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RESEARCH ARTICLE

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Neonatal experiences with ethanol intoxication modify respiratory and thermoregulatory plasticity and affect subsequent ethanol intake in rats

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Abstract

Different studies have focused on the deleterious consequences of binge-like or chronic exposure to ethanol during the brain growth spurt period (third human gestational trimester) that in the rat corresponds to postnatal days (PDs) 3–10. The present study analyzed behavioral and physiological disruptions caused by relatively brief binge-like exposures (PDs 3, 5, and 7) with an ethanol dose lower (3.0 g/kg) than those frequently employed to examine teratological effects during this stage in development. At PD 9, pups were exposed to ethanol doses ranging between .0–3.0 g/kg and tested in terms of breathing patterns and thermoregulation. At PDs 11 and 12, ethanol intake was examined. The main findings were as follows: i) pre-exposure to the drug resulted in brief depressions in breathing frequencies and an exacerbated predisposition toward apneic episodes; ii) these effects were not dependent upon thermoregulatory alterations; iii) early ethanol treatment increased initial consumption of the drug which also caused a marked hypothermia that appeared to regulate a subsequent decrement in ethanol consumption; and iv) ethanol exposure retarded overall body growth and even one exposure to the drug (PD 9) was sufficient to reduce brain weights although there were no indications of microcephaly. In conjunction with studies performed during the late gestational period in the rat, the results indicate that relatively brief binge-like episodes during a critical window of brain vulnerability disrupts the respiratory network and exacerbates initial acceptance of the drug. In addition, ethanol treatments were not found to induce tolerance relative to respiratory and thermal disruptions.

KEYWORDS
breathing disruptions, early ethanol intoxication, ethanol, intake rat, neonate, thermoregulation

1 | INTRODUCTION

Beyond ethanol's teratogenic effects leading to pathological entities known as Fetal Alcohol Syndrome or Fetal Alcohol Spectrum Disorders (Riley, Infante, & Warren, 2011; Riley & McGee, 2005), it has become evident that fetal experience with the drug usurps sensory, perceptual, and learning mechanisms that impact upon subsequent recognition of its chemosensory attributes, sensitivity to its motivational properties and the predisposition to use and abuse this psychotropic agent (for reviews, see: Abate, Pueta, Spear, & Molina, 2008; Chotro, Arias, & Laviola, 2007; Foltran, Gregori, Franchin, Verduci, & Giovannini, 2011). Preclinical, clinical, and epidemiological research has mainly addressed this problem via the analysis of alcohol-related problems emanating from drug exposure during the entire gestational period or during prenatal stages proximal to the time of birth.

A significant number of studies conducted in rats have centered its attention during late gestation; a stage equivalent to the second gestational human trimester (Abate, Spear, & Molina, 2001;

Cullere et al., 2015; Fabio et al., 2013; March, Abate, Spear, & Molina, 2009). In contrast, very few animal studies have addressed the consequences of early ethanol exposure upon ethanol's acceptance during the first week of postnatal development; a stage comparable to the third human gestational trimester and characterized by a brain growth spurt (Bayer, Altman, Russo, & Zhang, 1993; Tran, Cronise, Marino, Jenkins, & Kelly, 2000).

In regard to this stage of development, the pre-existing literature has concentrated its efforts in the analysis of adverse consequences of high ethanol doses or "binge-like" patterns of exposure; such as growth retardation, microcephaly, neuronal loss associated with apoptotic neurodegeneration, neuronal connectivity, motor coordination deficits, hyperactivity, social communication, etc. (Chen, Maier, Parnell, & West, 2003; Kelly, Goodlett, & Hannigan, 2009; Lewis, Wellmann, & Barron, 2007; West, Goodlett, Bonthius, & Pierce, 1989). Relative to the consequences of early ethanol exposure upon subsequent drug acceptance, Arias and Chotro (2006) intragastrically administered 3.0 g/kg ethanol during PDs 7 and 8. Three days later, animals showed an enhanced palatability to the drug. This effect was no longer observed if the administrations occurred during PDs 10 and 11. In addition, Lopez and Molina (1999) also found heightened infantile ethanol in pups pretreated during PDs 6–12 with 2.0 and 3.0 g/kg ethanol.

One of the goals of the present study was to analyze ethanol intake as a function of repeated drug administration during the brain growth period (PDs 3–9). Intake tests were sequentially conducted during PDs 11 and 12. At this age, ethanol intoxication generates hypothermia; a physiological disruption known to significantly mediate conditioned ethanol-related aversions (Hunt, Molina, Rajachandran, Spear, & Spear, 1993; Hunt, Molina, Spear, & Spear, 1990). Therefore, it was possible to expect that heightened intake during the first test may trigger hypothermic responsiveness and hence affect subsequent ethanol intake.

The study was also conceived to analyze if "binge-like" ethanol exposure during PDs 3, 5, and 7 also exerts thermoregulatory and respiratory disruptions and whether these alterations vary as a function of progressive administration of the drug. During early developmental stages, tolerance to the hypothermic effects of ethanol does not develop (Abate, Pepino, Spear, & Molina, 2004; Hunt et al., 1993; respectively). Relative to the respiratory consequences of ethanol, there is now evidence that chronic administration of the drug during gestation and lactation, severely affects breathing plasticity. This regimen of administration reduces brainstem-dependent respiratory rhythmic activity in the progeny and sensitizes juveniles to the depressant effects of ethanol upon phrenic and hypoglossal nerve activity (Dubois, Naassila, Daoust, & Pierrefiche, 2006; Dubois, Kervern, Naassila, & Pierrefiche, 2013). In vitro studies also show a significant depression in the respiratory-related hypoglossal nerve output caused by ethanol (Gibson & Berger, 2000). Recently, we conducted a study where ethanol (2.0 g/kg) was administered during the last four gestational days. Prenatal ethanol treatment was sufficient to sensitize neonates to the depressive effects of ethanol upon respiration (Cullere et al., 2015). The design of the present study allowed investigating if early sequential exposure to the drug (PDs 3, 5, and 7) also affects respiratory plasticity and whether this phenomenon is linked with thermoregulatory disruptions. It should be noted that prenatal or neonatal hypothermia can cause respiratory arrests (Duxbury, 2001).

As a function of the pertinent references previously summarized (e.g., Arias & Chotro, 2006), one prediction of the present study was heightened ethanol intake as a consequence of neonatal ethanol exposure. According to our prior experience (Cullere et al., 2015; Macchione et al., 2016) it was also expected that ethanol administration would systematically depress breathing during PDs 3, 5, and 7 and probably would affect the respiratory network after repeated drug administration (PD 9). The study was also conceived to analyze the impact of ethanol-related thermoregulatory disruptions upon respiratory processes and/or its effects when neonates re-experience the drug in an intake assessment.

2 | MATERIALS AND METHODS

2.1 | Subjects

A total of 110 Wistar neonate rats, representative of 13 litters were employed. Rats were born and reared at the vivarium of the Instituto de Investigación Médica M y M Ferreyra (INIMEC-CONICET-UNC, Argentina). The colony room was illuminated on a 12 hr light/dark cycle (lights on: 08:00–20:00) at an ambient temperature and humidity of 22 ± 1 °C and 45%, respectively. Births were daily examined and the day of parturition was considered postnatal day 0 (PD0). At PD1, each litter was randomly culled to 10 pups (five males and five females, whenever possible). Throughout days pups were kept with their dams in standard cages that contained water and food ad libitum (ACA Nutrición, Buenos Aires, Argentina).

All experimental treatments were in accordance with the Guide for Care and Use of Laboratory Animals (National Research Council, 1996) and were approved by the Institutional Animal Care and Use Committee of our institution (CICUAL-INIMEC-CONICET-UNC). To reduce confounds between litter and treatment effects (Holson & Pearce, 1992) no more than one male and one female per litter were assigned to the same experimental condition.

2.2 | General experimental procedures

During PDs 3, 5, and 7, pups were removed from their maternal cages and were placed in pairs in individual cages partially filled with clean corncob and kept at 32–34 °C with heating pads for 30 min. Subsequently, they were weighed (±.01 g) and received intragastric administrations of either 3.0 or .0 g/kg ethanol. Pups representative of seven litters were subjected to ethanol administrations while pups corresponding to the remaining six litters received vehicle. The differential drug treatment across litters was performed to avoid interactions between sober and intoxicated pups since social learning relative to the sensory and toxic effects of ethanol is likely to occur (Eade & Youngentob, 2009, 2010; Fernandez-Vidal & Molina, 2004). At PD 9, pups representative of both prior treatments either received no specific

treatment (untreated—UT-) or .0, .75, 1.5, 3.0 g/kg of ethanol. Respiratory patterns were registered 30–35 min following each specific treatment. Body temperatures were recorded at PDs 3, 5, and 7 prior to drug administration procedures as well as at the beginning and the end of breathing evaluations. Similar temperature recordings were used at PD 9. At PDs 11 and 12, pups were tested in terms of ethanol consumption and temperatures were recorded prior and after each specific test. At PD12, pups were humanely sacrificed in order to determine blood and brain ethanol concentrations (BECs and BrECs).

2.3 | Ethanol doses and administration procedures

Ethanol was administered intragastrically (i.g.) via a 12-cm length of polyethylene-10 tubing (PE-10 Clay Adams, Parsippany, NJ) attached to a 1 ml syringe (Becton Dickinson, Rutherford, NJ) with a 27-gauge needle. Ethanol doses of .75, 1.5, and 3.0 g/kg resulted from the administration of a volume equivalent to .015 ml per gram of body weight of 6.3, 12.6% or 25.2% (v/v) ethanol (Porta Hnos, Córdoba, Argentina) solutions, respectively. An equivalent volume of tap water was administered as vehicle (.0 g/kg). Pups were gently intubated in approximately 5 s, and the solutions were then slowly delivered over 3–4 s into the stomach.

2.4 | Determination of breathing and apnea frequencies

The breathing and apnea frequencies were determinated through a whole body plethysmograph (Model 10G equipped with the software "Breath Medidor de Respiración," Itcom, Córdoba, Argentina). The apparatus was built to record breathing patterns of very small organisms weighing between 6 and 28 g. It consists of two identical transparent and hermetic Plexiglas chambers (5 × 10 × 5 cm), that are interconnected via a polyurethane hose system. The hose system allows injection and extraction of equivalent amounts of air in both chambers in order to maintain constant and equivalent pressures. One of the chambers is used as a testing device while the other serves as a reference box in terms of flow/air pressure. The plethysmograph records air pressure/flow rate differences between the testing and reference chambers. These differences activate a pressure sensor (AWM2100 Honeywell) with the capability of recording one complete breathing event every 1×10^{-7} s. The plethysmograph records the breathing response every 1.5 s. These scores are transformed to mean breaths per minute. The apneic episodes were also measured. The criterion used to determine an apneic episode was the interruption of air flow for at least two normal respiratory cycles (.5 s or more; Julien, Joseph, & Bairam, 2010).

For each session, unrestrained awake pups were introduced into the chambers and the lids were closed. One minute after that pups were individually placed inside the chamber, respiratory responses were measured during five consecutive minutes. The minute of delay at the beginning of the test was used to allow air pressure stabilization in the chamber.

An air conditioner kept the room temperature at 22 ± 1 °C during experimental sessions. The temperature was kept at 31–32 °C inside the plethysmograph chambers, similar to their maternal nest, through heating pads placed beneath the chambers (Julien et al., 2010). The overall procedure has been previously used to evaluate breathing disruptions as a function of pre- and postnatal ethanol exposure (Cullere et al., 2015; Macchione et al., 2016; respectively).

2.5 | Body temperature measurements

Body temperatures were non-invasively registered through a thermal infrared imaging camera ("Flir Exx Series," Boston FLIR System, Inc., Boston, MA). As mentioned, thermal measurements were taken prior to ethanol treatment, immediately before and after each plethysmograph recording (PDs 3, 5, 7, and 9) as well as prior and following each ethanol intake tests (PDs 11 and 12).

2.6 | Ethanol intake test

At PDs 11 and 12, pups were removed from their maternal cages and they were intraorally implanted with a cannula that allowed liquid infusions. Subsequently, pups were anogenitally stimulated with a cotton swab to promote urination and defecation, weighed to the nearest .01 g/kg and individually placed in holding cages where they had access to an ethanol solution during 40 min. Pups received eight intraoral infusions of 5.0% v/v ethanol. This procedure was possible through the use of an infusion pump (KD Scientific, Model 200, Holliston, MA). Each liquid pulse had a duration of 1 min while the interpulse duration was equivalent to 4 min. The overall amount of liquid infused was equivalent to 4% of the average pre-infusion body weight. Post-infusion weights were calculated after the intake test and percent body weight change was calculated. Infant ethanol intake scores were also calculated in terms of absolute grams of ethanol per kilogram of body weight (g/kg). These scores were calculated as a function of body weight gain during the intake test session and in accordance with the concentration of the ethanol solution used. Similar procedures have been extensively used in prior studies that demonstrate its validity in terms of adequate evaluation of intake of different tastants as well as its sensitivity to determine conditioned taste preferences or aversions as well as operant responsiveness regulated via gustatory reinforcers (Arias & Chotro, 2006; Cullere et al., 2015; Lopez & Molina, 1999; Pautassi, Truxell, Molina, & Spear, 2008; Pueta, Rovasio, Abate, Spear, & Molina, 2011).

2.7 | Determination of blood and brain ethanol concentrations (BECs and BrECs)

Immediately after the intake test at PD12, pups were decapitated. Blood samples were collected from the trunk and held in plastic heparinized tubes, while brains were quickly removed from the skulls. Both types of samples were frozen at −70 °C until determination of ethanol concentrations via head-space gas chromatographic analyses (Hewlett-Packard, model 5890, Palo Alto, CA). Each brain sample was weighed (±.001 g), pre-diluted in miliQ water (.5 g/ml) and sonicated (Sonics Vibra Cells, Newtown, CT) for 15 s. Subsequently, 100 µl of brain samples, as well as of blood, were individually placed in microvials

containing 20 µl of a butanol solution (51 mg/100 ml) that served as an internal standard. The microvials were sealed in dry ice, were later incubated at 60 °C for 30 min and analyzed with the gas chromatograph as it has been previously described (Cullere et al., 2015; Pepino, Abate, Spear, & Molina, 2002). Both BECs and BrECs were expressed as milligrams of ethanol per deciliter of body fluid (mg/dl = mg%).

2.8 | Experimental design and data analysis

As can be observed in the preceding methodological sections, the independent factors under consideration were ethanol treatment at PDs 3, 5, and 7 (.0 or 3.0 g/kg ethanol), ethanol treatment during PD 9 (untreated, .0, .75, 1.5, or 3.0 g/kg ethanol) and gender (male or female). Extensive preliminary analysis of the data indicated that at PD 9, pups receiving no treatment or vehicle (untreated or .0 g/kg) representative of groups pretreated with .0 or 3.0 g/kg at PDs 3, 5, and 7 were never found to differ in any of the dependent variables under consideration. The mean values and standard errors for each group (.0 g/kg and untreated, respectively) at PD 9 were as follows: breathing frequencies, 239.66 ± 6.47 and 248.57 ± 6.22 breaths/min; apneic episodes, 1.04 ± .30 and .62 ± .29; body temperature before respiratory recordings, 35.17 ± .21 and 34.87 ± .20 °C and after 34.72 ± .19 and 34.62 ± .18 °C each plethysmograph recordings. Hence, both groups were collapsed for each specific drug treatment corresponding to PDs 3, 5, and 7. The first dependent variable under analysis was body weight across the entire experiment (PDs 3, 5, 7, 9, 11, and 12). Since at PD12 pups were sacrificed to process brain and blood ethanol concentrations, brain weights as well as the ratio (%) existing between this parameter and overall body weight were also statistically analyzed. The mentioned ratio serves to determine possible effects of drug treatment in terms of microcephaly (Chen et al., 2003).

The dependent variables in terms of breathing patterns were mean respiration rates and apneic frequencies per minute. Between-within analysis of variance were performed at PDs 3, 5, and 7 where ethanol treatment (.0 or 3.0 g/kg) and gender (male and female) served as independent factors and PDs (3, 5, and 7) as well as minutes corresponding to each particular evaluation (1–5 min) served as repeated measures. At PD 9, similar dependent variables were subjected to a between-within ANOVA defined by prior ethanol treatment at PDs 3, 5, and 7 and ethanol treatment at PD 9 (untreated, .0, .75, 1.5, or 3.0 g/kg). Once again, minutes of evaluation served as repeated measures. Thermoregulatory processes at PDs 3, 5, and 7 were analyzed using a four-way mixed ANOVA. Ethanol treatment at PDs 3, 5, and 7 and gender served as the between factors, whereas PDs and time of temperature recordings (before and after plethysmograph assessments) served as the within-group factors. At DP9 a four-way mixed ANOVA was also performed for body temperatures. Ethanol treatment at PDs 3, 5, and 7, gender and ethanol treatment at PD 9 served as between factors; time of temperature recordings served as repeated measures.

Ethanol intake scores were subjected to a four-way mixed ANOVA where ethanol treatment at PDs 3, 5, and 7 and PD 9, and gender served as independent factors; while days of evaluation (PDs 11 and 12) served as a repeated measures. Brain and blood ethanol concentrations at PD 12 were analyzed as a function of prior drug treatments throughout the course of the experiment. Whenever necessary, each significant main effect or significant interactions was further processed using Newman–Keuls posthoc tests ($p < .05$). According to the nature of the dependent variable/s under consideration, posthoc tests were performed using between, within or between-within pooled error terms. All the statistical analyses were performed using the STATISTICA 8.0 software.

3 | RESULTS

3.1 | Body and brain weights

Table 1 depicts pup body and brain weights across days of experiment as function of gender and ethanol treatment at PDs 3, 5, and 7. Data corresponding to body weights across days was analyzed via a between-within ANOVA (ethanol treatment at PDs 3, 5, and 7 × ethanol treatment at PD 9 × postnatal days × gender). As expected body weights increased as a function of age; $F(5,465) = 4369.03$, $p < .0001$. Ethanol treatment at PDs 3, 5, and 7 also exerted a significant main effect and significantly interacted with postnatal days [$F(1,93) = 4.23$, $p = .0426$ and $F(5,465) = 5.74$, $p < .0001$, respectively]. Newman–Keuls tests showed that pups treated with vehicle or 3.0 g/kg ethanol at the beginning of the experiment (PDs 3 and 5) showed similar body weights. At PD 7 and until the end of the experiment, pups originally treated with ethanol exhibited significantly lower body weights relative to vehicle controls.

TABLE 1 Pup's body and brain weights across days

Ethanol treatment at PDs 3, 5, and 7 (g/kg)	Gender	Body weight						Brain (PD12)	
		PD3	PD5	PD7	PD9	PD11	PD12	Weight (g)	Brain/Body (%)
.0	Males	9.78 ± .25	12.82 ± .40	17.11 ± .50	21.78 ± .57	26.74 ± .69	28.81 ± .70	1.128 ± 0.020	3.94 ± .05
	Females	9.83 ± .22	13.22 ± .27	17.31 ± .35	21.65 ± .56	27.27 ± .51	29.05 ± .49	1.107 ± .013	3.83 ± .06
3.0	Males	9.90 ± .17	12.62 ± .27	16.39 ± .35	20.66 ± .46	26.01 ± .50	28.33 ± .56	1.069 ± .011	3.80 ± .07
	Females	9.66 ± .14	12.34 ± .26	16.00 ± .31	20.38 ± .42	25.25 ± .56	27.56 ± .57	1.024 ± .010	3.77 ± .09

Values are expressed as means ± SEMs.

Brain weights at PD 12 were found to significantly vary as a function gender, ethanol treatment at PDs 3, 5, and 7 as well as ethanol treatment at PD 9 [$F(1,93) = 6.74$, $p = .0110$; $F(1,93) = 28.57$, $p < .0001$ and $F(3,93) = 3.86$, $p = .0118$, respectively]. Female rats exhibited significantly lower brain weights relative to male counterparts (means ± standard errors: 1.061 ± .009 g and 1.095 ± .011 g, respectively). Pups treated with ethanol at PDs 3, 5, and 7 were also found to show significantly lower brain weights relative to vehicle controls (means ± standard errors:1.046 ± .009 g and 1.121 ± .010 g, respectively). In the case of the main effect relative to ethanol treatment at PD 9, Newman–Keuls posthoc comparisons showed that pups receiving the highest ethanol dose (3.0 g/kg) had smaller brain weights relative to all the remaining conditions. The mean values and standard errors for each ethanol treatment group were as follows: .0 g/kg, 1.09 ± .01 g; .75 g/kg, 1.10 ± .02 g; 1.5 g/kg, 1.10 ± .01 g; and 3.0 g/kg, 1.04 ± .01 g. When examining the ratio between brain and body weights, the corresponding ANOVA did not showed significant main effects or interactions. Across groups, the percent relation between brain and body weights was 3.83 ± .04%. According to this data, under the present regimen of ethanol administration, microcephaly was not observed.

3.2 | Breathing frequencies and apneas during drug pretreatment (PDs 3, 5, and 7) and test (PD9)

Figure 1 illustrates the effects of ethanol treatment (.0 or 3.0 g/kg) upon respiratory rates during PDs 3, 5, and 7. During the initial stage of the study, the between-within ANOVA [ethanol treatment (.0 or 3.0 g/kg) × gender (female or male) × postnatal days (3, 5, and 7) × minutes of evaluation (1–5)] indicated significant main effects of ethanol treatment: $F(1,103) = 11.21$, $p = .0011$; PDs: $F(2,206) = 17.95$, $p < .0001$; minutes of evaluation: $F(4,412) = 28.81$, $p < .0001$; as well as a significant interaction between ethanol treatment and minutes of evaluation: $F(4,412) = 6.32$, $p < .0001$. According to posthoc tests, breathing frequencies were significantly higher in animals administered with .0 g/kg (219.18 ± 3.56 breaths/min) relative to those administered with 3.0 g/kg ethanol (203.40 ± 3.09 breaths/min). Breathing frequencies were also significantly higher at PDs 5 (219.62 ± 3.39 breaths/min) and 7 (219.00 ± 4.43 breaths/min) relative to the ones recorded at PD 3 (195.33 ± 2.66 breaths/min). Relative to the main effects of ethanol treatment at PDs 3, 5, and 7 and minutes of evaluation, and its significant interaction, Newman–Keuls tests showed that during the first 2 min of the assessment breathing frequencies were significantly higher (223.57 ± 2.58 and 213.00 ± 2.45 breaths/min, minutes 1 and 2, respectively) than during the remaining minutes of the evaluation (207.49 ± 2.48, 207.13 ± 2.80, and 205.24 ± 2.93 breaths/min, minutes 3, 4, and 5, respectively). In addition, pups treated with 3.0 g/kg during minutes 1 and 2 exhibited significantly lower breathing frequencies relative to vehicle counterparts. This interaction has been depicted in Figure 1.

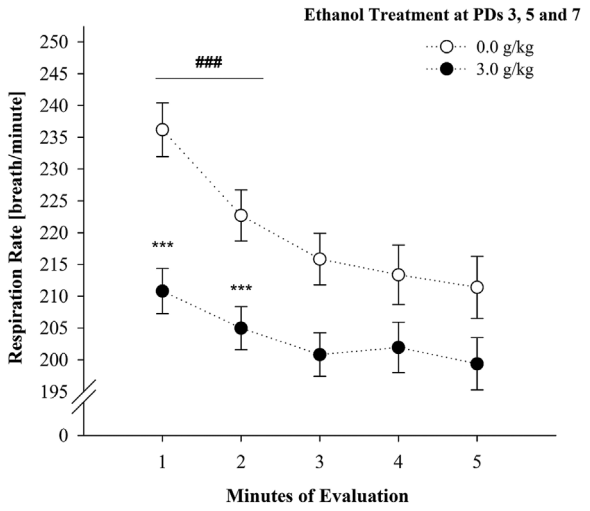

FIGURE 1 Respiration frequencies (breaths/min) at PDs 3, 5, and 7 as a function of ethanol treatment (.0 or 3.0 g/kg). ***Indicates significant differences between ethanol-treated and vehicle-treated animals. ###Indicates significant differences across minutes of evaluation; $p < .0001$. Vertical lines indicate standard errors of the means (SEMs)

A similar ANOVA was conducted to analyze frequency of apneas. Apneas at PDs 3, 5, and 7 were not significantly affected by any of the variables under consideration or the interaction between them: ethanol treatment [$F(1,103) = .12$, $p = .7255$], gender [$F(1, 103) = 3.61$, $p = .0601$], PDs [$F(2, 206) = 2.12$, $p = .1223$] or minutes of evaluation [$F(4,412) = 1.23$, $p = .2968$]. The highest F value corresponding to the interactions under analysis was equivalent to 1.10 ($p = .3535$; ethanol treatment at PDs 3, 5, and 7 × minutes of evaluation).

At PD 9, the corresponding between-within ANOVA (ethanol treatment at PDs 3, 5, and 7 × ethanol treatment at PD 9 × gender × minutes of evaluation) showed that breaths per minute significantly varied as a function of ethanol treatment at PD9 [$F(3,94) = 3.86$, $p = .0118$], minutes of evaluation [$F(4,376) = 30.53$, $p < .0001$] and the following two-way interactions: ethanol treatment at PDs 3, 5, and 7 × minutes of evaluation [$F(4,376) = 3.13$, $p = .0150$] and ethanol treatment at PD 9 × minutes of evaluation [$F(12,376) = 4.02$, $p < .0001$].

When considering the interaction between ethanol pretreatment at PDs 3, 5, and 7 and minutes of evaluation, posthoc tests showed that pups pre-exposed to 3.0 g/kg ethanol showed lower breathing rates relative to pups pretreated with vehicle. This difference attained significance at the beginning of the test (minute 1; Figure 2a).

When pups were evaluated at PD 9, the between-within ANOVA (ethanol treatment at PDs 3, 5, and 7 × ethanol treatment at PD 9 × gender × minutes of evaluation) indicated a significant main effect of ethanol treatment at PDs 3, 5, and 7; $F(1,94) = 4.66$, $p = .0334$. As can be observed in Figure 2b, pups pretreated with 3.0 g/kg showed significantly higher frequencies of apneic episodes relative to vehicle pretreated counterparts.

As could be expected breaths per minute also varied as a function of the ethanol dose administered at PD 9. Relative to the interaction between ethanol treatment at PD 9 and minutes of evaluation, posthoc comparisons indicated that, at commencement of testing, breathing frequencies of vehicle animals were significantly higher than those recorded in pups treated with 1.5 g/kg ethanol (minute 2) or

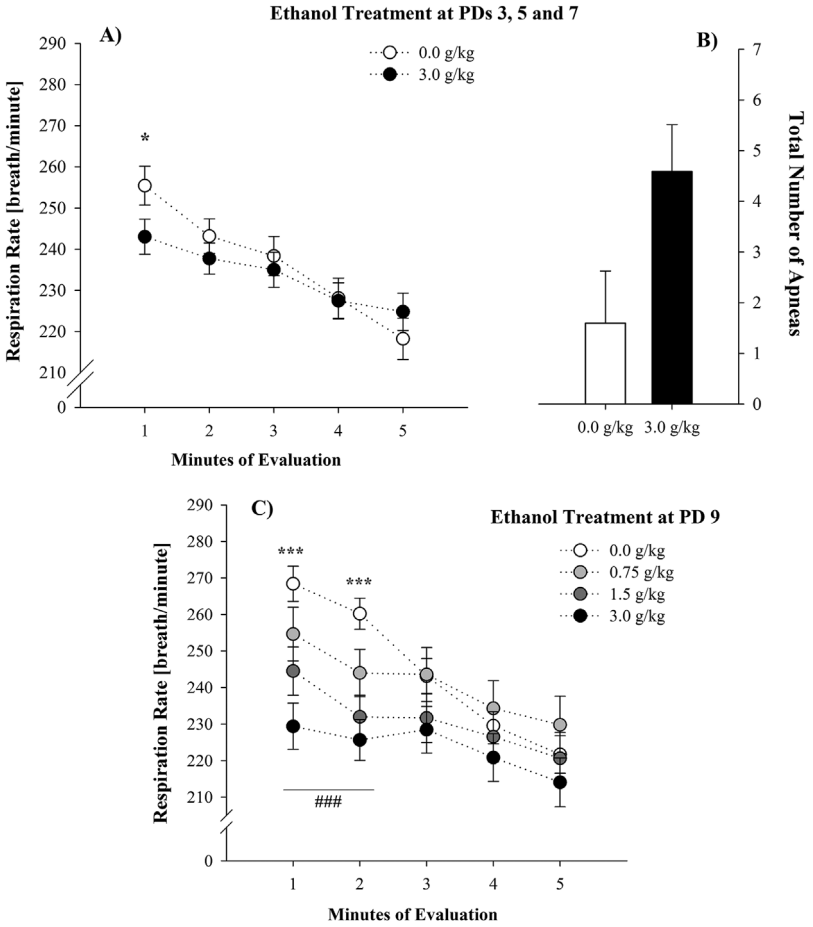

FIGURE 2 A) Respiration frequencies (breaths/min) at PD 9 as a function of ethanol treatment at PDs 3, 5, and 7 (.0 or 3.0 g/kg). *Indicates a significant difference between ethanol-treated and vehicle-treated animals, $p < .05$. Data have been collapsed across gender and ethanol treatments at PD9. B) Overall number of apneas at PD 9 as a function of ethanol treatment at PDs 3, 5, and 7. Ethanol-treated pups showed significantly higher levels of breathing disruptions than vehicle-treated animals, $p < .05$. Data have been collapsed across gender, minutes of evaluation, and ethanol treatments at PD 9. C) Respiration frequencies as a function of ethanol treatment (.0, .75, 1.5, or 3.0 g/kg ethanol) at PD9. ***Indicates a significant difference between vehicle-treated animals and pups treated with 3.0 g/kg (minutes 1–2), and between vehicle-treated animals and those treated with 1.5 g/kg ethanol (minute 2). ###Indicates a significant difference between minutes 1 and 2 regarding minutes 4 and 5 of the evaluation ($p < .0001$). Vertical lines indicate standard errors of the means (SEMs)

3.0 g/kg ethanol (minutes 1 and 2). In all groups, breathing frequencies progressively decreased as a function of the passage of time (see Figure 2c).

3.3 | Thermal responsiveness during drug pretreatment (PDs 3, 5, and 7) and test (PD 9)

As stated, at PDs 3, 5, and 7, body temperatures of pups treated with .0 or 3.0 g/kg ethanol were recorded immediately prior to drug administration (baseline) as well as before and after being exposed to the plethysmograph. The corresponding between-within ANOVA (ethanol treatment × postnatal days × gender × time of recording) indicated significant main effects of ethanol treatment and time of recording [$F(1,103) = 4.10$, $p = .0462$ and $F(2,206) = 16.90$, $p < .0001$, respectively]. PDs significantly interacted with time of recording and the 3-way interaction between ethanol treatment, postnatal days and time of recording also achieved significance [$F(4,412) = 5.10$, $p = .0005$ and $F(4,412) = 2.50$, $p = .0406$; respectively]. The locus of 3-way interaction was examined via posthoc tests indicating that body temperatures prior to drug treatment increased across days. At PD 7, baseline thermal values were significantly higher than at commencement of the experiment (PD 3). At this initial stage, no significant differences were encountered between pups exposed to ethanol or vehicle. At PD 5, pups treated with ethanol showed a significant level of hypothermia when comparing temperatures recorded before and after breathing assessments. At PD 7, pups treated with ethanol had significantly lower body temperatures before and after being evaluated in the plethysmograph relative to vehicle controls. Apparently, the hypothermic effects of ethanol gradually increased during the course of the initial phase of the experiment (Figure 3a).

At PD 9, body temperature was analyzed taking into account ethanol pretreatment (.0 or 3.0 g/kg ethanol), the ethanol dose administered during this particular day (.0, .75, 1.5, or 3.0 g/kg), gender and time of recording (pre-drug administration time—baseline—, before and after plethysmograph recordings). Time of temperature

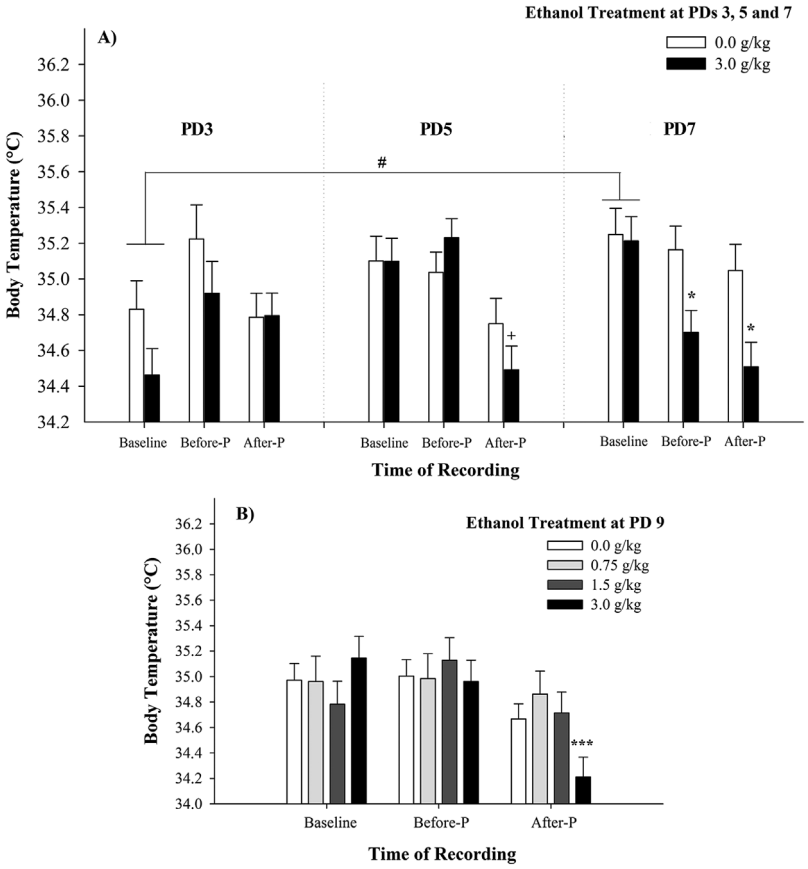

FIGURE 3 Body temperatures at PDs 3, 5, and 7 were recorded prior to drug treatment (baseline) and immediately before and after each plethysmograph recording (before-P and after-P; respectively). A) #Indicates a significant difference between baseline thermal values at PD 7 relative to baseline scores at PD 3. +Indicates a significant difference in ethanol-treated pups at PD 5 between body temperature recorded after plethysmograph relative to the temperatures previously recorded. At PD 7, prior and after being evaluated in the plethysmograph, pups treated with ethanol had significantly lower temperatures relative to vehicle controls and baseline values (*$p < .05$). B) ***Indicates a significant difference in body temperatures between the group of animals given 3.0 g/kg ethanol and all the remaining groups ($p < 0.0001$). Vertical lines indicate standard errors of the means (SEMs)

recording as well as the interaction between this factor and ethanol treatment at PD 9 exerted significant effects [$F(2,188) = 19.30$, $p < .0001$ and $F(6,188) = 4.40$, $p = .0004$, respectively]. Posthoc tests indicated that pups administered with 3.0 g/kg showed a significant reduction in body temperatures after breathing evaluations relative to those recorded in all the remaining drug conditions and recording times (Figure 3b).

3.4 | Ethanol intake and thermal responsiveness at PDs 11 and 12

Ethanol intake scores [percent body weight gains (BWG) and absolute grams of ethanol per kilogram of body weight (g/kg)] were significantly affected by PDs of evaluation and the interaction between this factor and ethanol treatment at PDs 3, 5, and 7; $F(1,93) = 15.33$, $ps = .0002$ and $F(1,93) = 9.19$, $ps = .0031$; respectively. As can be observed in Figure 4a, pups pretreated with ethanol consumed significantly higher levels of the drug during the first evaluation session (PD 11) relative to vehicle controls. During the second test (PD 12) intake scores of subjects originally administered with ethanol were significantly lower relative to the preceding day of evaluation and very similar to those recorded in vehicle counterparts.

As stated, body temperatures were also recorded before and after each intake test. The corresponding ANOVA showed significant effects of time of recording [$F(1,93) = 44.45$, $p < .0001$] and of the following interactions: postnatal days of evaluation × time of recording [$F(1,93) = 8.88$, $p < .0037$]; ethanol treatment at PDs 3, 5, and 7 × postnatal days of evaluation [$F(1,93) = 15.77$, $p < .0001$] and ethanol treatment at PDs 3, 5, and 7 × postnatal days of evaluation × time of recording [$F(1,93) = 4.49$, $p = .0368$]. Newman–Keuls tests were performed to clarify the locus of the 3-way interaction. During both postnatal days, body temperatures following the intake test were significantly lower relative to baseline values. At PD 11, the thermal decrement observed in pups pretreated with ethanol at PDs 3, 5, and 7 was significantly higher than the one recorded in subjects pretreated with vehicle (Figure 4b).

Blood and brain ethanol levels (mg%) obtained after the second intake test were not found to significantly vary across the different groups. Overall values were as follows: BECs, 126.16 ± 5.96 mg% and BrECs, 123.16 ± 6.02 mg%.

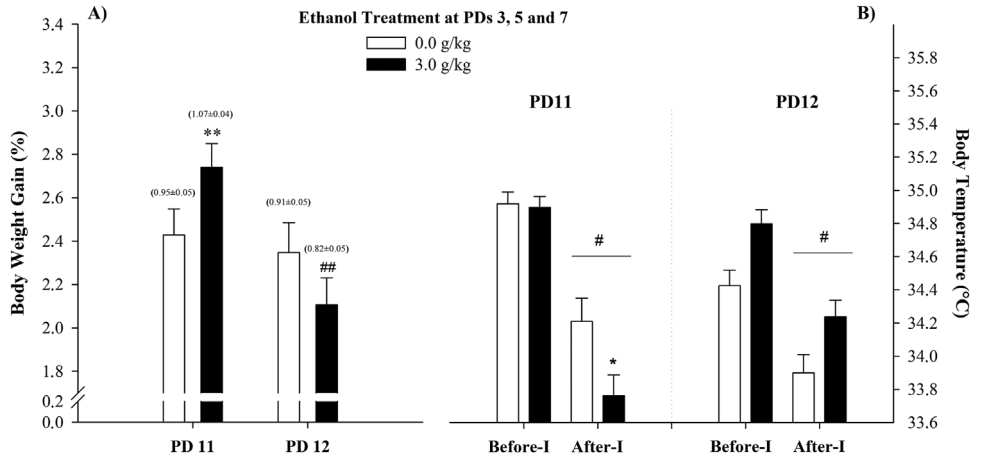

FIGURE 4 A) Ethanol consumption scores (percent body weight gain) as a function of prior exposure to vehicle or ethanol at PDs 3, 5, and 7. Numbers between parentheses indicate absolute ethanol intake in terms of g/kg (mean ± SEM). Prior exposure to the drug resulted in a significant increase in alcohol consumption at PD 11 (**$p < .01$). At PD 12, ethanol pretreated pups showed significant intake decrements when compared with the values obtained at PD 11 (##$p < .01$). B) Body temperatures before and after each ethanol intake test (before-I and after-I, respectively). Temperatures after each intake test were significantly lower relative to the ones recorded prior to each intake assessment. (#$p < .05$). At PD 11, ethanol treated pups during PDs 3, 5, and 7 exhibited heightened hypothermia relative to the corresponding vehicle controls (*$p < .05$). Vertical lines indicate standard errors of the means (SEMs)

4 | DISCUSSION

This study examined physiological and behavioral consequences of binge-like ethanol exposure in rats during a stage in development (PDs 3–9) equivalent to the third human gestational trimester. Pups were treated with an ethanol dose equivalent to .0 or 3.0 g/kg at PDs 3, 5, and 7 and at PD 9 they either received no treatment (untreated), vehicle (.0 g/kg ethanol) or one of the following ethanol doses: .75, 1.5, or 3.0 g/kg. Prior studies have been conducted with higher levels of ethanol exposure in terms of overall daily dosage (ranging between 3.3 and 9.8 g/kg) and the number of days of treatment (PDs 4–9 or even 4–10). Under these circumstances, microcephaly accompanied by neuronal loss is frequently observed (Chen, Andersen, & West, 1994; de Licona et al., 2009; Maier, Chen, Miller, & West, 1997; Pierce & West, 1986; West et al., 1989). These disruptions are accompanied by functional deficits such as altered habituatory patterns to novel stimuli (Morasch & Hunt, 2009), memory deficits arising from the examination of trace conditioning procedures (Hunt & Barnet, 2015), lessened preference of sweet solutions (Barron, Razani, Gallegos, & Riley, 1995), alterations in social communication patterns operationalized through ultrasound vocalizations (Wellmann, Lewis, & Barron, 2010), etc. Under the present experimental circumstances, ethanol exposure at PDs 3, 5, and 7 resulted in an overall reduction in body and brain weights as observed at PD 12 but the treatment was not sufficient to cause microcephaly (Table 1). Animals that only received ethanol at PD9 were unaffected in terms of overall body weights. Nevertheless, a single exposure to the highest ethanol dose (3.0 g/kg) at PD9 was sufficient to reduce brain weights. In mice, during the brain growth spurt period, a single and higher ethanol dose (4.4 g/kg) results in microencephaly and neuronal loss, but only when there exists a genetic deficiency in terms of the neuroprotective properties of nitric oxide (de Licona et al., 2009). According to the present results, genetically heterogenous rats are susceptible to brain growth underdevelopment following a single binge-like ethanol exposure. It appears necessary to conduct studies based on interspecific comparisons and as suggested by the study of de Licona et al. (2009), to determine species variations in neuroprotective factors such as the capability to synthesize nitric oxide.

When focusing on the functional effects of ethanol at PD 9, dose-dependent disruptions were observed relative to breathing rates and thermoregulation. The two highest doses (1.5 and 3.0 g/kg) depressed respiratory frequencies while the higher dose resulted in an abrupt reduction in body temperature. Effects such as sensitization or tolerance were not evident as a function of prior ethanol experiences. Yet, such experiences were sufficient to cause breathing alterations at PD 9. Pups administered with ethanol at PDs 3, 5, and 7 were observed to exhibit a significant respiratory depression at the beginning of the test as well as an increase in apneic episodes (Figure 2a and b, respectively). Two hypothesis, not mutually exclusive, seem pertinent when analyzing these alterations. The first hypothesis is related with short- and long-lasting effects of ethanol upon factors that regulate neonatal breathing processes; a phenomenon that has received increasing attention given the association between fetal alcohol exposure and Sudden Infant Death Syndrome (Bailey & Sokol, 2011; Burd, Klug, & Martsolf, 2004; Iyasu et al., 2002; O'Leary, Jacoby, Bartu, D'Antoine, & Bower, 2013). In rats, chronic ethanol exposure starting before mating and continuing throughout gestation and lactation severely reduces brainstem-dependent respiratory rhythmic activity (Dubois et al., 2006, 2013). There is also evidence of an altered serotonergic system which plays a critical role in cardio-respiratory control. Fetal alcohol exposure decreases 5HT neuronal number as well as serotonin brain levels (Idrus & Thomas, 2011; Valenzuela, Puglia, & Zucca, 2011). Chronic prenatal ethanol exposure also alters compensatory respiratory processes that occur following hypoxia in

neonate rats (Dubois et al., 2013). In in vitro studies, it is observed that a significant depression in the respiratory-related hypoglossal nerve output is caused by ethanol (Gibson & Berger, 2000). These alterations cannot be ruled out when considering the present results. A second hypothesis related with the early plasticity of the respiratory network should also be considered. Salient stimuli explicitly paired with ethanol-related breathing disruptions (dose: 2.0 g/kg during late pregnancy or early postnatal life) later elicit conditioned respiratory depressions and increase the probability of apneas (Cullere et al., 2015; Macchione et al., 2016). Although in the present study this learning process was not explicitly investigated, it is necessary to take into account that respiratory depressions caused by ethanol at PDs 3, 5, and 7 (Figure 1) were recorded in a distinct context. At PD 9, all pups re-experienced this context while being sober or intoxicated. Under this condition, pups pretreated with ethanol did exhibit an initial breathing depression and approximately a 2.5-fold increase in the number of apneas relative to pups pretreated with vehicle. Hence, the possibility exists relative to learning arising from the original association between specific environmental cues and the drug's unconditioned respiratory effects.

Relative to thermal responsiveness, the results are in agreement with those reported by Hunt et al. (1993) in older rat pups. At PD 9, there were no indications that drug pretreatment generated tolerance. In the rat, mature levels of thermoregulation are attained at the end of the third postnatal week (Leon, 1986) and even when considering adults, tolerance to ethanol-induced hypothermia requires at least 5–7 exposures to the drug (Pohorecky, Brick, & Carpenter, 1986). Apparently, the organism has to detect the thermal imbalance produced by the drug in order to generate compensatory mechanisms and regain thermal homeostasis (Hunt et al., 1993; Poulos & Cappell, 1991). The levels of ethanol exposure as well as the state of maturation of the thermoregulatory system within the context of the present experiment appear to represent critical factors that impede the development of tolerance. When considering the state of maturation, the thermoregulatory response to ethanol (hypothermia) during the second postnatal week is markedly lower than the one observed 1 week later (Spiers & Fusco, 1991). Beyond this phenomenon, it is important to note that prenatal or neonatal thermal disruptions such as hypothermia can cause respiratory arrests (Duxbury, 2001). As stated, one of the goals of this study was to analyze whether ethanol-induced breathing disruptions were associated with thermal alterations. The results relative to these physiological alterations argue against such an association. In addition to the specific results communicated in terms of breathing and thermal responsiveness, we also performed correlational analyses (Pearson's correlation coefficients, $p < .05$) to determine the possible strength of the association under consideration. No significant correlations were observed when considering the total amount of pups evaluated at PD 9 or when considering each particular group defined by the factorial design (data not shown).

When considering ethanol drinking at PDs 11 and 12, it was clear that sequential exposure to the drug at PDs 3, 5, and 7 promoted heightened intake values at PD 11. One exposure to a given ethanol dose (.75, 1.5, or 3.0 g/kg) at PD 9 was not sufficient to increase ethanol consumption. These results are in agreement with those reported in studies indicating a series of factors that modulate ethanol acceptance due to early exposure to the drug. Late in gestation, repeated exposure to the drug sensitizes the organism to its positive reinforcing effects (Nizhnikov, Molina, Varlinskaya, & Spear, 2006). Additionally, near term fetuses and neonates associate ethanol's chemosensory cues perceived in the amniotic fluid or via nonmetabolic elimination processes, such as alveolar excretion or salivation (Arias & Chotro, 2006; Molina, Chotro, & Spear, 1989), with the central motivational properties of the state of intoxication. This associative learning results in heightened acceptance of ethanol solutions or the drug's gustatory psychophysical equivalents (Dominguez, Lopez, Chotro, & Molina, 1996; Dominguez, Lopez, & Molina, 1998; Fabio et al., 2013; March et al., 2009). These effects are related with specific characteristics of ethanol metabolism during early development. At this stage, catalase brain activity reaches peak levels relatively to subsequent stages in development (Del Maestro & McDonald, 1987). This enzymatic system transforms ethanol into acetaldehyde which is a critical factor regulating the drug's central reinforcing effects (March, Abate, & Molina, 2013a; March, Abate, Spear, & Molina, 2013b; Nizhnikov, Molina, & Spear, 2007). On the contrary, peripheral accumulation of the metabolite, known to exert aversive grastrointestinal effects (Quertemont, 2004), is highly limited in the neonate due to hepatic immaturity (Kelly, Bonthius, & West, 1987). Given the balance between central and peripheral accumulation of the drug, it appears that in neonates the overall metabolic process favors the positive motivational properties of ethanol and/or of acetaldehyde (March et al., 2013). It is also necessary to take into account that ethanol at PD 11 in pups pre-exposed to the drug, significantly decreased during the following day. During the second postnatal week of life, the balance between central and peripheral acetaldehyde is different from the one observed earlier. In older pups the capability to metabolize ethanol through the central catalase system markedly decreases while peripheral accumulation of acetaldehyde increases due to an enhanced liver metabolic capability (Del Maestro & McDonald, 1987; Kelly et al., 1987, respectively). This pharmacokinetic profile appears to favor the acquisition of conditioned ethanol aversions in the older pups (Arias & Chotro, 2006; Molina, Pautassi, Truxell, & Spear, 2007; Pautassi, Godoy, Spear, & Molina, 2002). Hence, pups that consumed higher ethanol levels at PD 11 due to prior exposure to the drug may also experience negative peripheral consequences of ethanol or of its principal metabolite leading to a conditioned chemosensory aversion. A second factor also argues in favor of the differences observed in terms of ethanol consumption between PDs 11 and 12 in pups with prior history with the drug. These animals exhibited high levels of hypothermia after the first intake test (Figure 4c). Hypothermia is a critical factor in the acquisition of ethanol-related conditioned aversions (Cunningham & Niehus, 1993; Hunt, Spear, & Spear, 1991). This physiological disruption may be due to the high levels of ethanol consumption at PD 11 and/or a conditioned isodirectional thermal response caused by ethanol intoxication. The latter possibility is supported by studies indicating that pups associate physiological effects of the drug with its sensory cues perceived via non metabolic processes such as alveolar excretion, salivation, perspiration, urination (Bachmanov et al., 2003). At PD 11,

the intake test implies re-exposure to ethanol's sensory cues that may trigger an isodirectional thermal conditioned response relative to the original unconditioned effects of the drug (hypothermia as observed at PDs 5 and 7).

Beyond these considerations, the empirical findings of the present study, indicate that during a stage in development equivalent to the third human gestational trimester, relatively few exposures to ethanol affects the integrity of the respiratory network independently from thermoregulatory alterations. The drug also exerts negative consequences in terms of overall body growth and the weight of the brain. However, it appears that higher doses and levels of exposure are required to result in microcephaly. In conjunction with prior studies centered on this particular as well as an earlier ontogenetic stage (late gestation in the rat equivalent to the second human gestational trimester) the results also indicate that sequential ethanol intoxication increases ethanol affinity at least when the organism has the opportunity to ingest the drug for the first time. None of these effects seems to be associated with changes in central or ethanol metabolism as suggested by results attained at PD12 when ethanol pre-exposed and naive infants ingested similar levels of the drug. As a whole, these preclinical results should reinforce efforts to prevent ethanol intoxication during pregnancy and particularly binge-like drinking episodes independent of their frequency during a critical window of brain vulnerability.

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