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Adolescent alcohol exposure alters the central brain circuits known to regulate the stress response

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Abstract

Adolescent alcohol exposure (AAE) may exert long-term effects on the adult brain. Here, we tested the hypothesis that the brain regions affected include the rat hypothalamic-pituitary-adrenal (HPA) axis. Specifically, we examined the consequences of AAE [postnatal days (PND) 28–42] on the HPA axis-related brain circuitry of male rats challenged with an intragastric (ig) administration of alcohol in young adulthood (PND 61–62). Adolescent rats were exposed to alcohol vapors, while controls did not receive the drug. The mean blood alcohol level in adolescence on PND 40 was 212.8 ± 5.7 mg %. Using immunohistochemistry and *in situ* hybridization procedures, we measured signals for *c-fos* and corticotropin releasing factor (CRF) in the paraventricular nucleus (PVN) of the hypothalamus, as well as signals for *c-fos* and phenylethanolamine N-methyltransferase (PNMT) in the adrenergic brain stem regions (C1 and C2). PVN CRF mRNA expression was significantly blunted in AAE rats tested at PND 61–62, compared to their controls. These animals also displayed a significant increase in the mean number of PNMT-ir cells/brain stem section in the C2 area. Collectively, these results suggest that exposure to alcohol vapors during adolescence exerts long-term effects on the ability of the PVN to mount a response to an acute alcohol administration in young adulthood, possibly mediated by medullary catecholamine input to the PVN.

Keywords

Adolescent; alcohol; stress; CRF; catecholamines

INTRODUCTION

Adolescents appear to be less sensitive to the aversive properties of alcohol than their adult counterparts and are also more sensitive to its positive rewarding effects (Spear and Varlinskaya, 2010). In addition, adolescents may potentially differ from adults in the way that their brain is altered by alcohol since, during this critical age, the central nervous system undergoes maturational changes in many regions, including the prefrontal cortex,

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hippocampus and hypothalamus (Van Eden et al., 1990, Choi and Kellogg, 1992, Wolfer and Lipp, 1995, Choi et al., 1997, Dumas and Foster, 1998, Crews et al., 2000). Indeed, studies of the consequences of exposure to alcohol during adolescence show that this drug has a major influence upon long-term brain development and function. For example, Evrard and colleagues (2006) observed that six weeks of drinking, beginning in late adolescence, induced morphological changes in astrocytes and neurons. These authors also assessed the ability of the brain to return to a basal state following exposure to alcohol during adolescence and found that abstinence only partially improved the alterations caused by adolescent drinking. Furthermore, there is evidence of age-related differences in cortical electroencephalogram (EEG) measurements such that this drug affects power in EEG frequency bands of adolescent rats that were not influenced in adults (Pian et al., 2008). Other studies that have examined adolescent vulnerabilities to chronic intermittent alcohol exposure suggested that young animals exposed to this drug potentially have altered brain development through long-lasting effects on motor function and memory formation (White et al., 2000, White et al., 2002).

Overall, these studies show that brain alterations and damage occur after alcohol exposure at a young age. However, the detrimental effects of adolescent intermittent alcohol exposure on the brain stress-response system remain unknown. Recent epidemiological studies report that binge drinking by 12- to 14-yr-old adolescents significantly increases the probability that they will abuse alcohol upon reaching adulthood (Pitkanen et al., 2005). In view of the critical role that the hypothalamic-pituitary-adrenal (HPA) axis is thought to play in the establishment and maintenance of alcohol abuse (Le et al., 2000, Sarnyai et al., 2001, Le and Shaham, 2002, Olive et al., 2003), and of the observation that alcohol exposure during adolescence renders some animals or individuals more prone to initiation of ethanol drinking or alcohol dependency when they are older (Grant and Dawson, 1997, Barron et al., 2005, Maldonado-Devincci et al., 2010), it is reasonable to propose that this phenomenon is, at least in part, mediated through altered HPA axis activity. We therefore decided to investigate the potential consequences of adolescent alcohol exposure on some of the adult brain structures that regulate this axis. Specifically, we measured responses of the central circuits known to regulate the stress response system such as the paraventricular nucleus (PVN) of the hypothalamus and the adrenergic brain stem regions (C1 and C2), which provide catecholamine inputs to the PVN.

Alcohol causes significant changes in corticotropin releasing factor (CRF) activity in the PVN (Rivier et al., 1984, Rivier et al., 1990). While in adult rodents, acute treatment with the drug significantly up-regulates PVN CRF gene expression (Ogilvie et al., 1998, Lee et al., 2004), repeated exposure blunts the subsequent response of the HPA axis to alcohol or other stimuli (Lee et al., 2000a, Lee et al., 2001, Rivier and Lee, 2001, Lee and Rivier, 2003). Although, the precise mechanisms responsible for this phenomenon have not yet been elucidated, we know that the catecholaminergic brain stem neurons known to project to the PVN, such as the C1 and C2 regions (Cunningham et al., 1990, Phillipson and Bohn, 1994), are activated by stressors (Dayas et al., 2001), as indicated by changes in the co-localization of phenylethanolamine *N*-methyltransferase (PNMT) and *c-fos* (Choi et al., 2008). A recent study performed in our laboratory also indicated that adult female rats exposed to footshocks following prenatal alcohol exposure (Choi et al., 2008) showed an increase in the activity of neurons in the C1 region of the brain stem. In view of these findings, we investigated the response of adrenergic brain stem regions in adult animals that had been exposed to alcohol as adolescents. Specifically, we measured the effect of adolescent [postnatal days (PND) 28–42] alcohol exposure via vapor chambers on PVN and brain stem responses to an acute alcohol challenge [intragastric (ig) injection via ig cannula] administered when the animals reached PND 61–62.

MATERIALS AND METHODS

Animals

Eleven male Sprague Dawley rats (Harlan, San Diego, CA, USA) were exposed to intermittent alcohol vapors and 8 were air-exposed for a total of 19 animals. The animals were housed 3 rats per standard plastic cage with wood chip bedding. They were provided standard rat chow and water *ad libitum* throughout the study. Exposure to alcohol vapors took place during the light cycle of a 12 h light/12 h dark cycle with lights off at 1800. All experiments met the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Institute on Alcohol Abuse and Alcoholism, 2004) and were approved by The Salk Institute Animal Care and Use Committee (IACUC).

Intermittent alcohol vapor chambers

The vapor chamber system that was used in this study was provided by La Jolla Alcohol Research, Inc. (La Jolla, CA, USA, <http://www.ljari.com>) and has been described in detail previously (Lee et al., 2000a). Briefly, 3 animals per cage were exposed to alcohol vapors daily for 6 h (0700 – 1300) for 15 days (PND 28–42), after which time, they were returned to the housing racks in a clean cage; controls were not exposed to alcohol. Blood samples were collected from the tail (0.1 – 0.2 ml) of all animals on PND 40 to determine blood alcohol levels (BALs). BALs were always undetectable in control animals, but tail bleeding was conducted in order to account for any stress experienced during this procedure.

Blood alcohol levels

An analox AM 1 analyzer (Analox Instruments Ltd., Lunenburg, MA, USA) was used to measure BALs in 5 μ L samples (Lee et al., 2000a). The precision of this assay is 1 to 2%, sensitivity is 0.1 mg/100 mL, and the curve is linear up to 400 mg/100 mL.

Intragastric cannulation and alcohol injection

Upon reaching PND 53, the rats were implanted with an ig catheter using isoflurane anesthesia [see (Ogilvie et al., 1997) for methods], and were allowed to recover from surgery for 7–8 days before experimentation. They were then housed individually to prevent chewing of exteriorized cannula. On the day of the experiment (PND 61–62), the animals were placed in opaque buckets with wood chip bedding in a quiet room with extension cannulae connected such that the animals could be injected without being handled or stressed. They were left undisturbed for 2 h to allow hormone levels to return to basal prior to receiving the vehicle (sterile water) or alcohol via the ig cannula. Because of the large volume administered, injections were slowly infused over a 5 min period. In our hands, the ig administration of the volumes required for a 4.5 g/kg alcohol dose, in relation to an equivalent amount of control substance, does not induce *c-fos* expression in any of the brain regions studied. All animals were sacrificed 2 h after the start of the ig injections.

The alcohol challenge dose (4.5 g/kg) was diluted to $\leq 20\%$ prior to administration. It corresponds to that previously used in our laboratory (Lee et al., 2001, Rivier and Lee, 2001, Lee and Rivier, 2003, Rivier et al., 2003, Seo and Rivier, 2003) and only causes mild intoxication. The animals remain fully ambulatory.

Perfusion and brain collection

All animals were given an intraperitoneal injection of chloral hydrate (Ogilvie et al., 1997) followed by transcardial perfusion with 0.9% NaCl for 2 to 5 min and 4% cold paraformaldehyde (PFA) for 18 min. Brains were placed in 4% PFA until transfer to 10% sucrose in perfusion solution overnight followed by sectioning by microtome.

Immunohistochemistry and in situ hybridization

Perfused brains were cut at a thickness of 30 μ m in the coronal plane. All sections were maintained in an antifreeze solution (50% 0.1 M phosphate buffered saline, 20% glycerol, 30% ethylene glycol) at -20°C until analysis. Every fourth section throughout the rostral-caudal extent of the PVN and brain stem was used for analysis. Each immunohistochemistry (IHC) staining or *in situ* hybridization was performed using brains obtained from alcohol treatment and control animals.

The general method of double DAB IHC staining for each antibody was used on free-floating sections as described previously in our laboratory (Choi et al., 2008). The primary antibody used for all stainings was a rabbit anti-*c-fos* antibody (1:10,000, Calbiochem, San Diego, CA, USA) and one of the following antibodies was used as the second primary antibody: rabbit anti-CRF (1:13,000, gift from W. Vale, The Salk Institute, La Jolla, CA, USA) or sheep anti-PNMT (1:7,500, Chemicon/Millipore, Billerica, MA, USA). The resulting stains gave the first antibody (*c-fos*) black stain and the second antibody (CRF or PNMT) brown stain. The black (*c-fos*) stain indicates activated nuclei whereas the brown CRF- or PNMT-ir stain shows cytoplasmic signals. For each animal ($n = 5 - 7/\text{treatment}$), individual and colocalized immuno-labeled cells were counted using a 20X dry objective in three to six sections throughout each brain region examined and the mean was obtained. Ultimately, the overall mean for the animals in each treatment are provided. The immunostained PVN and medullary sections were captured using a Leitz Orthoplan 2 microscope (Wetzlar, Germany) coupled with the Optronics MicroFire camera and PictureFrame software (Goleta, CA, USA) that was connected to a PC computer.

In situ hybridization was performed according to a previously published protocol (Simmons et al., 1989) that was adapted and described extensively (Lee et al., 2000a). Briefly, autoradiographic localization of CRF mRNA signals was obtained by using ^{35}S -labeled cRNA probes and densitometric analysis was carried out using the same exposure time on brain sections mounted onto slides that were dipped in nuclear emulsion. A Nikon optical system with the Eclipse E600 microscope (Nikon Instruments Inc., Melville, NY, USA), a Microcolor filter (Model RGB-MS-C, CRI Inc., Boston, MS, USA) and CoolSNAP_{fx} camera (Photometrics, Tucson, AZ, USA) coupled to a PC computer and Image Pro Plus software (version 4.5.029, Media Cybernetics Inc., Bethesda, MD, USA) was used to obtain the densitometric analyses of the autoradiographic signals. Gray level measurements (optical density) were taken under dark-field illumination of hybridized sections in the PVN. Autoradiograph signals for CRF mRNA were measured in both sides of the brain throughout the PVN, and mean values for all animals ($n = 5 - 7/\text{treatment}$) were determined in three sections for each rat.

Statistical Analysis

All data are provided as the mean $\pm$ standard error of the mean (SEM). Two-tailed t-tests were performed in Prism (Version 4.0, Graphpad Software Inc., La Jolla, CA, USA). Statistical significance was accepted for $p < 0.05$.

RESULTS

Intermittent alcohol vapors

On PND 40, the mean BAL of alcohol-exposed animals during adolescence ($n = 11$) was 212.8 ± 5.7 mg %. In young adult rats exposed to an ig alcohol challenge (4.5 g/kg), the mean BAL was 306.7 ± 24.5 mg % for those that had received vapors and 274.2 ± 32.3 mg % for controls ($p > 0.05$). As illustrated in Fig. 1, exposure to alcohol vapors during adolescence significantly ($p = 0.006$) blunted CRF mRNA expression in the PVN of young

adult rats acutely injected with alcohol, relative to controls. In contrast, there was no statistical difference between treatments in the number of colocalized *c-fos*-ir and CRF-ir cells or CRF-ir cells in the PVN (Table 1). Thirdly, when adolescent alcohol-exposed (AAE) rats were injected with alcohol on PND 61–62, we also found a significant ($p = 0.045$) increase in the number of PNMT-ir cells/section in the C2 region of the brain stem (Fig. 2). However, there was only a trend in the number of colocalized *c-fos*-ir and PNMT-ir cells/section in this medullary area ($p = 0.0775$, Fig. 2). Finally, there were no statistical differences in the number of colocalized *c-fos*-ir and PNMT-ir or PNMT-ir cells/section in the C1 region of the brain stem of young adult rats subjected to an ig alcohol challenge (Table 2).

DISCUSSION

The purpose of the work presented here was to investigate possible long-term effects of AAE on the brain circuits involved in the response to stressors. We report that AAE rats administered the drug through vapors displayed a significant blunting of their PVN CRF neuronal response to an acute alcohol challenge in early adulthood, while a study from our laboratory that examined animals that self-administered alcohol during adolescence did not show these changes (Allen et al., 2011). At least in the acute paradigms of alcohol administration we previously used in adult rats, we observed measurable activation of the PVN in response to one vapor session or an alcohol injection, but not when rats self-administered the drug (Ogilvie et al., 1997). While there are differences in brain responses to investigator-controlled versus self-administered alcohol (Moolten and Kornetsky, 1990), the fact that vapors or ig alcohol injection induce higher BALs, in comparison to the self-administration model used previously in our laboratory (Allen et al., 2011), had led us to speculate that this might represent a factor in the lack of HPA axis activation observed in animals that freely ingested alcohol (Ogilvie et al., 1997). Similarly, the present work suggests that differences in BALs between the two AAE models we used (alcohol vapors and operant self-administration), may participate in the long-term effects found following exposure to alcohol vapors in adolescence. However, this hypothesis will require further testing. It should also be noted that in addition to the current set of experiments, the only presently available data relevant to the consequences of prolonged alcohol exposure in young rodents on the stress response brain circuitry, pertain to our unpublished observations (S. Lee and C. Rivier) that PVN CRF mRNA expression of juvenile male rats was lower on day 15 of vapor treatment, compared to day 1. This corresponds to the well-known habituation of the HPA axis to prolonged alcohol exposure [see for example (Rivier et al., 1990, Spencer and McEwen, 1990, Rivier, 1995, Rivier, 1997, Zhou et al., 2000, Rivier and Lee, 2001, Silva et al., 2002)].

From a neuroendocrine point of view, it is well known that rats exposed to alcohol during embryonic development display significant changes in their HPA axis when they reach adulthood (Taylor et al., 1982, Taylor et al., 1984, Weinberg, 1988, 1992b, a, Lee and Rivier, 1996, Weinberg et al., 1996, Gabriel et al., 2000, Lee et al., 2000b). As well, juvenile rodents born to dams exposed to alcohol during gestation also displayed increased ethanol intake (Chotro and Arias, 2003, Honey and Galef, 2004), and it has been proposed that fetal exposure to alcohol has rewarding properties for the offspring (Spear and Molina, 2005). We had previously reported that prenatal alcohol exposure caused a significant increase in the activity of adrenergic neurons in the C1 region of the brain stem when the animals were exposed to footshocks in adulthood (Choi et al., 2008). We have also recently found that an alcohol challenge, administered to young adult rats that had self-administered alcohol during adolescence, decreased the activity of the C3 adrenergic brain stem region (Allen et al., 2011). In the present study, we observed statistically significant changes in the activity of C2 adrenergic neurons of adult animals exposed to alcohol vapors when adolescents. The C1

and C2 regions respond to stress (Dayas et al., 2001) and project to the parvocellular region of the PVN where CRF perikarya reside (Sawchenko and Swanson, 1981, Cunningham et al., 1990, Chan and Sawchenko, 1995). It is therefore possible that the changes we found in the adrenergic brain stem neurons of adult rats exposed to alcohol in adolescence may modulate the decreased ability of hypothalamic areas to respond to stressors. This hypothesis is supported, for example, by the findings of Li and colleagues (1996), in which the activation of medullary aminergic neurons is a secondary consequence of a footshock stressor that is mediated by a descending projection of the PVN.

Collectively, the results presented here suggest that exposure to alcohol vapors during adolescence influences the ability of the HPA axis to respond to an acute drug challenge in young adult rats. This is in contrast to a study we recently reported that indicated that adolescent alcohol self-administration did not induce comparable changes in adult HPA axis activity (Allen et al., 2011). While provocative, the finding of an altered number of PNMT-ir cells/section in the C2 medullary area in this model points to the requirement for future research to clarify the role, if any, of the catecholaminergic brain stem region in modulating the long-term effects of alcohol on neuroendocrine or other functions. Finally, there is convincing evidence that the HPA axis influences drug abuse (Koob and Kreek, 2007). As, contrary to acute treatment, prolonged exposure to alcohol dampens the activity of this axis [see for example (Rivier, 1995, Lee et al., 2000a)], we had previously suggested the possibility that individuals who abuse alcohol might do so, at least in part, in an attempt to regain alterations in HPA axis function (Lee and Rivier, 1997). Therefore, it will be of interest to not only extend the present finding on the consequences of AAE on adult HPA axis activity, but also to determine whether adult alcohol intake is modified following AAE using the vapor chamber model and other addiction models that are characterized by altered neuroendocrine function following alcohol exposure.

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Figure 1.

Exposure to intermittent alcohol vapors during adolescence blunts CRF mRNA expression in the PVN of young adult male rats administered an alcohol challenge (4.5 g/kg, via ig catheter), compared to air-exposed adolescent controls. A: Dark-field photomicrographs show coronal sections through the PVN 2 h after alcohol challenge in adulthood. Magnification, 100X; B: Mean $\pm$ SEM CRF mRNA expression (optical density as arbitrary units expressed as % of control) of 5 – 7 rats/treatment, ★: ★ $p < 0.01$.

Figure 2.

Exposure to intermittent alcohol vapors during adolescence increases the number of PNMT immunoreactive cells in the C2 region of the brain stem of young adult rats administered an alcohol challenge (4.5 g/kg, via ig catheter), compared to controls. In addition, although not statistically significant, there was an increasing trend in the number of colocalized *c-fos* and PNMT immunoreactive cells. A: Double immunohistochemical procedure stained *c-fos* black nuclei and PNMT brown cytoplasm. Display panels compare the two conditions and the pictures were taken with a 20X dry objective (scale bar = 10 μ m) and the inset a 40X dry objective; B: Mean $\pm$ SEM number of colocalized *c-fos* and PNMT (top) or PNMT (bottom) positive cells/section, $n = 5 - 7$ rats/treatment, ★: $p < 0.05$.

Table 1

The number of CRF and co-localized *c-fos* and CRF immunoreactive cells in the PVN of young adult rats exposed to alcohol vapors on PND 28–42 then acutely challenged with alcohol on PND 61–62.

Adolescent Treatment	Number of CRF-ir cells per section in PVN	Number of colocalized <i>c-fos</i> -ir and CRF-ir cells per section in PVN
Air Exposed Control Rats	226.2 ± 13.5	46.2 ± 7.9
Alcohol Vapor Exposed Rats	233.4 ± 29.7	42.3 ± 3.8

Intermittent vapor exposure to alcohol during adolescence does not influence the number CRF or colocalized *c-fos* and CRF immunoreactive (ir) cells in the PVN of adult rats subjected to an alcohol challenge in adulthood (4.5 g/kg alcohol via ig catheter). Values are mean ± SEM, n = 5 – 7 rats/treatment.

Table 2

The number of PNMT and colocalized *c-fos* and PNMT immunoreactive cells in the C1 region of the brain stem of young adult rats exposed to alcohol vapors on PND 28–42 then acutely challenged with alcohol on PND 61–62.

Adolescent treatment	Number of PNMT-ir cells per section in C1	Number of colocalized <i>c-fos</i> -ir and PNMT-ir cells per section in C1
Air Exposed Control Rats	111.3 ± 8.8	18.7 ± 4.8
Alcohol Vapor Exposed Rats	122.3 ± 11.4	22.7 ± 2.9

Intermittent vapor exposure to alcohol during adolescence does not influence the number of PNMT or colocalized *c-fos* and PNMT immunoreactive (ir) cells in the C1 region of the brain stem of adult rats subjected to an alcohol challenge in adulthood (4.5 g/kg alcohol via ig catheter). Values are mean ± SEM, n = 5 – 7 rats/treatment.