Progress in Neuro-Psychopharmacology & Biological Psychiatry 74 (2017) 15–30

Contents lists available at ScienceDirect

Progress in Neuro-Psychopharmacology & Biological Psychiatry

journal homepage: www.elsevier.com/locate/pnp

Long-term ethanol self-administration induces ΔFosB in male and female adolescent, but not in adult, Wistar rats

Aranza Wille-Bille [a], Soledad de Olmos [a], Leonardo Marengo [a,b], Florencia Chiner [a,b], Ricardo Marcos Pautassi [a,b,*]

[a] Instituto de Investigación Médica M. y M. Ferreyra, INIMEC – CONICET-UNC, Córdoba C.P. 5000, Argentina
[b] Facultad de Psicología, Universidad Nacional de Córdoba (UNC), Córdoba, Argentina

ARTICLE INFO

Article history:
Received 19 October 2016
Received in revised form 25 November 2016
Accepted 29 November 2016
Available online 03 December 2016

Keywords:
Adolescent
Adult
Ethanol drinking
Ethanol-induced locomotor activity
ΔFosB

ABSTRACT

Early-onset ethanol consumption predicts later development of alcohol use disorders. Age-related differences in reactivity to ethanol's effects may underlie this effect. Adolescent rats are more sensitive and less sensitive than adults to the appetitive and aversive behavioral effects of ethanol, respectively, and more sensitive to the neurotoxic effects of experimenter-administered binge doses of ethanol. However, less is known about age-related differences in the neural consequences of self-administered ethanol. ΔFosB is a transcription factor that accumulates after chronic drug exposure and serves as a molecular marker of neural plasticity associated with the transition to addiction. We analyzed the impact of chronic (18 two-bottle choice intake sessions spread across 42 days, session length: 18 h) ethanol [or only vehicle (control group)] self-administration during adolescence or adulthood on the induction of ΔFosB in several brain areas, anxiety-like behavior, and ethanol-induced locomotor activity and conditioned place preference (CPP) in Wistar rats. Adolescent rats exhibited a progressive escalation of ethanol intake and preference, whereas adult rats exhibited a stable pattern of ingestion. Few behavioral differences in the open field or light-dark test were observed after the intake test. Furthermore, ethanol self-administration did not promote the expression of ethanol-induced CPP. There were, however, large age-related differences in the neural consequences of ethanol drinking: a significantly greater number of ethanol-induced ΔFosB-positive cells was found in adolescents vs. adults in the prelimbic cortex, dorsolateral striatum, nucleus accumbens core and shell, and central amygdala nucleus capsular and basolateral amygdala, with sex-related differences found at central amygdala. This greater ethanol-induced ΔFosB induction may represent yet another age-related difference in the sensitivity to ethanol that may put adolescents at higher risk for problematic ethanol use.

1. Introduction

Epidemiological studies (Windle and Zucker, 2010) have indicated that the greater prevalence of alcohol use disorders (AUD) occurs in late adolescence and young adulthood, and that the earlier adolescents begin to drink, the greater the likelihood of these psychiatric disorders (Kim et al., 2016). A seminal, longitudinal, study assessed the occurrence of alcohol dependence 10 years after onset of alcohol use and reported dependence in 14% of early drinkers (age of onset 11–12 years) compared with only 2% in those that began drinking after age 19 (i.e., late drinkers) (DeWit et al., 2000). Jenkins et al. (2011) found a four-fold increase in the risk for AUD in women who began drinking at age 16 compared with those who began drinking after age 16. Still unknown, however, is whether early-onset alcohol use plays a causal role in the subsequent probability of AUD or whether it is only an etiological marker of a preexisting condition (Buchmann et al., 2009). This cements the need for studies assessing age-related differences in the acute and chronic effects of self-administered alcohol.

Pre-clinical studies have contributed to our understanding of why adolescents progress more rapidly than adults from the onset of alcohol consumption to AUD. Animal studies have indicated that adolescent rats are, compared with adults, significantly less sensitive to several aversive effects of alcohol (further referred to as ethanol) (Saalfield and Spear, 2016) that likely deter from the escalation of alcohol consumption (Spear and Swartzwelder, 2014). Moreover, Guerri's group showed that intermittent ethanol exposure during adolescence caused neuroinflammation (Pascual et al., 2007), epigenetic changes (Pascual et al., 2012), and alterations in dopaminergic pruning (Pascual et al., 2009) that were associated with subsequent cognitive dysfunction and enhanced ethanol intake.

Less is known about age- and sex-related differences in the neural consequences of voluntary, self-administered ethanol. ΔFosB is a transcription factor that is induced in several brain areas, and unlike Fos, ΔFosB accumulates after chronic drug exposure and persists long after its withdrawal (Nestler, 2015). This transcription factor has been suggested to serve as a molecular marker of neural plasticity associated with the transition to addiction. ΔFosB, via dimerization with Jun family

* Corresponding author at: Instituto de Investigación Médica M. y M. Ferreyra, INIMEC – CONICET, Friuli 2434, Córdoba C.P. 5016, Argentina.
E-mail address: rpautassi@gmail.com (R.M. Pautassi).

http://dx.doi.org/10.1016/j.pnpbp.2016.11.008
0278-5846/

proteins and subsequently the formation of an AP-1 complex, significantly alters the expression of genes that regulate drug-related responses, such as those that encode opioid ligands (Li et al., 2010).

After initial reports of the reliable induction of ΔFosB following forced, chronic ethanol exposure (Perrotti et al., 2008), subsequent studies analyzed the expression of ΔFosB after voluntary ethanol self-administration. Ethanol consumption by male Sprague-Dawley rats (Li et al., 2010) was associated with robust ΔFosB induction in the nucleus accumbens core (AcbC), dorsolateral striatum (DLS), and lateral orbital cortex (LO) but not the nucleus accumbens shell (AcbSh), dorsomedial striatum (DMS), or medial prefrontal cortex. ΔFosB induction has been suggested to be selective for medium spiny neurons that express dynorphin/substance P, which are abundant in accumbal and striatal regions (Nestler et al., 2001).

The notion that ΔFosB is a neural marker of vulnerability for AUD was illustrated by a study that employed hybrids of female mice that spontaneously exhibited distinct ethanol drinking patterns (Ozburn et al., 2012). BxN mice exhibited a reduction of ethanol preference, whereas BxF mice maintained relatively high preference when challenged with high concentrations of ethanol in two-bottle choice tests. Exposure to low concentrations of ethanol induced a stable level of ethanol consumption in both hybrids. These phenotypes presented different mappings of ΔFosB. For example, BxN mice, which exhibited sustained alcohol preference, had greater levels of ΔFosB than BxF counterparts in the ventral tegmental area and amygdala.

We followed a similar strategy as the one used by Ozburn et al. (2012) but took advantage of the natural variability in ethanol consumption that occurs during ontogeny in rats (Truxell et al., 2007). The main aim was to analyze the impact of prolonged, chronic ethanol self-administration during adolescence and adulthood (18 two-bottle daily sessions, spread across 42 days, 1st session on postnatal day 25 or 80, for adolescents or adults, respectively) on the induction of ΔFosB in several brain areas, in male and female Wistar rats, with a focus on the mesocorticolimbic pathway. We hypothesized greater ethanol drinking, with probable sex-related differences (Doremus et al., 2005), or a steeper ethanol self-administration acquisition curve in adolescents vs. adults. Either pattern was expected to be associated with greater ΔFosB induction, with probable regional differences. Ethanol-induced locomotor activity and anxiety-like behavior were also assessed at termination of the ethanol intake protocol.

The present study included male and female subjects. An important aim was the analysis of sex differences in the neural effects of ethanol. Among college drinkers, women are more likely than men to progress to AUD (Perkins, 2002) and to exhibit some of the negative consequences of ethanol intoxication (Read et al., 2004). Despite this, and other, evidence, and recent initiatives to balance sex representation in basic studies (McCullough et al., 2014), women/female data remains underrepresented (Retson et al., 2015). Female rodents usually exhibit, when compared to male counterparts, greater seeking and intake of several drugs, including ethanol (for review, see Sanchis-Segura and Becker, 2016). These sex effects have been traditionally explained by differential levels of circulating gonadal hormones that, in turn, modulate the impact of these drugs upon central transmitter systems (Becker et al., 2012).

After establishing that ethanol drinking enhanced adolescent, but not adult, ΔFosB expression across most of the mesocorticolimbic pathway, and that this effect was fairly similar in males and females (Experiment 1), Experiment 2 tested – in adolescents females only and via a conditioned place preference (CPP) procedure – the hypothesis that this long-term exposure to ethanol drinking may affect the appetitive, motivational effects of ethanol.

2. Material and methods

2.1. Experimental design

A 2 (age: adolescence vs. adulthood) × 2 (sex: male vs. female) × 2 (condition during self-administration: access to ethanol and vehicle or access only to vehicle [experimental and control groups, respectively]) factorial design was employed in Experiment 1 (9–11 animals in each group). Experiment 2 was conducted in adolescent females only as was defined by a 2 (condition during self-administration: experimental or control) × 2 [conditioning group: sandpaper or ethylene vinyl acetate (EVA) as the excitatory conditioned stimulus (CS^+) during the CPP procedure] factorial design. Each of the four groups of Exp. 2 was composed by 6 subjects.

2.2. Subjects

One-hundred and four outbred Wistar rats (Experiment 1: 41 males and 39 females; Experiment 2: 24 females) were used. The animals were born and reared at INIMEC-CONICET-UNC (Córdoba, Argentina). The experiments were carried out in accordance with the Declaration of Helsinki and the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the NIH and the EU.

2.3. Intake procedures (Experiments 1 and 2)

The ethanol intake tests were similar to those described by Fabio et al. (2015). The tests began on postnatal day (PD25) (adolescents) or PD80 (adults) and lasted 6 weeks. Each week, intake sessions began at 3:00 PM on Monday, Wednesday, and Friday and lasted until 9:00 AM the next day. Control animals were given two vehicle-containing bottles. The experimental subjects were given one bottle that contained the ethanol solution and one bottle that contained vehicle. The ethanol bottle contained 5% ethanol that was mixed with 1% or 0.5% (w/v) sucrose during the first and second testing weeks, respectively, and 5% ethanol mixed with plain water during weeks 3–6. Control rats were given two bottles of 1% or 0.5% (w/v) sucrose (weeks 1 and 2, respectively) or plain water (weeks 3–6). Ethanol intake is expressed as grams per kilogram (g/kg) ingested, and the percentage of ethanol intake preference [(ethanol intake/overall liquid intake) × 100] and overall fluid intake (ml/100 g of body weight) were calculated.

2.4. Immunohistochemical measurement of ΔFosB levels and image analysis (Experiment 1)

Forty-eight hours after the last ethanol intake session, subgroups of animals ($n = 5$ of each sex/age/condition [experimental or control]) underwent the perfusion protocol. These animals were selected based on the highest mean ethanol consumption (g/kg) during the last week of testing within each condition (see insets in Fig. 3). Brain fixation was conducted as described in Fabio et al. (2015). During the final step of fixation, the brains were placed in 30% sucrose for at least 72 h. Then, four series of 40 μm coronal sections were obtained using a freezing microtome (Microme Hm 30).

The sections were first incubated for 1 h at room temperature in a solution of 3% hydrogen peroxide (H_2O_2) and 10% methanol in PBS 0.01 M to quench endogenous peroxidase. Thereafter, the sections were washed three times in 0.01 M PBS and incubated in a blocking solution of 5% normal horse serum (NHS) for 1 h. After blocking, sections were directly incubated for 48 h at 4 °C with a polyclonal antibody against FosB (1:1500, sc-48, Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted in 0.01 M PBS containing 1% NHS. The FosB antibody was raised against the residues 75–150 of the FosB molecule and has been demonstrated, by western blotting analyses, that proteins with a molecular weight corresponding to ΔFosB can be recognized (Perrotti et al., 2005; Perrotti et al., 2004). Following incubation in the primary antibody, sections were washed three times in 0.01 M PBS and incubated for 2 h in biotinylated secondary antibody (Jackson Laboratories, West Grove, PA, USA); diluted 1:500 in 0.01 M PBS containing 1% NHS, washed 3 times in 0.01 M PBS, and followed by an avidin-biotin-peroxidase complex (Vectastain ABC kit, Vector Laboratories) for 1 h at room temperature. Sections were incubated for 5 min with a solution

containing 0.05% 3-3′-diamino-benzidine tetra hydrochloride (DAB, Sigma) and 0.01% H_2O_2.

The photographs were acquired with an Axicam ERc 5s Microscope camera (Zeiss, Jena, Germany) connected to a Primo Star iLed microscope. The counting used a predefined area of an identical size (0.16 mm^2) and shape for each brain region maintaining constant background intensity across different areas. Automated counts of positive nuclei were obtained from each area, using FIJI Is Just Image J software (Schindelin et al. 2012). Data from three sections were selected per animal from each brain region and averaged for the statistical analysis. The coronal coordinates (Paxinos, 2007) for each section were as follows: accumbens shell and core (AcbSh and AcbC) 2.28 mm, 2.16 mm, and 2.04 mm from bregma; prelimbic prefrontal area (PrL) 4.20 mm, 3.72 mm, and 3.24 mm from bregma, lateral orbital cortex (LO) 4.20 mm, 3.72 mm, and 3.24 mm from bregma; dorsolateral striatum (DLS) and dorsomedial striatum (DMS) 2.28 mm, 2.16 mm, and 2.04 mm from bregma; and basolateral amygdaloid nucleus, anterior part (BLA) and central amygdaloid nucleus capsular part (CeC) 2.28 mm, 2.16 mm, and 2.04 mm from bregma. The approximate anterior/posterior levels where the slices were taken are shown in Fig. 1. Representative photomicrographs of each structure (4× magnification) are shown in Fig. 2. Of the five brains that were perfused for each condition, only four underwent the protocol.

2.5. Measurement of ethanol-induced locomotor activity and anxiety-like behavior (Experiment 1)

The rats that did not undergo perfusion were tested for ethanol-induced locomotor activity 48 h after the ethanol intake protocol ended. The locomotor-stimulating effects of ethanol were measured as the distance traveled in an open field. On PD72 (adolescents) or PD127 (adults), the rats were tested for anxiety-like behavior in the light/dark box (LDB) test (Cancela et al., 1995).

For the locomotor activity test, the animals were treated with 2.5 g/kg ethanol (i.p., 0.01 ml of 21% ethanol per gram of body weight) and immediately placed in a dimly lit Plexiglas open field (60 cm × 60 cm × 60 cm) equipped with photocell beams that divided the open field into sections. Proprietary software provided a minute-by-minute measure of distance traveled (centimeters).

The LDB test lasted 5 min and was conducted in an apparatus (42 cm × 25 cm × 25 cm) that had two compartments [one black and dark compartment (17.5 cm × 25 cm × 25 cm, 0 lx) and one white compartment (24.5 cm × 25 cm × 25 cm) illuminated by a 60 W white light bulb providing 400 lx] that were separated by a divider with an opening (6.5 cm × 6.5 cm) at floor level. The illumination was measured with a digital lux meter (LX1010B, Ecutool SA, China). The following variables were recorded: time spent in the illuminated compartment (in seconds), latency to enter the black compartment (in seconds), and locomotion (i.e., number of transfers from one compartment to the other compartment).

2.6. Conditioned place preference (Experiment 2)

Experiment 2 employed only adolescent females, which underwent 6 weeks of two-bottle intake tests (ethanol and vehicle; experimental group, $n = 12$). A control group exposed to only vehicle bottles ($n = 12$) was included as well. The self-administration protocol was the same at that described for Experiment 1, no procedural changes were made between experiments. The CPP apparatus was rectangular (20 cm × 24 cm × 70 cm) and made of Plexiglas. During conditioning a removable guillotine was used to divide the chamber into two compartments (20 cm × 24 cm × 35 cm each), one had black walls and a sandpaper floor (SAND; coarse: 60, Norton, Rio Grande do Sul, Brazil) whereas the other compartment had light orange walls and a floor made of copolymer of EVA (Cordoba Goma SA, Cordoba, Argentina). During the habituation and test the chamber featured a section lined with SAND, a section lined with EVA and an intermediate zone (about 20% of the total surface) floored with black Plexiglas.

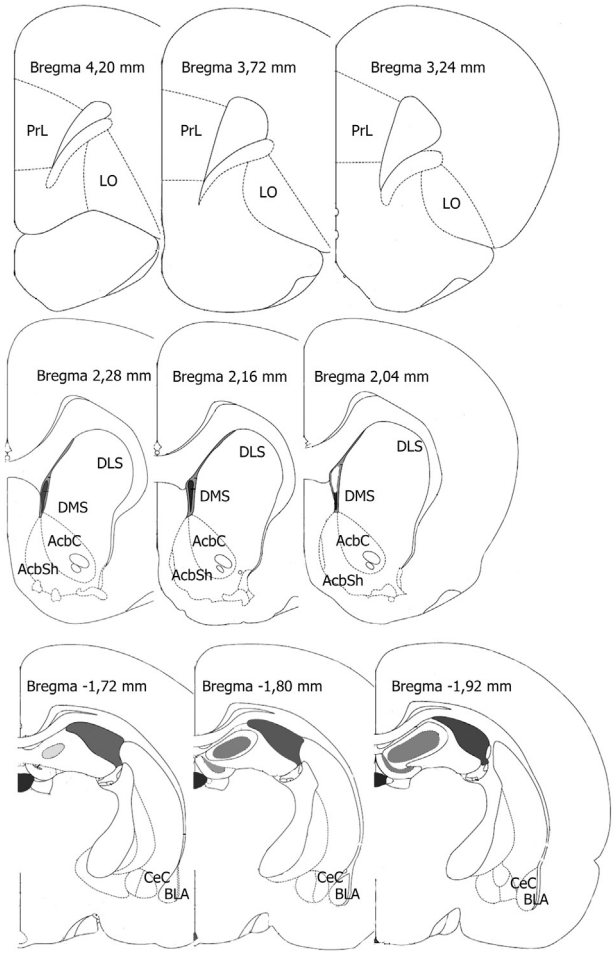


Fig. 1. Diagram of the brain sections analyzed, based on Paxinos (2007). The figures represent anterior/posterior levels relative to bregma of the selected areas. Localizations in the prelimbic cortex (PrL), orbitolateral cortex (LO), nucleus accumbens core (AcbC), nucleus accumbens shell (AcbSh), dorsolateral striatum (DLS), dorsomedial striatum (DMS), basolateral amygdala (BLA), and central amygdala nucleus capsular (CeC) are indicated by the corresponding legend.

The CPP procedure began 72 h after termination of the last ethanol intake session and involved one habituation session, four daily conditioning sessions defined by sequential exposure to CS^- and CS^+ stimuli, and one test session. This is a modified, abbreviated version of a standardized protocol for the assessment of the motivational effects of ethanol in mice (Cunningham et al., 2006). During the habituation, the rats received a saline injection (volume: 1 ml/g of body weight, 0.89% v/v) and then were placed in the central compartment of the apparatus for 12 min with free access to all of the compartments. This session served to familiarize the animals with the apparatus, the stress associated with the injection, and provided a measure of preconditioning preference for the SAND and EVA stimuli.

Conditioning began 1 day after habituation and involved 4 daily conditioning sessions. At 9:00 AM of each day, the animals were weighed and then received a saline injection. Immediately afterwards, half of the rats were placed for 12 min in the SAND compartment, and half were placed for 12 min in the EVA compartment. The ethanol administration was given 20 min after the saline trial had ended. Specifically, 20 min after the end of the saline trial animals received an administration of ethanol (0.75 g/kg, volume: 1 ml/g of body weight) and were immediately placed for 12 min in the CPP compartment lined with the alternative tactile stimulus. That is, those animals that had been placed over sandpaper after the saline injection now were placed in the

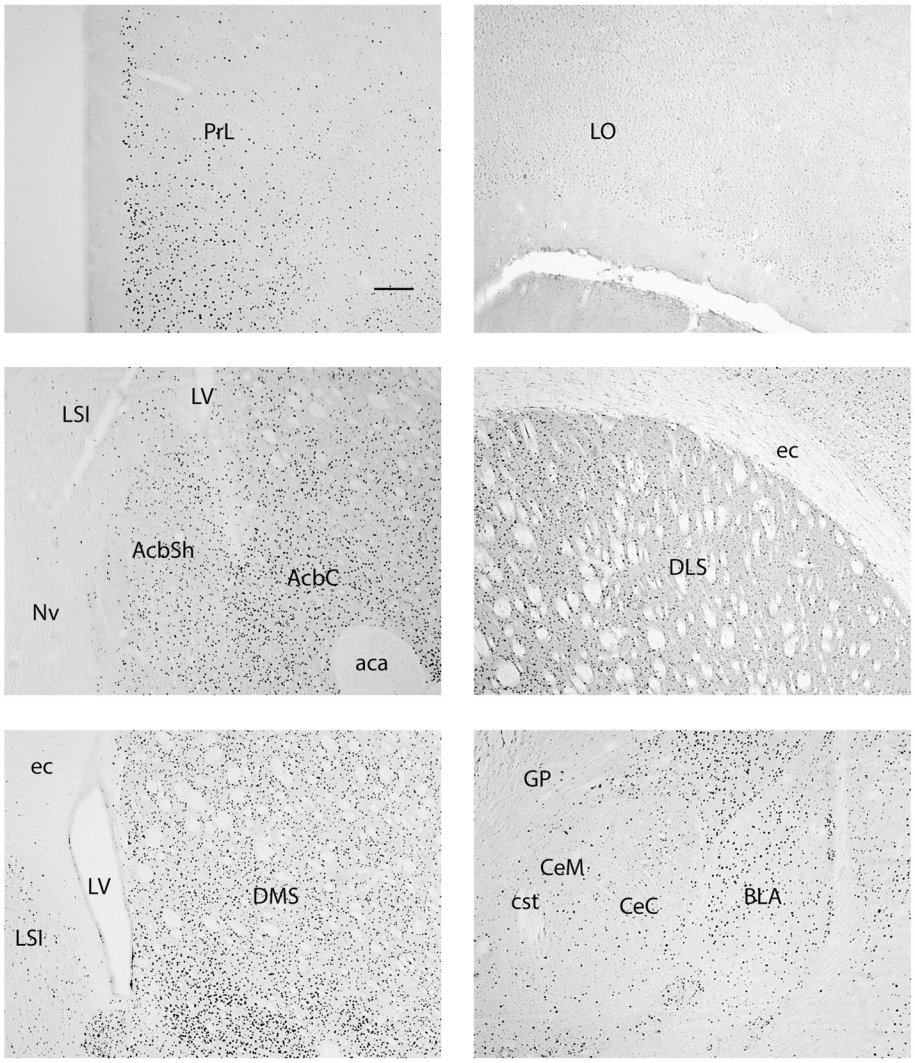


Fig. 2. Representative photomicrographs of the brain areas analyzed in this study (4×). (A, B) Prelimbic cortex (PrL) and orbitolateral cortex (LO), respectively. (C) Nucleus accumbens core (AcbC) and shell (AcbSh). (D, E) Dorsolateral (DLS) and dorsomedial (DMS) striatum, respectively. (F) Basolateral amygdala (BLA) and central amygdala nucleus capsular (CeC). Surrounding areas are also shown and abbreviated as the following: LV, lateral ventricle; aca, anterior commissure ant; NV, navicular nucleus basal forebrain; LSI, lateral septal nucleus intermediate; ec, external capsule; CeM, central amygdala nucleus medial; GP, globus pallidus; cst, commissural stria terminalis. Scale bar = 200 μm.

compartment lined with the smooth EVA; whereas animals that had been exposed to EVA after saline were introduced in a sandpaper-covered compartment. In other words, for half of the subjects the excitatory conditioned stimulus (CS^+) was the sandpaper whereas for the other half the CS^+ was the EVA covered chamber. The animals returned to their homecage after termination of each daily session. The dose of ethanol was chosen based on studies indicating that doses in the 0.5–1.25 g/kg range can be effective to induce conditioned preferences in rats (Fernandez-Vidal et al., 2003; Pautassi et al., 2008; Philpot et al., 2003).

The test was conducted 24 h after the termination of conditioning. No injection was given during the test. The rats were gently introduced to the central zone and had free access to all of the compartments of the apparatus for 12 min. All of the sessions were video recorded. Researchers who were blind to the training conditions of the rats rated the video records for time spent in the different compartments of the apparatus. The CPP paradigm was adapted from previous studies from our lab (Acevedo et al., 2013) indicating that the model detected – when using a biased design in which sandpaper was always the CS^+ – conditioned place preference and aversion induced by ethanol, albeit at doses (1.0 and 2.0 g/kg, respectively) other than that employed in the current study.

2.7. Data analysis

Body weight (g) during the self-administration protocol was analyzed separately in adolescents and adults using a Condition × Sex × Day repeated-measures analysis of variance (RM ANOVA). The absolute (g/kg) and percent preference for ethanol during the intake sessions were assessed by independent ANOVAs in the experimental, ethanol-drinking groups and in the subgroup of ethanol-drinking animals that underwent perfusion. The intake of vehicle (ml/100 g of body weight) was analyzed in control animals. Each analysis considered Sex and Age (only in Experiment 1, subjects of Experiment 2 were only female adolescents) as between-subjects factors and Intake Session (1–18) as the within-subjects factor. In Experiment, the percent increase in absolute ethanol intake (g/kg) from week 1 (average intake across the three sessions) to week 6 was calculated and analyzed by an Age × Sex ANOVA. Finally, overall fluid consumption (ml/100 g) across sessions was analyzed by a RM ANOVA.

The variables assessed in the LDB test and locomotor activity test were analyzed via separate factorial ANOVAs (Age × Sex × Condition). Similar ANOVAs were used to analyze the raw number of ΔFosB-positive cells in each brain structure. We also analyzed the percent change in ΔFosB immunoreactivity compared with the vehicle-drinking condition for each age group: (number of positive cells in a given ethanol-

drinking group × 100)/mean number of positive cells of control, vehicle-drinking group of the same age. Separate 2 (Age) × 2 (Sex) factorial ANOVAs were used to analyze relative changes in ΔFosB immunoreactivity in each structure.

A two-way mixed ANOVA [between factor: group (Experimental or Control); repeated measure factor: time spent on each surface (EVA or sandpaper)] was first conducted on the habituation, pre-conditioning, absolute CPP scores. This preliminary analysis assured that there was no bias or innate preference for any of the textures used during the conditioning. One of the dependent variables for the CPP test was the total amount of time (seconds and percent time) spent on the sandpaper CS during the 12-minute test. In this variety of CPP, the emergence of a place preference is revealed by the difference between the groups in which sandpaper or EVA served as the CS^+. Ethanol-induced conditioned place preference would be revealed if the group in which sandpaper served as CS^+ exhibits significantly more time spent over sandpaper than the group in which EVA served as the CS^+. Therefore, absolute and percent time spent over sandpaper were analyzed by means of a 2 × 2 ANOVA [group (Experimental or Control) × conditioning group (sandpaper or EVA as CS^+)].

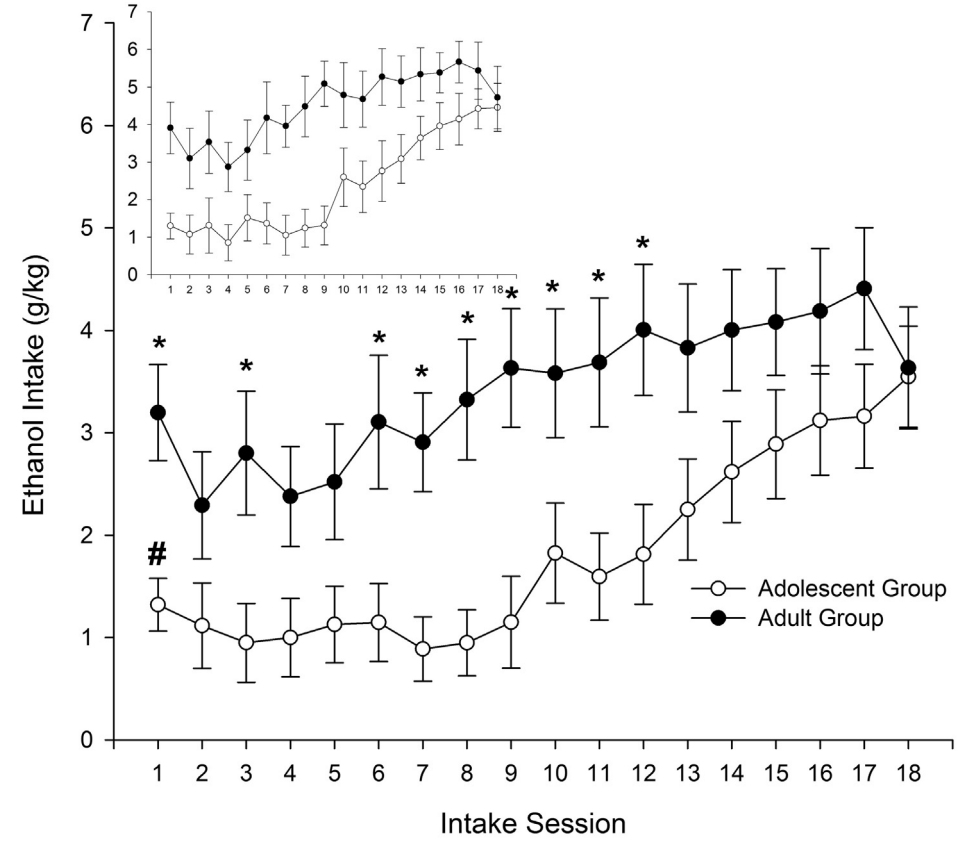
Then, the absolute (in seconds) and percent time spent in the CS^+ compartment (regardless this was the sandpaper or EVA covered chamber) during the test were separately assessed using independent *t*-tests (grouping factors: Experimental-Control, 12 subjects in each condition). The percent time was calculated as the following: ([total time spent in CS^+ compartment] / [total time spent in CS^+ compartment + total time spent in CS^- compartment]) × 100. Similar tests analyzed absolute and percent difference scores (time spent in the CS^+ during test − time spent in the CS^+ during habituation). Significant differences between Experimental and Control groups in these analyses would have indicated a modulatory effect of the history of ethanol self-administration on ethanol-induced CPP.

Spearman rank-order correlations (r_S) were calculated for the overall sample of Experimental subjects (i.e., those that drank ethanol during the self-administration sessions), regardless of age, and individually for each age group. The correlations were determined between the total amount of ethanol ingested (g/kg) across the 18 self-administration sessions or the percent increase in ethanol consumption (g/kg) between the first and the last session, and the raw number of ΔFosB-positive cells in each brain structure. The main aim was to correlate ethanol intake with ΔFosB induction. Specifically, we wanted to determine whether greater ethanol drinking across sessions or greater escalation in ethanol intake was positively correlated with ethanol-induced induction of ΔFosB. Spearman rank, instead of Pearson product-moment, correlation was chosen because the scores for percent increase in ethanol consumption between sessions 1 and 18 exhibited high skewness and kurtosis (2.68 and 6.70, respectively) and a non-normal distribution, as indicated by Kolmogorov-Smirnov and Lilliefors ($p < 0.01$). Correlations were not partitioned by sex.

Significant main effects or interactions between factors were subsequently analyzed using sequential ANOVAs and Tukey's post hoc test. There is a lack of appropriate post hoc tests to analyze interactions that involve between × within factors (Winer et al., 1991). Therefore, to better control for the numerous comparisons made, significant between × within factor interactions were analyzed via planned comparisons and only when supported by our a priori hypothesis. The α

Table 1
Body weight (g), volume of vehicle consumed (ml/100 g body weight), and overall fluid intake (ml/100 g of body weight) in adolescent and adult rats exposed to only vehicle (control groups) or a bottle of ethanol and a bottle of vehicle (experimental groups) in 18 two-bottle choice sessions. The data are presented as weekly averages (mean ± SEM).

		Adolescents				Adults			
		Control		Experimental		Control		Experimental	
		Females	Males	Females	Males	Females	Males	Females	Males
Week 1	Body weight (g)	85.41 ± 7.14	93.00 ± 7.84	77.87 ± 7.29	90.23 ± 10.47	262.50 ± 13.28	437.07 ± 14.64	257.43 ± 8.08	416.90 ± 11.97
	Vehicle intake (ml/100 g)	33.82 ± 4.36	34.07 ± 3.51	29.40 ± 5.47	26.80 ± 4.39	51.70 ± 6.12	37.23 ± 4.60	35.66 ± 7.23	21.18 ± 2.29
	Overall intake (ml/100 g)	–	–	32.75 ± 5.04	29.25 ± 4.81	–	–	44.39 ± 6.09	26.63 ± 1.77
Week 2	Body weight (g)	125.67 ± 6.84	149.03 ± 8.68	117.17 ± 7.15	145.77 ± 12.49	271.20 ± 14.81	462.20 ± 15.32	267.47 ± 8.07	437.00 ± 12.38
	Vehicle intake (ml/100 g)	26.86 ± 3.37	24.23 ± 2.91	18.75 ± 3.34	18.79 ± 1.17	40.18 ± 3.41	29.91 ± 3.60	32.19 ± 6.79	23.32 ± 2.40
	Overall intake (ml/100 g)	–	–	22.51 ± 2.86	20.63 ± 1.85	–	–	40.64 ± 5.16	28.55 ± 2.04
Week 3	Body weight (g)	161.19 ± 7.20	210.52 ± 8.77	155.00 ± 7.00	202.53 ± 13.48	286.33 ± 14.18	478.97 ± 16.72	274.27 ± 8.92	460.90 ± 12.67
	Vehicle intake (ml/100 g)	21.30 ± 2.57	20.11 ± 1.62	16.17 ± 2.48	16.84 ± 1.31	26.03 ± 3.22	19.54 ± 2.40	16.82 ± 4.19	10.85 ± 2.11
	Overall intake (ml/100 g)	–	–	18.62 ± 1.97	19.50 ± 1.77	–	–	20.85 ± 1.99	14.62 ± 0.84
Week 4	Body weight (g)	189.07 ± 7.74	265.97 ± 10.31	180.40 ± 6.82	255.60 ± 14.69	293.37 ± 14.34	501.03 ± 19.28	279.37 ± 8.91	477.57 ± 12.49
	Vehicle intake (ml/100 g)	19.24 ± 2.39	18.27 ± 1.14	12.71 ± 2.59	13.01 ± 1.60	20.78 ± 1.33	18.26 ± 2.30	12.27 ± 4.26	8.04 ± 2.53
	Overall intake (ml/100 g)	–	–	17.93 ± 1.42	16.74 ± 1.17	–	–	23.37 ± 2.57	16.21 ± 1.40
Week 5	Body weight (g)	211.52 ± 8.52	331.58 ± 10.85	203.17 ± 6.75	316.67 ± 16.27	299.90 ± 14.45	516.93 ± 21.57	287.20 ± 9.54	494.87 ± 13.07
	Vehicle intake (ml/100 g)	21.37 ± 2.40	17.49 ± 1.38	9.32 ± 2.20	10.78 ± 1.71	22.06 ± 1.83	17.35 ± 2.54	13.35 ± 4.00	5.66 ± 1.58
	Overall intake (ml/100 g)	–	–	17.09 ± 1.27	16.28 ± 1.36	–	–	24.30 ± 2.45	15.08 ± 0.88
Week 6	Body weight (g)	236.37 ± 12.49	374.15 ± 11.41	219.97 ± 5.82	354.67 ± 17.59	306.73 ± 15.41	525.13 ± 22.59	293.07 ± 9.91	503.73 ± 11.99
	Vehicle intake (ml/100 g)	20.57 ± 1.96	16.42 ± 1.63	7.13 ± 2.37	8.14 ± 1.50	22.06 ± 2.08	16.26 ± 2.22	10.90 ± 4.04	5.4545 ± 1.50
	Overall intake (ml/100 g)	–	–	16.84 ± 1.48	15.23 ± 1.04	–	–	21.92 ± 2.59	15.33 ± 1.18



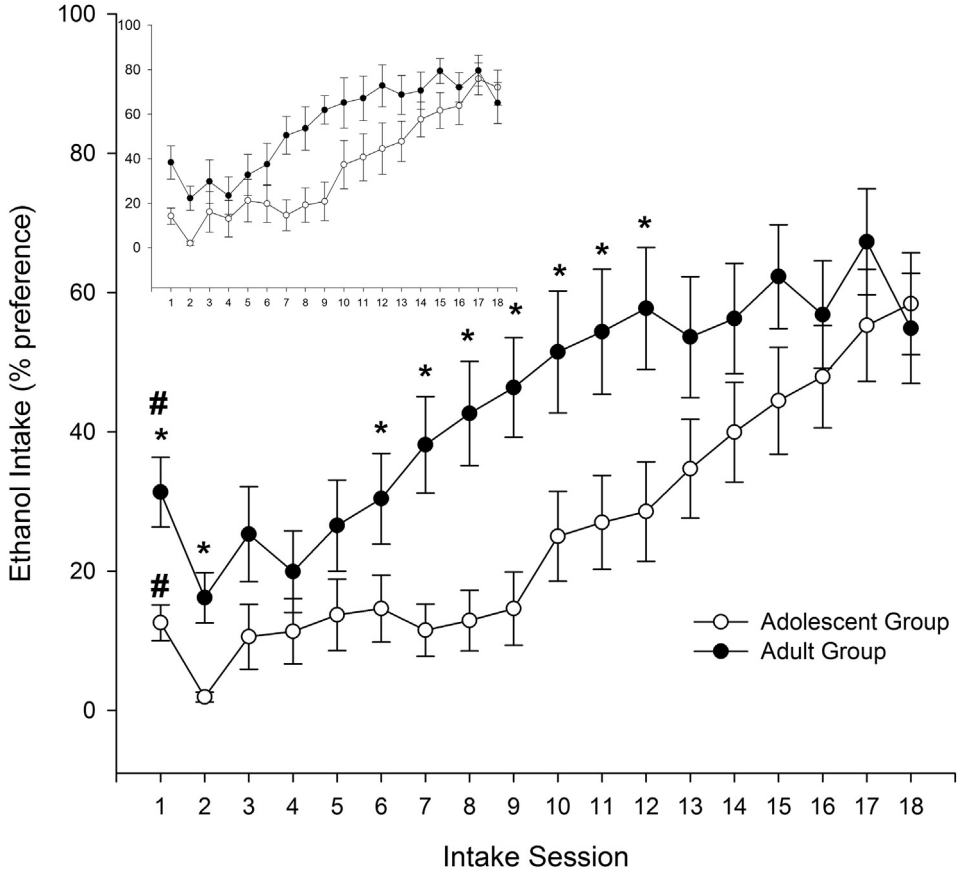


Fig. 3. Ethanol intake (g/kg and percent preference in the upper and lower panels, respectively) in adolescent and adult rats, in Experiment 1. The larger panels depict mean intake (g/kg and percent preference) across the entire sample of animals, and the insets depict intake in the subgroup of animals that were selected for brain perfusion and assessment of ΔFosB immunoreactivity. The asterisk indicates a significant difference between the adolescent and adult groups in a given intake session. The pound sign indicates a significant difference in either absolute (g/kg) or percent ethanol intake between session 1 and session 18 within each age group. Data were collapsed across sex and are presented as mean ± SEM.

level was 0.05, and the partial eta squared (η^2p) is presented as a measure of effect size.

3. Results

3.1. Experiment 1

Body weights in adolescents were similar across sex at the beginning of testing, but males exhibited greater body weight than females beginning at the second week onward (significant main effect of Sex and significant Sex × Day interaction: $F_{1,36} = 48.28$, $p < 0.0001$, $\eta^2p = 0.57$, and $F_{17,612} = 1697.11$, $p < 0.0001$, $\eta^2p = 0.98$, respectively). Adult male rats were significantly heavier than females ($F_{1,36} = 201.46$, $p < 0.0001$, $\eta^2p = 0.85$). Both sexes exhibited an increase in body weight across sessions, albeit the increase was significantly higher in males than in females (significant main effect of Day and significant Sex × Day interaction: $F_{17,612} = 135.32$, $p < 0.0001$, $\eta^2p = 0.79$, and $F_{17,612} = 20.71$, $p < 0.0001$, $\eta^2p = 0.36$, respectively). Experimental condition during the intake test did not significantly affect body weights in adults or adolescents or in males or females. Body weights (averaged across each testing week) can be found in Table 1.

The analysis of absolute and percent ethanol intake in adolescents and adults yielded significant main effects of Age ($F_{1,36} = 7.30$, $p < 0.05$, $\eta^2p = 0.17$, and $F_{1,36} = 5.70$, $p < 0.05$, $\eta^2p = 0.14$, respectively) and significant Age × Day interactions ($F_{17,612} = 1.79$, $p < 0.05$, $\eta^2p = 0.05$, and $F_{17,612} = 2.36$, $p < 0.005$, $\eta^2p = 0.06$, respectively). As shown in Fig. 3, the levels of ethanol consumption (upper panel) and preference (lower panel) were greater in adults than in adolescents at the beginning (e.g., session 1: 3.20 ± 0.50 g/kg [31.34% ± 5.01%] vs. 1.32 ± 0.25 g/kg [12.59 ± 2.58%], respectively) and middle (e.g., session 9: 3.63 ± 0.68 g/kg [46.36% ± 7.15%] vs. 1.15 ± 0.45 g/kg [14.62% ± 5.25%], respectively) sessions of testing, but this difference gradually faded away. By the end of testing, ethanol consumption and preference were similar for both ages. Planned comparisons indicated significant differences in g/kg consumed between age groups in session 1 and across sessions 3–12. Adolescents and adults differed in percent ethanol preference in sessions 1 and 2 and sessions 6–12. Moreover, planned comparisons indicated that g/kg of ethanol consumed significantly increased from the first to the last session in adolescents but not in adults and that the percent ethanol preference increased significantly from the first to the last session in both age groups. The corresponding ANOVA indicated that the percent increase in absolute ethanol intake (g/kg) from week 1 (average intake across the three sessions) to week 6 was significantly higher in adolescents (426.82 ± 86.91 g/kg) than in adults (182.92 ± 35.90 g/kg; significant main effect of age, $F_{1,12} = 12.29$, $p < 0.005$, $\eta^2p = 0.51$).

The analysis of ethanol intake in the subgroup of animals that underwent perfusion (see insets in Fig. 3) yielded a similar profile as the one found for the complete sample of subjects. The ANOVAs revealed significant main effects of age and significant Age × Day interactions (e.g., g/kg: $F_{1,16} = 11.05$, $p < 0.005$, $\eta^2p = 0.41$, and $F_{17,272} = 1.85$, $p < 0.05$, $\eta^2p = 0.10$, respectively).

The analysis of vehicle intake (ml/100 g of body weight; see Table 1) in control animals revealed a significant main effect of Day and a Day × Age interaction ($F_{17,612} = 35.31$, $p < 0.001$, $\eta^2p = 0.50$, and $F_{17,612} = 4.41$, $p < 0.001$, $\eta^2p = 0.11$, respectively). Planned comparisons indicated that adolescents and adults consumed similar quantities of vehicle, with the exception of sessions 3 and 4, in which adults drank more vehicle than adolescents.

The ANOVA of overall fluid intake (ml/100 g of body weight) yielded significant main effects of Sex, Day, and Age ($F_{1,72} = 10.19$, $p < 0.005$, $\eta^2p = 0.12$, $F_{1,72} = 6.12$, $p < 0.05$, $\eta^2p = 0.08$, and $F_{17,1224} = 67.45$, $p < 0.0001$, $\eta^2p = 0.48$, respectively). The Sex × Age, Day × Age, and Day × Sex interactions were also significant ($F_{1,72} = 4.53$, $p < 0.005$, $\eta^2p = 0.06$, $F_{17,1224} = 1.70$, $p < 0.0001$, $\eta^2p = 0.02$, and $F_{17,1224} = 10.06$, $p < 0.0001$, $\eta^2p = 0.12$, respectively). Planned comparisons indicated that adult female rats consumed more than adolescent female rats and male rats of both ages. These comparisons also indicated that adults drank more fluid than adolescents in sessions 3–5 and that females drank more than males in all of the sessions, with the exception of sessions 9–10. Descriptive data are shown in Table 1.

The ANOVA for distance traveled in the open field indicated a significant main effect of Sex ($F_{1,31} = 4.35$, $p < 0.05$, $\eta^2p = 0.12$) and a significant Sex × Age interaction ($F_{1,31} = 6.85$, $p < 0.05$, $\eta^2p = 0.18$). The post-hocs indicated that distance traveled in the adolescent, but not in the adult, rats was significantly higher in females than in males. Distance traveled was not affected by Experimental Condition. The latency to enter the bright compartment in the LDB test was not affected by Age or Experimental Condition, whereas the number of transfers between compartments was significantly greater in adolescent females than in any of the other groups ($F_{1,31} = 9.20$, $p < 0.005$, $\eta^2p = 0.23$). The analysis of the time spent in the white compartment revealed a significant Sex × Condition interaction ($F_{1,31} = 9.66$, $p < 0.05$, $\eta^2p = 0.24$). Males that drank ethanol during the intake tests spent significantly more time in the white compartment than male controls that drank vehicle or females that drank ethanol (see Table 2).

Fig. 4 illustrates ΔFosB immunoreactivity across brain regions. The ANOVA for the LO did not reveal significant main effects or interactions. The analysis for the PrL yielded a significant main effect of Age and a significant Age × Condition interaction ($F_{1,24} = 13.09$, $p < 0.005$, $\eta^2p = 0.35$, and $F_{1,24} = 4.54$, $p < 0.05$, $\eta^2p = 0.16$, respectively). Tukey's post hoc test revealed significantly greater ΔFosB immunoreactivity in ethanol-drinking adolescents than in ethanol-drinking adults and adults exposed only to vehicle. Fig. 5 illustrates these results via representative photomicrographs.

Separate ANOVAs for ΔFosB immunoreactivity in the AcbC and AcbSh yielded very similar results. Neural activation in the AcbC and AcbSh was significantly greater in females than in males ($F_{1,24} = 17.51$, $p < 0.001$, $\eta^2p = 0.42$, and $F_{1,24} = 7.69$, $p < 0.05$, $\eta^2p = 0.42$, respectively). The mean and SEM for females vs. males was the following: 22.09 ± 1.99 vs. 13.98 ± 1.76 (AcbC) and 18.82 ± 2.22 vs. 12.56 ± 1.75 (AcbSh). The analysis revealed main effects of Age (AcbC: $F_{1,24} = 22.90$, $p < 0.05$, $\eta^2p = 0.49$; AcbSh: $F_{1,24} = 22.90$, $p < 0.05$, $\eta^2p = 0.49$) and significant Age × Condition interactions (AcbC: $F_{1,24} = 8.74$, $p < 0.01$, $\eta^2p = 0.27$; AcbSh: $F_{1,24} = 7.70$, $p < 0.05$, $\eta^2p = 0.25$). As shown in Fig. 4 and confirmed by the post hoc tests for both the AcbC and AcbSh, ethanol-drinking but not control adolescents exhibited significantly greater ΔFosB immunoreactivity than their adult counterparts. These patterns are evident in Fig. 6.

The ANOVA of ΔFosB immunoreactivity in the DLS (see Fig. 4) yielded a significant main effect of Sex ($F_{1,24} = 5.40$, $p < 0.05$, $\eta^2p = 0.15$), with females exhibiting greater neural activation than males (3.02 ± 0.48 vs. 1.59 ± 0.45). The Age × Condition interaction was also significant ($F_{1,24} = 5.23$, $p < 0.05$, $\eta^2p = 0.18$), but the post hoc tests did not indicate significant differences between groups.

Females, particularly those in the adolescent group, exhibited significantly greater ΔFosB immunoreactivity in the DMS, although this effect was similar in the experimental and control conditions (significant main effect of Sex and significant Sex × Age interaction: $F_{1,24} = 28.87$, $p < 0.001$, $\eta^2p = 0.55$, and $F_{1,24} = 5.45$, $p < 0.05$, $\eta^2p = 0.18$; descriptive data not shown). Adolescent female rats also exhibited significantly greater immunoreactivity in the amygdala (both BLA and CeC) compared with males and adults (significant main effects of Sex and Age; BLA: $F_{1,24} = 11.31$, $p < 0.005$, $\eta^2p = 0.32$, and $F_{1,24} = 17.26$, $p < 0.05$, $\eta^2p = 0.42$, respectively; CeC: $F_{1,24} = 7.24$, $p < 0.05$, $\eta^2p = 0.23$, and $F_{1,24} = 18.56$, $p < 0.05$, $\eta^2p = 0.44$, respectively; descriptive data not shown). Perhaps more importantly, separate ANOVAs of ΔFosB immunoreactivity in the DMS, BLA, and CeC yielded significant Age × Condition interactions ($F_{1,24} = 12.08$, $p < 0.005$, $\eta^2p = 0.33$, $F_{1,24} = 13.69$, $p < 0.005$, $\eta^2p = 0.36$, and $F_{1,24} = 10.34$, $p < 0.005$, $\eta^2p = 0.30$, respectively). Across these areas, the corresponding post hoc tests confirmed that ethanol-drinking adolescents exhibited significantly greater

Table 2
Distance traveled in the open field during a 5-min test, latency to enter the dark compartment (in seconds), time spent in the light compartment (in seconds), and number of transfers between compartments in the 10-min light-dark box (LDB) test. Both tests were conducted 48 h after the end of chronic ethanol self-administration. Adolescents and adults were exposed to only vehicle (control groups) or a bottle of ethanol and a bottle of vehicle (experimental groups) in 18 two-bottle choice sessions. The data are expressed as mean ± SEM.

	Adolescents				Adults			
	Control		Experimental		Control		Experimental	
	Females	Males	Females	Males	Females	Males	Females	Males
Distance traveled in the OF (cm)	5193.25 ± 902.06	3069.67 ± 433.54	5587.00 ± 654.52	4301.20 ± 255.41	4273.50 ± 295.64	4874.80 ± 653.02	4671.20 ± 364.01	4456.20 ± 282.16
Latency to enter into the dark LDB compartment (s)	8.00 ± 1.73	10.17 ± 3.65	9.00 ± 1.14	54.00 ± 38.54	14.75 ± 5.39	11.00 ± 2.47	17.20 ± 4.94	28.20 ± 2.37
Time spent on the brightly lit LDB compartment (s)	33.25 ± 2.50	21.83 ± 13.27	46.40 ± 5.80	79.20 ± 38.69	147.75 ± 41.76	59.80 ± 32.94	61.20 ± 13.44	184.80 ± 46.74
Number of transfers between LDB compartments	6.50 ± 0.96	1.83 ± 0.83	7.40 ± 1.03	5.00 ± 0.71	3.75 ± 1.25	3.60 ± 0.81	3.20 ± 0.58	5.20 ± 1.71

ΔFosB immunoreactivity than the other groups, which not differ from each other. The bar graph in Fig. 4 depicts these age-related patterns, and representative photomicrographs are shown in Fig. 5 (DMS) and Fig. 7 (BLA and CeC).

The ANOVAs of the percent change in ΔFosB immunoreactivity were consistent across most of the brain regions analyzed. A main effect of Age (indicative of adolescents exhibiting significantly greater ethanol-induced relative changes in ΔFosB immunoreactivity than adults) was observed in the LO, PrL, AcbC, AcbSh, DLS, DMS, and BLA: ($F_{1,12} = 5.23$, $p < 0.05$, $\eta^2p = 0.33$, $F_{1,12} = 4.64$, $p \leq 0.005$, $\eta^2p = 0.36$, $F_{1,12} = 18.92$, $p < 0.001$, $\eta^2p = 0.30$, $F_{1,12} = 19.94$, $p < 0.0001$, $\eta^2p = 0.30$, $F_{1,12} = 16.23$, $p < 0.005$, $\eta^2p = 0.30$, $F_{1,12} = 19.94$, $p < 0.0001$, $\eta^2p = 0.30$, $F_{1,12} = 17.20$, $p < 0.005$, $\eta^2p = 0.30$, and $F_{1,12} = 16.32$, $p < 0.005$, $\eta^2p = 0.30$, respectively). A significant main effect of Sex (indicative of females exhibiting significantly greater immunoreactivity than males) was found in DLS ($F_{1,12} = 7.29$, $p < 0.05$, $\eta^2p = 0.38$). In the CeC, a greater percentage of ethanol-induced ΔFosB immunoreactivity was observed in adolescent females but not adolescent males compared with controls (significant Age × Sex interaction: $F_{1,12} = 6.34$, $p < 0.05$, $\eta^2p = 0.35$). The relative is shown in the insets in Figs. 5–7, and in Fig. 8.

Table 3 depicts Spearman rank correlation scores (r_S) between the ethanol intake measures and the raw number of ΔFosB-positive cells in each brain structure. The analysis conducted in the overall sample of subjects indicated that the percent increase in ethanol consumption (g/kg, between individual scores measured at session 1 and 18), but not the total amount of ethanol ingested across the self-administration sessions, were significantly and positively correlated with induction of ΔFosB at the following areas: PrL, LO, AcbC, AcbSh, DMS, BLA and CeC ($r_S = 0.53, 0.61, 0.65, 0.70, 0.60, 0.74, 0.60$ and 0.69, respectively). In the adolescent group ΔFosB at DMS was positively associated with the total amount of ethanol ingested across the sessions ($r_S = 0.74$), whereas no significant association was found in the sub-group of adult rats. Scatterplots depicting the association between percent increase in ethanol consumption (g/kg, between individual scores measured at session 1 and 18) and the raw number of ΔFosB-positive cells at CeC or AcbSh, as a function of age, are provided as Supplementary Fig. 1.

3.2. Experiment 2

The ANOVA for the body weights registered prior to each self-administration test revealed a significant main effect of Day, $F_{1,36} = 48.28$, $p < 0.0001$, $\eta^2p = 0.97$. The adolescent females exhibited a steady increase in body weight across sessions, which was fairly similar in Experimental rats that ingested ethanol (week 1 average: 78.56 ± 3.73, week 2: 123.22 ± 4.44, week 3: 159.72 ± 4.77, week 4: 185.69 ± 5.75, week 5: 207.39 ± 6.91, week 6: 225.66 ± 8.25) and in control counterparts than drank only vehicle (week 1 average: 74.72 ± 3.59, week 2: 116.97 ± 4.55, week 3: 150.33 ± 4.95, week 4: 173.77 ± 5.99, week 5: 195.80 ± 6.04, week 6: 208.39 ± 6.77).

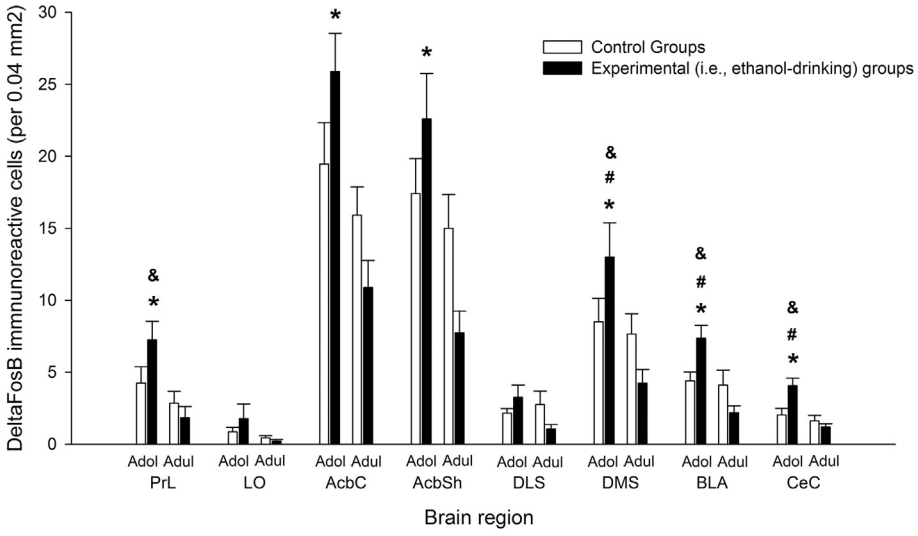


Fig. 4. ΔFosB-immunoreactive (ΔFosB-ir) cells in selected brain areas [prelimbic cortex (PrL), orbitolateral cortex (LO), nucleus accumbens core (AcbC) and shell (AcbSh), dorsolateral (DLS) and dorsomedial (DMS) striatum, basolateral amygdala (BLA) and central amygdala nucleus capsular (CeC)] of adolescent and adult rats exposed to two-bottle choice tests on PD25-67 or PD80-122, respectively. The asterisk indicates a significant difference between the adolescent and adult groups that were exposed to ethanol during the intake sessions (i.e., experimental groups). The pound sign indicates a significant difference between the experimental and control groups in a given age (i.e., adolescents or adults). The data are expressed as the number of ΔFosB-ir cells per 0.04 mm^2. Data were collapsed across sex. Vertical bars represent the SEM.

As can be seen in Fig. 9, ethanol intake patterns were very similar to those recorded in the adolescents females of Experiment 1. The ANOVA yielded a significant main effect of Day ($F_{17,87} = 8.67$, $p < 0.001$, $\eta^2p = 0.44$, and $F_{17,87} = 16.68$, $p < 0.001$, $\eta^2p = 0.60$; absolute and percent ethanol intake, respectively). As can be observed in Fig. 9, the levels of ethanol consumption (upper panel) and preference (lower panel)

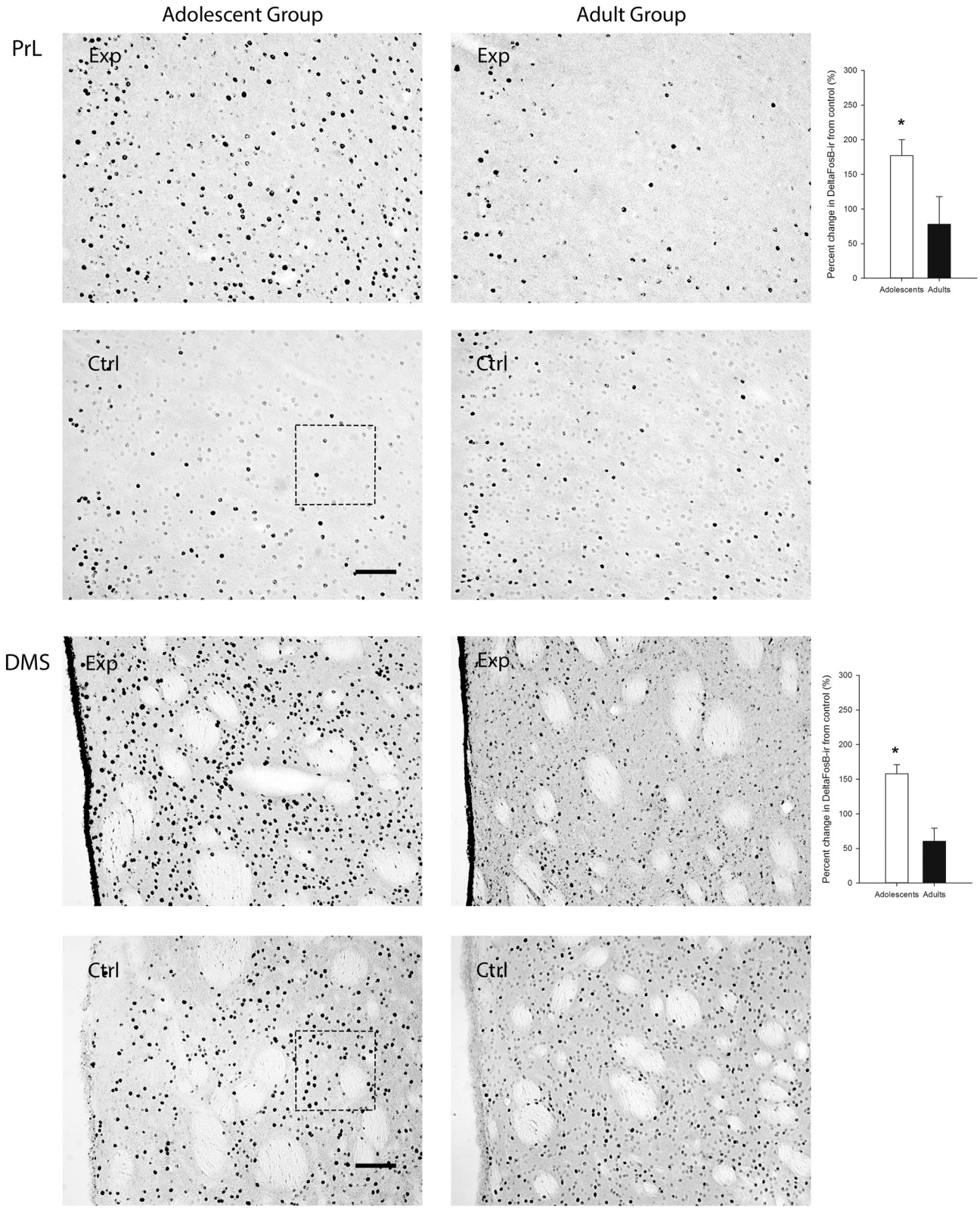


Fig. 5. Representative photomicrographs of ΔFosB-immunoreactive (ΔFosB-ir) cells in the prelimbic cortex (PrL) and dorsomedial striatum (DMS) (upper and lower panels, respectively) in adolescent and adult rats exposed to only vehicle (control groups) or a bottle of ethanol and a bottle of vehicle (experimental groups) in 18 two-bottle choice sessions. The inset bar graphs illustrate the percent change in ΔFosB immunoreactivity relative to control. The asterisk indicates a significant percent change in ΔFosB immunoreactivity between adolescents and adults. Scale bar = 100 μm. The dashed square shows the quantified area.

remained stable during testing weeks 1, 2 and 3. Planned comparisons indicated that g/kg of ethanol consumed significantly increased in the first intake session of week 4 (i.e., session 10), compared to the previous sessions, and remained stable afterwards. Percent ethanol preference also increased significantly in the first intake session of week 4 (i.e., session 10), compared to the previous sessions, and achieved its peak at

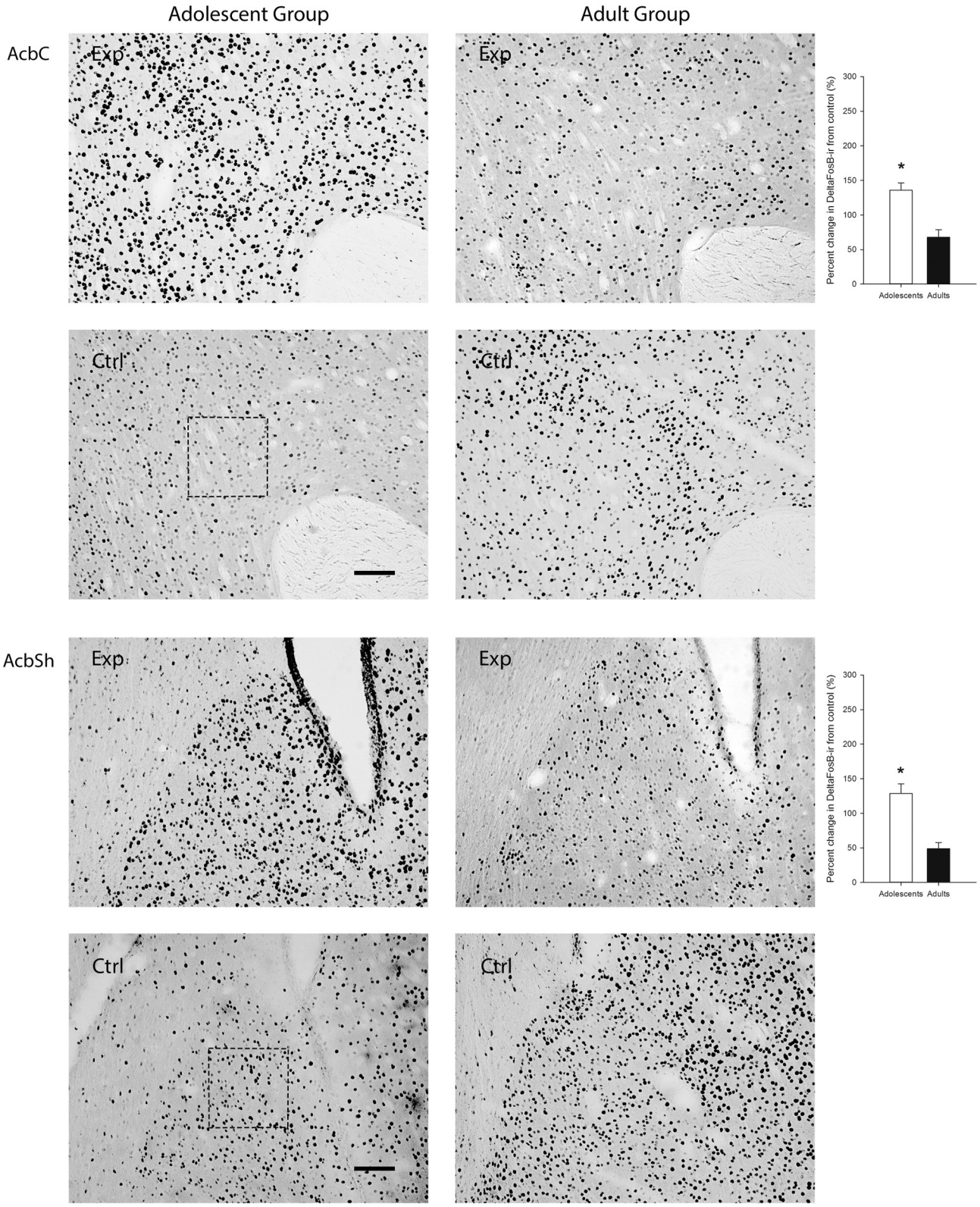


Fig. 6. Representative photomicrographs of ΔFosB-immunoreactive (ΔFosB-ir) cells in the nucleus accumbens core (AcbC) and shell (AcbSh) (upper and lower panels, respectively) in adolescent and adult rats exposed to only vehicle (control groups) or a bottle of ethanol and a bottle of vehicle (experimental groups) in 18 two-bottle choice sessions. The inset bar graphs illustrate the percent change in ΔFosB immunoreactivity relative to control. The asterisk indicates a significant percent change in ΔFosB immunoreactivity between adolescents and adults. Scale bar = 100 μm. The dashed square shows the quantified area.

sessions 17–18. Percent ethanol intake during these last two sessions was significantly greater than that observed in sessions 1–13. The analysis of overall fluid consumption (ml/100 g) across sessions indicated similar liquid consumption in adolescents that ingested ethanol compared to those that drank only vehicle. Average weekly mean and SEM was 32.56 ± 2.53, 27.88 ± 1.28, 19.86 ± 0.93, 20.44 ± 0.62, 18.29 ±

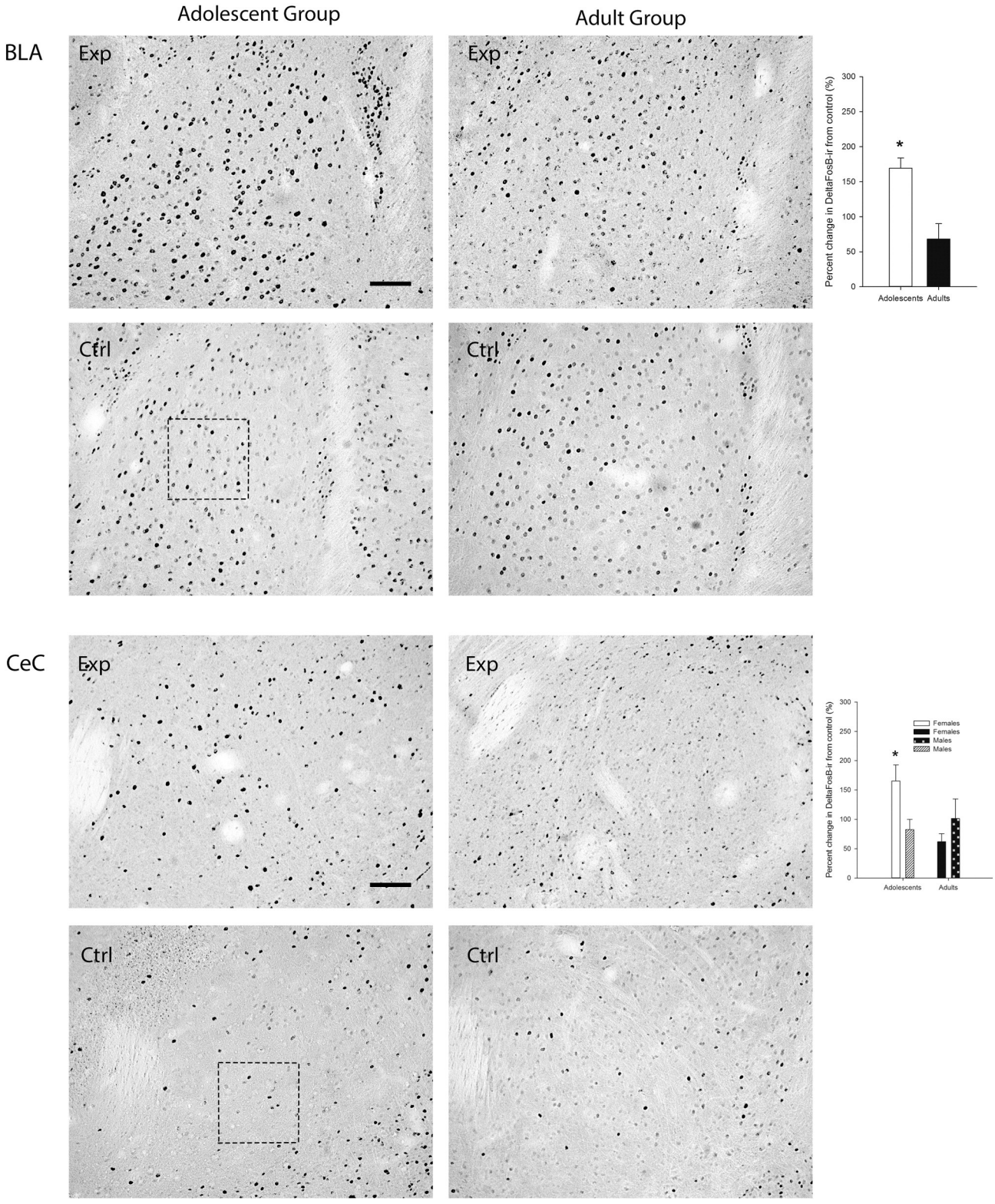


Fig. 7. Representative photomicrographs of ΔFosB-immunoreactive (ΔFosB-ir) cells in the basolateral (BLA) and central amygdala nucleus capsular (CeC) (upper and lower panels, respectively) in adolescent and adult rats exposed to only vehicle (control groups) or a bottle of ethanol and a bottle of vehicle (experimental groups) in 18 two-bottle choice sessions. The inset bar graphs illustrate the percent change in ΔFosB immunoreactivity relative to control. The asterisk indicates a significant percent change in ΔFosB immunoreactivity between adolescents and adults. The pound sign indicates a significant percent change in ΔFosB immunoreactivity between adolescent females and adult females. Scale bar = 100 μm. The dashed square shows the quantified area.

1.43 and 18.44 ± 2.36 (weeks 1–6, Control group); and 29.46 ± 4.05, 27.02 ± 2.37, 18.19 ± 1.13, 20.47 ± 1.69, 19.49 ± 1.45 and 19.60 ± 1.24 (weeks 1–6, Experimental group).

A two-way mixed ANOVA between factor: group (Experimental or Control); repeated measure factor: time spent on each surface (EVA or sandpaper) conducted on habituation scores revealed a lack of significant main effects or significant interactions. This indicated that the rats spent the same amount of time in each of the compartments of the CPP apparatus, during the pre-test (i.e., there was no unspecific, pre-conditioning texture bias).

The corresponding ANOVA indicated that neither the history of ethanol self-administration nor the conditioning group assignment (i.e., sandpaper or EVA as the CS^+) exerted a significant effect on absolute or percent time spent on the sandpaper CS at test. The interaction between these factors was also not significant. Moreover, the *t*-tests for absolute or percent time spent on the CS+ at test, and the *t*-tests for absolute (s) and percent difference scores, indicated no differences between the females that had ingested ethanol chronically and those that had been exposed to only vehicle. Mean and SEM absolute (s) and percent time spent on the CS+ compartment at test was 275.27 ± 35.68 and 48.24 ± 6.45 (control, vehicle-exposed group); and 273.17 ± 36.28 and 48.48 ± 6.39 (experimental, ethanol-exposed group). Absolute and percent time spent on each surface during habituation and test can be found in Fig. 10.

4. Discussion

The main new finding of the present study was that chronic ethanol ingestion resulted in greater ΔFosB induction in male and female adolescents, compared with their adult-drinking counterparts, in most of the brain areas analyzed (Exp. 1). A significantly greater number of ethanol-induced ΔFosB-positive cells was found in adolescents vs. adults in the PrL, DMS, AcbC, AcbSh, BLA, and CeC. This large, age-related, difference in the neural consequences of chronic ethanol ingestion did not promote, however, the expression of ethanol-induced conditioned place preference (Exp. 2), although it is difficult to draw definitive conclusions from the CPP data, since a single dose of ethanol was used. An important result was that, at most of the brain areas analyzed, the induction of ΔFosB was predicted by the percent increase in ethanol consumption (i.e., the change in ethanol intake, measured from the initial to the last self-administration session), but not by the total amount of ethanol ingested across the study. This suggest that the level of ΔFosB induction is not exclusively dependent on the amount of ethanol ingested, but instead is favored by escalating patterns of ethanol-self-administration.

Compared with adults, we expected adolescents to exhibit a steeper drinking acquisition curve (Vetter et al., 2007). The adolescents did exhibit a progressive escalation of ethanol intake and preference, whereas adults exhibited a much more stable pattern of intake, particularly in terms of the absolute ingestion of ethanol per body weight. Contrary to our expectations, adults drank significantly more ethanol than adolescents during the first weeks of testing. Although this difference could be attributable to differential reactions to the sweet component of the alcoholic beverage, previous work found significantly greater consumption of sweetened 5% ethanol in adolescents than in adults (Walker et al., 2008). Moreover, in the present study, the age difference persisted during weeks 3 and 4 of testing when the animals were offered 5% ethanol mixed with plain water. These age-related differences were specific for ethanol drinking. Vehicle and overall intake were very similar across age, and significant differences between adolescents and adults were only observed in a few, isolated sessions.

The pattern of ethanol intake contrasts with studies conducted in animal models that reported greater ethanol intake in adolescents than in adults (Doremus et al., 2005; García-Burgos et al., 2009; Tambour et al., 2008; Vetter et al., 2007). Some studies, however, found no age-related differences in ethanol intake (Doherty and Gonzales, 2015; Schramm-Sapyta et al., 2010). Importantly, we tested ethanol intake mainly during the dark phase. Walker et al. (2008) found significantly greater intake of sweetened 5% ethanol in adolescent vs. adult Wistar rats during the light phase but similar ethanol intake at both ages during the dark phase. Also, Siegmund et al. (2005) reported a similar profile as the one found in the present study.

Broadly speaking, across the ethanol-exposed and vehicle-only groups, ΔFosB induction was much more robust at nucleus accumbens (both AcbC and AcbSh) and to a lesser extent in the amygdala and DMS compared with the other brain areas analyzed. This finding is consistent with previous studies in which ΔFosB expression after non-pharmacological treatments (Perrotti et al., 2004) was observed in the AcbC and BLA but not elsewhere.

As described, adolescents exhibit significant and large ethanol-induced increases in ΔFosB induction, when compared with adults. Yet the age difference in ethanol-induced ΔFosB expression is even more striking when focusing on the percent change in ethanol-induced ΔFosB induction relative to controls. In this measurement, the age difference that favored adolescents emerged in all areas, with adolescents exhibiting an approximately three-fold relative increase in ΔFosB compared with adults (i.e., ~50% vs. 150% across most areas). The PrL, DMS,

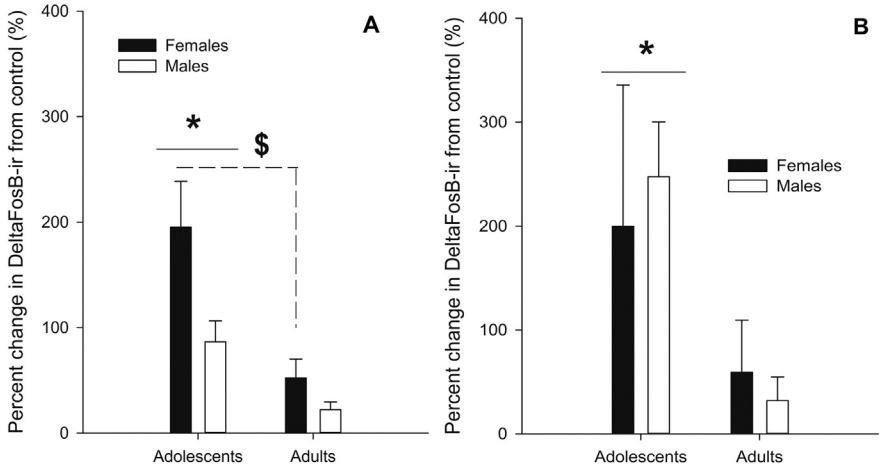


Fig. 8. Percent change in ΔFosB immunoreactivity, relative to control, in the dorsolateral striatum (DLS), and lateral orbital cortex (LO) (A and B panels, respectively) in adolescent and adult rats exposed to a bottle of ethanol and a bottle of vehicle (experimental groups) in 18 two-bottle choice sessions. The asterisk indicates a significant percent change in ΔFosB immunoreactivity between adolescents and adults. The currency sign indicates a significant percent change in ΔFosB immunoreactivity between female and male rats. Vertical bars represent the SEM.

Table 3
Spearman rank correlations (r_S) between the total amount of ethanol ingested across the 18 self-administration sessions or the percent increase in ethanol consumption between the first and the last self-administration session, and the raw number of ΔFosB-positive cells in each brain structure. Correlations were calculated for the overall sample of experimental subjects, and individually for each age group.

		PrL	LO	AcbC	AcbSh	DLS	DMS	BLA	CeC
Overall	A	−0.42	−0.42	−0.36	−0.42	−0.05	−0.29	−0.43	−0.44
	B	**0.53**	**0.61**	**0.65**	**0.70**	0.39	**0.60**	**0.74**	**0.60**
Adolescents	A	0.42	0.52	0.50	0.43	0.59	**0.74**	0.57	0.07
	B	−0.36	0.07	0.19	−0.11	−0.22	0.02	−0.11	−0.11
Adults	A	−0.63	−0.01	−0.02	−0.19	−0.01	−0.04	−0.38	−0.16
	B	0.23	0.10	0.00	0.24	0.13	0.12	0.51	0.01

Note: A = total amount of ethanol ingested (g/kg) across the 18 self-administration sessions; B = percent increase in ethanol consumption (g/kg) between the first and the last self-administration sessions (i.e., sessions 1 and 18, respectively). **Note 2:** significant correlations ($p < 0.05$) are expressed in bold. **Note 3:** PrL is prelimbic prefrontal area, LO is lateral orbital cortex, AcbSh and AcbC are accumbens shell and core, respectively; DLS is dorsolateral striatum; DMS is dorsomedial striatum; BLA is basolateral amygdaloid nucleus; and Cec is central amygdaloid nucleus capsular part (CeC).

AcbC, AcbSh, BLA, and CeC for the most part play an important role in the rewarding effects of drugs of abuse. The AcbSh is involved in the hedonic assessment of rewards and provides incentive value to stimuli that precede these rewards, thus resulting in the formation of habits that may perpetuate drug-seeking behavior (Di Chiara et al., 2004). The AcbC and DMS are associated with motor aspects of reward responses (Hu et al., 2013; Ortiz-Pulido et al., 2016), whereas the amygdala, particularly the CeC, has been shown to undergo neuroadaptations after chronic drug use that result in greater anxiety and sensitivity to stress (Koob, 2009). The PrL has been implicated in the reinstatement of drug self-administration (Millan et al., 2011).

Studies have revealed a plethora of differences between adolescents and adults in the response to the acute effects of ethanol. Adolescents are more sensitive to the social facilitating effects of ethanol (Varlinskaya and Spear, 2002) but are less sensitive to the aversive (Saalfield and Spear, 2016), sedative (Fernandez et al., 2016) and social impairing effects of the drug that may serve as deterrents to compulsive ethanol use [for review and references, see (Doremus-Fitzwater and Spear, 2016)]. The greater ethanol-induced ΔFosB induction that was found in adolescents in the present study may represent yet another age-related difference in the sensitivity to ethanol, in this case to the chronic effects of intermittent access to the drug, that may put young individuals at risk for problematic ethanol use. ΔFosB protein accumulates in medium spiny neurons in the Acb (Lobo et al., 2013) and decreases the expression of dynorphin (i.e., the endogenous ligand of κ opioid receptors), thus increasing the effects of dopamine and motivational impact of natural and drug rewards (Zachariou et al., 2006). Activation of the κ opioid system induces a dysphoric, aversive state. Consistent with this, animals that exhibit an increase in ΔFosB expression also present greater responsiveness to the appetitive effects of drugs of abuse and lower responsiveness to the aversive effects of κ opioid receptor activation (Zachariou et al., 2006).

The motivational effects of ethanol are regulated by the activation of dopaminergic neurons in the ventral tegmental area (VTA) that project to the AcbSh (Koob et al., 1998). The system is controlled, at least partially, by dynorphinergic neurons that liberate dynorphin on dopamine neurons in the VTA and reduce their firing (Xiao et al., 2007). Based on the present results, one hypothesis is that chronic ethanol self-administration during adolescence but not during adulthood causes an accumulation of ΔFosB in key areas of the reward system, notably the Acb, which alters the function of the kappa opioid system and ultimately results in greater ethanol intake. Under this framework, the level of ethanol intake during the first drinking session, although modest, would induce some induction of ΔFosB in the adolescent brain, which in turn would further increase the level of ethanol drinking in subsequent weeks. This may explain why adolescents escalated their ethanol intake, whereas adults maintained a constant level of ingestion, regardless of the initial age-related differences in ethanol intake.

Further insights into the conditions that lead to the ethanol-induced accumulation of ΔFosB are provided by dissecting neural patterns in the adult rats in the present study. Ethanol-drinking adults exhibited lower ΔFosB induction than ethanol-drinking adolescents, and they failed to exhibit significant accumulation of the protein compared with their

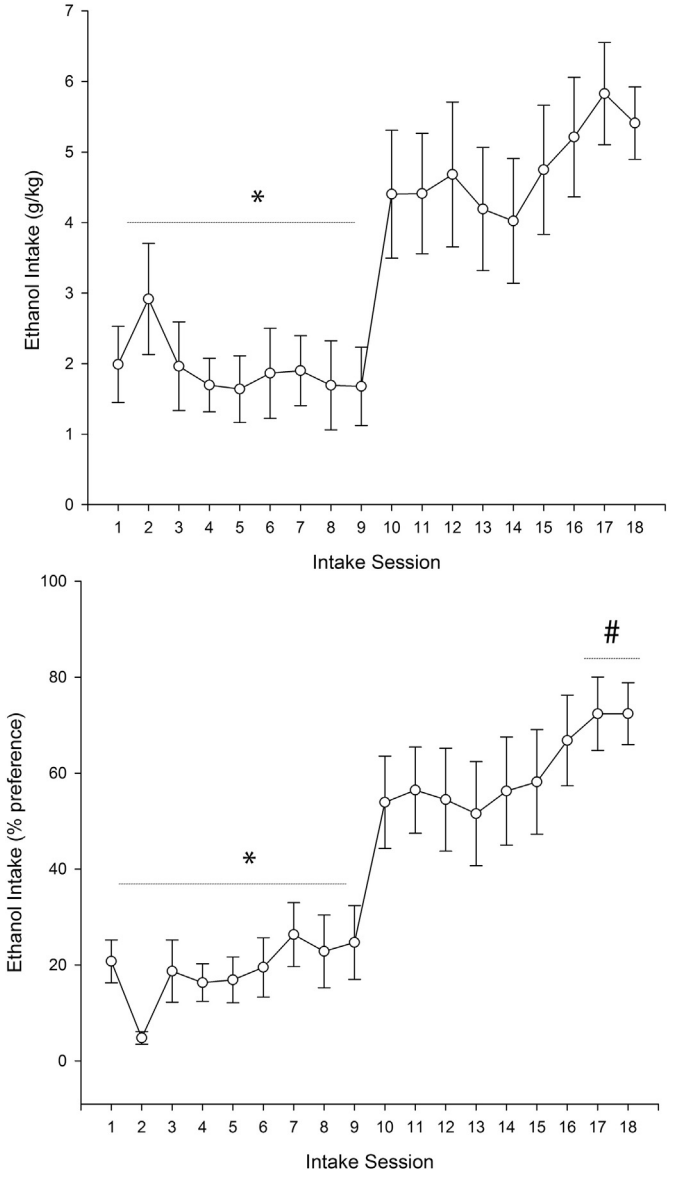


Fig. 9. Mean ethanol intake (g/kg and percent preference in the upper and lower panels, respectively) in adolescent female rats, in Experiment 2. The asterisk indicates that ethanol intake (g/kg and % preference) was significantly greater in sessions 10–18 than in sessions 1–9. The pound sign indicated that percent ethanol preference registered in sessions 17–18 was significantly greater than that registered in sessions 10–13. Vertical bars represent the SEM.

same-age, vehicle-drinking controls. This contrasts with findings from Li et al. (2010), who found robust ΔFosB immunoreactivity in the AcbC, AcbSh, DLS, and LO. In that previous study, Sprague-Dawley rats were exposed to intermittent access to 20% ethanol for 15 sessions. Unlike the relatively flat pattern of ethanol acceptance that was exhibited by adult Wistar rats in the present study, the drinking paradigm that was employed by Li et al. caused an escalating pattern of ethanol intake over 2–3 sessions. Drinking conditions that favor an escalating pattern of ethanol-self-administration may be more likely to induce ΔFosB accumulation. This is, of course, just a hypothesis; but it is intriguing that, in the present study, ethanol-induced ΔFosB accumulation in the overall sample of subjects was significantly and positively associated with the percent increase in ethanol acceptance (measured from the beginning to the end of self-administration), but not with the total amount of ethanol ingested throughout the self-administration sessions. Also interesting is that ethanol intake in the adolescent subjects followed a fairly stable pattern during self-administration weeks 1–3 and only began to increase in the first session of week 3, which was the first time that animals had a 2-day withdrawal from unsweetened ethanol. This hints at the possibility that the escalating pattern of ethanol intake, exhibited by the adolescents, was ignited by the first, 2-day withdrawal from unsweetened ethanol.

Even though previous studies have indicated sex-related differences in ethanol intake, we observed fairly similar intake patterns across males and females. This could relate to the use of a relatively low concentration of ethanol [i.e., 5%, as opposed to the ≥10% used in rat (Doremus et al., 2005) or mice (Lopez and Laber, 2015) studies that yielded sex-related differences in ethanol intake]. An intriguing result was that, across the ethanol-exposed and vehicle-only groups, females exhibited greater ΔFosB than males at most areas (i.e, AcbC, AcbSh, DLS, DMS, BLA and CeC), a finding consistent with recent reports (Retson et al., 2015). Also, despite ingesting similar amounts of ethanol, the relative ethanol-induced ΔFosB accumulation in the capsular subdivision of the amygdala (CeC), a key area in the regulation of anxiety and stress responses, was significantly greater in adolescent females than in adolescent or adult males. This is important because it meets previous findings (Pandey et al., 2015) in which rats exposed to intermittent ethanol exposure throughout adolescence [PD 28–41, 2 g/kg ethanol (2 days on/off)] exhibited, when tested at adulthood, increased anxiety-like behavior and decreased dendritic spine density in CeC [also see (Kyzar et al., 2016)]. Also, a recent study (Retson et al., 2015) exposed adult male and female Sprague–Dawley rats to a liquid diet containing ethanol, for 14 days. Ethanol-consuming animals drank an average of 14 g/kg/day and exhibited, when compared to pair-fed adult controls, significantly greater absolute number of ΔFosB positive cells at CeC. The effect found by Retson et al. (2015), however, was much greater in males than in females. The present study, which yielded lower levels of daily ethanol ingestion than those found in Retson et al. (2015), found greater (absolute) number of ΔFosB positive cells at CeC in adolescent, but not in adult, males and females, suggesting a complex interaction between age, sex and level of ethanol exposure in the neural consequences ethanol at this area of the amygdala.

The excitement brought by the age- and sex-related differences found in ΔFosB accumulation has to be considered in the context of important limitations. We did not measure ΔFosB in all animals that underwent the two-bottle choice tests. Instead, we subjected a subgroup of animals to the immunohistochemistry procedure, which exhibited relatively higher levels of ethanol acceptance at both ages. Another caveat is that the behavioral screening for anxiety and ethanol-induced simulation that was conducted at end of the intake protocol

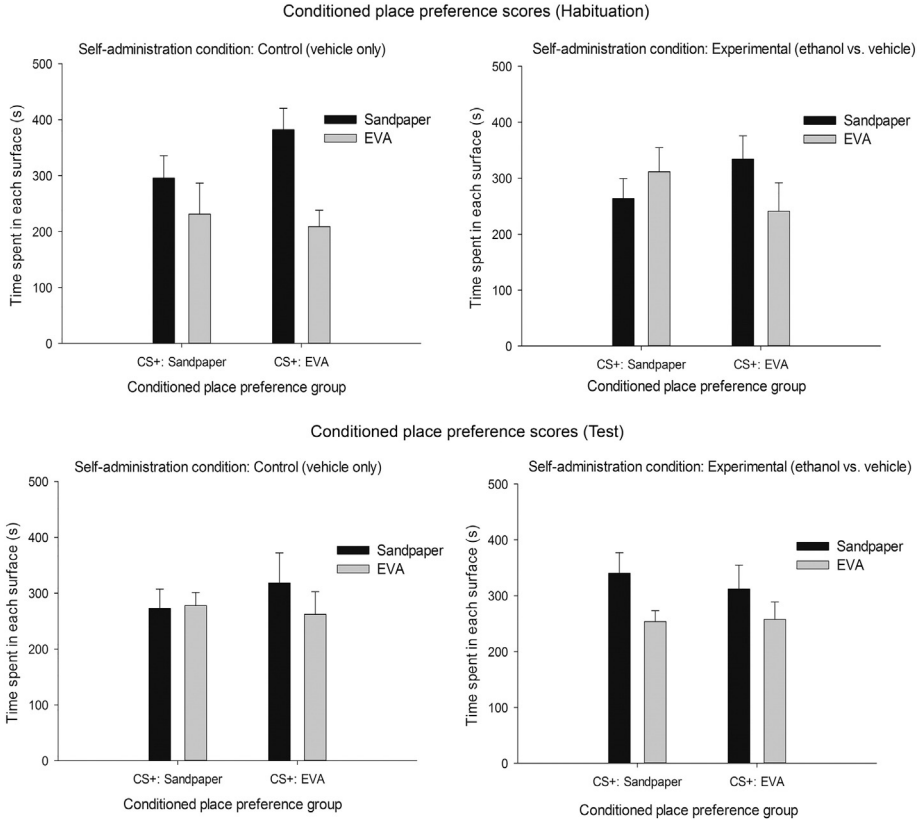


Fig. 10. Absolute time spent (s) on the sandpaper and ethylene vinyl acetate (EVA) lined chambers, during the habituation and test phases (duration of each phase: 12 min) of the conditioned place preference procedure (CPP, Experiment 2). The adolescent, female, rats, were exposed to only vehicle (control groups) or a bottle of ethanol and a bottle of vehicle (experimental groups) in 18 two-bottle choice sessions. These two-bottle intake sessions were conducted before CPP training in which sandpaper or EVA served as the excitatory conditioned stimulus (CS+). Specifically, the animals were given four pairings between the pharmacological effects of ethanol administration (0.75 g/kg) and the sandpaper or the EVA-lined chamber. For half of the subjects the CS+ was the sandpaper whereas for the other half the CS+ was the EVA covered chamber. Vertical bars represent the SEM.

did not reveal, for the most part, differences between ages or conditions. Ethanol-drinking males exhibited significantly more time in the anxiety-inducing compartment than the rest of the groups, a result that could indicate ethanol-induced disinhibition. This effect, however, was similar across age. Yet perhaps the biggest limitation is that ethanol self-administration resulted in greater induction of ΔFosB in the adolescents, yet did not favor the emergence of ethanol-induced CPP in this group. This was not completely unexpected, as it has proven difficult to find reliable CPP by ethanol in rats, with most studies reporting conditioned aversions at doses ≥ 2.0 g/kg. We only tested one ethanol dose, though, and trained the adolescents in an abbreviated variant of the typical CPP conditioning put forward by Cunningham et al. (2006). It is possible that differences between control and experimental subjects would have emerged if we had tested higher doses likely to exert aversive effects or if we had employed the biased CPP paradigm of Acevedo et al. (2013).

The likelihood of problematic alcohol use is dramatically lower in individuals who delay the onset of alcohol drinking until 15 or 16 years of age (DeWit et al., 2000; Jenkins et al., 2011), highlighting the importance of analyzing the neural correlates of early- vs. late-onset ethanol exposure. The present results suggest that subjects that begin drinking during adolescence and continue doing so throughout that stage of development, as opposed to those that begin in adulthood, are more likely to exhibit brain changes in areas of the reward circuit. These changes may put them at an increased risk for the development of ethanol-related disorders.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.pnpbp.2016.11.008.

Author contribution

Pautassi designed the study, with significant input by De Olmos, and wrote the protocol. Wille-Bille, Chiner and Pautassi run the ethanol intake tests and run the statistical analysis. Chiner, Pautassi and Marengo run the behavioral assays. Wille-Bille wrote the first draft of the manuscript. Wille-Bille and De Olmos run the immunohistochemical analyses. All authors contributed and approved the final manuscript.

Declaration of interest

We declare having no competing interest nor conflict of interest related to our MS or its results.

Acknowledgements

This work was supported by grants PICT 2012, PIP2013-2015 and SECyT-UNC 2014–2015 to RMP.

References

Acevedo, M.B., Nizhnikov, M.E., Spear, N.E., Molina, J.C., Pautassi, R.M., 2013. Ethanol-induced locomotor activity in adolescent rats and the relationship with ethanol-induced conditioned place preference and conditioned taste aversion. Dev. Psychobiol. 55, 429–442.

Becker, J.B., Perry, A.N., Westenbroek, C., 2012. Sex differences in the neural mechanisms mediating addiction: a new synthesis and hypothesis. Biol. Sex Differ. 3, 14.

Buchmann, A.F., Schmid, B., Blomeyer, D., Becker, K., Treutlein, J., Zimmermann, U.S., et al., 2009. Impact of age at first drink on vulnerability to alcohol-related problems: testing the marker hypothesis in a prospective study of young adults. J. Psychiatr. Res. 43, 1205–1212.

Cancela, L.M., Bregonzio, C., Molina, V.A., 1995. Anxiolytic-like effect induced by chronic stress is reversed by naloxone pretreatment. Brain Res. Bull. 36, 209–213.

Cunningham, C.L., Gremel, C.M., Groblewski, P.A., 2006. Drug-induced conditioned place preference and aversion in mice. Nat. Protoc. 1, 1662–1670.

DeWit, D.J., Adlaf, E.M., Offord, D.R., Ogborne, A.C., 2000. Age at first alcohol use: a risk factor for the development of alcohol disorders. Am. J. Psychiatry 157, 745–750.

Di Chiara, G., Bassareo, V., Fenu, S., De Luca, M.A., Spina, L., Cadoni, C., et al., 2004. Dopamine and drug addiction: the nucleus accumbens shell connection. Neuropharmacology 47 (Suppl. 1), 227–241.

Doherty, J.M., Gonzales, R.A., 2015. Operant self-administration of sweetened ethanol and time course of blood ethanol levels in adolescent and adult male Long-Evans rats. Alcohol. Clin. Exp. Res. 39, 485–495.

Doremus-Fitzwater, T.L., Spear, L.P., 2016. Reward-centricity and attenuated aversions: an adolescent phenotype emerging from studies in laboratory animals. Neurosci. Biobehav. Rev.

Doremus, T.L., Brunell, S.C., Rajendran, P., Spear, L.P., 2005. Factors influencing elevated ethanol consumption in adolescent relative to adult rats. Alcohol. Clin. Exp. Res. 29, 1796–1808.

Fabio, M.C., Macchione, A.F., Nizhnikov, M.E., Pautassi, R.M., 2015. Prenatal ethanol increases ethanol intake throughout adolescence, alters ethanol-mediated aversive learning, and affects mu but not delta or kappa opioid receptor mRNA expression. Eur. J. Neurosci. 41, 1569–1579.

Fernandez-Vidal, J.M., Spear, N.E., Molina, J.C., 2003. Adolescent rats discriminate a mild state of ethanol intoxication likely to act as an appetitive unconditioned stimulus. Alcohol 30, 45–60.

Fernandez, M.S., Fabio, M.C., Miranda-Morales, R.S., Virgolini, M.B., De Giovanni, L.N., Hansen, C., et al., 2016. Age-related effects of chronic restraint stress on ethanol drinking, ethanol-induced sedation, and on basal and stress-induced anxiety response. Alcohol 51, 89–100.

García-Burgos, D., González, F., Manrique, T., Gallo, M., 2009. Patterns of ethanol intake in preadolescent, adolescent, and adult Wistar rats under acquisition, maintenance, and relapse-like conditions. Alcohol. Clin. Exp. Res. 33, 722–728.

Hu, A., Lai, M., Wei, J., Wang, L., Mao, H., Zhou, W., et al., 2013. The effect of electroacupuncture on extinction responding of heroin-seeking behavior and FosB expression in the nucleus accumbens core. Neurosci. Lett. 534, 252–257.

Jenkins, M.B., Agrawal, A., Lynskey, M.T., Nelson, E.C., Madden, P.A., Bucholz, K.K., et al., 2011. Correlates of alcohol abuse/dependence in early-onset alcohol-using women. Am. J. Addict. 20, 429–434.

Kim, M.J., Mason, W.A., Herrenkohl, T.I., Catalano, R.F., Toumbourou, J.W., Hemphill, S.A., 2016. Influence of early onset of alcohol use on the development of adolescent alcohol problems: a longitudinal binational study. Prev. Sci. PMID: 27699620 http://dx.doi.org/10.1007/s11121-016-0710-z.

Koob, G.F., 2009. Brain stress systems in the amygdala and addiction. Brain Res. 1293, 61–75.

Koob, G.F., Sanna, P.P., Bloom, F.E., 1998. Neuroscience of addiction. Neuron 21, 467–476.

Kyzar, E.J., Zhang, H., Sakharkar, A.J., Pandey, S.C., 2016. Adolescent alcohol exposure alters lysine demethylase 1 (LSD1) expression and histone methylation in the amygdala during adulthood. Addict. Biol.

Li, J., Cheng, Y., Bian, W., Liu, X., Zhang, C., Ye, J.H., 2010. Region-specific induction of FosB/DeltaFosB by voluntary alcohol intake: effects of naltrexone. Alcohol. Clin. Exp. Res. 34, 1742–1750.

Lobo, M.K., Zaman, S., Damez-Werno, D.M., Koo, J.W., Bagot, R.C., DiNieri, J.A., et al., 2013. DeltaFosB induction in striatal medium spiny neuron subtypes in response to chronic pharmacological, emotional, and optogenetic stimuli. J. Neurosci. Off. J. Soc. Neurosci. 33, 18381–18395.

Lopez, M.F., Laber, K., 2015. Impact of social isolation and enriched environment during adolescence on voluntary ethanol intake and anxiety in C57BL/6J mice. Physiol. Behav. 148, 151–156.

McCullough, L.D., de Vries, G.J., Miller, V.M., Becker, J.B., Sandberg, K., McCarthy, M.M., 2014. NIH initiative to balance sex of animals in preclinical studies: generative questions to guide policy, implementation, and metrics. Biol. Sex Differ. 5, 15.

Millan, E.Z., Marchant, N.J., McNally, G.P., 2011. Extinction of drug seeking. Behav. Brain Res. 217, 454–462.

Nestler, E.J., 2015. FosB: a transcriptional regulator of stress and antidepressant responses. Eur. J. Pharmacol. 753, 66–72.

Nestler, E.J., Barrot, M., Self, D.W., 2001. DeltaFosB: a sustained molecular switch for addiction. Proc. Natl. Acad. Sci. U. S. A. 98, 11042–11046.

Ortiz-Pulido, R., Hernandez-Briones, Z.S., Tamariz-Rodriguez, A., Hernandez, M.E., Aranda-Abreu, G.E., Coria-Avila, G.A., et al., 2016. Effect of electrolytic lesion of the dorsomedial striatum on sexual behaviour and locomotor activity in rats. Neurologia.

Ozburn, A.R., Mayfield, R.D., Ponomarev, I., Jones, T.A., Blednov, Y.A., Harris, R.A., 2012. Chronic self-administration of alcohol results in elevated DeltaFosB: comparison of hybrid mice with distinct drinking patterns. BMC Neurosci. 13, 130.

Pandey, S.C., Sakharkar, A.J., Tang, L., Zhang, H., 2015. Potential role of adolescent alcohol exposure-induced amygdaloid histone modifications in anxiety and alcohol intake during adulthood. Neurobiol. Dis. 82, 607–619.

Pascual, M., Blanco, A.M., Cauli, O., Miñarro, J., Guerri, C., 2007. Intermittent ethanol exposure induces inflammatory brain damage and causes long-term behavioural alterations in adolescent rats. Eur. J. Neurosci. 541–550.

Pascual, M., Boix, J., Felipo, V., Guerri, C., 2009. Repeated alcohol administration during adolescence causes changes in the mesolimbic dopaminergic and glutamatergic systems and promotes alcohol intake in the adult rat. J. Neurochem. 108, 920–931.

Pascual, M., Do Couto, B.R., Alfonso-Loeches, S., Aguilar, M.A., Rodriguez-Arias, M., Guerri, C., 2012. Changes in histone acetylation in the prefrontal cortex of ethanol-exposed adolescent rats are associated with ethanol-induced place conditioning. Neuropharmacology 62, 2309–2319.

Pautassi, R.M., Myers, M., Spear, L.P., Molina, J.C., Spear, N.E., 2008. Adolescent but not adult rats exhibit ethanol-mediated appetitive second-order conditioning. Alcohol. Clin. Exp. Res. 32, 2016–2027.

Paxinos, G., 2007. The rat brain in stereotaxic coordinates. AcadPress, San Diego.

Perkins, H.W., 2002. Surveying the damage: a review of research on consequences of alcohol misuse in college populations. J. Stud. Alcohol Suppl. 91-100.

Perrotti, L.I., Bolanos, C.A., Choi, K.H., Russo, S.J., Edwards, S., Ulery, P.G., et al., 2005. DeltaFosB accumulates in a GABAergic cell population in the posterior tail of the ventral tegmental area after psychostimulant treatment. Eur. J. Neurosci. 21, 2817–2824.

Perrotti, L.I., Hadeishi, Y., Ulery, P.G., Barrot, M., Monteggia, L., Duman, R.S., et al., 2004. Induction of deltaFosB in reward-related brain structures after chronic stress. J. Neurosci. 24, 10594–10602.
Perrotti, L.I., Weaver, R.R., Robison, B., Renthal, W., Maze, I., Yazdani, S., et al., 2008. Distinct patterns of DeltaFosB induction in brain by drugs of abuse. Synapse 62, 358–369.
Philpot, R.M., Badanich, K.A., Kirstein, C.L., 2003. Place conditioning: age-related changes in the rewarding and aversive effects of alcohol. Alcohol. Clin. Exp. Res. 27, 593–599.
Read, J.P., Wood, M.D., Lejuez, C.W., Palfai, T.P., Slack, M., 2004. Gender, alcohol consumption, and differing alcohol expectancy dimensions in college drinkers. Exp. Clin. Psychopharmacol. 12, 298–308.
Retson, T.A., Hoek, J.B., Sterling, R.C., Van Bockstaele, E.J., 2015. Amygdalar neuronal plasticity and the interactions of alcohol, sex, and stress. Brain Struct. Funct. 220, 3211–3232.
Saalfield, J., Spear, L., 2016. The ontogeny of ethanol aversion. Physiol. Behav. 156, 164–170.
Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.Y., White, D.J., Hartenstein, V., Eliceiri, K., Tomancak, P., Cardona, A., 2012 Jun 28. Fiji: an open-source platform for biological-image analysis. Nat Methods 9 (7):676–682. http://dx.doi.org/10.1038/nmeth.2019.
Segura, C., Becker, J.B., 2016. Why we should consider sex (and study sex differences) in addiction research. Addict Biol. 21 (5):995–1006. http://dx.doi.org/10.1111/adb.12382.
Schramm-Sapyta, N.L., DiFeliceantonio, A.G., Foscue, E., Glowacz, S., Haseeb, N., Wang, N., et al., 2010. Aversive effects of ethanol in adolescent versus adult rats: potential causes and implication for future drinking. Alcohol. Clin. Exp. Res. 34, 2061–2069.
Siegmund, S., Vengeliene, V., Singer, M.V., Spanagel, R., 2005. Influence of age at drinking onset on long-term ethanol self-administration with deprivation and stress phases. Alcohol. Clin. Exp. Res. 29, 1139–1145.
Spear, L.P., Swartzwelder, H.S., 2014. Adolescent alcohol exposure and persistence of adolescent-typical phenotypes into adulthood: a mini-review. Neurosci. Biobehav. Rev. 45, 1–8.
Tambour, S., Brown, L.L., Crabbe, J.C., 2008. Gender and age at drinking onset affect voluntary alcohol consumption but neither the alcohol deprivation effect nor the response to stress in mice. Alcohol. Clin. Exp. Res. 32, 2100–2106.
Truxell, E.M., Molina, J.C., Spear, N.E., 2007. Ethanol intake in the juvenile, adolescent, and adult rat: effects of age and prior exposure to ethanol. Alcohol. Clin. Exp. Res. 31, 755–765.
Varlinskaya, E.I., Spear, L.P., 2002. Acute effects of ethanol on social behavior of adolescent and adult rats: role of familiarity of the test situation. Alcohol. Clin. Exp. Res. 26, 1502–1511.
Vetter, C.S., Doremus-Fitzwater, T.L., Spear, L.P., 2007. Time course of elevated ethanol intake in adolescent relative to adult rats under continuous, voluntary-access conditions. Alcohol. Clin. Exp. Res. 31, 1159–1168.
Walker, B.M., Walker, J.L., Ehlers, C.L., 2008. Dissociable effects of ethanol consumption during the light and dark phase in adolescent and adult Wistar rats. Alcohol 42, 83–89.
Windle, M., Zucker, R.A., 2010. Reducing underage and young adult drinking: how to address critical drinking problems during this developmental period. Alcohol Res. Health 33, 29–44.
Winer, B., Brown, D.R., Michels, K.M., 1991. Statistical principles in experimental design. 3rd ed. McGraw Hill, New York.
Xiao, C., Zhang, J., Krnjevic, K., Ye, J.H., 2007. Effects of ethanol on midbrain neurons: role of opioid receptors. Alcohol. Clin. Exp. Res. 31, 1106–1113.
Zachariou, V., Bolanos, C.A., Selley, D.E., Theobald, D., Cassidy, M.P., Kelz, M.B., et al., 2006. An essential role for DeltaFosB in the nucleus accumbens in morphine action. Nat. Neurosci. 9, 205–211.