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# PRENATAL ETHANOL EXPOSURE ALTERS ETHANOL-INDUCED FOS IMMUNOREACTIVITY AND DOPAMINERGIC ACTIVITY IN THE MESOCORTICOLIMBIC PATHWAY OF THE ADOLESCENT BRAIN

**M. C. FABIO,** [a,b] **L. M. VIVAS** [a] **AND R. M. PAUTASSI** [a,b]*

[a] *Instituto de Investigación Médica M. y M. Ferreyra (INIMEC – CONICET-Universidad Nacional de Córdoba), Friuli 2434, Córdoba C.P. 5000, Argentina*

[b] *Facultad de Psicología, Universidad Nacional de Córdoba, Enfermera Gordillo s/n, Córdoba C.P. 5000, Argentina*

**Abstract—Prenatal ethanol exposure (PEE) promotes alcohol intake during adolescence, as shown in clinical and pre-clinical animal models. The mechanisms underlying this effect of prenatal ethanol exposure on postnatal ethanol intake remain, however, mostly unknown. Few studies assessed the effects of moderate doses of prenatal ethanol on spontaneous and ethanol-induced brain activity on adolescence. This study measured, in adolescent (female) Wistar rats prenatally exposed to ethanol (0.0 or 2.0 g/kg/day, gestational days 17–20) or non-manipulated (NM group) throughout pregnancy, baseline and ethanol-induced cathecolaminergic activity (i.e., colocalization of c-Fos and tyrosine hydroxylase) in ventral tegmental area (VTA), and baseline and ethanol-induced Fos immunoreactivity (ir) in nucleus accumbens shell and core (AcbSh and AcbC, respectively) and prelimbic (PrL) and infralimbic (IL) prefrontal cortex. The rats were challenged with ethanol (dose: 0.0, 1.25, 2.5 or 3.25 g/kg, i.p.) at postnatal day 37. Rats exposed to vehicle prenatally (VE group) exhibited reduced baseline dopaminergic tone in VTA; an effect that was inhibited by prenatal ethanol exposure (PEE group). Dopaminergic activity in VTA after the postnatal ethanol challenge was greater in PEE than in VE or NM animals. Ethanol-induced Fos-ir at AcbSh was found after 1.25 g/kg and 2.5 g/kg ethanol, in VE and PEE rats, respectively. PEE did not alter ethanol-induced Fos-ir at IL but reduced ethanol-induced Fos-ir at PrL. These results suggest that prenatal ethanol exposure heightens dopaminergic activity in the VTA and alters the response of the mesocorticolimbic pathway to postnatal ethanol exposure. These effects may underlie the enhanced vulnerability to develop alcohol-use disorders of adolescents with a history of *in utero* ethanol exposure.** 

**Key words: adolescent, rat, prenatal ethanol, dopamine, mesocorticolimbic pathway, Fos.**

## INTRODUCTION

Epidemiological studies indicate that early alcohol exposure, including that resulting from maternal ingestion of alcohol (hereinafter referred as ethanol) during gestation, significantly modulates the transition from moderate toward problematic alcohol use (Baer et al., 2003; Alati et al., 2008). To date, several preclinical studies have confirmed that prenatal ethanol exposure (PEE) promotes alcohol intake during infancy (Chotro and Arias, 2007; Diaz-Cenzano and Chotro, 2010), adolescence (Diaz-Cenzano and Chotro, 2010; Fabio et al., 2013) and adulthood (Abel et al., 1981; Randall et al., 1983; Nash et al., 1984). Nevertheless, few studies analyzed the effects of moderate doses of prenatal ethanol on spontaneous and ethanol-induced brain activity on adolescence (Vilpoux et al., 2009).

Ethanol exerts a myriad of effects when administered during sensitive periods of development (Jang et al., 2005; Gil-Mohapel et al., 2014; Brolese et al., 2014). Significant effects of PEE have been found within the mesocorticolimbic pathway, which begins in the ventral tegmental area (VTA) and projects to the nucleus accumbens, and ultimately to the prefrontal cortex. Exposure of ethanol via liquid diet on days 6–15 of pregnancy altered ethanol-induced dopamine release in the nucleus accumbens and striatum (Blanchard et al., 1993). In this study, adult rats that had been exposed to vehicle *in utero* showed increased dopamine release following 0.5 g/kg ethanol, but not after 1.0 g/kg ethanol; whereas PEE counterparts exhibited ethanol-induced dopamine release only after receiving 1.0 g/kg. PEE seems to impair the normal postnatal development of the DA system, resulting in a reduction in the spontaneous activity of dopamine neurons in the VTA, which is first evident during adolescence (Choong and Shen, 2004). In another study, adolescent rats that had been exposed to 4.5 g/kg/day ethanol throughout pregnancy exhibit alterations in Fos-ir in nucleus accumbens Shell (AcbSh) (Jang et al., 2005).

*Correspondence to: R. M. Pautassi, Instituto de Investigación Médica M. y M. Ferreyra (INIMEC – CONICET), Friuli 2434, Córdoba C.P. 5000, Argentina. Tel: +54-351-4681465x218; fax: +54-351-4695163.

E-mail address: rpautassi@gmail.com (R. M. Pautassi).

*Abbreviations:* AcbC, nucleus accumbens core; AcbSh, nucleus accumbens shell; ANOVA, analysis of variance; GD, gestational day; IL, infralimbic prefrontal cortex; ir, immunoreactivity; KOR, kappa opioid receptor; NHS, normal horse serum; NM, non-manipulated during gestation; PB, phosphate buffer; PD, postnatal day; PEE, prenatal ethanol exposure (a.k.a., ethanol-exposed during gestation); PrL, prelimbic prefrontal cortex; PVN, paraventricular nucleus; TH, tyrosine hydroxylase; VE, vehicle-exposed during gestation; VTA, ventral tegmental area.

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We observed that moderate PEE (2.0 g/kg, intragastrically [i.g.]) during late gestation [gestational days (GD) 17–20] significantly increased ethanol intake and altered the expression of Fos-ir in the pre-frontal infralimbic cortex (IL, Fabio et al., 2013) of adolescent Wistar rats. The IL cortex is associated with the ability to extinguish associative learning induced by natural rewards and drugs of abuse (Millan et al., 2011). Furthermore, animals exposed to high doses of ethanol (6.0 g/kg/day, i.g.) during almost the entire gestational period exhibited persistent abnormalities in the dorsal striatum, as well as altered balance between dopaminergic receptors 1 and 2 (D1 and D2, respectively). In a similar model of prenatal ethanol exposure, Randall and Hannigan (1999) found that animals exposed to 3.0 g/kg i.g. during GDs 8–20 had less sites of union for D2 receptors in the ventral striatum, an effect that was reverted by the dopaminergic agonist methylphenidate. It has also been found that hippocampus culture cells had less amount of D1 receptors as a product of prenatal ethanol (Naseer et al., 2014). PEE also incremented the amount of dopaminergic transporter in the striatum (Kim et al., 2013) and altered the sensitivity to the effects of apomorphine on locomotor activity (Becker et al., 1995).

These alterations of the mesocorticolimbic functioning may underlie the greater sensitivity for ethanol-induced reward (Pautassi et al., 2012) and the greater ethanol intake (Fabio et al., 2013, 2015), observed after PEE. In an intriguing experiment, rats untreated during gestation exhibited ethanol-induced conditioned place preference at doses of 0.5, but not at 1.0 or 2.0 g/kg ethanol; whereas PEE rats (2.0 g/kg, i.g.) exhibited place preference at 1.0 and 2.0, but not at 0.5 g/kg, ethanol (Pautassi et al., 2012). This shift in the dose–response curve for ethanol-induced reward is associated with greater ethanol intake during adolescence, and reduced synaptosomal kappa opioid receptor (KOR) expression in the nucleus accumbens, amygdala and hippocampus (Nizhnikov et al., 2014). KORs in the nucleus accumbens are mostly presynaptical and inhibit dopamine release. A reduction in KOR functioning after PEE may enhance the sensitivity of the dopaminergic system to drugs of abuse.

The present study analyzed: (a) baseline and ethanol-induced cathecolaminergic activity in VTA, and (b), baseline and ethanol-induced neural activity in nucleus accumbens core and shell (AcbC and AcbSh, respectively) and prelimbic (PrL) and IL prefrontal cortex. We studied the expression patterns of Fos-ir cells in AcSh and the colocalization of c-Fos and tyrosine hydroxylase (TH), as a marker of cathecolaminergic activity, in VTA after an ethanol injection.

The measurements of Fos-ir and Fos/TH-ir cells were conducted in adolescent female Wistar rats that had been exposed to moderate doses of ethanol (2.0 g/kg/day, i.g.) or only vehicle (i.e., 0.0 g/kg ethanol) during GDs 17–20 [ethanol- and vehicle-exposed (PEE and VE) groups, respectively], or that were left untreated throughout pregnancy [non-manipulated (NM) group]. On postnatal day (PD) 37 the animals were challenged with ethanol (1.25, 2.5 or 3.25 g/kg) or vehicle (i.e., 0.0 g/kg). The hypotheses were that PEE animals would exhibit greater baseline cathecolaminergic activity in VTA, and that they would be more responsive than VE or NM counterparts to the ethanol-induced neural activation at VTA, AcbC and AcbSh. Additionally, it was possible that PEE would decrease baseline and ethanol-induced neural activity at IL or PrL. Prior research indicates that ethanol pre-exposure decreases Fos-ir or Fos/TH-ir in the medial prefrontal cortex (Fabio et al., 2013; Boutros et al., 2014).

The rationale for focusing on the adolescent period is that ethanol initiation (DeWit et al., 2000) and the transition into problematic ethanol consumption (Windle and Zucker, 2010) mainly occur during this developmental stage. Moreover, rats consume more ethanol during adolescence as compared to other developmental stages (García-Burgos et al., 2009), and this difference is more pronounced in females than in males (Doremus et al., 2005). Age of ethanol initiation is critical to discriminate between those that will progress to ethanol abuse or dependence, from those that will keep controlled drinking. Subjects that began drinking before age 15, approximately, are at increased risk of problematic drinking (Pilatti et al., 2014), and this could be further exacerbated by PEE. Moreover, it has been shown that the risk of developing ethanol-related problems among college students that drink ethanol is higher in females than in males (Perkins, 2002; Fernández-Solá, 2007). According to our previous studies, the effects of the prenatal ethanol exposure here employed (i.e., 2.0 g/kg, GDs 17–20) on ethanol intake, ethanol-induced conditioned preference and functionality of the KOR system are similar in males and females (Pautassi et al., 2012; Fabio et al., 2013).

## EXPERIMENTAL PROCEDURES

### Materials and methods

*Subjects.* We employed 48 female adolescent Wistar rats, aged 37 days, which were derived from 12 litters (four PEE, four VE and four NM litters). The rationale for conducting the experiment at PD37 was that the postnatal development of dopamine D1 and D2 receptors peaks at around PD 35–40 (Tarazi and Baldessarini, 2000). This is followed by a gradual and significant elimination of receptors (often interpreted as pruning of ‘‘excessive’’ receptors) until adulthood, when stable levels are achieved. Our aim was, therefore, to assess baseline and ethanol-induced cathecolaminergic and neural activity during the peak of development of D1 and D2 dopaminergic receptors.

Animals were born and reared in a temperature-controlled vivarium at the Instituto de Investigaciones Médicas M. y M. Ferreyra (INIMEC-CONICET-UNC, Córdoba, Argentina). The colony was kept under a 12-h light/12-h dark cycle (lights onset at 0800). Female rats were time-mated to provide subjects for this study and were maintained in standard maternity cages with food and water *ad libitum*. Dams remained undisturbed until the beginning of prenatal treatment on GD17. Births were examined daily, and the day of parturition was considered PD0. Weaning was performed at PD21.

At PD28 animals from the same litter were housed in same-sex groups of 4. All experiments complied with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) and were approved by the Institutional Animal Care and Use Committee at INIMEC-CONICET. All efforts were made to minimize the number of animals used and their suffering

*Prenatal and postnatal ethanol treatment.* From GD17 to GD20 pregnant dams received one daily i.g. administration of 0.015 ml/g of a 16.8% v/v ethanol solution (Porta Hnos Co, Córdoba, Córdoba, Argentina; vehicle: tap water; ethanol dose: 2.0 g/kg; PEE Group) or a similar volume of vehicle (VE group), or remained untreated (NM group). At PD 37, the offspring of the PEE, VE and NM dams received an i.p. administration of vehicle (i.e., 0.0 g/kg) or a dose of 1.25, 2.5 or 3.25 g/kg of ethanol. These doses were the result of administering 0.01 ml/g of a 7, 14 or 18.2% v/v ethanol solution. The i.g. administration was performed by gently introducing into the oral cavity of the dam a piece of PE 50 polyethylene tubing, which in turn was connected to a 10-cc syringe. Intraperitoneal injections were executed between the diaphragm and the genitalia, approximately.

*Assessment of Fos-ir in AcSh, AcbC, PrL and IL; and Fos/TH-ir in VTA.* Ninety minutes after intubation with the different doses of ethanol or vehicle on PD37, the adolescents were anesthetized with i.p. injections of Chloral hydrate (dose: 0.001 ml/g of a 30% v/v solution) and perfused transcardially with 0.9% heparinized saline (10 U/ml) and 4% paraformaldehyde (PFA) in 0.1-M phosphate buffer (PB; pH 7.4). Brains were left overnight in the skull and subsequently removed and placed in 30% sucrose for at least 72 h. Frozen brains were then sectioned through a freezing microtome. Four series of 40-μm sections were obtained and placed in 0.1-M PB. Three series were stored at −20 °C with a conservative solution and employed in other studies. The fourth one, in turn, was immediately used for immunohistochemistry. Peroxidase reaction was blocked by incubating the brains for 60 min in a solution composed of 1% hydrogen peroxide, 10% methanol and 0.01 PB. Brains were then washed three times in PB and incubated in a blocking solution of 5% normal horse serum (NHS, Invitrogen, New Zealand) for 1 h. Afterward, the brain sections were first incubated free-floating overnight at room temperature and under continuous agitation, with a rabbit monoclonal antibody against the c-Fos protein (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:2000 in PB containing 0.3% Triton X-100 plus 1% of NHS. Brains were washed three times in 0.01 PB, incubated 120 min with biotinylated donkey anti-rabbit secondary antibody (Jackson Laboratories, West Grove, PA, USA) diluted 1:500 in 1% NHS, and washed again three times in PB. The sections were then incubated for 120 min with the avidin–biotin–peroxidase complex (ABC Elite Kit; Vector Labs, Burlingame, CA, USA) diluted in 1% NHS. Sections were subsequently incubated for 5 min with a solution containing 0.05% 3–3 = -diamino-benzidine tetra hydrochloride (DAB, Sigma Aldrich, St. Louis, MO, USA) and 0.01% hydrogen peroxidase + 0.5 ml de ClCo 0.5% + 0.5 ml de ClNi 0.5% in order to obtain a black c-Fos mark (please see Fig. 3). After revealing for c-Fos, we repeated the protocol using an antibody against TH (Millipore, Billerica, MA, USA) diluted 1:1000. The bodies of dopaminergic neurons of the mesocorticolimbic circuit are located in the VTA (Koob and Nestler, 1997). It is known that 60–75% of VTA neurons are dopaminergic, and the rest are, mostly, GABAergic (Barrot et al., 2012). Therefore, it can be assumed that TH staining in the present study represents dopaminergic neurons in VTA. To distinguish the TH mark from the c-Fos mark, we revealed the sections with a solution containing 0.05% 3–3 = -diamino-benzidine tetra hydrochloride (DAB, Sigma Aldrich, St. Louis, MO, USA) to obtain a brown staining. The sections were then mounted on a gelatinized slide, dehydrated and covered with DPX.

Three slices were selected per animal in each of the brain regions under analysis (see Fig. 1). Following the

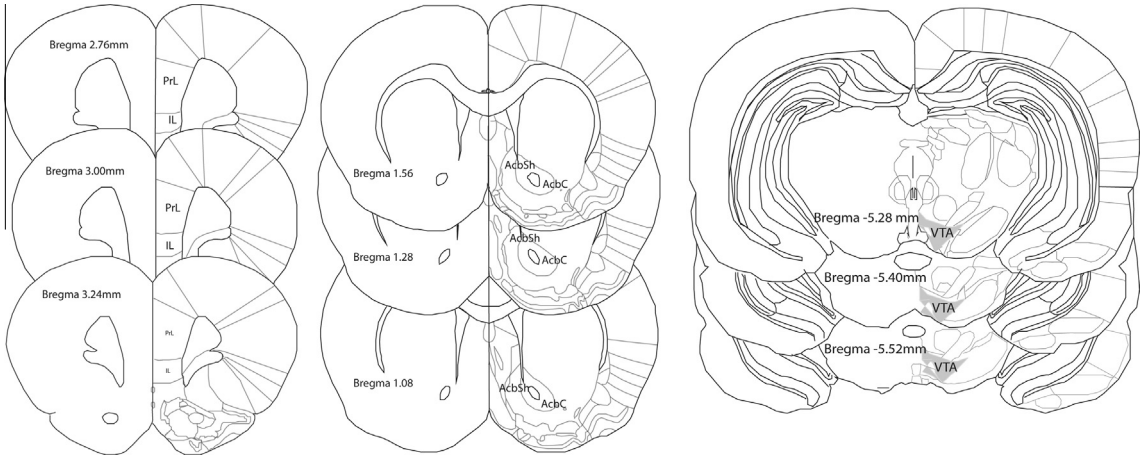


**Fig. 1.** Schematic diagram of brain sections analyzed in this study, based on Paxinos (2007). Figures represent antero-posterior levels to the bregma of selected areas. Localization of the considered section of ventral tegmental area (VTA), nucleus accumbens shell (AcbSh), nucleus accumbens core (AcbC), infralimbic cortex (IL) and prelimbic cortex (PrL) are indicated with the corresponding legend, and highlighted anatomically when necessary.

delineation of Paxinos (2007), slices for AcbSh and AcbC were taken from bregmas 1.68, 1.44 and 1.08 mm (i.e., plates 19, 21 and 24, respectively), and the slices for VTA were taken from bregmas −5.28, −5.40 and −5.52 mm (i.e., plates 77–79, respectively). PrL and IL were taken from bregmas 3.24, 3.00 and 2.76 mm, respectively (i.e., plates 10–12). A Primo Star iLed microscope, equipped with an Axicam ERc 5s Microscope camera (Zeiss, Jena, Germany), was used to acquire the photographs. The mean number of cells with activated nuclei in each structure was counted by means of the software FIJI Is Just Image J (Schindelin et al., 2012). In the VTA, a cell was considered to be Fos/TH-ir if double-labeled with both TH and c-Fos. In the remaining structures a cell was considered positive for c-Fos if expressing the typical black mark in the cell nuclei. Preliminary analysis indicated that the pattern of c-Fos and TH immunoreactivity (ir) was similar across the different plates measured. This is, analyses of variance (ANOVAs) that included Slice as a repeated measure factor did not yield significant main effects of Slice or significant Slice × Treatment (pre- or post-natal) interactions, across all structures. Data from the three sections, therefore, were averaged for the subsequent statistical analysis.

### Experimental designs and statistical analysis

A 3 [prenatal treatment at GDs 17–20 (PEE, VE or NM)] × 4 [postnatal treatment at PD37 (0.0, 1.25, 2.5 or 3.25 g/kg)] factorial design was used. Each of the 12 groups was composed by four subjects. Therefore, sample size was four for most of the groups and structures analyzed, although in a few cases – notably at AcbC – we were unable to find slices at the appropriate bregma and only three samples per group were available. Potential baseline differences between prenatal treatments, in Fos/TH-ir activation at VTA and Fos-ir expression at AcbSh, AcbC, PrL and IL were analyzed via one-way ANOVAs (comparative factor between groups: Prenatal treatment). These analyses, which considered the mean number of immunoreactive cells as the dependent variable, included only animals from the postnatal control groups (i.e., administered 0.0 g/kg ethanol).

To analyze ethanol-induced Fos/TH-ir activity at VTA, and ethanol-induced Fos-ir at AcbC, AcbSh, PrL and IL we conducted separate factorial ANOVAs [between factors: Prenatal treatment (PEE, VE or NM) and Postnatal ethanol treatments (1.25, 2.5, 3.25 g/kg)]. The dependent variable was, following previous studies (e.g., Larson et al., 2010), the relative change of neural activity, as compared with the specific control (i.e., the vehicle-treated group) of each prenatal treatment. In other words, results are expressed as percentage of saline. This measure, which is commonly used in studies that assesses drug- or stress-induced neural activation (Harbuz and Jessop, 1999; Caster and Kuhn, 2009), shows relative increases in c-Fos induced by ethanol and helps account for differential levels of response in the basic, control condition. This relative change was calculated as follows: (number of positive cells in a given

**Table 1.** Mean number of cells exhibiting C-Fos immunoreactivity in ventral tegmental area (VTA), nucleus accumbens core (AcbC), nucleus accumbens shell (AcbSh), prelimbic (PrL) or infralimbic (IL) areas; and mean number of cells exhibiting tyrosine hydroxylase (TH) immunoreactivity or colocalization of C-Fos and tyrosine hydroxylase (Fos/TH-ir) in VTA. The measurements were conducted in adolescent rats that had been exposed to 2.0 g/kg ethanol (prenatal ethanol-exposed, PEE) or vehicle (i.e., 0.0 g/kg; prenatal vehicle-exposed, VE) during gestational days 17–20, or that were untreated throughout pregnancy (prenatal non-manipulated, NM). On postnatal day 37, the adolescents were challenged with 0.0, 1.25, 2.5 or 3.25 g/kg of ethanol (i.p.) 90 min before brains extraction. Data are expressed as mean ± standard error of the mean.

| Prenatal treatment | Prenatal non-manipulated, NM group | | | | Prenatal vehicle-exposed, VE group | | | | Prenatal ethanol-exposed, PEE Group | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0.0 g/kg | 1.25 g/kg | 2.5 g/kg | 3.25 g/kg | 0.0 g/kg | 1.25 g/kg | 2.5 g/kg | 3.25 g/kg | 0.0 g/kg | 1.25 g/kg | 2.5 g/kg | 3.25 g/kg |
| VTA, C-Fos | 101.92 ± 21.45 | 107.00 ± 18.03 | 44.78 ± 12.40 | 57.42 ± 18.11 | 25.25 ± 9.03 | 96.17 ± 17.35 | 33.83 ± 9.86 | 62.08 ± 12.29 | 61.00 ± 15.20 | 95.33 ± 39.87 | 103.25 ± 3.25 | 71.83 ± 15.38 |
| VTA, TH | 150.92 ± 16.48 | 136.50 ± 17.68 | 101.67 ± 23.24 | 117.67 ± 13.20 | 96.08 ± 4.88 | 156.00 ± 9.30 | 87.25 ± 13.24 | 106.00 ± 8.35 | 116.33 ± 8.96 | 145.58 ± 28.94 | 139.75 ± 10.23 | 105.58 ± 11.33 |
| VTA, Fos/TH-ir | 102.08 ± 21.33 | 114.33 ± 13.40 | 45.00 ± 12.55 | 57.75 ± 18.40 | 24.92 ± 9.22 | 91.33 ± 17.04 | 33.00 ± 10.07 | 60.42 ± 14.06 | 60.58 ± 15.76 | 95.33 ± 39.87 | 96.83 ± 4.12 | 71.58 ± 15.21 |
| AcbC, C-Fos | 3.67 ± 1.35 | 9.41 ± 1.84 | 5.56 ± 2.31 | 7.67 ± 0.51 | 6.83 ± 3.18 | 7.92 ± 1.82 | 7.22 ± 1.47 | 10.75 ± 3.79 | 11.00 ± 3.68 | 6.50 ± 1.32 | 5.75 ± 1.13 | 5.91 ± 1.64 |
| AcbSh, C-Fos | 16.58 ± 9.87 | 57.89 ± 10.66 | 18.75 ± 4.66 | 25.83 ± 4.40 | 24.25 ± 5.42 | 30.75 ± 2.84 | 38.25 ± 7.11 | 41.08 ± 6.01 | 19.17 ± 6.86 | 27.83 ± 5.11 | 37.33 ± 9.72 | 24.25 ± 5.80 |
| IL, C-Fos | 22.00 ± 12.52 | 48.50 ± 15.55 | 32.83 ± 6.19 | 17.67 ± 2.69 | 29.25 ± 7.95 | 36.75 ± 7.48 | 31.92 ± 6.53 | 28.08 ± 6.27 | 24.42 ± 8.90 | 31.83 ± 6.89 | 31.42 ± 11.35 | 24.25 ± 4.85 |
| PrL, C-Fos | 15.83 ± 5.20 | 55.33 ± 3.42 | 16.50 ± 1.94 | 15.96 ± 2.51 | 13.33 ± 3.30 | 25.92 ± 1.02 | 17.92 ± 3.58 | 22.42 ± 5.41 | 14.33 ± 2.91 | 29.92 ± 5.63 | 26.25 ± 8.68 | 27.67 ± 4.94 |

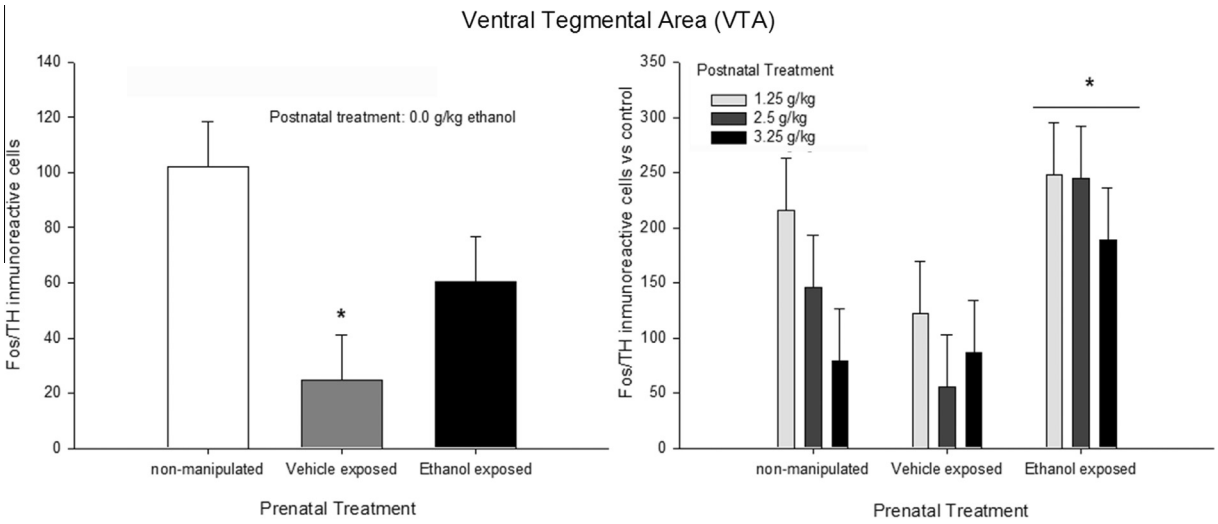


**Fig. 2.** Baseline (left panel) and ethanol-induced (right panel) Fos/TH immunoreactivity in the ventral tegmental area (VTA) of adolescent rats that had been exposed to 2.0 g/kg of ethanol (prenatal ethanol-exposed), vehicle (i.e., 0.0 g/kg; prenatal vehicle exposed) or were left undisturbed (prenatal non-manipulated) during gestational days 17–20. On postnatal day 37 the animals were challenged with ethanol (1.25, 2.5 or 3.25 g/kg ethanol dose, i.p.) or vehicle (i.e., 0.0 g/kg). The left panel depicts the number of Fos/TH immunoreactive cells of animals given vehicle (0.0 g/kg) 90 min before brains extraction (control group). A significant main effect of Prenatal treatment was revealed by the ANOVA, and post hocs indicated a significant difference between prenatal non-manipulated and prenatal vehicle-exposed groups (shown by the asterisk). The right panel depicts Fos/TH immunoreactive cells of adolescents challenged with 1.25, 2.5 or 3.25 g/kg of ethanol 90 min before brains extraction, expressed as the relative change (%) of Fos/TH immunoreactivity, compared with the specific vehicle-treated control (i.e., 0.0 g/kg group) of each prenatal treatment. The ANOVA indicated a main significant effect of prenatal treatment, with prenatal ethanol-exposed animals exhibiting greater double staining than the other groups (indicated by the horizontal line and the asterisk).Vertical bars indicate the standard error of the means.

ethanol-treated group * 100)/mean of the control group from the same prenatal treatment. The raw, untransformed data, for each structure analyzed can be found in Table 1.

To confirm that a given group was exhibiting ethanol induced Fos/TH-ir or Fos-ir activation (or depression), we conducted a *t*-test for single means against a user-defined constant. The constant was 100%, which represents the score of the control group. This test indicated if, in a given group challenged with ethanol postnatally, scores for Fos-ir or Fos/TH-ir differed from the control group treated with only vehicle at the challenge. We also calculated the total number of TH-containing neurons and the percentage of these neurons that showed Fos-ir. These variables were analyzed through separate factorial ANOVAs that included Prenatal treatment and Postnatal treatment as between factors.

The loci of statistical significant main effects or interactions were further analyzed using Fisher's post hoc test. Across analyses, alpha level was kept at $p < .05$

## RESULTS

### Baseline differences as a function of prenatal treatments

The ANOVAs for baseline prenatal differences in the number of Fos/TH-ir cells indicated a significant effect of Prenatal treatment [$F_{(2,\ 9)} = 5.68$, $p < .05$]. The post hoc analyses indicated that, compared with the NM group, VE animals exhibited significantly less basal cathecolaminergic activation. These results suggest that the mere prenatal manipulation (i.e., the procedures associated with the intragastric administration) during GD 17–20 reduced basal dopaminergic tone in VTA; an effect that seems to be inhibited – at least partially- by prenatal ethanol. This result has been depicted in the left panel of Fig. 2. Microphotographs illustrating this result can be observed in the left panel of Fig. 3.

The total number of neurons at the VTA that expressed TH, regardless that they co-expressed or not c-Fos, was significantly greater in adolescents challenged with 1.25 g/kg ethanol, and this effect was similar across prenatal treatments [significant main effect of Postnatal treatment; $F_{(3,\ 35)} = 3.99$, $p < .05$]. The percentage of TH-containing neurons expressing Fos-ir in VTA was not affected by the pre or postnatal treatments. The ANOVA indicated lack of significant main effects or significant interactions. Mean ± SEM (%) in groups given 0.0, 1.25, 2.5 and 3.25 g/kg ethanol postnatally were as follows: 65.70 ± 7.68, 85.62 ± 7.88, 42.82 ± 3.26 and 42.97 ± 10.81 (NM group); 27.64 ± 11.26, 57.54 ± 8.18, 38.85 ± 9.80 and 55.90 ± 10.53 (VE group); and 50.46 ± 12.79, 50.03 ± 16.32, 70.95 ± 8.16 and 65.50 ± 7.44 (PEE group).

The ANOVAs for baseline Fos-ir in AcbSh, AcbC, PrL and IL indicated a similar number of positive neurons across control groups from NM, VE and PEE conditions. Mean ± SEM for control groups in NM, VE and PEE conditions can be observed in the left panels of Figs. 4, 6, 8 and 9 (AcbSh, AcbC, IL, PrL and IL, respectively).

### Ethanol-induced acute neural and dopaminergic activity as a function of prenatal treatments

Ethanol-induced dopaminergic activity in VTA was greater in PEE than in VE or NM animals [significant main effect of Prenatal treatment: $F_{(2,\ 27)} = 6.54$, $p < .01$; post hoc

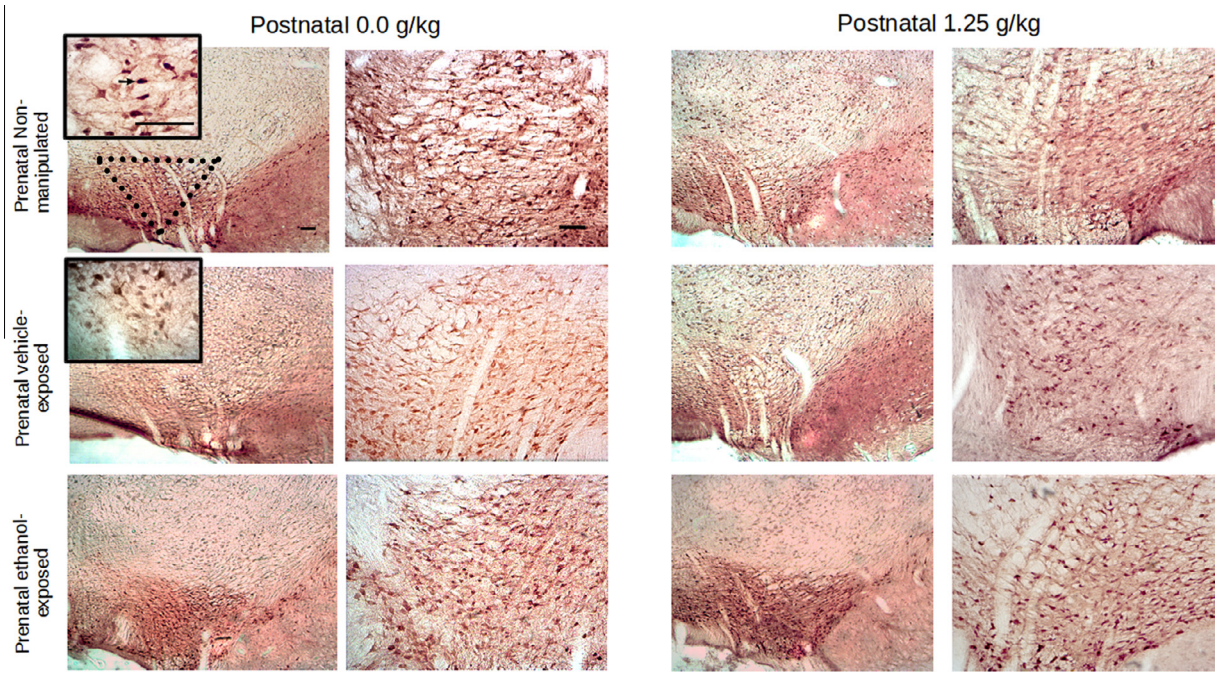


**Fig. 3.** Microphotographs illustrating Fos/TH immunoreactivity in ventral tegmental area (VTA) of adolescent rats that had been exposed to 2.0 g/kg ethanol (prenatal ethanol-exposed) or vehicle (i.e., 0.0 g/kg; prenatal vehicle-exposed) during gestational days 17–20), or that were untreated throughout pregnancy (prenatal non-manipulated). On postnatal day 37 the animals were challenged with 0.0, 1.25, 2.5 or 3.25 g/kg of ethanol (i.p.), 90 min before brain extraction. In this representative figure only results from animals postnatally treated with 0.0 or 1.25 g/kg ethanol are illustrated. Microphotographs taken at 40× (squared-inset), 4× (left columns and 10× (right columns) resolution are shown. The horizontal size bar represents 100 μm. The region quantified has been highlighted through the dashed lines.

tests < .05] (see Fig. 2, right panel). The facilitative effect of PEE on ethanol-induced Fos/TH-ir seemed to be fairly similar across all ethanol doses; the ANOVA indicated that the Prenatal treatment × Ethanol dose interaction was not significant. This ANOVA indicated significant differences between PEE and VE or NM groups, but did not indicate if these groups exhibit, regardless of their differences, a significant dopaminergic activation (or

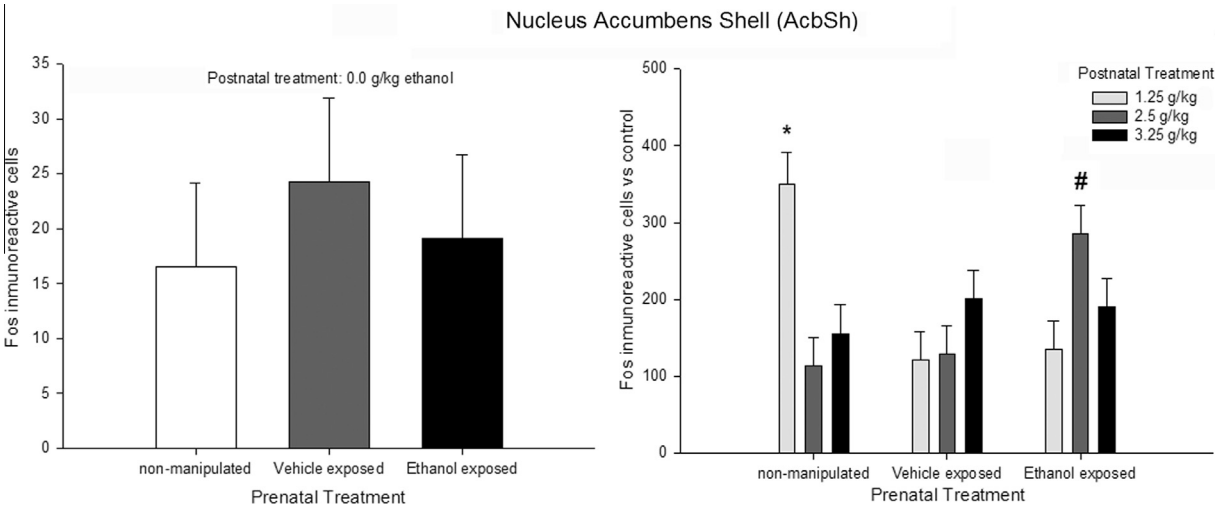


**Fig. 4.** Baseline (left panel) and ethanol-induced (right panel) Fos immunoreactivity in nucleus accumbens shell (AcbSh) of adolescent rats that had been exposed to 2.0 g/kg ethanol (prenatal ethanol-exposed) or vehicle (i.e., 0.0 g/kg; prenatal vehicle-exposed) during gestational days 17–20), or that were untreated throughout pregnancy (prenatal non-manipulated). On postnatal day 37 the animals were treated with ethanol (1.25, 2.5 or 3.25 g/kg) or vehicle (i.e., 0.0 g/kg). The left panel depicts the number of Fos immunoreactive cells of animals given vehicle (0.0 g/kg) 90 min before brains extraction (control group). The ANOVA indicated no significant differences in baseline Fos immunoreactivity. The right panel depicts Fos immunoreactive cells of adolescents challenged with 1.25, 2.5 or 3.25 g/kg of ethanol (i.p.) 90 min before brains extraction, expressed as the relative change (%) of Fos immunoreactivity, compared with the specific vehicle-treated control (i.e., 0.0 g/kg group) of each prenatal treatment. The ANOVA and subsequent post hoc tests indicated that 1.25 g/kg ethanol induced greater c-Fos activity in prenatal non-manipulated than in the prenatal vehicle or prenatal ethanol exposed animals. The 2.5-g/kg ethanol induced significantly greater activation in prenatal ethanol exposed than in prenatal non-manipulated or prenatal vehicle controls. These effects are indicated by the asterisk and the pound sign, respectively. Vertical bars indicate the standard error of the means.

depression) against their own control group. To confirm this, *t* tests against the theoretical value of 100% were conducted, one for each prenatal treatment. The *t* tests indicated significant dopaminergic activation in PEE animals [$t_{(11)} = 3.72$, $p < .05$], but no significant effect in VE or NM groups. Fig. 2 (right panel) depicts mean and SEM number of Fos/TH-ir-positive neurons as a function of pre- and post-natal conditions. Fig. 3, in turn, illustrates these results via representative microphotographies of NM, VE and PEE animals treated with the 1.25-g/kg ethanol dose.

The ANOVA for ethanol-induced Fos-ir in AcbSh showed a significant interaction between Prenatal and Postnatal treatment [$F_{(4,\ 26)} = 7.56$, $p < .001$] (see Fig. 4, right panel). Post hoc tests indicated that, among adolescents given postnatal administration of 1.25 g/kg ethanol, NM animals showed significantly greater Fos-ir than either PEE or VE groups. The post hoc tests also revealed that PEE animals challenged with 2.5 g/kg exhibit heightened Fos-ir, compared to VE and NM counterparts. Ethanol-induced Fos-ir in AcbSh did not significantly differ across prenatal treatments. Both *t* tests for the NM-1.25 g/kg and the PEE-2.5 g/kg groups neared significance (both, $t = 0.06$). Fig. 5 present microphotographs illustrating this pattern of results.

The ANOVA for ethanol-induced Fos-ir in AcbC revealed a significant main effect of Prenatal treatment [$F_{(2,\ 24)} = 6.58$, $p < .01$]. The post-hocs revealed, as shown in Fig. 6 (right panel), significantly lower Fos-ir in PEE than in NM counterparts. The *t* tests against the constant of 100% (one for each prenatal treatment), indicated a significant depression of neural activity in PEE animals [$t_{(11)} = -2.70$, $p < .05$], but neither neural activation or depression in VE or NM groups. Fig. 7 present microphotographs illustrating the results.

Ethanol-induced Fos-IR at IL was not affected by pre- or post-natal treatments. The ANOVA yielded no significant main effects or significant interactions (Fig. 8, right panel).

The ANOVA for ethanol-induced Fos-ir at PrL revealed significant main effects of Prenatal treatment [$F_{(2,\ 26)} = 14.09$, $p < .001$] and Postnatal treatment

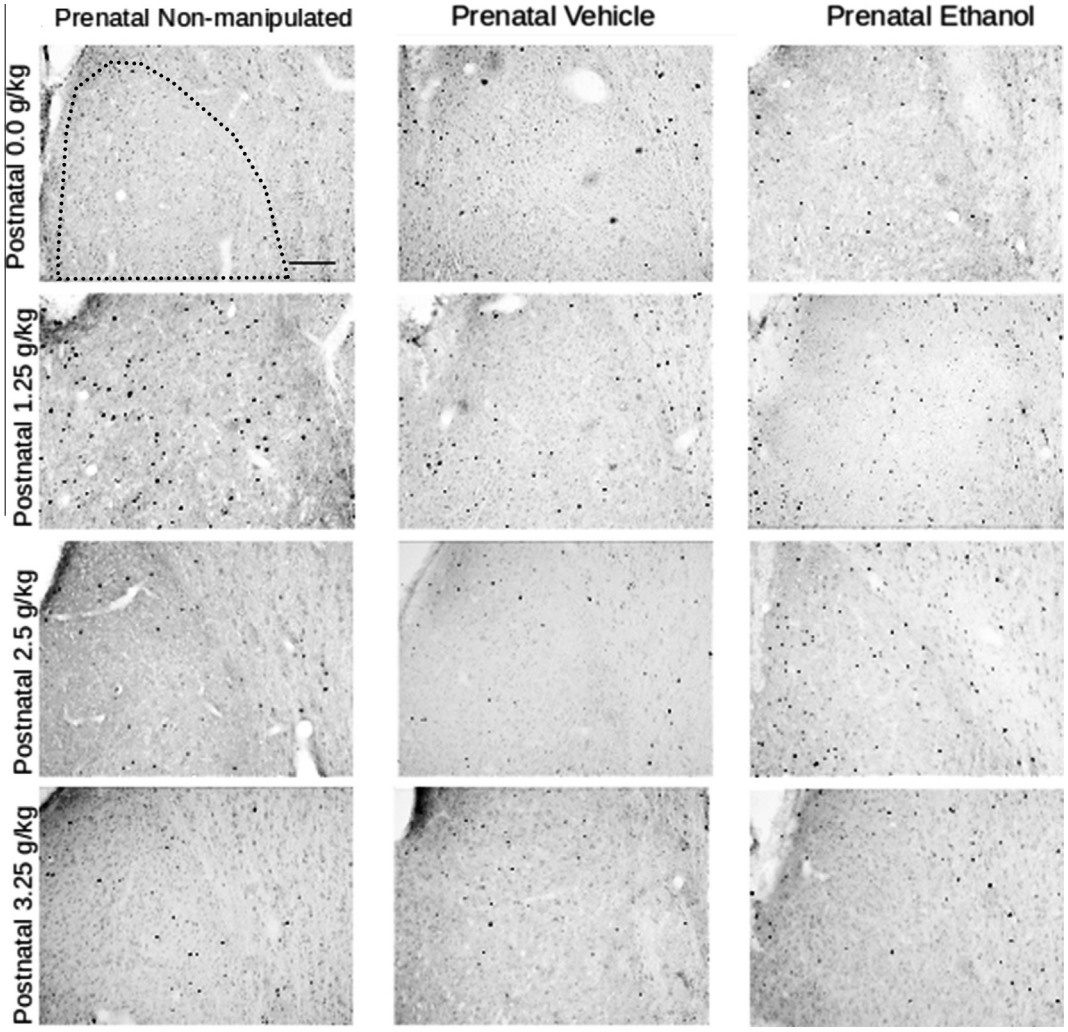


**Fig. 5.** Microphotographs illustrating neurons exhibiting Fos-immunoreactivity in the nucleus accumbens shell (AcbSh) of adolescent rats that had been exposed to 2.0 g/kg of ethanol (prenatal ethanol-exposed), vehicle (prenatal vehicle exposed) or were left undisturbed (prenatal non-manipulated) during gestational days 17–20. On postnatal day 37 the adolescents were challenged with 0.0, 1.25, 2.5 or 3.25 g/kg of ethanol (i.p.) 90 min before brain extraction. The horizontal size bar represents 100 μm. The region quantified has been highlighted through the dashed lines.

[$F_{(2,\ 26)}$ = 16.84, $p$ < .001]. The Prenatal × Postnatal treatment interaction also achieved significance [$F_{(4,\ 26)}$ = 10.28, $p$ < .001]. The post hoc tests indicated significantly greater Fos-ir in NM than in PEE or VE adolescents, following the 1.25-g/kg ethanol dose [$F_{(2,\ 8)}$ = 39.83, $p$ < .05]. No differences between these treatments were observed after 1.25 or 3.25 g/kg. The *t*-test confirmed that the NM-125 g/kg group exhibited significant ethanol-induced Fos-ir activation. These results are depicted in Fig. 9 (right panel) and Fig. 10.

## DISCUSSION

The present study was motivated by the need to understand the mechanisms underlying the greater ethanol intake, found in adolescents exposed to ethanol *in utero* (Fabio et al., 2013). We measured ethanol-induced Fos-ir in AcbC, AcbSh, PrL and IL after several ethanol doses and conducted a double staining for c-Fos and TH in VTA. The aim was to analyze potential differences in neural and dopaminergic activity between prenatal treatments defined by exposure to ethanol or vehicle during late gestation, or by lack of any explicit prenatal treatment. The main results were that prenatal ethanol heightened ethanol-induced dopaminergic activation in VTA, when compared to VE or NM counterparts; and changed the pattern of ethanol-induced c-Fos activation in AcbSh and AcbC. These are key areas in the mediation of ethanol's motivational effects (McBride et al., 1999).

The magnitude of the heightened ethanol-induced cathecolaminergic activity in VTA, observed in PEE animals, was similar across all ethanol doses. At AcbSh, in turn, PEE adolescent animals required, when compared to NM controls, a larger dose of ethanol (i.e., 3.25 vs. 1.25 g/kg) to show increased neural activity in this area. These results are important because they meet the hypothesis that PEE alters the sensitivity to the reinforcing effects of ethanol. Nizhnikov et al. (2006) assessed ethanol-induced attachment to an artificial nipple shortly after birth and found that PEE (1.0 g/kg, on GDs 17–20) increased the range of ethanol doses that the rat pups found reinforcing.

It is possible that PEE resulted in the development of tolerance to the drug. Blanchard et al. (1993) found heightened dopamine release in control rats after 0.5 g/kg ethanol, but not after 1.0 g/kg ethanol; whereas rats exposed to ethanol *in utero* exhibited ethanol-induced dopamine release only after 1.0 g/kg ethanol. A similar result, yet regarding a behavioral manifestation of ethanol's effects, was observed by Pautassi et al. (2012). In that study, animals exposed to ethanol during late gestation (2.0 g/kg on DGs 17–20) showed conditioned place preference for 1.0 and 2.0 g/kg doses of ethanol, whereas control subjects, untreated during gestation showed conditioned place preference for a lower ethanol dose (i.e., 0.5 g/kg) but not for the higher doses. Moreover, several studies have found that chronic ethanol exposure desensitizes c-Fos-induced responses (Vilpoux et al., 2009). Chang et al. (1995) observed robust ethanol-induced Fos-ir in paraventricular hypothalamic nucleus and in the central amygdala of rats given acute exposure to 3.0 g/kg i.p., yet this was inhibited in animals pre-exposed to ethanol. Other studies (Ryabinin et al., 1997) gave rats two weeks of ethanol exposure and still found Fos-ir in several brain areas (e.g., orbital and insular cortices, bed nucleus of the stria terminalis, among

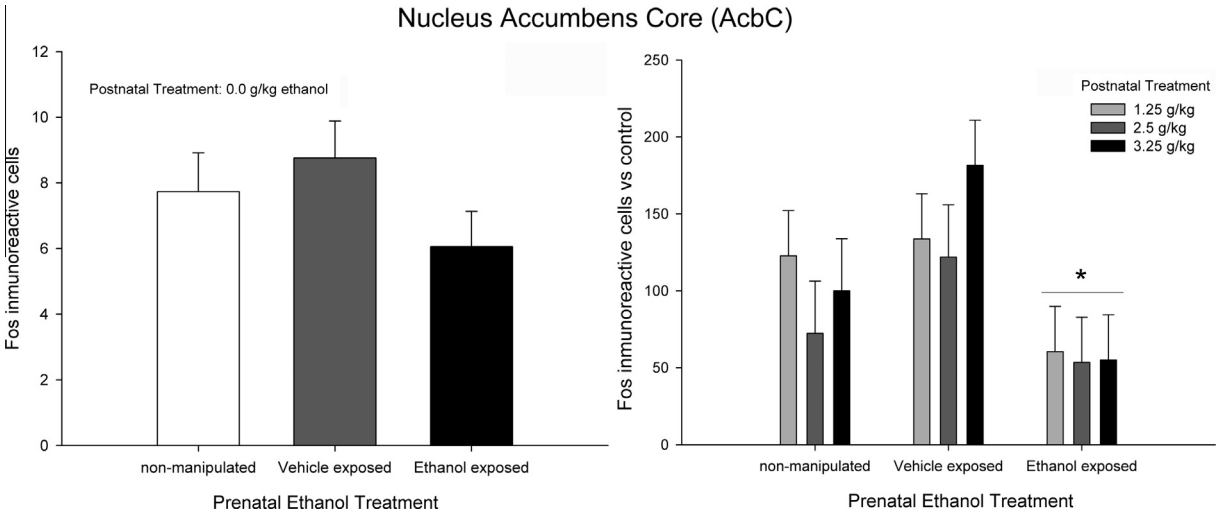


**Fig. 6.** Baseline (left panel) and ethanol-induced (right panel) Fos immunoreactivity in nucleus accumbens core (AcbC) of adolescent rats that had been exposed to 2.0 g/kg ethanol (prenatal ethanol-exposed) or vehicle (i.e., 0.0 g/kg; prenatal vehicle-exposed) during gestational days 17–20), or that were untreated throughout pregnancy (prenatal non-manipulated). On postnatal day 37 the animals were treated with ethanol (1.25, 2.5 or 3.25 g/kg) or vehicle (i.e., 0.0 g/kg). The left panel depicts the number of Fos immunoreactive cells of animals given vehicle (0.0 g/kg) 90 min before brain extraction (control group). The ANOVA indicated no significant differences in baseline Fos immunoreactivity. The right panel depicts Fos immunoreactive cells of adolescents challenged with 1.25, 2.5 or 3.25 g/kg of ethanol (i.p.) 90 min before brains extraction, expressed as the relative change (%) of Fos immunoreactivity, compared with the specific vehicle-treated control (i.e., 0.0 g/kg group) of each prenatal treatment. The ANOVA and subsequent post hoc tests indicated that prenatal ethanol-exposed animals showed significantly less Fos immunoreactivity induced by the postnatal ethanol challenge, than animals non-manipulated or exposed to vehicle during pregnancy (as indicated by the horizontal bar and asterisk). Vertical bars indicate the standard error of the means.

others), yet the magnitude of the response was substantially diminished when compared to animals given the drug for the first time. Similarly, in the present study postnatal ethanol administration induced significant Fos-ir at one the pre-frontal structures measured (i.e., PrL), yet this neural response was not observed in PEE or VE subjects. When results from prior work and from the present study are taken together, it can be concluded that a PEE-induced, desensitization of acute responsivity to ethanol is a persistent phenomenon that can be observed at multiple levels of measurement, in neural activity measures as well as in behavioral manifestation of ethanol-induced reward.

It is noteworthy that, in the present work, the effects of gestational ethanol exposure on the ethanol-induced Fos-ir at nucleus accumbens were region-dependent. Specifically, unlike the enhanced response to the drug found in AcbSh, PEE adolescents exhibited ethanol-induced neural depression in the AcbC. This finding is consistent with previous studies indicating that core and shell process different aspects of reward (e.g., Ito et al., 2004; Peciña et al., 2006). AcbSh (which projects mainly to limbic regions such as the ventral part of the bed nucleus of the stria terminalis and VTA, Zahm and Heimer, 1993) seems to be particularly important for the initial hedonic assessment of natural rewards or of drugs of abuse, and for providing motivational incentive to stimuli that accompany the drug's post-absorptive effects (Di Chiara et al., 2004). Pharmacological inhibition of rostral AcSh (i.e., Bregma 2.08–1.07 mm, we analyzed Bregmas 1.56–1.08 mm) altered place preference induced by food ingestion and the palatability of natural gustatory reinforcers (Reynolds and Berridge, 2003). The AcbC, on the other hand, mainly projects to structures associated in motor responses (e.g., substantia nigra; Zahm and Heimer, 1993). It is thus not surprising

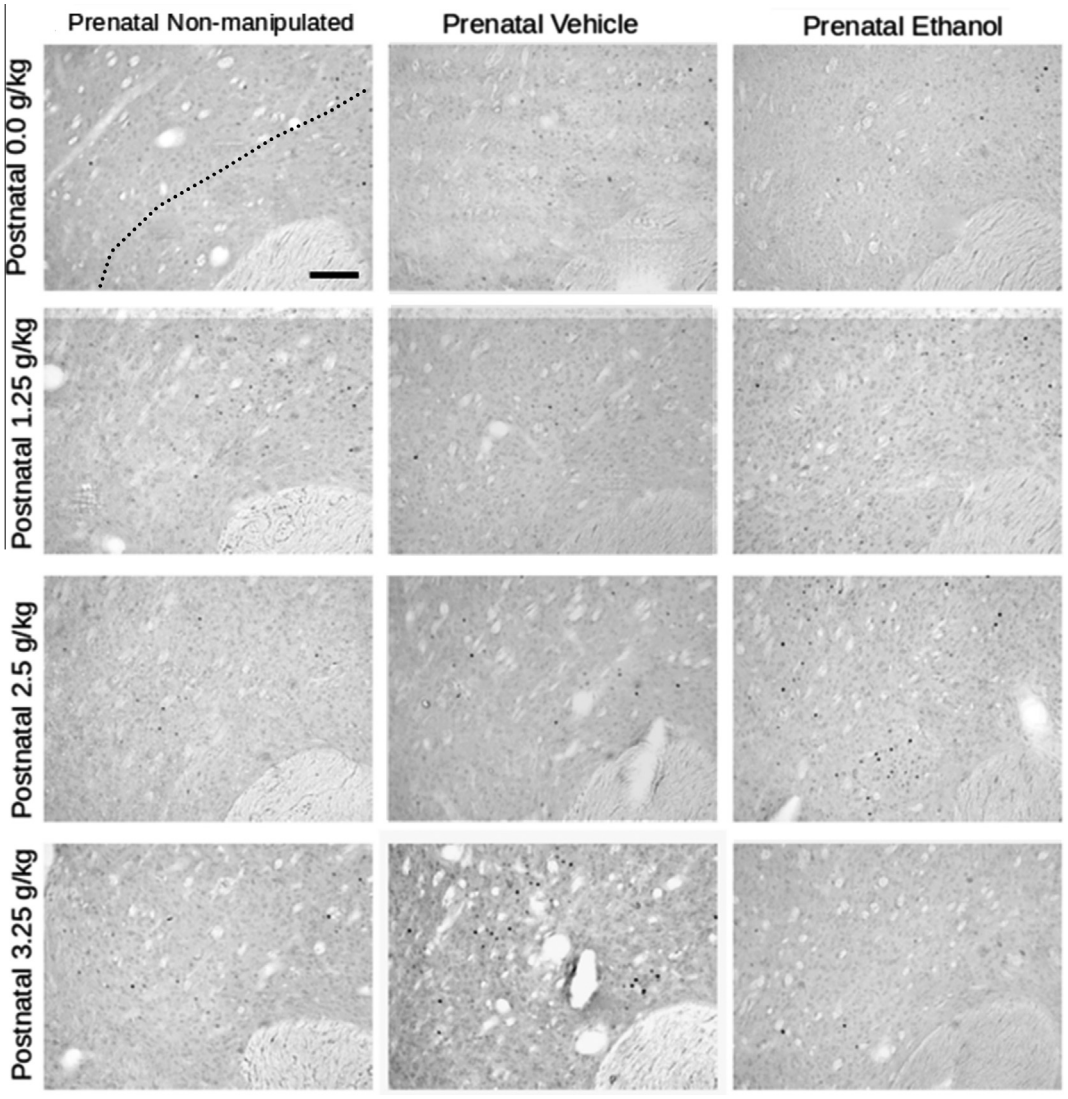


**Fig. 7.** Microphotographs illustrating neurons exhibiting Fos-immunoreactivity in nucleus accumbens core (AcbC) of adolescent animals that had been exposed to 2.0 g/kg ethanol (prenatal ethanol-exposed) or vehicle (i.e., 0.0 g/kg; prenatal vehicle-exposed) during gestational days 17–20), or that were untreated throughout pregnancy (prenatal non-manipulated). On postnatal day 37, the adolescents were challenged with 0.0, 1.25, 2.5 or 3.25 g/kg of ethanol (i.p.) 90 min before brains extraction. The horizontal size bar represents 100 μm. The region quantified has been highlighted through the dashed lines.

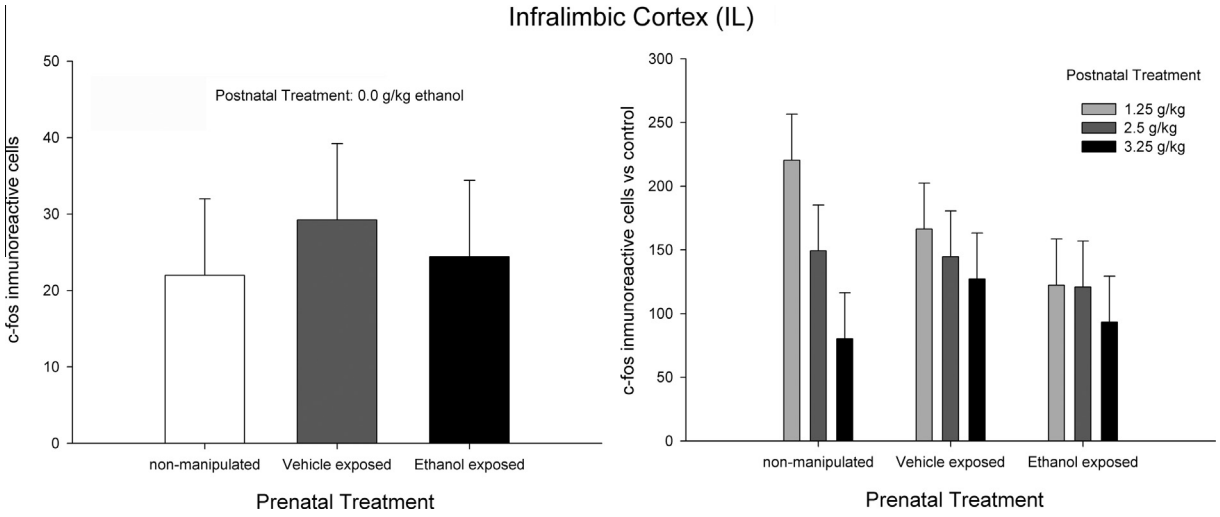


**Fig. 8.** Baseline (left panel) and ethanol-induced (right panel) Fos immunoreactivity in the infralimbic cortex (IL) of adolescent rats that had been exposed to 2.0 g/kg ethanol (prenatal ethanol-exposed) or vehicle (i.e., 0.0 g/kg; prenatal vehicle-exposed) during gestational days 17–20), or that were untreated throughout pregnancy (prenatal non-manipulated). On postnatal day 37 the animals were treated with ethanol (1.25, 2.5 or 3.25 g/kg) or vehicle (i.e., 0.0 g/kg). In the left panel, data from animals that were given vehicle (0.0 g/kg) 90 min before brains extraction (control group) is depicted. The right panel depicts Fos-immunoreactive cells of adolescents challenged with 1.25, 2.5 or 3.25 g/kg of ethanol (i.p.) 90 min before brains extraction, expressed as the relative change (%) of Fos immunoreactivity, compared with the specific vehicle-treated control (i.e., 0.0 g/kg group) of each prenatal treatment. The ANOVAs indicated that Fos immunoreactivity was not affected by pre- or post-natal ethanol treatment. Vertical bars indicate the standard error of the means.

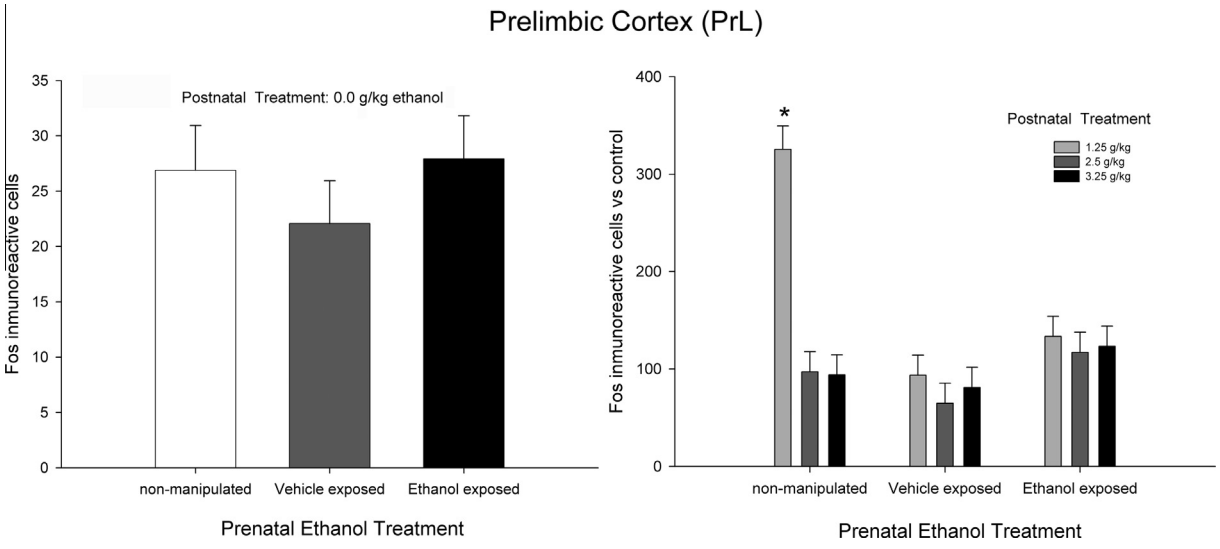


**Fig. 9.** Baseline (left panel) and ethanol-induced (right panel) Fos immunoreactivity in prelimbic cortex (PrL) from adolescent female rats that had been exposed to 2.0 g/kg ethanol (prenatal ethanol-exposed) or vehicle (i.e., 0.0 g/kg; prenatal vehicle-exposed) during gestational days 17–20), or that were untreated throughout pregnancy (prenatal non-manipulated). On postnatal day the animals were treated with ethanol (1.25, 2.5 or 3.25 g/kg) or vehicle (i.e., 0.0 g/kg). In the left panel, data from animals that were given vehicle (0.0 g/kg) 90 min before brains extraction (control group) is depicted. The ANOVA indicated no significant differences in baseline Fos immunoreactivity. In the right panel, values from adolescents challenged with 1.25, 2.5 or 3.25 g/kg of ethanol (i.p.) 90 min before brains extraction are expressed as the relative change (%) of Fos immunoreactivity (Fos-ir), compared with the specific control (i.e., 0.0 g/kg) of each prenatal treatment are shown. The ANOVA indicated that, during the challenge, the ethanol dose of 1.25 g/kg induced greater Fos-ir than the remaining doses, in prenatal non-manipulated animals only. The asterisk indicates this significant effect.

the greater involvement of AcbC in motor aspects of reward (Malenka et al., 2009), such as the sensitized motor response that occurs after intermittent administration of ethanol in mice (Faria et al., 2008). An intriguing work, that supports this notion of functional differences within the nucleus accumbens, measured dopamine release after an audio-visual cue that had been paired with sucrose (Cacciapaglia et al., 2012). The researchers found a significantly larger dopamine release in the AcbSh than in the AcbC.

The possibility of PEE inducing tolerance to the postnatal effects of the drug seems to clash with the overall heightened dopaminergic activity found in the VTA of PEE animals. Moreover, reduced baseline level

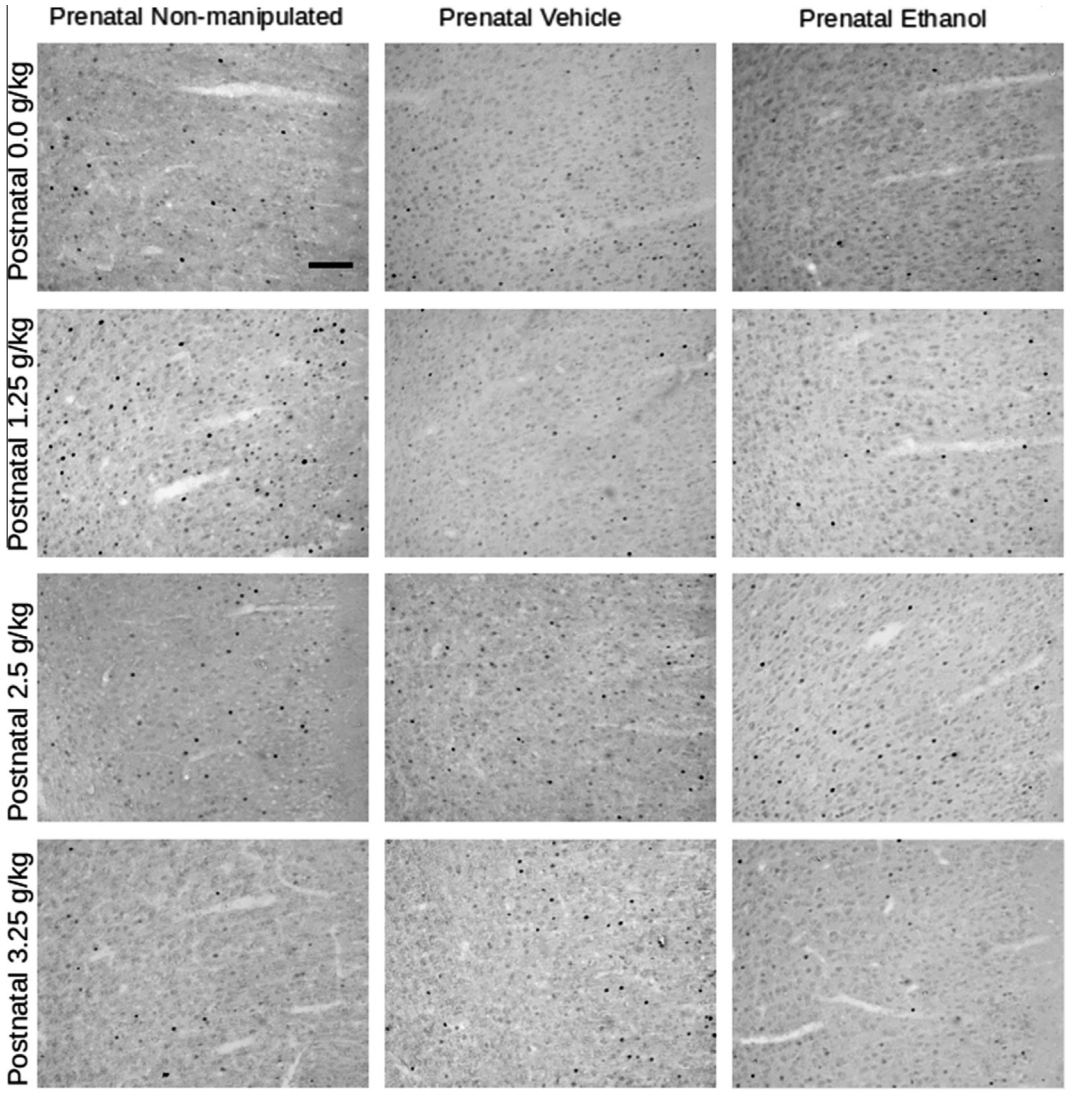


**Fig. 10.** Microphotographs illustrating neurons exhibiting Fos-immunoreactivity in prelimbic cortex (PrL) of adolescent female animals that had been exposed to 2.0 g/kg of ethanol (prenatal ethanol-exposed) or vehicle (prenatal vehicle-exposed) during gestational days 17–20, or that remained untreated throughout pregnancy (prenatal non-manipulated). On postnatal day 37, the adolescents were challenged with 0.0, 1.25, 2.5 or 3.25 g/kg of ethanol (i.p.) 90 min before brains extraction. The horizontal size bar represents 100 μm. The whole region represented in each panel was quantified for Fos-immunoreactivity.

of dopaminergic activity has been reported in other prenatal ethanol exposure models (Rubio et al., 1996; Choong and Shen, 2004; Carneiro et al., 2005). Our work and those studies exhibit significant differences in the magnitude of ethanol dosing, in the length of the prenatal treatment, and perhaps more important in the methods and area of analysis for dopaminergic activity. For instance, Carneiro et al. (2005) found decreased dopaminergic binding in the hippocampus and striatum in the offspring of dams given daily administration of ethanol 30 days before mating and throughout gestation and breastfeeding. It also has to be considered that tolerance can develop for some, but not for all effects of ethanol (Hunt et al., 1993). The present results can be framed within a model that postulates that the level of VTA dopaminergic activity in ethanol-experienced subjects is context dependent (Leyton and Vezina, 2014). According to this model, ethanol-preexposed animals may exhibit dopamine activity similar to that of controls or even dopaminergic hipoactivation. Yet, these animals would exhibit heightened dopaminergic activity when re-exposed to the drug or to ethanol-related cues.

What are the mechanisms by which PEE changes later ethanol sensitivity? It has been found that PEE reverses the valence of KOR activation from aversive to appetitive and alters the effects of kappa antagonism on ethanol drinking (Nizhnikov et al., 2014). The protein Δ-Fos-B, the truncated form of c-Fos, accumulates in median spiny neurons of the nucleus accumbens and decreases the expression of dynorphin (the endogenous ligand of KORs), thus increasing the functionality of the dopaminergic system and the motivational impact of drug rewards (Zachariou et al., 2006). It can be speculated that PEE causes the accumulation of delta-Fos B in the

nucleus accumbens, which in turn alters the kappa system and ultimately results in greater ethanol-induced neural activity, reward and intake. This is, of course just a hypothesis, and more work should be devoted to scrutinize the mechanisms underlying PEE effects.

Unlike previous studies (Fabio et al., 2013), we added an untreated dam control (i.e., NM group), along with animals born to dams given vehicle during late gestation (i.e., VE group). The inclusion of these groups is critical to parse out if the effects of PEE are specifically due to ethanol's pharmacological effects or merely a side-effect of the stress induced by the manipulations required to administer the drug. This turned out to add significantly to our understanding of prenatal ethanol effects. NM controls exhibited a substantial amount of c-Fos activation in VTA, a result likely the result of the stress of postnatal manipulation that habituated in the VE group. More in detail, compared to NM controls, VE animals exhibited reduced dopaminergic activity in VTA and failed to exhibit ethanol-induced c-Fos in this area, at any of the doses tested. This pattern of results fits nicely with previous findings. It has been found that VE, but not NM, animals failed to exhibit ethanol-induced place preference (Pautassi et al., 2012). The daily handling given to VE animals involved exposure to aversive stimuli, including intubations and brief, yet significant, restraint. These procedures can be considered mild unpredictable stressors, similar to those given to dams undergoing a schedule of prenatal stress (Lee et al., 2007; Harmon et al., 2009). In the present study, prenatal stress blunted the baseline level of dopaminergic activity at VTA, and reduced the responsivity of this system to ethanol intoxication. This makes the increased neural responding to ethanol of PEE animals even more noteworthy. Animals given ethanol *in utero* were exposed to the same stressors as VE counterparts (i.e., handling, intubation). PEE not only inhibited the reduced baseline level of dopaminergic activity, apparently induced by prenatal stress in the VE adolescents, but also heightened neural responding to the postnatal ethanol challenge.

The addition of the NM group also helped clarify the pattern of ethanol-induced Fos-ir at PrL. Animals untreated during pregnancy exhibited heightened Fos-ir in the medial prefrontal cortex following postnatal administration of 1.25 g/kg ethanol. This response was absent in PEE and in VE animals. Therefore, it is prudent to conclude that this apparent desensitization is due to prenatal stress and not specific to ethanol exposure during gestation. In addition, in our previous study (Fabio et al., 2013) we found reduced baseline Fos-IR in IL after gestational exposure to the drug, yet in the present work the IL was spared of any effect of PEE. This discrepancy likely relates to differences in the strains or in the sex of the rats employed in these studies (outbred Wistar rats vs. inbred WKAH/Hok rats; and males vs. females). This highlights the limitation of the present study having used only females. Although the PEE protocol here employed apparently exerts similar effects upon the ethanol intake of adolescent male and female rats (Fabio et al., 2013, 2015), future studies should employ animals of both sexes.

Another caveat is that, although it is known that Fos interacts with members of the Jun family, we employed a single marker of neural activity. This issue hinders the interpretation of the data. It would have been interesting to observe the differential effects of the postnatal ethanol challenge in these two markers, across the prenatal treatments. Previously, Zoeller and Fletcher (1994) found that ethanol (3.0 g/kg) increased c-Fos mRNA in the paraventricular nucleus (PVN) but decreased C-Jun mRNA in PVN and hippocampus.

## CONCLUSIONS

The results suggest that brief prenatal ethanol exposure alters the response of the mesocorticolimbic pathway to postnatal ethanol exposure. The implications of these results are important, as heightened dopaminergic activity in the VTA is linked to vulnerability to develop alcohol-use disorders (Nestler, 2013). In turn, desensitization to the effects of ethanol in areas related to processing of reward may put PEE subjects at risk of problematic ethanol consumption. PEE adolescents may need greater doses of ethanol to experience the same reinforcing effects of ethanol than VE or NM counterparts.

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