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Chronic intermittent ethanol exposure produces persistent anxiety in adolescent and adult rats

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Abstract

Background—Ethanol dependence and tolerance in the adult are marked by increased function of NMDA receptors and decreased function of $GABA_A$ receptors that coincides with altered receptor subunit expression in specific brain regions. Adolescents often use ethanol at levels greater than adults, yet the receptor subunit expression profiles following chronic intermittent ethanol (CIE) exposure in adolescents are not known. Persistent age-dependent changes in receptor subunit alterations coupled with withdrawal-related anxiety may help explain the increase in alcohol abuse following adolescent experimentation with the drug.

Methods—Adolescent and adult rats received 10 intraperitoneal administrations of 4.0 g/kg ethanol or saline every 48 hours. At either 24 hours or 12 days after the final exposure, anxiety-like behavior was assessed on the elevated plus maze and tissue was collected. Western blotting was used to assess changes in selected NMDA and $GABA_A$ receptor subunits in whole cortex and bilateral hippocampus.

Results—CIE exposure yields a persistent increase in anxiety-like behavior in both age groups. However, selected NMDA and $GABA_A$ receptor subunits were not differentially altered by this CIE exposure paradigm in adolescents or adults.

Conclusions—CIE exposure produced persistent anxiety-like behavior, which has important implications for alcohol cessation. Given the reported behavioral and neuropeptide expression changes in response to this dose of ethanol, it is important for future work to consider the circumstances under which these measures are altered by ethanol exposure.

Keywords

Ethanol; Adolescence; Anxiety; GABA; NMDA

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Introduction

Adolescence is marked by unique sensitivity to certain effects of ethanol, including reduced sensitivity to ethanol-induced motor impairments (Hefner & Holmes, 2007; Spear & Varlinskaya, 2005 for review; White et al., 2002), which has biological roots in age-dependent cerebellar electrophysiology and PKCγ expression (Van Skike et al., 2010). Ethanol-induced motor impairments are used as feedback cues to attenuate ethanol consumption (Spear & Varlinskaya, 2005), and perhaps as a result of their reduced sensitivity to ethanol-induced motor impairments, adolescents and young adults show greater rates of binge and heavy drinking compared to adults over age 25 (SAMHSA, 2011). Of particular concern is the association between a young age at initial intoxication and increased rates of alcohol use disorders in adulthood (Adam et al., 2011; Ehlers et al., 2006; Hingson et al., 2006).

Given that withdrawal-related anxiety is associated with relapse (Fox et al., 2007; Leite & Nobre, 2012; Roberts et al., 1999; Willinger et al., 2002), one of the primary goals of this study is to assess the persistence of anxiety-like behavior associated with CIE exposure and withdrawal in adolescent and adult rats. Additionally dependence, tolerance, and withdrawal symptoms in the adult rat are marked by increased function of NMDA receptors (Grant et al., 1990; Krystal et al., 1998; Leite & Nobre, 2012; Sanna et al., 1993) and attenuated function of $GABA_A$ receptors (Grobin et al,. 1998; Kang et al., 1998; Kang et al., 1996; Morrow, 1995), which coincide with altered receptor subunit expression (Grobin et al., 1998; Kang et al., 1996; Morrow, 1995; Pian et al., 2010). However, the effects of CIE exposure on receptor subunit expression in adolescence are not yet completely known, and likely differ from the adult. For instance, 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., 1995; Smothers et al., 1997). Additionally $GABA_A$ receptor subunits, which are altered due to chronic ethanol use (Grobin et al., 1998; Kang et al., 1996; Morrow, 1995), are differentially expressed throughout adolescence compared to adulthood (Yu et al., 2006).

Due to age-dependent expression of NMDA and $GABA_A$ receptor subunits, it is likely that receptor subunit expression would be differentially modulated by CIE exposure in adolescents compared to adults. However, only one study has addressed the age-dependent changes in NMDA receptor subunit expression following CIE exposure (Pian et al., 2010). Additionally, much previous research conducted on $GABA_A$ receptor subunit changes in the adult has focused on rapidly inducing physical dependence by administering large quantities of ethanol (e.g. Devaud et al., 1997; Matthews et al., 1998). Therefore, this study quantifies the changes in anxiety-like behavior and receptor subunit expression produced by CIE exposure and withdrawal during adolescence and adulthood. Additionally, the study utilizes a within-subjects design to help elucidate a potential molecular mechanism underlying the adolescent-specific increased risk of developing future alcohol use disorders.

Materials and Methods

Animals

Twenty-four adolescent (PD 28 on arrival) and 24 adult (~PD 120 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 began. At the start of the experiment, adolescents (PD 30) weighed 112.2 ± 4.8g and adults (PD 122) weighed 430.9 ± 6.7g. All animals in the 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., 2003b). All procedures were approved by the Baylor University Institutional Animal Care and Use Committee.

Chronic Intermittent Ethanol Exposure and Withdrawal Conditions

Adolescent and adult animals were administered 4.0 g/kg 20% w/v ethanol (CIE exposure group) or equivalent volume saline (chronic intermittent saline (CIS) exposure group) via intraperitoneal injection every 48 hours for 20 days, for a total of 10 intoxications and withdrawals. During treatment, all animals in the CIE exposure group had unrestricted access to food and water; however, animals in the CIS control group were weight yoked to the CIE-treated rats of the same age to control for ethanol-induced weight suppression (Silvers et al., 2003b).

After this treatment, animals in the CIE and CIS conditions were further divided into two separate groups based on time past final ethanol administration: 24 hours and 12 days (Silvers et al., 2003b). These time conditions determined when behavioral testing and tissue harvesting occurred. Adolescent animals were PD 49 and PD 60 and adult animals were approximately PD 139 and PD 150 at 24 hours and 12 days post ethanol administration, respectively.

Blood Ethanol Concentration

During chronic intermittent ethanol exposure blood ethanol concentrations (BECs) were determined 30 minutes after the intraperitoneal 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. Blood from CIE-treated rats was analyzed with the Analox AM1 Alcohol Analyser (Analox Instruments Ltd., United Kingdom).

Anxiety Assessment: Elevated Plus Maze

Anxiogenesis associated with CIE administration in both withdrawal conditions was measured using the elevated plus maze (EPM). The test was conducted under dim lighting conditions, with open arms measuring 4 lux and closed arms measuring 2 lux. The maze, elevated approximately 50 cm from the ground, 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 elevated plus maze task, 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 closed arm entries was calculated by dividing closed entries by open and closed entries, excluding entries through the central arena.

Protein Detection: Western Blotting

Immediately following the EPM trial, each animal was taken to a different room and sacrificed via rapid decapitation without anesthesia. Whole cortex and bilateral hippocampus were rapidly dissected over ice, transported in dry ice, and stored at −80°C until assayed.

P2 fractions were made as previously described (Van Skike et al., 2010). Briefly, tissue was homogenized in 0.32M sucrose in phosphate-buffered saline (PBS) and centrifuged at 1,000 × *g* for 10 minutes. The supernatant was centrifuged again at 12,000 × *g* for 20 minutes and the pellet was resuspended in PBS and stored at −80°C until assayed.

Western blots were conducted as previously described (Van Skike et al., 2010). Protein concentration for each P2 fraction was determined with a Bradford assay. Twenty μg of protein per well was loaded into Tris-Glycine gels (8% to 16%), counterbalanced across conditions. Proteins were separated by SDS–PAGE and electroblotted to polyvinylidenedifluoride membranes (Life Technologies, Carlsbad, CA). The membranes were then targeted with one of three following antibody combinations diluted in blocking buffer (50mL PBS, 25μL Tween-20, 0.5g milk powder) and applied on separate days: 1) $GABA_A$ α1 (1:1000; Millipore, Billerica, MA, USA; AB5946), β-Actin (1:7500; Millipore; MAB1501), and NMDA NR2A (1:500; Millipore; 07–632), 2) $GABA_A$ β2,3 (1:500; Millipore; MAB341), β-Actin, and NMDA NR2B (1:1000; Millipore; 06–600), 3) NMDA NR1 (1:500; Millipore; 05–432), β-Actin, and $GABA_A$ α4 (1:500; Abcam, Cambridge, MA, USA; AB117080). The appropriate horseradish peroxidase (HRP)-conjugated secondary antibody targeted against either a rabbit (1:7500–1:10,000; Millipore; AP132P,), mouse (1:5000–1:7500; Santa Cruz Biotechnology, Dallas, TX, USA; sc-2005), or goat (1:7500; Abcam; AB97110) host was applied and peptide labeling was detected with Thermo Scientific SuperSignal West Pico Chemiluminescent Substrate and exposed to X-ray film under non-saturating 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 differing protein sizes and order of application minimized any ambiguity that might have arisen from any residual expression.

Statistical Analysis

Repeated two-way ANOVAs (chronic treatment x day) were performed separately for each age group to assess the effectiveness of pair feeding (n = 12 per age group). BEC values obtained during CIE exposure were analyzed with a repeated measures ANOVA to assess the effect of repeated ethanol exposure on BEC in adolescent and adult rats (n = 12 per age group). Since the EPM has been shown to have inconsistent results when comparing different age groups (Doremus-Fitzwater et al., 2009), separate two-way ANOVAs (chronic treatment x withdrawal condition) for adolescent (n = 6) and adult (n = 6) animals were conducted for percent open time, percent closed time, and percentage of closed entries to assess anxiety-like behavior associated with ethanol withdrawal. Alterations in peptide expression were assessed by conducting separate age-dependent two-way ANOVAs (chronic treatment x withdrawal condition) for all six different subunits of interest normalized to actin for each of the two brain regions (n = 6 for all analyses). All comparisons were made within blots.

Results

Weight Yoking

Separate two-way repeated ANOVAs showed equivalent weights between CIS treated rats and their age-matched CIE treated counterparts (adolescents, F (1, 22) = 0.48, p = 0.50, n.s.; adults, F (1, 22) = 0.51, p = 0.48, n.s.; data not shown), indicating that pair feeding was effective in both age groups. Over the course of the chronic intermittent administration procedure, all adolescent rats gained weight (F (9, 22) = 323.30, p < 0.001). Conversely, all adult rats lost weight throughout the chronic intermittent administration protocol (F (1, 22) = 51.36, p < 0.001; data not shown).

CIE Exposure Progressively Reduces BEC

BECs were determined 30 minutes after CIE exposure on administration days 1, 5, and 10. CIE exposure in adolescent and adult rats led to progressively decreasing BECs throughout chronic treatment (F (2, 22) = 9.46, p < 0.001, Figure 1), which did not differ by age (F (1, 22) = 0.30, p = 0.59, n.s., Figure 1).

Anxiogenesis produced by CIE exposure is long-lasting

In adolescent rats, both CIE treatment and time post-withdrawal produced significant anxiogenesis. 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 2A) and time post-withdrawal (F (1, 20) = 9.77, p < 0.01; Figure 2A). 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 2C) and time post-withdrawal (F (1, 20) = 13.31, p < 0.01; Figure 2C). Although CIE-treated adolescent rats displayed suppressed movement on the EPM at both withdrawal time points (F (1, 20) = 7.86, p < 0.05; data not shown), the percentage of closed arm entries did not differ between treatment groups, but increased between withdrawal periods (F (1, 20) = 6.31, p < 0.05; Figure 2E).

Similarly, CIE treatment in adult rats increases anxiety-like behavior on the EPM. 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 2B). Time spent in the closed arms of the EPM was increased by CIE treatment (F (1, 20) = 69.93, $p < 0.001$; Figure 2D) and time post-withdrawal (F (1, 20) = 4.43, $p < 0.05$; Figure 2D). Although CIE treatment persistently suppressed total movement in the EPM by adult rats (F (1, 20) = 17.19, $p < 0.001$; data not shown), the percentage of total arm entries was higher in CIE-treated adults compared to control CIS-treated adult rats (F (1, 20) = 11.31, $p < 0.01$; Figure 2F).

Receptor Subunits in the Cortex and Hippocampus are Unchanged

There were no age-related differences in any of the NMDA or $GABA_A$ subunits in CIS treated animals only. However, there was a main effect of time past withdrawal in cortical NR2A expression (F (1, 20) = 5.66, $p < 0.05$; Table 1), such that NR2A expression in adolescents and adults decreased 13.7% from 24 hours to 12 days past withdrawal.

NMDA or $GABA_A$ subunits tested in the whole cortex and hippocampus were not affected by CIE treatment, time after withdrawal, or their interaction in adolescent and adult rats (Figures 3, 4, 5, and 6; Tables 2 and 3).

Discussion

Results from the current study indicate CIE exposure in adolescent and adult rats produces a profound increase in anxiety-like behavior in both age groups, which persists after 12 days of abstinence. The long-lasting increase in anxiety-like behavior may be mediated by CIE-induced increases in hippocampal allopregnanolone levels (Silvers et al., 2006), which can affect neuronal excitability (Belelli & Lambert, 2005). It is possible that the effect of CIE on anxiety is both age and dose dependent. However, the previous work that utilized high BEC, coupled with the work from Conrad and Winder (2011) who used ethanol vapor resulting in lower BEC levels of approximately 150–200 mg/dL, produced similar results, which suggests that CIE-induced anxiety is not dose dependent. Clearly additional research is needed on these topics.

Interestingly, adolescent rats exhibit increased anxiety-like behavior as time after withdrawal progressed from 24 hours to 12 days across both CIE and CIS treatment conditions. We speculate this increase in anxiety-like behavior between the two time points could possibly be due to maturational increases in amygdalocortical projection density and synapse type (Cunningham et al., 2002), along with alterations of hypothalamic-pituitary-adrenal (HPA) axis function, reactivity to stress, and hormonal alterations specific to adolescence and emerging adulthood (Spear, 2000 for review). Additionally, adolescent rats are susceptible to certain stressors that can have a lasting impact on the HPA axis, such as transport from the animal supplier (Wiley & Evans, 2009). Future studies should analyze corticosterone levels to gain greater insight into the increased anxiety-like behavior in CIS treated adolescent rats that was observed in this study.

The weights of the CIS-treated animals were successfully yoked to the CIE-treated animals of the appropriate age, making it unlikely that differential weight gain contributed to these persistent changes in anxiety-like behavior. While adolescents did gain weight throughout the chronic intermittent administration procedure, high-dose intraperitoneal CIE exposure has been shown to prevent normal weight gain in adolescent rats (Silvers et al., 2003b), suggesting that the observed growth pattern for adolescent rats was suppressed. Conversely, all adults lost weight throughout the chronic exposure paradigm, which is consistent with other binge-like ethanol administration paradigms (Majchrowicz, 1975). This effect could have been partially due to the increased ethanol-induced 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. Results from the present study indicate that high dose chronic intermittent ethanol exposure is detrimental to proper weight maintenance in both adolescent and adult rats, potentially due to inadequate nutritional intake.

Adolescent and adult rats treated with CIE exhibited progressively decreasing BECs in response to repeated 4.0 g/kg intraperitoneal administrations of ethanol, which is indicative of metabolic tolerance found in similar models of administration (Silvers et al., 2003b).

However, there were no changes in receptor subunit expression, which is curious when compared to previously published literature. Since we calculated our *a priori* power analysis using the large effects found in previous literature, the current study was underpowered to detect the smaller changes that we found. The differences between the current study and previous work could be due to several factors that are important to consider, including the quantity of alcohol received over the entire duration of the study, the presence or absence of withdrawal periods during chronic exposure, route of administration, and the timing of receptor subunit assessment after the final ethanol exposure. For instance, chronic ethanol has been shown to upregulate NMDA receptor subunits NR1, NR2A, and NR2B in the whole cortex and hippocampus by approximately 35% 1 hour after chronic ethanol, with all subunits returning to baseline after 48 hours withdrawal (Kalluri et al., 1998). However, animals in this study were administered 9–15 g/kg ethanol per day for six days via gavage, for a grand total of between 54–90 g/kg ethanol, with no clear withdrawal periods (Kalluri et al., 1998). In Pian et al (2010), researchers administered 14 hours of ethanol vapor exposure every day for two weeks, for a total of 196 hours of ethanol vapor exposure, and found age-dependent changes in NMDA receptor subunit expression in frontal cortex and hippocampus at both 24 hours and 2 weeks of ethanol withdrawal. Prior research with $GABA_A$ subunits found a 12–18% increase in different cortical layers, as well as a 26–46% increase in α4 mRNA in different subregions of the hippocampus at 48 hours withdrawal, using a 60-dose CIE procedure, where animals received 355 g/kg ethanol over the entire duration of the study (Mahmoudi et al., 1997). Others have found dramatic α1 and α4 subunit expression alterations in the cortex (Devaud et al., 1997), but not hippocampus (Matthews et al., 1998) following 14 days of ethanol exposure via liquid diet at 6–8 hours withdrawal. Animals in these studies consumed between 10–12 g/kg ethanol per day with no clear withdrawal periods, totaling 140–168 g/kg ethanol over two weeks. In contrast, the present work delivered 40 g/kg of intraperitoneal ethanol over 20 days, with assessments at 24 hours and

12 days of ethanol withdrawal, and we did not find any receptor subunit changes using this model. Comparing these studies highlights the importance of the exposure paradigm, including total ethanol exposure, route of administration, the inclusion or absence of withdrawal periods, and the timing of neuropeptide assessments. Based on the current body of research, it appears as though most of the changes in NMDA and $GABA_A$ receptor subunit expression occur within 24 hours of the final chronic ethanol exposure (Devaud et al., 1997, Mahmoudi et al., 1997, Matthews et al., 1998, Pian et al., 2010). However, there is some indication that the expression of certain NMDA subunits can still be affected by chronic intermittent ethanol exposure after an abstinence period of up to two weeks (Pian et al., 2010), although the quantity of subunits that show persistent versus immediate changes are more limited.

A significant limitation of the current study is that receptor subunit alterations were assessed only from whole cortex and bilateral hippocampus. Long-term potentiation in the extended amygdala, including the bed nucleus of the stria terminalis, is persistently suppressed by CIE exposure during adolescence, which co-occurs with increased anxiety-like behavior on the EPM (Conrad & Winder, 2011). Additionally, CIE exposure increases the functional expression of NR2B-containing receptors in the ventral bed nucleus of the stria terminalis (Kash et al., 2009). Therefore, CIE may produce age-dependent alterations in NR2B expression in the extended amygdala that may be related to the persistent increases in anxiety-like behavior after CIE exposure and protracted abstinence. Additionally, future research should address potential age-dependent alterations in other neurotransmitter systems, including receptor subunit expression and electrophysiological function, especially as it relates to the youth-specific increase in the development of future alcohol use disorders. Age-dependent alterations of corticotropin-releasing factor (CRF) and CRF_1 receptors, particularly within the amygdala, may help to better explain the persistent increase in anxiety-like behavior after CIE treatment in adolescent and adult rats presented herein. CRF-like immunoreactivity in the amygdala is elevated for at least six weeks following protracted ethanol withdrawal from a liquid ethanol diet (Zorrilla et al., 2001). Given the importance of CRF alterations in the maintenance and progression of addiction (reviewed in Zorrilla et al., 2014), age-dependent alterations produced by ethanol of this system may help explain the correlation between alcohol use disorders and early age of initial exposure.

This study establishes that CIE exposure produces profound increases in the anxiety-like behavior of adolescent and adult rats, which are persistent after a 12 day period of abstinence. The persistent behavioral changes have important implications for discontinuing alcohol consumption in both adolescence and adulthood. Although adolescents are less sensitive to the negative aspects of alcohol use (Spear & Varlinskaya, 2005), adolescents have significant and persistent anxiety produced by alcohol withdrawal. Since withdrawal-related anxiety is associated with relapse (Fox et al., 2007; Leite & Nobre, 2012; Roberts et al., 1999; Willinger et al., 2002), the main implication of our finding is that both adolescents and adults experience persistent withdrawal-induced increases in anxiety which may underlie treatment difficulties across the lifespan.

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References

Adam A, Faouzi M, Gaume J, Gmel G, Daeppen JB, Bertholet N. Age of first alcohol intoxication: association with risky drinking and other substance use at the age of 20. Swiss Med Wkly. 2011; 141:w13226. [PubMed: 21720968]

Belelli D, Lambert JJ. Neurosteroids: endogenous regulators of the GABAA receptor. Nat Rev Neurosci. 2005; 6:565–575. [PubMed: 15959466]

Boyd KN, Kumar S, O'Buckley TK, Morrow AL. Chronic ethanol exposure produces tolerance to elevations in neuroactive steroids: mechanisms and reversal by exogenous ACTH. J Neurochem. 2010a; 115(1):142–152. [PubMed: 20633208]

Boyd KN, Kumar S, O'Buckley TK, Porcu P, Morrow AL. Ethanol induction of steroidogenesis in rat adrenal and brain is dependent upon pituitary ACTH release and de novo adrenal StAR synthesis. J Neurochem. 2010b; 112(3):784–796. [PubMed: 20021565]

Broadwater M, Varlinskaya EI, Spear LP. Chronic intermittent ethanol exposure in early adolescent and adult male rats: effects on tolerance, social behavior, and ethanol intake. Alcoholism: Clinical and Experimental Research. 2011; 35(8):1392–1403.

Chandler LJ, Newsom H, Sumners C, Crews F. Chronic ethanol exposure potentiates NMDA excitotoxicity in cerebral cortical neurons. J Neurochem. 1993; 60(4):1578–1581. [PubMed: 8455043]

Chin VS, Van Skike CE, Berry RB, Kirk RE, Diaz-Granados JL, Matthews DB. Effect of acute ethanol and acute allopregnanolone on spatial memory in adolescent and adult rats. Alcohol. 2011; 45(5):473–483. [PubMed: 21600728]

Conrad KL, Winder DG. Altered anxiety-like behavior and long-term potentiation in the bed nucleus of the stria terminalis in adult mice exposed to chronic social isolation, unpredictable stress and ethanol beginning in adolescence. Alcohol. 2011; 45(6):585–593. [PubMed: 21194878]

Cunningham MG, Bhattacharyya S, Benes FM. Amygdalo-cortical sprouting continues into early adulthood: Implications for the development of normal and abnormal function during adolescence. Journal of Comparative Neurology. 2002; 453(2):116–130. [PubMed: 12373778]

Devaud LL, Fritschy JM, Sieghart W, Morrow AL. Bidirectional alterations of GABA(A) receptor subunit peptide levels in rat cortex during chronic ethanol consumption and withdrawal. Journal of Neurochemistry. 1997; 69(1):126–130. [PubMed: 9202302]

Doremus-Fitzwater TL, Varlinskaya EI, Spear LP. Effects of pretest manipulation on elevated plus-maze behavior in adolescent and adult male and female Sprague-dawley rats. Pharmacology Biochemistry and Behavior. 2009; 92(3):413–423.

Ehlers CL, Slutske WS, Glider DA, Lau P, Wilhelmsen KC. Age at first intoxication and alcohol use disorders in southwest California Indians. Alcoholism: Clinical and Experimental Research. 2006; 30(11):1856–1865.

Fox HC, Bergquist KL, Hong KI, Sinha R. Stress-induced and alcohol cue-induced craving in recently abstinent alcohol-dependent individuals. Alcoholism: Clinical and Experimental Research. 2007; 31:395–403.

Grant KA, Valverius P, Hudspith M, Tabakoff B. Ethanol withdrawal seizures and the NMDA receptor complex. European Journal of Pharmacology. 1990; 176(3):289–296. [PubMed: 2158451]

Grobin AC, Matthews DB, Devaud LL, Morrow AL. The role of GABAA receptors in the acute and chronic effects of ethanol. Psychopharmacology. 1998; 139(1):2–19. [PubMed: 9768538]

Hefner K, Holmes A. An investigation of the behavioral actions of ethanol across adolescence in mice. Psychopharmacology. 2007; 191:311–322. [PubMed: 17206494]

Hingson RW, Heeren T, Winter MR. Age at drinking onset and alcohol dependence: age at onset, duration, and severity. Arch Pediatr Adolesc Med. 2006; 106(7):739–746. [PubMed: 16818840]

Insel TR, Miller LP, Gelhard RE. The ontogeny of excitatory amino acid receptors in rat forebrain--I. N-methyl-D-aspartate and quisqualate receptors. Neuroscience. 1990; 35:31–43. [PubMed: 1972786]

Johnston MV. Neurotransmitters and vulnerability of the developing brain. Brain Development. 1995; 17(5):301–306. [PubMed: 8579213]

Kalluri HSG, Mehta AK, Ticku MJ. Up-regulation of NMDA receptor subunits in rat brain following chronic ethanol treatment. Molecular Brain Research. 1998; 58(1–2):221–224. [PubMed: 9685652]

Kang MH, Spigelman I, Olsen RW. Alteration in the sensitivity of GABA(A) receptors to allosteric modulatory drugs in rat hippocampus after chronic intermittent ethanol treatment. Alcoholism, Clinical and Experimental Research. 1998; 22(9):2165–2173.

Kang M, Spigelman I, Sapp DW, Olsen RW. Persistent reduction of GABA(A) receptor-mediated inhibition in rat hippocampus after chronic intermittent ethanol treatment. Brain Research. 1996; 709(2):221–228. [PubMed: 8833758]

Kash TL, Baucum AJ II, Conrad KL, Colbran RJ, Winder DG. Alcohol exposure alters NMDAR function in the bed nucleus of the stria terminalis. Neuropsychopharmacology. 2009; 34:2420–2429. [PubMed: 19553918]

Krystal JH, Petrakis IL, Webb E, Cooney NL, Karper LP, Namanworth S, Stetson P, Trevisan LA, Charney DS. Dose-related ethanol-like effects of the NMDA antagonist, ketamine, in recently detoxified alcoholics. Archives of General Psychiatry. 1998; 55(4):354–360. [PubMed: 9554431]

Leite LE, Nobre MJ. The negative effects of alcohol hangover on high-anxiety phenotype rats are influenced by the glutamate receptors of the dorsal midbrain. Neuroscience. 2012; 213:93–105. [PubMed: 22516019]

Mahmoudi M, Kang MH, Tillakaratne N, Tobin AJ, Olsen RW. Chronic intermittent ethanol treatment in rats increases GABA(A) receptor alpha4-subunit expression: possible relevance to alcohol dependence. J Neurochem. 1997; 68(6):2485–2492. [PubMed: 9166743]

Majchrowicz E. Induction of physical dependence upon ethanol and the associated behavioral changes in rats. Psychopharmacologia. 1975; 43:245–254. [PubMed: 1237914]

Matthews DB, Devaud LL, Fritschy JM, Sieghart W, Morrow AL. Differential regulation of GABA(A) receptor gene expression by ethanol in the rat hippocampus versus cerebral cortex. Journal of Neurochemistry. 1998; 70(3):1160–1166. [PubMed: 9489737]

McDonald JW, Johnston MV, Young AB. Differential ontogenetic development of three receptors comprising the NMDA receptor/channel complex in the rat hippocampus. Exp Neurol. 1990; 110:237–247. [PubMed: 2174375]

Morrow AL. Regulation of GABAA receptor function and gene expression in the central nervous system. International Review of Neurobiology. 1995; 38:1–41. [PubMed: 8537199]

Pian JP, Criado JR, Milner R, Ehlers CL. N-methyl-d-aspartate receptor subunit expression in adult and adolescent brain following chronic ethanol exposure. Neuroscience. 2010; 170(2):645–654. [PubMed: 20603193]

Prendergast MA, Little HJ. Adolescence, glucocorticoids and alcohol. Pharmacol Biochem Behav. 2007; 86(2):234–245. [PubMed: 16930684]

Rivier C, Lee S. Effect of repeated exposure to alcohol on the response of the hypothalamic-pituitary-adrenal axis of the rat: II. Role of the length and regimen of alcohol treatment. Alcohol Clin Exp Res. 2001; 25(1):106–111. [PubMed: 11198704]

Roberts MC, Emsley RA, Pienaar WP, Stein DJ. Anxiety disorders among abstinent alcohol-dependent patients. PsychiatrServ. 1999; 50:1359–1361.

SAMHSA. Substance Abuse and Mental Health Services Administration. NSDUH Series H-41, HHS Publication No. (SMA) 11–4658. Rockville, MD: Substance Abuse and Mental Health Services Administration; 2011. Results from the 2010 National Survey on Drug Use and Health: Summary of National Findings.

Sanna E, Serra M, Cossu A, Colombo G, Follesa P, Cuccheddu T, Concas A, Biggio G. Chronic Ethanol Intoxication Induces Differential Effects on GABAA and NMDA Receptor Function in the Rat Brain. Alcoholism: Clinical and Experimental Research. 1993; 17(1):115–123.

Shen H, Gong QH, Aoki C, Yuan M, Ruderman Y, Dattilo M, Williams K, Smith SS. Reversal of neurosteroid effects at α4β2δ GABAA receptors triggers anxiety at puberty. Nat Neurosci. 2007; 10(4):469–477. [PubMed: 17351635]

Silveri MM, Spear LP. Characterizing the ontogeny of ethanol-associated increases in corticosterone. Alcohol. 2004; 32:145–155. [PubMed: 15163565]

Silvers JM, Tokunaga S, Berry RB, White AM, Matthews DB. Impairments in spatial learning and memory: ethanol, allopregnanolone, and the hippocampus. Brain Res Brain Res Rev. 2003a; 43(3):275–284. [PubMed: 14629930]

Silvers JM, Tokunaga S, Mittleman G, Matthews DB. Chronic intermittent injections of high-dose ethanol during adolescence produce metabolic, hypnotic, and cognitive tolerance in rats. Alcoholism, Clinical and Experimental Research. 2003b; 27(10):1606–1612.

Silvers JM, Tokunaga S, Mittleman G, O'Buckley T, Morrow AL, Matthews DB. Chronic intermittent ethanol exposure during adolescence reduces the effect of ethanol challenge on hippocampal allopregnanolone levels and Morris water maze task performance. Alcohol. 2006; 39(3):151–158. [PubMed: 17127134]

Smothers CT, Mrotek JJ, Lovinger DM. Chronic ethanol exposure leads to a selective enhancement of N-methyl-D-apartate receptor function in cultured hippocampal neurons. J Pharmacol Exp Ther. 1997; 283(3):1214–1222. [PubMed: 9399996]

Spear LP. The adolescent brain and age-related behavioral manifestations. Neuroscience and Biobehavioral Reviews. 2000; 24:417–463. [PubMed: 10817843]

Spear LP, Varlinskaya EI. Adolescence. Alcohol sensitivity, tolerance, and intake. Recent Developments in Alcoholism. 2005; 17:143–159. [PubMed: 15789864]

Van Skike CE, Botta P, Chin VS, Tokunaga S, McDaniel JM, Venard J, Diaz-Granados JL, Valenzuela CF, Matthews DB. Behavioral effects of ethanol in cerebellum are age dependent: potential system and molecular mechanisms. Alcoholism, Clinical and Experimental Research. 2010; 34(12):2070–2080.

White AM, Truesdale MC, Bae JG, Ahmad S, Wilson WA, Best PJ, Swartzwelder HS. Differential effects of ethanol on motor coordination in adolescent and adult rats. Pharmacology, Biochemistry, and Behavior. 2002; 73:673–677.

Wiley JL, Evans RL. To breed or not to breed? Empirical evaluation of drug effects in adolescent rats. International Journal of Developmental Neuroscience. 2009; 27(1):9–20. [PubMed: 19041390]

Willinger U, Lenzinger E, Hornik K, Fischer G, Schönbeck G, Aschauer HN, Meszaros K. Anxiety as a Predictor of Relapse in Detoxified Alcohol-Dependent Patients. Alcohol and Alcoholism. 2002; 37(6):609–612. [PubMed: 12414556]

Yu Z-Y, Wang W, Fritschy J-M, Witte OW, Redecker C. Changes in neocortical and hippocampal GABAA receptor subunit distribution during brain maturation and aging. Brain Research. 2006; 1099(1):73–81. [PubMed: 16781682]

Zorrilla EP, Logrip ML, Koob GF. Corticotropin releasing factor: a key role in the neurobiology of addiction. Frontiers in Neuroendocrinology. 2014; 35:234–244. [PubMed: 24456850]

Zorrilla EP, Valdez GR, Weiss F. Changes in levels of regional CRF-like-immunoreactivity in plasma and corticosterone during protracted drug withdrawal in dependent rats. Psychopharmacology. 2001; 158:374–381. [PubMed: 11797058]

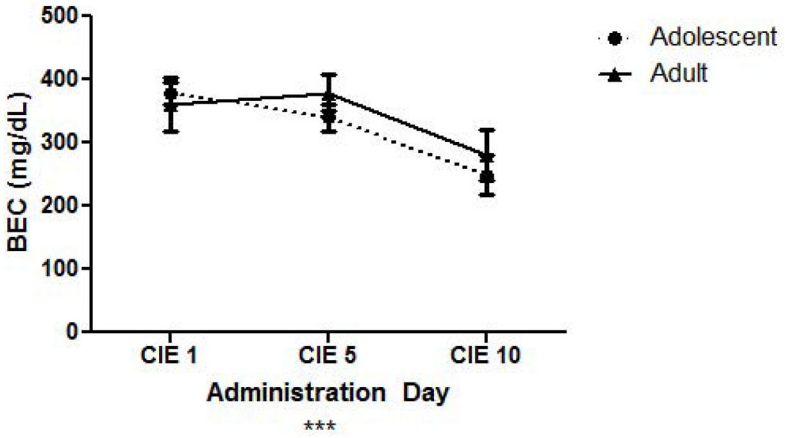

Figure 1.

Repeated i.p. ethanol administration yields progressively decreasing blood ethanol concentrations in adolescent and adult rats. Data are mean ± SEM; ***p<0.001, main effect of administration day.

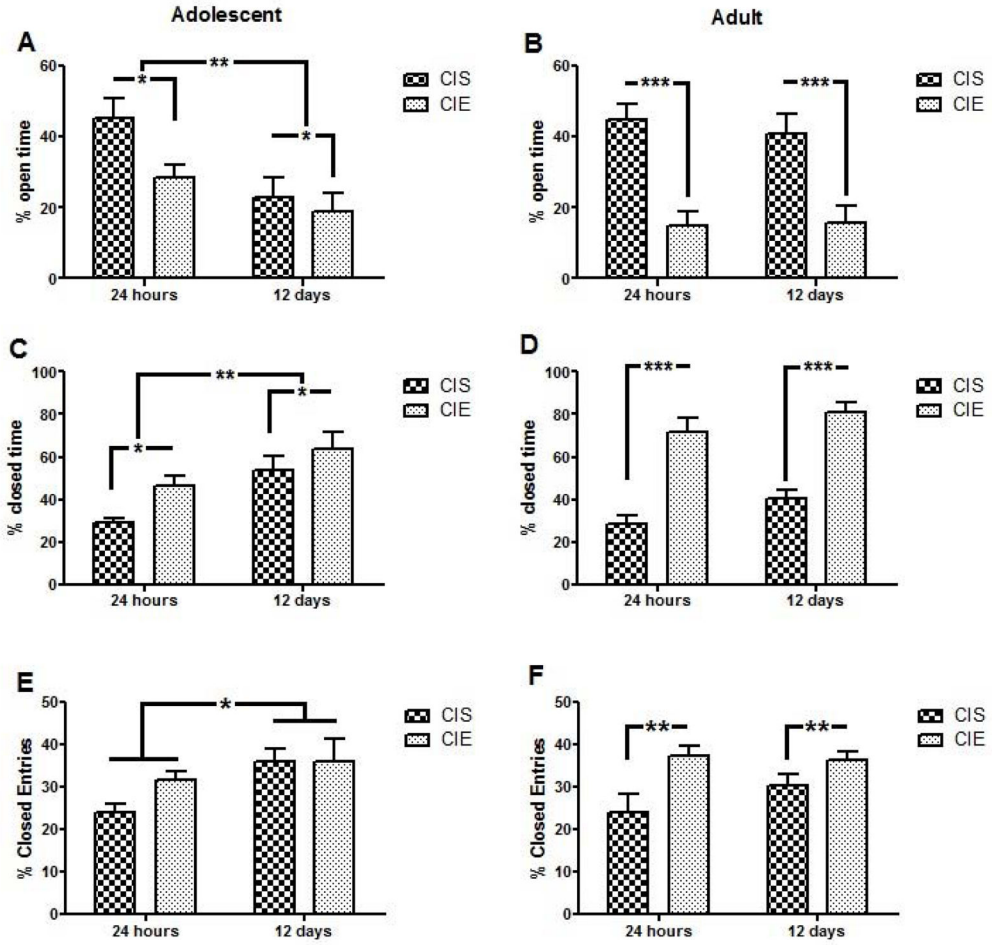

Figure 2.

Withdrawal and protracted abstinence from chronic intermittent ethanol persistently increases anxiety in adolescent and adult rats. Percentage of time spent in open arms in A) adolescents and B) adults; percent closed time in C) adolescents and D) adults; percentage of closed entries in E) adolescent and F) adult rats. Data are mean ± SEM; *p<0.05, **p<0.01, ***p<0.001.

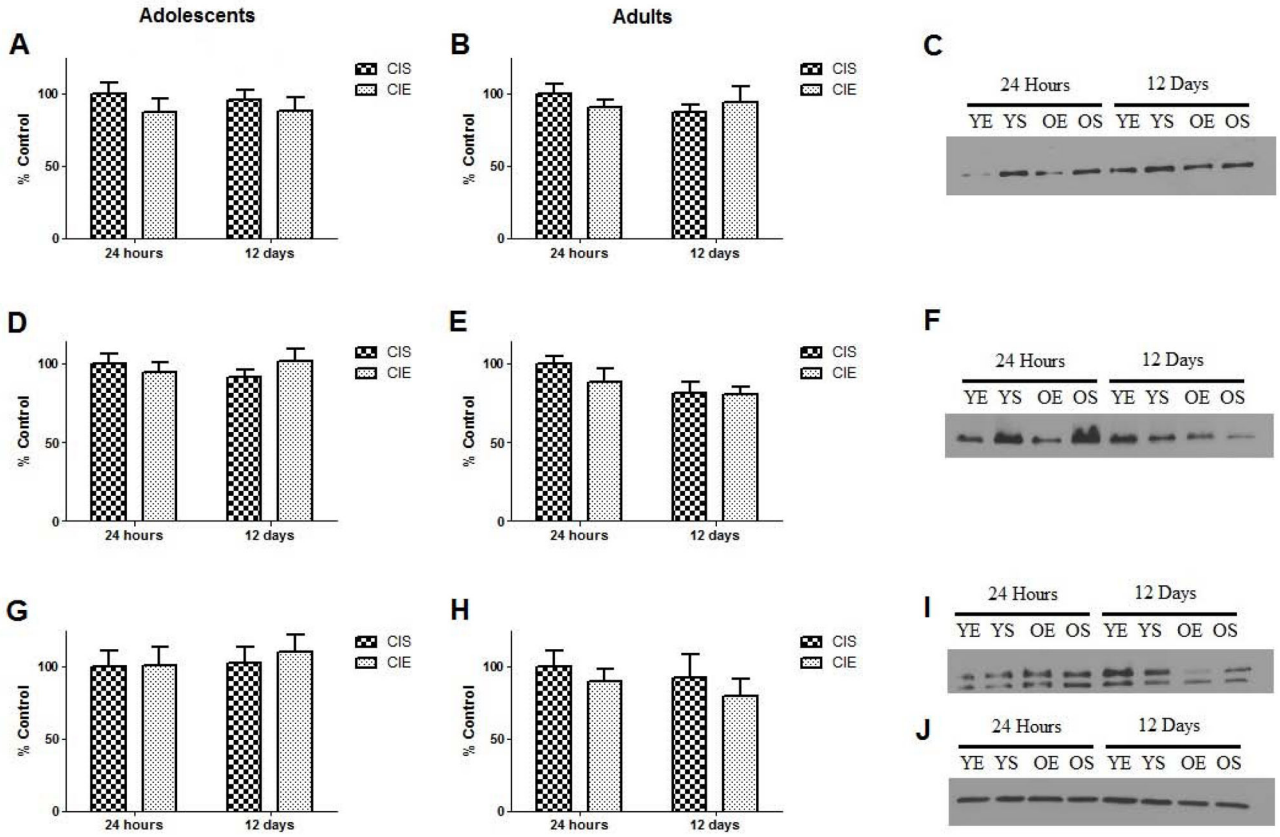

Figure 3.

Cortical NMDA receptor subunit expression. NR1 expression in A) adolescent and B) adult rats, with C) representative Western blot at 130 kDa. NR2A expression in D) adolescent and E) adult rats, with F) representative NR2A blot at 170 kDa. NR2B expression for G) adolescents and H) adults, with I) representative NR2B, top line at 180 kDa and J) representative β-actin at 43 kDa. All measurements are normalized to actin and taken in triplicate. Figures are normalized to CIS at 24 hours withdrawal. Data are presented as mean ± SEM. YE = Adolescent (Young) CIE, YS = Adolescent CIS, OE = Adult (Old) CIE, OS = Adult CIS.

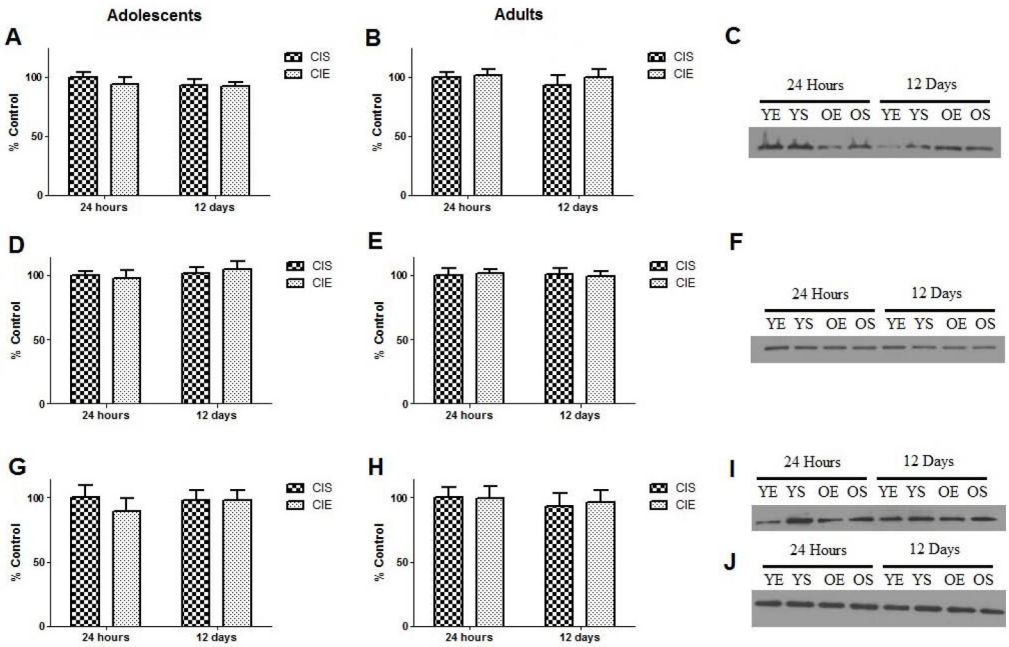

Figure 4.

Cortical $GABA_A$ receptor subunit expression. $GABA_A$ β2,3 expression in A) adolescent and B) adult rats, with C) representative Western blot at 55 kDa. D) Adolescent and E) adult α1 expression and F) representative Western blot at 51 kDa. G) Adolescent and H) adult α4 expression and I) representative α4 at 62 kDa, with J) representative β-actin at 43 kDa. All measurements normalized to actin and taken in triplicate. Figures are normalized to CIS at 24 hours withdrawal. Data are presented as mean ± SEM. YE = Adolescent (Young) CIE, YS = Adolescent CIS, OE = Adult (Old) CIE, OS = Adult CIS.

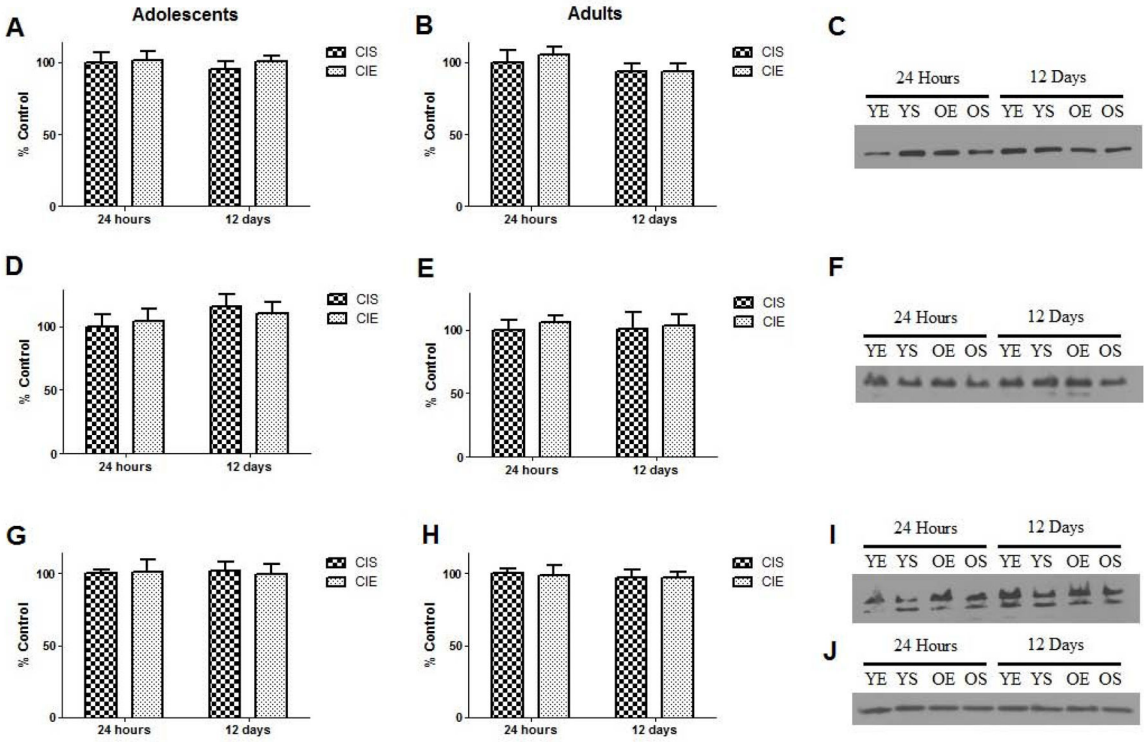

Figure 5.

NMDA subunit expression in the hippocampus. NR1 expression in A) adolescent and B) adult rats, with C) representative Western blot at 130 kDa. D) Adolescent and E) adult NR2A expression and F) representative Western blot at 170 kDa. G) Adolescent and H) adult NR2B expression and I) representative blot, top line at 180 kDa, with J) representative β-actin at 43 kDa. All measurements normalized to actin and taken in triplicate. Figures are normalized to CIS at 24 hours withdrawal. Data are presented as mean ± SEM. YE = Adolescent (Young) CIE, YS = Adolescent CIS, OE = Adult (Old) CIE, OS = Adult CIS.

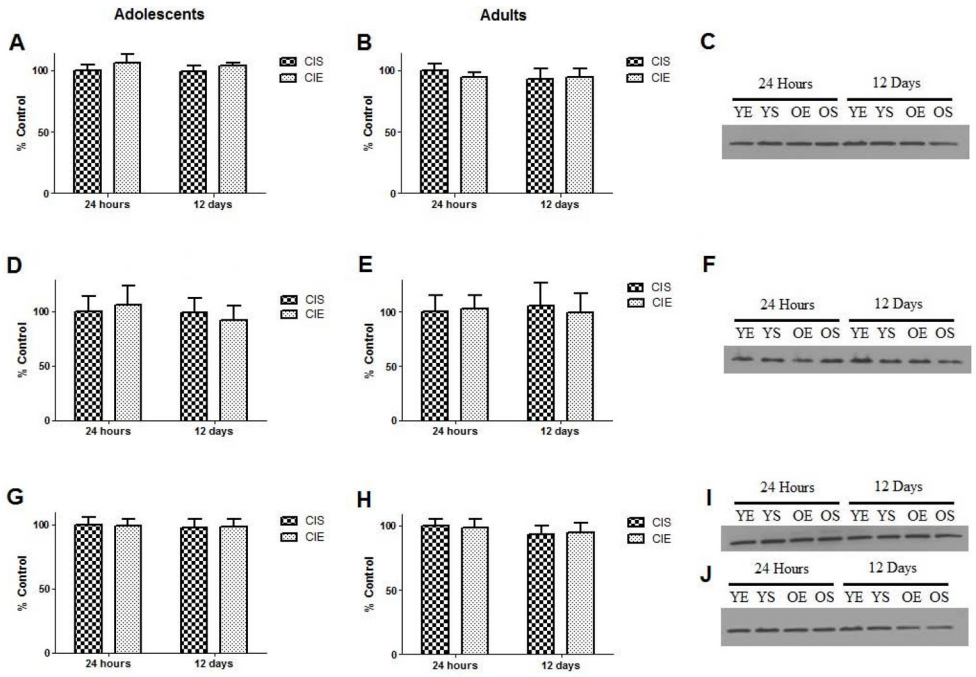

Figure 6.

$GABA_A$ receptor subunit expression in the hippocampus. $GABA_A$ β2,3 expression in A) adolescent and B) adult rats, with C) representative Western blot at 55 kDa. D) Adolescent and E) adult α1 expression and F) representative Western blot at 51 kDa. G) Adolescent and H) adult α4 expression and I) representative α4 at 62 kDa, with J) representative β-actin at 43 kDa. All measurements normalized to actin and taken in triplicate. Figures are normalized to CIS at 24 hours withdrawal. Data are presented as mean ± SEM. YE = Adolescent (Young) CIE, YS = Adolescent CIS, OE = Adult (Old) CIE, OS = Adult CIS.

Table 1

Age-dependent peptide expression in CIS treated animals. Data are expressed as mean percent control ± SEM. Controls are set to the timepoint matched adult.

		24 hours		12 days		
		Adolescent PD 49	**Adult PD 129**	**Adolescent PD 60**	**Adult PD 150**	**Effects**
Cortex	NR1	109.9 ± 6.6	100 ± 6.6	121.0 ± 7.1	100 ± 6.6	n.s.
	NR2A	96.2 ± 2.2	100 ± 4.4	112.0 ± 6.7	100 ± 9.0	* 24h vs 12d, p<0.05
	NR2B	92.2 ± 3.0	100 ± 10.3	108.6 ± 6.0	100 ± 17.5	n.s.
	α1	100.9 ± 4.8	100 ± 5.7	100.0 ± 3.3	100 ± 4.0	n.s.
	β2,3	105.3 ± 3.8	100 ± 4.0	106.8 ± 6.0	100 ± 8.9	n.s.
	α4	102.1 ± 6.6	100 ± 7.9	111.2 ± 11.9	100 ± 10.9	n.s.
Hippocampus	NR1	103.2 ± 7.7	100 ± 8.6	104.1 ± 7.6	100 ± 6.0	n.s.
	NR2A	96.5 ± 6.1	100 ± 7.2	116.3 ± 10.8	100 ± 13.3	n.s.
	NR2B	96.1 ± 1.2	100 ± 10.7	101.4 ± 6.7	100 ± 6.0	n.s.
	α1	106.5 ± 3.6	100 ± 15.5	104.5 ± 6.6	100 ± 20.5	n.s.
	β2,3	91.8 ± 3.4	100 ± 5.4	101.1 ± 7.4	100 ± 9.6	n.s.
	α4	98.0 ± 4.2	100 ± 5.2	103.2 ± 7.3	100 ± 7.1	n.s.

Table 2

Cortical expression levels. Data are expressed as mean percent control (CIS, 24 hours) ± SEM.

		Cortex			
		CIE		CIS	
		24 hours	12 days	24 hours	12 days
Adolescent	NR1	87.4 ± 9.4	88.0 ± .9.0	100.0 ± 7.5	95.7 ± 6.8
	NR2A	94.7 ± 6.2	101.5 ± 7.8	100.0 ± 6.3	91.6 ± 4.5
	NR2B	100.4 ± 13.2	110.0 ± 11.7	100.0 ± 10.7	102.3 ± 11.0
	α1	97.5 ± 6.1	104.5 ± 6.2	100.0 ± 2.9	101.3 ± 4.7
	β2,3	94.2 ± 5.9	92.3 ± 3.7	100.0 ± 4.1	93.3 ± 4.6
	α4	89.1 ± 9.9	97.9 ± 8.0	100.0 ± 9.8	97.8 ± 7.9
Adult	NR1	90.6 ± 4.7	94.4 ± 10.5	100.0 ± 6.6	86.9 ± 5.7
	NR2A	88.0 ± 8.5	80.3 ± 4.7	100.0 ± 4.4	81.0 ± 7.3
	NR2B	89.8 ± 7.9	79.6 ± 11.5	100.0 ± 10.3	91.8 ± 16.1
	α1	101.6 ± 3.5	99.0 ± 4.1	100.0 ± 5.7	101.1 ± 4.1
	β2,3	101.9 ± 5.2	99.7 ± 6.8	100.0 ± 4.0	93.5 ± 8.3
	α4	99.4 ± 9.8	96.5 ± 9.1	100.0 ± 7.9	93.3 ± 10.2

Table 3

Hippocampal expression levels. Data are expressed as mean percent control (CIS, 24 hours) ± SEM.

		Hippocampus			
		CIE		CIS	
		24 hours	12 days	24 hours	12 days
Adolescent	NR1	101.8 ± 6.3	100.7 ± 4.2	100.0 ± 6.7	95.5 ± 5.0
	NR2A	103.8 ± 9.8	110.5 ± 8.7	100.0 ± 9.5	115.4 ± 9.7
	NR2B	101.0 ± 8.9	99.6 ± 7.0	100.0 ± 2.5	101.6 ± 6.7
	α1	106.1 ± 18.2	91.9 ± 13.3	100.0 ± 13.8	99.2 ± 13.6
	β2,3	105.6 ± 7.5	103.7 ± 2.4	100.0 ± 4.5	99.5 ± 4.1
	α4	98.6 ± 5.6	98.0 ± 6.2	100.0 ± 5.5	97.3 ± 7.1
Adult	NR1	105.5 ± 5.4	93.7 ± 5.1	100.0 ± 8.6	94.0 ± 5.6
	NR2A	106.2 ± 5.3	103.0 ± 9.4	100.0 ± 7.2	100.3 ± 13.3
	NR2B	98.8 ± 6.6	97.3 ± 4.0	100.0 ± 3.6	97.1 ± 5.8
	α1	103.2 ± 12.5	99.9 ± 17.3	100.0 ± 15.5	105.3 ± 21.6
	β2,3	94.6 ± 4.0	94.8 ± 6.6	100.0 ± 5.4	92.8 ± 8.9
	α4	98.4 ± 6.5	95.0 ± 7.0	100.0 ± 5.2	93.3 ± 6.6