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Age differences in the manifestation & neurobiology of negative affect during alcohol withdrawal in a mouse model of binge drinking

A dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy in Psychological & Brain Sciences

by

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Abstract

Age differences in the manifestation & neurobiology of negative affect during alcohol withdrawal in a mouse model of binge drinking

By Kaziya Mai Lee

The research presented in this dissertation characterizes the manifestation and neurobiological underpinnings of negative affect during alcohol withdrawal in adult and adolescent mice using an animal model of voluntary binge drinking. A history of binge drinking elicits changes in glutamate-related protein expression within regions of the extended amygdala relevant to emotional regulation. Studying withdrawal-induced changes in protein expression may provide insight into the mechanisms mediating age-dependent differences in the negative affective consequences of alcohol abuse. The results of this work demonstrate that, similar to the human population, adult and adolescent mice differ both in their pattern of alcohol consumption and also in the consequences of that consumption. Although adolescent animals typically consume larger quantities of alcohol, adolescents are less sensitive than adults to negative affect during early withdrawal. However, adolescents binge drinkers show an emergence of negative affect later in adulthood, as well as elevated alcohol consumption. Moreover, these studies identify specific changes in protein expression within two brain regions critically involved in both emotion and addiction neurobiology- the central nucleus of the amygdala (CEA) and the nucleus accumbens shell (AcbSh). Withdrawal from binge drinking is associated with increased neuronal activity within the AcbSh, concomitant with increased glutamate-related protein expression in adult animals. Conversely, decreased expression of protein indices of Homer2-dependent mGluR signaling

was observed within the CEA of both adult and adolescent animals. Reversing these changes using neuropharmacological or virus-mediated transgene delivery approaches reduced behavioral signs of withdrawal-induced anxiety. Together, these studies identify neurobiological substrates and pathophysiology of relevance to the negative reinforcing properties of alcohol and characterize ontogenetic differences in the behavioral and neurobiological consequences of alcohol abuse.

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Chapter 1:

General Introduction

Alcohol abuse in the human population

Alcohol is the most widely used psychoactive drug in the United States. Excessive alcohol consumption is a significant societal problem associated with a variety of negative outcomes in the form of economic expense, global disease burden, and psychological distress. Excessive alcohol consumption is also one of the leading causes of preventable death nationally (Stahre et al., 2014) and contributes to an estimated 3.3 million deaths globally per year (World Health Organization, 2014). Binge drinking is the most common pattern of excessive alcohol consumption, with over 38 million Americans engaging in binge drinking an average of four times per month (Centers for Disease Control and Prevention, 2013). Binge drinking is especially prevalent in adolescents and young adults, and over 90% of alcohol consumed by underage individuals is in the form of binge drinks (Centers for Disease Control and Prevention, 2016). Although the majority of binge drinkers do not meet the diagnostic criteria for alcoholism (Esser et al., 2014), binge drinking is a significant risk factor for alcoholism.

Frequent binge drinking is associated with feelings of irritability, insomnia, confusion, anxiety, depression, and general dysphoria during withdrawal even in non-dependent individuals (Hasin et al., 2007; Knight et al., 2002; Substance Abuse and Mental Health Services Administration, 2011). Although addiction research operationally defines the term 'withdrawal' differently in various contexts, for the purposes of the work described in this dissertation, withdrawal simply refers to the absence of drug. Feelings of dysphoria during withdrawal from binge drinking promote re-engaging in the behavior in order to alleviate these symptoms and this perpetuation of the binge-withdrawal cycle is theorized to accelerate the transition to addiction (Driessen et al., 2001; Hall & Zador, 1997; Koob, 2013;

Koob & Le Moal, 1997). Many alcoholics report that anxiety reduction is a key motivator for drinking (Kushner, Abrams, & Borchardt, 2000) and in the clinical population, anxiety and depression during abstinence are significant sources of negative reinforcement and negative affect is one of the strongest predictors of relapse in abstinent individuals (Baker et al., 2004; Cloninger, 1987; Koob & Le Moal, 1997; Martinotti et al., 2008).

Alcohol and Adolescence

Binge drinking is particularly prevalent among adolescents and young adults. Under-aged individuals ages 12-20 years old account for 11% of all alcohol consumed in the U.S. (CDC, 2016) and over 90% of this alcohol is consumed in the form of binge drinks. Adolescence is a dynamic period of cognitive, social, and emotional maturation. During this transition from childhood to adulthood, adolescents show distinct behavioral and psychological differences, compared to adults (Dahl, 2004). For instance, adolescents show less positive responses to stimuli of low or moderate incentive value, which is thought to drive adolescents to seek-out stronger reinforcement by engaging in impulsive, risky behaviors such as drug use (Novier et al., 2015). Additionally, adolescents have higher basal levels of anxiety and depression and are more vulnerable to stress (Spear, 2002), which may contribute to the negative reinforcing properties of alcohol and encourage drug use. Indeed, research has found that perceived stress is one of the strongest predictors of adolescent substance use (Myers & Brown, 1990; Pohorecky, 1991; Wagner, 1993).

Adults and adolescents differ both in their pattern of alcohol consumption and also the consequences of that consumption, especially during the “hangover” period of early withdrawal. Although adolescents typically consumer larger quantities of alcohol per

drinking episode than adults, they appear resilient to many of the adverse effects of alcohol that serve as modulatory cues to curb excessive consumption such as locomotor incoordination, subjective intoxication, sedation, and many "hangover" symptoms including, anxiety and dysphoria (Brasser & Spear, 2002; Spear & Varlinskaya, 2005; Varlinskaya & Spear, 2004). In contrast, adolescents appear more sensitive to the positive rewarding effects of alcohol that serve as primary reinforcers of drinking. This combination of permissive/ facilitative factors is theorized to drive high alcohol consumption in adolescents (Spear & Varlinskaya, 2005). Although more extensively studied in animal models, these age-related differences and are consistent with reports of greater tolerance and less severe withdrawal symptoms in human adolescent drinkers (Deas et al., 2000; Filstead et al., 1989; Martin & Winters, 1998; Winters et al., 1999).

Evidence from both human and animal research suggests that chronic alcohol exposure may have differential effects on adolescent compared to adult drinkers, likely due to their distinct states of neurodevelopment. Adolescent binge drinking is associated with a variety of anatomical, behavioral, and cognitive impairments. Animal studies have shown that adolescent alcohol exposure is associated with structural anomalies in brain regions including the orbitofrontal cortex, cerebellum, and thalamus (Coleman et al., 2014), memory impairments (White, Matthews, et al., 2000), and increased anxiety- and depression-related behaviors (Slawecki et al., 2004). These findings are similarly represented in the human literature. Frequent under-aged binge drinking is associated with decreased verbal, non-verbal, and visuospatial functioning (Brown et al., 2000), memory impairments (Chin et al., 2010; Zeigler et al., 2005), decreased hippocampal and prefrontal volume (De Bellis et al., 2000; DeBellis et al., 2013), and fMRI evidence of abnormal functional changes (Ewing et

al., 2014). During adulthood, adolescent binge drinkers show increased incidence of anxiety and depression, as well as symptoms of antisocial and borderline personality disorder (McKenzie et al., 2011; Rohde et al., 2001). Importantly, engaging in under-aged binge drinking is one of the strongest predictors of substance abuse problems and addiction later in life (Beseler, 2008; Chassin et al., 2002; Gruber et al., 1996). Specifically, youth who start drinking before age 15 years are over five times more likely to develop alcohol dependence later in life than those who begin drinking at or after age 21 years (SAMHSA, 2004; 2011, 2013, 2014). Thus, adolescent binge drinkers show distinct behavioral and neurobiological consequences of binge drinking that contribute to increased vulnerability to addiction and negative affect in adulthood. Given the serious adverse outcomes associated with adolescent binge drinking, combined with the alarming prevalence of this behavior, it is important to better understand the negative affective consequences of adolescent binge drinking.

Negative affect in laboratory animals

Similar to humans, laboratory animals exhibit increased behavioral signs of anxiety and depression following a history of alcohol abuse and this is observed across a variety of assays designed to measure animal behaviors associated with an anxious or depressive state (Kliethermes, 2005). In humans, anxiety is often associated with feelings of negative arousal such as apprehension, tension, irritability, agitation nervousness, and worry (Kazdin, 2000). In laboratory animals- primarily rodents- a variety of assays have been developed to measure animal behaviors associated with an anxiety-like state. One subtype of anxiety tests is based on rodents' natural aversion to open and exposed spaces. These assays include the elevated-plus or elevated-zero maze, the light-dark box and open field test. As rodents are prey

animals, they exhibit a natural avoidance of unprotected areas that leave them vulnerable to predation. However, this aversion is also at odds with an innate exploratory drive, as rodents are also foraging animals (Finlay & Sengelaub, 1981; Lima & Dill, 1990). A behavioral shift showing increased bias toward 'safer' areas of the test apparatus and a reduced exploration of more open and vulnerable areas, relative to control animals, is interpreted as increased anxiety (Bourin & Hascoet, 2003; File et al., 2004; Kulkarni et al., 2007; Tovote et al., 2015). Another subtype of anxiety tests assays measure neophobia-related behavior, defined as an avoidance of, or aversion to, novel, unfamiliar objects (Misslin & Ropartz, 1981). Decreased interaction with, and exploration of, a novel object, relative to controls, is interpreted as a sign of anxiety (Griebel et al., 1993; Hoplight et al., 2005; Misslin & Ropartz, 1981; van Gaalen & Steckler, 2000). If there are several unfamiliar objects that are not easily avoidable, rodents may also engage in defensive burying behavior - a protective reflex to mitigate the potential threat of the novel object(s) (Bhatnagar et al., 2003; Peacock & Wong, 1982). Although defensive burying tests often involve the use of a shock probe, particularly when conducted in rats (Cueto-Escobedo et al., 2013; File et al., 2005), animals will also engage in defensive burying behavior in response to less noxious stimuli such as marbles (Dey et al., 2016; Gaikwad & Parle, 2011; Saadat et al., 2006; Savy et al., 2015; Shinomiya et al., 2005; Skalisz et al., 2004).

In contrast to the numerous animal assays of anxiety, there exist relatively few validated tests of depression, which has been attributed to the unique and complex features of human depression. In contrast to anxiety, the complex symptomology of depression has fewer behavioral analogs in laboratory animals (Krishnan & Nestler, 2011; Overstreet, 2012). However, animal assays are able to measure 'behavioral despair' (Casarotto et al., 2010). In

the Porsolt forced swim test (Porsolt et al., 1978; Porsolt et al., 2001) and the tail suspension test (Can et al., 2012), reduced swimming or climbing behavior is thought to reflect the hopelessness and helplessness associated with depression (Alloy et al., 1988). Other depression tests measure anhedonia, defined as reduced reinforcement or reward value of previously rewarding stimuli (Loas, 1996; Willner et al., 1992), which is a symptom of depression (American Psychiatric Association, 2013; Gorwood, 2008). For example, animals typically exhibit a high preference for sucrose-sweetened liquid over plain water and a reduction in this preference, relative to control animals, is interpreted as a sign of anhedonia (Papp et al., 1991; Serchov et al., 2016). Another test of anhedonia involves intracranial self-stimulation (ICSS), in which animals learn to operantly self-administer electrical stimulation to areas such as the lateral hypothalamus, medial forebrain bundle, and ventral tegmental area. This stimulation is rewarding and positively reinforces operant responding (Vlachou & Markou, 2011). Increases in ICSS threshold required to positively reinforce and sustain operant responding is thought to reflect diminished reward value of the stimulation and thus an anhedonic state (Carlezon & Chartoff, 2007). However, ICSS is less commonly used than other assays as it is an invasive procedure requiring intracranial surgery and may therefore interfere with other behaviors and tests (Tedford et al., 2014).

There exists debate among animal researchers as to whether or not it is inappropriately anthropomorphic to assign human emotional states to laboratory animals. However, these behavioral assays not only share face validity with aspects of the human condition (Bourin, 2015), but they have also been pharmacologically validated based on the ability of known anxiolytic and anxiogenic drugs to consistently alter behavior in these tests. For example, benzodiazepine drugs are classic anxiolytics (Tallman et al., 1980) and have

been shown to reduce anxiety-related behaviors in the light-dark box (Crawley & Goodwin, 1980), elevated plus and zero maze (File & Pellow, 1985; Shepherd et al., 1994), and also reduce neophobia (Griebel et al., 1993) and defensive burying (Broekkamp et al., 1986; De Boer & Koolhaas, 2003). Conversely, anxiogenic drugs such as inverse benzodiazepine agonists and HPA-activating compounds increase anxiety-like behaviors in these tests (Dunn & Berridge, 1990; Griebel et al., 1993; Lacosta et al., 1999; Pellow & File, 1986; Shepherd et al., 1994; Tsuda et al., 1988). Additionally, these tests have shown predictive validity as screens for novel anxiolytic and antidepressant drugs. Therefore, valuable insight can be gained through the use of animal tests of emotionality.

Alcohol-induced neuroadaptations & anxiety

Alcohol withdrawal-induced negative affect is thought to be a consequence of the acute anxiolytic effects of alcohol, observed in both humans and animals (Fernandez del Moral et al., 1989; Gaikwad & Parle, 2011; Gilman et al., 2008; Kushner et al., 1996; Stewart et al., 1993). This effect is attributed to alcohol's potentiation of GABAergic (Sundstrom-Poromaa et al., 2002), mesolimbic dopamine (DA) (Di Chiara, 1997), and endogenous opioid signaling (Ghozland et al., 2005), in addition to suppression of excitatory glutamatergic (Danysz et al., 1992) and corticotropin-releasing factor (CRF) signaling (Fernandez del Moral et al., 1989). With repeated exposure, alcohol imposes an allostatic load that elicits neurobiological counteradaptations in order regain homeostatic balance (Koob, 2008). These compensatory changes directly oppose the acute effects of alcohol, which leads to hyperactive glutamate and CRF signaling and hypoactive GABA and DA signaling during periods of withdrawal. Increased glutamate (Bergink et al., 2004) and CRF

(Heilig & Koob, 2007), as well as decreased DA (Ma & Zhu, 2014) and opioid signaling (Lutz & Kieffer, 2013), are all associated with negative affective states.

Negative affect during withdrawal is mediated primarily by alcohol-induced dysregulation of signaling within subcortical regions of the extended amygdala (Koob, 1999, 2003). The extended amygdala is a basal forebrain macrostructure consisting of the central nucleus of the amygdala (CEA), the bed nucleus of the stria terminalis (BNST), and the shell subregion of the nucleus accumbens (AcbSh) that is critically involved in emotional processing and affective regulation (Alheid, 2003; Alheid & Heimer, 1988). The central nucleus of the amygdala (CEA) consists primarily of GABAergic neurons (McDonald, 1982) is considered a 'striatum-like' nucleus, due to its morphological, physiological, and biochemical similarities to medium spiny neurons of the striatum (Martina et al., 1999; Schiess et al., 1999). Although the CEA shares reciprocal connectivity with both the BNST and the AcbSh, the CEA is more functionally related to the BNST (Gungor et al., 2015; Walker et al., 2009). Both the CEA and BNST are primarily innervated by glutamatergic projections from the basolateral amygdala (BLA), which receives sensory information from the cortex and thalamus (Lee et al., 2013). Together the CEA and BNST facilitate the formation of emotionally salient memories and serve similar, but complementary, functions in the expression of anxiety/fear-related behaviors through projections to brainstem structures (Lee et al., 2013; Radke, 2009; Sah, 2010; Walker et al., 2009). For example, inactivation studies show that the CeA, but not the BNST, mediates the expression of conditioned fear responses to discrete sensory cues (Hitchcock & Davis, 1991; Ledoux et al., 1988b). Conversely, inactivation of the BNST, but not the CEA, decreases contextual fear responses to environmental cues (Duvarci et al., 2009; Sullivan et al., 2004).

The nucleus accumbens (Acb), as a whole, is considered a limbic-motor interface that converts motivational affective states (mediated primarily by the shell subregion) to functional behavioral outputs (mediated primarily by the core subregion) (Levita et al., 2012; Salgado & Kaplitt, 2015). The AcbSh is thought of as a transitional zone linking the ascending striatopallidal system and the descending 'central' extended amygdala (i.e. the CEA and BSNT) (Heimer et al., 1997; Koob, 1999; Zahm, 1999). Although there is evidence that the AcbSh may have some direct connectivity with the CEA (Fudge et al., 2002), albeit limited (Heimer et al., 2008; Zahm et al., 1999), the AcbSh is known to share reciprocal connectivity with the BNST (Heimer et al., 1991; Kirouac & Ganguly, 1995). Together, the CEA, BNST, and AcbSh act as a relay station between the brainstem, thalamus, and the cortex and coordinate complex emotional processes (Alheid, 2003). As such, dysregulation within extended amygdala structures is implicated in both affective disorders such as anxiety and depression (Davis et al., 2010; Jennings et al., 2013; Shackman & Fox, 2016; Vyas et al., 2003) and also the negative reinforcing properties of alcohol and other drugs of abuse (Gilpin, 2014; Gilpin & Koob, 2008; Koob, 1999; Koob & Le Moal, 2001; Shackman & Fox, 2016).

Animal studies provide extensive evidence for dysregulation within extended amygdala circuitry during withdrawal from binge drinking (e.g. Gilpin, 2014; Gilpin et al., 2015; Gilpin & Koob, 2008; Koob, 1999, 2003, 2013; Koob et al., 1998 for review). Previous work in the Szumlinski laboratory has focused extensively on alcohol-induced plasticity within the AcbSh and CEA. Prior studies have shown that not only are these regions consistently sensitive to alcohol-induced changes in glutamate-related protein expression but also that these proteins are functionally relevant to voluntary alcohol consumption. For

example, binge drinking upregulates the expression of mGlu1, GluN2B, Homer2a/b and phospholipase C (PLC) within the CEA (Cozzoli et al., 2014), with similar upregulation of Homer2a/b, GluN2B, and mGlu5 also observed within the AcbSh (Cozzoli et al., 2012; Cozzoli et al., 2009). Further, inhibition of group 1 mGluR and kinase (i.e. PLC and PI3K) activity within the CEA and AcbSh reduces binge alcohol consumption via Homer2-dependent pathways (Cozzoli et al., 2015; Cozzoli et al., 2014; Lum et al., 2014). Thus, protein indices of Homer2-dependent mGluR signaling are theorized to be influential mediators of alcohol's rewarding/reinforcing properties.

These previous studies of the CEA and AcbSh have focused primarily on signaling pathways of relevance to voluntary alcohol consumption. However, the AcbSh and CEA are also integrally involved in emotionality via extended amygdala circuitry. Imbalances between excitatory and inhibitory transmission within extended amygdala structures are implicated in the etiology of mood disorders such as anxiety and depression, independent of prior alcohol abuse (Burns & Teesson, 2002; Driessen et al., 2001; Miguel-Hidalgo et al., 2010; Rosenberg, 2008; Shackman & Fox, 2016; Smith & Randall, 2012). Alcohol-induced neuroadaptations within extended amygdala circuitry also mediate symptoms of affective dysregulation during withdrawal from binge drinking and a shared neurobiological basis may contribute to the high comorbidity between alcohol abuse and mood disorders. Thus, the studies presented in this dissertation were intended to expand upon our laboratory's prior work to assess the role of alcohol-induced plasticity within the CEA and AcbSh in withdrawal-induced negative affect.

Animal studies of withdrawal-induced negative affect

There exist an abundance of animal studies on the negative affective consequences of alcohol withdrawal (reviewed in Kliethermes, 2005). However, nearly all of these studies employed noncontingent administration paradigms (e.g., vapor inhalation, injection, or gavage) or contingent models based prolonged access to adulterated solutions such as alcohol-containing liquid diet (McBride & Li, 1998; McClearn, 1988; Sinclair, 1979; Spanagel, 2000; Tabakoff & Hoffman, 2000). These studies have shown that both rats and mice exhibit signs of anxiety- and depression-related behaviors as early as 2 hr withdrawal (Valdez et al., 2002) that persist for at least 4 months or longer (Santucci et al., 2008). While noncontingent methods are effective at producing physical dependence and robust effects upon behavioral indices of negative affect during withdrawal, such models arguably lack face validity and often rely on higher doses than animals would consume voluntarily (Becker, 2000; Egli, 2005; Freund, 1975; Hitzemann, 2000; Kirchhoff & Chester, 2013; McMillen, 1997; Spanagel, 2000). Additionally, there is overwhelming evidence that contingent vs. noncontingent drug administration elicits distinct neurochemical, and behavioral effects (Jacobs et al., 2003; Kippin et al., 2006; LeBlanc et al., 2014; Lecca, Cacciapaglia, et al., 2007; Lecca, Valentini, et al., 2007; Lominac et al., 2012; Markou & Koob, 1991). This evidence is primarily derived from psychomotor stimulant research, as few studies have examined this phenomenon with regards to alcohol. However, one study found differential effects of voluntary vs. involuntary alcohol exposure during adolescence on subsequent drinking in adulthood. Specifically, *involuntary* exposure to binge-like alcohol via systemic injections reduced voluntary drinking in adulthood, which was not present in animals with a history of voluntary binge drinking during adolescence (Gilpin, 2012). Overall, there exist

extremely limited studies of emotional dysregulation following a history of voluntary alcohol consumption in non-physiologically dependent animals, despite evidence that moderate levels of binge drinking are also capable of producing symptoms of negative affect during periods of abstinence (Crabbe et al., 2011; Kampov-Polevoy et al., 2000).

Specific aims

Given the gaps in our extant knowledge concerning the emotional consequences of voluntary binge drinking, the studies presented in this dissertation the effects of alcohol on behavioral measures of anxiety and depression during withdrawal within the context of a modified version of the well-established "Drinking in the Dark" (DID) murine model of binge drinking (e.g. Cozzoli et al., 2012; Crabbe et al., 2009; Moore & Boehm, 2009; Rhodes et al., 2005). These studies were designed to address the following specific aims: 1) determine the affective consequences of binge alcohol drinking during long and short-term withdrawal in adult animals and also evaluate the effects of alcohol exposure on cellular activity within regions of the extended amygdala. 2) Elucidate age-related differences in withdrawal-induced negative affect between adult and adolescent binge drinkers. 3) Validate an alternative interpretation of behavior in the Porsolt Forced Swim Test based on observations of hyperanxious adult animals during acute withdrawal. 4) Assess of the adult consequences of adolescent binge drinking. 5) Determine if withdrawal-induced anxiety can be reduced or enhanced via systemic administration of an mGlu5 negative and positive allosteric modulator, respectively. 6) Evaluate the contribution of mGlu5 signaling within the AcbSh specifically to withdrawal-induced anxiety via intracranial administration of mGlu5 antagonist; and 7) determine if reversing alcohol-induced downregulation of Homer2 in the

CEA reduces withdrawal-induced negative affect. Together, these studies provide insight into the affective consequences of region-specific protein changes that may contribute to the ontogenetic differences in the behavioral and neurobiological consequences of alcohol abuse.

Chapter 2:

Binge Alcohol Drinking Elicits Persistent Negative Affect in Mice

1. Introduction

Binge drinking is the most common pattern of excessive alcohol consumption, with over 38 million Americans admitting to binge drinking an average of four times per month (Centers for Disease Control and Prevention, 2013). Binge drinking is defined as a pattern of alcohol intake sufficient to produce a blood alcohol concentration (BAC) of ≥80mg% in a 2-hr period (National Institute on Alcohol Abuse and Alcoholism, 2004). Frequent binge drinking is a significant risk factor for the development of alcoholism (Dawson et al., 2005). As such, characterizing the neurobiological impact of binge drinking is crucial for understanding the consequences of this pattern of excessive alcohol consumption, which will aid in the development of pharmacotherapies for this prevalent form of alcohol-use disorder.

The elevated anxiety and depression during alcohol withdrawal is an aversive state frequently reported in the human population (Driessen et al., 2001) that is theorized to be a compelling source of negative reinforcement fueling compulsive drug-seeking behavior and relapse (Koob, 2008). However, confounding subject factors render it difficult to discern whether or not binge-drinking history is a sufficient antecedent to a negative affect state in humans. Moreover, it is impossible to study the cellular and molecular mechanisms underpinning emotional disturbances during alcohol withdrawal in a systematic, controlled, manner through studies of humans. As such, we have gained the majority of our insight into the psychobiological impact of alcohol from studies of animal models of alcohol use disorders.

Emotional dysregulation during alcohol withdrawal is largely attributed to changes within the extended amygdala (e.g. Koob, 1999). The extended amygdala is a basal forebrain macrosystem that acts as a subcortical relay station between the brainstem, thalamus, and

cortical areas and includes the bed nucleus of the stria terminalis (BNST), the shell of the nucleus accumbens (AcbSh), and the central nucleus of the amygdala (CEA) (Alheid, 2003). These areas are heavily implicated in the processing of anxiety, fear, depression, reward, and reinforcement and alcohol-induced changes within this macrosystem are implicated in the emotional disturbances that can occur during alcohol abstinence (Koob & Le Moal, 2001).

As for withdrawal-induced negative affect, the vast majority of our understanding of how alcohol withdrawal impacts extended amygdala function of relevance to emotional dysregulation has been derived from studies employing non-contingent alcohol administration or the use of adulterated alcohol diets (Tabakoff & Hoffman, 2000). However, consistent with this prior work, a dysregulation of extended amygdala function is reported during alcohol withdrawal in animal models of binge drinking. For instance, increased indices of glutamate transmission within extended amygdala structures are observed at 24 hrs following a month-long history of binge drinking (Cozzoli et al., 2012; Cozzoli et al., 2014), while changes in the expression of genes related to synaptic transmission and neuronal plasticity occur within the AcbSh and CeA of binging rodents during early withdrawal (i.e., 1-6 hrs post-binge) (Freeman et al., 2013; McBride et al., 2010). Thus, binge alcohol drinking appears to induce neuroadaptations within the extended amygdala similar to those reported in traditional models of alcohol dependence (Ward et al., 2009). However, to the best of our knowledge, no study to date has related binge drinking-induced changes in extended amygdala function to the manifestation of negative affect during withdrawal from binge drinking.

Given the gaps in the extant literature, we conducted an immunohistochemical analysis of the expression of the transcription factor Egr-1 within the extended amygdala and

adjacent structures to correlate cellular activity (Kaczmarek & Robertson, 2002) with the manifestation of anxiety and depressive behavior during withdrawal from binge drinking. We hypothesized that a history of binge drinking would augment behavioral indices of negative affect and that withdrawal-induced negative affect would be associated with increased cellular activity within the extended amygdala.

2. Materials and Methods

2.1 Subjects

This study used 60 adult C57BL/6J (B6) male mice that were 8 weeks of age at onset of drinking and weighed 25-30g (Jackson Laboratories, Sacramento, CA). Animals were randomly divided into an alcohol-drinking group (n=30; hereafter referred to as Alcohol mice) and a water-drinking group (n=30; hereafter referred to as Water mice) and then individually housed in standard, Plexiglas cages, under a 12-hour-reverse light/dark cycle (lights off at 10am), in a temperature-controlled vivarium (23°C). Food and water were available *ad libitum*, with the exception of the 2-hr alcohol drinking period, during which time the home cage water bottle was removed. All experiments were conducted in compliance with the National Institutes of Health *Guide for Care and Use of Laboratory Animals* (NIH Publication No. 80–23, revised 2010) and approved by the IACUC of the University of California, Santa Barbara.

2.2 Drinking-in-the Dark (DID) Procedures

To elicit 'binge-like' alcohol consumption, we employed a modified version of the DID model, which results in alcohol intakes between 3.5-5.0 g/kg alcohol in a 2-hr period

and yields blood alcohol concentrations (BACs) in excess of 80mg% (e.g. Rhodes et al., 2005). Three hours after lights out, home cage water bottles were replaced with sipper tubes containing a 20% (v/v) unsweetened alcohol solution in filtered tap water and mice were allowed to drink for 2 hrs, at which point the alcohol bottles were removed and the home cage water bottles were replaced. Control animals received an identical sipper tube of filtered tap water *in lieu* of alcohol. For practical reasons, mice were subjected to these drinking conditions 5 days per week (M-F) over the course of 6 weeks (total drinking days = 30). Each day, the amount of alcohol consumed was calculated by bottle weight immediately before and after the drinking period. All animals, both alcohol and water drinkers, were weighed 3x per week throughout the drinking period. Unfortunately, technical difficulties with our Analox Analyzer precluded our ability to determine BACs in this study. Thus, BACs were estimated from observed intakes and based on the results of published correlational analyses in B6 mice (Crabbe et al., 2009; Rhodes et al., 2005; Rhodes et al., 2007), as conducted previously (Cozzoli et al., 2009).

2.3 Behavioral testing

We administered a 2-day test battery to assay for alcohol withdrawal-induced changes in behavior in both the short-term and long-term (respectively 1-2 vs. 21-22 days following last alcohol presentation; n=15/group/withdrawal time-point). At both time-points, testing for affect began with an overnight test for sucrose preference and the remaining tests were conducted across the following 2 days. On the first test day after sucrose preference testing, mice were assayed first in a light/dark shuttle box or novel object encounter (order randomized across cohorts), followed by a 15-min swim test. The 15-min swim test occurred

at the end of the first day to allow mice time to recuperate, as per our IACUC's request. On the second test day, mice were tested first on the elevated plus maze or for marble burying (order randomized across cohorts), followed by a 5-min swim retest and animals were sacrificed and brain tissue was harvested immediately upon completion of the swim retest. All tests were conducted under standard ambient lighting and the details of the procedures employed for each of these paradigms are provided below.

2.3.1 Sucrose preference. Anhedonia, an absence of pleasure from previously enjoyable activities, is a characteristic symptom of depression in humans**.** Low sucrose preference is a well-established index of anhedonia in animal models (Katz, 1982) and thus, we examined for the effects of early versus late withdrawal from binge drinking on sucrose preference in our mice. For this, animals were given overnight access to 2 identical sipper tubes, one contained 5% sucrose and the other contained tap water. The bottles were weighed prior to being placed on the home cage at 17:00 h. Sixteen hours later (09:00 h the next day), the bottles were removed from the home cage and weighed to determine the total volume consumed, as well as the relative preference for sucrose.

2.3.2 Light/dark shuttle box. The light/dark shuttle box test was used to assess exploratory and anxiety-like behaviors (Bourin & Hascoet, 2003; Crawley, 1985). Animals were placed into a polycarbonate box measuring 46 cm long × 24 cm high × 22 cm wide containing 2 distinct environments for a 15-minute trial. Half of the box was white and uncovered, the other half black and covered, and these 2 environments were separated by a central divider with an opening. The animals were first placed on the dark side and the

latency to enter the light side, number of light-side entries, and total time spent in the light-side of the shuttle box were recorded using Any-maze™ tracking software (Stoelting co, Wood Dale, IL). Increased reluctance to venture into the light, uncovered, side was interpreted as an index of anxiety.

2.3.3 Novel object test. To test reactivity to a novel object as an index of neophobia-related anxiety (Dulawa et al., 1999; Misslin & Ropartz, 1981), animals were placed in an activity arena measuring 46 cm long×42 cm wide×40 cm high. In the center of the arena was placed a novel, inedible, object (candlestick holder; measuring approximately 6cm in diameter×12cm high). The animals' interaction with the novel object was observed during a 2-minute trial. The number of contacts, total time spent in contact with the novel object, and fecal count were recorded by a trained observer who was blind to the drinking condition of the animals.

2.3.4 Porsolt forced swim test. Floating behavior during the Porsolt forced swim test serves as an index of behavioral despair in laboratory animals (Porsolt, Bertin, et al., 1977) and is a model with high predictive validity for the clinical efficacy of anti-depressant drugs (Porsolt, Bertin, et al., 1977). On day 1 of behavioral testing, each animal was placed into a 26-cm diameter pool of room-temperature water, deep enough so animals were unable to touch the bottom. Behavior was monitored every 30 seconds by a trained observer for 15 min. On day 2 of behavioral testing (approximately 24 hrs following the first swim test), the animals were re-exposed to the pool for a 5-min retest session, in which behavior was monitored every 30 seconds. On both test days, the animal's behavior at each observation

was classified as floating (all 4 limbs completely immobile), treading (minimal limb motion with no forward movement), or swimming (active paddling with forward movement). Only 1 behavior was recorded per observation. The latency to first float was also recorded using a stopwatch.

2.3.5 Elevated plus maze. The elevated plus maze is a well-established paradigm in which to measure anxiety in laboratory animals, with high predictive validity for anxiolytic drugs (Karl et al., 2003; Walf & Frye, 2007). Animals were placed on the center intersection of a 4-arm radial plus maze with 2 white open arms and 2 black walled arms 24cm high. Each arm measured 123cm long × 5cm wide. Latency to first open-arm entry, number of open-arm entries, and total time spent in an open arm were monitored for the 4-minute trial by a trained observer who was blind to the drinking history of the mice. Differences in the amount of time spent in an open versus enclosed arm were also used to assess anxiety.

2.3.6 Marble burying. The marble burying test was used to measure anxiety-induced defensive burying (Njung'e & Handley, 1991). In our paradigm, 12 square glass pieces ($2.5cm^2 \times 1.25cm$ tall) were placed in the animals' home cage, 6 at each end. Latency to start burying the marbles was determined by a blind observer using a stopwatch and the total number of marbles buried following a 20-minute trial was recorded.

2.4 Brain tissue collection

At the conclusion of behavioral testing on day 2 (i.e., immediately following the 5-min swim re-test), mice were euthanized with an overdose of Euthasol® (Virbac Animal

Health, Fort Worth, TX) and perfused transcardially with 10 ml of phosphate-buffered saline (PBS) followed by 10 ml of a 4% paraformaldehyde solution. Brains were extracted and post-fixed for 24 hrs in 2% paraformaldehyde in PBS, then stored in PBS containing 30% (w/v) sucrose until cryosectioning. A Leica CM1800 cryostat (Leica Microsystems Inc., Buffalo Grove, IL) was used to collect 20 μm slices of brain tissue along the coronal plane, which were then mounted onto Superfrost Plus microscope slides (Fisher Scientific, Chino, CA). Using the Paxinos & Franklin (Paxinos & Franklin, 2004) mouse brain atlas as a guide, the following areas were sampled (co-ordinates relative to Bregma): the AcbSh and AcbC (+1.98-0.86 mm), the dorsal BNST (+0.74-0.82 mm) and the CeA and BLA (-0.94-2.06 mm). The AcbC and the BLA are functionally and anatomically associated with the extended amygdala, but are not considered part of this macrosystem. Thus, these subregions were included in our analysis to determine whether or not any observed changes in cellular activity were exclusive to the extended amygdala. Additional water-drinking and alcohol-drinking groups were included in this study to control for the effects of behavioral testing upon basal Egr1 levels. These controls were subjected to identical drinking and withdrawal schedules as water/alcohol mice but did not undergo behavioral testing prior to brain collection.

2.5 Immunohistochemistry

Egr1 (also known as Zif268, krox-24, and NGFI-A) is a transcription factor encoded by an immediate early gene and is commonly used as a marker of localized brain activation in laboratory animals (Herdegen & Leah, 1998; Kaczmarek & Robertson, 2002). Egr1 is uniquely advantageous over other common immediate early genes (e.g., c-Fos) due to its constitutive expression, making it sensitive to either increases or decreases in activation. To

assess Egr1 levels throughout the extended amygdala, brain tissue slides were prepared using the ABC method (Su et al., 2013). Slides were washed twice with Tris-buffered saline (0.05 M, pH 7.6 at room temperature) between each of the different treatments. Sections were treated with 0.25% Triton X-100 (Sigma #X-100, St. Louis, MO, USA) and 5% dimethyl sulfoxide (Sigma D-5879), and then incubated for 1 hr in 20% normal horse serum (NHS; Sigma G6767) + 1% bovine serum albumin (BSA-Fract V; Fisher Scientific, Los Angeles, CA, USA, BP1605-100) to block non-specific binding. Slides were then incubated for 24 hrs in a rabbit Egr1 primary antibody 1:1000 (c-19 anti-Egr1; Santa Cruz Biotechnology, Santa Cruz, CA, USA) + 0.5% Triton X-100 + 1% NHS. Next, sections were incubated for 1 hr in the secondary anti-rabbit IgG antibody (Vector Laboratories BA110, Burlingame, CA, USA), and for 30 min in the avidin–biotin horseradish peroxidase complex (Elite Vectastain Universal ABC Kit, Vector Laboratories PK6200, Burlingame, CA, USA). Staining was visualized using the chromogen 3,3'-diaminobenzidine (DAB) (Vector Laboratories Peroxidase Substrate Kit SK-4100). Following staining, sections were dehydrated and cover-slipped. Egr1-positive (Egr1+) cell counts were recorded by visual inspection at 40× magnification, restricted to an area defined by a 0.25 mm^2 grid by experimenters blind to the treatment of the animals. Cell counts for each region were averaged across 3 different adjacent sections per animal.

2.6 Statistical analyses

For each week of alcohol consumption, the average alcohol intake (expressed as g/kg body weight) was determined and data were analyzed using a within-subjects ANOVA with repeated measures on the Week factor (6 levels), with Fisher LSD post-hoc pairwise

comparisons. A t-test comparison was performed on the final body weight measurement to determine if there was a significant difference between alcohol- and water-drinking animals. Statistical analyses of all behavioral testing data were conducted using between-subjects two-way analyses of variance (ANOVAs). Cell count data were analyzed with a 2 X 2 X 2 ANOVA to compare group differences based on treatment, behavioral testing, and withdrawal duration. ANOVAs were followed by post-hoc t-test comparisons, when appropriate. α=0.05 for all ANOVAs and t-tests. Correlational analyses were conducted between Egr1+ cell counts in discrete regions and individual behavioral measures to determine if cellular activation of a particular region was related to behavioral signs of anxiety or depression. Correlational analyses were also conducted between brain regions to assess regional co-activation using cell counts from both behaviorally tested and untested alcohol/water-drinking animals in order enhance statistical power. Bonferroni adjusted alpha levels for each set of correlational comparisons. Statistical outliers were identified using the widely accepted ±1.5*IQR rule (Krzywinski & Altman, 2014; Navidi, 2006; Tukey, 1977) and excluded from analyses. Any animals identified as outliers during behavioral analysis were excluded from the cell count analysis. All calculations were performed using SPSS v.21 statistical software (IBM, 2012).

3. Results

3.1. Animal attrition

Over the course of the study, 5 animals were lost from the initial 30. One Alcohol and one water mouse were found dead during drinking procedures from no apparent cause. Two Water mice had to be euthanized due to congenital malocclusion and one Water

mouse died shortly after arrival of apparent dehydration. This resulted in the following group sizes by the end of the behavioral component of this study: Alcohol-short-term withdrawal= 14, Alcohol-long-term withdrawal= 15, Water-short-term withdrawal= 12; Water-long-term withdrawal= 14. During tissue preparation, we encountered technical difficulties with cryostat slicing, which resulted in a significant reduction in the sample sizes for the BNST tissue. Issues related either to a malfunctioning cryostat or weak immunostaining contributed to lowering the sample sizes employed in our analysis of other brain regions.

3.2. Alcohol intake

The B6 mice in this study consumed on average 4.0 ± 0.06 g/kg/2 h over the entire 6-week alcohol drinking period with very low variability. Although blood alcohol levels were not assayed in this study, this level of intake is predicted to result in BACs ≥80 mg%, based on published results (Crabbe et al., 2009; Rhodes et al., 2005). Importantly, there was no difference in the average amount of alcohol consumed between animals tested for behavior at 1-day (average= 4.06 ± 0.08 g/kg/2hr) versus 21-days withdrawal (average= 4.07 ± 0.08 g/kg/2hr) [$t(28)=0.304$, $p>0.05$]. There was no significant difference in body weight between alcohol- and water-drinking animals ($p> 0.05$).

3.3 Behavioral Results

3.3.1 Sucrose preference. No group differences in sucrose intake or preference were observed following the overnight sucrose preference test (2-way ANOVAs, *p's*>0.05).

3.3.2 Novel object test. Alcohol mice exhibited less exploration of a novel object

during withdrawal compared to Water controls. Alcohol mice also made fewer contacts with the object (Fig. 1A) [Treatment effect: $F(1,48)=12.74$, $p=0.001$] and spent less time interacting with the object (Fig. 1B) [Treatment effect: $F(1,44)=6.31$, $p=0.016$] during the 2-minute trial. Importantly, the main treatment effect was independent of withdrawal duration (for both variables, no withdrawal effects or interactions, *p's*>0.05), indicating that the hyper-anxious state persisted into protracted withdrawal. However, there were no significant group differences in the latency to first contact the novel object or in fecal count (data not shown; 2-way ANOVAs, all *p's*>0.05).

3.3.3 Elevated plus maze. Alcohol mice showed a significantly longer latency to first open-arm entry compared to Water controls, independent of withdrawal duration (Fig. 1C) [Treatment effect: $F(1,46)=7.60$, $p=0.008$; other *p*'s>0.05]. However, there were no group differences in the total number of open-arm entries, total entries, or total time spent in an open arm (data not shown; 2-way ANOVAs, *p's*>0.05).

3.3.4 Marble burying. Alcohol mice exhibited an increase in marble burying compared to Water controls, independent of withdrawal duration (Fig. 1D) [Treatment effect: $F(1,51)=13.24$, $p=0.001$; other *p's*>0.05]. However, there were no significant group differences in the latency to begin burying (data not shown; 2-way ANOVA, *p's*>0.05).

3.3.5 Light/dark shuttle box. Binge drinking influenced the number of entries into the light-side of a light/dark shuttle box, but the magnitude of this effect varied with

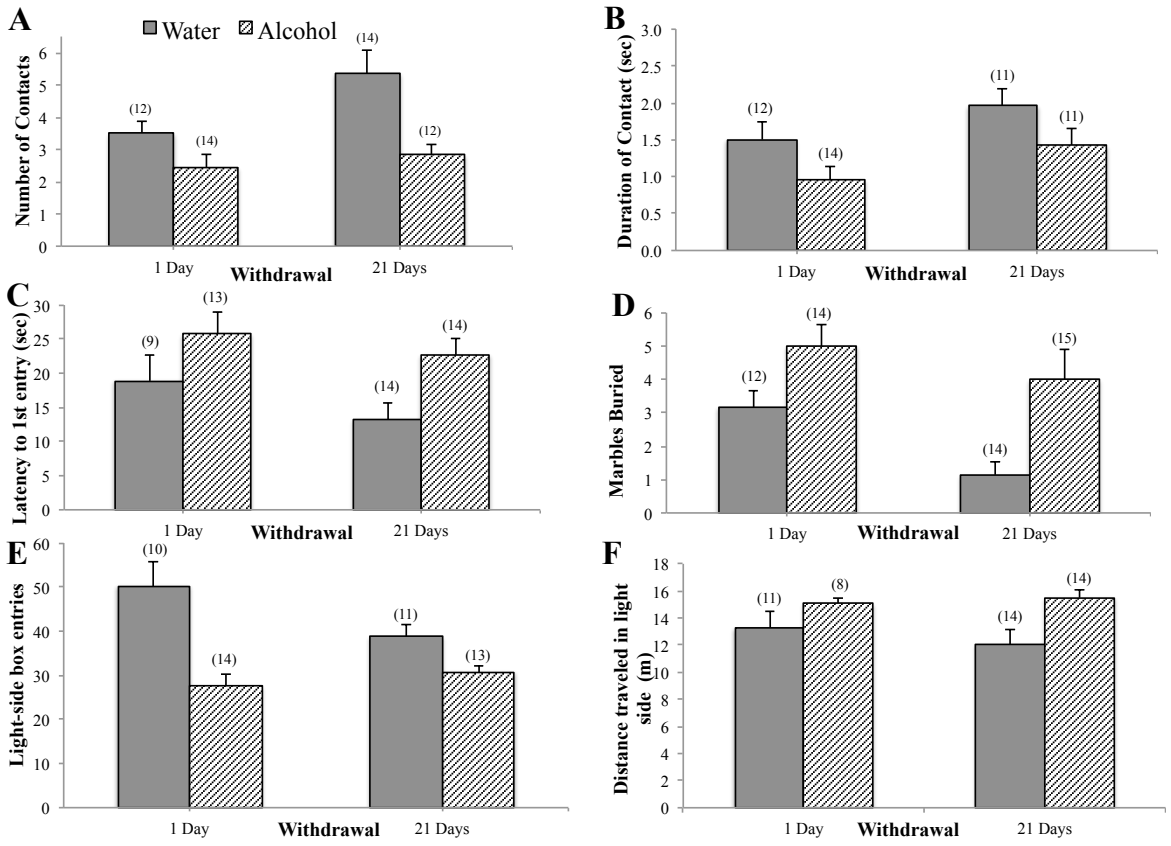

Figure 2.1. Withdrawal from binge drinking increases behavioral signs of anxiety across a variety of paradigms. Compared to water-drinking controls (Water), mice with a 30-day history of binge drinking (Alcohol): ***(A)*** made fewer contacts with the object and ***(B)*** spent less time interacting with the novel object in a 2-min novel object test. Alcohol mice also ***(C)*** exhibited a longer latency (in sec) to first enter into the open-arm in a 5-min elevated plus-maze test, ***(D)*** buried more marbles in a 20-min marble-burying test, ***(E)*** made fewer light-side box entries in a 5-min light-dark shuttle box test and ***(F)*** travelled a greater distance in the light side of the shuttle box. The heightened behavioral signs of anxiety were observed in Alcohol mice during both short- (1-day) and long-term (21 days) withdrawal. Data represent mean ± SEM of the number of animals indicated in parentheses. Each graph depicts a main effect of alcohol, $p < 0.05$ vs. water control.

withdrawal (Fig. 1E) [Treatment effect: $F(1,44)=22.27$, $p<0.001$; Treatment X Withdrawal: $F(1,44)=5.10$, $p=0.029$]. Deconstruction of the significant interaction for light-side entries along the Withdrawal factor indicated that the Alcohol mice made fewer light-side entries than Water controls at both withdrawal time- points [Short-term: $t(19)=3.742$, $p=0.001$; Long-term: $t(25)=2.496$, $p=0.023$], with a more prominent group difference during short-term withdrawal, as is apparent in Fig. 1E. While there were no time-dependent differences within the Alcohol mice [$t(22)=1.13$, $p>0.05$], Water mice showed a trend for a withdrawal effect [$t(22)=1.953$, $p=0.064$). This latter result was likely the source of the interaction revealed by the 2-way ANOVA. The cause of this trend is uncertain, but for the purposes of this study, it is most relevant that Alcohol mice made fewer light-side entries at both time points and their behavior was consistent between the time-points. Overall, Alcohol mice also traveled a greater distance in the light side compared to water controls (Fig. 1F) [Treatment effect: $F(1,43)=6.9$, $p=0.012$; interaction: $p>0.05$]. However, there were no group differences in the latency to first light-side entry or the total time spent in the light-side (data not shown; 2-way ANOVAs, *p's*>0.05).

3.3.6 Porsolt forced swim test. Alcohol mice exhibited a shorter latency to first float than Water mice during the 15-minute trial on test day 1 (Fig. 2A) [Treatment effect: $F(1,44)=10.38$, $p=0.002$]. However, there were no group differences in swimming, treading, or floating observed during this initial test (data not shown; 2-way ANOVAs, p's>0.05). During the 5-minute re-exposure test on day 2, Alcohol mice showed significantly more swimming (Fig. 2B) [Treatment effect: $F(1,45)=16.32$, $p<0.001$] and less treading than Water controls (Fig. 2C) [Treatment effect: $F(1,51)=5.77$, $p=0.02$]. Again, the treatment differences

did not vary as a function of withdrawal duration (no main effect or interaction, p's>0.05). There were no group differences in latency to first float or total number of floats on this re-exposure test (data not shown; 2-way ANOVAs, p's>0.05).

3.4. Correlational analyses between binge drinking and behavioral measures.

To determine whether or not the binge alcohol intake of the mice during the 30-day drinking period predicted the behavioral outcomes in our test battery, we conducted correlational analyses between the average alcohol intake (in g/kg) and each of our behavioral measures using a Bonferroni adjusted alpha level of 0.005 per test (0.05/10). Despite alcohol-exposed animals exhibiting greater indices of anxiety than water controls across a variety of measures (Figs. 1-2), the average amount of alcohol intake across the 30 days of drinking correlated with only a few behavioral outcomes (Fig. 3), likely due to the very low variability in intake. Nevertheless, higher alcohol intake was significantly correlated with the amount of swimming behavior during the re-exposure swim test (Fig. 3A) [$r(28)=0.56$, $p=0.006$] and there was a notable trend associating alcohol intake with the total number of marbles buried in the marble burying test (Fig. 3B) [$r(29)=0.46$, $p= 0.006$]. No other behavioral outcomes were correlated in a statistically significant manner with alcohol intake (*p's*>0.05; data not shown).

3.5 Immunohistochemistry

3.5.1 Amygdala. Overall, Alcohol mice showed significantly higher Egr1+ cell counts in the CEA than water controls (Fig. 4A,B) [main treatment effect: F(1,56)= 24.49,

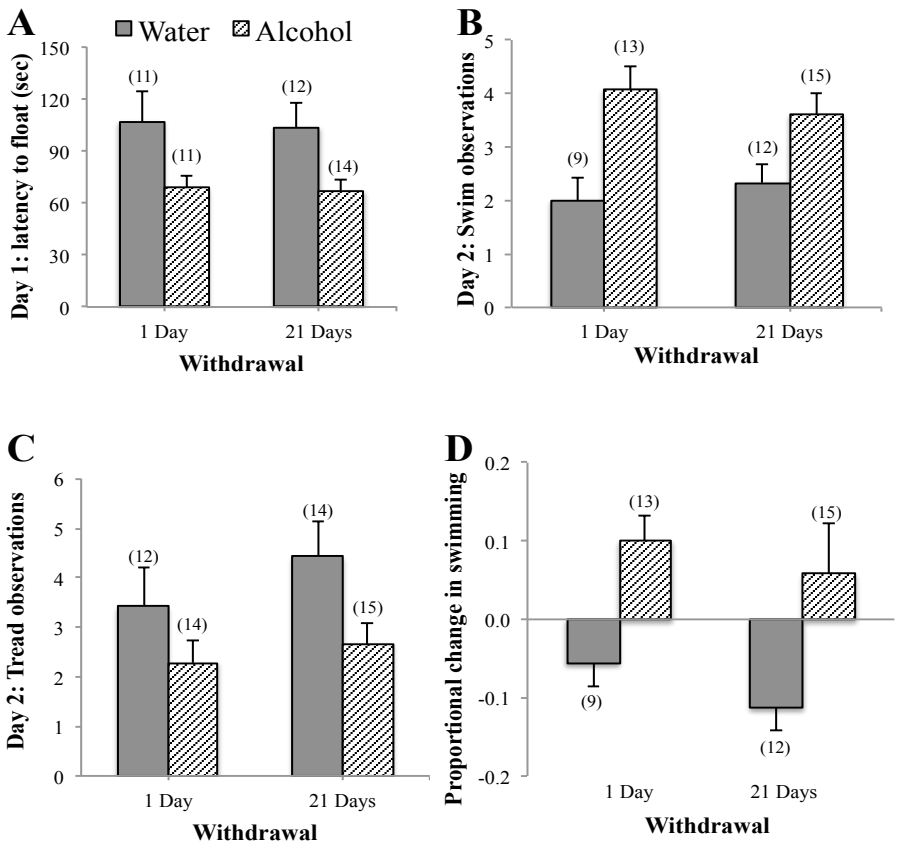

Figure 2.2. Withdrawal from binge drinking produces mixed effects on measures of behavioral despair. ***(A)*** During the first session of the Porsolt swim test, mice with a 30-day history of binge drinking (Alcohol) displayed a shorter latency to first float during the 15 min trial, compared to water-drinking controls (Water) and this effect was observed during both short- (1-day) and long-term (21 days) withdrawal. When assayed 24 hours later in a 5-min re-exposure test, mice with a history of binge drinking exhibited ***(B)*** more swimming behavior and ***(C)*** less treading behavior than water controls. ***(D)*** Alcohol mice also showed a significant increase in the proportion of swim observations between the 2 swim tests, while water-drinking mice displayed a test-dependent reduction in the proportion of swim observations. Data represent mean ± SEM of the number of animals indicated in parentheses. Each graph depicts a main effect of alcohol, $p < 0.05$ vs. water control.

p<0.001; n=8/group]. This alcohol effect was independent of behavioral testing as indicated by no main Testing effect or interactions with the Testing factor (*p*'s>0.05). As behavioral testing did not influence the number of Egr1+ cell counts, the elevated cellular activity within the CEA of Alcohol mice was likely due to their history of binge drinking and not a drinking-induced vulnerability to the cellular effects of testing stress. The alcohol-induced increase in Egr1 staining was present in the CEA as early as 24 hrs into withdrawal and persisted unchanged for at least 22 days into withdrawal, as indicated by no main Withdrawal effect or interactions with the Withdrawal factor (Fig. 6A; *p's*>0.05). In contrast to the CEA, there were no significant effects of binge drinking, behavioral testing, or withdrawal duration on Egr1+ cell counts in the BLA (Fig. 4D,E; 3-way ANOVA, *p*'s>0.05).

3.5.2 BNST. As observed in the CEA (Fig. 6)], overall, there were significantly more Egr1+ cells in the dorsal BNST of binge-drinking versus water-drinking animals (Fig. 5A,C) [main treatment effect: $F(1,40)=28.95$,$p<0.001$; n=6/group]. However, in contrast to the CEA, we observed a significant interaction between treatment and behavioral testing with respect to Egr1+ cell counts in the BNST [treatment by testing interaction: $F(1, 40)=5.53$, $p=0.02$], indicating that the binge alcohol-water differences in cellular activity within this region depended upon behavioral testing. Post-hoc t-test comparisons of tested versus untested animals separately for each withdrawal time-point confirmed that a history of binge drinking increased cellular activity in the BNST, regardless of behavioral testing, although the magnitude of the treatment difference was greater in the behaviorally tested animals (Fig. 5B) [no testing: $t(22)=2.29$, $p=0.03$; testing: $t(22)=4.87$, $p<0.001$]. While inspection of Fig. 5B suggested opposite influences of behavioral testing procedures upon the number of

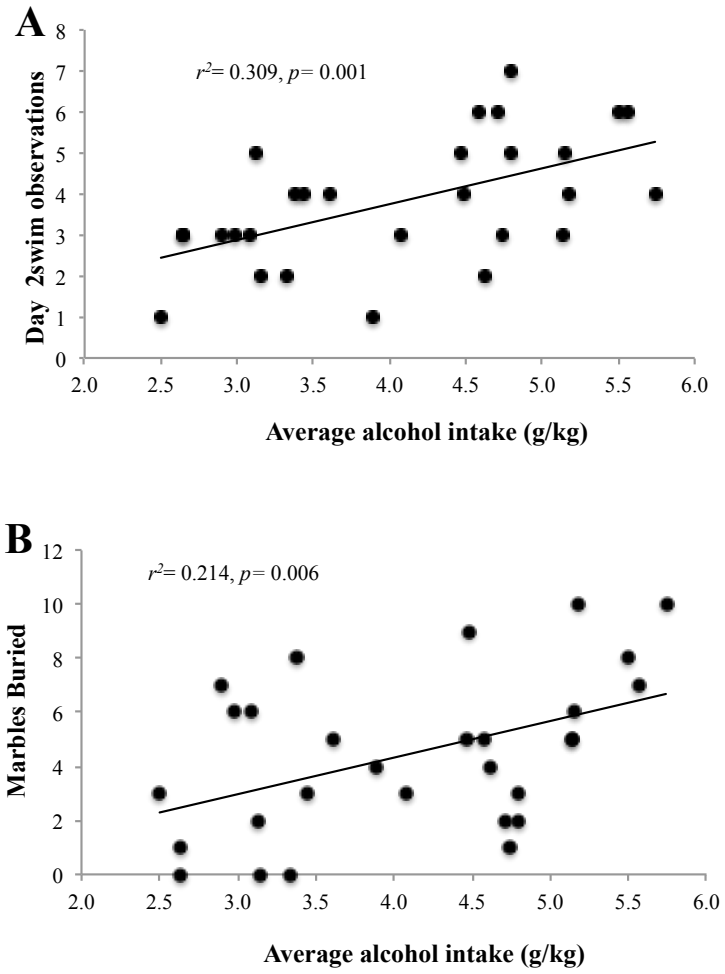

Figure 2.3. Binge alcohol intake correlates with the intensity of behavioral signs of negative affect in withdrawal. Using a Bonferroni adjusted alpha of .005, the correlational analyses between the average total amount of alcohol intake exhibited by the B6 mice across the 30-day drinking period and our behavioral measures of anxiety and behavioral despair revealed a significant correlation with ***(A)*** the swimming behavior on day 2 of the forced swim test. ***(B)*** There was also a notable trend associating alcohol intake with the total number of marbles buried. For sufficient statistical power, the data were collapsed across both withdrawal time-points for this analysis.

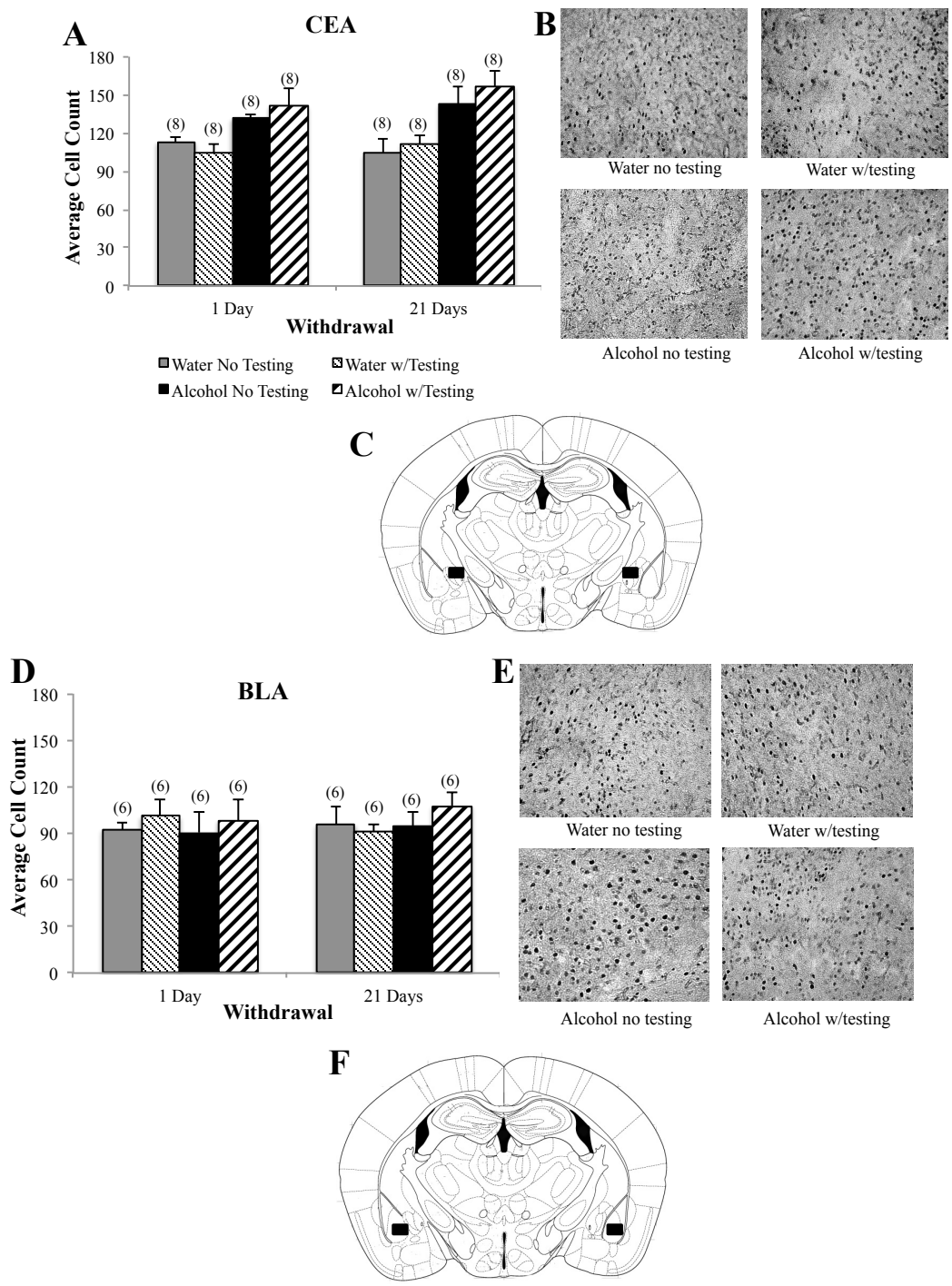

Figure 2.4. Subregional differences in the effects of withdrawal from binge drinking upon Egr1 expression in the amygdala. ***(A)*** Summary of the average Erg+ cell counts within the CeA exhibited by water-drinking controls (Water) and B6 mice with a 30-day

history of binge drinking (Alcohol) during short-term (1-day) and long-term (21 days) withdrawal. Compared to Water mice, Alcohol mice exhibited higher Egr1+ cell counts in the CEA, irrespective of the withdrawal time-point or whether or not the mice were subjected to our behavioral test battery (Testing vs. No Testing) prior to tissue collection. ***(B)*** Representative micrographs of Egr1 immunostaining within the CeA of the four treatment groups, indicating greater Egr1 immunostaning in Alcohol versus Water mice. ***(C)*** Schematic illustrating a coronal section through the amygdala, highlighting (black square) the size and location of the sampling region used to assay the number of Egr1+ cells within the CeA. ***(D)*** In contrast to the CeA, Egr1 expression within the BLA was unaffected by either alcohol treatment or behavioral testing. ***(E)*** Representative micrographs of Egr1 immunostaining within the BLA indicating comparable immunostaining within the four treatment groups. ***(F)*** Schematic illustrating a coronal section through the amygdala, highlighting (black square) the size and location of the sampling region used to assay the number of Egr1+ cells within the BLA. The data in panels A and D represent mean ± SEM of the number of animals indicated in parentheses.

Egr1+ cells within the BNST of alcohol-exposed (increase) and water-exposed (decrease) mice, t-test comparisons of tested versus untested animals for each treatment group separately indicated that the effects of behavioral testing were not statistically reliable [1 day: t(10)=1.90, p= 0.11; 21 days: t(10)=0.93, p= 0.38]. Also in contrast to the CEA (Fig. 4A), Egr1+ cell counts within the BNST were higher overall at 21 days versus 1 day withdrawal (Fig. 5A) [main time effect: F(1,40)=16.78, p<0.001]. This time-dependent difference in cell activity was independent of both treatment group and behavioral testing (no interactions with either factor, *p's*>0.05) and, thus, may reflect some age-related change in BNST activity.

3.5.3 Acb. Unlike the CEA and BNST, we failed to detect any influence of prior alcohol experience upon Egr1 expression within either the AcbC or AcbSh (Fig. 6; no main effects of, or interactions with, the treatment factor, *p's*>0.05]. Moreover, we failed to detect any withdrawal-dependent change in Egr1+ cell counts within either subregion (no main effects of, or interactions with, the time factor, *p's*>0.05). However, we did detect significant main effects of behavioral testing upon Egr1 levels in both the AcbSh (Fig. 6A,B) [F(1,36)=5.28, p=0.03; n=6/group] and the AcbC (Fig. 6D,E) [F(1,40)=8.01, p=0.007; n=6/group]], with behaviorally tested animals exhibiting higher Egr1+ cell counts than untested controls, regardless of drinking history or withdrawal duration (no interactions with the testing factor, *p's*>0.05).

3.6 Correlational Analyses of Egr1 Expression Between Brain Regions of Interest.

Within-subjects correlational analyses were conducted using a Bonferroni adjusted alpha of 0.008 (0.05/6) to compare Egr1 activation in the CEA, BNST, AcSh, and AcbC to

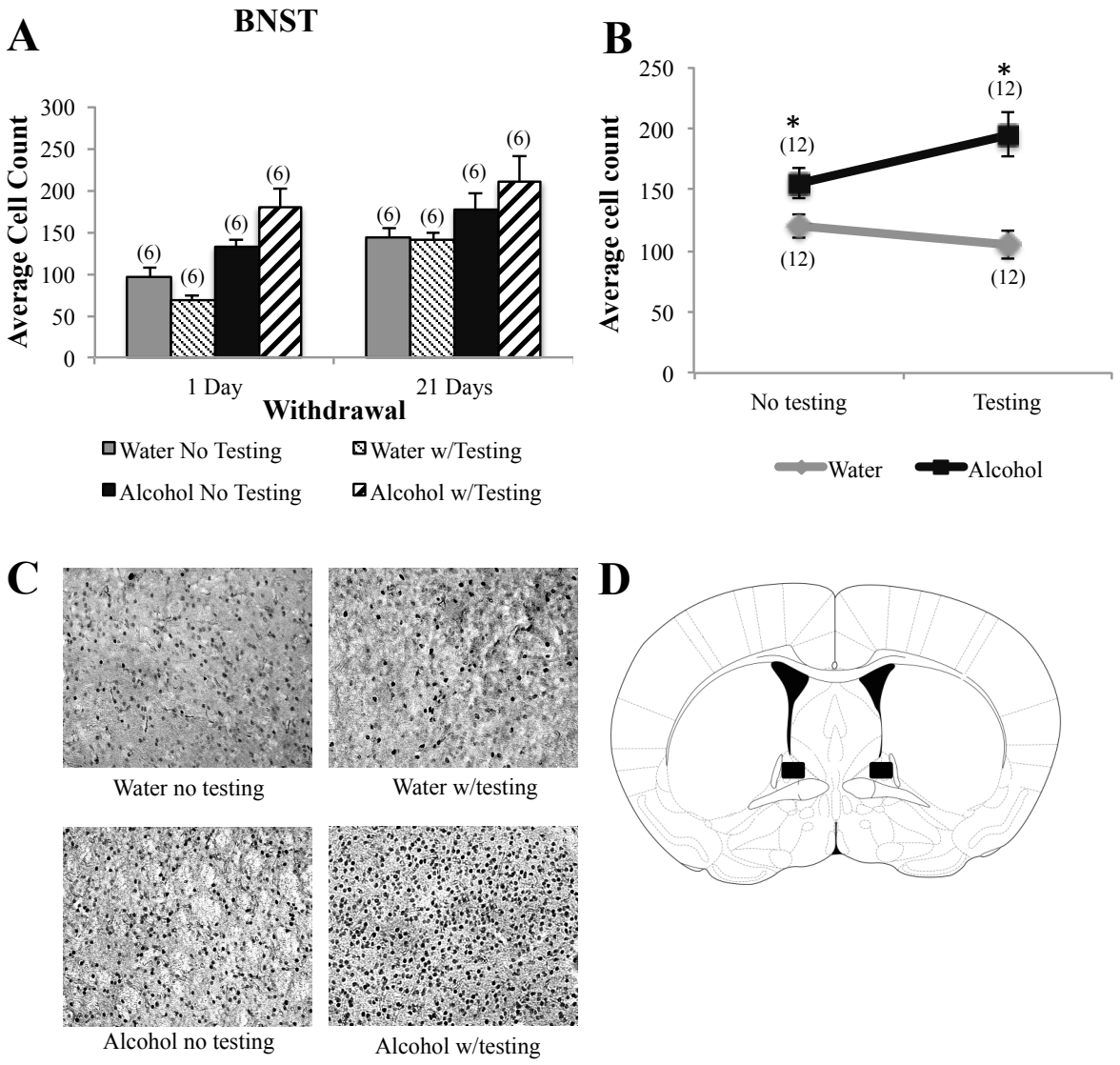

Figure 2.5. Egr1 expression in the BNST depends upon treatment and testing. ***(A)*** Compared to water controls (Water), B6 mice with a 30-day history of binge drinking (Alcohol) exhibited higher Egr1+ cell counts in the BNST. Overall, animals had higher Egr1 expression during long-term withdrawal (21 days), compared to short-term withdrawal (1 day). ***(B)*** Graphical depiction of the significant interaction effect between treatment group and behavioral testing collapsed across both withdrawal time points, illustrating that behavioral testing exerted a greater stimulatory effect on Egr1 expression in Alcohol mice

compared to Water controls. The data in panels A and B represent mean ± SEM of the number of animals indicated in parentheses. *Denotes treatment effect, $p<0.05$. ***(C)*** Representative micrographs of Egr1 immunostaining within the BNST of the four treatment groups, indicating the combinatorial effect of binge drinking history and behavioral testing upon Egr1 immunostaining within the BNST. ***(D)*** Schematic illustrating a coronal section through the BNST, highlighting (black square) the size and location of the sampling region used to assay the number of Egr1+ cells.

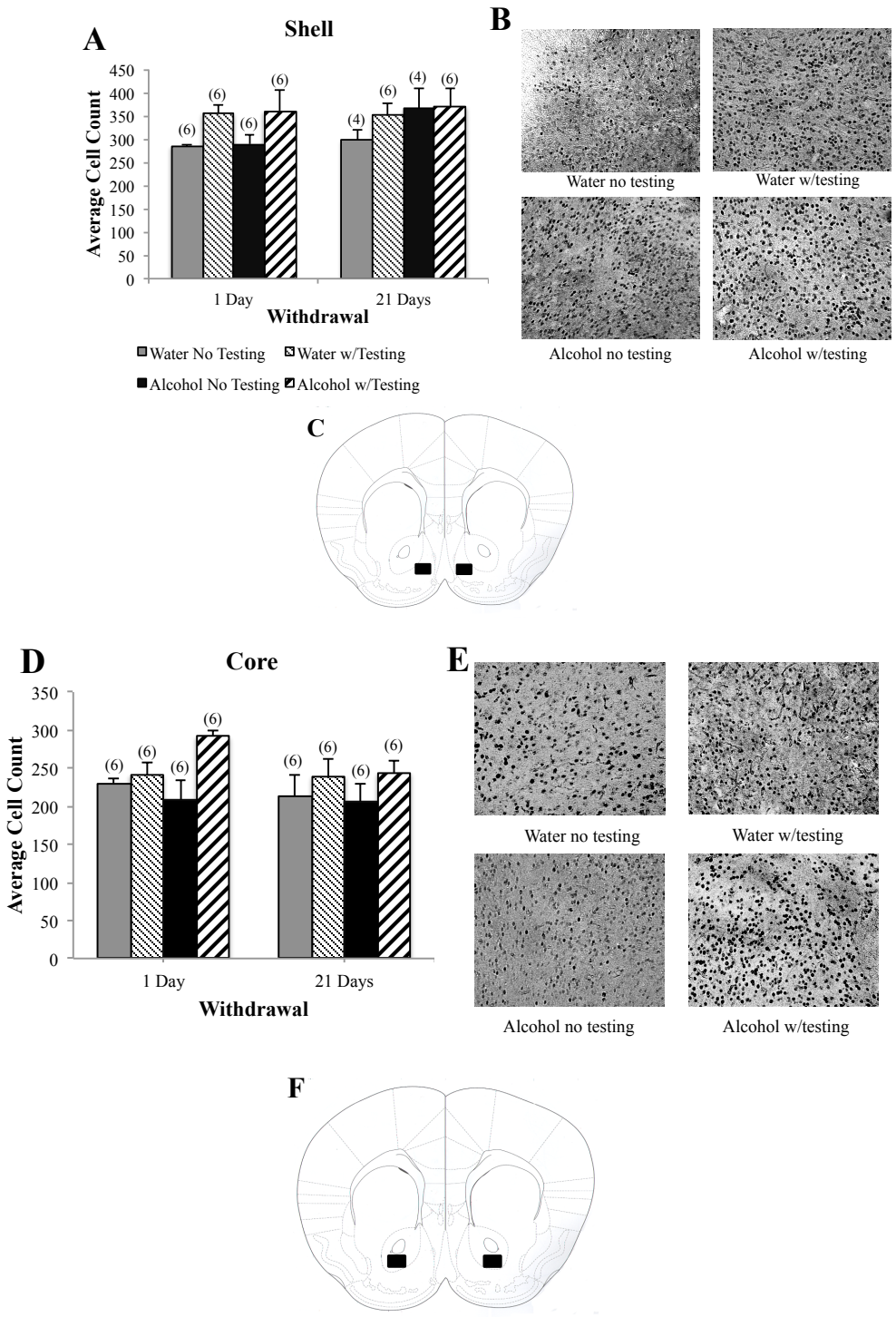

Figure 2.6. Egr1 expression within the Acb is regulated by behavioral testing. ***(A)*** Both water-drinking controls (Water) and mice with a 30-day history of binge drinking (Alcohol)

exhibited higher Egr1+ cell counts within the AcbSh if they underwent behavioral testing procedures (Testing) and this main effect of the behavioral testing was apparent in animals during both short- (2-day) and long-term (22 days) withdrawal. ***(B)*** Representative micrographs of Egr1 immunostaining within the AcbSh of the four treatment groups, indicating higher immunostaining in behaviorally tested mice. ***(C)*** Schematic illustrating a coronal section through the striatum, highlighting (black square) the size and location of the sampling region used to assay the number of Egr1+ cells within the AcbSh. ***(D)*** A similar pattern of group differences was observed within the AcbC. ***(E)*** Representative micrographs of Egr1 immunostaining within the AcbC of the four treatment groups, indicating higher immunostaining in behaviorally tested mice. ***(F)*** Schematic illustrating a coronal section through the striatum, highlighting (black square) the size and location of the sampling region used to assay the number of Egr1+ cells within the AcbC. The data in panels A and D represent mean ± SEM of the number of animals indicated in parentheses.

examine for inter-region relations in Egr1 levels. This analysis was conducted across all animals for which we obtained tissue sections from all five regions of interest, irrespective of drinking history or behavioral testing in order to obtain sufficient statistical power. We observed a significant correlation for Egr1+ cell counts between the AcbC and AcbSh (Fig. 7A) [$r(34)= 0.455$, $p= 0.006$] and a positive trend between cell counts within the CEA and those within the BNST (Fig. 7B) [$r(33)= 0.412$, $p= 0.017$]. However, Egr1+ cell counts within the CEA and BNST were not correlated with those observed in either the AcbC or AcbSh (*p's*>0.008).

3.7. Correlational analyses between binge alcohol intake and Egr1 expression.

To determine whether or not a relation existed between alcohol intake during the 30-day drinking period and the activational state of our brain regions of interest and drinking behavior, we also conducted within-subjects correlational analyses between the average alcohol intake and Egr1+ cell counts within the CEA, BLA, BNST, AcbSh and AcbC. Using a Bonferroni adjusted alpha of 0.01 (0.05/5), we found that alcohol intake was positively correlated with Egr1+ cell counts only within the BNST (Fig. 10A) [$r(31)=0.462$, $p=0.009$]. Egr1+ cell counts within other regions were not significantly correlated with alcohol intake [*p*'s> 0.05].

3.8. Correlational Analyses between Egr1 expression and behavioral measures.

To determine whether or not a relation existed between the activational state of our brain regions of interest and negative affect, we also conducted within-subjects correlational

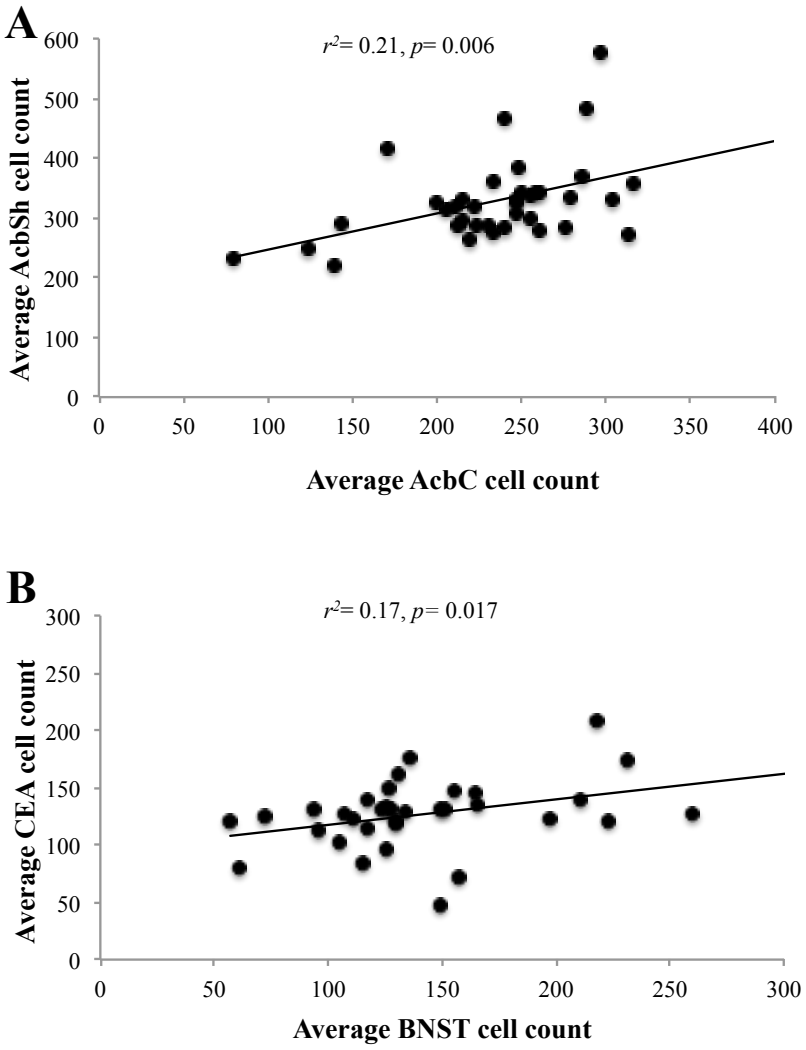

Figure 2.7. Egr1 activation is correlated across multiple extended amygdala structures. Correlational analyses were conducted between the average number of Egr1+ cell counts observed within our brain regions of interest to examine for concordant activation within these structures. ***(A)*** Concurrent activation was also observed between the AcbC and AcbSh. ***(B)*** Egr1 activation within the CEA showed a positive correlational trend with activation in the BNST. The statistical results of the analyses are represented in each individual panel.

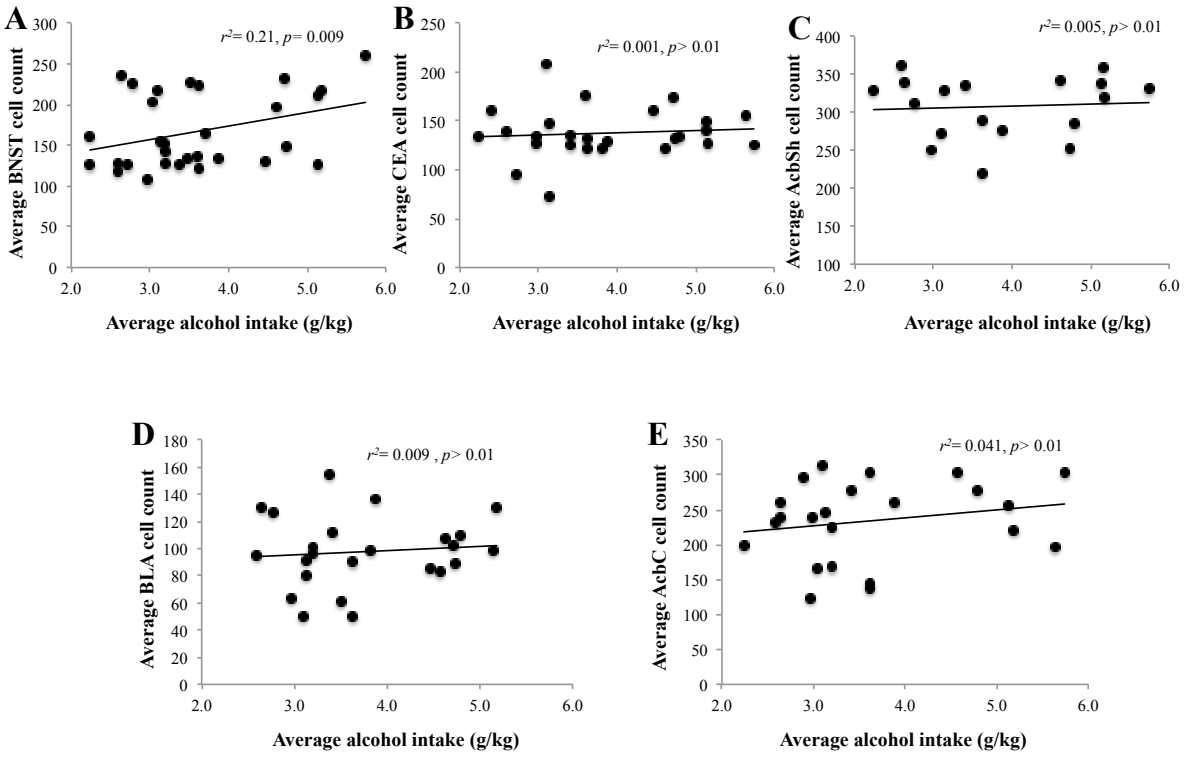

Figure 2.8. Binge alcohol intake relates to the intensity of Egr1 activation within BNST. The results of correlational analyses between the average total amount of alcohol intake exhibited by the B6 mice across the 30-day drinking period and the number of Egr1+ cell counts within ***(A)*** BSNT, ***(B)*** CeA, ***(C)*** AcbSh, ***(D)*** BLA, and ***(E)*** AcbC revealed a significant predictive relationship for the BNST only, accounting for approximately 20% of the variation in Egr1 activation. For sufficient statistical power, the data were collapsed across both withdrawal time-points for this analysis. The data represent the data from 22-31 mice and the statistical results are presented in each individual panel.

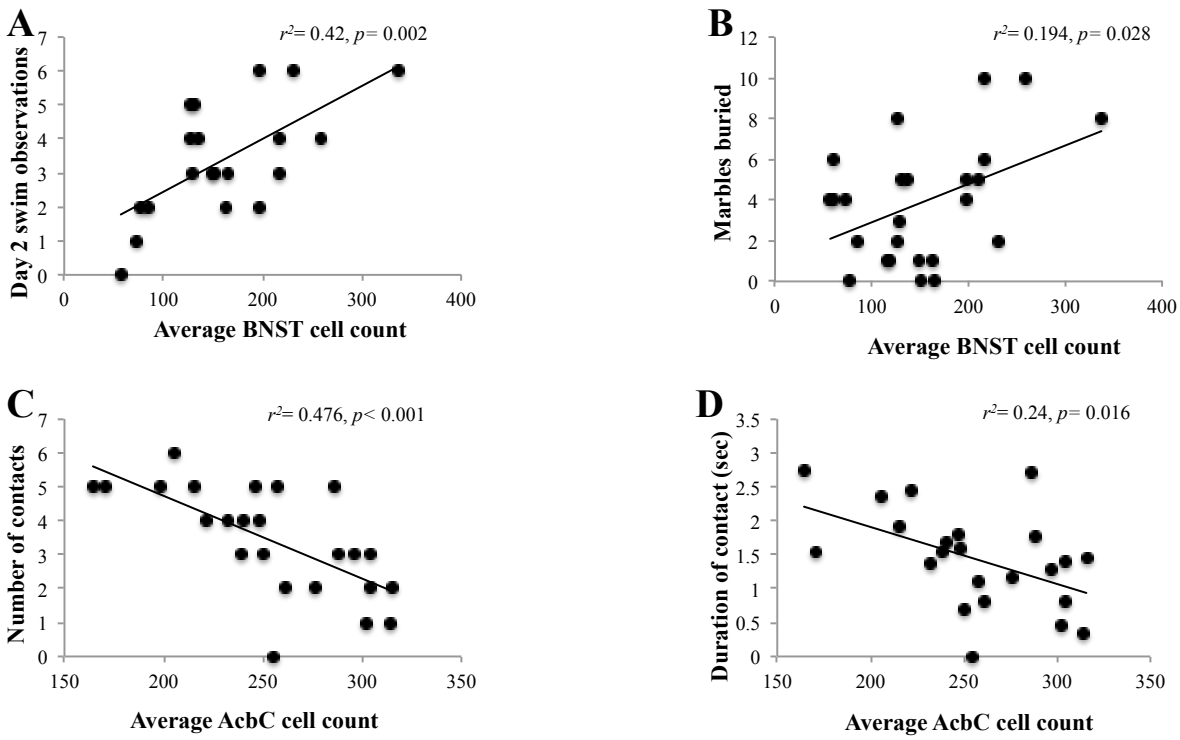

Figure 2.9. Activation of extended amygdala structures predicts behavioral indices of anxiety. ***(A-B)*** The statistically significant results of correlational analyses between different behavioral results from our test battery and the number of Egr1+ cell counts within BSNT. ***(C-D)*** The statistically significant results of correlational analyses between different behavioral result from our test battery and the number of Egr1+ cell counts within the AcbC. The data represent the data from 18-24 mice and the statistical results are presented in each individual panel.

analyses between the average Egr1+ cell counts within the CEA, BLA, BNST, AcbSh and AcbC and our different behavioral outcomes from the test battery using a Bonferroni adjusted alpha of 0.005 (0.05/10) for each region. Analyses indicated a strong positive relation between Egr1+ cell counts within the BNST and the number of swim observations during the re-exposure test (Fig. 9A) [$r(20)=0.65$, $p=0.002$] and a positive trend between BNST Egr1+ cell count and the number of marbles buried (Fig. 9B) [$r(25)=0.44$, $p=0.028$]. In contrast to the BNST, there were no predictive relations between Egr1 expression within the CEA or BLA and any of our behavioral measures (data not shown). In the novel object test, analyses indicated a negative relationship between cellular activation within the AcbC and the number of object contacts (Fig. 9C) [$r(24)=-0.69$, $p<0.001$] and a negative trend with total contact duration (Fig. 9D) [$r(23)=-0.49$, $p=0.016$]. There were no significant relations observed between Egr1+ cell counts within the AcbSh and any of our behavioral measures (data not shown).

4. Discussion

Many alcoholics report that anxiety reduction is a key motivator for drinking (Schuckit & Hesselbrock, 1994). Therefore, increased anxiety during withdrawal serves as a compelling source of negative reinforcement that substantially increases the likelihood of relapse during abstinence from chronic alcohol abuse (Kushner et al., 2000). Individuals who regularly engage in heavy episodic drinking do not necessarily meet the diagnostic criteria for alcoholism (Centers for Disease Control and Prevention, 2013); however, frequent binge drinking is a significant risk factor for the development of alcoholism and can produce symptoms during withdrawal similar to those seen with alcohol dependence (Hasin et al.,

2007; Substance Abuse and Mental Health Services Administration (SAMHSA), 2012). Herein, we show that withdrawal from a chronic (30-day) history of binge drinking increases a number of behavioral indices of negative affect, most notably anxiety-related measures, in mice. Moreover, the negative affect observed in our binge-drinking mice manifested as early as 24 hrs into withdrawal and persisted, unchanged, for at least 3 weeks following the last drinking episode. These data demonstrate that repeated bouts of alcohol intake under limited access conditions produces enduring changes in emotionality. Thus, the dysphoria caused by cessation of binge drinking likely promotes re-engaging in the behavior to alleviate these aversive symptoms and perpetuation of this binge-withdrawal cycle likely contributes to the transition from binge drinking to alcoholism.

4.1 Withdrawal from binge drinking elevates indices of anxiety

Although it was unfortunate and suboptimal that we were unable to analyze BACs for these animals due to equipment malfunction, there is precedent for estimating resultant BAC based on intake levels (Cozzoli et al., 2015; Gorin-Meyer et al., 2007; Rhodes et al., 2005). Additionally, alcohol consumption was sufficient to elicit signs of behavioral dysregulation during withdrawal- which was the primary focus of this study. For example, this 'binge-like' alcohol consumption produced anxiogenic effects during withdrawal, as evidenced by treatment-dependent differences in behavior on a variety of tests. For one, alcohol-drinking animals showed greater reluctance to interact with a novel object compared to water controls, suggesting heightened levels of neophobia-induced anxiety (Dulawa et al., 1999; Misslin & Ropartz, 1981). Consistent with this, Alcohol mice exhibited increased defensive burying in the marble burying test and this result was correlated with average alcohol intake during the

30-day drinking period. These present results are in line with a previous study in rats showing decreased interaction with a novel object during the first 10-30 hrs of withdrawal from a 4-day alcohol liquid diet (7-13 g/kg/day) (Knapp et al., 1993). However, in that previous study, the hyper-anxious effect dissipated by 70 hrs into withdrawal, while in the present study, the hyper-anxious state of the binge drinking mice persisted for at least 21 days. Although there are a number of different procedural variables between the present study and that prior (Knapp et al., 1993) (including species, adulteration of alcohol, daily duration of alcohol access, number of drinking days), we argue that the relatively brief duration of alcohol exposure in the prior rat study (4 days) was likely insufficient to elicit the enduring behavioral changes observed following more chronic drinking procedures.

Arguably, the longer latency to first open arm entry displayed by Alcohol mice on the EPM is not likely physiologically relevant nor sufficient to suggest a hypersensitivity to the aversive properties of open/brightly-lit areas. However, more meaningful evidence of an increased anxiogenic response to these aversive properties was observed in the light/dark shuttle box test, with alcohol-drinking mice exhibiting fewer light-side entries at both withdrawal time-points. Alcohol-drinking mice traveled a greater distance in the light side, compared to water controls, despite their fewer entries. As there were no group differences in the total time spent in the light side, these data suggest that binging mice exhibited greater hyperactivity during each entry into this aversive environment, compared to water-drinking animals. Although debate exists in the literature pertaining to the interpretation of locomotor activity in aversive environments (Hilakivi et al., 1989; Kliethermes, 2005; Rasmussen et al., 2001; Wright et al., 1991); independent of this interpretational challenge, both hypo- and

hyperactive psychomotor dysregulation are symptoms reported in humans during alcohol cessation (Saitz & O'Malley, 1997), as well as in those suffering from anxiety disorder (Tasman et al., 2015, pp. 1076-1080) and certain depression subtypes (Sobin & Sackeim, 1997). Thus, our data for both neophobia and motor hyperactivity are consistent with, and extend, extant data from humans and laboratory animals to a murine model of binge alcohol drinking and provide further predictive validity for our modified DID procedure as a model of alcohol dependence.

4.2 Withdrawal from binge drinking produces little change in indices of depression

This study found limited evidence of withdrawal-induced depressive symptoms, as indicated by a shorter latency to float in the Porsolt forced swim test for alcohol-drinking mice on the first, but not on the second, day of testing. While a decreased latency to float and increased floating behavior are both traditionally considered indicative of greater behavioral despair in this paradigm (Porsolt et al., 2001), no Alcohol-Water differences were noted for the latency to first float during the 5-minute re-exposure to the pool on day 2 of testing and alcohol-drinking mice showed significantly more swimming and less treading than water controls on this re-exposure test. In considering the traditional interpretation of floating behavior in the forced swim test as reflecting behavioral despair, these between-test results seem counterintuitive given the negative affective state manifested by Alcohol mice in the aforementioned anxiety tests. However, one can reconcile this apparent discrepancy in findings by considering the adaptive value of conserving energy by floating when re-exposed to a previously inescapable swim stressor. In this regard, the increase in floating/reduced swimming exhibited by water controls during the re-exposure test may reflect a more

adaptive and appropriate response upon recall of the futility of their prior escape efforts. By this rationale, the increase in swimming exhibited by alcohol-drinking mice during the re-exposure test appears to be an anomalous, potentially maladaptive response. This could indicate some sort of memory impairment (Depablo et al., 1989; West, 1990) or perhaps a sensitized behavioral response to a previously stressful situation that might reflect heightened anxiety (Blanchard et al., 2001; Foa et al., 1992; Polani, 2004). A memory deficit does not seem as likely, since the Alcohol animals increased their swimming behavior as a function of testing. Given the aforementioned evidence that a history of binge drinking produces a persistent increase in anxiety, Alcohol mice may be more sensitive to inescapable stressor-induced behavioral sensitization, particularly given that the increase in swimming during the swim re-test was positively correlated with the amount of alcohol intake. These interpretations are purely speculative and the impact of binge drinking history upon the subsequent development of repeated stressor-induced behavioral sensitization requires more direct examination.

Although group differences were noted in the Porsolt swim test, we failed to detect alcohol-water differences in the sucrose preference test – a test widely employed for assessing depression-related anhedonia in rodents (Katz, 1982). However, our data showed only a trend for a subject factor interaction at 1 day withdrawal, but no influence of prior binge history upon this measure in protracted withdrawal. Many studies have shown a relationship between alcohol consumption and increased preference for sweet solutions in both laboratory animals (Gosnell & Krahn, 1992; Stewart et al., 1994) and humans (Kampov-Polevoy et al., 1997; Kranzler et al., 2001), which may be especially prevalent in early withdrawal. Although the elevated sucrose preference in the AD animals at 1-day withdrawal

did not quite reach statistical significance, AD animals failed to exhibit depression-related behavior in this assay. While it is possible that a lack of significant group differences may reflect insensitivity of the test as conducted, taken together, the results of both the forced swim and sucrose preference tests argue a more robust effect of binge drinking history upon anxiety versus depressive measures.

4.3 Withdrawal from binge drinking increases cellular activity in the CEA, but not the BLA

Using the transcription factor Egr1 to index cellular activity (Herdegen & Leah, 1998), we observed elevated Egr1 in the CEA during withdrawal from binge drinking that was independent of withdrawal duration or behavioral testing. These findings argue that a history of binge drinking is sufficient to produce an enduring increase in the activity of this extended amygdala structure and are consistent with its role in regulating excessive drug taking (Cozzoli et al., 2015; Gilpin & Roberto, 2012; Liu et al., 2011; Lowery-Gionta et al., 2012). Despite the robust effects of withdrawal from binge drinking upon the cellular activity in the CEA, we failed to detect significant changes in Egr1 levels within the adjacent BLA- a finding consistent with other studies showing a region-specific role of the amygdala in underpinning alcoholism-related behaviors. For example, experimental CEA lesions significantly reduce the anxiogenic effects of restraint stress and also decrease voluntary consumption in rats, while BLA lesions produce no change in these behaviors (Summarized in Moller et al., 1997). Moreover, consistent with the present findings for Egr1, a chronic history of alcohol drinking, under either continuous access (Obara et al., 2009) or our modified DID binge procedures (Cozzoli et al., 2014), increases protein indices of

glutamatergic signaling in the CEA, but not in the BLA. While we have yet to assay for the long-term glutamatergic consequences of a chronic history of binge drinking upon subregional differences in glutamate protein expression within amygdala, the up-regulation of glutamate-related protein expression within the CEA persists for at least 4 weeks into alcohol withdrawal in rodents consuming alcohol under continuous access conditions (Cozzoli et al., 2014; Obara et al., 2009). Notwithstanding the possibility that a chronic history of binge drinking changes GABA, glucocorticoid, or other neuropeptide function within the CEA (as reported to occur in rodents withdrawn from high-dose, non-contingent, alcohol vapor exposure (Roberto et al., 2012)), the extant immunoblotting literature (Cozzoli et al., 2014), coupled with the present results for Egr1 expression provide descriptive evidence that a history of binge drinking produces enduring increases in the activity of the CEA, which might reflect a disruption of the balance between inhibitory and excitatory signaling within this amygdala subregion to elicit withdrawal-induced dysphoria.

4.4 Withdrawal from binge drinking increases cellular activity in the BNST

The BNST exhibits reciprocal interconnections with the CEA (Dong et al., 2001) and consistent with this neuroanatomical connectivity, we observed a similar alcohol withdrawal-induced increase in Egr1 expression in the BNST, as that observed within the CEA and a predictive relation existed between Egr1 expression between these 2 regions. Additionally, the amount of alcohol intake exhibited by the mice during the 30-day drinking period was positively correlated with the BNST level of Egr1. The BNST exhibits hyper-excitability in response to both acute and repeated stress, as indicated by evoked c-fos induction (Funk et al., 2006; Kovacs, 1998). Consistent with stressor-induced activation of

the BNST, BNST Egr1 levels were positively correlated with behavioral reactivity in the forced swim test. However, in contrast to the CEA, the withdrawal-induced increase in the number of BNST Egr1+ cells was greater in animals subjected to our behavioral test battery than in those that were test-naïve. Thus, factors associated with behavioral testing have a combinatorial effect with alcohol-withdrawal upon the cellular activity of this region and it remains to be determined whether or not the influence of behavioral testing upon BNST Egr1 levels reflects the stressful nature of the behavioral test (and thus, a stressor response) and/or the motor activity associated with exploration/attempts to escape during testing. Nevertheless, it is clear from the present data that, akin to results derived from more conventional models of alcoholism, a chronic history of binge drinking increases the basal activity of BNST neurons and also renders them more sensitive to the effects of stressors on cellular activity within this region. Given prior functional evidence that the BNST regulates alcoholism-related behaviors (Kash, 2012; Olive et al., 2002; Silberman & Winder, 2013), the hyper-reactivity of the BNST observed herein likely contributes heavily to the behavioral manifestation of anxiety during withdrawal from binge drinking and the continued propensity to binge drink when alcohol is next presented.

4.5 Withdrawal from binge drinking does not affect cellular activity within the Acb.

The Acb serves as an interface between limbic structures that process emotion and motivation and motor regions governing approach/avoidance behaviors. The AcbSh and AcbC are both anatomically and functionally distinct (Ito & Hayen, 2011), although both Acb subregions have been implicated in governing different aspects of alcohol reward and alcohol reward-related learning (Chaudhri et al., 2010) and prior immunoblotting studies

indicated changes in excitatory neurochemistry within these subregions in alcohol-experienced animals (Cozzoli et al., 2012; Cozzoli et al., 2009; Lum et al., 2014; Obara et al., 2009; Szumlinski, Ary, Lominac, et al., 2008). Further, dysphoric states during withdrawal are mediated by changes in Acb neurotransmission, with alcohol-induced reductions in monoaminergic function theorized to contribute to the depressive effects of alcohol withdrawal.(Cozzoli et al., 2009; Karkhanis et al., 2015; Szumlinski, Ary, Lominac, et al., 2008), Moreover, we know that a chronic history of binge drinking elevates indices of glutamatergic signaling within the Acb (Cozzoli et al., 2012; Lum et al., 2014). Thus, we hypothesized at the outset of this study that a history of binge drinking would augment Acb cellular activity, particularly within the AchSh, and this increased activity would correlate with behavioral indices of negative affect. However, (1) we failed to detect any alcohol-dependent changes in Egr1 expression within either Acb subregion and (2) binge-drinking history did not relate to Egr1 expression, irrespective of subregion. However, when all mice were considered collectively, Egr1 expression within the AcbC, but not the AcbSh, was associated with higher indices of anxiety in the novel object and elevated plus-maze tests and the causative nature of this relation requires further investigation. Interestingly, Egr1 expression within Acb subregions was up-regulated by behavioral testing and there was no subregional distinction in this regard.. Moreover, when all mice were considered collectively, Egr1 expression within the AcbC was associated with higher indices of anxiety in the novel object and elevated plus-maze tests. Thus, cellular hyperactivity was not restricted to the AchSh subregion integrated within the extrahypothalamic stress circuit. Whether or not this observation reflects the motor demand of the swim test conducted just prior to tissue collection, recollection of the prior swim test (or both) cannot be discerned in the present

study. However, the fact that Egr1 cell counts in the shell and core subregions were similarly increased in both alcohol-experienced and –naïve mice is consistent with the notion that the Acb integrates motivationally relevant stimuli with motor output (Cardinal et al., 2003), but does not provide any support for the regulation of this capacity by prior alcohol experience/withdrawal or for increased cellular activity within Acb subregions as requisite for the manifestation of negative affect during alcohol withdrawal. Across all the animals, the positive correlation between AcbC and ACbSh activation does suggest that these regions are activated concurrently when mice are exposed to an inescapable stressor, which again, is in line with the nature of the behavioral test conducted just prior to tissue collection. While the AcbSh is part of the extended amygdala/extrahypothalamic stress subcircuit and is modulated by BNST's projection to the VTA (Silberman et al., 2013), the discrepancies in the patterns of Egr1 expression observed across the CEA, BSNT and AcbSh argue that the entire extended amygdala does not activate in unison during the behavioral manifestation of alcohol withdrawal-induced negative affect and/or concurrent activation between these structures is not necessary for the manifestation of alcohol withdrawal-induced anxiety. Additionally, it is important to note that although we found significant correlations between measures of negative affect, Egr1 expression, and alcohol intake, the r^2 values ranged from 0.17-0.47. This suggests that while these factors may be statistically related, there is still a great deal of variability that is not accounted for and we are not claiming that any of these factors are the sole determinants of their covariates. Nonetheless, these correlations are of interest in understanding the relationship between alcohol intake, withdrawal-induced negative affect, and subcortical activation.

4.6 Proposed mechanisms underpinning the persistent negative affective state produced by alcohol withdrawal

Immediate early genes (IEGs) such as Egr1 participate in a transcription-level cellular response that is activated transiently and rapidly in response to a wide variety of stimuli. IEGs are a non-specific index of cellular activity and as such, increased levels of Egr1+ cells cannot be used to determine the precise nature of activity. A prudent next step would be to determine the upstream mediators and the downstream consequences of this increase in Egr1 expression. For instance, binge drinking-induced changes in Egr1 expression may be related to an upregulation of stress hormone signaling either by hormone-mediated effects upon Egr1 transcription or by Egr1-dependent transcription of stress hormone-related proteins. There is a preponderance of evidence showing increased corticotropin-releasing hormone (CRH) signaling throughout the extended amygdala in response to alcohol consumption. An upregulation of the CRH system is heavily implicated in alcohol dependence and has been linked to the negative affective states experienced during periods of alcohol abstinence (Gilpin, 2012; Kiefer & Wiedemann, 2004; Sarnyai et al., 2001; Spanagel et al., 2014; Valdez et al., 2002). Increased CRH signaling in the CEA and BNST specifically is implicated in withdrawal-induced anxiety. The CEA is a major source of CRH innervation of the BNST and the BNST itself is densely populated with CRH-expressing cell bodies. Antagonism of CRF receptors in the CEA and BNST is sufficient to reduce anxiogenic-like effects of alcohol withdrawal and decrease subsequent consumption (Baldwin et al., 1991; Funk et al., 2007; Huang et al., 2010; Rassnick et al., 1993; Valdez et al., 2004). Additionally, elevated CRH levels in the BNST during alcohol withdrawal are reduced by

subsequent alcohol intake (Olive et al., 2002), providing a neurophysiological basis for the negative reinforcing properties of withdrawal-induced affective dysregulation.

Anxiety during withdrawal and its enduring role in drug seeking and stress-induced has been demonstrated consistently in both humans and animals (Driessen et al., 2001; Koob, 2013; Schuckit & Hesselbrock, 1994; Silberman et al., 2009; Smith & Aston-Jones, 2008; Thevos et al., 1991). However, it is important to note that alcohol withdrawal studies in animals are typically conducted in using models of chronic alcohol dependence. In the human population, binge drinking is the most prevalent form of alcohol abuse (Centers for Disease Control and Prevention, 2013), yet very little is know about the psychological consequences of this behavior. The present study indicates that this pattern of consumption is capable of producing enduring affective disturbances during abstinence, consistent with those seen in chronic alcoholism. It therefore seems reasonable to speculate that binge drinking causes similar changes to extended amygdala structures, mediated by dysregulation of the HPA axis and CNS hyperexcitability.

4.7 Conclusion

The present study demonstrates that voluntary binge drinking in an animal model is capable of producing emotional dysregulation after as little as 30 days of alcohol exposure. The emotional dysregulation has a rapid onset, persists into protracted withdrawal, and is associated with increased cellular activity in the CEA and BNST, but in the Acb. Further investigation is warranted to better understand the psychophysiological consequences of binge drinking and how this pattern of behavior may contribute to the transition to addiction.

Chapter 3:

Adolescent mice are resilient to alcohol withdrawal-induced anxiety and changes in indices of glutamate function within the nucleus accumbens

1. Introduction

The nucleus accumbens (Acb) is a basal forebrain structure critically involved in learning, motivation, and reinforcement (Salgado & Kaplitt, 2015). The Acb is composed of the shell and core subregions, which are both anatomically and functionally distinct. The outer shell (AcbSh) subregion is believed to govern the primary positive reinforcing properties of rewarding stimuli (Salgado & Kaplitt, 2015). The AcbSh is part of the extended amygdala, a basal forebrain macrosystem critically involved in emotional processing and regulation (Alheid, 2003), which often undergoes maladaptive plasticity as a result of chronic drug abuse (Koob, 2003). The medial core (AcbC) subregion of the Acb is involved in initiating motivated behavior and mediates the motor 'seeking' behaviors associated with a reinforcing stimulus. The AcbC is involved in associative learning and plays a central role in the development and maintenance of operant conditioning. Through its connectivity with the basal ganglia, the AcbC serves as a motor interface in coordinating motivationally salient input with a behavioral output (Corbit et al., 2001)

The Acb is well-characterized with regards to its role in addiction, as virtually all drugs of abuse increase activation of the Acb (Quintero, 2013). Rewarding stimuli, including alcohol and other drugs of abuse, cause an increase in extracellular dopamine and glutamate within the Acb (Ding et al., 2013; Szumlinski et al., 2007), which over time can mold neural circuitry and cause drug-related stimuli to become more salient (Britt et al., 2012). With repeated use, synaptic plasticity within the Acb has been shown to underlie the maintenance and escalation of drug use (Quintero, 2013), as well as craving and the propensity for relapse during withdrawal (Bauer et al., 2013).

In addition to its role in appetitive motivation, the Acb is also involved in aversive motivation (Salamone, 1994). Acb dysfunction has been implicated in a variety of neuropsychiatric disorders characterized by pathologically high negative affect, including bipolar disorder, obsessive-compulsive disorder, anxiety, and depression (Salgado & Kaplitt, 2015; Shirayama & Chaki, 2006). In rodents, an increase in Acb glutamate is associated with the manifestation of depressive behaviors (Rada et al., 2003) and reducing Acb activation via NMDA antagonism has anxiolytic effects (Martinez et al., 2002). As such, Acb stimulation is emerging as a promising target for the treatment of both anxiety and depression in the clinical population (Bewernick et al., 2010; Nauczyciel et al., 2013; Sturm et al., 2003). In contrast to the increased excitation of Acb projection neurons typically associated with rewarding stimuli (Britt et al., 2012; Kalivas & Nakamura, 1999; Stuber et al., 2011), it has been shown that glutamatergic excitation of GABAergic interneurons suppresses neurotransmission within the Acb and elicits an aversive state (Qi et al., 2016). Therefore, given the role of the Acb in the regulation of negative affective states, the Acb is also a possible substrate for the aversive properties of drug withdrawal.

Although excessive alcohol consumption at any age is associated with adverse outcomes, underage binge-drinking specifically is a significant public health concern. Individuals ages 12-20 years old account for 11% of all alcohol consumed in the U.S. (CDC, 2016) and over 90% of this alcohol is consumed in the form of binge-drinking episodes. The Acb undergoes substantial development during adolescence, as do its major regulatory glutamatergic projections from the prefrontal cortex (PFC), which become fully established and strengthened during this critical developmental period (Arain et al., 2013). Insufficient prefrontal control over subcortical activation during adolescence is theorized to underlie a

preference for activities requiring low effort but yielding high excitement such as substance use (Kelley et al., 2004). Subcortical hyper-activation also creates a bias towards bottom-up emotional processing, which could contribute to the increased vulnerability to anxiety and depression during adolescence (Casey, Jones, et al., 2008).

Given the immature developmental state of the Acb during adolescence, it is reasonable to speculate that there may also be age-dependent glutamatergic effects of binge-drinking within this region that relates to age-dependent differences in sensitivity to alcohol withdrawal-induced anxiety. Therefore, frequent binge-drinking during the vulnerable developmental period of adolescence can have enduring psychological and neurobiological consequences that may be unique to adolescents compared to their adult counterparts.

The present study investigated the relation between protein indices of glutamate neurotransmission within the Acb and alcohol withdrawal-induced anxiety. Due to the relatively brief duration of mouse adolescence (Brust et al., 2015), all animals were subjected to a 14-day drinking period. This drinking period is similar to that employed in other studies of adolescent alcohol exposure (Brunell & Spear, 2005; O'Tousa et al., 2013; Spear, 2000b) and enables the extension of prior immunoblotting work (Cozzoli et al., 2012; Cozzoli et al., 2015; Cozzoli et al., 2014) to a shorter history of binge-drinking in adult animals. Approximately 24 hrs following the final drinking period, animals underwent behavioral testing or tissue collection. We predicted that a history of binge-drinking in adult mice would produce signs of negative affect and increased glutamate-related protein expression in Acb subregions, notably the AcbSh, given its limbic functions. Based on the evidence that adolescents are resilient to many of the aversive properties of alcohol and its withdrawal (Spear & Varlinskaya, 2005), we predicted that symptoms of negative affect would be

attenuated or absent in adolescent animals relative to adults. We also hypothesized that this behavioral resilience might be associated with a resistance to binge-induced changes in protein indices of glutamate function within the Acb of adolescent bingers.

2. Material and Methods

2.1 Subjects

This study used 2 separate cohorts of animals- 1 for behavioral testing and 1 for immunoblotting. As a previous study from our laboratory revealed effects of our behavioral testing procedures themselves on cellular activation within Acb subregions (Lee et al., 2015), a separate cohort of animals was used to generate tissue for immunoblotting in order to assess the effects of binge-drinking on protein expression, independent of behavioral-testing confounds. Both cohorts of animals were exposed to identical drinking procedures and each consisted of 48 C57BL/6J (B6) male mice (Jackson Laboratories, Sacramento, CA) that were either 8 weeks (adults; n=24) or 4 weeks (adolescents; n=24) of age at onset of drinking. Within each age group, animals were randomly divided into an alcohol-drinking group (n= 12) and a water-drinking group (n=12) and then individually housed in standard, Plexiglas cages, under a 12-h-reverse light/dark cycle (lights off at 10 am), in a temperature-controlled vivarium (23◦ C). Food and water were available *ad libitum*, with the exception of the 2-h alcohol-drinking period, during which time the home cage water bottle was removed. All experiments were conducted in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (NIH Publication No. 80-23, revised 2014) and approved by the IACUC of the University of California, Santa Barbara.

2.2 Drinking-in-the-dark (DID) procedures

Animals were subjected to 14 consecutive days of binge-drinking under modified 4-bottle Drinking-in-the-Dark (DID) procedures. While earlier studies employed 30-day binge drinking regimens (Cozzoli et al., 2009; Lee et al., 2015), alcohol-access was restricted to 14 days in this study to correspond with the estimated length of adolescence in the mouse (Spear, 2000). Each day, animals were given simultaneous access to 5, 10, 20, and 40% (v/v) unsweetened ethanol solutions for 2 h beginning at 3 h into the dark phase of the circadian cycle, which corresponds to the time of peak fluid intake. A multi-bottle presentation has been shown to increase voluntary intake compared to a single bottle (Cozzoli et al., 2014; Gustafsson & Nylander, 2006; Henniger et al., 2002; Tordoff & Bachmanov, 2003) as employed in our preceding study, which was especially important given our abbreviated drinking period. The amount of alcohol consumed was calculated daily by bottle weight immediately before and after the drinking period. Control animals received an identical sipper tube of filtered tap water *in lieu* of alcohol. All animals, both alcohol and water drinkers, were weighed 3x per week throughout the drinking period.

Submandibular blood samples were collected from all alcohol-drinking animals on day 10, immediately following the 2-h drinking period. The timing of the blood collection was selected to ensure that the animals' intakes had stabilized, while also allowing ample time for recovery prior to behavioral testing. Blood alcohol concentrations (BACs) were determined using an Analox alcohol analyzer (model AM1, Analox Instruments USA, Lunenburg, MA, USA) as per the manufacturer's instructions.

2.3 Behavioral testing

A 2-day behavioral test battery commenced approximately 24 h following the final alcohol presentation and consisted of a novel object test and the Porsolt forced swim test (FST) on day 1 and the marble burying test on day 2. These tests were selected based on the results of our prior study demonstrating robust effects of alcohol withdrawal upon the various dependent measures in these paradigms (Lee et al., 2015). All animals completed the novel object test before beginning the FST in order to allow animals to rest between assays. Given the size of our cohorts and the availability of testing equipment, it was not possible to complete all the behavioral testing in a single day. The order of testing was based on considerations regarding the duration of each trial, as well as the instruction from our IACUC which included avoiding additional testing following the FST in order to allow the animals time to fully recover.

2.3.1 Novel object. To test reactivity to a novel object as an index of neophobia-related anxiety (Dulawa et al., 1999; Misslin & Ropartz, 1981), animals were placed in an activity arena measuring 46 cm long × 42 cm wide × 40 cm high. In the center of the arena was placed a novel, inedible, object (we used a patterned ceramic candlestick holder; measuring approximately 6 cm in diameter × 12 cm high). Using AnyMaze™ tracking software (Stoelting Co., Wood Dale, IL), a zone was designated around the novel object and was used to monitor the animals' interaction with the novel object during the 2-min trial. The number of contacts and total time spent in contact with the novel object, as well as total distance traveled within the activity arena, were recorded.

2.3.2 Porsolt forced swim test. Floating behavior during the Porsolt forced swim test serves as an index of behavioral despair in laboratory animals (Porsolt, Bertin, et al., 1977) and is a model with high predictive validity for the clinical efficacy of anti-depressant drugs (Porsolt, Le Pichon, et al., 1977). Each animal was placed into an 11-cm diameter cylindrical container filled with room-temperature water such that animals were unable to touch the bottom. The latency to first exhibit immobility (defined as no horizontal or vertical displacement of the animal's center of gravity for 5s+), total time spent immobile, and the numbers of immobile episodes were monitored during a 6-min period using AnyMaze™ tracking software (Stoelting Co., Wood Dale, IL).

2.3.3 Marble-burying. The marble-burying test was used to measure anxiety-induced defensive burying (Njung'e & Handley, 1991). In our paradigm, 12 square glass pieces (2.5 cm2 × 1.25 cm tall) were placed in the animals' home cage, 6 at each end. Animals were then left undisturbed for 15min. At the end of the trial, a blind observer recorded the number of marbles at least 75% buried.

2.4 Brain tissue collection

Animals not subjected to behavioral testing were decapitated approximately 24 h following the final alcohol presentation to mirror the time-frame of that employed in the behavioral study. The brain was cooled on ice and sectioned in 1 mm-thick slices, along the coronal plane, at the level of the striatum using ice-cold razor blades. The AcbSh and AcbC were bilaterally sampled from the slice located approximately 1.18mm anterior to Bregma, as

depicted in the mouse brain atlas of Paxinos & Franklin (2004), using a 18-gauge biopsy needle (as depicted in Fig. 1).

2.5 Immunoblotting

Immunoblotting was performed on whole tissue homogenates from the AcbSh and AcbC subregions, following procedures similar to those employed previously by our group (Ary et al., 2013; Cozzoli et al., 2014; Goulding et al., 2011; Lum et al., 2014). Samples were homogenized in a medium containing RIPA buffer (Boston BioProducts, Ashland, MA), Complete Mini-tab Protease Inhibitor Cocktail tablet, sodium fluoride, sodium orthovanadate phosphatase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). For analysis, 15 ul of homogenized sample was subjected to SDS-polyacrylamide gel electrophoresis using Tris–Acetate gradient gels (3–8%) (Invitrogen, Carlsbad, CA) and transferred to polyvinylidene difluoride membranes. Gels were run such that each membrane contained 3 samples from each age/drinking group. Membranes were preblocked with tris-buffered saline containing 0.1% (v/v) Tween 20 and 5% (w/v) nonfat dried milk powder for 1 hr before overnight incubation with the following rabbit primary antibodies: mGlu1 (Synaptic Systems, Göttingen, Germany; 1:1000 dilution), mGlu5 (Millipore, Temecula, CA; 1:1000 dilution), GluN2A and GluN2B (both from Calbiochem, San Diego, CA; 1:1000 dilution), CAMKII (Millipore, Temecula, CA; 1:1000 dilution) and Thr286 phosphorylated CAMKII (pCAMKII) (Cell Signaling Technology, Beverly, MA; 1:1000 dilution), PKCε (Santa Cruz Biotechnology, Dallas, TX; 1:500 dilution) and Ser729 phosphorylated PKCε (pPKCε) (Santa Cruz Biotechnology, Dallas, TX; 1:500 dilution), and calnexin (Enzo Life Sciences, Farmingdale, NY; 1:1000 dilution) for standardization.

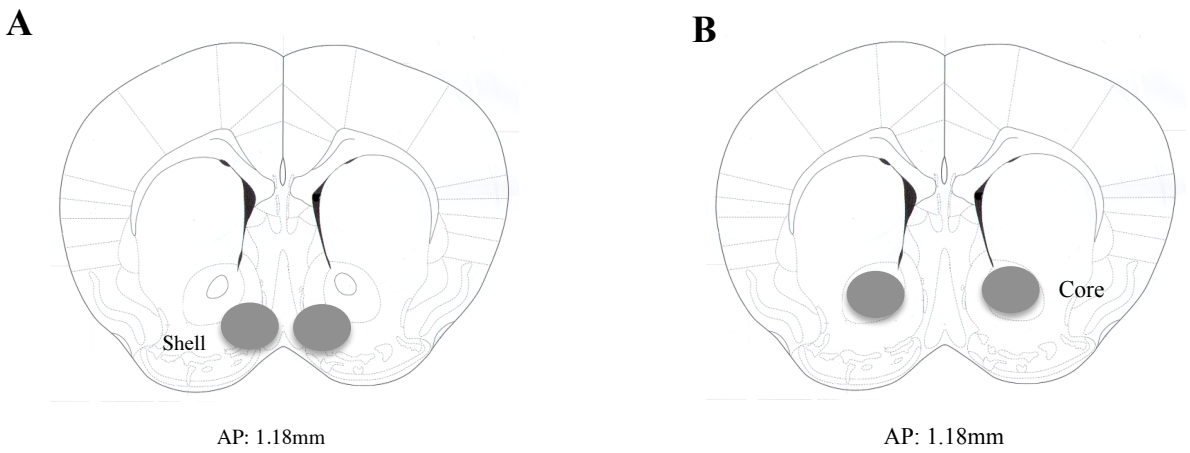

Figure 3.1. Immunoblotting sample placement. Schematic illustrating a coronal section through the Acb, highlighting the size and location of the bilateral sampling region used to assay protein changes within the **(A)** AcbSh and **(B)** AcbC.

Membranes were washed, incubated with a horseradish peroxidase-conjugated goat anti-rabbit secondary anti-body (Jackson ImmunoResearch Laboratories, West Grove, PA; 1:100000 dilution) for 90 min, washed again, and immunoreactive bands were detected by enhanced chemiluminescence (ECL Plus; Amersham Biosciences, Inc., Piscataway, NJ). Levels of immunoreactivity were quantified by integrated density using Image J (NIH, Bethesda, MD) and standardized to each animal's respective calnexin signal. These values were then averaged across the adult water control samples within each gel (n=3/gel) and all bands on that gel were normalized as percent of the average control value. To obtain an index of kinase activation, the density × area measurements for each phospho-protein was also normalized to that of its corresponding non-phosphorylated protein prior to expressing the data as a percent average of the controls on each gel.

Our proteins of interest were selected based on previous work from our lab demonstrating that binge-drinking history upregulates these protein indices of excitatory neurotransmission including mGlu1/5, NR2A/B, and the downstream effector protein kinase C epsilon (PKCε) (Cozzoli et al., 2015; Cozzoli et al., 2009; Goulding et al., 2011; Szumlinski, Ary, Lominac, et al., 2008), which are believed to promote a "pro-alcoholic" phenotype (Cozzoli et al., 2012; Kalivas et al., 2009; Szumlinski, Ary, Lominac, et al., 2008). For the present study, we also included plasticity-related calcium/calmodulin-dependent protein kinase II (CAMKII) in our analysis due to its recent implication in the maintenance of alcohol consumption, as well as negative affective states (Easton, Lucchesi, Lourdusamy, et al., 2013; Zhao et al., 2015). Prior work from our group indicates that alcohol-drinking history increases glutamate-related protein expression selectively within the AcbS (Cozzoli et al., 2012; Cozzoli et al., 2015; Cozzoli et al., 2009; Goulding et al., 2011; Szumlinski, Ary,

Lominac, et al., 2008). Thus, we measured protein expression within both the Acb shell and core in the present study with the expectation that the AcbS would show a greater number and larger changes in protein levels, relative to the adjacent, but functionally distinct, AcbC.

2.6 Statistical analysis

Statistical analyses of all behavioral and immunoblotting data were conducted using between-subjects two-way analyses of variance (ANOVAs), along with planned comparisons to assess group differences based on treatment and age using Fisher's LSD tests for simple main effects. $\alpha = 0.05$ for all analyses. Statistical outliers were identified using the $\pm 1.5 \times IQR$ rule and excluded from analyses. No more than 2 outliers were present per group, resulting in n's of 10-12 per age/drinking group. All calculations were performed using SPSS v.21 statistical software (IBM, 2012).

3. Results

3.1 Alcohol consumption

The ANOVA revealed a significant between-subjects effect of age [$F(1,22)=7.54$, $p=0.012$], with adolescent animals consuming significantly more alcohol than adults across the entire 14-day drinking period (Fig. 2A). The analysis of the blood samples collected immediately following the 2-hr drinking period on day 10 yielded an average BAC of 94.12 ± 10.83 mg/dl in adult mice exhibiting an average alcohol intake of 6.97±0.57 g/kg and an average BAC of 141.17 ± 10.23 mg/dl in adolescents exhibiting an average alcohol intake of 8.82±0.56 g/kg. Based on the NIAAA criteria of >80mg/dl BAC (National Institute on Alcohol Abuse and Alcoholism, 2004), both age groups were engaged in binge drinking.

However, adolescent mice binge-drank more alcohol [t(22)=2.32, p=0.029] with higher resulting BACs [t(22)=2.13, p=0.043; Fig 2B] compared to their adult counterparts on day 10. There was no significant difference in alcohol intake or body weight between the animals tested for behavior versus those used for immunoblotting.

3.2 Behavioral testing

3.2.1 Novel object test. Adolescent animals were more interactive and hyperactive in the novel object test, compared to adults (Fig. 3). Adolescents made more object contacts [age effect: F(1,44)=7.47, p=0.009; Fig. 3A] and spent more time in contact with the novel object during the 2-min trial [age effect: F(1,43)=14.46, p<0.001; Fig. 3B], irrespective of their prior binge-drinking history (no Treatment effects or interactions for either variable, p's>0.05). Adolescents also traveled a greater overall distance in the enclosure [age effect: F(1,42)=19.36, p<0.001; Fig. 3C], although alcohol-drinking animals of both ages were hypoactive compared to water-drinking controls [Treatment effect: F(1,42)=10.74, p=0.002; interaction: p>0.05].

3.2.2 Marble burying test. In the marble burying test, an age X treatment interaction [F(1,43)= 4.10, p=0.049; Fig.4A] was detected. Deconstruction of the interaction revealed that adult alcohol drinkers buried more marbles than age-matched water controls [LSD p=0.042], while adolescent alcohol drinkers trended toward burying less marbles [LSD p=0.09].

3.2.3 Forced Swim Test. As both adult and adolescent alcohol-drinkers showed

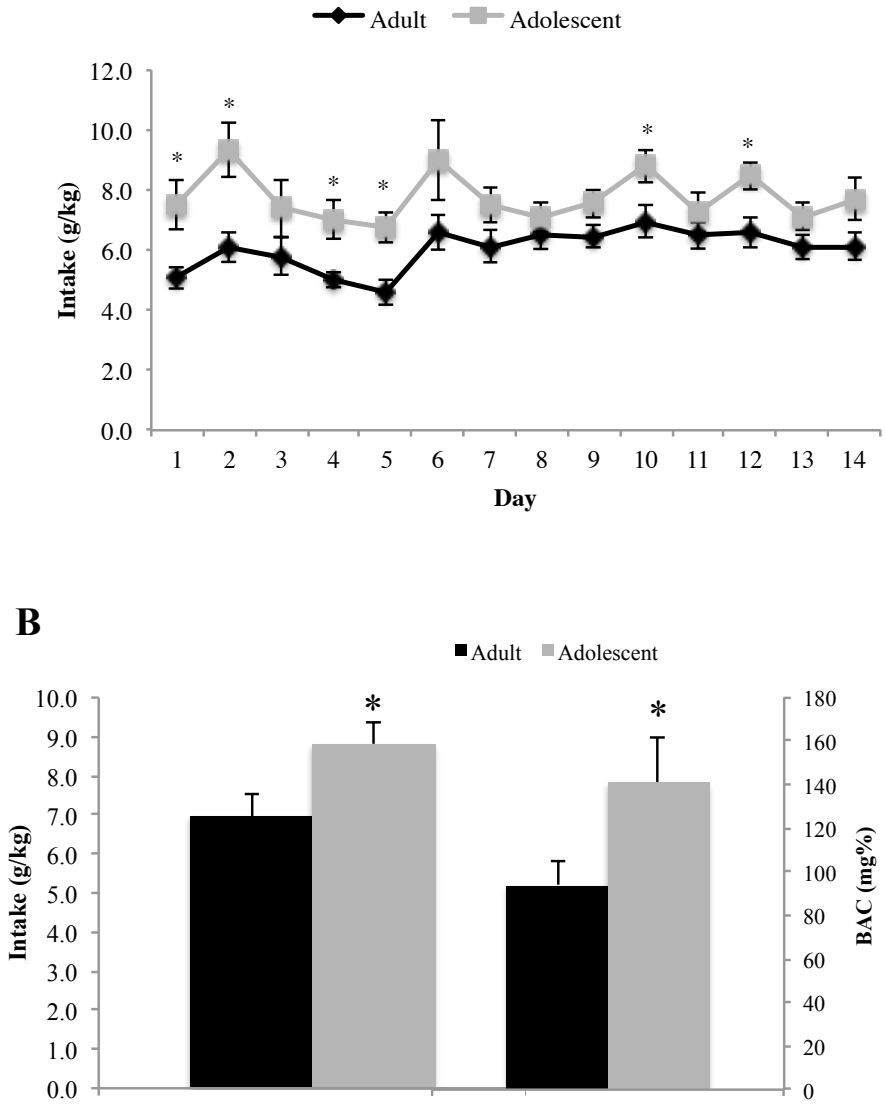

Figure 3.2. Adolescents consume more alcohol than adults. (A) Over the 14-day drinking period, adolescent animals consumed significantly more alcohol than adults ($p<0.05$). The average intake across all 14 days was 6.13 ± 0.19 g/kg for adults and 7.68 ± 0.23 g/kg for adolescents. **(B)** Blood samples were collected on day 10 of drinking, immediately following the 2hr drinking period. Adolescent animals consumed more alcohol on this day and had a higher average BAC than their adult counterparts. The data represent the means ± SEMs of 10-12 mice/group, excluding statistical outliers. *$p<0.05$ vs. adults.

comparable reductions in immobility, compared to their respective controls, the data from this test were collapsed across age to highlight the alcohol-water difference (Fig. 4B). Alcohol-drinkers spent less time immobile [Treatment effect: $F(1,43)=4.31$, $p=0.043$] and had fewer immobile episodes [Treatment effect: $F(1,42)=4.33$, $p=0.044$], compared to water drinkers. There were no age-related differences in behavior or interactions between age and prior drinking history (all p's>0.05).

3.3 Western blotting

3.3.1 Accumbens shell. The positive experimental outcomes from the immunoblotting study of the AcbSh are presented in Fig. 5. Significant age X treatment interactions were detected for mGlu1 [$F(1,40)=5.17$; $p=0.028$; Fig. 5A], mGlu5 [$F(1,41)=6.58$; $p=0.014$; Fig. 5B], and GluN2B [$F(1,41)=5.11$; $p=0.029$; Fig. 5C]. LSD analysis of simple main effects revealed that water-drinking adolescents had higher basal mGlu1 ($p=0.03$), and mGlu5 expression ($p=0.04$) compared to water-drinking adults. Additionally, adult bingers exhibited a significant alcohol-induced increase in mGlu1 ($p=0.033$), mGlu5 ($p=0.042$), and GluN2b ($p=0.037$) at 24 h withdrawal. In contrast, adolescent mice showed no alcohol-induced change in mGlu1, mGlu5, or GluN2b (all p's>0.05). Non-significant immunoblotting results are summarized in Table 1.

3.3.2 Accumbens core. The positive experimental outcomes from the immunoblotting study of the AcbC are presented in Fig. 6. In the AcbC, alcohol-drinking animals showed an age-independent increase in GluN2B [Treatment effect: $F(1,430=4.61$; $p=0.038$; no Age effect or interaction, p's>0.05; Fig. 6A]. We also observed an age X

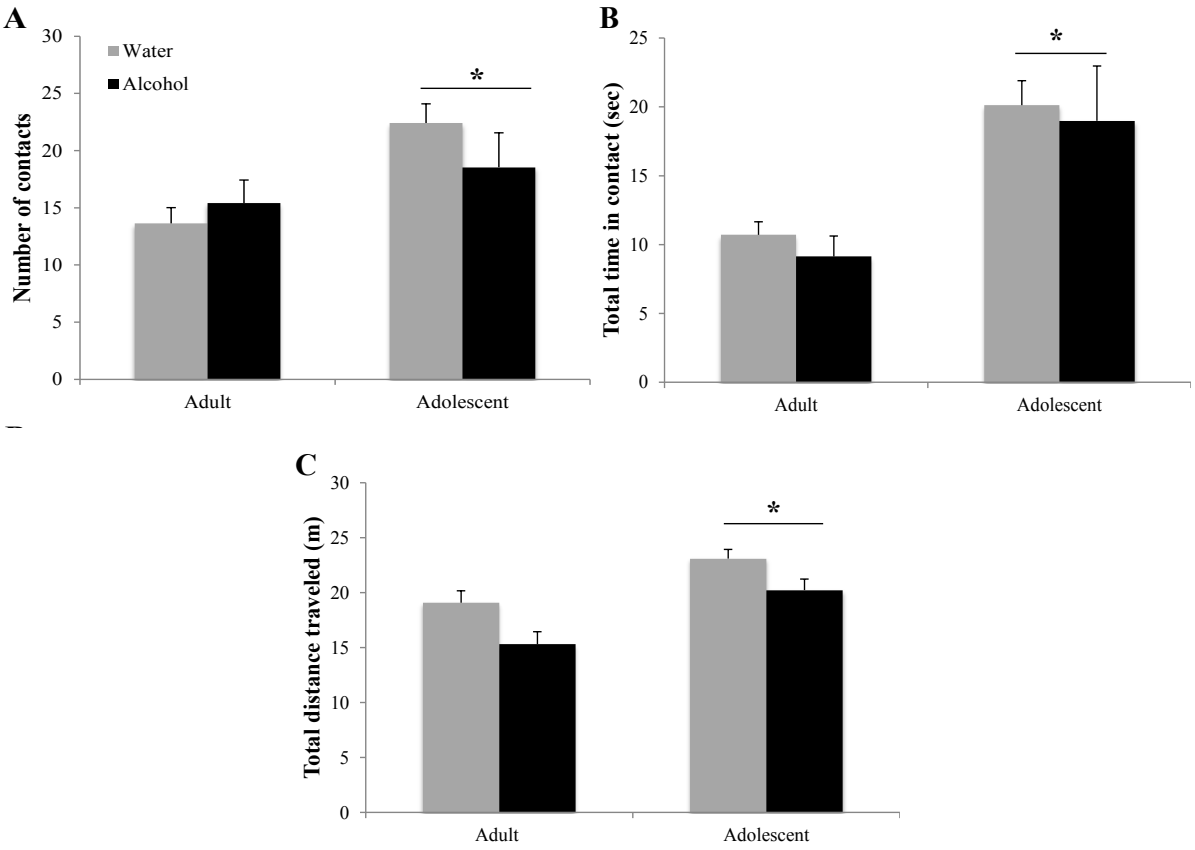

Figure 3.3. Differences in the novel object test are age-dependent but alcohol-insensitive. **(A)** Adolescent mice made more contacts with the novel object and **(B)** spent more total time in contact with the object during the 2min trial. **(C)** Adolescent animals also showed general locomotor hyperactivity compared to adults. The data represent the means ± SEMs of 10-12 mice/group. *$p<0.05$ vs. adults.

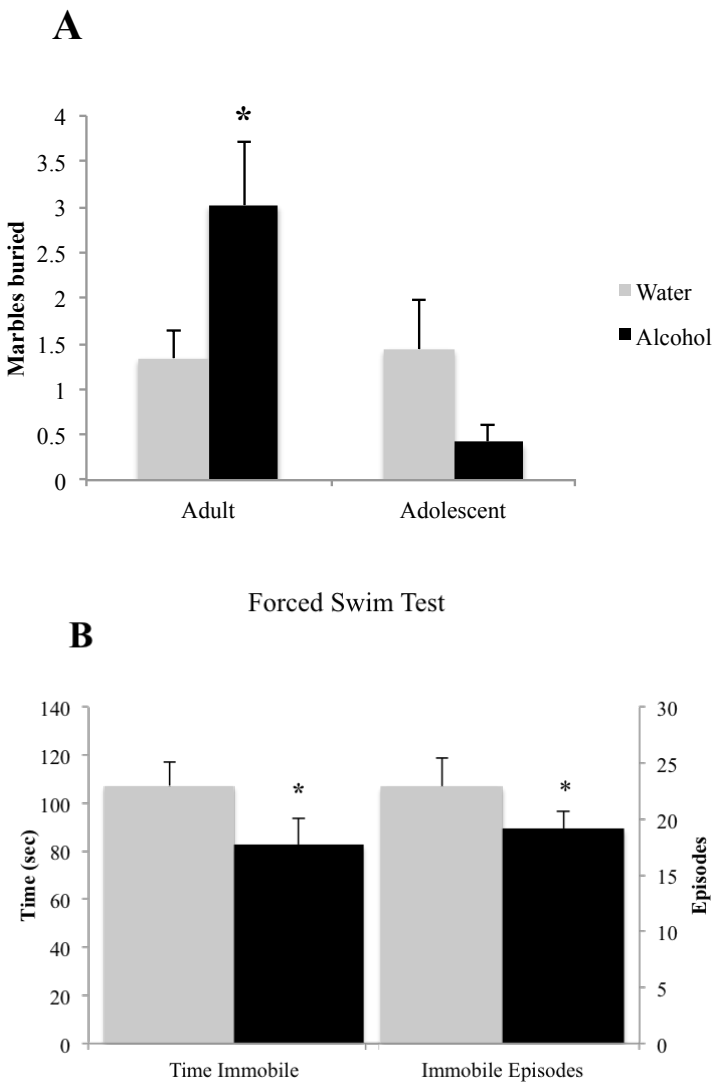

Figure 3.4. Withdrawal from binge drinking produces mixed effects on behavioral measures of negative affect. **(A)** In the marble burying test, there was an age X treatment interaction showing that adult drinkers buried more marbles compared to their water-drinking counterparts while adolescents trended toward burying less. **(B)** In the forced swim test, an age-independent main treatment effect showed that alcohol-drinking animals showed significantly reduced immobility compared to water drinkers, both in number of immobile episodes and total time spent immobile. The data represent the means ± SEMs of 10-12 mice/group. *$p<0.05$ vs. respective water control.

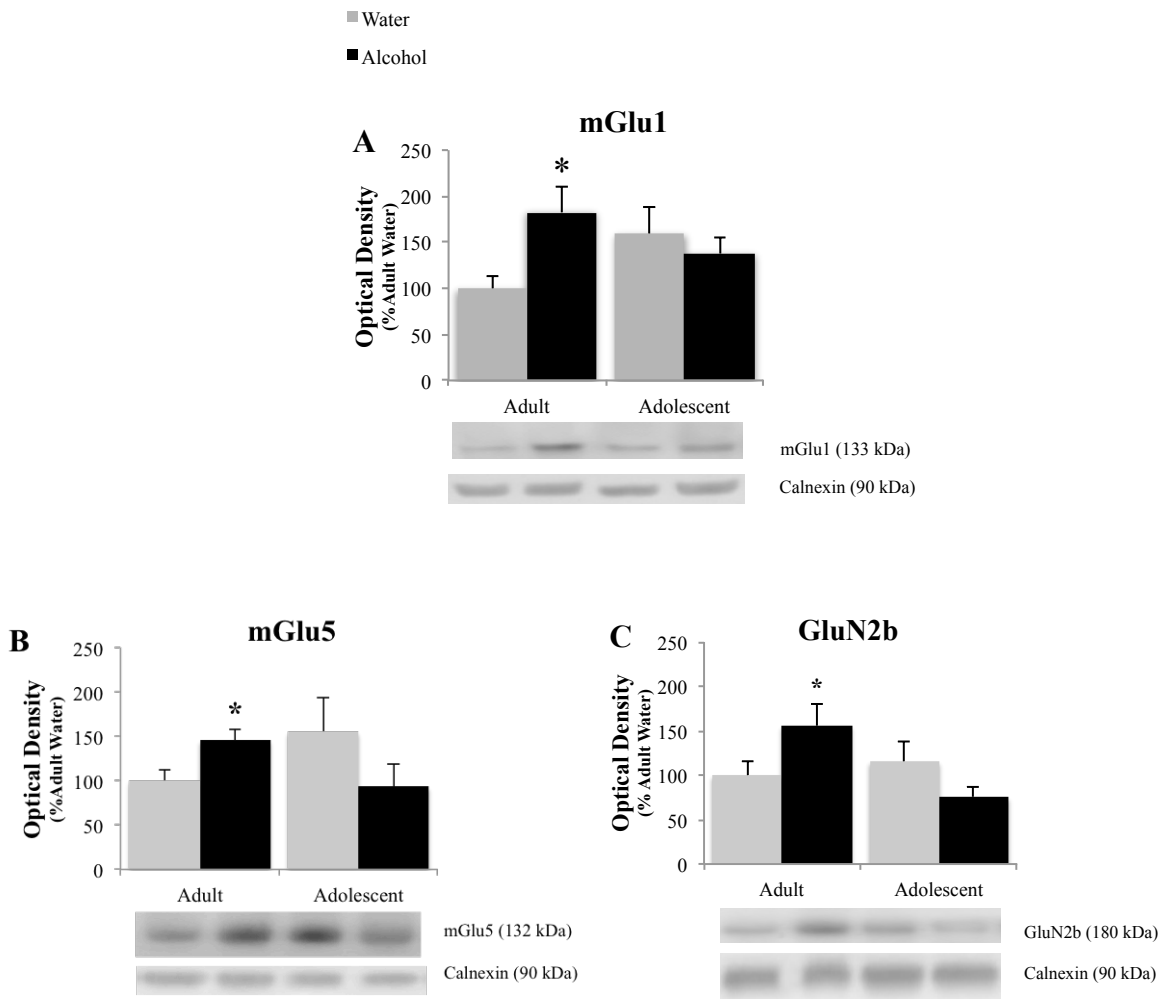

Figure 3.5. Adult animals are more vulnerable than adolescents to binge-induced increases in protein expression within the AcbSh. Adult drinkers showed a significant increase in **(A)** mGlu1, **(B)** mGlu5, and **(C)** GluN2B at 24hr withdrawal that was not present in adolescent drinkers. The data in panels A-C represent mean ± SEM of 10-12 mice/group; *$p<0.05$ vs. respective water control.

Table 3.1. Summary of non-significant immunoblotting results from the AcbSh and AcbC. (A) In the AcbSh, there was a trend toward an alcohol-induced increase in GluN2A in adult bingers and a decrease in PKCε in adolescent bingers. **(B)** In the AcbC, there was a trend toward an alcohol-induced increase in pCAMKII in adult bingers. The data represent mean ± SEM of 10-12 mice; #$p<0.10$ vs. respective water control.

	Adults		**Adolescents**	
AcbSh	**Water**	**Alcohol**	**Water**	**Alcohol**
GluN2A	100.0 ± 18.8	#170.48 ± 29.1	154.9 ± 35.6	141.3 ± 25.1
PKCε	100.0 ± 10.1	127.67 ± 23.7	118.5 ± 18.6	#80.9 ± 6.2
pPKCε	100.0 ± 11.0	99.7 ± 14.7	86.2 ± 11.8	67.4 ± 9.4
CAMKII	100.0 ± 9.5	97.2 ± 17.0	115.7 ± 14.4	116.3 ± 26.6
pCAMKII	100.0 ± 5.6	117.2 ± 25.9	105.7 ± 16.6	128.9 ± 15.9
AcbC				
mGlu1	100.0 ± 16.4	85.3 ± 13.1	88.0 ± 7.3	90.2 ± 6.3
mGlu5	100.0 ± 4.9	108.2 ± 24.9	127.3 ± 19.3	107.0 ± 17.1
GluN2A	100.0 ± 7.3	138.4 ± 24.5	120.9 ± 12.5	118.7 ± 24.9
PKCε	100.0 ± 12.6	99.9 ± 10.0	115.3 ± 12.0	107.0 ± 16.4
pCAMKII	100.0 ± 18.8	#161.3 ± 28.1	100.9 ± 13.5	97.0 ± 6.6

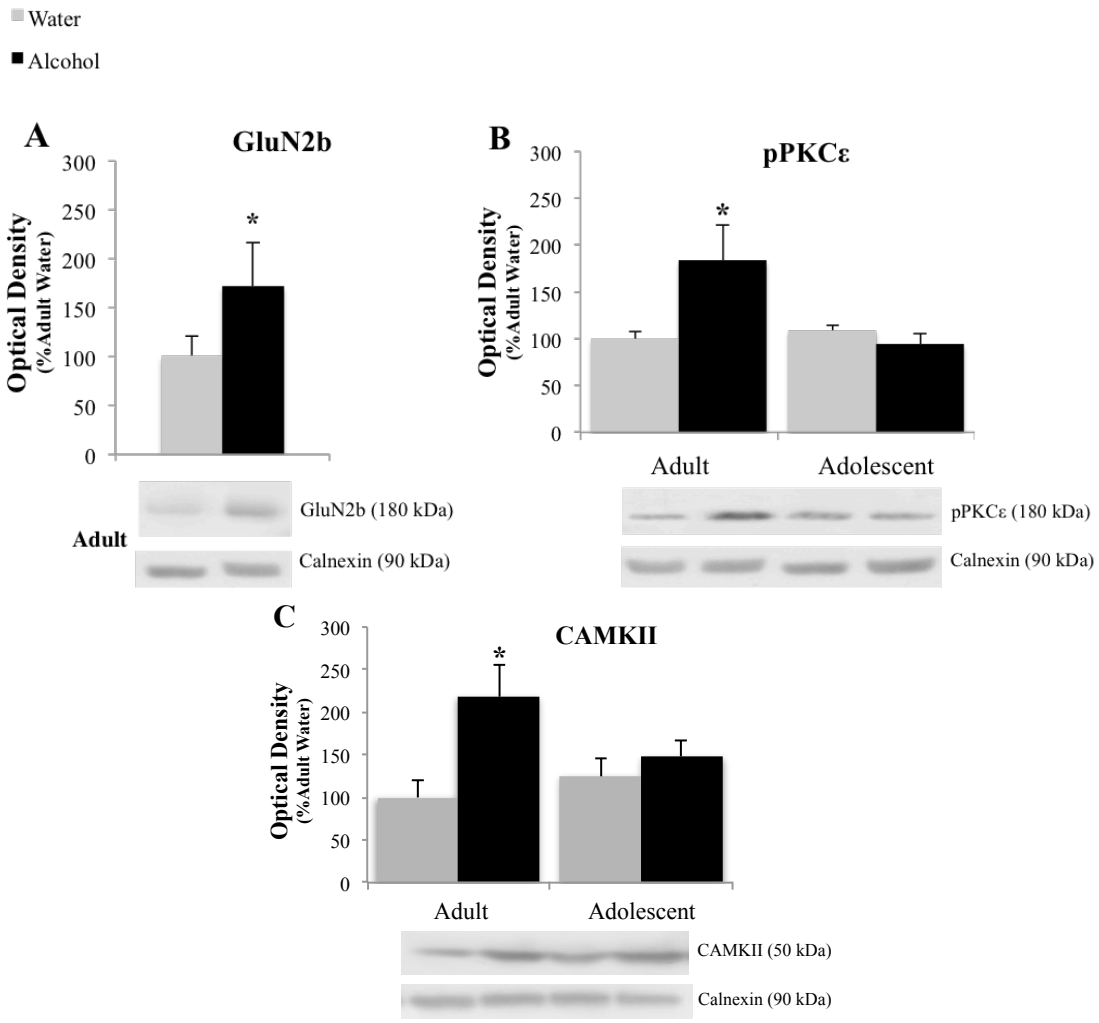

Figure 3.6. Adult animals are more vulnerable than adolescents to most binge-induced increases in protein expression within the AcbC. (A) A main treatment effect of alcohol showed a binge-induced increase in GluN2b independent of age. **(B)** Adult drinkers showed a significant increase in pPKCe at 24hr withdrawal that was not present in adolescent drinkers, which resulted in a similar increase in the phospho: total PKCe ratio (data not shown). **(C)** The ANOVA showed a main treatment effect of alcohol on CAMKII expression. However, further analysis revealed that this effect was primarily due to the significant increase in alcohol-drinking adults. The data in panels A-C represent mean ± SEM of 10-12 mice/group; *$p<0.05$ vs. respective water control.

treatment interaction for total pPKCε [F(1,40)=4.22; p=0.047; Fig. 6B], which mirrored the results of the activated:total PKCε ratio [F(1, 43)=4.12; p=0.049] reflecting an alcohol-induced increase in pPKCε in adult drinkers (LSD p=0.049) that was not present in adolescent drinkers (LSD p>0.05). The 2-way ANOVA also revealed an alcohol-dependent increase in CAMKII [treatment effect: F(1, 41)= 7.44; p=0.009; Fig. 6C] and although, the age X treatment interaction was shy of statistical significance (p=0.07), inspection of the results argue that the treatment effect is being driven primarily by the data from the alcohol-drinking adults. Indeed, LSD planned comparisons showed a significant increase in adult drinkers compared to water controls [t(21)=2.80; p=0.011], but no significant difference between alcohol- and water-drinking adolescents (p>0.05).

4. Discussion

Cessation of excessive alcohol consumption often results in a dysphoric state to which adolescent drinkers appear less susceptible (Spear & Varlinskaya, 2005). This age-dependent insensitivity to the affective consequences of early alcohol withdrawal is apparent in both humans and animal models of alcoholism (Doremus et al., 2003; Spear & Varlinskaya, 2005). The underlying neurobiological mechanisms contributing to adolescent resilience to withdrawal-induced dysphoria is not well understood, particularly within the context of the most prevalent pattern of alcohol drinking exhibited by adolescents - binge drinking. As such, the present study employed behavioral and immunoblotting procedures to determine the interactions between the subject factors of age and binge-drinking history with respect to emotionality and indices of glutamate transmission within Acb subregions. While correlational in nature, the study outcomes provide novel evidence that the age of binge-

drinking onset is an important subject factor that contributes to both alcohol withdrawal-induced negative affect and changes in Acb glutamate, the causal relation between which will be a major focus of future studies.

Consistent with prior literature characterizing adolescent drinking behavior in both humans (National Institute on Alcohol Abuse and Alcoholism, 2002) and laboratory rats (Doremus et al., 2005; Spear & Varlinskaya, 2005; Vetter et al., 2007), adolescent mice in our study consumed significantly more alcohol than adults across the 14-day drinking period, with higher resulting BACs following the 2-h drinking session. Notably, both adult and adolescent animals attained intoxicating 'binge' levels of drinking, however that of the adolescents was significantly greater (National Institute on Alcohol Abuse and Alcoholism, 2015).

4.1 Behavioral dysregulation at 24 h withdrawal from binge-drinking

When assayed at 24 h withdrawal, the adult bingers in this study exhibited hyper-anxiety in the marble burying test, which is consistent with our previous data showing an increase in marble burying in adult mice with a 30-day history of binge drinking (Lee et al., 2015). This suggests that even a relatively brief, 2-week period, of binge-drinking is sufficient to elicit a negative affective state in adult animals. In contrast, despite consuming more alcohol and achieving higher BACs, adolescent alcohol-drinkers showed resilience to withdrawal-induced anxiety in this paradigm, as indicated by no alcohol-induced increase in marble burying. These results corroborate those of other studies showing resilience to the anxiogenic effects of acute alcohol withdrawal in adolescent rats when assayed by the elevated plus maze and social interaction test (Doremus et al., 2003; Varlinskaya & Spear,

2004). Thus, the results of the present study complement existing work in the field by extending these findings to a mouse model of voluntary binge-drinking and demonstrating the sensitivity of an additional behavioral assay to the anxiogenic effects of alcohol withdrawal.

The adolescent mice in our study were overall more interactive and hyperactive in the novel object test compared to adult mice. Elevated novelty-preference and novelty-induced locomotor hyperactivity in adolescents versus adults is associated with greater impulsivity and a predisposition toward sensation-seeking (Stansfield & Kirstein, 2006). In humans, these traits are strongly predictive of engaging in risk-taking behaviors and substance abuse in human adolescents (MacPherson et al., 2010; Spear, 2000a, 2010). Given that locomotor suppression is a common symptom of acute alcohol withdrawal (Kliethermes, 2005; Kliethermes et al., 2004), it is not surprising that both adult and adolescent bingers exhibited less locomotor activity in the novel object test, compared to their respective age-matched water controls. Although, overall, adolescents showed greater locomotor hyperactivity, compared to adults, both age groups were susceptible to this alcohol withdrawal-induced hypo-activity. This outcome contrasts with that from our prior study in which adult mice binge-drank alcohol for 30 days and indicates that, unlike marble burying, the novel object test does not appear sensitive to changes in anxiety induced by a 14-day history of binge-drinking and it remains to be determined whether the subject factors of age and binge-drinking interact in this paradigm in animals with more prolonged history of alcohol consumption.

However, consistent with our previous study in adults (Lee et al., 2015), all binging animals, irrespective of age, exhibited hyper-activity in the forced swim test, as evidenced by

reduced time spent immobile and a lower number of immobile episodes, relative to water controls. Although the FST is typically used as an assay of depression, based on increased floating behavior (Porsolt et al., 2001), we have reliably observed a decrease in floating behavior in animals with a history of binge-drinking. We have previously interpreted this hyperactive swimming as reflecting panic in alcohol-withdrawn mice, given that panic is often characterized in laboratory animals using measures of motivated escape in which the animal is actively engaged in fleeing from aversion or a perceived proximal threat (Campos et al., 2013; Sena et al., 2003). In humans, panic is considered an anxiety-related condition with a distinct presentation and symptom profile (American Psychiatric Association, 2013; Craske et al., 2010). Panic disorder shares a high comorbidity with alcohol use disorders (Cowley, 1992; Marshall, 1997) and furthermore, frequent bouts of intoxication and withdrawal are capable of eliciting neuroadaptations that may precipitate symptoms of panic (Cosci et al., 2007).

Despite the somewhat unconventional interpretation of our FST data, the fact that these differences can be observed as early as 24 h following a 2-week history of binge-drinking argues that this assay is particularly sensitive to this pattern of alcohol consumption. However, as both binge-drinking adolescents and adults exhibited a similar behavioral profile in this assay, withdrawal-induced hyperactivity in the FST does not appear to be sensitive to differences in the age of binge-drinking onset. It is also plausible that the failure to observe an age-dependent effect of binge-drinking history in this test reflects the severity of the stressor, which is both psychological and physiological in nature and potentially life-threatening (versus encountering novel, but benign, objects such as marbles). These data indicate that binging adolescents are not wholly impervious to alcohol withdrawal-induced

anxiety of a panic-like nature and that factors associated with the nature of stressor may be critical in determining whether or not binging adolescents exhibit withdrawal-induced behavioral dysregulation.

4.2 Changes in protein expression within the Acb during withdrawal from binge-drinking

To complement our behavioral data and expand upon our prior work (Lee et al., 2015), we examined changes in protein expression within the Acb, a structure known to be sensitive to drug-induced neuroadaptations. We know that alcohol-induced dysregulation of excitatory signaling within the Acb is highly implicated in the maintenance and escalation of alcohol consumption, including binge-drinking (Cozzoli et al., 2012; Cozzoli et al., 2015; Cozzoli et al., 2009; Lum et al., 2014; Szumlinski, Ary, Lominac, et al., 2008; Szumlinski et al., 2007; Szumlinski et al., 2005); however, virtually no studies have assessed the role of the Acb in withdrawal-induced negative affect despite this structure's involvement in emotional circuitry.

4.2.1 Glutamate receptor expression in the AcbSh parallels withdrawal-induced anxiety. Similar to our previous studies in which mice were subjected to months-long drinking procedures (Cozzoli et al., 2012; Cozzoli et al., 2015; Szumlinski, Ary, Lominac, et al., 2008), we found that binge-alcohol experience significantly increased mGlu1/5 and GluN2b within the AcbSh. However, we failed to replicate previous work showing an increase in AcbSh PKCε priming (Cozzoli et al., 2015). This discrepancy are likely attributable to differences in the duration of binge-exposure (14 vs. 30 days) between the 2

studies, suggesting that alcohol-induced protein changes are, not surprisingly, experience-dependent and manifest differentially over the course of brief to prolonged exposure. The increased expression of mGlu1, mGlu5, and GluN2b expression in the AcbSh of adult bingers paralleled the hyper-anxious behaviors displayed by adult bingers in the marble burying test and FST. Given the well-established role of glutamate in anxiety (Bergink et al., 2004; Koltunowska et al., 2013; Kotlinska & Bochenski, 2008; Simon & Gorman, 2006; Swanson et al., 2005), these results were consistent with our hypothesis that withdrawal-induced anxiety would be associated with increased protein indices of glutamatergic transmission within the AcbSh, thereby further implicating AcbSh excitability in withdrawal-induced negative affect. The AcbSh also receives significant glutamatergic innervation from the amygdala, which is highly susceptible to alcohol-induced perturbation and is known to mediate many aspects of withdrawal-induced negative affect (Christian et al., 2012; Gilpin et al., 2015). Therefore, increased glutamate-related protein expression could render the AcbSh hypersensitive to excitatory innervation from the amygdala and perpetuate alcohol-induced dysfunction within the emotional circuitry of the extended amygdala.

Consistent with the literature (reviewed in Crews et al., 2007), the adolescent water-drinking controls exhibited higher basal glutamate receptor expression in the AcbSh, compared to control adults. These receptors have been shown to be important in all aspects of alcohol consumption including drug-seeking, maintenance and escalation of intake, and relapse (reviewed in Gonzales & Jaworski, 1997; Kalivas et al., 2009; Tsai et al., 1995). Our lab has previously shown higher basal mGlu1 expression in the AcbSh of two distinct lines of mice selectively bred to binge-drink high amounts of alcohol (Cozzoli et al., 2012; Cozzoli et al., 2009). Therefore, it is likely that hypersensitivity of these ‘pro-binge’ receptors is an

underlying factor contributing to the greater alcohol consumption seen in adolescent versus adult mice. Interestingly, the binge-induced increases in receptor expression seen in adult animals were not present in the adolescent bingers. These results paralleled the behavioral data from the marble burying test and provide additional evidence in support of adolescent resilience to binge-induced behavioral and neurobiological abnormalities.

4.2.2 Kinase expression in the AcbC parallels withdrawal-induced anxiety. Adult bingers showed an increase in CAMKII and activated PKCε in the AcbC at 24 h withdrawal, which also tracked with the behavioral data from the marble burying test. Similar to the results from the AcbSh, these protein changes were not present in adolescent bingers. Although there were no changes seen in mGlu1/5 receptor expression, both PKCε and CAMKII are downstream substrates of group1 mGlu activation and have both been implicated in alcohol-induced neural adaptations (Lee & Messing, 2008). PKCε is an emergent target of interest in the treatment of alcoholism given its role in the maintenance and escalation of drinking (Cozzoli et al., 2015; Gass & Olive, 2009; Lesscher et al., 2009) and its ability to influence hypnotic sensitivity to alcohol (Choi et al., 2002). PKCε is also of interest in the treatment of anxiety due to its ability to regulate GABA receptor function (Gordon, 2002). Additionally, animal studies that show PKCε knockout mice are less anxious than wild types (Hodge et al., 2002). Therefore, it is plausible that an alcohol-induced increase in activated PKCε may contribute to a hyperanxious state during withdrawal.

CAMKII is a critically important regulator of glutamatergic signaling. CAMKII interacts directly with both metabotropic and ionotropic glutamate receptors and plays an essential role in controlling receptor function, trafficking, and localization (Mao et al., 2014).

As such, CAMKII is a protein marker of synaptic plasticity and is essential for long-term potentiation. CAMKII-dependent modulation of AMPA receptor trafficking within the Acb is theorized to contribute to the maladaptive plasticity resulting from other drugs of abuse (Pierce & Wolf, 2013; Scheyer et al., 2016). Studies have shown that CAMKII plays a significant role in addiction as a mediator between accumbal DA and glutamate (Anderson et al., 2008). Accordingly, CAMKII is associated with craving and relapse for a variety of drugs including morphine (Liu et al., 2012), cocaine (Easton et al., 2014), and alcohol (Zhao et al., 2015). Alcohol has been shown to increase CAMKII-dependent phosphorylation of AMPA receptors within the Acb (Cannady et al., 2016) and elevated Acb CAMKII is theorized to contribute to the reinforcing properties of alcohol (Easton, Lucchesi, Lourdusamy, et al., 2013; Easton, Lucchesi, Mizuno, et al., 2013). Although the involvement of Acb CAMKII in emotional processes has not been well-defined, CAMKII is thought to play a role in anxiety through its enhancement of AMPA and NMDA activity, as both AMPA and NMDA blockade within the Acb has anxiolytic effects (Martinez et al., 2002).

In addition to these kinase changes, we also found an age-independent increase in GluN2b in the AcbC, which resembled the behavioral changes seen in the FST. Given the role of the AcbC as a "limbic-motor interface" integrating motivation and action (Mogenson et al., 1980), the increased glutamate receptor expression in the AcbC could render animals hypersensitive to stressful environmental conditions and primed to flee from aversive or threatening situations in a panic-like state. In support of this interpretation, a study characterizing the behavior of mGlu2 knockout mice showed that these animals displayed hyperlocomotion under the stressful conditions of the FST, which coincided with enhanced glutamate signaling in the Acb (Morishima et al., 2005). However, it is also possible that

these AcbC protein changes reflect adaptations related to conditioned aspects of drug reinforcement, many of which are mediated by the AcbC. For example, AcbC NMDA receptors are necessary for alcohol conditioned place-preference (Gremel & Cunningham, 2009) and AcbC NMDA signaling has been shown to mediate aversion-resistant alcohol consumption (Seif et al., 2013). Additionally, increased glutamatergic transmission within the AcbC is associated with cue-induced reinstatement of alcohol seeking (Gass et al., 2011).

Individual housing conditions are capable of eliciting behavioral and/or neurochemical changes (Brain, 1975; Goldsmith et al., 1978; Hilakivi et al., 1989), particularly in adolescent animals (Amiri et al., 2015; Robbins et al., 1996; Weintraub et al., 2010). Although individual housing could be a potential confounding factor for our results, other researchers have demonstrated that elevated alcohol consumption and resilience to withdrawal-induced anxiety in adolescent animals is not a function of isolation stress/individual housing (Brunell & Spear, 2005). Additionally, our lab has completed subsequent unpublished replicates of this study design in which animals were group housed and only separated during the drinking period. These experiments have yielded comparable alcohol intake and behavioral data. Although tissue samples were also collected from these animals, the immunoblotting has not yet been processed. Therefore, it will be interesting to see what, if any, effect single-housing stress has on binge-induced changes in protein expression.

Although our binge-induced changes in kinase-related proteins did not perfectly align with receptor changes in either the AcbSh or Acb, changes in total protein expression do not always correspond to changes in receptor function, nor do they necessarily indicate the behavioral relevance of the receptor for alcohol intake. Indeed, our laboratory has detected

alcohol-induced changes in Acb levels of mGlu5 in some (Fig. 4C; Goulding et al., 2011; Cozzoli et al., 2012), but not all studies (Szumlinski et al., 2007), which may reflect differences in the subregions examined, route of administration/drinking paradigm employed. Nevertheless, intact mGlu5 function within Acb subregions, notably the AcbSh, is important for binge-drinking behavior (Besheer et al., 2010; Cozzoli et al., 2009; Sinclair et al., 2012). Therefore, behavioral differences could be driven by changes in receptor function that are not reflected in changes in total protein expression. Given the present observation of an age-related difference in AcbSh mGlu5 expression, it is important for follow-up studies to causally relate to the manifestation of hyper-anxiety during early alcohol withdrawal to mGlu5 function within Acb subregions and determine age-related differences therein.

It is noteworthy that the AcbSh and AcbC show distinct profiles of binge-induced protein changes. These regions, while highly interconnected, are both functionally and anatomically distinct and serve unique roles in the neurobiology of drug abuse (Di Chiara, 2002; Quintero, 2013; Salgado & Kaplitt, 2015). Given that the Acb does not respond in unison to binge-induced dysregulation, exploring the functional significance and of these region-specific consequences of binge drinking is worthy of future investigation. It is also important to acknowledge that these neurobiological changes found in the Acb may not be functionally related to our behavioral data and perhaps a different brain region such as the amygdala or BNST is the primary mediator of these changes. This study provides a necessary initial characterization of distinct, age-dependent differences in alcohol-induced neuroadaptations and withdrawal phenotype in adult and adolescent male mice following a relatively brief history of binge drinking. However, further research is necessary to establish

a causal relationship between alcohol-induced changes in the Acb and withdrawal-induced negative affect.

4.3 Conclusions

In this study, we demonstrate that a 2-week voluntary binge-drinking experience is sufficient to increase behavioral signs of anxiety in adult male mice, concomitant with increased indices of excitatory neurotransmission within Acb subregions. Despite exhibiting higher basal glutamate receptor expression and greater alcohol intake than adult mice, adolescents appear more resilient than adults to particular affective and neurobiological consequences of binge drinking during early withdrawal. Given that glutamatergic synapses in the Acb are not yet fully developed in adolescence, this immaturity may be protective and render them less susceptible to alcohol-induced perturbation compared to adult animals, as was demonstrated in the present study. However, engaging in binge drinking during adolescence could adversely affect the maturation of this system and shape developing neural circuitry in such a way that creates a predisposition to addiction later in life. Additionally, this study presents an intriguing possibility for the involvement of excitatory signaling within the Acb in withdrawal-induced anxiety in adult bingers, which warrants further investigation. Thus, ontogenetic differences exist in vulnerability to alcohol-induced neuroplasticity within Acb that could contribute to age-related differences in binge drinking behavior and future addiction vulnerability.

Chapter 4:

Anxiolytic effects of buspirone and MTEP in the Porsolt Forced Swim Test during withdrawal from binge drinking

1. Introduction

The Porsolt Forced Swim Test (FST) was first described by Dr. Roger Porsolt in the late 1970s and has been used traditionally as a behavioral screen with high predictive validity for the clinical efficacy of anti-depressant drugs (Porsolt, Bertin, et al., 1977). Compounds with anti-depressant properties have been shown to reduce immobile floating behavior and increase active swimming in the FST (Porsolt, Le Pichon, et al., 1977). Researchers have also used the FST as a behavioral model for depression in which increased immobility is associated with a depressive state (Porsolt et al., 2001; Porsolt, Le Pichon, et al., 1977). Immobile floating behavior is thought to reflect the hopelessness or helplessness associated with depression [i.e., behavioral despair] (Alloy et al., 1988).

Our laboratory has previously employed the FST in a series of studies designed to assay the affective consequences of binge-like alcohol consumption (Lee et al., 2015; Lee et al., 2016). Chronic alcohol consumption, even at moderate levels, is known to elicit a dysphoric state during withdrawal (Wilson, 1988), as reported both in humans (Driessen et al., 2001; Kiefer et al., 2002; Schuckit & Hesselbrock, 1994) and a variety of animal models (Emmett-Oglesby et al., 1990; Getachew et al., 2008; Knapp et al., 1998; Valcheva-Kuzmanova et al., 2007). Our studies demonstrated that even a 2-week binge-drinking history is sufficient to increase anxiety-related behaviors in adult animals during early (24hrs) withdrawal across various behavioral assays, including the light-dark box and defensive marble burying test (Lee et al., 2015; Lee et al., 2016). However, we have also reported that the increase in anxiety-related behaviors consistently coincides with *reduced* immobility in the FST. These results were unexpected, as according to traditional interpretations, this decrease in floating behavior would suggest an anti-depressant effect of

alcohol withdrawal, which is counter-intuitive based on the characterization of alcohol withdrawal described in the extant clinical and basic science literature highlighted above. Based on this collection of observations, we speculated that the reduced immobility exhibited by alcohol-withdrawn mice in the FST (Lee et al., 2015, 2016) might reflect psychomotor hyper-reactivity to an acute swim stressor, analogous to a panic-like fight or flight response (Graeff, 2007).

Panic is a state characterized by intense fear and anxiety and is frequently accompanied by elevated heart rate, perspiration, shortness of breath, dizziness, and shaking (American Psychiatric Association, 2013). In the clinical population, panic is frequently a symptom of generalized anxiety disorder but can also itself constitute a discrete anxiety disorder subtype (Craske et al., 2010). Interestingly, there is also an association between alcohol use disorders and panic in the human population (e.g. Cosci et al., 2007), which, theoretically, might be expressed in animal models of alcohol abuse. In laboratory animals, panic is commonly quantified with assessments of stimulus-provoked flight, freezing, and/or defensive attack, typically in response to an unconditioned predator stimulus (Blanchard et al., 2003). It is plausible that the acute stress of the FST and the unconditioned fear of drowning presents a similar survival threat, leading to a manifestation of a prolonged fight or flight response in anxious animals (Polani, 2004). Indeed, other reports comparing behavior in the elevated plus-maze and FST noted more active struggling behavior (defined as "a presence of energetic escape-directed movements") in the FST in animals characterized as "high anxiety" on the plus-maze, compared to "low anxiety" counterparts (Ferre et al., 1994).

Through our investigation of the neurobiological correlates of alcohol withdrawal, we identified an increase in mGlu5 receptor protein expression within the nucleus accumbens

shell (AcbSh) as a potential mediator of withdrawal-induced hyper-anxiety (Lee et al., 2016). In support of this notion, mGlu5 antagonists such as 2-methyl-6-(phenylethynyl)-pyridine (MPEP) and and 1-(3-Chlorophenyl)-3-(3-methyl-5-oxo-4H-imidazol-2-yl)urea (fenobam), and negative allosteric modulators such as 3-[(2-methyl-1,3-thiazol-4-yl)ethynyl]pyridine (MTEP) exert anxiolytic effects in various animals (Busse et al., 2004; Porter et al., 2005; Tatarczynska et al., 2001; Varty et al., 2005). Thus, we tested the effects of reducing mGlu5 signaling upon behavior in the FST during early alcohol withdrawal compared to the prototypical anxiolytic buspirone (Batool, 2007), a 5-HT_{1a} partial agonist with efficacy in alleviating symptoms of alcohol withdrawal-induced anxiety (Lal et al., 1991).

2. Materials and Methods

Experimental procedures were similar to those described in our previous studies of alcohol withdrawal-induced anxiety (Lee et al., 2015; Lee et al., 2016) and are briefly summarized below.

2.1 Subjects

This study used 60 adult male C57BL/6J mice (Jackson Laboratories, Sacramento, CA) that were 8 weeks of age at the onset of drinking. Animals were housed in groups of 4 in standard Plexiglas cages, in a temperature-controlled vivarium (23◦C), under a 12 hr reverse light/dark cycle (lights off at 10 am). Animals were identified using small animal ear tags (Stoelting, Wood Dale, IL). Food and water were available *ad libitum*, with the exception of the 2-hr alcohol- drinking period. The study had a 2 (alcohol or water) X 3 (vehicle, buspirone, or MTEP) factorial design, with n=10/group. All experiments were

conducted in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (NIH Publication No. 80–23, revised 2014) and approved by the IACUC of the University of California, Santa Barbara.

2.2 Drinking-in-the-Dark (DID) Procedures

Half of the animals were subjected to 14 consecutive days of binge-drinking under modified 3-bottle Drinking-in-the-Dark (DID) procedures, while control animals were given a single bottle of water. Alcohol access was restricted to 14 days in this study to be consistent with our more recent studies of the ontogeny of binge-drinking (Lee et al., 2016). Each day prior to the drinking period, animals were separated into individual cages and allowed to acclimate for approximately 45 min. Beginning 3 hr into the dark phase of the circadian cycle, corresponding to the peak time of daily fluid intake (Rhodes et al., 2005), animals were given concurrent access to 10, 20 and 40% (v/v) unsweetened ethanol solutions for 2 hr. Animals were returned to their original group housing at the conclusion of the 2-hr drinking period. While our prior work employed 4-bottle drinking procedures (offering mice access also to a sipper tube containing 5% ethanol), alcohol intake from this solution is negligible, compared to that of 10, 20 and 40% ethanol (Cozzoli et al., 2014; Lee et al., 2015; Lee et al., 2016). Thus, the 5% concentration was not included in the present study. The amount of alcohol consumed each day was calculated by bottle weight immediately before and after the drinking period and expressed as a function of the animal's body weight (in kg). All animals, both alcohol and water drinkers, were weighed 3x per week throughout the drinking period.

2.2.1 Blood alcohol sampling. Submandibular blood samples were collected from all alcohol-drinking animals on day 10 of drinking, immediately following the 2-hr drinking period. The scheduling of the blood sampling was selected to ensure that the animals' intakes had stabilized, while also allowing ample time for recovery prior to behavioral testing. Blood alcohol concentrations (BACs) were determined using an Analox alcohol analyzer (model AM1, Analox Instruments USA, Lunenburg, MA), according to the manufacturer's instructions.

2.3 Drugs

Treatments were administered systemically via intraperitoneal injection, 30 min prior to the onset of behavioral testing. Ten animals from each drinking group received a 5 mg/kg injection of buspirone hydrochloride (Sigma-Aldrich, Atlanta, GA) in sterile water. This buspirone dose was selected for study as it elicits anxiolytic effects in other drug-related behavioral paradigms (Ettenberg & Bernardi, 2006; Nishimura et al., 1993; Risbrough et al., 2003). An additional 10 animals from each drinking group received a 3 mg/kg injection of the mGlu5 antagonist MTEP in sterile water. This MTEP dose was selected for study based on evidence of anxiolytic efficacy in rodents (Klodzinska et al., 2004; Pietraszek et al., 2005; Stachowicz et al., 2007). The remaining animals served as vehicle controls and received injections of sterile water (vol=0.01 ml/g).

2.4 Behavioral testing

Behavioral testing commenced approximately 24 hr following the final alcohol presentation and thus occurred during the circadian dark phase.

2.4.1 Porsolt Forced Swim Test. Each animal was placed into an 11-cm diameter cylindrical container filled with room-temperature water deep enough that animals were unable to touch the bottom of the enclosure. The latency to first exhibit immobility (defined as no horizontal or vertical displacement of the animal's center of gravity for ≥5 s), total time spent immobile, and the numbers of immobile episodes were monitored throughout the entire 6-min trial duration using AnyMaze^{TM} tracking software (Stoelting Co., Wood Dale, IL, USA), as conducted previously (Lee et al., 2016).

2.4.2 Locomotor activity. Following the FST, animals were allowed to dry off and recover for 20 min before being assessed for generalized locomotor effects of the anxiolytic treatments. Animals were placed in a polycarbonate box measuring 24cm long X 23cm wide X 24cm high and the total distance traveled was monitored for during a 15-min trial using Any-maze™ tracking software (Stoelting Co., Wood Dale, IL). Locomotor testing occurred within 20 min post-FST and thus, occurred at approximately 60 min following drug injection.

2.5 Statistical Analysis.

A one-way analysis of variance (ANOVA) was conducted first to ensure that there were no differences in alcohol intake between the 3 treatment groups. To determine the relationship between alcohol intake and resulting BACs, a Pearson's correlational analysis was conducted. Behavioral data were analyzed using between-subjects, two-way ANOVAs with Tukey's multiple comparison tests; α=0.05. All calculations and analyses were performed using SPSS v.21 statistical software (IBM, 2012).

3. Results

3.1 Alcohol consumption

Across the entire 14-day drinking period, animals had an average alcohol intake of 4.00±0.05 g/kg, which has been demonstrated to results in BACs >80mg/dL (Cozzoli et al., 2009; Rhodes et al., 2005). On day 10 of drinking, the animals averaged an intake lower than the 14-day average (3.37±0.16 g/kg) and this intake resulted in an average BAC of 73.19±3.84 mg/dl. Importantly, there was a significant positive correlation between intake and BAC (r=0.45, p=0.013; Fig 1). There were no significant differences in alcohol consumption across the 3 treatment groups [F(2,27)=0.84, p=0.44].

3.2 FST Behavior

3.2.1 Withdrawal-induced reduction in immobility. In the FST, we replicated our prior observations that early alcohol withdrawal reduces immobility in adult male mice (Lee et al., 2015; Lee et al., 2016). The ANOVA revealed a significant main alcohol effect for all 3 dependent behavioral measures. Overall, alcohol-drinking mice spent less time immobile [F(1, 54)=11.89, p=0.001; Fig. 2A], exhibited fewer immobile episodes [F(1, 54)=21.33, p<0.001; Fig. 2B], and a longer latency to first immobility [F(1,54)=6.57, p=0.013; Fig.2C], compared to water controls. More specifically, Tukey's post-hoc tests showed that vehicle-treated alcohol-drinking mice spent significantly less time immobile (p=0.026, Fig 2A), had fewer immobile episodes (p=0.001, Fig 2B) and a longer latency to first immobility (p=0.024, Fig 2C) compared to vehicle-treated water control animals, thus confirming the presence of alcohol withdrawal-induced behavioral differences.

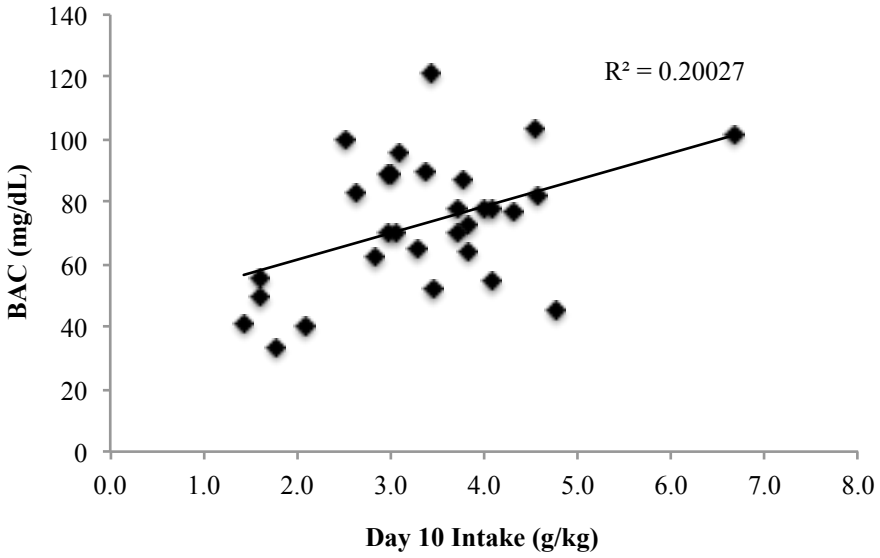

Figure 4.1. BAC and alcohol consumption. On day 10, animals consumed an average of 3.37±0.16 g/kg with a resulting BAC of 73.19±3.84 mg/dl, n=30. There was a significant positive correlation between intake and BAC (r=0.45, p=0.013).

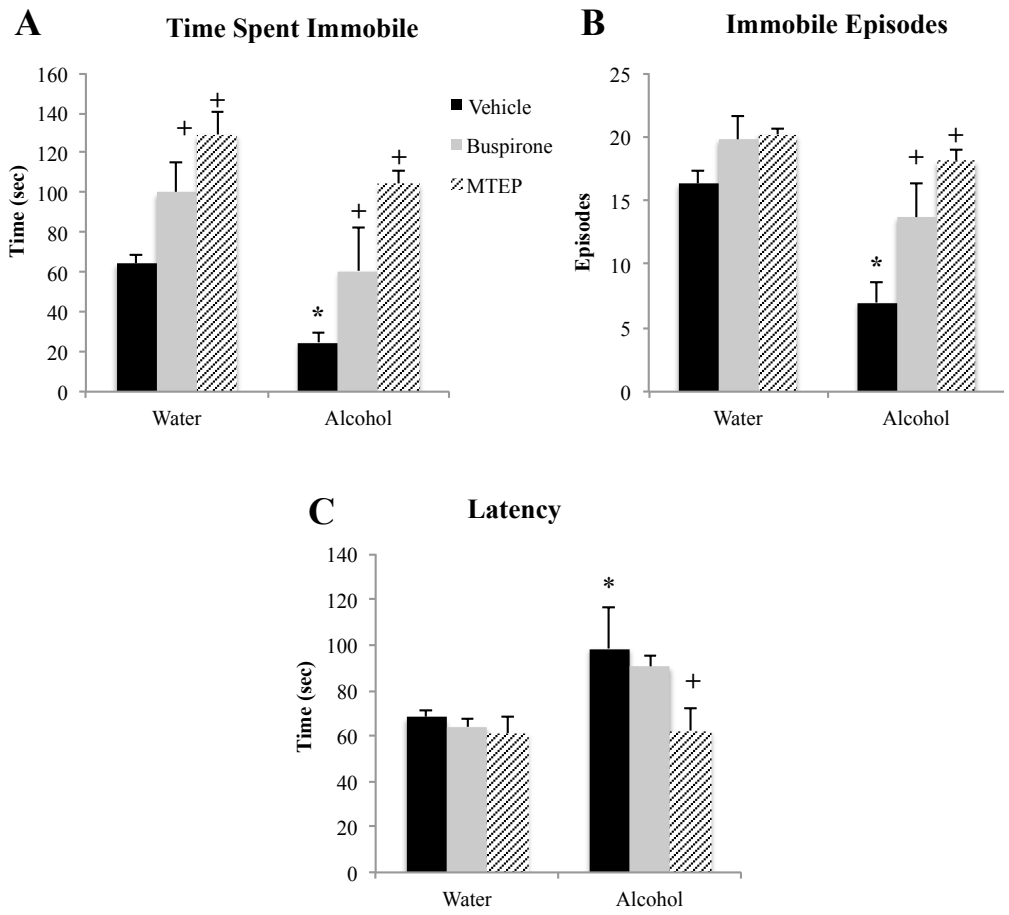

Figure 4.2. Withdrawal from binge drinking decreases immobility in the FST, an effect reversed by anxiolytics. **(A)** Vehicle-treated alcohol drinkers spent significantly less time immobile compared to vehicle-treated water controls. Buspirone and MTEP treatment significantly increased time immobile in both water and alcohol drinkers. **(B)** Vehicle-treated bingers had less immobile episodes compared to vehicle-treated water controls. Buspirone and MTEP treatment significantly increased immobile episodes in alcohol but not water drinkers. **(C)** Vehicle-treated bingers a longer latency to first immobility compared to vehicle-treated water controls. MTEP but not buspirone reduced latency in bingers only. Neither MTEP nor buspirone affected latency in water drinkers. *Tukey's $p<0.05$ vs water control; +$p<0.05$ vs vehicle treatment; n=10/treatment group.

3.2.2 Anxiolytic effects on immobility. There was a significant effect of treatment on time spent immobile [$F(2, 54)=17.57$, $p<0.001$; Fig. 2A]. Relative to vehicle treatment, both buspirone and MTEP significantly increased time spent immobile and this effect was apparent in alcohol- and water-drinking mice alike (buspirone: for alcohol, $p=0.042$; for water, $p=0.044$; MTEP: for water, $p=0.001$; for alcohol, $p<0.001$). A direct comparison of the effects of buspirone versus MTEP upon the time immobile exhibited by alcohol-drinking mice indicated a significantly greater effect of MTEP ($p=0.014$) and the data for water controls also exhibited a similar trend toward significance ($p=0.09$).

There was also a significant effect of treatment on total immobile episodes [$F(2, 54)=12.26$, $p<0.001$, Fig. 2B]. Buspirone and MTEP both increased immobile episodes in alcohol-drinking mice, relative to vehicle treatment ($p=0.014$ and $p<0.001$, respectively), although their effects on this measure were not statistically significant in the water controls ($p=0.11$ and $p=0.08$, respectively). As observed for the time spent immobile, MTEP increased the number of immobile episodes in alcohol-drinking mice to a greater extent than buspirone ($p=0.04$), with no drug-related differences noted for water controls ($p>0.10$).

There was a strong trend toward a main treatment effect on latency to immobility [$F(2,54)=2.88$, $p=0.06$] and inspection of Fig. 2C suggested that this trend was driven exclusively by an effect of MTEP upon this measure. As the results above suggested that MTEP was a more effective anxiolytic in alcohol-drinking mice than buspirone, Tukey's post-hoc tests were employed and determined that MTEP did, in fact, exert a statistically significant effect upon the latency to immobility ($p=0.008$), while buspirone did not ($p=0.54$). Neither compound altered the latency to the first immobile episode in water controls (p's>0.10).

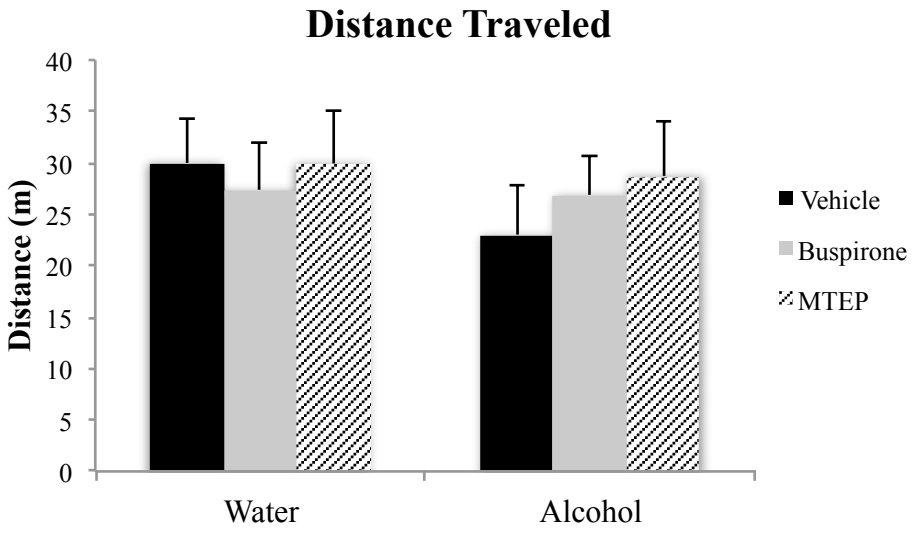

Figure 4.3. General locomotor activity. There were no significant differences between any of the groups in distance traveled in the activity monitor (p's>0.05; n=10/treatment group).

3.2.3 General locomotor activity. There were no significant effects of alcohol or anxiolytic treatment on the total distance traveled, when assessed following the FST (one-way ANOVA $p>0.10$; Fig. 3).

4. Discussion

In this study, we replicated our previous findings (Lee et al., 2015; Lee et al., 2016) showing decreased immobility in the FST during early withdrawal following a 14-day period of binge-drinking, as indicated by a longer latency to first become immobile, as well as reduced time spent immobile and reduced number of immobile episodes. Although the average BAC obtained on day 10 of drinking (73.19±3.84 mg/dL) was slightly under the 80 mg/dL specified in the official NIAAA definition of binge drinking (National Institute on Alcohol Abuse and Alcoholism, 2004; Ramboz et al., 1998), alcohol intake was lower on this day than the average alcohol intake observed across the entire 2-week drinking period (day 10: 3.37±0.16 vs. average: 4.00±0.05 g/kg). Importantly, the BACs on day 10 of drinking were significantly correlated with alcohol intake on that day and the average alcohol intake for the entire drinking period was above that reported to result in BACs above the 80 mg/dL NIAAA criterion for binge-drinking (e.g. Cozzoli et al., 2014; Lee et al., 2015; Lee et al., 2016; Rhodes et al., 2005). Thus, while the animals may not have achieved BACs > 80 mg/dL every day during the 2-week drinking period, the alcohol intakes (and resultant BACs) were nonetheless sufficient to elicit signs of behavioral dysregulation during withdrawal, as manifested by reduced immobility/increased swimming in the FST. Although other common methods of high alcohol exposure (e.g. vapor inhalation, gavage, liquid diet, injection) may be capable of eliciting more robust withdrawal symptoms, the present data

add to our prior evidence that a history of voluntary alcohol consumption is sufficient to elicit negative affective consequences in mice, while maintaining ethological validity.

In this study, treatment with the prototypical anxiolytic buspirone, as well as the non-conventional anxiolytic MTEP, reversed the effect of alcohol withdrawal upon swimming behavior and increased immobility. Moreover, consistent with the results of a prior report for buspirone (Kitamura & Nagatani, 1996), both buspirone and MTEP reduced some signs of immobility also in alcohol-naïve, water-drinking, controls. Our finding that buspirone and MTEP pretreatment did not impact the behavior of water controls on all measures obtained from the FST is in line with the results of other studies demonstrating minimal or absent effects of anxiolytic treatment on the behavior of non-anxious animals (Horváth et al., 2015; Landgraf & Wigger, 2002; Liebsch et al., 1998; Naslund et al., 2015). Importantly, our FST results for buspirone, in particular, strongly support our initial hypothesis that the reduction in immobility/increased swimming behavior observed consistently by our laboratory in alcohol-withdrawn animals (Fig.2; Lee et al., 2015, 2016) reflects an anxiety-related response.

Neither buspirone nor MTEP altered locomotor activity, when assessed following the 6-min FST. This negative outcome argues against the drug-induced increase in immobility/reduction in swimming behavior observed in the FST being attributable to non-specific motor effects of the doses administered. The fact that the effects of both buspirone and MTEP on the specific dependent variables examined in the FST were either weaker or absent in water controls versus alcohol-withdrawn mice also argues that the drug doses administered were not hypnotic or motor-impairing. It is also unlikely that our results were

confounded by an alcohol or treatment-related learning/ memory deficit, as animals were tested in a single trial and the tank diameter is such that it is readily apparent that there is no means of escape or platform to shelter upon. Therefore, we conclude that reduced immobility/increased swimming in the FST reflects the anxiogenic effects of alcohol withdrawal, possibly indicative of panic-like behavioral hyper-reactivity.

In support of our interpretation and consistent with the reduction in swimming produced by acute treatment with buspirone (see also Kitamura & Nagatani, 1996) and/or MTEP in both alcohol-withdrawn and alcohol-naïve mice, the anxiolytic benzodiazepine diazepam is also reported to significantly increase the duration of immobility, while the anxiogenic drug beta-CCE conversely reduces immobility (Nishimura et al. 1989). Furthermore, mGlu2 knock-out (KO) mice, reported to exhibit elevated anxiety-like behaviors on more conventional anxiety tests such as the open field test and elevated plus maze (Ceolin et al., 2011), also exhibit decreased immobility in the FST compared to wild-type mice, but do not differ from wild-type mice with respect to behavior in another test with predictive validity for anti-depressant efficacy – the tail suspension test (Morishima et al., 2005). A similar correlation between anxiety-like behavior and reduced immobility in the FST has also been reported in $5HT1_a$ KO mice (Ramboz et al., 1998). Thus, both behavioral pharmacological and genetics evidence argue that reduced immobility/increased swimming in the FST can reflect increased anxiety in rodent models.

It is interesting to note that, at the doses tested herein, MTEP treatment was more effective than buspirone at increasing immobility in alcohol-withdrawn animals on all 3 dependent variables. Other studies have also shown MTEP to out-perform buspirone in anxiety tests such as fear-potentiated startle (Brodkin et al., 2002) and the anti-conflict test

(Goldberg et al., 1983). Together, the present findings, coupled with evidence from other laboratories (Kotlinska & Bochenski, 2008; Kumar et al., 2013), suggest that mGlu5 inhibitors may be more effective anxiolytics than conventional treatments such as buspirone, particularly for alleviating alcohol withdrawal-induced anxiety. Early withdrawal from a history of voluntary alcohol-drinking increases mGlu5 signaling throughout the extended amygdala (Cozzoli et al., 2012; Cozzoli et al., 2014; Cozzoli et al., 2009; Lee et al., 2016; Obara et al., 2009; Szumlinski, Ary, Lominac, et al., 2008)– a neurocircuit critically involved in regulating the anxiogenic/negative affective properties of drug withdrawal (Koob, 2003, 2013), and recent correlative evidence suggests a relationship between the manifestation of alcohol withdrawal-induced hyper-anxiety and mGlu5 expression, at least within the AcbSh (Lee et al., 2016).

The present data for MTEP argue that the upregulation of mGlu5 signaling during early withdrawal from voluntary alcohol consumption may be causally related to withdrawal-induced anxiety, implicating increased mGlu5 signaling in the neurochemical imbalance driving the hyper-anxious state during alcohol withdrawal. However, while the mGlu5 negative allosteric modulator fenobam demonstrates anxiolytic efficacy comparable to benzodiazepines, with an improved side-effect profile with respect to its hypnotic and alcohol-promoting effects (Goldberg et al., 1983; Pecknold et al., 1982; Porter et al., 2005), amnesic and psychotomimetic side effects currently limit its clinical utility (Jacob et al., 2009; Palucha & Pilc, 2007). Thus, additional research into the anxiolytic potential of mGlu5 antagonists could provide a beneficial clinical tool for the treatment of anxiety disorders and substance abuse-related anxiety.

As a final point of discussion, it is important to clarify that the intention of this study was not to invalidate the FST as a predictive animal model for anti-depressant efficacy. Rather, we conducted this study to draw attention to alternative interpretations of the behavioral outcomes from this assay and to highlight the importance of testing animals in a variety of paradigms with predictive validity for both anxiolytic and anti-depressant action, as the results from one assay alone are often subject to interpretational debate (see, for examples, Olausson et al., 1999, 2000; Ouagazzal et al., 1999; Pellow, 1986; Pellow et al., 1985 for debate over interpretation of behavior in the elevated plus-maze and other exploratory animal models). As such, results should be considered carefully, particularly within the context of the animals' history, and additional measures/assays should be included whenever possible to facilitate distinction between anxiety- vs. depression-related behavioral phenotypes in the FST. For examples, other assays with predictive validity for anti-depressant efficacy [e.g, the sucrose preference test (Katz, 1982), tail suspension test (Steru et al., 1985), or intracranial self-stimulation (Vogel et al., 1990)], and tests with predictive validity for anxiolytic efficacy [e.g., elevated plus maze (Walf & Frye, 2007), light-dark box (Bourin & Hascoet, 2003), defensive burying (File et al., 2004) or conflict test (Moreira et al., 2006)] are well-validated options for consideration when trying to interpret behavioral changes within the FST.

In conclusion, this study provides evidence that reduced immobility in the FST may reflect anxiety, validated by a subsequent increase in immobility following anxiolytic treatment. Within this interpretational context, we also conclude that systemic mGlu5 blockade has robust anxiolytic properties in alcohol-withdrawn animals, providing cause-

effect evidence implicating increased mGlu5 signaling in the etiology of withdrawal-induced anxiety. As withdrawal-induced anxiety acts as a motivator of subsequent drinking, further research into the formulation of mGlu5 inhibitors for alleviating withdrawal-induced anxiety could yield additional non-benzodiazepine anxiolytic options, with lower abuse liability, for patients with a history of alcohol abuse.

Chapter 5:

Negative affect and elevated alcohol intake incubate during protracted withdrawal from binge-drinking in adolescent, but not adult, mice

1. Introduction

Adolescence is a critical period of accelerated neurodevelopment, which occurs between the ages of approximately 11-21 years in humans and conservative estimates of adolescence in rodents range from postnatal days (PNDs) 28-42 (Spear, 2000b). During adolescence, there is a dramatic reduction of gray matter as the cortex undergoes synaptic pruning, and a proliferation of white matter from ongoing myelination of axons, leading to extensive remodeling of the structure and function of the brain (e.g. Gogtay et al., 2004; Sowell et al., 2003). These processes are essential for refining excitatory and inhibitory connectivity and stabilizing synapses within corticofugal projections that exert control over subcortical hyperactivation (Arain et al., 2013; Casey et al., 2011; Sturman & Moghaddam, 2011). Thus, adolescents typically exhibit increased impulsivity, sensation/novelty seeking, risk-taking, and mood swings, compared to adults (Casey, Getz, et al., 2008; Spear & Swartzwelder, 2014; Sturman & Moghaddam, 2011).

Drug experimentation is also common during the adolescent stage of development, with alcohol being the most commonly used substance among adolescents (Kelley et al., 2004; Lopez et al., 2008). Indeed, underage alcohol-drinking is a serious public health concern, with 7.7 million individuals between the ages of 12-20 reporting drinking alcohol within the past month (published by the Center for Behavioral Health Statistics and Quality, 2016). Over 90% of alcohol consumed by underage drinkers is in the form of binge-drinking episodes (NIAAA, 2017), i.e. consumption sufficient to achieve a blood alcohol concentration (BACs) ≥80 mg/dL (approximately 4-5 drinks) in a 2-hr period (NIAAA, 2004). Additionally, research has consistently shown that adolescent drinking is one of the

strongest predictors of substance abuse problems and addiction later in life (Chassin et al., 2002; Grant & Dawson, 1997; Tapert & Schweinsburg, 2005).

In both humans and animal models, adolescents typically consume larger quantities of alcohol than adults per drinking episode and adolescents also respond differently to alcohol than their adult counterparts (Novier et al., 2015; Spear & Varlinskaya, 2005; White, Truesdale, et al., 2002). Adult drinkers often show pronounced signs of acute withdrawal following a binge episode, including headaches, anxiety, agitation, lethargy, gastrointestinal distress, in severe cases even withdrawal-induced seizures (Knapp et al., 1998). In contrast, both clinical and preclinical data show that adolescents tend to be less sensitive than adults both to the negative properties of acute intoxication such as sedation, motor impairment, and hypothermia, as well as the 'hangover' symptoms seen in adults during withdrawal (Anderson et al., 2010; Doremus et al., 2003; Little et al., 1996; Schramm-Sapyta et al., 2010; Varlinskaya & Spear, 2004; White, Truesdale, et al., 2002). At the same time, adolescents show increased sensitivity to the pleasurable, reinforcing properties of alcohol such as positive reward and social facilitation (Doremus-Fitzwater et al., 2010; Pautassi et al., 2008; Ristuccia & Spear, 2008). Blunting of the aversive consequences that typically serve as negative feedback to inhibit excessive consumption, along with enhancement of the positive incentive properties of alcohol, are theorized to promote high alcohol consumption in both human and animal adolescents (Spear & Varlinskaya, 2005).

Binge-drinking is the most toxic pattern of excessive alcohol consumption and has been shown to produce a 'kindling' effect (Ballenger & Post, 1978; Becker, 1998), whereby

repeated cycles of acute intoxication followed by periods of abstinence intensify withdrawal-induced neurotoxicity (Begleiter & Porjesz, 1979; Duka et al., 2004; Overstreet et al., 2002). Frequent binge-drinkers can rapidly develop tolerance to the subjective intoxicating effects of alcohol, leading to an escalation of intake and brain exposure to harmful concentrations of alcohol (Gruber et al., 1996; Hoffman & Tabakoff, 1989; Tabakoff et al., 1986). This is particularly concerning, as research suggests that adolescents are uniquely susceptible to neurotoxic insult resulting from chronic alcohol exposure and can suffer potentially life-long dysfunction resulting from perturbed maturation of prefrontal control over subcortical circuitry, particularly within regions involved in emotionality (Casey & Jones, 2010; Crews et al., 2016).

Studies have revealed persistent alcohol-induced neurobiological changes within the extended amygdala - the subcortical macrostructure integrally involved in governing diverse emotional states (Alheid, 2003; Jennings et al., 2013; Shackman & Fox, 2016). The extended amygdala consists of the central nucleus of the amygdala (CEA), bed nucleus of the stria terminalis (BNST), and shell subregion of the nucleus accumbens (AcbSh). These structures are highly vulnerable to drug-induced plasticity and dysregulation within the extended amygdala circuitry is known to underlie many of the negative reinforcing properties of withdrawal that fuel the cycle of addiction (reviewed in Baker et al., 2004; Koob, 2003). Mood disorders such as anxiety and depression, are also thought to be related to abnormal corticofugal development resulting in insufficient regulatory control over subcortical regions involved in emotion and motivation, for example the AchSh and CEA (Andersen & Teicher, 2008). Common underlying neuropathology could account for the high comorbidity between

alcohol abuse and mood disorder, which is especially prominent amongst those with a history of drinking during adolescence. In fact, adolescent alcohol use disorder is one of the strongest predictors of major depressive disorder in adulthood (Briere et al., 2014; Grant & Dawson, 1997).

Consistent with existing human and animal research, previous work from our lab has demonstrated that adolescent mice exhibit minimal signs of negative affect during early (24hr) withdrawal, and are also resistant to changes in protein expression within the Acb (Lee et al., 2016) using the Drinking-in-the-Dark animal model of voluntary binge-drinking (Rhodes et al., 2005; Rhodes et al., 2007; Thiele & Navarro, 2014). For example, we demonstrated recently that, in contrast to adult binge-drinking mice that exhibit robust anxiety-like behavior during early (24 h) withdrawal across several conventional behavioral tests of negative affect (e.g., light-dark shuttle box, novel object encounter, Porsolt swim test, elevated plus-maze), adolescent binge-drinking mice resemble water-drinking controls (Lee et al. 2015, 2016). In the present study, we sought to expand these findings to assess the adult consequences of adolescent binge-drinking on negative affect and subsequent alcohol-drinking. Based on the human literature (Briere et al., 2014; Chassin et al., 2002; Grant & Dawson, 1997), we predicted that when tested during adulthood (i.e. in protracted withdrawal), adolescent drinkers would show signs of alcohol-induced negative affect and increased alcohol consumption. Other studies of this nature have typically employed alcohol-naïve animals as the control group; however, we also wanted to compare adolescent drinkers to animals with equivalent drinking experience during adulthood, in order to specifically isolate the unique effects of alcohol during adolescence from the non-age-dependent effects

of alcohol, more generally. Based on the high-risk nature of adolescent binge-drinking reported clinically, we speculated that the withdrawal-induced hyper-anxiety manifested in adulthood would be more pronounced in animals with a prior history of binge-drinking during adolescence, than in animals with a prior history of drinking during adulthood. To complement the behavioral data, we also collected brain tissue samples from the AcbSh and CEA for immunoblotting, as these extended amygdala structures exhibit hyperactivity in adult mice during withdrawal from binge-drinking (Lee et al., 2015), as well as increases in protein indices of glutamate transmission that promote binge-alcohol intake (e.g., Cozzoli et al., 2009, 2011, 2014, 2015). We sampled tissue also from the adjacent nucleus accumbens core (AcbC) and the basolateral amygdala (BLA) to examine the subregional specificity of any observed protein effects. These adjacent subregions share connectivity and proximity with the extended amygdala but are not considered parts of this macrosystem, thus enabling us to determine whether or not any observed changes in protein expression were specific to the extended amygdala. If adult and adolescent drinkers do indeed show distinct withdrawal phenotypes, these differences could be reflected in divergent alcohol-induced protein changes within extended amygdala structures.

2. Materials and Methods

Experimental procedures were similar to those in our previous studies (Lee et al., 2015; Lee et al., 2016) and are briefly summarized below. All experiments were conducted in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (NIH Publication No. 80–23, revised 2014) and approved by the IACUC of the University of California, Santa Barbara.

2.1 Subjects

The animals used in this study were male C57BL/6J mice (Jackson Laboratories, Sacramento, CA). Animals were housed in groups of 4 in standard Plexiglas cages, in a temperature-controlled vivarium (23◦C), under a 12hr reverse light/dark cycle (lights off at 10 am). Food and water were available *ad libitum*, except during the 2hr alcohol- drinking period. Adolescent drinkers ($EtOH^{adolescents}$) began drinking at PND28, spanning the approximate period of early-mid adolescence in mice (Brust et al., 2015; Spear, 2000b), and underwent behavioral testing in adulthood on PND70, after 28 days withdrawal (i.e. protracted withdrawal). Adult drinkers ($EtOH^{adults}$) were PND56 at drinking onset and consisted of two subgroups: one group was behaviorally tested at PND70, after 1 day withdrawal ($wd1EtOH^{adults}$), to match the age of the aforementioned $EtOH^{adolescents}$ mice and control for known age-related differences in basal behavior and protein expression (Spear, 2010). In a follow-up experiment, an additional group of adult mice was added to the study and tested for behavior on PND98 (i.e., after 28-days withdrawal; $wd28EtOH^{adults}$), to control for the effects of a 28-day withdrawal period upon behavior/protein expression. All control animals (PND70 and PND98) received only water prior to behavioral testing. Sample sizes were n=9 for all groups. The experimental timeline for behaviorally tested animals is summarized in Fig. 1.

A separate cohort of animals (n=12/ group) was used to generate brain tissue for immunoblotting, as a previous study from our laboratory showed that behavioral testing procedures induced cellular activation within Acb subregions (Lee et al., 2015). These

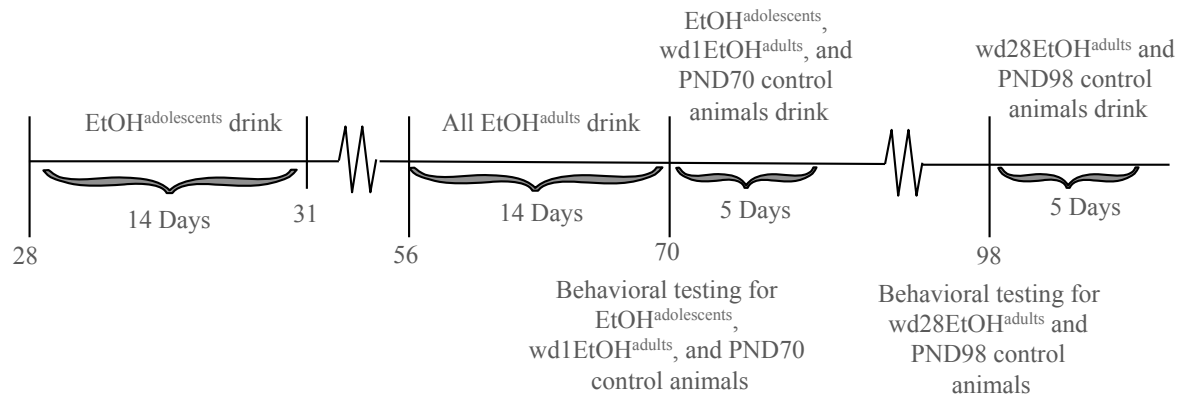

Figure 5.1. Procedural time-line of the experiments. Summary of the timing of the binge-drinking and testing procedures for comparing the protracted effects of a history of binge-drinking during adolescence ($EtOH^{adolescents}$) or adulthood ($EtOH^{adults}$) upon behavioral measure of negative affect and subsequent alcohol intake. wd1 and wd28 denote, respectively, 1 and 28 days withdrawal.

animals were subjected to the same drinking procedures as the animals in the behavioral experiment, but were sacrificed on PND70 or PND98 to obtain brain tissue, in lieu of behavioral testing.

2.2 Drinking-in-the-Dark (DID) Procedures

2.2.1 Initial alcohol exposure. All alcohol-experienced animals were exposed to 14 consecutive days of binge-drinking under our 4-bottle Drinking-in-the-dark (DID) procedures (see Lee et al., 2016). Alcohol-access was restricted to 14 days in order to correspond with the estimated length of early-mid adolescence in mice (Spear, 2000b), when developmental changes are most prolific (Spear, 2010). The Drinking-in-the-Dark protocol is a widely accepted model of binge-drinking that has been shown to elicit high voluntary alcohol consumption in laboratory animals (Crabbe et al., 2009; Rhodes et al., 2005). Each day prior to the drinking period, animals were separated into individual cages and allowed to acclimate for approximately 45 min. Beginning 3 hr into the dark phase of the circadian cycle, the peak time of daily fluid intake (Rhodes et al., 2005), animals were given simultaneous access to 5, 10, 20 and 40% (v/v) unsweetened ethanol solutions for 2 hrs. The positioning of the bottles on the cage was randomized each day. Expanding the traditional 1-bottle DID protocol to include 4 bottles of differing concentration has been shown to elicit even higher voluntary intakes (Cozzoli et al., 2014; Gustafsson & Nylander, 2006; Henniger et al., 2002; Tordoff & Bachmanov, 2003), as animals are able to sample from all the bottles and consume whichever concentration they find most palatable. This being said, the immunoblotting results for the CEA that ensued from our study of mice drinking under the 4-bottle procedure (see below) prompted us to conduct a follow-up immunoblotting study in

which mice were presented with a single bottle containing 20% (v/v) alcohol for 2 hrs/day for 14 days. In either case, the amount of alcohol consumed each day was calculated by bottle weight immediately before and after the drinking period. All animals, both alcohol and water drinkers, were weighed 3x per week throughout the drinking period.

2.2.2 Blood alcohol sampling. Submandibular blood samples were collected on drinking day 10, immediately following the 2-hr drinking period. The scheduling of the blood sampling was selected to ensure that the animals' intakes had stabilized, while also allowing ample time for recovery prior to behavioral testing. BACs were determined using an Analox alcohol analyzer (model AM1, Analox Instruments USA, Lunenburg, MA).

2.2.3 Subsequent drinking in adulthood. Beginning approximately 24 hr following behavioral testing, all animals, including previously alcohol-naïve water drinkers, were subjected to 5 additional days of DID procedures in order to relate prior alcohol experience, age of first exposure, and affective state to alcohol consumption in adulthood.

2.3 Behavioral testing

Behavioral testing consisted of the marble burying test, which was followed by the Porsolt forced swim test (FST). Both of these procedures were demonstrated to be particularly sensitive to the effects of alcohol withdrawal in our previous studies of mice (Lee et al., 2015; Lee et al., 2016). The order of testing was based on recommendations from our IACUC discouraging additional testing following the FST to allow animals to fully recover.

2.3.1 Marble burying. The marble-burying test was used as a measure of anxiety-induced defensive burying, as an increase in burying-related behavior serves as an index of anxiety (Umathe et al., 2008; Young et al., 2006) In our paradigm, 12 square glass pieces (2.5 cm^2 X 1.25 cm tall) were placed in the animals' home cage, six at each end. Animals were then left undisturbed for 15 min and video recorded for later analysis. At the end of the trial, a blind observer recorded the number of marbles at least 75% buried. Later, a blind observer reviewed the video footage and the latency to begin burying and the total time spent burying was recorded using a stopwatch.

2.3.2 Porsolt Forced Swim Test. The FST is a common measure of depression-like behaviors in laboratory animals, based on changes in active swimming (Porsolt, Bertin, et al., 1977; Porsolt et al., 2001; Porsolt, Le Pichon, et al., 1977). Each animal was placed into an 11-cm diameter cylindrical container filled with room-temperature water such that animals were unable to touch the bottom. The latency to first exhibit immobility (defined as no horizontal or vertical displacement of the animal's center of gravity for 5^+s), total time spent immobile, and the numbers of immobile episodes were monitored during a 6-min period using AnyMaze™ tracking software (Stoelting Co., Wood Dale, IL, USA).

2.3.3 Sucrose Preference test. The sucrose preference test is a common assay of anhedonia (e.g. Serchov et al., 2016), used to model depression in laboratory animals (Katz, 1982; Willner et al., 1992). Upon conclusion of the marble burying and forced swim test, animals were returned to the colony room and presented with overnight access to 2 identical sipper tubes, one containing 5% sucrose and the other containing plain water. Bottles were

weighed prior to being placed on the home cage at approximately 16:00h and again after removal at 09:00 h the following day. Change in bottle weight was used to determine the volume consumed and a relative sucrose preference was calculated as the volume of sucrose consumed/ total fluid volume consumed.

2.4 Brain Tissue Collection

Animals in the immunoblotting study were rapidly decapitated approximately 24 hr following the final alcohol presentation to mirror the time-frame of the behavioral testing. Brains were removed and cooled on ice, then sectioned in 1 mm-thick coronal slice at the level of the striatum and amygdala. The AcbSh and CeA were bilaterally sampled from the slice located approximately 1.18 mm and -1.22 mm relative to Bregma, respectively, as shown in the mouse brain atlas of Paxinos and Franklin (2004), using a 18-gauge biopsy needle (depicted in Figures 6 & 7).

2.5 Immunoblotting

Western blotting was performed on whole tissue homogenates from the AcbSh and AcbC (AP +1.18mm), and CeA and BLA (AP -1.34mm) (location relative to bregma, as depicted in Paxinos & Franklin, 2004) following procedures identical to those described in Lee et al. (2016). The following primary antibodies and concentrations were used: mGlu1 (Synaptic Systems, Göttingen, Germany; 1:1000 dilution), mGlu5 (Millipore, Temecula, CA, USA; 1:1000 dilution), Homer2b (Millipore, Temecula, CA; 1:1000 dilution), and calnexin (Enzo Life Sciences, Farmingdale, NY; 1:1000 dilution) for standardization. Homer2b is a postsynaptic density scaffolding protein that regulates signaling of Group 1 metabotropic

glutamate receptors (mGluRs) (Szumlinski et al., 2005). Together, these proteins were selected for study based on our laboratory's prior work identifying them as relevant to alcohol-induced neuroplasticity (Cozzoli et al., 2012; Cozzoli et al., 2015; Cozzoli et al., 2014; Cozzoli et al., 2009; Goulding et al., 2011; Lee et al., 2015; Lum et al., 2014; Obara et al., 2009; Quadir et al., 2015; Szumlinski, Ary, Lominac, et al., 2008; Szumlinski et al., 2007; Szumlinski et al., 2005).

2.6 Statistical Analysis

Alcohol intake data from the 14-day drinking period were analyzed with a repeated measures analysis of variance (ANOVA), with drinking age ($EtOH^{adolescents}$ or $wd1EtOH^{adults}$) as the between-subjects factor and day (14 days) as the within-subjects repeated measure to screen for potential group differences in alcohol consumption, which could confound alcohol-induced behavioral and neurobiological changes. The 5-day intake data were similarly analyzed with a drinking age ($EtOH^{adolescents}$, $wd1EtOH^{adults}$, or no prior experience) X day (5) repeated-measures ANOVA. A repeated measures ANOVA was also used determine if there was an effect of age/prior alcohol experience on the preference for a particular alcohol concentration, with drinking age ($EtOH^{adolescents}$, $wd1EtOH^{adults}$, or no prior experience) as the between-subjects factor and both day (14 or 5 levels) and concentration (5%, 10%, 20% or 40%) as the within-subjects factors.

Behavioral data for animals tested at PND70 were analyzed using between-subjects ANOVAs, with drinking ($EtOH^{adolescents}$ or $wd1EtOH^{adults}$) as the between-subjects factor, and Tukey's post-hoc comparisons when appropriate; $\alpha=0.05$. All comparisons between

wd28EtOH^{adults} and age-matched control animals were conducted using independent samples t-tests with Bonferroni corrections for multiple comparisons, as these animals were run as a separate follow-up to the animals tested at PND70. Paired-samples t-tests were used to compare the average consumption during the first and second rounds of drinking in alcohol-experienced animals.

The immunoblotting data for the animals subjected to our 4-bottle-choice drinking procedures were analyzed using a drinking age (EtOH^{adolescents}, wd1EtOH^{adults}, or no prior experience) univariate ANOVA, while that for the animals subjected to our single-bottle procedure were analyzed using unpaired-samples t-tests. For all analyses, statistical outliers were identified using the ±1.5*IQR rule and omitted from analyses. There were no statistical outliers excluded from the behavioral data. Outlier exclusion resulted in n's of 10-12 per group for the immunoblotting data (the specific n's for individual analyses are reported in the figure legends). All statistics and calculations were performed using SPSS v.21 statistical software (IBM, 2012).

3. Results

3.1 Alcohol consumption

Although the repeated measures ANOVA showed no between-subjects differences in the total amount of alcohol consumed by EtOH^{adolescents} , wd1EtOH^{adults}, and wd28EtOH^{adults} across the initial 14-day drinking period [$F(2, 24)=0.14$, $p=0.87$], there was a significant age X day within-subjects interaction [$F(78, 845)=6.11$, $p<0.001$]. Further analysis revealed that over days 1-7, wd1EtOH^{adults} drank more alcohol than EtOH^{adolescents} ($p=0.001$) but the

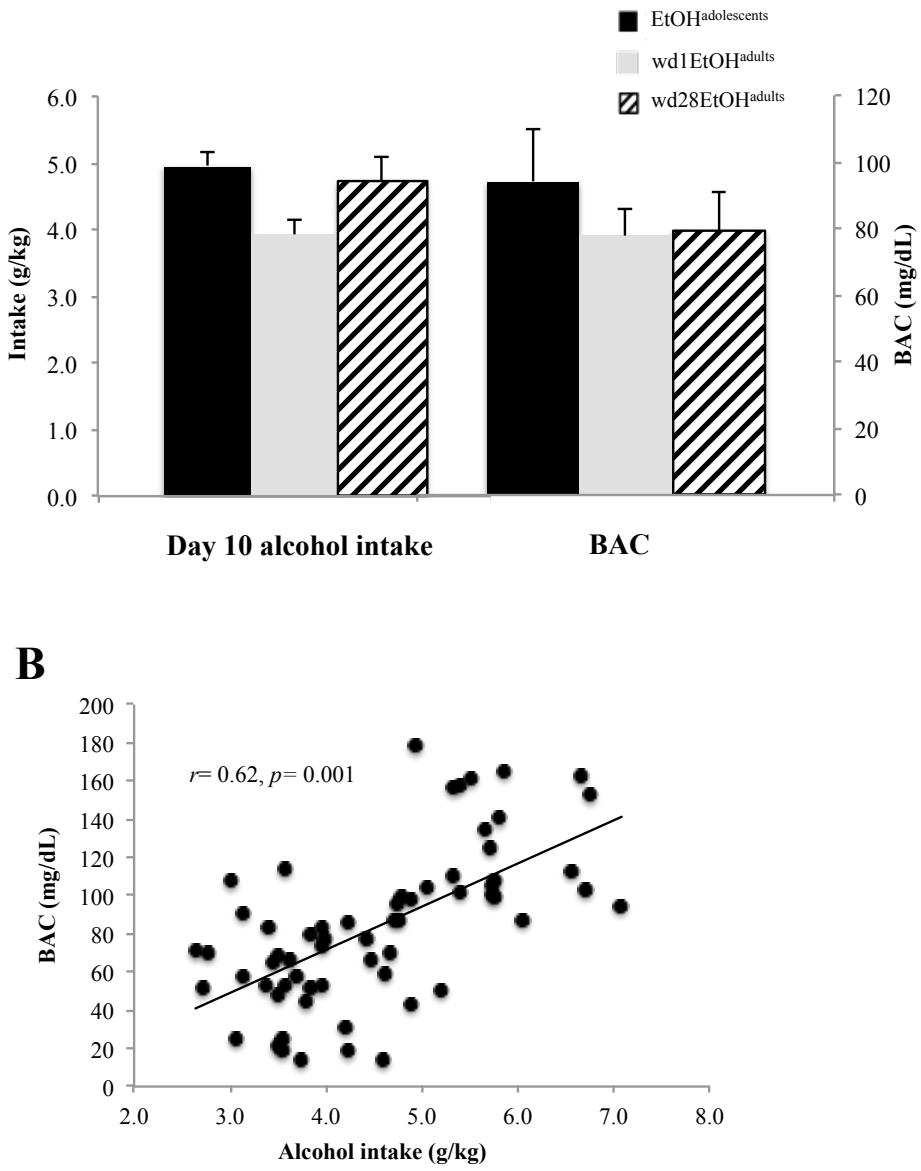

Figure 5.2. Day 10 BAC sampling. **(A)** Average alcohol intake and BAC by group, averaged across both behavioral testing and immunoblotting animals (n=21/group). **(B)** Alcohol intake was significantly correlated with BAC. Data depict all alcohol-drinking animals (n=63).

converse occurred over days 8-14 ($p<0.001$). This shift was reflected by a similar drinking-age X concentration X day interaction [$F(39,624)=4.62$, $p<0.001$] for concentration preference, with $EtOH^{adolescents}$ exhibiting greater preference for lower concentration during the first week and a shift to a preference for higher concentration during the second week compared to $wd1EtOH^{adults}$ (see Table 1). The repeated measures ANOVA also showed a significant drinking-age X concentration interaction [$F(3,48)=3.317$, $p=0.028$] and post-hoc analysis revealed that $wd1EtOH^{adults}$ had a lower preference for the 5% concentration and a higher preference for the 20% concentration compared to $EtOH^{adolescents}$ ($p=0.03$ and $p=0.049$, respectively). There was a trend toward higher preference for the 40% in adolescents compared to adults ($p=0.078$). During the subsequent 5-day drinking period, there were no significant main effects or interactions between age/ prior alcohol experience or concentration (p's> 0.10).

There were no differences in alcohol intake amongst the animals used for tissue collection [$F(2,32)=0.39$, $p=0.68$] and an overall analysis of all alcohol-drinking animals revealed no differences between cohorts used for behavioral testing or tissue collection [$F(5,56)=0.69$, $p=0.63$; summarized in Table 2].

3.2 Blood alcohol concentrations

As the ANOVA revealed no significant differences in alcohol consumption between behavioral testing and immunoblotting animals, day 10 intakes and BACs were collapsed across both cohorts within each drinking group (Fig. 2A). On day 10 of drinking, $EtOH^{adolescents}$ consumed an average 4.96 ± 0.21 g/kg of alcohol with a resulting BAC of

Table 5.1: Summary of group differences in the preference for different alcohol concentrations during the 2-week drinking period. Results of post-hoc analysis of the day X age X concentration interaction [$F(39,624)=4.62$, $p<0.001$] in $EtOH^{adolescents}$ and $wd1EtOH^{adults}$; n=9/group.

Day 1	• $EtOH^{adolescents}$ had a higher 5% and lower 40% preference, compared to $wd1EtOH^{adults}$ ($p<0.001$ and $p=0.014$, respectively)
Day 2	• $EtOH^{adolescents}$ had a higher 10% and a lower 20% preference, compared to $wd1EtOH^{adults}$ ($p=0.047$ and $p=0.003$, respectively)
Day 3	• $EtOH^{adolescents}$ had a higher 40% preference, compared to $wd1EtOH^{adults}$ ($p=0.037$)
Day 4	• No differences
Day 5	• $EtOH^{adolescents}$ had a higher 5% and 10% ($p=0.009$ and $p= 0.007$, respectively), but lower 20% & 40% preference, compared to $wd1EtOH^{adults}$ ($p=0.005$ and $p<0.001$, respectively)
Day 6	• No differences
Day 7	• $EtOH^{adolescents}$ had a higher 5%, but lower 20%, preference, compared to $wd1EtOH^{adults}$ ($p=0.002$ and $p=0.014$, respectively)
Day 8	• No differences
Day 9	• $EtOH^{adolescents}$ had a higher 5%, but lower 10%, preference, compared to $wd1EtOH^{adults}$ ($p=0.005$ and $p=0.022$, respectively)
Day 10	• $EtOH^{adolescents}$ had a higher 5% preference, compared to $wd1EtOH^{adults}$ ($p=0.001$)
Day 11	• $EtOH^{adolescents}$ had a lower 10%, but higher 40%, preference, compared to $wd1EtOH^{adults}$ ($p=0.002$ and $p=0.037$, respectively).
Day 12	• $EtOH^{adolescents}$ had a higher 40% preference, compared to $wd1EtOH^{adults}$ ($p<0.001$)
Day 13	• $EtOH^{adolescents}$ had a higher 10%,ower 20%, higher 40% ($p=0.048$, $p=0.002$, and $p=0.014$), compared to $wd1EtOH^{adults}$
Day 14	• $EtOH^{adolescents}$ had a lower 10% and 20%, but higher 40%, preference, compared to $wd1EtOH^{adults}$ ($p=0.04$, $p=0.031$, $p<0.001$)

Table 5.2: Summary of the average total alcohol intake exhibited by mice with a 14-day history of binge-drinking during adolescence ($EtOH^{adolescents}$), or during adulthood (wd1 or $wd28EtOH^{adults}$). Note that there were no significant group differences in alcohol intake across the 14-day drinking period.

	Behavioral testing animals	**Immunoblotting animals**
$EtOH^{adolescents}$	4.16 ± 0.10	4.48 ± 0.15
$wd1EtOH^{adults}$	4.05 ± 0.14	4.46 ± 0.08
$wd28EtOH^{adults}$	4.12 ± 0.10	4.40 ± 0.12

94.18 ± 9.25 mg/dL; wd1EtOH^{adults} consumed an average of 3.93 ± 0.22 g/kg with a resulting BAC of 77.73 ± 8.46, and wd28EtOH^{adults} consumed an average of 4.72 ± 0.25 g/kg with a resulting BAC of 79.47 ± 9.05 mg/dL. BAC was significantly correlated with alcohol consumption when sampled on day 10 of drinking (r=0.62, p=0.001, n=63; Fig. 2B).

3.3 Sucrose preference

The ANOVA showed significant group differences in sucrose preference [F(2,24)=20.01, p<0.001; Fig. 3] and posthoc analysis revealed that while wd1EtOH^{adults} showed increased sucrose preference (p=0.003 compared to alcohol-naïve control animals), EtOH^{adolescents} showed decreased preference (p=0.04).

3.4 Marble burying

In the marble burying test, there were significant group differences in total time spent burying [F(2,24)=11.82, p<0.001; Fig. 4A], the latency to start burying [F(2,24)= 4.15, p=0.028; Fig. 4B], and total number of marbles buried [F(2,24)=9.76, p=0.001; Fig. 4C]. Both wd1EtOH^{adults} and EtOH^{adolescents} spent more time burying compared to water controls (p=0.04 and p<0.001, respectively). EtOH^{adolescents} also had a shorter latency to start burying *(*p=0.022) and buried more marbles overall (p=0.001). However, wd1EtOH^{adults} did not differ significantly from controls on these factors (p's>0.1). There were no differences between wd28EtOH^{adults} and age-matched control animals on any behavioral factor tested (p's> 0.10, non-significant results are summarized in Table 3).

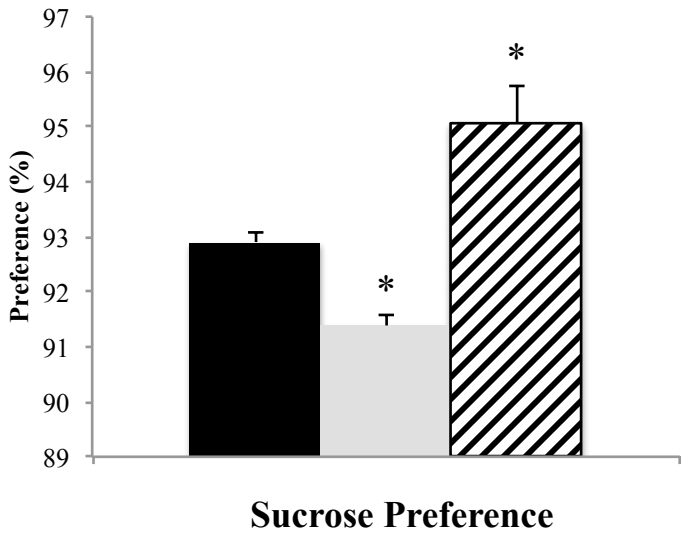

Figure 5.3. Altered sucrose preference following alcohol drinking. EtOH^{adolescents} showed significantly reduced sucrose preference compared to water control animals while wd1EtOH^{adults} showed increased preference. *$p<0.05$ vs water controls. Data represent mean + SEM, n=9/group.

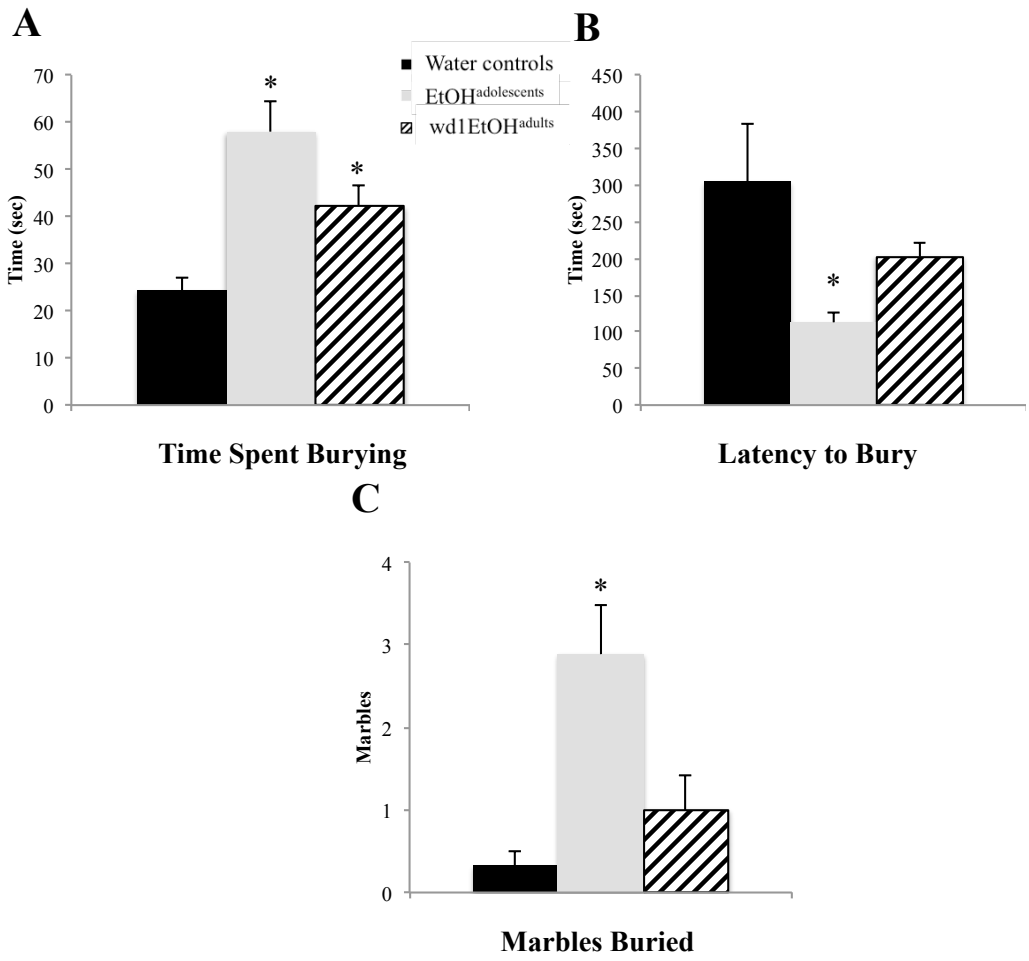

Figure 5.4. Increased marble burying following alcohol drinking. (A) Both EtOH^{adolescents} and wd1EtOH^{adilts} spent significantly more time burying marbles compared to control animals. **(B)** EtOH^{adolescent} also had a shorter latency to start burying and **(C)** buried more marbles overall compared to both control animals and wdEtOH^{adults}. *$p<0.05$ vs water controls. Data represent mean + SEM, n=9/group.

Table 5.3: Behavioral results from adult drinkers during protracted withdrawal. When tested 3 weeks following the end of their 14-day drinking session, no significant differences in behavior was observed between mice with a history of binge alcohol-drinking during adulthood (wd28EtOHadults) and their age-matched water controls. Data represent mean ± SEM, n=9/group

	PND98 water controls	**wd28EtOH^{adults}**
Marbles buried	1.44 ± 0.60	2.00 ± 0.40
Time spent burying (sec)	32.67 ± 4.56	40.51 ± 5.32
Latency to bury (sec)	119.44 ± 16.19	105.78 ± 14.26
FST immobile episodes	20.77 ± 1.19	19.77 ± 0.92
Time spent immobile (sec)	111.86 ± 6.13	110.35 ± 11.34
Latency to first immobility (sec)	51.53 ± 7.09	54.68 ± 3.70
Sucrose preference	93.87 ± 0.21	93.44 ± 0.25
5-day drinking average (g/kg)	3.09 ± 0.17	3.63 ± 0.25

3.5 Forced swim test

In the FST, there were group differences found for the number of immobile episodes [$F(2,24)=3.94$, $p=0.033$; Fig. 5A], total time spent immobile [$F(2,24)= 17.49$, $p<0.001$; Fig. 5B], and the latency to first immobility [$F(2,24)=38.81$, $p<0.001$; Fig. 5C]. Both wd1EtOH^{adults} and EtOH^{adolescents} had significantly fewer immobile episodes ($p=0.04$ and $p=0.02$, respectively) compared to control animals, but EtOH^{adolescents} spent significantly more time immobile *(*$p=0.008$), while adults spent less ($p=0.04$). EtOH^{adolescents} also had a shorter latency to first immobility *(*$p<0.001$) but wd1EtOH^{adults} did not ($p>0.20$). Despite having fewer immobile episodes, EtOH^{adolescents} spent more time immobile, compared to control animals, thus reflecting an overall increase in immobility with longer time spent immobile per episode.

3.6 Re-exposure drinking

During the subsequent 5-day drinking period following behavioral testing, the repeated measures ANOVA showed a significant effect of prior alcohol experience [$F(2,24)=20.92$, $p<0.001$; Fig. 6A&B]. Post hoc tests showed that both wd1EtOH^{adults} and EtOH^{adolescents} consumed more alcohol than first-time drinkers [wd1EtOH^{adults} $p=0.034$, EtOH^{adolescents} $p<0.001$]. Adittionally, EtOH^{adolescents} drank more than wd1EtOH^{adults} ($p=0.003$). Both wd1EtOH^{adults} and EtOH^{adolescents} also exhibited higher average alcohol consumption overall compared to their previous 14-day average (EtOH^{adolescents} $t(8)= 3.53$, $p=0.001$, wd1EtOH^{adults} $t(8)=7.12$, $p<0.001$; Bonferroni $\alpha= 0.025$). There was no difference in intake between wd28EtOH^{adults} and PND98 water control animals [wd28EtOH^{adults}: M= 3.63, SEM= 0.14; PND98 water controls: M= 3.09, SEM= 0.11; $t(16)=1.73$, $p=0.10$]

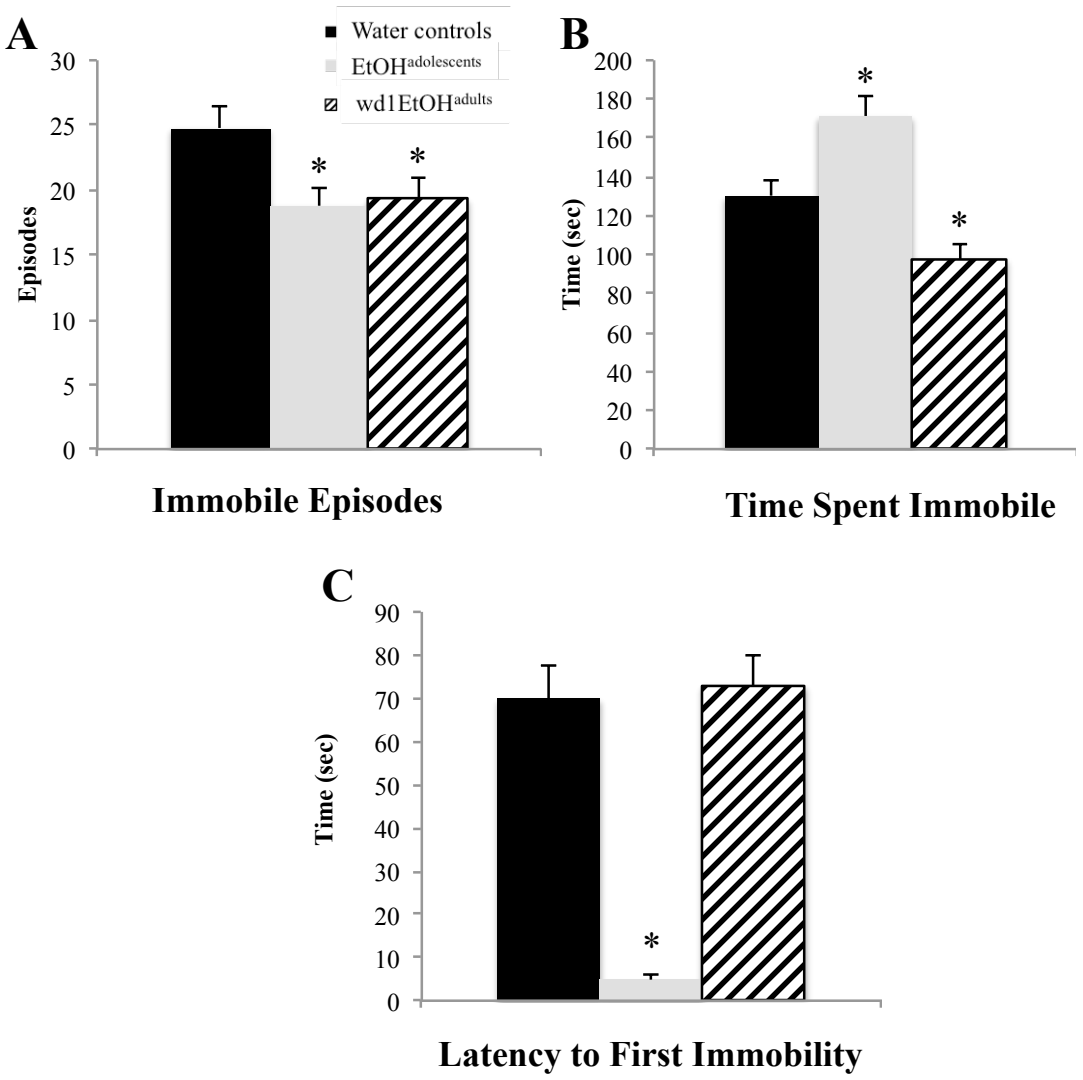

Figure 5.5. Altered FST behavior following alcohol drinking. (A) Both EtOH^{adolescents} and wd1EtOH^{adilts} had fewer immobile episodes than water controls. **(B)** EtOH^{adolescents} spent more time immobile compared to control animals, while wd1EtOH^{adults} spent less time immobile. **(C)** EtOH^{adolescents} had a shorter latency to first immobility, although wd1EtOH^{adults} did not differ significantly from control animals. *p<0.05 vs water controls. Data represent mean + SEM, n=9/group.

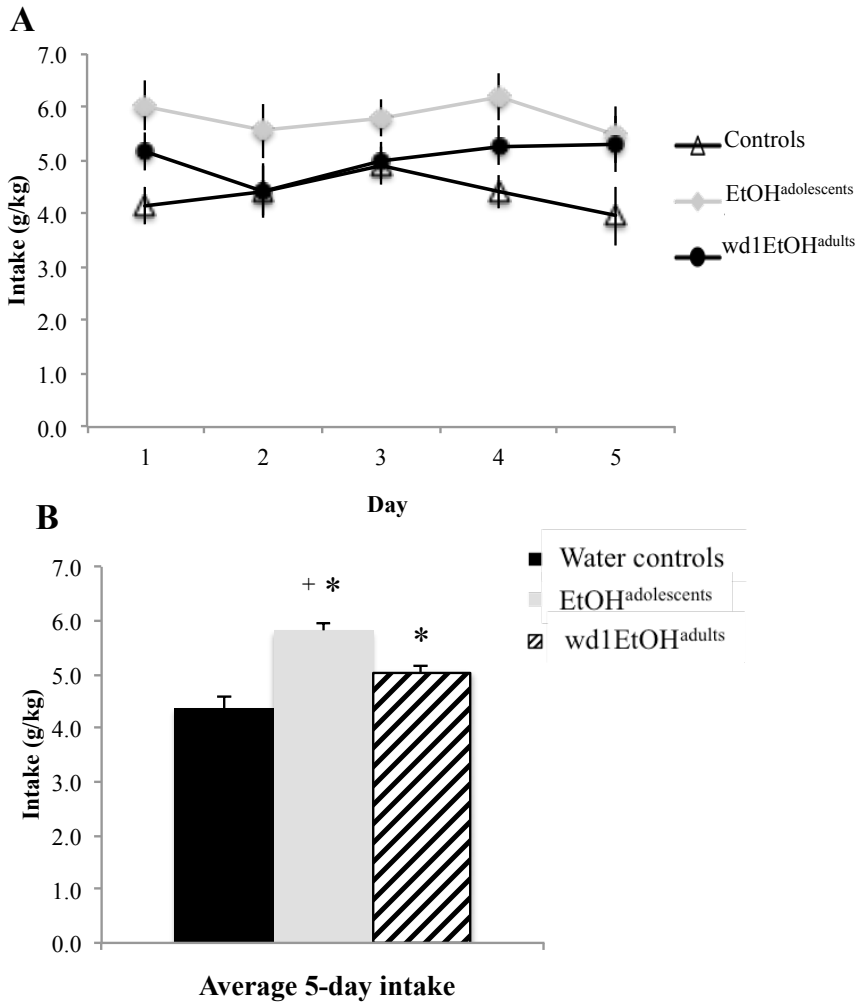

Figure 5.6. Increased consumption in alcohol-experienced animals. (A) Across the 5-day drinking period following behavioral testing, all alcohol-experienced animals consumed more alcohol than first-time drinkers. **(B)** When averaged across day, $EtOH^{adolescent}$ consumed significantly more than $wd1EtOH^{adults}$. *$p<0.05$ vs water controls, +$p<0.05$ vs $wd1EtOH^{adults}$. Data represent mean ± SEM, n=9/group.

and no increase in intake between the 14- and 5-day drinking period in wd28EtOH^{adults} [t(8)=1.85, p>0.10].

3.7 Immunoblotting

In the AcbSh, there were significant group differences in mGlu1 expression [F(2,31)=3.71, p=0.03; Fig. 7A] and mGlu5 [F(2,32)=4.15, p=0.02; Fig. 7B]. Posthoc analysis showed that EtOH^{adolescents} had increased mGlu1 expression relative to water controls (p=0.04), with a similar trend seen in wd1EtOH^{adults} (p=0.09). wd1EtOH^{adults}, but not EtOH^{adolescents}, showed a significant increase in mGlu5 expression (p=0.02 and p>0.10, respectively). There were no group differences in Homer2b expression within the AcbSh (non-significant immunoblotting results are from the AcbSh and CeA are summarized in Table 4).

There were significant group differences in mGlu1 expression within the CeA [F(2, 33)=6.32, p=0.005; Fig.8A] and Homer 2b [F(2,30)= 5.97, p=0.007; Fig. 8B]. Posthoc testing showed that both EtOH^{adolescents} and wd1EtOH^{adults} had decreased Homer2b expression relative to water controls (p=0.007 and p=0.04, respectively). EtOH^{adolescents}, but not wd1EtOH^{adults}, showed a significant decrease in mGlu1 relative to water controls (p=0.04 and p=0.53, respectively). There were no group differences found in mGlu5 expression (Table 4). There were no significant differences in mGlu1, mGlu5, or Homer2 within the AcbC or BLA (Table 4; all p's> 0.10).

Finally, when the immunoblotting data for the adult mice drinking under our single-bottle paradigm were compared, we replicated the reduction in CeA expression of mGlu1 in alcohol-experienced animals versus water-drinking Controls [t(18)=3.05, p= 0.006; Fig.9A]

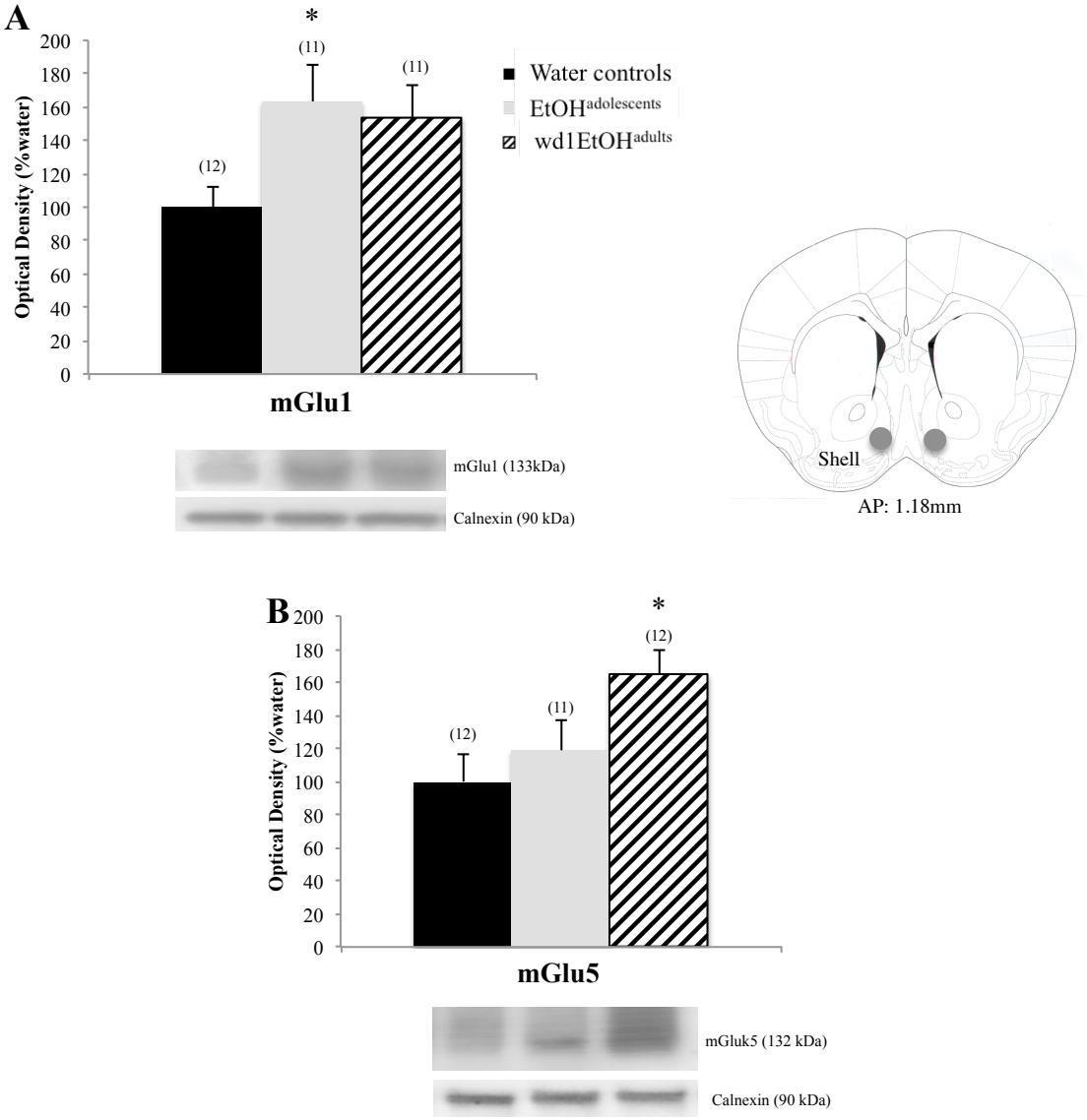

Figure 5.7. Alcohol-induced increases in mGluR expression within the AcbSh. (A) EtOH^{adolescents} showed a significant increase in mGlu1 expression within the AcbSh, with a similar trend in wd1EtOH^{adults}. **(B)** wd1EtOH^{adults} showed a significant increase in mGlu5, with no change observed in EtOH^{adolescents}. *p<0.05 vs water controls. Data represent mean + SEM of of the number of animals indicated in parentheses.

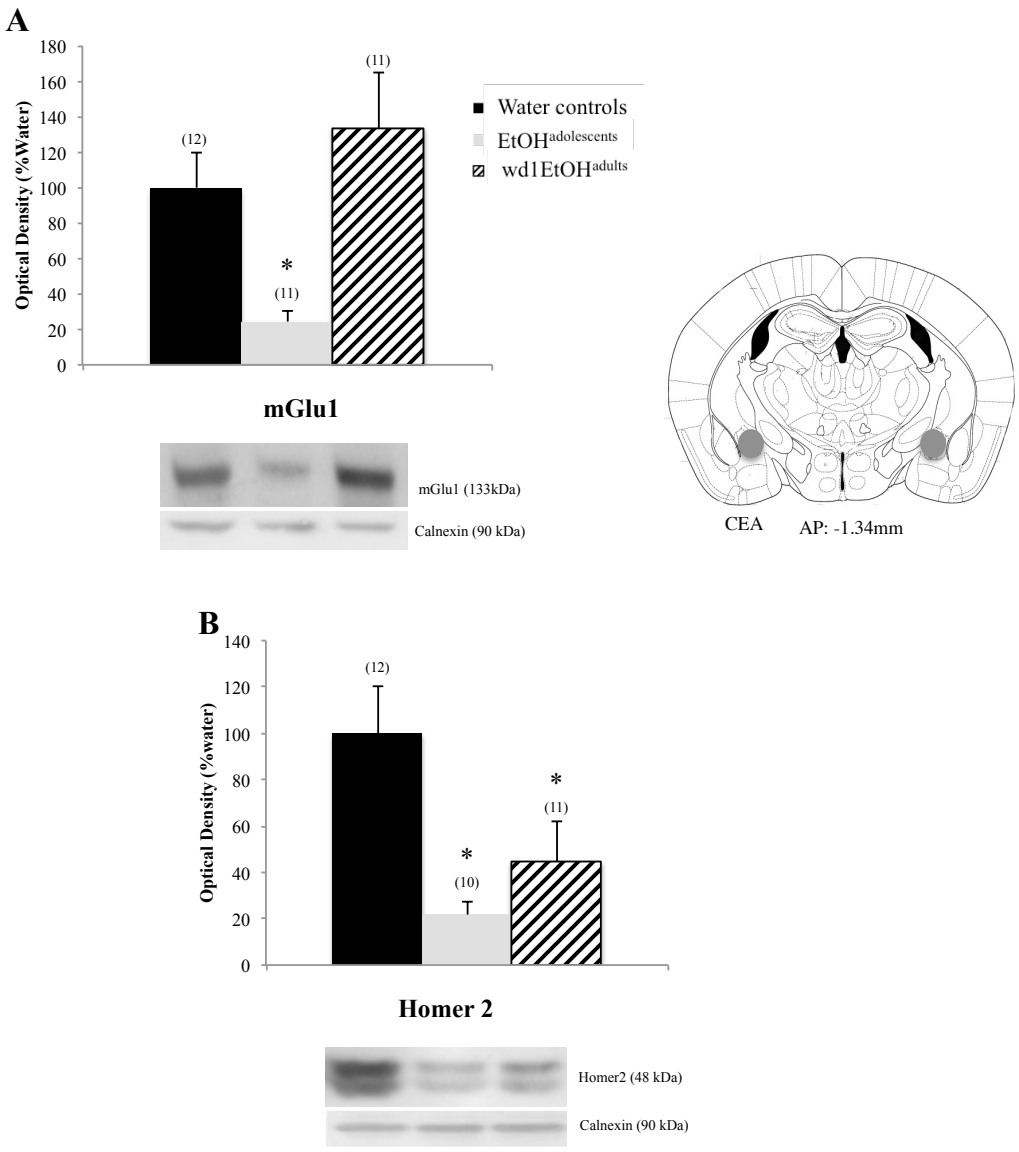

Figure 5.8. Alcohol-induced decreases in glutamate-related protein expression within the CeA. (A) EtOH^{adolescents}, but not wd1EtOH^{adults}, showed a significant decrease in mGlu1 expression within the CeA. **(B)** Both EtOH^{adolescent} and wd1EtOH^{adults} showed a significant decrease in homer 2 expression *p<0.05 vs water controls. Data represent mean + SEM of of the number of animals indicated in parentheses.

Table 5.4: Summary of non-significant immunoblotting results. There were no significant differences in protein expression in adult drinkers following 28-days withdrawal (wd28EtOH^{adults}) compared to age-matched water control animals. Data represent mean ± SEM, n=10-11/group.

	PND70 water controls	**EtOH^{adolescents}**	**wd1EtOH^{adults}**	**PND98 water controls**	**wd28EtOH^{adults}**
AcbSh: mGlu1				100 ±16.33	97.33 ±16.59
AcbSh: mGlu5				100 ± 21.07	117.34 ± 25.34
AcbSh: Homer2	100 ± 24.36	66.62 ± 8.07	126.86 ± 21.00	100 ± 16.94	73.78 ± 9.97
CEA: mGlu1				100 ± 32.63	76.48 ± 17.69
CEA: mGlu5	100 ± 12.12	198.81 ± 18.27	185.46 ± 14.76	100 ± 26.03	108.69 ± 22.60
CEA: Homer2				100 ± 24.78	71.31 ± 17.98
AcbC: mGlu1	100 ± 15.63	105.86 ± 15.90	92.05 ± 12.24	100 ± 18.14	80.39 ± 20.88
AcbC: mGlu5	100 ± 18.53	108.31 ± 24.17	113.36 ± 31.79	100 ± 11.30	115.52 ± 12.96
AcbC: Homer2	100 ± 19.42	116.98 ± 24.87	107.71 ± 18.91	100 ± 24.12	93.65 ± 13.10
BLA: mGlu1	100 ± 19.38	85.35 ± 17.36	116.25 ± 20.89	100 ± 15.88	81.98 ± 17.36
BLA: mGlu5	100 ± 14.07	75.80 ± 17.45	92.44 ± 17.37	100 ± 14.15	128.47 ± 26.05
BLA: Homer2	100 ± 13.28	86.09 ± 14.85	94.76 ± 16.15	100 ± 14.02	84.39 ± 15.06

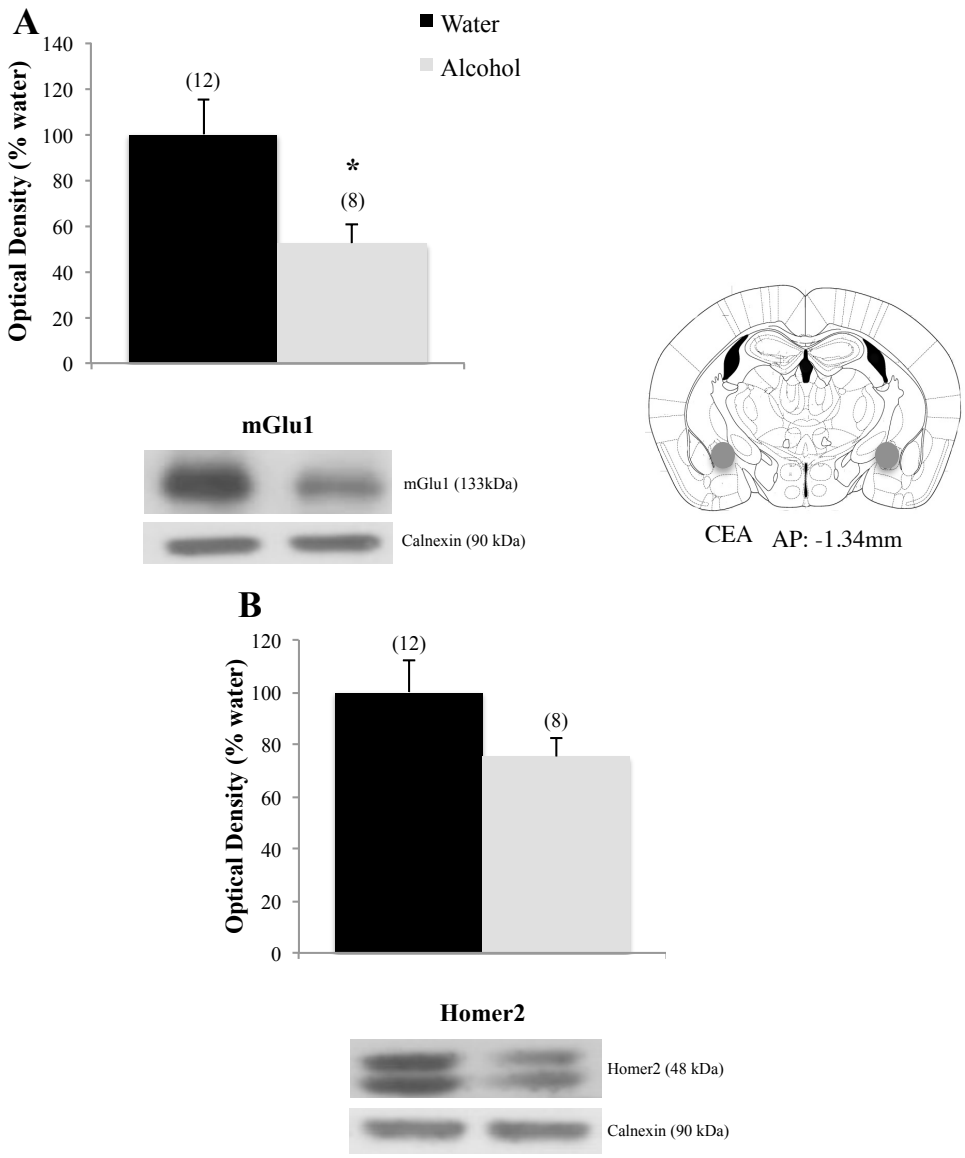

Figure 5.9. Decreases in glutamate-related protein expression within the CeA following single-bottle drinking. In a 14-day pilot study of single-bottle (20% EtOH) drinking in adults, animals consumed an average of 3.12 ± 0.18 g/kg. **(A)** Alcohol drinkers showed a significant decrease in mGlu1 expression within the CEA at 24 hr withdrawal. **(B)** A similar negative trend was observed in Homer 2 expression. *p<0.05 vs water controls. Data represent mean + SEM of of the number of animals indicated in parentheses. Unequal sample sizes due to sample availability, not outlier exclusion.

and also observed a trend toward reduced CeA Homer2 expression [t(18)=1.86, p=0.079; Fig. 9B].

4. Discussion

4.1 Drinking- age-dependent behavioral differences during withdrawal

In prior work, we showed that adult mice with a binge-drinking history exhibit robust negative affect in the light-dark box, marble burying test, and FST during early (24 hr) withdrawal that are not apparent in adolescent drinkers (Lee et al., 2016). In the present study, we assayed the behavior of adolescent drinkers during protracted withdrawal and uncovered distinct age-related differences in the time-course and presentation of withdrawal-induced negative affect in adolescent versus adult drinkers. Replicating our previous findings, wd1EtOH^{adults} showed signs of hyperanxiety during early withdrawal, as indicated by increased marble burying and decreased immobility in the FST. We have consistently observed decreased immobility in adult drinkers during early withdrawal, which we have interpreted as anxiety-related hyperactivity in response to an acute stressor (Lee et al., 2015; Lee et al., 2016). wd1EtOH^{adults} also showed increased sucrose preference compared to water control animals, which is not surprising given that studies have shown increased preference for sweet/sugary drinks amongst both humans (Kampov-Polevoy et al., 1997; Kranzler et al., 2001) and animals (Gosnell & Krahn, 1992; Katz, 1982; Stewart et al., 1994) with a history of chronic alcohol consumption.

These results support the presence of hyperanxiety, but not depression, in wd1EtOH^{adults}. However, these alcohol-induced behavioral differences dissipated during the course of withdrawal and by day 28, wd28EtOH^{adults} showed no significant differences compared to PND98 water control animals. This latter finding is particularly interesting as

we reported previously that a 30-day history of binge-drinking during adulthood produces a persistent increase in negative affect across a large number of assays and behavioral measures (Lee et al., 2015). As the drinking period employed in this study was only 14 days, our collection of work indicates that not only the severity (see Lee et al., 2016), but also the persistence, of alcohol withdrawal-induced hyper-anxiety varies as a function of the chronicity of binge alcohol-drinking in adults, with more chronic drinking experience eliciting more robust and enduring pharmacodynamic changes that drive the elevated negative affective state.

In contrast to adults with a 2-week binge-drinking history, $EtOH^{adolescents}$ exhibited signs of both hyperanxiety and depression during protracted withdrawal. In fact, $EtOH^{adolescents}$ demonstrated increased burying behavior across all measures in the marble-burying test and exhibited greater immobility in the FST, relative to $wd1EtOH^{adults}$. Although general locomotion was not assessed in this study, it is unlikely that the FST results are attributable to suppressed locomotor activity, given the vigorous burying behavior exhibited in the marble-burying test. Based on conventional interpretations of the FST, this increased immobility is indicative of depressive-like behavior. Consistent with this interpretation, $EtOH^{adolescents}$ also showed significantly lower sucrose preference relative to both $wd1EtOH^{adults}$ and water control animals, supporting the presence of an anhedonic state. Interestingly, the difference in sucrose preference between $EtOH^{adolescents}$ and $wd1EtOH^{adults}$ suggests that an alcohol-induced preference for sweet liquids is either absent in $EtOH^{adolescents}$ or is masked by the manifestation of anhedonia.

All alcohol-experienced animals consumed significantly more alcohol during the subsequent 5-day drinking period compared to their 14-day baseline average. Interestingly, $EtOH^{adolescents}$ consumed significantly more than $wd1EtOH^{adults}$, despite the fact that $wd1EtOH^{adults}$ were earlier in withdrawal, when the presence of an alcohol deprivation effect is typically more pronounced (Melendez et al., 2006; Vengeliene et al., 2014). These data provide additional evidence that early alcohol experience predisposes individuals to higher alcohol consumption in adulthood and may thus accelerate the transition to chronic alcohol abuse and addiction.

The present data, combined with our prior work (Lee et al., 2016), argue that a history of binge-drinking during adolescence *does* elicit a robust negative affective state, but that the manifestation of this state is dependent upon an incubation period during withdrawal. These results are consistent with others reported in the preclinical literature. For example, Pandey et al. (2015) showed increased anxiety-like behavior in the light-dark box and elevated-plus maze and elevated alcohol consumption in rats at approximately 50 days withdrawal following adolescent alcohol exposure. In contrast to our previous findings, this prior study also showed evidence of increased anxiety at 24 h withdrawal in adolescent animals. However, given that alcohol was administered via IP injection, it is possible that there was an alcohol X stress interaction due to the stress related to the route of alcohol delivery.

Although the dissipation of withdrawal signs in $wd28EtOH^{adults}$ during protracted withdrawal could be attributed to the relatively short 14-day drinking history, as our lab and others have shown persistent dysfunction following more prolonged alcohol exposure (Lee et

al., 2015; Santucci et al., 2008; Valdez et al., 2003). However, this difference nonetheless demonstrates that, compared to adults, adolescent drinkers are hypersensitive to persistent dysfunction following even brief periods of binge-drinking. Such findings suggest that the neural dysfunction underpinning the emotional hyper-reactivity observed in adult mice with a prior adolescent drinking history undergoes an incubation- or sensitization-like process, which likely relates to alterations in the developmental trajectory of corticofugal afferents governing emotional control.

4.2 Changes in glutamate-related protein expression within the AcbSh and CeA.

Consistent with previous immunoblotting studies, wd1EtOH^{adults} showed increased mGlu5 expression in the AcbSh at 24hr withdrawal, with a similar positive trend in mGlu1 (Cozzoli et al., 2014; Lee et al., 2016; Obara et al., 2009). Although adolescent binge-drinkers do not exhibit increased Group 1 mGluR expression in early withdrawal (Lee et al., 2015), adolescent drinkers in the present study showed a significant increase in mGlu1, but not mGlu5, during protracted withdrawal. These results are consistent with evidence implicating the importance of Group 1 mGluRs within the AcbSh in drug-taking, including the positive reinforcing properties of alcohol (Gass & Olive, 2008), as well as the initiation, maintenance, and escalation of intake (Cozzoli et al., 2012; Cozzoli et al., 2015; Cozzoli et al., 2009; Griffin et al., 2014; Kalivas et al., 2009; Lum et al., 2014). Therefore, these changes could underlie the increased alcohol consumption seen during the subsequent 5-day drinking period. However, given that these protein changes coincided with the emergence of behavioral dysfunction, increased group 1 mGluR expression could also be relevant to withdrawal-induced negative affect. Additionally, the lack of differences in the AcbC and

BLA demonstrate that these changes in protein expression are specific to extended amygdala subregions implicated in emotion.

The AcbSh receives significant glutamatergic input from the amygdala, which is known to mediate many of the negative reinforcing properties of alcohol withdrawal (Christian et al., 2012; Gilpin et al., 2015). Additionally, the Acb itself also has a role in negative affective states (Lim et al., 2012; Salamone, 1994; Shirayama & Chaki, 2006). There has been increased interest in the role of glutamatergic signaling within the Acb in aversive states such as anxiety, depression, and withdrawal-induced negative affect. For example, it has also been shown that an intra-AcbSh glutamate microinjection increases signs of depression in the FST, while inhibiting glutamate is antidepressant (Rada et al., 2003). Glutamatergic antagonism also alleviates the depressive, hypo-dopaminergic state during alcohol withdrawal (Rossetti et al., 1991). Therefore, the alcohol-induced increase in mGluR protein expression shown in the present study could render the AcbSh hypersensitive to glutamate-induced perturbation.

Within the CeA, $EtOH^{adolescents}$ exhibited decreased mGlu1 expression and both $EtOH^{adolescents}$ and $wd1EtOH^{adults}$ showed decreased Homer2b expression during withdrawal. While these results are consistent with post-mortem studies in human alcoholics demonstrating reduced glutamate receptor isoform expression within the CeA (Jin et al., 2014), they contrast with published data from our group (Cozzoli et al., 2014; Obara et al., 2009) and others (e.g. Roberto et al., 2004; Rossetti & Carboni, 1995; Zhu et al., 2007) indicating an increase in glutamate-related signaling within the CeA during alcohol

withdrawal. Comparable to our findings in the Acb, there were no significant changes in the BLA control region. This is consistent with previous studies from our lab (Cozzoli et al., 2014; Obara et al., 2009) and further substantiates the regional specificity of the changes observed herein. At the present time, it remains to be determined whether or not our inability to replicate our prior results from the CeA of binge-drinking C57BL/6J mice (i.e., Cozzoli et al., 2014) reflected procedural differences related to the total duration of alcohol-access (14 vs. 30 days) or to the number of bottles presented during alcohol-access (4 vs. 1). However, the results of a pilot immunoblotting study in our laboratory suggest the former, as a 2-week history of access to a single 20% alcohol bottle also reduced mGlu1 within the CeA of wd1EtOH^{adults} at 24 hr withdrawal, with a similar negative trend in Homer2 (Fig. 9).

The functional relevance of the observed reduction in CeA mGlu1/Homer2 expression remains to be determined, particularly considering that negative affect is classically associated with amygdalar hyperactivation (Davis & Whalen, 2001; Shackman & Fox, 2016). However, optogenetic evidence supports a causal relationship between reduced glutamatergic signaling within the CeA and negative affective states (Tye et al., 2011). Under basal conditions, glutamatergic inputs from the BLA excite GABAergic medium spiny neurons within the lateral subdivision of the CeA, which in turn exerts feed-forward inhibition onto the adjacent medial subdivision of the CeA, the output region which mediates autonomic and behavioral responses associated with anxiety and fear through projections to the brainstem (Davis & Whalen, 2001; Gilpin et al., 2015; Hilton & Zbrozyna, 1963; LeDoux et al., 1988a). Inhibition of this BLA projection reduces glutamatergic input to the CeA and increases anxiety-related behaviors, whereas stimulation of this projection is anxiolytic (Tye

et al., 2011). Additionally, low glutamatergic input produces asynchronous firing of GABAergic neural networks within the amygdala (Zhang et al., 2012). This asynchronous firing is associated hyperanxious behaviors that can be reversed by treatment with a group 1 mGluR agonist, which restores both neuronal synchronicity within the CeA and emotionality.

As the present study assayed protein expression in whole-cell homogenates, the site-specificity of these changes (i.e., subcellular location or cell phenotype) remains to be determined. Nevertheless, the work of Tye (2011) and Zhang (2012) support the possibility that reduced glutamate-related protein expression within the CeA, induced by a 2-week history of binge-drinking, may contribute to the manifestation of a hyper-anxious state in adult mice during early withdrawal. Furthermore, such a cause-effect relationship suggests that a time-dependent reduction in mGlu1/Homer2b-signaling within this region contributes to the apparent incubation of negative affect in mice with a prior history of binge-drinking during adolescence. In support of this possibility, no changes in glutamate receptor expression were observed within either the AcbSh or CeA in binge-experienced adult mice during protracted withdrawal (i.e., at a time when affective responding has normalized). As such, neuropharmacological and site-directed transgene delivery studies are currently on-going in our laboratory to directly assess the functional relationship between reduced glutamate signaling within the CeA and alcohol withdrawal-induced hyper-emotionality within the context of short-term binge-drinking.

4.3 Conclusions

This study provides further basic science evidence to support a causal relationship between adolescent binge-drinking and negative outcomes manifested during protracted withdrawal in adulthood. Despite apparent insensitivity to the negative affective consequences of drinking during acute withdrawal, this study indicates that adolescent binge-drinkers are uniquely vulnerable to the latent maladaptive effects of alcohol upon emotionality that manifest in later withdrawal and shows that even a 2-week history of binge-drinking during the adolescent phase of neurodevelopment can have profound and enduring effects upon negative affect and subsequent drinking behavior, which are temporally related to molecular anomalies within brain regions regulating emotionality and negative reinforcement. This combination of negative affect and increased drinking likely contributes to the predisposition toward alcohol abuse and alcoholism later in life. Alcohol-induced dysregulation within extended amygdala structures regions offers a potential neurobiological correlate for the high comorbidity between substance abuse and mood disturbances. Additional research is necessary to characterize the progression and duration of these changes throughout the course of withdrawal in order to further our understanding of the ontogenetic differences in the etiology of alcoholism and its high rate of comorbidity with affective disorders.

Chapter 6:

mGlu5-dependent modulation of anxiety during withdrawal from binge drinking in adult and adolescent male mice

1. Introduction

Both clinical and preclinical studies consistently report that chronic binge alcohol-drinking is associated with symptoms of negative affect and dysphoria during periods of abstinence. Binge-drinking is defined as a pattern of consumption sufficient to elevate blood alcohol concentrations (BAC) to ≥80 mg/dl, which relates to approximately 4-5 drinks in a 2-h period (NIAAA, 2004). Frequent binge-drinkers typically develop tolerance to the hedonic rewarding properties of alcohol, leading to an escalation of intake in order to reach a desired level of subjective intoxication. However, elevated consumption coupled with frequent bouts of intoxication/ withdrawal exacerbates the severity and duration of subsequent withdrawal symptoms (Ballenger & Post, 1978; Becker & Hale, 1993; Carrington et al., 1984). Over time, withdrawal-induced negative affect fuels the transition to addiction by shifting the primary motivation for drinking from positive to negative reinforcement in order to alleviate this aversive state during periods of abstinence.

While both adults and adolescents engage in binge-drinking, it is especially prevalent amongst adolescents (CDC, 2016). In fact, over 90% of alcohol consumed by underage drinkers is in the form of binge-drinking episodes (NIAAA, 2017). Adolescents typically consume larger quantities of alcohol than adults, yet adolescents are reportedly less susceptible to the negative consequences of acute intoxication (e.g., locomotor incoordination and sedation) and adolescents also experience fewer 'hangover'-like symptoms such as withdrawal-induced anxiety and dysphoria (Doremus et al., 2003; Spear & Varlinskaya, 2005; Varlinskaya & Spear, 2004; White, Truesdale, et al., 2002). Recent work in our laboratory has successfully recapitulated these age-related differences in withdrawal-induced negative affect using a mouse model of voluntary binge-drinking. We have shown

that adult alcohol-drinking mice exhibit increased behavioral indices of anxiety during early (24 h) withdrawal, which coincided with increased expression of metabotropic glutamate receptor 5 (mGlu5) within the nucleus accumbens shell (AcbSh) (Lee et al., 2016). In contrast, adolescent drinkers were resilient to both withdrawal-induced hyperanxiety and increased mGlu5 expression within the AcbSh during early withdrawal.

The AcbSh is a component of the extended amygdala, and drug-induced dysregulation within this circuitry is known to mediate many of the negative affect consequences of drug abuse (reviewed in Gilpin et al., 2015; Koob, 2003). Therefore, we hypothesized that the observed binge-induced increase in mGlu5 could be a causal mechanism involved in withdrawal-induced anxiety. Additionally, an age-dependent insensitivity to alcohol-induced upregulation of mGlu5 signaling in adolescent drinkers could constitute a neurobiological basis for their resilience to withdrawal-induced hyperanxiety. Indeed, glutamatergic dysregulation is implicated in the etiology of both addiction (reviewed in Cleva & Olive, 2012; Holmes et al., 2013; Kalivas et al., 2009; Tsai et al., 1995) and anxiety (reviewed in Bergink et al., 2004; Simon & Gorman, 2006; Swanson et al., 2005); group1 mGlu receptor antagonists attenuate behavioral measures in animal models of both disorders (Cozzoli et al., 2012; Cozzoli et al., 2014) Klodzinska et al., 2004; Kumar et al., 2013; (Lou et al., 2014; Sinclair et al., 2012) and exhibit anxiolytic efficacy in human clinical trials (Pecknold et al., 1982; Porter et al., 2005). A mutual basis of glutamatergic dysfunction could contribute to the high comorbidity between addiction and affective disorders.

In the present study, we assessed the functional significance of mGlu5 signaling in withdrawal-induced anxiety in adult and adolescent binge-drinking mice using the mGlu5 negative allosteric modulator 3-[(2-Methyl-1,3-thiazol-4- yl)ethynyl]pyridine (MTEP), and

the positive allosteric modulator 3-Cyano-N-(1,3-diphenyl-1H-pyrazol-5-yl)benzamide (CDPPB). We predicted that MTEP treatment would reduce early withdrawal-induced anxiety in adult drinkers, while treatment with the CDPPB should exacerbate alcohol-induced mGlu5 hyperactivation and increase anxiety. In adolescent alcohol-drinking mice, we hypothesized that increasing mGlu5 signaling with CDPPB would elicit a hyperanxious, adult-like phenotype during early withdrawal. This would suggest that withdrawal-induced anxiety is mediated by a common underlying mechanism in both adult and adolescent bingers, and a resistance to alcohol-induced neuroadaptations of mGlu5 could underlie the resilience to withdrawal-induced negative affect seen in adolescent drinkers. Based on the evidence supporting the anxiolytic properties of mGlu5 antagonism in both humans and laboratory animals (e.g. Kotlinska & Bochenski, 2008; Kumar et al., 2013; Varty et al., 2005), we also anticipated an anxiolytic effect of MTEP treatment in alcohol-naïve animals, although to a lesser extent than hyperanxious adult mice in alcohol withdrawal.

2. Materials and Methods

The binge-drinking and behavioral testing procedures employed herein were nearly identical to those used in previous studies in our lab (Lee et al., 2015; Lee et al., 2016) and are summarized briefly below. All procedures were conducted in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (NIH Publication No. 80–23, revised 2014) and approved by the IACUC of the University of California, Santa Barbara.

2.1 Subjects

The animals used in this study were male C57BL/6 mice that were either PND 28 (adolescents) or PND 56 (adults) at the onset of drinking. Animals were housed in a climate-controlled vivarium under a reverse light/dark cycle (lights off at 10am) in groups of 4 per cage. Animals were identified using small animal ear tags (Stoelting, Wood Dale, IL). Food and water were available *ad libitum*, except during the 2-h alcohol-drinking period. The study consisted of 2 age groups (adults and adolescents), 2 drinking groups (alcohol or water), and 3 treatment groups (MTEP, CDPPB, or vehicle); n=11/group.

2.2 Drinking-in-the-Dark (DID) Procedures

Half of the animals from each age group were subjected to 14 consecutive days of binge-drinking under 3-bottle DID procedures. Control animals received a single water bottle only. Alcohol-access was restricted to 14 days in order to correspond to the estimated duration of early-mid adolescence in mice (Spear, 2000b) and to maintain consistency across age groups. Each day prior to the drinking period, animals were separated into individual drinking cages and allowed to acclimate for approximately 45 min. Animals were then given concurrent access to 10, 20, and 40% (v/v) unsweetened ethanol solutions for 2 h, beginning 3 h into the circadian dark cycle- the time of peak daily fluid intake (Rhodes et al., 2005). At the conclusion of the drinking period, animals were returned to their original group cages. The amount of alcohol consumed each day was calculated by bottle weight immediately before and after the drinking period and expressed as a function of the animal's body weight (in kg). All animals, both alcohol and water drinkers, were weighed 3x per week throughout the drinking period.

2.2.1 Blood alcohol sampling. Submandibular blood samples were collected from all alcohol-drinking animals on day 11 of drinking, immediately upon conclusion of the 2-h drinking period. The scheduling of the blood sampling was selected to ensure that the animals' intakes had stabilized, while also allowing ample time for recovery prior to behavioral testing. BACs were determined using an Analox alcohol analyzer (model AM1, Analox Instruments USA, Lunenburg, MA).

2.3 Drugs

This study used a high 30 mg/kg dose of both MTEP (Sigma Aldrich; St. Louis, MO) and CDPPB (NIMH C-918; Bethesda, MD) dissolved in 90% sterile water: 10% Tween-80 (Sigma Aldrich; St. Louis, MO), injection vol=0.01 ml/g. This dose was selected from the high end of the dose-range typically reported to be behaviorally effective in the literature. For example, anxiolytic effects of MTEP have been reported at 20 mg/kg (Klodzinska et al., 2004) and 30 mg/kg achieves 100% receptor occupancy (Busse et al., 2004), while a 30mg/kg dose of CDPPB reverses amphetamine-induced prepulse inhibition deficits (Kinney et al., 2005). Based on the results obtained at the 30 mg/kg MTEP dose, an additional follow-up replicate of animals was treated with a low 3 mg/kg dose of MTEP in order to establish a dose-response relationship, as conducted previously in studies by the Szumlinski laboratory (Cozzoli et al., 2014; Cozzoli et al., 2009). Animals from each drinking group were sub-divided into their drug or vehicle treatment groups and were injected intraperitoneally at 30 min prior to the onset of behavioral testing for emotionality.

2.4 Behavioral testing

Drug administration and subsequent behavioral testing commenced approximately 24 h following the final alcohol presentation and thus occurred during the circadian dark phase.

2.4.1 Light-dark box. The light/dark shuttle box test was used to anxiety-like behaviors (Bourin & Hascoet, 2003; Crawley, 1985). Animals were placed into a polycarbonate box measuring 46cm long×24cm high×22cm wide containing 2 distinct environments; half of the box was white and uncovered and the other half black and covered, separated by a central divider with an opening. Animals started on the dark side and the latency to enter the light side, number of light-side entries, and total time spent in the light side of the shuttle box were recorded during a 15-min trial using Any-maze^{TM} tracking software (Stoelting Co., Wood Dale, IL). This apparatus was also used to assess general locomotor activity by measuring the total distance traveled during the trial.

2.4.2 Marble-burying. The marble-burying test was used to measure anxiety-induced defensive burying (Nicolas et al., 2006). In our paradigm, 10 square glass pieces (2.5 cm2 × 1.25 cm tall) were placed in the animals' home cage, 5 at each end. The total number of marbles 75^{+}% buried at the end of the 20-min trial was recorded. Trials were video recorded and later scored by a blind observer with a stopwatch for the latency to begin burying marbles and the total time spent burying.

2.4.3 Porsolt Forced Swim Test. Each animal was placed into an 11-cm diameter cylindrical container and the latency to first exhibit immobility (defined as no horizontal or

vertical displacement of the animal's center of gravity for ≥5 s), total time spent immobile, and the numbers of immobile episodes were monitored throughout the entire 6-min trial period using AnyMaze^{TM} tracking software (Stoelting Co., Wood Dale, IL, USA).

2.5 Statistical Analyses.

A repeated measures ANOVA was used to analyze intake data for all alcohol-drinking animals to determine age differences in alcohol consumption across the 14-day drinking period. Pearson's correlational analysis was conducted to determine the relationship between alcohol intake and resulting BACs from blood samples collected on day 11 of drinking. A repeated measures ANOVA was also performed on adults and adolescents separately to ensure there were no intake differences between treatment groups. Adult and adolescent behavioral data were analyzed independently using two-way ANOVAs with Tukey's post hoc multiple comparison tests where appropriate; α=0.05. Data from the initial study of 30 mg/kg MTEP and CDPPB treatment were analyzed independently from the lower 3 mg/kg MTEP dose, as the lower dose animals were run as a separate follow-up approximately 2 months after the original cohort. All calculations and analyses were performed using SPSS v.21 statistical software (IBM, 2012).

3. Results

3.1 Alcohol intake

Adults consumed an average of 4.15 ± 0.17 g/kg and adolescents an average of 6.10 ± 0.32 g/kg across the 14-day drinking period. The repeated measures ANOVA revealed a significant age effect showing that adolescents consumed significantly more alcohol, overall,

compared to adults [F(1,76)=28.83, $p<0.001$]. On day 11 of drinking, adults consumed an average of 4.23 ± 0.18 g/kg with a resulting BAC of 80.91 ± 5.94 mg/dl and adolescents consumed an average of 5.16 ± 0.22 g/kg with a resulting BAC of 88.84 ± 5.85 g/kg (Fig. 1A). Collapsed across age, intake was significantly correlated with BAC ($r=0.504$, $p<0.001$; Fig. 1B). Within each age group, there were no significant differences in alcohol intake amongst the animals slated to receive vehicle, MTEP or CDPPB [Adults: F(3,38)=0.84, $p=0.47$; adolescents: F(3,38)=0.48, $p=0.70$]

3.2 Alcohol, CDPPB and high-dose MTEP in adult drinkers

3.2.1. Porsolt Forced Swim Test. There was a significant drinking X treatment interaction in the time spent immobile in the FST [F(2,53)=9.19, $p<0.001$; Fig. 2A]. *Post-hoc* comparisons showed that in vehicle-treated animals, alcohol-drinking mice spent less time immobile, compared to water controls ($p=0.042$). In alcohol-drinking mice, MTEP-treated animals spent more time immobile, compared to vehicle treatment ($p=0.015$), while CDPPB-treated animals spent less time immobile ($p=0.043$). Overall, there was a trend toward fewer immobile episodes in alcohol-drinking mice versus water controls [F(1,57)=3.31, $p=0.07$; Fig. 2B]. Vehicle-treated, alcohol-drinking mice also exhibited fewer immobile episodes, compared to water controls ($p=0.045$). In water controls, CDPPB treatment reduced immobile episodes, relative to vehicle treatment ($p=0.025$). Alcohol-drinking mice also exhibited a longer latency to exhibit immobility, compared to water drinkers [main effect F(1, 52)=10.24, $p=0.002$; Fig 2C]; however, this difference was driven primarily by a significant increase in immobility latency in the alcohol-experienced mice treated with CDPPB (vs. vehicle: $p=0.018$).

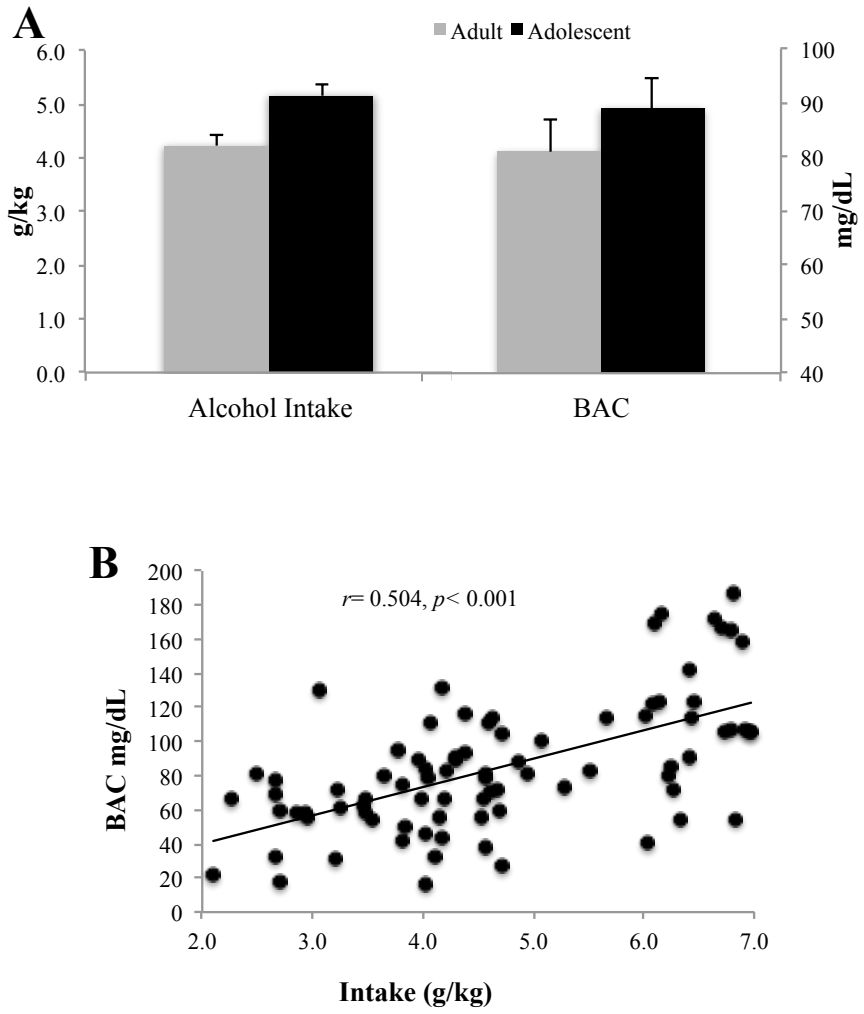

Figure 6.1. Alcohol Intakes and BACs. Blood was collected on drinking day 11 for BAC analysis. **(A)** Adults consumed an average of 4.23 ± 0.18 g/kg with a resulting BAC of 80.91 ± 5.94 mg/dL and adolescents consumed an average of 5.16 ± 0.22 g/kg with a resulting BAC of 88.84 ± 5.85 g/kg. Data represent mean + SEM. **(B)** Collapsed across age, alcohol intake was significantly correlated with BAC on day 11.

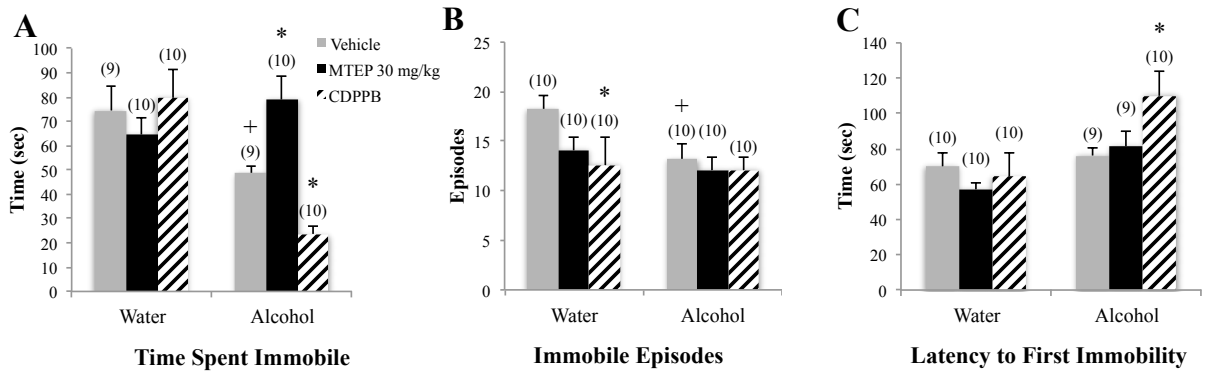

Figure 6.2. Forced swim test, CDPPB and high-dose MTEP in adult drinkers. **(A)** There was a significant drinking X treatment interaction in the time spent immobile. In vehicle-treated animals, alcohol drinkers spent less time immobile compared to water drinkers. In alcohol drinkers, MTEP-treated animals spent more time immobile compared to vehicle treatment while CDPPB-treated animals spent less. **(B)** In vehicle-treated animals, alcohol drinkers had fewer immobile episodes compared to water drinkers. In water drinkers, CDPPB treatment reduced immobile episodes compared to vehicle treatment. There was also a trend toward reduced immobile episodes in MTEP-treated water drinkers. **(C)** Alcohol drinkers had a longer latency to first immobility compared to water drinkers; however, this difference was driven primarily by a significant increase in CDPPB-treated alcohol drinkers compared to vehicle treatment. *$p<0.05$ vs. vehicle treatment within same drinking group, +$p<0.05$ vs. vehicle-treated water drinkers. Data represent mean + SEM of the number of animals indicated in parentheses.

3.3.2 Light-dark box. In the light-dark box, there was a significant drinking X treatment interaction for the number of light-side entries [$F(2,56)=4.92$, $p=0.011$; Fig. 3A]. In vehicle-treated animals, alcohol-drinking mice made fewer light-side entries than water controls ($p=0.017$). CDPPB treatment decreased light-side entries in both water- and alcohol-drinking mice, compared to their respective vehicle-treated groups ($p<0.001$ and $p=0.019$, respectively). MTEP treatment increased light-side entries, compared to vehicle in alcohol mice only ($p<0.001$). There was a significant drinking X treatment interaction in the time spent on the light side [$F(2,56)=11.45$, $p<0.001$; Fig. 3B]. In vehicle-treated animals, alcohol-drinking mice spent less time on the light side, compared to water controls ($p=0.028$). In alcohol-drinking mice, MTEP treatment increased light-side entries, while CDPPB treatment decreased entries versus vehicle treatment ($p=0.039$ and $p=0.034$, respectively). In water controls, both MTEP and CDPPB treatment decreased the time spent on the light side, compared to vehicle (p's<0.001). A significant treatment effect in the latency to first light-side entry [$F(2,54)=8.79$, $p<0.001$; Fig. 3C] indicated a longer latency to first light-side entry in CDPPB-treated animals, compared to vehicle ($p<0.001$). Visual inspection of the data suggested that this main effect was driven primarily by a significant increase in the latency exhibited by CDPPB-treated water controls ($p<0.001$). There were no effects of alcohol or treatment upon the distance traveled by the animals in the light-side of the apparatus (p's>0.1; Fig. 3D).

3.2.3 Marble-burying. In the marble burying test, there was a significant drinking X treatment interaction in the number of marbles buried [$F(2,56)=11.45$, $p=0.022$; Fig. 4A]. MTEP treatment decreased the number of marbles buried in both water- and alcohol-drinking

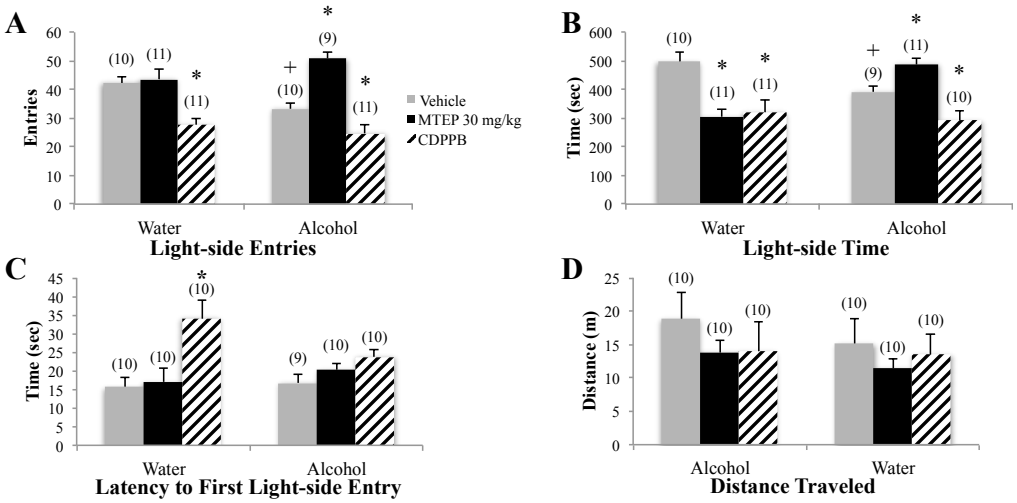

Figure 6.3. Light-dark box, CDPPB, and high-dose MTEP in adult drinkers. **(A)** There was a significant drinking X treatment interaction in the number of light-side entries. CDPPB treatment decreased light-side entries in both water and alcohol drinkers compared to vehicle, while MTEP treatment increased light-side entries compared to vehicle in alcohol drinkers only. Vehicle-treated alcohol drinkers made fewer light-side entries compared to water drinkers. **(B)** There was a significant drinking X treatment interaction in the time spent on the light side. Vehicle-treated alcohol drinkers spent less time on the light side compared to vehicle-treated water drinkers. In alcohol drinkers, MTEP treatment increased light-side entries while CDPPB treatment decreased entries compared to vehicle treatment. In water drinkers, both MTEP and CDPPB treatment decreased time spent on the light side. **(C)** A significant treatment effect showed a longer latency to first light-side entry in CDPPB-treated animals compared to vehicle. Visual inspection of the data shows that this main effect is driven primarily by a significant increase in CDPPB-treated water drinkers. **(D)** There were no effects of alcohol or treatment on distance traveled. *p<0.05 vs. vehicle treatment within same drinking group, +p<0.05 vs. vehicle-treated water drinkers. Data represent mean + SEM of the number of animals indicated in parentheses.

mice (p=0.049 and p<0.001, respectively). In vehicle-treated animals, there was also a trend toward more marbles buried by alcohol mice, compared to water controls (p=0.06). A significant treatment effect [F(2,57)=8.35, p=0.001; Fig. 4B] revealed decreased time spent burying in MTEP-treated animals, relative to vehicle (p=0.001). In vehicle-treated animals, there was also a trend towards more time spent burying in alcohol-drinking mice, compared to water controls (p=0.061). There was also a treatment trend in the latency to start burying F(2,50)=8.35, p=0.058; Fig. 4C], driven primarily by a significant increase in latency in the MTEP-treated alcohol mice (vs. vehicle: p=0.006). In vehicle-treated animals, alcohol-drinking mice had a shorter latency to start burying, compared to water controls (p=0.03).

3.3 Alcohol and low-dose MTEP in adult drinkers

3.3.1 Porsolt Forced Swim Test. In the follow-up study employing 3mg/kg MTEP, there was a significant drinking X treatment interaction in the time spent immobile in the FST [F(1,33)=4.29, p=0.046; Fig. 5A]. Vehicle-treated alcohol-drinking mice spent less time immobile, compared to their respective water control (p=0.039) and MTEP treatment significantly increased the time spent immobile in water controls only (p=0.018). Alcohol-drinking mice exhibited fewer immobile episodes, overall, compared to water controls [main drinking effect: F(1, 36)=11.52, p=0.002; Fig. 5B]. There were no effects of alcohol or treatment on the latency to first immobility (p's>0.1; Fig. 5C).

3.3.2 Light-Dark Box. In the light-dark box, there was a significant drinking X treatment interaction for the number of light-side entries [F(1,35)=5.21, p=0.029; Fig. 6A]. In vehicle-treated animals, alcohol-drinking mice made fewer light-side entries, compared to

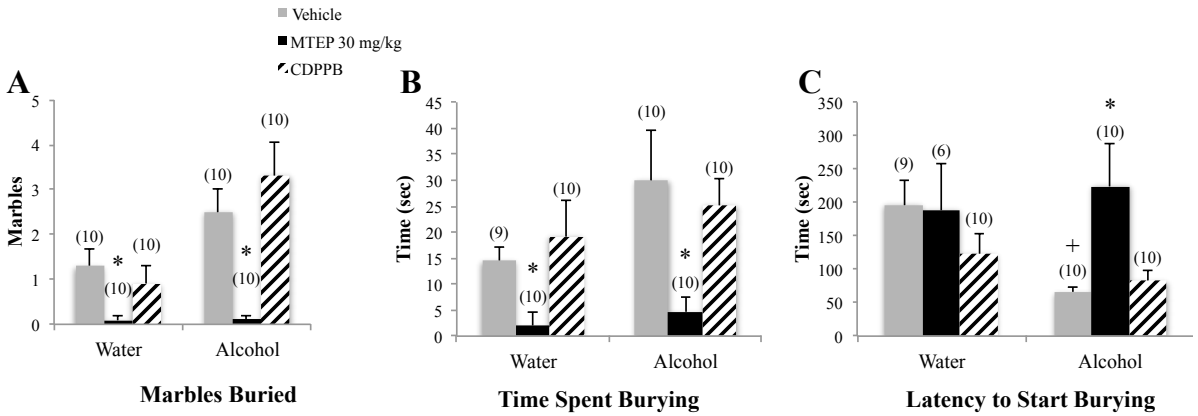

Figure 6.4: Marble burying, CDPPB, and high-dose MTEP in adult drinkers. **(A)** There was a significant drinking X treatment interaction in the number of marbles buried. MTEP treatment decreased the number of marbles buried in both water and alcohol drinkers. There was also a trend toward more burying in vehicle-treated alcohol drinkers compared to water drinkers. **(B)** A significant treatment effect showed decreased burying in MTEP-treated animals compared to vehicle. There was also a trend toward more time spent burying in vehicle-treated alcohol drinkers compared to water drinkers. **(C)** There was a trend toward a treatment effect in latency to start burying, driven primarily by a significant increase in MTEP-treated alcohol drinkers compared to vehicle. Also, vehicle-treated alcohol drinkers had a shorter latency to start burying compared to water drinkers. The small sample size in MTEP-treated water drinkers was due to 4 animals that did not engage in any burying and therefore had no latency data. *$p<0.05$ vs. vehicle treatment within same drinking group, +$p<0.05$ vs. vehicle-treated water drinkers. Data represent mean + SEM of the number of animals indicated in parentheses.

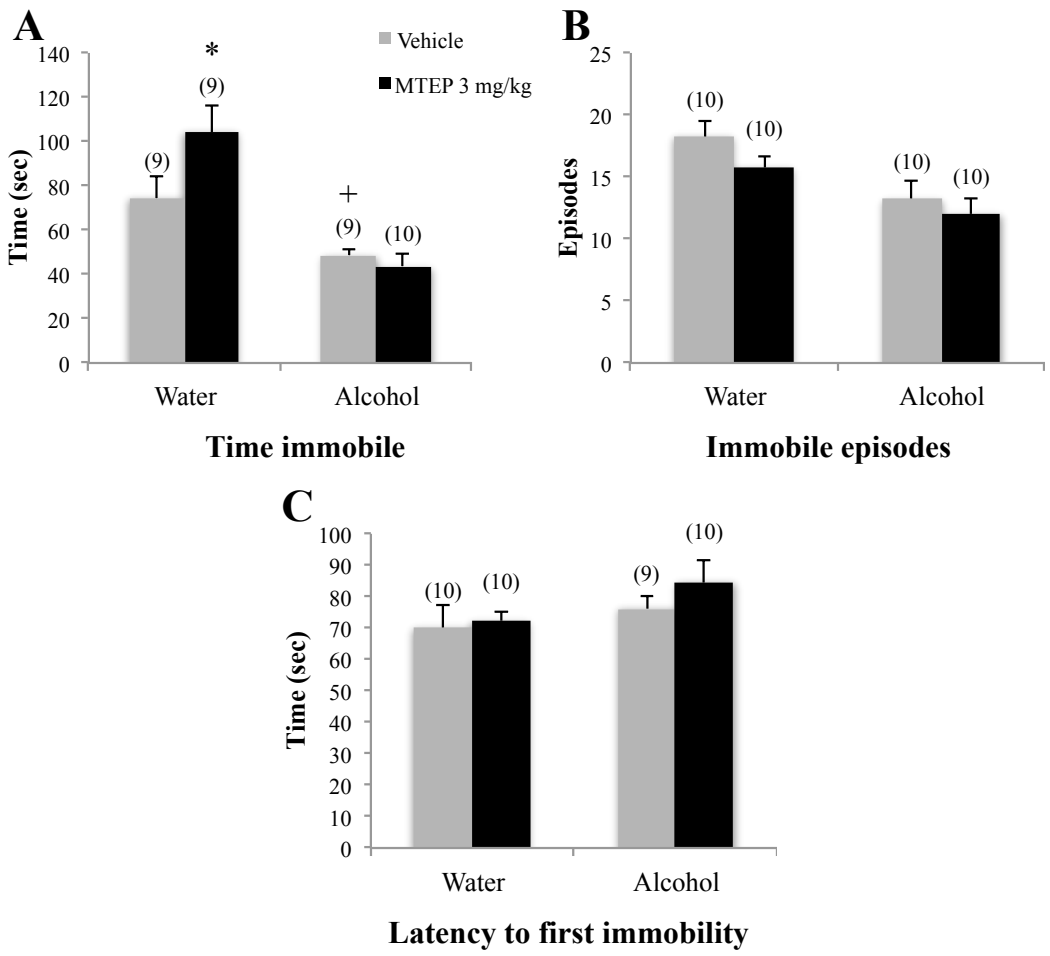

Figure 6.5: FST and low-dose MTEP in adult drinkers. **(A)** There was a significant drinking X treatment interaction in the time spent immobile. Vehicle-treated alcohol drinkers spent less time immobile compared to vehicle-treated water drinkers and MTEP treatment significantly increased time spent immobile in water drinkers only. **(B)** Alcohol drinkers had fewer immobile episodes overall compared to water drinkers. **(C)** There were no effects of alcohol or treatment on distance traveled. *p<0.05 vs. vehicle treatment within same drinking group, +p<0.05 vs. vehicle-treated water drinkers. Data represent mean + SEM of the number of animals indicated in parentheses.

water controls (p=0.006). MTEP treatment significantly increased light-side entries in water controls only (p=0.006). Overall, alcohol-drinking mice spent less time in the light side, relative to water-drinking mice [main drinking effect: F(1,35)=16.60, p<0.001; Fig. 6B] and MTEP-treated animals spent more time on the light-side compared to their vehicle-treated counterparts [main treatment effect: F(1,35)=6.56, p=0.015]. In vehicle-treated animals, alcohol-drinking mice spent less time on the light-side versus water controls (p=0.018), but MTEP treatment increased light-side time in water controls only (p=0.034). There were no drinking or treatment effects on the latency to first light-side entry (p's>0.1; Fig. 6C) or distance traveled in the light-side (Fig. 6D).

3.3.3 Marble-burying. In the marble burying test, alcohol-drinking mice buried more marbles than water controls [main drinking effect: F(1,36)=13.67, p=0.001; Fig. 7A] and MTEP treatment significantly reduced burying compared to vehicle [main treatment effect: F(1,36)=6.97, p=0.012]. In water controls only, MTEP treatment reduced the number of marbles buried relative to vehicle (p=0.030). Alcohol-drinking mice also exhibited a shorter latency to start burying, compared to water controls [main drinking effect: F(1,35)=23.55, p<0.001; Fig. 7B]. In vehicle-treated animals, alcohol-drinking mice exhibited a shorter latency to start burying versus water controls (p<0.001). There was also a trend toward more time spent burying in alcohol versus water mice [drinking trend: F(1,34)=3.49, p=0.07; Fig. 7C].

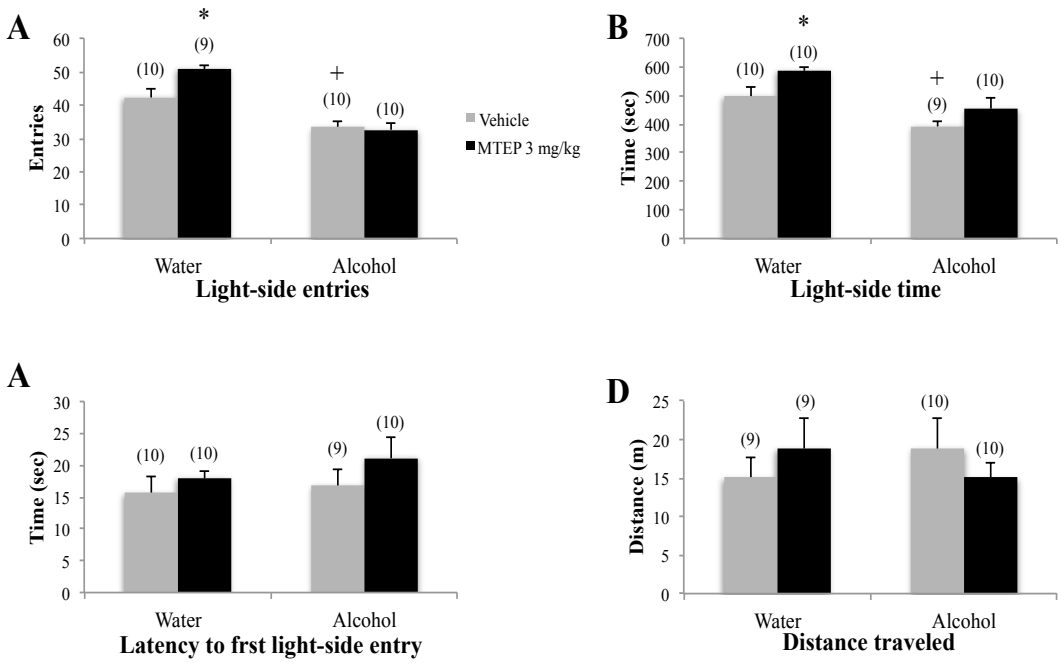

Figure 6.6. Light-dark box and low-dose MTEP in adult drinkers. **(A)** There was a significant drinking X treatment interaction in the number of light-side entries. Vehicle-treated alcohol drinkers made fewer light-side entries compared to vehicle-treated water drinkers. MTEP treatment significantly increased light-side entries in water drinkers. **(B)** Alcohol drinkers spent less time in the light side overall compared to water and MTEP-treated animals spent more time on the light side compared to vehicle. In vehicle-treated animals, alcohol drinkers spent less time on the light side compared to water drinkers. Also, MTEP treatment increased light-side time in water drinkers only. **(C)** There were no drinking or treatment effects on the latency to first light-side entry or **(D)** distance traveled. *$p<0.05$ vs. vehicle treatment within same drinking group, +$p<0.05$ vs. vehicle-treated water drinkers. Data represent mean + SEM of the number of animals indicated in parentheses.

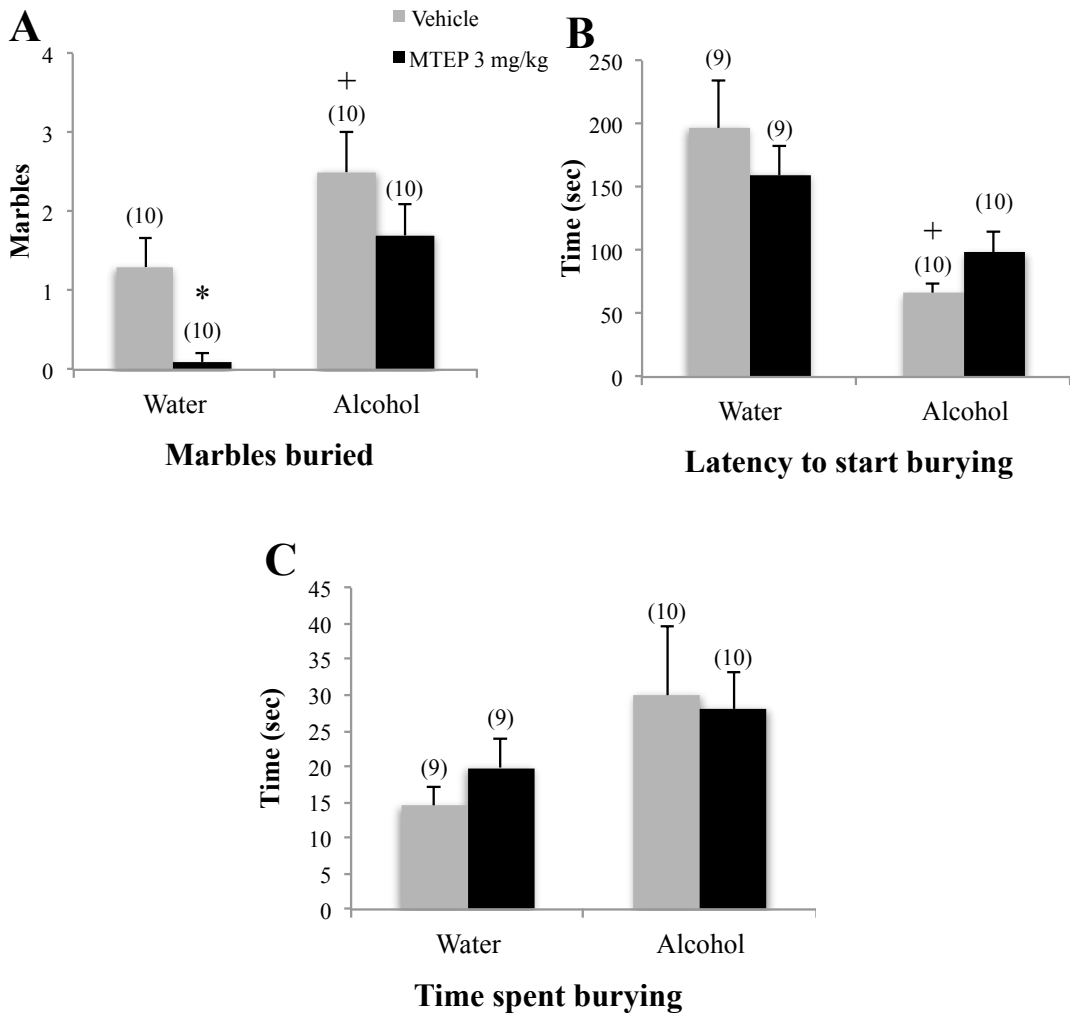

Figure 6.7: Marble burying and low-dose MTEP in adult drinkers. **(A)** Alcohol drinkers buried more marbles than water drinkers and MTEP treatment significantly reduced burying compared to vehicle. In water drinkers, MTEP treatment reduced the number of marbles buried compared to vehicle treatment. **(B)** Alcohol drinkers overall had a shorter latency to start burying compared to water drinkers. In vehicle-treated animals, alcohol drinkers had a shorted latency to start burying compared to water drinkers. **(C)** There was a trend toward more time spent burying in alcohol drinkers compared to water drinkers. *p<0.05 vs. vehicle treatment within same drinking group, +p<0.05 vs. vehicle-treated water drinkers. Data represent mean + SEM of the number of animals indicated in parentheses.

3.4 Alcohol, CDPPB and high-dose MTEP in adolescent drinkers

3.4.1 Porsolt Forced Swim Test. In adolescent animals, there was a treatment trend in the total time spent immobile in the FST [$F(2,58)=2.45$, $p=0.09$; Fig. 8A], which appeared to be driven primarily by a significant decrease in the time spent immobile in CDPPB-treated water controls versus their respective vehicle group ($p=0.008$). There were no significant group differences in the number of immobile episodes or latency to first exhibit immobility (p's>0.1; Fig. 8B & 8C).

3.4.2 Light-Dark Bo*x.* In the light-dark box, there were no significant group differences in the number of light-side entries, time spent on the light side, latency to first light-side entry, or distance traveled (p's>0.1; Fig. 9A-D).

3.4.2 Marble-Burying. In the marble burying test, there was a significant treatment effect in adolescent mice [$F(2,59)=3.23$, $p=0.005$; Fig. 10A], with MTEP-treated animals burying fewer marbles, compared to CDPPB-treated animals ($p=0.002$). Only in water controls did CDPPB treatment increase the number of marbles buried (vs. vehicle: $p=0.019$). There was a significant treatment effect in the total time spent burying [$F(2,59)=22.75$, $p<0.001$; Fig. 10B]; MTEP-treated animals spent less time burying, compared to both vehicle and CDPPB treatment ($p=0.001$ and $p<0.001$, respectively) and CDPPB-treated animals spent more time burying than vehicle ($p=0.002$). MTEP treatment reduced time spent burying in both water- and alcohol-drinking mice, relative to their respective vehicle-treated animals ($p=0.029$ and $p=0.011$, respectively). In contrast, CDPPB treatment increased time spent urying in both water- and alcohol-drinking animals, relative to their

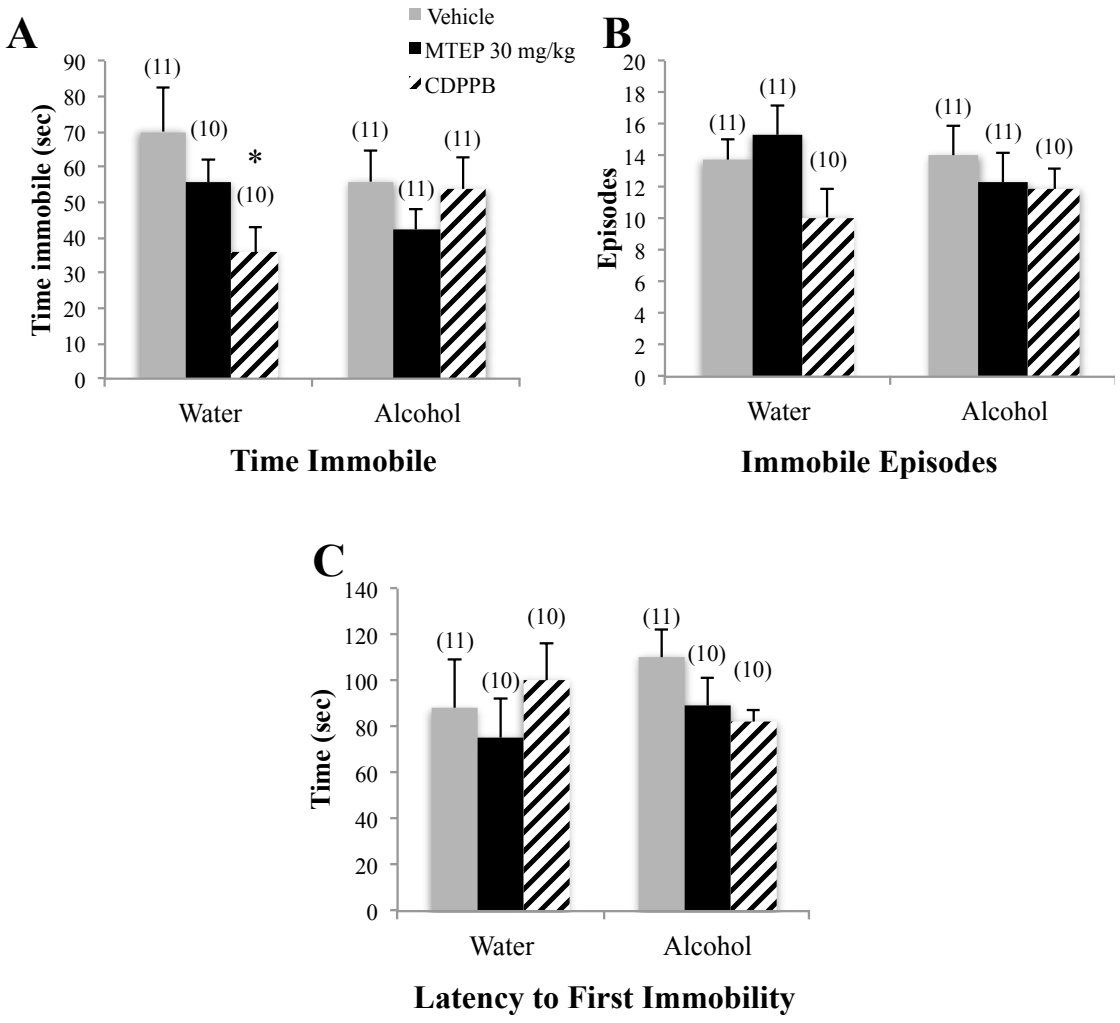

Figure 6.8. FST, CDPPB, and high-dose MTEP in adolescent drinkers. (A) There was a trend toward a drinking X treatment interaction in the total time spent immobile, attributable primarily to a significant decrease in CDPPB-treated water drinkers compared to vehicle treatment. There were no group differences in **(B)** the number of immobile episodes or **(C)** the latency to first immobility. *$p<0.05$ vs. vehicle treatment within same drinking group. Data represent mean + SEM of the number of animals indicated in parentheses.

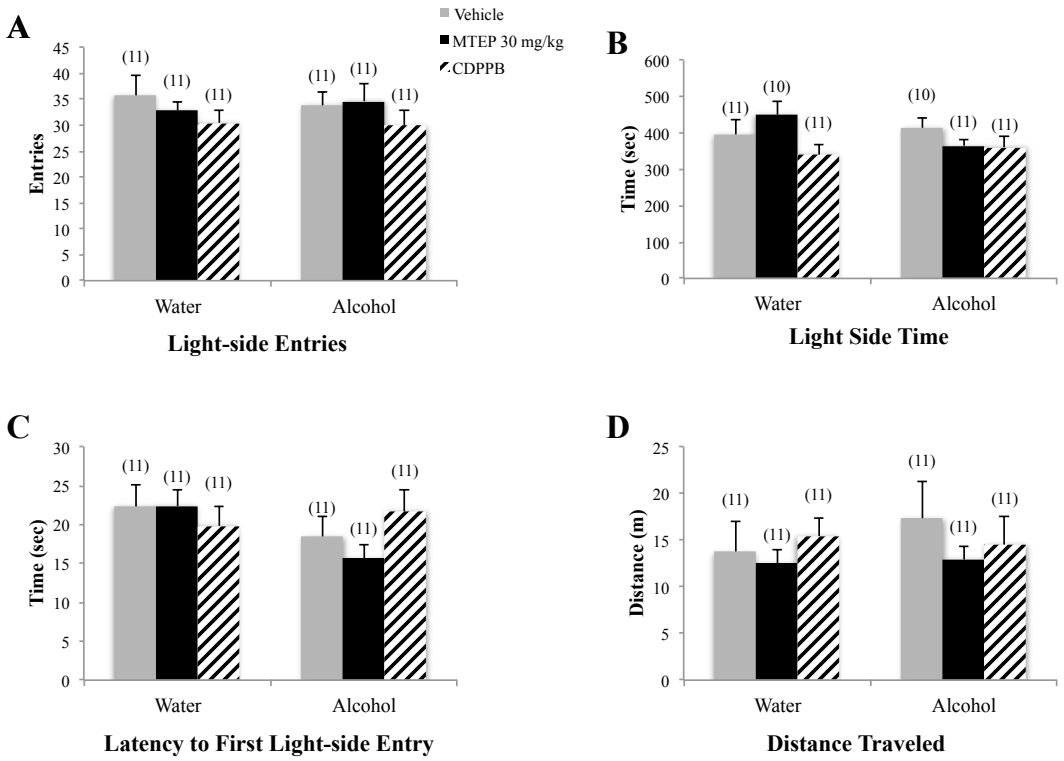

Figure 6.9. Light-dark box, CDPPB, and high-dose MTEP in adolescent drinkers. There were no group differences in any of the dependent variables measured. Data represent mean + SEM of the number of animals indicated in parentheses.

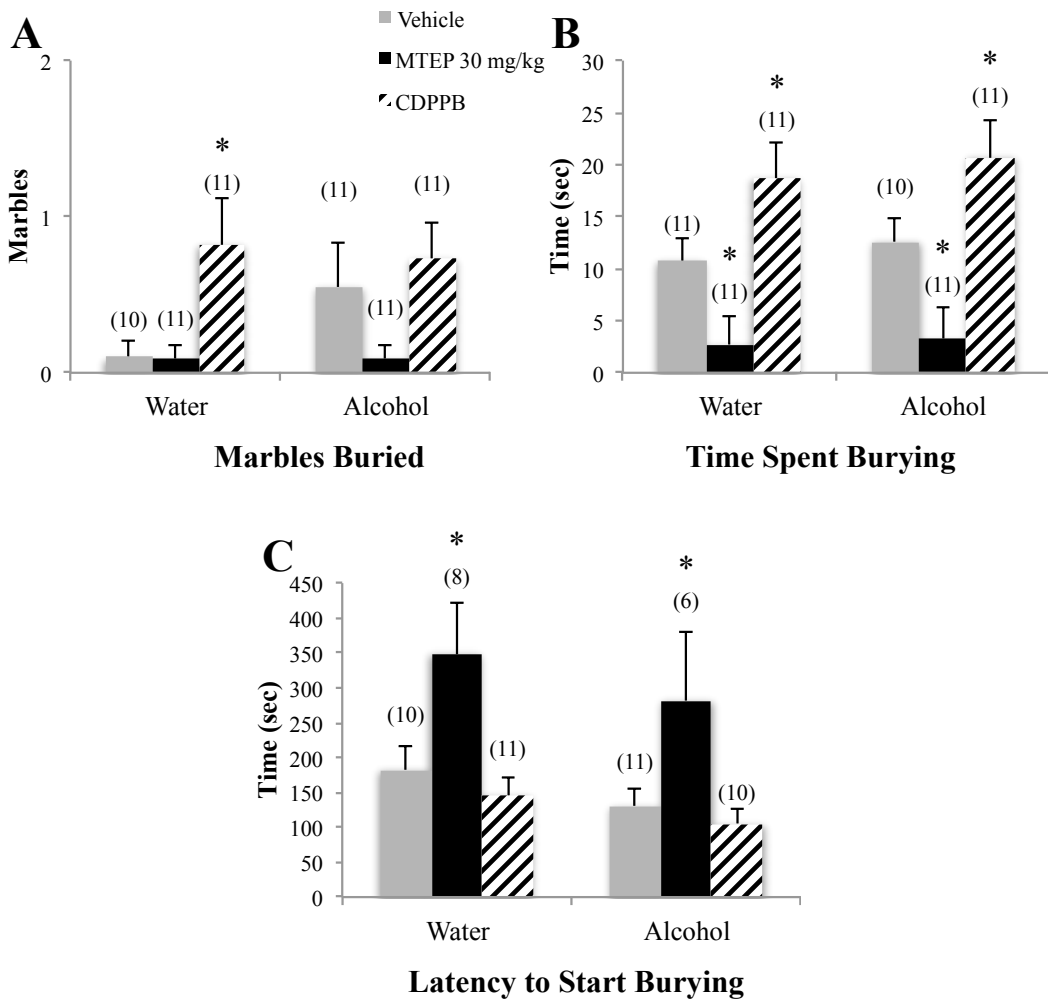

Figure 6.10. Marble burying, CDPPB, and high-dose MTEP in adolescent drinkers. (A) A main treatment effect showed that CDPPB-treated animals buried more marbles overall compared to MTEP treatment, independent of drinking group. This group difference is primarily driven by a significant increase in CDPPB-treated water drinkers specifically. **(B)** In both water and alcohol drinkers, CDPPB-treated animals buried more marbles compared to vehicle-treated animals while MTEP-treated animals buried less. **(C)** MTEP treatment increased latency to start burying overall compared to vehicle and CDPPB treatment in both water and alcohol drinkers. *$p<0.05$ vs. vehicle treatment within same drinking group. Data represent mean + SEM of the number of animals indicated in parentheses.

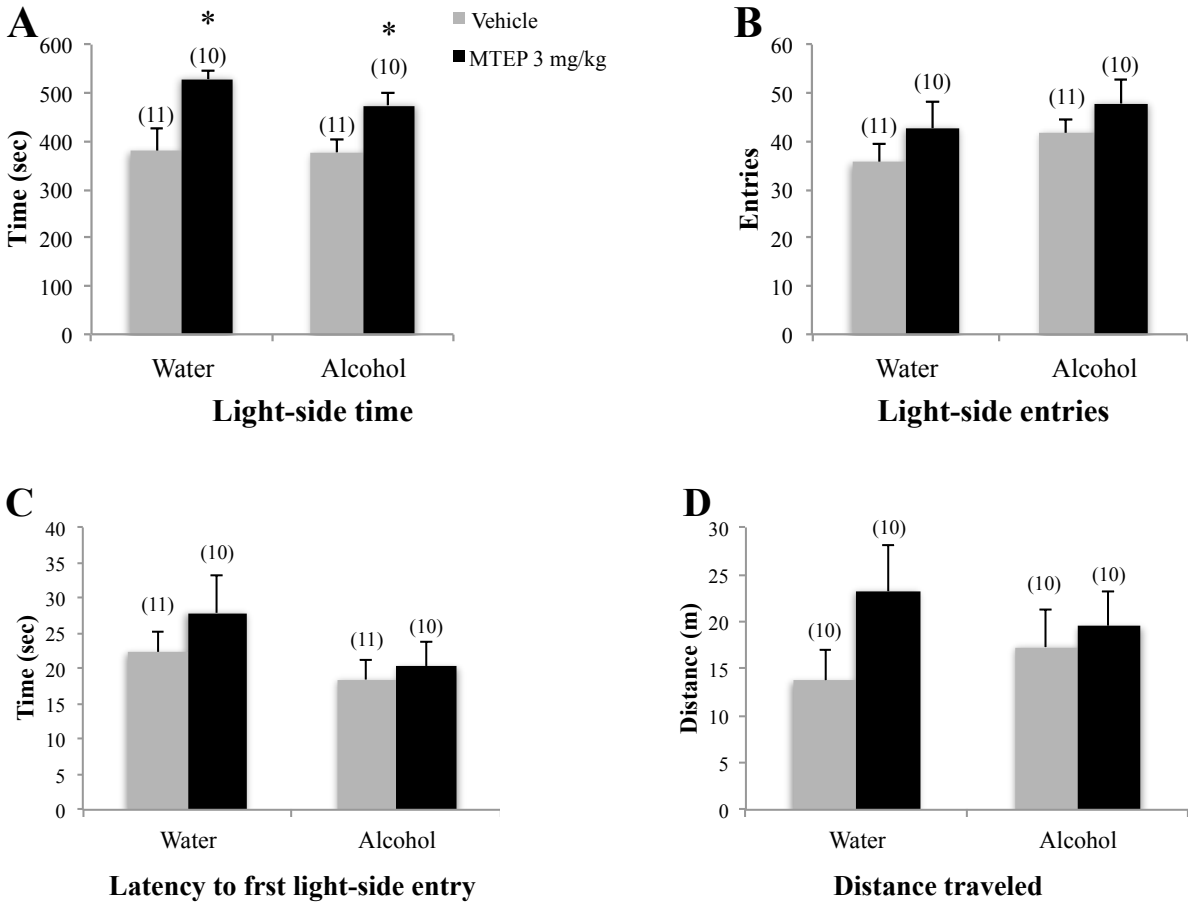

Figure 6.11. Light-dark box, CDPPB, and low-dose MTEP in adolescent drinkers. (A) In both water and alcohol drinkers, MTEP-treated animals spent more time on the light side compared to vehicle treatment. There were no group differences in **(B)** the number of light-side entries, **(C)** latency to first light-side entry, or **(D)** distance traveled. **p*<0.05 vs. vehicle treatment within same drinking group. Data represent mean + SEM of the number of animals indicated in parentheses.

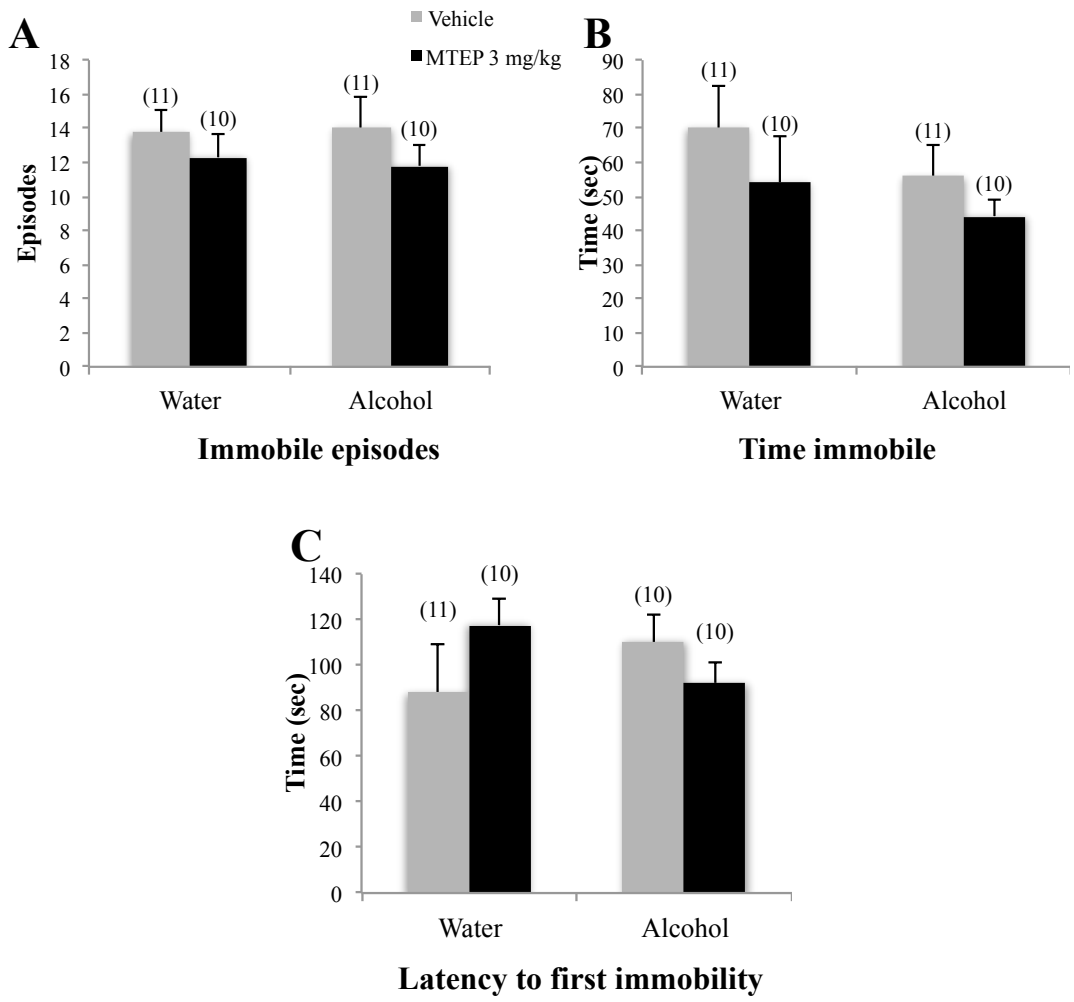

Figure 6.12. FST, CDPPB, and low-dose MTEP in adolescent drinkers. There were no group differences in any of the dependent variables measured. Data represent mean + SEM of the number of animals indicated in parentheses.

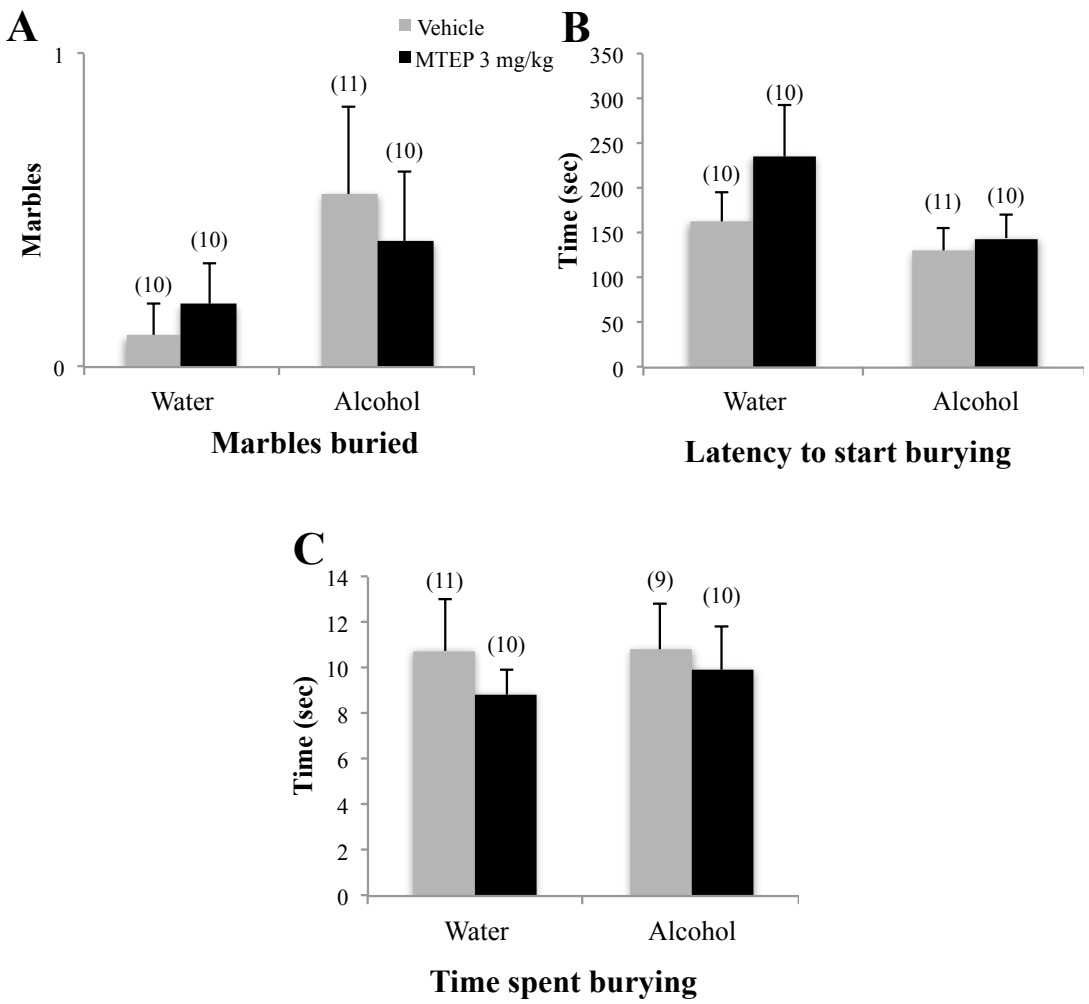

Figure 6.13. Marble burying, CDPPB, and high-dose MTEP in adolescent drinkers. There were no group differences in any of the dependent variables measured. Data represent mean + SEM of the number of animals indicated in parentheses.

respective vehicle- treated groups (p=0.025 and p=0.028, respectively). We also observed a significant treatment effect for the latency to start burying [F(2,50)=9.25, p<0.001; Fig. 10C]; MTEP-treated animals had a longer latency to start burying, compared to both vehicle and CDPPB treatment (p=0.001 and p<0.001, respectively). Further, MTEP treatment increased latency to bury in both water- (p=0.01) and alcohol-drinking mice (p=0.028), relative to respective vehicle-treated animals.

3.5 Alcohol and low-dose MTEP in adolescent drinkers

In the light-dark box, MTEP-treated animals spent more time on the light side, overall, compared to vehicle [main treatment effect: F(1,38)=14.69, p<0.001; Fig. 11A]. However, there were no significant group differences in the number of light-side entries, latency to first light-side entry, or total distance traveled in this paradigm [p's>0.1, Fig. 11B-D]. Further, there were no significant group differences in any of the dependent measures in the FST or the marble-burying test [p's>0.1, Fig. 12&13].

4. Discussion

Consistent with a recent study by our group (Lee et al., 2017), our 3-bottle alcohol-drinking protocol was successful at eliciting high voluntary alcohol consumption, with both adult and adolescent animals drinking at 'binge' levels (BACs ≥80 mg/dl). Extending prior work (Lee et al., 2016), this study demonstrated causal role for alcohol-induced mGlu5 upregulation in the manifestation of withdrawal-induced anxiety in adult, but not adolescent, binge drinkers.

4.1 Alcohol, CDPPB, and MTEP in adult animals

We successfully replicated previous results showing that a 2-week binge-drinking experience is sufficient to increase anxiety–like behavior in adult mice during early (24 h) alcohol withdrawal across various paradigms, including the FST, light-dark box and marble-burying tests. Although immobility in the FST is traditionally interpreted as reflecting behavioral despair (Porsolt, Bertin, et al., 1977; Porsolt et al., 2001), alcohol-withdrawn mice consistently exhibit reduced immobility in this assay (Lee et al., 2015, 2016, 2017), which can be reversed by pretreated with either typical or atypical anxiolytics (including MTEP) (Lee, Coelho, Sern, et al., 2017). Consistent with this recent report (Lee et al., 2017), treatment with 30 mg/kg MTEP reduced withdrawal-induced anxiety, as indicated by increased light-side time and entries, reduced marble-burying and increased latency to start burying, as well as increased time spent immobile in the FST and in all cases, the expression of behavior was comparable to that exhibited by water controls.

An anxiolytic effect of 30 mg/kg MTEP was also observed on some measures in alcohol-naïve adults; 30 mg/kg MTEP decreased marble-burying and the time on the light-side, but had no effect in the FST. These results are more, rather than less, consistent with prior reports of an anxiolytic effect of mGlu5 antagonism in other rodent models (e.g. Busse et al., 2004; Klodzinska et al., 2004; Varty et al., 2005). This being said, the anxiolytic effects of some group 1 mGlu antagonists exhibit an inverted U-shape dose-response curve in alcohol-naïve animals (Belozertseva et al., 2007; Koltunowska et al., 2013; Varty et al., 2005), suggesting that the lack of efficacy of the 30 mg/kg MTEP dose in water controls might be outside the therapeutic range for alcohol-naïve animals (i.e., animals without an alcohol-induced upregulation of mGlu5). Indeed, replicating the study with a lower 3 mg/kg

MTEP dose revealed robust anxiolysis in alcohol-naïve adults, evidenced by increased light-side time and entries, reduced marble burying, and increased time immobile in the FST. However, the 3 mg/kg MTEP dose failed to affect behavior in alcohol-experienced mice, indicating that a higher antagonist dose is required to counteract the anxiogenic effects of alcohol withdrawal presumably due to mGlu5 upregulation (Lee et al., 2016).

There was a modest anxiogenic effect of CDPPB treatment in adult animals. CDPPB decreased light-side time and entries, independent of alcohol experience, and also decreased immobility across various factors in the FST. However, there were no effects of CDPPB treatment in the marble burying test. Thus, increasing mGlu5 signaling appears to be moderately anxiogenic; however, alcohol-induced mGlu5 upregulation is not likely the sole mediator of withdrawal-induced anxiety, as the anxiety exhibited by alcohol-naïve animals treated with CDPPB was still less than that exhibited by alcohol-drinking controls infused with vehicle. Therefore, increased mGlu5 signaling is a necessary, but not sufficient, mediator of withdrawal-induced anxiety in adult animals.

4.2 Alcohol, CDPPB, and MTEP in adolescent animals

Consistent with the literature (Doremus et al., 2005; Spear & Varlinskaya, 2005; SAMHSA, 2008; Vetter et al., 2007), adolescent animals consumed significantly higher quantities of alcohol compared to adult drinkers across the 14-day drinking period. As observed in our previous work (Lee et al., 2016), despite the high alcohol intake, adolescent mice were resilient to withdrawal-induced behavioral dysfunction during early withdrawal, as demonstrated by a lack of differences between vehicle-treated alcohol drinkers and water controls. CDPPB treatment showed a slight anxiogenic effect in adolescent animals,

increasing the total time spent burying in both water- and alcohol-drinking mice. CDPPB treatment also increased the number of marbles buried and reduced time immobile in water-drinking animals. However, we were largely unsuccessful in eliciting a robust, 'adult-like' anxiogenic withdrawal phenotype in alcohol-experienced adolescents by stimulating mGlu5 signaling. Whether or not the failure of CDPPB to instigate hyper-anxiety during alcohol withdrawal in adolescent mice relates to age-dependent inefficiency in mGlu5 signaling or in CDPPB pharmacokinetics (or perhaps interactions between these factors and prior alcohol experience) remains to be determined.

In previous studies, we failed to detect an effect of early alcohol withdrawal upon mGlu5 expression in adolescent mice (Lee et al., 2016). Thus, it is perhaps not surprising that MTEP exerted relatively little effect upon behavior in adolescent mice and that the effects of MTEP were comparable between water- and alcohol-drinking adolescents. The 30 mg/kg MTEP dose decreased time spent marble burying and increased latency to start burying in both alcohol and water drinkers, but had no effect in the FST or light-dark box. Even when treated with the lower 3 mg/kg MTEP dose, the only MTEP effect observed in adolescent mice was an increase in light-side time. The relatively weak effect of MTEP in adolescent vs. adult mice furthers the notion that mGlu5 signaling may be less efficient in the younger animals and/or that the regulation of anxiety in adolescent animals is independent of mGlu5 signaling.

As a final point of consideration, due to the abundance of mGluRs located within the vertebrate retinal (Connaughton, 1995; Gerber, 2003; Thoreson & Witkovsky, 1999) and reports that mGlu5 antagonists can affect vision (Daw et al., 2004; Sidorov et al., 2015), an initial concern might be that perhaps some of the observed behavioral effects of MTEP or

CDPPB could be attributable to visual changes or the anxiogenic effects of impaired vision. However, this is not likely the case, as no reports could be found in the literature reporting visual distortions resulting from systemic administration of either mGlu agonists or antagonists at doses similar to those employed herein.

4.3 Conclusions

Binge alcohol-drinking during adulthood results in mGlu5 upregulation, which is causally related to the manifestation of anxiety-like behaviors during withdrawal in a mouse model of binge-drinking. Pharmacologically reversing this upregulation results in a dose-dependent reduction in anxiety-like behaviors. In contrast, adolescent mice with a history of binge-drinking are resistant to both alcohol-induced mGlu5 upregulation and withdrawal-induced anxiety, despite consumer larger quantities of alcohol. Allosteric modulation of mGlu5 signaling produces minimal effects in adolescent animals, independent of prior alcohol experience. These results point to the existence of age-specific underlying mechanisms mediating the ontogeny of anxiety and alcohol withdrawal-induced negative affect. As it relates to humans conditions, this study highlights the importance of age-specific considerations in the pharmacotherapeutic treatment of both anxiety and substance abuse disorders in the clinical population.

Chapter 7:

mGlu5 blockade within the nucleus accumbens shell reduces alcohol withdrawal-induced anxiety in adult and adolescent mice.

1. Introduction

Dysregulation of glutamate signaling is a fundamental feature of drug abuse and addiction. Virtually all drugs of abuse, including alcohol, nicotine, psychostimulants, and opiates alter glutamatergic signaling within the nucleus accumbens (Acb), a brain region critically involved in reward, reinforcement, and incentive salience (Kalivas et al., 2005; Quintero, 2013; Reid et al., 2000; Scofield et al., 2016). Group 1 metabotropic glutamate receptors (mGlu subtypes 1 and 5) are particularly susceptible to drug-induced adaptation (Gass & Olive, 2008). Activation of these receptors triggers intracellular signaling pathways, which can result in enduring changes in cellular excitability and gene expression (Cleva & Olive, 2012; Kumar et al., 2012). Chronic drug abuse can elicit a persistent hyperexcitable state within the Acb that is as associated with increased drug salience, craving, and relapse (Cornish & Kalivas, 2000; Di Chiara, 2002; Tzschentke & Schmidt, 2003). Thus, drug-induced changes in cellular activity due to dysregulation of group 1 mGluR signaling represent a fundamental component of the plasticity underlying the neurobiological basis of drug addiction.

Alcohol is the most commonly abused drug in the U.S. with over 17.7 million Americans ages 12 and over reporting dependence or problematic patterns of abuse (SAMHSA, 2012). Binge-drinking is the most prevalent form of alcohol abuse and is a significant risk factor for alcohol addiction (CDC, 2016; Dawson et al., 2005). Animal studies have repeatedly demonstrated the importance of group 1 mGluRs within the Acb in mediating many aspects of alcohol abuse, including the subjective interoceptive effects of alcohol, the maintenance and escalation of voluntary alcohol consumption/self-

administration, and reinstatement of alcohol seeking (Besheer et al., 2009; Cozzoli et al., 2012; Cozzoli et al., 2015; Cozzoli et al., 2009; Schroeder et al., 2005; Sinclair et al., 2012).

Chronic binge drinking often leads to feelings of anxiety, depression, and general dysphoria during periods of abstinence, which are theorized to facilitate the transition to addiction by acting as a source of negative reinforcement, encouraging further alcohol consumption in order to alleviate this aversive state (Baker et al., 2004; Gilpin & Koob, 2008; Koob, 2003; Stewart et al., 2001). Alcohol abuse has a remarkably high comorbidity with mood disorders such as anxiety and depression (Briere et al., 2014; Compton et al., 2007; Driessen et al., 2001; Hasin et al., 2007; Kushner et al., 1990) and it has been suggested that a shared basis of glutamatergic dysfunction could contribute to this high comorbidity (Schwartz et al., 2007; Silberman et al., 2009).

Decades of research provide a detailed examination of the AcbSh and the positive reinforcing/hedonic properties of alcohol (reviewed in Quintero, 2013). However, the shell region of the Acb is also a component of the extended amygdala, the subcortical macrosystem that mediates emotional processing and regulation, consisting of the central nucleus of the amygdala, the bed nucleus of the stria terminalis, and the shell subregion of the Acb (AcbSh) (Alheid, 2003). Disruption of glutamatergic signaling within the extended amygdala is implicated in anxiety and other mood disorders (Bergink et al., 2004; Cortese & Phan, 2005). Although activity within AcbSh is implicated in morphine and heroin withdrawal-induced anxiety (Lou et al., 2014; Radke & Gewirtz, 2012) and GABA agonist administered directly into the AcbSh reduces anxiety (Lopes et al., 2012), very little is known regarding the role of glutamate signaling within the AcbSh in alcohol withdrawal-induced negative affect specifically.

In the human population, binge-drinking is especially common among adolescents and young adults. In fact, over 90% of the alcohol consumed by underage drinkers is in the form of binge drinking episodes (NIAAA, 2017). As such, we have explored age-related differences in the manifestation and underlying neurobiology of withdrawal-induced negative affect in adult and adolescent mice with a history of voluntary binge-drinking. Consistent with the human data, animal studies have shown that adolescents tend to consume larger quantities of alcohol, yet show attenuated signs of acute withdrawal compared to adult drinkers, including withdrawal-induced negative affect (Doremus et al., 2003; Lee et al., 2016; Little et al., 1996; Schramm-Sapyta et al., 2010; Spear & Varlinskaya, 2005; Varlinskaya & Spear, 2004). However, despite this apparent resilience, adolescent drinkers may be more susceptible impairments later in life due to alcohol-induced disruption of ongoing neurodevelopment during adolescence. Consistent with this hypothesis, we showed recently that, although adolescent binge-drinkers show minimal signs of affective dysregulation during early (24 h) withdrawal, hyper-anxiety and depression-like behaviors increase during protracted (4 weeks) withdrawal (Lee, Coelho, Solton, et al., 2017). In contrast, the robust increase in anxiety-related behaviors observed during early withdrawal dissipates in adults during protracted withdrawal. Additionally, western-blotting indicated that the increase in anxiety-like behavior exhibited by adult binge-drinking mice coincided with increased mGlu5 expression within the AcbSh.

The present study is a continuation of this prior work examining the functional role of mGlu5 in withdrawal-induced negative affect. Based on evidence that systemic administration of an mGlu5 antagonist can reverse withdrawal-induced hyperanxiety in adult binge-drinking mice (Lee, Coelho, Class, et al., 2017), the present study used

neuropharmacological approaches to directly assess the effects of mGlu5 blockade within the AcbSh on withdrawal-induced anxiety in both adult and adolescent mice. We hypothesized that blocking mGlu5 hyperactivation within the AcbSh would decrease anxiety-like behaviors in adult binge-drinking mice during early withdrawal. Although adolescent binge-drinking mice do not exhibit a withdrawal-induced increase in total mGlu5 protein expression within the AcbSh (Lee et al., 2016), such findings do not preclude a functional alteration in mGlu5 that could underpin the incubated hyperanxious state exhibited by these mice during protracted withdrawal. Indeed, we demonstrated previously that binge-drinking-induced changes in AcbSh mGlu5 expression are dissociable from function (Cozzoli et al., 2009). Therefore, we deemed it prudent to test the effects of MTEP in adolescent binge-drinking mice during protracted withdrawal to determine the potential contribution of this receptor to the incubation of negative affect in these animals and provide a more definitive conclusion regarding age-related differences in the role for mGlu5 in the neurobiological effects of chronic alcohol exposure.

2. Materials and Methods

Binge-drinking and behavioral testing procedures used in this experiment were identical to those employed previously in our lab (Lee et al., 2016; Lee, Coelho, Sern, et al., 2017) and are briefly summarized below. All procedures were conducted in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (NIH Publication No. 80–23, 2011) and approved by the IACUC of the University of California, Santa Barbara.

2.1 Subjects

The animals in this study were male C57BL/6J mice, purchased from The Jackson Laboratory (Sacramento, CA). Mice were allowed 7 days to acclimate to our vivarium and were either PND 28 (adolescents) or PND 56 (adults) at the onset of drinking. Animals were identified using small animal ear tags (Stoelting, Wood Dale, IL) and housed in age-matched groups of 4 in a climate-controlled vivarium under a reverse light/dark cycle (lights off at 10am). Food and water were available *ad libitum*, except during the 2-h alcohol drinking period. The study design consisted of 2 age groups (adults and adolescents), 2 drinking groups (alcohol or water), and 3 treatment groups (MTEP 1 μg, MTEP 10 μg, or vehicle); n=12/group.

2.2 Drinking-in-the-Dark (DID) Procedures

Half of the animals from each age group were subjected to 14 consecutive days of binge-drinking under 3-bottle DID procedures. Control animals received water only. In order to maintain consistency across age groups, alcohol access was restricted to 14 days for all animals, corresponding to the approximate duration of early-mid adolescence in mice (Spear, 2000b). Each day, animals were separated into individual cages, allowed 45 min to habituate to the drinking cage and then given concurrent access to 10, 20, and 40% (v/v) unsweetened ethanol solutions for 2 h, beginning 3 h into the circadian dark cycle (Rhodes et al., 2005). The amount of alcohol consumed each day was calculated by bottle weight immediately before and after the drinking period and expressed as a function of the animal's body weight (g/kg). All animals, both alcohol and water drinkers, were weighed 3x per week throughout the drinking period.

Submandibular blood samples were collected from all alcohol-drinking animals on day 11 of drinking, immediately upon conclusion of the 2-h drinking period. The scheduling of the blood sampling was selected to ensure that the animals' intakes had stabilized, while also allowing ample time for recovery prior to behavioral testing. Blood alcohol concentration (BAC) was determined using an Analox alcohol analyzer (model AM1, Analox Instruments USA, Lunenburg, MA).

2.3 Behavioral testing

Based on our previous work showing age differences in the emergence of a withdrawal phenotype (early withdrawal in adults vs protracted withdrawal in adolescents), both adult and adolescent animals were behaviorally tested at PND 70. Even though all animals were tested in adulthood, 'adult' and 'adolescent' refer to the age of their alcohol-drinking experience. The behavioral testing battery consisted of the light-dark box, marble burying test, and Porsolt forced swim test (FST). These assays have been consistently sensitive to alcohol withdrawal-induced behavioral differences in our prior studies (exactly as conducted in Lee et al., 2016; Lee, Coelho, Sern, et al., 2017). In the light-dark box, the dependent measures were: the number of light-side entries, latency to first light-side entry, total time spent on the light side, and distance traveled. In the marble burying test, the dependent measures were: the number of marbles buried, latency to first begin burying, and total time spent burying. In the FST, the dependent measures were: the number of immobile episodes, latency to first immobile episode, and total time spent immobile. Behavioral testing was conducted during the animals' circadian dark phase.

2.4 Surgical procedures

Both adult and adolescents underwent surgery at the same age, approximately 3 weeks prior to behavioral testing (PND 42-48). Thus, surgery occurred prior to the 14-day drinking period in adults and afterward in adolescents. In addition to maintaining consistency in the surgery timing across age groups, this also allowed us to avoid the practical limitations of performing head cap surgeries on small juveniles. Surgical procedures were similar to those conducted previously in our lab (e.g. Cozzoli et al., 2012; Lum et al., 2014). Under isoflurane anesthesia, animals were implanted with bilateral indwelling guide cannulae (20-gauge, 10 mm long) positioned 2mm above AcbSh, based on coordinates from the mouse brain atlas of Paxinos and Franklin (2004): AP: +1.3; ML: ±0.5 mm; DV: −2.3 mm from Bregma. Cannulae were secured to the skull surface using dental resin (as conducted previously e.g. Cozzoli et al., 2012; Lum et al., 2014) and dummy cannulae (24 gauge; 10 mm long) were placed inside the guide cannulae to protect from contamination. Animals were allowed to recover for a minimum of 7 days before further experimentation.

2.5 Intracranial drug infusion procedures

This study used both a high 10 μg/side and low 1 μg/side dose of MTEP (Sigma Aldrich; St. Louis, MO), dissolved in sterile water, to establish a dose-response relationship. These doses were selected based on prior studies by our group (Cozzoli et al., 2014; Cozzoli et al., 2009). On the day of behavioral testing (PND 70), animals from each drinking group (alcohol or water) received a microinfusion of MTEP, or sterile water vehicle. Bilateral infusions (volume = 0.25 μl/side) were delivered simultaneously to the AcbSh via 33-gauge injector (12 mm long) at a rate of 0.25 μl/min for 60 sec. Injectors were left in place for an

additional 60 sec to allow for drug diffusion. Animals were then returned to their home cages for approximately 15 min before the start of testing, to allow for maximal drug efficacy.

2.6 Immunohistochemistry

Upon completion of behavioral testing, animals were euthanized with an overdose of Euthasol® (Virbac AH, Fort Worth, TX) and transcardially perfused with phosphate-buffered saline (PBS), followed by 4% paraformaldehyde. Brains were removed and post-fixed for 24 h in 2% paraformaldehyde in PBS, then cold-stored in cryoprotectant (30% ethylene glycol, 30% glycerol in PBS to prevent ice crystal formation) until slicing. Tissue was sectioned (40 μm) along the coronal plane on a vibratome at the level of the Acb (as depicted in Paxinos & Franklin, 2004). Immunohistochemistry was performed to detect expression of Egr1, an immediate early gene (IEG) encoded inducible transcription factor commonly used as a marker of localized brain activation in laboratory animals (Lee, 2014). Egr1 has higher constitutive expression compared to other common IEGs such as c-fos, making it sensitive to both increases and decreases in expression (Herdegen & Leah, 1998). Thus, changes in Egr1 expression were used to determine the effects of alcohol withdrawal and MTEP treatment on neuronal activation within the AcbSh. Tissue sections were stained using standard immunohistochemical procedures, as described in Lee et al. (2015), using a rabbit Egr1 primary antibody (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) and visualized with 3,3′-diaminobenzidine (DAB). Following staining, sections were dehydrated and cover-slipped.

2.6.1 Immunohistochemical analysis. Slides were viewed with a Nikon Eclipse E800 microscope equipped with a Hamamatsu CCD camera (model C4742-95). Images were acquired at 40X magnification using MetaMorph® software (Molecular Devices, Sunnyvale, CA). DAB staining intensity was quantified using ImageJ (NIH Image, National Institutes of Health, Bethesda, MD; available online at http://rsbweb.nih.gov/ij/), using the 'mean gray' function. The mean gray intensity value of the entire image was divided by an internal background control value for each animal obtained from a region lacking any Egr1+ cells, yielding a normalized measure (arbitrary units) of staining intensity (similar to as described in Hartig, 2013; Jensen, 2013; Nguyen DH, 2013). A left- and right-side measurement was summed for each animal. In addition to an analysis of staining, slides were also examined for proper microinjector placement. Only animals with injector cannulae located within the boundaries of the AcbSh were included in the statistical analyses of the data (see Fig. 7).

2.7 Statistical analysis

A repeated measures ANOVA was used to analyze intake data for all alcohol-drinking animals to assess age differences in alcohol consumption across the 14-day drinking period. A Pearson's correlational analysis was conducted to determine the relationship between alcohol intake and resulting BACs sampled on day 11 of drinking. A repeated measures ANOVA was also performed on adults and adolescents independently to ensure there were no intake differences between treatment groups.

Behavioral data were analyzed separately within adults and adolescents using Tukey-Kramer multiple comparison tests. Although most commonly used as a post hoc test, an ANOVA is not a prerequisite and Tukey's HSD can be used as a stand-alone test, as it

provides conservative protection against Type I error while maximizing statistical power (Cardinal & Aitken, 2006; Hayter, 1984; Kramer, 1956). This approach is particularly powerful, and often considered preferable to the traditional ANOVA (Games, 1971; Hancock & Klockars, 1996; Rosnow & Rosenthal, 1989; Ruxton & Beauchamp, 2008; Wilkinson & Inference, 1999), in studies that contain multiple *a priori* comparisons of interest. Based on the hypotheses in this study, MTEP-treated animals were compared to vehicle-treated animals within each drinking group, as we predicted that MTEP treatment would have an anxiolytic effect, and vehicle-treated water drinkers were compared with vehicle-treated alcohol drinkers to confirm the presence of withdrawal-induced negative affect. This approach was also used to analyze the immunohistochemistry data and we predicted that MTEP would reduce Egr1 expression in the AcbSh compared to vehicle treatment in both water and alcohol drinkers. The effect of alcohol withdrawal on Egr1 expression among vehicle-treated animals was also assessed.

Statistical outliers were identified using the ±1.5×IQR rule and excluded from analyses. All data depicted in figures represent mean ± SEM of the number of the number of animals indicated in parentheses; *p*-values less than 0.05 were considered to be significant for all tests. Tukey-Kramer analyses were performed in Microsoft Excel using add-on StatPlus$^{6.0}$ and all other calculations and analyses were performed using SPSS v.21 statistical software (IBM, 2012).

3. Results

3.1 Animal exclusion

Animals were omitted entirely from data analysis based on incorrect microinjector placement, difficulties encountered during staining, and attrition due to surgical complications. Final sample sizes for analysis were 9-11 per group (see Table 1 for detailed summary).

3.2 Alcohol intake

Adults consumed an average of 3.88 ± 0.20 g/kg and adolescents an average of 5.00 ± 0.21 g/kg across the 14-day drinking period. The repeated-measures ANOVA showed a significant effect of age, indicating that adolescent consumed more alcohol than adults across the 14-day drinking period [$F(1,58)=14.93$, $p<0.001$]. On day 11 of drinking, adults consumed an average of 3.69 ± 0.31 g/kg resulting in an average BAC of 69.93±3.17 mg/dl and adolescents consumed an average of 4.94 ± 0.34 g/kg resulting in an average BAC of 78.29 ± 3.01 mg/dl. Thus, by definition, the alcohol intake exhibited by either age group did not satisfy the criterion for "binge-drinking". Although the correlation between alcohol intake and resulting BAC on day 11 did not quite reach significance, there was a strong statistical trend ($r=0.242$, $p=0.062$). The repeated-measures ANOVA within each age independently revealed no significant between-subjects effect of treatment [adults: $F(2,28)=0.70$, $p=0.507$; adolescents: $F(2,26)=1.11$, $p=0.341$]

3.3 Intracranial MTEP in adults during early alcohol withdrawal

3.3.1 Light-dark box. In vehicle-treated animals, alcohol-drinking mice made fewer light-side entries, compared to water controls ($p=0.001$; Fig. 1A). 1 μg/side MTEP

Table 7.1. Summary of final sample sizes

	Water			Alcohol		
	Vehicle	MTEP 1 μg/side	MTEP 10 μg/side	Vehicle	MTEP 1 μg/side	MTEP 10 μg/side
Adults	11	11	10	10	10	11
Adolescents	11	9	10	10	9	10

significantly increased the number of light-side entries in alcohol-drinking mice, compared to vehicle treatment (p=0.039). There was no effect of 1 μg/side MTEP in water drinkers and no effect of 10 μg/side in either alcohol or water drinkers (p's >0.10). There was a similar pattern of result in the time spent on the light side. In vehicle-treated animals, alcohol-drinking mice spent less time on the light side, compared to water drinkers (p<0.001; Fig. 1B), and in alcohol-drinking mice specifically, 1 μg/side MTEP significantly increased the time spent on the light side, compared to vehicle treatment (p=0.004). However, there was no effect of 1 μg/side MTEP in water drinkers and no effect of 10 μg/side in either alcohol or water drinkers (p's >0.10). There were no significant effects of MTEP treatment within water or alcohol-drinking animals on latency to first light-side entry and no effect of alcohol among vehicle-treated animals (p's >0.10; Fig. 1C). There were also no significant group differences in the total distance traveled (p's>0.01; Fig. 1D).

3.3.2 Forced swim test. In vehicle-treated animals, alcohol-drinking mice spent less time immobile, compared to water drinkers (p=0.008; Fig. 2A) and also had fewer immobile episodes compared to water drinkers (p=0.036; Fig. 2B). However, neither dose of MTEP had an effect in water or alcohol drinkers (p's>0.10). There were no significant differences in the latency to first immobility (p's>0.10; Fig. 2C).

3.3.3 Marble burying. In the vehicle treatment group, alcohol-drinking animals buried more marbles compared to water controls (p=0.023; Fig. 3A). Alcohol-drinking mice treated with 1 μg/side MTEP buried fewer marbles compared to vehicle (p=0.016), but there

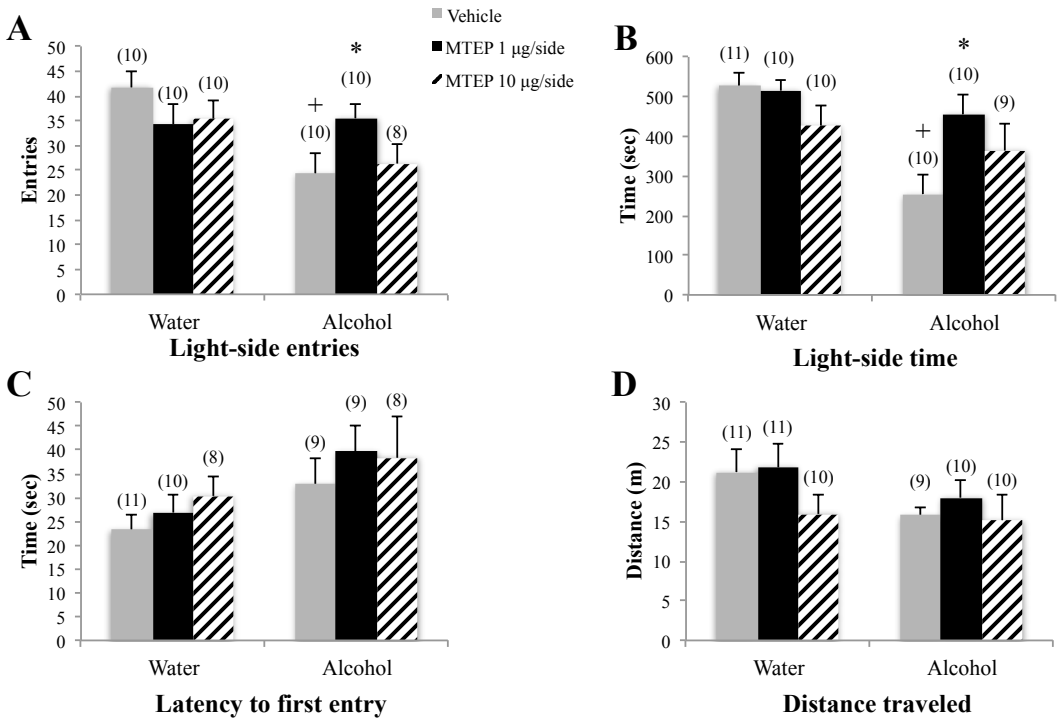

Figure 7.1. **Effects of MTEP and adult alcohol experience in the light-dark box. (A)** Vehicle-treated alcohol drinkers made fewer light-side entries compared to water drinkers. Alcohol drinkers treated with 1 µg/side MTEP showed a significant increase in light-side entries compared to vehicle treatment. **(B)** Similarly, vehicle-treated alcohol drinkers also spent less time on the light side compared to water drinkers and 1 µg/side MTEP significantly increased light-side time compared to vehicle treatment. **(C)** There were no significant group differences in the latency to first light-side entry or **(D)** distance traveled. *$p<0.05$ treatment effect within same drinking group, +$p<0.05$ drinking effect within same treatment group.

was no effect of 1 μg/side MTEP in water-drinking animals and no effect of 10 μg/side in either the water or alcohol group. In the alcohol group, animals treated with 1 μg/ side MTEP spent less time marble burying compared to vehicle (p=0.037; 3B) and there was also a trend toward more time spent burying in vehicle-treated alcohol-drinking mice, compared to water controls (p=0.061), but there was no effect of 1 μg/side MTEP in the water group and no effect of 10 μg/side in either the water or alcohol group (p's> 0.10). In both the water and alcohol group, 1 μg/side MTEP significantly increased latency to begin burying (p=0.046 and p=0.021, respectively; Fig. 3C). However, there was no effect of 10 μg/side MTEP and no effect of alcohol among vehicle-treated animals (p's> 0.10).

3.4 Intracranial MTEP in adolescent drinkers during protracted alcohol withdrawal

3.4.1 Light-dark box. In vehicle-treated animals, alcohol-drinking mice made fewer light-side entries, compared to water-drinking controls (p=0.004; Fig. 4A), but there were no effects of MTEP in either alcohol or water drinkers (p's> 0.10). In vehicle-treated animals, alcohol-drinking mice also spent less time on the light side, compared to water controls (p=0.045; Fig. 4B). In alcohol-drinking mice specifically, 10 μg/side MTEP significantly increased light-side time, compared to vehicle (p=0.006). However, there was no effect of 10 μg/side MTEP in water drinkers and no effect of 1 μg/side in either alcohol or water drinkers (p's> 0.10). There was a trend toward longer latency to first light-side entry in vehicle-treated alcohol-drinking mice, compared to vehicle-treated water control animals (p=0.065; Fig. 4C), but no effects of MTEP in either the alcohol or water group (p's> 0.10). There were also no significant differences in the distance traveled (p's>0.10; Fig. 4D).

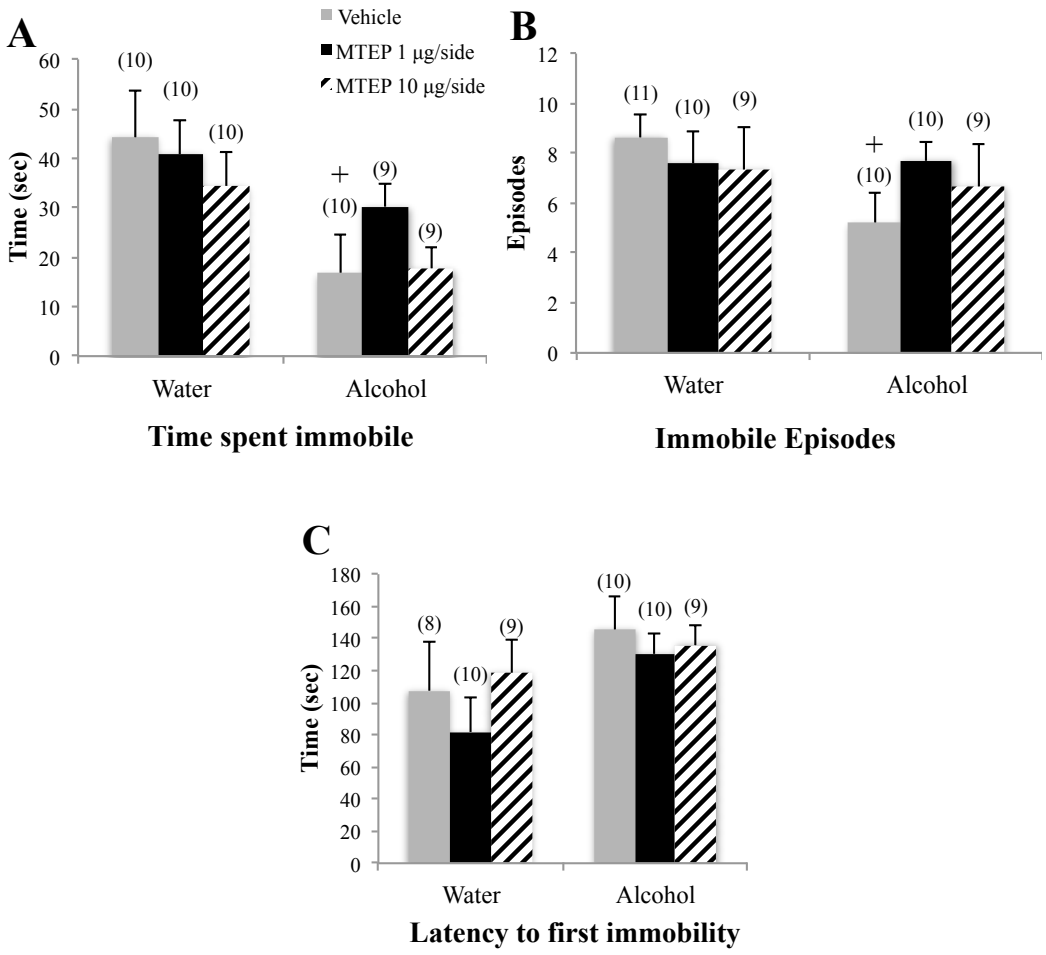

Figure 7.2 Effects of MTEP and adult alcohol experience in the FST. (A) Vehicle-treated alcohol drinkers specifically spent less time immobile compared to water drinkers. However, there were no treatment effects of MTEP in either water or alcohol drinkers. **(B)** There were no significant group differences in the number of immobile episodes or **(C)** latency to first immobility +$p<0.05$ drinking effect within same treatment group.

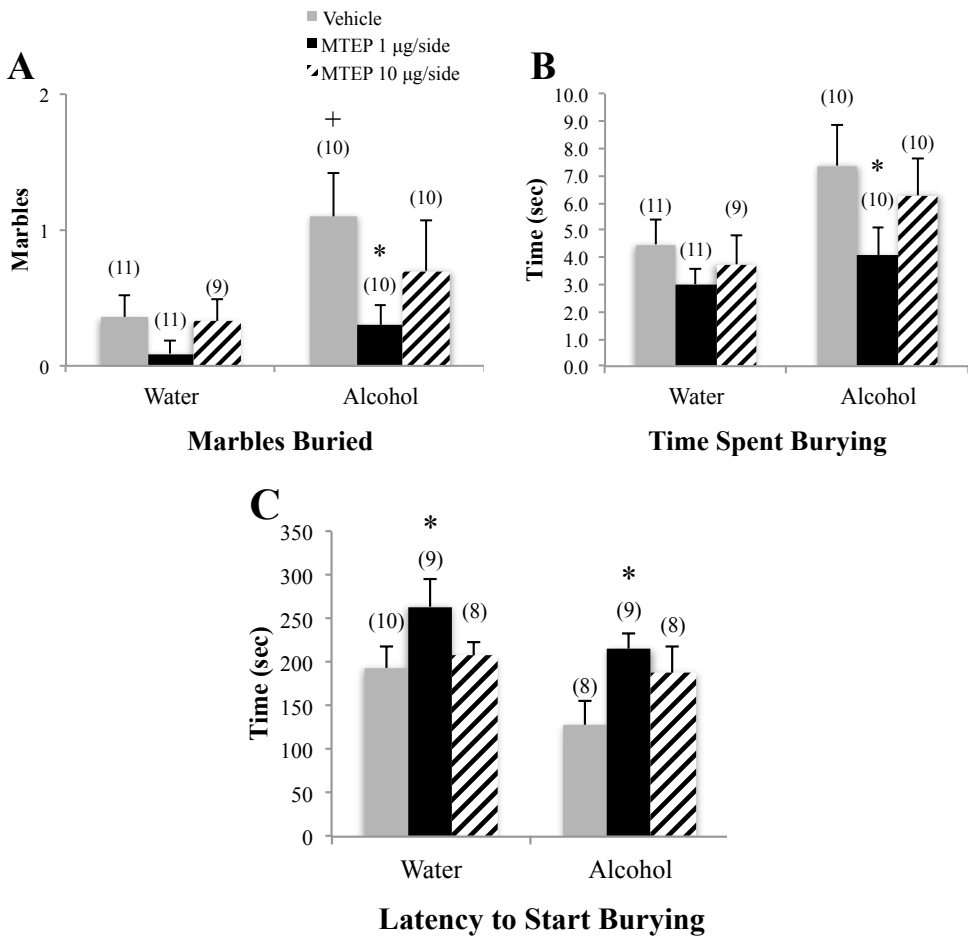

Figure 7.3 Effects of MTEP and adult alcohol experience in the marble burying test. **(A)** Vehicle-treated alcohol drinkers buried more marbles than vehicle-treated water drinkers. Within alcohol drinkers specifically, 1 µg/side MTEP significantly reduced marble burying compared to vehicle treatment. **(B)** There was a trend toward more time spent burying in vehicle-treated alcohol drinkers compared to water drinkers (p=0.061). In alcohol drinkers specifically, 1 µg/side MTEP significantly reduced time spent burying compared to vehicle treatment. **(C)** There was a trend toward a shorter latency to start burying in vehicle-treated alcohol drinkers compared to water drinkers. 1 µg/side MTEP significantly increased latency within each drinking group compared to vehicle treatment. *p<0.05 treatment effect within same drinking group, +p<0.05 drinking effect within same treatment group.

3.4.2 Forced swim test. In vehicle-treated animals, alcohol-drinking mice spent more time immobile, compared to water-drinking controls (p=0.034; Fig. 6A) and also had a shorter latency to first immobility ((p=0.006; Fig. 6B). However, there were no effects of MTEP treatment on time spent immobile or latency to first immobility (p's>0.10). There were no significant group differences in the number of immobile episodes in the FST (p's>0.10; Fig. 6C).

3.4.3 Marble burying. In vehicle-treated animal, alcohol-drinking mice buried more marbles, compared to water-drinking controls (p=0.043; Fig. 6A). There were no effects of MTEP on the number of marbles buried in either the alcohol or water group (p's>0.10). In vehicle-treated animal, alcohol-drinking mice also spent more time burying compared to water-drinking controls (p=0.043; Fig. 7B). In alcohol-drinking mice specifically, 10 μg/side MTEP significantly reduced the time spent burying, compared to vehicle-treated alcohol-drinking mice (p=0.03). There were no significant differences in the latency to start burying (p's>0.10; Fig. 7C).

3.5 Intracranial MTEP and Egr1 expression within the AcbSh

In vehicle-treated adult animals, alcohol-drinking mice had higher Egr1 expression versus water controls (p=0.001; Fig. 8). Both 1 μg/side and 10 μg/side MTEP significantly reduced Egr1 expression in both water- and alcohol-drinking animals relative to vehicle infusion (water group: 1 μg/side, p=0.041; 10 μg/side, p=0.004. Alcohol group: 1 μg/side, p=0.014; 10 μg/side, p<0.001).

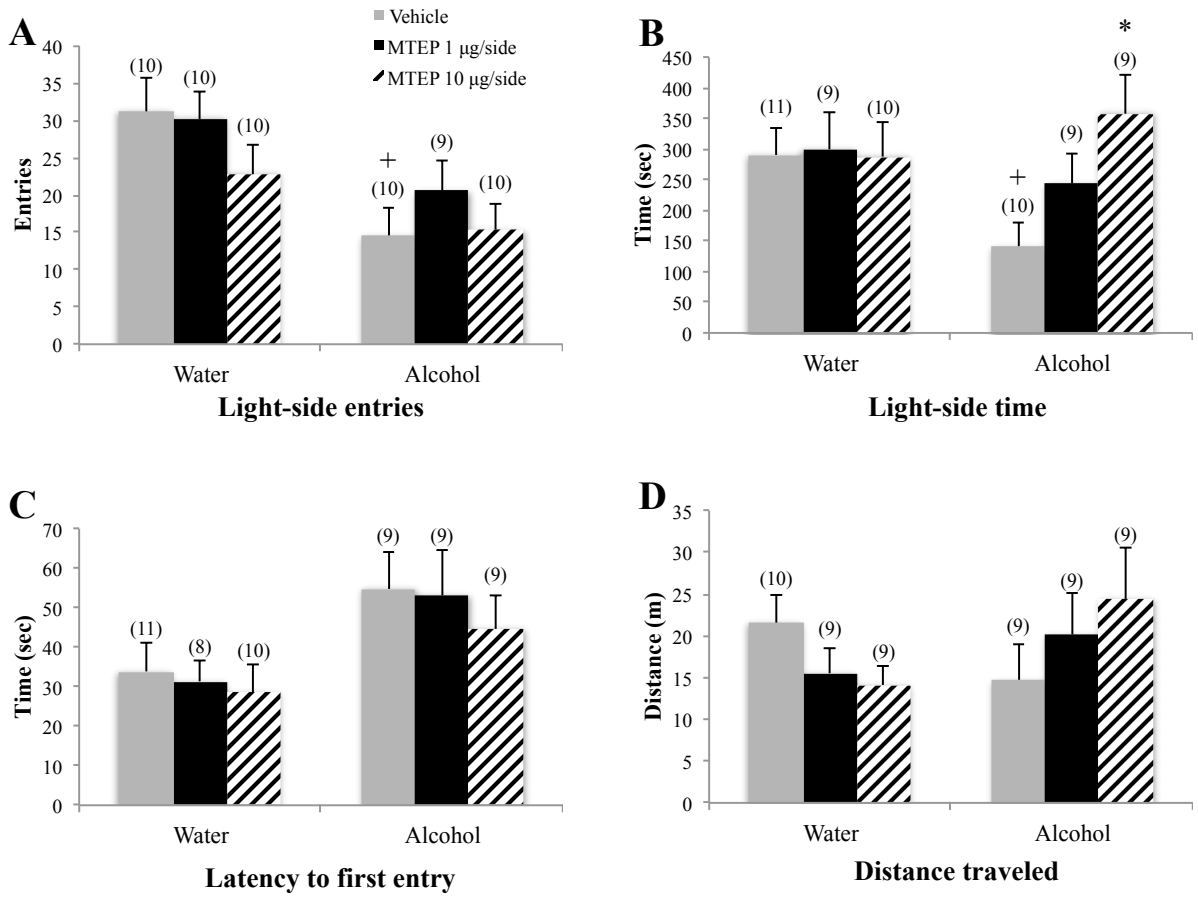

Figure 7.4 Effects of MTEP and adolescent alcohol experience in the light-dark box. **(A)** Vehicle-treated alcohol drinkers made fewer entries compare to vehicle-treated water drinkers. **(B)** Vehicle-treated alcohol drinkers specifically spent less time on the light side compared to water drinkers. In alcohol drinkers only, 10 µg/side MTEP significantly increase light-side time compared to vehicle treatment. **(C)** Within vehicle-treated animals, there was a trend toward longer latency to first light-side entry in alcohol drinkers compared to water drinkers (p=0.065). **(D)** There were no significant differences in distance traveled. *p<0.05 treatment effect within same drinking group, +p<0.05 drinking effect within same treatment group.

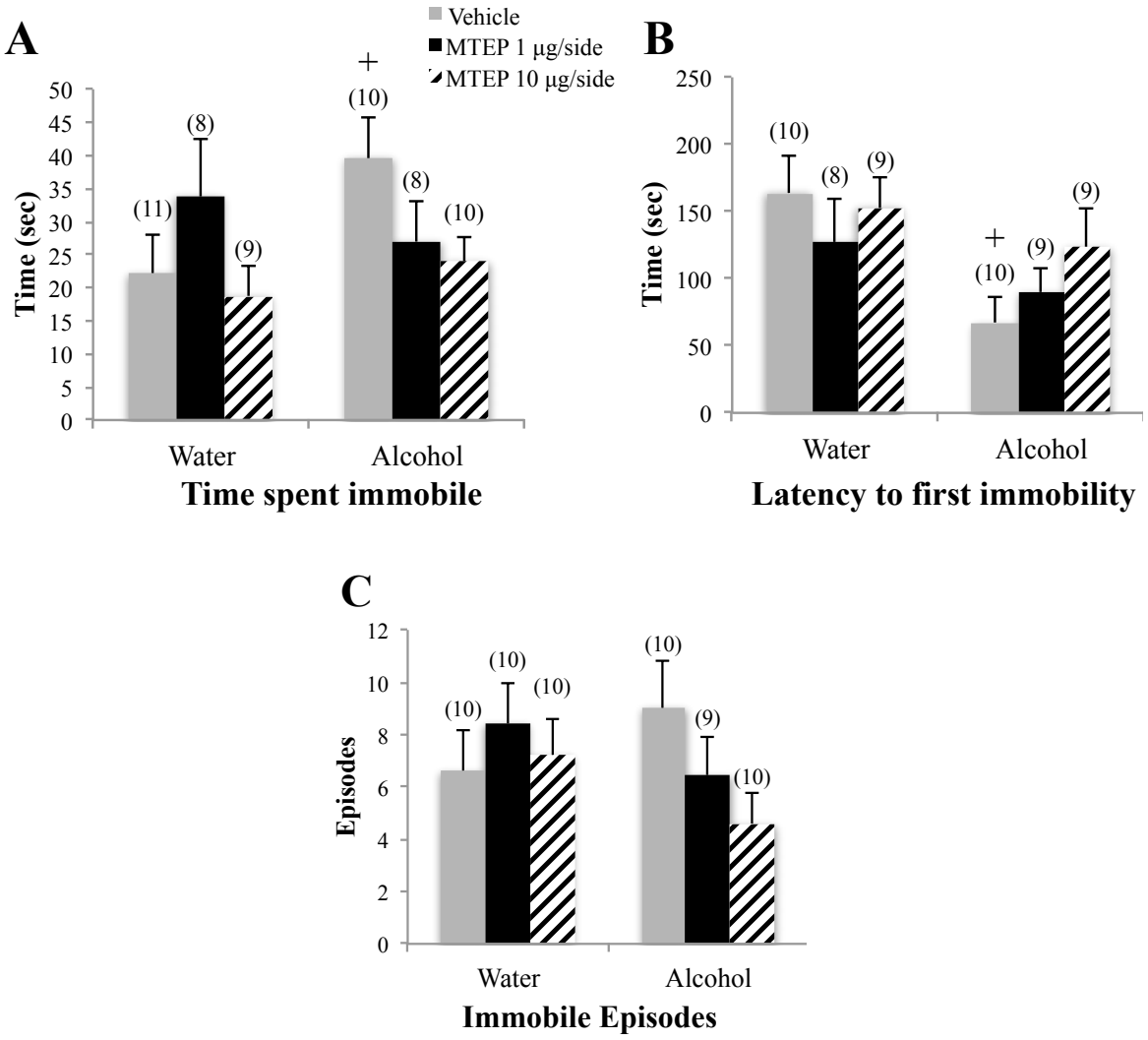

Figure 7.5 Effects of MTEP and adolescent alcohol experience in the FST. **(A)** Vehicle-treated alcohol drinkers spent more time immobile compared to water drinkers. However, there were no effects of MTEP treatment. **(B)** Alcohol drinkers overall had a shorter latency to first immobility compared to water drinkers and specifically, vehicle-treated alcohol drinkers had a shorter latency to first immobility compared to vehicle-treated water drinkers. However, there were no effects of MTEP treatment. **(C)** There were no group differences in the total number of immobile episodes. +$p<0.05$ drinking effect within same treatment group.

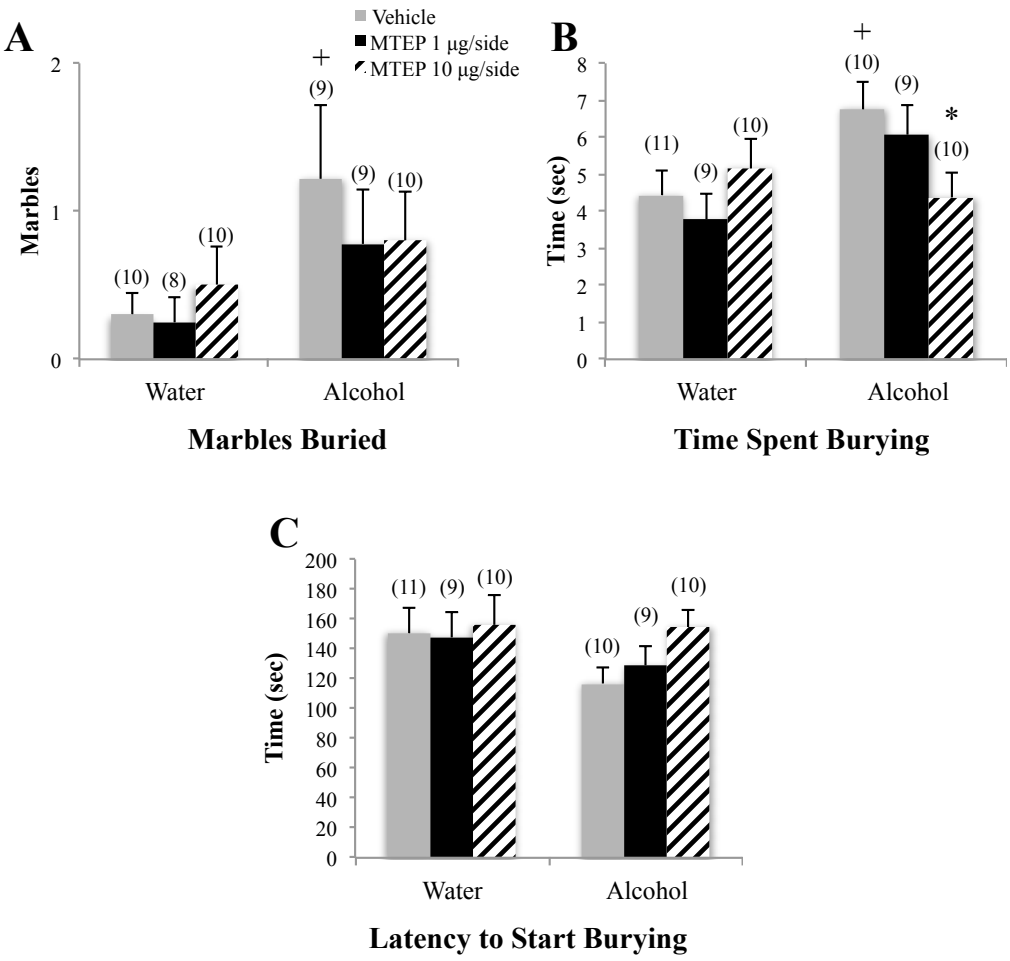

Figure 7.6 Effects of MTEP and adolescent alcohol experience in marble burying test. **(A)** Alcohol drinkers buried more marbles overall compared to water drinkers. Additionally, vehicle-treated alcohol drinkers specifically buried more than vehicle-treated water drinkers. **(B)** Alcohol drinkers spent more time burying compared to water drinkers and vehicle-treated alcohol drinkers specifically spent more time burying compared to vehicle-treated water drinkers. 10 µg/side MTEP significantly reduced time spent burying compared to vehicle treatment in alcohol drinkers only. **(C)** There were no significant group differences in the latency to begin marble burying. *$p<0.05$ treatment effect within same drinking group, +$p<0.05$ drinking effect within same treatment group.

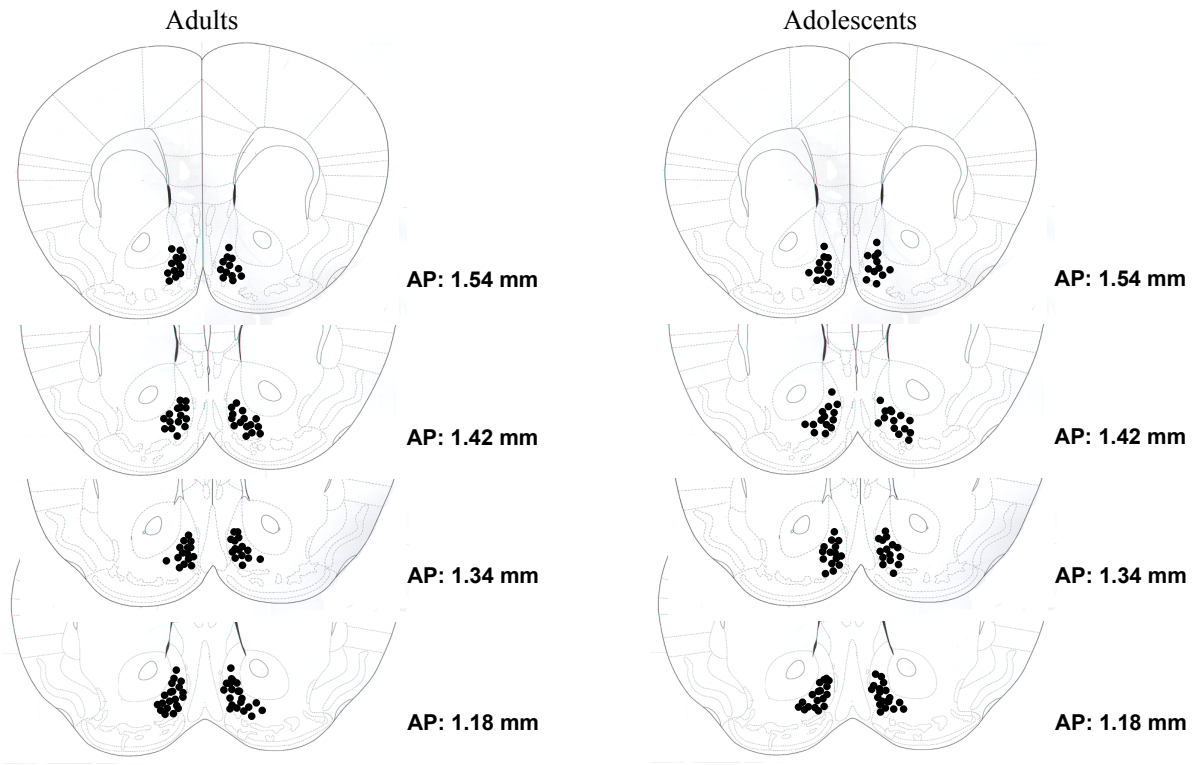

Figure 7.7. Representative depiction of microinjector tip placements aimed at the shell of the nucleus accumbens in **(A)** adults, n=63, and **(B)** adolescents, n= 59.

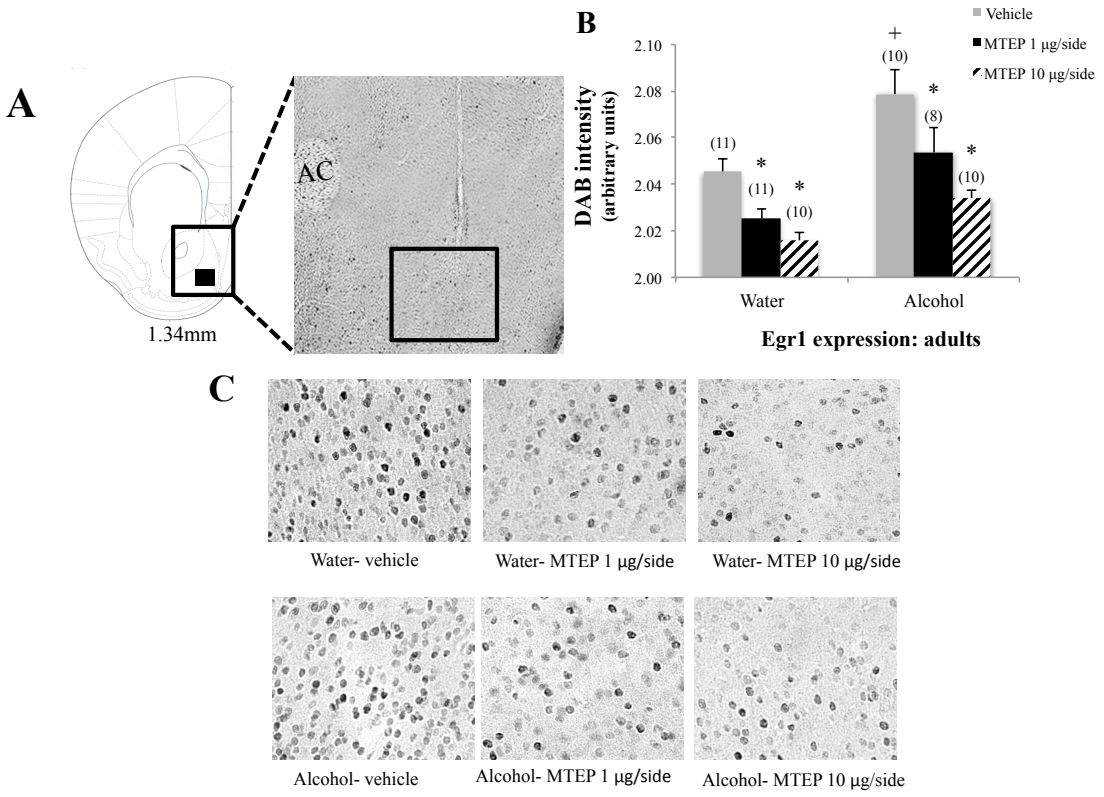

Figure 7.8 Effects of MTEP and adult alcohol experience on Egr1 expression within the AcbSh. (A) A representative anatomical depiction of a coronal section through the striatum, highlighting (black square) the approximate size and location of the sampling region used to assay the number of Egr1+ cells within the AcbSh. **(B)** Alcohol drinkers showed higher Egr1 within AcbSh overall compared to water drinkers. Among vehicle-treated animals specifically, alcohol drinkers showed higher Egr1 expression compared to water drinkers. Both 1 µg and 10 µg/side MTEP significantly reduced Egr1 expression compared to vehicle, independent of drinking group. Both MTEP doses also significantly reduced Egr1 expression within each drinking group compared to vehicle treatment. **(C)** Representative micrographs of Egr1 immunostaining within the AcbSh in each of the 6 treatment groups. *$p<0.05$ treatment effect within same drinking group, +$p<0.05$ drinking effect within same treatment group.

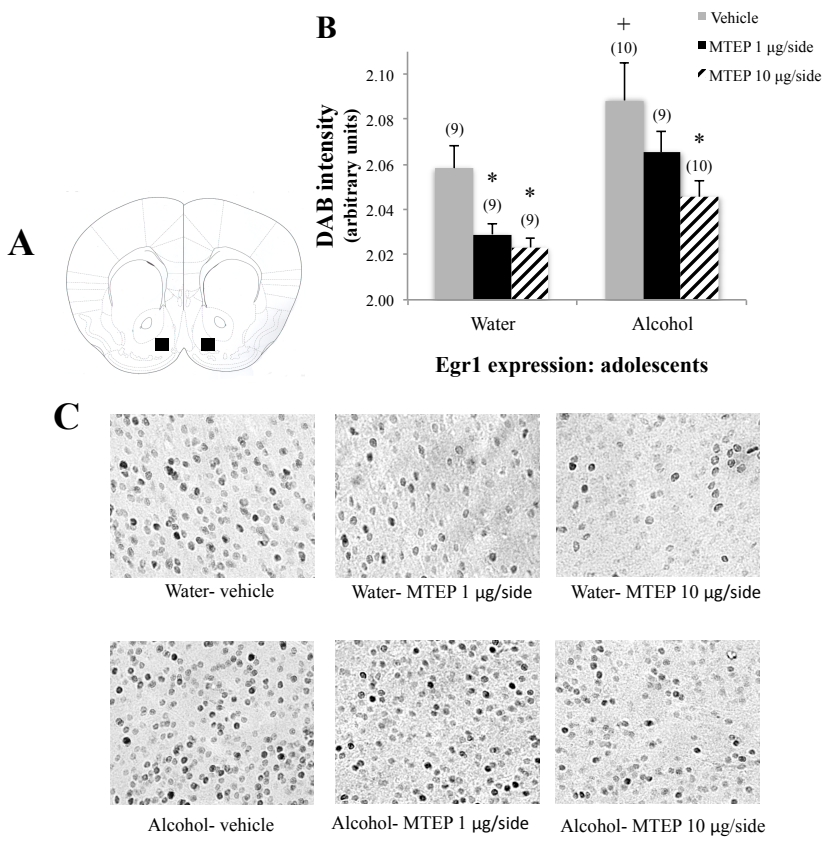

Figure 7.9 Effects of MTEP and adolescent alcohol experience on Egr1 expression within the AcbSh. **(A)** A reference schematic of the approximate size and location of the sampling region. **(B)** Alcohol drinkers showed higher Egr1 within AcbSh overall compared to water drinkers. In vehicle-treated animals specifically, alcohol drinkers showed higher Egr1 expression compared to water drinkers. Both 1 µg and 10 µg/side MTEP significantly reduced Egr1 expression compared to vehicle, independent of drinking group. Both MTEP doses also significantly reduced Egr1 expression in water drinkers. However, in alcohol drinkers, the reduction was only significant at the 10 µg/side MTEP dose. **(C)** Representative micrographs of Egr1 immunostaining within the AcbSh in each of the 6 treatment groups. *$p<0.05$ treatment effect within same drinking group, +$p<0.05$ drinking effect within same treatment group.

In vehicle-treated adolescents, alcohol-drinking mice exhibited higher Egr1 expression compared to water-drinking controls (p=0.009; Fig. 9). In water-drinking animals specifically, both 1 μg/side and 10 μg/side MTEP reduced expression relative to vehicle (p=0.046 and p=0.018, respectively). However, in alcohol drinkers, the reduction in expression was significant only at the 10 μg/side dose (p=0.003), but not 1 μg/side (p=0.20).

4. Discussion

Drug-induced plasticity within the AcbSh is a critical component of addiction neurobiology subserving drug reward, craving, and relapse. Although the AcbSh is a component of the emotional circuitry of the extended amygdala, relatively little is known regarding the role of the AcbSh in negative affective states during drug withdrawal. This study used intracranial administration of MTEP to demonstrate a modulatory role of mGlu5 within the AcbSh in anxiety-like behavior during withdrawal following a 14-day history of voluntary alcohol-drinking.

4.1 Withdrawal-induced negative affect in adult and adolescent alcohol drinkers.

Although the BACs obtained on day of 11 of drinking did not quite reach the criterion for binge-drinking (i.e., 80 mg/dl), the average alcohol consumption on day 11 was slightly lower than the overall 14-day average for both adults and adolescents. Despite this, alcohol consumption was sufficient to elicit behavioral signs of negative affect during withdrawal, as demonstrated previously in animals drinking at "near-binge" levels (Lee et al., 2015; Lee, Coelho, Sern, et al., 2017). The presence of withdrawal-induced negative affect was confirmed based on planned comparisons between vehicle-treated alcohol-drinking mice and

their water-drinking counterparts within each age group. Adult alcohol-drinking animals exhibited increased anxiety-like behaviors during early withdrawal on all 3 assays, as indicated by reduced interaction with the light side in the light-dark box, increased marble burying activity, and decreased immobility in the FST versus their water controls. We have repeatedly observed reduced immobility in the FST in adult alcohol-drinking mice during early withdrawal and have pharmacologically validated that this behavior reflects a hyper-anxious state as it can be reversed upon system administration of both prototypical and atypical anxiolytic drugs (Lee, Coelho, Sern, et al., 2017).

Consistent with our previous results (Lee et al., 2016; Lee et al., 2017-frontiers) and those reported in the literature (Doremus et al., 2005; Spear, 2000b; Spear & Varlinskaya, 2005), adolescents consumed significantly higher quantities of alcohol, compared to their adult counterparts under identical drinking conditions. As also reported recently (Lee, Coelho, Solton, et al., 2017), during protracted withdrawal (PND 70, i.e. early adulthood), mice with a history of drinking during adolescence also exhibited a negative affective state, indicated by reduced interaction with the light side in the light-dark box, and increased marble burying activity, compared to their vehicle-treated water controls. As reported previously by our group (Lee et al., 2017 Frontiers), adolescent alcohol-drinking mice exhibited increased immobility in the FST when tested in protracted withdrawal – a phenotype opposite that of alcohol-drinking adults. Increased immobility or floating in the FST is conventionally interpreted as reflecting behavioral despair or a depression-like state (Porsolt et al., 1977) and consistent with the notion that adolescent-onset drinking augments depressive-like features in early adulthood, increased immobility coincides with signs of

anhedonia as indicated by reduced sucrose preference (Lee, Coelho, Solton, et al., 2017). Taken together, we theorize that the opposite behavior expressed in the FST by adolescent- versus adult-onset binge-drinking reflects age-dependent differences in alcohol withdrawal phenotype, with adult-onset mice presenting predominantly with hyper-anxiety and adolescent-onset mice presenting a combination of hyper-anxious and depressive-like features.

4.2 Behavioral effects of MTEP in alcohol-experienced animals.

The effects of intra-AcbSh MTEP exhibited an interesting drinking age-by-dose effect in our study. In adult animals, the 1 μg/side MTEP dose reduced indices of hyper-anxiety in alcohol-drinking mice only (e.g., MTEP increased interaction with the light side of the light-dark box and reduced marble-burying activity). These data extend our prior indication of the anxiolytic efficacy of systemic MTEP treatment during alcohol withdrawal in adult animals (Lee, Coelho, Class, et al., 2017) by implicating the AchSh as one site important for the anxiolytic actions of mGlu5 inhibition during alcohol withdrawal. Further, these neuropharmacological results provide direct cause-effect evidence that alcohol-induced increases in AchSh mGlu5 expression/function observed during early withdrawal in adult mice (Lee et al., 2016) contribute to their hyper-anxious state. Although an intra-AchSh infusion of high-dose mGlu5 antagonist is effective at reducing binge alcohol intake by mice (Cozzoli et al., 2012) and to reduce behavioral indices of alcohol reinforcement (Besheer et al., 2010; Besheer et al., 2009; Gass & Olive, 2009; Sinclair et al., 2012), the 10 μg/side dose did not alter anxiety-like behavior on any of our measures in adult-onset alcohol drinking mice. This negative result for the higher MTEP dose is in line with prior reports, including

our own, suggesting that the MTEP dose-response function may exhibit an inverted U-shaped dose-response function, with higher inhibitor doses sometimes being anxiogenic (Belozertseva et al., 2007; Koltunowska et al., 2013; Lee, Coelho, Class, et al., 2017; Steckler et al., 2005; Varty et al., 2005).

In contrast to the adult-onset drinkers, the 1 μg/side MTEP dose was ineffective in mice with an adolescent-onset drinking history, while the 10 μg/side MTEP dose exerted some anxiolytic effects (e.g., increased light-side time and decreased time spent marble-burying) in these animals. The age-related differences in the effects of intra-AcbSh MTEP are also reminiscent of our prior findings for systemic MTEP treatment (Lee, Coelho, Class, et al., 2017) , in which the MTEP dose-anxiolysis response function was shifted to the right in mice with adolescent- versus adult-onset binge-drinking experience, indicating that a history of adolescent-onset alcohol-drinking reduces MTEP's anxiolytic efficacy. As in our prior study (Lee, Coelho, Class, et al., 2017), the age-related effects of intra-AcbSh MTEP are likely unrelated to nonspecific locomotor effects as neither dose influenced the distance traveled by either adolescent or adult alcohol-drinking mice in any of our assays. As we recently showed that adolescent-onset binge-drinking does not elevate the total protein expression of mGlu5 within the AcbSh during either early (Lee et al., 2016) or late withdrawal (Lee et al., 2017-Frontiers), the apparent shift to the right in the dose-response function for MTEP-induced anxiolysis in adolescent-onset drinking mice likely reflects a time-dependent increase in mGlu5 function, which could readily be attributed to increased expression of one of its major functionally-relevant scaffolding proteins Homer2 (Lee et al., 2016, Lee et al., 2017-Frontiers). While the precise mechanism(s) accounting for the age-related differences in the temporal expression of alcohol withdrawal-induced hyper-anxiety,

as well as MTEP's anxiolytic efficacy, remain to be determined, our data to date (Lee, Coelho, Class, et al., 2017, present study) have clinical ramifications for anxiolytic dosing based on the age of binge-drinking onset.

It is noteworthy that neither MTEP dose influenced emotional responsiveness in alcohol-naïve animals of either drinking age, indicating that while mGlu5 within the AcbSh clearly regulates alcohol withdrawal-induced hyper-anxiety, these receptors play little role in basal anxiety. The selective effects of MTEP for anxiety-like behavior during alcohol withdrawal argue in favor of its potential utility as a therapeutic agent for minimizing emotional distress during alcoholism recovery. Further, unlike conventional anxiolytic drugs that produce positive subjective effects in unaffected individuals, which contribute to their recreational use and abuse (e.g., benzodiazepines), the failure of MTEP, administered either systemically (Lee, Coelho, Class, et al., 2017), or intra-AcbSh (present study), to influence behavioral indices of emotionality in drug-naïve mice suggests very low abuse liability. It is also interesting to note that an intra-AcbSh MTEP did not influence behavior in the FST in either adolescent or adult mice with a prior history of alcohol-drinking. Admittedly, the water-alcohol difference in behavior manifested on the FST was smaller in this study than that reported by our group previously (Lee, Coelho, Class, et al., 2017; Lee et al., 2016; Lee, Coelho, Solton, et al., 2017), which may have limited our ability to detect an MTEP effect. However, systemic MTEP treatment did not influence FST behavior in our recent report in which adult-onset drinking mice exhibited robust hyperanxiety (Lee, Coelho, Class, et al., 2017). As both systemic (Lee, Coelho, Class, et al., 2017) and intra-AcbSh MTEP altered anxiety-like behavior in the marble-burying and light-dark box tests, the failure of MTEP to influence FST behavior may reflect the physical versus psychological nature of the stressor.

4.3 Effects of MTEP and alcohol withdrawal on Egr1 induction within the AcbSh

Alcohol withdrawal increased Egr1 induction within the AcbSh of both adult- and adolescent mice, coinciding with the presence of hyper-anxiety. These results contrast with those from our earlier report in which we failed to detect alcohol-induced changes in Egr1 expression within the AcbSh of adult mice during either early (1 day) or protracted (21 days) withdrawal (Lee et al., 2015). However, it should be noted that the discrepancies in findings might be related to a number of procedural differences between the past and present studies that include: duration of drinking history (6 weeks vs. 14 days), age of animals when euthanized (PND 99 vs PND 70), and drinking protocol (single 20% alcohol-bottle vs. multi-bottle-choice), which render these outcomes difficult to compare. Notably, the present results are more in-line with other published studies reporting increased expression of other IEGs such as *c-fos* within the AcbSh during early withdrawal from alcohol, with alcohol experience ranging from a single alcohol-exposure (Kozell et al., 2005) to as long as 5 months of drinking (George et al., 2012). In fact, acute alcohol withdrawal has been shown to induce similar patterns of *c-fos* immunoreactivity within specific brain regions, including the AcbSh, that also respond to anxiety-provoking stimuli such as an air-puff challenge (Knapp et al., 1998) and aversive foot shocks (Duncan et al., 1996). Additionally, anxiogenic doses of caffeine and yohimbine also induce *c-fos* activation within the AcbSh (Baldwin et al., 1989; Singewald et al., 2003). Therefore, the increased Egr1 expression observed in alcohol-withdrawn mice herein is likely functionally related to the increase in anxiety-related behaviors. If so, it is particularly noteworthy that Egr1 expression was elevated in adolescent-onset drinkers even after 28 days of alcohol abstinence, providing further

evidence that a 2-week history of binge-like alcohol drinking during adolescence produces enduring changes within the activational state of the addiction-related neurocircuitry, which could drive alcohol's incentive motivational properties and maintain compulsive alcohol-drinking (Lee et al., 2016).

Both MTEP doses decreased Egr1 expression within the AcbSh, irrespective of the alcohol experience of the animals. These results were expected, based on other studies showing decreased *c-fos* IEG induction following treatment with MTEP and another mGlu5 inhibitor, MPEP (Besheer et al., 2009; Bianchi et al., 2003; Edling et al., 2007; Shin et al., 2015). Both 1 μg/side and 10 μg/side MTEP significantly reduced Egr1 expression in adult-onset alcohol-experienced mice. In adolescent-onset alcohol-experienced animals, the 10 μg/side dose similarly reduced Egr1 expression; however, the lower 1 μg/side dose did not significantly reduce Egr1 expression compared to vehicle. Importantly, the age-by-dose interactions with respect to the anxiolytic effect of MTEP were reminiscent of a similar pattern of inhibitor effects upon AcbSh Egr1 expression. For example, 1 μg/side MTEP significantly reduced Egr1 expression and showed an anxiolytic effect in adult-onset alcohol-drinking mice, but this dose did not significantly affect Egr1 expression or anxiety in adolescent-onset alcohol-experienced animals. Only the 10 μg/side dose resulted in significant reduction in Egr1 expression and also showed behavioral efficacy in adolescent-onset alcohol-experienced animals. It is somewhat surprising that a disconnect was observed in adult-onset drinkers with respect to the effects of the 10 μg/side MTEP dose upon behavior (no effect) and AcbSh Egr1 expression (reduction). However, high doses of mGlu5 antagonist are also effective at reducing binge-alcohol drinking (Cozzoli et al., 2012), as well as the positive reinforcing properties of alcohol (Besheer et al., 2010; Gass & Olive, 2009;

Sinclair et al., 2012) when infused intra-AcbSh and thus, our present immunoblotting results may have relevance for understanding how mGlu5 inhibitors exert their "anti-alcoholism" effects.

In contrast to our previous study (Lee et al., 2015), we did not include behavioral testing control animals due to time and resource considerations related to the intracranial surgeries. All Egr1 data in the present study were obtained from behaviorally tested animals, which allowed for direct inferences to be drawn between Egr1 expression and our observed behavioral changes. However, based on previous data (Lee et al., 2015), it is also possible that the testing procedures could elicit changes in Egr1 expression. Of particular concern would be a potential interaction between behavioral testing alcohol drinking history; for example, a greater susceptibility to testing stress in alcohol-experienced animals, associated with a greater increase in Egr1 expression. However, our prior results also showed that the activational effects of behavioral testing within Acb subregions were independent of drinking history (Lee et al., 2015). Therefore, while we cannot discount this potential confound entirely, these previous findings help to mitigate the severity of this concern.

As a final point of clarification, the focus of the present study was the AcbSh specifically, given its role in extended amygdala circuitry, and thus its potential involvement in the negative affective consequences of alcohol abuse. We did not probe the Acb core and do not seek to make any claims regarding the neuroanatomical selectivity of our reported changes in Egr1 expression. Rather, the objective was to assess the functional relevance of a previously observed change in protein expression within the AcbSh as it relates to withdrawal-induced negative affect and in doing so, possibly shed light on ontogenetic differences in the neurobiological and affective consequences of alcohol abuse.

4.4 Conclusions

The results of the present study highlight an important role for mGlu5 within the AcbSh in the age-related manifestation of hyper-anxiety during alcohol withdrawal. Adolescent-onset alcohol-drinking appeared to render mice less sensitive to MTEP's anxiolytic effects than adult-onset drinking, extending earlier indications that the age of drinking-onset is a critical subject factor influencing the behavioral sequelae of excessive drinking, including the severity of affective symptoms and therapeutic responsiveness. Further, we provide confirmatory evidence that heavy drinking during adolescence produces enduring behavioral and neurobiological changes within extended amygdala structures that potentially contribute to an increased vulnerability to addiction and/or affective disorders later in life.

Chapter 8:

Homer2 within the central nucleus of the amygdala gates withdrawal-induced anxiety in a mouse model of binge-drinking

1. Introduction

Binge-drinking is the most prevalent form of alcohol abuse in the United States, with approximately 1 in 6 American engaging in binge drinking an average of 4 times per month (Centers for Disease Control and Prevention, 2013). This pattern of consumption is especially prevalent in adolescents and over 90% of the alcohol consumed by underage individuals is in the form of binge drinks (Centers for Disease Control and Prevention, 2016). Although the majority of binge-drinkers do not meet the criteria for dependence (Esser et al., 2014), frequent binge-drinking is one of the strongest risk factors for alcoholism (Dawson et al., 2005; Hasin & Beseler, 2009; Saha et al., 2007). Chronic binge-drinking often results in the development of tolerance to the pleasurable/hedonic effects of acute alcohol that serve as positive reinforcers of drinking, leading to an escalation of intake (reviewed in Koob & Moal, 1997). Simultaneously, repeated bouts of intoxication and withdrawal can lead to increasing severity of withdrawal symptoms during periods of abstinence, including negative affective consequences such as anxiety, depression, agitation, and general dysphoria. Over time, withdrawal-induced negative affect in chronic binge-drinkers is theorized to facilitate the transition to addiction in non-dependent individuals by shifting the primary motivation for drinking from positive to negative reinforcement (Koob, 2013; Koob & Le Moal, 2001).

The negative affective consequences of alcohol withdrawal are primarily associated with drug-induced adaptations within extended amygdala circuitry- consisting of the bed nucleus of the stria terminalis (BNST), the shell subregion of the nucleus accumbens (AcbSh), and the central nucleus of the amygdala (CEA). Negative affect is classically associated with an increase in amygdalar activation (Beesdo et al., 2009; Davis & Whalen, 2001; Peluso et al., 2009; Shackman & Fox, 2016) and the CEA dysfunction is critically

implicated in alcohol withdrawal-induced negative affect (reviewed in detail by Gilpin et al., 2015). Chronic alcohol exposure induces glutamatergic plasticity via both pre- and post-synaptic adaptations (Lack et al., 2007; Lovinger & Roberto, 2013; Siggins et al., 2005; Stuber et al., 2010). These changes contribute to CEA hyperactivation during withdrawal, which is associated with negative affective states.

Prior research has shown that drug-induced restructuring of glutamatergic synapses often involves Homer proteins (reviewed in Szumlinski, Ary, & Lominac, 2008). Homer proteins are intracellular scaffolding proteins located abundantly in the post-synaptic density that exert regulatory control over glutamatergic signaling (Shiraishi-Yamaguchi & Furuichi, 2007). The 'long-form' Homer isoforms (Homer1b/c/d, Homer2a/b, and Homer3) are constitutively expressed throughout many addiction-relevant brain regions including the Ach, PFC, and the amygdala (Ary et al., 2013; Soloviev et al., 2000; Verpelli et al., 2012), where they directly influence glutamatergic signaling by regulating the trafficking, distribution, and function of group 1 metabotropic glutamate receptors (mGluRs) and NMDA receptors (Brakeman et al., 1997; Tu et al., 1998).

Alcohol-induced synaptic plasticity within the extended amygdala circuitry is theorized to involve, in part, increases in the expression of Homer proteins - Homer2 isoforms in particular (Cozzoli et al., 2012; Cozzoli et al., 2014; Cozzoli et al., 2009; Cui et al., 2013; Haider et al., 2015; Lee et al., 2016; Lum et al., 2014; Obara et al., 2009; see also Szumlinski, Ary, & Lominac, 2008 for review). *Homer2* knockout mice are alcohol-averse and –intolerant and these phenotypes are reversed via adeno-associated viral vector (AAV)-mediated Homer2 restoration within the nucleus accumbens shell (AcbSh). Likewise, AAV-mediated over-expression of Homer2 within the AcbSh augments alcohol reward,

reinforcement and behavioral sensitization (Szumlinski et al., 2005), while Homer2 knock-down in this region reduces binge-drinking, without influencing water or sweet solution intake (Cozzoli et al., 2009, 2012). Within the CEA, AAV-mediated Homer2 knockdown significantly reduces binge-drinking, particularly of high alcohol concentrations (Szumlinski, Ary, Lominac, et al., 2008) and CEA Homer2 maintains voluntary binge-drinking through group 1 mGluR-dependent pathways involving phospholipase C and PKCε (Cozzoli et al., 2014). Taken together, our prior work argued an important role for Homer2-dependent argued that a Homer2-dependent hyper-glutamatergic state during alcohol withdrawal promotes the positive reinforcing properties of alcohol to drive excessive intake (Cozzoli et al., 2015; Cozzoli et al., 2014).

In contrast to its role in regulating the positive reinforcing properties of alcohol, we know very little regarding Homer2's potential role in the negative reinforcing properties of this drug. *Homer2* knockout mice exhibit wild-type levels of basal emotionality (as well as cognitive, sensorimotor and gross motor function) (Szumlinski et al., 2004, 2005) and do not exhibit stress-alcohol cross-sensitization of locomotor activity (Quadir et al., 2016), but no study to date has examined how Homer2 impacts alcohol-induced changes in affect. Recent western blotting data from our laboratory showed that the manifestation of negative affect during alcohol withdrawal closely aligned with *reductions* in Homer2 protein expression within the CEA. For example, mice with a 14-day history of binge- drinking during adulthood show increased anxiety and CEA Homer2 expression during acute (24 h) withdrawal and both effects dissipate with the passage of time (Cui et al., 2013; Szumlinski, Ary, & Lominac, 2008). In contrast, adolescent binge-drinkers show negligible signs of behavioral dysfunction during acute withdrawal and no change in CeA Homer2 expression

(Lee et al., 2015; Lee, Coelho, Sern, et al., 2017), but a marked negative affective state incubates in these animals during protracted (4 weeks) withdrawal and the mice exhibit hyper-anxiety, as well as increased CEA H2 expression, when tested as young adults (Lee et al., 2016). Importantly, irrespective of the age of drinking onset, withdrawal-induced negative affect is positively related to increased subsequent alcohol consumption. Our prior behavioral data are consistent with other clinical and preclinical literature regarding the enduring consequences of adolescent alcohol exposure indicating increased mood disorders, cognitive impairment, and excessive alcohol consumption (Lee, Coelho, Solton, et al., 2017) and our correlational immunoblotting findings argue a potential role for alcohol withdrawal-induced changes in CEA Homer2 expression in the manifestation of a negative affective state, including the incubation of a negative affective state in those with a history of adolescent-onset binge-drinking.

In the present study, we sought to determine if the observed reduction in CEA Homer2 was functionally relevant to the manifestation of anxiety and elevated alcohol consumption during withdrawal using an adeno-associated virus (AAV)-mediated gene transfer approach to overexpress Homer2 locally within the CEA (H2-cDNA). We hypothesized that reversing the reduction in CEA Homer2 expression produced by a 2-week history of binge-drinking should reduce anxiety in adult and adolescent alcohol-experienced animals, during early and protracted withdrawal, respectively. Moreover, if withdrawal-induced anxiety is a motivating factor of subsequent drinking, we hypothesized also that Homer2 ‘rescue’ should block withdrawal-induced drinking.

2. Materials and Methods

The binge-drinking and behavioral testing procedures in this study were identical to those used previously in our lab (Lee et al., 2015, 2016) and are briefly summarized below. The timeline of experimental procedures is outlined in Figure 1. All procedures were conducted in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (NIH Publication No. 80–23, revised 2014)(McBride et al., 2005; Nixon & McClain, 2010; Spear, 2014; Tapert & Schweinsburg, 2005) (McBride et al., 2005; Nixon & McClain, 2010; Spear, 2014; Tapert & Schweinsburg, 2005) (McBride et al., 2005; Nixon & McClain, 2010; Spear, 2014; Tapert & Schweinsburg, 2005) and approved by the IACUC of the University of California, Santa Barbara.

2.1 Subjects & study design

This study used male C57BL/6J mice (Jackson Laboratory, Sacramento, CA) that were either PND 28 (adolescents) or PND 56 (adults) at the onset of drinking. Animals were housed in age-matched groups of 4 per cage in a climate-controlled vivarium under a reverse light/dark cycle (lights off at 10am), with food and water available *ad libitum* except during the 2-h alcohol drinking period. The study design consisted of 2 age groups (adults or adolescents), 2 drinking groups (alcohol or water), and 2 AAV groups (H2-cDNA or GFP); n=12/group.

2.2 Viral transfection

2.2.1 Viral vectors. The AAVs were obtained from the laboratory of Dr. M. Klugmann (University of New South Wales, Sydney, Australia), as in our prior transgenic

studies kb cytomegalovirus immediate early enhancer/chicken β-actin (CBA) promoter (H2-cDNA). This AAV has been previously validated via western blotting to successfully overexpress H2 [illegible](e.g. Cozzoli et al., 2012; Cozzoli et al., 2009; Szumlinski et al., 2006; Szumlinski, Ary, Lominac, et al., 2008). *Renilla* green fluorescent protein (GFP) was used as an AAV control.green fluorescent protein (GFP) was used as an AAV control.

2.2.2 Craniotomy & virus infusion. Both adults and adolescents underwent surgery at the same age (PND 42-48), approximately 3 weeks prior to behavioral testing to allow for maximal transfection (Ary et al., 2013; Haider et al., 2015). Thus, surgery occurred prior to the 14-day drinking period in adults and afterward in adolescents. Under isoflurane gas anesthesia, 33-gauge injector cannulae (12 mm long; threaded through a 24-gauge adapter for stability) were used to deliver bilateral infusions H2-cDNA or GFP directly into the CEA [AP:−1.25; ML: ±2.70; DV: −2.70 mm from Bregma, according to Paxinos & Franklin (Klugmann & Szumlinski, 2008)]. Infusions were delivered at a rate of 0.05 µl/min for 5 minutes (total vol=0.25 µl/side). Injector cannulae were left in place for an additional 5 minutes before removal. The incision site was closed with a small animal wound clip. Mice were left to recover, undisturbed (with the exception of post-operative monitoring and routine cage maintenance) for a minimum of 7 days before further experimentation.

2.3 Multi-Bottle-Choice Drinking-in-the-Dark (DID) Procedures

Half of the animals from each drinking-age group were subjected to 14 consecutive days of binge-drinking under 3-bottle DID procedures. Alcohol access was restricted to 14 days for all animals, corresponding to the approximate duration of early-mid adolescence in

mice (Spear, 2000). Each day, animals were separated into individual cages, allowed to habituate to the drinking cage for a minimum of 45 min and then given simultaneous access to bottles containing 10, 20, and 40% (v/v) unsweetened ethanol solutions for 2 h, beginning 3 h into the circadian dark cycle (Lee et al., 2017c; Rhodes et al., 2005). Control animals received water only. Daily alcohol consumption was calculated by weighing the bottles immediately before and after the drinking period and expressed as a function of the animal's body weight (g/kg). All animals, both alcohol and water drinkers, were weighed 3x per week throughout the drinking period. Submandibular blood samples were collected from all alcohol-drinking animals on day 11 of drinking, immediately upon conclusion of the 2-h drinking period. Blood alcohol concentration (BAC) was determined using an Analox alcohol analyzer (model AM1, Analox Instruments USA, Lunenburg, MA).

2.4 Behavioral testing

Based on our previous work showing robust anxiety in adult drinkers during acute (24-h) alcohol withdrawal and adolescent drinkers during protracted (4-weeks), both adult and adolescent drinkers and (water-drinking controls) were behaviorally tested at PND 70 as indicated in Figure 1. Thus, behavioral testing was conducted during adulthood for both age groups. However, in this study, the terms 'adult' and 'adolescent' refer to the age of alcohol exposure. Behavioral testing was conducted during the animals' circadian dark phase and consisted of the light-dark box, marble burying test, and Porsolt forced swim test (FST) (exactly as conducted in Lee et al., 2016, 2017c). In the light-dark box, the dependent measures were: the number of light-side entries, latency to first light-side entry, total time spent on the light side, and the total distance traveled. Decreased interaction with the light

side compared to control animals was interpreted as a sign of increased anxiety. Distance traveled provided an index of general locomotor activity. In the marble burying test, the dependent measures were: the number of marbles buried, latency to first begin burying,

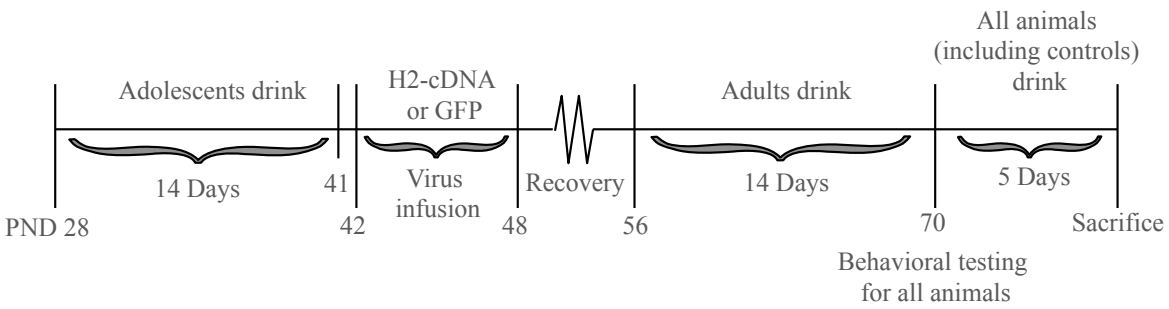

Figure 8.1. Experimental timeline for this study of the effects of infusing AAVs into the CEA of mice with a history of binge-drinking during either adulthood or adolescence upon the manifestation of withdrawal-induced anxiety (tested on PND 70) and subsequent drinking.

and total time spent burying. Increased burying behavior was interpreted as a sign of increased anxiety. In the FST, the dependent measures were: the number of immobile episodes, latency to first immobile episode, and total time spent immobile. In prior work, we have observed an age-dependent effect of alcohol withdrawal in this assay, with adults and adolescents showing, respectively, decreased and increased immobility during withdrawal. Thus, the FST is a sensitive assay of alcohol-induced affective dysregulation that is capable of detecting age by alcohol interactions in behavior

2.5 Subsequent drinking in adulthood

Approximately 24 h following the conclusion of behavioral testing, all animals, including previously alcohol-naïve animals, were subjected to an additional 5 days of drinking (following the same procedures as the initial 14-day exposure) in order to assess the effects of prior alcohol experience and H2-cDNA on alcohol consumption in adulthood. Additionally, the drinking behavior of previously-naïve H2-cDNA alcohol control animals allowed us to characterize the effects of Homer2 overexpression on binge-drinking in adulthood, independent of previous alcohol experience.

2.6 Brain tissue collection & verification of AAV transduction

Approximately 24 h following the final alcohol presentation of the 5-day DID, animals were euthanized with an overdose of Euthasol® (Virbac AH, Fort Worth, TX) and transcardially perfused with phosphate-buffered saline (PBS), followed by 4% paraformaldehyde, as described previously (2004). Brains were removed and cold-stored in cryoprotectant (30% ethylene glycol, 30% glycerol in PBS) until slicing. Tissue was

sectioned (40 μm) along the coronal plane on a vibratome at the level of the amygdala (e.g. Lee et al., 2015). Immunohistochemistry was performed using standard procedures employed previously in our laboratory (as depicted in Paxinos & Franklin, 2004) Tissue sections from H2-cDNA infused mice were stained with an antibody against the hemagglutinin (HA) tag using a mouse anti-HA primary antibody (Biolegend, San Diego, CA; 1:1000 dilution), followed by biotinylated anti-mouse secondary IgG (Vector Laboratories, Burlingame, CA; 1:2,000 dilution) and visualized with with 3,3′-diaminobenzidine (DAB). Following staining, sections were mounted on slides, dehydrated, and cover-slipped. GFP transfection of control animals was detected using a GFP tag antibody (Invitrogen, Carlsbad, CA; 1:200 dilution) and fluorescence microscopy. Slides were examined and photographed using a Nikon Eclipse E800 microscope equipped with a Hamamatsu CCD camera (model C4742-95) and MetaMorph® imaging software (Molecular Devices, Sunnyvale, CA). Any animals that failed to show positive AAV transfection within the CEA (Fig. 2) were excluded from the final analysis of the data.

2.7 Statistical analysis

A repeated-measures ANOVA was used to analyze intake data for all alcohol-drinking animals with age as the between-subject factor in order to determine if there was a difference in alcohol consumption between adults and adolescents across the 14-day drinking period. Adult and adolescent alcohol drinkers were analyzed independently via repeated-measures ANOVA with AAV as the between-subject factor to assess for any intake differences between H2-cDNA and GFP animals. A Pearson's correlational analysis was conducted for all alcohol-drinking animals to determine the relationship between alcohol

intake and resulting BACs sampled on day 11 of drinking. The 5-day drinking data were analyzed separately within each drinking-age group using a repeated-measures ANOVA and Tukey-Kramer multiple comparison tests to compare the effects of H2-cDNA to GFP within alcohol-experienced and -inexperienced animals.

All behavioral data were analyzed separately within each drinking-age group using Tukey-Kramer multiple comparison tests. A significant ANOVA is not a prerequisite for planned pairwise comparison procedures such as Tukey's HSD, which provides conservative protection against Type I error while maximizing statistical power (e.g. Cozzoli et al., 2009; Goulding et al., 2011; Szumlinski, Ary, Lominac, et al., 2008; Szumlinski et al., 2004; Szumlinski et al., 2005). This approach is particularly powerful, and often considered preferable to the traditional ANOVA (Cardinal & Aitken, 2006; Hayter, 1984; Kramer, 1956), in studies such as this that contain multiple *a priori* comparisons of interest. Specifically, we were interested in between-group comparisons of GFP versus H2-cDNA animals within each drinking group and alcohol versus water within GFP animals (to assess the effect of alcohol, independent of transgenic manipulation). Dependent samples t-tests were conducted separately in alcohol-experienced adult and adolescent drinkers for within-group comparisons of average intake during the initial 14-day drinking period with intake during the subsequent 5-day drinking period.

Statistical outliers were identified using the ±1.5×IQR rule and excluded from analyses. All data depicted in figures represent mean ± SEM of the number of the number of animals indicated in parentheses; α=0.05, though statistical trends (p<0.10) are also reported. Calculations and analyses were performed using add- StatPlus$^{6.0}$ for Microsoft Excel and SPSS v.21 statistical software (IBM, 2012).

3. Results

3.1 Animal exclusion

Animals were omitted entirely from data analysis based unsuccessful or misplaced viral transduction, staining complications rendering it impossible to confirm viral transduction, and attrition due to surgical complications. Final sample sizes for analysis were 9-12 per group and the group sizes are indicated in all figures.

3.2 Alcohol consumption

Across the entire 14-day drinking period, adult animals consumed an average of 2.96 ± 0.12 g/kg and adolescents an average of 6.12 ± 0.19 g/kg. The repeated-measures ANOVA showed a significant between-subjects effect of age [$F(1,42)=48.42$, $p<0.001$], but no within-subject effect of Day or Age by Day interaction (p's>0.05). Thus, adolescents drank significantly more alcohol than adults across the entire 14-day drinking period and adults maintained low levels of alcohol intake throughout. Within each age group, the repeated-measures ANOVA showed no significant difference in alcohol consumption between adult animals previously infused with GFP or H2-cDNA [GFP: 3.01 ± 0.20 g/kg vs. H2-cDNA: 2.93 ± 0.22 g/kg; $p=0.793$] and there was also no differences in the alcohol intake between adolescent mice subsequently infused with GFP or H2-cDNA [GFP: 5.96 ± 0.35 g/kg vs. H2-cDNA: 6.26 ± 0.32 g/kg; $F(1,21)=0.426$, $p=0.521$]. On day 11 of drinking when blood was sampled, adolescent alcohol drinkers had an average alcohol consumption of 6.25 ± 0.29 g/kg with a resulting BAC of 105.49 ± 4.84 mg/dl, which is above the 80 mg/dl NIAAA criterion for binge-drinking. Unfortunately, adult animals had an average alcohol

Table 8.1: Summary of the final sample sizes employed in the statistical analyses of the data.

	Water		**Alcohol**	
	GFP	***cDNA***	***GFP***	***cDNA***
Adults	11	11	12	11
Adolescents	12	9	11	10

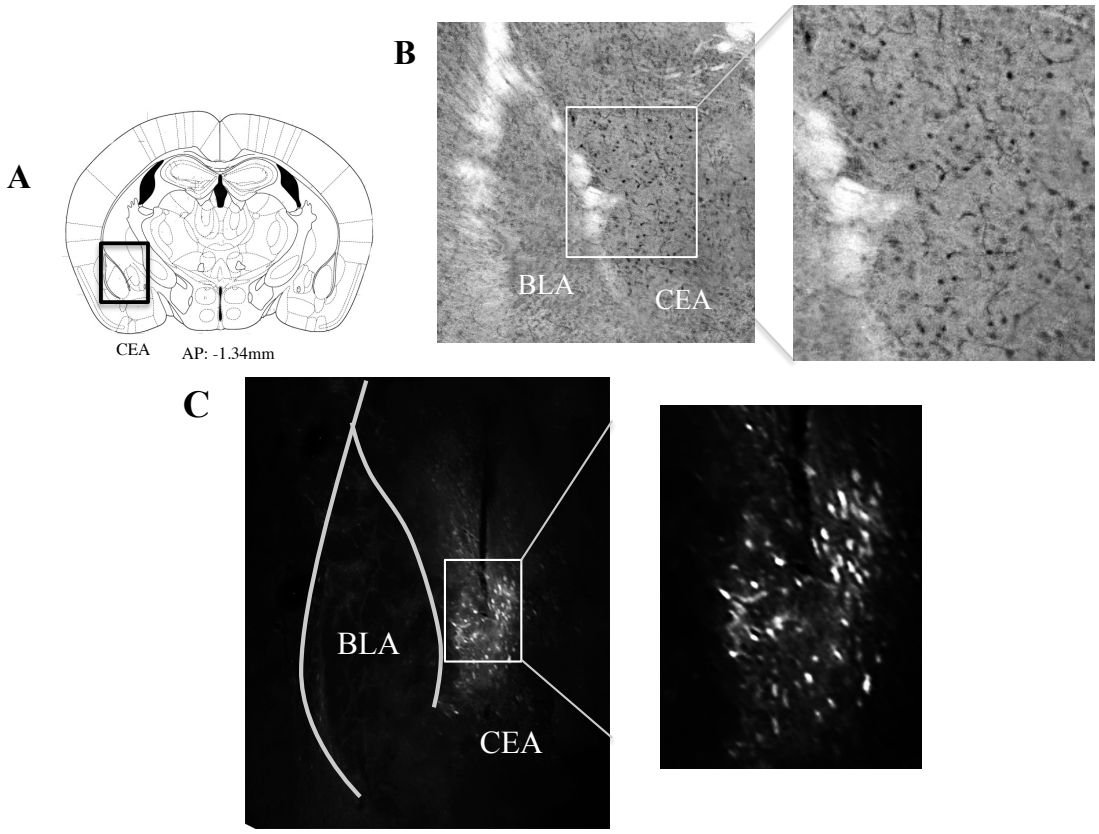

Figure 8.2 Verification of viral transduction of neurons selectively within the CEA. **(A)** Illustration of the sampling region used to verify the subregional selectivity of AAV transduction. Representative 20X micrographs of immunostaining for **(B)** AAV-transfected HA-tagged Homer2b and **(C)** AAV-transfected GFP control. The 40X inserts indicate that transduction occurred within both the cell bodies and processes of the neurons.

consumption of 2.78 ± 0.17 g/kg with a resulting BAC of 57.27 ± 3.54 mg/dl and thus, were not engaged in binge-drinking. Alcohol intake was significantly correlated with BAC across all animals (r=0.892, p<0.001). While we were concerned that the low levels of alcohol intake by adult mice would be insufficient to elicit changes in affect, little is known regarding the behavioral or biological consequences of repeated low-dose alcohol consumption and thus, we retained the adult animals in the study.

3.3 Effects of alcohol and CEA Homer2 in adolescent animals during protracted withdrawal

When tested as adults on PND70 in the light-dark box, GFP alcohol-drinking mice spent less time on the light side (p=0.004; Fig. 3A), made fewer light-side entries (p=0.019; Fig. 3B), and showed a trend toward a longer latency to first light-side entry (p=0.079; Fig. 3C), compared to GFP water-drinking mice, supporting greater anxiety-like behavior during protracted withdrawal in adolescent-onset alcohol-drinking mice. In alcohol-drinking mice specifically, H2-cDNA significantly increased light-side time (p=0.039) and also showed a strong positive trend toward more light-side entries (p=0.052) compared to GFP, but no effect was observed on the latency to first light-side entry p>0.10). Thus, CEA Homer2 over-expression blunts the negative affective state produced by a history of adolescent-onset alcohol-drinking. In water-drinking mice, the effects of H2-cDNA were mixed in the light-dark box test; relative to GFP-infused mice, H2-cDNA significantly decreased light-side time (p=0.002) and also showed a trend toward increased latency to first light-side entry (p=0.062), but had no effect on light-side entries (p>0.10). There were no significant group

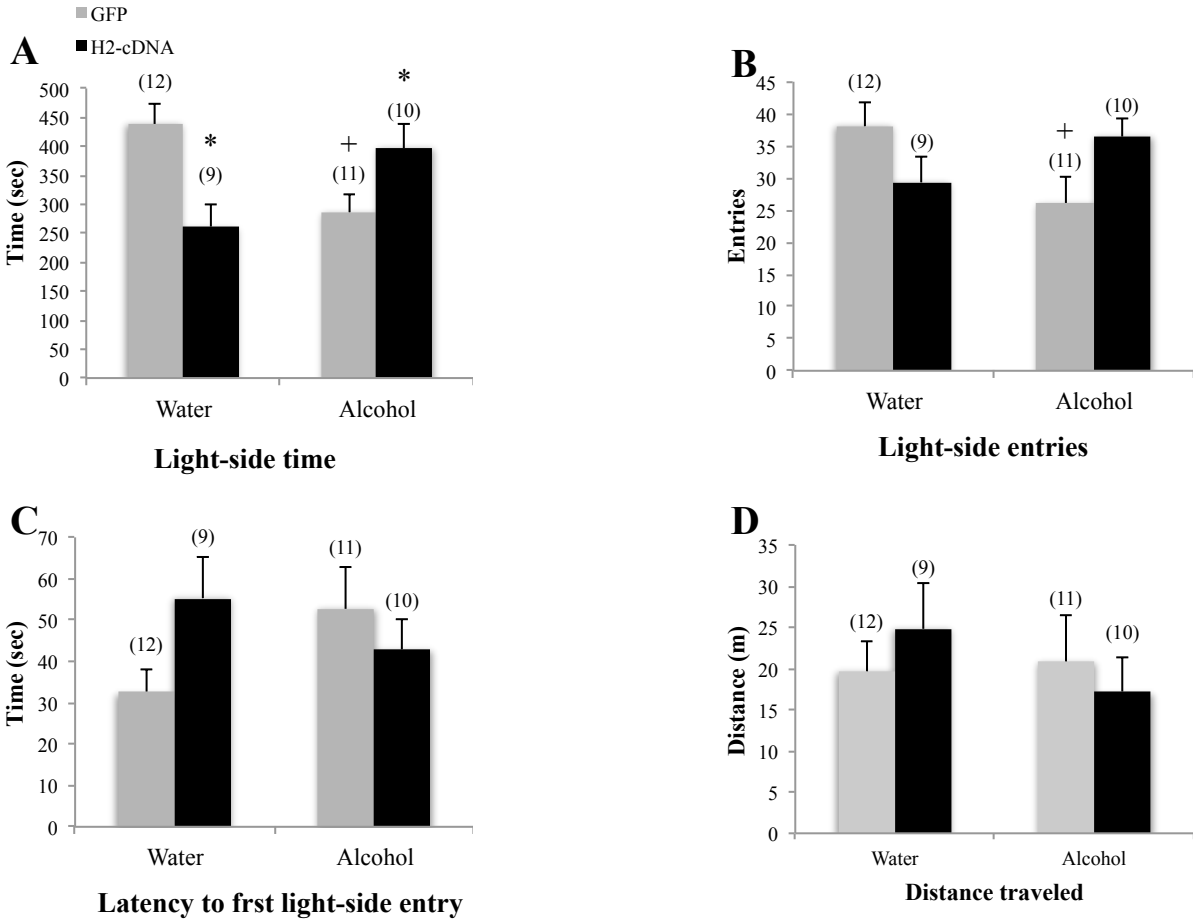

Figure 8.3 The effects of adolescent alcohol experience and intra-CEA infusion of Homer2-cDNA (H2-cDNA) on **(A)** the time on the light side (sec), **(B)** the number of light-side entries, **(C)** the latency to the first light entry (sec) and **(D)** the total distance traveled in the light-side in a light-dark box test (m). In GFP-infused animals (grey bars), alcohol-experienced mice (Alcohol) exhibited signs of hyper-anxiety, but no change in locomotor activity. H2-cDNA infusion (black bars) reversed the hyper-anxiety exhibited by alcohol-experienced mice, while this same treatment produced mixed effects in water-drinking mice. + $p<0.05$ vs. respective water control (adolescent drinking effect). * $p<0.05$ vs. respective GFP control (H2-cDNA effect).

differences in total distance traveled (*p*'s>0.10; Fig. 3D) indicating that neither a history of adolescent binge-drinking or CEA Homer2 over-expression influences gross motor activity.

In the marble burying test, GFP alcohol-drinking animals buried more marbles (p=0.008; Fig. 4A) and spent more time burying (p=0.039; Fig. 4B), compared to GFP water-drinking animals, confirming the presence of an anxiogenic state in control mice. In alcohol-drinking mice, H2-cDNA significantly decreased marbles buried (p=0.015) and showed a negative trend toward less time spent burying (p=0.077), compared to GFP alcohol-drinking animals, but there was no effect on the latency to start burying (p>0.10). In water-drinking animals, H2-cDNA increased marbles buried (p=0.025) and time spent burying (p=0.044) compared to GFP water-drinking animals. There were no significant group differences in the latency to begin burying (p's>0.10; Fig. 4C). Thus, as observed in the light-dark back, the effects of an intra-CEA H2-cDNA infusion upon an animal's affective state varied as a function of adolescent-onset drinking experience.

In GFP-infused animals. alcohol-drinking mice spent more time immobile compared to their water-drinking animals (p=0.039; Fig. 5A), had a shorter latency to first immobility (p=0.024; Fig. 5B), and showed a positive trend toward more immobile episodes (p=0.059; Fig. 5C) in the FST. In water-drinking animals, there was a trend toward less time spent immobile in H2-cDNA animals compared to their GFP controls (p=0.088). However, there was no other effect of H2-cDNA in either the water or alcohol-drinking groups (*p*'s>0.10). Thus, CEA Homer2 expression does not appear to play a major role in regulating depressive-like behaviors in the FST.

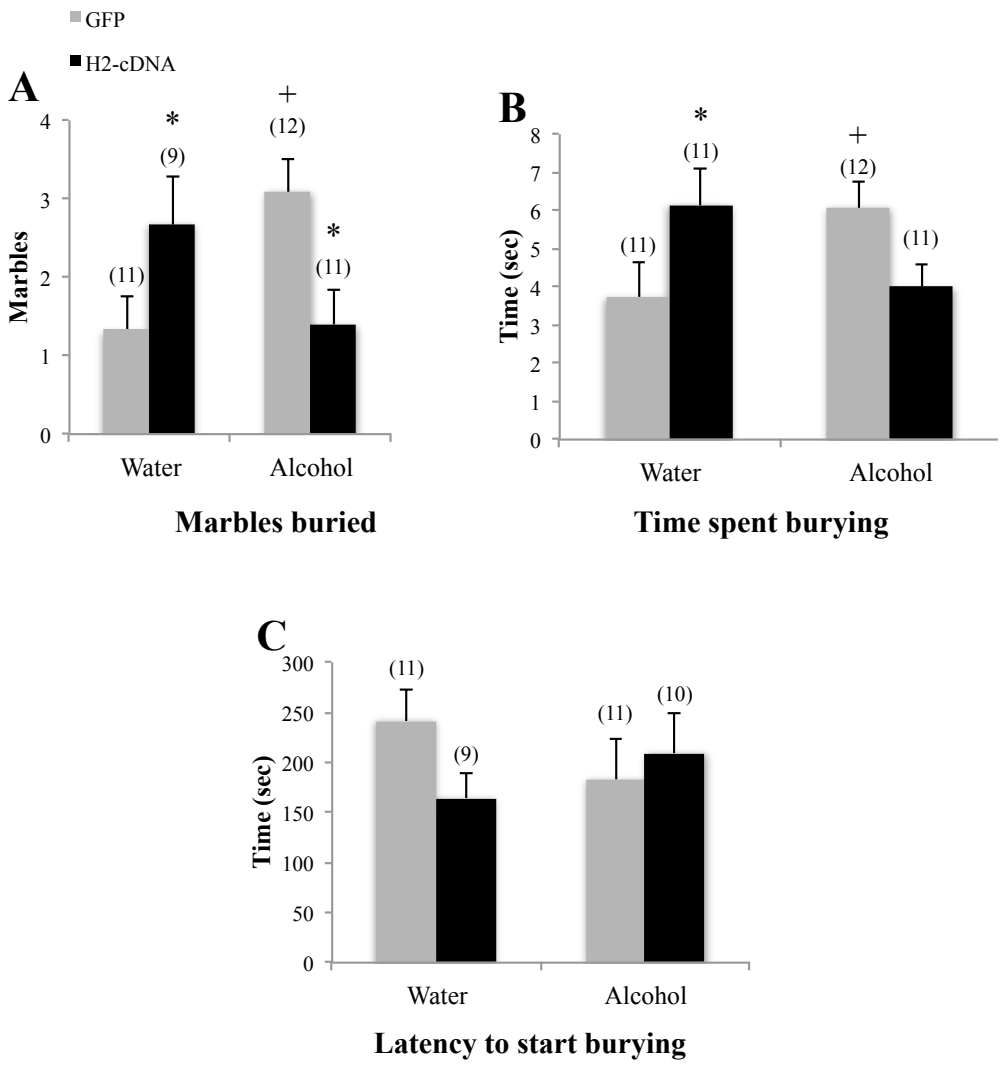

Figure 8.4 The effects of adolescent alcohol experience and intra-CEA infusion of Homer2-cDNA (H2-cDNA) on **(A)** the number of marbles buried, **(B)** the time spent burying (sec), **(C)** the latency to begin burying (sec) in a marble-burying test. In GFP-infused animals (grey bars), alcohol-experienced mice (Alcohol) exhibited some signs of hyper-anxiety. H2-cDNA infusion (black bars) reversed the hyper-anxiety exhibited by alcohol-experienced mice, and produced an anxiogenic effect in water-drinking mice. + $p<0.05$ vs. respective water control (adolescent drinking effect). * $p<0.05$ vs. respective GFP control (H2-cDNA effect).

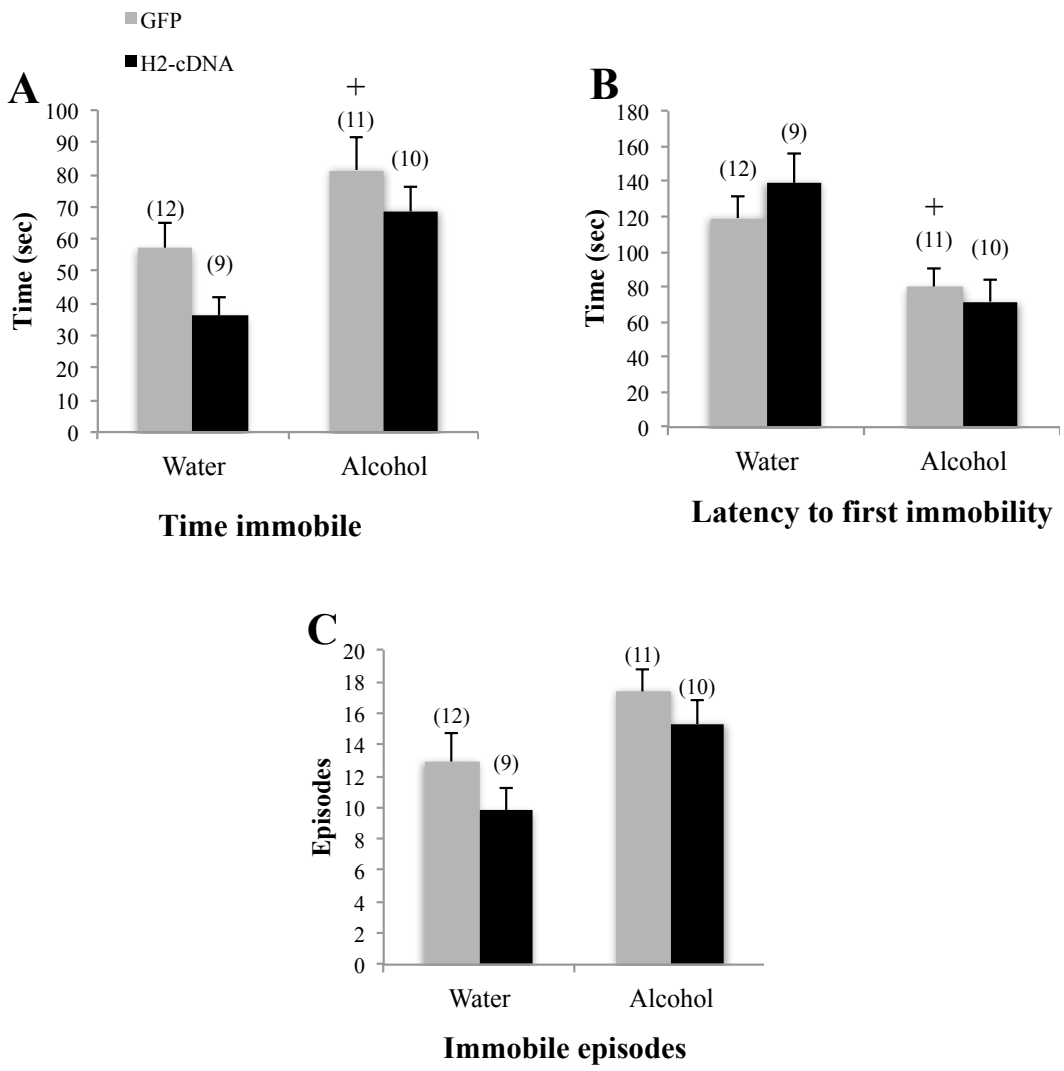

Figure 8.5 The effects of adolescent alcohol experience and intra-CEA infusion of Homer2-cDNA (H2-cDNA) on **(A)** the time spent immobile (sec), **(B)** the latency to first immobile episode (sec), **(C)** the number of immobile episodes in a Porsolt swim test. In GFP-infused animals (grey bars), alcohol-experienced mice (Alcohol) exhibited signs of depressive-like behavior. H2-cDNA infusion (black bars) did not significantly influence behavior of either alcohol- or water-drinking (Water) animals. + $p<0.05$ vs. respective water control (adolescent drinking effect).

3.4 Effects of alcohol and CEA Homer2 in adult animals during early withdrawal

Not surprisingly given their low levels of alcohol intake (see Sect. 3.1.), adult mice infused with GFP did not exhibit signs of anxiety-like behavior on any of our assays (Table 1 and Fig.6 grey bars; p's>0.065). Likewise, there were also no significant effects of H2-cDNA infusion on the behavior of the alcohol-experienced animals (*p*'s>0.10; Table 1 and Fig.6, black vs. grey bars).

However, we did observe some effects of intra-CEA H2-cDNA infusion on the behavior of water-drinking animals and the significant findings are presented in Fig. 6 (left columns),. Specifically, H2-cDNA animals spent less time on the light side compared to GFP (p=0.028; Fig. 6A), with a similar negative trend for light-side entries (p=0.075; data not shown), but no effects on the latency to first light-side entry (p>0.10) or distance traveled (p>0.10). In the marble-burying test, H2-cDNA animals spent more time burying compared to GFP (p=0.023; Fig. 6B), but no group differences were observed for the number of marbles buried or the latency to start burying (*p'*s>0.10). In the FST, H2-cDNA-infused animals spent less time immobile compared to GFP controls (p=0.042; Fig. 6C) and there was also a trend toward fewer immobile episodes (p=0.062), but no effect on the latency to first immobility in either drinking group (*p'*s>0.10).

3.5 Effect of H2-cDNA on alcohol consumption in alcohol-experienced and alcohol-inexperienced animals

During the subsequent 5-day drinking period, adolescent alcohol-experienced GFP-infused animals drank more alcohol compared to their alcohol-inexperienced GFP counterparts (p=0.013, Fig. 7A), suggesting the maintenance of a more "adolescent-like"

Table 8.2: Summary of the negative results regarding the effects of adult-onset binge-drinking in GFP-infused controls, as well as the effects of intra-CEA H2-cDNA infusion upon our behavioral measures observed in early withdrawal. The data represent the means ± SEMs of the number of mice indicated in parentheses. . #$p<0.10$ vs. water-GFP.

Test	**Dependent measure**	**Water**		**Alcohol**	
		GFP (11)	H2-cDNA (11)	GFP (12)	H2-cDNA (11)
Light-dark box	Light-side entries	36.70 ± 2.84	29.36 ± 3.22#	32.42 ± 2.30	28.90 ± 2.83
	Time spent on light side	Fig. 6A			
	Latency to first light-side entry	37.07 ± 5.85	44.75 ± 8.63	34.34 ± 8.73	38.81 ± 4.86
Marble burying	Marbles buried	1.18 ± 0.30	1.82 ± 0.72	1.55 ± 0.39	1.09 ± 0.34
	Time spent burying	Fig. 6B			
	Latency to begin burying	106.57 ± 21.44	70.28 ± 14.95	79.30 ± 16.15	94.48 ± 27.11
Forced swim test	Immobile episodes	18.91 ± 2.52	12.18 ± 2.02#	15.17 ± 2.08	14.00 ± 2.73
	Time spent immobile	Fig. 6C			
	Latency to first immobility	72.85 ± 8.42	94.28 ± 9.88	67.33 ± 8.99	87.86 ± 8.24

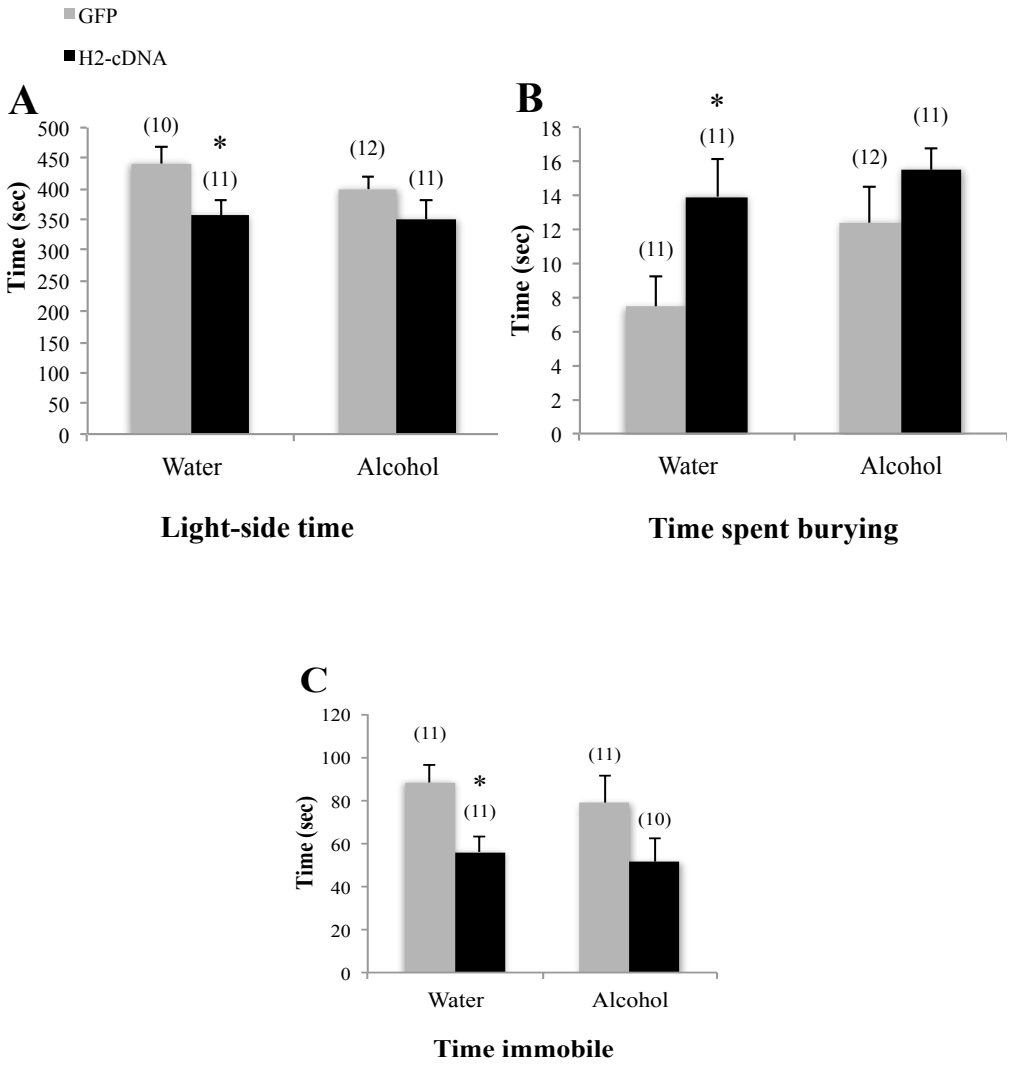

Figure 8.6. The effects of adult alcohol experience and intra-CEA infusion of Homer2-cDNA (H2-cDNA) on **(A)** the time spent in the light-side of the light-dark box (sec), **(B)** the time spent marble-burying (sec), **(C)** the total time spent immobile in the FST (sec). The remaining data are presented in Table 1. In GFP-infused animals (grey bars), alcohol-experienced mice (Alcohol) failed to exhibit signs of anxiety- or depressive-like behavior. H2-cDNA infusion (black bars) produced an anxiogenic effect in water-drinking controls (Water), but did not significantly influence behavior in alcohol-experienced animals. * $p<0.05$ vs. GFP control (cDNA effect).

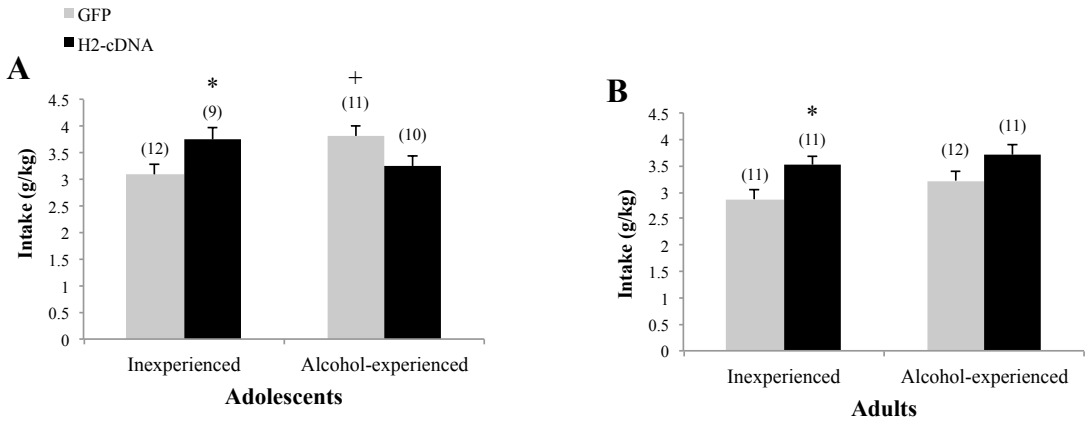

Figure 8.7. The effects of alcohol experience and intra-CEA infusion of H2-cDNA on the alcohol intake exhibited by mice with a prior history of drinking either water (Inexperienced) or alcohol (Alcohol-experienced) during **(A)** adolescence or **(B)** adulthood. In GFP-infused animals (grey bars), adolescent-onset alcohol-experienced mice exhibited greater alcohol intake relative to first-time drinkers. This alcohol history effect was less pronounced in adult mice. H2-cDNA infusion (black bars) augmented the alcohol intake exhibited by first-time drinkers but did not significant alter alcohol intake alcohol-experienced animals. + $p<0.05$ vs. respective Inexperienced control (drinking history effect. * $p<0.05$ vs. GFP control (cDNA effect).

drinking phenotype in the alcohol-experienced adolescent controls. H2-cDNA significantly increased intake compared to GFP controls in alcohol-inexperienced mice (p=0.029). In contrast, H2-cDNA tended to reduce alcohol consumption in alcohol-experienced adolescent mice (p=0.057; Fig.7A). There was no difference in intake between adult alcohol-experienced and -inexperienced animals infused with GFP (p>0.10; Fig. 7B). However, as observed for alcohol-inexperienced adolescent mice, H2-cDNA significantly increased alcohol intake (p=0.019; Fig. 7B), with a similar positive trend observed in alcohol-experienced animals (p=0.066; Fig. 7B). Dependent samples t-tests conducted within each drinking group showed that both H2-cDNA [t(9)=8.86, p's<0.001] and GFP [t(10)=6.31, p<0.001] animals with adolescent alcohol experience drank significantly less alcohol on average during the subsequent drinking period compared to the initial 14-day drinking period. This likely reflects the well-characterized ontogeny of alcohol-intake (Games, 1971; Hancock & Klockars, 1996; Rosnow & Rosenthal, 1989; Ruxton & Beauchamp, 2008; Wilkinson & Inference, 1999), given that the 2nd round of drinking occurred during adulthood (starting on PND 71). In contrast, there was a significant escalation of alcohol intake in H2-cDNA adult alcohol-drinking mice [t(10)=3.68, p=0.004], but not in their GFP-infused counterparts [t(11)=1.12, p=0.288].

4. Discussion

Replicating our prior work (Lee et al., 2016; Spear & Varlinskaya, 2005) and other reports in the literature (Lee, Coelho, Class, et al., 2017; Lee et al., 2016; Lee, Coelho, Solton, et al., 2017), adolescent animals in the present study consumed significantly more alcohol across the 14-day drinking period than adults and were, by definition, engaged in

binge-drinking. However, the average alcohol intake and resultant BACs in adult animals were below the levels typically observed under in our multi-bottle, limited alcohol-access, binge-drinking models (Doremus et al., 2005; Spear & Varlinskaya, 2005; Vetter et al., 2007). While infrequent, it is not unprecedented for an occasional cohort of animals to exhibit low alcohol intakes; however, to date, we have been unable to pin-point a specific cause for this anomaly. Nevertheless, the adult mice in this study provided an opportunity to extend our earlier indication that the severity of alcohol withdrawal-induced negative affect is proportional to the amount of alcohol consumed during adulthood (e.g. Cozzoli et al., 2015; Lee, Coelho, Class, et al., 2017; Lee et al., 2016; Lee, Coelho, Solton, et al., 2017) by demonstrating that a repeated history of low-dose alcohol consumption (~ 2g/kg/day) produces negligible signs of withdrawal-induced negative affect nor does it increase the subsequent propensity to binge-drink. Thus, while precluding our ability to assay the effects of CEA Homer2 over-expression upon withdrawal-induced negative affect in adult-onset drinkers, the present results nonetheless argue that repeated low-dose alcohol consumption in adulthood is insufficient to elicit the neurobiological adaptations that drive alcohol withdrawal-induced changes in affect.

Also replicating our prior work (Lee et al., 2015; Lee, Coelho, Class, et al., 2017; Lee, Coelho, Solton, et al., 2017), a history of binge alcohol-drinking elicited a hyper-anxious state during withdrawal, which is theorized to contribute to the negative reinforcing properties of alcohol abuse (Lee et al., 2015; Lee, Coelho, Class, et al., 2017; Lee et al., 2016; Lee, Coelho, Solton, et al., 2017). This negative affective state is associated with reduced Homer2 expression within the CEA (Koob & Le Moal, 1997; Koob & Moal, 1997) and the present data for adolescent-onset binge-drinking mice provide novel evidence that

this neuroadaptation modulates alcohol withdrawal-induced anxiety, and offers insight into the potential psychological mechanism through which CEA Homer2 overexpression augments binge-alcohol intake (Lee, Coelho, Solton, et al., 2017).

4.1 Homer2 over-expression reverses the hyper-anxious phenotype of adolescent-onset drinking mice during protracted withdrawal.

Replicating the results from our previous reports (Cozzoli et al., 2015), animals with adolescent alcohol-experience showed robust signs of negative affect during protracted withdrawal, as indicated by increased indices of anxiety-like behavior in the light-dark box and marble-burying test and increased signs of depression-like 'behavioral despair' (i.e. increased immobility) in the FST. The fact that adolescent-onset drinkers do not exhibit overt signs of negative affective during early withdrawal (Lee, Coelho, Class, et al., 2017; Lee et al., 2016; Lee, Coelho, Solton, et al., 2017) argue that this negative affective state, and its neurobiological underpinnings, incubate during protracted withdrawal (Lee et al., 2016). Indeed, mice with a history of adolescent-onset binge-drinking exhibit a number of protein changes within the AcbSh and CeA in protracted withdrawal that are not manifest in early withdrawal (Lee, Coelho, Solton, et al., 2017). Further, many of the protein changes observed during protracted withdrawal in adolescent-onset drinkers resemble those exhibited by adult-onset drinkers during early withdrawal when they manifest a hyper-anxious phenotype (Lee et al., 2016; Lee, Coelho, Solton, et al., 2017). One such change that we pursued in the present study is reduced Homer2 expression within the CEA (Lee et al., 2016; Lee, Coelho, Solton, et al., 2017). This neuroadaptation is opposite that reported in our prior

immunoblotting studies of mice with a 30-day history of binge-drinking (Lee, Coelho, Solton, et al., 2017), but its functional relevance has not been pursued in our prior work.

Herein, an intra-CEA infusion of H2-cDNA, administered following a 2-week history of binge-drinking during adolescence, exerted an anxiolytic effect, as indicated by a reversal of some of the signs of anxiety-like behavior in the light-dark box and marble-burying tests. Notably, however, the cDNA-mediated reversal of hyper-anxiety in these assays was not complete; some measures appeared to be more sensitive to the effects of CEA Homer2 over-expression than others, arguing, not unexpectedly, that the withdrawal-induced decrease in CEA Homer2 expression is not the sole mediator of withdrawal-induced negative affect. Consistent with this notion, H2-cDNA infusion did not alter any dependent measure in the FST, despite the manifestation of depressive-like signs in GFP-infused controls. The disparate findings between our assays of anxiety- versus depression-like behaviors may relate to the nature of the stressor (physical versus psychological) or the possibility that H2-cDNA selectively gates output from the CEA to mesocorticolimbic structures driving withdrawal-induced anxiety, but not depressive-like states. Nevertheless, it is important to note that not only did intra-CEA H2-cDNA infusion reverse the hyper-anxious phenotype of adolescent-onset drinkers, it also attenuated their subsequent alcohol consumption. These data provide novel evidence to support an important role for CEA Homer2 expression in gating the negative reinforcing properties of alcohol that drive excessive intake. Given the limitations of our study of adult alcohol-experienced animals, an obvious future direction is to replicate this aspect of the experiment in an adult cohort that exhibits binge-levels of alcohol consumption and determine whether or not reversing the reduction in CEA Homer2 expression observed during early withdrawal in adult-onset drinkers can attenuate the

manifestation their hyper-anxious phenotype. Further, future studies should probe how Homer2 influences CEA output, as well as characterize the Homer2-dependent molecular interactions within the CEA that drive that output, in animals with histories of adolescent- versus adult-onset drinking to better understand their unique temporal patterning of withdrawal-induced negative affect (Cozzoli et al., 2014).

4.2 CEA Homer2 over-expression drives hyper-anxiety and alcohol consumption in alcohol-naïve mice.

Fascinatingly, the capacity of CEA H2-cDNA over-expression to reverse the hyper-anxious phenotype of adolescent-onset binge-drinkers did not reflect some general anxiolytic effect of AAV infusion. In fact, intra-CEA H2-cDNA infusion was anxiogenic in both water-drinking adolescent and adult controls, as evidenced in all three behavioral assays. In both cases, CEA Homer2 over-expression produced an anxiety-like phenotype in alcohol-naïve mice akin to that reported in alcohol withdrawn animals (Lee, Coelho, Solton, et al., 2017). Further, consistent with the prior evidence that AchSh Homer2 over-expression augments alcohol reward and reinforcement (Szumlinski et al., 2005, 2008) and our previous indication that CEA Homer2 drives binge-drinking behavior (Cozzoli et al., 2014), CEA Homer2 over-expression also augmented the alcohol intake exhibited by mice drinking alcohol for the first time. It is interesting to note that the effects of H2-cDNA on affective behavior and subsequent drinking in adult alcohol-experienced were in the same direction as the effects observed in water-drinking controls, but with a lesser magnitude (i.e., the GFP-cDNA effects were not statistically significant in the adult alcohol-experienced animals). While speculative at this time, it is possible that repeated low-dose alcohol consumption may

have reduced CEA Homer2 expression to an extent enough to counteract the anxiogenic effects of H2-cDNA infusion, but not to the extent required to promote anxiety or increase consumption. Nevertheless, the present data are the first to demonstrate that CEA Homer2 over-expression during late adolescence/early adulthood is sufficient to both induce hyper-anxiety and augment subsequent binge-alcohol consumption, raising the possibility that the large alcohol-induced increase in CEA Homer2 expression observed during early withdrawal in mice with a more chronic (30-day) history of binge-drinking (Cozzoli et al., 2014) might drive directly the negative reinforcing properties of alcohol.

4.3 Reconciling disparities in the role for CEA Homer2 in regulating anxiety

Homer2 is glutamate receptor scaffolding protein that regulates various aspects of glutamate transmission within extended amygdala structures, ranging from the maintenance of basal extracellular glutamate content to the activational state of specific intracellular signaling pathways and its expression is generally considered necessary for normal excitatory neurotransmission (for reviews, Szumlinski et al., 2008). Consistent with this reasoning, the anxiogenic effects of Homer2 overexpression within the CEA of alcohol-naïve mice are in line with an abundant literature implicating amygdalar hyper-activation in clinical anxiety, depression and withdrawal-induced negative affect (Lee et al., 2015; Lee, Coelho, Class, et al., 2017; Lee et al., 2016; Lee, Coelho, Solton, et al., 2017, present study). However, our 2-week binge-drinking paradigm reduces CEA Homer2 expression (Lee et al., 2017c) and herein, H2-cDNA infusion exerted an anxiolytic effect on some measures in mice with a prior history of adolescent-onset binge-drinking. While the precise relation between the chronicity/amount of binge-drinking and the direction of the effect of alcohol withdrawal

upon CEA Homer2 expression requires a considerable parametric study, the disparities in results across our studies argue that either increased or decreased CEA Homer2 expression can result in the manifestation of a negative affective state that augments subsequent alcohol-taking, likely via shifts in group 1 mGlu receptor function.

Indeed, several lines of evidence support the notion that an imbalance in the relative expression of different Homer isoforms results in behavioral and neurochemical anomalies of relevance to a number of different neuropsychiatric disorders, including depression and addiction (Davidson, 2002; Davis, 1992; Gilpin et al., 2015; Ketter et al., 1996; Servan-Schreiber et al., 1998; Stein et al., 2007). As inducible *Homer1* gene products (e.g., Homer1a) act as dominant negatives to disrupt constitutive Homer isoform binding to interacting partners and alter mGlu and NMDA receptor function (e.g., Kammermeier et al., 2000; Park et al., 2013; Xiao et al., 1998), the majority of this work has focused on the interplay between constitutively expressed versus inducible *Homer1* gene products (e.g., Klugmann et al., 2005; Lominac et al., 2005; Szumlinski et al., 2006; Tappe and Kuner, 2009). Even within this line of Homer1-related work, both over- or under-expression of Homer1a results in comparable cognitive dysfunction in fear-conditioning models (Ary et al., 2013; Gould et al., 2015; Szumlinski et al., 2006), which is a finding in-line with our disparate results for CEA Homer2 expression and anxiety.

To date, we have failed to detect any alcohol-induced changes in the expression of constitutively expressed Homer1 isoforms in the brain across a variety of alcohol delivery paradigms (Banerjee et al., 2016; Mahan et al., 2012). Thus, either an increase or a decrease

in CEA Homer2 levels will be predicted to imbalance the relative expression of Homer1 versus Homer2 isoforms in this region. Less well-studied than the effects of imbalancing different *Homer1* gene products, we have reported previously that cocaine-induced changes in the relative levels of constitutively expressed *Homer1* and *Homer2* gene products, at least within the prefrontal cortex, are critical for gating the rewarding and reinforcing properties of this drug (Ary et al., 2013; Gould et al., 2013). Thus, it is entirely possible that any alcohol-induced disruption in the Homer1-Homer2 balance within the CEA is sufficient to produce a negative affective state that motivates alcohol consumption. Indeed, Homer2 is more occlusive than Homer1 with respect to the efficiency through which Group1 mGlu receptors signal to voltage-gated ion channels *in vitro* (Cozzoli et al., 2012; Cozzoli et al., 2014; Cozzoli et al., 2009; Goulding et al., 2011; Lee et al., 2015; Lee, Coelho, Solton, et al., 2017; Obara et al., 2009; Szumlinski, Ary, Lominac, et al., 2008) notably, to inhibit Cav2.2 and Cav2.3 (Kammermeier & Worley, 2007), but it remains to be determined how this property relates to Homer2 regulation of group1 mGlu function *in vivo*, let alone behavior. Given that capacity of an intra-CEA infusion of mGlu1 and mGlu5 inhibitors to regulate binge-drinking require Homer2 (Cozzoli et al., 2014), and systemic treatment with mGlu5 inhibitors blunt alcohol withdrawal-induced negative affect (Beqollari & Kammermeier, 2013; Kammermeier, 2008; Kammermeier et al., 2000; Won et al., 2009), future work will focus on better understanding the biochemical consequences of manipulating CEA Homer2 expression *in vivo* in relation to the negative reinforcing properties of alcohol.

4.5 Conclusions

In conclusion, the present study provides novel insight into the role of CEA Homer2 in basal and alcohol-induced increases in anxiety, as well as alcohol consumption. Together these data provide evidence of an anxiogenic and pro-drinking effect of Homer2 overexpression in the CEA. Conversely, restoring CEA Homer2 levels in animals with adolescent alcohol experience exerts a protective effect against withdrawal-induced anxiety and escalated alcohol consumption in adulthood. If relevant to humans, these data argue idiopathic or alcohol-induced changes in CEA Homer2 expression as important for the etiology of negative affect, the negative reinforcing properties of alcohol and the ontogeny of alcohol abuse.

Chapter 9:

General Discussion

There are many psychological aspects of drug withdrawal that could contribute to the increase in anxiety reported by human alcohol abusers. There is also speculation that hyperanxiety often *precedes* alcohol abuse in humans and may motivate the initiation of alcohol consumption (Kaplow et al., 2001; McKenzie et al., 2011). However, through the use of animal models, it is possible to disentangle the psychopharmacological effects of alcohol withdrawal from psychosocial factors and pre-existing psychological conditions. The studies presented in this dissertation provide evidence of a causal relationship between excessive alcohol consumption and subsequent affective dysregulation and highlight the association between alcohol-induced changes in glutamate-related protein expression and anxiety during abstinence. In addressing the original aims of these studies, we found that 1) adult animals show persistent signs of negative affect during both acute (24hr) and protracted (3 weeks) withdrawal following a 30-day history of binge drinking under single-bottle DID conditions. The presence of negative affect was associated with increased cellular activity within the CEA and BNST. 2) Adolescent animals consume larger quantities of alcohol than adults under 4-bottle DID conditions. In contrast to adults, adolescent binge drinkers are resilient to affective dysregulation during acute withdrawal following 14 days of drinking. Additionally, adults show increases in glutamate-related protein expression within the accumbens during acute withdrawal that are absent in adolescent drinkers. 3) Based on consistent observation of decreased immobility in the FST in adult animals during acute withdrawal coinciding with increased anxiety on other behavioral assays, we demonstrated that this reduction in immobility is a sign of anxiety that can be reversed with both a traditional and atypical anxiolytic drug. 4) Despite apparent resilience during acute withdrawal, negative affect incubates in adolescent binge drinkers and emerges during protracted withdrawal (i.e. in

adulthood), characterized by both anxiety- and depression-like symptoms. Additionally, animals with adolescent drinking experience exhibit higher alcohol consumption during protracted withdrawal, compared to animals with adulthood drinking experience. This negative affective state was associated with increased group 1 mGluR expression within the AcbSh but reduced H2 and mGlu1 expression within the CEA. 5) Withdrawal-induced anxiety is significantly reduced via systemic mGlu5 antagonism, while mGlu5 positive allosteric modulation is mildly anxiogenic. 6) Intracranial administration of mGlu5 antagonist within the AcbSh specifically has anxiolytic effects in adult drinkers during acute withdrawal, but minimal effects in adolescent drinkers during protracted withdrawal; and 7) reversal of alcohol-induced downregulation of H2 within the CEA has an anxiolytic effect and curbs excessive alcohol consumption in adolescent drinkers during protracted withdrawal, but has an anxiogenic, drinking-enhancing effect in animals without prior alcohol experience.

Unexpected decreases in protein indices of Homer-mediated mGluR signaling within the CEA

The observed decreases in mGlu1 and Homer2 expression observed within the CEA were surprising, based on previous findings from our laboratory and others, which typically show an increase during withdrawal (see Chapter 5 for more detail). However, our other studies provided evidence that withdrawal-induced anxiety is likely mediated by a net hyperactive state, based on the robust anxiolytic effects of systemic mGlu5 antagonism. Additionally, alcohol withdrawal was associated with increased cellular activity (as indicated by immediate early gene expression) within the CEA, BNST, and AcbSh. Although less

likely, it is also possible that this protein downregulation is a unique consequence of a 14-day drinking period, as the present IEG data, as well as earlier data from our laboratory (e.g., Cozzoli et al., 2014) from the CEA were obtained following 30 days of drinking. Thus, it would be informative to track the time course of these changes in glutamate-related proteins and IEG expression and also vary the duration of drinking history in order to provide a more comprehensive characterization of the overall activational state of extended amygdala structures at different stages of withdrawal.

Although reversing the alcohol-induced decrease in H2 expression in the CEA appeared to have anxiolytic effects in adolescent drinkers during protracted withdrawal, we do not know that this manipulation resulted in an overall increase in glutamatergic activation. Rather, these anxiolytic effects are more likely mediated by changes in the downstream consequences of glutamatergic signaling (as discussed in Chapter 8). To that end, more precise techniques such as electrophysiology or microdialysis could possibly provide a more nuanced depiction of the net outcome of the observed protein changes on CEA physiology and output. Additionally, it would be interesting to identify at what point in time between acute and protracted withdrawal does H2 expression begin to decline within the CEA of adolescent drinkers and conversely, to recover in adult drinkers, and whether this time-course and/or direction of effect varies depending on the duration of drinking history.

Nevertheless, a possible mechanism by which a decrease in glutamatergic tone could contribute to an increase in anxiogenic output could be due to internal dysregulation within the CEA. The CEA can be further subdivided into a lateral (CEA_L) and medial (CEA_M) region, which serve different functions and have distinct connectivity (Ciocchi et al., 2010). The CEA is largely controlled by incoming glutamatergic projections from the basolateral

amygdala (BLA) to the CEA_L, relaying information sensory information from the cortex (Krettek & Price, 1978; Savander et al., 1995). The CEA_L exerts inhibitory control over the CEA_M, which is the major output nucleus of the amygdala and projects to regions that mediate fear and anxiety responses to relevant environmental stimuli (Hopkins & Holstege, 1978; Pape & Pare, 2010; Zimmerman et al., 2007). As the glutamatergic synapses of the CEA are primarily localized to the CEA_L, impaired sensitivity to regulatory afferents due to decreased glutamate-related protein expression could results in a disinhibition of the anxiogenic outputs from the CEA_M. As the present studies did not distinguish CEA_L vs. CEA_M, further characterization of intra-CEA microcircuitry may help clarify this seemingly paradoxical decrease in CEA H2 protein expression.

Given that the BLA is the primary source of glutamatergic innervation to the CEA, future studies should seek to characterize the effects of alcohol in this region. The present studies did not pursue the BLA, as we did not find consistent evidence of withdrawal-induced changes in protein expression in our western blotting data (Chapter 3) or in our laboratory's previous studies of the glutamatergic consequences of binge drinking (Cozzoli et al., 2014; Obara et al., 2009). However, we know from prior experiments that there are likely changes in protein function that occur independent of changes in overall expression levels [e.g. intra-CeA mGlu5 blockade reduced alcohol intake, despite no alcohol-induced change in protein expression, suggesting an increase in CEA mGlu5 function following a history of binge drinking (Cozzoli et al., 2014)]. The BLA is not only connected with the CEA, but also shares projections to the Acb and BNST (LaLumiere et al., 2005; Stamatakis et al., 2014; Stuber et al., 2011; Vyas et al., 2003), thus the BLA is integrally connected with all regions of the extended amygdala and is a primary source of glutamatergic innervation

(Gilpin et al., 2015; Kim et al., 2013). Therefore, alcohol-induced dysregulation within the BLA would likely impinge upon the activity throughout the entire extended amygdala.

Future studies should also track the persistence of negative affect and excessive alcohol consumption across the lifespan in animals with a history of adolescent binge drinking to determine if these changes are life-long. If these changes are permanent, this would suggest irreversible malformation of corticofugal and/or mesocorticolimbic circuitry due to alcohol-related derailment of normal development. Additionally, it should be assessed whether there are differential effects resulting from exposure during early, mid, or late adolescence, as a study in non-human primates showed that rhesus macaques exhibit distinct patterns of consumption associated with difference phases of adolescence (Helms et al., 2014). From an ontogenetic standpoint, it would also be interesting to expand these studies to compare the affective consequence of alcohol exposure at various stages of development from prenatal through post-reproductive age.

Proposed mechanisms of affective dysregulation in adolescent vs. adult binge drinkers

Affective dysregulation following adult binge drinking appears to be the result of classic drug-induced plasticity resulting from repeated bouts of intoxication and withdrawal, eliciting an allostatic shift to compensate for the acute effects of alcohol [e.g. an inhibition of excitatory glutamatergic signaling and potentiation of inhibitory GABAergic signaling (Roberto et al., 2003; Siggins et al., 2005; Strawn & Cooper, 2002)]. During early withdrawal, animals experience a transient imbalance between excitatory and inhibitory signaling in the absence of alcohol (Esel, 2006), one consequence of which are symptoms of negative affect. However, given that underlying neuroanatomical connectivity is still

relatively intact, animals are able to revert to a "normal" default state over time as the duration of abstinence increases [although, while this is the case following a relatively brief drinking history (e.g. 14 days), the capacity to rebound may diminish with increased chronicity of alcohol abuse].

In adolescence, PFC development is delayed relative to subcortical circuitry (Sturman & Moghaddam, 2011), allowing cognitive processing to be dominated by mesolimbic regions (Andersen & Teicher, 2008). This bias toward 'bottom-up' processing is thought to underlie the greater susceptibility to mood disturbances, poor emotional regulation, increased impulsivity, sensation/novelty seeking, and risk-taking that is characteristic of adolescence (Arain et al., 2013; Bava & Tapert, 2010; Casey, Getz, et al., 2008; Pine et al., 2001; Spear, 2010; Sturman & Moghaddam, 2011). Neurotypical adolescent development is associated with extensive remodeling of the structure and function of the brain. There is a proliferation of glutamatergic synapses at puberty, followed by substantial pruning shortly thereafter (Huttenlocher 1984; Insel et al. 1990; Rakic et al. 1994). This results in a dramatic reduction of gray matter, as well as an increase in white matter from ongoing myelination of axons to reinforce residual connections (Gogtay et al., 2004; Sowell et al., 2003). It is through this process of synapse stabilization (i.e. decreased redundancy and increased efficiency of neural function), fine-tuning of excitatory and inhibitory neurotransmission, and development of effective connections between cortical and subcortical circuitry (Keshavan et al., 2014) that the brain reaches its mature state (Arain et al., 2013; Bava & Tapert, 2010; Casey, Getz, et al., 2008; Spear, 2000a, 2010).

Interestingly, in contrast to adults, adolescents show minimal signs of dysfunction during early withdrawal from binge drinking. However, given that the adolescent brain is

already in a constant state of flux, this may enhance flexibility and facilitate compensatory neuroadaptation to chronic alcohol exposure. Combined with the hyper-emotionality that is already characteristic of adolescence, these factors could contribute to an apparent resilience to alcohol-induced affective dysregulation during early withdrawal, which, for the animals in our studies, occurred during late adolescence (Brust et al., 2015; Spear, 2000b). It is not until animals reach adulthood that the detriments of adolescent binge drinking become apparent, which could account for the delayed 'incubation' of negative affect in adolescent drinkers.

The human and animal literature frequently reports that adolescent binge drinking is associated with a persistent 'adolescent-like' behavioral and neurobiological phenotype, both basally and in response to alcohol (reviewed in Spear & Swartzwelder, 2014). This is attributed to an alcohol-induced arrest of the normal developmental trajectory described above (Matthews et al., 2008; Varlinskaya et al., 2014; White, Bae, et al., 2002; White, Ghia, et al., 2000). Adolescent binge drinking is associated defective neuroanatomical connectivity that can result in life-long deficits even after extended periods of abstinence (Lopez et al., 2008; McBride et al., 2005; Nixon & McClain, 2010; Novier et al., 2015). In contrast to adults, adolescents lack a functionally mature configuration upon which to revert. Thus, adolescent are more susceptible to profound and enduring consequences of binge drinking following a comparable duration of exposure (Dahl, 2004; Hunt, 1993; Spear, 2014).

Although the precise mechanisms responsible for this derailment of normal development are still unclear, it is theorized that adolescent binge drinking may cause dysfunctional (i.e. excessive or inadequate) pruning of glutamatergic synapses, resulting in impaired cortical restructuring/connectivity (Selemon, 2013; Squeglia et al., 2009). A failure to establish and strengthen synapses of corticofugal projections may permanently weaken

control over subcortical hyperactivation (Arain et al., 2013; Casey et al., 2011; Sturman & Moghaddam, 2011). This weakened control may also be further exacerbated by alcohol-induced inhibition of neuronal complexity and decreased density of dendritic arborization of projections from regulatory regions such as the mPFC and hippocampus (Hamilton et al., 2010; Sala et al., 2003). Other underlying mechanisms could involve alcohol-induced epigenetic reprogramming and altered gene expression, leading to persistent imbalances of excitatory/inhibitory signaling and impaired or maladaptive synaptic plasticity (Goyal & Raichle, 2013; Kofink et al., 2013). Together, these factors likely account for the negative affect and excessive alcohol consumption observed in adolescent drinkers during protracted withdrawal.

In summary, binge drinking during the sensitive neurodevelopmental period of adolescence is uniquely detrimental compared to binge drinking in adulthood. This is supported by clinical data showing increased susceptibility to alcohol-induced neuroadaptations in adolescent drinkers, which are associated with a persistent vulnerability to substance abuse problems and affective dysregulation throughout life (Brown et al., 2000; Brumback et al., 2015; Carrara-Nascimento et al., 2011; Chaby et al., 2015; Clark & Tapert, 2008; Crews et al., 2007; Dahl, 2004).

Interpretational considerations

There may exist some ambiguity as to whether the behavioral changes adult animals at 24 hr post-drinking can be attributed to *withdrawal* specifically, or if these could be lingering effects of acute alcohol. Although alcohol is no longer present in blood or urine at 24 hr (NIAAA, 1997), there are indirect oxidative biomarkers of alcohol use that remain in

the body for up to a week after cessation (Abudu, 2008). However, there is evidence to suggest that the effects of alcohol withdrawal are in fact present at 24 hr. In animal studies, signs of physiological withdrawal (i.e. handling-induced convulsion) are present at 4 hr withdrawal (Goldstein & Pal, 1971) and anxiogenic effects have been reported at 12 hr (Lal et al., 1991). In humans, dependent individuals begin to exhibit physiological signs of withdrawal as early as 6 hr after cessation (Trevisan et al., 1998) and anxiety is often observed around 12 hr (Peyser, 1993). Therefore, there is precedent in the literature to postulate that the behavioral effects reported in our studies are indeed a consequence of alcohol withdrawal.

Multiple behavioral tests were included in our studies to provide corroboration for our findings and facilitate interpretation of the data. For example, had we used only the FST, our results from this assay would suggest that acute alcohol withdrawal has anti-depressant effects in adult animals, based on traditional interpretations of decreased immobility. The fact that we observed robust signs of anxiety in our other tests that suggested conventional interpretations of behavior in the FST may not be appropriate in this circumstance. While the use of multiple tests certainly has its advantage, it can also lead to interpretational ambiguity when behavioral changes are not observed consistently across all tests. Part of this inconsistency is likely attributable to differential sensitivity of the tests to behavioral changes following a moderate drinking history (compared to dependence-inducing high exposure). For example, preliminary pilot studies revealed that the open field tests was not a particularly sensitive assay for our purposes and routinely showed no alcohol-water differences, despite the presence of group differences in other tests. Therefore, we did not pursue the use of this assay in our studies.

Even after narrowing our tests down to the 3 that showed the most reliable sensitivity (light-dark box, marble-burying and FST), there were also occasional inconsistencies observed between tests in some of our studies. For example, in Chapter 7 and 8, anxiolytic effects of our manipulations were observed in the light/dark box and marble burying test, but not the FST. We speculated that this could be due to the acute physiological nature of the swim stressor (compared to the more mild, psychological nature of the other stressors), which is likely to elicit greater activation of HPA- and locomotor-related circuitry (Dal-Zotto et al., 2000; Gong et al., 2015; Pitman et al., 1990). Additionally, all animals were subjected to the same order of behavioral tests without counterbalancing and thus the FST was always the last test administered. This decision was based primarily on the recommendation of our IACUC to avoid further testing immediately following the FST. Therefore, it is possible that drug treatment effects could have dissipated or that alcohol-experienced animals may be more susceptible to compounding carry-over stress effects from the preceding tests in a way that differentially affected their behavior. However, it is not uncommon in this field of research for behavior to change non-uniformly across multiple validated tests of anxiety (discussed in Belzung & Le Pape, 1994; Bourin, 2015). For example, a study found that although various benzodiazepine agonists significantly reduced neophobia in mice in response to a novel environment, 5-HT-targeted drugs with known anxiolytic properties did not alter neophobia-related behavior (Griebel et al., 1993).

Further challenges arose from the inclusion of adolescent animals, which are known to exhibit marked behavioral differences compared to adults, primarily with regards to impulsivity. Behavioral differences related to impulsivity may be difficult to distinguish from anxiety-related behavioral differences. For instance, increased interaction with a novel object

could be interpreted as either increased impulsivity or decreased anxiety (or perhaps both). However, impulsivity and anxiety are theorized to be mediated by separate motivational systems (Fowles, 1987); impulsivity is attributed to a release of approach behavior by behavioral activating systems, while passive avoidance results from a suppression of approach behavior by behavioral inhibition systems (Gray, 1987). Therefore, it can be challenging to disentangle the contributions of competing behavioral processes when interpreting the data.

Although we used the marble burying test repeatedly due to its consistent sensitivity to alcohol-related behavioral differences, there is debate within the field over the interpretation of this assay. Some researchers argue that the behavior measured by this test is actually digging, rather than burying, as mice exhibit strong species-typical digging and burrowing instincts (Deacon, 2006; Gyertyan, 1995). Thus, this assay has been used to study perseverative, compulsive-like behavior related to obsessive-compulsive disorder (OCD) (Angoa-Perez et al., 2013; Thomas et al., 2009). However, not only is anxiety a symptom of OCD (American Psychiatric Association, 2013) but OCD also shares a high comorbidity with anxiety disorders (Carter et al., 2004; Nutt & Malizia, 2006), which could account for the positive correlation between burying/digging and OCD-related behaviors. Published literature provides persuasive evidence supporting the use of the marble burying assay as an anxiety-related test. In addition to our work, other studies have shown increased marble burying during alcohol withdrawal (Umathe et al., 2008) as well as following chronic restraint stress (Kedia & Chattarji, 2014). Importantly, marble burying behavior is successfully reduced by traditional and putative anxiolytic drugs, independent of locomotor effects (Cryan & Sweeney, 2011; Jimenez-Gomez et al., 2011; Nicolas et al., 2006; Njung'e

& Handley, 1991). However, anxiogenic drugs do not consistently increase burying behavior (Jimenez-Gomez et al., 2011; Njung'e & Handley, 1991). Although this assay may not be an isomorphic model of anxiety *per se*, it is nonetheless a valuable screening tool. Additionally, the utility of this assay as an anxiety test does not necessarily preclude its application in studies of compulsive-like behavior. Rather, it merely indicates that burying behavior may not be selective/specific to anxiety, which further reaffirms the inclusion of multiple behavioral assays in our work.

We encountered additional interpretational complications with our behavioral data when dependent measures within the same test did not all change in unison. Over the course of our experiments, a fairly consistent pattern emerged showing differential sensitivity among our dependent variables. The measure of 'total time' spent engaging in a behavior was typically the most sensitive and robust measure while 'latency to begin' that behavior was the least sensitive. This pattern was observed both between assays and across studies. Perhaps in future studies it would be preferable to use 'time' as the primary dependent and only analyze 'latency' and 'episodes' as secondary variables if there is significance in order to avoid the appearance of experimenter bias in interpretation of the data.

Some critics of animal-based anxiety research have also raised concern that virtually all traditional anxiety tests are measures of state anxiety related to a potentially threatening situation rather than a measure of persistent, non-adaptive trait anxiety as is characteristic of generalized anxiety disorders in humans (Bourin, 2015; Calhoon & Tye, 2015; Spielberger, 2010). However, others argue that both state and trait anxiety are relevant to drug abuse. While high trait anxiety may be a risk factor for addiction vulnerability (Butler et al., 2014;

Comeau et al., 2001) and animals selectively bred for high alcohol preference exhibit high trait anxiety (Landgraf & Wigger, 2003), state anxiety is a predictor of stress-induced relapse and escalation of intake (Fox et al., 2007; Simioni et al., 2012; Sinha et al., 2011). Most importantly, the neural circuitry of anxiety identified in rodent models using these tests is consistent with that observed in humans (Adhikari, 2014; Kim et al., 2013; Lister, 1990; Yassa et al., 2012), emphasizing the translational and ethological validity of these methods. Nevertheless, in future studies, it would also be advantageous to supplement behavioral data with physiological stress measures such as serum corticosterone (Johnston et al., 1988) or respiratory rate (Kim et al., 2013) that serve as biomarkers of anxiety (Martin, 1961). Additionally, certain neurotrophic factors such as BDNF, NGF, and TrkA/B and also neuroinflammatory cytokines such as IL-1β, IL-6, and TNF-α have been identified as promising biomarkers of depression (Stepanichev et al., 2014) and could be a useful tool for distinguishing between anxiety and depression with greater sensitivity than behavioral methods.

Study limitations

The experiments described in this dissertation utilized a different approach than the majority of published studies examining alcohol withdrawal-induced negative affect. While the voluntary DID model is advantageous for extending the findings of noncontingent, dependency studies to a model with higher face validity, this approach is not without its pitfalls. Our studies demonstrate that the manifestation of withdrawal-induced negative affect is dependent on the magnitude of alcohol exposure (Chapter 8). Therefore, an inability to control alcohol intake inevitably leads to natural variability both between animals and within

animal across days. Consequently, animals may show more modest severity of withdrawal-related effects compared to consistent daily exposure to doses equivalent to animals' overall average intakes. Nevertheless, this approach provides insight into the consequences of more moderate binge drinking during the transitional phase between casual use and dependence, while also controlling for behavioral and neurobiological differences resulting from contingent versus noncontingent drug administration.

Another concern regarding our drinking model could be the use of unflavored ethanol dilutions rather than sweetened solutions. Relatively few people drink straight grain alcohol, and this arguably reduces the face validity of the model, and the addition of sweetener could help to augment consumption. However, in addition to the historical justification for using unadulterated alcohol solution (Rhodes et al., 2005), there are additional considerations that make our approach preferable. For one, sweet solutions are themselves reinforcing (Sclafani & Ackroff, 2003) and induce neuronal activation within the Acb (Roop et al., 2002; Tukey et al., 2013). Sucrose, a common sweetening agent, influences satiety signals due to its caloric value (Low et al., 2014), which could influence consumption. There is also evidence that sweetening alcohol with sucrose may, paradoxically, adversely affect resulting BACs despite increasing consumption. A study showed that although the addition of sucrose increased operant responding for alcohol, compared to unsweetened solution, resulting BACs following sucrose-sweetened alcohol were significantly lower per gram of alcohol consumed, compared to the unsweetened solution (Roberts et al., 1999). While calorically neutral artificial sweeteners such as saccharin or aspartame could serve as an alternative to sucrose, studies suggests that these artificial sweeteners may be associated with increased anxiety and depression (Ashok et al.; Guo et al., 2014; Rycerz & Jaworska-Adamu, 2013). When

contending with the variability already inherent in a voluntary drinking model, adding an additional, potentially confounding variable such as sweetener to the design further complicates the interpretation of the resulting data. However, future studies would benefit from examining whether or not the presence of sweetener has an effect on alcohol withdrawal-induced negative affect in a voluntary drinking paradigm.

An additional limitation of these studies is that all of our experiments were conducted in C57BL/6 mice, which is the most common mouse strain used for studies of alcohol abuse due to their propensity to voluntarily consume alcohol (Crabbe et al., 2010; Hwa et al., 2011; Rhodes et al., 2007). The tradeoff is that the use of an inbred strain precludes examination of potential genetic influences, which are known to contribute to drinking behavior in humans (Enoch & Goldman, 2002; Goodwin, 1985; Ray et al., 2010; Verhulst et al., 2015). Of particular relevance are variations in genes related to GABA, CRF, NPY, endogenous opioids that are implicated in both alcohol consumption and the anxiolytic effects of alcohol and therefore likely contribute to the anxiogenic effects of withdrawal (Ciccocioppo, 2013; Froehlich et al., 2000; Putman et al., 2016; Radke et al., 2014). Given the abundance of literature indicating the importance of genetics in alcohol use disorders, it would be beneficial for future studies to include outbred rodent strains that have been selectively bred to be alcohol preferring such as AA, P, HAD, and sP rats (Colombo et al., 2006; Eriksson, 1968; Li et al., 1993; Murphy et al., 2002) and HAP mice (Grahame et al., 1999). These animals better reflect the genetic diversity present in the human population.

Another potential criticism of these studies is that the reported ‘age-dependent’ effects are attributable to higher levels of alcohol exposure resulting from the elevated

consumption typically observed in adolescent animals and humans. While this may admittedly be a contributing/exacerbating factor, it is certainly not the sole explanation. In fact, a significant portion of animal studies reporting similar adolescent findings such as resilience to acute withdrawal symptoms (Brasser & Spear, 2002; Doremus et al., 2005; Little et al., 1996; Varlinskaya & Spear, 2004), persistent behavioral and neurobiological dysregulation in adulthood (Carrara-Nascimento et al., 2011; Pascual et al., 2009; Sircar & Sircar, 2005), and excessive subsequent alcohol consumption (Pandey et al., 2015; Pascual et al., 2009) employ involuntary methods of administration (e.g. vapor inhalation, gavage, IP injection) that allow for strict control over dosing. Thus, consistent age-related differences in animals with equivalent alcohol exposure help to ameliorate concern over this potential confound.

A related consideration is that adolescent alcohol exposure could enhance metabolism of alcohol, as chronic consumption has been reported to increase alcohol metabolism (Cederbaum, 2012; Lieber & DeCarli, 1972; Lumeng & Li, 1986). Such metabolic tolerance could theoretically account for the higher alcohol consumption exhibited by adolescent-onset binge drinkers when they are tested in adulthood (Chapters 5 and 8). However, if the elevated consumption in adult animals with adolescent alcohol experience was solely due to increased metabolic 'fitness' (i.e. metabolic tolerance), this would not account for the ability of our H2 manipulation to bi-directionally modulate intake in experienced vs. inexperienced animals, as an intra-CEA infusion of H2 should have no direct effect on alcohol metabolism. Although there is evidence that adolescent animals may show enhanced alcohol elimination compared to adults (Walker & Ehlers, 2009), the higher consumption in adolescent animals in our study during the initial exposure period was correlated with higher resulting BACs even after 11

days of drinking. Additionally, other studies have shown that elevated alcohol consumption in adult animals with adolescent alcohol experience also corresponds with higher resulting BACs compared to control animals (Alaux-Cantin et al., 2013; Pascual et al., 2009), which provides further evidence that the elevated consumption is not merely a function of improved alcohol metabolism. Nonetheless, in the future studies, additional blood sampling would be prudent in order to monitor circulating alcohol levels and control for this potential source of variability.

Finally, many of the studies described in this dissertation did not probe adjacent control regions, as considerations related to time and financial expense compelled us to limit our focus specifically to regions of the extended amygdala implicated in emotion. Our objective was to determine the relevance of particular region-specific protein anomalies observed following a history of binge drinking that could be causally related to the manifestation of negative affect during withdrawal. However, future studies should include adjacent regions in order to evaluate the neuroanatomical selectivity of the reported alcohol-induced changes and experimental manipulations. These studies should also be extended to include females (which is the focus of current ongoing work in the laboratory), as females are known to differ in patterns of alcohol consumption, as well as susceptibility to mood disorders (Breslau et al., 1995; Nolen-Hoeksema, 2004; Thomasson, 1995; Zilberman et al., 2003).

General insight into affective regulation

By observing the effects of our manipulations in water control animals, these studies provided insight into the regulation of basal anxiety and depression. Interestingly, despite the

anxiolytic effects of systemic and intracranial MTEP observed in alcohol-withdrawn animals, there were minimal treatment effects overall in water control animals. In fact, the only treatment that showed an anxiolytic effect was the systemic administration of low-dose MTEP. These results are in line with many studies reporting minimal or absent anxiolytic treatment effects in non-anxious animals (Horváth et al., 2015; Landgraf & Wigger, 2002; Liebsch et al., 1998; Naslund et al., 2015). Based on these results, it would be interesting to further explore potential distinctions in the etiology of withdrawal-induced anxiety, compared to anxiety precipitated by other means (e.g. chronic stress, social defeat, stimulant withdrawal, etc.). For example, the results of our transgenic study (Chapter 8) demonstrated that both augmentation and depletion of Homer2 within the CeA is capable of producing similar behavioral symptomology, despite divergent etiology.

Additionally, none of the manipulations employed in the described experiments had any effect on the depression-like behavior observed in adolescent animals during protracted withdrawal, suggesting the existence of distinct circuitry/biochemistry mediating anxiety vs. depression during withdrawal. It has been shown that clinical anxiety and depression have distinct neurobiological profiles, despite overlapping symptomology (Kemp & Felmingham, 2008; Maletic et al., 2007; Martin et al., 2009). For example, depressed individuals show decreased left prefrontal EEG activity and anxious individuals show increased right frontal EEG activity, compared to health control subjects, and these two brain regions are involved in approach and withdrawal behaviors, respectively (Davidson et al., 2000; Debener et al., 2000; Diego et al., 2001; Mathersul et al., 2008). Functional brain imaging studies show distinct regional blood flow patterns, suggesting that major depression is associated with hyperactivity in the ventromedial and lateral orbital PFC and hypoactivity in the dorsolateral

PFC, compared to both anxious individuals and healthy controls (Drevets, 1998). Given these distinctions, it is plausible that the same is true of withdrawal-induced anxiety vs. depression. Disentangling the similarities and differences between uncomplicated clinical anxiety or depression versus withdrawal-induced negative affect could improve and accelerate treatment outcomes by directly addressing the specific underlying causes. A detailed understanding of the neurobiology of emotional regulation is critical for the development of relevant pharmacotherapies in service of more comprehensive treatment options for alcohol use disorders, anxiety, and depression.

Potential implications

Although the research presented in this dissertation was not designed specifically for translational/clinical applications, these findings may have implications for the treatment of substance abuse and/or mood disorders. The anxiolytic effects of systemic MTEP in Chapter 6 support the therapeutic potential of mGluR-modulating drugs. Indeed, mGluRs are already a target of interest for both addiction (Harris et al., 2002; Hovelso et al., 2012; Olive, 2009) and mood disorders (Harvey & Shahid, 2012; Palucha & Pilc, 2007; Swanson et al., 2005). In contrast to the numerous pharmacological tools available for targeting mGluRs, the manipulation of Homer proteins has typically relied on genetic and viral interventions and there are currently no known Homer-specific drugs. However, protein-protein interactions are a current target of interest for novel therapeutics due to reduced side-effect profiles compared to conventional receptor-targeting drugs (Klussmann et al., 2008) and could someday be used in the treatment of substance abuse and/or mood disorders.

There is a substantial unmet need for treatment of alcohol use disorders in the U.S., which is particularly prevalent among individuals with comorbid mood disorders (Davis et al., 2008; Kaufmann et al., 2014). This unmet need is even more profound in adolescents and young adults. One study found that between the years 2003 and 2010 approximately 90% of adolescents with an alcohol or illicit drug disorder did not receive treatment. The unmet need was greatest for alcohol use disorders specifically (Mericle et al., 2015).The results of our studies suggest that adolescents may differ from adults in both the symptomology and underlying neurobiology of alcohol withdrawal-related complications and, given the enduring changes associated with adolescent binge drinking, these individual may require longer treatment support and ongoing monitoring, compared to adults. Consideration should be given to subject factors of age, level of intake, and duration of drinking history when determining the most appropriate treatment approach to maximize prognosis.

Concluding remarks

In summary, the series of experiments presented in this dissertation provide insight into the underlying neurobiology involved in withdrawal-induced negative affect following a history of binge drinking and also identify adult vs. adolescent age differences therein. Our data complement existing literature showing alcohol-induced increases in glutamate-related protein expression within the AcbSh and extends these findings to implicate the AcbSh as a potential substrate of withdrawal-induced anxiety. Additionally, we characterized a novel role of Homer proteins within the CEA in the mediation of anxiety and alcohol consumption. Together, the results of these studies add additional dimension to our general understanding of the neurobiological and behavioral consequences of alcohol abuse.

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