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PRENATAL BINGE-LIKE ALCOHOL EXPOSURE ALTERS BRAIN AND SYSTEMIC RESPONSES TO REACH SODIUM AND WATER BALANCE

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Abstract—The aim of the present work is to analyze how prenatal binge-like ethanol exposure to a moderate dose (2.0 g/kg; group Pre-EtOH) during gestational days (GD) 17–20 affects hydroelectrolyte regulatory responses. This type of exposure has been observed to increase ethanol consumption during adolescence (postnatal day 30–32). In this study we analyzed basal brain neural activity and basal-induced sodium appetite (SA) and renal response stimulated by sodium depletion (SD) as well as voluntary ethanol consumption as a function of vehicle or ethanol during late pregnancy. In adolescent offspring, SD was induced by furosemide and a low-sodium diet treatment (FURO + LSD). Other animals were analyzed in terms of immunohistochemical detection of Fra-like (Fra-LI-ir) protein and serotonin (5HT) and/or vasopressin (AVP). The Pre-EtOH group exhibited heightened voluntary ethanol intake and a reduction in sodium and water intake induced by SD relative to controls. Basal Na and K concentrations in urine were also reduced in Pre-EtOH animals while the induced renal response after FURO treatment was similar across prenatal treatments. However, the correlation between urine volume and water intake induced by FURO significantly varied across these treatments. At the brain level of analysis, the number of basal Fra-LI-ir was significantly increased in AVP magnocellular neurons of the paraventricular nucleus (PVN) and in 5HT neurons in the dorsal raphe nucleus (DRN) in Pre-EtOH pups. In the experimental group, we also observed a significant increase in Fra-LI along the nucleus of the solitary tract (NTS) and in the central extended amygdala nuclei. In summary, moderate Pre-EtOH exposure produces long-lasting changes in brain organization, affecting basal activity of central extended amygdala nuclei, AVP neurons and the inhibitory areas of SA such as the NTS and the 5HT-DRN. These changes possibly modulate the above described variations in basal-induced drinking behaviors and renal regulatory responses. © **2015 Published by Elsevier Ltd. on behalf of IBRO.**

Key words: prenatal ethanol exposure, sodium balance, serotonergic neurons, vasopressinergic neurons.

INTRODUCTION

It has been widely demonstrated that the effects of prenatal alcohol exposure on offspring are mainly related to the amount of drug consumed and to the period of pregnancy in which exposure occurs. A recent meta-analytical study shows that, despite the well-known consequences of high prenatal alcohol exposure during most of the pregnancies (Bailey and Sokol, 2008; U.S. Department of Health and Human Services, 2000), which include fetal alcohol syndrome and other fetal alcohol spectrum disorders (FASDs), the effects of mild to moderate prenatal alcohol exposure on neurodevelopment and neurophysiological order are inconsistent in the literature (Flak et al., 2014). Mild or moderate drinking patterns are more frequent in the pregnant population and therefore it is important to determine whether these patterns induce behavioral and physiological disruptions in the progeny. In the United States, for example, from 1991 through 2005, 12% of pregnant women reported consuming at least one alcoholic drink a month (Center of Disease Control and Prevention, 2009).

Our previous studies with rats showed that administration of mild-to-moderate doses of ethanol (2 g/kg) in pregnant females (gestational days 17–20) has behavioral consequences in the offspring. This prenatal binge-like ethanol exposure increases alcohol intake during infancy and adolescence (Molina et al., 1995; Chotro and Spear, 1997; Chotro and Arias, 2003; Spear

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Abbreviations: 5HT, serotonergic system; 5-HT-ir, 5-HT immunoreactivity; AP, area postrema; AVP, vasopressinergic system; BNSTL, lateral division of the bed nucleus of the stria terminalis; CD, sham-depleted rats; CeA, central amygdaloid nucleus; CVOs, circumventricular organs; DAB, diaminobenzidine hydrochloride; DRN, dorsal raphe nucleus; DRV, ventral subdivisions of DRN; Fra-LI, Fra like immunoreactivity; FURO, Furosemide; FURO + LSD, Furosemide and low-sodium diet; GD, gestational day; LPBN, the lateral parabrachial nucleus; MnPO, median preoptic nucleus; Na, sodium; NHS, normal horse serum; NTS, nucleus of the solitary tract; OT, immunoreactivity; OVLT, organum vasculosum of the lamina terminalis; PaLM, lateral magnocellular group; PaMM, medial magnocellular group; PB, phosphate buffer; PDN, postnatal day; Pre-EtOH, prenatally exposed animals; Pre-Water, prenatally control animals; PVN, paraventricular nucleus; SA, sodium appetite; SD, sodium depletion; SFO, subfornical organ; SON, supraoptic nucleus; Veh, vehicle.

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and Molina, 2005; Fabio et al., 2013). In addition and as indicated by Flak et al. (2014), there is increasing evidence that early exposure to moderate ethanol doses affect neural plasticity and consequently has negative physiological and neurological effects throughout the life span of an organism.

Thirst and sodium appetite (SA) are the motivational states leading to the search for and consumption of water and sodium in order to reestablish hydroelectrolyte balance. When body sodium depletion (SD) occurs, hypovolemia and hyponatremia activate the renin–angiotensin–aldosterone system (''RAAS''). This system stimulates vasoconstriction and releases aldosterone (ALDO) and vasopressin (AVP) into the bloodstream, thus increasing renal reabsorption of sodium and water to restore the volume of the extracellular space (Vivas et al., 2013). We have previously investigated the brain areas and neurochemical systems involved in the control of SA following SD (Franchini and Vivas, 1999; Franchini et al., 2002; Godino et al., 2007; Margatho et al., 2015). In these studies, the CVOs of the lamina terminalis, subfornical organ (SFO) and organum vasculosum of the lamina terminalis (OVLT), were found to be activated (as shown by Fos immunoreactivity; Fos-ir) during SA stimulation. On the other hand, the brainstem nuclei (such as the nucleus of the solitary tract (NTS), area postrema (AP) and the lateral parabrachial nucleus (LPBN)) and the serotonergic (5HT) neurons in the dorsal raphe nucleus (DRN) were also activated during the inhibition or satiety phase of SA.

It has been demonstrated that the neural circuit involved in the control of both ethanol and sodium consumption behaviors shares common pathways and neurochemical systems. For example, the bed of the stria terminalis and the central amygdala nucleus that form part of the extended amygdala complex are involved in the modulation of ethanol preference and SA (Johnson et al., 1999; Ryabinin et al., 1997). In addition, the AVP and 5HT neurochemical central systems participate in the control of hydroelectrolyte homeostasis and alcohol abuse (Druse et al., 1991; Sari et al., 2001; Knee et al., 2004; Kim et al., 2005; Bird et al., 2006; Sanbe et al., 2008; Oreland et al., 2011).

It has also been shown that prenatal ethanol exposure affects the central AVP and 5HT systems. Previous studies in prenatally ethanol-exposed animals have shown a reduction in synthesis, storage, and release of AVP in response to hyperosmolality and hemorrhage (Knee et al., 2004; Bird et al., 2006). Moreover, effects of *in utero* ethanol exposure produced: (a) decreases of 5HT and tryptophan hydroxylase expression within the DRN of rat offspring (Kim et al., 2005); (b) reductions in the number of 5HT DRN neurons and the density of serotonergic fibers in the forebrain (Sari et al., 2001), and (c) a decline of 5-HT1A receptors in the brain stem and cortex (Druse et al., 1991). These results were obtained using high-to-moderate ethanol doses administered for prolonged periods of time during pregnancy; a procedure known to induce serious teratological alterations.

The aim of the present study is to determine the effect of prenatal binge-like ethanol exposure (2 g/kg) during gestational days 17–20, a procedure known to increase postnatal ethanol affinity (Molina et al., 1995; Chotro and Spear, 1997; Chotro and Arias, 2003; Spear and Molina, 2005; Fabio et al., 2013), upon hydroelectrolyte regulatory responses. Specifically we evaluated sodium intake and renal responsiveness induced by body SD during adolescence. In addition, we also examined the impact of prenatal ethanol exposure upon neuroanatomical substrates via immunohistochemical detection of Fra-LI, alone or combined with 5HT and AVP at the brainstem and forebrain levels, respectively.

EXPERIMENTAL PROCEDURES

Subjects

All animals employed in this study were Wistar-derived rats born and reared at the vivarium of the Instituto Ferreyra (INIMEC-CONICET-UNC), Córdoba, Argentina. The animal colony was kept at 22–24 °C and under artificial lighting conditions (lights on 08:00–20:00 h). Maternal-enriched lab chow (Cargill, Buenos Aires, Argentina) and water were available *ad libitum*. Vaginal smears of adult female rats were microscopically analyzed on a daily basis. On the day of proestrus, females (pre-pregnancy body weight: 200–300 g) were housed during the dark cycle with males (three females per male). Vaginal smears were checked the following morning (10:00–12:00 h) and the day of sperm detection was considered as gestational day 0 (GD 0). Births were checked daily (10:00–12:00 h) and the day of parturition was considered as postnatal day 0 (PD 0). On PD 1, each litter was randomly culled to eight pups (four males and four females, whenever possible). Pregnant females or litters were individually placed in standard maternity cages filled with wood shavings.

At all times, animals used in this study were maintained and treated according to the guidelines for animal care established by the Guide for Care and Use of Laboratory Animals (National Institutes of Health, Institute of Laboratory Animal Resources, 1996).

Maternal treatments

From GDs 17 to 20, pregnant females were weighed and intragastrically intubated with 2.0 g/kg ethanol (Pre-EtOH group). This dose was delivered on a daily basis and was achieved by administering 0.015 ml/g of a 16.8% v/v ethanol solution. The vehicle (Veh) used was room temperature tap water. Control females (Pre-Water group) were administered with this Veh. The ethanol dose and the days of administration were selected on the basis of prior studies demonstrating fetal chemosensory and interoceptive processing of the drug under similar experimental circumstances and the general lack of deleterious effects of ethanol upon different infantile gross morphological and behavioral parameters (Abate et al., 2008; Molina et al., 1995; Domínguez et al., 1996, 1998; Pueta et al., 2005). Intragastric intubations were performed employing a polyethylene cannula (PE 50; Clay Adams, Parsippany, New Jersey, U.S.A.) attached to a disposable 5-ml syringe.

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Offspring treatment

At PD 30, periadolescents derived from dams given late prenatal ethanol or Veh (Pre-EtOH and Pre-Water group, respectively) were individually allocated to metabolic cages and had home-cage adaptation sessions to graded tubes filled with distilled tap water and sodium chloride (NaCl 2%) for 3 days. During these sessions, animals were evaluated in terms of spontaneous sodium and water ingestion and basal urine output. Sham sodium depletion (CD) with a corresponding drinking test (2% NaCl and water access during 1 h) was also performed at this time. The adaptation session was meant to facilitate the transition between drinking from standard bottles to drinking from the novel tubes of sodium chloride and water. After adaptation, the animals were submitted to SD using furosemide combined with a low-sodium diet (FURO + LSD) to induce SA. In order to confirm the effect of prenatal ethanol exposure on the increased alcohol consumption during adolescence, another group of offspring were allocated to metabolic cages but had access to graded tubes filled with distilled tap water and ethanol (EtOH 3% v/v).

To analyze the stimulation of the brain and the possible different neurochemical systems changes induced by prenatal ethanol treatment in adolescent offspring, we run the other group of dams and male offspring at PD30 (without any postnatal treatment or adaptation) but derived from dams exposed to ethanol or Veh during late pregnancy (Pre-EtOH and Pre-Water group, respectively) were perfused for immunohistochemical detection of Fra as a chronic neuronal activation marker in combination with AVP or 5HT.

SA studies. SA was stimulated by acute treatment with the diuretic natriuretic furosemide (FURO, Astra, USA Inc.) in combination with a sodium-deficient diet (ICN, CostaMesa, CA, USA). Animals were injected subcutaneously with FURO (10 mg/kg in isotonic saline Veh). Immediately after treatment, they were placed in clean individual metabolic cages and for the next 24 h were given access only to distilled water and a sodium-deficient diet. Sham sodium depletion (CD) was performed by injecting isotonic saline (NaCl 0.9%) subcutaneously in combination with a normal sodium diet for 24 h. Then, food was removed and the animals had access to fluids in a two-bottle preference test in which they were offered water and 2% of NaCl solution. The volume of ingested fluids was recorded for 1 h and expressed as ml/100 g of body weight.

Urine electrolyte assays. Urine was collected at baseline and after 24 h FURO administration in both groups. The urine was centrifuged and 1 ml extracted and stored at −20 °C. Sodium, potassium and chloride concentration of these samples was analyzed by flame photometry (Hitachi 911, automatic analyzer).

Immunohistochemistry. Animals were perfused for immunohistochemical detection of Fra and AVP or Fra and 5-HT. For this purpose, the groups of rats were anesthetized with thiopentone (100 mg/kg ip) and perfused transcardially with ~100-ml normal saline followed by ~400 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.2). The brains were removed, fixed in the same solution overnight, and then stored at 4 °C in PB containing 30% sucrose. Coronal sections were cut into two series of 40 μm using a freezing microtome and were placed in a mixture of 10% H_2O_2 and 10% methanol until oxygen bubbles ceased appearing. They were then incubated in 10% normal horse serum (NHS) in PB for 1 h to block non-specific binding sites.

All the series of free-floating sections from each brain were first processed for Fra immunoreactivity using an avidin–biotin–peroxidase procedure. The sections of the midbrain were then also stained for 5-HT immunoreactivity (5-HT-ir), and those from the hypothalamus for AVP (AVP-ir). The staining procedures followed the double-labeling procedures described in Godino et al., 2005. Briefly, the free-floating sections were incubated overnight at room temperature in a rabbit anti-fra using an affinity-purified rabbit polyclonal antibody (c-fos K-25, Santa Cruz Biotechnology, SantaCruz, CA, USA) recognizing amino acids 128–152 in the NH2-terminal region of Fos. This antibody recognizes Fos, Fos-B, Fra-1, and Fra-2 proteins, diluted 1:10,000 in PB containing 2% NHS (Gibco, Auckland, NZ) and 0.3% Triton X-100 (Sigma Chemical Co., St. Louis, MO, USA). The sections were then washed with PB and incubated with biotin-labeled anti-rabbit immunoglobulin and the avidin–biotin–peroxidase complex (Vector Laboratories Inc., Burlingame, CA, USA, 1:200 dilutions in 1% NHS-PB) for 1 h at room temperature. The peroxidase label was detected using diaminobenzidine hydrochloride (DAB, Sigma Chemical Co., St. Louis, MO, USA) intensified with 1% cobalt chloride and 1% nickel ammonium sulfate. This method produces a blue-black nuclear reaction product. The series of Fos-labeled sections, also processed for immunocytochemical localization of 5HT and AVP, were incubated for 72 h at 4 °C with their corresponding antibodies: polyclonal rabbit anti-5HT antibody (ImmunoStar Inc, WI, USA, dilution 1:10,000) and polyclonal rabbit anti-AVP antibody (Calbiochem, dilution: 1:25,000). After incubation, the sections were rinsed and incubated with biotin-labeled anti-rabbit immunoglobulin and an avidin–biotin–peroxidase complex for 1 h at room temperature. Cytoplasmic 5-HT-ir and AVP-ir were detected with unintensified DAB that produces a brown reaction product. Finally, the free-floating sections were mounted on gelatinized slides, air-dried overnight, dehydrated, cleared in xylene and placed under a coverslip with DePeX (Fluka, Buchs, Switzerland).

Cytoarchitectural and quantitative analysis. The brain nuclei exhibiting Fra-LI were identified and delimited according to the rat brain atlas of Paxinos and Watson (1997). The different subnuclei of paraventricular nucleus (PVN) analyzed in the present work were counted at two different levels, the medial magnocellular (PaMM) and the lateral magnocellular (PaLM) (distance from the bregma

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of the corresponding plates: −1.30 mm and, −1.8 mm). The distance from the bregma of the corresponding plates is indicated between brackets: Supraoptic nucleus (SON, −1.3 mm), SFO (−0.92 mm), OVLT (−0.20 mm), median preoptic nucleus (MnPO, −0.30 mm), central amygdaloid nucleus (CeA, −22.3 mm), bed nucleus of the stria terminalis, laterodorsal subdivision (BSTLD, −20.26 mm), DRN (−8.00 mm), LPBN (−9.3 mm), NTS (−13.24) and AP (−13.68 mm).

Fra-LI nuclei were quantified using a computerized system including a Zeiss microscope equipped with a DC 200 Leica digital camera attached to a contrast enhancement device. Images were digitalized and analyzed using Scion Image PC, based on the NIH 1997 version. Fra-li cells in each section were counted by setting a size range for cellular nuclei (in pixels) and a threshold level for staining intensity. Representative sections in each group were acquired at exactly the same level, with the aid of the Adobe Photoshop Image Analysis Program, version 5.5. The counting was done in six animals of each condition, and was repeated at least twice on each section analyzed, to ensure that the number of profiles obtained was similar. The investigator who conducted the counting of Fra-LI cells was blinded to the experimental condition.

Experimental design

As shown in Fig. 1, we performed prenatal ethanol treatment during the last days of gestation with a daily i. g. infusion of ethanol (Pre-EtOH group) or water Veh (Pre-Water group). The offspring were analyzed at different times after weaning. Initially, a group of subjects (PD 30) were intracardially perfused to perform the immunohistochemical analysis of a chronic neuronal activation marker, Fra, alone or combined with 5HT and AVP, in order to analyze which areas previously involved in the control of ethanol abuse and hydroelectrolyte balance suffer neuroadaptive changes from early alcohol exposure ($n = 6$). At PD31, two groups of rats, representative from each prenatal treatment, were separated and the first subgroup was used to confirm or not our previous results showing that prenatal ethanol exposure increases alcohol consumption in adolescents (Pre-EtOH $n = 27$ and Pre-Water $n = 24$). For this purpose an ethanol intake 2-bottle choice test was performed. The percentage of ethanol preference was also calculated by dividing the ethanol consumption by the total fluid consumption (ethanol plus water). The other subgroup was placed in metabolic cages. In the adaptation period, spontaneous or basal consumption of sodium (2% NaCl) and water (Pre-EtOH $n = 16$ and Pre-Water $n = 13$) as well as basal urine output (Pre-EtOH $n = 14$ and Pre-Water $n = 9$) were measured during 3 days. The first day we also performed a sham sodium depletion (CD) consisting in the same manipulation (sc injection of isotonic saline) but with access to a normal sodium diet, and 24 h later we recorded the amount of sodium (2% NaCl) and water consumed during 1 h. On PD 34 animals were sodium depleted using the FURO + LSD protocol and the water intake and urine output evoked by furosemide during 24 h was analyzed. Finally, 24 h after Furo + LSD, when SA was stimulated, we offered an intake test with sodium (2% NaCl) and water access (1 h) and recorded the volume drunk from each solution (Pre-EtOH $n = 16$ and Pre-Water $n = 13$).

Statistical analysis

The data of urine electrolytes and chronic brain activity pattern and the activity of the AVP and 5HT systems were analyzed using Student's *t*-tests. MANOVAs were employed to analyze ethanol, water or total fluid consumption (mls). These MANOVAs were defined by prenatal treatment and gender as independent factors

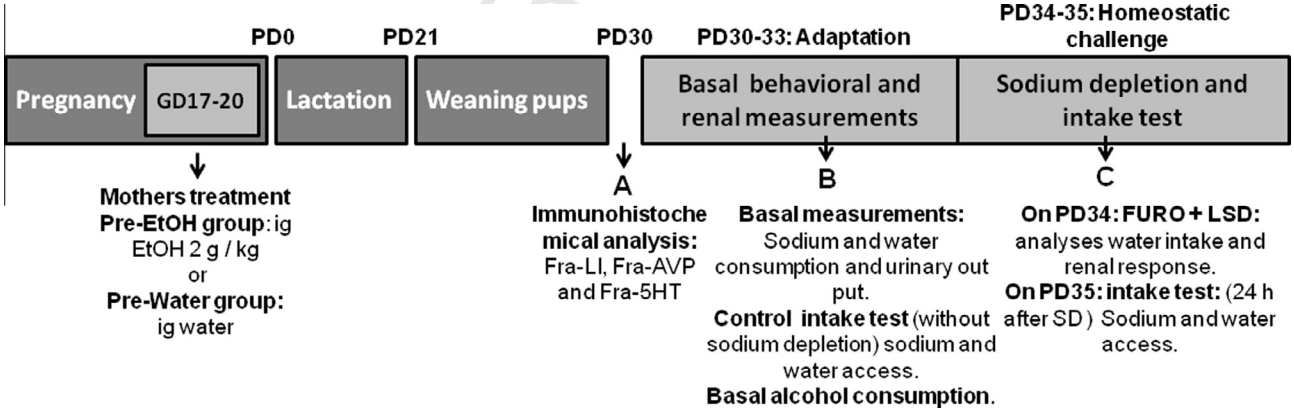

Fig. 1. Schematic diagram showing the sequence of procedures applied to pregnant females and their corresponding offspring. Mothers were treated during late gestation with a daily i.g. infusion of 2.0 g/kg ethanol (Pre EtOH group) while controls received water (Pre-Water group). Postnatal evaluations of the offspring were conducted at different times after weaning: (A) Offspring representatives from each prenatal treatment were perfused without any postnatal treatment in order to perform the immunohistochemical detection of Fra-LI, Fra-AVP and Fra-5HT (PD30), (B) other periadolescents (PDs 30–33) were placed in individual metabolic cages to measure the basal consumption of sodium (2% w/v NaCl) and water and the basal urinary output during three consecutive days, (C) additional periadolescents (PDs 30–33) were subjected to voluntary ethanol intake tests (drinking tubes containing 3% v/v ethanol or tap water) and (d) at PD 34, the offspring described in item "B" were sodium depleted using FURO + LSD protocol and we analyzed during 24 h after treatment the water intake and urinary output evocated by furosemide. Finally, on PD 35, 24 h Furo + LSD we offered an intake test with sodium (2% NaCl) and water access (1 h).

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and testing days as repeated measures. A similar inferential analysis was employed when considering sodium consumption (mls/100 g body weight). Post hoc comparisons were made using the Tukey test with an alpha set at 0.05. The correlation of water intake and urine volume induced by furosemide was analyzed using Pearson's correlation coefficients. For descriptive purposes all the results are presented as group mean values ± standard errors of the means (SEMs).

RESULTS

Baseline brain pattern of Fra-LI and double-immunolabeled (Fra-5-HT and Fra-AVP) cells in adolescent pups prenatally treated with EtOH

Pattern of Fra-AVP-positive neurons along the magnocellular neurons of the hypothalamus. Prenatal treatments did not significantly affect Fra-AVP-positive neurons in the SON (Fig. 2). However, the magnocellular subdivisions of the paraventricular nucleus (PaLM and PaMM: lateral and medial magnocellular subdivisions, respectively) showed a significant increase in Fra-AVP-positive cells in the animals prenatally exposed to ethanol in comparison to the control group (PaLM: $t_{(10)} = -2.30$, $P < 0.05$ and PaMM: $t_{(10)} = -3.42$, $P < 0.05$).

Pattern of Fra-5HT-positive neurons along the dorsal raphe nucleus. As shown in Fig. 3, there was a significant difference in the Fra-LI serotonergic-positive cells along the DRN as a function of prenatal treatment ($t_{(10)} = -2.34$, $P < 0.05$). Prenatal ethanol exposure relative to Veh controls was found to significantly increase basal chronic activation of 5HT neurons in the DRN.

Pattern of Fra-LI-positive neurons in other nuclei involved in sodium intake regulation. We also analyzed the pattern of basal chronic activity along the lamina terminalis (4A), brain stem nuclei (4B) and central extended amygdala (4C), areas known to be implicated in the control of ethanol consumption and hydroelectrolyte balance (Grobe et al., 2004; Godino et al., 2007; Vilpoux et al., 2009; Margatho et al., 2015). The lamina terminalis consists of the MnPO and two

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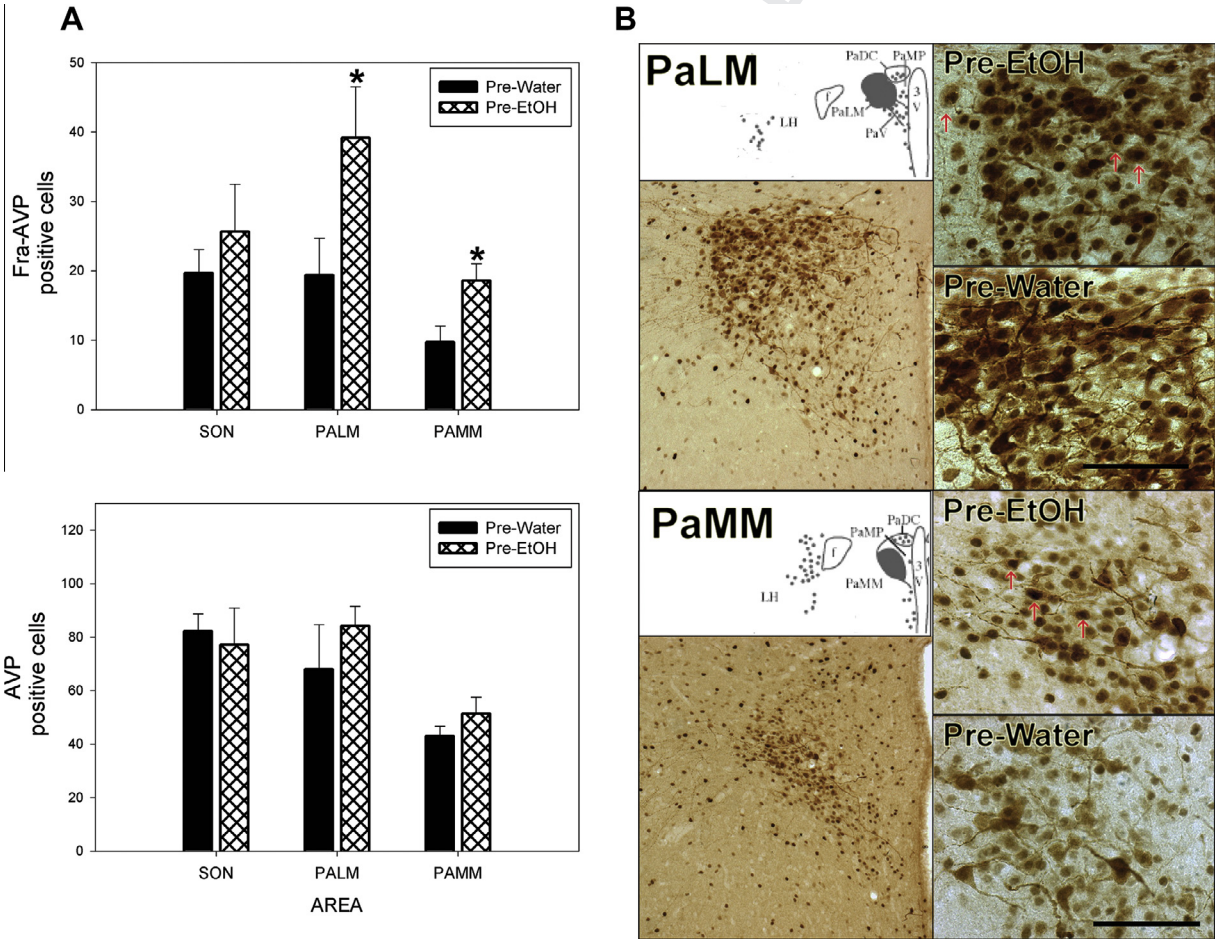

Fig. 2. (A) Average number of double-immunolabeled Fra-vasopressin (AVP) neurons (upper graph) and average number of AVP-positive neurons (down graph) in the supraoptic nucleus and magnocellular subdivision of paraventricular nucleus: lateral (PaLM) and medial (PaMM) subdivisions in adolescence prenatally treated with EtOH (plaid bars) and control pups (Pre-Water: black bars) on PD 30 without any postnatal treatment. Test *t* analysis. Values are means ± SE; $n = 6$. $^{*}P < 0.05$. (B) Pattern of double immunoreactivity cells (Fra-AVP) within PaLM and PaMM in animals prenatally treated with EtOH or water. Scale bar = 100 μm.

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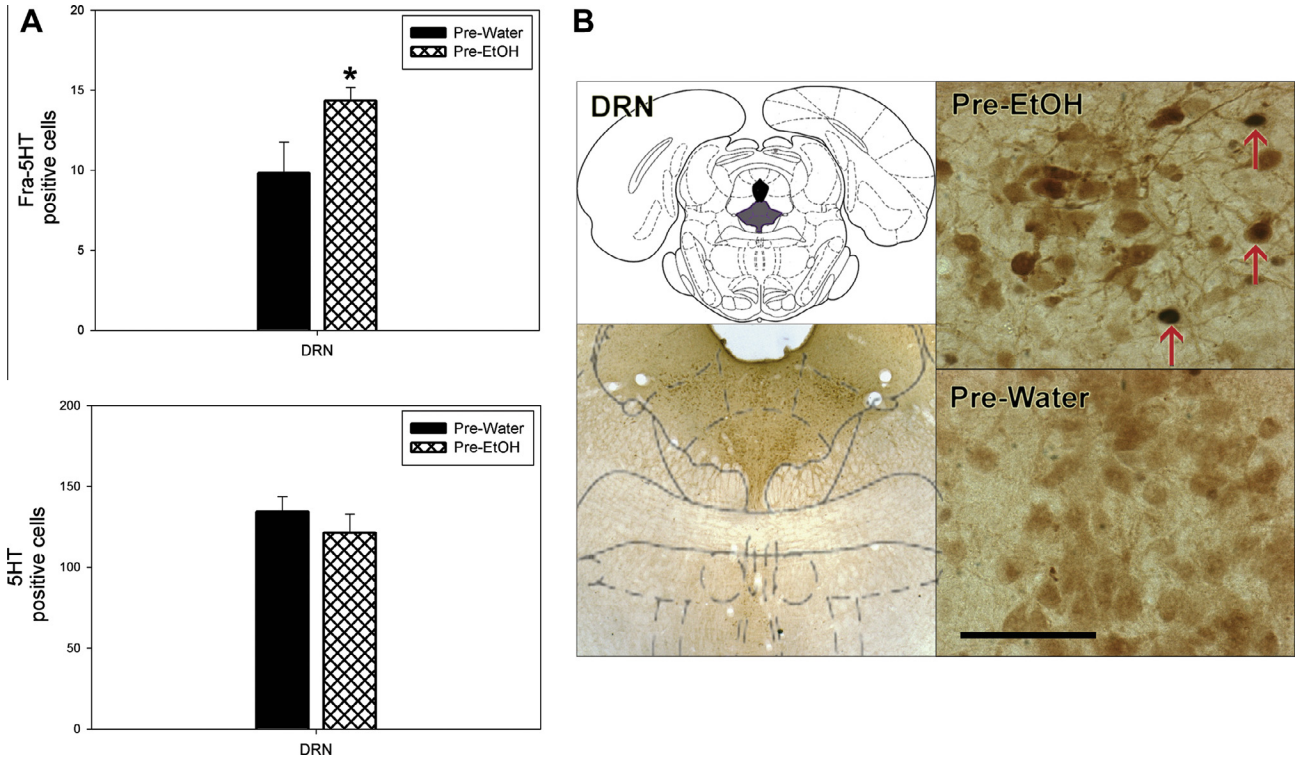

Fig. 3. (A) Average number of double-immunolabeled Fra-serotonin (5HT) neurons (upper graph) and average number of 5HT-positive neurons (down graph) in the dorsal raphe nucleus (DRN) in adolescence prenatally treated with EtOH (plaid bars) and control pups (Pre-Water: black bars) on PD 30 without any postnatal treatment. Test *t* analysis. Values are mean ± SE; $n = 6$. $^{*}P < 0.05$. (B) Pattern of double immunoreactivity cells (Fra-5HT) within DRN animals prenatally treated with EtOH or water. Scale bar = 100 μm.

circumventricular organs: OVLT and the SFO. We found no significant difference in these structures (Fig. 4A).

As shown in Fig. 4B, along the brainstem extension, we found a significant increase in Fra-LI immunoreactivity in the NTS (NTS, $t_{(10)} = -4.90$, $P < 0.001$) in ethanol-exposed animals. In the LPBN, Fra-LI immunoreactivity showed a tendency to increase in the Pre-EtOH group, but it did not reach a statistically significant level ($t_{(10)} = -1.88$, $P < 0.05$). Finally, the AP, a brainstem circumventricular organ, did not show significant differences in relation to prenatal manipulation.

The central extended amygdala consists of the CeA and BSTLD, which are also implicated in drinking behaviors (Margatho et al., 2015). We found significant increases in Fra-LI immunoreactivity in these areas in adolescents prenatally exposed to ethanol relative to controls (CeA: $t_{(10)} = -5.6$, $P < 0.001$ and BSTLD: $t_{(10)} = -3.69$, $P < 0.05$), Fig. 4C.

Baseline drinking behavior and urine output in adolescent pups prenatally treated with EtOH

Voluntary ethanol consumption. In agreement with our previous work (Fabio et al., 2013), our present study demonstrates that prenatal binge-like ethanol exposure during late gestation significantly increases voluntary ethanol consumption during adolescence. Fig. 5A shows the volume of 3% v/v alcohol, water and total fluid drunk by adolescents prenatally treated with ethanol or water during the two-bottle test. Pups prenatally exposed to ethanol exhibited a significant increase in terms of 3% v/v ethanol consumption (prenatal treatment factor: $F_{(1, 47)} = 17.301$, $P < 0.001$) and a significant reduction in water intake during these days (prenatal treatment factor: $F_{(1, 47)} = 10.666$, $P < 0.05$ and repeated measures: $F_{(2, 94)} = 6.2856$, $P < 0.05$). Total fluid consumption was not different between groups. Regarding the spontaneous ethanol intake we did not observe any significant differences in the sex factor (sex factor: $F_{(1, 47)} = 0.26$, $P = 0.6$ and Sex X prenatal treatment interaction $F_{(1, 47)} = 0.045$, $P = 0.8$). The analysis of percent ethanol preference (Fig. 5B) shows how brief gestational ethanol exposure can increase alcohol preference during adolescence (prenatal treatment factor: $F_{(1, 47)} = 14.040$, $P < 0.001$ and repeated measures: $F_{(2, 94)} = 3.2848$, $P < 0.05$).

Spontaneous or need-free 2% NaCl and water consumption. Spontaneous or need-free NaCl intake during the 3 days of adaptation showed no significant differences between the groups prenatally treated with ethanol or with water ($F_{(1, 46)} = 0.61938$, $P = 0.443$). Non significant differences across prenatal treatments were also attained when considering water consumption: $F_{(1, 46)} = 0.53$, $P = 0.47$) or total fluid intake (Current effect: $F_{(1, 46)} = 0.002$, $P = 0.96$). Regarding the spontaneous sodium intake we did not observe any significant differences in the sex factor (sex

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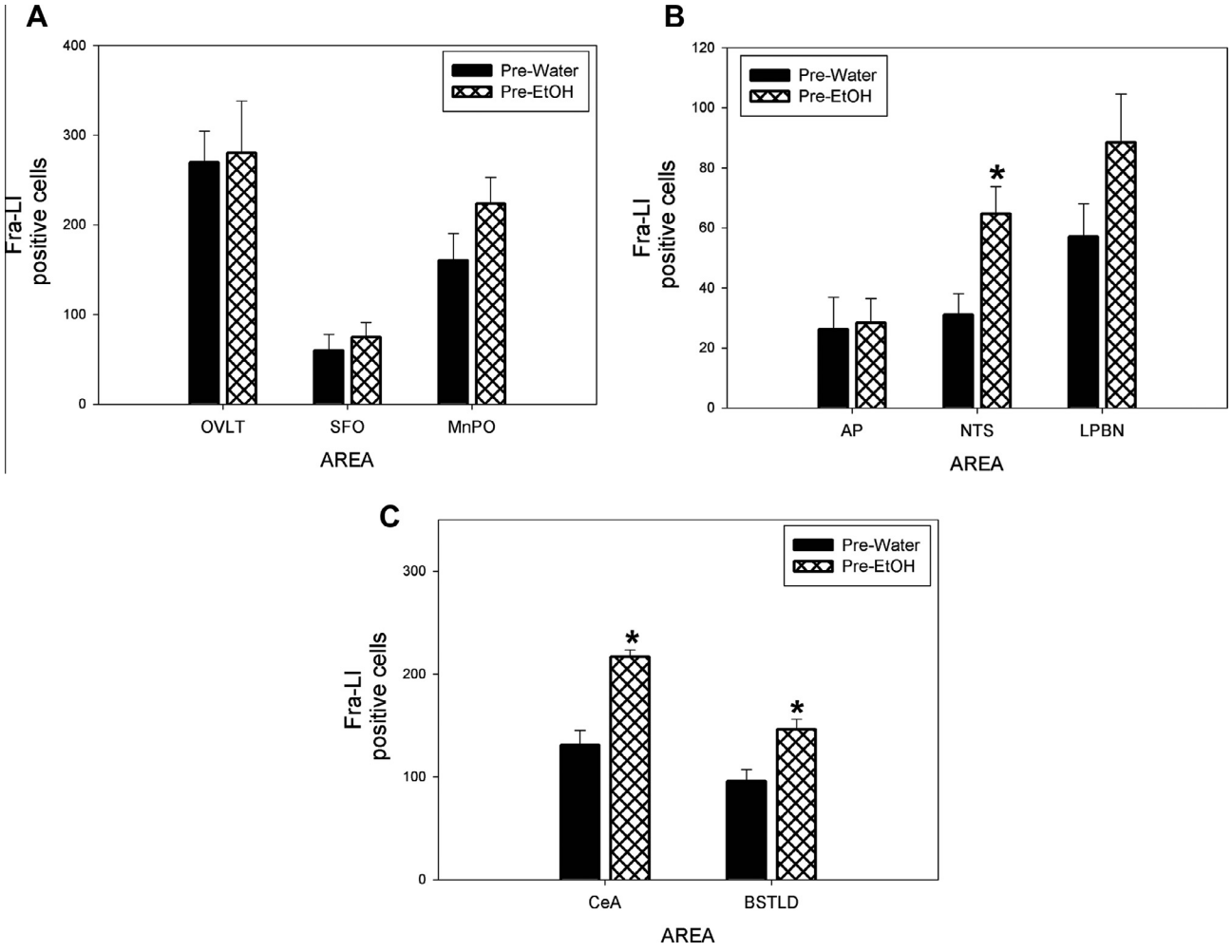

Fig. 4. (A) Average umber of Fra-LI-positive neurons along the lamina terminalis nuclei (OVLT, SFO and MnPO), (B) brainstem nuclei (AP, NTS and LPBN) and in (C) Central extended amygdala nuclei (CeA and BSTLD) in adolescence prenatally treated with EtOH (plaid bars) and control pups (Pre-Water: black bars) on PD 30 without any postnatal treatment. Test *t* analysis. Values are mean ± SE; $n = 6$. $^{*}P < 0.05$.

factor: $F_{(1,\ 46)} = 0.77$, $p = 0.38$ and Sex X prenatal treatment interaction: $F_{(1,\ 46)} = 0.76$, $P = 0.39$).

Basal urine output. As shown in Table 1, periadolescents prenatally exposed to ethanol, exhibited significant reductions in sodium and potassium concentrations in basal urine (Na: Current effect: $F_{(1,\ 19)} = 5.29$, $P < 0.05$ and K: $F_{(1,\ 19)} = 6.72$, $P < 0.05$). We did not observe any significant difference across the sex in these basal urine ion concentration (Sodium concentration: $F_{(1,\ 19)} = 2.01$, $P = 0.17$ and Potassium concentration: $F_{(1,\ 19)} = 0.051$, $P = 0.82$). However, across prenatal treatments, there were no differences in urine chloride concentration or in the total excretion of sodium, potassium and chloride. The urine volume was not different between the groups.

Homeostatic challenge: SD induced by FURO + LSD in adolescents prenatally treated with EtOH

Renal response induced by furosemide treatment. We studied the renal response after FURO treatment, which induces SD and stimulates SA 24 h later. The renal response 24 h after FURO administration was not significantly different in the pre-EtOH group in relation to the water control group (Table 2). We did not observe any statistically significant differences in these urinary ions according to sex factor.

Correlation between urine volume and water intake induced by SD. We analyzed the correlation between urine volume and water intake induced by SD (furo + LSD) in both prenatal groups. There was a positive and significant correlation between these factors in control animals (Pre-Water: $r = 0.476$, $n = 58$, $P < 0.05$). However, this correlation was not evident in Pre-EtOH animals (Pre.EtOH: $r = 0.17$, $n = 65$, $P =$ ns). Moreover, the difference between both correlation coefficients was significant (Fisher r-to-z transformation, $z = 1.83$, $P < 0.05$) (Fig. 6). This may indicate that the Pre-EtOH rats are less responsive to a hydroelectrolyte challenge such as SD induced by FURO treatment. Regarding sex factor we did not observe a significant difference between females and males in the urine

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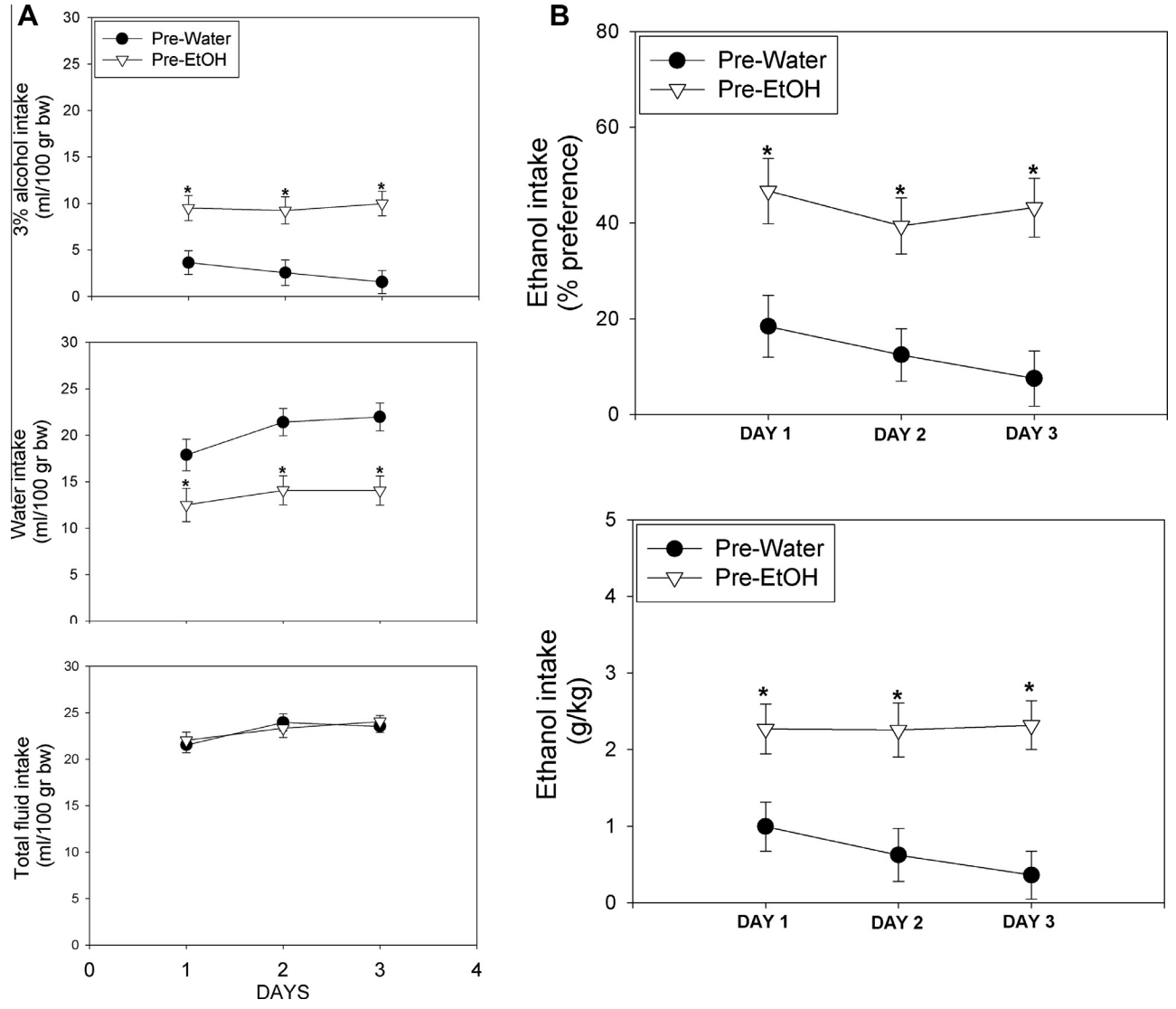

Fig. 5. (A) Daily consumption of 3% alcohol solution (ethanol concentration: 3% v/v mix in distilled water v/v), water and total fluid during 3 days in prenatally ethanol-exposed and control offspring. (B) Absolute ethanol intake (g/kg) and percent ethanol preference scores (upper and lower panels, respectively) during adaptation days in prenatally ethanol-exposed (n = 27) and control (n = 24) offspring. Data were collapsed across sex (male or female). One-way ANOVA repeated measures. Values are mean ± SE. $^{*}P < 0.05$.

volume and water intake induced by furosemide treatment correlation.

2% NaCl and water consumption induced by SD. As shown in Fig. 7, prenatally ethanol-exposed animals consumed significantly less than 2% NaCl and water after SD induced by FURO + LSD in relation to the Pre-Water group and non-sodium depleted groups. The statistical analysis was performed using repeated measures (cumulative volume of sodium and water drunk during the intake tests performed before and after SD). The MANOVA indicated a significant interaction between prenatal treatment, SD and time of assessment [$F_{(2,\ 120)} = 5.11$, $P < 0.05$] on sodium intake. In the case of water consumption, a similar significant interaction was observed [$F_{(2,\ 120)} = 13.72$, $P < 0.001$]. As we expect we observed a significant increase in the sodium intake after SD procedure. However, the Tukey post hoc analysis demonstrated that after SD there is a significant reduction in the sodium consumption in the prenatally ethanol-exposed rats in comparison to Pre-Water group at 30 and 60 min. According to our previous results we did not find significant differences in sex factor on water and sodium consumption.

Sodium balance. Sodium balance is the difference between the sodium excretion after FURO treatment and the 2% NaCl intake induced by SD. The analysis of the sodium balance shows a significant difference in relation to prenatal manipulation ($t_{(21)} = 2.55$, $P < 0.05$; Table 3). Control subjects have a positive sodium balance in contrast to the Pre-EtOH group, which barely reached balance due to the lower consumption of sodium. We did not find any significant difference regarding the sex on sodium balance.

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Table 1. Basal urinary measures in Pre-Water and Pre-EtOH

	Unit	Pre-Water	Pre-EtOH
Urine volume	ml. · 100 g bw^{-1} · 24 h	14.3 (±1.14)	13.3 (±1.50)
Na^+ excretion	meq. · 100 g bw^{-1}	1.44 (±0.08)	1.26 (±0.15)
Cl^- excretion	meq. · 100 g bw^{-1}	2.21 (±0.14)	2.14 (±0.29)
K^+ excretion	meq. · 100 g bw^{-1}	1.17 (±0.11)	0.95 (±0.12)
Na^+ concentration	meq.l.-24 h	123.4 (±11.17)	101.26 (±4.03)*
Cl^- concentration	meq.l.-24 h	100.7 (±16.1)	76.8 (±5.02)
K^+ concentration	meq.l.-24 h	189.9 (±8.19)	176.9 (±8.98)*
		$n = 9$	$n = 14$

Values are means ± SE.
* $P < 0.05$ significantly different between groups.

Table 2. Renal response in Pre-Water and Pre-EtOH after sodium depletion induced by Furosemide

	Unit	Pre-Water	Pre-EtOH
Urine volume	ml. · 100 g bw^{-1} · 24 h	20.15 (±1.35)	20.30 (±1.73)
Na^+ excretion	meq. · 100 g bw^{-1}	0.66 (±0.14)	0.65 (±0.19)
Cl^- excretion	meq. · 100 g bw^{-1}	0.91 (±0.08)	1.04 (±0.07)
K^+ excretion	meq. · 100 g bw^{-1}	0.55 (±0.045)	0.45 (±0.042)
Na^+ concentration	meq.l.-24 h	33.6 (±3.73)	37.7 (±3.75)
Cl^- concentration	meq.l.-24 h	45.1 (±5.05)	58.4 (±4.58)
K^+ Concentration	meq.l.-24 h	26.72 (±2.21)	24.04 (±1.34)
		$n = 10$	$n = 14$

Values are means ± SE.
* $P < 0.05$ significantly different between groups.

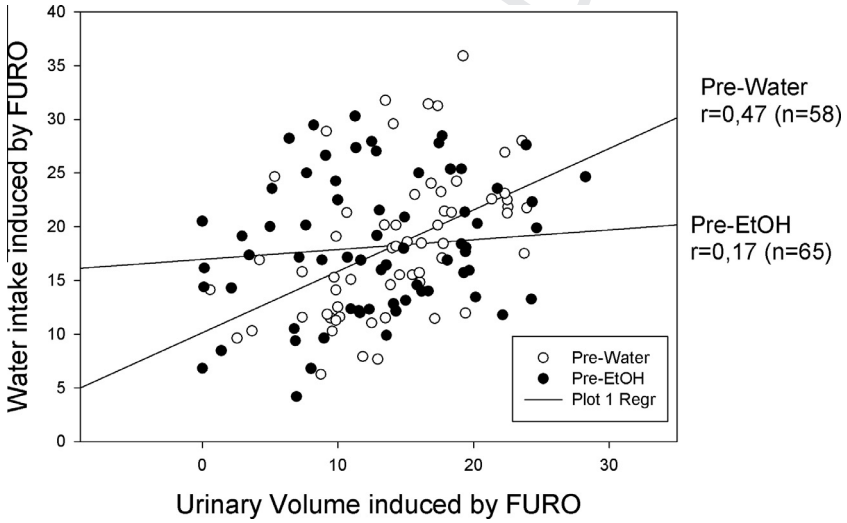

Fig. 6. Graph representing the correlation between water intake and urinary volume induced by Furosemide treatment in prenatally ethanol-exposed (black circles) and control (white circles) adolescent rats. Pearson's analysis. * $P < 0.05$.

DISCUSSION

The present study demonstrates the disruptive effects of a dose (2 g/kg) of ethanol during late gestation, considered "safe" in terms of gross teratological effects (Abate et al., 2008), on the homeostatic response under a SD challenge. Our results show that adolescents prenatally exposed to ethanol consume lower amounts of sodium and water after body SD, thus barely reaching sodium balance in relation to a control group that is in positive balance. Prenatal binge-like ethanol administration also affected the renal response, reducing basal urine sodium and potassium concentrations and therefore affecting the correlation between the urinary volume and water intake induced by FURO. Our results also show that early ethanol exposure is enough to increase basal chronic neuronal activation in adolescents along the NTS and in the 5HT cells in the DRN, both involved in the inhibition of SA and gustatory neural responses (Houpt et al., 1998; Franchini et al., 2002; Godino et al., 2007, 2013). Moreover, we observed a significant increase in Fra-LI immunoreactivity in AVP magnocellular subdivisions of the PVN, involved in neurohypophyseal AVP release, in prenatally ethanol-exposed rats

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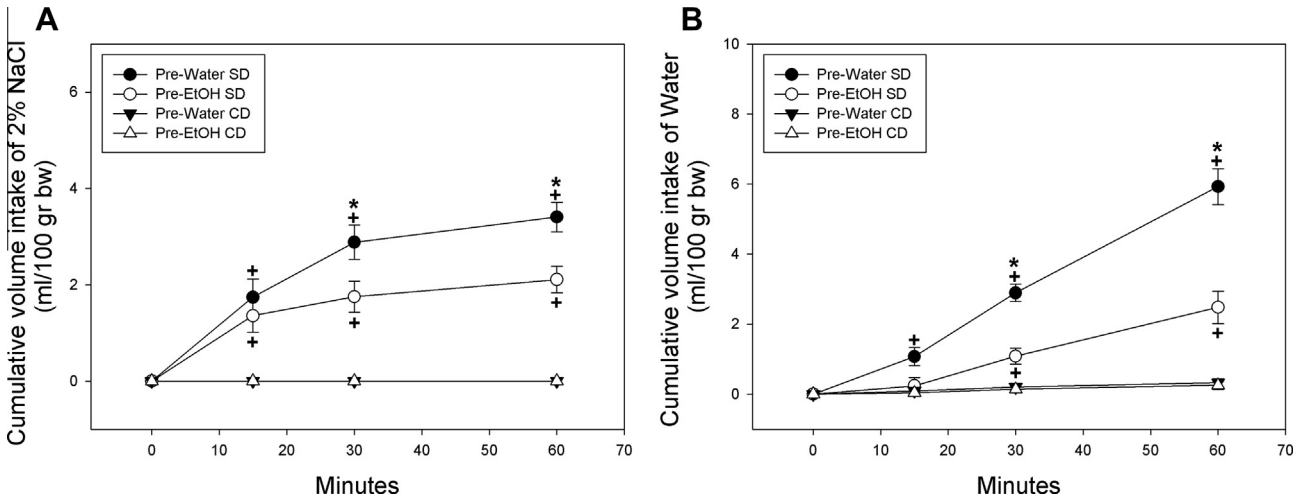

Fig. 7. Time course of the effect of prenatal ethanol exposure on induced sodium and water intake (panels A and B, respectively). Black control and white pre-EtOH, circles represent the intake after sodium depletion by the combined treatment of FURO + LSD and triangles after sham sodium depletion. First, the offspring were subjected to a Veh s.c. injection and 24 h later a two-bottle (distilled water/2% NaCl solution) access during the next 60 min (sham depletion, triangles). After a 3-day-recovery period, the offspring were again subjected to sodium depletion by the combined treatment of FURO + LSD. Twenty-four hours later they were subjected to the intake test protocol. Data are expressed as ml/100 g of body weight. All tests were carried out between 9:00 and 15:00 h. $^{+}P < 0.05$ differences in relation to control or sham-depleted group. $^{*}P < 0.05$ differences in relation to Pre-EtOH group. $n = 13$ pre-Water and $n = 16$ pre-EtOH.

Table 3. Sodium balance in Pre-EtOH and Pre-Water animals

Group	Sodium exit Na^+ excretion, meq. 100 g body $wt^{-1} \cdot 24\ h^{-1}$	Sodium enter Sodium intake, meq. 100 g body $wt^{-1} \cdot 24\ h^{-1}$	Sodium balance	*n*
Pre-Water	0.663 ± 0.027	1.165 ± 0.085	0.482 ± 0.097	9
Pre-EtOH	0.653 ± 0.037	0.721 ± 0.11*	0.078 ± 0.021*	14

Values are means ± SE.
* $P < 0.05$ significantly different from control group.

(Knee et al., 2004; Bird et al., 2006). Additionally, we found a significant increase in Fra-LI immunoreactivity in the central extended amygdala nuclei (CeA and BSTLD) involved in the modulation of fear, anxiety and drinking behaviors (Roberto et al., 2012; Johnson et al., 1999; Kash, 2012).

In the present study we used the immunohistochemical detection of Fra-LI as a chronic neuronal marker in adolescent offspring prenatally exposed to alcohol without postnatal treatment, in order to analyze whether there are persistent changes in basal activity induced by early ethanol exposure. The K-25 antibody recognizes all proteins of the Fos family. However, Fos typically has a peak at 30–90 min, decreasing at 2 h and is essentially absent at 4 h after stimulation. Fra appears 1–3 h after stimulation and may persist up to 4 weeks later (Morgan and Curran, 1989; Rosen et al., 1994; Vahid-Ansari and Leenen, 1998).

Our study showed how prenatal ethanol animals have a lower compensatory response after SD that induces SA behavior. We found that control animals reached a positive sodium balance after sodium intake induced by FURO treatment, but that the Pre-EtOH group reached a comparatively low level during the sodium access. As an osmotic consequence of this, these animals also drank significantly less water during the intake test, in order to make an isotonic cocktail and to reestablish the extracellular volume (Stricker et al., 1992).

We also observed increased neuronal activity in the NTS and 5HT cells of the DRN from the Pre-EtOH treatment. Both areas are frequently involved in the inhibition of SA. As previously described by Houpt et al. (1998) and later confirmed by our studies (Franchini and Vivas, 1999; Godino et al., 2007; Margatho et al., 2015), there was increased activity within different levels of the NTS after rats drank sodium solution. Additionally, NTS/AP-lesioned rats drank larger volumes of concentrated saline solutions than control animals after furosemide treatment (Ogihara et al., 2009). Our previous works also showed 5-HT system involvement in the inhibition of SA, since the activity of DRN serotonergic cells increased after sodium consumption induced by SD when the animals were in the process of re-establishing body sodium status levels (Franchini et al., 2002; Godino et al., 2007, 2010, 2013). Consistent with these studies, our recent results indicate that sodium overload increases the firing frequency of 5HT-DRN neurons during extracellular recording (Godino et al., 2013). We can thus postulate that the increased Fra-LI immunoreactivity in the NTS and 5HT neurons of the DRN possibly produces a tonic inhibition that slows the effect of stimulatory signals of SA after SD.

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Our present results demonstrate that basal sodium and potassium urine concentration is reduced in prenatally ethanol-exposed adolescents, but we did not find differences in basal sodium consumption. We also observed, in these animals, a lower correlation between the urinary response induced by furosemide (diuretic and natriuretic effect) and the water intake induced by the water loss produced by furosemide treatment. Similarly, Assadi (1990) found that, among six children diagnosed with fetal alcohol syndrome, none were able to concentrate their urine after 12 h of water deprivation. Their data suggested that a large factor in this alteration may be of renal origin but plasma AVP was not measured in their studies (Assadi, 1990). Our own data regarding renal response after furosemide treatment did not show significant differences between the Pre-Water and Pre-EtOH groups and the softer prenatal ethanol exposure of our model, and therefore we were able to rule out the possibility that a renal histological change could explain urinary baseline differences. On the other hand, in the present study we also demonstrate a significant increase in neuronal activity in chronic AVP cells in the PVN nucleus produced by prenatal ethanol exposure, and its activity was still present in adolescents when we performed the hydroelectrolyte challenge.

Vasopressin is a neurohormone that is released into the blood at the neurohypophysis level. The AVP released acts in the kidney promoting tubular reabsorption of the water. The increased amount of water in plasma produces an osmotic dilution of electrolytes, which may explain the reduced basal urine sodium and potassium concentration and the lower responsivity in the water balance after furosemide treatment. Chronic consumption of alcohol has been shown to significantly reduce the number of AVP-producing neurons in the rat and humans (Madeira et al., 1993; Harding et al., 1996). Most recently, prenatal exposure to a moderate dose of ethanol during most of the gestation (7–22 GD) produced decreases in the AVP response after osmotic and hemorrhage stimulation and a reduction in the pituitary AVP and hypothalamic AVP mRNA content during the adulthood of the offspring (Knee et al., 2004; Bird et al., 2006). The decrease in the basal urine sodium and potassium concentration observed in our study was possibly the result of an increased threshold of AVP stimulation produced by the early activation of AVP neurons, which increased the set point to induce AVP neurohypophyseal release. In agreement with this, Knott et al. (2002) provide a possible mechanism for prolonged ethanol exposure to produce a calcium channel plasticity that can explain at least a portion of the behavioral tolerance resulting from changes in sensitivity of peptide hormone release.

Besides its involvement in the control of water balance, the AVP system also modulates a variety of behaviors such as anxiety, aggression, and bonding (Bester-Meredith et al., 1999; Lim and Young, 2004; Wigger et al., 2004; Caldwell et al., 2008; Veenema et al., 2010). Two AVP1 receptor subtypes have been described: the AVP1a vasopressin receptor (AVPr1a) and the AVP1b vasopressin receptor (AVPr1b), which are expressed in the brain (Ostrowski et al., 1994). A recent study shows that AVPr1a knockout mice displayed an increase in ethanol consumption and preference (Sanbe et al., 2008). AVP inhibits the release of glutamate from the presynaptic terminal via AVPr1a, and the increased glutamate due to loss of the inhibitory effect in AVPr1a KO mice may play an important role in ethanol preference behavior (Sanbe et al., 2008).

Taken together, the vasopressinergic-related evidence enables us to speculate that prenatal ethanol treatment affects either the tonic response of AVP neurons (reducing glutamatergic inhibition (Sanbe et al., 2008)) or their threshold of stimulation (changing the calcium channel sensitivity (Knott et al., 2002)), thus explaining the increased ethanol preference and the differential hydrosaline homeostasis control observed in the present study.

Our present data also confirm increases in alcohol intake after an early ethanol experience during adolescence (Fabio et al., 2013). This increase could be associated with the augmented basal activity found along the extended amygdala nuclei and brainstem nuclei such as NTS and DRN. Studies indicate that the oral sensations produced by ethanol consist of both appetitive (sweet) and aversive (bitter taste and oral irritation) components (Youngentob and Glendinning, 2009). In accordance with our present result, it has been demonstrated that the administration of a moderate dose of alcohol ($1.5\ g\ kg^{-1}$) led to induction of c-Fos expression in the regions important for reinforcing as well as aversive properties of drugs. These include the extended amygdala, the PVN of the hypothalamus, the NTS and several neocortical areas (Ryabinin et al., 1997). The NTS has a group of sweet-responsive gustatory neurons (Brasser et al., 2010). The early and chronic activation of this area possibly changes the threshold of the activation of these cells modulating the reinforcing property of ethanol in animals pre-exposed to the drug and other unsweet solutions like hypertonic sodium chloride, provided in the present study. Also, it has been demonstrated that an early experience can affect the 5HT brain circuit promoting later increased alcohol consumption and ethanol-preferring rats also show a decrease in components of the 5HT central system (Druse et al., 1991; Sari et al., 2001; Kim et al., 2005; Oreland et al., 2011). In this sense, the increased Fra-LI in DRN serotonergic neurons in animals with a history of ethanol experience possibly determined an increased alcohol preference in the long term.

The extended amygdala consists of a series of extensively interconnected limbic structures including the CeA and the bed nucleus of the stria terminalis (BNST) (Johnson et al., 1999). These structures are critical regulators of behavioral and physiological activation associated with anxiety and addiction processes (Oreland et al., 2011). Additionally, numerous reports have suggested that these regions are involved in increased drinking behavior associated with chronic alcohol exposure and withdrawal (Roberto et al., 2012; Kash, 2012). In agreement with these reviews, we observed a significant increase in Fra-LI immunoreactivity in CeA and BSTLD nuclei. These data possibly explain, at least

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partially, the increase in ethanol consumption during adolescence in prenatally ethanol-exposed rats.

Together these results suggest that the early and brief ethanol experience induced chronic changes in several areas of the brain and also in specific neurochemical systems that affect the behavioral and homeostatic responses under different challenges in adolescence. These data also show a common neuroanatomical substrate that modulates differential animal behavior under different contexts or experimental paradigms.

It is important to consider the relevance of the alcohol dose used in the current studies in relation with human alcohol consumption. First, we selected this dose and the period of ethanol administration because our previous studies indicate that they do not produce gross and macroscopical changes in terms of several parameters, such as: placenta weight, umbilical cord length, offspring body weight, weight and/or size of the olfactory bulbs, cerebral hemispheres, and cerebellum (Domínguez et al., 1996). Similar prenatal manipulations, in terms of gestational period and ethanol doses did not significantly affect olfactory bulb size and cell number in the granular cell layer of the main olfactory bulb of infant rats (PD seven) (Pueta et al., 2011). Alternative morphological parameters, like body or lung weights and microscopic analyses of the diameters of the bronchioles and the pulmonary artery were also found to be unaffected by prenatal ethanol (Cullere et al., 2015). However, the vast literature sustains the notion that a binge-like daily exposure to 2.0 k/kg of ethanol during late gestation (GDs 17–20) exerts a significant impact on later ethanol-seeking and intake behaviors by the offspring in infantile and adolescent stages (Molina et al., 1995; Chotro and Spear, 1997; Chotro and Arias, 2003; Spear and Molina, 2005; Abate et al., 2008; Fabio et al., 2013). Secondly, maternal blood alcohol levels reached 150 mg/dl and in the fetus 120 mg/dl, at 60 min after administration during late gestation (GD: 17–20, Domínguez et al., 1996). These levels of blood alcohol are similar to those achieved by human mothers, who reported binge ethanol consumption patterns during pregnancy and their child exhibited impaired neuropsychological outcomes (Flak et al., 2014).

In summary, these results suggest that an early binge-like ethanol exposure (2 g/kg) during a discrete period (GD 17–20) produces long-lasting changes in brain organization, affecting basal activity of vasopressinergic neurons, the inhibitory areas of SA such as NTS and 5HT-DRN, and central extended amygdala structures. These central changes seem to modulate drinking behavior and renal regulatory responses after a homeostatic challenge.

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