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New evidence of ethanol's anxiolytic properties in the infant rat

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Abstract

Ethanol induces appetitive, aversive, and anxiolytic effects that are involved in the development of ethanol use and dependence. Because early ethanol exposure produces later increased responsiveness to ethanol, considerable effort has been devoted to analysis of ethanol's appetitive and aversive properties during early ontogeny. Yet, there is a relative scarcity of research related to the anxiolytic effects of ethanol during early infancy, perhaps explained by a lack of age-appropriate tests. The main aim of this study was to validate a model for the assessment of ethanol's anxiolytic effects in the infant rat (postnatal days 13– 16). The potentially anxiolytic effects of ethanol tested included: i) amelioration of conditioned place aversion, ii) ethanol intake in the presence of an aversive conditioned stimulus, iii) the inhibitory behavioral effect in an anxiogenic environment, and iv) innate aversion to a brightly illuminated area in a modified light/dark paradigm. Ethanol doses employed across experiments were 0.0, 0.5, and 2.0 g/kg. Results indicated that a low ethanol dose (0.5 g/kg) was effective in attenuating expression of a conditioned aversion. Ethanol intake, however, was unaffected by simultaneous exposure to an aversive stimulus. An anxiogenic environment diminished ethanol-induced locomotor stimulation. Finally, animals given 0.5 g/kg ethanol and evaluated in a light/dark box showed increased time spent in the illuminated area and increased latency to escape from the brightly lit compartment than rats treated with a higher dose of ethanol or vehicle. These new results suggest that ethanol doses as low as 0.5 g/kg are effective in ameliorating an aversive and/or anxiogenic state in preweanling rats. These behavioral preparations can be used to assess ethanol's anxiolytic properties during early development.

Keywords

anxiolysis; ethanol motivational properties; conditioned aversion; light/dark test; infant rat

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Introduction

A number of studies have shown that infant rats exhibit a particular sensitivity to ethanol's motivational effects (reviewed in Abate, Pueta, Spear, & Molina, 2008; Chotro, Arias, & Laviola, 2007; Pautassi, Nizhnikov, & Spear, 2009; Spear & Molina, 2005). Aside from their importance in helping our understanding of the ontogeny of response to ethanol, preweanling rats have proven valuable for assessing ethanol-related effects. These infants acquire ethanol-induced first- and second-order conditioning, readily express ethanol-mediated taste conditioning, are sensitive to ethanol-induced locomotor activation and ethanol-mediated operant responding, and consume relatively high amounts of the drug without initiation procedures (Pautassi et al., 2009). In addition, infant rats are sensitive to the biphasic motivational properties of ethanol. Conditioned preferences or aversions are established as a function of ethanol dose and post-administration time (Molina, Pautassi, Truxell, & Spear, 2007). Appetitive reinforcing effects are present after the administration of a low and even a relatively high dose of ethanol (0.5 or 2.0 g/kg, respectively) during the ascending limb of blood ethanol concentration (BEC). When BECs reach approximately 210 mg% (with 2 g/kg ethanol intoxication; post-administration time: 37.5 min), aversive rather than appetitive motivational properties of ethanol are prevalent.

Motivational properties of ethanol are not restricted to its appetitive or aversive effects. The drug also produces anti-anxiety effects similar to those found in anxiolytic drugs. For instance, Wilson, Burghardt, Ford, Wilkinson, & Primeaux (2004) compared the anxiolytic effects of the benzodiazepine agonist diazepam and ethanol in rats. This study found that both ethanol and diazepam (a GABA-A agonist) caused dose-dependent increases in time spent in the open arms of an elevated plus maze and also reduced burying behavior in the prod-burying task (Wilson et al., 2004). These potentially negative reinforcing effects of ethanol seem to play an important role in modulating patterns of ethanol use and abuse (Koob et al., 2004). Effects of ethanol on anxiety have been repeatedly reported in clinical literature (Kushner, Abrams, & Borchardt, 2000) as well as in animal literature (Wilson et al., 2004). Nevertheless, this claim rarely has received careful experimental attention in developing animals. To our knowledge, few experimental preparations have been designed to specifically test negative reinforcement from ethanol in infant rats. The adult literature dealing with this phenomenon is based on techniques not suitable for tests early in life.

A few studies nevertheless provide information that can usefully be applied to tests of negative reinforcement in preweanling rats. For instance, Molina, Serwatka, Enters, Spear, & Spear (1987) found, in infant rats, that although ethanol impaired acquisition or expression of conditioned aversion to a visual cue paired to footshock, ethanol had no effect on conditioned aversion to an olfactory stimulus similarly paired with footshock. In both experiments, rats exposed to ethanol were given similar CS-US pairings (CS: conditioned stimulus; US: unconditional stimulus) to induce conditioning. The authors suggest that alcohol may impair some aspects of learning but spare others, depending perhaps on the particular sensory modality to be conditioned (i.e., visual vs. olfactory). McKinzie, Lee, Bronfen, Spear, & Spear (1994) indicated that ethanol is also capable of impairing retrieval processes of aversive memories. It is notable that in these studies, animals were conditioned and/or tested 30 min after administration of ethanol, when peak ethanol blood levels are

achieved and when it often is easier to find aversive rather than appetitive ethanol hedonic effects.

More recently, through a devaluation procedure, Pautassi, Sanders, Miller, Spear, & Molina (2006) tested ethanol's anxiolytic effects in preweanlings by measuring ethanol's devaluation of an aversive memory. In this study, after conditioning of an aversion to an odor (CS; lemon odor), infant rats were exposed to the US (citric acid), paired with administration of ethanol. Pairing the US with moderate to low ethanol doses ameliorated the otherwise strong aversion to the CS. This effort seemed to depend on the temporal course of the intoxication. A reduction in the magnitude of the aversive response was observed only if the US and the post-absorptive effects of ethanol were paired 5 min (but not 25 min) after ethanol administration (Pautassi et al., 2006). Similarly, Pautassi, Nizhnikov, Molina, Boehm, & Spear (2007) studied ethanol's effects on distress calls induced in the preweanling rat by intraoral infusion of citric acid. Upon comparison with the effects of midazolam (MDZ; a fast-acting GABA-A agonist known to have anxiolytic effects), they found similar calming effects of 0.5 g/kg ethanol and 0.09 mg/kg MDZ. Surprisingly, ethanol but not MDZ was capable of attenuating a conditioned aversion through the devaluation procedure, indicating that these effects of ethanol may not be GABA-mediated and instead, appetitive effects of ethanol could underlie this devaluation effect (Pautassi et al., 2007). These results imply value in seeking a more effective model for assessing anxiolytic effects in early ontogeny. The present experiments tested four apparently anxiolytic effects of low and moderate ethanol doses (0.5 and 2.0 g/kg). The first experiment tested ethanol's amelioration of a conditioned avoidance. The second experiment assessed ethanol intake in the presence of an aversive conditioned stimulus. The third experiment tested effects of ethanol on exploration of a novel anxiogenic environment, to determine the value of ethanol-induced locomotor stimulation as an index of ethanol anti-anxiety effects. The fourth experiment examined behavior in a modified light/dark box, using a test adapted from those used previously for adult rats.

General method

Subjects

Sprague–Dawley infant rats (postnatal days [PDs] 13–16) were used across all experiments (99 in Experiment 1; 112 in Experiment 2; 88 in Experiment 3; and 63 in Experiment 4). These animals were born and reared in the vivarium at the Center for Development and Behavioral Neuroscience (AAALAC-accredited facility, in Department of Psychology, Binghamton University, Binghamton, NY, USA). Births were examined daily, and day of birth was considered Postnatal Day 0 (PD 0). The colony was maintained at 22–24 °C under a 14 h/10 h light/dark cycle. The experiments were approved by the Binghamton University Institutional Review Committee for the Use of Animal Subjects and complied with the NIH Guide for the Care and Use of Laboratory Animals (National Institutes of Health, 1996).

Drug preparation and administration procedures

For Experiments 1 and 2, the kappa receptor agonist U62,066E (Sigma-Aldrich; St. Louis, MO) was used as an aversive US. The drug was dissolved in saline (NaCl, 0.9% v/v) and

administered at a dose of 0.0, 0.5, or 1.0 mg/kg. The injection volume was kept at 0.01 mL/g and saline was used as vehicle. U62,066E (hereafter referred as: U62) was administered via intraperitoneal (i.p.) injection. Injections took less than 5 sec and were performed in the region situated between the diaphragm and the genitalia.

Across experiments, different concentrations of ethanol solutions were employed (190 proof ethanol, Pharmaco; Brookfield, CT; vehicle: distilled water) to establish doses of 0.0, 0.5, or 2.0 g/kg. The volume administered was equivalent to 0.015 mL/g of body weight of 0.0, 4.2, or 16.8% ethanol solution, respectively. Pups assigned to the control condition (0 g/kg) received only vehicle (water). Ethanol solutions were given by intragastric (i.g.) administration using a 10-cm length of polyethylene tubing (PE-10) attached to a 1 mL syringe with a 27 G × 1/2 inch needle. This tubing was gently introduced through the mouth and slowly pushed into the stomach. The entire procedure took less than 20 sec per pup. For Experiment 2, a 0.0 or 5.0% v/v ethanol solution was used for intake assessment.

Conditioned Place Aversion (CPA) Test (Experiment 1)

Phase 1 (Conditioning)—Pups were separated from their dams on PD 14, placed in pairs in a holding cage for 2 h, voided and weighed. The mean weight of all subjects was calculated and used as a benchmark for the volume of the i.p. injection of U62 (0.0, 0.5, or 1.0 mg/kg). Kappa activation is a stress-inducing event (Bruchas, Land, & Chavkin, 2010) and kappa agonists were found to be aversive (Anderson, Morales, Spear, & Varlinskaya, 2013; Pautassi, Nizhnikov, Acevedo, & Spear, 2012). For this reason, we decided to employ U62 as an unconditional aversive stimulus (US). For conditioning (pairing of rough texture with consequences of U62), 5 min after i.p. injection, animals were placed into a Plexiglas® container (9 × 15 cm) in which the bottom was lined with a piece of rough sandpaper (coarse: 50, Gatorgrit; Fairborn, OH) (conditioned stimulus: CS), where they remained for 15 min. Conditioning was conducted in a dim illumination environment (10 lux). Immediately after conditioning, pups were returned to the holding chambers. Two hours post-injection, pups were returned to their dams. On PD 15, pups received the same conditioning treatment as on PD 14.

Phase 2 (Testing)—A 2-way tactile preference test was performed at PD 16. After a 2 h maternal separation, pups were intubated with 0.0, 0.5, or 2.0 g/kg of ethanol. This manipulation was intended to test ethanol-induced anxiolytic effects and amelioration of a conditioned aversion. Five minutes after intubation, pups were placed in a Plexiglas® box floored with sandpaper (the CS used during conditioning) on one side and a novel texture (a smooth cardboard-like floor; the reverse side of the sandpaper sheet) on the other. The test (duration: 5 min) was conducted in a dim illumination environment (10 lux) and the testing chambers were situated in a sound-attenuating compartment (53 × 58 × 43 cm; VersaMax Animal Activity Monitoring System). Position of the animals in the testing chambers was detected by consecutive photocell beam interruptions and translated by the VersaMax software into time spent (in seconds) and distance travelled (in cm) in each side of the chamber. Using many doses of the aversive US and many ethanol doses would allow us to compare the effectiveness of ethanol-induced anxiolysis as a function of intensity of the

conditioned aversion. We expected that higher doses of the US would be accompanied by less ethanol-induced suppression of its avoidance.

Tests of Conditioned Place Aversion and Intake (Experiment 2)

The second experiment tested whether exposure to a conditioned aversive stimulus would produce changes in ethanol intake patterns during infancy. On PD 13, infants were separated from their dams, cannulated, and placed in pairs in a holding cage (45 × 20 × 20 cm). Intraoral cannulation was performed using a procedure extensively described in previous studies (Chotro & Arias, 2003; Miranda-Morales, Molina, Spear, & Abate, 2012; Miranda-Morales, Spear, Nizhnikov, Molina, & Abate, 2012; Spear, Specht, Kirstein, & Kuhn, 1989). The entire procedure took less than 5 sec per pup. These cannulas were later used for intraoral infusion of solutions during the intake test. After the 2-h separation, pups were assigned to the treatment defined by the solution infused: 0.0 or 5.0% ethanol. Before the intake test, pups' bladders were voided by gentle brushing of the anogenital area. Body weights were then registered. Each subject's cannula was connected to a length of PE-50 tubing that in turn was connected to a 5 mL syringe operated by an automatic infusion pump (KD Scientific, Holliston, MA). The subjects were placed into a Plexiglas® container divided into eight sections measuring 7 × 15 cm each. The bottoms of these containers were lined with absorbent paper and slightly heated (26–27 °C). The total volume administered to each subject was equivalent to 5.5% of their body weight and was infused at a constant rate for 15 min. These infusion parameters allowed pups to either accept or reject the infused solution. Immediately following the intraoral infusion, pups were disconnected from the tubing and weighed to estimate consumption scores. Ninety minutes later, pups were returned to their dams.

On PDs 14 and 15, the Phase 1 of CPA was performed as mentioned in *Experiment 1*. Pups were assigned to the treatments defined by: solution consumed at PD 13 (water or 5% ethanol) and intensity of the aversive US (0.0, 0.5, or 1.0 mg/kg of U62). The CS employed for CPA was similar to the one used for Experiment 1: sandpaper (coarse: 50, Gatorgrit; Fairborn, OH).

On PD 16, pups were evaluated in an intake test similar to the one performed on PD 13. Pups were assigned to the treatments defined by: solution consumed at PD 13 (water or 5% ethanol), intensity of the aversive US at PD 14–15 (0.0, 0.5, or 1.0 mg/kg of U62), and solution to consume at PD 16 (water or 5% ethanol). Pups were weighed before and after intake to estimate consumption scores. The intake test at PD 16 was performed in the presence of the CS (sandpaper) that had been experienced previously, during the conditioned aversion. Water and ethanol intake were estimated through the percentage of body weight gained (% BWG) during the test: ([(post-test weight − pre-test weight)/pre-test weight] × 100).

Locomotor activity assessment in different lighting conditions (Experiment 3)

On PD 16, pups were separated from the dams and placed in pairs in holding chambers for 1 h. Locomotor activity was then evaluated for 5 min in a testing environment that consisted of an open-field chamber with Plexiglas® walls (42 × 42 × 30 cm; VersaMax Animal

Activity Monitoring System, Accuscan Instruments; Columbus, OH). The chambers were equipped with a light which could be turned on or off (intensity: 0 or 50 lux). Locomotion was detected by interruption of eight pairs of intersecting photocell beams evenly spaced along the walls of the testing environment. This equipment was situated in sound-attenuating chambers (53 × 58 × 43 cm) equipped with a fan for ventilation and background noise. Consecutive photocell beam interruptions were translated to distance traveled in cm by the VersaMax software. This dependent variable takes into account the path of the animal and is an accurate indicator of ambulatory activity. Immediately after the locomotor activity, test pups were returned to their home cage. Prior to this evaluation of locomotor behavior, animals were intubated with ethanol at 0.0, 0.5, or 2.0 g/kg; a fourth group was not intubated. Five minutes after ethanol administration, animals were tested in the chambers with the light turned on or off.

Light/dark test

Animals were evaluated at PD 16 in a modified light/dark box. The evaluation chamber consisted of a Plexiglas® box (30 × 24 × 24 cm) divided into two compartments connected through a small hole (4 cm) located at the middle of the separating wall. The dark compartment (no light: 0 lux) had black walls; the illuminated compartment had light from the top (200 lux). In this test, animals were placed into the lit compartment and had the opportunity to move freely through both compartments. At PD 16, pups were separated from the dam and placed into holding chambers for 1 h. Prior to evaluation, animals were intubated with ethanol (0.0, 0.5, 2.0 g/kg) or remained untreated. Five minutes after ethanol administration, pups were placed in a corner of the brightly illuminated compartment. The location where animals were placed remained constant across the experiment. Animal behavior in the brightly illuminated compartment was video recorded for 5 min, and then pups were returned to their dams. Taking into account the revision of this test by Bourin & Hascoët (2003), the variables analyzed were time spent in the illuminated compartment, latency to enter into the dark compartment, number of side changes, and frequency of leaning out (or peeking out) from the dark compartment. A decrease in rate of leaning out of the dark compartment appears to be a consistent effect of standard anxiety-inducing drugs (Lapin, 1999). Entry into a compartment was defined as the placement of all four paws in the compartment (Bourin & Hascoët, 2003).

Experimental design and data analysis

Across experiments, no more than one subject from each sex was assigned to the same treatment condition in a given litter. Data were analyzed with the analysis of variance (ANOVA). The loci of significant main effects or two-way interactions were analyzed with Fisher's LSD *post hoc* comparisons. A rejection criterion of $p < 0.05$ was adopted for all statistical analysis in the present study. Planned comparisons ($p < 0.05$) were used whenever *a priori* hypotheses were postulated. The final group size for each experiment was as follows: Experiment 1: 10–12 animals by group; Experiment 2: 8–10 animals by group; Experiment 3: 11 animals by group; Experiment 4: 14–16 animals by group. Preliminary statistical considerations indicated that across variables and experiments, gender did not enter into any significant main effects or interactions with the remaining factors. Therefore, data were collapsed across sex for all of the subsequent analyses. The lack of sex effects was

not unexpected: previous studies working with ethanol-related effects in rats of the present age (Arias & Chotro, 2005; Chotro & Arias, 2003; Miranda-Morales, Molina, et al., 2012; Miranda-Morales, Spear, et al., 2012) usually have found no significant effects of gender or interaction with other factors.

Results

Experiment 1. Results are depicted in Fig. 1. A 3 × 3 ANOVA (U62 dose: 0.0, 0.5, or 1.0 mg/kg; ethanol dose: 0.0, 0.5, or 2.0 g/kg) was employed to process time spent on the CS paired previously with the aversive US (U62). Time spent on the CS was significantly affected by the dose of U62 ($F_{(2,90)} = 7.6$, $p < 0.001$). Animals that received pairings of U62, at 0.5 and 1.0 mg/kg doses, with sandpaper exhibited conditioned place aversion. In addition, the different doses of U62 employed, considered as different intensities of an aversive US, tended to exert a dose aversive-response profile ($p = 0.06$). Subsequent planned comparisons revealed that animals injected with 1.0 mg/kg of U62 continued to show significant conditioned place aversion even under the effects of ethanol. Pairing of U62 0.5 mg/kg with sandpaper, generated conditioned aversion to the CS but only in animals intubated with water (vehicle) pre-test. Administration of 0.5 g/kg ethanol produced an opposite profile in pups pretreated with U62 0.5 mg/kg, increasing significantly the time spent on the aversive CS when compared with its own internal control (comparison with U62 0.5 mg/kg – water: $p < 0.01$), and bringing responding up to the level of all U62 0.0 mg/kg controls. Pups given 2.0 g/kg ethanol tended to show a profile similar to that of pups given 0.5 g/kg ethanol (comparison with U62 0.5 mg/kg – water: $p = 0.07$). Analysis of the U62 0.0 mg/kg group indicated that ethanol administration by itself did not modify basal preference for the CS.

When analyzing locomotor activity (LMA) of infants, a two-way ANOVA indicated a main effect of ethanol dose ($F_{(2,90)} = 9.13$, $p < 0.001$). The highest dose of ethanol stimulated locomotor activity, denoted as a significantly higher LMA of those animals when compared with rats given 0.5 or 0.0 g/kg ethanol. There was no significant effect of ethanol-induced LMA for animals treated with 0.5 g/kg ethanol.

In summary, infants acquired a conditioned aversion to a surface paired with activation of kappa opioid receptors by U62. Both doses of U62 were effective in exerting aversion to the sandpaper. In addition, ethanol at 0.5 g/kg was capable of ameliorating the expression of the conditioned aversion, but only when the CPA was generated with a low dose of U62. The anxiolytic effect of 0.5 g/kg ethanol dose was not related to its stimulating properties: there was no effect of ethanol 0.5 g/kg on locomotor activity of these animals. Moreover, the activating effect of the high ethanol dose (2.0 g/kg) did not preclude expression of aversion to 1.0 mg/kg U62 dose.

Experiment 2. Intake profiles at PD 13 were analyzed through a one-way ANOVA. As can be observed in Table 1, PD 13 animals consumed significantly more ethanol than water ($F_{(1,110)} = 31.69$, $p < 0.001$). At PD 16, after CPA conditioning, intake scores were analyzed through a two-way ANOVA (solution consumed at PD 13 × intensity of the aversive US). The analysis revealed a significant main effect of solution consumed at PD 13 ($F_{(1,50)} =$

4.64, $p < 0.05$). A significant reduction in ethanol consumption was observed in those animals that experienced ethanol at PD 13. Conditioned aversion to sandpaper had no significant effect on ethanol intake when the animals consumed the drug while exposed to the aversive CS. None of the factors under analysis significantly affected water consumption at PD 16.

In summary, results from Experiment 2 indicate that infant rats showed avoidance of a texture stimulus previously associated with an aversive state (see Results from *Experiment 1*); exposure to that texture (i.e., sandpaper) while the animals had the opportunity to consume ethanol did not modify ethanol or water intake profiles.

Experiment 3. The LMA profile of animals is detailed in Table 2. The dependent variable was analyzed through a two-way ANOVA (lighting condition: 0 or 50 lux × ethanol dose: 0.0, 0.5, 2.0 g/kg or untreated). The analysis revealed significant effects of both factors. Lighting of the chamber significantly attenuated LMA of infants ($F_{(1,80)} = 8.11$, $p < 0.006$). Ethanol administration affected motor activity in the opposite direction: the dose of 2.0 g/kg significantly increased LMA of the animals when compared with any of the other ethanol-treated groups ($F_{(3,80)} = 8.97$, $p < 0.001$). The interaction of the factors did not attain significance. These results showed no synergism between the effects of lighting and ethanol on LMA. Instead, these treatments had opposite effects.

Experiment 4. As can be observed in Figure 2, the ANOVA employed to analyze time spent in the brightly illuminated area (Panel A) revealed a significant effect of ethanol treatment ($F_{(3,59)} = 3.09$, $p < 0.05$). *Post hoc* analyses indicated that animals under the effects of 0.5 g/kg ethanol spent more time in the illuminated area than all the other treated groups. (Comparison against the 0.0 g/kg ethanol group revealed a borderline significance: $p = 0.09$). Latency to enter to the dim area (Panel B) was also significantly affected by ethanol treatment ($F_{(3,59)} = 3.54$, $p < 0.02$); animals from the 0.5 g/kg ethanol group exhibited greater latency to enter into the dim compartment than the remaining groups. (Comparison with the 0.0 g/kg ethanol group again showed a trend, $p = 0.1$). Frequency of side changes and leaning-out behavior were not significantly affected by ethanol treatment (see Panels C and D).

All together, these results indicate a significant anxiolytic effect of 0.5 g/kg ethanol in the light/dark paradigm. This dose was effective in increasing the elapsed time in an anxiety-producing area and yielded higher latency to enter the dim compartment.

Discussion

The present experimental preparations revealed ethanol's anxiolytic effects in some measures (Experiments 1 and 4) but not others (Experiments 2 and 3) during early ontogeny. *Experiment 1* indicated attenuation of a conditioned aversive response (sandpaper avoidance) in those animals tested under the effects of a low ethanol dose (0.5 g/kg). *Experiment 2* indicated no evident changes in ethanol consumption when animals consumed the drug while exposed to an aversive CS. In *Experiment 3*, no synergism could be observed between ethanol-induced LMA and the effects of environment illumination on locomotor

activity. *Experiment 4* indicated that 0.5 g/kg ethanol was effective in increasing the elapsed time in the illuminated area and yielded higher latency to enter the dim area.

As mentioned earlier, a previous study found a reduction in a conditioned aversion response when infant rats received ethanol during the retrieval process (pre-testing) (McKinzie, Lee, Bronfen, Spear, & Spear, 1994). A main difference between that study and the present one was the post-ethanol time of testing. Here, animals were tested 5 min after ethanol intubation, during the rising phase of intoxication in which positive motivational effects of the drug are observed (Arias, Mlewski, Molina, & Spear, 2009; Molina et al., 2007; Molina, Ponce, Truxell, & Spear, 2006; Pautassi et al., 2009). In contrast, McKinzie et al. (1994) evaluated pups 30 min after ethanol administration. At that time, ethanol achieves peak blood levels (Arias et al., 2009; Molina et al., 2006) which are usually accompanied by aversive ethanol post-absorptive effects (Molina et al., 2007). In the present study, amelioration of conditioned aversion was evident with 0.5 g/kg ethanol when the aversion had been induced with 0.5 mg/kg U62. Conditioned aversion induced by 1.0 mg/kg U62 could not be ameliorated with any dose of ethanol. This main result may be contrasted usefully with those obtained by Molina et al. (1987) and by McKinzie et al. (1994), who point out possible impairment of learning due to ethanol administration. In our case, that explanation seems unlikely to be useful, taking into account that if ethanol's apparent anxiolytic effects were due instead to impairment in learning or in recognition of stimuli, they should be evident with the 2.0 g/kg dose, but this was not observed. In rat pups, Lopez, Spear, & Molina (1996) also found that a 2.0 g/kg ethanol dose failed to alter aversive olfactory conditioning. In addition, when the intensity of the conditioned aversion was great, no anxiolytic effect of 0.5 g/kg ethanol could be observed. Moreover, this ethanol-related effect was not influenced by the locomotor stimulating effect of ethanol during the rising phase of intoxication, which was evident only with 2.0 g/kg ethanol (Arias et al., 2009).

A possible explanation for the results obtained in *Experiment 1* is that appetitive motivational effects of 0.5 g/kg ethanol may have counteracted the aversive value of the CS. This possibility seems to be likely as preweanling rats do express appetitive effects of ethanol to stimuli paired with post-absorptive effects of this ethanol dose (in neonatal rats: Nizhnikov, Molina, Varlinskaya, & Spear, 2006; in infant rats: Molina et al., 2007; Pautassi, Nizhnikov, Acevedo, & Spear, 2012). A second hypothesis points to the analgesic effects of ethanol. Engel & Hård (1987) observed that ultrasonic vocalizations (USVs) induced by maternal separation were dose-dependently reduced by the same low ethanol dose (0.5 g/kg), and this effect was reversed by using picrotoxin, a GABAergic antagonist. Pautassi et al. (2006) used a devaluation paradigm to indicate that ethanol's post-absorptive consequences were effective in reducing the aversive value of an innately aversive stimulus. Experiencing the aversive US under the effects of ethanol rendered the original US (citric acid) less aversive, which in turn decreased the capability of the CS to elicit escape or avoidance responses. These results may be interpreted as ethanol exerting anxiolytic effects early in infancy.

The aim of *Experiment 2* was to test whether exposure to a conditioned aversive stimulus would produce changes in ethanol intake patterns during infancy. In addition, it explores whether ethanol intake experience at PD 13, prior to conditioning, would help animals to

retain information about ethanol before the ethanol intake test at PD 16. As was observed in the results of *Experiment 1*, the CPA protocol was successful in generating conditioned aversion to sandpaper in infants. Nonetheless, *Experiment 2* indicated that exposure to an aversive cue did not modify ethanol intake patterns, even if animals had experienced ethanol before. Contrary to the present results, aversive conditioning with footshock as US has resulted in heightened ethanol intake (Serwatka, Molina, & Spear, 1986) in weanling rats. Furthermore, disruption of infant/dam interactions results in exacerbated ethanol consumption in the offspring, probably due to the anxiolytic effects of the drug (Fahlke et al., 2000; Huot, Thrivikraman, Meaney, & Plotsky, 2001). Nonetheless, Ponce, Pautassi, Spear, & Molina (2004) found that nociceptive stimulation (footshock) was not effective in modulating ethanol intake patterns in adolescent rats. Even though animals had the opportunity to retain information pertaining to ethanol before intake at PD 16, results indicated a clear reduction in ethanol consumption at PD 16 in animals consuming ethanol at PD 13. Molina & Chotro (1989) found similar results in younger infant rats. A single administration of 3.0 g/kg ethanol to 11-day-old rats promoted a later reduction of ethanol intake compared to control animals. Molina & Chotro (1989) proposed that ethanol cues eliminated through salivation or respiration were paired with the aversive post-ingestive consequences of the ethanol dose employed, resulting in an acquired taste aversion toward ethanol. As Hunt, Lant, & Carroll (2000) discussed, repeated early experiences with ethanol-related cues in a context of the home environment may be necessary to promote later increase in ethanol acceptance. As discussed previously, experience with ethanol, intensity, modality, and duration of stressful experiences are important factors when assessing the impact on later initiation of ethanol consumption.

Ethanol-induced locomotor activity (LMA) has been proposed as another measure of ethanol-induced reinforcement (Quoilin, Didone, Tirelli, & Quertemont, 2010). Recent studies indicate that preweanling rats are highly sensitive to ethanol-induced LMA, particularly when tested during the initial rising phase of the blood ethanol curve (Arias, Molina, Mlewski, Pautassi, & Spear, 2008). The present study tried to evaluate whether this variable, as a correlate of ethanol reinforcement, could also be used as an index of ethanol's anti-anxiety effects. *Experiment 3* tested whether ethanol-induced LMA could counteract effects of illumination in activity chambers. Exposure to sudden darkness has been reported to increase motor activity and to decrease anxiety of rodents observed either in the open-field or in the elevated plus-maze (Bert, Felicio, Fink, & Nasello, 2005; Nasello, Machado, Bastos, & Felicio, 1998). Our results indicated that both darkness and ethanol treatment facilitated locomotor stimulation. However, there was no synergism between the effects of these two modulating factors. It could be suggested that the anxiolytic effect produced by sudden exposure to dim light (Bert et al., 2005; Nasello et al., 1998) acted in concert with the (possibly) anxiolytic effect induced by acute ethanol administration, thereby inhibiting the well-known phenomenon of anxiety-induced hypolocomotion in the open-field apparatus (Broadhurst, 1960) and potentiating ethanol-induced LMA (effects that were significant for 2.0 g/kg animals; see Table 2).

A common procedure across the present experiments was to separate animals from the home cage before testing, a treatment well known to induce stress in infants (Levine, 2001, 2005;

Stanton, Gutierrez, & Levine, 1988; Suchecki, Mozaffarian, Gross, Rosenfeld, & Levine, 1993). In addition, unpublished data from our laboratory indicate that i.g. administration significantly decreases rectal temperature of infant rats. Even though in Experiments 2 and 3 ethanol did not appear to produce an anxiolytic effect, it is possible that maternal separation stress and any other stress associated with the procedures employed here (i.e., the intubation procedure) may have interacted in some way with the behavioral assays, altering ethanol's ability to produce clear anxiolytic effects.

The light/dark test is based on the common aversion of rodents to brightly illuminated areas and on the spontaneous exploratory behavior of rodents in response to mild stressors, in this case, novel environment and light (Crawley & Goodwin, 1980). Results from *Experiment 4* indicated that 0.5 g/kg ethanol exerted anxiolytic effects, reflected in an increased time spent in the brightly illuminated area and increased latency to enter into the dark area. Disinhibition of the suppression of behavior is shown by the time taken for the animal to move from the lit area (control pups placed in the lit area moved rapidly into the dim area). After ethanol treatment, anxiolysis with this measure was reduced below that of control pups. This result cannot be explained by an effect of ethanol on locomotor activity. As can be seen in results of *Experiments 1 and 3*, the 0.5 g/kg ethanol dose did not modify locomotor activity compared to that of control or untreated animals. There were no anxiolytic effects in terms of leaning-out behavior, even though this behavior had appeared to be a constant effect of anxiety-inducing drugs in at least one study (Lapin, 1999). Number of side changes or transitions between compartments was also unaffected by ethanol treatment. Costall, Jones, Kelly, Naylor, & Tomkins (1989) found that amphetamine enhanced transitions between compartments, but did not induce an increase in time spent in the dark compartment. Bourin & Hascoët (2003) noted that not all the variables show the same consistency across studies. As discussed before, the effect of this low ethanol dose could be driven by its appetitive motivational properties and consequent competition with an aversive state or by anxiolytic properties of ethanol, to ameliorate any anxiogenic state. To our knowledge, this is the first study to employ this test in infant rats as a measure of ethanol's anxiolytic effects.

In this study, different measures were employed to analyze ethanol's anxiolytic effects. The common denominator between experiments in which a positive anxiolytic ethanol result was observed was ethanol's effective dose. A dose as low as 0.5 g/kg of ethanol, but not a higher one, was effective in ameliorating a conditioned aversion (Experiment 1) and increasing the time elapsed in an anxiogenic environment (Experiment 4). The motivational value of this ethanol dose would help to explain this similar outcome across experiments. This dose has a positive hedonic value capable of inducing conditioned preferences and has been shown to decrease aversive responses (Molina et al., 2007; Pautassi, Melloni, Ponce, & Molina, 2005; Pautassi et al., 2007; Pautassi et al., 2006). In contrast, even though a 2.0 g/kg ethanol dose has been found to exert conditioned preferences in some cases (Molina et al., 2007), this dose failed to induce any anti-anxiety effect even when several test measures were employed. A third important point mentioned by Molina et al. (2007) is that while the motivational value of a low ethanol dose remains constant across post-administration times, appetitive hedonic effects seem to be also present after the high dose of 2.0 g/kg ethanol

during the ascending limb of intoxication, but when ethanol content reaches peak values, aversive rather than appetitive motivational properties of ethanol seem to prevail. Finally, the present study adds new tests to the literature to be used during early stages of developments to inquire about drug-induced anxiolysis.

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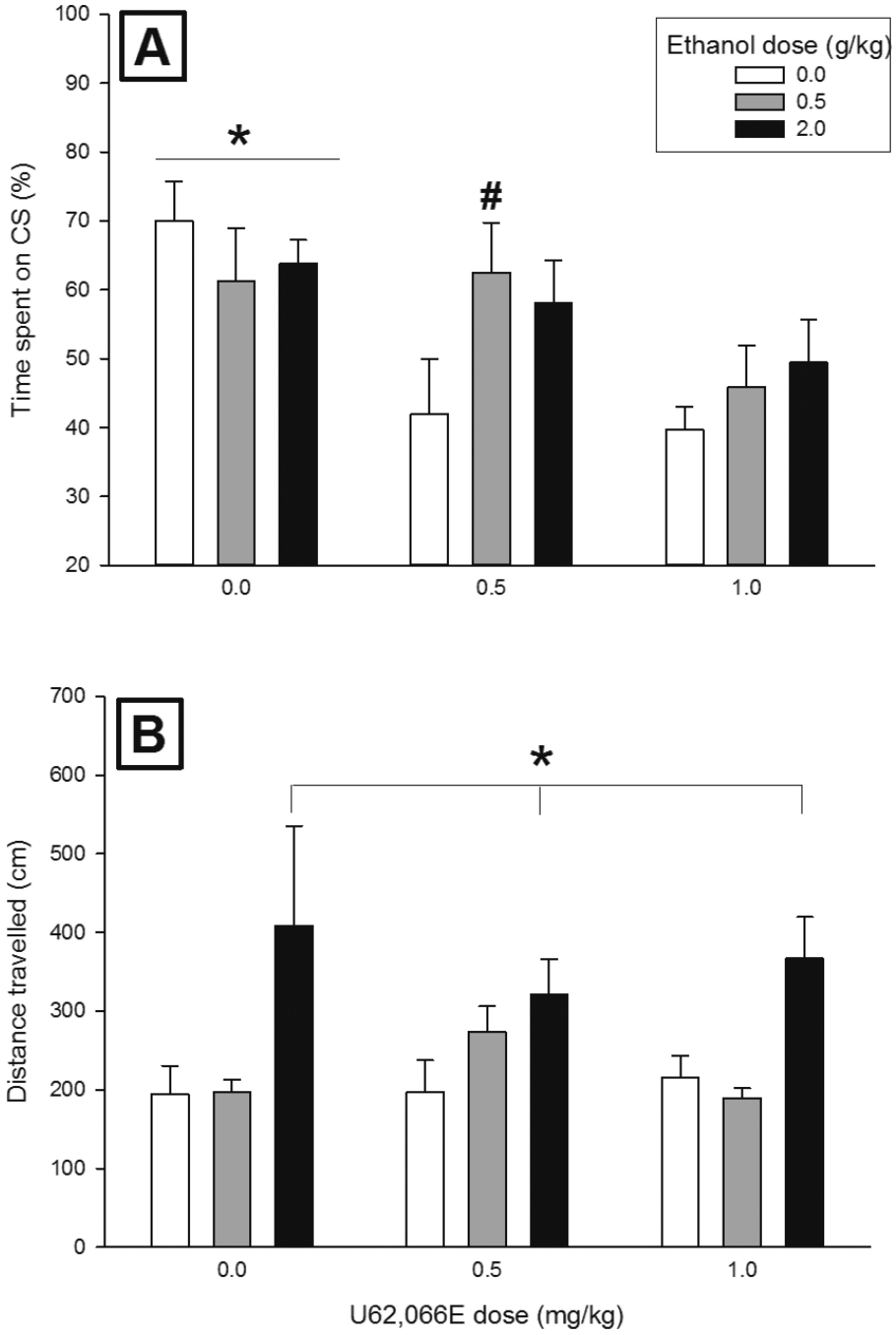

Figure 1.

U62-induced conditioned aversion in infant rats (doses employed: 0.0, 0.5, or 1.0 mg/kg). Panel A expresses the percent time spent in the CS compartment during the test as a function of ethanol treatment during testing (0.0, 0.5, or 2.0 g/kg). The asterisk (*) sign indicates significant differences between 0 mg/kg U62 and the remaining U62 doses. The pound (#) sign indicates significant differences between 0.5 g/kg ethanol and 0.0 g/kg ethanol, for 0.5 mg/kg U62 condition. Panel B depicts locomotor activity of PD16 rats during the two-texture preference test as a function of ethanol treatment (0.0, 0.5, or 2.0 g/kg) and US

employed during conditioned aversion (U62 at 0.0, 0.5, or 1.0 mg/kg). The asterisk (*) sign indicates significant differences between 2 g/kg ethanol and the corresponding vehicle-treated group. Values represent mean ± SEM.

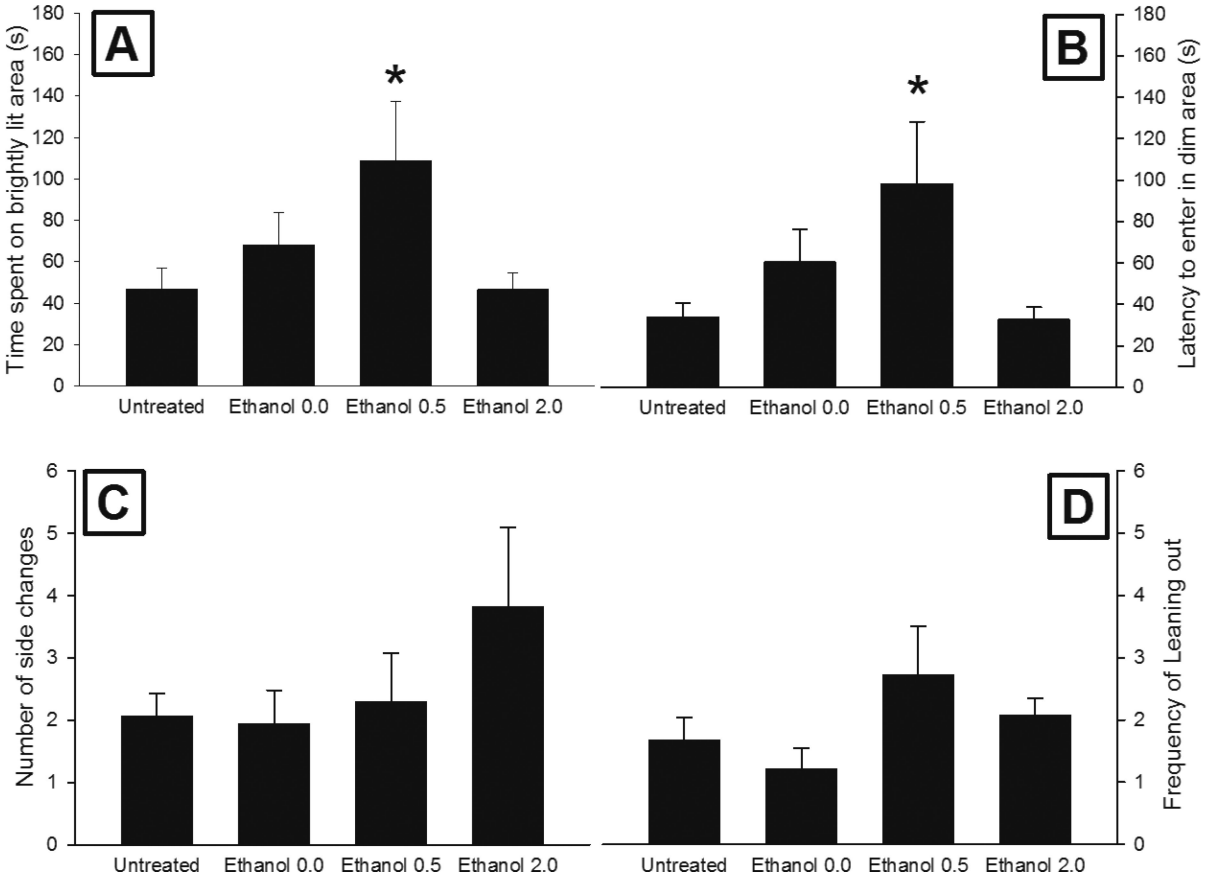

Figure 2.

Ethanol effects on infant rats tested in an adapted light/dark box (ethanol doses: 0.0, 0.5, 2.0 g/kg or untreated). Panel A depicts time spent (in seconds) in the illuminated compartment; panel B represents the latency to enter into the dim compartment (expressed in seconds); panel C depicts the number of compartment changes during the test, and panel D represents the frequency of leaning-out behavior during the test. The asterisk (*) sign indicates significant differences between 0.5 g/kg ethanol and the remaining conditions. Values represent mean ± SEM.

Table 1

Intake scores at PD13 of 5% ethanol or water (expressed in % BWG) and intake scores at PD16 of 5% ethanol or water, as a function of aversive US (U62,066E) employed in CPA at PD14-15. Values represent mean ± SEM.

PD13 Intake Test		PD14-15 CPA (U62,066E dose)	PD 16 Intake test	
			5% Ethanol	Water
5% Ethanol	1.90 ± 0.12	**0.0 mg/kg**	1.69 ± 0.16	1.41 ± 0.14
		0.5 mg/kg	1.05 ± 0.17	0.94 ± 0.09
		1.0 mg/kg	1.51 ± 0.30	1.49 ± 0.19
Water	1.13 ± 0.07	**0.0 mg/kg**	1.94 ± 0.15	1.58 ± 0.16
		0.5 mg/kg	1.83 ± 0.24	1.39 ± 0.15
		1.0 mg/kg	1.68 ± 0.27	1.22 ± 0.15

Table 2

Locomotor activity of infant rats (expressed as distance travelled, in cm) in different lighting conditions (0 or 50 lux) as a function of ethanol treatment (0.0, 0.5, or 2.0 g/kg). Values represent mean ± SEM.

Ethanol Treatment	Lighting Condition	
	0 lux	50 lux
Untreated	469.36 ± 65.02	360.81 ± 31.51
0.0 g/kg	584.72 ± 57.25	438.09 ± 33.59
0.5 g/kg	570.09 ± 66.49	403.91 ± 73.02
2.0 g/kg	954.73 ± 173.85	690.09 ± 91.68