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$G\alpha_q$-ASSOCIATED SIGNALING PROMOTES NEUROADAPTATION TO

ETHANOL AND WITHDRAWAL-ASSOCIATED HIPPOCAMPAL DAMAGE

DISSERTATION

Dissertation submitted in partial fulfillment
of the requirements for the degree of Doctor of Philosophy in the
College of Arts and Sciences
at the University of Kentucky

By
Anna R. Reynolds

Lexington, Kentucky

Director: Dr. Mark A. Prendergast, Professor of Psychology

Lexington, Kentucky

2015

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ABSTRACT OF DISSERTATION

$G\alpha_q$-ASSOCIATED SIGNALING PROMOTES NEUROADAPTATION TO ETHANOL AND WITHDRAWAL-ASSOCIATED HIPPOCAMPAL DAMAGE

Prolonged, heavy consumption of alcohol produces marked neuroadaptations in excitatory neurotransmission. These effects are accelerated following patterns of intermittent heavy drinking wherein periods of heavy consumption are followed by periods of abstinence. Studies have shown that neuroadaptive changes in the glutamatergic N-methyl-D-aspartate (NMDA) receptor produces excitotoxicity during periods of withdrawal; however, upstream targets were not adequately characterized. The present studies sought to identify these targets by assessing the role of group 1 metabotropic glutamate receptors (mGluR) and intracellular calcium in promoting cytotoxicity of hippocampal cell layers *in vitro*. It was hypothesized that ethanol-induced activity of mGluR1-and-5 contributes to hippocampal cytotoxicity and promotes the behavioral effects of withdrawal *in vivo*. In order to identify and test this theory, rat hippocampal explants were co-exposed to chronic intermittent ethanol exposure with or without the addition of a group 1 mGluR antagonist to assess cytotoxicity in neuronal cell types. In a second study, adult male rodents were co-exposed to chronic intermittent ethanol exposure with or without the addition of an mGluR5 antagonist to assess the role of these receptors in the development of dependence as reflected in withdrawal behaviors. Together, these studies help to identify and screen toxicity of putative pharmacotherapies for the treatment of ethanol dependence in the clinical population.

KEYWORDS: ethanol dependence, metabotropic glutamate receptors, intracellular calcium, signal transduction

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$G\alpha_q$-ASSOCIATED SIGNALING PROMOTES NEUROADAPTATION TO ETHANOL AND WITHDRAWAL-ASSOCIATED HIPPOCAMPAL DAMAGE

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CHAPTER ONE: General Introduction

1.1. Background and Significance

Alcohol misuse and abuse is a widespread problem in the United States of America. According to the National Survey on Drug Use and Health, just over half of the population (52.1%), or an estimated 135.5 million individuals over the aged of 12, can be characterized as being a current consumer of alcohol (Substance Abuse and Mental Health Services Administration [SAMHSA], 2014). Of this number, nearly 60 million individuals can be characterized as binge drinkers and 16 million individuals as heavy drinkers (SAMHSA, 2014; Figure 1). Binge drinking is currently defined as an event in which one's blood ethanol level exceeds 0.08% (National Institute on Alcohol Abuse and Alcoholism, 2004). Binge drinking as separate from alcohol use disorders is a quite recent issue. The first volume of the *Oxford English Dictionary* (letters A and B) was published in 1888 and defined "binge" as a verb meaning "to fawn; to cringe." This would indicate that such drinking practices were not considered a noteworthy issue or fell under the catch-all of "alcoholism." However, in a 1933 supplement, the dictionary included in the definition a noun meaning "a heavy drinking bout," as well as another verb meaning "to drink heavily, 'soak'" (see Crabbe et al., 2011 for an elegant review).

The American Psychological Association (APA) previously defined binge drinking as the consumption of four or five drinks in one occasion (for females and males, respectively; DSM-IV). Given that alcoholic beverages vary in the amount of alcohol in a drink, this definition could be misinterpreted. It does not allow for quantification via a biological dose of alcohol consumed, and while it does account for other factors that could influence the effects of alcohol (e.g., gender), it does not account for others (e.g., body mass index). The APA updated terminology in 2013 in the fifth

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edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-V) to combine the terms “alcohol abuse” and “alcohol dependence” under a more encompassing definition of “alcohol use disorder.” This term allows for identification without resorting to alcohol units consumed (APA, 2013). In order to be characterized with an alcohol use disorder, an individual’s alcohol-related behaviors should include at least two of the following eleven possible symptoms: consuming larger amounts than intended, unsuccessful efforts to control drinking, significant amount of time spent on alcohol-related activities, craving, failure to fulfill obligations, continued use despite negative consequences, failure to participate in social activities, recurrent use in the presence of hazardous domains, health conditions are exacerbated by alcohol use, tolerance, and presence of withdrawal symptoms (APA, 2013).

These updated criterion provided by the APA also characterize the severity of alcohol use disorders into one of three categories: mild (i.e., presence of two or three symptoms), moderate (i.e., presence of four or five symptoms), and severe (i.e., presence of six or more symptoms (APA, 2013). The National Institute on Alcohol Abuse and Alcoholism (NIAAA) has a more detailed definition of binge drinking as a pattern (i.e., repetitive consumption) in which the total amount of alcohol in the blood (i.e., blood alcohol concentration) exceeds 0.08% (i.e., 80 mg%; NIAAA, 2004). This definition allows for a clearly defined quantification of binge drinking so as to more prudently assess consequences of this pattern of consumption; it also provides a foundation for physiological investigations on the subject (for a review, see Crabbe et al., 2011).

While the definition of binge or heavy drinking as a pathology continues to be debated, the SAMHSA 2014 survey clearly notes a self-identified desire for intervention

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with an estimated 18 million Americans reporting wanting treatment for an alcohol use disorder in 2012 (alcohol abuse and dependence on alcohol). The survey also notes that a very small percentage of individuals (1.5 million) actually received treatment at a treatment facility (SAMHSA, 2014). Currently, there are four medications approved by the United States Food and Drug Administration (FDA) for the treatment of alcohol dependence: oral naltrexone, extended-release naltrexone, disulfiram, and acamprosate. In a nine-month randomized trial, abstinence was higher for disulfiram (250 mg/day) than for topiramate (150 mg/day), an anticonvulsant medication currently being assessed for treatment of alcohol dependence (De Sousa et al., 2008). Findings from another trial indicate that topiramate (25–300 mg/day) is more efficacious in promoting abstinence than naltrexone (50 mg/day) or acamprosate (333 mg/day) (Narayana et al., 2008), while other studies indicate that topiramate (50–400 mg/day) and naltrexone (50 mg/day) are similarly effective for the treatment of alcohol dependence (Flórez et al., 2008; Flórez et al., 2011). Given the fact that only a small percentage of individuals who are unhappy with their drinking actually receive help, and that efficacy for relapse prevention outside of the clinic is only modestly effective, pharmaceutical interventions are needed to address this gap in care, as they may prove to be effectively both in terms of time and economics of treatment because alcohol abuse and misuse is a widespread phenomenon.

Despite many preventative efforts (e.g., community-based services), the estimated economic burden in the United States for excessive alcohol use, including binge drinking, heavy drinking, underage consumption, and consumption by pregnant women was estimated at $223.5 billion (e.g., Bouchery et al., 2006; Center for Disease Control and Prevention, 2012). This figure includes costs for acute injuries, chronic health problems,

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property damage, fetal alcohol syndrome, and loss of productivity. Notably, binge drinking is responsible for the majority of these costs (76.1%). The *American Journal of Preventative Medicine* suggests that responsible individuals and their families cover an estimated 42% of the total costs associated with the excessive use of alcohol and the United States government carries an approximate burden of 42% of these costs (Sacks et al., 2013). Given that these figures are an approximation, the remainder of costs is not yet accounted for. In the state of Kentucky, for example, the governmental economic burden for alcohol-related damage and disease is an estimated $0.86 per drink (Sacks et al., 2013). Precise economic costs are difficult to figure for a drug that is responsible for not only individual physical damage but also has a ripple effect of damage to both self and others physically and psychologically while often causing material damage as well; however, within the context of these studies, it can be stated that the abuse of alcohol is a widespread problem in the United States and the costs associated with its damage (both direct and collateral) are significant. Future efforts to control this problem, then, need to face it on a variety of fronts, both social and medical, including the development of putative pharmacotherapies for the treatment of alcohol use disorders.

1.2. Alcohol Withdrawal: Effects on Brain and Behavior

Prolonged, excessive alcohol consumption (i.e., 4 or 5 drinks on one occasion) is known to promote brain atrophy. The effects include significant decreases in the number of cortical neurons (Harper, 1987), widening of ventricles (Bergman et al., 1980; Carlen et al., 1978; Ron, 1977), cortical degeneration (Epstein, 1977), and widening of sulci and fissures (Bergman et al*.,* 1980). In one study, males with a history of alcohol use disorders displayed significant decreases of anterior hippocampal volume (Sullivan et al.,

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1995). In another study, individuals with a history of withdrawal-induced seizure activity from alcohol dependence demonstrated significant decreases in the volume of temporal matter when compared with individuals who had not experienced a seizure during detoxification from alcohol (Sullivan et al., 1996). However, it has also been shown that decreases in the volume of white matter recover quite significantly following protracted abstinence (Pfefferbaum et al., 1995). Thus, given proper treatment, the effects of alcohol use disorders may be diminished.

Alcohol use disorders have also been shown to produce neurocognitive consequences, and low levels of blood flow in the brain have been associated with decreases in cognitive function. For example, significant relationships between scores on neuropsychological assessments and density of gray matter (e.g., hippocampus, cortices, and cerebellum) have been reported in alcohol-dependent participants (Chanraud et al., 2007). In another study, alcohol-dependent female participants displayed decreases in verbal and nonverbal working memory, gait, and balance (Sullivan & Marsh, 2003). Other studies have demonstrated decreases in assessments measuring visuospatial abilities, psychomotor speed, gait, and balance (for reviews, see Kleinknecht & Goldstein, 1972; Luhar et al., 2013; Sullivan et al., 2000).

Studies employing retrospective analyses of in-patient chart records of adults suggest that multiple withdrawals, or periods of abstinence, are associated with increases in rate, intensity, and duration of withdrawal-induced seizures. Gross and colleagues (1972) found a significant relationship between seizures experienced during acute withdrawal and re-admittance for acute withdrawal. Ballenger and Post (1978) demonstrated a significant relationship between years of abuse, amount of alcohol

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consumed per day, and severity of withdrawal behavioral symptoms. Indeed, the authors suggest that withdrawal effects increase in a step-wise fashion (Ballenger & Post, 1978). Brown and colleagues (1988) conducted a series of studies assessing the relationship between multiple periods of withdrawal and rate of seizures in a sample of 50 alcohol-dependent individuals where a significant association between multiple prior withdrawals (i.e., five or more) and rate of seizures was observed. These findings suggest that the neurological consequences associated with alcohol use disorders increase progressively in individuals with a history of multiple detoxifications or withdrawals.

The adolescent brain is particularly vulnerable to the effects of alcohol (Prendergast & Little, 2007), and binge drinking during adolescence or young adulthood can be predictive of dependence later on in life (Grant & Dawson, 1997). Magnetic resonance imaging (MRI) has shown that occipital lobe cortical gray matter increases steadily into the early twenties (Giedd et al., 1999), and binge drinking at an early age is predictive of brain matter volume atrophy (e.g., cerebellum and brain stem; Pfefferbaum et al., 1992). Duka and colleagues (2004) suggest that the negative consequences of chronic alcohol abuse and multiple detoxifications in adults are similar to consequences observed in young-adult binge drinkers (for a review, see Duka et al., 2004; Duka & Townshend, 2004). Young-adult female binge drinkers demonstrated increases in errors of commission on the Gordon Diagnostic System Vigilance task (Townshend & Duka, 2005) and demonstrated significant increases in errors on the spatial working memory task compared to age-matched non-binge drinkers. In sum, these findings suggest that patterns of binge-like consumption of alcohol can produce certain long-lasting cognitive

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effects in young-adult social drinkers that are similar to those observed in chronic alcohol-dependent adults.

Collectively, these studies demonstrate that patterns of binge-like alcohol consumption and multiple detoxifications or withdrawals, predict worse neurologic outcomes in the clinical population. The purpose of the present report is to identify potential mechanisms associated with promoting these consequences using rodent models of chronic, intermittent ethanol exposure (i.e., CIE) *in vitro* and *in vivo*.

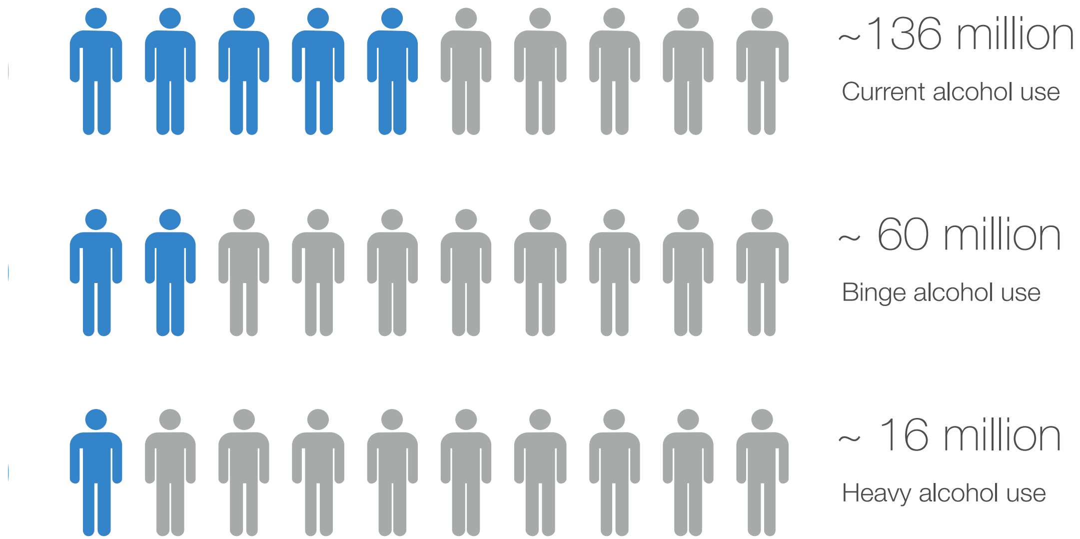

Figure 1. Alcohol abuse and misuse is a widespread phenomenon in the United States (SAMSHA, 2014). According to the National Survey on Drug Use and Health, an estimated 60 millions report current binge alcohol use.

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CHAPTER TWO: Study One: Protein Kinase Activation and Cytotoxicity of Intermittent Ethanol *in Vitro*

2.1. Introduction

Goddard and colleagues (1969) conducted a seminal series of studies employing electroencephalogram (EEG) techniques to assess the effects of repeated, subthreshold stimulations to areas of the limbic system on epileptic-like convulsions in rodents (Goddard et al., 1969). In these studies, delivery of initial electrical stimulations did not produce significant alterations in EEG activity or epileptic-like behavioral activity. But significant spikes in EEG activity and convulsions were observed following repeated, daily, subthreshold stimulation of areas of the limbic system. The authors concluded that spikes in EEG activity and epileptic-like activity were "kindled" in areas of the limbic system. These effects have since been observed in many different types of preclinical laboratory animals (Epsztein et al., 2008; Loscher et al., 1998; Sutala et al., 1988).

Other studies have also examined the effects of kindling in other areas of the brain, such as the amygdala. Indeed, it is suggested that repeated electrical stimulation to the amygdala can produce subsequent yet spontaneous spikes in EEG activity in the hippocampus, as well as seizure activity (McNamara et al., 1988). These findings are particularly interesting in light of the notion that the dentate gyrus, the primary granule cell layer of the hippocampus, is known to demonstrate a unique reorganization after kindling in these areas. For example, McNamara (1988) provided evidence that the sprouting of axons produced by neurons in the granule cell layer and mossy fiber tract of the pyramidal cell layer of the CA3 is associated with decreases in the seizure threshold. Notably, although neurons in the granule cell layer die following electrical stimulation to

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the hippocampus, these effects are followed by subsequent neuronal proliferation (Parent et al., 1997). These studies are the foundation for the hypothesis that multiple periods of withdrawal could serve as a kindling stimulus in the development of cytotoxicity following chronic, intermittent ethanol (CIE) exposure *in vitro*.

2.2. Experimental Rationale

Prior work has suggested that multiple cycles of CIE produces consistent and significant decreases of neuron-specific nuclear protein (NeuN) and thionine staining of Nissl bodies in each of the primary cell layers of the hippocampal formation (i.e., the granule cell layer of the dentate gyrus and the pyramidal cell layers of the CA1 and CA3; Reynolds et al., 2015). These effects were prevented following exposure to the NMDA-receptor antagonist (2R)-amino-5-phosphonovaleric acid (APV) at a 40µM concentration. These effects highlight what is classically referred to as "NMDA-receptor-mediated excitotoxicity" (Reynolds et al., 2015).

Excitotoxicity is conceptualized as the overactivation or overstimulation of amino acid receptor complexes producing subsequent neuronal cell death (Olney et al., 1986). Choi (1987, 1992) identified the NMDA receptor as the likely candidate for producing these excitotoxic effects *in vitro* and described this event as being mediated by an excessive influx of extracellular calcium and the subsequent activation of phospholipases, endonucleases, and proteases. This possibility is consistent with the work of others who have shown that chronic ethanol exposure increases calcium influx through the NMDA receptor (DeWitte et al., 2003), increases the sensitivity of the NMDA receptor (Lovinger et al., 1993), increases production of NMDA-receptor complexes (Floyd et al., 2003), and

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increases synaptic clustering of the NMDA receptor at the synapse (Carpenter-Hyland et al., 2004).

Other studies have shown neuroadaptive changes in second messenger systems using a CIE treatment regimen in cortical neurons of 75 mM ethanol for 14 hours followed by 10 hours of withdrawal that is repeated a total of five times and terminated by either a two- or five-day period of withdrawal (i.e., Qiang et al., 2007). Western blot and immunoblot analyses have shown that CIE produced selective increases in GluN1 and GluN2B subunit expression on the surface membrane (Qiang et al., 2007). However, exposure to KT-5720 (i.e., 1 μM)—a protein kinase A (PKA) inhibitor—prevented increases in GluN1 and partially prevented increases in GluN2B expression (Qiang et al., 2007). These data are consistent with studies conducted by Carpenter-Hyland and colleagues (2004) suggesting changes in the NMDA-receptor synaptic clustering is dependent on the activity of PKA. These findings suggest a role for PKA signaling in the overactivation of the NMDA receptor and subsequent cytotoxicity; however, this relationship is not yet fully understood. The purpose of the present studies, therefore, is to examine the distinct role that PKA-dependent NMDA-receptor activation has in promoting hippocampal cytotoxicity produced by CIE *in vitro*.

2.3. Methods

Organotypic hippocampal slice culture preparation. Whole brains from eight-day-old Sprague-Dawley rats (Harlan Laboratories; Indianapolis, IN) were aseptically removed and placed in culture dishes containing frozen dissecting medium composed of Minimum Essential Medium (MEM; Invitrogen, Carlsbad, CA), 25 mM HEPES (Sigma, St. Louis, MO), and 50 μM streptomycin/penicillin (Invitrogen). Bilateral hippocampi were

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removed and carefully transferred onto plates containing a chilled culture medium composed of dissecting medium, distilled water, 36 mM glucose (Fisher, Pittsburg, PA), 25% Hanks' Balanced Salt Solution (HBSS; Invitrogen), 25% (v/v) heat-inactivated horse serum (HIHS; Sigma), and 0.05% streptomycin/penicillin (Invitrogen). Excess hippocampal tissue was detached using a stereoscopic microscope, and unilateral hippocampi were sectioned at 200 μM using a McIlwain Tissue Chopper (Mickle Laboratory Engineering Co. Ltd., Gomshall, UK). Three hippocampal slices with intact cell layers were plated onto Millicell-CM 0.4 μM biopore membrane inserts containing 1 mL of pre-incubated culture medium and placed in a six-well culture plate. Excess culture medium was extracted off the top of each biopore membrane insert and the harvested tissue was maintained in an incubator at 37°C with a gas composition of 5% CO_2/95% air for five days prior to any experimental manipulations so that each hippocampal slice could adequately adhere to the membrane. Each culture well plate generated eighteen intact hippocampal slices. Care of all animals was carried out in agreement with the University of Kentucky's Institutional Animal Care and Use Committee.

Ethanol exposure and withdrawal. As Figure 2 shows, previous studies have suggested that exposure to CIE produces cytotoxicity in each of the cell layers of the hippocampal formation (Reynolds et al., 2015). The present experiments employed this model of CIE to delineate probable mechanistic underpinnings associated with the hippocampal cytotoxicity produced by this particular pattern of ethanol exposure. After five days *in vitro*, male and female hippocampal slice cultures were randomly transferred to plates

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containing either 1 ml of the culture medium (control) or medium containing a binge-like ethanol concentration (i.e., 0 and 50 mM) for five days with or without the addition of PKA inhibitor KT-5720 (1μM). At 11 days *in vitro*, cultures were removed from their three respective treatment groups and transferred to plates containing 1 ml of fresh ethanol-naïve culture medium for a 24-hour ethanol withdrawal period. These treatments were repeated a total of three times in consecutive order. Subsets of hippocampi were exposed to the CIE treatment regimen described above, but exposure to KT-5720 occurred only during withdrawal and not during ethanol exposure. During each five-day exposure period, ethanol and control-treated cultures were maintained inside Ziploc bags filled with 5% CO_2/95% air and water bath solutions containing either distilled water (50 ml) for control plates or distilled water (50 ml) containing ethanol (50 mM) for ethanol-treated plates. On *in vitro* day 23, cultures were fixed for immunohistochemistry and histology. This CIE treatment regimen is shown in Figure 3.

Immunohistochemistry and histology. Cultures were fixed by placing 1 ml of 10% formalin solution on the top and bottom of each well for 30 minutes before being washed twice with phosphate buffered saline (PBS) and stored at 4°C until immunohistochemistry was initiated. NeuN (Fox-3) is found in nearly all post-mitotic neurons (Kim et al., 2009; Mullen et al., 1992), and thus cultures were labeled with NeuN to assess cytotoxicity in each primary cell layer of the hippocampal formation: pyramidal cell layers of the cornu ammonis (CA1 and CA3) and granule cell layer of the dentate gyrus. Fixed inserts were transferred to a plate containing 1 ml of permeabilization (wash) buffer (200 ml PBS [Invitrogen], 200 μL Triton X-100 [Sigma], 0.010 mg Bovine

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Serum [Sigma]) with 1 ml of buffer added to the top of each well for 45 minutes to permeate cell membranes. Tissue was then incubated with the primary monoclonal antibody mouse anti-NeuN (1:200; Sigma) for 24 hours. Inserts were then washed with PBS and incubated for 24 hours with goat anti-mouse fluorescein isothiocyanate (FITC; 1:200; Sigma). Histological staining using thionine was conducted to confirm immunohistochemical findings. Thionine is a monochromatic dye known to bind to Nissl substance(s) located on cytoplasmic RNA (Kadar et al., 2009) and DNA content of all cell nuclei (Scott & Willett, 1996). Following immunohistochemistry, tissues were exposed to a 0.2% thionine stock solution for five minutes followed by a two-minute dehydration period with 70% ethanol before being washed twice and imaged. Cultures were imaged with SPOT software 4.0.2 (advanced version) for Windows (W. Nuhsbahm Inc.; McHenry, IL, USA) through a 5x objective with a Leica DMIRB microscope (W. Nuhsbahm Inc.; McHenry, IL, USA) connected to a computer and captured with a SPOT 7.2 color mosaic camera (W. Nuhsburg). For immunohistochemical studies, FITC fluorescence was detected with a band-pass filter at 495 nm (520 nm emission).

Statistical analyses. Statistical analyses were conducted to assess the influence of PKA in the development of ethanol dependence. Effects were considered significant at $p<0.05$. Study One was conducted two times using two different rat litters. All immunohistochemical and histological data were converted to percent control and then combined for ease of data interpretation. A one-way analysis of variance (ANOVA) was conducted for all *in vitro* techniques proposed with treatment as the factor for each hippocampal cell layer (i.e., CA1, CA3, and dentate gyrus). This statistical strategy is

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based on results of preliminary data suggesting that sex is not a factor influencing the effects produced by repeated binge-like ethanol. Post-hoc comparisons were conducted if a significant effect of treatment was detected using a protracted Fisher's Least Significant Difference (LSD). These planned comparisons were used to make pairwise comparisons between means. For graphical representation of thionine data, mean data from each condition were converted using the formula ([x-100]-100*[-1]) so as to express data on the same scale used for the immunohistochemical data. N=25–47 for immunohistochemical data and 20–24 for histological data per primary cell layer of the hippocampal formation.

2.4. Results

Study One examined the influence of PKA on the development of ethanol dependence in rat hippocampal explants exposed to a PKA inhibitor either during ethanol exposure or during withdrawal. Hippocampi exposed to three cycles of CIE demonstrated a 19% decrease of NeuN immunoreactivity in the CA1 ($F[5,228] = 6.26$, $p<0.001$; Figure 4), a 31% decrease of NeuN immunoreactivity in the CA3 ($F[5,228] = 11.46$, $p<0.001$; Figure 5), and a 25% decrease of NeuN immunoreactivity in the dentate gyrus ($F[5,228] = 10.82$, $p<0.001$; Figure 6). Exposure to KT-5720 (1 μM) during periods of withdrawal did not prevent this loss of NeuN immunoreactivity in any hippocampal subregion (Figures 4, 5, and 6), while exposure to KT-5720 (1 μM) during periods of ethanol exposure significantly attenuated this effect in the CA1 ($p<0.001$; Figure 4), CA3 ($p < 0.05$; Figure 5), and dentate gyrus ($p<0.05$; Figure 6).

Thionine staining of slices yielded similar findings, as exposure to KT-5720 (1 μM) significantly attenuated the losses of thionine staining in the CA1 ($F[3, 88] = 12.06$,

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$p<0.001$; Figure 7), CA3 ($F[3, 88] = 86.82$, $p<0.001$; Figure 8), and dentate gyrus ($F[3, 88] = 7.91$, $p<0.001$; Figure 9). Notably, exposure to KT-5720 (1 μM) during periods of withdrawal modestly attenuated the loss of thionine staining ($p<0.05$; Figure 7) in the CA1; however, this effect was not observed in the CA3 or dentate gyrus. Moreover, exposure to KT-5720 in ethanol-naïve slices produced significant decreases of NeuN immunoreactivity ($p<0.05$, data not shown) and thionine staining ($p<0.05$, data not shown) as compared to control values in the CA3. This effect was not demonstrated in the CA1 or dentate gyrus subregions of the hippocampal formation. Representative images are found in Figure 10.

2.5. Discussion

The present studies found that multiple cycles of CIE produced cytotoxicity in hippocampal slice cultures, as reflected by significant decreases of NeuN immunoreactivity and thionine staining. These findings are not unexpected, as we had previously found that exposure to 50 mM ethanol for five days *in vitro* followed by a single ethanol withdrawal period did not result in significant decreases of NeuN immunoreactivity or thionine staining in any hippocampal subregion (Reynolds et al., 2015). Other studies have also found that exposure to ethanol (50 mM) followed by a single period of withdrawal did not produce excitotoxicity *in vitro* (Butler et al., 2009; Self et al., 2005), but chronic exposure to this concentration of ethanol produced a heightened sensitivity of hippocampal glutamatergic receptors systems to agonists (Self et al., 2004). In a recent study, it was found that exposure to three cycles of CIE exposure in hippocampal slices produced consistent and significant decreases of NeuN immunoreactivity and thionine staining in the pyramidal cell layers of the CA1 and CA3

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and the granule cell layer of the dentate gyrus. These data are consistent with findings in which exposure to CIE produced deficits, such as cytotoxicity in cortical neurons (Nagy & Laszlo, 2002) and increased seizure susceptibility (Kokka et al., 1993), EEG activity (Veatch & González, 1996), and hippocampal neurodegeneration *in vivo* (Collins et al., 1998; Zhao et al., 2013). The present findings expand on this literature by characterizing the distinct roles of ethanol exposure and PKA on hippocampal injury produced by CIE exposure. In a previous study, exposure to 50 mM ethanol for 18 consecutive days (in the absence of any withdrawal) did not produce significant loss of NeuN immunoreactivity or thionine staining in any hippocampal subregion (Reynolds et al., 2015), but NMDA receptors were overactivated during periods of withdrawal (Reynolds et al., 2015). The present findings extend upon these results by demonstrating that neuroadaptations in the NMDA receptor, which likely confer sensitivity to cytotoxicity, are mediated, in part, via PKA prior to withdrawal. This finding suggests the importance of PKA in promoting neuroadaptations in NMDA-receptor activity following CIE exposure (i.e., 50 mM).

In general, CIE exposure produced a more modest reduction of NeuN immunofluorescence than thionine staining. While the reasons for these effects are unknown, it is likely that they reflect inherent differences regarding the neuronal selectivity of these immunohistochemical and histological markers (Kadar et al., 2009; Mullen et al., 1992; Scott & Willett, 1996; Wolf et al., 1996). The more significant decreases in thionine observed in the current study could reflect a loss of neuronal and non-neuronal cell types. For example, prior work has shown that a single exposure to ethanol withdrawal produces significant decreases of glial fibrillary acidic protein immunofluorescence in the rat hippocampus (Wilkins et al., 2006), and astrocytes are

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known to express each of the known NMDA-receptor subunits and are relatively vulnerable to classic NMDA-receptor-mediated excitotoxicity (Lee et al. 2010). Therefore, the present findings suggest that both neurons and glia (i.e., astrocytes) may demonstrate sensitivity to NMDA-receptor-dependent cytotoxicity following CIE. Notably, these cytotoxic effects are ubiquitous in nature, as they can be observed in each examined cell layer of the hippocampal formation (i.e., CA1, CA3, and dentate gyrus). Indeed, prior studies have demonstrated that the pyramidal cell layer of the CA1 is selectively vulnerable to the excitotoxic effects of ethanol withdrawal (Prendergast et al. 2004). It is, therefore, likely that the CIE exposure treatment regimen employed in the present study produced a greater extent of withdrawal-related cytotoxicity via spreading cytotoxicity of the pyramidal cell layer to the granule cell layer. This notion is supported by the work of Guiterrez and Heinemann (1999) which demonstrated *de novo* sprouting of mossy fiber tracts from granule cells to pyramidal cells.

The role of PKA in the overactivation of NMDA receptors. NMDA receptors are heteromeric ionotropic glutamatergic receptors comprised of an obligatory NR1 and combination of NR2 (A–D) subunits (Dingledine et al., 1999; for a review, see Paoletti & Neyton, 2007). During withdrawal from chronic ethanol exposure, activation of these receptors can lead to excessive calcium ($Ca2^{+}$) influx and subsequent cell death via activation of phospholipases, endonucleases, and proteases (i.e., excitotoxicity; Choi, 1992). Following CIE exposure, neuroadaptative changes in NMDA receptors can further potentiate $Ca2^{+}$-mediated excitotoxicity. In one study, for example, exposure to CIE produced increases in NMDA-receptor-mediated excitatory post-synaptic potentials

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(EPSPs) in the pyramidal cell layer of the CA1 of the rat hippocampus (Nelson et al, 2005).

Ethanol exposure indirectly enhances NMDA-receptor function through PKA-dependent phosphorylation (Carpenter-Hyland et al., 2004) and the subsequent trafficking of NMDA receptors from the endoplasmic reticulum to the synapse (Mu et al., 2003). The reasons for these effects are associated with cAMP-response element-binding protein (CREB) activation and subsequent increases in gene expression of the GluN2B subunit (Rani et al., 2005). Previous studies examining the modulatory effects of ethanol on GABA suggest it has a role in the release of intracellular $Ca2^{+}$ in the activation of PKA (Kelm et al., 2008). These alterations in PKA activity are associated with decreases in the sedative effects of ethanol and voluntary ethanol consumption (Thiele et al., 2000; Wand et al., 2001). The present studies examined the functional role of PKA-dependent overactivation of NMDA receptors. Co-exposure to ethanol with the PKA inhibitor KT-5720 for three cycles of CIE significantly attenuated the loss of mature neurons. By contrast, exposure to KT-5720 during periods of EWD did not attenuate these effects. These findings suggest that the CIE regimen produces increases in PKA activity, which promotes the overactivation of NMDA receptors during periods of withdrawal.

The selectivity of protein kinase inhibitors is debatable. Given that there are an estimated 500 protein kinases encoded in the human genome and that they each are belong to one super-family, it is no surprise that protein kinase inhibitors may target more than one protein kinase. In one study, for example, specificities of 60 compounds were assessed against a panel of 70–80 protein kinases (Bain et al., 2007). While this study made recommendations for specific protein kinase inhibitors that target specific kinases,

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such as mitogen-activated protein kinase (MAPK) (e.g., BIRB 0796) and phosphoinositide-3 kinases (e.g., PPI and Src inhibitor-1 collectively), recommendations to specifically target other kinases (e.g., PKA) were not made. However, other studies have employed KT-5720 in experimental procedures to specifically identify the role of PKA in various physiological processes (e.g., Almami et al., 2014; Clarysse et al., 2014; Rodriguez-Duran & Escobar, 2014; Sun et al., 2014; Xin et al., 2014). Together, these studies suggest that conclusions pertaining to the use of KT-5720 should be drawn cautiously.

Collectively, the present study and prior work suggest that the concomitant neuroadaptations in protein kinase activation (e.g., PKA) and increased function of NMDA receptors are likely associated with both the behavioral and neurodegenerative effects observed following multiple bouts of heavy ethanol consumption (Duka et al., 2003; Duka et al., 2004; Sullivan et al., 1996) and in the development of tolerance.

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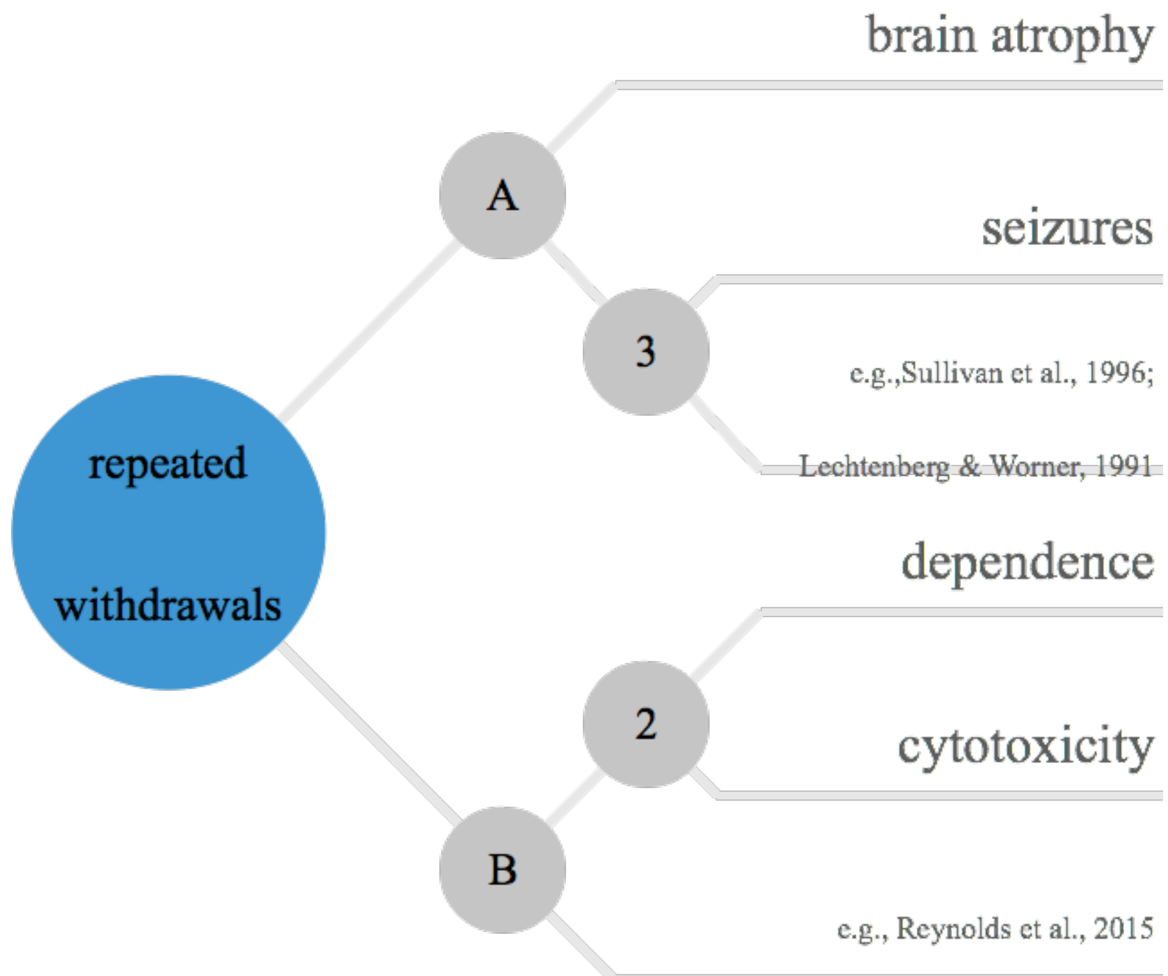

Figure 2. Experimental rationale for assessing mechanisms of damage associated with multiple withdrawals in organotypic hippocampal slice cultures.

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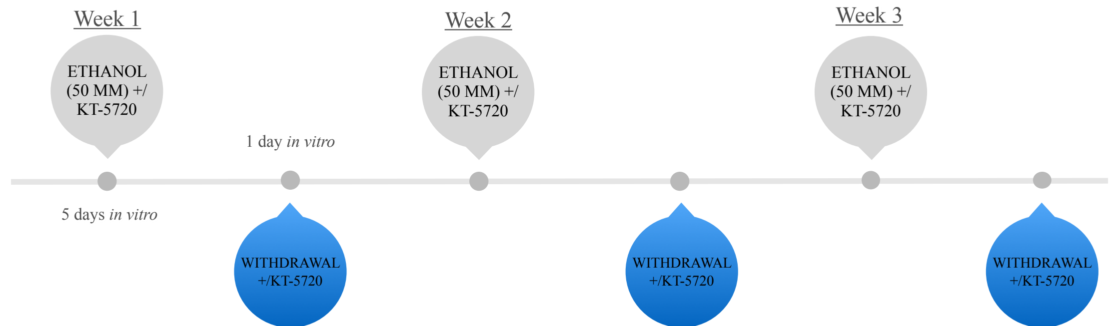

Figure 3. Rat hippocampal explants were exposed to ethanol (50 mM) for five days *in vitro,* followed by a 24-hour period of withdrawal and repeated three times. KT-5720, a protein kinase inhibitor was applied to ethanol-enriched medium or ethanol-free medium to assess the influence of protein kinase A activity in promoting NMDA-receptor-mediated cytotoxicity produced by CIE.

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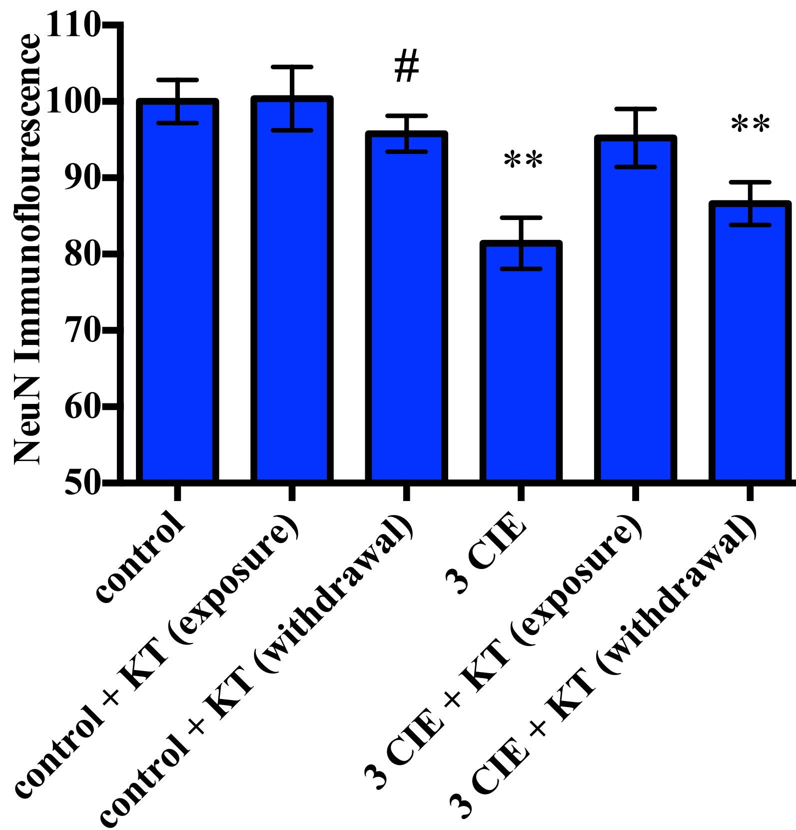

Figure 4. Co-exposure to KT-5720 (1 µM) and ethanol prevented the loss of NeuN immunoreactivity within the CA1 region produced by CIE. **p <0.001 vs control; #p <0.05 vs ethanol

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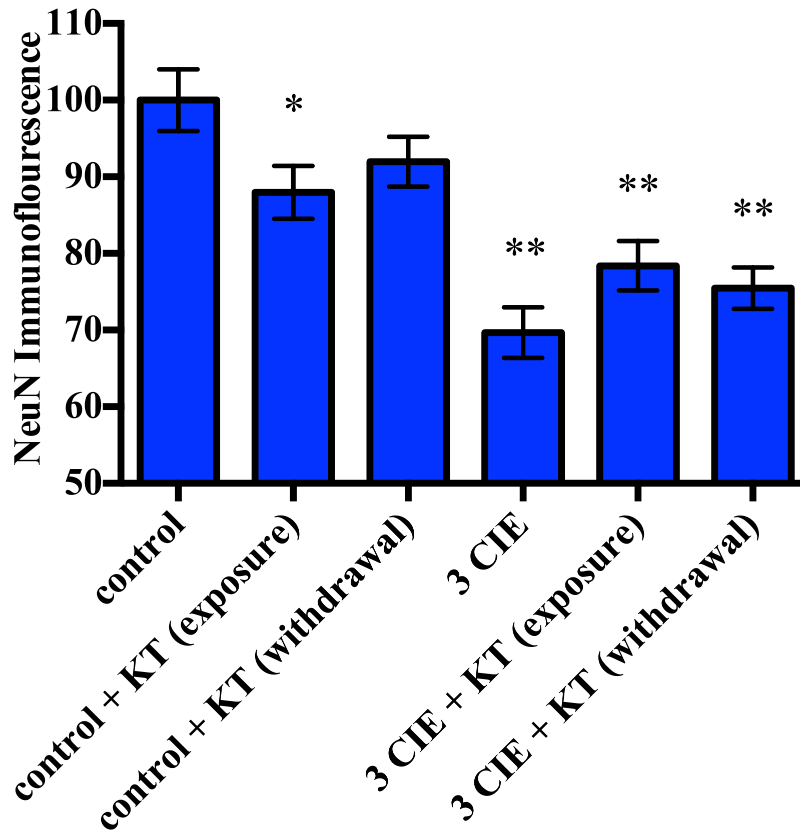

Figure 5. Co-exposure to KT-5720 and CIE attenuated the loss of NeuN immunofluorescence within the CA3 region. *p <0.05 vs control; **p <0.001 vs control

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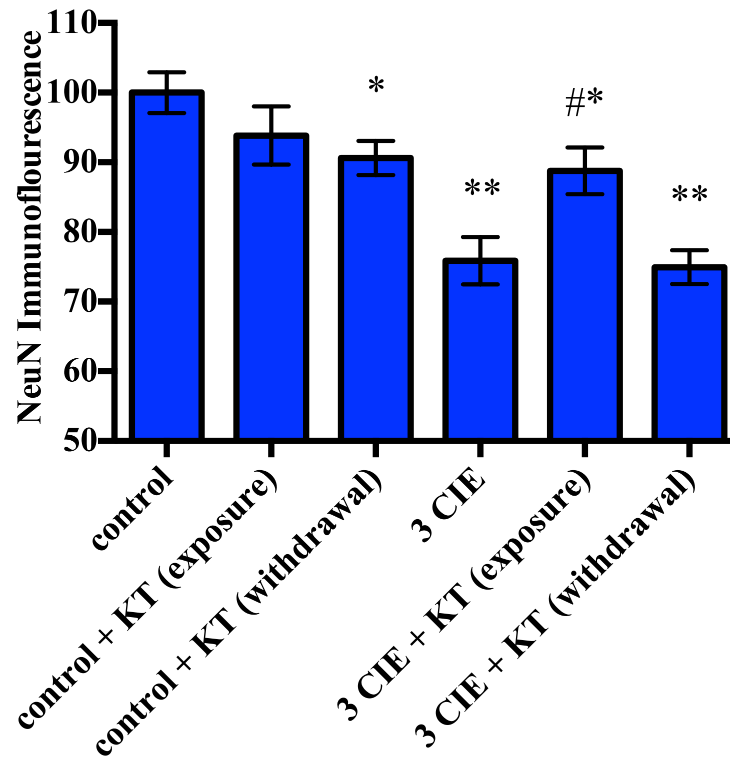

Figure 6. Co-exposure to KT-5720 and CIE prevented the loss of NeuN immunofluorescence within the dentate gyrus. *p <0.05 vs control; **p <0.001 vs control; #p <0.05 vs ethanol

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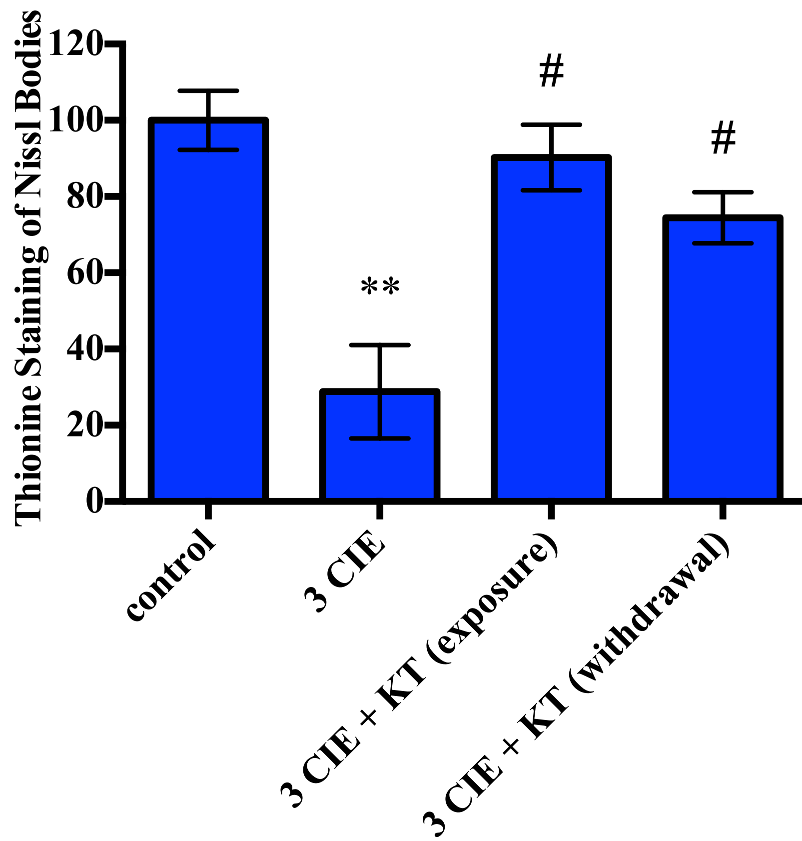

Figure 7. Co-exposure to KT-5720 and CIE prevented the loss of thionine staining within the CA1. **p <0.001 vs control; #p <0.05 vs ethanol

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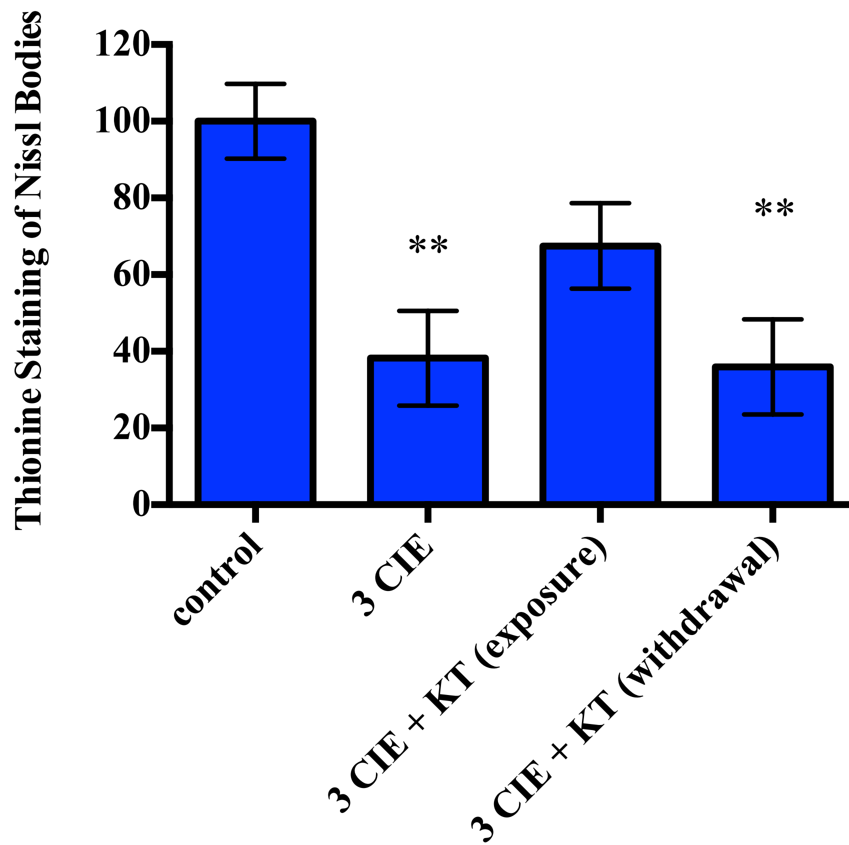

Figure 8. Co-exposure to KT-5720 and CIE attenuated the loss of thionine staining within the CA3. **p <0.001 vs control

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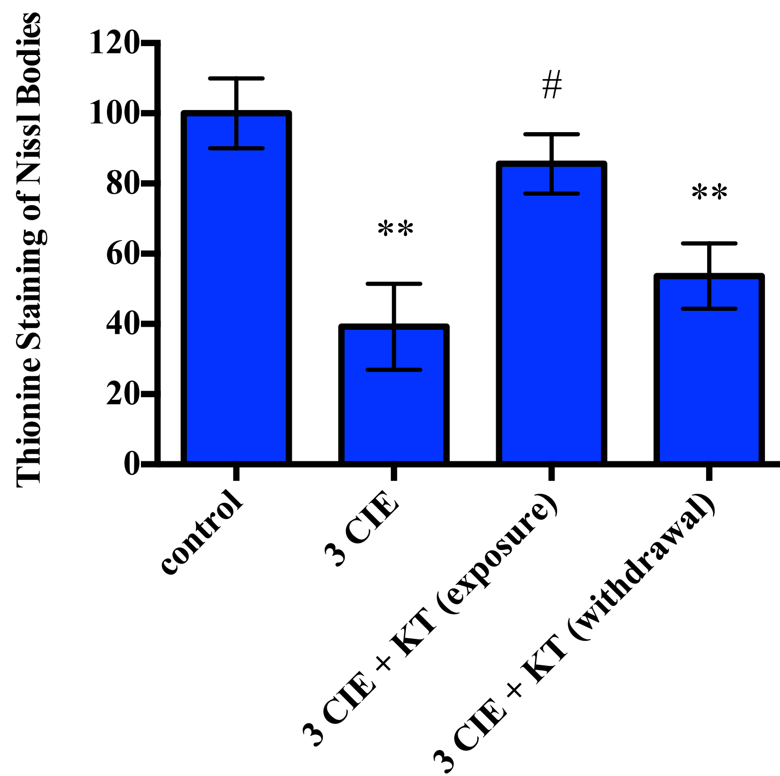
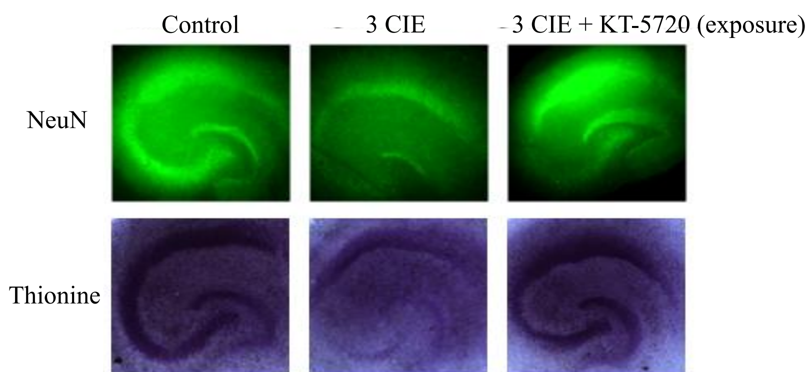


Figure 9. Co-exposure to KT-5720 and CIE prevented the loss of thionine staining within the dentate gyrus. **p <0.001 vs control; #p <0.05 vs ethanol



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CHAPTER THREE: Study Two: Effects of Chronic, Intermittent Ethanol on Withdrawal Behavior and Working Memory *in Vivo*

3.1. Introduction

Neuroadaptations and cognitive impairment associated with multiple alcohol detoxifications that are observed in humans (Duka et al., 2003) may also be demonstrated in rodent models (Stephens & Duka, 2008). This means that rodents can be used for preclinical assessment to assess metabolic and/or physical tolerance to ethanol after short-term exposure to ethanol (Self et al., 2009). Rodents are particularly valuable in these studies as they quickly develop effects from ethanol. In previous preclinical studies, repeated administration of high doses of ethanol have been used to evoke physical dependence in rodents. Factors considered when evaluating the effects of ethanol dependence in these studies typically include duration and dose (Majchrowicz, 1975; Sircar & Sircar, 2005; Wallgren et al., 1973). In one study, for example, a four-day, binge-like exposure to high doses of ethanol produced rapid intoxication. This was followed by repeated administration of high doses of ethanol for four consecutive days creating ethanol dependence rather rapidly (Majchrowicz, 1975). Once dependence was developed and blood ethanol levels declined, behavioral effects of withdrawal were evident. These effects included, but were not limited to, hypoactivity, tremors, wet dog shakes, spastic rigidity, and convulsions (Majchrowicz, 1975). Becker and colleagues (2000) concluded that the behavioral effects of withdrawal could be classified into one of three categories: 1) overactivation of the autonomic nervous system (e.g., tachycardia, increased blood pressure, sweating, and vomiting); 2) CNS hyperexcitability (e.g.,

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anxiety and seizures); and 3) disturbances related to sensation and perception (e.g., hallucinations and delirium).

Work in our laboratory observed cognitive impairments and withdrawal behavioral effects in rodents exposed to ethanol three times per day (3–5 g/kg) for four days (Self et al., 2009). Rodents in this study rapidly developed metabolic tolerance to ethanol, which was reflected in significant decreases in peak blood ethanol levels from ~187.69 mg/dl (Day Two) to ~100 mg/dl (Day Four; Self et al., 2009). Long-term ethanol administration can also be used to examine dependence in rodents. In one study, for example, ethanol administration (~6%) for 14 consecutive days produced significant increases in NMDA GluN2A and GluN2B subunits in the rodent hippocampus (Devaud & Alele, 2004). In another study, rodents exposed to ethanol (~6%) for a prolonged period of time (13 months) followed by a six-week period of withdrawal demonstrated significant decreases in spatial working memory compared to dependent rodents that did not experience this period of withdrawal (Alele & Devaud, 2005). In sum, these studies demonstrated that both short-term and long-term administration of ethanol withdrawal may be used to evoke physical dependence, tolerance, and behavioral effects of withdrawal in preclinical laboratory animals.

Other studies have assessed mechanisms involved in the associated damage of multiple withdrawals, as rodents are known to display predictable increases in the severity of withdrawal symptoms in a manner similar to the clinical population (Stephens et al., 2001; Stephens et al., 2005). Studies using rodents have shown that CIE exposure impairs metabolic processes (Clemmensen et al., 1988), creates kindling of the cortices (McCown & Breese, 1990), and causes hyperexcitability of the limbic structures of the

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brain (Adinoff et al., 1994). Other studies have shown that CIE exposure increases the effects of kindling within the amygdala (Carrington et al., 1984; Pinel, 1980; Pinel & Van Oot, 1975). Notably, exposure to CIE produces increases in electroencephalogram (EEG) activity during periods of withdrawal (Duka et al., 2004), and these effects were most evident following multiple periods of withdrawal (Ballenger & Post, 1978).

Given these data, it is not surprising that CIE exposure produces predictable increases in the rate, intensity, and duration of seizures during periods of withdrawal (e.g., Stevens et al. 2001; Veatch & Becker, 2002), decreases the development of subsequent long-term potentiation (e.g., Stephens et al., 2005), and causes neurodegeneration of the hippocampal formation (e.g., Collins et al., 1998; Corso et al., 1998; Zhao et al., 2013). In one study, for example, mice exposed to three cycles of vaporized ethanol for 16 hours a day, followed by eight hours of ethanol withdrawal demonstrated significant increases in handling-induced convulsions and EEG activity. The administration of MK-801, a noncompetitive NMDA-receptor antagonist, (i.e., 0.1–0.3 g/kg) during periods of withdrawal attenuated these behavioral indices of withdrawal (Veatch & Becker, 2005).

Collectively, these findings suggest that the effects of CIE exposure increase in a step-wise fashion and are mediated, in part, by activation of the NMDA receptor. Further, these data demonstrate that there is predictive validity in cell culture models of alcohol dependence that translate to the live animal.

3.2. Experimental Rationale

Work in our laboratory has demonstrated that intra-cornu ammonis 1 administration of the human immunodeficiency virus-1 transcription factor produces

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activation of NMDA receptors during withdrawal and subsequent persisting spatial learning deficits in rodents (Self et al., 2009). These effects were prevented following the administration of MK-801, an NMDA-receptor antagonist. Other studies have shown that a CIE treatment regimen activates microglia and produces spatial learning and memory deficits associated with neurodegeneration of limbic structures (Zhao et al., 2013). These findings are consistent with the cognitive consequences readily observed in the clinical population following prolonged, alcohol abuse (Kril et al., 1997; Sullivan et al., 2000; Sullivan et al., 2005). The present study was designed to assess the effects of CIE exposure on withdrawal behavior and working memory in a live animal model of ethanol dependence.

3.3. Methods

Subjects. Twenty-two adult, male Sprague-Dawley rats (i.e., 300–325 grams; Harlan Laboratories, Indianapolis, IN) were housed individually and allowed to acclimate to the animal colony for two days following arrival. Subjects were then handled for two minutes per day for three consecutive days prior to experimentation. Subjects were allowed *ad libitum* access to food and water throughout the entire duration of the experiment. Food weight data were collected at 0800 hour once per week. Each animal was weighed at 0800 hour on days Monday through Friday, prior to ethanol administration. A mortality rate of 5% occurred to complications with the gavage procedure.

Ethanol administration and withdrawal. Previous studies conducted in our laboratory have shown that CIE exposure produces hippocampal cytotoxicity in cell cultures (Reynolds et al., 2015). The present study sought to extend these *in vitro* findings to

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assess the effects of CIE on withdrawal behavior and working memory. In these studies, subjects were administered ethanol (4 g/kg) via intragastric gavage twice daily at 0800 and 1600 for five consecutive days followed by a two-day period of withdrawal; this was repeated either a total of two (Figure 11) or three times (Figure 11). One day after the last period of withdrawal, cognitive function was assessed for one week in the Morris Water Maze. This study aimed to achieve peak blood ethanol levels of approximately 200 mg/dl. The justification for the desired peak blood ethanol levels achieved of 200 mg/dl is based on a previous study that suggested ethanol-induced sedation occurs between 340 and 190 mg/dl, but ataxia occurs between 570 and 250 mg/dl in the rat (Majchrowicz, 1975). The effective dose (i.e., ED50) for hypnotic effects of ethanol are approximately 1.80 g/kg in the rodent (Garfield & Bukusoglu, 1996). Experimental timelines for Study Two are depicted in Figure 11.

Behavioral effects of withdrawal. Subjects exposed to CIE and an isocaloric diet were observed and rated for behavioral effects of withdrawal. In this portion of the study, subjects were administered ethanol for five consecutive days, followed by two days of withdrawal; this was repeated a total of two times (i.e., two cycles of CIE) or three times (i.e., three cycles of CIE). During each period of withdrawal, subjects were monitored 16 hours after the last ethanol administration (i.e., 0900 hour) for behavioral effects of withdrawal. Assessment of withdrawal behavior occurred in a square Plexiglas chamber for two minutes. During this time, two experimenters who were blinded to experimental conditions rated the physical effects of withdrawal using a modified behavioral scale that has been used in other studies (Majchrowicz, 1975; Self et al., 2009). Severity of

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withdrawal symptoms was assessed using a 10-point discrete scale adapted from previous studies (e.g., Majchrowicz, 1975; Self et al., 2009). These effects were rigidity, tremor, stereotypy, retropulsion, dystonic gait, hypoactivity, aggression, splayed paws, vocalization, and seizure (Figure 12).

Morris Water Maze. Two days following CIE administration, subjects were tested for five consecutive days in the Morris Water Maze to assess cognitive function. This protocol was published in a previous study by our laboratory (Self et al., 2009) and by others (Morris, 1984). The apparatus was an open field pool 157.48 cm in diameter and 34.29 cm in height that was divided into five quadrants (platform, N, S, E, and W). The removable platform (10.16 cm in diameter) was consistently positioned 1 cm below the surface of the water and 30 cm from the pool wall in the northeast quadrant. Water was maintained at between 19°C and 21°C and dyed black with non-toxic black powdered paint so that the platform was hidden to sight. Each day of testing consisted of four 60-second trials in which the animal located the platform via navigation through the pool. In the event that the animal located the platform during the trial, they were allowed to briefly rest on the platform for 15 seconds before being returned to their home cage for five minutes. In the event that the animal did not navigate to the platform during the 60-second trial, they were gently guided to the platform and allowed to rest for 15 seconds before being returned to their home cage for five minutes. On Day Five, animals completed a probe trial in which the platform was removed and the animal had 30 seconds to navigate the pool. Following this probe test, animals were returned to their home cages. During each of the trials, a video monitor analyzer (EZ Mapper; AccuScan

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Instrument, Inc) was used to assess 1) latency to platform (seconds), 2) distance travelled (centimeters), and 3) velocity (centimeters per second).

Analysis of blood ethanol levels. In order to assess blood ethanol levels (BELs), approximately 200 µL of tail blood was collected in two Fisherbrand heparinized micro-hematocrit capillary tubes (Fisher Scientific) on Day Four of Weeks One, Two, and Three. Next, samples were centrifuged for four minutes using an Analox benchtop centrifuge (Analox Instruments) with blood plasma collected and placed into a 0.65 mL Costar microcentrifuge tube (Fisher Scientific). Samples were stored at -80°C until further analyses of BELs using an Analox AM1 apparatus. This device contains a Clark-type amperometric oxygen electrode to directly measure levels of molecular oxygen. When molecular oxygen is present, the enzyme alcohol oxidase is oxidized into two byproducts: acetaldehyde and hydrogen peroxide. This device, therefore, indirectly measures BELs, as oxygen consumption is directly proportional to ethanol concentrations in each blood plasma sample.

Statistical analyses. Statistical analyses were conducted to assess the effects of CIE on body weight, food consumption, and behavioral effects of withdrawal, as well as on cognitive performance and BELs. Effects were considered significant at $p<0.05$. Body weight data were analyzed by a two-factor repeated-measure ANOVA with day and treatment as factors. Food consumption data were analyzed by a two-factor repeated-measure ANOVA with week and treatment as factors. Behavioral effects of withdrawal were analyzed by a two-factor repeated-measure ANOVA with day and treatment as factors. In the Morris Water Maze, 1) latency (seconds), 2) distance (centimeters), and 3)

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velocity (centimeters per second) were analyzed using separate two-factor repeated-measure ANOVAs with day and treatment. Probe trial data from the Morris Water Maze was analyzed by a one-factor ANOVA treatment (control and ethanol). BELs were analyzed using a two-factor repeated-measure ANOVA with day and treatment (control and ethanol) as factors. Planned comparisons were conducted if a significant effect of day or treatment or an interaction between these two factors was detected using Tukey's Honestly Significant Difference (HSD). These planned comparisons were used to make conservative pairwise comparisons between means.

3.4. Results

Body weight and food consumption. ANOVA revealed a significant interaction of day and treatment ($F[1,109] = 10.325$, $p<0.001$) in subjects exposed to two cycles of CIE (n=5) or an isocaloric diet (n=6). Figure 13 shows that significant decreases in body weight (grams) were detected in subjects that were administered ethanol starting on Day Three of the CIE treatment regimen. These decreases in body weight were accompanied by decreases in food consumption, as ANOVA revealed a significant interaction of week and treatment ($F[1,21] = 36.834$, $p<0.001$) in subjects exposed to two cycles of CIE. Figure 14 shows that while there was no main effect of week in subjects administered a control diet, subjects in the ethanol condition showed increases in food consumption on the second week, as compared to the first week ($F[1,21] = 65.799$, $p<0.001$). Further, as Figure 14 shows, subjects administered ethanol consumed less food in Week One ($F[1,109] = 30.300$, $p<0.001$) and Week Two ($p< 0.05$; Tukey's HSD) compared to subjects that received an isocaloric diet.

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ANOVA also revealed a significant interaction of day and treatment ($F[1,164] = 7.272$, $p<0.001$) in subjects exposed to three cycles of CIE (n=5) or an isocaloric diet (n=6). Figure 15 shows that significant decreases in body weight (grams) were detected in subjects that were administered ethanol starting on Day One of the CIE treatment regimen. These decreases in body weight were accompanied by decreases in food consumption, as ANOVA revealed a significant interaction of week and treatment ($F[1,21] = 5.590$, $p<0.05$) in subjects exposed to three cycles of CIE. Figure 16 shows that while there was no significant effect of treatment on food consumption for Week One ($p> 0.05$; Tukey's HSD), subjects administered ethanol demonstrated significant decreases in food consumption compared to subjects that received an isocaloric diet in Week Two ($p< 0.001$; Tukey's HSD) and Week Three ($p< 0.001$; Tukey's HSD).

Behavioral effects of withdrawal. We assessed the influence of CIE on the development of dependence, as reflected by behavioral effects of withdrawal. ANOVA revealed a significant main effect of treatment ($F[1,21] = 8.578$, $p<0.05$). Figure 17 shows that subjects administered ethanol had significant increases in behavioral effects of withdrawal compared to subjects that received an isocaloric diet. There was not a significant main effect of day in subjects exposed to two cycles of CIE.

By contrast, ANOVA revealed a significant interaction of day and treatment ($F[1,164] = 17.430$, $p<0.001$) in subjects exposed to three cycles of CIE (n=5) or an isocaloric diet (n=6). Figure 18 shows that significant increases were detected on Day Two and Day Three of withdrawal in subjects that received ethanol administration. This

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figure also shows that the most robust increases in withdrawal behavior occurred on Day Three of withdrawal ($p<0.05$; Tukey's HSD) in ethanol-dependent subjects.

Morris Water Maze. ANOVA revealed a significant main effect of day on latency ($F[1,36] = 45.267$, $p<0.001$) and distance ($F[1,36] = 47.682$, $p<0.001$) in subjects exposed to two cycles of CIE (n=5) or an isocaloric diet (n=6). Figures 19 and 20 show that latency to platform and distance travelled were significantly decreased as a function of day in subjects that received ethanol and an isocaloric diet, respectively. ANOVA also revealed a significant interaction of day and treatment on velocity ($F[1,36] = 2.706$, $p<0.05$). Figure 21 shows that ethanol administration significantly decreased velocity on Day Two ($p<0.05$; Tukey's HSD), Day Four ($p<0.05$; Tukey's HSD), and Day Five ($p<0.05$; Tukey's HSD). This effect was not observed on Day One or Day Three ($p>0.05$; Tukey's HSD). There were no significant effects of platform crosses at the 15-second interval or total platform crosses during the probe trial (Figure 22).

ANOVA revealed a significant main effect of day on latency ($F[1,36] = 34.951$, $p<0.001$) and distance ($F[1,36] = 135.627$, $p<0.001$) in subjects exposed to three cycles of CIE (n=5) or an isocaloric diet (n=6). Figures 23 and 24 show that latency to platform and distance travelled were significantly decreased as a function of day in subjects that were administered ethanol and an isocaloric diet, respectively. By contrast, ANOVA revealed a significant interaction of day and treatment on velocity ($F[1,36] = 2.020$, $p<0.05$). Figure 25 shows that ethanol administration significantly increased velocity on Day Four ($p<0.05$; Tukey's HSD). This figure also shows that these increases were not

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observed on Days One, Two, Three, or Five (*p*>0.05; Tukey's HSD) in subjects exposed to three cycles of CIE.

Blood ethanol levels. ANOVA revealed significant main effects of day (F[1,12] = 25.352, p<0.05) and treatment (F[1,12] = 6.287, p<0.05) on BELs in subjects exposed to three cycles of CIE (n=5). Figure 26 shows that peak BELs achieved were less than 250 mg/dl or slightly less than three times the legal driving limit.

3.5. Discussion

In the present report, subjects administered CIE demonstrated significant decreases in body weight, as compared to subjects administered a control diet. Given that these decreases in body weight were accompanied by decreases in food consumption, the sedative effects of binge-like ethanol administration likely produced these effects. These findings are not unexpected and are consistent with previous findings in which binge-like ethanol exposure results in weight loss in preclinical laboratory animals (Broadwater et al., 2011A; Broadwater et al., 2011B; Matthews et al., 2008; Roberto et al., 2010).

The present study demonstrates that exposure to CIE produces physical withdrawal. These findings are consistent with prior work demonstrating prototypical withdrawal-like behaviors (for reviews, see Becker, 2012 & 2013; Botia et al., 2015; N'Gouemo et al., 2015; Pérez & DeBiasi, 2015; Van Skike et al., 2015). In one study, for example, adult male rodents administered binge-like ethanol had seizures during withdrawal following a startle stimulus (N'Gouemo et al., 2015). The present findings also demonstrate that severity of withdrawal is significantly increased on the third consecutive withdrawal as compared to prior withdrawals. These findings are consistent

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with prior studies suggesting that CIE exposure produces a progressive intensification in the physical manifestation of withdrawal in rodents (Veatch & Becker, 2002). Notably, other studies have suggested that a history of binge-like ethanol intake does not alter seizure susceptibility (Cox et al., 2013). The lack of agreement between studies may be a reflection of the level of intoxication and/or the route of administration. With this said, the present findings are consistent with data derived from studies that indicated that previous periods of detoxification predict worse neurologic outcomes (Lechtenberg & Worner, 1991, 1992; for a review, see Duka et al., 2004). Gross and colleagues (1972), for example, found that individuals with five prior detoxifications were more likely to experience a seizure during subsequent detoxification (Gross et al., 1972). Collectively, these findings suggest that rodent models of CIE exposure have been productive in illustrating that the severity of withdrawal symptoms increases in a predictable and step-wise fashion.

In the present study, moderate severity of withdrawal symptoms could reflect more moderate BELs that resulted from lower ethanol doses. Peak BELs remained below 230 mg/dl at each time point examined at 90 minutes following a 4 g/kg dose. By contrast, Majchrowicz (1975) employed a model of binge-like ethanol administration in which ethanol was administered in fractional doses of 9–15 g/kg/day. While ethanol-induced sedation was achieved throughout the entire intubation period, withdrawal symptoms were present only when levels of ethanol in the blood were lower than 100 mg/dl. Majchrowicz (1975) noted that mortality occurred during both periods of intoxication and periods of withdrawal, and other studies have modified Majchrowicz's binge model in order to minimize mortality rates (e.g., Vetreno & Crews, 2015). In a

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more recent study, BELs significantly correlated with withdrawal severity in adolescent and adult rats (Morris et al., 2010).

In the present study, we also examined the effects of CIE exposure on cognitive performance in the Morris Water Maze, a test developed by Morris (1981) who suggested that rodents use proximal cues and distal cues to navigate through their environment. The current study employed both distal and proximal cues within the Morris Water Maze: local stimuli (e.g., shapes on each wall of testing room) were present during each trial and testing period and the platform remained hidden in the same location (i.e., northwest quadrant). Another study conducted by Morris (1984) demonstrated that tasks in the water maze are dependent on higher cortical brain structures, such as the hippocampus, and that while rodents with a hippocampal lesion can navigate to a visible platform using proximal cues, they cannot navigate to a hidden platform using distal cues. This conclusion is consistent with a study by Watson and Lashly (1915) who described inherent differences between proximal and distal spatial localization (quoted in Rudy et al., 1987, pp. 239–240):

> In "proximal" orientations, a goal object is visible or audible, or detectable by smell and so can be approached from a distance. Learning is straight forward as it requires no more than the operation of a motor "taxis" system toward a significant conditional or unconditional cue. In "distal" orientations, however, the goal object is invisible, inaudible, and cannot be detected by smell. No "local" cues, where "local" implies cues that are spatially concurrent with the goal object, are evadable to guide the animal

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to the goal. Thus any "directionality" behavior can only be achieved by learning about the spatial location of the goal relative to the distal cues.

In the present study, we found that latency to platform (i.e., measured in seconds) and distance travelled (i.e., measured in centimeters) were significantly decreased as a function of day in subjects that were administered either an ethanol or an isocaloric diet for two or three cycles of CIE exposure. Given that the Morris Water Maze is a task used to examine spatial reference memory, typically defined as a hippocampal-dependent function (Buzsáki & Moser, 2013; Foster et al., 2012; Moser et al., 1995; Vorhees & Williams, 2006), the present findings suggest that CIE exposure does not interfere with a rodent's ability to use spatial localization to navigate through its environment. Reversal learning in the Morris Water Maze is thought to assess more subtle differences (Vorhees & Williams, 2006) and tap into other processes, such as executive function (D'Hooge & De Deyn, 2001; Robbins & Arnsten, 2009). In one study, for example, no differences between experimental treatment groups were identified in a cohort of middle-age rats that were taught to locate a hidden platform (Latimer et al., 2014); however, when a spatial reversal task was employed (i.e., task required subjects to locate a hidden platform in a new location on one day and remember the location after several days), significant differences among treatment groups were detected (Latimer et al., 2014). While future studies could assess more subtle differences in executive function using the reversal learning task employed by Porter and colleagues (Latimer et al., 2014), the findings here suggest that physical dependence can be achieved by employing a model of CIE exposure and that spatial navigation remains intact in adult rodents affected by this dependence.

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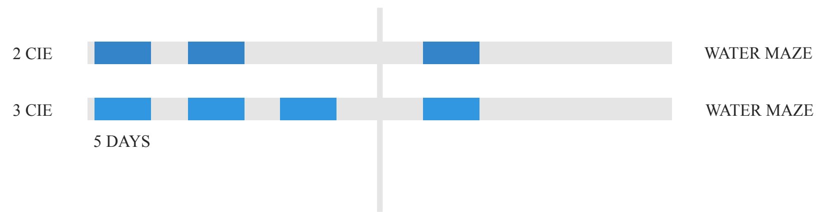

Figure 11. Representative experimental timelines showing that subjects were exposed to ethanol (4 g/kg) twice daily for five days, followed by two days of withdrawal, and repeated either a total of two times (i.e., two CIE) or three times (i.e., three CIE).

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Figure 12. Withdrawal behavior was rated on a 10-point scale modified from prior reports in our laboratory (Sharrett-Field et al., 2013). Behaviors ranged from mild (e.g., hypoactivity, rigidity, aggression, and stereotypy) to more moderate (e.g., dystonic gait, retropulsion, splayed paws, and tremor) to severe (e.g., vocalizations and seizure).

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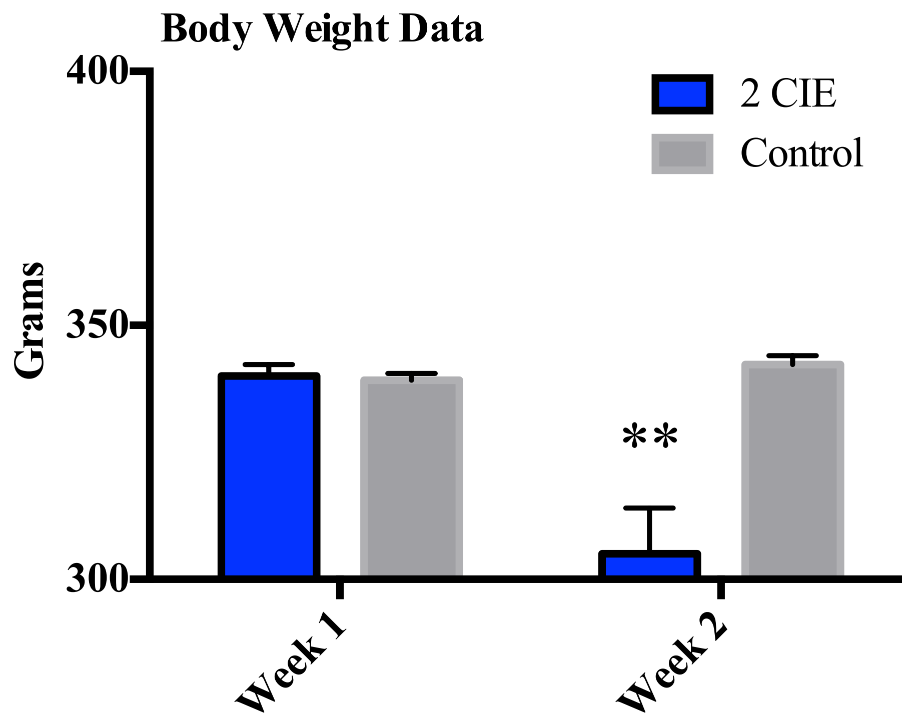

Figure 13. Changes in body weight in subjects exposed to ethanol (n=5) or an isocaloric diet (n=6) for two cycles of CIE. X-axis: days in Week One and Week Two. Data points show mean body weight in grams for subjects exposed to ethanol (blue bar) or an isocaloric diet (gray bar). Two asterisks indicate a significant day-by-treatment interaction.

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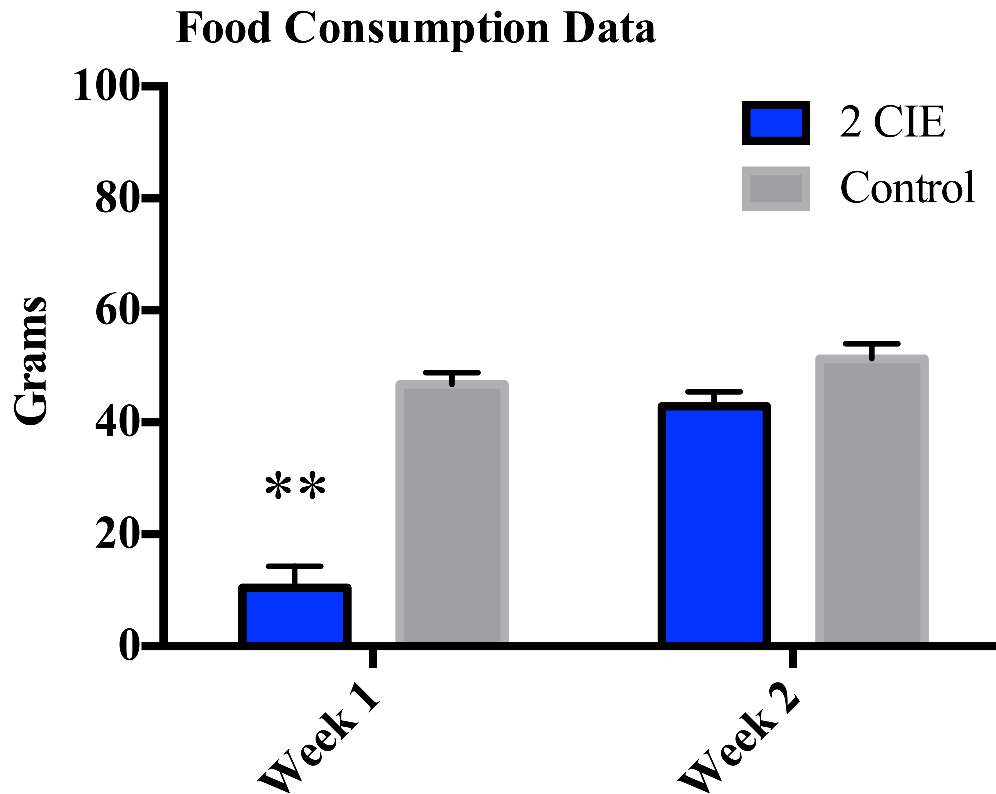

Figure 14. Changes in food consumption in subjects exposed to ethanol (n=5) or an isocaloric diet (n=6) for two cycles of CIE. X-axis: week. Data points show mean food consumption in grams for subjects exposed to ethanol (blue bar) or an isocaloric diet (gray bar). Two asterisks indicate a significant day-by-treatment interaction.

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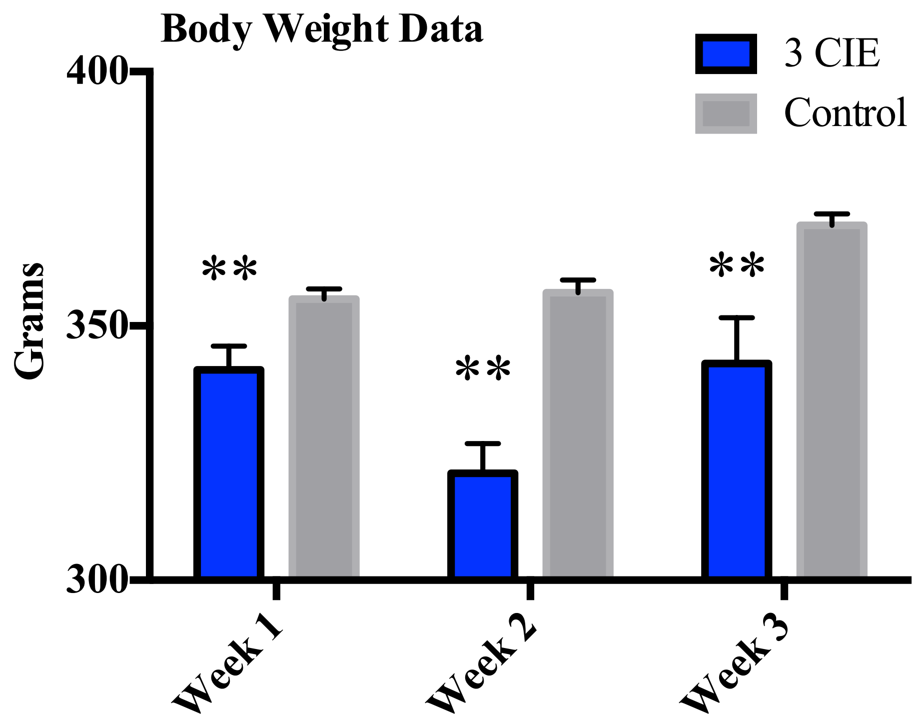

Figure 15. Changes in body weight in subjects exposed to ethanol (n=5) or an isocaloric diet (n=6) for three cycles of CIE. X-axis: days in Week One (left), Week Two (middle), and Week Three (right). Data points show mean body weight in grams for subjects exposed to ethanol (blue bar) or an isocaloric diet (gray bar). Two asterisks indicate a significant day-by-treatment interaction.

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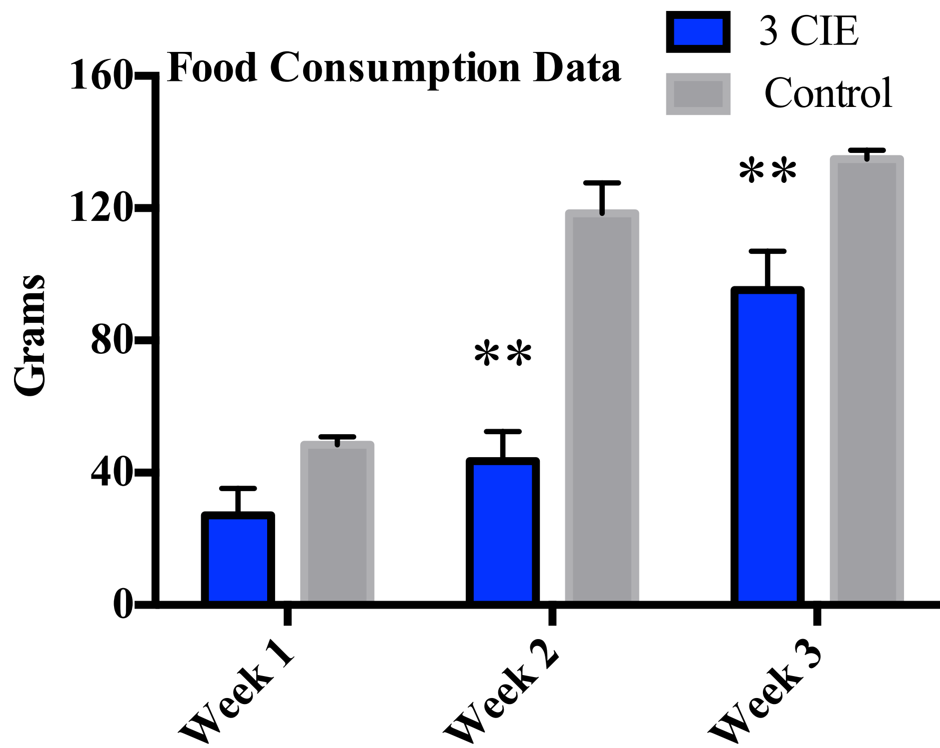

Figure 16. Changes in food consumption in subjects exposed to ethanol (n=5) or an isocaloric diet (n=6) for three cycles of CIE. X-axis: week. Data points show mean food consumption in grams for subjects exposed to ethanol (filled circle) or an isocaloric diet (empty circle). Two asterisks indicate a significant day-by-treatment interaction.

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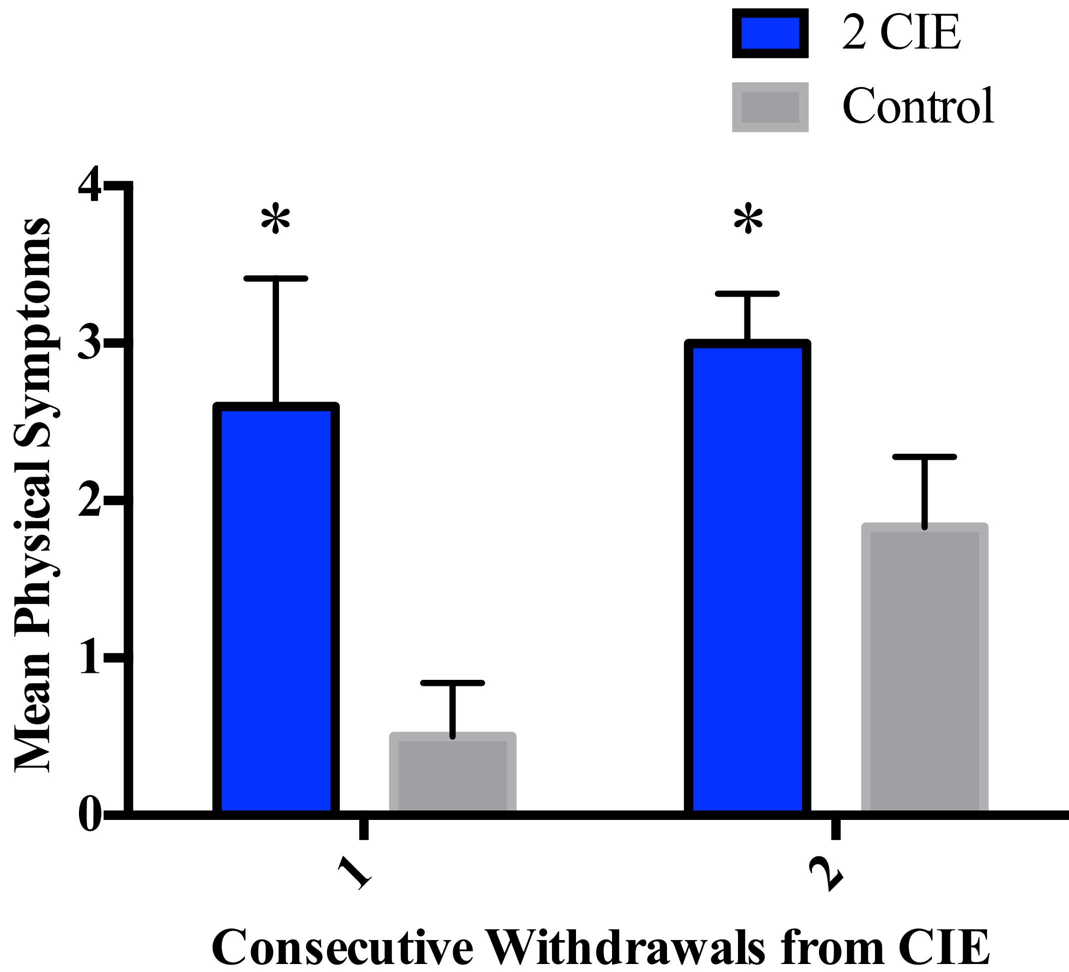

Figure 17. Behavioral effects of withdrawal in subjects exposed to ethanol (n=5) or an isocaloric diet (n=6) for two cycles of CIE. X-axis: day. Data points show mean scores in withdrawal behavior for subjects exposed to ethanol (blue bar) or an isocaloric diet (gray bar). An asterisk indicates that is a significant difference between ethanol and control subjects.

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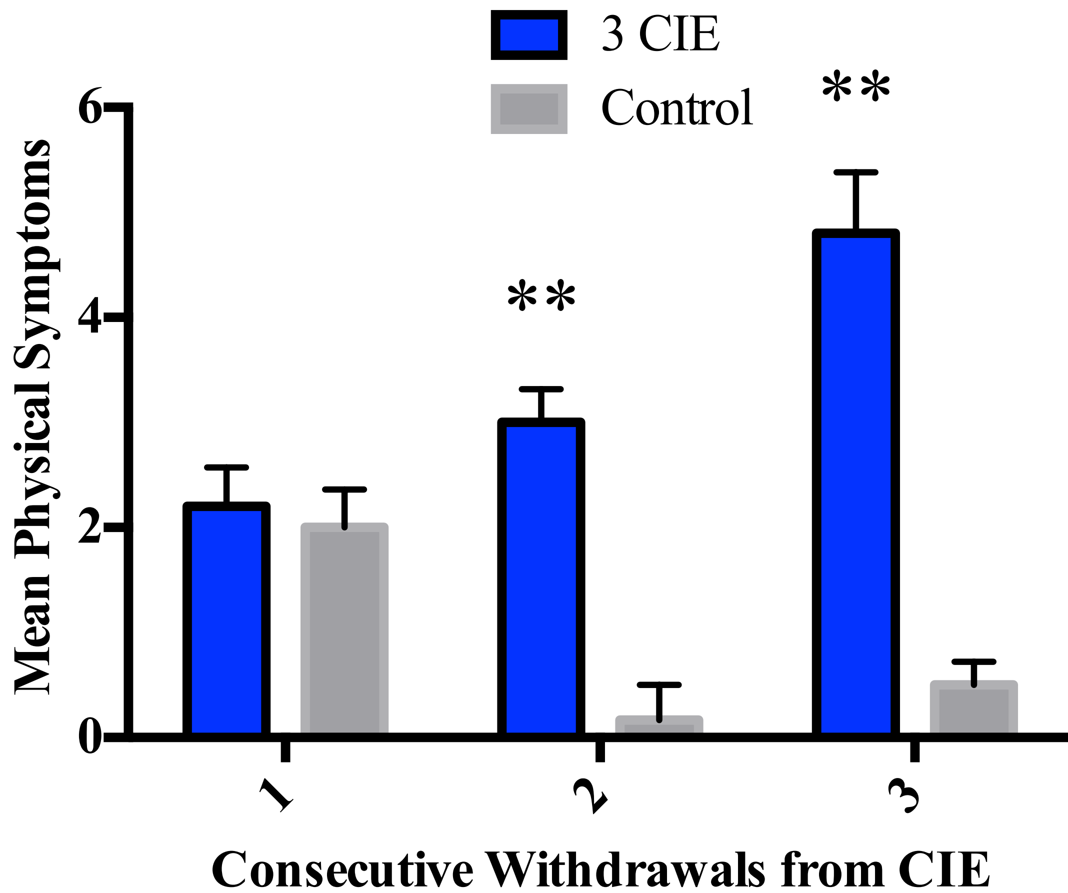

Figure 18. Behavioral effects of withdrawal in subjects exposed to ethanol (n=5) or an isocaloric diet (n=6) for three cycles of CIE. X-axis: day. Data points show mean scores in withdrawal behavior for subjects exposed to ethanol (blue bar) or an isocaloric diet (gray bar). Two asterisks indicate a significant interaction between day and treatment.

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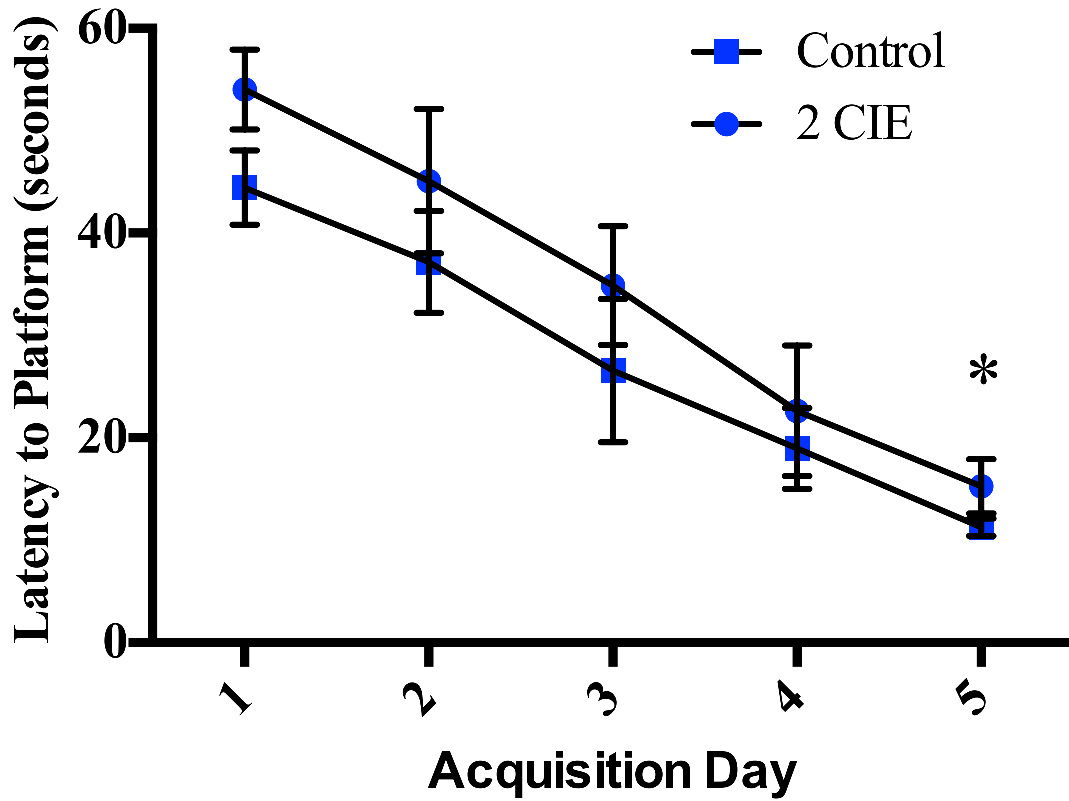

Figure 19. Cognitive performance was assessed in subjects exposed to ethanol (n=5; filled circle) or an isocaloric diet (n=6; filled square) for two cycles of CIE. X-axis: day. Data points show mean scores in latency to platform. One asterisk indicates that there is a significant difference between acquisition days.

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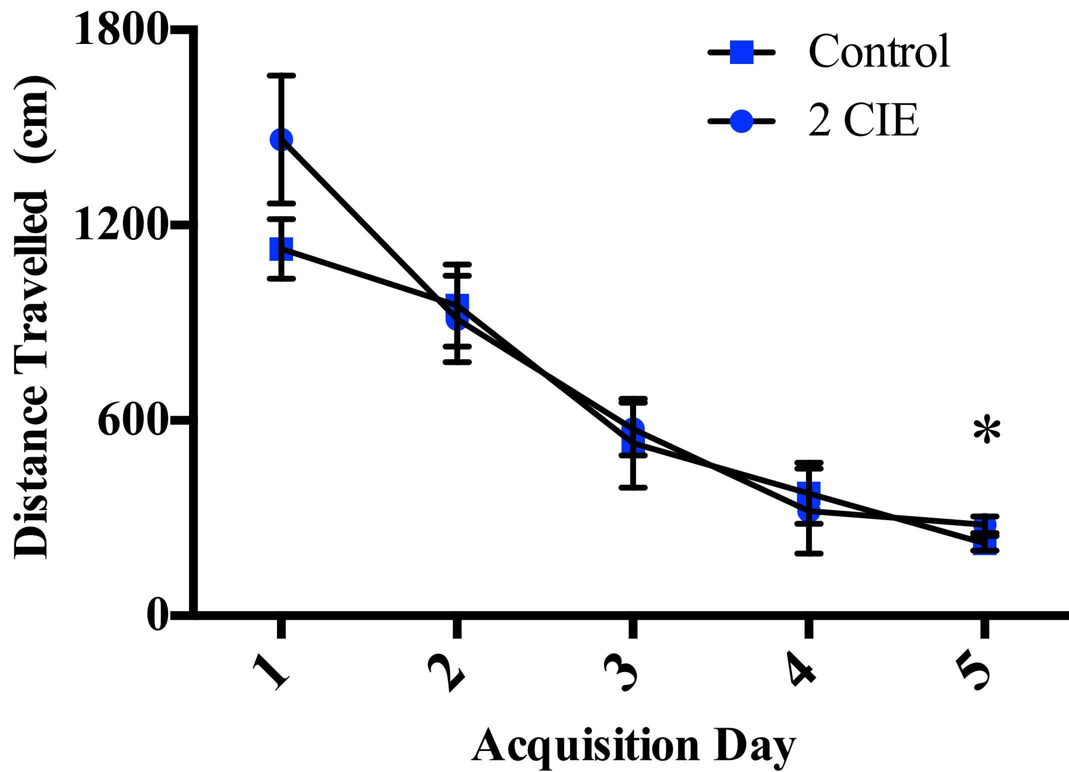

Figure 20. Cognitive performance was assessed in subjects exposed to ethanol (n=5; filled circle) or an isocaloric diet (n=6; filled square) for two cycles of CIE. X-axis: day. Data points show mean scores in distance travelled. One asterisk indicates that there is a significant difference between acquisition days.

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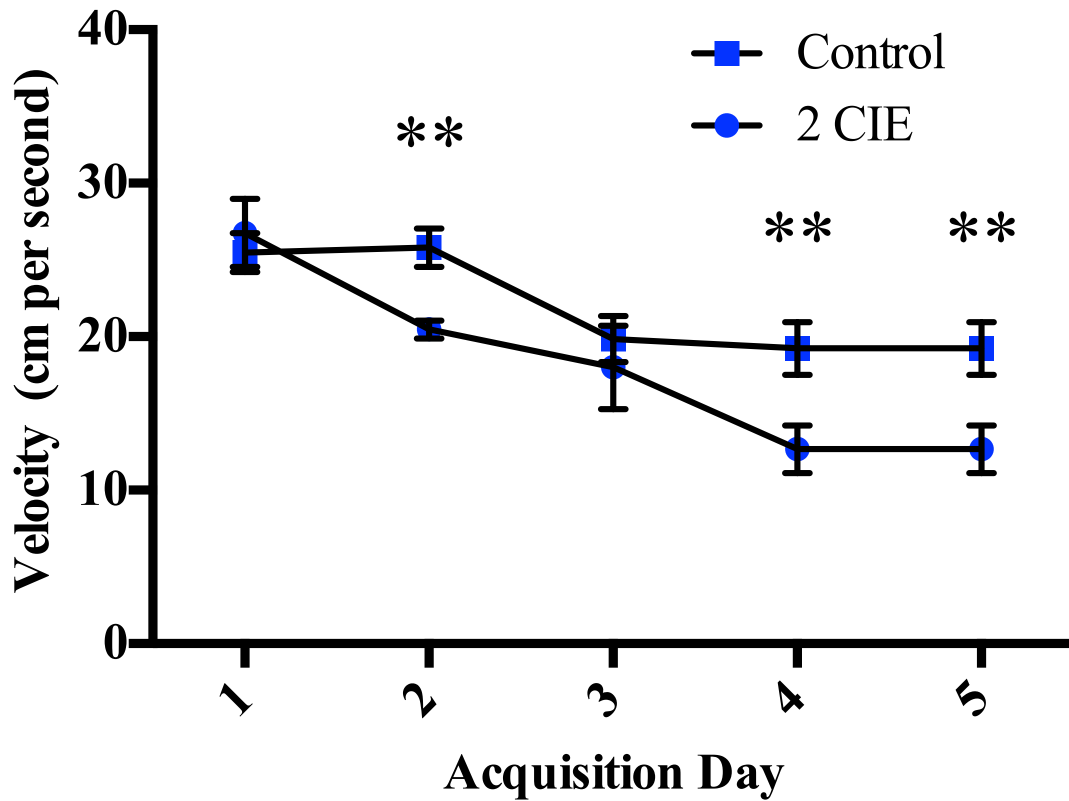

Figure 21. Cognitive performance was assessed in subjects exposed to ethanol (n=5; filled circle) or an isocaloric diet (n=6; filled square) for two cycles of CIE. X-axis: day. Data points show mean scores in velocity. Two asterisks indicates that there is a significant difference between treatment groups.

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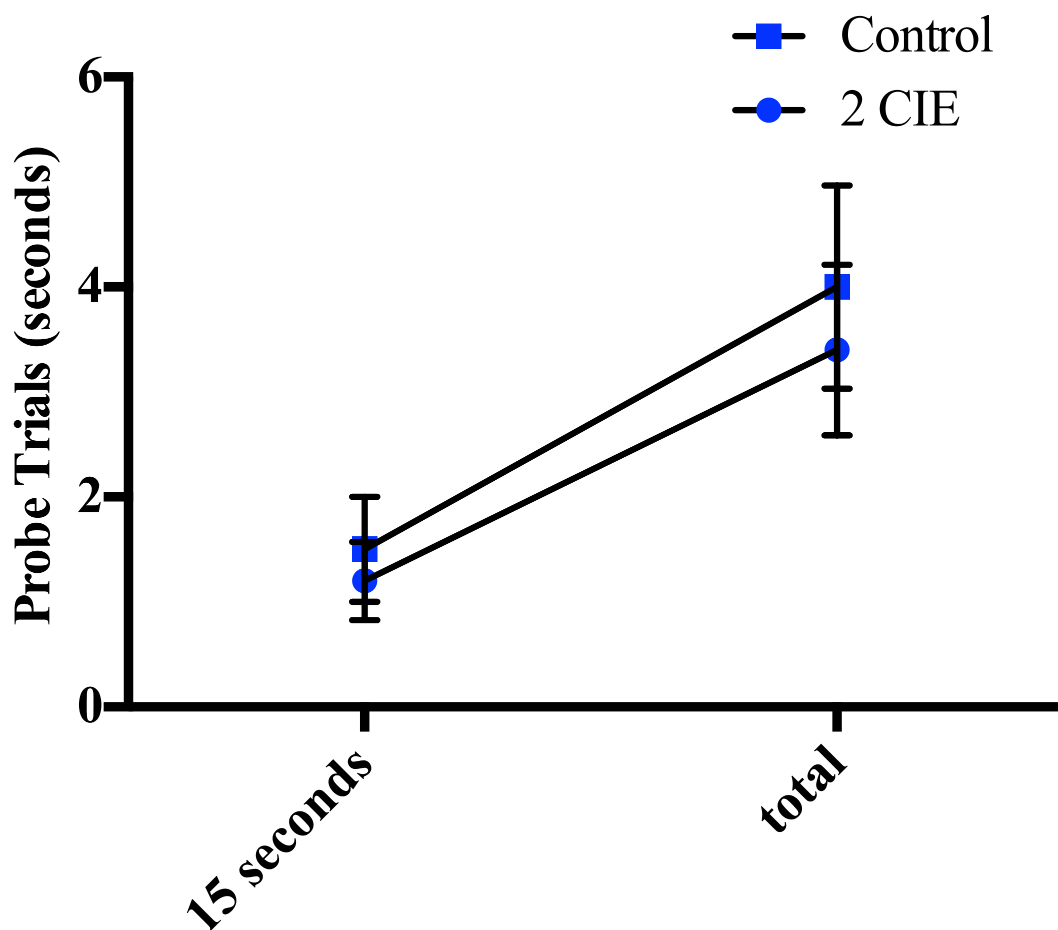

Figure 22. Working memory assessment in subjects exposed to ethanol (n=5) or an isocaloric diet (n=6) for two cycles of CIE. X-axis: day. Data points show mean scores in platform crosses during the probe trial for subjects exposed to ethanol (filled circle) or an isocaloric diet (filled square).

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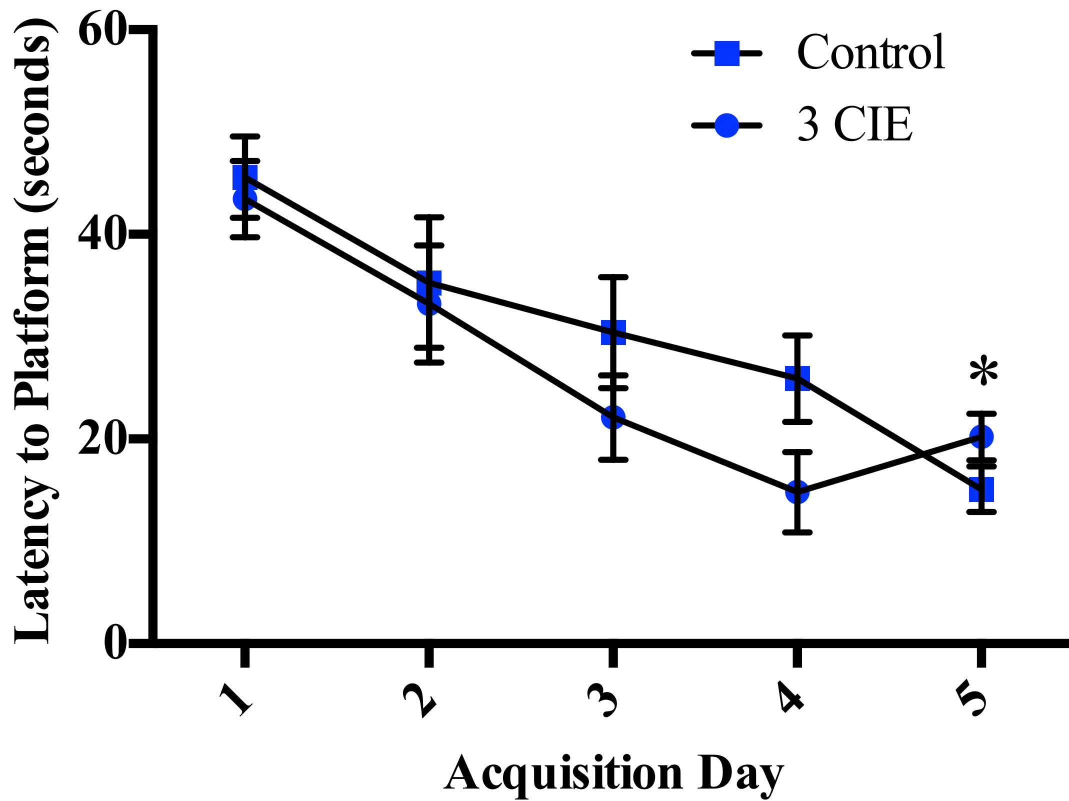

Figure 23. Cognitive performance was assessed in subjects exposed to ethanol (n=5; filled circle) or an isocaloric diet (n=6; filled square) for three cycles of CIE. X-axis: day. Data points show mean scores in latency to platform. One asterisk indicates that there is a significant difference between acquisition days.

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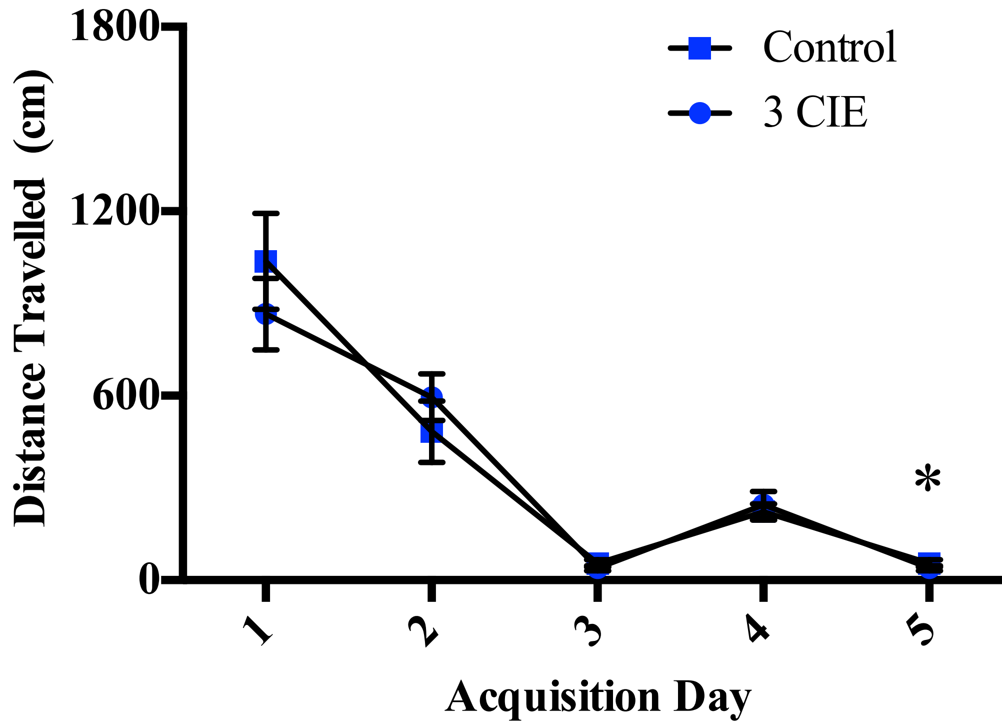

Figure 24. Cognitive performance was assessed in subjects exposed to ethanol (n=5; filled circle) or an isocaloric diet (n=6; filled square) for three cycles of CIE. X-axis: day. Data points show mean scores in distance travelled. One asterisk indicates that there is a significant difference between acquisition days.

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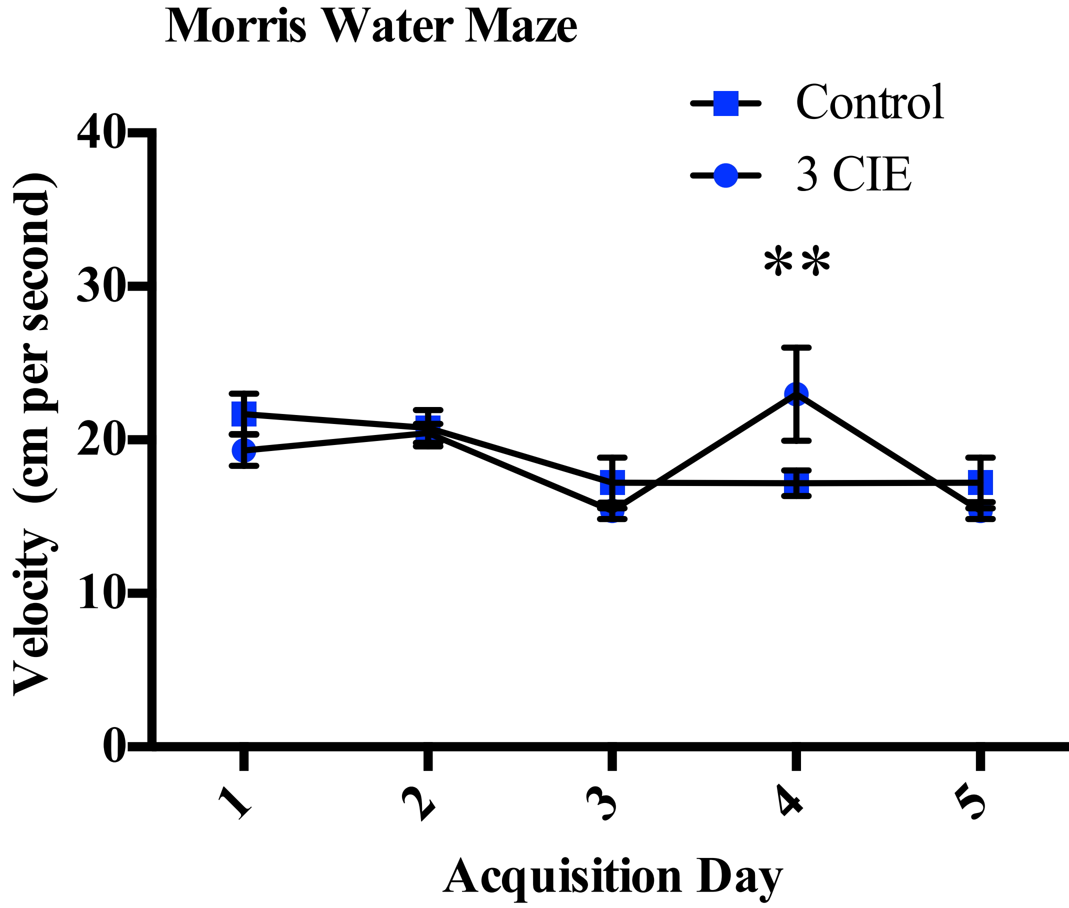

Figure 25. Cognitive performance was assessed in subjects exposed to ethanol (n=5; filled circle) or an isocaloric diet (n=6; filled square) for three cycles of CIE. X-axis: day. Data points show mean scores in velocity. Two asterisks indicate that there is a significant difference between groups.

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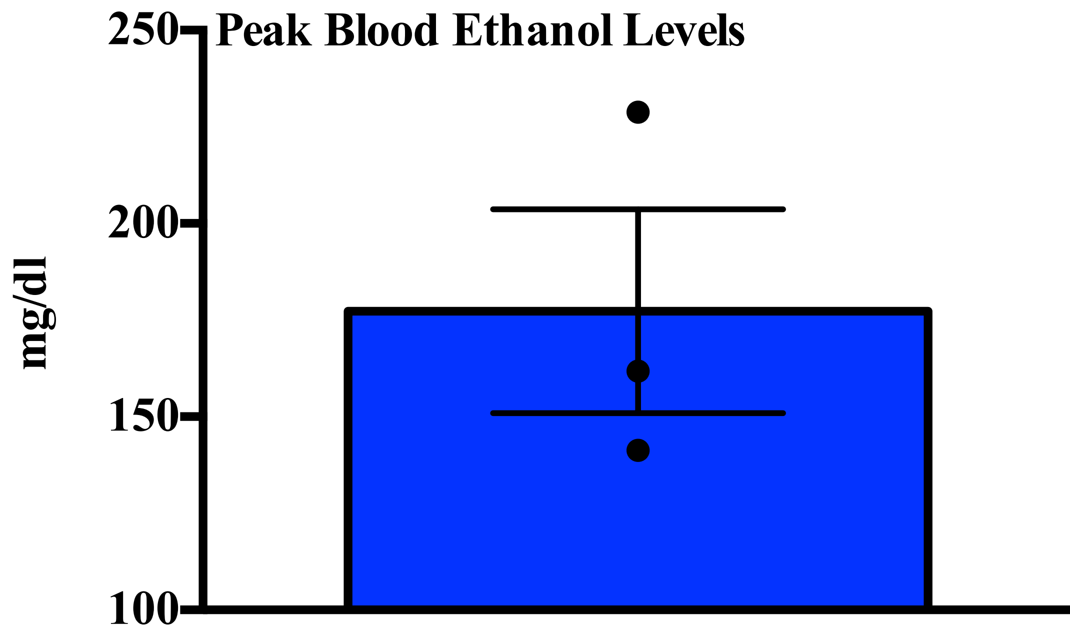

Figure 26. Peak BELs in subjects exposed to ethanol (n=5) for three cycles of CIE. X-axis: day. Data points show mean levels determined at 90 minutes post ethanol administration on Week One, Week Two, and Week Three.

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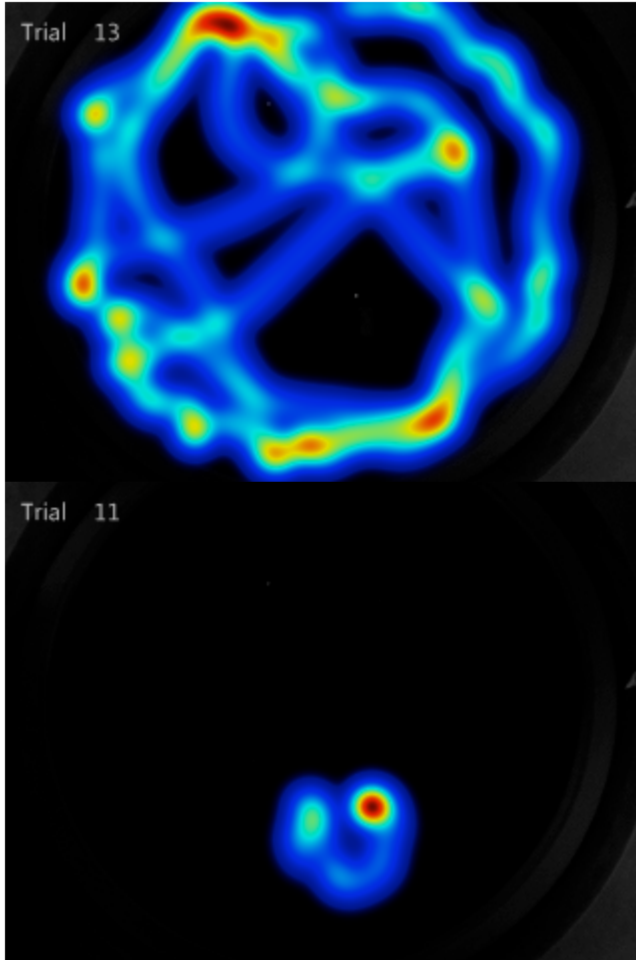

Figure 27. Representative heat map images depicting distance travelled in Morris Water Maze decreased significantly on Day Five (bottom image), as compared to Day One (top image).

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CHAPTER FOUR: Study Three: Influence of mGluR1/5-containing Receptors and Intracellular Calcium in Development of Ethanol Dependence *in Vitro*

4.1. Introduction

Group 1 metabotropic glutamate receptors. Glutamate is the primary excitatory neurotransmitter in the mammalian central nervous system. There are two distinct classes of glutamate receptors: ionotropic glutamate receptors (e.g., N-methyl-D-aspartate [NMDA] receptors) and metabotropic glutamate receptors (i.e., mGluRs). While ionotropic glutamate-type receptors were first characterized as containing ligand-gated ion channels (Cotman et al., 1989), mGluRs were later identified as large guanine nucleotide-binding protein (G-protein)-coupled receptors involved in the modulation of excitatory neurotransmission (Nakanishi, 1994). Studies using molecular cloning and cross-hybridization techniques have identified eight known subtypes of mGluRs classified into three groups based on sequence homology, signal transduction, and pharmacological action (Nakanishi, 1994; for a review, see Conn & Pin, 1997). Group 1 mGluRs (i.e., mGluR1 and mGluR5) are $G\alpha_q$-linked to phospholipase C and can stimulate phosphoinositide hydrolysis (Abe et al., 1992; Houamed et al., 1991; Pin et al., 1992). These receptors are known to be involved in a myriad of biological processes, including regulation of second messengers (Miller et al., 1995; Schoepp & Conn, 1993; Schoepp et al., 1994), ion channels, such as potassium channels (Charpak et al., 1990; Guérineau et al., 1994), and neuronal excitability (Davies et al., 1995; Gereau & Conn, 1995). These diverse effects are likely mediated in part via the wide distribution of group 1 mGluRs throughout the brain and spinal cord (Baude et al., 1993; Fotuhi et al., 1993; Martin et al., 1992; Petralia, 1997).

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Group 1 mGluRs interact closely with intracellular scaffolding proteins, such as Homer proteins. A study using immunofluorescent techniques found that Homer1b retains group 1 mGluRs at the endoplasmic reticulum when these proteins are coexpressed (Roche et al., 1989). Ango et al. (2000) found that the mGluR5 is found in dendrites and axons when cotransfected with Homer1a whereas these receptors are concentrated at cell bodies when transfected alone in cerebellar granule cells. Further, immunocytochemical and western blot analyses show that Homer proteins can actually bind and activate group 1 mGluRs independent of agonist application (Ango et al., 2001; for a review, see Spooren et al., 2001).

Group 1 mGluRs are also known to be modulated by extracellular agonists, which may indirectly affect function of these receptors. Cozzoli and colleagues (2009) found that binge-like ethanol administration produced significant increases in Homer2a/b expression as well as Homer2-phosphatidylinositol 3-kinase (PI3K) signaling in the nucleus accumbens. These effects were attenuated with administration of mGluR5 antagonist MPEP and PI3K antagonist Wortmannin. Notably, pretreatment with mGluR5 antagonist MPEP dose-dependently reduced binge ethanol consumption while mGluR1 antagonist (hydroxyimino)cyclopropa[b]chromen-1a-carboxylate ethyl ester (CPCCOEt) did not alter ethanol self-administration. Collectively, these studies suggest that $G\alpha_q$-coupled group 1 mGluRs influence the voluntary intake of ethanol in rodents. However, the functional influence of group 1 mGluRs in promoting the development of ethanol dependence is not fully understood. Further, the role of activated mGluR1-containing receptors and mGluR5-containing receptors is not fully characterized with regards to ethanol withdrawal-induced cytotoxicity. Thus, the present study examined the functional

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relationship between ethanol-associated activation of group 1 mGluRs and subsequent and persisting withdrawal-induced NMDA-receptor-dependent cytotoxicity following CIE. This study closely followed a model from an earlier study (Reynolds et al., 2015).

Inositol-triphosphate-mediated release of intracellular calcium. Calcium is a ubiquitous intracellular signal known to control a cadre of cellular processes (e.g., neurotransmitter release and synaptic plasticity) and is also modulated by ethanol (Berridge et al., 2000). The primary activator of intracellular calcium is calcium itself (i.e., calcium induced calcium release); however, inositol triphosphate (IP3) can also engage the IP3 receptors to release calcium from internal stores (Verkhratsky, 2002), and exposure to ethanol can release intracellular calcium from receptor complexes located at the endoplasmic reticulum, such as the IP3 receptor (Daniell & Harris, 1989). Other studies suggest that exposure to ethanol produces presynaptic release of calcium from internal stores prior to γ-Aminobutyric acid (GABA) neurotransmission in cerebellar neurons (Kelm et al., 2007) and that GABAergic neurotransmission is dependent on the release of intracellular calcium in dopaminergic neurons (Thiele et al., 2009).

During periods of withdrawal from chronic ethanol exposure, excessive extracellular calcium influx via the NMDA receptor can produce excitotoxicity (Prendergast et al., 2004) and neurodegeneration (Lau & Tymianski, 2010). The mGluR5 is also implicated in the neurotoxic effects of withdrawal. In one study, for example, exposure to SIB-1893, the competitive mGluR5 antagonist, attenuated cytotoxicity produced by withdrawal in cultured organotypic hippocampal slices (Harris et al., 2003). The inhibition of intracellular calcium mobilization via antagonists for the IP3, sigma-1

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receptor, and ryanodine receptors can attenuate excitotoxicity, and in some cases, provide neuroprotection in various models of neurodegenerative disorders, such as HIV-1 Tat (Haughey et al., 2001) and stroke (Shen et al., 2008). Previous studies examining the modulatory effects of ethanol on GABA implicate a role for the release of intracellular calcium in the activation of PKA (Kelm et al., 2008). These alterations in PKA activity are associated with decreases in the sedative effects of ethanol and voluntary ethanol consumption (Thiele et al., 2000; Wand et al., 2001).

4.2. Experimental Rationale

These findings suggest a role for mGluR5 in the behavioral effects of ethanol and, thus, in the development of acute alcohol dependence. They also suggest a potential role for intracellular calcium-initiating alterations in the NMDA receptor that potentiate cytotoxicity during ethanol withdrawal. A linking of these mechanisms and the primary hypothesis tested is shown in Figure 28.

4.3. Methods

Organotypic hippocampal slice culture preparation. Whole brains from eight-day-old Sprague-Dawley rats (Harlan Laboratories; Indianapolis, IN) were aseptically removed and placed in culture dishes containing frozen dissecting medium composed of Minimum Essential Medium (MEM; Invitrogen, Carlsbad, CA), 25 mM HEPES (Sigma, St. Louis, MO), and 50 μM streptomycin/penicillin (Invitrogen). Bilateral hippocampi were then removed and carefully transferred onto plates containing chilled culture medium composed of dissecting medium, distilled water, 36 mM glucose (Fisher, Pittsburg, PA), 25% Hanks' Balanced Salt Solution (HBSS; Invitrogen), 25% (v/v) heat-inactivated horse serum (HIHS; Sigma), and 0.05% streptomycin/penicillin (Invitrogen). Excess

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hippocampal tissue was carefully detached using a stereoscopic microscope and unilateral hippocampi and then sectioned at 200 µM using a McIlwain Tissue Chopper (Mickle Laboratory Engineering Co. Ltd., Gomshall, UK). Three hippocampal slices with intact cell layers were plated onto Millicell-CM 0.4 µM biopore membrane inserts containing 1 mL of pre-incubated culture medium and placed in a six-well culture plate. Excess culture medium was extracted off the top of each biopore membrane insert and the harvested tissue were maintained in an incubator at 37°C with a gas composition of 5% CO_2/95% air for five days prior to any experimental manipulations so that each hippocampal slice could adequately adhere to the membrane. Each culture well plate generated eighteen intact hippocampal slices. Care of all animals was carried out in agreement with the University of Kentucky's Institutional Animal Care and Use Committee.

Ethanol exposure and withdrawal. A previous study found that exposure to CIE produced NMDA-receptor-mediated hippocampal cytotoxicity (Reynolds et al., 2015). This study suggested that these effects are produced by the activation of these receptor complexes during periods of withdrawal, but the neuroadaptive changes that take place prior to withdrawal are not yet fully understood. The present study employed this model of CIE exposure to delineate probable mechanistic underpinnings associated with the development of ethanol dependence. The timeline of the study is detailed in Figure 29.

After five days *in vitro*, male and female hippocampal slice cultures were randomly transferred to plates containing either 1 ml of the culture medium (control) or medium containing a binge-like ethanol concentration (i.e., 0 and 50 mM) for five days

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with or without the addition of 1) mGluR1 antagonist (CPCCOEt; 0.5–3 μM); 2) mGluR5 antagonist (SIB-1893; 20–200 μM); 3) Xestospongin C (0–1 μM), a membrane-permeant inhibitor of IP3-mediated calcium release; 4) BD1047 (0–100 μM), a selective antagonist at the sigma-1 receptor; or 5) dantrolene (0–30 μM), a ryanodine receptor antagonist (Kim et al., 2010; Tarragon et al., 2012; Taylor & Tovey, 2010; Wang et al., 2004). At 11 days *in vitro*, cultures were removed from their respective treatment groups and transferred to plates containing 1 ml of fresh ethanol-naïve culture medium for a 24-hour ethanol withdrawal period. These treatments were repeated a total of three times in consecutive order. During each five-day exposure period, ethanol and control-treated cultures were maintained inside Ziploc bags filled with 5%CO_2/95% air and water bath solutions containing either distilled water (50 ml) for control plates or distilled water (50 ml) containing ethanol (50 mM) for ethanol-treated plates. At 23 days *in vitro*, cultures were fixed for immunohistochemistry.

Immunohistochemistry. Cultures were fixed by placing 1 ml of 10% formalin solution on the top and bottom of each well for 30 minutes, washing twice with phosphate buffered saline (PBS), and storing at 4°C until immunohistochemistry was initiated. NeuN (Fox-3) is found in nearly all post-mitotic neurons (Kim et al., 2009; Mullen et al., 1992), and thus these cultures were labeled with NeuN to assess cytotoxicity in each primary cell layer of the hippocampal formation—pyramidal cell layers of the cornu ammonis (CA1 and CA3) and granule cell layer of the dentate gyrus. Fixed inserts were transferred to a plate containing 1 ml of permeabilization (wash) buffer (200 ml PBS [Invitrogen], 200 μL Triton X-100 [Sigma], 0.010 mg Bovine Serum [Sigma]), with 1 ml

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of buffer added to the top of each well for 45 minutes to permeate cell membranes. Tissue was then incubated with the primary monoclonal antibody mouse anti-NeuN (1:200; Sigma) for 24 hours. Inserts were then washed with PBS and incubated for 24 hours with goat anti-mouse fluorescein isothiocyanate (FITC; 1:200; Sigma). Cultures were imaged with SPOT software 4.0.2 (advanced version) for Windows (W. Nuhsbahm Inc.; McHenry, IL, USA) through a 5x objective with a Leica DMIRB microscope (W. Nuhsbahm Inc.; McHenry, IL, USA) connected to a computer and captured with a SPOT 7.2 color mosaic camera (W. Nuhsbahm). In these immunohistochemical studies, FITC fluorescence was detected with a band-pass filter at 495 nm (520 nm emission).

Statistical analyses. Statistical analyses were conducted to assess the influence of group 1 mGluRs and intracellular calcium in the development of ethanol dependence. Effects were considered significant at $p<0.05$. This study was conducted two times using two different rat litters. All immunohistochemical data were converted to percent control and then combined for ease of data interpretation. A one-way ANOVA was conducted for all *in vitro* techniques with treatment as the factor for each hippocampal cell layer (i.e., CA1, CA3, and dentate gyrus). This statistical strategy was based on the results of preliminary data suggesting that sex is not a factor influencing the effects produced by repeated binge-like ethanol. Planned comparisons were conducted if a significant effect of treatment was to make pairwise comparisons between means detected using Fisher's LSD.

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4.4. Results

Noncompetitive mGluR1 antagonist CPCCOEt. In the pyramidal cell layer of the CA1, ANOVA analyses revealed a main effect of treatment ($F[5,102]= 3.574$, $p=0.002$). Figure 30 shows that CIE exposure produced a 30% decrease in NeuN immunoreactivity. Exposure to CPCCOEt (0.5, 1, and 3 μM) did not significantly alter these effects in ethanol-naïve or ethanol-exposed hippocampi (Fisher's post hoc; $p > 0.05$). In the pyramidal cell layer of the CA3, ANOVA revealed a main effect of treatment ($F[5,102]= 5.938$, $p<0.001$). Figure 31 shows that decreases in NeuN immunoreactivity were significantly attenuated in hippocampi exposed to the highest concentration of CPCCOEt examined (3 μM) in the CA3 despite evidence that this compound is toxic alone. A similar pattern was observed in the dentate gyrus, as ANOVA revealed a main effect of treatment ($F[5,102]= 5.699$, $p<0.001$) in this hippocampal cell layer. Loss of NeuN immunoreactivity was spared in hippocampi co-exposed to CIE and the highest concentration of CPCCOEt (i.e., 3 μM) in the granule cell layer of the dentate gyrus (Figure 32). Representative images of NeuN immunofluorescence of control hippocampi, hippocampi exposed to ethanol, and hippocampi co-exposed to ethanol and CPCCOEt (3 μM) are found in Figure 33.

Noncompetitive mGluR5 antagonist SIB-1893. In the pyramidal cell layer of the CA1, ANOVA analyses revealed a main effect of treatment ($F[5,200]= 3.167$, $p<0.05$) where CIE exposure produced significant decreases in NeuN immunofluorescence within the CA1 subregion (see Figure 34). Exposure to SIB-1893 (20, 100, and 200 μM) prevented these effects (Fisher's post hoc; $p > 0.05$; Figure 34) despite SIB-1893 being toxic to ethanol-naïve slices. In the pyramidal cell layer of the CA3, ANOVA revealed a main

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effect of treatment ($F[5,102]= 3.385$, $p<0.05$). Figure 35 shows that decreases in NeuN immunoreactivity were significantly attenuated in hippocampi exposed to the lowest concentration of SIB-1893 (20 μM) examined in the CA3. A similar pattern was observed in the dentate gyrus, as ANOVA revealed a main effect of treatment ($F[7,201]=6.831$, $p<0.001$) in this hippocampal cell layer. Loss of NeuN immunoreactivity was spared in hippocampi co-exposed to binge-like ethanol and 20 μM of SIB-1893 in the granule cell layer of the dentate gyrus (Figure 36). Representative images are found in Figure 37.

Inhibitor of IP3-mediated calcium release Xestospongin C. In the pyramidal cell layer of the CA1, ANOVA analyses revealed a main effect of treatment ($F[3,133]= 21.910$, $p<0.001$) where CIE produced significant decreases in NeuN immunofluorescence within the CA1 subregion (see Figure 38). Exposure to Xestospongin C (0.5 μM) prevented these effects (Fisher's post hoc; $p >0.05$; Figure 38) despite Xestospongin C being neuroprotective to ethanol-naïve slices. In the pyramidal cell layer of the CA3, ANOVA revealed a main effect of treatment ($F[3,133]= 10.1325$, $p<0.001$). Figure 39 shows that decreases in NeuN immunoreactivity were significantly attenuated in hippocampi exposed to Xestospongin C (0.5 μM) examined in the CA3 despite Xestospongin C being neuroprotective to ethanol-naïve slices. A similar pattern was observed in the dentate gyrus, as ANOVA revealed a main effect of treatment ($F[3,133]=14.891$, $p<0.001$) in this hippocampal cell layer. Loss of NeuN immunoreactivity was spared in hippocampi co-exposed to CIE and 0.5 μM of Xestospongin C in the granule cell layer of the dentate gyrus despite Xestospongin C being neuroprotective to ethanol-naïve slices (Figure 40).

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Sigma-1 receptor antagonist BD-1047. In the pyramidal cell layer of the CA1, ANOVA analyses revealed a main effect of treatment (F[5,91]= 5.848, p<0.001) where CIE produced significant decreases in NeuN immunofluorescence within the CA1 subregion (see Figure 41). Exposure to BD-1047 (20 µM) prevented these effects (Fisher's post hoc; p >0.001; Figure 41) despite BD-1047 (20 µM) being neuroprotective to ethanol-naïve slices. In the pyramidal cell layer of the CA3, ANOVA revealed a main effect of treatment (F[5,91]= 6.023, p<0.001). Figure 42 shows that decreases in NeuN immunoreactivity were significantly attenuated in hippocampi exposed to BD-1047 (20 µM) examined in the CA3 despite BD-1047 being neuroprotective to ethanol-naïve slices. A similar pattern was observed in the dentate gyrus, as ANOVA revealed a main effect of treatment (F[5,91]=6.381, p<0.001) in this hippocampal cell layer. Loss of NeuN immunoreactivity was spared in hippocampi co-exposed to CIE and 20 µM of BD-1047 in the granule cell layer of the dentate gyrus despite BD-1047 being neuroprotective to ethanol-naïve slices (Figure 43).

Ryanodine receptor antagonist dantrolene. In the pyramidal cell layer of the CA1, ANOVA analyses revealed a main effect of treatment (F[3,91]= 5.848, p<0.001) where CIE exposure produced significant decreases in NeuN immunofluorescence within the CA1 subregion (see Figure 44). Exposure to dantrolene (5 µM) did not prevent these effects (Fisher's post hoc; p >0.001; Figure 44) despite dantrolene (5 µM) being toxic to ethanol-naïve slices. In the pyramidal cell layer of the CA3, ANOVA revealed a main effect of treatment (F[3,91]= 6.023, p<0.001). Figure 45 shows that decreases in NeuN immunoreactivity were significantly attenuated in hippocampi exposed to dantrolene (5

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μM) examined in the CA3 despite dantrolene being toxic to ethanol-naïve slices. While ANOVA revealed a main effect of treatment ($F[3,91]=6.381$, $p<0.001$) in the dentate gyrus hippocampal cell layer, loss of NeuN immunoreactivity was not spared in hippocampi co-exposed to CIE and 5 μM of dantrolene in the granule cell layer of the dentate gyrus (Figure 46).

4.5. Discussion

Effects of mGluR1 antagonist CPCCOEt on hippocampal cytotoxicity produced by CIE exposure. The present study suggests that CIE exposure produces significant hippocampal cytotoxicity characterized by loss of NeuN immunofluorescence in the primary cell layers of the hippocampal formation. In the study, exposure to higher concentrations of CPCCOEt significantly attenuated the loss of NeuN immunoreactivity in the CA3 and dentate subregions of the hippocampal formation. Thus, the loss of NeuN immunoreactivity could reflect a down-regulation of NeuN protein expression. However, in a previous study using an identical model of CIE, we found that long-lasting (seven-day) ethanol withdrawal-induced decreases in NeuN immunoreactivity correlated with decreases in thionine, a cellular marker (Reynolds et al., 2015). Thus, the exact cause of these effects that adversely affect "physiological status" (Weyer & Schilling, 2003) may reflect mGluR1 activation of downstream signaling cascades, as stimulation of hippocampal mGluR1s can activate second messenger effectors, such as mitogen-activated protein kinase (Berkeley & Levey, 2003; Gallagher et al., 2004), and gene expression can then be altered via CREB activation, producing increases in NMDA-receptor subunit GluN2B (Rani et al., 2005). These neuroadaptive changes to NMDA receptors likely confer sensitivity to cytotoxicity produced by ethanol exposure. This

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interpretation is supported by the work of Ticku and colleagues (Qiang et al., 2007) who demonstrated the upregulation of GluN1 and GluN2B subunits in cortical neurons exposed to binge-like ethanol *in vitro* (Qiang et al., 2007). Further, earlier work suggested that this model of ethanol dependence reflects increases in NMDA-receptor activation upon withdrawal, and thus the activity of group 1 mGluRs may contribute to this specific neuroadaptation to ethanol (Reynolds et al., 2015).

It has been shown that mGluR1s are differentially expressed in neurons throughout various areas of the brain and spinal cord (Shigemoto et al., 1993). In the hippocampus, for example, levels of mGluR1 expression are highest in the granule cell layer of the dentate gyrus and pyramidal cell layer of the CA3 (Davies et al., 1995; Miller et al., 1995; Petralia et al., 1997). In the present study, mGluR1 antagonist CPCCOEt did not alter NeuN immunofluorescence in the pyramidal CA1 cell layer of the hippocampus, which is consistent with a previous study that found that immunolabeling of mGluR1s was not detectable in the pyramidal cell layer of the CA1 in the rat hippocampus (Shigemoto et al., 1993). In sum, the present findings extend upon prior work suggesting a region-specific functional role for the mGluR1 in the development of hippocampal cytotoxicity within the CA3 and dentate gyrus.

Effects of mGluR5 antagonist SIB-1893 on hippocampal cytotoxicity produced by CIE exposure. Noncompetitive mGluR5 antagonists (e.g., SIB-1893) bind to the 7-transmembrane domain and alter conformational changes without affecting the extracellular ligand binding site (for a review, see Spooren et al., 2001). Other studies have suggested that mGluR5 antagonists are neuroprotective in various models of

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neurodegeneration (Battalglia et al., 2002; Chapman et al., 2000; Harris et al., 2003). In the present study, exposure to noncompetitive mGluR5 antagonist SIB-1893 prevented hippocampal cytotoxicity produced by CIE exposure. Other studies have shown that acute exposure to SIB-1893 prevented ethanol withdrawal-induced loss of propidium iodide, a marker of cytotoxicity, in the CA1 subregion of the mature hippocampus (Harris et al., 2003). The mGluR5 is, therefore, important for both ethanol intoxication and withdrawal. An earlier *in vivo* study found that the mGluR5 antagonist MPEP mitigated increases in Homer2 signaling that were produced by binge-like ethanol exposure and ethanol-induced plasticity (Cozzoli et al., 2009), and other studies have shown that MPEP dose-dependently decreased operant ethanol self-administration and GluN1 and GluN2 mRNA expression in the cingulate cortex while CPCCOEt did not alter voluntary ethanol self-administration (Hodge et al., 2006). Other studies have shown the efficacy of MPEP in attenuating ethanol reinstatement elicited by drug-associated cues (Bäckström et al., 2004). Blednov and Harris (2008), for example, demonstrated that MPEP (10 mg/kg) attenuates ethanol withdrawal behavior in rodents if administered prior to ethanol administration but not following.

It is notable that in the current study mGluR5 antagonist SIB-1893 exposure in the presence of ethanol prevented the hippocampal NeuN loss produced by binge-like ethanol. The reasons for the neuroprotective effects of SIB-1893 are likely associated with the effects of mGluR5s on NMDA-receptor activity in the modulation of glutamatergic tone. For example, NMDA-receptor activity is influenced by the activation of the mGluR5 in the hippocampus via second messenger effector protein kinase C *in vitro* (Chen et al., 2011). Other studies have suggested that Ca^{2+}/calmodulin-dependent

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protein kinase IIα (CaMKIIα) binds to the intracellular C terminus of the mGluR5 to produce a release of calcium from intracellular stores and that subsequent phosphorylation of adjacent GluN2Bs can potentiate NMDA-receptor activity (Jin et al., 2013). Together, these studies suggest that the neuroprotective effects of mGluR5 antagonist SIB-1893 include the modulation of glutamatergic tone via downstream effects at the NMDA receptor.

Effects of blocking IP3-mediated release of intracellular calcium on hippocampal cytotoxicity. Calcium is a ubiquitous intracellular signal responsible for controlling numerous cellular processes. At rest, cells have a basal calcium concentration of 100 nM. When activated, cells have a calcium concentration of 1000 nM (for a review, see Berridge et al., 2003). Internal stores of calcium are held within membrane systems of the endoplasmic reticulum (ER) or sarcoplasmic reticulum of muscle cells (Berridge, 1993; Clapham, 1995). Release of calcium from these internal stores is controlled by various channels, such as the inositol-1,4,5-trisphosphate (IP3) receptor and ryanodine receptor families (Clapper et al., 1987). The present study examined the efficacy of Xestospongin (i.e., C), a macrocyclic bis-1-oxaquinolizidines characterized as a potent blocker of IP3-mediated calcium release on the vesicles of the ER membrane (Gafni et al., 1997), against toxicity produced by CIE. We found that application of Xestospongin C for three consecutive cycles of CIE actually increased levels of NeuN immunofluorescence in the rat hippocampus. The reasons for these effects are unknown, but likely reflect inherent characteristics of the slice culture preparations, such as damage to the ER, as IP3 channels are regulated by several factors and cytosolic actions of calcium can either be

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stimulatory or inhibitory (for a review see Berridge et al., 2003; Verkhratsky, 2002). Therefore, the present findings suggest a functional implication of blocking IP3-mediated calcium release in organotypic hippocampal slice cultures so as to preserve calcium homeostasis. These data are similar to a previous study conduced in our laboratory demonstrating the efficacy of acute Xestospongin application during withdrawal from methamphetamine (Smith et al., 2008).

Given that there is a 10-fold increase of IP3 receptors in the cerebellum as compared to the hippocampus (Smith, 1987) and a robust shrinking of the cerebellum observed in alcohol-dependent individuals (Chanraud et al., 2007; Sullivan et al., 2000; for a review, see Rosenbloom & Pfefferbaum 2008), future studies could assess the influence of these receptor complexes in other areas of the brain implicated in having neurological consequences in individuals with an alcohol use disorder, such as the cerebellum.

Collectively, these findings suggest that while ethanol application mediates a release of intracellular calcium in the hippocampus (Daniell & Harris; 1989, Mironov & Hermann, 1995) that may reflect a decrease of IP3 mRNA (Rodríguez et al., 1996; Simonyi et al., 1996), preparation of organotypic hippocampal slices may also inherently cause calcium dysregulation from the ER.

Effects of sigma-1 receptor antagonism on the hippocampal cytotoxicity produced by CIE. The sigma-1 receptor is characterized as a calcium-sensitive, ligand-regulated chaperone of the ER. Under basal conditions, the sigma-1 receptor is located on the ER/mitochondrial membrane interface. Here the sigma-1 receptor forms a

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macromolecular complex with glucose regulated protein (i.e., BiP). Calcium depletion of the ER (via ligand activation) initiates dissociation of these two chaperone proteins. Unbound sigma-1 receptors can then bind to IP3 (type 3) receptors, preventing degradation by proteasomes. The dissociation of the sigma-1 receptor from BiP also allows for the sigma-1 receptor to stabilize aggregated IP3 receptors on the ER/mitochondria interface membrane and translocation of IP3 receptors to ER periphery following prolonged calcium depletion (i.e., ER stress). Notably, the unbound sigma-1 receptor can potentiate prolonged IP3 receptor-mediated $Ca2^{+}$ signaling to the mitochondria for checks and balances of ER calcium homeostasis (Hayashi & Su, 2007; for a review, see Hayashi et al., 2009). In the present study, we assessed the influence of the chaperone sigma-1 protein in promoting cytotoxic effects of CIE using a sigma-1 receptor antagonist. We found that blocking this receptor prevented the loss of NeuN immunofluorescence produced by three consecutive cycles of CIE. We also found that chronic blockade of the sigma-1 receptor significantly increased levels of NeuN immunofluorescence in ethanol-naïve hippocampi in a manner similar to Xestospongin C.

Given that the sigma-1 receptor is highly concentrated in the hippocampus (Alonso et al., 2000), it is not surprising that the sigma-1 receptor is known to alleviate alterations in learning and memory produced by exogenous substances (Maurice & Su, 2009; Meunier et al., 2006). In one study, for example, hippocampal neurons from rodents exposed to CIE followed by a period of withdrawal demonstrated an inhibited EPSP-to-spike coupling potentiation curve in the pyramidal cell layer of the CA1 (Sabeti et al., 2011). Exposure to a selective sigma-1 receptor antagonist BD1047 (1 µM)

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resulted in a return to normal EPSP spike potentiation. *In vivo*, sigma-1 receptor antagonism is known to attenuate cognitive deficits produced by CIE (10 mg/kg BD 1047; Meunier et al., 2006) and reduce ethanol consumption (1 mL/kg NE-100; Sabino et al., 2009). Western blot analyses found that CIE produced an upregulation of sigma-1 receptor protein expression in the hippocampus (Sabeti et al., 2011). Notably, these effects were regulated via a sigma-1 receptor antagonist (10 mg/kg BD 1047). Functional polymorphisms in the sigma-1 receptor gene were also associated with a propensity for alcoholism in a case-controlled study (Miyatake et al., 2004). In sum, these studies suggest that targeting the sigma-1 receptor may have clinical implications for behavioral effects associated with prolonged ethanol consumption.

The sigma-1 receptor is also known to be involved in mediating NMDA glutamate-type receptor activity in the hippocampal formation. For example, in one study, exposure to the high affinity sigma-1 receptor agonist ditolylguanidine (DGT) produced increases in NMDA-mediated neuronal firing in the pyramidal cell layer of the hippocampus (Monnet et al., 1990). In another study, exposure to sigma-1 receptor antagonist haloperidol produced suppression of these agonist-dependent responses to NMDA (Monnet et al., 1992). It is known that withdrawal can produce CNS hyperexcitability and/or seizure activity following prolonged exposure to ethanol (Prendergast et al., 2004; Puz & Stokes, 2005; for a review, see Prendergast & Mulholland, 2012) and that these effects are augmented following exposure to multiple withdrawals in rodent models of CIE (Stevens et al., 2001; Veatch & Becker, 2002, 2005) and in the clinical population (Ballenger & Post, 1978; Shaw et al., 1998; Wojnar et al., 1999). Further, neuroadaptations in NMDAR function and/or expression are implicated in

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potentiating excitotoxicity during periods of withdrawal from CIE exposure (Carpenter-Hyland et al., 2004; Rani et al., 2005; Qiang et al., 2007). Thus, in addition to these NMDA-receptor-dependent neuroadaptations, the sigma-1 receptor may modulate these effects. In one study, exposure to ifenprodil, the selective NMDA-receptor antagonist for the NR2B complex, was found to be efficacious in alleviating seizures during periods of withdrawal in rodents (Malinowska et al., 1999). This is notable, as in addition to affinity for NMDA receptors, ifenprodil also has a high affinity for sigma-1 receptors (Hanner et al., 1996; for a review, see Debonnel & de Montigny, 1996). Collectively, these studies suggest that blocking the sigma-1 receptor attenuates classic excitotoxicity *in vitro*. Future studies could assess the efficacy of blocking this chaperone protein during withdrawal from CIE, or drug-taking behaviors (e.g., ethanol self-administration and psychomotor stimulants), *in vivo.*

Effects of ryanodine receptor antagonist dantrolene on the hippocampal cytotoxicity produced by CIE. In the present study, we found that blocking the ryanodine receptor with dantrolene was not effective in attenuating hippocampal cytotoxicity when co-applied with ethanol for three consecutive cycles of CIE. The reasons for these effects are unknown but could reflect the relatively low concentrations of dantrolene employed. Future studies could use higher concentrations of dantrolene in organotypic hippocampal slice culture preparations. Given previous studies that have shown that while the NMDA receptors can trigger calcium release from the ryanodine receptor (Isokawa & Bradley, 2006) and ethanol application inhibits NMDA receptors (Lovinger et al., 1989), future

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studies could assess the effects of blunting ryanodine receptor activation during periods of withdrawal from CIE *in vitro* and *in vivo*.

Using this study's model, it has been shown that ethanol withdrawal-induced hippocampal cytotoxicity is NMDA-receptor dependent (Reynolds et al., 2015), and the present findings extend upon the current literature by characterizing the distinct roles that mGluR1- and mGluR5-containing receptors have in promoting the development of ethanol dependence prior to NMDA-receptor dependent withdrawal-associated cytotoxicity. Collectively, these findings suggest that the concomitant neuroadaptations in group 1 mGluRs and NMDA receptors are likely associated with both the behavioral and neurodegenerative effects observed following multiple bouts of heavy ethanol consumption (Duka et al., 2003; Duka et al., 2004; Sullivan et al., 1996) and in the development of dependence. Group 1 mGluRs—in particular the mGluR5—may, therefore, be a therapeutic target for treatment of alcohol use disorders. *In vivo* examination of this notion is discussed in Chapter 5.

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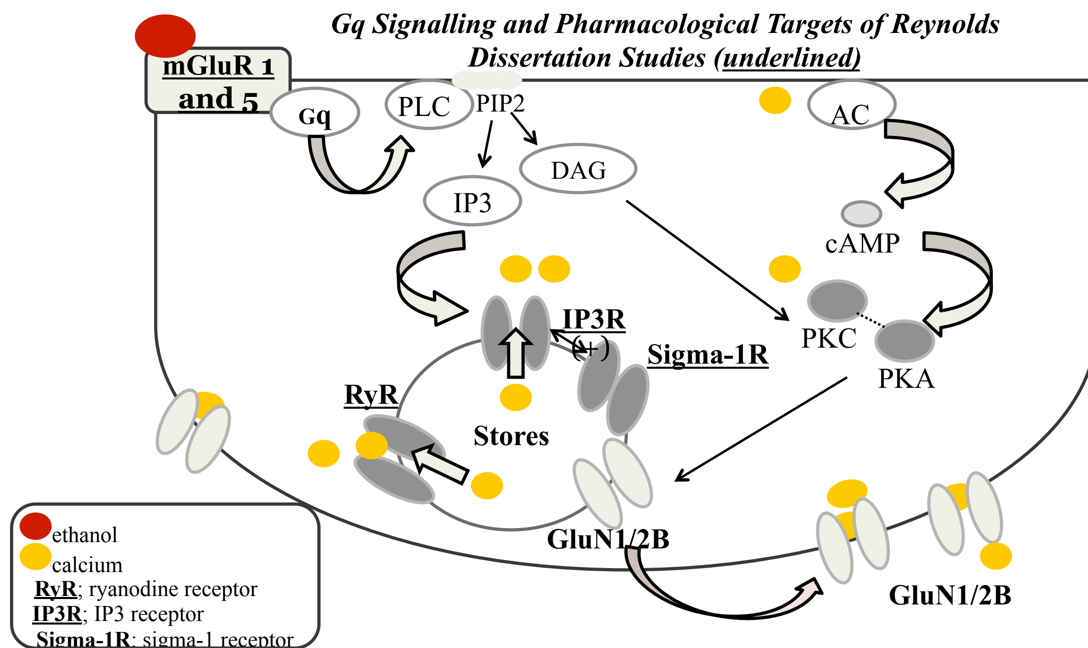

Figure 28. Pictorial representation of the hypothesis that ethanol stimulates group 1 mGluR promoting release of intracellular calcium from IP3-mediated stores. Calcium also stimulates the adenylate cyclase signal transduction pathway that can produce coordinated phosphorylation of NMDA receptors (i.e., GluN1 and GluN2B) by PKA and PKC and subsequent trafficking of these receptor complexes from extrasynaptic to synaptic sites. During withdrawal, excessive calcium influx through these synaptic receptors activates phospholipases, endonucleases, and proteases to produce cytotoxicity and cell death.

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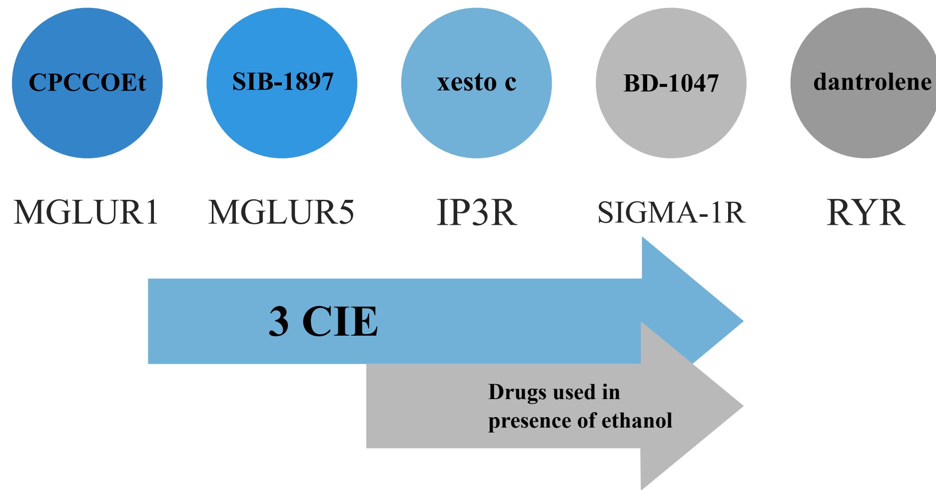

Figure 29. Rat hippocampal explants were exposed to 50 mM ethanol for five days followed by 24-hours of withdrawal with the cycle repeated three times (i.e., 3 CIE) with or without the addition of one of five antagonists.

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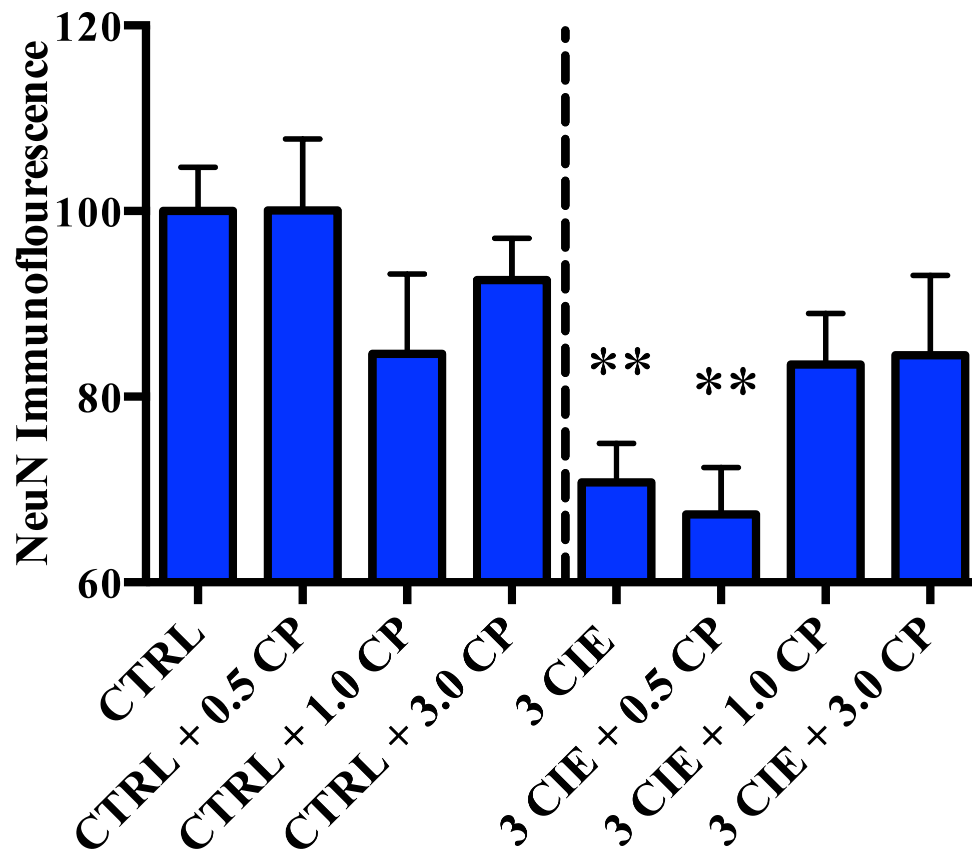

Figure 30. Effects of noncompetitive mGluR1 antagonist CPCCOEt (0.5–3 µM) on NeuN immunofluorescence in the pyramidal CA1 cell layer of the rat hippocampus. Data are presented as percent control of the mean +/- the SEM. ** = statistical significance ($p<0.001$) compared to control hippocampi.

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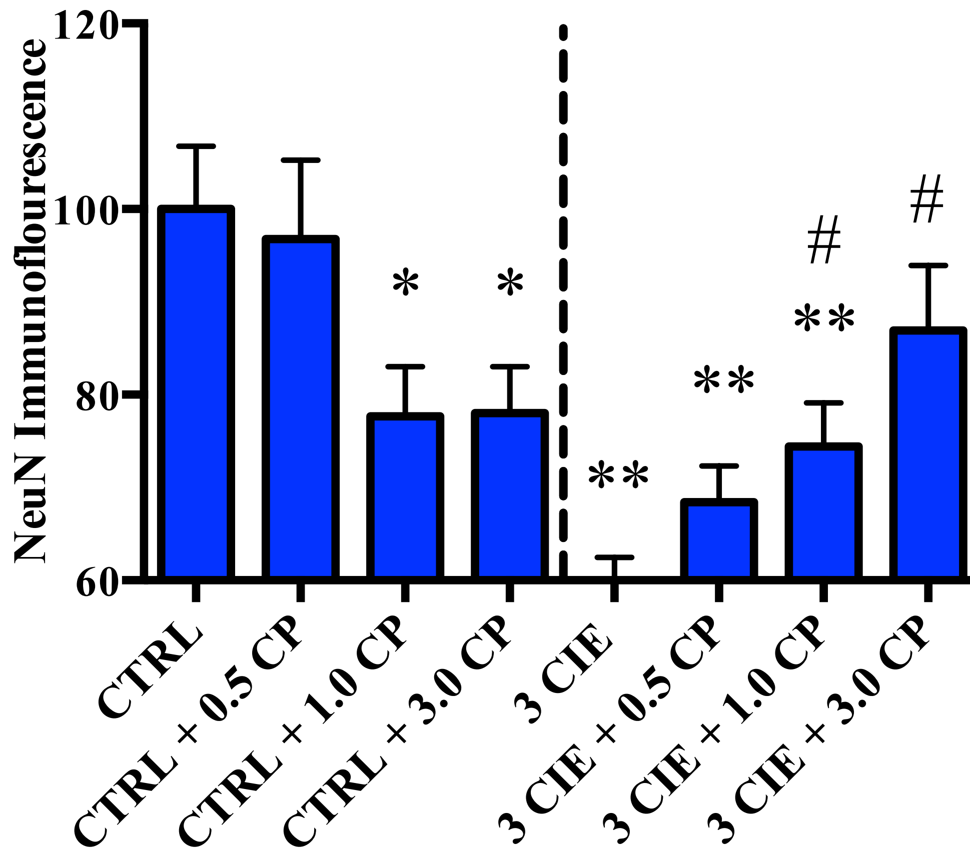

Figure 31. Effects of noncompetitive mGluR1 antagonist CPCCOEt (0.5–3 μM) on NeuN immunofluorescence in the pyramidal CA3 cell layer of the rat hippocampus. Data are presented as percent control of the mean +/- the SEM. * = statistical significance ($p < 0.05$) compared to control hippocampi. ** = statistical significance ($p < 0.001$) compared to control hippocampi. # = statistical significance ($p < 0.05$) compared to ethanol hippocampi.

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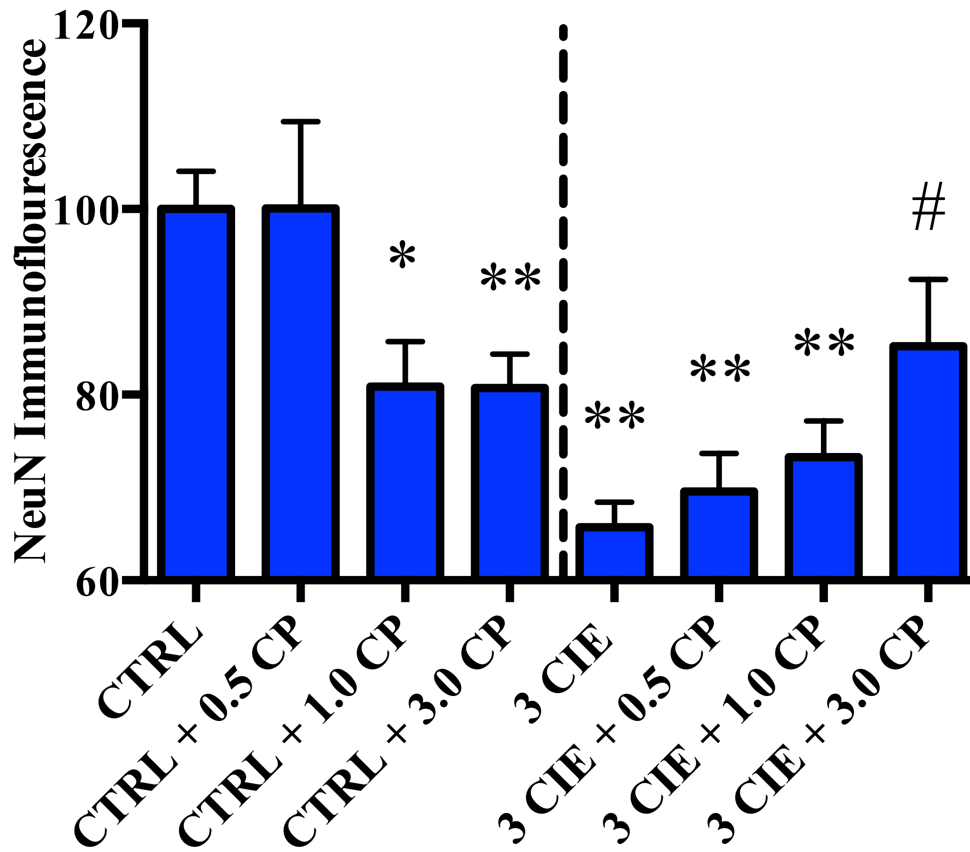

Figure 32. Effects of noncompetitive mGluR1 antagonist CPCCOEt (0.5–3 μM) on NeuN immunofluorescence in the granule cell layer of the dentate gyrus. Data are presented as percent control of the mean +/- the SEM. * = statistical significance ($p < 0.05$) compared to control hippocampi. ** = statistical significance ($p < 0.001$) compared to control hippocampi. # = statistical significance ($p < 0.05$) compared to ethanol hippocampi.

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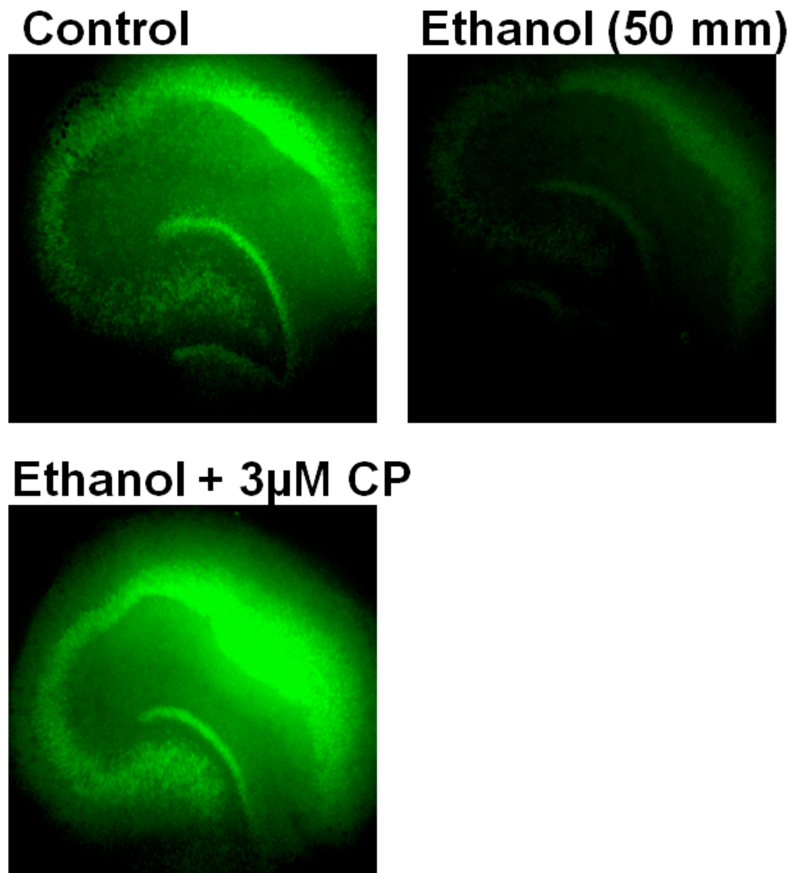

Figure 33. Representative images of hippocampi exposed to ethanol-naïve media (control) or ethanol media (50 mM) or hippocampi co-exposed to 3.0 µM CPCCOEt and ethanol (50 mM).

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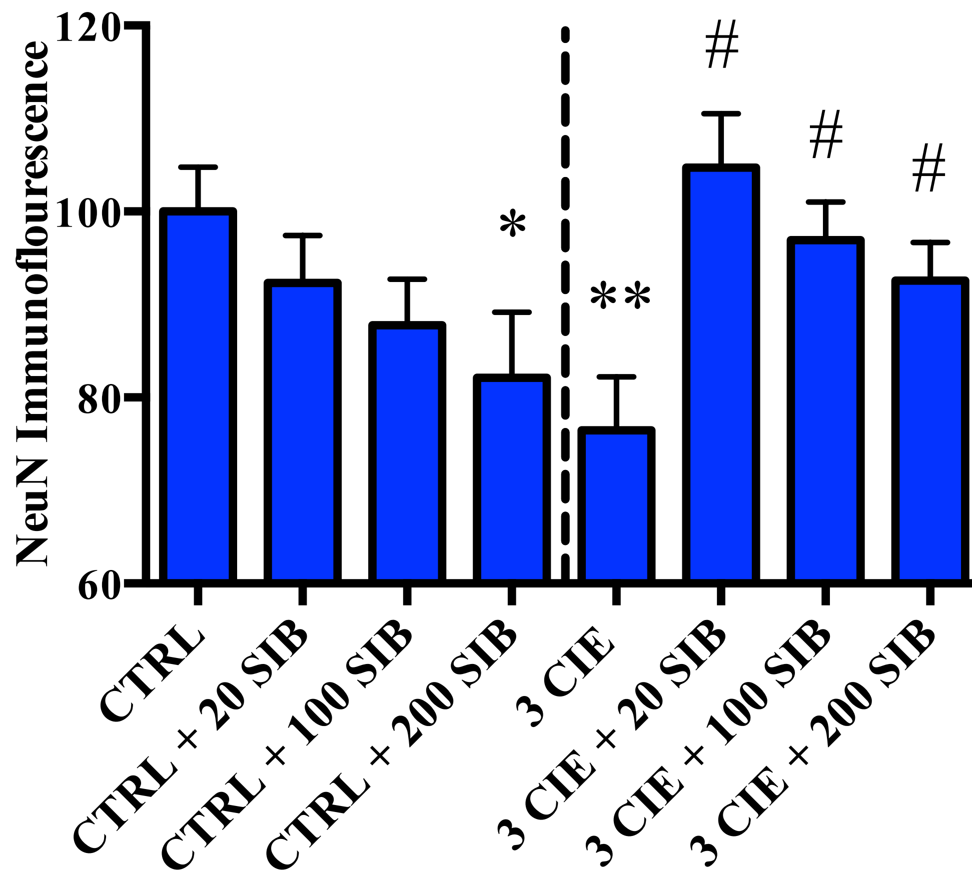

Figure 34. Effects of mGluR5 antagonist SIB-1893 on NeuN immunofluorescence in the primary cell layer of the CA1. Data are presented as percent control of the mean +/- the SEM. * = statistical significance ($p < 0.05$) compared to control hippocampi. ** = statistical significance ($p < 0.001$) compared to control hippocampi. # = statistical significance ($p < 0.05$) compared to ethanol hippocampi.

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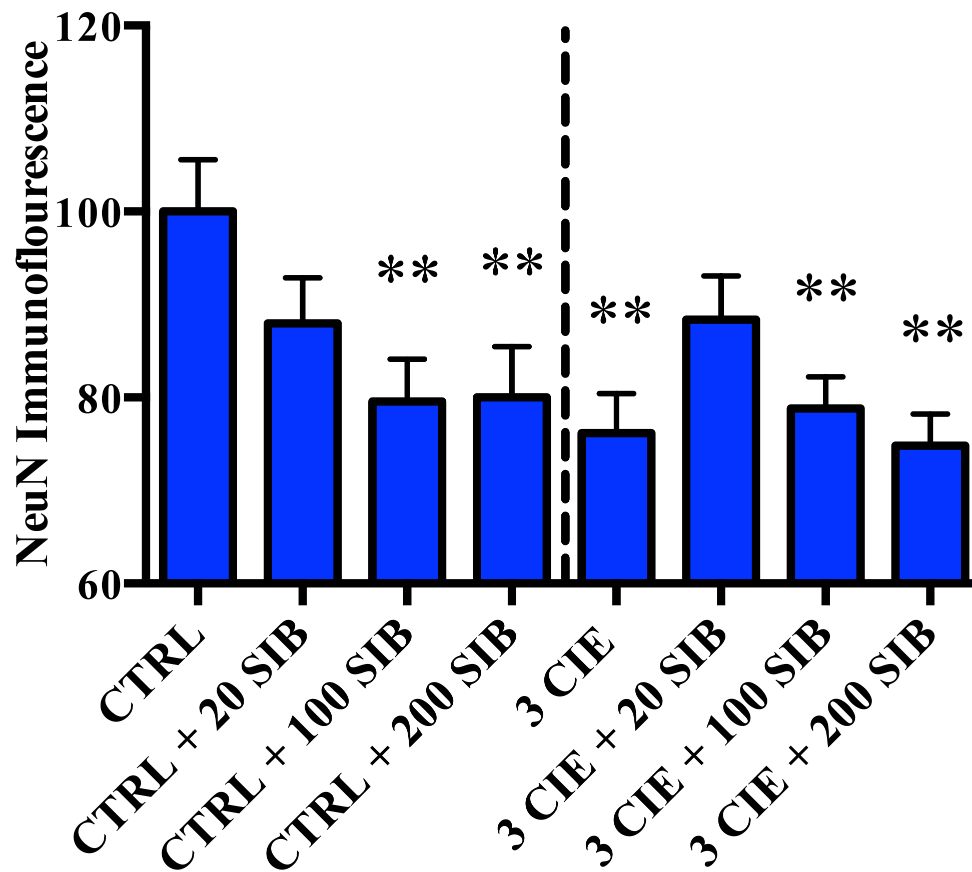

Figure 35. Effects of mGluR5 antagonist SIB-1893 on NeuN immunofluorescence in the primary cell layer of the CA3. Data are presented as percent control of the mean +/- the SEM. ** = statistical significance ($p < 0.001$) compared to control hippocampi.

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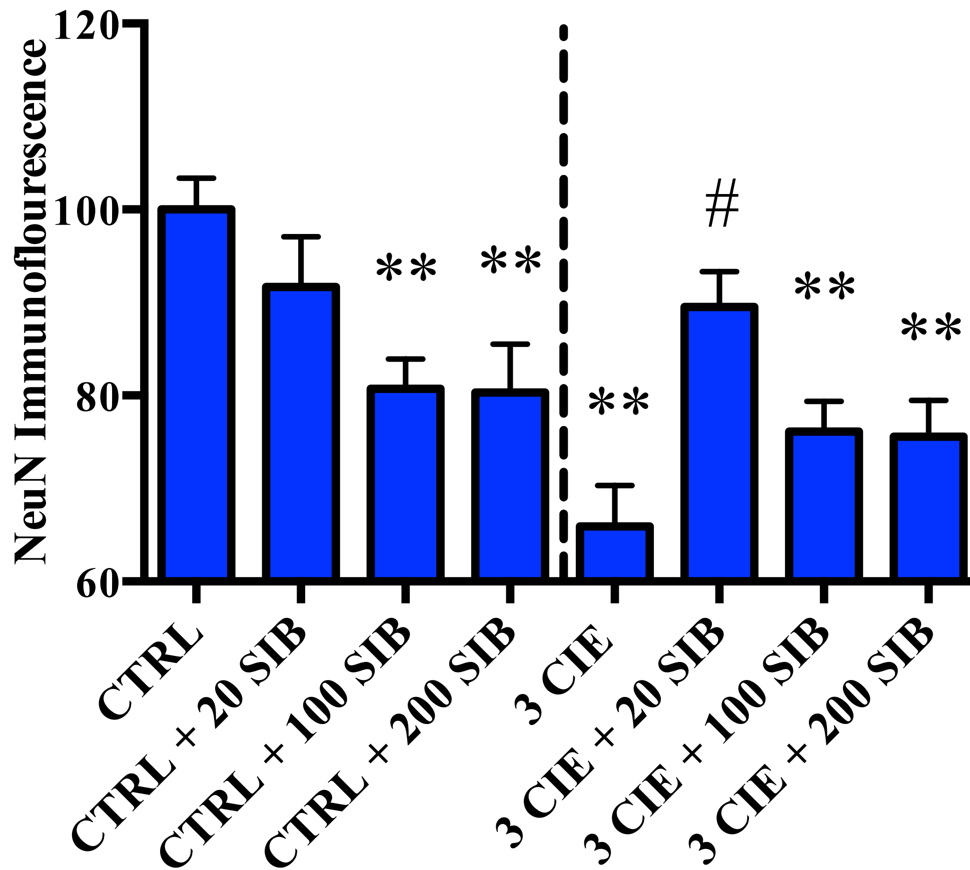

Figure 36. Effects of mGluR5 antagonist SIB-1893 on NeuN immunofluorescence in the granule cell layer of the dentate gyrus. Data are presented as percent control of the mean +/- the SEM. ** = statistical significance ($p < 0.001$) compared to control hippocampi. # = statistical significance ($p < 0.05$) compared to ethanol hippocampi.

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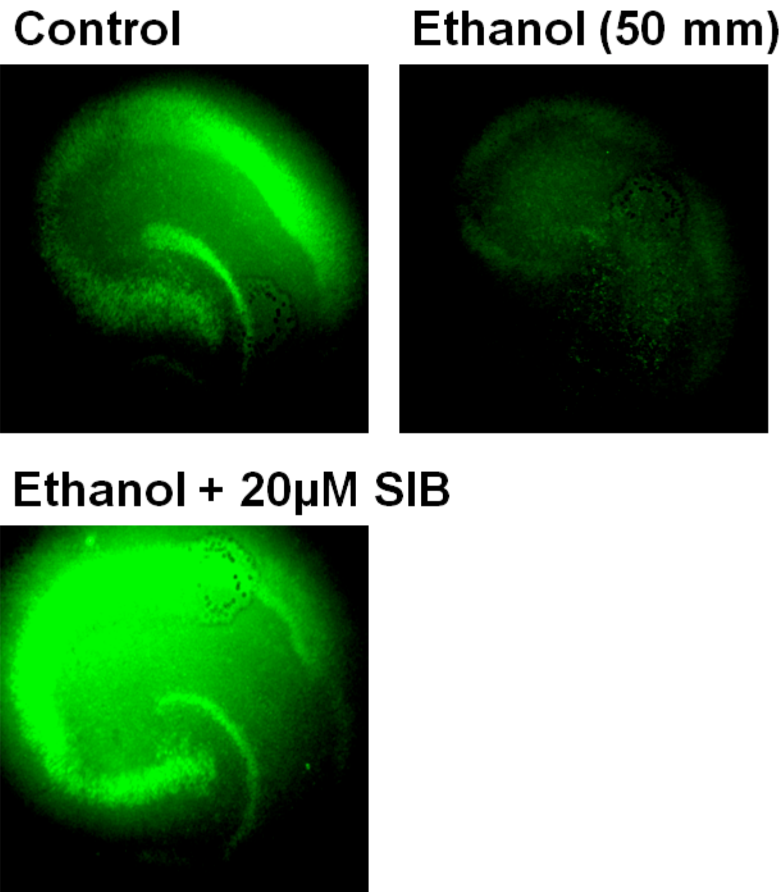

Figure 37. Representative images of hippocampi exposed to ethanol-naïve media (control) or ethanol media (50 mM) or hippocampi co-exposed to 20 µM SIB-1897 and ethanol (50 mM).

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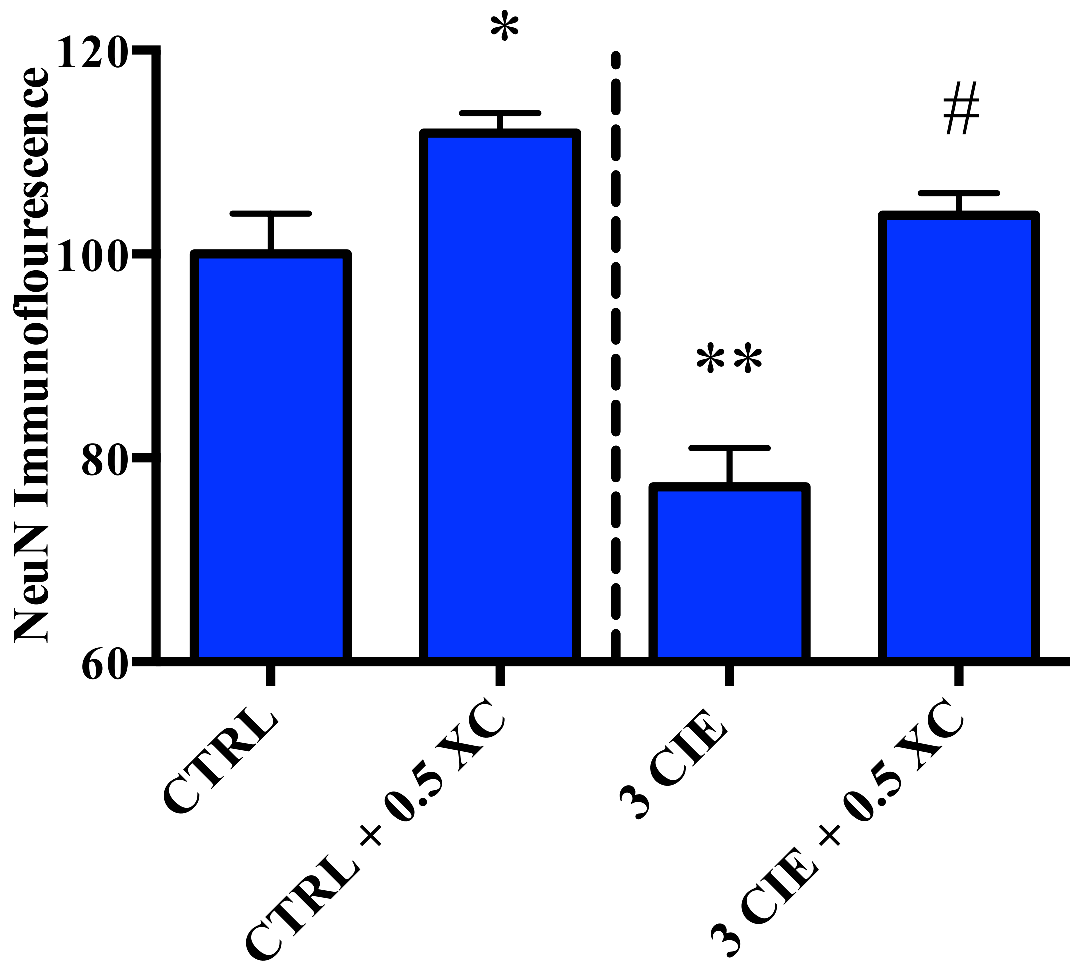

Figure 38. Effects of inhibitor of IP3-mediated calcium release (0.5 μM) on NeuN immunofluorescence in the pyramidal CA1 cell layer of the rat hippocampus. Data are presented as percent control of the mean +/- the SEM. * = statistical significance ($p < 0.05$) compared to control hippocampi. ** = statistical significance ($p < 0.001$) compared to control hippocampi. # = statistical significance ($p < 0.05$) compared to ethanol hippocampi.

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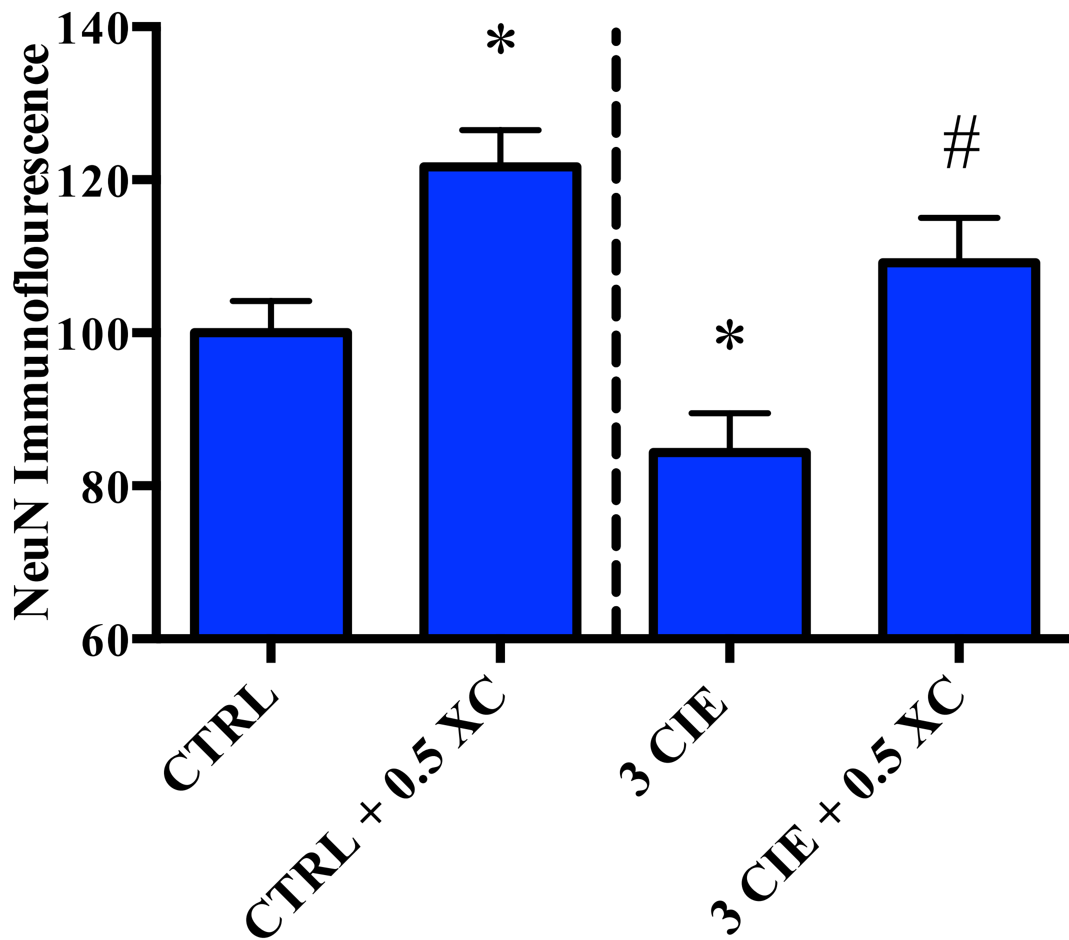

Figure 39. Effects of inhibitor of IP3-mediated calcium release (0.5 μM) on NeuN immunofluorescence in the pyramidal CA3 cell layer of the rat hippocampus. Data are presented as percent control of the mean +/- the SEM. * = statistical significance ($p < 0.05$) compared to control hippocampi. ** = statistical significance ($p < 0.001$) compared to control hippocampi. # = statistical significance ($p < 0.05$) compared to ethanol hippocampi.

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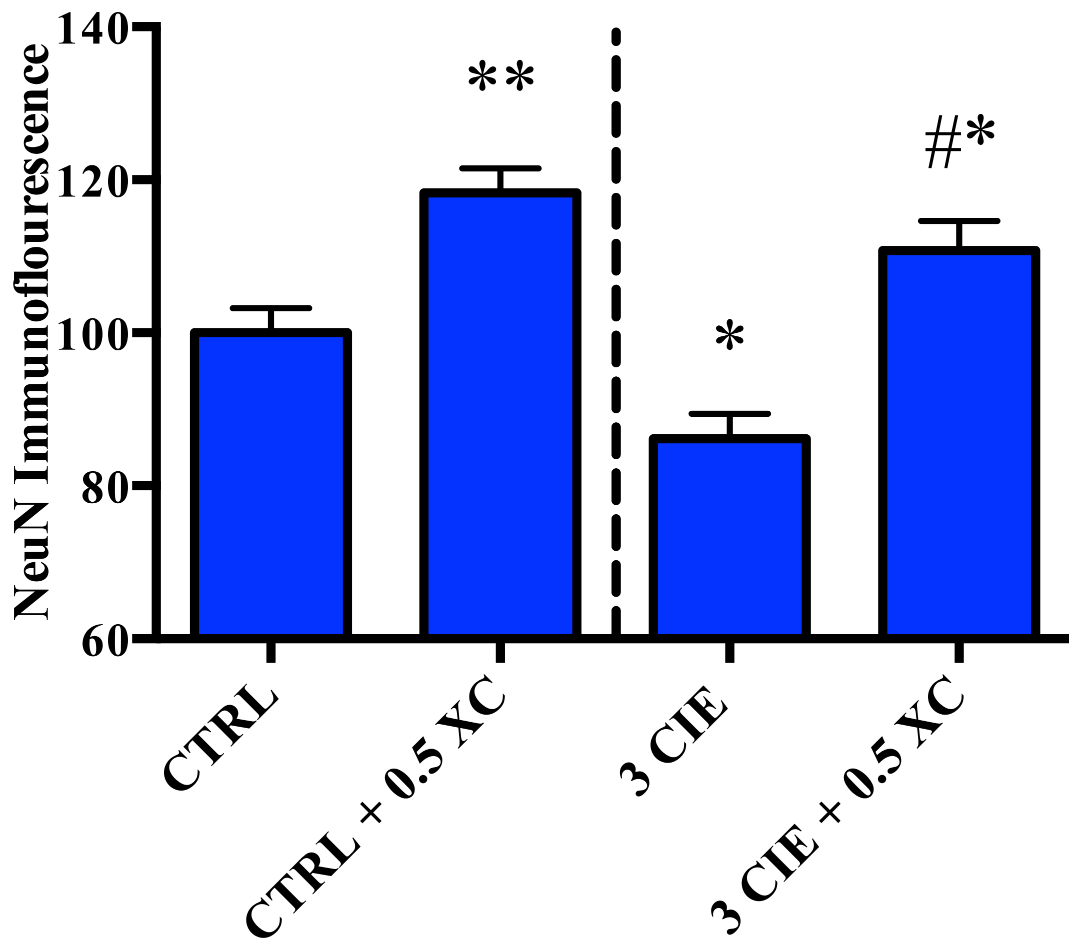

Figure 40. Effects of inhibitor of IP3-mediated calcium release (0.5 µM) on NeuN immunofluorescence in the granule cell layer of the dentate gyrus. Data are presented as percent control of the mean +/- the SEM. * = statistical significance ($p < 0.05$) compared to control hippocampi. ** = statistical significance ($p < 0.001$) compared to control hippocampi. # = statistical significance ($p < 0.05$) compared to ethanol hippocampi.

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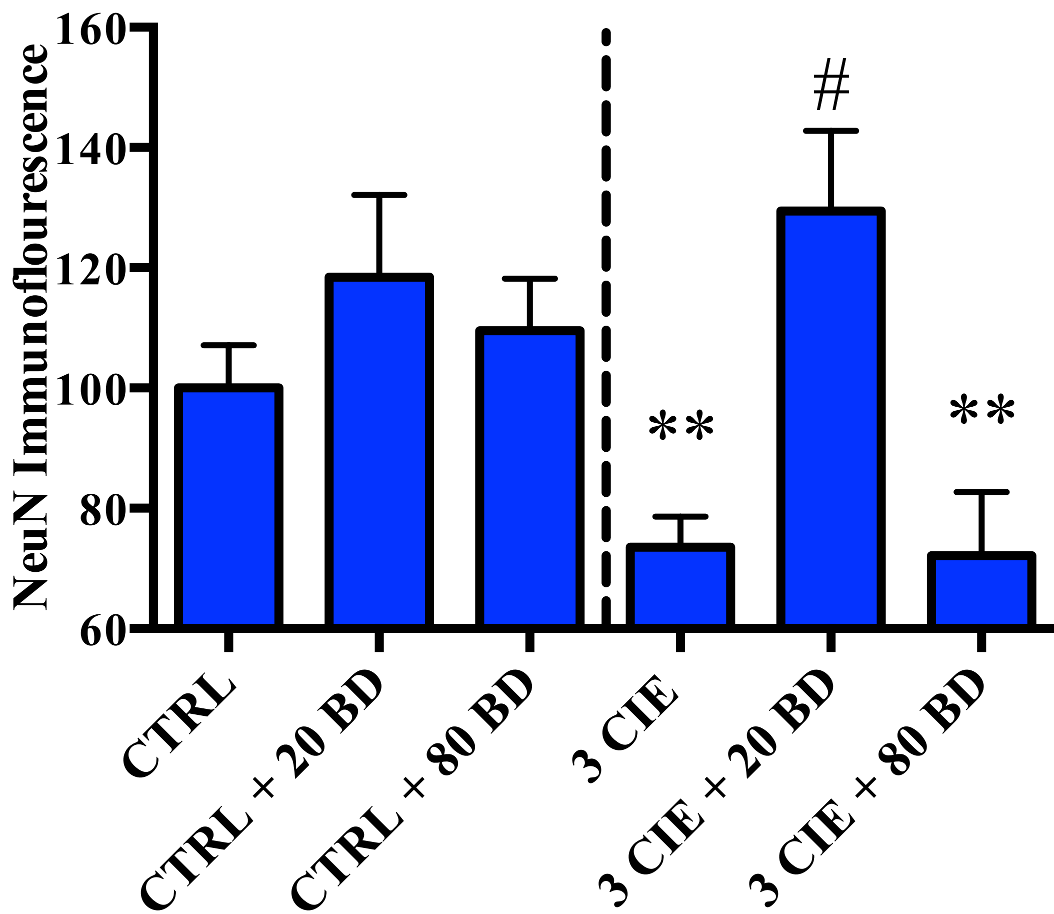

Figure 41. Effects of sigma-1 receptor antagonist BD-1047 (20 and 80 μM) on NeuN immunofluorescence in the CA1 hippocampal cell layer. Data are presented as percent control of the mean +/- the SEM. ** = statistical significance ($p < 0.001$) compared to control hippocampi. # = statistical significance ($p < 0.05$) compared to ethanol hippocampi.

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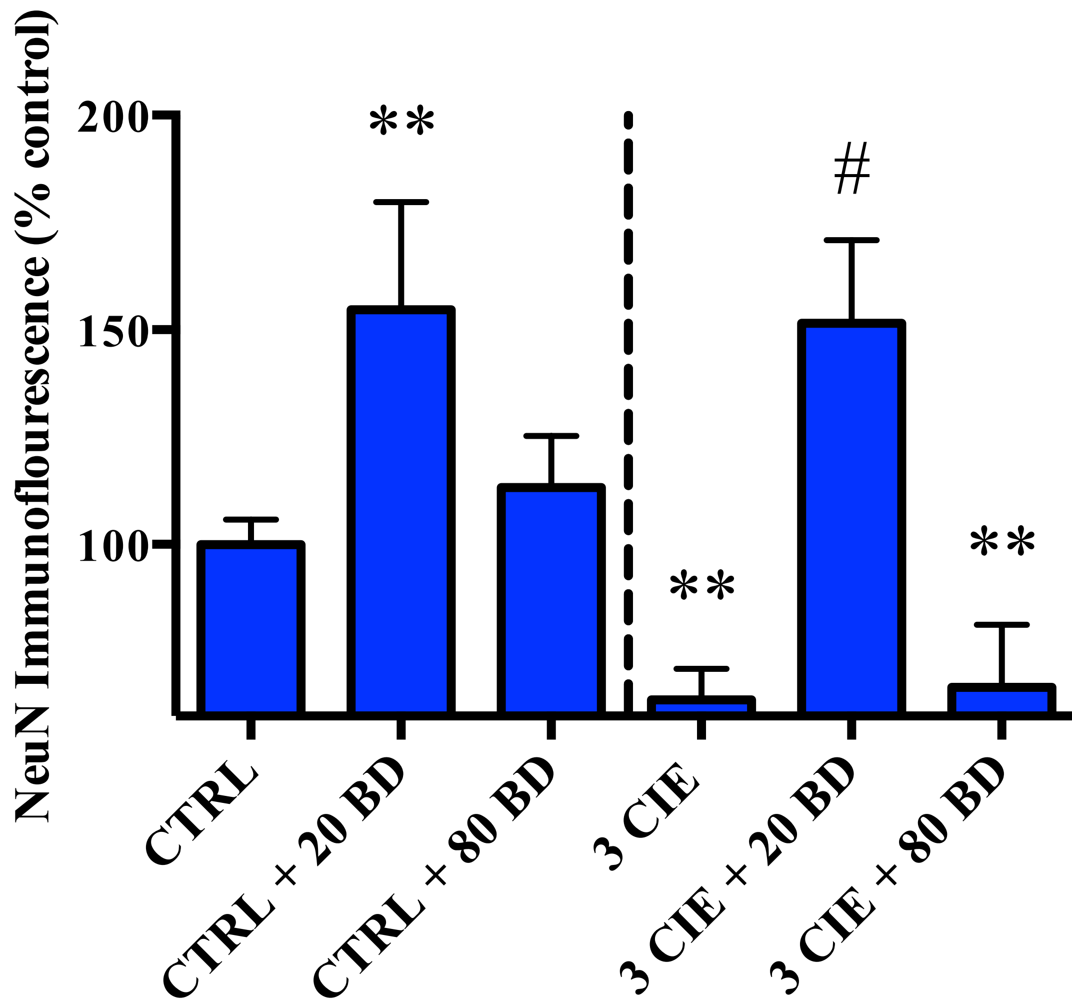

Figure 42. Effects of sigma-1 receptor antagonist BD-1047 (20 and 80 μM) on NeuN immunofluorescence in the CA3 hippocampal cell layer. Data are presented as percent control of the mean +/- the SEM. ** = statistical significance ($p < 0.001$) compared to control hippocampi. # = statistical significance ($p < 0.05$) compared to ethanol hippocampi.

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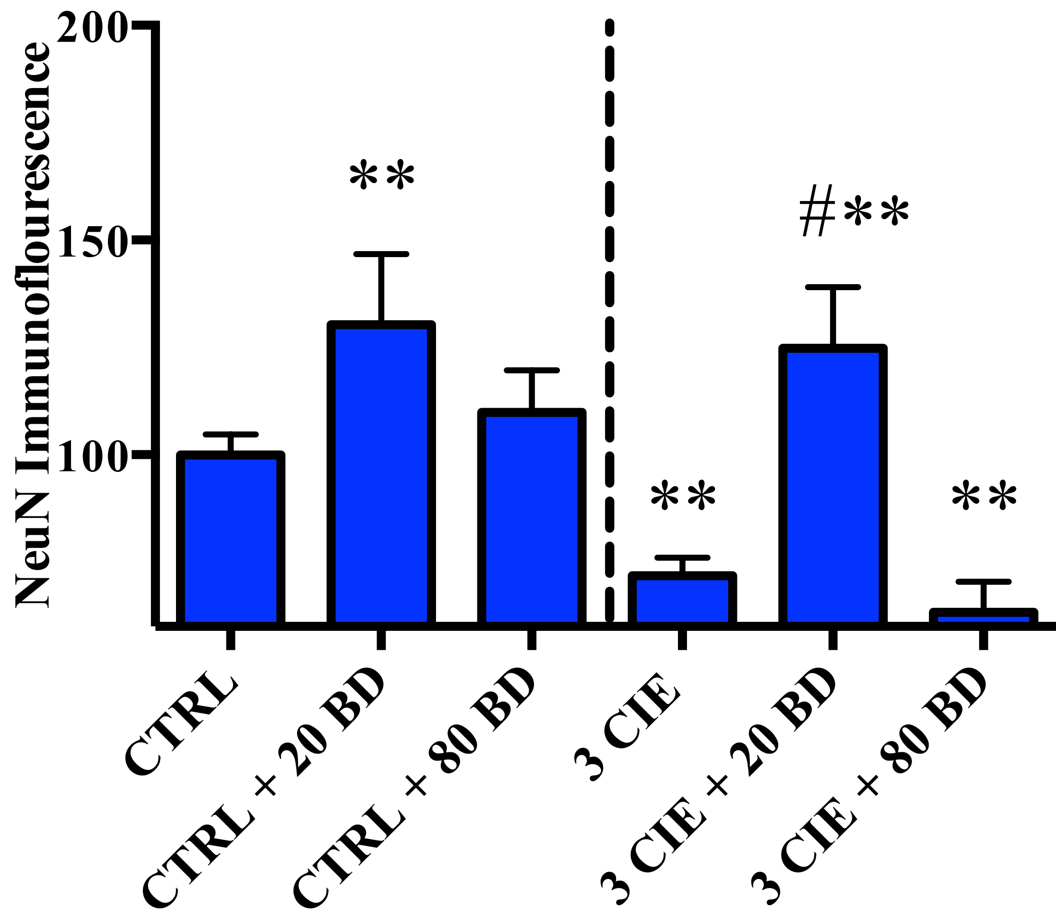

Figure 43. Effects of sigma-1 receptor antagonist BD-1047 (20 and 80 μM) on NeuN immunofluorescence in the dentate gyrus hippocampal cell layer. Data are presented as percent control of the mean +/- the SEM. ** = statistical significance ($p < 0.001$) compared to control hippocampi. # = statistical significance ($p < 0.05$) compared to ethanol hippocampi.

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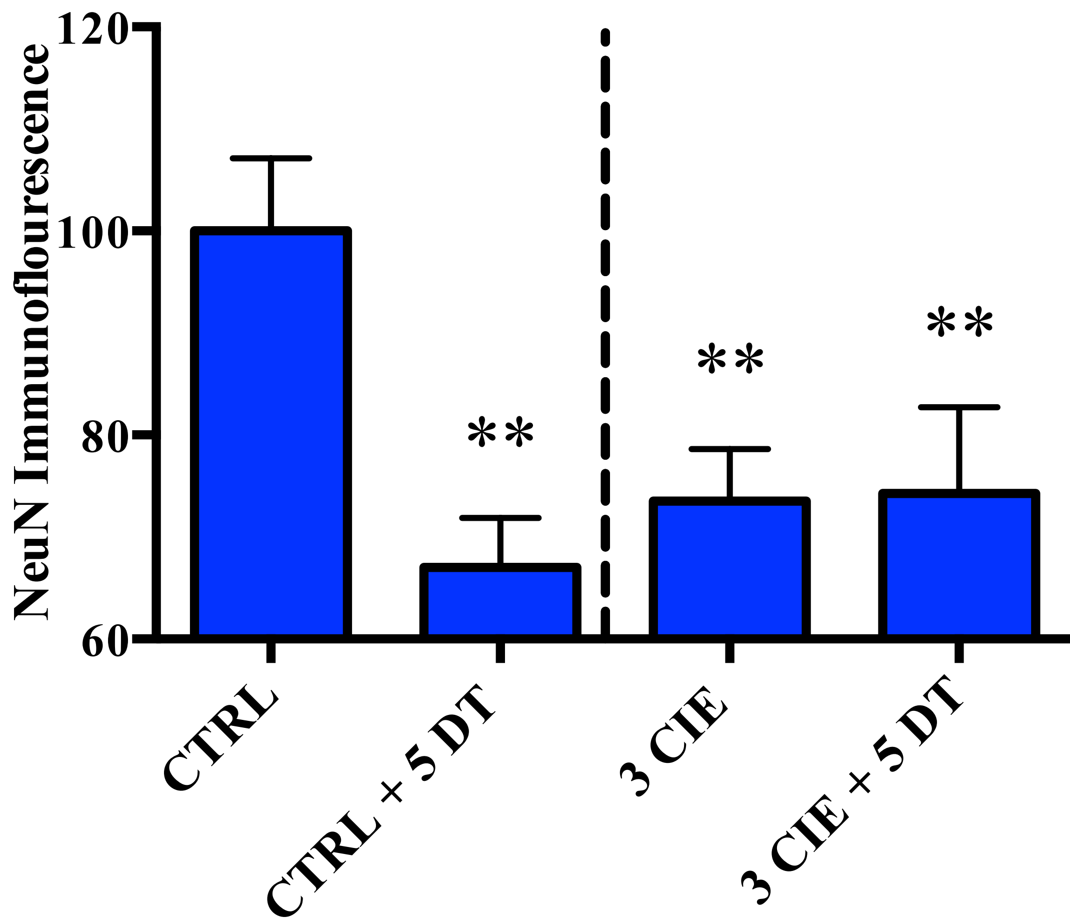

Figure 44. Effects of ryanodine receptor antagonist dantrolene (5 μM) on NeuN immunofluorescence in the CA1 hippocampal cell layer. Data are presented as percent control of the mean +/- the SEM. ** = statistical significance ($p < 0.001$) compared to control hippocampi.

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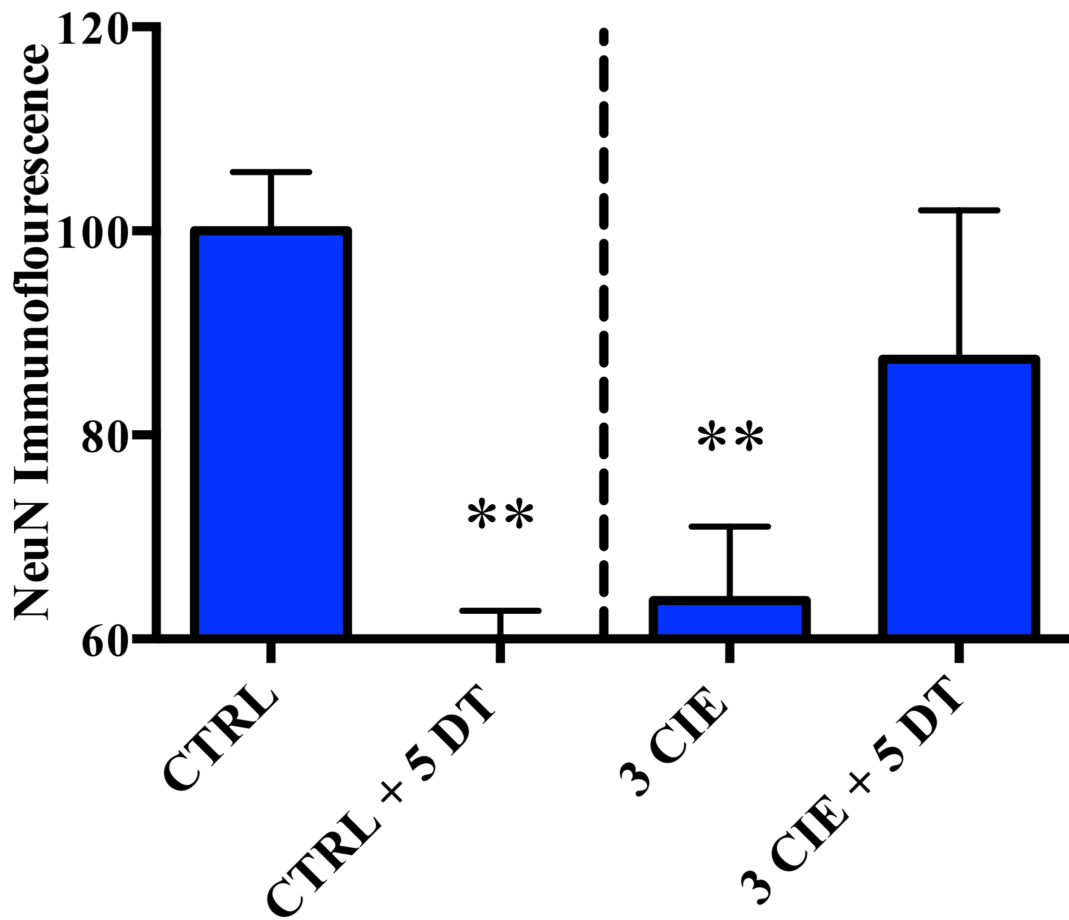

Figure 45. Effects of ryanodine receptor antagonist dantrolene (5 μM) on NeuN immunofluorescence in the CA3 hippocampal cell layer. Data are presented as percent control of the mean +/- the SEM. ** = statistical significance ($p < 0.001$) compared to control hippocampi.

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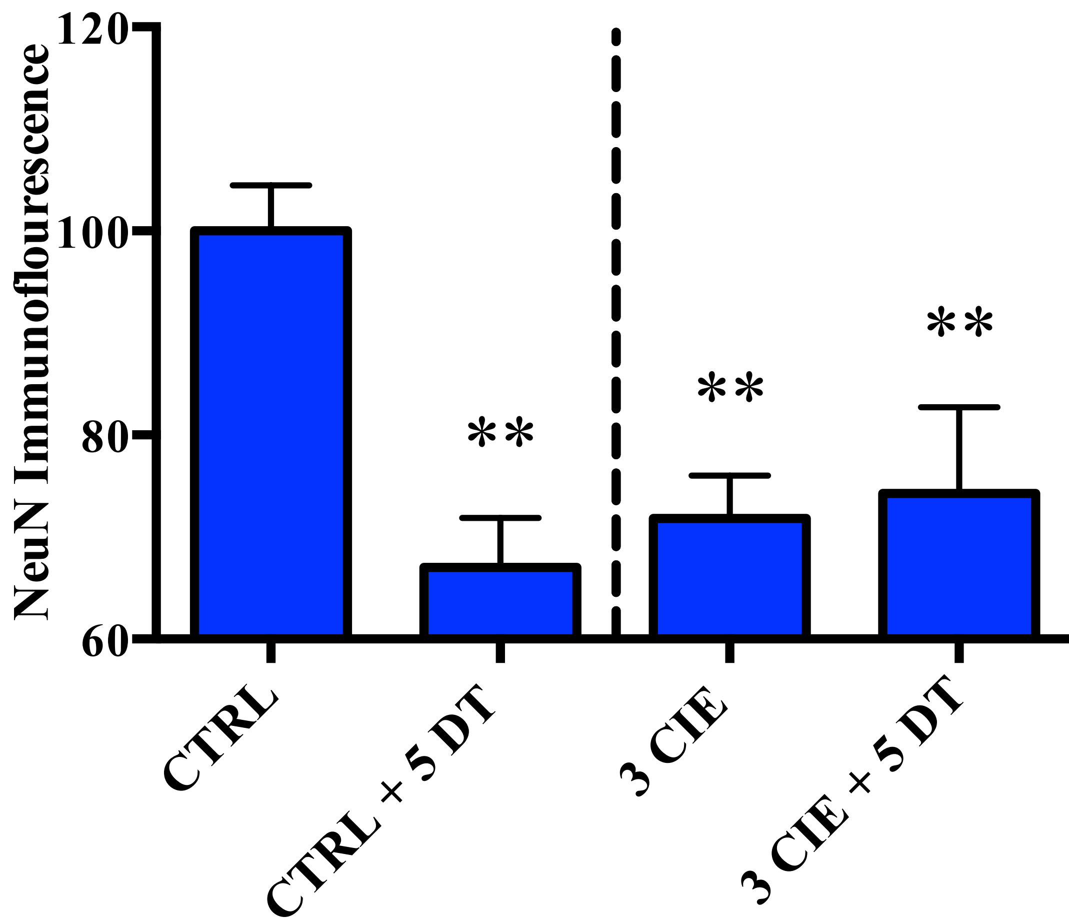

Figure 46. Effects of ryanodine receptor antagonist dantrolene (5 μM) on NeuN immunofluorescence in the dentate gyrus hippocampal cell layer. Data are presented as percent control of the mean +/- the SEM. ** = statistical significance (p <0.001) compared to control hippocampi.

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CHAPTER FIVE: Influence of mGluR5-containing Receptors in the Development of Ethanol Dependence *In Vivo*

5.1. Introduction

Rodent models of CIE have been productive in that they display parallel behavioral and neurodegenerative effects to those observed in the clinical population. These effects include increases in duration of seizures during withdrawal from ethanol (Stephens et al., 2001; Veatch & Becker, 2002), cognitive deficits (Badanich et al., 2011; Duka et al., 2003; Zhao et al., 2013), and hippocampal neurodegeneration (Collins et al., 1998; Corso et al., 1998; Zhao et al., 2013). Notably, rodents exposed to a CIE regimen displayed significant increases in handling-induced convulsions and spontaneous electroencephalogram activity (Veatch & Becker, 2005), and these effects were reduced following the administration of noncompetitive NMDA-receptor antagonist MK-801 during periods of ethanol withdrawal. Other studies have found that protein kinase A and C-dependent increase in GluN1 and GluN2B surface expression in cortical neurons exposed to chronic and intermittent ethanol (Qiang et al., 2007), and that the NMDA-receptor containing GluN1/2N2B subunits confer sensitivity to excitotoxicity (e.g., via increased channel opening) (Ran et al., 2003; for a review, see Prendergast & Mulholland, 2012). It has also been noted that trafficking of these receptors from the ER to the synapse can occur (Mu et al., 2003), further potentiating cell death.

Chronic exposure to ethanol also modulates G_q-coupled group 1 mGluR, which can stimulate IP3-mediated release of intracellular calcium (Conn et al., 1997) or activate downstream second messenger signaling pathways (Olive et al., 2009) in rodents and in the clinical population (e.g., Lominac et al., 2006). As an example, *in vivo*

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experimentation has shown binge-like ethanol consumption produced increased signaling of the mGluR5, and the administration of an mGluR5 antagonist significantly attenuated binge-like ethanol consumption in this rodent model of scheduled, high-alcohol consumption (Cozzoli et al., 2009). In another study, administration of an mGluR5 antagonist mitigated withdrawal manifestations and the sedative-like behavioral effects of ethanol if the mGluR5 antagonist was injected prior to, but not following, ethanol exposure (Blendov et al., 2008). Notably, human studies have proposed a link between genetic alterations in the mGluR5 and a propensity for alcohol abuse (Schumann et al., 2008).As noted in Chapter 4, blocking mGluR1-and-5 containing receptors attenuates the damage to hippocampal neuronal integrity produced by CIE *in vitro*.

5.2. Experimental Rationale

Recent efforts have been made to understand the adverse behavioral and neurodegenerative effects that are produced by CIE exposure. The mechanistic underpinnings associated with these effects, however, are not fully characterized. The model of CIE exposure proposed in these studies mimics patterns of binge-like consumption using a binge-like ethanol concentration (i.e., 50 mM[~230 mg/dl]) that can be readily achieved in the clinical population. The use of an *in vivo* model of CIE exposure allowed for an examination of the role of the mGluR5 in the development of dependence.

5.3. Methods

Subjects. Thirty-two adult, male Sprague-Dawley rats (i.e., weighed in at 300–325 grams upon arrival; Harlan Laboratories, Indianapolis, IN) were housed individually and allowed to acclimate to the animal colony for two days following arrival. Subjects were

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then handled for two minutes per day for three consecutive days prior to experimentation. Subjects were allowed *ad libitum* access to food and water throughout the entire duration of the experiment. Food weight data were collected at 0800 hour each week. Each animal was weighed at 0800 hour on days Monday through Friday, prior to ethanol administration. A mortality rate of 5% was observed due to complications with the intragastric gavage procedure.

Ethanol exposure and withdrawal. The experiments included in this study were designed to extend the *in vitro* findings described previously to a live animal model of CIE. In these studies, subjects were administered ethanol (4 g/kg) via intragastric gavage twice daily at 0800 and 1600 for five consecutive days with or without the addition of 3 mg/kg 2-Methyl-6-(phenylethynyl)pyridine (MPEP). MPEP is a group 1 metabotrophic glutamate receptor antagonist that has been used previously as a neuroprotective compound in several models of drug and alcohol dependence, as well as in several models of neurodegenerative disorders. Given that nasogastric gavage administration of MPEP (10 mg/kg) for one month reduced Parkinsonian symptoms in non-human primates (Morin et al., 2013), we chose to deliver this compound to subjects orally. During periods of withdrawal, subjects received no experimental manipulations; this was repeated for three consecutive cycles. The experimental timeline for this study is depicted in Figure 47.

Withdrawal behavior. Following each five-day ethanol administration period, subjects were not administered ethanol for two consecutive days (i.e., ethanol withdrawal). During

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the third period of withdrawal, subjects were monitored for 16 hours after the last ethanol administration (i.e., 0900 hours) for physiological manifestations of withdrawal. The rationale for the timing of these behavioral manifestations is based on the study described in Chapter 3 suggesting robust withdrawal behaviors are observed on the third period of withdrawal. Assessment of withdrawal behavior occurred in a square Plexiglas chamber for two minutes. During this time, an experimenter blinded to experimental conditions rated physical effects of withdrawal using a modified behavioral scale that was described in detail previously in our laboratory (Majchrowicz, 1975; Self et al., 2009) using a 10-point discrete scale (i.e., all or nothing). This scale includes the following behaviors: rigidity, tremor, stereotypy, retropulsion, dystonic gait, hypoactivity, aggression, splayed paws, vocalization, and seizure.

Analysis of blood ethanol levels. In order to assess BELs, approximately 200 µL of tail blood was collected into two Fisherbrand heparinized micro-hematocrit capillary tubes (Fisher Scientific) on Day Two of Weeks One, Two, and Three. Next, samples were centrifuged for four minutes using an Analox benchtop centrifuge (Analox Instruments) with blood plasma collected and placed into a .65 mL Costar microcentrifuge tube (Fisher Scientific). Samples were stored at -20°C until further analyses of BELs using an ethanol assay kit (i.e., colorimetric; Abcam [Cambridge, United Kingdom]) for Week 1 and the Analox for Weeks 2 and 3 (please see Chapter 3 for a detailed description of Analox methodology). With regards to the ethanol ELISA assay, alcohol oxidase oxidizes ethanol to generate hydrogen peroxide that reacts with the probe included in the assay to generate color. All standards, background wells, and sample wells were run in duplicate

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per manufacturer's instructions, and 10 μL of blood plasma were incubated at room temperature for 60 minutes with reaction enzymes in a 96-well plate (Corning, New York). Optical density was measured using a Beckman Coulter DTX 880 Multimodal Detector (Lagerhausstrasse, Austria) using Beckman Coulter Multimode Detection Software (v.20.0.12). Absorbance was detected at 595 nm for standards, background wells, and sample wells. Mean absorbance for each value was determined and then averaged so as to yield one measurement for each sample. Mean absorbance of the blank (i.e., standard with 0 nmol/well ethanol) was subtracted from each standard and sample value to yield the corrected absorbance value, and a standard curve was generated based on values of standards. Concentrations of ethanol in samples were then determined via sample amount from standard curve (i.e., Sa; nmol) divided by the sample volume added into the sample well (i.e., Sv; μL) using this formula: *Sa/Sv*.

Statistical analyses. Statistical analyses were conducted to assess the effects of CIE on body weight, behavioral effects of withdrawal, and BELs. Effects were considered significant at $p<0.05$. Body weight data were analyzed by a two-factor repeated-measure ANOVA with day and treatment (control and ethanol) as factors. Behavioral effects of withdrawal and BELs were analyzed by a two-factor repeated-measure ANOVA with diet and drug as factors. Planned comparisons were conducted if a significant effect of day or treatment or an interaction of these two factors was detected using Bonferroni. These planned comparisons were used to make conservative pairwise comparisons between means.

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5.4. Results

Body weight. ANOVA revealed a significant interaction of day and diet ($F[3,50] = 12.69$, $p<0.001$) in subjects exposed to three cycles of CIE or an isocaloric diet with or without the addition of mGluR5 antagonist MPEP. Figure 48 shows that significant decreases in body weight (grams) were detected in subjects that were administered ethanol compared to subjects administered an isocaloric diet. This figure also shows that MPEP administration did not have an effect on body weight in subjects exposed to CIE or an isocaloric diet.

Behavioral effects of withdrawal. Subjects were administered CIE (or an isocaloric control diet) for three consecutive weeks with or without the addition of an oral and selective mGluR5 antagonist MPEP (3 mg/kg) to evaluate the influence of this particular receptor subtype in the development of ethanol dependence. ANOVA revealed a significant diet-by-drug interaction ($F[1,28] = 44.87$, $p<0.001$). Figure 49 shows that subjects administered ethanol had significant increases in behavioral effects of withdrawal compared to subjects that received an isocaloric diet. This figure also shows that oral administration of mGluR5 antagonist MPEP significantly attenuated the physical manifestations of withdrawal in ethanol-dependent rats.

Blood ethanol levels. ANOVA revealed a significant main effect of drug ($F[1,7] = 11.77$, $p<0.05$) on BELs in ethanol-dependent subjects. Figure 50 shows that MPEP administration modestly reduced BELs from during Weeks 1, 2, and 3 of the CIE treatment regimen. . ANOVA also revealed a significant main effect of week (F[1,14] =

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35.00, $p<0.001$) on BELs in ethanol-dependent subjects. Figure 50 also shows that average BELs on Weeks 2 and 3 were significantly lower as compared to Week 1.

5.5. Discussion

The present report examined the influence of the mGluR5 receptor in the development of ethanol dependence using oral administration of MPEP in adult, ethanol-dependent and ethanol-naïve male rodents. MPEP is a mGluR5 antagonist that has been used previously as a neuroprotective compound in several models of drug and alcohol dependence, as well as in several models of neurodegenerative disorders (for a review, see Nickols & Conn, 2014). In the present report, subjects administered CIE demonstrated significant decreases in body weight, as compared to subjects administered a control diet. As described in Chapter 3, the sedative effects of binge-like ethanol administration likely produced these effects. This notion is supported by the work of others demonstrating that administration of binge-like ethanol produces decreases in body weight (e.g., Broadwater et al., 2011A; Broadwater et al., 2010B; Matthews et al., 2008; Roberto et al., 2010). Worthwhile to note is that while chronic MPEP administration (3 mg/kg/day) did not produce significant changes in body weight in ethanol-dependent rats or ethanol-naïve rats, significant attenuation of BELs were observed in ethanol-dependent rats. These findings suggest safety and tolerability of this compound in adult, male rodents and perhaps indicate efficacy of MPEP in attenuating the reinforcing properties of ethanol via reducing BELs.

The present study demonstrates that CIE administration produces physical dependence as manifested in physical symptoms of withdrawal. The reasons for these effects are unknown but could reflect the ability of MPEP administration to reduce BELs

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during Weeks 1, 2, and 3 of the first week of the CIE treatment regimen. Perhaps the ability of MPEP to reduce withdrawal behavior in ethanol-dependent rats is a function of pharmacokinetic considerations, rather than pharmacokinetic considerations. Nonetheless, in general, the most consistent features of withdrawal-like behavior in the current study were retropulsion (e.g., backing into a corner), dystonic gait, extreme tail rigidity, and severe head tremors. While no seizures were observed in the current report, withdrawal behaviors related to motor abnormalities were more consistent across subjects. For example, moderate to severe head tremors were observed in nearly all ethanol-dependent subjects. These findings are consistent with the work of others who unequivocally demonstrate hallmark characteristics of central nervous system hyperexcitability during periods of withdrawal from binge-like ethanol exposure (for reviews, see Becker, 2012, 2013; Botia et al., 2015; N'Gouemo et al., 2015; Pérez & DeBiasi, 2015; Van Skike et al., 2015).

Notably, chronic and oral administration of mGluR5 antagonist MPEP significantly attenuated the behavioral effects of withdrawal in the present report, such as sparing the extreme tail rigidity observed during the third consecutive period of withdrawal in ethanol-dependent subjects administered CIE. These findings are consistent with prior work demonstrating the efficacy of MPEP against neurodegenerative diseases. In one study, for example, nasogastric gavage administration of MPEP (10 mg/kg) for one month reduced Parkinsonian symptoms in non-human primates (Morin et al., 2013). Indeed, this prior study shows that MPEP is orally available and is consistent with the findings of the current study with regards to the bioavailability of MPEP. It is notable that MPEP spared motor dystonias (e.g., dystonic

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gait and retropulsion) in the current report during withdrawal from CIE in ethanol-dependent rodents. These findings are consistent with the work of others who have demonstrated the efficacy of MPEP for motor abnormalities known to be produced by the benchmark treatment of Parkinson disease via L-3,4-dihydroxyphenylalanine (l-DOPA) administration (Morin et al., 2013A), as well as the efficacy of chronic MPEP administration to attenuate neurodegeneration and mitochondrial dysfunction produced by 6-hydroxydopamine (6-OHDA) exposure in the nigrostriatum in rodents (Ferringo et al., 2015). Others have shown that an "effective" dose of MPEP (i.e., 3 mg/kg/day) reduced cognitive impairment produced by dopaminergic striatal lesioning in rodents (DeLonibus et al., 2009). The reasons for these effects are unknown, but could include the ability of MPEP to normalize basal glutamate levels in areas of the limbic system, such as the basal ganglia (Morin et al., 2013B).

Cozzoli and colleagues (2009) found that binge-like ethanol administration produced significant increases in Homer2a/b expression as well as Homer2-phosphatidylinositol 3-kinase (PI3K) signaling in the nucleus accumbens. These effects were significantly blocked with MPEP. In the same study, pretreatment with mGluR5 antagonist MPEP dose-dependently reduced binge ethanol consumption in rodents (Cozzoli et al., 2009). Others have shown that MPEP administration (10 mg/kg) attenuated ethanol withdrawal anxiety-like behaviors (Kumar et al., 2013). Other studies have shown a reduction in ethanol reinstatement following MPEP administration elicited by drug-associated cues (Bäckström et al., 2004), as well as attenuation of ethanol withdrawal-elicited behaviors in rodents (Blendov & Harris, 2008).

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Previous studies have suggested that MPEP administration attenuates the reinforcing properties of others drugs of abuse, such as psychomotor stimulants. For example, MPEP administration was found to reduce cocaine self-administration (Kenny et al., 2005) and cocaine reinstatement (Kumaresan et al., 2009) in rodents and non-human primates (Lee et al., 2005; Platt et al., 2008). Similarly, MPEP has been shown to attenuate the reinforcing properties of nicotine (e.g., DeSousa & Markou, 2011), as well as ketamine and heroin (Van der Kam et al, 2007). Collectively, these studies demonstrate the safety and efficacy of MPEP in various models of disease, such as neurodegeneration and addiction.

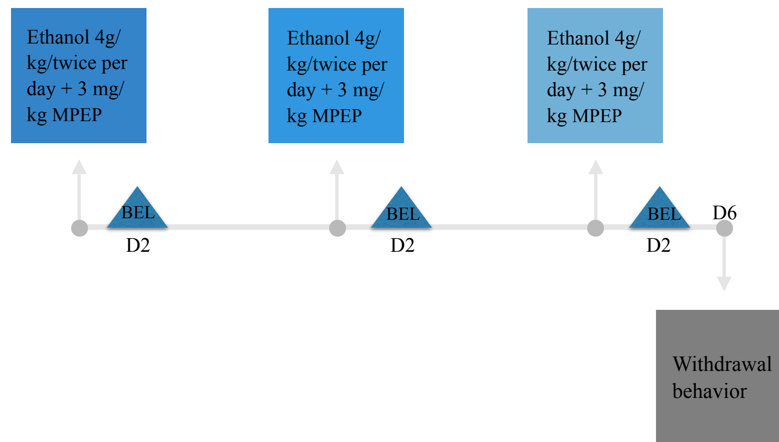

Figure 47. Representative experimental timelines depicting subjects were exposed to ethanol (4 g/kg) with or without the addition of MPEP (3 mg/kg) twice daily for five days followed by two days of withdrawal and repeated three times (i.e., 3 CIE).

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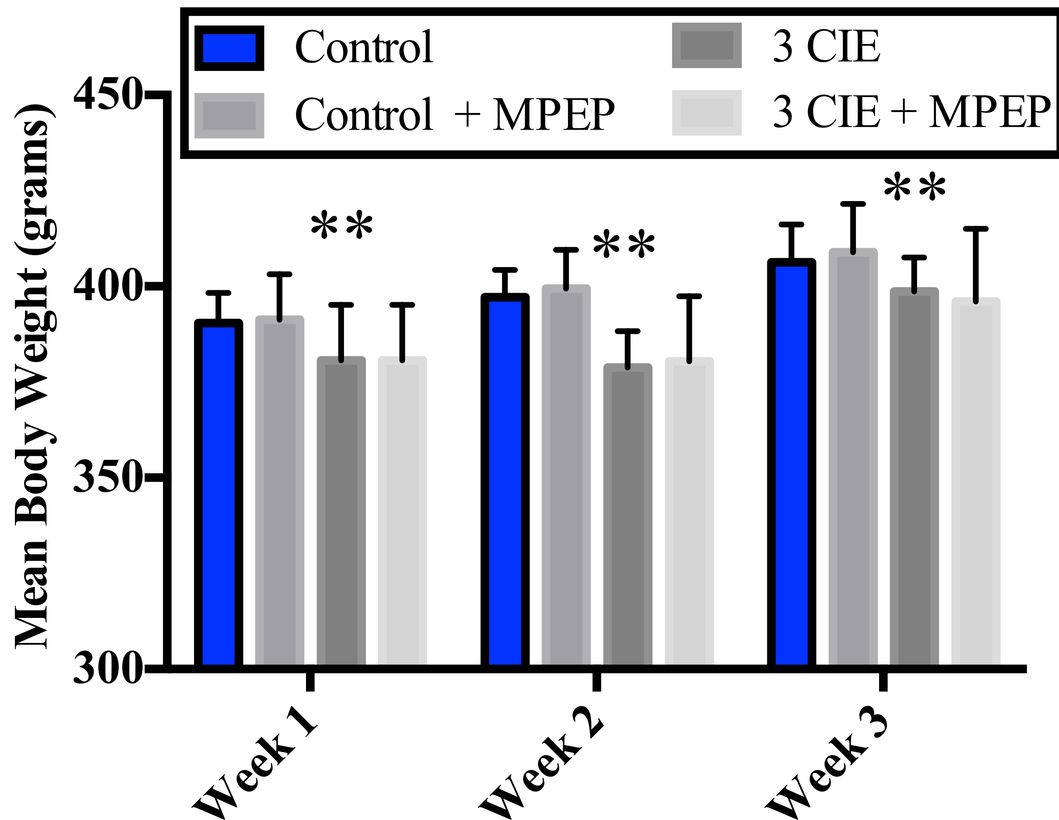

Figure 48. Changes in body weight in subjects exposed to ethanol or an isocaloric diet with or without the addition of mGluR5 antagonist MPEP (3 mg/kg) for three cycles of CIE. X-axis: days in Week One, Week Two, and Week Three. Data points show mean body weight in grams. Two asterisks indicate that there is a significant day-by-treatment interaction. N=7 for control subjects; N=8 for control with MPEP; N=6 for ethanol subjects; N=8 for subjects administered ethanol and MPEP

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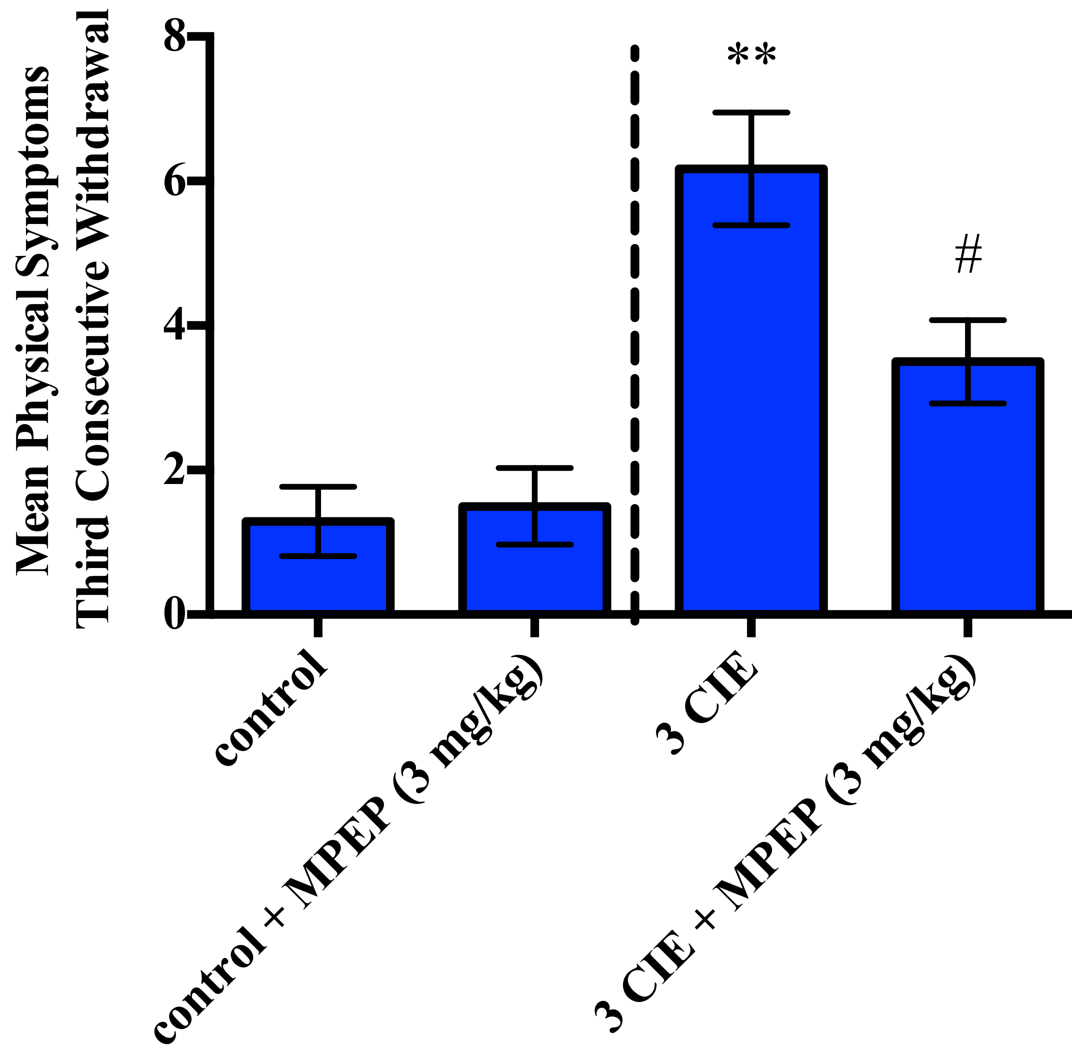

Figure 49. Behavioral effects of withdrawal in subjects exposed to ethanol or an isocaloric diet with or without the administration of MPEP for three cycles of CIE. X-axis: day. Data points show mean scores in withdrawal behavior observed during the third consecutive withdrawal from CIE. Two asterisks indicate that there is a significant diet-by-drug interaction. A pound sign indicates that there is a main effect of MPEP on withdrawal behavior in ethanol-dependent rats. N=7 for control subjects; N=8 for control with MPEP; N=6 for ethanol subjects; N=8 for subjects administered ethanol and MPEP.

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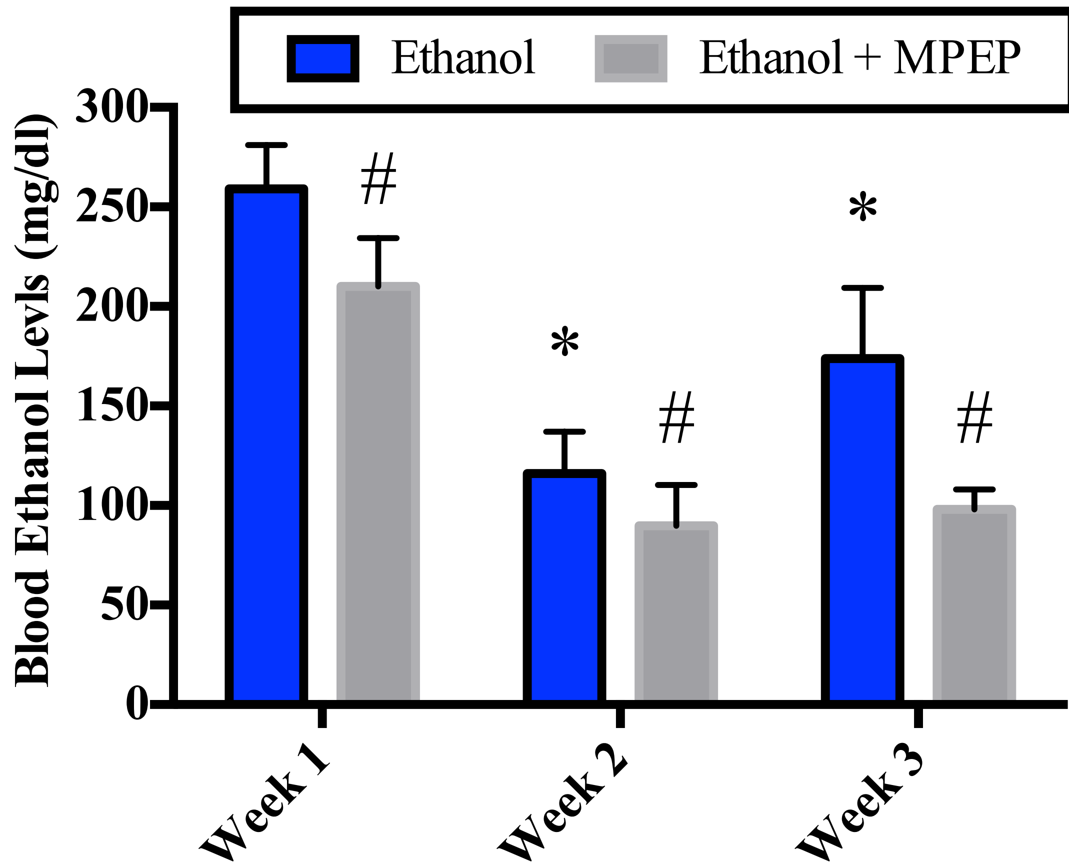

Figure 50. Peak BELs in ethanol-dependent subjects on Weeks One, Two, and Three of the CIE treatment regimen. X-axis: Week. Data points show average BELs determined at 90 minutes post ethanol administration on Day Two of Weeks One, Two, and Three. Two asterisks indicate that there is a significant main effect of Week. A pound sign indicates that there is a main effect of MPEP on BELs in ethanol-dependent rats. N=4 for ethanol subjects; N=5 for ethanol with MPEP.

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Chapter Six: A General Discussion and Future Pharmacotherapies for Treatment Alcohol Use Disorders

Alcohol use disorders are a widespread public health problem in the Unites States (SAMSHA, 2014). While approximately 18 million individuals self-report the desire to seek treatment for an alcohol use disorder (e.g., alcohol abuse and dependence on alcohol), less than 10% of these individuals reported obtaining receiving formal treatment at a facility (SAMHSA, 2014). Currently, there are four medications approved by the United States FDA for the treatment of alcohol dependence: oral naltrexone, extended-release naltrexone, disulfiram, and acamprosate (Gastfriend, 2014; Hartung et al., 2014; for a review see Kraemer, 2014; Kufahl et al., 2014). Indeed these drugs have been moderately effective in the treatment of alcohol use disorders (Blodgett et al., 2014; Guglielmo et al., 2015; Jonas et al., 2014; Liang & Olsen, 2014). While the reasons for the limited efficacy of current medications indicated for treatment of alcohol use disorders are unknown, it is likely that many individuals desire to control consumption rather than abstain completely. Recent research efforts, therefore, have been aimed at identifying and developing putative pharmacotherapies for the treatment of alcohol use disorders so as to address this gap in care by so as to tailor medications development toward the needs of the individual. Putative pharmacotherapies for the treatment of alcohol use disorders will be discussed below, followed by closing remarks of this dissertation.

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Anticonvulsants

Anticonvulsant medications (e.g., topiramate, gabapentin, and baclofen) are a potential candidate class of compounds for the treatment of alcohol use disorders, due in part, to enhancement of inhibitory neurotransmission and perhaps their limited abuse potential. In general, many of these studies compared candidate compounds to medications with an indication for treatment of alcohol use disorders so as to have a solid reference point (e.g., disulfiram, naltrexone, and acamprosate). In one study, topiramate (0–300 mg/day) reduced the number of self-assessed heavy drinking days (Johnson et al., 2007). In another study, for example, topiramate (25–300 mg/day) promoted abstinence in alcohol-dependent individuals as compared to naltrexone (50 mg/day) or acamprosate (333 mg/day) (Narayana et al., 2015). Consistent with this notion, several randomized clinical trials suggest moderate efficacy of topiramate (25–300 mg) for treatment of alcohol use disorders (Baltieri et al., 2008; Fernandez Miranda et al., 2007; Johnson et al., 2003; Johnson et al., 2004; Ma et al., 2006; Rubio et al., 2009). Yet in another study, rates of abstinence were higher for disulfiram (250 mg/day) than for topiramate (150 mg/day) (De Sousa et al., 2008) while others have found similar efficacy between topiramate (50–400 mg/day) and naltrexone (50 mg/day) (Flórez et al., 2008; Flórez et al., 2011). In general, the efficacy of topiramate for the treatment of alcohol use disorders is comparable to those marketed for this indication.

Gabapentin, another anticonvulsant medication, has been more recently assessed for the treatment of alcohol use disorders. In one study, for example, gabapentin (900 and 1800 mg/day) promoted abstinence in individuals with an alcohol use disorder (Mason et al., 2014). In two other studies, however, gabapentin (100-1200 mg/day) was not efficacious in protracted abstinence (Arias et al., 2010; Anton et al., 2009; Furieri &

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Nakamura-Palacious, 2007). Zonisamide (50–300 mg/day), another anticonvulsant drug, significantly attenuated subjective effects measures of "craving", a subjective feeling thought to precede relapse (Rubio et al., 2010) however these effects are modest. Other anticonvulsant medications (e.g., oxcarbazepine and tiagabine) are thought to not be effective in promoting abstinence (Croissant et al., 2006 and Paparrigopoulos et al., 2010). Interestingly, baclofen, a medication indicated for treatment of skeletal muscle spasms, reduced amount of alcohol consumed (Flannery et al., 2004), as well as attenuated rates of relapse (Addolorato et al., 2002; Addolorato et al., 2011). Yet in another study, baclofen administration was not efficacious for treatment of alcohol use disorders (Garbutt et al., 2010).

Collectively, these studies demonstrate that there is modest efficacy of anticonvulsants for the treatment of alcohol use disorders in a clinical setting. Perhaps the efficacy of these medications that mediate GABAergic tone could be examined for the treatment of acute but severe alcohol withdrawal in combination with benzodiazepines in patients so at to prevent known neurocognitive complications and cross-dependence on benzodiazepines.

Antidepressants

Antidepressants (e.g., sertraline, gabapentin, and baclofen) are a potential candidate class of compounds for the treatment of alcohol use disorders, due in part, to high rates of comorbid alcohol use disorders and psychological disorders. In one series if studies, sertraline (200 mg/day) promoted protracted abstinence (Pettinati et al., 2001; Pettinati et al., 2004). In another series of studies, sertraline (50–200 mg/day) decreased drinking in late-onset individuals with an alcohol use disorder, but actually increased

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drinking in those individuals with an early-onset of an alcohol use disorder (Kranzler et al., 2011; Kranzler et al., 2012). Similar findings with regards to time of onset have been observed with ondansetron, a serotonin 5-HT3 receptor antagonist and antiemetic (Kranzler et al., 2003; Roache et al., 2008) or in individuals with altered function of serotonin transport (Johnson et al., 2011). Other candidate medications with current indications for depression (e.g., fluvoxamine and escitalopram) were not shown to be efficacious for treatment of alcohol use disorders when administered alone (Chick et al., 2004; Stella et al., 2008) while metadoxine (3000 mg/day), a 5HT2B antagonist, was efficacious in promoting relapse in one study (Guerrini et al., 2006).

Collectively, these findings suggest that there are individual differences (e.g., early- versus late-onset and polymorphisms in serotonin transport) in the efficacy of antidepressants for the treatment of alcohol use disorders. Drug combination therapies have been more effective than monotherapy with these serotonergic agents. Perhaps screening of patients for polymorphisms in serotonergic transporter could yield insight into the most effective medication for the treatment-seeking individual.

Antipsychotics

Antipsychotic medications (e.g., aripiprazole, flupenthixol, olanzapine, tiapride, lisuride and amisulpride) recently examined for therapeutic potential in the treatment of alcohol use disorders are not effective (Anton et al., 2008; Bender et al., 2007; Guardia et al., 2004; Marra et al., 2002; Schmidt et al., 2002; Weisbeck et al., 2001; Weisbeck et al., 2003). The reasons for these effects are unknown, but could reflect adverse side effects of antipsychotic medications.

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Opioid Receptor Antagonist Nalmefelene

European countries have recently approved nalmefelene, a partial kappa receptor agonist, for the treatment of alcohol dependence in patients who seek treatment for controlled consumption. While results from several large-scale randomized clinical trials suggest nalmefelene (20–40mg/day), in combination with cognitive therapy, significantly reduces drinking (Mann et al., 2013; Gual et al., 2013; Van de Brink et al., 2013), further examination of this medication is warranted before any definitive conclusions can be drawn.

Collectively, these large-scale clinical trials demonstrate that there is an unequivocal need to identify and develop putative pharmacotherapies for the treatment of alcohol use disorders. Drug combination therapies might be most effective for treatment of alcohol use disorders, particularly in individuals with altered serotonergic transport function, co-morbid psychological disorders, or individuals whom seek treatment to control drinking rather than abstain completely.

Summary

Alcohol-related disorders arise from a variety of factors (e.g., early- and late-onset, genetics, and comorbidities with other psychological disorders) and are complicated by corresponding factors (e.g., physiological and psychological). Further, the patient desire to seek treatment adds a final complication (e.g., full abstinence or controlled consumption). Pharmacotherapy alone will not answer the large-scale problem of alcohol use disorders in the United States but has the potential to reduce rates of consumption and relapse in individuals seeking treatment. Patterns of intermittent consumption (e.g., multiple withdrawals and binge drinking) are commonly demonstrated

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by dependent individuals, and neurological, behavioral, cognitive, and effects of sensitization or kindling to multiple withdrawals occurs in humans and rodent models of CIE exposure. The studies in this dissertation sought to add to the field by identifying underlying mechanisms associated with hippocampal cytotoxicity *in vitro* that contribute to the behavioral effects of withdrawal *in vivo*.

The studies in this dissertation found that neuroadaptations in protein kinase activity occur in the presence of binge-like ethanol exposure prior to the onset of withdrawal in the rat hippocampus. This protein kinase activation in response to ethanol application contributes to neuroadaptations in glutamatergic tone, promoting cytotoxicity of hippocampal pyramidal and granule cell layers. Next, this *in vitro* model of CIE exposure was translated into a model of CIE exposure *in vivo* so as to examine the effects of presumed hippocampal excitotoxicity on cognitive performance in the Morris Water Maze. While physical dependence was achieved in subjects administered CIE (i.e., confirmed by a significant presence of physical withdrawal characteristics), spatial learning and memory remained intact in ethanol-dependent rats. It is reasonable to conclude, therefore, that the specific task used for cognitive assessment was not sensitive enough to the relatively modest hippocampal cytotoxicity reliably detected in our *in vitro* assay. Future studies should implement a reversal task in the Morris Water Maze to detect more subtle differences in reference memory before definitive conclusions can be made. A series of antagonist studies that assessed a specific signal transduction cascade (e.g., G-protein group 1 mGluRs and release of intracellular calcium from the ER) identified that Gαq proteins (i.e., mGluR1-and-5-containing receptors) are involved in the development of dependence *in vitro* and *in vivo*. For example, selective mGluR1 and

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mGluR5 antagonist exposure in the presence of ethanol restored hippocampal neuronal integrity *in vitro*. The oral administration mGluR5 antagonist in the presence of ethanol limited the severity of withdrawal from CIE administration *in vivo.* In general, these findings suggest that organotypic hippocampal slice cultures are a good model for initial high-throughput screening of compounds and the identification of mechanisms involved in the development of ethanol dependence. Specifically, these data suggest that blocking group 1 mGluRs is effective in reducing the rate in which development of dependence occurs in rodents. However, this effect could be due to pharmacokinetic interactions due to decreases of BELs as compared to a pharmacodynamics effect. These data also suggest that concentration of these metabotropic glutamate receptors localized in the hippocampus mediate the cytotoxic effects of CIE exposure that contribute to the behavioral effects of ethanol withdrawal.

From these results, putative pharmacotherapies that modulate glutamatergic tone may be more thoroughly evaluated with regards to treatment of alcohol use disorders. In particular, pharmacological manipulations of group 1 mGluRs in the presence of binge-like ethanol warrant further study. These understandings may contribute to political, social, and individual success by providing more effective options for the treatment of alcohol use disorders in the clinical population.

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ANNA R. REYNOLDS
Curriculum vitae

June 2015

EDUCATION

2013 MS, Department of Psychology, University of Kentucky

2009 BA, Department of Psychology, University of Kentucky

RESEARCH EXPERIENCE

2010-present Neuropharmacology of drugs of abuse in vitro and in vivo, Psychology Department and Spinal Cord and Brain Injury Research Center (Dr. Mark Prendergast), University of Kentucky

2009–2010 Studies of vertebrate genome evolution, Department of Biology and Spinal Cord and Brain Injury Research Center (Dr. Randall Voss), University of Kentucky

2008–2009 Human psychopharmacology of drugs of abuse (Senior Honors thesis under the supervision of Dr. Craig Rush), Laboratory of Human Behavioral Pharmacology, Department of Psychology and Behavioral Sciences, University of Kentucky

GRANTS AND FELLOWSHIPS

2014–present National Institute on Drug Abuse (NIDA) T32–DA035200, University of Kentucky (Dr. Craig Rush)

2013–2014 National Institute on Drug Abuse (NIDA) T32–DA16176, University of Kentucky (Dr. Linda Dwoskin)

PUBLICATIONS

Refereed Journals Articles
*** denotes corresponding author**

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Reynolds AR, Saunders MS, Brewton HW, Winchester SW, Elgumati IE, & Prendergast MA. Acute oral administration of the novel, competitive and selective glucocorticoid receptor antagonist ORG 34517 reduces the severity of ethanol withdrawal and related hypothalamic- pituitary-adrenal axis activation (In press: *Drug and Alcohol Dependence*).

Wang X, **Reynolds AR**, Elshahawi SI, Shaaban KA, Ponomareva LV, Saunders MA, Elgumati IS, Zhang Y, Copley GC, Hower JC, Sunkara M, Morris AJ, Kharel MK, Van Lanen SG, Prendergast MA, Thorson JS (2015). Terfestatins B and C, New p-Terphenyl Glycosides Produced by Streptomyces sp. RM-5-8. *Organic Letters*.

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Voss SR, Kump DK, Putta S, Pauly N, **Reynolds AR**, Henry RJ, Basa S, Walker JA & Smith JJ (2011). Origin of amphibian and avian chromosomes by fission, fusion, and retention of ancestral chromosomes. *Genome Research*. 21(8):1306–12.

Submitted Manuscripts

Van Skike CE, Casey EM, Maggio SE, **Reynolds AR**, Bardo MT, Dwoskin LP, Prendergast MA, Nixon, K. Critical Needs in Medications Development for Cessation of Alcohol and Nicotine Polysubstance Abuse (Submitted to *Progress in Neuropsychopharmacology and Biological Psychiatry*).

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Berry JN, Sharrett-Field LJ, **Reynolds AR** & Prendergast MA. Corticosterone enhances N-methyl-D-aspartate receptor signaling to promote ventral tegmental area neurotoxicity in the reconstituted mesolimbic dopamine pathway (Submitted to *Synapse*).

Manuscripts in Preparation

Prendergast MA, Butler TR, Smith KJ, Little HJ, & **Reynolds AR**. The novel, competitive glucocorticoid receptor antagonist ORG34517 reduces the severity of withdrawal from repeated binge-like ethanol administration (In preparation for *Alcoholism: Clinical and Experimental Research*).

Berry JN, Butler TR, Sharrett-Field LJ, **Reynolds AR** & Prendergast MA. Exposure to corticosterone during ethanol exposure and withdrawal augments the loss of synaptophysin immunoreactivity in a NR2B–dependent manner (In preparation for *Alcoholism: Clinical and Experimental Research*).

Poster Presentations

Reynolds AR, Berry JN, Sharrett-Field LJ & Prendergast MA (2014). Development of ethanol dependence requires activation of MGlua 1-and-5 containing receptors. Bluegrass Chapter of SfN Neuroscience Day, Lexington, KY.

Reynolds AR, Berry JN, Sharrett-Field LJ, Saunders MA & Prendergast MA (2014). Ethanol exposure stimulates the mGluR5 receptor prior to withdrawal to promote cytotoxicity of chronic, intermittent ethanol. Abstract submitted for poster presentation, *Research Society on Alcoholism*, Bellevue, Washington.

Reynolds AR, Berry JN, Sharrett-Field LJ & Prendergast MA (2013). Chronic intermittent t-ethanol exposure produces a loss if mature neurons in hippocampal slice cultures. Abstract for poster presentation, *Research Society on Alcoholism*, Orlando, FL; Bluegrass Chapter of SfN Neuroscience Day, Lexington, KY.

Berry JN, Sharrett-Field LJ, **Reynolds AR** & Prendergast MA (2013). Co-exposure to corticosterone and ethanol augments the loss of synaptophysin in a NR2B-dependent manner. Abstract for poster presentation, *Research Society on Alcoholism*, Orlando, FL.

Sharrett-Field LJ, Berry JN, **Reynolds AR** & Prendergast MA (2013). Startle reactivity predicts and is altered by intermittent access to ethanol in the outbread long evans

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rat. Abstract for poster presentation, *Research Society on Alcoholism*, Orlando, FL.

Berry JN, Sharrett-Field LJ, **Reynolds AR** & Prendergast MA (2012). Chronic corticosterone sensitizes the mesolimbic dopamine reward pathway to excitatory NMDA receptor activity. Abstract for poster presentation*, Society for Neuroscience*, New Orleans, LA.

Sharrett-Field LJ, Butler TR, Berry JN, **Reynolds AR** & Prendergast MA (2012). Short term ethanol exposure induces calpain dependent α–spectrin proteolysis and neurodegeneration in the hippocampus. Abstract for poster presentation, *Research Society on Alcoholism*, San Francisco, CA.

Berry JN, Sharrett-Field LJ, Butler TR, **Reynolds AR** & Prendergast MA (2011). Time–dependence of cysteine protease activation following excitotoxic hippocampal injury. Abstract for poster presentation*, Society for Neuroscience*, Washington, D.C.

TEACHING EXPERIENCE

2014	Co-Instructor, Psychology 459, "Neuropharmacology: Drugs and Behavior," Psychology Department, University of Kentucky
2013	Guest Lecture, Psychology 311, "Sleep and Consciousness," Psychology Department, Eastern Kentucky University
2012	Guest Lecture, Psychology 496, "Endocannabinoids and Drugs of Abuse," Psychology Department, University of Kentucky
2011	Guest Lecture, Psychology 215 "Research Methods," Psychology Department, University of Kentucky
2010–2012	Teaching Assistant, Psychology Department, University of Kentucky

LEADERSHIP

2014–present	Graduate Student Executive Committee, Experimental Psychology Representative, Department of Psychology, University of Kentucky
2014	Undergraduate Student Research Mentor, Summer Training in Alcohol Research, University of Kentucky

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2013–present	Undergraduate Student Research Mentor for Psychology and Biology Majors, University of Kentucky
2013–present	Community Outreach, Brain Awareness for local Elementary Schools
2013–2014	Executive Committee, Graduate Student Representative, Bluegrass Society for Neuroscience
2011	Seminar Coordinator, Behavioral Neuroscience and Pharmacology, University of Kentucky

PROFESSIONAL MEMBERSHIPS/AFFILIATIONS

2011–present	Research Society on Alcoholism
2010–present	Bluegrass Chapter of the Society for Neuroscience
2008	Psi Chi National Honors Society
2005	Phi Eta Sigma Honors Society

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