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ADOLESCENT INTERMITTENT ETHANOL EXPOSURE ENHANCES ETHANOL ACTIVATION OF THE NUCLEUS ACCUMBENS WHILE BLUNTING THE PREFRONTAL CORTEX RESPONSES IN ADULT RAT

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Abstract

The brain continues to develop through adolescence when excessive alcohol consumption is prevalent in humans. We hypothesized that binge drinking doses of ethanol during adolescence will cause changes in brain ethanol responses that persist into adulthood. To test this hypothesis Wistar rats were treated with an adolescent intermittent ethanol (AIE; 5 g/kg, i.g. 2 days on–2 days off; P25–P54) model of underage drinking followed by 25 days of abstinence during maturation to young adulthood (P80). Using markers of neuronal activation c-Fos, EGR1, and phophorylated extracellar signal regulated kinase (pERK1/2), adult responses to a moderate and binge drinking ethanol challenge, e.g., 2 or 4 g/kg, were determined. Adult rats showed dose dependent increases in neuronal activation markers in multiple brain regions during ethanol challenge. Brain regional responses correlated are consistent with anatomical connections. AIE led to marked decreases in adult ethanol PFC (prefrontal cortex) and blunted responses in the amygdala. Binge drinking doses led to the nucleus accumbens (NAc) activation that correlated with the ventral tegmental area (VTA) activation. In contrast to other brain regions, AIE enhanced the adult NAc response to binge drinking doses. These studies suggest that adolescent alcohol exposure causes long-lasting changes in brain responses to alcohol that persist into adulthood.

Keywords

adolescence; alcohol; networks; frontal cortex; development

INTRODUCTION

Human studies find that adolescent brain maturation involves the transition of the immature child brain to the mature networks that characterize the adult brain (Giedd et al., 1999; Giedd, 2004). Unfortunately, adolescence is also a time when individuals initiate alcohol use and abuse (Spear, 2011). Studies find heavy episodic drinking in 5% of 8th grade, 14% of 10th grade, and 22% of 12th grade individuals (Johnston et al., 2013). This heavy drinking

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pattern increases in college with 44% of students reporting binge drinking every 2 weeks, and 19%reporting more than three binge drinking episodes per week (Wechsler et al., 1995; O'Malley et al., 1998). Routine binge drinking might lead to long-term changes in adult neurobiology due to the heightened neural plasticity and structural development that characterizes the adolescent brain (Crews et al., 2007). An earlier age of drinking onset (i.e., 11–14 years of age) increases the risk of developing an alcohol use disorder later in life (DeWit et al., 2000). Further, adolescent binge drinking is associated with diminished impulse inhibition (White et al., 2011), reduced attentional functioning (Koskinen et al., 2011), deficits in visuospatial ability (Giancola et al., 1998; Tapert et al., 2002), and impaired executive functioning (White et al., 2011). Although adolescents binge drink, the pattern is intermittent, and not daily. To investigate the impact of adolescent binge drinking on adult brain we treated rats with a model of intermittent binge drinking, i.e., adolescent intermittent ethanol (AIE) administration. Following AIE treatment, animals were abstinent during maturation to young adulthood. Alterations in the adult brain responses to alcohol challenge were used to test the hypothesis that AIE would induce long-term alterations in adult neuronal responses to ethanol.

Immediate early genes (IEGs) are genes that are transiently and rapidly induced in activated neurons. c-Fos protein, a product of an immediate early gene (c-Fos), is a transcription factor thought to play a role in neuronal adaptations and brain plasticity (Chaudhuri, 1997; Filipkowski et al., 2000) as a marker of neuronal activation (Brown et al., 1992; Liste et al., 1997). Transcription of c-Fos is triggered by intracellular calcium and cAMP (Cyclic adenosine monophosphate), which are increased through activation of NMDA (N-methyl-D-aspartic acid) receptors, L-type calcium channels, and/or other excitatory receptors, neuronal activity where action potentials are coincident with synaptic activity (Bito et al., 1997). Another transcription factor that marks neuronal activation and long-term potentiation is EGR-1 (Cole et al., 1989) which along with phophorylated extracellar signal regulated kinase (pERK1/2), a kinase that marks neuronal activation (Gao and Ji, 2009), provide induction of neuronal activation that are implicated in long-term neuronal plasticity (Hiroi et al., 1999) and provide insights into neural signaling that may be altered by AIE. Ethanol- and stress-induced increases in brain c-Fos are independent of circulating levels of CORT (Helmreich et al., 1996; Ryabinin et al., 1999; Hansson et al., 2003) suggesting changes in neuronal activation markers provide direct insights into neuronal and neurocircuit changes in plasticity that may be altered by AIE.

Many studies have investigated ethanol induction of neuronal c-Fos and EGR1 and found that numerous and similar brain regions are activated by systemic ethanol exposure of rats and mice (Ryabinin et al., 1997, 1999; Knapp et al., 2001; Vilpoux et al., 2009). Ethanol activation of brain neurons likely occur through multiple mechanisms including directional pharmacological effects on neurons as well as through alterations in excitability through altered neuronal networks. Comparison of c-Fos responses to acute systemic ethanol with responses to intraventricular injections suggests direct ethanol effects on brain regions contributing to reward and feeding, with circuits regulating aversion and feeding behavior contributing to the broad neuronal activation during peripheral ethanol challenge in naïve rats (Crabbe et al., 1983; Crankshaw et al., 2003). Chronic ethanol administration leads to tolerance of c-Fos induction in many brain regions showing that loss of response is

associated to a reduction in the aversive qualities of alcohol during chronic exposure (Hansson et al., 2008). We investigated the ethanol response of multiple brain regions involved in decision making, learning, reward, and negative affect using immediate early gene expression. We report here that AIE followed by abstinent maturation to adulthood markedly blunts the adult mPFC (medial prefrontal cortex) response to alcohol, but enhances the nucleus accumbens (NAc) response to alcohol.

EXPERIMENTAL PROCEDURES

Animals

Twelve timed-pregnant Wistar rats, young mothers at the same age, were ordered from Harlan Laboratories, Inc. (Indianapolis, IN, USA) under a protocol approved by the Institutional Animal Care and Use Committees at the University of North Carolina. Timed-pregnant dams were allowed to acclimate to our vivarium (University of North Carolina at Chapel Hill) at embryonic day 17 (E17), and pups were bred and reared to avoid pre-existing variation. Efforts were made to reduce environmental variation. All animals were maintained at 22 °C under 12:12-h light/dark cycles with free access to food and water. On the day following birth (postnatal day 1, P1), litters were culled to 10 pups (including male and female pups at this time). On weaning at P21, male offspring was pair-housed with a same-sex, same-age non-littermate and body weight match assigned to two experimental groups, control and ethanol (average 4–5 male rats used from each litter). This study was done in all males to avoid the confounds of sex differences in puberty and hormone cycles. Fig. 1A illustrates the intermittent treatment protocol (e.g., 2 days alcohol, 2 days off) with ethanol (5 g/kg, 25% ethanol w/v, i.g.) or water from early adolescence until well after puberty (P25–P54); the control group was administered the same volume of water. Body weight of animals, measured every four days was shown in Fig. 1B. Ethanol treatment stopped on P54. There was no treatment until postnatal day 80 (P80). At P80, the ethanol group was subdivided into three groups with body weight match (n = 7–8/each group); two groups of them were separately challenged with 2 or 4 g/kg i.g. ethanol (AIE-challenge 2 g/kg and AIE-challenge 4 g/kg groups), and the third one was administered with the same volume of water (AIE group). The water control group was subdivided into three adult-matched groups (n = 7–8/each group) identical to the adolescent ethanol-treated animals. Animals were handled at least 7 days before challenge day (P80), and sacrificed 2 h after either acute ethanol or water treatment.

Blood ethanol concentration

Tail blood samples were collected 1 h after ethanol treatment (5 g/kg i.g.) at P38 and P54, and 2 h after acute ethanol treatment (2 or 4 g/kg i.g.) at P80 (Fig. 1B). Blood ethanol concentrations (BEC) were measured using a GM7 Analyser (Analox, London, UK).

Tissue Collection and Preparation

Rats were deeply anesthetized with an overdose of sodium pentobarbital, and transcardially perfused with 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde (in 0.1 M phosphate buffer, pH 7.4). Brains were removed, and post-fixed for 24 h in 4% paraformaldehyde at 4 °C, and embedded with paraffin. Coronal sections

(from the entire rostral to caudal of the whole brain) were obtained at a thickness of 10 μm in 1:20 series.

Histology procedure

All tissue sections embedded with paraffin were deparaffinized, rehydrated and retrieved with antigen retrieval buffer. Briefly, sections were incubated in 0.6% hydrogen peroxide (H_2O_2) for 30 min, and blocked in 3% goat serum (0.25% Triton X-100, Sigma, Saint Louis, MO) for 1 h at room temperature prior to 48 h for c-Fos (Ab-5)(4–17)(1:10,000, PC38; Calbiochem, EMD Chemicals, Inc., San Diego, CA, USA), and overnight for EGR1 (1:200, #4153; Cell Signaling Technology, Inc., Danvers, MA, USA) and phospho-p44/42 MAPK (ERK1/2) (1:200, #4370, Cell Signaling Technology, Inc., Danvers, MA, USA) at 4 °C. Then, sections were rinsed in PBS, and incubated with biotinylated secondary anti-rabbit antibody (Vector Laboratories Inc., Burlingame, CA, USA) for 1 h at room temperature. Subsequently, avidin–biotin complex (Vector ABC kit, Vector Laboratory Inc., Burlingame, CA, USA) was applied for 1 h at room temperature, and positive neurons were visualized using nickel-enhanced diaminobenzidine (DAB, D5637, Sigma, Sigma–Aldrich CO. LLC, Saint Louis, MO, USA) reaction.

Double fluorescent staining for c-Fos and other cell markers was done using a mix of anti-mouse c-Fos (1:5000, sc-166940, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and either rabbit anti-Tbr1 (1:200, ab31940, Abcam, Cambridge, MA, USA) or rabbit anti-EGR1 (1:200, #4153; Cell Signaling Technology, Inc.) or rabbit anti-Iba1 (1:1000, 019-19741, Wako Chemicals, Richmond, VA, USA) for 48 h at 4 °C. Then, sections were incubated in the dark for 1 h with the following secondary antibodies (1:200, Alexa Fluors: a mix of goat anti-mouse 594 and goat anti-rabbit 488; or goat anti-mouse 488 and goat anti-rabbit 594, Molecular Probes, Eugene, OR, USA). All sections were cover slipped with anti-fade mounting reagent (Life Technologies, Grand Island, NY, USA).

Quantification

The number of single c-Fos, EGR1, and pERK1/2-positive neurons was quantified by image analysis software as previously described (Crews et al., 2004). Briefly, Bioquant Nova Advanced Image Analysis (R&M Biometric, Nashville, TN) was used for image capture and analysis. Images were captured by using an Olympus BX50 Microscope and Sony DXC-390 video camera linked to a computer. The positive neurons were counted within the region of interest and expressed as cells per square millimeter with 2–3 sections per animals. Both left and right hemispheres of an individual brain subregion of each animal were counted, and the average value per mm^2 was used. The location brain regions of interest and abbreviations are shown below: PrL, prelimbic cortex (+2.70 mm); IL, infralimbic cortex (+2.70 mm); OFC, orbital frontal cortex (+2.70 mm); Cg1, cingulate cortex, area 1 (+1.60 mm); Cg2, cingulate cortex, area 2 (+1.60 mm); M2, secondary motor cortex (+1.60 mm); Pir, piriform cortex (+1.60 mm); AcbSh, nucleus accumbens shell (+1.60 mm); AcbC, nucleus accumbens Core (+1.60 mm); dBNST, dorsal bed nucleus of stria terminalis (−0.30 mm); MDL, mediodorsal thalamic nucleus, lateral part (−2.12 mm); PVA, paraventricular thalamic nucleus, anterior part (−2.12 mm); DG, the dentate gyrus (−3.30 mm); CeA, central nucleus of amygdala (−3.30 mm); BLA, basolateral nucleus of amygdala (−3.30 mm); LaA, lateral nucleus of

amygdala (−3.30 mm); Ent, entorhinal cortex (−3.30 mm); PRh, perirhinal cortex (−3.30 mm); EW, Edinger–Westphal nucleus (−5.20 mm); aVTA, anterior ventral tegmental area (−5.20 mm); pVTA, posterior ventral tegmental area (−5.80 mm) (Fig. 2).

Confocal analyses were conducted using LesicaSP2 AOBS Upright Laser Scanning Confocal in the Michael Hooker Microscopy Facility of the University of North Carolina. In PrL, 50–100 c-Fos+ neurons per sample were analyzed for co-labeling with Tbr1+, and EGR1+. In the NAc, 50–100 c-Fos+ neurons per sample were analyzed for adjacent microglia with Iba+. The percentages of co-labeling in PrL or adjacent microglia to c-Fos+ cells numbers were calculated.

Statistical analysis

All values (including body weight and positive cells) were reported as mean±SEM and analyzed using analysis of variance (ANOVA) (IBM SPSS Statistics 19). The change of body weights were analyzed using a mixed ANOVA (group × day) with day as the repeated measure. Significant effects of adolescence intermittent ethanol on the body weight were used with Student's *t*-test. For the effect of adolescence intermittent ethanol on the expression of c-Fos-positive cells, ANOVA was used to test statistical significance, and followed by comparison of each group mean with Fisher's LSD (least significant difference) or Independent-Samples T-Test where appropriate. For the effect of adolescence intermittent ethanol on the expression of EGR1- or pERK1/2 - positive cells and co-labeled cells, a one-way ANOVA was used to test statistical significance, and by comparison of each group mean with Fisher's LSD where appropriate.

RESULTS

"Adolescent human intermittent binge drinking was modeled using Wistar rats and assessed in early adulthood, P80 (Fig. 1). BEC during AIE treatment were at binge drinking levels, being 204±17 mg/dl one hour after the P38 dose and were 210±17 mg/dl one hour after the P54 dose. All rats gained weight over the course of the experiment, although ethanol-treated rats showed a drop in body weight during ethanol treatment, the body weight normalized within a few days of abstinence. There were no significant differences in body weight between AIE and controls in young adulthood, e.g., P80, after 25 days of abstinence (Fig. 1). All rats appeared normal throughout the experiment consistent with AIE binge exposure not causing observable changes in health.

To investigate the impact of AIE treatment on adults neurocircuits responding to ethanol markers of neuronal activation were assessed in multiple brain regions (Fig. 2). Expression of c-Fos was assessed in young adult rats (P80) following adolescent water or AIE treatment (P25–P54). Rats were acclimatized to handling to avoid handling-induced changes that could confound changes in ethanol challenge responses. Basal control c-Fos expression and the response to ethanol were determined in both water and AIE groups resulting in four study groups, adolescent water treatment – control water challenge (control group), adolescent water treatment – ethanol challenge (control-challenge group), AIE treatment – water challenge (AIE group) and AIE treatment – ethanol challenge (AIE-challenge). Doubling the ethanol dose from 2 to 4 g/kg i.g. resulted in a twofold difference in BAC at 2

h., e.g., of 73±7 mg/dl and 135±14 mg/dl ethanol, respectively (Fig. 1B). The 25-day no-treatment period of maturation between AIE treatment and the ethanol challenge (P55–P80) is designed to assess persistent long-term changes in the brain after maturation to adulthood following adolescent ethanol exposure. This design provides insights into long-lasting changes in brain responses to moderate as well as binge drinking doses of ethanol following adolescent exposure.

Cortex

Multiple cortical areas showed c-Fos responses (Figs. 2 and 3 and Table 1). Frontal cortical (FC) areas including prelimbic (PrL mPFC) and infralimbic medial prefrontal cortex (IL mPFC) as well as OFC had low basal c-Fos that increased several fold during ethanol challenge in young adult ethanol-naïve rats (Water during adolescence with adult ethanol challenge, control-challenge, Fig. 3). The largest increase in c-Fos in ethanol-challenged naïve rats was in PrL mPFC which showed a near maximal c-Fos increase at 2 g/kg ethanol (Fig. 3), that included a significant increase in c-Fos+/Tbr1+ pyramidal neurons in PrL mPFC (Fig. 4). Interestingly, OFC showed an ethanol dose-dependent increase in c-Fos, with 4 g/kg ethanol treatment doubling c-Fos expression over 2 g/kg (Fig. 3). The OFC c-Fos response significantly correlated with BEC across the control-challenge, 2 and 4 g/kg ethanol doses [$F(1,9) = 13.00$, $r = 0.787$, $p = 0.007$]. Both PrL and OFC show a significant dose × group interaction ($p < 0.001$, see Fig. 3 legend for F statements). EGR1 expression, another marker of neuronal activation, also increased several fold in all three PFC regions of control-challenge rats (4 g/kg, i.g. Fig. 5). These findings indicate AIE alters prefrontal cortical neuron responses to ethanol, although subregions show differing dose responses.

AIE treatment did not change basal c-Fos (Fig. 3) or EGR1 (Fig. 5) expression, but did reduce pERK1/2 (Fig. 6), another indicator of neuronal activation (Gao and Ji, 2009). Baseline c-Fos and EGR1 were not changed by AIE; however, pERK1/2 was markedly reduced by AIE treatment to a low level comparable to that found in naïve controls challenged with ethanol (Fig. 6). Ethanol challenge after AIE reversed the acute pERK1/2 PrL response in naïve controls, although the most dramatic effect was the animals showed lower pERK1/2 in all control-challenge groups. In OFC of AIE rats the 2 g/kg dose of ethanol, e.g., AIE-challenge, did not significantly increase c-Fos and at the high binge drinking dose of 4 g/kg the c-Fos OFC response in AIE-challenge rats was lower than the 2 g/kg c-Fos response in the ethanol-naive control-challenge rats (Fig. 3). In the OFC, EGR1 increased in control-challenge rats (4 g/kg, Fig. 5), but not in AIE-challenged rats. OFC pERK1/2 baseline was reduced about 90% to near zero in AIE-treated rats, mimicking the acute reduction found with ethanol challenge of naïve rats, and AIE challenge showed no pERK1/2 response (4 g/kg, Fig. 6). IL mPFC also showed an AIE induced loss of c-Fos, and EGR1 as well as a reversal of response in AIE-challenged animals (Figs. 3 and 5). Thus, AIE treatment caused a marked tolerance to adult PFC neuronal activation markers c-Fos and EGR1 as well as a marked reduction in baseline pERK1/2. These findings are all consistent with decreased PFC neuronal responses to ethanol following AIE.

Other cortical brain regions showed blunted responses to ethanol challenge. Piriform cortex showed a large increase in c-Fos at the 2 g/kg ethanol challenge dose that was not changed

by AIE, although AIE blunted the 4 g/kg ethanol-challenge response similar to IL responses [Table 1, effect of group: $F(1,36) = 7.31$, $p = 0.01$; dose: $F(2,36) = 7.21$, $p = 0.002$; interaction of group and dose: $F(2,36) = 2.04$, $p = 0.144$]. Both PRh and entorhinal cortex (Ent) are temporal lobe structures in humans. Both the 2 and 4 g/kg ethanol treatments induced a dose-dependent increase in c-Fos that was blunted in the high-challenge dose group following AIE treatment [Table 1, PRh, effect of group: $F(1,35) = 6.28$, $p = 0.017$; dose: $F(2,35) = 21.13$, $p < 0.001$; interaction of group and dose: $F(2,35) = 1.70$, $p = 0.197$; Ect, effect of group: $F(1,35) = 24.87$, $p < 0.001$; dose: $F(2,35) = 40.16$, $p < 0.001$; interaction of group and dose: $F(2,35) = 10.62$, $p < 0.001$]). In contrast, cingulate cortical areas 1 and 2 showed increased c-Fos at 2 g/kg that was not further increased by 4-g/kg challenge and was not altered by AIE treatment. Thus, AIE impacts various cortical areas differently with PrL and OFC ethanol-challenge responses markedly blunted by AIE, whereas cingulate cortical responses to ethanol challenge are not altered by AIE (Table 1).

Amygdala

Amygdala subregions also showed variation in c-Fos neuronal activation markers across the two doses of ethanol challenge (Fig. 7). The CeA, BLA and LA all showed significant increases in c-Fos expression following the 2 g/kg ethanol challenge in naïve control-challenge rats. BLA showed progressive dose–response increases, whereas CeA and LA showed near maximal responses at the 2-g/kg dose. AIE-treated animals showed a marked tolerance at the 2-g/kg ethanol-challenge dose with no BLA c-Fos response in AIE animals until the 4-g/kg dose. The BLA showed a dose × group interaction ($p < 0.003$, Fig. 7, see legend for F statement). The CeA and LA had blunted c-Fos response following AIE at the 2 g/kg dose, but not at the 4-g/kg challenge dose. This pattern of response was also found in the dBNST and Edinger–Westphal nuclei (Table 2). Thus the BLA showed a persistent tolerance to ethanol challenge after AIE, whereas other extended amygdala limbic regions showed a persistent tolerance to 2-g/kg ethanol challenge, but not the binge drinking doses of 4-g/kg ethanol.

Nucleus accumbens-VTA

We next assessed ethanol challenge-induced increases in c-Fos in reward-associated ventral-striatum-nucleus accumbens and ventral tegmental area dopamine (DA) neurons. We divided VTA into anterior and posterior subregions since it is known to have different responses to ethanol (Rodd et al., 2004) as well as neurocircuitry (Bromberg-Martin et al., 2010). Anterior (aVTA) and posterior (pVTA) subregions showed similar responses in ethanol-naïve control-challenge animals, with c-Fos markedly increased in both VTA subregions by the 2 and 4 g/kg ethanol challenge (Table 2). AIE did not change the aVTA c-Fos challenge response, but eliminated the pVTA response to challenge, and an ANOVA showed that there was a significant effect of subregion [$F(1,38) = 9.94$, $p = 0.03$] (Table 2). In the pVTA subregion, there were a significant effect of group [$F(1,40) = 37.51$, $p < 0.001$], dose [$F(2,40) = 23.28$, $p < 0.001$] and interaction of group and dose [$F(2,40) = 13.34$, $p < 0.001$], and not in the aVTA. The VTA projects into nucleus accumbens (NAc), which we divided into ventral striatal nucleus accumbens shell (vsNAc Sh) and ventral striatal nucleus accumbens core (vsNAc C). The low-dose (2 g/kg) ethanol challenge in ethanol-naïve animals increased c-Fos in aVTA and pVTA, and vsNAc C without effect in vsNAc Sh.

Ethanol challenge with the binge drinking dose, e.g., 4 g/kg, in naïve control-challenge rats significantly increased c-Fos several fold in vsNAc Sh, whereas the ethanol-increased c-Fos in vsNAc C did not increase beyond the 2 g/kg ethanol response similar to aVTA and pVTA. AIE-treated rats did not show a persistent tolerance-like effect, but AIE animals showed enhanced responses to the binge drinking dose challenge in vsNAc C and vsNAc Sh (Fig. 8, Table 2). At the binge drinking dose, both vsNAc Sh and vsNAc C showed marked increases in c-Fos following AIE exposure than in naïve controls. There were significant dose × group interactions [p=0.022] in the vsNAc Sh and vsNAc C [p=0.040] (Fig. 8, see legend for F statement). Previous studies have found AIE induced persistent changes in neuroimmune gene expression (Vetreno and Crews, 2012; Vetreno et al., 2013) and have hypothesized that microglial neuroimmune gene expression contributes to alcohol dependence (Crews et al., 2011) prompting an assessment of microglial markers in vsNAc. Co-localization of neuronal and microglial markers indicated that only neurons show c-Fos+ IR with no Iba1+ colocalization (Fig. 9B). However, Iba1+ microglia were found to be adjacent to c-Fos+ neurons and AIE increased (48%, p<0.05) the association of c-Fos+ neurons with Iba1+ microglia in the binge-challenge group that showed an increase in c-Fos+ neurons (Fig. 9). Interestingly, there was a correlation between the total c-Fos+ IR and numbers of c-Fos+ neurons with adjacent Iba1+ microglia in the control group [F(1,4)=13.664, R=0.906, p=0.034], but only a trend in the AIE-challenge group [F(1,4)=4.637, R=0.779, p=0.120] (Fig. 9C). These findings suggest AIE increases NAc responses to binge drinking doses of ethanol.

To gain insight into the impact of AIE on neural circuits we measured the correlation of Fos + neurons between brain regions known to connect anatomically (Table 3, Fig. 10, George et al., 2014). The connection of VTA DA neurons to NAc neurons has been extensively studied and there was a very strong correlation of Fos+ cells between both aVTA and pVTA with the vsNAc C in ethanol-naïve adults during the binge drinking challenge, but not the lower ethanol challenge dose (Table 2, rFig. 10B, C). Interestingly, the low-dose ethanol challenge increased Fos+ cells in vsNAc core that correlated with PrL mPFC, but not VTA (Table 3, Fig. 10A, B). AIE treatment altered correlations of Fos+ neurons during the ethanol challenge. The highly significant correlations between VTA-vsNAc core were no longer statistically significant following AIE at the binge drinking challenge (4 g/kg), however a highly significant correlation, (e.g., =0.99; p=0.001 Table 3, Fig. 10C) between aVTA and vsNAc shell emerged suggesting an AIE-induced persistent change in ethanol activation of this pathway. The increase in c-Fos+ responses in both vsNAc shell and vsNAc core following AIE are consistent with increased ethanol activation of adult brain regions associated with reward following AIE. Although the binge drinking challenge dose of ethanol revealed only VTA–vsNAc correlations, multiple brain regions showed correlated responses during the low dose of ethanol (Table 3, Fig. 10A, B). OFC correlated with dBNST and CeA with LaA in both naïve and AIE-treated rats following 2 g/kg ethanol even though AIE markedly blunted the responses. Interestingly, AIE treatment disrupted EW correlations with CeA and LaA and PrL mPFC correlations with CeA and vsNAc core. The PrL mPFC c-Fos activation was markedly blunted following AIE treatment and resulted in correlations between PrL mPFC with BLA and vsNAc shell. These findings suggest that

AIE results in persistent alterations in the brain response to ethanol challenge and the neuronal networks correlating neuronal activation across brain regions.

DISCUSSION

We report in this manuscript that a model of adolescent ethanol exposure, e.g., AIE leads to long-lasting changes in the adult brain response to ethanol as determined using c-Fos and EGR1 markers of neuronal activation. Our findings in ethanol-naïve adult Wistar rats are largely consistent with previous studies using various ethanol challenges of rats and mice (Ryabinin et al., 1999; Horn et al., 2008; May et al., 2008; Vilpoux et al., 2009) including ethanol dose-response curves as reported here (Ryabinin et al., 1997; Knapp et al., 2001). We report here that AIE results in a marked loss of the adult frontal cortical neuronal responses to ethanol challenge, a modest right shift in CeA and BLA dose responses and a significant increase in nucleus accumbens maximal responses at high ethanol doses. In PrL and OFC of mature rats 25 days after adolescent ethanol exposure c-Fos responses to ethanol are markedly reduced compared to ethanol-naïve rats. In PrL AIE exposure eliminated ethanol challenge-induced increases in c-Fos in Tbr1+ frontal cortical projection neurons and EGR1+ neurons. PrL pERK1/2 was reduced by AIE and acute ethanol consistent with a marked change in FC and frontal cortical responses to ethanol. Although the control response to ethanol may involve novelty ethanol is known to pharmacologically alter GABAergic neurotransmission that could impact PFC responses. Another possibility is that AIE habituates ethanol stress-like responses in PFC and studies of repeated stress habituation find FC among other brain regions show stressor- specific c-Fos habituation (Girotti et al., 2006; Weinberg et al., 2010; Herman, 2013). These findings are similar to those of the Heilig's group using adult Wistar rats which found a chronic relapsing alcoholic model in adult Wistar rats resulted in a blunting of the c-Fos (e.g., c-Fos-mRNA) response in mPFC (IL) to acute ethanol challenge and a twofold increase in voluntary ethanol drinking following weeks of abstinence (Hansson et al., 2008). We found a marked decrease in both c-Fos and EGR-1 mPFC (PrL>IL) responses after 25 days of abstinent maturation to an adulthood following AIE. The mechanisms for the reduced c-Fos response are likely complex, however, studies in mice using a similar AIE procedure found adolescent alcohol exposure causes long-lasting increases of histone acetylation in the promoters of c-Fos, FosB and BDNF in the PFC (Pascual et al., 2012). Histone acetylation of c-Fos in mPFC could result in the reduced mPFC response. AIE induced long-lasting changes in neuroimmune signaling following AIE including brain expression of Toll-like receptor 4 (TLR4) and the TLR agonist HMGB1 (Vetreno and Crews, 2012; Vetreno et al., 2013). Guerri's lab has multiple reports finding that anti-inflammatory drugs (Pascual et al., 2007) or mice lacking the neuroimmune Toll-like receptor 4 do not respond to ethanol activation of glia, ethanol stimulated NAc DA release and changes in adult ethanol behavioral responses (Alfonso-Loeches et al., 2010; Pascual et al., 2009, 2011). Adolescent alcohol exposure induction of brain neuroimmune genes and pathology does not occur in transgenic mice lacking TLR4 receptors (Pascual et al., 2014) including loss of ethanol activation of kinases and mobilization of HMGB1 from nuclear to cytosolic fractions (Montesinos et al., 2015). Although the mechanisms of adolescent alcohol treatment induced blunting of PFC c-Fos responses is not clear, alterations in histone acetylation and neuroimmune gene induction

persist after adolescent ethanol treatment and are likely to contribute to the long-lasting changes in adult brain PFC responses.

In contrast to FC, AIE increased adult c-Fos responses to ethanol in NAc. We found modest increases in NAc Core c-Fos+ with our moderate dose of 2 g/kg, i.g., however, the binge drinking challenge dose (4 g/kg) resulted in marked increases in c-Fos expression in the NAc Core and Shell in all groups, with AIE-treated animals showing significantly more c-Fos+ cells than naïve controls. Although we found an increase in microglia associated with NAc c-Fos+ neurons, AIE is known to cause long-term increases in neuroimmune genes associated with microglia (Vetreno and Crews, 2012; Vetreno et al., 2013) and additional studies are needed to determine if this is related to the AIE-induced increases. The increase in the NAc core and shell c-Fos responses to ethanol challenge is consistent with increased activation of reward-associated brain regions. Interestingly, in both ethanol-naïve controls and AIE-treated rats the VTA and NAc c-Fos responses were correlated only during the high binge drinking challenge dose and not the moderate dose. Previous studies have reported adolescent rats (postnatal day [PND] 45) have significantly more NAc DA than adults and ethanol exposure during adolescence increases adult extracellular NAc DA as well as adult conditioned place preference to alcohol (Badanich et al., 2007; Maldonado-Devincci et al., 2010). Further, studies of young adult Wistar rats following an AIE exposure similar to our treatment protocol found AIE increased alcohol preference, doubled nucleus accumbens microdialysate DA as well as increasing alcohol challenge induced DA metabolites consistent with enhanced DA responses to ethanol in adults following AIE (Pascual et al., 2009). Although we did not assess ethanol drinking, multiple studies have reported increased ethanol drinking and preference in adults following adolescent alcohol exposure (Gulley and Juraska, 2013; Spear and Swartzwelder, 2014). These findings suggest adolescent alcohol exposure results in long-lasting increased activation of the NAc shell and core to binge drinking doses of ethanol.

Systemic ethanol administration induces brain c-Fos due to direct neuronal effects as well as neuronal circuit induced increases in c-Fos (Canales, 2004). To gain insight into adult neurocircuitry during ethanol challenges, c-Fos responses were correlated across brain regions (George et al., 2012). Interestingly, only VTA–NAc responses in both groups were correlated during the binge drinking ethanol challenge dose. The moderate ethanol challenge dose found PrL c-Fos correlated with NAc c-Fos and CeA c-Fos. OFC correlated with dBNST with the moderate challenge dose. The EW is well characterized as an ethanol-responsive brain region being uniquely activated by both ethanol administration and ethanol self-administration (Ryabinin et al., 1997). EW contains urocortin neurons that are linked in a complex manner to stress responses and have been hypothesized to contribute the positive reinforcing effects of ethanol (Ryabinin et al., 2012). We found EW, CeA and LA all showed maximal c-Fos responses to the moderate dose ethanol challenge in naïve adult rats with EW correlating with CeA and LaA. The EW has been hypothesized to modulate social behaviors (Schank et al., 2012) and the ability of moderate ethanol doses to inhibit social anxiety is consistent with correlations of EW, CeA and LaA responses. Although these correlations between brain regions are statistically significant, we have not assessed all brain regions, and how clearly the correlations reflect neurocircuitry-driven changes in c-Fos is uncertain. Regardless, the findings presented in this study indicate that AIE treatment has markedly

altered the adult responses of multiple brain regions to ethanol challenge suggesting both pharmacological and neurocircuitry responses to ethanol are altered in adults following adolescent ethanol exposure.

The studies reported here focus on ethanol pharmacological responses that include ethanol-activated neurocircuitry. Repeated ethanol self-administration alters ethanol responses increasing reinforcement by psychological processes that shift neurocircuits to cues in association with escalation of drinking. George et al. (2012), investigated c-Fos in adult Wistar rats drinking ethanol and found correlations between dorsal prefrontal cortex (dPFC) c-Fos (PrL) and NAc c-Fos as well as dPFC-CeA c-Fos during drinking (George et al., 2012), similar to our correlations found with our moderate (2 g/kg, i.g.) ethanol challenge dose, which achieved similar BEC, e.g., 50–70 mg% BAC. Both studies find abstinence following intermittent ethanol exposure dysregulates adult PrL-CeA responses. Dysregulation of the mPFC and CeA were associated with long-term cognitive impairment, increased anxiety-like behavior and escalation of alcohol intake, consistent with this dysfunction contributing to increase drinking. However, the studies differ in mPFC c-Fos responses to re-exposure to alcohol following abstinence; we found a decrease to ethanol challenge whereas George et al. found an increase with renewed alcohol drinking. The difference in c-Fos responses is possibly explained by psychological adaptations that escalate drinking in association with increased c-Fos in a specific population of mPFC GABAergic and CRFergic interneurons (George et al., 2012). Other studies investigating self-administration of alcohol and 30 days of abstinence find increased mPFC c-Fos responses to reinstatement cues (Wedzony et al., 2003). Increased mPFC cue activation of inhibitory interneurons would reduce pyramidal projection neuron excitability, reducing PFC connectivity, similar to our pharmacological challenge response findings of decreased c-Fos in mPFC pyramidal neurons. Although we did not phenotype all of our mPFC c-Fos neurons, we did find c-Fos+ Tbr1 glutamatergic projection neurons and EGR1+ responses, associated with neuroplasticity (Thomas et al., 2003) are completely lost following AIE. These studies suggest PFC is a key brain region involved in ethanol adaptation and that adolescent exposure leads to PFC dysfunction that persists for long periods of abstinence including maturation to adulthood.

A clear understanding of the mechanisms of the change in adult ethanol-challenge responses following AIE will require additional studies. The AIE-reduced mPFC response to adult ethanol challenge contrasts with the increased nucleus accumbens response to ethanol challenge. Previous studies have found long-term changes in the expression of neuroimmune signaling molecules including HMGB1, a cytokine-like protein released from neurons (Maroso et al., 2011; Crews, 2012). AIE treatment has been found to cause an increase in brain expression of HMGB1 and its receptors, TLR4 and RAGE, as well as other neuroimmune signaling cytokines into adulthood that correlates with age of drinking onset (Vetreno and Crews, 2012; Vetreno et al., 2013). CCL2 is a cytokine we found increased in post-mortem human alcoholic brain, including Nucleus accumbens, (He and Crews, 2008) and it has been found to regulate the release of glutamate, GABA and DA (Rostene et al., 2007). It is possible that neuroimmune gene induction in PFC blunts ethanol responses of projection glutamate neurons, whereas neuroimmune genes expression in NAc potentiates excitation of GABAergic projection neurons. Guerri’s lab has multiple reports finding that

anti-inflammatory drugs (Pascual et al., 2007) or mice lacking the neuroimmune Toll-like receptor 4 do not respond to ethanol induction of neuroimmune genes, ethanol-stimulated NAc DA release and changes in adult ethanol behavioral responses (Pascual et al., 2009, 2011; Alfonso-Loeches et al., 2010). Taken together, these studies are consistent with ethanol induction of neuroimmune genes during adolescence and persistence into adulthood contributes to changes in adult brain responses to ethanol.

In summary, we find that intermittent binge drinking doses of ethanol during adolescence alter the adult brain response to ethanol. Adult rats following 25 days of abstinence following adolescent exposure show marked reductions in prefrontal cortex (PFC) markers of neuronal activation during ethanol challenge that contrasts with significant increases in NAc neuronal activation markers. These studies suggest that adolescent alcohol exposure causes long-lasting changes in brain responses to alcohol that persist into adulthood that could contribute to the development of alcoholism.

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Abbreviations

AIE	adolescent intermittent ethanol
ANOVA	analysis of variance
aVTA	anterior ventral tegmental area
BECs	blood ethanol concentrations
BLA	basolateral nucleus of amygdala
CeA	central nucleus of amygdala
DA	dopamine
DAB	nickel-enhanced diaminobenzidine
dBNST	dorsal bed nucleus of stria terminalis
dPFC	dorsal prefrontal cortex
Ent	entorhinal cortex
EW	Edinger–Westphal nucleus
FC	frontal cortex
IL	infralimbic cortex
LaA	lateral nucleus of amygdala
mPFC	medial prefrontal cortex
NAc C	nucleus accumbens core
NAc Sh	nucleus accumbens shell

OFC	orbitalfrontal cortex
PBS	phosphate-buffered saline
pERK1/2	phophorylated extracellar signal regulated kinase
PFC	prefrontal cortex
PRh	perirhinal cortex
PrL	prelimbic cortex
PVA	paraventricular thalamic nucleus, anterior part
pVTA	posterior ventral tegmental area
vsNAc C	ventral striatal nucleus accumbens core
vsNAc Sh	ventral striatal nucleus accumbens shell

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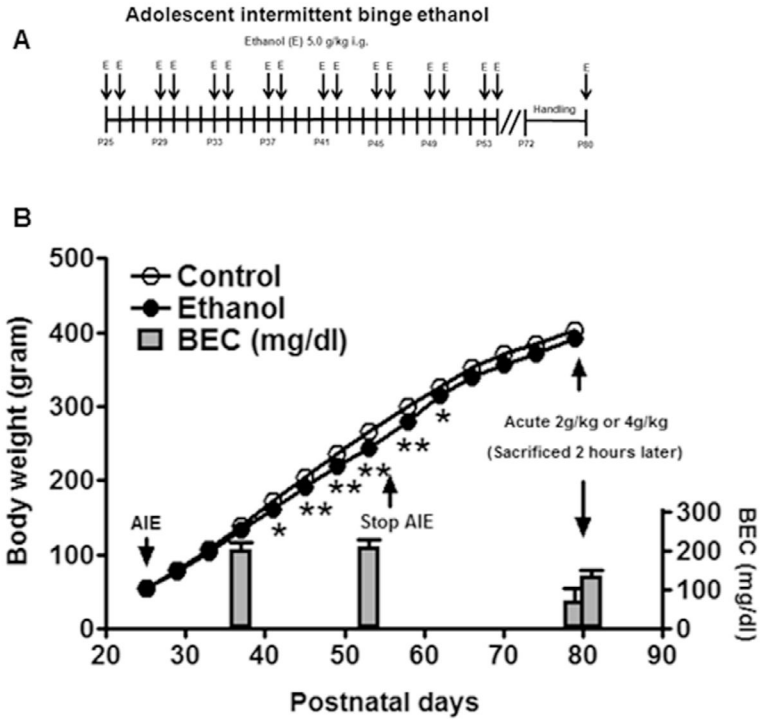

Fig. 1.

The experimental design (A) and body weight (B), blood ethanol concentration (BEC, B) of Wistar male rats. (A) The timeline of the experimental design. Adolescent intermittent ethanol (AIE) started at postnatal days 25 (P25). Adolescent animals were intermittently administered with either water or ethanol (5 g/kg, 25% ethanol w/v, i.g.) with 2 days on and 2 days off during adolescence (P25-P54). At P80, the ethanol group was subdivided into three groups; two groups of them were separately challenged with 2 or 4 g/kg ethanol (i.g., AIE-challenge), and another group was administered with the same volume water (AIE). Water group also was reassigned to three groups (control, control-challenge 2 and 4 g/kg), and treated as the ethanol group. Animals were handled at least 7 days before the challenge day (P80), and sacrificed 2 h after acute treatment. (B) Body weight was measured every four days during the procedure. A mixed ANOVA with day as the repeated measure revealed a significant day × group interaction [$F(13,871) = 2.471$, $p = 0.003$]. There was no difference in the mean of body weight between control and ethanol group depending on body weight match assigned to the experimental groups at P25. The body weight of ethanol group rats significantly decreased at P41 to P62 (16 to 37 days after beginning treatment at P25). $^{*}p < 0.05$, $^{**}p < 0.01$ compared with the control. BEC was measured 1 h after treating with ethanol (5 g/kg, i.g.) at P38 and P54, and 2 h after acute ethanol (2 or 4 g/kg, i.g.) at P80. Each point is mean±SEM (n = 32–37/group).

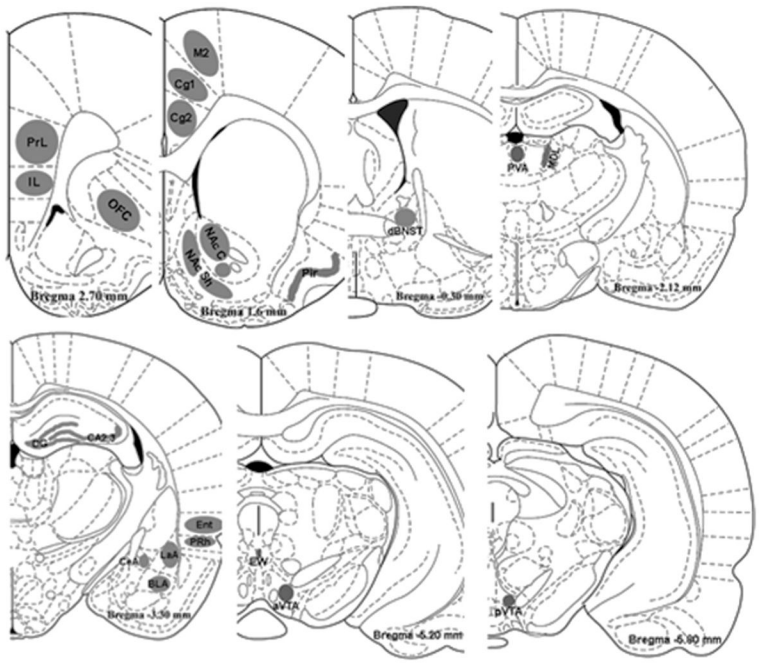

Fig. 2.

Brain regions of interest were studied by the histological method. C-Fos- or EGR1-positive cells of shaded areas were counted. Rat atlas panels reprinted from Paxinos and Watson (Paxinos and Watson, 1998). IL, infralimbic cortex; PrL, prelimbic cortex; OFC, orbitalfrontal Cortex; Cg1, cingulate cortex, area 1; Cg2, cingulate cortex, area 2; M2, secondary motor cortex; Pir, piriform cortex; NAc Sh, nucleus accumbens shell; NAc C, nucleus accumbens Core; dBNST, dorsal bed nucleus of stria terminalis; DG, the dentate gyrus; CA2/3, cornu Ammonis 2 and 3; CeA, central nucleus of amygdala; BLA, basolateral nucleus of amygdala; LaA, lateral nucleus of amygdala; Ent, entorhinal cortex; PRh, perirhinal cortex; PVA, paraventricular thalamic nucleus, anterior part; MDL, mediodorsal thalamic nucleus, lateral part; EW, Edinger–Westphal nucleus; aVTA, anterior ventral tegmental area; pVTA posterior ventral tegmental area. We divided VTA in posterior and anterior subdivisions, e.g., pVTA and aVTP respectively. Studies of intracranial self-administration of ethanol find that rats self-administer ethanol into the pVTA, but not the aVTA, (Rodd-Henricks et al., 2000; Rodd et al., 2004; Quertemont et al., 2005) and voluntary ethanol intake by nondependent P rats significantly increased the number of spontaneously active dopamine (DA) neurons in the pVTA (Morzorati et al., 2010) suggesting these subdivisions of VTA differ in ethanol response.

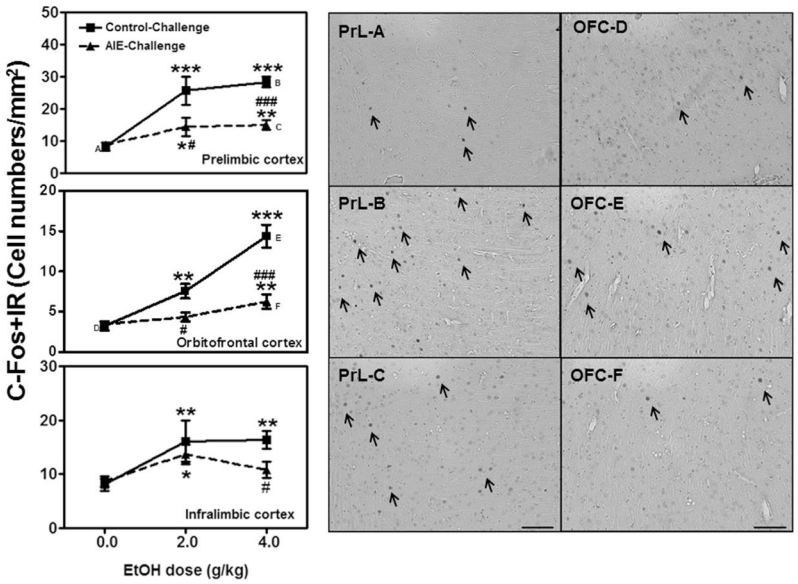

Fig. 3.

Effects of adolescent intermittent ethanol (AIE, 5 g/kg, i.g.) on ethanol (2 or 4 g/kg, i.g.)-induced c-Fos expression in the prelimbic (PrL), orbitofrontal (OFC) and infralimbic (IL) cortex of the adult rat brain. Figures on the left side: there was a significant dose × group interaction [$F(2,35) = 8.77$, $p = 0.001$] in the PrL and [$F(2,36) = 18.98$, $p < 0.001$] in the OFC. Ethanol (2 or 4 g/kg, i.g., control-challenge) significantly induced the increase of c-Fos expression in the PrL, OFC and IL of rat brain at P80. $^{**}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with control-challenge to AIE-challenge group at 0.0 g/kg point (, control or AIE) in group respectively. Adolescent intermittent ethanol (AIE) blunted the increase by acute ethanol (2 or 4 g/kg, i.g., AIE-challenge). $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ compared separately with the water–ethanol (control-challenge) group at either 2.0 or 4.0 g/kg point. Data were expressed as the numbers of c-Fos-positive cells, each point is mean ±S.E.M. per mm^2 ($n = 5$–8/group). Right panels: less c-Fos-positive cells expressed for the control group in the PrL (PrL-A, photomicrography from brain section of control-challenge group at 0.0 g/kg point, see marker "A" in the left top figure; " " control) and OFC (OFC-D, photomicrography from brain section of control-challenge group at 0.0 g/kg point, see marker "D" in the left middle figure; " " control),). Ethanol induced the increase of c-Fos expression in both the regions (PrL-B, photomicrography from brain section of control-challenge group at 4.0 g/kg point, see marker "B" in the left top figure and OFC-E, see marker "E" in the left middle figure; " " control-challenge), and the increases were blocked by AIE (PrL-C, photomicrography from brain section of AIE-challenge group at 4.0 g/kg point, see marker "C" in the left top figure and OFC-F, see marker "F" in the left middle figure; " " AIE-challenge). Scale bar = 50 μm.

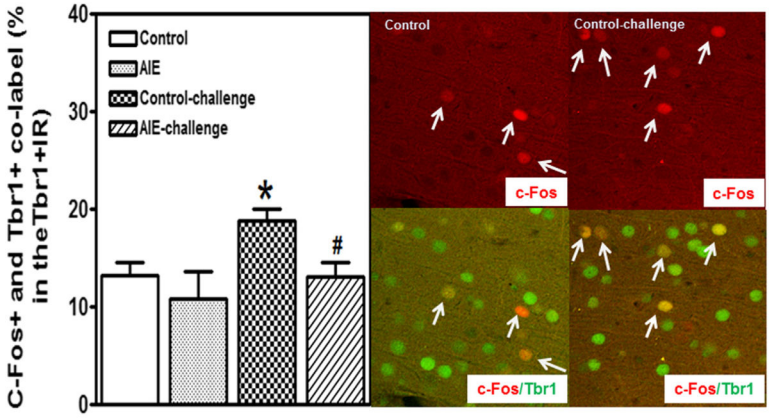

Fig. 4.

Expression of the transcription factor protein Tbr1 and c-Fos in the Prelimbic cortex (PrL) of adult rat brain. Figure on the left side: 13.23±1.33% (control) and 18.88±1.08% (AIE) of Tbr1+ IR were c-Fos+ IR, ethanol (4 g/kg, i.g., control-challenge) resulted in 42.74±8.18% of increase of c-Fos+ IR in the Tbr1+ IR cells. AIE treatment blocked the increase (30.40±7.45% AIE-challenge). Right side: photomicrography of confocal imagines in the PrL, c-Fos (red) and Tbr1 (green), were shown in right panel (original magnification × 80).

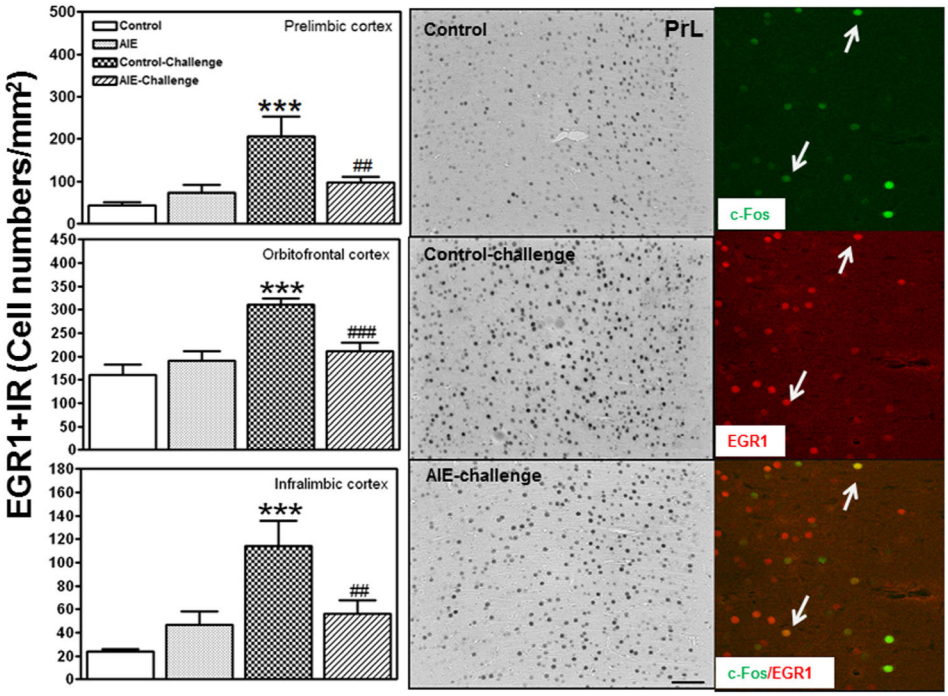

Fig. 5.

Effects of adolescent intermittent ethanol (AIE, 5 g/kg, i.g.) on ethanol (4 g/kg, i.g)-induced EGR1 expression in the prelimbic (PrL), orbitofrontal (OFC) and infralimbic (IL) cortex of adult rat brain. Ethanol (4 g/kg, i.g., control-challenge) significantly induced the increase of EGR1 expression in the PrL, OFC and IL cortex at P80. $^{***}p < 0.001$ compared with control group. AIE blunted the increase by acute ethanol (4 g/kg, i.g., AIE-challenge). $^{##}p < 0.01$, $^{###}p < 0.001$ compared with the control-challenge group (left side). Middle panels show the presence of EGR1-positive cells in the PrL (Immunohistochemical staining, Scale bar = 50 μm), and co-labeling with c-Fos-positive cells (right side: immunofluorescence staining, original magnification × 20). There was about 31.94±3.57% c-Fos (green) and EGR1 (red) co-labeling in the c-Fos-positive cells of the PrL area in the control group. Ethanol (4 g/kg, i.g., control-challenge) increased the co-label cells to 64.68±3.09% ($p < 0.001$). AIE blocked the increase of the co-label cells (47.59±4.52%, $p < 0.05$). Each point is mean±SEM ($n = 5–7$/group).

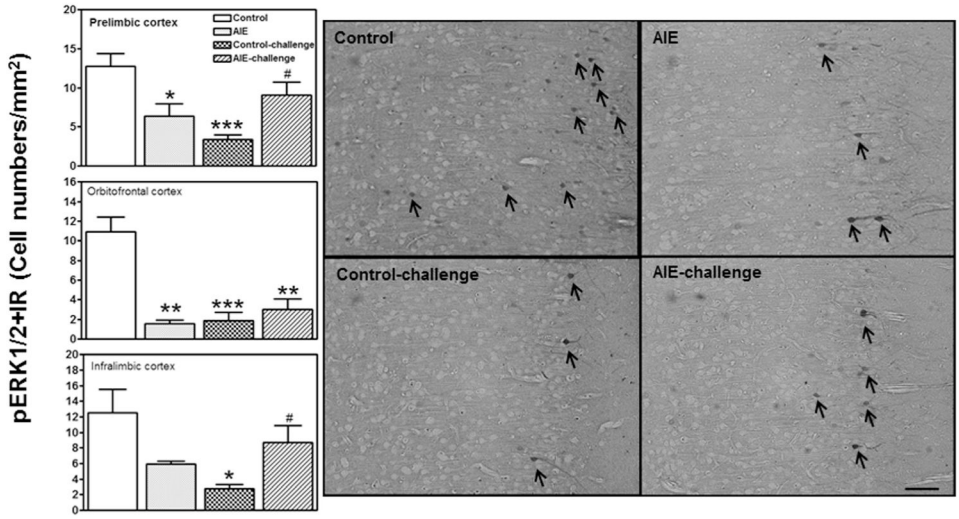

Fig. 6.

Effects of adolescent intermittent ethanol (AIE, 5 g/kg, i.g.) on ethanol (4 g/kg, i.g)-induced pERK1/2 expression in the prelimbic (PrL), orbitofrontal (OFC) and infralimbic (IL) cortex of the adult rat brain. Ethanol (4 g/kg, i.g., control-challenge) significantly induced the decrease of pERK1/2 expression in the PrL (73±4%), OFC (83±7%) and IL (78±4%) at P80. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with control group. AIE blunted the decrease by acute ethanol (4 g/kg, i.g., AIE-challenge) in the PrL and IL. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with control-challenge group (left side). Right panels show the presence of pERK1/2-positive cells in the PrL (Immunohistochemical staining, Scale bar = 50 μm), each point is mean±SEM (n = 5–7/group).

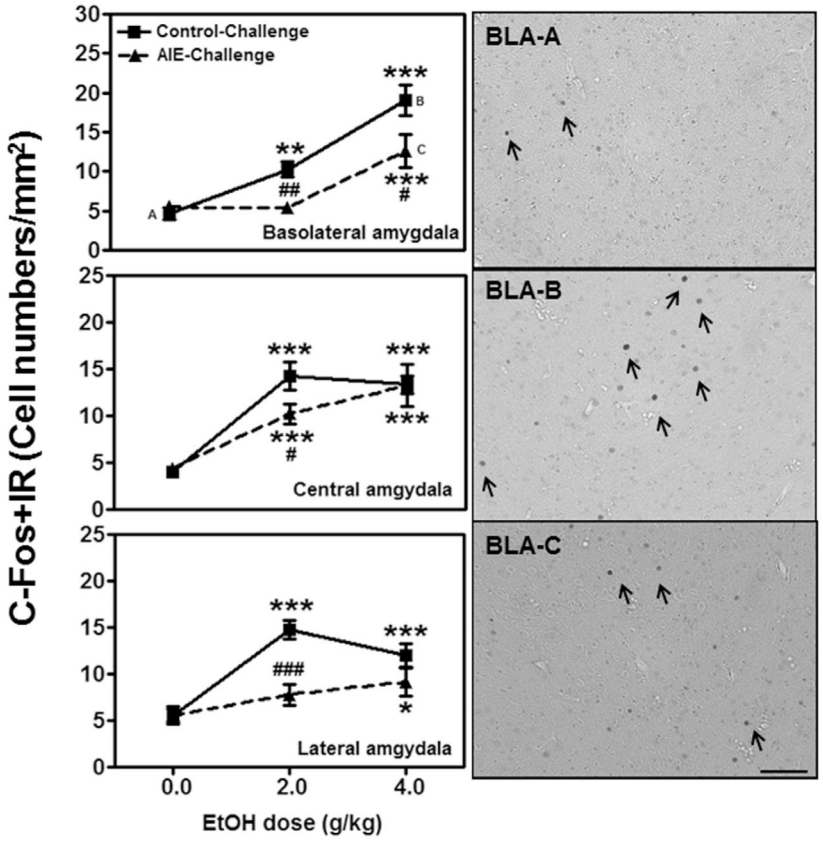

Fig. 7.

Effects of adolescent intermittent ethanol (AIE, 5 g/kg, i.g.) on ethanol (2 or 4 g/kg, i.g.)-induced c-Fos expression in the amygdala of the adult rat brain. There was a significant region × dose × group interaction [$F(4,64)=3.640$, $p=0.010$] between basolateral (BLA), central (CeA) and lateral (LaA) amygdala. There were dose × group interaction [$F(2,39)=6.935$, $p=0.003$] in the BLA (left top), and [$F(2,39)=6.195$, $p=0.005$] in the LaA (left below). Ethanol (-●- 2 or 4 g/kg, i.g., control-challenge) significantly induced the increase of c-Fos expression in the BLA, CeA and LaA at P80. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ compared with the control-challenge to the AIE-challenge group at 0.0-g/kg point (-●-, control or -▲- AIE) in group respectively. AIE blunted the increase by acute ethanol (-▲- 2 or 4 g/kg, i.g., AIE-challenge). $^{\#}p<0.05$, $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$ compared with control-challenge group at either 2- or 4-g/kg point (control-challenge). Each point is mean±SEM per mm^2 (n=6–9/group). Right panels: less c-Fos-positive cells were expressed in the control group in the BLA (BLA-A, photomicrograph from the brain section of the control-challenge group at 0.0-g/kg point, see marker “A” in the left top figure; “ -●- ” control); the expression of c-Fos was increased by acute ethanol (BLA-B, photomicrograph from the brain section of the control-challenge group at 4-g/kg point, see marker “B”; “ -●- ” control-challenge). Adolescent AIE blocked the increase (BLA-C: Photomicrograph from the brain section of AIE-challenge group at 4-g/kg point, see marker “C”; “ -▲- ” AIE-challenge). Scale bar=50 μm.

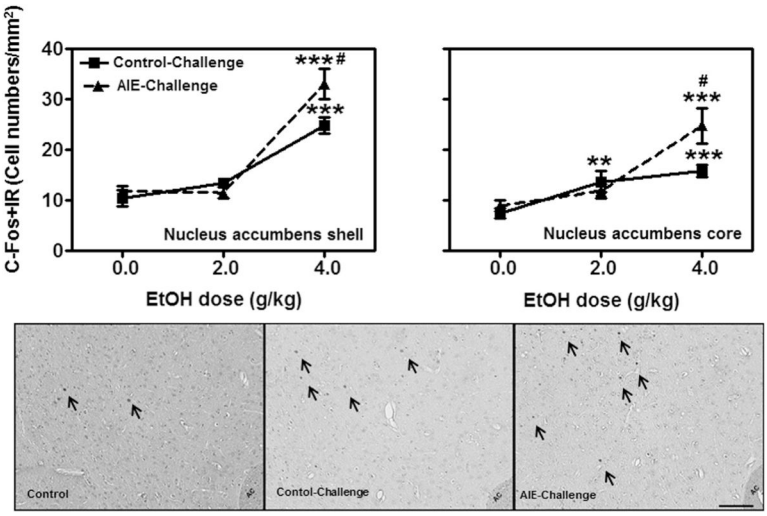

Fig. 8.

Effects of adolescent intermittent ethanol (AIE, 5 g/kg, i.g.) on ethanol (2 or 4 g/kg, i.g.)-induced c-Fos expression in the nucleus accumbens (NAc) shell and core of the adult rat brain. There were a significant dose × group interaction [$F(2,40)=4.181$, $p=0.022$] in the nucleus accumbens shell (top left) and [$F(2,42)=3.467$, $p=0.040$] in the core (top right). Ethanol (⊸2.0 or 4.0 g/kg, i.g., control-challenge) significantly induced the increase of c-Fos expression in both the core and shell (The bottom photomicrograph, Scale bar=50 μm). $^{**}p<0.01$, $^{***}p<0.001$ compared with control-challenge to AIE-challenge group at 0.0 g/kg point (⊸, control or ⊸ AIE). AIE augmented ethanol-induced c-Fos expression with 4 g/kg dose (⊸, AIE-challenge). $^{\#}p<0.05$ compared with control-challenge group at 4.0 g/kg point. Each point is mean±SEM per mm^2 (n=6–9/group).

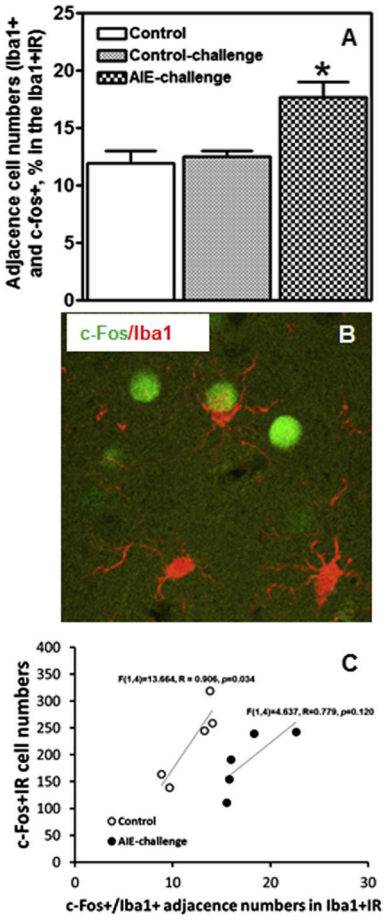

Fig. 9.

Effects of adolescent intermittent ethanol (AIE, 5 g/kg, i.g.) on adjacent cell numbers of c-Fos+ with Iba1+ co-labeling in the nucleus accumbens (NAc). There were 11.92±1.11% c-Fos+ cells adjacent Iba1+ in control, 17.65±1.35% in AIE-challenge (4 g/kg, 48% increase, $p<0.05$) in Iba1+ IR (A). Photomicrography of confocal imagines in the NAc, c-Fos (green) and Iba1 (red), were shown in the middle panel (original magnification × 160). There was a correlation between c-Fos+ IR and c-Fos+/Iba+ adjacent cell numbers in the control group [$F(1,4)=13.664$, $p=0.034$], but not in the AIE-challenge group [$F(1,4)=4.637$, $p=0.120$] (C).

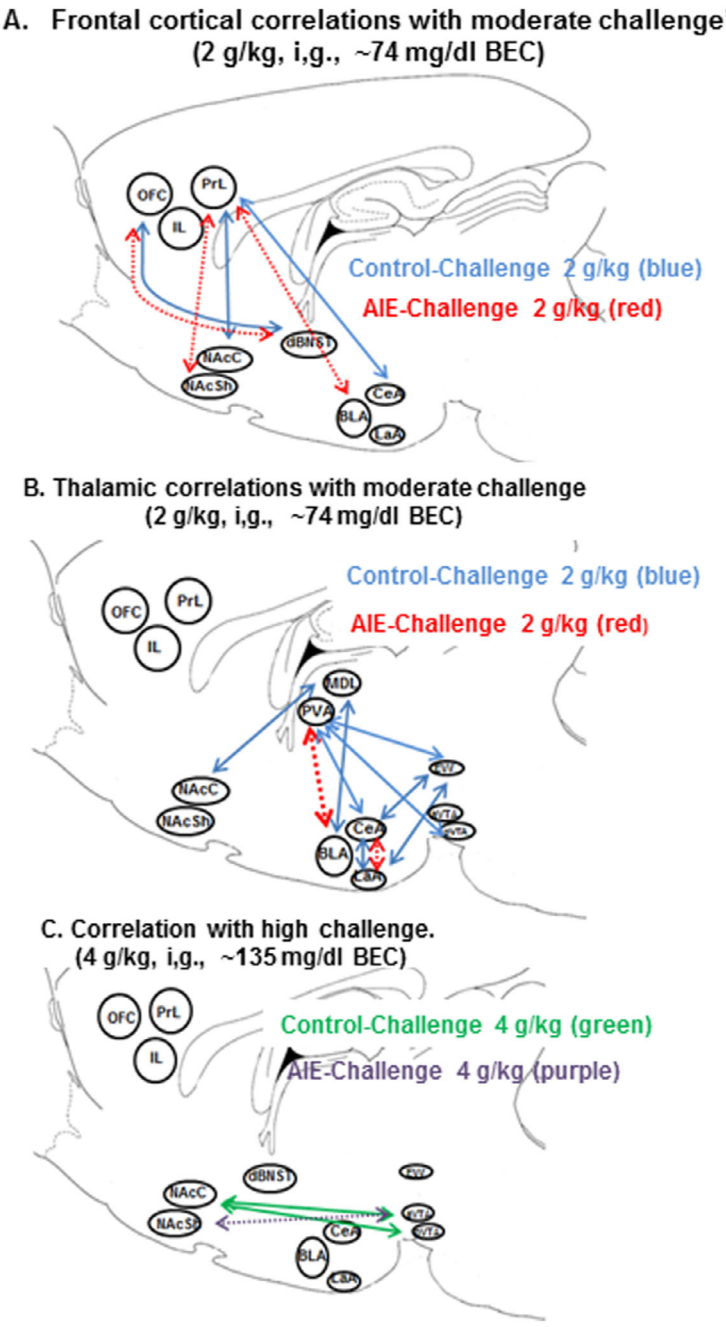

Fig. 10.

Correlations of c-Fos increase across brain regions. Brain regions with significant Pearson correlations of ethanol-induced neuronal activation c-Fos responses across anatomically connected brain regions. Only statistically significant correlations ($p < 0.05$ or $p < 0.01$) are shown. A and B Moderate Ethanol challenge: control-challenge (2 g/kg, i.g., blue solid line) and, AIE-challenge (2 g/kg, red-dashed line). C High Ethanol Challenge, Control-challenge (4 g/kg; i.g., green solid line) and AIE-challenge (4 g/kg; i.g., purple-dashed line). Note how AIE markedly altered the moderate ethanol challenge thalamic correlations. Also with the

high ethanol challenge the only significant correlations found are VTA and NAc, two reward-related brain regions.

Table 1

Effects of adolescent intermittent ethanol (AIE, 5 g/kg, i.g.) on ethanol (2 or 4 g/kg, i.g.)-induced cortical regions known to show ethanol responses and those that contribute to neuronal networks.

Cortex	Group	EtOH doses (g/kg)		
		0.0	2.0	4.0
Prelimbic	Control-challenge	8.20±1.22	25.67±4.35***	28.41±1.60***
	AIE-challenge	8.84±1.02	14.43±2.28*#	15.02±1.44**###
Infralimbic	Control-challenge	8.07±1.09	16.12±3.82**	16.40±1.64**
	AIE-challenge	8.67±0.71	13.68±1.43*	10.84±1.70#
Obitofrontal	Control-challenge	3.25±0.57	7.57±0.89**	14.36±1.44***
	AIE-challenge	3.47±0.43	4.36±0.54*	6.26±0.87**###
Cingulate, area 1	Control-challenge	6.67±0.80	11.60±1.44***	9.91±0.68*
	AIE-challenge	6.65±1.11	13.93±1.54**	14.27±2.19**
Cingulate, area 2	Control-challenge	8.76±1.04	14.74±2.54*	14.53±1.66*
	AIE-challenge	10.05±1.07	16.01±0.55**	15.12±2.56*
Secondary motor	Control-challenge	4.02±0.75	7.15±0.80*	10.26±0.82***
	AIE-challenge	4.27±0.60	7.00±1.54	12.17±0.95***
Piriform	Control-challenge	17.27±1.84	28.90±2.48**	30.76±2.82***
	AIE-challenge	16.06±2.94	22.92±3.40	18.51±3.05#
Perirhinal	Control-challenge	2.58±0.38	5.30±0.71**	7.12±0.96***
	AIE-challenge	2.20±0.32	4.49±0.91**	4.62±0.44**#
Entorhinal	Control-challenge	2.30±0.24	8.88±0.95***	9.57±1.25***
	AIE-challenge	2.74±0.32	5.09±0.70***#	4.89±0.40**##

The regions were the prelimbic (PrL), infralimbic (IL), orbitofrontal (OFC), cingulate, area 1 (Cg1) and area 2 (Cg2), secondary motor (M2), piriform (Pir), perirhinal (PRh) and entorhinal (Ent) cortex. Ethanol (2 or 4 g/kg, i.g., control-challenge) significantly induced the increase of c-Fos expression in all cerebral cortex of interest.

* $p < 0.05$,

** $p < 0.01$ and

*** $p < 0.001$ compared with control-challenge to AIE-challenge group at 0.0 g/kg point in group, respectively. Adolescent intermittent ethanol (AIE) blunted the increase by acute ethanol (2 or 4 g/kg, i.g., AIE-challenge) in the PrL, IL, OFC, Pri, PRh and Ent.

# $p < 0.05$,

$p < 0.05$ and

$p < 0.05$ compared with control-challenge group at 2.0 or 4.0 g/kg point. However, there were no effect of AIE on ethanol-induced increase in the Cg1, Cg2 and M2. Data were expressed as the numbers of c-Fos-positive cells, all values were the mean ±SEM per mm^2 (n = 6–9/group)

Table 2

Effects of adolescent intermittent ethanol (AIE, 5 g/kg, i.g.) on ethanol (2 or 4 g/kg, i.g.)-induced multiple brain regions' response to alcohol. Efforts were made to include limbic, reward and stress-related brain regions as well as regions previously shown to be c-Fos responsive to ethanol. The regions were the nucleus accumbens shell (NAc Sh) and core (NAc C), granule cell layer (GCL), *Cornu Ammonis* area 3 (CA3), basolateral nucleus of amygdala (BLA), central nucleus of amygdala (CeA), lateral nucleus of amygdala (LaA), dorsal bed nucleus of stria terminalis (dBNST), paraventricular thalamic nucleus, anterior part (PVA), mediodorsal thalamic nucleus, lateral part (MDL), Edinger–Westphal nucleus (EW), anterior (aVTA) and posterior (pVTA) ventral tegmental area. Ethanol (2 or 4 g/kg, i.g., control-challenge) significantly induced the increase of c-Fos expression in all regions of interest except the GCL and CA3.

Brain region	Group	EtOH doses (g/kg)		
		0.0	2.0	4.0
Nucleus accumbens shell	Control-challenge	10.48±1.55	13.34±0.38	24.90±1.57***
	AIE-challenge	11.89±0.98	11.49±1.05	33.03±3.83***#
Nucleus accumbens core	Control-challenge	7.41±1.04	13.73±2.12**	15.87±1.19***
	AIE-challenge	8.93±1.01	11.85±0.70	24.83±3.50***#
Granule cell layer	Control-challenge	11.81±0.88	9.69±0.97	7.33±1.57
	AIE-challenge	11.49±1.30	11.86±2.14	10.10±1.03
Cornu Ammonis 3	Control-challenge	4.94±0.74	5.44±0.19	5.92±1.61
	AIE-challenge	5.61±0.76	7.07±1.53	8.58±1.34
Amygdala BLA	Control-challenge	4.65±0.75	10.09±0.98**	19.02±1.20***
	AIE-challenge	5.51±0.44	5.38±0.65##	12.66±2.12***#
Amygdala CeA	Control-challenge	4.51±0.52	14.26±1.49***	13.35±0.77***
	AIE-challenge	4.45±0.35	10.24±1.08***#	13.25±2.00***
Amygdala LaA	Control-challenge	5.55±0.66	14.82±0.98***	12.02±1.25***
	AIE-challenge	5.58±0.88	7.80±1.10###	9.16±1.54*
dBNST	Control-challenge	2.46±0.29	8.58±0.97***	7.41±0.85***
	AIE-challenge	3.25±0.26	5.07±0.88*#	8.21±1.01***
Paraventricular thalamic nucleus	Control-challenge	35.60±3.28	90.33±12.99**	90.58±6.84***
	AIE-challenge	36.67±2.75	47.20±5.22#	75.05±5.71***
Mediodorsal thalamic nucleus, lateral	Control-challenge	4.18±0.79	19.86±6.22*	37.06±6.24**
	AIE-challenge	6.10±0.95	7.60±2.04	16.23±3.49*#
Edinger–Westphal	Control-challenge	3.57±0.68	32.22±3.63***	32.44±5.76***
	AIE-challenge	3.81±0.41	18.78±3.64**#	34.57±5.11***
Anterior VTA	Control-challenge	4.11±0.60	16.79±2.32***	10.09±1.23*
	AIE-challenge	6.82±1.28	17.09±3.99**	9.55±2.43
Posterior VTA	Control-challenge	4.89±0.56	13.74±1.38***	9.67±0.96***
	AIE-challenge	5.02±0.77	5.35±0.21###	4.85±0.32###

$^{*}p < 0.05$,

$^{**}p < 0.01$ and

$^{***}p < 0.001$ compared with control-challenge to AIE-challenge group at 0.0 g/kg point in group, respectively. Adolescent intermittent ethanol (AIE) exposure significantly increased 4 g/kg ethanol-induced (AIE-challenge) c-Fos expression in both NAc Shell and Core. However, there were opposite effects of AIE on ethanol-induced c-Fos increase in the subregions of the amygdala (BLA, CeA and LaA), dBNST, PVA, MDL, EW and pVTA. C-Fos expressions were markedly increased in both aVTA and pVTA by the 2 and 4 g/kg ethanol challenge. AIE eliminated the challenge response in the pVTA, but not change in the aVTA.

$^{\#}p<0.05$,

$^{\#\#}p<0.05$ and

$^{\#\#\#}p<0.05$ compared with controlchallenge group at 2.0 or 4.0 g/kg point. Data were expressed as the numbers of c-Fos-positive cells, all values were the mean ± SEM per mm^2 (n = 6–9/group)

Table 3

Pearson correlations across brain regions on ethanol-induced c-Fos expression. Two ethanol challenge doses, e.g., 2 and 4 g/kg are shown. Both water controls and adolescent intermittent ethanol (AIE, 5 g/kg, i.g.) groups known anatomical connections were assessed and the F and p values determined. Only correlations of $p < 0.05$ or $p < 0.01$ between brain region are shown. There were no correlations to be shown as "–" in the table

Group	Brain regions	Brain regions							
		NAc Sh	NAc C	BLA	CeA	LaA	dBNST	EW	pVTA
Control-challenge (2 g/kg)	OFC	–	–	–	–	–	$F(1,5) = 7.38$, $p < 0.05$	–	–
AIE-challenge (2 g/kg)	OFC	–	–	–	–	–	$F(1,5) = 8.64$, $p < 0.05$	–	–
Control-challenge (2 g/kg)	PrL		$F(1,4) = 74.65$, $p < 0.01$	–	$F(1,4) = 11.48$, $p < 0.05$	–	–	–	–
AIE-challenge (2 g/kg)	PrL	$F(1,5) = 8.66$, $p < 0.05$		$F(1,5) = 12.64$, $p < 0.05$	-	–	–	–	–
Control-challenge (2 g/kg)	PVA	–	–	–	$F(1,5) = 22.58$, $p < 0.01$	–	–	$F(1,5) = 8.47$, $p < 0.05$	$F(1,5) = 11.79$, $p < 0.05$
AIE-challenge (2 g/kg)	PVA	–	–	$F(1,5) = 12.91$, $p < 0.05$	–	–	–	–	–
Control-challenge (2 g/kg)	MDL	–	–	$F(1,5) = 20.99$, $p < 0.01$	–	–	$F(1,5) = 7.53$, $p < 0.05$	–	–
AIE-challenge (2 g/kg)	MDL	–	–	–	–	–	–	–	–
Control-challenge (2 g/kg)	EW	–	–	–	$F(1,4) = 14.43$, $p < 0.05$	$F(1,4) = 57.31$, $p < 0.01$	–	–	–
AIE-challenge (2 g/kg)	EW	–	–	–	–	–	–	–	–
Control-challenge (4 g/kg)	aVTA	–	$F(1,5) = 9.10$, $p < 0.05$	–	–	–	–	–	–
AIE-challenge (4 g/kg)	aVTA	$F(1,4) = 128.88$, $p < 0.001$	–	–	–	–	–	–	–
Control-challenge (4 g/kg)	pVTA	–	$F(1,6) = 8.41$, $p < 0.05$	–	–	–	–	–	–
AIE-challenge (g/kg)	pVTA	–	–	–	–	–	–	–	–