# THE EFFECT OF A NEUROACTIVE STEROID ANALOG AND AN EXTRASYNAPTIC $GABA_A$ RECEPTOR AGONIST ON ETHANOL CONSUMPTION AND SEEKING IN MICE

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## LIST OF ABBREVIATIONS

10E – 10% (v/v) ethanol solution

10S – 10% (w/v) sucrose solution

2S - 2% (w/v) sucrose solution

3α-HSD - 3α-hydroxysteroid dehydrogenase

3β-HSD - 3β-hydroxysteroid dehydrogenase

5-HT - serotonin type 3

5α-R - 5α-Reductase

aCSF – artificial cerebral spinal fluid

ADE – alcohol deprivation effect

ALLO – allopregnanolone; 3α,5α tetrahydroprogesterone

AMPA - alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

AUD - alcohol use disorder

BEC – blood ethanol concentration

CA1 - *Cornu Ammonis* area 1

CA3 - *Cornu Ammonis* area 3

CeA – central nucleus of the amygdala

CNS – central nervous system

CPP – conditioned place preference

D1 receptor – dopamine receptor type 1

D2 receptor– dopamine receptor type 2

DHP – dihydroprogesterone

DMSO - dimethylsulfoxide

EPSC - excitatory postsynaptic currents

FIN – finasteride

FR – fixed ratio

GABA – γ-aminobutyric acid

GAN - ganaxolone

IBI – interbout interval

ICV - intracerebroventricular

i.p. - intraperitoneally

MBR – mitochondrial benzodiazepine receptor

mg/dl – milligrams per deciliter

mg/kg – milligrams per killigrams

mIPSC – mini inhibitory postsynaptic current

MSN – medium spiny neuron

NAc – nucleus accumbens

nACh - nicotinic acetylcholine

NAS. – neuroactive steroid

NIAAA - National Institute on Alcohol Abuse and Alcoholism

NMDA - N-methyl-D-aspartate

P450scc – P450 side chain cleavage

PFC – prefrontal cortex

PKA – protein kinase A

PKC – protein kinase C

RR – response requirement

sd – standard deviations

StAR – steroid acute regulatory

STN – subthalamic nuclei

THIP - 4,5,6,7-tetrahydroisoxazolo-[5,4-c]pyridine-3-ol hydrochloride; gaboxadol

THP - tetrahydroprogesterone

VTA – ventral tegmental area

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## OVERALL ABSTRACT

Alcohol use disorders (AUDs) are a group of heterogenous disorders of which no single animal model can capture the entirety of the disease. This dissertation examined the effect of a neuroactive steroid (NAS) analog ganaxolone (GAN) on measures of ethanol seeking and intake across multiple procedures in male C57BL/6J mice. NAS can act at both synaptic and extrasynaptic γ-aminobutyric acid$_A$ ($GABA_A$) receptors, and it is unclear if the effects of GAN on ethanol intake are due to actions at synaptic $GABA_A$ receptors, extrasynaptic $GABA_A$ receptors, or both. In order to examine how activation of extrasynaptic $GABA_A$ receptors might be contributing to alterations in ethanol intake across the different procedures, I also examined the effect of gaboxadol (THIP), a $GABA_A$ receptor agonist with preference for extrasynaptic receptors, on ethanol seeking and consumption. My hypothesis was that both GAN and THIP would alter components of ethanol seeking and consumption, indicating a role for NAS and extrasynaptic $GABA_A$ receptor activation in altering ethanol intake, and that the nucleus accumbens (NAc) shell would be a brain region contributing to this effect.

In Chapter 2, a continuous-access procedure was used in order to examine the time-related effects of GAN and THIP across 24-hours of ethanol access. In this experiment, GAN promoted the onset of ethanol consumption while decreasing overall ethanol intake. THIP produced decreases in intake that were present for the first 5 hours of access, and it also increased the latency to complete the first ethanol bout. These experiments revealed interesting divergent and comparable effects between GAN and THIP, particularly during the first hours of access, suggesting that GAN was likely exerting its effects on ethanol intake via an action at both synaptic and extrasynaptic $GABA_A$ receptors.

In Chapter 3, the first set of studies utilized a 2-hour limited-access procedure to take advantage of the high ethanol intakes and maximal drug effects during this shorter time period. This experiment revealed a dose-dependent decrease in ethanol intake with GAN, due to a trend for a decrease in bout frequency. Consistent with the continuous-access study, THIP dose-dependently decreased ethanol intake, and this was primarily due to a decrease in bout frequency and a delay in the onset of the first ethanol bout.

Additionally, Chapter 3 utilized an operant procedure, in which lever pressing and subsequent drinking were temporally separated, in order to dissect the effects of GAN and THIP on appetitive versus consummatory behavior. GAN caused a slight, non-significant increase in both the appetitive and consummatory phases of self-administration, consistent with a possible role in promoting ethanol seeking and initiation of intake. To examine the specificity of this effect, I also tested the effects of GAN on sucrose self-administration and found that GAN promoted sucrose seeking but did not systematically alter sucrose intake, indicating that GAN may promote general seeking behavior. THIP on the other hand, decreased both the appetitive and consummatory phases of ethanol and sucrose self-administration, indicating a lack of specificity for ethanol seeking and consumption in this procedure. Importantly, this procedure again revealed divergent roles of GAN and THIP, primarily on ethanol seeking, indicating that GAN's actions at synaptic $GABA_A$ receptors may be particularly important for promoting ethanol seeking.

Chapter 4 examined whether activation of $GABA_A$ receptors in the NAc shell by GAN or THIP could account for the effects that were observed following the systemic injections of these drugs which were reported in Chapter 3. GAN infused into the NAc

shell led to a decrease in ethanol intake that was primarily due to a decrease in bout frequency. Dorsal controls showed that effects of GAN were localized to the NAc shell. Infusions of THIP into the NAc shell also decreased ethanol intake, but the use of multiple cohorts revealed important order effects of the drug. Locomotor activity studies confirmed that there were no sedative effects following intra-NAc infusion with the highest dose of either drug that could have contributed to the decreases in ethanol intake observed. In total, these results suggest that the activation of $GABA_A$ receptors in the NAc shell by GAN or THIP is sufficient to account for the effects of systemic GAN and THIP on ethanol intake that were observed in the limited-access procedure.

Finally, in Chapter 5, ethanol seeking was measured with the reinstatement procedure. Following extinction of operant ethanol self-administration, lever pressing was measured in the absence of gaining access to the ethanol reinforcer. GAN reinstated extinguished ethanol-reinforced responding, measured by an increase in pressing on the previously active lever. However, THIP did not alter reinstatement of ethanol seeking. Consistent with Chapter 3, these findings suggest that activation of extrasynaptic receptors at the GABA binding site is not sufficient to promote reinstatement of ethanol seeking.

Collectively, these studies showed that GAN altered ethanol consumption by increasing appetitive ethanol seeking and initial ethanol intake, while suppressing overall ethanol intake. These studies also showed that THIP decreased both ethanol seeking and consumption across multiple procedures**.** Together these studies suggest that activation of extrasynaptic receptors alone, at the GABA binding site, is not sufficient to promote ethanol seeking in these procedures. The results demonstrate that alteration of NAS levels, as well as activation of extrasynaptic $GABA_A$ receptors, influence the

consumption of ethanol in mice. Importantly, the results also document the sufficiency of the NAc shell in mediating the effects on ethanol intake observed following systemic injection of GAN and THIP. These studies contribute to literature examining the mechanisms underlying ethanol intake and will hopefully aid in the ability to understand and treat AUDs. Data with GAN and THIP may be especially useful in light of their increased use in clinical trials, particularly for diseases (i.e. post-traumatic stress disorder, depression, nicotine dependence) that may have a high comorbidity with AUDs.

# CHAPTER 1: GENERAL INTRODUCTION

It is estimated by the National Institute on Alcohol Abuse and Alcoholism (NIAAA) that 18 million Americans suffer from an alcohol use disorder (AUD). AUDs are defined by alcohol craving, loss of control of use, physical dependence such as withdrawal effects, and tolerance to alcohol. The NIAAA estimates that AUDs cost the US billions of dollars each year in health-related costs, loss of productivity, and treatment-related costs, but despite the enormous costs to society, the mechanisms underlying alcohol abuse and potential treatments remain poorly understood. There are currently four treatments approved by the U.S. Food and Drug Administration for AUDs: disulfiram (Antabuse), naltrexone (Trexan), acamprosate (Campral), and ondansetron (Zofran). Yet even with treatment, abstinence rates at the three month mark remain less than 50% (e.g., Kieffer et al., 2003).

Part of the challenge in treating AUDs is that alcohol (ethanol) acts at many different receptor systems. For example, ethanol has been shown to act as an agonist at nicotinic acetylcholine (nACh) (Narahashi et al., 1999), glycine (Mihic et al., 1997) and serotonin type 3 ($5HT_3$) receptors (Lovinger and Zhou, 1998), as an antagonist at N-methyl-D-aspartate (NMDA) (Lovinger et al., 1989), alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) (Valenzuela et al., 1998), and kainate receptors (Valenzuela et al., 1998), as an inhibitor of potassium (Koboyashi et al., 1999) and calcium channels (Wang et al., 1994), and as a positive allosteric modulator at $\gamma$-aminobutyric $acid_A$ ($GABA_A$) receptors (Mihic et al., 1997). The action at the latter is the focus of the present studies. A better understanding of mechanisms by which activity of $GABA_A$ receptors contributes to ethanol seeking and consumption, particularly across multiple different models, could improve the therapeutic tools available to treat AUDs.

**$GABA_A$ receptor: subunits and binding sites**

The $GABA_A$ receptor is a pentameric receptor that gates a chloride channel and is comprised from a pool of at least 19 possible subunits: $\alpha_{1-6}$, $\beta_{1-3}$, $\gamma_{1-3}$, δ, ε, π, θ, and $\rho_{1-3}$. $GABA_A$ receptor subunit mRNA and protein distribution across the brain has been studied extensively for rats (Pirker, et al., 2000; Wisden et al., 1992; Schwarzer et al., 2001; Fritschy and Mōhler, 1995), and has recently been shown to be remarkably similar in C57BL/6NCrl male mice (Horntägl, et al., 2013). The most common stoichiometry is 2α:2β:γ, which makes up about 90-95% of the $GABA_A$ receptors in the brain (McKernan and Whiting, 1996). The most abundant arrangement is a $\gamma_2$ subunit in combination with $\alpha_{1-3}$ and $\beta_{2-3}$ subunits. These receptors are located in the synaptic cleft, where they mediate phasic inhibition (Prenosil et al., 2006). Phasic inhibition occurs following action potential-dependent vesicular release of GABA from the presynaptic cell (Fatt and Katz, 1953). GABA, which is present at nM to μM concentrations, then binds at the postsynaptic cell, which causes a conformational change of the channel (Mody et al., 1994). The conformational change opens the channel, allowing chloride to flow down its concentration gradient, which in most cases leads to chloride flowing into the cell. The influx of chloride hyperpolarizes the postsynaptic cell, decreasing the ability of an action potential to occur (e.g., Cherubini, 2010).

In the rat, in a small subset of receptors (<5%), the γ subunit is replaced by a δ subunit (McKernan and Whiting, 1996), which limits the location of the channel exclusively to the extrasynaptic space (Nusser et al., 1998; Sun et al., 2004). This subunit is most commonly paired with $\alpha_4$ or $\alpha_6$ and $\beta_{2-3}$ subunits and contributes exclusively to tonic inhibition (Stell and Mody, 2002). Tonic inhibition results when a nM concentration of ambient GABA binds to high-affinity, slowly-desensitizing receptors, leading to a steady, sustained opening of channels (Mody, 2001; Farrant and Nusser et

al., 2005). The source of this low concentration of GABA may be the result of action potential-dependent diffusion of GABA from the synapse or action potential-independent quanta (single vesicles) being released (Fatt and Katz, 1952; Kaneda et al., 1995). Tonic inhibition can provide up to 75% of the total inhibition of the cell, which is vital for limiting excitability of the cell (Hamann et al., 2002).

In addition to influencing the subcellular localization of the receptor, the specific subunit composition also contributes to the affinity and efficacy that $GABA_A$ receptors have for GABA as well as several classes of modulators that bind to distinct and interacting allosteric sites (Fig. 1.1) (Mody and Pearce, 2004). For example, GABA and other agonists of the $GABA_A$ receptor (e.g., muscimol) bind to the interface of the α and β subunits to open the channel and increase chloride conductance (Lehoullier and Ticku, 1989; Akabas, 2004). Despite not directly binding to the δ or γ subunit, the presence of the δ subunit confers higher sensitivity to GABA than presence of the γ subunit (You and Dunn, 2007). Importantly, $GABA_A$ receptor agonists, such as muscimol, do not require GABA in order to alter chloride conductance. $GABA_A$ receptor antagonists (e.g., bicuculline) have no intrinsic activity, and can either competitively block the site where the agonists bind (e.g., bicuculline) or non-competitively block the chloride pore (e.g., pictrotoxin), both of which decrease GABA-mediated chloride flux (Allan and Harris, 1986).

Benzodiazepines are positive allosteric modulators of the $GABA_A$ receptor that bind to a distinct site at the interface of the α and γ subunits (Sigel and Buhr, 1997). Receptors containing the $\alpha_1$ and γ subunits show the highest affinity for benzodiazepines, whereas receptors containing the $\alpha_4$ or $\alpha_6$ in combination with the δ subunit are benzodiazepine insensitive (Rudolph and Möhler, 2004). Upon

benzodiazepine binding, the receptor undergoes a conformational change, which allows for enhanced binding of GABA and therefore increases the frequency of channel opening (Hevers and Lüddens, 1998). Thus, benzodiazepine agonists potentiate the action of GABA at synaptic $GABA_A$ receptors, and they require the presence of GABA for activity. Benzodiazepine antagonists block the site where the benzodiazepine binds, but have no intrinsic activity (Ehlert et al., 1982). Inverse agonists also bind to the benzodiazepine site and block benzodiazepine binding, but the binding can diminish GABA-mediated constitutive activity of the channel, leading to the opposite effect as the benzodiazepine (Kemp et al., 1987).

Barbiturates are another class of $GABA_A$ receptor-acting drugs that can act as positive allosteric modulators at a separate binding site. They bind to the β subunit and, in the presence of GABA, cause a conformational change which increases the duration of channel opening. Thus by allowing greater amounts of chloride to flow through the channel per opening, barbiturates increase the efficacy of GABA (Serafini et al., 2000). At higher concentrations, barbiturates can directly activate the channel even in the absence of GABA (Fisher and Fisher, 2010).

**The $GABA_A$ receptor and neuroactive steroids**

Traditionally, steroids were thought to bind exclusively to steroid receptors, where they act in the nucleus to alter gene transcription and produce effects on the order of hours to days. In 1941, Hans Selye identified a rapid anesthetic effect of the synthetic steroid, alphaxalone, and proposed an alternative mechanism whereby steroids could bind to membrane receptors to rapidly alter excitability of the cell on the order of seconds to minutes (Selye, 1941; also Craig 1968). Evidence that steroids specifically acted at the $GABA_A$ receptor was later revealed by biochemical studies showing that

alphaxalone enhanced GABA-stimulated chloride conductance (Harrison and Simmonds, 1984). These findings were extended to show that endogenous steroids were potent allosteric modulators of $GABA_A$ receptors (Morrow et al., 1987). These steroids have since been termed either "neurosteroids," referring to the fact that they can be made *de novo* in the brain (Baulieu, 1991), or "neuroactive steroids," referring to their ability to exert rapid activity in the central nervous system (CNS) (Paul and Purdy, 1992). The present work focuses on neuroactive steroids (NAS), referring to steroids that act in the CNS but may be made either peripherally in steroidogenic organs (which include the gonads and the adrenal gland) or *de novo* in the brain (Paul and Purdy, 1992).

NAS can be either positive or negative allosteric modulators of the $GABA_A$ receptor (referred to collectively as GABAergic NAS). Positive allosteric modulators can enhance GABA binding and efficacy and include the 3α-reduced NAS (see Fig. 1.2) (Lan and Gee, 1994). Negative modulators of the channel diminish GABAergic signaling and include the 3β-reduced NAS as well as sulfated derivatives of NAS (Majewska, 1992). Binding studies have revealed a NAS binding site distinct from benzodiazepines or barbiturates (Gee et al., 1988). The binding site for NAS is thought to be embedded within the lipophilic plasma membrane; therefore NAS may be able to access the receptor from either inside or outside of the cell or via lateral diffusion in the plasma membrane (Mitchell et al., 2008; Akk et al., 2005). In the nM concentration range, NAS bind to the α subunit, where they can increase the duration or frequency of $GABA_A$ receptor opening (Hosie et al., 2006, 2007, 2009). When present in the low μM concentration range, they can bind to the α/β subunit interface to directly activate the channel (Hosie et al., 2006). At supraphysiological levels (≈100 μM to mM), they begin to have non-specific effects at NMDA (Park-Chung et al., 1997), 5-$HT_3$ (Wetzel et al., 1998), nACh (Bullock et al., 1997), or sigma receptors (Su et al., 1998).

The focus of the present work is the NAS allopregnanolone (ALLO), the most potent endogenous positive allosteric modulator of the $GABA_A$ receptor known to date (Lambert et al., 1995). Electrophysiological studies have revealed specific mechanisms by which ALLO's actions at the $GABA_A$ receptor can affect neuronal signaling. At nM concentrations, ALLO can bind to the postsynaptic $GABA_A$ receptor, prolonging decay time and increasing the efficacy of GABA (Belelli and Herd, 2003). ALLO can also act presynaptically to increase GABA (Haage et al., 2002; Park et al., 2011) or glutamate (Kim et al., 2011) release, or extrasynaptically to increase tonic inhibition (Belelli et al., 2002; Stell et al., 2003). In addition to acting as a positive allosteric modulator, if ALLO is present at low μM concentrations, which may be achieved during parturition (Concas et al., 1999), it can also directly activate the receptor (Majewska et al., 1998).

**Steroidogenesis**

As with other NAS, ALLO is derived from cholesterol (Fig. 1.2). Cholesterol is first transported to the mitochondrial membrane by steroidogenic acute regulatory (StAR) protein where the mitochondrial benzodiazepine receptor (MBR) then transports it inside the mitochondria. Following translocation, the cytochrome P450 side chain cleavage (P450scc) enzyme converts cholesterol to pregnenolone. Outside of the mitochondria, pregnenolone is metabolized to progesterone by 3β-hydroxysteroid dehydrogenase (HSD). Progesterone is the precursor to several different steroid pathways. Progesterone can be reduced by 5α-reductase (5α-R) or 5β-R in an irreversible reaction producing 5α-dihydroprogesterone (DHP) and 5β-DHP, respectively (of note, 5α-R is thought to be the primary form of the reductase enzyme in rodents; Karavolis, 1976; Roselli and Snipes, 1984). Each of these intermediates can be metabolized by either 3α-HSD or 3β-HSD in a reversible reaction. The four products resulting from this two-step reaction therefore are 3α,5α-tetrahydroprogesterone (THP)

(ALLO), 3α,5β-THP (pregnanolone), 3β,5α-THP (epiallopregnanolone), and 3β,5β-THP (epipregnanolone). Alternative metabolism of progesterone includes conversion to androstenedione or deoxycorticosterone, and subsequent production of downstream 3α/β,5α/β-reduced NAS. Importantly, the 3α-reduced NAS are positive modulators (with ALLO being the most potent; Lambert et al., 1995). The 3β-reduced NAS are generally thought to be inactive, but because they compete for the same binding site as the 3α-NAS, they can have an antagonist property (Park-Chung et al., 1999).

Steroidogenesis, resulting in increased levels of circulating ALLO and other NAS, is dynamically increased by pregnancy (Concas et al., 1998), stressors (Barbaccia et al., 2001; Paul and Purdy, 1992; Purdy et al., 1991), and ethanol exposure. For example, ALLO levels were increased in the brain and/or plasma of rodents following ethanol injections (VanDoren et al., 2000; Barbaccia et al., 1999; Boyd et al., 2010a; Gabriel et al., 2004; Morrow et al., 1999; O'Dell et al., 2004; Porcu et al., 2010), in the brains of mice following oral ethanol consumption (Finn et al., 2004; Eva et al., 2008), and in the plasma of humans following intoxicating levels of ethanol intake (Torres and Ortega., 2003, 2004). However, there have also been examples of a lack of an increase in plasma levels of ALLO following ethanol exposure in humans, non-human primates, and mice (Holdstock et al., 2006; Porcu et al., 2010).

In the brain, the increased levels of ALLO following ethanol exposure may be due to increased peripheral levels or to *de novo* synthesis in the brain, either in neurons or glia. There is a lack of a correlation between ethanol-induced ALLO levels in the plasma and brain, suggesting independent synthesis in the brain of rats (VanDoren et al., 2000). Although plasma and cortical increases in ALLO levels (following ethanol administration) in rats appear dependent on the adrenals (Porcu et al., 2004; Follesa et al., 2006; O'Dell

et al., 2004; Cook et al., 2013), the hippocampus shows signs of *de novo* synthesis (Sanna et al., 2004; Porcu et al., 2004). For example, *in vitro* studies demonstrated an increase in ALLO levels following ethanol application to hippocampal slices (Sanna et al., 2004), and hippocampal ALLO levels were increased by ethanol in slices from adrenalectomized rats (Follesa et al., 2006), consistent with the notion that neurosteroidogenesis is induced by ethanol even in the absence of a peripheral source. Similarly, enzymatic machinery necessary for this reaction has now been documented in many limbic brain regions in the rat including the hippocampus, nucleus accumbens (NAc), and cortex (Agis-Balboa et al., 2006). Additionally, in the rat, levels of StAR protein (Boyd et al., 2010a) and StAR, P450scc, 5α-R, and 3α-HSD mRNA were increased in the cortex following ethanol exposure, providing one potential mechanism for local changes in neurosteroidogenesis (Kim et al., 2003).

**The $GABA_A$ receptor and ethanol**

Not only does ethanol increase the production of some NAS that act at the $GABA_A$ receptor, ethanol itself is thought to be a modulator of the $GABA_A$ receptor, although the mechanism (i.e., a direct binding site versus indirect actions) remains largely inconclusive (Allan et al., 1991; Harris et al., 2008; Kumar et al., 2004). The link between the $GABA_A$ receptor and ethanol is primarily supported by behavioral studies, which have shown that activation of the $GABA_A$ receptor leads to many behavioral properties similar to the behavioral effects of ethanol, such as anxiolysis, sedation, muscle-relaxation, motor incoordination, and anti-convulsant properties (e.g., Breese et al., 2006; Lüddens and Korpi, 1995). Additionally, $GABA_A$ receptor antagonists attenuate many of these ethanol-induced behaviors (Frye and Breese, 1982; Givens and Breese 1990; Martz et al., 1983), suggesting that these ethanol-induced behaviors may be, at least in part, mediated through the $GABA_A$ receptor.

In addition to mimicking and altering ethanol-like behaviors, systemic administration of $GABA_A$ receptor-acting drugs have been shown to alter ethanol intake in rodent models, further supporting a link between the $GABA_A$ receptor and ethanol. Systemic administration of $GABA_A$ receptor antagonists (or benzodiazepine site antagonists or inverse agonists) have consistently been shown to decrease ethanol consumption in rat models (Petry, 1997; June et al., 1991, 1994, 1996, 1998; Samson et al., 1987, 1989; Buczek et al., 1998; Hyytiä and Koob, 1995; Chester and Cunningham, 2002). Experiments with $GABA_A$ receptor agonists, on the other hand, have been less conclusive. For example, the $GABA_A$ receptor agonist muscimol decreased 30-minute operant self-administration of ethanol (Petry, 1997), but low doses of the benzodiazepine site agonist, chlordiazepoxide, increased 30-minute operant drinking in rats (Petry, 1997). These differences highlight the importance of using a range of doses to target specific brain regions and receptor subtypes, but in general support the idea that the $GABA_A$ receptor plays a prominent role in altering ethanol consumption.

**The $GABA_A$ receptor, ALLO, and ethanol**

Behaviorally, ALLO shares many characteristics with other $GABA_A$ receptor acting drugs, specifically ethanol, in that it produces anticonvulsant, sedative, anxiolytic, muscle relaxant, and ataxic effects (Gaisor et al., 1999; Finn et al., 1997b; Gee et al., 1988; Kokate et al., 1994). In fact, sensitivity to many of the behavioral effects of ethanol administration can be attributed to the rise in NAS levels and can be uncovered by specifically blocking steroidogenesis. In the rat, the production of ALLO (and other NAS) peaks approximately 40-80 minutes after ethanol injection (VanDoren et al., 2000), and many behavioral properties of ethanol are diminished in this time period if the animals are pretreated with enzyme inhibitors such as finasteride (FIN). FIN blocks the 5α-R enzyme (see Fig 1.2), decreasing the production of 5α-reduced NAS such as

ALLO, therefore decreasing ethanol's steroidogenic effect. For example, the ethanol-induced anticonvulsant (VanDoren et al., 2000) and antidepressant (Hirani et al., 2002) effects were prevented when rats were pretreated with FIN. It should be noted that other ethanol-induced behaviors, such as muscle relaxation and motor incoordination, were not altered by blocking steroidogenesis (Khisti et al., 2004), indicating that the production of NAS contribute to some, but not all, ethanol-induced behaviors.

In addition to the behavioral effects, electrophysiological studies have demonstrated that ethanol-induced increases in NAS are physiologically relevant to alter neuronal signaling. An *in vivo* electrophysiology study showed that an ethanol injection to rats inhibited the firing rate of medial septum diagonal band neurons, and that this effect was blocked with FIN pretreatment (VanDoren et al., 2000). Similarly, application of ethanol to hippocampal slices increased $GABA_A$ receptor-mediated mini inhibitory postsynaptic current (mIPSC) amplitude and decay time, but pretreatment with FIN blocked only the delayed, and not the initial effect of ethanol application (Sanna et al., 2004). This dissection of the time course of ethanol effects revealed a dual action of ethanol; the first being an immediate, direct effect of ethanol itself on $GABA_A$ receptor function, and the second being the delayed, indirect effect, which was dependent on the increase in levels of GABAergic NAS such as ALLO (Sanna et al., 2004).

The steroidogenic effect of ethanol appears to be important, not only to the behavioral and electrophysiological effects of ethanol, but also for the interoceptive properties of ethanol. In humans, inhibiting NAS synthesis with FIN decreased the subjective effects of ethanol (Pierucci-Lagha, et al., 2005). Although FIN decreases the production of all 5α-reduced NAS, there is evidence that ALLO may be at least one of the NAS contributing to the discriminative properties of ethanol. Systemic injections of

ALLO substituted for ethanol in drug discrimination studies in rats (Bowen et al., 1999) and non-human primates (Grant et al., 1996, 2008). Site-specific studies have revealed that ALLO infused into the NAc core, but not the CA1 region of the hippocampus, substituted for ethanol in a drug discrimination procedure in rats (Hodge et al., 2001), providing insight into at least one of the brain regions where the NAS environment may contribute to the subjective stimulus effect of ethanol. Further, in female non-human primates, ALLO substituted better for ethanol during the luteal versus follicular phase, when circulating NAS are naturally higher (Grant et al., 1997; Green et al., 1999). In conjunction, these studies suggest that the levels of circulating or exogenous NAS, such as ALLO, may impact some of the stimulus effects of ethanol.

The overlap of the behavioral, physiological, and subjective properties between ethanol and ALLO raises the question whether the production of ALLO may underlie some of ethanol's reinforcing effects. Data examining whether ALLO may have reinforcing or rewarding properties have been inconclusive. For example, both rats and mice orally self-administered ALLO in a 2-bottle choice procedure, to levels shown to produce anxiolytic effects in mice (Sinnott et al., 2002b; Finn et al., 2003), and mice showed conditioned place preference (CPP) for ALLO (Finn et al., 1997a). Additionally, monkeys intravenously self-administered pregnanolone (Rowlett et al.,1999), the 5$\beta$-isomer of ALLO with similar pharmacological properties as ALLO (Engel et al., 2001; Shannon et al., 2005a, 2005b). However, rats would not orally self-administer ALLO in an operant procedure and there has been a report of conditioned place aversion (CPA) following intracerebroventricular (ICV) infusions of high ALLO doses in rats, which could reflect either species or dose differences (Sinnott et al., 2002b; Beauchamp et al., 2000). Further, neither ALLO nor FIN altered ethanol-induced CPP in DBA/2J mice (Gabriel et

al., 2004; Murphy et al., 2006), complicating the understanding of how steroidogenesis alters the rewarding or reinforcing values of ethanol.

**ALLO and ethanol intake**

Studies examining the effect of exogenous ALLO on ethanol consumption have generally demonstrated a biphasic effect, with low doses of ALLO increasing ethanol intake and high doses of ALLO decreasing ethanol intake. In C57BL/6J male mice with a 2-hour 2-bottle choice between an ethanol solution or water, a physiological dose of ALLO (3.2 mg/kg ≈ 34 ng/ml plasma concentration) increased ethanol intake, whereas a supraphysiological dose of ALLO (24 mg/kg ) decreased ethanol intake. However, when examining the number of licks on the ethanol sipper across each 20-minute bin in the 2 hour session, an effect of intermediate ALLO doses was also uncovered during the first 80 minutes of the session (Ford et al., 2005b). This experiment highlighted the benefit of using lickometers to examine, on a finer time-scale, the time points during the session in which ALLO was having its effect. Further, in an operant self-administration procedure, ICV infusions of ALLO produced a biphasic effect that was limited to the first 10 minutes of the 30 minute session (Ford et al., 2007a). Similar effects have been uncovered in rats trained to lever press for access to ethanol, where a biphasic effect of ALLO on ethanol reinforced lever pressing was observed throughout the 30-minute session but was most pronounced during the first run (series of consecutive responses; Janak et al., 1998). Finally, in both rats and mice, ALLO led to reinstatement of ethanol seeking as measured by non-reinforced pressing on a lever previously contingent with receiving access to an oral ethanol solution (Finn et al., 2008; Nie and Janak, 2003). As a whole, these studies highlight an effect of ALLO to influence ethanol seeking and consumption, while they also underscore the transient nature of the effects.

The transient effect of ALLO is in part attributable to its relatively short half-life, which is reported to be approximately 30 minutes in mice and 4 hours in humans (Mellon et al., 1990; Timby et al., 2006). The short half-life is primarily due to the vulnerability of ALLO to oxidation by 3α-HSD back to its immediate precursor 5$\alpha$-DHP (Fig. 1.2), which has no action at $GABA_A$ receptors but acts at intracellular progesterone receptors (Rupprecht et al., 1993). The short half-life of ALLO is also influenced by the rapid metabolism of ALLO via conjugation, for example, by hydroxylation, glucuronidation, or sulfation, to more polar molecules that are less active (or have opposite activity) and can be more easily excreted by the body (Havlíková et al., 2006; Johannson et al., 2002; Park-Chung et al., 1999; Garzón et al., 1989). Additionally, a transient effect of ALLO may be due in part to desensitization of the $GABA_A$ receptor, which could lead to a decreased response of the $GABA_A$ receptor in the continued presence of ALLO and GABA (Bright et al., 2011).

The present studies circumvent some of the transient nature of ALLO's effects by using ganaxolone (GAN), a synthetic analog of ALLO, which is structurally identical with the exception of an added 3β-methyl group (Figure 1.3). This additional methyl group makes GAN resistant to oxidation by 3α-HSD and enhances the half-life by about 3 to 4-fold (Reddy et al., 2000), while maintaining the primary pharmacological and behavioral profile of the NAS (Carter et al., 1997; Ungard et al., 2000). Studies examining how GAN alters ethanol intake have been scarce, with just one study in rats showing that systemic GAN led to a biphasic effect on operant lever pressing for access to an ethanol solution (Besheer et al., 2010). Despite the paucity of research examining how GAN may alter ethanol intake, GAN has been used for over a decade in epilepsy research and is currently in Phase II clinical trials for use as an antiepileptic. The present studies

aim to contribute to a better understanding of how this longer-acting synthetic GABAergic NAS will affect ethanol seeking and consumption in a variety of procedures.

**Extrasynaptic $GABA_A$ receptors: ethanol and ALLO**

The search for a binding site for ethanol on the $GABA_A$ receptor has been largely inconclusive. Studies showing that single or double amino acid mutations in $GABA_A$ receptor subunits ($\alpha_1$, $\beta_1$, and $\rho$) are sufficient to diminish or abolish ethanol potentiation of the receptor suggest that there is a binding pocket for ethanol within the $GABA_A$ receptor (Wick et al., 1998; Mihic et al., 1997; Harris et al., 2008). However, the specific subunit that ethanol binds to in $\alpha\beta\gamma$ or $\alpha_{4/6}\beta\delta$ $GABA_A$ receptors is still controversial, and the affinity that certain subunits (or combinations of subunits) of the $GABA_A$ receptor have for ethanol has been a topic of intense debate in the field (Lovinger and Homanics, 2007). *In vitro* evidence from oocytes expressing recombinant receptors has shown that δ subunit-containing $GABA_A$ receptors exhibit potentiation of the $GABA_A$ receptor with as little as 1-3 mM ethanol. This is in contrast to non-δ containing $GABA_A$ receptors, which require up to 100-300 mM of ethanol to show potentiation of $GABA_A$ receptor function (Mody et al., 2007; Olsen et al. 2007; Santhakumar et al., 2007). Given that 17 mM ethanol corresponds to a blood ethanol concentration (BEC) of 80 mg/dl, considered a legally intoxicated level in humans, these results indicate that δ subunit-containing $GABA_A$ receptors might be an important *physiological* target for ethanol. Unfortunately other laboratories have been unsuccessful at replicating some of the above findings (Borghese et al., 2007; Korpi et al., 2007), complicating the understanding of how ethanol acts at extrasynaptic $GABA_A$ receptors.

Data with δ subunit knockout mice have contributed to the understanding of the role that the δ subunit plays in mediating the behavioral effects of ethanol. Delta-subunit

knockout mice self-administered less ethanol than their wild-type counterparts, without differing in quinine or saccharin consumption. The knockout mice also showed a reduced ethanol-induced anticonvulsant effect and a lower ethanol withdrawal effect following chronic exposure (measured with handling-induced convulsions), when compared to their wild-type littermates (Mihalek et al., 2001). However, there was no change in the anxiolytic, hypothermic, or acute tolerance of ethanol in knockout versus wild-type mice, and the knockout mice could be trained to discriminate for ethanol versus saline in drug discrimination studies (Shannon et al., 2004). Together these studies suggest that the δ subunit is important in mediating some, but not all, of the behavioral effects of ethanol.

In addition to differing in ethanol-induced behaviors, δ subunit knockout mice also showed altered NAS sensitivity. The knockout mice had a reduced anticonvulsant and anxiolytic effect of GAN, as well as a reduced sedative effect to other positive GABAergic NAS (pregnanolone and alphaxalone) (Mihalek et al., 1999). However, it should be noted that data with δ subunit mice can be hard to interpret in light of compensatory changes in expression of other subunits. Normally in the forebrain, the δ subunit is paired with the $\alpha_4$ subunit extrasynaptically (Sur et al., 1999). Consequently, in the δ subunit knockout mouse, the $\alpha_4$ subunit was also down-regulated across many brain regions including the thalamus, cortex, hippocampus, and caudate-putamen (Peng et al., 2002). There was also an upregulation in $\gamma_2$ protein across these regions, as well as an upregulation in $\alpha_1$ subunit expression in the caudate-putamen (Peng et al., 2002). In the cerebellum, the δ subunit pairs with the $\alpha_6$ subunit extrasynaptically (Quirk et al., 1994). Although total $\alpha_6$ subunit expression was unchanged in the knockout mice, there was an increase in co-assembly of the $\alpha_6$ subunit with $\gamma_2$ and $\beta_3$, both of which were upregulated in the knockouts (Tretter et al., 2001). The cellular result of an increase in

$\alpha_6 \gamma_2 \beta_3$ receptors was a faster decay time of mIPSCs, resulting in a net decrease in GABAergic inhibition in the cerebellum (Spigelman et al., 2002). In light of these changes, it is difficult to know whether alterations in ethanol and NAS responses in the δ subunit knockout mice are due to the decreases in the δ subunit or due to the corresponding changes in the expression of other subunits.

Despite confounds in drawing conclusions from the knockout mice, the enhanced sensitivity of the δ subunit to NAS is supported by *in vitro* studies. In oocytes expressing human recombinant $GABA_A$ receptors, electrophysiological recordings have shown that the δ subunit-containing $GABA_A$ receptors exhibited greater sensitivity in the ability of ALLO to potentiate the GABA response, compared to non-δ subunit-containing $GABA_A$ receptors (Belelli et al., 2002; Brown et al., 2002).

Given that the δ subunit may be an important target for NAS and ethanol, it remains to be examined whether extrasynaptic $GABA_A$ receptors represent a target by which NAS such as ALLO or GAN may alter the effects of ethanol. Unfortunately, studies specifically examining whether ALLO or GAN acts at extrasynaptic receptors as a mechanism to alter ethanol intake are lacking. Currently, there is no pharmacological tool to selectively block extrasynaptic $GABA_A$ receptors. Therefore, I examined the contribution of extrasynaptic $GABA_A$ receptors to ethanol consumption and seeking by examining the effects of an extrasynaptic $GABA_A$ receptor agonist across various procedures that model aspects of the appetitive (i.e., seeking) and consummatory phases of ethanol self-administration. This was done using THIP (gaboxadol), a $GABA_A$ receptor agonist that binds at the GABA site and, when present in nM to low μM concentrations, selectively acts at δ subunit-containing $GABA_A$ receptors (Stórustovu and Ebert, 2006). I reasoned that overlapping or divergent effects between GAN and

THIP across parameters of ethanol intake or seeking could provide indirect insights into the contribution of GAN's actions at synaptic versus extrasynaptic mechanisms on particular ethanol consumption behaviors.

The effect of THIP on ethanol intake had been examined in four previous studies in rats and mice. Work in rats showed that THIP increased the acquisition (Boyle et al., 1992; Smith et al., 1992) and maintenance (Boyle et al., 1993) of 24-hour ethanol intake, primarily via increases in bout size, frequency, and duration. However, these studies used a high dose of THIP (16 mg/kg). Based on a study showing that 15 mg/kg THIP led to similar behavioral effects in both wild-type and $\alpha_4$ knockout mice (most commonly extrasynaptic and paired with the δ subunit) (Chandra et al., 2006), both synaptic and extrasynaptic receptors were likely activated at that dose. On the other hand, a study in mice using doses from 0-16 mg/kg THIP found that 8 mg/kg and 16 mg/kg THIP decreased ethanol intake in the drinking in the dark procedure (Moore et al., 2007). Given that 10 mg/kg THIP alters behaviors in wild-type but not $\alpha_4$ knockout mice (Chandra et al., 2006) and led to CNS concentrations in the low µM range (Cremers and Ebert, 2007), which was selective for extrasynaptic $GABA_A$ receptors *in vitro* (Stórustovu and Ebert, 2006), it seems plausible that doses under 10 mg/kg THIP generate concentrations of THIP in the brain that would be selective for extrasynaptic receptors. The second primary aim of the present experiments was therefore to extend findings with THIP to other procedures (continuous access, limited-access, and operant self-administration), and to use a range of doses that would include selective and non-selective doses for extrasynaptic $GABA_A$ receptors.

**Region-specific $GABA_A$ receptor manipulations and ethanol intake**

In an attempt to elucidate the contradictory findings following the administration of systemic $GABA_A$ receptor agonists and antagonists on ethanol intake, site specific infusions into various regions of the limbic circuitry have sought to examine the contribution of specific brain regions to ethanol intake. However, as with the systemic studies, most region-specific studies have reported a decrease in ethanol intake regardless of whether the $GABA_A$ receptor is activated or inhibited. For example, a $GABA_A$ receptor antagonist (SR 95531) infused into the central nucleus of the amygdala (Hyytiä and Koob, 1995), a benzodiazepine inverse agonist (RY023) infused into the CA1 or CA3 region of the hippocampus (June et al,. 2001), and a $GABA_A$ receptor agonist (muscimol) infused into the prefrontal cortex (PFC) (Samson et al., 2001) all have been reported to decrease ethanol intake across different procedures in rats.

In the ventral tegmental area (VTA), a non-competitive $GABA_A$ receptor antagonist (pictrotoxin) decreased ethanol consumption in rats (Nowak et al., 1998). Interestingly, $GABA_A$ receptor antagonists (picrotoxin and bicuculline) infused into the VTA have been shown to increase dopamine levels in the NAc (Ikemoto et al., 1997; Westerink et al., 1996). Therefore, one interpretation of the data was that the increase in VTA dopamine firing with picrotoxin led to a left-ward shift in the dose-response curve, thereby decreasing the ethanol intake necessary to yield a similar CNS effect (Nowak et al., 1998). A VTA infusion of a $GABA_A$ receptor agonist (muscimol), on the other hand, did not alter overall intake, but prolonged the time until the rats terminated their consumption (Hodge et al., 1996). Contradictingly, muscimol infused into the VTA had also been shown to increase dopamine levels in the NAc (Kalivas et al., 1990), complicating the understanding of how the VTA to NAc projections ultimately alter ethanol intake and what the underlying mechanism might be.

In the NAc, a $GABA_A$ receptor agonist (muscimol), antagonist (bicuculline), and benzodiazepine inverse agonist have all been shown to decrease ethanol intake in rats (Hodge et al., 1995; June et al., 1998). However, analysis of the pattern of intake between the direct agonist and antagonist revealed that they altered intake by divergent methods; whereas the agonist led to early termination of the operant session, the antagonist led to a non-significant decrease in response rate throughout the session (Hodge et al., 1995). Although the mechanisms leading to these different measures (early termination versus maintenance) remain unknown, this experiment highlights the importance of examining patterns of intake in order to elucidate seemingly paradoxical findings. Although somewhat unsatisfactory in providing a clear mechanism or circuitry, in total, these studies highlight the contribution of the $GABA_A$ receptor in the NAc and other limbic brain regions in mediating ethanol self-administration.

**Reward Circuitry: NAc**

As evidenced above, there are likely many brain regions in which the $GABA_A$ receptor contributes to ethanol consumption. In fact, a CA1 infusion of ALLO decreased ethanol intake in rats (Martin-Garcia, 2007). I chose to focus on the NAc for a number of reasons. In addition to evidence that the $GABA_A$ receptor in the NAc may regulate ethanol consumption, a $GABA_A$ receptor agonist (muscimol) infused locally in the NAc substituted for ethanol in a drug discrimination procedure in rats (Hodge et al., 1996), indicating that $GABA_A$ receptor activation in the NAc may be important for perceiving some of the subjective stimulus effects of ethanol. Further, ALLO infused into the NAc substituted for the discriminative effects of ethanol in rats (Hodge et al., 2001), providing rationale that the NAS environment in this brain region may be an important component for perceiving the stimulus effects of ethanol. Immunohistochemistry showed that ALLO was endogenously located in the NAc in rats (Saalman et al., 2007; Cook et al., 2013)

and both 5α-R and 3α-HSD colocalized in the NAc with glutamic acid decarboxylase positive neurons (GAD; a marker for GABA; Agis-Balboa et al., 2006), indicating that machinery necessary for *de novo* synthesis is also present in the NAc GABAergic neurons.

Tracing studies have enhanced our understanding of the major afferents and efferents of the NAc. However, most of these studies have been performed in rats, and precisely how this information extrapolates to mice or humans remains to be examined. Data from the rat studies have revealed that the NAc is a major part of the reward circuitry which links together limbic and motor functions. It receives dopaminergic afferents from the VTA and glutamatergic afferents from the PFC, hippocampus, thalamus, and amygdala, allowing it to integrate motivational, executive, contextual, and affective information. Minor inputs also include GABAergic and glutamatergic projections from the VTA, GABAergic projections from the ventral pallidum, serotonergic projections from the median raphe nucleus, and noradrenergic projections from the nucleus of the solitary tract and locus coeruleus (Groenewegen et al., 1993, 1999; Heimer, 1991; Heinz et al., 2009).

GABAergic medium spiny neurons (MSNs), named for their medium soma size (5-20 μm) and spiny dendrites, make up about 90-95% of the neurons in the NAc (Kawaguchi et al., 1995). The MSNs in the NAc are projection neurons that are generally divided into two distinct classes, depending on where they project to in the brain (Figure 1.4). The first group of neurons projects primarily back to the midbrain via a direct route and are therefore named the “direct” pathway (Gerfen, 1990). These GABAergic cells colocalize with dynorphin and substance P and contain the dopamine receptor type 1 (D1 receptor) (Gerfen, 1990; Hubert and Kuhar, 2006). D1 receptors are

$G_s$-linked (excitatory) (Jose et al., 1995), and therefore dopamine release onto these cells increases excitatory postsynaptic currents (EPSCs), activating the cell (André et al., 2010). The second main class of MSNs projects via the ventral pallidum and subthalamic nuclei and then back to the midbrain, therefore making up the "indirect" pathway (Gerfen, 1990). The ventral pallidal cells also project via the thalamus to cortical areas, comprising a cortico-striato-pallido-thalamo-cortical loop (Le Moine and Bloch, 1995; Gangarossa et al., 2013; see Figure 1.4). These GABAergic cells colocalize with enkephalin and contain the dopamine receptor type 2 (D2 receptor) (Gerfen, 1990). Importantly, the D2 receptors are $G_{i/o}$-linked (inhibitory) (Jose et al., 1995) and therefore dopamine acting at these cells decreases the firing of those cells (André et al., 2010). The populations of D1 receptor- versus D2 receptor-containing cells are thought to be mostly divergent in terms of their projections, but there may also be cells (up to 17% in the shell) containing both D1 and D2 receptors that could belong to either pathway (Bertran-Gonzales et al., 2008).

In addition to the 2 populations of projection cells of the NAc, there are also at least 5 classes of aspiny interneurons: cholinergic, GABAergic/neuropeptide Y (NPY), GABAergic /somatostatin, GABAergic /calretinin, and GABAergic /parvalbumin (Kawaguchi et al., 1995). The cholinergic cells, which have a larger soma (20-60 μm), large dendritic trees, and are aspiny, contain D2 receptors (Wang, et al., 2006). The GABAergic interneurons are medium in size (~7-35 μm), aspiny (Kawaguchi, 1995), and are thought to be absent of D1 or D2 receptors (Bertran-Gonzalez et al., 2004). The GABAergic interneurons primarily synapse onto the soma of the MSNs and are important for controlling local inhibition (Smith and Bolam, 1990).

Histological and biochemical studies have contributed to further division of the NAc into the shell and core. The shell maintains more reciprocal connections with limbic regions such as the extended amygdala (a collection of forebrain subnuclei comprised of the centromedial nucleus of the amygdala, subpallidal regions, the bed nucleus of the striata terminalis, as well as the most posterior region of the NAc shell (Alheid and Heimer, 1988)). On the other hand, the core maintains projections with motor related regions such as the dorsal striatum (Usuda et al., 1998). Although there are no afferents exclusive to the shell versus the core, the inputs to the NAc maintain a general medial-lateral, dorsal-ventral, and rostral-caudal topography. For example, more ventral regions of the cortex and hippocampus project to the shell versus the more dorsal regions of the cortex and hippocampus project to the core (Angulo and McEwen et al., 1994). Caudal portions of the amygdala project to the shell versus more rostral portions of the amygdala project to the core. Additionally, medial VTA projects primarily to the shell, whereas the lateral VTA projects primarily to the core (Groenewegen et al., 1993, 1999; Heimer 1997).

Similar to the inputs, the outputs of the NAc are also heavily topographically organized. For example, the core sends projections to dorsolateral portions of the ventral pallidum, whereas the shell sends projections to the medial regions of the ventral pallidum, and these regions go on to have distinct roles and projections. Although both the shell and core receive input from the VTA, the shell primarily sends projections back to the VTA as well as to the substantia nigra compacta, whereas the core projects back to the midbrain more laterally (to the substantia nigra reticulata). This creates spiraling feedback loops, allowing limbic structures to influence motor structures (Seasack and Grace, 2010). The shell is also unique from the core in that it sends projections to the lateral preoptic area, extended amygdala, and lateral hypothalamus, which may

contribute to some of the divergent functions between the shell and core (Heimer et al., 1991).

The shell region of the NAc may be particularly important for ethanol reinforcement. A $GABA_A$ receptor agonist targeted into the shell decreased ethanol intake in rats (Stratford and Wirthshafter, 2011) and rats self-administered ethanol into the shell but not the core (Engelmann et al., 2009). There has been a further division of the shell into lateral, intermediate, and medial regions (Groenewegen et al., 1999). Electrolytic lesions of the medial shell decreased limited-access ethanol, but not sucrose, intake in rats (Dhaher et al., 2009).

Further clarification suggests that extrasynaptic $GABA_A$ receptors may play a unique role in the contributions of the medial shell to the reinforcing effects of ethanol. Indeed, δ subunit immunohistochemical staining is seen throughout the NAc in both rats (Pirker et al., 2000) and mice (Horntägl et al., 2013), and immunohistochemistry studies in C57BL/6J mice have shown that the δ subunit colocalizes with both D1 and D2 receptor-containing MSNs, as well as choline acetyltransferase, somatostatin, and NPY positive interneurons in the NAc (Maguire et al., 2014). However, viral-mediated knockdown of the $\alpha_4$ (predominately paired with δ) or the δ subunit in the NAc shell, but not NAc core, decreased ethanol consumption and preference in rats (Nie et al., 2011; Rewal et al., 2009, 2011), and this effect was specific to the medial dorsal region of the shell, as δ subunit knockdown in the lateral or intermediate shell had no effect (Nie et al., 2011). Some of this divergence likely is due to distinctions in inputs and outputs. Aside from topographical distinctions within this region, the medial portion of the shell sends outputs to the lateral hypothalamus, which is known to be involved in consummatory behaviors (Groenewegen et al, 1999; Maldonado-Irizarry et al., 1995; Wayner et al.,

1971; Atrens, et al., 1983). Together these studies suggest that the medial shell of the NAc, and in particular the extrasynaptic $GABA_A$ receptors there, might be an important target for mediating the reinforcing effects of ethanol.

**Goals of the Dissertation**

The present studies used GAN, a synthetic GABAergic NAS, and THIP, a preferential extrasynaptic $GABA_A$ receptor agonist, to elucidate some of the behavioral mechanisms associated with ethanol seeking and consumption. AUDs represent a group of extremely heterogeneous disorders and therefore no single model can capture the totality of the disease. For all studies used in the dissertation, male C57BL/6J mice were utilized. An inbred strain is advantageous to decrease variability resulting from genetic heterogeneity, and specifically, C57BL/6J mice self-administer greater amounts of ethanol than other inbred strains (Yoneyama et al., 2008). Although limitations are discussed in Chapter 6, male mice were chosen based on previous studies showing that male mice were more sensitive than female mice to manipulations of ALLO on altering ethanol intake (Ford et al., 2008a; Finn et al., 2010).

The first goal was to use multiple ethanol intake procedures in mice to examine the manner in which GAN alters ethanol seeking and patterns of ethanol consumption. I hypothesized that GAN would alter parameters of ethanol intake across a variety of procedures, which would provide support for a role of the NAS environment in contributing to ethanol intake. The second goal was to examine the contribution of extrasynaptic $GABA_A$ receptors on these measures with the use of THIP. I also hypothesized that THIP would alter ethanol intake across procedures, demonstrating that activation of extrasynaptic $GABA_A$ receptors is an important component in mediating appetitive and consummatory aspects of ethanol intake.

In Chapter 2, a continuous-access 2-bottle choice procedure aimed to examine time-related effects of systemic injections of GAN, FIN, and THIP on ethanol intake. The continuous-access procedure provides face validity to the human condition in that the ethanol is always available to the animal, as is likely the case for the human alcoholic population, where alcohol is widely accessible. A benefit to this procedure was the use of lickometers, which count the number of licks the mice make on each sipper on a sub-second time scale. By examining pattern of licks, we could examine not only the overall intake, but also the pattern of intake over 24-hours, as well as any shift in patterns as a result of the drug treatment. The goal was to examine intake and hourly patterns for a NAS positive allosteric modulator (GAN), and to compare any similarities and differences with an agonist with preference for extrasynaptic receptors (THIP) and with a 5α-R inhibitor (FIN; used to decrease endogenous GABAergic NAS levels).

Because Chapter 2 revealed that the drug effects occurred primarily during the first hours of the session, and hourly analysis revealed interesting divergent effects of GAN and THIP during this time, the goal of Chapter 3 was to examine the effects of these two drugs in a limited-access procedure. The limited-access session is advantageous because mice have been shown to self-administer high amounts of intake in a short time period, hence achieving BECs that meet or exceed the definition of a binge level of intoxication, set at 80 mg/dl by NIAAA. This procedure also allowed for a direct comparison to published effects with ALLO (Ford et al., 2005b). In addition to examining the hourly intakes as in Chapter 2, we also examined bout parameters. A bout has been operationally defined in the Finn laboratory as at least 20 licks with no more than 60 seconds separating successive licks (Ford et al., 2005b, 2008a). Similar to examining licks over time, bout analysis is a supplementary approach to detect subtle changes in patterns of intake. Importantly, based on human data, patterns of intake may

be a better predictor of the efficacy of a drug treatment than considering only overall intake (Anton et al., 2004). Additionally, as evidenced by studies reporting bout changes which I referred to above (e.g. Hodge et al., 1995), this analysis can uncover differences in intake following drug treatment that would not be evident solely based on examining overall intake.

As another approach to examine the effects of GAN and THIP on ethanol intake, studies in Chapter 3 also examined the effects of systemic injections of these drugs in an operant procedure. The operant procedure allowed us to measure the amount of lever pressing performed in order to gain access to the ethanol solution. In these experiments, mice were trained to lever press using a fixed ratio (FR) requirement, where mice were allowed a brief (30 second) access to the ethanol solution and a stimulus light each time a set number of lever presses was made. The FR schedule is advantageous in that it allows for multiple pairings of the lever pressing with the ethanol solution and any conditioned cues (stimulus light) per session. Although this procedure is advantageous for maintaining relatively high rates of responding, a disadvantage is that the mice were observed to drink from the sipper only about 50% of the time that they actually had access (Ford et al., 2007b). This schedule also is confounded by the fact that after the first access to ethanol in the session, subsequent pressing may be influenced by any stimulant or sedative effects of the ingested ethanol. For these reasons, the effects of GAN and THIP were tested after the mice were switched to a response requirement (RR) schedule. The RR schedule requires the animal to complete the appropriate number of lever presses up front, followed by 30 minutes of continuous access to the solution. In this way, it is possible to temporally separate out the appetitive phase, measured by the number of lever presses or latency to complete the lever pressing requirement, and the consummatory phase, measured by the total intake

(Samson et al., 1998). Importantly, data from the Finn laboratory has found that mice self-administered approximately twice as much ethanol on an RR versus FR schedule (Ford et al., 2007b), and use of lickometers again allowed for an examination of bout parameters. While the previous experiments assessed the role of GAN and THIP on ethanol intake, the goal of these studies was to elucidate how these drugs influenced the seeking and consummatory components of self-administration. Additionally, to examine the specificity of these effects on ethanol seeking and consumption, a separate group of mice was used to test the effects of GAN and THIP on sucrose seeking and subsequent intake. My prediction was that GAN and THIP would alter both appetitive and consummatory aspects of self-administration, and that I would see similar effects between the two drugs. Based on previous studies showing that ALLO also increased sucrose seeking (Finn et al., 2008), I predicted that there would be non-specific effects of these drugs on sucrose seeking and intake as well.

To expand on the first part of Chapter 3, Chapter 4 attempted to elucidate a brain region that might be important for the systemic effects of GAN and THIP that were observed in the limited-access two-bottle choice procedure. Experiments in Chapter 4 utilized microinfusions of the drugs into the NAc shell in order to determine whether activation of $GABA_A$ receptors in that brain region were sufficient to account for the drugs' effects on ethanol intake observed following systemic injections in Chapter 3. My hypothesis was that activation of $GABA_A$ receptors in the NAc shell would be sufficient to account for the decreases in ethanol intake following systemic injection of GAN and THIP.

To expand on the differences observed between GAN and THIP on ethanol-reinforced responding in Chapter 3, Chapter 5 measured the effect of GAN and THIP on ethanol seeking using a reinstatement procedure. Importantly, the ethanol seeking in

this procedure differed from that in Chapter 3 in that it was measured in the absence of the reinforcer, and was measured following a period of ethanol abstinence. The mice were originally trained to lever press for access to an ethanol solution, similar to that in Chapter 3. Mice then underwent extinction, where lever pressing no longer led to ethanol access or any other scheduled outcome. Finally, ethanol seeking during the reinstatement session was assessed by measuring the number of lever presses the mice performed following injections of GAN or THIP. I predicted that similar to ALLO, GAN would reinstate ethanol seeking. The goal with THIP was to test whether preferential activation of extrasynaptic $GABA_A$ receptors was sufficient to reinstate the lever pressing behavior. Because data from Chapter 3 suggested that THIP may decrease ethanol seeking, I predicted that THIP would not promote reinstatement of ethanol seeking.

In total, these experiments attempted to define a role for the NAS analog GAN to alter ethanol intake across procedures and to demonstrate an effect on both ethanol consummatory and seeking behaviors. These studies also attempted to elucidate the contribution of extrasynaptic $GABA_A$ receptors to different measures of ethanol seeking and consumption. Finally, I aimed to demonstrate whether the NAc shell might be an important neuroanatomical substrate in some of these effects, with the goal of increasing the understanding of a possible mechanism and brain area by which NAS or extrasynaptic $GABA_A$ receptors may alter ethanol intake.

**Figure 1.1. The pentameric $GABA_A$ receptor showing binding sites of $GABA_A$ receptor-acting molecules at a synaptic $GABA_A$ receptor**

A typical synaptic $GABA_A$ receptor is made up 2α:2β:γ subunits. Benzodiazepines bind to the interface of the α and γ subunit at the synaptic $GABA_A$ receptor. In about 5% of receptors, the γ is replaced with a δ subunit, which limits the channel exclusively to the extrasynaptic space. In either class of receptor, GABA binds to the interface of the α and β subunit, barbiturates bind to the β subunit, and neuroactive steroids bind to the α subunit.

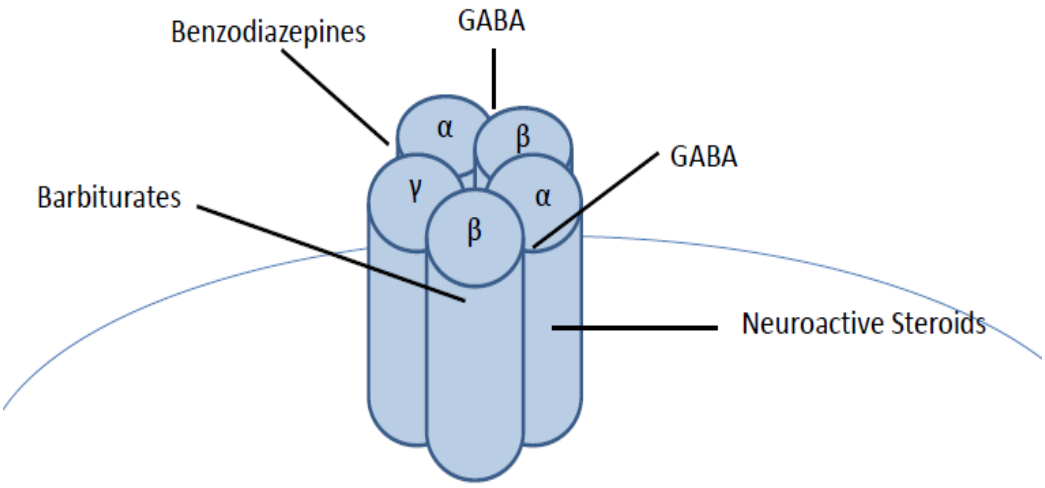


**Figure 1.2. Steroidogenesis pathway**

Multiple steroidogenesis pathways are shown, with the pathway by which cholesterol can be metabolized to allopregnanolone or its 3α/β,5α/β isomers outlined in red. DHP = dihydroprogesterone; DHT = dihydrotestosterone; THDOC = tetrahydroxydeoxycorticosterone; DHDOC = dihydroxydeoxycorticosterone; HSD=hydroxysteroid dehydrogenase; DHEA = dehydroepiandrosterone; A = androstanedione. StAR protein = steroidogenic acute regulatory protein; MBR = mitochondrial benzodiazepine receptor. Adapted from Girdler et al., 2012.

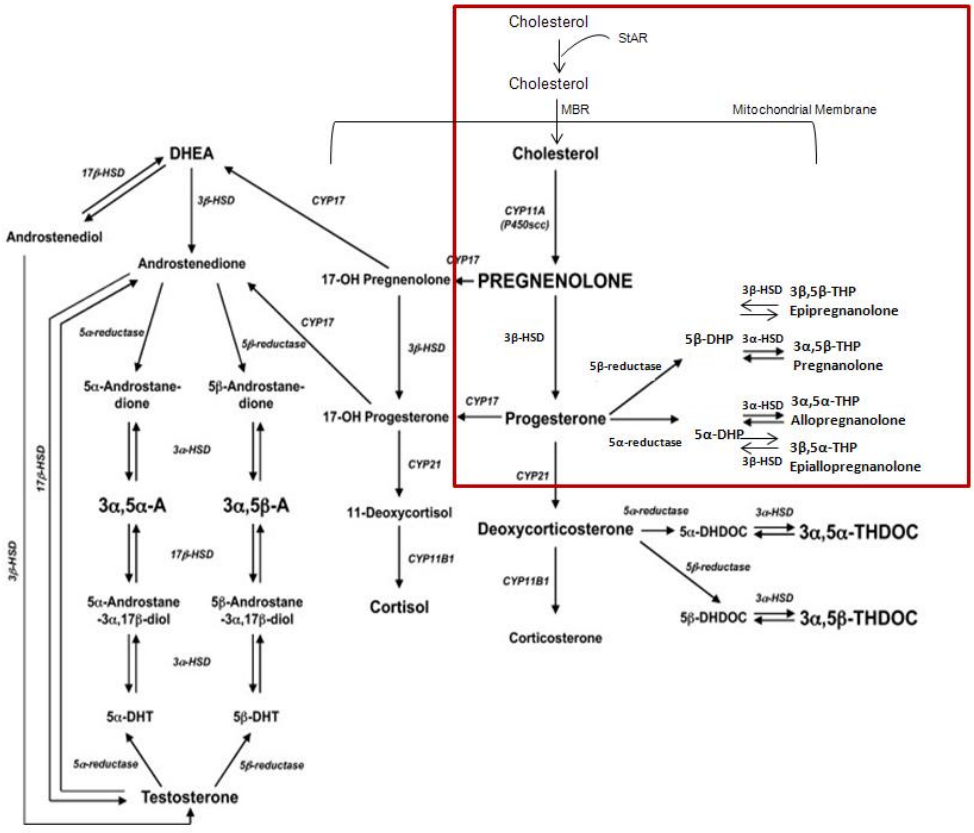
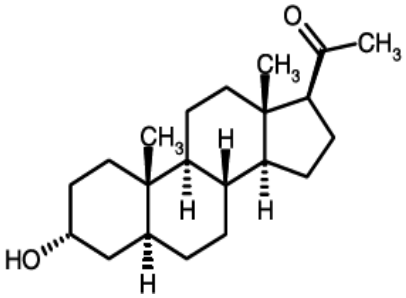


**Figure 1.3. Molecular structure of allopregnanolone (ALLO) and ganaxolone (GAN)**

The molecular structure for ALLO is shown in A. The molecules are structurally identical with the exception of a 3β-methyl group present on GAN, shown in B.

A) ALLO




B) GAN

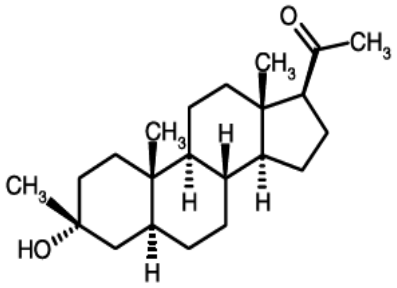
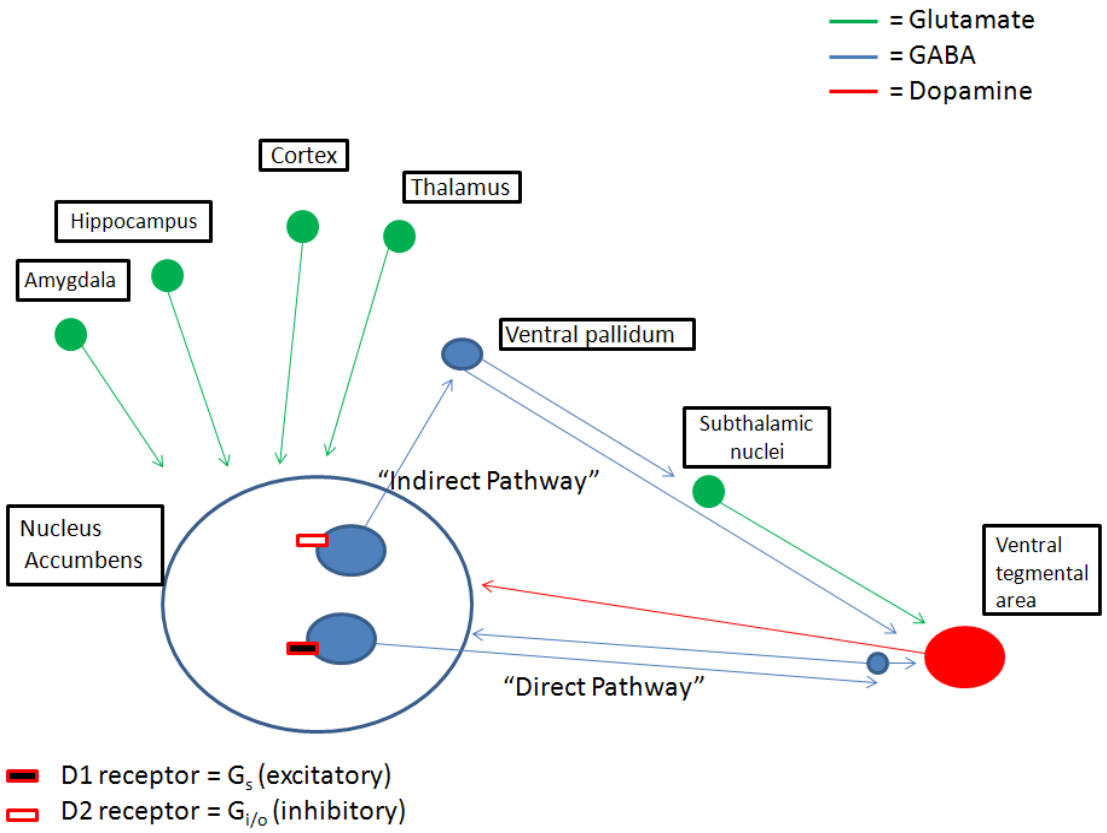


**Figure 1.4. Simplified reward circuitry showing the major NAc inputs and outputs**

The figure shows the four major glutamatergic inputs from the amygdala, thalamus, hippocampus, and cortex, as well as dopaminergic and GABAergic input from the VTA. The major outputs are also shown. The D1 receptor-containing GABAergic cells project primarily back to the ventral tegmental area and are termed the "direct" pathway. The D2 receptor-containing GABAergic cells project to the ventral pallidum, which then project back to the VTA (either monosynaptically or via the subthalamic nuclei) to make up the "indirect" pathway.



# CHAPTER 2: ALTERATION OF 24-HOUR ETHANOL DRINKING IN MICE VIA MODULATION OF THE $GABA_A$ RECEPTOR WITH GANAXOLONE, FINASTERIDE, AND THIP

This chapter has been reformatted for inclusion in this dissertation from:

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**Abstract**

Background: NAS and other $GABA_A$ receptor modulating compounds have been shown to alter ethanol intake, although their mechanisms of action remain unclear. The present study examined how patterns of 24-hour ethanol drinking in mice were altered with the synthetic GABAergic NAS (GAN), with an inhibitor of NAS synthesis (FIN), or with a $GABA_A$ receptor agonist with some selectivity at extrasynaptic receptors (THIP).

Methods: Male C57BL/6J mice had continuous access to a 10% v/v ethanol solution (10E) or water. Using lickometer chambers, drinking patterns were analyzed among mice treated in succession with GAN (0, 5, 10 mg/kg), FIN (0 or 100 mg/kg), and THIP (0, 2, 4, 8, 16 mg/kg).

Results: GAN shifted drinking in a similar but extended manner to previous reports using low doses of the NAS ALLO; drinking was increased in hour 1, decreased in hours 2 and 3, and increased in hours 4 and 5 post-injection. THIP (8 mg/kg) and FIN both decreased 10E drinking during the first 5 hours post-injection by 30% and 53%, respectively, while having no effect on or increasing water drinking, respectively. All 3 drugs altered initiation of drinking sessions in a dose-dependent fashion. FIN increased and GAN decreased time to first lick and first bout. THIP (8 mg/kg) decreased time to first lick but increased time to first bout and attenuated first bout size.

Conclusions: The present findings support a role for the modulation of ethanol intake by NAS and $GABA_A$ receptor acting compounds and provide insights as to how drinking patterns are shifted. The ability of THIP to alter 10E drinking suggests that extrasynaptic $GABA_A$ receptors may be involved in the modulation of ethanol intake. Further, the consistent results with THIP to that seen previously with high doses of ALLO suggest

that future studies should further examine the relationship between NAS and extrasynaptic $GABA_A$ receptors, which could provide a better understanding of the mechanism by which NAS influence ethanol intake.

**Introduction**

Ethanol has been shown to act on many different receptor types, one of which is the $GABA_A$ receptor (Deitrich et al., 1989; Grobin et al., 1998; Kumar et al., 2009). The $GABA_A$ receptor is a ligand-gated inhibitory receptor, composed of 5 subunits. The specific combination of subunits, together with the sub-cellular localization of the receptor, give it a unique physiology and pharmacology (Mody and Pearce, 2004). Ethanol has a dual pharmacological action on $GABA_A$ receptors; the first is a rapid and direct positive modulation of receptor activity, and the second is an indirect, delayed, positive modulation of the receptor that electrophysiological studies suggest is due to the production of the NAS, ALLO following ethanol exposure (Sanna et al., 2004). Supporting this hypothesis, previous studies have shown that ethanol consumption leads to physiologically relevant increases in ALLO levels in the brains of C57BL/6J male mice (Finn et al., 2004). ALLO is one of the most potent endogenous positive modulators known for $GABA_A$ receptors (Lambert et al., 1995), and it shares physiological characteristics with ethanol in that it has anesthetic, anticonvulsant, sedative-hypnotic, anxiolytic, locomotor-stimulant, and muscle-relaxant properties (Finn et al., 1997b; Gasior et al., 1999). Therefore, clarifying the relationship between NAS and alcohol could be vital to a better understanding of alcohol's mechanism of action.

Physiologically-relevant doses of exogenous ALLO have been shown to dose-dependently increase ethanol drinking in rodents, and mice show conditioned place preference for ALLO, indicating that the production of this NAS may play a role in

ethanol reinforcement or reward (Finn et al., 1997a; Ford et al., 2005b; Janak and Gill, 2003; Janak et al., 1998). ALLO is metabolically reduced from progesterone by a two-step reaction, with 5α-R being the rate-limiting enzyme in this reaction. Thus, one way to decrease brain ALLO levels is with FIN, a 5α-R inhibitor (Finn et al., 2006). In a 2-hour access study, injections of FIN in C57BL/6J male mice have been shown to decrease ethanol drinking, further indicating NAS and other $GABA_A$ acting compounds as tools for altering ethanol intake (Ford et al., 2005a, 2008a, 2008b).

To elucidate how ALLO modulates ethanol intake, previous studies in the Finn laboratory have used lickometer circuits to examine the temporal patterns of ethanol drinking in 2-hour access studies. Although these studies uncovered transient alterations in ethanol consumption in male mice injected with ALLO or FIN ( Ford et al., 2005b), it is possible that in addition to a simple suppression or elevation in drinking, these drugs could be leading to a shift in the pattern of drinking across 24 hours. The current study used lickometer chambers to examine the drinking patterns of C57BL/6J male mice that had continuous access to ethanol and were treated in succession with three different $GABA_A$ receptor modulating drugs: GAN, FIN, and THIP.

While previous studies have shown that injections of low doses of ALLO increased ethanol drinking in mice while injections of high doses of ALLO decreased ethanol drinking, intake levels recovered after about 80 minutes post-treatment, possibly due to the rapid metabolism of ALLO (Ford et al., 2005b). To overcome the transient nature of these effects, we used GAN, a synthetic analogue of ALLO with an added methyl group, making it more metabolically stable (Hogenkamp et al., 1997). We predicted that GAN would lead to similar changes in ethanol consumption as that seen with ALLO, but because of its slower metabolism, the effect would be more prolonged.

Previous studies have examined the effects of 7 consecutive days of FIN treatment, but suppression of drinking was transient across the two-hour access period when given at a dose of 50 mg/kg in male mice and non-existent when given to female mice (Ford et al., 2005a, 2008a). On the other hand, 100 mg/kg FIN was effective at altering ethanol intake in female mice and at impeding acquisition of ethanol drinking in male mice in 2-hour limited access paradigms (Ford et al., 2008a, 2008b). Therefore, we chose to use the higher dose of FIN to examine how effects of this enzyme inhibitor would extend into a 24-hour time frame. In line with the notion that inhibition of ALLO synthesis decreases ethanol intake, we predicted that the higher dose of FIN would reveal a more pronounced and prolonged suppression of ethanol drinking than observed previously (Ford et al., 2005a, 2008b), and that this would be evident in a paradigm where mice were allowed continuous access to ethanol.

The present study also examined the patterns of ethanol drinking upon administration of THIP, a $GABA_A$ receptor agonist that has some selectivity for receptors containing the δ subunit (Herd et al., 2009). Receptors containing the δ subunit in combination with $\alpha_4$ or $\alpha_6$ occur almost exclusively outside of the synapse (Farrant and Nusser, 2005). NAS such as ALLO have a high affinity for extrasynaptic receptors, but it is unclear whether their modulation of ethanol's actions is via these extrasynaptic sites (Brown et al., 2002; Fodor et al., 2005; Mihalek et al., 1999). It is also controversial whether ethanol acts through extrasynaptic receptors (Borghese et al., 2006; Sundstrom-Poromaa et al., 2002). Therefore, we used THIP to examine the role of the extrasynaptic receptors in the modulation of ethanol drinking. We predicted that, similar to that seen with the $GABA_A$ agonist muscimol, THIP would decrease consumption of ethanol (Hodge et al., 1995; Petry, 1997; Samson and Chappell, 2001). Together, the goal of these experiments was to further examine the manner in which NAS alter the

consumption and pattern of ethanol drinking, in order to provide insight regarding a possible mechanism that NAS might be acting through to affect ethanol consumption.

## Materials and Methods

### *Animals*

Twenty-four C57BL/6J male mice of approximately 8 weeks of age at the start of experiments were used (Jackson Laboratory, Bar Harbor, ME). Mice were individually housed and acclimated to a reverse light/dark schedule (12/12hour; lights off at 1500) for 3 weeks prior to the start of experiments. All mice were provided *ad libitum* access to rodent chow and tap water in lickometer chambers (see below). Mice were weighed and handled daily on all acclimation and experimental days. All procedures were approved by the local Institutional Animal Care and Use Committee and complied with NIH guidelines.

### *Two-bottle Preference Procedure in Lickometer Chambers*

Lickometer chambers were used as previously described (Ford et al., 2005a, 2005b, 2008a, 2008b). Briefly, the chamber was made up of a 4-walled plexiglas insert that was positioned on an elevated wire grid floor inside a shoebox cage with bedding. The insert contained a hinged top and two small holes where the sippers of the drinking bottles entered into the cage. The wire floor of the chamber and each sipper formed an open electrical circuit that was connected to a lickometer device (MED Associates Inc., St. Albans, VT). Each time the circuit was completed, a lick was recorded for that individual animal. Lickometers were interfaced to an IBM computer running MED-PC IV software (MED Associates Inc.) to record time-stamped licks for each individual animal and each fluid presented.

Mice had approximately 23 hours access to 10E in one bottle and tap water in a second bottle for the duration of experiments (except when given only water as noted below). The 10E bottles were counterbalanced between the left and right sides across lickometer chambers to minimize the influence of side preference. Each individual animal had its ethanol bottle on the same side for the duration of the experiment to allow consistent drinking routines to become established. We have used this strategy consistently in our previous lickometers studies (Ford et al., 2005a, 2005b, 2008a, 2008b).

*Drugs*

An ethanol solution (10E; Pharmco Products, Brookfield, CT) was prepared by dilution of a 200 proof stock in tap water. GAN was purchased from Dr. Robert Purdy (VA Research Foundation, San Diego, CA) and was solubilized in a solution of 3% (v/v) dimethyl sulfoxide (DMSO, Mallinckrodt Baker, Inc., Paris, KY) and 20% (w/v) 2-hydroxypropyl-β-cyclodextrin (β-cyclodextrin, Cargill Inc., Cedar Rapids, IA) in saline. FIN [1,(5α)-androstan-4-aza-3-one-17β-(N-tert-butyl-carboxamide)] was purchased from Steraloids Inc. (Newport, RI) and was solubilized into a 20% (w/v) β-cyclodextrin solution in saline. THIP (4,5,6,7-tetrahydroisoxazolo-[5,4-c]pyridine-3-ol hydrochloride) was purchased from Tocris Bioscience (Ellisville, MO) and dissolved in 0.9% saline. Drugs were injected IP in a volume of 0.01 ml/g body weight.

*Acclimation*

Mice were first acclimated to the lickometer chambers by singly housing them in the chambers with access to food and water for one week. They were then given continuous ethanol access for another week, followed by injections of the 20% β-cyclodextrin solution along with continuous 10E access for another week. This

procedure acclimated the mice to the chambers and handling, and it was determined they had reached a stable 10E intake (defined as less than 10% variation for three consecutive days) before experiments began. Over the course of five months, all mice were administered multiple treatments in the following order: GAN, FIN or vehicle, and THIP.

*Experiment 1: Effects of GAN on 10E Intake*

Each day, bottles were removed and weighed to determine intake, and mice were weighed and injected with 20% β-cyclodextrin to acclimate them to handling. Immediately after injection, mice were placed back in the lickometer cages, and bottles were returned 30 minutes later so that access began immediately prior to the start of the dark cycle.

Once drinking was stable across 3 consecutive days of 20% β-cyclodextrin injections, all mice were given an injection of 3% DMSO/20% β-cyclodextrin (vehicle; 0 mg/kg GAN). There were 4 intervening days with 20% β-cyclodextrin injections, and on the $5^{th}$ day all mice received 5 mg/kg GAN in 3% DMSO/20% β-cyclodextrin. Following 6 more days of 20% β-cyclodextrin injections, all mice received 10 mg/kg GAN in 3% DMSO/20% β-cyclodextrin. Baseline, which is shown for non-statistical comparative purposes, was calculated by averaging each of the three 20% β-cyclodextrin days that immediately preceded a drug or vehicle day. The current doses were chosen based on a pilot study in male Withdrawal Seizure-Resistant mice where a 10 mg/kg dose of GAN produced a 30% decrease in ethanol intake over 24 hours (Finn and Ford, unpublished).

*Experiment 2: Effects of FIN and FIN Withdrawal on 10E Intake*

Mice were given a 3-week drug washout period where they were given daily vehicle injections (20% β-cyclodextrin) and continuous access to 10E. Mice again showed stable 10E intake with less than 10% variability across three consecutive days. Next, 10E was removed, and mice were given access to food and water for 19 days. The purpose of the 10E washout period was to examine the effect of FIN on the re-acquisition of 10E intake. Baseline was calculated by averaging the intake on the last 3 days prior to removal of 10E, and mice were group-matched for 10E intake on these days so that 14 mice were assigned to the FIN group and 10 mice were assigned to the vehicle group. Each day, bottles were weighed, and access to 10E resumed 24 hours after the first FIN injection (24 hour pretreatment time). The FIN group received daily injections of 100 mg/kg FIN for 7 days, followed by 7 days of vehicle injections (i.e., FIN withdrawal). The vehicle group received vehicle injections daily for the duration of the experiment.

Previous studies from the Finn laboratory have shown that effects of FIN differ across treatment phase (Ford et al., 2008a, 2008b). For this reason, data were split into four treatment phases: baseline (baseline days 1-3), acute FIN (FIN injections days 1-3), chronic FIN (FIN injections days 4-7), and withdrawal (FIN withdrawal days 1-7). In past studies, acute and chronic FIN withdrawal have been treated as separate phases (Ford et al., 2005a, 2008a). In the current study, however, a 2-way ANOVA with withdrawal phase and hour (through hour 6) as repeated measures revealed no significant withdrawal phase by hour interaction [$F(5,65) = 0.763$; $p = 0.580$]. The two withdrawal phases were therefore collapsed for simplicity and are referred to jointly as “withdrawal”.

*Experiment 3: Effects of THIP on 10E Intake*

After FIN treatment, mice were given a 10E washout period with access to food and water for one week. This was followed by a week of continuous 10E access, where animals were weighed and received saline injections daily. Again mice showed stable 10E intake over 24 hours with less than 10% variability across 3 consecutive days prior to any THIP treatment.

As before, bottles were weighed daily. Injections were given 30 minutes prior to the 10E bottles being put back in the chamber so that 10E access began just prior to the start of the dark cycle. A within-subjects design was used for THIP treatment. Mice were balanced for 10E intake and assigned to 1 of 2 groups, and mice previously treated with FIN were split evenly between the two groups. Group 1 received one dose of THIP per week in the following order: 16 mg/kg, 8 mg/kg, 4 mg/kg, 2 mg/kg. Group 2 received one dose of THIP per week in the following order: 8 mg/kg, 16 mg/kg, 2 mg/kg, 4 mg/kg. Mice were injected with saline on all intervening days and data for baseline (0 mg/kg) was calculated by averaging the 4 vehicle days that immediately preceded a treatment day. One mouse was removed at the start of the experiment due to excessive weight loss (vehicle-treated in experiment 2).

*Statistical Analysis*

The 10E dose (g/kg) consumed was calculated based on the grams of 10E depleted and the mouse's body weight on that day. Total fluid intake was the amount of 10E plus water consumed. Ethanol preference ratios were calculated from the 10E intake divided by total fluid intake. Cumulative and hourly 10E and water licks, as well as bout parameters were recorded using MED-PC IV software, and SoftCR version 4 (MED Associates, Inc.) was used to access the time-stamped data. As defined

previously, a 10E bout was defined as a minimum of 20 licks with no more than a 60-second pause between successive licks (Ford et al., 2005b).

All statistical analyses were performed using SYSTAT 11. For GAN treatment, a two-way analysis of variance (ANOVA) was used with dose (0 m/kg, 5 mg/kg,10 mg/kg) and hour (1, 2, 3, 4, 5, 6) as repeated measures. For THIP treatment, a two-way ANOVA was used with dose (0 m/kg, 2 mg/kg, 4 mg/kg, 8 mg/kg, 16 mg/kg) and hour (1, 2, 3, 4, 5, 6) as repeated measures. For FIN treatment, a two-way ANOVA was used with phase (baseline, acute FIN, chronic FIN, and withdrawal) and hour (1, 2, 3, 4, 5, 6) as repeated measures. In all cases, a significant interaction warranted follow-up with one-way ANOVAs at each hour with dose or phase as the repeated measure. In the event of a significant ANOVA, pair-wise differences were determined by the Fisher's Least Significant Difference multiple comparisons procedure against baseline. For all analyses, statistical significance was set at $p \leq 0.05$. Graphs were made using GraphPad Prism 4 for Windows.

## Results

*Correlation Between Licks and g/kg 10E Consumed*

Prior to any drug treatment, animals drank an average of 15.30 ± 0.35 g/kg over 24 hours and showed less than 10% variability between days on 5 consecutive days. As shown in Fig. 2.1A, during this time, a significant positive correlation was found between 10E licks and 10E g/kg consumed ($r$ (24) = 0.694; $p < 0.001$) supporting the validity of the lickometer system for estimating fluid intake across time. The average licks/ml 10E was also calculated prior to any drug treatment, and data were removed from any lick analysis if an animal's licks/ml fell outside 2 standard deviations (sd) of the average (1295 ± 976). Generally, discordances occur when an animal leans against the sipper

so that liquid leaks but licks are not recorded, or when an animal paws at a piece of the sipper so that many licks are recorded but no liquid is lost.

A three-day average intake (g/kg 10E) was calculated before the start of each new experiment. The pre-drug g/kg 10E intakes for all 24 mice were: GAN baseline = 15.2 ± 0.69, FIN baseline = 16.6 ± 0.71, THIP baseline = 13.2 ± 0.73. These represent normal fluctuations in baseline 24-hour 10E intake in male C57BL/6J mice across the five months of the studies. Body-weight, recorded in grams, increased throughout the duration of the experiments: GAN baseline = 23.2 ± 0.34, FIN baseline = 24.7 ± 0.35, THIP baseline = 25.9 ± 0.37.

*Experiment 1: Effects of GAN on 10E Intake*

Before GAN treatment, mice exhibited stable 24-hour drinking with less than 10% variability of 24-hour 10E intake over three consecutive days. Table 2.1 shows results of GAN on 24-hour parameters. All statistical analyses are against the within-subjects vehicle injection, and baseline measurements are provided for non-statistical comparative purposes. There was a significant effect of GAN on 24-hour g/kg 10E intake [$F(2,44) = 6.447$; $p = 0.004$] and 24-hour 10E licks [$F(2,44) = 3.252$; $p = 0.048$]. There was no effect of GAN on overall water licks or preference ratio over 24 hours.

The greatest 10E consumption by mice occurs at the beginning of the dark cycle. This has been previously reported and can be seen graphically in the review by Finn et al. (2010). Visual inspection of our data showed that this was in fact the time period where the greatest differences in drinking occurred in the present study (Fig. 2.1B). Therefore, to avoid floor effects from low drinking at the end of the dark cycle and throughout the light cycle, and to utilize time periods when the drug would be expected to be at physiologically relevant concentrations, we chose to focus on the drug effects

during the first 6 hours of the dark cycle. For the three 20% β-cyclodextrin days prior to the DMSO vehicle or any drug treatments, a 2-way ANOVA with day and hour (through hour 6) as repeated measures revealed no effect of day and no day by hour interaction of drinking prior to GAN or vehicle treatment, indicating stable drinking immediately prior to treatment.

Examining the first 6 hours of the dark cycle during vehicle, 5 mg/kg, and 10 mg/kg GAN treatment, a two-way ANOVA with dose and hour as repeated measures revealed a significant interaction [$F(10,220) = 4.424$; $p < 0.001$]. To examine the interaction, one-way ANOVA's were then performed at each hour with dose as a repeated measure. Figure 2.2 shows the effects of GAN at each of the first 6 hours of 10E access. Again, all statistical analyses are against the within-subjects vehicle treatment, and baseline values (the average of the three 20% β-cyclodextrin days that immediately preceded a vehicle or drug day) are given for non-statistical comparisons. Statistical analysis showed a dose dependent effect of GAN on 10E licks during hour 1 [$F(2,44) = 5.494$; $p = 0.008$], hour 2 [$F(2,44) = 6.841$; $p = 0.003$], hour 3 [$F(2,44) = 6.328$; $p = 0.004$], hour 4 [$F(2,44)= 3.799$; $p = 0.030$], and hour 5 [$F(2,44)= 4.804$; $p = 0.013$]. The effect of GAN was recovered by hour 6. See Fig. 2.2 A-F for a summary of 10E licks by hour. During hours 1 through 6, there was only a significant effect on water licks during the first hour [$F(2,46) = 9.073$; $p = 0.001$], with 5 mg/kg ($p = 0.017$) and 10 mg/kg GAN ($p = 0.001$) enhancing water licks versus vehicle (data not shown).

First bout characteristics were examined in order to better elucidate the initiation and pattern of drinking (see Table 2.1). A significant effect of GAN dose on latency to first lick [$F(2,44) = 11.115$; $p < 0.001$] was seen; 5 mg/kg GAN ($p = 0.004$) and 10 mg/kg GAN ($p = 0.002$) were associated with a significant decrease in latency. Analysis also

showed a significant effect of GAN dose on latency to first bout [$F(2,42) = 12.351$; $p < 0.001$], with a decrease following 5 mg/kg GAN ($p = 0.005$) and 10 mg/kg GAN ($p = 0.001$). There was no effect of GAN on first bout size.

*Experiment 2: Effects of FIN and FIN Withdrawal on 10E Intake*

The vehicle group was included to allow us to dissociate the effects of reacquisition from the effects of FIN. There was a significant phase by treatment by hour interaction [$F(15,330) = 1.932$; $p = 0.020$], confirming that FIN altered the reacquisition of 10E differently than vehicle across the first 6 hours among the phases of FIN treatment. Among the vehicle group, there was a transient effect of reacquisition in that the vehicle treated mice increased their intake during the withdrawal phase during hour 1 (data not shown). It can be seen from the vehicle error bars in Figure 2.3 that little variability occurred aside from this during each of the first 6 hours throughout the rest of the vehicle treatment. Due to the small and transient effects of reacquisition, the vehicle data are averaged for each hour and are shown in Figure 2.3 for non-statistical comparisons.

The rest of the analyses were performed between phases against the within-subjects baseline among the FIN treated mice. During the three baseline days preceding the removal of 10E and prior to any FIN treatment, mice exhibited stable 24-hour drinking with less than 10% variability in 24-hour 10E intake. During hours 1-6 of these days, there was some slight variability. A 2-way ANOVA for the 3 baseline days showed that there was a day by hour interaction. Post-hoc analysis showed that this was due to significantly higher drinking on baseline day 1 hour 1, baseline day 2 hour 5, and baseline day 3 hour 4 when analyzed across FIN animals. Even with these fluctuations, the averages for each hour still were not significantly different than pre-GAN

hourly baselines. Given that these fluctuations were not systematic, and because the nature of this study used averages for all phases of the study, we do not believe that our decision to collapse the baseline data changed our interpretation of the study.

See Table 2.2 for a summary of FIN effects on 24-hour parameters. Among FIN-treated animals, there was a significant effect of phase on 24-hour g/kg 10E consumed [$F(3,39) = 16.535$; $p < 0.001$], 24-hour 10E licks [$F(3,39) = 3.625$; $p = 0.021$], 24-hour 10E preference ratio [$F(3,39) = 15.022$; $p < 0.001$], and 24-hour water licks [$F(3,39) = 29.18$; $p < 0.001$].

Figure 2.3 shows the effects of FIN phase at each of the first 6 hours of 10E access. A two-way ANOVA examining the first 6 hours of drinking and treatment phase as repeated measures revealed a significant interaction between hour and phase [$F(15,195) = 3.591$; $p < 0.001$]. To elucidate the interaction, one-way ANOVAs were performed at each hour with treatment phase as a repeated measure. There was an effect of phase for FIN-treated mice in hour 1 [$F(3,39) = 6.439$; $p = 0.001$], hour 2 [$F(3,39) = 7.791$; $p < 0.001$], hour 3 [$F(3,39) = 9.61$; $p < 0.001$], hour 4 [$F(3,39) = 3.30$; $p = 0.03$], and hour 5 [$F(3,39) = 4.577$; $p = 0.008$]. The effect was recovered by hour 6. In general, acute FIN treatment decreased 10E licks through hour 4. Chronic FIN treatment decreased 10E licks in hour 1 only, and the withdrawal phase had no effect on 10E licks at any hour. See Fig. 2.3 A-F for a summary of hourly 10E licks.

During hours 1 through 6, water licks were slightly, though non-significantly, increased in hour 1, and significantly increased in hours 2 ($p = 0.001$), 3 ($p < 0.001$), and 4 ($p < 0.001$) of acute FIN treatment, suggesting a lack of general fluid suppression by FIN (data not shown). There was no effect of treatment group on body weight.

Bout analysis, also shown in Table 2.2, shows a significant effect of phase on latency to first lick [$F(3,39) = 12.40$; $p < 0.001$] with acute FIN (191%, $p < 0.001$) and chronic FIN (118%, $p < 0.001$) associated with a significant increase in latency. There was also a significant effect of phase on latency to first bout [$F(3,39) = 14.492$; $p < 0.001$] with acute FIN (423%, $p = 0.001$) and chronic FIN (164%, $p < 0.001$) associated with an increase in latency. Importantly, there was no change in latency to first water lick during either of these phases (data not shown), indicating that decreases in 10E consumption were likely not due to general locomotor suppression. There was no effect of phase on first 10E bout size.

*Effects of THIP on 10E Intake*

Throughout the THIP portion of the experiment, there were 4 animals whose data were excluded from 10E lick analyses due to sensitivity issues with lickometer circuits, which led to a discordance between licks and g/kg 10E consumed. There were two instances of a 10E bottle leak and one instance where a water bottle weight was not recorded. A 2-way ANOVA with day and hour as repeated measures revealed no effect of day and no day by hour interaction for the first 6 hours of drinking for three days prior to THIP treatment, indicating stable drinking immediately prior to THIP treatment.

Because group 1 and group 2 of the THIP experiment received THIP doses in a different order, but the doses were not administered in a perfect pseudo-random design, statistical analysis was done to examine whether there was an order effect of the doses. A three-way mixed model ANOVA with group as a between-subjects factor and dose and hour (through hour 6) as repeated measures revealed no main effect of group [$F(1,17) = 2.314$; $p = 0.147$], no interaction between group and dose [$F(4,68) = 1.948$; $p = 0.112$], no interaction between group and hour [$F(5,85) = 1.601$; $p = 0.169$], and no 3-

way interaction of group by dose by hour [F(20,340) = 0.950; $p = 0.524$]. This confirmed alack of a group effect, so data were collapsed between groups for all further analysis.

Analysis of the 24-hour data, shown in Table 2.3, revealed a significant effect of THIP dose on g/kg 10E consumed [F(4,80) = 9.622; $p < 0.001$]. Specifically, there was a significant decrease in g/kg 10E consumed with 16 mg/kg THIP versus baseline ($p < 0.001$). There was also a significant effect of dose on 24-hour 10E licks [F(4,72) = 9.565; $p < 0.001$]. Post hoc analysis showed an increase in licks with 2 mg/kg ($p = 0.011$) and 4 mg/kg THIP versus baseline ($p = 0.049$) and a decrease in licks with 8 mg/kg ($p = 0.043$) and 16 mg/kg THIP versus baseline ($p < 0.001$). There was a significant effect of 10E preference across doses [F(4,76) = 4.182; $p = 0.004$], with 8 mg/kg ($p = 0.013$) and 16 mg/kg ($p = 0.002$) THIP decreasing 10E preference. There was no effect of dose on the 24-hour water licks.

Figure 2.4 shows the effects of THIP at each of the first 6 hours of 10E access. A 2-way ANOVA performed over the first six hours of the dark cycle, with treatment dose and hour as repeated measures, revealed a significant interaction [F (20,360) = 2.252; $p = 0.002$]. To elucidate the interaction, a one-way ANOVA was performed at each hour with dose as a repeated measure until drinking returned to baseline. There was a significant effect of dose at hour 1 [F(4,72) = 6.953; $p < 0.001$], hour 2 [F(4,72) = 19.902; $p < 0.001$] hour 3 [F (4,72) = 17.467; $p < 0.001$], hour 4 [F(4,72) = 8.257; $p < 0.001$], and hour 5 [F(4,72) = 2.951; $p = 0.026$]. The effect of dose was absent at hour 6 (Fig. 2.4F). See Fig. 2.4 A-F for a summary of 10E licks by hour. Water licks were decreased by the 16 mg/kg dose of THIP at hours 1 and 2, reflective of a sedative effect of this dose. Water licks were unaffected in hours 1 and 2 and enhanced during hours 3 and 4 with

the 8 mg/kg dose THIP, indicating selectivity for decreasing 10E intake during this time. Water intake during hours 1-6 was unaffected after 2 or 4 mg/kg THIP (data not shown).

Analysis of first bout characteristics, shown in Table 2.3, revealed an effect of dose on latency to first lick [$F(4,84) = 18.856$; $p < 0.001$], with 4 mg/kg (72%, $p = 0.001$) and 8 mg/kg (53%, $p = 0.001$) associated with a decrease and 16 mg/kg (134%, $p < 0.001$) associated with an increase compared to baseline. There was also a significant dose effect on latency to first bout [$F(4,80) = 16.215$; $p < 0.001$], with 8 mg/kg (103%, $p = 0.002$) and 16 mg/kg (317%, $p < 0.001$) associated with an increase. Finally, a significant dose effect on first bout size was revealed [$F(4,80) = 3.322$; $p = 0.018$]. Post-hoc analysis showed that first bout size was reduced following 8 mg/kg THIP (33%, $p = 0.001$).

**Discussion**

Growing evidence suggests that manipulation of NAS levels, or other modulations at the $GABA_A$ receptor, can influence voluntary 10E intake. Here we show that in a 24-hour two-bottle choice paradigm, C57BL/6J male mice drank significantly less in the first 5 hours of 10E access following FIN or THIP administration. This is in accordance with previous data from the Finn laboratory showing that high doses of NAS, or the inhibition of their synthesis, decreases ethanol intake in mice in a limited access session (Ford et al., 2005a, 2005b, 2008a, 2008b). In addition, GAN shifted 10E intake so that drinking was increased in the first hour, decreased in the next two hours, and increased in hours 4 and 5, illustrating a similar, but extended pattern to that seen with low doses of ALLO (Sinnott et al., 2002a). GAN, FIN, and THIP also altered first lick and first bout latency, and THIP decreased first bout size. Importantly, some human studies have indicated that total ethanol consumption is an insufficient predictor of the

successfulness of therapeutic strategies (Bobak et al., 2004; Gmel et al., 2001); rather, the efficacy of treatment is reliant on drinking patterns (Anton et al., 2004; O'Malley et al., 2002). The differences in pharmacology between these drugs may underlie their differential effects on the bout parameters and patterns of consumption and may improve therapeutic approaches to alcohol use.

*GAN Alters Ethanol Intake Patterns*

GAN is a synthetic analog of the NAS ALLO, but contains an added methyl group, making it more resistant to metabolism than ALLO (Hogenkamp et al., 1997). Previous work with NAS has shown a bimodal effect of ALLO on alcohol intake in mice: low doses of ALLO that produce physiological concentrations of plasma ALLO increased ethanol intake in a 2-hour access procedure, while high doses of ALLO that produce supraphysiological plasma concentrations decreased ethanol intake (Finn et al., 2010; Ford et al., 2005b). Specifically, the findings with GAN are consistent with data showing that low doses of ALLO dose-dependently increased ethanol intake in the first hour of access, and dose dependently decreased ethanol intake in the second hour of access (Sinnott et al., 2002a). This increase in ethanol intake in the first hour is also consistent with data showing that low doses of ALLO increased operant lever presses for ethanol in a 30-minute limited access session and increased reinstatement for 10E in rodents in an operant procedure (Finn et al., 2008; Janak and Gill, 2003; Janak et al., 1998; Nie and Janak, 2003).

The effects of GAN in the present study lasted 5 hours into ethanol access in contrast to effects with ALLO, which persisted for about 80 minutes (Ford et al., 2005b). Given that injections of ALLO and GAN when given at 1, 3, or 10 mg/kg resulted in comparable behavioral and anticonvulsant properties on a battery of tests, it is possible

that these doses of GAN and ALLO exhibit comparable doses on other indexes such as their effect on ethanol intake (Beekman et al., 1998; Gasior et al., 1997). If this is indeed the case, it would not be surprising that GAN affected 10E drinking in a similar but extended manner as has been shown for low doses of ALLO (Sinnott et al., 2002a). It is important to keep in mind, however, that differences in pretreatment times and drug administration in relation to the dark cycle may complicate direct comparisons between the effects of GAN with those seen with ALLO in previous studies.

The effects of GAN on locomotor activity were not examined in the present study, however, Besheer et al., (2010) found no change in locomotion with the dose reported to increase self-administration in rats (1 mg/kg), and sedation only at a dose (30 mg/kg) much higher than that used in the present study and which decreased drinking in their study (Besheer et al., 2010).

*FIN Decreases Ethanol Intake*

Consistent with the drinking suppression reported in the 2-hour access studies that examined FIN effects on ethanol maintenance and acquisition (Ford et al., 2005a, 2008b), acute FIN administration in the current study decreased 10E intake in male mice by 53% during the first five hours of reacquisition to 10E continuous access. No effects of FIN withdrawal on 10E intake were observed during the 6 hourly measurements (Figure 2.3) in the present study, consistent with results in female C57BL/6J mice with the 100 mg/kg FIN dose (Ford et al., 2008a). It should be noted that the overall effects of acute FIN on 10E reacquisition were significant when compared with the animals' baseline values (Table 2.2) or versus values in the vehicle controls (not shown), which were slightly lower than baseline. Thus, even though the vehicle group did not exhibit an alcohol deprivation effect upon 10E reacquisition, the significant suppression of ethanol

intake upon reacquisition indicates that FIN may be a useful therapeutic tool to prevent relapse among alcoholics and should be examined further.

Although we did not directly measure ALLO levels in these animals, previous work in the Finn laboratory has shown that 100 mg/kg FIN decreased endogenous plasma levels of ALLO by over 80% in naïve C57BL/6J male mice when given at a 24-hour pre-injection time (Finn, unpublished). However, in our recent examination of the effects of FIN on the acquisition of 10E intake, we found that ALLO levels were suppressed by 28% following the 7$^{th}$ FIN injection (50 mg/kg) and the 2 hour 10E drinking session, and that 10E intake was suppressed by 43% (Ford et al., 2008b). We did not observe a similar relationship with the 100 mg/kg FIN dose, but it should be noted that interpretation of these data from Ford et al. (2008b) has the potential confound that ALLO measurements were conducted after limited access 10E intake, which has been shown to significantly increase brain ALLO levels in male C57BL/6J mice (Finn et al., 2004). Thus, the putative involvement of GABAergic NAS in the FIN-induced suppression of 10E intake is likely more complex than simply due to a concomitant suppression of ALLO levels following FIN treatment and will require additional studies that can more accurately measure several GABAergic steroid levels at various periods during the time-course of FIN treatment and ethanol consumption.

In order to have a 24-hour pre-injection time, animals were injected immediately prior to the preceding drinking session. FIN at this dose causes noticeable sedative effects that one could argue are underlying the decrease in 10E intake observed (Gabriel et al., 2004). However, the substantial increase in water licks during these same time points (almost a two-fold increase in hour 1 and a 5-6-fold increase in hours 2, 3, and 4; data not shown) suggests that sedative effects were not the primary factor in

decreasing 10E intake. Additionally, although FIN increased latency to first 10E lick, it had no effect on latency to first water lick, indicating that FIN administration did not cause an inability to locomote to the bottles. It is possible that the increase in water intake during FIN treatment was mediated by FIN's decrease of deoxycorticosterone (DOC) metabolism, another substrate of 5α-R, as high concentrations of DOC have been shown to increase water intake in rats (Cooney and Fitzsimons, 1996). Future studies are necessary to determine the manner by which FIN increased water intake.

There was no difference in weight gain between FIN and vehicle treated animals, suggesting a lack of general food and fluid suppression and also suggesting there were not differences in general metabolism between groups. It is also unlikely that FIN's effects are a result of changes in ethanol metabolism; a previous study showed that FIN had no effect on BECs following injections of ethanol in mice (Gorin-Meyer et al., 2007).

*THIP Decreases Ethanol Intake*

Currently, the effect of THIP on ethanol drinking in rodents is not clear, with some groups reporting an increase (Boyle et al., 1993) and others reporting a decrease (Moore et al., 2007). We reasoned that if THIP affected ethanol drinking in a similar manner to ALLO or GAN, it could provide clues for a possible mechanism explaining how NAS alter ethanol drinking behavior. Specifically, THIP was used in the present study to reveal the influence of extrasynaptic $GABA_A$ receptors on ethanol drinking. Although ethanol is known to modulate $GABA_A$ receptors (Deitrich et al., 1989; Harris and Mihic, 2004), the localization and subunit specificity of the receptors acted on by ethanol remains highly controversial. A majority of *in vitro* studies showing potentiation at $GABA_A$ receptors by ethanol only see this effect when using doses on the order of 100 mM to 300 mM, 2-3 times what is seen in humans even with extreme intoxication

(Borghese et al., 2006; Casagrande et al., 2007; Mehta et al., 2007). Recent reports have revealed a subclass of $GABA_A$ receptors located exclusively in the extrasynaptic membrane that are responsive to doses of ethanol on the magnitude of 1-30 mM, in the range attained by humans after alcohol consumption (Olsen et al., 2007; Sundstrom-Poromaa et al., 2002; Wallner et al., 2003). These extrasynaptic receptors are thought to be predominantly composed of a δ subunit in combination with $\alpha_4$ or $\alpha_6$ and $\beta_2$ or $\beta_3$ subunits and are responsible for tonic $GABA_A$ receptor-mediated inhibition (Farrant and Nusser, 2005; Nusser et al., 1998; Stell et al., 2003). This is in contrast to synaptic receptors, which are responsible for phasic GABAergic inhibition and are predominantly composed of $\gamma_2$ along with β and $\alpha_1$, $\alpha_2$, or $\alpha_3$ subunits (Farrant and Nusser, 2005). The sensitivity to low doses of ethanol make extrasynaptic receptors a plausible pharmacological target of ethanol (Kumar et al., 2009). The potentiation of these receptors with small doses of ethanol, however, is not consistently seen between laboratories (for a review on the subject see Borghese and Harris, 2007). Similar to ethanol, NAS have been shown to have a high affinity for the δ subunit of the $GABA_A$ receptor, raising the intriguing possibility that endogenous NAS could be modulating ethanol intake via extrasynaptic $GABA_A$ receptors (Brown et al., 2002; Fodor et al., 2005; Mihalek et al., 1999). Using THIP, we show that a drug that acts preferentially on extrasynaptic receptors was able to affect ethanol drinking, suggesting that this is a functionally relevant pathway to explore further.

In the present study, the highest dose of THIP (16 mg/kg) greatly suppressed 10E intake but also led to a very noticeable sedative effect and suppressed water intake. It is possible that this high dose activated synaptic receptors as well as extrasynaptic receptors. On the other hand, 8 mg/kg THIP (see Figure 2.4) selectively decreased 10E drinking by 30% during the first 5 hours of access. Importantly this dose of THIP caused

a decrease in latency to first 10E lick (Table 2.3) and first water lick (not shown), indicating a lack of sedative effects confounding the decreases in drinking. It also appeared that the 2 mg/kg and 4 mg/kg dose of THIP did not sedate the animals, as these doses caused no change and a decrease, respectively, in latency to both first water lick and first 10E lick.

It has been shown that rats injected with a 10 mg/kg subcutaneous dose of THIP had peak CNS concentrations of 2.9 µM and that this concentration of THIP selectively activated extrasynaptic $GABA_A$ receptors (Cremers and Ebert, 2007). Additionally, the peak concentration was reached 30 minutes post injection (Cremers and Ebert, 2007). Given that we had a pre-injection time of 30 minutes, that we used a lower dose (8 mg/kg) than Cremers and Ebert (2007), and that the injection took place in a species with a faster metabolism than rats, the peak levels of THIP in the current study were likely to be less than 2.9 µM, implicating a role for extrasynaptic $GABA_A$ receptors in modulating ethanol intake. Our dose-response effect also is consistent with a study showing that a 10 mg/kg injection of THIP in $GABA_A$ receptor $\alpha_4$ subunit knockout mice failed to induce sedative, analgesic, and ataxic behaviors seen in wild-type mice after THIP injection (Chandra et al., 2006). The lack of effect of the 10 mg/kg dose of THIP in the knockout mice suggests that at this dose, THIP was affecting these behaviors via extrasynaptic receptors. On the other hand, the same study found that a 15 mg/kg injection of THIP caused ataxia, analgesia, and sedation in both wild-type and knockout mice, consistent with the idea that our 16 mg/kg dose of THIP likely activated more than just extrasynaptic receptors (Chandra et al., 2006). Interestingly, the low doses of THIP increased 24-hour 10E licks while high doses decreased licks, mimicking the biphasic effect seen with NAS.

There is evidence for δ-subunit localization of $GABA_A$ receptors in the thalamus, cerebellum, dorsal and ventral striatum (including the NAc), olfactory bulb, and cerebral cortex of the rat, and in the hippocampus, primarily in the dentate gyrus, of humans and rats (Brooks-Kayal et al., 1999; Fodor et al., 2005; Pirker et al., 2000). The localization in the NAc is especially interesting as this area has well-documented GABAergic involvement in ethanol consumption (Hodge et al., 1995; Hyytiä and Koob, 1995). Additionally, ALLO infused into the NAc fully substituted for ethanol, suggesting that NAS present in this region may be important for some of the subjective effects of ethanol (Hodge et al., 2001). A recent report also showed that deletion of $GABA_A$ receptor $\alpha_4$ subunits in the NAc shell decreased ethanol consumption in rats (Rewal et al., 2009). Given the combination of these data, it is possible that NAS acting at extrasynaptic $GABA_A$ receptors in the NAc may be contributing to some of ethanol's reinforcing effects.

The hippocampus also may play an important role in ethanol consumption, and ethanol consumption has been shown to be modulated by NAS in this region. Voluntary consumption of ethanol is highly correlated with a change in c-fos expression in the hippocampus of C57BL/6J mice (Ryabinin et al., 2003). Additionally, infusions of ALLO into this brain region decreased voluntary ethanol consumption in rats (Martin-Garcia et al., 2007). Further, there is evidence that voluntary ethanol consumption might be controlled in part by extrasynaptic receptors in this region; administration of a benzodiazepine inverse agonist selective for the $\alpha_5$ subunit, which is an extrasynaptic subunit particularly enriched in this brain area, decreased ethanol intake when administered systemically or infused directly into the hippocampus (Cook et al., 2005; June et al., 2001). Future studies should further examine the relationship between NAS and extrasynaptic $GABA_A$ receptors, which could provide a better understanding of both the mechanism and the brain regions involved in NAS modulation of alcohol intake.

*Bout Analysis*

Bout characteristics were differentially affected by GAN, FIN, and THIP in the current study. As noted earlier, elucidating patterns of drinking may be an important component to better understanding and treating alcohol-related disorders. Similar to what was observed in previous studies, FIN decreased overall voluntary ethanol intake in mice ( Ford et al., 2005a, 2008b). As has been reported, high doses of ALLO and FIN both decreased alcohol drinking, but did so by different mechanisms. Earlier work showed that whereas ALLO dose-dependently altered bout frequency, FIN decreased bout size (Ford et al., 2005a, 2005b). In the current study, first bout characteristics were assessed to provide clues as to how mice were initiating alcohol sessions. Here, FIN increased latency to first lick and latency to first bout, consistent with a delay in the initiation of ethanol drinking. GAN also affected first bout characteristics; 5 and 10 mg/kg GAN decreased both latency to first lick and latency to first bout, suggesting that GAN facilitated the initiation of ethanol drinking. Interestingly, 8 mg/kg THIP decreased time to first lick, while increasing time to first bout. First bout size was only affected by 8 mg/kg THIP, which led to a decrease. This indicates that the 8 mg/kg THIP dose might be affecting the initiation of drinking quite differently than either of the two other drugs. Namely, it appears that with this dose, animals were taking their first lick sooner than they had with vehicle, but their first completed bout was smaller and more delayed. The way these drugs work to alter bout parameters warrants further investigation, as they could increase our knowledge of how alcohol drinking patterns are initiated and maintained.

*Limitations and Potential Confounds*

Although all mice received an acute 5 mg/kg dose of GAN one week prior to an acute dose of 10 mg/kg GAN, and all mice received GAN before FIN or THIP treatment, we do not believe there was a pharmacological order effect of the drugs. To minimize confounds of previous treatment, treatment groups were matched for 10E intake at the beginning of testing each new drug. Additionally, animals treated with FIN and vehicle were then balanced across the THIP groups. As mentioned, baseline 10E intake prior to the initiation of testing each drug did not differ from the normal fluctuations in 24 hour 10E consumption that we observe in male C57BL/6J mice across time, making it unlikely that drug carryover effects altered subsequent ethanol intake.

With 24-hour access procedures, it is difficult to pick a time to measure BECs without knowing patterns of ethanol intake. Since the goal of these studies was to measure patterns of intake, we did not introduce the disruption of measuring BEC during a particular hour post-injection. With limited access procedures, we have already shown that administration of ALLO and FIN did not alter ethanol metabolism, as the change in BEC corresponded to the change in ethanol intake in these studies (Ford et al., 2005a, 2005b). Thus, it is unlikely that the effects of FIN on ethanol intake in the present study were due to an indirect effect on ethanol metabolism. Nonetheless, because we did not measure BECs in the present studies, we cannot rule out that the effect of GAN and THIP on 10E intake was due to an indirect effect on ethanol metabolism.

The present experiments were performed only in C57BL/6J male mice. Previous work from the Finn laboratory has shown that female C57BL/6J mice were less sensitive to the behavioral and pharmacological effects of ALLO and FIN administration on ethanol intake (Finn et al., 2010; Ford et al., 2008a). For this reason, we decided to assess the robustness of the effects in males first, with the goal of

extending the findings in female mice given continuous 10E access in future experiments.

Although the present study illustrates selectivity of these $GABA_A$ receptor acting compounds for ethanol versus water, their effects on other sweet or calorie-containing solutions is currently unknown. There have been mixed reports of ALLO's effects on sucrose or saccharin intake, which may reflect a species difference or procedural differences, such as concurrent versus separate access to the sweetened solution and ethanol (Sinnot et al., 2002a; Janak and Gill, 2003). A general effect of GAN, FIN, and THIP on calorie-containing or sweet solutions therefore cannot be ruled out and remain the subject of ongoing investigation in the Finn laboratory.

An additional consideration is that progesterone, testosterone and deoxycorticosterone are all metabolized by 5α-R. Since FIN can block the conversion of these three steroids to their GABAergic NAS derivatives, there may be a complex interaction between FIN, ethanol, and their subsequent interaction at $GABA_A$ receptors (Finn et al., 2006). Therefore, we cannot rule out the contribution of other 5α-reduced steroids to the alterations in 10E consumption observed with FIN.

*Conclusions*

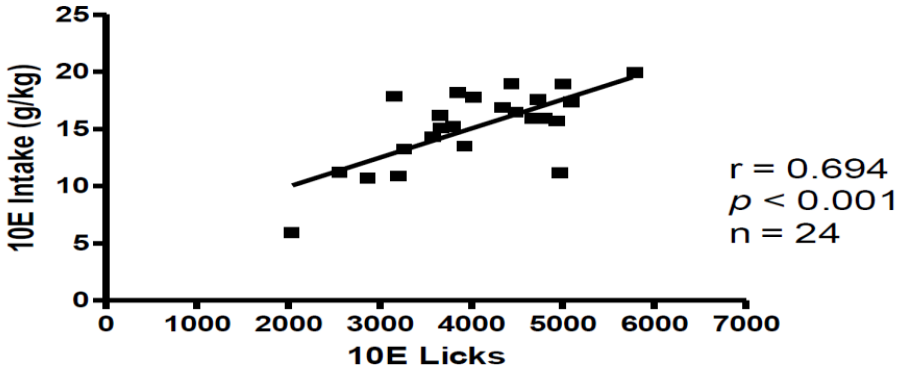
Even in light of the caveats mentioned above, the characterization of the alterations in 10E intake patterns with GAN, FIN, and THIP strongly support a role for the modulation of ethanol intake by NAS and $GABA_A$ receptor-active compounds. A better understanding of the manner in which NAS and $GABA_A$ receptor active compounds alter drinking patterns may greatly increase our understanding and ability to treat alcohol-related disorders. Future studies should continue to look at this relationship

and further characterize how extrasynaptic receptors may be involved.

Figure 2.1. 10E lick correlation and hourly pattern

A) Positive correlation between 24-hour 10E intake (g/kg) and licks on the 10E bottle. Data points show the average 10E licks and ethanol g/kg consumed for each mouse (n = 24) for the five baseline days preceding the first drug treatment. Solid line represents linear regression of the data. $p < 0.001$ as determined by a Pearson bivariate correlation analysis. B) Licks on the 10E bottle shown for hours 1 through 24 on a baseline day where mice were pretreated with 20% β-cyclodextrin. Data is duplicated for hours 25 through 48 for visual continuity. Shaded areas show when lights are off.

A)




B)

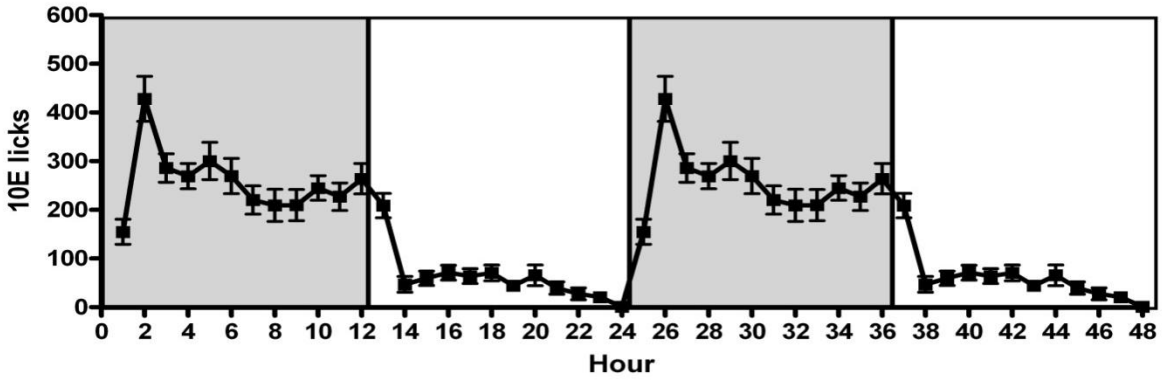


**Figure 2.2. Effects of GAN on the first 6 hours of ethanol access.**

The number of licks of 10E with each dose of GAN are shown for hour 1 (A), hour 2 (B), hour 3 (C), hour 4 (D), hour 5 (E), and hour 6 (F). A collapsed mean of the baseline days immediately preceding a drug day is shown for non-statistical comparative purposes. One animal's data was removed from all 10E lick analysis due to a discordance between licks and g/kg consumed. Vertical bars represent the mean ± SEM for 23 mice. +$p \leq 0.10$, *$p \leq 0.05$, **$p \leq 0.01$ versus within subjects vehicle (0 mg/kg).

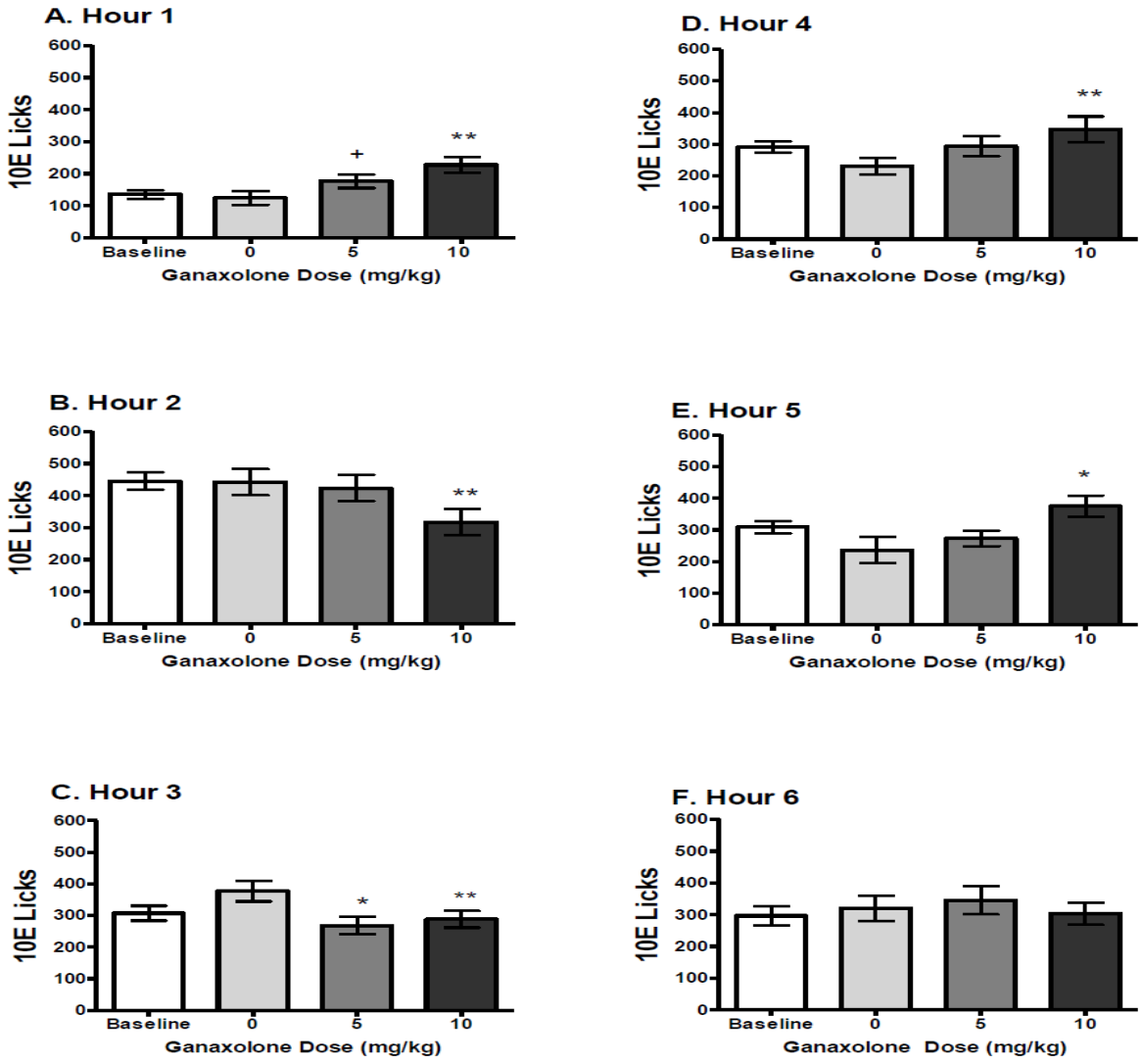


**Figure 2.3. Effects of FIN on the first 6 hours of ethanol access**

Total 10E licks during each phase of FIN (100 mg/kg) treatment are shown for hour 1 (A), hour 2 (B), hour 3 (C), hour 4 (D), hour 5 (E), and hour 6 (F). A collapsed mean of the last three 10E-access days before FIN treatment are shown as baseline. Vehicle treated animals' data were collapsed across treatment phase and are graphed for non-statistical comparative purposes. Vertical bars represent the mean ± SEM for 14 mice (FIN) or 10 mice (vehicle). +$p \leq 0.10$, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ versus within subjects baseline. Wdrl = withdrawal

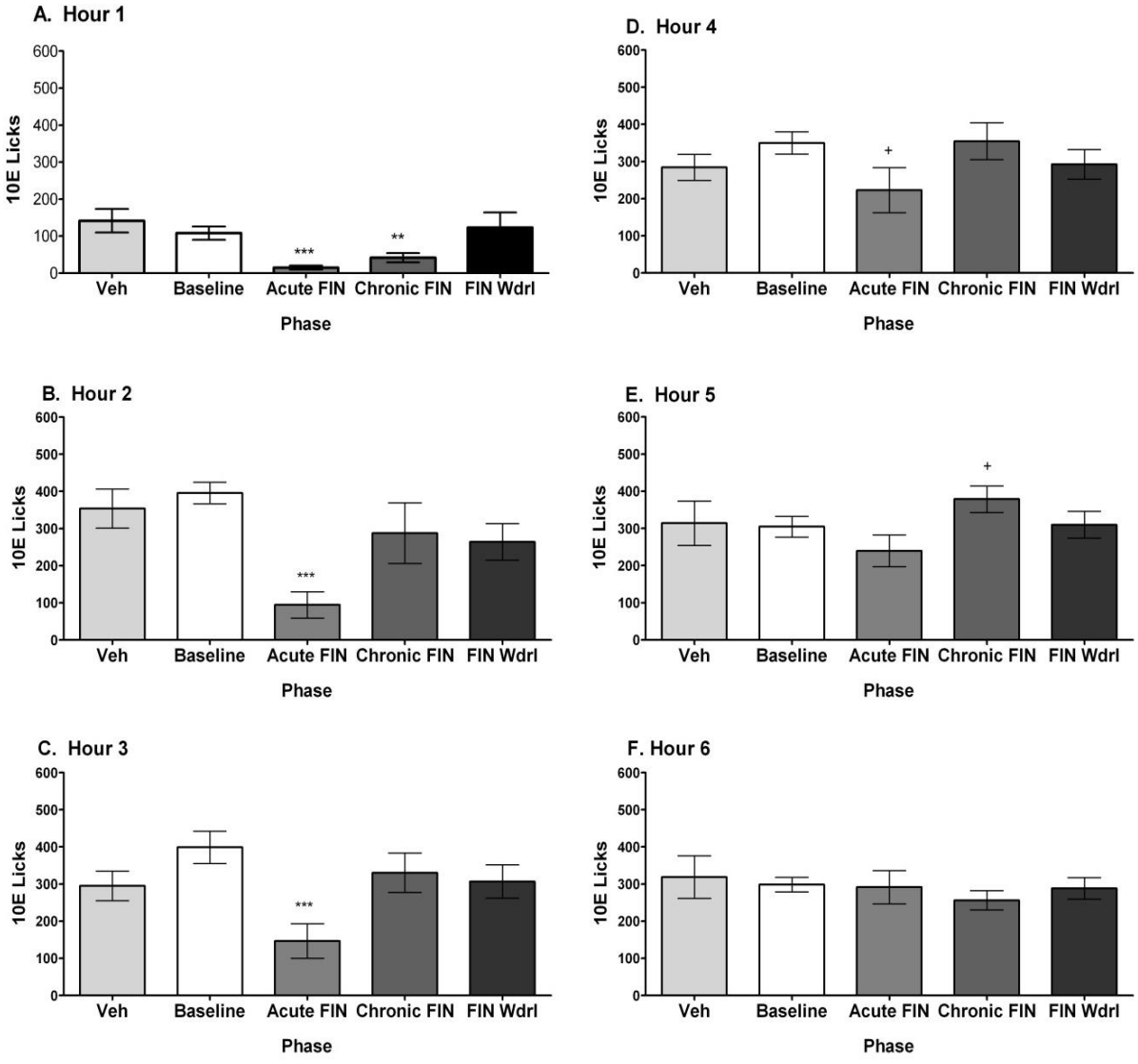


**Figure 2.4. Effects of THIP on the first 6 hours of ethanol access**

The number of licks of 10E with each dose of THIP are shown for hour 1 (A), hour 2 (B), hour 3 (C), hour 4 (D), hour 5 (E), and hour 6 (F). A collapsed mean of the vehicle days immediately preceding a drug day are shown as 0 mg/kg. Four animals were removed from all 10E lick analysis due to discordance between licks and g/kg 10E consumed on a drug or baseline day. Vertical bars represent the mean ± SEM for 19 mice. +$p \leq 0.10$, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ versus 0 mg/kg.

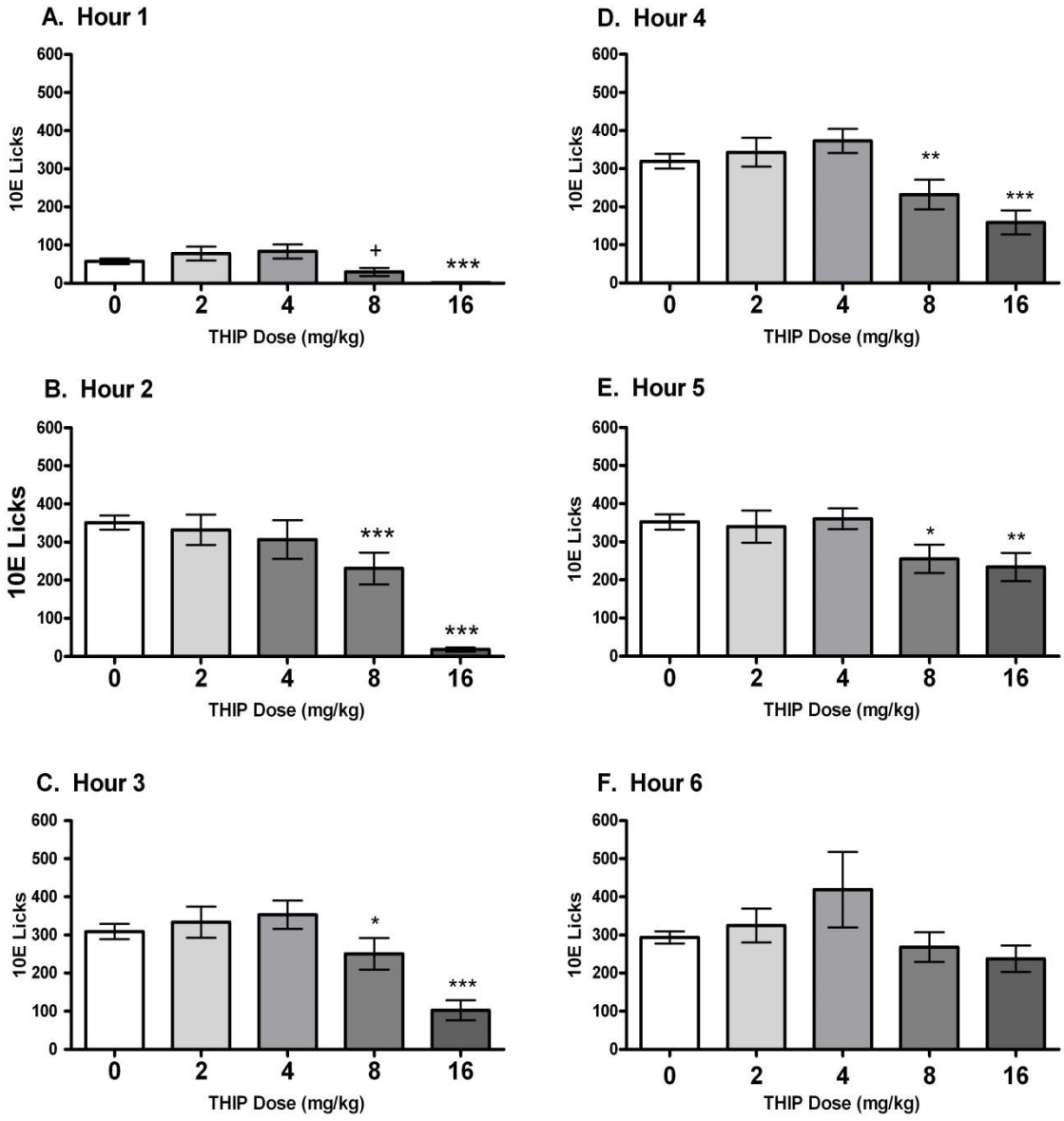


**Table 2.1. Effect of GAN on 24-hour measures and bout parameters**

Each mouse received all GAN doses. Baseline was calculated by averaging each of the three days that immediately preceded a drug or vehicle day and is shown for non-statistical comparative purposes. One animal's data was removed from the 10E lick analysis because there was discordance between his licks and g/kg 10E. Values represent the mean ± SEM for 23 mice. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ versus within subjects vehicle treatment.

| | **Baseline** | **Vehicle** | **5 mg/kg GAN** | **10 mg/kg GAN** |
|---|---|---|---|---|
| 10E dose (g/kg) | 14.55 ± 0.67 | 15.59 ± 0.76 | 13.98 ± 0.75 ** | 14.18 ± 0.81 * |
| 10E licks | 3983 ± 200 | 3981 ± 212 | 3942 ± 198 | 4308 ± 235 * |
| Preference ratio | 0.81 ± 0.04 | 0.82 ± 0.05 | 0.81 ± 0.05 | 0.82 ± 0.05 |
| Water licks | 1046 ± 229 | 972 ± 222 | 1068 ± 264 | 1059 ± 214 |
| Latency to first lick (minutes) | 10.2 ± 5.7 | 20.8 ± 5.7 | 2.9 ± 1.4 ** | 0.8 ± 0.3 ** |
| Latency to first bout (minutes) | 30.8 ± 6.1 | 36.3 ± 8.9 | 7.4 ± 3.3 ** | 0.8 ± 0.3 *** |
| First bout size | 87 ± 7 | 102 ± 9 | 105 ± 13 | 107 ± 12 |

**Table 2.2. Effect of FIN and FIN withdrawal on 24-hour measures and bout parameters**

Mice were given 7 days of 100 mg/kg FIN or vehicle followed by 7 days of vehicle (i.e. FIN withdrawal). Values represent the mean ± SEM for 14 mice for FIN treatment and 10 mice for vehicle treatment. Data for the vehicle-treated group were collapsed across all phases and are shown for non-statistical comparison. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ versus within subjects baseline.

| | **Vehicle** | **Baseline** | **Acute FIN** | **Chronic FIN** | **FIN withdrawal** |
|---|---|---|---|---|---|
| 10E dose (g/kg) | 14.48 ± 1.48 | 16.62 ± 0.91 | 10.08 ± 1.46 *** | 12.48 ± 1.48 ** | 13.22 ± 1.36 * |
| 10E licks | 4025 ± 357 | 4254 ± 276 | 3195 ± 446 * | 3775 ± 445 | 3627 ± 391 |
| Preference ratio | 0.73 ± 0.08 | 0.84 ± 0.06 | 0.47 ± 0.08 *** | 0.56 ± 0.07 ** | 0.64 ± 0.07 * |
| Water licks | 1338 ± 372 | 734 ± 346 | 3233 ± 536 *** | 2847 ± 492 *** | 1870 ± 386 *** |
| Latency to first lick (minutes) | 32.1 ± 9.4 | 31.9 ± 6.4 | 92.7 ± 12.6 *** | 69.4 ± 6.8 *** | 50.7 ± 7.4 |
| Latency to first bout (minutes) | 51.3 ± 16.1 | 37.1 ± 6.53 | 193.9 ± 36.6 *** | 97.8 ± 11.6 *** | 66.9 ± 12.6 |
| First bout size | 95 ± 9 | 95 ± 9 | 85 ± 12 | 100 ± 20 | 91± 8 |

**Table 2.3. Effect of THIP on 24-hour measures and bout parameters**

Each mouse received all THIP doses. Baseline (0 mg/kg) was calculated by averaging each of the four vehicle treatment days that immediately preceded a drug day. Four animals were removed from all 10E lick analysis due to discordance between licks and g/kg 10E consumed on one day. Values represent the mean ± SEM for 23 mice (10E dose; preference ratio; water licks), 19 mice (10E licks; latency to first lick; latency to first bout; first bout size). *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ versus 0 mg/kg.

| | **0 mg/kg THIP** | **2 mg/kg THIP** | **4 mg/kg THIP** | **8 mg/kg THIP** | **16 mg/kg THIP** |
|---|---|---|---|---|---|
| 10E dose (g/kg) | 13.01 ± 0.80 | 13.28 ± 0.91 | 13.05 ± 0.94 | 12.20 ± 0.93 | 10.21 ± 0.96 *** |
| 10E licks | 3921 ± 233 | 4294 ± 295 * | 4378 ± 310 * | 3498 ± 290 * | 3077 ± 277 *** |
| Preference ratio | 0.79 ± 0.04 | 0.76 ± 0.05 | 0.76 ± 0.05 | 0.71 ± 0.05 * | 0.68 ± 0.06 ** |
| Water licks | 1182 ± 236 | 1356 ± 348 | 1095 ± 249 | 1296 ± 243 | 1363 ± 294 |
| Latency to first lick (minutes) | 37.8 ± 4.9 | 31.7 ± 8.2 | 10.4 ± 5.5 *** | 17.5 ± 3.9 *** | 88.5 ± 11.5 *** |
| Latency to first bout (minutes) | 49.2 ± 6.2 | 34.9 ± 8.9 | 36.8 ± 11.2 | 99.9 ± 12.9 ** | 205 ± 37 *** |
| First bout size | 85 ± 4 | 100 ± 10 | 70 ± 10 | 57 ± 7 *** | 72 ± 11 |

# CHAPTER 3: EFFECT OF GANAXOLONE AND THIP ON OPERANT AND LIMITED-ACCESS ETHANOL SELF-ADMINISTRATION

This chapter has been reformatted for inclusion in this dissertation from:

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**Abstract**

The endogenous NAS, ALLO, has a similar pharmacological profile as ethanol, and it alters ethanol intake in rodent models. Recent evidence suggests that $GABA_A$ receptor ligands may regulate ethanol intake via effects at both synaptic and extrasynaptic receptors. Additionally, recent evidence suggests that δ subunit-containing extrasynaptic $GABA_A$ receptors may confer high sensitivity to both ethanol and NAS. The purpose of the present study was to determine the effects of GAN (an ALLO analog) and THIP (a $GABA_A$ receptor agonist preferential for the extrasynaptic δ subunit) on ethanol intake, drinking patterns, and bout characteristics in operant and limited-access intake procedures. In separate studies, the effects of GAN (0 - 10 mg/kg) and THIP (2 – 16 mg/kg) were tested in C57BL/6J male mice provided with two-hour access to a two-bottle choice of water or 10E or trained to lever press to acquire 30 minutes of access to 10E. GAN had no overall significant effect on operant ethanol self-administration, but tended to decrease the latency to consume the first bout. In the limited-access procedure, GAN dose-dependently decreased ethanol intake. THIP dose-dependently decreased ethanol intake in both paradigms, altering both the consummatory and appetitive processes of operant self-administration as well as shifting the drinking patterns in both procedures. THIP also decreased latency to acquire a sucrose reinforcer and decreased sucrose intake. These results add to literature suggesting time-dependent effects of NAS to promote the onset, and to subsequently decrease, ethanol drinking behavior, and they support a role for extrasynaptic $GABA_A$ receptor activation in ethanol self-administration.

**Introduction**

The $GABA_A$ receptor is one target of ethanol in the central nervous system (CNS; e.g., Kumar et al., 2009; Wallner et al., 2006), and many pharmacological agents that alter $GABA_A$ receptor function have been shown to alter ethanol intake in preclinical models (e.g., Chester and Cunningham, 2002; Finn et al., 2010). Additional evidence for a contribution of $GABA_A$ receptor signaling to ethanol drinking behaviors is provided by recent work utilizing site-specific knockdown of select $GABA_A$ receptor subunits. Knockdown of the $\alpha_1$ subunit in the ventral pallidum (Yang et al., 2011) and of the $\alpha_2$ subunit in the central nucleus of the amygdala (Liu et al., 2011) significantly decreased binge alcohol intake. Studies targeting extrasynaptic $GABA_A$ receptors found that knockdown of the $\alpha_4$ (Rewal et al., 2011, 2009) and $\delta$ (Nie et al., 2011) subunit in the NAc shell significantly reduced moderate alcohol intake. Collectively, the available data suggest that $GABA_A$ receptors may regulate ethanol intake via effects at both synaptic and extrasynaptic receptors although the extent of the contribution of each class of receptors remains unclear.

AUDs encompass a class of complex traits and behaviors including alcohol craving, escalation of alcohol use, tolerance, and development of physical dependence, making it difficult for animal models to capture the totality of this human disease. Therefore, it is important to test any potential therapeutic compound across a variety of procedural paradigms and animal models. Our recent work showed a shift in ethanol drinking patterns in mice across 24 hours following administration of THIP (a $GABA_A$ receptor agonist with selectivity for extrasynaptic receptors containing $\delta$ subunits) (Chandra et al., 2006; Herd et al., 2009) and GAN (a synthetic analogue of the NAS, ALLO) (Carter et al., 1997); the drinking pattern changes following THIP and GAN were consistent with the involvement of synaptic and extrasynaptic $GABA_A$ receptors in the modulation of moderate ethanol intake (Ramaker et al., 2011 (Chapter 2)). Thus, the

purpose of the present study was to extend the examination of these two potent modulators of the $GABA_A$ receptor for their effects on limited-access ethanol consumption in two different paradigms: operant self-administration and 2-bottle choice limited-access drinking.

The NAS ALLO is the most potent endogenous positive modulator of the $GABA_A$ receptor identified to date (e.g., Belelli and Lambert, 2005), and ALLO levels are increased in the periphery and brain following ethanol intake or administration (Barbaccia et al., 1999; Finn et al, 2004; Torres and Ortega 2004, 2003; VanDoren et al., 2000; but also see Holdstock et al., 2006; Porcu et al, 2010). ALLO has been shown to generalize to the discriminative stimulus effects of ethanol (Bowen et al., 1999; Grant et al., 1996), suggesting that the two compounds share similar subjective effects. ALLO also exhibits a similar behavioral, physiological, and pharmacological profile as ethanol. For instance, both compounds have anxiolytic, anticonvulsant, sedative-hypnotic, and muscle relaxant properties (Finn and Gee, 1994; Finn et al., 1997, Gasior et al., 1999). This relationship suggests that manipulation of NAS levels may alter the reinforcing properties of ethanol, with subsequent effects on ethanol intake. Indeed, multiple rodent studies have shown that exogenous ALLO administration biphasically alters voluntary ethanol consumption (Finn et al., 2010; Ford et al, 2007a, 2005b; Janak et al., 1998; Janak and Gill, 2003; Sinnott et al., 2002a). On the other hand, administration of the 5α-R inhibitor FIN, which decreases the metabolism of progesterone to ALLO, has been shown to decrease ethanol consumption (Ford et al., 2005a, 2008b). The inhibition of ethanol-induced increases in ALLO production by FIN or adrenalectomy has also been shown to block some of the behavioral and physiological properties that normally follow ethanol administration (e.g., Khisti et al., 2003; Sanna et al., 2004; VanDoren et al.,

2000), further indicating that the increase in NAS levels following ethanol intake may impact sensitivity to some of ethanol's reinforcing and behavioral properties.

One limitation to administering ALLO in an experimental procedure is its short half-life, which leads to a transient pharmacological effect ($T_{½}$ in plasma ~30 minutes in rodents and peak levels in brain within 10 minutes; maximum behavioral effect at 15 min post-injection is reduced to 50% effectiveness at 60-90 minutes post-injection; Mellon et al., 2008; Kokate et al., 1994). One way to circumvent this problem is by using GAN, a synthetic analogue with an added methyl group that extends the half-life to 3-4 fold that of ALLO, while retaining the primary neuropharmacological properties of this NAS (Belelli and Herd, 2003; Carter et al., 1997). Though the research to date is limited, systemic GAN administration has been shown to alter ethanol consumption in two rodent models. The first used rats in an operant self-administration paradigm (Besheer et al., 2010) and the second used mice in a 24-hour 2-bottle choice study (Ramaker et al., 2011 (Chapter 2)). These studies support a similar biphasic effect on ethanol intake to that seen with ALLO, namely an enhancement of ethanol intake with low doses of the drug and suppression of ethanol intake with high doses of GAN. Therefore, the first goal of the present studies was to expand on these earlier findings by examining the effect of GAN in an operant self-administration and a 2-hour limited-access ethanol consumption procedure in mice.

The $GABA_A$ receptor is a pentaheteromeric chloride channel comprised of a combination of subunits consisting of $\alpha_{1\text{-}6}$, $\beta_{1\text{-}3}$, $\gamma_{1\text{-}3}$, δ, ε, θ, or π. The specific combination of subunits that make up the channel alter the pharmacology, physiology, and subcellular localization of the receptors (Farrant and Nusser, 2005; Mody and Pearcy, 2004). For example, δ subunit-containing receptors are thought to occur

exclusively outside of the synapse and to contribute to GABA-mediated tonic inhibition, in contrast to synaptic receptors, which are responsible for phasic inhibition (Nusser et al., 1998). *In vitro* studies of the effects of ethanol on extrasynaptic $GABA_A$ receptors have produced inconsistent results. Some studies have shown a highly selective effect of ethanol at δ subunit-containing receptors, while other studies have been unable to replicate this effect (Borghese et al., 2006; Korpi et al., 2007; Olsen et al., 2007; Santhakumar et al., 2007). Recent *in vivo* work has shown that selectively modulating these extrasynaptic receptors either pharmacologically (Boyle et al., 1993, 1992; Moore et al., 2007; Ramaker et al., 2011 (Chapter 2)), with viral knockdown (Nie et al., 2011; Rewal et al., 2009, 2011), or with subunit-specific knockout (Mihalek et al., 2001, 1999) can alter ethanol consumption, indicating that these receptors may be important in mediating ethanol's reinforcing effects.

Evidence shows that although ALLO can potentiate both tonic and phasic inhibition, extrasynaptic δ subunit-containing receptors may be especially sensitive to ALLO (Belelli and Lambert, 2005; Brown et al., 2002; Fodor et al., 2005). To better isolate the contribution of extrasynaptic $GABA_A$ receptors to ethanol's reinforcing effects (which could eventually elucidate the contribution of each class of receptors to NAS effects on ethanol intake), the second aim of this study was to utilize THIP, an agonist with selectivity for δ subunit-containing receptors, to examine the role of enhanced tonic inhibition on operant and limited-access consumption in mice.

In addition to examining effects on total ethanol intake, the present studies aimed to capture patterns of drinking that are not reflected by analysis of total consumption alone. In the current operant self-administration experiment, the use of an RR schedule (see Methods) enabled us to procedurally separate the appetitive and the consummatory

phases of self-administration (e.g., Ford et al., 2007b; Samson et al., 1998). Further, analysis of bout and bin data in both procedures allowed for the examination of the shifts in the patterns of drinking and provided further insight into drug-induced effects on the microarchitecture of the drinking episodes. This may have important therapeutic relevance as human studies have shown that the pattern of drinking, for example, the size and frequency of drinking bouts, rather than overall intake, may be more informative when assessing problems associated with alcohol use and therapeutic efficacy of drugs (Anton et al., 2004; Boback et al., 2004; Gmel et al., 2001). Thus, the present studies add to the current literature by providing insight into the regulatory mechanisms in which GAN and THIP alter ethanol intake.

## Materials and Methods

### *Animals*

Fifty-four C57BL/6J male mice, approximately 8 weeks of age at the start of experiments, were used (Jackson Laboratory, Bar Harbor, ME). Twenty-four mice were single-housed in lickometer chambers (Exp. 1; see below) and acclimated to a reverse 12-hour light: 12-hour dark cycle (lights off at 0600). Thirty mice were pair-housed in standard shoebox cages (Exp. 2; see below) and acclimated to a 12-hour light: 12-hour dark cycle (lights on at 0600). All mice were provided *ad libitum* access to rodent chow and water (except where noted). Experiments were run Monday through Friday, and mice were weighed and handled daily on those days. All efforts were made to minimize animal suffering and to reduce the number of mice used. All procedures were approved by the local Institutional Animal Care and Use Committee and complied with NIH guidelines.

### *Drugs*

Ethanol (200 proof; Pharmco Products, Brookfield, CT) and sucrose (Sigma-Aldrich Company, St. Louis, MO, USA) solutions were prepared by dilution in tap water. GAN was purchased from Dr. Robert Purdy (VA Research Foundation, San Diego, CA) and was solubilized in a solution of 3% DMSO (Mallinckrodt Baker, Inc., Paris, KY) and 20% β-cyclodextrin (Cargill Inc., Cedar Rapids, IA) in Millipore water. THIP was purchased from Tocris Bioscience (Ellisville, MO) and dissolved in saline. Drugs were injected intraperitoneally (i.p) in a volume of 0.01 ml/g body weight.

*Apparatus*

Lickometer chambers were used as previously described by the Finn laboratory (Ford et al., 2005a, 2005b, 2008a, 2008b; Ramaker et al., 2011 (Chapter 2)). Briefly, the chamber was comprised of a 4-walled plexiglas insert that was positioned on an elevated wire grid floor inside a shoebox cage with bedding. The insert contained a hinged top and two small holes where the sippers of the drinking bottles protruded into the cage. The wire floor of the chamber and each sipper formed an open electrical circuit that was connected to a lickometer device (MED Associates Inc., St. Albans, VT). Each time the circuit was closed by animal contact, a lick was recorded for the respective sipper. Lickometers were interfaced to an IBM computer running MED-PC IV software (MED Associates Inc.) to record time-stamped licks for each individual animal and each fluid presented.

Operant self-administration sessions were carried out in eight sound and light-attenuating mouse chambers as described previously (Ford et al., 2007a, 2007b). Each chamber contained a stainless steel grid floor, house light, 2 retractable levers with a stimulus light above each one, and a retractable sipper apparatus. The retractable sipper consisted of a 10 ml graduated pipette with a double ball-bearing metal sipper

tube. Each metal sipper was connected to a lickometer circuit, which interfaced to a computer operating with MED-PC software (Med-Associates Inc.).

Experiment 1: Two-hour Two-bottle Preference Procedure in Lickometer Chambers

Acclimation

Mice were acclimated to lickometer chambers for 2 weeks where they had *ad libitum* access to food and two 50 ml bottles of water. For the rest of the experiment, the 50 ml water bottles were removed and weighed 1.5 hours into the dark cycle, the respective injection was given, and 30 minutes later each mouse was given access to both a 10 ml bottle of 10E and a 10 ml bottle of tap water for the next 2 hours. The 10E bottles were counterbalanced between the left and right sides across lickometer chambers to minimize the influence of side preference; each individual animal had its ethanol bottle on the same side for the duration of the experiment to allow consistent drinking routines to become established. We have used this strategy consistently in our previous lickometers studies (Ford et al., 2005a, 2005b, 2008a, 2008b; Ramaker et al., 2011 (Chapter 2)). At the conclusion of the 2 hours of access, fluid consumed was measured (to the nearest 0.05 ml), and the 10 ml bottles were replaced with 50 ml water bottles. To control for leakage, two control cages were set up with no animals present, and the amount lost from those bottles was averaged and subtracted from the amount consumed from each bottle. Mice received 2 hours of access to 10E or water for approximately 5 weeks, and were then divided into two groups, balanced for their ethanol intake and ethanol bottle side. Starting during the 8$^{th}$ week, group 1 (GAN group) received daily 20% β-cyclodextrin injections at 30 minutes prior to the session start, while group 2 (THIP group) received daily saline injections at 30 minutes prior to the session start. Daily 22-hour water intake was also measured throughout the

experiment in order to assess any alterations in overall fluid balance following drug treatment.

*Experiment 1a: GAN*

Prior to drug treatment, mice (group 1; n = 12) received 14 days of i.p. 20% β-cyclodextrin injections in order to habituate them to injection and handling stress. Mice then received a weekly injection of GAN in 3% DMSO/20% β-cyclodextrin in the following order: 0 mg/kg, 5 mg/kg, 10 mg/kg, and 0 mg/kg. 20% β-cyclodextrin was given on all intervening days to maintain stable drinking routines under injection conditions. Vehicle drinking values were calculated by averaging the two vehicle treatment (0 mg/kg, which contained 3% DMSO/20% β-cyclodextrin) days.

*Experiment 1b: THIP*

Following 14 days of saline injections, separate mice (group 2; n = 12) were given a weekly injection of THIP in the following order: 8 mg/kg, 4 mg/kg, 2 mg/kg, and 16 mg/kg. Saline was given on all intervening days. Vehicle values (0 mg/kg) were calculated by averaging the 4 saline days that immediately preceded each THIP treatment day.

*Experiment 2: Operant self-administration of ethanol or sucrose*

*Acquisition*

Self-administration was assessed with the "sipper" procedure (Ford et al., 2007b; Samson et al., 1998). For 10 days mice were water restricted for 16 hours preceding the training sessions and were trained to respond for 10% sucrose (10S) on a FR 1 schedule during 60-minute sessions. A response on the "active" lever resulted in

retraction of both levers, the house light turning off and the respective stimulus light turning on for 5 seconds, and the sipper extending into the chamber for 60 seconds. Responses on the “inactive” lever were recorded but had no scheduled consequence. The position of the active lever was counterbalanced between chambers. Sipper access was reduced from 60 to 30 seconds, and session length was decreased from 60 to 30 minutes over successive training sessions. After the initial 10 sessions, fluid restriction was lifted and the FR schedule was gradually increased across sessions until mice were consistently responding on FR 4. At this point, the animals were divided into two groups. An ethanol-reinforced group had 10E added to the 10S solution and then sucrose was faded out in a step-wise manner to eventually yield a 10E solution (3 sessions each with 10S/10E, 5S/10E, and 2.5S/10E solutions). The sucrose-reinforced group had the 10S solution incrementally reduced to eventually yield a 2% sucrose solution (2S; 3 sessions each with 10S, 7.5S, 5S, 2.5S). Once the final concentration of each reinforcer solution was reached, the FR schedule was further raised in a step-wise fashion (3-4 sessions each at FR 4, FR 6, FR 8), and then a RR schedule was instituted, whereby all appetitive responding preceded a consummatory phase that involved 30 minutes of continuous fluid access. The RR schedule was also raised in a step-wise fashion (3-4 sessions each at RR 8, RR 10, RR 12, RR 14, RR 16). The final contingency was RR 16, where mice had up to 20 minutes to make 16 responses on the active lever, followed by 30 minutes of fluid access.

*Experiment 2a: GAN*

Mice were maintained on the RR 16 schedule for 3 weeks prior to any treatment to establish baseline drinking. During the third week, they received daily 20% β-cyclodextrin injections at 30 minutes prior to the start of the session to habituate them to

handling. At this time, 9 mice in the 10E group and 14 mice in the 2S group consistently earned the reinforcer and licked over 100 times per session and were therefore included in the experiment. Mice who licked less than 100 licks on any baseline or pretreatment days (~ 0.25 g/kg) were excluded from statistical analysis as we estimated that this was the minimum an animal could drink to experience a pharmacological effect of the 10E (Ford et al., 2007a). Every 2-3 days, all mice received a systemic injection of GAN in 3% DMSO/20% β-cyclodextrin in the following order: 0 mg/kg, 5 mg/kg, 10 mg/kg, 0 mg/kg. 20% β-cyclodextrin was given on all intervening days. Vehicle data was calculated by averaging the two vehicle treatment (0 mg/kg) days.

*Experiment 2b: THIP*

For 2 weeks following the conclusion of the GAN study, the same mice were weighed and run in a daily session without injections. After 2 weeks, daily saline injections with a 30-minute pretreatment time were resumed. In the week preceding THIP treatment and on all saline days, 12 mice in the 10E group and 17 mice in the 2S group consistently earned the reinforcer and licked over 100 times per session and were therefore used in the statistical analysis. One day each week, the saline injection was replaced with an injection of THIP in the following order: 2 mg/kg, 4 mg/kg, and 8 mg/kg. Vehicle (0 mg/kg) data was calculated by averaging the 3 saline days that immediately preceded the THIP treatment days.

*BECs*

BECs were assessed at the end of the 2-hour lickometer experiment on a drug and vehicle day for each group (after 5 mg/kg GAN and the final vehicle treatment for group 1; after 4 mg/kg THIP and the final baseline treatment for group 2). A 20 µL sample was taken from the retro orbital sinus of each mouse. Blood samples were

processed according to previously published methods (Finn et al., 2007). Briefly, each blood sample was added to 500 µL of a solution containing 4 mM *n*-propanol (internal standard) in deionized water, vortexed, and analyzed using head-space gas chromatography. Six pairs of external standards with known ethanol concentrations (from 0.5 to 3.0 mg/ml) were used in parallel to construct a standard curve from which unknown concentrations were interpolated. Blood samples were not taken in the operant mice as these cohorts were especially sensitive to any handling outside of the daily systemic injections.

*Statistical Analysis*

Ethanol intake (g/kg) was calculated based on the ml of 10E solution depleted and the pre-session body weight of each mouse. Patterns of 10E and water licks, as well as ethanol bout parameters were recorded using MED-PC IV software, and SoftCR version 4 (MED Associates, Inc.) was used to access the time-stamped data. A 10E bout was defined as a minimum of 20 licks with no more than a 60 second pause between successive licks (Ford et al., 2008, 2005a, 2005b; Ramaker et al., 2011 (Chapter 2)). A similar strategy was used for the analysis of the 2S data. All statistical analyses were performed using SYSTAT 11. For intake and bout analysis, a repeated-measures ANOVA was used with dose as the factor. In the event of a significant main effect, pair-wise differences against vehicle were determined by the Fisher's Least Significance Difference multiple comparisons test. For bin analysis, a two-way repeated-measures ANOVA factored by bin and dose was conducted. For bin and bout analysis, data were excluded from statistical analysis for any animal whose licks/ml fell outside 2.5 sd from the average across 5 pretreatment days. For all analyses, statistical

significance was set at $p \leq 0.05$. Graphs were made using GraphPad Prism 4 for Windows.

## Results

Experiment 1a: Effect of GAN on limited access ethanol intake in lickometer chambers

*Intake*

There was no difference between vehicle days for g/kg ethanol intake ($p = 0.993$), and so the data on these two days were collapsed. On the average of the 2 vehicle days, mice drank 3.39 ± 0.18 g/kg ethanol. Repeated-measures ANOVA revealed an effect of GAN treatment to significantly alter 10E intake versus vehicle [$F(2,22) = 7.69$, $p < 0.01$]; specifically, 10E intake was significantly decreased following 10 mg/kg GAN ($p < 0.01$; Fig. 3.1A). During the 2 hours of 2-bottle choice drinking, mice exhibited strong preference for the ethanol solution (>97% ethanol preference on all days). Two-hour water intake measurements were: 0.02 ± 0.01 ml (39 ± 13 licks) following 0 mg/kg GAN, 0.03 ± 0.01 ml (22 ± 7 licks) following 5 mg/kg GAN, and 0.02 ± 0.01 ml (32 ± 9 licks) following 10 mg/kg GAN. Because 2-hour water intake was negligible and below the level of reliable detection, statistical analyses were not performed on these data and they could not be used as a measurement of non-specific effects on fluid intake. There was a dose effect of GAN on 22-hour water intake [$F(2,22) = 4.13$, $p < 0.05$], with a slight increase following both doses (Table 3.1). BECs at the end of the 2-hour limited access session were 1.37 ± 0.17 mg/ml after DMSO vehicle and 1.22 ± 0.17 mg/ml after 5 mg/kg GAN. There was a significant positive correlation between BEC and g/kg consumed ($r = 0.61$, $n = 24$, $p = 0.001$; data not shown).

*Bin analysis*

Seven data points (out of 48) throughout the entirety of the experiment were removed from bin and bout analysis due to discordances in licks per ml correlation. For the remainder of the animals, licks and g/kg intake were highly correlated across all treatment days (Pearson's r ranged from 0.86 to 0.95; $p \leq 0.001$ for all days; data not shown). There was a dose-dependent effect of GAN on total 10E licks [$F(2,16) = 12.17$, $p = 0.001$; Table 3.1], consistent with the g/kg data. To better understand the pattern of ethanol intake, we examined lick patterns by sorting the data into 20-minute bins (Fig. 3.1B). There was a dose effect of GAN, consistent with the overall lick data, and no treatment by time interaction, suggesting that the GAN-induced decreases in 10E intake were consistent throughout the 2 hour time period (dose effect: [$F(5,40) = 4.37$; $p < 0.01$]). Based on our *a priori* hypothesis that GAN and ALLO would promote the onset of drinking, even when there was no change or a decrease in overall intake (Ford et al., 2005b; Ramaker et al., 2011 (Chapter 2), Sinnott et al, 2002a), we examined the first 20 minutes of the session, split into 5-minute bins. The main effect of GAN dose did not reach statistical significance across the first 20-minute bin and there was no significant dose by bin interaction (data not shown), suggesting that GAN did not differentially alter intake across time throughout the first 20-minute bin.

*Bout analysis*

Analysis with ANOVA revealed a trend for GAN to decrease bout frequency [$F(2,16) = 3.29$, $p = 0.06$], suggesting that the decrease in intake was primarily due to a 20% reduction in the number of bouts. There was no significant effect of GAN on latency to first bout, latency to first lick, first bout size, average bout size, average lick rate, average bout length, or average inter-bout interval (IBI; see Table 3.1).

*Experiment 1b: Effect of THIP on limited access ethanol intake in lickometer chambers*

*Intake*

In the absence of THIP treatment (average of 4 vehicle days), mice drank an average of 3.08 ± 0.21 g/kg over the 2-hour period. There was no difference on g/kg ethanol intake on these days ($p = 0.432$) and so data were collapsed across these days. THIP dose dependently decreased 2-hour 10E intake [$F(4,44) = 31.63$, $p < 0.001$; Fig. 3.2A]. During the 2 hours of 2-bottle choice drinking, mice exhibited strong preference for the ethanol solution (>98% ethanol preference on all days). Water intake was: 0.013 ± 0.005 ml (65 ± 18 licks) following 0 mg/kg THIP, 0.001 ± 0.001 ml (36 ± 13 licks) following 2 mg/kg THIP, 0.004 ± 0.009 ml (62 ± 19 licks) following 4 mg/kg THIP, 0.008 ± 0.006 ml (106 ± 46 licks) following 8 mg/kg THIP, and 0.004 ± 0.004 ml (27 ± 14 licks) following 16 mg/kg THIP. As in the GAN study, water intake was below the level of reliable detection, and therefore not used in statistical analyses. There was a dose dependent increase in the 22-hour water intake following the conclusion of the 2-hour drinking session after THIP administration [$F(4,44) = 2.53$, $p = 0.05$; Table 3.2]. BECs upon the conclusion of a limited access session were 1.25 ± 0.21 mg/ml after one saline day and 0.98 ± 0.17 mg/ml after 4 mg/kg THIP. There was a significant positive correlation between BEC and g/kg consumed ($r = 0.80$, $n = 24$, $p < 0.001$; data not shown).

*Bin analysis*

Four data points (out of 84) throughout the entirety of the experiment were removed from bin and bout analysis due to discordances in licks per ml data. For the remaining data points, there was a significant correlation between licks and g/kg on each baseline and drug day (Pearson's r ranged from 0.76 to 0.95; $p \leq 0.01$ for all days; data not shown). There was a dose-dependent effect of THIP on total 10E licks [$F(4,36) =$

10.29, $p < 0.001$; Table 3.2], consistent with the g/kg intake data. To better understand the pattern of ethanol intake, we examined lick patterns by 20-minute bins (Fig. 3.2B). Examining the data in 20-minute bins revealed a significant dose by bin interaction [$F(20,180) = 1.65$, $p < 0.05$]. Main effect analysis showed a significant effect of dose during bins 1 (minutes 0-20, $p < 0.001$), 2 (minutes 20-40, $p < 0.001$), 3 (minutes 40-60, $p = 0.001$), 4 (minutes 60-80, $p = 0.05$), and 6 (minutes 100-120, $p = 0.005$). Post hoc tests revealed that the 16 mg/kg THIP dose exerted a suppressive effect on 10E intake across bins 1-4. The 8 mg/kg THIP dose significantly decreased 10E intake during bin 1. There was a trend for an increase in licks during bin 1 with the 4 mg/kg THIP dose, and this dose significantly decreased licks during bin 4. Further analysis of the first 20 minutes examined by 5-minute intervals revealed a bin by dose interaction [$F(12,108) = 2.19$; $p < 0.005$]. Post hoc analysis revealed a dose effect during the first 5 minutes [$F(4,36) = 6.91$, $p < 0.001$] with 8 m/kg ($p < 0.01$) and 16 mg/kg ($p < 0.001$) significantly decreasing licks. There was also a dose effect during minutes 10-15 [$F(4,36) = 3.28$, $p < 0.001$] with a trend for a decrease in licks at 8 mg/kg ($p = 0.10$) and 16 mg/kg THIP ($p = 0.08$; data not shown).

*Bout analysis*

Following treatment with 16 mg/kg THIP, 5 mice did not drink a complete bout so their lick data was removed from all bout analysis (except bout frequency). THIP significantly increased latency to first 10E lick [$F(4,20) = 34.38$, $p < 0.001$] and latency to first 10E bout [$F(4, 20) = 35.57$, $p < 0.001$; Table 3.2]. The 16 mg/kg THIP dose increased latency to first lick and first bout and caused some noticeable sedative effects. Interestingly, 8 mg/kg THIP increased latency to first bout but had no effect on latency to first lick (Table 3.2), suggesting a lack of a confounding sedative effect. Further, 4

mg/kg THIP led to no change in latency to first bout, and a trend for a decrease in latency to first lick, again indicating a lack of a sedative effect at this dose. THIP significantly altered bout frequency [$F(4, 36) = 17.50$, $p < 0.001$] and IBI [$F(4,20) = 5.92$, $p < 0.001$]. There was no effect of THIP on average bout size, average bout length, lick rate, or first bout size.

*Experiment 2a: Effect of GAN on operant self-administration of ethanol or sucrose*

Pretreatment intakes were 0.70 ± 0.03 g/kg ethanol and 0.40 ± 0.03 g/kg sucrose for the 10E- and 2S-reinforced mice, respectively. There was no difference between the 2 vehicle days on g/kg intake for 2S or 10E and so in each case, data were collapsed to give the 0 mg/kg GAN data.

Analyses of the 10E-reinforced mice indicated that GAN did not affect 10E intake to the level of significance, but caused a slight dose-dependent increase in intake (Fig. 3.3A); 5 mg/kg GAN increased 10E intake by 21% and 10 mg/kg GAN increased 10E intake by 33%. Latency to reinforcer (Fig. 3.3B) was decreased by 25% and 43% with 5 mg/kg and 10 mg/kg GAN, but this did not reach the level of significance. There was, however, a trend for GAN to decrease latency to first bout [$F(2,16) = 2.65$, $p = 0.10$; Table 3.3], consistent with a modest increase in the initiation of drinking. GAN also significantly reduced IBI [$F(2,12) = 5.91$, $p < 0.05$]. There was no effect of GAN on average bout size, first bout size, average lick rate, bout frequency, or average bout length.

Analyses of the 2S-reinforced mice indicated that GAN significantly altered 2S intake [$F(2,26) = 4.83$, $p < 0.05$]. However, no dose was significantly different than vehicle; 5 mg/kg GAN decreased 2S intake by 11%, whereas 10 mg/kg GAN increased 2S intake by 12% (Fig. 3.3A). There was a dose-dependent effect of GAN to decrease

the latency to acquire the reinforcer [F(2,26) = 9.14, $p$ = 0.001], which was decreased by 55% and 64% with 5 mg/kg and 10 mg/kg GAN, respectively (Fig. 3.3B). Similar to the effect on latency to acquire the reinforcer, GAN pretreatment decreased latency to the first bout [F(2,26) = 6.46, $p < 0.01$] and increased first bout size [F(2,26) = 4.604, $p < 0.05$], consistent with an enhancement in the initiation of drinking (see Table 3.3). There was also a significant effect of GAN on bout frequency [F(2,26) = 6.46, $p < 0.01$]. There was no effect of GAN on average bout size, lick rate, IBI, or average bout length.

*Experiment 2b: Effect of THIP on operant self-administration of ethanol or sucrose*

Pretreatment intakes were 0.81 ± 0.08 g/kg ethanol and 0.41 ± 0.03 g/kg sucrose in the 10E- and 2S-reinforced mice, respectively. For both groups of mice, there was a dose-dependent effect of THIP on appetitive responding, which prevented some mice from attaining the reinforcer (see Table 3.4).

For the mice that earned the reinforcer, THIP also altered the consummatory phase of 10E self-administration; THIP dose-dependently reduced g/kg 10E intake [F(2,18) = 16.49 $p < 0.001$; Fig. 3.4A]. The 12% decrease in intake following 2 mg/kg THIP was not significant, but 4 mg/kg THIP significantly decreased 10E intake by 38%. THIP also produced a dose-dependent increase in the latency to acquire the 10E reinforcer [F(2,18) = 10.94, $p$ = 0.001; Fig. 3.4B] and in latency to first bout [F(2,18) = 16.08, $p < 0.001$; Table 3.4]. The decrease in 10E intake could not be attributed to one specific parameter, but there was a trend for THIP to decrease first bout size [F(2,18) = 2.89; $p$ = 0.08; Table 3.4]. There was no effect of THIP on average bout size, bout frequency, average lick rate, average bout length, or IBI.

Intake of 2S was also significantly altered by THIP [F(2,26) = 9.39, $p$ = 0.001; Fig. 3.4A], with post-hoc tests revealing that 4 mg/kg THIP significantly decreased 2S

intake by 29%. For the mice that earned the reinforcer, THIP significantly increased latency to acquire the reinforcer [$F(2,26) = 6.44$, $p = 0.001$; Fig. 3.4B] and latency to first bout [$F(2,26) = 5.39$, $p < 0.01$; Table 3.4]. The decrease in intake appeared to be due to a decrease in average bout size [$F(2,26) = 7.02$, $p < 0.01$]. THIP also exerted significant effects on IBI [$F(2,26) = 7.02$, $p < 0.01$], first bout size [$F(2,26) = 6.39$; $p < 0.01$], and bout frequency [$F(2,26) = 4.10$, $p < 0.05$]. There was no effect of THIP on average bout length or lick rate.

**Discussion**

The current study demonstrates that a NAS analogue (GAN) and a $GABA_A$ receptor agonist with preferential activity at extrasynaptic receptors (THIP) alter ethanol intake in mice, as measured with limited-access 2-bottle choice or operant self-administration paradigms. These studies expand on the existing literature by providing analysis of lick patterns and shifts in drinking patterns following administration of GAN and THIP, as well as providing insights into the appetitive and consummatory contributions of these drugs to ethanol self-administration.

Previous studies have shown a biphasic effect of ALLO on ethanol intake under limited access procedures; that is, low physiologically relevant doses of ALLO increased ethanol intake while supraphysiological doses of ALLO decreased ethanol intake (e.g., Finn et al., 2010; Ford et al., 2007a, 2005a; Janak et al., 1998). Besheer et al. (2010) reported a similar biphasic effect of GAN on operant self-administration in rats trained on a concurrent FR schedule. They found that 1 mg/kg and 3 mg/kg GAN produced a non-significant increase in ethanol intake (g/kg estimated by number of reinforcers) of 62% and 25%, respectively. Whereas the 10 mg/kg GAN dose produced a non-significant decrease in ethanol intake of 30% (Besheer et al., 2010), we found that 10 mg/kg GAN

caused a significant 22% decrease in 2-hour ethanol intake in the present study. This pattern of decrease in ethanol consumption is consistent with data from a continuous access study in mice, where the 10 mg/kg GAN dose significantly decreased ethanol intake during the 2nd and 3rd hour of ethanol access (Ramaker, et al, 2011 (Chapter 2)). Besheer and colleagues (2010) also found that 30 mg/kg GAN significantly decreased ethanol intake by 87%, but that it also produced a motor-impairing effect. Thus, in order to prevent the motor impairment seen at high doses, the present studies used a more restricted dose-response range. Lick analysis at these doses revealed an effect of GAN throughout the entire 120-minute period, consistent with similar but extended actions as previously reported following ALLO administration (Ford et al., 2005b). A previous lickometer study using ALLO found that high doses of ALLO primarily decreased intake via reductions in bout frequency (Ford et al., 2005b). In the current study there was a trend for a reduction in bout frequency following GAN administration and a slight increase in time between bouts. These findings are consistent with our earlier work with ALLO and suggest that the significant decrease in 10E intake following GAN was primarily due to a 20% reduction in the number of bouts.

It should be noted that within the current 30-minute operant self-administration sessions, ethanol intake was modestly increased by administration of 5 and 10 mg/kg doses of GAN (↑ 21% and 33%, respectively). This result is consistent with a significant increase in ethanol intake during the 1st hour of continuous ethanol access (Ramaker et al., 2011 (Chapter 2)). Interestingly, previous studies have shown that ALLO promoted the initiation of drinking even at doses that produced no change or decreases in overall ethanol intake (Ford et al., 2005b; Ramaker et al., 2011 (Chapter 2); Sinnott et al., 2002a). For example, ALLO injections in mice led to an increase in ethanol intake in the first hour of access and a decrease in the second hour (Sinnott et al., 2002a). Likewise,

a subsequent study that utilized lickometers (Ford et al., 2005b) revealed that ALLO exerted a biphasic and time-dependent effect on ethanol intake, with increases in ethanol intake observed within the first 60 minutes of ethanol access (and in most cases, within the first 20 minutes of access). With this in mind, the slight increase in ethanol intake in the 30-minute operant session is therefore consistent with only capturing the initial increase in ethanol intake. In harmony with this idea was the current finding in the operant procedure that GAN tended to decrease latency to first bout, and appeared to modestly increase the appetitive drive to consume ethanol, as seen by a non-significant 25% and 43% decrease in time to acquire the reinforcer with 5 and 10 mg/kg GAN, respectively.

In addition to the different lengths of ethanol access in the current experiments, it is also possible that the different times of testing within the light /dark cycle contributed to the seemingly differing effects of GAN between the two current experimental paradigms. Analysis of the circadian rhythm of ALLO and other NAS has shown that brain levels peak at the start of the dark cycle in rodents (Corpéchot et al., 1997). Because the limited-access experiment was conducted during the early phase of the circadian dark cycle (which should correspond to higher endogenous levels), it is possible that the addition of exogenous GAN produced the equivalent of supraphysiological levels of NAS with a resultant decrease in 10E intake (Finn et al., 2010; Ford et al., 2005b). However, the operant experiment was run during the light cycle when endogenous ALLO levels should be at their lowest. Although speculative, the addition of exogenous GAN at this time point may have resulted in NAS levels on the ascending portion of the biphasic dose and time response curve. Thus, it is possible that the divergent time of testing during the circadian cycle and interaction with fluctuations in endogenous NAS levels, as well as the different length of experimental

sessions, contributed to the dissimilar direction of GAN's effect on 10E intake. Overall, the present results with GAN in two different procedures and lengths of ethanol access, in conjunction with existing information on the effects of GAN and ALLO on ethanol intake, provide strong support for time-dependent effects of NAS to promote the onset, and to subsequently decrease, ethanol drinking behavior.

Research examining the role of extrasynaptic $GABA_A$ receptors in ethanol sensitivity and reinforcement has produced inconclusive results. Some *in vitro* studies suggest that these extrasynaptic receptors exhibit enhanced sensitivity to ethanol, while others have failed to show a direct action of physiologically-relevant levels of ethanol (Borghese et al., 2006; Korpi et al., 2007; Olsen et al., 2007; Santhakumar et al., 2007). The δ subunit is thought to occur primarily outside of the synaptic space (Nusser et al., 1998), making it a potential target of pharmacological intervention to exclusively alter tonic inhibition. Mice deficient in the δ subunit showed decreases in ethanol intake and preference, a decrease in ethanol's anticonvulsant effect, and decreases in NAS sensitivity (Mihalek et al., 1999, 2001), indicating that the δ subunit may be important for some of the reinforcing and behavioral properties of both ethanol and NAS. Because ALLO and GAN can act at both synaptic and extrasynaptic $GABA_A$ receptors, experiments with THIP aimed to isolate the effects on ethanol intake that were attributable to actions at extrasynaptic $GABA_A$ receptors. If both THIP and GAN altered the microarchitecture of ethanol intake in a similar manner, this would provide some indirect evidence that NAS were altering ethanol intake via an action at extrasynaptic $GABA_A$ receptors.

There have been contradictory results related to the effects of THIP on ethanol consumption. Early work (Boyle et al., 1992, 1993) found that 16 mg/kg THIP increased

the acquisition and intake of ethanol in male Long Evans rats. We recently found that 8 and 16 mg/kg THIP significantly decreased ethanol intake in C57BL/6J mice during the first 5 hours of a continuous access procedure (Ramaker et al., 2011 (Chapter 2)). A decrease in 1-hour binge ethanol intake following 8 and 16 mg/kg THIP also was reported in C57BL/6J mice (Moore et al., 2007). The current limited-access study showed no effect on 2-hour ethanol intake with 2 or 4 mg/kg THIP, and a decrease in intake with 8 and 16 m/kg THIP, while the current operant self-administration study revealed a significant decrease in ethanol intake following administration of 4 mg/kg THIP (and as mentioned previously, administration of 8 mg/kg resulted in only one mouse gaining access to the 10E sipper). The effect of the high THIP doses (e.g., 8 and 16 mg/kg) to suppress ethanol intake in various mouse models is therefore consistent across studies. Since the 4 mg/kg THIP dose only decreased operant ethanol self-administration, it is possible that the instrumental responding that was required to gain access to the ethanol solution enhanced the sensitivity to the behavioral effects of this dose of THIP. Consistent with the 38% decrease in ethanol self-administration there was a 192% increase in the latency to acquire the reinforcer and a 238% increase in the latency to complete the first ethanol bout.

One potential explanation for this sensitivity shift between experiments is that local levels of GABA and extrasynaptic receptor density can alter the potency of THIP and NAS (Houston et al., 2012). Thus, although speculative, it is possible that the different experimental parameters (i.e., instrumental versus free access) employ slightly divergent brain regions and neural networks and that local differences in GABA levels or receptor density may contribute to differences seen in sensitivity to drugs across paradigms. Another potential explanation for the variation between procedures in the effect of the 4 mg/kg THIP dose may be a shift in the dose-response curve due to

differences in sensitivity to pharmacological manipulation across intake levels. Importantly, at the end of the 2-hour lickometer study, mice achieved physiologically relevant BECs of > 100 mg/dl that are associated with the criteria for binge drinking (i.e., > 80 mg/dl), whereas data from our laboratory suggest that BECs likely would be much lower following a 30-minute operant self-administration session (e.g., average BECs of 30 or 37 mg/dl, range of BECs from 0 – 107 mg/dl; Ford et al., 2007a, 2007b). Further work is necessary to elucidate shifts in sensitivity between paradigms, but together, the current studies corroborate earlier findings of a suppressive effect of THIP on ethanol intake and support a role for the involvement of extrasynaptic $GABA_A$ receptors in ethanol consumption.

One of the advantages of the current experiments was the ability to examine bouts of consumption and individual lick patterns that have not been represented in previous paradigms that merely measure overall intake. Additionally, the RR schedule contingency provided insight into the appetitive and consummatory processes and their respective contributions to changes in ethanol intake. Specifically, in the limited-access study, the latency to first bout was increased following 8 and 16 mg/kg THIP, indicating a decrease in the initiation of drinking following THIP treatment. Also, latency to acquire the reinforcer (and also latency to first bout) was increased by 4 mg/kg THIP in the operant study. Together these studies indicate that the decreases in intake following THIP in the current procedures may be partly explained by THIP's effect to decrease the appetitive drive to consume ethanol, as well as to decrease the onset of ethanol consumption.

Potential complications that arise with the use of $GABA_A$ receptor-specific drugs involve sedative and non-specific motor effects that may confound interpretations of

effects on ethanol drinking behavior. We deliberately limited our range of GAN doses to test, based on locomotor suppressive effects that were reported following 30 mg/kg GAN (Besheer et al., 2010). With regard to THIP, ataxia has been reported following 5 mg/kg THIP in CF1 mice (Madsen et al., 2011), while another study showed an effect of THIP on ataxia after 10 and 30 mg/kg, but not 5 mg/kg THIP (Herd et al., 2009). In the present studies, locomotor activity was not directly measured, and low baseline responding on the inactive lever (<1 per animal per session) precluded its use as a tool to assess non-specific motor effects, so we cannot exclude an effect of THIP-elicited motor impairment on the intake of ethanol in the operant paradigm. However, given that there was no effect on latency to first lick following 8 mg/kg THIP and a trend for a *decrease* in latency to first lick at 4 mg/kg THIP, it is unlikely that the decrease in intake in the lickometer experiment was confounded by locomotor suppression at either of these doses. On the other hand, administration of the 16 mg/kg THIP dose caused observable sedative effects that likely contributed to the decrease in ethanol intake.

Another consideration in the interpretation of the THIP data is whether the doses employed produced concentrations that would selectively activate extrasynaptic $GABA_A$ receptors. In the present study, injections of 16 mg/kg THIP caused a strong sedative effect and likely led to activation of both synaptic and extrasynaptic receptors, consistent with data showing that 15 mg/kg THIP caused ataxia, analgesia, and sedation in both wild-type and $\alpha_4$ subunit (paired with δ) knockout mice (Chandra et al., 2006). On the other hand, injections of THIP doses of up to 10 mg/kg in rats produced peak CNS levels within minutes, and levels were in the low micromolar range by 30 minutes post-injection (Cremers and Ebert, 2007). Given that these low micromolar THIP concentrations (2-5 μM) act very selectively, if not exclusively, at δ subunit containing receptors (Stórustovu and Ebert, 2006), we believe that the 2 – 8 mg/kg doses of THIP that were employed in

the present study exerted selective effects at extrasynaptic $GABA_A$ receptors. Importantly, these studies extend existing information to indicate that doses of THIP that act preferentially at extrasynaptic $GABA_A$ receptors modulate ethanol intake in multiple paradigms, including a 24-hour access and limited-access 2-bottle choice procedure, a single bottle drinking in the dark procedure, and operant self-administration procedure (Moore et al., 2007; Ramaker et al., 2011(Chapter 2)).

One important consideration in studying drugs that affect ethanol intake is the generalizability to other calorie containing solutions or to sweetened solutions. In the present operant self-administration study, GAN appeared to enhance the appetitive phase of sucrose self-administration, based on the significant decrease in the latency to acquire the reinforcer. While sucrose intake was not systematically affected by GAN in the present study, a study in NSA mice found a biphasic effect of GAN on sucrose intake (↑ with a 4 mg/kg dose, no change with 8 mg/kg, and ↓ at a 16 mg/kg dose, Vanover et al., 2000). Thus, use of a larger range of doses in the present study may have revealed significant effects on sucrose intake. None-the-less, this is the first study directly comparing the effect of GAN on ethanol self-administration to that of a non-drug natural reinforcer. Similarly, there is also conflicting evidence on the specificity of ALLO to alter intake of sucrose and saccharin, which may be influenced by caloric differences, species differences (rat versus mouse), procedural differences (operant versus 2-bottle choice), or availability of a concurrent reinforcer (Janak and Gill 2003; Sinnott et al., 2002a). There is some evidence to suggest that local increases in GABA concentration or administration of $GABA_A$ receptor agonists stimulate feeding behavior (Kelley et al., 2005; Stratford and Kelley, 1997). Therefore a non-specific effect on consumption by GABAergic drugs could be due to the stimulation of systems that trigger increases in caloric intake. Similarly, the effect of THIP to decrease intake in the present study was

not specific to ethanol self-administration, as intake of sucrose and latency to acquire the sucrose reinforcer were altered by THIP in a comparable manner to that of ethanol. Consistent with this, Moore et al. (2007) also found that 8 and 16 mg/kg THIP decreased 1-hour water intake in mice. To our knowledge, this is the first study examining the generalizability of THIP to intake of a sweetened reinforcer. However, data following brain region-specific knockdown of extrasynaptic $GABA_A$ receptor subunits ($\alpha_4$ and δ) have illustrated that the subunit knockdown produced a selective decrease in ethanol intake that did not extend to sucrose intake in rats (Nie et al., 2011; Rewal et al., 2009, 2011). Clearly, further studies examining the specificity of the regulatory role of extrasynaptic $GABA_A$ receptors on ethanol self-administration versus a natural reinforcer are necessary.

*Conclusions*

The present results add to an abundance of data showing a relationship between NAS levels, ethanol consumption, and ethanol-induced behaviors. Further, these data corroborate a growing body of literature suggesting that extrasynaptic $GABA_A$ receptors may be an important target of ethanol and may regulate ethanol intake. Further investigation into the contributions of NAS and extrasynaptic $GABA_A$ receptors to the reinforcing properties of ethanol are necessary. GAN is currently in phase II clinical trials for treatment of convulsions and post-traumatic stress disorder. Until recently, THIP was in phase III clinical trials for use as a treatment for insomnia, but it currently is in a phase II clinical trial as a combination therapy with escitalopram in major depressive disorder. Therefore, continued investigation of these (or similarly acting) drugs as possible therapeutic agents is valuable. A more complete profile of the manner in which

GAN and THIP affect ethanol reinforcement and intake in various preclinical models could lead to novel therapeutic strategies for the treatment of alcohol use disorders.

**Figure 3.1. Effect of systemic GAN on 2-hour 10E intake**

Effect of GAN on 2-hour intake of 10E, measured in A) g/kg and as B) number of licks per 20-minute bin. The 0 mg/kg treatment is the average of two vehicle injections. Values represent the mean ± SEM for 12 mice. $^{**}p \leq 0.01$ versus within-subjects vehicle.

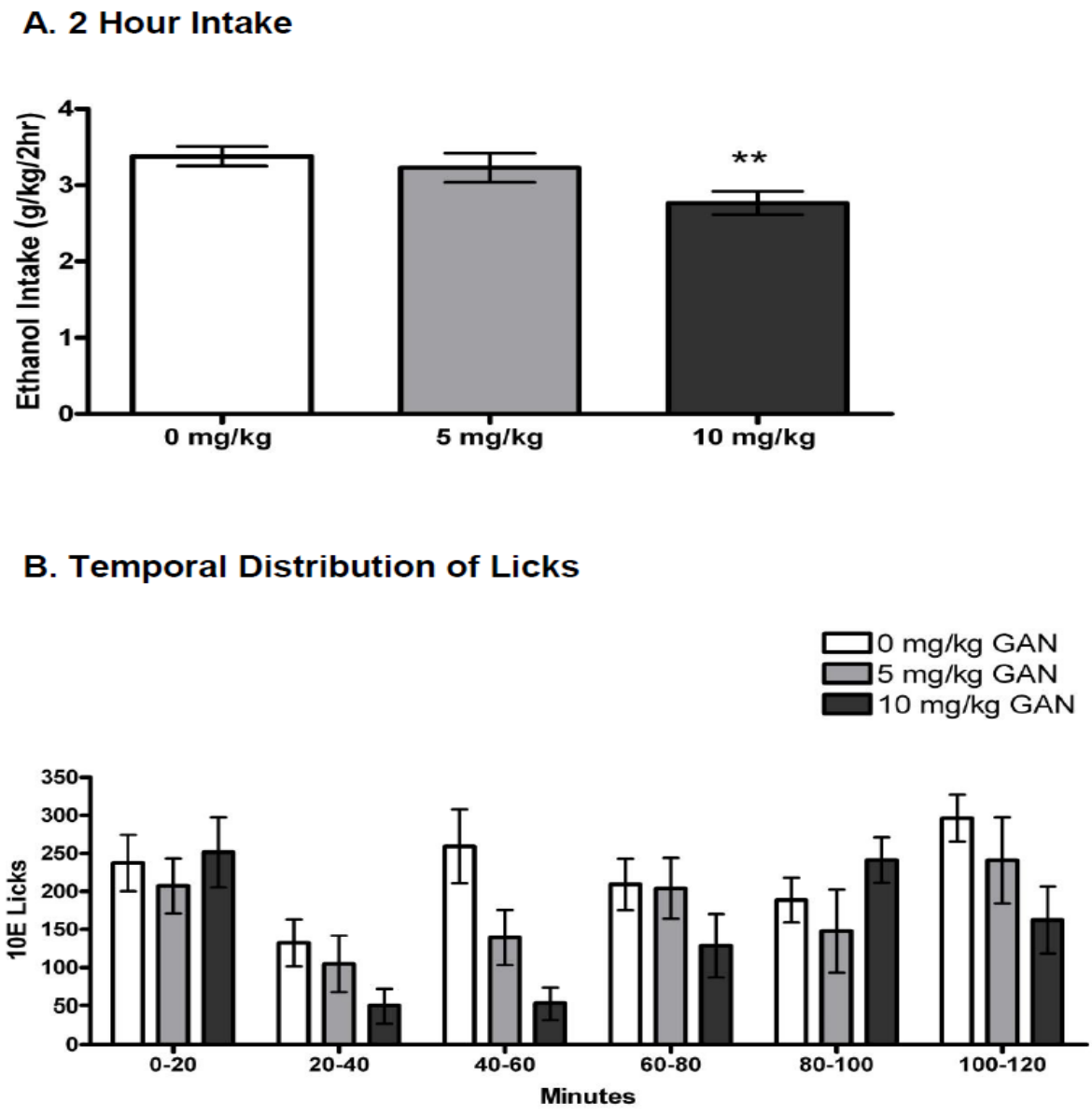

**Figure 3.2. Effect of systemic THIP on 2-hour 10E intake**

Effect of THIP on 2-hour intake of 10E, measured in A) g/kg and as B) number of licks per 20-minute bin. The 0 mg/kg treatment is the average of the four saline days that immediately preceded a drug day. Values represent the mean ± SEM for 12 mice. $^{+}p <$ 0.10, $*p < 0.05$, $**p \leq 0.01$, $***p \leq 0.001$ versus within-subjects vehicle.

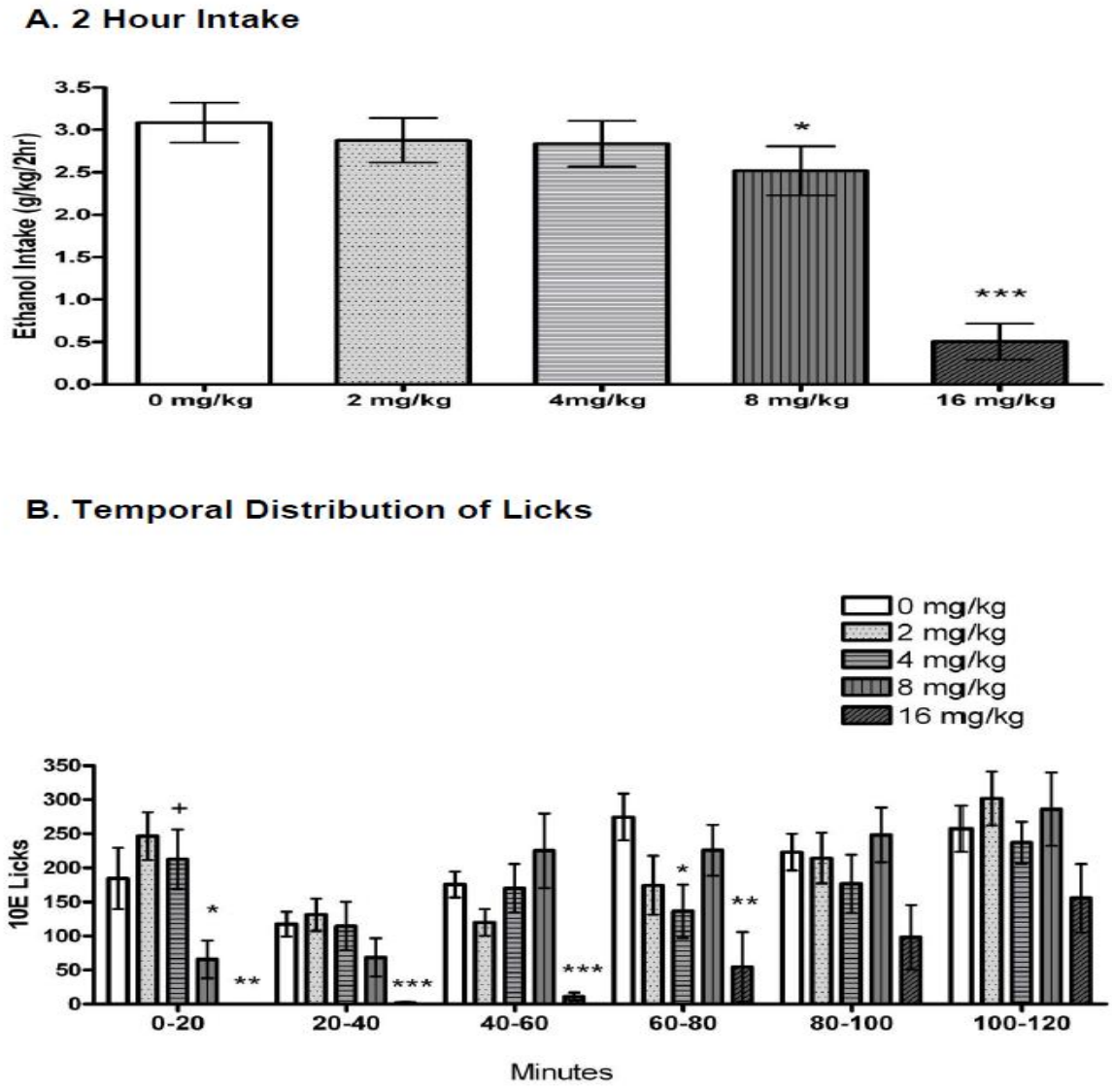

**Figure 3.3. Effect of GAN on operant self-administration**

Effect of GAN on 30-minute operant self-administration of 10E or 2S, measured in A) g/kg. B) The effect of GAN on latency to complete the RR 16 and acquire access to the reinforcer is shown in seconds. Values represent the mean ± SEM for 14 mice (10E) and 9 mice (2S). *$p \leq 0.05$, **$p \leq 0.01$ versus within-subjects vehicle.

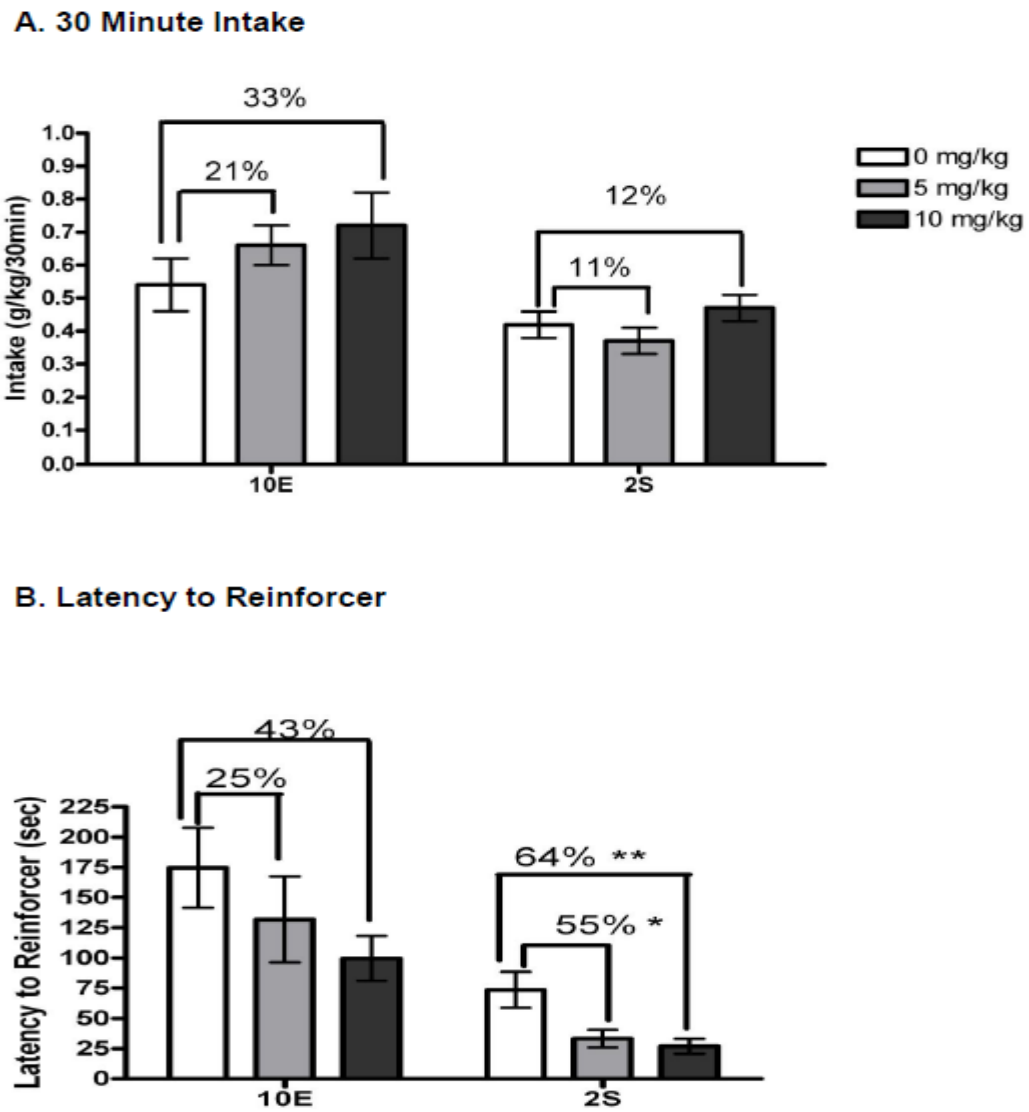

**Figure 3.4. Effect of THIP on operant self-administration**

Effect of THIP on 30 minute operant self-administration of 10E or 2S measured in A) g/kg. B) The effect of THIP on latency to complete the RR 16 and acquire access to the reinforcer is shown in seconds. Values represent the mean ± SEM for 17 mice (10E) and 12 mice (2S) following vehicle (see Table 4 for n/group following each dose of THIP). *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ versus within-subjects vehicle.

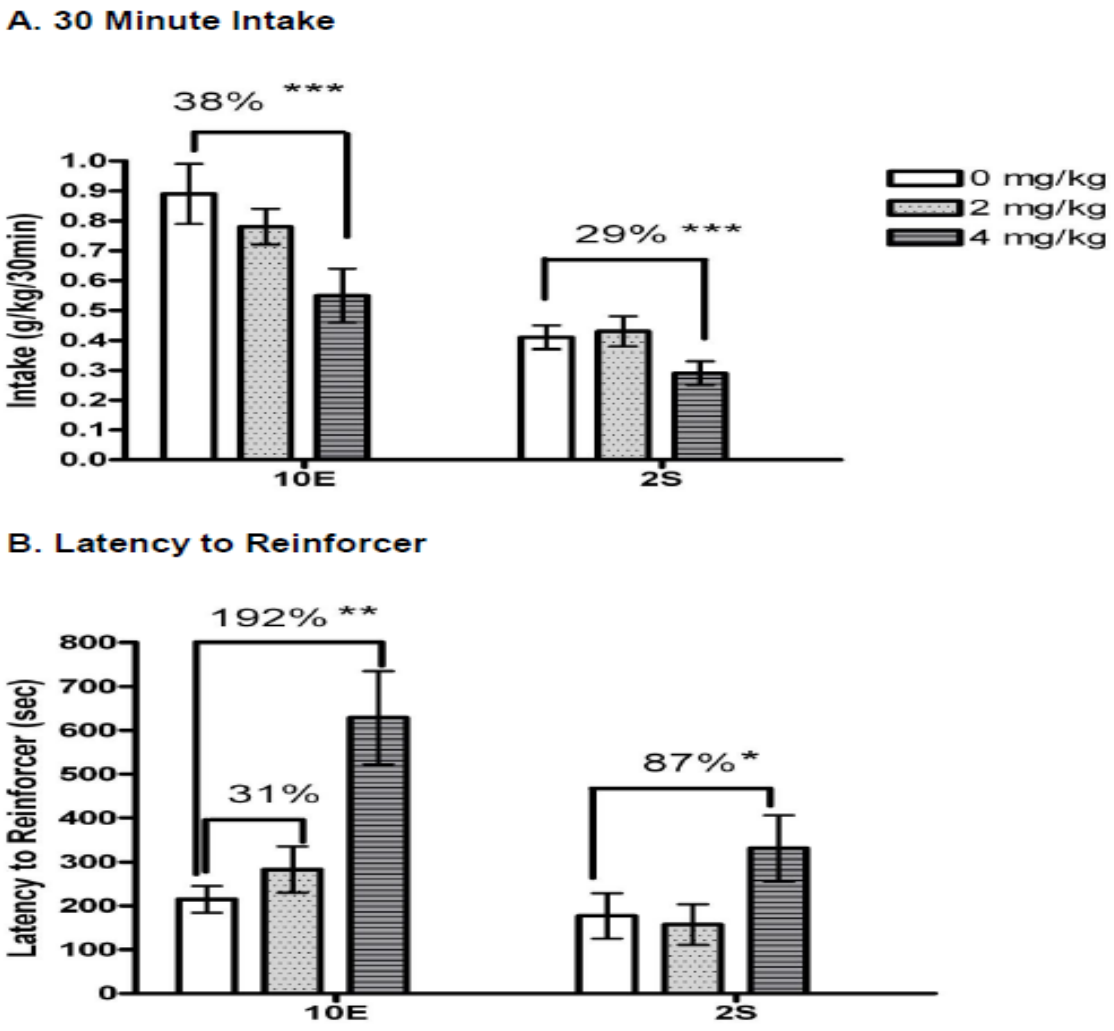

**Table 3.1. Effects of GAN on 10E bout parameters for 2-hour limited access**

Values represent the mean ± SEM for 12 mice depicted in Fig. 3.1. First and average bout size = licks/bout; bout frequency = bouts/session. $^{+}p \leq 0.10$, $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$ versus within-subjects vehicle.

| **DOSE GAN** | **0 mg/kg** | **5 mg/kg** | **10 mg/kg** |
|---|---|---|---|
| 2-hr 10E licks | 1322 ± 91 | 1043 ± 94** | 886 ± 93*** |
| 22-hr water intake (ml) | 3.25 ± 0.2 | 3.85 ± 0.2* | 3.58 ± 0.2+ |
| Latency to first lick (min) | 1.7 ± 0.7 | 4.7 ± 3.9 | 3.4 ± 2.3 |
| Latency to first bout (min) | 2.5 ± 1.1 | 4.7 ± 3.9 | 7.7 ± 4.4 |
| First bout size | 152 ± 23 | 133 ± 15 | 177 ± 20 |
| Ave bout size | 113 ± 6 | 116 ± 10 | 108 ± 17 |
| Bout frequency | 12.1 ± 1.1 | 9.6 ± 1.2 | 9.5 ± 1.7 |
| Ave lick rate (licks/min) | 366 ± 16 | 346 ± 21 | 324 ± 24 |
| Ave bout length (s) | 34.7 ± 4 | 34.0 ± 5 | 35.0 ± 8 |
| IBI (min) | 10.8 ± 1.0 | 14.8 ± 3.0 | 14.1 ± 1.9 |

**Table 3.2. Effects of THIP on 10E bout parameters for 2-hour limited access**

Values represent the mean ± SEM for mice depicted in Fig. 3.2. First and average bout size = licks/bout; bout frequency = bouts/session. n = 12, but note that only 7 mice could be analyzed at 16 mg/kg THIP due to an absence of bouts for the other 5 mice. $^{+}p \leq 0.10$, $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$ versus within-subjects vehicle.

| **DOSE THIP** | **0 mg/kg** | **2 mg/kg** | **4 mg/kg** | **8 mg/kg** | **16 mg/kg** |
|---|---|---|---|---|---|
| 2-hr 10E licks | 1246 ± 112 | 1072 ± 98 | 1047 ± 113 | 1120 ± 168 | 321 ± 123*** |
| 22-hr water intake (ml) | 3.32 ± 0.1 | 3.30 ± 0.2 | 3.10 ± 0.2 | 3.64 ± 0.1** | 3.68 ± 0.2* |
| Latency to first lick (min) | 3.0 ± 1.3 | 5.4 ± 3.0 | 0.43 ± 0.2$^{+}$ | 4.2 ± 1.8 | 56.4 ± 8.5*** |
| Latency to first bout (min) | 4.9 ± 1.6 | 5.4 ± 3.0 | 5.3 ± 3.6 | 26.6 ± 7.5* | 80.1 ± 13.8*** |
| First bout size | 78 ± 12 | 79 ± 11 | 102 ± 32 | 71 ± 17 | 72 ± 29 |
| Ave bout size | 80 ± 5 | 83 ± 7 | 76 ± 5 | 81 ± 4 | 101 ± 31 |
| Bout frequency | 15.5 ± 1.0 | 13.0 ± 0.9* | 13.6 ± 1.1 | 13.4 ± 1.7 | 3.7 ± 1.4*** |
| Ave lick rate (licks/min) | 282 ± 21 | 327 ± 256 | 277 ± 26 | 282 ± 22 | 264 ± 44 |
| Ave bout length (s) | 45.1 ± 5.3 | 35.6 ± 6.6 | 40.1 ± 5.0 | 38.3 ± 4.9 | 54.3 ± 26 |
| IBI (min) | 7.5 ± 0.6 | 9.2 ± 0.8 * | 8.4 ± 0.7 | 7.5 ± 1.1 | 4.4 ± 1.0$^{+}$ |

**Table 3.3. Effects of GAN on 10E and 2S bout parameters for operant self-administration**

Values represent the mean ± SEM for mice depicted in Fig. 3.3. First and average bout size = licks/bout; bout frequency = bouts/session. $^{+}p \leq 0.10$, $*p \leq 0.05$, $**p \leq 0.01$ versus within-subjects vehicle.

| **Reinforcer** | **10E** | | | **2S** | | |
|---|---|---|---|---|---|---|
| **Dose GAN** | **0 mg/kg** | **5 mg/kg** | **10 mg/kg** | **0 mg/kg** | **5 mg/kg** | **10 mg/kg** |
| No. mice that earned reinforcer | 9 | 9 | 9 | 14 | 14 | 14 |
| Latency to first bout (s) | 191 ± 32 | 133 ± 35 | 100.8 ± 18 | 70.8 ± 16 | 38.4 ± 7$^{+}$ | 25.8 ± 5** |
| Ave bout size | 62 ± 10 | 94 ± 20 | 76 ± 10 | 132 ± 20 | 100 ± 14 | 178 ± 38 |
| Bout frequency | 3.2 ± 0.6 | 2.9 ± 0.5 | 3.2 ± 0.5 | 5.8 ± 0.5 | 6.5 ± 0.5 | 4.9 ± 0.5$^{+}$ |
| Ave lick rate (licks/min) | 222 ± 26 | 145 ± 54 | 98 ± 21 | 80 ± 16 | 101 ± 20 | 86 ± 26 |
| Ave bout length (min) | 0.83 ± 0.2 | 1.5± 0.5 | 1.5 ± 0.1 | 3.4 ± 0.5 | 2.84 ± 0.4 | 4.6 ± 0.7 |
| IBI (min) | 8.4 ± 1.4 | 3.0 ± 0.9* | 5.2 ± 1.0$^{+}$ | 2.6 ± 0.3 | 3.0 ± 0.9 | 1.9 ± 0.2 |
| First bout size | 83 ± 14 | 120 ± 20 | 126 ± 16 | 307 ± 41 | 301 ± 59 | 497 ± 107***** |

**Table 3.4. Effects of THIP on 10E and 2S bout parameters for operant self-administration**

Values represent the mean ± SEM for mice depicted in Fig. 3.4. First and average bout size = licks/bout; bout frequency = bouts/session. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ versus within-subjects vehicle.

| **Reinforcer** | **10E** | | | | **2S** | | | |
|---|---|---|---|---|---|---|---|---|
| **Dose THIP** | **0 mg/kg** | **2 mg/kg** | **4 mg/kg** | **8 mg/kg** | **0 mg/kg** | **2 mg/kg** | **4 mg/kg** | **8 mg/kg** |
| No. mice that earned reinforcer | 12 | 11 | 11 | 1 | 17 | 17 | 14 | 3 |
| Latency to first bout (s) | 234 ± 35 | 286 ± 53 | 792 ± 126** | --- | 198 ± 54 | 157 ± 57 | 340 ± 78* | --- |
| Ave bout size | 67 ± 5 | 62 ± 6 | 53 ± 6 | --- | 126 ± 19 | 142 ± 30 | 70 ± 9***** | --- |
| Bout frequency | 4.5 ± 0.5 | 4.6 ± 0.6 | 3.7 ± 0.5 | --- | 5.8 ± 0.5 | 5.5 ± 0.6 | 6.6 ± 0.5 | --- |
| Ave lick rate (licks/min) | 153 ± 30 | 103 ± 42 | 152 ± 37 | --- | 110 ± 18 | 106 ± 20 | 126 ± 23 | --- |
| Ave bout length (min) | 1.2 ± 0.2 | 1.5 ± 0.3 | 0.84 ± 0.2 | --- | 9.0 ± 5.9 | 4.1 ± 1.0 | 1.9 ± 0.3 | --- |
| IBI (min) | 4.8 ± 0.8 | 5.1 ± 0.8 | 7.7 ± 1.7 | --- | 3.4 ± 0.6 | 2.4 ± 0.4***** | 3.4 ± 0.6 | --- |
| First bout size | 96 ± 10 | 92 ± 10 | 66 ± 12 | --- | 264 ± 33 | 266 ± 54 | 138 ± 33* | --- |

# CHAPTER 4: EFFECT OF NUCLEUS ACCUMBENS SHELL INFUSIONS OF GANAXOLONE OR THIP ON ETHANOL INTAKE

This chapter has been modified from a submitted manuscript:

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**Abstract**

ALLO is an endogenous NAS thought to alter the reinforcement value of ethanol. Additionally, extrasynaptic $GABA_A$ receptors may be a particularly sensitive target of ethanol and NAS. Previous work and results in chapter 3 showed that systemic injections of an ALLO analog, GAN, or an extrasynaptic $GABA_A$ receptor agonist, THIP, altered ethanol intake in male mice with limited access to ethanol, but the underlying brain areas are unknown. The present studies tested whether the NAc shell was a sufficient brain region in mediating this effect. C57BL/6J mice were given a 2-hour 2-bottle choice of 10E or water, and ethanol intake was measured following site-specific infusions of GAN or THIP. Decreases in limited-access intake of ethanol were seen following site-specific infusions of either drug into the NAc shell, although there appeared to be important order effects of THIP. Significant changes in intake were absent when the drugs were infused dorsally to the target site (GAN) or into the lateral ventricle (THIP). These data support a role for the NAS environment and extrasynaptic $GABA_A$ receptor activation in the NAc shell to influence ethanol consumption in mice.

## Introduction

ALLO is an endogenous NAS that acts as a potent positive modulator at the $GABA_A$ receptor. ALLO levels are increased in the plasma and brains of rodents following oral or systemic ethanol administration (VanDoren et al., 2000; O'Dell et al., 2004; Porcu et al., 2010; Finn et al., 2004; Eva et al., 2008). Systemic or ICV infusions of ALLO have been shown to alter ethanol intake in a variety of procedures in rodents, indicating that ALLO may alter some of the reinforcing effects of ethanol (Ford et al., 2005b, 2007a; Sinnot et al., 2002a; Janak et al., 1998; Janak and Gill, 2003). Recently, work with GAN, a synthetic analog of ALLO, demonstrated similar but longer-duration

effects on ethanol intake in rodents as had been demonstrated with ALLO (Besheer et al., 2010; Ramaker et al., 2011, 2012 (Chapters 2 and 3)). GAN is structurally identical to ALLO with the exception of an added 3β methyl group, extending the half-life without changing the primary pharmacological or behavioral properties (Carter et al., 1997; Ungard et al., 2000). However, the brain regions underlying the effect of GAN or ALLO to mediate ethanol intake remains unknown.

The NAc has been postulated to be an important brain area mediating ethanol intake (Koob, 1992; Hodge et al., 1995). In particular, rats self-administered ethanol into the NAc shell, but not the core, and electrolytic lesion of the shell decreased ethanol intake (Englemann et al., 2009; Dhaher et al., 2009). A $GABA_A$ receptor agonist or antagonist infused into the NAc shell also decreased ethanol intake (Stratford and Wirtshafter, 2011; Hyytia and Koob, 1995; Eiler and June, 2007,) indicating that $GABA_A$ receptors in the NAc shell may underlie some of ethanol's reinforcing effects.

$GABA_A$ receptors are pentameric chloride channels made up from a pool of at least 16 possible subunits: $\alpha_{1-6}$, $\beta_{1-3}$, $\gamma_{1-3}$, δ, ε, π, and θ. The most common stoichiometry is 2α:2β:γ, but in about 5% of $GABA_A$ receptors in the brain, the γ is replaced with a δ subunit (McKernan and Whiting, 1996). The δ subunit is most often paired with $\alpha_{4-6}$ and $\beta_{2-3}$ and is located exclusively in the extrasynaptic space, where it is thought to contribute solely to tonic inhibition (Farrant and Nusser et al., 2005). *In vitro* work suggests that $GABA_A$ receptors containing the δ subunit may show enhanced sensitivity to ethanol and NAS (Mody et al., 2007; Olsen et al. 2007; Santhakumar et al., 2007; Belelli et al., 2002). Similarly, δ-subunit knockout mice showed reduced sensitivity to NAS and ethanol and self-administered less ethanol than their wild type counterparts (Mihalek et al., 1999, 2001). Additionally, a $GABA_A$ receptor agonist with selectivity for

the δ subunit, THIP, altered ethanol consumption in mice (Moore et al., 2007; Ramaker et al., 2011, 2012 (Chapters 2 and 3)). The brain areas underlying this effect are unknown, but knockdown of the $\alpha_4$ or δ subunit in the NAc shell, but not the core, decreased ethanol intake in rats (Rewal et al., 2009, 2011; Nie et al., 2011). These studies suggest that extrasynaptic $GABA_A$ receptors, specifically in the NAc shell, may be an important component of ethanol consumption.

Immunoreactivity for ALLO, as well as the two enzymes needed for metabolism of progesterone to ALLO, have been detected in the NAc (Saalman et al., 2007; Cook et al., 2013; Agis-Balboa et al., 2006). ALLO infused into the NAc substituted for the discriminative effects of ethanol (Hodge et al., 2001), providing rationale that the NAS environment in this brain region was important for perceiving the stimulus effects of ethanol. However, the role of NAS in the NAc to alter ethanol intake has not been reported.

The present study aimed to determine the importance of the NAS environment in the NAc shell on ethanol consumption by locally infusing various doses of GAN. Given the suggestion that NAS such as ALLO may preferentially act at extrasynaptic $GABA_A$ receptors, and given the role of the extrasynaptic receptors in the NAc shell on ethanol consumption, the second goal was to determine the effect of preferentially activating extrasynaptic $GABA_A$ receptors in the NAc shell on ethanol consumption by locally infusing various doses of THIP. The specific hypothesis was that activation of $GABA_A$ receptors in the NAc shell would be sufficient to mimic the alterations in ethanol consumption in mice that had been observed following systemic injections of GAN and THIP in a limited-access 2-bottle choice procedure (Ramaker et al., 2012 (Chapter 3)).

## Materials and Methods

### Animals

Male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) of approximately 8 weeks of age at the start of the study, were used for all studies. For drinking studies, mice were singly housed and acclimated to lickometer chambers on a reverse 12-hour light:12-hour dark cycle (lights off at 0600) for a week prior to any surgery or ethanol access. All mice were provided *ad libitum* access to rodent chow and water throughout the experiments. Experiments were run Monday through Friday, and mice were weighed and handled daily on those days. For locomotor studies, mice were pair housed with *ad libitum* access to food and water and were maintained on a 12-hour light:12-hour dark cycle (lights on at 0600). All efforts were made to minimize animal suffering and to reduce the number of mice used. All procedures were approved by the local Institutional Animal Care and Use Committee and complied with NIH guidelines.

### Apparatus

Lickometer chambers have been described previously (Ford et al., 2005a; 2005b, 2008; Ramaker et al., 2011, 2012 (Chapters 2 and 3)). Briefly, lickometer chambers are composed of a 4-walled plexiglas insert positioned on an elevated wire grid floor inside a shoebox cage with bedding. The insert contains a hinged top and two small holes where the sippers of the drinking bottles protrude into the cage. The wire floor of the chamber and each sipper forms an open electrical circuit that is connected to a lickometer device (MED Associates Inc., St. Albans, VT). Every time an animal makes contact with the sipper, an electrical circuit is completed and a lick is recorded. Lickometers were interfaced to an IBM computer running MED-PC IV software (MED Associates Inc.) to record time-stamped licks for each individual animal and each fluid presented.

*Locomotor chambers:*

Locomotor chambers were described in detail previously (Phillips et al., 1995; Gubner et al., 2013). Sixteen automated locomotor chambers (40 x 40 x 30 cm; AccuScan Instruments Inc., Columbus, OH, USA) were used, which were equipped with 8 photocell beams 2 cm above the floor. Horizontal distance traveled (cm) was recorded with VERSADAT software (AccuScan Instruments Inc.). A fan in each chamber provided ventilation and background noise, and each chamber contained a 3.3 W incandescent light bulb during activity testing.

Drugs

Ethanol (200 proof; Pharmco Products, Brookfield, CT) solutions were prepared by dilution in tap water. GAN was purchased from Dr. Robert Purdy (VA Research Foundation, San Diego, CA). To make the GAN solution, GAN was weighed to the required amount per concentration and mixed with 200 mg 20% β- cyclodextrin (Cargill Inc., Cedar Rapids) in a 2.0 ml Eppendorf Safe-Lock tube. The tube and contents were weighed and the drug and host were then complexed in 1.0 ml of 200 proof ethanol (Decon Labs, King of Prussia, PA) for 1 hr at ambient temperature. The solvent was then evaporated off using a Speed-Vac Concentrator until the weight of the tube was within +/- 1% of the original weight, resulting in a single transparent crystal of GAN complexed into 20% β- cyclodextrin. Immediately prior to use, the complex was solubilized into 1.0 ml of 3% DMSO (Mallinckrodt Baker, Inc., Paris, KY) in high-purity water, yielding a 3% DMSO/20% β-cyclodextrin solution with GAN at the necessary concentration to achieve infusions of doses ranging from 10 – 500 ng/side. Vehicle, or 0 ng, was a 3% DMSO/20% β-cyclodextrin solution for GAN experiments. THIP was purchased from Tocris Bioscience (Ellisville, MO) and dissolved in artificial cerebral

spinal fluid (aCSF) at concentrations to achieve infusions of doses ranging from 10 – 1000 ng. Vehicle, or 0 ng, was aCSF for THIP experiments.

Surgery

For NAc shell placements, mice were kept under isoflurane anesthesia and implanted with 2 independent guide cannulae (26. Ga. Stainless steel, 12.0 mm long) aimed at the nucleus accumbens shell. Coordinates used were A/P: +1.34, L/M: ± 0.5, D/V -2.75 and the tips of the injectors extended 2.0 mm beyond the guide shaft. For ICV placements, mice were implanted with a unilateral guide cannula at A/P: -0.46, L/M: + or - 1.2, D/V -2.0 and the tips of the injectors extended 0.5 mm past the guide shaft. Stylets were kept in the cannulae to keep them free of debris. Mice were allowed a minimum of a week to recover from surgery before being used in any experiment.

Drug infusions

Mice were gently restrained by hand, the microinjectors were lowered to the target site, and 200 nL of fluid was delivered over 60 seconds. Injectors remained in place for an additional 30 seconds before being removed and replaced with stylets (Gililland-Kaufman et al., 2008; Tanchuck et al., 2013).

General Drinking Experiment

For the 22-hours outside of the 2 hour period of ethanol-access, two 50-ml water bottles were kept on each cage. Two hours into the dark cycle, the 50-ml water bottles were removed from the cages and weighed to assess overall 22 hour fluid intake. Mice were weighed, or weighed and infused with drug (see Drug infusion above), and immediately returned to their cage. Each mouse was given access to both a 10 ml bottle

of 10E and a 10 ml bottle of tap water for the next 2 hours. The 10E bottles were counterbalanced between the left and right sides across lickometer chambers to minimize the influence of side preference; each individual animal had its 10E bottle on the same side for the duration of the experiment to allow consistent drinking routines to become established. At the conclusion of the 2 hour period of 10E access, fluid consumed was measured (to the nearest 0.05 ml), and the 10 ml bottles were replaced with the 50 ml water bottles. To control for leakage, bottles were placed on two control cages with no animals present, and the amount lost from those bottles was averaged and subtracted from the amount consumed from each bottle.

*Experiment 1: Effect of NAc shell infusion of GAN on 10E intake*

For the GAN drinking experiments, a total of 15 C57BL/6J mice with confirmed placements in the NAc shell were used across 4 different experimental passes. In pass 1 (n = 2 confirmed placements), animals had been used in a previous experiment and had a total of 11 weeks of 10E drinking experience before surgery and 3 weeks of 10E access following surgery. Of these 2 animals, 1 had a previous history of systemic THIP and 1 had a previous history of systemic GAN; there were 7 weeks separating their last systemic injection from their first infusion. GAN was infused weekly in the following order, with baseline 10E intake re-established before each infusion: 0 ng (pre), 50 ng, 100 ng, 10 ng, 500 ng, 0 ng (post) GAN. Because 10 ng was only given to 2 of the animals and did not appear to cause an effect on 10E intake, this dose was not repeated in subsequent experimental passes and not analyzed. In pass 2 (n = 4 confirmed placements), animals had 5 weeks of 10E access prior to surgery. Mice had a week of recovery from surgery, followed by 4 more weeks of 10E access prior to any drug treatment. GAN was infused weekly in the following order, with baseline 10E intake re-

established before each infusion: 0 ng (pre), 500 ng, 100 ng, 50 ng, 1000 ng, 0 ng (post). Data with 1000 ng GAN were not analyzed, as we had trouble getting this high dose into solution, and it continually clogged injectors, making complete infusions questionable. For passes 3 and 4 (n = 5 and 4 confirmed placements), animals underwent surgery prior to any 10E experience. This was done to minimize the length of experiments in view of the fact that in the previous 2 passes, there was no change in baseline intake before or after surgery. Mice had a week of recovery from surgery followed by 5 weeks of 10E access before any infusions. GAN was infused weekly in the following order, with baseline intake re-established before each infusion: 0 ng (pre), 50 ng, 100 ng, 500 ng, 0 ng (post). Across all cohorts, there was no difference on intake between 0 ng (pre) and 0 ng (post) so these data were collapsed. There was no effect of pass and no cohort by pass interaction. Overall, collapsed data among the 4 passes was analyzed using repeated-measures ANOVA with four levels: 0 ng (average), 50 ng, 100 ng, 500 ng and a total of 13 animals.

*Experiment 2: Effect of NAc shell infusion of THIP on 10E intake*

For THIP drinking experiments, a total of 13 mice with confirmed placements were analyzed across 3 different experimental passes. In pass 1 (n = 5), animals had been used in a previous experiment and had a total of 11 weeks of 10E drinking experience before surgery and 3 weeks of 10E access following surgery. Of these, 2 had a previous history of systemic THIP and 3 had a previous history of systemic GAN; there were 7 weeks separating their last systemic injection from their first infusion. THIP was infused weekly in the following order, with baseline 10E intake re-established before each infusion: 0 ng (pre), 50 ng, 100 ng, 500 ng, 10 ng, 0 ng (post). Because the 10 ng dose led to no change in intake from vehicle, and we wanted to minimize the number of

infusions these animals received, 10 ng was not repeated in the subsequent experimental passes. In pass 2 (n = 4), animals had 5 weeks of 10E access prior to surgery. They had a week to recover from surgery, followed by 4 more weeks of 10E access before the first infusion was given. THIP was infused weekly in the following order, with baseline 10E intake re-established before each infusion: 0 ng (pre), 500 ng, 100 ng, 250 ng, 0 ng (post). The 250 ng dose was not repeated in the next experimental pass. For pass 3 (n = 4), animals underwent surgery prior to any ethanol experience. This was done to minimize the length of experiments in view of the fact that in the previous 2 passes there was no change in baseline intake before and after surgery. Mice had a week of recovery followed by 5 weeks of 10E access before any infusions. THIP was infused weekly in the following order, with baseline 10E intake re-established before each infusion: 0 ng (pre), 50 ng, 100 ng, 500 ng, 0 ng (post). There was no difference between 10E intake for 0 ng (pre) versus 0 ng (post) for each cohort, so these data were collapsed as the 0 ng.

*Experiment 3: Effect of ICV administration of GAN or THIP on 10E intake*

For the ICV experiments, animals underwent surgery prior to any 10E experience. Mice had 5 weeks of 10E access before any infusions. GAN was infused weekly in the following order, with baseline 10E intake re-established before each infusion: 0 ng, 500 ng, 0 ng. A week later the same mice received weekly infusions of THIP: 0 ng, 500 ng, 1000ng, 0 ng. There were 10 mice with confirmed placements. Note that vehicle was 3%DMSO/20%β-cyclodextrin for GAN and aCSF for THIP.

*Experiment 4: Effect of NAc shell infusion of GAN or THIP on locomotor activity:*

A separate cohort of 11 mice with confirmed bilateral NAc shell cannulae placements were used to test the locomotor effects of the highest dose of each drug.

Mice were tested during their light cycle and were tested at the same time each day. On day 1, mice were acclimated to the locomotor chambers for 2 hours. On day 2, mice were again placed in locomotor chambers for 2 hours to assess "baseline" locomotion. On day 3, separate groups of mice were given a bilateral infusion of either 0 ng THIP (n = 6) or 500 ng THIP (n = 5) and immediately placed in the activity chambers. Drug-induced locomotor activity was determined by subtracting horizontal locomotor (cm) activity on day 2 from activity on day 3 (Phillips et al., 1995; Palmer et al., 2002). There was no difference in "baseline" activity on day 2 between mice that subsequently received an infusion of 0 versus 500 ng THIP. A week later (day 4), mice were given either 0 ng GAN (n = 5) or 500 GAN (n = 6) balanced on previous drug infusion. Drug-induced locomotor activity was determined by subtracting activity on day 2 from activity on day 4. Again there was no difference in "baseline" activity on day 2 between mice that subsequently received 0 versus 500 ng GAN.

*BECs*:

Blood samples were taken at the conclusion of the 2-hour access period in a subset of the animals on a baseline (no infusion) day. Blood samples were collected and analyzed as previously described (Finn et al., 2007). Briefly, a 20 µL sample was taken from the orbital sinus of each mouse. Each blood sample was then added to 500 µL of a solution containing 4 mM *n*-propanol (internal standard) in deionized water, vortexed, and analyzed using head-space gas chromatography. A standard curve was constructed using 6 pairs of external standards with known ethanol concentrations (from 0.5 to 3.0 mg/ml), and concentrations of samples were interpolated from this curve.

*Histological Confirmation*:

At the conclusion of pass 1 for each of the NAc shell GAN and THIP drinking studies, mice were euthanized, and whole brains were removed and placed in 2% paraformaldehyde overnight. Brains were then transferred to 20% sucrose, and 30% sucrose for 24 hours each before sectioned into 35 µm sections using a Leica cryostat. Slices were mounted on glass slides and stained with thionin and photographed with an IM50 imaging system (Leica Microsystems Imaging Solutions Ltd). This was done to visualize whether 5 to 6 repeated infusions contributed to any excessive damage or glial scarring (Fig. 4.1D).

At the conclusion of all other drinking studies, as well as the locomotor studies, mice were infused with 17 mg/ml methylene blue dye in 20% β-cyclodextrin in a manner identical to that used for the drinking and locomotor studies (Fig. 4.1E). Following euthanasia, intact whole brains were rapidly removed from the skull and flash frozen in isopentane and stored at -80° Celsius until sectioned. Brains were sectioned into 35 µm sections using a Leica cryostat, mounted on glass slides and photographed with an IM50 imaging system (Leica Microsystems Imaging Solutions Ltd). For all studies, only placements confirmed by 2 independent investigators as bilateral hits in the NAc shell or a unilateral placement into the lateral ventricle (ICV) were used.

*Statistical Analysis:*

Any animal without a confirmed placement (bilateral NAc shell or ICV) was excluded from the respective analysis. Ethanol intake (g/kg) was calculated based on the ml of 10E solution depleted and the pre-session body weight of each mouse. Total fluid intake (TFI) was measured by adding the ml of water and 10E consumed during the 2-hour access along with the 22-hour change in weight of the 50-ml water bottles. Patterns of 10E licks, as well as 10E bout parameters, were recorded using MED-PC IV

software, and SoftCR version 4 (MED Associates, Inc.) was used to access the time-stamped data. A bout was defined as a minimum of 20 licks with no more than a 60-second pause between successive licks (Ford et al., 2008, 2005a, 2005b; Ramaker et al., 2011, 2012 (Chapters 2 and 3)). All statistical analyses were performed using SYSTAT 11. Graphs were made using GraphPad Prism 4 for Windows.

For each measure, any data point over 2 sd from the average was removed. Lick and bout data were removed for any animal's data where the average licks/ml fell over 2 sd outside of the average. For all other data points, across all GAN and THIP infusion days, there was a significant correlation between g/kg consumed and 10E licks recorded from the lickometer ($r = 0.66$ to $0.97$; $p = 0.02$ to $<0.001$). For drinking experiments, the effect of drug was analyzed using repeated-measures ANOVA. For bin analysis, a two-way repeated measures ANOVA factored by bin and dose was conducted, and in the event of a significant interaction, one-way ANOVAs were performed. In the event of a significant main effect, pairwise differences against vehicle were determined by the Fisher's Least Significance Difference multiple comparisons test. For all analyses, statistical significance was set at $p \leq 0.05$.

## Results

### *GAN*

#### *NAc GAN: 10E intake*

Baseline 10E intakes (the average of all non-infusion days that immediately preceded a drug day) are shown in Table 4.1 and did not statistically differ between any of the studies. Confirmed placements for mice receiving GAN (n = 15) are shown in Fig. 4.1A. Four data points were removed as statistical outliers for being >2 sd *above* the mean (1 each at 0 ng (pre), 50, 100, and 500 ng) and 2 mice missed an infusion: 1 at 50

and 1 at 100 ng due to a clogged cannula. BECs on a non-infusion day were 61 ± 18 mg/dl (g/kg = 2.51 ± 0.25; n = 6). GAN decreased g/kg ethanol intake over the 2-hour session [F (3,33) = 2.944; *p* = 0.047; Fig. 4.2A], with 500 ng GAN significantly reducing intake (*p* = 0.025). There were 3 data points where licks per ml fell > 2 sd below the average (this generally occurs when accumulation of food near the sipper prevents the lickometer from completing a circuit), and 3 data files that did not save. Due to multiple missing data points, bout data were analyzed as between-subjects ANOVAs. There was a significant effect of GAN on bout frequency [F (3,45) = 3.315; *p* = 0.035; Fig. 4.2B], with a decrease following 100 ng (*p* = 0.018) and 500 ng (*p* = 0.008). There was also an effect on GAN on early termination of the session F (3,45) = 6.578 ; p = 0.05], with the last bout ending sooner with 500 ng GAN ( p = 0.008). There was no effect of GAN on any other bout parameters measured (Table 4.2).

Analyzing 10E licks, there was a significant dose by hour interaction [F(3,18) = 7.277; *p* = 0.002; Fig. 4.2C]. Specifically, there was a dose effect during hour 2 [F(3,18) = 7.277; *p* = 0.002], where both 100 ng (p = 0.032) and 500 ng (p = 0.001) decreased 10E licks (Fig. 4.2C). To better understand how GAN might be shifting the patterns of licks, 10E licks were split into 20 minute bins (Fig. 4.2D). Analysis revealed a significant bin by dose interaction [F(15,90) = 2.733; *p* = 0.002]. There was a significant effect of GAN during minutes 0 – 20 [F(3,18) = 3.580; *p* = 0.035] and minutes 20 – 40 [F(3,18) = 3.952; *p* = 0.025], with only 100 ng significantly decreasing licks (p = 0.031) during minutes 20 – 40.

Dorsal control GAN: 10E Intake

Nine mice had injector placements that were located dorsally to the NAc shell, which was used as a control region (Fig. 4.1C; n = 6 from ascending dose groups; n = 3

from descending dose group). Average g/kg 10E intake following vehicle infusion was not statistically different from that of the NAc shell experiment. There was 1 outlier removed from the 500 ng group (>2 sd above average intake). There was no effect of GAN on overall 10E intake [F(3,21)=0.740; p = 0.540; Fig. 4.3A]. There was one lick/ml outlier removed from the 500 ng group. There was no effect of GAN on bout frequency (Fig. 4.3B) or any other bout parameter whether assessed by between-subjects or repeated-measures ANOVAs (data not shown). There was an hour by dose interaction when assessing hourly licks [F(3,18)= 4.822; *p* = 0.003], but post-hoc ANOVAs revealed no dose effect at either hour. There was no bin by dose effect when assessing licks by 20 minute bins (data not shown).

1 Shell + 1 Core GAN: 10E Intake

In a subset of mice (n = 9), there were placements consisting of an injector targeting the shell on one side of the brain and targeting the core on the other side. These 9 mice comprised n = 8 from cohort 1 and n = 1 from cohort 3. There was one missed infusion each in 0 ng (pre), 50 ng, 100 ng, and 0 ng (post). Average g/kg 10E intake following vehicle infusion was not statistically different from that of the NAc shell experiment. There was no significant effect of GAN infusion on 10E intake [F(3,18)= 1.627; *p* = 0.218; Fig. 4.4A]. There also was no effect of GAN infusion on bout frequency (*p* = 0.602; Fig. 4.4B), average bout size, first bout size, or termination of final bout (data not shown). Contrary to bilateral shell placement, there was a significant effect of GAN to decrease latency to first bout [F(3,15)= 4.808; *p* = 0.015; Fig. 4.4C] with a decrease following 50 ng (*p* = 0.021) and 100 ng (*p* = 0.043) and a trend for a decrease following 500 ng (*p* = 0.095). There was also a significant effect of GAN on latency to first lick [F(3,15)= 4.156; *p* = 0.021; Fig 4.4D] with a decrease following 50 ng

($p$ = 0.023) and a trend for a decrease following 100 ($p$ = 0.062) and 500 ng ($p$ = 0.084). There was no hour by dose and no bin by dose interaction when analyzing across 20-minute bins (data not shown).

*ICV GAN: 10E Intake*

There was a significant effect of GAN to decrease g/kg 10E intake from vehicle ($t_9$ =2.758; p = 0.022; Fig. 4.5A). Bout analysis revealed that 500 ng GAN significantly decreased bout frequency ($t_9$ = 3.126; $p$ = 0.012; Fig. 4.5B). No other bout parameter was significantly altered by ICV GAN (data not shown). Data were analyzed hourly, and by 20 minute bins, but there was no bin by dose interaction in either case (Fig. 4.5C and 4.5D).

*NAc GAN: locomotor activity*

There was no difference between a NAc shell infusion of 0 ng (n = 5) versus 500 ng GAN (n = 6) on locomotor activity over the 2 hour session (0 ng = 3809 ± 2020 cm versus 500 ng = 2026 ± 2947 cm; data not shown). There was no bin by dose interaction when analyzing by 20 minute bins (Fig. 4.6).

*THIP*

*NAc THIP: 10E intake*

Placements for the NAc drinking study are shown in Figure 4.1C. Initially, with the first two cohorts, there was no significant cohort by dose interaction so data were collapsed. The results of these data are shown in Figure 4.7. For these data, 0, 100, and 500 ng were analyzed since these 3 doses were repeated in both cohorts. Two mice missed an infusion: 1 at 100 ng and 1 at 500 ng due to a clogged cannula (both from

cohort 1). BECs on a non-infusion day were 91 ± 21 mg/dl (3.01 ± 0.41 g/kg; n = 5). THIP significantly decreased g/kg 10E intake [F(3,9) = 8.75; *p* = 0.005; Fig. 4.7A], with 500 ng THIP significantly decreasing intake (*p* = 0.004). Despite 2 mice not administering a complete bout following 500 ng, there was no effect of THIP on bout frequency (Fig. 4.7B) or any other bout parameter measured (data not shown). There was no significant dose by hour interaction (Fig. 4.7C). Analyzing 10E licks by 20-minute bins, there was a significant dose by bin interaction [F(10,60) = 2.419; *p* =0.017; Fig. 4.7D], but subsequent post-hoc tests did not reveal a significant dose effect for THIP in any 20 min bin.

Because the 50 ng dose of THIP was only tested in one cohort, and visual inspection of the data hinted at an interesting potential increase in intake with this dose (Fig 4.8A), another cohort was added to examine whether there was also an effect with 50 ng that was not detectable due to the low power. For this reason, another cohort was run that received 0, 50, 100, and 500 ng. However, the inclusion of this cohort uncovered an important order effect of the THIP doses as revealed by a dose by cohort interaction that was just shy of significance [F(4,16) = 2.935; *p* = 0.054]. For this reason, data are also analyzed and discussed by cohort. There was no effect of pre versus post vehicle infusions on 10E intake, and doses are analyzed versus the vehicle average, but vehicle pre versus post are shown separately to better illustrate how order affected intake. Additionally, doses are shown in the order in which they were administered, and all doses received by that cohort were analyzed.

Fig. 4.8A depicts 10E intake for each cohort. In cohort 1 (n = 5) there was a significant dose effect [F(4,8) = 4.512; *p* = 0.034], with 500 ng decreasing 10E intake (*p* = 0.010). In cohort 2 (n = 4) there was a trend for a dose effect [F(3,9) = 3.390; *p* = 0.067]. In cohort 3, there was again a significant effect of THIP dose [F(3,9) = 17.811; *p*

= 0.001], where the 500 ng dose decreased intake ($p$ = 0.025). There was no effect of THIP dose on bout frequency with any cohort alone, likely due to the small group size. These data are shown in Fig. 4.8B. However, visual inspection suggests that there also could be a contribution of dose and order, which is supported by a dose by cohort interaction when analyzing just the three repeated doses (0, 100, 500 ng) [$F(4,12 = 3.351$; $p = 0.041$].

The remainder of the bout data are shown in Table 4.3. Due to missing data and some mice not completing a whole bout in cohorts 1 and 3, those cohorts were analyzed with a between-subjects ANOVA. (cohort 1: missed infusion for 100 ng and 500 ng, and one instance with 50 ng where licks did not record; cohort 3: 1 instance where licks did not record, 1 had no licks on the 10E bottle, and one did not complete a full bout following 500 ng). Since there were no data points missing from cohort 2, and to maximize power with an n of only 4, this cohort was analyzed using a within-subjects ANOVA. The only bout parameter that was significant was an effect of dose on bout size in cohort 3 [$F(3,10) = 4.979$; $p = 0.023$; Table 4.3], where there was a significant decrease following 100 ng ($p = 0.010$). There were also trends for dose effects on first bout size in cohort 3 and termination of the final bout for cohorts 2 and 3.

Licks split by 20-minute bin are shown for each cohort in Fig. 4.8C. For cohort 1, there was a significant bin by dose interaction [$F(20,20) = 7.356$; $p < 0.001$]. There was a significant effect of THIP infusion in minutes 0 – 20 [$F(4,4) = 10.847$; $p = 0.020$], with a trend for an increase in licks following 10 ($p = 0.091$) and 50 ng ($p = 0.075$). There was also an effect of THIP dose during minutes 100 – 120 [$F(4,4) = 24.586$; $p = 0.004$], with a significant decrease following every dose ($p = 0.01$ to 0.001). For cohort 2, there was a significant bin by dose effect [$F(15,45) = 2.307$; $p = 0.016$], but there was only a trend for a dose effect during minutes 100 – 120. For cohort 3, there was also a

significant bin by dose effect [F(15,45) = 9.645; $p < 0.001$]. There was a trend for an effect of THIP dose in minutes 20 – 40. THIP dose exerted a significant effect on 10E intake during minutes 60 – 80 [F(3,9) = 5.298; $p = 0.022$], with a trend for a decrease following 500 ng ($p = 0.095$) and during minutes 100 – 120 [F(3,9) = 21.582; $p < 0.001$], with a significant decrease following 50 ($p = 0.028$), 100 ($p = 0.016$), and 500 ng ($p = 0.017$).

*ICV THIP: 10E Intake*

There was no significant effect of an ICV infusion of THIP on 10E intake [F(2,18) = 1.068; $p = 0.214$; Fig. 4.9A], despite the 30% decrease in 10E intake following 1000 ng THIP. There was no effect of THIP on 10E total licks, and no bin by dose interaction when analyzed across hours or 20-minute bins (data not shown). There was no effect of THIP on bout frequency (Fig. 4.9B) or any other bout parameter (data not shown).

*NAc THIP: locomotor activity*

There was a significant increase in 2-hour locomotor activity following NAc shell THIP [F(1,9) = 20.864; $p = 0.001$]. Total cm distance traveled (subtracting out baseline activity on day 2) was 0 ng = -3758 ± 3377 and 500 ng = 20,366 ± 4138; data not shown. The locomotor effects of THIP infusion into the NAc shell were analyzed over 20 minute bins in order to compare the time periods that THIP altered 10E intake to that when it might be altering locomotor activity. There was a significant dose by bin interaction [F (5,45) = 3.183; $p = 0.015$; Fig. 4.10]. Post-hoc t-tests revealed a significant effect of THIP to increase activity during minutes 20 – 40 ($p = 0.010$), minutes 40 – 60 ($p = 0.001$), minutes 60 – 80 ($p = 0.013$), and minutes 80 – 100 ($p = 0.049$). There was a trend for an increase in activity during minutes 0 – 20 ($p = 0.098$) and minutes 100 – 120 ($p = 0.056$).

## Discussion

The present studies suggest that NAc shell infusions of GAN and THIP can account for the systemic effects of these drugs on limited-access ethanol consumption in mice. Analysis of locomotor behavior suggests that the decreases in 10E intake were not due to a sedative effect of the GAN or THIP infusions. However, intra-NAc THIP produced locomotor activation, so we cannot rule out the influence of a competing behavior in the interpretation of the effects of THIP infusion on ethanol intake.

These present results are consistent with data showing the importance of the $GABA_A$ receptor in the NAc shell on ethanol consummatory behaviors (Stratford and Wirtshafter, 2011; Hyytiä and Koob, 1995; Eiler and June, 2007). $GABA_A$ receptors, including those containing the δ-subunit, are present on MSNs of both the direct and indirect pathways, as well as several classes of NAc interneurons (Maguire et al., 2014). Studies using optogenetics or DREADDs (Designer Receptors Exclusively Activated by Designer Drugs) suggest that activation of the direct pathway increases the rewarding value of some drugs of abuse, while activating the indirect pathway has the opposite effect (Ferguson et al., 2011; Lobo and Nestler, 2011). Although it is unclear in the present studies if GAN and THIP are decreasing intake by increasing or decreasing the rewarding value of ethanol, it is possible that GAN and THIP could be altering ethanol reinforcement by directly decreasing the excitability of one or both of these classes of cells.

With GAN, there was an overall decrease in 10E intake primarily driven by a decrease in bout frequency concomitant with early termination of the last bout, but no change in onset of intake. With GAN, we have now seen similar changes in the 2-hour 2-bottle choice procedure following systemic (Ramaker et al., 2012 (Chapter 3)), ICV,

and NAc shell infusions, with a 22%, 21%, and 25% decrease in intake following the highest dose administered, respectively. We have also seen similar quantitative differences in bout frequency across these 3 modes of GAN administration, highlighting the sufficiency of the NAc shell in these effects. On the other hand, given a similar effect of ICV and NAc GAN (where concentrations of available GAN are expected to be much more diffuse following a unilateral ICV infusion of 500 ng versus a bilateral infusion of 500 ng localized to the NAc), one interpretation is that although the NAc shell is sufficient, there are likely other brain areas that would also be sufficient to see this effect. This is consistent with the fact that ALLO immunoreactivity is seen in many limbic structures in the brain (Saalmann et al., 2007), and has been shown to be altered locally in many of these regions following acute ethanol injection (Cook et al., 2013). Future studies should look at other regions which may be sufficient for an effect of GAN to decrease ethanol intake, including the PFC, NAc core, paraventricular nucleus, and amygdala.

Placements were verified with a dye of a similar molecular weight as GAN, in order to assess the extent of diffusion of the drug, in addition to solely visualizing injector placement. Although we cannot rule out diffusion to other brain areas, the dorsal control suggests that there is some regional specificity to the observed effects. Since there is no bilateral core placement to compare to, limited information can be gleaned from the subset of mice with a unilateral core plus a unilateral shell placement. However, it is of interest that the decrease in intake and bout frequency seen following the bilateral shell GAN infusion is absent in mice that received GAN with one placement in each of the shell and the core. This could indicate that a unilateral placement in the shell is not sufficient to obtain the effect, or it is possible that the shell and core have opposing effects on these measures, which cancel out when targeting both. Differences between

the bilateral shell placements and the 1 shell + 1 core placements also provides some indirect evidence that the NAc shell placement was mostly contained within the shell and had limited diffusion to other areas such as the core. It is interesting that GAN altered latency to first bout and first lick in the mice with a unilateral shell and unilateral core hit, but these measures were unaffected in the bilateral shell group. These measures could reflect a motor component to ethanol seeking and would be consistent with the idea that the core is thought to be relatively more involved in locomotor functions than the shell (Maldonado-Irizarry and Kelley, 1994).

The NAc shell appears to regulate at least some of the effects of THIP on ethanol intake, although these results need to be interpreted with caution due to important order effects that were uncovered. The apparent sensitization to repeated THIP infusions is curious in light of data showing no effect or tolerance to multiple THIP injections (Voss et al., 2003; Chandra et al., 2006). The sensitization did not appear to be a consequence of mechanical damage from repeatedly lowering the injector, as intake following the post vehicle infusion did not differ from the pre vehicle infusion. The primary effect appeared to be driven by the fact that the 500 ng THIP dose was much more efficacious at decreasing drinking and bout frequency when it was given last versus first. However, this does not appear to be strictly an order effect; when 10 ng THIP was given as the 4$^{th}$ infusion, there was no effect on ethanol intake. Thus, it appears that there may be both a THIP dose and order effect that contributes to the decrease in intake.

The sufficiency of the NAc shell in mediating the effects of THIP on ethanol intake would be consistent with work showing that knockdown of the δ subunit in the shell decreased ethanol self-administration in rats (Nie et al., 2011). Specifically, this effect was localized to the medial, but not the lateral or intermediate shell (Nie et al.,

2011). Since the δ subunit is located throughout the entire NAc (Schwarzer et al., 2001), it will be of interest to test the sufficiency of the NAc core, as well as other regions with high δ subunit expression, including the thalamus, cerebellum, and cortex (Pirker et al., 2000) that may be underlying the effect of systemic THIP on ethanol intake.

Given that that systemic injections of THIP decreased limited-access ethanol intake (Ramaker et al., 2012), the lack of a significant effect following ICV infusion was unexpected. However, δ-subunit-containing $GABA_A$ receptors make up only about 5% of total $GABA_A$ receptors in the brain (McKernan and Whiting, 1996) and expression is low or absent in many brain areas (Hörtnagl et al., 2013). Thus, it is possible that ICV THIP became too diluted as it diffused and was sub-threshold upon reaching the NAc or other relevant brain regions to alter 10E intake. Interestingly, the smaller effect of THIP to decrease intake following infusion into the ventricles versus directly into the NAc provides confidence that the observed outcomes following intra-NAc shell infusions of THIP were not an artifact of inadvertently hitting the ventricles, a potential concern when targeting the shell.

Although our aim was not to limit placements to the medial portion of the shell or to compare it to other areas of the shell, it is of interest that visual inspection of our placements revealed that our placements were primarily centered in this area, and consideration of medial shell projections may be important for the interpretation of our results. The medial NAc shell is in part distinguished from the rest of the shell by its reciprocal projections with the lateral hypothalamus (Groenewegen et al., 1999), an area with well-established roles in ingestive behaviors (Wise et al., 1974; Berridge and Valenstein, 1991). For this reason, future studies examining the specificity of this effect

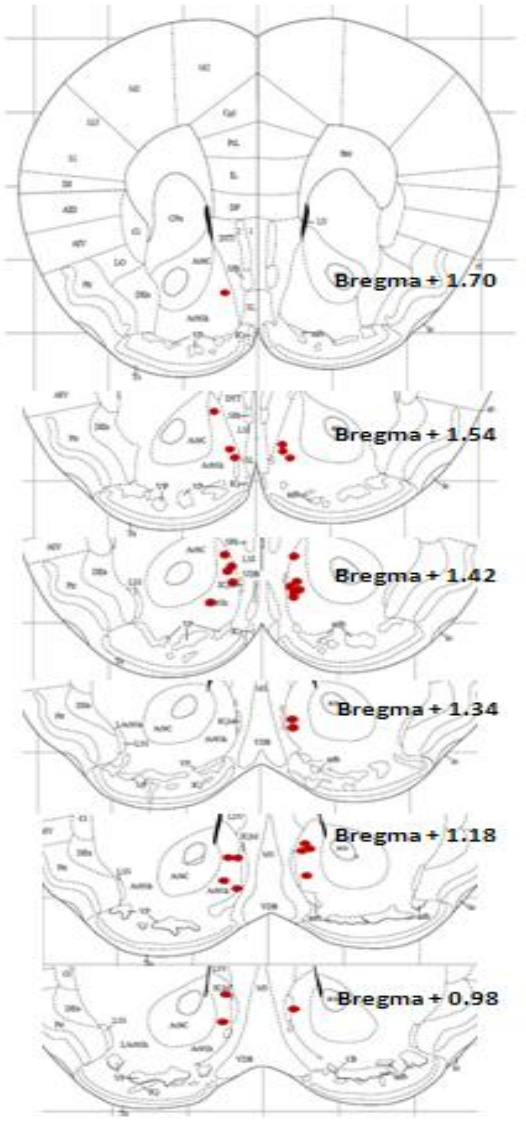
on ethanol versus other natural reinforcers, such as sucrose or saccharin will be important.

The results of this study show that the NAc shell is a sufficient brain region in mediating ethanol consumption by either a NAS or extrasynaptic $GABA_A$ receptor agonist. This adds to the growing literature that extrasynaptic $GABA_A$ receptors in this region regulate ethanol intake, and that local changes in the NAS environment may be an important mediator of ethanol intake.

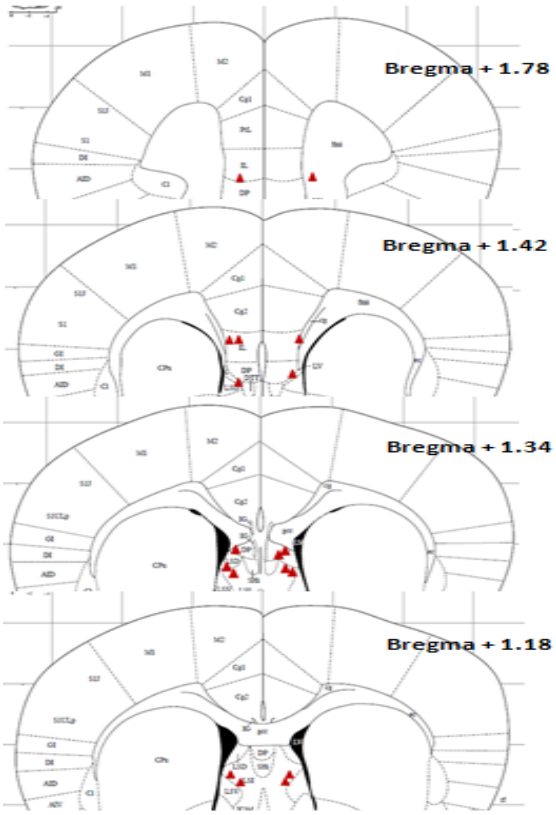
**Figure 4.1. Placements**

Injector tip locations for mice used in the drinking studies for A) NAc shell infused with GAN (n = 15) B) dorsal controls infused with GAN (n = 9) and C) NAc shell infused with THIP (n =13). D) Thionin staining was performed in a subset of mice to assess whether 6 microinfusions caused excessive damage or scarring. E) Representative image of a NAc shell placement visualized using methylene blue dye.

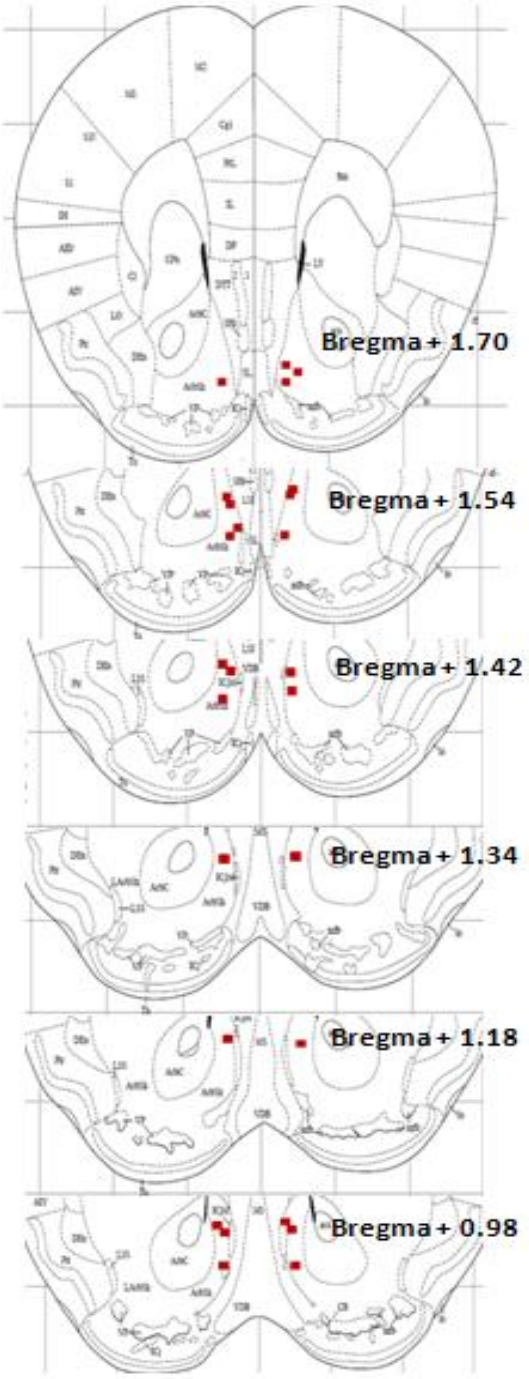
A) GAN NAc Shell Placements



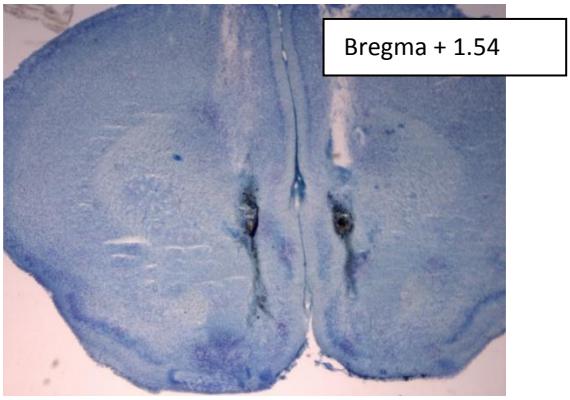

B) GAN Dorsal Placements



C) THIP NAc Shell Placements




D) Representative NAc Shell Placement




E) Representative NAc Shell Placement

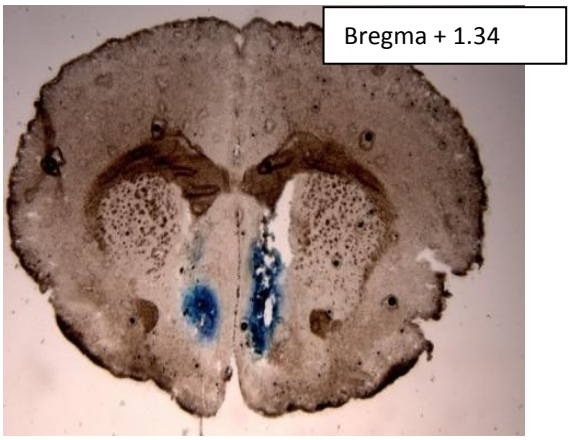


**Figure 4.2. Nac shell GAN: 10E Intake**

Effect of NAc shell GAN on A) total 2-hour 10E intake, B) average bout frequency, C) 10E licks analyzed by hour, and D) 10E licks split into 20-minute bins. Values represent mean ± SEM for 15 mice. 0 ng represents the average of the pre and post vehicle infusion. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ relative to 0 ng.

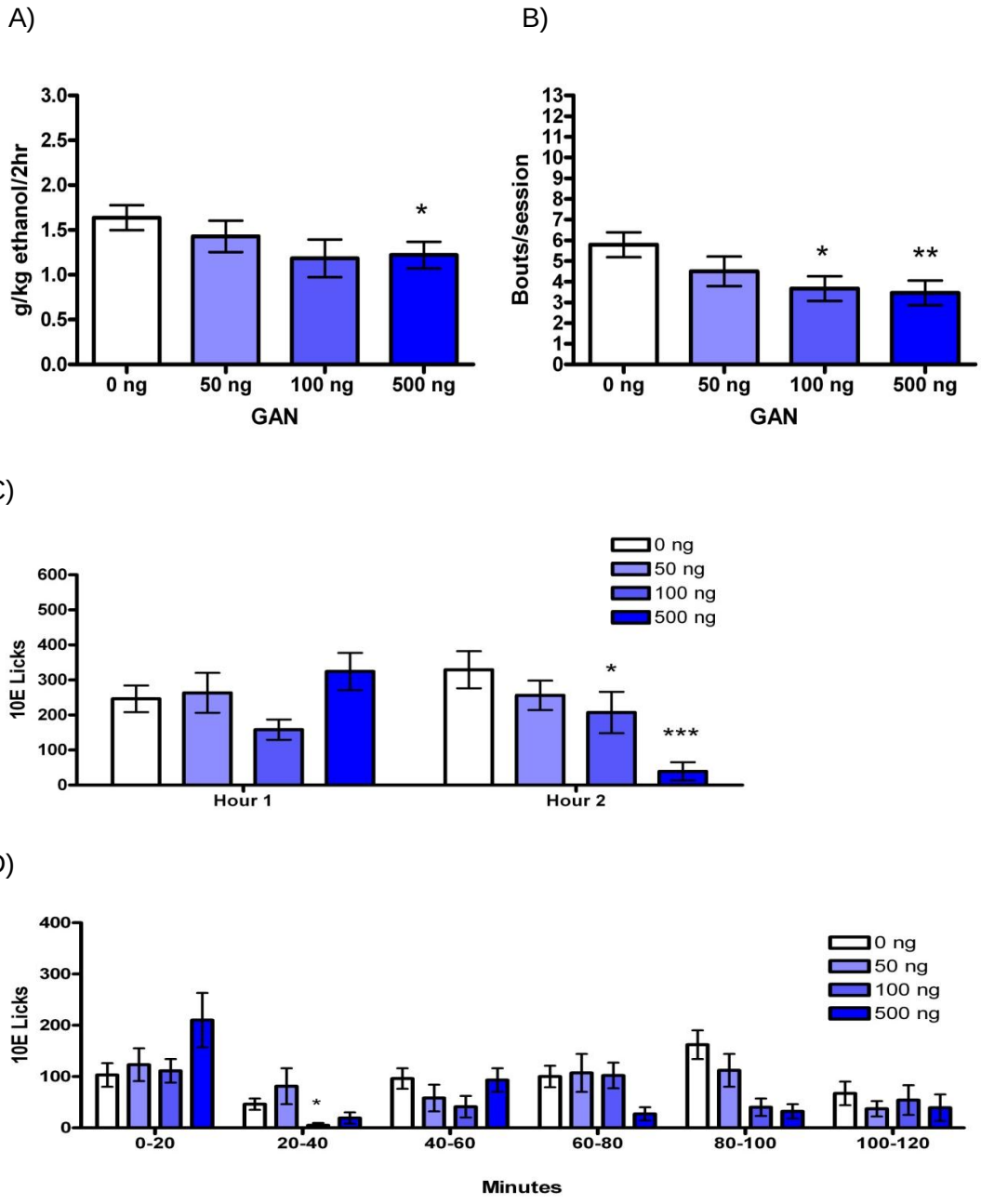


**Figure 4.3. Dorsal GAN: 10E Intake**

Effect of GAN into regions dorsal to the NAc shell on A) total 2-hour 10E intake and B) average bout frequency. 0 ng represents the average of the pre and post vehicle infusion. Injector placements are shown in Figure 4.1B. Values represent mean ± SEM for 9 mice.

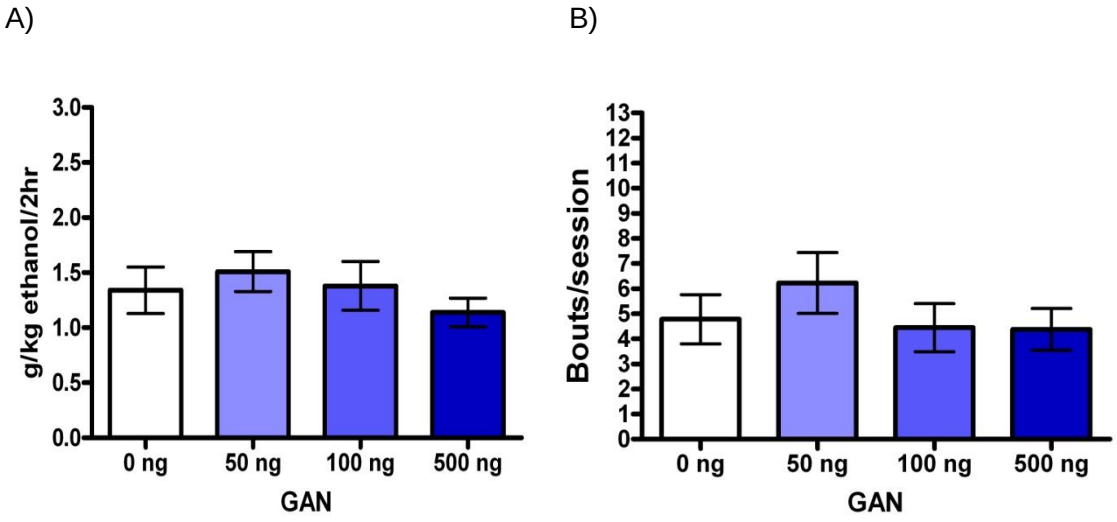


**Figure 4.4. Unilateral NAc Shell + Unilateral NAc core: 10E Intake**

Effect of intra-NAc GAN, with one injector placed in the NAc shell and one in the NAc core, on A) total 2-hour 10E intake, B) average bout frequency, C) latency to first bout, and D) latency to first lick. 0 ng represents the average of the pre and post vehicle infusion. Values represent mean ± SEM for 9 mice. +$p \leq 0.10$, *$p \leq 0.05$.

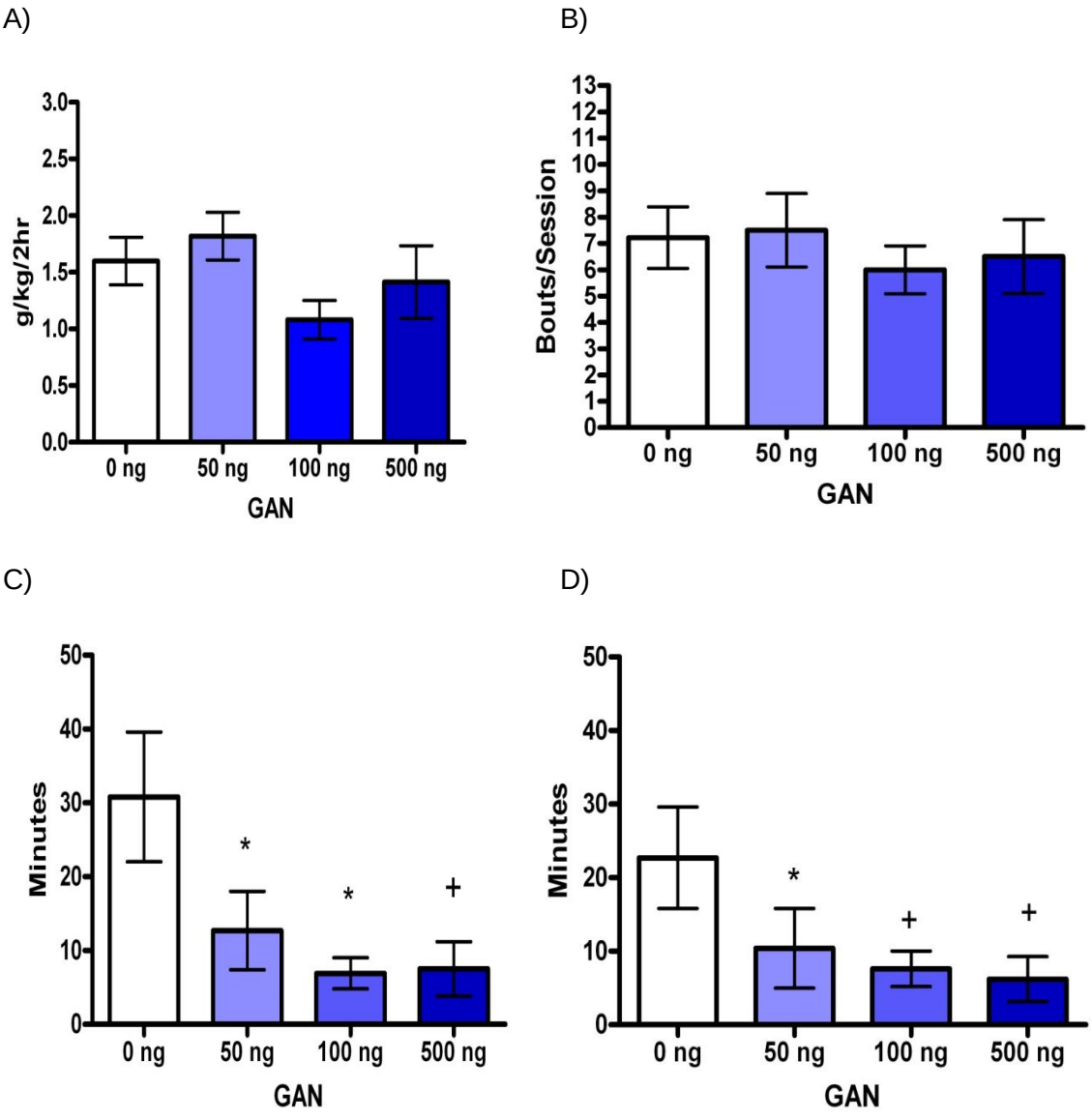
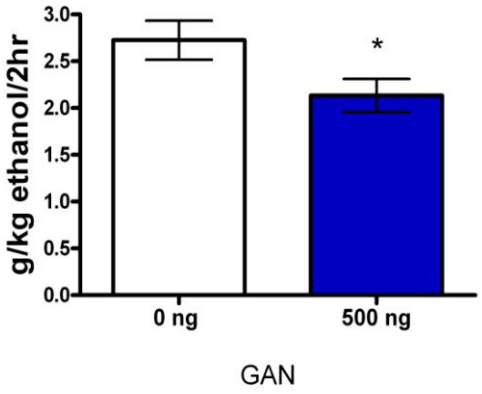


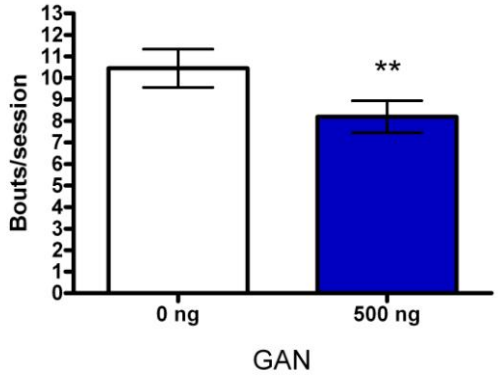
**Figure 4.5. ICV GAN: 10E Intake**

Effect of ICV GAN on A) total 2-hour 10E intake, B) average bout frequency, C) 10E licks analyzed by hour, and D) 10E licks split into 20-minute bins. Values represent mean ± SEM for 10 mice. 0 ng represents the average of the pre and post vehicle infusion. *$p \leq 0.05$, **$p \leq 0.01$ relative to 0 ng.

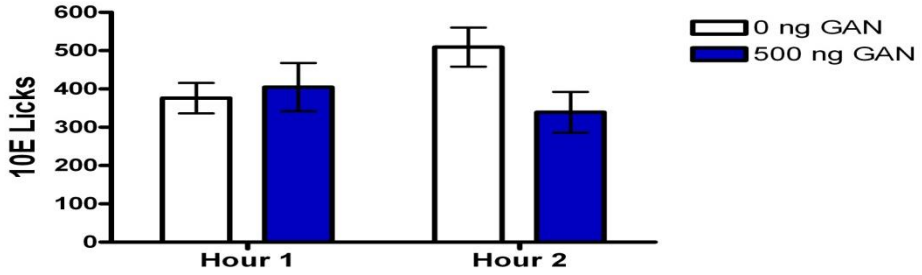
A)




B)




C)




D)

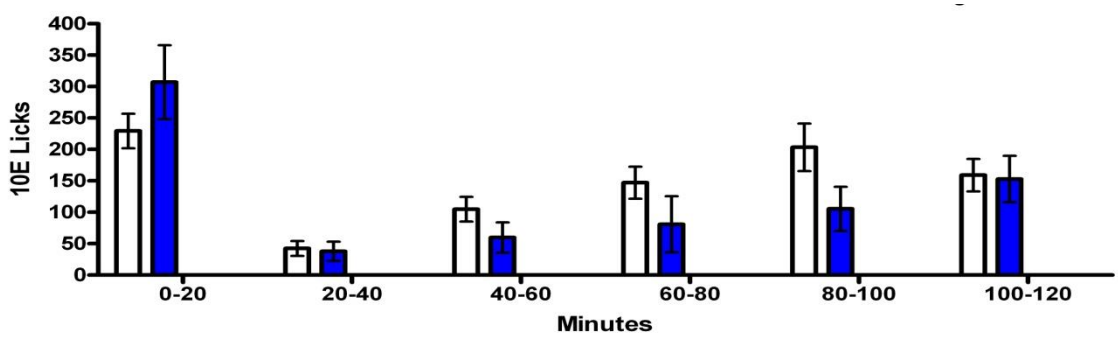


**Figure 4.6. Nac shell GAN: Locomotor Activity**

Horizontal locomotor activity following a NAc shell infusion of 0 or 500 ng GAN. Values represent total cm moved following the infusions, subtracting out cm moved on a baseline day. Values represent mean ± SEM for n = 5 for 0 ng GAN and n = 6 for 500 ng GAN.

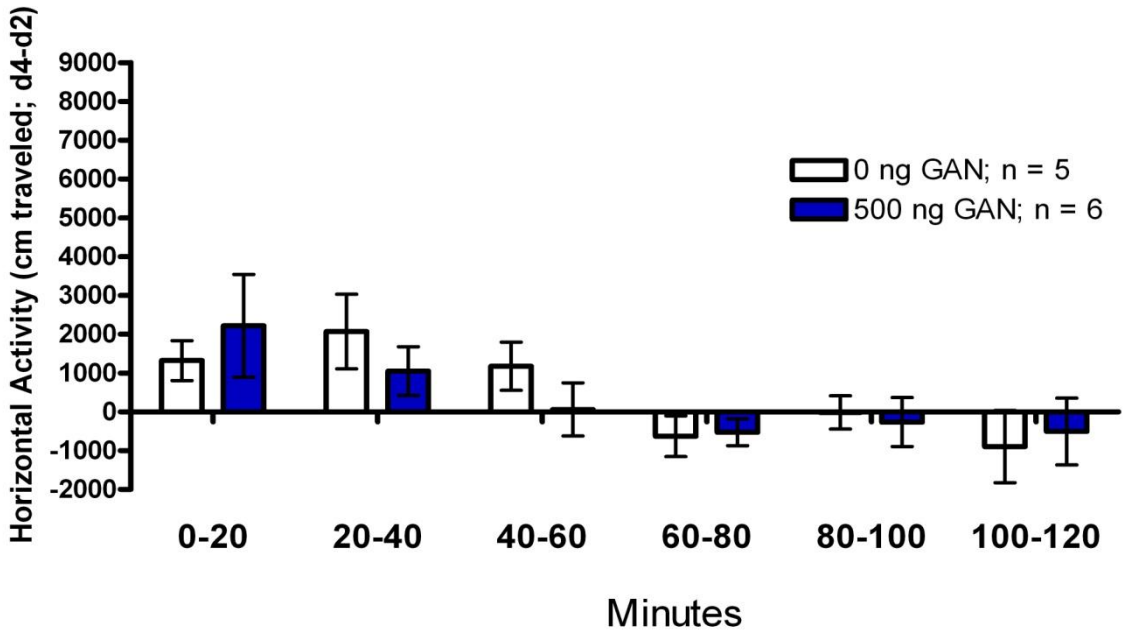
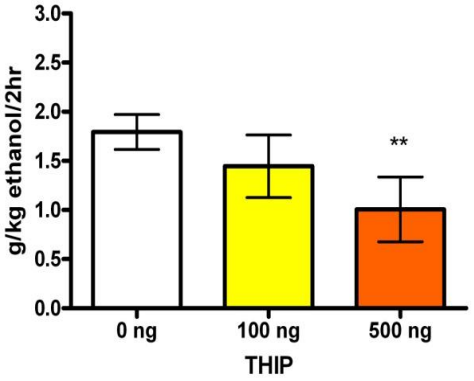


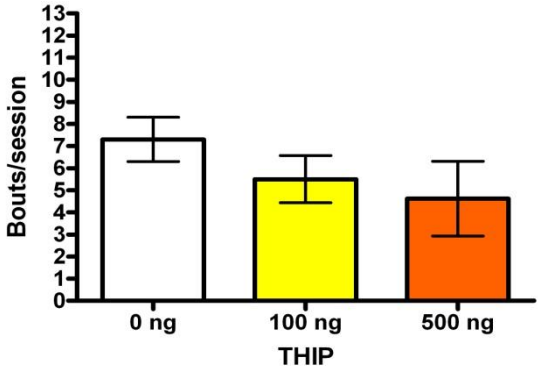
**Figure 4.7. NAc shell THIP: 10E Intake**

Effect of NAc shell THIP on A) total 2-hour 10E intake, B) average bout frequency, C) 10E licks analyzed by hour, and D) 10E licks split into 20-minute bins. Values represent mean ± SEM for n = 9 mice from cohorts 1 and 2. 0 ng represents the average of the pre and post vehicle infusion. $^{**}p \leq 0.01$ relative to 0 ng.

A)




B)




C)


D)

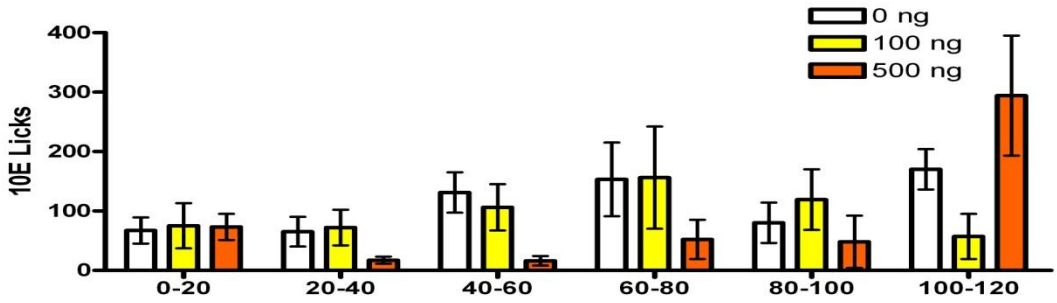
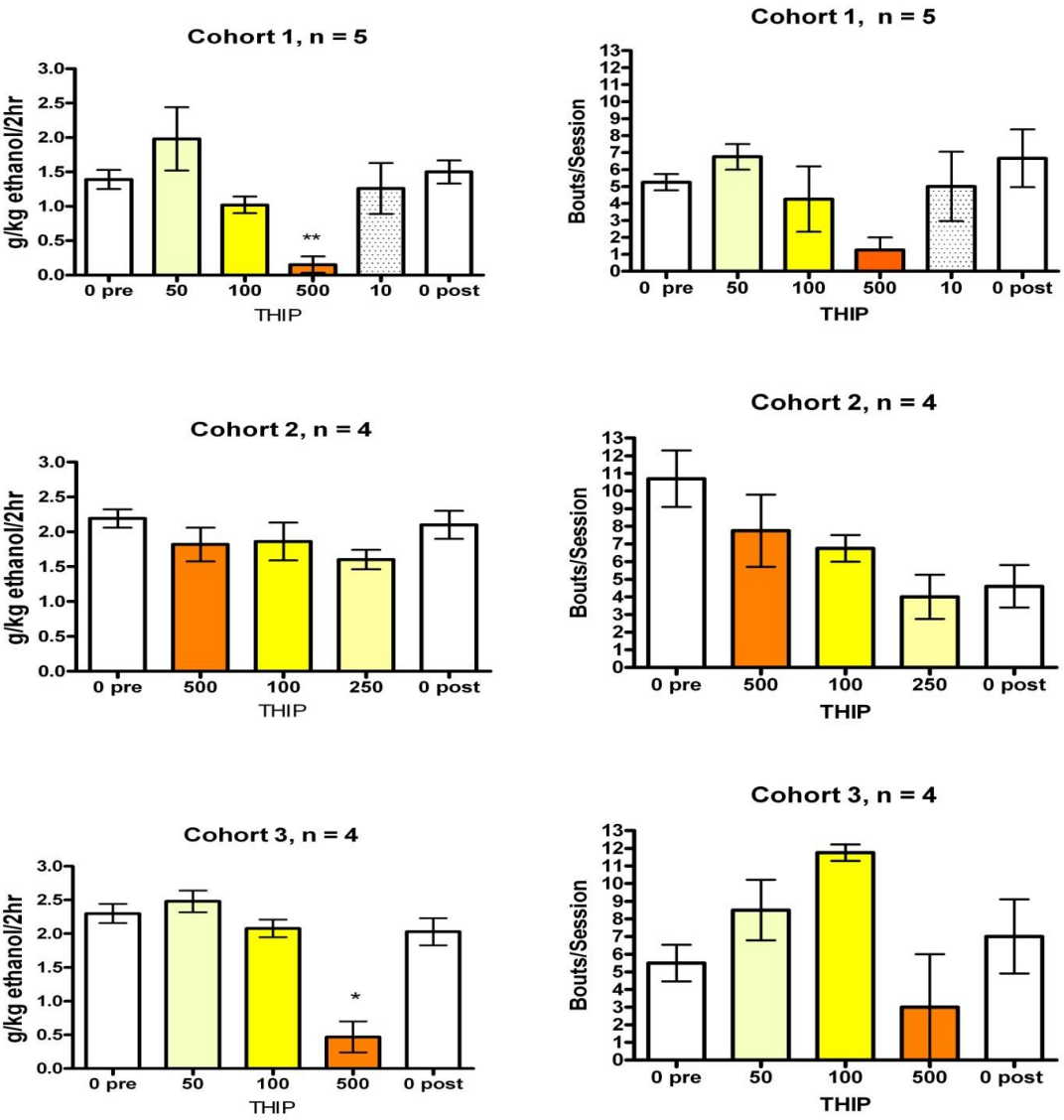


**Figure 4.8. NAc shell THIP: 10E Intake by cohort**

Effect of NAc shell THIP for each cohort on A) total 2-hour 10E intake, B) average bout frequency, C) 10E licks split into 20-minute bins. Values represent mean ± SEM for each dose. 0 ng represents the average of the pre and post vehicle infusion. +$p \leq 0.10$, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ relative to 0 ng (ave).

A) B)



C)

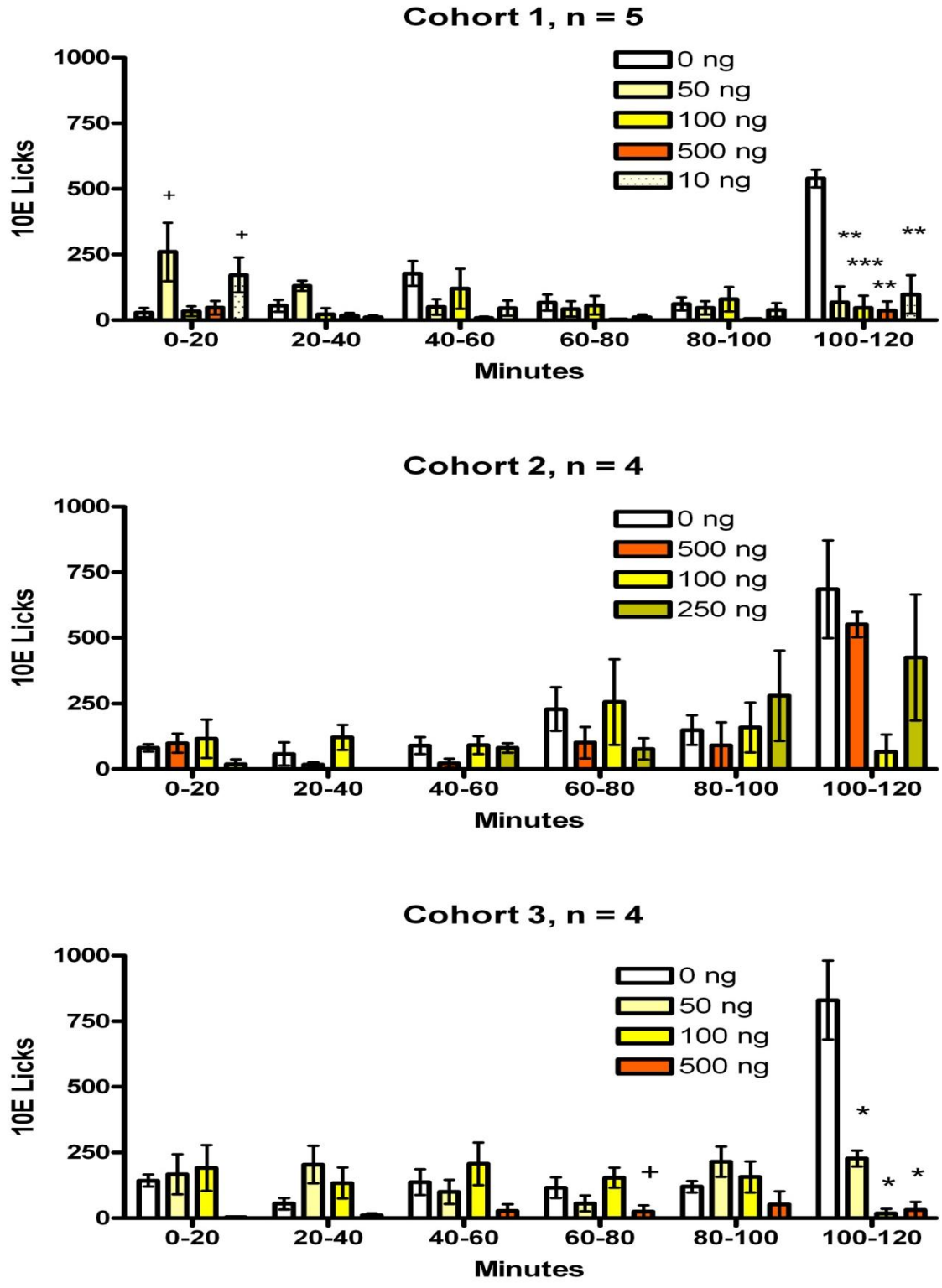

Cohort 1, n = 5
0 ng
50 ng
100 ng
500 ng
10 ng
10E Licks
1000
750
500
250
0
0-20
20-40
40-60
60-80
80-100
100-120
Minutes
Cohort 2, n = 4
0 ng
500 ng
100 ng
250 ng
10E Licks
1000
750
500
250
0
0-20
20-40
40-60
60-80
80-100
100-120
Minutes
Cohort 3, n = 4
0 ng
50 ng
100 ng
500 ng
10E Licks
1000
750
500
250
0
0-20
20-40
40-60
60-80
80-100
100-120
Minutes

**Figure 4.9. ICV THIP: 10E Intake**

Effect of ICV THIP on A) total 2-hour 10E intake and B) average bout frequency. Values represent mean ± SEM for 10 mice. 0 ng represents the average of the pre and post vehicle infusion.

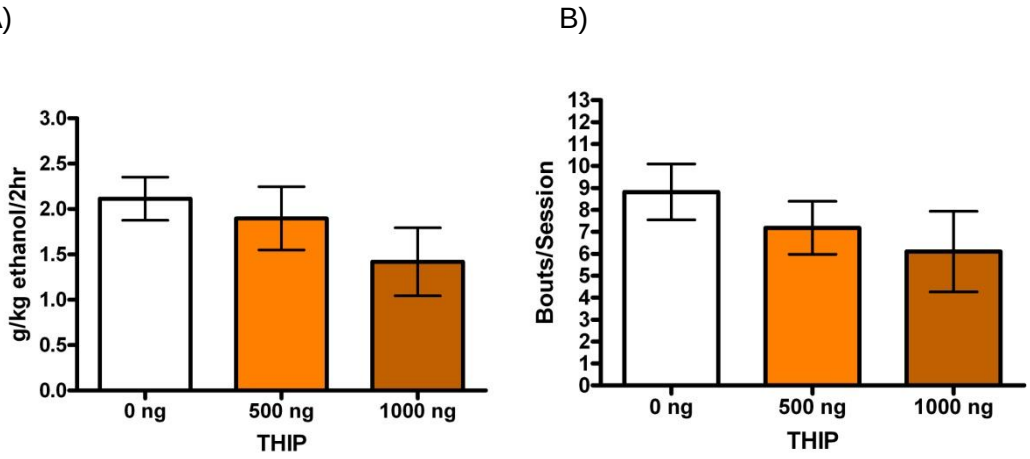



**Figure 4.10. NAc shell THIP: Locomotor Activity**

Horizontal locomotor activity following a NAc shell infusion of 0 or 500 ng THIP. Values represent total cm moved following the infusions, subtracting out cm moved on a baseline day. Values represent mean ± SEM for each dose. +$p \leq 0.10$, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ using 2-sample t-test.

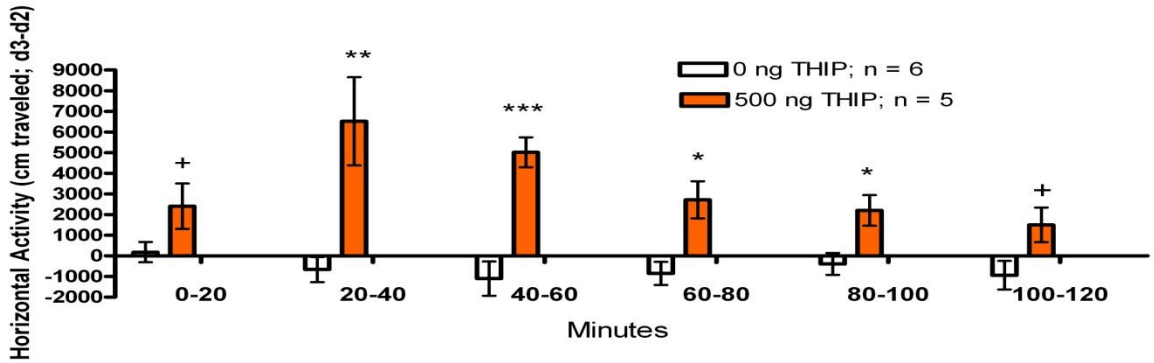


**Table 4.1. Baseline 10E intakes for each study.**

Data are mean ± SEM g/kg/2-hours, defined as the average of all days that immediately preceded a drug day. Baseline intakes did not statistically differ between any of the studies.

| NAc shell GAN | Dorsal GAN | 1 shell + 1 core GAN | ICV GAN | NAc shell THIP | ICV THIP |
|---|---|---|---|---|---|
| 2.44 ± 0.15 | 2.34 ± 0.29 | 2.40 ± 0.19 | 2.81 ± 0.17 | 2.45 ± 0.23 | 2.68 ± 0.18 |

**Table 4.2. Effect of NAc shell GAN on 10E bout parameters.**

Values represent mean ± SEM for each dose. 0 ng represents the average of the pre and post vehicle infusion.

| Dose GAN | **0 ng** | **50 ng** | **100 ng** | **500 ng** |
|---|---|---|---|---|
| 2-hr 10E licks | 575 ± 75 | 519 ± 72 | 355 ± 70 | 423 ± 72 |
| 22-hr water intake (ml) | 3.3 ± 0.4 | 3.2 ± 0.4 | 3.0 ± 0.3 | 2.6 ± 0.5 |
| Total fluid intake (ml) | 3.9 ± 0.4 | 3.7 ± 0.5 | 3.4 ± 0.3 | 3.1 ± 0.5 |
| Average bout size (licks) | 103 ± 9 | 121 ± 10 | 106 ± 11 | 117 ± 15 |
| Latency to first lick (min) | 14.5 ± 5 | 18.1 ± 8 | 20.5 ± 10 | 9.7 ± 3 |
| Latency to first bout (min) | 16.7 ± 4 | 21.7 ± 8 | 21.5 ± 10 | 10.1 ± 3 |
| First bout size (licks) | 102 ± 12 | 132 ± 20 | 99 ± 11 | 122 ± 22 |
| Termination of last bout (min) | 106 ± 10 | 91 ± 8 | 90 ± 8 | 54 ± 11 |

**Table 4.3. Effect of NAc shell THIP on 10E bout parameters for each cohort.**

Values represent mean ± SEM. 0 ng is the average of pre and post vehicle. n.d = not determined. +$p \leq 0.10$, *$p \leq 0.05$, **$p \leq 0.01$.

| Dose THIP | Cohort | 0 ng | 10 ng | 50 ng | 100 ng | 250 ng | 500 ng | ANOVA p-value |
|---|---|---|---|---|---|---|---|---|
| **2-hr 10E licks** | 1 | 541 ± 34 | 378 ± 141 | 759 ± 196 | 363 ± 180 | n. d. | 63 ± 141 | 0.134 |
| | 2 | 804 ± 143 | n. d. | n. d. | 813 ± 158 | 526 ± 232 | 749 ± 33 | 0.190 |
| | 3 | 802 ± 152 | n. d. | 971 ± 116 | 864 ± 37 | n. d. | 152 ± 138 **+** | 0.004 ** |
| **22-hr water intake (ml)** | 1 | 3.0 ± 0.6 | 2.4 ± 0.3 | 2.6 ± 0.6 | 1.7 ± 0.6 | n. d. | 3.4 ± 0.3 | 0.568 |
| | 2 | 3.6 ± 0.3 | n. d. | n. d. | 3.5 ± 0.5 | 3.6 ± 0.6 | 4.2 ± 0.2 | 0.510 |
| | 3 | 3.4 ± 0.2 | n. d. | 3.7 ± 0.3 | 3.1 ± 1.1 | n. d. | 3.1 ± 0.8 | 0.908 |
| **Total fluid intake (ml)** | 1 | 3.7 ± 0.3 | 2.8 ± 0.3 | 3.4 ± 0.6 | 2.1 ± 0.8 | n. d. | 3.4 ± 0.4 | 0.869 |
| | 2 | 4.2 ± 0.4 | n. d. | n. d. | 4.2 ± 0.5 | 4.2 ± 0.7 | 4.8 ± 0.2 | 0.549 |
| | 3 | 4.1 ± 0.3 | n. d. | 4.5 ± 0.3 | 3.8 ± 1.1 | n. d. | 3.2 ± 0.8 | 0.619 |
| **Ave bout Size (licks)** | 1 | 91 ± 7 | 138 ± 36 | 88 ± 8 | 87 ± 21 | n. d. | 68 ± 30 | 0.342 |
| | 2 | 104 ± 14 | n. d. | n. d. | 121 ± 19 | 123 ± 26 | 112 ± 30 | 0.925 |
| | 3 | 137 ± 17 | n. d. | 130 ± 33 | 72 ± 4 ***** | n. d. | 83 | 0.023 ***** |
| **Latency to first lick (min)** | 1 | 31 ± 10 | 9 ± 8 | 15 ± 8 | 24 ± 16 | n. d. | 12 ± 2 | 0.541 |
| | 2 | 17 ± 8 | n. d. | n. d. | 15 ± 6 | 20 ± 8 | 3 ± 2 | 0.153 |
| | 3 | 15 ± 5 | n. d. | 7 ± 6 | 9 ± 8 | n. d. | 22 ± 5 | 0.568 |
| **Latency to first bout (min)** | 1 | 31 ± 10 | 9 ± 8 | 15 ± 8 | 26 ± 16 | n. d. | 14 ± 6 | 0.525 |
| | 2 | 17 ± 8 | n. d. | n. d. | 16 ± 6 | 39 ± 10 | 10 ± 8 | 0.514 |
| | 3 | 15 ± 5 | n. d. | 7 ± 6 | 9 ± 8 | n. d. | 43 | 0.140 |
| **First bout size (licks)** | 1 | 99 ± 13 | 145 ± 56 | 136 ± 20 | 76 ± 28 | n. d. | 58 ± 11 | 0.468 |
| | 2 | 85 ± 33 | n. d. | n. d. | 126 ± 36 | 54 ± 12 | 78 ± 19 | 0.414 |
| | 3 | 153 ± 23 | n. d. | 101 ± 34 | 78 ± 11 | n. d. | 22 | 0.071 **+** |
| **Termination of last bout (min)** | 1 | 114 ± 2 | 70 ± 22 | 57 ± 25 | 79 ± 11 | n. d. | 85 ± 32 | 0.648 |
| | 2 | 117 ± 1 | n. d. | n. d. | 88 ± 10 | 101 ± 2 | 116 ± 2 | 0.056 **+** |
| | 3 | 111 ± 3 | n. d. | 114 ± 1 | 95 ± 5 | n. d. | 114 | 0.065 **+** |

# CHAPTER 5: GANAXOLONE, BUT NOT THIP, REINSTATES ETHANOL SEEKING IN MICE

This chapter has been modified from a submitted manuscript:

Ramaker MJ, Ford MM, Phillips TJ, Finn DA. Differences in the reinstatement of ethanol seeking with ganaxolone and THIP. Submitted to Psychopharmacology. January 24, 2014.

Acknowledgements

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**Abstract**

*Rationale*: The endogenous NAS ALLO has previously been shown to induce reinstatement of ethanol seeking in rodents. ALLO is a positive allosteric modulator at both synaptic and extrasynaptic $GABA_A$ receptors. The contribution of each class of $GABA_A$ receptor in mediating reinstatement of ethanol seeking is unknown.

*Objective*: One aim of the present study was to determine whether GAN, a longer-acting synthetic analog of ALLO, also promoted reinstatement of ethanol seeking. A second aim was to examine whether preferentially activating extrasynaptic $GABA_A$ receptors with the selective agonist THIP was sufficient to reinstate responding for ethanol in mice.

*Methods*: Male C57BL/6J mice were trained to lever press for access to 10E, using a sucrose fading procedure. Following extinction of the lever pressing behavior, systemic THIP (0, 4 and 6 mg/kg) and GAN (0, 10, and 15 mg/kg) were tested for their ability to reinstate ethanol-appropriate responding in the absence of 10E access. Locomotor studies examined whether changes in general activity accounted for responding in the reinstatement procedure.

*Results*: GAN reinstated ethanol seeking, but THIP did not. Similar quantitative increases in locomotor activity following both drugs suggested that drug-induced locomotor stimulation could not explain the increased ethanol-appropriate responding following GAN or lack of responding following THIP.

*Conclusion*: The results of this study suggest that differences in the pharmacological activity of GAN and THIP at the $GABA_A$ receptor contribute to the dissimilarity in reinstatement of ethanol seeking by the two drugs.

## Introduction

The NIAAA estimates that nearly 18 million Americans suffer from an AUD. AUDs are considered chronic relapsing disorders (Leshner, 1997; Volkow and Li, 2005). Nearly 90% of dependent individuals relapse at least once in a 4-year span (Polich et al. 1980), highlighting the need for a better understanding of the mechanisms underlying relapse. Although relapse is difficult to model in animals, the reinstatement model provides a measure of drug seeking during abstinence from the drug (de Wit and Stewart 1981; Shaham, 2003). The model has predictive validity in that re-exposure to drugs, drug-related cues, and stressors, all of which can provoke craving and possibly relapse in humans (de Wit 1996; Childress et al., 1993; Sinha, 2001), also promote reinstatement of drug-seeking in animals (Epstein et al., 2006).

ALLO is an endogenous NAS that can be increased in the brain and plasma by stress, estrus, pregnancy, and ethanol (Purdy et al., 1991; Genazzani et al., 1995; Concas et al., 1998; VanDoren et al., 2000; Barbaccia et al., 2001; Finn et al., 2004). Due to its potent positive modulation at $GABA_A$ receptors, ALLO shares many behavioral properties with ethanol, such as anxiolysis, sedation, and anticonvulsant properties (Kumar et al., 2009). ALLO substitutes for ethanol in drug discrimination procedures, indicating that it shares subjective stimulus properties with ethanol (Bowen et al., 1999; Grant et al., 1996, 2008). Exogenous ALLO also time and dose-dependently alters ethanol intake and reinstates ethanol seeking in rodents (Finn et al., 2008; Ford et al., 2005; Sinnott et al., 2002; Janak et al., 1998; Janak and Gill, 2003; Nie and Janak, 2003), suggesting that it plays a role in both ethanol seeking and consumption.

Although ALLO may have clinical benefit in multiple disease states (epilepsy, premenstrual dysphoric disorder, depression, traumatic brain injury; clinicaltrial.gov), the

therapeutic potential of ALLO is limited by its short half-life (Timby et al., 2006). GAN is a synthetic analog of ALLO with an added methyl group that renders it more resistant to metabolism (Nohria and Giller, 2007). Although its primary pharmacological and behavioral properties are unaltered (Carter et al., 1997; Ungard et al., 2000), the half-life of GAN is 3-4 times that of ALLO (Reddy and Rogawski, 2000). The use of GAN in clinical trials has broadened in the last decade, to include potential treatment of epilepsy, post-traumatic stress disorder, and smoking cessation (clinicaltrial.gov).

$GABA_A$ receptors are chloride channels composed of 5 subunits from a pool of at least 16 possible subunits: $\alpha_{1-6}$, $\beta_{1-3}$, $\gamma_{1-3}$, δ, ε, π, and θ. Importantly, inclusion of the δ subunit limits the location of the receptor to the extrasynaptic space, where it is postulated to contribute exclusively to tonic inhibition (Farrant and Nusser, 2005). *In vitro* work using recombinant receptors suggests that δ subunit-containing $GABA_A$ receptors may be particularly sensitive to the GABA-modulatory effects of ALLO (Belelli, et al. 2002). Similarly, δ subunit-containing $GABA_A$ receptors have been proposed to be a more sensitive target of physiologically-relevant doses of ethanol than non-δ subunit-containing $GABA_A$ receptors (Olsen et al., 2007; Mody et al., 2007; but also Borghese and Harris, 2007). Consistent with the importance of the δ subunit in the actions of NAS and ethanol, δ subunit knockout mice showed reduced sensitivity to some of the behavioral effects of both NAS and ethanol, and the knockout mice self-administered less ethanol than their wild-type littermates (Mihalek et al., 1999, 2001). Although GAN and ALLO can act at both synaptic and extrasynaptic $GABA_A$ receptors (Belelli and Herd, 2003), the contribution of each class of receptors to the reinstatement of ethanol seeking has not been examined.

The primary aim of the present studies was to determine whether GAN reinstates ethanol seeking in mice, as previously demonstrated with ALLO (Finn et al., 2008). The second aim of the study was to examine whether preferentially activating extrasynaptic $GABA_A$ receptors with THIP, which binds at the GABA site of the $GABA_A$ receptor, is sufficient to induce reinstatement of ethanol seeking. Locomotor tests were performed with each drug to elucidate whether changes in responding were accounted for by changes in general locomotor activity.

**Methods**

Animals

Male C57BL/6J mice, approximately 8 weeks of age at the start of experiments, were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were pair-housed for the reinstatement study or group-housed for the locomotor study. Mice were provided *ad libitum* access to rodent chow and water (except where noted). Mice were maintained on a 12-hour light: dark cycle (lights on at 0600), and all experiments were run during the animals' light cycle. All efforts were made to minimize animal suffering and to reduce the number of mice used. All procedures were approved by the local Institutional Animal Care and Used Committee and complied with NIH guidelines.

Drugs

The ethanol (200 proof; Pharmco Products, Brookfield, CT) solution was prepared by dilution in tap water. GAN was purchased from Tocris Bioscience (Ellisville, MO) and was solubilized in 20% β-cyclodextrin (Cargill Inc., Cedar Rapids, IA) in Millipore water. THIP was purchased from Tocris Bioscience and dissolved in saline.

For all experiments, drugs were injected i.p. in a volume of 0.01 ml/g body weight and with a 30 minute pretreatment time.

## Apparatus

Reinstatement sessions were carried out in eight operant conditioning chambers, consisting of a 21" L x 13.75" D x 5.0" H inner chamber located inside a sound and light-attenuating chamber, as described previously (Finn et al., 2008; Ford et al., 2007a, 2007b; Ramaker et al., 2012 (Chapter 3)). Each chamber contained a house light, 2 retractable levers with a stimulus light above each one, a retractable sipper apparatus, and a stainless steel grid floor. The retractable sipper was made from a 10 ml graduated pipette with a double ball-bearing metal sipper tube. Each metal sipper was connected to a lickometer circuit, which interfaced to a computer operating with MED-PC software (Med-Associates Inc.) to record time-stamped lever presses (and licks during the self-administration portion of the experiment).

## Acquisition

Self-administration was acquired with the “sipper” procedure (Ford et al., 2007a, 2007b; Samson et al., 1998). For 12 days, 32 mice were mildly fluid restricted and were trained to respond for access to 10% (w/v) sucrose (10S) on an FR1 schedule. Sessions were 60 minutes long, and every time the animal made a response on the “active” lever, both levers retracted, the house light turned off and the respective stimulus light turned on for 5 seconds, and the sipper extended into the chamber for 60 seconds. Responses on the “inactive” lever were recorded but had no scheduled consequence. The position of the active lever was counterbalanced between chambers. Sipper access was reduced from 60 to 30 seconds, and session length was decreased from 60 to 30 minutes over successive training sessions. The FR was increased by 1

every 3-4 sessions until mice were on an FR4 schedule. At this point, which was a month into training, 10E was added to the solution. The sucrose was also faded out at this time in a step-wise manner (3 sessions each with 10S/10E, 5S/10E, and 2.5S/10E solutions), to eventually yield a 10E solution. The FR schedule was then raised in a step-wise fashion (3-4 sessions each at FR4, FR6, FR8), and then a RR schedule was instituted. On the RR schedule, mice had 20 minutes to make the required number of lever presses, followed by 30 minutes of continuous 10E access. This schedule has been shown to increase the amount of fluid that the mice consume versus an FR schedule (Ford et al., 2007b). Mice were maintained on the RR8 schedule for approximately three weeks. At that time, because intake was still low, mice were exposed to a week of continuous access to 10E and water in the homecage. Intake during this time was 11.5 ± 0.4 g/kg per day for 5 days. Then, mice were put back on the RR8 schedule for 8 more weeks, where intake substantially increased. In total, mice had been self-administering 10E (in the absence of sucrose) for 16 weeks prior to the initiation of the extinction sessions.

Extinction and reinstatement

There were 12 mice that consistently earned the reinforcer and consumed ethanol [≥ 4 out of 5 days of acquiring the reinforcer and taking ≥100 licks on the sipper (approximately equal to 0.25 g/kg)] in the final week of 10E self-administration. These mice were used for the extinction and reinstatement phases of the study. Extinction consisted of a 30-minute session where presses on both the previously inactive and active lever were recorded, but there was no scheduled consequence (the house light remained on, the cue light did not come on, the levers did not retract, and no sipper came in). Mice then underwent 16 days of extinction, and during the last 4 extinction

sessions, mice were pretreated with saline at 30 minutes prior to the session start in order to habituate them to injections. When presses on the previously active lever were consistently below 25% of the previous criterion of 8, mice were then tested for the ability of 4 or 6 mg/kg THIP to reinstate lever pressing. Doses were given in ascending order in a within-subject design with baseline re-established between doses. Vehicle was given on all intervening days, and "0 mg/kg" was calculated by averaging the 2 days that immediately preceded a drug day (there were no significant differences between active or inactive lever presses on these 2 baseline days). Mice received 4 days off following the last THIP injection, and then extinction sessions were resumed with pretreatment of 20% β-cyclodextrin at 30 minutes prior to the session start. Beginning ten days after the last THIP injection, mice were tested with 10 and 15 mg/kg GAN in ascending order in a within-subject design, with baseline re-established between doses. Again, there were no statistical differences between active or inactive lever presses on the 2 days that immediately preceded a drug day, so these values were collapsed to give the "0 mg/kg" value.

BECs

During the final week of 10E self-administration, orbital blood samples were taken at the conclusion a 30-minute session. A 20 µL sample was taken from the orbital sinus of each mouse. Each blood sample was then added to 500 µL of a solution, which contained 4 mM *n*-propanol (internal standard) in deionized water. The solution was vortexed and analyzed using head-space gas chromatography (Finn et al., 2007). Concentrations of samples were interpolated from a standard curve, which was constructed using 6 pairs of external standards with known ethanol concentrations (from 0.5 to 3.0 mg/ml).

## Locomotor Activity

Locomotor chambers are described in detail previously (Phillips et al., 1995; Gubner et al., 2013). Briefly, sixteen automated locomotor chambers (40 x 40 x 30 cm; AccuScan Instruments Inc., Columbus, OH, USA) were used, which were equipped with 8 photocell beams located 2 cm above the floor. VERSADAT software (AccuScan Instruments Inc.) was used to calculate horizontal distance traveled (cm). Each chamber contained a fan to provide ventilation and background noise, and each chamber contained a 3.3 W incandescent light bulb during activity testing.

A separate group of naïve mice (n = 29) were used for the locomotor studies. On days 1 (habituation) and 2 (baseline), mice received an i.p. injection of 20% β-cyclodextrin. Thirty minutes later, they were placed in the locomotor chambers and horizontal activity was measured for 30 minutes. On day 3, separate groups of mice received an injection of 0 mg/kg (n = 10), 10 mg/kg (n = 10), or 15 mg/kg (n = 9) GAN at 30 minutes prior to the session start, and activity was again recorded for 30 minutes. There was no difference between groups for day 2 baseline activity. Drug-induced locomotor activity for each animal was calculated by subtracting its individual baseline (day 2) activity from its activity following drug injection (day 3). A week later, mice were again tested in the locomotor chambers after a 30 minute pretreatment with saline (days 1 and 2). On the third day, mice were balanced on previous treatment, and separate groups were tested following a 30 minute pre-treatment with 0 mg/kg (n = 10), 4 mg/kg (n = 10), or 6 mg/kg (n = 9) THIP. Drug-induced locomotor activity was calculated by subtracting each individual animal's baseline activity (day 2 saline; no difference between groups) from activity following drug injection (day 3).

## Statistical Analysis

To examine extinction, a 2-way repeated measures ANOVA was conducted, with lever type (active vs. Inactive) and session as factors. Post-hocs analyzed effect of each lever separately across days. Based on previous results with ALLO where ALLO significantly affected pressing on both levers, but post-hocs were only significant for the active lever (Finn et al., 2008), *a priori* planned comparisons analyzed active and inactive lever presses separately using one-way Repeated Measures ANOVAs to analyze the reinstatement tests. In the event of a significant main effect of dose, paired t-tests versus vehicle were performed using Fisher's Least Significant Difference test. For locomotor activity data, between-subjects ANOVAs assessed the effect of dose on overall activity. Activity data were analyzed in 5-minute bins using a 2-way ANOVA, with dose as a between-groups measure and time as the repeated measure. Post-hoc t-tests were performed only in the event of a significant result (time x dose interaction and then main effect of dose). The significance criterion was set at $p \leq 0.050$ for all experiments. A single outlier was removed from the analysis of active lever presses following reinstatement with 10 mg/kg GAN; this outlier was greater than 2 sd above the group mean.

**Results**

Extinction and Reinstatement

Prior to the initiation of extinction sessions, mice self-administered 10E for approximately 16 weeks. During the final week of self-administration, BECs measured in 10 mice were 26 ± 9 mg/dl (range 0 – 70 mg/dl). Intake on this day was 0.71 ± 0.14 g/kg (range 0 – 1.37 g/kg), yielding a significant correlation between intake and BEC ($r = 0.78$, $p = 0.008$). Throughout extinction, there was a significant interaction between active and inactive lever presses across sessions [$F(16,192) = 14.157$; $p < 0.001$; Fig.

5.1]. There was an effect of session for presses on the active lever [$F(16,192) = 15.034$; $p < 0.001$], but not for the inactive lever. During extinction, mice showed an initial extinction "burst" over their initial criterion of 8 presses (i.e. session 0 in Fig. 5.1) on the previously active lever ($p < 0.001$), and active lever presses remained significantly elevated through day 3. On days 13 through 16, pressing on the previously active lever was significantly below the original criterion of 8 lever presses (*ps* < 0.05).

During reinstatement, there was no effect of THIP to increase lever pressing on either the previously active [$F(2,22) = 0.793$, $p = 0.465$] or the previously inactive lever $F(2,22) = 1.023$, $p = 0.376$; Fig. 5.2). However, pretreatment with GAN significantly increased presses on the previously active lever [$F(2,20) = 3.575$, $p = 0.047$; Fig. 5.3], with an increase following 10 mg/kg ($p = 0.025$) and 15 mg/kg ($p = 0.008$). There was also a trend for an effect of GAN on inactive lever presses [$F(2,22) = 2.973$, $p = 0.07$; Fig. 5.3).

Locomotor Activity

To test whether sedative effects could account for the lack of reinstatement with THIP, mice were tested in locomotor activity chambers following an injection of 0, 4, or 6 mg/kg THIP injection. The effect of THIP on horizontal activity was not significant when data were collapsed across the 30-minute session (Fig. 5.4A). When examined by 5-minute bins, there was a trend for a bin by dose interaction [$F(2,26) = 2.306$; $p = 0.097$; Fig. 5.4B].

To examine whether locomotor stimulant effects could explain the increase in lever pressing with GAN, mice were tested in the locomotor chambers following treatment with either 0, 10, or 15 mg/kg GAN. There was a trend for an effect of GAN to increase locomotor activity across the entire 30-minute session ($F(2,26) = 2.923$; $p =$

0.072; Fig. 5.5A). Analyzing by 5-minute bins revealed a significant bin by dose interaction [$F(10,130) = 3.152$; $p < 0.001$; Fig 5.5B]. During minutes 0 – 5, there was a main effect of dose [$F(2,26) = 8.218$ $p = 0.002$]; both 10 mg/kg ($p = 0.022$) and 15 mg/kg ($p < 0.001$) GAN significantly increased activity during this time. There was also an effect of dose during minutes 5 – 10 [$F(2,26) = 4.722$ $p = 0.018$], with a trend for an increase following 10 mg/kg ($p = 0.051$) and a significant increase following 15 mg/kg ($p = 0.006$). There was a trend for an increase in activity during minutes 10 – 15 [$F(2,26) = 2.923$; $p = 0.072$].

**Discussion**

In the present study, mice had a history of more than 4 months of lever pressing for 10E access. Even after more than 3 weeks of abstinence, during which time lever pressing did not result in 10E access or conditioned cue exposure, GAN increased ethanol seeking behavior, as measured by the number of presses on the previously active lever. This is consistent with data showing that GAN increased appetitive ethanol seeking prior to gaining access to ethanol (Ramaker et al., 2012 (Chapter 3)) and that ALLO increased ethanol seeking in reinstatement procedures (Nie and Janak, 2003; Finn et al., 2008). THIP on the other hand, did not reinstate ethanol seeking, consistent with a previous finding that it decreased ethanol-reinforced lever pressing (Ramaker et al., 2012 (Chapter 3)).

Although GAN did cause locomotor stimulation, which could have contributed to the increases in lever pressing during the reinstatement tests, its effects were only significant with regard to the previously active lever. Furthermore, visual comparison between the locomotor data for THIP and GAN shows that THIP induced similar quantitative increases in locomotor activity as GAN across all time-points. The inability

of THIP to reinstate lever pressing, despite similar hyperactivity, suggests that a locomotor stimulant effect of a drug is not sufficient to explain a reinstatement of extinguished lever pressing. In support of this, Besheer et al. (2010) showed that 1 mg/kg GAN in rats increased ethanol-reinforced responding in the absence of a general effect on locomotor activity, indicating that GAN can have effects on ethanol-reinforced responding separable from its general locomotor effects. With THIP, there were not sedative effects that could explain the lack of reinstatement with THIP. Although we cannot rule out that a competing behavior (activation) interfered with lever pressing behavior, again, the similar quantitative responding as that observed with GAN suggests that this level of activation did not prevent lever pressing behavior.

One interpretation of the differential effect between GAN and THIP is that activation of extrasynaptic $GABA_A$ receptors alone is not sufficient to promote ethanol reinstatement. It is possible that GAN's activity at synaptic $GABA_A$ receptors, or the combined actions at synaptic and extrasynaptic $GABA_A$ receptors, is necessary to promote ethanol seeking. However, another important consideration is that pharmacological differences between GAN and THIP in their ability to potentiate $GABA_A$ receptor function [positive allosteric modulator (GAN) versus direct agonist (THIP)] may also contribute to the differential effect of these drugs on ethanol reinstatement. Examination of reinstatement of ethanol seeking with other $GABA_A$ receptor-acting drugs such as muscimol, which acts at the GABA binding site at both synaptic and extrasynaptic $GABA_A$ receptors, could help elucidate whether it is the action at synaptic versus extrasynaptic receptors or the action at the NAS site (Hosie et al., 2006) versus the GABA site that differentiate the effect of these drugs on ethanol reinstatement.

Although the mechanism by which GAN, but not THIP, reinstated ethanol seeking is not clear, due to shared stimulus properties between ALLO and ethanol (Bowen et al., 1999; Grant et al., 1996, 2008), GAN may have acted similar to a drug prime. This is in line with studies showing that non-contingent priming injections of a drug, or agents with similar pharmacological or stimulus properties of a drug, reinstated seeking for that drug (de Wit and Stewart,1981; de Wit and Stewart ,1983; Stewart 1983). THIP did not substitute for ethanol (1.5 g/kg) in a drug discrimination study (Shelton and Grant, 2002), suggesting that, unlike GAN, THIP does not share discriminative stimulus effects with ethanol. Additionally, mice lacking the δ subunit of the $GABA_A$ receptor could discriminate between ethanol and saline (Shannon et al., 2004), indicating that this subunit is not necessary for the discriminative properties of ethanol. On the other hand, there is not always a direct relationship between drug discrimination and reinstatement (Shaham et al., 2003), and there could be alternative explanations as to why GAN, but not THIP, promoted reinstatement.

Although the specificity of GAN to enhance reinstatement of ethanol seeking was not tested in the present study, ALLO promoted reinstatement of sucrose seeking in mice, but not in rats (Finn et al., 2008; Nie and Janak, 2003). Given that ALLO also decreased latency to feed in rats (Holmberg et al., 2013), it is possible that ALLO and GAN may have more general effects on seeking behavior, particularly for calorie-containing substances. On the other hand, ALLO *decreased* yohimbine- and drug-induced reinstatement of cocaine and methamphetamine seeking, respectively, in rats, at doses that did not induce sedation (Holtz et al., 2012; Anker et al., 2009), indicating that ALLO or GAN-induced enhancement of ethanol seeking does not generalize to all drugs of abuse or reinforcers. It should be noted that in the case of reinstatement of cocaine and methamphetamine seeking, the effect of ALLO was only present in female

mice (Holtz et al., 2012; Anker et al., 2009), highlighting important potential interactions with sex hormones that remain to be elucidated in the case of the reinstatement of ethanol seeking.

The inability of THIP to reinstate ethanol seeking is consistent with data showing that it decreased ethanol-reinforced responding in an operant self-administration procedure (Ramaker et al., 2012 (Chapter 3)). The 4 mg/kg dose was chosen based on the data showing that it decreased both the appetitive and consummatory phases of self-administration in an operant procedure (Ramaker et al., 2012 (Chapter 3)). Importantly, the 4 and 6 mg/kg doses of THIP correspond to low μM concentrations in the CNS, concentrations thought to act selectively on tonic inhibition (Cremers and Ebert, 2007). The locomotor data indicate that these doses are not causing a sedative effect that would inhibit the animal's ability to lever press, but that they actually induce a slight statistically non-significant locomotor stimulant effect. Although it is possible that increasing the dose may have uncovered an effect of THIP to promote reinstatement, use of 8 mg/kg prevented 11 of 12 mice from attaining an ethanol reinforcer in a prior study (Ramaker et al., 2012 (Chapter 3)), consistent with the idea that THIP may decrease ethanol seeking. Future studies should test the effect of THIP to decrease stress- or cue-induced reinstatement, as a floor effect in the current procedure limited the ability to detect a decrease if one existed.

An important consideration is that mice in the present study had an extended history of ethanol intake followed by a period of ethanol abstinence. Removal of ethanol following chronic exposure can lead to alterations in $GABA_A$ receptor subunit expression, specifically a down-regulation in δ subunit surface expression (Liang et al., 2007) and a subsequent decrease in THIP-mediated tonic current (Liang et al., 2006). Although

there are major differences between the present study and that used by Liang and colleagues (2006, 2007) in terms of timing and exposure to ethanol (60 exposures to 5 or 6 g/kg ethanol injections in Liang et al. versus 4 months of self-administered ethanol of < 1 g/kg), consideration of this potential effect is important. A possible reduction in δ subunit expression may have limited the ability of THIP to induce effects in the reinstatement procedure. On the other hand, mice in the locomotor study were naïve. Consequently, it is possible that an extensive ethanol experience would also have blunted any locomotor effects of THIP.

Because these studies all utilized systemic injections, they do not provide insight into the brain areas underlying the ability of GAN to promote reinstatement of ethanol seeking. In human studies, the ventromedial PFC and anterior cingulate are both hypoactive in abstinent, recovering alcoholics, and this hypofunction is related to increased craving and possibly relapse (Seo et al., 2013). Although speculative, it is possible that GAN could have acted in the PFC or interacting brain areas, and that this hypofunction could have contributed to increased ethanol seeking. Future studies using site-specific infusions, particularly aimed at the PFC, could help elucidate some of the underlying brain mechanisms contributing to ethanol reinstatement.

In conclusion, THIP did not promote reinstatement of ethanol seeking in the present study, consistent with data showing that THIP decreased ethanol-reinforced responding in an operant procedure (Ramaker et al., 2012 (Chapter 3)). These data suggest that direct activation of extrasynaptic $GABA_A$ receptors at the GABA site is not sufficient to induce ethanol seeking in the reinstatement procedure. On the other hand, GAN promoted reinstatement of ethanol seeking, consistent with data showing that GAN increased ethanol seeking in an operant self-administration procedure (Ramaker et al.,

2012 (Chapter 3)) and that ALLO promoted ethanol seeking in a reinstatement procedure (Finn et al. 2008). Further work is necessary to examine whether neurosteroidogenesis could represent a point of therapeutic intervention in preventing relapse in humans. These data are informative, considering the increasing use of GAN and THIP in clinical trials across multiple disease states (clinicaltrials.gov). For diseases that have high comorbidity with AUDs (such as smoking and post-traumatic stress disorder), the potential effect of GAN on alcohol seeking is important to consider when using this drug in a clinical setting.

**Figure 5.1. Lever pressing behavior during extinction**

Lever presses during extinction are shown for both the previously active and inactive levers. Presses on either lever during this time had no scheduled consequence. Values represent mean ± SEM of 12 mice. $+p \leq 0.10$, $*p \leq 0.05$, $***p \leq 0.001$ versus pre-extinction (session 0) active lever presses.

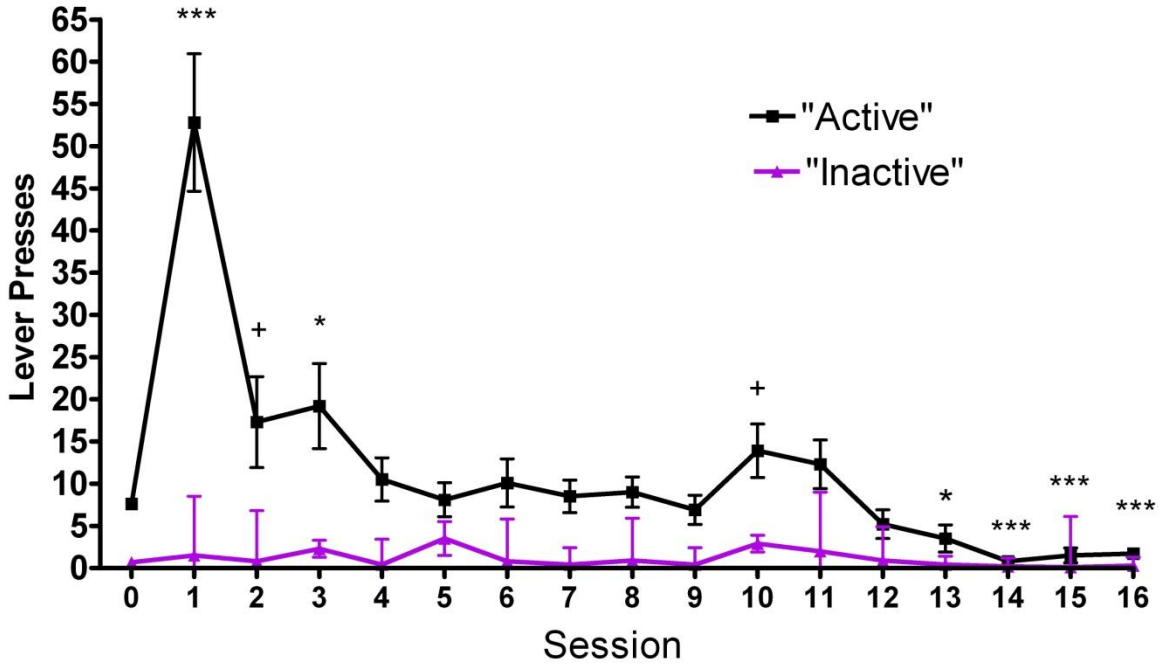


**Figure 5.2. THIP did not reinstate extinguished ethanol-reinforced responding**

Pretreatment with THIP did not alter lever pressing on either the previously active or inactive lever. Values represent mean ± SEM for the mice depicted in Figure 5.1.

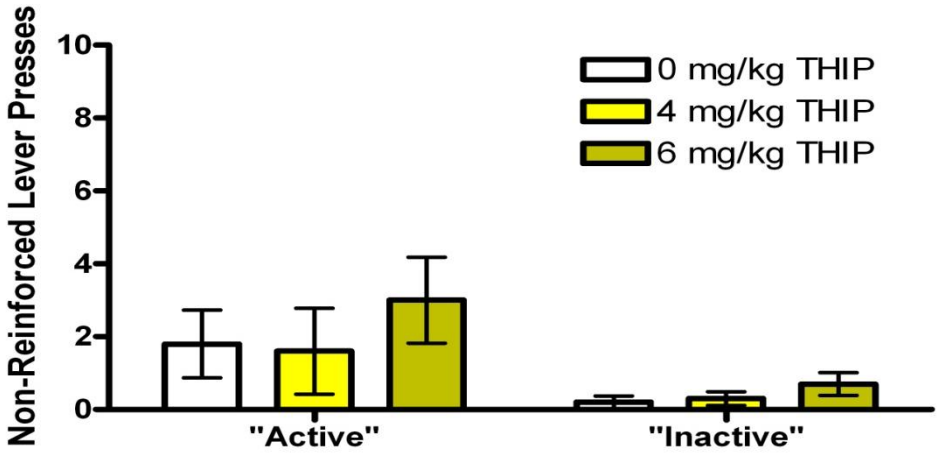



**Figure 5.3. GAN reinstates extinguished ethanol-reinforced responding**

GAN increased lever pressing on the lever previously active lever, with a trend for increased responding on the previously inactive lever. Values represent mean ± SEM for the mice depicted in Figures 5.1 and 5.2. *$p \leq 0.05$, **$p \leq 0.01$.

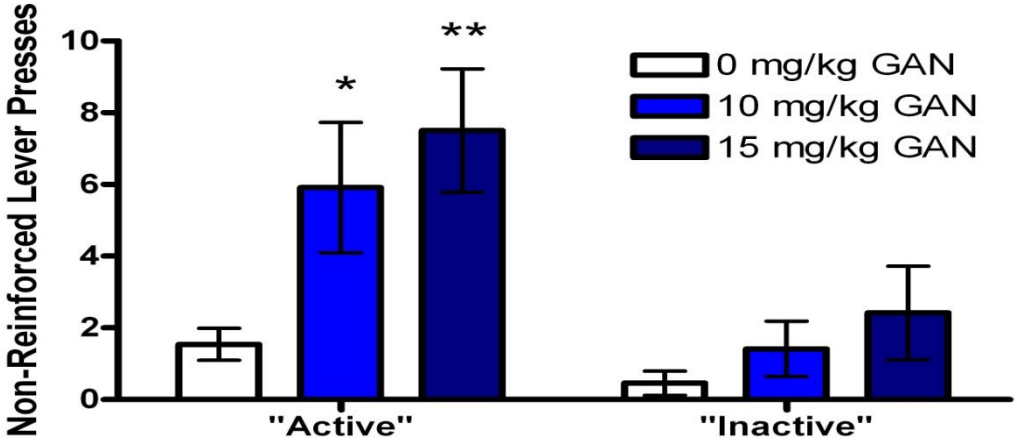
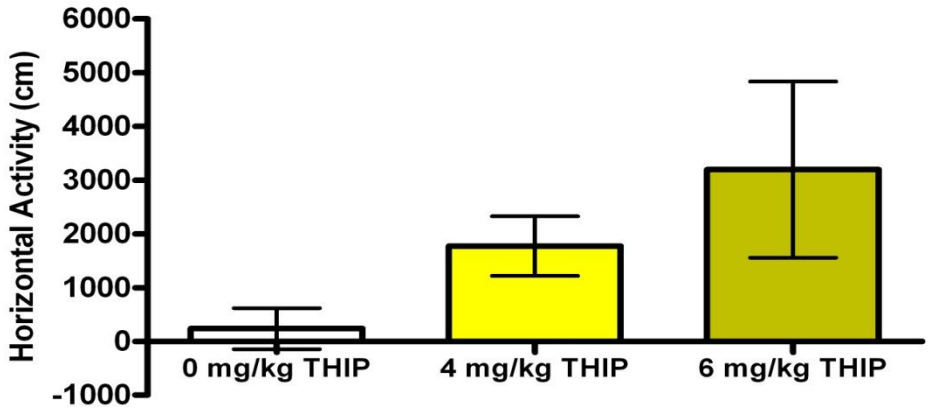


**Figure 5.4. Locomotor effects of THIP**

Locomotor effects of THIP A) across the total 30-minute session and B) split into 5-minute bins. Separate groups of mice were treated with 0, 4, or 6 mg/kg THIP at 30 minutes prior to the locomotor session. To calculate the drug-induced locomotor activity for each animal, the baseline activity following a saline injection was subtracted from activity following their drug treatment. Values represent mean ± SEM for 9 – 10 per group.

A)




B)

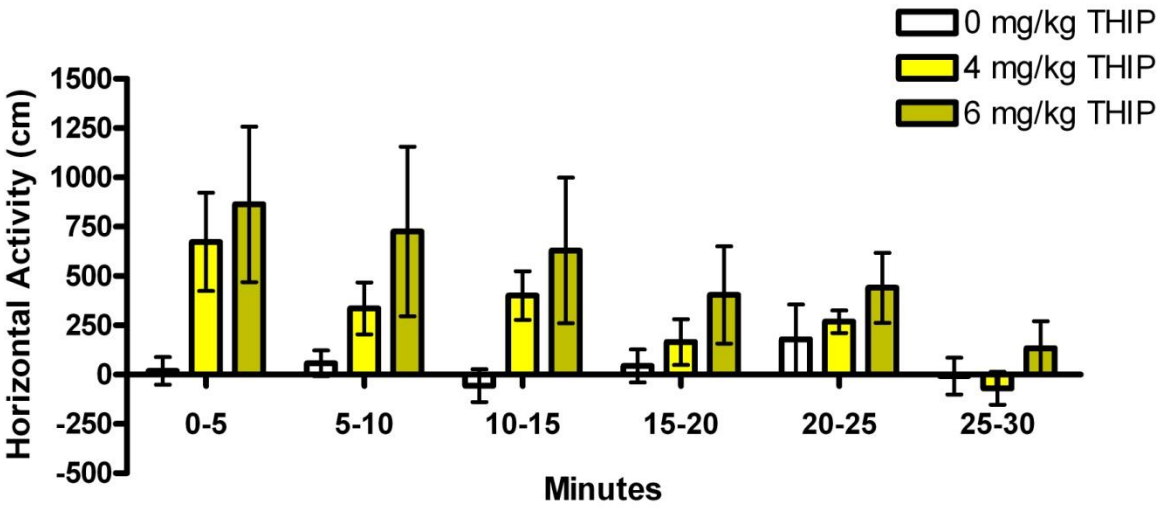
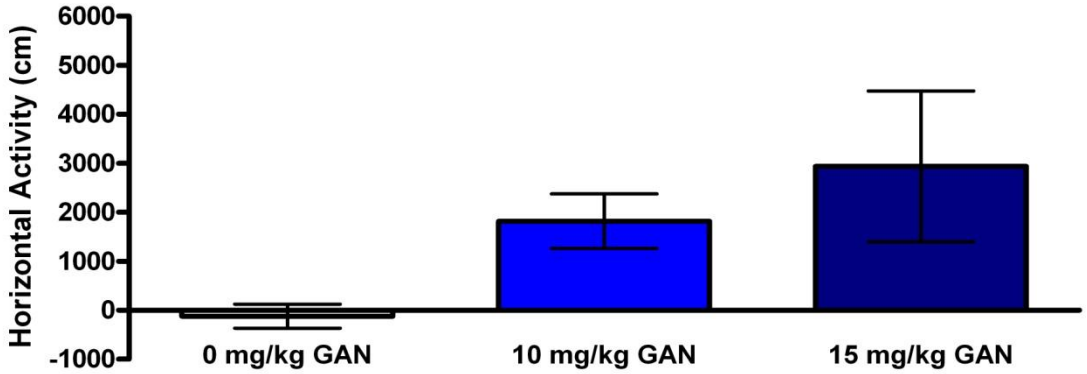


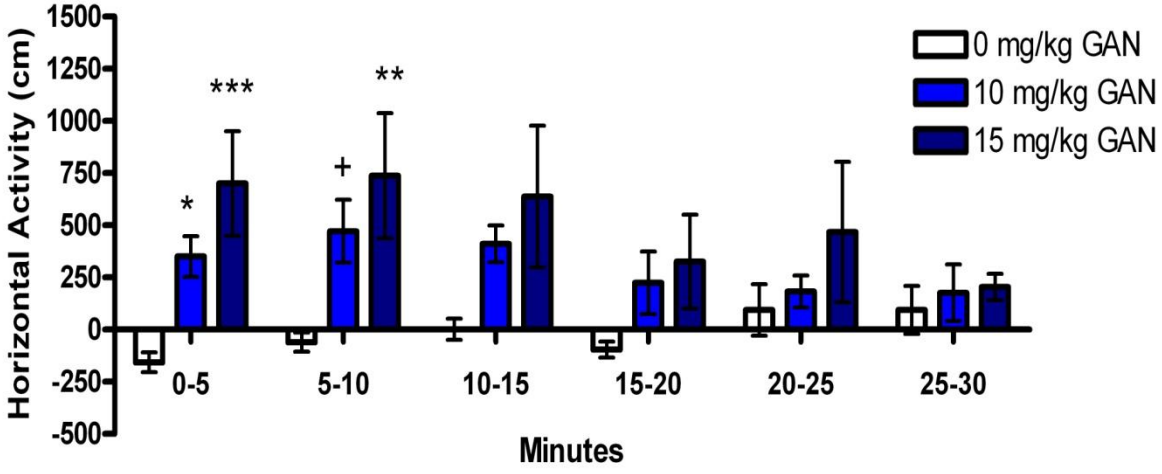
**Figure 5.5. Locomotor effects of GAN**

Locomotor effects of GAN A) across the total 30-minute session and B) split into 5-minute bins. Separate groups of mice were treated with 0, 10, or 15 mg/kg GAN at 30 minutes prior to the locomotor session. To calculate the drug-induced locomotor activity, baseline activity for each animal following 20% β-cyclodextrin on day 2 was subtracted from the activity following drug treatment on day 3. Values represent mean ± SEM for 9 – 10 per group. $+p \leq 0.10$, $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$.

A)




B)



# CHAPTER 6: GENERAL DISCUSSION

## Initial hypothesis

The present studies aimed to use GAN, a synthetic GABAergic NAS, and THIP, a preferential extrasynaptic $GABA_A$ receptor agonist, to elucidate some of the behavioral mechanisms associated with ethanol seeking and consumption. I hypothesized that GAN would alter parameters of ethanol intake across a variety of procedures, which would provide support for a role of the NAS environment in mediating ethanol intake. I also hypothesized that THIP would alter ethanol intake across procedures, demonstrating that activation of extrasynaptic $GABA_A$ receptors contributes to the appetitive and consummatory aspects of ethanol self-administration. Finally, I predicted that intra-NAc shell infusions of GAN and THIP would be sufficient to account for the decreases in limited-access ethanol intake that were observed following systemic injection of GAN and THIP.

## Summary of findings

In Chapter 2, I used a 24-hour 2-bottle choice procedure to show that the ALLO analog, GAN, increased initial ethanol intake, followed by an overall suppression of ethanol intake in a continuous-access procedure. The effect with GAN lasted for 5 hours, confirming that it could be used to examine longer effects on ethanol consumption than had been previously observed with ALLO. In Chapter 3, I used a 2-hour 2-bottle choice procedure to capitalize on the time-frame in which the drug effects were most pronounced and to take advantage of higher BECs acquired by the mice in this procedure versus those typically attained in a continuous-access procedure. In this experiment systemic injections of GAN decreased overall 2-hour ethanol intake by

trending to decrease bout frequency. In Chapter 4, the same 2-hour 2-bottle choice procedure was used in mice that had bilateral cannulae targeted to the NAc shell. Intra-NAc shell GAN decreased10E consumption, and the decrease in 10E intake was again characterized by a decrease in bout frequency, as well as early termination of the session. The similarity in 10E intake between systemic and intra-NAc shell GAN suggests that activation of $GABA_A$ receptors in the NAc shell by GAN is sufficient to regulate 10E limited-access consumption. Control placements confirmed that there was some site-specificity to this effect as there was no change in overall intake or bout frequency in dorsal controls or in placements with one injector terminating in the shell and one terminating in the core. In Chapter 3, I also used an operant procedure to examine appetitive seeking and subsequent consumption of ethanol. These experiments revealed that administration of GAN led to a non-significant, 43% decrease in latency to attain the reinforcer, followed by a non-significant, 33% increase in consumption of ethanol, consistent with a role for GAN to slightly increase ethanol seeking and initial intake. Sucrose controls showed that GAN also promoted sucrose seeking, but did not systematically alter sucrose intake, indicating some non-specificity in the seeking behavior induced by GAN. In Chapter 5, I again examined ethanol seeking, but in the absence of the ethanol reinforcer, with the use of a reinstatement procedure. GAN reinstated extinguished ethanol-reinforced lever pressing, consistent with a role for GAN to increase ethanol seeking. In total, and consistent with previous literature using ALLO, GAN appeared to primarily increase ethanol seeking and initial ethanol intake while decreasing overall consumption.

With the extrasynaptic $GABA_A$ receptor agonist, THIP, I first examined the effects of this drug on 10E intake across 24-hours in Chapter 2. I found that THIP suppressed ethanol intake for the first 5 hours of a continuous-access procedure. In Chapter 3, in a

2-hour limited access procedure whereby mice were consuming binge levels of ethanol (BEC ≥ 80 mg/dl as defined by NIAAA), I again observed a suppressive effect of THIP on total intake, where it both delayed the onset to the first bout and decreased the total number of bouts in the 2-hour session. In Chapter 4, I found that activation of $GABA_A$ receptors in the NAc shell with THIP contributed to the decreases in 10E intake observed in the systemic study, but there was a pronounced influence of the order in which THIP infusions were given (discussed in "Order effects with THIP"). I found that THIP decreased both seeking and consumption of ethanol in an operant procedure (Chapter 3), but this effect appeared non-specific as the same effect was observed in mice self-administering sucrose. Data from Chapter 5 showed that THIP did not promote reinstatement of ethanol seeking following extinction of a learned behavior to attain ethanol. In total, these studies showed a general suppressive effect of THIP on measures of ethanol consumption, with weaker evidence for a suppression in ethanol seeking.

**General interpretations**

Because GAN can act at both synaptic and extrasynaptic $GABA_A$ receptors, THIP was used to examine how activation of extrasynaptic $GABA_A$ receptors contributes to ethanol seeking and intake. Because THIP preferentially acts at δ subunit-containing $GABA_A$ receptors (discussed in "Doses of THIP" below), via binding at the GABA binding site, the observation of effects following administration of GAN, but not THIP, implies that solely activating extrasynaptic $GABA_A$ receptors with a direct agonist is not sufficient to alter that behavior. Overall, these studies showed divergent effects between GAN and THIP, particularly for ethanol seeking and initiation of ethanol intake. These results provide evidence that the effect of GAN on those measures were likely due to its actions,

at least in part, at *non*-δ subunit-containing $GABA_A$ receptors (i.e. It may have required the combination of actions at δ and non- δ subunit-containing $GABA_A$ receptors, but actions at δ subunit-containing $GABA_A$ receptors alone was not sufficient). On the other hand, overall reductions in consumption were similar following GAN and THIP across multiple bout parameters, indirectly supporting the hypothesis that there is some overlap in their mechanisms of action, and that decreases on overall ethanol intake by GAN may be attributable, at least in part, to activation of extrasynaptic $GABA_A$ receptors. An alternative explanation is that the location of the activated receptor is not important for the seeking or consummatory behaviors, but that activation of the receptor at the GABA site (THIP) versus modulation of the receptor at the NAS site (GAN) is what differentiated the effects of these two drugs on ethanol seeking behavior.

An important interpretational consideration is that the present experiments do not allow me to determine if the decreases in consumption following GAN or THIP are due to a change in the rewarding value of ethanol. For example, a decrease in consumption could indicate that the ethanol has a greater rewarding value, and so a comparable CNS effect is attained with lower quantities of ethanol. On the other hand, a decrease in consumption could reflect a decrease in the rewarding effects of ethanol, where a reduced rewarding value of the ethanol may lead to a decreased motivation to consume it. There are also alternative, and not mutually exclusive, explanations in which GAN or THIP could have caused aversion or had no effect on reward.

One speculative hypothesis, based on the findings in my dissertation work and incorporation of other literature, is that steroidogenesis following ethanol intake may contribute to the rewarding experience of ethanol consumption. Therefore, once a threshold level of ethanol-induced NAS is reached, ethanol intake is terminated.

Further, I hypothesize that activation of $GABA_A$ receptors containing the δ subunit is an important component to the signal of ethanol reward. Therefore activation of the δ subunit with THIP may decrease ethanol intake by acting at some of the same receptor targets as ethanol or ethanol-induced NAS such as ALLO.

**Revised hypothesis: ethanol seeking**

In the present experiments, the ethanol seeking and initial intake following GAN administration could be explained by ALLO's shared stimulus properties with ethanol (Bowen et al., 1999; Grant et al., 1996, 2008). For example, previous studies have suggested that activation of the same sites that elicited positive incentive effects with drug-taking will promote seeking of that drug (Bozarth and Wise, 1981). Therefore, by acting on the same reward circuitry targeted by ethanol, GAN may have induced ethanol seeking. Further, a priming effect could explain not only seeking, but also initial intake of ethanol. For example, in rats that could freely administer d-amphetamine, a priming injection of non-contingent d-amphetamine during their self-imposed abstinent period (their light cycle), led to an increase in self-administration of d-amphetamine during that time (Pickens and Harris, 1968). Likewise, in the present experiments, injections of GAN during periods when the mice were not drinking (i.e., 30 minutes before session start) may have activated circuits normally activated by ethanol, thereby initiating seeking and onset of ethanol intake (Chapters 2 and 3).

A noticeable divergence in the results was that GAN promoted ethanol seeking, while THIP did not. If there is a link between drug discrimination and drug priming, the lack of increased ethanol seeking with THIP would be consistent with the observation that THIP did not substitute for ethanol in a drug discrimination procedure (Shelton and Grant, 2002), and therefore would not be expected to act as an ethanol prime.

Additionally δ subunit knockout mice can discriminate between ethanol and saline (Shannon et al., 2004), indicating that this subunit is not necessary for the detection of the discriminative properties of ethanol. Data from non-human primates suggest that benzodiazepine-sensitive $GABA_A$ receptor subunits $\alpha_2$, $\alpha_3$, or $\alpha_5$ contributed to the stimulus properties of ethanol, particularly at a 1 g/kg ethanol training dose (Helms et al., 2008, 2009), a dose consistent with that administered by the mice in the present operant experiments (Chapters 3 and 5), and an ethanol dose which shares discriminative properties with ALLO in rats and non-human primates (Bowen et al., 1999; Grant et al., 2008). Additionally, pregnanolone (the 5β-isomer of ALLO, which shares pharmacological properties with ALLO and GAN; Shannon et al., 2005b) substitutes for ethanol in both δ subunit knockout and wild-type mice (Shannon et al., 2004), providing further evidence that GAN's actions at *non*-δ subunit-containing $GABA_A$ receptors likely contribute to the shared stimulus properties of NAS with ethanol. Therefore non-δ subunit-containing $GABA_A$ receptors may underlie GAN's ability to increase ethanol seeking.

An important consideration, however, is that the differing results on ethanol seeking with GAN and THIP may not be due to a difference in action at non-δ subunit-containing $GABA_A$ receptors. Rather, GAN's action as a positive allosteric modulator versus THIP's action as a direct agonist likely also contribute to the divergent behavioral effects in terms of drug discrimination and measures of ethanol seeking, consistent with data showing that muscimol (direct $GABA_A$ receptor agonist that would be expected to bind to both δ and non-δ subunit-containing $GABA_A$ receptors) does not substitute for ethanol in drug discrimination procedures (Grant et al., 2000; Shelton and Balster, 1994). It would be informative to examine the effect of systemic muscimol on reinstatement of ethanol seeking. If muscimol, like THIP, does not reinstate ethanol

seeking, it would suggest that direct activation of the $GABA_A$ receptor (regardless of its location at synaptic or extrasynaptic sites) is not sufficient to reinstate ethanol seeking. Alternatively, if muscimol did reinstate ethanol seeking, it would indirectly suggest that only activating extrasynaptic $GABA_A$ receptors (regardless of whether by direct activation or positive modulation) is not sufficient to reinstate ethanol seeking.

**Revised Hypothesis: ethanol consumption**

Despite the increased ethanol seeking and initial intake induced by GAN, it generally led to a reduction in overall consumption, which likely was regulated by a different mechanism than seeking. According to my hypothesis, ethanol intake would cause an increase in NAS levels in the brain, expected to peak about 30-80 minutes after ethanol intake (Finn et al., 2004; VanDoren et al., 2000); the increased NAS would contribute to reward, and therefore once satiety was reached, the mice would subsequently terminate their intake. A pretreatment of GAN, therefore, may have acted in concert with ethanol-induced NAS levels to signal reward, and may have contributed to signals of satiety sooner and with lower amounts of ethanol consumed. This hypothesis would be consistent with the decreases in overall consumption with GAN that I observed in all of the procedures that lasted longer than 30 minutes, as well as the early termination of the session that I observed with GAN in Chapter 4. The one exception to an overall reduction in consumption was the 30-minute operant procedure in Chapter 3, where levels of ethanol-induced NAS would not be expected to peak until after the session was over. Another important consideration is that in the 30 minute session, drinking may not have been high enough to elicit steroidogenesis. At least with systemic injections of ethanol, there appeared to be a threshold of at least 1.5 g/kg ethanol that was necessary to increase levels of ALLO and steroidogenic machinery

such as adrenal levels of StAR protein in the rat (Boyd et al., 2010). Thus, results from this 30-minute operant procedure were likely less influenced by ethanol-induced steroidogenesis.

Across procedures, THIP also decreased ethanol consumption. My primary hypothesis, again based on my data and the available literature, was that similar to GAN, THIP might decrease ethanol consumption by increasing the rewarding effects of ethanol. The hypothesis that activation of the δ subunit may be an important component of ethanol reward is consistent with interpretations from studies using δ subunit knockout mice or δ subunit knockdown in the NAc. Rodents in those studies consistently self-administered less ethanol than their wild-type littermates or controls, without differing in saccharin, quinine, or sucrose intake (Nie et al., 2011; Mihalek et al., 2001). One interpretation from those studies was that ethanol (or subsequent NAS) actions at the δ subunit normally contributed to a signal of ethanol reward; in the absence of the NAS or ethanol target (i.e. the δ subunit), the ethanol was less rewarding and so the animals self-administered less (Nie et al., 2011; Mihalek et al., 2001). In the case of my studies, one possibility is that by acting at δ subunit-containing $GABA_A$ receptors, THIP could be causing a left-ward shift in the dose-response curve (i.e., less ethanol or subsequent NAS levels would be needed to activate the same number of δ subunit containing $GABA_A$ receptors).

**Proposed mechanism**

If steroidogenesis contributes to the rewarding value of ethanol consumption, it follows that ALLO/GAN should act somewhere within the reward circuitry to alter physiological signals of reward. It is well documented that, similar to many drugs of abuse, injection (Imperato and Di Chiara, 1986; Yoshimoto et al., 1991; Di Chiara and

Imperato, 1985) or oral administration of ethanol in rodents (Weiss et al., 1993) and humans (Boileau et al., 2003) increases levels of dopamine in the NAc. In addition to the NAc, increases in dopamine levels have been reported in the medial PFC following ethanol injection (Dazzi et al., 2002), consistent with the idea that ethanol directly or indirectly increases VTA dopaminergic cell firing and increases extracellular dopamine levels in terminal areas (Tateno and Robinson, 2011; Brodie et al., 1990). There is also evidence that ALLO and other 5α-R NAS may modulate the actions of ethanol on the mesolimbic dopamine system. For example, progesterone pretreatment that increased cortical ALLO levels caused a left-ward shift in the dose-response curve for ethanol induction of dopamine output in the mPFC (Dazzi et al., 2002). Further, the ability of ethanol to increase extracellular dopamine levels in the mPFC was blocked with FIN, suggesting that an ethanol-induced increase in NAS influenced the effect of ethanol on dopamine levels in the mPFC (Dazzi et al., 2002). Besides influencing ethanol-induced dopamine release, NAS also may directly elicit an increase in extracellular dopamine levels as ICV infusions of ALLO (31 ng in 5 µL) increased NAc dopamine levels (Rogué-Pont et al., 2002). Collectively, the results of these studies are consistent with the idea that NAS may contribute to ethanol-induced or ethanol-independent alterations in mesolimbic dopamine signaling.

Consistent with the notion that ALLO or GAN may cause physiological changes associated with reward, a single systemic injection of GAN (30 mg/kg) increased AMPA:NMDA receptor ratio on cell bodies in the VTA (Vashchinkina, et al., 2013), a common physiological change observed with many drugs of abuse, including ethanol, morphine, cocaine, amphetamine, and nicotine, as well as stress (Saal et al., 2003). The behavioral implications of an increased AMPA:NMDA receptor ratio are not known. However the increase in AMPA:NMDA ratio seems to be a common consequence of

many drugs of abuse, even those with very different underlying molecular mechanisms, whereas non-addictive therapeutics such as fluoxetine (antidepressant) or carbamazepine (antiseizure medication) do not induce this change (Saal et al., 2003). Therefore the change in AMPA: NMDA ratio is generally thought to reflect plasticity that is associated with abuse liability (Saal et al., 2003). It can be summarized, then, that like drugs of abuse, GAN causes molecular changes that enhance the strength of excitatory afferents onto dopamine cells in the VTA. Interestingly, the increase in the AMPA:NMDA ratio following a single GAN injection was absent in δ subunit knockout mice, suggesting an integral link between GAN's action at the δ subunit and molecular changes associated with a putative rewarding property of GAN (Vashchinkina, et al., 2013).

In line with the notion that THIP may increase the rewarding value of ethanol, possibly by acting at some of the same sites as ethanol acts at to increase dopamine levels, intravenous THIP administered to rats increased the firing of VTA dopamine neurons *in vivo* (Waszczak and Walters, 1980). Similar to GAN, a single injection of 6 mg/kg THIP in mice increased the AMPA:NMDA receptor ratio in the VTA (Vashchinkina et al., 2012), indicating that THIP may alter the potential strength of subsequent incoming excitatory afferents onto dopamine cells in the VTA.

**Proposed circuitry**

From a neural circuit point of view, my model for the effects of GAN or THIP on the ethanol consummatory behaviors predicts that these drugs could act at multiple points in the reward circuit, but would ultimately act to increase the ethanol-induced dopamine signal in the NAc. This could result from disinhibiting the VTA dopamine neurons or from directly altering the excitability of the NAc cells that dopamine acts on. In my systemic studies, there are many brain areas that could be contributing to the

effect of either of these drugs on ethanol consumption, and the microinfusion studies suggest that the NAc shell is one important neuroanatomical target.

Figure 6.1 represents a hypothetical model of how GAN/THIP may modulate ethanol-induced dopamine reward in the NAc. If the end result is to disinhibit the VTA, it is unlikely that the drugs infused into the NAc are doing so via actions on the D1 receptor-expressing GABAergic cells that directly project back to the VTA. Data show that these NAc projections primarily synapse on to non-dopaminergic cells in the VTA, likely the GABAergic interneurons (Xia et al., 2011). The net result of GAN/THIP acting on the NAc "direct" cells would be to disinhibit the GABAergic interneurons in the VTA, consequently dampening the dopamine cell firing. Therefore it is likely that more indirect circuitry is involved, either via the "direct" cells and incorporating other levels of feedback or via the "indirect" pathway. For example, it is possible that the inhibitory actions of GAN/THIP on the D2 receptor-containing cells disinhibited the ventral pallidal cells. Disinhibition of pallidal cells would reduce glutamatergic output from the subthalamic nuclei, thalamus, or cortex (among other areas) back to the VTA. If these inputs also synapse onto VTA GABAergic interneurons, this would therefore provide one way to indirectly disinhibit dopamine cell firing (Fig 6.1C). Multiple points in the circuit also provide feedback, which could in turn alter the response. Even within the NAc, there are local interneurons and axon collaterals that synapse onto other projection neurons (Tunstall et al., 2002). These connections could serve to disinhibit a projection cell, thereby increasing rather than decreasing GABA release in the presence of GAN or THIP. This feedback mechanism would reverse all of the situations above, and if GAN/THIP is acting on the direct projections back to the VTA, this could cause increased dopamine cell firing. Admittedly, this model is speculative, and it would require many follow-up studies to confirm the mechanisms. One useful study would be a

microdialysis experiment to confirm if NAc or systemic injections of GAN or THIP do in fact increase dopamine levels in the NAc as this scenario would predict.

It is also possible that, in addition to or instead of increasing dopamine release in the NAc, GAN or THIP may have influenced the ethanol-induced dopamine signal in the NAc by directly altering the excitability of the NAc neurons (Fig. 6.1). The D1 and D2 receptors in the NAc are generally thought to have opposing effects on behavior. Evidence with DREADDs and optogenetics suggest that activation of direct pathway D1 receptor containing cells increases the rewarding effects of some drugs of abuse while activation of the indirect pathway blocks the rewarding effects, as measured by amphetamine sensitization and cocaine CPP (Ferguson et al., 2011; Lobo and Nestler, 2011). Consistent with this hypothesis, knockdown of the D1 receptor in the NAc shell decreased ethanol intake, ethanol locomotor sensitization, and ethanol-CPP (Bahi and Dreyer, 2012). One possibility therefore, is that ethanol normally acted to increase dopamine release in the NAc, and thus activated D1 receptor-positive cells and inhibited D2 receptor-positive cells, both of which would be rewarding (Fig. 6.1B). GAN/THIP may have acted to increase inhibition of the D2 receptor-positive cells (Fig. 6.1C), making the incoming dopamine signal more rewarding (i.e., now more D2 receptor-positive cells are inhibited with the same amount of dopamine present).

The above scenario is dependent on GAN and THIP preferentially acting at D2 versus D1 receptor-positive cells. Although the δ subunit is present on both D1 and D2 receptor-positive cells in the NAc (Maguire et al., 2014), quantification has not been performed and it is possible that a greater expression on D2 cells could lead to enhanced responsivity of these cells to THIP or NAS.

Additionally, the basal phosphorylation state of the $GABA_A$ receptors in one class of cells versus the other could also impact differences in the sensitivity to subsequently administered THIP, GAN, or ethanol. In the event that phosphorylation is altered, the direction of the change on $GABA_A$ receptor sensitivity is contradictory and seems to depend on the brain region, cell type, and kinase involved, as well as local GABA levels, and which subunit is phosphorylated (e.g. reviewed in Song and Messing, 2005). For example, phosphorylation of the β3 subunit by protein kinase A (PKA) enhanced phasic GABA-stimulated currents in HEK cells transfected with recombinant $GABA_A$ receptors (McDonald et al., 1998), whereas phosphorylation of the β3 subunit by protein kinase C (PKC) in cultured cortical neurons decreased receptor activity (Brandon et al., 2000). PKA activation also increased tonic $GABA_A$ receptor current in transfected HEK cells, but only in the presence of low GABA concentrations (Tang et al., 2010).

Evidence that ALLO or THIP may induce PKC activity is indirectly supported by studies showing that co-administration of a PKC inhibitor blocked ALLO- or THIP-induced $GABA_A$ receptor trafficking (Kuver et al., 2012). Additionally, because ethanol can activate PKA and PKC, which can have opposing action on $GABA_A$ receptor action (Carlson et al., 2013; Kumar et al., 2012), there could be a complex interaction between ethanol, phosphorylation, and sensitivity of the receptor to GAN, THIP, or subsequent ethanol intake.

It is not known which cells within the NAc endogenously contain ALLO. In future studies, I could use immunohistochemistry to co-label for ALLO as well as the D1 or D2 receptor to elucidate whether ALLO is located in D1 receptor-containing cells or D2 receptor-containing cells (or neither or both). Interneurons could also be immunostained for ALLO and their respective neuropeptide, which would further elucidate which cellular

populations within the NAc contain endogenous ALLO. Notably, even if ALLO is found only in one population of cells, it would not rule out the possibility that exogenous GAN acted on other classes of cells in my experiments. Rather, the presence of endogenous ALLO in a particular cellular population would increase the likelihood that those cells contain the $GABA_A$ receptor subtypes and other machinery necessary for GAN/ALLO to produce a physiological outcome.

One experiment that I could do to examine whether the GAN/THIP is increasing or decreasing the rewarding value of ethanol is to use a progressive ratio schedule. By examining the breakpoint (the maximum number of lever presses emitted by the mice to attain access to ethanol), I could measure whether pretreatment with GAN/THIP increases (interpreted as an increase in the rewarding value of ethanol) or decreases (interpreted as a decrease in reward) the breakpoint for ethanol. Alternatively, the self administration experiments could be repeated at various concentrations of an available ethanol solution. Because I would anticipate an inverted-U shaped dose-response curve, this could help elucidate whether I am examining changes in intake on the ascending or descending limb of a dose-response curve, and therefore, whether changes in intake with GAN/THIP at various concentrations of ethanol correspond to a leftward (interpreted as more rewarding) or rightward (interpreted as less rewarding) shift of the dose-response curve.

**Potential brain areas underlying GAN or THIP effects on ethanol consumption**

Although the microinfusion study (Chapter 4) focused on the NAc shell, the shell is likely not the only brain region underlying the central effect of GAN or THIP on ethanol intake, and future studies could use site-specific microinfusions targeted to other regions to test their sufficiency to alter ethanol intake. Given that δ subunit expression is highest

in the cerebellum, thalamus, cortex, and is present throughout the dorsal and ventral striatum (Pirker et al., 2000; Wisden et al., 1992; Hörntagl, et al., 2013), these regions would be candidates for further examination of brain regions underlying the effects of THIP. I would be particularly interested in the PFC based on human data showing that ethanol's actions in the PFC account for deficits in executive function (indicating that ethanol acts in the PFC physiologically) and that hypoactivity is observed in the PFC of chronic alcohol users (Abernathy et al., 2010). Consistent with a role for the PFC in ethanol consumption, inhibiting the PFC with muscimol decreased ethanol intake in rats (Samson and Chappell, 2001). Therefore, I would predict that increasing tonic inhibition in the PFC with THIP would also induce alterations in ethanol intake in the 2-hour 2-bottle choice procedure.

There are also many potential brain regions where alterations in ALLO levels may be important to modulate ethanol intake. Endogenous ALLO is widely distributed throughout the brain, with the densest labeling in the hippocampus, cortex, olfactory bulb, and striatum (Saalman et al., 2007). Additionally, ALLO levels in PFC, hippocampus (CA1and polymorph layer of the dentate gyrus), paraventricular nucleus, NAc core/shell boundary, central nucleus of the amygdala, and bed nucleus of the striata terminalis were dynamically altered by an acute ethanol injection (Cook et al., 2013). In addition to regional differences in the localization of ALLO, variations in the potency of ALLO have been shown across different regions of the brain (Gee, 1988). Regional variations in the potency of ALLO may have resulted from differences in enzyme levels and NAS metabolism across brain regions, alterations in $GABA_A$ receptor density and/or subunit compositions, or post-translational modifications, such as phosphorylation of the receptors (Belelli and Herd, 2003). Regardless of the cause, regional differences in the biosynthesis and regulation of ALLO activity across many brain regions, particularly

following ethanol administration, suggest that many brain regions may act in concert and/or be sufficient to mediate ALLO's effect on ethanol reinforcement.

Although pilot data from the Finn laboratory indicated that ALLO infusions into the VTA did not alter ethanol intake in a limited-access procedure (Ford and Finn, unpublished), it would be of interest to determine if intra-VTA GAN does alter intake. If GAN alters ethanol intake in the same procedure, it would suggest that endogenous enzyme levels (particularly 3α-HSD, which rapidly converts ALLO back to its immediate precursor 5α-DHP) in this region limited the effectiveness of ALLO. Using GAN is advantageous to extend the temporal effects of ALLO, and GAN and ALLO have been shown to have very similar pharmacological and behavioral effects (Mascia et al., 2002; Carter et al., 1997). However, GAN cannot just be considered a longer-acting ALLO. Rather, due to regional differences in the enzymes that metabolize ALLO, it follows that the effects of ALLO and GAN would be expected to be most similar in brain regions where 3α-HSD levels are low, and differences would be most prominent in brain regions where 3α-HSD levels are high. For example, in the CA1, ALLO and GAN had identical effects on mIPSC decay time (Belelli and Herd, 2003). However, in the dentate gyrus, the effect of GAN was much more pronounced than that of ALLO. When ALLO was applied in conjunction with a 3α-HSD enzyme inhibitor (therefore making it act more like GAN in that it was not oxidized by 3α-HSD), mIPSC decay times were similar (Belelli and Herd, 2003). These data suggest that regional differences in enzyme activity can alter the efficacy of ALLO versus GAN, and highlight the importance of testing GAN in brain regions even in the case that ALLO was ineffective.

A brain area of interest to test the effects of THIP versus GAN would be the central nucleus of the amygdala (CeA). A $GABA_A$ receptor antagonist infused into this

region decreased the CeA contribute to ethanol intake. Further, ALLO immunohistochemistry was decreased in this area following an acute ethanol injection (Cook et al., 2013), indicating that ALLO is dynamically altered here as a result of ethanol exposure. Thus, I would predict that microinjection of GAN might alter ethanol intake. However, since there is weak or no labeling for the $GABA_A$ receptor δ subunit in the CeA (Wisden, et al., 1992; Hörntagl, et al., 2013; Pirker et al., 2000), I predict that THIP infused into this region would not alter ethanol intake. Therefore if intra-CeA GAN alters intake, but THIP does not, it would suggest that GAN could alter intake via non-δ subunit-containing $GABA_A$ receptors.

**Alternative hypotheses**

Although my hypothesis posits that GAN and THIP decreased ethanol consumption by increasing the rewarding properties of ethanol, there are multiple alternative, and not mutually exclusive, hypotheses that could explain the decreases in ethanol intake following THIP or GAN administration. Possibilities include that GAN and/or THIP: (1) acted to increase general reward that was not specific to ethanol; (2) decreased the rewarding value of ethanol; (3) caused an ethanol aversion; or (4) altered the reinforcing properties of ethanol by modulating a neurotransmitter or neuropeptide other than dopamine.

One potential weakness of my hypothesis is that ALLO/GAN can also alter seeking or consumption of non-ethanol reinforcers. Given that ALLO may directly alter dopamine release (in addition to its ability to modulate ethanol-induced dopamine release) (Rogué-Pont et al., 2002) it may not be surprising that ALLO can modulate the incentive value of other reinforcers. GAN and ALLO increased sucrose seeking in the operant procedure (Chapter 3) and in a reinstatement model (Finn et al., 2008),

respectively. Therefore, the effect of ALLO/GAN on ethanol seeking cannot merely be attributed to its similar stimulus properties to ethanol, but possibly due to its actions on overlapping motivational circuits as natural reinforcers. A more general effect of ALLO on seeking behavior is consistent with a study showing that ALLO decreased latency to feed in rats (Holmberg et al., 2013). Although I found no systematic effect of GAN on sucrose consumption in Chapter 3, previous studies have found that ALLO increased saccharin (during hour 1 of 2-hour intake; Sinnott et al., 2002a) and chow intake (Chen et al., 1996; Holmberg et al., 2013; Reddy and Kulkarni, 1998), and intrahippocampal ALLO decreased glucose intake (Martin-Garcia, et al., 2007). Additionally, ALLO decreased cocaine self-administration (Anker et al., 2010), indicating that ALLO may alter the reinforcing effects of natural reinforcers or other drugs of abuse besides ethanol. In fact, cortical and plasma ALLO increases have been reported following injections of nicotine, morphine, or ∆9-tetrahydrocannibal (Concas et al., 2006; Grobin et al., 2005), supporting a more general link between drugs of abuse and neurosteroidogenesis. Consistent with behavioral data showing that rodents self-administered (Sinnott et al., 2002b) and showed CPP (Finn et al., 1997a) for ALLO, it is possible that ALLO may have some general rewarding properties and that neurosteroidogenesis is a broad mechanism contributing to drug or natural reward. Thus, it is still possible that ALLO alters ethanol reward, but it may do so via a more general mechanism, rather than due to ALLO and ethanol's overlapping pharmacological targets at the $GABA_A$ receptor.

An alternative hypothesis to my primary hypothesis is that GAN or THIP could be *decreasing* the rewarding effect of ethanol, which could also result in a decrease in consumption. The fact that neither ALLO nor FIN alters ethanol-CPP makes this hypothesis particularly difficult to support or reject (Gabriel et al., 2004; Murphy et al.,

2006). There are no reports examining how THIP affects ethanol-CPP, which could be informative in determining if THIP increases or decreases the rewarding effects of ethanol. Studies examining the effect of THIP on cocaine reward have found that systemic or NAc core-infused THIP decreased cocaine-enhanced cocaine-CPP (Maguire et al., 2014). Further, THIP applied directly to the NAc slice decreased the resting potential of the MSNs and required greater input of current to induce an action potential (Maguire et al., 2014). Therefore, a potential mechanism for NAc-infused THIP is that THIP could decrease the excitability of the NAc cells to an extent that even in the presence of increased dopamine release into the NAc (as may be expected with ethanol intake or subsequent NAS production), an action potential could not occur. The decreased excitability would be particularly important for the D1 receptor-positive output cells, where THIP could be preventing those cells from firing and therefore decreasing the rewarding signal of ethanol. One way to examine whether THIP is selectively acting at D1 or D2 receptor-positive cells within the NAc to alter ethanol intake would be to repeat the experiments in transgenic mice where the $\alpha_4$ subunit is selectively knocked out in either D1 or the D2 receptor-positive cells of the NAc (Gong et al., 2007; Maguire et al., 2014). If THIP only alters ethanol intake in one of the transgenic mouse lines, it would suggest that THIP acted selectively via that class of cells to alter ethanol consumption.

Another potential explanation by which GAN or THIP may have decreased ethanol intake is that either drug may have aversive properties that could have induced an ethanol aversion. Although there have been reports of conditioned place aversion following systemic GAN or ICV ALLO injections, these studies used high doses, 30 mg/kg and 5-25 μg (in 10 μL), respectively (Vashchinkina et al., 2013; Beauchamp et al., 2000), the latter of which decreased NAc and mPFC dopamine levels (Motzo et al.,

1996). Additionally, given that GAN caused initial increases in ethanol intake in my experiments and previous studies (Janak et al., 1998; Sinnott et al., 2002a; Ford et al, 2005b), and that ALLO increased food intake in many studies (Chen et al., 1996; Holmberg et al., 2013; Reddy and Kulkarni, 1998), it seems unlikely that GAN and ALLO have general aversive properties. The operant experiment with THIP, on the other hand, showed that it decreased both sucrose and ethanol self-administration (Chapter 3). Further, previous studies showed that neither mice nor baboons will self-administer THIP intravenously and mice showed conditioned place aversion following 6 mg/kg THIP, which raises the possibility that THIP could be decreasing ethanol intake by eliciting an aversion (Vashchinkina et al., 2012). Given that baseline intakes remained stable throughout the course of individual experiments, it seems unlikely that a general aversion resulted from GAN or THIP. Conditioned taste aversion studies with each of these drugs could help elucidate whether either drug has general aversive properties that could contribute to the decreases in ethanol intake that I observed.

It should be noted that my primary hypothesis (that GAN and THIP increased ethanol reward) is based on the idea that ethanol will increase dopamine levels in the NAc (Gonzales et al., 2004), and that an increase in NAc dopamine release signals reward. However, this has been challenged by studies showing that there can be an absence of an increase in NAc dopamine levels following ethanol administration, or that the rise in NAc dopamine may occur prior to the rise in NAc ethanol levels following oral administration (Doyon et al., 2003). Additionally, even if there is a dopamine increase, it may not be a signal of reward but may instead signal anticipation for a reward (Schultz, 1997), salience-based learning (Waelti et al., 2001), or even aversion (Abercrombie et al., 1989; Imperato et al., 1992). Consistent with a divergence between ethanol, dopamine, and putative rewarding effects, lesions of the dopamine system in rats failed

to alter lever pressing for ethanol (Rassnick et al., 1993), suggesting that the dopamine system was not necessary for the reinforcing effects of ethanol in that procedure.

There are a number of ways that GAN or THIP could be altering ethanol intake independent of any downstream effects on dopamine, including influencing the release of various neuropeptides or neurotransmitters whose release could be altered by the presence of $GABA_A$ receptors. Specifically in the NAc, GAN or THIP's actions on D1 receptor-positive cells could decrease dynorphin and Substance P (present in the D1 receptor-positive "direct" neurons; Curran and Watson, 1995) release. Additionally, actions on D2 receptor-positive cells could decrease enkephalin (present in the D2 receptor-positive "indirect" neurons; Curran and Watson, 1995) release. It is also possible that GAN or THIP could influence the release of other neurotransmitters, either downstream in the circuit, or directly by a presynaptic mechanism. For example, ALLO applied to a hippocampal slice acted presynaptically to increase glutamate or GABA release (Haage et al., 2002; Kim et al., 2003). THIP applied to NAc slices *decreased* presynaptic GABA release, although the source of that GABA was not known (interneurons, axon collaterals, or afferent GABA terminals; Anstee et al., 2013). On the other hand, THIP has been shown to act presynaptically in the VTA to *increase* GABA release (Xiao et al., 2007). Therefore, it is possible that GAN or THIP could have acted presynaptically to alter GABA, glutamate, (or possibly even norepinephrine or serotonin,) release, and that this action could have contributed to alterations in ethanol intake.

**Order effects with GAN**

Although a within-subjects design is ideal to limit the number of animals used, there are important considerations with regard to the effects of multiple GAN injections. Studies where rats were injected twice a day with GAN (7 mg/kg) for a week found no change in the anticonvulsant or ataxic effects of GAN over time (Reddy and Rogawski,

2000), indicating a lack of tolerance to the effects of GAN. There was, however, a development of cross tolerance to diazepam, suggesting there may have been some down-regulation or post-translational modifications (i.e., phosphorylation) to a benzodiazepine-sensitive subunit such as $\gamma_{1\text{-}2}$ or $\alpha_1$ (Reddy and Rogawski, 2000). Similarly, 5 days of GAN exposure to cerebellar granule cells did not alter mRNA levels for any of the 9 $GABA_A$ receptor subunits examined, but withdrawal from GAN produced increases in $\alpha_2$, $\alpha_4$, and $\alpha_5$ subunits, and decreases in $\alpha_1$ and $\gamma_2$ subunits (Mascia et al., 2002). These data are similar to work showing that 48-hour exposure and subsequent removal of ALLO alters $GABA_A$ receptor subunit expression, importantly, increasing expression of the $\alpha_4$ and δ subunits in the hippocampus (Smith et al., 2007). Given that GAN was administered in a within-subjects design, and the δ subunit may be especially sensitive to GAN or ethanol, this point is important to consider. In culture, these changes peaked 3-6 hours after GAN removal and were reversed within 24-hours (Mascia et al., 2002). Because I allowed 7 days between GAN injections, this effect was hopefully mitigated. However, it is unclear how the *in vitro* data corresponds to the exposure and plasticity *in vivo*, so physiological changes occurring after one or multiple GAN infusions cannot be ruled out.

Using repeated injections of GAN is also a concern in terms of changes that may be induced to non-$GABA_A$ receptors, which could in turn affect subsequent ethanol reinforcement. As indicated, a single injection of GAN altered the AMPA:NMDA receptor ratio on dopaminergic cell bodies in the VTA and this effect lasted at least 6 days (Vashchinkina et al., 2013). However, these changes were not present until 6 hours post-injection, suggesting that these potential alterations would not have contributed to the changes in ethanol intake within the immediate 2-hour session. Additionally, the change in AMPA:NMDA receptor ratio was limited to high doses of GAN (30 mg/kg, but

not 10 mg/kg; Vashchinkina et al., 2013). Thus, because ethanol intake always returned to baseline by the next day, I believe that I was either using doses of GAN below the threshold to induce the AMPA:NMDA receptor changes, or that this change was not sufficient to alter the reinforcing properties of subsequent ethanol intake.

**Order effects with THIP**

Similar to GAN, molecular changes can result from even an acute injection of THIP. A single injection of 6 mg/kg THIP altered AMPA:NMDA receptor ratios in the VTA for at least 6 days (Vashchinkina et al., 2012). Although 6 mg/kg THIP was within the range that I used, there were no changes in basal ethanol intake in these experiments, making it unlikely that potential changes in AMPA:NMDA receptor expression altered the reinforcing value of subsequent ethanol.

There are also concerns with molecular changes to the $GABA_A$ receptor that may occur following repeated THIP injections. Repeated THIP injections led to tolerance to the ataxic (Voss et al., 2003), but not the hypnotic or anticonvulsant (Ebert et al., 2008) properties of THIP. These data suggest that there may be a down-regulation in expression or activity of the δ subunit in some brain areas, such as the cerebellum, but not in other areas such as the thalamus or hippocampus. Whether or not repeated administration of THIP causes a down-regulation of $GABA_A$ receptor δ subunit expression in other brain areas, such as the NAc, is unknown. However, down-regulation of δ subunit-containing $GABA_A$ receptors would cause THIP to be less effective as more doses were given.

In the present studies, examining data by cohort in the NAc study (Chapter 4) revealed substantial order effects of the THIP doses. However, contrary to a tolerance, there appeared to be a sensitization to THIP. There has been a report of an up-

regulation of the δ subunit in the hippocampus following chronic ethanol exposure (Follesa et al., 2006). If this extends to the NAc, it could represent one potential explanation for a sensitization effect with THIP (see "Plasticity following chronic ethanol exposure" below for a further discussion on the potential changes following chronic ethanol intake).

The sensitization with THIP did not appear to be purely a non-specific mechanical consequence of repeatedly lowering the injectors, since intake following the final vehicle infusion did not differ from the first vehicle infusion. The sensitization was primarily evident when observing intakes following the 500 ng THIP dose; when 500 ng THIP was given as the 3$^{rd}$ THIP infusion (cohorts 1 and 3), it decreased intake to a much greater extent than when it was given as the 1$^{st}$ THIP infusion (cohort 2). However, when 10 ng THIP was given as the 4$^{th}$ THIP infusion, it did not decrease intake from vehicle, indicating that dose, and not just number of infusions, influenced the change in ethanol intake. Similarly, although 100 ng THIP was always given as the 2$^{nd}$ infusion, it seemed equally efficacious at decreasing intake whether it was preceded by the 50 or 500 ng THIP dose, complicating a clear understanding of how previous doses influenced intake with subsequent doses. The effect of dose on bout frequency also appeared to be affected by order and dose in a complex manner. The bout frequency in cohort 2 appeared predominately determined by the number of infusions; it was not dose-dependent and the vehicle (post) bout frequency remained low. However, in cohorts 1 and 3, the effect on bout frequency appeared more affected by dose than order, and did not remain low with the 10 ng or vehicle post infusions. Thus, it is unclear exactly how order interacted with the dose administered (i.e., whether just the number of previous THIP infusions or the specific doses in the previous infusions mattered) to influence intake. In order to dissect the influence of repeated infusions and dose, it would be

informative to give 3 repeated infusions of the highest (500 ng) dose to directly compare the effects of the first and third infusion across infusions within the same mice. It would also be interesting to use immunohistochemistry to compare protein levels of the δ subunit following one or more THIP infusions to determine if the δ subunit is up-regulated, as would be expected in a sensitized response.

Despite the fact that the influence of order calls into question the dose-dependency of the effect of NAc-THIP on ethanol intake, in all cases, 500 ng THIP suppressed ethanol intake versus vehicle, even with small sample sizes (ns = 4 or 5). Thus, it can be concluded that NAc THIP does influence ethanol intake to a similar degree as in the systemic study, although the influence of multiple doses remains to be examined.

**Plasticity following chronic ethanol exposure**

Another concern in long-term ethanol studies is that ethanol exposure or subsequent removal can induce molecular changes that could influence the sensitivity of the system to GAN, THIP, or subsequent ethanol intake. For example, $GABA_A$ receptor δ subunit expression in hippocampal cells was decreased following ethanol exposure and subsequent ethanol removal both *in vitro* and in a chronic intermittent ethanol model (Liang et al., 2006, 2007). The decrease in the δ subunit expression was associated with $GABA_A$ receptor $\alpha_4$ subunits being trafficked to the synapse and paired with $\gamma_2$ subunits (Liang et al., 2006). On the other hand, the $GABA_A$ receptor δ subunit mRNA was *increased* following both chronic ethanol (5 days) and ethanol withdrawal (3-6 hours) in hippocampal neurons (Follesa, et al., 2006). The latter group found no change in $GABA_A$ receptor δ subunit expression following chronic ethanol applied to cerebellar granule cells but an upregulation upon removal (Follesa et al., 2006).

Additionally, chronic ethanol exposure in non-human primates has been associated with reduced mRNA expression of the $GABA_A$ receptor $\alpha_2$, $\alpha_{4,}$ $\beta_1$, $\beta_{3,}$ and $\gamma_{1\text{-}3}$ subunits in the orbital frontal cortex and a reduction in the expression of $\beta_1$, $\beta_{2,}$ $\gamma_1$, and δ subunits in the dorsal lateral PFC (Hemby et al., 2006). If δ subunit containing $GABA_A$ receptors are especially sensitive to GAN and THIP (compared to non-δ subunit-containing $GABA_A$ receptors), testing these drugs later in the experiment when the δ subunit expression is potentially lower could be a concern. However, because mice had access to ethanol for at least 4 weeks prior to any drug testing, many of these changes may have occurred prior to the first drug exposure. All drinking experiments in this dissertation were at least 4 months long, and all studies induced a period of forced abstinence, whether it was for 22-hours or 23.5 hours until the next session. Thus, plasticity resulting from chronic ethanol intake or ethanol removal, whether at the level of the $GABA_A$ receptor or other neurotransmitter systems, cannot be ruled out.

Another consideration is that chronic ethanol exposure can induce changes to basal NAS levels, the steroidogenic ability of ethanol, or the sensitivity of $GABA_A$ receptors to NAS. Cortical ALLO levels were significantly lower in ethanol-dependent versus non-dependent male rats (Janis et al., 1998) and mice (Tanchuck et al., 2009). Although dependence was not measured in any of the present studies, it is possible that there were similar changes in basal NAS levels with long-term ethanol consumption. Further, ethanol dependence reduced the neurosteroidogenic effect of an ethanol challenge injection (Boyd et al., 2010b), likely due to the down regulation of 5α-R and 3β-HSD that has been demonstrated in chronic ethanol exposure models (Cagetti et al., 2004; Finn et al., 2004; Srikanth et al., 1998; Tanchuck et al., 2009). With regard to $GABA_A$ receptor sensitivity to NAS in dependent animals, the anticonvulsant effect of ALLO was heightened during chronic ethanol withdrawal (Devaud et al., 1995; Finn et

al., 2000) in certain rodent genotypes. However, there have also been reports of a reduced sensitivity to the hypnotic effect of alphaxalone in dependent rats (Cagetti et al, 2003) and tolerance to the anticonvulsant effect of ALLO in genotypes with severe ethanol withdrawal (Finn et al., 2000, 2006). These changes in NAS sensitivity likely reflect regionally specific changes in $GABA_A$ receptor expression or activity. Again, changes in subunit expression or activity would be expected to alter the sensitivity to injections of GAN or THIP, as well as subsequent ethanol intake.

Taken together, given the potential concerns with order-effects of GAN and THIP, as well as plasticity from long-term ethanol exposure, future studies could benefit from counterbalancing drug dose order within cohorts. This could also potentially eliminate an infusion, as vehicle would not need to be administered twice.

**Dose of GAN**

Previous data have demonstrated a biphasic effect of exogenous ALLO to alter ethanol intake in mice (Finn et al., 2010). For example, a low dose of exogenous ALLO (3.2 mg/kg) that led to a physiological level of plasma ALLO (~100 nM 60 minutes post-injection) increased ethanol intake in mice (Ford et al., 2005a; Finn et al., 1997b); similarly, a low dose of GAN (1 mg/kg) increased ethanol-reinforced responding in rats (Besheer et al., 2010). On the other hand, a high dose of exogenous ALLO (24 mg/kg) that led to supraphysiological levels (~1500 nM 60 minutes post-injection) decreased ethanol intake (Finn et al., 1997b; Ford et al., 2005a) and a high dose of GAN (30 mg/kg) decreased ethanol-reinforced responding in rats. Intermediate doses of ALLO and GAN caused intermediate effects on ethanol intake and ethanol-reinforced responding (Finn et al., 2010; Besheer et al., 2010). Pertinent to the present studies, injections of 5 and 10 mg/kg ALLO corresponded to plasma levels of approximately 300

and 600 nM, respectively, 30 minutes post-injection (Finn et al., 1997b). Thus, the doses used in my studies (5-15 mg/kg GAN) likely correspond to supra-physiological levels, consistent with the observation that high doses of ALLO and GAN caused subtle, non-significant alterations or decreases in ethanol intake (Finn et al., 2010; Ford et al., 2005b; Besheer et al., 2010). Therefore, it is likely that I did not see a biphasic effect of systemic GAN on ethanol consumption in the current experiments due to the fact that I did not test a dose lower than 5 mg/kg.

In terms of the intra-NAc GAN doses, it is also likely that I used doses corresponding to high concentrations. In a previous experiment, unilateral ICV infusions of ALLO led to a slight increase in intake with 50 ng, and a slight decrease in intake with 400 ng in the first 10 minutes (Ford et al., 2007a). Additionally hippocampal ALLO (2000 ng) led to a decrease in ethanol intake in rats (Martin-Garcia, et al., 2007). Therefore, it is likely that my infusions of 50 ng – 500 ng (especially considering that they were bilateral and locally infused) all corresponded to high doses, and were all on the descending portion of the dose-response curve. I also cannot rule out that these doses resulted in high enough local concentrations (~10 μM) to directly gate the $GABA_A$ receptor (Majewska et al., 1998), or to exert effects on non-$GABA_A$ receptors such as NMDA (Park-Chung et al., 1997), 5-$HT_3$ (Wetzel et al., 1998), nACh (Bullock et al., 1997), or sigma receptors (Su et al., 1998). Although I only tested 10 ng GAN in 2 animals (Chapter 4), it is possible that this or a lower dose of GAN may have revealed an increase in intake consistent with the biphasic effect of ALLO and GAN on ethanol intake that has been shown in studies that used a wider range of doses (Ford et al., 2005b, 2007a; Besheer et al., 2010).

**Doses of THIP**

One concern with THIP is that I used doses greater than what would be expected to be specific for extrasynaptic $GABA_A$ receptors. Although THIP is often referred to as δ-specific, it would be more accurately described as δ-selective (i.e., preferring), in that it shows about a 10 to 100-fold higher affinity at δ subunit-containing $GABA_A$ receptors versus non-δ subunit-containing $GABA_A$ receptors (Stórustovu and Ebert, 2006). As mentioned previously, doses up to about 10 mg/kg THIP seem to be selective based on the lack of behavioral effects in $GABA_A$ receptor $\alpha_4$ knockouts below this dose (Chandra et al., 2006). This approximated selective dose of 10 mg/kg is also consistent with a study examining how systemic injections corresponded to CNS concentrations, and how selective the subsequently-generated CNS concentrations were at δ and non-δ subunit-containing $GABA_A$ recombinant receptors *in vitro* (Cremers and Ebert, 2007; Stórustovu and Ebert, 2006; see discussion in Chapters 2 and 3). Another consideration is that in the event that THIP does bind to non-δ subunit-containing $GABA_A$ receptors, its efficacy would be relatively small compared to the efficacy THIP had at δ subunit-containing $GABA_A$ receptors; the THIP $E_{max}$ at $\alpha_4\beta_3\delta$ and $\alpha_6\beta_3\delta$ $GABA_A$ receptors was 215% and 309% of the GABA response, respectively, while the $E_{max}$ of THIP at all of the non-δ subunit-containing $GABA_A$ receptors measured was less than 100% of the GABA response (Stórustovu and Ebert, 2006). Taken together this indicates that THIP is likely binding at δ-subunit containing $GABA_A$ receptors in my systemic studies (except for 16 mg/kg THIP), but if THIP did bind to non-δ subunit-containing receptors, the relative contribution based on the efficacy would be relatively minor.

In the microinfusion studies, it is difficult to know how the doses of drugs given correspond to the concentration that is then available at the synapse or perisynaptically. However, a study using comparable doses of systemic and intra-NAc core THIP (8

mg/kg and 700 ng/500 nl/side) showed that in both cases THIP altered cocaine CPP in wild-type mice, but that these effects were absent in the $GABA_A$ receptor $\alpha_4$ subunit knockout mice (Maguire et al., 2014). These results imply specificity for $\alpha_4\delta$ subunit-containing $GABA_A$ receptors at these THIP doses (since knockout of $\alpha_4$ greatly reduces δ subunit expression at the plasma membrane in forebrain regions; Peng et al., 2013). However, it is possible that these doses did act at synaptic receptors and influenced a behavior those authors were not measuring. One way to measure the specificity of the doses I used in my studies would be to use the $GABA_A$ receptor δ subunit knockout mice or a viral vector to knock down δ subunit-containing $GABA_A$ receptors in the NAc, and then test the effect of THIP on ethanol intake. If THIP fails to alter ethanol intake in the absence or knockdown of the δ subunit, this would imply that its actions were via δ subunit-containing $GABA_A$ receptors. On the other hand, an effect of THIP on ethanol intake in the absence of the δ subunit would suggest that THIP had actions at synaptic $GABA_A$ receptors.

**Sex differences**

The present studies were conducted only in male mice. Previous work from the Finn laboratory has shown that female C57BL/6J mice were less sensitive than males to the steroidogenic effects of ethanol to increase brain ALLO levels (Finn et al. 2004). The female mice were also insensitive to the effect of systemic ALLO to alter ethanol intake (Ford et al., 2008a). Interestingly, females are *more* sensitive to the anticonvulsant effects of NAS, indicating that the lack of ethanol alterations was not a result of general NAS insensitivity (for review see Finn et al., 2010). One potential explanation for the inability of ALLO to alter ethanol intake in female mice could be a faster metabolism of ALLO, possibly due to increased 3α-HSD levels in brain areas responsible for ALLO-induced alterations on ethanol intake. It would be of interest therefore to test the more

stable analog GAN, which could prove to be effective in altering ethanol intake in female mice. Ongoing studies in the Finn laboratory are examining the effect on ethanol drinking of both GAN and THIP in female mice.

**Locomotor and other off-target effects of THIP or GAN**

In control experiments in Chapter 5, I saw slight but non-significant increases in locomotor activity following 4 and 6 mg/kg THIP in a 30 minute session; I also saw an increase in activity in the first 20 minutes of a 2-hour session following 8 mg/kg THIP (Appendix). Further, with systemic GAN, there was a significant increase in activity during the first 20 minutes of a 30-minute session. It is unclear why intra-NAc THIP, but not intra-NAc GAN, caused locomotor activation in the current studies (Chapter 4), given that systemic injection of both drugs caused similar quantitative increases in activity (Chapter 5). One possibility is that the dose of intra-NAc GAN was sub-threshold to alter activity. In support of this, previous work from the Finn laboratory showed that 400 ng ALLO infused into the lateral ventricle did not alter locomotor activity (Ford et al., 2007a) and that doses of at least 1 µg of ALLO infused into the VTA were needed to cause locomotor stimulation (Phillips & Finn, unpublished; discussed in Ford et al., 2005a).

Although the increases in activity across the studies confirm that sedative effects are not contributing to the decreases in ethanol intake at these doses, as stated earlier, it does not rule out that an increase in activity could have interfered with drinking. Further, with the systemic GAN, I cannot rule out that initial locomotor activation effects are responsible for the seeking and initial increases in intake. It is possible, therefore, that initial increases due to increased locomotion shifted the drinking, and the animals reached satiety sooner, therefore decreasing drinking at later time-points (i.e., a left-ward shift). It would be helpful to find a dose of GAN that does not cause locomotor

activation or sedation to examine if changes in ethanol intake are still present in the absence of an effect on locomotor activity. In rats, Besheer et al. (2010) found that 1 mg/kg GAN increased ethanol-reinforced responding in the absence in a change in locomotor activity. Perhaps by testing a GAN dose lower than the 5 mg/kg used in the current studies, I could uncover a biphasic effect on ethanol intake (i.e., an increase in ethanol intake) without a change in locomotor activity. Alternatively, a higher dose (such as 20 or 25 mg/kg GAN) may still cause decreases in ethanol intake, but may be between doses that would cause activation or sedation (since 30 mg/kg caused both decreases in ethanol-reinforced responding and locomotor sedation in rats (Besheer et al., 2010)).

In addition to locomotor effects, there are other consequences of GAN or THIP treatment that I did not measure, and which could have contributed to the decrease in intake. For example, both GAN and THIP can produce anxiolytic and anti-depressant effects (Christensen, et al., 2012; Gulinello et al., 2003; Reddy, 2010), and it is possible that effects on ethanol intake could be secondary to these behaviors.

**Relevance to humans**

The general goal of these studies was to provide insight into the mechanisms underlying ethanol seeking and intake, which could potentially be useful in the treatment of AUDs. Information from rodent studies clearly needs to be interpreted with caution with regard to how it relates to humans. In the 2-hour access study, mice were consuming about 2-3 g/kg of ethanol. Using the NIAAA definition that 14 g of ethanol defines one drink, and considering a 150 lb (68 kg) individual, 2 g/kg/2-hours would be the equivalent of about 10 drinks in 2 hours. Clearly there are differences between a mouse and human, in particular the higher metabolism in mice, which makes this

translation less meaningful. Calculating based on the BEC achieved by the mice in the 2 hour procedure (~ 100 mg/dl), this BEC would correspond to approximately 4 drinks over 2 hours for a 150 lb man (according to the National Clearinghouse for Alcohol and Drug Information). Therefore a ~25% decrease in intake, as I saw across the 2-hour experiments with the highest doses of GAN and with systemic THIP (8 mg/kg), would correspond to a decrease from about 4 drinks to 3 in a 2-hour span.

From a public health perspective, information using GAN and THIP are particularly important in light of their increasing use in clinical trials across multiple disease states. GAN is currently in clinical trials for epilepsy, post-traumatic stress disorder, attention and anxiety deficits in Fragile X syndrome, and to aid in smoking cessation. THIP is being explored primarily as a somniac and for adjunct treatment in depression (clinicaltrials.gov). AUDs share very high comorbidity with many of these diseases, compared to the prevalence of these diseases in the general population. For alcohol dependent individuals, the prevalence of depression is 28% (Kessler, 1996), the prevalence of post-traumatic stress disorder is 8% (Regier et al., 1990), and the prevalence of nicotine dependence is 45% (Falk et al., 2006). Particularly in view of my data that GAN may increase alcohol seeking, consideration of the effect that GAN or THIP may have on individuals being treated for one of these diseases with a comorbid AUD is of critical importance.

**Final conclusions**

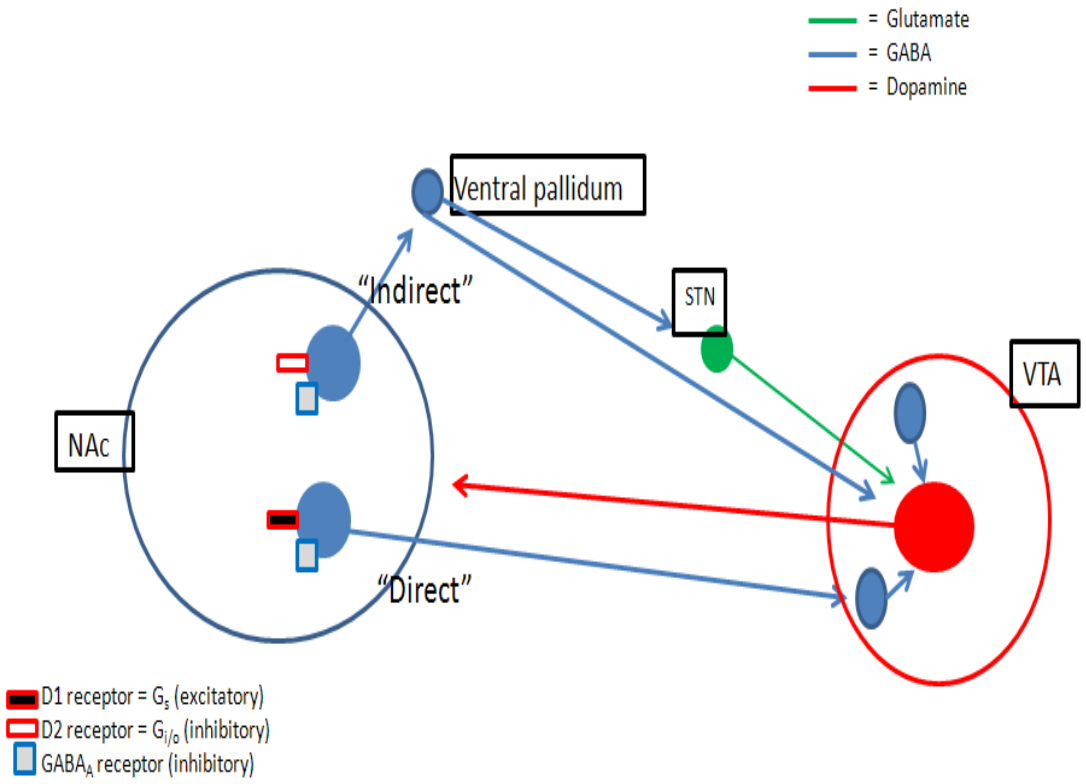
The demonstration that a drug alters ethanol seeking or intake across many procedures is important in order to understand the means by which it ultimately alters ethanol intake. Across the present studies, GAN increased ethanol seeking and initial intake, while decreasing overall consumption. THIP decreased both ethanol seeking

and consumption, although there were non-specific effects on sucrose self-administration in both of these cases. These studies demonstrated the importance of the NAc in mediating ethanol intake. In conjunction, these studies support a role of the NAS environment, as well as extrasynaptic $GABA_A$ receptor activation in altering ethanol intake. Future work is necessary to better elucidate the mechanism by which NAS and extrasynaptic $GABA_A$ receptors alter ethanol reinforcement, as well as the possible interaction between the two.

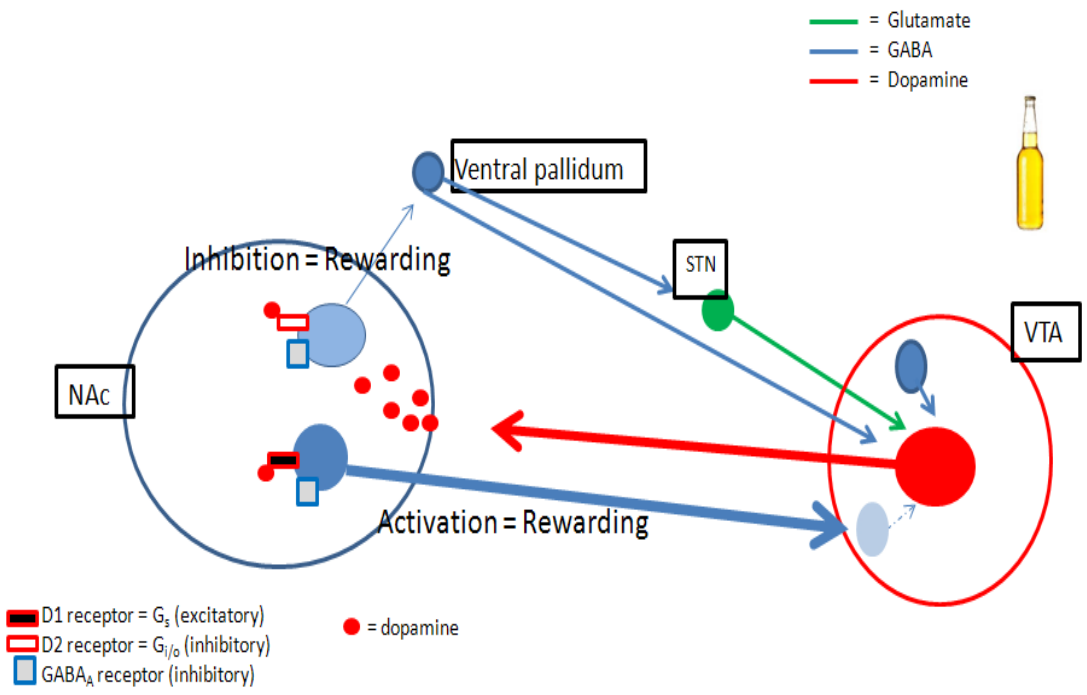
**Figure 6.1. A hypothetical model for how GAN or THIP may increase ethanol reward by mediating the dopamine signal in the NAc.**

A) Simplified reward circuitry showing the direct and indirect output pathways of the NAc. B) With ethanol intake and subsequent dopamine release in the NAc, dopamine would act to 1) activate D1 receptor-positive cells and 2) inhibit D2 receptor-positive cells, both of which may increase reward. C) Pretreatment with GAN or THIP could inhibit both D1 and D2 receptor-positive cells, but importantly at D2 receptor-positive cells, this would further inhibit the cells and increase reward. Thicker/darker lines in B and C versus A indicate more excitation, while thinner/dashed lines indicate more inhibition. STN = subthalamic nuclei;

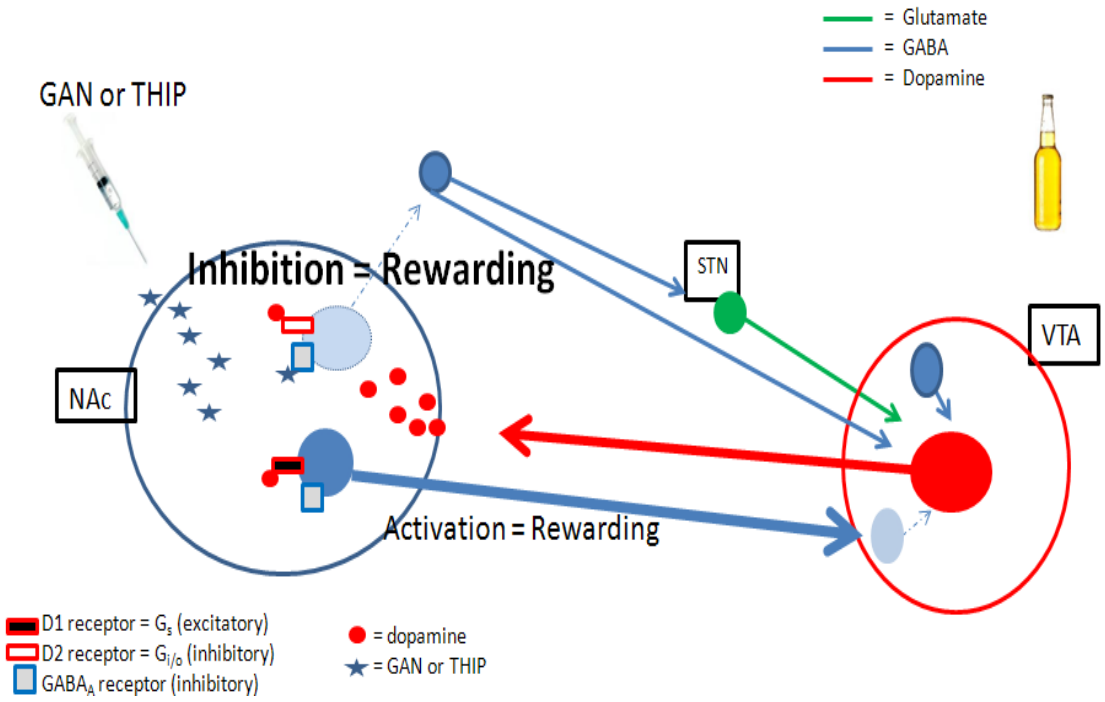
A) Normal state



B) With ethanol intake and subsequent dopamine release




C) With NAc pretreatment of GAN or THIP



## APPENDIX: Pilot experiment testing the effect of THIP on ethanol intake following ethanol abstinence (i.e., the alcohol deprivation effect)

### Introduction

To supplement the reinstatement data in Chapter 5, I attempted to look at the effects of THIP on ethanol intake in mice following a period of abstinence. One disadvantage of the reinstatement model presented in Chapter 5 is that, because ethanol seeking was measured without access to the reinforcer, the model lacks face validity with the human relapse condition, where alcohol seeking would be expected to be followed by alcohol consumption. That is, because reinstatement was measured in the absence of the ethanol, it cannot be assumed that an alteration in ethanol seeking would necessarily correspond to an alteration in subsequent consumption, information which is vital to ascertain any therapeutic benefit of a treatment. Further, due to a floor effect of non-reinforced lever pressing in Chapter 5, a potential decrease in ethanol seeking could not be detected.

The alcohol deprivation effect (ADE) is defined as an increase in alcohol (ethanol) intake following a period of deprivation or abstinence (e.g., Heyser et al., 1997; Spanagel and Hölter, 2000). The ADE model has strong predictive validity in that drugs which reduce craving and relapse in humans, such as naltrexone and acamprosate, successfully block the ADE in rats (e.g., Heyser et al., 1997; Spanagel and Hölter, 2000). In Chapters 2 and 3, I showed that systemic injections of THIP (an agonist preferential for δ subunit-containing $GABA_A$ receptors) decreased ethanol seeking and intake in an operant procedure and decreased ethanol intake in a limited- and continuous-access procedure. The goal of this pilot experiment was to examine whether THIP would alter ethanol intake following a 2-week abstinence period in mice with a

history of chronic ethanol intake. I predicted that mice would show an increase in intake upon re-exposure to the ethanol solution (i.e., show an ADE), and that the ADE would be blocked by THIP pretreatment.

Additionally, to assess whether locomotor effects of 8 mg/kg THIP could account for the results of the present experiment, or the results in Chapter 2 or 3 using this dose, separate mice were tested for locomotor activity following an injection of either 0 or 8 mg/kg THIP.

**Methods**

C57BL6/J male mice (n = 19) that had been part of a previous experiment (referred to as "cohort 2" in Chapter 4) were used. Briefly, in that experiment, mice had a 2-bottle choice of a 10E or water, where access began 2 hours into the dark cycle and was available for 2 hours. The mice had a total of 16 weeks of 10E access, during which time they were treated with 5 microinfusions of various doses of GAN or 4 doses of THIP targeted to the NAc shell. Following the conclusion of that experiment, mice underwent 2 weeks of ethanol abstinence. During this time, mice had *ad libitum* access to food and water, but were not handled or treated with any drug.

On the day termed "post-abstinence," mice were weighed and injected i.p. at 30 minutes prior to 10E access with an acute injection of either 0 or 8 mg/kg THIP, and a no-injection group was included to account for the fact that the mice were not handled during those 2 weeks and were not habituated to i.p. injections. Groups were assigned and balanced on previous NAc shell treatment and on previous ethanol intake (See Fig. A.1. for pre-abstinence intakes for each group). Group one received no injection [n = 5 (n = 2 former GAN, n = 3 former THIP)]. Group two received injections of 0 mg/kg THIP [n = 7 (n = 4 former GAN, n = 3 former THIP)]. Group three received 8 mg/kg THIP [n =

7 (n = 4 former GAN, n = 3 former THIP)]. The dose of THIP was chosen based on the fact that it prevented 11 of 12 mice from attaining access to a 10E reinforcer in an operant procedure, interpreted as a decrease in ethanol seeking, and also led to a decrease in intake in a 2-hour and 24-hour 2-bottle choice procedure (Chapters 2 and 3).

For the locomotor study, 29 C57BL6/J male mice that had been used in a previous experiment (used for locomotor testing of systemic THIP and GAN in Chapter 5) were tested. Two weeks following their last injection, they underwent the same 3 day protocol as I used in Chapter 5. Briefly, on days 1 and 2, all mice received a saline injection 30 minutes prior to the session. Horizontal locomotor activity was then measured for 2 hours using VERSADAT software to measure cm traveled. On day 3, mice were balanced on prior treatment and treated with either 0 mg/kg THIP (n = 14) or 8 mg/kg THIP (n = 15) prior to testing in the locomotor chambers. Drug-induced locomotor activity was measured by subtracting out baseline activity (day 2) from activity following the injection on day 3. There was no difference between groups for day 2 activity.

**Results**

On the first day of reintroduction to 10E, ethanol intake was measured following no injection, an acute vehicle, or an acute THIP injection. Contrary to my prediction, the mice did not show an ADE upon re-exposure to the ethanol solution following abstinence (i.e., they did not show an increase in intake compared to their pre-abstinence baseline). Rather, intake actually decreased for all treatment groups following abstinence [$F(1,16) = 22.493$; $p = 0.001$; Fig. A.1]. Further, there was no abstinence by treatment interaction, indicating a lack of treatment to differentially alter pre-abstinence versus

post-abstinence intake. Water intake was negligible (< 0.05 ml for all groups), so it could not be statistically analyzed due to a floor effect.

Despite the absence of an effect of THIP on overall 10E intake, I examined the number of 10E licks across 20-minute bins to elucidate whether THIP altered the pattern of drinking during the post-abstinence drinking session. Bin analysis revealed a treatment by bin interaction [$F(10,70) = 2.626$; $p = 0.009$; Fig. A.2]. (Two data files did not save. Therefore n = 5 (no-injection), n = 6 (0 mg/kg), and n = 6 (8 mg/kg THIP) for bin and bout data). There was a significant effect of treatment in bin 1 (0 – 20 minutes) [$F(2,14) = 3.876$; $p = 0.046$]. Post-hoc t-tests revealed that 8 mg/kg THIP decreased 10E licks when compared to the no-injection group ($p = 0.016$), but there was no difference versus 0 mg/kg THIP. There was a trend for an effect of treatment in bin 4 (60 - 80 minutes; $p = 0.065$) and a significant effect of treatment during bin 5 (80 - 100 minutes) [$F(2,14) = 4.519$; $p = 0.031$]. Post-hoc analysis revealed that 8 mg/kg THIP significantly increased 10E licks versus 0 mg/kg THIP ($p = 0.011$) and tended to increase 10E licks versus no-injection ($p = 0.063$) during bin 5 (80 – 100 minutes).

Consistent with the shift in drinking patterns revealed in the bin analysis, there was a significant effect of treatment on latency to first bout [$F(2,14) = 3.816$; $p = 0.050$; Table A.1]. THIP delayed the time to first bout (increase in latency) compared to no-injection ($p = 0.019$), and there was a trend for an increase when compared to vehicle ($p = 0.087$). There was no significant effect on latency to first lick, bout frequency, average bout size, or first bout size (Table A.1).

There was no effect of THIP on total 2-hour activity [0 mg/kg: 58 ± 1154 cm and 8 mg/kg THIP: 688 ± 2151 cm; $p = 0.802$ (data not shown)]. However, when analyzing the data by 20-minute bins, there was a significant bin by dose interaction [$F(5,135) =$

3.961; $p = 0.002$; Fig. A.3]. Specifically, during minutes 0 – 20, there was a significant increase in activity with 8 mg/kg THIP ($p = 0.036$).

**Discussion**

The goal of this study was to determine whether an acute injection of THIP would prevent ADE in mice subjected to chronic (16 weeks) ethanol drinking, followed by 2 weeks of ethanol abstinence. Given that THIP did not decrease ethanol consumption compared to either of the control conditions, this experiment suggests that THIP may not be an effective tool to decrease ethanol intake following a period of abstinence. However, because this experiment had several confounds, conclusions from the study must be interpreted with caution.

The primary confounds of this experiment were the previous drug history of the mice, the small group sizes, and the lack of an ADE expressed by the mice. To help minimize the impact of drug history on the results, groups were balanced on past drug infusions. However, one cannot rule out that plasticity resulting from previous drug treatment could contribute to the lack of effectiveness of THIP in the present study. For example, if the previous drug regimen led to a down-regulation of δ subunit-containing $GABA_A$ receptors, this would decrease the ability of THIP to have an effect.

Secondly, the addition of a non-injection group enabled me to account for the effect of injection stress in these animals, but this lowered group size as a result. Therefore it is possible that larger sample sizes may have uncovered effects of THIP treatment. However, a power analysis revealed a very small effect size (cohen's d = .39), and based on this small effect size, I would need over 50 mice per group to detect significance, indicating that it is likely correct not to reject the null hypothesis.

Finally, although ADE is not necessarily a requirement for relapse, the absence of an ADE in the present experiment calls into question the predictive validity of my results, and hence the lack of effectiveness following THIP administration is difficult to interpret. It should be noted that although expression of the ADE is reported in rats (Sinclair, 1972), and has also been observed in monkeys (Kornet et al., 1990) and humans (Burish et al., 1981), ADE reports have been less consistent in mice (Salimova and Salimova, 1993a, 1993b). Notably, a lack of an ADE in C57BL/6J mice has been reported previously, where the same procedures that elicited an ADE in C57BL/6NCrl mice failed to do so in C57BL/6J mice (Khisti et al., 2006). Another possible reason I did not observe an ADE in the present study is due to my experimental setup. There is no single ADE procedure established, but it is possible I may have been more likely to uncover an ADE following a continuous access paradigm or if multiple alcohol deprivation cycles had been used (Spangel et al., 1999).

The above confounds aside, it is interesting to speculate as to why our data did not reveal a role for THIP in reducing ethanol consumption following abstinence in the present study, despite previous data in Chapters 2 and 3 showing that THIP decreased appetitive and consummatory aspects of ethanol self-administration. As discussed in Chapter 6 (Plasticity following chronic ethanol exposure), ethanol exposure and subsequent removal may lead to a down-regulation of the δ subunit and a concurrent decreased potency of THIP (Liang et al., 2006). Therefore, it is possible that down-regulation of the δ subunit in one or more areas of the brain following ethanol deprivation could explain THIP's lack of effect in the current procedure.

The locomotor testing revealed a stimulatory effect of THIP during the first 20 minutes. The increase in activity in this timeframe is interesting given that this dose

*increased* the latency to the first bout in this and all other procedures in this dissertation. As with the other locomotor data, I cannot rule out that the increase in activity may be causing a competing behavior during this time, but the lack of sedation confirms that the decreases in intake and increases in latency to drink with this dose were not to a locomotor suppressive effect.

Despite not revealing a role for THIP on overall ethanol intake following abstinence, the present study may reveal roles for THIP important to other aspects of ethanol reinforcement. Notably, THIP increased latency to the first bout without altering the time for the mice to initially contact the sipper (latency to first lick; Table A.1). Similar to the increase in latency to first bout, THIP appeared to cause a delay in the drinking pattern as evidenced by the bin analysis. The shift in intake could be interesting in light of human data exploring the efficacy of drug treatments based on patterns of ethanol intake (Anton et al., 2006). Although speculative, it is possible that THIP's ability to delay intake could be advantageous to alter overall drinking when combined with another treatment. For example, in a clinical laboratory setting, individuals pretreated with either placebo or naltrexone were offered an initial drink, followed by either an immediate or delayed access to more alcohol. The number of drinks consumed was only reduced by naltrexone in the group where a delay was mandated; on the other hand, there was no effect of delay on number of drinks consumed in the placebo group (Anton et al, 2004). Given that THIP seemed to promote a delay, but no change in overall intake in the current experiment, it would be interesting to examine the efficacy of naltrexone following THIP treatment in a relapse model. Although drug effects become difficult to tease apart when given concurrently, given ethanol's actions across many receptor systems, it is plausible that two or more drugs targeting different receptor

systems are necessary to decrease ethanol intake in certain ethanol reinforcement models.

**Figure A.1. Effect of THIP on 10E intake after ethanol abstinence**

The average intake of the 3 days prior to abstinence are shown as the "pre-abstinence" intakes for each group. Note that there was no treatment during "pre-abstinence" for any group. After 2 weeks of abstinence, mice were pretreated with either no-injection (n = 5), 0 mg/kg THIP (n = 7), or 8 mg/kg THIP (n = 7), injected i.p. at 30 minutes prior to ethanol access. Intakes following treatment are shown as the "post-abstinence" intake for each group. ***$p \leq 0.001$ for pre-abstinence versus post-abstinence intake.

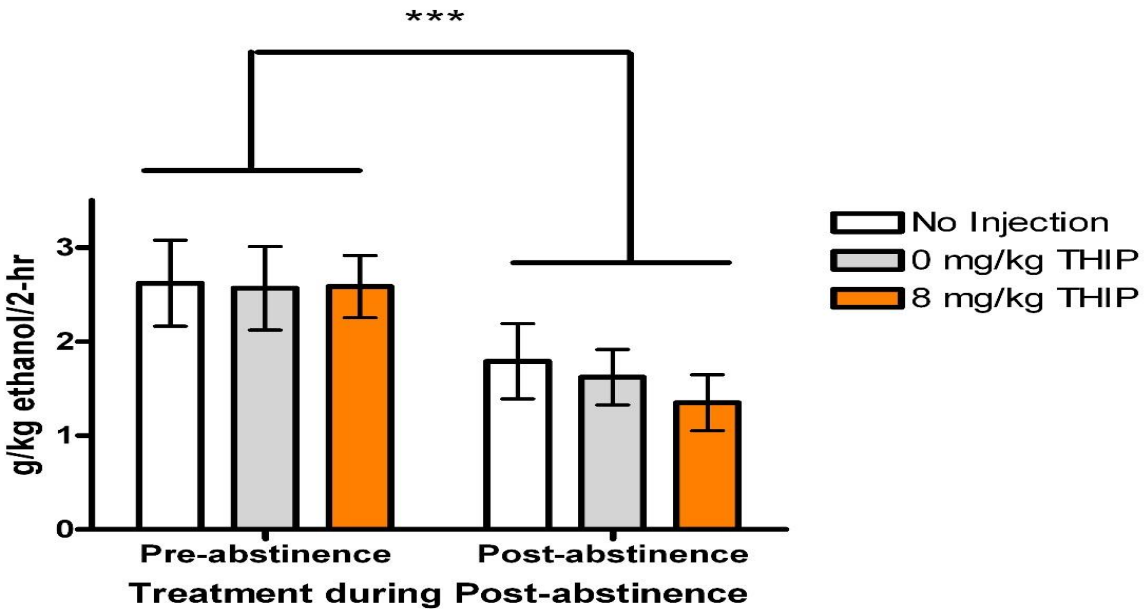


**Figure A.2. Post-abstinence 10E licks**

10E licks analyzed by 20-minute bins on the first day of "post-abstinence" following no-injection, 0 mg/kg, or 8 mg/kg THIP. #$p \leq 0.05$ versus no-injection, *$p \leq 0.05$ versus 0 mg/kg THIP.

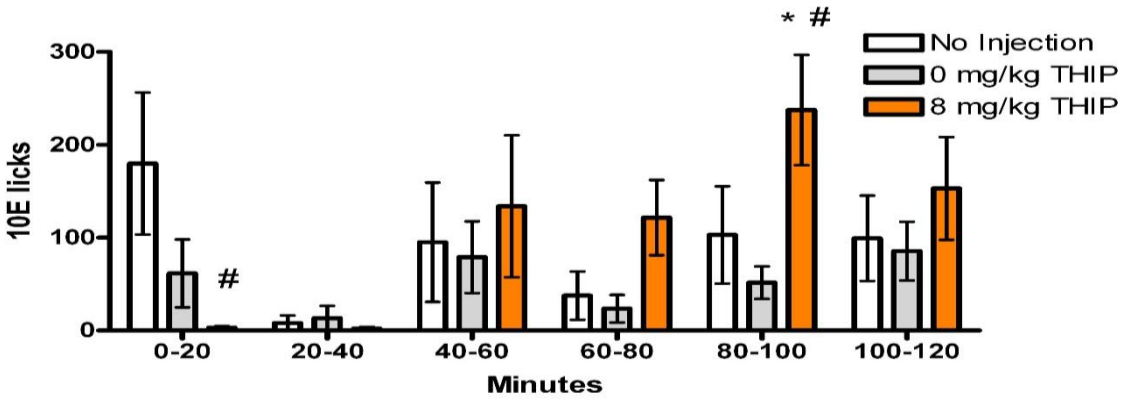



**Figure A.3. Systemic THIP: 2-hour locomotor activity**

Horizontal locomotor activity following a systemic injection of 0 or 8 mg/kg THIP. Values represent total cm moved following the infusions, subtracting out cm moved on the day prior following a saline injection. Values represent mean ± SEM for each dose. *$p \leq 0.05$,

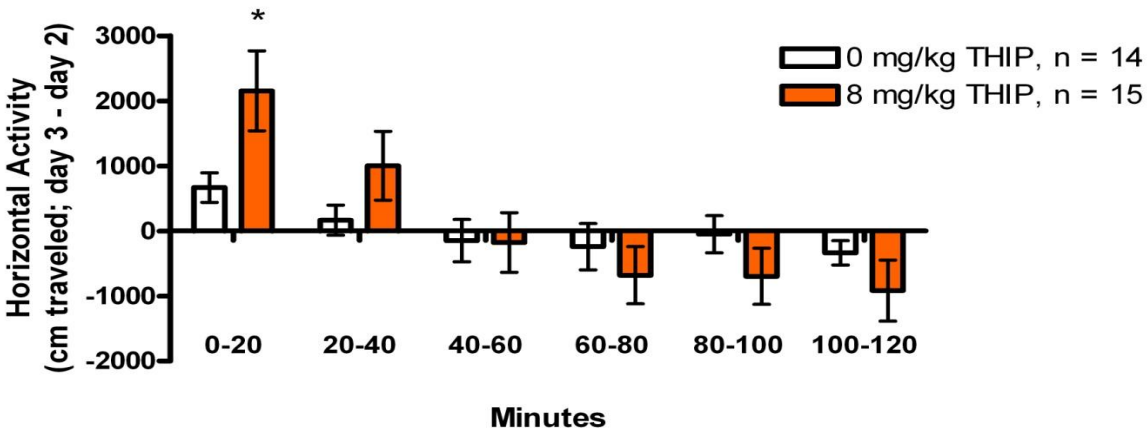


**Table A.1. Post-abstinence 10E bout parameters**

10E bout parameters following no-injection, 0 mg/kg, or 8 mg/kg THIP upon reintroduction to 10E after 2 weeks of abstinence. #$p \leq 0.05$ versus no-injection, +$p \leq 0.10$ versus 0 mg/kg.

| **Treatment** | **No-Injection** | **0 mg/kg THIP** | **8 mg/kg THIP** |
|---|---|---|---|
| **Bout Frequency** | 6.6 ± 3.0 | 4.5 ± 1.5 | 7.0 ± 1.2 |
| **Bout Size** | 102 ± 34 | 61 ± 9 | 88 ± 9 |
| **Latency to First Lick (Min)** | 3.4 ± 2 | 18 ± 16 | 13 ± 8 |
| **Latency to First Bout (Min)** | 15 ± 10 | 29 ± 20 | 61 ± 6 # + |
| **First Bout Size** | 104 ± 97 | 48 ± 7 | 132 ± 64 |

# REFERENCES


Abernathy K, Chandler LJ, Woodward JJ. (2010) Alcohol and the prefrontal cortex. Int Rev Neurobiol. 91:289–320.

Abramian AM, Comenencia-Ortiz E, Vithlani M, Tretter EV, Sieghart W, Davies PA, et al. (2010) Protein kinase C phosphorylation regulates membrane insertion of GABAA receptor subtypes that mediate tonic inhibition. J Biol Chem. 285(53):41795–805.

Agís-Balboa RC, Pinna G, Pibiri F, Kadriu B, Costa E, Guidotti A. (2007) Down-regulation of neurosteroid biosynthesis in corticolimbic circuits mediates social isolation-induced behavior in mice. Proc Natl Acad Sci. 104(47): 18736–18741.

Akabas MH. (2004) $GABA_A$ receptor structure-function studies: a reexamination in light of new acetylcholine receptor structures. Int Rev Neurobiol. 62:1–43.

Akk G, Shu H-J, Wang C, Steinbach JH, Zorumski CF, Covey DF, et al. (2005) Neurosteroid access to the GABAA receptor. J Neurosci. 25(50):11605–13.

Alheid GF, Heimer L. (1988) New perspectives in basal forebrain organization of special relevance for neuropsychiatric disorders: the striatopallidal, amygdaloid, and corticopetal components of substantia innominata. Neuroscience. 27(1):1–39.

Allan AM, Harris RA. (1986) γ-aminobutyric acid and alcohol actions: neurochemical studies of long sleep and short sleep mice. Life Sci. 39(21):2005–15.

André VM, Cepeda C, Cummings DM, Jocoy EL, Fisher YE, William Yang X, et al. (2010) Dopamine modulation of excitatory currents in the striatum is dictated by the expression of D1 or D2 receptors and modified by endocannabinoids. Eur J Neurosci. 31(1):14–28.

Angulo JA, McEwen BS. (1994) Molecular aspects of neuropeptide regulation and function in the corpus striatum and nucleus accumbens. Brain Res Brain Res Rev. 19(1):1–28.

Anker JJ, Holtz NA, Zlebnik N, Carroll ME. (2009) Effects of allopregnanolone on the reinstatement of cocaine-seeking behavior in male and female rats. Psychopharmacology (Berl). 203(1):63–72.

Anker JJ, Zlebnik NE, Carroll ME. (2010) Differential effects of allopregnanolone on the escalation of cocaine self-administration and sucrose intake in female rats. Psychopharmacology (Berl). 212(3):419–29.

Anstee QM, Knapp S, Maguire EP, Hosie AM, Thomas P, Mortensen M, et al. (2013) Mutations in the Gabrb1 gene promote alcohol consumption through increased tonic inhibition. Nat Commun. 4:2816.

Anton RF, Drobes DJ, Voronin K, Durazo-Avizu R, Moak D. (2004) Naltrexone effects on alcohol consumption in a clinical laboratory paradigm: temporal effects of drinking. Psychopharmacology (Berl) 173(1-2):32-40.

Atrens DM, Marfaing-Jallat P, Le Magnen J. (1983) Ethanol preference following hypothalamic stimulation: relation to stimulation parameters and energy balance. Pharmacol Biochem Behav. 19(4):571–5.

Barbaccia ML, Affricano D, Trabucchi M, Purdy RH, Colombo G, Agabio R, et al. (1999) Ethanol markedly increases “GABAergic” neurosteroids in alcohol-preferring rats. Eur J Pharmacol. 384(2-3):R1–2.

Barbaccia ML, Serra M, Purdy RH, Biggio G. (2001) Stress and neuroactive steroids. Int Rev Neurobiol. 46:243–72.

Baulieu EE. (1991) Neurosteroids: a new function in the brain. Biol Cell. 71(1-2):3–10.

Beauchamp MH, Ormerod BK, Jhamandas K, Boegman RJ, Beninger RJ. (2000) Neurosteroids and reward: allopregnanolone produces a conditioned place aversion in rats. Pharmacol Biochem Behav. 67(1):29–35.

Beekman M, Ungard JT, Gasior M, Carter RB, Dijkstra D, Goldberg SR, Witkin JM. (1998) Reversal of behavioral effects of pentylenetetrazol by the neuroactive steroid ganaxolone. J Pharmacol Exp Ther 284(3):868-77.

Belelli D, Casula A, Ling A, Lambert JJ. (2002) The influence of subunit composition on the interaction of neurosteroids with $GABA_A$ receptors. Neuropharmacology. 43(4):651–61.

Belelli D, Herd MB. (2003) The contraceptive agent Provera enhances $GABA_A$ receptor-mediated inhibitory neurotransmission in the rat hippocampus: evidence for endogenous neurosteroids? J Neurosci 23:10013-10020.

Belelli D, Lambert JJ. (2005) Neurosteroids: endogenous regulators of the $GABA_A$ receptor. Nat Rev Neurosci 6:565-575.

Berridge KC, Valenstein ES. (1991) What psychological process mediates feeding evoked by electrical stimulation of the lateral hypothalamus? Behavioral Neuroscience. 105(1):3–14.

Bertran-Gonzalez J, Bosch C, Maroteaux M, Matamales M, Hervé D, Valjent E, et al. (2008) Opposing patterns of signaling activation in dopamine D1 and D2 receptor-expressing striatal neurons in response to cocaine and haloperidol. J Neurosci. 28(22):5671–85.

Besheer J, Lindsay TG, O'Buckley TK, Hodge CW, Morrow AL. (2010) Pregnenolone and ganaxolone reduce operant ethanol self-administration in alcohol-preferring p rats. Alcohol Clin Exp Res 34(12):2044-52.

Bianchi L, Ballini C, Colivicchi MA, Della Corte L, Giovannini MG, Pepeu G. (2003) Investigation on acetylcholine, aspartate, glutamate and GABA extracellular levels from ventral hippocampus during repeated exploratory activity in the rat. Neurochem Res. 28(3-4):565–73.

Bobak M, Room R, Pikhart H, Kubinova R, Malyutina S, Pajak A, et al. (2004) Contribution of drinking patterns to differences in rates of alcohol related

problems between three urban populations. J Epidemiol Community Health. 58(3):238-42.

Boileau I, Assaad J-M, Pihl RO, Benkelfat C, Leyton M, Diksic M, et al. (2003) Alcohol promotes dopamine release in the human nucleus accumbens. Synapse. 49(4):226–31.

Borghese CM, Harris RA. (2007) Studies of ethanol actions on recombinant δ-containing γ-aminobutyric acid type A receptors yield contradictory results. Alcohol. 41(3):155-62.

Borghese CM, Storustovu S, Ebert B, Herd MB, Belelli D, Lambert JJ, et al. (2006) The δ subunit of γ-aminobutyric acid type A receptors does not confer sensitivity to low concentrations of ethanol. J Pharmacol Exp Ther. 316(3):1360-8.

Bowen, CA, Purdy, RH, Grant, KA. (1999) Ethanol-like discriminative stimulus effects of endogenous neuroactive steroids: effect of ethanol training dose and dosing procedure. J Pharmacol Exp Ther 289:405-411.

Boyd KN, Kumar S, O'Buckley TK, Porcu P, Morrow AL. (2010a) Ethanol induction of steroidogenesis in rat adrenal and brain is dependent upon pituitary ACTH release and de novo adrenal StAR synthesis. J Neurochem. 112(3):784–96.

Boyd KN, Kumar S, O'Buckley TK, Morrow AL. (2010b) Chronic ethanol exposure produces tolerance to elevations in neuroactive steroids: mechanisms and reversal by exogenous ACTH. J Neurochem. 115(1):142–52.

Boyle AE, Smith BR, Amit Z. (1992) Microstructural analysis of the effects of THIP, a $GABA_A$ agonist, on voluntary ethanol intake in laboratory rats. Pharmacol Biochem Behav. 43:1121-1127.

Boyle AE, Segal R, Smith BR, Amit Z. (1993) Bidirectional effects of GABAergic agonists and antagonists on maintenance of voluntary ethanol intake in rats. Pharmacol Biochem Behav. 46:179-182.

Bozarth MA, Wise RA. (1981) Heroin reward is dependent on a dopaminergic substrate. Life Sci. 29(18):1881–6.

Breese GR, Criswell HE, Carta M, Dodson PD, Hanchar HJ, Khisti RT, et al. (2006) Basis of the gabamimetic profile of ethanol. Alcohol Clin Exp Res. 30(4):731-44.

Bright DP, Renzi M, Bartram J, McGee TP, MacKenzie G, Hosie AM, et al. (2011) Profound desensitization by ambient GABA limits activation of δ-containing GABAA receptors during spillover. J Neurosci. 31(2):753–63.

Brodie MS, Shefner SA, Dunwiddie TV. (1990) Ethanol increases the firing rate of dopamine neurons of the rat ventral tegmental area in vitro. Brain Res. 508(1):65-9.

Brooks-Kayal AR, Shumate MD, Jin H, Lin DD, Rikhter TY, Holloway KL, et al. (1999) Human neuronal γ-aminobutyric $acid_A$ receptors: coordinated subunit mRNA

expression and functional correlates in individual dentate granule cells. J Neurosci. 19(19):8312-8.

Brown N, Kerby J, Bonnert TP, Whiting PJ, Wafford KA. (2002) Pharmacological characterization of a novel cell line expressing human $\alpha_4\beta_3\delta$ $GABA_A$ receptors. Br J Pharmacol. 136(7):965-74.

Buczek Y, Lê AD, Sellers EM, Tomkins DM. (1998) Effect of pentylenetetrazole on ethanol intake, ethanol kinetics, and social behavior in male Wistar rats. Alcohol Clin Exp Res. 22(2):428–36.

Bullock AE, Clark AL, Grady SR, Robinson SF, Slobe BS, Marks MJ, et al. (1997) Neurosteroids modulate nicotinic receptor function in mouse striatal and thalamic synaptosomes. J Neurochem. 68(6):2412–23.

Burish TG, Maisto SA, Cooper AM, Sobell MB. (1981) Effects of voluntary short-term abstinence from alcohol on subsequent drinking patterns of college students. J Stud Alcohol. 42(11):1013–20.

Cagetti E, Liang J, Spigelman I, Olsen RW. (2003) Withdrawal from chronic intermittent ethanol treatment changes subunit composition, reduces synaptic function, and decreases behavioral responses to positive allosteric modulators of GABAA receptors. Mol Pharmacol. 63(1):53-64.

Cagetti E, Pinna G, Guidotti A, Baicy K, Olsen RW. (2004) Chronic intermittent ethanol (CIE) administration in rats decreases levels of neurosteroids in hippocampus, accompanied by altered behavioral responses to neurosteroids and memory function. Neuropharmacology. 46(4):570-9.

Carlson SL, Kumar S, Werner DF, Comerford CE, Morrow AL. (2013) Ethanol activation of protein kinase A regulates GABAA α1 receptor function and trafficking in cultured cerebral cortical neurons. J Pharmacol Exp Ther. 345(2):317–25.

Carter RB, Wood PL, Wieland S, Hawkinson JE, Belelli D, Lambert JJ et al. (1997) Characterization of the anticonvulsant properties of ganaxolone (CCD 1042; 3α-hydroxy-3β-methyl-5α-pregnan-20-one), a selective, high-affinity, steroid modulator of the γ-aminobutyric $acid_A$ receptor. J Pharmacol Exp Ther. 280:1284-1295.

Casagrande S, Cupello A, Pellistri F, Robello M. (2007) Only high concentrations of ethanol affect $GABA_A$ receptors of rat cerebellum granule cells in culture. Neurosci Lett. 414(3):273-6.

Chandra D, Jia F, Liang J, Peng Z, Suryanarayanan A, Werner DF, et al. (2006) $GABA_A$ receptor alpha 4 subunits mediate extrasynaptic inhibition in thalamus and dentate gyrus and the action of gaboxadol. Proc Natl Acad Sci. 103(41):15230-5.

Chen SW, Rodriguez L, Davies MF, Loew GH. (1996) The hyperphagic effect of 3α-hydroxylated pregnane steroids in male rats. Pharmacol Biochem Behav. 53(4):777-82.

Cherubini E. (2012) Phasic $GABA_A$-Mediated Inhibition. Jasper's Basic Mechanisms of the Epilepsies [Internet]. 4th ed. Bethesda (MD): National Center for Biotechnology Information (US); Available from:http://www.ncbi.nlm.nih.gov/books/NBK98155/

Chester JA, Cunningham CL. (2002) $GABA_A$ receptor modulation of the rewarding and aversive effects of ethanol. Alcohol. 26(3):131-43.

Childress AR, Hole AV, Ehrman RN, Robbins SJ, McLellan AT, O'Brien CP. (1993) Cue reactivity and cue reactivity interventions in drug dependence. NIDA Res Monogr. 137:73–95.

Christensen T, Bétry C, Mnie-Filali O, Etievant A, Ebert B, Haddjeri N et al. (2012) Synergistic antidepressant-like action of gaboxadol and escitalopram. European Neuropsychopharmacology. 22(10): 751-760.

Cook JB, Foster KL, Eiler WJ, 2nd, McKay PF, Woods J, 2nd, Harvey SC, et al. (2005) Selective $GABA_A$ α5 benzodiazepine inverse agonist antagonizes the neurobehavioral actions of alcohol. Alcohol Clin Exp Res. 29(8):1390-401.

Cooney AS, Fitzsimons JT. (1996) Increased sodium appetite and thirst in rat induced by the ingredients of liquorice, glycyrrhizic acid and glycyrrhetinic acid. Regul Pept. 66(1-2):127-33.

Concas A, Mostallino MC, Porcu P, Follesa P, Barbaccia ML, Trabucchi M, et al. (1998) Role of brain allopregnanolone in the plasticity of γ-aminobutyric acid type A receptor in rat brain during pregnancy and after delivery. Proc Natl Acad Sci. 95(22):13284-9.

Concas A, Follesa P, Barbaccia ML, Purdy RH, Biggio G. (1999) Physiological modulation of $GABA_A$ receptor plasticity by progesterone metabolites. Eur J Pharmacol. 375(1-3):225–35.

Concas A, Sogliano C, Porcu P, Marra C, Brundu A, Biggio G. (2006) Neurosteroids in nicotine and morphine dependence. Psychopharmacology (Berl). 186(3):281-92.

Corpéchot C,Collins BE, Carey MP, Tsouros A, Robel P, Fry JP. (1997) Brain neurosteroids during the mouse oestrous cycle. Brain Res. 766: 276-80.

Craig CR, Deason JR. (1968) Anticonvulsant activity of steroids, specificity of structure. Arch Int Pharmacodyn Ther. 172(2):366-72.

Cremers T, Ebert B. (2007) Plasma and CNS concentrations of Gaboxadol in rats following subcutaneous administration. Eur J Pharmacol. 562(1-2):47-52.

Curran EJ, Watson SJ Jr. (1995) Dopamine receptor mRNA expression patterns by opioid peptide cells in the nucleus accumbens of the rat: a double in situ hybridization study. J Comp Neurol. 361(1):57-76.

Dazzi L, Serra M, Seu E, Cherchi G, Pisu MG, Purdy RH, et al. (2002) Progesterone enhances ethanol-induced modulation of mesocortical dopamine neurons: antagonism by finasteride. J Neurochem. 83(5):1103-9.

Deitrich RA, Dunwiddie TV, Harris RA, Erwin VG. (1989) Mechanism of action of ethanol: initial central nervous system actions. Pharmacol Rev. 41(4):489-537.

de Wit H, Stewart J. (1981) Reinstatement of cocaine-reinforced responding in the rat. Psychopharmacology (Berl). 75(2):134-143.

de Wit H, Stewart J. (1983) Drug reinstatement of heroin-reinforced responding in the rat. Psychopharmacology (Berl). 79(1):29–31.

de Wit, Harriet. (1996) Priming effects with drugs and other reinforcers. Experimental and Clinical Psychopharmacology. 4(1): 5-10.

Devaud LL, Purdy RH, Morrow AL. (1995) The neurosteroid, 3α-hydroxy-5α-pregnan-20-one, protects against bicuculline-induced seizures during ethanol withdrawal in rats. Alcohol Clin Exp Res. 19(2):350-5.

Dhaher R, Finn DA, Oberbeck DL, Yoneyama N, Snelling CC, Wu W, et al. (2009) Electrolytic lesions of the medial nucleus accumbens shell selectively decrease ethanol consumption without altering preference in a limited access procedure in C57BL/6J mice. Pharmacology, Biochemistry, and Behavior. 92(2): 335-34.2

Di Chiara G, Imperato A. (1985) Ethanol preferentially stimulates dopamine release in the nucleus accumbens of freely moving rats. Eur J Pharmacol. 115(1):131-2.

Doyon WM, York JL, Diaz LM, Samson HH, Czachowski CL, Gonzales RA. (2003) Dopamine activity in the nucleus accumbens during consummatory phases of oral ethanol self-administration. Alcohol Clin Exp Res. 27(10):1573-82.

Ebert B, Anderson NJ, Cremers TI, Rasmussen S, Vogel V, Fahey JM, et al. (2008) Gaboxadol -- a different hypnotic profile with no tolerance to sleep EEG and sedative effects after repeated daily dosing. Pharmacol Biochem Behav. 90(1):113-22.

Ehlert FJ, Ragan P, Chen A, Roeske WR, Yamamura HI. (1982) Modulation of benzodiazepine receptor binding: insight into pharmacological efficacy. Eur J Pharmacol. 78(2):249-53.

Eiler WA II, June HL. (2007) Blockade of $GABA_A$ receptors within the extended amygdala attenuates $D_2$ regulation of alcohol-motivated behaviors in the ventral tegmental area of alcohol-preferring (P) rats. Neuropharmacology. 52(8):1570-1579.

Engel SR, Purdy RH, Grant KA. (2001) Characterization of discriminative stimulus effects of the neuroactive steroid pregnanolone. J Pharmacol Exp Ther. 297(2):489–95.

Engleman EA, Ding ZM, Oster SM, Toalston JE, Bell RL, Murphy JM et al. (2009) Ethanol is self-administered into the nucleus accumbens shell, but not the core: evidence of genetic sensitivity. Alcohol Clin Exp Res. 33(12): 2162-2171.

Epstein DH, Preston KL, Stewart J, Shaham Y. (2006) Toward a model of drug relapse: an assessment of the validity of the reinstatement procedure. Psychopharmacology (Berl). 189(1):1–16.

Eva C, Mele P, Collura D, Nai A, Pisu MG, Serra M, et al. (2008) Modulation of neuropeptide Y and Y1 receptor expression in the amygdala by fluctuations in the brain content of neuroactive steroids during ethanol drinking discontinuation in Y1R/LacZ transgenic mice. Journal of Neurochemistry. 104(4):1043-1054.

Falk DE, Yi H, Hiller-Sturmhöfel S. (2006) An epidemiologic analysis of co-occurring alcohol and tobacco use and disorders: findings from the National Epidemiologic Survey on Alcohol and Related Conditions.Alcohol Res Health. 29(3):162-71.

Farrant M, Nusser Z. (2005) Variations on an inhibitory theme: phasic and tonic activation of $GABA_A$ receptors Nature Reviews Neuroscience. 6(3):215-229

Fatt P, Katz B. (1953) The effect of inhibitory nerve impulses on a crustacean muscle fibre. J Physiol. 121(2):374-89.

Ferguson SM, Eskenazi D, Ishikawa M, Wanat MJ, Phillips PEM, Dong Y, et al. (2011) Transient neuronal inhibition reveals opposing roles of indirect and direct pathways in sensitization. Nat Neurosci. 14(1):22-4.

Finn DA, Gee KW. (1994) The estrus cycle, sensitivity to convulsants and the anticonvulsant effect of a neuroactive steroid. J Pharmacol Exp Ther. 271:164-70.

Finn DA, Phillips TJ, Okorn DM, Chester JA, Cunningham CL. (1997a) Rewarding effect of the neuroactive steroid 3α-hydroxy-5α-pregnan-20-one in mice. Pharmacol Biochem Behav. 56(2):261-4.

Finn DA, Roberts AJ, Lotrich F, Gallaher EJ. (1997b) Genetic differences in behavioral sensitivity to a neuroactive steroid. J Pharmacol Exp Ther. 280(2):820-8.

Finn DA, Gallaher EJ, Crabbe JC. (2000) Differential change in neuroactive steroid sensitivity during ethanol withdrawal. J Pharmacol Exp Ther. 292(1):394-405.

Finn DA, Roberts AJ, Long S, Tanchuck M, Phillips TJ. (2003) Neurosteroid consumption has anxiolytic effects in mice. Pharmacol Biochem Behav. 76(3-4):451-62.

Finn DA, Sinnott RS, Ford MM, Long SL, Tanchuck MA, Phillips TJ. (2004) Sex differences in the effect of ethanol injection and consumption on brain allopregnanolone levels in C57BL/6 mice. Neuroscience. 123(4):813-9.

Finn DA, Beadles-Bohling AS, Beckley EH, Ford MM, Gililland KR, Gorin-Meyer RE, et al. (2006) A new look at the 5α-reductase inhibitor finasteride. CNS Drug Rev. 12(1):53-76.

Finn DA, Snelling C, Fretwell AM, Tanchuck MA, Underwood L, Cole M et al. (2007) Increased drinking during withdrawal from intermittent ethanol exposure is blocked by the CRF receptor antagonist D-Phe-CRF(12-41) Alcohol Clin Exp Res. 31(6): 939-949.

Finn DA, Mark GP, Fretwell AM, Gililland-Kaufman KR, Strong MN, Ford MM. (2008) Reinstatement of ethanol and sucrose seeking by the neurosteroid allopregnanolone in C57BL/6 mice. Psychopharmacology. (Berl) 201(3):423-33.

Finn DA, Beckley EH, Kaufman KR, Ford MM. (2010) Manipulation of GABAergic steroids: Sex differences in the effects on alcohol drinking- and withdrawal-related behaviors. Horm Behav. 57(1):12-22.

Fisher MT, Fisher JL. (2010) Activation of α6-containing $GABA_A$ receptors by pentobarbital occurs through a different mechanism than activation by GABA. Neurosci Lett. 471(3):195-9.

Follesa P, Biggio F, Talani G, Murru L, Serra M, Sanna E, et al. (2006) Neurosteroids, $GABA_A$ receptors, and ethanol dependence. Psychopharmacology (Berl). 186(3):267-80.

Fodor L, Biro T, Maksay G. (2005) Nanomolar allopregnanolone potentiates rat cerebellar $GABA_A$ receptors Neurosci Lett. 383(1-2):127-30.

Ford MM, Nickel JD, Finn DA. (2005a) Treatment with and withdrawal from finasteride alter ethanol intake patterns in male C57BL/6J mice: potential role of endogenous neurosteroids? Alcohol. 37(1):23-33.

Ford MM, Nickel, JD, Phillips TJ, Finn DA. (2005b) Neurosteroid modulators of $GABA_A$ receptors differentially modulate ethanol intake patterns in male C57BL/6J mice. Alcohol Clin Exp Res. 29(9):1630-1640.

Ford MM, Mark GP, Nickel JD, Phillips TJ, Finn DA. (2007a) Allopregnanolone influences the consummatory processes that govern ethanol drinking in C57BL/6J mice. Behavioural Brain Research. 179(2):265-272.

Ford MM, Fretwell AM, Mark GP, Finn DA. (2007b) Influence of reinforcement schedule on ethanol consumption patterns in non-food restricted male C57BL/6J mice. Alcohol. 41:21-29.

Ford MM, Beckley EH, Nickel JD, Eddy S, Finn DA. (2008a) Ethanol intake patterns in female mice: influence of allopregnanolone and the inhibition of its synthesis. Drug Alcohol Depend. 97(1-2):73-85.

Ford MM, Yoneyama N, Strong MN, Fretwell A, Tanchuck M, Finn DA. (2008b) Inhibition of 5alpha-reduced steroid biosynthesis impedes acquisition of ethanol drinking in male C57BL/6J mice. Alcohol Clin Exp Res. 32(8):1408-16,

Franck J, Jayaram-Lindström N. (2013) Pharmacotherapy for alcohol dependence: status of current treatments. Current opinion in neurobiology. 23(4):692-699.

Fritschy JM, Mohler H. (1995) $GABA_A$-receptor heterogeneity in the adult rat brain: differential regional and cellular distribution of seven major subunits. J Comp Neurol. 359(1):154–94.

Frye GD, Breese GR. (1982) GABAergic modulation of ethanol-induced motor impairment. J Pharmacol Exp Ther. 223(3):750-6.

Gabriel KI, Cunningham CL, Finn DA. (2004) Allopregnanolone does not influence ethanol-induced conditioned place preference in DBA/2J mice. Psychopharmacology. (Berl) 176(1):50-6.

Gangarossa G, Espallergues J, de Kerchove d'Exaerde A, El Mestikawy S, Gerfen CR, Hervé D, et al. (2013) Distribution and compartmental organization of GABAergic medium-sized spiny neurons in the mouse nucleus accumbens. Front Neural Circuits. 7:22.

Garzón P, Navarro-Ruíz A, García-Estrada J, Gallegos A. (1989) Steroid conjugate formed by human endometrium. Arch Invest Med. 20(2):113-22.

Gasior M, Carter RB, Goldberg SR, Witkin JM. (1997) Anticonvulsant and behavioral effects of neuroactive steroids alone and in conjunction with diazepam. J Pharmacol Exp Ther. 282(2):543-53.

Gasior M, Carter RB, Witkin JM. (1999) Neuroactive steroids: potential therapeutic use in neurological and psychiatric disorders.Trends Pharmacol Sci. 20(3):107-12.

Gee KW. Steroid modulation of the GABA/benzodiazepine receptor-linked chloride ionophore. Mol Neurobiol.1988;2(4):291-317.

Genazzani AR, Palumbo MA, de Micheroux AA, Artini PG, Criscuolo M, Ficarra G, et al. (1995) Evidence for a role for the neurosteroid allopregnanolone in the modulation of reproductive function in female rats. Eur J Endocrinol. 133(3):375–80.

Gerfen CR, Young WS 3rd. (1988) Distribution of striatonigral and striatopallidal peptidergic neurons in both patch and matrix compartments: an in situ hybridization histochemistry and fluorescent retrograde tracing study. Brain Res. 460(1):161-7.

Gerfen CR, Engber TM, Mahan LC, Susel Z, Chase TN, Monsma FJ Jr, et al. (1990) D1 and D2 dopamine receptor-regulated gene expression of striatonigral and striatopallidal neurons. Science. 250(4986):1429-32.

Girdler SS, Lindgren M, Porcu P, Rubinow DR, Johnson JL, Morrow AL. (2012) A history of depression in women is associated with an altered GABAergic neuroactive steroid profile. Psychoneuroendocrinology. 37(4):543-53.

Givens BS, Breese GR. (1990) Site-specific enhancement of γ-aminobutyric acid-mediated inhibition of neural activity by ethanol in the rat medial septal area. J Pharmacol Exp Ther. 254(2):528-38.

Gmel G, Klingemann S, Muller R, Brenner D. (2001) Revising the preventive paradox: the Swiss case. Addiction. 96(2):273-84.

Gorin-Meyer RE, Wiren KM, Tanchuck MA, Long SL, Yoneyama N, Finn DA. (2007) Sex differences in the effect of finasteride on acute ethanol withdrawal severity in C57BL/6J and DBA/2J mice. Neuroscience. 146(3):1302-15.

Gong S, Doughty M, Harbaugh CR, Cummins A, Hatten ME, Heintz N, et al. (2007) Targeting Cre recombinase to specific neuron populations with bacterial artificial chromosome constructs. J Neurosci. 27(37):9817-23.

Gonzales RA, Job MO, Doyon WM. (2004) The role of mesolimbic dopamine in the development and maintenance of ethanol reinforcement. Pharmacol Ther. 103(2):121-46.

Grant KA, Azarov A, Bowen CA, Mirkis S, Purdy RH. (1996) Ethanol-like discriminative stimulus effects of the neurosteroid 3α-hydroxy-5α-pregnan-20-one in female Macaca fascicularis monkeys. Psychopharmacology (Berl). 124:340-346.

Grant KA, Azarov A, Shively CA, Purdy RH. (1997) Discriminative stimulus effects of ethanol and 3α-hydroxy-5α-pregnan-20-one in relation to menstrual cycle phase in cynomolgus monkeys (Macaca fascicularis). Psychopharmacology (Berl). 130(1):59-68.

Grant KA, Waters CA, Green-Jordan K, Azarov A, Szeliga KT. (2000) Characterization of the discriminative stimulus effects of $GABA_A$ receptor ligands in Macaca fascicularis monkeys under different ethanol training conditions. Psychopharmacology (Berl). 152(2):181–8.

Grant KA, Helms CM, Rogers LSM, Purdy RH. (2008) Neuroactive steroid stereospecificity of ethanol-like discriminative stimulus effects in monkeys. J Pharmacol Exp Ther. 326(1):354-61.

Green KL, Azarov AV, Szeliga KT, Purdy RH, Grant KA. (1999) The influence of menstrual cycle phase on sensitivity to ethanol-like discriminative stimulus effects of $GABA_A$-positive modulators. Pharmacol Biochem Behav. 64(2):379–83.

Grobin AC, Matthews DB, Devaud LL, Morrow AL. (1998) The role of $GABA_A$ receptors in the acute and chronic effects of ethanol. Psychopharmacology (Berl). 139(1-2):2-19.

Grobin AC, VanDoren MJ, Porrino LJ, Morrow AL. (2005) Cortical 3α-hydroxy-5α-pregnan-20-one levels after acute administration of ∆9-tetrahydrocannabinol, cocaine and morphine. Psychopharmacology (Berl). 179(3):544-50.

Groenewegen HJ, Berendse HW, Haber SN. (1993) Organization of the output of the ventral striatopallidal system in the rat: ventral pallidal efferents. Neuroscience. 57(1):113-42.

Groenewegen HJ, Wright CI, Beijer AV, Voorn P. (1999) Convergence and segregation of ventral striatal inputs and outputs. Ann N Y Acad Sci. 877:49-63.

Gubner NR, McKinnon CS, Reed C, Phillips TJ. (2013) Accentuating effects of nicotine on ethanol response in mice with high genetic predisposition to ethanol-induced locomotor stimulation. Drug Alcohol Depend. 127(1-3):108–14.

Gulinello M, Gong QH, Smith SS. (2003) Progesterone withdrawal increases the anxiolytic actions of gaboxadol: role of α4βδ $GABA_A$ receptors. Neuroreport 14(1): 43-46.

Haage D, Druzin M, Johansson S. (2002) Allopregnanolone modulates spontaneous GABA release via presynaptic $Cl^-$ permeability in rat preoptic nerve terminals. Brain Res. 958(2):405-13.

Harris RA, Mihic SJ. (2004) Alcohol and inhibitory receptors: unexpected specificity from a nonspecific drug. Proc Natl Acad Sci. 101(1):2-3.

Harris RA, Trudell JR, Mihic SJ. (2008) Ethanol's molecular targets. Sci Signal. 1(28):7.

Harrison NL, Simmonds MA. (1984) Modulation of the GABA receptor complex by a steroid anaesthetic. Brain Res. 323(2):287-92.

Harney SC, Frenguelli BG, Lambert JJ. (2003) Phosphorylation influences neurosteroid modulation of synaptic GABAA receptors in rat CA1 and dentate gyrus neurones. Neuropharmacology. 2003 Nov;45(6):873–83.

Havlíková H, Hill M, Kancheva L, Vrbíková J, Pouzar V, Cerny I, et al. (2006) Serum profiles of free and conjugated neuroactive pregnanolone isomers in nonpregnant women of fertile age. J Clin Endocrinol Metab. 91(8):3092-9.

Heimer L, Zahm DS, Churchill L, Kalivas PW, Wohltmann C. (1991) Specificity in the projection patterns of accumbal core and shell in the rat. Neuroscience. 41(1):89-125.

Heimer L, Alheid GF, de Olmos JS, Groenewegen HJ, Haber SN, Harlan RE, et al. (1997) The accumbens: beyond the core-shell dichotomy. J Neuropsychiatry Clin Neurosci. 9(3):354-81.

Heinz A, Beck A, Grüsser SM, Grace AA, Wrase J. (2009) Identifying the neural circuitry of alcohol craving and relapse vulnerability. Addict Biol. 14(1):108-18.

Hemby SE, O'connor JA, Acosta G, Floyd D, Anderson N, McCool BA, et al. (2006) Ethanol-induced regulation of $GABA_A$ subunit mRNAs in prefrontal fields of cynomolgus monkeys. Alcohol Clin Exp Res. 30(12):1978-85.

Helms CM, Rogers LSM, Waters CA, Grant KA. (2008) Zolpidem generalization and antagonism in male and female cynomolgus monkeys trained to discriminate 1.0 or 2.0 g/kg ethanol. Alcohol Clin Exp Res. 32(7):1197–206.

Helms CM, Rogers LSM, Grant KA. (2009) Antagonism of the ethanol-like discriminative stimulus effects of ethanol, pentobarbital, and midazolam in cynomolgus monkeys reveals involvement of specific GABA(A) receptor subtypes. J Pharmacol Exp Ther. 331(1):142–52.

Herd MB, Belelli D, Lambert JJ. (2007) Neurosteroid modulation of synaptic and extrasynaptic $GABA_A$ receptors. Pharmacol Ther. 116(1):20-34.

Herd MB, Foister N, Chandra D, Peden DR, Homanics GE, Brown VJ, et al. (2009) Inhibition of thalamic excitability by 4,5,6,7-tetrahydroisoxazolo[4,5-c]pyridine-3-ol: a selective role for δ-$GABA_A$ receptors. Eur J Neurosci. 29(6):1177-87.

Hevers W, Lüddens H. (1998) The diversity of $GABA_A$ receptors. Pharmacological and electrophysiological properties of $GABA_A$ channel subtypes. Mol Neurobiol. 18(1):35-86.

Heyser CJ, Schulteis G, Koob GF. (1997) Increased ethanol self-administration after a period of imposed ethanol deprivation in rats trained in a limited access paradigm. Alcohol Clin Exp Res. 21(5):784–91.

Hirani K, Khisti RT, Chopde CT. (2002) Behavioral action of ethanol in Porsolt's forced swim test: modulation by 3α-hydroxy-5α-pregnan-20-one. Neuropharmacology. 43(8):1339-50.

Hodge CW, Chappelle AM, Samson HH. (1995) GABAergic transmission in the nucleus accumbens is involved in the termination of ethanol self-administration in rats. Alcohol Clin Exp Res. 19(6):1486-93.

Hodge CW, Haraguchi M, Chappelle AM, Samson HH. (1996) Effects of ventral tegmental microinjections of the $GABA_A$ agonist muscimol on self-administration of ethanol and sucrose. Pharmacol Biochem Behav. 53(4):971-7.

Hodge CW, Nannini MA, Olive MF, Kelley SP, Mehmert KK (2001) Allopregnanolone and pentobarbital infused into the nucleus accumbens substitute for the discriminative stimulus effects of ethanol. Alcohol Clin Exp Res. 25(10):1441-7.

Hogenkamp DJ, Tahir SH, Hawkinson JE, Upasani RB, Alauddin M, Kimbrough CL, et al. (1997) Synthesis and in vitro activity of 3β-substituted-3α-hydroxypregnan-20-ones: allosteric modulators of the $GABA_A$ receptor. J Med Chem. 40(1):61-72.

Holdstock L, Penland SN, Morrow AL, de Wit H. (2006) Moderate doses of ethanol fail to increase plasma levels of neurosteroid 3α-hydroxy-5α-pregnan-20-one-like immunoreactivity in healthy men and women. Psychopharmacology. 186:442-450.

Holmberg E, Bäckström T, Johansson M, Löfgren M, Haage D. (2013) Allopregnanolone induces a diurnally dependent hyperphagic effect and alters feeding latency and duration in male Wistar rats. Acta Physiol. 208(4):400-9.

Holtz NA, Lozama A, Prisinzano TE, Carroll ME. (2012) Reinstatement of methamphetamine seeking in male and female rats treated with modafinil and allopregnanolone. Drug Alcohol Depend. 120(1-3):233–7.

Hörtnagl H, Tasan RO, Wieselthaler A, Kirchmair E, Sieghart W, Sperk G. (2013) Patterns of mRNA and protein expression for 12 $GABA_A$ receptor subunits in the mouse brain. Neuroscience. 236:345-72.

Hosie AM, Wilkins ME, da Silva HMA, Smart TG. (2006) Endogenous neurosteroids regulate $GABA_A$ receptors through two discrete transmembrane sites. Nature. 444(7118):486-9.

Hosie AM, Wilkins ME, Smart TG. (2007) Neurosteroid binding sites on $GABA_A$ receptors. Pharmacol Ther. 116(1):7-19.

Hosie AM, Clarke L, da Silva H, Smart TG. (2009) Conserved site for neurosteroid modulation of $GABA_A$ receptors. Neuropharmacology. 56(1):149-54.

Houston CM, McGee TP, MacKenzie G, Troyano-Cuturi K, Rodriguez PM, Kutsarova E, et al. (2012) Are extrasynaptic $GABA_A$ receptors important targets for sedative/hypnotic drugs? J Neurosci. 32:3887-3897.

Hubert GW, Kuhar MJ. (2006) Colocalization of CART peptide with prodynorphin and dopamine D1 receptors in the rat nucleus accumbens. Neuropeptides. 40(6):409-15.

Hyytiä P, Koob GF. (1995) $GABA_A$ receptor antagonism in the extended amygdala decreases ethanol self-administration in rats. Eur J Pharmacol. 283(1-3):151-9.

Ikemoto S, Kohl RR, McBride WJ. (1997) $GABA_A$ receptor blockade in the anterior ventral tegmental area increases extracellular levels of dopamine in the nucleus accumbens of rats. J Neurochem. 69(1):137–43.

Imperato A, Di Chiara G. (1986) Preferential stimulation of dopamine release in the nucleus accumbens of freely moving rats by ethanol. J Pharmacol Exp Ther. 239(1):219–28.

Janak PH, Redfern JE, Samson HH. (1998) The reinforcing effects of ethanol are altered by the endogenous neurosteroid, allopregnanolone. Alcohol Clin Exp Res. 22(5):1106-12.

Janak PH, Gill. (2003) Comparison of the effects of allopregnanolone with direct GABAergic agonists on ethanol self-administration with and without concurrently available sucrose. Alcohol. 30(1):1-7.

Janis GC, Devaud LL, Mitsuyama H, Morrow AL. (1998) Effects of chronic ethanol consumption and withdrawal on the neuroactive steroid 3α-hydroxy-5 α -pregnan-20-one in male and female rats. Alcohol Clin Exp Res. 22(9):2055–61.

Jose PA, Yu PY, Yamaguchi I, Eisner GM, Mouradian MM, Felder CC, et al. (1995) Dopamine D1 receptor regulation of phospholipase C. Hypertens Res. 18 Suppl 1:S39–42.

June HL, Lummis GH, Colker RE, Moore TO, Lewis MJ. (1991) Ro15-4513 attenuates the consumption of ethanol in deprived rats. Alcohol Clin Exp Res. 15(3):406–11.

June HL, Murphy JM, Mellor-Burke JJ, Lumeng L, Li TK. (1994) The benzodiazepine inverse agonist RO19-4603 exerts prolonged and selective suppression of ethanol intake in alcohol-preferring (P) rats. Psychopharmacology (Berl). 115(3):325–31.

June HL, Murphy JM, Hewitt RL, Greene TL, Lin M, Mellor-Burke JJ, et al. (1996) Benzodiazepine receptor ligands with different intrinsic efficacies alter ethanol intake in alcohol-nonpreferring (NP) rats. Neuropsychopharmacology. 14(1):55–66.

June HL, Torres L, Cason CR, Hwang BH, Braun MR, Murphy JM. (1998) The novel benzodiazepine inverse agonist RO19-4603 antagonizes ethanol motivated behaviors: neuropharmacological studies. Brain Res. 784(1-2):256–75.

June HL, Harvey SC, Foster KL, McKay PF, Cummings R, Garcia M, et al. (2001) $GABA_A$ receptors containing α5 subunits in the CA1 and CA3 hippocampal fields regulate ethanol-motivated behaviors: an extended ethanol reward circuitry. J Neurosci. 21(6):2166-77.

Kalivas PW, Duffy P, (1990) Eberhardt H. Modulation of A10 dopamine neurons by γ-aminobutyric acid agonists. J Pharmacol Exp Ther. 253(2):858–66.

Kawaguchi Y, Wilson CJ, Augood SJ, Emson PC. (1995) Striatal interneurones: chemical, physiological and morphological characterization. Trends Neurosci. 18(12):527–35.

Kelley AE, Baldo BA, Pratt WE, Will MJ. (2005) Corticostriatal-hypothalamic circuitry and food motivation: integration of energy, action and reward. Physiol Behav. 86:773-795.

Kemp JA, Marshall GR, Wong EH, Woodruff GN. (1987) The affinities, potencies and efficacies of some benzodiazepine-receptor agonists, antagonists and inverse-agonists at rat hippocampal $GABA_A$-receptors. Br J Pharmacol. 91(3):601–8.

Kessler RC, Nelson CB, McGonagle KA, Edlund MJ, Frank RG, Leaf PJ. (1996) The epidemiology of co-occurring addictive and mental disorders: implications for prevention and service utilization. Am J Orthopsychiatry. 66(1):17–31.

Khisti RT, VanDoren MJ, O'Buckley T, Morrow AL. (2003) Neuroactive steroid 3α-hydroxy-5α-pregnan-20-one modulates ethanol-induced loss of righting reflex in rats. Brain Res. 980:255-265.

Khisti RT, VanDoren MJ, Matthews DB, Morrow AL. (2004) Ethanol-induced elevation of 3α-hydroxy-5α-pregnan-20-one does not modulate motor incoordination in rats. Alcohol Clin Exp Res. 28(8):1249–56.

Khisti RT, Wolstenholme J, Shelton KL, Miles MF. (2006) Characterization of the ethanol-deprivation effect in substrains of C57BL/6 mice. Alcohol. 40(2):119–26.

Kiefer F, Jahn H, Tarnaske T, Helwig H, Briken P, Holzbach R, et al. (2003) Comparing and combining naltrexone and acamprosate in relapse prevention of alcoholism: a double-blind, placebo-controlled study. Arch Gen Psychiatry. 60(1):92–9.

Kim BG, Cho JH, Choi IS, Lee MG, Jang IS. (2011) Modulation of presynaptic $GABA_A$ receptors by endogenous neurosteroids. Br J Pharmacol. 164(6):1698–710.

Kim HJ, Ha M, Park CH, Park SJ, Youn SM, Kang SS, et al. (2003) StAR and steroidogenic enzyme transcriptional regulation in the rat brain: effects of acute alcohol administration. Brain Res Mol Brain Res. 115(1):39–49.

Kobayashi T, Ikeda K, Kojima H, Niki H, Yano R, Yoshioka T, et al. (1999) Ethanol opens G-protein-activated inwardly rectifying K+ channels. Nat Neurosci. 2(12):1091–7.

Kokate TG, Svensson BE, Rogawski MA. (1994) Anticonvulsant activity of neurosteroids: Correlation with $\gamma$-aminobutyric acid-evoked chloride current potentiation. J Pharmacol Exp Ther. 270:1223-1229.

Koob GF. (1992) Neural mechanisms of drug reinforcement. *Ann N Y Acad Sci.* 654:171-191.

Kornet M, Goosen C, Van Ree JM. (1990) The effect of interrupted alcohol supply on spontaneous alcohol consumption by rhesus monkeys. Alcohol. 25(4):407–12.

Korpi ER, Debus F, Linden AM, Malecot C, Leppa E, Vekovischeva O, et al. (2007) Does ethanol act preferentially via selected brain $GABA_A$ receptor subtypes? The current evidence is ambiguous. Alcohol. 41:163-176.

Kumar S, Fleming RL, Morrow AL. (2004) Ethanol regulation of γ-aminobutyric $acid_A$ receptors: genomic and nongenomic mechanisms. Pharmacol Ther. 101(3):211–26.

Kumar S, Porcu P, Werner DF, Matthews DB, Diaz-Granados JL, et al. (2009) The role of $GABA_A$ receptors in the acute and chronic effects of ethanol: a decade of progress. Psychopharmacology (Berl). 205(4):529-64.

Kumar S, Ren Q, Beckley JH, O'Buckley TK, Gigante ED, Santerre JL, et al. (2012) Ethanol Activation of Protein Kinase A Regulates GABA(A) Receptor Subunit Expression in the Cerebral Cortex and Contributes to Ethanol-Induced Hypnosis. Front Neurosci. 6:44.

Lambert JJ, Belelli D, Hill-Venning C, Peters JA. (1995) Neurosteroids and $GABA_A$ receptor function. Trends Pharmacol Sci. 16(9):295–303.

Lan NC, Gee KW. (1994) Neuroactive steroid actions at the $GABA_A$ receptor. Horm Behav. 28(4):537-44.

Lê AD, Quan B, Juzytch W, Fletcher PJ, Joharchi N, Shaham Y. (1998) Reinstatement of alcohol-seeking by priming injections of alcohol and exposure to stress in rats. Psychopharmacology (Berl). 135(2):169–74.

Lehoullier PF, Ticku MK. (1989) The pharmacological properties of GABA receptor-coupled chloride channels using 36Cl-influx in cultured spinal cord neurons. Brain Res. 487(2):205–14.

Le Moine C, Bloch B. (1995) D1 and D2 dopamine receptor gene expression in the rat striatum: sensitive cRNA probes demonstrate prominent segregation of D1 and D2 mRNAs in distinct neuronal populations of the dorsal and ventral striatum. J Comp Neurol. 355(3):418–26.

Leshner AI. (1997) Addiction is a brain disease, and it matters. Science. 278(5335):45–7.

Liang J, Zhang N, Cagetti E, Houser CR, Olsen RW, Spigelman I. (2006) Chronic intermittent ethanol-induced switch of ethanol actions from extrasynaptic to synaptic hippocampal $GABA_A$ receptors. J Neurosci. 26(6):1749–58.

Liang J, Suryanarayanan A, Abriam A, Snyder B, Olsen RW, Spigelman I. (2007) Mechanisms of reversible $GABA_A$ receptor plasticity after ethanol intoxication. J Neurosci. 27(45):12367–77.

Liu J, Yang AR, Kelly T, Puche A, Esoga C, June Jr HL, et al. (2011) Binge alcohol drinking is associated with $GABA_A$ α2-regulated Toll-like receptor 4 (TLR4) expression in the central amygdala. Proc Natl Acad Sci. 108:4465-4470.

Lobo MK, Nestler EJ. (2011) The striatal balaning act in drug addiction: distinct roles of direct and indirect pathway medium spiny neurons. Front Neuroanat. 5:41.

Lovinger DM, White G, Weight FF. (1989) Ethanol inhibits NMDA-activated ion current in hippocampal neurons. Science. 243(4899):1721–4.

Lovinger DM, Zhou Q. (1998) Alcohol effects on the 5-HT3 ligand-gated ion channel. Toxicol Lett. 100-101:239–46.

Lovinger DM, Homanics GE. (2007) Tonic for what ails us? high-affinity $GABA_A$ receptors and alcohol. Alcohol. 41(3):139–43.

Lüddens H, Korpi ER. (1995) Biological function of $GABA_A$/benzodiazepine receptor heterogeneity. J Psychiatr Res. 29(2):77–94.

Lundgren P, Strömberg J, Bäckström T, Wang M. (2003) Allopregnanolone-stimulated GABA-mediated chloride ion flux is inhibited by 3β-hydroxy-5α-pregnan-20-one (isoallopregnanolone). Brain Res. 982(1):45–53.

Madsen KK, Ebert B, Clausen RP, Krogsgaard-Larsen P, Schousboe A, White HS. (2011) Selective GABA transporter inhibitors tiagabine and EF1502 exhibit mechanistic differences in their ability to modulate the ataxia and anticonvulsant action of the extrasynaptic $GABA_A$ receptor agonist gaboxadol. J Pharmacol Exp Ther. 338:214-219.

Maguire EP, Macpherson T, Swinny JD, Dixon CI, Herd MB, Belelli D, et al. (2014) Tonic Inhibition of Accumbal Spiny Neurons by Extrasynaptic α4βδ GABAA Receptors Modulates the Actions of Psychostimulants. J Neurosci. 34(3):823–38.

Maldonado-Irizarry CS, Swanson CJ, Kelley AE. (1995) Glutamate receptors in the nucleus accumbens shell control feeding behavior via the lateral hypothalamus. J Neurosci. 15(10):6779–88.

Majewska MD. (1992) Neurosteroids: endogenous bimodal modulators of the $GABA_A$ receptor. Mechanism of action and physiological significance. Prog Neurobiol. 38(4):379–95.

Martin-Garcia E, Darbra S, Pallares M. (2007) Intrahippocampal allopregnanolone decreases voluntary chronic alcohol consumption in non-selected rats. Prog Neuropsychopharmacol Biol Psychiatry. 31(4):823-31.

Martz A, Deitrich RA, Harris RA. (1983) Behavioral evidence for the involvement of γ-aminobutyric acid in the actions of ethanol. Eur J Pharmacol. 89(1-2):53–62.

Mascia MP, Biggio F, Mancuso L, Cabras S, Cocco PL, Gorini G, et al. (2002) Changes in $GABA_A$ receptor gene expression induced by withdrawal of, but not by long-term exposure to, ganaxolone in cultured rat cerebellar granule cells. J Pharmacol Exp Ther. 303(3):1014–20.

Maurice T, Su TP, Privat A. (1998) Sigma1 receptor agonists and neurosteroids attenuate B25-35-amyloid peptide-induced amnesia in mice through a common mechanism. Neuroscience. 83(2):413–28.

McKernan RM, Whiting PJ. (1996) Which $GABA_A$-receptor subtypes really occur in the brain? Trends Neurosci. 19(4):139–43.

Mehta AK, Marutha Ravindran CR, Ticku MK. (2007) Low concentrations of ethanol do not affect radioligand binding to the δ-subunit-containing $GABA_A$ receptors in the rat brain. Brain Res. 1165:15-20.

Mellon SH, Gong W, Schonemann MD. (2008) Endogenous and synthetic neurosteroids in treatment of Niemann-Pick Type C disease. Brain Res Rev. 57:410-420.

Mihalek RM, Banerjee PK, Korpi ER, Quinlan JJ, Firestone LL, Mi ZP, et al. (1999) Attenuated sensitivity to neuroactive steroids in γ-aminobutyrate type A receptor delta subunit knockout mice. Proc Natl Acad Sci. 96(22):12905-10.

Mihalek RM, Bowers BJ, Wehner JM, Kralic JE, VanDoren MJ, Morrow AL, et al. (2001) $GABA_A$ receptor δ subunit knockout mice have multiple defects in behavioral responses to ethanol. Alcohol Clin Exp Res. 25(12):1708–1718.

Mihic SJ, Ye Q, Wick MJ, Koltchine VV, Krasowski MD, Finn SE, et al. (1997) Sites of alcohol and volatile anaesthetic action on $GABA_A$ and glycine receptors. Nature. 389(6649):385–9.

Mitchell EA, Herd MB, Gunn BG, Lambert JJ, Belelli D. (2008) Neurosteroid modulation of $GABA_A$ receptors: molecular determinants and significance in health and disease. Neurochem Int. 52(4-5):588–95.

Mody I, De Koninck Y, Otis TS, Soltesz I. (1994) Bridging the cleft at GABA synapses in the brain. Trends Neurosci. 17(12):517–25.

Mody I. (2001) Distinguishing between $GABA_A$ receptors responsible for tonic and phasic conductances. Neurochem Res. 26(8-9):907–13.

Mody I, Pearce RA. (2004) Diversity of inhibitory neurotransmission through $GABA_A$ receptors. Trends Neurosci. 27:569-575.

Mody I, Glykys J, Wei W. (2007) A new meaning for "Gin & Tonic": tonic inhibition as the target for ethanol action in the brain. Alcohol. 41(3):145–153.

Moore EM, Serio KM, Goldfarb KJ, Stepanovska S, Linsenbardt DN, Boehm SL, $2^{nd}$. (2007) GABAergic modulation of binge-like ethanol intake in C57BL/6J mice. Pharmacol Biochem Behav. 88(1):105-13.

Morrow AL, Suzdak PD, Paul SM. (1987) Steroid hormone metabolites potentiate GABA receptor-mediated chloride ion flux with nanomolar potency. Eur J Pharmacol. 142(3):483–5.

Morrow AL, Janis GC, VanDoren MJ, Matthews DB, Samson HH, Janak PH, et al. (1999) Neurosteroids mediate pharmacological effects of ethanol: a new mechanism of ethanol action? Alcohol Clin Exp Res. 23(12):1933–40.

Mortensen M, Ebert B, Wafford K, Smart TG. (2010) Distinct activities of GABA agonists at synaptic- and extrasynaptic-type $GABA_A$ receptors. J Physiol. 588(8):1251–68.

Motzo C, Porceddu ML, Maira G, Flore G, Concas A, Dazzi L, et al. (1986) Inhibition of basal and stress-induced dopamine release in the cerebral cortex and nucleus accumbens of freely moving rats by the neurosteroid allopregnanolone. J Psychopharmacol. 10(4):266–72.

Murphy NP, Sakoori K, Okabe C. (2006) Lack of evidence of a role for the neurosteroid allopregnanolone in ethanol-induced reward and c-fos expression in DBA/2 mice. Brain Res. 1094(1):107–18.

Narahashi T, Aistrup GL, Marszalec W, Nagata K. (1999) Neuronal nicotinic acetylcholine receptors: a new target site of ethanol. Neurochem Int. 35(2):131–41.

Nie H, Janak PH. (2003) Comparison of reinstatement of ethanol- and sucrose-seeking by conditioned stimuli and priming injections of allopregnanolone after extinction in rats. Psychopharmacology (Berl). 168(1-2):222-8.

Nie H, Rewal M, Gill TM, Ron D, Janak PH. (2011) Extrasynaptic δ-containing $GABA_A$ receptors in the nucleus accumbens dorsomedial shell contribute to alcohol intake. Proc Natl Acad Sci. 108:4459-4464.

Nohria V, Giller E. (2007) Ganaxolone. Neurotherapeutics: the journal of the American Society for Experimental NeuroTherapeutics. 4(1):102-105.

Nowak KL, McBride WJ, Lumeng L, Li TK, Murphy JM. (1998) Blocking $GABA_A$ receptors in the anterior ventral tegmental area attenuates ethanol intake of the alcohol-preferring P rat. Psychopharmacology (Berl). 139(1-2):108-16.

Nusser Z, Sieghart W, Somogyi P. (1998) Segregation of different $GABA_A$ receptors to synaptic and extrasynaptic membranes of cerebellar granule cells. J Neurosci. 18(5):1693-703.

O'Dell LE, Alomary AA, Vallée M, Koob GF, Fitzgerald RL, Purdy RH. (2004) Ethanol-induced increases in neuroactive steroids in the rat brain and plasma are absent in adrenalectomized and gonadectomized rats. Eur J Pharmacol. 484(2-3):241–7.

Olsen RW, Hanchar HJ, Meera P, Wallner M. (2007) $GABA_A$ receptor subtypes: the "one glass of wine" receptors. Alcohol. 41(3):201-9.

O'Malley SS, Krishnan-Sarin S, Farren C, Sinha R, Kreek MJ. (2002) Naltrexone decreases craving and alcohol self-administration in alcohol-dependent subjects and activates the hypothalamo-pituitary-adrenocortical axis. Psychopharmacology (Berl). 160(1):19-29.

Palmer AA, McKinnon CS, Bergstrom HC, Phillips TJ. (2002) Locomotor activity responses to ethanol, other alcohols, and GABA-A acting compounds in forward- and reverse-selected FAST and SLOW mouse lines. Behavioral neuroscience. 116(6): 958-967.

Park HM, Choi IS, Nakamura M, Cho JH, Lee MG, Jang IS. (2011) Multiple effects of allopregnanolone on GABAergic responses in single hippocampal CA3 pyramidal neurons. Eur J Pharmacol. 652(1-3):46–54.

Park-Chung M, Wu FS, Purdy RH, Malayev AA, Gibbs TT, Farb DH. (1997) Distinct sites for inverse modulation of N-methyl-D-aspartate receptors by sulfated steroids. Mol Pharmacol. 52(6):1113–23.

Park-Chung M, Malayev A, Purdy RH, Gibbs TT, Farb DH. (1999) Sulfated and unsulfated steroids modulate γ-aminobutyric $acid_A$ receptor function through distinct sites. Brain Res. 830(1):72-87.

Paul SM, Purdy RH. (1992) Neuroactive steroids. FASEB J. (6):2311-22.

Peng Z, Hauer B, Mihalek RM, Homanics GE, Sieghart W, Olsen RW, et al. (2002) $GABA_A$ receptor changes in δ subunit-deficient mice: altered expression of α4 and γ2 subunits in the forebrain. J Comp Neurol. 446(2):179-97.

Peng Z, Zhang N, Chandra D, Homanics GE, Olsen RW, Houser CR. (2013) Altered Localization of the δ Subunit of the $GABA_A$ Receptor in the Thalamus of α4 Subunit Knockout Mice. Neurochem Res. [Epub Ahead of print]

Petry NM. (1997) Benzodiazepine-GABA modulation of concurrent ethanol and sucrose reinforcement in the rat. Exp Clin Psychopharmacol. 5(3):183-94.

Phillips TJ, Huson M, Gwiazdon C, Burkhart-Kasch S, Shen EH. (1995) Effects of acute and repeated ethanol exposures on the locomotor activity of BXD recombinant inbred mice. Alcohol Clin Exp Res. 19(2): 269-278.

Pickens R, Harris WC. Self-administration of d-amphetamine by rats. Psychopharmacologia. 1968;12(2):158-63.

Pierucci-Lagha A, Covault J, Feinn R, Nellissery M, Hernandez-Avila C, Oncken C, et al. (2005) GABRA2 alleles moderate the subjective effects of alcohol, which are attenuated by finasteride. Neuropsychopharmacology. 30(6):1193-203.

Pirker S, Schwarzer C, Wieselthaler A, Sieghart W, Sperk G. (2000) $GABA_A$ receptors: immunocytochemical distribution of 13 subunits in the adult rat brain. Neuroscience. 101(4):815-850.

Polich JM, Armor DJ, Braiker HB. (1980) Patterns of alcoholism over four years. Journal of studies on alcohol. 41(5):397-416.

Porcu P, Sogliano C, Ibba C, Piredda M, Tocco S, Marra C, et al. (2004) Failure of γ-hydroxybutyric acid both to increase neuroactive steroid concentrations in adrenalectomized-orchiectomized rats and to induce tolerance to its steroidogenic effect in intact animals. Brain Res. 1012(1-2):160-8.

Porcu P, O'Buckley TK, Alward, SE, Song SC, Grant KA , de Wit H, Morrow AL. (2010) Differential effects of ethanol on serum GABAergic 3α,5α/3α,5β neuroactive steroids in mice, rats, cynomolgus monkeys, and humans. Alcohol Clin Exp Res. 34:432-442.

Prenosil GA, Schneider Gasser EM, Rudolph U, Keist R, Fritschy J-M, Vogt KE. (2006) Specific subtypes of $GABA_A$ receptors mediate phasic and tonic forms of inhibition in hippocampal pyramidal neurons. J Neurophysiol. 96(2):846-57.

Purdy RH, Morrow AL, Moore PH Jr, Paul SM. (1991) Stress-induced elevations of γ-aminobutyric acid type A receptor-active steroids in the rat brain. Proc Natl Acad Sci. 88(10):4553-7.

Quirk K, Gillard NP, Ragan CI, Whiting PJ, McKernan RM. (1994) Model of subunit composition of γ-aminobutyric acid A receptor subtypes expressed in rat cerebellum with respect to their α and γ/δ subunits. J Biol Chem. 269(23):16020-8.

Ramaker MJ, Ford MM, Fretwell AM, Finn DA. (2011) Alteration of ethanol drinking in mice via modulation of the $GABA_A$ receptor with ganaxolone, finasteride, and gaboxadol. Alcohol Clin Exp Res. 35(11):1994-2007.

Ramaker MJ, Strong MN, Ford MM, Finn DA. (2012) Effect of ganaxolone and THIP on operant and limited-access ethanol self-administration. Neuropharmacology. 63(4):555–564.

Rassnick S, Stinus L, Koob GF. (1993) The effects of 6-hydroxydopamine lesions of the nucleus accumbens and the mesolimbic dopamine system on oral self-administration of ethanol in the rat. Brain Res. 623(1):16-24.

Reddy DS, Kulkarni SK. (1998) The role of $GABA_A$ and mitochondrial diazepam-binding inhibitor receptors on the effects of neurosteroids on food intake in mice. Psychopharmacology (Berl). 137(4):391-400.

Reddy DS, Rogawski MA. (2000) Chronic treatment with the neuroactive steroid ganaxolone in the rat induces anticonvulsant tolerance to diazepam but not to itself. J Pharmacol Exp Ther. 295(3):1241-8.

Reddy DS. (2010) Neurosteroids: endogenous role in the human brain and therapeutic potentials. Progress in Brain Research. 186: 113-137.

Regier DA, Farmer ME, Rae DS, Locke BZ, Keith SJ, Judd LL, et al. (1990) Comorbidity of mental disorders with alcohol and other drug abuse. Results from the Epidemiologic Catchment Area (ECA) Study. JAMA. 264(19):2511-8.

Rewal M, Jurd R, Gill TM, He DY, Ron D, Janak PH. (2009) α4-containing $GABA_A$ receptors in the nucleus accumbens mediate moderate intake of alcohol. The Journal of Neuroscience 29(2): 543-549.

Rewal M, Donahue R, Gill TM, Nie H, Ron D, Janak PH (2011) α4 subunit-containing $GABA_A$ receptors in the accumbens shell contribute to the reinforcing effects of alcohol. Addict Biol. 17:309-321.

Roberto M, Madamba SG, Moore SD, Tallent MK, Siggins GR. (2003) Ethanol increases GABAergic transmission at both pre- and postsynaptic sites in rat central amygdala neurons. Proc Natl Acad Sci. 100(4):2053–8.

Rougé-Pont F, Mayo W, Marinelli M, Gingras M, Le Moal M, Piazza PV. (2002) The neurosteroid allopregnanolone increases dopamine release and dopaminergic response to morphine in the rat nucleus accumbens. Eur J Neurosci. 16(1):169-73.

Rowlett JK, Winger G, Carter RB, Wood PL, Woods JH, Woolverton WL. (1999) Reinforcing and discriminative stimulus effects of the neuroactive steroids pregnanolone and Co 8-7071 in rhesus monkeys. Psychopharmacology (Berl). 145(2):205-12.

Rudolph U, Möhler H. (2004) Analysis of $GABA_A$ receptor function and dissection of the pharmacology of benzodiazepines and general anesthetics through mouse genetics. Annu Rev Pharmacol Toxicol. 44:475-98.

Rupprecht R, Reul JM, Trapp T, van Steensel B, Wetzel C, Damm K, et al. (1993) Progesterone receptor-mediated effects of neuroactive steroids. Neuron. 11(3):523–30.

Rupprecht R, Holsboer F. (1999) Neuroactive steroids: mechanisms of action and neuropsychopharmacological perspectives. Trends Neurosci. 22(9):410-6.

Ryabinin AE, Galvan-Rosas A, Bachtell RK, Risinger FO. (2003) High alcohol/sucrose consumption during dark circadian phase in C57BL/6J mice: involvement of hippocampus, lateral septum and urocortin-positive cells of the Edinger-Westphal nucleus. Psychopharmacology (Berl). 165(3):296-305.

Saal D, Dong Y, Bonci A, Malenka RC. (2003) Drugs of abuse and stress trigger a common synaptic adaptation in dopamine neurons. Neuron. 37(4):577–82.

Saalmann YB, Kirkcaldie MTK, Waldron S, Calford MB. (2007) Cellular distribution of the $GABA_A$ receptor-modulating 3α-hydroxy,5α-reduced pregnane steroids in the adult rat brain. Journal of Neuroendocrinology. 19(4): 272-284.

Salimov RM, Salimova NB. (1993a) L-glutamate abolishes differential responses to alcohol deprivation in mice. Alcohol. 10(4):251–7.

Salimov RM, Salimova NB. (1993b) The alcohol-deprivation effect in hybrid mice. Drug Alcohol Depend. 32(2):187–91.

Samson HH, Tolliver GA, Pfeffer AO, Sadeghi KG, Mills FG. (1987) Oral ethanol reinforcement in the rat: effect of the partial inverse benzodiazepine agonist RO15-4513. Pharmacol Biochem Behav. 27(3):517–9.

Samson HH, Haraguchi M, Tolliver GA, Sadeghi KG. (1989) Antagonism of ethanol-reinforced behavior by the benzodiazepine inverse agonists Ro15-4513 and FG 7142: relation to sucrose reinforcement. Pharmacol Biochem Behav. 33(3):601-8.

Samson HH, Slawecki CJ, Sharpe AL, Chappell A. (1998) Appetitive and consummatory behaviors in the control of ethanol consumption: a measure of ethanol seeking behavior. Alcohol Clin Exp Res. 22(8):1783-1787.

Samson HH, Chappell A. (2001) Muscimol injected into the medial prefrontal cortex of the rat alters ethanol self-administration. Physiol Behav. 74(4-5):581-7.

Sanna E, Talani G, Busonero F, Pisu MG, Purdy RH, Serra M, Biggio G. (2004) Brain steroidogenesis mediates ethanol modulation of $GABA_A$ receptor activity in rat hippocampus. J Neurosci. 24(29):6521-30

Santhakumar V, Wallner M, Otis TS. (2007) Ethanol acts directly on extrasynaptic subtypes of $GABA_A$ receptors to increase tonic inhibition. Alcohol. 41:211-221.

Schwarzer C, Berresheim U, Pirker S, Wieselthaler A, Fuchs K, Sieghart W, et al. (2001) Distribution of the major γ-aminobutyric $acid_A$ receptor subunits in the basal ganglia and associated limbic brain areas of the adult rat. The Journal of Comparative Neurology. 433(4): 526-549.

Selye H. (1941) On the hormonal activity of a steroid compound. Science. 94(2430):94.

Seo D, Lacadie CM, Tuit K, Hong K-I, Constable RT, Sinha R. (2013) Disrupted ventromedial prefrontal function, alcohol craving, and subsequent relapse risk. JAMA psychiatry. 70(7):727-739.

Serafini R, Bracamontes J, Steinbach JH. (2000) Structural domains of the human $GABA_A$ receptor $\beta_3$ subunit involved in the actions of pentobarbital. J Physiol. 3:649-76.

Sesack SR, Grace AA. (2010) Cortico-Basal Ganglia reward network: microcircuitry. Neuropsychopharmacology. 35(1):27-47.

Shaham Y, Stewart J. (1995) Effects of restraint stress and intra-ventral tegmental area injections of morphine and methyl naltrexone on the discriminative stimulus effects of heroin in the rat. Pharmacology, biochemistry, and behavior. 51(2-3):491-498.

Shaham Y, Shalev U, Lu L, de Wit H, Stewart J. (2003) The reinstatement model of drug relapse: history, methodology and major findings. Psychopharmacology (Berl). 168(1-2):3–20.

Shannon EE, Shelton KL, Vivian JA, Yount I, Morgan AR, Homanics GE, et al. (2004) Discriminative stimulus effects of ethanol in mice lacking the γ-aminobutyric acid type A receptor δ subunit. Alcohol Clin Exp Res. 28(6):906–13.

Shannon EE, Purdy RH, Grant KA. (2005a) Discriminative stimulus effects of 5.6 mg/kg pregnanolone in DBA/2J and C57BL/6J inbred mice. Alcohol. 37(1):35–45.

Shannon EE, Porcu P, Purdy RH, Grant KA. (2005b) Characterization of the discriminative stimulus effects of the neuroactive steroid pregnanolone in DBA/2J and C57BL/6J inbred mice. J Pharmacol Exp Ther. 314(2):675–85.

Shelton KL, Balster RL. (1994) Ethanol drug discrimination in rats: substitution with GABA agonists and NMDA antagonists. Behav Pharmacol. 5(4 - 5):441–51.

Shelton KL, Grant KA. (2002) Discriminative stimulus effects of ethanol in C57BL/6J and DBA/2J inbred mice. Alcohol Clin Exp Res. 26(6):747–57.

Shen H, Gong QH, Yuan M, Smith SS. (2005) Short-term steroid treatment increases δ $GABA_A$ receptor subunit expression in rat CA1 hippocampus: pharmacological and behavioral effects. Neuropharmacology. 49(5):573–86.

Sigel E, Buhr A. (1997) The benzodiazepine binding site of $GABA_A$ receptors. Trends Pharmacol Sci. 18(11):425–9.

Sinclair JD. The alcohol-deprivation effect. (1972) Influence of various factors. Q J Stud Alcohol. 33(3):769–82.

Sinha R. How does stress increase risk of drug abuse and relapse? (2001) Psychopharmacology (Berl). 158(4):343–59.

Sinnott RS, Phillips TJ, Finn DA. (2002a) Alteration of voluntary ethanol and saccharin consumption by the neurosteroid allopregnanolone in mice. Psychopharmacology (Berl). 162(4):438-447.

Sinnott RS, Mark GP, Finn DA. (2002b) Reinforcing effects of the neurosteroid allopregnanolone in rats. Pharmacol Biochem Behav. 72(4):923-9.

Smith AD, Bolam JP. (1990) The neural network of the basal ganglia as revealed by the study of synaptic connections of identified neurones. Trends Neurosci. 13(7):259-65.

Smith BR, Robidoux J, Amit Z. (1992) GABAergic involvement in the acquisition of voluntary ethanol intake in laboratory rats. Alcohol. 27(3):227-31.

Smith SS, Shen H, Gong QH, Zhou X. (2007) Neurosteroid regulation of $GABA_A$ receptors: Focus on the α4 and δ subunits. Pharmacol Ther. 116(1):58-76.

Song M, Messing RO. (2005) Protein kinase C regulation of $GABA_A$ receptors. Cell Mol Life Sci. 62(2):119–27.

Spanagel R, Hölter SM. (1999) Long-term alcohol self-administration with repeated alcohol deprivation phases: an animal model of alcoholism? Alcohol. 34(2):231–43.

Spanagel R, Hölter SM. (2000) Pharmacological validation of a new animal model of alcoholism. J Neural Transm. 107(6):669–80.

Spanagel R. (2009) Alcoholism: a systems approach from molecular physiology to addictive behavior. Physiol Rev. 89(2):649-705.

Spealman RD, Barrett-Larimore RL, Rowlett JK, Platt DM, Khroyan TV. (1999) Pharmacological and environmental determinants of relapse to cocaine-seeking behavior. Pharmacology, biochemistry, and behavior. 64(2):327-336.

Spigelman I, Li Z, Banerjee PK, Mihalek RM, Homanics GE, Olsen RW. (2002) Behavior and physiology of mice lacking the $GABA_A$-receptor δ subunit. Epilepsia. 43 Suppl 5:3-8.

Stell BM, Mody I. (2002) Receptors with different affinities mediate phasic and tonic $GABA_A$ conductances in hippocampal neurons.J Neurosci. 22(10):RC223.

Stell BM, Brickley SG, Tang CY, Farrant M, Mody I. (2003) Neuroactive steroids reduce neuronal excitability by selectively enhancing tonic inhibition mediated by δ subunit-containing $GABA_A$ receptors. Proc Natl Acad Sci. 100(24):14439-44.

Stewart J. (1983) Conditioned and unconditioned drug effects in relapse to opiate and stimulant drug self-adminstration. Prog Neuropsychopharmacol Biol Psychiatry. 7(4-6):591–7.

Stewart J, de Wit H. (1987) Reinstatement of drug-taking behavior as a method of assessing incentive motivational properties of drugs. In Methods of assessing the reinforcing properties of abused drugs. (pp 211-227) New York: Springer-Verlag.

Stratford TR, Kelley AE. (1997) GABA in the nucleus accumbens shell participates in the central regulation of feeding behavior. J Neurosci. 17:4434-4440.

Stratford TR, Wirtshafter D. (2011) Opposite effects on the ingestion of ethanol and sucrose solutions after injections of muscimol into the nucleus accumbens shell. Behavioural Brain Research. 216(2):514-518.

Stórustovu SI, Ebert B. (2006) Pharmacological characterization of agonists at δ-containing $GABA_A$ receptors: functional selectivity for extrasynaptic receptors is dependent on the absence of $\gamma_2$. J Pharmacol Exp Ther. 316:1351-1359.

Stretch R, Gerber GJ. (1973) Drug-induced reinstatement of amphetamine self-administration behaviour in monkeys. Canadian journal of psychology. 27(2):168-177.

Sun C, Sieghart W, Kapur J. (2004) Distribution of α1, α 4, γ2, and δ subunits of $GABA_A$ receptors in hippocampal granule cells. Brain Res. 1029(2):207-16.

Sundstrom-Poromaa I, Smith DH, Gong QH, Sabado TN, Li X, Light A, et al. (2002) Hormonally regulated $\alpha_4\beta_2\delta$ $GABA_A$ receptors are a target for alcohol. Nat Neurosci. 5(8):721-2.

Sur C, Farrar SJ, Kerby J, Whiting PJ, Atack JR, McKernan RM. (1999) Preferential coassembly of α4 and δ subunits of the γ-aminobutyric $acid_A$ receptor in rat thalamus. Mol Pharmacol. 56(1):110–5.

Tanchuck MA, Cozzoli DK, He I, Kaufman KR, Snelling C, Crabbe JC, et al. (2013) Local changes in neurosteroid levels in the substantia nigra reticulata and the ventral tegmental area alter chronic ethanol withdrawal severity in male withdrawal seizure-prone mice. Alcohol Clin Exp Res. 37(5):784–93.

Tateno T, Robinson HPC. (2011) The mechanism of ethanol action on midbrain dopaminergic neuron firing: a dynamic-clamp study of the role of $I_h$ and GABAergic synaptic integration. J Neurophysiol. 106(4):1901–22.

Timby E, Ballard M, Nyberg S, Spigset O, Andersson A, Porankiewicz-Asplund J, et al. (2006) Pharmacokinetic and behavioral effects of allopregnanolone in healthy women. Psychopharmacology (Berl). 186(3):414-24.

Torres JM, Ortega E (2003) Alcohol intoxication increases allopregnanolone levels in female adolescent humans. Neuropsychopharmacology 28:207-1209.

Torres JM, Ortega E. (2004) Alcohol intoxication increases allopregnanolone levels in male adolescent humans. Psychopharmacology (Berl). 172:352-355.

Tretter V, Hauer B, Nusser Z, Mihalek RM, Höger H, Homanics GE, et al. (2001) Targeted disruption of the $GABA_A$ receptor δ subunit gene leads to an up-regulation of $\gamma_2$ subunit-containing receptors in cerebellar granule cells. J Biol Chem. 276(13):10532-8.

Tunstall MJ, Oorschot DE, Kean A, Wickens JR. (2002) Inhibitory interactions between spiny projection neurons in the rat striatum. J Neurophysiol. 88(3):1263-9.

Ungard JT, Beekman M, Gasior M, Carter RB, Dijkstra D, Witkin JM. (2000) Modification of behavioral effects of drugs in mice by neuroactive steroids. Psychopharmacology (Berl). 148(4):336-43.

Usuda I, Tanaka K, Chiba T. (1998) Efferent projections of the nucleus accumbens in the rat with special reference to subdivision of the nucleus: biotinylated dextran amine study. Brain Res. 797(1):73-93.

Valenzuela CF, Bhave S, Hoffman P, Harris RA. (1998) Acute effects of ethanol on pharmacologically isolated kainate receptors in cerebellar granule neurons: comparison with NMDA and AMPA receptors. J Neurochem. 71(4):1777–80.

VanDoren MJ, Matthews DB, Janis GC, Grobin AC, Devaud LL, Morrow AL. (2000) Neuroactive steroid 3α-hydroxy-5α-pregnan-20-one modulates electrophysiological and behavioral actions of ethanol. J Neurosci. 20:1982-1989.

Vanover KE, Suruki M, Huber M, Wilent WB, Carter RB. (2000) Neuroactive steroids attenuate cocaine-induced sucrose intake in rats, but not cocaine-induced hyperactivity in mice. Psychopharmacology (Berl). 149:269-276.

Vashchinkina E, Panhelainen A, Vekovischeva OY, Aitta-aho T, Ebert B, Ator NA, et al. (2012) GABA site agonist gaboxadol induces addiction-predicting persistent changes in ventral tegmental area dopamine neurons but is not rewarding in mice or baboons. J Neurosci. 32(15):5310-20.

Vashchinkina E, Manner AK, Vekovischeva O, Hollander B den, Uusi-Oukari M, Aitta-aho T, et al. (2013) Neurosteroid agonist at $GABA_A$ receptor induces persistent neuroplasticity in VTA dopamine neurons. Neuropsychopharmacology. [Epub ahead of print].

Volkow ND, Li T-K. (2005) Drugs and alcohol: treating and preventing abuse, addiction and their medical consequences. Pharmacol Ther. 108(1):3

Voss J, Sánchez C, Michelsen S, Ebert B. (2003) Rotarod studies in the rat of the $GABA_A$ receptor agonist gaboxadol: lack of ethanol potentiation and benzodiazepine cross-tolerance. Eur J Pharmacol. 482(1-3):215-22.

Waelti P, Dickinson A, Schultz W. (2001) Dopamine responses comply with basic assumptions of formal learning theory. Nature. 412(6842):43–8.

Wallner M, Hanchar HJ, Olsen RW. (2003) Ethanol enhances $\alpha_4\beta_3\delta$ delta and $\alpha_6\beta_3\delta$ γ-aminobutyric acid type A receptors at low concentrations known to affect humans. Proc Natl Acad Sci. 100(25):15218-23.

Wallner M, Hanchar HJ, Olsen RW. (2006) Low dose acute alcohol effects on $GABA_A$ receptor subtypes. Pharmcol Ther. 112:513-528.

Wang X, Wang G, Lemos JR, Treistman SN. (1994) Ethanol directly modulates gating of a dihydropyridine-sensitive Ca2+ channel in neurohypophysial terminals. J Neurosci. 14(9):5453–60.

Waszczak BL, Walters JR. (1980) Intravenous GABA agonist administration stimulates firing of A10 dopaminergic neurons. Eur J Pharmacol. 66(1):141–4.

Wayner MJ, Greenberg I, Carey RJ, Nolley D. (1971) Ethanol drinking elicited during electrical stimulation of the lateral hypothalamus. Physiol Behav. 7(5):793-5.

Weiss F, Lorang MT, Bloom FE, Koob GF. (1993) Oral alcohol self-administration stimulates dopamine release in the rat nucleus accumbens: genetic and motivational determinants. J Pharmacol Exp Ther. 267(1):250-8.

Westerink BH, Enrico P, Feimann J, De Vries JB. (1998) The pharmacology of mesocortical dopamine neurons: a dual-probe microdialysis study in the ventral tegmental area and prefrontal cortex of the rat brain. J Pharmacol Exp Ther. 285(1):143-54.

Wetzel CH, Hermann B, Behl C, Pestel E, Rammes G, Zieglgänsberger W, et al. (1998) Functional antagonism of gonadal steroids at the 5-hydroxytryptamine type 3 receptor. Mol Endocrinol. 12(9):1441-51.

Wick MJ, Mihic SJ, Ueno S, Mascia MP, Trudell JR, Brozowski SJ, et al. (1998) Mutations of γ-aminobutyric acid and glycine receptors change alcohol cutoff: evidence for an alcohol receptor? Proc Natl Acad Sci. 95(11):6504-9.

Wisden W, Laurie DJ, Monyer H, Seeburg PH. (1992) The distribution of 13 $GABA_A$ receptor subunit mRNAs in the rat brain. I. Telencephalon, diencephalon, mesencephalon. J Neurosci. 12(3):1040-62.

Wise RA. (1974) Lateral hypothalamic electrical stimulation: does it make animals “hungry”? Brain Res. 67(2):187-209.

Xia Y, Driscoll JR, Wilbrecht L, Margolis EB, Fields HL, Hjelmstad GO. (2011) Nucleus accumbens medium spiny neurons target non-dopaminergic neurons in the ventral tegmental area. J Neurosci. 31(21):7811-6.

Xiao C, Zhou C, Li K, Ye J-H. (2007) Presynaptic $GABA_A$ receptors facilitate GABAergic transmission to dopaminergic neurons in the ventral tegmental area of young rats. J Physiol. 580(3):731-43.

Yang AR, Liu J, Yi HS, Warnock KT, Wang M, June Jr HL, et al. (2011) Binge drinking: in search of its molecular target via the $GABA_A$ receptor. Front Neurosci. 108:4465-4470.

Yoneyama N, Crabbe JC, Ford MM, Murillo A, Finn DA. (2008) Voluntary ethanol consumption in 22 inbred mouse strains. Alcohol. 42(3):149-60.

Yoshimoto K, McBride WJ, Lumeng L, Li TK. (1992) Alcohol stimulates the release of dopamine and serotonin in the nucleus accumbens. Alcohol. 9(1):17-22.

You H, Dunn SMJ. (2007) Identification of a domain in the δ subunit (S238-V264) of the α4β3δ $GABA_A$ receptor that confers high agonist sensitivity. J Neurochem. 103(3):1092-101.