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PRENATAL ETHANOL EXPOSURE INCREASES ETHANOL INTAKE AND REDUCES C-FOS EXPRESSION IN INFRALIMBIC CORTEX OF ADOLESCENT RATS

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Abstract

Prenatal ethanol exposure significantly increases later predisposition for alcohol intake, but the mechanisms associated with this phenomenon remain hypothetical. This study analyzed (Exp. 1) ethanol intake in adolescent inbred WKAH/Hok Wistar rats prenatally exposed to ethanol (2.0 g/kg) or vehicle, on gestational days 17–20. Subsequent Experiments (2, 3 and 4) tested several variables likely to underlie the effect of gestational ethanol on adolescent ethanol preference, including ethanol-induced locomotor activation (LMA), ethanol-induced emission of ultrasonic vocalizations (USVs) after exposure to a rough exteroceptive stimulus, and induction of the immediate early gene C-fos in brain areas associated with processing of reward stimuli and with the retrieval and extinction of associative learning. Prenatal ethanol induced a two-fold increase in ethanol intake. Adolescents exhibited significant ethanol-induced LMA, emitted more aversive than appetitive USVs, and postnatal ethanol administration significantly exacerbated the emission of USVs. These effects, however, were not affected by prenatal ethanol. Adolescents prenatally exposed to ethanol as fetuses exhibited reduced neural activity in infralimbic cortex (but not in prelimbic cortex or nucleus accumbens core or shell), an area that has been implicated in the extinction of drug-mediated associative memories. Ethanol metabolism was not affected by prenatal ethanol. Late gestational exposure to ethanol significantly heightened drinking in the adolescent offspring of an inbred rat strain. Ethanol-induced LMA and USVs were not associated with differential ethanol intake due to prenatal ethanol exposure. Prenatal ethanol, however, altered basal neural activity in the infralimbic prefrontal cortex. Future studies should analyze the functionality of medial prefrontal cortex after prenatal ethanol and its potential association with predisposition for heightened ethanol intake.

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Keywords

adolescent; ethanol; prenatal exposure; C-fos; ultrasonic vocalization; LMA

1. Introduction

Problematic use of ethanol during adolescence is a worldwide health problem. In the U.S., lifetime prevalence of alcohol consumption is 72.3% in adolescents (Johnston et al, 2009). Ethanol use and abuse during adolescence is also a major problem in European and Latin-American countries. A study revealed risky drinking in one-quarter of Spanish college students (Mota et al., 2010), similar to the number identified for Argentinian students (SEDRONAR, 2011).

Epidemiological (Baer et al., 1998; 2003, Yates et al, 1998) and animal studies have indicated that prenatal ethanol exposure increases the probability of alcohol-use disorders later in life. The overall finding after in-utero ethanol exposure in rats (for reviews see Abate et al., 2008; Chotro et al., 2007; Spear & Molina, 2005) has been increased ethanol intake in preweanling (e.g., Dominguez et al., 1998) or adult subjects (Nash et al., 1984). There have been fewer studies that tested this effect during adolescence, particularly using restricted developmental timing of exposures that better mimic moderate patterns of ethanol exposure. Some studies have found heightened adolescent ethanol intake in rats reared by dams given ethanol during late gestation (Chotro & Arias, 2003; Díaz-Cenzano & Chotro, 2010). Other studies, in turn, failed to show significant effects of prenatal ethanol exposure on adolescent intake (Reyes et al., 1985), or required substantial postnatal ethanol exposure for the effect to emerge (Honey & Galef, 2003).

The mechanisms underlying the effects of prenatal ethanol on later ethanol preference remain largely hypothetical. It is possible that fetal ethanol promotes either familiarization to ethanol's sensory properties (i.e., the "mere exposure" hypothesis; Spear and Molina, 2005), or associative learning between the chemosensory cues of ethanol and the appetitive, pharmacological component of the drug (Abate et al., 2008; Arias & Chotro, 2005). Another possibility is that prenatal alcohol makes subjects more sensitive to the appetitive effects of ethanol or less sensitive to ethanol's aversive consequences. Early work indicated that prenatal ethanol induced tolerance to the hypothermic effects of ethanol (Abel et al., 1981), which has been identified as one component of the aversive state of intoxication. Another early study (Becker et al., 1995) suggested that prenatal experience with ethanol could enhance the behavioral stimulant effect of ethanol, which is considered a proxy for ethanol's positive rewarding effects

The aim of the present study was to further analyze the effects of prenatal ethanol exposure on subsequent responsiveness to ethanol during adolescence. To our knowledge the few studies that reported a facilitative effect of adolescent ethanol intake following prenatal ethanol exposure have employed outbred rat strains. It was, therefore, important to analyze if this phenomenon could generalize to an inbred strain (Experiment 1). After establishing that this gestational ethanol exposure reliably enhances ethanol acceptance in inbred rats, subsequent experiments tested several variables likely to underlie the effect of prenatal ethanol exposure on adolescent ethanol preference, including acute effects of ethanol upon locomotor and emotional patterns. Experiment 2 analyzed -- in adolescents prenatally exposed or not exposed to ethanol -- spontaneous and ethanol-induced locomotor activity (LMA) as well as the emission of ultrasonic vocalizations (USVs) when animals were exposed to a novel and rough exteroceptive cue.

A few studies have assessed the effects of novelty or ethanol exposure in neuronal activity of the adolescent brain (Vilpoux et al., 2011). To our knowledge, none of these studies have analyzed if prenatal exposure to moderate ethanol doses can modulate ethanol-induced neuronal activity. In Experiments 2 and 3 the brains of adolescents exposed or not to ethanol during pregnancy were examined for neuronal activation in areas of the mesocorticolimbic pathway related to the reward system (Faria et al., 2008) and involved in the extinction of associative learning (Millan et al., 2011). Specifically, the immediate early gene (IEG) *C-fos*, indicative of changes in cellular activity, was measured in infralimbic and prelimbic cortex (IL and PrL, respectively), nucleus accumbens core and nucleus accumbens shell (AcbC and AcbSh, respectively).

Blood ethanol levels (BELs) in adolescents reared by dams given ethanol or vehicle during gestation were also examined (Experiment 4).

2. Materials and Methods

2.1 Experimental designs and hypotheses

Experiment 1 assessed the effects of alcohol exposure during late gestation [gestational days (GDs) 17–20] on ethanol consumption at adolescence [postnatal days (PDs) 36–39], in an inbred rat strain [Wistar-King Apteckman Hokkaido (WKAH/Hok)]. The few studies that have assessed the effects of prenatal ethanol on adolescent ethanol intake have used outbred stocks of rats (Diaz-Cenzano & Chotro, 2010). Therefore, the rationale for using WKAH/Hok rats was to assess if the facilitative effect of prenatal ethanol on later adolescent intake generalizes to an inbred strain. Moreover, it has been suggested that experiments that use inbred rats are better equipped to detect subtle effects and less prone to have type II errors (Festing, 2010).

The experimental design of Experiment 1 was a 2 (prenatal treatment: ethanol or vehicle, PE or PV groups, respectively) \times 2 (sex: male or female) factorial, with 7–8 data points in each group. To control for potential litter effects, when more than one male and one female of a same litter were tested, their data were averaged across sex. The hypothesis was that prenatal ethanol exposure would significantly increase ethanol drinking at adolescence.

In Experiment 2, spontaneous or ethanol-induced motor activity (LMA) and ultrasonic vocalizations (USVs) in adolescence were measured in animals prenatally exposed to alcohol or vehicle. Experiment 2 was defined by a 2 (prenatal treatment: PE or PV) \times 2 (sex) \times 3 (postnatal treatment: ethanol, vehicle or untreated) factorial, with 6–8 animals in each group. Preweanling rats emit USVs when stressed or isolated from familiar conspecifics (Blumberg & Alberts, 1990; Kraebel et al., 2002). USVs have been more often studied in older rats after aversive stimuli and are typically found in the 20–30 khz range, but these animals also emit USVs in the 50–60 khz range following appetitive stimuli (Panksepp & Burdof, 2003). LMA was selected as a dependent variable on the basis of previous research indicating its association with ethanol-mediated reinforcement (Acevedo et al., 2012) and ethanol intake (Tarragón et al., 2012). Animals with greater spontaneous LMA exhibit, when compared with counterparts featuring lower LMA, enhanced ethanol intake (Bisaga & Kostowski, 1993) and an attenuation of ethanol-induced conditioned taste aversion (Arias et al., 2009). Sensitivity to ethanol-induced LMA during adolescence has been also associated with greater predisposition for ethanol intake (Acevedo et al., 2010).

Based on previous studies (Shea et al., 2012), we expected to find enhanced spontaneous and ethanol-induced motor behavior after prenatal ethanol. We also expected greater emission of USVs after the postnatal intubation with 2.5 g/kg ethanol and a reduction in the anxiogenic effect of this intubation in animals that had been exposed to the drug in-utero. USV emission

was further categorized as aversive or appetitive (20–30 or 50–60 khz range). An additional hypothesis was that ethanol-induced appetitive USVs would be increased by prenatal exposure to ethanol.

In Experiment 2, five subjects were randomly selected from each group. The brains of these animals were preserved to examine C-fos activation in IL, PrL, AcbC and AcbSh, following exposure to the novel environments of the open-field and the sandpaper-lined chamber employed for USV measurement. Substantial C-fos expression is known to occur after exposure to novel stimuli (Ryabinin et al. 1997). The main aim of Experiment 2 was to assess if overall level of novelty-induced C-fos expression was altered by prenatal ethanol exposure and whether postnatal ethanol also altered this pattern of IEG expression.

Experiment 3 replicated the prenatal and postnatal treatments of Experiment 2. Animals, however, did not undergo behavioral testing or exposure to new environments prior to injections, brain preservation and subsequent immunohistochemical analysis. Due to the lack of sex-dependent effects in the previous Experiments, only males were employed and groups had 4–5 animals.

BELs after 2.5 g/kg ethanol were measured in Experiment 4, which employed a 2 (prenatal treatment: PE or PV) \times 2 (sex) \times 3 (post-administration time of sampling: 30, 90 or 120 min) factorial, with 5 to 7 animals in each group.

2.2 Subjects

A total of 237 Wistar rats (WKAH/Hok) inbred strain, 119 males and 60 females], representative of 35 litters, were employed in Experiments 1, 2 and 4. In Experiment 1, 61 adolescent animals (28 male and 33 female) PD 36 at the beginning of the experiment were obtained from 16 female Wistar rats [8 pre-treated with ethanol (PE litters) and 8 treated with vehicle (i.e., water) during late gestation (PV litters)]. This experiment employed only 2 males and 2 females from a given litter. Three animals were lost due to procedural errors.

Experiment 2 employed 103 animals (52 males, 51 females) representative of 9 and 10 PE and PV litters, respectively, whereas 73 rats (34 males and 39 females, derived from 10 PE and 10 PV litters) were used in Experiment 4. Thirty male adolescent outbred Wistar rats, representative of 5 PE and 5 PV litters, were employed in Experiment 3. An outbred Wistar strain was used in Experiment 3 because the supplier was not able to provide an inbred stock of animals.

Animals were born and reared in a temperature-controlled vivarium of the Instituto de Investigaciones Médicas M. y M. Ferreyra (INIMEC-CONICET, Córdoba, Argentina). The colony room 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 (Cargill, Buenos Aires) and water ad-libitum. Dams remained undisturbed until the beginning of prenatal treatment on GD 17 (GD 0 presence of sperm in vaginal smear). Births were examined daily, and the day of parturition was considered PD0. Weaning was performed at PD21; at that instance animals from the same litter were housed in same-sex groups of 4 until the end of the experiment. Experimental procedures 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.

2.3 Prenatal treatment (Experiments 1, 2, 3 and 4)

From GD17 to GD20 pregnant dams received one daily intragastric (i.g.) administration of 0.015 ml/g of a 16.8% v/v ethanol solution (vehicle: tap water; ethanol dose: 2.0 g/kg) or a

similar volume of vehicle. The i.g. administrations were performed by gently introducing a polyethylene cannulae (PE 50; Clay Adams, Parsippany, New Jersey, USA) through the oral cavity into the stomach. The tubing was connected to a 5cc syringe mounted with on a 21 G needle (Becton Dickinson & Co., Rutherford, N.J). The whole procedure took approximately 20 sec per animal. The dose was selected based on previous studies that suggested its effectiveness in promoting heightened postnatal ethanol acceptance (Díaz-Cenzano & Chotro, 2010) and greater ethanol-induced reinforcement (Pautassi et al., 2012).

2.4 Adolescence Intake Test (Experiment 1)

On PD 35, animals derived from dams given ethanol or vehicle during late gestation had a 24-hr home-cage adaptation session to graded tubes filled with tap water. During this session, animals were housed in groups of four and exposed to four graded tubes per cage. The reason to expose the animals to four graded tubes was to provide a 1:1 ratio between number of animals and number of available tubes in each cage. The adaptation session was meant to facilitate the transition between drinking from the standard bottles to drinking from the novel tubes of ethanol intake sessions. During ages PD 36–39, voluntary ethanol consumption was tested using a standardized two-bottle ethanol intake test protocol (see Acevedo et al., 2010; Pepino et al., 2004; Ponce et al., 2004; 2008). Animals underwent daily 2-h intake sessions preceded by a 22-h water deprivation period. Rats were weighed before each session. During drinking sessions animals were placed in standard individual wire mesh cages with two graded tubes, one filled with tap water and the other with the ethanol solution (3% v/v on the first day and increasing 1% on subsequent days until reaching 6% v/v on the last testing day on PD39). After each test period rats were returned to their home cages. The volume consumed from each tube was assessed at 20, 60 and 120 min. Tubes were never touched or removed during intake tests. The position of the ethanol and water tubes was varied across sessions to prevent place-preference effects. The dependent variables under analysis were ethanol intake in grams per kilogram (g/kg) and percent selection of ethanol [(consumption of ethanol/overall liquid ingestion) × 100].

This intake protocol has been extensively used to detect the influence of early alcohol exposure, mediated by maternal consumption during breastfeeding (Pepino et al., 2004; Ponce et al., 2011), operant-self administration (Ponce et al., 2008) or passive intubations (Acevedo et al., 2010), on later alcohol acceptance. Preliminary studies conducted in our lab (Fabio et al., unpublished) indicate that, without liquid deprivation, adolescent Wistar rats drink very little ethanol, even when ethanol is provided in an intermittent basis. Ethanol acceptance can be facilitated by adding sweeteners (e.g., sucrose) to the ethanol solution, yet this adds the possibility that rats might respond for sensory or caloric properties of the sweetener rather than for ethanol. The deprivation schedule employed in the present work promotes fast absorption and distribution of ethanol and therefore rapid perception of its postabsorptive effects (Ponce et al., 2004; Pepino et al., 2004).

2.5 Adolescence alcohol administration procedures (Experiments 2, 3 and 4)

On PD 36, Animals from PE or PV groups received an i.g. administration of a 0.015 ml/g of a 21% v/v ethanol solution (vehicle: tap water; ethanol dose: 2.5 g/kg; hereinafter referred as 2.5 Group), a similar volume of vehicle (0.0 group) or were left untreated (UT group). The intubations were accomplished following similar procedures as those utilized during prenatal treatment. The ethanol dose was selected on the basis of previous studies (Acevedo et al., 2010) that suggested its effectiveness in promoting ethanol intake and inducing significant ethanol-induced locomotor activity in adolescents.

2.6 Assessment of ethanol-induced locomotor activity (LMA) and ultrasonic vocalizations (USVs) (Experiment 2)

Five minutes after intubation, animals were tested for motor activity in square chambers (50×50×50cm) connected to an automatic activity monitoring system (ITCOMM, Córdoba, Argentina). Each chamber was surrounded by photocell beams that generated a matrix. The system tracked the rat's movement in real-time basis and recorded the distance traveled (cm) during a 5-min test (i.e., post-administration time 5–9 mins). Vertical movement of the animal broke a photocell beam located about 12 cms from the floor of the chamber. Total number of these beams broken was considered a measure of rearing. Dependent variables are expressed in a minute-by-minute basis (i.e., five 1-min bins). Testing was conducted in a room equipped with two fluorescent lamps located in the center of the room, about 2.5 meters above the testing chambers. Immediately following the motor activity test, animals were individually placed into a sound-attenuating chamber (Med Associates, St Albans, VT) with sandpaper lining the floor (60 grit, Norton, Rio Grande do Sul, Brazil). Exposure to the rough sandpaper surface was meant to be a mild stressor. Previous pilot studies had indicated total absence of USV emissions in similarly aged adolescent rats tested in a plain Plexiglas chamber. The sound-attenuating chambers were located in the same room as the automatic activity monitoring system. Ultrasonic vocalizations were recorded for 5 min using a ANL 817-1B vocalization detector (Med Associates, St Albans, VT). Vocalizations in the range 20–30 khz and 40–50khz were separately measured and analyzed to generate total number of USVs for each range across the five-min test.

2.7 Assessment of C-fos activity (Experiments 2 and 3)

The immunohistochemistry protocol was similar to that described in de Olmos et al. (2009) and Faria et al. (2008). Ninety minutes after intubation with 2.5 g/kg ethanol the adolescents were anesthetized with i.p injections of Chloral hydrate (dose: 0.7 mls/100 mg of body weight) and perfused transcardially with 0.9% heparinized saline 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 40µm sections were obtained and placed in 0.1-M PB. Two series were stored at 4°C and employed in other studies. The other series, in turn, was immediately used for immunohistochemistry. Peroxidase reaction was blocked by incubating the brains for 60 min in a solution composed of 1% H₂O₂, 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 hour. Afterwards, 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) 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 60 min with biotinylated donkey anti-rabbit secondary antibody (Jackson Laboratories, West Grove, PA) diluted 1:500 in 1% NHS, and washed again three times in PB. The sections were then incubated for 60 min with the avidin–biotin–peroxidase complex (ABC Elite Kit; Vector Labs, Burlingame, CA) diluted in 1% NHS. Sections were subsequently incubated for 5 min with a solution containing 0.05% 3–3'-diamino-benzidine tetra hydrochloride (DAB, SigmaAldrich, St. Louis, MO, USA) and 0.01% hydrogen peroxidase. The sections were then mounted on a gelatinized slide, dehydrated and covered with DPX glue.

Three slices were selected per animal in each of the brain regions under analysis (please see Figure 1). Following the delineation of Paxinos and Watson (2007), slices for IL and PrL were taken at bregmas 3.34, 3.00 and 2.76 mm; whereas AcbC and AcbSh were taken from bregmas 1.68, 1.44 and 1.08 mm. 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). Data from the three sections were averaged for the subsequent statistical analysis.

2.8 Determination of Blood Ethanol levels (Experiment 4)

Animals were given 2.5 g/kg of ethanol and trunk blood ethanol samples (2 ul) were taken 30, 90, or 120 minutes after ethanol administration, through a heparinized capillary tube. Only one sample was collected from each animal. The vials containing the blood were stored at -70°C for later analysis. BELs were obtained through head-space gas-chromatography (Hewlett Packard 5890, Wilmington, DE). Blood samples were incubated into a hot water bath (60°C) for 30 min. The volatile component was then injected into the chromatographer through gas-tight syringes (Hamilton Co., Reno, NV). The temperature of the column, oven and detector was set at 60°C , 150°C and 250°C , respectively. The carrier gas was nitrogen (speed: 15 ml/min). BELs were expressed as milligrams of ethanol per deciliter of body fluid (mg/dl = mg %).

2.9 Statistical Analysis

Body weights, overall liquid intake (ml/100g), ethanol intake on a gram per kilogram basis and percent selection were examined using separate two-way mixed analyses of variance (ANOVAs). Prenatal treatment (water or ethanol) and sex were between-group factors, and days of assessment (sessions 1, 2, 3, and 4) were repeated measures. Maximum daily amount of absolute ethanol intake was analyzed through a factorial ANOVA (prenatal treatment and sex). This variable reflects the highest level of ethanol consumption (g/kg) achieved by each animal across testing days.

Ethanol-induced motor activity [distance traveled (cm), Experiment 2] was analyzed through a three-way mixed ANOVA, which included the between factors sex, prenatal treatment and postnatal ethanol treatment (0.0 g/kg ethanol, 2.5 g/kg ethanol or untreated). Bin of assessment (bins 1–5) was considered a within subject measure. A similar ANOVA was employed to analyze rearing scores. Total number of ultrasonic vocalizations in each band (20–30 khz and 40–50khz) across the test was separately analyzed through an ANOVA that included sex, prenatal treatment and postnatal ethanol treatment as between factors.

The dependent variable for the immunohistochemistry studies in Experiments 2 and 3 was the total number of C-fos positive cells in each brain structure (IL, PrL, AcbC AcbSh). Number of positive cells in each brain region was analyzed through separate factorial ANOVAs (sex×prenatal treatment×postnatal treatment, Experiment 2; prenatal treatment×postnatal treatment, Experiment 3).

BELs were analyzed through a factorial ANOVA [sex × prenatal treatment × sampling time (30, 90, and 120 minutes after ethanol administration)].

Across analysis, the loci of significant main effects or interactions were further examined using pair-wise comparisons (Tukey's *post hoc* tests or planned comparisons). Tukey was used to further analyze main effects or interactions comprising “between” factors. Orthogonal planned comparisons were used to analyze the significant interactions involving between-by-within factors. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1 Experiment 1

The ANOVA for body weight indicated a significant main effect of day of assessment, $F_{3, 81} = 17.21$; $p < .001$. Body weight was significantly higher in PD36 than in the subsequent testing days and was not affected by prenatal manipulations or sex. The mean \pm SEM body weight (g) in animals that were exposed to ethanol during gestation was 85.36 ± 7.01 , 80.21 ± 11.77 , 77.84 ± 11.00 and 79.15 ± 12.72 , whereas the mean \pm SEM in subjects reared by vehicle-treated dams was 84.82 ± 9.95 , 80.33 ± 7.24 , 78.35 ± 6.88 and 78.02 ± 6.77 in postnatal days 36, 37, 38 and 39, respectively.

Overall liquid intake (ml/100g) was found to be similar across prenatal conditions and exhibited a significant increase across test days, $F_{3, 81} = 11.07$; $p < .001$. Across prenatal treatment and sex, overall intake values were as follows: PD 36, 6.32 ± 0.32 ; PD 37, 7.68 ± 0.32 ; PD 38, 8.55 ± 0.48 and PD 39, 8.35 ± 0.39 .

Means and standard error for ethanol intake across sessions are depicted in figure 2 (left panel: gram per kilogram; right panel: percent preference). Intake on a gram per kilogram basis was fairly constant across sessions, with a peak on session 2 when animals were exposed to a 4% v/v ethanol solution. Percent preference for ethanol was higher in the first two sessions and seemed to decrease during the last sessions. Prenatal ethanol treatment significantly heightened ethanol consumption across sessions and this facilitative effect of prenatal ethanol was fairly similar for both measures of ethanol intake. The ANOVAs confirmed these impressions. The ANOVAs for ethanol intake measured in g/kg and percent preference revealed significant main effects of prenatal treatment ($F_{1, 27} = 9.86$; $p < .005$, $F_{1, 27} = 11.82$; $p < .005$, respectively) and session ($F_{3, 81} = 3.38$; $p < .005$, $F_{3, 81} = 6.54$; $p < .001$, respectively). Post-hoc comparisons indicated significantly greater consumption of ethanol in animals exposed to ethanol during gestation than in counterparts reared by vehicle-treated dams. Ethanol intake was significantly greater in the second test session than in the remaining sessions.

Prenatal ethanol also affected the maximum amount of absolute ethanol intake on a given day. This variable reflects the highest daily level of ethanol consumption (g/kg) achieved by each animal across testing days. The ANOVA yielded a significant main effect of prenatal treatment, $F_{1, 27} = 7.67$; $p < .05$. Adolescents exposed to ethanol in utero exhibited significantly higher maximum intake scores (0.84 ± 0.08 g/kg) than control counterparts (0.52 ± 0.08 g/kg).

3.2 Experiment 2

This Experiment tested acute responsiveness to ethanol, in terms of LMA and emission of USVs, in adolescent rats exposed to ethanol prenatally through the drug administration schedule that, in Experiment 1, resulted in greater ethanol intake. The brains of a sub-sample of these animals were analyzed for expression of the immediate early gene C-fos.

The ANOVA for LMA scores indicated significant main effects of postnatal ethanol treatment and bin of assessment [$F_{2, 78} = 28.46$; $p < .0001$, $F_{4, 312} = 57.55$; $p < .0001$] as well as a significant interaction between these factors, $F_{8, 312} = 2.90$; $p < .005$. As depicted in Figure 3 and confirmed by planned comparisons, ethanol induced significant behavioral stimulation during the first four minutes of testing. Untreated and vehicle-treated controls exhibited fairly similar LMA scores across test. The ANOVA for rearing scores revealed only a significant main effect of bin of assessment, $F_{4, 312} = 48.74$; $p < .0001$. Rearing scores (mean \pm SEM) in testing bins 1, 2, 3, 4 and 5 were as follows: 16.59 ± 1.17 , 12.31 ± 0.98 , 7.78 ± 0.93 , 5.94 ± 0.86 , 6.21 ± 1.04 and 16.00 ± 1.18 , 11.73 ± 0.93 , 8.38 ± 0.89 , $7.18 \pm$

0.82, 5.42 ± 0.99 , for adolescents exposed to ethanol or vehicle as fetuses. Prenatal treatment exerted no significant main effect nor was it involved in significant interactions.

As expected, animals emitted many more aversive than appetitive USVs when exposed to the rough sandpaper surface. Overall frequency of USVs was 13.88 ± 1.60 and 2.84 ± 0.43 , for the aversive and appetitive band respectively.

Postnatal administration of 2.5 g/kg ethanol induced a two-fold increase in the emission of USVs in the aversive range (20–30 khz), suggesting that this high ethanol dose had a pharmacological effect 10 minutes following administration, perhaps anxiogenic, that added to the stress of the testing situation (see figure 4, top panel). This effect of ethanol seemed similar across adolescents exposed to ethanol in utero or exposed to only vehicle. The ANOVA confirmed these impressions and yielded only a significant main effect of postnatal ethanol treatment, $F_{2,78} = 10.22$; $p < .0001$. Post-hoc tests indicated significantly greater aversive USV emission in adolescents given 2.5 g/kg shortly before testing than in counterparts treated with vehicle or those that remained untreated. The two latter groups, in turn, did not differ between each other.

The bottom panel of Figure 4 depicts frequency of emission of appetitive USVs (50–60 khz range). Postnatal treatment with 2.5 g/kg significantly enhanced the emission of these vocalizations, $F_{2,78} = 10.22$; $p < .0001$. Although visual inspection seems to indicate that this facilitative effect was greater in adolescents exposed to the drug in utero than in non-exposed controls, the two-way interaction between prenatal and postnatal ethanol treatment did not achieve significance, $F_{2,78} = 2.50$; $p = .09$.

Exposure to the open field and UVS test chamber seemed to result in a substantial number of C-fos positive cells in all brain structures. Postnatal ethanol treatment neither reduced nor enhanced this novelty-induced C-fos expression. Moreover, prenatal ethanol did not exert a significant main effect nor was it involved in any significant interaction in C-fos expression at PrL, AcbC or AcbSh. Interestingly, a significant main effect of prenatal ethanol exposure was detected when analyzing infralimbic cortex, $F_{1,47} = 6.79$; $p < .05$. Adolescents with a history of ethanol exposure in the womb exhibited significantly fewer positive C-fos cells in IL than counterparts derived from vehicle-treated dams. Overall mean was 40.39 ± 5.38 and 71.66 ± 9.68 , for PE and PV adolescents, respectively. Table 1 presents the number of C-fos-positive cells in each brain area under analysis as a function of prenatal and postnatal treatments. The top panel of figure 5 shows images of C-fos positive nuclei in IL of adolescent rats with or without prenatal exposure to ethanol and given ethanol, vehicle or no treatment (i.e., untreated group) on postnatal day 36.

3.3 Experiment 3

The result replicated the pattern observed in the previous Experiment. The ANOVA for C-fos positive cells in infralimbic cortex yielded a significant main effect of prenatal treatment, $F_{1,20} = 4.84$; $p < .05$. PE adolescents exhibited significantly less neuronal activation in this area than PV counterparts (22.32 ± 4.66 vs. 38.16 ± 4.43 , respectively) and this pattern was not significantly altered by postnatal treatment. The ANOVAs for PrL, AcbC AcbSh revealed neither significant main effects nor significant interactions involving sex, prenatal treatment or postnatal treatment. Table 1 depicts number of C-fos-positive cells for each brain area as a function of prenatal and postnatal treatments. Fig. 5 (lower panel) depicts C-fos positive nuclei in IL of adolescent rats as a function of prenatal and postnatal treatments.

3.4 Experiment 4

Adolescents exposed to ethanol or vehicle during gestation exhibited similar blood ethanol levels after intubation with 2.5 g/kg ethanol. The ANOVA revealed a lack of significant

main effects or significant interactions between sex, prenatal treatment or sampling interval. Blood ethanol level (mean \pm SEM) in terms of mg% at 30, 90 and 120 min post administration was as follows: 126.74 ± 6.04 , 119.23 ± 16.35 , 114.63 ± 11.25 and 120.71 ± 11.22 , 117.81 ± 9.87 , 112.08 ± 13.44 , for adolescent exposed to ethanol or vehicle during late gestation.

4. Discussion

Brief exposure to ethanol during late gestation (2.0 g/kg, daily intubations on GDs 17–20) significantly enhanced ethanol intake in the adolescent offspring of inbred WKAH/Hok Wistar rats. Prenatal ethanol induced a substantial, two-fold increase in adolescent drug intake when measured on a gram-per-kilogram basis and in percent selection. Maximum daily level of intoxication achieved throughout testing was also significantly higher in adolescents derived from ethanol-exposed dams.

These results are congruent with studies indicating heightened ethanol consumption in preweaning rats after moderate ethanol exposure during late gestation (e.g. Abate et al., 2008; Chotro et al., 2007). The present study adds that the facilitative effect of prenatal ethanol on ethanol intake occurs clearly in adolescent inbred rats, whereas most studies focused their attention in early infancy and employed outbred strains (e.g., Dominguez et al., 1998).

There has been, however, some evidence of prenatal ethanol significantly enhancing alcohol intake in adolescent, outbred Wistar rats, assessed through one- or two-bottle intake tests. Chotro & Arias (2003) found heightened adolescent ethanol intake in rats reared by dams given ethanol (1–2 g/kg) on gestational days 17–20. The effect was gender and dose-dependent, with males exhibiting enhanced ethanol intake after 2.0, but not 1.0 g/kg, ethanol, and vice versa for females. Díaz-Cenzano & Chotro (2010) observed that just two administrations of ethanol on gestational days 19–20 increased ethanol intake when measured a few days after weaning, at postnatal day 26–27. Some interesting comparisons can be drawn between these studies and the present results. Ethanol intake in terms of percent preference of fluids available during tests was very similar in Chotro & Arias (2003) and in the first testing day of the present study (i.e., around 30%). Unlike Chotro & Arias (2003), however, we found that the facilitative effect of gestational exposure to ethanol on later ethanol intake was similar in male and females.

It is unlikely that gross morphological teratology could account for the increased ethanol intake observed in the present work. There were no body weight differences across prenatal treatments and previous studies indicate that daily, prenatal ethanol intubations from gestational days 17 to 20 did not alter maximum cerebral hemisphere width or length, body weight of the fetuses, placenta weight, cerebellar width (Dominguez et al., 1996), nor did it alter cell number in the granular cell layer or in the main olfactory bulb (Pueta et al., 2011). Moreover, the offspring of dams exposed to moderate doses of ethanol during late gestation exhibit normal patterns of habituation when exposed to chemosensory stimuli (Abate et al., 2000; Arias & Chotro, 2005), suggesting that this prenatal ethanol protocol does not alter processing of basic sensory stimulation or learning abilities. Teratogenic effects expressed in deficits in habituation to novel stimuli have been reported after prenatal exposure to ethanol, yet they were associated with relatively high ethanol doses, chronically administered during the brain growth spurt period (Hofmann et al., 2005).

Differences observed in this study might be hypothesized to be due to a change in ethanol metabolism following prenatal ethanol exposure. Behavioral and neurochemical examination of ethanol's effects was followed by a pharmacokinetic experiment which

indicated that blood ethanol metabolism was not affected by prenatal exposure to the drug. This pharmacokinetic study also indicated that ethanol levels induced by intubation with 2.5 g/kg ethanol remained stable across the 30–120 min post-administration interval. The mode of drug administration used can explain this pattern. When relatively high doses of ethanol are administered intragastrically the blood ethanol curve exhibits a more delayed and persistent peak than that induced by intraperitoneal administration. These results are consistent with those found in outbred Wistar rats (Walker & Ehlers, 2008).

The 22-h water deprivation period before the drinking procedure likely promoted stress. Moreover, water deprivation probably resulted in rapid perception of ethanol's pharmacological effects, due to a greater rate of ethanol's absorption and distribution. Therefore, it is possible that heightened ethanol drinking following prenatal ethanol exposure was driven by the negative reinforcing, anxiolytic effects of ethanol (Kushner et al., 2000; Pautassi et al., 2006).

Experiment 2 tested adolescents with or without prenatal ethanol exposure in terms of several variables likely to underlie the facilitated intake effect observed in Experiment 1, including acute effects of ethanol upon locomotor and emotional reactivity patterns. Emotional reactivity was measured in terms of emission of ultrasonic vocalizations following exposure to a mildly aversive stimulus (i.e., a novel environment featuring rough-textured flooring). Ethanol-induced locomotion was selected on the basis of previous research indicating some overlap between the neurobiological systems mediating drug-induced behavioral stimulation and those involved in drug-mediated reinforcement (Tzschentke & Schmidt, 2000). Ethanol induced significant behavioral stimulation during the onset of the post-administration interval, when blood ethanol levels are rising and the appetitive effect of ethanol is presumably maximal, yet this effect was similar across prenatal treatments. Similar to the present study, Arias et al. (2008) assessed acute sensitivity to ethanol-induced locomotion in preweanling rats derived from dams given 0.0 or 2.0 g/kg ethanol on GDs 17–20. Ethanol induced motor activating and depressing effects, and acute tolerance to the sedative effect was observed. Similar to the present findings with adolescent rats (Experiment 2), however, these effects on infant rats were not affected by prenatal ethanol exposure.

Our expectation of greater emission of aversive than appetitive USVs after exposure to the rough sandpaper texture was confirmed and postnatal intubation with 2.5 g/kg ethanol significantly exacerbated the emission of both appetitive and aversive USVs, although the effect seemed greater for the aversive vocalizations. As in Pautassi et al. (2006), the 2.5 g/kg ethanol dose seemed to exert aversive or anxiogenic effects that summated with the mild stress of the test. This effect of ethanol, however, was not affected by exposure to the drug in-utero. One caveat of the present study is that a single ethanol dose was used during testing. It is possible that prenatal effects of ethanol would have been seen using other ethanol doses.

Immediate early genes, such as C-fos, indicate neural response after several stimuli, including exposure to novelty. For instance, rats exposed to a novel chamber exhibited a two-fold increase of C-fos activity in several brain areas, when compared to home cage controls (Ryabinin et al., 1997). In Experiment 2 rats exposed to the novel environments of the motor activity arena and the USV chamber box exhibited substantial C-fos expression across the brain areas analyzed. Between-experiment comparisons indicate a two-fold greater number of positive C-fos cells in Experiment 2 than in Experiment 3, in which animals were not exposed to distinctive novel stimulation. New information derived from the present study is that adolescents prenatally exposed to ethanol exhibited a significant reduction in the number of C-fos positive cells in the infralimbic prefrontal cortex, but not in

pre-limbic cortex nor in nucleus accumbens core or shell, when compared to counterparts exposed to vehicle in utero. It could be argued that this effect may be an artifact derived from the substantial manipulations experienced by the animals during LMA and USV tests. This explanation, however, seems unlikely, as significantly less C-fos labeling in PE than in PV adolescents was replicated in Experiment 3, when neither behavioral testing nor exposure to new environments occurred.

The infralimbic cortex (IL) has a key role in the extinction of learned associations induced by conventional reinforcers or drugs (for reviews see Millan et al., 2011). It has been observed that electrical stimulation of IL neurons reduces conditioned fear (Vidal-Gonzalez et al., 2006). Pharmacological activation of IL resulting from microinjections of D1 or D2-like antagonists prevented cocaine reinstatement (Sun et al., 2005), but inactivation of IL through muscimol and baclofen facilitated cocaine-induced reinstatement (Peters et al., 2008). Similarly, infralimbic infusion of an N-methyl-D-aspartate receptor antagonist made subjects more resistant to the extinction of conditioned fear task (Burgos-Robles, 2007) but had no effect on its acquisition. Moreover, expression of extinction has been associated with protein synthesis (Santini et al., 2004) and greater number of positive C-fos cells in IL (Knapska & Maren, 2009). In concert with these previous studies, the main result of Experiments 2 and 3 (i.e., reduced basal neural activity in infralimbic cortex after gestational ethanol) suggests that moderate prenatal ethanol exposure may be associated with extinction deficits during adolescence. The implications may be particularly important when framed under theories (Spear and Molina, 2005) suggesting that prenatal ethanol induces associative learning between the odor/taste of ethanol and the appetitive effects of the drug.

Previous studies indicate that acute ethanol intoxication induces dose-dependent C-fos activation in several brain areas, including prefrontal cortex and nucleus accumbens, whereas desensitization in the amplitude of the response is often observed after chronic exposure to ethanol (Chang et al., 1995; Faria et al. 2008). Based on these studies it could be hypothesized that prenatal ethanol exposure may have resulted in a desensitization of ethanol-induced C-fos activation. On the present study, however, ethanol did not affect IEG expression in any of the areas under analysis, neither in Experiment 2 nor in Experiment 3. Some procedural differences could explain this lack of effect. These previous studies (Chang et al., 1995; Faria et al. 2008) employed i.p. ethanol injection, whereas i.g. intubations were employed in the present work. These two modes of administration are known to result in significantly different blood ethanol levels (Nizhnikov et al., 2009) and different patterns of C-fos induction (Vilpoux et al., 2009). Moreover, according to previous studies (Walker and Ehlers, 2009) BELs induced by doses around 3.0 g/kg peak earlier for i.p. than for i.g. administration (15 vs. 30 min post-administration, respectively) and, therefore, it is likely that the timing of C-fos measurement in the present study was not optimal for detection of ethanol-induction of C-fos.

In summary, late gestational exposure to moderate doses of ethanol significantly heightened drinking in the adolescent offspring of an inbred rat strain. Emotional and unconditioned motor responses mediated by ethanol were not directly associated with differential ethanol intake resulting from prenatal ethanol exposure. Prenatal ethanol, however, altered basal neural activity in infralimbic prefrontal cortex, an area implicated in the extinction of drug-mediated associative memories. These results are relevant when considering that ethanol seeking, which commonly begins during adolescence (DeWit et al., 2000), is significantly regulated by drug-associated cues (Hyman, 2005). Future studies should further analyze the functionality of medial prefrontal cortex after prenatal ethanol exposure as well as its potential association with predisposition for heightened ethanol intake.

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Research Highlights

- Prenatal ethanol exposure heightened drinking in adolescent inbred rats.
- Postnatal ethanol induced motor stimulation.
- Postnatal ethanol increased emission of appetitive and aversive ultrasonic vocalizations.
- Unconditioned effects of ethanol were not affected by prenatal ethanol exposure.
- Prenatal ethanol reduced basal neural activity in infralimbic prefrontal cortex.

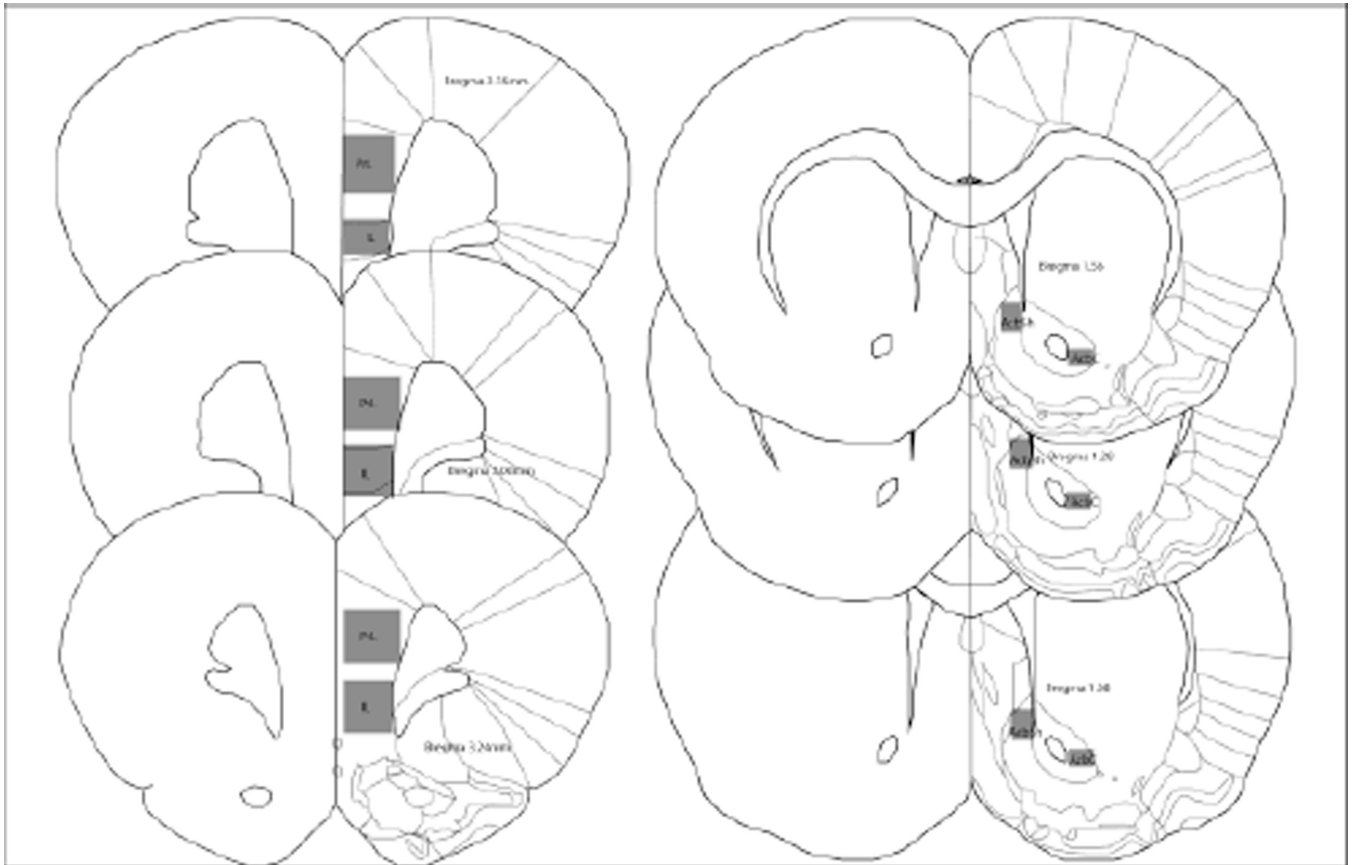


Figure 1. Schematic diagrams of brain sections adapted from the rat brain atlas of Paxinos & Watson (2007). The figures represent the approximate antero-posterior levels (to Bregma) where select brain regions were analyzed. The location and size of the analyzed area for each region [PrL (prelimbic cortex), IL (infralimbic cortex), AcbSh (accumbens nucleus, shell) and AcbC (accumbens nucleus, core)] is indicated with a grey square.

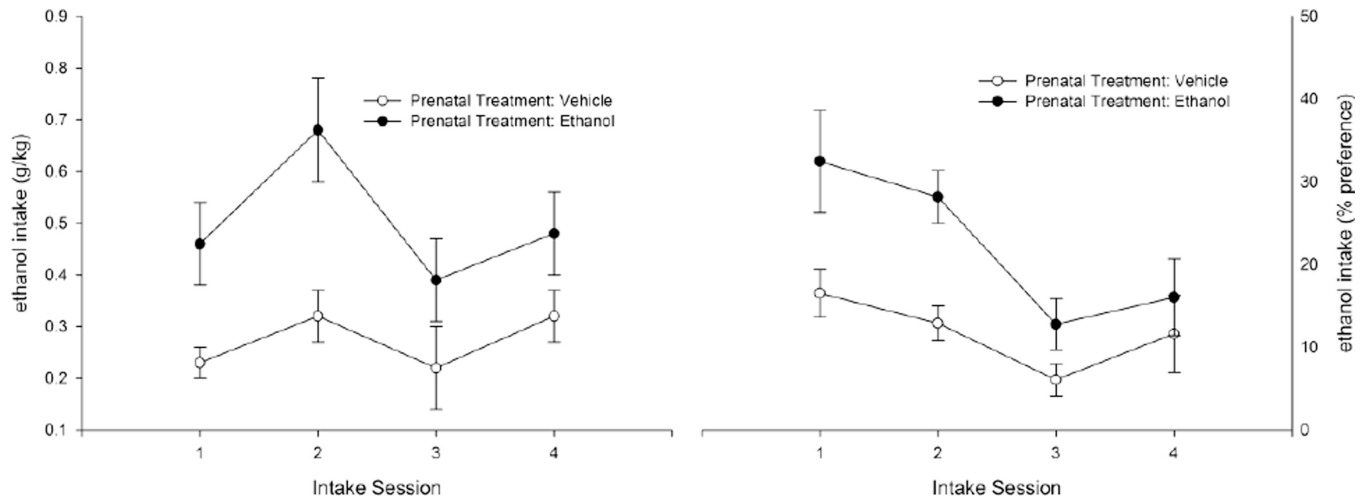


Figure 2.

Absolute ethanol intake (g/kg) and percent ethanol preference scores (left and right panels, respectively) in adolescent rats as a function of intake test session (sessions 1, 2, 3 and 4) and prenatal treatment experienced during gestational days 17 to 20 (2.0 g/kg or vehicle: tap water, mediated by maternal intoxication). In each daily intake test (duration: 120 min) animals had access to a bottle of water and a bottle of ethanol (ethanol concentration: 3, 4, 5 or 6% v/v, sessions 1 to 4; respectively). Data were collapsed across sex (male or female). The sex factor did not exert a significant main effect or significantly interact with the remaining variables. The statistical analysis indicated that prenatal ethanol treatment significantly heightened ethanol consumption across sessions and that this effect was similar for both measures of ethanol intake. Vertical lines indicate SEM.

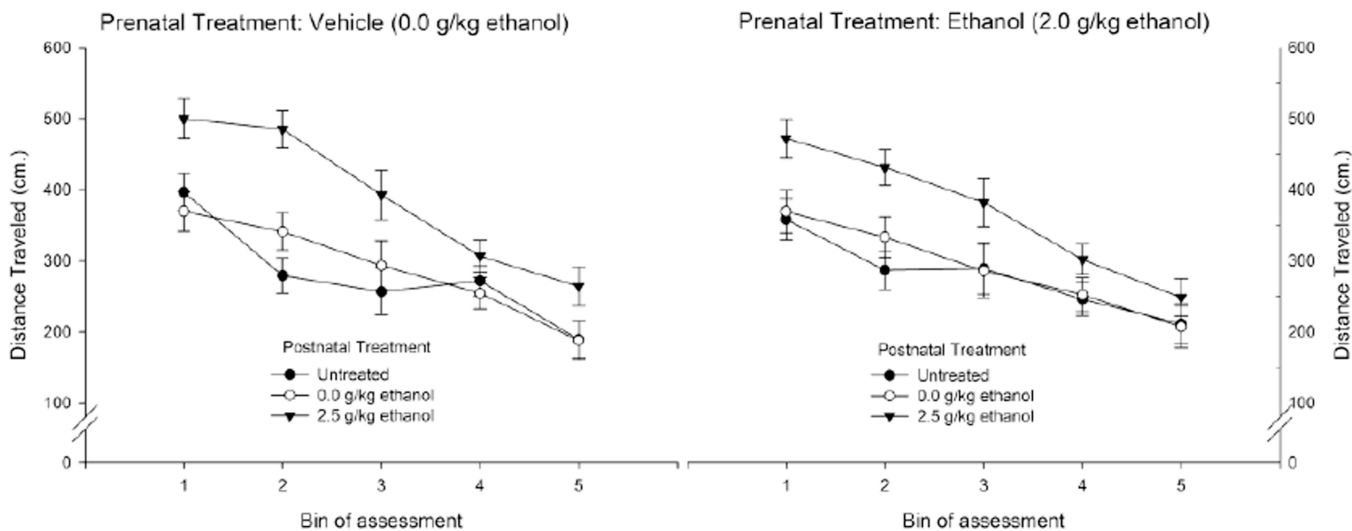


Figure 3. Locomotor activity (distance traveled, cm) in male and female adolescent rats derived from dams given ethanol (2.0 g/kg) or vehicle during late gestation. On postnatal day 36, the adolescents were given ethanol (2.5 g/kg, i.g.) or its vehicle (tap water) or remained untreated before locomotor activity was measured during post-administration time 5–9 min (bins 1–5, corresponding to testing minutes 5, 6, 7, 8 and 9). Data were collapsed across sex (male or female). The sex factor did not exert a significant main effect or significantly interact with the remaining variables. The statistical analysis indicated that postnatal ethanol induced significant locomotor activity during the first 4 minutes of testing, and that this effect was similar for animals exposed to ethanol or vehicle during late gestation. The vertical bars indicate SEM.

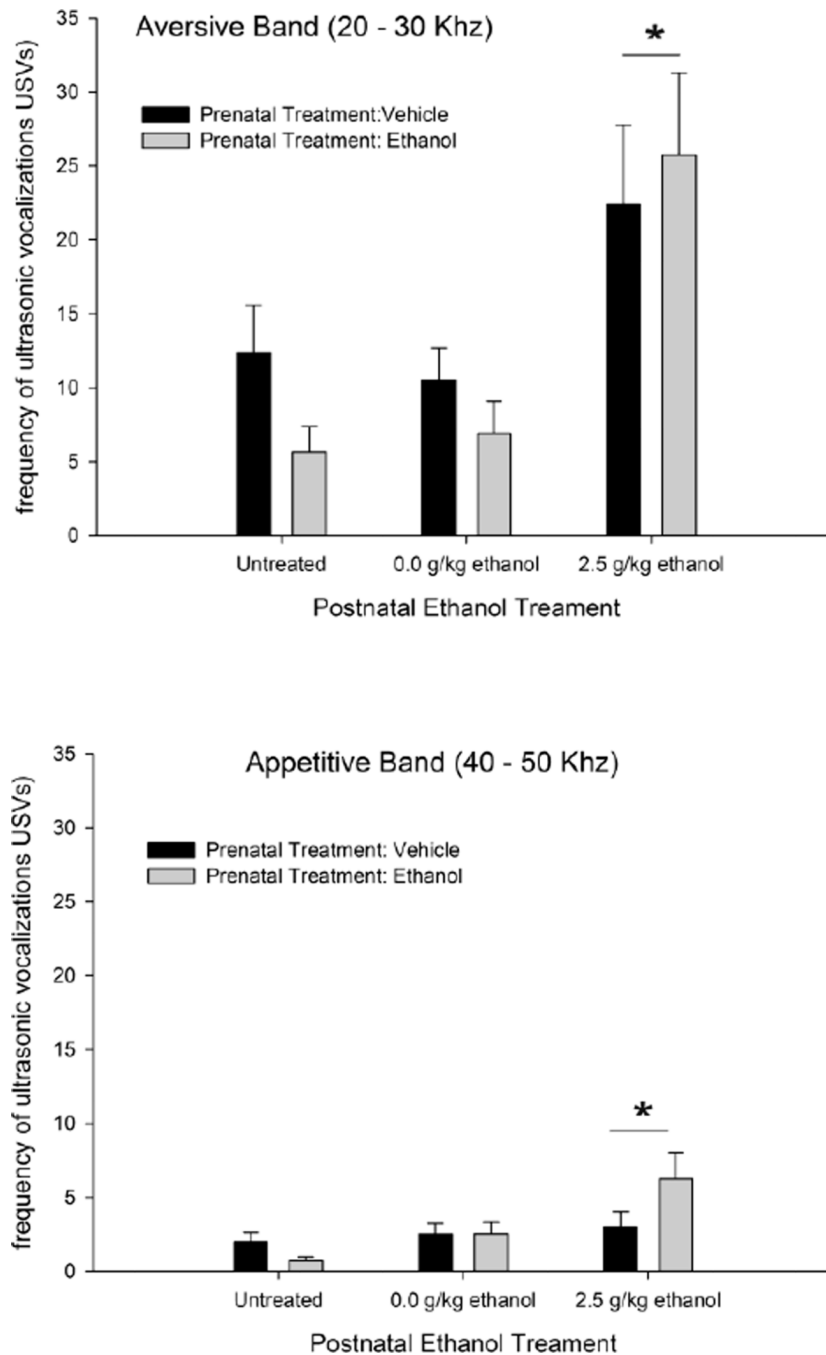


Figure 4.

Frequency of emission of aversive and appetitive ultrasonic vocalizations (USVs) in the 20–30 khz range and in the 50–60 khz range (top and bottom panels, respectively), in male and female adolescent rats derived from dams given ethanol (2.0 g/kg) or vehicle during gestational days 17 to 20. On postnatal day 36, adolescents were given ethanol (2.5 g/kg, i.g.) or vehicle (0.0 g/kg, tap water) or remained untreated. Locomotor activity was assessed during post-administration time 5–9 min and emission of USVs was measured during post-administration time 10–14 min. Data were collapsed across sex (male or female). The sex factor did not exert a significant main effect or significantly interact with the remaining variables. Asterisks (*) indicate significant differences between the group administered

ethanol on postnatal day 36 and the groups administered vehicle or untreated ($p < 0.05$). The vertical bars indicate SEM.

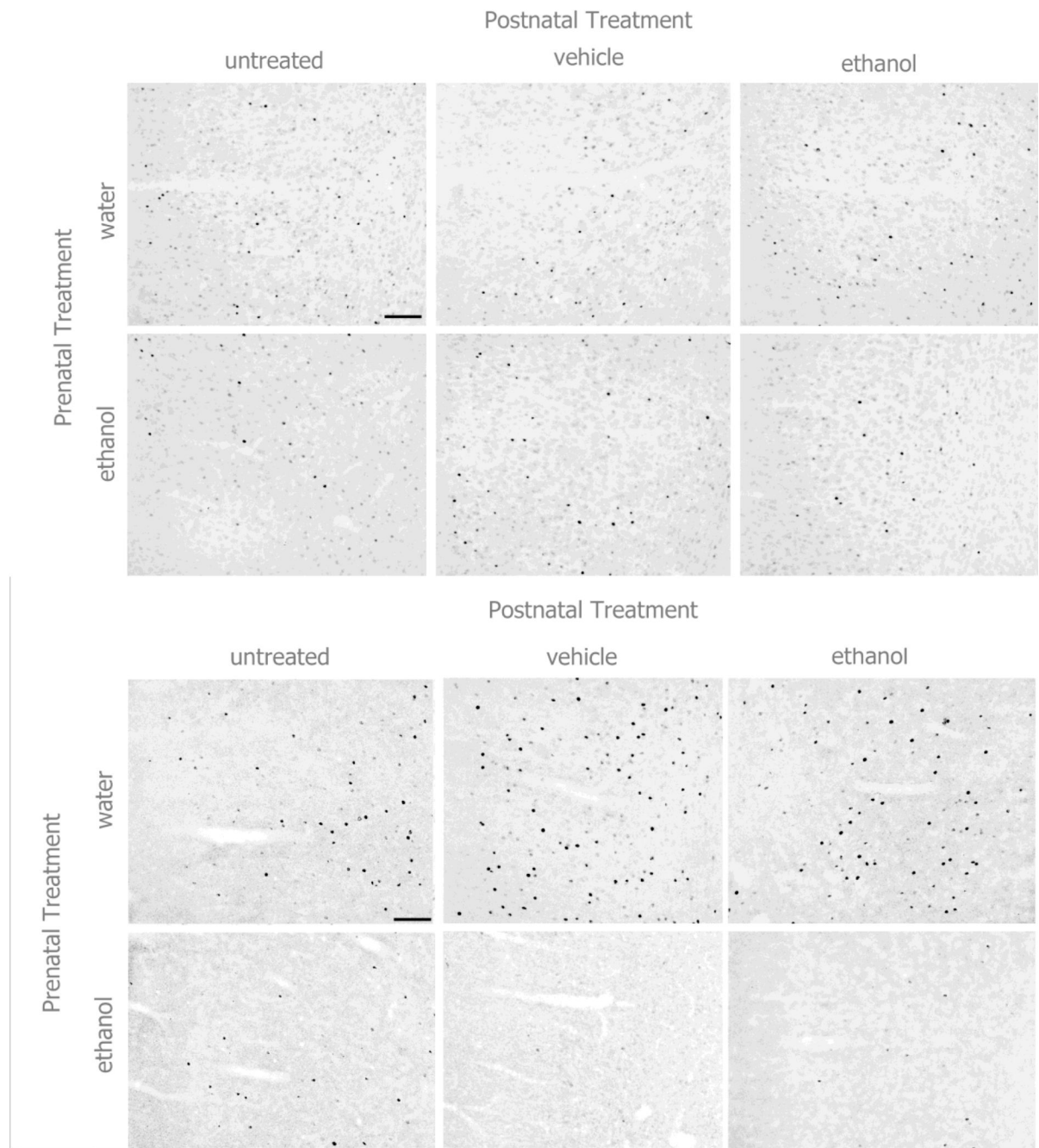


Figure 5.

Photograph of brain sections from adolescent rats employed in Experiment 2 and 3 (upper and lower panels, respectively). Photographs were taken at the level of the prefrontal infralimbic cortex. Rats were derived from dams given ethanol (2.5 g/kg) or vehicle administrations on gestational days 17 to 20. On postnatal day 36 rats were given 2.5 g/kg ethanol, vehicle (i.e., 0.0 g/kg), or were untreated. In Experiment 2, but not in Experiment 3, adolescents were tested for ethanol-induced motor activation and emission of ultrasonic vocalizations before preservation of the brains. In both experiments the statistical analysis indicated decreased C-fos in adolescent rats exposed to ethanol in-utero when compared to adolescents derived from dams given only vehicle during gestation.

Table 1

Number of C-fos-positive cells in infralimbic and prelimbic prefrontal cortex (IL and PrL, respectively), and in nucleus accumbens core and shell (AcbC and AcbSh, respectively), in Experiments 1 and 2 as a function of prenatal treatment (daily administrations of 0.0 or 2.0 g/kg ethanol, i.g., on gestational days 17–20) and postnatal treatment during adolescence (0.0 or 2.5 g/kg ethanol, i.g., or no treatment). Values express mean \pm SEM.

Prenatal Condition	Postnatal Condition	Experiment 2					Experiment 3				
		IL	PrL	AcbC	AcbSh	IL	PrL	AcbC	AcbSh		
Prenatal Vehicle (0.0 g/kg, i.g., Gestational Days 17–20)	Untreated group	60.65 \pm 14.41	39.58 \pm 4.97	2.87 \pm 0.56	13.20 \pm 3.01	39.55 \pm 9.94	30.57 \pm 9.89	3.40 \pm 1.06	6.13 \pm 2.53		
	0.0 g/kg ethanol group	64.55 \pm 13.99	54.25 \pm 15.05	2.87 \pm 0.74	9.17 \pm 1.35	34.20 \pm 5.93	41.47 \pm 13.91	17.00 \pm 13.73	8.70 \pm 2.04		
	2.5 g/kg ethanol group	83.16 \pm 23.07	44.92 \pm 10.62	2.41 \pm 0.92	11.83 \pm 1.84	40.73 \pm 8.13	30.28 \pm 3.49	3.60 \pm 1.24	6.93 \pm 2.45		
Prenatal Ethanol (2.0 g/kg, i.g., Gestational Days 17–20)	Untreated group	29.71 \pm 5.19	27.67 \pm 5.51	3.80 \pm 2.69	8.92 \pm 2.03	15.21 \pm 4.63	16.92 \pm 6.00	3.50 \pm 0.78	1.67 \pm 0.95		
	0.0 g/kg ethanol group	43.03 \pm 8.08	34.96 \pm 7.24	5.24 \pm 2.83	15.17 \pm 3.99	29.56 \pm 13.31	50.67 \pm 13.31	3.33 \pm 1.07	9.33 \pm 1.76		
	2.5 g/kg ethanol group	47.91 \pm 12.16	31.25 \pm 5.97	6.15 \pm 3.11	15.27 \pm 6.04	24.00 \pm 7.61	35.63 \pm 5.95	2.17 \pm 1.38	11.67 \pm 4.26		