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Persisting changes in basolateral amygdala mRNAs after chronic ethanol consumption

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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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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