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Intra-nucleus accumbens shell injections of R(+)- and S(-)-baclofen bidirectionally alter binge-like ethanol, but not saccharin, intake in C57Bl/6J mice

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INTRA-NUCLEUS ACCUMBENS SHELL INJECTIONS OF R(+)- AND S(-)-  
BACLOFEN BIDIRECTIONALLY ALTER BINGE-LIKE ETHANOL, BUT NOT  
SACCHARIN, INTAKE IN C57BL/6J MICE

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For my family, with love.

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## ABSTRACT

Kasten, Chelsea R. M.S., Purdue University, May 2014. Intra-nucleus accumbens shell injections of R(+)- and S(-)-baclofen bidirectionally alter binge-like ethanol, but not saccharin, intake in C57Bl/6J mice. Major Professor: Stephen L. Boehm, II.

It has been proposed that the GABA<sub>B</sub> receptor subtype plays a role in alcoholism and alcohol use disorders (AUDs) (Cousins et al., 2002; Agabio et al., 2012). Specifically, the GABA<sub>B</sub> agonist baclofen has been looked at extensively in clinical and pre-clinical studies. In various animal models of chronic and intermittent consumption, baclofen has been shown to both increase (Petry, 1997; Smith et al., 1999; Czachowski et al., 2006; Moore et al., 2007) and decrease (Colombo et al., 2000; 2002; 2005; Stromberg, 2004; Moore et al., 2009) drinking. A critical issue in determining pharmacological effects of a drug is using the appropriate animal model. The drinking-in-the-dark (DID) model, developed by Rhodes et al. (2005, 2007), produces high levels of drinking in a binge-like paradigm and has been used to assess many pharmacological targets (e.g. Kamdar et al., 2007; Gupta et al., 2008; Moore et al., 2007; 2009).

While DID produces high-levels of binge drinking, it is unclear what areas of the brain are involved in this behavior. A direct way to target areas that are believed to be involved in the circuitry of particular behaviors is through microinjection of drugs (Kiiianmaa et al., 2003). Of particular recent interest involving motivated behaviors and addiction is the nucleus accumbens (Acb) (Everitt & Robbins, 2005); specifically the

accumbens shell (AcbSh) (e.g. Rewal et al., 2009, 2012; Nie et al., 2011; Leriche et al., 2008).

The current study aimed to investigate the role of GABA<sub>B</sub> receptors in the AcbSh by examining the ability of two different enantiomers of baclofen to alter ethanol and saccharin intake in male C57BL/6J (B6) mice. B6 mice underwent bilateral cannulation surgery targeting the AcbSh. After 48 hours of recovery time, animals began a five day Drinking-in-the-Dark (DID) procedure where they received 20% ethanol or 0.2% saccharin for two hours, three hours into the dark cycle, each day. Throughout the five drinking sessions, animals were kept in home-cage locomotor activity chambers to monitor activity throughout the drinking cycle. Day 4 drinking was immediately preceded by a mock microinjection, whereas Day 5 drinking was immediately preceded by a drug microinjection. Microinjection of one of five doses of baclofen was given in ng/side dissolved in 200 µl of aCSF (aCSF alone, 0.02 R(+)-, 0.04 R(+)-, 0.08 S(-)-, or 0.16 S(-)-). Intake was recorded every twenty minutes on Days 4 and 5. Retro-orbital sinus blood samples were taken from ethanol animals immediately following the Day 5 drinking period to determine blood ethanol concentrations (BECs).

A one-way ANOVA on total Day 4 ethanol consumption revealed no baseline differences between dose groups. A one-way ANOVA on total Day 5 ethanol consumption revealed that the 0.04 R(+)- baclofen dose reduced total drinking, but the 0.16 S(-)- baclofen dose increased total drinking ( $p$ 's<0.05). This pattern was reflected in the BECs; 0.04 R(+)- baclofen reduced BECs, whereas 0.16 S(-)- baclofen increased BECs ( $p$ 's<0.05). These results were also time-dependent, with R(+)-baclofen reducing drinking in the first 20 minutes of the session and S(-)- increasing drinking in the last 40

minutes of the session. There were no effects on saccharin intake. An issue with the locomotor activity boxes led to unreliable locomotor activity counts. However, because there were no drug effects on saccharin consumption, it was concluded that locomotor effects did not contribute to the decreases or increases in ethanol consumption. These results further implicate the role of GABAB receptors in modulating ethanol intake. The bidirectional effects shown highlight the importance of considering enantioselective drug effects when interpreting data. Finally, these results also support previous conclusions that the AcbSh plays an important role in modulating use of drugs of abuse, but not other reinforcers.

## CHAPTER 1 INTRODUCTION

### 1.1 Neural Circuitry

GABA and ethanol interactions have been repeatedly supported through a broad spectrum of research. For example, extrasynaptic GABA<sub>A</sub> receptors are known to be sensitive to moderate doses of ethanol (Wallner et al., 2003; Rewal et al., 2009), ethanol affects GABA function through interactions with neural circuits that influence GABA release both pre- and post-synaptically (Breese et al., 2006; Roberto et al., 2003), and that such interactions between GABA and ethanol are regionally specific within the brain (Breese et al., 2006; Rewal et al., 2009; Rewal et al., 2012; Nie et al., 2011; Leriche et al., 2008; Koob et al., 1998).

Generally, the focus of acute ethanol reinforcement has been in areas that are involved in the dopaminergic system and include prevalent GABA receptors. These areas include the mid-brain, basal forebrain, amygdala, and the Acb. Leriche et al. (2008) sought to characterize GABA neurons in the neurocircuitry known to be involved in motivated behavior, including the medial prefrontal cortex, central division of the extended amygdala and the Acb. Male Sprague-Dawley rats were given seven days of intra-gastric distilled water with administration of distilled water or a 2.5 g/kg dose of ethanol on the eighth day. Brains were taken two hours later and Fos-positive cells were counted in six areas of interest. The ethanol animals showed increased Fos-positive cells

as compared to the water and intra-gastric naïve animals in the prelimbic cortex, infralimbic cortex, AcbSh, bed nucleus of the stria terminalis, and lateral central amygdala. The Acb core showed no difference in Fos-positive cells between groups. GAD mRNA hybridization was then done to quantify reactive GABA cells in these areas. In the AcbSh, more than 80% of the Fos-reactive cells were GABAergic, solidifying the important role GABA neurons of the AcbSh play in response to ethanol.

Generally, the Acb is known for its involvement in reward, pleasure, addiction, aggression, and fear (Ikemoto & Panskepp, 1999). The AcbSh is specifically identified as playing an important role in locomotor stimulation directed at acquiring food and drug reinforcers, as well as motivation to obtain reinforcers (Everitt and Robbins, 2005). Multiple neurons, receptors, and neurotransmitters populate the AcbSh. These include a small population (~1-2%) of acetylcholine (ACh) neurons, most of which are interneurons, a large population of dopaminergic neurons, NMDA and AMPA receptors, and GABAergic medium spiny neurons (Di Chiara et al., 1994). Dopaminergic feedback loops between the AcbSh and VTA, Acb core, and dorsal striatum are considered to be important in addiction circuitry, with the Acb receiving dopaminergic projections to its GABAergic cells (Koob et al., 1998) The AcbSh also receives glutamatergic inputs from the prefrontal cortex and hippocampus and GABAergic outputs to the hypothalamus, brain stem, and ventral globus pallidus (Everitt & Robbins, 2005). While Everitt & Robbins (2005) discusses the GABAergic projection from the ventral globus pallidum, first characterized by Dray and Oakley (1978) and further reviewed by Mogenson and Nielsen (1983), the model neglects to show the strong GABAergic projection from the

Acb to the ventral tegmental area (VTA) (Kalivas et al., 1993), The VTA and ventral pallidus have been directly implicated in various addiction paths (Bardo, 1998) (**Figure 1**).

To date, few studies have looked at the ability of directly interfering with GABA in the AcbSh to affect ethanol intake in preclinical models of consumption. The following studies have seen promising results on the ability of intra-AcbSh extrasynaptic GABA<sub>A</sub> receptors to moderate ethanol intake and the reinforcing effects of ethanol. Rewal et al. (2009) interfered with GABA<sub>A</sub> receptors containing the  $\alpha_4$  subunit in the Acb core or shell using a viral microinjection which reduced  $\alpha_4$  expression through mRNA interference. Viral knockdown of  $\alpha_4$  in the Acb shell, but not core, reduced ethanol consumption and active lever pressing as compared to the sham virus injected group. Sucrose consumption and active lever pressing was not altered following viral injections into the core or shell. Nie et al. (2011) saw similar reductions in ethanol drinking following interference of extrasynaptic  $\delta$ -containing GABA<sub>A</sub> receptors from viral vector injections targeting  $\delta$ -mRNA expression. Again, viral knockdown of  $\delta$  in the shell, but not core, reduced ethanol consumption compared to the sham group. These results also did not extend to sucrose consumption, suggesting that the role of the AcbSh in reinforcement seeking is specific to drugs of abuse.

As previously mentioned, GABA is known to be widely prevalent within the Acb, and GABA<sub>B</sub> receptors represent a substantial target. An entire third of the GABA receptors in the Acb are GABA<sub>B</sub> receptors (Bowery et al., 1987). In the rat brain, the Acb has one of the highest levels of  $\mu\text{mol}$  of GABA/g of tissue (Balcom et al., 1975). Within the human brain the Acb falls into middle ground for levels of GABA binding sites

quantified in femtomoles (fmoles) out of regions in the cortex and subcortical nuclei (Lloyd et al., 1977). The cerebellar cortex binds the highest fmoles of GABA per mg of protein at 328.1, the substantia alba binds the lowest at 1.7 fmoles, and the Acb binds 58.1 fmoles. A higher level of GABA terminals in the Acb has also been seen in two strains of alcohol preferring rats versus their respective non-preferring strains eight weeks after the end of a four week drinking period (Hwang et al., 1990). The authors propose that, as long-term GABA fluctuations are not related to drinking itself, it appears that the GABA increase is specific to alcohol preference seen in P and HAD rats as well as B6 mice. However, Hwang et al. (1990) took no baseline measures of GABA. Therefore GABA levels may have increased following drinking or held constant from baseline to post-drinking. This still suggests that there are differences in GABA receptor terminals in the Acb between animals that do and do not prefer ethanol.

All of this information taken together suggests multiple things. 1) There is an important interaction between GABA and ethanol. 2) GABA is widely prevalent in the Acb, with both GABA<sub>A</sub> and GABA<sub>B</sub> receptors being present. 3) Altering extrasynaptic GABA<sub>A</sub> receptors in the AcbSh leads to reductions in drinking. Further, GABA<sub>A</sub> and GABA<sub>B</sub> receptors are known to “cross-talk” to influence each other’s binding properties and signal activities (Balasubramanian et al., 2004). While the mechanisms of this cross-talk are obscure, and a review of the literature is beyond the scope of this project, knowing that GABA<sub>A</sub> and GABA<sub>B</sub> receptors interact further supports the hypothesis that GABA<sub>B</sub> receptors are involved in alcohol consumption. This project sought to identify the role that GABA<sub>B</sub> receptors of the AcbSh play in ethanol consumption, with the

hypothesis that directly altering intra-AcbSh GABA<sub>B</sub> receptor activation via microinjection of a GABA<sub>B</sub> agonist will alter ethanol consumption

### 1.2 Baclofen

The GABA<sub>B</sub> agonist baclofen has been of great interest in treating AUDs, although the only approved uses are as a muscle relaxant and to treat spasticity in neurological disorders (Iversen et al., 2009). GABA<sub>B</sub> receptors are heterodimer complexes comprised of mutually dependent B1 and B2 subunits (White et al., 1998). As a direct agonist, baclofen binds to the B1 subunit of active and inactive receptors as GABA would (Pin et al., 2004; Urwyler et al., 2003). GABA<sub>B</sub> receptors are located pre- and postsynaptically. Presynaptic receptor activation inhibits neurotransmitter release via modulation of high-voltage-activated Ca<sup>++</sup> channels. Such suppression may increase or decrease system inhibition, as autoreceptors are also present that decrease the release of GABA itself. Overall inhibition of neuronal excitability is left to postsynaptic GABA<sub>B</sub> receptors through G-coupled protein activation that leads to K<sup>+</sup> channel activation and down regulation of adenylyl cyclase (Bernard et al., 2001; Agabio et al., 2012).

Baclofen comes in three forms; an R-enantiomer, S-enantiomer, and the racemate, which is a combination of both R- and S-baclofen. While baclofen has been shown to be a promising prospect for treatment of AUD both pre-clinically and clinically, it is very rarely specified which form of baclofen has been used. In the following studies, clinical and NIH trials would have used the racemic compound. Apart from the preliminary data, it is unclear which compounds were used in preclinical drinking models.

In European populations, baclofen rapidly suppresses the alcohol withdrawal syndrome (AWS) and delirium tremens, symptoms which did not recur after

discontinuation of the drug in an open clinical study (Addolorato et al., 2003). Baclofen has also been shown to be just as effective as diazepam in reducing AWS symptoms in alcohol-dependent patients with moderate to severe AWS (Addolorato et al., 2002). Baclofen has been shown to reduce craving and anxiety while increasing alcohol abstinence in alcohol-dependent subjects (Addolorato et al., 2000). Finally, baclofen has been shown to be a safe medication for patients with liver cirrhosis compared to other anti-craving drugs that possibly worsen liver disease, such as naltrexone (Addolorato et al., 2009). None of these studies have addressed whether baclofen in itself is an addictive drug.

While many reviews highlight the inability of baclofen to produce effects in non-European populations, few clinical studies have been done in US populations to investigate baclofen's alleviation of AUD symptoms. One initial open label study found that baclofen was well-tolerated as it reduced anxiety, craving, number of drinks per drinking day, and number of heavy drinking days while increasing number of abstinent days (Flannery et al., 2004). However, these conclusions were drawn from a final sample size of 4 individuals. A much larger US clinical trial run a few years later showed once again that baclofen was well-tolerated, but was unable to increase the number of abstinent days in heavy drinkers, time to first drink during drinking bouts, or time to relapse to heavy drinking over the placebo condition (Garbutt et al., 2010). Conversely, one small clinical study showed the ability of baclofen to reduce alcohol withdrawal symptoms in hospital inpatients at high risk for AWS (Lyon et al., 2011). Current FDA trials include recruiting participants to test the safety and efficacy of baclofen for treating alcohol withdrawal symptoms, abstinence during and after treatment, and to reduce heavy

drinking. Baclofen is also being investigated for use as a combination therapy with lorazepam to reduce withdrawal symptoms, and as a combination with naltrexone to reduce symptoms that naltrexone does not treat, like anxiety and irritability (clinicaltrials.gov; search term “lioresal”).

Pre-clinically, baclofen has been shown to reduce the “alcohol deprivation effect” in sP rats (Colombo et al., 2003; 2006), reduce operant responding and amount of self-administered alcohol in fixed ratio operant paradigms (Anstrom et al., 2003; Janak et al., 2003; Besheer et al., 2004; Maccioni et al., 2005; Liang et al., 2006; Walker et al., 2007), lower the breakpoint of responding for ethanol in a progressive ratio procedure in alcohol preferring rats (Maccioni et al., 2008), and suppress extinction responding via systemic injections (Colombo et al., 2003). Studies looking specifically at baclofen’s effect on alcohol intake have shown mixed results. Decreases (Colombo et al., 2000; 2002; 2005; Stromberg, 2004) and increases (Petry, 1997; Smith et al., 1999; Czachowski et al., 2006) have been seen in drinking following acute or chronic systemic injections of baclofen in rodents. Our lab has previously seen an increase of consumption in B6 mice with acute systemic injections of baclofen (Moore et al., 2007). We have also seen a decrease in drinking when baclofen was microinjected into the anterior VTA, but no effect when injected into the posterior VTA (Moore et al., 2009).

As mentioned, while these studies have all used baclofen, they do not specify which isomer of baclofen has been used. The racemic compound of baclofen, used in clinical studies and approved for use for anti-spasticity, can be separated into two different enantiomers. The R-enantiomer is 100 times more active than the S-enantiomer (Spahn et al., 1987; Zhang et al., 2011). Many chemical studies looked at the synthesis of

each baclofen compound, yet there are not many studies that quantify what each compound does behaviorally. Olpe et al. (1978) sought to compare the R- and S-enantiomers with the racemate of baclofen in multiple tests. They found that the racemate and the R-enantiomer of baclofen dose-dependently inhibited the patellar, flexor, and linguo-mandibular reflexes and lowered blood pressure in cats. The S-enantiomer had no effects on reflexes even at the highest doses, and had minimal effects on blood pressure. The racemic baclofen was actually less potent than the R-enantiomer at reducing the patellar reflex, whereas racemic and R-baclofen were equipotent at reducing the flexor and linguo-mandibular reflexes. Both the racemate and R-enantiomer of baclofen dose-dependently protected mice from electroshock-induced seizures, while S-enantiomer doses of up to 100 mg/kg had no effect on convulsions. Further, Paredes and Agmo (1989) showed S-baclofen to be ineffective at inhibiting sexual behavior, even when given at a dose 40 times higher than the minimally effective R-baclofen dose. The racemic compound was half as active as the R- enantiomer, yet both reduced sociosexual-specific locomotor activity and motor execution while S-baclofen remained completely inactive. Fromm et al. (1990) showed that it takes a S-baclofen dose 20 times larger than the least effective R-baclofen dose to depress response of the trigeminal nucleus oralis and that S-baclofen actually reduces response to R-baclofen in the periphery, but not the CNS.

Such results question the effects of the S-enantiomer, as it appears to have no effect, lowered effects, or even antagonistic effects on various behaviors and reflexes. Olpe et al. (1978) suggest that the less active S-enantiomer may play a role in potentiating the action of the R-enantiomer without being active itself, yet Fromm et al. (1990) show that not only is S-baclofen active at higher doses, they also believe it inhibits

the actions of R-baclofen in certain cases. The enantioselective actions of baclofen are different depending on the behavior being observed, highlighting the need to consider enantioselective aspects of drug action when considering therapeutic uses of the drug (Jamali et al., 1989) and choosing an appropriate model of behavior to therapeutically test drugs (Fromm et al., 1990).

Preliminary studies within our lab have shown an effect of both R(+)- and S(-)-baclofen on ethanol and saccharin consumption. B6 mice were exposed to a 20% ethanol solution for two hours, three hours into the dark cycle, for 5 days. On Day 4 a habituation saline injection was given and on Day 5 a dose of baclofen was given. Animals were matched by intake for dose groups on Day 5. Injections took place immediately before bottles on, with intake readings at 1hr and 2hrs on Days 4 and 5. Retro-orbital sinus blood samples were collected on Day 5 immediately after access. Dose-responses determinations were also performed using a 0.32% saccharin solution in place of the 20% ethanol solution.

R(+)- baclofen was given in doses of 0, 1, 3, or 10mg/kg. Following a significant one-way ANOVA, Dunnett's post-hoc tests revealed the 10mg/kg dose of baclofen reduced total 2hr ethanol intake and ethanol intake in the first hour compared to the 0mg/kg group ( $p$ 's < .05). This reduction of intake was reflected in the pattern of blood ethanol concentrations (BECs), with the 10mg/kg dose reducing BECs ( $p$  < .05). BECs were strongly correlated with total 2 hour ethanol intake;  $r(39) = .922$ ,  $p < .001$  (data not shown). R(+)- baclofen also reduced total 2hr saccharin intake at the 10mg/kg dose ( $p$  < .05). This dose effect was not specific to hour; the 10mg/kg dose reduced intake at 1hr and 2hr readings ( $p$ 's < .05).

S(-)- baclofen was given in a dose of 0, 1, 3, 10, or 30mg/kg on Day 5 of drinking. The higher 30mg/kg dose was included due to the lower potency of the S(-)-baclofen enantiomer. Whereas R(+)-baclofen decreased ethanol intake, S(-)-baclofen increased ethanol consumption in the second hour of access at the 3 and 10mg/kg dose ( $p < .05$ ), and trended towards increasing intake at the 1mg/kg dose ( $p = .05$ ). No significant effects on total ethanol drinking or BECs were evident. Also contrary to R(+)-baclofen, S(-)-baclofen did not affect total or hourly saccharin intake . These results show that the baclofen enantiomers work in opposing directions while altering ethanol intake.

### 1.3 Drinking-in-the-Dark

To target the neural circuitry that may be involved in this drinking phenomenon, microinjection work was carried out using the same DID paradigm used in the preliminary data. This model offers many advantages and few disadvantages. DID is a method that achieves high levels of drinking in a binge-like paradigm by introducing ethanol three hours into the dark cycle with a two hour access period (Rhodes et al., 2005; 2007). The DID paradigm allows for many advantages over 24-hour access drinking. In a relatively short period of time B6 mice reliably ingest enough ethanol to reach pharmacologically relevant BECs of over 1mg/ml, as evidenced in the preliminary data. These levels are met without having to implement food or water restriction. This, combined with the simplicity of the design, allows the experimenter to easily assess drinking in a high volume of animals quickly and efficiently while collecting reliable and meaningful data (Rhodes et al., 2005; 2007). Ethanol intake using DID procedures is moderately to strongly correlated with two-bottle choice preference drinking, ethanol-

induced hypothermia, and locomotor activity, and negatively correlated with conditioned taste aversion (Rhodes et al., 2007)

Because DID produces pharmacologically relevant ethanol intakes and behavioral and physiological changes it has been used as a model to screen potential AUD treatments. Kamdar et al. (2007) used the DID paradigm to show that naltrexone reduced ethanol drinking, but not water or sugar water consumption, while the dopaminergic drug GBR 12909 reduced ethanol and sugar water consumption. The glutamatergic drugs acamprosate and MPEP have been shown to dose-dependently reduce ethanol drinking while only reducing water and sugar water intake at higher doses in the DID paradigm (Gupta et al., 2008). The GABA system has also been shown to play a role in fluid intake under DID procedures. The GABA<sub>A</sub> agonists gaboxadol (THIP) and muscimol have been shown to reduce ethanol and water intake, while the GABA<sub>B</sub> agonist baclofen specifically increased ethanol consumption without affecting water intake (Moore et al., 2007).

The current study aimed to investigate the role of GABA<sub>B</sub> receptors in the AcbSh in ethanol consumption in B6 mice. This goal was undertaken by establishing DID, then giving microinjections of either R(+)- or S(-)- baclofen directly into the AcbSh with the hypotheses that R(+)- baclofen would decrease ethanol consumption and S(-)- baclofen would increase ethanol consumption. I elaborate further on these hypotheses in the specific aims section.

#### 1.4 Specific Aims

1. *Reduce established drinking by microinjecting R(+)- baclofen into the AcbSh.*

Preliminary data have shown that R(+), the more potent enantiomer of the baclofen

racemate, decreases ethanol and saccharin consumption following acute systemic injection (unpublished data).

2. *Increase established drinking by microinjecting S(-)- baclofen into the AcbSh.*

Preliminary data have shown that S(-), the less potent enantiomer of the baclofen racemate, increases ethanol, but not saccharin, consumption following acute systemic injection (unpublished data).

## CHAPTER 2 EFFECTS OF INTRA-ACBSH BACLOFEN ON ETHANOL AND SACCHARIN INTAKE AND LOCOMOTION

### 2.1 Materials and Methods

#### 2.1.1 General Design

The design of this project is shown in Table 1. In brief, male B6 mice underwent surgery for bilateral cannulation of the AcbSh. Following 48 hours or longer of recovery, mice began a five-day DID procedure where 20% ethanol or 0.2% saccharin was available for two hours, three hours into the dark period each day. On Day 5, animals received one of five possible microinjection doses; 0.02 or 0.04 $\mu$ g R(+)-baclofen, 0.08 or 0.16 $\mu$ g S(-)-baclofen, or artificial cerebrospinal fluid (aCSF) (Fig. 2).

#### 2.1.2 Subjects

Male B6 mice were bred in-house. Breeders for our colony were obtained from Jackson Labs (Bar Harbor, ME) and replaced every few months by new breeder pairs purchased from Jackson Labs. A total of 127 animals were used in this study. Within each reinforcer group (saccharin or ethanol) the same aCSF control group (dose = 0.0  $\mu$ g) was used for each drug (R- or S- baclofen) to reduce the number of animals used in this project (see **Fig. 2**).

Animals received food at all times and water ad lib apart from during implementation of DID. Lights were kept on a reverse light-dark schedule with lights off at 8am. Animals were group housed until the time of surgery, after which time they were

individually housed. Animals were at least 58 days of age at the time of surgery, and at least 60 days of age at the time DID started. Procedures were approved by the IUPUI School of Science Institutional Animal Care and Use Committee and conformed to the Guide for the Care and Use of Laboratory Animals (The National Academic Press, 2003).

### 2.1.3 Drugs

Ethanol (190 proof) was obtained from Pharmco, Inc (Brookfield, CT). The 20% v/v ethanol solution was prepared with tap water. Saccharin ( $\geq 99\%$ ) was obtained from Sigma Aldrich (St. Louis, MO) and was dissolved in tap water to make a 0.2% saccharin solution. R(+)- and S(-)- baclofen was obtained from Sigma Aldrich (St. Louis, MO). Baclofen was dissolved in aCSF (Sigma Aldrich, St. Louis, MO) to doses of 0.02 and 0.04  $\mu\text{g}$  for R(+)-baclofen, and 0.08 and 0.16  $\mu\text{g}$  for S(-)-baclofen. Justification of these doses is discussed below. Unadulterated aCSF was used as the 0.0  $\mu\text{g}$  control group. Drug was delivered in a volume of 200 nl per side with a flow rate of 382 nl/min.

Our goal in choosing doses for this study was to find the highest drug dose that did not cause an effect on ethanol intake, as well as the lowest dose that did cause an effect on ethanol intake. Therefore, all doses were piloted in ethanol drinking animals following the same methodology discussed below.

Initial R(+)-baclofen doses for this study were based on those used by Boehm et al. (2002), which were chosen for their ability to cause ethanol-induced locomotion when ethanol was given via i.p. injections, as well as Moore et al. (2009), which showed that both a 0.01 and 0.02  $\mu\text{g}$  dose of baclofen inject into the anterior VTA were able to reduce drinking. Three doses of R(+)-baclofen were piloted ( $n=3$  for each pilot group); 0.01, 0.02, and 0.04  $\mu\text{g}/\text{side}$ . The 0.04  $\mu\text{g}/\text{side}$  dose appeared to reduce drinking, and was

therefore kept as a final dose. Neither 0.01 nor 0.02  $\mu\text{g}/\text{side}$  appeared to reduce drinking, so 0.02  $\mu\text{g}/\text{side}$  was chosen as the secondary dose because it was the highest dose to not produce an effect.

S(-)-baclofen doses were expected to be higher than R(+)-baclofen doses, because S(-) is the less potent enantiomer. Piloting appropriate doses for S(-)-baclofen began by using the highest agreed-upon dose of 0.32  $\mu\text{g}/\text{side}$  (n=3). This dose appeared to incapacitate the animals, and was therefore cut in half to 0.16  $\mu\text{g}/\text{side}$ . The 0.16  $\mu\text{g}/\text{side}$  appeared to potentially increase drinking in the pilot group (n=3), and was kept as one of the two final doses. To choose the second final dose, 0.08  $\mu\text{g}/\text{side}$  and 0.24  $\mu\text{g}/\text{side}$  doses were piloted (n=3 for each group). The 0.24  $\mu\text{g}/\text{side}$  did not appear to increase ethanol intake more than the 0.16  $\mu\text{g}/\text{side}$  dose, therefore the 0.08  $\mu\text{g}/\text{side}$  dose was chosen because it appeared to be the highest dose that would not affect intake.

These pilot doses led to final doses of 0.02 and 0.04  $\mu\text{g}/\text{side}$  of R(+)-baclofen and 0.08 and 0.16  $\mu\text{g}/\text{side}$  of S(-)-baclofen. Animals used to pilot the final doses were included in the final group sizes for these doses.

Dose groups were randomly assigned when possible. Due to microinjection pump availability, a maximum of two dose groups could be tested at a time.. When five or more animals were tested, they were split into two dose groups that were matched by Day 4 drinking. Animals were tested in every dose group before groups were filled out. Means for Day 4 drinking were kept constantly updated to make sure that baseline drinking did not differ as animals were being assigned groups throughout the course of the study.

#### 2.1.4. Surgery

The shell of the Acb was targeted (coordinates of X:  $\pm 0.63$ , Y: 1.18, Z: 2.0) through bilateral cannulation. Surgical procedures followed those currently outlined in our approved IACUC protocol, as described in Moore et al. (2009). Briefly, mice were anesthetized using a ketamine/xylazine cocktail (1mL ketamine, 0.1 mL xylazine, and 8.9 mL sterile saline). Once anesthetized, the animal was placed in a Kopf stereotaxic alignment system (Tujunga, CA). The animal's head was shaved and a small incision made from approximately bregma to lambda. The skull was sterilized with 100% ethanol and Nolvasan three times. Animals' eyes were kept moist with commercially available tear ointment. For accuracy, the distance between bregma and lambda was measured and divided by 4.21mm, the published average distance between lambda and bregma for B6 mice (Frankling and Paxinos, 1997). The previously mentioned coordinates were then adjusted by that percentage and two holes were drilled at the adjusted coordinates for bilateral placement of guide cannula. A third hole was drilled for placement of a screw to anchor the skull cap, which was placed after the cannulae were simultaneously lowered into the brain to a depth of 2mm. After placement of the cannula and screw. Stoelting clear dental cement (Fisher Science, Hampton, NH) was applied to the exposed skull to secure the guide cannulae and protect the skull. After the cement dried, animals were placed in a cage (with a special flat top to accommodate the cannulae) on a heating pad until they recovered from the anesthesia. Bedding, food, and water were reintroduced once the animal was up and moving then the animals was returned to the reverse light-dark room.

### 2.1.5. DID Procedures

Following surgery, animals had at least 48 hours of recovery before the introduction of DID. The length of recovery time was varied between 48 hours to six days for the first few cohorts tested, and it was found that 48 hours of recovery led to optimal consumption during DID following surgery. During the recovery time, the cannulae stylets were removed daily to prevent obstruction. While daily restraint was necessary to move the stylets, timed restraint to habituate the animal to the microinjection procedure began on Day 3 of DID (Table 1). Stylet removal and restraint occurred immediately prior to bottles-on time to acclimate animals to the restraint stress of the microinjection.

All drinking took place in home-cage locomotor boxes (Opto M3 13" x 9" Mouse Cages, Columbus Instruments, Columbus, OH). DID procedures were slightly modified from that of Rhodes et al. (2005, 2007) and Moore et al. (2007). Three hours into the dark cycle animals received two hours of access to either a 20% ethanol solution or a 0.2% saccharin solution in 10mL ball-bearing sipper tubes for five days, with access to only water for the remaining 22 hours. The saccharin group was included to act as a control group for drug effects on alternative reinforcers. The home cage locomotor boxes were used to account for competing locomotor sedation. As previously mentioned, baclofen shows a sedative effect in ethanol-naïve animals (Cryan et al., 2004). Using the home-cage monitors allowed a count of the animals' movement to speak to any effects of sedation by allowing characterization of the animals' typical movement patterns and pattern of movement following drug injection.

Restraint took place on Day 3 and mock microinjections took place on Day 4. Drug was administered via microinjection on Day 5. In aim 1, R(+)- baclofen was microinjected with the hypothesis that it would reduce ethanol consumption. In aim 2, S(-)- baclofen was microinjected with the hypothesis that it would increase ethanol consumption. On Days 1-3, fluid volumes were recorded immediately before and after fluid access. On Days 4 and 5, fluid volumes were recorded immediately before and after fluid access, as well as every 20 minutes during fluid access to capture the time course of the drug effects on Day 5.

#### 2.1.6. Intra-AcbSh Microinjections

Guide cannulae, stylets and tubing to make microinjectors were obtained from Small Parts Inc. (Miami Lakes, FL). Guide cannulae were made of 25-gauge stainless steel tubing, cut to 15 mm. Stainless steel wire (0.0095 inch) was used to make stylets to be placed inside the guide cannulae to prevent obstruction. Microinjectors were made from two sections of stainless steel tubing: a 30-mm section of 32-gauge tubing was inserted into a 30-mm section of 25-gauge tubing so that 18 mm extended. The two pieces were soldered together and checked for leaks.

Two segments of PE-20 tubing were attached to each microinjector and loaded with aCSF or one of the four baclofen doses. The other ends of the tubing were fitted over two 10- $\mu$ l Hamilton glass syringes (Hamilton Co., Reno, NV, USA) filled with distilled water. The syringes were placed on a Cole-Parmer (74900-Series) dual infusion pump. For the microinjection, mice were grasped by the scruff of the neck using the thumb and forefinger of the left hand. The microinjectors were slowly inserted and held in place by the thumb and forefinger of the right hand, extending 3mm past the guide

cannula to hit the AcbSh. Fluid was microinjected at a rate of 382 nl/min, which took about 40 seconds to inject the 200nl of fluid. Microinjectors were held in place for another 30 seconds to allow time for the drug to diffuse from the microinjector tips. The microinjectors were then slowly removed and animals were placed back into their cages.

#### 2.1.7. Blood Ethanol Concentrations

On the fifth day of access, retro-orbital sinus blood samples were collected from every animal given access to ethanol immediately following bottle removal using 50  $\mu$ L heparinized microcapillary tubes to verify intake. Samples were immediately placed in the centrifuge and plasma and decanted. The samples were stored at  $-80^{\circ}\text{C}$  until time of assay, at which point they were analyzed with an Analox Alcohol Analyzer (Analox Instruments, Lunenburg, MA) to determine BEC.

#### 2.1.8. Histological Verification of Cannulae Placement

Immediately following the end of drinking on Day 5, animals were euthanized by cervical dislocation. Brains were extracted and flash-frozen using methylbutane ( $-25^{\circ}\text{C}$ ). Brains were stored in the  $-20^{\circ}\text{C}$  freezer until they were sliced using a cryostat, thaw-mounted on slides, thionin stained, and cover slipped. Placement of cannulae was inspected and verified by two different blind experimenters.

#### 2.1.9. Statistical Analysis

Statistical analyses only included animals with histologically verified bilateral hits of the AcbSh (detailed in Tables 2 and 3). Ethanol and saccharin results were analyzed separately. Within the ethanol and saccharin groups, R(+)- and S(-)- baclofen were analyzed together because they shared a control group. Significance level for all overall

analyses was set at  $p < .05$ , with all post-hoc tests being corrected for multiple comparisons.

Drinking acquisition on Days 1-4 was assessed using a one-way analysis of variance (ANOVA) with days as the within-subject factor. Tukey's post-hoc tests were used when warranted. A one-way ANOVA was used to analyze total Day 4 drinking and locomotor activity with dose as the between-subjects factor to verify that total baseline drinking and locomotion following mock microinjection did not vary between groups. Following this verification, dose effects on total Day 5 drinking and locomotion were assessed using a one-way ANOVA with dose as the between-subjects factor. For the ethanol animals, a one-way ANOVA with dose as the between-subjects factor was also used to assess dose effects on BEC following intake, as well as a bivariate Pearson's correlation between total intake and BEC. Post-hoc independent samples t-tests were used for all necessary post-hoc tests on total 2hr consumption and BEC data.

To assess for dose-effects on time course of intake and locomotion, a two-way repeated measures ANOVA with dose as the between-subjects factor and time as the within-subjects factor was used. Day 4 intake and locomotion was assessed first to verify that there were no baseline differences in time course between each dose group. Following this verification, Day 5 data was assessed. One-way ANOVAs were used at each time-point with dose as the between subjects factor as post-hoc tests for a significant two-way ANOVA. Tukey's post-hoc tests were used for any significant one-way ANOVA effect.

Due to a problem with the locomotor equipment that was discovered midway through the experiments (discussed below), more analyses were used to properly assess

the locomotor activity data. Total Days 1-5 locomotion for each dose independently was analyzed by a one-way ANOVA, with Tukey's used as the post-hoc test. A two-way repeated measures ANOVA with Day and time-bin as within-subjects factors was used to analyze Day 4 and Day 5 locomotion for each dose independently. This analysis was also used to compare drinking data to locomotor data. Paired samples t-tests were used as post-hocs for repeated measures tests.

## 2.2 Results

### 2.2.1. Ethanol Consumption

Animal usage is detailed in **Table 2**. Seventy-eight total animals were included in all aspects of the ethanol portion of this study. Out of those animals, 52 completed the study and were in the appropriate dose groups, with 44 of those animals being successful hits, giving a hit rate of 84.62% within this subset of animals. Only the 44 successful animals are included in the following analyses.

The ANOVA on Days 1-4 acquisition of drinking revealed a significant effect;  $F(3,175) = 3.21, p < .05$  (**Fig. 3A**). Tukey's post-hoc revealed that Day 2 drinking was significantly higher than Day 3 drinking ( $p < .05$ ). Total Day 4 drinking following the mock injection did not differ between groups;  $F(4,43) = .322, p > .05$ , data not shown. There were significant dose effects on total Day 5 ethanol intake following the microinjection;  $F(4,43) = 10.14, p < .001$ . Independent samples t-tests corrected for multiple comparisons revealed that the 0.04  $\mu\text{g}$  R(+)-baclofen group drank significantly less than the aCSF group, whereas the 0.16  $\mu\text{g}$  S(-)-baclofen group drank significantly more than the aCSF group ( $p$ 's  $< .05$ ) (**Fig. 3B**). This pattern was replicated in the BEC data, with the 0.04 R(+)- baclofen group showing significantly lower BECs than the

aCSF group, while the 0.16 S(-)- baclofen group showed significantly higher BECs immediately following ethanol intake ( $p$ 's < .05) (**Fig. 3C**). BECs and total two hour ethanol consumption were strongly correlated;  $r(43) = 0.834, p < .001$  (**Fig. 3D**).

The time course of ethanol intake was then statistically compared. For both analyses, Greenhouse-Geisser statistics are reported because Mauchly's Test for Sphericity was significant ( $p$ 's < .05) A dose\*time bin repeated measures ANOVA on Day 4 time course intake verified that there were no baseline differences between dose groups;  $F(11.38, 110.90) = 1.169; p > .05$  (**Fig. 4A**). The time course of ethanol intake following micro-injection on Day 5 was significantly different between dose groups;  $F(16.12, 153.18) = 1.931, p > .05$ . One-way ANOVAs at each time-point revealed that differences between doses existed at the 0 to 20, 81 to 100, and 101 to 120 minute time bins ( $p$ 's < .05). Tukey's post-hocs revealed that at 0 to 20 minutes. The 0.04  $\mu\text{g}$  R(+)- group drank significantly less than the aCSF group ( $p < .05$ ). At 81 to 100 minutes. The 0.16 $\mu\text{g}$  S(-) group drank significantly more than the 0.02 and 0.04  $\mu\text{g}$  R(+)- groups ( $p$ 's < .05). At the 101 to 120 minute time point, the 0.16  $\mu\text{g}$  S(-)- group drank significantly more than the aCSF, 0.02, and 0.04  $\mu\text{g}$  R(+)- groups ( $p$ 's < .05) (**Fig. 4B**).

While the following statistical tests were not agreed upon in the project proposal, repeated measures ANOVAs analyzing the time course of drinking between Day 4 and Day 5 for each dose independently were used to confidently compare drinking to locomotor activity. The ANOVAs revealed no main effects of time or day, nor was there an interaction for the aCSF and 0.16 S(-)- groups ( $p$ 's > .05) (**Fig. 5A & 5E**). The 0.02 R(+)- group showed a main effect of day and time, with animals drinking less on the microinjection day and drinking being lower in the middle of the drinking session ( $p$ 's

< .05). There would have been an effect of day\*time bin, except Mauchly's Test for Sphericity was significant ( $p = .049$ ) and Greenhouse-Geisser statistics indicated no interaction was present;  $F(1.99,15.918) = 2.99, p = .079$  (**Fig. 5B**). There was a main effect of day for the 0.04 R(+)- group, with animals drinking less overall on the microinjection day ( $p < .05$ ). There was no main effect of time ( $p > .05$ ). Again, a significant interaction of Day\*Time bin was reduced by a significant Mauchly's Test for Sphericity ( $p < .05$ ), with the Greenhouse-Geisser correction not being significant;  $F(1.858,13.077) = 3.591, p = .06$  (**Fig. 5C**). The 0.08 S(-)- group displayed no main effect of day, but a main effect of time, with higher drinking in the first and last 20 minute bins ( $p < .05$ ). There was an interaction of day\*time;  $F(5,40) = 3.635, p < .01$ . Post-hoc, paired samples t-tests revealed that drinking on the microinjection day was significantly lower only at the 0-20 minute point;  $t(8) = 4.218, p < .01$  (**Fig. 5D**).

### 2.2.2. Ethanol Locomotion

Midway through the experiment a malfunction in the Opto M3 home-cage activity monitors was noticed. The malfunction appeared to be a degradation in the equipment, with photobeam scanners slowly failing over time, resulting in inaccurate locomotor counts, with distance travelled being lower than what it should have been because broken photobeams were not registering locomotor counts. The slow breakdown of the system eventually resulted in complete failure, at which time a representative from Columbus Instruments came to replace a majority of the system. All of the ethanol aCSF, R(+)- 0.02, and R(+)- 0.04 animals as well as three of the S(-)- 0.16 animals, were tested before the equipment was fixed. Four S(-) 0.08 animals do not have locomotor data because the activity boxes had completely failed at the time of their testing. For the saccharin animals,

six aCSF, five R(+)- 0.02, and five R(+)- 0.04 animals were tested before the activity boxes were fixed. Further, because the failure was time dependent, the closer an animal was tested to the point of complete system failure, the more inaccurate their locomotor count may have been. Therefore, there is low confidence in the accuracy of those activity counts, especially as there is no way to account for when the system started to degrade and how it affected activity counts over time. Because of this issue, statistical analyses that focused on each independent dose group were used to minimize the variance due to equipment error in the data. A one-way ANOVA on Days 1-5 drinking, as well as a repeated measures ANOVA on Day 4 and 5 time course drinking, analyzed effects each dose independently. These analyses minimized equipment error variance by focusing on the *relative* difference between a few days' time, where there would be minimal changes in equipment malfunction. In other words, comparing all Day 5 locomotion back to the previous day's locomotor activity accounts for the *absolute* differences in locomotor scores between when the equipment was and was not working properly.

As an example, a group tested before the equipment was fixed may have had a Day 4 score of 900 counts and a Day 5 score of 800 counts. A group tested after the fix may have had a Day 4 count of 12,000 and a Day 5 count of 11,900. So the absolute difference between groups on Day 5 is likely to be inaccurate at 11,000 counts, whereas the relative differences between each group for Day 4 and Day 5 is more accurate at 100 counts each. Although the statistical analyses analyzed within groups will be used for discussion of the overall findings because they are the most credible, between dose groups differences were statistically analyzed to uphold the expectations of the committee.

One-way ANOVAs showed no differences between dose groups on total Day 4 baseline locomotor scores or total Day 5 test locomotor scores ( $p$ 's > .05), data not shown. The day 4 locomotion dose\*time bin ANOVA revealed no baseline differences between dose on the time course of locomotor activity. There were no main effects of time or dose ( $p$  > .05), data not shown. Using Greenhouse-Geisser statistics due to a significant Mauchly's Test for Sphericity ( $p$  < .001), there was not a significant interaction between time bin and dose or main effects on Day 5 locomotion ( $p$ 's > .05) (**Fig. 7A**).

ANOVAs on Day 1-5 locomotor data for each dose revealed a significant effect of day for the aCSF, R(+)- 0.02, and S(-)- 0.08 dose groups ( $p$ 's < .01). Total locomotion on each day for the R(+)- 0.04 and S(-)- 0.16 groups was not significantly different (**Figs. 6C and 6E**). Tukey's post-hoc tests revealed that for the aCSF group, locomotion on Days 4 and 5 was significantly lower than on Day 1 ( $p$ 's < .05) (**Fig. 6A**). For the R(+)- 0.02 group, locomotion on Days 3, 4, and 5 was significantly lower than Day 1 and 2 locomotion ( $p$ 's < .05) (**Fig. 6B**). For the S(-)- 0.08 group, locomotion on Day 3 was lower than on Day 1, and Day 5 locomotion was lower than Days 1 and 2 (**Fig. 6D**).

To confidently assess the time course of locomotor behavior, Day 4 time course locomotion was compared to Day 5 time course locomotion. There were no significant day\*time-bin interactions or main effects of time or dose for the aCSF, S(-)- 0.08, or S(-)- 0.16 dose groups (**Figs. 7B, 7E, and 7F**, respectively). There was no significant main effect of time or day, but a significant interaction of day\*time-bin for the R(+) 0.02 dose group;  $F(5,40) = 3.014$ ,  $p < .05$ . Post-hoc paired samples t-tests revealed that locomotor activity on the microinjection day (Day 5) was significantly lower than on the mock injection day (Day 4) only in the first 20-minute bin (**Fig. 7C**). There were no other

significant differences at each time-bin ( $p$ 's  $> .05$ ). There was no significant main effect of time or day, but a significant interaction of day\*time-bin for the R(+)- 0.04 dose group;  $F(1.98,15.84) = 4.448, p < .05$ . Greenhouse-Geisser statistics are reported because Mauchly's Test for Sphericity was significant ( $p = .001$ ). Post-hoc paired samples t-tests revealed that there were significant differences in locomotor activity during the 0-20 and 21-40 minute time bins, with the animals moving less on the microinjection day ( $p$ 's  $< .05$ ) (**Fig. 7D**).

### 2.2.3. Saccharin Consumption

Animal usage is detailed in **Table 3**. Forty-nine total animals were included in all aspects of the saccharin portion of this study. Out of those animals, all completed the study and were in dose groups included in the study. Of the 49 animals, 42 were successful bilateral hits of the AcbSh, giving a hit rate of 85.71% within the saccharin subset of animals. Only the successfully hit animals were included in the following analyses.

The ANOVA on Days 1-4 acquisition data revealed no significant effect of day on acquisition of drinking;  $F(3,45) = 2.206, p > .05$  (**Fig. 8A**). Total two-hour saccharin drinking on Day 4 revealed no baseline differences in drinking between groups;  $F(4,41) = 2.043, p > .05$ , data not shown. There were no effects of drug dose on total Day 5 drinking;  $F(4,41) = 0.981, p > .05$  (**Fig 8B**).

The time course of saccharin intake was then statistically compared using a two-way repeated measures ANOVA on Day 4 and Day 5 dose\*time bins. There were no baseline differences in the time course of saccharin intake on Day 4;  $F(13.85,128.14) = 1.194, p > .05$  (**Fig. 9A**). Further, there were no differences in time course of intake on

Day 5;  $F(14.89,137.74) = 1.096$ ,  $p > .05$  (**Fig. 9B**). Greenhouse-Geisser statistics are reported for both of these repeated measures ANOVAs, as Mauchly's Test of Sphericity was significant for each test ( $p$ 's  $< .01$ ).

Although there was no dose\*time bin interaction, repeated measures ANOVAs were used to mirror locomotor activity analyses comparing Day 4 and Day 5 time course of drinking did reveal significant effects. The aCSF group displayed a main effect of day and time on drinking ( $p$ 's  $< .05$ ), with consumption being lower on Day 5 and drinking being lower as time went on (**Fig. 10A**). There was no interaction between day\*time bin ( $p > .05$ ). The 0.02 R(+) group showed no effect of day or an interaction of day\*time, but showed a main effect of time ( $p < .05$ ) (**Fig. 10B**). The 0.04 R(+)- group had no main effects or interactions ( $p$ 's  $> .05$ ) (**Fig. 10C**). The 0.08 S(-)- and 0.16 S(-)- groups revealed only an effect of day ( $p < .05$ ), with animals consuming less saccharin following the microinjection versus the mock injection (**Fig. 10D and 10E**, respectively).

#### 2.2.4. Saccharin Locomotion

As previously discussed, locomotor data was analyzed in two separate ways – to satisfy the agreed-upon statistics for this project as well as accurately characterize any changes in locomotor activity. To satisfy the agreed-upon statistics, Day 4 and Day 5 total locomotor data was analyzed by an ANOVA. There were no differences between doses in Day 4 baseline total locomotor data;  $F(4,41) = 1.722$ ;  $p > .01$ . There was a significant effect of dose on total Day 5 locomotor data;  $F(4,41) = 6.85$ ,  $p < .01$ . Post-hoc t-tests revealed that the R(+)- 0.04 group displayed significantly higher total locomotor scores than all other dose groups ( $p$ 's  $< .05$ ). Total locomotor activity data is not shown. Time course data were analyzed by a dose\*time bin ANOVA on Day 4 and Day 5

locomotion. There was no interaction between time bin and dose on baseline locomotor data;  $F(11.65,104.88) = 0.757$ ,  $p > .05$ . There was a significant interaction of time bin\*dose on Day 5 locomotor scores;  $F(8.95,82.811) = 2.682$ ,  $p < .01$ . Post-hoc one-way ANOVAs on each time bin revealed differences at the 61-80, 81-100, and 101-120 minute time bins. Tukey's post hoc tests revealed that the R(+)- 0.04 group showed significantly higher locomotor scores than all groups at the 61-80 minute time point, and higher scores than the aCSF, S(-)- 0.08, and S(-)- 0.16 groups at the 81-100 and 101-120 minute time bins ( $p$ 's  $< .05$ )\* (**Fig. 12A**).

One-way ANOVAs on Day 1-5 locomotor data for each dose revealed a significant effect of day for all dose groups except for the R(+)- 0.02 group (**Fig. 11B**). Tukey's post-hoc tests revealed that Day 1 locomotor scores for the aCSF group were significantly higher than Days 2-5. Day 2 was also higher than Day 5 locomotor scores (**Fig. 11A**). For the R(+)- 0.04 group, Day 1 scores were significantly higher than Days 3 and 4. Day 2 scores were also significantly higher than Days 3, 4, and 5 (**Fig. 11C**). For the S(-)- 0.08 group, Day 1 locomotion was significantly higher than Days 4 and 5, Day 2 locomotion was significantly higher than Days 3, 4, and 5, and Day 3 locomotion was significantly higher than Day 5 (**Fig. 11D**). For the S(-)- 0.16 group, Day 2 locomotor scores were significantly higher than Days 1, 4, and 5. Day 3 locomotor scores were also higher than Day 5 scores (**Fig. 11E**).

To confidently assess the time course of locomotor behavior, Day 5 time course locomotion was compared to Day 4 time course locomotion for each group independently. Greenhouse-Geisser statistics are reported when Mauchly's Test for Sphericity was significant ( $p < .05$ ). For the aCSF group, there was only a main effect of day, with

locomotion being lower on the micro-injection day regardless of time bin ( $p < .05$ ) (**Fig. 12A**). There was also only a main effect of day for the S(-)- 0.08 group, with less locomotor activity following the microinjection ( $p < .05$ ) (**Fig. 12D**). There were no main effects or interactions of day\*time bin for the R(+)- 0.02, R(+)- 0.04, or S(-)- 0.16 groups (**Figs. 12B, 12C, and 12E**, respectively). While it appears in the graphs that there may have been some interactions in the locomotor data, these effects were not significant due to the use of Greenhouse-Geisser statistics for the R(+)- 0.04 and S(-)- 0.08 ( $p$ 's  $< .05$ ). The S(-)- 0.16 dose group did not reach a significant interaction ( $p = .077$ ).

## CHAPTER 3 DISCUSSION

### 3.1. Summary

Overall, these data show that intra-AcbSh microinjection of R(+)- and S(-)-baclofen bidirectionally affect ethanol intake, but not saccharin consumption. Total ethanol intake is decreased by the high dose 0.04  $\mu$ g R(+)-baclofen/side, but increased following microinjection of the high dose 0.16 $\mu$ g S(-)-baclofen /side. These drug effects also show a distinct time course of action, which is only partially reflected by changes in locomotor activity following microinjections of the drug.

### 3.2 Locomotion

The ethanol and saccharin data taken together suggest that locomotor activity is not predictive of reinforcer intake and vice versa. If the reduction of ethanol intake by the high R(+)- dose was due to sedative effects, there also would have been a reduction in saccharin intake at the same dose. Therefore consumption data should be considered independent of the locomotor behavior. Further complicating the locomotor/consumption story is that the Acb itself plays a role in locomotor activity. Bilateral ablation of the Acb by 6-OHDA in rats abolished the locomotor response typically seen to a systemic amphetamine injection (Kelly et al., 1975). In a Pavlovian response paradigm, ablating the AcbSh completely abolished the effect of intra-Acb amphetamine injections on potentiating conditioned reinforcer responding without affecting Pavlovian or

instrumental conditioning (Parkinson et al., 1999). Parkinson et al., (1999) found that intra-Acb amphetamine injections in rats with an ablated core still showed increased potentiation of responding, but it was not specific to the reinforced lever, suggesting that alterations in locomotor activity are related to the AcbSh, but not core, and are not specific to goal-related activity. Increases of dopamine in the Acb have also been shown to increase locomotor activity, either through direct dopamine microinjection (e.g. Pijnenburg & van Rossum, 1973), or via alteration by another drug, such as nicotine (e.g. Benwell & Balfour, 1992) or cocaine (e.g. Delfs et al, 1990). This interaction is especially important, as baclofen may moderate locomotor activity via interaction with dopamine in a non-goal specific manner, yet possibly in a dose- and enantiomer-specific fashion. The role of dopamine in the AcbSh and its interaction with GABA will be discussed further on in this thesis. As already mentioned, the minimal locomotor effects observed do not represent a 1-to-1 relationship with ethanol or saccharin intake or effects on other behaviors, such as Pavlovian or instrumental response (Parkinson et al., 1999).

### 3.3. Stereospecific Drug Action

The major highlight of these results is the stereospecific, bidirectional effects of the baclofen enantiomers on ethanol intake. The systemic work discussed in the rationale of this paper demonstrate that baclofen has these stereospecific effects when administered systemically, but this work specifically implicates the AcbSh as a target of interest for ethanol consumption. The specific role of the shell in addiction pathology will be discussed further on, with the current focus on a few other drugs that have shown stereospecific effects, which may elucidate the role of the separate baclofen enantiomers. Isozymes, which are enzymes that are functionally identical yet differ in amino acid

sequencing (Hunter & Markert, 1957), have been shown to be expressed differently across brain regions in monkeys, rats, and humans, potentially as a function of their binding affinity (Perez-Torres et al., 2000). The baclofen enantiomers are similar to isozymes in the sense that they are structurally different, yet functionally identical because they work as agonists at GABA<sub>B</sub> receptors. Stereospecific binding has been shown with other neurotransmitters and drugs in the rat brain, including norepinephrine and amphetamine (Coyle & Snyder, 1969).

General differences in brain area binding are not a sufficient explanation in these experiments, where bidirectional behavior was observed following direct microinjection into one area of the brain. It is possible that the baclofen enantiomers are binding differentially in the same areas of the brain, and this may be the case in the AcbSh. For instance, Hill et al. (1988) showed that the orientation of aromatic and amine groups to the drug molecule of type 1 antiarrhythmic drugs is an important determinant of how the drug binds to the cardiac sodium channel of myocytes taken from Sprague Dawley heart tissue. Further, the antidepressant drug rolipram shows stereospecific high and low binding affinity (Schneider et al., 1986). Low-affinity binding of rolipram was demonstrated to be specific to one protein component of (R)-rolipram, which does not bind at high affinity sites (Rocque et al., 1997). Yet the high affinity rolipram binding sites mitigate the antidepressant effects of the drug (Zhang et al., 2006). However, the racemate and separate enantiomers of rolipram produce the same “feeling” or state of the animal, as they all substitute for each other in discriminative stimulus tasks (Schneider et al., 1995). Tangentially, the discriminatory stimulus findings of rolipram are interesting, as it has been shown that baclofen does not substitute for ethanol (Shelton & Balster,

1994). What is unknown is whether the enantiomers and racemic substitute for each other, or if the R(+)- enantiomer substitutes for ethanol, thereby reducing ethanol intake.

Mecamylamine, a nicotinic parasymphathetic ganglionic blocker, is of great interest when working to interpret the current findings. Mecamylamine (Inversin) is used clinically to treat moderately severe to severe high blood pressure, yet was shown to be effective in reducing the rewarding effects of alcohol, alcohol-induced mood alterations, craving to consume alcoholic beverages, and decrease BECs following alcohol administration in a very small group of participants (N=17) that were not alcohol or nicotine dependent (Blomqvist et al., 2002). The separate enantiomers of mecamylamine have been relatively well studied. Nickell et al. (2013) reviewed the enantio-specific differences in behavior. S(+)- is a more effective anxiolytic than R(-)- in the light/dark box, forced swim test, and social interaction tests. S(+)- has also been shown to be more effective at blocking nicotine-induced seizures and decreasing spontaneous locomotor activity, yet both enantiomers are equally efficacious at altering nicotine-induced antinociception. Although R(-)- and S(+)- mecamylamine show no differences in affinity for nicotinic acetylcholine receptors (nAChRs) in rat whole membranes or in the ability of nicotine to alter binding, it has been suggested that S(+)- mecamylamine may work better at targeting nAChRs that are associated with anxiolytic and antidepressant drug effects, while also displaying less side effects than R(-)- (Nickell et al., 2013). Interestingly, S(+)- mecamylamine is more efficacious at inhibiting low sensitivity nAChRs than R(-)- while also working as a positive allosteric modulator at high sensitivity nAChRs (Fedorov et al., 2009). Receptor sensitivity is associated with affinity for nicotine and calcium permeability. Low sensitivity receptors show low

affinity for nicotine but high calcium permeability, while high sensitivity receptors display high nicotine affinity and low calcium permeability (Tapia et al., 2007).

Taken together, the actions of baclofen may depend on whether high and low affinity GABA<sub>B</sub> receptors exist, whether R(+)- and S(-)- baclofen binding is preferential to one type of receptor over the other, and whether this affinity, sensitivity, or binding preference is related to behavioral effects of the drug. Unfortunately, the literature on baclofen binding does not really help to understand how the compounds used in the current study act at receptors. Binding of baclofen relies not only on the absolute configuration of the drug (d/l or S/R), but also on the rotation of the molecule (+ or -). Waddington and Cross (1980) showed that d- and l-baclofen (S- and R-, respectively) display no differences in displacing GABA in low-affinity crude membranes or in high-affinity Triton membranes. Both enantiomers were much less effective at displacing GABA at high-affinity sites. Conversely, Bowery et al. (1983) looked at displacement of racemic baclofen in crude rat membrane. They found that GABA and (-)baclofen were equally able to displace the racemic mixture, whereas (+)baclofen displaced it to a much lesser extent. Drew et al. (1984) showed that, in the rat cerebellum, (-)baclofen binds more readily than the racemic mixture, and that it appears to bind to (-)baclofen selective sites. Yet Haas et al., (1985) showed that hyperpolarization of hippocampal CA1 cells is activated by R-, and not S-, baclofen. Further complicating issues, Karbon et al. (1984) suggest that there are anatomically distinct high and low affinity GABA<sub>B</sub> binding sites, and that low affinity sites are co-localized with cerebral cortical noradrenergic nerve terminals in the cow brain. While Waddington and Cross (1980) suggest that the non-GABAergic effects of baclofen reside with the R- configuration, Karbon et al. (1984)

suggest that it is the (-)baclofen isomer that interacts with norepinephrine to potentiate the cAMP response in the rat cerebral cortex. At best, these literature sources suggest that the R- absolute configuration and the (-) molecular rotation appear to be important for binding affinity and interaction with other receptor systems in the brain. Yet, the mixtures used in this study combine the R- configuration with the (+) rotation, and the S- configuration with the (-) rotation, leading to an extremely muddled interpretation of binding and affinity results. In short, while it appears that R- and S- show no differences in binding to GABA<sub>B</sub> receptors, they may also be acting differentially with other systems. Contrarily, the (-) rotation is as efficacious as GABA at displacing racemic binding and also may be acting with other systems in the brain. Therefore, these specific compounds need to be looked at for binding and receptor selectivity before much headway can be made on their potential effects and interactions within the brain at a receptor level.

#### 3.4. Dopamine Involvement

Dopamine in the Acb has long been posited to play an important role in addiction pathology (Di Chiara et al., 1994). The “incentive arousal” view of dopamine response suggests that dopamine is not responsible for the behavioral reaction to the stimuli, but rather works as an amplifier signal for the stimuli – moderating whether or not the stimuli receives a response (Di Chiara et al., 2004). To elicit dopamine transmission in the AcbSh, a taste stimulus must not only have positive valence associated with it, but it must also be novel (Bassareo et al., 2002). It is believed that this normal dopamine response to a novel, positive stimulus becomes maladaptive in response to drugs of abuse (Di Chiara et al., 1994, Bassareo et al., 2003). For instance, dopaminergic responses are not elicited by presentation of sucrose following an active lever press (Carrillo & Gonzales, 2011),

and are not elicited by intraoral 20% sucrose infusion (Bassareo et al., 2002), even though when paired with ethanol both of these stimuli produce a dopaminergic response in the Acb. More specifically, Bassareo et al. (2003) gave rats a taste stimulant (20% sucrose + chocolate flavor), the taste stimulant + 10% ethanol, or 10% ethanol alone intraorally and looked at dialysate dopamine in the AcbSh. All three solutions showed an initial dopamine spike in response to the taste, with both ethanol solutions showing a secondary spike in dopamine related to the physiological effects of ethanol. To observe whether the dopaminergic response to ethanol was maladaptive, Bassareo et al. (2003) gave animals a chocolate and ethanol intraoral infusion pretreatment. Twenty-four hours later, a second oral ethanol infusion was given. Dialysate dopamine response showed that the initial dopamine response related to taste was absent, but the secondary dopamine response related to ethanol was stronger. These results suggest a habituation to the dopaminergic response to the tastant, but a maladaptive persistence in the response to the intracerebral effects of ethanol that the authors relate as a mechanism in developing behavioral responses to addictive drugs. Such effects have also been seen in the AcbSh with many other drugs of abuse. IV cocaine has been shown to increase extracellular dopamine in the shell, but not core of the Acb (Pontieri et al., 1996). Rats given the chance to self-administer cocaine have shown a trend for increased dopamine release in the shell over the core (Ito et al., 2000) and a statistically significant increase in dopamine levels in the shell over the core (Lecca et al., 2007). Long-term nicotine treatment in rats has been shown to decrease  $\alpha 6\beta 2$  nAChRs mediated dopamine release and the number of receptors in the AcbSh (Perez et al., 2013). Ahn and Phillips (2013) showed that amphetamine increased Acb dialysate dopamine efflux by 2000-2500%. These increases

were blocked by the dopamine transporter blockers GBR 12909 and methylphenidate. Further, mu opioid stimulation via DAMGO has been shown to be as effective as enhanced dopamine stimulation in increasing “wanting” of a sucrose solution, defined as cue-triggered response in a Pavlovian-Instrumental Transfer paradigm (Peciña & Berridge, 2013). Together, these results suggest that drugs of abuse and pharmacological interventions that do not directly target dopamine may interfere with the function of the dopamine system, thereby reducing or increasing salience of drug abuse.

### 3.5. GABA Involvement

GABA is known to be largely present in the Acb, as well as to interact with ethanol and dopamine. Exposure to a single intra-gastric dose of ethanol showed increased activation of cells in the NAC shell, but not core over rats given a dose of water (Lerich et al., 2008). Of the reactive neurons, 80% were GABAergic. An increase in GABA receptors in the Acb has been seen in two strains of high alcohol preferring rats following four weeks of ethanol access (Hwang et al., 1990). As previously mentioned, one third of GABA receptors in the Acb are GABA<sub>B</sub> receptors (Bowery et al., 1987). Extrasynaptic GABA<sub>A</sub> receptors of the shell have been shown to play a role in ethanol intake (Nie et al., 2011; Rewal et al., 2009), and these results of the current experiments support the role of AcbSh GABA<sub>B</sub> receptors in ethanol intake. GABA and dopamine of the Acb are known to interact with each other. Within the Acb, 4% of tyrosine hydroxylase labeled, primarily dopaminergic, terminals were located on the soma of GABAergic neurons and another 14% formed junctions with GABAergic dendrites (Pickel et al., 1988). Through action on dopamine receptors located on GABAergic neurons, dopamine agonists have been shown to inhibit GABA release in the Acb (Beart

et al., 1979; De Belloche & Gardiner, 1983). At low doses, neurotensin administered into the Acb has been shown to increase GABA release while decreasing dopamine release, whereas high doses have been shown to increase both GABA and dopamine (Tanganelli et al., 1994).

Therefore, it is proposed that the GABAergic actions of each baclofen enantiomer may be interacting with dopamine or other receptor types to exert effects on ethanol consumption. For example, Reynolds & Berridge (2001) showed that injection of muscimol (GABA<sub>A</sub> agonist) into the rostral AcbSh increased positive reinforcement feeding behavior but not defensive treading, whereas more caudal injections increased negative defensive treading behavior but did not affect feeding behavior. The authors suggest that this change in behavior may be dependent upon differential GABAergic interactions in each area, with more dopamine D1, D2, and opioid enkephalin mRNA being present in the rostral shell, but more cholecystinin, ACh, vasopressin, oxytocin, and norepinephrine and serotonin innervation in the caudal shell.

Such interactions may explain the lack of drug effects seen on saccharin intake. If the actions of baclofen in the AcbSh are dependent upon interactions with cue-induced dopamine release, with the cue in this case being the daily introduction of the bottle, and there are no dopaminergic responses to the saccharin bottle on Day 5 (as would be suggested by Carrillo & Gonzales, 2011; Bassareo et al., 2002; 2003), baclofen would not be able to elicit effects on saccharin intake, as seen in this study. A secondary explanation may be a transfer of control of saccharin intake to a secondary area of the brain. Control of intake of a tastant that elicits an AcbSh dopaminergic response on Day 1 due to positive valence and novelty may be transferred to another brain area once the tastant is

no longer considered novel, as would be the case on Day 5 of intake. Bassareo et al. (2002) offer support for this theory by demonstrating that habituation of the dopamine response to the chocolate tastant occurred in the AcbSh, but not in the Acb core or the prefrontal cortex. Therefore, neurological control of non-addictive substances may be transferred to one of these two areas or elsewhere once novelty of the substance has worn off, suggesting that baclofen injections directly into the NAC core or prefrontal cortex on Day 5 may reduce saccharin intake. A third explanation of why baclofen was unable to reduce saccharin intake is the possibility that animals were willing to overcome drug effects to consume saccharin.

A secondary consideration for interpreting the data is the GABAergic projections from the AcbSh. GABAergic efferents from the AcbSh project to the ventral pallidum and the VTA (Kalivas et al., 1993; Dray & Oakley, 1978). Both of these areas are believed to be involved in reinforcer directed behavior. Lesions of the anterior and posterior pallidum significantly reduce development of conditioned place preference for sucrose in rats kept at 90% of their free-feeding weight compared to sham lesioned animals (McAlonan et al., 1993). Lesioning the ventral pallidum also produces decreases in self-administration of cocaine and heroin (Hubner & Koob, 1990), while injections of mu or delta specific opiate receptor agonists into the caudal ventral pallidum have been shown to increase intracranial self-stimulation responses (Johnson et al., 1993; Johnson & Stellar, 1994).

A review by Kalivas (1993) reports unpublished data suggesting that 35-55% of projections from the Acb to the VTA are GABAergic, and that these efferent projections originate in the shell of the Acb, not the core. These projections synapse on both

dopaminergic and non-dopaminergic neurons, including GABAergic interneurons of the VTA. Kalivas suggests that inhibition of these GABA interneurons via GABAergic projections from the AcbSh and the pallidum may result in disinhibition of the VTA dopamine cells. Conversely, stimulation of D1 receptors by GABA projections from the AcbSh augments GABA release within the VTA and stimulates firing of GABAergic interneurons.

There is some literature on how baclofen acts within the VTA. Tanner (1979) showed that baclofen microinjected into the VTA increased dialysate dopamine levels in the AcbSh. Kalivas et al. (1990) have also shown a similar effect; baclofen injected into the dopaminergic A10 region that is part of the VTA reduced dialysate dopamine response in the Acb to a systemic injection of mu opiate agonist in rats. Interestingly, inhibition of dopamine release in the VTA appears to be specific to GABA<sub>B</sub> receptors. Klitenick et al. (1992) showed that the GABA<sub>A</sub> agonist muscimol delivered directly into the VTA increased dialysate dopamine, whereas application of baclofen decreased dialysate dopamine. The authors propose that GABA<sub>A</sub> receptors inhibit GABAergic inputs to dopamine neurons due to their presynaptic location, whereas GABA<sub>B</sub> receptors, which are located on dopaminergic neurons, are directly inhibiting dopamine release. Moore & Boehm (2009) showed that direct injection of baclofen into the anterior, but not posterior, VTA reduced binge-like ethanol intake in the same DID paradigm used in this study. Again, these studies support that GABA is somehow interacting with dopamine to modulate ethanol consumption.

### 3.6. Drawbacks to DID

When interpreting the differences between drug effects on ethanol and saccharin intake, it is important to keep in mind that DID only accounts for consumption of the reinforcer. It does not account for the reinforcing value, as an operant paradigm would. This poses a problem for assessing whether the chosen reinforcers, ethanol and saccharin, are similarly reinforcing and whether drug effects are truly reinforcer specific, or if they are due to increased motivation to obtain saccharin. While mice freely ingest saccharin and a 0.2% solution is often used as a standard reinforcer control group (Lush, 1984; Risinger et al., 1998; Yoneyama et al., 2008), minimal data exists on the reinforcing quality of different saccharin concentrations as seen in operant paradigms. One study (Risinger et al., 1998) found that animals responded 2.5 times more in an FR4 operant paradigm for a 0.2% w/v saccharin solution than a 20% v/v ethanol concentration.

At a 0.2% w/v concentration, B6 mice have been shown to have between a 50% (Lush, 1984) to a 94.5% preference (Yoneyama et al., 2008). Preference, however, does not speak to how reinforcing a solution is. An animal may find a solution to be preferable, but may not actually work for that solution. This case is seen in Risinger et al. (1998); 0.2% saccharin and 20% ethanol have similar preference ratios, yet B6 mice work harder to receive saccharin. In an FR4 paradigm, B6 mice will respond 400 times for ethanol but respond 1000 times for saccharin in a 23 hr operant session. When 20% ethanol was given in a 0.2% saccharin, vehicle responding increased to almost 600 ethanol lever presses. The authors suggest that their results show that some of the effects in responding may be due to ethanol intoxication, while some are due to the higher reinforcing nature of saccharin, as seen by the increase in ethanol responding when saccharin was included.

Overcoming negative drug effects to consume saccharin would explain why animals drank even after receiving R(+)-baclofen, but it does not explain why S(-)-baclofen did not produce an increase in saccharin consumption. Further, systemic R(+)-baclofen reduced saccharin intake, so a blanket statement that R(+)-baclofen is unable to reduce saccharin consumption is unsatisfactory.

Therefore, the best explanation is that the AcbSh is not responsible for the control of saccharin intake, likely due to a combination of a transfer of control to another brain region and the possible lack of dopamine response on Day 5. To test whether there is a transfer of control from the shell to the core, or elsewhere, baclofen injections could be given into the AcbSh on Day 1 to see if they block saccharin intake when dopamine is likely present as a response to the novelty of saccharin. Other regions of the brain, such as the Acb core and the prefrontal cortex, could also be targeted for Day 5 microinjections to see if they reduce saccharin and/or ethanol consumption.

### 3.7. Conclusions

In conclusion, the two aims of this study – to modulate consummatory behavior via intra-AcbSh injections of R(+)- and S(-)-baclofen – were met. Further, the hypothesis that R(+)- and S(-)-baclofen would bidirectionally alter ethanol intake, but not saccharin intake, was upheld. It is not likely that the effects of baclofen on consummatory behavior are related to locomotor activity, as activity counts did not track exclusively with consummatory behavior and should not be expected to, as an animal spending more time at the bottle would be more likely immobile. Unfortunately, due to equipment failure, using a simple ANOVA to show that groups do not differ in locomotor activity is not possible. Further, dopamine in the AcbSh plays a role in locomotor activity, and how that

role affected activity in these experiments cannot be identified. Overall, GABAergic interactions with dopamine in the shell may have contributed to the drug effects seen in this study. Maladaptive dopamine release in the AcbSh has been shown to occur with consumption of drugs of abuse, like ethanol, compared to dopamine response to simple tastants, like saccharin. This maladaptive dopamine release may be necessary for altering consummatory behavior, which may be why there is an effect of drug on ethanol, but not saccharin intake. When administered systemically, R(+)- baclofen reduces saccharin intake. Systemic administration may lead to these drugs acting elsewhere, like the Acb core or the prefrontal cortex where there is still release of dopamine in response of the tastant. The stereospecific effects of baclofen on ethanol intake may be related to a myriad of enantioselective properties like those seen in other compounds. These include differential binding to high/low affinity receptors which may influence differential receptor effects or which behaviors receptors may modify. Future studies within our lab could aim to target other brain areas, including the Acb core and the prefrontal cortex, to determine if injections of baclofen into these areas also affects ethanol intake and modulates saccharin intake. Work could be done using real-time PCR to assess differences in mRNA production following administration of the different enantiomers, whereas Western blotting could be used to assess protein changes. Further, immunohistochemistry could be used to identify if these enantiomers are binding differentially in the brain. Simple drug discrimination experiments should be used to assess if the three baclofen compounds substitute for each other, and whether each enantiomer substitutes for ethanol. Of great interest, but outside of the scope of our lab, is using microdialysis in awake behaving animals to assess how injections of R(+)- and S(-

)- baclofen alter dopamine release in the shell. Currently, the Gonzales lab at the University of Texas at Austin, is working to develop microdialysis technique to identify GABA release in the brain (personal communication with Dr. Rueben Gonzales). Such advances in technology would present an amazing opportunity to advance the understanding of how GABA acts alone in the AcbSh to influence reinforcer consumption, and how it is interacting with dopamine and other neurotransmitters. Overall, these results continue to implicate the important role of the GABA<sub>B</sub> receptor in influencing ethanol intake. They also reinforce the notion that drugs exert enantioselective actions on behaviors, and that these actions are extremely important in considering the efficacy or potency of a drug for clinical treatment. This study has led to many more questions that are of great importance to the scientific field that can be answered in future research.

## TABLES

## TABLES

Table 1. Timeline of Experiment

Surgery	Recovery >48 Hours	DID Day 1	DID Day 2	DID Day 3	DID Day 4	DID Day 5
	Check cannula	Check cannula	Check cannula	30 second restrain stress	Mock microinjection	Microinjection

Table 2. Detailed breakdown of animals included in the ethanol experiments. \*Includes exploratory doses of R+ 0.01, S- 0.24, S- 0.32. Hit rates were taken (9/9), but are not included in this table.\*\*Animals which were lost before group assignment due to skull cap (3) or surgery error (8).\*\*\*Animals lost after group assignment due to unused dose, compromised skull cap, failure to acquire ethanol intake, histology error, and in one case death following microinjection.

Dose	Hit	Miss	Other Loss***	Total
aCSF	8	1	0	9
R+ 0.02	9	3	9	12
R+ 0.04	9	2	4	15
S- 0.08	9	0	1	10
S- 0.16	9	2	1	12
Unused dose*	n/a	n/a	9	9
Other**	n/a	n/a	11	11
<b>Total</b>	<b>44</b>	<b>8</b>	<b>26</b>	<b>78</b>

Table 3. Detailed breakdown of animals used in saccharin experiments. Asterisk (\*) indicates animals lost after group assignment due to compromised skull cap, failure to acquire saccharin intake, or histology error.

Dose	Hit	Miss	Other Loss*	Total
aCSF	9	1	0	10
R+ 0.02	7	3	0	10
R+ 0.04	8	2	0	10
S- 0.08	10	0	0	10
S- 0.16	8	1	0	9
<b>Total</b>	<b>42</b>	<b>7</b>	<b>0</b>	<b>49</b>

## FIGURES

## FIGURES

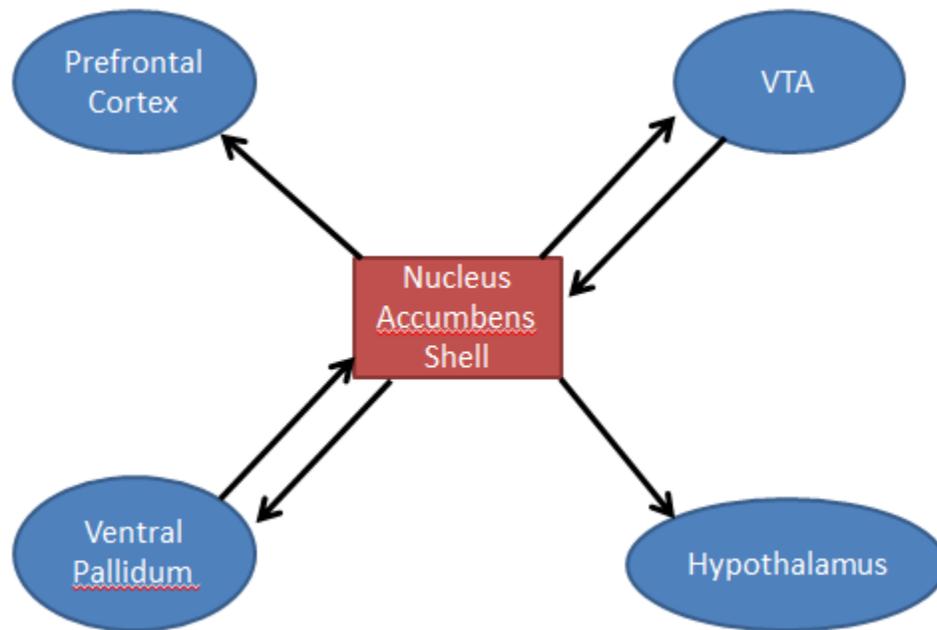


Figure 1. GABAergic projections to and from the AcSh.

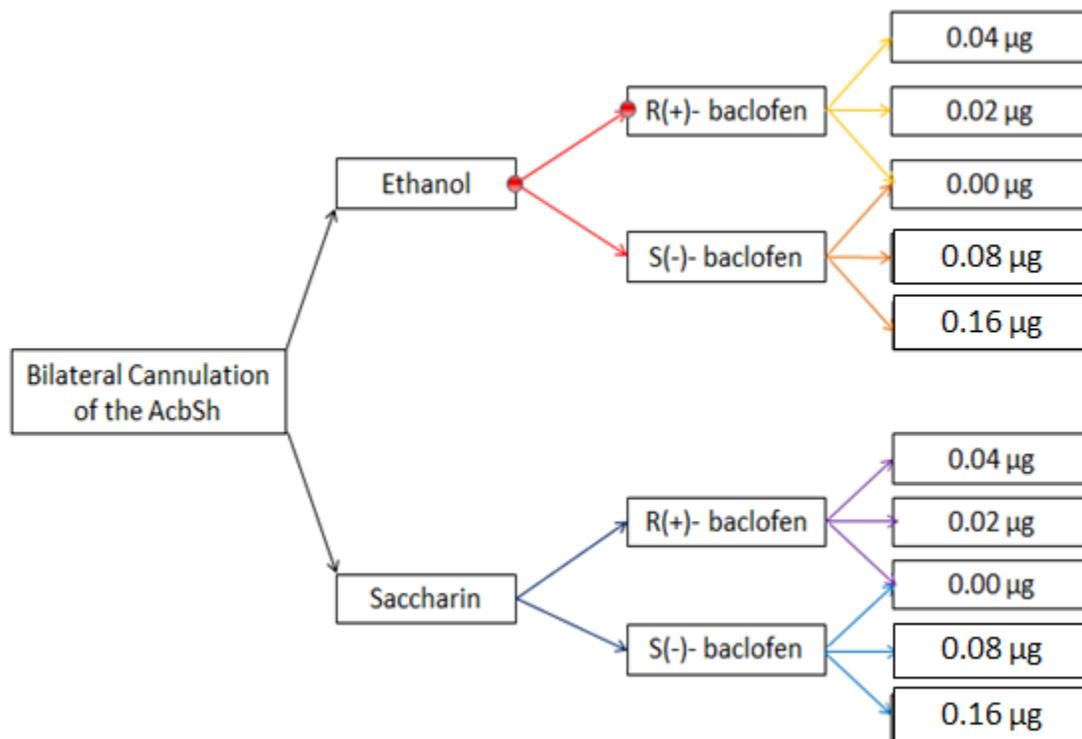


Figure 2. Groups included in this project.

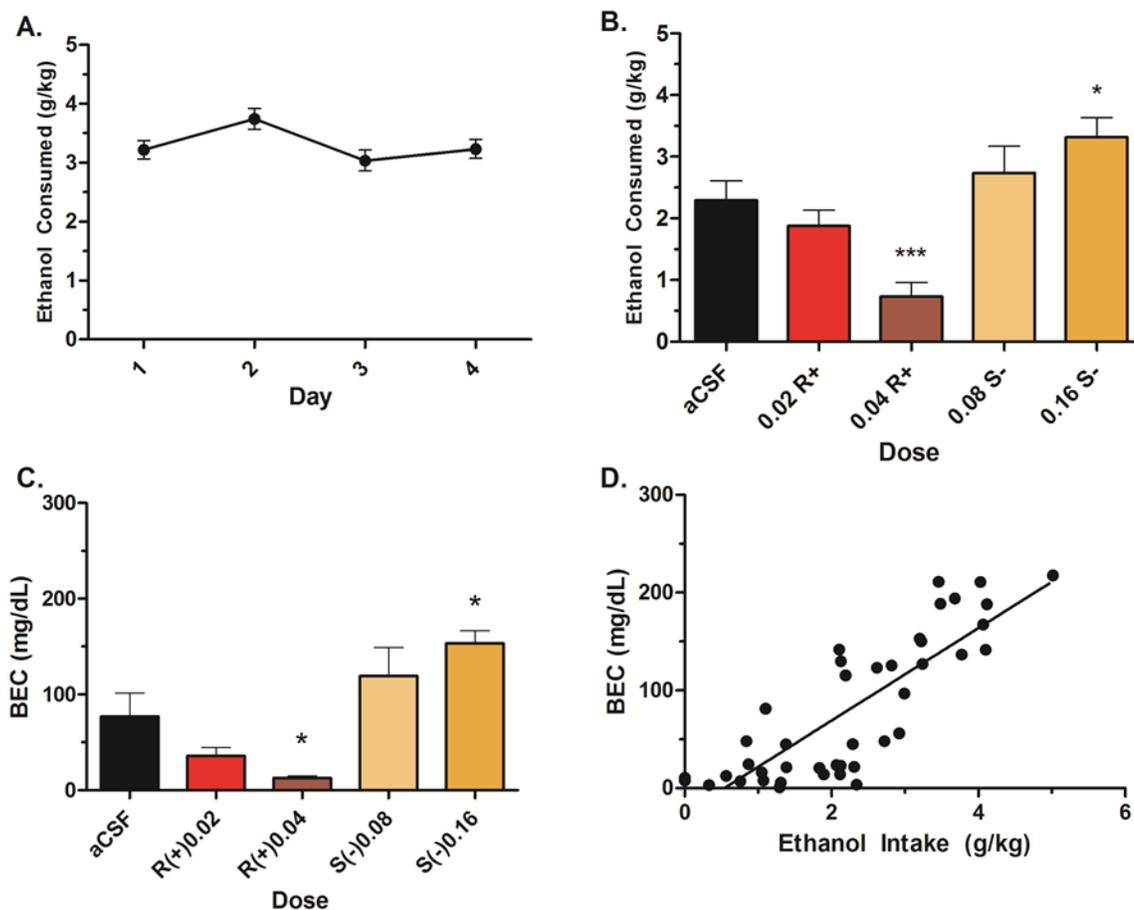


Figure 3. Microinjection of R(+)- and S(-)-baclofen into the AcbSh bidirectionally affects ethanol consumption. A) Acquisition of ethanol drinking. B) Total two hour ethanol consumption is reduced by a 0.04 R(+)-baclofen dose, but increased by a 0.16 S(-)-baclofen dose compared to an aCSF microinjection. C) This bidirectional effect is also represented in the BECs immediately following intake on Day 5. D) BECs were strongly and significantly correlated with total ethanol intake. Asterisk (\*) indicates significantly different than the aCSF group at  $p < .05$ . Asterisk (\*\*\*) indicates significantly different than the aCSF group at  $p < .001$ .  $n$ 's = 8-9 per group.

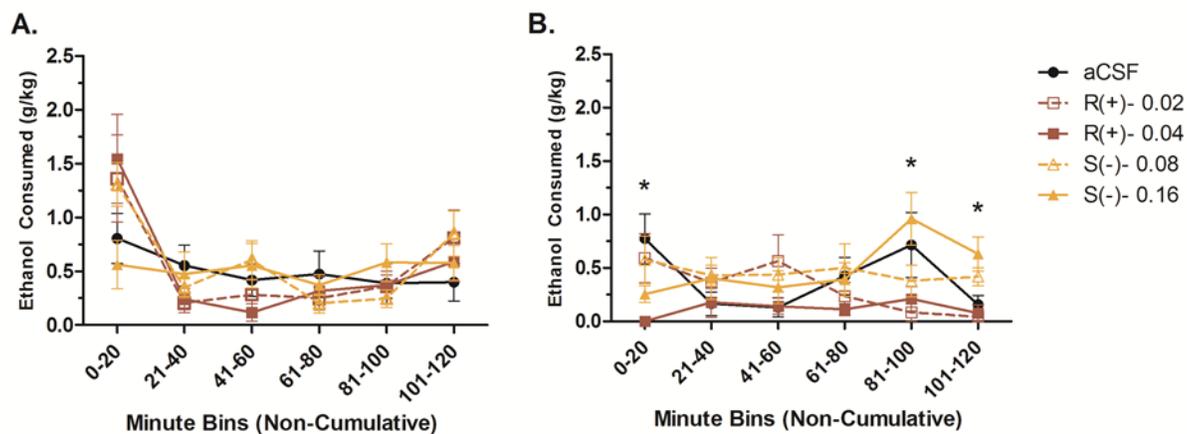


Figure 4. Time course of ethanol intake following mock and microinjections. A) Following the mock injection, the time course of ethanol intake did not differ between dose groups. B) Following the microinjection, the time course of ethanol intake was significantly different between dose groups. Asterisk (\*) indicates a significant follow up ANOVA  $p < .05$ ,  $n$ 's = 8-9 per group.

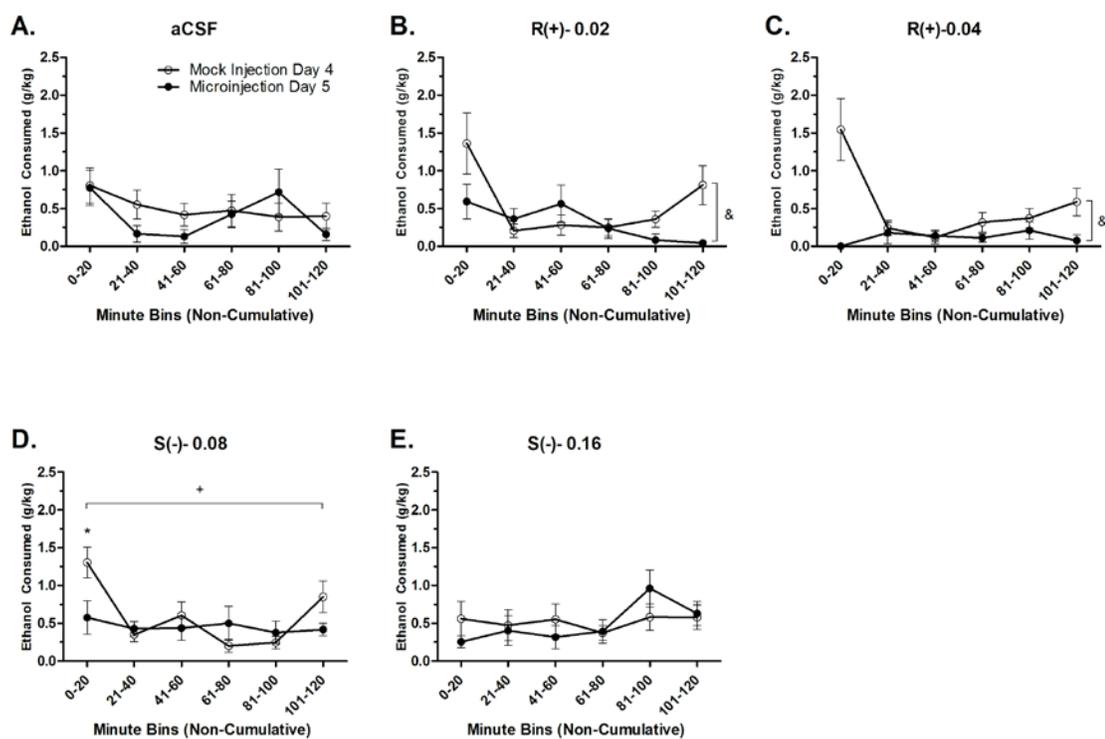


Figure 5. Time course of ethanol intake following the mock and microinjections for the aCSF (n=8) (A), R(+)- 0.02 (n=9) (B), R(+)- 0.04 (n=9) (C), S(-)- 0.08 (n=9) (D), and S(-)- 0.16 (n=9) (E) groups. Asterisk (\*) indicates a significant difference between the mock and microinjection intake at that time point,  $p < .05$ . Plus sign (+) indicates a main effect of time,  $p < .05$ . Ampersand (&) indicates a main effect of day,  $p < .05$ .

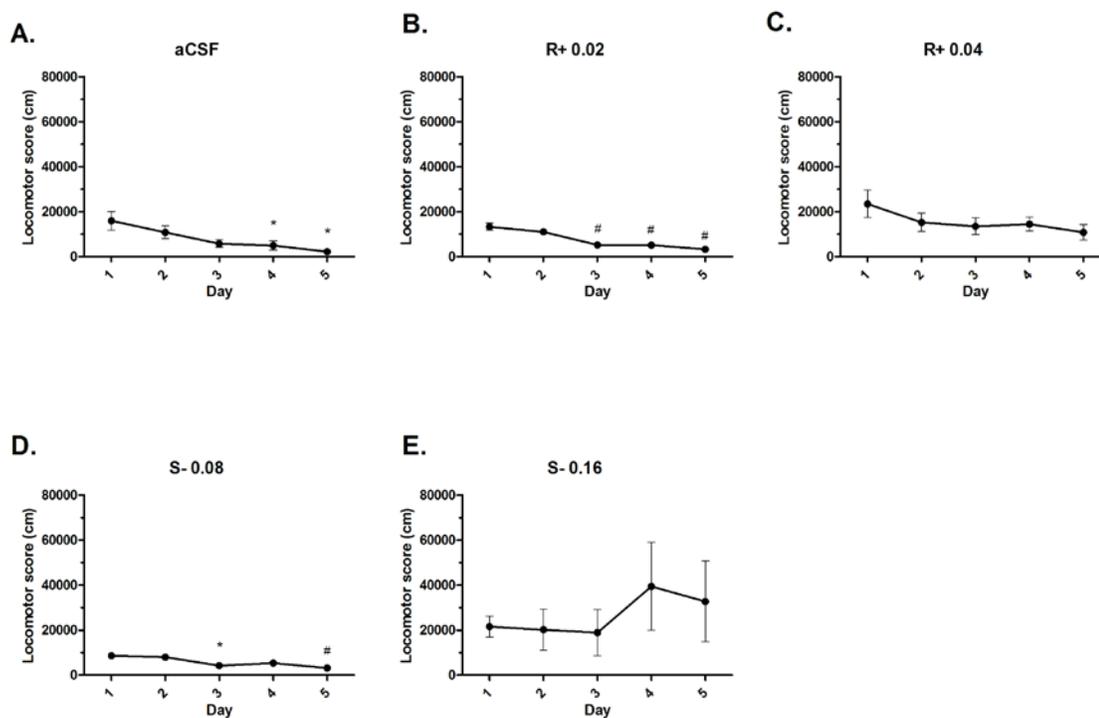


Figure 6. Daily total two hour locomotor data for the aCSF (A), R(+)-0.02 (B), R(+)-0.04 (C), S(-)-0.08 (D), and S(-)-0.16 (E) groups. Asterisk (\*) indicates significantly different than Day 1,  $p < .05$ . Pound sign (#) indicates significantly different than Days 1 and 2,  $p < .05$ ,  $n$ 's = 8-9.

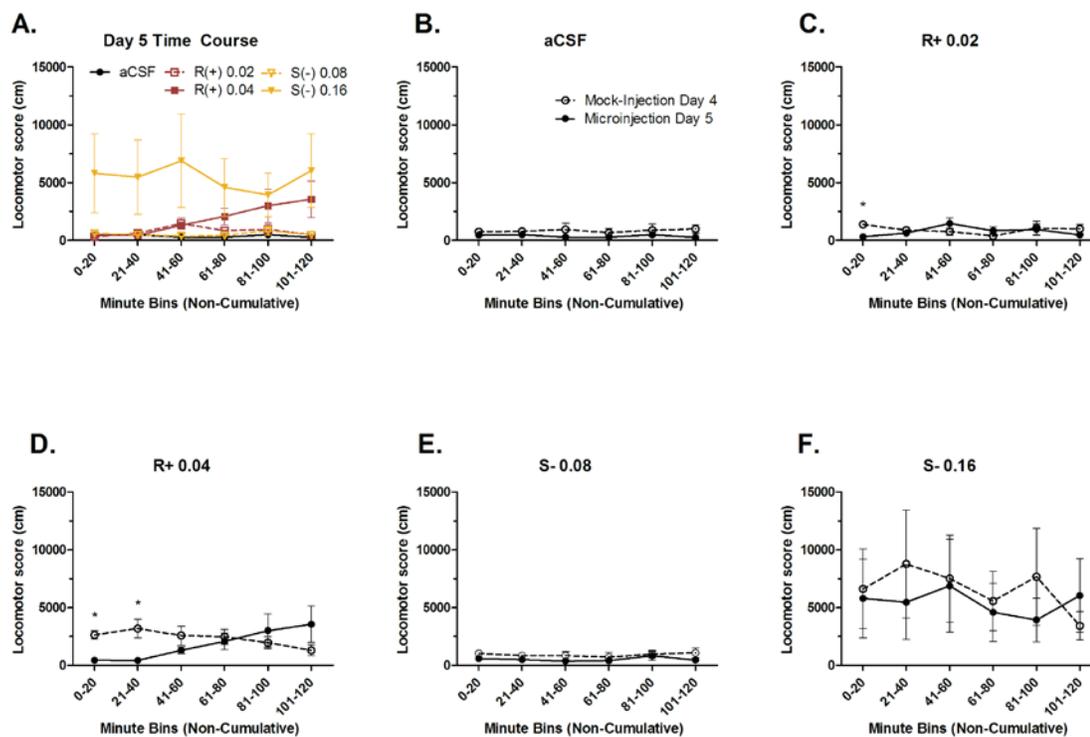


Figure 7. Time course of locomotor activity in the ethanol animals. A) Time course of activity for all groups following Day 5 microinjection. Time course of locomotor activity following mock- compared to micro-injection days is shown for the aCSF (B), R(+)- 0.02 (C), R(+)- 0.04 (D), S(-)- 0.08 (E), and S(-)- 0.16 (F) groups. Asterisk (\*) indicates that locomotor activity following mock and micro-injection is significantly different,  $p < .05$ ,  $n$ 's = 8-9 per group.

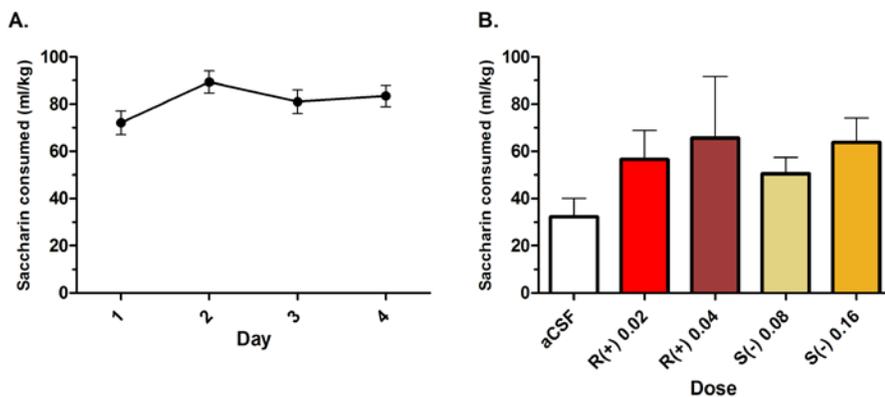


Figure 8. Effects of microinjection of R(+)- and S(-)-baclofen in the AcbSh on saccharin intake. A) Acquisition of saccharin intake. B) Microinjections of R(+)- and S(-)- baclofen do not alter ethanol intake compared to a microinjection of aCSF (n's = 7-10 per group).

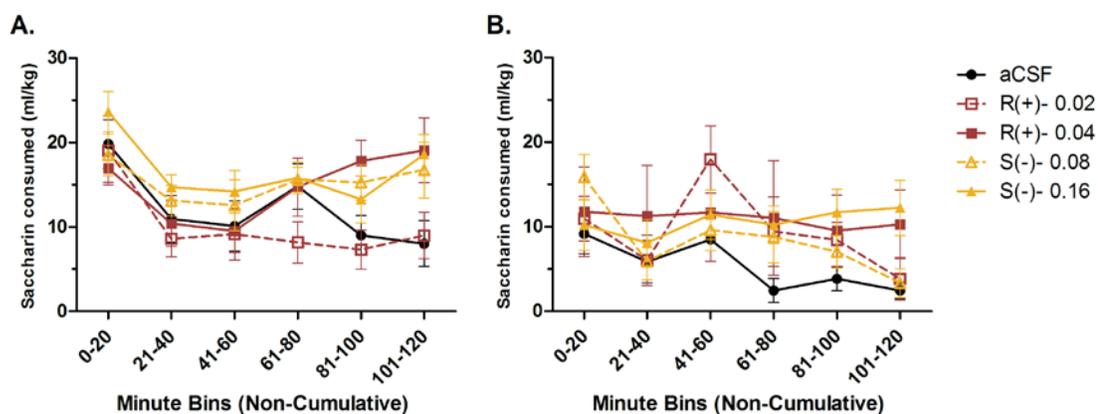


Figure 9. Time course of saccharin intake following the Day 4 mock injection (A) and the Day 5 microinjection (B) showed no significant differences between dose groups (n's = 7-10 per group).

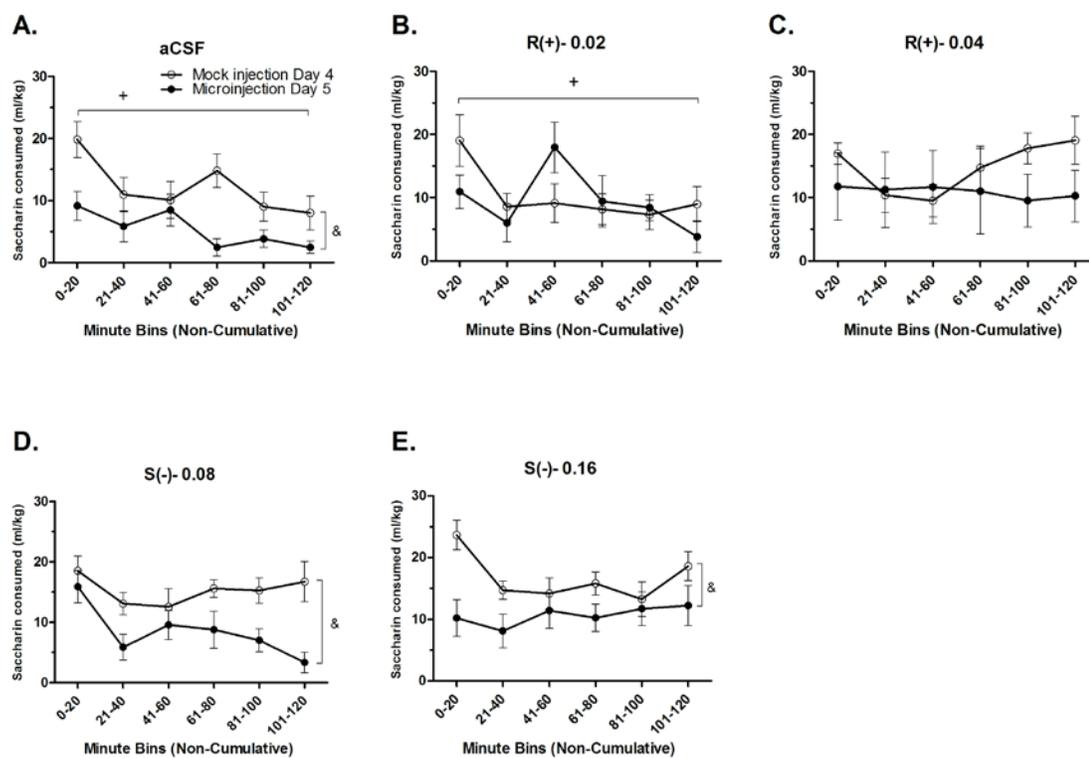


Figure 10. Saccharin consumption following mock (Day 4) versus microinjection (Day 5) for the aCSF ( $n=9$ ) (A), R(+)- 0.02 ( $n=7$ ) (B), R(+)- 0.04 ( $n=8$ ) (C), S(-)- 0.08 ( $n=10$ ) (D), S(-)- 0.16 ( $n=8$ ) (E) groups. Plus sign (+) signifies a main effect of time,  $p < .05$ . Ampersand (&) signifies a main effect of day,  $p < .05$ .

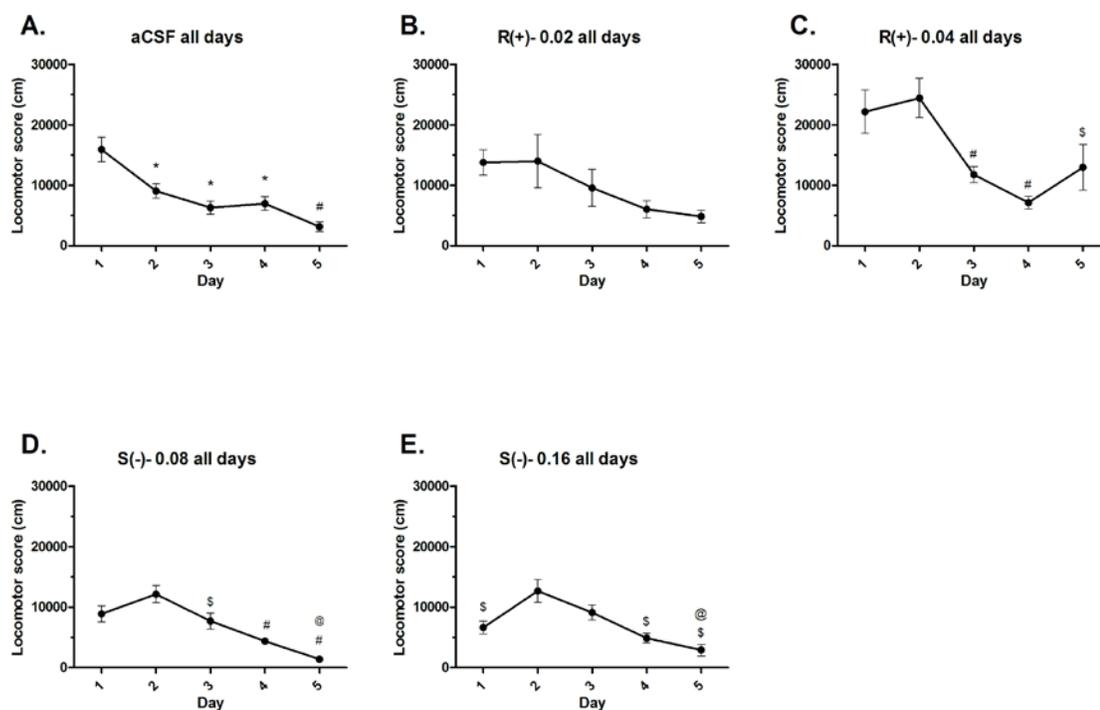


Figure 11. Daily two hour locomotor data for the aCSF (A), R(+)- 0.02 (B), R(+)- 0.04 (C), S(-)- 0.08 (D), and S(-)- 0.16 (E) groups. Asterisk (\*) indicates significantly lower than Day 1, dollar sign (\$) indicates significantly lower than Day 2, at sign (@) indicates significantly lower than Day 3, and pound sign (#) indicates significantly lower than Days 1 and 2, n's = 7-10 per group.

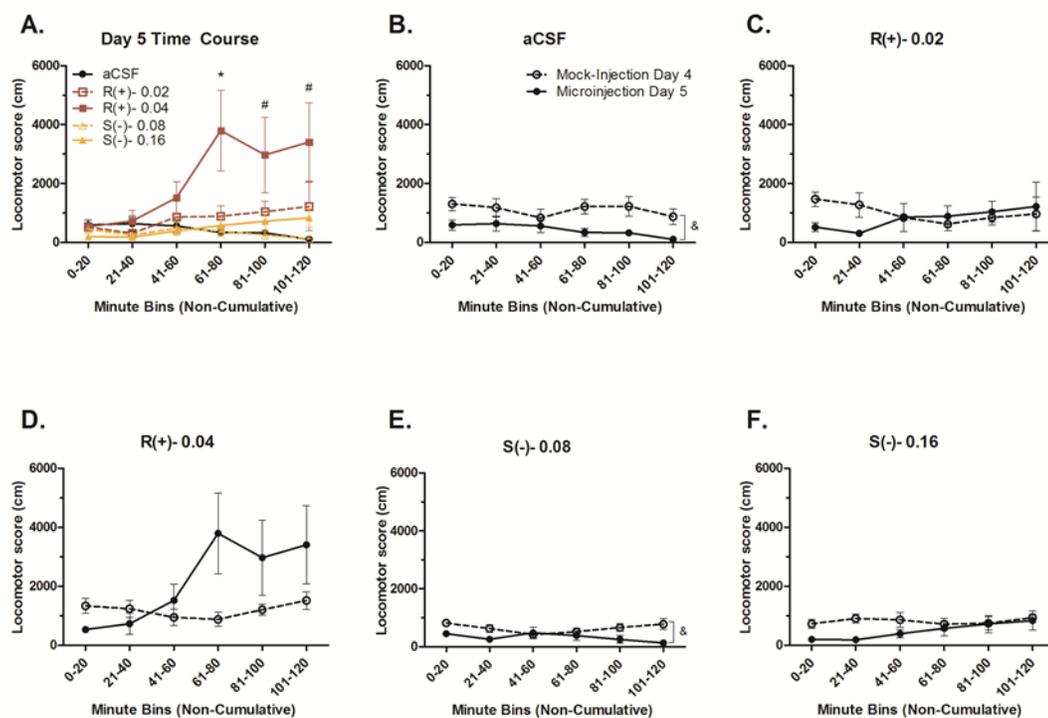


Figure 12. The time course of locomotor data for each group following microinjection is graphed in Panel A. Asterisk (\*) indicates that the R(+)- 0.04 group is displaying significantly more activity than all other groups. Pound sign (#) indicates that the R(+)- 0.04 group is displaying significantly more activity than the aCSF, S(-)- 0.08, and S(-)- 0.16 groups. The time course of locomotor activity following mock- and microinjections for aCSF (B), R(+)- 0.02 (C), R(+)- 0.04 (D), S(-)- 0.08 (E), and S(-)- 0.16 (F) groups is also shown. Ampersand (&) indicates a main effect of day, n's = 7-10 per group

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Psi Chi International Honor Society in Psychology (2009-present)

Publications

Siviy SM, Deron LM, Kasten CR (2011) *Serotonin, motivation, and playfulness in the juvenile rat*. *Developmental Cognitive Neuroscience*, 1; 606-616.

Matson LM, Kasten CR, Boehm II SL, Grahame NJ (2013) *Selectively bred crossed High Alcohol Preferring mice drink to intoxication and develop functional tolerance, but not locomotor sensitization during free-choice ethanol access*. Alcoholism: Clinical and Experimental Research.

Kasten, CR, Blasingame, SN, Boehm II, SL (In Preparation) *Bidirectional enantioselective effects of baclofen in binge-like and chronic models of ethanol intake*.

### Poster presentations

*Kasten CR, Henning A, Siviy SM (2010) Chronic mild stress does not alter ethanol consumption in Sprague-Dawley rats. Gettysburg College Celebration, Gettysburg, PA.*

*Kasten CR, Siviy SM (2011) Comparing the effects of social vs isolated housing during adolescence on ethanol consumption in adulthood in Wistar rats. Gettysburg College Celebration, Gettysburg, PA.*

*Kasten CR, Vore AS, Linsenhardt DN, Melon LC, Boehm II SL (2012) Free-choice ethanol access and daily ethanol injections do not alter locomotor response in crossed high-alcohol preferring mice. Alcoholism: Clinical and Experimental Research, 36: P0055*

*Kasten CR, Boehm II SL (2013) R(+)- baclofen attenuates ethanol intake in High Alcohol Preferring Line 1 mice. Genes, Brain, and Behavior, 12: P-23*

*Kasten CR, Boehm II SL (2013) R(+)- and S(-)-baclofen bidirectionally alter binge-like ethanol intake in C57Bl/6J mice. Alcoholism: Clinical and Experimental Research, 37: P0663*

*Matson LM, Kasten CR, Buckingham A, Tombers E, Boehm SL II, Grahame N (2013) Crossed High Alcohol Preferring (cHAP) mice exhibit stimulation and ataxia during early ethanol access, and develop functional tolerance during free-choice ethanol access.*

### Research Experience

Graduate Research Assistant, Dr. Stephen Boehm (August 2011-present)  
Addiction Neuroscience Lab, IUPUI, IN

Undergraduate Research Assistant, Dr. Steven Siviy (August 2006-May 2007)  
Behavioral Neuroscience Lab, Furman University, SC

### Teaching Experience

Lab instructor (August 2012-December 2012; August 2013-present)  
Research Methods in Psychology, IUPUI

Teaching Assistant, Dr. Cristine Czachowski (January 2013-May 2013)  
Learning, IUPUI

Teaching Assistant, Dr. Nicholas Grahame (January 2013-May 2013)  
Behavioral Neuroscience, IUPUI

Teaching Assistant, Dr. Sandra Hellyer (August 2012-December 2012)  
Child and Adolescent Psychology, IUPUI

Teaching Assistant, Dr. Debbie Herold (January 2012-May 2012)  
Introduction to Psychology, IUPUI

Teaching Assistant, Gina Seaton, MS (August 2011-December 2011)  
Tests and Measurements, IUPUI

Teaching Assistant, Jennifer Steiner, MS (August 2011-December 2011)  
Stress and Health, IUPUI