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MOTIVATIONAL EFFECTS OF INTRAORALLY-INFUSED ETHANOL IN RAT PUPS IN AN OPERANT SELF-ADMINISTRATION TASK

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Abstract

Motivational effects of self-administered ethanol have rarely been studied in preweanling rats due primarily to the lack of age-appropriate operant tasks. The present experiments assessed the hedonic effects of intraoral ethanol in infant rats self-administered by activating a touch sensor. On postnatal day (PD) 13 pups were pre-exposed to the drug's pharmacological and/or sensory effects. Operant sessions were conducted during PDs 14-16 (Experiments 1 and 2). Paired animals were placed in chambers equipped with a touch-sensitive disk and received an intraoral infusion of ethanol (3 or 5% v/v, 5 μ l) after each sensor contact. Yoked controls were equated for number and distribution of ethanol infusions but had no control over the contingency between operant behavior and intraoral infusion. In Experiment 2, training trials were preceded by a non-reinforced phase. Paired pups performed fewer operant responses than controls and decreased their operant responses across sessions. These results suggest that intraoral self-administered ethanol has an aversive hedonic value in two-week old rats. Operant behavior seems to have been associated with aversive orosensory effects derived from intraoral ethanol infusion.

Keywords

self-administered ethanol; operant learning; ethanol taste; infant rat; aversion

1. Introduction

Animal models are useful tools for the analysis of ethanol abuse and dependence. Ethanol can serve as an appetitive reinforcer, causing animals to approach stimuli signaling the drug's postabsorptive effects [1]. Several selected mouse lines as well as inbred and heterogeneous mice strains and also genetically selected lines of rats prefer a texture associated with postabsorptive effects of ethanol [2-3]. Depending on dosage, ethanol also mediates conditioned taste preference or aversions [4-5]. These conditioning models employ experimenter-administered ethanol, mainly through intraperitoneal (i.p.) or intragastric (i.g.)

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routes. These drug-administration methods are different in many ways from the oral self-administration occurring in humans. Due to pharmacokinetic and pharmacodynamic factors (e.g., different peak drug levels and slower rate of drug accumulation and distribution), the route of administration has critical effects on the behavioral consequences of ethanol or any other drug [6]. Yet, this factor is often dismissed, even in studies aimed to provide meaningful pre-clinical results. Thus, to better assess the pathological consumption of ethanol observed in humans, self-administration models in experimental animals are usually more desirable [7].

However, the assessment of the positive reinforcing effects of ethanol by means of self-administration models faces several caveats. At the foremost, the orosensory effects of ethanol seem to constitute a “taste barrier” for the consumption of the fluid. Ethanol consumption and/or preference is low when heterogeneous rats are used in these tests. While animals can show preference for relatively low concentrations of oral ethanol (1 to 5%), consumption decreases dramatically when higher concentrations are employed. [8-9]. Kiefer et al. [10] observed substantial aversive orofacial reactions in naive rats intraorally stimulated with ethanol (10 % v/v). Aversive responsiveness decreased when animals were given extensive prior experience with intraoral ethanol. Employing newborn rats, Petrov et al. [11] found that these animals readily consumed 5% v/v ethanol through a surrogate nipple, but consumption of the drug decreased when employing 10% v/v ethanol. The authors suggested that the newborn rat perceived the higher concentration of the drug as less palatable. Indeed, enhanced consumption of high concentrations of ethanol can be observed in rats lacking gustatory cortex or olfactory bulbs [12].

To overcome the apparent aversiveness of the taste of ethanol, techniques have been designed to acclimate animals to the sensory effects of the drug. In the sucrose-substitution procedure animals are offered a sweet solution mixed with progressively increased concentrations of ethanol [13]. After sufficient training, rats exhibit substantial consumption of high concentrations (40%) of uncontaminated ethanol. Self-administration of ethanol is also enhanced after prolonged pre-exposure to the drug. Passively infusing rats with ethanol on a chronic basis is associated with later operant ethanol self-administration patterns equivalent to 4 g/kg/day [14].

These techniques have been successful in inducing ethanol self-administration in adult rats. Yet, there are very few studies aimed at analyzing the hedonic effects of self-administered ethanol in infant (preweanling) rats. On the other hand, expression of motivational effects of ethanol has been repeatedly found in preweanlings when assessed by means of conditioning models. Pups easily learn to reject a taste paired with the postabsorptive effects of ethanol [15] and are also sensitive to appetitive postabsorptive consequences of low doses of the drug [16]. Yet, it is still not clear if these young animals will self-administer ethanol in an operant preparation and, if so, what mechanisms underlie this phenomenon. In an operant self-administration paradigm, access to ethanol is dependent upon execution of a given behavior, such as pressing a lever [17]. This void in the ontogenetic literature can be explained by a lack of appropriate age-related procedures. Initiation and training techniques commonly employed in adult rodents are not suitable for younger animals. When assessing the ontogeny of ethanol's intraoral self-administration, the brevity of infancy (postnatal days 1–21; PDs 1–21) as well as teratological effects likely to result from prolonged exposure to the drug [18-19] preclude the use of extensive training or long-term preexposure strategies. To analyze ethanol self-administration in infancy we are in need of techniques that employ minimal or no shaping procedures. The issue of comparable behavioral dependent variables across developmental stages is also critical to allow longitudinal studies of ethanol acceptance and reinforcement. Such an ontogenetic approach is indeed crucial to understand the patterns of consumption observed in adulthood [20-21]. Animal experimental studies define infancy as a developmental stage characterized by an increased affinity for ethanol intake and high sensitivity to the

reinforcing effects of the drug [for a comprehensive review, see 22]. Furthermore, exposure to ethanol during both prenatal and early postnatal life predicts ethanol abuse later in life, as observed in clinical [23-25] and experimental animal studies [22,26].

The aim of the present work was to assess motivational effects of intraoral ethanol in preweanling rats by means of a novel operant conditioning task. Brief pre-exposure manipulations aimed to facilitate pup's initiation in ethanol self-administration were employed in conjunction with procedures designed to minimize ethanol's effects upon exploratory behavior. The operant strategy examined ethanol self-administration through the probability of execution of a very simple behavior, contacting a touch sensitive disk located in the floor of the operant cage. Unlike traditional lever-pressing models, this strategy is suited for the behavioral repertoire of the young rat. The preparation requires no shaping procedures. Also, behaviors needed to obtain the reinforcer are part of the exploratory pattern of the preweanling and are expressed throughout the ontogeny of the rat, hence allowing for developmental studies.

2. Experiment 1

Our goal was to assess whether intraorally delivered ethanol would affect the probability of preweanlings contacting a touch-sensitive disk located in the floor of the experimental chamber. Aversion to ethanol's orosensory effects (i.e., odor, taste, burning sensation) has been hypothesized to be a barrier precluding oral self-administration [12]. Also, pre-exposure to ethanol could facilitate later expression of appetitive learning mediated by the drug [27-28]. In the present experiment, animals were pre-exposed to the orosensory effects of ethanol, its postabortive effects, or both. Then they were given training sessions in which an intraoral infusion of ethanol was delivered after each contact with the sensor chip.

2.1. Methods

2.1.1 Subjects—Ninety-six Sprague-Dawley derived pups representative of 12 litters born and reared at the Center for Developmental Psychobiology (Binghamton University, NY, USA) were used. Births were examined daily and the day of parturition was considered as PD 0. Pups were housed with the dam in maternity cages with free access to water and food. The colony was kept at 22 – 24 °C and a 12-h light-dark cycle was used. At the start of the experiment (PD 13) animals had a mean body weight of 31.0 ± 2.5 g. Experimental procedures complied with the Guide for Care and Use of Laboratory Animals [29].

2.1.2 Apparatus—Custom-made operant chambers ($10 \times 10 \times 12$ cm) constructed with clear Plexiglas and floored with aluminum were used. The floor had been abraded to prevent the animal from slipping. In one corner was a raised metal disk attached to a touch sensitive computer chip. Each chamber was connected to a computer equipped with custom-made software that received data from the operant chambers every tenth of a second. When a paired animal came in contact with the raised disk, the computer activated a syringe-infusion pump (AutoPump, Kashinsky 5/2000, Binghamton, NY) connected to its cage as well as a similar pump corresponding to the pertinent yoked control. The software registered the total number of sensor contacts. A sensor touch was not registered until the animal released the sensor from the previous touch. A circuit board supporting six pairs of infrared photo emitters-receptors surrounded the chamber on all sides. The photo beams crossed the chamber, allowing the software to divide the cage in four sections. Hence, the location of the animal in these sections was continuously tracked. Overall amount of activity (number of broken infrared beams) was also recorded.

2.1.3 Experimental design—A 4 (preexposure treatment on PD 13: intragastric ethanol, intragastric water, intragastric ethanol + oral ethanol or oral ethanol only) $\times$ 2 (ethanol concentration: 3 or 5%) $\times$ 2 (learning condition) experimental design was used. Learning

condition refers to the nature of the contingency between behavior and delivery of the reinforcer [target behavior either paired or unpaired (yoked control) with reinforcer delivery]. No more than one animal per litter was assigned to each particular experimental treatment. Each of the 16 conditions derived from the experimental design was composed by 6 animals.

2.2 Procedures

As depicted in Figure 1a, the experimental protocol was divided into 3 stages:

Stage I: pre-exposure (PD 13, Fig. 1a)—Pups were removed from the maternal cage and placed in pairs in holding cages lined with clean pine shavings. Temperature in these cages was kept at 32–34 °C through the use of heating pads. All pups were immediately implanted with an intraoral polyethylene cannula, as described elsewhere [15]. This procedure is minimally stressful to the pups [30] and has been a useful tool to assess both intake and orofacial responsiveness to a variety of tastants in rat pups [31]. One hour after cannulation, pups were weighed to the nearest 0.01 g (Sartorius, Gottingen, Germany) and randomly assigned to one of four groups according to the nature of pre-exposure procedures. One group of preweanlings received an intragastric administration of 0.5 g/kg ethanol. This dose was achieved by administering 0.015 ml of a 4.2 % v/v alcohol solution per gram of body weight (190-proof Ethanol, Pharmaco, Brookfield). A second group was treated with a similar volume of vehicle. A third group was briefly exposed to the chemosensory effects of oral ethanol in addition to the i.g. delivery of 0.5 g/kg ethanol. Specifically, 5 min after the i.g. administration, these animals were given intraoral infusions of ethanol (7% v/v, volume of each pulse: 5 uls; rate of delivery: 1.8 sec on - 58.2 seconds off). The solution was delivered via an infusion pump (Harvard Apparatus syringe pump, Natick, MA) connected to the subject's intraoral cannula. Animals were placed into individual boxes fitted with a cotton floor (15 × 7 × 14 cm). Two 15 min sessions were conducted. Hence, total amount of ethanol delivered throughout this stage was 150 uls. The interval between sessions was 30 min. Pups assigned to the fourth pre-exposure condition were given only the intraoral ethanol infusion. This group was untreated in terms of i.g. administration.

In this, as well as in the following experiment, the vehicle for intraorally infused ethanol was distilled water. This vehicle was chosen to minimize the likelihood of pup's response being altered by potential extraneous orosensory stimuli that may be present in tap water. On the other hand, tap water was employed as a vehicle when ethanol was intragastrically administered.

Cannulae were removed following pre-exposure treatments and pups were returned to the holding chambers where they remained for 45 min before being returned to their respective maternal chambers. This time interval was meant to allow for clearance of the drug before being reunited with the dam. According to previous work [32], a 45 min interval is enough to allow a significant decrease in the effects of the doses of ethanol achieved in the present study. Dosage used for the i.g. administration as well as concentration and schedule of ethanol intraoral stimulation were based on previous work [27]. In that study, ethanol self-administration in preweanlings was facilitated by preexposure to the drug's orosensory and pharmacological effects.

Stage II: Operant Training (PD's 14–16, Fig. 1a)—Pups were intraorally cannulated and placed in pairs in heated holding chambers (32–34 °C), where they remained undisturbed for 60 min. Pup's cannulae were then attached to the infusion pump (AutoPump, Kashinsky 5/2000, Binghamton, NY). Animals were individually placed into the operant chambers where they remained for 20 min. Whenever paired subjects (P pups) performed the target behavior (contacting the touch sensitive disk), an intraoral pulse of ethanol (5 uls of 3 or 5% v/v ethanol,

pulse duration: 1.8 sec) was delivered. The schedule of reinforcement employed can be described as a fixed ratio 1. In yoked controls (Y pups) ethanol infusion was completely independent from their own sensor-touching behavior. Animals were randomly assigned to pairs comprised by a P and a Y pup (each one in different boxes), both receiving the infusion when the paired animal contacted the sensor. No attempt was made to shape the behavior of the animals. The procedure was derived from unpublished work conducted in our laboratory, showing substantial sensor-touching behavior in infants when reinforced with 10% w/v sucrose.

Training sessions were conducted in a dimly illuminated room, different from the one where pre-exposure had taken place. Indirect lighting was provided by two portable fluorescent lamps (118 v, 60 Hz, 20 w) positioned on the floor, about 2 meters away from the conditioning chambers. After each trial, animals remained in the holding chambers for 45 min before being reunited with their mother.

Stage III: Extinction (PD 17, Fig. 1a)—Implementing an extinction stage is particularly important in drug operant studies. Many drugs, including ethanol, have alternative nonmotivational effects (i.e., motor stimulatory effects) that can interact with learning expression. These factors are less likely to interfere when conducting an extinction session [33]. In the present experiment, extinction was defined by withholding the reinforcer following the target response. Pups were placed in the operant chambers and their cannulae were connected to the infusion pump. The pump was turned on but the syringes were empty. Therefore, no reinforcer was available during the 20 min session.

2.3 Data Analysis

General Considerations—The Analysis of Variance (ANOVA) assumes that each observation of a between-factor design must be completely independent [34]. This is not the case for the data derived from the paired and yoked conditions used in the present study. Consequently, whenever learning condition (paired or yoked) was included in an ANOVA design, it was regarded as a within-measure factor.

Preliminary analysis of the operant behavior data as well as of the activity patterns during training and extinction consistently showed no significant main effects or interactions involving pre-exposure manipulations. Pre-exposure was found not to interact with learning condition or with other factors in terms of consumption of ethanol. Hence, descriptive and inferential analysis of the data has been performed by collapsing across this factor. In the current and subsequent experiment, the loci of significant main effects or interactions were further examined through follow-up ANOVAs and by means of post-hoc comparisons (Fisher's Least Mean Significant tests, type I error set at .05).

Operant Behavior—A 2 (ethanol concentration: 3 or 5% v/v) $\times$ 2 (learning condition: paired or yoked) $\times$ 4 mixed ANOVA (day of assessment: PDs 14, 15, 16 or 17) served to analyze frequency of sensor contacts during each 20 min training session. Maximal operant score on a given training day was analyzed by a 2-way mixed ANOVA (learning condition $\times$ ethanol concentration).

Ethanol Intake—Infant ethanol intake patterns were calculated in terms of absolute grams of ethanol per kilogram of body weight (g/kg). These scores were calculated as a function of body weight gain during the training session and in accordance with the concentration of the ethanol solution used as a reinforcer. Intake scores were analyzed using a 4-way mixed ANOVA defined by ethanol concentration, learning condition and day of assessment (PDs 14, 15, 16).

When naive rats have access to ethanol they later exhibit changes in ethanol seeking and acceptance patterns [9,35]. To assess whether pups might have developed an aversion to the taste of ethanol we used the following approach. The method of oral ethanol delivery (intraoral cheek cannulation) allowed pups to actively consume or reject the amount of drug self-administered. This permitted obtaining a measure of percentage ethanol actually ingested during PDs 14-16. In each session body weight gains were calculated by subtracting the posttraining weight from the pretraining weight. Volume of ethanol self-infused by each pup was derived by means of total number of target behaviors performed. Percent ethanol consumption scores were then calculated $\{[(\text{postsession weight} - \text{presession weight}) \times 100] / [(\text{total number of operant responses} \times 5) / 1000]\}$. These values were subjected to a 2 (learning condition) $\times$ 3 (day of assessment) mixed ANOVA.

Locomotive Patterns—Overall locomotion across PDs 14-17 (total number of beams crossed) was analyzed by means of a 2 (ethanol concentration) $\times$ 2 (learning condition) $\times$ 4 (day of assessment) mixed ANOVA.

Exploratory Patterns—The chamber's photobeams virtually divide the operant chambers into four quadrants, allowing the computer to track the pup's location on a continuous basis. Exploratory patterns were scrutinized by indexing time spent in the quadrant containing the touch-sensitive disk relative to time spent in the opposite quadrant. This location index was calculated as follows: Time spent in Target Quadrant / (Time spent in Target Quadrant + Time spent in quadrant opposite to target). Given equal preference for both sections, this index is 0.5. If more time is spent in the opposite section relative to the target quadrant, the index will be lower than 0.5, and if less time is spent in the alternative section the coefficient will be higher than 0.5. Due to apparatus malfunction, data for eight pairs of animals were not recorded. Location scores across training and extinction were analyzed by means of a 3-way mixed ANOVA (ethanol concentration $\times$ learning condition $\times$ day of assessment). To analyze the loci of significant main effects or interactions, separate repeated measures ANOVA for each learning condition were conducted (within factor: day of assessment). That is, analyses regarding location scores were performed either between experimental conditions or within-group across sessions.

2.4 Results and Discussion

Operant Behavior—P pups exhibited more operant responses than Y controls on PD 14. This difference was no longer observable on PD 15 and seemed to reverse during PD 16 (Figure 1b). In addition, responding in both conditions decreased between PDs 14-15 and PDs 15-16. ANOVA and subsequent post-hocs confirmed these observations. The ANOVA indicated a significant main effect of day of assessment, $F(3, 138) = 17.42$; $p < 0.0001$. An interaction between day of assessment and learning condition was also observed, $F(3, 138) = 3.20$; $p < 0.05$. Pups in the paired condition had significantly more operant responses than their Y controls on PD 14, while the opposite difference showed a borderline significance ($p = 0.051$) on PD 16. Concentration of ethanol (3 or 5%) did not affect operant scores nor significantly interacted with the remaining factors.

Ethanol Intake—Pups receiving 5% ethanol registered the highest levels of drug intake, achieving an average of 0.27 g/kg during PD 14. Also, higher ethanol intake was observed on PD 14 and 15 than on PD 16. Main effects of ethanol concentration and day of assessment were observed, $F(1, 46) = 7.27$; $p < 0.01$; $F(2, 92) = 21.33$; $p < 0.0001$; respectively. Learning condition did not affect drug intake. Total ethanol intake (g/kg) across days is shown in figure 2a.

Percentage of infused ethanol actually ingested significantly decreased across days, $F(2, 92) = 5.09$; $p < 0.01$. No significant effect of ethanol concentration was found. Post-hoc tests indicated that consumption of ethanol was significantly lower on PD 16 than on PD 14 and 15. Mean and standard error across days for paired and yoked conditions was as follows: Day 1: $P = 53.00 \pm 3.14$, $Y = 54.95 \pm 3.10$; Day 2: $P = 55.10 \pm 3.23$, $Y = 50.95 \pm 4.10$; PD 16: $P = 39.24 \pm 3.71$, $Y = 45.55 \pm 4.01$.

Locomotive Patterns—ANOVA analysis indicated a significant main effect of day of assessment, $F(3, 126) = 22.66$; $p < 0.0001$. As indicated by post-hocs, level of locomotion (total number of beam breaks per session) was greater on PD 15 than on PD 14 regardless of experimental condition. A decrease was then observed on PD 16 and 17. Also, P pups showed an increased number of beam breaks relative to Y controls, but only when they were intraorally infused with 5% ethanol. Specifically, ANOVA analysis revealed an effect of learning condition $F(1, 42) = 9.41$; $p < 0.005$. This effect was tempered by a significant learning condition $\times$ ethanol concentration interaction, $F(1, 42) = 9.53$; $p < 0.005$. The locus of this effect was analyzed by subsequent follow-up ANOVAs for each ethanol concentration. While no effect of learning condition was found in pups responding for 3% ethanol, P animals given 5% ethanol exhibited greater numbers of beams broken than Y pups given a similar ethanol concentration, $F(1, 21) = 14.13$; $p < 0.005$. Descriptive data relative to activity patterns can be found in Table 1.

Exploratory Patterns—ANOVA for relative time spent in the quadrant containing the sensor indicated a significant main effect of ethanol concentration, $F(1, 42) = 10.88$, $p < 0.005$. The interaction between learning condition and day of assessment was also significant, $F(3, 126) = 3.28$, $p < 0.05$, although tempered by a 3-way interaction between these factors and ethanol concentration, $F(3, 126) = 2.90$, $p < 0.05$. Further one-way, within-groups ANOVAs indicated a progressive increase across sessions for the yoked condition, $F(3, 126) = 3.64$, $p < 0.05$. The within-group analysis for P pups indicated a significant main effect of ethanol concentration as well as an interaction between this factor and day of assessment, $F(1, 42) = 16.82$, $p < 0.001$; $F(3, 126) = 3.15$, $p < 0.05$. Relative preference was significantly higher for P animals given 3% ethanol than for P pups given 5% ethanol as the reinforcer during PDs 15 and 16. Location scores are depicted in Figure 3.

When taken together, these results suggest that oral ethanol exerted differential motivational effects in the operant task. An increased number of target responses was observed during the initial training session (PD 14) in those animals given a positive contingency between sensor touching and intraoral ethanol. Nonetheless, as training progressed, P pups displayed behaviors indicative of aversive effects of the drug. They exhibited a reduced number of operant responses on PD 16. Percent consumption of total ethanol self-administered decreased across sessions. These results indicate that pups are likely to have developed conditioned avoidance to the taste of ethanol. Interestingly, enhanced locomotor activity was observed in P animals given the highest ethanol concentration (5%). The target behavior under analysis --touching a sensor located in the floor of a relatively small cage-- seems highly sensitive to unconditional effects of the drug on exploratory patterns. This raises the possibility that the operant results might have been modulated to a certain extent by activating locomotive effects of ethanol. This possibility was further analyzed in the following experiment.

3. Experiment 2

Experiment 1 suggests that intraoral ethanol exerted appetitive effects at commencement of training followed by aversive effects during subsequent sessions. Ethanol also seemed to exert activating effects that might interact with these motivational effects. To more systematically assess these phenomena, a similar operant task (i.e., intraoral infusion of ethanol correlated

with sensor touching) was employed in the present experiment. A major procedural change was that a 20-min non-reinforced phase preceded each daily operant session. This non-reinforced session aimed to minimize the possibility of operant performance being altered by possible activating effects of ethanol on normal exploration patterns. It is known that animals exhibit an unconditioned increase in locomotion when exposed to a novel environment. Also, this response is likely to interact with activating effects of drug administration [36]. This interaction can be minimized by previous experience with the testing environment. Such non-reinforced passive exposure can presumably attenuate the unconditional locomotor response otherwise elicited by the testing environment [37]. To further reduce the likelihood of ethanol's stimulatory effects on locomotion, only responsiveness for 3% ethanol was examined. In Experiment 1 ethanol's activating effects were particularly apparent in animals given pairings between sensor touching and 5% ethanol. Hence, employment of the latter concentration was discarded in the present experiment.

The initial, non-reinforced session and the subsequent operant training took place under differential illumination conditions. While operant training was conducted under dim light conditions (as in Experiment 1), the testing room was brightly illuminated during the initial non-reinforced exposure. Delivery of ethanol under particular illumination conditions was intended to help pups achieve a better discrimination of the contingency under analysis and therefore increase operant performance across days. In other words, bright and dim lighting conditions served as discriminative contexts signaling ethanol availability and non-availability, respectively (also referred as S+ and S− stimuli) [38]. Finally, to improve perception of the correlation between sensor touching and drug delivery, the sensor was made more salient by placing a distinctive tactile cue surrounding it (see Methods).

Non-reinforced exposure to the experimental chambers as well as delivery of the reinforcer under particular S+ cues was meant to clarify whether initial high levels of responding displayed by P pups in Experiment 1 reflects a positive hedonic value of ethanol. In the present study pre-exposure manipulations were restricted to intraoral infusions of either water or ethanol. Previous work indicate that familiarization with ethanol's sensory cues can facilitate appetitive learning mediated by intraoral infusion of the drug [27].

3.1 Methods

3.1.1 Subjects—Thirty-two Sprague-Dawley pups were used. These animals had a mean body weight of 28.8 $\pm$ 3.2 g and were representative of eight litters born and raised at the vivarium of the Center for Developmental Psychobiology. Housing and breeding conditions replicated those described in Experiment 1.

3.1.2 Apparatus—The apparatus was similar to that described in Experiment 1. The only difference was that a ring of sandpaper (diameter 1.5 cms, coarse: 50, Gatorgrit, USA) surrounded the sensor.

3.1.3 Experimental design—A 2 (pre-exposure treatment) $\times$ 2 (learning condition) factorial design defined the experiment. During pre-exposure, animals received intraoral infusions of ethanol (7%) or vehicle (distilled water). The second factor took into account whether the delivery of the reinforcer was contingent or not with the target behavior under analysis [Groups paired (P) and yoked (Y), respectively]. Eight animals composed each of the four groups thus created.

3.2. Procedures

A three-stage procedure similar to the one employed in Experiment 1 was used. Briefly, during PD 13 (Pre-exposure) animals were given two 15-min pre-exposure trials. During each trial

they received 15 infusions of either ethanol (7% v/v) or water [5 μ ls each infusion; duration: 1.8 sec; intertrial interval: 30 min].

During PDs 14 to 16, pups were placed in the operant chambers for 20 min (non-reinforced session, phase 1 of the daily operant trial). During this stage, the pump – which produces a mild but still audible clicking noise – was off. No reinforcer was available. This phase allowed pups to become familiar with the chambers. Locomotion and sensor touching were recorded throughout the 20 min non-reinforced session. This phase took place under bright room illumination (four fixed 25-w fluorescent lamps located in the wall opposite to the operant chambers plus two 20 w portable fluorescent lamps positioned in the floor). Following termination of the non-reinforced session (phase 1), pups were removed from the operant chamber and returned to their holding cages located in a different room. After 30 min they were weighed to the nearest 0.01 g, their cannulae connected to the infusion pumps and again placed in the operant chambers. Commencement of the training session (phase 2) and availability of the reinforcer occurred under dim illumination (see Experiment 1 for specifications). During the 20 min training trial each sensor contact of a P pup was reinforced with an intraoral infusion of ethanol (3% v/v, pulse duration: 1.8 s). Pups were weighed at the end of the trial and placed in their holding chambers for 45 min before being returned to their maternal cages.

A 20-min extinction session took place on PD 17. Pups were placed in the operant chambers (under dim lighting conditions) 60 min after being cannulated. Behavioral responding was recorded but no reinforcer was available.

3.3 Data Analysis

Operant behavior across training and extinction sessions was analyzed by means of a 2 (pre-exposure treatment: ethanol or water) $\times$ 2 (learning condition: paired or yoked) $\times$ 4 (day of assessment: PDs 14, 15, 16 and 17) mixed ANOVA. Sensor touching during the non-reinforced session (PDs 14-16) was analyzed by a 2 (preexposure treatment) $\times$ 2 (learning condition) $\times$ 3 (day of assessment) mixed ANOVA.

A 2 (pre-exposure treatment) $\times$ 2 (learning condition) $\times$ 3 (day of assessment) mixed ANOVA served to analyze ethanol intake (g/kg) and percentage of ethanol consumed from the total amount of drug delivered.

Maximal number of sensor contacts in a given training day was analyzed by means of a 2-way ANOVA (pre-exposure $\times$ learning condition).

Overall activity scores (number of beams broken) during non-reinforced and training sessions (PD 14-16) were analyzed with a 2 (pre-exposure treatment) $\times$ 2 (learning condition) $\times$ 3 (day of assessment) $\times$ 2 (recording time: phase 1 or 2) ANOVA. The non-reinforced session was not conducted on PD 17. Hence, analysis of motor scores during PD 17 required a separate 2 (pre-exposure) $\times$ 2 (learning condition) ANOVA. Finally, a three-way ANOVA (pre-exposure treatment $\times$ learning condition $\times$ day of assessment) served to analyze location scores (time spent in the target quadrant relative to time spent in the opposite quadrant) across PD's 14-17.

3.4 Results and Discussion

Sensor touching during the initial non-reinforced session was less for P animals than for Y controls [$F(1, 15) = 7.36$; $p < 0.05$], particularly on PDs 15 and 16 (i.e., following initial training on PD14). Means and standard errors for total number of target responses in P and Y conditions when collapsed across pre-exposure manipulations were as follows: PD 14: P = $50.3.4 \pm 11.06$, Y = 54.6 ± 8.5 ; PD 15: P = 49.4 ± 6.6 , Y = 71.6 ± 8.1 ; PD 16: P = 48.3 ± 6.7 , Y = 73.5 ± 12.3 .

Operant behavior scores during training (PDs 14-16) and extinction (PD 17) sessions were not significantly affected by pre-exposure manipulations. As depicted in Figure 4, P animals had fewer operant responses than controls. This was particularly evident during the last day of training as well as during extinction. A significant main effect of learning condition was indicated by the analysis, $F(1, 14) = 23.98$, $p < 0.0005$, an effect tempered by a significant interaction with day of assessment, $F(3, 42) = 4.12$; $p < 0.05$. Further analysis of the interaction via post-hoc tests revealed no differences between P and Y groups during the initial training session (PD 14). On PD 15 there was a trend for P animals to perform less target behavior than controls, with the pertinent pair-wise comparison indicating a borderline significance ($p = .07$). This difference reached significance during PD 16 and during extinction. Moreover, within-group comparisons indicated that amount of target operant behavior decreased across days in P pups. Specifically, significantly fewer sensor contacts were recorded in PDs 16 and 17 than in PDs 14-15. This pattern was not observed in Y animals, whose number of sensor contacts remained stable across training and extinction.

No significant main effects or interactions were detected when analyzing ethanol consumption across days (g/kg/20 min, Fig. 2b) or percent consumption of total ethanol infused. Maximal number of responses in a given day was significantly higher in Y than in P pups, $F(1, 14) = 11.16$, $p < 0.005$. Mean numbers of maximal responses for both conditions were as follows: $P = 94.1 \pm 5.5$, $Y = 114.9 \pm 4.8$.

Across PDs 14-16, pups displayed more motor activity during the training phase than during the initial non-reinforced session [$F(1, 14) = 6.04$, $p < 0.05$]. Locomotion during extinction was not affected by the factors under analysis. Table 1 depicts activity levels for P and Y pups across days.

ANOVA analysis for relative location scores (time spent in the section of the cage where the sensor was located relative to time spent in the opposite quadrant of the cage, Figure 5) yielded a significant main effect of learning condition [$F(1, 14) = 7.45$, $p < 0.05$]. This effect was tempered by a significant interaction with day of assessment [$F(3, 42) = 3.77$, $p < 0.05$]. Post-hoc tests indicated that location scores in P and Y animals were not significantly different during the two initial days of training (PDs 14 and 15), but on PDs 16 and 17 paired pups had significantly lower preference scores when compared with Y counterparts. A significant interaction between pre-exposure treatment and learning condition was also found, $F(1, 14) = 4.89$; $p < 0.05$. To further analyze the locus of this effect, location scores were analyzed by means of one-way mixed ANOVAs executed for each learning condition. Although prior exposure had no effect in P animals, the corresponding ANOVA for Y pups indicated that ethanol-pre-exposed animals had higher relative location scores than similar Y pups that had been intraorally infused with water during the pre-exposure phase, $F(1, 7) = 8.76$, $p < 0.05$.

In summary, the introduction of a non-reinforced phase preceding each training session as well as the addition of discriminative cues signaling the availability of ethanol did not induce the expression of positive reinforcing effects of oral ethanol in P animals. On the contrary, these pups showed several behaviors suggesting that oral ethanol exerted aversive effects. Relative to controls, P animals exhibited a reduced number of target responses during training sessions. Maximal number of responses in a given trial was also lower in P pups relative to Y counterparts. Paired subjects also showed a progressive decrement in the predisposition to engage in sensor-touching behavior as the experiment progressed. Significantly fewer target responses in P animals than in controls were also observed during the non-reinforced sessions and during extinction.

4. General Discussion

It has often been recognized that animal models of ethanol self-administration offer more utility in understanding ethanol consumption patterns in humans [7]. The present study assessed the reinforcing effects of self-administered ethanol in 14-17-day old rats. No evidence of positive reinforcing effects of oral ethanol was observed. Target responding in pups reinforced with ethanol was less than in controls given equivalent --but noncontingent-- drug infusions. These findings are consistent with research conducted both in infants [27,39] and in heterogeneous adult rats with no prior experience with ethanol [40,41]. Previously, Domínguez et al. had found substantial lever pressing for milk but much less when milk was supplemented with 6.0% v/v ethanol, a pattern that remained similar across the course of early ontogeny (PDs 3-4, 9-10 and 15-16) [39]. Similarly, Ponce et al. reported that mixing sucrose with 6% v/v ethanol --either from the very start of training or when using a progressive substitution schedule-- attenuated the reinforcing effects of the sweet tastant in 14-day old rats [27]. In conjunction with these and previous experiments [8,10,11,12], the present study indicates that pups seem to reject orosensory attributes of ethanol. These effects seem to constitute a barrier limiting ethanol intake, precluding the analysis of its potential positive hedonic effects.

This conclusion does not disregard that oral ethanol may exert positive postabsorptive reinforcing effects in alternative self-administration models, characterized by high levels of intake in short periods of time [42]. Yet, operant paradigms, including the sensor-touching technique here employed, are constrained by the delay between intake of the drug and onset of its postabsorptive effects. Animals undergoing operant training are usually offered small quantities of oral ethanol, requiring an extensive period of time for the drug to reach pharmacological relevant blood ethanol levels. Hence, the response under operant training is likely to be associated with the immediate and probably aversive orosensory effects of EtOH and not with its delayed central effects [40-41].

In Experiment 1, less target responding in P pups than in Y controls was found in a relatively late stage of training (PD 16, third training session). When each training session was preceded by a non-reinforced phase (Experiment 2), reduced engagement in ethanol self-administration was exhibited by P pups during the second training session. In both experiments, paired groups decreased their operant responses across daily test sessions. Interestingly, less response in P pups than controls was observed even in sessions when the reinforcer was withheld (Experiment 2: extinction sessions at PD 17 and non-reinforced sessions at PDs 15-16). This indicates that the pattern of target responding in P pups is not due to detrimental effects of the drug upon sensory and/or motor capabilities of the infant. Rather, amount of operant responding seems to be regulated by a specific ethanol-induced motivational learning.

Animals also exhibited a marked decrease in ethanol consumption across trials, an effect that possibly indicates the development of conditioned aversion to self-administered ethanol. Seemingly, P animals associated the target operant behavior with aversive effects derived from intraoral ethanol administration. These aversive effects could be directly related to the sensory attributes of ethanol (taste, odor and/or trigeminal components) [12] or to aversive sensory aspects inherent to the intraoral infusion procedure (e.g., liquid temperature or forced liquid delivery into the oral cavity) [43]. An alternative interpretation also deserves mentioning. Ethanol-mediated conditioned taste aversions are usually found when employing ethanol doses higher than 1 g/kg [5]. Yet, in preweanlings, even lower ethanol doses (0.4-0.5 g/kg) have successfully induced such aversions [32,44]. The latter doses are still higher than the maximum ethanol intake registered in the present study. Also, ethanol ingestion equivalent to those found in Experiments 1 and 2 yielded appetitive reinforcement in neonate rats [45]. Moreover, it has been recently found that low-dose ethanol (0.25 g/kg) failed to induce conditioned taste avoidance in 14-day old rats [46]. Still, the possibility of ethanol's central aversive effects

playing a role in the reduced operant behavior found in P animals cannot be completely disregarded.

Appetitive effects of low to moderate doses of intragastrically administered ethanol have been observed in 14-day old pups [16]. Human pathological consumption of ethanol also indicates that oral intake of the drug should possess reinforcing effects. In Experiment 1, P animals made more target behavior than controls on the first training day. This effect is likely to be interpreted in terms of appetitive effects of ethanol. Yet, this result was not only transient but also small in magnitude and not sensitive to changes in ethanol concentration. In drug operant studies performance is not only affected by the hedonic effects of the drug serving as the reinforcer, but also by concurrent sedative or activating effects of the drug upon general activity and locomotive patterns [33]. Hence, it is possible that the enhanced number of presses in P animals during PD 14 was due to non-specific activating effects of the drug. In other words, the initial high number of target responses on PD14 in P animals could just be the result of ethanol exacerbating the normal pattern of exploration in these animals. Results obtained in Experiment 1 support this possibility. P pups, particularly those receiving 5% ethanol, exhibited more locomotion than Y controls or P pups given a lower ethanol concentration. Also, when target responding was measured in the absence of ethanol delivery (PD 17, extinction trial), there was no evidence that the effects of the drug had been rewarding.

Additional proof that activating locomotive effects of ethanol modulated these apparent ethanol positive effects was provided in Experiment 2. This differed from Experiment 1 by including a non-reinforced phase conducted before each operant training trial. No behavior indicative of appetitive effects of ethanol was found in Experiment 2. On the contrary, behaviors characteristic of aversion were more clearly expressed: P pups exhibited fewer target responding than control pups as early as in the 2nd training session and also during the non-reinforced phases.

Experiment 2 yielded differential locational scores for P and Y subjects. Specifically, between-groups comparisons indicated that P pups displayed less relative time spent in the section of the operant cage containing the sensor than Y counterparts. This behavioral pattern was particularly apparent in those pups that had been stimulated with ethanol during the initial pre-exposure phase (see Figure 5, right panel). A conclusive interpretation of these results requires further experiments explicitly controlling the contingency between specific discrete cues and postabsorptive effects of ethanol. However, this behavioral outcome indicates an interesting feature of the operant technique here presented, namely, the possibility of measuring exploratory patterns associated with ethanol self-administration.

In summary, intraorally delivered ethanol seems to exert aversive effects when used in an operant self-administration task early in infancy. Ethanol-associated operant responding was lower for P pups than for controls, particularly when procedures minimized ethanol's activating effects. The aversiveness of the orosensory attributes (taste, olfactory or trigeminal components) of ethanol appear to be the main factor underlying these effects. These results are relevant to understand hedonic effects of intraorally delivered ethanol. They also provide a strong basis for future studies aimed at analyzing neurobiological determinants of self-administration in preweanlings and its sensitivity to genetic and environmental variables. Infancy, in both humans and animal models, is a developmental stage characterized by a high sensitivity to ethanol's motivational effects [22] and exposure to the drug during this period increases likelihood for later ethanol abuse [21-25]. The present study also reflects significant progress towards establishing an operant task suited to the behavioral repertoire of the infant rat. The brevity of the novel contingency-dependent preparation allows its use early in ontogeny when developmental changes are occurring rapidly. The technique allows determination of ethanol consumption as well as locomotor and exploratory patterns associated with ethanol

seeking and intake. Finally, behaviors needed to obtain the reinforcer do not require previous shaping procedures and are exhibited throughout the development of the rat. Hence, this paradigm may be useful in elucidating changes in motivational effects of ethanol across the life span [20].

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Figure 1.

1a. Methods for the analysis of reinforcing properties of intraorally delivered ethanol in infant rats (Experiment 1). Stage I (preexposure, postnatal day 13, PD 13), pups were given an intragastric (i.g.) administration of ethanol (0.5 g/kg) or water (0.0 g/kg). A third group was intraorally stimulated with oral ethanol (7% v/v, 30 infusions of 5 ul each) in addition to the i.g. delivery of 0.5 g/kg ethanol. In the fourth pre-exposure group pups were given only the same intraoral ethanol infusion. Stage II (operant training, PDs 14-16), pups underwent daily sessions (20 min) in which each contact with a dime-sized disk yielded an intraoral infusion of ethanol (3 or 5% v/v, 5 ul). Yoked controls equated for ethanol exposure (but not in regards to contingency on behavior) were employed. Stage III (extinction, PD 17), pups underwent a 20-min session in which the reinforcer was withheld following the target response. **1b.** Mean number of target operant responses (sensor contacts) in Experiment 1 during training (postnatal days 14-16, PDs 14-16) and extinction (PD 17) sessions as a function of the contingency

between behavior and delivery of the reinforcer [target behavior either paired or unpaired (yoked control) with reinforcer availability]. Data has been collapsed across ethanol concentration and pre-exposure manipulations. These factors did not affect operant behavior scores. The vertical dashed line in the y-axis indicates that PD 16 was the last day that ethanol was delivered. Vertical lines represent standard errors of the mean.

Figure 2.

Ethanol consumption scores (g/kg) in Experiments 1 and 2 (Figs. 2a and 2b, respectively) during daily 20 min operant sessions (postnatal days 14, 15 and 16, PDs 14-16) as a function of learning condition (paired or yoked) and ethanol concentration (3 or 5% v/v). Vertical lines represent standard errors of the mean.

Figure 3.

Location scores (time spent in the section of the cage where the sensor was located relative to time spent in the opposite quadrant) in Experiment 1 during training (postnatal days 14-16, PDs 14-16) and extinction (PD 17) sessions as a function of learning condition (Paired or Yoked) and ethanol concentration (3 or 5%, v/v). The vertical dashed line in the y-axis indicates that PD 16 was the last day that ethanol was delivered. Vertical lines represent standard errors of the mean.

Figure 4.

Mean number of target operant responses (sensor contacts) in Experiment 2 during training (postnatal days 14-16, PDs 14-16) and extinction (PD 17) sessions as a function of the contingency between behavior and delivery of the reinforcer [target behavior either paired or unpaired (yoked control) with reinforcer availability]. The vertical dashed line in the y-axis indicates that PD 16 was the last day that ethanol was delivered. Vertical lines represent standard errors of the mean.

Figure 5.

Location preference scores (time spent in the section of the cage where the sensor was located relative to time spent in the opposite quadrant) in Experiment 2 during training (postnatal days 14-16, PDs 14-16) and extinction (PD 17) sessions as a function of learning condition (Paired or Yoked) and nature of the preexposure treatment (Ethanol or Water). The vertical dashed line in the y-axis indicates that PD 16 was the last day that ethanol was delivered. Vertical lines represent standard errors of the mean

Table 1

Locomotive Patterns (number of beam breaks)

	Experiment 1				Experiment 2			
	PD 14	PD 15	PD 16	PD 17	PD 14	PD 15	PD 16	PD 17
Paired (3% EtOH)	1425.32 ± 103.49	1578.68 ± 102.65	1284.96 ± 119.1	1025.96 ± 97.28	Habit. 1385.4 ± 118.0	Habit. 1489.6 ± 98.5	Habit. 1349.2 ± 120.9	-----
					Training 1538.1 ± 125.2	Training 1764.1 ± 119.4	Training 1404.8 ± 155.1	Ext. 1391.1 ± 76.3
Yoked (3% EtOH)	1455.55 ± 113.3	1518.05 ± 118.3	1302.18 ± 115.6	1022.05 ± 73.3	Habit. 1324.4 ± 95.1	Habit. 1607.4 ± 98.3	Habit. 1382.9 ± 180.5	-----
					Training 1416.1 ± 156.0	Training 1686.6 ± 118.9	Training 1676.0 ± 156.3	Ext. 1386.1 ± 150.1
Paired (5 % EtOH)	1505.41 ± 104.3	1608.59 ± 113.3	1168.5 ± 98.3	1036.21 ± 92.4	-----	-----	-----	-----
Yoked (5 % EtOH)	1035.96 ± 101.8	1249.23 ± 109.1	933.14 ± 88.8	882.00 ± 101.0	-----	-----	-----	-----

Values represent mean +/- SEMs.