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EFFECT OF CENTRAL AMYGDALA *GABRA2* EXPRESSION ON ANXIETY AND ALCOHOL'S ANXIOLYTIC CAPACITY IN C57BL/6J MICE

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ABSTRACT

Smoker, Michael P. M.S., Purdue University, May 2016. Effect of Central Amygdala *Gabra2* Expression on Anxiety and Alcohol's Anxiolytic Capacity in C57BL/6J Mice. Major Professor: Stephen L. Boehm, II.

The *GABRA2* gene, which encodes the α2 subunit of $GABA_A$ receptors, is one of the genes most frequently associated with alcohol-related behavior in human studies (Demers, Bogdan, & Agrawal, 2014). Polymorphisms in *GABRA2* have been found to be associated with alcohol dependence, changes in drinking frequency, and alcohol's stimulating and euphoric effects (Arias et al., 2014; Dick et al., 2014; Edenberg et al., 2004). However, the *GABRA2*-alcohol relationship may not be direct, as anxiety and impulsiveness have been found to be mediating factors (Enoch, Schwartz, Albaugh, Virkkunen, & Goldman, 2006; Villafuerte, Strumba, Stoltenberg, Zucker, & Burmeister, 2013).

Comorbidity of anxiety and alcohol use disorders is both prevalent and clinically relevant (J. P. Smith & Randall, 2012), and $GABA_A$ receptors play a significant role in each. Benzodiazepines, primary pharmacologic treatments for anxiety disorders and alcohol withdrawal, facilitate signaling at $GABA_A$ receptors, and their anxiolytic effects appear to depend on the presence of α2 subunits in these receptors (Low et al., 2000). The amygdala is widely implicated in both anxiety disorders as well as addiction (Janak & Tye, 2015), and its central nucleus is an important mediator of responses to both

alcohol- and stress-related stimuli (Roberto, Gilpin, & Siggins, 2012), some of which may be related to *GABRA2* expression within this region (Jin et al., 2014).

The aim of the current study was to explore the role of *Gabra2* (mouse ortholog of *GABRA2*) expression within the central nucleus of the amygdala (CeA) in anxiety-related behavior and alcohol's anxiolytic effects in mice. C57BL/6J (B6) mice underwent surgery for bilateral infusion of GFP-tagged lentivirus targeting *Gabra2* or a scramble control lentivirus into the CeA. Following 12-13 days of recovery, mice were assessed for anxiety-like behavior in the elevated plus maze (EPM) naïve or following IP injection of 0, 0.75, or 1.5 g/kg ethanol. After assessment, brains were extracted and sectioned through the CeA. Finally, GFP was quantified, the CeA was collected via laser microdissection, and α2 protein was quantified via ELISA.

In mice expressing GFP in the CeA, α2 protein concentrations were lower for Virus mice relative to Control mice. The EPM was anxiogenic, and alcohol was found to be anxiolytic. In naïve mice, while there was no difference between Control mice and Virus mice on any behavioral measure, there were significant correlations between CeA α2 protein concentration and time spent in closed arms as well as both total and average time spent in open arms. In mice receiving injection of 0, 0.75, or 1.5 g/kg ethanol, there was a main effect of dose on several behavioral measures, but no interaction between viral condition and dose, and only a main effect of viral condition on average time spent in closed arms. There were no significant correlations between CeA α2 protein concentration and behavioral measures within any injected dose. These results are consistent with *GABRA2*-anxiety associations and effects of Gabra2 manipulation on anxiety-like behavior. Furthermore, they suggest that CeA α2 protein concentration is

positively related to basal anxiety, which could affect alcohol use through various routes. However, these results also suggest that CeA α2 protein concentration is not related to alcohol's anxiolytic capacity, at least when acutely administered in alcohol-naïve animals.

CHAPTER 1 INTRODUCTION

1.1 *GABRA2* & Alcohol-Related Behavior

Alcohol is a widely consumed drug, and its abuse can lead to negative individual and social consequences. Many of alcohol's neurobehavioral effects result from its interaction with GABA signaling within the central nervous system, and in particular with $GABA_A$ receptors. These receptors are ligand-gated and composed of five protein subunits, and their functional properties can vary depending on subunit combinations. Among these possible protein subunits is α2, which is encoded by the *GABRA2* gene, one of the genes most frequently associated with alcohol-related behavior in human studies (Demers et al., 2014).

Some of the earliest evidence for a *GABRA2*-alcohol association was with respect to alcohol dependent individuals. In a sample of individuals with a family history of alcohol dependence, Edenberg et al. (2004) found 31 single nucleotide polymorphisms (SNPs) within the *GABRA2* gene to be associated with dependence. Furthermore, Soyka et al. (2008) found an association between a single, 8-SNP haplotype in *GABRA2* and dependence between treatment-seeking dependent and non-dependent individuals. These results support a relationship between *GABRA2* polymorphisms and chronic alcohol consumption.

GABRA2 has also been associated with alcohol-related behavior outside of dependence. In a longitudinal study examining drinking frequency, allelic variation at 6 SNPs within *GABRA2* was associated with a greater increase in drinking frequency during the transition from adolescence to adulthood (Dick et al., 2014). Additionally, several studies have found associations between single or multiple *GABRA2* SNPs and the subjective effects of acutely administered alcohol. These include increased stimulation and euphoria (Arias et al., 2014), increased happiness and vigor (Haughey et al., 2008), and decreased negative effects (Uhart et al., 2013) following oral administration, as well as increased stimulation and liking following IV administration (Haughey et al., 2008). Taken as a whole, the evidence points to a significant relationship between *GABRA2* and alcohol-related behavior, including both chronic use and acute subjective effects.

As manipulation of *GABRA2* in humans is not currently feasible, non-human animals can be extremely useful, and studies with rodents also support the relationship between *GABRA2* and alcohol. Alterations in α2 expression have been found following selection for alcohol consumption and preference, with greater α2 mRNA expression in the nucleus accumbens (NAc) of HAP vs LAP mice (Boehm, unpublished) and greater α2 protein expression in the CeA of P vs NP rats (Liu et al., 2011). That these rodents were alcohol-naïve suggests that α2 alterations might play a role in alcohol consumption or preference.

Experimental manipulation of α2 expression also supports its role in alcohol-related behavior. Boehm II et al. (2004) found that α2 knock-out mice had a reduction in the duration of alcohol-induced loss-of-righting reflex (LORR), a measure of alcohol's

hypnotic effects. Furthermore, α2 knock-out mice showed a reduction in alcohol consumption and preference, but this was only the case in females. However, their (non-significant) reduced preference for bitter taste (quinine) may also have contributed to this effect. Although not part of this study, α2 knock-out mice have also been found to show a reduction in conditioned taste aversion (CTA) to alcohol (Blednov, unpublished). In a more refined approach to assessing the role of α2 subunits in alcohol-induced behavioral responses, Blednov et al. (2011) used knock-in mice containing point mutations at specific amino acids which yielded functional, but alcohol-insensitive α2-containing $GABA_A$ receptors. These knock-in mice showed both decreases and increases in alcohol consumption and preference depending on the paradigm (2-bottle choice, 1-bottle DID, or 2-bottle DID), access schedule (continuous or limited) and sex. Overall, there was no alcohol-drinking assessment that yielded a lack of effect in knock-in mice. In addition, these mice showed an increase in the duration of alcohol-induced LORR and a decrease in alcohol-induced CTA and locomotion. While the results of these studies suggest a role of α2-containing receptors in multiple alcohol-related behaviors, the implications are not completely clear as there were differences in effects based on study, paradigm, and sex. Furthermore, an effect of compensatory structural or functional changes within these congenital knock-out/in mice cannot be ruled out.

1.2 Anxiety's Mediation of *GABRA2* & Alcohol-Related Behavior

Both human and rodent data suggest, relatively consistently, a relationship between *GABRA2* and alcohol-related behavior. However, this relationship may not always be direct, as human studies indicate that there may be important mediating factors, including anxiety and impulsiveness (Enoch et al., 2006; Villafuerte et al., 2013),

with anxiety being of particular interest for this study. Enoch et al. (2006) used two distinct populations, Finnish and Plains American Indian, to investigate a potential *GABRA2*-alcohol-anxiety relationship. *GABRA2* SNPs were associated with alcoholism in both populations, replicating previous findings. Furthermore, risk (of alcohol dependence)-conferring haplotype configurations within *GABRA2* were associated with harm avoidance (a measure of trait anxiety) but not associated with alcoholism. Harm avoidance was higher in alcoholics, and haplotype frequencies varied with level of harm avoidance, but only for alcoholics. These results indicate that some of the relationship between *GABRA2* haplotype and alcoholism can be revealed when anxiety is taken into consideration. Additionally, it has been suggested that anxiety may partly explain the discrepancy between the ability of high- and low-risk *GABRA2* alleles to differentiate the effects of various psychosocial treatments in alcoholics (Bauer et al., 2007).

1.3 *GABRA2* & Anxiety-Related Behavior

Given the prevalence and clinical relevance of comorbidity of anxiety and alcohol use disorders (J. P. Smith & Randall, 2012), as well as the significant role that $GABA_A$ receptors play in these disorders, it would not be surprising if *GABRA2* played a role in both, and possibly even their overlap. Studies in rodents with global α2 expression manipulation tend to support its role in anxiety, but results aren't unequivocal. α2 knock-out mice have been found to display more risk assessment and longer latencies to explore a novel environment during free-choice exploration, as well as less time in light and more time in the tunnel during a light/dark (L/D) choice situation (Koester et al., 2013). In addition, mice with alcohol-insensitive α2-containing receptors have displayed a reduced

percentage of open arm entries in the elevated plus maze (EPM) (Blednov et al., 2011). These results indicate a role for α2 expression in basal anxiety.

With respect to drug-induced anxiolysis, the results are mixed. Boehm II et al. (2004) found no significant difference in alcohol's anxiolytic effects in the EPM in α2 knock-out mice, and the same was true of mice with alcohol-insensitive, α2-containing receptors (Blednov et al., 2011). However, when looking at studies with mutant mice containing point mutations rendering $GABA_A$ receptors containing specific subunits (α1, α2, or α3) insensitive to modulation by benzodiazepines, the evidence for an α2-anxiolysis relationship is strong. Low et al. (2000) found that only α2 mutant mice showed no effect of diazepam on time in light in the L/D test and open arm time and entries in the EPM. Furthermore, only α2 mutant mice have shown a consistent lack of effect of diazepam and chlordiazepoxide in EPM and fear-potentiated startle assessments without affecting locomotor activity (K. S. Smith, Engin, Meloni, & Rudolph, 2012). Benzodiazepines are effective anxiolytics in non-mutated mice, and while multiple $GABA_A$ α-subunits are implicated in a number of benzodiazepines' behavioral effects, it appears that α2-containing $GABA_A$ receptors play a principal role in mediating their anxiolytic effects. That global α2 expression manipulations in rodents tend to alter the anxiolytic properties of benzodiazepines but not alcohol might suggest a drug-specific relationship between α2 expression and anxiolysis. However, evidence from region-specific studies (discussed below) suggests that targeted investigation of the role of α2 expression in alcohol's anxiolytic effects is warranted.

1.4 $GABA_A$ Involvement in CeA-Mediated Behavior

The amygdala is widely implicated in both anxiety disorders and addiction, and its circuitry is well conserved across species, allowing for meaningful translational research (Janak & Tye, 2015). Based on location and connectivity, the CeA is an important mediator of responses (physiological and behavioral) to alcohol- and stress-related stimuli (Roberto et al., 2012), leading to the possibility of behaviorally-relevant, alcohol-anxiety interactions in this region. With respect to alcohol, acute administration in rats or mice increases c-Fos expression in the CeA (McBride, 2002), and CeA lesions have been shown to reduce alcohol intake in a 2-bottle choice procedure (Moller, Wiklund, Sommer, Thorsell, & Heilig, 1997). In rats, infusion of a $GABA_A$ antagonist (SR5531) into the CeA has been shown to decrease responding for alcohol in an operant choice task at lower concentrations than infusion into other brain regions (Hyytia & Koob, 1995); infusion of a selective, α1 $GABA_A$ benzodiazepine mixed agonist/antagonist (βCCt) into the CeA has been shown to decrease responding for alcohol, with and without sucrose (Foster et al., 2004); and infusion of a $GABA_A$ agonist (muscimol) into the CeA has been shown to substitute for alcohol in a discrimination task (Hodge & Cox, 1998). With respect to anxiety, lesions of the CeA in rats have been shown to abolish the anxiogenic effects of restraint stress in the EPM (Moller et al., 1997). Furthermore, rats infused with muscimol or midazolam in the CeA have shown a reduction in a number of anxiety-related behaviors in the EPM, and these effects were not present after infusions in the basolateral amygdala (Carvalho, Moreira, Zanoveli, & Brandao, 2012; Moreira, Masson, Carvalho, & Brandao, 2007). These results indicate a role for the CeA broadly, and $GABA_A$ receptors within the CeA specifically, in

subjective and behavioral responses to alcohol as well as in the exhibition of anxiety-related behaviors.

Alcohol- and anxiety-related responses/behaviors also appear to interact within the CeA based on the following studies assessing CeA-mediated anxiety-related behaviors in the context of prior alcohol exposure. Sharko, Kaigler, Fadel, and Wilson (2013) split rats into high- and low-drinking groups after multiple, limited-access alcohol sessions and found an increase in the percent time in open arms in the EPM in the high-drinking group as well as higher c-Fos activation in the CeA, which was correlated with both alcohol intake and percent time in open arms. Rassnick, Heinrichs, Britton, and Koob (1993) infused a corticotropin-releasing factor (CRF) antagonist into the CeA (and intracerebroventricularly) and assessed anxiety in the EPM after withdrawal from either an alcohol- or sucrose-based liquid diet. CRF antagonism was found to be anxiolytic, but only after infusion into the CeA, and only in alcohol-dependent rats. The fact that the aforementioned effects were only found in animals with prior (high) alcohol experience suggests the possibility that alcohol-related alterations in the CeA are relevant to expression of anxiety-related behavior.

1.5 *GABRA2* & the CeA

GABRA2 is expressed at relevant levels in the CeA of multiple species. There is moderate to strong expression of α2 protein in the rat CeA (Pirker, Schwarzer, Wieselthaler, Sieghart, & Sperk, 2000) and α2 mRNA and protein expression in the mouse CeA (Hortnagl et al., 2013), and concerning $GABA_A$ subunit distribution in the (extended) amygdala, α2 protein expression is most prominent in the CeA (and NAc, BNST) while α1 protein expression is most prominent in the lateral amygdala (and VP).

With respect to alcohol, post-mortem analysis of CeA tissue from humans has revealed decreases in mRNA expression of a number of glutamate receptor subunits, but only one GABA receptor subunit, α2, in alcoholics compared to controls (Jin et al., 2014). Additionally, both alcohol-naïve and alcohol-exposed P rats have been shown to have increased expression of α2 (and α1) protein compared to NP rats in the CeA (Liu et al., 2011). Although these human and rat results differ in direction and expression measure, they both indicate a relationship between α2 expression in the CeA and a chronic alcohol consumption phenotype. With respect to anxiety, diazepam-modulated inhibitory post-synaptic currents have been shown to be mediated by α2-containing receptors in the CeA and α1- and α2-containing receptors in the lateral/basolateral amygdala in mutant mice with diazepam-insensitive α1-, α2-, or α3-containing $GABA_A$ receptors (Marowsky, Fritschy, & Vogt, 2004). This goes beyond the effects of the lesioning of, and $GABA_A$ agonism within, the CeA on anxiety to provide evidence for the importance of α2 subunits in the CeA in regulating benzodiazepine-induced inhibition and related anxiolysis.

In what appears to be the only study with direct manipulation of α2 expression specifically in the CeA including an assessment of behavior, Liu et al. (2011) infused P rats with virally-mediated, small interfering RNA (siRNA) targeting either α1 or α2 subunit expression in the CeA, NAc, or ventral pallidum (VP) and assessed them on an operant, multiple-scheduled-access procedure. siRNA infusions resulted in a drastic reduction from baseline responding for alcohol (but not sucrose) when targeting α2 in the CeA and when targeting α1 in the VP, providing strong evidence for subunit-specific, subregion-specific effects within the extended amygdala. Interestingly, alcohol and

sucrose responding were both lower on the first day of assessment post-surgery; however, while sucrose responding returned immediately thereafter, alcohol responding slowly returned to baseline over the course of several days, which could suggest a stress-alcohol-α2 interaction within the CeA.

In summary, *GABRA2* appears to play a role in both alcohol- and anxiety-related behavior. The CeA is an important substrate for the mediation of these behaviors, and α2-containing $GABA_A$ receptors within this region appear to play an important role in each. The prospect that this subunit's expression within the CeA might play a role in their interaction (alcohol-induced anxiolysis) is intriguing. However, there appear to have been no studies manipulating α2 expression exclusively in the CeA and assessing its effects on basal anxiety and alcohol-induced anxiolysis. The current study sought to fill this gap.

1.6 Specific Aims

1. *Determine the appropriate parameters and dosage for assessing anxiety and alcohol's anxiolytic effects in the elevated plus maze in C57BL/6J mice.*
2. *Assess the effects of virally-mediated, Gabra2 knock-down in the central nucleus of the amygdala on basal anxiety and alcohol's anxiolytic effects in the elevated plus maze in C57BL/6J mice.*

CHAPTER 2 EFFECT OF CENTRAL AMYGDALA *GABRA2* EXPRESSION ON ANXIETY AND ALCOHOL'S ANXIOLYTIC CAPACITY

2.1 Materials and Methods

2.1.1 General Design

In brief, male B6 mice in Aim 1 were assessed in the EPM naïve or following IP injection of 0, 0.75, 1.0, or 1.5 g/kg ethanol to determine the appropriate parameters for subsequent assessment with viral manipulation. Male B6 mice in Aim 2 underwent surgery for bilateral infusion of GFP-tagged lentivirus targeting *Gabra2* or a scramble control lentivirus into the CeA. Following 12-13 days of recovery, mice were assessed in the EPM naïve or following IP injection of 0, 0.75, or 1.5 g/kg ethanol. After assessment, brains were extracted and sectioned through the CeA. Finally, GFP was quantified, the CeA was collected via laser microdissection, and α2 protein was quantified via ELISA.

2.1.2 Subjects

For Aim 1, a total of 60 male B6 mice were obtained from Jackson Labs (Bar Harbor, ME) at 8 weeks of age. For Aim 2, a total of 104 male B6 mice were obtained from Jackson Labs at 8 weeks of age, and of these, 6 mice died during or after surgery and 2 mice were not used due to cagemate death, leaving a total of 96 mice used for experimentation. Mice were 9-10 weeks of age at the time of surgery and 11-12 weeks of age at the time of behavioral assessment. All mice had ad libitum access to food and water for the duration of the experiment and were pair-housed (except for 5-6 days during

recovery from surgery) under a 12-hour, reverse light/dark schedule, with lights off at 8:00 am. 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. Surgery

Mice were anesthetized with a 0.01 ml/g IP injection of a ketamine/xylazine cocktail (10:1:89 ratio of ketamine (100 mg/ml), xylazine (100 mg/ml), and 0.9% sterile saline, respectively). Once anesthetized, mice had their heads shaved and were placed in a Kopf stereotaxic alignment system (Tujunga, CA). Mice's eyes were kept moist with commercially available tear ointment. A small incision was made from approximately bregma to lambda, and the skull was sterilized with 70% alcohol and Nolvasan disinfectant. For accuracy, the distance between bregma and lambda was measured and divided by 4.21 mm, the published average distance between lambda and bregma for B6 mice (Franklin and Paxinos, 1997), to create a scaling factor for each mouse. The targeted coordinates for the CeA (-1.20 mm A/P, ±2.80 mm M/L, and -4.85 mm D/V) were adjusted by the scaling factor, and one hole was drilled in the skull in each hemisphere above the CeA. A microinjection syringe (Hamilton Co., Reno, NV) was twice lowered into the CeA to bilaterally infuse a GFP-tagged lentivirus targeting *Gabra2* (Virus) via short hairpin RNA (shRNA) or a GFP-tagged scramble control lentivirus (Control) (Sigma-Aldrich, Inc., St. Louis, MO). An illustration and description of RNA interference via shRNA are given in **Figure 1**. Viruses were diluted 1:1 with 0.9% sterile saline, as exploratory surgery indicated this reduces extra-regional incorporation. This produced final titers of 2.4×10^9 and 2.2×10^9 TU/ml for Virus and Control lentiviruses,

respectively. 0.15 µl of virus was infused at a rate of 0.1 µl/min and allowed to diffuse for an additional 10 minutes. The microinjector was raised 0.05 mm between infusion and diffusion, as exploratory surgery indicated this reduces residual virus on the microinjector tip. Between infusions, the syringe pump (World Precision Instruments, Sarasota, FL) was run until virus was visible in order to confirm patency. Following infusions, incisions were sealed with VetBond skin glue (3M Animal Care, St. Paul, MN), and mice received 0.01 ml/g SC injections of buprenorphine (0.03 mg/ml) and rimadyl (5 mg/ml) for analgesia and anti-inflammation, respectively. Following surgery, mice were single-housed in a biohazard-approved, filter-top cage for 5-6 days of recovery and subsequently pair-housed with their original cagemate, with 4 exceptions. 2 mice were housed with an age-matched, surgery-naïve mouse, and 2 mice were housed with each other due to the death of their original cagemate during surgery.

2.1.4. EPM Assessment

The testing room was separated by a canvas curtain into two sections, one under red light (the same conditions as the vivarium) and the other, containing the EPM, under red light plus dim ambient lighting. The EPM (Med Associates, Inc., St. Albans, VT) was black in color, was 74.5 cm above the ground, and had arms measuring 76.0 cm in length. The closed arms had walls measuring 20.5 cm, and the open arms had no walls, but a slight lip on the sides but not ends. The luminance across testing was 26±2, 5±1, and 33±2 lux for Aim1 and 43±1, 6±1, and 54±4 lux for Aim 2, for the center, closed arms, and open arms, respectively. Luminance was increased slightly in Aim 2 in order to enhance the EPM's anxiogenic nature.

Previous work in our lab has shown viral incorporation and significant effects on alcohol consumption at 2 weeks post-infusion (Boehm, unpublished). Therefore, mice were assessed in the EPM following 12-13 days of recovery from surgery. Mice were transported to the testing room 5 minutes prior to lights off (8:00 am) and allowed to habituate in the non-maze portion of the testing room for at least 1 hour prior to assessment. Prior to placement on the EPM, each mouse was weighed and placed in an empty holding cage for 10 minutes. During this time, injected mice were taken just outside the testing room for 30 seconds to receive an IP injection of either 0, 0.75, or 1.5 g/kg of 20% ethanol in 0.9% sterile saline (Aim 1 also included a 1.0 g/kg dose). All injections took place 10 minutes prior to placement in the EPM. Mice were placed in the center of the EPM, facing an open arm, and allowed to explore for 5 minutes while being video-recorded by a camera mounted directly overhead. All condition-dose combinations were represented on each testing day except for 2 instances.

Following assessment, the number of fecal boli were counted, and the EPM was cleaned with Clidox-S. Retro-orbital sinus blood samples were collected in Aim 1 from a subset of mice in each injection condition (n's = 8) to determine BEC resulting from IP administration of ethanol. Videos were later scored for entries and time spent in center, closed arms, and open arms as well as the number of head dips made. Mice were considered to have entered an arm when all 4 paws transitioned from outside to inside an arm and considered to be in an arm as long as all 4 paws remained inside. Mice were considered to have made a head dip when the shoulders moved forward and the head was facing downward over the edge of the EPM. Head dips were differentiated as protected when initiated from outside of an open arm.

2.1.5. Histology

Approximately 2-4 hours after assessment in the EPM, mice's brain were extracted, flash frozen in 2-methylbutane at < -40° C, and stored at -80° C until processed. Brains were sliced in a cryostat at -16° C. Serial sections through the CeA at 35 µm thickness were mounted to PEN-Membrane slides, dehydrated with ascending concentrations of ethanol (70% - 5 sec, 95% - 5 sec, 100% - 20 sec), and stored at -80° C until microdissection. For sections containing the CeA, tissue was laser microdissected using the following technique. The area expressing GFP was encircled under 5x magnification with fluorescence using laser microdissection software (Leica Biosystems Inc., Buffalo Grove, IL), which calculates area in nm2. Bilateral CeA was then laser microdissected under 5x magnification in bright field while referencing a mouse brain atlas (Paxinos & Franklin, 1997), and the tissue was captured in 1x RIPA buffer (1x RIPA buffer with 100 µl/ml of 10x PI and 10 µl/ml of 20x PMSF). The remaining area expressing GFP was again encircled and calculated under 5x magnification with fluorescence. Bilateral CeA tissue was collected from all sections whether or not they expressed GFP fluorescence. The pre- and post-microdissection GFP area calculations were used to estimate the amount of GFP expressing tissue within and without the CeA. Tissue samples were homogenized manually, and total protein concentration was calculated for each. Samples were assessed via ELISA (MyBioSource, Inc., San Diego, CA), in triplicate and with 60 µg of total protein loaded per sample, for quantification of α2 protein concentration according to the manufacturer's suggested procedures. An illustration and description of the sandwich ELISA assay are given in **Figure 2**. A total of 4 ELISA assays were needed to assess all samples, with all 3 samples from each brain

run within one assay, and the mean value of samples from each brain was used as the α2 protein concentration.

2.1.6. Statistical Analysis

Statistical significance for all analyses was set at $p < .05$. For Aim 1, confirmation of the anxiogenic nature of the EPM was assessed using only naïve mice. Both a single-sample t-test comparing % of entries in open arms to 50% and a paired-samples t-test comparing time spent in the open vs. closed arms were run. To determine appropriate doses for future assessment in Aim 2, the following measures were submitted to a one-way ANOVA with dose (0, 0.75, 1.0, 1.5 g/kg) as the lone factor, using only mice receiving injection: % of entries in open arms, time spent in open arms, and total arm entries. Each ANOVA was followed by Dunnett's test using the 0 g/kg dose as the reference.

For Aim 2 histological analyses, only mice expressing GFP in the CeA were included, and mice with total protein and/or α2 protein concentration values ≥ 3 SD from the overall mean were excluded (see **Table 1** for n's). Consistency of ELISA output was assessed using a one-way ANOVA on α2 protein concentrations, with ELISA pass as the lone factor, for Control mice only. Because there was a significant effect of ELISA pass on α2 protein concentrations, $F(3,27) = 3.84$, $p = .021$, mean α2 protein concentrations for all mice were normalized via z-score transformations based on distributions within their respective ELISA pass (**Fig. 5A**). Normalized (z-score) values were used to represent α2 protein concentrations for all subsequent analyses. A one-tailed, independent samples t-test was used to test the prediction that α2 protein concentrations would be lower in the Virus relative to the Control condition.

For Aim 2 behavioral analyses, only mice expressing GFP in the CeA were included, and mice with ≤ 1 open arm entry or an environmental confound (noise during assessment) were excluded (see **Table 1** for n's). To limit the influence of general activity levels on measures of arm entries, the % of entries in open arms ((open/total) x 100) was calculated for each mouse. In addition, % of head dips protected ((protected/total) x 100) was calculated for each mouse. Lastly, latency to first arm entry and fecal boli were largely unrelated to other EPM measures and were not analyzed further (**Table 2**). The following behavioral measures were used as dependent variables for analyses: total arm entries; % of entries in open arms; total and average time spent in the center, closed arms, and open arms; head dips; and % of head dips protected. To assess the effect of viral condition on basal anxiety, these measures were each submitted to an independent-samples t-test comparing Control to Virus using only naïve mice. To assess both alcohol's anxiolytic capacity and potential augmentation by viral condition, these measures were each submitted to a two-way ANOVA with viral condition and dose as factors using only mice receiving 0, 0.75, or 1.5 g/kg ethanol injections. Main effects of dose were followed by Dunnett's test using the 0 g/kg dose as the reference, and main effects of viral condition were followed by Bonferroni-corrected t-tests comparing Control to Virus within each dose.

For Aim 2 brain-behavior correlations, all mice were included, with the following exceptions: mice with total protein and/or α2 protein concentration values ≥ 3 SD from the overall mean and mice with ≤ 1 open arm entry or an environmental confound (see **Table 1** for n's). The relationships between α2 protein concentrations and behavioral

measures in the EPM, as well as the relationships between the various EPM behavioral measures, were assessed using Pearson correlations.

2.2 Results

2.2.1. Aim 1

The anxiogenic nature of the EPM was confirmed for this experiment by mice making significantly fewer open arm entries than would be expected by chance (50%), $t(11) = -4.15$, $p = .002$ and by mice spending significantly less time in open vs. closed arms, $t(11) = 6.35$, $p < .001$ (data not shown). The anxiolytic capacity of alcohol was confirmed by a significant increase in the % of entries in open arms, $F(3, 38) = 4.57$, $p = .008$, with Dunnett's test indicating that all doses differed significantly from 0 g/kg (p's $< .048$) (**Fig. 3A**); however, alcohol was not found to affect either time spent in open arms, $F(3, 38) = 1.63$, $p = .199$ or total arm entries, $F(3, 38) = 2.54$, $p = .071$ (**Figs. 3B** and **3C**, respectively). Based on these results, the 0.75 and 1.5 g/kg doses were chosen for use in Aim 2. These doses produced mean BEC values of 74.58 and 151.62 mg%, respectively.

2.2.2. Histology

Representative images showing both successful and unsuccessful viral incorporation with reference to a mouse brain atlas (Paxinos & Franklin, 1997) are shown in **Figure 4**. As shown in **Figure 5B**, a one-tailed, independent-samples t-test revealed a decrease in normalized α2 protein concentrations for Virus mice relative to Control mice, $t(62) = 1.81$, $p = .038$, thus confirming the effectiveness of the virus in reducing expression of the target protein. An assessment of the potential impact of dose on these

normalized protein concentrations via two-way ANOVA yielded no significant effect of dose or dose x condition interaction (p's > .470, data not shown).

2.2.3. EPM Assessment

The anxiogenic nature of the EPM was confirmed for this experiment by mice making significantly fewer open arm entries than would be expected by chance (50%), $t(10) = -4.97$, $p = .001$ and by mice spending significantly less time in open vs. closed arms, $t(10) = 4.61$, $p = .001$ (**Figs. 6B** and **7B/C**, respectively). Regarding basal anxiety in naïve mice, there were no significant differences between Control mice and Virus mice on any behavioral measure (p's > .376) (**Figs. 6**, **7**, and **8**). Regarding alcohol's anxiolytic capacity in mice receiving 0, 0.75, or 1.5 g/kg ethanol injections, two-way ANOVA's yielded a significant main effect of viral condition for average time spent in closed arms, $F(1, 44) = 5.23$, $p = .027$, with average time reduced in Virus mice relative to Control mice (**Fig. 7E**). Post hoc independent-samples t-tests yielded no significant differences between Virus mice and Control mice at any given dose (p's > .104). In contrast to viral condition, a significant main effect of dose was found for several behavioral measures including % of entries in open arms, $F(2, 44) = 9.73$, $p < .001$, time spent in center, $F(2, 44) = 7.10$, $p = .002$, time spent in open arms, $F(2, 44) = 3.60$, $p = .036$, head dips, $F(2, 44) = 7.20$, $p = .002$, and % of head dips protected, $F(2, 44) = 4.98$, $p = .011$ (**Figs. 6B**, 7**A**, **7C**, **8A**, and **8B**, respectively). Follow-up Dunnett's tests using the 0 g/kg dose as the reference revealed that both the 0.75 and 1.5 g/kg doses increased % of entries in open arms ($p = .03$ and $p < .001$, respectively). However, it was only the 1.5 g/kg dose that decreased time spent in center ($p = .001$), increased time spent in open arms ($p = .022$), increased head dips ($p = .002$), and decreased % of head dips

protected (p = .011). There were no significant interactions between viral condition and dose for any behavioral measure (p's > .186) (**Figs.6**, **7**, and **8**).

2.2.4. Brain-Behavior Correlations

Pearson correlations were run within each dose (and naïve) to further explore the potential relationship between CeA α2 protein concentration and behavior. For naïve mice, normalized α2 protein concentration was significantly positively correlated with time spent in closed arms, $r(22)$ = .449, p = .036 and significantly negatively correlated with both time spent in open arms overall $r(22)$ = -.519, p = .013 and on average $r(22)$ = -.463, p = .03 (**Figs. 9A**, **9B**, and **9C**, respectively). There were no significant correlations between these measures in mice receiving 0, 0.75, or 1.5 g/kg ethanol (p's > .068) (**Table 2**).

CHAPTER 3 DISCUSSION

3.1. EPM & Alcohol's Anxiolytic Capacity

The current EPM parameters were confirmed to be anxiogenic in completely naïve mice (Aim 1) as well as mice having undergone surgery (Aim 2). As expected, alcohol was found to be an effective anxiolytic in both Aims; however, this effect was more pronounced in Aim 2. In addition to differential histories, naive (Aim 1) vs. surgery (Aim 2), EPM luminance was increased for Aim 2, and each aim was run as a separate experiment, so a direct comparison of alcohol's anxiolytic capacity between them may not be appropriate. However, these parameters did provide an appropriate context for the assessment of the effect of CeA *Gabra2* expression on basal anxiety and alcohol's anxiolytic capacity.

3.2. Virally-Mediated Gabra2 Knock-Down

Several mice in this experiment exhibited no GFP expression, yet all mice had visible needle track marks in or near the CeA. Lack of expression could have been due to clogged/blocked microinjector tips or ineffective viral incorporation. Although, considering the majority of mice exhibited GFP expression, ineffective incorporation seems much less likely. Quantification of the area of GFP expression within the CeA was intended to provide an indirect measure of the amount/degree of viral incorporation. While there should have been no relationship between CeA GFP expression and α2

protein concentration in Control mice, a negative relationship was expected for Virus mice, as more incorporation should have produced a greater knock-down. On the contrary, there was a positive correlation between CeA GFP expression and α2 protein concentration in Virus mice. Both the inability to measure GFP expression in three dimensions and the inability to account for the intensity of fluorescence could have been responsible for this unexpected result. Therefore, GFP expression was useful as an indicator of viral incorporation but not as a measure of amount/degree of incorporation. Overall, lentivirus targeting *Gabra2* was effective in reducing α2 protein concentrations relative to a scramble control lentivirus.

3.3. CeA *Gabra2* Expression & Basal Anxiety

The current study found no significant difference on any behavioral measure between naïve Control and Virus mice. However, when looking at naïve mice collectively, there was a significant relationship between CeA α2 protein concentration and multiple behavioral measures. This discrepancy could be accounted for by the fact that the statistically significant reduction in α2 protein concentrations seen for Virus mice relative to Control mice may not have been behaviorally significant. Taken together, the results from naïve mice support a role for CeA *Gabra2* expression in basal anxiety, specifically a positive relationship between CeA α2 protein concentration and anxiety-like behavior. In addition to the significant correlations, the relationships between α2 protein concentration and behavior were all consistently in this direction (except average time spent in closed arms), and although not statistically significant, the difference in means between Control mice and Virus mice for these behaviors were also consistently in the same direction (except head dips).

The current demonstration of a *Gabra2*-anxiety relationship is in line with several previous studies. In humans, haplotype configurations within *GABRA2* have been found to be associated with harm avoidance, the tendency to be anxious and fearful (Enoch et al., 2006). Of course these haplotypes don't indicate a specific change in α2 protein concentration, and these individuals likely had varied environmental histories. Similarly, compared to wild-type mice, α2 knock-out mice show alterations in anxiety-like behavior, specifically increased risk-assessment in and latency to explore a novel environment as well as decreased time in light in a L/D test (Koester et al., 2013). That this negative relationship between *Gabra2* expression and anxiety-like behavior is opposite in direction to the current results could be due to a number factors including the congenital and global nature of the *Gabra2* manipulation, the type of assessment used, and the different strain of mouse used (129X1/SvJ). Nevertheless, it supports a *Gabra2*-anxiety relationship in animals with controlled environmental histories. The current results also implicate α2 protein concentration specifically in the CeA in affecting anxiety-like behavior. Additional support for this relationship comes from the observation that naïve P rats have elevated CeA α2 protein concentrations compared to NP rats (Liu et al., 2011) and also display greater anxiety-like behavior in the EPM (R. B. Stewart, Gatto, Lumeng, Li, & Murphy, 1993). Although there are likely several structural/functional differences between P and NP rats, the difference in CeA α2 protein concentrations is significant (~100% increase) and the direction of its relationship to behavior is the same as in the current study. Finally, while the current results can't rule out other brain-related factors as contributing to the observed behavioral differences, the fact that B6 mice are inbred, resulting in relatively homogeneous brain structure/function,

and that viral manipulation of the CeA produced significant alterations in α2 protein concentrations specifically in this region strengthens the interpretation that CeA *Gabra2* expression is positively related to basal anxiety.

3.4. CeA *Gabra2* Expression & Alcohol's Anxiolytic Capacity

Although alcohol was clearly found to be anxiolytic in this study, affecting a number of EPM measures in a predictable fashion, there was no interaction between viral condition and dose on any measure and only a main effect of viral condition across doses on one measure, average time spent in closed arms. That this behavior alone, and not in conjunction with behaviors typically reported in EPM studies (e.g. time spent in open arms and % of entries in open arms), was affected by viral condition decreases confidence in the interpretation that a reduction in α2 protein concentration is anxiogenic across these doses. In fact, mean group differences for this behavior are most pronounced at the 0.75 and 1.5 g/kg doses, yet correlations indicate a null relationship and a weak relationship in the opposite direction for these doses, respectively. Furthermore, increases in average time spent in closed arms could be explained by behaviors not necessarily related to anxiety, like increases in grooming. Finally, there were no significant relationships between α2 protein concentration and behavioral measures within any dose. Taken together, these results suggest that alterations in CeA *Gabra2* expression do not affect alcohol's anxiolytic capacity.

A lack of effect of *Gabra2* expression on alcohol's anxiolytic capacity is consistent with a lack of effect seen in both α2 knock-out mice (Boehm II et al., 2004) and knock-in mice containing alcohol-insensitive α2-containing $GABA_A$ receptors (Blednov et al., 2011). However, this is in contrast to the role of *Gabra2* expression in

mediating the anxiolytic actions of benzodiazepines, as mice with benzodiazepine-insensitive α2-containing $GABA_A$ receptors fail to show an anxiolytic response to these drugs in a number of behavioral assessments (Low et al., 2000; K. S. Smith et al., 2012). In addition, while modulation of inhibitory post-synaptic currents by diazepam in the CeA appears to be mediated by α2-containing $GABA_A$ receptors (Marowsky et al., 2004), the results of the current study do not suggest a role for CeA α2 subunits in alcohol's pharmacologic actions. This difference could be related to, among other things, alcohol's enhancement of inhibitory GABAergic transmission in the CeA via both pre- and post-synaptic mechanisms (Roberto, Madamba, Moore, Tallent, & Siggins, 2003) or alcohol's interactions with anxiogenic CRF and anxiolytic neuropeptide Y (NPY) signaling in the CeA (Gilpin, Herman, & Roberto, 2015). Taken together, these studies suggest a drug- or drug class- specific relationship between α2 subunits and anxiolysis.

3.5. Conclusions, Limitations, & Future Directions

The current study supports the idea that CeA α2 protein expression is positively related to anxiety. Enoch et al. (2006) found that risk (of alcohol dependence)-conferring *GABRA2* haplotypes were associated with trait anxiety, which was increased in alcoholics, and one could speculate that these individuals had increased CeA *GABRA2* expression. However, post-mortem analysis of CeA tissue from alcoholics has revealed decreased *GABRA2* mRNA expression (Jin et al., 2014). These findings can be reconciled when considering that a substantial change in CeA α2 protein expression and corresponding change in basal anxiety in either direction could lead to increases in alcohol use via different routes. Increased anxiety, associated with an increase in CeA α2 protein expression, would lead to an increase in the opportunity for alcohol to provide

relief and acquire negatively-reinforcing properties. This negative-reinforcement route is direct and fairly intuitive, and it is consistent with several models of human alcoholism (J. P. Smith & Randall, 2012). On the other hand, decreased anxiety, associated with a decrease in CeA α2 protein expression, might lead to alcohol use through a more indirect route via a relationship with externalizing behaviors, which are commonly found to be comorbid with substance use disorders (SUDs) (Hofmann, Richey, Kashdan, & McKnight, 2009). In a direct assessment of the relationship between anxiety, externalizing problems, and SUDs, Hofmann, Richey, Kashdan, and McKnight (2009) found that while having either an anxiety disorder or externalizing problems increased the probability of having a SUD, the probability of having a SUD for individuals with externalizing problems was decreased for those with a comorbid anxiety disorder compared to those without. Thus anxiety can moderate the relationship between externalizing problems and substance use, with decreased anxiety being associated with greater substance use in those with externalizing problems.

Interestingly, both *GABRA2* and the amygdala have been associated with externalizing behaviors. With respect to *GABRA2*, a risk (of alcohol dependence)-conferring *GABRA2* genotype has been found to be associated with a greater likelihood of maintaining elevated expression of externalizing behaviors across the transition from adolescence to adulthood (Dick et al., 2009). With respect to the amygdala, preschool externalizing behavior has been shown to predict decreased amygdala volume in adolescence in males (Caldwell et al., 2015), and adolescents with conduct disorder have also been shown to have decreased amygdala volume (Wallace et al., 2014). While there is growing evidence that *GABRA2*, anxiety, externalizing behavior, and alcohol use all

share some degree of interrelation, the exact nature of these relationships is not clear. Future studies could further investigate the *GABRA2*-anxiety relationship by assessing (central) amygdala structure/connectivity or activity in response to anxiogenic cues/situations as a function of *GABRA2* genotype in individuals prior to the initiation of alcohol use. In addition, a longitudinal study assessing *GABRA2* genotype in relation to measures of anxiety and externalizing behaviors before and after the initiation of alcohol use might further elucidate the impact and potential interaction of these *GABRA2*-associated phenotypes in relation to alcohol use.

In contrast to basal anxiety, the current study does not support the idea that CeA α2 protein expression is related to alcohol's anxiolytic capacity. However, one limitation of the current study was that it assessed this capacity with only a single acute exposure. Several studies indicate that chronic exposure to alcohol can alter basal GABAergic transmission and response to GABAergic drugs in the CeA (Koob, 2004), and it appears that these alterations might affect anxiety-like behavior. Sharko et al. (2013) assessed anxiety-like behavior in the EPM using rats with water or low- or high-drinking alcohol histories. High-drinking rats had greater CeA c-Fos expression, and CeA c-Fos expression was associated with anxiety-like behavior in alcohol- but not water-drinking animals, suggesting alcohol-induced alterations in the relationship between CeA activity and anxiety. Withdrawal from chronic alcohol exposure can also augment CeA functioning with respect to anxiety, as infusion of a CRF antagonist in the CeA has been shown to be anxiolytic in alcohol-dependent but not non-dependent animals 8 hours post-exposure (Rassnick et al., 1993). Lastly, Liu et al. (2011) found a decrease in alcohol consumption in rats with virally-mediated CeA *Gabra2* knock-down which occurred just

3 days after invasive brain surgery, which could be interpreted as a change in the negatively-reinforcing capacity of alcohol. Given that these rats had 21 days of drinking history prior to surgery, alcohol-induced changes in CeA functioning could play a role in this finding, and it remains to be seen how CeA *Gabra2* manipulation would affect drinking in alcohol-naïve animals.

Taken together, the aforementioned studies provide evidence for alcohol history as being an important factor in the CeA-alcohol-anxiety relationship and provide rationale for future investigations. Surprisingly, it appears that an assessment of the effect of alcohol infusion directly into the CeA on anxiety-like behavior has not been conducted. Performing this assessment in both alcohol-naïve and chronically alcohol-exposed animals would help to clarify the role of the CeA in alcohol's anxiolytic capacity and address potential augmentation of this role based on prior exposure. In addition, replicating the current study in chronically exposed mice would indicate whether or not the relationship between CeA *Gabra2* expression and anxiety-like behavior is still present after a history of alcohol exposure and whether or not a history of alcohol exposure alters the lack of relationship between CeA *Gabra2* expression and alcohol's anxiolytic capacity.

One major limitation of the current study that has yet to be mentioned is that only male mice were used, yet gender differences do exist for many of the behaviors discussed. Females have a higher prevalence of anxiety disorders than males even though age of onset and duration tend not to differ (McLean, Asnaani, Litz, & Hofmann, 2011). In the context of alcohol, females and males with high anxiety sensitivity differ in the degree to which the nature of a stressor impacts the acute effects of consumed alcohol

(Zack, Poulos, Aramakis, Khamba, & MacLeod, 2007). With respect to *GABRA2*, in individuals with a risk (of alcohol dependence)-conferring *GABRA2* genotype, increased levels of positive life events serves as a protective factor for males but not females (Perry et al., 2013). Finally, α2 knock-out and knock-in mice have shown sex-dependent differences in alcohol consumption (Blednov et al., 2011; Boehm II et al., 2004). Given these, and other, gender differences, especially regarding anxiety and alcohol use, an important future study would be to replicate the current experiment in female mice.

In summary, CeA α2 protein expression does not appear to be related to alcohol's anxiolytic capacity but does appear to be positively related to basal anxiety. The CeA may be a region where changes in α2 protein expression resulting from *GABRA2* polymorphisms could affect basal anxiety, which in turn could affect alcohol use through various routes. Investigating the role that these subunits play in GABAergic transmission within the CeA and its effect on efferent signaling at downstream targets in response to anxiogenic or stressful conditions could lead to a better understanding of mechanisms underlying the relationship between anxiety and alcohol use.

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TABLES

TABLES

Table 1. Group sizes overall and for specific analyses.

		All	Exclusions			Used for Analyses		
			GFP	Protein	Behavior	Brain	Behavior	Correlations
Control	NI	11	2	0	-	9	-	-
	0	12	6	0	-	6	-	-
	0.75	12	4	0	-	8	-	-
	1.5	12	3	1	-	8	-	-
Virus	NI	12	3	1	-	8	-	-
	0	12	2	1	-	9	-	-
	0.75	13	4	1	-	8	-	-
	1.5	12	3	1	-	8	-	-
Total		96	27	5	-	64	-	-

		All	Exclusions			Used for Analyses		
			GFP	Protein	Behavior	Brain	Behavior	Correlations
Control	NI	11	2	-	0	-	9	-
	0	12	6	-	0	-	6	-
	0.75	12	4	-	0	-	8	-
	1.5	12	3	-	0	-	9	-
Virus	NI	12	3	-	0	-	9	-
	0	12	2	-	1	-	9	-
	0.75	13	4	-	0	-	9	-
	1.5	12	3	-	0	-	9	-
Total		96	27	-	1	-	68	-

		All	Exclusions			Used for Analyses		
			GFP	Protein	Behavior	Brain	Behavior	Correlations
Control	NI	11	-	0	0	-	-	11
	0	12	-	0	0	-	-	12
	0.75	12	-	0	1	-	-	11
	1.5	12	-	2	0	-	-	10
Virus	NI	12	-	1	0	-	-	11
	0	12	-	1	1	-	-	10
	0.75	13	-	0	0	-	-	13
	1.5	12	-	1	1	-	-	10
Total		96	-	5	3	-	-	88

Note: Mice with multiple exclusions are listed in their leftmost occurring exclusion column.

Table 2. Pearson correlations between behavioral measures overall (Top) and between normalized (Z-Score) CeA α2 expression and behavioral measures in naïve mice and within each ethanol dose (Bottom). Behavioral measures in parentheses were excluded from additional analyses. ^$p < .05$, *$p < .01$, **$p < .001$ uncorrected for multiple comparisons. Bold values are significant at the more stringent criteria of $p < .01$.

	Entries Total	%Entries OA	Time Center	Time CA	Time OA	Avg. Time Center	Avg. Time CA	Avg. Time OA	(1st Entry Latency)	Head Dips	% Protected HD	(Fecal Boli)
Entries Total	1											
%Entries OA	-0.01	1										
Time Center	**0.31** *	**-0.52** **	1									
Time CA	-0.13	**-0.61** **	0.12	1								
Time OA	-0.05	**0.75** **	**-0.59** **	**-0.87** **	1							
Avg. Time Center	**-0.49** **	**-0.38** **	**0.59** **	0.06	**-0.34** *	1						
Avg. Time CA	**-0.67** **	0.20	**-0.39** **	**0.43** **	-0.15	0.17	1					
Avg. Time OA	**-0.51** **	0.24 ^	**-0.39** **	**-0.56** **	**0.65** **	0.26 ^	0.02	1				
(1st Entry Latency)	-0.11	0.05	0.24 ^	-0.10	-0.03	**0.43** **	0.04	0.19	1			
Head Dips	0.21	**0.57** **	-0.22 ^	**-0.49** **	**0.51** **	-0.27	-0.09	0.14	0.03	1		
% Protected HD	0.03	**-0.64** **	**0.60** **	**0.45** **	**-0.66** **	**0.43** **	-0.11	**-0.36** *	0.06	**-0.47** **	1	
(Fecal Boli)	-0.21 ^	-0.05	-0.19	0.05	0.05	0.10	0.09	**0.35** *	0.12	-0.16	0.02	1
CeA α2 - NI	0.27	-0.37	0.19	0.45 ^	-0.52 ^	-0.01	-0.17	-0.46 ^	-0.22	-0.32	0.22	0.03
CeA α2 - 0 g/kg	0.09	0.19	0.15	-0.23	0.09	0.11	-0.07	0.09	0.34	-0.01	-0.12	0.25
CeA α2 - 0.75 g/kg	0.01	-0.19	-0.08	0.25	-0.16	-0.12	0.00	-0.11	0.36	-0.26	0.13	-0.14
CeA α2 - 1.5 g/kg	-0.42	-0.19	-0.15	0.19	-0.11	0.15	0.28	0.05	0.07	-0.12	0.22	-0.01

FIGURES

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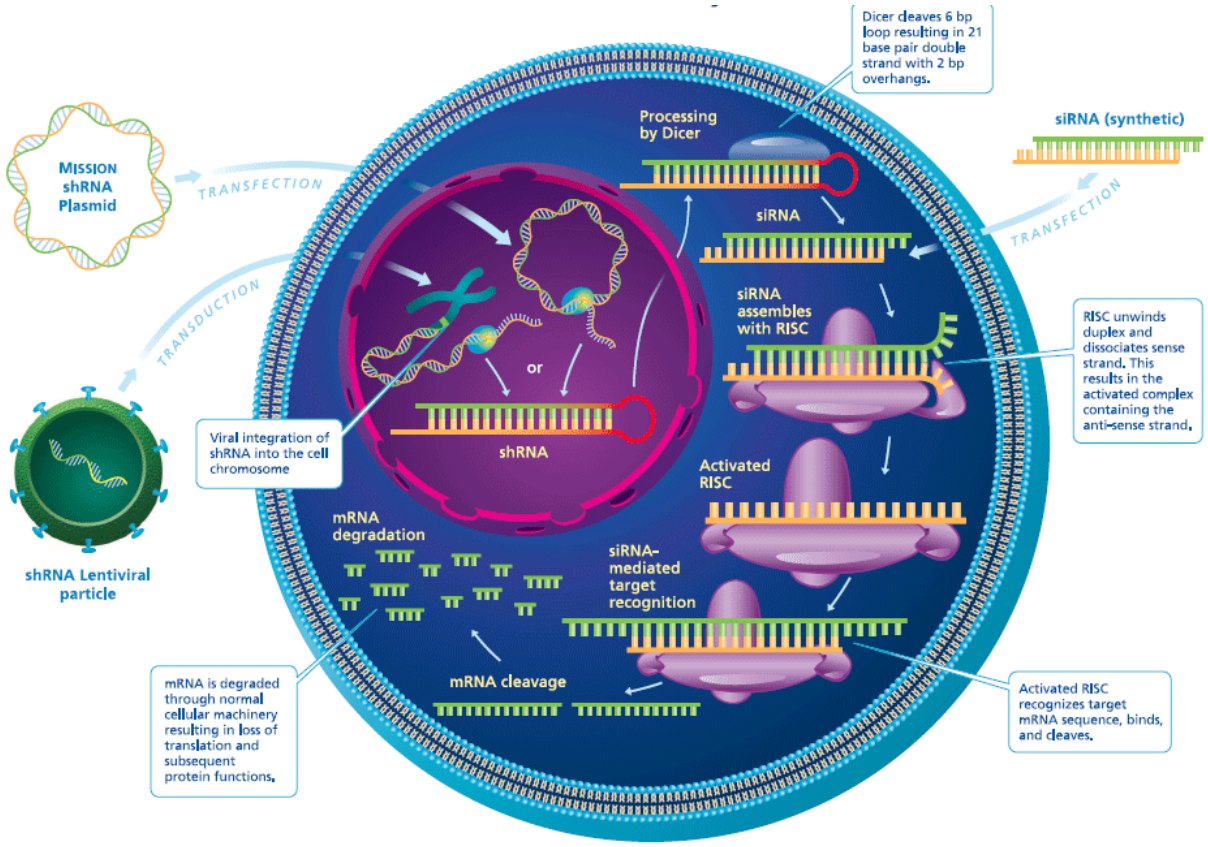

Figure 1. RNA interference via shRNA. Illustration taken from (Jolliff, 2007) and description synthesized from (Hutvagner & Zamore, 2002; Moore, Guthrie, Huang, & Taxman, 2010; S. A. Stewart et al., 2003). Lentiviral transduction delivers a *Gabra2*-targeting genetic sequence which is integrated with a cell's DNA. The cell is induced to synthesize a double-stranded RNA (dsRNA) comprised of sense and anti-sense sequences linked by a short hairpin loop (shRNA), with the anti-sense sequence being complementary to endogenous *Gabra2* mRNA. The enzyme Dicer cleaves shRNA into small interfering RNA (siRNA) which is incorporated into a protein complex to form an RNA-induced silencing complex (RISC). RISC uses siRNA as a guide to cleave mRNA that is complementary to the siRNA, in this case *Gabra2*.

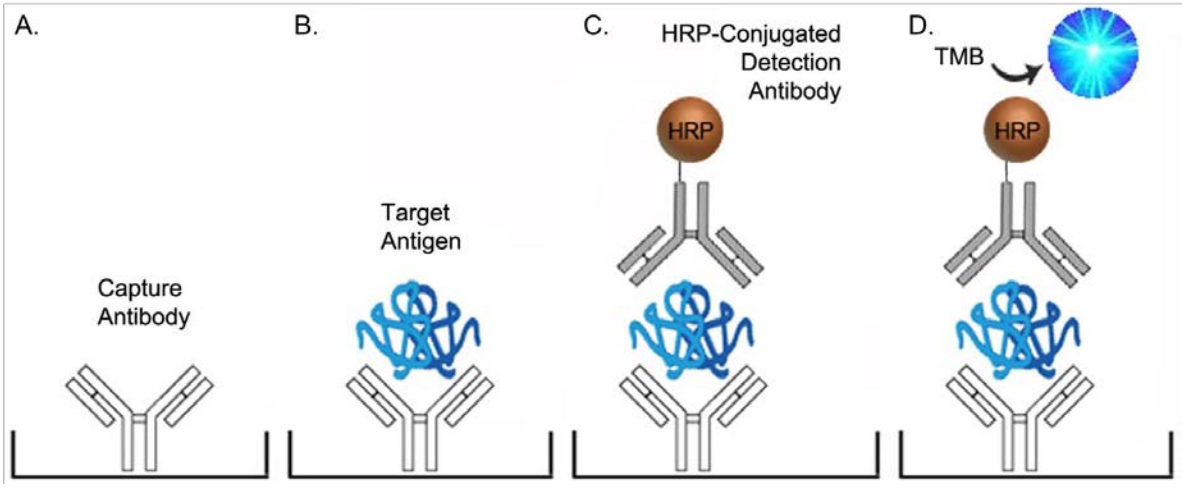

Figure 2. Sandwich ELISA procedure. Illustration adapted from LifeSpan Biosciences, Inc. (lsbio.com). A) The wells on each ELISA plate come pre-coated with a *Gabra2*-specific capture antibody (monoclonal, developed using rats). B) Samples and standards are loaded into wells and incubated, and the excess liquid is removed. α2 protein should remain bound to the capture antibody. C) A second *Gabra2*-specific detection antibody (polyclonal, developed using rabbits), conjugated with horseradish peroxidase (HRP), is added to each well and incubated, and the wells are subsequently washed of unbound antibody. D) HRP substrate solution, containing tetramethylbenzidine, is added to each well and incubated, resulting in blue color within each well proportional to α2 protein concentration. A stop solution is then added, ceasing color development. Finally, the optical density of each sample is determined via spectrophotometry at the 450nm wavelength using a microplate reader.

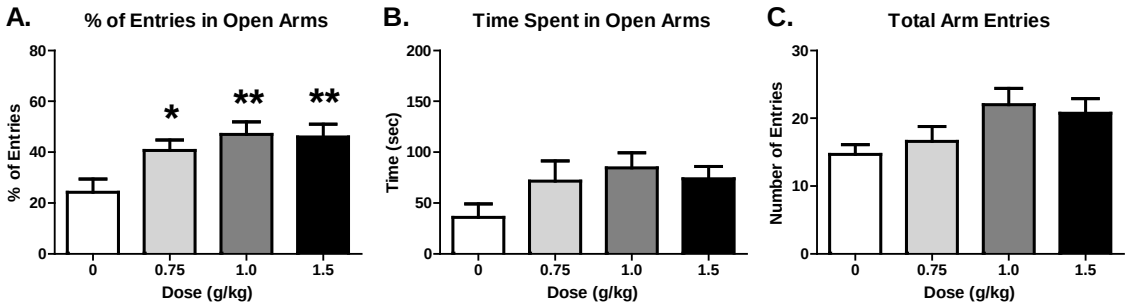

Figure 3. EPM behaviors in mice receiving 0, 0.75, 1.0, or 1.5 g/kg ethanol in Aim 1 (n's = 10, 12, 9, 11 (left to right)). A) All doses of ethanol increased % of entries in open arms. However, there was no significant effect of dose on either (B) time spent in open arms or (C) total arm entries. *$p < .05$, **$p < .01$ vs. 0 g/kg.

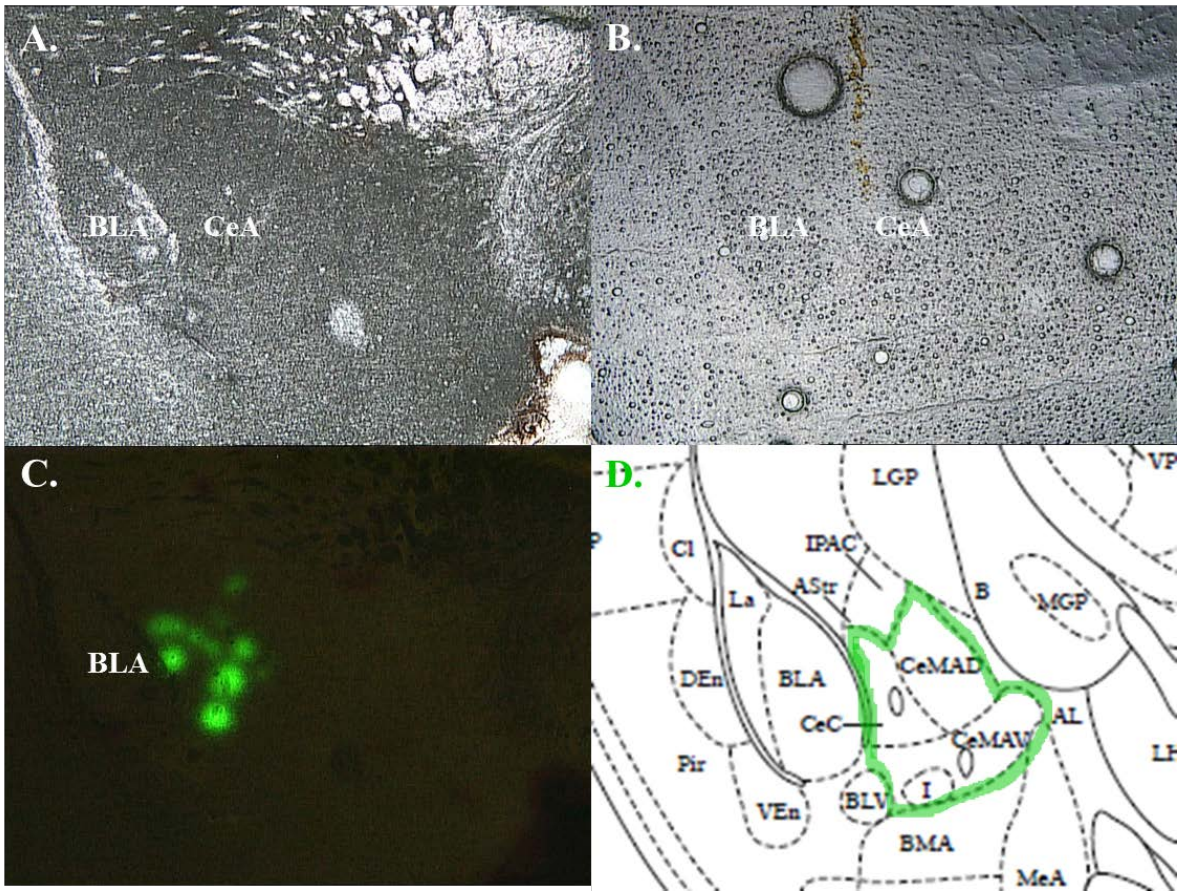

Figure 4. Lentiviral incorporation in the CeA. A) CeA and surrounding areas under 5x magnification in bright field. C) The same section under 5x magnification with fluorescence showing GFP expression in the CeA. B) A section under 5x magnification showing accurate microinjector placement in the CeA; however, this brain showed no GFP expression. D) Mouse brain atlas with the CeA highlighted for reference.

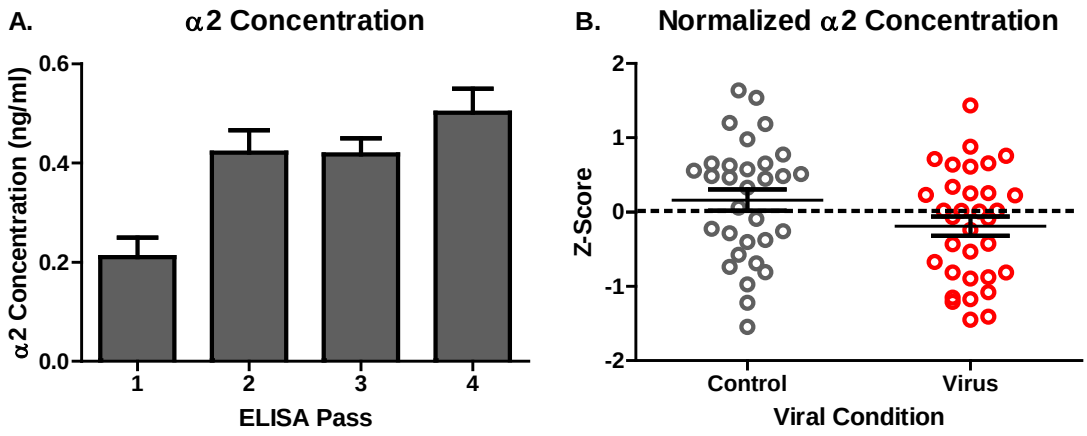

Figure 5. CeA α2 protein concentrations. A) CeA α2 protein concentrations differed across ELISA passes (n's = 3, 8, 8, 12 (left to right)). B) Normalized (Z-Score) α2 concentrations were lower for Virus mice than Control mice (n's = 31, 33 (left to right)).

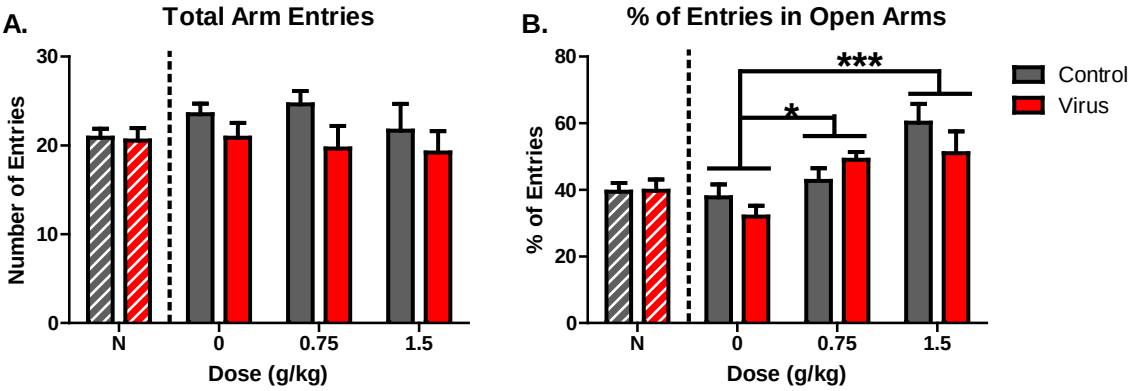

Figure 6. Arm entries made by naïve mice and mice receiving 0, 0.75, or 1.5 g/kg ethanol (n's = 9, 9, 6, 9, 8, 9, 9, 9 (left to right)). A) There was no effect of viral condition or dose on total arm entries for naïve mice or mice receiving injection. B) In naïve mice or mice receiving injection, there was no effect of viral condition on % of entries in open arms. However, % of entries in open arms was increased for mice receiving 0.75 or 1.5 g/kg ethanol. $*p < .05$, $***p < .001$ vs. 0 g/kg.

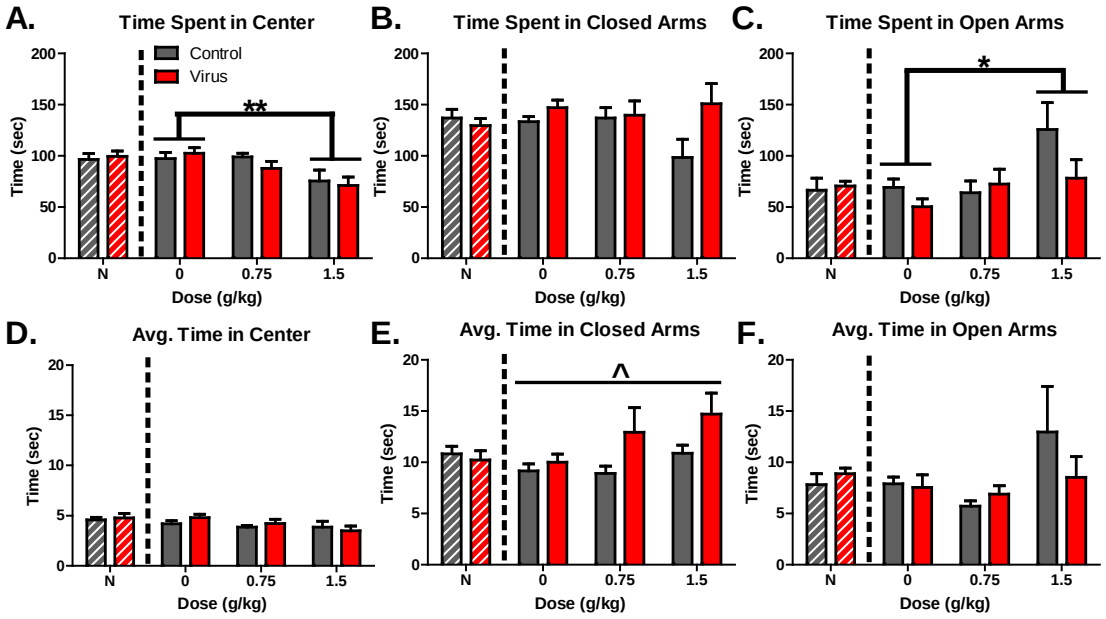

Figure 7. Total and average time spent in center, closed arms, and open arms by naïve mice and mice receiving 0, 0.75, or 1.5 g/kg ethanol (n's = 9, 9, 6, 9, 8, 9, 9, 9 (left to right)). In naïve mice, there was no effect of viral condition on any time-related measure (A-F). In mice receiving injection, 1.5 g/kg ethanol reduced time spent in center (A) and increased time spent in open arms (C). Also in mice receiving injection, Virus mice showed a trend ($p = 0.059$) towards increased time spent in closed arms (B) and showed an increase in average time spent in closed arms (E) compared to Control mice. There was no effect of viral condition or dose on average time spent in center (D) or open arms (F). *$p < .05$, **$p < .01$ vs. 0 g/kg, ^$p < .05$ vs. Control.

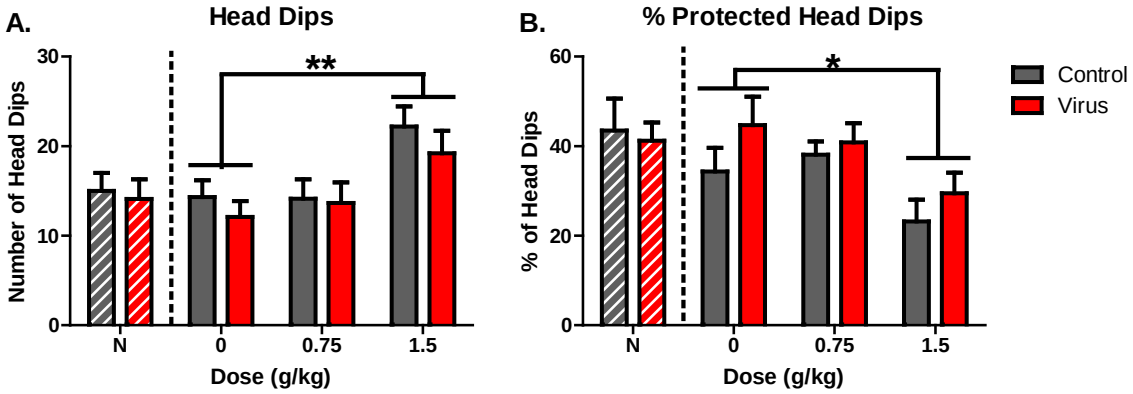

Figure 8. Head dips and % protected head dips made by naïve mice and mice receiving 0, 0.75, or 1.5 g/kg ethanol (n's = 9, 9, 6, 9, 8, 9, 9, 9 (left to right)). In naïve mice, there was no effect of viral condition on head dips or % protected head dips (A-B). However, in mice receiving injection, 1.5 g/kg ethanol increased head dips (A) and decreased % protected head dips (B). *$p < .05$, **$p < .01$ vs. 0 g/kg.

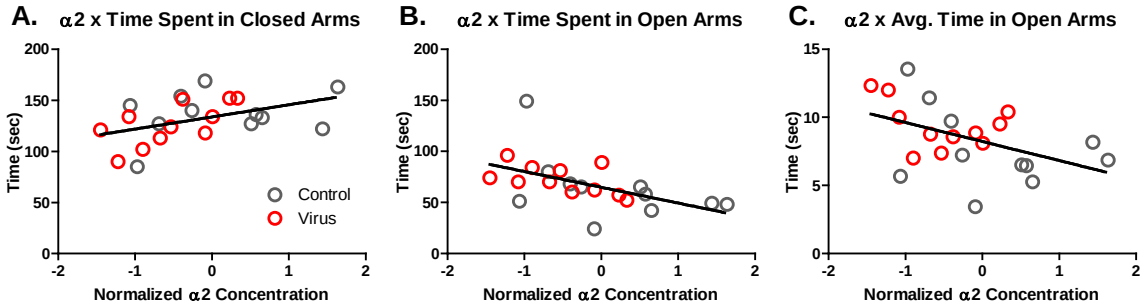

Figure 9. Correlations between α2 protein concentration and behavioral measures in naïve mice. Normalized (Z-Score) α2 protein concentration was positively correlated with time spent in closed arms (A) and negatively correlated with both total (B) and average (C) time spent in open arms. Regression lines incorporate both Control and Virus data points (n = 11 for each viral condition

VITA

VITA

Michael P. Smoker

Education

1999 – 2003	**B.S., Mathematics** Marian University, Indianapolis, IN
2011 – 2013	**Non-Degree, Psychology** IUPUI, Indianapolis, IN
2013 – Present	**Graduate Student, Addiction Neuroscience** IUPUI, Indianapolis, IN

Teaching Experience

2004 – 2005	Mathematics Teacher North Vermillion Jr/Sr High School, Cayuga, IN
2008 – 2009	Adjunct Mathematics Instructor Ivy Tech Community College, Indianapolis, IN
2005 – 2010	Teacher/Tutor Sylvan Learning Center, Indianapolis, IN & Phoenix, AZ
Spring 2012	Teaching Assistant – Behavioral Neuroscience
Summer 2012	Teaching Assistant – Statistics
Fall 2013	Teaching Assistant – Behavioral Neuroscience, Statistics
Spring 2014	Instructor – Statistics Lab Teaching Assistant – Drugs and Behavior
Summer 2014	Teaching Assistant – Alcoholism and Drug Abuse
Fall 2014	Teaching Assistant – Foundations of Neuroscience, Learning
Spring 2015	Instructor – Statistics Lab
Summer 2015	Instructor – Statistics Lab Teaching Assistant – Behavioral Neuroscience, Foundations of Neuroscience

Research Experience

2011 – 2012 **Research Assistant – Simon Katner, Ph.D.**
Institute of Psychiatric Research, IUPUI, Indianapolis, IN

- Handling/weighing rats
- Solution/equipment preparation – ethanol, nicotine, aCSF, HPLC mobile phase, pH, sipper tubes, microdialysis probes
- Taking/running samples – microdialysate, tail blood, nicotine extraction, HPLC
- Data recording/analysis – HPLC, Excel
- Stereotaxic surgery and cannula implantation
- Histology – cryostat slicing, probe placement verification

2012 – 2013 **Research Assistant – Christopher Lapish, Ph.D.**
Department of Psychology, IUPUI, Indianapolis, IN

- Handling/weighing rats, IP injections, euthanization
- Solution preparation – ethanol, sucrose
- Behavioral tasks – catalepsy, social interaction, 2-way drinking, delay discounting
- Delay discounting – development, experimental design, programming (Med PC)
- Data recording/analysis – Excel, R

2013 – Present **Graduate Student – Stephen Boehm II, Ph.D.**
Department of Psychology, IUPUI, Indianapolis, IN

- Handling/weighing mice, IP injections, retro-orbital blood sampling, vaginal-cell sampling, brain extraction, tissue punching, euthanization
- Microscope cell visualization, BEC analysis, histology, laser microdissection
- Drug preparation/administration
- Stereotaxic surgery, cannula implantation, site-specific microinjection

Graduate Student – Christopher Lapish, Ph.D.
Department of Psychology, IUPUI, Indianapolis, IN

- Delay discounting in P and Wistar rats

Awards

Research Society on Alcoholism Student Merit Award 2015 ($230)
School of Science Graduate Student Council Travel Award 2015 ($500)

Published Manuscripts

Katner SN, Toalston JE, **Smoker MP**, Rodd ZA, McBride WJ, & Engleman EA (2015). Time-course of Extracellular Nicotine and Cotinine Levels in Rat Brain Following Administration of Nicotine: Effects of Route and Ethanol Co-administration. *Psychopharmacology 232(3),* 551-560.

Linsenbardt DN, **Smoker MP**, Janetsian SJ, & Lapish CC (In Revision). Impulsivity in Rodents with a Genetic Predisposition for Excessive Alcohol Consumption is Associated with a Lack of a Prospective Strategy.

Fritz BM, Kasten CR, Quoilin C, **Smoker MP**, Boehm SL (Submitted). Binge Co-Consumption of Caffeine and Ethanol Increases Ethanol Intake in Adolescent and Adult Mice and Produces Age-Specific Motor Effects.

Published Abstracts – Poster Presentations

Smoker MP, Linsenbardt DN, Oberlin BG, & Lapish CC (2013). P rats show greater discounting than Wistars in a sucrose pellet-reinforced delay discounting task. Poster presented at the Indianapolis Chapter of the Society for Neuroscience conference, Indianapolis, IN.

Smoker MP, Linsenbardt DN, Lukic NA, Oberlin BG, & Lapish CC (2014). P rats show greater discounting than Wistars in a sucrose pellet-reinforced delay discounting task. Poster presented at the Research Society on Alcoholism Conference, Bellevue, WA.

Smoker MP, Linsenbardt DN, & Lapish CC (2014). Differences in impulsivity and prospective-like memory between P rats and Wistars in a sucrose pellet-reinforced delay discounting task. Poster presented at the Indianapolis Chapter of the Society for Neuroscience conference, Indianapolis, IN.

Smoker MP, Zhang Y, Liang T, & Boehm SL (2015). Effect of Gabra2 knockdown in the nucleus accumbens shell on ethanol intake and preference in C57BL/6J mice. Poster presented at the Research Society on Alcoholism Conference, San Antonio, TX.