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Ethanol-induced tolerance and sex-dependent sensitization in preweanling rats

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HIGHLIGHTS

- The same ethanol dose induced locomotor sensitization and tolerance in infant rats.
- Only males displayed sensitization induced by ethanol.
- Tolerance was observed when the training and testing contexts coincided.
- Preexposure to the context attenuates the acute stimulating effect of ethanol.

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ABSTRACT

According to genetic studies, the acute stimulating effect of ethanol seems to be associated with an increased predisposition to consume large quantities of ethanol. Ethanol-induced stimulation has been rarely reported in adult rats. However, infant rats, particularly during the second postnatal week of life, are highly sensitive to ethanol-induced behavioral activation. They also consume more ethanol than in later ontogenetic stages. In adult mice repeated ethanol experience usually results in sensitization to the stimulating effect of ethanol, while tolerance is the predominant result in rats. The present study was designed to explore in rats whether repeated exposure to ethanol during infancy modifies subjects' sensitivity to the stimulating effect of the drug, either increasing or decreasing its magnitude (i.e. sensitization or tolerance, respectively). Furthermore, we also explored the possible context-modulation of these effects. In two experiments, subjects were trained with water or ethanol (2.5 g/kg) between postnatal days (PDs) 8 and 12 (Experiment 1) or between PDs 14 and 18 (Experiment 2), and tested in response to water or ethanol two days later. In these experiments we identified three variables that critically modulate the effect of the repeated ethanol exposure: sex, context and age. Ethanol exclusively and consistently induced locomotor sensitization in males trained outside of the testing context (Experiments 1a and 1b), while tolerance to the stimulating effect of ethanol was observed in males and females trained in the testing context (Experiment 1a). In Experiment 2 tolerance was detected in females trained outside of the testing context. Finally, experience with the testing context during training strongly attenuated the stimulating effect of ethanol in the older subjects (Experiment 2). These results show that the same ethanol treatment can produce opposite effects (tolerance or sensitization) and demonstrate the involvement of Pavlovian conditioning in the development of tolerance. Furthermore, sex was revealed as an important factor to take into consideration in the analysis of chronic experience with ethanol during infancy. We can conclude that specific ontogenetic stages can be used to study the biological determinants underlying both ethanol-induced tolerance and sensitization, and the environmental modulators of these effects.

1. Introduction

Ethanol-induced locomotor stimulation in laboratory rats has been considered a valuable tool for studying the motivational effects of this drug [28,64,83]. In rodents, the expression of this ethanol effect depends on the complex interaction of a variety of environmental and genetic factors, including the rodent species, individual differences (sex, age, behavioral traits) and variables related to the prior experience with both the drug itself and the environment in which the drug effects are experienced, among others [46,59]. The specific biological correlates of the behavioral activation induced by ethanol have mainly been studied in mice, since this rodent species shows this effect under a wider range of experimental conditions than rats [59]. Usually, when adult rats are injected with ethanol using similar parameters to those used with mice, the effect produced tends to be one of sedation rather than stimulation [25,46]. However, the locomotor activating effect of ethanol has been consistently observed in genetically selected rats that consume

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high amounts of ethanol [1,18,55,61], which may indicate a positive correlation between subjects' sensitivity to the stimulating effect of ethanol and their predisposition to ingest the drug.

Similarly to that observed with other drugs of abuse, repeated experience with ethanol can result in an increased (sensitization) or reduced (tolerance) sensitivity to some of its specific effects. From different theoretical perspectives, both tolerance and sensitization induced by ethanol (or by other drugs) have been linked to the development of dependence [39,66,72,75]. The way biological and environmental factors interact to modulate these opposite drug effects are still not fully understood, and also depend on the specific behavioral or physiological index analyzed. For example, even with the same ethanol treatment, tolerance to one ethanol effect (rearing or ataxia) and sensitization to another (locomotor activity) have been observed in the same animal [52]. While ethanol-induced locomotor sensitization is easily observed in mice, in rats this effect is infrequent [58], and even when behavioral activation is observed after ethanol administration, rats rapidly develop tolerance to this effect [9,34].

The importance of studying these effects of ethanol (i.e. tolerance and sensitization) during infancy is based on a considerable amount of converging evidence from human and laboratory studies, which have shown that early experience with ethanol is an important determinant of responsiveness to the drug in later stages of ontogeny [23,74]. This association highlights the importance of understanding those factors that modulate the outcome of early experience with the drug. In many studies, the infant rat has been characterized by an increased responsiveness to the acute effects of ethanol, showing particular sensitivity to this drug's motivational and motor stimulating effects, especially during the first two postnatal weeks [2,5,53,54]. This profile is accompanied by a clear predisposition to consume relatively large amounts of ethanol [68,78]. Beyond this, however, few studies have focused on sensitivity to ethanol during infancy after repeated experiences with the drug [7,9,24,35,71].

Another argument to justify the study of tolerance and sensitization during infancy is that these effects have been described in different animal models as being context-dependent, which means that when subjects are evaluated in a different context from the one in which they were trained, the effects are attenuated or eliminated [16,70,75]. Context effects are particularly important in studies with infant rats, because their capacity to retain context learning is a matter of current debate (see, for example, [37,60]). The involvement of classical conditioning in ethanol-induced sensitization or tolerance has been observed in adult rodents by measuring different behavioral indexes, the most common one being hyperlocomotion in sensitization studies with mice [21], and the hypothermic and depressant effects of ethanol in the study of tolerance [27,44,82]. Although few additional studies have reported tolerance to the locomotor stimulating effect of ethanol in rats [9,34], the context-dependence of this effect has not been analyzed. In infant rats, locomotor sensitization induced by psychostimulants has been described as context-independent [40,47,48], although other authors have found context-specific locomotor sensitization induced by cocaine during this ontogenetic period [77].

The present study was designed to explore whether the locomotor response to ethanol is increased (sensitization) or reduced (tolerance) in pups repeatedly exposed to the drug during the infantile period. The protocol used in the experiments described below also enabled us to explore the possible context-modulation of these ethanol effects during the second (Experiment 1) or third (Experiment 2) postnatal weeks of life.

2. Experiment 1a

In this first experiment we focused our attention on the second postnatal week of life. Our interest in this specific stage of development stems from studies which have observed heightened sensitivity to ethanol-induced behavioral activation during this period [5], along with a predisposition to consume large amounts of ethanol [68]. Interestingly, it has been shown that the development of locomotor sensitization induced by ethanol may be associated with an increased consumption of the drug in mice with high initial affinity of ethanol [42]. Therefore, it is likely that ethanol-induced sensitization can also be detected at this early stage of development. In a previous study we demonstrated that ethanol (2.5 g/kg) can produce biphasic locomotor effects, increasing activity soon after ethanol administration (5–10 min) and attenuating exploration 30 min after administration [4]. In Experiment 1a we explored the effect of a repeated ethanol treatment, between PDs 8 and 12, on the locomotor response induced by ethanol after two days of withdrawal (PD15). In order to capture the biphasic locomotor effects of the drug, independent samples of subjects were trained and tested at two post-administration intervals, 5–10 or 30–35 min. Finally, the influence of context learning on the acute and chronic effects of ethanol was also explored. We previously found that prior experience with the testing context attenuates the stimulating effect of ethanol, and that this ethanol effect critically depends on novelty of the testing environment [4]. Considering also that environmental novelty is an important factor that can influence the development and expression of sensitization, it can be expected that detection of ethanol-induced sensitization in our experimental model may be benefited if subjects have not experience with the testing context before testing.

2.1. Materials and methods

2.1.1. Subjects

For Experiment 1a, a total of 169 male and female Wistar pups were used, 108 animals representative of 18 litters corresponding to post-administration time 5–10 min, and 61 animals from 8 litters for post-administration time 30–35 min. In all the experiments, no more than one subject of each sex from a given litter was assigned to the same treatment condition, to avoid overrepresentation of a particular litter in any treatment. Animals were born and reared at the vivarium of the Instituto de Investigación Médica Mercedes y Martín Ferreyra, INIMEC–CONICET-UNC, under conditions of constant room temperature (22 ± 1.0 °C), on a 12 h light–12 h dark cycle. Births were examined daily and the day of parturition was termed postnatal day 0 (PD0). Subjects were 8 days old at the start of the experiment. All procedures were approved by the National Department of Animal Care and Health (SENASA — Argentina) and were in compliance with the National Institute of Health's general guidelines for the Care and Use of Laboratory Animals.

2.1.2. Apparatus

In Experiment 1a, all animals were tested in a circular open field (30 cm diameter), with a white plastic wall and floor. A piece of cotton impregnated with almond odor (almond scent, 1 ml of a 0.1% solution v/v, Esencias del Boticario, Córdoba, Argentina) was placed on the top of the open field. The odor was included as a feature of the context in order to favor contextual learning in the infant rat. This context was also used for training subjects from the Context condition (see Procedures section). In all experiments, locomotor activity was estimated through an index that was calculated by counting the number of quadrants that the subject crossed during the training or testing session. For this purpose, the floor of the open field was divided into four quadrants. Training and testing sessions were videotaped, and were later evaluated by a researcher blind to the treatments, who counted the number of quadrants crossed. Every time a pup passed its head and forepaws across one of the lines that divided the quadrant, a quadrant was considered to have been crossed.

2.1.3. Procedures

2.1.3.1. Training phase. This phase took place between PDs 8 and 12 (one session per day). On PD 8 subjects were assigned to one of the two context conditions (Context or No-Context). In each training session, pups from both conditions were separated from their mothers and placed in

pairs in a holding cage (25 cm × 23 cm × 23 cm) partially filled with clean wood shavings. The floor of the cage was maintained at 36 °C (±1 °C) through the use of a heating pad. One hour later, pups' body weights were individually recorded and they immediately received an intragastric (i.g.) administration of water or ethanol (2.5 g/kg). This ethanol dose was selected because it has been found to consistently induce locomotor stimulation in preweanling rats [4,5]. The volume administered was equivalent to 0.015 ml per gram of bodyweight of a 21% (v/v) ethanol solution. Pups assigned to the vehicle control group received the same volume of tap water. Intragastric administrations were performed using a 10-cm length of polyethylene tubing (PE-10 Clay Adams, Parsippany, New Jersey) attached to a 1 ml syringe with a 27G × 1/2 needle. This tubing was gently inserted through the mouth and slowly guided into the stomach. The entire procedure took less than 20 s per pup. After the i.g. administration, pups from the Context condition were placed for 5 min in the open field (see Apparatus section), where their behavior was videotaped for further analysis of locomotor activity. Locomotor activity was measured independently at two post-administration intervals, 5 to 10 or 30 to 35 min after the i.g. administration. Finally, after the 5-min session, all subjects were returned to their home cages. Pups of the No-Context condition remained in pairs in the holding cage during the same period of time. The two time periods for testing were selected because previous studies have shown that the ethanol dose administered induces opposite locomotor effects at these intervals, stimulating locomotion during the first interval (5–10 min), and inhibiting behavior (sedation) during the second (30–35) [4]. This would allow us to study the effect of the repeated experience with ethanol on these biphasic ethanol effects.

2.1.3.2. Testing phase. After two days of withdrawal, on PD 15, pups from both conditions (Context and No-Context) were evaluated in response to water or ethanol in terms of locomotor activity. Procedures were identical to those used for training, with a few exceptions. Firstly, the locomotor activity of all subjects (from both context conditions) was assessed in the same context, namely the one used for training subjects from the Context condition. Secondly, half of the subjects were assessed in response to the same treatment (water or ethanol) received during training, while the remaining half were tested after being administered with the alternative treatment; i.e., those trained with water received ethanol during testing, while those trained with ethanol received water during testing. All subjects were tested at the same post-administration interval as during training (5 or 30 min after i.g. administration).

2.1.4. Experimental design and statistics

The experimental design is a mixed one, with five between-group variables: Training treatment (Water vs Ethanol), Testing treatment (Water vs ethanol), Context (Context vs No-Context), Sex (Male vs female) and Post-administration time (5 vs 30 min). The dependent variable analyzed was locomotor activity, which was estimated through the total number of crosses during the 5-min test. Training scores were analyzed by means of a mixed ANOVA. The between-group factors were Training treatment, Post-administration time and Sex, while Day (5 days of training) was the only within-group variable. Due to the complexity of the experimental design, in order to simplify the analysis of the testing data, a preliminary ANOVA was used to explore locomotor activity scores from subjects given water during testing, in order to see whether this behavioral index varied across sex, or whether it was affected by the training experience with the testing context (habituation) or ethanol (conditioned locomotor responses) (see Results section). This ANOVA did not find any significant effect or interaction between the different variables included in the analysis (Training treatment, Context or Sex). Hence, scores from animals given water during testing were condensed into two control groups, one for each post-administration time. The definitive ANOVA used for the analysis of locomotor activity scores includes three between-group factors: Sex, Post-administration time, and Group. This last variable (Group) included five independent experimental conditions (Control, W–E Context, W–E No-Context, E–E Context, E–E No-Context). The first letter of the names of the experimental groups indicates training treatment with water (W) or ethanol (E), while the second letter indicates the testing treatment (W or E). In this, as well as in the following experiments, the loci of the significant main effects or interactions were further explored using post-hoc tests (Newman–Keuls), with an alpha level set at 0.05.

2.2. Results

2.2.1. Training

Fig. 1 shows locomotor activity scores as a function of Training treatment (W or E), Post-administration time (5 or 30 min) and Day, for subjects trained in the testing context. The ANOVA (Training treatment by Post-administration time by Sex by Day) revealed significant main effects of Training treatment [$F(1,71) = 30.89$, $p < 0.05$] and Day [$F(4,284) = 47.43$, $p < 0.05$]. The interaction between these factors also achieved statistical significance [$F(4,284) = 3.99$, $p < 0.05$]. Post-hoc tests revealed that ethanol increased locomotion on every single training day. In sum, these results show the typical stimulating effect of ethanol throughout the training phase. The ANOVA detected no main effects of Sex, nor any interactions between this variable and the other variables analyzed.

2.2.2 . Testing

Fig. 2 shows the locomotor activity scores from all groups that received Water during testing (E–W and W–W groups). As mentioned earlier, a preliminary 4-way ANOVA (Training treatment by Context by Post-administration time by Sex) failed to reveal any significant main effect or interaction between factors, ruling out habituation to the context or expression of conditioned locomotor responses induced by ethanol at this age. Therefore, in order to simplify the statistical analysis, all subjects treated with Water during testing were divided into two Control groups, one for each post-administration time (5 and 30 min).

Fig. 3 shows the locomotor activity scores as a function of Group (Control, W–E Context, E–E Context, W–E No-Context or E–E No-Context), Post-administration time (5 or 30 min) and Sex. The ANOVA revealed a significant main effect of Group [$F(4,149) = 21.79$, $p < 0.05$], and a significant interaction between Group and Sex [$F(4,149) = 3.55$, $p < 0.05$]. Post-hoc tests revealed that, regardless Sex, subjects given ethanol for the first time at testing (groups W–E Context and W–E No-Context) scored higher in locomotor activity than the Control and E–E Context groups. Although the interaction Group by Post-administration time did not reach the statistical significance, these differences were only supported by post-hoc test in the first testing interval (5–10 min). Interestingly, in none of the post-administration intervals did the E–E Context group (males or females) differ from the Control group in terms of locomotion.

Finally, males trained and tested with ethanol from the No-Context condition (E–E No-Context) displayed more locomotor activity than the remaining conditions (including the W–E No-Context), an effect observed in both post-administration times (i.e. sensitization). In contrast, in females, scores from the E–E No-Context group differed from those from the Control condition, but were statistically similar to those from the W–E No-Context group (Fig. 3).

In short, the present data indicate that ethanol induces locomotor activation in pups throughout the second week of life, a result consistent with previous data. Interestingly, subjects' response to chronic treatment with ethanol was modulated by context and sex. Tolerance was observed in males and females (during the first testing interval) when the training and testing contexts coincided, while sensitization was observed exclusively in males trained in a different context than the testing one, regardless the post-administration time.

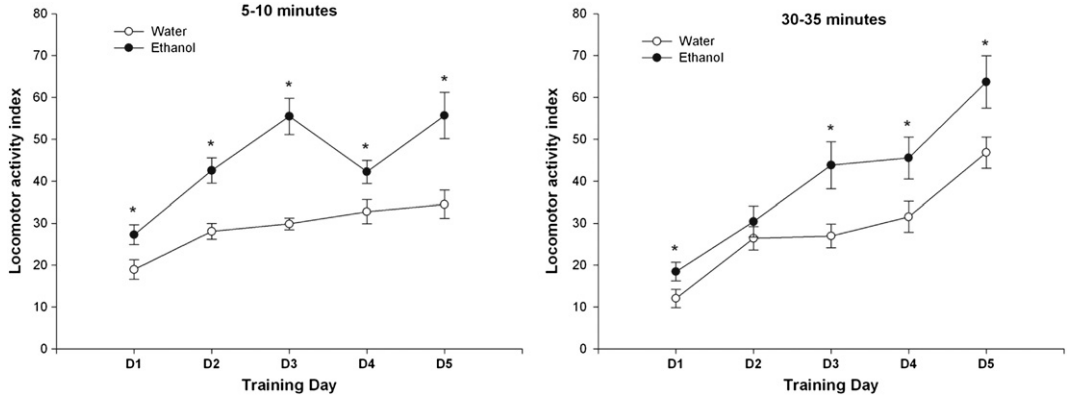

Fig. 1. Locomotor activity levels during the training days (days 1 to 5) in subjects trained in the testing context 5–10 (left) or 30–35 (right) min after ethanol administration. * indicates significant differences from the Water control group, $p < 0.05$. Vertical lines illustrate standard errors of the mean.

3. Experiment 1b

During training, subjects that expressed locomotor sensitization to ethanol received a slightly different treatment from those which showed tolerance. Specifically, during the training session, subjects from the No-context condition were kept in pairs in the holding cage for 5 min in which those from the Context condition were placed in the open field. This procedure implies that, during the five training sessions, these subjects were not completely isolated and were not exposed to novelty, since the holding cage shares some characteristics with the home-cage (for example, they both contain wood shavings). Experiment 1b was designed to explore whether or not the sensitization observed in Experiment 1a was dependent on these procedures.

3.1. Subjects

Experiment 1b was carried out using 20 pups representative of 10 litters. Subjects were 8 days old at the start of the experiment.

3.2. Apparatus

In Experiment 1b, the context used during training consisted of a rectangular black plastic cage (27 cm × 11 cm × 16 cm), with a white floor and an alternative odor cue (orange scent, 1 ml of a 0.075% solution v/v, Esencias Bangladesh, Buenos Aires, Argentina) on the roof. During testing, locomotor activity was assessed in the same circular open field as in Experiment 1a.

3.3. Procedures

To achieve the goal of this experiment, we trained subjects in an alternative and distinctive context (see Apparatus) during the training phase, rather than keeping them in pairs in the holding cage. All other procedures were similar to those used in Experiment 1a, with a few variations. Firstly, subjects were only trained and tested 5–10 min after i.g. administration, because in Experiment 1a behavioral effects were comparable during the two time periods explored. Secondly, for this experiment, only subjects from the W–E and E–E groups were used, because in Experiment 1a, locomotion of subjects given water during testing was not affected by their prior experience with ethanol or the context. All subjects were trained with water or ethanol (2.5 g/kg) in the new context between PDs 8 and 12, and on PD 15 they were tested in the circular open field used for Experiment 1a in response to ethanol (2.5 g/kg). Consequently, sensitization will be inferred from differences between the E–E and W–E groups. The present experiment was also designed to corroborate the greater predisposition of males than females to express sensitization.

3.4. Experimental design and statistics

The experimental design was based on the factorial combination of Group (W–E or E–E) and Sex. The locomotor activity scores obtained during the 5-min test were analyzed using a 2 (Group) × 2 (Sex) ANOVA.

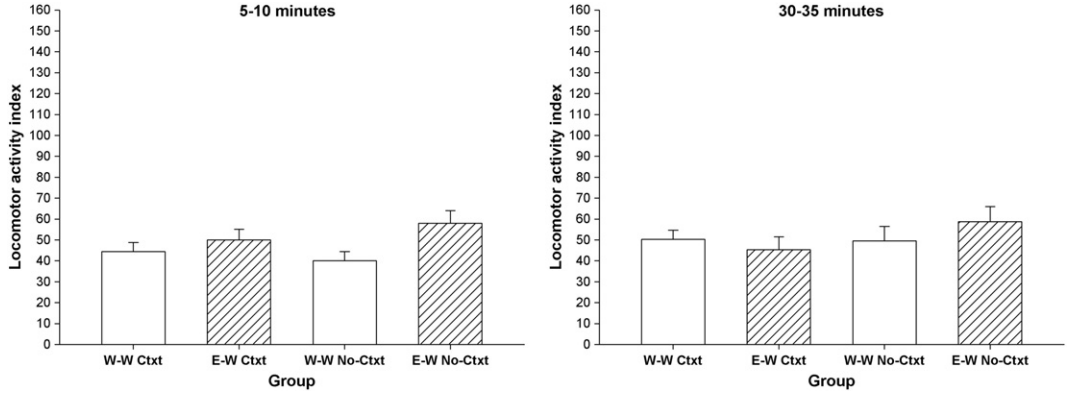

Fig. 2. Locomotor activity levels from pups given Water at testing, as a function of Group and Post-administration time [5–10 (left) or 30–35 (right)]. Vertical lines illustrate standard errors of the mean.

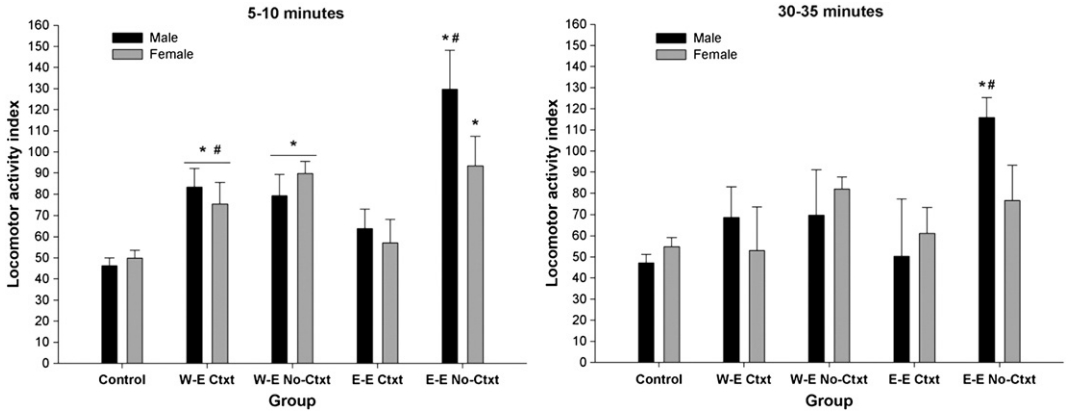

Fig. 3. Locomotor activity scores during the 5-min testing as a function of Group, Sex, and Post-administration interval. * indicates significant differences from Controls, $p < 0.05$. # represents significant differences from the respective context control condition, $p < 0.05$. Vertical lines illustrate standard errors of the mean.

3.5. Results

Fig. 4 shows the locomotor activity scores as a function of Group (W–E or E–E) and Sex. The ANOVA (Group by Sex) revealed that the interaction between these factors reached statistical significance level, [F (1, 16) = 7.28, $p < 0.05$]. Subsequent post-hoc tests revealed that males that had received ethanol during both training and testing (E–E) scored higher for locomotor activity than males receiving ethanol for the first time during testing (W–E). Consistently with the results from Experiment 1a, this difference was not observed in female rats. This finding corroborates the two main conclusions drawn in relation to sensitization from the previous experiment: firstly, ethanol-induced sensitization is context-independent in preweanling rats, and secondly, this effect is exclusively displayed by males, at least with our ethanol protocol.

4. Experiment 2

This experiment was conducted to explore whether the results from Experiment 1a are specific to the age group studied in that experiment, or can still be observed in a later ontogenetic period during infancy. Sensitivity to the stimulating and motivational effects of ethanol seems to vary across the first two postnatal weeks of life. For example, rats are less sensitive to the stimulating effect of ethanol by the third than during the second postnatal week of life [5,9]. Moreover, predisposition to consume ethanol decreases after a peak around PDs 10 and 12 [68,78].

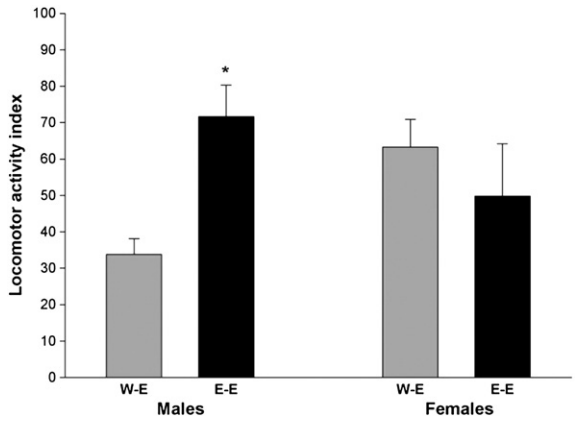

Fig. 4. Locomotor activity scores as a function of Training Treatment and Sex (Experiment 1b). Subjects were trained in a different context than the one used at testing. * indicates significant differences from Controls, $p < 0.05$. Vertical lines illustrate standard errors of the mean.

The aversive and appetitive effects of ethanol also seem to change during infancy, with younger rats being more sensitive to the appetitive and less sensitive to the aversive effects of ethanol [2,22,36]. If development or expression of tolerance or sensitization is functionally linked to motivational properties, then it is likely that predisposition to develop and express these ethanol effects will also vary throughout infancy.

4.1. Subjects

122 preweanling rats, derived from 16 litters, were used for Experiment 2. Subjects were 14 days old at the beginning of the experiment.

4.2. Apparatus

In Experiment 2, training (for the Context condition) and testing (for all subjects) was performed in an open field similar to that used in Experiment 1. The only difference was the diameter (38 cm), which in this case was adapted to the size of older preweanling rats.

4.3. Procedures

Procedures were similar to those used in Experiment 1a, although in this case training was carried out between PDs 14 and 18, and subjects were tested after two days of withdrawal, on PD 21. For this experiment, locomotor activity was assessed 5–10 min after i.g. administration, and the open field size was adapted to the age of the subjects (see Apparatus).

4.4. Experimental design and statistics

The experimental design for Experiment 2 consisted of four between-group variables: Training treatment (Water vs. Ethanol), Testing treatment (Water vs. ethanol), Context (Context vs. No-Context) and Sex (Male vs. female). The dependent variable analyzed was locomotor activity, which was estimated on the basis of the total number of crosses during the 5-min test. Training scores were analyzed by a mixed ANOVA (Training Treatment by Sex by Day). Similar to Experiment 1, a preliminary ANOVA was conducted to explore the locomotor activity scores obtained by subjects given water during testing, with the aim of reducing the complexity of the statistical analysis. In this case, this analysis revealed a significant main effect of Context, with subjects from the Context condition scoring lower for locomotor activity than those from the No-Context one. Hence, scores from animals given water during testing were condensed into two control groups, one for each Context condition. Consequently, the ANOVA used for testing the scores included three between-group factors (Sex, Context and Group). The variable Group had three levels (Control, W–E and E–E).

4.5. Results

4.5.1. Training

Fig. 5 shows the locomotor activity means as a function of Training Treatment (Water or Ethanol) and Day, for pups trained in the testing context. The ANOVA (Training treatment by Sex by Day) revealed significant main effects of Training Treatment [$F(1, 56) = 9.68$, $p < 0.05$] and Day [$F(4, 224) = 30.14$, $p < 0.05$]. The interaction between these two factors was also significant [$F(4, 224) = 16.42$, $p < 0.05$]. According to post-hoc tests, the only significant difference between subjects given ethanol and water controls was observed on day 1.

4.5.2. Testing

Fig. 6 contains the locomotor activity scores of subjects tested in Experiment 2 in response to water. As explained in the Experimental design and statistics section, the three-way ANOVA (Training treatment by Context by Sex) revealed a significant main effect of Context, [$F(1, 49) = 6.34$, $p < 0.05$], showing that subjects trained in the testing context moved less than those from the No-context condition (i.e. habituation).

Since Training treatment did not exert a significant effect on or interact with the other factors, subjects given water during testing were divided into two control conditions, one for each Context condition. Locomotor activity scores were analyzed using a 3-way ANOVA including Group (Control, W–E or E–E), Context (No-Context or Context) and Sex as between-group factors. This ANOVA revealed significant main effects of Group [$F(2, 110) = 6.93$, $p < 0.05$], and Context [$F(1, 110) = 21.97$, $p < 0.05$]. This latter effect indicates an attenuation of locomotion in rats trained in the testing context. The ANOVA also detected a significant interaction between Group and Sex, [$F(2, 110) = 3.72$, $p < 0.05$]. According to post-hoc tests, in females, locomotor activity scores from the W–E group were significantly higher than those in the E–E and Control groups. Scores from these latter two groups were statistically equivalent. This result is compatible with the tolerance observed in Experiment 1a, but in this case this effect was only observed in females that were trained outside of the testing context. Scores obtained by subjects from the Context condition did not differ as a function of Group. No differences between groups were observed with scores from males (Fig. 7).

5. Discussion

The present study was designed to explore the effects of chronic exposure to ethanol during infancy on the locomotor effect of this drug. The results revealed, firstly, that infant rats have a heightened sensitivity to the locomotor stimulating effect of ethanol, particularly during the second postnatal week of life, and secondly, that the same repeated experience with the drug can influence this ethanol effect in two opposite ways, increasing or attenuating its magnitude. Through these experiments, we identified three main factors that critically modulate the chronic effect of ethanol during infancy. The first one is experience with the context, since in Experiment 1a the stimulation induced by ethanol was completely abolished when training with ethanol occurred in the testing context (i.e. conditioned tolerance). The other two important variables were sex and age, because only males trained during the second post-natal week exhibited locomotor sensitization to the excitatory effect of ethanol (Experiments 1a and 1b), while females trained during the third postnatal week showed context-independent tolerance to this ethanol effect (Experiment 2).

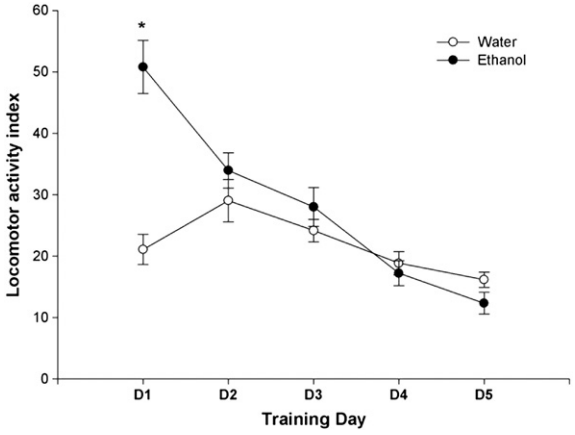

Fig. 5. Locomotor activity during training phase in subjects trained in the testing context across training days (Days 1 to 5) with Water or Ethanol (Experiment 2). * indicates significant differences from the Water group, $p < 0.05$. Vertical lines illustrate standard errors of the mean.

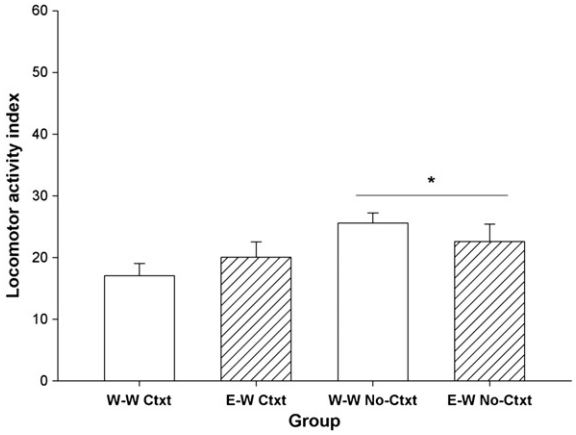

Fig. 6. Locomotor activity scores from subjects given Water at testing, as a function of Group (Experiment 2). * indicates significant differences between groups from the context and no-context conditions, $p < 0.05$. Vertical lines illustrate standard errors of the mean.

In the present experiments a high ethanol dose (2.5 g/kg) was found to increase locomotion during the second and third postnatal weeks. This result is consistent with previous findings [4,5,10], and contrasts with what was typically reported with adult rats, which usually display locomotor suppression in response to similar or even lower ethanol doses [25]. In previous studies we found biphasic locomotor effects of ethanol during this ontogenetic period, with subjects showing stimulation soon after being treated with ethanol and sedation approximately 30 min after ethanol treatment [4,7]. In Experiment 1a, in order to explore the effect of prior experience with ethanol on these biphasic ethanol effects, we decided to assess subjects at two post-administration intervals, 5–10 and 30–35 min after ethanol administration. Surprisingly, ethanol did not induced sedation in the second interval. The lack of sedation in this experiment may be related to the shape and size of the open field used, which is known to affect animals' exploratory activity [81]. In the few studies in which ethanol was found to suppress locomotion during infancy, subjects were evaluated in a smaller, square-shaped open field. The rodents spend time exploring the corners of these square open fields, which also may influence locomotion indexes. Since novelty is an important modulator of ethanol-induced activation in infant rats [4], it seems plausible that smaller open fields also facilitate habituation to novelty and foster the expression of the sedative effects of ethanol [7]. In addition to the shape and size of the open field, maternal separation during the training sessions may also contribute to the lack of ethanol-mediated sedation and to the expression of behavioral activation induced by ethanol during the second post-administration interval [4,9]. Maternal separation is a known stressor, particularly during the first two postnatal weeks [43], and the locomotor stimulating effect of ethanol is critically modulated by the stress response [10].

The acute stimulating effect of ethanol in older pups (on PD 21, Experiment 2) was much weaker than in younger rats (PD 15, Experiment 1a), which is consistent with previous findings showing that this ethanol effect is stronger during the second postnatal week than later on

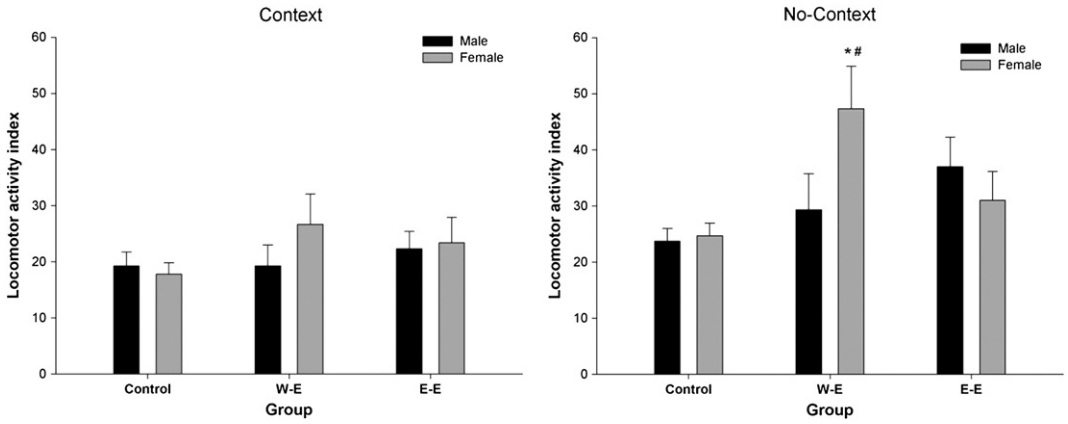

Fig. 7. Locomotor activity levels at testing as a function of Group, Context and Sex. * indicates significant differences from the Control group, $p < 0.05$. # represents significant differences from the respective context control condition, $p < 0.05$. Vertical lines illustrate standard errors of the mean.

in infancy [5]. Another difference between the results of Experiments 1a and 2 was the way in which context preexposure influenced acute response to ethanol. The influence of preexposure to the testing context on the stimulating effect of ethanol was only evident in 21-day-old rats (Experiment 2). This result shows the importance of novelty for the stimulating effect of ethanol, a result that is consistent with prior evidence found by our research group [4]. Although in Experiment 1a no habituation to the context was observed, and experience with the testing context during training did not affect locomotor response to ethanol, it was clear that infants did acquire and retain contextual information, since they expressed context-dependent tolerance.

In Experiment 1a, training with ethanol in the testing context completely abolished the stimulating effect of ethanol (i.e. tolerance), in a sex-independent manner. This effect was previously found in older preweanling [9] or adult [34] rats. To our knowledge, this is the first time evidence has been found of tolerance to ethanol during the second postnatal week of life. This effect was observed only in the first testing interval (5–10 min) in which subjects given ethanol for the first time showed more locomotor activity than those trained with ethanol in the testing context before testing. Interestingly, since this effect was not observed in subjects that were trained with ethanol in a context different from the testing one, it can be concluded that tolerance was conditioned. In our protocol, subjects were tested after two days of withdrawal, and in Experiment 1a no evidence of tolerance was observed during training, with ethanol stimulating locomotion in each session. This result suggests that these withdrawal days are necessary for the expression of tolerance, or alternatively that this effect requires at least five days of training with ethanol during this ontogenetic period.

Older infant female rats (Experiment 2) also showed tolerance to this ethanol effect. In this case it is likely that tolerance was developed faster, and may already start occurring during training, because ethanol lost its capacity to stimulate behavior after the first training session (i.e. rapid tolerance), although additional control groups would be required to support such conclusion. In the same experiment context preexposure strongly reduced the acute locomotor response to ethanol during testing, with this effect almost impeding our assessment of tolerance in animals trained in the testing context. Interestingly, at this age, tolerance was observed in females trained outside the testing context. This may indicate that mere experience with ethanol is sufficient to promote such an effect, although we cannot rule out the possibility that other cues may have acquired control upon the ethanol effects during training, such as, for example, those related to the injection.

Sensitization to ethanol-induced behavioral activation was exclusively and consistently observed in male rats trained during the second postnatal week (Experiment 1a and 1b), and only when subjects had no experience with the testing context before testing, a result indicative of the importance of environmental novelty in this effect. Ethanol-induced sensitization has been more frequently reported in mice [58]. In contrast, in adult rats this effect is not easy to find, and sometimes requires the selection of specific ethanol doses during training and testing (below 1 g/kg) or the selection of subpopulations of rats which are highly responsive to novelty [33]. In some cases, genetic studies have related this ethanol effect to high ethanol consumption [31], a correlation that seems also to be supported from an ontogenetic perspective, since infants, particularly during the second postnatal week, tend to consume more ethanol than later on in infancy, adolescence or adulthood [68,78]. Moreover, during adulthood, genetically heterogeneous rat strains are more predisposed to sedation than to stimulation after ethanol treatment [46], and chronic exposure to ethanol tends to produce tolerance to the stimulating effect rather than sensitization [34]. It has been shown that infants can develop and express locomotor sensitization in response to other drugs, such as amphetamine or cocaine [40,47,48,77]. This indicates that mechanisms supporting this learning phenomenon are matured early in development. However, we acknowledge that ontogenetic conclusions about sensitivity to ethanol need to be taken with caution, especially if the procedures used across age groups differ. For example, in most studies with infants, ethanol is delivered intragastrically and subjects are tested without prior habituation to the context, while in studies with adult rats, ethanol is given intraperitoneally and the context in which locomotor activity is assessed is not novel. This latter procedural issue is particularly important, because as discussed above, the stimulating effect of ethanol during infancy depends on novelty [4] and more precisely, it is critically modulated by stress [10]. Novelty seems to be a necessary factor for the stimulating effect of ethanol during infancy, but it is not sufficient alone, and it is the combination of novelty and stress that is critical to the expression of this ethanol effect during infancy. This conclusion is supported by results showing that subjects without maternal separation did not display stimulation after an ethanol challenge and testing in a novel environment [10]. Hence, maternal separation is required for this ethanol effect. Considering the importance of stress in the acute stimulating effect of ethanol, during infancy this effect can, to a certain extent, be considered a cross-sensitization effect between stress and ethanol.

As mentioned, we observed stronger stimulation in the younger (Experiment 1a) than in the older (Experiment 2) rats, a difference consistent with previous observations in our laboratory. Moreover, sensitivity to locomotor sensitization seems to parallel this ontogenetic profile, since younger rats, but not older ones, showed this effect. These differences may be causally linked to variations which occur rapidly during the preweanling period in a variety of neurochemical systems involved in ethanol's activating effects during this ontogenetic period [3,6,8] and ethanol-induced sensitization in adult mice [15,56]. For example, opioid, GABA B and dopamine receptors undergo important changes in number, relative density and even function during the second and

third postnatal weeks of life [30,73,76,79]. Interestingly, there are also crucial differences in the central and peripheral metabolism of ethanol and its metabolites during the first two postnatal weeks. Brain catalase activity in rats progressively falls from gestation to adulthood, and during the first two postnatal weeks the activity of this enzyme decreases by about 50% [26]. Moreover, the peripheral ethanol metabolism becomes faster as infant rats grow older [32,38]. This is important because the stimulating effect of ethanol [57] as well as the locomotor sensitization [20] induced by this drug have been linked to the activity of the brain catalase system, while the accumulation of peripheral metabolites, such as acetate, positively influences the sedative effect of this drug [19]. In fact, sensitization in adult rats has also been observed when ethanol is administered centrally (intra cerebrum ventricular) [19,51], suggesting that the peripheral metabolism of ethanol may obstruct the development of behavioral sensitization. Overall, these neurochemical and metabolic ontogenetic differences may interact and help to explain why rats are more predisposed to show locomotor stimulation induced by ethanol, and ethanol-induced locomotor sensitization, during the second postnatal week of life than in later stages of ontogeny.

Debate still continues regarding whether or not infant rats can show long-term retention of context learning. Evidence against long-term context memory during infancy comes from studies showing poor retention of contextual fear conditioning [67,69], or lack of context modulation of interference learning [84,85]. However, a considerable number of studies have reported positive evidence of long-term contextual learning and context effects during infancy [11,12,29,49,50,60,62,63]. An analysis of the procedures used in this second set of studies reveals that positive results (i.e. long-term contextual memory retention) were usually obtained when contexts were enriched by explicit odors, their salience probably being increased by adjusting the sensory content of the context to the perceptual capacities of the preweanling rat [12,63]. Due to these antecedents, the context that we used in our experiments was enriched with an explicit odor, and perhaps as a result of this, various evidence of context learning was found: in Experiment 1, tolerance to the stimulating effect of ethanol was context-dependent, and in Experiment 2, preexposure to the context attenuated the locomotor stimulating effect of ethanol in females. Additionally, in this experiment, long-term retention of habituation to the context was also observed, since control subjects trained in the testing context scored significantly lower in locomotor activity than those trained outside this context. These results demonstrate that even as early as during the second postnatal week of life, infant rats can encode and retain contextual information.

Surprisingly, one of the critical factors modulating our results was sex. Firstly, sex-differences were consistent in the expression of sensitization, because only males displayed this effect, and secondly, males and females also differed in their sensitivity to the acute and chronic ethanol effect in Experiment 2. In this experiment tolerance was only observed in females. The influence of sex in our results was unexpected, because little evidence of sex differences has been reported during this ontogenetic period, and less still in relation to sensitivity to ethanol [41,80]. Since there are few antecedents, it may be too soon to postulate hypotheses to explain the differences observed between males and females in our study. The most remarkable sex difference was the absence of sensitization in females. This result indicates either that females are resistant to this ethanol effect, or that our parameters were not sensitive enough to detect this in females. Since the stress response seems to be critically involved in behavioral sensitization induced by ethanol [45,65], one possible explanation of the differences in sensitivity to ethanol-induced sensitization observed between males and females may be related to sex differences in the stress-response, although few studies have found sex differences in response to stressors during infancy [13,14,17].

Our results suggest that the ontogenetic model summarizes findings that are analogous to observations derived from genetic analyses of sensitivity to ethanol. Furthermore, the present exploratory study allowed us to describe some circumstances and factors that critically modulate the chronic effect of ethanol during infancy. Our results raise many questions about the biological mechanisms underlying the influence of context, age and sex on sensitivity to developing tolerance or sensitization, as well as about the persistence of these effects, their ontogenetic specificity and the possible relationship between these ethanol effects and ethanol consumption or reinforcement. These questions will be addressed in future studies.

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