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Effects of ethanol exposure in a familiar or isolated context during infancy on ethanol intake during adolescence

Roberto Sebastián Miranda-Morales[1,2]* | Beatriz Haymal[1] | Ricardo M. Pautassi[1,2]

[1] Instituto de Investigación Médica Mercedes y Martín Ferreyra (INIMEC-CONICET-Universidad Nacional de Córdoba), Córdoba, Argentina

[2] Facultad de Psicología, Universidad Nacional de Córdoba, Córdoba, Argentina

*Correspondence
Roberto Sebastián Miranda-Morales, Instituto de Investigación Médica M. y M. Ferreyra. Friuli 2434, Córdoba, 5016, Argentina.
Email: smiranda@immf.uncor.edu

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Abstract

Early exposure to ethanol affects ethanol intake later in life. This early experience encompasses exposure to social stimuli and the pharmacological and orosensory properties of ethanol. The specific contribution of each type of stimulus to subsequent ethanol intake remains unknown. We assessed the intake of various concentrations of ethanol in a familiar or isolated context during infancy and the lingering effects of this experience on ethanol intake during adolescence. On postnatal day 3 (PD3), PD7, and PD11, rats were given 5% ethanol or water in a nursing or isolated context (Experiments 1 and 2). Intake tests (ethanol vs. water) were conducted during adolescence. Experiment 2 matched the amount of fluid ingested during infancy in both contexts and subsequently tested ethanol consumption during adolescence. The results revealed a facilitative effect of the nursing context on fluid intake during the tests in infancy. Pups stimulated with ethanol but not water in the isolated context exhibited an increase in ethanol consumption during adolescence. This effect disappeared when the isolated infants were matched to receive the same amount of ethanol ingested by their nursed counterparts. In Experiment 3, isolated infant rats were exposed to different ethanol concentrations (.0%, 2.5%, 5.0%, and 10.0%), and drug consumption was tested during adolescence. This exposure increased adolescent ethanol intake, regardless of the alcohol concentration (Experiment 3). The common denominators that resulted in enhanced ethanol intake during adolescence were preexposure to ethanol via active consumption of the drug that induced a low-to-moderate level of intoxication in an isolated context.

KEYWORDS
adolescent, ethanol, home environment, infant, nursing

1 | INTRODUCTION

Epidemiological studies have shown that ethanol exposure during prenatal development or early infancy at levels that do not result in overt dysmorphology increases the probability of alcohol-use disorders later in life (Baer, Barr, Bookstein, Sampson, & Streissguth, 1998; Baer, Sampson, Barr, Connor, & Streissguth, 2003; Yates, Cadoret, Troughton, Stewart, & Giunta, 1998). The mechanisms that underlie this effect of ethanol exposure, however, are poorly understood. Exposure to ethanol during infancy can occur during breastfeeding after maternal ethanol ingestion. Ethanol levels in milk following maternal ingestion are similar to those in maternal blood (Guerri & Sanchis, 1986; Lawton, 1985; Pepino, Kraebel, López, Spear, & Molina, 1998). Ethanol alters the flavor of milk, which in turn can affect suckling behavior and provide an opportunity for early learning of ethanol's chemosensory properties. Human babies detect relatively low concentrations of ethanol in maternal milk (50 mg/100 ml), reflected by changes in suckling behavior and overall milk consumption (Mennella, 1997; Mennella & Beauchamp, 1991, 1993).

Rats pups that are reared by a dam that is chronically intoxicated with ethanol were shown to be more likely than controls to exhibit heightened behavioral manifestations of distress (Molina, Pepino, Johnson, & Spear, 2000) and avoid a texture that is paired with ethanol odor (Molina et al., 2000). These pups were also more and less likely to acquire conditioned aversion to and conditioned preference for, respectively, ethanol's chemosensory properties (Pepino et al., 1998;

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Pepino, Spear, & Molina, 2001). These effects are likely the consequence of ethanol intoxication that disrupts the quality of maternal care. Ethanol's odor and taste that are detected in the ingested milk become a signal for the lack of proper maternal care; hence, pups exhibit conditioned aversion to these cues.

Rat pups that were reared by a dam that was chronically intoxicated with ethanol also exhibited heightened ethanol intake (Ponce, Pautassi, Spear, & Molina, 2004), which persisted into adolescence (Pepino, Abate, Spear, & Molina, 2004; Ponce, Pautassi, Spear, & Molina, 2011). A similar outcome was found when 12- and 16-day-old infant rats were intraorally stimulated with a milk/ethanol compound while suckling from an anesthetized dam (Hunt, Kraebel, Rabine, Spear, & Spear, 1993). These pups subsequently exhibited enhanced ethanol intake, which was likely attributable to the pups' associating the inherently appetitive suckling condition (unconditional stimulus) with the odor and taste of alcohol (conditional stimulus). The critical role of suckling in this outcome was confirmed by the observation that ethanol intake was unaffected in control pups that were concurrently exposed to ethanol's flavor and milk infusions but devoid of the possibility of suckling (Hunt et al., 1993).

Altogether, ethanol exposure during nursing results in the acquisition of memories that, despite some of them having negative hedonic value, can enhance ethanol intake later in life. One limitation of this conclusion, however, was provided by the elegant study by Honey and Galef (2003). These researchers found that exposure to an ethanol-consuming dam during nursing enhanced ethanol consumption during adolescence, but only when the pups also had access to ethanol during the weaning period (up to postnatal day 26 [PD26]), suggesting that maternal/pup exposure to ethanol alone is insufficient, by itself, to enhance ethanol consumption during adolescence. Instead, subsequent reexposure to ethanol during the juvenile period may be needed to reactivate the memory that is acquired during nursing. Moreover, the previous studies (i.e., Hunt et al., 1993; Pepino et al., 2004; Ponce et al., 2004, 2011) had the caveat of lacking control groups of pups that were exposed to ethanol in a context that was completely different from the one during nursing. These studies also did not explore the long-term effects of experience with the drug during nursing.

In summary, early exposure to ethanol in the womb or during the first weeks of life affects ethanol intake later in life. This early experience encompasses exposure to social stimuli and the pharmacological and orosensory properties of ethanol. The specific contribution of each type of stimulus on later ethanol intake, however, remains unknown. We assessed the intake of various concentrations of ethanol during infancy in a familiar nursing context or in an isolated context. The lingering effects of this experience on ethanol intake was then assessed during adolescence. The inclusion of an isolated group that was also exposed to ethanol should help determine the specific contribution of each context to ethanol acceptance. The hypothesis was that ethanol exposure in the nursing context but not in the isolated context would increase ethanol intake during adolescence. The protocol minimized the inherent problems that are associated with giving alcohol to lactating dam, such as alterations in maternal behavior (Pueta, Abate, Haymal, Spear, & Molina, 2008) or milk production and letdown that in turn affects the offspring pattern of feeding (Mennella, Jagnow, & Beauchamp, 2001). Our procedure allowed precise control of the level of ethanol exposure and can be combined with treatments in which pups are reared throughout infancy by chronically intoxicated dams (Pepino et al., 2004).

2 | GENERAL METHODS

2.1 | Subjects

A total of 257 Wistar rats that were derived from 39 litters were used for the experiments (Experiment 1: 49 males and 45 females derived from 16 litters; Experiment 2: 40 males and 33 females derived from 10 litters; Experiment 3: 46 males and 44 females derived from 13 litters). These animals were born and reared in the vivarium of the Instituto M. M. Ferreyra (INIMEC-CONICET, Universidad Nacional de Córdoba, Córdoba, Argentina). Births were examined daily, and the day of birth was considered PD0. On PD1, the litters were culled to 10 pups (five males and five females, whenever possible). The pups were housed with their dams in standard maternity cages with free access to water and food. On PD21, the offspring were weaned from their dams and housed in same-sex groups. The colony was maintained at 22 ± 1 °C under a 12 hr/12 hr light/dark cycle. The experiments were approved by the Ministry of Animal Care of INIMEC-CONICET, Universidad Nacional de Córdoba, and complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Research Council, 2011).

2.2 | Data analysis

Data from each experiment were analyzed using repeated-measures analysis of variance (ANOVA). Least significant difference (LSD) pairwise *post hoc* tests were conducted to analyze significant main effects and significant interactions. The partial eta squared ($\eta^2 p$) was used to estimate effect size. The alpha level was set to .05, and Statistica software was used to compute descriptive and inferential statistics. Figure 1 presents a timeline of the procedures that were employed in Experiments 1–3.

2.3 | Experiment 1

This experiment examined ethanol intake in preweanling rats in the presence or absence of the dam (i.e., nursing vs. isolated conditions). The long-lasting effects of this experience were evaluated during adolescence when the animals had the opportunity to consume ethanol in a two-bottle choice test.

2.3.1 | Methods

Ethanol exposure during early infancy in a nursing context

On PD3, the infant rats were removed from the home cage and cannulated. Intraoral cannulation was performed using a procedure that was described in previous studies (Miranda-Morales, Molina, Spear, & Abate, 2010; Spear, Specht, Kirstein, & Kuhn, 1989). The entire procedure took less than 5 s per pup. The cannulas were later

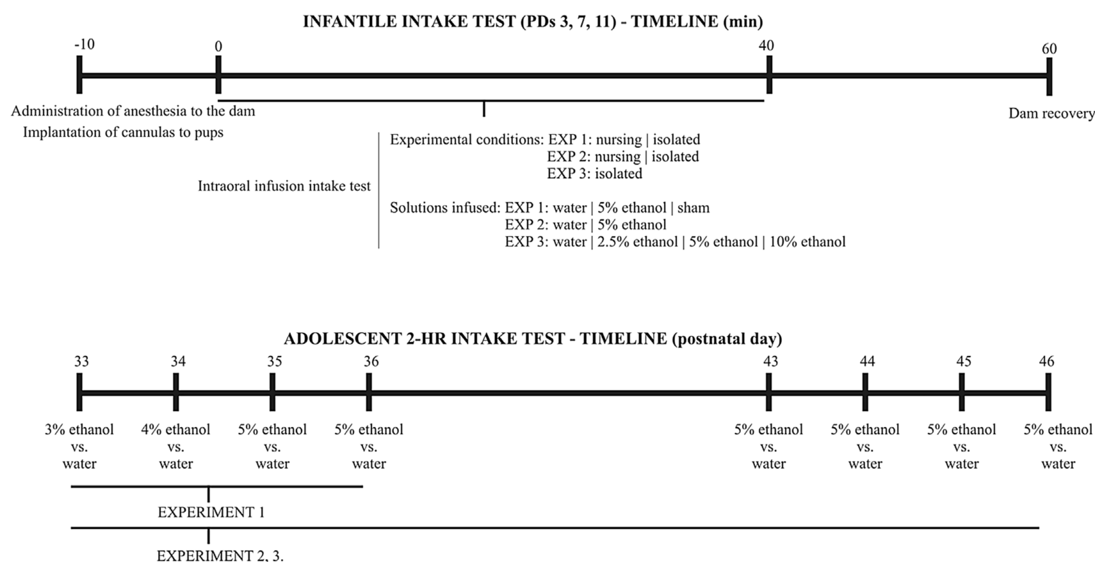

FIGURE 1 Methods employed for assessing ethanol acceptance during infancy in a familiar (nursing) or isolated context and the lingering effects of ethanol pre-exposure at infancy upon adolescence ethanol consumption. The intake test at infancy took place at postnatal days (PD) 3, 7, and 11. The adolescent intake test took place at PDs 33–36 (Experiment 1) or PDs 33–36 and PDs 43–46 (Experiments 2 and 3)

used to infuse the solutions during the intake test. The infants were kept in a holding chamber that was partially filled with wood shavings and maintained at 32–34 °C by a heating pad until the time of testing. During early infancy, pups are still unable to self-regulate body temperature. The use of these pads is a standard procedure that is widely used in protocols that test infant rats (Arias & Chotro, 2005; Miranda-Morales et al., 2010; Miranda-Morales, Molina, Spear, & Abate, 2012; Miranda-Morales, Nizhnikov, Waters, & Spear, 2014; Pautassi, Nizhnikov, & Spear, 2012). The dam's weight was recorded (± .01 g; Ohaus Scout Pro, Pinebrook, NJ). The dams were then intraperitoneally anesthetized with .1 ml/kg ketamine/xylazine (90 mg/kg ketamine, 2 mg/kg xylazine). Immediately afterward, the infants were stimulated in the anogenital region by gently stroking with a cotton swab (to promote defecation and void their bladders) and subsequently weighed. Stimulation of the anogenital area is a standard procedure that is used before intake tests during infancy. It is performed to prevent urination/defecation that could confound intake measurements (Arias & Chotro, 2005; Arias, Pautassi, Molina, & Spear, 2010a; Miranda-Morales et al., 2010, 2012). The inclusion of a sham condition ensured that the anesthesia effectively blocked maternal milk letdown. Two sham groups were included: one was tested in the nursing condition, and the other was tested in the isolated condition. The dam was positioned on her side so that her ventrum was exposed. Figure 2 depicts the setup that was used for the intraoral infusion test during infancy.

The infant rats were individually placed next to the dam's ventrum in the maternity chamber (nursing group) or into an individual Plexiglas chamber ($15 \times 7 \times 15$ cm^3) that was maintained at 32–34 °C (isolated group). Each subject's cannula was connected to polyethylene-50 tubing that was connected to a 3 ml syringe that was operated by an automatic infusion pump (KD Scientific, Holliston, MA). The duration of the intake test was 40 min. The animals were given .0% or 5.0% w/v ethanol solution (vehicle: distilled water). The infusions were administered every 4, 3, or 2 min at a constant rate of .04, .06, or .08 ml/min on PD3, PD7, and PD11, respectively. Across days, the total volume infused was 5.5% of the infant's body weight. These intervals and fluid volumes were similar to those employed by Hunt et al. (1993) and approximated those of milk letdown in a non-anesthetized dam (Cramer & Blass, 1983; Lincoln, 1973). Most of the animals in the nursing condition rapidly attached to the nipple. Notably, this modality of intraoral infusion allows pups ample control of the amount of fluid ingested. Previous studies indicated that the level of intake can be modulated by drugs (Miranda-Morales et al., 2010) and conditioning treatment (Pautassi, Arias, Molina, & Spear, 2008) that alter palatability. Animals can control ingestion by regulating taste reactivity responses (Arias & Chotro, 2005). The emission of mouthing and tongue protrusions helps ingest the fluid, whereas gaping, chin rubbing, and passive drips allow for partial avoidance of the solution (Arias et al., 2010b).

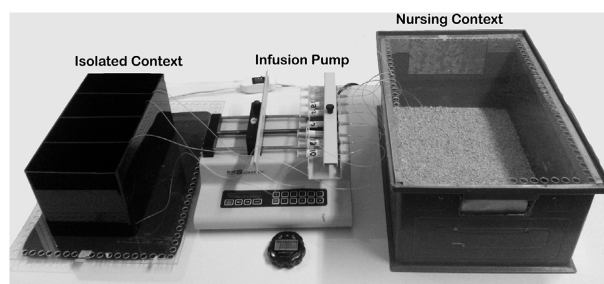

FIGURE 2 Apparatus employed for ethanol exposure to Wistar infant rats in a nursing or isolated context, during postnatal days 3, 7, and 11. Infants were placed either next to the anesthetized dam's ventrum in a standard housing cage (nursing context), or in an individual Plexiglas chambers ($15 \times 7 \times 15$ cm, isolated context), kept at 32–34 °C via a heating pad. A syringe infusion pump was placed between the two contexts and delivered ethanol or water through an intraoral cheek cannula

According to previous studies, ethanol exposure during infancy was conducted on PD3, PD7, and PD11. Pepino et al. (2001, 2004) assessed maternal behavior in dams that received ethanol intubation on PD3, PD5, PD7, PD9, PD11, and PD13. Pepino also tested the offspring a few days later for ethanol intake. We shortened Pepino's 6-day protocol after we performed preliminary experiments, in which we found that dams developed tolerance to anesthesia after PD7.

After the intake test, the pups were weighed, and the percentage of body weight gain during the test was calculated as the following: ([*posttest weight* − *pretest weight*]/*pretest weight*) × *100*. Ethanol consumption in terms of grams per kilogram (g/kg) of ethanol ingested during the intake test was calculated as the following: ([(*posttest weight* − *pretest weight*) × (*.05* × *100*)] × *.81*)/(*pretest weight* × *1,000*). Dams begin to recover from anesthesia approximately 60 min after the injection (20 min after the intake test). This procedure was repeated on PD7 and PD11. In summary, the pups were exposed to intraoral infusions of ethanol or vehicle, either in a nursing context or in isolation. On PD21, the animals were weaned from the dam and housed in same-sex groups.

Adolescent homecage ethanol consumption test

Voluntary ethanol consumption was tested using a modified version of a standardized two-bottle choice ethanol intake protocol (Fabio, Macchione, Nizhnikov, & Pautassi, 2015; Pepino et al., 2004; Ponce, Pautassi, Spear, & Molina, 2008). The animals underwent daily 2 hr intake tests (PD33-36), preceded by 22 hr in which they had access to 50% of the water they usually consume. On PD31, the animals were individually housed, although they could see and smell (but not touch) a conspecific of the same sex through a plastic divider. On this first day of the experiment, the animals were weighed, and they had access to a single bottle (50 ml capacity) that was filled with tap water. Twenty-four hours later, on PD32, the bottle was refilled with 50% of the water that the animal would regularly drink, taking into account intake scores on PD31. On PD33-36, the animals had access to two 100 ml bottles, one filled with tap water and the other filled with an ethanol solution (3% v/v on PD33, 4% on PD34, and 5% on PD35-36). The volume consumed from each bottle was assessed by subtracting the weight of the bottle after the intake test from the volume recorded before the test. The position of the ethanol and water bottles was varied across tests to prevent place-preference effects. After each daily intake test, the bottles were removed and replaced with a bottle filled with 50% of the water that the animal would regularly drink. The dependent variables were ethanol (g/kg) and water (ml/100 g) intake and total fluid consumption (ethanol + water intake scores, ml/100 g). This intake protocol has been extensively used to detect the influence of early alcohol exposure that is mediated by maternal consumption during pregnancy (Fabio et al., 2015), maternal breastfeeding (Pepino et al., 2004), operant self-administration (Ponce et al., 2008), or passive intubations (Acevedo, Molina, Nizhnikov, Spear, & Pautassi, 2010) on subsequent alcohol acceptance.

Experimental design and data analysis

Experiment 1 assessed ethanol intake during early infancy and during adolescence and used a 2 (infant testing condition: nursing or isolation) × 2 (solution received: 5.0% ethanol or vehicle) × 2 (sex: male or female) factorial design with an isolated (sham) control condition. The percentage of body weight gain (%bwtg) during the tests that were conducted during infancy was analyzed using a 4-way mixed analysis of variance (ANOVA; sex × solution received × testing condition × day of evaluation [PD3, PD7, and PD11]). The variables that were analyzed in the intake test during adolescence included overall intake scores (water + ethanol, expressed in ml/100 g), water intake scores (ml/100 g), and ethanol intake scores (g/kg). These variables were independently analyzed using a 4-way mixed ANOVA (sex × solution infused during infancy [5% ethanol, vehicle or sham] × testing condition during infancy [nursing or isolation] × day of evaluation [PD33, PD34, PD35, and PD36]).

We performed *t* tests for a single mean against a user-defined constant equal to zero (i.e., 0% bwtg) to confirm that the pups in the sham condition did not receive significant amounts of milk from anesthetized dams on PD3, PD7, and PD11.

2.4 | Results

2.4.1 | Preliminary analysis

The *t* tests, one for each of the intake tests that were conducted on PD3, PD7, and PD11 indicated that sham animals in the isolated condition lost significant weight during the 40 min intake test (PD3: $t = -2.17$; PD7: $t = -5.46$; PD11: $t = -9.38$; all $p < .001$). Sham animals in the nursing condition did not differ significantly from zero on PD3 or PD7 but significantly lost weight on PD11 ($t = -5.63$, $p < .001$). These results confirm the effectiveness of the anesthesia to inhibit milk letdown and were further scrutinized by performing an ANOVA for sham animals only (between-subjects factor: Testing condition; within-subjects factor: Days of evaluation), indicating that sham-isolated animals lost significantly more weight than their nursed sham counterparts ($F_{1,27} = 12.52$, $p < .01$, $\eta^2p = .32$). The day × testing condition interaction was also significant ($F_{2,54} = 3.38$, $p < .05$, $\eta^2p = .11$). The *post hoc* test indicated that on PD3 and PD7, isolated animals lost more weight than nursed pups ($p < .01$). On PD11, however, both groups lost a similar percentage of weight. These results (Figure 3) indicate that the anesthetic did not allow sufficient maternal milk letdown to significantly increase the pups' body weight.

2.4.2 | Intake scores at infancy

The ANOVA of the %bwtg that was achieved during the infancy test yielded significant main effects of testing condition and day of evaluation ($F_{1,57} = 126.05$, $p < .001$, $\eta^2p = .69$, and $F_{2,114} = 20.72$, $p < .001$, $\eta^2p = .26$, respectively). As shown in Figure 3, pups in the nursing condition consumed significantly more of either fluid than their isolated counterparts. The solution received × day of evaluation interaction was significant ($F_{2,114} = 6.42$, $p < .01$, $\eta^2p = .10$). The *post hoc* tests revealed significantly greater intake of ethanol versus water on PD7 and PD11 but not on PD3.

2.4.3 | Intake scores at adolescence

The ANOVAs of overall intake and water intake scores indicated a significant main effect of day of evaluation ($F_{3,246} = 14.08$, $p < .001$,

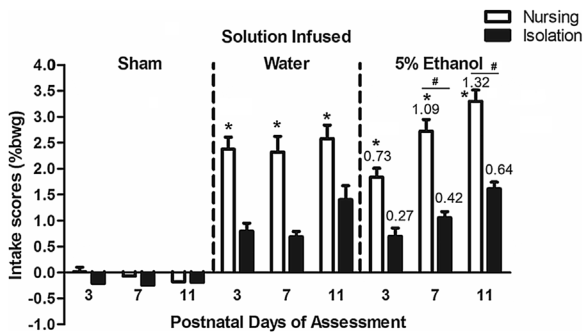

FIGURE 3 Intake scores (expressed as percentage of body weight gained, %bwtg) of infant rats intraorally stimulated with water or 5% ethanol, in Experiment 1. Intake tests occurred on postnatal days 3, 7, and 11, in a nursing or in an isolated context. The sham groups did not received water or ethanol during the test, yet were exposed to the same testing conditions as were the other groups. Data has been collapsed across sex, which did not exert a significant effect upon the intake scores. Values represent mean ± SEM. The values over the bars indicate gram per kilogram (g/kg) of ethanol ingested by pups stimulated with 5% ethanol. The asterisks (*) signs indicate the significant main effect of test condition (nursing vs. isolated). The pound (#) signs indicate significant differences between ethanol and water-infused groups, in a given postnatal day of assessment

$\eta^2p = .15$, and $F_{3,246} = 8.20$, $\eta^2p = .09$, $p < .001$, for total intake and water intake scores, respectively). Total intake scores and water intake scores were significantly greater on the second and third days, respectively, than on the other days and significantly lower on the fourth day than on the other days.

The ANOVA of g/kg ethanol ingested indicated significant main effects of testing condition during infancy and day of evaluation ($F_{1,82} = 3.99$, $p < .05$, $\eta^2p = .05$, and $F_{3,246} = 11.42$, $p < .001$, $\eta^2p = .12$, respectively). As depicted in Figure 4, adolescent animals that had been isolated during the infancy intake test consumed significantly more ethanol than those in the nursing condition. Ethanol intake significantly increased on the second testing day and remained at that level across the remaining sessions. Visual inspection of Figure 4 suggests that the difference in ethanol intake between animals that were isolated versus nursed during the tests at infancy was greater in those that received ethanol during the tests compared with those that received water or were given the sham treatment. The testing condition at infancy × solution infused during infancy interaction was not significant.

These results indicate an immediate facilitative effect of the nursing context on fluid intake during infancy, which was fairly similar between ethanol and water. Additionally, the preweanling animals exhibited an overall relative preference for ethanol over water. The acute, facilitative effect of the early nursing experience dissipated by adolescence. During this stage, a significant, promoting effect of having been tested in isolation during infancy was observed, which was specific for upon ethanol ingestion.

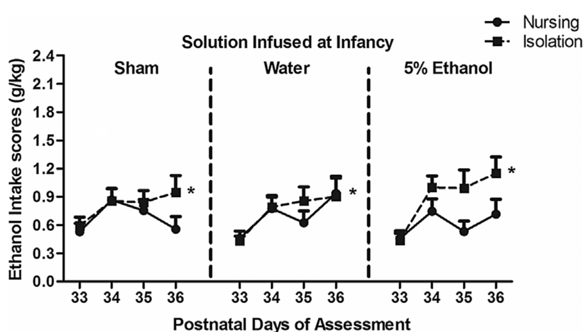

FIGURE 4 Ethanol intake scores (g/kg) of adolescent rats in Experiment 1, as a function of day of assessment (intake test sessions at postnatal day 33, 34, 3, and 36), solution received at infancy (water, 5% ethanol, or sham) and test condition at infancy (nursing or isolation). Data has been collapsed across sex, which did not exert a significant effect upon the intake scores. Values represent mean ± SEM. The asterisks (*) indicate the significant main effect of test condition at infancy

2.5 | Experiment 2

The main result of Experiment 1 was that brief episodes of maternal separation during infancy increased ethanol ingestion during adolescence. Unclear, however, was whether isolation was solely responsible for this result, or was isolation plus the specific level of intoxication that was derived from infant ethanol intake responsible for these effects. Experiment 2 was designed to answer this question. The intake protocol during infancy was similar to the one in Experiment 1, but we added a brief yet important manipulation at the end of each intraoral infusion test on PD3, PD7, and PD11. We matched, via experimenter-administered intubations, the levels of fluid that was ingested by the animals in the nursing and isolated conditions. After each intake session during infancy, the animals were supplemented with ethanol or water to achieve the same level of body weight gain as their counterparts in the other testing condition. The animals in the isolated condition were mostly supplemented because the nursing condition induced (at the group level) greater solution intake than in the isolated condition.

The expectations of this experiment were the following. If maternal isolation was the only factor that led to greater ethanol intake during adolescence, then supplementation should avoid such effects. Thus, Experiment 2 should yield a pattern of adolescent ethanol intake that was similar to the one found in Experiment 1. However, if greater ethanol intake during adolescence was caused by isolation in conjunction with a specific level of ethanol intoxication that was achieved during the infant intake test, then supplementation should inhibit the promoting effect of isolation on adolescent ethanol consumption.

2.5.1 | Methods

The intake test during infancy was similar to the one described in Experiment 1. After the termination of each intake test on PD3, PD7, and PD11, however, a protocol for ethanol/water supplementation was implemented. Animals in the isolated and nursing conditions were paired in same-sex couples that were stimulated with the same fluid (5% ethanol or water). Each couple was composed of one pup from the nursing condition and one pup from the isolated condition. The

experimenter calculated the body weight that was gained by each member of the couple (e.g., .5% and 1.0%bwtg in the isolated and nursed animals, respectively). The member of the couple that exhibited the lower body weight gain (in our example above, the animal in the isolated condition) received an intragastric administration of the same solution that was previously ingested. The volume of the intubation was the one that was required to match the level of ingestion of the other member of the couple. The member that did not receive supplementation still received a sham intubation to control for potential activating/stressful effects of the manipulation. Supplementation was performed, for the most part, in pups in the isolated condition, with the exception of PD3, when a few animals in the nursing condition drank less. The aim of supplementation was to match the body weight gain of the animals that were tested in the different contexts.

The consumption test during adolescence was similar to the one described in Experiment 1. The animals were tested daily for ethanol and water intake on PD33-36 in 2 hr daily tests. To increase the possibility of detecting differences in intake patterns between groups, a second intake session of four additional days (5% ethanol vs. water) was conducted 1 week after termination of the first session on PD43-46.

Experimental design and data analysis

Experiment 2 analyzed intake scores during early infancy and adolescence and employed a 2 (testing condition during infancy: nursing or isolation) × 2 (solution received during infancy: 5.0% ethanol or vehicle) × 2 (sex: male or female) factorial design. There was no sham condition.

Intake scores (expressed as a percentage of body weight gain) during the test at infancy were analyzed using a 4-way mixed ANOVA (sex × solution received × testing condition × day of evaluation [PD3, PD7, and PD11]). In adolescent animals, total intake, water intake, and ethanol intake scores were independently analyzed using a 4-way mixed ANOVA (sex × solution infused during infancy × testing condition during infancy × day of evaluation [PD33-36 and PD43-46]).

2.5.2 | Results

Infant intake scores

The analysis of drinking patterns during early infancy yielded a significant main effect of testing condition ($F_{1,65} = 79.41$, $p < .001$, $\eta^2p = .55$). As shown in Figure 5, pups in the nursing condition drank significantly more of both solutions than their isolated counterparts. The day of evaluation × solution received interaction also achieved significance ($F_{2,130} = 6.9$, $p < .01$, $\eta^2p = .10$). The *post hoc* tests revealed greater preference for water versus ethanol on PD3 but similar ethanol and water intake scores on PD7 and PD11.

The levels of ethanol and water intake that are shown in Figure 5 are those that were observed after each intake test (i.e., before supplementation). After the test, supplementation was performed. Supplemented animals were, for the most part, the isolated animals, which received ethanol or water supplementation that resulted in the same drinking levels as those achieved by nursed animals.

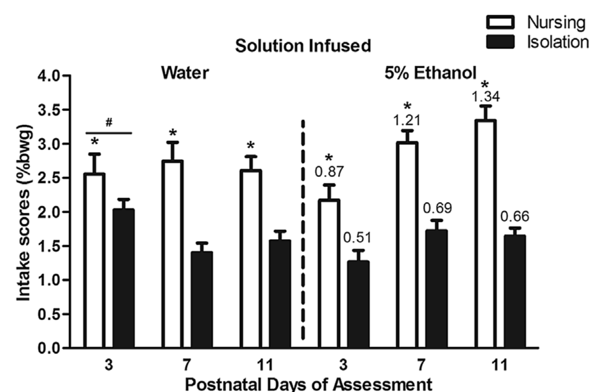

FIGURE 5 Intake scores (expressed as percentage of body weight gained, %bwtg) of infant rats intraorally stimulated with water or 5% ethanol in Experiment 2. Intake tests occurred on postnatal days 3, 7, and 11, in a nursing or in an isolated context. The values over the bars indicate gram per kilogram (g/kg) of ethanol ingested by pups stimulated with 5% ethanol. After each test, animals that had been stimulated with the same fluid (5% ethanol or water) were paired in same-sex couples. Each couple was composed by one pup from the nursing condition and one pup from the isolated condition. The member of the couple exhibiting the lower %bwtg received an intragastric administration of the same solution previously ingested. The volume of the intubation was that needed to match the level of ingestion of the other member of the couple. The aim was to match the %bwtg of the animals tested in the different contexts. Data have been collapsed across sex, which did not exert a significant effect upon the intake scores. Values represent mean ± SEM. The asterisk (*) signs indicate the significant main effect of test condition. The pound (#) sign indicates significant differences between ethanol and water-infused groups, in postnatal day 3

Adolescent intake scores

The ANOVAs of total intake and water intake scores indicated a significant main effect of day of evaluation ($F_{7,455} = 37.10$, $p < .001$, $\eta^2p = .36$, and $F_{7,455} = 9.00$, $p < .001$, $\eta^2p = .12$, for total intake and water intake scores, respectively). Relative to the scores that were found on the first testing day (PD33), adolescents had significantly higher total intake scores on the second day of testing and significantly higher water intake scores on the third day of testing. Furthermore, significant decreases in total intake and water intake scores were observed on the first day of testing in the second session (PD43) compared with the last day of testing in the first session (PD36).

The ANOVA of ethanol intake scores (g/kg) indicated significant main effects of solution infused during infancy and day of evaluation ($F_{1,65} = 6.38$, $p < .025$, $\eta^2p = .09$, and $F_{7,455} = 10.75$, $p < .001$, $\eta^2p = .14$, respectively). As depicted in Figure 6, animals that drank ethanol during infancy consumed significantly less ethanol than animals that drank water. Ethanol intake scores significantly increased on the second and third days of testing compared with the initial level on PD33. After reaching a peak on PD34-35, ethanol intake scores significantly decreased on the fourth day of testing and then continued to gradually decrease across subsequent test days. This pattern was fairly similar in adolescents from both infant testing conditions. A significant sex × days interaction was observed ($F_{7,455} = 3.24$, $p < .01$,

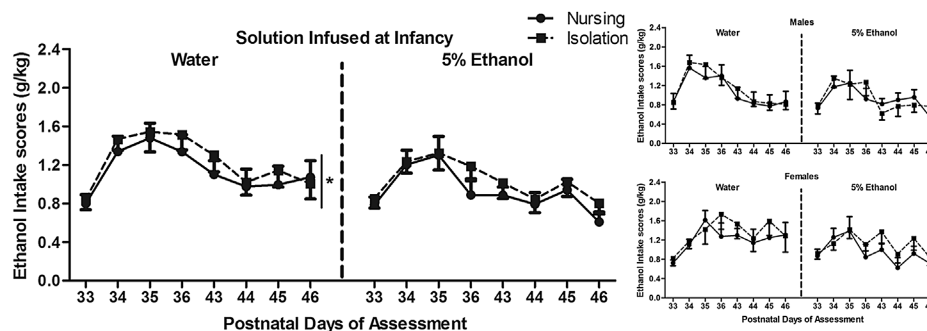

FIGURE 6 Ethanol intake scores (g/kg) of adolescent rats in Experiment 2, as a function of day of assessment (intake test sessions at postnatal days 33–36 and 43–46), solution received at infancy (water or 5% ethanol) and test condition at infancy (nursing or isolation). In the main panel data have been collapsed across sex. The small panels on the right depicts ethanol intake scores across days of adolescent animals as a function of sex (males and females). Values represent mean ± SEM. The asterisk (*) sign indicates the significant main effect of solution infused at infancy (water vs. 5% ethanol)

$\eta^2 p$ = .05). As can be observed in the small panels of Figure 6, on PD43, PD45, and PD46, the females drank significantly more ethanol than males.

Similar to Experiment 1, the nursing condition promoted greater intake scores than the isolated condition during the infant intake test. Supplementation, which was conducted mainly in pups from the isolated condition, significantly changed the pattern of adolescent ethanol intake that was observed in Experiment 1. In Experiment 1 (when supplementation was not performed), isolated animals drank more than nursed animals. In Experiment 2 (when supplementation was performed), animals that were isolated or nursed during infancy exhibited the same level of consumption. These results suggest that experimenter-administered supplementation inhibited the promoting effect of isolation. Moreover, animals that were exposed to ethanol during infancy drank less ethanol when tested during adolescence compared with their water-exposed counterparts.

2.6 | Experiment 3

Experiment 2 suggested that exposure to moderate (i.e., >1.0 g/kg) levels of ethanol during early infancy results in an attenuation of ethanol consumption during adolescence. This possibility warranted further exploration because it seems to contradict or limit the notion that ethanol exposure during infancy (Bannoura, Kraebel, Spear, & Spear, 1998; Ponce et al., 2008) increases the subsequent predisposition to ethanol intake. In Experiment 3, preweanling rats in the isolated condition were stimulated with the same ethanol concentration (5%) as in Experiment 2 or different ethanol concentrations (2.5% or 10%). These manipulations resulted in different levels of ethanol intoxication during infancy. The effect of this experience on adolescent ethanol drinking was subsequently assessed.

2.6.1 | Methods

The infant intake test was the same as the one that was used for the isolated condition in Experiment 1, and the pups were exposed to one of four ethanol concentrations (.0, 2.5, 5.0, or 10.0%). The two-bottle choice test during adolescence was the same as the one that was described in Experiment 2, with two 4-day sessions (ethanol vs. water).

Blood alcohol levels

We measured the level of intoxication during the intake tests that were conducted during infancy and adolescence. A separate group of male and female infant animals (derived from five litters) was subjected to the intake test under conditions of isolation on PD3, PD7, and PD11. Trunk blood samples were obtained from these animals 10 min after the intake test on PD11. During adolescence, trunk blood samples (.5 ml) were collected 10 min after termination of the last test on PD 46, and blood alcohol levels (BALs) were determined. Blood samples were collected in heparinized capillary tubes (Fisherbrand, natelson blood collecting tubes; .25 ml capacity) and stored in Eppendorf tubes at −80 °C until the determination of BALs. Blood samples were subjected to head-space gas chromatography (Pepino et al., 1998). At least two 100 µl samples of blood were analyzed for each subject. The previously collected fluids were distributed into 100 µl samples in a cold chamber at 4 °C. The samples were then incubated in a water bath at 60 °C for 30 min. Gas-tight syringes (Hamilton, 1 ml capacity) were used to collect the volatile component of the samples and inject them into the gas chromatograph (Hewlett-Packard, Model 5890). The column (Carbowax 20M; 10 m × .53 mm × 1.33 µm film thickness), injector, and detector temperatures were 60, 150, and 250 °C, respectively. Nitrogen served as the carrier gas (flow rate, 15 ml/min). Blood alcohol levels were computed using linear regression analysis of known standards. The value for each subject was averaged across samples of a given bodily fluid. All of the values are expressed as milligrams of ethanol per deciliter (mg/dl = mg%).

Experimental design and data analysis

Experiment 3 analyzed ethanol intake during early infancy and adolescence and employed a 4 (ethanol solution during infancy: .0%, 2.5%, 5.0%, or 10.0%) × 2 (sex: male or female) factorial design. Body weight gain in infants was analyzed using a 3-way mixed ANOVA (sex × ethanol solution received × day of evaluation [PD3, PD7, and PD11]). A similar ANOVA was used to analyze g/kg of ethanol ingested by the pups that were given the different ethanol solutions. Overall intake, water intake, and ethanol intake scores in adolescents were analyzed using independent 3-way mixed ANOVAs (sex × solution infused during infancy × day of evaluation [PD33-36 and PD43-46]). Blood alcohol levels that were achieved on PD11 and PD46 were

independently analyzed using a factorial ANOVA (sex × ethanol solution infused during infancy (2.5%, 5.0%, or 10.0% ethanol]).

2.6.2 | Results

Infant intake scores

The analysis of %bwtg during infancy yielded significant main effects of solution and day of evaluation ($F_{3,76} = 3.56$, $p < .025$, $\eta^2 p = .12$, and $F_{2,152} = 21.5$, $p < .001$, $\eta^2 p = .22$, respectively). The solution × day interaction achieved significance ($F_{6,152} = 4.96$, $p < .001$, $\eta^2 p = .16$). As shown in Figure 7A, the *post hoc* tests revealed that on PD3, the consumption of water or 2.5% ethanol was significantly higher than the consumption of 5% or 10% ethanol. On PD 7, the intake of 5% ethanol was significantly higher than the intake of water. On PD11, water intake scores were significantly lower than those of the three ethanol solutions (all $p < .01$), and the consumption of 5% ethanol was significantly higher than the consumption of 2.5% ethanol ($p < .05$).

The ANOVA of g/kg ethanol ingested during infancy revealed significant main effects of solution and day of evaluation ($F_{2,63} = 121.02$, $p < .001$, $\eta^2 p = .79$, and $F_{2,126} = 42.43$, $\eta^2 p = .40$, $p < .001$, respectively). The solution × day of evaluation interaction achieved significance ($F_{4,126} = 10.20$, $p < .001$, $\eta^2 p = .24$). As shown in Figure 7B, animals that were given 2.5% ethanol ingested similar levels of ethanol (g/kg) during the 3 days of evaluation. Animals that were given 5% and 10% ethanol consumed significantly more ethanol across days, and animals that received 10% ethanol consumed significantly more ethanol than the other animals across days. On PD7 and PD11, 5% ethanol consumption scores were significantly higher compared with 2.5% ethanol.

The ANOVA of BALs that were achieved during the last intake test on PD11 indicated a significant main effect of ethanol concentration ($F_{2,24} = 39.17$, $p < .001$, $\eta^2 p = .77$). The BALs that were attained by ingesting 10% ethanol were significantly higher than those that were attained after ingesting of 5% ethanol, which in turn was significantly greater than those attained after ingesting 2.5% ethanol (all $p < .001$). A significant positive correlation was found between g/kg ethanol consumed on PD11 and BALs ($r = .87$, $p < .001$). Table 1 summarizes the BALs that were achieved after the PD11 intake test.

Adolescent intake scores

The ANOVAs of overall intake and water intake during adolescence indicated a significant main effect of day of evaluation ($F_{7,364} = 31.22$, $p < .011$, $\eta^2 p = .37$, and $F_{7,364} = 16.19$, $p < .001$, $\eta^2 p = .24$, respectively). Overall intake and water intake were greater on the second day than on the first day, and significant decreases in both overall intake and water intake scores were observed at the beginning of the second session compared with the last day of the first session.

The ANOVA of g/kg ethanol ingested during adolescence indicated significant main effects of solution given during infancy and day of evaluation ($F_{3,52} = 4.02$, $p < .025$; $\eta^2 p = .19$, and $F_{7,364} = 12.03$, $p < .001$, $\eta^2 p = .19$, respectively). Importantly, the solution given during infancy × day of evaluation interaction was significant ($F_{21,364} = 1.77$, $p < .025$, $\eta^2 p = .09$). As depicted in Figure 8, on PD35 and PD36, ethanol intake was significantly higher in adolescents that had been given ethanol during infancy (2.5%, 5%, and 10%) than in the controls that were preexposed to water. Animals that were preexposed to 5% or 10% ethanol during infancy exhibited greater ethanol intake scores than water controls by the end of the second intake session (i.e., PD46 in the 5% ethanol group and PD44 and PD46 in the 10% ethanol group).

Blood alcohol levels that were achieved after the 2 hr intake test on PD46 were unaffected by the factors solution given during infancy and day of evaluation. Table 1 summarizes the BALs that were achieved after the PD46 intake test.

In summary, the preweanling animals exhibited relative preference for ethanol over water during the intake tests that were conducted on PD3, PD7, and PD11. This experience, in turn, significantly modulated the subsequent ingestion of ethanol. Adolescent animals that were exposed to ethanol during infancy, particularly those that were stimulated with 5% or 10% ethanol, exhibited greater ethanol intake than un-exposed controls.

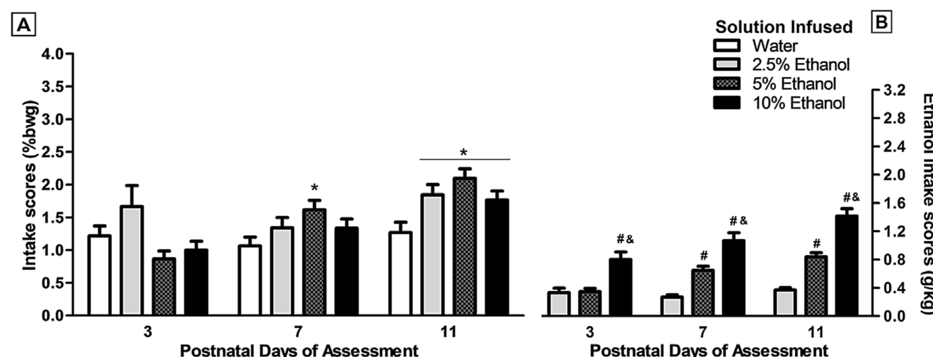

FIGURE 7 Intake scores (expressed as percentage of body weight gained, %bwtg, Panel A) and ethanol intake scores (g/kg, Panel B) of infant rats intraorally stimulated with ethanol (.0, 2.5, 5.0, or 10.0%) in Experiment 3. Intake tests occurred on postnatal days 3, 7, and 11, in an isolated context. Data have been collapsed across sex, which did not exert a significant effect upon the intake scores. Values represent mean ± SEM. The asterisk (*) signs indicate significant difference between a given ethanol-exposed group and the water-exposed control group, in postnatal day 7 or 11. The pound (#) signs indicate significant differences between a given group and the 2.5% ethanol-exposed group. The ampersand (&) signs indicate significant differences between the 10% ethanol-exposed group and the 5% ethanol-exposed group, in a given postnatal day of assessment

TABLE 1 Blood alcohol levels (expressed as mg/dl) after termination of the infantile intake test at PD 11 and the adolescent intake test at PD 46, as a function of solution received at the infantile intake test (water or 2.5, 5.0, or 10.0% ethanol)

Solution received at infancy	BALs at PD 11	BALs at PD 46
Water	—	8.72 ± 1.51
2.5% ethanol	52.06 ± 5.50	23.93 ± 7.27
5.0% ethanol	146.42 ± 9.35	18.57 ± 7.71
10.0% ethanol	242.28 ± 23.63	22.72 ± 10.86

3 | DISCUSSION

One of the aims of the present study was to assess ethanol intake in a nursing context versus ethanol intake in a context that was devoid of the maternal presence or cues. Experiments 1 and 2 found a substantial, promoting effect of the nursing context on fluid consumption. Pups that were tested in the nursing context exhibited a twofold increase in fluid acceptance (i.e., both ethanol and water) compared with pups that were tested in isolation. Animals that were given ethanol in the nursing context and those that were isolated and provided 10% ethanol achieved a remarkably high level of intoxication by the last day of testing (i.e., ~1.4 g/kg, in which pups that were stimulated with 10% ethanol achieved BALs of 242.28 mg/dl). Pups initially exhibited relatively lower preference for ethanol over water, but this preference reversed by the end of testing. By the end of the second week, pups consumed more of the ethanol solution than water. This pattern was remarkably similar to a previous procedure (i.e., consumption-off-the-floor test) that, similar to our "nursing context," allows pups to drink independently of the dam (Sanders & Spear, 2007).

The ultimate aim of the present study, however, was to assess the lingering effects of ethanol preexposure during infancy on ethanol

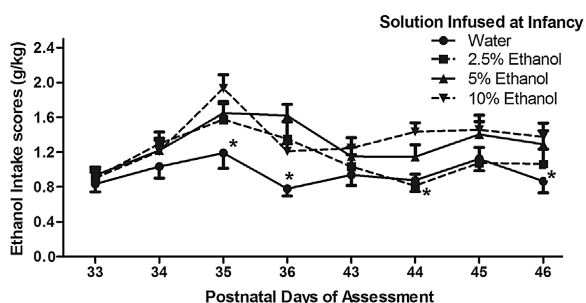

FIGURE 8 Ethanol intake scores (g/kg) of adolescent rats in Experiment 3, as a function of day of assessment [daily intake test sessions at postnatal days (PDs) 33–36 and 43–46] and ethanol solution received at infancy (.0, 2.5, 5.0, or 10.0%). Data have been collapsed across sex, which did not exert a significant effect upon the intake scores. Values represent mean ± SEM. The asterisk (*) signs indicate significant differences between animals infused with water at infancy and the remaining groups (PDs 35 and 36), between animals infused with 5% or 10% ethanol and the water-infused animals (PD44), and between animals infused with 10% ethanol and the water-infused animals (PD46)

acceptance during adolescence. We sought to determine the ways in which the mode and level of intoxication during preexposure modulates subsequent ethanol intake. Adolescence is a developmental stage during which independent alcohol consumption patterns usually begin and are likely determined (Pilatti, Godoy, Brussino, & Pautassi, 2013). Our hypothesis that rats would exhibit greater ethanol intake during adolescence after preexposure to ethanol in the nursing context was not proven. Instead, the pups that were isolated during the tests at infancy exhibited the highest ethanol consumption during adolescence. However, when the amount of ethanol that was received was matched for the two modes of exposure, an attenuation of adolescent ethanol intake was observed. This suggests a "sweet point" at which the combination of isolation and a given amount of ethanol intoxication facilitates greater ethanol intake during adolescence. The facilitating effect of exposure to ethanol in isolation was observed in pups that were stimulated with a relatively wide range of ethanol concentrations (2.5–10%).

We expected that alcohol preexposure within the nursing context would exert a greater promoting effect on adolescent ethanol intake compared with preexposure in isolation, which is likely attributable to an association between innate appetitive suckling behavior and ethanol's odor or taste. However, this was not the case. Animals that were preexposed to ethanol in the isolated context exhibited the highest ethanol intake scores during adolescence. One possibility is that the stress that is associated with repeated isolation may have increased adolescent ethanol intake. Early-life stress in rodents can increase the self-administration of drugs of abuse and induce a conditioned place preference (Jaworski, Francis, Brommer, Morgan, & Kuhar, 2005; Michaels & Holtzman, 2008; Moffett et al., 2007). Arias et al. (2010) found that isolated infants exhibited greater sensitivity to the stimulant effects of ethanol compared with their counterparts that remained in pairs or with their dam. Several studies have reported an increase in anxiety-like behavior and greater ethanol intake in adult rodents after early maternal separation (Cruz, Quadros, da Planeta, & Miczek, 2008; Huot, Thrivikraman, Meaney, & Plotsky, 2001). Thus, ethanol intake in animals that are isolated during infancy would be driven by negative reinforcement (i.e., an anxiolytic-like effect of the drug), which has been described in preweanling rats (Miranda-Morales, Nizhnikov, Waters, & Spear, 2014). Intriguingly, a similar hypothesis has been proposed to explain why preweanling rats that are exposed to ethanol during breastfeeding via maternal intoxication (which reduces the quality of maternal care) exhibit enhanced ethanol consumption later during infancy (Pepino et al., 2004; Pueta, Rovasio, Abate, Spear, & Molina, 2011) or adolescence (Pepino et al., 2004; Ponce et al., 2008).

The stress response during early ontogeny is quite different from the one that is observed in adults. The levels of hypothalamic corticotropin-releasing factor are low, and the adrenal response to stress is minimal during the first two postnatal weeks in the rat (Levine, 2001; Sapolsky & Meaney, 1986). This developmental period is known as the stress hyporesponsive period (Levine, 2001; Sapolsky & Meaney, 1986). However, infants are still capable of releasing corticosterone (CORT) when they are exposed to certain stressors, particularly under conditions of maternal isolation.

Brief handling can alter the functioning of the hypothalamic-pituitary-adrenal axis in young rats (Stamatakis et al., 2008), and exposure to a novel environment can increase CORT release in maternally isolated 12-day-old rats (Stanton, Wallstrom, & Levine, 1987). A previous study found that handling or intraperitoneal or intragastric administration (i.e., the mode of administration that was used in the present study) did not alter CORT release in 2-week-old rats (Pautassi, Nizhnikov, & Spear, 2012). In that previous study, only the relatively high ethanol dose of 2.0 g/kg increased CORT release (Pautassi et al., 2012). Thus, one postulation is that the isolated rats in the present study may have experienced a stress response and thus higher CORT release.

Another hypothesis that does not necessarily contradict the negative reinforcement hypothesis is that increased familiarity (i.e., habituation) with ethanol through preexposure to the drug may have been counteracted or blocked by the nursing context but potentiated by isolation. Thus, the biological relevance of mother-pup contact and the overall maternal environment (Meaney, 2001) may have interfered with the acquisition of familiarity with ethanol or prevented potential appetitive associations between ethanol's flavor and the pharmacological effects of the drug. The main difference between the present study and other studies that reported an increase in adolescent ethanol intake after being reared by an ethanol-intoxicated dam (Honey & Galef, 2003; Pepino et al., 2001, 2004; Ponce et al., 2008) is that in the previous studies the pups experienced the flavor of ethanol through the dam's milk. As mentioned by Mennella and Beauchamp (1997), both the act of suckling and milk are inherently reinforcing to nurslings.

In Experiment 1 in the present study, unclear was whether isolation was solely responsible for the increase in adolescent ethanol consumption or whether isolation plus the specific level of intoxication that was derived from the ethanol that was consumed during infancy increased subsequent ethanol intake during adolescence. Passive ethanol intubation was performed in Experiment 2 to match isolated pups with regard to the level of intoxication that was achieved by their corresponding nursed controls. This manipulation resulted in a general reduction of adolescent ethanol consumption in both the isolated and nursing conditions. This result indicates the relevance of the specific levels of ethanol intoxication and the ways in which these levels are achieved (i.e., active consumption vs. passive intubation) and may suggest that supplemented animals developed an aversion to ethanol, possibly because of the pharmacological levels that were achieved during infancy. Although conditioned taste aversion studies usually employ ethanol doses ≥ 2.0 g/kg, doses of ethanol as low as 1.2 g/kg have been found to exert aversive responses in preweanling animals (Hunt, Molina, Spear, & Spear, 1990). This is rare, however, and the literature indicates that pups that are younger than 10–12 days old express aversion to ethanol only after receiving doses ≥ 3 g/kg (Hunt, Spear, & Spear, 1991). Moreover, the g/kg ethanol that was ingested by infants that were administered 10% ethanol in Experiment 3 was similar to the level reached by infants that consumed ethanol in the nursing context in Experiments 1 and 2. In Experiment 3, these levels of intoxication increased rather than decreased ethanol intake during adolescence. Notably, according to Ponce et al. (2008), preexposure to ethanol during infancy via oral self-administration increases subsequent ethanol intake, but passive intraoral administration does not.

The common denominators that increased ethanol intake during adolescence in the present study were preexposure to ethanol via active consumption during infancy in an isolated context, which likely induces stress. The mere asocial exposure of pups to ethanol during nursing was insufficient to enhance voluntary ethanol consumption during adolescence. The term "asocial exposure" is used to refer to pups' exposure to ethanol and ethanol-related cues that are experienced from a source that is different from their dams, although there may be social aspects of such events because of the presence of littermates (Honey & Galef, 2003). Maternal exposure during gestation and lactation, combined with asocial access to ethanol during weaning, has been shown to result in substantial voluntary ethanol consumption in adolescent rats (Honey & Galef, 2003).

In summary, the present results suggest that preexposure to ethanol in the home environment may not to be sufficient to enhance the predisposition to ethanol intake during adolescence, but such preexposure plus early stressful experiences (i.e., parental deprivation, negligent parental care, and adverse socioeconomic conditions) may do so. This predisposition to ethanol intake may be exacerbated by even earlier exposure to ethanol (e.g., during pregnancy; see Fabio et al., 2013) or continued exposure to ethanol during early adolescence (Miller & Spear, 2006).

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