ABSTRACT

Chronic Intermittent Ethanol Treatment Yields Persistent Increases in Anxiety and Receptor Subunit Changes in Adolescent and Adult Rats

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$GABA_A$ and NMDA receptors are involved in the behavioral effects of ethanol; however, the age-dependent molecular mechanisms associated with the effects of chronic ethanol have yet to be fully elucidated. Adolescence is marked by unique sensitivity to certain effects of ethanol, including distinct consumption patterns, increased prevalence of consumption during young adulthood compared to that of abstaining adolescents, and increased risk for development of future alcohol use disorders. In the adult, tolerance and dependence are marked by attenuated function of $GABA_A$ receptors and increased function of NMDA receptors, but the receptor subunit expression profiles for adolescents following binge-like ethanol exposure are not yet completely known. Since tolerance and withdrawal appear to be age dependent, it is likely that receptor subunit expression is differentially altered following chronic ethanol exposure in adolescence compared to adulthood. Additionally, chronic ethanol exposure and its withdrawal can alter behavior. Especially relevant to the maintenance and persistence of consumption behaviors are alterations in anxiety. Anxiety levels can often be used to predict relapse in detoxified

alcohol-dependent patients long after ethanol cessation, therefore it is important to determine the persistence of the anxiogenic effects of ethanol withdrawal and how alterations in receptor subunits may interact with withdrawal-induced anxiogenesis. Given that very little research has focused on age dependent anxiogenesis and subunit alterations following chronic ethanol consumption, this project will investigate multiple withdrawal induced changes in anxiety and its persistence in adolescent and adult rats. Additionally, the persistence of any changes in receptor subunit expression will be assessed using the same animals from the anxiety data. This multimodal, within-subjects design allows for the direct exploration of the relationship between behavioral and molecular alterations due to chronic ethanol exposure.

Chronic Intermittent Ethanol Treatment Yields Persistent Increases in Anxiety and Receptor Subunit Changes in Adolescent and Adult Rats

by

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The end of a thing is better than its beginning (Ecclesiastes 7:8a).

CHAPTER ONE

Introduction

Overview

Adolescence is marked by unique sensitivity to certain effects of ethanol, including reduced sensitivity to ethanol-induced motor impairments (Hefner & Holmes, 2007; Lisenbardt et al., 2009; Little et al., 1996; Pian et al., 2008; Ristuccia & Spear, 2008; Silveri & Spear, 2001; Spear & Varlinskaya, 2005 for review; Varlinskaya & Spear, 2002; White et al., 2002), which has biological roots in age-dependent neuronal electrophysiology and protein expression (e.g. Van Skike et al., 2010). Ethanol-induced motor impairments are used as feedback cues to attenuate ethanol consumption (Spear & Varlinskaya, 2005), and perhaps partially as a result of their altered sensitivity, adolescents and young adults show greater rates of binge and heavy drinking over non-binge consumption compared to adults over age 25 (SAMHSA 2011). Other factors contributing to the higher proportion of binge consumption during youth compared to adulthood include high sensation seeking (Sargent et al., 2010), peer influence, alcohol use expectancies, social facilitation, and social context (reviewed in Courtney & Polich, 2009). The many contributing factors indicate alcohol use during adolescence is a complex issue. Indeed, ethanol use among adolescents is a consistent and global problem, with 23.1% of American youths aged 12 to 17 reporting past month ethanol consumption in 2010 (SAMHSA 2011), which is comparable to rates reported in previous years. However, this number may be significantly underreported, as a study of over 10,000 students spanning 25 European countries found the number of students aged

15-16 reporting past month alcohol use to be 82% in 2007 (Hibell et al., 2009). Regardless of the overall drinking rates, over half of ethanol consumption in American underage drinkers is accounted for by binge and heavy binge drinking (SAMHSA 2011).

Binge Drinking

Binge drinking is more harmful to overall health compared to moderate drinking. For instance, weekend binge drinking elevates atherosclerotic plaque volume in the carotid artery of mice compared to daily moderate alcohol exposure, which lowers plaque volume even compared to non-alcohol receiving controls (Liu et al., 2011). Both binge and regular moderate alcohol consumption elevate plasma high-density lipoprotein (HDL). However, low-density lipoprotein (LDL) plasma level alterations are dependent on the pattern of consumption. LDL is decreased with regular moderate consumption, and elevated following binge drinking (Liu et al., 2011). This LDL elevation increases the risk of heart disease: a 10% increase in plasma LDL produces a 20% increased risk for atherosclerosis (Wood et al., 1998). Conversely, moderate alcohol consumption ranging from 0.5 to 2 standard drinks per day has consistently been reported to reduce coronary heart disease-related risks by 30-35% in humans compared to non-drinkers (for review see Krenz & Korthuis, 2012). Another health-related factor is body mass index (BMI), which is positively correlated with number or alcoholic drinks per drinking day. Participants reporting less frequent drinking days, perhaps indicative of binge-pattern consumption, had the highest BMIs (Breslow & Smothers, 2005). Additionally, mice that were exposed to binge ethanol weighed more than mice that received daily ethanol, even though both groups received the same number of drinks per week (Liu et al., 2011). Binge drinking also produces neuropsychological deficits compared to moderate

consumption. Participants who consumed alcohol in binges had greater acute functional tolerance, or tolerance that develops within a single drinking episode, to ethanol-induced motor impairments. In contrast, participants who were moderate drinkers did not regain motor precision during the ethanol challenge. Neither group developed acute functional tolerance to inhibitory control, suggesting that people who binge drink recover their ability to execute behaviors while intoxicated, but do not recover the ability to inhibit behavior. This may account for the continued ethanol consumption, while intoxicated, among people who engage in binge drinking (Fillmore & Weafer, 2012). Finally, binge drinking is also more detrimental than continuous ethanol exposure, increasing the propensity for future ethanol consumption (Lopez & Becker, 2005) and diminishing ethanol-induced taste aversion (Diaz-Granados & Graham, 2007) more quickly in mice exposed to binge ethanol. From these studies, it is obvious that binge alcohol consumption is more damaging to overall health compared to any other pattern of consumption and abstinence.

Binge pattern drinking is most prevalent during adolescence and young adulthood (SAMHSA, 2011), which presents several other concerns, as adolescents seem to be more vulnerable to developing future alcohol use disorders even when compared to younger adults. A young age at initial intoxication is associated with future risky drinking behaviors and alcohol use problems and disorders (Adam et al., 2011; Ehlers et al., 2006; Hingson et al., 2006). While the rates of alcohol consumption do decline with increasing age (SAMHSA, 2011), one cannot assume that adolescents with drinking problems will simply "age out" of problematic drinking behaviors, as many studies have uncovered significant associations of early onset drinking and subsequent heavy alcohol

use well into adulthood (Casswell et al., 2002; Muthen & Muthen, 2000; Pitkanen et al., 2005). Specifically, a 15- year longitudinal study of young Australians reveals the rate of binge drinking during young adulthood is higher among those who engaged in adolescent-onset binge drinking compared to those who abstained from binge drinking during adolescence (Degenhardt et al., 2013). Additionally, animal models reveal that ethanol exposure during the peri-adolescent period results in persistent neurobehavioral changes that mitigate conditioned aversion to ethanol in adulthood (Diaz-Granados & Graham, 2007). This suggests there is something in the biology of the adolescent that makes this age group more vulnerable to developing alcohol use disorders. Therefore, the current study will use chronic intermittent ethanol (CIE) exposure, which utilizes multiple periods of intoxication and withdrawal, to model binge drinking in adolescent and adult rats in an attempt to elucidate the age-dependent risk in developing future alcohol use disorder.

The age-dependent risk in developing alcohol problems may have an ontogenetic basis. Significant neural alterations are occurring during adolescence, including myelination of axons, synaptic pruning (Sowell et al., 2001; Sowell et al., 2004), and modification of neurotransmitter systems (Insel et al., 1990; McDonald et al., 1990; Yu et al., 2006). Specifically, in adolescent rats, there is a transient overproduction of NMDARs in the cortex and hippocampus (Insel et al., 1990; McDonald et al., 1990), which may make adolescents more vulnerable to excitotoxic insults within the same brain regions (Johnston, 1995), such as that produced by repeated ethanol exposure and withdrawal (Chandler et al., 1993; Smothers et al., 1997). Additionally, $GABA_A$ receptor subunits, which are altered due to chronic alcohol use (Grobin et al., 1998; Kang et al.,

1998; Kang et al., 1996; Morrow, 1995), are differentially expressed throughout adolescence compared to adulthood (Yu et al., 2006). While the exact mechanism underlying these persistent changes in behavior and function of the central nervous system is not yet fully understood, much evidence implicates the involvement of NMDA and $GABA_A$ receptors. Therefore, this study will quantify the changes produced by CIE exposure and variable withdrawal time points in adolescent and adult rats to help uncover part of the molecular mechanism underlying the youth-dependent increased risk of developing future alcohol use disorders. Additionally, receptor subunit expression and multiple withdrawal induced anxiety will be collected from the same animal, allowing for the direct exploration of a potential link between receptor subunit changes and behavior.

Anxiety and Alcohol Use

Chronic ethanol yields hyperexcitability of the central nervous system during withdrawal and abstinence, which has several behavioral consequences, including increased risk for withdrawal seizures (Lopez et al., 2011) and increased anxiety (Gatch & Lal, 2001; Grobin et al., 1998; reviewed in Kumar et al., 2009; Noone et al., 1999; Roberts et al., 1999; Willinger et al., 2002). From a recovery standpoint, anxiety is among the most troublesome withdrawal symptoms as it is associated with relapse (Fox et al., 2007; Noone et al., 1999; Roberts et al., 1999; Willinger et al., 2002). For instance, patients with alcoholism are approximately three times more likely to report drinking to terminate feelings of anxiety or depression than drinking to alleviate physical withdrawal symptoms (Hershon, 1977). Additionally, anxiety and alcohol use disorders are highly comorbid (Grant et al., 2004; Marquenie et al., 2007), and patients dually diagnosed with alcoholism and anxiety disorders experience more severe alcohol withdrawal symptoms

(Johnston et al., 1991). One of the primary aims of the proposed study is to assess the development of anxiety associated with chronic intermittent ethanol (CIE) withdrawal in adolescent and adult rats and correlate such anxiety development to receptor subunit alterations within subjects.

In preclinical models the quantification of anxiety requires the use of behavioral analysis. A standard protocol used to measure anxiety in rodents is the elevated plus maze (Pellow et al., 1985). The apparatus is elevated above the ground and consists of 4 arms of equal length that intersect at right angles. Two of the opposing arms are enclosed by opaque walls, while the other two opposing arms are open. The ratio of entries to and time spent in open and closed arms provides the index for anxiety: rats with higher anxiety show a greater preference for the closed arms (Landgraf & Wigger, 2002; Leite & Nobre, 2012; Pellow & File, 1986).

Animal models also mirror the comorbidity between anxiety and alcohol use in humans. In rats selectively bred to exhibit high or low anxiety-related behaviors, rats in ethanol withdrawal increase ethanol intake. However, high-anxiety rats increased their intake over three times as much compared to the low-anxiety rats (Leite & Nobre, 2012). This effect can be partially prevented when given an NMDAR antagonist (Leite & Nobre, 2012), suggesting NMDA plays a critical role in conferring withdrawal-induced anxiety. In light of this research, all NMDAR subunits of interest in the hippocampus and cortex are hypothesized to be positively correlated with increased anxiety-like behavior. Most relevant to this project, the anxiogenic effects of chronic ethanol withdrawal have been shown in animal models using the elevated plus maze (Baldwin et al., 1991; File, 1994; Gatch et al., 1999; Zhang et al., 2007; Zhao et al., 2011). We predict that CIE will result

in a persistent increase of anxiety-like behavior in rats on the elevated plus maze at both 24 hours and 12 days after withdrawal (Noone et al., 1999; Valdez et al., 2002). However, this finding may be attenuated in adolescent rats (Doremus-Fitzwater & Spear, 2007).

Additionally, $GABA_AR$ α1 subunit appears to be important in modifying central nervous system hyperexcitability (Kralic et al., 2002; Werner et al., 2009). Cortical expression of $GABA_AR$ α1 is decreased following chronic ethanol exposure (Devaud et al., 1997; Matthews et al., 1998); therefore, we predict $GABA_AR$ α1 will be inversely correlated with anxiety. The $GABA_AR$ α4 subunit does not appear to be important in determining anxiety levels, as α4 knock-out mice show no baseline or ethanol-induced behavioral differences on the elevated plus maze compared to wild types (Chandra et al., 2008). Therefore the potential positive correlation between anxiety and cortical $GABA_AR$ α4 subunit expression after chronic ethanol exposure is exploratory and its importance is likely minimal. The $GABA_AR$ β2,3 subunit may play a role in withdrawal seizure susceptibility (Blednov et al., 2003b) and manifestation of withdrawal symptoms (Sanchis-Segura et al., 2007). Since this receptor subunit is upregulated in the cortex, but not hippocampus, following chronic ethanol exposure, we predict the $GABA_AR$ β2,3 subunit will positively correlate with anxiety. These subunit alterations underlie the increased NMDAR and decreased $GABA_AR$ function following chronic alcohol exposure, which plays a role in the development of ethanol dependence and manifestation of withdrawal.

Indeed, the glutamatergic and GABAergic neurotransmitter systems regulate many of the same behavioral dimensions involved in anxiety disorders (Millan, 2003),

and both of these systems are modified following chronic ethanol exposure. Over 70 years ago, C. K. Himmelsbach postulated that physiological mechanisms in the brain and body are striving to maintain homeostasis, and these mechanisms are responsible for drug tolerance, and also withdrawal symptoms in the absence of the drug (Himmelsbach, 1941). This concept of drug-induced neuroadaptation provides a simple schematic to understand basic alterations in receptor function that underlies ethanol dependence. For instance, acute ethanol inhibits NMDAR function (Lovinger et al., 1989; White et al., 1990), while chronic exposure increases receptor function (Grant et al., 1990; Krystal et al., 1998; Sanna et al., 1993). Also in accordance with Himmelsbach's theory, $GABA_A$ receptor function is enhanced following acute ethanol (Kumar, 2009; Lovinger, 1997) and attenuated after chronic exposure (Grobin et al,. 1998; Kang et al., 1998; Kang et al., 1996; Morrow, 1995). Although not the only neurotransmitter systems altered by chronic ethanol, much research has focused on the amino acid transmitter systems and their role in behavioral tolerance and dependence.

The NMDAR is a glutamatergic, ligand gated ion channel complex that is highly permeable to calcium ions, as well as other monovalent cations like sodium and potassium. Acute ethanol inhibits NMDAR-mediated Ca2+ currents (Lovinger et al., 1989; White et al., 1990) by decreasing the open channel probability and mean open time of the NMDAR (Wright et al., 1996). Perhaps as a compensatory mechanism of regulation, chronic ethanol exposure increases receptor function (Grant et al., 1990; Krystal et al., 1998; Sanna et al., 1993) as well as the density of NMDAR binding sites (Gulya et al., 1991; Hu & Ticku, 1995; Snell et al., 1991). Additionally, withdrawal from chronic ethanol exposure increases synaptic NMDAR expression and activation

(Hendrickson et al., 2007), which can lead to neurotoxicity of the hippocampus and cortex, as well as seizures, upon ethanol withdrawal (Chandler et al., 1993; Davidson et al., 1995; Grant et al., 1990; Hoffman, 1995; Thomas & Morrisett 2000).

NMDA Receptors and Ethanol

The chronic ethanol induced increase in NMDAR function is presumably due to differential upregulation of NMDAR subunit protein levels (Follesa & Ticku, 1995; Kalluri et al., 1998). Seven NMDAR subunits have been identified: NR1, NR2A-D, and NR3A&B, which assemble primarily as tetraheteromeric complexes from two NR1 subunits with either two NR2 subunits or a combination of NR2 and NR3 subunits (reviewed in Traynelis et al., 2010). The NMDAR NR1 subunit is widely expressed and is required for the functional expression of NR2-containing NMDARs (Du et al., 2011). Of the NR2 subunits, NR2A- and NR2B- containing receptors are preferentially sensitive to ethanol inhibition compared to NR2C- and NR2D- containing NMDARs (Masood et al., 1994). Additionally, receptor subunit composition determines the channel kinetics of the receptor. For instance, NR2A- and NR2B- containing receptors generate ion channels permeable to calcium ions, while NR2C- and NR2D- containing receptors form ion channels with preferential permeability for sodium ions (Perez-Otano et al., 2001). In light of this research, NMDAR NR1, NR2A, and NR2B subunits appear to be the most important in conferring increased NMDAR function after chronic ethanol exposure.

Chronic ethanol exposure transiently elevates NR2A and NR2B subunit mRNA levels in hippocampus and cortex (Follesa & Ticku, 1995), and also transiently increases the protein expression of subunits NR1, NR2A, and NR2B in rat cortex and hippocampus (Kalluri et al., 1998). However, withdrawal seems to be an important factor in

determining subunit alterations, as studies that employ withdrawal (i.e. CIE exposure) have divergent findings (Nagy & Laszlo, 2002; Pian et al., 2010). To date, there are relatively few studies that address the effects of CIE exposure on NMDAR subunit alterations, especially during different periods of development. More ontogenetic studies are needed to determine how age-dependent subunit regulation produced by CIE exposure affects behavior, and to determine if CIE-induced alterations are reversible. For instance, increased NMDA NR1, NR2A, and NR2B subunit expression in the cortex and hippocampus is reversible in adult rats (Kalluri et al., 1998; Pian et al., 2010). However, in adolescent rats, withdrawal from CIE vapor exposure leaves NR1 and NR2A subunit expression elevated in the hippocampus (Pian et al., 2010). This may suggest the adolescent hippocampus is particularly vulnerable to persistent ethanol-induced molecular (Pian et al., 2010), cellular (Fleming et al., 2012), and certain behavioral (Kuzmin et al., 2012) alterations. One aim of the proposed study is to uncover the age-dependent effects of CIE withdrawal on NMDAR subunit expression, as well as the age-dependent reversibility of these alterations after a period of abstinence. Additionally, receptor changes will be correlated with a withdrawal-related behavioral measure at both time points to better understand how the receptor subunit alterations translate into altered anxiety levels.

NMDAR Subunit-Specific Predictions

The NMDAR NR1 subunit is widely expressed and is required for the functional expression of NR2-containing NMDARs (Du et al., 2011). NMDAR NR1 heterozygous mice exhibit increased ethanol consumption and reduced withdrawal severity compared to wildtypes. However these findings are slightly confounded by the concurrent

alteration of NMDAR NR2 subunit expression (Du et al., 2011). Several studies have found that chronic ethanol exposure increases both mRNA (Follesa & Ticku, 1995) and protein expression (Kalluri et al., 1998) of the NMDAR NR1 subunit, but recent data suggests that subunit alterations may be dependent on the ethanol administration pattern and time since the last ethanol administration (Pian et al., 2010). Not all research findings are consistent; however, we predict that in adult rats, CIE will result in increased NR1 expression in the hippocampus and cortex because NMDAR function is conferred by upregulation of NMDAR subunits and NR1 protein expression has been found to be increased in the same brain regions following chronic ethanol exposure (Kalluri et al., 1998), we predict that in adult rats, CIE will result in increased NMDAR NR1 expression in the hippocampus and cortex. Consistent with previous research, NMDAR NR1 subunit expression is predicted to return to control levels after a period of abstinence (Kalluri et al., 1998). Behaviorally, NMDAR NR1 subunit expression may be important in determining ethanol consumption preference (Du et al., 2011) and adolescent rats voluntarily consume more ethanol than adults (Doremus et al., 2005). Therefore, we predict CIE will have a greater impact on NMDAR NR1 subunit upregulation in the cortex and hippocampus of adolescent rats. Additionally, chronic alcohol use during adolescence yields an age-specific vulnerability to persistent changes in ethanol-related responses (Diaz-Granados & Graham, 2007), especially to subtle alterations within the hippocampus (Fleming et al., 2011; Kuzmin et al., 2012; Pian et al., 2010). In light of this research, we predict the upregulation of NMDAR NR1 subunit will be persistent in the cortex and hippocampus of adolescent rats.

NMDAR NR2A subunit expression is transiently increased in the hippocampus and cortex following chronic ethanol consumption (Kalluri et al., 1998; Sultana & Babu, 2003). Based on this research, we hypothesize CIE exposure in adult rats will transiently increase NMDAR NR2A expression in the hippocampus and cortex. NMDAR NR2A knockout mice display greater ethanol-induced motor impairments and abnormal ethanol-induced conditioned place preference (Boyce-Rustay & Holmes, 2006). Regarding the former finding, adolescents are less sensitive to motor impairments (Spear & Varlinskaya, 2005), so we predict CIE administration in adolescent rats will have a greater impact on cortical upregulation of the NMDAR NR2A subunit. Adolescent rats also exhibit differential sensitivity to ethanol-related reward (Boyce-Rustay & Holmes, 2006; Dickinson et al., 2009), as induction of conditioned place preference to ethanol requires a higher dose for adolescent mice compared to adults (Dickinson et al., 2009). Hippocampal NMDA receptors are involved in drug-induced conditioned place learning (Keleta & Martinez, 2012). Therefore, CIE administration might have less of an impact on hippocampal upregulation of NMDAR NR2A subunit in adolescent rats. Based on research showing adolescents have a greater vulnerability for the development of future alcohol use problems, we predict these NMDAR NR2A subunit alterations will be persistent after a period of abstinence.

The NMDAR NR2B subunit has received much attention regarding ethanol dependence. For example, subunit selective antagonists, which inhibit the neurotoxic effects (Nagy et al., 2004) and seizure susceptibility (Malinowska et al., 1999) associated with alcohol withdrawal, and are being considered in the treatment of alcohol dependence (Nagy, 2004). The expression of this subunit is increased in the hippocampus and cortex

following chronic ethanol exposure (Kalluri et al., 1998), therefore we hypothesize that CIE exposure in adult rats will increase NMDAR NR2B expression in the hippocampus and cortex, and this effect will be reversible. An NR2B subunit selective antagonist attenuates ethanol-induced deficits in balance, hyperactivity, and spatial retention (Lewis et al., 2011). This suggests the NMDAR NR2B subunit may be important in conferring ethanol-induced behavioral deficits. Adolescent rats are less sensitive to ethanol-induced motor impairments (Spear & Varlinskaya, 2005), so CIE administration is hypothesized to have an attenuated impact on NMDAR NR2B subunit expression in adolescent rats. Our lab has found no age-dependent differences in spatial memory impairments (Chin et al., 2011; Novier et al., 2012), we hypothesize CIE exposure will upregulate hippocampal NMDAR NR2B expression similarly in adolescent and adult rats. However, research suggests chronic ethanol exposure persistently alters certain aspects of hippocampal function (Fleming et al., 2011; Kuzmin et al., 2012; Pian et al., 2010). We hypothesize altered NMDAR NR2B expression in adolescent rats will persist following a period of abstinence.

$GABA_A$Rs and Ethanol

The other amino acid transmitter system that has been implicated in ethanol-related behavioral tolerance and dependence is GABA. Chronic alcohol exposure alters GABAergic neurotransmission, especially through the $GABA_A$ receptor. The $GABA_A$ receptor has 19 different subunits: α1-6, β1-4, γ1-3, δ, ε, θ, π, and ρ1-3 and assembles as a heteropentamer (Nestler et al., 2009) with a central chloride ion pore (Nestler et al., 2009). Acute alcohol positively modulates $GABA_A$ receptor function (reviewed in Kumar, 2009; Lovinger, 1997). Conversely, tolerance and dependence in the adult are

marked by attenuated function of $GABA_A$ receptors (Grobin et al., 1998; Kang et al., 1998; Kang et al., 1996; Morrow, 1995), which is accompanied by altered $GABA_A$ receptor subunit protein expression (Devaud et al., 1997; Matthews et al., 1998). However, there is a dearth of research regarding $GABA_AR$ subunit expression alterations at other developmental time points. One aim of the proposed study is to uncover the age-dependent effects of CIE withdrawal on $GABA_AR$ subunit expression, as well as the age-dependent reversibility of these alterations after a period of abstinence. Additionally, receptor changes will be correlated with a withdrawal-related behavioral measure at both time points to better understand how the receptor subunit alterations translate into altered ethanol withdrawal symptoms.

$GABA_AR$ Subunits

The $GABA_AR$ β2,3 subunit is ubiquitously expressed throughout the brain; however, its significance is currently unknown. The $GABA_AR$ β2 subunit may affect allosteric modulation of $GABA_A$ receptor function and may contain a residue, Asn 265, which contributes to an intrasubunit alcohol binding site (McCracken et al., 2010). Additionally, $GABA_AR$ β2 may play a role in determining withdrawal seizure susceptibility, as $GABA_AR$ β2 null mutant mice had higher withdrawal scores compared to wild-type mice (Blednov et al., 2003b). The $GABA_AR$ β3 subunit may play a role in chronic tolerance, with knock-in mice having enhanced tolerance to the sedative and withdrawal effects of ethanol (Sanchis-Segura et al., 2007). A clear role for this subunit and the functional importance of ethanol-induced changes in expression is currently lacking. However, previous research indicates $GABA_AR$ β2,3 expression is increased in

the cortex (Devaud et al., 1997), but unchanged in the hippocampus (Matthews et al., 1998).

The $GABA_AR$ α1 subunit peptide is the most abundant α subunit in the adult brain and is expressed in approximately 50% of all $GABA_A$ receptors (Duggan & Stephenson, 1990). Receptors containing this subunit typically contribute to synaptic-mediated phasic inhibition (Olsen & Sieghart, 2009). Behaviorally, $GABA_AR$ α1 subunit expression has been implicated in contributing to the locomotor-stimulating (June et al., 2007) and sedative/hypnotic (Blednov et al., 2003a) effects of ethanol, such that $GABA_AR$ α1 expression positively correlates with ethanol-induced sedation (Blednov et al., 2003a). Since repeated exposure yields tolerance to the sedative/hypnotic effects of alcohol (Silvers et al., 2003), it follows that chronic alcohol results in a decrease in α1 subunit expression within the cortex (Devaud et al., 1997; Matthews et al., 1998). However, there is no change in hippocampal expression of $GABA_AR$ α1 (Matthews et al., 1998), which is seemingly intuitive since this subunit appears to be involved in determining motor impairments.

$GABA_A$ receptors can also be located extrasynaptically, mediating tonic inhibition. In extrasynaptic $GABA_A$ receptors, $GABA_AR$ α4 is the predominant subunit that is expressed (Olsen & Sieghart, 2009). These receptors are touted to be highly sensitive to ethanol, mediating the intoxication produced by low doses of ethanol (Wallner et al., 2006). This subunit has been implicated in conferring ethanol consumption and preference, as reduction of $GABA_A$ α4-containing receptors by RNA interference decreases these behaviors (Rewal et al., 2009). Chronic ethanol exposure increases $GABA_AR$ α4 expression in the cortex (Matthews et al., 1998) and hippocampus

(Mahmoudi et al., 1997), but only after long periods of exposure within hippocampus (Matthews et al., 1998).

$GABA_AR$ Subunit-Specific Hypotheses

These effects on chronic ethanol induced alterations of $GABA_A$ subunit expression in adults have been well documented; however, it does not appear that any investigations thus far have considered the potential age-dependent alterations of $GABA_A$ receptor subunits. Both behavioral tolerance (Broadwater et al., 2011; Varlinskaya & Spear, 2007) and withdrawal (Acheson et al., 1999; Chung et al., 2008; Clark et al., 2002) resulting from chronic ethanol exposure have been found to be age-dependent. Since tolerance and dependence are marked by changes in the function (Grobin et al., 1998; Kang et al., 1998; Kang et al., 1996; Morrow, 1995) and subunit expression (Devaud et al., 1997; Mahmoudi et al., 1997; Matthews et al., 1998) of $GABA_A$ receptors, it is therefore likely that there would be age-dependent subunit alterations produced by chronic intermittent ethanol exposure.

The role of the $GABA_AR$ β2,3 subunit in behavior is not well known; however, expression is ubiquitous and does not appear to be age-dependent (Fritschy et al., 1994). Based on previous research in adults, we hypothesize that $GABA_AR$ β2,3 subunit expression will increase in the cortex (Devaud et al., 1997) but remain unchanged in the hippocampus of adult rats (Matthews et al., 1998). $GABA_AR$ β2,3 subunit may play a role in withdrawal seizure susceptibility (Blednov et al., 2003b). Seizure susceptibility has been found to be age-dependent; however the results are inconclusive as adolescents have been found to be both more (Chung et al., 2008) and less (Acheson et al., 1999)

sensitive to ethanol withdrawal seizures. Therefore it is difficult to make age-specific predictions regarding $GABA_AR$ β2,3 subunit expression based on the behavioral findings.

In the adult, $GABA_AR$ α1 expression is hypothesized to decrease in the cortex, but not hippocampus (Devaud et al., 1997; Matthews et al., 1998). Since $GABA_AR$ α1 expression is associated with ethanol-induced sedation (Blednov et al., 2003a) and adolescents are inherently less sensitive to this effect (Spear & Varlinskaya et al., 2005; Van Skike et al., 2010), adolescents may have further reduced α1 expression compared to adults within the cortex, but not hippocampus, which is also likely reversible. Compared to adults, adolescents have an attenuated development of chronic tolerance to ethanol-induced sedation; therefore there will likely be an interaction of age and CIE exposure, such that the reduction of $GABA_AR$ α1 expression in adult cortex will be greater than the decrease seen in adolescent rats. Previous research in our lab demonstrates tolerance to the sedative effects of ethanol are persistent (Silvers et al., 2003), so we predict $GABA_AR$ α1 subunit changes will persist after a period of abstinence in adolescent and adult rats.

Finally, consistent with previous research, we predict CIE will increase $GABA_AR$ α4 subunit expression in the adult cortex (Matthews et al., 1998). The α4 subunit is positively correlated with ethanol consumption (Rewal et al., 2009). Adolescents voluntarily consume more ethanol compared to adults (Doremus et al., 2005; Walker et al., 2008), so adolescents may have increased cortical α4 expression compared to adults. We hypothesize these effects will be long lasting, especially in adolescent rats. For instance, alcohol use during adolescence increases future risk for alcohol use problems, more so than when the first exposure occurs during adulthood (Adam et al., 2011; Ehlers et al., 2006; Hingson et al., 2006), and previous work from our lab shows ethanol

exposure during peri-adolescence attenuates the aversive properties of alcohol into adulthood (Diaz-Granados & Graham, 2007). Hippocampal expression of $GABA_AR$ α4 subunit is also upregulated (Mahmoudi et al., 1997), but only after long periods of exposure (Matthews et al., 1998). Therefore, we predict that hippocampal $GABA_AR$ α4 subunit expression in adults will be comparable to control levels in this study. Because low-dose ethanol was found to produce no functional age-dependent spatial memory impairments (Novier et al., 2012) and α4-containing $GABA_A$ receptors mediate the behavioral actions of low-dose ethanol (Wallner et al., 2006), $GABA_AR$ α4 expression in the hippocampus is predicted to be similar in adolescent and adult rats. This subunit is not expected to change following a period of abstinence.

In sum, adolescence is characterized by unique sensitivities to the effects of acute ethanol, which may help to permit the preference for binge ethanol consumption during this developmental period. Additionally, this age group is especially vulnerable to developing future alcohol use disorders, which may result from differential regulation of the amino acid neurotransmitter systems. Although chronic ethanol induced NMDA and $GABA_A$ receptor subunit alterations have been well documented in adult rats, there is very little information regarding age-dependent subunit regulation. The differential regulation of these neurotransmitter systems may have significant implications related to the age-dependent variability associated with chronic ethanol exposure, such as differential development of behavioral tolerance manifestation of alcohol withdrawal symptoms. Some of these effects might be more persistent in adolescent rats, which may help to explain the enhanced susceptibility of adolescents in developing future alcohol use disorders.

CHAPTER TWO

Methods

Animals

Twenty-four adolescent (PD 28 on arrival) and 24 adult (PD 118 on arrival) male Sprague-Dawley rats obtained from Harlan (Indianapolis, IN) were pair-housed in an IACUC-approved animal colony maintained on a 12:12 light/dark cycle (lights on 7:00 to 19:00) at Baylor University. Animals were given two days to acclimate before any experimental procedures begin. All animals in the chronic intermittent ethanol (CIE) exposure group (see below) received *ad libitum* access to food and water throughout all experimental procedures; however, the weight of the control groups were yoked to the weight of the age-appropriate CIE group to control for any potential nutritional deficiencies due to CIE-inhibited weight gain (Silvers et al., 2003). Repeated two-way ANOVAs (CI treatment x day) were performed separately for each age group to assess the effectiveness of weight-yoking. All procedures were approved by the Institutional Animal Care and Use Committee of Baylor University.

Chronic Intermittent Ethanol Exposure and Withdrawal Conditions

Adolescent and adult animals were randomly assigned to receive either chronic intermittent ethanol (CIE) or the control treatment of chronic intermittent saline (CIS). Chronic treatment spanned PD 30-48 for adolescent rats or PD 120-138 for adult rats. During this time, 4.0 g/kg 20% w/v ethanol or equivalent volume saline was delivered via intraperitoneal (i.p.) injection every 48 hours for 20 days, for a total of 10 intoxications and withdrawals.

After this treatment, animals in the CIE and CIS conditions were further divided into ethanol withdrawn (24 hours after final CIE/CIS exposure, n=6 per condition [age x CI treatment]) and abstinence (12 days after final CIE/CIS exposure, n=6 per condition [age x CI treatment]) conditions (Silvers et al., 2003). These time conditions determined when behavioral testing and tissue harvesting occurred.

Blood Ethanol Concentration

During chronic intermittent ethanol exposure blood ethanol concentrations (BECs) were determined 30 minutes after i.p. injection on the first, fifth, and tenth ethanol administration. To control for restraint and procedural induced stressors, all rats, both CIE- and CIS-treated, were restrained in a clear Plexiglas tube, the tip of the tail was nicked, and 50 μL of blood was collected. Since CIS-treatment was predicted *a priori* to produce a BEC of 0 and was included as a sham control procedure, only blood from the CIE-treated rats was analyzed with the Analox AM1 Alcohol Analyser protocol. To assess the development of tolerance in adolescent and adult rats, BEC values were analyzed with a repeated measures ANOVAs.

Anxiety Assessment: Elevated Plus Maze

Anxiogenesis associated with chronic intermittent ethanol administration in both ethanol withdrawn and abstinent animals was measured by the elevated plus maze. The test was conducted under dim lighting conditions, with open arms measuring 4 lux and closed arms measuring 2 lux. The maze was elevated approximately 50 cm from the ground, and consists of four arms 50 cm in length and 11 cm wide arranged at right angles. The closed arms, on opposing sides of the maze, have opaque walls 40 cm high that extend the length of the arm.

Animals were transported and given 30 minutes to acclimate in the adjacent staging room prior to testing. At the beginning of an anxiety test, the animal was placed in the central arena facing the northern open arm, and explored freely for 5 minutes without the researcher present. Each session was recorded using a ceiling-mounted camera onto VHS tape. The tape was subsequently scored and animals were considered to have made entry into an arm when all four paws were on that arm. A stopwatch was used to record the time spent in the open and closed arms and the total number of entries was also recorded. Percentage of time spent in the open or closed arms was quantified by taking the target arm and dividing by time spent on open plus closed arms, excluding time spent in the central arena. Separate two-way ANOVAs (CI treatment x withdrawal condition) for adolescent and adult animals were used to analyze the number of entries and time spent in the open and closed arms.

Protein Detection: Western Blotting.

Tissue Collection

Immediately following the EPM trial, each animal was taken to a different room and sacrificed via unanaesthetized rapid decapitation. Whole cortex and bilateral hippocampus were rapidly dissected over ice, transported in dry ice, and stored at -80ºC until assayed

Tissue Preparation

P2 fractions of the individual brain regions of individual animals were prepared by homogenizing the tissue sample in 0.32 M sucrose in phosphate-buffered saline (PBS), followed by centrifugation at 1,000 x g for 10 minutes. The resulting supernatant

was centrifuged again at 12,000 x g for 20 minutes. This pellet (P2) was resuspended in PBS and stored at -80ºC.

Western Blot Analysis

Each P2 fraction was assessed with a Bradford assay to determine protein concentration. Equivalent amounts of protein (20 µg) were loaded into Tris-Glycine gels (8% to 16%), counterbalanced across conditions. Proteins were separated by SDS–PAGE and electroblotted to polyvinylidene difluoride (PVDF) membranes (Life Technologies, Carlsbad, CA). The membranes were then targeted with one of the following antibody combinations diluted in blocking buffer and applied over 3 separate days (50 ml PBS, 25 µL Tween-20, 0.5 g milk powder): 1.) $GABA_A$ α1 (Millipore AB5946, 1:1000 dilution), β-Actin (Millipore MAB1501, 1:7500), and NMDA NR2A (Millipore 07-632, 1:500), 2.) $GABA_A$ β2,3 (Millipore MAB341, 1:500), β-Actin, and NMDA NR2B (Millipore 06-600, 1:1000), 3.) NMDA NR1 (Millipore 05-432, 1:500), β-Actin, and $GABA_A$ α4 (Abcam AB117080, 1:500). The appropriate horseradish peroxidase (HRP)-conjugated secondary antibody targeted against either a rabbit (Millipore AP132P, 1:7,500-1:10,000), mouse (Santa Cruz Biotechnology sc-2005, 1:5,000-1:7,500), or goat (Abcam AB97110, 1:7,500) host was applied and peptide labeling was detected with Thermo Scientific SuperSignal West Pico Chemiluminescent Substrate and exposed to X-ray film under nonsaturating conditions. Densitometric measurements were made within blots with NIH Image J software. All measurements were normalized to β-Actin expression within blots to verify equivalent protein loading and transfer. Blots were not stripped between antibody applications; however, the

application order minimized any ambiguity that might have arisen from any residual expression.

Alterations in peptide expression was assessed by conducting separate age-dependent two-way repeated ANOVAs (CI treatment x withdrawal condition) for all six of the different subunits of interest for each of the two brain regions.

Correlation between Anxiety and Subunit Expression.

Since anxiety and subunit expression data were collected from the same animal, we correlated anxiety scores (percent closed time on the EPM) with peptide expression changes (optical density normalized to β-Actin) using separate Pearson correlations for each subunit of interest.

CHAPTER THREE

Results

Weight Yoking

Food restriction was effective in matching the weights of the adolescent rats receiving control CIS treatment with adolescent rats that received CIE (F (1, 22) = 0.48, p = 0.50, n.s., Figure 1). However, it is important to note that all adolescents gained weight throughout the chronic intermittent administration procedure (F (9, 22) = 323.30, p < 0.001, Figure 1).

Similarly in adults, food restriction was effective in yoking the weights of rats receiving CIS with those receiving CIE (F (1, 22) = 0.51, p = 0.48, n.s., Figure 1). However, unlike adolescent animals, adult rats in both groups lost weight throughout the chronic intermittent administration procedure (F (1, 22) = 51.36, p < 0.001, Figure 1).

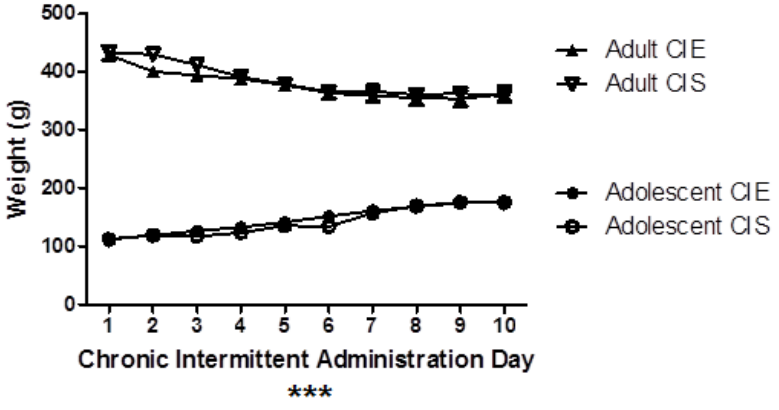

Figure 1. The weights of age-matched control animals were successfully yoked to the treatment group through food restriction. Error bars denote SEM, and are included but are often within the size of the marker. *** denotes p < 0.001.

CIE Yields Progressively Reduced BECs to Subsequent Ethanol

During chronic intermittent ethanol exposure blood ethanol concentrations (BECs) were determined 30 minutes after CIE exposure on administration days 1, 5, and 10. Both adolescent and adult rats developed tolerance to subsequent ethanol administrations, as indicated by reduced blood ethanol concentrations throughout chronic treatment (F (2, 22) = 9.46, $p < 0.001$, Figure 2). Adolescent and adult rats developed tolerance at a similar rate, as there was no evidence of any interaction between age and administration day (F (1, 22) = 0.30, $p = 0.59$, n.s., Figure 2).

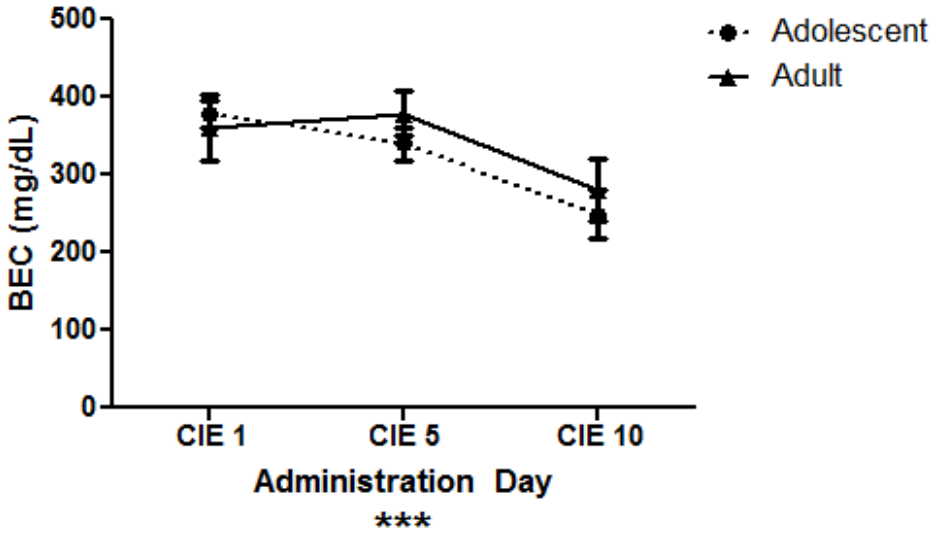

Figure 2. Adolescent and adult rats developed tolerance to subsequent ethanol administrations at similar rates. Error bars denote SEM. *** indicates p < 0.001.

CIE Produces Persistent Anxiogenesis in Adolescent and Adult Rats

In adolescent rats CIE treatment and increasing time past withdrawal was anxiogenic. The percentage of time spent on the open arms of the EPM was decreased by CIE treatment (F (1, 20) = 4.34, $p = 0.05$; Figure 3A) and time post-withdrawal (F (1, 20) = 9.77, $p < 0.01$; Figure 3A). Similarly, the percentage of time spent on the closed arms

of the EPM was increased by CIE treatment (F (1, 20) = 5.67, $p < 0.05$; Figure 4A) and time post-withdrawal (F (1, 20) = 13.31, $p < 0.01$; Figure 4A). Additionally, adolescent rats treated with CIE displayed suppressed movement in the EPM at both withdrawal time points (F (1, 20) = 7.86, $p < 0.05$; Figure 5A).

Similarly, CIE treatment and time post-withdrawal increased anxiety on the EPM in adult rats. Specifically, CIE treatment decreased the percentage of time spent in the open arms of the EPM at both 24 hours and 12 days post-withdrawal (F (1, 20) = 33.85, $p < 0.001$; Figure 3B). Time spent in the closed arms of the EPM was increased by CIE treatment (F (1, 20) = 69.93, $p < 0.001$; Figure 4B) and time post-withdrawal (F (1, 20) = 4.43, $p < 0.05$; Figure 4B). Additionally, CIE treatment persistently suppressed total movement in the EPM by adult rats (F (1, 20) = 17.19, $p < 0.001$; Figure 5B).

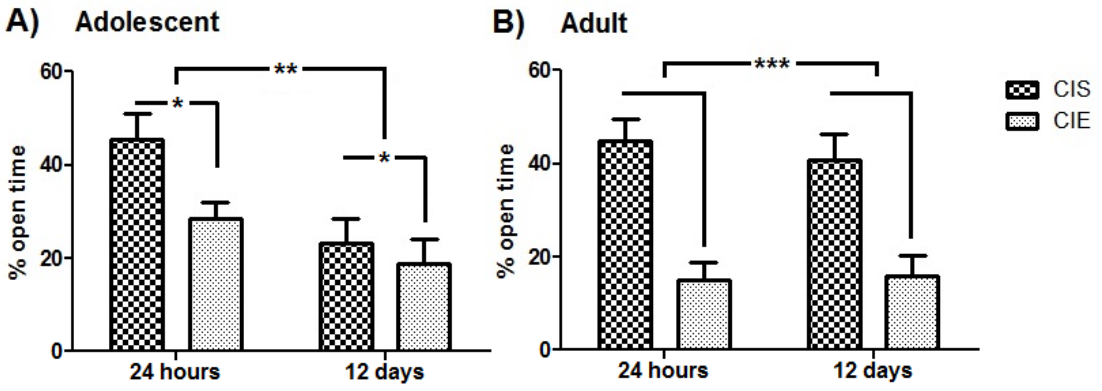

Figure 3. CIE and withdrawal from CIE increase anxiolytic behavior on the EPM as indicated by decreased percentage of time spent on open arms. A) Adolescent rats display increased anxiety due to CIE treatment as well as time post-withdrawal. B) Adult rats treated with CIE display increased anxiety at both 24 hours and 12 days post-withdrawal. Error bars denote SEM. * signifies $p < 0.05$, ** signifies $p < 0.01$, *** signifies $p < 0.001$.

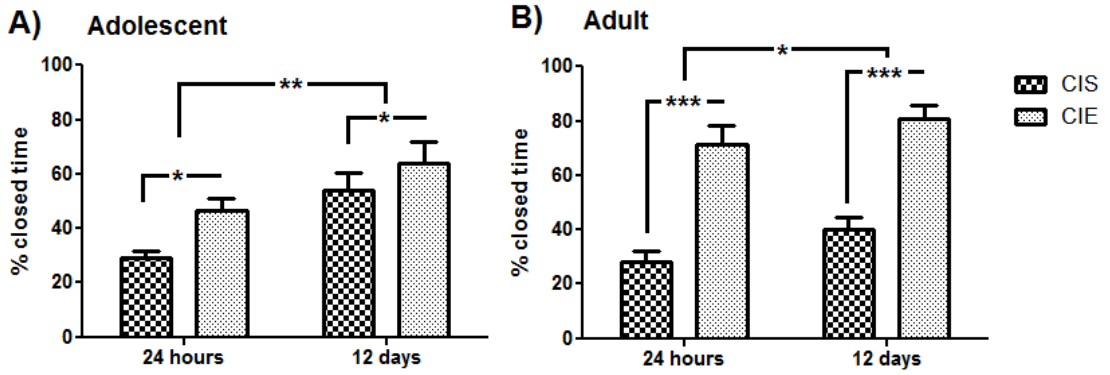

Figure 4. CIE and its withdrawal increase anxiolytic behavior on the EPM as indicated by increased percentage of time spent on the closed arms. A) Adolescent and B) adult rats display increased anxiety due to CIE treatment and time post-withdrawal. Error bars denote SEM. * p signifies < 0.05, ** signifies p < 0.01, *** signifies p < 0.001.

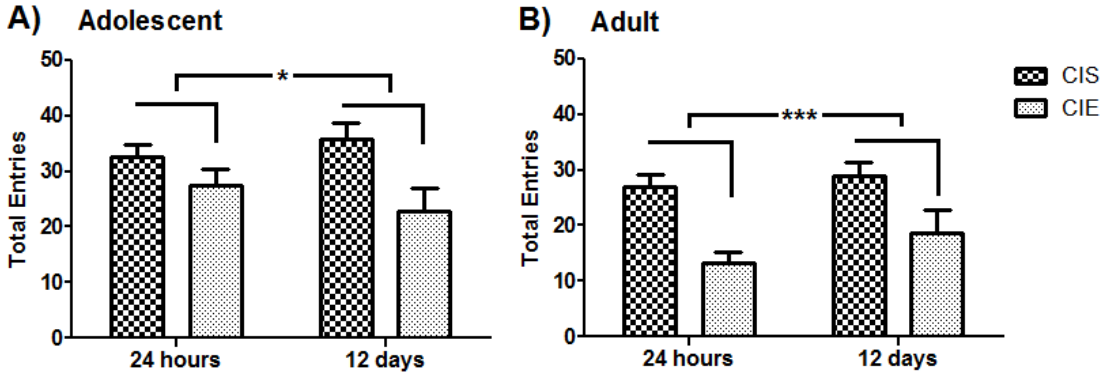

Figure 5. CIE and its withdrawal decrease overall movement on the EPM, indicating an increase in anxiety. A) Adolescent and B) adult rats treated with CIE show suppressed movement both 24 hours and 12 days post-withdrawal. Error bars denote SEM. * signifies p < 0.05, *** signifies p < 0.001.

Receptor Subunit Changes in the Cortex

NMDAR Subunits

CIE treatment in adolescent rats significantly decreased NR1 expression in whole cortex by 10.15% (F (1, 10) = 5.35, $p < 0.05$, Figure 6A), and this effect was still present 12 days after the final withdrawal. Conversely, there was no effect of CIE treatment (F

(1, 10) = 0.05, $p = 0.83$), time past withdrawal (F (1, 10) = 0.25, $p = 0.63$), or their interaction (F (1, 10) = 3.81, $p = 0.08$) in adult rats.

Cortical expression of NR2A in adolescent rats was not significantly affected by CIE treatment (F (1, 10) = 0.16 $p = 0.70$), time (F (1, 10) = 0.01, $p = 0.91$), or their interaction (F (1, 10) = 1.79, $p = 0.21$, Figure 6C). In adult rats, cortical expression of NR2A was not significantly altered by CIE treatment (F (1, 10) = 2.94, $p = 0.12$), time (F (1, 10) = 2.51, $p = 0.14$), or their interaction (F (1, 10) = 2.39, $p = 0.15$, Figure 6D).

Cortical NR2B expression in adolescent rats was not significantly affected by CIE treatment (F (1, 10) = 0.51, $p = 0.49$), time (F (1, 10) = 0.15, $p = 0.71$), or the interaction of CIE treatment and time (F (1, 10) = 0.42, $p = 0.53$, Figure 6E). Similarly in adult rats, NR2B expression was not significantly affected by CIE treatment (F (1, 10) = 3.52, $p = 0.09$), time (F (1, 10) = 0.35, $p = 0.57$), or the interaction of CIE treatment and time (F (1, 10) = 0.03, $p = 0.87$, Figure 6F).

Representative blots for these NMDAR subunits can be found in Figure 7 on the next page.

$GABA_AR$ Subunits

Cortical expression of $GABA_A$ β2,3 in adolescent rats was not affected by CIE treatment (F (1, 10) = 1.13, $p = 0.31$), time (F (1, 10) = 0.56, $p = 0.47$), or their interaction (F (1, 10) = 0.60, $p = 0.46$, Figure 8A). Expression of β2,3 in adult rats treated with CIE significantly increased 4.09% (F (1, 10) = 7.82, $p < 0.05$, Figure 8B), which did not change with time (F (1, 10) = 0.24, $p = 0.63$).

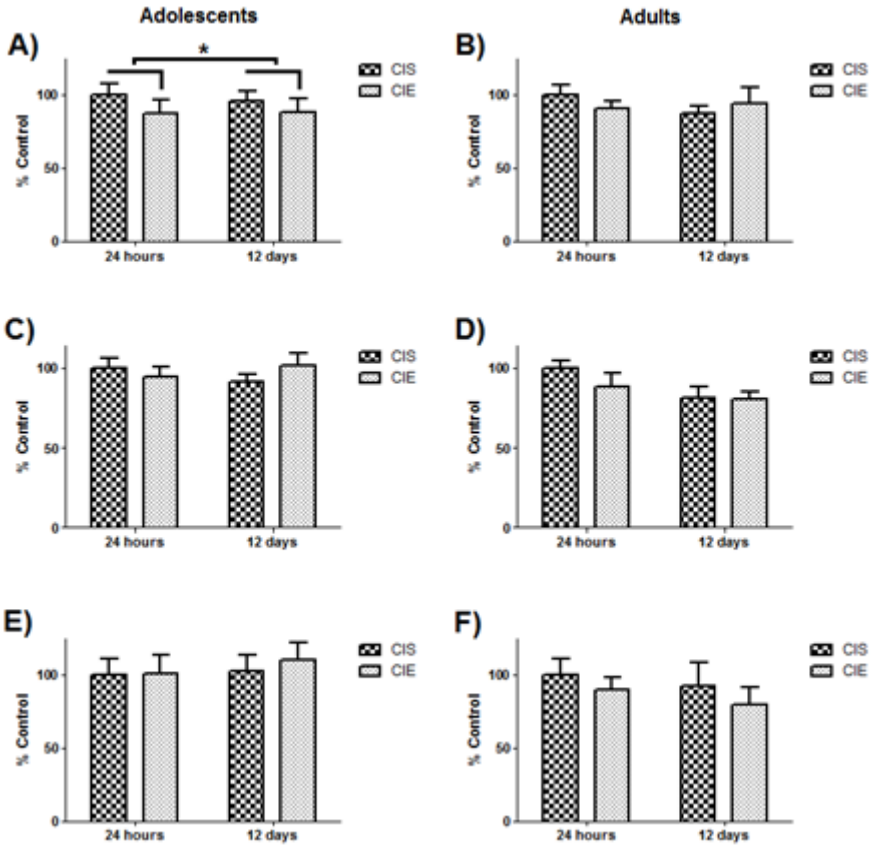

Figure 6. NMDA subunit expression from whole cortex of adolescent and adult rats. A) CIE exposure reduces NR1 expression in adolescent rats, but B) no effect on adult rats. Cortical NR2A expression is not changed in C) adolescent or D) adult rats. NR2B expression is unaltered in E) adolescent and F) adult rats. Figures normalized to CIS at 24 hours withdrawal. Error bars denote SEM, * signifies $p < 0.05$.

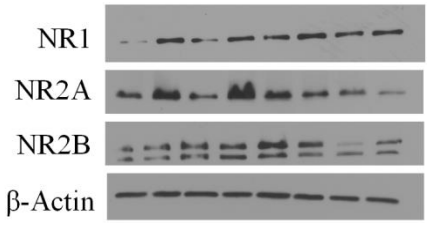

Figure 7. Representative blots for the NMDA subunits using cortical tissue. All gels are loaded the same way: Lane 0 – Ladder (not present on developed blots), lane 1 - Adolescent CIE at 24 hours withdrawal, 2 – Adolescent CIS at 24 hours withdrawal, 3 – Adult CIE at 24 hours withdrawal, 4 – Adult CIS at 24 hours withdrawal, 5 – Adolescent CIE at 12 days withdrawal, 6 – Adolescent CIS at 12 days withdrawal, 7 – Adult CIE at 12 days withdrawal, 8 – Adult CIS at 12 days withdrawal, 9 – negative control using sample buffer (not present on developed blots).

Expression of α1 in the adolescent cortex was not affected by CIE treatment (F (1, 10) = 0.02, p = 0.90), time (F (1, 10) = 0.40, p = 0.55), or their interaction (F (1, 10) = 1.01, p = 0.34, Figure 8C). Similarly α1 expression in adults was unaltered by CIE treatment (F (1, 10) = 0.02, p = 0.89), time (F (1, 10) = 0.01, p = 0.91), or their interaction (F (1, 10) = 0.90, p = 0.36, Figure 8D).

Adolescent cortical expression of the $GABA_A$ α4 subunit was not affected by CIE treatment (F (1, 10) = 1.62, p = 0.23), time past withdrawal (F (1, 10) = 0.07, p = 0.79), or their interaction (F (1, 10) = 1.67, p = 0.22, Figure 8E). Similarly α4 expression in adult cortex was unaltered by CIE treatment (F (1, 10) = 0.12, p = 0.74), time (F (1, 10) = 0.15, p = 0.71), or their interaction (F (1, 10) = 0.25, p = 0.63, Figure 8F).

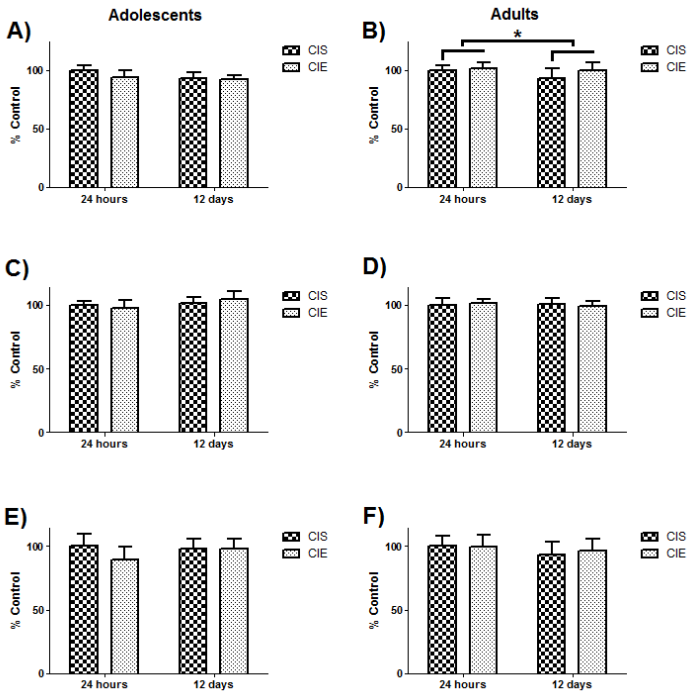

Figure 8. Cortical $GABA_A$ receptor subunit expression. A) No change in adolescent β2,3 expression. B) Adult β2,3 expression is increased by CIE exposure. C) Adolescent and D) adult α1 expression is unaltered. E) Adolescent and F) adult cortical α4 expression is unaltered. Figures normalized to CIS at 24 hours withdrawal. Error bars denote SEM, * signifies $p < 0.05$.

Representative blots for these $GABA_AR$ subunits in the cortex are located in Figure 9 below.

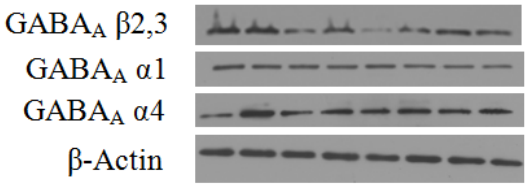

Figure 9. Representative blots for $GABA_A$ subunits using cortical tissue. All gels are loaded the same way: Lane 0 – Ladder (not present on developed blots), lane 1 - Adolescent CIE at 24 hours withdrawal, 2 – Adolescent CIS at 24 hours withdrawal, 3 – Adult CIE at 24 hours withdrawal, 4 – Adult CIS at 24 hours withdrawal, 5 – Adolescent CIE at 12 days withdrawal, 6 – Adolescent CIS at 12 days withdrawal, 7 – Adult CIE at 12 days withdrawal, 8 – Adult CIS at 12 days withdrawal, 9 – negative control using sample buffer (not present on developed blots).

Receptor Subunit Changes in the Hippocampus

NMDAR Subunits

Hippocampal NR1 expression in adolescent rats was not affected by CIE treatment (F (1, 10) = 0.88, p = 0.37), time (F (1, 10) = 0.16, p = .70), or their interaction (F (1, 10) = 0.20, p = 0.66, Figure 10A). In adult rats, a similar pattern of peptide expression pattern was found. Expression of NR1 was unaffected by CIE treatment (F (1, 10) = 0.44, p = 0.52), time past withdrawal (F (1, 10) = 1.23, p = .29), or any interaction (F (1, 10) = 0.54, p = 0.48, Figure 10B).

Peptide expression of NR2A in the adolescent hippocampus was unaffected by CIE treatment (F (1, 10) = 0.04, p = 0.85), time (F (1, 10) = 0.72, p = .41), or their interaction (F (1, 10) = 2.26, p = 0.16, Figure 10C). Similarly, expression of NR2A in the adult hippocampus was not altered by CIE treatment (F (1, 10) = 1.46, p = 0.25), time (F (1, 10) = 0.01, p = 0.91), or any interaction (F (1, 10) = 0.22, p = 0.65, Figure 10D).

Hippocampal expression of NR2B in adolescent rats was not significantly affected by CIE treatment (F (1, 10) = 0.01 p = 0.93), time (F (1, 10) = 0.00, p = 0.99), or their interaction (F (1, 10) = 0.08, p = 0.79, Figure 10E). In adult rats, hippocampal expression of NR2B was not significantly altered by CIE treatment (F (1, 10) = 0.03, p = 0.87), time (F (1, 10) = 0.11, p = 0.75), or their interaction (F (1, 10) = 0.06, p = 0.81, Figure 10F).

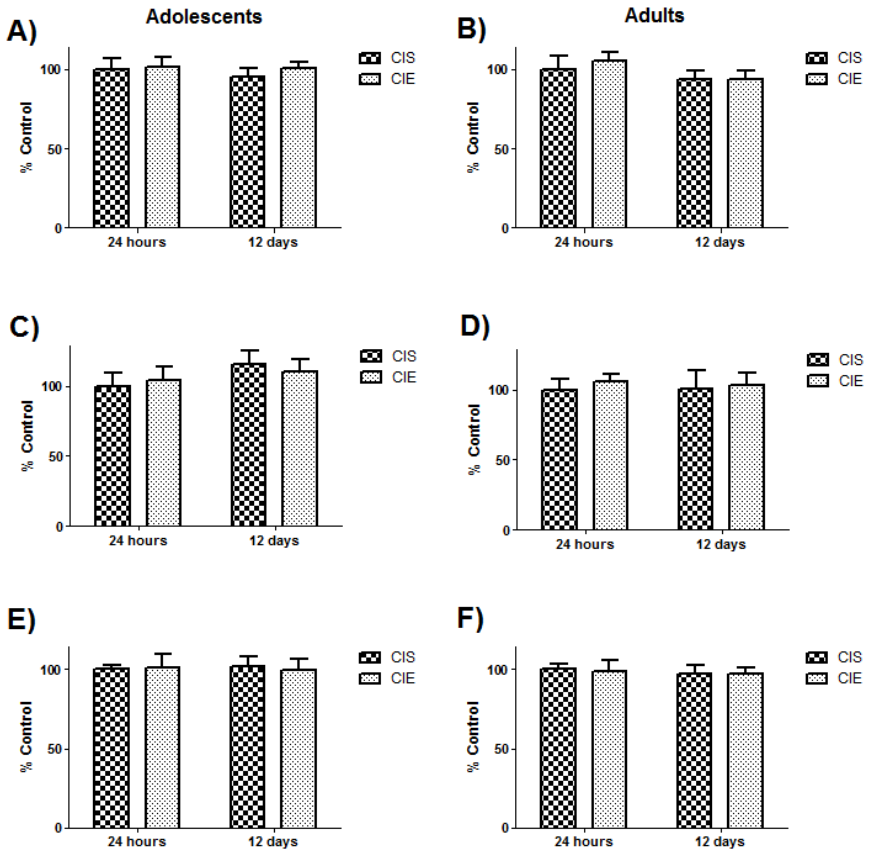

Figure 10. NMDA subunit expression in the hippocampus. NR1 expression is unchanged in A) adolescent and B) adult rats. NR2A expression is unaltered in C) adolescent and D) adult rats. NR2B expression is not affected in E) adolescent or F) adult rats. Figures are normalized to CIS expression at 24 hours withdrawal. Error bars denote SEM.

Representative blots for hippocampal NMDA subunits can be found in Figure 11 on the next page.

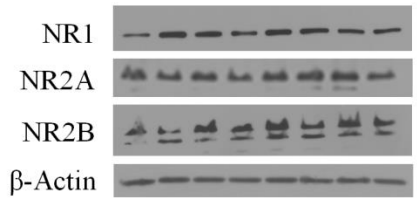

Figure 11. Representative blots for NMDA subunits using tissue from the hippocampus. All gels are loaded the same way: Lane 0 – Ladder (not present on developed blots), lane 1 - Adolescent CIE at 24 hours withdrawal, 2 – Adolescent CIS at 24 hours withdrawal, 3 – Adult CIE at 24 hours withdrawal, 4 – Adult CIS at 24 hours withdrawal, 5 – Adolescent CIE at 12 days withdrawal, 6 – Adolescent CIS at 12 days withdrawal, 7 – Adult CIE at 12 days withdrawal, 8 – Adult CIS at 12 days withdrawal, 9 – negative control using sample buffer (not present on developed blots).

$GABA_AR$ Subunits

Hippocampal $GABA_A$ β2,3 expression in adolescent rats was not altered by CIE treatment (F (1, 10) = 1.69, p = 0.22), time past withdrawal (F (1, 10) = 0.05, p = 0.83), or their interaction (F (1, 10) = 0.05, p = 0.83; Figure 12A). Similarly in adult hippocampus, β2,3 expression was unaltered by CIE treatment (F (1, 10) = 0.37, p = 0.56), time (F (1, 10) = 0.16, p = 0.70), or their interaction (F (1, 10) = 1.78, p = 0.21; Figure 12B).

In adolescent rats, hippocampal α1 peptide expression was altered by the interaction of CIE treatment and time past withdrawal (F (1, 10) = 5.58, $p < 0.05$, Figure 12C), such that in adolescent rats treated with control CIS, α1 peptide expression levels remained consistent between the 24 hour and 12 day withdrawal periods. However, adolescent rats treated with CIE showed a 6.12% increase in α1 expression at 24 hours post-withdrawal compared to time-matched controls, while α1 expression was decreased 7.32% in CIE treated rats at 12 days post-withdrawal compared to controls at this time point. In contrast, expression of hippocampal α1 in adult rats was unchanged by CIE

treatment (F (1, 10) = 0.13, p = 0.72, time past withdrawal (F (1, 10) = 0.00, p = 0.97), or their interaction (F (1, 10) = 1.96, p = 0.19; Figure 12D).

Hippocampal α4 expression in adolescent rats was not affected by CIE treatment (F (1, 10) = 0.01, p = 0.92), time after final withdrawal (F (1, 10) = 0.04, p = 0.84), or the interaction of the two (F (1, 10) = 0.10, p = 0.75; Figure 12E). Similarly, α4 peptide expression in the hippocampus of adult rats was unaltered by CIE treatment (F (1, 10) = 0.00, p = 0.99), withdrawal (F (1, 10) = 0.33, p = 0.58), or their interaction (F (1, 10) = 0.64, p = 0.44; Figure 12F).

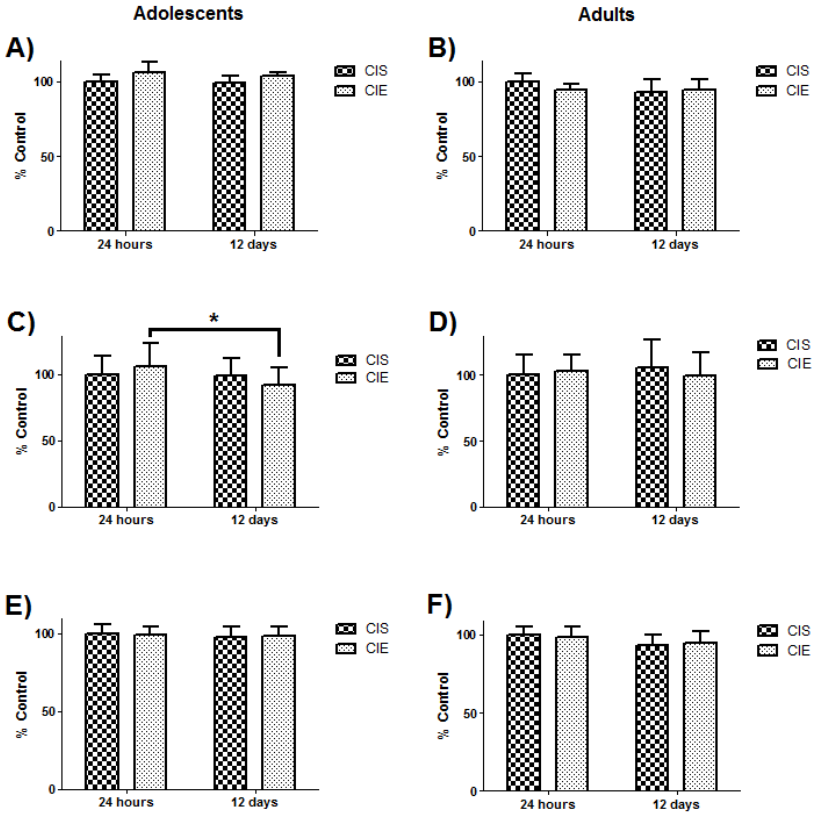

Figure 12. $GABA_A$ receptor subunit expression in the hippocampus. β2,3 expression is unaltered in A) adolescent and B) adult rats. C) Time past withdrawal decreases hippocampal α1 expression in CIE-treated adolescents, but not in CIS-treated adolescents. D) Adult α1 expression is unaltered. Hippocampal α4 expression remains stable in E) adolescent and F) adult rats. Figures are normalized to CIS treatment at 24 hours withdrawal. Error bars denote SEM, * indicates $p < 0.05$.

Representative blots for $GABA_A$ subunits within the hippocampus can be found in Figure 13 below.

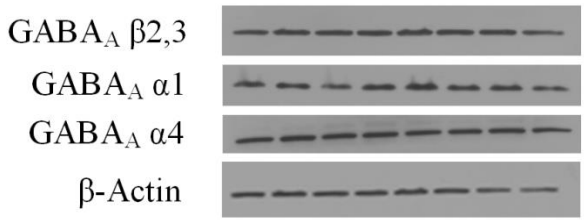

Figure 13. Representative blots for $GABA_A$ subunits using hippocampal tissue. All gels are loaded the same way: Lane 0 – Ladder (not present on developed blots), lane 1 - Adolescent CIE at 24 hours withdrawal, 2 – Adolescent CIS at 24 hours withdrawal, 3 – Adult CIE at 24 hours withdrawal, 4 – Adult CIS at 24 hours withdrawal, 5 – Adolescent CIE at 12 days withdrawal, 6 – Adolescent CIS at 12 days withdrawal, 7 – Adult CIE at 12 days withdrawal, 8 – Adult CIS at 12 days withdrawal, 9 – negative control using sample buffer (not present on developed blots).

Correlations between Anxiety and Receptor Subunit Changes

There were no significant correlations between anxiety and any of the subunits that had significant changes produced by chronic drug treatment, time past withdrawal, or the interaction of drug and time. Specifically, in adolescent rat cortex, NR1 expression does not correlate with anxiety (r = -0.06, p = 0.79; Figure 14A). In adult rat cortex, β2,3 expression does not correlate with anxiety (r = 0.09, p = 0.67, Figure 14B). Similarly, in adolescent hippocampus, α1 expression does not correlate with anxiety (r = -0.02, p = 0.92, Figure 14C).

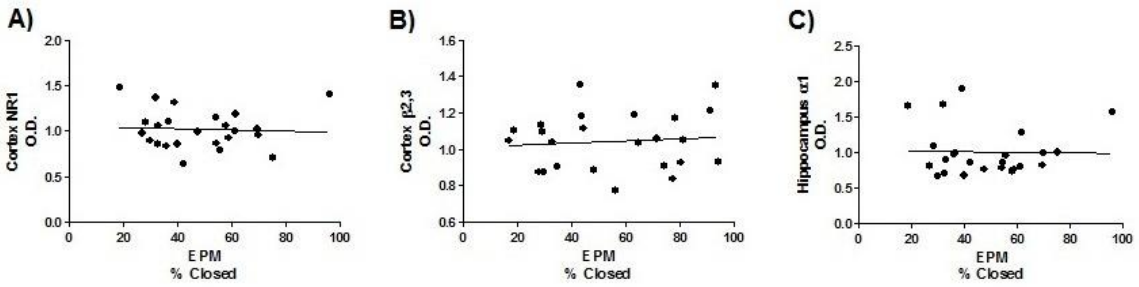

Figure 14. Correlations between anxiety and receptor subunit changes. There is no correlation between anxiety and A) adolescent cortical NR1 expression, B) adult cortical β2,3 expression, or C) adolescent hippocampal α1 expression.

CHAPTER FOUR

Discussion

Results from the current study indicate that CIE exposure in adolescents and adults produces progressively decreasing BECs in response to the same 4.0 g/kg dose of ethanol, perhaps indicative of metabolic tolerance. Additionally, CIE exposure in both age groups alters body weight (Silvers et al., 2003); because of this, the weight of the CIS (control) group was yoked to the CIE treatment group of the appropriate age. While adolescents did gain weight throughout the chronic intermittent administration procedure, our lab has previously demonstrated that high-dose intraperitoneal CIE exposure prevents normal weight gain in adolescent rats (Silvers et al., 2003), suggesting that the CIS-treated adolescents would have gained even more weight in the absence of food restriction. Conversely, adults that received CIE treatment, and thereby those receiving CIS treatment, lost weight over the chronic exposure paradigm. This effect could have been partially due to the increased motor impairments of adult rats compared to adolescents (Van Skike et al., 2010), specifically regarding the increased hypnotic effects (Broadwater et al., 2011), which may have inhibited the movements required to reach and access the food bin. To minimize weight loss in adult rats, future studies may benefit from distributing food pellets throughout the cage, incorporating a nutritionally complete liquid diet, or even intragastric administration of a supplemental diet. Some studies administer binge-like intragastric ethanol mixed with Vanilla Ensure Plus to minimize mortality (Liput et al., 2013), so a different method of CIE administration might be beneficial to help control for nutritional deficiencies that were present in both adolescent

and adult rats in this study. Current results indicate that high dose chronic intermittent ethanol exposure contributes to inadequate nutritional intake and is therefore detrimental to proper weight maintenance in both adolescent and adult rats.

Furthermore, CIE exposure yields increased anxiety in both adolescents and adults, which persists after 12 days of abstinence. Interestingly, along with the CIE-induced increase in anxiety, adolescent rats also have increased anxiety as time progresses from 24 hour to 12 days withdrawal in both treatment conditions. We speculate this increase in anxiety between the two time points is likely due to increased hippocampal allopregnanolone levels in adolescent rats 12 days following the chronic intermittent saline and ethanol treatment protocol (Silvers et al., 2006). Importantly, chronic saline or ethanol does not produce a differential alteration of adolescent hippocampal allopregnanolone levels at postnatal day 50 (Silvers et al., 2006), roughly the equivalent of the current study's 24 hour withdrawal time point. Allopregnanolone is a potent $GABA_A$ receptor modulator that is capable of eliciting rapid changes in neuronal excitability (Belelli & Lambert, 2005) and can elicit similar behavioral effects as those produced by ethanol (Chin et al., 2011; Silvers et al., 2003). Acute i.p. ethanol administration higher than 1.5 g/kg increases plasma and brain levels of allopregnanolone, and this effect is dependent on adrenocorticotrophic hormone (ACTH) release from the pituitary and *de novo* adrenal synthesis of steroidogenic acute regulatory (StAR) protein (Boyd et al., 2010b). However, chronic ethanol administration via liquid diet does not alter allopregnanolone levels (Boyd et al., 2010a; Silvers et al., 2006), indicating that tolerance develops to ethanol-induced neurosteroid production and may contribute to the behavioral tolerance produced by chronic ethanol administration. The

loss of neurosteroid production by ethanol appears to be mediated by blunted ACTH release (Boyd et al., 2010a), which remains blunted following a prolonged period of abstinence (Rivier & Lee, 2001). Given that the hypothalamic-pituitary-adrenal axis of adolescence is thought to be less responsive compared to adults (Prendergast & Little, 2007; Silveri & Spear, 2004), it is likely that the neurosteroid-mediated response to alcohol may be different in adolescents compared to adults. For example, administration of exogenous allopregnanolone exerts unique effects on adolescent behavior: whereas allopregnanolone administration decreases anxiety in pre-adolescent and adult animals, it is actually anxiogenic in adolescents (Shen et al., 2007). These data suggest that the increase in anxiety produced by 12 days of non-treatment in adolescent rats could likely be due to elevated levels of endogenous allopregnanolone (Silvers et al., 2006).

The persistent increase in anxiety of adolescent and adult rats co-occurs with some changes in NMDA and $GABA_A$ receptor subunit expression in the cortex and hippocampus. Ethanol tolerance and dependence in the adult are marked by increased function of NMDA receptors (Kalluri et al., 1998) and attenuated function of $GABA_A$ receptors (Kang et al., 1998). As such, age-dependent alterations in NMDA and $GABA_A$ receptor subunit composition following alcohol exposure may be associated with the youth-specific increased risk for developing future ethanol dependence. Indeed, NMDA subunits NR1, NR2A, and NR2B in the frontal cortex and hippocampus show age-specific modulation by two weeks of CIE vapor exposure and withdrawal (Pian et al., 2010). Additionally, the current work has demonstrated there are also some slight age-dependent and brain region dependent variations in both NMDA and $GABA_A$ receptor subunits in the whole cortex and hippocampus that persist even after 12 days of

abstinence from alcohol. These studies indicate that there may be age-specific changes in addiction neurocircuitry that may help to explain the disparate rates of addiction between adolescent and adult onset alcohol use. However, much of the data from the current study did not support the *a priori* hypotheses regarding the changes in receptor subunit expression, as well as their relationship to anxiety-like behavior on the EPM. For instance, in the current study NMDA NR1 subunit expression in adolescent cortex was decreased 10% at 24 hour and 12 days by CIE exposure compared to CIS exposure, with no change in the adult; additionally, there was no change in either age group in the hippocampus. Previous research indicates that chronic ethanol exposure increases NR1 protein expression in cortex and hippocampus (Kalluri et al., 1998), but more recent studies, including the current work suggest that subunit alterations may be dependent on the ethanol administration pattern and time since the occurrence of the last ethanol exposure (Pian et al., 2010). More specifically, the former study administered a priming dose of 5.0 g/kg ethanol via intragastric intubation, followed by variable subsequent doses based on the presence or absence of ataxia and loss of righting reflex. These doses were administered three times a day for six days, and rats received between 9-15 g/kg ethanol over a 24 hour period (Kalluri et al., 1998). Importantly, the timing of the doses suggests animals did not experience any substantial withdrawal, which appears to be an important factor in conferring certain receptor subunit changes. For instance, more recent studies employed a chronic intermittent pattern of ethanol exposure, to model binge drinking, which includes clear ethanol withdrawal periods. This pattern of ethanol administration, along with the time past the final ethanol exposure, appears to modify receptor subunits in a different pattern than what previous work with chronic ethanol

exposure would indicate. Specifically, 24 hours following CIE vapor withdrawal, NR1 subunit expression is decreased in the frontal cortex of adults, but not adolescents. After two weeks, NR1 expression returns to control levels in both adolescents and adults (Pian et al., 2010). In the adolescent hippocampus, NR1 expression is decreased at 24 hours following withdrawal, and increased after 2 weeks. In contrast, adult hippocampal NR1 expression is not changed (Pian et al., 2010). This pattern of expression is similar to the results yielded from the current study: NR1 expression is decreased 10% by CIE exposure at both 24 hours and 12 days past final CIE exposure in the cortex of adolescent rats, with no significant change in the adult cortex. In the hippocampus, we found no significant changes in either adolescents or adults.

Similarly with NR2A expression, prior studies using chronic ethanol administration found transient increases of NR2A expression in the cortex and hippocampus (Kalluri et al., 1998; Sultana & Babu, 2003). However, the changes were only reported immediately following the final chronic ethanol administration (i.e. 0 hours withdrawal), and not at 48 hours withdrawal from chronic ethanol exposure (Kalluri et al., 1998). Once again, chronic intermittent ethanol exposure appears to produce different results: there is no change in NR2A expression at 0 hours, 24 hours, or 2 weeks following final CIE exposure in adolescent frontal cortex, but adult cortical expression is decreased at 0 hours, increased at 24 hours, and back to control levels at 2 weeks (Pian et al., 2010). In the adolescent hippocampus, NR2A expression is decreased at 0 and 24 hours, but increased after 2 weeks withdrawal. In contrast, adult hippocampal NR2A expression is increased only at 0 hours withdrawal, and returns to control levels at both 24 hours and 2 weeks withdrawal (Pian et al., 2010). Once again, our results are more

consistent with the study that employed CIE exposure, although only with respect to the null findings. We did not find any changes in NR2A expression in the cortex or hippocampus of adolescent or adult rats.

Chronic ethanol exposure increases cortical and hippocampal expression of the NR2B subunit at 0 hours withdrawal, with no change from control at 48 hours withdrawal (Kalluri et al., 1998). Keeping with the previous trends, CIE exposure produces a different pattern of receptor subunit expression. Previous data indicates NR2B expression in the adolescent frontal cortex is unchanged; whereas adult expression is decreased at 0 and 24 hours, with elevated expression at 2 weeks withdrawal (Pian et al., 2010). This is similar to what the current study found: NR2B expression at 24 hours and 12 days withdrawal is unchanged in the cortex and hippocampus of both adolescent and adult rats.

The only NMDAR subunit change present was a 10% decrease in NR1 expression in adolescent rats treated with CIE. Given that the NMDAR is usually assembled as a dimer of dimers with NR1 and a pair of the NR2-containing subunits, with NR1 required for the functional expression of NR2-containing NMDARs (Du et al., 2011), it is interesting to speculate how NR1 changed without a simultaneous change in the NR2 subunits. One possibility is that NR2C or NR2D, which were not included in the present study, could have also decreased. However, NR2A- and NR2B- containing receptors are preferentially sensitive to ethanol inhibition compared to NR2C- and NR2D- containing NMDARs (Masood et al., 1994), indicating that NR2C and NR2D are perhaps not as important in conferring ethanol-induced receptor subunit composition changes compared to NR2A and NR2B subunits. Another possibility is that NR1 subunits are expressed on

the cell surface in the absence of NR2 (Okabe et al., 1999; Standley et al., 2000), with shorter C-terminal splice variants more likely to be expressed on the surface than longer splice forms (Okabe et al., 1999). Additionally, NR1 subunits can coassemble to form a homomeric receptor, which form a ligand binding site for glycine but not glutamate (Grimwood et al., 1995). Changes in receptor subunit assembly, composition, and unassembled NR1 subunit cell surface expression may help to explain the isolated change in NR1 without a corresponding change in NR2A or NR2B subunit expression found in the present work.

Based on previous research in adults, $GABA_AR$ β2,3 subunit expression was hypothesized to increase in the cortex (Devaud et al., 1997) and remain unchanged in the hippocampus of adult rats (Matthews et al., 1998), which is consistent with the results of the current study. Adult rats had a 4% increase in β2,3 expression in the whole cortex produced by CIE exposure at both 24 hours and 12 days withdrawal. While significant, this effect is probably not pharmacologically relevant. Behaviorally, the β2,3 subunit has been implicated in withdrawal seizure susceptibility (Blednov et al., 2003b). However, the *a priori* hypothesis regarding adolescent β2,3 expression was difficult to make because adolescents have been found to be both more (Chung et al., 2008) and less (Acheson et al., 1999) sensitive to ethanol withdrawal seizures. In the current study, there was no change in adolescent cortical or hippocampal expression of β2,3.

We were perhaps most surprised that CIE exposure did not alter cortical expression of α1 in adolescent or adult rats due to previous reports of chronic ethanol consumption resulting in a 40% decrease of α1 expression in adults (Devaud et al., 1997). Interestingly, the only effect we found for alteration of α1 was in the hippocampus, which

was predicted to remain unchanged: in the adolescent hippocampus, there is an interaction of chronic intermittent condition and time past withdrawal, such that CIS-treated rats had similar α1 expression levels from 24 hours to 12 days past final administration, but CIE-treated rats had a 14% decrease from 24 hours to 12 days. There is no change in α1 expression in the hippocampus of adult rats. Once again, the discrepancies are probably best explained by ethanol administration protocol, including the total amount of alcohol received. In the previous study, rats were administered chronic ethanol via liquid diet, consuming 10-12 g/kg per day for 14 days, for a total of at least 140 g/kg (Devaud et al., 1997). Animals in the current study received 10 administrations of 4 g/kg i.p. ethanol every 48 hours for a total of 40 g/kg ethanol during CIE exposure. Therefore, the total amount of alcohol received over the treatment period likely contributed to the disparate results between the current and previous work.

We hypothesized that cortical α4 expression would be increased following ethanol exposure in the adult (Matthews et al., 1998). Ethanol consumption and α4 expression has been shown to be positively correlated (Rewal et al., 2009) and adolescents voluntarily consume more ethanol than adults (Doremus et al., 2005; Walker et al., 2008); therefore, we predicted that adolescents would also have increased α4 expression in the cortex. However, in the present study, neither adolescents nor adults had altered α4 subunit expression in the whole cortex. Previous research indicated that chronic exposure increased α4 expression in the hippocampus, but only after 40 days of ethanol exposure (Matthews et al., 1998). Therefore, we predicted that there would be no changes produced by CIE exposure in adult rats. Additionally, based on previous data from the lab indicating no age-dependent difference in ethanol-induced spatial memory

impairments (Novier et al., 2012), we predicted adolescent α4 expression would also be unchanged following CIE exposure in the hippocampus. The present results were consistent with our hypotheses: CIE exposure produced no alterations in α4 expression in adolescent or adult rats at 24 hours or 12 days following the final chronic exposure.

One of the strengths of the current study was that behavior and receptor subunit analyses were conducted on the same animal, allowing for the exploration of a direct relationship between receptor subunit expression and anxiety-like behavior. However, none of the significant changes produced by CIE exposure were correlated with anxiety on the EPM. We expected several receptor subunits to correlate with anxiety levels; however, our hypotheses were based on prior research that found large receptor changes associated with chronic ethanol exposure in the cortex and hippocampus. Since our animals were exposed to less alcohol than administered in previous research, changes in receptor subunits that correlate with anxiety may have come from brain regions that are more traditionally involved in anxiety, rather than gross cortex and hippocampus, to provide a more sensitive and direct measure of receptor subunit alterations related to anxiety. Ethanol-induced dysregulation of the extended amygdala, including the amygdala and bed nucleus of the stria terminalis (BNST), is a critical region for processing and integrating stress and reward (Koob, 2008), especially as it relates to addiction. CIE exposure during adolescence results in blunted long-term potentiation in the dorsolateral BNST during adulthood and alterations of EPM anxiety (Conrad & Winder, 2011). Additionally, NR2B subunits are required for long-term potentiation and acute ethanol inhibition in the dorsolateral BNST (Wills et al., 2012), and CIE exposure increases the functional expression of NR2B-containing receptors in the ventral BNST

(Kash et al., 2009). Since CIE exposure can produce persistent alterations of BNST functioning that co-occur with changes in EPM anxiety, and a link between BNST functioning and the NR2B subunit has been established, it may be worthwhile to investigate NMDAR subunit expression in the BNST as it relates to multiple withdrawal-induced anxiety.

Additionally, another possible explanation for the lack of significant associations is that the subunits that were significantly changed were not as associated with anxiety as compared to some of the other subunits that were not significantly altered following CIE exposure. For example, α1 expression was decreased in CIE, but not CIS, treated adolescent rats, and although $GABA_A$ α1 has been shown to be important in modifying central nervous system hyperexcitability (Kralic et al., 2002; Werner et al., 2009), it has also been shown to be associated with ethanol-induced sedation (Blednov et al., 2003a). Perhaps the correlation between α1 subunit expression and sedation might have been stronger than the correlation between α1 and EPM anxiety. Additionally, β2,3 appears to have some role in withdrawal seizure susceptibility (Blednov et al., 2003b), which is not specifically related to anxiolytic behavior on the EPM. The present work found a very slight, but significant increase in β2,3 expression produced by CIE exposure in the adult cortex that was not correlated with EPM anxiety. Finally, NMDAR expression and activation is increased following withdrawal from chronic ethanol exposure (Hendrickson et al., 2007), and the increased function of these receptors is presumably due to differential upregulation of NMDAR subunit protein levels (Follesa & Ticku 1995; Kalluri et al., 1998). The present work found a decrease in NR1 subunit expression in the adolescent cortex produced by CIE exposure that was not correlated with EPM anxiety

levels. The NR1 receptor subunit is probably not a pharmacologically meaningful drug target because of its ubiquitous expression, specifically regarding the necessity of the NR1 subunit for the functional expression of the NR2 subunit-containing NMDARs (Du et al., 2011). Instead, the NR2B subunit has received the majority of attention regarding ethanol dependence: subunit selective antagonists inhibit the neurotoxic effects (Nagy et al., 2004) and seizure susceptibility (Malinowska et al., 1999) associated with alcohol withdrawal and are therefore being considered in the treatment of alcohol dependence (Nagy, 2004). Therefore, other receptor subunits that we measured might have been more directly involved in conferring an increase in anxiety-like behavior, and might be more pharmacologically relevant drug targets than the receptor subunit alterations we found in the present work.

One subunit to consider for future research that we did not include in the current study is $GABA_A$ α2, which has been shown to mediate anxiety (McEown & Treit, 2013; Smith et al., 2012). However, this effect might not be detectible with our current protocol for tissue harvesting because this effect may be localized to the ventral hippocampus (McEown & Treit, 2013); therefore, it might be beneficial to utilize a technique where different regions of the hippocampus can be differentiated. Additionally, polymorphisms in the GABRA2 gene that encodes the $GABA_A$ α2 subunit have been found to be linked to chronic alcohol dependence (Edenberg et al., 2004). Therefore the α2 subunit may be of particular interest, especially as it relates to ethanol withdrawal induced anxiety and the comorbidity between anxiety and alcohol use disorders. Additionally, ethanol-induced increases in synaptic NR1 clustering to synapses is protein kinase A (PKA) dependent (Carpenter-Hyland et al., 2004), and PKA

has been implicated in the development of tolerance produced by ethanol exposure, as disruptions in PKA signaling in mice blocks tolerance to ethanol-induced sedation and increases ethanol consumption (Thiele et al., 2000). Therefore, PKA activity might also be an area for future research, especially as it pertains to age-dependent motor responses to ethanol and alterations of future ethanol consumption in adolescent and adult rats.

Another consideration for future research that the current project has brought to light is that the timing, duration, and amount of ethanol exposure is important for conferring the direction and magnitude of receptor subunit changes. Compared to previous research, the magnitude of our effects were rather small, as many subunits that have been shown to be largely affected by chronic ethanol in previous studies were unaffected by the current ethanol administration protocol. Specifically, the rats in the current study were exposed to significantly less alcohol compared to previous studies where larger receptor subunit changes were found. Previous research used a chronic ethanol administration where rats consumed between 9-15 g/kg ethanol via intragastric intubation (Kalluri et al., 1998) or liquid diet (Devaud et al., 1997; Matthews et al., 1998) and found large changes in NMDA and $GABA_A$ receptor subunits, respectively. An important consideration from the latter two studies is that a conservative estimate of ethanol received is 140 g/kg over 14 days. Additionally, the former study indicates time past ethanol exposure may also influence receptor subunit alterations, as NMDA receptor subunit changes were found only at 0 hours following withdrawal, and not after 48 hours withdrawal (Kalluri et al., 1998). Another prior study employed a 60-dose CIE protocol where animals received 5 g/kg ethanol via intragastric intubation every other day for exposures 1 through 5, which increased to 6 g/kg ethanol every day for exposures 6

through 60 (Mahmoudi et al., 1997), for a total of 355 g/kg ethanol received over the entire duration of the study. A recent study used an ethanol vapor administration paradigm where rats received ethanol 14 hours per day with a 10 hour withdrawal, for 14 consecutive days (Pian et al., 2010). This study found some evidence of differing results in NMDA receptor subunit alterations compared to the prior chronic exposure studies, as a result of CIE exposure and potentially as a result of exposure to less alcohol overall than in previous studies. Rats in the current study were exposed to much less alcohol, as they received a total of 40 g/kg over 10 i.p. administrations spaced 48 hours apart. Inherent in this administration paradigm is that the BECs spike quickly, with prolonged periods of ethanol withdrawal due to the 48 hour spacing in between doses. Although the BECs are lower in the ethanol vapor study, the BECs are maintained at this more moderate level for 14 hours with a shorter 10-hour withdrawal period. Unfortunately, the revelation regarding the importance of the assumptions underlying differing ethanol administration protocols occurred *post hoc*, and caused our *a priori* power analyses to be insufficient to detect some of our effects, due to an overestimation of effect sizes based on the previous research. There were a few results that were of marginal significance, $p < 0.10$, and were trending in the way that previous research with CIE exposure would have indicated; however, we lacked the power to be able to successfully detect those results.

Animal models have demonstrated that adolescents respond uniquely to ethanol compared to adults. These age-dependent differences, ranging across all stages and patterns of ethanol administration, seem to indicate that adolescents who consume alcohol have an age-specific biological predisposition toward the development of alcohol

use disorders compared to those experiencing an adult-onset of alcohol use. For instance, mounting evidence suggests that human adolescents are especially vulnerable to alcohol-induced neurotoxicity (Squeglia et al., 2009). Animal models indicate CIE exposure during adolescence yields persistent reductions in hippocampal volume into adulthood, even after 10 weeks of abstinence (Ehlers et al., 2013b). This long-lasting reduction in hippocampal morphology is likely due to reduced neural stem cell proliferation and decreased survival of newborn neurons (Morris et al., 2010), which are also present after nearly 7 weeks of abstinence (Ehlers et al., 2013a). Furthermore, the decrease in measures of neurogenesis is correlated with an increase in disinibitory behavior in the open field conflict test (Ehlers et al., 2013a). These studies clearly indicate that early CIE exposure restricted to the adolescent developmental period produces long-lasting alterations of brain and behavior that persist into adulthood.

This study establishes that CIE exposure at a significantly lower overall dose than previously considered produces profound increases in the anxiety levels of adolescent and adult rats, which are persistent after a 12 day period of abstinence. Additionally, these elevations in anxiety levels co-occur with several long-lasting changes in NMDA and $GABA_A$ receptor subunit expression in the cortex and hippocampus. The changes in neuropeptide expression found in this study, as well as that from other published data indicate that CIE exposure has age-dependent effects on receptors involved in addiction neurocircuitry that may help to explain the increased rate of addiction when alcohol use begins during adolescence, compared to alcohol use beginning during adulthood.

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