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SYNAPTIC EFFECTS OF ETHANOL ON THE AGRANULAR INSULAR CORTEX

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SYNAPTIC EFFECTS OF ETHANOL ON THE AGRANULAR INSUNLAR CORTEX

by

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Dedication

To those that suffer internally and invisibly. The scientific community at large is trying our hardest to help, and although our task is great and our techniques are insufficient, at very least we are training ourselves through our science and our relationships to value empathy with and compassion for each other.

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Abstract

SYNAPTIC EFFECTS OF ETHANOL ON THE AGRANULAR INSULAR CORTEX

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The purpose of this study was to provide the first examination of whether the agranular insular cortex (AIC) is a target for the actions of ethanol. Initially, we wished to determine whether basic excitatory and inhibitory synaptic transmission in the AIC are sensitive to acute ethanol. Therefore, we collected brain slices from ethanol-naïve adult male mice, obtained whole-cell recording configuration in layer 2/3 AIC pyramidal neurons, and bath-applied ethanol at pharmacologically relevant concentrations during electrophysiological assays of glutamatergic and γ-amino-butyric acid (GABA)ergic synaptic transmission and plasticity. We found that ethanol inhibited electrically evoked N-methyl-D-aspartate receptor (NMDAR)-mediated excitatory postsynaptic currents (EPSCs) in a concentration-related fashion, and had little effect on other electrophysiological parameters of glutamatergic and (GABA)ergic synaptic transmission. Synaptic conditioning (low-frequency stimulation for 15 min at 1 Hz) induced a form of long-term depression (LTD) that was inhibited by a nonselective NMDAR antagonist (DL-2-amino-5-phosphonovaleric acid), and also by acute, intoxicating concentrations of ethanol. We then wished to determine whether NMDAR-dependent LTD in the AIC is

sensitive to chronic ethanol. We utilized the Becker-Lopez chronic intermittent ethanol (CIE) exposure model to model chronic ethanol experience, and found that CIE vapor exposure disrupted the ability to induce NMDAR-dependent LTD in the AIC. Then, we wished to determine whether NMDAR-dependent LTD and other glutamatergic synaptic properties in the AIC are differentially modulated by conditions that promote aspects of alcohol use disorder (AUD) relative to conditions that do not. Therefore, groups of ethanol-drinking mice underwent either extended CIE (Drinking + CIE) or air (Drinking + Air) to generate AUD-like and non AUD-like phenotypes, respectively, before undergoing electrophysiological recordings testing for LTD and other glutamatergic synaptic properties. Drinking + CIE and Drinking + Air groups no longer displayed NMDAR-dependent LTD, but Drinking + CIE groups displayed a reduced NMDA/AMPA ratio relative to Drinking + Air groups. To verify the reduction in NMDA/AMPA ratio between Drinking + CIE and Drinking + Air groups, as well as determine whether modest amounts of ethanol drinking alter NMDA/AMPA ratio, we replicated the experiment with an additional ethanol-naïve handling control group. We found that ethanol drinking alone reduces NMDA/AMPA ratio relative to ethanol naïve animals. Finally, to verify whether AIC NMDAR-dependent LTD was not an age-dependent phenomenon, we tested for the presence of LTD in an additional ethanol-naïve group age-matched to the Drinking + CIE and Drinking + Air groups (≥14 weeks). We no longer observed the presence of LTD. These data indicate that glutamatergic transmission is sensitive to acute and chronic ethanol in AIC 2/3 pyramidal neurons, and that even moderate, non AUD-like ethanol drinking may alter age-dependent glutamatergic synaptic plasticity mechanisms.

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CHAPTER 1:

BACKGROUND

ALCOHOL

An alcohol is any carbon-based compound that contains a hydroxyl (-OH) group. Ethanol is the two-carbon alcohol that is used for human consumption. Ethanol is produced via fermentation; any type of carbohydrate can be converted into glucose which can then be fermented with yeast to produce ethanol and carbon dioxide. Ethanol has been produced for human consumption for thousands of years, and its production has been considered an indicator of the transition from hunter-gatherer to agricultural societies (Chrzan, 2013). Historically, ethanol has been consumed for its nutritional, medicinal, spiritual, and pharmacological effects.

ALCOHOL USE

Drinking ethanol is ubiquitous to our society as it is interwoven in our culture and involved in several aspects of life. In the United States, 82% of people aged 12 and older were estimated to have ever consumed ethanol, and 65.9% of those had used alcohol in the past year (Koob et al., 2014). Moreover, more than 50% of Americans over the age of 18 consume ethanol regularly (CDC, 2012). The large majority of drinkers consume ethanol for its positive subjective effects, including anxiolysis (relief from anxiety), mild euphoria, social disinhibition, and tension reduction. This general enhancement in emotional state due to the pharmacological effects of ethanol increases the likelihood of future ethanol consumption, a psychological concept called positive reinforcement. The positive reinforcing aspects of ethanol consumption are the motivating factors for initial ethanol

use, as ethanol consumption is generally accepted to begin in a social environment for the pleasurable effects of drinking.

However, a minority of the individuals who drink ethanol escalate their consumption, and become preoccupied with drinking to a level that is detrimental to their health. The terms for this condition have evolved over time, and have included alcoholism, alcohol abuse, and alcohol dependence. However, the most recent iteration of the Diagnostic and Statistical Manual of Mental Disorders, the DSM-V (2013), denotes this condition as Alcohol Use Disorder (AUD). Once an individual has an AUD, the motivational locus of control behind ethanol drinking may have shifted. While ethanol was originally consumed in a controlled fashion for its positively reinforcing, pleasurable effects, those individuals with AUD now likely consume ethanol in an uncontrolled fashion in order to alleviate negative emotional states, such as anxiety and irritability. Thus, individuals with AUD suffer from a life of preoccupation with and recovery from ethanol drinking and its associated detrimental health effects.

ALCOHOL USE DISORDER

The general preoccupation with ethanol consumption characteristic of AUD is exemplified in the DSM-V (2013). The diagnostic criterion for AUD is the following list: 1) Excessive use for longer than intended, 2) unsuccessful efforts to reduce use, 3) significant time allotted to use or recovery from ethanol use, 4) craving, 5) use or recovering from use caused home, job, or school problems, 6) use despite it causing trouble with family or friends, 7) reducing social, occupational, or recreational activities to use, 8) use in situations in which it is physically hazardous, 9) use despite its having depressing or anxiogenic (anxiety-producing) effects or adding to other health problems, 10) escalated use to obtain desired effect, and 11) withdrawal symptoms in the absence of use. This 11-question inventory can be collapsed into the three hallmarks of substance use disorder: 1) Compulsion to seek and take a drug, 2) loss of control in limiting intake, and 3) the emergence of a negative emotional state (e.g. anxiety, irritability) when access to the drug

is prevented. As a spectrum disorder, the degree of severity is contingent upon the number of questionnaire items to which an individual responds positively. The presence of 2-3 of the criteria indicate a mild AUD, the presence of 4-5 of the criteria indicate a moderate AUD, and the presence of 6 or more of the 11 criteria indicate a severe AUD.

PREVALENCE AND COST OF ALCOHOL USE DISORDER

AUD is a prevalent and costly health problem. Of the 170.4 million of the United States population (aged 12 and older) to have consumed ethanol in the past year, 16.7 million, or 9.8% met the diagnostic criteria for an AUD (Koob et al., 2014). This problem is stratified around the late-adolescent and early-adulthood periods, as approximately 1/3 of 18-24 year olds in the United States meet the criteria for AUD (National Institute on Alcohol Abuse and Alcoholism [NIAAA], 2005). Complications from excessive ethanol consumption were responsible for 1 in 10 deaths among working-age adults between 2006 and 2010, and cost the United States an estimated $249 billion in 2010 (Sacks et al., 2015). An analysis of the "actual" causes of death, which investigated the nongenetic modifiable behavioral factors that contributed to death in the United States in 1990 & 2000, showed ethanol consumption was the third leading actual cause of death, behind only (1) tobacco use and (2) poor diet and physical inactivity (Mokdad et al., 2004). These included ethanol-related crashes, various types of cancer linked to ethanol use, cardiovascular complications such as stroke and hypertensive heart disease, and chronic liver disease and cirrhosis. The economic impact of AUD is substantial, and a global issue. An analysis which investigated the risk factors for loss of years to disease and disability in 2012 found that ethanol use is the third-largest risk factor worldwide, behind only (1) childhood underweight and (2) unsafe sex. This investigation additionally found that ethanol use is responsible for 2.5 million deaths per year, which is almost 4% of all global deaths (World Health Organization., 2009).

CANONICAL ADDICTION MOTIVATIONAL FRAMEWORK

The development of AUDs (and other substance use disorders) is accepted to occur via neurobiological changes in brain regions and brain circuits implicated in motivational processes (Koob et al., 2014). Therefore, AUD can be thought of as disrupted motivational processing for ethanol. The motivational changes characteristic of AUD develop over the course of the three stage model of ethanol use leading to AUD: 1) Binge/Intoxication, 2) Withdrawal/Negative Affect, and 3) Preoccupation/Anticipation (Koob et al., 2014). The neuroadaptations which occur at each stage in the model increase the likelihood an AUD, and through repeated cycles this three-part process ultimately produce an AUD.

In the initial, Binge/Intoxication stage, the positively reinforcing pharmacological effects of ethanol leads the drinker to consume ethanol to levels of intoxication. Once intoxication occurs, the removal of ethanol from the brain regions and circuits implicated in the positive reinforcing effects of ethanol and motivational valence produces the Withdrawal/Negative Affect stage. At this stage, changes occur to motivational systems, and therefore symptoms which oppose the acute positively reinforcing effects of ethanol occur. For example, the socially disinhibiting, tension reducing effects of acute ethanol are now typically replaced with general irritability and anxiety. Ethanol-withdrawal induced anxiety is a common symptom following a binge exposure to ethanol (Koob et al., 2014). Following the Withdrawal/Negative Affect stage, the Preoccupation/Anticipation stage of AUD occurs, in which obtaining and drinking ethanol gains priority and the individual becomes more sensitive to conditioning effects associated with ethanol use and availability. It is important to note that this model for AUD is imperfect: Although history of ethanol exposure is a contributing component of AUD, the development of AUDs are accepted to occur due to a myriad of factors including but not limited to social, environmental, genetic, and ethanol exposure history (Koob et al., 2014). Nevertheless, several repeated cycles of the aforementioned 3-stage model produce an AUD in a minority of individuals in which healthy functioning is affected by the preoccupation with and use of ethanol.

ANIMAL MODELS OF ALCOHOL USE DISORDER

Psychiatric disorders, on the whole, are complex, multidimensional deviations of healthy functioning which are constantly evolving and contain various subtypes and diverse etiology (Edwards and Koob, 2003). It is therefore thought that using an animal model to generate the complete syndrome of any psychiatric disorder is highly unlikely, if not impossible. Moreover, animal models rely only on an animal's behavior to inform us of its internal state. Since the characteristics of many psychiatric disorders, including AUD, involve complex internal states that are often in disagreement with external behavior (i.e. an individual with AUD who does not drink despite cravings), animal models have limited utility investigating the subjective aspects of AUD (Naqvi and Bechara, 2010). Nonetheless, addiction scientists can generate observable aspects of AUD in animals, which can then be used for neurobiological investigation.

There are several animal models which have construct validity with the 3 stages of the addiction cycle (Binge/Intoxication, Withdrawal/Negative Affect, Preoccupation/Anticipation). These models have proven not only necessary for a comprehensive understanding of the addiction cycle, but also useful for screening potential treatment compounds for individuals with AUD. In particular, rodent models of the binge/intoxication stage have proven to have excellent predictive validity to ethanol intake in humans. Multiple FDA-approved medications for AUD were developed by using animal models before being successfully implemented in humans (Spanagel, 2017).

Historically, animal researchers have focused their investigations on the initial, positively reinforced aspects of ethanol drinking and its neurobiological substrates. Although investigations of this field have proven to be of vital importance for developing treatments, positively reinforced drinking mechanisms do not model all aspects of the pathology of AUD. Since the overwhelming majority ($\approx 90\%$) of alcohol drinkers do not have an AUD, it is clear that understanding only the positively reinforcing effects of initial ethanol use has limited utility. A more precise way to parse out AUD-like rather than

initial ethanol consumption is to investigate the neurobiological and behavioral differences between animals that drink ethanol that display aspects of AUD from animals that drink ethanol and do not display aspects of AUD. One such way to do this is to use the Becker-Lopez chronic intermittent ethanol (CIE) two-bottle choice (2BC) exposure model (Becker and Lopez 2004; Griffin, 2014; Griffin et al., 2009; Renteria et al., 2017).

The Becker-Lopez CIE 2BC exposure model is a mouse model of AUD that uses bouts of passive ethanol exposure followed by withdrawal to increase volitional ethanol drinking. In this model, mice initially drink ethanol for several weeks via 2BC drinking in which one bottle of contains water, and the other bottler contains 15% ethanol (v/v). Then, mice are then treated with bouts of chronic intermittent ethanol (CIE) vapor targeting blood ethanol concentrations (≈35-45 mM). Each bout contains a 4-day period during which an animal is treated with ethanol vapor for 16 hours per day followed by an 8 hour period of withdrawal. Following each bout mice undergo a 3 day period of withdrawal.

Because of the long duration of ethanol exposure, physiological processes occur to counteract the effects of ethanol and maintain homeostasis. As a result of these physiological processes, a new homeostatic set point emerges (allostasis), such that removal of ethanol is now a disruptive event. Once allostasis occurs, an animal is now dependent on ethanol for normal physiological functioning, and therefore increases its volitional ethanol intake following withdrawal. Therefore, the Becker-Lopez CIE/2BC model allows us to model two hallmarks of AUD: 1) Escalated intake, and 2) Withdrawal symptoms after ceasing use. This validated model of increased volitional ethanol drinking permits researchers to investigate neuroadaptations as alcohol drinking becomes dependent (Becker and Lopez, 2004; Renteria et al., 2017; Griffin et al., 2009). Importantly, the control group in this study identically drinks ethanol via 2BC drinking, but is not treated with CIE and therefore does not undergo withdrawal and develop dependence. Therefore, this control group of animals does not typically display escalated intake or withdrawal symptoms after ceasing ethanol use, and can therefore be thought of as a model of non AUD-like ethanol consumption.

MEDICATION OPTIONS

A hallmark of SUD and AUD is their chronically relapsing nature; 90% of individuals with AUD will begin drinking ethanol again to intoxicating levels following an extended period of abstinence (National Institute on Alcohol Abuse and Alcoholism [NIAAA], 1989). Current pharmacological and behavioral treatments for AUDs have achieved only moderate success decreasing the vulnerability to relapse in individuals with AUD in abstinence (Scofield et al., 2016). Therefore, a more comprehensive understanding of the neuroadaptations produced by chronic ethanol exposure in unexplored brain regions of addiction-relevance might yield improved targets for pharmacotherapy. Because the addiction cycle is composed of the 3 stage cycles, it is now even suggested that it is possible to use different drugs to treat different aspects of the addiction cycle (Koob et al., 2014). There are currently three medications approved by the FDA to treat AUD: disulfiram, acamprosate, and naltrexone.

Disulfiram (Antabuse) is an acetaldehyde dehydrogenase inhibitor that is targeted to decrease alcohol drinking and relapse via an aversion reaction. Ethanol is metabolized in the liver mainly via an oxidation pathway; alcohol is oxidized into acetaldehyde thru alcohol dehydrogenase. Then, acetaldehyde dehydrogenase oxidizes acetaldehyde into acetic acid, and then into water and carbon dioxide. Disulfiram's inhibition of acetaldehyde dehydrogenase therefore causes a build-up of acetaldehyde in the blood. High blood levels of acetaldehyde cause an aversive, flushing reaction along with tachycardia, nausea, and vomiting. Together these symptoms make ethanol consumption aversive, which therefore limits drinking. Although disulfiram has efficacy limiting ethanol consumption in individuals with AUD, there are major issues with compliance. Individuals with AUD, by definition, have a compulsive preoccupation with experiencing the effects of ethanol. Since Disulfiram limits this experience, individuals with AUD are likely to stop taking their medication. There have been attempts to enhance compliance

with via the development of slow-releasing disulfiram implants (Allen & Litten; 1992). However, these have proven only modestly effective.

Acamprosate (Campral) is used in AUD for relapse prevention in abstinent individuals with AUD. It has shown to be decrease the risk to relapse, but not affect the craving for ethanol in individuals with AUD (Umhau et al., 2011; Spanagel, 2014). The exact molecular mechanism responsible for the actions of acamprosate has still not yet been determined. It was originally hypothesized to normalize the excitatory and inhibitory signaling imbalances in the brain caused by chronic ethanol via its modulatory actions on the N-methyl-D-aspartate (NMDA) receptor or through action on metabotropic glutamate receptors (mGluRs) (Spanagel and Vengeliene, 2013; De Witte et al., 2005; Mann et al, 2008; Rammes et al, 2001; Harris et al., 2002). However, there has been some additional recent work showing that acamprosate works via normalizing calcium homeostasis (Spanagel et al., 2014). Overall, acamprosate has proven to be a moderately effective treatment strategy for maintaining abstinence with individuals with (Rösner et al., 2010).

Naltrexone is used in AUD for relapse prevention via reducing ethanol craving. Naltrexone is a μ opioid receptor antagonist that has been shown to reduce the positively reinforcing properties of alcohol in several animal models of ethanol drinking (Koob et al., 2014). Although data from the animal laboratory directly indicates that naltrexone reduces ethanol drinking by its actions on ethanol reward, it has shown efficacy in reducing alcohol craving components of AUD (Volpicelli et al., 1992). It has been shown that naltrexone is useful in reducing alcohol craving and the number of heavy drinking days relative to placebo in those individuals with AUD (Streeton and Whelan, 2001).

In addition to the three FDA-approved medications to treat AUD, there are several FDA-approved medications for other conditions that have shown efficacy reducing ethanol drinking in animal models of AUD. These medications are approved for a variety of different health issues, including but not limited to SUD (varenicline), nausea/vomiting (ondansetron), seizure disorders (gabapentin, topiramate), muscle spasms (baclofen), and even metabolic syndrome (fenofibrate). Clinical trials for these drugs are necessary, and in some cases, ongoing.

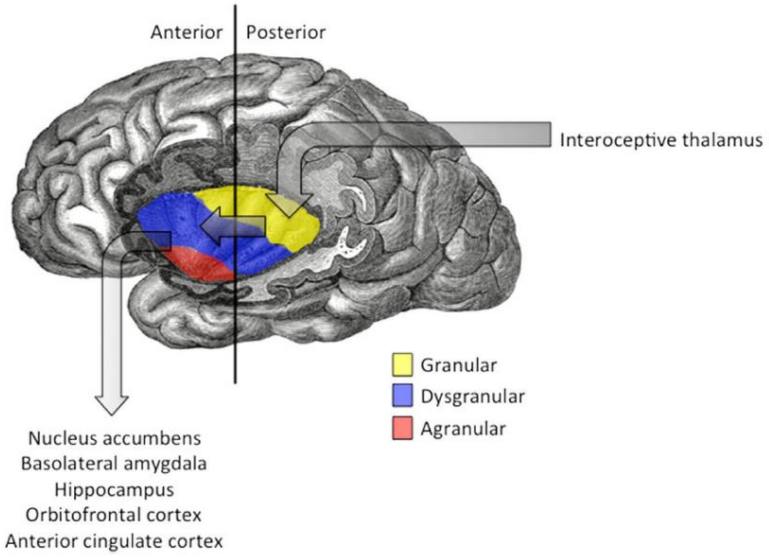
Finally, while the FDA-approved medications for AUD at present have limited efficacy, a larger component hindering successful treatment of individuals with AUD are factors inhibiting treatment-seeking. Only approximately 20% of people with AUDs seek any kind of treatment (Cohen et al., 2007; Dawson et al., 2006). This is thought to be attributed to the public perception and stigma attached to AUD relative to other mental illnesses, and doubts of the efficacy of treatment (Schomerus et al., 2011; Saunders et al., 2006). Moreover, some individuals with AUD have reservations about complete abstinence as the recovery goal, which many find neither feasible nor desirable (Copeland, 1997). Even more compelling is the fact that the current FDA-approved medications for AUD are prescribed in less than 9% of individuals who report needing help with an alcohol problem (Kranzler and Soyka, 2018). This is thought to occur because of the historically high proportion of treatment-seeking individuals do so via community-based addiction treatment centers which either have a lack of knowledge about AUD medications or concerns of their modest effects (Weisner et al., 1995; Cohen et al., 2007). Taken together, basic neurobiological research is needed to understand the pathology of AUD and screen potential treatment compounds; however, approaches to increase treatment seeking and education of addiction treatment centers are likely more immediately beneficial to improving clinical outcomes.

INSULAR CORTEX AND ADDICTION

The insular cortex (INS) is a cortical brain region composed of three structurally and functionally distinct subregions arranged posterior to anterior: the granular insular cortex (GIC), the dysgranular insular cortex (DIC), and the agranular insular cortex (AIC) (Figure 1; Morel et al., 2013). Sensory information from the viscera is transmitted to the GIC via a spinothalamic pathway via reciprocal connections with thalamic nuclei (Craig, 2002). Then, in a process called *interoception*, this information is relayed anteriorly and ventrally into DIC, and then into the AIC, which is considered the interoceptive hub. Thus, the INS integrates internal information about the physiological condition of bodily tissues

such as temperature, touch quality, urinary bladder and stomach distension, and sends this information to cortical areas of conscious thought so that an organism may make the appropriate decisions to ensure homeostasis (Craig, 2002; Craig, 2009). As an interoceptive brain region, the INS has major functions in the learning and experiencing of body-induced internal states such as awareness, taste memory, and pain perception (Brooks et al., 2005; Craig et al., 2000; Zhuo, 2008; Henderson et al., 2007).

Figure 1: Diagram of the insular cortex



The insular cortex, its subdivisions and major inputs/outputs. Reproduced under the creative commons license from *Naqvi et al. Ann N Y Acad Sci 1316 (2014) 53-70.*

INSULAR CORTEX AND ADDICTION: HUMAN STUDIES

Research in humans indicates that the INS and maladaptive interoceptive processing are involved in conscious craving and potentially relapse components of

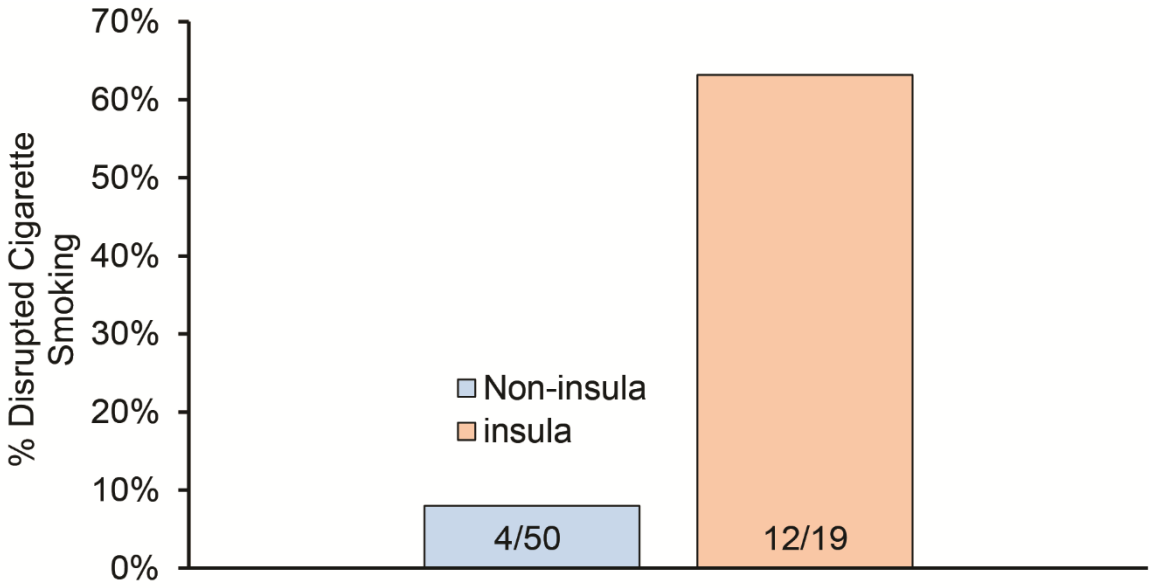
addiction; however, the specific role of the INS in these processes has not yet been clearly delineated. The seminal study investigating the role of the INS in addiction suggests the INS mediates drug craving; smokers with INS damage underwent a "disruption" of cigarette smoking, in which they lost the subjective urges to smoke (Figure 2; Naqvi et al., 2007). Additional investigations of INS damage and cigarette smoking behavior have largely corroborated these findings, indicating a clear role for INS functioning in the maintenance of addiction to cigarette smoking (Suner-Soler et al., 2012; Abdolahi et al., 2015; Gaznick et al., 2014; Abdolahi et al., 2017). Several functional imaging studies support this "urge" hypothesis; human addicts in withdrawal displayed a positive correlation between INS activity and subjective drug urges when exposed to drug cues. Importantly, this relationship was observed across several drugs of abuse, and largely not observed in recreational drug users (Table 1; Naqvi and Bechara, 2010).

These data suggest that INS neural networks sensitize during the development of addiction to mediate drug craving (Droutman et al., 2015). From these studies, amongst others, Naqvi and Bechara (2010) have created a working model for the INS and drug addiction. They suggest that after long term drug use, environmental cues originally paired with drug use activate interoceptive memories of the bodily effects of drugs in the INS. These interoceptive memories are processed from the INS to other cortical regions such as the anterior cingulate cortex and are thought to contribute to the feeling of conscious urges to take drugs. From this perspective, INS activity is an integrator between the bodily effects of drugs and environmental cues and plays a role in the conscious perception of drug craving.

Conversely, there are other human data which contradicts this model. Imaging studies have shown reduced gray matter in the INS in drug users, and reduced INS activity in decision-making tasks in drug users predicts relapse to methamphetamine use (Droutman et al., 2015). This data is mirrored by the alcohol research community. Interoceptive awareness, which has been taken to indicate healthy INS functioning, has been shown to be reduced in individuals with AUD relative to individuals without AUD (Ateş Çöl et al., 2016). Moreover, this investigation found an inverse relationship between

interoceptive awareness and ethanol craving. These latter findings indicate that that INS activity *protects* against drug relapse. Nevertheless, on the whole, there exists ample evidence that the INS plays a modulatory role in drug craving and relapse in complex and multidimensional manner(s). Since the INS is composed of three anatomically and functionally distinct subregions, each containing different neurocircuitry for mediating its respective functions, we reason that the disparate findings in the human literature indicate multiple roles for the INS in craving and relapse. Therefore, it is likely that the role of the INS in craving and relapse depends upon the functional subdivision and/or circuit that were not observable in prior studies with standard imaging techniques.

Figure 2: Insula damage disrupts cigarette smoking



Bar graph of the seminal study investigating INS damage and cigarette smoking. Damage to brain regions other than the INS resulted in a disruption of cigarette smoking in 4/50 individuals (blue). Damage to the INS resulted in a disruption of cigarette smoking in 12/19 individuals (orange). This finding has been replicated in several studies. Adapted with permission from *Naqvi et al. Science 315 (2007) 531-534.*

Table 1: Studies investigating brain activity during exposure to drug cues

Study	Drug	Insula	OFC/ VMPFC	ACC	DLPFC	Amygdala	VS	HF
McBride et al. (2006)	Cigarettes	L	L	L, R	L			
Franklin et al. (2007)	Cigarettes	L	R		L	L, R	L, R	L, R
Brody et al. (2002)	Cigarettes	L, R	L, R	L, R	L, R	L		
Brody et al. (2007)	Cigarettes	L, R		L, R	L			
McClernon et al. (2005)	Cigarettes	L, R		L, R	L, R			
Lee et al. (2005)	Cigarettes	R	L	L	R			
Wang et al. (2007)	Cigarettes	R	R	R	R	R	R	L, R
Kilts et al. (2004)	Cocaine	L		R		L, R	R	
Bonson et al. (2002)	Cocaine	L	L		R	L		
Kilts et al. (2004)	Cocaine	L, R	L, R	L		L, R	R	
Wang et al. (1999)	Cocaine	L, R	L, R					
Garavan et al. (2000)	Cocaine	R		L	L, R			
Wexler et al. (2001)	Cocaine	R		L, R	L			
Myrick et al. (2004)	Alcohol	L, R	L, R	L, R			L, R	L
Tapert et al. (2004)	Alcohol	L, R		L	L			
Sell et al. (1999)	Heroin	L	L	L, R				

The table above are a meta-analysis of functional imaging studies correlating brain activity and subjective drug urges. In nearly all of the studies, individuals are exposed to drug-associated stimuli and asked to rate their subjective urges while brain activity is measured via PET or FMRI scan. Adapted with permission from *Naqvi and Bechara, Brain Structure Function 214 (2010) 435-450.*

INSULAR CORTEX AND ADDICTION: RODENT STUDIES

The INS is relatively unexplored in comparison to other mesocorticolimbic brain regions, but has been shown to mediate the motivational properties of drugs of abuse. A comprehensive listing of all studies of our knowledge investigating the INS and addiction-relevant behavior in rodents is presented in Table 2 (adapted from Naqvi et al., 2014). Since some studies did not focus specifically on the 3 anatomical and functional subdivisions of the INS, the subdivisions were collapsed into anterior and posterior for simplicity. Anterior contains more of the AIC-DIC subdivisions and posterior contains

DIC-GIC, respectively. As indicated below, the majority of studies used INS inactivation techniques during conditioned place preference or aversion paradigms or self-administration studies to determine whether the INS mediates motivational properties of drugs of abuse. Conditioned place preference for amphetamine, morphine, and nicotine are sensitive to INS inactivation, suggesting INS activity mediates some of the rewarding aspects of these drugs (Contreras et al., 2007; Scott and Hiroi, 2011; Contreras et al., 2012; Li et al., 2013). Moreover, conditioned place aversion for nicotine and morphine are also sensitive to INS inactivation, suggesting INS activity mediates some of the aversive aspects of nicotine and morphine (Scott and Hiroi, 2011; Li et al., 2013) Drug self-administration studies have shown that INS activity mediates seeking behavior for drugs of abuse (Di Pietro et al., 2008; Hollander et al., 2008; Forget et al., 2010; Kutlu et al., 2013; Pushparaj et al., 2013; Pushparaj et al., 2015). Taken together, these investigations suggest that the INS may mediate the recall of both rewarding and aversive aspects of past drug use, and may play a role in motivation to approach or avoid drugs of abuse.

INSULAR CORTEX AND ADDICTION: RODENT STUDIES: ALCOHOL

Whether the INS mediates the motivational properties of ethanol has not yet been sufficiently determined. Table 2 highlights the sole 6 studies (in shading) investigating the INS and addiction-relevant ethanol behaviors (Seif et al., 2013; Pushparaj et al., 2015; Jaramillo et al., 2016; Jaramillo et al., 2017; Jaramillo et al., 2018; Lasek et al., 2017). Pushparaj and colleagues found that operant ethanol self-administration in rats was reduced by inactivation of the GIC subregion, suggesting that this subregion of the INS mediates initial, positively reinforced aspects of ethanol use (Pushparaj et al., 2015). The following 5 studies suggest that the more anterior AIC subregion mediates the interoceptive, discriminative stimulus effects of ethanol as well as positively and negatively reinforced motivational aspects of ethanol consumption. Seif and colleagues elegantly modeled compulsive drug self-administration by teaching rats to self-administer ethanol despite it being adulterated with the bitter tastant quinine or paired with a foot shock. Optogenetic

inactivation of glutamatergic terminals from the AIC to the NAc core reduced self-administration resistant to quinine adulteration and to a foot shock. Notably, they found that self-administration without adulteration with quinine or paired with a foot shock was unchanged by inactivation of these terminals (Seif et al., 2013). This finding suggests a role for AIC circuitry mediating compulsive aspects of alcohol use characteristic of AUD. Jaramillo et al. (2016) found that temporary pharmacological inactivation of the AIC reduced the discriminative stimulus/interoceptive effects of ethanol. Since the discriminative stimulus/interoceptive cues of a drug are thought to be predictive of drug-related behaviors and potentially abuse potential, this finding suggests AIC functioning is linked to alcohol abuse (Koob and Volkow, 2010; Solinas et al., 2006). A follow-up investigation found that temporary inhibition of the AIC and AIC inputs to the NAc core via Designer receptors exclusively activated by Designer Drugs (DREADDS) both *enhanced* the sensitivity to the interoceptive effects of ethanol in subsequent operant and pavlovian procedures (Jaramillo et al., 2017). Although these effects are in opposition to earlier findings, they nonetheless add evidence that AIC activity and its output may alter the interoceptive effects of ethanol. Finally, Jaramillo and colleagues found the first evidence that AIC and its output are involved in ethanol reinforcement. Temporary inhibition of the AIC via DREADDS increased ethanol self-administration, and temporary inhibition of the AIC inputs to the NAc core via DREADDS decreased self-administration (Jaramillo et al., 2018). Finally, Lasek and colleagues have recent evidence suggesting the AIC is directly recruited in animal models of advanced drinking. Perineuronal nets (PNN), extracellular matrix structures that play roles in synaptic stabilization and plasticity, are increased in the AIC after binge ethanol exposure (Chen et al., 2015). Digesting the PNN with chondroitinase ABC in the AIC reduced ethanol consumption in a model of binge drinking. Importantly, the reduction in ethanol consumption was greatest for quinine adulterated ethanol (Lasek at al., 2017). These data indicate that binge ethanol exposure increases AIC PNN expression, which then mediates both binge drinking and aversion resistant drinking. Taken together, the 6 aforementioned studies investigating the INS and addiction-relevant ethanol behaviors suggest that the INS plays roles in both initial ethanol

drinking and more pathological forms of ethanol drinking depending upon the subregion and/or circuit of investigation.

Table 2: Rodent studies of the insula in addiction-relevant behavior

Study	Drug	Insular Region	Species	Manipulation	Behavioral Effects
Contreras et al. 2007	Amphetamine	Posterior	Rat	Lidocaine Injection	Reversibly abolished CPP Reversibly abolished lithium-induced malaise
Hollander et al. 2008	Nicotine	Anterior and Posterior Combined	Rat	Hypocretin receptor blockade	Reduced self-administration
Forget et al. 2010	Nicotine	Posterior	Rat	GABA agonist inactivation	Reduced self-administration Prevented drug and cue-induced reinstatement of self-administration
Scott and Hirori 2011	Nicotine	Anterior and Posterior Combined	Mouse	Excitotoxic lesion	Disrupted nicotine-cue approach Spared withdrawal-cue avoidance
Contreras et al. 2012	Amphetamine	Anterior, Posterior	Rat	Protein synthesis inhibition	Abolished retrieval of CPP No effect on CPP
Pushparaj et al. 2013	Nicotine	Posterior	Rat	Electrical inhibition	Reduced self-administration Prevented drug and cue-induced reinstatement of self-administration
Seif et al. 2013	Alcohol	Anterior input to nucleus accumbens	Rat	Optogenetic inhibition	Disruption of alcohol intake when paired with aversive consequence Sparing of alcohol intake in absence of aversive consequence
Pushparaj and Le Foll 2015	Alcohol	Posterior	Rat	GABA agonist inactivation	Reduced self-administration and oral alcohol intake
Di Pietro et al. 2008	Cocaine	Anterior	Rat	D1 agonist D1 antagonist	D1 agonism had no effect D1 antagonism reduced cocaine self-administration
Kutlu et al. 2013	Nicotine	Anterior	Rat	D1 antagonist D2 antagonist	D1 antagonist reduced self-administration D2 antagonist had no effect
Li et al. 2013	Morphine	Anterior, Posterior	Rat	GABA agonist inactivation	No effect on CPP, Impaired CPA Disrupted CPP, Impaired CPA
Jaramillo et al. 2016	Alcohol	Anterior	Rat	GABA agonist inactivation	Reduced interoceptive sensitivity to alcohol
Lasek et al. 2018	Alcohol	Anterior	Mouse	Chondroitinase ABC	Digestion of perineuronal nets attenuates alcohol consumption, and this effect is most pronounced for alcohol drinking under aversive conditions
Venniro et al. 2017	Methamphetamine	Anterior input to central amygdala	Rat	DREADD inhibition	Decreased relapse to methamphetamine seeking after voluntary abstinence
Jaramillo et al. 2017	Alcohol	Anterior, Anterior input to nucleus accumbens	Rat	DREADD inhibition	Both potentiated sensitivity to interoceptive effects of alcohol in operant and pavlovian tasks
Jaramillo et al. 2018	Alcohol	Anterior, Anterior input to nucleus accumbens	Rat	DREADD inhibition	Silencing anterior increased self-administration Silencing input to accumbens decreased self-administration

The table above lists all of the rodent studies investigating the INS and addiction. Each study is broken down by investigated drug of abuse, INS subregion, species (rat or mouse), and INS manipulation method. The investigations of alcohol (6) are highlighted in gray. Adapted from *Naqvi et al. Ann N Y Acad Sci 1316 (2014) 53-70.*

SYNAPTIC PLASTICITY

Synaptic plasticity mechanisms are the means by which neural networks adapt to strengthen or weaken their connections to form the basis of information storage and are thought of as mechanisms of learning and memory (Kauer and Malenka, 2007; Kandel et al., 2014). Maladaptive drug-induced forms of synaptic plasticity are accepted to contribute to addictive behaviors (Luscher and Malenka, 2011). Extensive work from our laboratory has used the Becker-Lopez CIE-2BC model to investigate glutamatergic synaptic plasticity mechanisms in the nucleus accumbens encoding aspects of AUD such as escalated drinking (Jeanes et al., 2011; Jeanes et al., 2014; Renteria et al., 2017; Renteria et al., 2018). Specifically, our laboratory has observed a form of NMDAR-dependent long-term depression (LTD) in the nucleus accumbens shell that is disrupted over the time course of CIE-induced escalated drinking. These data suggest that disrupted accumbal LTD contribute, in part, to the expression of ethanol dependence (Jeanes et al., 2011; Renteria et al., 2018).

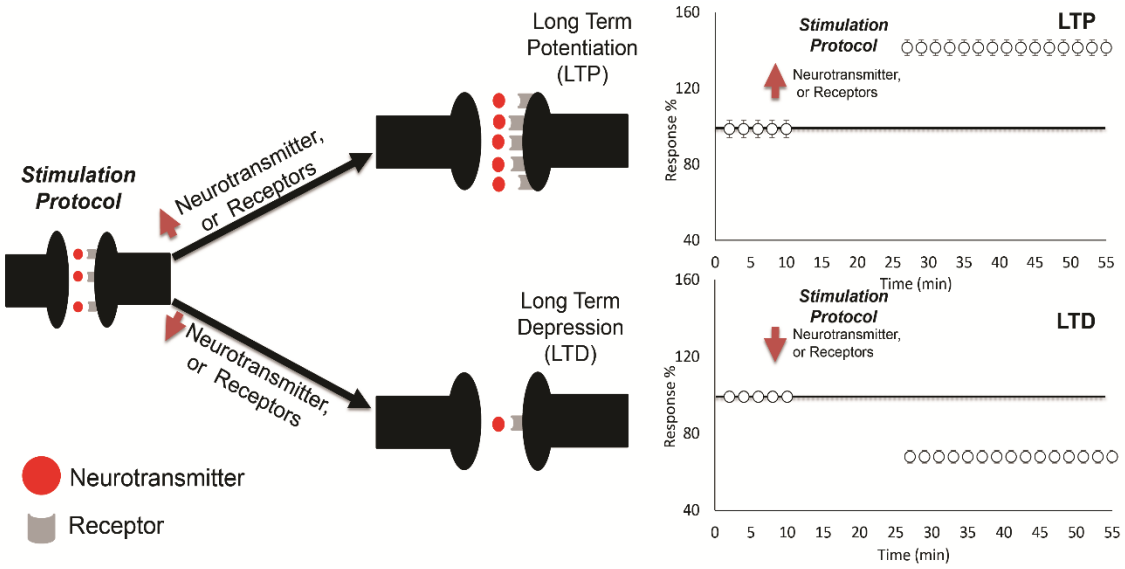
THE AIC AND SYNAPTIC PLASTICITY

Of the subregions of the INS, the chemoarchitecture, circuitry, and function of the anterior most region, the AIC, makes it of high relevance for addiction researchers (Craig 2002; Craig 2009). The AIC contains high levels of D1 receptors as well as μ-opioid receptors, canonically associated with initial positive drug motivation and reward (Hurd et al., 2001; Baumgärtner et al., 2006). Moreover, the AIC also contains high expression of CRF1 receptors, implicated negative drug reinforcement (Van Pett et al., 2000). Also, there are prominent reciprocal connections to mesolimbic regions heavily implicated in motivated behavior and addiction such as the nucleus accumbens, the central amygdala, the locus coeruleus, and the rostromedial tegmental nucleus (Chikama et al., 1997; Kapp et al., 1985; Lavezzi and Zahm, 2011; Uematsu et al., 2015). We wished to determine

whether the actions of ethanol on the glutamate system in the AIC disrupts homeostatic interoceptive mechanisms and promotes aspects of AUD. Therefore, the major focus of the investigation is to determine 1) Whether ethanol is a direct synaptic target for the actions of acute ethanol, 2) Whether functioning of the INS is disrupted by chronic ethanol exposure.

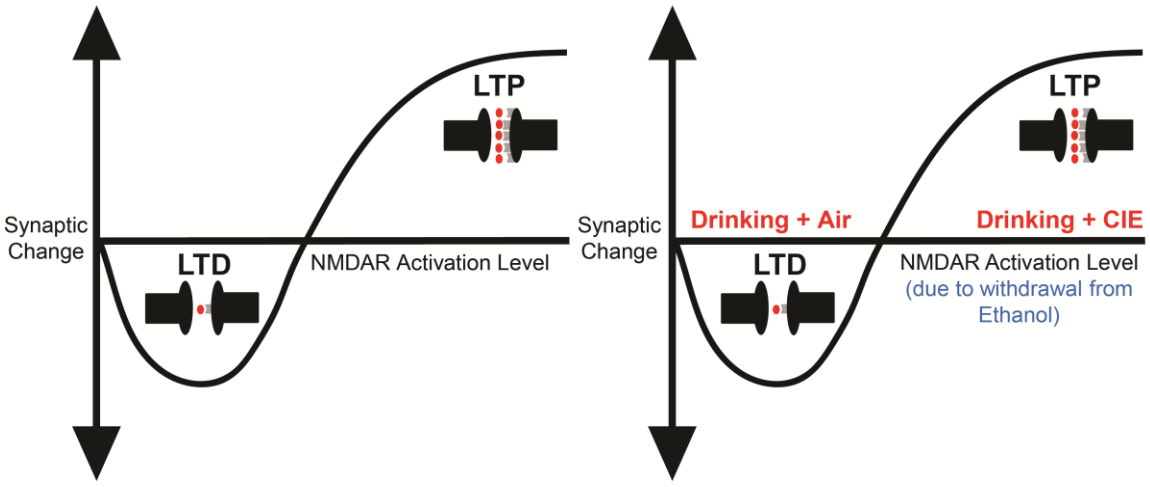
Our NMDAR-dependent LTD design was based off of the Artola-Brocher-Singer rule of canonical NMDAR-dependent synaptic plasticity. This rule states that the polarity of synaptic plasticity depends upon ability of the postsynaptic membrane to reach sequential thresholds and regulate postsynaptic calcium levels (Artola & Singer, 1993; Cho et al., 2001). In this framework, postsynaptic calcium levels determine expression of plasticity; low concentrations of postsynaptic calcium cause LTD, medium postsynaptic calcium causes no change in synaptic strength, and that higher postsynaptic calcium levels cause a long-term potentiation (LTP) (Figure 3; Figure 4; Cho 2001). It has been shown in several investigations that chronic ethanol and withdrawal enhances NMDAR functioning (Roberto and Varodayan, 2017). Therefore, since our two groups of animals, Drinking + CIE and Drinking + Air, will undergo significantly differential ethanol exposure, we predicted that they will display differential expression of synaptic plasticity. Taken together we predicted that disruptions in AIC NMDAR-dependent plasticity indicate the transition from low-risk drinking to AUD-like drinking.

Figure 3: Schematic of the expression of synaptic plasticity



The figure above represents how NMDAR-dependent synaptic plasticity is generated in a slice electrophysiology experimental preparation. Electrophysiologists stimulate the postsynaptic neuron (stimulation protocol) at a certain frequency with a stimulating electrode to mimic *in vivo* presynaptic glutamatergic input. The membrane potential of the postsynaptic neuron is typically slightly depolarized from the resting membrane potential to remove the voltage-dependent magnesium blockade of NMDARs. Higher presynaptic stimulation frequencies typically result in the insertion of AMPARs or enhanced presynaptic glutamate release, causing the expression of long term potentiation (LTP). Lower stimulation frequencies typically result in the removal of AMPARs or reduced presynaptic glutamate release, causing the expression of long term depression (LTD).

Figure 4: Schematic of NMDAR-dependent synaptic plasticity and our hypothesis



The figure above represents the canonical mechanism for NMDAR-dependent synaptic plasticity (left) and our experimental prediction (right). The polarity of NMDAR-dependent synaptic plasticity is dependent upon postsynaptic calcium influx. Higher presynaptic stimulation frequencies typically cause a large NMDAR activation, leading to large amounts of postsynaptic calcium influx and the activation of proteins causing LTP. Lower presynaptic stimulation frequencies typically cause a lower NMDAR activation, leading to smaller amounts of postsynaptic calcium influx and the activation of proteins causing LTD. From this model, and the fact that chronic ethanol has shown to enhance NMDAR activation levels, we predicted a difference in the expression of plasticity between Drinking + Air and Drinking + CIE groups. Since Drinking + CIE mice experience more chronic ethanol action and withdrawal, we predicted a more LTP-like phenotype in Drinking + CIE relative to Drinking + Air mice.

Chapter 2:

Ethanol Modulates Glutamatergic Transmission and NMDAR-Mediated Synaptic Plasticity in the Agranular Insular Cortex

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Authors: Joel Shillinglaw, Richard Morrisett, Regina Mangieri. Joel Shillinglaw and Richard Morrisett conceived and designed experiments. Joel Shillinglaw performed the experiments. Joel Shillinglaw and Regina Mangieri analyzed the data and interpreted the results. Joel Shillinglaw and Regina Mangieri wrote the paper.

ABSTRACT

The agranular insular cortex (AIC) has recently been investigated by the alcohol field because of its connectivity to and modulatory control over limbic and brainstem regions implicated in alcohol use disorder (AUD), and because it has shown involvement in animal models of alcohol drinking. Despite evidence of AIC involvement in AUD, there has not yet been an examination of whether ethanol modulates glutamatergic and γ-amino-butyric acid (GABA)ergic synaptic transmission and plasticity in the AIC. Characterizing how the synaptic transmission and plasticity states of AIC cortical processing neurons are modulated by acute ethanol will likely reveal the molecular targets by which chronic ethanol alters AIC function as alcohol drinking transitions from controlled to problematic. Therefore, we collected brain slices from ethanol-naïve adult male mice, obtained whole-cell recording configuration in layer 2/3 AIC pyramidal neurons, and bath-applied ethanol at pharmacologically relevant concentrations during electrophysiological assays of glutamatergic and GABAergic synaptic transmission and plasticity. We found that ethanol inhibited electrically evoked N-methyl-D-aspartate receptor (NMDAR)-mediated excitatory postsynaptic currents (EPSCs) in a concentration-related fashion, and had little effect on evoked α-amino-3-hydrox-5-methylisoxazole-4-propionic acid-type receptor (AMPAR)-mediated EPSCs. Ethanol had no effect on spontaneous excitatory postsynaptic currents (sEPSCs) or inhibitory

$GABA_AR$-mediated postsynaptic currents (sIPSCs). We found that synaptic conditioning (low-frequency stimulation for 15 min at 1 Hz) induced a form of long-term depression (LTD) of evoked AMPAR-mediated EPSCs. The ability to induce LTD was inhibited by a nonselective NMDAR antagonist (DL-2-amino-5-phosphonovaleric acid), and also by acute, intoxicating concentrations of ethanol. Taken together these data suggest that the glutamate, but not GABA system in the AIC is uniquely sensitive to ethanol, and that in particular NMDAR-mediated processes in the AIC may be disrupted by pharmacologically relevant concentrations of ethanol.

INTRODUCTION

Despite several years of preclinical research investigating the mechanisms underlying the transition from controlled to problematic alcohol drinking in order to develop future therapeutic approaches, alcohol use disorder (AUD) remains one of the most prevalent and costly health problems in the United States (Sacks et al., 2015; Stahre et al., 2014). Current pharmacological and behavioral treatments have achieved only moderate success largely due to their inability to decrease the vulnerability to relapse in abstinent addicts (Scofield et al., 2016). Therefore, investigating the neural networks implicated in the craving and relapse components of AUD is vital to developing more efficacious treatment options. Recent research suggests that deficits in interoceptive processing, or the processing and integration of physiological bodily states, may in part contribute to craving and relapse components of substance use disorders (Paulus and Stewart, 2014).

The agranular insular cortex (AIC) is a brain region implicated in interoceptive processing, and altered AIC function and output to subcortical limbic regions has been shown to mediate alcohol intake in animal models of AUD (Seif et al., 2013; Jaramillo et al., 2016; Jaramillo et al., 2017; Jaramillo et al., 2018). Yet despite evidence for altered AIC function and output in AUD, there has been no investigation of whether the AIC is an ethanol-sensitive brain region in which basic synaptic functions are modulated by

pharmacologically relevant concentrations of acute ethanol. It is widely accepted that the synapse is sensitive to ethanol, and that ethanol's major pharmacodynamic effects occur at least in part via its modulatory actions on the major fast excitatory and inhibitory neurotransmitter systems, glutamate and gamma-aminobutyric acid (GABA), respectively. However, these synaptic actions have been shown to be both brain region and concentration-dependent (Nie et al., 1994; Lovinger et al., 1990; Roberto et al, 2003; Weitlauf et al., 2008; Badanich et al., 2013; Kash et al., 2008).

Moreover, the synaptic targets of acute ethanol have generally displayed opposing or compensatory effects after chronic ethanol exposure in animal models designed to mimic long-term alcohol abuse (Lovinger and Roberto, 2013). These compensatory effects of chronic ethanol on synaptic receptors have been shown to encode long-term alterations in glutamatergic and GABAergic transmission and to underlie, in part, aspects of AUD such as withdrawal, tolerance and dependence (Jeanes et al., 2011; Renteria et al., 2017; Lovinger & Kash., 2015; Lovinger and Roberto, 2013). We therefore suggest that any synaptic target in the AIC that is sensitive to acute ethanol may be a target by which chronic ethanol disrupts AIC function as chronic ethanol shifts alcohol drinking from controlled to problematic.

For these reasons we investigated the effects of acute ethanol on pharmacologically isolated glutamatergic and GABAergic synaptic transmission, and an N-methyl D-aspartate-type glutamate receptor (NMDAR)-dependent glutamatergic synaptic plasticity in mouse AIC. We decided to investigate layer 2/3 pyramidal neurons since layer 2/3 of the cortex is generally considered the intracortical processing layer. We found that NMDAR-mediated currents were inhibited by pharmacologically relevant concentrations of ethanol. Conversely, alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid-type glutamate receptor (AMPAR)-mediated currents were insensitive to ethanol. Ethanol had no effect on spontaneous excitatory postsynaptic currents (sEPSCs) or spontaneous inhibitory γ-amino-butyric acid receptor (GABAR)-mediated postsynaptic currents (sIPSCs). Our investigated form of synaptic plasticity, NMDAR-dependent long-term depression (LTD), was sensitive to pharmacologically relevant

concentrations of ethanol. These findings are the initial demonstration that the AIC is a direct synaptic target for the actions of ethanol, and that glutamatergic transmission and plasticity, but not GABAergic transmission, is sensitive to pharmacologically relevant concentrations of acute ethanol.

METHODS

Preparation of Brain Slices

Mice were briefly anesthetized with isoflurane, euthanized by decapitation, and then brains were rapidly extracted and placed in ice-cold oxygenated artificial cerebrospinal fluid (ACSF) containing the following (in mM): 210 Sucrose, 26.2 $NaHCO_3$, 1 NaH_2PO_4, 2.5 KCl, 11 dextrose, bubbled with 95% O_2/5% CO_2. Coronal slices (230 to 250 µm thick) containing the most anterior portion of the AIC (anterior-posterior = +2.46 to +1.54) were then collected in ice-cold oxygenated ACSF using a Leica VT1000S vibrating microtome (Leica Corp., Bannockburn, IL). Slices were then transferred into an incubation solution containing the following (in mM): 120 NaCl, 25 $NaHCO_3$, 1.23 NaH_2PO_4, 3.3 KCl, 2.4 $MgCl_2$, 1.8 $CaCl_2$, 10 dextrose, continuously bubbled with 95% O_2/ 5% CO_2; 32°C, and maintained in this solution at least 45 min prior to recording.

Patch-Clamp Electrophysiology

Whole cell voltage clamp recordings were made in layer 2/3 pyramidal AIC neurons from anterior-posterior = +2.46 to +1.54. Pyramidal neurons were identified based on morphology (large, pyramidal shape) using a MRK200 Modular Imaging system (Siskiyou Corporation, Grants Pass, OR) mounted on a vibration isolation table. Passive electrical membrane properties for each cell at the beginning and end of each

experiment are provided in Tables 1-7. Recordings were made in ACSF containing (in mM): 120 NaCl, 25 $NaHCO_3$, 1.23 NaH_2PO_4, 3.3 KCl, 1.2 $MgSO_4$, 2.0 $CaCl_2$, and 10 dextrose unless otherwise noted, bubbled with 95% O_2/ 5% CO_2; 32°C, controlled by an in-line bath heather (Warner Instruments, Hamden, CT). The bath ACSF perfused brain slices at a rate of 2.0 mL/min. Recording electrodes (thin-wall glass, WPI Instruments, Sarasota FL) were made using a P-97 Flaming/Brown model micropipette puller (Sutter Instruments, San Rafael, CA) which produced electrodes of resistances from 3-6 MΩ. Series resistance (Rs) was monitored throughout the duration of each experiment and cells with Rs of over 30 MΩ or that changed over 20% over the course of the experiment were excluded from the analysis.

All chemicals, unless otherwise noted, were obtained from Sigma-Alrich or Tocris Bioscience with the exception of ethanol, which was obtained from Pharmco-Aaper. Multiple cells per brain slice were sometimes recorded from in glutamatergic and GABAergic transmission experiments, but only when the first cell recorded from was assigned to the time and sham solution exchange control condition. Thus, the final recording for each brain slice occurred once the slice was exposed to ethanol. For LTD experiments, only one cell per brain slice was used.

Evoked Glutamatergic Transmission

For evoked excitatory (NMDAR-mediated) postsynaptic currents, recording electrodes were filled with (in mM): 120 $CsMeSO_4$, 15 CsCl, 8 NaCl, 10 HEPES, 0.2 EGTA, 10 TEA-Cl, 4 Mg-ATP, 0.3 Na-GTP, 0.1 Spermine, and 5 QX-314-Cl. DNQX (20 µM) was added to the recording ACSF to block AMPA receptors, along with picrotoxin (50 µM) to block $GABA_A$ receptors. The recording ACSF for evoked NMDAR-mediated experiments contained 1.0 mM $MgSO_4$, and EPSCs were evoked by local stimulation while holding the postsynaptic membrane voltage at -40 mV for 2.4 seconds. For evoked excitatory (AMPAR-mediated) postsynaptic currents, recording

electrodes were filled with (in mM): 120 K-gluconate, 10 KCl, 10 HEPES, 2 $MgCl_2$, 1 EGTA, 2 Mg-ATP, and 0.3 Tris-GTP. DL-APV (100 µM) was added to the recording ACSF to block NMDA receptors, along with picrotoxin (50 µM). Neurons in evoked AMPAR-mediated experiments were held at -70 mV for the entirety of the experiment. For both evoked AMPAR-mediated and NMDAR-mediated postsynaptic current experiments, standard evoked EPSCs were established for at least 8 min (at 0.025 Hz) to ensure stable recordings, followed by 10 min periods of ethanol treatment and ethanol washout. Additional validation experiments were conducted to confirm that the currents under investigation were mediated by the receptors of interest. Evoked NMDA-mediated synaptic currents were reduced in amplitude by ≈ 78% by 100 µM DL-APV, and evoked AMPAR-mediated synaptic currents were reduced in amplitude by ≈ 93% by 20 µM DNQX (data not shown).

Spontaneous Glutamatergic and GABAergic Transmission

For spontaneous excitatory postsynaptic currents (sEPSCs), recording electrodes were filled with (in mM): 135 $KMeSO_4$, 12 NaCl, 0.5 EGTA, 10 HEPES, 2 Mg-ATP, and 0.3 Tris-GTP. Picrotoxin (50 µM) was added to the recording ACSF. For spontaneous inhibitory postsynaptic currents (sIPSCs), recording electrodes were filled with (in mM): 120 CsCl, 10 HEPES, 2 $MgCl_2$, 1 EGTA, 2 Mg-ATP, 0.3 Tris-GTP, and 1 QX-314. Kynurenic acid (1 mM) was added to the recording ACSF to block AMPA and NMDA receptors. For both spontaneous excitatory and inhibitory postsynaptic current experiments, neurons were held at -70 mV for 10 min to ensure stable recordings, followed by 10 min periods of ethanol treatment and ethanol washout. Additional validation experiments were conducted to confirm that the currents under investigation were mediated by the receptors of interest. sEPSCs were reduced in frequency by ≈ 93% by 1 mM kynurenic acid, and sIPSCs were reduced in frequency by ≈ 95% by 50 µM picrotoxin (data not shown).

Synaptic Plasticity

For LTD synaptic plasticity experiments, recording electrodes were filled with (in mM): 120 K-gluconate, 10 KCl, 10 HEPES, 2 $MgCl_2$, 1 EGTA, 2 Mg-ATP, and 0.3 Tris-GTP. Neurons were held at -70 mV for the entirety of the experiment, and the ACSF was supplemented with picrotoxin (50 µM). Standard evoked EPSCs were established for at least 10 min (at 0.025 Hz) to ensure stable recordings, and then followed by a low-frequency stimulation protocol consisting of 1 Hz stimulation for 15 min. Evoked EPSCs were then monitored for a 30 min post-stimulation period at 0.025 Hz to test for the expression of LTD.

Data Acquisition & Analysis

All currents were acquired using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA), filtered at 1 kHz, and digitized at 10-20 kHz via a Digidata 1440A interface board using pClamp 10.2 (Axon Instruments). In spontaneous experiments, sEPSCs and sIPSCs were recorded for 30 min and separated into 198 consecutive sweeps; events greater than 5 pA and 10 pA were analyzed in sEPSC and sIPSC experiments, respectively, for mean frequency and mean amplitude. For all evoked experiments, postsynaptic currents (100-200 pA) were evoked via either theta glass electrode or a stainless steel bipolar stimulating electrode (MX21AES, FHC, Inc., Bowdoin, ME, United States) placed approximately 500 µm dorsomedial to the cell body (Figure 1).

For all experiments investigating acute ethanol on GABAergic and glutamatergic transmission, we used two approaches to statistical analysis. First, a General Linear Model Repeated Measures in IBM SPSS Advanced Statistics 23 was used, with time or phase of the experiment as the repeated measure, and treatment condition (ethanol concentration) as the between-groups factor. For evoked currents, we analyzed the entire time course of the experiment, with 28 levels of the repeated measure (time), and 4 levels (eNMDAR) or 5 levels (eAMPAR) of the between-groups factor (treatment condition). For spontaneous currents, we used phase of the experiment as the repeated measure (3

levels: baseline, treatment, and washout) and treatment condition as the between-groups factor (2 levels). When sphericity within groups was violated (as indicated by Mauchly's test), the Greenhouse-Geisser adjusted degrees of freedom and p values were reported in the text, rounded to the nearest whole number. Second, we also analyzed the effects of ethanol on evoked and spontaneous currents without the use of a repeated measure. We used a 1-way between groups ANOVA to compare treatment conditions during a particular phase of the experiment – either the treatment phase (for evoked NMDAR) or the washout phase (for evoked AMPAR). These were followed by Bonferroni-corrected multiple comparisons. For spontaneous currents, we performed between groups analysis (t-test) to compare treatment conditions during just the treatment phase. GraphPad Prism 8.0 was used to analyze LTD experiments. The expression of LTD was determined by comparing the 20 to 30 min period after the low-frequency stimulation protocol to the 10 min baseline period. Statistical significance from baseline for within each treatment group was defined as $p < 0.05$ using a one-sample t test. Group comparisons for LTD experiments were made using a one-way ANOVA and Bonferroni post hoc test. Statistical significance for all experiments was defined as $p < 0.05$.

Mice

Ethanol-naïve *Drd1a*-tdTomato BAC transgenic male mice (MMRRC:030512-UNC) of at least 7 weeks of age were used for all experiments. Briefly, an existing colony of *Drd1a*-tdTomato mice (Ade et al., 2011; initial breeding pairs obtained from The Jackson Laboratory, Stock No. 016204) was maintained by backcrossing mice onto a C57BL/6J background in which only one parent carried the *Drd1a*-tdTomato transgene (as described in Mangieri et al., 2017). Mice were group-housed (up to five mice per cage) in standard cages (7.5" x 11.5" x 5") with Sani-Chips wood bedding (PJ Murphy) at 22°C with a 12:12 light: dark cycle (lights off at 9:30AM). Water and standard chow (LabDiet®5LL2 Prolab RMH1800) were available ad libitum, and all experimental

procedures were approved by the University of Texas Institutional Animal Care and Use Committee.

RESULTS

Ethanol-sensitivity of evoked NMDAR-type glutamatergic transmission

NMDAR-mediated currents were evoked for 8 min to ensure steady baseline responses before slices were perfused with an ethanol-containing ACSF for a 10 min treatment period, followed by a 10 min ethanol washout period (Figure 2). When analyzed over the entire 28 min experiment, we observed that the effect of time on NMDAR-mediated EPSC amplitude was not uniform, but varied with the treatment condition (two-way repeated measures ANOVA, main effect of time: $F(3,86) = 4.7$, $p = 0.004$; time x treatment interaction: $F(12,86) = 2.3$, $p = 0.013$), indicating that the changes in EPSC amplitude over the course of the experiment were not due to time alone. This conclusion was further supported by a significant between-groups effect during the treatment period ($F(4,29) = 8.1$, $p < 0.001$), when higher concentrations of ethanol (≥ 40 mM) all displayed significant reduction of peak NMDAR-mediated response relative to control (Figure 2C).

Ethanol-sensitivity of evoked AMPAR-type glutamatergic transmission

Ethanol has been shown to inhibit NMDAR-mediated currents across several brain regions, but has also been shown to inhibit AMPAR-mediated currents (Lovinger and Roberto, 2013). In order to test the sensitivity of AMPAR-mediated currents to ethanol, we tested whether ethanol modulated evoked AMPAR-mediated transmission. Therefore, neurons were voltage-clamped at -70 mV, and EPSCs were evoked in the presence of 100 µM DL-APV and 50 µM picrotoxin to isolate AMPAR-mediated currents. Following 8 min of recording to ensure steady baseline responses, neurons were

perfused with an ethanol-containing ACSF for a 10 min treatment period, followed by a 10 min ethanol washout period.

We observed no effect of time or time x treatment condition interaction on AMPAR-mediated EPSC amplitude over the entire 28 minute experiment (Figure 3; two-way repeated measures ANOVA, main effect of time: $F(2,37) = 1.9$, n/s; time x treatment interaction: $F(6,37) = 1.7$, n/s). In the 60 mM and 80 mM treatment concentrations, there appeared to be a delayed enhancement of peak AMPAR-mediated EPSCs during the last 2 min of the 10 min washout period (Figure 3C). However, one-way ANOVA comparing treatment conditions during this time period indicated these enhancements were not statistically significant ($F(3,20) = 1.44$, n/s).

Ethanol-sensitivity of sEPSCs

As a final assay of whether ethanol modulates glutamatergic transmission onto layer 2/3 AIC pyramidal neurons, we tested whether acute ethanol modulates spontaneous EPSCs (sEPSCs) in the AIC. Ethanol has been shown to reduce presynaptic glutamate release in multiple brain regions (Lovinger and Roberto, 2013). We assumed that any ethanol-induced changes in sEPSC frequency would be indicative of changes in presynaptic glutamate release, while ethanol-induced changes in sEPSC mean amplitude would be indicative of changes in postsynaptic sensitivity to glutamate (Siggins et al., 2005). Therefore, neurons were voltage-clamped at -70 mV, and sEPSCs were recorded in the presence of 50 μM picrotoxin to yield glutamate receptor-mediated spontaneous currents. Following 10 min of recording to ensure steady baseline responses, neurons were perfused with an ethanol-containing ACSF for a 10 min treatment period, followed by a 10 min ethanol washout period.

For the effect of ethanol on mean frequency of sEPSCs, we observed that an effect of time on sEPSC frequency did not vary by treatment condition (Figure 4; two-way repeated measures ANOVA, main effect of time: $F(2,30) = 4.2$, $p = 0.025$; time x treatment interaction: $F(2,30) = 1.41$, n/s). We also directly compared the two treatment

conditions (control vs. 50 mM ethanol) during just the treatment phase of the experiment, but this analysis also did not indicate a statistically significant effect of ethanol: $t(15) = 1.756$, n/s. Thus, although sEPSC frequency appeared to decrease with the application of 50 mM ethanol, the magnitude of this change was not different than that observed in the control treatment group. For the effect of ethanol on mean amplitude of sEPSCs, we observed no effect of time or interaction of time with treatment condition (Figure 4; two-way repeated measures ANOVA, main effect of time: $F(2,30) = 2.3$, n/s; time x treatment interaction: $F(2,30) = 0.84$, n/s), nor a difference in treatment conditions during the treatment period: $t(15) = 1.607$, n/s.

Ethanol-sensitivity of sIPSCs

In addition to our investigation of the effects of acute ethanol on glutamatergic transmission, we wished to characterize whether ethanol modulates GABAergic transmission in the AIC. Acute ethanol has been shown to modulate GABAergic transmission in several brain regions and experimental preparations (Lovinger and Roberto, 2013). Therefore, we tested whether acute ethanol modulated $GABA_AR$-mediated spontaneous IPSCs (sIPSCs) in the AIC. Neurons were voltage-clamped at -70 mV, and sIPSCs were recorded in the presence of 1 mM kynurenic acid to block glutamatergic transmission. Following 10 min of recording to ensure steady baseline responses, neurons were perfused with an ethanol-containing ACSF for a 10 min treatment period, followed by a 10 min ethanol washout period.

For the effect of ethanol on mean frequency of sIPSCs, we observed no effect of time on mean frequency of sIPSCs and no time x treatment condition interaction (Figure 5; two-way repeated measures ANOVA, main effect of time: $F(1,18) = 1.52$, n/s; time x treatment interaction: $F(1,18) = 0.24$, n/s). We also found no difference between treatment conditions (control vs. 50 mM ethanol) during the treatment phase: $t(15) = 0.031$, n/s. For the effect of ethanol on mean amplitude of sIPSCs, we observed no effect of time on mean amplitude of sIPSCs and no time x treatment condition interaction

(Figure 5; two-way repeated measures ANOVA, main effect of time: $F(2,30) = 0.43$, n/s; time x treatment interaction: $F(2,30) = 1.79$, n/s). A separate comparison of just the treatment phase of the experiment also did not reveal a statistically significant difference between control and 50 mM ethanol: $t(15)=1.058$, n/s.

LTD in AIC layer 2/3 in ethanol-naïve mice

We found that in the presence of 50 µM picrotoxin, local low frequency stimulation (1 Hz for 15 min) induced long-term depression (LTD) of evoked EPSCs in layer 2/3 AIC pyramidal neurons (Figure 6A, B; one-sample t-test, $t = 4.622$, $p = 0.0007$). To investigate whether the reduction in EPSC magnitude observed was due to either presynaptic changes in glutamatergic release or postsynaptic changes in glutamate receptor sensitivity, we measured paired-pulse ratios (2 pulses, 50 ms apart) before the 10 min of baseline recording and after the 30 min of post-stimulation in a separate group of neurons. Neurons tested for paired-pulse ratios displayed equivalent post-conditioning EPSC amplitudes to that seen in naïve control neurons (Figure 6B; unpaired t-test, $t = 0.05$, n/s). We observed no change in paired-pulse ratios before and after the induction of LTD, indicating that LTD in layer 2/3 AIC neurons is not due to changes in presynaptic glutamate release (Figure 6C, D; paired t-test, $t = 2.484$, n/s).

LTD in AIC layer 2/3 in ethanol-naïve mice is NMDAR-dependent and ethanol-sensitive

An investigation from Liu and colleagues was the initial demonstration and investigation of LTD in the mouse INS (Liu et al., 2013). They found via field potential recordings that low frequency stimulation in adult mouse INS can induce LTD of evoked excitatory postsynaptic potentials that depends upon NMDAR activation. Since this is the first investigation of whole cell LTD in the INS, we wished to determine whether our observed form of LTD similarly depended upon NMDAR activation. Bath application of the nonselective NMDA receptor antagonist DL-APV (100 µM) blocked the

expression of LTD (Figure 7; one-sample t-test, t = 0.154, n/s). Acute ethanol has been shown to modulate the expression NMDAR-dependent forms of synaptic plasticity in multiple brain regions (McCool, 2011). Therefore, we next tested whether acute pharmacologically relevant concentrations of ethanol modulate the expression of layer 2/3 AIC pyramidal neuron LTD. Bath application of multiple concentrations of ethanol (20, 40, 60 mM) did not differ in their ability to block the expression of AIC LTD (Figure 8; one-way ANOVA, F(2,17) = 0.16, n/s).

Figure 5: Representative diagram of recording site and bipolar stimulating electrode placement

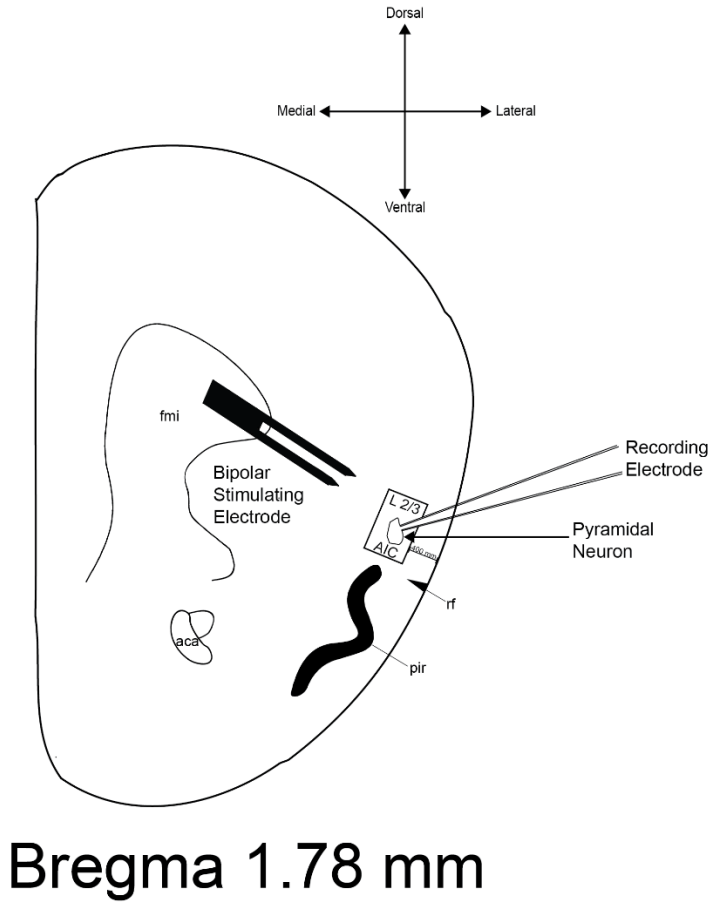

Figure 5. Representative diagram of recording site and bipolar stimulating electrode placement. The boundaries of the area from which neurons were selected for recordings

are inside the black box. The boundaries of the stimulating electrode are located ≈ 500 µm dorsomedial to the recording electrode. aca = anterior commissure, AIC = agranular insular cortex, fmi = forceps minor of the corpus callosum, pir = piriform cortex, rf = rhinal fissure.

Figure 6: Ethanol inhibits evoked NMDAR-mediated EPSCs in AIC layer 2/3 pyramidal neurons

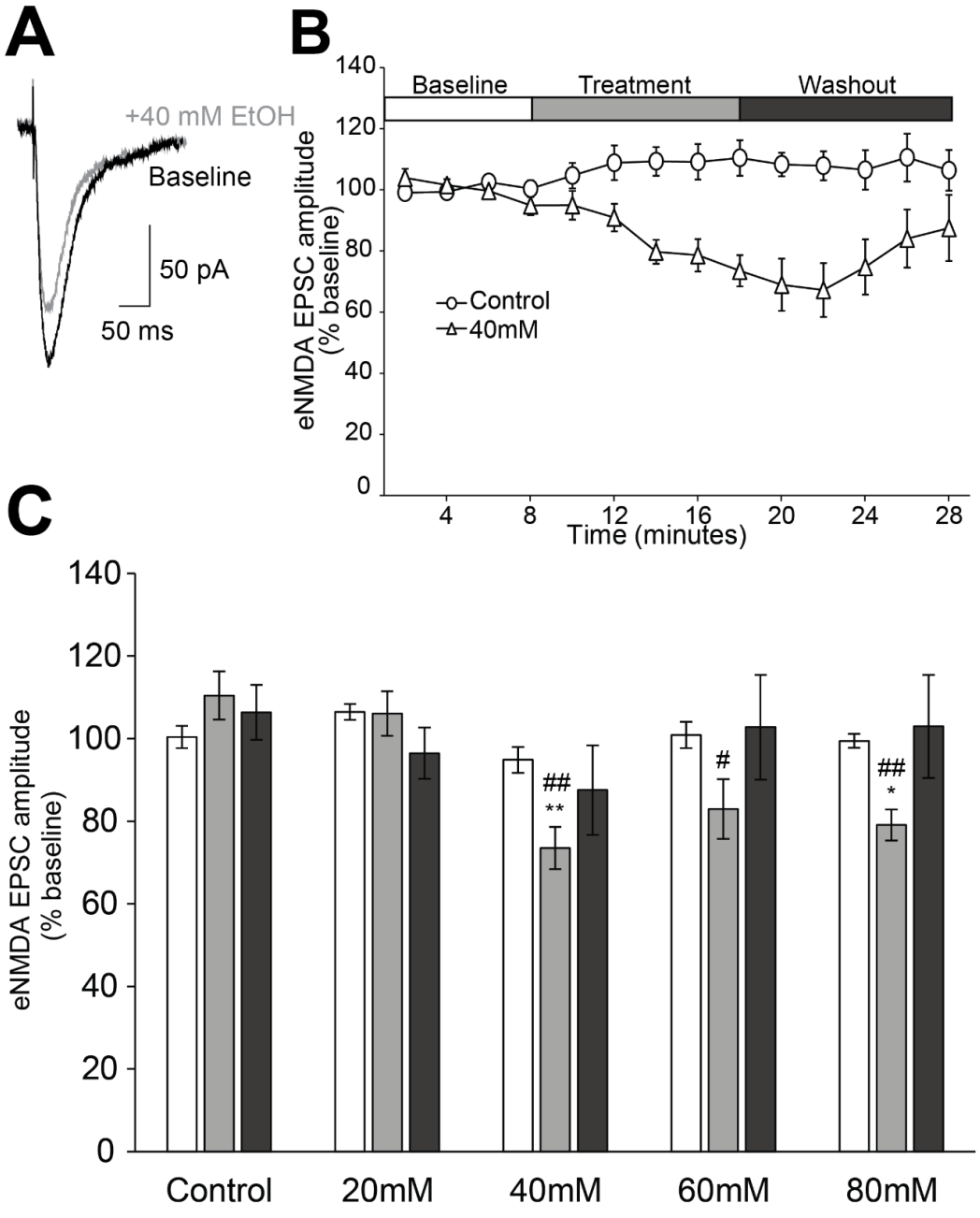

Figure 6. Ethanol inhibits evoked NMDAR-mediated EPSCs in AIC layer 2/3 pyramidal neurons. (**A**) Representative traces from a single neuron showing evoked NMDAR-mediated EPSCs before and after treatment of acute ethanol (40 mM). (**B**) Normalized timecourse of evoked NMDAR EPSC (eNMDA) responses in either sham solution exchange (open circles) or 40 mM ethanol application (open triangles) conditions. The white bar displays the 8 min baseline period, the gray bar displays the 10 min treatment period, and the dark gray bar shows the 10 min washout period. (**C**) Bars show average eNMDA EPSC amplitudes during the last 2 min of each period of the experiment (Baseline, Treatment, Washout), expressed as a percentage of the entire 8 min baseline average. Values are expressed as averages ± S.E.M. *, $p < 0.05$ compared to control during treatment. **, $p < 0.01$ compared to control during treatment. #, $p < 0.05$ compared to 20 mM during treatment. ##, $p < 0.01$ compared to 20 mM during treatment (Control, n = 4 neurons/4 slices/2 mice; 20 mM, n = 8 neurons/8 slices/4 mice; 40 mM, n = 7 neurons/7 slices/4 mice; 60 mM, n = 7 neurons/7 slices/4 mice; 80 mM, n = 8 neurons/8 slices/4 mice).

Figure 7: Ethanol has no effect on evoked AMPAR-mediated EPSC amplitudes in AIC layer 2/3 pyramidal neurons

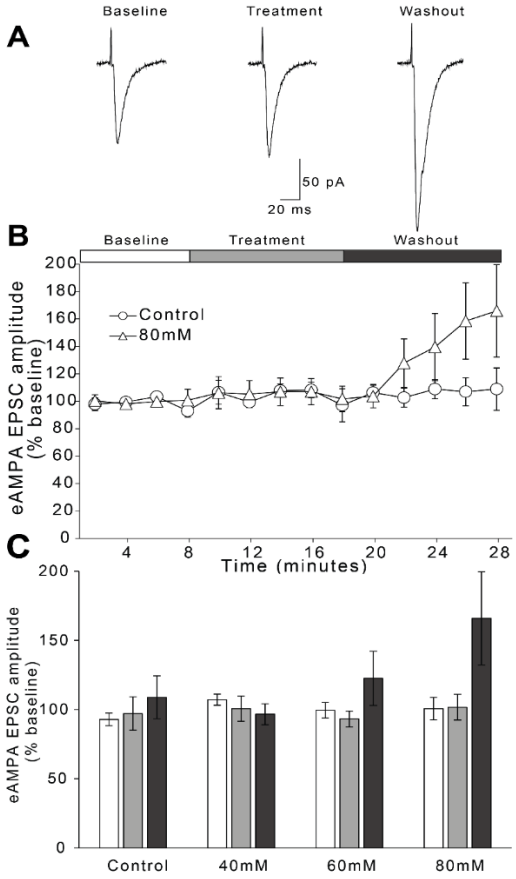

Figure 7. Ethanol has no effect on evoked AMPAR-mediated EPSC amplitudes in AIC layer 2/3 pyramidal neurons. (**A**) Representative traces from a single neuron showing evoked AMPAR-mediated EPSCs before and after treatment with acute ethanol (80 mM). (**B**) Normalized timecourse of evoked AMPAR EPSC (eAMPA) responses in either sham solution exchange (open circles) or 80mM ethanol application (open triangles) conditions. The white bar displays the 8 min baseline period, the gray bar displays the 10 min treatment period, and the dark gray bar shows the 10 min washout period. (**C**) Bars show average eAMPA amplitudes during the last 2 min of each period of the experiment (Baseline, Treatment, and Washout), expressed as a percentage of the entire 8 min baseline average. Values are expressed as averages ± S.E.M (Control, n = 4 neurons/4 slices/3 mice; 40 mM, n = 6 neurons/6 slices/4 mice; 60 mM, n = 5 neurons/5 slices/4 mice; 80 mM, n = 9 neurons/9 slices/7 mice).

Figure 8: Ethanol has no effect on spontaneous EPSCs in AIC layer 2/3 pyramidal neurons

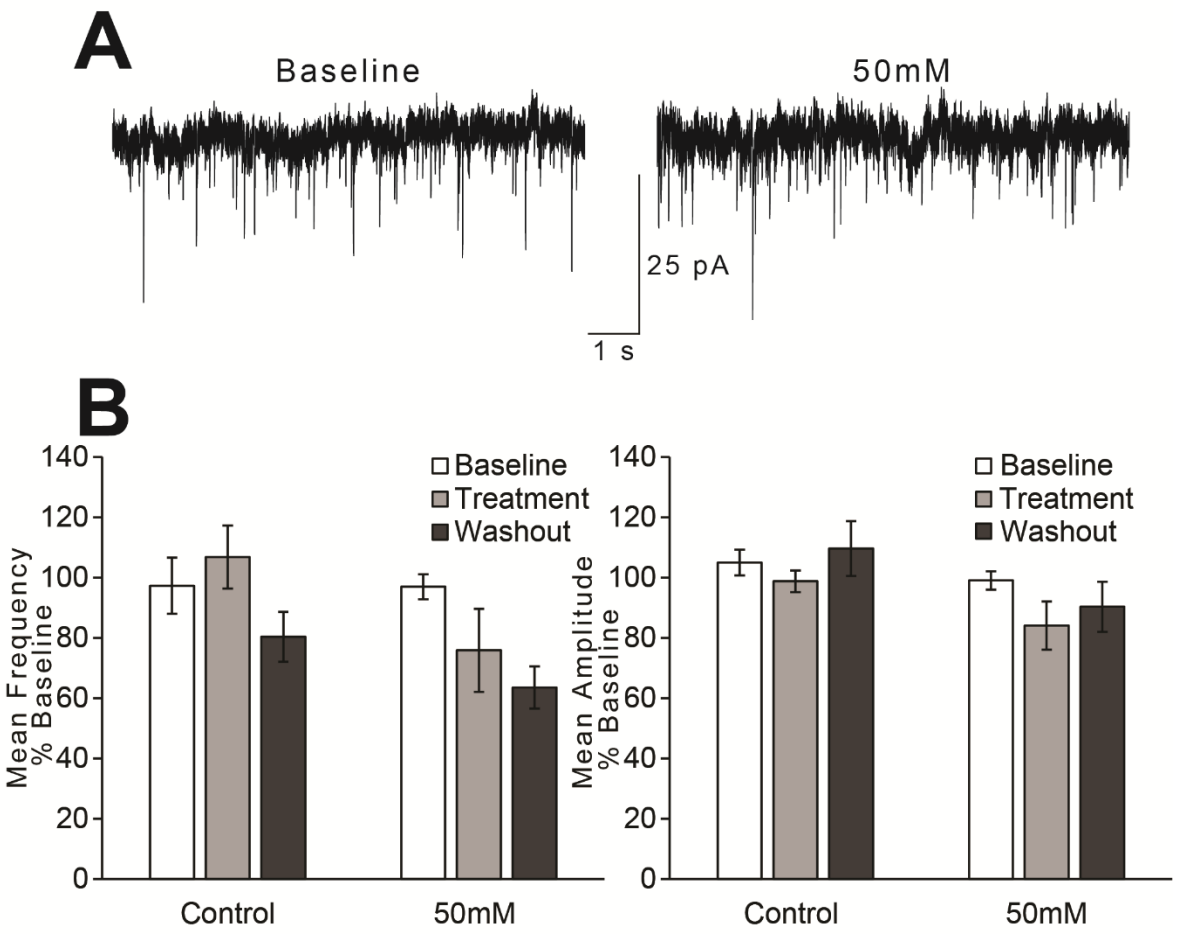

Figure 8. Ethanol has no effect on spontaneous EPSCs in AIC layer 2/3 pyramidal neurons. (**A**) Representative traces from a single neuron showing spontaneous EPSCs (sEPSCs) before and after treatment with acute ethanol (50 mM). (**B**) Summary charts showing mean frequency (left) and amplitude (right) of sEPSCs during the last 2 min of each period of the experiment (Baseline, Treatment, and Washout), expressed as a percentage of the entire 10 min baseline average. Values are expressed as averages ± S.E.M (Control, n = 8 neurons/8 slices/6 mice; 50 mM, n = 9 neurons/9 slices/7 mice).

Figure 9: Ethanol has no effect on spontaneous GABA IPSCs in AIC layer 2/3 pyramidal neurons

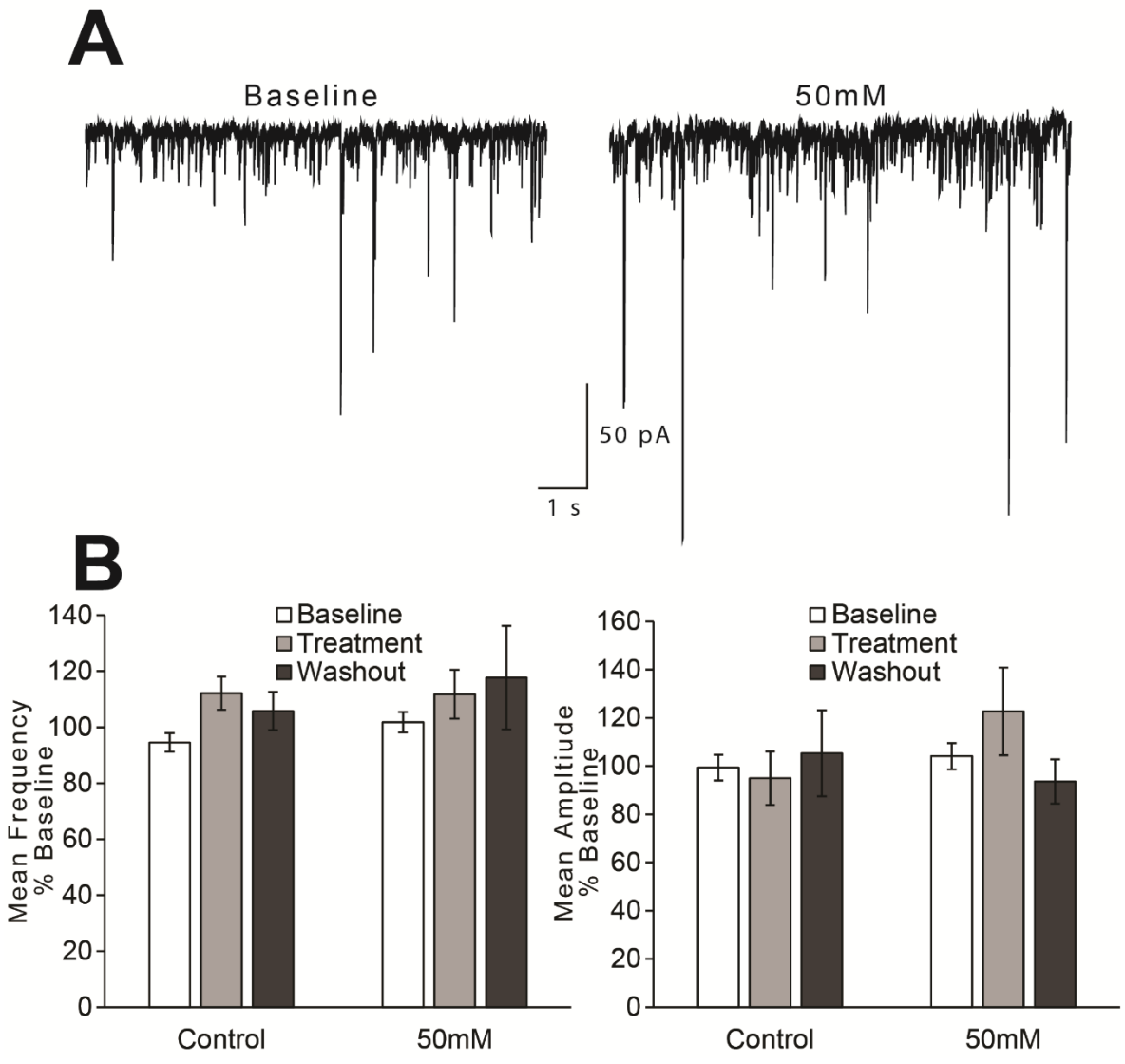

Figure 9. Ethanol has no effect on spontaneous GABA IPSCs in AIC layer 2/3 pyramidal neurons. (**A**) Representative traces from a single neuron showing spontaneous IPSCs (sIPSCs) before and after treatment with acute ethanol (50 mM). (**B**) Summary charts showing mean frequency (left) and amplitude (right) of sIPSCs during the last 2 min of each period of the experiment (Baseline, Treatment, and Washout), expressed as a percentage of the entire 10 min baseline average. Values are expressed as averages ± S.E.M (Control, n = 6 neurons/6 slices/6 mice; 50 mM, n = 11 neurons/11 slices/9 mice).

Figure 10. Low-frequency stimulation produces LTD at glutamatergic synapses onto AIC layer 2/3 pyramidal neurons

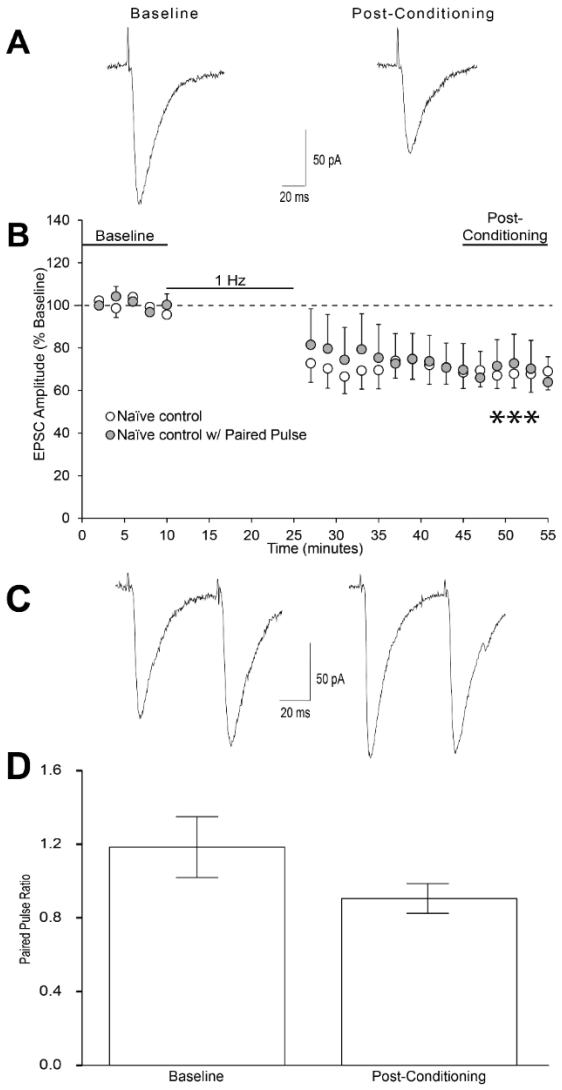

Figure 10. Low-frequency stimulation produces LTD at glutamatergic synapses onto AIC layer 2/3 pyramidal neurons. (**A**) Representative traces from a single neuron showing evoked EPSCs before and 20-30 min after low-frequency conditioning stimulation protocol (900 pulses at 1 Hz while holding the neuron at -70 mv). (**B**) Conditioning stimulation induced long-term depression of evoked EPSCs onto AIC layer 2/3 pyramidal neurons of ethanol-naïve mice (12 neurons/12 slices/9 mice, ***p = 0.0007 compared to baseline). (**C)** Representative traces from a single neuron showing evoked paired pulse ratios before baseline and after the post-conditioning period. (**D**) Bar graph representing the mean PPR ± S.E.M. before baseline and after the post-conditioning period. PPR was determined by dividing the amplitude of EPSC2 by EPSC1 for each sweep. Average PPRs before baseline and after post-conditioning were not significantly different (n = 6 neurons/6 slices/4 mice; paired t-test, t = 2.48, n/s). Values are expressed as averages ± S.E.M.

Figure 11. NMDA receptors are required for AIC layer 2/3 pyramidal neuron LTD expression

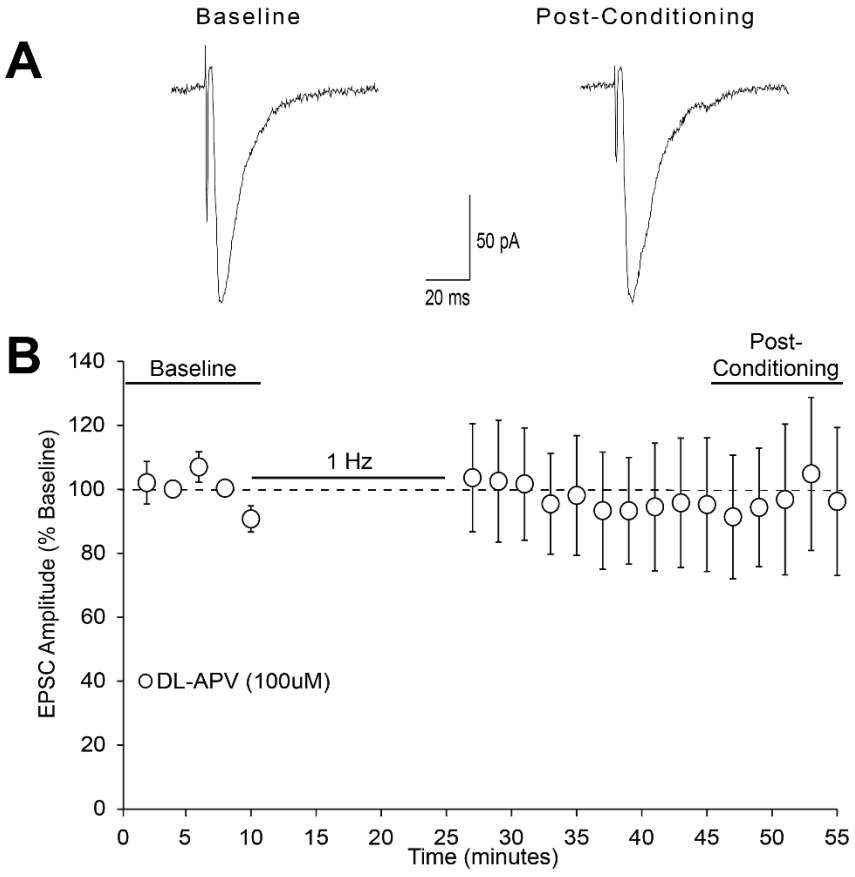

Figure 11. NMDA receptors are required for AIC layer 2/3 pyramidal neuron LTD expression. (**A**) Representative traces from a single neuron showing evoked EPSCs before and 20-30 min after low-frequency conditioning stimulation protocol in the presence of the nonselective NMDA receptor antagonist DL-APV (100 µM). (**B**) Conditioning stimulation did not induce LTD expression in the presence of DL-APV (100 µM), (7 neurons/7 slices/6 mice, $p > 0.05$ versus baseline). Values are expressed as averages ± S.E.M.

Figure 12. *In vitro* ethanol exposure blocks AIC layer 2/3 pyramidal neuron LTD expression

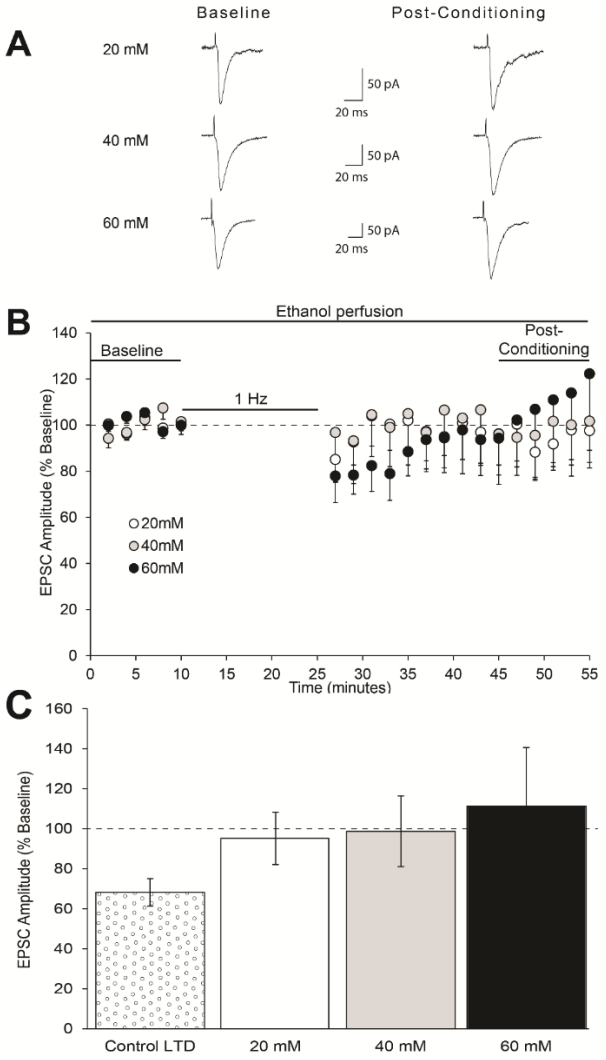

Figure 12. *In vitro* ethanol exposure blocks AIC layer 2/3 pyramidal neuron LTD expression. (**A**) Representative traces from a single neuron of each ethanol group showing evoked EPSCs before and 20-30 min after low-frequency conditioning stimulation protocol

in the presence of acute ethanol. (**B**) Conditioning stimulation did not induce LTD expression in the presence of acute ethanol (20, 40, or 60 mM). (**C**) Bar graph representing the average post-conditioning (min 45-55) EPSC amplitude as percentage of baseline for each ethanol concentration. Control LTD value from prior experiment (Figure 6) shown for comparison. Values are expressed as averages ± S.E.M (Control LTD, n = 12 neurons/12 slices/9 mice; 20 mM, n = 7 neurons/7 slices/5 mice; 40 mM, n = 6 neurons/6 slices/5 mice; 60 mM, n = 7 neurons/7 slices/5 mice).

DISCUSSION

Ethanol has multiple effects on the glutamate system

The major findings of this investigation are that acute ethanol has significant effects on glutamatergic transmission and glutamatergic synaptic plasticity in layer 2/3 AIC pyramidal neurons, but little to no effect on GABAergic transmission at the concentration tested (50 mM). Recordings from brain slice preparations across multiple brain regions have generally shown an inhibitory effect of acute ethanol on glutamatergic transmission (Lovinger and Roberto, 2013). This effect is largely attributed to ethanol's inhibitory actions on postsynaptic NMDARs (Ron and Wang, 2009). Since acute ethanol has been shown to modulate glutamatergic transmission across several brain regions and experimental preparations (Lovinger and Roberto, 2013), we wished to determine whether ethanol modulates glutamatergic intracortical processing in the AIC. A commonly replicated synaptic effect of ethanol across multiple brain regions has been its inhibitory action on postsynaptic NMDARs, as ethanol has generally been found to have a concentration-dependent inhibition of NMDAR-mediated transmission (Ron and Wang, 2009). Moreover, ethanol's inhibitory effects on NMDARs and disruption of NMDAR-dependent signaling processes have been shown to be major canonical mechanisms by which chronic ethanol disrupts healthy brain functioning; NMDAR-dependent synaptic mechanisms of learning and memory have generally been shown to be disrupted by chronic alcohol use and implicated in alcohol-related phenotypes (Ron and Wang, 2009). For these reasons we tested whether ethanol inhibited postsynaptic

NMDARs in layer 2/3 AIC pyramidal neurons. Our investigation determined that ethanol modestly inhibited evoked NMDAR-mediated currents in the AIC in a concentration-related manner. Such a finding complements research in other brain regions which identifies NMDARs as a modest (≈ 25% inhibition) ethanol-sensitive target in cortical neurons (Lovinger and Roberto, 2013). However, it is important to consider that no statistically significant inhibition of evoked NMDAR-mediated EPSCs was observed at 20 mM, an intoxicating ethanol concentration. Therefore, our data, at initial consideration, suggest that the action of ethanol on NMDARs in the AIC is a modest effect observable only at highly intoxicating concentrations (≥ 40 mM) of ethanol.

However, it is possible that ethanol's action on AIC NMDARs *in vivo* occurs at lower ethanol concentrations and at greater peak inhibition levels than what we observed in the current study due the limitations of a brain slice preparation. For example, a well-established modulator of the degree of ethanol inhibition on evoked NMDAR-mediated responses is the ACSF Mg^{2+} concentration, as studies have shown that higher Mg^{2+} concentrations increase the sensitivity of NMDARs to ethanol (Ron and Wang, 2009; Carlton et al., 1998). Prior research investigating ethanol's inhibitory effect on NMDARs utilizing expression systems has shown that the degree of ethanol inhibition of NMDARs is Mg^{2+}-dependent (Jin et al., 2008). Our experimental design utilized a concentration of Mg^{2+} (1.0 mM) that has been shown to produce significant inhibition of evoked NMDAR currents of pyramidal neurons in the basolateral amygdala (Carlton et al., 1998). Since normal cerebrospinal Mg^{2+} concentration in healthy people is estimated to be around 1.48 mM, due to enhanced free Mg^{2+} in human cerebrospinal fluid, AIC NMDARs may be more sensitive to the inhibitory effects of ethanol under physiological conditions than under those of our brain slice preparation (Banki et al., 1985).

Nonetheless, even if the modest level of inhibition observed only at higher ethanol concentration levels (40, 60, 80 mM) in this study fully replicate *in vivo* conditions, we still maintain that this inhibitory effect is a significant phenomenon by which chronic ethanol exposure likely elicits long-term alterations AIC functioning. NMDARs that display sensitivity to acute ethanol inhibition generally enhance their functioning in

response to chronic ethanol exposure as a compensatory mechanism due to ethanol's chronic inhibition, which results in aberrations from homeostatic NMDAR-dependent signaling processes (Roberto and Varodayan, 2017). Prior research from our laboratory, among others, has shown that these long term alterations due chronic ethanol exposure lead to robust changes in expression of NMDAR-dependent plasticity states and ethanol-related behavior (Abrahao et al., 2013; Jeanes et al., 2011; Jeanes et al., 2014). Thus, our findings suggest that NMDARs and NMDAR-mediated signaling processes in layer 2/3 AIC pyramidal neurons are ethanol-sensitive targets likely to underlie alterations in AIC function after chronic ethanol exposure. Since layer 2/3 is the intracortical processing layer of the AIC, our data suggest that general intracortical processing in the AIC as well as its output to downstream brain regions are sensitive to disruption by chronic ethanol.

In order to test the sensitivity of AMPAR-mediated glutamatergic transmission to ethanol, we examined whether evoked AMPAR-mediated currents were sensitive to ethanol. Our investigation found that evoked AMPAR-currents were insensitive to intoxicating concentrations of ethanol, except for a non-statistically significant delayed enhancement nearly twenty minutes after the initial bath application of ethanol at a concentration nearly lethal (80 mM) to intolerant individuals. As such, these negative results on evoked AMPAR-mediated currents are indicative of a selective postsynaptic action of ethanol. However, as a final test of ethanol action on presynaptic glutamate release, we measured whether acute ethanol modulated sEPSCs. We found that the significantly intoxicating concentration of ethanol (50 mM) did not change the mean frequency or mean amplitude of sEPSCs, indicative of no changes in glutamate release probability.

In summary, these findings contribute to the abundance of literature indicating that the effects of acute ethanol on glutamatergic transmission in brain slice preparations are brain-region specific and concentration dependent. Acute ethanol has been shown to generally reduce glutamatergic transmission (Lovinger and Roberto, 2013). However, investigations of acute ethanol on glutamatergic transmission in some brain regions such as the ventral tegmental area, somatosensory cortex, and central amygdala show an ethanol-

induced enhancement of glutamatergic transmission (Lu and Yeh, 1999; Xiao et al., 2009; Silberman et al., 2015; Herman et al., 2016).

Ethanol has little action on $GABA_A$ transmission

Similar to the glutamate system, modulatory effects of ethanol on $GABA_A$-mediated transmission in brain slice preparations have depended upon the brain region investigated as well as the ethanol concentration used (Nie at al., 1994, Roberto et al., 2004, Lu and Yeh, 1999). Acute ethanol has generally, but not always been shown to increase GABAergic transmission by both pre and postsynaptic mechanisms (Siggins et al., 2005; Lovinger and Roberto, 2013). However, some studies have shown that GABAergic transmission in cortical regions is relatively insensitive to acute ethanol (Proctor et al., 1992; Soldo et al., 1998, Weitlauf and Woodward, 2008). The current investigation did not show any effects of ethanol on spontaneous $GABA_A$-mediated transmission. We therefore conclude from our investigation that an intoxicating concentration of ethanol has little, if any, effect on spontaneous $GABA_A$-mediated transmission onto layer 2/3 AIC pyramidal neurons.

Ethanol disrupts NMDAR-dependent synaptic plasticity

Since the disrupted processing of interoceptive stimuli has been suggested to play a role in drug and alcohol use disorders, and synaptic plasticity mechanisms are accepted as underlying aspects of learning and memory, we wished to investigate the effect of ethanol on long-term synaptic plasticity in intracortical processing layers of the AIC. We initially found that acute ethanol inhibits NMDARs in the AIC, and so we hypothesized that any NMDAR-dependent long-term synaptic plasticity measures onto layer 2/3 AIC pyramidal neurons would likely be disrupted by acute ethanol. Therefore, we investigated LTD as a long term synaptic plasticity mechanism onto layer 2/3 AIC pyramidal neurons.

Synaptic plasticity mechanisms are the means by which neural networks adapt to strengthen and weaken their connections to form the basis of information storage and are thought of as mechanisms of learning and memory (Kauer and Malenka, 2007; Kandel et al., 2014). Such synaptic plasticity mechanisms in mesolimbic, addiction-relevant brain regions have been shown to be disrupted by drug experience and are thought to encode for and contribute to future drug and alcohol use (Luscher and Malenka, 2011; Lovinger and Kash, 2015). Since the AIC and its output have been shown, in animal models, to be involved in more advanced, pathological forms of alcohol drinking, we reasoned that ethanol-induced changes in AIC processing and its output may mediate the changes in interoceptive functioning that are implicated in AUD. Therefore, we decided to investigate plasticity mechanisms in AIC layer 2/3 pyramidal neurons. We performed the first demonstration of LTD using whole cell configuration in the IC. Using a 1 Hz, low-frequency stimulation protocol, we found a reduction in EPSC magnitude (LTD) of approximately 34%. This form of LTD was NMDAR-dependent and likely mediated by a postsynaptic mechanism. Since prior investigation in this study had determined an inhibitory effect of ethanol on NMDARs in AIC 2/3 pyramidal neurons, we reasoned that ethanol may, through its actions on NMDARs, inhibit the expression of our discovered NMDAR-dependent LTD mechanism. We found that AIC LTD was similarly inhibited by several intoxicating concentrations of acute ethanol (20, 40, 60 mM), indicating that this NMDAR-dependent plasticity state is highly sensitive to intoxicating concentrations of ethanol.

It is noteworthy that while 20 mM ethanol prevented the expression of LTD, this concentration of ethanol did not inhibit evoked NMDAR-mediated currents. We suggest there are at least three reasons why this could be so. First of all, the difference in ACSF Mg^{2+} concentration between LTD experiments (1.2 mM) and evoked NMDAR-mediated current experiments (1.0 mM) suggests that NMDARs were sensitive to lower concentrations of ethanol in LTD experiments than in evoked NMDAR-mediated experiments, as higher Mg^{2+} concentrations increase the sensitivity of NMDARs to ethanol (Ron and Wang, 2009). Secondly, it is possible that ethanol inhibits our

uncovered form of synaptic plasticity via an alternative molecular target than NMDARs. Ethanol in acute preparations has a wide array of molecular targets, and has been shown to inhibit the expression of forms of LTD via its inhibitory action on synaptic metabotropic glutamate receptors (mGluRs) (Belmequenai et al., 2008; Carta et al., 2006; Su et al., 2010; Zorumski et al., 2014). Generally, the major postsynaptic forms of LTD have been shown to be either NMDAR or mGluR-dependent, but some require both NMDARs and mGluRs (Collingridge et al., 2010). Therefore, it is possible that our uncovered form of AIC LTD was additionally mGluR-dependent, and that acute ethanol inhibited its expression, at least in part, via its inhibitory actions on mGluRs. Finally, NMDARs have metabotropic actions; thus it is possible that this APV-sensitive LTD is not mediated by ion flux (Dore et al., 2016).

AIC synaptic plasticity, pain, and alcohol use disorder

Recent research suggests that the neurobiological substrates for pain disorders and addiction overlap, and that adaptations in brain regions involved in chronic pain contribute to alcohol use disorder (Egli et al., 2012). Multiple animal models have implicated NMDAR-depending signaling processes in the INS as targets encoding for chronic pain: The ability to induce INS NMDAR-dependent long-term potentiation and the ability to induce INS NMDAR-dependent LTD in *ex vivo* slice preparations were each shown to be lost in animal models of chronic pain (Qiu et al., 2013; Liu and Zhuo, 2014). This evidence of disrupted INS NMDAR-dependent signaling processes in chronic pain considered alongside ethanol's widely demonstrated disruption of NMDAR-dependent signaling processes suggests that NMDAR-dependent signaling processes in the INS may be shared mechanisms by which both pain and ethanol change INS function. In the present work, we verified that layer 2/3 of the AIC is an additional region in which acute ethanol modulates NMDAR function, and we observed that NMDAR-dependent plasticity in the AIC is sensitive to intoxicating concentrations of ethanol used to develop alcohol dependence in animal models. Thus, together these findings suggest that

processing in the AIC is sensitive to acute ethanol disruption, and that synaptic mechanisms thought to mediate pain-related interoceptive changes in the AIC can also be disrupted by acute ethanol. This is the initial investigation of the molecular mechanisms by which alcohol exposure may change healthy AIC functioning in the development of AUD.

Chapter 3:

Chronic Ethanol Experience Modulates Glutamatergic Synaptic Transmission in the Agranular Insular Cortex

This chapter written in the style for submission to Frontiers in Pharmacology

Authors: Joel Shillinglaw, Heather Aziz, Daniela Carrizales, Richard A. Morrisett, Regina A. Mangieri. Joel Shillinglaw, Richard Morrisett, and Regina Mangieri conceived and designed experiments. Joel Shillinglaw performed the experiments. Joel Shillinglaw analyzed the data and interpreted the results. Joel Shillinglaw and Regina Mangieri wrote the paper. Heather Aziz conducted breeding of animals and assisted with animal behavior. Daniela Carrizales assisted with animal behavior.

ABSTRACT

The Agranular Insular Cortex (AIC) is implicated in alcohol use disorder (AUD), and pharmacologically relevant concentrations of acute ethanol modulate N-methyl-D-aspartate receptor (NMDAR)-mediated glutamatergic synaptic transmission and plasticity onto layer 2/3 AIC pyramidal neurons. However, despite this evidence indicating NMDAR-dependent signaling is a synaptic target for acute ethanol in the AIC, it is not known whether NMDAR-dependent synaptic plasticity is a target by which chronic ethanol alters AIC function as alcohol drinking transitions from controlled to problematic. Therefore, we utilized the Chronic Intermittent Ethanol (CIE) vapor exposure model to model chronic ethanol exposure in adult male mice, and then tested for the expression of NMDAR-dependent long-term depression (LTD) of AMPAR-mediated signaling onto layer 2/3 AIC pyramidal neurons. The ability to induce LTD *ex vivo* was inhibited by limited CIE exposure, but not equivalent chambering experience with air. Groups that underwent extended exposure to ethanol via three bouts of CIE or Air, coupled with volitional ethanol drinking, no longer displayed LTD. We tested for the expression of LTD in an ethanol-naïve group of mice of the same age at which extended exposure to ethanol experiments were conducted (≥14 weeks), and were unable to induce LTD, indicating AIC NMDAR-dependent LTD is an age-sensitive phenomenon. We next tested whether

extended exposure to ethanol modulates other glutamatergic synaptic properties, and found that the ratio of NMDAR to α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid-type receptor (AMPAR)-mediated EPSCs to be reduced in Drinking + CIE relative to Drinking + Air groups. In a follow-up investigation, we found that ethanol exposure (Drinking + CIE & Drinking + Air groups) reduced the NMDAR/AMPAR relative to an ethanol-naïve, handling & age-matched control group. Taken together these data indicate that glutamatergic transmission in the AIC is sensitive to chronic ethanol, and that changes in glutamatergic signaling in the AIC may occur by even moderate alcohol drinking. Moreover, these data add to the growing body of literature indicating that some synaptic mechanisms of learning and memory are at least partially age-dependent processes, which should be considered during the design and interpretation of experiments.

INTRODUCTION

The Agranular Insular Cortex (AIC) is involved in interoceptive processing, and deficits in AIC functioning and interoceptive processing are implicated in alcohol use disorder (AUD) (Craig et al., 2002; Craig et al., 2009; Naqvi and Bechara., 2010; Paulus and Stewart, 2014; Ateş Çöl et al., 2016). Despite evidence for altered AIC functioning and/or output in animal models of AUD (Seif et al., 2013; Jaramillo et al., 2016; Jaramillo et al., 2017; Jaramillo et al., 2018), there has been minimal investigation of the effects of ethanol on synaptic transmission in the AIC. The synaptic effects of ethanol in brain regions of addiction-relevance contribute to various neural and behavioral components of AUD (Lovinger and Roberto, 2013). Recent research from our laboratory has demonstrated that N-methyl-D-aspartate receptor (NMDAR)-mediated synaptic transmission and plasticity onto AIC layer 2/3 pyramidal neurons is sensitive to acute ethanol at concentrations achieved during alcohol drinking (Shillinglaw et al., 2018).

Prior work in several brain regions has shown that chronic ethanol produces long-term alterations in the synaptic targets of acute ethanol (Lovinger and Roberto, 2013; Roberto and Varodayan, 2017). Moreover, the long-term alterations in the functioning of synaptic receptors have been shown to encode for altered brain function and alcohol-related

behavioral phenotypes such as dependence and tolerance (Jeanes et al., 2011; Renteria et al., 2016; Lovinger & Kash., 2015; Lovinger and Roberto, 2013). We hypothesized that NMDAR-dependent synaptic plasticity is a target by which chronic ethanol exposure disrupts AIC functioning, and therefore interoceptive processing, over the development of AUD.

For these reasons we utilized the Chronic Intermittent Ethanol (CIE) Exposure Model to model in vivo chronic ethanol exposure in mice, and then tested whether CIE modulates NMDAR-dependent synaptic plasticity onto layer 2/3 AIC pyramidal neurons. The ability to induce LTD was inhibited by limited CIE experience. We then wished to determine whether ethanol experience sufficient to enhance volitional alcohol drinking and produce ethanol dependence and withdrawal modulates NMDAR-dependent plasticity (Becker and Lopez 2004; Griffin et al., 2014; Griffin et al., 2009; Renteria et al., 2018). Therefore, we tested whether extended CIE (3-bouts) coupled to volitional two-bottle choice (2BC) drinking (Drinking + CIE) differentially modulates NMDAR-mediated synaptic plasticity relative to an identically treated group but with no CIE exposure (Drinking + Air). Drinking + CIE and Drinking + Air groups both displayed the inability to induce LTD. Since age has been shown to modulate the expression of different types of glutamatergic synaptic plasticity, we tested for the expression of LTD in an ethanol-naïve group of mice at the same age at which extended exposure to ethanol experiments were conducted (≥14 weeks), and found we were unable to induce LTD. We additionally tested whether extended exposure to CIE alters non NMDAR-mediated prominent glutamatergic electrophysiological properties (Renteria et al., 2017; Renteria et al., 2018; Hopf and Mangieri, 2018). We found that Drinking + CIE and Drinking + Air groups showed no difference in paired pulse ratio or rectification index, but that the Drinking + CIE group showed a decreased NMDA/AMPA ratio relative to the Drinking + Air group.

In order to validate this finding, as well as investigate whether drinking even moderate amounts of ethanol drinking alter NMDA/AMPA ratios, we replicated the extended exposure experiment with an additional ethanol-naïve, handling & age-matched control group. We found that Drinking + CIE and Drinking + Air groups displayed a

reduction in NMDA/AMPA ratio relative to the ethanol-naïve, handling & age-matched control group. These findings demonstrate that glutamatergic synaptic transmission in the AIC is sensitive to chronic ethanol, and suggest that even small amounts of alcohol drinking may alter glutamatergic synaptic transmission in the AIC. These findings also add to the literature indicating that aspects of synaptic physiology are age-dependent beyond the transition into early adulthood, and that the age of mice, even beyond the onset of adulthood, should be considered in experimental design (McCutcheon and Marinelli, 2009; Jackson et al., 2017).

Methods

MICE

Drd1a-tdTomato BAC transgenic male mice (MMRRC:030512-UNC) were used for all experiments. Drd1a-tdTomato mice (Ade et al., 2011; initial breeding pairs obtained from The Jackson Laboratory, Stock No. 016204) were generated by backcrossing mice onto a C57BL/6J background in which only one parent carried the *Drd1a*-tdTomato transgene (Mangieri et al., 2017). Mice were housed in standard cages (7.5" x 11.5" x 5") with Sani-Chips wood bedding (PJ Murphy) at 22°C with a 12:12 light: dark cycle (lights off at 9:30AM). Water and standard chow (LabDiet®5LL2 Prolab RMH1800) were available ad libitum, and all experimental procedures were approved by the University of Texas Institutional Animal Care and Use Committee.

CHRONIC INTERMITTENT ETHANOL EXPOSURE

For all CIE experiments (limited & extended), mice underwent exposure to ethanol vapor using the Becker-Lopez chronic intermittent ethanol (CIE) exposure model. Mice were exposed to ethanol via the CIE exposure model, a well-validated passive exposure model which has been shown to induce intoxicating blood alcohol concentrations over extended amounts of time. Briefly, a stock solution of 95% ethanol was placed inside

sealed flask and volatized by bubbling air. The resulting ethanol vapor was combined with an additional stream of air which was delivered to mice contained in mouse vapor chamber units containing an airtight top, a vapor inlet, and an exhaust outlet (Allentown Inc., Allentown, NJ). Mice received intraperitoneal injections of a loading dose of ethanol (20% v/v, 1.5g/kg) and pyrazole (1 mM, 68.1 mg/kg) in phosphate buffered saline (PBS) immediately prior to placement in chamber units to obtain a target BEC of 150-200 mg/dl. Both doses and flow rates were adjusted to keep mice in target BEC range. Air control mice were handled identically but injected with a solution of pyrazole in PBS before being placed into mouse vapor chamber units not connected an ethanol flask. Ethanol-exposed mice were exposed to ethanol vapor for 16 hours followed by 8 hours of withdrawal, in which mice are not exposed to ethanol and blood ethanol concentrations (BEC) return to zero (data not shown). This 4 consecutive day exposure period constitutes 1-bout of CIE. Limited CIE (1-bout) experiments were conducted 24 hours into withdrawal (Figure 13). Extended CIE (3-bout) experiments were conducted from 5-8 days to correlate glutamatergic changes with volitional ethanol consumption (Figures 14, 15, 16).

BLOOD ETHANOL CONCENTRATIONS

Immediately after each chambering day, tail blood samples were taken and BECs were calculated using a gas chromatograph (Bruker 430-GC) equipped with a flame ionization detector and CombiPAL autosampler (Bruker Corporation, Fremont, CA). Two, 5 µL tail blood samples were collected and added to two, 10 mL vials containing 45 µLs of saturated sodium chloride solution. After samples were heated to 65 °C, ethanol vapor was absorbed from the samples with the solid-phase micro extraction fiber (SPME; 75 µm CAR/PDMS, fused silica; Supelco, Bellefonte, PA). A capillary column was the stationary phase (30 m x 0.53 mm x 1 µm film thickness; Agilent Technologies, Santa Clara, CA), and helium was used in the mobile phase (at a flow rate of 8.5 mL/min). CompassCDS Workstation software (Bruker Corporation, Fremont, CA) was used to analyze ethanol peaks, and external ethanol standards were used for calibration.

PREPARATION OF BRAIN SLICES

Mouse brains were rapidly extracted and coronal slices containing the most anterior portion of the AIC were collected via established laboratory protocols (Shillinglaw et al., 2018). Briefly, mice were first anesthetized by inhalation of isoflurane and then euthanized by decapitation. Brains were then quickly extracted from the skull and submerged in ice-cold oxygenated artificial cerebrospinal fluid (ACSF) containing the following (in mM): 210 Sucrose, 26.2 $NaHCO_3$, 1 NaH_2PO_4, 2.5 KCl, 11 dextrose, bubbled with 95% O_2/5% CO_2. Coronal slices (250 to 270 µm thick) containing AIC were collected using a Leica VT1000S vibrating microtome (Leica Corp., Bannockburn, IL), which were then placed into an incubation solution containing the following (in mM): 120 NaCl, 25 $NaHCO_3$, 1.23 NaH_2PO_4, 3.3 KCl, 2.4 $MgCl_2$, 1.8 $CaCl_2$, 10 dextrose, continuously bubbled with 95% O_2/ 5% CO_2; 32°C, for at least 45 min for recovery prior to recording.

Patch-Clamp Electrophysiology

Whole cell recordings were taken from layer 2/3 pyramidal AIC neurons identified via morphology (large, pyramidal-like shape) using a MRK200 Modular Imaging system (Siskiyou Corporation, Grants Pass, OR) mounted on a vibration isolation table. Recordings were made in ACSF containing (in mM): 120 NaCl, 25 NaHCO3, 1.23 93 NaH2PO4, 3.3 KCl, 1.2 MgSO4, 2.0 CaCl2, and 10 dextrose unless otherwise noted, bubbled with 94 95% O2/ 5% CO2; 32°C, controlled by an in-line bath heather (Warner Instruments, Hamden, CT). Picrotoxin (50 µM) was included in the recording ACSF to block $GABA_A$ receptor-mediated synaptic currents. Brain slices were perfused at a rate of 2.0 mL/min. A P-97 Flaming/Brown model micropipette puller (Sutter Instruments, San Rafael, CA) was used to make recording electrodes (thin-wall glass, WPI Instruments, Sarasota FL) of resistances from 3-6 MΩ. For all experiments, recording electrodes were

filled with (in mM): 120 CsMeSO4, 15 CsCl, 8 NaCl, 10 HEPES, 0.2 EGTA, 10 TEA-Cl, 4 Mg-ATP, 0.3 Na-GTP, 0.1 spermine, and 5 QX-314-Cl. Series resistance (Rs) was monitored throughout each experiment, and cells with Rs of over 30 MΩ or that changed over 20% over the course of the experiment were excluded from the analysis. One cell per brain slice was used for LTD experiments, and multiple cells per brain slice were sometimes used for other glutamatergic assays. All chemicals were obtained from either Sigma-Aldrich or Tocris Bioscience with the exception of ethanol, which was obtained from Pharmco-Aaper.

DATA ACQUISITION AND ANALYSIS

An Axopatch 200B amplifier (Axon Instruments, Foster City, CA) was used to acquire all currents, which were filtered at 1 kHz, and digitized at 10-20 kHz via a Digidata 1440A interface board using pClamp 10.2 (Axon Instruments). Excitatory postsynaptic currents (EPSCs) were evoked with a stainless steel bipolar stimulating electrode (MX21AES, FHC, Inc., Bowdoin, ME, United States) placed approximately 500 µm dorsomedial to the cell body (Shillinglaw et al., 2018). For LTD experiments, EPSCs were evoked for at least 10 min (at 0.025 Hz) to ensure stable recordings, followed by a low-frequency stimulation protocol consisting of 1 Hz stimulation of 15 min. Evoked EPSCs were then monitored for a 30 min post-stimulation period at 0.025 Hz to test for the expression of LTD. Neurons were held at -70 mV for the entirety of LTD experiments. Data were collapsed into 2 min bins and the expression of LTD was calculated by comparing the 20 to 30 min period after the low-frequency stimulation protocol to the 10 min baseline period. Statistical significance from baseline for within each treatment group was defined as $p < 0.05$ using a one-sample t test. For paired-pulse ratios, ppr were acquired by applying two stimuli of equal intensity, separated by an interstimulus interval (ISI) of 50 ms and calculated as a ratio of EPSC 2/EPSC 1. NMDA/AMPA ratios were calculated as the ratio of the EPSC recorded at +40 mV, 50 ms after stimulation (NMDA) and the peak EPSC recorded at -80 mV (AMPA). Rectification index was calculated as

the EPSC amplitude at +40 mV/EPSC amplitude at -80 mV in the presence of 100 μM DL-APV. Group comparisons were made using a 1-way ANOVA and Bonferroni post hoc test. Statistical significance for all experiments was defined as $p < 0.05$.

RESULTS

Sensitivity of LTD to limited CIE

It has been shown that chronic ethanol modulates synaptic plasticity across multiple brain regions which encodes for behavioral aspects of AUD (Jeanes et al., 2011; McCool, 2011; Abrahao et al., 2013; Lovinger and Kash, 2015). Prior research from our laboratory has observed that even limited exposure to CIE (1-bout) induces robust alterations in the polarity of glutamatergic synaptic plasticity of medium spiny neurons in the nucleus accumbens shell in a cell-type specific manner (Jeanes et al., 2014; Renteria et al., 2016). Since the CIE model has shown that cycles of ethanol vapor generally result in enhanced volitional ethanol consumption, these results are taken to indicate that even limited CIE exposure is sufficient to alter glutamatergic synaptic plasticity mechanisms underlying aspects of escalated drinking, withdrawal, and dependence (Becker and Lopez, 2004; Griffin et al., 2009; Griffin, 2014; Lopez and Becker, 2005). We therefore investigated whether limited CIE (1-bout) similarly modulates the expression of AIC glutamatergic LTD. Layer 2/3 AIC pyramidal neurons prepared from mice 24 hours after limited CIE (1-bout) displayed differences in excitatory transmission in response to the low frequency stimulation paradigm (1 Hz for 15 min) previously shown to induce NMDAR-dependent LTD of evoked AMPAR-mediated EPSCs (Shillinglaw et al., 2018). We found that LTD was produced in layer 2/3 AIC pyramidal neurons in ethanol-naïve air mice (Figure 13; one-sample t-test, $t = 3.265$, $p = 0.0138$). However, using this same stimulation paradigm, we were unable to induce LTD in CIE mice 24 hours into withdrawal (Figure 13; one-sample t-test, $t = 0.680$, $p = 0.5118$).

Sensitivity of LTD to extended CIE

We additionally wished to determine whether extended CIE experience, which has been shown to enhance volitional ethanol drinking and produce ethanol dependence and withdrawal, differentially modulates LTD relative to an identical ethanol-drinking group without CIE experience (Becker and Lopez, 2004; Griffin et al., 2009; Griffin, 2014; Lopez and Becker, 2005). Therefore, additional groups of mice underwent 3 bouts of either CIE or air exposure coupled with 2BC drinking sessions (Drinking + CIE and Drinking + Air groups, respectively; Figure 14). Layer 2/3 AIC pyramidal neurons were recorded from 5-8 days after the final CIE/Air bout to relate glutamatergic changes to the time point at which 2BC drinking occurs (Figure 14). The Drinking + CIE mice displayed the inability to induce LTD (Figure 2; one-sample t-test, $t = 1.146$, $p = 0.2894$). Additionally, the Drinking + Air mice displayed the inability to induce LTD (Figure 14; one-sample t-test, $t = 1.162$, $p = 0.2834$).

Sensitivity of LTD to Age

The absence of LTD in the Drinking + Air group was a surprise, as similar studies of NMDAR-LTD in the NAc found LTD was still present in Drinking + Air groups (Renteria et al., 2018; Kircher et al., 2019). NMDAR-dependent LTD of AMPAR transmission in the AIC has not been extensively characterized and the prior work on this form of plasticity in the AIC was done using animals that were younger in age than that of the extended ethanol exposure experiment (Shillinglaw et al., 2018). Thus, the absence of LTD in both the Drinking + CIE and the Drinking + Air group was unexpected. Research has shown that synaptic physiology and the expression of plasticity can change over the course of an animal's life from adolescence to adulthood, and even beyond the emergence of early adulthood (McCutcheon and Marinelli, 2009; Jackson et al., 2017; Barnes, 1979, Lynch et al., 2006; Foster, 1999). Mice from the limited CIE (1-bout) experiment were 8-11 weeks, and mice from the extended CIE (3-bout) experiment were at least 14 weeks of

age. Therefore, as a control experiment, we tested whether we could reliably induce LTD onto AIC layer 2/3 pyramidal neurons in mice that were at least 15 weeks of age. Low frequency stimulation onto layer 2/3 AIC pyramidal neurons prepared from ethanol-naïve mice of at least 15 weeks of age did not induce LTD (Figure 15; one-sample t-test, $t = 1.231$, $p = 0.2385$).

Sensitivity of other glutamatergic properties to extended CIE

Since LTD was not reliably expressed in mice beyond early adulthood, we determined whether extended CIE experience differentially modulates other indices of glutamatergic synaptic transmission and plasticity relative to an identical ethanol-drinking group without CIE experience. Therefore, additional groups of mice underwent 3-bouts of either CIE or Air exposure both coupled with 2BC drinking sessions (Drinking + CIE and Drinking + Air groups, respectively; Figure 16). Layer 2/3 AIC pyramidal neurons were recorded from 5-8 days after the final CIE/Air bout to relate glutamatergic changes to the time point at which 2BC drinking occurs (Figure 16). We found no differences in paired pulse ratio between Drinking + CIE and Drinking + Air mice (Figure 16; unpaired t-test, $t = 0.07125$, $p = 0.9441$). We also found no differences in rectification index between Drinking + CIE and Drinking + Air mice (Figure 16; unpaired t-test, $t = 1.21$, $p = 0.2364$). Finally, we wished to observe whether there were any changes in NMDA/AMPA ratio. We did, however, observe a significant reduction in the NMDA/AMPA ratio in the Drinking + CIE relative to the Drinking + Air mice (Figure 16; unpaired t-test, $t = 2.932$, $p = 0.0098$).

Sensitivity of NMDA/AMPA to Handling + Air, Drinking + Air, and Drinking + CIE

Since this is the initial investigation of chronic ethanol on glutamatergic transmission the AIC, we wished to validate our reduced NMDA/AMPA ratio by replicating this finding in additional groups of mice. We also wished to determine whether drinking ethanol for weeks without CIE as conducted in the Drinking + Air groups alters

NMDA/AMPA ratios relative to ethanol-naïve mice. Therefore, we replicated the extended CIE experiment with additional groups of Drinking + Air and Drinking + CIE mice, along with a concurrent ethanol-naïve Handling + Air control group. We observed a statistically effect of group (Figure 17; one-way ANOVA, $F(2, 27) = 0.4457$, $p = 0.0110$]. However, we did not observe any differences between Drinking + Air and Drinking + CIE groups using a Bonferroni post hoc multiple comparisons test. It is therefore possible that any glutamatergic alterations in the AIC could occur due to non-pathological ethanol drinking rather than CIE-induced dependence.

Figure 13. Limited exposure to CIE inhibits the ability to induce LTD at glutamatergic synapses onto AIC layer 2/3 pyramidal neurons.

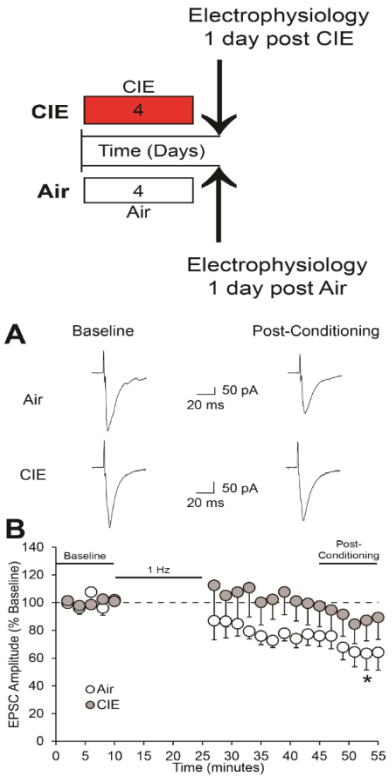

Figure 13. Limited exposure to CIE exposure inhibits the ability to induce LTD at glutamatergic synapses onto AIC layer 2/3 pyramidal neurons. (A) Representative traces from a single neuron of each group showing evoked EPSCs before and 20-30 min after conditioning stimulation protocol (900 pulses at 1Hz while holding the neuron at -70 mV). (B) Conditioning stimulation induces LTD expression in Air-treated, but not CIE-

treated mice 24 hours into withdrawal (Air, n = 8 neurons/8 slices/8 mice, *p = 0.01 compared to baseline; CIE, n = 11 neurons/11 slices/8 mice). Values are expressed as averages ± S.E.M

Figure 14. Extended exposure to CIE and ethanol drinking does not alter the expression of NMDAR-mediated LTD relative to an Air and ethanol drinking control group in AIC layer 2/3 pyramidal neurons.

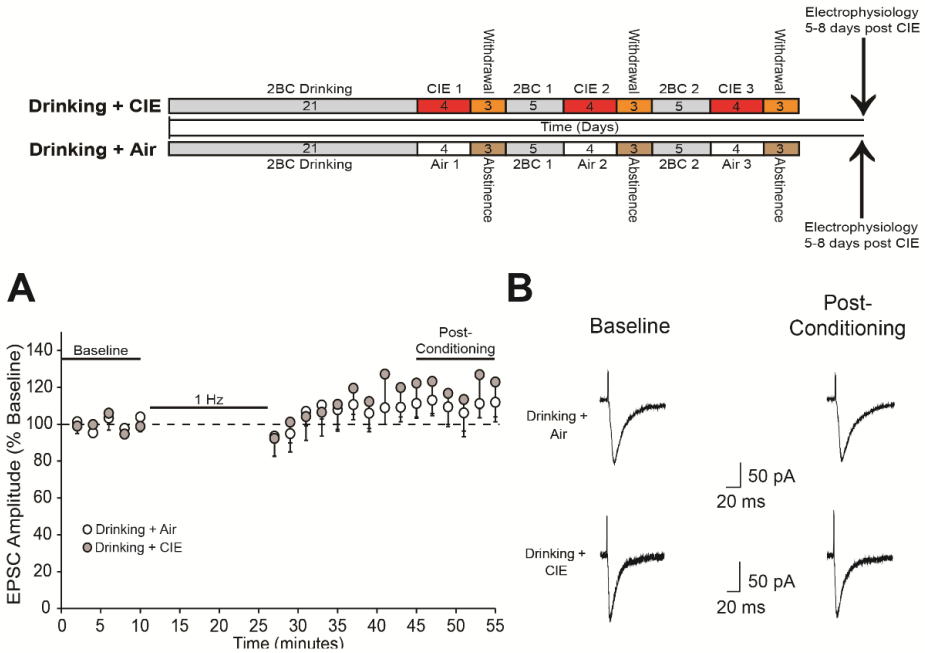

Figure 14. Extended exposure to CIE and ethanol drinking does not alter the expression of NMDAR-mediated LTD relative to an Air and ethanol drinking control group in AIC layer 2/3 pyramidal neurons. (A) Conditioning stimulation does not induce LTD of evoked EPSCs onto AIC layer 2/3 pyramidal neurons of Drinking + Air or Drinking + CIE mice (Drinking + Air, n = 8 neurons/8 slices/6 mice; Drinking + CIE, n = 8 neurons/8 slices/7 mice). (B) Representative traces from a single neuron of each group showing evoked EPSCs before and 20-30 min after low-frequency conditioning stimulation protocol. Values are expressed as averages ± S.E.M.

Figure 15. Conditioning stimulation does not induce LTD at glutamatergic synapses onto AIC layer 2/3 pyramidal neurons in ethanol-naïve mice at least 14 weeks old.

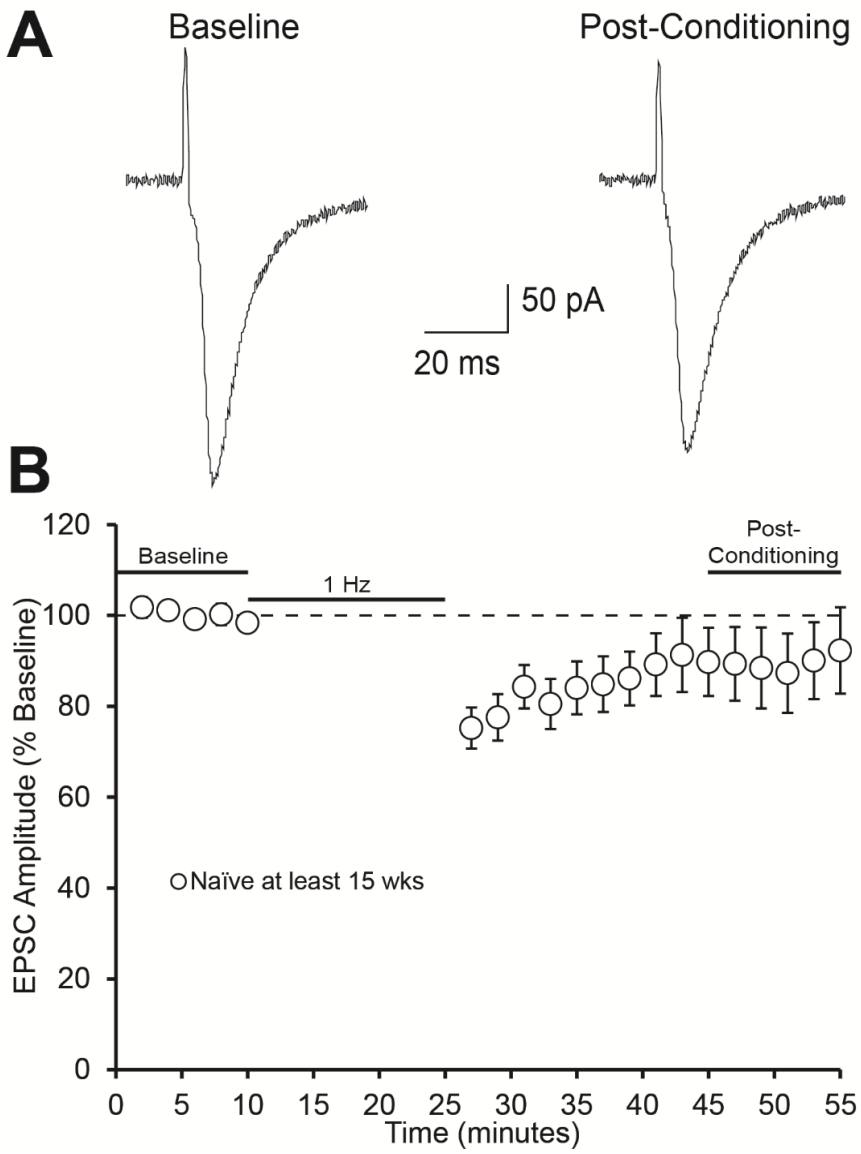

Figure 15. Conditioning stimulation does not induce LTD at glutamatergic synapses onto AIC layer 2/3 pyramidal neurons in ethanol-naïve mice at least 14 weeks old. (A) Representative traces from a single neuron showing evoked EPSCs before and 20-30 min after low-frequency conditioning stimulation protocol. (B) Conditioning stimulation did

not induce LTD expression in at least 15 weeks old (n = 15 neurons/15 slices/9 mice). Values are expressed as averages ± S.E.M.

Figure 16. Extended exposure to CIE and ethanol drinking reduces NMDA/AMPA ratio, but does not alter the expression of other prominent glutamatergic properties relative to an Air and ethanol drinking control group in AIC layer 2/3 pyramidal neurons.

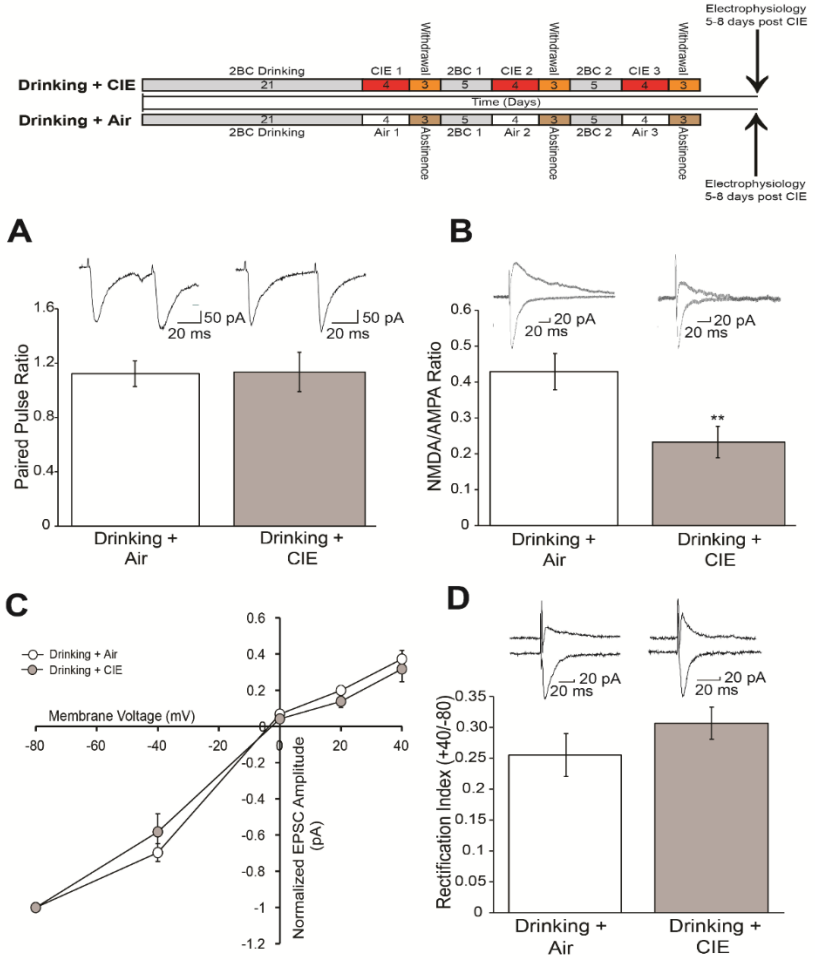

Figure 16. Extended exposure to CIE and ethanol drinking reduces NMDA/AMPA ratio, but does not alter the expression of other prominent glutamatergic electrophysiological properties relative to an Air and ethanol drinking control group in AIC layer 2/3 pyramidal neurons. (A) Paired Pulse Ratio (second evoked EPSC amplitude/ first evoked EPSC amplitude) does not change between Drinking + Air and Drinking + CIE mice (Drinking + Air, n = 8 neurons/7 slices/7 mice; Drinking + CIE, n = 9 neurons/8 slices/4 mice). (B) NMDA/AMPA Ratio (NMDAR component evoked at +40 mV and 50 ms after stimulus offset/AMPAR component evoked at -80 mV) is decreased in Drinking + CIE relative to

Drinking + Air mice (Drinking + Air, n = 9 neurons/9 slices/5 mice; Drinking + CIE, n = 9 neurons/9 slices/5 mice, **p = 0.0098 vs Drinking + Air). (C) No change is observed in the current voltage (IV) relationship of AMPAR mediated EPSCs (in the presence of 100 µM DL-APV) between Drinking + Air and Drinking + CIE mice (Drinking + Air, n = 10 neurons/10 slices/6 mice; Drinking + CIE, n = 6 neurons/6 slices/4 mice). (D) No change in the rectification index (Evoked EPSC amplitude at +40 mV/EPSC amplitude at -80 mV in the presence of 100 µM DL-APV) is observed between drinking + Air and Drinking + CIE mice (Drinking + Air, n = 13 neurons/13 slices/6 mice; Drinking + CIE, n = 16 neurons/15 slices/8 mice). Values are expressed as averages ± S.E.M.

Figure 17. Extended ethanol drinking reduces NMDA/AMPA ratio relative to an ethanol-naïve, age-matched and handling control group in AIC layer 2/3 pyramidal neurons.

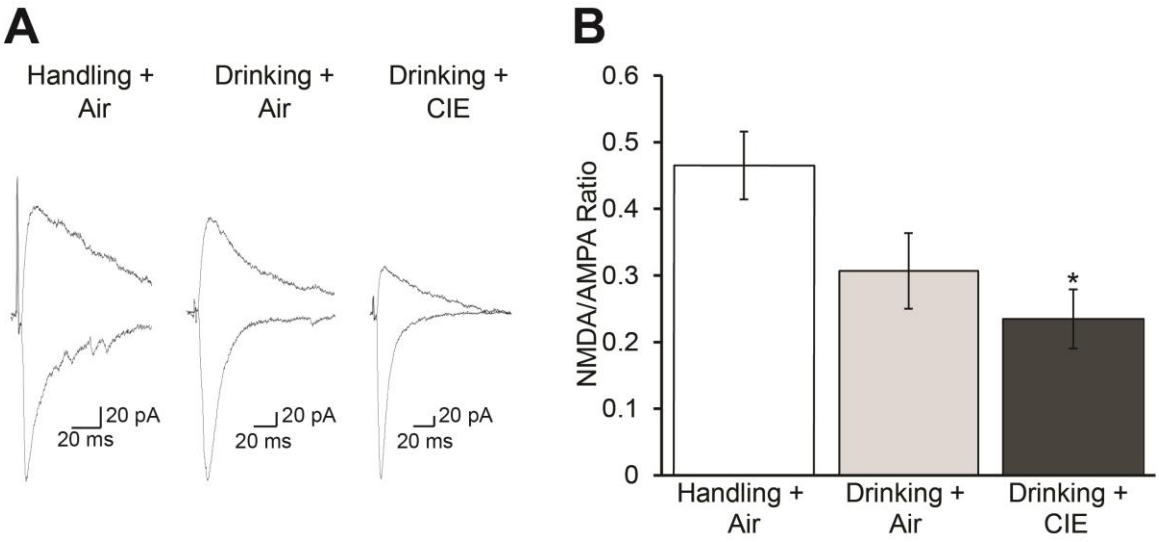

Figure 17. Extended ethanol drinking reduces NMDA/AMPA ratio relative to an ethanol-naïve, age-matched and handling control group in AIC layer 2/3 pyramidal neurons. (A) Representative traces from a single neuron of each group showing evoked NMDA/AMPA Ratio (NMDAR component evoked at +40 mV and 50 ms after stimulus offset/AMPAR component evoked at -80 mV). (B) NMDA/AMPA Ratio is decreased in Drinking + CIE mice relative to Handling + Air mice (Handling + Air, n = 14 neurons/9 slices/5 mice; Drinking + Air, n = 8 neurons/5 slices/2 mice; Drinking + CIE, n =8 neurons/4 slices/3 mice; *p = 0.01 vs. Handling + Air. Values are expressed as averages ± S.E.M.

Discussion

AIC LTD is sensitive to limited CIE

Chronic ethanol exposure has been shown to disrupt the healthy functioning of cortical regions via its long-term neuroadaptations to the electrophysiological properties of neurons (Kroener et al., 2012; Pleil et al., 2015; Nimitvilai et al., 2016). These neuroadaptations are thought to potentially reveal how various cognitive disruptions observed in individuals with AUD, such as deficits in response inhibition and reversal learning, occur (Kroener et al., 2012; Nimitvilai et al., 2016). It has been shown that alterations in AIC functioning and interoception are involved in aspects of AUD (Naqvi and Bechara, 2010; Paulus and Stewart, 2014; Ateş Çöl et al., 2016). Therefore, since our previous investigation showed that NMDAR-dependent synaptic transmission and plasticity are targets for acute ethanol, we tested whether the chronic actions of ethanol on NMDAR-dependent synaptic plasticity is a mechanism by which chronic ethanol alters AIC function.

We found that limited (1-bout) CIE inhibits the ability to induce LTD onto AIC layer 2/3 pyramidal neurons 24 hours into withdrawal (Figure 13). Since the Becker-Lopez CIE model reliably produces aspects of AUD, we hypothesized that disruptions in NMDAR-dependent LTD may be a signature of altered AIC functioning and interoception as ethanol consumption shifts from controlled to problematic (AUD-like). Such a finding wound not be without precedence, as several investigations have found disruption of NMDAR-dependent plasticity in brain regions of addiction interest that are related to ethanol-related behavior (McCool 2011; Abrahao et al., 2013; Jeanes et al., 2011; Jeanes et al., 2014; Renteria et al., 2017). However, it was also possible that the disruption in AIC LTD is not related to ethanol-related behavior, and rather a more general effect of ethanol withdrawal unrelated to the pathology of AUD. Indeed, Spiga and colleagues first suggested that the disruption of NMDAR-dependent LTD might be a ubiquitous mechanism at excitatory synapses across several brain regions as a consequence of ethanol withdrawal (Spiga, 2014).

AIC LTD is age-sensitive

We wished to better determine whether the disruption of AIC NMDAR-dependent LTD is linked to ethanol-related behavior. We next tested whether extended ethanol experience with the Becker-Lopez CIE model reliably shown sufficient to produce behavioral aspects of AUD differentially modulates NMDAR-dependent LTD from mice that drink ethanol without behavioral aspects of AUD (Becker and Lopez 2004; Griffin et al., 2014; Griffin et al., 2009; Renteria et al., 2017). To do so, mice that drank ethanol were either given 3-bouts of exposure to CIE (Drinking + CIE) or 3-bouts of exposure to Air (Drinking + Air) and tested *ex vivo* for the expression of LTD (Figure 14). Previous investigations from our laboratory have shown that withdrawal from CIE switches the polarity of synaptic plasticity from LTD to long-term potentiation (LTP) due to enhanced NMDAR functioning in the nucleus accumbens shell (Jeanes et al., 2011; Jeanes et al., 2014; Renteria et al., 2016). Therefore, we hypothesized that several bouts of CIE would result in either the continued inability to induce LTD, or the switch in polarity of plasticity to long-term potentiation (LTP) in Drinking + CIE mice. Since Drinking + Air mice would not undergo bouts of chronic ethanol vapor, we predicted that Drinking + Air mice would express LTD. We found, surprisingly, that both Drinking + CIE and Drinking + Air groups no longer displayed LTD in response to conditioning stimulation (Figure 14).

The absence of LTD in both groups was indeed unexpected, and could occur due to several reasons. However, one preliminary difference we wished to rule out between the limited and extended exposure experiments was the age difference between mice. Mice in the limited, 1-bout CIE exposure LTD experiment ranged from 8-11 weeks of age and mice extended access experiment were ≥14 weeks. There is a wealth of literature indicating that synaptic properties are at least partly age-dependent (McCutcheon and Marinelli, 2009; Jackson et al., 2017). It is generally thought that adulthood starts for mice around week 8, and so at first impression the differences in using mice from different periods of adulthood may not appear important for experimental design (Spear, 2000; Laviola et al., 2003). However, there is evidence that synaptic effects change even over the course of adulthood (Barnes, 1979, Lynch et al., 2006; Foster, 1999). Moreover, we were the initial group to

uncover AIC NMDAR-dependent LTD in whole-cell configuration, and did not initially test for age-dependent plasticity effects. In ethanol-naïve mice that were at least 15 weeks old, we no longer observed LTD, indicating that the ability to induce AIC NMDAR-dependent LTD is, at least in part, an age-dependent phenomenon (Figure 15). Therefore, using the Becker-Lopez model of extended ethanol exposure and drinking with mice of ≈ 15 weeks of age for our LTD assay is not an appropriate test to perform after this model of extended ethanol exposure.

Extended chronic ethanol exposure and ethanol drinking reduce AIC NMDA/AMPA

Since our LTD assay was not feasible for determining differences in glutamatergic synaptic transmission in mice of ≈ 15 weeks, additional indices of glutamatergic synaptic transmission and plasticity were conducted. We found that Drinking + CIE mice show a significantly reduced NMDA/AMPA ratio relative to Drinking + Air mice. Since exposure to chronic ethanol has frequently been shown to enhance glutamatergic transmission via increasing NMDAR function and NMDAR-mediated signaling, we were initially surprised by this finding (Lovinger and Roberto, 2013; Cebers et al., 1999; Grover et al., 1998; Gulya et al., 1991; Roberto and Varodayan, 2017). Moreover, although there is evidence that chronic ethanol enhances the expression and functioning of AMPA receptors, it has generally been shown to be at a lower level than that of NMDA (Lovinger and Roberto, 2013; Smothers et al., 1997; Chandler et al., 1997; Roberto and Varodayan, 2017). Therefore, at initial glance, our findings were perplexing as we expected to observe an enhanced NMDA/AMPA ratio due to increased NMDAR expression and function accompanied by potential, but less significant, changes in AMPAR expression and function. However, additional demonstrations are showing that the effects of chronic ethanol on postsynaptic glutamate receptors depend upon the brain region investigated and the time point after ethanol withdrawal (Kroener et al., 2012; Kim et al., 2014; Trantham-Davidson et al., 2014; Mcguier et al., 2015; Nimitvilai et al., 2016).

Since chronic ethanol has been shown to alter AMPAR-dependent aspects of glutamatergic synaptic transmission, we also wished to determine whether extended CIE exposure with ethanol drinking modulates aspects of AMPAR-dependent glutamatergic synaptic transmission onto the AIC (Hopf and Mangieri, 2018). Acute ethanol is generally accepted to be an inhibitor of excitatory signaling, while chronic ethanol has been shown to increase excitatory drive by increasing glutamatergic transmission onto neurons (Lovinger and Roberto, 2013). We found that chronic ethanol did not change paired pulse ratios (PPR) at a 50 ms interval, indicating that chronic ethanol exposure with ethanol drinking does not alter glutamate release probability onto AIC 2/3 pyramidal neurons. As a final assay of glutamatergic changes, we tested for changes in the rectification index to determine whether there were differences in the expression of calcium-permeable AMPA receptors (CPARS) which have been shown to be increased in the nucleus accumbens and implicated in motivational aspects of AUD such as craving. We found no changes in the I/V curve or rectification index, indicating no changes in the expression of CPARs. Additional follow-up electrophysiological studies of isolated NMDAR-mediated and AMPAR-mediated input-output curves to determine which component of the NMDA/AMPA ratio is changed at this timepoint is warranted. Additionally, complementary investigations of biochemical data to determine whether changes in glutamatergic NMDAR and AMPAR subunit composition accompany this investigation are also warranted.

Our main finding was that drinking ethanol alters the NMDA/AMPA ratio, a commonly used index of glutamatergic synaptic transmission and plasticity, onto layer 2/3 AIC pyramidal neurons. Therefore, it is likely that any synaptic changes onto layer 2/3 AIC pyramidal neurons occur during the initial aspects of ethanol drinking before extended ethanol exposure may occur. It is important to relate the glutamatergic synaptic plasticity effects observed due to even moderate (Drinking + Air) ethanol exposure in this experiment with the literature referencing the behavioral manifestations of glutamatergic synaptic plasticity mechanisms in the cortex. For example, glutamatergic synaptic plasticity mechanisms in the INS have been shown to encode for phenotypes produced by models of

neuropathic pain, as well as regulate the rate of conditioned taste aversion learning (Qiu et al., 2013; Liu and Zhuo, 2014; Rodríguez-Durán et al., 2017). From this point of view, it would be an invaluable investigation to determine whether the glutamatergic synaptic changes due to ethanol exposure even from our limited (Drinking + Air) paradigm are enough to modulate the expression of neuropathic pain as well as the extinction of conditioned taste aversion (Qiu et al., 2013; Liu and Zhuo, 2014; Rodríguez-Durán et al., 2017). Moreover, ethanol has well-documented aversive properties, and the reduction of the aversive aspects of ethanol consumption have been increasingly recognized as a component of the addition cycle for ethanol. It has been shown that a single 24 hour experience two-bottle choice paradigm enhances ethanol consumption adulterated with the bitter tastant quinine, but that it does not change quinine palatability (Lei et al., 2016). Therefore, it could be that ethanol exposure, at even early, moderate levels, disrupts glutamatergic synaptic plasticity mechanisms in the INS that encode for aversive interoceptive mechanisms including, but not limited to, alcohol consumption.

Chapter 4:

Concluding remarks and future directions

The findings in this dissertation are the first piece of evidence that the AIC is a direct synaptic target for the actions of ethanol. Of the major components of GABAergic and glutamatergic synaptic transmission, NMDAR-type glutamatergic transmission is sensitive to pharmacologically relevant concentrations of acute ethanol. The NMDA/AMPA ratio, a marker for glutamatergic synaptic strength and plasticity, is altered by even limited ethanol consumption. Together these data indicate that the AIC is an ethanol-sensitive brain region in which changes during exposure to ethanol are likely to occur.

There is a great need to better understand how the brain adapts to conditions that produce aspects of AUD. Despite several years of preclinical research investigating the neural mechanisms underlying aspects of AUD, there are still few effective pharmacotherapies for AUD treatment. More basic research investigating how neural circuits of motivational valence become disrupted over the development of AUD will likely lead to treatment targets, and more efficacious treatment compounds. To that effect, there needs to be a more comprehensive investigation of how chronic ethanol changes brain regions and circuits implicated in the three stages of the addiction cycle: 1) binge/intoxication of ethanol exposure, 2) ethanol withdrawal, and 3) preoccupation/anticipation with ethanol.

It is generally accepted that the development of addiction occurs over iterations of a three-stage, interdependent cycle: Initial ethanol use to binge/intoxication levels in which use is maintained by positive reinforcement mechanisms, withdrawal/negative affect in which the absence of ethanol produces negative affective states such as anxiety and dysphoria, and preoccupation/anticipation stage in which the motivation for ethanol drinking becomes increasingly ubiquitous in an individual's life (Koob et al., 2014). Individuals within the preoccupation/anticipation stage are therefore more likely to begin

drinking ethanol to binge/intoxicating levels, and thus begin the cycle again. Several cycles over time are generally accepted to generate, at least in part, an AUD. For this reason it is vital to comprehensively investigate how alcohol changes brain regions and circuits implicated in each aspect of the addiction cycle.

While a comprehensive review of the pharmacological effects of ethanol on brain circuits implicated in each aspect of AUD is outside the scope this dissertation, it can be summarized as follows: Initial ethanol use to binge/intoxicating levels is thought to be maintained via brain structures connected by the medial forebrain bundle reward system with focus on the ventral tegmental area, nucleus accumbens, amygdala, prefrontal cortex, and hippocampus (Koob et al., 2014). Dopaminergic afferents from the ventral tegmental area to these structures, termed the "reward circuit," have been implicated in the rewarding and positive reinforcing aspects of several drugs of abuse, including ethanol. Withdrawal from binge ethanol places the individual within the withdrawal/negative affect stage, in which neuroadaptations occur in some of these same brain structures as well as brain regions implicated in anxiety and dysphoria such as the bed nucleus of the stria terminalis and the extended amygdala. After repeated cycles of binge and withdrawal, glutamatergic afferents from cortical regions onto these midbrain structures play a large role in advanced aspects of AUD in the preoccupation/anticipation stage of ethanol use.

Until recently, despite evidence for altered INS activity and interoception in individuals with AUD, the INS has been largely overlooked by researchers investigating the addiction cycle (Naqvi and Bechara, 2010; Ateş Çöl et al., 2016). Animal research has suggested that the INS and disrupted interoceptive states contribute to the addiction cycle for AUD in a multidimensional manner (Pushparaj et al., 2015; Jaramillo et al 2016; Jaramillo et al., 2017; Jaramillo et al 2018; Naqvi and Bechara, 2010). Since chronic ethanol has been shown to contribute to AUD via disrupting homeostatic brain functioning in other brain regions, our overarching hypothesis was that chronic ethanol may disrupt INS processing and promote aspects of AUD.

While it had been determined that the INS has a role in the behavioral actions of ethanol, it had not yet been determined whether the INS is a direct target for ethanol's actions (Pushparaj et al., 2015; Jaramillo et al 2016; Jaramillo et al., 2017; Jaramillo et al 2018). Historically, elucidating the pharmacology of ethanol has proven difficult; its molecular structure yields low binding affinity for several cellular components. However, more recent research has shown its main behavioral mechanism of action to be via its actions on various ethanol-sensitive proteins (Harris et al., 2008). Despite a diverse set of ethanol-sensitive molecular targets, an extant body of research has indicated that the synapse is among the most sensitive of sites of ethanol's actions. This research indicates that a major component of the behavioral effects of ethanol is due to its actions on the ligand-gated ion channels responsible for major fast inhibitory and excitatory synaptic transmission, GABA and glutamate (Lovinger and Roberto 2013). However, these synaptic effects of ethanol have shown to be both brain region and concentration dependent, ostensibly due to receptor subunit composition and ion channel phosphorylation state (Nie et al., 1994; Lovinger et al., 1990; Roberto et al, 2003; Weitlauf et al., 2008; Badanich et al., 2013; Lovinger and Roberto., 2013; Kash et al., 2008; Shillinglaw et al., 2018).

We therefore investigated whether synaptic components of GABAergic and glutamatergic transmission in the AIC were targets for acute ethanol. We investigated the AIC subregion for several reasons. Its chemoarchitecture contains addiction-relevant receptor systems, D1Rs and CRF1Rs, indicated in positive reinforcement as well as stress signaling. Additionally, the AIC has reciprocal connections to limbic regions of significant relevance to AUD. Moreover, glutamatergic efferents from the AIC to midbrain and limbic structures have been shown to mediate compulsive and relapse components of drugs of abuse (Seif et al., 2013; Venniro et al., 2017). Of the cell types and layers of the AIC, we investigated layer 2/3 pyramidal neurons because layer 2/3 is typically considered the intracortical processing layer.

We found NMDARs in the AIC are sensitive to ethanol at blood ethanol concentrations achieved while drinking ethanol, a phenomenon consistent with results

from several other brain regions (Ron and Wang, 2009). Long-term changes in synaptic functioning are thought to be mechanisms of information storage and learning and memory in the central nervous system, and NMDARs have been shown to be major regulators of long-term information storage in the CNS (Kandel et al., 2014;). Therefore, we suggested that NMDAR-dependent mechanisms of long-term information storage and memory in the AIC are sensitive to ethanol. After generating and validating an NMDAR-dependent LTD in whole cell configuration, we determined that ethanol disrupted NMDAR-dependent mechanisms of information storage in the AIC at $\geq$ 20 mM. Therefore, NMDAR-type glutamate transmission in the AIC is sensitive to acute ethanol.

Chronic ethanol has been shown to produce long-term alterations in functioning of the synaptic targets of acute ethanol and involvement in AUD behavioral phenotypes. Therefore, we wished to investigate how chronic ethanol modulates NMDAR-dependent LTD in the AIC. In an experiment designed to test the initial effects of chronic ethanol on LTD, we found that four days of ethanol vapor exposure was sufficient to disrupt NMDAR-dependent LTD in layer 2/3 AIC pyramidal neurons 24 hours into withdrawal. We concluded that since ethanol vapor exposure generates aspects of AUD, the disruption of AIC LTD may predict an AUD-like phenotype. However, to fully test for differences in an AUD phenotype versus an ethanol drinking non-AUD drinking phenotype, we tested for differences in NMDAR-dependent LTD in CIE-treated (Drinking + CIE) vs. Air-treated (Drinking + Air) mice after extended bouts. We found that both groups no longer displayed LTD. In addition, we wished to test whether our NMDAR-dependent LTD mechanism was an age dependent phenomenon. Mice in the 1 bout CIE study were from 8-11 weeks, and mice in the extended drinking paradigm were at least 14 wks. We observed an inability to induce LTD in an additional group of ethanol naïve mice at least 14 weeks old, indicating this form of synaptic plasticity is at least partially an age-dependent phenomenon. Therefore, the aforementioned investigation of differences in LTD in Drinking + CIE vs. Drinking + Air mice is not valid.

Since chronic ethanol has produced differences in several aspects of glutamatergic transmission in other brain regions, we then investigated whether other glutamatergic

electrophysiological parameters were modulated in low-risk (Drinking + Air) and AUD-like (Drinking + CIE) mice. We found that the NMDA/AMPA ratio was decreased in AUD-like mice relative to low-risk mice. To further validate this finding and determine whether ethanol drinking in a low-risk phenotype alters NMDA/AMPA ratio relative to ethanol-naïve animals, we replicated the experiment with an additional ethanol-naïve handling control and found that ethanol drinking alone reduces NMDA/AMPA ratio relative to ethanol naïve animals. These data indicate that even moderate amount of ethanol consumption may be enough to alter glutamatergic synaptic plasticity mechanisms.

The findings in this dissertation are the initial investigation of whether acute and chronic ethanol exposure modulates synaptic transmission in the INS. The findings, on the whole, indicate that the glutamate system in the AIC is sensitive to ethanol, and that NMDAR-dependent signaling mechanisms in the AIC are sensitive to ethanol. These investigations together with the prior literature provide two crucial points: That the INS regulates ethanol intake, and that the INS and its activity are directly modulated by ethanol. Therefore, the INS and its circuitry should be considered in the context of future investigations, and potentially in the context of AUD treatment targets.

It is important to note that it has not yet been demonstrated whether ethanol's action on the INS contributes to how the INS regulates ethanol intake. Amongst other phenomena, Jaramillo and colleagues found that reducing activity in the INS substitutes for the discriminative stimulus/interoceptive effects of ethanol (Jaramillo et al., 2016). Our data have shown that of the components of basic GABAergic and Glutamatergic transmission, NMDARs are sensitive to acute ethanol. Therefore, since NMDARs are excitatory receptors which are inhibited by ethanol in the INS, and that reducing the activity of the INS substitutes for the interoceptive effects of ethanol, the most parsimonious hypothesis is that the actions of ethanol on NMDARS in the INS produces, at least in part, the discriminative effects of ethanol. Future investigations of NMDARs in the INS and ethanol drinking are warranted.

The recent development of techniques over the past decade that permit manipulation of *in vivo* brain function in a cell-type and/or circuit-specific manner may

have translational potential. One of these techniques, Designer Receptors Exclusively Activated by Designer Drugs (DREADDS), entails virally expressing mutated G protein-coupled receptors in brain cells of interest. The expression of these mutated G-protein coupled receptors allows for the selective activation or inactivation of brain cells of interest by an otherwise inert ligand, clozapine N-oxide (CNO). To date, DREADDS have been used to control neuronal activity in the brain, and investigate various aspects of psychiatric diseases, including several animal models of aspects of AUD (Cheng and Wang, 2019). It is therefore conceivable that DREADDS may have efficacy treating psychiatric illnesses such as AUD. DREADDS have already been safely expressed in nonhuman primates to modulate brain function and behavior (Eldridge et al., 2016). Moreover, the most common viral approach to expressing a DREADD in the brain, an adeno-associated virus, is already approved for use in humans in gene therapies (Eldridge et al., 2016; Nagai et al., 2016; Pignataro et al., 2018). Therefore, it is possible that DREADDS could be used in lieu of current FDA-approved invasive psychiatric techniques such as deep brain stimulation once precise targets for psychiatric disorders are elucidated by preclinical research.

AUD is a multifaceted condition characterized by problematic preoccupation with, consumption of, and recovery from ethanol. One of the subjective aspects of AUD which has gained recent interest to the addiction research community is craving. Craving has recently been included as an AUD diagnosis criterion in the most recent iteration of the Diagnostic and Statistical Manual of Mental Disorders, the DSM-V (2013). This inclusion is empirically supported, as craving has proven to be associated with severity of AUD, and relapse to drinking following treatment (Chakrovorty et al., 2010; MacKillop et al., 2010; Ramo & Brown, 2008; Zywiak et al., 1996). In addition, craving has proven to be valid as a diagnostic criterion for AUD as well, as craving has shown to be positively corrected with indices of drinking, and adverse consequences of alcohol abuse (Murphy et al., 2014). The inclusion of a subjective craving component of AUD adds an additional impetus to elucidating the neurobiological substrates underlying ethanol craving. Research from human and rodent laboratories indicate INS activity is involved in components of drug and ethanol craving and relapse behavior (Naqvi and Bechara 2010; Pushparaj and Lefoll,

2010; Venniro et al., 2017). A more complete understanding of INS circuitry and cell-type specific involvement in cue-induced craving may have therapeutic potential. A common-sense, if not overly simplistic idea, would be a thorough characterization of INS cells involved in cue-induced craving. Virally expressing either an inhibitory or excitatory DREADD into these cells (depending on how the disease state alters their function) could be a final safety net for individuals with AUD. Oral CNO could be taken prophylactically before the individual goes into situations in which previous ethanol cues, are readily available and likely to cause cravings.

The discoveries in this dissertation provide the first direct evidence of ethanol on AIC synaptic transmission and plasticity. Interoceptive processing has recently gained appreciation as a vital, under-investigated component of the addiction cycle. Therefore, future investigations of the INS are warranted.

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