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Neonatal sensitization to ethanol-induced breathing disruptions as a function of late prenatal exposure to the drug in the rat: Modulatory effects of ethanol's chemosensory cues

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HIGHLIGHTS

- Ethanol depresses respiration rates in newborn rats.
- Late gestational alcohol exposure sensitizes ethanol-induced breathing depression.
- Ethanol odor potentiates early ethanol-related disruptions upon respiration.

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ABSTRACT

Preclinical and clinical studies have systematically demonstrated abrupt changes in fetal respiratory patterns when the unborn organism is exposed to the effects of maternal ethanol intoxication. In subprimates, chronic exposure to this drug during gestation and infancy results in marked alterations of the plasticity of the respiratory network. These alterations are manifested in terms of an early incapability to overcome deleterious effects of hypoxic events as well as in terms of sensitization to ethanol's depressant effects upon breathing patterns. It has also been demonstrated that near term rat fetuses process ethanol's chemosensory cues when the drug contaminates the amniotic fluid and that associative learning processes occur due to the temporal contiguity existing between these cues and different ethanol-related physiological effects. In the present study during the course of late gestation (gestational days 17–20), pregnant rats were intragastrically administered with either 0.0 or 2.0 g/kg ethanol. Seven-day-old pups derived of these dams were evaluated in terms of respiration rates (breaths/min) and apneas when subjected to different experimental conditions. These conditions were defined by postnatal exposure to the drug (intragastric administrations of either 0.0, 0.5, 1.0 or 2.0 g/kg ethanol), postadministration time of evaluation (5–10 or 30–35 min) and olfactory context at test (no explicit ambient odor or ethanol ambient odor). The results, obtained via whole body plethysmography, indicated that brief prenatal experience with the drug sensitized the organisms to ethanol's depressant effects particularly when employing the higher ethanol doses. In turn, presence of ethanol odor at test potentiated the above mentioned respiratory alterations. Prenatal treatment with ethanol was not found to alter pharmacokinetic profiles resulting from postnatal exposure to the drug or to affect different morphometric parameters related with lung development. These results indicate that even brief exposure to the drug during late gestation is sufficient to sensitize the organism to later disruptive effects of the drug upon breathing responsiveness. These deficits are potentiated through the re-exposure to the olfactory context perceived in utero which is known to be associated with ethanol's unconditioned effects. As a function of these observations it is possible to suggest a critical role of fetal sensory and learning capabilities in terms of modulating later ethanol-related breathing disruptions.

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1 . Introduction

The teratological effects of ethanol, involving craneofacial anomalies, neurobehavioral alterations and growth retardation, do not cover all the possible consequences of early exposure to the drug. Beyond Fetal Alcohol Syndrome, intrauterine experiences with ethanol promote short and long term effects related with alcohol seeking, intake and preference patterns [1,2]. Preclinical and epidemiological research has indicated that these effects not only occur when utilizing high ethanol doses or chronic exposure to the drug that are known to result in gross morphological abnormalities [3].

The olfactory systems (principal, accessory and trigeminal) of different mammals, including humans, allow fetal detection of volatile substances present in the amniotic fluid [4–6]. Mere fetal familiarization with odorants changes subsequent detection and preference patterns of the stimuli perceived in utero. This non-associative learning process affects highly structured behaviors such as nipple attachment and lactation and even complex social patterns implying non-nutritive interactions with the mother or other conspecifics [7–10].

Fetuses exposed to subthreshold ethanol doses relative to its gross teratological properties, form memories relative to the chemosensory components of the drug. Rats that sense alcohol in the amniotic fluid will later react, behaviorally and autonomically, to the presence of this odorant [11,12]. When the drug is administered to the rat dam during the last four gestational days (blood and amniotic fluid ethanol levels ranging between 40–120 mg%) pups will later prefer the odor and they behaviorally react to this stimulus as alcohol-naïve pups respond to a biological odorant such as the amniotic fluid [13,14]. Analogous findings have been observed in healthy human babies delivered by mothers exhibiting moderate drinking patterns during pregnancy [15].

Fetal alcohol-related memories can also be established via associative learning processes. Numerous studies indicate that the near term fetus associates different chemosensory cues with ethanol's interoceptive or unconditioned effects [16,17]. From a correlational perspective the magnitude of ethanol-induced physiological disruptions (hypothermia) in the womb are highly predictive of neonatal responsiveness to the drug's chemosensory cues [18]. In neonates, and probably modulated by acetaldehyde central production and the involvement of the opiate system, ethanol exerts appetitive reinforcing effects [19–24]. Intrauterine pavlovian conditioning using ethanol as an unconditioned stimulus results in preferences to conditioned cues that signal ethanol intoxication [16,17]. In addition, it has been reported that prenatal ethanol exposure sensitizes the organism to the positive reinforcing effects of the drug [25]. Besides, it would be incorrect to state that relatively low doses of ethanol administered during short periods of time, do not exert short or long-lasting alterations in the developing organism [26].

Exposure to alcohol during prenatal development has also been associated with significantly reduced amniotic fluid volume and shortened umbilical cord length [27,28]. Umbilical cord length is a good indicator of fetal movement and provides direct evidence that maternal alcohol ingestion affects spontaneous fetal activity while suppressing breathing movements [29]. Fetal breathing movements (FBMs) are known to be present approximately 30% of the time in the near term human fetus and they represent a critical factor for normal development in different mammalian species [30,31]. Maternal human consumption of only two glasses of wine during late gestation disrupts fetal organization of behavioral states (particularly active sleep) and exerts a dramatic suppression of breathing activity [32–34]. The depressant effects of the drug upon FBMs have been systematically reported in humans and ewes [34–38]. The fact that fetal alcohol exposure is a risk factor for Sudden Infant Death Syndrome [39,40], has stimulated research focusing on the deleterious effects of the drug upon the respiratory system and its plasticity. In rats, chronic ethanol exposure (starting before mating and continuing throughout gestation and lactation) reduces brainstem-dependent respiratory rhythmic activity in the progeny and sensitizes juveniles to the depressant effects of acute ethanol upon phrenic and hypoglossal nerve activity [41]. Chronic prenatal ethanol exposure also affects neonatal rats in terms of compensatory respiratory processes that occur following hypoxia. Rather than observing long term facilitation of breathing after low oxygen exposure, neonates with a prenatal alcohol history exhibit long term depression of respiratory activity [42]. In the neonate rat, in vitro studies indicate significant depression in the respiratory-related hypoglossal nerve output caused by ethanol [43].

As mentioned, near term rat fetuses rapidly sensitize to the drug's chemosensory properties and its pharmacological effects and they are capable of acquiring associative memories comprising these factors. Under the consideration of these processes and the systematic reports of ethanol's effects upon FBMs, the present study (particularly Experiment 1) was guided by the following questions: i) will relatively short lasting near term fetal experiences with ethanol alter subsequent neonatal breathing frequencies? ii) is this type of exposure sufficient to generate either tolerance or sensitization to the drug's effects upon neonatal breathing or to disruptions such as apnea? and iii) is it possible that neonatal re-exposure to ambient ethanol odor modulates neonatal breathing patterns under the state of sobriety or intoxication? As can be observed the second question implies two opposite possibilities: development of tolerance or sensitization. As stated above, sensitization has been observed in juvenile rats after chronic ethanol exposure during gestation and lactation [41]. Yet, in near term sheep, development of tolerance to ethanol-induced suppression of FBMs occurs following short-term maternal administration of a moderate Ethanol dose (1 g/kg) [44].

Experiment 2 was conducted to determine if prenatal treatment with ethanol affected pharmacokinetic profiles in pups subjected to similar doses and postadministration times as those examined in Experiment 1. A third experiment examined whether prenatal exposure to the drug altered morphometric characteristics of the lungs of the neonates under consideration.

2. Material and methods

2.1.1. Subjects

Animals employed in this study were Wistar-derived rats born and reared at the vivarium of the Instituto Ferreyra (INIMEC-CONICET-UNC, Argentina). The animal colony was kept at 22–24 °C and under artificial lighting conditions (lights on: 08:00–20:00 h). Maternal lab chow (Cargill, 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 (body weights: 200–300 g) were housed overnight with males. Vaginal smears were checked the following morning and the day of sperm detection was considered as gestational day 0 (GD 0). Pregnant females were individually placed in maternity cages partially filled with wood shavings. Day of parturition was considered as postnatal day 0 (PD 0). At PD 1, litters were culled to 10 pups (5 males and 5 females whenever possible). 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 (Institute of Laboratory Animal Resources, 1996).

2.1.2. Drug treatment during gestation

From GDs 17 to 20, females were intragastrically intubated on a daily basis with either 0.0 (tap water) or 2.0 g/kg ethanol. The ethanol dose was achieved by administering 0.015 ml/g of a 16.8% v/v ethanol solution. Ethanol dosage and days of administration were selected based on prior studies showing fetal learning derived from the drug's sensory, physiological and behavioral effects and lack of deleterious effects of this treatment upon infantile gross morphological brain and body parameters, neuronal migration processes and sensory or perceptual and learning capabilities [3,13,14,45,46]. Intragastric intubations were

performed employing a polyethylene cannula (PE 50; Clay Adams, New Jersey) attached to a disposable 5-ml syringe. Maternal weights at GDs 17–20 and litter sizes at PD 0 were analyzed.

2.1.3. Infantile ethanol administration

At PD 7 pups were removed from their maternal cages and placed in pairs in isolation cages for 30 min. Immediately later, they were weighed (+/− 0.01 g) and administered with one of the following ethanol doses: 0.0, 0.5, 1.0 or 2.0 g/kg (i.g.). These doses were achieved by administering 0.015 ml/g of a 4.2, 8.4 or 16.8% v/v alcohol solution per gram of body weight, respectively. After administration, animals were placed in pairs in isolation chambers kept at 32–34 °C for 5 or 30 min before performing breathing or metabolic evaluations (Experiments 1 and 2; respectively). In Experiment 3 (morphometric lung analyses), 7-day-old pups received either 0.0 or 2.0 g/kg ethanol. Intragastric intubations were performed employing a polyethylene cannula (PE 10; Clay Adams, New Jersey) attached to a disposable 1-ml syringe.

2.1.4. Determination of breathing and apnea frequencies

In Experiment 1, whole body plethysmography (Plethysmograph Model 10G equipped with the following software: "Breath Medidor de Respiracion", Itcom, Argentina) was used to determine respiratory and apneic frequencies in unrestrained and awake pups. Two identical transparent and hermetic Plexiglas chambers (5 × 10 × 5 cm) define the apparatus. These chambers are interconnected via a polyurethane hose system resistant to pressurized air. One of the chambers is utilized as a testing device while the second chamber is considered as a reference box in terms of flow/air pressure. The hose system allows injection and extraction of equivalent amounts of air in both chambers in order to maintain constant and equivalent pressures. The hardware also allows determining the sensitivity of the recordings as a function of the weight of the animal that is being tested. The hardware was built to support respiratory recordings of animals weighing between 6 and 28 g. 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 recorded the respiratory response every 1.5 s. These values were then transformed to mean breaths per minute. The apparatus was also calibrated to record apneas. An apneic episode was defined as the interruption of air flow for at least two normal respiratory cycles (0.5 s or more) [47].

As mentioned, pups had differential histories of ethanol exposure during late pregnancy and they were evaluated under the effects of varying ethanol doses and postadministration times and under the presence or absence of ethanol odor. Five or 30 min following postnatal ethanol i.g. administration, pups were individually placed in the testing chamber.

For each test, two small balls of cotton (0.05 g each) were attached to the inside of the chamber lids. Depending on the olfactory treatment at test, these cotton swabs were embedded or not with 0.01 ml of 190% proof alcohol (Porta Hermanos, Argentina). Immediately after, the animals were introduced into the chambers, the lids were closed and the recording of the respiratory response was initialized.

After a 1 minute acclimatization period in the chamber, respiratory responses were registered for 5 min under the presence or absence of ethanol odor. In order to avoid infantile thermal disruptions, the temperature inside the plethysmograph was kept at 31–32 °C through the use of heating pads placed beneath the chambers [48]. An air conditioning system was also employed to maintain room temperature at 24 +/− 1 °C. Prior and after each individual evaluation, temperature inside the testing chamber was controlled.

2.1.5. Determination of blood ethanol concentrations (BECs)

In Experiment 2, pups were subjected to similar pre- and postnatal treatments as those employed in Experiment 1. After being placed in the plethysmograph, pups were sacrificed through decapitation. BECs were determined at 10 and 35 min after postnatal drug administration on PD 7 (0.5, 1.0 or 2.0 g/kg). These time periods correspond to the end of each breathing evaluation session that took place in Experiment 1. A total of 200 μl of trunk blood was collected from each pup. Each sample was fractioned to obtain two 100 μl sample which were placed in microvials containing 50 μl of a butanol solution (51 mg/100 ml) that served as an internal standard. Microvials were sealed and blood samples were frozen until determination of BECs through head-space gas chromatographic analyses (model 5890, Hewlett-Packard, Palo Alto, CA). The procedures and technical characteristics of this chromatographic analysis have been extensively described in prior studies (e.g. [49]). BECs corresponding to each pup were averaged across both blood samples. All values were expressed as milligrams of ethanol per 100 ml of blood (mg%).

2.1.6. Determination of lung morphometric characteristics

The morphometric analysis was performed in order to consider possible alterations caused by pre- and postnatal treatments upon lung weights and diameters of bronchioles and pulmonary arteries (Experiment 3). In this experiment we replicated the prenatal treatments employed in Experiments 1 and 2 but we only employed 0.0 or 2.0 g/kg ethanol in terms of postnatal treatments.

Thirty minutes following vehicle or ethanol administration, pups were euthanized by decapitation. Lungs were surgically removed, weighed and processed for histological examination. Paraffin-embedded sections of the lungs were cut in 4-mm sagittal sections and were stained with hematoxylin and eosin (H&E). A Primo Star iLed microscope, equipped with an Axicam ERc 5s Microscope camera (Zeiss, Jena, Germany), was used to obtain microphotographs and digital images were employed to determine bronchioles and pulmonary artery diameters (μm). Morphometric measurements were made using the digital imaging software Adobe Photoshop version CS2. Three fields per case were quantified and averaged and all measurements were made on coded slides, thereby blinding the observer to the case classification.

2.1.7. Experimental designs and statistical analysis

Descriptive data in tables and figures are expressed as means ± standard errors of the means (SEMs). In all experiments, only one male or female from a given litter were assigned to a given evaluation treatment. This quasirandom procedure was employed to avoid confusions between litter and treatment effects [50]. Efforts were also made in order to ensure an equivalent representation of sex in each specific group of animals. The number of litters and pups as well as the dependent variables under analyses will be specified in each experiment. All experiments included factorial designs primarily defined by pre- and postnatal treatments. Dependent variables corresponding to the different experiments (breathing and apnea frequencies, BECs and lung morphometric parameters) were inferentially examined through the use of between analyses of variance (ANOVAs). Despite the fact that a considerable number of male and female pups were employed in all experiments, preliminary analysis of all the dependent variables under analysis indicated that sex was never found to exert significant main effects or interactions. Hence, inferential analyses in all experiments were performed by collapsing sex across the different treatments. Between factors in Experiment 1 were: Prenatal Treatment (0.0 or 2.0 g/kg ethanol) × Postnatal Treatment (0.0, 0.5, 1.0 or 2.0 g/kg ethanol) × Postnatal Postadministration Time (5–10 or 30–35 min) × Odor Exposure during Testing (no odor or ethanol

ambient odor). Experiment 2 included similar factors with the only exception being that the postnatal dose 0.0 g/kg was not included. The ANOVAs employed in Experiment 3 (morphometric parameters) included the following between factors: Prenatal Treatment (0.0 or 2.0 g/kg ethanol) × Postnatal Treatment (0.0 or 2.0 g/kg ethanol). Main effects and interactions were considered to be statistically significant at $p < 0.05$. Due to the large number of groups defining the factorial designs (particularly those corresponding to Experiments 1 and 2) and in order to avoid type I errors, Bonferroni post-hoc tests were utilized to correct for repeated testing.

Despite the fact that a considerable number of male and female pups were employed in all experiments, preliminary analysis of all the dependent variables under analysis indicated that sex was never found to exert significant main effects or interactions. Hence, inferential analyses in all experiments were performed by collapsing sex across the different treatments.

2.2. Experiment 1

The main purpose of this experiment was to analyze neonatal breathing frequencies as a function of moderate levels of ethanol exposure during late gestation and neonatal re-exposure to the drug's chemosensory and/or intoxicating properties. As stated, evaluations took place either at commencement of the toxic state (postadministration time: 5–10 min) or when blood ethanol contents reach peak levels (postadministration time: 30–35 min; see Results of Experiment 2). Total frequency of apnea was also determined.

2.2.1. Subjects

A total of 46 pregnant females were employed. Half of these dams were prenatally treated with water while the remaining animals received 2.0 g/kg ethanol (GDs 17–20). At PD 7, pups were assigned to one of 32 groups defined by the factorial design [Prenatal Treatment (0.0 or 2.0 g/kg ethanol) × Postnatal Treatment (0.0, 0.5, 1.0 or 2.0 g/kg ethanol) × Postnatal Postadministration Time (5–10 or 30–35 min) × Odor Exposure during Testing (no odor or ethanol odor)]. A total of 398 pups were employed and the number of animals per group ranged between 10–15 pups.

2.2.2. Results: maternal and infantile body weights and litter size

Maternal weights at GDs 17–20 were analyzed through a two-way mixed analysis of variance (ANOVA) where prenatal treatment (0.0 or 2.0 g/kg ethanol) served as a between factor and days of treatment as repeated measures. As could be expected, body weights progressively increased during the course of late gestation. The ANOVA indicated a significant main effect of day [$F(3,102) = 227.83$, $p < 0.0001$] but no significant effects of prenatal treatment or of the interaction between the factors under consideration.

Litter sizes at PD 0 were analyzed as a function of prenatal treatments. A one-way ANOVA did not reveal significant differences between treatments. The number of pups born from vehicle and ethanol-treated dams were 9.17 ± 0.44 and 9.42 ± 0.41(mean ± SEM); respectively.

Body weights at PD 7 were similar across prenatal treatment and sex. Mean ± SEMs for the groups under consideration were as follows: prenatal water; males: 17.15 ± 0.09 g and females: 17.18 ± 0.08 g; prenatal 2.0 g/kg ethanol, males: 17.11 ± 0.09 g and females: 17.23 ± 0.10 g.

2.2.3. Results: respiration frequencies and apneas

Fig. 1a illustrates mean breaths per minute obtained across all treatments. The 4-way between ANOVA (prenatal treatment × postnatal ethanol dose × postadministration time × odor at test) indicated significant main effects of the following factors: prenatal treatment, $F(1,379) = 11.83$; postnatal ethanol dose, $F(3,379) = 41.75$; postadministration time, $F(1,379) = 53.36$ and odor at test, $F(1,379) = 5.72$; all p's < 0.025. The following two-way interactions also achieved significance: prenatal treatment × postnatal ethanol dose, $F(3,379) = 4.69$; postnatal ethanol dose × postadministration time, $F(3,379) = 3.62$ and postnatal ethanol dose × odor at test, $F(3,379) = 6.57$ (all p's < 0.025). Finally, two 3-way interactions were statistically significant: prenatal treatment × postnatal ethanol dose × postadministration time [$F(3,379) = 4.82$, $p < 0.005$] and prenatal treatment × postnatal ethanol dose × odor at test [$F(3,379) = 3.38$, $p < 0.025$]. These 3-way interactions have been depicted in Fig. 1b and c; respectively.

To better understand the locus of the 3-way interactions, Bonferroni post-hoc tests were performed. When focusing on the "prenatal treatment × postnatal ethanol dose × postadministration time" interaction, it was observed that pups with no prenatal ethanol history, tested only 5 min after ethanol administration, exhibited similar breathing frequencies across all postnatal doses. In these pups, when ethanol reached higher BECs (30–35 min after administration; see Experiment 2), a dose–response effect was evident. During this postadministration time, breathing frequencies of prenatally ethanol naïve pups administered with either 1.0 or 2.0 g/kg ethanol, showed a significant depression in breathing when compared to pups exposed to either 0.0 or 0.5 g/kg ethanol (all p's < 0.001). A different pattern emerged when focusing on pups subjected to ethanol exposure during late pregnancy. Shortly (5–10 min) after being treated with 1.0 or 2.0 g/kg ethanol, significant decrements in breathing were observed relative to neonates with similar prenatal history but postnatally administered with 0.0 or 0.5 g/kg ethanol (p's < 0.05). At the later stage of intoxication (30–35 min), pups prenatally exposed to ethanol and postnatally treated with 2.0 g/kg were significantly more depressed in terms of breathing than any other group tested at this time period (all p's < 0.05). These values were also significantly lower when compared to the ones exerted by a similar dose during the initial stage of the toxic process ($p < 0.0001$) (Fig. 1b).

When taking into account the "prenatal treatment × postnatal ethanol dose × odor" interaction, post-hoc analyses revealed the following effects. In water prenatally treated pups, under no explicit olfactory stimulation, the effects of postnatal ethanol doses (obviously collapsed across postadministration times) were not significant. Pups with a positive ethanol gestational experience administered with 1.0 or 2.0 g/kg ethanol and tested under no explicit ambient odor, exhibited significantly lower breathing rates when compared to age counterparts receiving either 0.0 or 0.5 g/kg ethanol (with or without prenatal ethanol experience, all p's < 0.025). When ethanol odor defined the testing condition, pups prenatally exposed to water and later treated with 1.0 or 2.0 g/kg ethanol differed from those treated with lower doses (all p's < 0.05). Presence of ethanol odor at test also potentiated the depressant effects of the 2.0 g/kg ethanol dose in pups with prior prenatal ethanol history. In this group of animals there was a dramatic depression of breathing (89.8 ± 19.0 breaths/min) that was significantly different from all the remaining groups (all p's < 0.0025) (Fig. 1c).

Frequencies of apneas are shown in Fig. 2a. These scores were significantly affected by the main effects of prenatal treatment [$F(1,379) = 21.09$, $p < 0.0001$], postnatal ethanol [$F(3,379) = 3.66$, $p < 0.025$] and postadministration time [$F(1,379) = 7.03$, $p < 0.01$]. The following 2-way significant interactions were observed: prenatal treatment × postnatal ethanol [$F(3,379) = 4.57$, $p < 0.005$] and prenatal treatment × postadministration time [$F(1,379) = 5.24$, $p < 0.025$]. A 3-way interaction involving prenatal status, postnatal ethanol dose and odor at test also attained significance [$F(2,379) = 3.08$, $p < 0.05$].

Relative to the interaction existing between prenatal status and postnatal postadministration time, post-hoc tests showed that apnea frequency in pups prenatally exposed to alcohol during the 30–35 min test, was significantly higher relative to all the remaining conditions (all p's < 0.005) (Fig. 2b). When considering the 3-way interaction comprising pre- and postnatal ethanol treatments and odor at test, it was

observed that pups with no prenatal ethanol experience, exhibited minimal number of apneas. This pattern of results was different in pups with positive prenatal ethanol experience. Post-hoc tests did not reflect significant differences across postnatal ethanol doses when these pups were tested under non-olfactory conditions. When ethanol odor was present, pups exposed to alcohol in utero and later administered with 2.0 g/kg ethanol, exhibited significantly higher frequencies of apnea relative to those treated with 0.0 or 0.5 g/kg ethanol under similar olfactory condition and relative to all groups composed by pups subjected to intrauterine water exposure (both p's < 0.01) (Fig. 2c).

2.3. Experiment 2: Determination of blood ethanol concentrations

In Experiment 2, BECs were determined in 7-day-old pups subjected to similar treatments as those employed in the previous study. The main goal was to determine if differential prenatal exposure to ethanol modifies postnatal levels of intoxication as operationalized through BECs which could in turn explain differences observed in breathing patterns. This experimental approach was defined through the use of similar prenatal treatments as those employed in Experiment 1. The remaining between factors were postnatal ethanol administration (0.5, 1.0 or 2.0 g/kg), postadministration time of evaluation (5–10 or 30–35 min) and ambient odor at test (no explicit odorant or ethanol odor).

2.3.1. Subjects

Eighteen pregnant females were utilized. Nine were treated with vehicle while the remaining animals received 2.0 g/kg ethanol (GDs 17–20). At PD 7 pups were assigned to one of 24 groups (n = 6–8 per group) while avoiding litter overrepresentation and pursuing equivalent sex representation. A total of 147 infants were used in this experiment.

2.3.2. Results

Litter sizes and infantile body weights were not affected by prenatal treatment. In terms of metabolic profiles, the ANOVA indicated that prenatal treatment and odor at test exerted no significant effects upon BECs nor did they significantly interact with any of the other factors. Main significant effects of postnatal ethanol dose [$F(2,123) = 112.79$] and postadministration time [$F(1,123) = 7.51$] were observed (both p's < 0.01). The interaction between these two factors was also significant [$F(2,123) = 8.09$, $p < 0.001$]. As can be observed in Table 1, BECs positively correlated with ethanol dosage. Post-hoc tests showed that at the initial stage of intoxication (5–10 min) BECs corresponding to 2.0 g/kg ethanol were already significantly higher than the values obtained with 0.5 or 1.0 g/kg. At the late stage of intoxication (30–35 min) BECs corresponding to the higher ethanol dose kept on rising and were significantly higher relative to the ones observed in all the remaining groups (Table 1).

2.4. Experiment 3: determination of morphometric lung characteristics

The morphometric analysis was performed in order to consider possible alterations caused by pre- and postnatal ethanol exposure in terms of lung wet weight and diameters of the bronchioles and the pulmonary artery. This experiment was conducted using four groups of pups. Animals were representative of similar prenatal treatments as those previously employed and on PD 7 they either received 0.0 or 2.0 g/kg ethanol. Pups were sacrificed 30 min after receiving either dose.

2.4.1. Subjects

At GDs 17–20, 7 pregnant females were i.g. administered with vehicle and 7 additional dams were treated with 2.0 g/kg ethanol. A total of 44 pups representative from these dams were used ("n" per group = 11 pups). Litter overrepresentation was avoided and each group had similar sex representation.

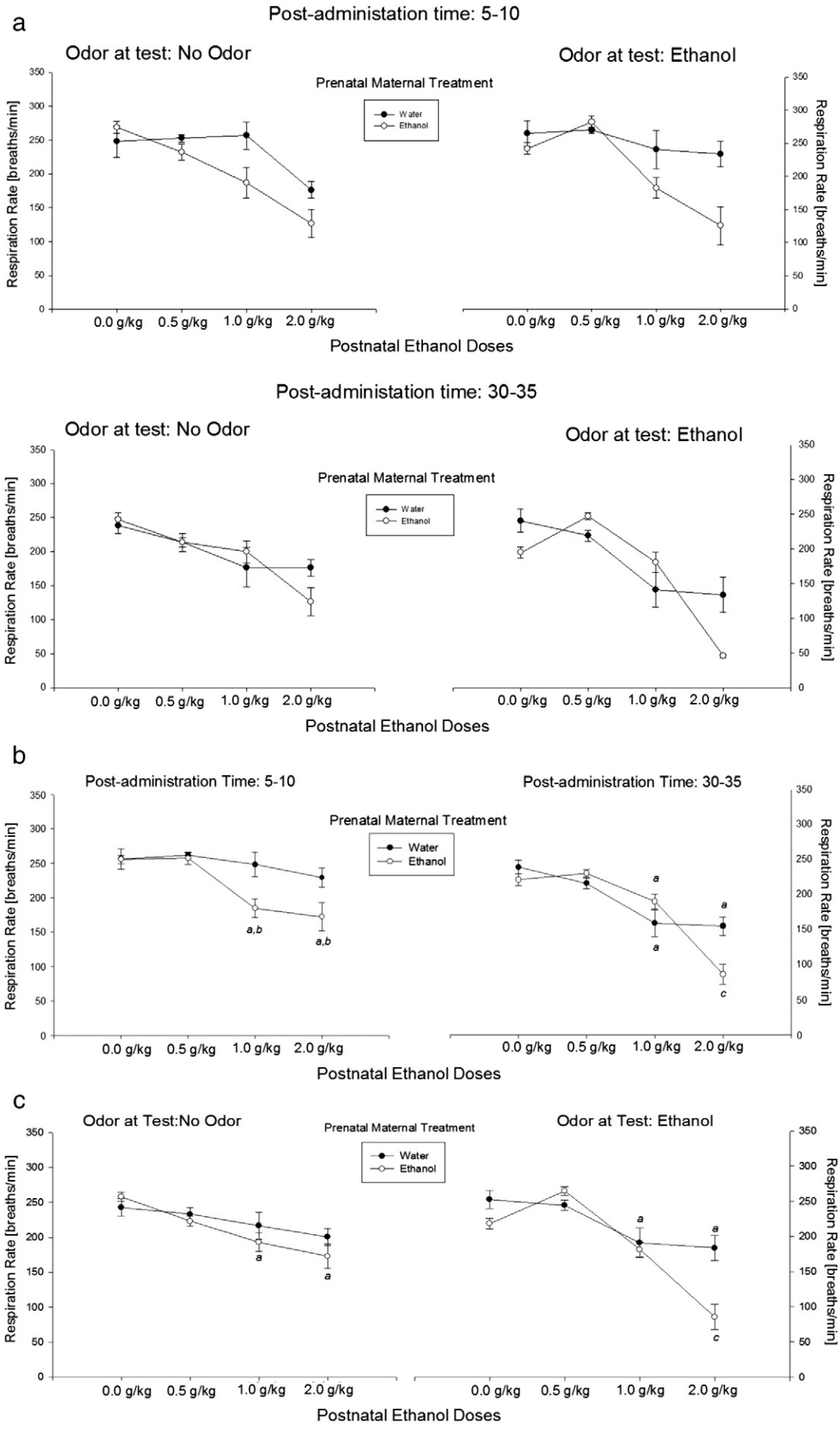
2.4.2. Results

Table 2 summarizes the results. With respect to lung morphometric parameters, no significant differences were found between the groups when analyzing wet lung weight (absolute and adjusted for body weight), diameters of bronchiolus and pulmonary artery.

3. Discussion

The results from the present study indicated that breathing frequencies negatively correlated with ethanol dosage during postnatal life (Experiment 1). In other words, the higher the ethanol dose given at test the lower the breathing rate. Yet, these effects were tempered by the interaction of several factors such as prenatal ethanol treatment, postadministration time and odor at test. The depressant effects of ethanol upon infantile breathing patterns were more clearly observed when BECs increased as a function of postadministration time; especially when focusing on the 2.0 g/kg postnatal ethanol dose (in terms of BECs see Experiment 2). When considering specific interactions, it was clear that pups prenatally exposed to ethanol were more sensitive to the depressant effects of postnatal administration of the drug. This sensitivity was evident when testing was conducted under the ambient presence of the drug's olfactory properties and the intoxicating effects of 2.0 g/kg ethanol. Yet, the process of sensitization was also present in pups with positive prenatal ethanol experiences when focusing on the initial stages of infantile intoxication (5–10 min) resulting from the administration of 1.0 and 2.0 g/kg ethanol. In this early toxic phase, sensitization was observed independently from the nature of the odorant presented (Fig. 1b). As has been the case in studies devoted to the motivational properties of ethanol, these results appear to indicate two processes that can independently or synergistically affect subsequent reactivity to the drug. Similar levels of prenatal ethanol exposure as those here employed have shown that sensitization occurs when postnatally employing relative small ethanol doses known to exert appetitive effects. This phenomenon not necessarily requires the presence of conditioned stimuli (e.g. the odor the drug) that the fetus originally associates with ethanol's unconditioned properties [51,25]. The second process is mainly linked with associative learning. In this case, the mentioned conditioned stimuli either elicit specific conditioned responses or potentiate later unconditioned consequences of the toxic state [51,52]. In other words, the present results indicate a similar profile in terms of possible differential learning effects upon respiration plasticity. Sensitization to the depressant effects of the drug due to postnatal ethanol experiences does occur

Fig. 1. a: Respiration frequency (breaths/min) as a function of prenatal treatment (0.0 or 2.0 g/kg), postnatal ethanol dose (0.0, 0.5, 1.0 or 2.0 g/kg), postadministration time (5–10 or 30–35 min) and odor at test (No Odor or Ethanol Odor). The figure illustrates mean values for the 32 groups defined by the corresponding factorial design. Vertical lines depict standard errors of the means (SEMs). b: Respiration frequency (breaths/min) as a function of the significant 3-way interaction existing between prenatal treatment, postnatal ethanol dose and postadministration time. Vertical lines depict standard errors of the means (SEMs). (a) Significantly different relative to controls treated postnatally with 0.0 or 0.5 g/kg ethanol. (b) Significantly different from pups prenatally treated with water and postnatally administered with 1.0 or 2.0 g/kg ethanol. (c) Significantly different from all the remaining groups. c: Respiration frequency (breaths/min) as a function of the significant 3-way interaction existing between prenatal treatment, postnatal ethanol dose and odor at test. Vertical lines depict standard errors of the means (SEMs). (a) Significantly different relative to controls treated postnatally with 0.0 or 0.5 g/kg ethanol. (c) Significantly different from all the remaining groups.


a
Post-administation time: 5-10
Odor at test: No Odor
Odor at test: Ethanol
Prenatal Maternal Treatment
Water
Ethanol
Respiration Rate [breaths/min]
0.0 g/kg 0.5 g/kg 1.0 g/kg 2.0 g/kg
Postnatal Ethanol Doses
Post-administation time: 30-35
Odor at test: No Odor
Odor at test: Ethanol
Prenatal Maternal Treatment
Water
Ethanol
Respiration Rate [breaths/min]
0.0 g/kg 0.5 g/kg 1.0 g/kg 2.0 g/kg
Postnatal Ethanol Doses
b
Post-administration Time: 5-10
Post-administration Time: 30-35
Prenatal Maternal Treatment
Water
Ethanol
a,b
a,b
a
a
a
c
Respiration Rate [breaths/min]
0.0 g/kg 0.5 g/kg 1.0 g/kg 2.0 g/kg
Postnatal Ethanol Doses
c
Odor at Test:No Odor
Odor at Test: Ethanol
Prenatal Maternal Treatment
Water
Ethanol
a
a
a
a
c
Respiration Rate [breaths/min]
0.0 g/kg 0.5 g/kg 1.0 g/kg 2.0 g/kg
Postnatal Ethanol Doses

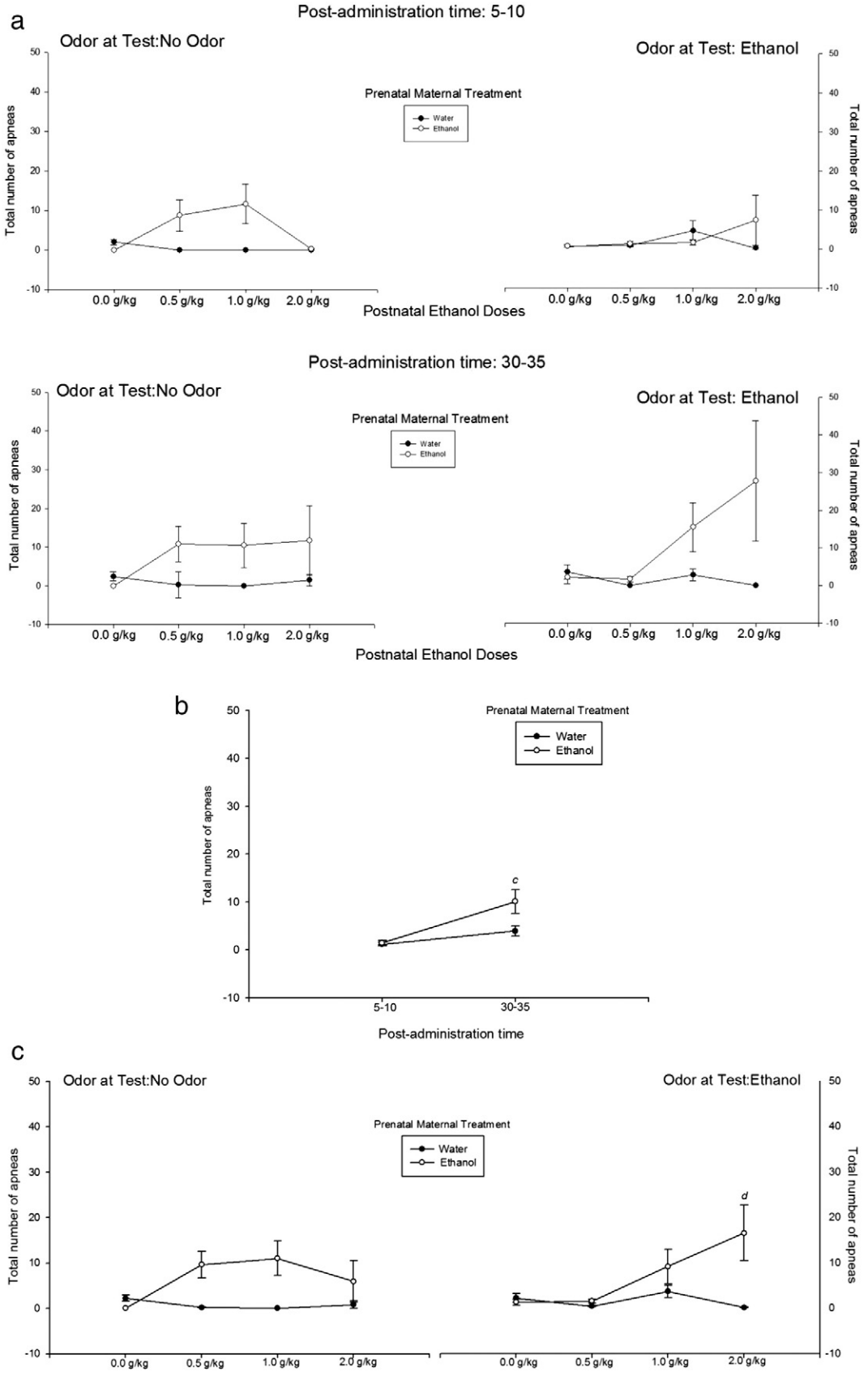


Fig. 2. a: Total number of apneas as a function of prenatal treatment (0.0 or 2.0 g/kg), postnatal ethanol dose (0.0, 0.5, 1.0 or 2.0 g/kg), postadministration time (5–10 or 30–35 min) and odor at test (No Odor or Ethanol Odor). The figure illustrates mean values for the 32 groups defined by the corresponding factorial design. Vertical lines depict standard errors of the means (SEMs). b: Total number of apneas as a function of the significant 2-way interaction existing between prenatal treatment and postadministration time. Vertical lines depict standard errors of the means (SEMs). (c) Significantly different from all the remaining groups. c: Total number of apneas as a function of the significant 3-way interaction existing between prenatal treatment, postnatal ethanol dose and odor at test. Vertical lines depict standard errors of the means (SEMs). (d) Significantly different from all groups prenatally exposed to water and those prenatally exposed to ethanol, tested under ethanol odor and postnatally administered with 0.0 or 0.5 g/kg.

early during the toxic state (Fig. 1b). In turn this phenomenon is potentiated when utilizing the highest infantile ethanol dose (2.0 g/kg) while pups inhale ethanol odor. In this last case, potentiation occurred during both postadministration intervals (Fig. 1c) but the effect was more pronounced when BECs were higher (30–35 min; Table 1). This heightened depression may obey to the similarity existing between the testing conditions and those inherent to fetal learning experiences with the drug. Following maternal ethanol administration, the drug progressively accumulates in the amniotic fluid and in the fetal blood and brain [3]. Maximal chemosensory recruitment and unconditioned respiratory effects are likely to occur in later rather than early stages of the process of fetal intoxication.

In terms of frequency of apneas, pups prenatally treated with water, under all postnatal conditions, rarely exhibited this respiratory disruption. A significant number of apneas were recorded in subjects prenatally exposed to ethanol, particularly during the 30–35 min test. The magnitude of these disruptions reached maximal levels in pups prenatally exposed to ethanol and tested under the effects of the highest ethanol dose (2.0 g/kg) and the presence of ambient ethanol odor. As can be observed the results are similar but not identical to those related with the drug's effects upon respiratory depression. As stated, in order to recruit heightened levels of apneas there were two necessary conditions: the presence of ethanol odor while infants experience high levels of intoxication (ethanol dose: 2.0 g/kg; postadministration time: 30–35 min yielding BELs ranging between 250–300 mg/dl).

These results appear not to be associated with differential pharmacokinetic profiles in terms of BECs across prenatal conditions. Experiment 2 only indicated dose dependent increases in BECs and that maximal levels of this dependent variable were registered in pups receiving the highest ethanol dose (2.0 g/kg) during the later stage of intoxication (30–35 min). The metabolic profiles are in agreement with prior studies aimed at determining effects of late gestational ethanol upon the drug's psychomotor stimulatory and depressant effects [53]. In the mentioned study similar gestational treatments were employed and infants were tested under the same postadministration times as those evaluated here after receiving either 0.5 or 2.5 g/kg ethanol. As was the case in the present study, BECs were not affected by prenatal history with the drug. Arias et al. [53] also utilized a methodological approach ("inverted ladder") that allowed the analysis of behavioral habituation during testing, biphasic motor effects of the drug (stimulatory versus depressant) and the interaction between these factors. The fact that prenatal treatment did not affect any of these processes is important in light of the present results. The null effects upon infantile activity patterns argues against the possibility that the current results indicating changes in breathing patterns, represent an epiphenomenon of prenatal effects upon non-associative learning processes that affect activity or upon differential psychomotor properties of the drug. Ethanol-related disruptions of thermal control may also represent a potential factor involved in the breathing alterations here reported. Indeed, similar prenatal treatments as those here utilized promote maternal ethanol hypothermia [18]. In turn, prenatal or neonatal hypothermia can cause acidosis, bradycardia, hypoglycemia and respiratory arrest [54]. Any of these factors or the combination between them, could act as an effective unconditioned stimulus. From this perspective, re-exposure to the toxic state or to cues originally associated with such a state may also generate specific conditioned responses or differential magnitudes of the unconditioned effect dependent upon processes of sensitization or tolerance. In other words, the present results in terms of respiratory plasticity may also be considered as an epiphenomenon of associative and/or pharmacodynamic processes linked with thermoregulatory effects of ethanol. This hypothesis cannot be completely discarded since in this study thermal regulation was not assessed. Yet, it is important to note that during the entire testing procedure, chambers were heated to avoid possible thermal disruptions caused by vehicle or drug administration or social isolation (see Material and methods). This strategy was similar to the one employed by Hunt et al. [55] when analyzing taste aversions dependent upon ethanol-induced hypothermia in infant rats. In her study, exposure to a heated chamber (32–34 °C) completely blocked hypothermia and the related aversive learning phenomenon. Despite these considerations, it is difficult to rule out possible coupling of thermal and respiratory processes that can explain the results of the present study. This difficulty also becomes evident when considering prior studies that show either postnatal tolerance [56,57] or sensitization [58] to the hypothermic effects of ethanol as a function of prenatal exposure to the drug.

Table 1
Blood ethanol concentrations (mg %) as a function of postnatal ethanol dosage and postadministration time.

	Postadministration time	
Postnatal ethanol doses	5–10 min	30–35 min
0.5 g/kg	52.88 ± 5.05	44.04 ± 2.44
1.0 g/kg	99.36 ± 10.86 (a)	105.03 ± 7.88 (a)
2.0 g/kg	200.69 ± 25.95 (a,b)	293.19 ± 14.95 (a,c)

Values are mean and standard errors (SEMs). (a) Significantly different relative to pups postnatally treated with 0.5 g/kg ethanol. (b) Significantly different relative to pups postnatally treated with 1.0 g/kg ethanol at either postadministration time. (c) Significantly different from all the remaining groups.

Brief ethanol exposure during late gestation was not found to impair body or lung weights at PD 7 (Experiments 1 and 3). In lambs, null effects of late prenatal ethanol exposure have been reported in terms of postnatal lung weight and volume and in terms of postnatal alveoralization processes [59,60]. In the present study the microscopic analyses of the diameters of the bronchioles and the pulmonary artery were also found to be unaffected by prenatal ethanol. This does not preclude other possible lung-related alterations. As reported by Sozo et al. [59,60] the immune status of the developing lung in lambs can be compromised by late gestational ethanol exposure; an effect that rapidly fades without further exposure to the drug. We cannot rule out that other effects of prenatal ethanol upon pulmonary processes may alter breathing patterns within the context of the present study (e.g. alterations in lung collagen deposition [61] or impaired pulmonary surfactant protein [62]). Yet, these alterations appear to be primarily related with susceptibility to respiratory infections in neonates prenatally exposed to ethanol.

From a functional perspective, sober pups tested without the presence of ambient ethanol odor, showed similar breathing frequencies and absence of apnea across prenatal treatments. In agreement with the findings of Dubois et al. [41], the depressant effects of acute infantile ethanol exposure increased following prenatal ethanol exposure (sensitization). There are marked differences between the prenatal treatments employed by these authors (chronic exposure throughout gestation and lactation) and those corresponding to the present study (late gestation). As described, within the framework of the present study the sensitization effect comprising prenatal status and postnatal ethanol was tempered by either the ambient olfactory nature of the test or by postnatal administration time. Under a variety of experimental circumstances, it has been demonstrated that both chronic [63–65] and late prenatal exposure [2,3,66] sensitizes the organism to the drug's olfactory and taste properties. It is also important to note that early in ontogeny, and under the state of intoxication pups perceive the chemosensory properties of the drug due to direct ethanol elimination (saliva, respiration or urination) or hematogenic stimulation of olfactory receptors [67,68]. Under these considerations, coupled with the fact that near term rat fetuses rapidly associate ethanol's sensory cues and different pharmacological effects of the drug, common mechanisms may underlie pre- and postnatal potentiation effects recruited under the explicit presence of ethanol odor or as a function of postadministration time. In the first case, the sensitization effect was clearly driven by the presence of the ambient odorant known to be perceived in utero and probably contingent with ethanol-related breathing disruptions. When focusing on pre- and postnatal interactions as a function of

Table 2
Lung morphometric parameters of pups at PD 7.

Prenatal EtOH treatment	Lung weight (g)		Bronchiolus diameter (μm)		Pulmonary artery diameter (μm)	
	Postnatal EtOH treatment		Postnatal EtOH treatment		Postnatal EtOH treatment	
	0.0 g/kg	2.0 g/kg	0.0 g/kg	2.0 g/kg	0.0 g/kg	2.0 g/kg
0.0 g/kg	0.34 ± 0.01	0.34 ± 0.01	87.7 ± 3.6	93.5 ± 4.4	42.3 ± 1.7	43.9 ± 3.5
2.0 g/kg	0.35 ± 0.01	0.36 ± 0.01	95.9 ± 3.2	98.0 ± 4.5	42.0 ± 2.0	38.9 ± 3.0

postadministration time, it is possible that direct elimination of the drug also recruits the chemosensory properties of ethanol leading to the described sensitization effect (see Fig. 1c). In other words, the significant interactions under consideration appear to have a common denominator; i.e. ethanol's chemosensory cues modulating the prenatal impact upon latter depressant effects of the drug. Probably the saliency of the olfactory stimulus increases when the odor is not only processed due to direct elimination of the drug but it is also present as an exteroceptive stimulus. This appears to be the case when focusing on the dramatic depressant effects in pups administered with 2.0 g/kg ethanol that were prenatally exposed to the drug and tested under the presence of ethanol odor. Similar considerations appear pertinent when focusing on the high levels of apnea associated with prenatal treatment and postadministration time or the interaction comprising prenatal and postnatal ethanol dosage as well as ambient odor (Fig. 2b and c).

Within this functional perspective, it can be expected that chemosensory signals contingent with ethanol-induced breathing depression will eventually become effective conditioned stimuli that can elicit alterations in the respiration network. In the present study, despite a trend in this direction, we were unable to detect significant changes in sober pups that differed as a function of prenatal status or odor at test (Prenatal ethanol–ethanol odor: 222.2 +/− 7.6; Prenatal ethanol–No Odor: 259.9 +/− 6.6; Prenatal Water–ethanol odor: 252.4 +/− 12.9 and Prenatal Water–No Odor: 242.7 +/− 12.6 breaths/min; Fig. 1c). To test the hypothesis of possible conditioned breathing responses, alternative experimental procedures analyzing the importance of contiguity or contingency between an olfactory cue and breathing alterations are required. These procedures have been developed when examining associative learning capabilities of the fetus comprising non-biological odorants and unconditioned effects of the drug related with its motivational properties [17] and in the case of conditioned breathing responses when employing perinatal associations between an odorant and maternal care [69]. Based on these experimental approaches, we have recently examined if ethanol odor, originally associated with ethanol intoxication, later elicits conditioned breathing changes in rats during a period of brain development [PDs 3–9] comparable with that of the human third trimester [70,71]. Following only 3 conditioning trials where the smell of the drug was explicitly associated with a significant respiratory depression caused by 2.0 g/kg ethanol (i.g.), the sensory cue elicited a conditioned isodirectional response [72]. This preliminary result, in combination with those here reported, argues in favor of associative learning mechanisms arising from the process of ethanol intoxication that can affect breathing patterns in the developing organism.

Tolerance rather than sensitization to ethanol-induced suppression of FBMs has been observed in near term fetal sheep [44]. Pregnant ewes received an intravenous 1 g/kg ethanol dose slowly delivered during the course of 1 h. After 14 days of treatment, tolerance developed in terms of FBMs. Beyond species differences, the procedures employed by Smith et al. [44] markedly differ in terms of dosage, route and number of administrations from those here employed. These differences can account for the opposite outcomes between studies. It is possible that prolonged exposure to a relatively low dose, probably yielding low BECs due to the nature of the administration procedure, will eventually result in the establishment of functional or metabolic tolerance. On the contrary, defying the mechanisms that control FBMs with higher ethanol doses (e.g. as the one here employed) may lead to a lack of plasticity of the respiration network that will latter overreact to the toxic effects of the drug. These observations do not preclude the possibility of sequential biphasic effects (sensitization followed by tolerance) as a function of repeated exposure to the drug (e.g. [73]). Yet, it is important to note that chronic gestational ethanol exposure in rats has also been shown to increase latter sensitivity to the drug's suppressive effects on breathing [41].

In the rat neonate sensitization to other effects of ethanol (positive reinforcement) have been reported following drug exposure during late gestation [25]. It has also been shown that moderate late prenatal exposure to the drug, facilitates subsequent sensory detection of minimal ethanol concentrations in maternal milk and exacerbates the consumption of the contaminated nutrient [3,74]. Epidemiological studies indicate that a significant proportion of women exhibiting ethanol ingestion during pregnancy will continue to drink during lactation (e.g. [75]). The human literature shows that sensory detection of ethanol occurs during each of these developmental stages and that these experiences are accompanied by physiological alterations such as the organization of sleep–wake patterning [15,76]. Alternative modes of ethanol administration in babies are also likely to expose the infant to the drug's sensory and physiological effects [2,77]. In conjunction with the comprehensive work of Dubois et al. [41,42,78], the results here reported emphasize the need to consider alterations in early breathing patterns that arise from the drug's unconditioned effects upon respiration even when ethanol exposure occurs during brief periods of time. Our results also include the notion that the sensory properties of the drug, probably mediated through associative learning processes, can represent a critical factor regulating ethanol-induced breathing depression accompanied with higher levels of respiratory dysfunctions in terms of apneas.

These results emphasize the notion that respiratory dysfunctions caused by ethanol in utero not necessarily require chronic exposure to high doses known to exert marked teratogenic effects upon the cardiorespiratory system or the central network involved in breathing control [41,42,79,80]. Prolonged neonatal breathing depression and the presence of apneic episodes (temporally related with hypoxemia and bradycardia) represent a risk factor in terms of hypoxic ischemic effects upon the developing human brain [81,82]. This observation logically implies the need to detect and control maternal, medical and environmental factors that cause or modulate such disruptions. The reported results, in conjunction with preclinical and epidemiological research indicating heightened alcohol abuse resulting from intrauterine exposure to even low to moderate ethanol doses, extend the notion of early life programming of later life disorders [83]. Obviously and as a consequence of this observation, it seems pertinent to still question the existence of safe amounts of prenatal ethanol exposure [3]. As observed, early learning mechanisms associated with intrauterine ethanol exposure can profoundly affect basic physiological processes such as neonatal respiratory patterns.

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