INVESTIGATING THE ROLE OF EXTRASYNAPTIC $GABA_A$ RECEPTORS LOCATED IN THE INFRALIMBIC CORTEX IN THE BINGE-LIKE ALCOHOL INTAKE OF MALE C57BL/6J MICE

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ABSTRACT

Fritz, Brandon Michael. M.S., Purdue University, May 2013. Investigating the Role of Extrasynaptic $GABA_A$ Receptors Located in the Infralimbic Cortex in the Binge-Like Alcohol Intake of Male C57BL/6J Mice. Major Professor: Stephen L. Boehm II, Ph.D.

Extrasynaptic $GABA_A$ receptors, often identified as those containing both α4 and δ subunits, appear to be a target for the actions of alcohol (ethanol) at relatively low concentrations, perhaps suppressing the activity of GABAergic interneurons which regulate activity in the mesolimbocortical circuit. Pharmacological studies in rodents using the δ-subunit selective agonist Gaboxadol (THIP) have found both promotional and inhibitory effects on alcohol consumption. The goal of this project was to determine the role of extrasynaptic $GABA_A$ receptors located in the infralimbic cortex (ILC) in the binge-like alcohol intake of male C57BL/6J (B6) mice. The ILC is of interest due to its demonstrated involvement in stress reactivity and alcohol exposure has been shown to interfere with extinction learning; impairments of which may be related to inflexible behavior (i.e. problematic alcohol consumption). Adult male B6 mice were bilaterally implanted with stainless steel guide cannulae aimed at the ILC and were offered limited access to 20% ethanol or 5% sucrose for 6 days. On day 7, mice were bilaterally injected with 50 or 100 ng THIP (25 or 50 ng per side

respectively) or saline vehicle into the ILC. It was found that the highest dose of THIP (100 ng/mouse) increased alcohol intake relative to vehicle controls, although control animals consumed relatively little ethanol following infusion. Furthermore, THIP had no effect on sucrose consumption ($p > 0.05$), suggesting that the effect of THIP was selective for ethanol consumption. Together, these findings suggest that the mice that consumed ethanol may have been particularly reactive to the microinfusion process relative to animals that consumed sucrose, perhaps because ethanol consumption was not as reinforcing as sucrose consumption. In addition, the observation that THIP effectively prevented the decrease in ethanol intake on day 7 induced by the microinjection process may be related to a role for the ILC in adaptive learning processes, which in turn, promote behavioral flexibility

INTRODUCTION

General Introduction

One of the most widely used and abused psychoactive compounds, alcohol (ethanol), has a major impact on public health. Bouchery and colleagues (2011) estimate the direct and indirect economic costs of alcohol abuse in the United States to be around $223.5 billion. In addition to great financial expense, the World Health Organization has implicated a role for alcohol in the deaths of around 2.5 million people worldwide each year and the Center for Disease Control (2004) estimates that number to be roughly 79,000 in the U.S. It is therefore important to research the consequences of alcohol consumption in order to better understand the risks they pose to the individual as well as society.

Many people drink alcoholic beverages in moderation and do so with no significant health risks. Consuming alcohol in a binge fashion, however, is associated with alcohol use disorder susceptibility (Chassin *et al.* 2002). The National Institute on Alcohol Abuse and Alcoholism (NIAAA) defines binge alcohol consumption as alcohol intake resulting in a blood alcohol content of 0.08 gram % or higher which can often be achieved by consuming 4 (females) or 5 (males) standard drinks within a 2 hour time frame. The accumulation of this much alcohol in one's system typically produces motor incoordination (ataxia)

and reduced inhibitions, posing potential threats to the drinker's safety as well as the safety of those around them. While many human studies have greatly contributed to our understanding of the neurobiology/neurochemistry of alcohol use, ethical constraints limit the detail and causal inferences of their findings. Animal models of high alcohol consumption are valuable resources which allow researchers to not only control environmental factors, but more directly explore the neurobiological actions of alcohol.

Modeling Binge Drinking in Mice

Perhaps one of the most popular animal models of high alcohol consumption is the inbred C57BL/6J (B6) mouse. Inbred mouse strains are derived by brother-sister mating for at least 20 generations, producing homozygosity at all alleles. One advantage of using an inbred strain is that variation due to genetic differences is no longer a factor, theoretically making the effect of a treatment more clear. Another advantage for alcohol researchers that this particular strain offers is that B6 mice will readily consume significant amounts of alcohol. In the first published study that explored whether genetic background could influence the propensity to consume alcohol in animals, McClearn and Rodgers (1959) found that preference for a 10% ethanol solution over water was influenced by the genetic background of various inbred mouse strains. The C57BL strain happened to be the highest consumer of alcohol and actually exhibited a preference for the compound over water, when offered a choice. From this point on, inbred mice with a C57BL background, in particular

C57BL/6J ('6J' meaning the 6th iteration from Jackson Laboratories) became the focus of many alcohol studies. What is also unique about B6 mice is that their alcohol intake is particularly impressive when employing a limited-access alcohol consumption paradigm. Although these mice exhibit a preference for 10% ethanol over water in a continuous access, 2-bottle choice paradigm, concerns that they do not consume enough ethanol to reach pharmacologically-relevant blood ethanol concentrations (BECs) using this paradigm have been noted (Oberlin *et al.* 2011) as their rate of intake nearly matches their rate of metabolism. However, Rhodes and colleagues (2005) found that when an alcohol solution was made the only fluid available to B6 mice for 2-4 hours each day, the concentration of ethanol could be increased to 20% (v/v) and resultant BECs were consistently greater than 0.08 gram %. This binge-like alcohol consumption model, 'Drinking in the Dark' (DID), takes advantage of the mice's most active circadian period by providing the 2-hour alcohol access session during this time (beginning ~3 hours into the dark cycle). The DID paradigm has been validated as a binge drinking model, as B6 mice exhibit responses to alcohol similar to those observed in humans that binge drink: behavioral intoxication following alcohol access (Moore *et al.* 2007; Rhodes *et al.* 2007) and the capacity to develop tolerance following repeated daily drinking bouts (Linsenbardt *et al.* 2011).

Neuropharmacology

In order to understand the effects of binge alcohol consumption at the biochemical level, it is important to explore the role of neurotransmitter systems in the observed binge-drinking phenotype. One way to investigate this is through neuropharmacology. Pharmacological studies in alcohol research have provided invaluable information about the composition and involvement of various neurotransmitter systems in the reinforcing and/or rewarding effects of alcohol. By systemically administering receptor-selective agonists or antagonists prior to ethanol exposure, the involvement of particular neurotransmitters and specific receptor subtypes can be implicated in various alcohol-related behaviors.

Microinjection of drugs into particular brain structures narrows the focus even further by pinpointing the role of neurotransmitter systems *in specific brain structures or regions*. For example, in our laboratory we have recently evaluated the effectiveness of *Ro15-4513*, a positive allosteric modulator of $GABA_A$ receptors, to reduce alcohol drinking in B6 mice using the DID paradigm (Melón & Boehm II 2011). Given systemically, it was found that *Ro15-4513* reduced alcohol consumption. When microinjected into the ventral tegmental area (VTA), however, infusion of drug into the posterior region (pVTA) was found to effectively reduce alcohol drinking, although injection into the anterior region (aVTA) had no effect. Therefore, it can be inferred that this very discrete brain region (pVTA) is an important target for the actions of *Ro15-4513* and therefore is likely involved in the reinforcing properties of alcohol consumption. This highly

specific technique will be useful for determining the role of a discrete region of the prefrontal cortex, described below, in binge-like alcohol consumption.

Gamma-Aminobutyric Acid

Involvement of the mesolimbic dopamine (DA) pathway in the rewarding and reinforcing properties of numerous drugs of abuse, including ethanol has been repeatedly shown (for review, see Pierce & Kumaresan 2006). Although dopamine has long been the focus of this pathway's role in substance use and abuse, the importance of other neurotransmitters is also becoming clear. The major source of inhibition in the central nervous system, Gamma-aminobutyric acid (GABA), has been shown to be involved in ethanol consumption (Boyle *et al.* 1993; Leggio *et al.* 2013; Melón & Boehm II 2011; Moore & Boehm II 2009; Moore *et al.* 2007; Ramaker *et al.* 2011; Samson & Chappell 2001) as well as number of responses to alcohol such as sedation and stimulation (Boehm II *et al.* 2004; Kruse *et al.* 2012). For example, knockout mice lacking various $GABA_A$ receptor subunits display significant differences in alcohol intake as well as alcohol-induced locomotion and anxiolysis, hypnotic sensitivity, and withdrawal severity (Boehm II *et al.* 2004) In our lab, we have previously demonstrated that systemic administration of $GABA_A$ and $GABA_B$ receptor agonists differentially alter alcohol intake in B6 mice using a one-hour limited access paradigm (Moore *et al.* 2007). Microinjecting GABAergic drugs in discrete brain regions will further our understanding of how this neurotransmitter system is involved in binge alcohol consumption.

Ionotropic $GABA_A$ Receptor and Ethanol

Inhibitory GABAergic signaling is achieved through both acute chloride ion flux via activation of the ionotropic $GABA_A$ receptor and the engagement of second messenger cascades via the activation of metabotropic $GABA_B$ receptors. The particularly malleable $GABA_A$ receptor is a pentameric structure, made up of 5 subunits (comprised of various combinations of α, β, γ, δ, ε, π, ρ subunits), and is therefore very diverse in both form and function. For example, $GABA_A$ receptor subtypes containing the α2 subunit have been associated with behavioral insensitivity to the sedative hypnotic effects of ethanol relative to other subtypes (Täuber *et al.* 2003). Acute alcohol has been shown to potentiate the natural internal chloride ion current produced by the binding of GABA to $GABA_A$ receptors (Allan & Harris 1986), hyperpolarizing the membrane and often resulting in inhibition. When exposed to alcohol chronically, the subunit composition of $GABA_A$ receptors can be altered. One example is that α4 subunits have been found to be upregulated in the hippocampus as a consequence of chronic alcohol exposure (Cagetti *et al.* 2003). In comparison to α1β2γ2 $GABA_A$ receptors, the α4-containing isoform exhibits reduced sensitivity to benzodiazepines (Whittemore *et al.* 1996). It is therefore apparent that these ionotropic receptors are important targets for the actions of alcohol and exposure can alter their pharmacological sensitivity both acutely and chronically.

4,5,6,7-Tetrahydroisoxazolo[5,4-c]pyridine-3-ol (Gaboxadol or THIP)

A unique $GABA_A$ agonist used in some of the studies discussed above, *4,5,6,7-Tetrahydroisoxazolo[5,4-c]pyridine-3-ol* (THIP), appears to enhance GABAergic activity in a particularly selective manner. THIP acts as a partial $GABA_A$ receptor agonist, promoting chloride ion flux, hyperpolarizing the immediate intracellular region of a neuron. Reports have suggested that THIP is highly selective, as a superagonist, for δ-subunit-containing $GABA_A$ receptors (Adkins *et al.* 2001; Brown *et al.* 2002; Meera *et al.* 2011). Thus far, δ subunits have been found solely in extrasynaptic regions (Farrant & Nusser 2005). Extrasynaptic receptors appear to produce tonic inhibitory currents and are highly responsive to low concentrations of both GABA and ethanol (< 30 mM) (Hanchar *et al.* 2005; Wei *et al.* 2004) which may suggest their role in the initiation of an excessive alcohol intake session. Although some earlier work has called into question the receptor selectivity of THIP (Ebert *et al.* 1997), a subsequent study from the same lab suggested that THIP principally activated extrasynaptic $GABA_A$ receptors (Ebert *et al.* 2002). Furthermore, a recent study investigating the molecular basis of THIP's actions concluded that δ-subunit-containing $GABA_A$ receptors are extremely sensitive to THIP as compared to receptors comprised of α4/6β3 subunits (found in the synapse) and therefore, these extrasynaptic receptors are likely the site of action for THIP's effect on behavior at the appropriate concentrations (Meera *et al.* 2011). Finally, both behavioral (Boehm II *et al.* 2006) and electrophysiological (Vashchinkina *et al.* 2012) experiments using $GABA_A$ receptor δ-subunit knockout mice have suggested that

these animals are far less sensitive to THIP, supporting the notion that this drug selectively activates extrasynaptic $GABA_A$ receptors.

THIP has been found to both promote (Boyle *et al.* 1993; Boyle *et al.* 1992) and inhibit (Moore *et al.* 2007; Ramaker *et al.* 2011) alcohol drinking in rodents. Potential reasons for this discrepancy may be due to the use of different species (rats vs. mice), different alcohol access schedules, different drinking paradigms (2-bottle choice vs. single bottle), different alcohol concentrations, or timing of drug administration (Moore *et al.* 2007). In addition, the studies by Boyle and colleagues assessed 24hr fluid intake on THIP test days whereas as Moore et al. measured fluid intake after 1 hour. THIP has a relatively short half life of under 2 hours (Schultz *et al.* 1981) and as a result, any decrease in alcohol intake THIP may have had in the studies by Boyle et al. may have been masked by an alcohol intake 'rebound'. In addition, Moore and colleagues (2007) noted that the action of $GABA_A$ agonists can be sedative and that this may explain why THIP was found to also reduce water intake which is why home cage locomotor activity was measured in the current study following THIP administration.

Infralimbic Cortex

In humans, various regions of the prefrontal cortex (PFC) are the evolutionarily 'highest order' brain regions that have been implicated in problematic alcohol use. In a review by Miller and Cohen (2001), it is argued that the PFC processes information in a top-down manner, incorporating information about experiences from a variety of brain regions, ultimately creating a neural

representation of a particular behavior which influences subsequent action. As such, disorders of behavioral regulation, such as addiction, may be marked by deficits in PFC function. Imaging studies have found that alcohol-dependent individuals displayed enhanced alcohol cue reactivity in the dorsolateral (dlPFC) (George *et al.* 2001) and medial PFC (mPFC) (Grüsser *et al.* 2004) and that the amount of alcohol consumed in relapse is positively associated with stronger cue reactivity when abstinent (Grüsser *et al.* 2004). Furthermore, lasting perturbations of the orbitofrontal cortex of abstinent drug and alcohol dependent subjects have been demonstrated (Volkow & Fowler 2000). These different regions are functionally and anatomically heterogeneous (Pandya *et al.* 1996; Petrides & Pandya 2002) and likely have different roles in drug and alcohol intoxication and dependence and it is therefore important to focus on subregions of the PFC to better understand their unique involvement in responses to alcohol.

The PFC of rodents also has anatomical/functional subdivisions (Sesack *et al.* 1989; Vertes 2004). As discussed above, microinjection of pharmacological agents into discrete brain regions in animal studies is a valuable technique to elucidate the role of neurotransmitter systems in binge-like alcohol consumption. The rodent mPFC has been demonstrated in drug- and alcohol-seeking behavior as well (Hodge *et al.* 1996; Sun & Rebec 2005; Van den Oever *et al.* 2008) and can be divided into functionally heterogeneous subregions: the prelimbic cortex (PLC) and the infralimbic cortex (ILC). One suggested mechanism for the role of the PLC in drug/alcohol seeking is that it sends excitatory glutamatergic projections to the core of the nucleus accumbens (NAcb). For example,

stimulating the PLC via DA microinfusion has been shown to reinstate responding for cocaine in rats that successfully underwent extinction (McFarland & Kalivas 2001) and a subsequent study from the same group demonstrated increased glutamate levels in the NAcb core during reinstatement that were no longer apparent following PLC inhibition via microinfusion of $GABA_A$ and $GABA_B$ agonists (McFarland *et al.* 2003). Furthermore, chronic alcohol exposure has been shown alter the glutamatergic signaling in the PLC as evidenced by changes in n-methyl-d-aspartate (NMDA) receptor subunit expression (Kroener *et al.* 2012) and increased apical dendritic length (Holmes *et al.* 2012). These findings suggest that glutamatergic transmission in the PLC is acted upon by various drugs of abuse, producing potentially deleterious alterations in form and function.

Less is known, however, about the role of another subregion of the mPFC, the ILC, in drug and alcohol consumption and reinforcement. Although physically adjacent, the ILC interestingly appears to act opposite of the PLC in drug reinforcement. Cocaine seeking is reduced and extinction learning is enhanced when the ILC is stimulated via the glutamate receptor agonist, 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid (AMPA) (Peters *et al.* 2008a). Concerning alcohol, lesions of the mPFC (PLC & ILC) have been shown impair the extinction of conditioned place preference for alcohol in mice (Groblewski *et al.* 2012). Chronic intermittent exposure (CIE) has been shown to impair conditioned fear extinction which was attributable to impaired NMDA receptor-mediated burst firing in the ILC (Holmes *et al.* 2012). Furthermore, CIE alcohol

exposure has been demonstrated to produce deficits in cognitive flexibility (as assessed by a set-shifting task) which the authors suggest may be due to alterations in synaptic plasticity, characterized by an increase in NMDA to AMPA receptor current ratio and an increase in mushroom-type dendritic spines in the PLC and ILC (Kroener *et al.* 2012). A recent study (Meinhardt *et al.* 2013) has also found support for a potential mechanism of ILC dysregulation in the ILC. After being exposed to CIE alcohol, rats unsurprisingly self-administered large amounts of alcohol. Interestingly, these animals displayed significantly reduced expression of the type-2 metabotropic glutamate receptor (mGluR2). When mGluR2 levels were rescued via viral mediated gene transfer, however, alcohol self-administration was greatly reduced. These findings clearly demonstrate that alcohol and other drugs of abuse also alter glutamatergic signaling in the ILC. There is virtually nothing known, however, about the involvement of GABAergic signaling processes in the ILC which may ultimately regulate downstream glutamate signaling. Furthermore, no study to date has evaluated the involvement of the ILC in binge-like alcohol consumption. Given that the ILC is involved the extinction of drug and alcohol responses and is functionally altered by CIE exposure, it stands to reason that the ILC must be acted upon, in some capacity, earlier on in drug or alcohol exposure as well. The present study seeks clarity for the role of the ILC in binge-like alcohol intake.

Study Rationale

Functional deficits in the ILC following chronic alcohol as well as other drugs have been demonstrated, potentially reflecting rigidity in behavior; it may therefore be a target of ethanol early on in habit formation. The overall objective of the current experiments was to determine the role, and specificity, of intra-ILC extrasynaptic $GABA_A$ receptor activation in binge-like alcohol intake. $GABA_A$ receptors are highly concentrated in the ILC (Bowery *et al.* 1987) and previous studies have demonstrated that both the $GABA_A$ agonist muscimol and $GABA_B$ agonist baclofen infused into the ILC have clear behavioral effects (Akirav *et al.* 2006; Peters *et al.* 2008a), validating the GABA system as a target in this region. Furthermore, although not extensively characterized, the $GABA_A$ receptor δ subunit is present throughout the rodent cortex (Fritschy & Mohler 1995), offering selective targeting of extrasynaptic $GABA_A$ receptors by THIP. Extrasynaptic $GABA_A$ receptors have been proposed to be a target for the actions of alcohol (Hanchar *et al.* 2005; Wei *et al.* 2004), and hypofunction of the ILC appears to be related to increased drug and alcohol seeking (Groblewski *et al.* 2012; Meinhardt *et al.* 2013; Peters *et al.* 2008a; Peters *et al.* 2008b). It may therefore be that THIP locally applied to the ILC will increase alcohol intake via enhanced δ-subunit-containing $GABA_A$ receptor inhibition in the ILC; the actions of which may be similar to alcohol itself.

Specific Aim 1. Assess the effect of intra-ILC microinfusions of THIP on binge-like alcohol intake and resultant BECs in male B6 mice

Both chronic and acute ethanol exposure affect the ILC and hypofunction/dysregulation in this region has been associated with impaired extinction learning and cognitive flexibility (Holmes *et al.* 2012; Kroener *et al.* 2012; Meinhardt *et al.* 2013). Furthermore, increased ILC activity has specifically been demonstrated in suppressing drug and alcohol seeking (Groblewski *et al.* 2012; Meinhardt *et al.* 2013; Peters *et al.* 2008a). It is therefore hypothesized that enhanced inhibitory currents mediated by extrasynaptic $GABA_A$ receptors via THIP microinfusion into the ILC will increase binge-like alcohol intake and resultant BECs. One reason for this prediction is that inhibition via THIP may simply make the animal more impulsive. Lesioning of the ILC increases impulsivity in rats (Chudasama *et al.* 2003) and human studies observing patients with lesions of the ventromedial prefrontal cortex also find increased impulsivity for reasons that are not yet clear (Bechara *et al.* 1994; Best *et al.* 2002). As proposed by Peters et al. (2009), decreased activity in the ILC may confer a ‘high-risk’ phenotype for addiction. Enhanced impulsivity in THIP-injected animals may increase alcohol intake relative to vehicle-infused animals following the stressful microinjection process because their behavior may be less inhibited. In addition, glutamatergic output from the ILC to shell of the nucleus accumbens appears to gate the behavioral activation needed for drug seeking (McFarland & Kalivas 2001; Peters *et al.* 2008a), therefore reducing the output of these neurons via THIP may increase alcohol consumption. As the neural

processes underlying alcohol seeking in the ILC and the expression of GABA receptor subtypes in this region are not well characterized, the findings of this experiment may offer valuable information concerning GABAergic mechanisms in the ILC that may influence binge-like alcohol intake.

Specific Aim 2: Assess the effect of intra-ILC microinfusions of THIP on sucrose intake in male B6 mice

While intra-ILC THIP may have an effect on alcohol intake, it is important to determine the specificity of this effect by monitoring intake of an alternate reinforcer. Preference for ethanol is genetically correlated with saccharin preference in animals (Kampov-Polevoy *et al.* 1999). Therefore, offering a sweet solution instead of alcohol is an appropriate alternative to determine the specificity of THIP's effects. Sucrose was chosen for the current study in order to maintain the caloric value of the fluid consumed. Due to the unique effects of alcohol exposure on the ILC described previously, intra-ILC THIP is not expected to have an effect on sucrose consumption.

MATERIALS AND METHODS

Animals

Adult (PND 60-100 at time of surgery) male C57BL/6J mice were either obtained from the Jackson Laboratory (Bar Harbor, ME) or bred on site in our colony at the IUPUI School of Science. The breeders for these in-house animals were originally procured from the Jackson Laboratory and it was ensured that breeding did not take place past two generations from the founder animals. Lighting was maintained on a reverse light-dark cycle with lights off at 0900 and the temperature and humidity of the room were held constant near 20°C and 50%, respectively. All mice were singly housed 7-10 days prior to surgery to allow sufficient time for acclimation to isolated housing. Food and water were available ad libitum, except during the 2-hour drinking access periods where either a 20% (v/v) ethanol in tap water solution or a 5% (w/v) sucrose in tap water solution was the only fluid available. All experiments were performed under a protocol approved by the IUPUI Institutional Animal Care and Use Committee.

Drugs and Drinking Solutions

The 20% (v/v) ethanol solution was made by diluting 190 proof ethanol (Pharmco Inc., Brookfield, CT) in tap water. The 5% (w/v) sucrose solution was

prepared by dissolving sucrose (Sigma Aldrich, St. Louis, MO) in tap water. THIP was purchased from Sigma Aldrich (St. Louis, MO) and dissolved in 0.9% physiological saline vehicle before microinjection.

Drinking in the Dark

Starting 3 hr into the dark cycle, animals had their water bottle removed and replaced by a modified 10 ml graduated cylinder drinking tube fitted with a stainless steel double ball bearing sipper (Ancare, Belmore, NY). Animals had access to either a 20% (v/v) ethanol solution or 5% sucrose (w/v) solution for 2 hours. During this limited access period, mice did not have access to their regular water bottles. Volume readings were taken immediately before and after the 2 hour access period.

Home Cage Locomotor Activity

Dual axis (X/Y) locomotor activity was recorded during the DID drinking sessions using an Opto M-3 Multi-Device Interface from Columbus Instruments Inc. (Columbus, OH). Two 33 cm photocell beam units (one emitter and one censor on either side of the cage consisting of 12 beams, spaced 2.54 cm apart) ran along the length of the cage, mounted 27 cm apart. Two more 24 cm units (consisting of 8 beams, spaced 2.54 cm apart) were positioned along the width of cage, mounted 32 cm apart. An ambulatory count was registered by the Opto-M3 software (version 1.4.0) when two consecutive photocell beams (0.32 cm diameter; 875 nm wavelength; 160 Hz scan rate) were interrupted. This option

filters out stereotypic activity, such as scratching, where a single beam is repeatedly interrupted and therefore only registers activity as the animal's body moves throughout the cage. Home cage activity was monitored to determine whether or not THIP infused into the ILC had a stimulatory or sedative effect which may have competed with the target behavior, drinking.

Microneurosurgery

Materials to manufacture stainless steel guide cannulae and stylets were obtained from Small Parts Inc. (Miami Lakes, FL). Guide cannulae were cut to 14.0 mm lengths from 25-guage stainless steel 316 hypodermic tubing. Stylets were manufactured from stainless steel wire which was inserted into the guide cannulae to ensure patency after implantation. Cannulae were aimed and bilaterally implanted 2 mm above the ILC via stereotaxic surgery (Model 1900; David Kopf Instruments; Tujunga, CA). Stereotaxic coordinates for the ILC were obtained from the Franklin and Paxinos (1997) mouse brain atlas (from bregma: ± 0.4 mm lateral, + 1.685 mm anterior, - 1.15 mm ventral). Animals were anesthetized using a ketamine and xylaxine cocktail (1% xlaxine/10% ketamine w/v in saline; i.p.). Once animals no longer exhibited a pedal withdrawal reflex, they were deemed fully anesthetized for surgery. A rostral-to-caudal incision was made along the midline of the scalp to expose the bregma and lambda sutures on the skull. The exposed area was cleansed using three alterations of Nolvasan surgical scrub (Fort Dodge, Overland Park, KS) and 100% ethanol applied by sterile cotton swabs. The distance from bregma to lambda for each mouse was

used to adjust ILC stereotaxic coordinates based on skull size in order to enhance accuracy. This was achieved by taking the bregma-lambda distance measurement and dividing it by the average distance published for the C57BL/6J mouse strain (4.21 mm), creating a ratio to apply to the aforementioned coordinates. After these calculations were made, two holes were drilled bilaterally through the skull for the cannulae and a third hole was drilled unilaterally for the addition of a stainless steel anchor screw. This third hole was carefully widened using a hand drill in order to accommodate the larger width of the screw. The cannulae were then lowered simultaneously through the bilateral holes to their calculated ventral depth. Dental cement was applied to the skull to mount all of the hardware in place. As the cement dried, all mice received an analgesic injection of buprenorphine (0.03 mg/kg; s.c.) and an anti-inflammatory injection of rimadyl (5 mg/kg; s.c.). Mice were left in place in the stereotax until the cement had dried and were then placed in an empty cage warmed by a heating pad until regaining consciousness.

Blood Ethanol Concentration Determination

Blood samples (25 µl) were spun down in a centrifuge and the plasma supernatant was siphoned off and transferred to 0.5 ml microcentrifuge tubes. Samples were stored at -80°C until determination of BEC in mg/dl by an Analox Alcohol Analyzer (Analox Instruments, Lunenburg, MA).

Histology

Animals were euthanized by CO_2 inhalation and brains were extracted and snap frozen in super-cooled 2-methylbutane (Fisher Scientific, Pittsburgh, PA) and stored at -80°C until sectioning. Thirty micrometer sections were sliced through the ILC using a CM 30505 Leica cryostat (Walldorf, Germany). Tissue was stained with 0.5% cresyl violet and examined with a Leica dissecting microscope for accurate cannula placement. Data from animals that had misplaced cannulae were not included in the analysis. A representative section illustrating accurate bilateral microinjection into the ILC can be seen in Figure 1A. The placements of microinjections for all animals in the current study can be seen in Figure 1B.

Intra-ILC Microinjections

Microinjectors were constructed from 25 and 32 gauge stainless steel hypodermic tubing which were cut to 30 and 25 mm lengths, respectively (Small Parts, Miami Lakes, FL). The 32 gauge segment was inserted into the 25 gauge segment until only 16 mm extended past the length of the 25 gauge cannula. The contact point of the two segments was cleaned by applying soldering flux and heating with a soldering iron. The pieces were soldered together and patency was verified by using a syringe to flush deionized water through a tube attached to the microinjector.

Microinjectors were connected to two 60 cm lengths of PE-20 tubing which were filled with saline vehicle or THIP. Two 10 µl glass syringes (Hamilton, Reno,

NV) were filled with double deionized water and the tips were inserted into the alternate ends of the tubing. The fluid displacement engaged by these syringes acted as propulsion for fluid infusion. On test day (day 7), the animal was restrained, stylets were removed, and microinjectors were slowly inserted 2 mm past the guide cannulae into the ILC. Infusion rate (382 nl/min) was controlled by a Cole-Parmer (74900-series) dual infusion pump. Microinjectors were left in place for 60 seconds after the infusion had completed to allow time for the fluid to diffuse away from the tips. The microinjectors were then slowly removed to avoid drawing the fluid back up into the cannulae and the animal was placed back in its cage and presented with ethanol or sucrose.

Experiment 1

The goal of Experiment 1 was to determine if intra-ILC THIP reduced ethanol intake and resultant BECs in male B6 mice following a 6-day DID regimen. The timeline for microinjection experiments is illustrated in Figure 2. Mice were habituated to isolate housing for 7-10 days prior to surgery. Cohorts of 4-7 were bilaterally implanted with cannulae aimed 2 mm above the ILC and allowed 2 days to recover. Stylets were adjusted during these recovery days to ensure patency. The DID procedure commenced the following day and continued for a total of 7 days. Animals were habituated to the microinjection handling procedure in escalating durations across days 1-6 of DID, immediately prior to ethanol access. Days 1-2 consisted of 30 seconds of restraint and stylet adjustment and restraint duration was increased to 60 seconds on days 3-4.

Days 5-6 increased restraint time to 90 seconds and included a ‘mock microinjection’ wherein stylets were removed and microinjectors were slowly inserted only 1 mm past the guide cannulae. On Day 7, animals received bilateral 200 µl infusions of THIP (25 or 50 ng/side or 50 and 100 ng total/mouse, respectively) or saline vehicle. Immediately following ethanol access on Day 7, periorbital blood samples (25 µl) were taken for later BEC analysis.

Experiment 2

Experiment 2 was carried out identically to Experiment 1, except animals were presented with a 5% (w/v) sucrose solution during the DID access periods. The effect of intra-ILC THIP on sucrose intake was evaluated to determine whether or not the observed effect on ethanol intake was specific to ethanol itself or generalized to an alternate reinforcer. Sucrose solution intake is reported below in ml/kg.

Statistical Analysis

Only animals with confirmed bilateral microinjection into the ILC were included in the analyses. For both experiments, fluid intake and home cage locomotor activity across the 6 microinjection habituation days were analyzed by a two-way, repeated measures ANOVA using group and day as factors. Fluid intake (Experiments 1 & 2) and BEC (Experiment 1) following access on day 7 were analyzed by a one-way ANOVA with group as the factor for both experiments. Total ambulatory activity during the test day was also analyzed via

a one-way ANOVA with group as the factor in both experiments. Tukey-Kramer post-hoc tests were conducted where applicable and the level of significance was set at $p < 0.05$. All analyses were carried out using Statistica 7 software (StatSoft, Tulsa, OK).

RESULTS

Experiment 1

The acquisition of 20% ethanol consumption across the 6-days of handling exposure and DID is presented in Figure 3A. These data are presented collapsed on drug dose group as ethanol consumption between groups was not significantly different ($F_{2,26} = 1.47$, $p = 0.248$). As such, any differences in ethanol consumption between groups following microinfusion can be attributed to the dose of THIP and not differences in baseline ethanol consumption. A main effect of day was found ($F_{5,130} = 4.021$, $p < 0.01$) with intake on day 5 following the first 'mock microinjection' being significantly lower than intake on days 3 and 4 ($p < 0.05$). Ethanol intake was deemed to have recovered on day 6 following the second 'mock microinjection' as intake on this day was not significantly different from any other days ($p > 0.05$). The groups were also not found to significantly differ in home cage locomotor activity across the 6 habituation days ($F_{2,26} = 0.091$, $p = 0.913$; Figure 4A). Following microinfusion on day 7, a main effect of dose was found ($F_{2,26} = 5.144$, $p < 0.05$) with the 100 ng dose group consuming significantly more ethanol than the vehicle controls ($p < 0.05$; Figure 3A). The analysis of BECs following DID access on day 7 revealed a main effect of dose ($F_{2,26} = 12.333$, $p < 0.001$) with the 100 ng THIP dose group reaching significantly

higher BECs (m = 90.96 ± 10.54) than the vehicle (m = 27.64 ± 8.439; $p < 0.001$) and the 50 ng THIP (m = 34.13 ± 8.952; $p < 0.01$) dose groups. Home cage locomotor activity following microinfusion was not significantly different between groups ($F_{2,26} = 1.647$, $p = 0.212$; Figure 4A).

Experiment 2

The acquisition of 5% sucrose consumption across the 6-days of handling exposure and DID is presented in Figure 3B. Sucrose consumption between THIP dose groups was not significantly different ($F_{2,19} = 0.321$, $p = 0.73$) and these data are therefore presented collapsed on dose group. A main effect of day was found ($F_{5,95} = 8.696$, $p < 0.001$) with intake on day 5 following the first 'mock microinjection' being significantly lower than intake on days 3 and 4 ($p < 0.05$). Sucrose intake also recovered on day 6 following the second 'mock microinjection' as intake on this day was not significantly different from any other days ($p > 0.05$). The groups were also not found to significantly differ in home cage locomotor activity across the 6 habituation days ($F_{2,19} = 1.045$, $p = 0.371$; Figure 4B).

Following microinfusion on day 7, THIP was found to have no effect on sucrose consumption ($F_{2,19} = 0.606$, $p = 0.556$; Figure 3B). Home cage locomotor activity following microinfusion was also not significantly different between groups ($F_{2,19} = 1.458$, $p = 0.257$; Figure 4B).

DISCUSSION

The objective of Experiment 1 was to assess the involvement of δ-subunit-containing $GABA_A$ receptors in the ILC in binge-like alcohol consumption via site-specific microinjections of the selective $GABA_A$ agonist, THIP. Experiment 2 sought to determine whether or not intra-ILC THIP's effect on binge-like ethanol intake was specific to ethanol by evaluating intake of the alternate reinforcer, sucrose, following microinfusion. The results suggest that THIP may act in some capacity to influence ethanol intake, but not sucrose consumption when microinjected into the ILC.

Specific Aim 1. Assess the effect of intra-ILC microinfusions of THIP on binge-like alcohol intake and resultant BECs in male B6 mice

In the present study, mice that received the 100 ng dose of intra-ILC THIP consumed significantly more alcohol than the saline vehicle group (Figure 3A) and attained significantly higher BECs than both the vehicle and 50 ng THIP dose groups. It is important to note, however, that the vehicle group on day 7 displayed a large reduction in ethanol consumption relative to day 6 and the day 7 intake of the 100 ng group was nearly equivalent to day 6 (Figure 3A). A paired t-test comparing baseline ethanol intake for animals assigned to the vehicle

group on day 6 to the intake on day 7 was not significant ($p > 0.05$); although group sizes were relatively small in this study and it is clear that ethanol intake in this group is lower than the overall baseline of all 31 animals (Figure 3A). However, this decrease in intake from day 6 to day 7 in the vehicle group that consumed sucrose was not observed (Figure 3B). Perhaps one interpretation of this observation is that the animals that consumed ethanol may have had particularly negative reactions to the microinfusion process. The previous alcohol exposure over the 6 habituation days of DID may have potentiated the anxiety of these animals in response to the insertion of the microinjectors into the brain. As can be seen in Figure 3, the first 'mock microinjection' on day 5 produced a steep decrease in alcohol intake. However, a steep decrease in sucrose intake was also observed on day 5 following the first 'mock microinjection'. Importantly, animals consuming sucrose did not show a decrease in fluid intake in response to a microinfusion of vehicle into the ILC. This finding may suggest that alcohol consumption across the first 6 days of DID differentially influenced the animals' response to the microinfusion.

The microinjection process is unquestionably stressful as the animals are restrained for a considerable period of time (~90 seconds) and have injection cannulae inserted into their brain. The ILC has been shown to be critical for fear extinction learning and heightened activity in this region enhances fear extinction (Milad & Quirk 2002) as well as suppresses the reinstatement of cocaine seeking following extinction (Peters *et al.* 2008a). Concerning alcohol, CIE has been shown to impair fear extinction in mice, perhaps related to impaired NMDA

receptor-mediated burst firing in the ILC (Holmes *et al.* 2012). Furthermore, CIE has been shown to produce deficits in a cognitive set-shifting task, perhaps reflective of impaired cognitive-behavioral flexibility (Kroener *et al.* 2012). The duration and degree of alcohol of exposure needed to induce functional deficits in the ILC is not clear, however. One study in rats found that after a single oral ethanol gavage (2.5 g/kg), immediate-early gene (IEG) expression in the ILC was 57% greater than water controls (Leriche *et al.* 2008). It may therefore be that ethanol exposure for the mice in the present study was sufficient to induce changes in the ILC. Furthermore, exposure to a single and repeated uncontrollable stressor (forced swim) has been shown to produce specific impairments in fear extinction accompanied with dendritic retraction in the ILC, but not the PLC (Izquierdo *et al.* 2006). Perhaps the combination of restraint stress and alcohol exposure in these mice resulted in synergistic alterations of the ILC which made them particularly reactive to the microinfusion on day 7.

A more parsimonious explanation of this observed decrease may be that the insertion of microinjectors into the ILC of mice sufficiently disrupts alcohol intake. To my knowledge, the few mPFC microinjection studies that have evaluated alcohol seeking and intake in non-dependent animals (which focus primarily on the PLC) have used rats, rather than mice (Hodge *et al.* 1996; Samson & Chappell 2001, 2003). This may pose an issue when attempting to determine the relative trauma of the insertion of microinjectors into the mPFC of rats versus mice as a substantially larger portion of the region is lesioned in mice compared to rats. Given that the mPFC is a region involved in higher-order

cognitive processes, perhaps the microinjection process more greatly perturbs the binge-like alcohol intake of C57BL/6J mice using the procedure of the current study. While previous microinjection studies from our lab have not observed a steep decrease in alcohol consumption in vehicle controls (Linsenbardt & Boehm II 2009; Melón & Boehm II 2011; Moore & Boehm II 2009), these studies examined an evolutionarily 'lower-order' brain region, the VTA. It may be that the mere presence of cannulae or the insertion or microinjectors into the mPFC perturbed higher order cognitive processes which more greatly impacted ethanol consumption or made the animal highly reactive to the microinjection procedure.

Another issue this raises is that the duration of alcohol exposure was far greater in the above studies and the animals were trained to make operant responses for alcohol. These experimental procedures could have more effectively solidified ethanol as a reinforcer as compared to voluntary intake alone. It may therefore be that 6 days of limited access alcohol consumption was not sufficient for the solution to be adequately reinforcing in order to overcome the stress of the microinjection procedure on day 7 in the current study. Future studies should evaluate whether or not a longer period of DID alcohol consumption overcomes the decrease in ethanol intake following ILC saline vehicle infusion observed in the current study.

Although the microinfusion itself may have decreased alcohol intake in vehicle control animals, interestingly, the 100 ng dose of intra-ILC THIP prevented this large decrease in consumption. I am hesitant to say that THIP *increased* alcohol intake as the intake of the 100 ng dose group was similar to

intake on day 6 (Figure 3A). Rather, it appears that the highest dose of THIP mitigated the negative response to the microinfusion process in the ethanol animals.

Interpretation of this finding is fairly convoluted as research has mostly focused on the role of the ILC in drug and alcohol extinction learning or seeking in animals that either are dependent or have had extensive drug experience (Meinhardt *et al.* 2013; Ovari & Leri 2008; Peters *et al.* 2008a; Peters *et al.* 2008b). Collectively, these studies predominantly suggest that increased activity in the ILC is associated with enhanced extinction learning and reduced drug and alcohol seeking. The mice used in the current study were non-dependent and little is known about the involvement of the ILC in alcohol related behaviors in non-dependent animals. In one recent study, non-dependent mice underwent place preference conditioning for alcohol and were found to have significantly greater IEG expression in both the ILC and PLC relative to animals that received unpaired alcohol injections (Groblewski *et al.* 2012). Interestingly, this increase was no longer apparent following extinction. Furthermore, lesions of the mPFC greatly impaired ethanol place preference extinction. These findings may suggest that the ILC is involved in processing contextual cues associated with alcohol intoxication, but may not necessarily be modulated by alcohol alone in non-dependent mice. Other research, however, has found that 10 hours after a single intragastric bolus of ethanol (2.5 g/kg), IEG expression was significantly increased in the ILC. Therefore, Groblewski and colleagues (2012) may not have observed increases in IEG expression in the ILC of mice in the unpaired injection

group as they collected tissue at a significantly later time point following the final alcohol exposure. Regardless, the experimental procedure of the current study provides many contextual cues (e.g. handling, experimenter odor, ethanol odor) which may be associated with access to ethanol and/or intoxication which could have facilitated neurobiological changes in the ILC.

Clearly, the ILC is involved in association learning related to drug and alcohol seeking in both experienced and relatively inexperienced animals. Furthermore, the numerous observations that perturbations of the ILC are associated with extinction resistance (Groblewski *et al.* 2012; Holmes *et al.* 2012; Peters *et al.* 2008a; Peters *et al.* 2008b), disrupted cognitive flexibility (Kroener *et al.* 2012), and impulsivity (Chudasama *et al.* 2003) suggest that dysfunction in the ILC may generally manifest as cognitive-behavioral inflexibility. In other words, the ILC may be an important hub for the incorporation of relevant contextual information to influence subsequent behavior in an adaptive fashion.

Perhaps enhanced tonic inhibition from δ-subunit containing $GABA_A$ receptors via THIP, potentially similar to ethanol, interfered with the animals' reactions to the microinfusion event as a negative experience, guided by output from the ILC, which would have subsequently interfered with alcohol intake (as seen in the vehicle control group). THIP has been proposed to enhance tonic inhibition at putative extrasynaptic $GABA_A$ receptors (Meera *et al.* 2011), as has ethanol (Hanchar *et al.* 2005; Wei *et al.* 2004) and it is possible that THIP acted upon the ILC similar to ethanol itself. The half-life of THIP has been demonstrated to be relatively short (~2 hours) following systemic administration

(Schultz *et al.* 1981). In the current study, THIP was directly infused into the brain which allows the drug to rapidly diffuse. Therefore, THIP's pharmacological efficacy was likely continuously decreasing across the 2-hour ethanol access period on day 7. THIP may have therefore primed the ILC via extrasynaptic $GABA_A$ receptor activation, similarly to ethanol which may have replaced and perpetuated the pharmacological activation initially produced by THIP. In order to clarify this hypothesis, future studies may evaluate the time course of ethanol consumption following intra-ILC THIP as well as the effect of $GABA_A$ agonists that are known to activate synaptic $GABA_A$ receptors, as it appears that only very high, anesthetic doses of ethanol potentiate synaptic $GABA_A$ receptors (Wallner *et al.* 2006).

Speculating about possible mechanisms whereby THIP may have interfered with adaptive learning processes in the ILC, preventing a decrease in ethanol intake, there may have been a region-specific decrease in excitatory glutamatergic output to the nucleus accumbens shell (NAcbSh). Previous research has demonstrated that glutamatergic projections from the ILC to the NAcbSh are necessary to maintain the extinction of operant responding for cocaine (Peters *et al.* 2008a). These excitatory projections synapse onto inhibitory GABAergic neurons in the NAcbSh which then project to the ventral pallidum, which may ultimately inhibit drug seeking (McFarland & Kalivas 2001). It has also been observed that blocking AMPA and kainite glutamate receptors in the NAcbSh produces a robust increase in feeding behavior (Maldonado-Irizarry *et al.* 1995). Therefore, glutamatergic input to the NAcbSh may be an important

mechanism by which the ILC regulates hedonic behaviors. Perhaps GABAergic interneurons, producing tonic inhibition via postsynaptic extrasynaptic $GABA_A$ receptors, regulate excitatory glutamate efferents in the ILC. By enhancing this effect via THIP, or potentially ethanol, it may be that glutamatergic neurons projecting to the NAcbSh were inhibited by extrasynaptic $GABA_A$ receptors, resulting in a decrease in excitatory output to the NAcbSh, reducing GABAergic inhibition of the ventral pallidum and ultimately, preventing a decrease in alcohol consumption following microinfusion. In vehicle animals, the ILC may have responded to the stressful microinfusion process in an adaptive manner by robust glutamatergic output to the NAcbSh, reducing ethanol consumption.

To evaluate whether or not THIP may have prevented a decrease in behavioral activation brought on by the microinfusion process, a follow-up analysis of home cage locomotor activity on day 7 was conducted. A two-way factorial ANOVA with fluid (sucrose or ethanol) and dose as factors found no significant interactive effect of these variables on activity on day 7 ($F_{2,45} = 0.32$, $p = 0.727$). Therefore groups were collapsed on fluid and a one-way ANOVA was run with dose as the factor. Interestingly, there was a main effect of dose ($F_{2,48} = 3.881$, $p = 0.027$) with animals receiving the 100 ng THIP dose being significantly more active than saline vehicle controls ($p < 0.05$). When carefully examining these data, it again appears that THIP may have prevented a decrease from baseline activity that was observed in the saline controls. As can be seen in Figure 4, the vehicle groups exhibit the least activity on test day and the 100 ng THIP dose groups appear unaltered from baseline on day 6. Taken together with

the drinking data (Figure 3), it appears that there may have been an inhibitory effect of the microinfusion process that the 100 ng THIP dose effectively prevented, supporting the hypothesis above. However, no decrease in consumption on day 7 was observed in the vehicle group that consumed sucrose (Figure 3B). Therefore, the amount of fluid consumption, in general, does not appear to be directly related to locomotor activity across these two experiments and the effect of THIP on ethanol intake may therefore not necessarily be related to a general effect of the compound on activity. It has been previously demonstrated in our lab that the amount of ethanol intake during DID is significantly positively related to activity in male B6 mice (Linsenbardt & Boehm 2012). It may therefore be that animals that consumed ethanol and received a 100 ng dose of THIP were more active due to their higher alcohol intake, not necessarily a THIP-induced increase in activity. Although, based on the main effect of dose on activity reported above, it is difficult to disentangle these possibilities. For clarification, future studies should address whether or not 100 ng THIP can increase activity in mice consuming water. If this was indeed observed, it would provide further evidence that intra-ILC THIP generally increases activity. Furthermore, efforts should be made to produce ethanol intake that is similar to pre-microinfusion levels in saline vehicle controls which may offer clarity as to whether intra-ILC THIP more directly affects locomotor activity, in general, or ethanol intake.

Speculation aside, the distribution of the $GABA_A$ receptor δ-subunit in the ILC is not known and it will be important to determine whether or not putative

extrasynaptic receptor subtypes containing this subunit are present in particular projection neurons or possibly regulatory interneurons. Furthermore, it is not known whether $GABA_A$ receptor activation in this region, in general, can alter ethanol intake. Nevertheless, THIP was found to influence ethanol intake in the current study, suggesting that the activation of δ-subunit-containing $GABA_A$ receptors in the ILC can influence binge-like alcohol intake. Future studies should address the synaptic versus extrasynaptic receptor question by employing synaptic $GABA_A$ receptor agonists. If findings from these studies suggested a unique action of THIP on binge-like ethanol intake, this may support a mechanism of ethanol action in the ILC.

Specific Aim 2: Assess the effect of intra-ILC microinfusions of THIP on sucrose intake in male B6 mice

The goal of Experiment 2 was to evaluate intra-ILC THIP's effect on sucrose intake in order to determine whether or not the effect generalized to an alternate reinforcer. It was found that THIP had no effect on sucrose intake (Figure 3B) or home cage locomotor activity (Figure 4B) relative to vehicle controls. It must be noted, however, that the decrease in fluid intake on day 7 that was observed in the ethanol-consuming vehicle group was not seen in animals that consumed sucrose. One reason for this observation may be that 5% (w/v) sucrose is actually more reinforcing than 20% (v/v) ethanol in these experiments. As noted previously, the brain region targeted in the current study involves higher-order neural processes and the lesions produced by cannula

implantation and microinjection may have interfered with the acquisition of ethanol consumption and its value as a reinforcer. Relative to previous microinjection studies from our lab (Linsenbardt & Boehm II 2009; Melón & Boehm II 2011; Moore & Boehm II 2009), ethanol intake over the acquisition period appears to have been lower in the current study. In addition, these previous studies did not see the large decrease in alcohol intake in the vehicle control group. Sucrose intake, however, was not affected by the microinjection process in both the current and previous studies. Therefore, it reasonable to propose that in the current study, sucrose may have had a greater reinforcing value relative to ethanol which may explain why a) ethanol consumption was lower in the present study relative to previous observations while sucrose intake was similar b) the vehicle control animals significantly reduced their ethanol intake following microinjection on day 7 while the sucrose animals did not and c) why intra-ILC THIP was seen to have an effect on animals that consumed ethanol but not sucrose.

Conclusions and Future Directions

The mPFC has been implicated in drug and alcohol seeking in both humans (Grüsser *et al.* 2004; Volkow & Fowler 2000) and animals (Groblewski *et al.* 2012; McFarland & Kalivas 2001; McFarland *et al.* 2003; Meinhardt *et al.* 2013; Ovari & Leri 2008; Peters *et al.* 2008a; Peters *et al.* 2008b) and this region governs higher-order neural processes related to drug and alcohol consumption which primarily appear to be related to associative learning. As there is functional

heterogeneity in this region (Pandya *et al.* 1996; Petrides & Pandya 2002; Sesack *et al.* 1989; Vertes 2004), efforts have been made to determine whether or not there are unique roles for the various subdivisions of the mPFC in drug and alcohol-related behaviors. In the animal literature, the majority of studies have focused on the involvement of the PLC (McFarland & Kalivas 2001; McFarland *et al.* 2003; Miller & Marshall 2004; Samson & Chappell 2001, 2003).

Virtually nothing is known, however, about the role of the ILC in voluntary binge-like alcohol intake. Of the few studies that have investigated a role for the ILC in alcohol-related behaviors, they have primarily focused on extinction learning (Groblewski *et al.* 2012; Holmes *et al.* 2012) or operant self-administration (Meinhardt *et al.* 2013). In addition, most of these studies examined subjects after forced, chronic exposure (Holmes *et al.* 2012; Kroener *et al.* 2012; Meinhardt *et al.* 2013), although there is some evidence that alcohol acts upon the ILC with subchronic exposure (Groblewski *et al.* 2012; Leriche *et al.* 2008). Furthermore, these studies primarily focused on glutamatergic processes in the ILC and virtually nothing is known about GABAergic processes in this region. Given the observations that both chronic and acute ethanol exposure affect the ILC and that hypofunction/dysregulation in this region has been associated with impaired extinction learning and cognitive flexibility, I hypothesized that THIP-induced inhibition in the ILC would increase alcohol intake. THIP was found to increase alcohol intake in the present study, but this was potentially due to a substantial decrease in intake in the vehicle control group. Nevertheless, THIP positively influenced ethanol intake relative to control

animals in a way that was not observed with sucrose intake. Taken together, one potential interpretation of these results, given that THIP and ethanol have been shown to potentiate the activity of δ subunit-containing $GABA_A$ receptors (Hanchar *et al.* 2005; Meera *et al.* 2011), is that an effect of THIP was only detected in animals consuming ethanol due to a synergistic interaction of both compounds in the animals' system. However, the follow-up analysis mentioned above found that intra-ILC THIP may have generally acted to increase activity in mice consuming either ethanol or sucrose. This argues that this potential interaction was not necessary for the drug to effectively produce a change in behavior.

On the basis of the aforementioned literature, I propose that the ethanol consumption history of these animals was not sufficient to achieve adequate reinforcing value in order to override the stress of the ILC microinjection process and local inhibition via THIP blunted the salience of the microinjection event, thereby resulting in ethanol intake that was similar to intake on day 6. These findings may support the proposed role of the ILC in adaptive learning and extend its involvement to binge-like alcohol intake. Given the observations of the current study in addition to the aforementioned literature, the ILC may be important in cognitive-behavioral flexibility as it relates to alcohol consumption. In other words, this region may process events associated with alcohol consumption and subsequently influence behavior in an adaptive manner. It may be that reduced activity in the ILC inhibits adaptive capacity and results in behavioral rigidity. As alcohol has been proposed to act on extrasynaptic $GABA_A$

receptors (Hanchar *et al.* 2005; Wei *et al.* 2004) similarly to THIP (Meera *et al.* 2011), perhaps alcohol may have an inhibitory role in the ILC that impairs the ability of the individual to adapt subsequent alcohol intake in response to salient contextual information.

In order to clarify the findings of the current study, future research should establish whether or not a longer duration of limited-access to alcohol consumption prevents a substantial decrease in alcohol intake in male C57BL/6J mice following intra-ILC microinfusion of saline vehicle. If, in fact, increasing alcohol exposure results in uninterrupted alcohol intake following microinfusion, this would support the notion that alcohol consumption was not as reinforcing in the current study as has been seen in previous investigations in our lab (Linsenbardt & Boehm II 2009; Melón & Boehm II 2011; Moore & Boehm II 2009). This may then suggest that future microinjection studies targeting the mPFC in mice should extend the alcohol consumption period past 7 days. Understanding when, over the course of limited-access alcohol exposure, these mice no longer show a decrease in intake following vehicle microinfusion may also be a relevant time point to evaluate markers of neural activity, such as c-fos, which may provide insight on the relationship between activity in the ILC and the rigidity of binge-like alcohol intake. Furthermore, producing such uninterrupted alcohol intake would clarify whether or intra-ILC THIP more directly influences activity in general or ethanol intake. In addition, it is not clear whether or not potentiating $GABA_A$ receptor activity in the ILC, in general, may have a similar effect on alcohol intake. Future work characterizing $GABA_A$ receptor δ-subunit

expression in the ILC and whether or not it co-localizes with certain projection neurons will offer clarity on the current findings. Microinjection studies evaluating $GABA_A$ agonists that are known to bind to synaptic $GABA_A$ receptor subtypes will also help elucidate whether the potentiation of extrasynaptic $GABA_A$ receptor function is a potential mechanism of action for ethanol in the ILC. Finally, it will be important to determine if the efficacy of intra-ILC THIP on alcohol intake is related to the extent of alcohol exposure as alcohol-induced changes in neurobiology may alter responsiveness to THIP.

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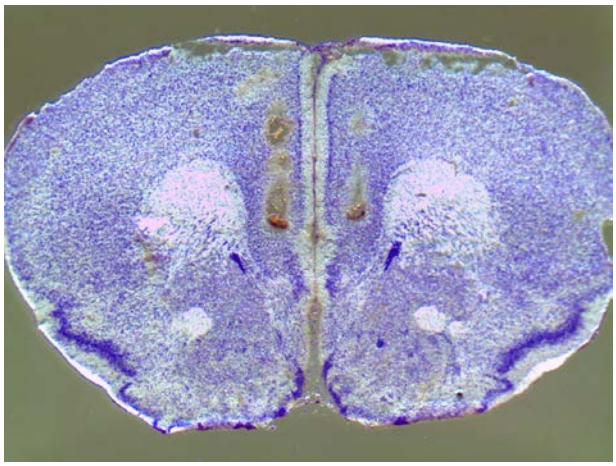
APPENDIX

APPENDIX: FIGURES

A



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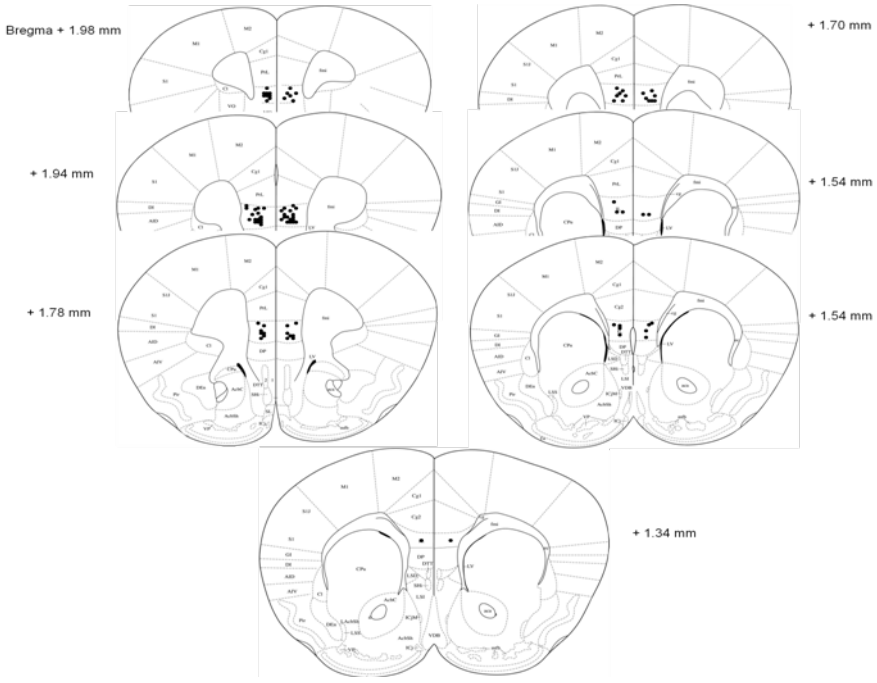

Figure 1: Representation of surgical accuracy. A) Representative cresyl violet-stained section depicting accurate bilateral insertion of microinjection cannulae into the ILC. B) Stereotaxic map illustrating the microinjection sites for all animals included in the analyses.

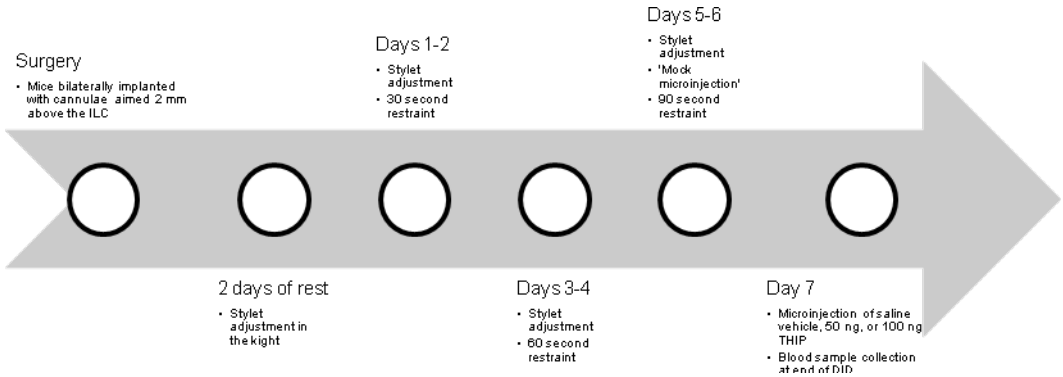

Figure 2: Flowchart of the microinjection experimental procedure.

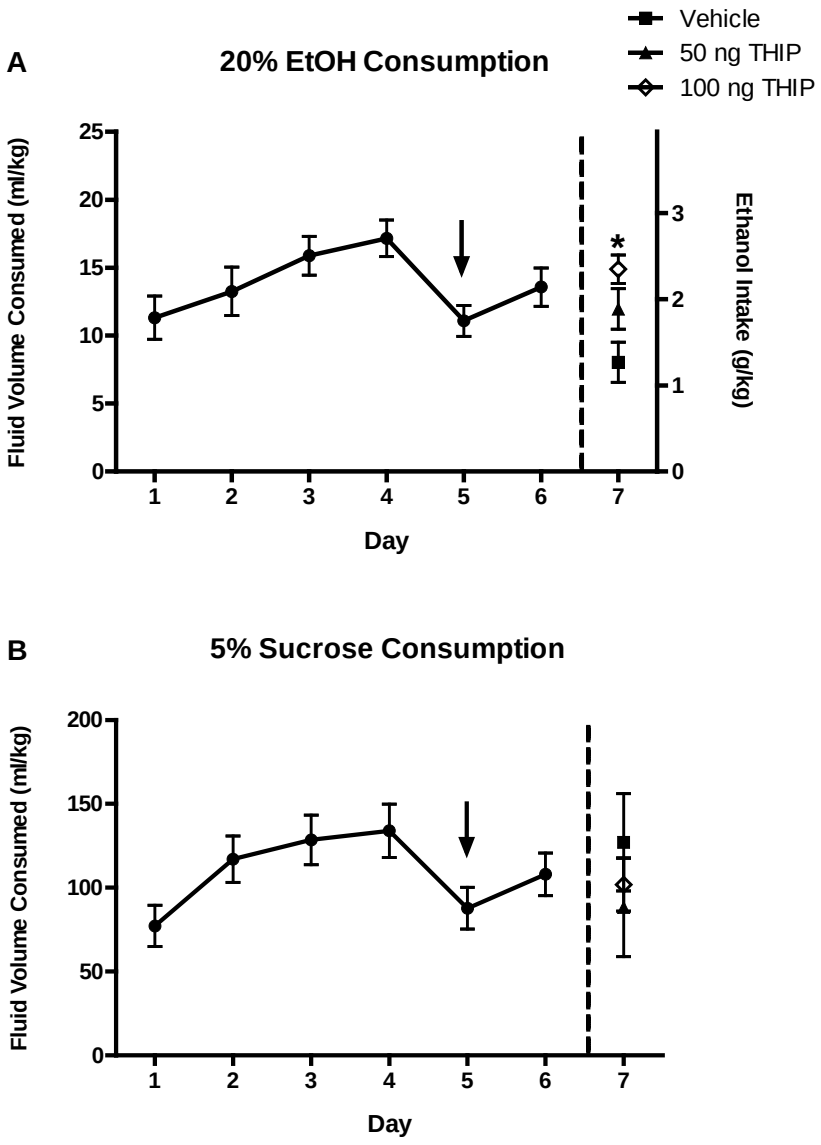

Figure 3: Fluid consumption. A) Consumption of 20% (v/v) ethanol and B) 5% (w/v) sucrose via DID across the 6 days of handling habituation and day 7, the microinjection test day. The data for days 1-6 are presented collapsed on THIP dose group as there were no significant differences ($p > 0.05$) between groups in fluid intake across days 1-6 (N = 31 for ethanol and N = 22 for sucrose). The arrow notes the first 'mock microinjection' on day 5. Data for day 7 are separated by dose group (n = 8-12 for ethanol and 6-10 for sucrose). *$p < 0.05$ vs. vehicle.

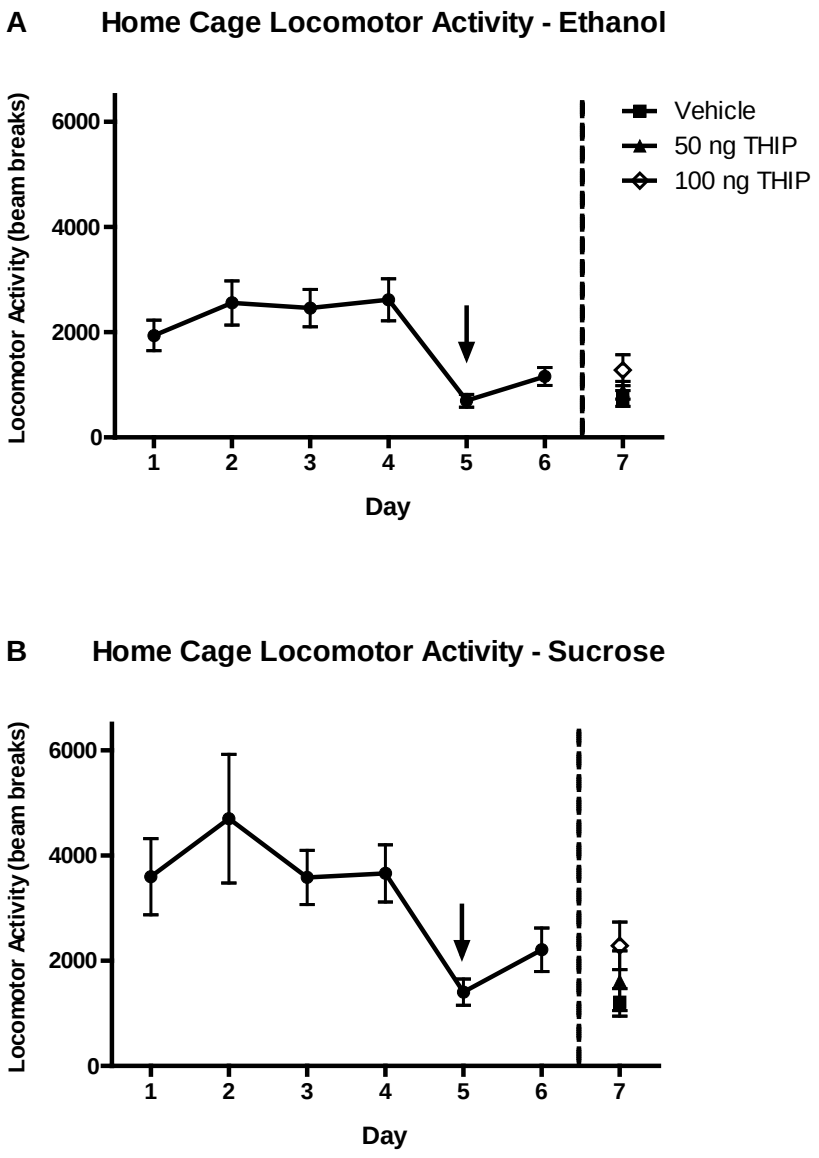

Figure 4: Home cage locomotor activity. Locomotor activity during each DID session while animals consumed A) 20% (v/v) ethanol and B) 5% (w/v) sucrose across the 6 days of handling habituation and day 7, the microinjection test day. The data for days 1-6 are presented collapsed on THIP dose group as there were no significant differences ($p > 0.05$) between groups in activity across days 1-6 (N = 31 for ethanol and N = 22 for sucrose). The arrow notes the first 'mock microinjection' on day 5. Data for day 7 are separated by dose group (n = 8-12 for ethanol and 6-10 for sucrose).