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ORIGINAL INVESTIGATION

Brief ethanol exposure and stress-related factors disorganize neonatal breathing plasticity during the brain growth spurt period in the rat

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Abstract

Rationale The effects of early ethanol exposure upon neonatal respiratory plasticity have received progressive attention given a multifactorial perspective related with sudden infant death syndrome or hypoxia-associated syndromes. The present preclinical study was performed in 3–9-day-old pups, a stage in development characterized by a brain growth spurt that partially overlaps with the 3rd human gestational trimester.

Methods Breathing frequencies and apneas were examined in pups receiving vehicle or a relatively moderate ethanol dose (2.0 g/kg) utilizing a whole body plethysmograph. The experimental design also considered possible associations between drug administration stress and exteroceptive cues (plethysmographic context or an artificial odor). Ethanol exposure progressively exerted a detrimental effect upon breathing frequencies. A test conducted at PD9 when pups were under the state of sobriety confirmed ethanol's detrimental effects upon respiratory plasticity (breathing depression).

Results Pre-exposure to the drug also resulted in a highly disorganized respiratory response following a hypoxic event, i.e., heightened apneic episodes. Associative processes involving drug administration procedures and placement in the plethysmographic context also affected respiratory plasticity. Pups that experienced intragastric administrations in close temporal contiguity with such a context showed diminished hyperventilation during hypoxia. In a 2nd test conducted at PD9 while pups were intoxicated and undergoing hypoxia, an attenuated hyperventilatory response was observed. In this test, there were also indications that prior ethanol exposure depressed breathing frequencies during hypoxia and a recovery normoxia phase.

Conclusion As a whole, the results demonstrated that brief ethanol experience and stress-related factors significantly disorganize respiratory patterns as well as arousal responses linked to hypoxia in neonatal rats.

Keywords Neonate · Stress · Learning · Ethanol · Hypoxia · Breathing

Introduction

Prenatal ethanol exposure is associated with a spectrum of short- and long-lasting detrimental morphological and functional effects. Growth retardation, including intrauterine growth restriction, spontaneous abortion, stillbirth, preterm delivery, as well as other perinatal alterations are strongly associated with the patterns of maternal ethanol consumption. Beyond possible gestational and birth disorders, cognitive and emotional/social disturbances, autism spectrum disorder, and sudden infant death syndrome (SIDS) have been frequently reported in studies analyzing embryonic and/or fetal exposure to the drug (Parazzini et al. 2003; Peadon et al. 2007; Bailey and Sokol 2011; Dubois et al. 2013; Haas et al. 2015). Most studies have focused on fetal alcohol syndrome and fetal alcohol spectrum disorders, commonly associated with high levels of ethanol exposure during gestation. Yet, a variety of studies argue against safe ethanol doses during early development (Wise 2002; Molina et al. 2007; Abate et al. 2008; Welch et al. 2016). Even low-to-moderate ethanol exposure during early ontogeny may have dramatic effects upon a broad

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spectrum of functional alterations involving synaptic plasticity, learning and memory mechanisms, social behavior, and respiratory patterns (Savage et al. 2002; Molina et al. 2007; Abate et al. 2008; Zucca and Valenzuela 2010; Cullere et al. 2015). In animal and human fetuses, ethanol-dependent effects such as bradycardia, acidosis, hypercapnia, hypothermia, hypoglycemia, and hypotonia have been detected when employing different dosing procedures (Abel 1981; Schröck et al. 1989; Duxbury 1990; Pepino et al. 1998; Abate et al. 2004; Parnell et al. 2007; Bake et al. 2012; Dubois et al. 2013). Some studies also indicate disruptions caused by relatively low and infrequent doses of ethanol upon the respiratory system and its plasticity in fetuses and neonates (Fox et al. 1978; Lewis and Boylan 1979). Maternal human ingestion of only two glasses of wine during late gestation are sufficient to significantly suppress fetal breathing movements (Brien and Smith 1991); an activity that under baseline conditions occurs 30–46% of the time in the near-term fetus (Lewis and Boylan 1979; Bocking 2003; Joshi and Kotecha 2007; Greer 2012). Similar alterations have been detected in near-term sheep following short-term administration of relatively low ethanol doses (0.3–1.0 g/kg; Vojcek et al. 1988; Smith et al. 1989, 1990).

Preclinical studies based on ethanol-related teratological models have also indicated severe breathing impairments in the arousal response to hypoxia in rats (Dubois et al. 2013; Sirieix et al. 2015). Generally, these models imply chronic and high levels of ethanol exposure where females are exposed to the drug via liquid diets during a long period of time (i.e., starting before gestation, throughout the entire gestational period, and during lactation). Logically, these models do not take into account ethanol-related effects caused by brief and moderate levels of exposure to the drug. Under normoxia and during neonatal, periadolescent and adult stages of development, ethanol-exposed rats exhibit marked decrements in breathing frequencies. Under hypoxic conditions, these decrements are also observed in conjunction with alterations comprising compensatory respiratory processes (Dubois et al. 2008; Kervern et al. 2009). These physiological alterations positively correlate with a depressant effect of the brainstem rhythmic activity and decrements in the activity of the phrenic and hypoglossal nerve response (Dubois et al. 2008; Kervern et al. 2009). The preceding preclinical and clinical investigations related with ethanol's effects upon breathing lead to the following relevant observations: (i) breathing disruptions represent a risk factor relative to hypoxic ischemic effects upon the developing brain (Pillekamp et al. 2007) and (ii) given a significant association between fetal ethanol exposure and the sudden infant death syndrome (Burd et al. 2004; O'Leary et al. 2013), scientific attention has been devoted towards the effects of the drug upon respiratory plasticity (Kinney et al. 2009; Dubois et al. 2013).

Depressant effects of moderate ethanol doses upon early breathing frequencies have been recently reported following few administrations of the drug during late gestation or early postnatal life (Cullere et al. 2015; Macchione et al. 2016; Acevedo et al. 2017b). In rats, these stages of development are analogous to the 2nd and 3rd human gestational trimesters, respectively (Dobbing and Sands 1973, 1979; Tran et al. 2000). Independently from the ontogenetic stage of administration, the studies indicated reductions in breathing frequencies and increments in apneic episodes in pups with prior history with the drug. Also, sequential ethanol exposure generated a sensitization process relative to ethanol's depressant effects (Cullere et al. 2015; Macchione et al. 2016). This sensitization process was intimately related with the emergence of a respiratory conditioned response elicited by stimuli explicitly associated with ethanol's unconditioned depressant effects. In the case of fetal alcohol exposure, the stimulus (conditioned stimulus (CS)) that elicits an isodirectional conditioned response relative to ethanol's depressant effects, is the odor of the drug (Cullere et al. 2015). Near-term fetuses process ethanol's chemosensory attributes when the drug contaminates the amniotic fluid and are capable of learning an association between the sensory components of the drug and physiological consequences of the state of intoxication (Abate et al. 2001, 2008; Molina et al. 2007; Cullere et al. 2015). When studies were conducted during the brain growth spurt period in rats (1st postnatal week of life), ethanol odor also served as an effective CS signaling the state of intoxication and later eliciting conditioned respiratory depressions (Macchione et al. 2016). A follow-up study indicated that the context (e.g., the plethysmographic context (pleth context)) where neonates were evaluated during the toxic episode may also act as an effective CS. In addition, this study suggested that different administration procedures (intracisternal or intragastric) may act as unconditioned stressors capable of mediating associative learning processes (Acevedo et al. 2017a, b). Neonatal-conditioned respiratory responses have also been detected when utilizing an artificial odorant as a CS (lemon) associated with the unconditioned properties of maternal care (Durand et al. 2003).

The overall goal of the present study is to analyze the consequences of brief ethanol exposure during the brain growth spurt in rats upon respiratory plasticity. The first experiment was designed to test the following two main hypotheses: (i) brief ethanol experiences (postnatal days (PDs) 3, 5, and 7; a stage comparable to the 3rd human gestational trimester) are capable of altering respiratory patterns when the organism is evaluated under the state of sobriety and when recreating the state of intoxication and (ii) these impairments are modulated by learning processes involving salient conditioned stimuli associated with depressant respiratory effects of the drug which are also modulated by stress-related events(e.g., intragastric administration procedures). As will be described, both hypotheses were evaluated through sequential testing procedures defined by an initial normoxia phase followed by hypoxia and recovery normoxia phases. The last two phases appear to be implicated in the pathophysiology of SIDS and to

our knowledge they have not been analyzed as a function of brief ethanol exposure during the brain growth spurt period (Paterson et al. 2006; Carlin and Moon 2017). A second experiment analyzed if ethanol pre-exposed pups (PD3–PD7) exhibited metabolic tolerance to the drug when were re-exposed to ethanol at PD9 and tested under normoxic or hypoxic conditions.

Materials and methods

Subjects

Wistar-derived rats born and reared at the vivarium of the Instituto Ferreyra (INIMEC-CONICET-UNC, Argentina) were employed. The animal colony was kept at 22–24 °C under artificial lighting conditions (lights on, 08:00–20:00 hours). On the day of proestrus, females (body weights, 200–250 g) were housed overnight with males and when pregnant they were housed in individual maternal cages. The day of parturition was considered postnatal day 0 (PD0). At PD1, litters were culled to ten pups (five males and five females whenever possible).

The "Principles of Laboratory Animal Care" were followed, and animals used in this study were maintained and treated according to Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research (National Research Council 2011) and also by the Institutional Committee of Laboratory Animal Use and Care of our institution (CICUAL-INIMEC-CONICET-UNC).

Basic experimental procedures

Experiment 1

Based on the original hypotheses, in this experiment, we analyzed the impact of relatively brief ethanol exposure (PD3, PD5, and PD7) upon subsequent breathing frequencies and respiratory alterations (apneas) in pups tested under sequential phases of air conditions (normoxia, hypoxia, and recovery normoxia) and while being under the state of sobriety or intoxicated (PD9). The experiment was also designed to establish whether ethanol or stress-related alterations in breathing plasticity are modulated by associative learning processes. To address this learning process, we employed an experimental strategy where drug administration procedures were paired or not with an exteroceptive context (i.e., pleth context). The pleth context was scented or not with a salient odorant. The odorant (0.5 ml of a 0.15% (*v*/*v*) orange solution; Esencias del Boticario, Córdoba, Argentina) was placed in a small ball of cotton (0.5 g) that was attached to the inside of the chamber lid.

At PD9, testing was conducted in the pleth context and under the presence of orange odor. The whole body apparatus that was utilized to record breathing frequencies and number of apneic episodes in neonates has been previously described (Cullere et al. 2015; Acevedo et al. 2017b). The criterion used to determine an apneic episode was the interruption of air flow for at least two normal respiratory cycles (0.5 s or more; Julien et al. 2010).

Neonatal conditioning phase A total of 97 pups representative of 13 litters were employed (10–13 pups per experimental group with an equivalent sex representation). Pups representative of seven litters were intragastrically (i.g.) administrated at PD3, PD5, and PD7 with 2.0 g/kg ethanol while the remaining animals (six litters) received 0.0 g/kg ethanol. The 2.0-g/kg ethanol dose was achieved by administering 0.015 ml/g of body weight of a 16.8% (*v*/*v*) ethanol solution (96% proof alcohol, Porta Hermanos, Cordoba, Argentina; vehicle: distilled water). Similar volumes of distilled water were utilized in the case of pups exposed to the 0.0-g/kg ethanol dose. The differential drug treatment across litters was performed to avoid interactions between non-intoxicated and intoxicated pups since social learning relative to the sensory and toxic effects of ethanol is likely to occur (Fernández-Vidal and Molina 2004; Eade and Youngentob 2009, 2010). Previous studies (Acevedo et al. 2017b) and unpublished preliminary experiments have been conducted to analyze specific effects relative to the i.g. intubation itself. The explicit comparisons were made relative to vehicle-treated animals and relative to pups treated with either 2.0 g/kg ethanol or even a higher dose than the one here employed (3.0 g/kg). These preliminary studies systematically indicated the absence of significant differences between non-intubated pups and those receiving vehicle during conditioning and testing phases analogous to the ones here employed. Hence, a non-intubated control group was not employed in the present series of experiments.

To address whether the pleth context or this stimulus compounded with a salient odor served as effective CSs signaling ethanol's unconditioned effects upon respiration, eight different groups were employed. These groups were derived from a 2 × 2 × 2 factorial design. One factor was defined by drug treatment (0.0 or 2.0 g/kg ethanol). A second factor was the temporal contiguity existing between drug administration and the experience within the pleth context (paired or unpaired). The third factor was defined by the presence or not of the orange scent in the pleth context (odor or no odor).

Pups subjected to the paired protocol were removed from their maternal cage and one male with one female were placed in cages partially filled with clean corncob. Pups were maintained in pairs to avoid undesirable effects of social isolation conditions (Pautassi et al. 2006; Arias et al. 2010). They remained in these cages, kept at 32–34 °C via heating pads, during 15 min. Pups were then weighed, i.g. administered with 0.0 or 2.0 g/kg ethanol, and they were later returned to

cages where they remained for 15 min. Immediately after, pups were individually introduced into the scented or unscented pleth context. Breathing and apnea frequencies were simultaneously recorded in four pups via a whole body plethysmographic system during 5 min (Fig. 1a). Data was averaged every 2.5 min of the test (bin). Hence, in a 5-min test, two 2.5 min bins were taken into account.

Pups subjected to the unpaired protocol were removed from their maternal cages, weighed, and placed in pairs (one male/one female) during 30 min in isolation cages under similar ambient conditions as those described for paired pups. Immediately after, breathing and apnea frequencies were individually recorded in the scented or unscented pleth context. Pups were then returned to their maternal cages where they stayed for 2.5 h. Unpaired pups were then i.g. administered with 0.0 or 2.0 g/kg ethanol and returned to their maternal cages (Fig. 1a).

As can be observed, paired pups were evaluated under the effects of vehicle or ethanol soon after being administered with these substances. The common denominator across these pups was the association between the administration procedure and the exposure to the scented or unscented pleth context. Unpaired pups were tested in the scented or unscented pleth context 2.5 h prior to the

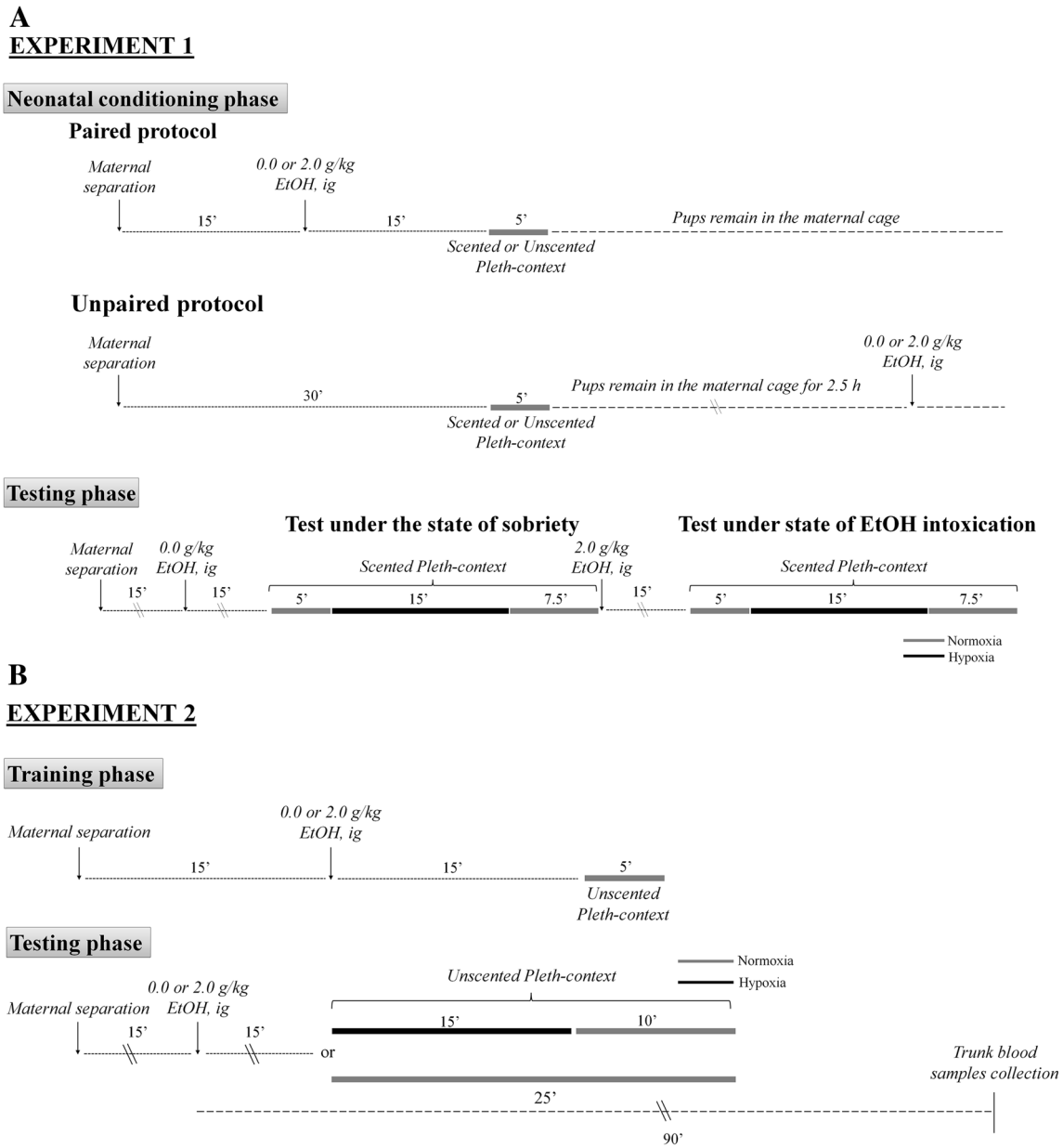

Fig. 1 Schematic illustration representing the experimental designs employed in Experiment 1 **(a)** and Experiment 2 **(b)**

administration of vehicle or ethanol; a delay aimed at avoiding the association between the possible stress of the administration procedure and exteroceptive cues such as the pleth context and/or orange odor. In paired pups, this possible stressor was in close temporal contiguity with the unscented or scented pleth context.

Testing day At PD9, all pups were removed from their maternal cages, placed in pairs in isolation cages during 15 min and i.g. administered with 0.0 g/kg ethanol. Fifteen minutes later, pups were evaluated in an orange-scented pleth context (Fig. 1a). Breathing frequencies and apneas were recorded during 27.5 min. This test was defined by the following sequential phases: initial normoxia (5 min; bins 1–2), hypoxia (15 min; bins 3–8), and recovery normoxia (7.5 min; bins 9–11). Plethysmographic chambers were saturated with room air (~ 21% O_2) during initial and recovery normoxia phases. During the hypoxic episode, chambers were saturated with oxygen-poor air (8% O_2 and 92% N_2, Praxair, Argentina SRL). The flow of room-air or hypoxic gas mixture delivered into the chamber was kept always constant. Subsequently, pups were administrated with 2.0 g/kg ethanol. Following 15 min in the isolation cages, they were again tested in the scented pleth context. The phases of this test under the state of intoxication were analogous to the ones employed when pups were tested under the state of sobriety.

Experiment 2

This experiment assessed possible changes in ethanol metabolism at PD9 as a function of prior ethanol exposure during the conditioning phase (PD3, PD5, and PD7). Additionally, possible changes in BELs at PD9 as a function of normoxic or hypoxic conditions were examined. Forty-eight pups representative of 11 litters were utilized (4–8 pups per experimental condition with an equivalent sex representation). As shown in Fig. 1b, pups were administered with 0.0 or 2.0 g/kg ethanol at PD3, PD5, and PD7 similarly to the design used in experiment 1. Fifteen minutes after of i.g. administration, pups were tested in an unscented pleth context during 5 min. At PD9, pups representative of the prior drug treatments, were either administered with 0.0 or 2.0 g/kg ethanol. Animals were tested in an unscented pleth context under one of the following air conditions: only normoxia during 25 min or hypoxia (15 min) followed by a period of recovery normoxia (10 min). Ninety minutes after the i.g. administration, pups were euthanized and trunk blood samples were collected to determine BELs. As can be observed, the experimental design was defined by the following factors: drug treatment at PD3, PD5, and PD7 (0.0 or 2.0 g/kg ethanol), drug treatment at PD9 (0.0 or 2.0 g/kg ethanol), and air conditions at test (only normoxia or hypoxia/recovery normoxia). The apparatus and methods employed to determine BELs via head-space gas chromatographic analyses have been described in detail in prior studies (Pueta et al. 2008; Cullere et al. 2015; Macchione et al. 2016).

Experimental designs and statistical analysis

Only one male and one female per litter were assigned to a given experimental condition. This *quasirandom* procedure was employed to avoid confusions between litters and treatment effects (Holson and Pearce 1992). Experiment 1 was defined by a factorial design where drug treatment (0.0 or 2.0 g/kg ethanol), temporal contiguity between the administration procedure and placement in the pleth context (paired or unpaired), olfactory stimulation within the pleth context (unscented or scented), as well as sex represented the between factors. Repeated measures were collected during the course of each specific breathing assessment (bins 1–2 at PD3, PD5, and PD7 or bins 1–11 at PD9). Between-within analyses of variance (ANOVAs) were utilized to process breathing and apnea frequencies. During the *conditioning phase* (PD3, PD5, and PD7), the ANOVA took into account the independent variables previously mentioned while age (PD3, PD5, and PD7) and bins within each test served as repeated measures. At test (PD9), variables that served as repeated measures were the state of intoxication (state of sobriety or ethanol intoxication) and bins (1–11).

Experiment 2 responds to a factorial design defined by sex, drug treatment during PD3, PD5, and PD7 (0.0 or 2.0 g/kg ethanol), drug treatment at PD9 (0.0 or 2.0 g/kg ethanol) and air-conditions (only normoxia or hypoxia/recovery normoxia). BELs at PD9 served as the dependent variable.

Whenever an ANOVA revealed a significant main effect of a between factor or the interaction comprising between factors, Newman-Keuls post hoc tests (alpha set at 0.05) were utilized to determine the locus of such an effect. In the case of significant interactions comprising between and within factors, follow-up ANOVAs and planned comparisons were utilized. These orthogonal comparisons were utilized since there is no unambiguous choice of appropriate error term for post hoc interactions involving between-within group significant interactions (Winer 1991).

Results

Experiment 1

Body weight gains at PD3, PD5, PD7, and PD9

According to the overall ANOVA, body weights during the temporal course of the experiment were found to

significantly differ as a function of sex, age, and drug treatment ($F_{(1,\ 81)} = 5.24$, $p = 0.0247$; $F_{(3,\ 243)} = 2313.3$, $p < 0.0001$; and $F_{(1,\ 81)} = 6.23$, $p = 0.0146$, respectively). These significant main effects were tempered by the following two-way interactions: sex × age and drug treatment × age ($F_{(3,\ 243)} = 4.64$, $p = 0.0036$ and $F_{(3,\ 243)} = 18.36$, $p < 0.0001$, respectively). According to planned comparisons, males and females exhibited no significant differences in body weights at commencement of the experiment (PD3). During the following days (PD5, PD7, and PD9), males always exhibited significantly higher weights relative to females. When taking into account the interaction between drug treatment and postnatal days, planned comparisons indicated that ethanol administration progressively affected the morphological index under examination. During the last days of the experiment (PD7 and PD9), body weights of ethanol-treated pups were significantly lower than those recorded in vehicle-exposed pups. As has been the case in multiple studies based on the brain growth spurt period, sequential ethanol administration progressively exerts a negative effect upon body weights (Macchione et al. 2016; Acevedo et al. 2017a, b). Percentage of increment of body weights between the PD3 and PD9 was also analyzed. Pups exposed to ethanol showed less percent increase relative to pups treated with vehicle ($F_{(1,\ 81)} = 18.90$, $p = 0.0001$). Table 1 depicts body weights as a function of drug treatment across age and the percentage increase of body weight between PD9 and PD3.

Breathing frequencies and apneas during the conditioning phase (PD3, PD5, and PD7)

Figure 2a illustrates average respiration frequencies every 2.5 min across factors that were found to exert significant main effects or to significantly interact (drug treatment, temporal contiguity, age, and bins). The overall ANOVA indicated significant main effects of the following factors: temporal contiguity between drug administration and pleth placement, age, and bin ($F_{(1,\ 81)} = 21.32$, $p < 0.0001$; $F_{(2,\ 162)} = 47.26$, $p < 0.0001$; $F_{(1,\ 81)} = 92.80$, $p < 0.0001$, respectively). The following two-way interactions also attained significance: drug treatment × temporal contiguity ($F_{(1,\ 81)} = 6.63$, $p = 0.0119$); drug treatment × age ($F_{(2,\ 162)} = 4.69$, $p = 0.0105$); drug treatment × bin ($F_{(1,\ 81)} = 6.12$, $p = 0.0154$); age × temporal contiguity ($F_{(2,\ 162)} = 6.47$, $p = 0.0020$); and age × bin ($F_{(2,\ 162)} = 11.64$, $p < 0.0001$). A three-way significant interaction comprising drug treatment, age, and bin was also observed ($F_{(2,\ 162)} = 4.47$, $p = 0.0129$). Relative to the interaction between drug treatment and temporal contiguity, Newman-Keuls post hoc tests indicated that paired pups that received 2.0 g/kg ethanol showed lower breathing frequencies relative to paired pups treated with vehicle or unpaired pups subjected to either drug treatment (Fig. 2b). When taken into account, the interaction between age and temporal contiguity, planned comparisons showed that in unpaired pups, breathing frequencies significantly increased across days. This was not the case in paired pups where breathing frequencies did not significantly increase during the PD5 and PD7. During these days, paired pups exhibited significantly less breathing frequencies relative to unpaired controls (Fig. 2c). The ANOVA also indicated a significant three-way interaction (drug treatment × age × bin). Follow-up two-way ANOVAs (drug treatment × bin) were conducted for each specific postnatal day. At PD3 and PD5, the only significant differences were related with a decrement in breathing scores during the 2nd bin relative to the 1st bin (habituation) ($F_{(1,\ 81)} = 13.01$, $p = 0.0005$ and $F_{(1,\ 81)} = 76.41$, $p < 0.0001$, respectively). At PD7, drug treatment and bin exerted significant main effects ($F_{(1,\ 81)} = 6.29$, $p = 0.0142$ and $F_{(1,\ 81)} = 55.42$, $p < 0.0001$, respectively), and these variables also significantly interacted ($F_{(1,\ 81)} = 9.24$, $p = 0.0032$). According to planned comparisons, ethanol-treated pups at commencement of the test (bin 1) exhibited significantly lower breathing frequencies relative to vehicle controls. In both groups, breathing frequencies were higher during the 1st bin relative to the 2nd bin (Fig. 2d).

Table 1 Body weights and percent increase in body weights corresponding to experiment 1 as a function of drug treatment (0.0 and 2.0 g/kg EtOH)

Age (postnatal day)	Experimental groups	0.0 g/kg EtOH	2.0 g/kg EtOH
PD3	Body weight (g)	9.30 ± 0.21	8.97 ± 0.19
PD5		12.36 ± 0.29	11.71 ± 0.27
PD7		16.20 ± 0.39	14.84 ± 0.36***
PD9		20.51 ± 0.46	18.39 ± 0.43***
PD9/PD3 (%)	Percent increase in body weights (%)	120.98 ± 2.64	105.24 ± 2.47***

Values are expressed as means ± SEMs

***$p < 0.001$, significant differences between ethanol and vehicle-exposed pups

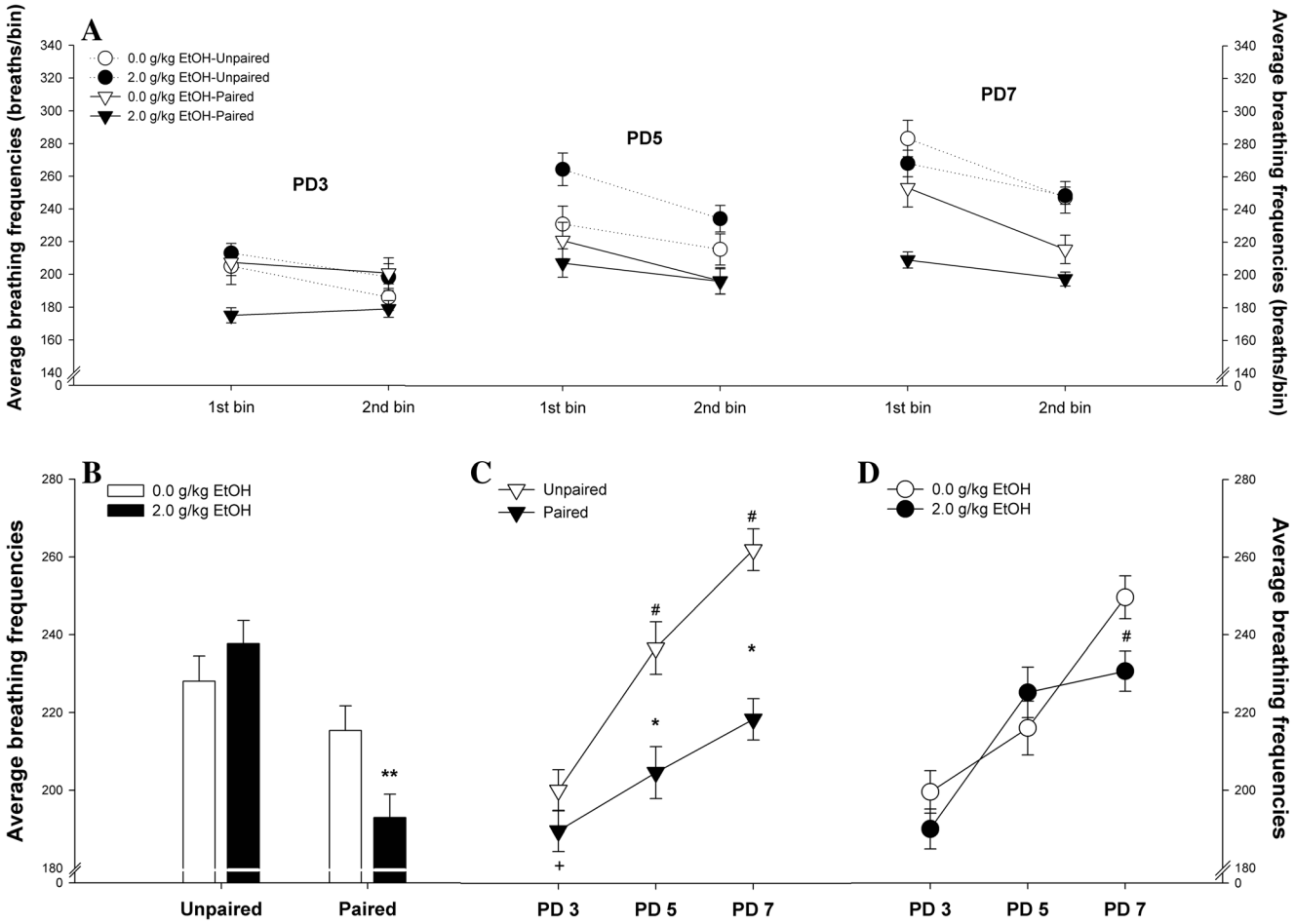

Fig. 2 **a** Average breathing frequencies(breaths/bin) during the conditioning phase (PD3, PD5, and PD7) as a function of drug treatment (0.0 or 2.0 g/kg EtOH), temporal contiguity between the administration procedure and placement in the pleth context (paired or unpaired) and bin (1 and 2). **b** Average breathing frequencies as a function of drug treatment and temporal contiguity between the administration procedure and placement in the pleth context. $^{**}p < 0.01$, significant differences between the paired-EtOH group relative to remaining groups. **c** Average breathing frequencies as a function of temporal contiguity between the administration procedure and placement in the pleth context and conditioning days. $^{\#}p < 0.05$, significant differences between postnatal days in the unpaired group. $^{+}p < 0.05$, significant differences between PD3 respect to PD5 and PD7 in paired pups. $^{*}p < 0.05$, significant differences between unpaired and paired groups at PD5 and PD7. **d** Average breathing frequencies during the conditioning phase as a function of drug treatment. $^{\#}p < 0.05$, significant differences between EtOH and vehicle groups at PD7. Vertical lines depict standard errors of the means (SEMs)

The between-within ANOVA performed to analyze apneic episodes during PD3, PD5, and PD7 indicated no significant main effects or significant interactions between factors.

Breathing frequencies and apneas during the testing phase (PD9): test under the state of sobriety

Two evaluations were performed for each subject. The first test was under the state of sobriety while the second assessment was performed when pups were intoxicated with 2.0 g/kg ethanol. Each of these testing procedures was defined by three sequential phases. The first phase was under normoxic conditions (bins 1 and 2). During the next 15 min, pups were subjected to hypoxia (bins 3–8). Following this phase, pups were returned to a normoxic condition for 7.5 min (bins 9–11).

There were clear overall differences in breathing frequencies as a function of the state of intoxication. As could be expected, breathing frequencies during the state of intoxication were significantly lower than those previously recorded under sobriety state. A preliminary ANOVA confirmed this phenomenon ($F_{(1, 81)} = 105.16$, $p < 0.0001$). Hence, the respiratory performance under each particular state (sobriety or intoxication) was processed via separate ANOVAs.

Figure 3a illustrates the overall results under the state of sobriety. The ANOVA indicated significant main effects corresponding to prior drug treatment and bin ($F_{(1, 81)} = 4.20$, $p = 0.0437$ and $F_{(10, 810)} = 194.72$, $p < 0.0001$, respectively). Pups pretreated with ethanol exhibited lower breathing frequencies relative to vehicle-exposed controls (Fig. 3b). Breathing frequencies varied during the temporal course of the testing procedure. During the initial portion of the test

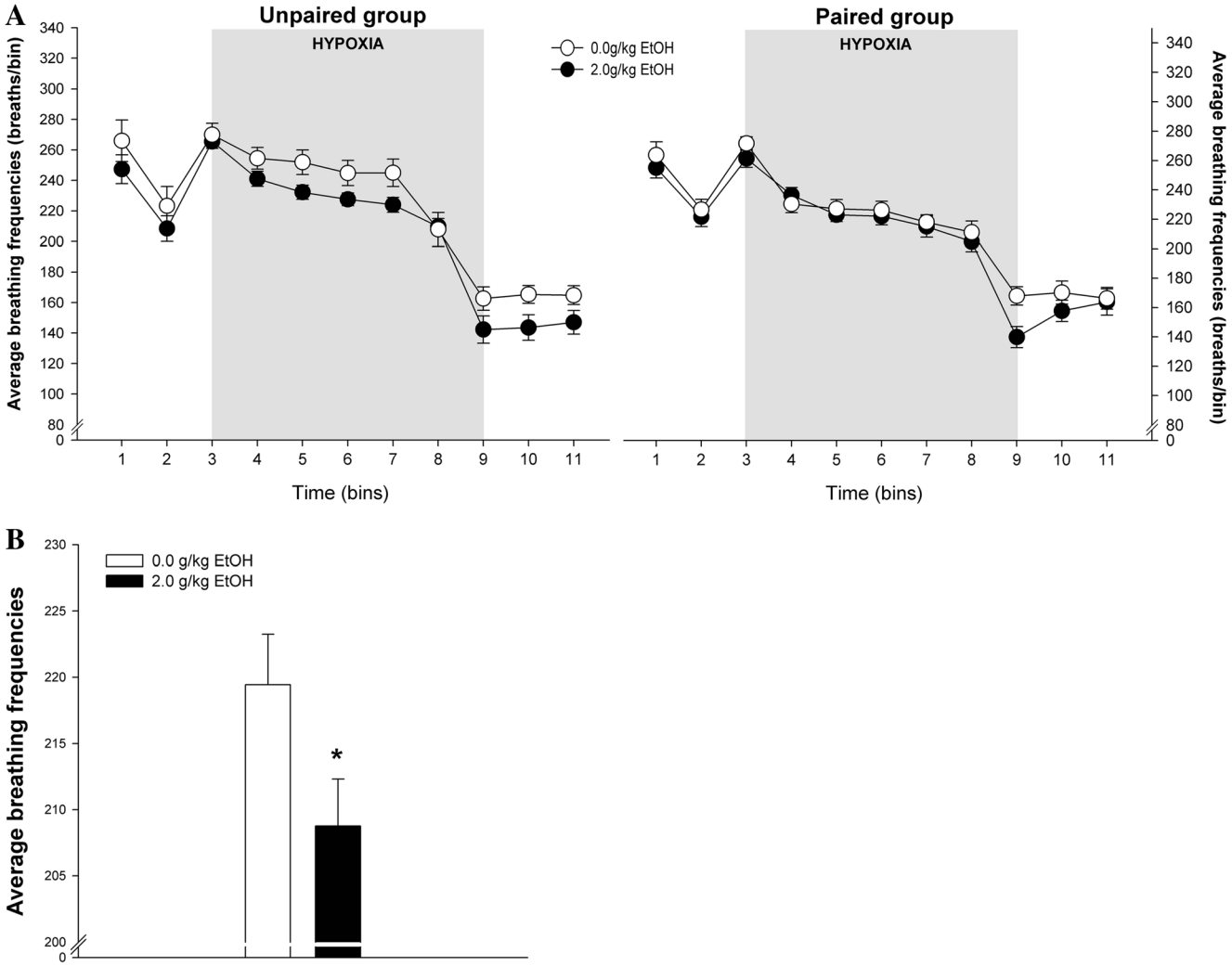

Fig. 3 **a** Average breathing frequencies during the testing phase (PD9) under the state of sobriety as a function of drug treatment (0.0 or 2.0 g/kg EtOH), temporal contiguity between the administration procedure and placement in the pleth context (paired or unpaired) and bin (1–11). Left, data of unpaired pups; right, data of paired pups. **b** Average breathing frequencies as a function of drug treatment. $^{*}p < 0.05$, significant differences between pups pretreated with 2.0 g/kg EtOH respect to pups pretreated with 0.0 g/kg EtOH. **c** Average breathing frequencies as a function of temporal contiguity between the administration procedure and placement in the pleth context and bin. $^{***}p < 0.0001$, significant differences between paired and unpaired groups. $^{\#}p < 0.0001$, significant differences between bins 3–7 (hyperventilation) relative to bin 2. **d** Average breathing frequencies as a function of sex and bin. $^{+}p < 0.05$, significant differences between males and females. **e** Frequency of apneic events at PD9 as a function of drug treatment during the conditioning phase and bin. $^{\#\#\#}p < 0.0001$, significant differences between recovery normoxia phase respect to the others bins. $^{**}p < 0.01$, significant differences between EtOH-pretreated pups respect to vehicle controls. **f** Frequency of apneic events as a function of sex and bin. $^{\&\&\&}p < 0.0001$, significant differences between the bins of the recovery normoxia phase respect to other bins. $^{++}p < 0.01$, significant differences between males and females (bin 9). Vertical lines depict standard errors of the means (SEMs)

(normoxia), the results were very similar to what was observed during PD3, PD5, and PD7. Breathing frequencies were high at commencement of the test (bin 1) relative to the 2nd bin (habituation). Under the initial effects of hypoxia (bin 3), pups were found to significantly increase their respiratory rate (hyperventilation) relative to the preceding normoxic bin (bin 2). Hyperventilation progressively decreased during the following bins under the state of hypoxia (bins 4–7). During the last bin under the effects of hypoxia (bin 8), breathing frequencies were significantly lower relative to those recorded during bins 3–7. When pups were returned to a normoxic condition (recovery normoxia, bins 9–11), respiration frequencies markedly decreased reaching levels even lower than those recorded during the initial normoxic condition (bins 1–2).

Temporal contiguity between i.g. administration and pleth placement was found to significantly interact with bin ($F_{(10, 810)} = 3.48$, $p = 0.0002$). Respiratory frequencies observed during the hypoxic phase varied as a function of temporal contiguity (Fig. 3c). Specifically, paired and unpaired groups showed similar levels of responding during the initial normoxic phase (bins 1 and 2) and when initiating the hypoxic

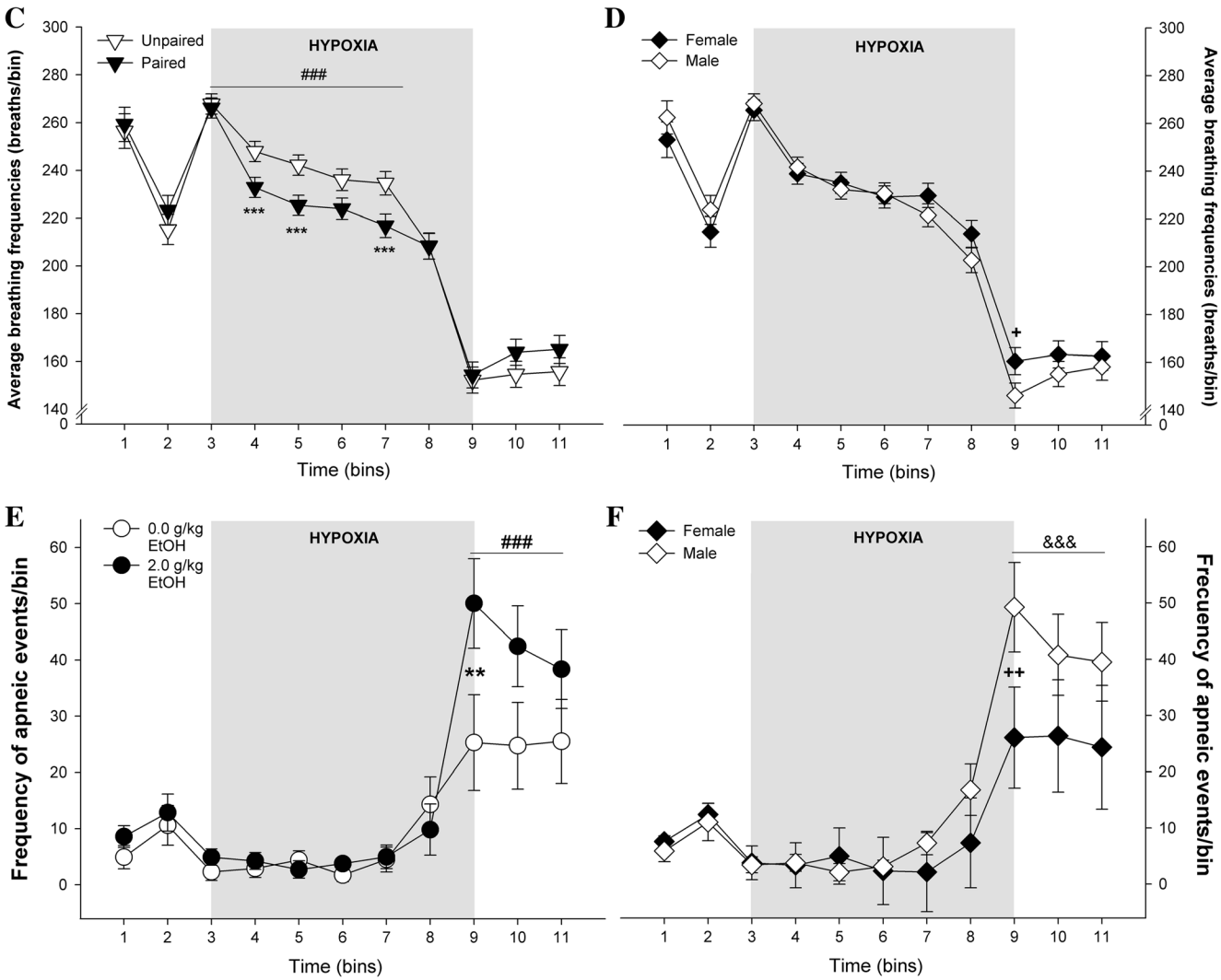

Fig. 3 continued.

condition (bin 3). Nevertheless, while hypoxia continued, paired pups showed significantly lower breathing frequencies relative to unpaired pups (bins 4, 5, and 7). Relative to hyperventilation, unpaired pups maintained significantly higher breathing frequencies (bins 4–7) relative to those recorded immediately before the hypoxic event (bin 2). This was not the case in paired pups. Their breathing frequencies during bins 4–7 were not significantly different from the ones recorded during bin 2. At the end of the hypoxic phase (bin8) and during the recovery normoxia phase (bins 9–11), these groups exhibited similar respiratory frequencies.

Sex and bin significantly interacted ($F_{(10,\ 810)} = 1.95$, $p = 0.0364$]. Planned comparisons indicated that, as soon as the hypoxic phase was terminated and pups were returned to a normoxia air condition (bin 9), males exhibited significant lower breathing frequencies relative to females (Fig. 3d).

When analyzing apneic episodes in neonates tested under the state of sobriety, the ANOVA showed a significant main effect of bin ($F_{(10,\ 810)} = 24.82$, $p < 0.0001$) which was tempered by the following two-way significant interactions: drug treatment × bin and sex × bin ($F_{(10,\ 810)} = 2.67$, $p = 0.0033$ and $F_{(10,\ 810)} = 2.50$, $p = 0.0058$, respectively). In the case of the drug treatment × bin interaction (Fig. 3e), planned comparisons revealed that neonates with a prior history with ethanol exhibited, relative to vehicle controls, a significant increase in apneas during the recovery normoxia phase, particularly during bin 9 (first normoxic bin after termination of the hypoxic stressor). The number of apneas in ethanol pre-exposed pups during the different bins that defined the recovery normoxia phase (bins 9–11) was also significantly higher than those recorded in these animals during the preceding bins of the testing procedure (1–8). This was not the case in pups pre-exposed to 0.0 g/kg ethanol. Although apneas also increased during the recovery normoxia phase in this last group of pups, apneic scores were not significantly different from those recorded in prior stages of the testing procedure (bins 1–8).

When taking into account the significant interaction between sex and bin (Fig. 3f), planned comparisons indicated that both sex groups exhibited significant increases in apneas during the 3 bins of the recovery normoxia phase (9–11) relative to all the preceding bins (1–8). Yet, in males, the frequency of apneas during the first bin (9) of the recovery normoxia phase was significantly higher than the one recorded in females.

Breathing frequencies and apneas during the testing phase (PD9): test under the state of ethanol intoxication

Due to technical problems related with breathing recordings, one animal (pretreated with water, paired, and exposed to an unscented chamber) was excluded when processing the data of the test conducted under the state of ethanol intoxication. The ANOVA showed that breathing frequencies were significantly affected by bin ($F_{(10,\ 800)} = 42.71$, $p < 0.0001$). This main effect was tempered by the following significant interactions: sex × bin ($F_{(10,800)} = 4.08$, $p < 0.0001$); odor × bin ($F_{(10,\ 800)} = 2.10$, $p = 0.0224$); and drug treatment × contiguity between drug administration and pleth context × bin ($F_{(10,\ 800)} = 2.19$, $p = 0.0164$). Males were found to exhibit significantly higher breathing frequencies relative to females during the initial normoxia phase (bins 1 and 2). In addition, pups that had experienced orange odor during PD3–PD7, showed higher breathing frequencies during the last bins of the hypoxic phase of the test (bins 7 and 8) relative to pups that had no prior experience with this scent.

As mentioned, the ANOVA revealed a significant three-way interaction (drug treatment × contiguity between drug administration and pleth context × bin). Follow-up two-way ANOVAs (drug treatment × bin) were conducted for each category composing the temporal contiguity factor (paired and unpaired). In the case of paired pups, only bin exerted a significant main effect ($F_{(10,\ 460)} = 18.89$, $p < 0.0001$). During initial normoxia breathing frequencies decreased during the 2nd bin relative to the preceding bin. This dependent variable significantly increased when pups were subjected to hypoxic conditions (bins 3–7 relative to bin-2). During the recovery normoxia phase (bins 9 and 11), breathing frequencies were significantly lower than those recorded at the beginning of the test (bin 1) and during most of the hypoxic phase (bins 3–6; Fig. 4). In unpaired pups, the two-way ANOVA showed a significant interaction between drug pre-exposure and bins ($F_{(10,\ 460)} = 2.03$, $p = 0.0285$). The temporal pattern previously described for paired animals was similar across unpaired conditions (main effect of bin: $F_{(10,\ 460)} = 24.75$, $p < 0.0001$). Nevertheless, unpaired pups pretreated with ethanol showed a significant depression in breathing frequencies relative to unpaired subjects pretreated with vehicle. This depression was observed during the last bins of the hypoxic phase (bins 7–8) and during the entire recovery normoxia phase.

Apneas were affected by bin ($F_{(10,\ 800)} = 3.53$, $p = 0.0001$). Apneic episodes were significantly higher at commencement of testing (bin 1) relative to all bins during the hypoxic phase (bins 3–8) and bin 10 corresponding to the recovery normoxia phase.

Experiment 2

Blood ethanol levels

Blood ethanol levels (BELs) at PD9 were analyzed in pups pretreated with vehicle or 2.0 g/kg ethanol (PD3, PD5, and PD7) and tested under the state of sobriety or intoxication. BELs were determined after pups were tested under normoxic or hypoxic conditions. In accordance with different studies (Barron et al. 1986; Lewis et al. 2007; Topper et al. 2015), no traces of ethanol were found in pups pre-exposed to the drug (PD3–PD7) and tested 48 h latter under the state of sobriety. Due to the lack of variance in these animals, the ANOVA only took into account pups that were tested when intoxicated. Prior drug treatment, sex and the air condition during the test or the interaction between these factors failed to significantly affect BELs at PD9 (Table 2). BELs obtained 90 min following ethanol administration were very similar to those obtained in prior studies; overall mean ± SEM: 216.07 ± 33.17 (Macchione et al. 2016).

Discussion

The present study examined the impact of ethanol and stress-related stimuli upon respiratory developmental plasticity in the rat during a stage in development that is equivalent to the 3rd human gestational trimester. Beyond this overall goal, the study was designed to evaluate whether contextual and/or olfactory cues can be associated with the effects of the drug upon respiration or alterations caused by stressors such as the administration procedure. In light of these possible associations, we examined, under different air conditions (normoxia, hypoxia, and recovery normoxia), the emergence of conditioned responses under states of sobriety or drug intoxication. To our knowledge, this study represents the first experimental approach leading towards an analysis of neonatal respiratory patterns under anomalous air conditions (hypoxia) as a function of the state of the organism (under the state of sobriety or when intoxicated with ethanol), of prior history with the drug, and/or potential stress-related associative processes.

The main results are (i) repeated early ethanol exposure reduced breathing rates in pups tested on PD9 soon after receiving ethanol, independently of olfactory cues associated with context; (ii) At PD7, pups exposed previously to ethanol (PD3 and PD5) exhibited depressed respiration whether or not they were given ethanol when tested; (iii) during the

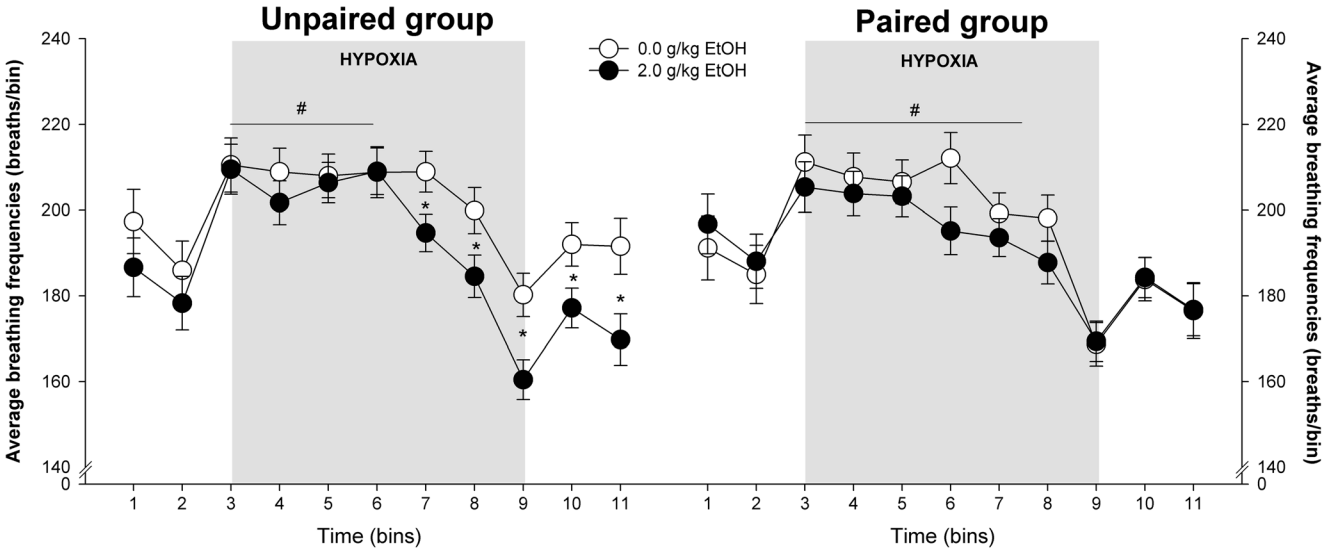

Fig. 4 Average breathing frequencies(breaths/bin) of unpaired (left panel) and paired pups (right panel) during the testing phase (PD9) under the state of intoxication as a function of drug treatment (0.0 or 2.0 g/kg EtOH) and bin (1–11). $^{\#}p < 0.05$, significant differences between hypoxic bins relative to the final bin (2) of the initial normoxic period. $^{*}p < 0.05$, significant differences between unpaired groups pretreated with vehicle or ethanol. Vertical lines depict standard errors of the means (SEMs)

conditioning phase, breathing depression was observed in pups that associated the pleth context with the intragastric administration procedure (paired groups); independently of the drug that was administered; (iv) when pups were tested under the state of sobriety, prior exposure to ethanol reduced breathing frequencies and increased apneic episodes immediately following hypoxia; (v) under a similar state (i.e., sobriety), prior explicit association between the administration procedure and the pleth context decreased breathing frequencies during hypoxia relative to pups that experienced both stimuli explicitly unpaired; (vi) at PD9, the depressant respiratory effects of ethanol intoxication were more pronounced in pups pre-exposed to ethanol that had never experienced the pleth context paired with this drug; and (vii) pretreatment with ethanol or exposure to hypoxia did not alter BELs in pups.

Chronic ethanol exposure during pregnancy and lactation, acts as a detrimental factor upon respiratory plasticity (Dubois et al. 2006, 2008; Kervern et al. 2009). There are marked differences between these studies and the one here reported. The preceding investigations were based on extensive periods of ethanol intoxication that start before mating and cover the entire stage of embryonic and fetal development. In such studies, animals were either evaluated when the protocol continued via the intoxication of the lactating female (Kervern et al. 2009) or after finishing ethanol exposure at weaning (Dubois et al. 2008). In none of these studies, respiratory plasticity was examined under the perspective of possible functional disruptions caused by ethanol- or stress-related stimuli that may respond to non-associative (sensitization or habituation) or associative learning processes. The present results indicate that brief experiences with a moderate ethanol dose (2.0 g/kg) later exert a negative impact upon neonatal respiratory plasticity. This negative impact was observed during the last day (PD7) of the conditioning phase or when pups were evaluated under the state of sobriety (PD9). These results emphasize the notion that brief experiences with the drug during the brain growth spurt period are sufficient to significantly affect respiratory plasticity. According to Acevedo et al. (2017a, b), pretreatment with a higher ethanol dose (3.0 g/kg) not only results in a significant respiratory

Table 2 Blood ethanol levels (BELs) at PD9

Drug treatment		Air conditions	
PD3–PD5–PD7	PD9	Only normoxia	Hypoxia/recovery normoxia
0.0 g/kg EtOH	0.0 g/kg EtOH	nd (4)	nd (4)
2.0 g/kg EtOH	0.0 g/kg EtOH	nd (4)	nd (4)
0.0 g/kg EtOH	2.0 g/kg EtOH	220.00 ± 18.41(8)	228.22 ± 9.35 (8)
2.0 g/kg EtOH	2.0 g/kg EtOH	211.48 ± 9.79 (8)	204.57 ± 6.46 (8)

Values are expressed as means ± SEMs. Number of animals in each group between brackets

nd, not determined

depression under a normoxic condition but also induces an exacerbated predisposition towards apneic episodes. With the dose here utilized (2.0 g/kg), we did not observe heightened apneic episodes during the initial normoxic phase of the testing procedure or when administering the drug during the conditioning phase. When contrasting the results here obtained with those reported by Acevedo et al. (2017a, b), it appears that there exists a dose-dependent effect relative to apneic disruptions caused by ethanol under normal air conditions. Yet, it is important to note that the present testing procedure was defined by three sequential phases related with the air conditions (normoxia-hypoxia-recovery normoxia). Following the hyperventilation caused by a hypoxic condition, all pups exhibited a marked posthypoxic breathing frequency decline that has also been observed in adult animals and that is related with a short-term depression of the phrenic nerve motor output (Powell et al. 1998; Dick and Coles 2000). This phenomenon was associated with an 11-fold increase in the number of apneas relative to those recorded in the initial normoxic phase of the test (Fig. 3e). This result is consistent with the observations of Julien et al. (2008) after exposing neonates to intermittent hypoxic events. Furthermore, the group pre-exposed to ethanol was the one that showed the highest level of apneic episodes during the recovery normoxic phase. In other words, pretreatment with a moderate ethanol dose as the one here utilized (2.0 g/kg) defies the integrity of the respiratory network following diminished oxygen levels in the inspired air. When taking into account a diminished ability to generate appropriate ventilatory and arousal responses following hypoxia, this result is particularly relevant. These failures are regarded as potential mechanisms underlying the development of respiratory disorders in human newborns which may also include SIDS (Gallego and Matrot 2010). Despite these observations, we cannot discard that the developing organism pre-exposed to ethanol, is capable of generating adaptive processes in response to the detrimental effects of the drug upon breathing. Homeostatic mechanisms, such as polycythaemia, serve to compensate the consequences of hypoxia in young rats pre-exposed to ethanol (Dubois et al. 2008).

Independently from the effects of ethanol upon breathing frequencies, close temporal contiguity between the administration procedure and pleth placement (paired groups) also exerted a significant role during the conditioning and the testing phase. It has been previously observed that either intracisternal, intragastric, or intraperitoneal administrations exert substantial stress-related effects during early postnatal life (Pautassi et al. 2007; Acevedo et al. 2017a, b). In the case of the first two modes of administration, the organism exhibits a subsequent unconditioned breathing depression that was also corroborated during the conditioning phase of the present experiment. When the administration procedure is not temporally associated with breathing evaluations within the plethysmograph (unpaired condition), respiratory frequencies are noticeable higher (Acevedo et al. 2017a). In this study, unpaired treatments were defined by a 2.5-h delay between pleth placement and the corresponding administration procedure. As illustrated in Fig. 2c, respiratory differences between paired and unpaired pups progressively increased across the conditioning phase. This phenomenon may reveal a sensitization effect of the stressor and/or a progressive emergence of a conditioned response elicited by the pleth context. Both learning phenomena (sensitization and conditioning) are intimately related since sensitization to a given unconditioned stimulus impedes habituation to a conditioned cue favoring learned associations between them (Çevik 2014; Macchione et al. 2016). At PD9, all pups were tested after being i.g. administered with vehicle. This similar condition across paired and unpaired pups probably diminished the possibility of observing stress-related conditioned or unconditioned responses during the initial normoxic phase that replicates the procedure employed during the conditioning phase. Yet, clear differences emerged immediately after pups started hyperventilating due to hypoxic air conditions. At this point in time, unpaired pups showed heightened and prolonged hyperventilation that lasted during most of the hypoxic phase. In paired animals, hyperventilation only occurred immediately after commencement of hypoxia (Fig. 3c). However, these animals failed to maintain this compensatory response during subsequent stages of the hypoxic phase. We have previously observed that stimuli that signal respiratory depressions later elicit isodirectional conditioned responses (Macchione et al. 2016). Therefore, it is possible that these learned responses, elicited by the pleth context, compete with respiratory requirements inherent to hypoxia. Another possibility is that hypoxia acts as a stress-related challenge facilitating the expression of prior learned respiratory responses; responses that are mediated by stressors such as the administration procedure. Prior studies indicate that infantile handling procedures as well as hypoxia augment neurohormonal correlates of the stress response such as corticosticosterone release (Sirieix et al. 2015; Harrell et al. 2015).

While pups were tested under the state of sobriety, a significant sexual dimorphism was observed during the recovery normoxia phase. Males exhibited lower respiratory frequencies relative to females and higher levels of apneas. This observation is in agreement with a heightened capability of women and female pups to adapt to hypoxia (Kryger et al. 1978; Pequignot et al. 1997). From a pathophysiological perspective females appear to be more resistant to hypoxia-associated syndromes during infancy and adulthood (Joseph et al. 2000).

At PD9, a second test was conducted under the state of ethanol intoxication. Breathing frequencies were lower than those recorded while pups were tested under the state of sobriety; a result that supports the depressant respiratory effects of the drug. Yet, we cannot discard that

breathing frequencies were also affected during the second test due to habituatory and/or extinction processes occurring during the first evaluation. Beyond this consideration and from a descriptive perspective, depressant effects of ethanol during the second test were evident during hypoxia; i.e., lower hyperventilation. When pups were tested under the state of sobriety, the percent increase in respiration frequencies during the initiation of the hypoxic episode relative to the last normoxic bin was equivalent to 24.98 ± 2.20% (data not shown). When these pups were evaluated while intoxicated the increment was 14.02 ± 1.37% (*t* test for dependent samples: $t = 4.0838$, $df = 96$, $p < 0.0001$). This phenomenon has also been observed when utilizing preclinical approaches based on acute or chronic ethanol exposure (Mayock et al. 2007; Dubois et al. 2013; Sabino et al. 2014). Also, when pups were evaluated under the state of intoxication, there were some significant effects related with prior conditions associated with the pleth context and drug treatment. In unpaired subjects, those pretreated with ethanol exhibited significant lower breathing frequencies during the last portion of the hypoxic phase and during all the recovery normoxia phase relative to counterparts pretreated with water. In other words, the detrimental effects upon respiratory plasticity that was evident when pups were tested under the state of sobriety, partially persisted during the test under the state of intoxication; a state where pups already evidenced an overall decrement in breathing frequencies. Taking into account the preceding observations, the results obtained when pups were intoxicated indicate that ethanol pre-exposure exerts a sensitization effect; it attenuates hyperventilatory compensatory responses and decreases breathing frequencies during the process of re-oxygenation.

During the conditioning phase of the experiment, an artificial scent (orange odor) was also present in some animals when they were placed in the pleth. This odorant was also used during testing. In paired pups, the conditioning procedure implies a compound conditioned stimulus given the simultaneous presence of the odor and contextual cues provided by the pleth context itself. In prior studies performed with infants, similar procedure seems to facilitate the expression of associative learning (Brasser and Spear 2004; Revillo et al. 2015). This was not the case in the present study. We only observed that when pups were tested under the state of intoxication, those that had been pre-exposed to orange odor had higher respiratory frequencies than those without prior olfactory experience. This result may reveal certain levels of habituation to the olfactory cue that later facilitate higher levels of respiratory frequencies. In prior studies, we have demonstrated that the scent of ethanol odor acts as an effective conditioned stimulus when paired with depressant respiratory effects of the state of ethanol intoxication (Macchione et al. 2016). Hence, the question arises relative to the lack of conditioning when utilizing orange odor. Ethanol's non-metabolic elimination processes (via alveolar excretion, urination, defecation) may explain these apparent contradictory results. These processes have been demonstrated to promote sensory processing of the drug in infants (Molina and Chotro 1989; Abate et al. 2008). In intoxicated pups, it is possible that the perception of ethanol-related chemosensory cues competed with the perception of an alternative exteroceptive odorant and therefore impeded the association of orange odor with the unconditioned consequences of ethanol upon respiration.

Experiment 2 clearly indicated that BELs in intoxicated-pups at PD9 were unaffected by prior history with the drug. These results indicate the absence of metabolic tolerance due to pre-exposure to the drug. They also show that the hypoxic episode did not accelerate the elimination rate of ethanol. Additionally, in accordance with prior studies (Lewis et al. 2007; Topper et al. 2015), no traces of ethanol were found in pups pre-exposed to the drug and tested under a state of sobriety. Therefore, the results obtained when pups were tested under the state of sobriety or while intoxicated are not dependent upon metabolic changes.

As a whole, this study indicates that a brief ethanol-related experience during the brain growth spurt period exerts a highly detrimental effect upon respiratory plasticity. The organism is prone to exhibit an overall decrement in breathing frequencies and a significant disorganization of respiratory plasticity after being exposed to a short-lasting hypoxic event. A failure in the arousal response after hypoxia, here operationalized through a significant increase in apneas, is considered as a critical factor leading to early brain damage, asphyxia, and even death (Mayock et al. 2007; Carlin and Moon 2017). In agreement with these findings, other studies have shown that (i) apneas are very common in newborn mammals, especially during sleep (Martin and Abu-Shaweesh 2005); (ii) given by the immaturity of the respiratory system, preterm infants have failures to initiate an appropriate arousal response to hypoxia that is accompanied with an increase in apneic episodes (Galland and Elder 2014); (iii) these failures may cause the sleep apnea syndrome (Harrington et al. 2002; Thach 2002; Waters and Tinworth 2005); and (iv) infants who died from SIDS previously showed alterations of their breathing pattern during sleep (Schechtman et al. 1991; Kahn et al. 1992), frequent episodes of obstructive and mixed sleep apneas (Kahn et al. 1988; Kato et al. 2001; Haas et al. 2015) and impairment in spontaneous and evoked arousal (Horne et al. 2004). In conjunction with these findings, the literature also indicates that within the multifactorial nature of SIDS, exogenous stressors play a significant role. According to the present data, stress-like events also impact upon respiratory plasticity and are likely to be associated with contextual cues that later elicit breathing alterations.

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