal/interoceptive cues associated with alcohol directly contribute to ongoing alcohol intake.

Given the importance of internal cues to modulate alcohol self-administration behavior, we hypothesized that silencing all outgoing projections of the IC and Rh, by systemically administered

CNO, would increase sensitivity to the interoceptive effects of a loading dose of alcohol. This hypothesis was based on the findings described in Chapters 2 and 3 showing that silencing these regions produces partial alcohol-like effects and potentiates the interoceptive effects of alcohol, respectively. However, contrary to our hypothesis silencing the IC or Rh did not affect alcohol self-administration under "control" conditions (i.e., water preload) and did not potentiate sensitivity to the alcohol preload doses. Although we demonstrate a role for the IC and Rh in modulating sensitivity to the interoceptive effects of alcohol (i.e., Chapters 2-3) the lack of findings suggests that silencing all activity including all outgoing projections of the IC and Rh does not modulate alcohol self-administration or the alcohol loading dose effect. Furthermore these findings demonstrate the complexity of the interoceptive effects in modulating alcohol-reinforced behavior, suggesting the possible recruitment of differential circuity or conversely more specific circuitry that is overshadowed by the overall silencing of the IC or Rh. interoceptive circuitry only under certain conditions. Additionally, we hypothesized that the control-mCherry group trained to self-administer alcohol and the hM4Di-mCherry group trained to self-administer sucrose would be unaffected by CNO. Indeed, silencing the IC did not disrupt alcohol self-administration behavior in the control-mCherry group, indicating no off target effects of CNO in non-DREADD expressing controls. Additionally, silencing the IC, did not affect sucrose self-administration in the hM4Di-mCherry group, indicating an alcohol reinforcer-specific role for the IC on self-administration. This finding is corroborated by other studies demonstrating no effect on sucrose self-administration following IC inhibition (Forget *et al*, 2010), an important finding, as the IC has been implicated in food-seeking and taste processing (Carleton *et al*, 2010; Kusumoto-Yoshida *et al*, 2015).

The Rh has been implicated in modulating behavioral flexibility (Cassel *et al*, 2013; Cholvin *et al*, 2013; Prasad *et al*, 2013)., however silencing of the Rh did not disrupt behavioral flexibility relative to outcome devaluation as demonstrated in this study. To our knowledge, our findings are the first to investigate and demonstrate no role for the Rh in outcome devaluation or general alcohol self-administration. However, the lack of effect of IC silencing was surprising, given that IC has been

implicated in modulating aspects of outcome value particularly in reference to internal states (Craig, 2009). Furthermore a study has shown that infusion of an NMDA antagonist, ifenprodil, into the IC disrupted devaluation during a food choice test (Parkes and Balleine, 2013). Interestingly, contralateral disconnection of the IC and AcbC by NMDA-induced lesions also disrupted specific satiety-induced outcome devaluation of food (Parkes *et al*, 2015). Additionally, contrary to the Rh, pharmacological inhibition of the IC has been shown to decrease alcohol self-administration (albeit modestly; Pushparaj and Le Foll, 2015) and self-administration of various other drugs of abuse (Droutman *et al*, 2015; Kutlu *et al*, 2013; Pushparaj and Le Foll, 2015). Given the lack of effects and limited literature of Rh role on drug-related behaviors, we decided to pursue IC→AcbC projections, as the overwhelming literature of the IC implicated that global silencing of the IC may have overshadowed a pathway specific role for the IC efferent projections.

Indeed specific silencing of the IC→AcbC projections dramatically decreased self-administration under "control" conditions (i.e., water pretreatment) and potentiated the effect of a 0.5 (g/kg) alcohol loading dose (an effect absent in the control-mCherry group), demonstrating for the first time a role of the IC→AcbC in modulating satiety-induced outcome devaluation of alcohol. Given that we have previously demonstrated that silencing IC→AcbC projections increases sensitivity of the interoceptive effects of alcohol, our findings suggest that IC→AcbC silencing produces an alcohol loading effect similar to a moderate (0.5 g/kg) loading dose. Meanwhile, in the presence of 0.5 g/kg alcohol loading dose, silencing of IC→AcbC projections increases sensitivity to the loading dose of alcohol (i.e., potentiates the decrease in alcohol self-administration). Together with the findings of Chapter 3, these findings implicate IC→AcbC projections as a site of action of alcohol, suggesting that alcohol acts by silencing IC→AcbC activity to induce and potentiate the interoceptive effects of alcohol which in turn can regulate alcohol self-administration. Interestingly, optogenetic silencing of IC→AcbC results in decreased aversion resistant compulsive alcohol drinking, with no effect on alcohol drinking not altered with quinine (Seif *et al*, 2013). This finding along with (Parkes *et al*, 2015)demonstrating a role for the IC→AcbC in outcome devaluation and

our present findings, implicate a specific role of the insular-striatal projections in modulating alcohol intake in conditions under strong interoceptive control. Furthermore, our findings demonstrate IC→AcbC silencing does not affect sucrose self-administration, and thus indicate reinforcer-specific modulation by the insular-striatal circuit. Although not tested in this study it will be important for future studies to investigate the specific role of Rh→AcbC projections, as global silencing of Rh may have also overshadowed the specific role of Rh efferent projections, as we observed with the IC. Additionally, it will be important to further investigate the role of the AcbC, as general inhibition of efferent projections to the AcbC may be responsible for the effects demonstrated through IC→AcbC silencing.

Together these data identify a role for the IC→AcbC circuit in modulating alcohol self-administration under "control" conditions (i.e., water pretreatment) and following satiation-induced devaluation of alcohol by an alcohol loading dose. Here, we consistently demonstrate that titration of alcohol self-administration is relative to the interoceptive effects of an alcohol loading dose. Further, silencing of the insular-striatal circuit decreases self-administration to levels relative to an alcohol loading dose. Thus, these findings elucidate the complex role of the IC while suggesting that alcohol acts by silencing the IC→AcbC circuit to decrease self-administration in an outcome value manner. Together with the previous data, these findings inform us of the complex IC and Rh structure while providing evidence of the critical nature of striatal circuitry in modulating alcohol self-administration while implicating a role on other alcohol-related behaviors (i.e., drug-seeking and -reinstatement).

Total Inactive Lever Responses (per test session)

Alcohol Loading Dose (g/kg, IG):	0		0.5		1.0	
Treatment:	Vehicle	CNO	Vehicle	CNO	Vehicle	CNO
Experiment 4.1						
Intra-Insula	2.0±0.6	0.9±0.3	0.8±0.4	0.9±0.4	1.2±0.5	1.1±0.4
Intra-Rhomboid	1.3±0.6	2.8±0.7	2.7±0.8	2.2±0.8	2.3±0.7	2.0±0.6
Experiment 4.2						
Intra-AcbC (hM4Di)	0.4±0.2	0.3±0.2	0.4±0.3	0.1±0.1	0.1±0.1	0.3±0.3
Intra-AcbC (control-mCherry)	1.4±0.9	0.6±0.6	1.1±0.5	0.1±0.1	0.4±0.3	0.4±0.3
Experiment 4.3						
Intra-Insula	1.7±1.1	2.2±0.9	-	-	-	-
Intra-AcbC	1.0±0.3	1.0±0.2	-	-	-	-

*p<0.05

Table 4.1- Inactive lever responses (mean ± S.E.M.).

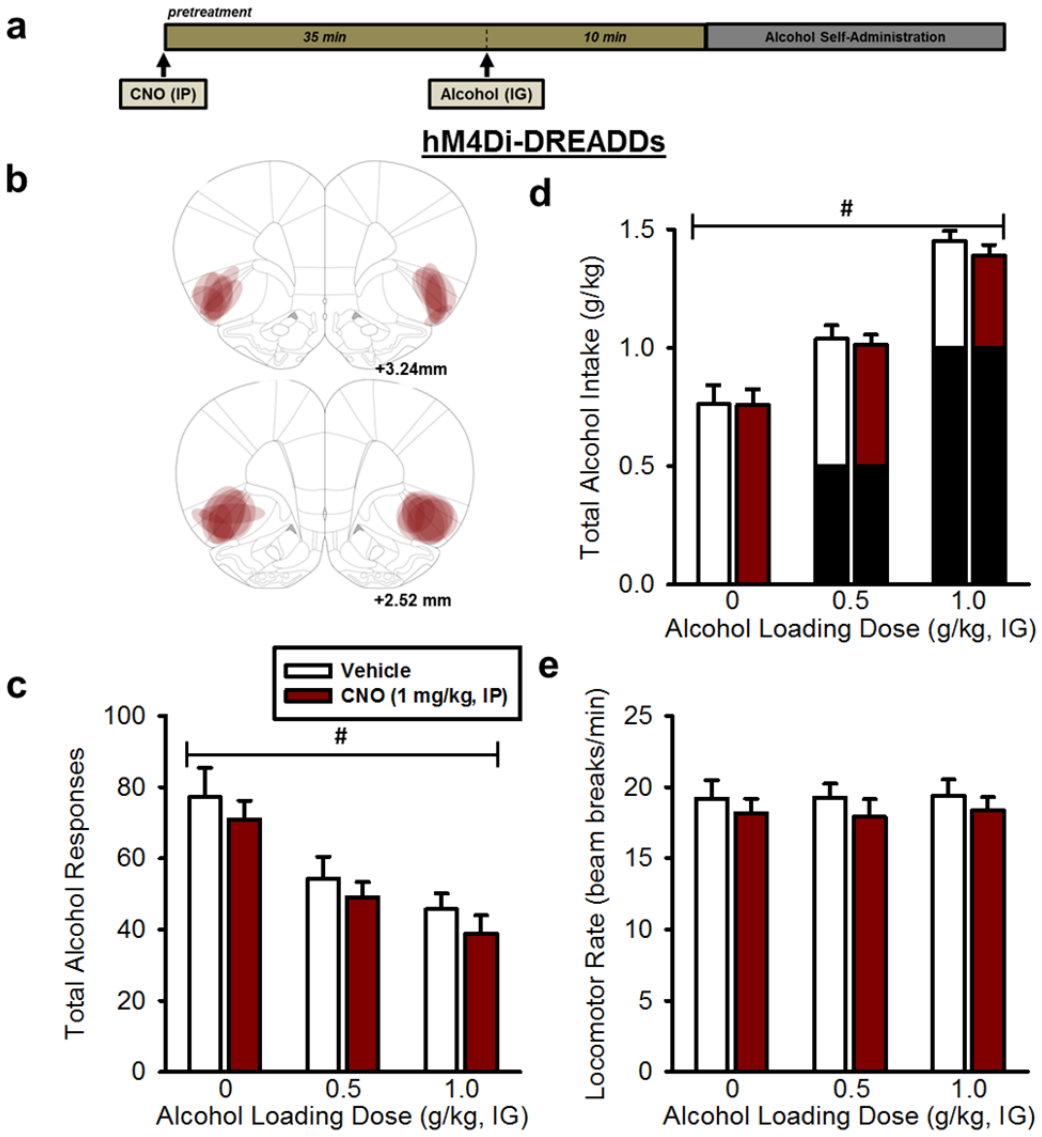

Figure 4.1- Chemogenetic silencing of IC does not modulate alcohol self-administration or the effect of the alcohol loading dose.

(**a**) Schematic diagram of test. (**b**) Intra-IC hM4Di-mCherry expression from individual rats trained to self-administer alcohol (n=13). (**c**) There was a significant decrease in total alcohol lever responses for the 30-min session following an alcohol loading dose. (**d**) Total alcohol exposure (g/kg; alcohol intake that was self-administered + experimenter-administered loading dose in black), was significantly increased by the alcohol loading dose. Silencing of IC by CNO had no effects. **(f)** Locomotor rate was unaffected. Values on graphs represent mean ± S.E.M. #Significant main effect of loading dose (two-way RM ANOVA, $p<0.05$).

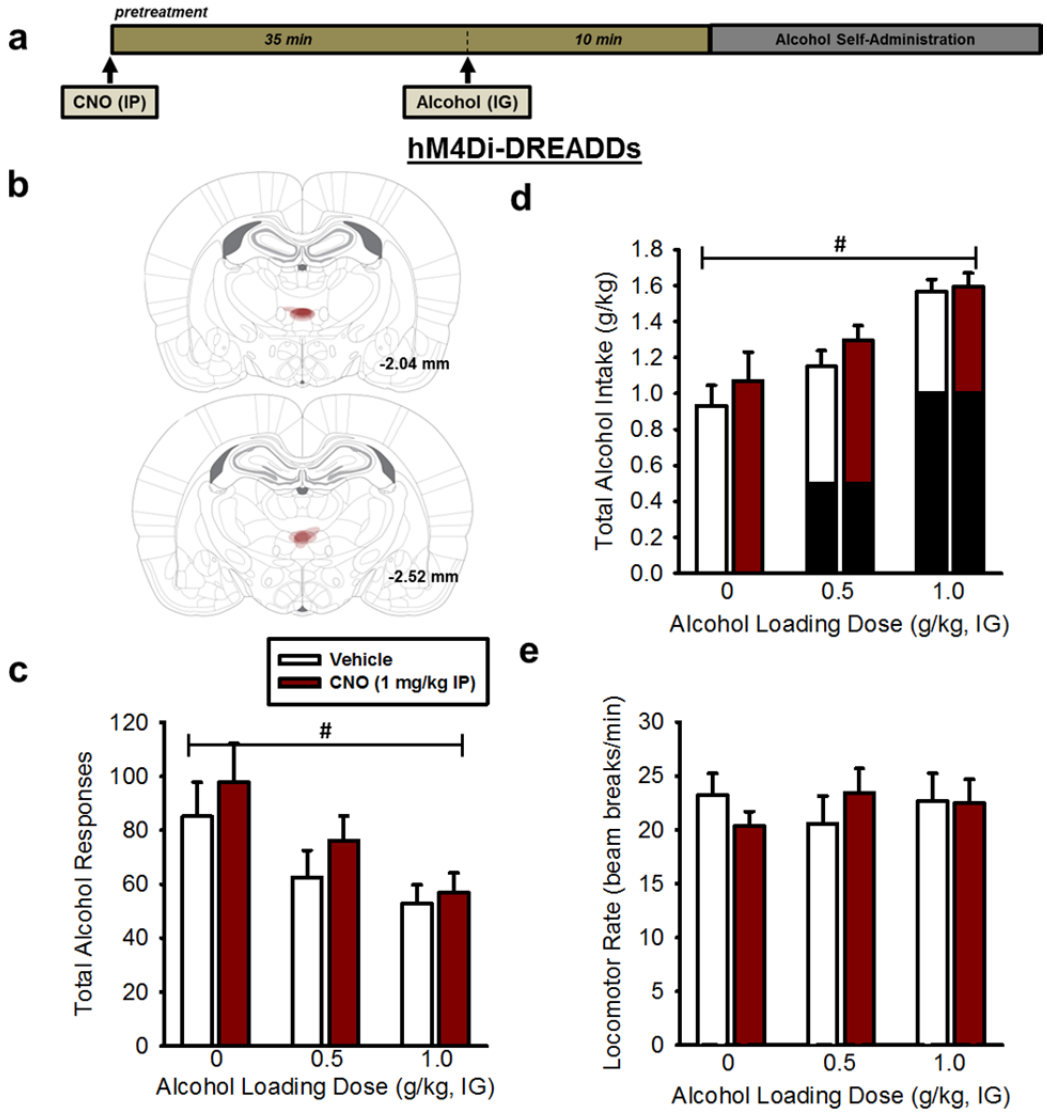

Figure 4.2- Chemogenetic silencing of Rh does not modulate alcohol self-administration or the effect of the alcohol loading dose.

(a) Schematic diagram of test. (**b**) Intra-Rh hM4Di-mCherry expression from individual rats trained to self-administer alcohol (n=9). (**c**) There was a significant decrease in total alcohol lever responses for the 30-min session following an alcohol loading dose. (**d**) Total alcohol exposure (g/kg; alcohol intake that was self-administered + experimenter-administered loading dose in black), was significantly increased by the alcohol loading dose. Silencing of IC by CNO had no effects. **(f)** Locomotor rate was unaffected. Values on graphs represent mean ± S.E.M. #Significant main effect of loading dose (two-way RM ANOVA, $p<0.05$).

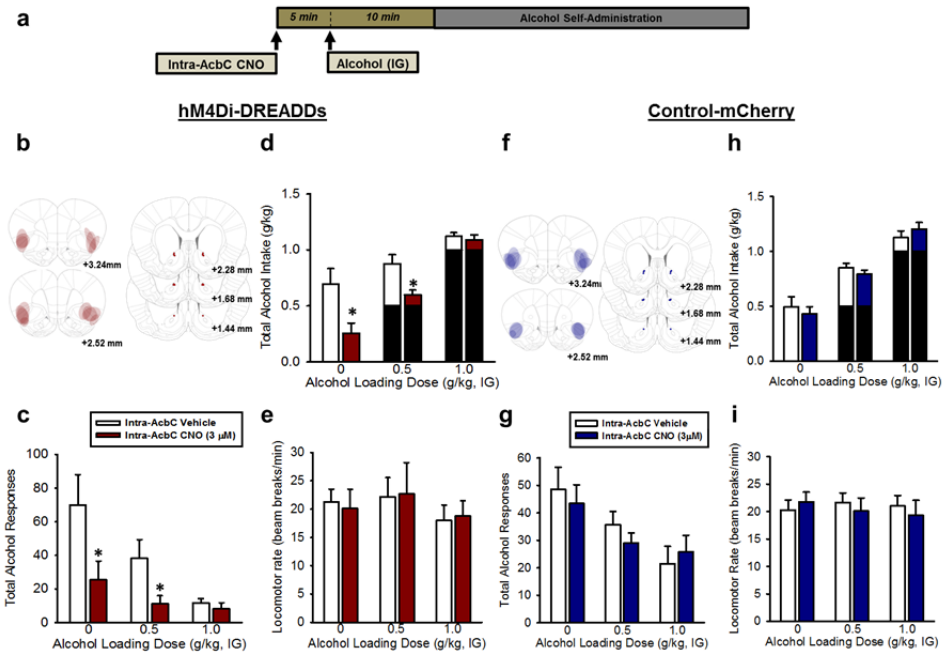

Figure 4.3- Chemogenetic silencing of IC→AcbC projections decreases alcohol self-administration and potentiates the effects of a moderate alcohol loading dose.

(a) Schematic diagram of test. (**b**) Intra-IC hM4Di-mCherry expression and bilateral AcbC injector tip placements (depicted as circles) from individual rats trained to self-administer alcohol (n=7). (**c**) There was a significant decrease in total alcohol lever responses for the 30-min session following an alcohol loading dose. (**d**) Total alcohol exposure (g/kg; alcohol intake that was self-administered + experimenter-administered loading dose in black), was significantly increased by the alcohol loading dose. Silencing of IC→AcbC, by CNO significantly decreased alcohol responses and alcohol intake following the water and 0.5 (g/kg) alcohol loading dose. **(e)** Locomotor rate was unaffected. (**f**) Intra-IC control-mCherry expression and bilateral AcbC injector tip placements (depicted as squares) from individual rats trained to self-administer alcohol (n=X). (**g**) There was a significant decrease in total alcohol lever responses for the 30-min session following an alcohol loading dose. (**h**) Total alcohol exposure (g/kg; alcohol intake that was self-administered + experimenter-administered loading dose in black), was significantly increased by the alcohol loading dose. Intra-AcbC CNO had no effects. **(i)** Locomotor rate was unaffected. Values on graphs represent mean ± S.E.M. *Significant difference from vehicle (t-test, $p<0.05$)

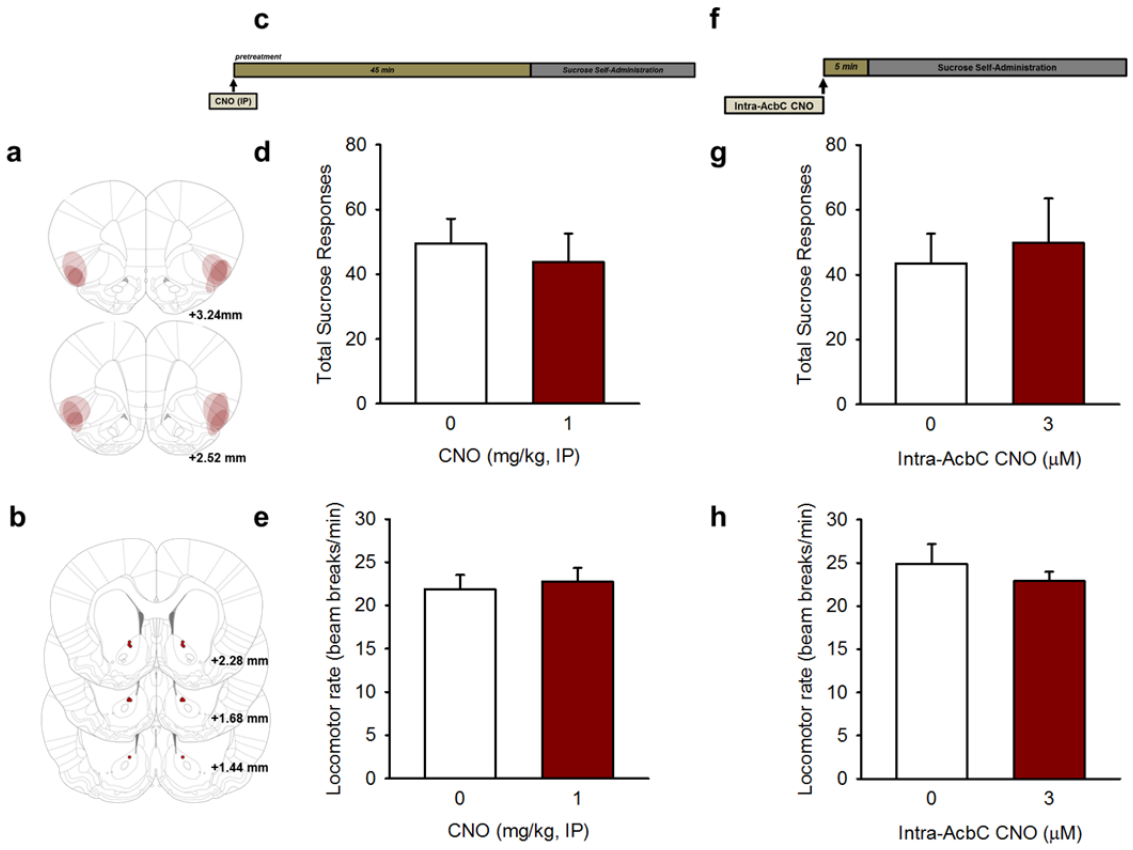

Figure 4.4- Chemogenetic silencing of IC or IC→AcbC projections does not modulate sucrose self-administration.

(**a**) Schematic diagram of test. (**b**) Intra-IC hM4Di-mCherry expression and bilateral AcbC injector tip placements (depicted as circles) from individual rats trained to self-administer sucrose (n=X). (**c**) Total sucrose lever responses across the 30-min session, demonstrate no effect of silencing IC by CNO (**d**) Silencing of IC→AcbC projections did not affect sucrose lever responses. **(f)** Locomotor rate was unaffected. Values on graphs represent mean ± S.E.M. (two-way RM ANOVA, $p<0.05$)

CHAPTER 5: FUNCTIONAL ROLE FOR CORTICAL/THALAMIC-STRIATAL CIRCUIT IN MODULATING RELAPSE-LIKE BEHAVIORS

INTRODUCTION

Traditionally, reinstatement of drug-seeking is examined after extinction of drug-reinforced behavior. Depending on the factors being investigated, extinction sessions occur in the absence of the drug and/or in the absence of drug-associated cues. During the reinstatement test non-contingent exposure to the drug and/or drug-associated cues leads to significantly more drug-associated behavior (i.e., previously drug-reinforced) compared to control manipulations; thereby demonstrating the power of the internal or external cues to drive reinstatement, respectively. For the present study, we sought to adapt the standard reinstatement test to examine behavior under seeking conditions but also under conditions in which the drug is available. The two-phase reinstatement test utilized in this study reintroduces alcohol-associated contextual stimuli (e.g., response-contingent light presentations) during the first phase, in the absence of alcohol (similar to a traditional reinstatement test; i.e., alcohol seeking phase). At the 10 min mark, a non-contingent delivery of alcohol signals the second phase of the reinstatement test during which alcohol is available. Therefore, this reinstatement procedure allows us to investigate both alcohol-seeking and reinstatement of alcohol self-administration within the same session. The goal of the present work is to use this reinstatement model to examine the effects of a loading dose of alcohol (i.e., alcohol pretreatment) on subsequent alcohol relapse-like behavior and will build on the previous chapters which demonstrate that the insular cortex (IC) and rhomboid thalamic nucleus (Rh) functionally modulate the interoceptive effects of alcohol. Thus, by focusing on regions known to modulate alcohol-induced internal cues, this study investigates the role of the IC and Rh in modulating sensitivity to a loading dose of alcohol on cue-induced relapse-like

behaviors (i.e., seeking and reinstatement of alcohol drinking) after extinction of alcohol-reinforced behaviors. Additionally, inclusion of testing under non-alcohol preload conditions also allows us to determine the role of the IC and Rh in modulating relapse-like behavior in general.

In relation to the potential IC involvement on relapse-like behaviors, clinical-imaging studies demonstrate increased IC activity in response to the interoceptive effects and cue-induced urges for alcohol (Droutman *et al*, 2015). Various preclinical studies demonstrate the role of the IC in modulating cue-induced relapse to cocaine, nicotine, morphine and amphetamine (Droutman *et al*, 2015). In relation to the Rh involvement on relapse-like behaviors, the Rh is a region proposed to regulate cue-induced behavior, particularly under conditions that require behavioral flexibility (Prasad *et al*, 2013). This is likely related to the central anatomical location of the Rh and the extensive connections with the cortex and limbic regions (Cassel *et al*, 2013; Cholvin *et al*, 2013; Prasad *et al*, 2013). Furthermore the Rh has also been implicated in cue-induced motivation, as lesions to the Rh result in decreased number of omitted responses and decreased latency to obtain reward (Prasad *et al*, 2013). Given that relapse-like behaviors are affected by disruptions in behavioral flexibility and are primed by drug-associated cues, we sought to investigate the role of the Rh and IC on modulating drug-induced relapse-like behaviors (i.e., using an alcohol loading dose) which remains largely unstudied. Additionally IC and Rh project to the nucleus accumbens core (AcbC), a region largely implicated in modulating the interoceptive effects of alcohol and various alcohol-related behaviors (Besheer *et al*, 2010; Chaudhri *et al*, 2008; Chaudhri *et al*, 2010; Gass *et al*, 2011; Griffin *et al*, 2014; Knapp *et al*, 2009; Rassnick *et al*, 1992a; Rassnick *et al*, 1992b).. Furthermore, Chapter 3 demonstrates that specific chemogenetic silencing of the IC→AcbC or Rh→AcbC increases sensitivity to alcohol, thus implicating a functional role for the insular/thalamic-striatal circuitry in modulating the discriminative stimulus effects of alcohol. Additionally Chapter 4 demonstrates chemogenetic silencing of IC→AcbC projections decrease alcohol self-administration and potentiate the effects of a loading dose of alcohol (i.e., decrease intake) further implicating a role for the insular/thalamic-striatal circuitry in alcohol-related behaviors.

Therefore the goal of the present work was to test the functional role of the IC, Rh, and the IC→AcbC in modulating the effects of a loading dose of alcohol on cue-induced seeking and reinstatement of alcohol self-administration. As such, a similar chemogenetic strategy (i.e., hM4Di Designer Receptors Exclusively Activated by Designer Drugs [DREADDs]), as in Chapter 4, was implemented to silence the IC, Rh or IC →AcbC projections. Thus following testing in Chapter 4, male Long Evans rats trained to self-administer alcohol were tested in the present study. Furthermore, to test if the functional role of these regions in regulating relapse-like behaviors is specific to alcohol reinforcement, the group of hM4Di-infused rats trained to self-administer sucrose previously used in Chapter 4 were also tested. Given that chemogenetic silencing of these regions and projections increases sensitivity to alcohol (Jaramillo *et al*, 2016) and specifically IC→AcbC decreases alcohol self-administration, we hypothesized that chemogenetic silencing of these regions and the projections to the AcbC, would increase sensitivity to the loading dose of alcohol resulting in decreased alcohol-seeking and –reinstatement of alcohol self-administration, with no effect on sucrose relapse-related behavior. Given the role of interoceptive effects as potent modulators of drug-related behaviors, understanding the circuitry of these internal cues and their functional role in modulating self-administration will be important to better understand the neural mechanisms driving alcohol relapse.

METHODS

Animals

In an effort to minimize the number of animals utilized, rats from Chapter 4 were used in this study after completion of testing. That is following testing in Chapter 4, rats were used for the next respective test in Chapter 5 (e.g., Experiment 4.1 then Experiment 5.1). Refer to Chapter 4 for details.

Behavioral Procedures

Self-administration training.

The self-administration training procedures are described in detail in Chapter 4.

Relapse-like behavior following extinction.

Following testing for the studies in Chapter 4, rats underwent additional baseline self-administration session, after which extinction training sessions began. Extinction sessions (30 min) were similar to self-administration sessions except responding on either lever had no programmed consequence (i.e., no cues were presented) and no reinforcer was available. Following 14 consecutive extinction sessions in which the extinction criterion was met (i.e., 80% decrease in alcohol-lever responding) rats underwent a seeking/reinstatement test. This 30 min test was divided into two phases (i.e., seeking and reinstatement phase). The first 10 min, FR2 lever responses resulted in cue presentation but no reinforcer delivery (seeking phase). At the 10 minute mark, there was a non-contingent presentation of the cues with a single alcohol delivery (0.1 ml). For the remaining 20 min of the session, alcohol was available and the session was identical to a standard self-administration session (reinstatement of self-administration phase). For all experiments a within-subject design was used, with the preload and CNO doses experienced in a random order, and rats underwent intervening extinction sessions (14 d) between each test.

Stereotaxic Surgery

These animals received DREADD injection and cannulae placements as described in Chapter 4.

Experimental Approach

Experiment 5.1: Examination of the functional role of IC or Rh on modulating the effects of an alcohol loading dose on relapse-like behavior, through chemogenetic silencing.

IC-silencing. After completion of prior testing (as documented in **Chapter 4** [**Experiment 4.1**]) and following stable self-administration behavior, rats with IC-infused hM4Di-DREADDs (n=12) underwent 14 extinction sessions. Then to determine the functional role of the IC in modulating the effects of an alcohol loading dose on alcohol-seeking and reinstatement of alcohol self-administration, rats underwent a seeking/reinstatement test. On this test day, rats received CNO (0, 3 mg/kg, intraperitoneal [IP]), 35 min prior to an alcohol loading dose (0, 1.0 g/kg, IG). 10 min after the loading dose, rats were placed in the chambers for the seeking/reinstatement test session.

Rh-silencing. After completion of prior testing (as documented in **Chapter 4** [**Experiment 4.1**]) and following stable self-administration behavior, rats with Rh-infused hM4Di-DREADDs (n=11) underwent the identical experimental protocol as used above, to determine the functional role of the Rh in modulating the effects of an alcohol loading dose on alcohol-seeking and reinstatement of alcohol self-administration.

Experiment 5.2: Examination of the functional role of IC→AcbC on modulating the effects of an alcohol loading dose on relapse-like behavior, through chemogenetic silencing.

After completion of prior testing (as documented in **Chapter 4** [**Experiment 4.2**]) and following stable self-administration behavior, rats implanted with bilateral AcbC cannulae and infused with hM4Di-DREADDs (n=9) or Control-mCherry (n=12) in the IC, underwent 14 extinction sessions. A similar experimental protocol as used in Experiment 2 (i.e., albeit CNO microinjection) was utilized to determine a functional role of IC→AcbC in modulating the effects of an alcohol loading dose on alcohol-seeking and –reinstatement. Rats received intra-AcbC infusion of CNO (0 or 3 μM/side) 5 min prior to an alcohol loading dose (0 or 1.0 g/kg, IG). Then 10 min later commenced the seeking/reinstatement test session.

Experiment 5.3: Examination of the functional role of IC or IC→AcbC on sucrose-seeking and –reinstatement, through chemogenetic silencing.

IC-silencing. After completion of prior testing (as documented in **Chapter 4** [**Experiment 4.3**]) and following stable sucrose self-administration behavior, rats infused with hM4Di-DREADDs (n=12) in the IC, underwent 14 extinction sessions. To test the role of IC-silencing on sucrose-seeking and reinstatement of sucrose self-administration, rats underwent a seeking/reinstatement test. The identical experimental protocol as used in Experiment 5.1 (i.e., albeit no alcohol-loading dose) was used. On test day, rats received CNO (0, 3 mg/kg, IP). Then 45 min later commenced the seeking/reinstatement test session.

Intra-AcbC silencing. Following stable sucrose self-administration behavior, rats underwent 14 extinction sessions. The identical experimental protocol as used in Experiment 5.2 (i.e., albeit no alcohol-loading dose) was used, to determine a functional role of the IC→AcbC in modulating the effects of sucrose-seeking and –reinstatement. On test day, rats received intra-AcbC infusion of CNO (0 or 3 μM/side). Then 5 min later commenced the seeking/reinstatement test session.

Microinjection Procedures for Viral Vectors, and Drug Infusions

These animals received microinjections of viral vectors and intra-AcbC CNO as described in Chapter 4.

Tissue Preparation for Viral Vector and Cannulae Confirmation

Tissue for these animals was prepared and analyzed as described in Chapter 4.

Drugs

Alcohol (95% w/v) was diluted in distilled water to a concentration of 20% (v/v) and administered IG, with volumes varied by weight to obtain the desired dose. For systemic administration CNO, injected at a volume of 3 ml/kg (NIDA Drug Supply Program, NC), was

dissolved in 1% dimethyl sulfoxide in water (v/v) or in aCSF for intracranial administration. The CNO doses were chosen based on previous work (Krashes *et al*, 2011; Roth, 2016; Stachniak *et al*, 2014) and pilot studies from our lab.

Data Analysis

For all experiments, within-subject treatments were assigned in a repeated measures design and analyzed using one-way or three-way RM ANOVA. Alcohol intake (g/kg) was approximated based on body weight and number of reinforcements delivered. Post hoc analysis (Tukey) was used to determine differences between specific treatment conditions, or planned comparisons (t-tests). Statistical significance was declared at $P \leq 0.05$.

RESULTS

Experiment 5.1: Examination of the functional role of IC or Rh on modulating the effects of an alcohol loading dose on relapse-like behavior, through chemogenetic silencing.

IC- silencing.

Bilateral hM4Di-mCherry expression is represented in **Figure 5.1b** (n=8). Four rats had inaccurate placements or inefficient hM4Di-DREADD infusions (i.e., no hM4Di-mCherry expression), and were not included in the analyses and in **Figure 5.1a-c**. Baseline self-administration performance (i.e., 2 sessions prior to initiation of extinction) is shown to the left of the x-axis break (**Fig. 5.1b**) as a visual reference (i.e., not included in the overall analyses). One-way RM ANOVA of alcohol lever responses across the 14 extinction sessions demonstrated a significant effect of extinction session [$F(7, 13)=27.09$, $p<0.001$]. Indeed through the 2^{nd} to 14^{th} extinction session, alcohol lever responses significantly decreased relative to the first extinction session (<0.001; **Fig. 5.1b**), demonstrating extinction of previously reinforced-behavior. There was no effect on inactive lever responses (**Fig 5.1.b**) or locomotor activity (**Table 5.1**) across extinction sessions.

Three-way RM ANOVA of alcohol lever responding during the seeking/reinstatement test session demonstrated a significant a main effect of time [$F(2,14)=21.01$, $p<0.000$], with the greatest alcohol lever responding at the 20 min time point relative to the other time points ($p<0.05$), indicating that non-contingent presentation of the alcohol-associated cues initiated responding (i.e., self-administration). There was no main effect of CNO or alcohol loading dose. Three-way RM ANOVA of locomotor rate during the seeking/reinstatement test session (**Table 5.3**) demonstrated a significant main effect of time [$F(2,14)=21.54$, $p<0.000$] and a significant alcohol by time interaction [$F(2,14)= 4.31$, $p\leq0.04$], with decreased locomotor activity at 20 and 30 min relative to 10 min ($p<0.05$). There was no main effect of CNO or three way interaction. Additionally, three-way RM ANOVA of inactive lever responses demonstrated no significant effects (**Table 5.2**).

IC-control.

Bilateral Control-mCherry expression is represented in **Figure 5.1d** (n=7). Baseline self-administration performance (i.e., 2 sessions prior to initiation of extinction) is shown to the left of the x-axis break (**Fig. 5.1b**) as a visual reference (i.e., not included in the overall analyses). One-way RM ANOVA of alcohol lever responses across the 14 extinction sessions demonstrated a significant effect of extinction session [$F(6, 13)=25.34$, $p<0.001$]. Indeed through the 3rd to 14th extinction session, alcohol lever responses significantly decreased relative to the first day of extinction ($t= <0.001$; **Fig. 5.1e**). There was no effect on inactive lever responses (**Fig 5.1e**) or locomotor across extinction sessions (**Table 5.1**).

The three-way RM ANOVA of alcohol responses (**Fig. 5.1f**) demonstrated a significant main effect of time [$F(2,12)=13.92$, $p=0.001$], with greatest responding at 20 min compared to 10 min ($p<0.05$), indicating that non-contingent presentation of the alcohol-associated cues initiated responding (i.e., self-administration). There was also a significant main effect of alcohol loading dose [$F(1,6)=7.02$, $p\leq0.04$], and a significant time by loading dose interaction [$F(2,12)= 4.59$, $p=0.03$], with decreased alcohol responding at 20 min with the alcohol loading dose relative to

vehicle ($p<0.05$). There was no significant main effect of CNO and no significant three way interaction. Three-way RM ANOVA of locomotor rate during the seeking/reinstatement test session (**Table 5.3**) demonstrated a significant main effect of phase [$F(2,14)=13.32$, $p<0.001$], with decreased locomotor activity at 20 and 30 min relative to 10 min ($p<0.05$). There was no effect of CNO or alcohol loading dose. Additionally, three-way RM ANOVA of inactive lever responses demonstrated no significant effects (**Table 5.2**). Together these results show the lack of modulation by CNO.

Rh- silencing.

hM4D-mCherry expression is represented in **Figure 5.3a** (n=9). Baseline self-administration performance (i.e., 2 sessions prior to initiation of extinction) is shown to the left of the x-axis break (**Fig. 5.3b**) as a visual reference (i.e., not included in the overall analyses). One-way RM ANOVA of alcohol lever responses across the 14 extinction sessions demonstrated a significant effect of extinction session [$F(8, 13)=18.48$, $p<0.001$]. Indeed through the 2nd to 14th extinction session, alcohol lever responses significantly decreased relative to the first day of extinction (<0.001). There was no effect on inactive lever responses (**Fig. 5.3b**) or locomotor (**Table 5.1**) across extinction sessions.

The three-way RM ANOVA of alcohol responses (**Fig. 5.3c**) demonstrated a significant main effect of time [$F(2,16)=41.53$, $p<0.000$], with greatest responding at 20 min compared to 10 min ($p<0.05$), indicating that non-contingent presentation of the alcohol-associated cues initiated responding (i.e., self-administration). There was also a significant main effect of alcohol loading dose [$F(1,8)=40.15$, $p<0.000$], and a significant time by loading dose interaction [$F(2,16)= 14.48$, $p<0.000$], with decreased alcohol responding at 20 min with the alcohol loading dose relative to vehicle ($p<0.05$). There was no significant main effect of CNO and no significant three way interaction. Three-way RM ANOVA of locomotor rate during the seeking/reinstatement test session (**Table 5.3**) demonstrated a significant main effect of time [$F(2,16)=17.68$, $p<0.000$] and a significant time by CNO interaction [$F(2,16)=5.81$. $p\leq0.01$], with decreased locomotor activity at 20 and 30 min

relative to 10 min ($p<0.05$). There was no effect of alcohol loading dose. Additionally, three-way RM ANOVA of inactive lever responses demonstrated no significant effects (**Table 5.2**). Together these results show no significant modulation by CNO.

Experiment 5.2: Examination of the functional role of IC→AcbC on modulating the effects of an alcohol loading dose on relapse-like behavior, through chemogenetic silencing.

Intra-AcbC silencing.

Bilateral hM4Di-mCherry expression and AcbC injector placements (red circles) are represented in **Figure 5.4a** (n=7). One rat did not complete testing and another rat had inefficient hM4Di-DREADD infusions (i.e., no hM4Di-mCherry expression), and are not shown nor included in **Figure 5a-c**. Baseline self-administration performance (i.e., 2 sessions prior to initiation of extinction) is shown to the left of the x-axis break (**Fig. 5b**) as a visual reference (i.e., not included in the overall analyses). One-way RM ANOVA of alcohol lever responses across the 14 extinction sessions demonstrated a significant effect of extinction session [$F(8, 13)=19.56$, $p<0.001$]. Indeed through the 1st to 14th extinction session, alcohol lever responses significantly decreased relative to the first extinction session (<0.001), demonstrating extinction of previously reinforced-behavior. There was no effect on inactive lever responses (**Fig. 5b**) or locomotor activity (**Table 5.1**) across extinction sessions.

The effect of silencing IC→AcbC projections on alcohol lever responses is illustrated in (**Fig. 5c**). The three-way RM ANOVA showed no significant main effects of time, alcohol loading dose, or CNO. However, there was a significant CNO dose, by alcohol loading dose, by time interaction [$F(2,12)=4.03$, $p\leq0.05$]. Under water loading dose vehicle conditions, there was significant reinstatement of alcohol lever responding as indicated by an increase in responding at the 20 min time point relative to the 10 (i.e., extinction; $p<0.05$). In contrast, this pattern of responding was not observed under the water loading dose and intra-AcbC CNO treatment, indicating that silencing the

IC→AcbC projection prevented reinstatement. Under vehicle conditions, there was a significant effect of alcohol loading dose at the 20 min time point, indicating that non-contingent presentation of the alcohol-associated cues initiated responding (i.e., self-administration). There was no effect of alcohol loading dose under the CNO condition. Three-way RM ANOVA of locomotor rate during the seeking/reinstatement test session (**Table 5.3**) demonstrated a significant main effect of time [$F(2,12)=20.71$, $p<0.000$], with decreased locomotor activity at 20 and 30 min relative to 10 min ($p<0.05$). There was no effect of CNO or alcohol loading dose. Additionally, three-way RM ANOVA of inactive lever responses demonstrated no significant effects (**Table 5.2**). Overall, these results show silencing of IC→AcbC projections prevented reinstatement and are unaffected by the alcohol loading dose.

Intra-AcbC control.

Bilateral control-mCherry expression and AcbC injector placements (blue circles) are represented in **Figure 5.4a** (n=7). Two rats did not complete testing and three rats had inefficient control-mCherry infusions (i.e., no hM4Di-mCherry expression), and are not shown nor included in **Figure 5a-c** Baseline self-administration performance (i.e., 2 sessions prior to initiation of extinction) is shown to the left of the x-axis break (Fig. 5.1b) as a visual reference (i.e., not included in the overall analyses). One-way RM ANOVA of alcohol lever responses across the 14 extinction sessions demonstrated a significant effect of extinction session [$F(6, 13)=10.59$, $p<0.001$]. Indeed through the 2nd to 14th extinction session, alcohol lever responses significantly decreased relative to the first day of extinction ($p<0.05$; Fig. 5.1e). There was no effect on inactive lever responses (Fig 5.1e) or locomotor across extinction sessions (Table 5.1).

The three-way RM ANOVA of alcohol responses (Fig. 5.1f) demonstrated a significant main effect of time [$F(2,10)=66.13$, $p<0.000$], with greatest responding at 20 min compared to 10 min ($p<0.05$), indicating that non-contingent presentation of the alcohol-associated cues initiated responding (i.e., self-administration). There was also a significant between-subjects main effect of

alcohol loading dose [$F(1,5)=53.50$, $p\leq0.01$], and a significant time by loading dose interaction [$F(2,10)= 50.78$, $p<0.000$], with decreased alcohol responding at 20 min with the alcohol loading dose relative to vehicle ($p<0.05$). There was no significant main effect of CNO and no significant three way interaction. Three-way RM ANOVA of locomotor rate during the seeking/reinstatement test session (Table 5.3) demonstrated a significant main effect of phase [$F(2,10)=10.44$, $p<0.004$], with decreased locomotor activity at 20 and 30 min relative to 10 min ($p<0.05$). There was no effect of CNO or alcohol loading dose. Additionally, three-way RM ANOVA of inactive lever responses demonstrated no significant effects (Table 5.2). Together these results show the lack of modulation by CNO.

Experiment 5.3: Examination of the functional role of IC and IC→AcbC on modulating the effects of relapse-like behavior on sucrose self-administration, through chemogenetic silencing.

Bilateral hM4Di-mCherry expression and AcbC injector placements (red circles) are represented in **Figure 5.4a** (n=6). Two rats did not complete testing and 4 rats had inefficient hM4Di-DREADD infusions (i.e., no hM4Di-mCherry expression), and are not shown nor included in **Figure 5a-c**. Baseline self-administration performance (i.e., 2 sessions prior to initiation of extinction) is shown to the left of the x-axis break (**Fig. 5b**) as a visual reference (i.e., not included in the overall analyses). One-way RM ANOVA of alcohol lever responses across the 14 extinction sessions demonstrated a significant effect of extinction session [$F(5, 13)=21.47$, $p<0.001$]. Indeed through the 2^{nd} to 14^{th} extinction session, alcohol lever responses significantly decreased relative to the first extinction session (<0.001), demonstrating extinction of previously reinforced-behavior. There was no effect on inactive lever responses (**Fig. 5b**) or locomotor activity (**Table 5.1**) across extinction sessions.

IC-silencing.

The effect of IC silencing on sucrose lever responses is illustrated in (**Fig. 5c**). The two-way RM ANOVA showed a significant a main effect of time [$F(2,10)=26.36$, $p<0.001$], with the greatest sucrose lever responding at the 20 min time point relative to the other time points ($p<0.05$), indicating that non-contingent presentation of the sucrose-associated cues initiated responding (i.e., self-administration). There was also a significant time by CNO interaction [$F(2,10)=3.95$, $p\leq0.05$], with decreased sucrose responding at 20 min with CNO relative to vehicle ($p<0.05$). Two-way RM ANOVA of locomotor rate during the seeking/reinstatement test session (**Table 5.3**) demonstrated a significant main effect of time [$F(2,10)=4.66$, $p\leq0.04$], with decreased locomotor activity at 30 min relative to 10 min ($p<0.05$). There was no effect of CNO. Additionally, two-way RM ANOVA of inactive lever responses demonstrated no significant effects (**Table 5.2**). Overall, these results show silencing of the IC attenuated reinstatement of sucrose self-administration.

Intra-AcbC silencing.

The effect of IC→AcbC silencing on alcohol lever responses is illustrated in (**Fig. 5c**). The two-way RM ANOVA of alcohol responses (Fig. 5.1f) demonstrated a significant main effect of CNO [$F(1,10)=9.60$, $p\leq0.03$], with decreased sucrose responding. There was no main effect of time or two-way interaction, thus indicating no effect by the non-contingent presentation of the sucrose-associated cues on responding. Two-way RM ANOVA of locomotor rate during the seeking/reinstatement test session (Table 5.3) demonstrated a significant main effect of time [$F(2,10)=43.48$, $p<0.001$], with decreased locomotor activity at 20 and 30 min relative to 10 min ($p<0.05$). There was no effect of CNO. Additionally, two-way RM ANOVA of inactive lever responses demonstrated no significant effects (Table 5.2). Together these results show the lack of modulation by CNO.

DISCUSSION

The present findings demonstrate the IC projections to the AcbC modulate reinstatement of alcohol self-administration in a reinforcer-specific manner, implicating a role for the insular-striatal circuitry in modulating relapse. Here we reliably demonstrate reinstatement of alcohol self-administration, following extinction of alcohol-reinforced behavior, in rats trained on an operant self-administration paradigm. Furthermore, we demonstrate alcohol pre-exposure (i.e., alcohol loading dose), blocks reinstatement of alcohol self-administration. Contrary to our hypothesis, chemogenetic silencing of IC or Rh did not affect relapse-like behaviors (alcohol-seeking or reinstatement of alcohol self-administration), but significantly attenuated reinstatement of sucrose self-administration. However, following specific chemogenetic silencing of IC→AcbC projections, reinstatement of alcohol self-administration was attenuated, but not alcohol-seeking. Furthermore, chemogenetic silencing of the IC→AcbC projections affected reward-related behavior (in general) during sucrose-seeking and reinstatement of sucrose-drinking, although statistically we did not observe reinstatement of behavior under vehicle conditions. Furthermore, these findings demonstrate the complexity of the insular circuitry in modulating previously-reinforced behavior. Additionally, these findings implicate a role for the AcbC and specifically insular-striatal circuit, in modulating the devaluation of behavior associated with alcohol-reinforcement, thus resulting in attenuated reinstatement of alcohol but not alcohol-seeking.

An important goal of this study was to investigate alcohol seeking and reinstatement of alcohol self-administration following extinction training and the effect of an alcohol loading dose on these relapse-like behaviors. Traditional alcohol reinstatement findings demonstrate that an acute loading dose of alcohol can "prime" previously extinguished alcohol responding (in the absence of alcohol, (Le *et al*, 1998; Samson and Chappell, 2002). However, previous work from our lab demonstrates decreased alcohol-seeking following a moderate alcohol loading dose of 1 g/kg

(Randall *et al*, 2015), an effect others have demonstrated to be independent of volume (Czachowski *et al*, 2006). Thus, we hypothesized that the interoceptive effects of an alcohol loading dose would decrease both seeking and reinstatement of alcohol self-administration. Contrary to our hypothesis the alcohol loading dose did not affect behavior under the "alcohol-seeking" conditions (albeit trends for a decrease). This is in contrast to our previous findings demonstrating a moderate alcohol loading dose decreased alcohol-seeking. However, in that work, the test was under "probe-extinction" conditions in which alcohol-reinforced behavior had not been extinguished (i.e., no extinction history), Another explanation for the discrepancy in findings is that in the present study alcohol-seeking behavior was examined for 10 min, which may explain the lack of a loading dose effect as the previous studies investigated alcohol seeking for 20-30 min (Czachowski *et al*, 2006; Randall *et al*, 2015). However in line with our hypothesis, here we demonstrate that following the 10 min under alcohol-seeking conditions, a non-contingent presentation of alcohol (0.1 ml) reliably reinstated of alcohol drinking. Furthermore, we demonstrate that an alcohol loading dose (i.e., alcohol pre-exposure) effectively blocked reinstatement of alcohol self-administration, an effect also shown by others following a self-administered alcohol loading dose (1 g/kg; Samson and Chappell, 2002). Together the present study and others, implicate a role for the interoceptive effects of an alcohol loading dose to generally decrease relapse-like behaviors likely related to satiation processes (Czachowski *et al*, 2006; Randall *et al*, 2015; Samson *et al*, 2003).

Another goal of the present study was to investigate the role of the IC and Rh in modulating relapse-like effects. Given that silencing the IC or Rh produces partial alcohol-like effects and potentiate the interoceptive effects of alcohol (Chapters 2 and 3, respectively), we hypothesized that chemogenetic silencing of these regions and the projections to the AcbC, would increase sensitivity to the loading dose of alcohol resulting in decreased alcohol-seeking and reinstatement of alcohol self-administration. Contrary to our hypothesis silencing all activity in the IC or Rh did not affect alcohol seeking or reinstatement of alcohol self-administration. Furthermore, silencing the Rh did not affect the ability for the alcohol loading dose to block alcohol-seeking and reinstatement of alcohol

drinking. Additionally, we hypothesized that the control-mCherry group trained to self-administer alcohol and the hM4Di-mCherry group trained to self-administer sucrose would be unaffected by CNO. Indeed, silencing the IC did not disrupt alcohol self-administration behavior in the control-mCherry group, indicating no off target effects of CNO in non-DREADD expressing controls. Interestingly, silencing the IC attenuated reinstatement of sucrose self-administration in the hM4Di-mCherry group, indicating a reinforcer-specific role of the IC (in general) on sucrose self-administration. These findings on the reinstatement of sucrose self-administration are in contrast to our findings demonstrating no role for the IC in modulating reinstatement of alcohol self-administration. Furthermore, given that we demonstrate no role of the IC in modulating ongoing sucrose self-administration (Chapter 5), together these findings implicate the IC in modulating previously extinguished reinforced-behavior, specific to sucrose or a non-drug reward.

The lack of modulation by the IC and Rh on alcohol-seeking and reinstatement of alcohol drinking is in line with our previous findings (Chapter 4) demonstrating no effect of IC or Rh silencing on ongoing alcohol self-administration. Thus, despite the suggested role for the Rh in modulating behavioral inhibition and motivation (Cassel *et al*, 2013; Cholvin *et al*, 2013; Prasad *et al*, 2013), our findings do not suggest a role for the Rh in modulating alcohol-seeking and reinstatement of alcohol self-administration and similar to the strategy in Chapter 4, we did not pursue examination of Rh→AcbC circuitry. However, given the findings in Chapter 3 implicating a role for the Rh→AcbC projections in modulating the interoceptive effects of alcohol, it will be important for future studies to investigate the potential role of this circuitry. Given that our previous findings demonstrate IC→AcbC silencing decreases alcohol self-administration and increases sensitivity to a moderate alcohol loading dose, the specific role of IC→AcbC was further investigated.

Silencing of IC→AcbC projections did not affect alcohol-seeking but did block reinstatement of alcohol drinking (an effect absent in the control-mCherry group), following the extinction of alcohol-reinforced responding. Although previous studies have demonstrated a role for the IC in modulating relapse-like behaviors of other drugs of abuse (Cosme *et al*, 2015; Hamlin *et al*, 2007;

Wu *et al*, 2014), this is the first study to demonstrate a specific role for the IC→AcbC in modulating reinstatement of alcohol self-administration behavior. The lack of modulation by IC→AcbC on alcohol-seeking, indicates a unique role for the circuit in modulating behavior following access to alcohol and unaffected by alcohol-associated external cues (e.g., light stimuli). Additionally, IC→AcbC silencing following the loading dose of alcohol, did not further potentiate reductions in reinstatement of self-administration, as behavior was likely at a floor effect. Although not tested, utilizing a lower loading dose (i.e., 0.5 g/kg) may have produced informative results as the effects of IC→AcbC silencing potentiated a 0.5 g/kg alcohol loading dose in Chapter 4.

Furthermore, our findings regarding IC→AcbC silencing on sucrose-seeking and reinstatement are difficult to interpret because under vehicle conditions, reinstatement of sucrose self-administration was absent (i.e., no significant reinstatement). The lack of sucrose reinstatement can be attributed to extensive testing history (i.e., Chapter 4 and 5 testing) which is also noted in the alcohol self-administration hMDi-mCherry group (Fig 5.3) by their low level of reinstatement (albeit significant). Together our findings demonstrate a role for the insular circuitry (in general) in modulating previously extinguished reinforcement-behavior reinstatement and, a specific role for the IC→AcbC projections (i.e., not general IC outgoing projections) in modulating alcohol self-administration and relapse-like behavior and further implicate an interoceptive specific role in outcome devaluation of alcohol related-behaviors.

Together these data identify a role for the IC→AcbC circuit in modulating reinstatement of alcohol self-administration but not alcohol-seeking. Here we consistently demonstrate reinstatement of alcohol drinking following previous extinction of reinforced behavior. Furthermore we demonstrate blunted reinstatement following an alcohol loading dose. The present findings show IC→AcbC silencing blunts reinstatement of alcohol drinking, an effect similar to the alcohol loading dose. Thus these findings, together with our previous studies demonstrating IC→AcbC silencing increases sensitivity to alcohol, implicate a role for the IC in modulating outcome value regarding alcohol-reinforced behaviors. Furthermore the lack of effects of global IC and Rh silencing

demonstrate the complex role of interoceptive effects and behavior. Together with the previous data, these findings inform us of the complex IC structure while providing evidence of the critical nature of insular-striatal circuitry in modulating alcohol-related behaviors.

Inactive Lever Responses (per 10 min)

Phase:	Seeking				Reinstatement							
Time (m):	*0-10*				*10-20*				*20-30*			
Loading Dose (1 g/kg, IG)	Water		Alcohol		Water		Alcohol		Water		Alcohol	
	Vehicle	CNO	Vehicle	CNO	Vehicle	CNO	Vehicle	CNO	Vehicle	CNO	Vehicle	CNO
Experiment 5.1												
Intra-Insula (hM4Di)	0.5±0.3	0.4±0.3	0.0±0.0	0.1±0.1	0.0±0.0	0.0±0.0	0.0±0.0	0.4±0.4	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Intra-Insula (control-mCherry)	0.3±0.2	1.0±0.4	0.7±0.5	0.1±0.1	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.1±0.1	0.0±0.0	0.0±0.0	0.0±0.0
Intra-Rhomboid	1.1±0.6	1.3±0.6	0.3±0.2	0.3±0.2	0.0±0.0	0.0±0.0	0.1±0.1	0.0±0.0	0.0±0.0	0.3±0.3	0.0±0.0	0.6±0.6
Experiment 5.2												
Intra-AcbC (hM4Di)	0.2±0.2	1.0±1.0	0.0±0.0	0.1±0.1	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.1±0.1	0.1±0.1
Intra-AcbC (control-mCherry)	0.0±0.0	0.0±0.0	0.3±0.3	0.3±0.3	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Experiment 5.3												
Intra-Insula	0.5±0.3	0.7±0.2	_	_	0.3±0.3	0.0±0.0	_	_	0.5±0.5	0.0±0.0	_	_
Intra-AcbC	0.3±0.2	0.0±0.0	_	_	0.0±0.0	0.0±0.0	_	_	0.0±0.0	0.0±0.0	_	_

Table 5.1- Inactive lever responses (mean ± S.E.M.).

Locomotor Beam Breaks (per 10 min)

Phase:	Seeking				Reinstatement							
Time (m):	*10*				*20*				*30*			
Loading Dose (1 g/kg, IG)	Water		Alcohol		Water		Alcohol		Water		Alcohol	
	Vehicle	CNO	Vehicle	CNO	Vehicle	CNO	Vehicle	CNO	Vehicle	CNO	Vehicle	CNO
Experiment 5.1												
Intra-Insula (hM4Di)	19.0±2.2	20.8±1.9	17.4±2.7	16.9±2.2	9.5±1.3#	10.0±1.4	12.7±1.6	12.9±1.6#	10.4±1.7	12.7±3.1	8.9±2.8#	10.2±1.6
Intra-Insula (control-mCherry)	23.9±2.6	14.6±3.4	15.1±3.1	19.6±2.9	13.2±2.7	13.8±5.3	19.5±4.0	14.9±3.5#	13.4±4.0	21.0±3.2	16.2±4.2	16.0±3.8
Intra-Rhomboid	21.9±2.1	12.9±1.1	12.0±1.9	19.0±1.9	14.1±5.6	9.2±1.0#	19.6±2.9	10.0±2.4#	12.0±2.1	19.1±1.6	19.2±3.4	15.0±2.6
Experiment 5.2												
Intra-AcbC (hM4Di)	25.8±2.9	35.9±5.6	26.7±3.3	24.2±3.6	15.4±2.2	19.9±3.0	28.7±5.8	14.6±2.8#	14.9±3.3	16.3±3.0	17.0±3.7	16.0±3.7
Intra-AcbC (control-mCherry)	29.8±3.9	25.8±4.2	37.3±7.9	23.8±7.8	15.1±5.6	17.7±2.5	12.4±1.0	10.9±2.7#	26.2±7.4	8.9±4.2#	23.5±3.5	5.9±2.9#
Experiment 5.3												
Intra-Insula	22.4±1.9	24.6±3.4	-	-	19.9±3.2	17.0±2.0	-	-	13.9±1.9	13.8±1.3	-	-
Intra-AcbC	30.4±4.2	35.8±4.6	-	-	18.1±2.7	20.1±3.6	-	-	18.0±3.4	15.3±3.0	-	-

Table 5.2- Locomotor rate (mean ± S.E.M.).

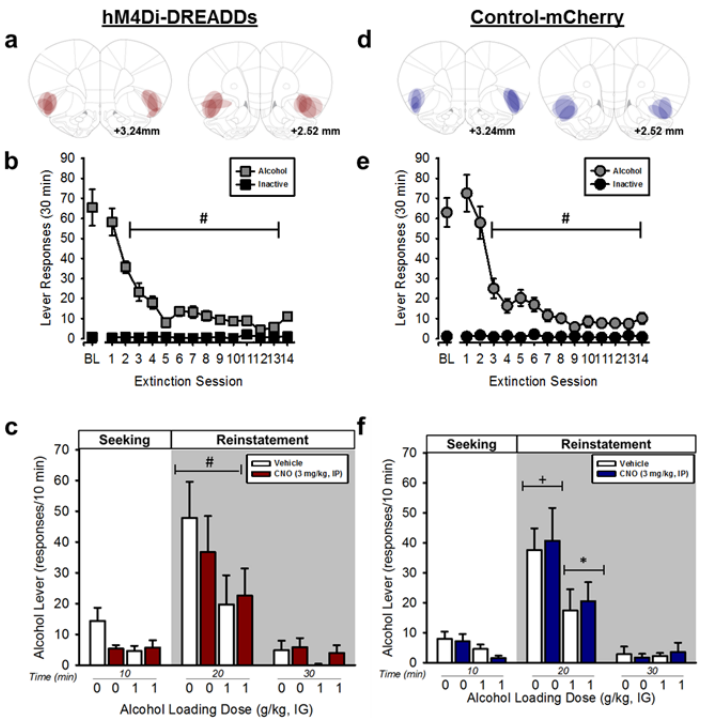

Figure 5.1- Chemogenetic silencing of IC does not modulate reinstatement of alcohol drinking or the effect of the alcohol loading dose.

(a) Intra-IC hM4Di-mCherry expression from individual rats trained to self-administer alcohol (n=8). **(b)** Lever responses across 14 extinction sessions, demonstrating extinction of previous alcohol reinforced-behavior. Baseline self-administration performance is shown to the left of the x-axis break. (**c**) Alcohol lever responses during the alcohol seeking/reinstatement test session, demonstrating increased responding following the availability of alcohol. There was no effect of CNO. **(d)** Intra-IC control-mCherry expression from individual rats trained to self-administer alcohol (n=7). **(e)** Lever responses across 14 extinction sessions, demonstrating extinction of previous alcohol reinforced-behavior. Baseline self-administration performance is shown to the left of the x-axis break. (**f**) Alcohol lever responses during the alcohol seeking/reinstatement test session, demonstrating increased responding following the availability of alcohol, with significantly less responding following the alcohol loading dose. There was no effect of CNO. #Significant main effect of time (two-way or three-way RM ANOVA, $p<0.05$). +Significant difference from 10 min (Tukey, $p<0.05$). *Significant difference from water loading dose (Tukey, $p<0.05$). Values on graphs represent mean ± S.E.M.

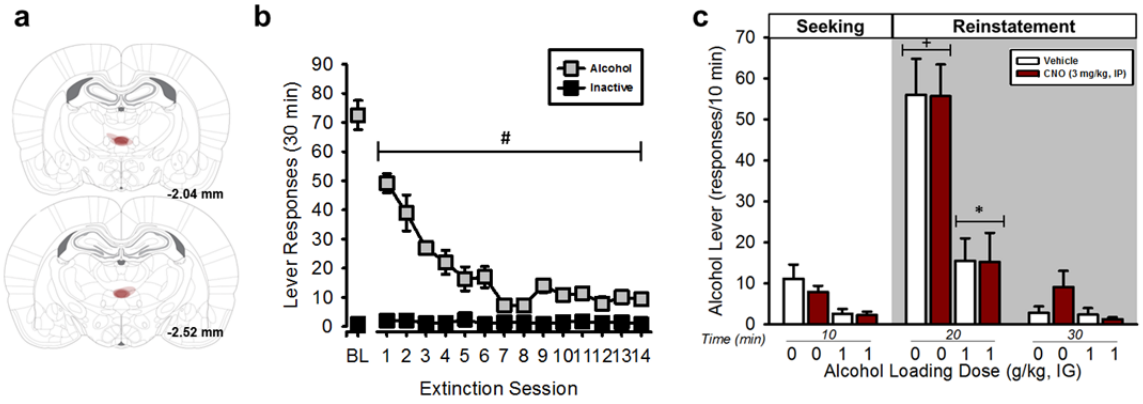

Figure 5.2- Chemogenetic silencing of Rh does not modulate reinstatement of alcohol drinking or the effect of the alcohol loading dose.

(a) Intra-Rh hM4Di-mCherry expression from individual rats trained to self-administer alcohol (n=9).

(b) Lever responses across 14 extinction sessions, demonstrating extinction of previous alcohol reinforced-behavior. Baseline self-administration performance is shown to the left of the x-axis break.

(**c**) Alcohol lever responses during the alcohol seeking/reinstatement test session, demonstrating increased responding following the availability of alcohol. There was no effect of CNO. #Significant main effect of time (two-way RM ANOVA, $p<0.05$). +Significant difference from 10 min (Tukey, $p<0.05$). *Significant difference from water loading dose (Tukey, $p<0.05$). Values on graphs represent mean ± S.E.M.

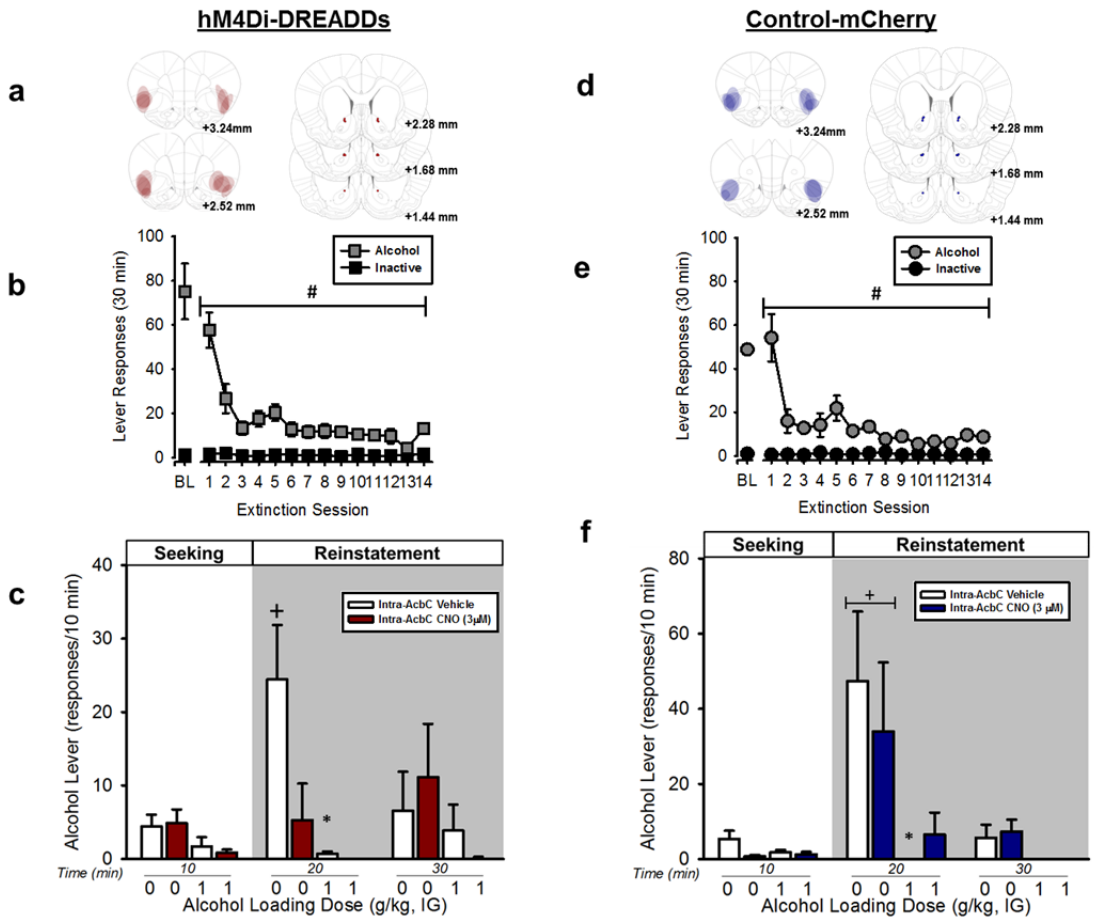

Figure 5.3- Chemogenetic silencing of IC→AcbC projections blocks reinstatement of alcohol drinking and the effect of the alcohol loading dose.

(a) Intra-IC hM4Di-mCherry expression from individual rats trained to self-administer alcohol (n=7).

(b) Lever responses across 14 extinction sessions, demonstrating extinction of previous alcohol reinforced-behavior. Baseline self-administration performance is shown to the left of the x-axis break.

(**c**) Alcohol lever responses during the alcohol seeking/reinstatement test session, demonstrating increased responding following the availability of alcohol, with significantly less responding following the alcohol loading dose under vehicle conditions. Silencing of IC→AcbC prevented reinstatement under the water loading dose and was unaffected by the alcohol loading dose. +Significant difference from 10 min (Tukey, $p<0.05$). *Significant difference from water loading dose (Tukey, $p<0.05$). Values on graphs represent mean ± S.E.M.

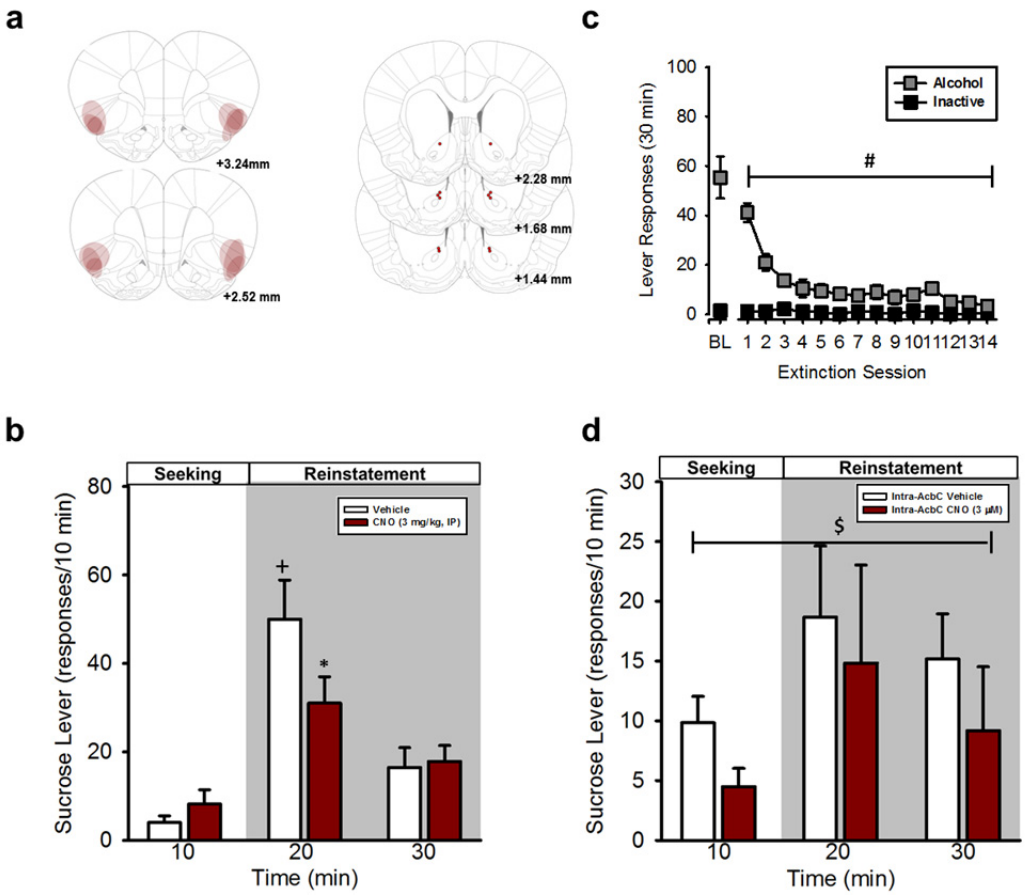

Figure 5.4- Chemogenetic silencing of IC or IC→AcbC projections attenuated reinstatement and general reward-related behavior, respectively.

(a) Intra-IC hM4Di-mCherry expression from individual rats trained to self-administer sucrose (n=6).

(b) Lever responses across 14 extinction sessions, demonstrating extinction of previous sucrose reinforced-behavior. Baseline self-administration performance is shown to the left of the x-axis break.

(**c**) Sucrose lever responses during the sucrose seeking/reinstatement test session, demonstrating increased responding following the availability of sucrose. Silencing of IC by CNO attenuated reinstatement of sucrose self-administration. (**d**) Lever responses across 14 extinction sessions, demonstrating extinction of previously reinforced-behavior. Baseline self-administration performance is shown to the left of the x-axis break. (**e**) Sucrose lever responses during the sucrose seeking/reinstatement test session, demonstrating increased responding following the availability of sucrose. Silencing of IC→AcbC, by intra-AcbC CNO had no effects. +Significant difference from 10 min (Tukey, $p<0.05$). Values on graphs represent mean ± S.E.M.

CHAPTER 6: OVERALL DISCUSSION

Despite the well-established role of drug-induced interoceptive effects as potent modulators of drug-intake and relapse (Naqvi and Bechara, 2010; Paulus and Stewart, 2014; Verdejo-Garcia *et al*, 2012), the brain circuitry modulating those effects and behaviors remains understudied. The current literature demonstrates a prominent role for the AcbC in modulating drug self-administration and reinstatement (Chaudhri *et al*, 2008; Chaudhri *et al*, 2010; Gass *et al*, 2011; Griffin *et al*, 2014; Rassnick *et al*, 1992a; Rassnick *et al*, 1992b) and suggests a central role for the AcbC in regulating the discriminative stimulus effects of alcohol (Besheer *et al*, 2003; Besheer *et al*, 2010; Besheer *et al*, 2009; Hodge and Alken, 1996; Hodge and Cox, 1998; Hodge *et al*, 2001b). The goal of the present dissertation was to broaden our understanding of the AcbC-related circuitry by investigating two brain regions with projections to the AcbC, the IC and Rh. Thus, the goal of **Aim 1** was to investigate the striatal circuitry modulating the interoceptive effects of alcohol. Additionally, titration of alcohol self-administration and relapse-like drinking is sensitive to the interoceptive effects produced by a loading dose of alcohol (i.e., alcohol pre-exposure; Czachowski *et al*, 2006; Randall *et al*, 2015; Samson *et al*, 2002; Samson *et al*, 2003). Therefore, the goal of **Aim 2** was to examine the striatal circuitry modulating sensitivity to the effects of an alcohol loading dose on alcohol self-administration, alcohol-seeking, and reinstatement of alcohol drinking. We hypothesized that silencing the insular/thalamic-striatal circuit would potentiate the interoceptive effects of alcohol. Moreover, we hypothesized that silencing activity in the insular/thalamic-striatal circuit would potentiate the effects of an alcohol loading dose on alcohol self-administration, seeking, and reinstatement of alcohol drinking (i.e., further decrease the behaviors). Overall, the present findings

demonstrate that suppression of the insular/thalamic-striatal circuit is important for the expression of the interoceptive effects of alcohol. Furthermore, we demonstrate the insular-striatal circuit modulates ongoing alcohol intake and reinstatement of alcohol self-administration. Together, results from the studies within the present dissertation provide a novel role for the insular/thalamic-striatal circuit in modulating sensitivity to alcohol and implicate the insular-striatal circuit in modulating alcohol-reinforced behavior.

INTEROCEPTIVE CIRCUITRY ON SELF-ADMINISTRATION BEHAVIOR

Numerous preclinical and clinical findings consistently demonstrate that the interoceptive effects produced by a low alcohol dose can prime alcohol-related behaviors, including craving, relapse, and additional or increased alcohol intake (Bigelow *et al*, 1977; de Wit and Chutuape, 1993; Gass and Olive, 2007; Hodgson *et al*, 1979; Kirk and de Wit, 2000; Le *et al*, 1998; Vosler *et al*, 2001). Conversely, likely related to processes such as satiation or devaluation, pretreatment with a high alcohol dose (i.e., loading dose as used in the present work) can decrease alcohol self-administration, alcohol-seeking, and relapse-like drinking (Czachowski *et al*, 2006; Randall *et al*, 2015; Samson *et al*, 2003). The present findings also demonstrate titration of self-administration and relapse-like drinking as a consequence of the interoceptive effects produced by a loading dose of alcohol. Furthermore, we demonstrate that inhibition of the insular/thalamic-striatal circuit enhances sensitivity to alcohol and inhibition of the insular-striatal circuit decreases ongoing and reinstatement of self-administration, likely by producing alcohol-like effects. However, despite the findings that silencing outgoing IC and Rh projections (i.e., systemic CNO) potentiate the interoceptive effects of alcohol, inhibition surprisingly had no effect on alcohol self-administration, alcohol-seeking, reinstatement of alcohol self-administration and did not potentiate the alcohol loading dose effect. This would suggest that interoceptive circuitry differentially recruit motivational circuits, resulting in different behavioral outputs. Only chemogenetic silencing of the IC→AcbC projections resulted in

decreased alcohol self-administration and attenuated reinstatement of alcohol self-administration, thus demonstrating the specific recruitment of insular-striatal interoceptive circuitry in modulating alcohol self-administration and relapse-like behaviors.

Insular-Striatal Circuit

The present findings demonstrate that chemogenetic silencing of the insular-striatal circuit increases sensitivity to alcohol and decreases ongoing and reinstatement of alcohol self-administration, which suggest that the insular-striatal circuit is a site of action for alcohol. This is corroborated by our findings demonstrating changes in neuronal activity within the IC and IC→AcbC following alcohol (vs water) in rats trained to discriminate alcohol and behavior/alcohol-naïve rats, respectively. Additionally, by investigating the role of the insular-striatal circuit across various behavioral paradigms (i.e., alcohol discrimination and self-administration), the present study allows us to obtain a general understanding of the insular-striatal circuit under different behavioral conditions. Together these findings implicate a role for IC→AcbC particularly in reference to behaviors affected by the interoceptive effects of alcohol and reward associations. Additionally, the present study demonstrates that chemogenetically silencing the IC→AcbC decreases alcohol self-administration of alcohol in a model of moderate alcohol self-administration. Conversely (Seif *et al*, 2013) demonstrates that optogenetic inactivation of the IC→AcbC decreases alcohol consumption (i.e., home-cage drinking) of adulterated alcohol (i.e., quinine) in a model of compulsive alcohol drinking. However, optogenetic inactivation of the IC→AcbC did not affect alcohol consumption under non-aversive conditions, which is in contrast to our findings in which we found decreased self-administration following chemogenetic silencing of IC→AcbC projections. The differences in our findings may be due to the different motivational processes recruited to obtain alcohol (i.e., self-administration vs. drinking) and the difference in alcohol history and intake between the two models (i.e., moderate vs. high). Despite the different results, together these findings further implicate the complex role of the insular-striatal circuit in modulating varying stages (i.e., moderate to compulsive)

and doses of alcohol intake. Moreover, the present study demonstrates that only specific suppression of the insular-striatal circuit (vs. all insular outgoing projections) decreases ongoing alcohol self-administration and attenuates reinstatement of alcohol self-administration. Together with the existing literature, our findings implicate that the insular-striatal circuit is necessary for modulating titration of alcohol drinking possibly related to a homeostatic balance (i.e., "ideal" interoceptive state) which may vary among models of alcohol-use.

Thalamic-Striatal Circuit

The present findings are the first to implicate a role for the Rh in modulating alcohol-like effects. Although, the present findings do not demonstrate neuronal response to alcohol (vs water) within the Rh→AcbC of behavior/alcohol-naïve rats, we do demonstrate a decrease in Rh neuronal activity in animals trained to discriminate alcohol, confirming Rh as a site of action for alcohol. Furthermore, we demonstrate that silencing activity within the Rh and Rh→AcbC potentiated sensitivity to the interoceptive effects of alcohol. However, contrary to our hypothesis, Rh did not modulate ongoing alcohol self-administration, seeking, or reinstatement of alcohol drinking. Together these findings demonstrate that modulation of the Rh circuitry does not directly affect alcohol-reinforced behavior, despite the role for the Rh in producing alcohol-like effects. Given our findings demonstrating the recruitment of interoceptive circuitry under different behavior conditions (e.g., all outgoing vs. site-specific IC projections), it may be a specific task or contingency that requires the recruitment of Rh and Rh→AcbC circuitry. For example, it will be interesting for future work to investigate the role of Rh→AcbC in modulating extinction learning given that the Rh is implicated in modulating cue-induced behavior, particularly under conditions that require behavioral flexibility (Prasad *et al*, 2013). As such, we hypothesize that silencing the Rh or Rh→AcbC during extinction learning may block extinction of alcohol-reinforced behavior. Furthermore, given that the Rh is proposed to integrate various inputs to affect psychological, affective, and cognitive functions required to induce behavioral flexibility in a changing environment (Cassel *et al*, 2013; Cholvin *et al*,

2013; Prasad *et al*, 2013), behavioral processes often associated with drug self-administration and relapse-like behavior, it will be important for future studies to investigate the role of the Rh in modulation of other drugs of abuse, and to examine the potential involvement in alcohol self-administration under other conditions.

STRIATAL IMPLICATIONS

In general, as reflected in the alcohol discrimination literature, pharmacological manipulations that result in CNS inhibition (e.g., $GABA_A$ agonists, NMDA antagonist) tend to have "alcohol-like" effects (Grant and Colombo, 1993b; Hiltunen and Jarbe, 1989; Hodge and Alken, 1996; Hodge and Cox, 1998; Hodge *et al*, 2001b). Specifically, general inhibition in the AcbC has been shown to modulate sensitivity to alcohol, as competitive $GABA_A$ agonists and noncompetitive NMDA antagonist fully substitute for the interoceptive effects of alcohol (Besheer *et al*, 2003; Hodge and Alken, 1996; Hodge and Cox, 1998; Hodge *et al*, 2001b). The findings in the present dissertation are in line with the existing literature as we demonstrate that pharmacologically inhibiting (i.e., with $GABA_A$ and $GABA_B$ agonists [muscimol+baclofen cocktail infusion]) the Rh or IC, regions with presumably glutamatergic projections to the AcbC, results in partial substitution for the discriminative stimulus effects of alcohol. Additionally, we demonstrated that chemogenetic silencing of the IC and Rh, and silencing the IC and Rh outgoing projections to the AcbC through DREADD-induced intrinsic G_i signaling, potentiates sensitivity to alcohol. Together, these findings implicate GABAergic and G_i signaling within the IC and Rh and specifically G_i signaling within the insular/thalamic-striatal circuits in modulating the interoceptive effects of alcohol. Given the previous literature, silencing IC/Rh→AcbC may enhance sensitivity to alcohol through a similar mechanism as seen following pharmacological inhibition of the AcbC which produced alcohol-like effects (Besheer *et al*, 2003; Hodge and Alken, 1996; Hodge and Cox, 1998; Hodge *et al*, 2001b).

Furthermore, we demonstrate that silencing the IC/Rh→AcbC increased sensitivity to alcohol and silencing the IC→AcbC decreased ongoing alcohol self-administration, likely due to its alcohol-

like effects. Although not investigated in the present study, future work will need to investigate the neuronal subpopulations in the AcbC that the IC and Rh projections innervate. Those studies could offer insight onto the differing roles of the IC→AcbC and Rh→AcbC in modulating alcohol-reinforcement as the IC and Rh could be innervating differential signaling processes. Presumably, the IC and Rh afferent inputs to the AcbC are glutamatergic. Thus, by chemogenetically silencing the IC and Rh projections to the AcbC, which enhance sensitivity to alcohol, we presumably decrease glutamatergic activity in the AcbC. Together with the literature, which demonstrates that general inhibition in the AcbC produces alcohol-like effects (Besheer *et al*, 2003; Hodge and Alken, 1996; Hodge and Cox, 1998; Hodge *et al*, 2001b) (similar to our findings following chemogenetic silencing of IC→AcbC and Rh→AcbC projections), our findings may suggest that the IC and Rh projections are synapsing on NMDA-expressing medium spiny neurons in the AcbC, which are the vast majority of striatal neurons (Kreitzer, 2009). Furthermore, considerable evidence exists demonstrating that depletion of dopamine in the nucleus accumbens can attenuate reinforced-behavior (Salamone and Correa, 2002). Thus, when chemogenetically silenced or following alcohol intake, it is possible that the IC recruits fewer AcbC processes thereby decreasing ongoing and reinstatement of alcohol self-administration. However, given our findings demonstrating alcohol self-administration is still occurring (albeit decreased) after silencing the insular-striatal circuit, other motivation/reward-related circuits are likely recruited to initiate alcohol self-administration. Therefore, by silencing the IC and Rh outgoing projections to the AcbC glutamatergic synapses onto the AcbC, and specifically silencing IC→AcbC projections may in turn decrease the dopaminergic output of the AcbC through decreased activity on dopaminergic medium spiny neurons (e.g., D1 and/or D2) resulting in decreased ongoing alcohol-reinforced behavior (Gonzales *et al*, 2004; Nieh *et al*, 2013). Furthermore, this hypothesis is in line with studies demonstrating that a long history of alcohol drinking results in NMDA receptor neuroadaptations that increase glutamatergic activity onto the AcbC from the IC, resulting in decreased compulsive drinking (Seif *et al*, 2013). Together, this suggests that a consequence of decreased glutamatergic tone in the AcbC is to inhibit ongoing alcohol drinking.

Given that only IC→AcbC silencing resulted in decreased alcohol-reinforced behavior, this implicates IC innervation of the indirect pathway on the striatum leading to the recruitment of downstream circuitry to inhibit ongoing alcohol-reinforced behavior, by specifically affecting the ongoing motivational drive for alcohol-reinforced behavior.

A limitation of the present work is that no other incoming striatal projections were investigated. As such, general inhibition of incoming glutamatergic projections to the AcbC may increase sensitivity to alcohol. Future work will need to examine other regions with glutamatergic projections to the AcbC to test circuit specificity (i.e., anatomical controls). For example, investigation for the precortical-striatal circuit in modulating sensitivity to alcohol would be informative, as various work has demonstrated dense mPFC to AcbC projections and implicated a role for the circuit in cued-reinstatement (McGlinchey *et al*, 2016). Additionally given the presence of dense reciprocal projections among the IC and Rh the possibility of cross talk within the regions cannot be ruled out. For example, pharmacological inhibition or chemogenetic silencing of all outgoing Rh projections could silence Rh→IC projections, which could indirectly silence IC→AcbC activity. However, the current results utilizing intra-AcbC CNO infusions provide strong evidence for potentiation of alcohol interoceptive effects by selectively silencing IC→AcbC or Rh→AcbC projections. Additionally, a limitation of the present work is that only one alcohol training dose (1 g/kg, IG) was examined in the discrimination studies. It is well documented that the interoceptive effects of alcohol are dose dependent, implicating a more prominent role for the GABAergic system at lower doses and a role for the NMDA system at higher doses (Grant and Colombo, 1993a; Kostowski and Bienkowski, 1999). Thus, it will be critical for future work to investigate the role of insular/thalamic-striatal circuit in modulating the interoceptive effects of higher and lower alcohol training doses. Furthermore, those studies could provide insight into the differential recruitment of insular-striatal circuitry under compulsive drinking conditions but not under control conditions, which is (Seif *et al*, 2013) in contrast to our findings demonstrating decreased self-administration of moderate alcohol doses.

OVERALL CONCLUSION

In conclusion, the present dissertation determined a novel and functional role for the insular/thalamic-striatal circuit in modulating sensitivity to alcohol and a role for the insular-striatal circuit in modulating ongoing alcohol-reinforcement. A major challenge in the alcohol field is to better understand how interoceptive states can serve as internal cues to modulate ongoing alcohol drinking and relapse. Therefore, this study employed chemogenetic and pharmacological techniques alongside behavioral paradigms to demonstrate that the insular/thalamic-striatal circuit is a site of action for alcohol. Here we demonstrate that silencing of the Rh and Rh→AcbC circuit increases sensitivity to alcohol in rats trained to discriminate alcohol. However in rats trained to self-administer alcohol, silencing the Rh did not affect alcohol self-administration, alcohol-seeking, or reinstatement of alcohol self-administration. Additionally, we demonstrate that IC and IC→AcbC circuit increases sensitivity to alcohol in rats trained to discriminate alcohol. Furthermore, we show that silencing the insular-striatal circuit decreases the reinforcing value of alcohol in rats trained to self-administer alcohol under ongoing and reinstatement of alcohol self-administration but not alcohol-seeking.

Importantly, this dissertation provides further evidence that drug-induced interoceptive effects can directly modulate behavior. However, despite our hypothesis, these findings implicate the complexity and behavioral specific circumstances under which the interoceptive circuitry is recruited. The findings demonstrate that not all interoceptive circuitry (e.g., all outgoing IC and Rh projections) directly affect alcohol-reinforced behavior (under the conditions tested). The present study implicates a specific role for the insular-striatal circuit in modulating the reinforcing effects of alcohol. The present findings taken within the context of the existing literature, allows us to conclude that the interoceptive effects of alcohol are, in part, produced by alcohol-induced inhibition of the insular/thalamic-striatal circuitry and, that specifically, inhibition of the insular-striatal circuit in turn decreases alcohol drinking. Specifically, alcohol may act by suppressing activity within the

insular/thalamic-striatal circuitry, and through alcohol-induced inhibition of the insular-striatal circuit, decrease activity within the AcbC, thus leading to decreased recruitment of downstream striatal circuitry modulating ongoing alcohol drinking. Given the complex nature of interoceptive effects in modulating drug-related behavior it will be necessary for future studies to further investigate interoceptive circuitry (i.e., insular/thalamic-striatal) and their role on modulating different aspects of behavior. Interestingly, the ability to detect interoceptive effects of alcohol in individuals with AUDs can be achieved, utilizing alcohol discrimination procedures, and results in decreased alcohol drinking (Kamien *et al*, 1993), which suggests the possibility of recruiting interoceptive circuitry (possibly through striatal inhibition) to stop ongoing drinking. Thus, behavioral therapy focusing on interoception could prove be a therapeutic strategy for treating and preventing AUDs.

REFERENCES

Besheer J, Cox AA, Hodge CW (2003). Coregulation of ethanol discrimination by the nucleus accumbens and amygdala. *Alcohol Clin Exp Res* **27**(3): 450-456.

Besheer J, Fisher KR, Cannady R, Grondin JJ, Hodge CW (2012a). Intra-amygdala inhibition of ERK(1/2) potentiates the discriminative stimulus effects of alcohol. *Behav Brain Res* **228**(2): 398-405.

Besheer J, Fisher KR, Durant B (2012b). Assessment of the interoceptive effects of alcohol in rats using short-term training procedures. *Alcohol* **46**(8): 747-755.

Besheer J, Fisher KR, Grondin JJ, Cannady R, Hodge CW (2012c). The effects of repeated corticosterone exposure on the interoceptive effects of alcohol in rats. *Psychopharmacology (Berl)* **220**(4): 809-822.

Besheer J, Fisher KR, Jaramillo AA, Frisbee S, Cannady R (2014). Stress hormone exposure reduces mGluR5 expression in the nucleus accumbens: functional implications for interoceptive sensitivity to alcohol. *Neuropsychopharmacology* **39**(10): 2376-2386.

Besheer J, Frisbee S, Randall PA, Jaramillo AA, Masciello M (2015). Gabapentin potentiates sensitivity to the interoceptive effects of alcohol and increases alcohol self-administration in rats. *Neuropharmacology* **101**: 216-224.

Besheer J, Grondin JJ, Cannady R, Sharko AC, Faccidomo S, Hodge CW (2010). Metabotropic glutamate receptor 5 activity in the nucleus accumbens is required for the maintenance of ethanol self-administration in a rat genetic model of high alcohol intake. *Biol Psychiatry* **67**(9): 812-822.

Besheer J, Grondin JJ, Salling MC, Spanos M, Stevenson RA, Hodge CW (2009). Interoceptive effects of alcohol require mGlu5 receptor activity in the nucleus accumbens. *J Neurosci* **29**(30): 9582-9591.

Besheer J, Hodge CW (2005). Pharmacological and anatomical evidence for an interaction between mGluR5- and GABA(A) alpha1-containing receptors in the discriminative stimulus effects of ethanol. *Neuropsychopharmacology* **30**(4): 747-757.

Besheer J, Stevenson RA, Hodge CW (2006). mGlu5 receptors are involved in the discriminative stimulus effects of self-administered ethanol in rats. *Eur J Pharmacol* **551**(1-3): 71-75.

Bevins RA, Besheer J (2014). Interoception and learning: import to understanding and treating diseases and psychopathologies. *ACS Chem Neurosci* **5**(8): 624-631.

Bigelow GE, Griffiths RR, Liebson IA (1977). Pharmacological influences upon human ethanol self-administration. *Adv Exp Med Biol* **85B**: 523-538.

Bossert JM, Marchant NJ, Calu DJ, Shaham Y (2013). The reinstatement model of drug relapse: recent neurobiological findings, emerging research topics, and translational research. *Psychopharmacology (Berl)* **229**(3): 453-476.

Cannady R, Grondin JJ, Fisher KR, Hodge CW, Besheer J (2011). Activation of group II metabotropic glutamate receptors inhibits the discriminative stimulus effects of alcohol via selective activity within the amygdala. *Neuropsychopharmacology* **36**(11): 2328-2338.

Carleton A, Accolla R, Simon SA (2010). Coding in the mammalian gustatory system. *Trends Neurosci* **33**(7): 326-334.

Cassel JC, Pereira de Vasconcelos A, Loureiro M, Cholvin T, Dalrymple-Alford JC, Vertes RP (2013). The reuniens and rhomboid nuclei: neuroanatomy, electrophysiological characteristics and behavioral implications. *Prog Neurobiol* **111**: 34-52.

Cauda F, Cavanna AE, D'Agata F, Sacco K, Duca S, Geminiani GC (2011). Functional connectivity and coactivation of the nucleus accumbens: a combined functional connectivity and structure-based meta-analysis. *J Cogn Neurosci* **23**(10): 2864-2877.

Ceunen E, Vlaeyen JW, Van Diest I (2016). On the Origin of Interoception. *Front Psychol* **7**: 743.

Chang WH, Lin SK, Lane HY, Wei FC, Hu WH, Lam YW*, et al* (1998). Reversible metabolism of clozapine and clozapine N-oxide in schizophrenic patients. *Prog Neuropsychopharmacol Biol Psychiatry* **22**(5): 723-739.

Chaudhri N, Sahuque LL, Cone JJ, Janak PH (2008). Reinstated ethanol-seeking in rats is modulated by environmental context and requires the nucleus accumbens core. *Eur J Neurosci* **28**(11): 2288-2298.

Chaudhri N, Sahuque LL, Schairer WW, Janak PH (2010). Separable roles of the nucleus accumbens core and shell in context- and cue-induced alcohol-seeking. *Neuropsychopharmacology* **35**(3): 783-791.

Chaudhri N, Woods CA, Sahuque LL, Gill TM, Janak PH (2013). Unilateral inactivation of the basolateral amygdala attenuates context-induced renewal of Pavlovian-conditioned alcohol-seeking. *Eur J Neurosci* **38**(5): 2751-2761.

Cho YT, Fromm S, Guyer AE, Detloff A, Pine DS, Fudge JL*, et al* (2013). Nucleus accumbens, thalamus and insula connectivity during incentive anticipation in typical adults and adolescents. *Neuroimage* **66**: 508-521.

Cholvin T, Loureiro M, Cassel R, Cosquer B, Geiger K, De Sa Nogueira D*, et al* (2013). The ventral midline thalamus contributes to strategy shifting in a memory task requiring both prefrontal cortical and hippocampal functions. *J Neurosci* **33**(20): 8772-8783.

Christiansen P, Townsend G, Knibb G, Field M (2017). Bibi ergo sum: the effects of a placebo and contextual alcohol cues on motivation to drink alcohol. *Psychopharmacology (Berl)* **234**(5): 827-835.

Clithero JA, Reeck C, Carter RM, Smith DV, Huettel SA (2011). Nucleus accumbens mediates relative motivation for rewards in the absence of choice. *Front Hum Neurosci* **5**: 87.

Cohen BM, Wan W, Froimowitz MP, Ennulat DJ, Cherkerzian S, Konieczna H (1998). Activation of midline thalamic nuclei by antipsychotic drugs. *Psychopharmacology (Berl)* **135**(1): 37-43.

Colpaert FC (1999). Drug discrimination in neurobiology. *Pharmacol Biochem Behav* **64**(2): 337-345.

Cosme CV, Gutman AL, LaLumiere RT (2015). The Dorsal Agranular Insular Cortex Regulates the Cued Reinstatement of Cocaine-Seeking, but not Food-Seeking, Behavior in Rats. *Neuropsychopharmacology* **40**(10): 2425-2433.

Craig AD (2002). How do you feel? Interoception: the sense of the physiological condition of the body. *Nat Rev Neurosci* **3**(8): 655-666.

Craig AD (2009). How do you feel--now? The anterior insula and human awareness. *Nat Rev Neurosci* **10**(1): 59-70.

Czachowski CL, Prutzman S, DeLory MJ (2006). Volume and dose effects of experimenter-administered ethanol preloads on ethanol seeking and self-administration. *Alcohol* **40**(1): 35-40.

Damasio A (2003). Feelings of emotion and the self. *Ann N Y Acad Sci* **1001**: 253-261.

Damasio AR (1996). The somatic marker hypothesis and the possible functions of the prefrontal cortex. *Philos Trans R Soc Lond B Biol Sci* **351**(1346): 1413-1420.

de Wit H, Chutuape MA (1993). Increased ethanol choice in social drinkers following ethanol preload. *Behav Pharmacol* **4**(1): 29-36.

Di Pietro NC, Mashhoon Y, Heaney C, Yager LM, Kantak KM (2008). Role of dopamine D1 receptors in the prefrontal dorsal agranular insular cortex in mediating cocaine self-administration in rats. *Psychopharmacology (Berl)* **200**(1): 81-91.

Ding DC, Gabbott PL, Totterdell S (2001). Differences in the laminar origin of projections from the medial prefrontal cortex to the nucleus accumbens shell and core regions in the rat. *Brain Res* **917**(1): 81-89.

Droutman V, Read SJ, Bechara A (2015). Revisiting the role of the insula in addiction. *Trends Cogn Sci* **19**(7): 414-420.

Duka T, Stephens DN, Russell C, Tasker R (1998). Discriminative stimulus properties of low doses of ethanol in humans. *Psychopharmacology (Berl)* **136**(4): 379-389.

Fernie G, Christiansen P, Cole JC, Rose AK, Field M (2012). Effects of 0.4 g/kg alcohol on attentional bias and alcohol-seeking behaviour in heavy and moderate social drinkers. *J Psychopharmacol* **26**(7): 1017-1025.

Filbey FM, Claus E, Audette AR, Niculescu M, Banich MT, Tanabe J*, et al* (2008). Exposure to the taste of alcohol elicits activation of the mesocorticolimbic neurocircuitry. *Neuropsychopharmacology* **33**(6): 1391-1401.

Forget B, Pushparaj A, Le Foll B (2010). Granular insular cortex inactivation as a novel therapeutic strategy for nicotine addiction. *Biol Psychiatry* **68**(3): 265-271.

Gass JT, Olive MF (2007). Reinstatement of ethanol-seeking behavior following intravenous self-administration in Wistar rats. *Alcohol Clin Exp Res* **31**(9): 1441-1445.

Gass JT, Sinclair CM, Cleva RM, Widholm JJ, Olive MF (2011). Alcohol-seeking behavior is associated with increased glutamate transmission in basolateral amygdala and nucleus accumbens as measured by glutamate-oxidase-coated biosensors. *Addict Biol* **16**(2): 215-228.

George MS, Anton RF, Bloomer C, Teneback C, Drobes DJ, Lorberbaum JP*, et al* (2001). Activation of prefrontal cortex and anterior thalamus in alcoholic subjects on exposure to alcohol-specific cues. *Arch Gen Psychiatry* **58**(4): 345-352.

Gonzales RA, Job MO, Doyon WM (2004). The role of mesolimbic dopamine in the development and maintenance of ethanol reinforcement. *Pharmacol Ther* **103**(2): 121-146.

Goudie AJ, Smith JA, Taylor A, Taylor MA, Tricklebank MD (1998). Discriminative stimulus properties of the atypical neuroleptic clozapine in rats: tests with subtype selective receptor ligands. *Behav Pharmacol* **9**(8): 699-710.

Grant KA (1999). Strategies for understanding the pharmacological effects of ethanol with drug discrimination procedures. *Pharmacol Biochem Behav* **64**(2): 261-267.

Grant KA, Barrett JE (1991). Blockade of the discriminative stimulus effects of ethanol with 5-HT3 receptor antagonists. *Psychopharmacology (Berl)* **104**(4): 451-456.

Grant KA, Colombo G (1993a). Discriminative stimulus effects of ethanol: effect of training dose on the substitution of N-methyl-D-aspartate antagonists. *J Pharmacol Exp Ther* **264**(3): 1241-1247.

Grant KA, Colombo G (1993b). Pharmacological analysis of the mixed discriminative stimulus effects of ethanol. *Alcohol Alcohol Suppl* **2**: 445-449.

Grant KA, Colombo G, Gatto GJ (1997). Characterization of the ethanol-like discriminative stimulus effects of 5-HT receptor agonists as a function of ethanol training dose. *Psychopharmacology (Berl)* **133**(2): 133-141.

Griffin WC, 3rd, Haun HL, Hazelbaker CL, Ramachandra VS, Becker HC (2014). Increased extracellular glutamate in the nucleus accumbens promotes excessive ethanol drinking in ethanol dependent mice. *Neuropsychopharmacology* **39**(3): 707-717.

Gu X, Hof PR, Friston KJ, Fan J (2013). Anterior insular cortex and emotional awareness. *J Comp Neurol* **521**(15): 3371-3388.

Hallock HL, Wang A, Shaw CL, Griffin AL (2013). Transient inactivation of the thalamic nucleus reuniens and rhomboid nucleus produces deficits of a working-memory dependent tactile-visual conditional discrimination task. *Behav Neurosci* **127**(6): 860-866.

Hamlin AS, Newby J, McNally GP (2007). The neural correlates and role of D1 dopamine receptors in renewal of extinguished alcohol-seeking. *Neuroscience* **146**(2): 525-536.

Helms CM, Rogers LS, Grant KA (2009). Antagonism of the ethanol-like discriminative stimulus effects of ethanol, pentobarbital, and midazolam in cynomolgus monkeys reveals involvement of specific GABA(A) receptor subtypes. *J Pharmacol Exp Ther* **331**(1): 142-152.

Hembrook JR, Mair RG (2011). Lesions of reuniens and rhomboid thalamic nuclei impair radial maze win-shift performance. *Hippocampus* **21**(8): 815-826.

Hembrook JR, Onos KD, Mair RG (2012). Inactivation of ventral midline thalamus produces selective spatial delayed conditional discrimination impairment in the rat. *Hippocampus* **22**(4): 853-860.

Hiltunen AJ, Jarbe TU (1989). Discriminative stimulus properties of ethanol: effects of cumulative dosing and Ro 15-4513. *Behav Pharmacol* **1**(2): 133-140.

Hodge CW (1994). Comparison of the discriminative stimulus function of ethanol following intracranial and systemic administration: evidence of a central mechanism. *Pharmacol Biochem Behav* **47**(3): 743-747.

Hodge CW, Alken AS (1996). Discriminative stimulus function of ethanol: role of GABAA receptors in the nucleus accumbens. *Alcohol Clin Exp Res* **20**(7): 1221-1228.

Hodge CW, Cox AA (1998). The discriminative stimulus effects of ethanol are mediated by NMDA and GABA(A) receptors in specific limbic brain regions. *Psychopharmacology (Berl)* **139**(1-2): 95-107.

Hodge CW, Cox AA, Bratt AM, Camarini R, Iller K, Kelley SP*, et al* (2001a). The discriminative stimulus properties of self-administered ethanol are mediated by GABA(A) and NMDA receptors in rats. *Psychopharmacology (Berl)* **154**(1): 13-22.

Hodge CW, Nannini MA, Olive MF, Kelley SP, Mehmert KK (2001b). Allopregnanolone and pentobarbital infused into the nucleus accumbens substitute for the discriminative stimulus effects of ethanol. *Alcohol Clin Exp Res* **25**(10): 1441-1447.

Hodgson R, Rankin H, Stockwell T (1979). Alcohol dependence and the priming effect. *Behav Res Ther* **17**(4): 379-387.

Hollander JA, Lu Q, Cameron MD, Kamenecka TM, Kenny PJ (2008). Insular hypocretin transmission regulates nicotine reward. *Proc Natl Acad Sci U S A* **105**(49): 19480-19485.

Ihssen N, Cox WM, Wiggett A, Fadardi JS, Linden DE (2011). Differentiating heavy from light drinkers by neural responses to visual alcohol cues and other motivational stimuli. *Cereb Cortex* **21**(6): 1408-1415.

Jann MW, Lam YW, Chang WH (1994). Rapid formation of clozapine in guinea-pigs and man following clozapine-N-oxide administration. *Arch Int Pharmacodyn Ther* **328**(2): 243-250.

Jaramillo AA, Randall PA, Frisbee S, Besheer J (2016). Modulation of sensitivity to alcohol by cortical and thalamic brain regions. *Eur J Neurosci* **44**(8): 2569-2580.

Jaramillo AA, Randall PA, Frisbee S, Fisher KR, Besheer J (2015). Activation of mGluR2/3 following stress hormone exposure restores sensitivity to alcohol in rats. *Alcohol* **49**(6): 525-532.

Jasinska AJ, Stein EA, Kaiser J, Naumer MJ, Yalachkov Y (2014). Factors modulating neural reactivity to drug cues in addiction: a survey of human neuroimaging studies. *Neurosci Biobehav Rev* **38**: 1-16.

Kamien JB, Bickel WK, Hughes JR, Higgins ST, Smith BJ (1993). Drug discrimination by humans compared to nonhumans: current status and future directions. *Psychopharmacology (Berl)* **111**(3): 259-270.

Kesner RP, Gilbert PE (2007). The role of the agranular insular cortex in anticipation of reward contrast. *Neurobiol Learn Mem* **88**(1): 82-86.

Kirk JM, de Wit H (2000). Individual differences in the priming effect of ethanol in social drinkers. *J Stud Alcohol* **61**(1): 64-71.

Knapp CM, Tozier L, Pak A, Ciraulo DA, Kornetsky C (2009). Deep brain stimulation of the nucleus accumbens reduces ethanol consumption in rats. *Pharmacol Biochem Behav* **92**(3): 474-479.

Koob GF, Volkow ND (2010). Neurocircuitry of addiction. *Neuropsychopharmacology* **35**(1): 217-238.

Kostowski W, Bienkowski P (1999). Discriminative stimulus effects of ethanol: neuropharmacological characterization. *Alcohol* **17**(1): 63-80.

Krashes MJ, Koda S, Ye C, Rogan SC, Adams AC, Cusher DS*, et al* (2011). Rapid, reversible activation of AgRP neurons drives feeding behavior in mice. *J Clin Invest* **121**(4): 1424-1428.

Kreitzer AC (2009). Physiology and pharmacology of striatal neurons. *Annu Rev Neurosci* **32**: 127-147.

Kufahl PR, Zavala AR, Singh A, Thiel KJ, Dickey ED, Joyce JN*, et al* (2009). c-Fos expression associated with reinstatement of cocaine-seeking behavior by response-contingent conditioned cues. *Synapse* **63**(10): 823-835.

Kusumoto-Yoshida I, Liu H, Chen BT, Fontanini A, Bonci A (2015). Central role for the insular cortex in mediating conditioned responses to anticipatory cues. *Proc Natl Acad Sci U S A* **112**(4): 1190-1195.

Kutlu MG, Burke D, Slade S, Hall BJ, Rose JE, Levin ED (2013). Role of insular cortex D(1) and D(2) dopamine receptors in nicotine self-administration in rats. *Behav Brain Res* **256**: 273-278.

Lasseter HC, Wells AM, Xie X, Fuchs RA (2011). Interaction of the basolateral amygdala and orbitofrontal cortex is critical for drug context-induced reinstatement of cocaine-seeking behavior in rats. *Neuropsychopharmacology* **36**(3): 711-720.

Layfield DM, Patel M, Hallock H, Griffin AL (2015). Inactivation of the nucleus reuniens/rhomboid causes a delay-dependent impairment of spatial working memory. *Neurobiol Learn Mem* **125**: 163-167.

Le AD, Quan B, Juzytch W, Fletcher PJ, Joharchi N, Shaham Y (1998). Reinstatement of alcohol-seeking by priming injections of alcohol and exposure to stress in rats. *Psychopharmacology (Berl)* **135**(2): 169-174.

Leong JK, Pestilli F, Wu CC, Samanez-Larkin GR, Knutson B (2016). White-Matter Tract Connecting Anterior Insula to Nucleus Accumbens Correlates with Reduced Preference for Positively Skewed Gambles. *Neuron* **89**(1): 63-69.

Li CL, Zhu N, Meng XL, Li YH, Sui N (2013). Effects of inactivating the agranular or granular insular cortex on the acquisition of the morphine-induced conditioned place preference and naloxone-precipitated conditioned place aversion in rats. *J Psychopharmacol* **27**(9): 837-844.

Loureiro M, Cholvin T, Lopez J, Merienne N, Latreche A, Cosquer B*, et al* (2012). The ventral midline thalamus (reuniens and rhomboid nuclei) contributes to the persistence of spatial memory in rats. *J Neurosci* **32**(29): 9947-9959.

Ma P, Liu H, Li H, Huang X, Chen L, Zhai H (2014). Involvement of the insular nitric oxide signaling pathway in the expression of morphine-induced conditioned place preference in rats. *Neuroreport* **25**(9): 641-646.

MacLaren DA, Browne RW, Shaw JK, Krishnan Radhakrishnan S, Khare P, Espana RA*, et al* (2016). Clozapine N-Oxide Administration Produces Behavioral Effects in Long-Evans Rats: Implications for Designing DREADD Experiments. *eNeuro* **3**(5).

Maffei A, Haley M, Fontanini A (2012). Neural processing of gustatory information in insular circuits. *Curr Opin Neurobiol* **22**(4): 709-716.

Maurel S, Schreiber R, De Vry J (1998). Role of 5-HT1B, 5-HT2A and 5-HT2C receptors in the generalization of 5-HT receptor agonists to the ethanol cue in the rat. *Behav Pharmacol* **9**(4): 337-343.

McGeorge AJ, Faull RL (1989). The organization of the projection from the cerebral cortex to the striatum in the rat. *Neuroscience* **29**(3): 503-537.

McGlinchey EM, James MH, Mahler SV, Pantazis C, Aston-Jones G (2016). Prelimbic to Accumbens Core Pathway Is Recruited in a Dopamine-Dependent Manner to Drive Cued Reinstatement of Cocaine Seeking. *J Neurosci* **36**(33): 8700-8711.

McLellan AT, Lewis DC, O'Brien CP, Kleber HD (2000). Drug dependence, a chronic medical illness: implications for treatment, insurance, and outcomes evaluation. *JAMA* **284**(13): 1689-1695.

Mizoguchi H, Katahira K, Inutsuka A, Fukumoto K, Nakamura A, Wang T*, et al* (2015). Insular neural system controls decision-making in healthy and methamphetamine-treated rats. *Proc Natl Acad Sci U S A* **112**(29): E3930-3939.

Mokdad AH, Marks JS, Stroup DF, Gerberding JL (2004). Actual causes of death in the United States, 2000. *JAMA* **291**(10): 1238-1245.

Mufson EJ, Mesulam MM (1984). Thalamic connections of the insula in the rhesus monkey and comments on the paralimbic connectivity of the medial pulvinar nucleus. *J Comp Neurol* **227**(1): 109-120.

Myrick H, Anton RF, Li X, Henderson S, Drobes D, Voronin K*, et al* (2004). Differential brain activity in alcoholics and social drinkers to alcohol cues: relationship to craving. *Neuropsychopharmacology* **29**(2): 393-402.

Naqvi NH, Bechara A (2009). The hidden island of addiction: the insula. *Trends Neurosci* **32**(1): 56-67.

Naqvi NH, Bechara A (2010). The insula and drug addiction: an interoceptive view of pleasure, urges, and decision-making. *Brain Struct Funct* **214**(5-6): 435-450.

Naqvi NH, Rudrauf D, Damasio H, Bechara A (2007). Damage to the insula disrupts addiction to cigarette smoking. *Science* **315**(5811): 531-534.

Nieh EH, Kim SY, Namburi P, Tye KM (2013). Optogenetic dissection of neural circuits underlying emotional valence and motivated behaviors. *Brain Res* **1511**: 73-92.

O'Brien CP, Childress AR, Ehrman R, Robbins SJ (1998). Conditioning factors in drug abuse: can they explain compulsion? *J Psychopharmacol* **12**(1): 15-22.

Ohtake T, Yamada H (1989). Efferent connections of the nucleus reuniens and the rhomboid nucleus in the rat: an anterograde PHA-L tracing study. *Neurosci Res* **6**(6): 556-568.

Palmatier MI, Wilkinson JL, Metschke DM, Bevins RA (2005). Stimulus properties of nicotine, amphetamine, and chlordiazepoxide as positive features in a pavlovian appetitive discrimination task in rats. *Neuropsychopharmacology* **30**(4): 731-741.

Parkes SL, Balleine BW (2013). Incentive memory: evidence the basolateral amygdala encodes and the insular cortex retrieves outcome values to guide choice between goal-directed actions. *J Neurosci* **33**(20): 8753-8763.

Parkes SL, Bradfield LA, Balleine BW (2015). Interaction of insular cortex and ventral striatum mediates the effect of incentive memory on choice between goal-directed actions. *J Neurosci* **35**(16): 6464-6471.

Paulus MP, Stewart JL (2014). Interoception and drug addiction. *Neuropharmacology* **76 Pt B**: 342-350.

Paulus MP, Tapert SF, Schulteis G (2009). The role of interoception and alliesthesia in addiction. *Pharmacol Biochem Behav* **94**(1): 1-7.

Paxinos G, Watson C (2007). *The rat brain in stereotaxic coordinates*, 6th edn. Academic Press/Elsevier: Amsterdam ; Boston ; .

Platt DM, Bano KM (2011). Opioid receptors and the discriminative stimulus effects of ethanol in squirrel monkeys: Mu and delta opioid receptor mechanisms. *Eur J Pharmacol* **650**(1): 233-239.

Prasad JA, Abela AR, Chudasama Y (2016). Midline thalamic reuniens lesions improve executive behaviors. *Neuroscience*.

Prasad JA, Macgregor EM, Chudasama Y (2013). Lesions of the thalamic reuniens cause impulsive but not compulsive responses. *Brain Struct Funct* **218**(1): 85-96.

Preston KL, Bigelow GE (1991). Subjective and discriminative effects of drugs. *Behav Pharmacol* **2**(4 And 5): 293-313.

Prus AJ, Wise LE, Pehrson AL, Philibin SD, Bang-Andersen B, Arnt J*, et al* (2016). Discriminative stimulus properties of 1.25mg/kg clozapine in rats: Mediation by serotonin 5-HT2 and dopamine D4 receptors. *Brain Res* **1648**(Pt A): 298-305.

Pushparaj A, Le Foll B (2015). Involvement of the caudal granular insular cortex in alcohol self-administration in rats. *Behav Brain Res* **293**: 203-207.

Quertemont E, Green HL, Grant KA (2003). Brain ethanol concentrations and ethanol discrimination in rats: effects of dose and time. *Psychopharmacology (Berl)* **168**(3): 262-270.

Randall PA, Cannady R, Besheer J (2016). The nicotine + alcohol interoceptive drug state: contribution of the components and effects of varenicline in rats. *Psychopharmacology (Berl)* **233**(15-16): 3061-3074.

Randall PA, Jaramillo AA, Frisbee S, Besheer J (2015). The role of varenicline on alcohol-primed self-administration and seeking behavior in rats. *Psychopharmacology (Berl)*.

Rassnick S, D'Amico E, Riley E, Pulvirenti L, Zieglgansberger W, Koob GF (1992a). GABA and nucleus accumbens glutamate neurotransmission modulate ethanol self-administration in rats. *Ann N Y Acad Sci* **654**: 502-505.

Rassnick S, Pulvirenti L, Koob GF (1992b). Oral ethanol self-administration in rats is reduced by the administration of dopamine and glutamate receptor antagonists into the nucleus accumbens. *Psychopharmacology (Berl)* **109**(1-2): 92-98.

Ray S, Hanson C, Hanson SJ, Bates ME (2010). fMRI BOLD response in high-risk college students (Part 1): during exposure to alcohol, marijuana, polydrug and emotional picture cues. *Alcohol Alcohol* **45**(5): 437-443.

Rose AK, Grunsell L (2008). The subjective, rather than the disinhibiting, effects of alcohol are related to binge drinking. *Alcohol Clin Exp Res* **32**(6): 1096-1104.

Roth BL (2016). DREADDs for Neuroscientists. *Neuron* **89**(4): 683-694.

Rowland NE, Barnett M (1992). Sham ingestion of alcohol in rats. *Alcohol* **9**(1): 75-77.

Ryabinin AE, Criado JR, Henriksen SJ, Bloom FE, Wilson MC (1997). Differential sensitivity of c-Fos expression in hippocampus and other brain regions to moderate and low doses of alcohol. *Mol Psychiatry* **2**(1): 32-43.

Salamone JD, Correa M (2002). Motivational views of reinforcement: implications for understanding the behavioral functions of nucleus accumbens dopamine. *Behav Brain Res* **137**(1-2): 3-25.

SAMHSA (2014). National Survey on Drug Use and Health (NSDUH).

Samson HH, Chappell A (2002). Reinstatement of ethanol seeking responding after ethanol self-administration. *Alcohol* **26**(2): 95-101.

Samson HH, Chappell A, Legg B (2002). Effects of self-administered alcohol or sucrose preloads on subsequent consumption in the rat. *J Stud Alcohol* **63**(1): 107-113.

Samson HH, Cunningham CL, Czachowski CL, Chappell A, Legg B, Shannon E (2004). Devaluation of ethanol reinforcement. *Alcohol* **32**(3): 203-212.

Samson HH, Denning C, Czachowski CL (2003). Effects of self-administered ethanol or water preloads on appetitive and consummatory behavior in the alcohol-preferring (P) rat. *J Stud Alcohol* **64**(1): 105-110.

Schechter MD (1989). Time-dependent effect of ethanol upon discrimination behavior. *Alcohol* **6**(6): 445-449.

Schechter MD (1997). Discrete versus cumulative dosing in dose-response discrimination studies. *Eur J Pharmacol* **326**(2-3): 113-118.

Schmued LC, Fallon JH (1986). Fluoro-Gold: a new fluorescent retrograde axonal tracer with numerous unique properties. *Brain Res* **377**(1): 147-154.

Seif T, Chang SJ, Simms JA, Gibb SL, Dadgar J, Chen BT*, et al* (2013). Cortical activation of accumbens hyperpolarization-active NMDARs mediates aversion-resistant alcohol intake. *Nat Neurosci* **16**(8): 1094-1100.

Sesack SR, Deutch AY, Roth RH, Bunney BS (1989). Topographical organization of the efferent projections of the medial prefrontal cortex in the rat: an anterograde tract-tracing study with Phaseolus vulgaris leucoagglutinin. *J Comp Neurol* **290**(2): 213-242.

Shelton KL, Grant KA (2002). Discriminative stimulus effects of ethanol in C57BL/6J and DBA/2J inbred mice. *Alcohol Clin Exp Res* **26**(6): 747-757.

Solinas M, Panlilio LV, Justinova Z, Yasar S, Goldberg SR (2006). Using drug-discrimination techniques to study the abuse-related effects of psychoactive drugs in rats. *Nat Protoc* **1**(3): 1194-1206.

Stachniak TJ, Ghosh A, Sternson SM (2014). Chemogenetic synaptic silencing of neural circuits localizes a hypothalamus-->midbrain pathway for feeding behavior. *Neuron* **82**(4): 797-808.

Stockwell TR, Hodgson RJ, Rankin HJ, Taylor C (1982). Alcohol dependence, beliefs and the priming effect. *Behav Res Ther* **20**(5): 513-522.

Stolerman I (1992). Drugs of abuse: behavioural principles, methods and terms. *Trends Pharmacol Sci* **13**(5): 170-176.

Stolerman IP, Childs E, Ford MM, Grant KA (2011). Role of training dose in drug discrimination: a review. *Behav Pharmacol* **22**(5-6): 415-429.

Tapert SF, Brown GG, Baratta MV, Brown SA (2004). fMRI BOLD response to alcohol stimuli in alcohol dependent young women. *Addict Behav* **29**(1): 33-50.

Thrasher MJ, Freeman PA, Risinger FO (1999). Clozapine's effects on ethanol's motivational properties. *Alcohol Clin Exp Res* **23**(8): 1377-1385.

Verdejo-Garcia A, Clark L, Dunn BD (2012). The role of interoception in addiction: a critical review. *Neurosci Biobehav Rev* **36**(8): 1857-1869.

Vertes RP (2002). Analysis of projections from the medial prefrontal cortex to the thalamus in the rat, with emphasis on nucleus reuniens. *J Comp Neurol* **442**(2): 163-187.

Vertes RP, Hoover WB, Do Valle AC, Sherman A, Rodriguez JJ (2006). Efferent projections of reuniens and rhomboid nuclei of the thalamus in the rat. *J Comp Neurol* **499**(5): 768-796.

Vertes RP, Linley SB, Hoover WB (2015). Limbic circuitry of the midline thalamus. *Neurosci Biobehav Rev* **54**: 89-107.

Vilpoux C, Warnault V, Pierrefiche O, Daoust M, Naassila M (2009). Ethanol-sensitive brain regions in rat and mouse: a cartographic review, using immediate early gene expression. *Alcohol Clin Exp Res* **33**(6): 945-969.

Vivian JA, Waters CA, Szeliga KT, Jordan K, Grant KA (2002). Characterization of the discriminative stimulus effects of N-methyl- D-aspartate ligands under different ethanol training conditions in the cynomolgus monkey (Macaca fascicularis). *Psychopharmacology (Berl)* **162**(3): 273-281.

Vosler PS, Bombace JC, Kosten TA (2001). A discriminative two-lever test of dizocilpine's ability to reinstate ethanol-seeking behavior. *Life Sci* **69**(5): 591-598.

WHO (2004). Global Status Report on Alcohol 2004.

Wiebking C, Duncan NW, Tiret B, Hayes DJ, Marjanska M, Doyon J*, et al* (2014). GABA in the insula - a predictor of the neural response to interoceptive awareness. *Neuroimage* **86**: 10-18.

Willcocks AL, McNally GP (2013). The role of medial prefrontal cortex in extinction and reinstatement of alcohol-seeking in rats. *Eur J Neurosci* **37**(2): 259-268.

Wright CI, Groenewegen HJ (1996). Patterns of overlap and segregation between insular cortical, intermediodorsal thalamic and basal amygdaloid afferents in the nucleus accumbens of the rat. *Neuroscience* **73**(2): 359-373.

Wu W, Li H, Liu Y, Huang X, Chen L, Zhai H (2014). Involvement of insular muscarinic cholinergic receptors in morphine-induced conditioned place preference in rats. *Psychopharmacology (Berl)* **231**(21): 4109-4118.

Wu X, Zhao N, Bai F, Li C, Liu C, Wei J*, et al* (2016). Morphine-induced conditioned place preference in rhesus monkeys: Resistance to inactivation of insula and extinction. *Neurobiol Learn Mem* **131**: 192-200.