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Early ethanol exposure in mice increases laterality of rotational side preference in the free-swimming test

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ABSTRACT

In order to test the hypothesis that early postnatal ethanol exposure has long lasting behavioral effects that include changes to the normal pattern of cerebral asymmetries, the free swimming test (FST) was used to study the behavior of adult Swiss mice (males and females) exposed to ethanol during the third trimester equivalent of human gestation. Animals received ethanol (5 g/Kg ip, ETOH group) or saline (CONT group) on alternate days from postnatal day (*P*) 2 to *P*8, and were submitted to 1 session of open field (OF) and 3 sessions of FST from *P*75 to *P*81. No differences between ETOH and CONT groups were observed in OF. However, the FST revealed significant differences between ETOH and CONT mice during the first session. The percentage of animals that presented strong turning preferences (especially to the right side) was higher in the ETOH group when compared with the CONT group. These data give support to the hypothesis that early ethanol exposure affects cerebral asymmetries and suggests that the FST is a useful tool to investigate the long-lasting effects of ethanol exposure during development.

1. Introduction

Ethanol is known to interfere with nervous system development disrupting processes such as neuronal proliferation, migration, differentiation, neurochemical signaling, formation of appropriate connectivity, and programmed cell death (Goodlett et al., 2005; Heaton et al., 2003; Luo and Miller, 1998; Welch-Carre, 2005). Accordingly, maternal alcohol use during pregnancy produces a range of long-lasting neurological and behavioral outcomes in the offspring (Spohr et al., 2007; Welch-Carre, 2005), commonly referred to as fetal alcohol spectrum disorder (FASD). Interestingly, even the establishment of cerebral asymmetries in humans seems to be affected by alcohol exposure during pregnancy, as demonstrated by *in vivo* imaging studies published in the last decade (Niccols, 2007; Riley et al., 2004; Sowell et al., 2008). For instance, alcohol-exposed subjects present a significant reduction of the cortical surface gray matter asymmetry in the temporal lobe (Riley et al., 2004), a smaller left hippocampus volume when compared to the right one (Riikonen et al., 1999), and a significant increase in the thickness of the right frontal cortex as compared to controls (Sowell et al., 2008). These abnormal patterns of lateralization may underlie some of the cognitive deficits observed in FASD (Niccols, 2007; Riley et al., 2004).

There is a large body of literature demonstrating that naïve rodents present cerebral asymmetries (Lent and Schmidt, 1993; Schwarting and Huston, 1996) and life-long sequelae of prenatal ethanol exposure similar to those observed in humans (Becker et al., 1996; Cudd, 2005; Hannigan, 1996; Kelly et al., 2000; Slawecki et al., 2004). However, there is a scant number of studies investigating the effects of early ethanol exposure on cerebral lateralization in rodents and, in all cases, the pups were exposed to ethanol during the prenatal period (Moreland et al., 2002; Zimmerberg et al., 1986; 1989; Zimmerberg and Reuter, 1989; Zimmerberg and Riley, 1986; 1988), which is considered a period equivalent to the first and second trimester of human gestation (Livy et al., 2003; Maier et al., 1999; Maier and West, 2001a,b). Moreover, most studies evaluated the effects of prenatal ethanol exposure on the offspring only during a short period after birth.

The rotational behavior (or circling) is a lateralized behavior that has been widely used as an experimental tool for the study of cerebral asymmetries both in humans (Bracha et al., 1987; Mohr et al., 2004) and in mice (Filgueiras, 2004; 2005; 2006; Krahe et al., 2001; Manhães et al., 2007; Schwarting and Huston, 1996). One method used to evaluate rotational behavior is the free-swimming test, a simple paradigm in which animals are forced to swim for a few minutes in a cylindrical container (Filgueiras, 2004; 2005; 2006; Krahe et al., 2001; Manhães et al., 2007; Schmidt et al., 1999). Besides its simplicity, the free-swimming test has a unique feature when compared to other circling paradigms: it is recorded in a more stressful environment where the animals spend most of their time swimming close to the wall of the container attempting to escape from a frightening test situation (Schmidt et al., 1999; West et al., 1986). This stressful testing situation may add an interesting characteristic to

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the free-swimming test for the behavioral analysis of the effects of early life ethanol exposure. It has been demonstrated that behavioral deficits which ameliorate with age in animals prenatally exposed to ethanol can re-emerge under challenging or stressful conditions (Gabriel et al., 2006; Hannigan et al., 1987).

Considering that the establishment of cerebral asymmetries in humans seems to be affected by alcohol exposure during pregnancy (Niccols, 2007; Riley et al., 2004; Riikonen et al., 1999; Sowell et al., 2008), in the present work, we tested the hypothesis that early life ethanol exposure is associated with altered patterns of cerebral asymmetries at adulthood. To do this, we analyzed the rotational behavior of adult mice exposed to ethanol during the third trimester equivalent of human gestation, a critical period for ethanol neurotoxicity (Coles 1994; Cudd, 2005; Ikonomidou et al., 2000; Olney, 2002; Slawecki et al., 2004), in the free-swimming test.

In addition to the free swimming test, mice were tested in the open field, a testing paradigm that has been widely used to assess behavioral consequences of early life ethanol exposure in rodents (Gilbertson and Barron, 2005; Melcer et al., 1994; Tran et al., 2000). Several studies using this test have failed to demonstrate behavior alterations after a considerable time has passed since early life ethanol exposure (Abel and Berman, 1994; Bond and Di Giusto, 1977; Dursun et al., 2006; Tran et al., 2000). These negative results might be linked to the fact that the open field is not a very stressful testing environment, particularly when compared to FST. Accordingly, by using both the FST and the open field test, we further intend to verify the role of stress in revealing long-lasting behavioral effects of early ethanol exposure.

2. Methods

2.1. Animal treatment

This study was conducted under the State University of Rio de Janeiro institutional approval. All experiments were carried out in compliance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health. The subjects were Swiss mice that were bred and maintained in our laboratory on a 12:12 h light/dark cycle (lights on: 2:00, lights off: 14:00) at a constant temperature (22 °C). Original breeding stock was purchased from Fundação Oswaldo Cruz (Fiocruz, Rio de Janeiro, RJ, Brazil) and four generations have been bred in our laboratory. Access to food and water was unrestricted. From *P*2 to *P*8 (*P*1 = birth day), litters either received ethanol (5 g/Kg IP, 25% in saline solution) or an equivalent volume of saline solution every other day. Treatment on alternate days was chosen since it mimics 'binge' drinking in humans, which is associated with severe cognitive and behavioral deficits (Maier and West, 2001a,b). In addition, ethanol treatment on alternate days induces marked neurological abnormalities in animal models of FASD (Medina et al., 2005; 2006; Medina and Ramoa, 2005). Moreover, this type of exposure requires less manipulation of the animals than daily treatments. In order to minimize the risk of injury to abdominal organs, a 28-gauge needle was carefully inserted to just penetrate the abdominal wall and reach the peritoneal cavity. Leakage from the injection site was minimized by slowly withdrawing the needle from the abdominal cavity. The great majority of the animals survived the saline and ethanol IP injections (90.9% of the mice treated with ethanol and 96.7% of the mice treated with saline). Although the survival rate was smaller in the ethanol group, this difference was slim and did not reach statistical significance (Fisher's Exact Test, $P = 0.28$). At *P*30, mice were weighed, separated by sex and allowed free access to food and water. To further verify if the weight gain was affected by IP injections, a separate group of Swiss mice (23 males and 24 females from 4 litters), in which the pups were not manipulated from birth to weaning, was weighted at *P*30. These animals were bred and maintained in our laboratory at the same conditions of the animals treated with saline or ethanol.

At *P*75 and *P*81, 60 mice (29 males and 31 females) from 6 litters treated with ethanol (ETOH group) and 59 mice (29 males and 30 females) from 6 litters treated with saline only (CONT group) were subjected to behavioral tests.

2.2. Open field

At *P*75, all animals were tested in the open field. The open field arena consisted of a polypropylene box (37.6 x 30.4 x 17 cm) in which the floor was divided into 16 same-sized rectangles (7.6 x 9.4 cm), 12 peripheral and 4 central. The experiments were conducted during the dark part of the daily cycle, 1–2 h after its onset. Each mouse was individually placed within the arena (in a standardized location, in one corner) and a piece of cardboard (90° L-shape) was used to fence the animal at the corner until the start of the test (about 10 s). The cardboard was then removed and the animal's behavior was recorded for 5 min with an overhead VHS video camera (height = 60 cm). At the end of each session, the animal was returned to its home cage and before another animal was placed in the open field arena, the floor and walls were washed with odorless liquid soap, rinsed thoroughly with tap water several times, and dried with a disposable paper towel. The location of the observer and the orientation of the open field apparatus did not vary from session to session.

Recorded images of the tests were used to analyze the behavior in open field. The observer was blind regarding the experimental treatments of individual animals. The ambulation was quantified on the basis of the number of rectangles crossed by the animal. Animals had to place all four legs on a given rectangle for a crossing to be counted. The following ambulation measures were evaluated: ambulation in the center (*C*), ambulation in the periphery (Pe), *C*/Pe ratio and total ambulation (*C* + Pe). In addition, considering that direct comparisons between the activity in the center and in periphery can be influenced by the fact that the number of rectangles in periphery is greater than that in center, the number of rectangles crossed in the center and in the periphery is respectively divided by 4 (*C*/4) and 12 (Pe/12).

2.3. Free-swimming test

The free-swimming test was performed from *P*77 to *P*81. Each mouse was tested for 5 min on 3 different days with a 48 h test–retest interval (testing sessions began 2 to 3 h after the dark cycle began). The test procedure is described in detail elsewhere (Filgueiras and Manhães, 2004; 2005). Briefly, each animal was placed in the center of a plastic container (diameter = 21 cm, height = 23 cm) filled with water (depth = 16 cm) at about 25 °C. The animal's behavior was continuously recorded throughout the testing session with an overhead VHS video camera (height = 60 cm). The animal's starting location, the location of the observer, and the orientation of the apparatus did not vary from session to session.

Video images of the tests were used to analyze turning behavior. The observer was blind regarding the experimental treatments of individual animals. A turn, either leftward (counterclockwise swimming) or rightward (clockwise swimming), was defined by using a 30° unit. To facilitate the scoring of the 30° turns, a transparent overlay with 30° axes was matched with the image of the circular apparatus on the screen of the video monitor. For each animal, the counting of any successive number of 30° turns in a particular direction was interrupted when the animal shifted its initial direction, when it ceased to move, or when it floated passively. Since a 30° turn was counted only when the animal performed an active movement, the period of time in which the animal remained immobile or floated passively in the water was excluded from the analysis. Most animals swam around the center of the testing apparatus. Whenever the turns

did not occur around the center of the apparatus, the animal's initial and final positions were recorded and the number of 30° turns was estimated with the aid of the transparent overlay with 30° axes. During sampling, we never observed a mouse dive or turn 360° around its own axis.

Turns to the right (R) or to the left (L), total angular activity ($R+L$) and turns to preferred side (PS) were calculated for each test. Lateralization was assessed by two measures: the percentage of turns to the right side [$\%R = 100\,R/(R+L)$] and the percentage of turns to the preferred side [$\%PS = 100\,PS/(R+L)$]. The direction of lateralization, indicating whether the animal preferred to rotate to the left or to the right, was assessed using the $\%R$. For each testing session, a particular mouse was assigned to a side preference group if it turned to one side more often than to the other (right turner, $\%R>50\%$; left turner, $\%R<50\%$). Consistent turners were defined as mice that did not change their preferred side of rotation throughout the three testing sessions. Therefore, consistency of laterality was defined independently of the magnitude of the differences between right and left preferences.

2.4. Blood Ethanol Concentration (BEC)

A separate group of mice was injected with ethanol or saline as described above. One, three, five and seven hours after the third injection (at $P6$), animals were decapitated and the blood collected from ethanol (1 and 3 h: $n=3$; 5 and 7 h: $n=2$) and saline (1, 3, 5 and 7 h: $n=1$) exposed mice. Blood was centrifuged at 3000 rpm for 10 min and supernatant stored at −20 °C until assayed. BEC was assessed using an enzymatic kit (Alcohol Reagent Set, Pointe Scientific Inc., Michigan, USA) in accordance with the manufacturer's recommendations.

2.5. Statistical analysis

In order to verify whether measurements in open field and in free-swimming test have acceptable inter-observer accuracy, the video images of a random sample of 15 cases were analyzed by two independent-observers blind of the results obtained by each other. The analyses of inter-observer accuracy were performed using Pearson correlation coefficients ($P<0.05$, one-tailed). One-tailed tests were used because the hypothesis of reliability cannot be accepted if the correlation coefficient is negative.

Preliminary analyses did not show any significant differences between males and females. In order to minimize the influence of litter effects (Wainwright, 1998), for all ANOVAS, we considered the average of values from animals of the same litter instead of using individual animal values. The Treatment (ETOH X CONT) was used as between-subjects factor. Significance was assumed at the level of $P<0.05$ (two-tailed). The analyses of open field data (C, Pe, C/Pe, $C+$Pe, $C/4$ and Pe/12) were carried out by separate univariate ANOVAs. Repeated measures analyses of variance (rANOVA) were performed for free-swimming data ($R+L$, PS and %PS) and for body weight data. Session and age were considered the within-subjects factors. Protected t-tests were used for *post-hoc* analyses.

Regarding rANOVAs, for simplicity, we will report results based only on the averaged univariate F tests. The univariate approach is considered more powerful than the multivariate criteria (Huynh and Feldt, 1976). However, each univariate test requires that the variances of all transformed variables for an effect to be equal and their covariances to be zero (Huynh and Feldt, 1976). Therefore, the extent to which the covariance matrices deviate from sphericity was estimated by Mauchly's test. Whenever the sphericity assumption was violated, we used the Greenhouse–Geisser correction, which adjusts the degrees of freedom, in order to avoid Type I errors.

The comparisons between ETOH and CONT groups involving the percentage of consistent turners as well as the percentage of side (left or right) consistent turners were made by the use of Chi-square (2 2) tests. The analysis of test–retest reliability was performed using Pearson correlation coefficients ($P<0.05$, two-tailed). In order to evaluate whether significant differences existed between ETOH and CONT mice regarding the entire distributions of %PS and $\%R$, the Kolmogorov–Smirnov two-sample test (K–S) was performed for each group pairs analyzed. Initially, the comparisons were conducted on the basis of scores derived from pooled data from the three testing sessions. Subsequently, analyses were conducted separately for each testing session.

3. Results

3.1. Offspring growth

Offspring weights during the injection period are shown in Table 1. The mean litter weights increased significantly from $P2$ to $P8$ ($F=595.7$; $df=3,30$; $P<0.001$). From $P2$ to $P8$, no differences were observed between the ETOH and CONT groups regarding weight gain or absolute weight at any age. At $P30$, the mean litter weights of the ETOH group (24.0 ± 0.6), CONT group (24.4 ± 1.1) and non-manipulated group (24.0 ± 2.0) did not differ (Univariate ANOVA, $F=0.6$, $df=2,10$, $P=0.56$).

3.2. Blood ethanol concentration

The average BEC was highest at 1 h after injection (174.5 ± 3.5 mg/dL), and decreased progressively (3 h: 161.8 ± 4.5 mg/dL; 5 h: 149.4 ± 8.8 mg/dL; 7 h: 141.4 ± 9.5 mg/dL). These BECs are within the range that a human fetus would be exposed to after maternal ingestion of a moderate to heavy dose of ethanol (Eckardt et al., 1998).

3.3. Open field data

We found a high inter-observer agreement (ambulation in the center: $r=0.991$, $df=14$, $P<0.001$; ambulation in the periphery: $r=0.994$, $df=14$, $P<0.001$). Based on these data, we concluded that the ambulation scores present acceptable inter-observer accuracy.

No differences were observed between alcohol treated and control animals (Table 2). In general, most of the activity in the open field took place in the periphery. For both ETOH and CONT groups, the ambulation in the periphery was significantly greater than in the center. Similar results were observed when ambulation values were divided by the correspondent number of rectangles in the center and in the periphery.

3.4. Free-swimming data

We found a high inter-observer agreement (30° right turns: $r=0.990$, $P<0.001$; 30° left turns: $r=0.997$, $P<0.001$). Based on these data, we concluded that a 30° unit yields data with acceptable inter-observer accuracy.

Regarding the consistency of laterality, the percentage of mice classified as side-consistent rotators in the ETOH group (68.3%) and in the CONT group (52.5%) was not significantly different from each other. The proportion of left-consistent turners and right-consistent turners did not differ between ETOH (left-consistent turners = 41.5%,

Table 1
Mean litter weights (g).

	P2	*P4*	*P6*	*P8*
CONT	2.1 ± 0.1	3.0 ± 0.1	4.0 ± 0.2	5.0 ± 0.2
ETOH	2.1 ± 0.1	2.9 ± 0.1	3.9 ± 0.1	4.9 ± 0.1

Values represent means ± SEM.

Table 2
Number of rectangles crossed in the center (*C*) and in the periphery (Pe).

	CONT group	ETOH group
Pe	$92.5 \pm 7.3^{***}$	$86.5 \pm 7.4^{***}$
C	16.7 ± 1.3	15.7 ± 2.4
Pe/12	$7.7 \pm 0.6^{***}$	$7.2 \pm 0.6^{***}$
C/4	4.2 ± 0.3	3.9 ± 0.6
C/Pe	0.2 ± 0.0	0.2 ± 0.0
C+Pe	109.2 ± 8.5	102.2 ± 9.0

Values are means ± SEM.
$^{***}P \leq 0.001$ center vs. periphery comparison.

right-consistent turners = 58.5%) and CONT (left-consistent turners = 48.4%, right-consistent turners = 51.6%) mice.

Regarding total angular activity ($R+L$) and turns to preferred side (PS), there were no differences between CONT and ETOH groups. We found that the amount of turning declines upon repeated testing sessions for both $R+L$ (Session effect: $F=92.0$; $df=2,20$; $P<0.001$) and PS (Session effect: $F=18.8$; $df=2,20$; $P<0.001$). Particularly, the turning activity on the first session was higher than on the second ($R+L$: $t=8.6$, $df=11$, $P<0.001$; PS: $t=3.3$, $df=11$; $P<0.01$) and the turning activity on the second session was higher than on the third session ($R+L$: $t=6.2$, $df=11$, $P<0.001$; PS: $t=4.1$, $df=11$; $P<0.01$).

Regarding the percentage of turns to the preferred side (%PS), there was a significant increase in the magnitude of laterality from the first (mean = 70.5, S.E.M. = 1.6) to the second (mean = 74.5, S.E.M. = 2.1) session (Session effect: $F=4.1$; $df=2,20$; $P<0.05$). No difference was observed between the second and third (mean = 74.8, S.E.M. = 2.1) sessions. Of note, there were marked differences between CONT and ETOH groups (Treatment effect: $F=5.3$; $df=1,10$; $P<0.05$). The average percentage of turns to the preferred side of mice in the ETOH group (mean = 76.4, S.E.M. = 2.3) was higher than that of the CONT group (mean = 69.9, S.E.M. = 1.6). Interestingly, the difference between CONT and ETOH groups was more pronounced for the first session (Fig. 1).

The Kolmogorov–Smirnov test comparing the distributions of %PS from CONT and ETOH groups indicated a significant difference only in the first session (*K–S*: $Z=2.2$, $P<0.001$). The ETOH mice turned preferentially more to one side (right or left) than CONT mice (Fig. 2). In accordance, 65.0% of the ETOH mice ($n=39$) presented strong turning preferences (%PS>70%) while this percentage was only 32.2% ($n=19$) in the CONT group. These frequencies were significantly different from each other ($\chi^2=12.8$; $df=1$, $P<0.001$).

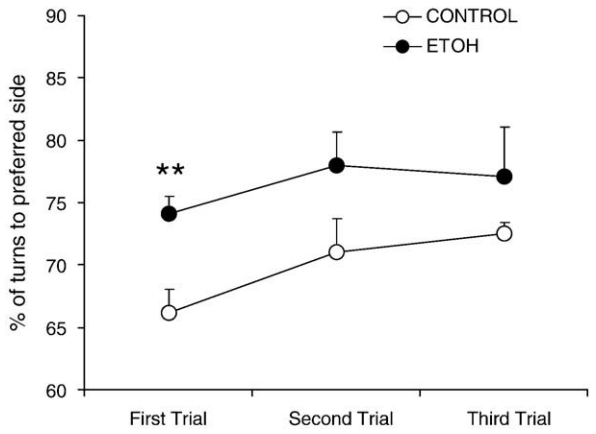

Fig. 1. Percentage of 30° turns to preferred side of adult mice exposed to ethanol (ETOH) or saline (CONT) during the first postnatal week upon repeated testing sessions. Note that the ETOH group is more lateralized than CONT group in the first session. The values are means (+S.E.). *T*-test: $^{**}P<0.01$.

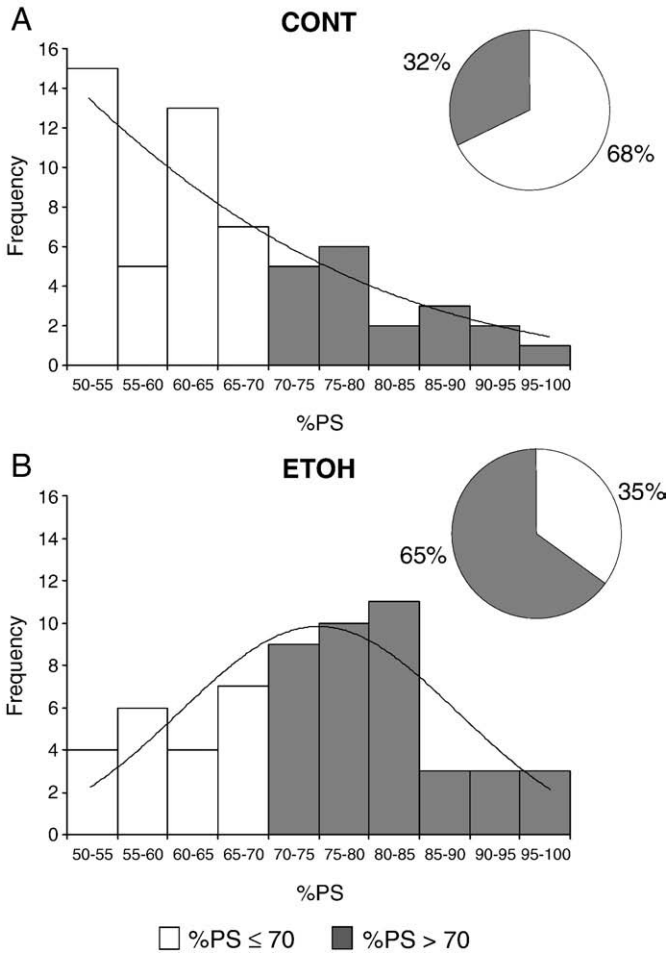

Fig. 2. Frequency distributions of percentage of 30° turns to preferred side (%PS) in the first session among mice exposed to ethanol (ETOH) or saline (CONT) during the first postnatal week. The lines in the histograms represent the Gaussian fitting curves. The pie-graphs show the percentage of animals with strong (%PS>70) and weak (%PS≤70) turning preferences. Regarding %PS, both histograms and pie-graphs show that the ETOH mice are more lateralized than CONT mice.

Regarding the distribution of percentage of turns to the right side (%*R*), a significant difference between CONT and ETOH groups was also observed only in the first session (*K–S*: $Z=1.5$, $P=0.02$). In the ETOH group, 35% of the animals ($n=21$) presented weak directional turning biases (%*R* between 30% and 70%) while this percentage was 68% ($n=40$) in the CONT group (Fig. 3). Fig. 3 also shows that, among animals with strong turning activity to one side (right or left), the percentage of animals with strong right-turning activity (%*R*>70%) in the ETOH group (42%, $n=25$) was higher than that observed in the CONT group (18%, $n=11$). These frequencies were significantly different from each other ($\chi^2=7.5$; $df=1$, $P<0.01$). The frequency of animals with strong turning preferences to the left (%*R*<30%) did not differ between ETOH (23%, $n=14$) and CONT (14%, $n=8$) groups ($\chi^2=1.9$; $df=1$, $P>0.15$).

Additionally, we investigated the test–retest reliability of the continuous variables. The Pearson correlation coefficients were always positive and significantly different from zero ($P<0.001$). There were no differences between CONT and ETOH mice.

4. Discussion

In the present study, mice were exposed to ethanol from *P*2 to *P*8 every other day, during the period that is equivalent to the third

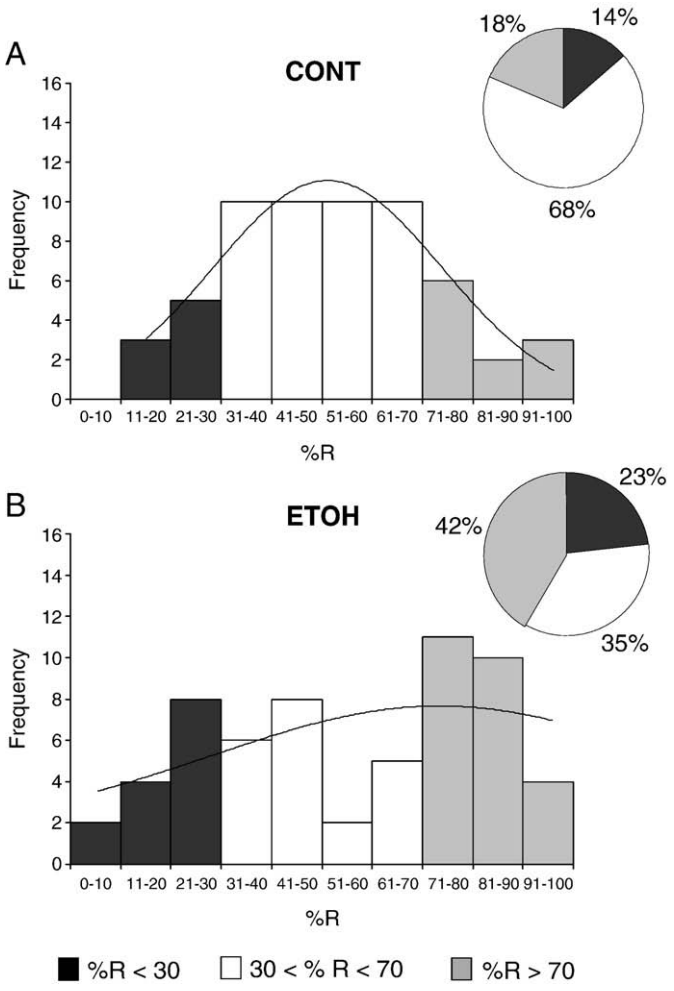

Fig. 3. Frequency distributions of the percentage of 30° turns to the right side (%*R*) in the first session among mice of CONT (A) and ETOH (B) groups. The lines in the histograms represent the Gaussian fitting curves. The pie-graphs show the percentage of animals with strong (%*R*>70% for right-turner and %*R*<30 for left-turner) and weak (%*R* between 30% and 70%) directional turning preferences. Both histograms and pie-graphs show that the number of animals with strong turning preferences to the right in the ETOH group are greater than that of the CONT group.

trimester of human gestation. At adulthood, long after the period of alcohol exposure, animals were subjected to the open field and the free-swimming test. Briefly, no differences were found between ETOH and CONT groups in the open field. In contrast, marked differences between ethanol-exposed and control mice were observed in the first session of the free-swimming test. In particular, the ethanol-exposed mice were more lateralized (preferentially to the right side) when compared to control animals.

Several techniques have been used to administer ethanol in neonatal rodents, such as: the artificial rearing (pup in the cup model), the oral intubation and the alcohol inhalation procedures. While reliable results have been obtained with each one of these approaches, each one has its own disadvantages and limitations. In all cases there are factors other than the ethanol exposure which could per se affect the neurobehavioral results. For instance, in the artificial rearing model (Diaz and Samson, 1980), gastric tubes are surgically implanted into neonates and the pups are subjected to isolation (from the mother and littermates), there is the obvious elimination of normal suckling behavior and pups' body temperature is artificially controlled. In the oral intubation model (Kelly and Lawrence, 2008), a flexible gavage canula is inserted through the oral cavity into the stomach and pups are submitted to frequent handling and maternal separation. In the inhalation model (Pal and Alkana, 1997), the ethanol vapor can irritate the respiratory tract system and induce stress in the mother and pups.

Considering that in the present study animals were exposed to ethanol via IP injections from *P*2 to *P*8, an invasive route that is not commonly used in third trimester FASD models, we were careful in verifying the safeness and feasibility of this route of administration. Here, we demonstrated that: 1) the great majority of the animals survived our protocol of four IP injections and the mortality rates did not differ between ETOH and CONT mice; 2) there were no significant differences between the animals injected with ETOH or saline solution regarding weight gain during the treatment period or at *P*30 when the animals were weaned; and 3) the mean body weight of litters injected with ethanol or saline did not differ from the mean body weight of litters in which the pups were not manipulated from birth to weaning.

Regarding our body weight results, the fact that ETOH did not affect weight gain during the treatment period (from *P*2 to *P*8) or at *P*30, when the animals were weaned, is in accordance with other studies carried out in rodents (O'Leary-Moore et al., 2006; Pal and Alkana, 1997; Karaçay et al., 2008). Pal and Alkana (1997) demonstrated, in Swiss–Webster mice, that daily exposure to ethanol vapor (BEC ranging from 160 to 290 mg/dL) from *P*2 to *P*14 did not result in significant body weight differences during the exposure period. Sprague–Dawley rats exposed to ethanol via intragastric intubations from *P*4 to *P*9 (ETOH dosing = 5 g/Kg, BEC = 320 ± 7 mg/dL) also did not present significant body weight differences at *P*21 when compared to non-intubated animals (O'Leary-Moore et al., 2006). By *P*25, there were no significant differences in body weights between C57BL/6 mice submitted either to IP injections of ethanol (4.4 g/kg/d, BEC around 310 mg/dL) or to saline during the third trimester equivalent period (Karaçay et al., 2008). It is important to note that in all these studies the maximum BEC was much higher than ours (our highest value was 174.5 ± 3.5 mg/dL).

Our data showing no differences between alcohol-treated and control animals in open field test are in accordance with several other studies that failed to demonstrate hyperactivity after a considerable time has passed since early-life ethanol exposure (Abel and Berman, 1994; Bond and Di Giusto, 1977; Driscoll et al., 1990; Dursun et al., 2006; Spohr et al., 2007; Tran et al., 2000). Longitudinal and cross-sectional studies have demonstrated that alcohol-related hyperactivity, at least as measured in the open-field, appears to be age-related. Ethanol exposed animals tested as juveniles typically are more active than controls (Melcer et al., 1994; Gilbertson and Barron, 2005; Tran et al., 2000), while mature subjects are not different (Abel and Berman, 1994; Bond and Di Giusto, 1977; Driscoll et al., 1990; Dursun et al., 2006; Spohr et al., 2007; Tran et al., 2000). Accordingly, rats exposed to ethanol during gestation displayed significantly greater locomotor activity in an open-field test at 28 and at 56 days of age, but not at 112 days of age (Bond and Di Giusto, 1977). In addition, mice exposed to ethanol on a single postnatal day (*P*7) presented spatial learning and memory impairments that were very severe at *P*30, less severe if testing was first performed at *P*75, and minimal in later adulthood (Wozniak et al., 2004). Therefore, in the present study, the absence of alcohol-induced hyperactivity during adulthood may be related to the fact that some effects of early alcohol exposure are significantly attenuated during subsequent development.

As indicated in previous studies (Filgueiras and Manhães, 2004; 2005; Manhães et al., 2007; Schmidt et al., 1999), the finding that the animals, irrespective of postnatal treatment, presented the highest total turning activity ($R+L$) in the first session of the free-swimming test is due to more pronounced escape-related behaviors. In fact, when first placed in the novel and stressful situation of the aquatic arena, the animal's behavior is typically characterized by vigorous swimming accompanied by frantic clawing at the side of the testing chamber (Krahe et al., 2001; Schmidt et al., 1999). The enhanced escape response randomizes the direction of turning, resulting in an

increased number of turns to the non-preferred side (PS), which, in turn, affects %PS.

Interestingly, a marked difference between ETOH and CONT groups regarding the %PS (higher in the former) was observed only in the first session of the free-swimming test. This result corroborates others showing that, under stressful or challenging situations, animals exposed to ethanol early in life present a wide range of behavioral alterations (Gabriel et al., 2006; Hannigan et al., 1987). Behavioral deficits induced by early ethanol exposure which diminish with age can reappear under stressful or challenging situations (Gabriel et al., 2006; Hannigan et al., 1987). There are studies showing that early ethanol exposure results in long-term effects on the organism's ability to respond and adapt to stressors, as measured by the hyperresponsiveness of the hypothalamic–pituitary–adrenal axis (Haley et al., 2006; Hofmann et al., 2007; Park et al., 2004) and altered emotional responses (Kodituwakku, 2007). Therefore, the differences between ETOH and CONT groups might be associated with a maladaptive response to stress. In fact, in the far less aversive paradigm of the open field test, there were no differences between ETOH and CONT groups, a finding that is consistent with previous studies (Abel and Berman, 1994; Bond and Di Giusto, 1977; Dursun et al., 2006; Tran et al., 2000). Similarly, circling behavior measured in dry land is not known to be affected by prenatal exposure to alcohol when tests are performed after a long alcohol-free period (Zimmerberg et al., 1986).

The absence of differences between ETOH and CONT groups regarding test–retest reliability and consistency of laterality in the free-swimming test is related to the fact that with the repetition of the sessions there is a habituation process, which results in a reduction in escape attempts from the stressful testing situation (Denenberg et al., 1990; Filgueiras and Manhães, 2004; 2005; Krahe et al., 2001; 2002; Manhães et al., 2007; Schmidt et al., 1999; West et al., 1986). It has been suggested that emotional responses related to fear are generally assumed to be highest during the initial exposure to a testing apparatus and then decrease as the animal gradually habituates to this environment (Leppänen et al., 2006). Our turning activity data corroborate this interpretation since both the overall turning activity ($R+L$) and the number of turns to preferred side (PS) diminish from session to session in both groups.

Our data also show that, in the first session of the free-swimming test, the percentage of animals that presented strong turning preferences to the right side was higher in the ETOH group. This result is in accordance with human studies showing that alcohol exposure during pregnancy affects the populational pattern of cerebral asymmetries (Niccols, 2007; Riikonen et al., 1999; Riley et al., 2004; Sowell et al., 2008). Although in the present study we did not measure biochemical and morphological asymmetries, it has been widely accepted that turning biases reflect dopaminergic asymmetries in the nigrostriatal system (Carlson and Glick, 1996; Glick and Shapiro, 1985; Nielsen et al., 1997; Schwarting and Huston, 1996). Of note, there are several studies showing that both striatal and rotational asymmetries are modulated by the prefrontal cortex (Carlson and Glick, 1996; Glick and Greenstein, 1973; Gonzalez et al., 2006; Nielsen et al., 1997). Interestingly, both the prefrontal cortex and the basal ganglia are significantly affected by early ethanol exposure (Barr et al., 2005; Cortese et al., 2006; Fryer et al., 2007; Kumral et al., 2005; Niccols, 2007). Therefore, our data suggest that, in mice, the ethanol exposure during the third trimester equivalent period of human gestation induces an alteration in the populational pattern of asymmetries favoring the right hemisphere. Accordingly, it was recently demonstrated that subjects with heavy prenatal alcohol exposure histories present a significant increase in the thickness of the right lateral frontal cortex as compared to controls (Sowell et al., 2008). Some studies have also described a more pronounced reduction of the left ventral portions of the frontal lobes (Niccols, 2007; Sowell et al., 2002), as well as of the left caudate nucleus in individuals exposed to alcohol during gestation (Cortese et al., 2006). Furthermore, in individuals with Fetal Alcohol Syndrome, an increase in blood supply to the right frontal region has been demonstrated (Riikonen et al., 1999). Finally, children prenatally exposed to alcohol present an elevated N-acetyl-aspartate to creatine (NAA/Cr) metabolite ratio, which has been associated with neural dysfunction in left caudate nucleus of FASD subjects when compared to the control group (Cortese et al., 2006).

5. Conclusion

Our data show that the rotational behavior of adult Swiss mice measured in the free-swimming test is affected by ethanol exposure during the first postnatal week. The finding that differences between ETOH and CONT groups were more pronounced in the first session gives support to the suggestion that stressful or challenging situations can bring out defective behaviors in early ethanol-exposed animals. The fact that the percentage of animals that presented strong turning preferences to the right side in the ETOH group was higher than that in the CONT group lends further support to the hypothesis that early ethanol exposure affects cerebral asymmetries. Furthermore, our results indicate that the free-swimming test is a useful tool to investigate the long-lasting consequences of early alcohol exposure.

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