Physiology & Behavior 96 (2009) 169–173

Contents lists available at ScienceDirect

Physiology & Behavior

journal homepage: www.elsevier.com/locate/phb

Persisting changes in basolateral amygdala mRNAs after chronic ethanol consumption

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ARTICLE INFO

Article history:
Received 9 February 2007
Received in revised form 29 August 2008
Accepted 25 September 2008

Keywords:
Basolateral amygdala
Ethanol
$GABA_A$
NMDA
GAD_{67}
CRF
Adolescence
In situ hybridization

ABSTRACT

Adolescent alcohol use is common and evidence suggests that early use may lead to an increased risk of later dependence. Persisting neuroadaptions in the amygdala as a result of chronic alcohol use have been associated with negative emotional states that may lead to increased alcohol intake. This study assessed the long-term impact of ethanol consumption on levels of several basolateral amygdala mRNAs in rats that consumed ethanol in adolescence or adulthood. Male Long-Evans rats were allowed restricted access to ethanol or water during adolescence (P28, $n=11$, controls=11) or adulthood (P80, $n=8$, controls=10) for 18 days. After a sixty day abstinent period, the brain was removed and sections containing the basolateral amygdala were taken. *In situ* hybridization was performed for $GABA_A$ α_1, glutamic acid decarboxylase (GAD_{67}), corticotropin releasing factor (CRF), and N-methyl-D-aspartate (NMDA) NR2A mRNAs. A significant decrease was observed in $GABA_A$ α_1, GAD_{67}, and CRF, but not NR2A, mRNAs in adult rats that consumed ethanol in comparison to controls. No significant changes were seen in adolescent consumers of ethanol for any of the probes tested. A separate analysis for each probe in the piriform cortex ascertained that the changes after ethanol consumption were specific to the basolateral amygdala. These results indicate that chronic ethanol consumption induces age-dependent alterations in basolateral amygdala neurochemistry.

1. Introduction

Adolescence is a neurodevelopmental stage marked by changes in both emotional and cognitive faculties [1]. Alcohol consumption is common in adolescence with 42% of 12–17 year olds reporting having used alcohol in their lifetime [2]. These figures are important since adolescent alcohol exposure increases the likelihood of adult dependence [3]. Numerous animal models have shown that alcohol exposure during adolescence produces differing neurobehavioral alterations compared with adults [4]. Few basic studies have assessed whether neurobehavioral alterations resulting from adolescent alcohol exposure extend into adulthood [5].

Recently, Bergstrom and colleagues [6] found that ethanol consumption during adolescence impaired expression of fear conditioning in adulthood. Corresponding adult ethanol consumption did not produce long-term alterations in fear conditioning, suggesting that lasting impairment of fear conditioning following ethanol consumption is age-dependent. Given that the amygdala plays an important role in the acquisition and consolidation of fear conditioning [7], lasting impairment of fear conditioning following adolescent ethanol exposure may be the result of ethanol's impact on amygdala neurochemistry.

Ethanol interacts with numerous neurotransmitter systems in the basolateral amygdala (BLA) [8], including the gamma-aminobutyric acid (GABA) family of receptors. Particularly high densities of $GABA_A$ receptors are found in the BLA [9], and activation of $GABA_A$ receptors has been shown to influence fear conditioning [10]. Chronic ethanol exposure has been shown to decrease $GABA_A$ α_1 subunit expression within the BLA [11]. Another marker of GABAergic expression, glutamic acid decarboxylase ($GAD_{65/67}$), a GABA synthesizing enzyme, has been associated with fear learning [12], and was found to be altered in the hippocampus and cerebral cortex at three days of ethanol withdrawal [13]. Whether $GABA_A$ α_1 or GAD_{67} mRNA in the BLA is altered following long-term discontinuation from chronic ethanol has not been determined.

The BLA contains high levels of corticotropin-releasing factor (CRF) receptors [14,15] and CRF signaling plays a role in amygdala dependent emotional learning and memory formation [16–18]. Increased CRF levels have been found following extended abstinence from ethanol in adult animals [19,20]. It is not known, however, how chronic ethanol consumption impacts CRF mRNA when ethanol is administered during the adolescent period.

Ethanol has an inhibitory effect on N-methyl-D-aspartate (NMDA) receptors [21,22] with heteromeric assemblies containing NR2A or NR2B receptor subunits being particularly sensitive to ethanol [23–25]. NMDA receptors in the lateral amygdala have been shown to play a unique role in fear learning [26] with selective blockade of the NR2A receptor in the amygdala preventing both fear conditioning and fear expression [27]. No work has yet assessed NR2A mRNA expression in the BLA following long-term discontinuation from chronic ethanol.

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doi:10.1016/j.physbeh.2008.09.019

The purpose of this study was to evaluate expression of $GABA_A$ α_1, GAD_{67}, CRF, and NR2A mRNAs in the BLA following protracted abstinence from chronic ethanol consumption in adolescence and adulthood. Animals were exposed to ethanol either during adolescence or adulthood and *in situ* hybridization was performed 60 days following discontinuation from ethanol. It was hypothesized that ethanol's impact on BLA mRNA levels would differ depending on the age of consumption, given the previous finding of age-dependent differences in fear conditioning from the same cohort [6].

2. Materials and methods

2.1. Subjects

Male Long-Evans hooded rats were obtained at postnatal day (P) 22 and P55–60 (Harlan, Indianapolis, IN). Rats were housed individually in metal wire-hanging cages and maintained at constant temperature (22–24 °C), on a 12 h L:D cycle (lights on at 7:00 am) with *ad libitum* food and water except when restricted during ethanol exposure. Rats were handled every third day by a trained animal care technician. Animal care was in accordance with George Mason University guidelines and National Institute of Health Guide for the Care and Use of Laboratory Animals.

2.2. Restricted access ethanol drinking

Adolescent male rats at P28 ($n=11$, ethanol group; $n=11$, control group) and adult male rats at P80 ($n=10$, ethanol group; $n=10$, control group) were used for this experiment.

A restricted access ethanol consumption procedure was used. Ethanol consumption was calculated for each rat as gram of ethanol per kilogram of body weight (g/kg). Rats were randomly assigned to ethanol (Pharmco, Brookfield, CT; 95%) or water (H_2O) groups. The drinking schedule lasted 18 days starting on P28 for the adolescent group and P80 for the adult group. Parameters measured daily were body weight (g), ethanol consumed in milliliters (mL), and water consumed (mL). Control (tap water [H_2O]) and experimental (ethanol) groups were both H_2O deprived 24 h prior to ethanol and throughout the 18-day schedule. Food was provided *ad libitum*. Each dosing period began at approximately 10:00 am with the animals in the experimental group receiving access to a bottle containing 10% ethanol solution (v/v). Rats received 1-h access to the ethanol bottle. After 1 h, the bottle was removed for 30 min. Following the 30 min delay, the rats were presented with H_2O for 30 min. To control for the potential dehydrating effects of reduced H_2O availability, the control group was yoked to the experimental group so that the control group was presented with approximately the same total fluid consumed as the experimental group. On P45 for adolescents and P97 for adults, rats were placed back on *ad libitum* H_2O for 30 days before behavioral testing in order to eliminate potential ethanol withdrawal effects. Following 30 days discontinuation from ethanol, rats were tested in an auditory fear conditioning behavioral paradigm. Methods and results have been described in a prior publication [6]. Sixty days after discontinuation from ethanol, rats were sacrificed for tissue processing.

2.3. Probes

The $GABA_A$ α_1 mRNA probe was a 48-mer probe (5′-CT GGT TGC TGT TGG AGC GTA AGT GTT GTT TTT CTT AAT AAG AGG ATC C-3′). The preproCRF mRNA probe was a 48-mer probe (5′ GAC ACC GCC CAA AGC CAG GAC GAT GCA GAG CGC GGC CAG CGC GCA CTG 3′) [28]. The GAD_{67} mRNA probe was a 48-mer probe (5′ TGG TAT TGG CAG TTG ATG TCA GCC ATT CGC CAG CTA AAC CAA TGA TAT 3′). A 48-mer probe was used as the control probe for all 48-mer probes (5′ AAT ACA CCG AGC GGT ACT CGA GGT GGT ACA TGT TGG GGT AGT AAA TAA 3′). The NR2A mRNA probe was a 32-mer probe (5′ TTC TGT GCT CAC GGC CAC CTC CAC CGT GTT AG 3′) [29]. A 32-mer mis-sense probe was used as the control probe (5′ GTG GCG AAA TCG TAG GGT CTA ACC GGG TAC GG 3′).

2.4. In situ hybridization

Adolescent male rats ($n=11$, ethanol group; $n=11$, control group) and adult male rats ($n=8$, ethanol group; $n=10$, control group) were used for *in situ* hybridization. Rats were sacrificed by guillotine decapitation 60 days after dosing was completed. Brains were quickly removed, frozen in powdered dry ice, and kept frozen in air-tight plastic freezer bags at −80 °C until cryostat-sectioning. Tissue was cryostat cut in 16 μm coronal sections (Sakura Finetek, Torrance, CA) and thaw-mounted on a series of gelatin-subbed glass slides for *in situ* hybridization. Slides with BLA represented were selected from corresponding histological slides (stereotaxic atlas coordinates from −2.30 to −2.80 mm Bregma [30]). A control area from the piriform cortex immediately adjacent to BLA was also selected from each slide to determine if a difference in mRNA between groups was specific to BLA. The boundaries of the BLA and piriform cortex were defined according to Paxinos and Watson [30].

Oligonucleotide probes (Oligo's Etc., Wilsonville, OR) were radiolabeled, using terminal deoxynucleotidyl transferase and deoxyadenosine [alpha-[35S]-thio] triphosphate, at the 3′ end, to a specific activity of $5–10\times10^5$ cpm/μl, according to the method described by Young [31].

Hybridization was carried out according to the method described by Young [31]. Briefly, warmed, dried sections were fixed in 4% formaldehyde/PBS for 5 min, rinsed in PBS, acetylated in 0.25% acetic anhydride/1 M triethanolamine hydrochloride (pH 8.0) for 10 min, dehydrated in graded ethanol, delipidated in chloroform for 5 min, rinsed in absolute and 95% ethanol, and air-dried. Hybridization buffer (50 μl) containing 50% formamide, 600 mM NaCl, 80 mM Tris–HCl (pH 7.5), 4 mM EDTA, 0.1% sodium pyrophosphate, 0.2% sodium dodecyl sulfate, 0.2 mg/mL sodium heparin, 100 mM dithiothreitol, 10% dextran sulfate, .01% cold polyadenylic acid, plus 1×10^6 cpm of labelled probe, was then pipetted onto each slide, and parafilm coverslips added. Slides were incubated at 37 °C overnight. Coverslips were then removed in SSC, and slides rinsed and collected in 1× SSC, and washed in 4 changes of 1× SSC at 60 °C for the 48-mer probes, or 50 °C for the 32-mer probes, for 15 min each, and 2 changes of room temperature 1× SSC for 30 min each. Slides were rinsed in water and 70% ethanol, and air-dried. All solutions used water treated with diethylpyrocarbonate (DEPC).

2.5. Autoradiography and image measurement

Biomax film (Eastman Kodak, New Haven, CT) was exposed to treated slides and ^{14}C standards (ARC Inc., St. Louis, MO) in a cassette, and then developed. Autoradiographic images of individual slides were converted to TIFF files with a flatbed scanner. Using NIH Image (Rasband, NIH), regions of interest (BLA and piriform cortex (Fig. 1)) were then sampled manually, with optical density interpolated along the calibration curve established from the standards.

2.6. Data analysis

SPSS (v. 13) for Windows was used for all statistical analyses. Comparisons for ethanol consumption were made by using a 2×6 (age by day) repeated measures analysis of variance (ANOVA), with day (3-day intervals) being the repeated measure. Independent sample *t*-tests were used to examine between-group differences at individual time points. A Bonferroni correction was used when appropriate. Comparisons for total liquid consumption was made using a 2×2×6 (treatment by age by day) repeated measures ANOVA with drug and age

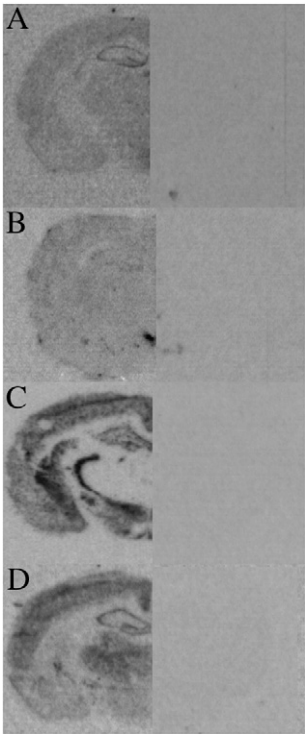

Fig. 1. All representative autoradiographs come from adult rats that consumed ethanol. These subjects are used for continuity and are representative of anatomical location and overall probe binding as qualitative differences due to age and control and experimental subjects cannot be visually determined. (A) Left image is representative slice containing basolateral amygdala (BLA) labeled with antisense NMDA NR2A oligoprobe. Right image is representative slice containing BLA labeled with 32-base mis-sense oligoprobe. (B) Left image is representative slice containing BLA labeled with antisense preproCRF oligoprobe. Right image is representative slice containing BLA labeled with control 48-base oligoprobe. (C) Left image is representative slice containing BLA labeled with antisense GAD_{67} oligoprobe. Right image is representative slice containing BLA labeled with control 48-base oligoprobe. (D) Left image is representative slice containing BLA labeled with antisense $GABA_A$ α_1 oligoprobe. Right image is representative slice containing BLA labeled with control 48-base oligoprobe.

representing the between groups factors and day (3-day intervals) being the repeated measure. A 2×2 (treatment by age) univariate ANOVA was conducted for each mRNA measured. For all analyses, p values less than or equal to 0.05 were considered significant.

3. Results

3.1. Ethanol consumption

Ethanol consumption data are reported in grams per kilogram (g/kg), averaged over blocks of three days. Repeated measures ANOVA showed no overall age×day interaction on ethanol consumption. Consumption levels ranged from 2.1 to 3.0 g/kg for adolescents and from 1.5 to 3.3 g/kg for adults, averaged over 18 days. Analysis of individual time points indicated that days 4 to 6 ($t[17]=3.3$; $p<0.05$) differed significantly by age. The mean daily consumption levels over the 18 day ethanol drinking period were 2.49 g/kg for adolescents and 2.3 g/kg for adults.

3.2. Total fluid consumption

Total fluid intake values were expressed as milliliters-per-kilogram-of-body-weight (mL/kg) averaged over 18 drinking days. Overall, adolescents consumed significantly more total fluid than adults ($F[1,38]=125.1$; $p<0.001$) with consumption levels ranging from 6.3 to 10.9 mL/kg for adolescents and from 4.0 to 6.7 mL/kg for adults. In addition, animals exposed to ethanol consumed more total fluid than

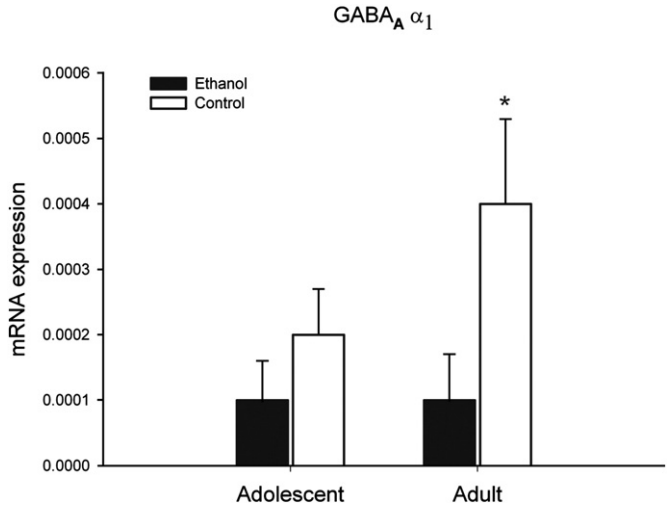

Fig. 2. $GABA_A$ α_1 mRNA receptor levels in basolateral amygdala in rats exposed to ethanol (EtOH) during adolescence (EtOH exposed, $n=11$; control, $n=11$) or adulthood (EtOH exposed, $n=8$, control, $n=10$). Adults dosed with EtOH showed significant decrease in $GABA_A$ α_1 mRNA compared to controls. Error bars = standard error of the mean.* = $p\leq.05$.

controls (7.9±.49>6.7±.39), although the difference was not statistically significant.

3.3. In situ hybridization

Univariate ANOVA was conducted between age and treatment for each mRNA measured. Rats that consumed ethanol during adulthood showed a significant decrease in $GABA_A\alpha_1$ receptor mRNA in the BLA ($F[1,16]=4.55$, $p<.05$; Fig. 2) and GAD_{67} mRNA ($F[1,17]=4.44$, $p\leq.05$; Fig. 3) in the BLA in comparison to controls. In addition, a correlation was found between the expression of $GABA_A\alpha_1$ mRNA and GAD_{67} mRNA in the BLA ($r=0.82$, $p<.05$) among adult ethanol consumers, but not controls. Ethanol consumption during adulthood was also associated with a significant decrease in overall preproCRF mRNA in the BLA ($F[1,17]=4.53$, $p<.05$) compared to controls (Fig. 4). Rats that consumed ethanol during adulthood were not different from controls for NR2A subunit mRNA in BLA (Fig. 5). There were no significant

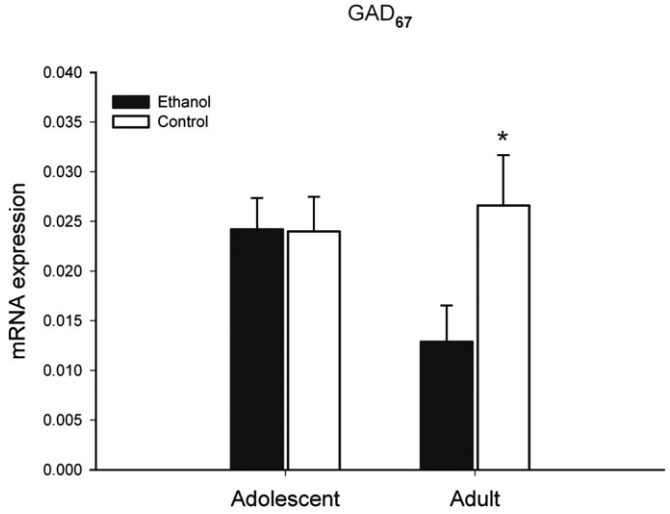

Fig. 3. GAD_{67} mRNA levels in basolateral amygdala in rats exposed to ethanol (EtOH) during adolescence (EtOH exposed, $n=11$; control, $n=11$) or adulthood (EtOH exposed, $n=8$, control, $n=10$). Adults dosed with EtOH showed significant decrease in GAD_{67} mRNA compared to controls. Error bars = standard error of the mean. * = $p\leq.05$.

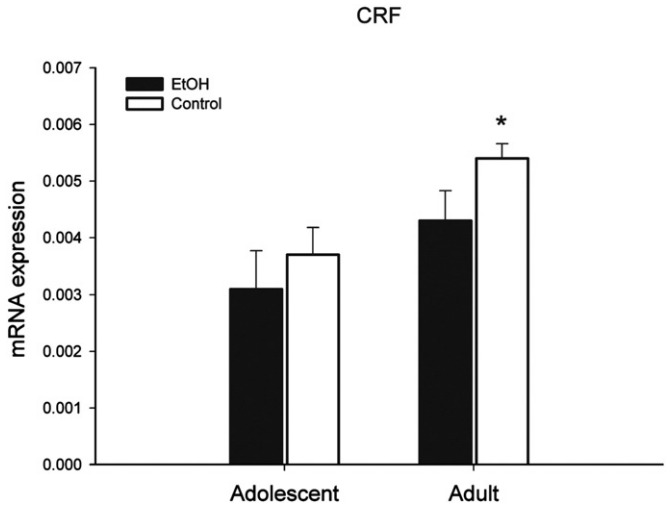

Fig. 4. preproCRF mRNA levels in basolateral amygdala in rats exposed to ethanol (EtOH) during adolescence (EtOH exposed, $n=11$; control, $n=11$) or adulthood (EtOH exposed, $n=8$, control, $n=10$). preproCRF mRNA was decreased in adults dosed with EtOH relative to controls. Error bars = standard error of the mean. * = $p \leq .05$.

changes in rats that consumed ethanol during adolescence in any of the mRNAs tested.

3.4. Control (piriform) region

A control region in the piriform cortex immediately adjacent to the BLA was analyzed for the CRF, GAD_{67} and $GABA_A\alpha_1$ mRNAs to examine neuroanatomical specificity of ethanol's effects on neurochemistry in the BLA. ANOVA revealed no significant differences in the piriform cortex for CRF or GAD_{67} mRNAs between animals based on either age or treatment condition, suggesting that the differences between adult rats that consumed ethanol and controls were unique to the BLA. In contrast, $GABA_A\alpha_1$ mRNA was higher in adolescent controls than adult controls in piriform cortex ($F[1,17]=7.95$, $p<.05$). There were no significant differences among ethanol consuming animals. These results indicate that the lasting neurochemical effects of ethanol in adults were distinct to the BLA.

4. Discussion

This experiment compared the lasting effects of limited-access ethanol drinking during adolescence and adulthood on $GABA_A\ \alpha_1$, GAD_{67}, CRF, and NR2A subunit mRNA levels in the BLA. It was hypothesized that adult mRNA levels would be significantly altered in animals after adolescent ethanol consumption. However, the data did not support this hypothesis. Chronic ethanol consumption failed to yield any discernible changes in mRNA levels in BLA of rats after ethanol consumption during adolescence. Instead, significant changes were found in $GABA_A\ \alpha_1$, GAD_{67}, and CRF mRNAs among rats that consumed ethanol during adulthood. Specifically, $GABA_A\ \alpha_1$, GAD_{67}, and CRF mRNAs were decreased in the BLA of the adult ethanol group compared to control.

mRNA levels were not assessed until sixty days following completion of the ethanol consumption and thirty days after impairment in auditory fear conditioning was found [6]. It is possible that alterations were initially present in rats that consumed ethanol during adolescence, but were reversed due to homeostatic processes during continued development of the adolescent brain [32,33]. Our findings correspond with those from Slawecki and colleagues [34] that reported no changes in CRF protein levels in the amygdala of adolescent rats after dosing with ethanol vapor for ten days and assaying for neuropeptides seven weeks later.

Adult rats that consumed ethanol exhibited decreased levels of $GABA_A\ \alpha_1$ subunit mRNA. These data coincide with several studies showing decreases in $GABA_A\ \alpha_1$ subunit mRNA and protein levels, particularly in the amygdala [11,35–37]. The current study extends previous data on the relationship between chronic ethanol and $GABA_A\ \alpha_1$ by showing $GABA_A\ \alpha_1$ mRNA decreases in the BLA following a significantly longer time interval between ethanol consumption and sacrifice than in previous studies. To our knowledge, this is the first study to show decreased GAD_{67} mRNA in the BLA following long-term discontinuation from chronic alcohol consumption. GAD_{67} is a GABA synthesizing enzyme and, ultimately, lower levels of GAD_{67} would be predicted to modify GABA output in the BLA.

CRF changes would be expected to co-occur with changes in $GABA_A\ \alpha_1$ mRNA, as CRF and GABA interactions have been shown in numerous regions, including the amygdala [38]. In the present study, the finding of long-lasting decreased CRF mRNA expression in the BLA of adults dosed with ethanol has varying support in previous research. While some studies have found no changes in amygdala CRF levels following ethanol exposure [34], Sommer et al. [20] recently showed that rats receiving chronic ethanol in a two-bottle paradigm exhibited increased CRF_1 and decreased CRF_2 levels in the BLA. Other evidence demonstrated that during the early phase of ethanol withdrawal CRF immunoreactivity decreased [19,39], but by 6 weeks, CRF levels were again increased above normal [19]. The present study is one of few studies examining the impact of ethanol on CRF in the BLA after extended (60 days) abstinence. Clearly, further studies are needed to unravel the long-term impact of ethanol on BLA CRF mRNA expression.

The present experiment found no changes in NMDA NR2A subunit mRNA levels in adults dosed with ethanol, a result that has mixed support in existing literature. Some research has found either modest or no effects on NR2A subunits after ethanol dosing in rodents in both BLA and central amygdala [40–42]. It is possible that ethanol's effects on NMDA receptors may act through NR2B subunits as a number of studies have found that ethanol alters the action and expression of these subunits [21,41,43].

The limited-access ethanol drinking schedule utilized in this experiment may have been differentially aversive in the two age groups, contributing to group differences in anxiety-like behavior. Adolescents have been shown to be particularly susceptible to stress [44] and some evidence suggests that certain brain regions, including the amygdala, exhibit differential stress activation relative to adults [45]. In the current study, the adolescent group exhibited increased ethanol consumption relative to the adult group, indicating that adolescents found ethanol less aversive than adults. Furthermore,

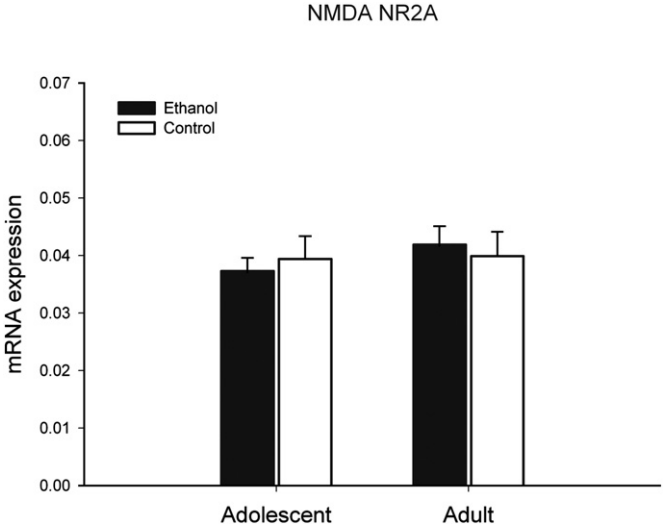

Fig. 5. NMDA NR2A mRNA subunit levels in basolateral amygdala in rats exposed to ethanol (EtOH) during adolescence (EtOH exposed, $n=11$; control, $n=11$) or adulthood (EtOH exposed, $n=8$, control, $n=10$). Neither age nor dosing group showed any differences in NR2A mRNA. Error bars = standard error of the mean.

stress resulting from dehydration was controlled for by a yoked control group that received the same volume of total fluid as the ethanol group. Adolescents in the ethanol group also drank significantly more fluid than the yoked controls, potentially counteracting any differences in dehydration. Another issue for concern is that the limited access drinking paradigm may have induced ethanol withdrawal. Withdrawal effects were not determined here and may warrant further study in relation to fear conditioning and mRNA expression in the amygdala.

This experiment demonstrated that chronic ethanol in a limited access drinking schedule contributes to the alteration of several mRNAs in the basolateral amygdala after an extensive time period following chronic ethanol dosing. The disconnect between lasting behavioral effects of adolescent, but not adult dosing [6], and lasting neurochemical effects of adult, but not adolescent, dosing is difficult to interpret. One possible explanation is that the behavioral effects observed by Bergstrom and colleagues [6] were mediated by neurochemical changes not measured here. The neurochemical changes reported here may then represent plastic changes in adults which do not themselves mediate observed behavioral changes in fear conditioning. It remains to be determined which specific neurotransmitter underlies impairment of fear conditioning found after adolescent ethanol dosing. Nevertheless, the present results indicate that ethanol exposure in adulthood altered $GABA_A$ α_1, GAD_{67}, and CRF mRNA expression in the amygdala; an effect that persisted for at least 60 days and was not observed following ethanol exposure in adolescence.

Acknowledgments

We thank Dr. Craig G. McDonald for editorial contributions and Juliza Chan for animal care. Research was supported by the Virginia Tobacco Settlement Foundation.

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