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Adolescent alcohol exposure alters the rat adult hypothalamic-pituitary-adrenal axis responsiveness in a sex-specific manner

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Abstract

Exposure to alcohol during adolescence exerts long-term effects on the adult brain stress circuits, causing many changes that persist into adulthood. Here we examined the consequences of adolescent intermittent ethanol [AIE, administered from postnatal day (PND) 28–42] on the hypothalamic-pituitary-adrenal (HPA) axis-related brain circuitry of rats challenged with an intragastric administration of alcohol in adulthood (PND 70–71). Both male and female adolescent rats were exposed to alcohol vapors, while controls did not receive the drug, to assess whether AIE alters adult alcohol response in a sex-specific manner. We demonstrated that AIE increased PVN *Avp* mRNA levels during late (PND 42) but not middle (PND 36) adolescence in males. While an alcohol challenge administered to 70–71-day-old rats increased *Crf* mRNA levels in males and *Avp* mRNA levels in females, AIE blunted both effects. These results suggest that AIE produced long-lasting changes in the responsiveness of the HPA axis to a subsequent alcohol challenge in a sex-specific manner. Furthermore, AIE altered adrenergic brain stem nuclei involved in stress responses in adulthood, resulting in increased numbers of phenylethanolamine N-methyltransferase (PNMT) neurons in male C2 and female C1 regions. This tended to enhance activation of the male C2 nucleus upon alcohol challenge. Collectively, these results suggest that AIE exerts long-term effects on the ability of the PVN to respond to an alcohol challenge in adulthood, possibly mediated by catecholaminergic input from the brain stem to the PVN.

Keywords

adolescent; alcohol; catecholamines; corticotropin-releasing factor; vasopressin

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Authors' Contributions

MLL, CR and SL conceptualized and designed the research; MLL, CL, SI, JV AND SL performed the experiments and analyzed the data; MLL, CR and SL wrote and edited the manuscript.

INTRODUCTION

Alcohol abuse and dependence are significant societal problems whose likelihood is greatly enhanced by starting alcohol consumption during adolescence (Dawson et al., 2008). Thus, determining the long-term impact of adolescent alcohol exposure is critical for developing interventions to treat alcohol-related disorders. One possible locus of persistent drug-induced adaptations is the hypothalamic-pituitary-adrenal (HPA) axis, the system that mediates responses to stressors. As the HPA response has been shown to change between adolescence and adulthood, it is thought that adolescence presents a vulnerable period for shaping the function of the adult HPA axis (Romeo, 2010). Both male and female rats initiating alcohol consumption in adolescence show greater stress-induced increases in alcohol intake in adulthood than similarly alcohol-experienced rats whose alcohol access commenced in adulthood (Siegmund et al., 2005, Fullgrabe et al., 2007). This enhanced susceptibility to stress-induced alcohol intake suggests that adolescent alcohol experience may permanently alter stress responses, with deleterious consequences for alcohol consumption in adulthood. Thus, understanding how adolescent alcohol exposure modifies adult stress responses is of great importance for developing therapies to treat individuals with early onset of alcohol drinking.

The HPA response to stressors is initiated by activation of neurons in the paraventricular nucleus (PVN) of the hypothalamus, triggering the release of corticotropin-releasing factor (CRF) and vasopressin (VP) into the pituitary gland (Herman and Cullinan, 1997). This causes release of adrenocorticotropic hormone (ACTH), which then acts on the adrenal gland to increase corticosterone (cort) production. The HPA axis is activated by an acute alcohol challenge in adult rats (Rivier et al., 1984, Rivier et al., 1990, Ogilvie et al., 1997), while repeated alcohol exposure and dependence lead to a reduction in the magnitude of alcohol-induced HPA activation (Lee and Rivier, 1997, Richardson et al., 2008). The HPA response to alcohol develops during the adolescent period, increasing in magnitude from postnatal day (PND) 28 and reaching a maximal level in adulthood, an effect much more prominent in females than in males (Silveri and Spear, 2004, Willey et al., 2012). Exposure of male rats to repeated alcohol injections during adolescence resulted in both blunted adult baseline cort levels and enhanced cort release following an alcohol challenge in adulthood (Przybycien-Szymanska et al., 2011). Cort responses to acute stressors in adolescents, as compared to adult rats, were significantly prolonged in both sexes (Romeo et al., 2004b, Romeo et al., 2006). However, stress responses in chronically stressed adolescent rats were blunted in males (Romeo et al., 2006, Weintraub et al., 2010) but enhanced in females (Weintraub et al., 2010). Together, these data suggest adolescence as a period of prolonged activity and enhanced plasticity of HPA axis responses that may significantly impact adult HPA axis function, with differential susceptibility of females and males to such long-term adaptations.

Additional studies support persistent effects of adolescent exposure to stressors, including alcohol, on HPA axis activity and anxiety-like behavior. Both isolated housing (Weintraub et al., 2010) and binge-like alcohol drinking (Gilpin et al., 2012) during adolescence reduced baseline anxiety-like behavior in adult male rats, as assessed by time spent on the open arms of the elevated plus maze (EPM). However, female rats did not display any significant changes in EPM anxiety-like behavior following adolescent isolation (Weintraub et al., 2010), but did show reduced anxiety-like behavior in adulthood during estrus if exposed to social stress in adolescence (McCormick et al., 2008). Despite the greater behavioral effect of adolescent isolation in adult males, females, but not males, displayed elevated baseline PVN *Avp* mRNA, with no changes in baseline *Crf* mRNA in either sex, as compared to rats housed socially during adolescence (Weintraub et al., 2010). Similarly, adolescent binge drinking did not alter baseline PVN CRF neuron number or activity in males (Allen et al.,

2011a), while alcohol injections administered to mimic a binge pattern in adolescence elevated both baseline and alcohol challenge-induced PVN *Crf* mRNA in adult male rats (Przybycien-Szymanska et al., 2011). These data support long-term effects of adolescent exposure to stressors, including alcohol, on adult stress responses and HPA axis activity, as well as possible sexual dimorphism in these effects. However, the fact that differences in PVN gene and protein expression do not directly align with behavioral effects of adolescent stress/alcohol history in adulthood suggests that long-term modifications in HPA axis activity may not be restricted to the PVN but rather may involve changes in afferent regions regulating PVN activation.

One source of projections that modulate PVN neuronal activity in response to stress is the catecholaminergic neurons in the brain stem, particularly the noradrenergic neurons in the locus coeruleus and regions A1–A2, as well as the adrenergic neurons of regions C1–C3 (Dayas et al., 2001). Previously, we showed that acute alcohol activates these brain stem nuclei in adult male rats (Lee et al., 2011). We have also demonstrated in male rats that adolescent binge drinking attenuated alcohol-induced activation of C3 neurons in adulthood (Allen et al., 2011a), while intermittent exposure to alcohol vapor throughout adolescence increased the number of phenylethanolamine N-methyltransferase (PNMT)-labeled neurons in the C2 region following an acute alcohol challenge in early adulthood [PND 60–61] (Allen et al., 2011b). Unlike these long-term changes resulting from adolescent alcohol experience, prenatal alcohol exposure enhanced the footshock stress responsiveness of C1 neurons in adulthood in females, but not males (Choi et al., 2008). Together, these data indicate that alcohol exposure during various developmental epochs may alter the responsiveness of brain stem catecholaminergic neurons, suggesting these neurons as a possible locus of the long-term effects of adolescent alcohol exposure on adult HPA axis function.

Taken together, these previous studies led to the hypothesis that adolescent intermittent ethanol (AIE) exposure would generate long-lasting changes in the HPA axis sensitivity to alcohol challenge, resulting in a reduced alcohol response in adulthood. We also theorized that the depressive effects of AIE on adult HPA axis function would result from decreased activation of either PVN neurons or the afferent C1–C3 adrenergic neurons upon alcohol challenge. Finally, we proposed that these persistent effects of AIE would differ by sex.

EXPERIMENTAL PROCEDURES

Animals

Male and female Sprague Dawley rats (Harlan, San Diego, CA, USA) were housed 3–4 per standard plastic cage with wood chip bedding, with standard rat chow and water available *ad libitum* throughout the study. Rats lived in a humidity- and temperature-controlled vivarium under a 12 h light/12 h dark light cycle with lights off at 1800. Each experimental group consisted of 5–7 animals. Experiments were performed during the early portion of the light cycle in the trough of the circadian HPA cycle (Atkinson and Waddell, 1997), to minimize the experimental impact of individual differences. All experiments met the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by The Salk Institute Animal Care and Use Committee.

Intermittent alcohol vapor exposure

Adolescent rats were exposed to alcohol vapor in an air-tight chamber system provided by La Jolla Alcohol Research, Inc. (La Jolla, CA, USA, http://www.ljari.com), as described in detail previously (Lee et al., 2000a, Lee et al., 2000b). Briefly, 3–4 animals per cage were exposed to alcohol vapor daily for 6 h (0700–1300; AIE) or air (Control) for the duration of

the 15-day adolescent period (PND 28–42), except where indicated for rats euthanized at an earlier time point. After each exposure, the rats were returned to the housing racks in clean cages. Blood alcohol levels (BALs) were sampled from one rat per cage every 2–3 days and alcohol drip rates adjusted, as necessary, to maintain BALs at approximately 200 mg%. Each rat was sampled twice during the exposure period, with the second sampling performed on the same day for all rats in the cohort (PND 39 for males, PND 41 for females). Experimental control rats were exposed to similar handling and tail nicking to standardize any procedural stress effects across all subjects. This exposure paradigm was used to generate controlled daily cycles of alcohol intoxication and withdrawal during the adolescent period. To analyze the time course of BAL and cort levels in response to vapor exposure during the adolescent period, rats were euthanized by rapid decapitation at the time points specified, relative to the onset of vapor exposure, and trunk blood collected. Control samples were collected in parallel, matched for time and age, but collapsed into a single group for analysis due to similar low basal cort levels in both age groups.

Blood alcohol levels

Blood samples (50–100 μl) were collected from the tails of all animals, including the air-exposed rats to control for any procedural stress effects. BALs were measured in 5 μl samples using an Analox AM 1 analyzer (Analox Instruments Ltd., Lunenburg, MA, USA) (Lee et al., 2000a, Lee et al., 2000b). The precision of this assay is 1–2%, the sensitivity is 0.1 mg/100 ml, and the curve is linear up to 400 mg/100 ml.

Corticosterone assay

Plasma cort levels were measured using a kit optimized for small sample volumes (MP Biomedicals, Inc., Orangeburg, NY, USA), according to manufacturer's instructions. The detection limit of this assay is 10 ng/ml.

Animal surgery and alcohol injection

Upon reaching PND 62–63, male and female rats were implanted with intragastric (ig) catheters under isoflurane anesthesia (Butler Animal Health Supply, Dublin, OH, USA) [see (Ogilvie et al., 1997) for methods], and were allowed to recover from surgery for 7–8 days before experimentation. Rats were housed individually post-surgery to prevent chewing of external cannulae. Despite individual housing, we did not observe differences in *c-fos* expression in any region analyzed between ig saline injected experimental control rats and group-housed absolute control rats that did not undergo experimental procedures on the test day, nor did we observe activation of *c-fos* expression in the PVN in either experimental or absolute controls (data not shown). Estrous cycles were synchronized in female rats on the third day prior to experimentation via subcutaneous injection of 2 μg of a GnRH analog, [D-Trp^{6}-Pro^{9}-Net-GnRH], at 9 am and 2 pm, such that female rats were in diestrus II at the time of experimentation, as previously described by ourselves (Lee et al., 2003, Richardson et al., 2006) and others (Roberts et al., 1998, Cottone et al., 2007, Parylak et al., 2012). On the day of the experiment (PND 70–71), the animals were singly housed 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, to prevent procedural stress. Rats were left undisturbed for 2 h to allow hormone levels to return to baseline (Lee and Rivier, 1997, Ogilvie et al., 1997, Rivier and Lee, 2001), then administered 4.5 g/kg alcohol (<20% v/v in water) or an equivalent volume of water via the ig cannula. The alcohol challenge dose corresponds to that previously used in our laboratory (Rivier and Lee, 2001, Lee and Rivier, 2003, Seo and Rivier, 2003). BALs at the time of euthanasia can be found in Table 1. Because of the large volume administered, injections were slowly infused over a 2-min period. Compared to absolute, non-infused controls, ig delivery of this volume of fluid did

not induce *c-fos* expression in any of the brain regions studied (P's>0.05, absolute control vs. control/vehicle group; data not shown).

Immunohistochemistry

Rats were euthanized at the end of the 6-h alcohol vapor session on PND 36 or PND 42, or 2 h after acute ig alcohol challenge on PND 70–71, based on our previous studies (Ogilvie et al., 1998). All animals were deeply anesthetized by an intraperitoneal injection of 35% chloral hydrate (Ogilvie et al., 1997) followed by transcardial perfusion with 0.9% NaCl for 2 min and 4% cold paraformaldehyde (PFA) for 18 min. Brains were placed in 4% PFA, then cryoprotected by overnight incubation in a 10% sucrose/4% PFA solution prior to coronal sectioning by microtome at a thickness of 30 μm. 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 reaction was performed using brains obtained from AIE-treated and control animals. Double DAB IHC staining was performed on free-floating sections as described previously (Choi et al., 2008, Allen et al., 2011b), with a rabbit anti-*c-fos* antibody (1:10,000, Calbiochem, San Diego, CA, USA) stained black and a sheep anti-PNMT antibody (1:7,500, Chemicon/Millipore, Billerica, MA, USA) stained brown. Sections were mounted on gelatin-coated sub slides, dehydrated, and coverslipped using DPX mounting medium (Electron Microscopy Sciences, Hatfield, PA). Negative controls without primary or secondary antibody were included. The black (*c-fos*) stain indicates activated nuclei whereas the brown PNMT-ir stain shows cytoplasmic signals. Individual and colocalized immuno-labeled cells were counted using a 20X dry objective in 3–6 sections throughout each brain region examined, and the average values per section for each rat were determined by brain region. The data are expressed as both the total number of PNMT-positive cells per section and as the number of Fos-positive cells colocalized with PNMT-positive cell bodies.

In situ hybridization

In situ hybridization was performed according to a previously published protocol that was adapted and described extensively (Lee et al., 2000a, Lee et al., 2000b). Briefly, autoradiographic localization of *Crf* and *Avp* mRNA signals was obtained 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. Autoradiograph signals for *Crf* or *Avp* mRNA levels were measured bilaterally for 3 sections containing the PVN. Data were calculated as average autoradioagraphic density per section for each rat.

Imaging

IHC and *in situ* sections were imaged using a Nikon optical system, the Eclipse E600 microscope (Nikon Instruments Inc., Melville, NY, USA), equipped with a Micro*Color filter (Model RGB-MS-C, CRI Inc., Woburn, MA, USA) and CoolSNAP camera (Photometrics, Tucson, AZ, USA), coupled to a PC. IHC images were obtained using a 20X objective, while autoradiographic images were captured under 100X magnification. 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.

Statistical analysis

All data are expressed as the mean ± standard error of the mean (SEM). Data were analyzed using two-way ANOVA with post-hoc comparisons via the Tukey method. Statistical

analyses were performed using Prism (Version 4.0, Graphpad Software Inc., La Jolla, CA, USA) and SigmaPlot (Version 11.0, Systat Software Inc., Chicago, IL, USA). Statistical significance was accepted for $P < 0.05$.

RESULTS

Effect of AIE on plasma corticosterone levels

In order to study the effects of adolescent alcohol exposure on the HPA axis, we utilized a model of AIE in which rats were exposed to alcohol vapor or air for 6 h per day from PND 28–42 (Allen et al., 2011b). On PND 30 and 36, plasma cort release and BALs were assessed in adolescent males at hourly intervals beginning 1.5 h after the onset of alcohol vapor exposure, or at the same time points for age-matched controls (Fig. 1). Analysis of plasma cort data by two-way ANOVA with the factors Time (1.5, 2.5, 3.5, 4.5 and 5.5 h post-vapor onset) and AIE Group (Control, AIE PND 30 and AIE PND 36) yielded main effects of Time ($F_{4,55}$=8.98, P<0.001) and AIE Group ($F_{2,55}$=10.42, P<0.001). Cort levels were elevated by 3.5 h into vapor exposure on PND 30 and by 4.5 h into vapor exposure on PND 36 vs. alcohol-naïve controls (Fig. 1A). The more rapid increase in plasma cort on PND 30, as compared to PND 36, manifested as an interaction between the factors Time and AIE Group ($F_{8,55}$=3.21, P<0.01). BALs also increased steadily across the 6-h vapor sessions on PND 30 and 36 (Fig. 1B, main effect of Time, $F_{4,30}$ = 122.83, P<0.001). Although BALs increased similarly over the 6-h period on both PND 30 and PND 36, an Age x Time Point interaction was observed ($F_{4,24}$=2.98, P<0.05) due to significantly higher BALs at the 2.5-h time point for PND 36 rats. This difference did not persist and was observed in the group showing delayed, rather than earlier, elevations in cort levels, suggesting that differences in plasma cort levels were independent of BAL discrepancies.

Effect of AIE on PVN *Crf* and *Avp* mRNA levels

As the observed elevation in cort levels indicated activation of the HPA axis by the daily AIE exposure, we hypothesized that AIE would also increase *Crf* and/or *Avp* mRNA levels in the PVN of male rats during the vapor exposure period. To test this, *Crf* and *Avp* mRNA levels were assessed in male rats at the end of the 6-h vapor exposure period on PND 36 and 42. As shown in Fig. 2, PVN *Avp* mRNA levels were significantly increased in AIE male rats at the end of vapor access on PND 42 (mean ± SEM BAL: 271.1 ± 9.1 mg/dl) but not PND 36 (mean ± SEM BAL: 285.8 ± 4.2 mg/dl). Analysis by two-way ANOVA showed a Group x Age interaction ($F_{1,12}$=6.39, P<0.05), with significantly higher *Avp* mRNA levels in the PND 42 AIE group as compared to both its respective control and PND 36 AIE (P's<0.01). PVN *Crf* mRNA levels of AIE rats were not significantly different from controls at either time point (PND 36: control, 100.0 ± 4.0%; AIE, 85.1 ± 7.5 %, P>0.05; PND 42: control, 100.0 ±16.4 %; AIE, 125.6± 14.2%, P>0.05). Together, these data show that AIE increased PVN *Avp* but not *Crf* gene expression at the end of a 6-h vapor exposure session only in late adolescent males.

Sex differences in AIE effects on the PVN *Crf* and *Avp* response to alcohol challenge in adulthood

Previously, we reported a dampened PVN *Crf* response to an alcohol challenge in young adulthood, at PND 60–61, in males exposed to AIE as compared to adolescent air-exposed controls (Allen et al., 2011b). We wanted to determine (1) whether this difference resulted from altered basal PVN gene expression in AIE-experienced adult rats, (2) whether differences in response to alcohol challenge in rats with a history of AIE persisted past early adulthood, and (3) whether males and females responded differentially to AIE. Rats were exposed to air (Control) or AIE (mean ± SEM peak BALs: male PND 39, 270.8 ± 5.6; female PND 41, 196.3 ± 4.4) until PND 42, then challenged on PND 70–71 with ig infusion

of 4.5 g/kg alcohol or vehicle. As shown in Figure 3, AIE did not significantly alter basal *Crf* mRNA levels on PND 70–71 in males (Fig. 3A,B), although alcohol challenge did increase *Crf* mRNA levels (two-way ANOVA, main effect of Adult Challenge, $F_{1,20}$=5.12, P<0.05; no significant effect of AIE, $F_{1,20}$=3.31, P=0.08). We found no interaction between the factors, suggesting that while AIE appeared to reduce the increase in PVN *Crf* mRNA levels observed following 4.5 g/kg alcohol challenge on PND 70–71, this effect was not significant ($F_{1,20}$=2.00, P=0.17). In contrast, there was no statistical difference in PVN *Avp* mRNA levels either at baseline or following alcohol challenge between control and vapor-exposed male rats (F's>2.68, P's>0.11; Fig 3C).

In contrast to AIE male rats, challenging females with alcohol in adulthood did not significantly modify PVN *Crf* mRNA levels, regardless of AIE history (F's<2.73, P's>0.11, Fig. 3D). Instead, following administration of alcohol on PND 70–71, female rats demonstrated a striking elevation in PVN *Avp* mRNA levels which was blunted by AIE exposure (Fig. 3E,F). Analysis by two-way ANOVA yielded an interaction between AIE and adult alcohol challenge ($F_{1,16}$=6.80, P<0.05) due to significant effects of adult alcohol challenge in controls (P<0.001) as well as AIE history in alcohol-challenged rats (P<0.05). Together, these results demonstrated differential effects of AIE on the adult PVN response to alcohol challenge in adult male and female rats.

Effects of AIE on adult alcohol responses in brain stem catecholaminergic neurons

Brain stem catecholaminergic neurons provide a significant input driving the activity of PVN neurons (Dayas et al., 2001). We hypothesized that the sex differences observed in the PVN might result from sex-specific effects of AIE and/or adult alcohol challenge on the medullary C1–C3 nuclei. We performed IHC to examine changes in neurons expressing PNMT, a final enzyme in adrenergic synthesis, following alcohol challenge on PND 70–71 in male and female rats with or without a history of AIE exposure. As shown in Table 2, AIE male rats showed elevated colocalization of *c-fos*-ir and PNMT-ir cells/section in the C1 medullary region on PND 70–71 (main effect of AIE, $F_{1,16}$=6.31, P<0.05). Alcohol challenge also increased the number of colocalized neurons (main effect of alcohol challenge, $F_{1,16}$=46.15, P<0.001), but there was no interaction between the factors ($F_{1,16}$=0.16, P=0.70) and no change in the total number of PNMT-positive cells (F's<1.64, P's>0.21). A similar trend for increase in alcohol challenge-induced c-fos/PNMT colocalization was observed in the C2 region of AIE-exposed male rats (Fig. 4; AIE, $F_{1,16}$=3.68, P<0.08; alcohol challenge, $F_{1,16}$=20.05, P<0.001; AIE x alcohol challenge interaction, $F_{1,16}$=3.68, P<0.08). Unlike C1, AIE significantly increased the number of PNMT-ir cells per section, regardless of adult alcohol challenge, in male rats (Fig. 4; main effect of AIE, $F_{1,16}$=12.59, P<0.005).

Based on the differential effects of adult alcohol challenge on PVN mRNA expression in male and female rats, we hypothesized disparate effects of AIE on brain stem PNMT neuron activation as well. As predicted, female rats demonstrated a significant AIE-dependent increase in the number of PNMT-ir neurons per section in the C1 region on PND 70–71 ($F_{1,17}$=17.33, P<0.001), but no AIE-related effect on the alcohol challenge-induced increase in *c-fos*/PNMT colocalization (Fig. 5; main effect of alcohol challenge, $F_{1,17}$=22.35, P<0.001; no effect of AIE or interaction, F's<0.04, P's>0.84). Although adult alcohol challenge activated C2 PNMT-expressing neurons in the brain stem of female rats (Table 3, $F_{1,17}$=5.24, P<0.05), no AIE-related significant statistical differences were observed (all F's<1.59, all P's>0.22 for AIE and AIE by Alcohol Challenge interaction). No significant differences were observed in the C3 region of either male or female rats, regardless of AIE exposure (Table 4). Together, these data demonstrate that AIE differentially altered medullary brain stem regions in adult male and female rats, both at baseline and following

an alcohol challenge, suggesting a central role for catecholamines in modulating the long-term consequences of AIE on the adult HPA axis.

DISCUSSION

The purpose of this study was to investigate long-term effects of AIE on the brain stress circuits that alter HPA axis activity, and to determine whether such effects differ by sex. Here we demonstrated that AIE increases *Avp* gene expression during late (PND 42) but not middle (PND 36) adolescence in the male. Unlike adolescence, alcohol exposure in the 70–71-day-old male altered *Crf*, but not *Avp*, mRNA levels. Conversely, adult females did not show significant changes in *Crf* mRNA levels following alcohol challenge, but rather displayed increased *Avp* mRNA levels. These effects were blunted by AIE, suggesting that alcohol exposure during adolescence produced long-lasting changes in the responsiveness of the HPA axis to subsequent alcohol challenge in a sex-specific manner. AIE also altered brain stem nuclei involved in stress responses in adulthood, with increased numbers of PNMT-immunoreactive neurons in male C2 and female C1 regions, resulting in enhanced activation of the male C2 nucleus upon alcohol challenge. Together, these data show sex-specific, persistent modifications of stress-responsive nuclei in rats exposed to intermittent alcohol during adolescence.

Modulation of alcohol responsiveness during adolescent maturation

The data herein demonstrate differential effects of alcohol exposure as adolescence progresses, with HPA axis responsiveness changing over adolescence. Specifically, PVN *Avp* gene expression increased only at PND 42, not PND 36, in males, despite similar BALs in both groups. The elevation in *Avp* in later adolescence agrees with results from Przbycien-Szymanska et al. (2010) following daily 3 g/kg i.p. alcohol treatments on PND 37–44, although that treatment regimen also generated elevated *Crf*, which was not seen in the present study. This discrepancy may be due to the more prolonged alcohol exposure utilized herein, both in number of days (beginning at PND 28 vs. PND 37) and in discrete alcohol exposure duration (6 h vapor session vs. single acute injection). Indeed, prolonged alcohol exposure generates dysregulation of the HPA axis in human alcoholics (Heuser et al., 1988, von Bardeleben et al., 1989), and the possibility exists that the greater severity of the 15-day vapor exposure paradigm may cause such an adaptation in the HPA axis. Alternatively, the difference may arise from the earlier onset of alcohol exposure in the periadolescent period. Nonetheless, the results indicate both that repeated alcohol exposure in later adolescence increased *Avp* and that greater magnitude of adolescent alcohol exposure may trigger an adaptation of the *Crf* response within the adolescent period.

In line with the change in alcohol vapor effects on *Avp* mRNA expression as adolescence progresses, developmental stage impacts the behavioral consequences of alcohol exposure. For example, alcohol increased open arm exploration in the elevated plus maze, suggestive of decreased anxiety-like behavior, in early but not late adolescence or adulthood in mice (Hefner and Holmes, 2007). Adolescent rats also failed to show the alcohol withdrawal-induced anxiety observed in adults (Doremus et al., 2003). These results are in line with studies that demonstrated reduced behavioral suppressive effects of alcohol in younger rats, including sedation/intoxication (Silveri and Spear, 2004, Pian et al., 2008), tolerance (Swartzwelder et al., 1998, Varlinskaya and Spear, 2006) and motor impairment (Silveri and Spear, 2004). These data demonstrate blunted sensitivity to the inhibitory and anxiogenic-like effects of alcohol, particularly during early adolescence, in agreement with PVN gene expression changes emerging only later in the adolescent period.

Sex differences in AIE effects on stress circuitry

While both male and female rats received equivalent alcohol treatment during adolescence, alterations in the response to adult alcohol challenge were quite different. Males demonstrated a slight attenuation of *Crf* responses and nonexistent *Avp* responses, similar to our previously reported findings in younger males (Allen et al., 2011b), while females showed inhibition of *Avp* activation by alcohol, without *Crf* effects. These data contrast directly with previous findings of elevated PVN *Crf* mRNA after an acute alcohol challenge in adult male rats with a history of adolescent alcohol injections (PND 37–42), but not in adolescent alcohol-naïve controls following the same adult alcohol challenge (Przybycien-Szymanska et al., 2011). The authors also showed reduced PVN *Avp* mRNA in control rats, but not adolescent alcohol-experienced rats, following adult alcohol challenge, whereas we did not see any significant *Avp* changes in adult males following acute alcohol challenge, regardless of AIE history. These discrepancies may result from differences in the adolescent alcohol exposure paradigms (PND 28–42 vapor vs. PND 37–44 injections), the routes of adult alcohol exposure (ig vs. intraperitoneal injection), or the time points analyzed (2 h vs. 1 h after adult alcohol challenge). Sex differences in gonadal maturation during the adolescent period, which occurs earlier in females than males, may also impact the sex differences observed in the effects of AIE on adult HPA responses, as differences in stress responses pre- and post-puberty (Viau et al., 2005), as well as variation in stress responses across the estrous cycle (Viau and Meaney, 1991), suggest that gonadal steroids modify HPA axis activity. Alternatively, alcohol exposure in adolescence could disrupt or delay puberty, as adult alcohol exposure reduces gonadal hormone levels (Cicero et al., 1978, Ogilvie and Rivier, 1997). However, removal of female sex hormones via ovariectomy (Romeo et al., 2004b) or augmentation of circulating testosterone to adult levels (Romeo et al., 2004a) in adolescent male rats did not alter HPA responses to stressors during early adolescence, suggesting that adolescent stress responses, including the observed AIE effects, may differ from adult responses via mechanisms independent of pubertal maturation.

Interestingly, rather than mirroring other studies investigating the adult impact of adolescent alcohol (Przybycien-Szymanska et al., 2011), our observations are more directly in line with findings reported following 6 months of alcohol liquid diet consumption in adulthood (Silva et al., 2009). Male rats exposed to 6-month alcohol intake showed significantly blunted numbers of CRF-positive neurons (Silva et al., 2009) and *Crf* mRNA levels (Silva and Madeira, 2012) in the PVN, with no significant change in VP-positive neurons (Silva et al., 2009), although a slight reduction in *Avp* mRNA levels was reported (Silva and Madeira, 2012). Conversely, females that consumed alcohol liquid diet for 6 months showed significant reductions in numbers of both CRF- and VP-positive PVN neurons (Silva et al., 2009), as well as decreases in both *Crf* and *Avp* mRNA levels (Silva and Madeira, 2012). Importantly, while withdrawal of alcohol access attenuated alcohol-induced changes in males exposed to the drug for 6 months, the inhibitions seen in female PVN were either maintained or enhanced during the withdrawal period (Silva et al., 2009, Silva and Madeira, 2012). Together, these studies suggest an increased sensitivity of the female PVN to permanent changes following prolonged alcohol exposure, and indicate that alcohol experience during the brief adolescent period may be as deleterious to PVN function as months of alcohol intake in adulthood.

In congruence with our previous study in young adult (PND 60–61) male rats (Allen et al., 2011b), AIE increased brain stem adrenergic neuronal PNMT expression and/or activation of PNMT-positive neurons. Specifically, PND 70–71 males showed elevated activation of C1 PNMT neurons and elevated C2 PNMT neuron numbers and alcohol responsiveness according to AIE history, while adult AIE females displayed elevated numbers of PNMT-ir neurons in C1. As these brain stem adrenergic nuclei project to the PVN (Cunningham et al., 1990), they are believed to participate in activation of the PVN; however, the increased

activity of the brain stem adrenergic cell groups contrasts with the decreased *Crf* and *Avp* mRNA levels observed in AIE PVN following adult alcohol challenge. This discrepancy may be due to circuit alterations generated by AIE, although the brain stem adrenergic nuclei have been suggested to play a role independent of the initial HPA axis activation, as unilateral transection of the ventral noradrenergic bundle did not alter footshock-induced PVN activation (Li et al., 1996).

Adolescence as a period of heightened vulnerability for the HPA axis

During adolescence, many neural circuits undergo maturation, leaving them highly susceptible to insult. Not surprisingly, adolescent alcohol exposure has been shown to generate a variety of long-lasting neuroadaptations, including prefrontal cortical modifications of synaptic structure (Crews et al., 2007) and neurotransmission-related gene expression (Coleman et al., 2011), inhibition of hippocampal neurogenesis in both rats (Crews et al., 2006) and nonhuman primates (Taffe et al., 2010), blunting of the alcohol-responsiveness of tonic inhibitory current in adult hippocampus (Fleming et al., 2012), altered EEG frequencies in the hippocampus and parietal cortex (Slawecki et al., 2001), and reduced numbers of CRF-expressing neurons in the central nucleus of the amygdala (Gilpin et al., 2012). Some changes elicited by adolescent alcohol exposure demonstrated sex-specific patterns, such as reduced glial cell numbers in the medial prefrontal cortex, an effect only observed in males (Koss et al., 2012). Together, these studies highlight the deleterious effects caused by adolescent alcohol exposure in a variety of neural systems, and suggest a role for sex in modifying alcohol's impact on adolescent brain development.

Differential stress response patterns in adolescent and adult rats, indicative of developmental changes in the stress system, accompany the progression to adulthood. Both male and female rats show prolonged cort responses to acute restraint stress during adolescence as compared to adulthood, independent of gonadal sex steroids (Romeo et al., 2004a, Romeo et al., 2004b). Acute stress exposure also triggers a greater activation of PVN neurons, as measured by c-*fos*-ir, in adolescent than adult male rats, particularly within cells that express CRF (Romeo et al., 2006), although we observed elevated *Avp*, not *Crf*, mRNA levels during adolescent alcohol exposure in male rats. Nonetheless, adolescent alcohol exposure in both males and females resulted in permanent perturbations of the adult HPA axis, particularly among females, who showed a striking inhibition of alcohol-induced PVN *Avp* mRNA expression. Additionally, the fact that our AIE males and females displayed elevated numbers of PNMT-positive neurons in brain stem areas C2 and C1, respectively, suggests the possibility that AIE modifies adult HPA responsiveness to alcohol challenge via modified connectivity to adrenergic neurons of the rostroventrolateral medulla. Determination of the specific PVN inputs responsible for the sex differences in AIE modulation of PVN responsiveness to alcohol challenge presents an important area for future study.

Implications for susceptibility to adult alcohol dependence following AIE

While the data clearly demonstrate alterations in the alcohol responsiveness of PVN *Avp* mRNA levels in females and, to a lesser degree, *Crf* mRNA levels in males following AIE experience, the role of these changes in shaping adult alcohol intake are currently unknown. Several studies have demonstrated elevated alcohol intake in adult rats (Pascual et al., 2009, Maldonado-Devincci et al., 2010, Gilpin et al., 2012) and mice (Moore et al., 2010) with binge-like patterns of adolescent alcohol exposure, suggesting the possibility that the observed changes in the stress circuitry of AIE rats might promote high alcohol intake in adulthood. Interestingly, alcohol-preferring P and HAD male rats showed higher levels of PVN *Avp* mRNA (Hwang et al., 1998) but not *Crf* mRNA (Hwang et al., 2004) than their low-drinking NP and LAD counterparts. VP has been demonstrated to support alcohol

tolerance in mice via activation of the VP 1B (V1B) receptor (Hoffman, 1994). In line with these results, alcohol-dependent, but not nondependent, male rats were sensitive to suppression of alcohol intake during withdrawal by treatment with a V1B inhibitor (Edwards et al., 2012). Interestingly, during abstinence, human alcoholics displayed prolonged depression of basal circulating VP levels, lasting at least 9 months after cessation of alcohol intake (Doring et al., 2003), possibly due to epigenetic modification of the *AVP* gene (Hillemacher et al., 2009). The CRF system has also been implicated in human alcohol use disorders, as genetic polymorphisms in the CRF-binding protein, which inhibits CRF interactions with its receptors, have been associated with elevated stress-induced alcohol craving in heavy drinkers (Ray, 2011). Together, these studies suggest possible involvement of both VP and CRF in the maintenance of alcohol use disorders, thus making both systems attractive targets for the development of pharmacotherapies to treat alcohol use disorders, particularly for those patients whose alcohol use begins in adolescence.

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HIGHLIGHTS

- Adolescent alcohol exposure alters the adult PVN response to alcohol challenge
- Adolescent alcohol increases the number of PNMT neurons in the adult brain stem
- Effects of adolescent alcohol on adult PVN and brain stem differ by sex

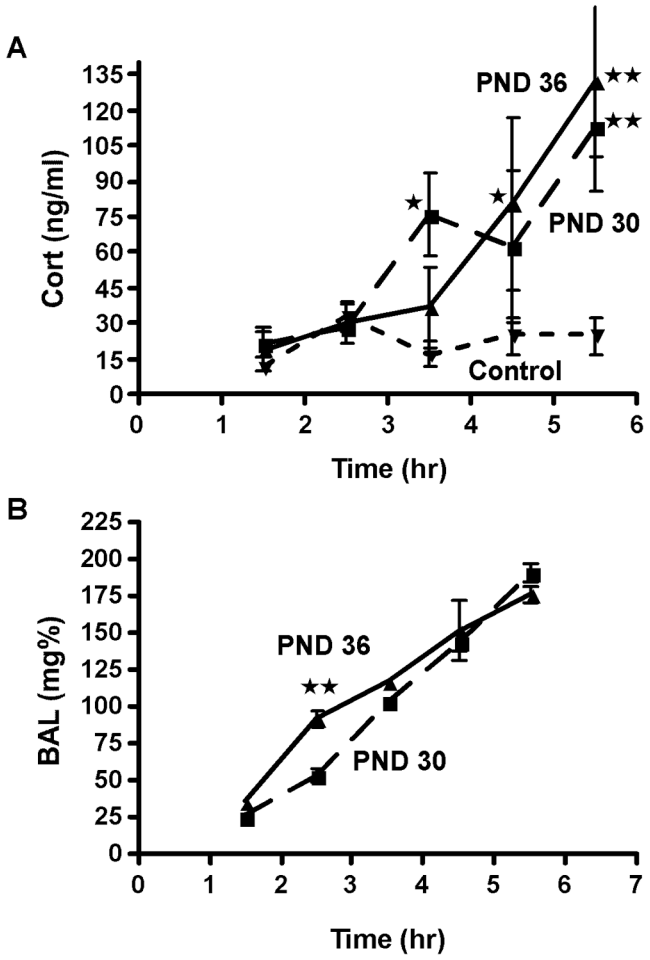

Figure 1.

Time course of elevated corticosterone (cort) and blood alcohol levels (BALs) during adolescent alcohol vapor exposure. Plasma samples were collected by rapid decapitation at 1-h intervals in male rats during a 6-h vapor exposure session or at the same time points in air-exposed controls on PND 30 and PND 36. Samples were analyzed to determine plasma cort levels (A) and BALs (B). Data are represented as mean ± SEM of 4–6 rats per time point. Inverted triangle with short dashed line, Control (collapsed across ages); Square with large dashed line, AIE PND 30; Triangle with solid line, AIE PND36. *, $P<0.05$, **, $P<0.01$, AIE vs. Control (A) or PND 30 vs. PND 36 (B).

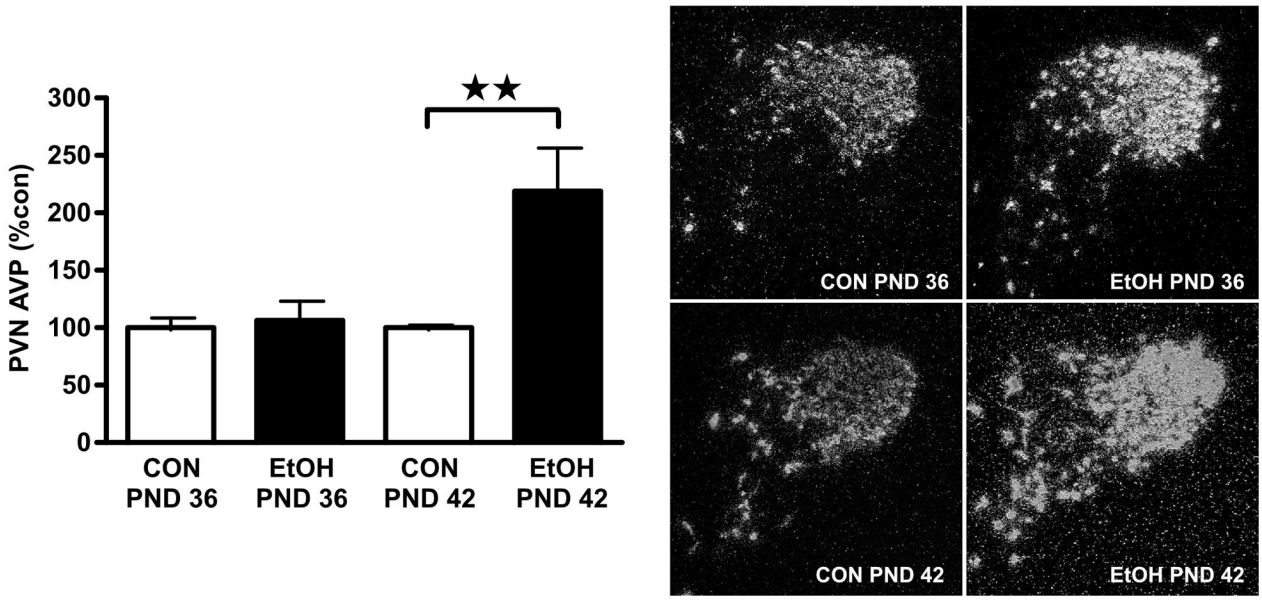

Figure 2.

Exposure to intermittent alcohol vapor during adolescence increased *Avp* mRNA in the PVN of male rats on PND 42 but not PND 36. Male rats were exposed to alcohol vapor for 6 h per day from PND 28 and brains collected for analysis at the conclusion of the 6 h vapor exposure period on PND 36 and PND 42. (Left panel) *Avp* mRNA levels were quantified using optical density (arbitrary units) and normalized to average values of age-matched controls. Data are expressed as mean ± SEM percent control of 4–6 male rats per group. **, $P<0.01$. (Right panel) Representative dark-field photomicrographs (100X magnification) of PVN *Avp* mRNA in alcohol vapor- (EtOH) and air-exposed (CON) rats euthanized on PND 36 and PND 42.

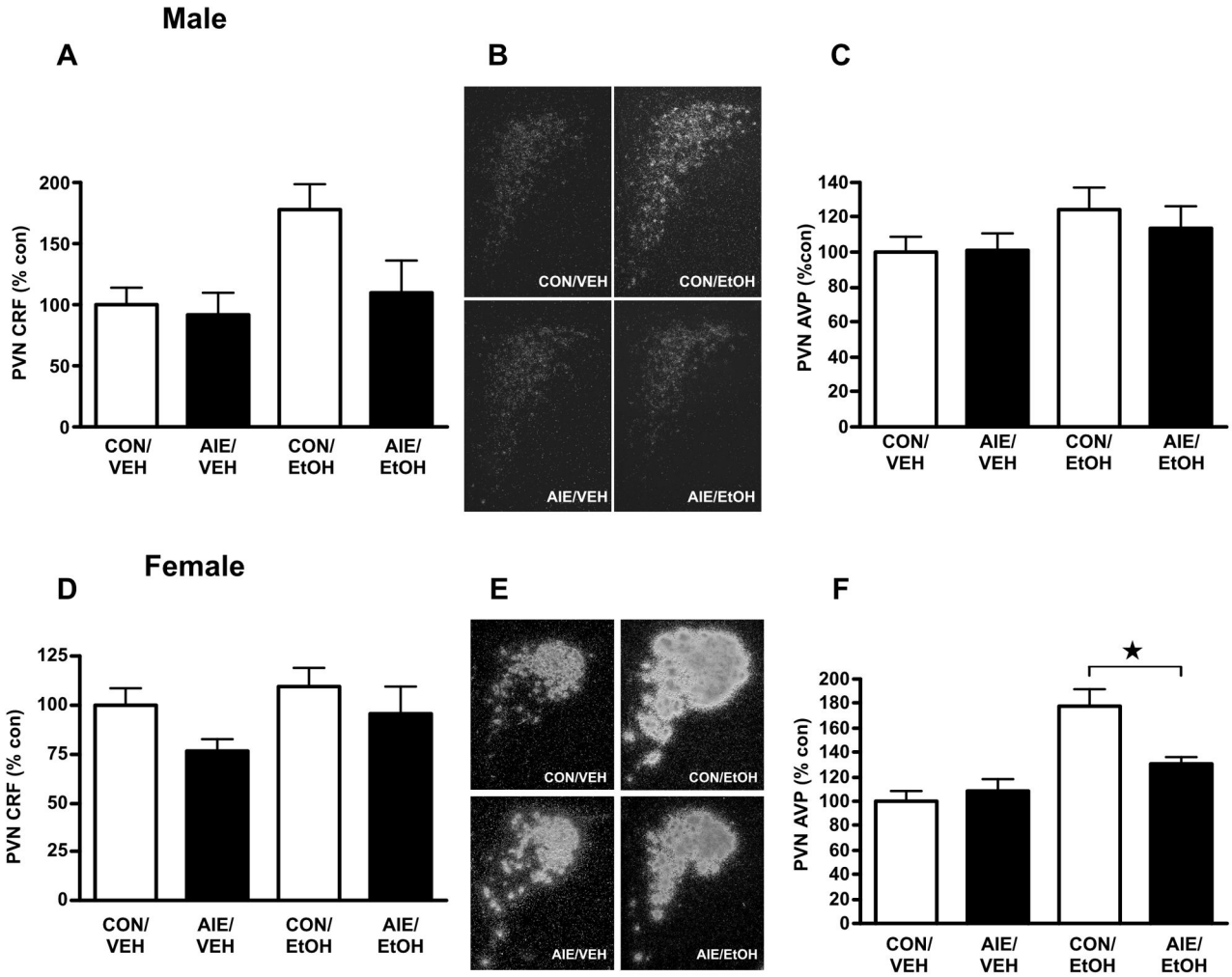

Figure 3.

Effects of intermittent alcohol vapor exposure during adolescence on *Crf* and *Avp* mRNA expression in the PVN of adult (PND 70–71) male (A–C) and female (D–F) rats following alcohol challenge. Control, air-exposed (CON) and adolescent intermittent ethanol-exposed (AIE) rats were euthanized on PND 70–71 2 h after administration of a 4.5 g/kg ig alcohol challenge (EtOH) or equivalent volume of water (VEH), and *in situ* hybridization was performed to quantify *Crf* and *Avp* mRNA levels. (A) Quantification of *CRF* mRNA by densitometric analysis of autoradiographic signals (arbitrary units) in male rats. (B) Representative dark-field photomicrographs (100X magnification) of PVN *Crf* mRNA in male rats. (C) Quantification of *Avp* mRNA by densitometric analysis of autoradiographic signals (arbitrary units) in male rats. (D) Quantification of *Crf* mRNA by densitometric analysis of autoradiographic signals (arbitrary units) in female rats. (E) Representative dark-field photomicrographs (100X magnification) of PVN *Avp* mRNA in female rats. (F) Quantification of *Avp* mRNA by densitometric analysis of autoradiographic signals (arbitrary units) in female rats. Data in histograms are expressed as mean ± SEM optical density expressed percent control relative to CON/VEH average for the given experiment from 4–6 rats per treatment. * $P<0.05$.

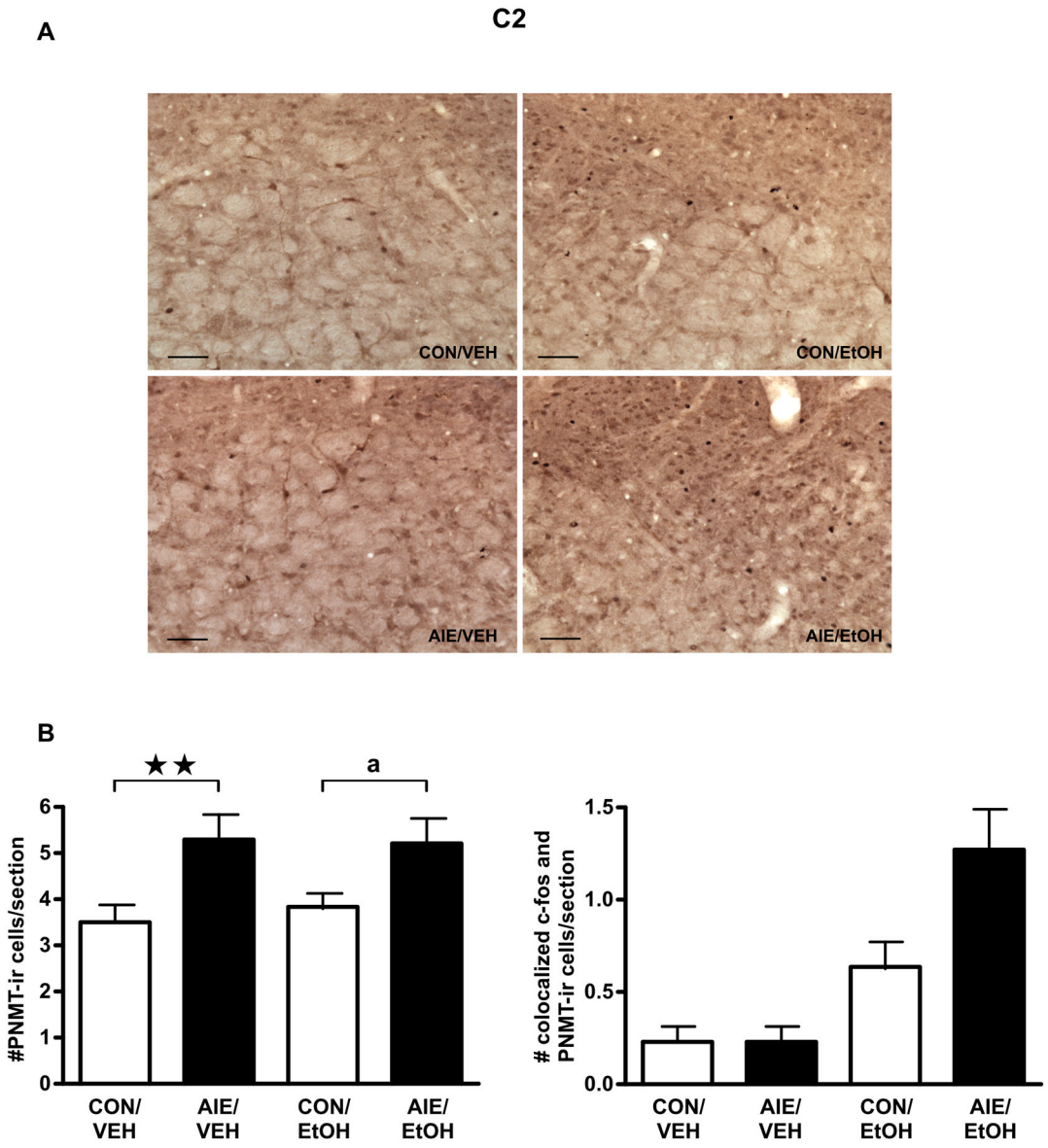

Figure 4.

Exposure to intermittent alcohol vapor during adolescence increased the number of colocalized *c-fos* and PNMT immunoreactive (ir) cells/section and the number of PNMT-ir cells/section in the C2 region of the brain stem of male rats following adult alcohol challenge. Male rats exposed in adolescence to intermittent ethanol (AIE) or air controls (CON) were euthanized 2 h after administration of a 4.5 g/kg ig alcohol challenge (EtOH), or equivalent volume of water (VEH). (A) Double immunohistochemical staining labeled *c-fos*-containing nuclei black and PNMT-containing cell bodies brown. Representative images were captured using a 20X dry objective (scale bar=100 μm). (B) Quantification of PNMT-positive cell numbers (left) and PNMT-positive cells containing *c-fos* staining (right) are expressed as mean ± SEM cells/section for 4–6 male rats per treatment. ** P=0.01, [a] P=0.053.

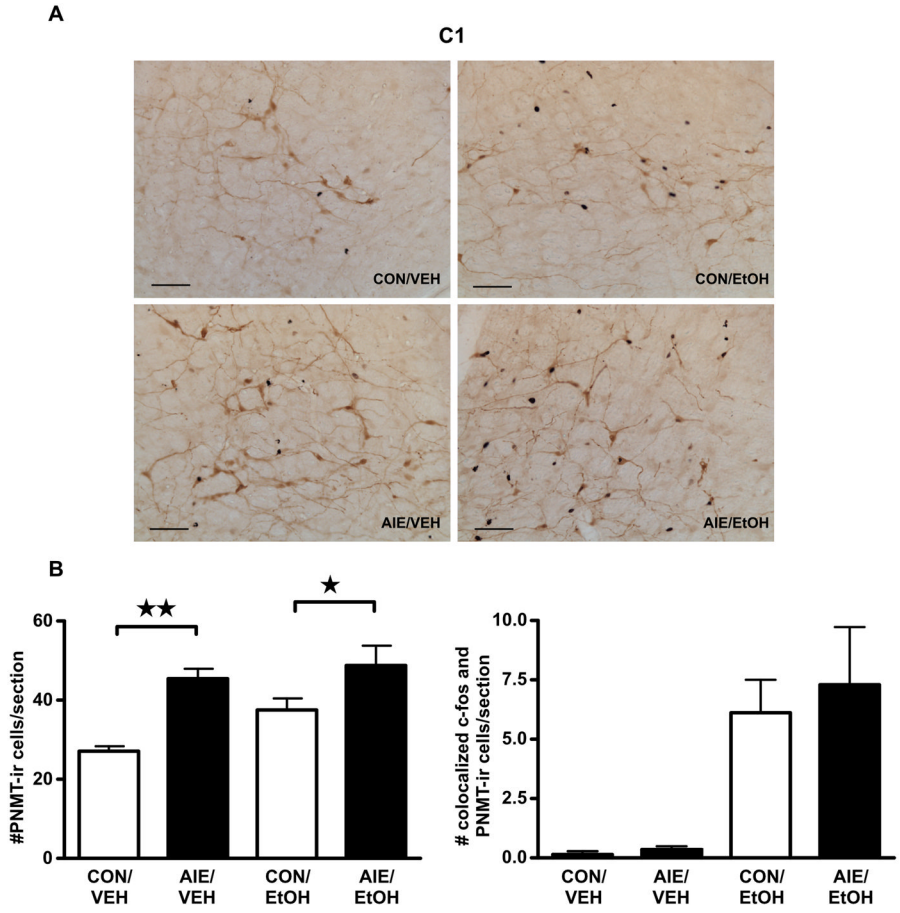

Figure 5.

Exposure to intermittent alcohol vapor during adolescence increased the number of colocalized *c-fos* and PNMT immunoreactive (ir) cells/section and the number of PNMT-ir cells/section in the C1′ region of the brain stem of female rats following adult alcohol challenge. Female rats exposed in adolescence to intermittent ethanol (AIE) or air controls (CON) were euthanized 2 h after administration of a 4.5 g/kg ig alcohol challenge (EtOH), or equivalent volume of water (VEH). (A) Double immunohistochemical staining labeled *c-fos*-containing nuclei black and PNMT-containing cell bodies brown. Representative images were captured using a 20X dry objective (scale bar=100 μm). (B) Quantification of PNMT-positive cell numbers (left) and PNMT-positive cells containing *c-fos* staining (right) are expressed as mean ± SEM cells/section for 4–6 female rats per treatment. * $P<0.05$.

Table 1

Blood alcohol levels at time of brain collection following acute intragastric alcohol administration on PND 70–71

Sex	Control	AIE
M	310.8 ± 27.3	332.8 ± 19.8
F	258.3 ± 11.6	293.4 ± 30.7

Blood alcohol levels are expressed as mean ± SEM mg alcohol per dl blood.

Table 2

Brain stem region C1 PNMT and c-*fos* expression in male rats exposed to AIE and challenged with alcohol on PND 70–71

Group	Treatment[a]	Number of PNMT-ir cells per section in C1	Number of colocalized c-*fos*-ir and PNMT-ir cells per section in C1
Male	Control/vehicle ig	68.6 ± 13.6	0.6 ± 0.2
	Control/alcohol ig	78.1 ± 11.8	4.3 ± 0.9[b]
	AIE/vehicle ig	79.2 ± 3.9	1.8 ± 0.4[c]
	AIE/alcohol ig	92.5 ± 6.4	6.0 ± 0.6[b,c]

[a]Male rats were exposed to alcohol vapor (AIE) or air (Control) from PND 28–42, then injected with alcohol (4.5 g/kg, ig) or vehicle at PND 70–71. Values are mean ± SEM, n=4–6 rats per group.

[b]$P<0.001$, alcohol challenge vs. vehicle.

[c]$P<0.05$, AIE vs. control.

Table 3

Brain stem region C2 PNMT and c-*fos* expression in female rats exposed to AIE and challenged with alcohol on PND 70–71

Group	Treatment[a]	Number of PNMT-ir cells per section in C2	Number of colocalized c-*fos*-ir and PNMT-ir cells per section in C2
Female	Control/vehicle ig	7.4 ± 0.4	0 ± 0
	Control/alcohol ig	8.7 ± 1.2	0.4 ± 0.2[b]
	AIE/vehicle ig	7.7 ± 0.9	0.1 ± 0.1
	AIE/alcohol ig	9.2 ± 1.1	1.2 ± 0.6[b]

[a]Female rats were exposed to alcohol vapor (AIE) or air (Control) from PND 28–42, then injected with alcohol (4.5 g/kg, ig) or vehicle at PND 70–71. Values are mean ± SEM, n=4–6 rats per group.

[b]$P<0.05$, alcohol challenge vs. vehicle.

Table 4

Brain stem region C3 PNMT and c-*fos* expression in female and male rats exposed to AIE and challenged with alcohol on PND 70–71

Group	Treatment[a]	PNMT-ir cells per section	Number of colocalized c-*fos*-ir and PNMT-ir cells per section in C3
Female	Control/vehicle ig	18.7 ± 2.0	0.1 ± 0.1
	Control/alcohol ig	17.8 ± 1.0	0.4 ± 0.3
	AIE/vehicle ig	16.6 ± 1.9	0.1 ± 0.1
	AIE/alcohol ig	18.3 ± 1.8	0.7 ± 0.4
Male	Control/vehicle ig	34.4 ± 4.1	0.3 ± 0.2
	Control/alcohol ig	21.0 ± 2.7	0.9 ± 0.5
	AIE/vehicle ig	24.6 ± 0.7	0.4 ± 0.1
	AIE/alcohol ig	21.8 ± 2.0	1.8 ± 0.4

[a]Male and female rats were exposed to alcohol vapor (AIE) or air (Control) from PND 28–42, then injected with alcohol ig (4.5 g/kg) or vehicle at PND 70–71. Values are mean ± SEM, n=4–6 rats per group.