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Research Article

Ethanol Perinatal Exposure Induces Behavioral Alterations and Ethanol Preference in Adolescent Wistar Rats

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Abstract Ethanol-perinatally-exposed (EPE) Wistar rats show central nervous system morphological alterations that persist until adulthood. The aim of this work was to study whether adolescent EPE rats present alterations in behavior and develop ethanol (EtOH) preference. For behavioral analyses, locomotor and exploratory activity, anxiety and long-term memory standardized tests were performed on 40 to 45 day-old male rats. Another group of rats chose to drink between EtOH and water using the model of preference between two bottles, one with EtOH 6% v/v and the other with water. During eight weeks, the volume of EtOH consumed/kg/day was registered and blood EtOH concentration was determined at the end of the experiment. Results in EPE rats show a decrease in aerial exploration and anxiety, while locomotor activity and long-term memory were not affected. Both male and female EPE rats preferred EtOH earlier than control rats, showing a highest level of blood EtOH concentration.

Keywords ethanol preference; adolescent rats; perinatal ethanol exposition

1. Introduction

In humans, exposure to EtOH during pregnancy can cause serious changes in morphological, behavioral, and cognitive development in children, which may also persist into adulthood [11,46]. Children with Fetal Alcohol Syndrome (FAS) have shown certain distinctive, persistent, and subtle patterns of cognitive dysfunction that become more pronounced with more complex psychological assessments [21]. Adolescents who perceive the drug as more rewarding may face higher risk of alcohol use disorders [33].

Studies in both humans and animals have extensively demonstrated the deleterious effects of maternal alcohol ingestion on the fetus. Although deficits produced by prenatal exposure to high levels of EtOH are most severe and have been documented most significantly in children with FAS, children prenatally exposed to lower levels of alcohol have been shown to frequently exhibit similar problems, especially those related to neurobehavioral impairment [22,23,43].

Different animal models have been performed to analyze FAS and its consequences in adulthood. However, studies on adult rats prenatally exposed to EtOH have shown controversial results. Some authors report an increase in locomotor hyperactivity as one of the most characteristic effects of in utero EtOH intoxication [45], while others have found no hyperactivity [1]. Also, rats prenatally exposed to EtOH show alterations in the rotary drum, which suggests a decrease in muscle strength and sensory-motor coordination [2], while sensory-motor functions in adult animals prenatally exposed to EtOH have not yet been investigated. Previous studies have found that adolescent mice [40] and infant rats [7,8] exhibit EtOH-induced locomotor activation. However, evidence for this effect in adolescent rats is scarce.

It has been observed that early experience with alcohol (passive or in the context of operant learning schemes) alter consumption evaluated at later stages of development [27,31,35]. Chotro and collaborators [15] indicate that when rats are exposed to EtOH during late gestation or infancy, even to a dose that induces EtOH aversion in adults, no effects are observed in terms of motor activity or odor preference, although these rats exhibit greater EtOH intake than controls without prenatal EtOH exposure.

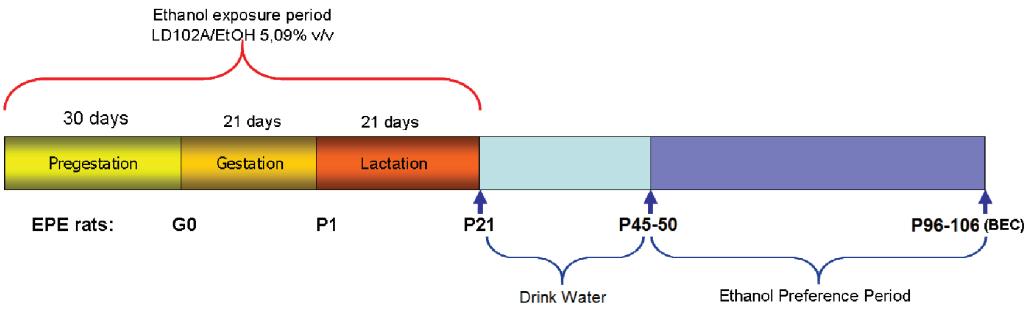
A growing number of studies using rodents consistently demonstrate that prenatal EtOH exposure induces increased intake of EtOH postnatally, as observed in studies in which EtOH was administered to the pregnant dam during most of gestation [30,47,48].

The aims of this work were to analyze the behavioral responses of adolescent Wistar rats exposed perinatally to EtOH at moderate doses, and study the development of alcohol preference.

2. Materials and methods

2.1. Animals

Twenty two adult nulliparous female Wistar rats (*Rattus norvegicus; Hsd: WI*) initially weighing 250–300 g were



Graph 1: Experimental procedure. G: gestational day; P: PND.

mated with 5 adult Wistar males, four females for each male. One month before mating (metabolic and taste acclimatization period) and during pregnancy and lactation, a group of twelve females assigned to the treated group (EPE) were fed the LD 102A liquid diet supplemented with 5.9% (w/w) EtOH. The control group (C; the ten remaining females) was fed the LD 102 control liquid diet. LD 102A powder is mixed with EtOH or maltodextrin to normalize calories between groups. After weaning, pups from each litter were separated according to sex and received standard food and water ad libitum. Rats were maintained under a 12 h light/dark cycle (lights on at 7 AM) at a room temperature of 23 °C. For behavioral studies, 40–45 post natal day (PND) male rats were used. For alcohol preference studies, another group of rats of 50 days of age was housed (two or three per cage and separated according to sex) and given the choice to drink either EtOH 6% v/v or water for eight weeks (Graph 1). All procedures complied with the National Institutes of Health guide for care and use of laboratory animals and were approved by the CICUAL of the School of Medicine, Universidad de Buenos Aires.

2.2. Morphological studies

Three males for each experimental group, EPE and C rats, were deeply anesthetized with Ketamine and Xylazine in doses of 10 mg/kg and 75 mg/kg, respectively, and perfused through the left ventricle, initially with saline solution added to 50 IU heparin and 0.05% (w/v) $NaNO_2$, and subsequently with a fixative solution containing 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer, pH 7.2 (PB). Brains were removed and postfixed in the same cold fixative solution for 2 h. Brains were then washed three times in cold PB containing 5% (w/v) sucrose, and left in the same washing solution for 18 h at 4 °C. After that, brains were cryoprotected by immersion in a solution containing 25% (w/v) sucrose in PB and stored at −20 °C until use. Coronal 40-μm-thick brain sections were cut using a cryostat (Leitz, Kryostat 1720 Digital) and mounted on gelatine-coated slides.

Brain sections were processed for toluidine blue staining following the same procedure we previously described [9]. Brain sections were rinsed three times in PB and, once in distilled water, they were exposed for 30 s to Toluidine Blue 0.5% (w/v) dissolved in an aqueous solution of sodium carbonate 2.5% (w/v). After 10 rinses in distilled water, sections were mounted on gelatine-coated slides, dehydrated and cover-slipped using Permount mounting media.

2.3. Behavioral studies

The effect of EPE was determined on motor activity, emotional reactivity, learning and memory in PND 40–45 pups. Standardized tests were performed with different experimental behavior paradigms: anxiety test (elevated plus maze), aerial exploration and locomotor activity test (open field) and memory test (conditioned taste aversion).

2.4. Elevated plus maze

Anxiety was tested through the elevated plus maze test. This maze consists of two opposite arms (50 cm × 10 cm) crossed with two opposite enclosed arms of the same dimension, with 40-cm-high walls. The arms are connected with a central square (10 cm × 10 cm) to give the apparatus a plus sign appearance. The elevated plus maze was kept elevated 50 cm above the floor in a dimly lit room. The rats were placed individually on the central square of the plus maze facing one enclosed arm. The time spent and the number of entries made by the rat during the next 5 min on the open and closed arms were recorded. An arm entry was defined as all four limbs on the arm.

2.5. Open field (OF)

The apparatus is a 50-cm-wide × 50-cm-long × 39-cm-high area with black plywood walls and wooden floor, divided into 9 squares by black lines. A novel environment exploration consists of a 5 min OF session. Ambulation (number of squares crossed) and rearing (number of times the animal stands on its hind legs) were observed. To make animals familiar with the area, a 30 min OF session was performed one day before the experiment [3,4].

2.6. Conditioned taste aversion (CTA)

Rats were kept in individual cages throughout the study, and withdrawn to administer injections of LiCl during training.

The behavioral paradigm included water deprivation during the first day, followed by consumption of 20 mL of water for 3 days, 20 min per day. On the fourth day, the rats were injected 0.15 M LiCl (0.64% w/v, 2% body weight), which causes severe abdominal pain; after 30 min, they were given to drink 20 mL saccharin solution 0.1 M for 20 min. During the fifth and sixth days, rats were given to drink 20 mL water for 20 min. The next day (day 7), time of testing, they were given 20 mL saccharin 0.1 M solution for 20 min again. The relationship between saccharin and abdominal pain was expected to cause a lower saccharin intake and performance was quantified as the saccharin consumption rate.

2.7. Preference to EtOH model

The experimental model of preference between two bottles, one with EtOH 6% v/v and the other with water, was performed in rats perinatally exposed to EtOH and control rats. Two or three rats were housed in each cage. The position of each bottle (with water or with EtOH) was randomly changed during the experimental period. During the eight weeks of study, the volume of EtOH and water consumed and rat weight were measured twice a week.

2.8. Determination of blood EtOH concentration (BEC)

At the end of the EtOH preference test, between 9 AM and 10 AM, a sample of blood was obtained from each rat to measure BEC, which was determined in a spectrophotometer by means of an enzymatic method with a specific QuantiChrom EtOH Assay Kit (BioAssay Systems Hayward, CA, USA).

2.9. Morphometric digital image analysis

All measurements were performed on coded slides under blinded and standardized conditions. The dorsolateral region of the cerebral cortex (CC) was the focus of morphometric studies. Digital images from the toluidine blue stain were taken with a Zeiss Axiophot microscope. CC thickness was measured with ImageJ software by means of a line drawn between the inner and outer limiting membranes, traced through the middle zone of the telencephalic vesicle. The thickness in each cortical layer and total CC thickness were measured in order to obtain a relative value with ImageJ software.

2.10. Data analysis

Four to six separate toluidine blue staining experiments were run. Individual experiments were composed of 6 to 10 tissue sections of each animal from each group. Five to ten CC were measured of each animal. Differences between animals within each group were not statistically significant.

For behavioral studies, between 10 and 12 PND 40–45 animals were used for each group, C and EPE. For the EtOH preference model, 16 to 20 Wistar rats were used from each group. For all experiments, reported values represent the mean ±SD of experiments performed. Differences among the means in the C and EPE groups were statistically analyzed by a "two-tailed" test. Statistical significance was set to a value of $P < .05$. For the statistical analysis, GraphPad Prism 4.00 software (GraphPad Software Inc., CA, USA) was used.

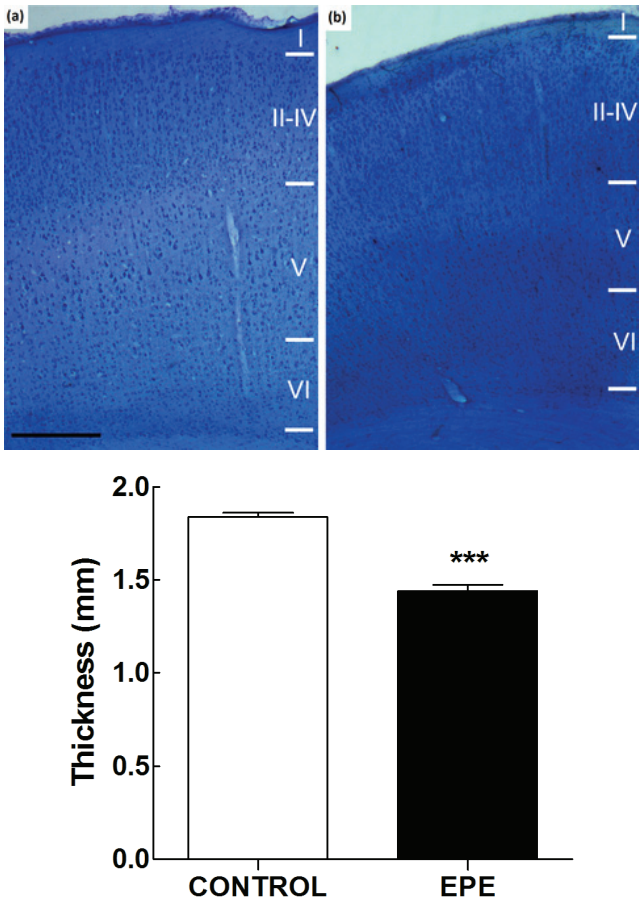


Figure 1: CC thickness in control (a) and EPE (b) adolescent rats. The bar in (a) (photography) is 400 μm. Graphic values are represented as the mean ±SD. $^{***}P < .001$.

3. Results

3.1. Study of cytoarchitecture in the adolescent CC perinatally exposed to EtOH

CC thickness was measured in adult PND 40–45 EPE rats and a significant decrease was found in the EPE group as compared to C group (C: 1.84 ± 0.04 vs. EPE: 1.44 ± 0.08, $P < .001$); see Figure 1.

3.2. Study of the relative thickness of layers in the adolescent CC perinatally exposed to EtOH

The thickness of layers I, II–IV, V, and VI of the CC was measured (Figure 2). The EPE only showed a significant

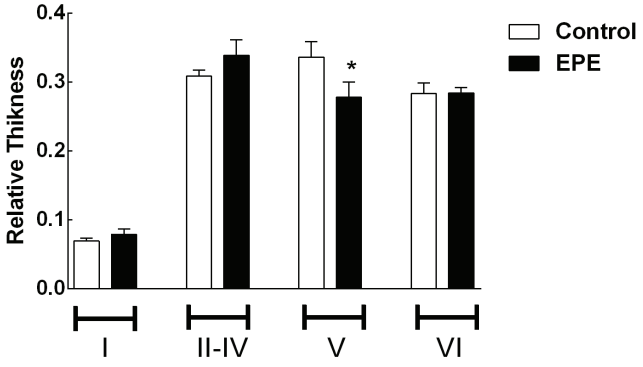


Figure 2: Thickness of cortical layers in C and EPE adolescent rats. Graphic values are represented as the mean ±SD. $^{**}P < .05$.

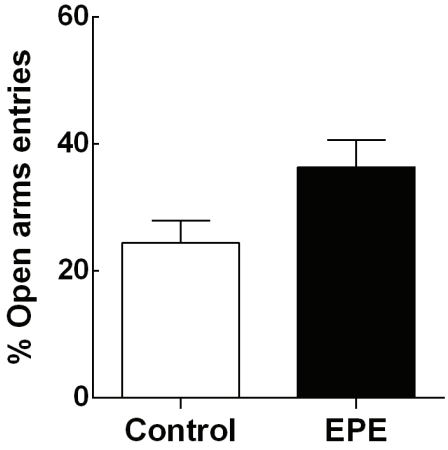


Figure 3: Study of EtOH effect on anxiety in C and EPE adolescent rats. Ratio, expressed as a percentage, between the number of entries in open arms and the total number of entries (open and closed branches). Graphic values are represented as the mean ±SD. $^{*}P < .05$.

decrease in the thickness of layer V (C: 0.33 ± 0.04 vs. EPE: 0.27 ± 0.05, $P < .05$), while no significant differences were observed between groups in layers I, II–IV, and VI. Layer V is the large pyramidal cell location, which corresponds to one of the main neuronal projections of the CC.

3.3. Behavioral studies

CC alterations can cause cognitive impairment and behavioral deficits in adulthood. In order to characterize and determine the behavior of EPE rats regarding anxiety, motor activity, and long-term memory, we performed the following standardized tests in male PND 40–45 rats exposed to EtOH.

3.3.1. Elevated plus maze

The elevated plus-maze test was performed to evaluate the effect of EtOH on anxiety. Between groups, there were no differences in the number of total entries in the arms, but the EPE group showed a higher number of entries into the open arms (C: 24.5 ± 12.26 vs. EPE: 36.4 ± 15.9, $P < .05$). This may suggest that EtOH exposure during gestation and lactation produces an anxiolytic effect in adolescent (Figure 3).

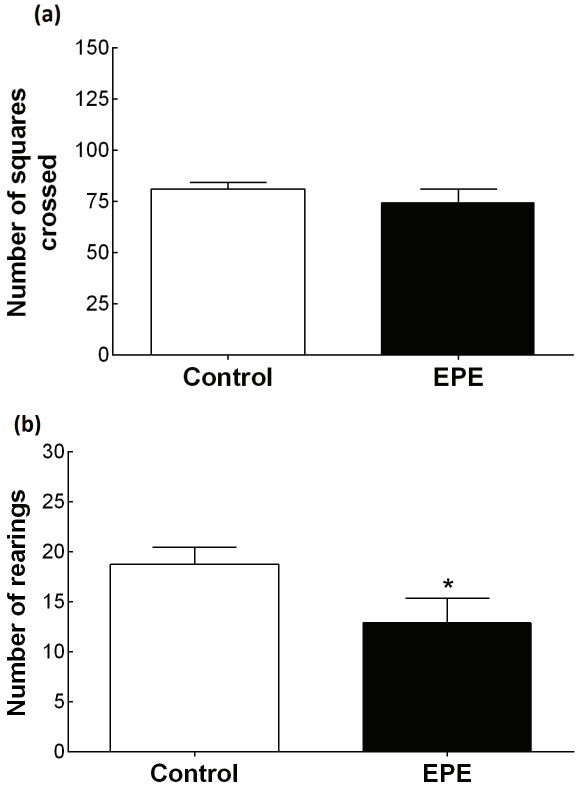


Figure 4: Study of the effect of EtOH on locomotor activity and aerial exploration in C and EPE adolescent rats. Number of squares crossed (a) and number of rearings (b). Graphic values are represented as the mean ±SD. $^{*}P < .05$.

3.3.2. Open field (OF)

From the moment of training, two parameters were evaluated: the number of crossings, to assess the effect of EtOH on locomotor activity, and the number of rearings, to assess aerial exploration. No differences were observed between groups in the number of crossings, which indicates that EtOH did not affect locomotor activity in adolescent who had been exposed perinatally (Figure 4(a)). In turn, the number of rearings was significantly lower in the EPE group (C: 18.74 ± 1.8 vs. EPE: 12.8 ± 2.5, $P < .05$), suggesting that EtOH clearly affected aerial exploration (Figure 4(b)).

3.3.3. Conditioned taste aversion (CTA)

Rats were subjected to a CTA paradigm, largely known to be dependent on the activation of the insular cortex. In this task, rats associate the consumption of saccharin with the intraperitoneal injection of a lithium chloride (LiCl) solution, which induces digestive malaise. As a consequence,

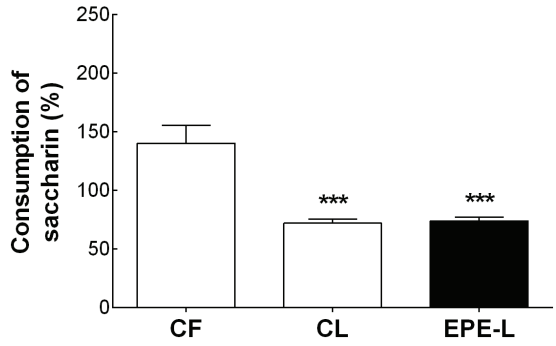


Figure 5: Study of the effect of EtOH on long-term memory in C and EPE adolescent rats. Graphic values are represented as the mean ±SD. $^{***}P < .001$. CF: control physiological solution, CL: control LiCl, EPE-L: EPE LiCl.

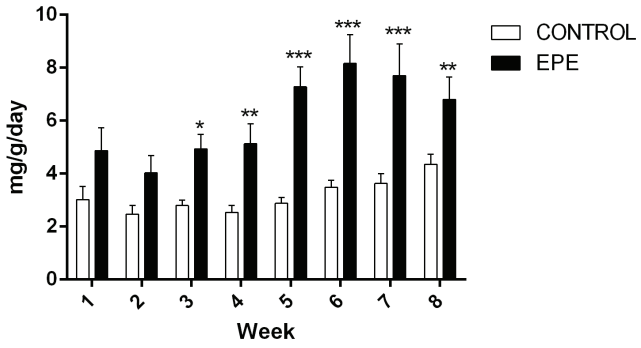


Figure 6: Study of preference to EtOH in C and EPE adolescent rats. Graphic values are represented as the mean ±SD. $^{***}P < .001$, $^{**}P < .01$, $^{*}P < .05$.

there is a decrease in saccharin consumption during the test session with respect to the acquisition session, which is used as a measure of aversion strength.

No significant differences were observed between the C and EPE groups (Figure 5), since the animals of both groups showed a significant decrease in the consumption of saccharin after the injection of LiCl, which suggests that EtOH affects long-term memory.

3.4. Study of preference to EtOH

EtOH-naïve rats showed two types of behavior toward alcohol during the period of study: (1) initial aversion followed by a clear preference after two weeks, (2) irregular preference during the entire period of the experiment (eight weeks, data not shown).

EtOH intake in Wistar rats was measured for about 8 weeks. The EPE group showed an increase in EtOH intake with respect to the C group (Figure 6). This response was statistically significant as from the third week.

Later, when the previous values were separated for male and female rats, both EPE groups showed a clear preference to EtOH as from the first week with respect to the C group.

This response was maintained throughout the study (Figure 7), although statistically significant differences were only observed between C-M and EPE-M at the third and fourth weeks, and between C-F and EPE-F at the sixth week. Notice that, as from the sixth week and at the end of the experimental period, the intake of females was higher than that of males.

3.5. Blood EtOH concentration (BEC)

BEC measurement was done separately in males and females at the end of the last week of the EtOH preference assay.

In controls, there were no significant differences in BEC between sexes (174 mg/dL female vs. 176 mg/dL male).

EPE rats, both male and female, showed a higher level of BEC with respect to control rats (223 mg/dL female; 246 mg/dL male). Again, no differences were observed between sexes in EPE rats.

4. Discussion

Underdeveloped CC is characteristic of FAS [32]. It has been shown that, by altering the migrating neuroblasts pertaining to the first migratory wave (that lately should give rise to cortical pyramidal neurons), moderate perinatal EtOH exposure can induce cortical dysplasia in the brains of rat offspring [10]. These observations suggest a sequence of toxic events that contribute to cortical dysplasia in offspring exposed to EtOH during gestation.

Studies in children exposed to alcohol during gestation show behavioral and cognitive alterations, including hyperactivity, impulsivity, and attention deficit, which could reflect an inability of the inhibitory response [41]. The effects of perinatal exposure to EtOH in PND 40–45 rats on the behavioral paradigms studied were mild. The results obtained from the elevated plus maze test show that EtOH causes a long-term anxiolytic effect, as rats were not exposed to EtOH after the lactation period. From the open field test, we observed that treatment only caused a decrease in aerial exploration but did not affect locomotor activity, perhaps due to the absence of EtOH in the blood. Given that the first 20 postnatal days, third trimester in human gestation, is the most vulnerable period for cognitive function [36], results from the conditioned taste aversion test suggest that perinatal exposure to EtOH does not affect sensory functions in PND 40–45 rats which have not been exposed to EtOH for the previous 20 days.

On the basis of these results, our data suggest that these alterations in behavioral responses are a consequence of cortical neurologic development disorders observed in children of alcoholic mothers, affected by FAS. These alterations are probably due to alterations in layer V of the CC, which persist until adulthood even when rats have

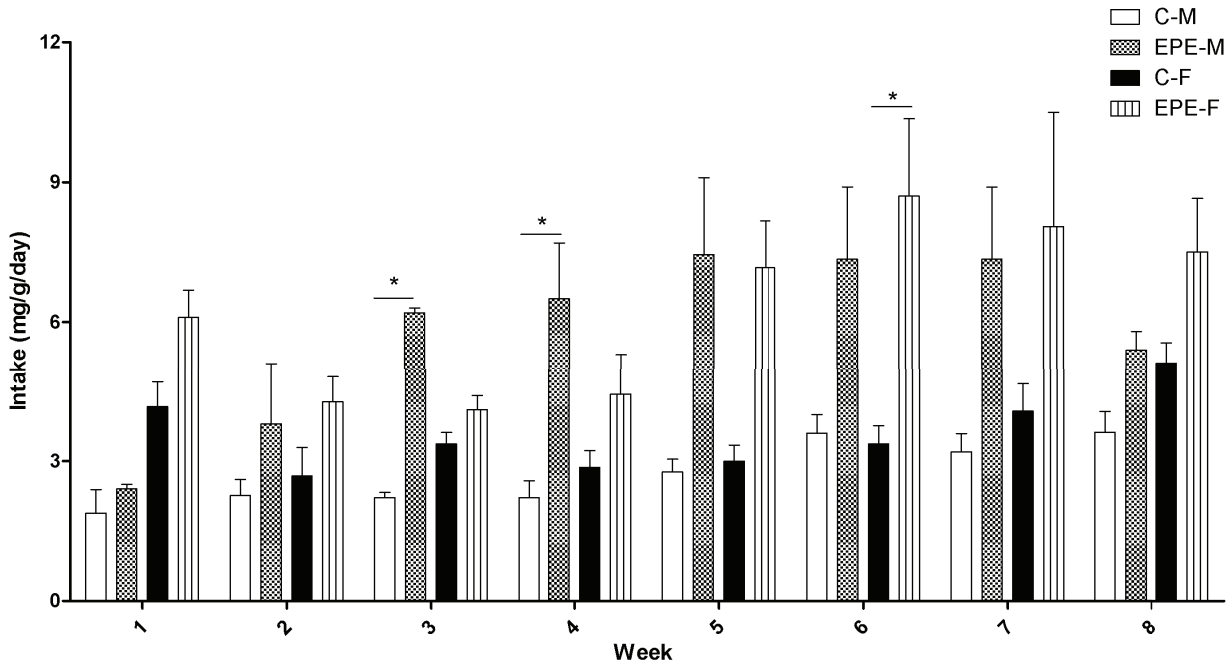


Figure 7: Study of preference to EtOH in C and EPE adolescent male and female rats. Graphic values are represented as the mean ±SD. $^{*}P < .05$. C-M: control male, EPE-M: EPE treatment male, C-F: control female, EPE-F: EPE treatment female.

no further exposure to EtOH. However, it is difficult to compare and relate the results of different studies, since the behavioral deficits observed involve the interaction of several factors such as the amount, duration, and pattern of EtOH consumption, maximum BEC reached, the degree of difficulty in the handling of animals by the operator, and the age, gender, and stress levels of tested animals [34]. There are discrepancies between the results related to gender differences in susceptibility to perinatal alcohol exposure. Some authors describe female rats as the most disadvantaged in spatial memory tasks [26], while others claim that males are more deficient [49]. Regardless of rat strain, sex, and age, chronic exposure to alcohol at the time of neonatal brain development (PND 4–9) appears to be the leading cause of permanent deficits in spatial learning [44], also demonstrated in this work in male rats exposed during the first postnatal days and before birth, during the gestational period. These results suggest that brain structures involved in spatial memory show increased vulnerability to the teratogenic effects of EtOH during development.

In the context of the previous discussion on the potential factors influencing gravity and resistance to the effects of EtOH, the present results may suggest an increased resistance of Wistar rats to the effects of perinatal exposure to EtOH, as pups were not exposed to EtOH from weaning until PND 40–45. In the present work, we observed behavioral responses (direct consequence of the alterations produced in the morphology of the CNS) in adolescent EPE rats without a new exposure to EtOH, which seem comparable

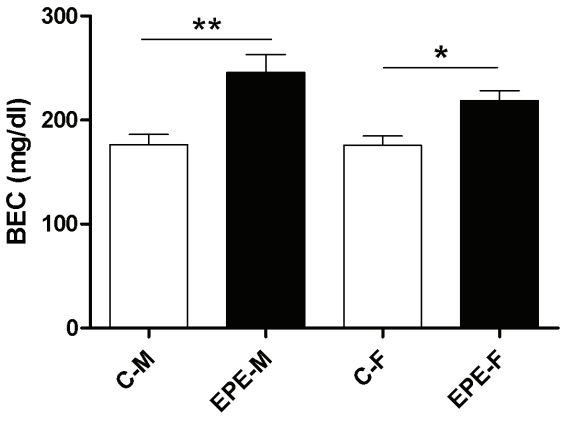


Figure 8: Blood EtOH concentration in C and EPE adolescent male and female rats. Graphic values are represented as the mean ±SD. $^{**}P < .01$, $^{*}P < .05$. C-M: control male, EPE-M: EPE treatment male, C-F: control female, EPE-F: EPE treatment female.

to Evrard's results in adolescent rats after a long period of abstinence [17], related to the persistence of morphological changes in neurons of the CC.

The attenuation of behavioral abnormalities induced by alcohol during CNS maturation may also occur due to some type of morphological and/or functional regeneration of the CNS, bearing in mind that it is still under development. Another study based on qualitative and quantitative hippocampal pyramidal cell evaluation revealed a marked

reduction in the length of dendrites in the CC of animals exposed to EtOH during the early postnatal period [16]. This study also demonstrated a significantly lower number of hippocampal neurons in rat pups exposed to alcohol in high doses [25]. Other authors reported a marked reduction in hippocampal dendritic density in young rats (PND 15) prenatally exposed to EtOH, but not in adulthood (PND 90) [19]. In our previous work, we showed morphological changes in hippocampus of pups prenatally exposed to EtOH using an animal model in which a solution of EtOH in drinking water was administrated to the mother [31].

Clinical and epidemiological studies indicate that prenatal exposure to EtOH is strongly associated with the risk for alcohol abuse in adolescent and young adult humans [4, 5]. In effect, gestational exposure in humans is perhaps the best predictor of later EtOH abuse during adolescence [42]. However, studies in animal models of prenatal or perinatal exposure to EtOH revealed that the level of alcohol intake during adolescence and/or adulthood depends on the animal model used for the study, as well as the period in which the animal was exposed to EtOH.

Using the same via of administration of EtOH during pregnancy (liquid diet), but using Long Evans Hooded rats, Glendinning et al. [20] showed that the effects of fetal EtOH exposure on EtOH intake in adolescence are mediated, in part, by changes in the quality components of EtOH, principally the odor. They demonstrated that a reduced aversion to the bitter taste and odor qualities of EtOH could account for 51% of the effect of fetal EtOH exposure on oral acceptability of EtOH (29% bitter taste and 22% olfaction) in Long Evans adolescent rats [47]. It stands out that Long Evans Hooded rats receiving EtOH from gestational day 11 to 20 reach a blood EtOH concentration of 150 mg/dL. In our previous work, Wistar rats received EtOH during a month previous to mating and in all the gestational period, reaching a blood EtOH concentration near 89 mg/dL at the end of gestation [10]. On the other hand, we obtained a higher blood concentration at the end of gestation, near 200 mg/dL in pregnant Wistar rats receiving EtOH intraperitoneally during gestation [9]. Thus, although the pups in this work received a low dose of EtOH during perinatal development, similar results were observed regarding a marked decrease in aversion to EtOH.

As well as our present results, results from other studies show that the administration of a 5.9% w/w EtOH dose to pregnant rats during gestation and lactation results in a clear increase in EtOH intake by the offspring. Several studies in rats have found that maternal administration of low or moderate doses of EtOH result in fetal perception of the chemosensory and toxic effects of EtOH. This prenatal experience with the drug enhances the palatability of EtOH's flavor and increases EtOH consumption during infancy and adolescence [6].

The capacity of the rat fetus to perceive chemosensory properties of the amniotic fluid and other substances present in their prenatal environment, from at least gestational day 17 until parturition, has been well documented [29, 38, 39]. This fetal ability has a direct relationship with postnatal responses toward those substances: contamination of the amniotic fluid with a certain flavor increases intake of that flavor later in life [37]. Animal studies about the effects of moderate or low doses of EtOH during the last gestational period indicate that, although no apparent teratological effects are evidenced with this treatment, the prenatal experience with EtOH may alter normal patterns of response to the drug along life. In this case, prenatal exposure to EtOH results in fetal learning about its sensory and toxic properties, which in turn is expressed during infancy, and even in adolescent periods, as an increase in EtOH intake and a preference for its flavor. In general, the outcome of this animal research is congruent with data from human studies showing infantile recognition of and preference for substances previously experienced [18, 24, 28].

There is a clinical evidence that humans exposed prenatally to moderate levels of EtOH have increased susceptibility to alcohol abuse in adolescence and childhood [12, 13]. Using animal models, the neurobiological basis of this effect has begun to be studied [7, 8, 14]. EtOH exposure during early adolescence significantly affects its affinity during late adolescence, and subpopulations of animals with differential susceptibility to EtOH effects are possible (i.e., high- and low-responders) [3].

These results contribute to the study of the relationship between EtOH perinatal exposure and later EtOH abuse problems. However, we still know very little about the mechanisms of possible CNS recovery from the damaging effects caused by perinatal exposure to EtOH. In addition to the close relationship between the morphological CC layer organization and learning processes, the behavioral tests performed in this study, such as the elevated plus maze and open field, are related to the hippocampus, while the aversion test is connected with the insular cortex. Therefore, future work will require a more detailed assessment of the effect of EtOH on these structures, and the role of cerebral structures related to addiction. The elucidation of these mechanisms is an interesting topic for future research.

5. Conclusion

Previous data from our laboratory demonstrated the impact of maternal EtOH consumption on the development of CNS in offspring, suggesting a sequence of toxic events that contribute to cortical dysplasia, altering the morphology of areas of the CNS involved in behavioral processes. The results described in this paper demonstrate that offspring may be affected by perinatal exposure to EtOH during a critical period of this perinatal development, which

may impact both behavior and alcohol preference during adolescence. Further research will enable us to begin to unravel the mechanisms underlying the effects of in utero EtOH exposure on offspring development.

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