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Exposure to alcohol during adolescence exerts long-term effects on stress response and the adult brain stress circuits

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Abstract

The hypothalamic-pituitary-adrenal (HPA) axis undergoes critical developments during adolescence. Therefore, stressors experienced during this period potentially have long-term effects on adult HPA axis function. We hypothesized that adolescent intermittent ethanol (AIE) exposure would affect adult HPA axis function, resulting in altered responses to an alcohol challenge in young adults or adults. To test these hypotheses, male rats were exposed to alcohol vapor for 6 h per day from post-natal day (PND) 28–42, then acutely challenged with alcohol intragastrically (3.2–4.5 g/kg) in young adults (PND 70) or adults (PND 90). Overall, we observed blunted HPA axis responses to an alcohol challenge due to AIE exposure. Specifically, AIE tended to inhibit the alcohol-challenge-induced increase in plasma corticosterone (CORT) concentrations in young adult and adult rats. As well, AIE significantly blunted the alcohol challenge-induced arginine vasopressin (*Avp*) mRNA expression in the paraventricular nucleus (PVN) of the hypothalamus of adult rats. Results of the present study are similar to what we have previously shown, that these changes in PVN responsiveness may result from AIE-induced alterations in adrenergic neurons in brain stem regions C1–C3 known to project to the PVN. AIE elevated the number of colocalized c-fos/phenylethanolamine N-methyltransferase (PNMT)-positive cell bodies in the C1 region of adult rats. Together, this data suggests that AIE exposure produces alterations in male HPA axis responsiveness to administration of an acute alcohol challenge that may be long-lasting.

Keywords

Alcohol; Corticosterone; *Crf*; *Avp*; PNMT

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INTRODUCTION

Alcohol drinking is generally initiated during adolescence when the brain is still developing. In comparison to adults, adolescents are less sensitive to the aversive properties of alcohol, while they are more sensitive to the positive rewarding effects of alcohol (Spear and Varlinskaya, 2010). Genetic vulnerability, stress exposure or previous alcohol use may exacerbate these sensitivities to the aversive and rewarding aspects of alcohol (Spear and Varlinskaya, 2010). The effects of exposure to alcohol during adolescence are varied and could contribute to increased alcohol consumption in adulthood; these consequential effects may involve changes in the mesolimbic dopaminergic and glutamatergic systems (Pascual et al., 2009), impairment of neurogenesis (Morris et al., 2010), and differences in brain damage than is found in adults (Crews et al., 2000). Interestingly, adolescents also seem to be more susceptible to the effects of stress on alcohol drinking (Siegmund et al., 2005, Füllgrabe et al., 2007). Our laboratory is interested in alcohol's effects on the central circuits known to regulate stress response – the hypothalamic-pituitary-adrenal (HPA) axis, which includes the paraventricular nucleus (PVN) of the hypothalamus and adrenergic brain stem regions that provide catecholamine inputs to the hypothalamus. It is widely known that alcohol exposure alters corticotropin releasing factor (*Crf*) activity in the PVN (Rivier et al., 1984, Rivier et al., 1990) with reported increases in PVN neuronal activity (Lee et al., 2000b) as well as corticosterone (CORT) release from the adrenal glands in response to alcohol consumption (Richardson et al., 2008). Our lab has also shown that alcohol causes changes in catecholaminergic cell numbers and activation following alcohol exposure (Logrip et al., 2013). However, it is important to distinguish between the response of a brain structure to alcohol itself, and the consequence of previous exposure to alcohol on the HPA axis' ability to respond to an additional stressor such as an alcohol challenge in young adulthood and adulthood. We have examined the effects of adolescence binge drinking (self-administration) and adolescent intermittent ethanol (AIE) exposure (vapor chambers) on the brain stress response in young adulthood. In adolescent self-administering binge-drinking animals, we found a decrease in the number of *Crf* cells in the young adult amygdala (Allen et al., 2011a), a brain region that is important in conveying the emotional component of the stress response (Pich et al., 1995). Additionally, we have shown that AIE exerts long-term effects on the ability of the PVN to respond to an alcohol challenge in young adulthood, possibly mediated by catecholaminergic input from the brain stem to the PVN as seen by changes in activation (measured via c-fos immunoreactivity) of phenylethanolamine N-methyltransferase (PNMT) neurons (Allen et al., 2011b, Logrip et al., 2013). These results prompted us to investigate whether AIE exposure similarly or differentially affects the response to a distinct acute stressor, an alcohol challenge in young adult and adult male rats.

EXPERIMENTAL PROCEDURES

Animals

Male Sprague Dawley rats (n = 45) were obtained from Harlan Laboratory (San Diego, CA, USA). Animals were housed three or four per cage with food and water *ad libitum* in a humidity- and temperature-controlled vivarium under a 12 h light/12 h dark light cycle with lights off at 1800. All experiments were carried out in the morning and 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 [post-natal day (PND) 28–42] were exposed to alcohol vapor in an airtight 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, 2000b). Rats were exposed to alcohol as vapor daily for 6 h (0700–1300; AIE) or air [Control (CON)] for the duration of the 15-day adolescent period (PND 28–42). After each daily exposure, the rats were returned to clean housing cages. Blood alcohol levels (BALs) were obtained and monitored on alternate days to maintain BALs at approximately 200 mg/dL. This exposure paradigm was used to generate controlled daily cycles of alcohol intoxication and withdrawal during the adolescent period as described previously (Logrip et al., 2013).

Blood alcohol levels

Blood samples for BAL measurements were obtained from the tails of all animals, including the air-exposed rats to control for any procedural stress effects. Blood plasma (5 μ L) was used to determine BALs via an Analox AM 1 analyzer (Analox Instruments Ltd., Lunenburg, MA, USA (Lee et al., 2000a, 2000b). The precision of this assay is 1–2%, the sensitivity is 0.1 mg/dL and the curve is linear up to 400 mg/dL.

Corticosterone assay

Plasma corticosterone levels were measured in unextracted plasma from iv blood according to manufacturer's instructions using a widely applied commercial double antibody RIA kit for rat and mouse samples (MPBiomedicals, Diagnostic Division, Orangeburg, NY, USA, catalog # 07120103). Specifically, blood was collected into chilled plastic tubes containing EDTA (0.6 mg/500 mL whole blood) and centrifuged. The resulting plasma was aspirated and a 5 μ L aliquot immediately diluted into 995 μ L corticosterone assay buffer, 1/200 final dilution per manufacturers instructions, and stored frozen until thawing once for CORT analysis. All samples from a single experiment were analyzed in the same CORT RIA and all samples displayed B/B₀ of 90–20%. The reliable range of the assay is 10 to 1000 ng/mL with an intra-assay coefficient of variance of <10%. This assay is highly specific for corticosterone; the cross-react with desoxycorticosterone is 0.34%, testosterone is 0.1% and cortisol is 0.05%.

Animal surgery and alcohol injection

AIE and control male rats were implanted with ig or iv catheters upon reaching PND 62–63 or PND 68–69, respectively, under isoflurane anesthesia (Butler Animal Health Supply, Dublin, OH, USA) [see (Ogilvie et al., 1997) for methods], and were allowed to recover from surgery in individual cages for 7–8 days (ig) or 1–2 days (iv) before experimentation. On the day of the experiment (PND 70 or 90), the animals were placed in individual buckets with wood chip bedding in a quiet room with extension cannulae connected to a syringe containing heparinized saline such that the animals could be injected without being handled, to prevent any procedural stress. They were free-moving and left undisturbed for 2–3 h in an

effort to acclimatize rats, then administered 3.2–4.5 g/kg alcohol (< 20% v/v in water) via the ig cannula. The alcohol challenge dose corresponds to that previously used in our laboratory (Lee et al., 2001, Lee and Rivier, 2003, Seo and Rivier, 2003). Injections were slowly infused over a 2-min period because of the large volume needed to be administered.

Acute Stressor

Plasma CORT concentration in young adult (PND 70) rats was determined to confirm a hormonal stress response to alcohol – a previously published stressor known to elicit changes in HPA-related brain circuitry [i.e., alcohol challenge; (Logrip et al., 2013)]: n = 6–7/group]. We also determined CORT concentration in adult animals (PND 70 or 90) to identify a hormonal stress response to an alcohol challenge; blood was collected from control (air: n = 4–7) and treatment (AIE: n = 4–7) male rats at 0, 60, 120, 180 and 240 min (PND 70) and at 0, 60, 120 and 210 min (PND 90) after an alcohol challenge (3.2–3.6 g/kg, ig). Finally, the effects of AIE exposure on the brain stress response to an alcohol challenge in adult animals was examined. Following an alcohol challenge in adulthood, the expression of *Crf* or arginine vasopressin (*Avp*) mRNA in the PVN of the same animals was examined (air: n = 4–5 and AIE: n = 6). In addition, the number of PNMT cells in the brain stem (C1–C3) region (air: n = 6 and AIE: n = 6) was studied.

Immunohistochemistry

Young adult and adult rats were deeply anesthetized by an ip 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. Briefly, double DAB immunohistochemistry (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 (1:7500, 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 c-fos-positive cells colocalized with PNMT-positive cell bodies.

In situ hybridization

As we observed the blunting effect of AIE on CORT responses to alcohol challenges, we further hypothesized that AIE would also decrease *Crf* and *Avp* mRNA expression in the PVN of adult male rats during the alcohol challenge. To test this, *Crf* and *Avp* mRNA levels were assessed in adult male rats at the end of the 2-h alcohol administration (4.5 g/kg, ig). *In*

situ hybridization was performed according to a previously published protocol [see (Lee et al., 2000a, 2000b) for method].

Imaging

A Nikon optical system, 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 was used to capture images for IHC and *in situ* sections. IHC images were obtained with 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

For all analyses which contained repeated measures over time or different brain regions 2-way ANOVA for repeated measures with Sidak's multiple comparisons test between groups at individual time points was used. All samples with $n > 4$ were tested for normal distribution employing the Shapiro-Wilk or Kolmogorov-Smirnov test and visual evaluation using QQ plots. Equality of variances was tested using the Brown-Forsythe method. Single moderate violations were considered acceptable taking in account the relatively high robustness of ANOVA against minor violations especially when equal sample sizes are employed. However, in order to reduce probability for type-I errors post-hoc tests were amended by non-parametric tests for comparisons containing non-normally distributed samples and by Welch-corrected t-tests when variances between samples were significantly unequal. For simple comparisons between two samples unpaired t-tests were used when samples displayed normal distribution (determined by Kolmogorov-Smirnov test) and equal variances (Brown-Forsythe). Otherwise they were amended by non-parametric (Mann-Whitney) or Welch-corrected t-tests. Statistics were performed using Microsoft Excel 2013 (Microsoft Corp., Redmond, WA, USA) and Graphpad Prism 6.0 (Graphpad Software Inc., La Jolla, CA, USA). For all tests $P < 0.05$ was considered significant. Where applicable, significant differences indicated in figures are based on corrected/non-parametric p-values.

RESULTS

Effect of AIE on plasma corticosterone (CORT) levels of young adult and adult male rats in response to an alcohol challenge

We hypothesized that exposure to alcohol during adolescence would have long-lasting effects on the adult HPA axis in response to an alcohol challenge. As predicted based on previous changes in the brain stress response (Logrip et al., 2013), the AIE group showed decreased plasma CORT concentrations in comparison to that of the control group at multiple time-points after ig alcohol injection in young adulthood (significant time $\times$ group interaction, $F(4,48)=2.679$ $p < 0.05$, $n=7$ per group; significant difference between groups at 180 min: $t=3.275$, $DF=60$, $P < 0.01$ (Sidak); $t=2.917$, $df=6.236$, $p < 0.05$ (Welch-corrected t-test); Fig. 1). Furthermore, we tested to see if the blunting effects of AIE that we observed in

young adult male rats were consistent with adult male rats as well (Fig. 2). As plasma CORT levels were measured at 60, 120, and 210 minutes after the injection of alcohol ig (3.2 g/kg), the AIE group again showed a significantly decreased CORT level compared to the control group (significant time $\times$ group interaction, $F(3,18)=3.276$, $P<0.05$, $n=4$ per group; significant difference between groups at 120 min: $t=2.829$, $DF=24$, $P<0.05$ (Sidak); Fig. 2, *top*). BALs, on the other hand, did not show any difference between AIE and control group, suggesting that differences in plasma CORT levels in adult male rats were independent of BAL discrepancies (Fig. 1 and 2, *bottom*). These results support the previous findings, from PVN *Crf* and *Avp* gene expression data that we published [see (Logrip et al., 2013)], suggesting that AIE blunts HPA axis activity. Together, these results show that AIE has a unique effect of inhibiting the HPA axis in response to an alcohol challenge.

Effect of AIE on PVN *Crf* and *Avp* mRNA levels of adult male rats in response to an alcohol challenge

As illustrated in Fig. 3, PVN *Avp* mRNA levels were significantly decreased in the AIE group ($t=2.416$, $df=9$, $P<0.05$, $n=5$ CON, $n=6$ AIE). However, PVN *Crf* mRNA levels showed no difference between AIE and control groups (Mann-Whitney $U=6$, $P>0.05$, $n=4$ CON, $n=6$ AIE). Together, these data support that AIE blunted PVN *Avp* gene expression at the end of a 2-h alcohol challenge in adult males.

Effect of AIE on brain stem PNMT levels of adult male rats in response to an alcohol challenge

Based on the observed effect of AIE on PVN *Crf* or *Avp* mRNA expression of adult male rats in response to an alcohol challenge, we hypothesized that AIE would affect brain stem PNMT neuron activation of adult male rats in response to an alcohol challenge as well. To test this, we performed c-fos/PNMT IHC on adult male rats' brain stem, following alcohol administration (4.5 g/kg, ig). As shown in Fig. 4, the AIE group showed significantly elevated colocalization of c-fos-ir and PNMT-ir cells/section in the C1 medullary region (significant brain region $\times$ group interaction, $F(2,20)=9.343$, $P<0.01$, $n=6$ per group; post-hoc comparison at C1: $t=3.566$, $DF=30$, $P<0.01$), in comparison to the control group. For both C2 and C3 regions of the brain stem, there was no statistically significant difference observed between AIE and control group in activation of PNMT-expressing neurons in the brain stem. Together, these data indicate that AIE differentially altered medullary brain stem regions in adult male rats 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 overall goal of this study was to determine if AIE exposure exerts long-term effects on stress response and brain stress circuits in adult animals subjected to a distinct acute stressor – alcohol challenge. In line with previous findings in young adult male rats exposed to alcohol during adolescence, we found that alcohol elicits a stress response (CORT) and that AIE exposure causes changes in the brain stress circuitry to an alcohol challenge in adult male rats. Abnormal stress responses could have repercussions, such as changes in immune

function (Segerstrom and Miller, 2004), which could lead to a decrease in overall health or survivorship.

Intermittent alcohol exposure during adolescence blunts the CORT response to an alcohol challenge. Interestingly, Przybycien-Szymanska et al. (2011) found the opposite result; animals exposed to ethanol (single dose or binge-like) during puberty (PND 37–44) had greater CORT concentrations than control animals and this finding suggested that when subjected to a stressor (ethanol), the HPA axis of young adult (PND 75) animals (Male Wistar rats) exposed to ethanol during puberty was more responsive. It is likely these differences are due to the more invasive method of ethanol administration (ip injection vs. ig cannulation administration) of the Przybycien-Szymanska et al. (2011) study as the intensity and type of stressor differs between the studies, with the less intense stress occurring with present methodology. Despite these discrepancies, it is evident that adolescent drinking can alter the adult HPA axis response to stress.

Exposure to intermittent alcohol vapor during adolescence significantly decreased *Avp* mRNA expression in the PVN of adult male rats following alcohol challenge in adulthood. In contrast, while Przybycien-Szymanska et al. (2011) found no significant difference in PVN *Avp* mRNA between control rats and young adult (PND 75) male rats with a history of alcohol injections during adolescence, the authors did find that adolescent alcohol-experienced rats had elevated PVN *Crf* mRNA after an acute alcohol challenge administered in young adulthood. Previous studies in our laboratory on AIE where young adult animals were challenged with alcohol identified the opposite result; Allen et al. (2011b) found a decrease in PVN *Crf* in young adult male (Sprague Dawley) rats (PND 61–62) rather than a decrease of *Avp* expression. Again, it is possible that the differences between our findings and those of the Przybycien-Szymanska et al. (2011) study are due to different methods of alcohol challenge. Taken together, these findings support our central hypothesis that AIE exerts long-term neuroendocrine effects.

Considering that we found a decrease in CORT concentration following an acute stressor in adult rats exposed to AIE, it is not surprising that we found a decrease in *Avp* mRNA levels in the PVN. Recently, Jasnic et al. (2013) found direct evidence of the role of *Avp* in regulating pituitary adrenocorticotrophic hormone secretion which correlated with CORT concentration in male Wistar rats. This result supports our finding that exposure to alcohol during adolescence decreases the ability of the brain to mount a stress response to an acute stressor in adulthood and this is likely due to the hypothalamic blunting of *Avp* which consequently decreases the adrenal release of CORT into circulation.

An alcohol challenge in adulthood following AIE exposure increased the stress response of adrenergic cells in the brain stem area which innervates the hypothalamus (see summary of studies in Table 1). Specifically, AIE increased PNMT neuron activation in the C1 region of the brain stem of adult male rats following adult alcohol challenge. As well, exposure to intermittent alcohol vapor during adolescence increased PNMT neuron activation and the number of PNMT-ir cells/section in the C2 region of the brain stem of male (Sprague Dawley) rats following young adult alcohol challenge (Logrip et al., 2013). Similarly, Allen et al. (2011a) found a decrease in PNMT neuron activation in the C3 region of the brain

stem of young adult male (Sprague Dawley) rats (PND 61–62) that voluntarily consumed alcohol in a binge-like manner during adolescence. Other researchers who examine the effects of stress on the heart, found an increase in PNMT expression in the ventricles of the heart of rats and suggested that this finding could provide a molecular mechanism that contributes to a modified physiological response, which is necessary for stress responsivity (Spasojevic et al., 2011). These multiple lines of evidence of changes in the catecholaminergic system suggest that central catecholamines play an important role in modulating the long-term consequence of AIE exposure on the HPA axis.

Overall, exposure to alcohol during adolescence alters (1) corticosterone release following an alcohol challenge, (2) *Avp* mRNA levels in the PVN after alcohol challenge, and (3) adrenergic cell activation in the brainstem following an alcohol challenge. All of these findings suggest that the HPA axis is vulnerable to the effects of alcohol during adolescence. Because the adolescent brain is still developing it is possible that alcohol permanently alters the brain during development and prevents it from being able to mount a stress response in the future. Based on our results, it may be that the adrenergic neurons that project to the PVN are activated in a greater number during alcohol consumption in adulthood, which then decreases the ability of the hypothalamus to respond to that stressor. Therefore, adults that were exposed to alcohol during adolescence may have irrevocably altered the ability of their brain to respond to stressful events (e.g., alcohol drinking in adulthood). However, future studies are required to further investigate the role of the HPA axis system and the catecholaminergic brain stem regions in modulating the future consequences of AIE exposure on adult's HPA axis system.

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Abbreviations

AIE	adolescent intermittent ethanol
Avp	arginine vasopressin
BAL	blood alcohol level
CON	control group
CORT	corticosterone
Crf	corticotropin releasing factor
HPA	hypothalamic-pituitary-adrenal
ir	immunoreactive
ig	intragastric
iv	intravenous

PVN	paraventricular nucleus
PNMT	phenylethanolamine N-methyltransferase
PND	post-natal day

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Highlights

- AIE blunts the young adult and adult corticosterone response to alcohol.
- AIE blunts paraventricular nucleus *Avp* expression response to alcohol challenge.
- AIE increases adult brain stem PNMT activation response to alcohol challenge.

Fig. 1.

Following an alcohol challenge in young adulthood (3.6g/kg via ig on PND 70) adolescent intermittent ethanol (AIE) exposure significantly decreased plasma CORT concentrations in male rats compared to the control group (CON) in young adulthood. There was no significant blood alcohol level (BAL) difference observed between AIE and control groups at any given time point in young adulthood. All male rats were exposed to alcohol vapor for 6 hours per day from PND 28 to PND 42, and blood samples were collected for analysis at each time point of interest. (**Top**) Data are expressed as mean $\pm$ SEM CORT concentration of 7 male rats per group. * $P < 0.05$. (**Bottom**) Data are expressed as mean $\pm$ SEM BAL of 7 male rats per group.

Fig. 2.

Following an alcohol challenge in adulthood (3.2g/kg via ig on PND 90), adolescent intermittent ethanol (AIE) exposure significantly decreased plasma CORT concentrations in male rats compared to the control group (CON) in adulthood. There was no significant blood alcohol level (BAL) difference observed between AIE and control groups at any given time point in adulthood. All male rats were exposed to alcohol vapor for 6 hours per day from PND 28 to PND 42, and blood samples were collected for analysis at each time point of interest. **(Top)** Data are expressed as mean $\pm$ SEM CORT level of 4 male rats per group. * P < 0.05. **(Bottom)** Data are expressed as mean $\pm$ SEM BAL of 4 male rats per group.

Fig. 3.

Exposure to intermittent alcohol vapor during adolescence significantly decreased *Avp* mRNA levels in the PVN of adult male rats (PND 90) compared to controls following an alcohol challenge. Male rats exposed to adolescent intermittent ethanol (AIE) vapor or air (CON) were perfused 2 h after administration of alcohol challenge (4.5 g/kg, ig), and *in situ* hybridization was performed to quantify *Crf* and *Avp* mRNA levels in the PVN. (A) Quantification of PVN *Crf* mRNA levels by densitometric analysis of autoradiographic signals (arbitrary units, left) and representative dark-field photomicrographs of PVN *Crf* mRNA levels in adult male rats on PND 90 (100x magnification, right). (B) Quantification of PVN *Avp* mRNA levels by densitometric analysis of autoradiographic signals (arbitrary units, left) and representative dark-field photomicrographs of PVN *Avp* mRNA levels in male rats on PND 90 (100x magnification, right). Data in histograms are expressed as mean $\pm$ SEM optical density, expressed as percent control relative to CON/EtOH (ethanol) average for the given experiment from 4–6 rats per treatment. * $P < 0.05$.

Fig. 4.

Exposure to intermittent alcohol vapor during adolescence significantly increased the number of colocalized c-fos and PNMT immunoreactive (ir) cells/section in the C1 region of the brain stem of male rats following alcohol challenge in adulthood (PND 90). Male rats exposed to adolescent intermittent ethanol (AIE) or air (CON) were perfused 2 h after administration of the alcohol challenge (4.5 g/kg, ig). (A) Double immunohistochemical staining labeled c-fos-containing nuclei black and PNMT-containing cell bodies brown in C1–C3 regions of the brain stem. Representative images were captured using a 20x dry objective (scale bar = 100 μ m). (B) Quantification of PNMT-positive cell numbers (top) and PNMT-positive cells containing c-fos staining (bottom) are expressed as mean $\pm$ SEM cells/section for 6 male rats per treatment. ** $P < 0.01$.

Table 1

Exposure to alcohol during adolescence significantly changes the number and activation of adrenergic cells [phenylethanolamine N-methyltransferase (PNMT)] in the brain stem area (which innervates the hypothalamus – the site of stress response initiation) following an alcohol challenge in young adult and adult male rats. Note: post-natal day (PND).

Treatment Group (Citation)	PNMT-ir cells per section	c-fos-ir/PNMT-ir cells per section
Vapor	C1: -	C1: -
<i>PND 61–62</i>	C2: ↑	C2: -
(Allen et al., 2011b)	C3: -	C3: -
Vapor	C1: -	C1: ↑
<i>PND 70–71</i>	C2: ↑	C2: -
(Logrip et al., 2013)	C3: -	C3: -
Vapor	C1: -	C1: ↑
<i>PND 90–91</i>	C2: -	C2: -
(Present Study)	C3: -	C3: -
Self-Administration	C1: -	C1: -
<i>PND 61–62</i>	C2: -	C2: -
(Allen et al., 2011a)	C3: -	C3: ↑