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Prenatal exposure to ethanol during late gestation facilitates operant self-administration of the drug in 5-day-old rats

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Abstract

Prenatal ethanol exposure modifies postnatal affinity to the drug, increasing the probability of ethanol use and abuse. The present study tested developing rats (5-day-old) in a novel operant technique to assess the degree of ethanol self-administration as a result of prenatal exposure to low ethanol doses during late gestation.

On a single occasion during each of gestational days 17–20, pregnant rats were intragastrically administered ethanol 1 g/kg, or water (vehicle). On postnatal day 5, pups were tested on a novel operant conditioning procedure in which they learned to touch a sensor to obtain 0.1% saccharin, 3% ethanol, or 5% ethanol. Immediately after a 15-min training session, a 6-min extinction session was given in which operant behavior had no consequence. Pups were positioned on a smooth surface and had access to a touch-sensitive sensor. Physical contact with the sensor activated an infusion pump, which served to deliver an intraoral solution as reinforcement (Paired group). A Yoked control animal evaluated at the same time received the reinforcer when its corresponding Paired pup touched the sensor.

Operant behavior to gain access to 3% ethanol was facilitated by prenatal exposure to ethanol during late gestation. In contrast, operant learning reflecting ethanol reinforcement did not occur in control animals prenatally exposed to water only. Similarly, saccharin reinforcement was not affected by prenatal ethanol exposure.

These results suggest that in 5-day-old rats, prenatal exposure to a low ethanol dose facilitates operant learning reinforced by intraoral administration of a low-concentration ethanol solution. This emphasizes the importance of intrauterine experiences with ethanol in later susceptibility to drug reinforcement. The present operant conditioning technique represents an alternative tool to assess self-administration and seeking behavior during early stages of development.

Keywords

prenatal ethanol exposure; late gestation; ethanol reinforcement; operant self-administration; infant rat

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Introduction

The motivational properties of ethanol can modulate appetitive and consummatory behaviors, particularly during early stages of ontogeny (Pautassi, Nizhnikov, & Spear, 2009). Epidemiological and preclinical studies have indicated that early experiences with ethanol produce a heightened affinity for ethanol in both humans and rats (Baer, Barr, Bookstein, Sampson, & Streissguth, 1998; Baer, Sampson, Barr, Connor, & Streissguth, 2003; Chotro, Arias, & Laviola, 2007; Spear & Molina, 2005). In this context, we have observed that neonatal rats exhibit positive responses to postabsorptive ethanol effects when very low ethanol doses (0.125 or 0.25 g/kg) are associated with a surrogate nipple (Nizhnikov, Molina, Varlinskaya, & Spear, 2006). Moreover, the range of ethanol doses capable of having reinforcing effects is increased (0.25, 0.5, and 0.75 g/kg) when those pups were prenatally exposed to ethanol (Nizhnikov et al., 2006). Near-term fetuses seem to rapidly associate olfactory cues present in the amniotic fluid with postabsorptive effects of ethanol (Abate, Pepino, Domínguez, Spear, & Molina, 2000; Abate, Pueta, Spear, & Molina, 2008). These studies have been partially confirmed in humans. Maternal intake of ethanol during pregnancy resulted in the fetus' detection of ethanol odor. Response patterns of 1- and 2-day-old babies to ethanol chemosensory cues appear to be modulated by levels of alcohol consumed by their mothers during pregnancy (Faas, Spontón, Moya, & Molina, 2000).

In the preclinical literature the use of operant techniques to evaluate the motivational effects of ethanol during early ontogeny has been recently documented in the literature (Bordner, Molina, & Spear, 2008; March, Abate, Spear, & Molina, 2009; Miranda-Morales, Molina, Spear, & Abate, 2010, 2012a; Miranda-Morales, Spear, Nizhnikov, Molina, & Abate, 2012b; Ponce, Pautassi, Spear, & Molina, 2008). In 1-day-old rats, ethanol's sensorial properties were sufficient to promote vigorous operant responses when ethanol ingestion was contingent upon operant behavior. Furthermore, postabsorptive ethanol effects attained with low levels of ethanol in blood (20 mg/dL) were sufficient to maintain a relatively high level of seeking behavior during an extinction session (Bordner et al., 2008). March et al. (2009) and Miranda-Morales et al. (2010) extended these results and indicated that prenatal experience with ethanol during the last stages of gestation increased the probability of executing these operant responses to obtain ethanol or a compound that mimics the sensory attributes of the drug. Other studies showed that this sensitivity to ethanol reinforcement during early ontogeny can also be observed during the second postnatal week of the infant rat. For instance, self-administration of ethanol was established in terms of operant responding in preweanling rats with no previous exposure to the drug (Miranda-Morales, Molina, et al., 2012a; Miranda-Morales, Spear, et al., 2012b; Ponce et al., 2008).

Considering the study of Arias, Spear, Molina, & Molina (2007), who showed rapid acquisition of operant conditioning in 5-day-old rat pups using milk as reinforcer, we aimed to test similar operant conditioning in rat pups (5 days old) using one of two alternative ethanol concentrations or saccharin as reinforcers. This operant conditioning was tested as a function of prenatal experience with low ethanol doses during the last stages of gestation. Results from the present study will help to determine if increased ethanol operant self-administration during very early stages of development is circumscribed to the neonatal period or can be extended to the later stages of development, and how prenatal ethanol experience may modulate these ethanol-related behaviors.

Materials and methods

Subjects

Sprague–Dawley rats were employed in the study (106 infant pups derived from 19 females). These animals were born and reared in the vivarium at the Center for Development and Behavioral Neuroscience (AAALAC-accredited facility, Binghamton University, Binghamton, NY, USA). For breeding, animals were housed in groups of 1 male and 2 females in wire mesh hanging cages. Every day female animals were checked for plugs and the day a plug was found was considered gestational day zero (GD0). Immediately after the plug was found the female was removed from the cage and individually housed in standard maternity cages lined with pine shavings. Cages were checked for births daily, and the day of birth was considered postnatal day zero (PD0). The colony was maintained at 22–24 °C under a 14 h/10 h light/dark cycle. The experiments were approved by the Binghamton University Institutional Review Committee for the Use of Animal Subjects and were in compliance with the NIH Guide for the Care and Use of Laboratory Animals (National Institutes of Health, 1996).

Prenatal Treatment

From GD17 to GD20 a randomly selected set of dams received a daily intragastric (i.g.) administration of a 12.6% v/v ethanol solution (volume of administration, 0.01 mL/g). This administration procedure resulted in an ethanol dose equivalent to 1.0 g/kg of body weight. A second group of females received an equivalent number of i.g. administrations but only with water, which served as the vehicle of ethanol. Female rats remained undisturbed until parturition. The ethanol dose selected for prenatal treatment was based on previous studies. Nizhnikov et al. (2006) reported that prenatal exposure to 1 g/kg ethanol during late gestation increased the range of ethanol doses found appetitively reinforcing in newborn rats. This study was performed in Sprague-Dawley rats, as the one employed for the present preparation.

Operant Conditioning Test

The apparatus employed for operant conditioning was similar to the one employed by Arias et al. (2007). Rat pups were placed in a semi-supine position over the internal cotton surface of a respirator mask (3M Particulate Respirator 8576). This holding “seat” was positioned over a metal support box. The angle between the pup's body and the surface of the box was equivalent to 40°. This position allowed the pup to rest its rear limbs over the filter of the respirator mask. Each pup was strapped and buckled into a spandex “vest” with a “v”-shaped neck designed to avoid restriction of head movements. Two holes (0.5 cm in diameter) in this vest allowed the pup's forelimbs to be free. The vest produced no apparent discomfort or major restriction of behavior. An articulated iron stand equipped with alligator clips allowed positioning of a touch-sensitive bronze sensor (4 cm long × 0.5 cm wide) 1.5 cm away from the pup's mouth and perpendicular to the base of the holding seat. The tip of this sensor was kept equidistant from each forepaw. Physical contact with the sensor activated an infusion pump (Kashinsky-Rozboril, Model 5/2000, Binghamton, NY) equipped with a 2-mL micrometer syringe (Gilmont Instruments; Barrington, IL) filled with a specific solution. The sensor was connected to a single channel charge-transfer sensor chip (Model E11x Evaluation Board; Quantum Research Group, Pittsburgh, PA) which in turn controlled the infusion pump. The pump was set to deliver 1 μL of solution whenever the sensor was activated (the schedule of reinforcement was set as a fixed ratio 1). The sensor chip was also connected to a device (Simple Logger II, Model L404, AEMC Instruments, USA; sensitivity: 1 response/0.01 s) which registered in real time the number of sensor contacts displayed by the animals. A section of PE50 polyethylene tubing was attached to a

needle fitted into the tip of the syringe, and the free end of the PE50 tubing was inserted into a thinner section PE10 tubing surgically implanted into the pup's cheek.

Across the experiment, intraoral infusion was conducted by means of polyethylene cannulas positioned in the pup's cheek. Intraoral cheek cannulation is minimally stressful in preweanlings (Spear, Specht, Kirstein, & Kuhn, 1989) and has been shown to be a useful tool for the assessment of responsiveness to tastants, early in life (Arias & Chotro, 2005; Arias et al., 2007; Bordner et al., 2008). This intraoral cannulation procedure has been extensively described in previous studies (for detailed information please see Abate, Spear, & Molina, 2001; Arias et al., 2007; Chotro & Arias, 2003; Miranda-Morales et al., 2010).

On PD5, pups were removed from their maternal cages, cannulated, and placed in holding cages (45 × 20 × 20 cm) partially filled with clean wood shavings for 3 h. The floor of the cage was maintained at 27 °C (± 1 °C) using a heating pad. Pups were evaluated in 2 sessions. The first was the 15-min training session in which animals received a solution (reinforcer) contingent upon their operant behavior (i.e., sensor contact). Immediately after, a 6-min extinction session was performed, in which no reinforcer was available after the execution of target behavior. Before commencement of the training session, pups were quasi-randomly assigned to either a Paired (P) or a Yoked (Y) condition group. Each P subject was evaluated with its corresponding Y control which, whenever possible, was matched in terms of sex and body weight. Prior to conditioning, the anogenital region of preweanlings was gently stroked with a cotton swab to stimulate defecation and void the subject's bladder. The animal's weight was then registered (± 0.01 g; balance model BP410; Sartorius Corporation, Edgewood, NY). Following these procedures, P and Y animals were placed in the corresponding conditioning devices and their respective cannulas were attached to the tubing exiting from the infusion pump. The touch-sensitive sensors were then placed near the head and forepaws of each subject. Total time invested in this placement procedure was approximately 2 min per animal. Whenever an experimental pup (P animals) touched the sensor, a 1-μL pulse of solution was delivered into its mouth as well as into the mouth of the corresponding Y control. Physical contacts of Y subjects with the sensor did not result in reinforcement. All pups received 4 priming pulses of solution at the beginning of the training session (1, 60, 120, and 180 s). Each priming pulse was equivalent to 2 μL. These pulses were administered independently of the motor activity rates of the subjects and were intended to familiarize them with the reinforcer and to minimally stimulate head and body movements. After termination of the extinction session, body weights were again recorded, cannulas were removed, and pups were returned to their mothers. The environment where the operant procedure took place was maintained at 27 °C (± 1 °C) using 3 heating pads located around the apparatus employed for operant conditioning.

Solutions employed as reinforcers were: 0.1% w/v saccharin; 3.0% ethanol, and 5.0% ethanol (190 proof ethanol, Pharmaco, Brookfield, CT; vehicle: distilled water). Consumption of reinforcers was estimated from variation in body weight and calculated according to the following equation: ([(post-conditioning weight − pre-conditioning weight)/(pre-conditioning weight)] × 100).

Experimental design and data analysis

Across experiments, no more than one subject from each sex in a given litter was assigned to the same treatment condition. The dependent variable for operant performance was number of sensor contacts executed by subjects. Data obtained during the operant task were analyzed with analysis of variance (ANOVA). Separate ANOVAs were executed to analyze training and extinction sessions. Each solution employed as reinforcer was analyzed using a separate ANOVA. Operant performance across training or extinction session was analyzed via a 2-way mixed ANOVA defined by the following factors: the between-group factor was

prenatal treatment (ethanol or water prenatal exposure), and conditioning (P or Y) served as a within-group factor. Consumption of reinforcers during training session was analyzed using a similar 2-way mixed ANOVA (prenatal treatment × conditioning).

The loci of significant main effects or 2-way interactions were further analyzed with Fisher's LSD *post hoc* comparisons. A rejection criterion of $p < 0.05$ was adopted for all statistical analyses in the present study.

Preliminary analysis of the experimental data included sex as a variable. This factor consistently failed to exert any significant main effect, or to interact with any other factor under consideration. Therefore, data were collapsed across sex for all subsequent analyses. The lack of sex effects was not unexpected: previous studies working with ethanol reinforcement in operant conditioning during early infancy (Bordner et al., 2008; March et al., 2009; Miranda-Morales et al., 2010; Miranda-Morales, Molina, et al., 2012a; Miranda-Morales, Spear, et al., 2012b) found no significant effect or interaction of gender with other factors under analysis.

Results

As can be seen in Figure 1A the ANOVA employed to assess 3% ethanol reinforcement during the training session indicated a significant main effect of conditioning [$F(1,15) = 11.95$, $p < 0.01$]. In addition, number of sensor contacts was also significantly affected by the interaction of the two factors [$F(1,15) = 8.64$, $p < 0.01$]. Fisher *post hoc* tests indicated that P animals prenatally exposed to ethanol executed significantly more operant responses for 3% ethanol than did their corresponding Y controls; no significant difference could be observed in P vs. Y animals from the prenatal water exposure condition. During the extinction session, when operant behavior did not result in ethanol reinforcement, the main effect of conditioning and the interaction between prenatal treatment and conditioning reached significance [$F(1,15) = 8.35$, $p < 0.025$, and $F(1,15) = 5.00$, $p < 0.05$, respectively]. *Post hoc* analysis indicated that P animals prenatally exposed to ethanol executed significantly more operant responses than did all the remaining groups (their respective Y controls, and water prenatally exposed-P or Y animals).

In terms of 3% ethanol consumption during the training session, it was observed that pups prenatally exposed to ethanol exhibited higher body weight gains relative to pups prenatally exposed to water. Nevertheless, the corresponding ANOVA did not show any significant main effect or a significant interaction between prenatal treatment and conditioning.

The statistical analysis of 5% ethanol reinforcement during training and extinction sessions did not show any significant effect of prenatal treatment, conditioning, or their interaction. Intake scores of 5% ethanol during the training session were not significantly affected by any of the factors or their interactions. Data from 5% ethanol reinforcement and intake are depicted in Figure 1B and Table 1, respectively.

The ANOVA employed to analyze saccharin reinforcement during training session found no significant effects of any of the factors considered. For extinction, the ANOVA revealed a significant main effect of conditioning [$F(1,16) = 5.81$, $p < 0.03$]. P animals displayed significantly more operant responses than Y counterparts did in the absence of saccharin reinforcement. The ANOVA employed to analyze saccharin intake scores indicated no significant effect of the factors or their interaction. Results are depicted in Figure 1C (operant responses) and Table 1 (intake scores).

Discussion

The main result of this study was that prenatal experience with a small amount of ethanol during late gestation facilitated operant responding for a low-concentration ethanol solution in 5-day-old pups. Contingency between operant behavior and ethanol reinforcement produced an increase in target behavior, a result not observed in yoked control pups. This effect was also evident during the extinction session when, in the absence of the reinforcer, Paired pups had higher levels of operant responding than Yoked controls. Of major importance, operant behavior toward ethanol reinforcement was only observed in those infant rats prenatally exposed to low ethanol doses during late gestation. When saccharin was employed as the reinforcer, prenatal ethanol's facilitating effect could not be observed. Even these animals did not show clear operant conditioning for saccharin reinforcement during training; P pups exhibited significantly more operant responding than Y controls during extinction, which indicated operant reinforcement. The differences in saccharin reinforcement observed across sessions could be due to a general increase in motor activity during a training session. For instance, Arias et al. (2007) employed two 15-min training sessions to evaluate milk operant reinforcement in 5-day-old pups, in order to observe differential responding between P and Y animals.

Consistent with previous studies with 1-day-old rats (March et al., 2009; Miranda-Morales et al., 2010), 3% ethanol solution served as a positive reinforcer only in pups that had previous experience with the drug during late gestation. However, unlike Bordner et al. (2008) with 1-day-old pups, we observed no ethanol reinforcement in 5-day-old pups that had not experienced ethanol prenatally. Infant pups also seem capable of responding differentially to 2 different concentrations of the drug. In this sense, the effects of pre-exposure to ethanol have been found to vary markedly, perhaps partially dependent on the developmental period during which the pre-exposure and the test takes place (Spear & Molina, 2005). Truxell, Molina, & Spear (2007) indicated a progressive decline in ethanol acceptance across ontogeny. The present results seem to indicate that at this infantile age, prenatal experience with the drug has a more relevant impact in the later acceptance of ethanol than a few minutes of neonatal exposure to the drug. This fact is supported by the results obtained in the present preparation. As depicted in Figure 1, 3% ethanol was highly reinforcing in animals prenatally exposed to ethanol, while no evidence of reinforcement could be observed in control animals prenatally exposed to water. On the other hand, reinforcement was not observed toward 5% ethanol in pups prenatally exposed to ethanol, while the prenatal control group tended to show an aversion to that solution, as evidenced by a decrease of responding in P pups when compared to Y controls.

As was discussed in earlier studies (March et al., 2009; Miranda-Morales et al., 2010), differences across prenatal treatments cannot account for differences in associative learning capabilities: with saccharin as the reinforcer, no effect of prenatal ethanol treatment could be found. Ethanol reinforcement is not better explained in terms of ethanol-induced hyperactivity or hyper-reactivity [which has been encountered in other studies, although when much higher doses of ethanol have been chronically administered during gestation (Abel, 1980; Anandam, Felegi, & Stern, 1980; Bond, 1988)].

Even when the measure employed for reinforcer intake was not an optimal index of consumption scores (animals were weighed after an intervening extinction session and not immediately after the training session), it allowed us to ascertain that animals effectively consumed the drug during reinforcement. In fact, values of intake scores descriptively showed a similar profile as observed in operant reinforcement: ethanol intake scores were higher in animals prenatally exposed to ethanol, but saccharin intake scores were similar across both prenatal groups. Previous studies (March et al., 2009) also failed to show that

ethanol intake patterns statically resembled operant activity patterns, as a function of prenatal experience with the drug. Nevertheless, palatability of the different reinforcers seemed to result in differential body weight gained. It seems that a low-concentration ethanol solution was better accepted than a higher one (5% ethanol) by animals prenatally exposed to ethanol. These results, even though they were not statistically supported through intake scores, did find support when considering number of operant responses. Another finding that deserves consideration is the fact that the ethanol intake profile seemed to differ as a function of conditioning treatment. This result has previously been found in neonates (Bordner et al., 2008; March et al., 2009) and infant rats (Ponce et al., 2008). For animals prenatally exposed to ethanol, contingency between operant behavior and reinforcer delivery seemed to promote higher levels of 3% ethanol intake, while at 5% ethanol the opposite effect was seen. This fact brings up the possibility that, at 5% ethanol, P animals may have rejected part of the reinforcement, which could be seen later in the low frequency “seeking behavior” phase during the extinction session. Rejection of 5% ethanol could be in part due to the palatability of the specific concentrated reinforcer. Newborns evaluated in a similar operant conditioning task showed rejection of 6% ethanol and, moreover, that solution was not found to increase operant performance, even in animals prenatally exposed to ethanol (March et al., 2009).

The heightened ethanol acceptance after prenatal exposure could be due to mere pre-exposure to the sensory attributes of the drug. Another plausible hypothesis is that fetuses can form an associative memory comprising ethanol chemosensory cues and its positive hedonic effects. Both hypotheses predict similar postnatal outcomes, i.e., higher affinity and greater potential for reinforcement from ethanol (Abate et al., 2008; Spear & Molina, 2005). A recent study emphasizes the possibility that associative learning could occur prenatally. Prenatal exposure to anise or vanilla during late gestation increased neither intake nor palatability of these tastants on PD14, but prenatal ethanol exposure did show an increased acceptance of ethanol during infancy (Díaz-Cenzano, Gaztañaga, & Chotor, 2013). These results seem to indicate that prenatal ethanol exposure not only represents for the fetus a chemosensory stimulus, but also represents the presence of a reinforcer, which is supposedly ethanol's pharmacological effects (Díaz-Cenzano et al., 2013).

In summary, the conditioning technique employed here represents an alternative tool for the ontogenetic analysis of ethanol-mediated learning and memory processes. This study supports the notion that ethanol exposure during fetal developmental influences later patterns of ethanol use (Abate et al., 2008; Spear & Molina, 2005). Prenatal exposure to a low ethanol dose such as 1.0 g/kg facilitates not only neonatal (1-day-old; March et al., 2009) operant learning, supported by intraoral administration of a low-concentration ethanol solution, but also infantile (5-day-old) operant learning as evidenced by increased operant behavior during the training session and the maintenance of seeking behavior during extinction.

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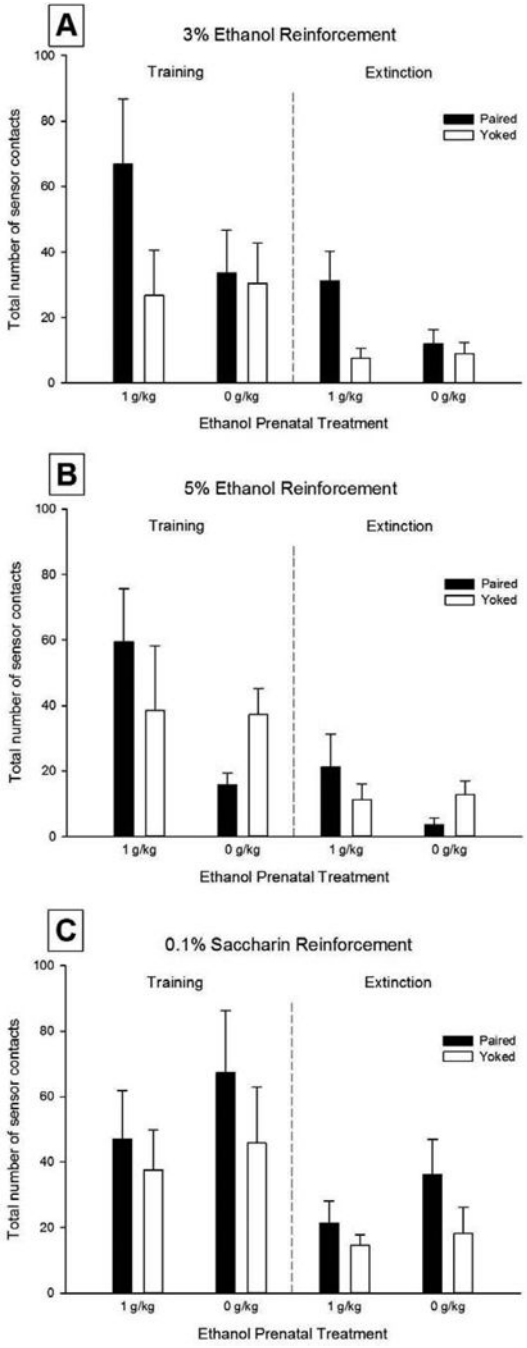

Figure 1.

Total number of operant responses (sensor contacts) toward 3% ethanol (Panel A), 5% ethanol (Panel B), or 0.1% saccharin (Panel C) reinforcement during training and extinction sessions, as a function of prenatal treatment (0 or 1 g/kg ethanol) and conditioning treatment (paired or yoked conditions). Values are expressed as mean ± standard error of the mean.

Table 1

Body weight gained during training session as a function of solution used as reinforcer, prenatal treatment, and conditioning treatment. Values represent mean ± SEM.

	Prenatal Treatment			
	1 g/kg Ethanol		Water	
Reinforcer	Paired	Yoked	Paired	Yoked
3.0% ethanol	0.21 ± 0.05	0.14 ± 0.04	0.14 ± 0.02	0.10 ± 0.03
5.0% ethanol	0.13 ± 0.04	0.24 ± 0.07	0.08 ± 0.03	0.06 ± 0.03
0.1% saccharin	0.16 ± 0.02	0.21 ± 0.04	0.19 ± 0.03	0.18 ± 0.05