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Prenatal ethanol increases sucrose reinforcement, an effect strengthened by postnatal association of ethanol and sucrose

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ABSTRACT

Late prenatal exposure to ethanol recruits sensory processing of the drug and of its motivational properties, an experience that leads to heightened ethanol affinity. Recent studies indicate common sensory and neurobiological substrates between this drug and sweet tastants. Using a recently developed operant conditioning technique for infant rats, we examined the effects of prenatal ethanol history upon sucrose self-administration (postnatal days, PDs 14–17). Prior to the last conditioning session, a low (0.5 g/kg) or a high (2.5 g/kg) ethanol dose were paired with sucrose. The intention was to determine if ethanol would inflate or devalue the reinforcing capability of the tastant and if these effects are dependent upon prenatal ethanol history. Male and female pups prenatally exposed to ethanol (2.0 g/kg) responded more when reinforced with sucrose than pups lacking this antenatal experience. Independently of prenatal status, a low ethanol dose (0.5 g/kg) enhanced the reinforcing capability of sucrose while the highest dose (2.5 g/kg) seemed to ameliorate the motivational properties of the tastant. During extinction (PD 18), two factors were critical in determining persistence of responding despite reinforcement omission. Pups prenatally exposed to ethanol that subsequently experienced the low ethanol dose paired with sucrose, showed higher resistance to extinction. The effects here reported were not associated with differential blood alcohol levels across prenatal treatments. These results indicate that fetal ethanol experience promotes affinity for a natural sweet reinforcer and that low doses of ethanol are also capable of enhancing the positive motivational consequences of sucrose when ethanol and sucrose are paired during infancy.

Introduction

Newborn and infant rats share many characteristics in terms of ethanol affinity with those observed in genetically selected alcohol-preferring rats. Pups self-administer highly concentrated alcohol solutions (15–30% v/v) without the need of initiation procedures (Spear & Molina, 2005). Ethanol has also been found to exert rapid positive and negative (anti-anxiety) reinforcing effects in newborns (Abate, Pueta, Spear, & Molina, 2008; Abate, Varlinskaya, Cheslock, Spear, & Molina, 2002; Chotro, Arias, & Laviola, 2007; Pautassi, Sanders, Miller, Spear, & Molina, 2006; Petrov, Varlinskaya, & Spear, 2001, 2003). Motor stimulating effects of ethanol, effects which seem to share neurobiological mechanisms with positive motivational properties of the drug, have been detected early in development (Arias, Molina, Mlewski, Pautassi, & Spear, 2008). The preclinical and epidemiological research indicate that the effects of early ethanol experiences persist, and strongly predict alcohol abuse in adolescents and adults (Abate et al., 2008; Bannoura, Kraebel, Spear, & Spear, 1998; Domínguez, López, Chotro, & Molina, 1996; Faden, 2006; Grant & Dawson, 1997; Molina, Domínguez, López, Pepino, & Faas, 1999; Spear & Molina, 2005; Windle, 2003).

The near-term rat fetus acquires and retains ethanol-related information. The organism senses the drug's chemosensory cues present in the amniotic fluid while low to moderate maternal ethanol administrations act as appetitive unconditioned stimuli (Abate et al., 2008; Chotro, Córdoba, & Molina, 1991; Chotro & Molina, 1990, 1992; Dominguez et al., 1996). Exposure to subthreshold levels of ethanol, in terms of teratogenic properties, sensitize the organism to the drug's positive reinforcing effects (Nizhnikov, Molina, Varlinskaya, & Spear, 2006). Fetal ethanol exposure affects later alcohol affinity and strengthens the predisposition to abuse other addictive agents, probably because of common neurobiological mechanisms (Arias & Chotro, 2005a,

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2005b; Bachmanov et al., 2003; Chotro & Arias, 2003; Scher, Richardson, Coble, Day, & Stoffer, 1988). When considering natural reinforcers such as sweet tastants, there are also common neurobiological processes underlying the positive consequences of ethanol and such tastants. Positive correlations have been observed between sweet preference and ethanol affinity in animals and humans with a biological predisposition toward alcoholism (Kampov-Polevoy, Eick, Boland, Khalitov, & Crews, 2004; Kampov-Polevoy, Garbutt, & Janowsky, 1999; Kampov-Polevoy, Tsoi, Zvartau, Neznanov, & Khalitov, 2001; Kampov-Polevoy et al., 2003; Lange, Kampov-Polevoy, & Garbutt, et al., 2010). Alcohol-preferring rats consume higher levels of sucrose and accept more highly concentrated solutions than alcohol-avoiding animals (Fortuna, 2010). The reinforcing effects of sweet tastants and of alcohol partially converge in terms of common mechanisms, implying central release of endogenous opiates and dopamine. When focusing on ethanol reinforcement, opiate antagonism during early life inhibits subsequent alcohol preference. Similarly, opiate antagonism in newborn rats blocks sucrose preference as well as its negative reinforcing effects (Cleary, Weldon, O'Hare, Billington, & Levine, 1996; Garbutt et al., 2009; Philopena, Greenberg, & Smith, 1996).

To our knowledge, there have been no systematic advances in the analysis of how early ethanol experience influences reinforcement by sweet substances. There are only two studies in which the effects of fetal or infantile ethanol exposure were evaluated through sucrose consumption tests. In both studies no clear conclusions were evident due to ceiling effects of sucrose consumption across groups (López & Molina, 1999; Molina et al., 1996).

The present study takes advantage of recently developed learning procedures for the infant rat. Utilizing exploratory patterns (e.g. nose-poking) in infants, we have developed operant conditioning procedures that require minimal amounts of training (Bordner, Molina, & Spear, 2008; Domínguez, Bocco, Chotro, Spear, & Molina, 1993; March, Abate, Spear, & Molina, 2009; Miranda-Morales, Molina, Spear, & Abate, 2012; Pautassi, Truxell, Molina, & Spear, 2008; Ponce, Pautassi, Spear, & Molina, 2006, 2008). In the present study, goals relevant to understanding effects of early ethanol exposure upon subsequent sucrose reinforcement capability were subjected to experimental analysis based on operant associative learning. Given the effects of late prenatal ethanol exposure upon the predisposition to use and abuse this drug, it was decided to expose rat pups to ethanol during the stage of nursing to evaluate: a) the effect of this exposure on the reinforcing capabilities of sucrose, b) whether these capabilities are modified through subsequent revaluation procedures in which the sweetened solution is associated with a low or high ethanol dose, and c) seeking behavior for sucrose as a function of prenatal and revaluation treatments through the use of an extinction procedure. For item "b" it is relevant that recent studies show that during commencement of a state of acute intoxication, a relatively low ethanol dose (0.5 g/kg) exerts profound positive reinforcing effects. In the case of utilizing a higher dose (2.5 g/kg), we have observed minor reinforcing effects (Molina, Ponce, Truxell, & Spear, 2006; Molina, Pautassi, Truxell, & Spear, 2007; Pautassi, Nizhnikov, & Spear, 2009).

It is well known that the representation of an unconditioned stimulus (US) in memory may undergo revaluation if subsequently paired with another stimulus with clear aversive or appetitive unconditioned effects. Infants show significant decrements in aversive responsiveness (US: citric acid) to a given conditioned stimulus (CS), when after conditioning, ethanol's anti-anxiety effects are paired with the original US (Pautassi et al., 2006). In the present experiment the revaluation procedure was meant to determine whether ethanol is capable of revaluating the appetitive consequences of sucrose and if this revaluation is dependent upon prenatal ethanol experience. The study was conducted through 4 sequential phases: i) prenatal vehicle or ethanol exposure during gestational days (GDs) 17–20, ii) operant conditioning using sucrose as a reinforcer during PDs 14–16, iii) a sucrose revaluation procedure where ethanol or vehicle were paired with the sweetened solution, and a subsequent operant session reutilizing sucrose as a reinforcer (PD 17), and iv) an operant extinction session in which sucrose was omitted (PD 18).

Material and methods

Subjects

Animals employed in this study were Wistar-derived rats born and reared at the vivarium of the Instituto Ferreyra (INIMEC-CONICET), Argentina. The animal colony was kept at 22–24 °C and under artificial lighting conditions. Maternal lab chow and water were available *ad libitum*. Vaginal smears of adult females were microscopically analyzed on a daily basis. On the day of proestrus, females (body weights: 200–300 g) were housed overnight with males. Vaginal smears were checked the following morning, and the day of sperm detection was designated GD 0. Day of parturition was designated PD 0.

Animals used in this study were maintained and treated according to the guidelines for animal care established by the Guide for Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996).

Drug treatments during gestation

Twenty dams were utilized. From GDs 17–20, females were intragastrically intubated once each day with either 2.0 g/kg ethanol or water (vehicle). The ethanol dose was achieved by administering 0.015 mL/g of a 16.8% v/v ethanol solution. Ethanol dosage and days of administration were selected based on prior studies showing fetal learning derived from the drug's motivational effects and a lack of deleterious effects of this dose of ethanol upon infantile morphological and behavioral parameters (Domínguez et al., 1996; Domínguez, López, & Molina, 1998; Molina, Chotro, & Domínguez, 1995; Pueta, Abate, Spear, & Molina, 2005).

Infantile intraoral cannulation procedures

On each experimental day (postnatal days, PDs 14–18), male and female pups were removed from their maternal cages and intraorally implanted with a polyethylene cannula to allow the intraoral infusion of liquid reinforcers. This procedure is minimally stressful in younger preweanlings (PD 4) than those here utilized, as operationalized through the release of corticosterone or growth hormone (Spear, Kucharski, & Miller, 1989). We cannot discard certain responsiveness to this apparent mild stressor that, from a procedural perspective, is consistent across groups. We have employed this procedure in a variety of studies and it appears that the cannulation procedure does not seem to overshadow basic sensory and learning capabilities (Dominguez et al., 1993, 1996; Hunt, Kraebel, Rabine, Spear, & Spear, 1993; Pepino, Kraebel, López, Spear, & Molina, 1998; Pepino, López, Spear, & Molina, 1999; Pueta et al., 2005).

The procedure is performed in only 20 s. The location of the cannula varied each conditioning day. In other words, we never cannulated the same cheek on 2 consecutive days. Cannulas were made from 7-cm sections of PE 10 polyethylene tubing (Clay-Adams, Parsippany, NJ). A small flange was created in one end of these devices. The unflanged end was attached to a curved 27-G ½

needle that was pulled through the medial internal surface of the cheek. The flanged end rested over the oral mucosa while the remainder exited from the mouth. Animals were then placed in pairs in isolation chambers kept at 32–34 °C for 6 h until the beginning of the conditioning or extinction sessions.

Operant conditioning sessions

Pups prenatally exposed to ethanol or vehicle were subjected to 3 sequential conditioning sessions (PDs 14–16) where nose-poking was reinforced or not reinforced with intraoral sucrose reinforcement. Six hours after intraoral cannulation, pups were introduced into the operant chambers. These chambers (20 × 20 × 20 cm) were constructed with black Plexiglas®. One of the lateral walls had a hole in it (diameter: 1 cm, distance between the center of the circumference and the floor: 1.5 cm; distance from the adjacent wall: 0.8 cm). A single-channel charge-transfer touch and proximity sensor chip (Model E11 × Evaluation Board; Quantum Research Group, Pittsburgh, PA) was located 1.5 cm away from the hole. The target behavior under training was nose-poke. When the nose of a paired subject touched the sensor chip, an infusion pump (Manostat Cassette R Pump, N.Y.) was activated. This pump delivered intraoral sucrose to the Paired animal as well as to a Yoked control (see below). Prior to each session (PDs 14–16) the anogenital region of each pup was gently stroked with cotton to stimulate defecation and urination. Infants' body weights were then registered (±0.01 g).

Conditioning began by individually placing a Paired pup and its corresponding Yoked control into the operant chambers. Twenty-nine pairs (Paired and Yoked) of pups corresponding to the vehicle prenatal treatment were utilized. In the case of ethanol prenatal exposure, 26 pairs were employed. Each session lasted 15 min. In the case of Paired pups, each nose-poke was reinforced with an intraoral infusion of 10% w/v sucrose (volume: 5 μL, pulse duration: 3 s, reinforcement schedule: fixed ratio 1). Nose-poking of Paired pups also resulted in simultaneous delivery of sucrose to the corresponding Yoked sibling. Hence, Yoked controls received the same reinforcement as Paired pups but had no control over the link between nose-poking and sucrose. Yoked controls also served to determine whether prenatal treatments had any specific effect upon spontaneous exploratory behaviors such as nose-poking.

Nose-poking frequency and latency to perform the first nose-poke were registered. Percent body weight gains during conditioning served to determine levels of sucrose intake ([(Postinfusion weight − preinfusion weight)/(preinfusion weight)] × 100). This variable was employed because pups are able to control the ingestion of fluids infused into their mouths (e.g. Domínguez et al., 1998; López & Molina, 1999; Molina et al., 2006). Experimenters controlling the conditioning sessions were blind relative to the treatments of the animals. After conditioning, pups were returned to the nursing cage.

Sucrose revaluation and subsequent conditioning sessions

On PD 17, animals were cannulated and kept in pairs in the isolation cages for 1 h. Pups were intragastrically administered with ethanol (0.0, 0.5, or 2.5 g/kg). These doses were achieved by administering 0.015 mL of a 0.0, 4.2, or 21% v/v ethanol solution. Five minutes later, pups were individually placed on cotton in individual transparent (Plexiglas®) chambers (20 × 10 × 15 cm). Sucrose (10% w/v) was delivered intraorally in a pulsating manner (3 s on, 57 s off; infusion rate, 0.5 mL/min) during 5 min. Five hours later, a new conditioning session, utilizing sucrose as a reinforcer, was conducted. This session was similar to those conducted during PDs 14–16.

Extinction session

On PD 18, animals were cannulated and kept in pairs in the isolation cages for 6 h. They were then exposed individually to the extinction session (5 min) inside the same Plexiglas® chambers utilized during conditioning. In this session the sweet reinforcer was withheld. The dependent variables under consideration were nose-poking frequency and latency to observe the first target behavior. Withholding the reinforcer facilitated the analysis of the duration of 2 behaviors that, according to prior literature, indicate appetitive (mouthing) or aversive reactivity (wall climbing) (Arias & Chotro, 2005a, 2005b). The relatively short length of this extinction procedure was determined as a function of previous studies indicating rapid decrements in the probability of emission of operant responses in young rats due to the omission of specific reinforcers (Arias, Spear, Molina, Molina, & Molina, 2007).

Determination of blood ethanol concentrations (BELs, mg/dL)

From a different set of animals (litters born from 20 females; 10 pre-treated with vehicle and 10 pre-treated with 2.0 g/kg ethanol), BELs were determined at 15 and 300 min after drug administration on PD 17 (0.5 or 2.5 g/kg). These time periods correspond to the end of the revaluation session and the end of the 4th conditioning session. Each group of animals ranged between 8 and 9 pups. Two 200-μL blood samples were obtained from each pup. BELs were determined through head-space gas chromatography analysis using procedures extensively described in prior studies (Pepino, Abate, Spear, & Molina, 2002; Pepino et al., 1998). The explicit intention of this experiment was to analyze whether prenatal treatments had any specific effects upon ethanol pharmacokinetics, which could in turn explain possible differences arising from ethanol revaluation procedures.

Design, inferential analysis, and litter representation

The first phase of the experiment (conditioning sessions during PDs 14–16) responds to a factorial design defined by 2 main factors: prenatal treatment (0.0 or 2.0 g/kg ethanol) and conditioning treatment (paired or yoked). Conditioning sessions imply repeated measures. Nose-poke frequencies, latencies to perform the first nose-poke in each session and body weight gains resulting from sucrose infusions were analyzed via 3-way between-within ANOVAs (prenatal treatment × conditioning procedure × days). *Post hoc* comparisons (Tukey's Honestly-Significant-Differences) served to further analyze the locus of significant interactions. These *post hoc* tests were also utilized following specific ANOVAs employed for the conditioning session after revaluation as well as during the extinction session.

During the morning of PD 17, each pair of pups (Paired and Yoked) representative of each prenatal treatment was assigned to a given ethanol dose employed during the upcoming revaluation procedure (0.0, 0.5, or 2.5 g/kg). Only one Paired and its corresponding Yoked control from a given litter were assigned to a given revaluation procedure. This quasi-random procedure was employed to avoid confusion between litter and treatment effects (Holson & Pearce, 1992). Performance scores following revaluation were statistically analyzed through a 3-way between-within ANOVA defined by prenatal, conditioning, and revaluation treatments. In the case of the extinction session a similar 3-way between-within ANOVA was utilized.

It is important to note that preliminary analysis of the data indicated that sex was never found to exert a significant main effect or to interact with other factors. Hence, this factor was collapsed across all the remaining treatments.

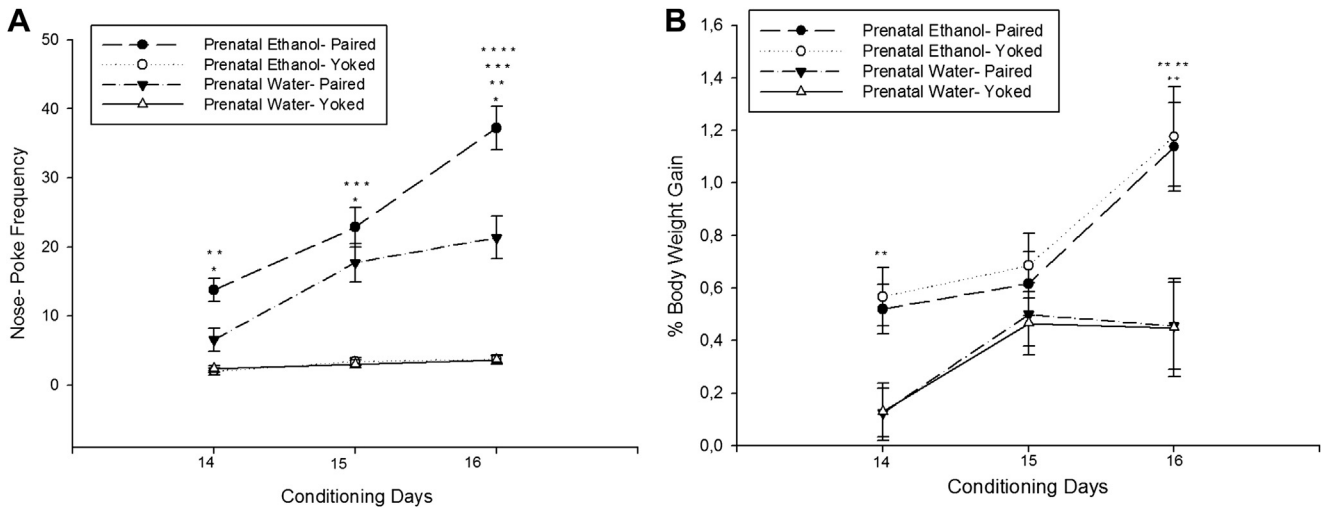

Fig. 1. A) Operant responses; B) % Body weight gain during PDs 14–16 as a function of prenatal treatment and conditioning status. Vertical bars indicate standard errors of the means. Asterisks indicate a significant difference from: *Yoked controls; **Water Prenatal Treatment; ***PD 14; ****PD 15.

Results

Litter size and infantile body weights

Prenatal treatments (0 or 2.0 g/kg ethanol) exerted no significant main effects upon the variables under consideration. At birth, litter sizes were as follows: Vehicle, 8.78 ± 0.31 pups and Ethanol, 9.34 ± 0.25 pups (mean ± SEMs). Infantile body weights at commencement of treatment were also not affected. Body weights significantly increased as a function of the passage of days, $F(4,92) = 4.81$, $p < 0.0001$.

Operant performance during conditioning (PDs 14–16)

The ANOVAs took into account prenatal treatment, conditioning procedure, and days as repeated measures. Nose-poke frequencies were significantly affected by prenatal treatment, conditioning status, and days of training: $F(1,59) = 12.96$, $F(1,59) = 179.62$, $F(2,118) = 33.33$, respectively, all p's < 0.001. The following interactions also attained significance: prenatal treatment × conditioning [$F(1,59) = 13.85$, $p < 0.001$] and conditioning × days [$F(2,118) = 29.62$, $p < 0.001$]. The 3-way interaction also reached statistical significance [$F(2,118) = 3.16$, $p < 0.05$]. *Post hoc* tests indicated that at commencement of training (PD 14), Paired and Yoked pups prenatally exposed to vehicle did not differ. In the case of prenatal ethanol treatment, nose-poking was significantly higher in Paired relative to Yoked pups. When focusing on the performance of Paired subjects prenatally exposed to ethanol, a significant increase in operant responding was observed across the 3 conditioning sessions. Paired pups exposed to water *in utero* showed a significant increase in responding in the 2nd and 3rd conditioning session relative to the initial training session. Yoked controls, independently from prenatal treatment, showed a minimal amount of responding. Paired subjects that experienced alcohol *in utero* always had higher levels of responding than Yoked pups. As can be observed, conditioning was effective in terms of increasing the probability of nose-poking execution as a function of the contingency between this behavior and sucrose reinforcement. Of major importance for the goals of this investigation, Paired subjects prenatally exposed to ethanol were more sensitive to the reinforcing effects of sucrose than ethanol-naïve paired infants. This phenomenon was already observed during initial training (PD 14) and it reached a maximum difference after 2 training trials (PD 16). These results have been depicted in Fig. 1A.

Latencies to exhibit the first target behavior were significantly affected by conditioning status [$F(1,59) = 68.84$], days [$F(2,118) = 9.72$], and the interaction between these two factors [$F(2,118) = 11.23$], all p's < 0.001. Latencies were high across all groups during the first session. In both Paired groups, latencies significantly decreased during the 2nd and 3rd sessions (all p's < 0.001). In Yoked pups, latencies were consistently high across

Table 1
Operant and intake patterns during PD 16.

	Prenatal and revaluation treatment			Prenatal and revaluation treatment		
	Water			Ethanol		
	0.0 g/kg	0.5 g/kg	2.5 g/kg	0.0 g/kg	0.5 g/kg	2.5 g/kg
Total operant responses						
Paired	23.72 ± 5.30	17.80 ± 5.57	22.3 ± 5.5	43.0 ± 5.61	33.40 ± 5.55	35.30 ± 5.56
Yoked	2.81 ± 1.01	2.60 ± 1.05	5.60 ± 1.04	4.3 ± 1.06	3.2 ± 1.06	3.60 ± 1.07
Latency of the 1st response						
Paired	86.27 ± 34.84	152.0 ± 36.54	163.9 ± 35.54	88.72 ± 36.54	103.4 ± 36.54	143.60 ± 36.54
Yoked	427.90 ± 100.9	583.5 ± 105.8	458.3 ± 106	456.9 ± 105.7	369.1 ± 105.8	411 ± 107.8
% Body weight gain						
Paired	0.45 ± 0.28	0.42 ± 0.3	0.48 ± 0.29	1.5 ± 0.31	1.02 ± 0.29	0.88 ± 0.3
Yoked	0.46 ± 0.29	0.35 ± 0.31	0.52 ± 0.31	2.07 ± 0.2	0.53 ± 0.31	0.91 ± 0.28

Values indicate mean ± standard error of the mean.

Table 2
Latencies [s] to perform the first nose-poke during PDs 14–18.

Prenatal Treatment-Learning Condition	PD 14	PD 15	PD 16	PD 17			PD 18[a]		
				Revaluation dose			Revaluation dose		
				0.0 g/kg	0.5 g/kg	2.5 g/kg	0.0 g/kg	0.5 g/kg	2.5 g/kg
Water-Paired	392.5 ± 34.4	188.1 ± 28.9	132.5 ± 20.7	164.4 ± 44.2	114.9 ± 46.4	138.3 ± 46.3	101.7 ± 56.1	263.3 ± 58.8	63.4 ± 58.9
Water-Yoked	428.5 ± 6.45	452.1 ± 56.1	487.9 ± 58.8	555.8 ± 94.6	291.3 ± 99.28	560.8 ± 99.2	348.2 ± 88.7	451.5 ± 93.3	264.9 ± 93.32
Alcohol-Paired	391.9 ± 34.6	102.16 ± 29.4	111.9 ± 29.4	111.7 ± 46.3	98.2 ± 46.36	214.9 ± 56.6	84 ± 58.8	48.7 ± 58.9	124.8 ± 65.8
Alcohol-Yoked	422 ± 57.39	390.36 ± 57.1	412.33 ± 59.8	332.2 ± 99.2	519.5 ± 99.2	630.9 ± 99.3	362.1 ± 93.32	443.4 ± 93.2	536.8 ± 104.3

Values indicate mean ± standard error of the mean.
[a] Indicates the revaluation doses that were previously employed during PD17.

days. These results again indicate robust conditioning mediated by sucrose (Table 2).

The analysis of sucrose consumption during PDs 14–16 indicated two main significant effects. Sucrose intake increased as a function of the passage of days [$F(2,118) = 6.93$, $p < 0.01$], a result which is consistent with progressively higher operant responding in Paired pups and also implies higher sucrose availability for Yoked controls. Also consistent with the fact that a prenatal history of alcohol exposure exacerbated operant responding mediated by sucrose, the ANOVA showed a significant main effect of gestational treatment [$F(1,59) = 14.87$, $p < 0.001$], i.e., pups prenatally exposed to ethanol drank significantly higher amounts of the sweetened solution (Fig. 1B).

In summary, sucrose served as a positive reinforcer that modified the probability of nose-poking when this behavior was contingent upon the sweet solution. Operant performance correlated positively with number of conditioning trials, an effect absent in Yoked controls. In turn, prenatal ethanol exposure resulted in heightened sensitivity to sucrose's reinforcing properties.

Post-revaluation conditioning session

As described, pairs of Paired and Yoked pups from each prenatal treatment were assigned to different revaluation procedures during PD 17 (sucrose associated either with 0.0, 0.5, or 2.5 g/kg ethanol). It is important to note that this new factor exerted no significant main effect or interactions relative to scores during the last training session (PD 16). Hence, there were no carryover differences in Paired and Yoked pups within each prenatal treatment that could affect the forthcoming revaluation procedures. Table 1 depicts all the dependent variables attained during PD 16. As can be observed, differences between groups were due only to prenatal and conditioning treatments.

Following sucrose-ethanol pairings, pups were again subjected to a sucrose-reinforced operant session. The ANOVA took into account the following factors: prenatal treatment, conditioning status, and revaluation procedures. In this session, nose-poking behavior was no longer affected by prenatal treatment. Revaluation procedures and conditioning status exerted main significant effects: $F(2,55) = 4.82$ and $F(1,55) = 100.00$, respectively, p's < 0.05. These two factors also significantly interacted: $F(2,55) = 4.37$, $p < 0.05$. According to *post hoc* tests, a low ethanol dose (0.5 g/kg) was capable of inflating the reinforcing capability of sucrose. Paired pups from both prenatal treatments treated with this dose exhibited significantly higher levels of operant responding when compared with all the remaining groups (all p's < 0.001). If water (0.0 g/kg) was paired with sucrose, the inflating effect was absent. Indeed, Paired and Yoked groups revaluated with water exhibited similar nose-poking frequencies relative to the one observed prior to revaluation treatment. When employing a high ethanol dose (2.5 g/kg) during revaluation, operant responsiveness was very low in Paired pups (significantly lower relative to Paired pups revaluated with either 0.0 or 0.5 g/kg). In summary, a low dose of the drug exacerbates sucrose's subsequent reinforcing capability while a high dose appears to decrease sucrose reinforcement (Fig. 2A).

During the post-revaluation session, it was again observed that latencies to perform the first operant-based exploratory response were significantly lower in Paired pups relative to Yoked controls: $F(1,55) = 72.28$, $p < 0.00001$ (Table 2). In terms of consumption of

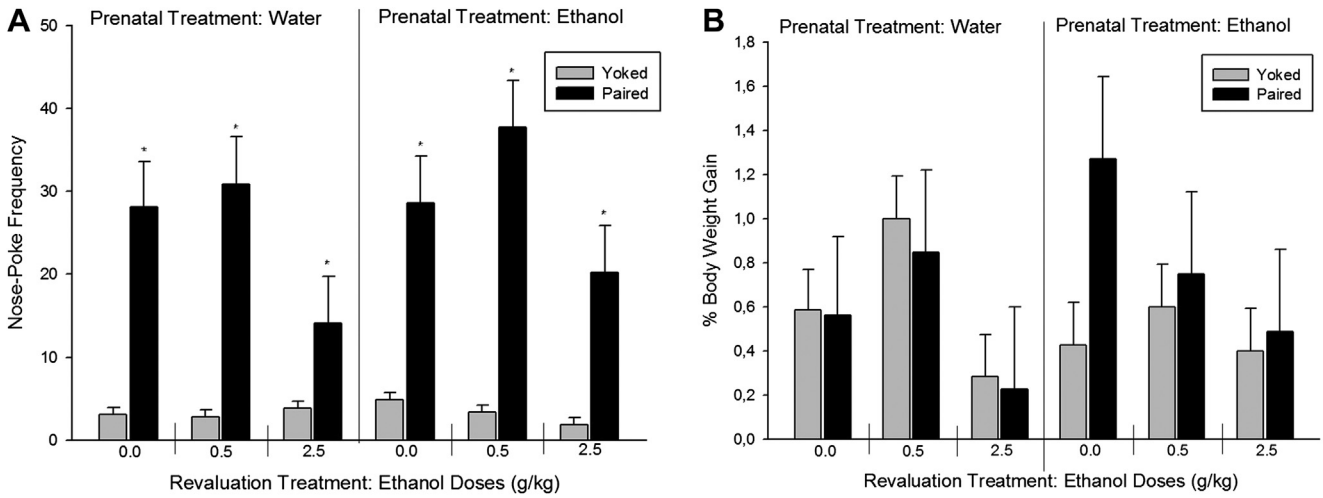

Fig. 2. A) Operant responses; B) % Body weight gain during PD 17 as a function of prenatal treatment, conditioning status, and revaluation treatment. Vertical bars indicate standard errors of the means. Asterisks indicate a significant difference from: *Yoked controls.

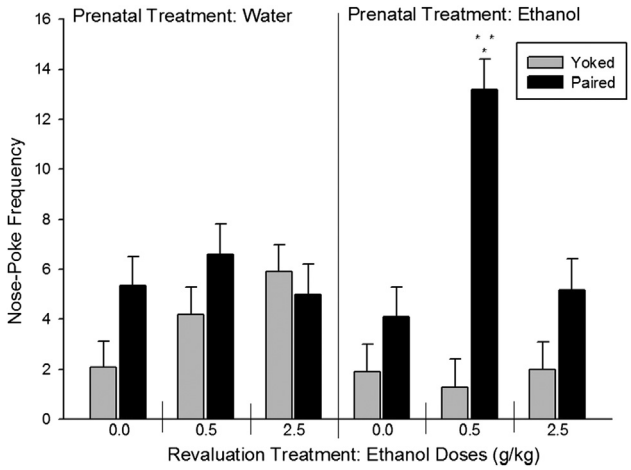

Fig. 3. Operant responses during PD 18 (extinction session) as a function of prenatal treatment, conditioning status, and revaluation treatment. Vertical bars indicate standard errors of the means. Asterisks indicate a significant difference from: *Yoked controls, **Other Paired groups.

the reinforcer (% body weight gain), the ANOVA did not show significant main effects or interactions (Fig. 2B).

Extinction session

When the reinforcer was withheld, the ANOVA (prenatal × conditioning × revaluation) indicated significant main effects of prior conditioning and revaluation treatments: $F(1,55) = 27.03$, $p < 0.00001$ and $F(2,55) = 7.84$, respectively, $p < 0.01$. The interactions prenatal × conditioning, revaluation × conditioning, and prenatal × conditioning × revaluation also attained significance: $F(1,55) = 8.70$, $F(2,55) = 6.35$, and $F(2,55) = 4.68$, respectively, all p's < 0.05. As can be observed in Fig. 3, Yoked pups were practically unresponsive. Paired pups prenatally exposed to ethanol that subsequently experienced the revaluation treatment with a 0.5 g/kg dose showed a significantly higher rate of responding when compared with any of the remaining groups, including pups prenatally exposed to water and revaluated with the same ethanol dose, all p's < 0.001. In other words, the inflating effect of sucrose reinforcement mediated by 0.5 g/kg ethanol observed during the post revaluation conditioning session (PD 17) was still observable during extinction but only in pups with a prenatal ethanol history. The persistence of this apparent inflating effect was no longer observed in Paired pups prenatally exposed to water (Fig. 3).

Latencies to exhibit the first nose-poke during extinction again revealed the impact of prior conditioning upon responsiveness. All Paired pups exhibited significantly lower latencies when compared with Yoked siblings: $F(1,53) = 45.95$, $p < 0.0001$ (Table 2).

A similar pattern of results relative to nose-poking frequency was observed when processing mouthing, a behavior associated with positive hedonic behavioral profiles. From a descriptive perspective, it appeared that prenatal treatment enhanced the inflation effect derived from the association of a low ethanol dose (0.5 g/kg) and sucrose. Nevertheless, the pertinent ANOVA did not show a 3-way interaction, as was the case with nose-poking. In this case, conditioning, revaluation, and the interaction between these factors exerted significant effects: $F(1,55) = 70.47$, $F(2,55) = 16.67$, and $F(2,55) = 15.98$, respectively, all p's < 0.0001. All Paired groups differed from the corresponding Yoked controls. Furthermore, Paired pups revaluated with 0.5 g/kg showed a significantly higher duration of mouthing when compared with Paired groups revaluated with either 0 or 2.5 g/kg ethanol.

When focusing on the presumably aversive behavior, wall-climbing, the ANOVA indicated a significant interaction between conditioning and revaluation, $F(2,55) = 3.77$, $p < 0.05$. In this case, wall-climbing duration was significantly higher in Paired subjects revaluated with the high ethanol dose (2.5 g/kg) relative to the corresponding Yoked controls, independently of prenatal treatment (all p's < 0.001). The interactions between conditioning and revaluation attained when analyzing the previously mentioned behaviors are depicted in Fig. 4.

In summary, the results indicate that sucrose-seeking behavior, operationalized through nose-poking persistence during extinction, was significantly affected by the interaction of 2 factors. Prenatal treatment with ethanol, coupled with a revaluation procedure in which a low ethanol dose (0.5 g/kg) was paired with sucrose, potentiated seeking behaviors when the sweet reinforcer was withheld. There were also indications that a behavior (mouthing) related with the acceptance and consumption of a positive reinforcer such as sucrose, was dependent upon the nature of preceding revaluation procedures. On the contrary, if sucrose was paired with a high ethanol dose (2.5 g/kg), a behavior

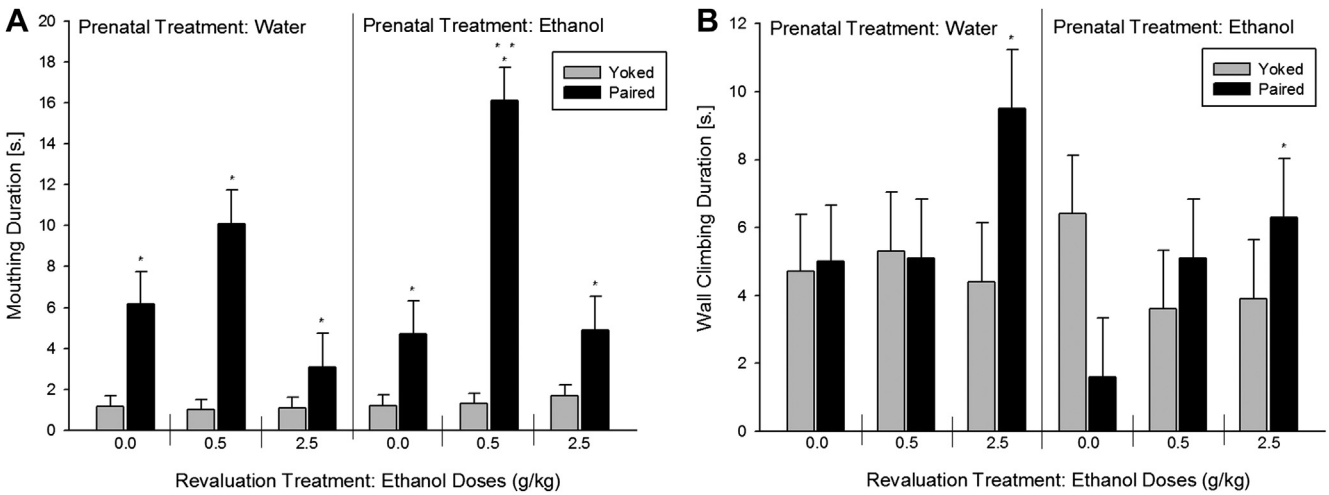

Fig. 4. A) Mouthing duration; B) Wall climbing duration during PD 18 (extinction session) as a function of prenatal treatment, conditioning status, and revaluation treatment. Vertical bars indicate standard errors of the means. Asterisks indicate a significant difference from: *Yoked controls, **Other Paired groups.

characteristic of aversion, wall climbing, was particularly evident in Paired pups.

Blood ethanol levels (BELs, mg/dL)

BELs varied as a function of dose, post-administration time and the interaction between these factors: $F(1,58) = 391.30$, $F(1,58) = 394.59$, and $F(1,58) = 159.96$, respectively, all p's < 0.001. Prenatal treatment did not significantly affect BELs and did not interact with any of the other factors. When utilizing a 0.5 g/kg dose, BELs at 15 min were equivalent to 35.07 ± 1.76 mg/dL. Minimal levels were detected 5 h later (1.62 ± 1.06). Fifteen minutes after administering the highest ethanol dose (2.5 g/kg), BELs were equivalent to 185.38 ± 7.76 mg/dL. Five hours later, BELs dropped to 34.68 ± 4.36 mg/dL.

General discussion

The main results of the present study can be summarized as follows. A) Sucrose was an effective positive reinforcer in infants, as expected. B) Prenatal exposure to ethanol potentiated the reinforcing capability of the sweet tastant. C) When using revaluation procedures in which sucrose was paired with a low or high ethanol dose, there were indications of differential hedonic effects. During the post-revaluation conditioning session, pups that experienced sucrose paired with 0.5 g/kg ethanol exhibited heightened operant responding. In this session there were also indications of a devaluation effect exerted by a high ethanol dose. Paired pups exposed to the association between sucrose and 2.5 g/kg ethanol exhibited reductions in operant performance relative to groups revaluated with 0.0 or 0.5 g/kg ethanol. D) During extinction, the inflating hedonic effects of 0.5 g/kg ethanol were still observable, particularly when focusing on responses indicative of sucrose-seeking behavior. This effect was more robust when pups were prenatally exposed to the drug. E) None of the effects related with prenatal ethanol history appear to depend upon differential BELs attained during the revaluation procedure.

These results strengthen the hypothesis that prenatal exposure to ethanol sensitizes the organism to the drug's reinforcing effects (Nizhnikov et al., 2006; Pautassi, Nizhnikov, Spear, & Molina, 2012). Moderate exposure to ethanol late in gestation was sufficient to potentiate appetitive responsiveness to sucrose when this natural reinforcer was later paired with a low ethanol dose (0.5 g/kg). The effect related with sucrose-seeking behavior was observable during the extinction session. Pups conditioned with sucrose exhibited marked seeking behavior of this reinforcer if the sucrose had been paired with a relatively low dose of ethanol. The effect was maximal when pups had a prior prenatal history with ethanol. Why was this effect not observed during the conditioning session that followed revaluation? As can be observed in Fig. 1 and Table 1, pups prenatally exposed to ethanol also exhibited heightened responding when reinforced with sucrose. Indeed, prenatal ethanol exposure potentiated the reinforcing capabilities of sucrose. It is possible that prior to revaluation, this group of animals had already reached a functional ceiling effect in terms of operant performance. This parametric obstacle is likely to be removed when explicitly omitting the reinforcer (extinction) and hence decreasing the probability of operant responding. When considering the effects of prenatal ethanol history, the results indicate subsequent heightened incentive value of sucrose while prior research shows a marked sensitization to ethanol doses capable of establishing appetitive conditioning (Nizhnikov et al., 2006). The summation of these effects might be responsible for the persistence of sucrose-seeking behavior during extinction.

Some effects of the revaluation procedure related with the use of a low ethanol dose were still observable during extinction across prenatal treatments. Mouthing was higher in Paired pups revaluated with 0.5 g/kg ethanol relative to pertinent Yoked controls. With revaluation that included presentation of a high ethanol dose (2.5 g/kg), Paired pups were more likely to exhibit aversive responding as indicated through heightened wall climbing. These behavioral patterns support the hypothesis that early in ontogeny, infant rats are sensitive to biphasic motivational effects of ethanol (Molina, Pautassi, et al., 2007).

Why does prenatal ethanol exposure sensitize the organism to the positive hedonic properties of a natural reinforcer such as sucrose? Is this effect relatively specific to sucrose or does it also apply to other reinforcers as well? There is clear evidence that late prenatal exposure to ethanol does not affect the rate of operant learning with natural reinforcers such as water or milk (Bordner et al., 2008; March et al., 2009). Also there has been no indication that this prenatal ethanol treatment suggests general alterations in terms of sensory detection and discrimination of different odors and tastes (Abate, Pepino, Domínguez, Spear, & Molina, 2000; Domínguez et al., 1998) or alterations in non-associative (Arias et al., 2008) or associative learning processes (Bordner et al., 2008; Domínguez et al., 1993; March et al., 2009). Yet, we have observed some hyperreactivity to novel stimuli as a function of prenatal ethanol exposure (Domínguez et al., 1996). This is a major problem when considering operant conditioning implying a given behavior and sensory stimulation provided by the intraoral infusion of a liquid. As mentioned, prenatal treatment does not affect behavioral performance when using reinforcers other than the one employed here. More importantly, Yoked controls did not differ in any of the experimental phases under analysis although they received the same sensory stimulation through intraoral infusions as Paired groups. This observation supports the notion that nose-poking does not simply increase as a function of stimulatory effects associated with the conditioning or prenatal status.

There are two hypotheses, not mutually exclusive, that should be taken into account when considering fetal alcohol sensitization to sucrose's reinforcing effects. From a psychophysical perspective, rats perceive ethanol as a tastant configured by sweet and bitter components (Bachmanov et al., 2003). Experiences with ethanol's chemosensory cues generalize to sucrose-quinine configurations (Kiefer & Lawrence, 1988). Near-term fetuses have been observed to process ethanol's chemosensory cues leading to heightened acceptance of these cues and those that define a sucrose-quinine solution (Domínguez et al., 1996). Although this sensory familiarization effect and its generalization appear to explain, at least partially, subsequent sucrose acceptance in this study, it seems inadequate when considering the fact that prenatal ethanol exposure facilitated the hedonic synergism between ethanol and sucrose during revaluation procedures. It is unlikely that pups process ethanol's chemosensory cues when receiving a low dose (0.5 g/kg) via i.g. administration (Molina & Chotro, 1989a). Prior studies show that direct elimination of the drug through alveolar excretion, saliva, or urine recruits sensory processing only when relatively large amounts (2–3 g/kg) of the drug are administered (Arias & Chotro, 2006; Molina & Chotro, 1989b). Lower doses do not reach levels of excretion capable of influencing sensory or perceptual processing. Yet we have found, through the use of operant and pavlovian procedures, that neonates and infants are highly sensitive to the interoceptive motivational properties of 0.5 g/kg ethanol (Bordner et al., 2008; Molina, Pautassi, et al., 2007; Nizhnikov et al., 2006). Interestingly, the positive hedonic effects of low to moderate ethanol doses are rapidly observed during early ontogeny, but not later in development (Pautassi et al., 2009). Recent studies indicate

that the reinforcing capability of ethanol early in life is strongly modulated by the release of endogenous opiates. Tests of infants with operant procedures clearly indicate that opiate antagonism blocks the reinforcing capability of low ethanol doses (Miranda-Morales et al., 2012). Similarly, fetal inhibition of opiate release blocks subsequent ethanol intake and preference patterns determined through prenatal exposure to the drug (Arias & Chotro, 2005a). When considering sucrose, central, but not peripheral, opiate antagonism decreases consumption (Garbutt et al., 2009). In light of these considerations it is possible that fetal sensitization to ethanol's reinforcing effects are summated or integrated with the appetitive consequences of sucrose through common mechanisms comprising endogenous opiate bioavailability.

In the present study, fetal ethanol exposure potentiated later revaluation effects of the drug. This potentiation, based on the explicit association with sucrose, was observed only with a low ethanol dose (0.5 g/kg). In the case of the higher dose (2.5 g/kg), evidence suggested that sucrose acceptance was instead reduced after revaluation. Infants are indeed sensitive to biphasic hedonic effects of ethanol, and generally, low doses exert positive or negative (anti-anxiety) reinforcement effects while aversive components of the state of intoxication are recruited with doses that exceed 2 g/kg (Molina, Pautassi, et al., 2007; Pautassi et al., 2009; Spear & Molina, 2005). After revaluation with 2.5 g/kg ethanol, paired pups decreased their level of operant responsiveness relative to a prior conditioning session (see Fig. 2). During extinction, Paired pups subjected to this high ethanol dose during revaluation also showed heightened levels of a behavior (wall climbing) consistent with memory for an aversive episode. This devaluation effect is likely the result of learned taste aversions comprising sucrose as a conditioned stimulus and high ethanol as an aversive unconditioned stimulus (Arias, Pautassi, Molina, & Spear, 2010). Notice that when considering devaluation, prenatal history with ethanol did not affect these aversion-like behaviors. This result is consistent with those of prior studies indicating that early experience magnifies the appetitive consequences of the drug but does not seem to affect its aversive consequences that appear to be modulated by central and peripheral mechanisms leading to the perception of gastrointestinal distress (Arias & Chotro, 2006; Nizhnikov et al., 2006; Pueta et al., 2005).

In conjunction with prior literature, the present results indicate that alcohol exposure in the near-term fetus which leads to maternal and fetal blood levels equivalent to 120 mg% (Domínguez et al., 1996) not only affects consumption patterns of this drug and other substances of abuse, but also likely sensitizes the organism to the reinforcing effects of sweet tastants. It is important to note that this level of ethanol exposure does not produce gross alterations in terms of overall body weight and size, the weight of different cerebral structures, cellular migration processes leading to the configuration of the olfactory bulbs, or evident deficits in sensory processing or basic learning capabilities (Domínguez et al., 1996; Pueta, Rovasio, Abate, Spear, & Molina, 2011). These effects appear to persist during infancy and are likely to affect early disposition to self-administer sucrose. Furthermore, the organism appears to maintain a positive hedonic content capable of increasing the natural reinforcing effects of sweet substances, substances that share common sensory or motivational neurobiological mechanisms with ethanol (Bachmanov et al., 2003; Molina, Spear, et al., 2007; Spear & Molina, 2005). These considerations are especially pertinent when considering recent literature indicating a genetic link between predisposition to use and abuse ethanol and sweet substances. The thorough work of Mennella, Pepino, Lehmann-Castor, and Yourshaw (2010) indicates that children with a positive family history of alcoholism are more likely to accept highly concentrated sweet solutions and to prefer sweet-tasting foods. Apparently, a similar link might be established when ethanol exposure represents a congenital rather than a genetic factor.

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