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Infant rats exhibit aversive learning mediated by ethanol's orosensory effects but are positively reinforced by ethanol's post-ingestive effects

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Abstract

Previous work suggest aversive and appetitive hedonic effects of intraorally delivered EtOH in pre-weanling rats. Pups are reluctant to perform an operant response when reinforced with intraoral EtOH infusions, a result suggesting aversive orosensory properties of EtOH. Yet, post-absorptive effects of ethanol seem capable of supporting appetitive conditioning. Two experiments were conducted to test this phenomenon. Both included a pre-exposure phase (postnatal day 13, PD13) comprising intraoral stimulation with water or EtOH. In Experiment 1, pups were given pairings between a tactile conditioned stimulus (CS) and intraoral infusions of EtOH or water. A subsequent tactile preference test revealed that pups spent significantly less on the EtOH-related CS relative to time spent on the alternative CS. In Experiment 2 pups were exposed to a texture CS (sandpaper) while intraorally infused with EtOH or during a later EtOH post-infusion interval. A tactile locational test conducted on PD16 indicated that EtOH-pre-exposed animals that experienced sandpaper paired with EtOH's post-absorptive effects exhibited a significant preference for the CS, even relative to a control group that experienced non-reinforced exposure to the tactile CS during conditioning. These results confirm that intraoral ethanol acts as an aversive tastant. A brief pre-exposure to EtOH allows later expression of appetitive learning mediated by the drug's post-ingestive effects.

Keywords: Intraoral ethanol; Ethanol pre-exposure; Tactile conditioning; Infant rat; Ethanol reinforcement; Learning; Ontogeny

1. Introduction

Assessment of ethanol (EtOH) motivational properties is critical for understanding patterns of use and abuse of this drug. Appetitive effects of EtOH increase drug-seeking and intake, while ethanol-mediated aversive effects reduce the probability of these behaviors (Cunningham, 1988).

Rat pups and heterogeneous non-selected adult rats show avoidance of a tactile cue previously paired with ethanol's pharmacological consequences, even when ethanol dosage is kept relatively low (Cunningham et al., 1993; Molina et al., 1996; Schechter and Krimmer, 1992). Emergence of ethanol-mediated tactile preferences has required extensive training and ethanol pre-exposure procedures (Bienkowski et al., 2001; Bozarth, 1990), the combination of ethanol with alternative reinforcers (i.e., morphine, Marglin et al., 1988) or, as found in infant rats, the use of second-order conditioning procedures (Molina et al., 2006, 2007).

Models of ethanol's motivational effects mainly employ intraperitoneal (i.p.) or intragastric (i.g.) routes of administration (Cunningham et al., 2000). Different routes of drug administration are associated with different peak drug levels and rates of drug accumulation (Kuczenski and Segal, 2005). Hence, it is not surprising that method of ethanol administration affects expression of its motivational effects. Ciccocioppo et al. (1999a) found that i.g. ethanol (0.7–1.5 g/kg) was ineffective in evoking taste aversions in selectively bred Marchigian Sardinian alcohol-preferring rats. Yet, when EtOH was delivered intraperitoneally doses as low as 0.7 g/kg induced conditioned taste aversion. Method of EtOH delivery also affected expression of ethanol-mediated conditioned place preference (Ciccocioppo et al., 1999b).

Both i.p and i.g methods of ethanol delivery allow systematic control of factors such as ethanol dosage, absorption, distribution

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and elimination rates. Yet, they do not model accurately patterns of ethanol consumption observed in humans. Alternative models, employing the intraoral (i.o.) route of administration are needed to achieve this goal (Samson and Li, 1988). Factors regulating ethanol intake patterns not only include EtOH's post-ingestive consequences but also olfactory, gustatory and tactile qualities associated with the drug (Kiefer and Dopp, 1989; Fidler et al., 2007). Yet, there have been few studies aimed at understanding hedonic properties of i.o. EtOH, particularly in pre-weanling animals. Assessment of hedonic consequences of oral EtOH has been mainly restricted to operant procedures and halted by the apparent aversiveness of the orosensory features of ethanol (Morrow et al., 1993) as well as by problems such as the delay between EtOH consumption and onset of its pharmacological effects (Samson et al., 1988).

Experiments examining whether behavior is affected by pairings of tactile or odor CSs with intraorally delivered EtOH have been mainly restricted to the neonate rat (in adult animals see: Eckardt, 1975). Employing a surrogate nipple preparation, Petrov et al. (2001) found that newborns readily consumed 0.1% v/v saccharin or 5% v/v EtOH, with significantly lower consumption scores observed for pups given 10% v/v EtOH. Yet, when subsequently exposed to the empty surrogate nipple, level of nipple attachment was greater for pups that previously consumed 10% EtOH from the nipple than for those that previously consumed 5% EtOH or 0.1% saccharin. These results suggested that newborns treated the higher EtOH concentration as less palatable but more reinforcing.

In infant rats (3, 9 or 15 days of age; Domínguez et al., 1993) operant responding for intraorally delivered milk dropped significantly when milk was contaminated with EtOH. Recently, Ponce et al. (2006) observed that infant rats significantly reduced amount of nose poking for sucrose after the intraorally-infused sweet reinforcer was contaminated with EtOH (6% v/v). In a follow-up experiment, pups that had been passively pre-exposed to a brief intraoral infusion of EtOH showed substantial ethanol-mediated operant responding (Ponce et al., 2006). We recently trained two-week old rats in a novel operant task in which intraoral infusion was contingent with touching of coined-shaped sensor (Pautassi et al., 2006). Fewer ethanol-related operant responses were observed in paired animals than in yoked controls during both training and extinction sessions. Paired pups also showed a progressive decrease in EtOH self administration across days. Presumably, pups associated the target operant behavior with aversive orosensory effects related to oral EtOH (i.e., taste, smell). However, yoked pups that had pre-exposure to EtOH's orosensory properties showed a location preference for the specific section of the operant cage associated with EtOH. The latter result suggests that delayed, post-absorptive effects of i.o EtOH may be appetitive.

We propose two main hypotheses relative to the hedonic effects of intraorally delivered ethanol in pre-weanlings: (a) Chemosensorial properties (taste, odor, etc) of oral EtOH constitute an aversive stimulus, and (b) the delayed, post-ingestive effects of oral ethanol exert appetitive effects in these animals, particularly after prior exposure to ethanol's orosensory and pharmacological consequences. The present study examined these hypotheses. Specifically, we wanted to test the possibility that, in infant rats, intraorally delivered EtOH would exert aversive acute effects during drug delivery but that the subsequent, post-absorptive effects of the administration may induce appetitive reinforcing consequences. In Experiment 1, animals were exposed to a tactile CS while experiencing either intraorally delivered EtOH or its vehicle. In Experiment 2 a tactile cue was paired with the actual intraoral EtOH stimulation or with the delayed post-ingestive effects of the administration. In both experiments, development of ethanol-mediated preferences or aversions towards the target texture was then assessed.

2. General methods

2.1. Subjects

One hundred and forty-eight Sprague–Dawley rat pups, representative of twenty litters born and reared at the Center for Developmental Psychobiology (Binghamton University, USA), were employed. Births were examined daily and the day of parturition was considered as PD 0. Pups were housed with the dam in standard maternity cages with free access to water and food. The colony was kept at 22–24 °C and a 12-hour light–dark cycle was used. At the start of the experiment (PD 13) animals had a mean body weight of 31±2.5 g. Experimental procedures complied with the Guide for Care and Use of Laboratory Animals (NIH, Institute of Laboratory Animal Resources, 1996) and were also approved by the local animal care committee.

2.2. Cannulation procedures

Procedures conducted during pre-exposure and conditioning required animals to be intraorally implanted with polyethylene tubing cannulae. Intraoral cheek cannulation is minimally stressful in pre-weanlings (Spear et al., 1989) and allows stimulation with a variety of tastants (Arias and Chotro, 2005). These cannulae were made from 6-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 1/2 precision glide. The needle was pulled through the medial internal surface of the cheek of the subject. Consequently, the flanged end of the cannulae rested over the oral mucosae while the remainder exited from the mouth. When not in use, the reminder of the cannulae was secured by means of a small cap made of PE 50 tubing.

2.3. Pre-exposure and intraoral infusion procedures

On PD 13, pups were separated from the dam and placed in pairs in holding cages kept warm (32–34 °C) through the use of heating pads. Pups were immediately implanted with an intraoral polyethylene cannulae. One hour later, the pup's anogenital region was gently stroked with a cotton swab in order to stimulate defecation and void the subject's bladder. This manipulation was conducted prior to pre-exposure and conditioning procedures. It minimized error in terms of intake

measurements caused by pups defecating and urinating. Pups were then weighed to the nearest 0.01 g (Sartorius, Gottingen, Germany) Next, they were intraorally stimulated with either EtOH (5% v/v) or its vehicle, distilled water. Fifteen 2-second pulses (5 µl per pulse, pulse duration: 1.8 s, interstimulus interval: 55 s) were delivered throughout the trial. Two of these trials were performed (intertrial interval: 30 min). Total amount of EtOH delivered during pre-exposure was 150 µl. When considering the average weight of our subjects (31 g), total intake of EtOH delivered might have induced a maximum EtOH dose of 0.19 g/kg, approximately. Taking into account previous work conducted at this age, (e.g., Molina et al., 2006) it could be estimated that blood ethanol levels (BELs) induced by the pre-exposure treatment can reach a maximal level of approximately 17 mg%.

Across experiments, intraoral infusions of EtOH or water were delivered via an infusion pump (Harvard Apparatus syringe pump, Hollinston, MA) connected to the subject's cannulae (see Fig. 1). At the end of infusion procedures, pups were disconnected from the pump and returned to the holding cages. They remained there for 90 min, prior to being reunited with their mother.

2.4. Testing procedures

Tactile preference assessments were conducted on PD 16. Pups were separated from the dam and placed in pairs in heated holding chambers. Tactile preferences were assessed 1 h later. Subjects were placed into a clear Plexiglas split-floor box (28 × 12 × 15 cm). Half of the floor was lined with sandpaper (coarse: 50, Gatorgrit, USA) while the remaining floor surface was covered with the smooth backside of a piece of sandpaper. Both textures were replaced in each new test. The testing procedure lasted 5 min and was conducted under red light. Time spent over each particular tactile section of the apparatus was recorded. The middle section of the apparatus (15% of the entire surface) was considered as a neutral area and not taken into account for data collection nor analysis.

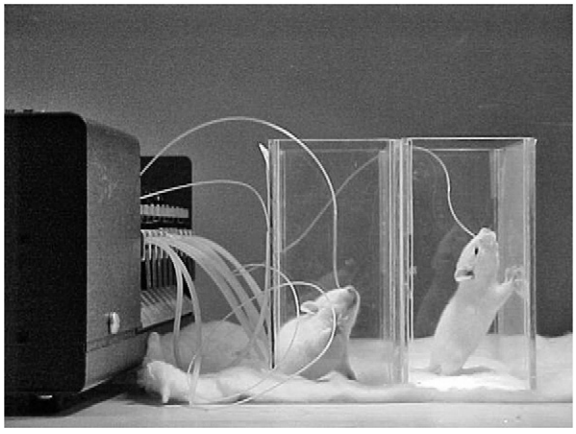

Fig. 1. Intraoral infusion procedure. Solutions (water or ethanol, 5%, v/v) were delivered in a pulsate pattern by means of an infusion pump connected to a cannulae positioned in the cheek of the animal.

3. Experiment 1

The present experiment examined whether intraoral infusion of EtOH acts as an aversive unconditional stimulus (US) in infant rats. If this is correct, a stimulus paired with intraoral EtOH should acquire aversive properties relative to a stimulus paired with intraoral infusion of a neutral sapid stimulus. On PD 14 and 15 animals received pairings of a given texture (sandpaper or the smooth backside of a sandpaper sheet) while intraorally infused EtOH or water. Water was chosen on the basis of previous research reporting a relatively neutral motivational value of this stimulus when tested for taste reactivity patterns in a variety of species, including monkeys as well as human and rodent infants (Ueno et al., 2004; Steiner et al., 2001; Petrov et al., 2001). In newborn and neonatal rats, water evoked more positive hedonic scores than bitter substances (i.e., quinine, Kozlov et al., 2006). Yet, number of positive reactions elicited by water is still lower than for sweet tastants such as sucrose (Nizhnikov et al., 2002). Similar results have been recently observed in our lab when assessing intake and behavioral responsiveness to water infusions in 15 day-old rats (Molina et al., 2006).

Pups were tested regarding their preference for tactile CSs in a two-way preference test (see General methods). The relative preference/avoidance observed towards the texture originally paired with the drug infusion was considered as an index of the hedonic properties of intraoral EtOH. In light of previous studies suggesting that pre-exposure to ethanol affects later ethanol-mediated learning (Ponce et al., 2006; Reid et al., 1985), pups were subjected to an initial phase where they were passively stimulated with i.o. ethanol or its vehicle. In summary, a salient texture was presented during infusion of a neutral stimulus whereas an alternative tactile CS was experienced in contiguity with oral EtOH. We expected that pups would learn to avoid the texture paired with the orosensory properties of EtOH.

3.1. Materials and methods

3.1.1. Experimental design

The experimental approach was defined by two independent factors. During pre-exposure pups received either intraorally infused EtOH or water (Groups PE and PW, respectively). The second factor took into account whether pups were exposed to the sandpaper CS during EtOH or water intraoral infusions. That is, a counterbalanced 2 × 2 factorial design was employed. Half of the pups were exposed to a tactile salient stimulus (sandpaper) while receiving oral EtOH and to a smooth surface (the reverse side of the sandpaper sheet) when infused with water (Groups SAND+). The reverse sequence of stimuli presentation (i.e., water infusions paired with sandpaper and oral EtOH paired with the smooth surface) took place in the remaining animals (Groups SAND−). Total number of animals in each of these four groups were as follows: PE/SAND−: 15, PE/SAND+: 11, PW/SAND−: 13, PW/SAND+: 11. To eliminate confounding of litter with treatment effects, no more than one subject from a given litter was assigned to the same

treatment condition. The number of males and females in each group was balanced.

3.1.2. Procedures

As depicted in Fig. 2A, the experimental protocol was divided into 3 phases: pre-exposure, conditioning and tactile preference assessment.

On PD 13, pre-exposure was conducted as described in Section 2.2. Conditioning sessions took place during PDs 14 and 15. Pups representative of both pre-exposure conditions (EtOH or Water) were assigned to one of two conditioning groups (SAND+ or SAND−, see Fig. 2A). These groups were exposed to a variant of the widely employed conditioned place preference paradigm (Fidler et al., 2004). In a place preference preparation, animals are given pairings between a set of environmental stimuli and the unconditional effects of a given drug or a non-drug treatment. Motivational learning is later assessed by measuring time spent in the vicinity of these stimuli relative to time spent in an alternative set of CSs (Tzschentke, 1998). In the present experiment, a tactile stimulus was paired with intraoral infusion of distilled water and an alternative tactile stimulus was paired with intraorally delivered EtOH. Conditioning procedures were as follows. Pups were cannulated and left undisturbed in standard holding cages for 60 min. Then animals were voided, weighed and placed in individual Plexiglas boxes (15 × 7 × 14 cm) lined either with a sandpaper floor (coarse: 50, Gatorgrit, USA) or with a smooth cardboard surface (Groups SAND− and SAND+, respectively). All textures were replaced in each new conditioning trial. In these boxes, pups were stimulated with intraoral infusions of distilled water (20 min). Specifically, they received 65 infusions of 5 µl each (duration of the infusion: 1.8 s). After completion of the water stimulation procedure pups were again weighed to determine water consumption scores and returned to their respective holding cages where they remained for 40 min. They were then placed again in clean, individual Plexiglas boxes. These receptacles were now lined with the alternative tactile stimulus. That is, those pups that had been originally stimulated with water while over sandpaper now were placed in a box lined with the smooth cardboard surface (Group SAND−). On the

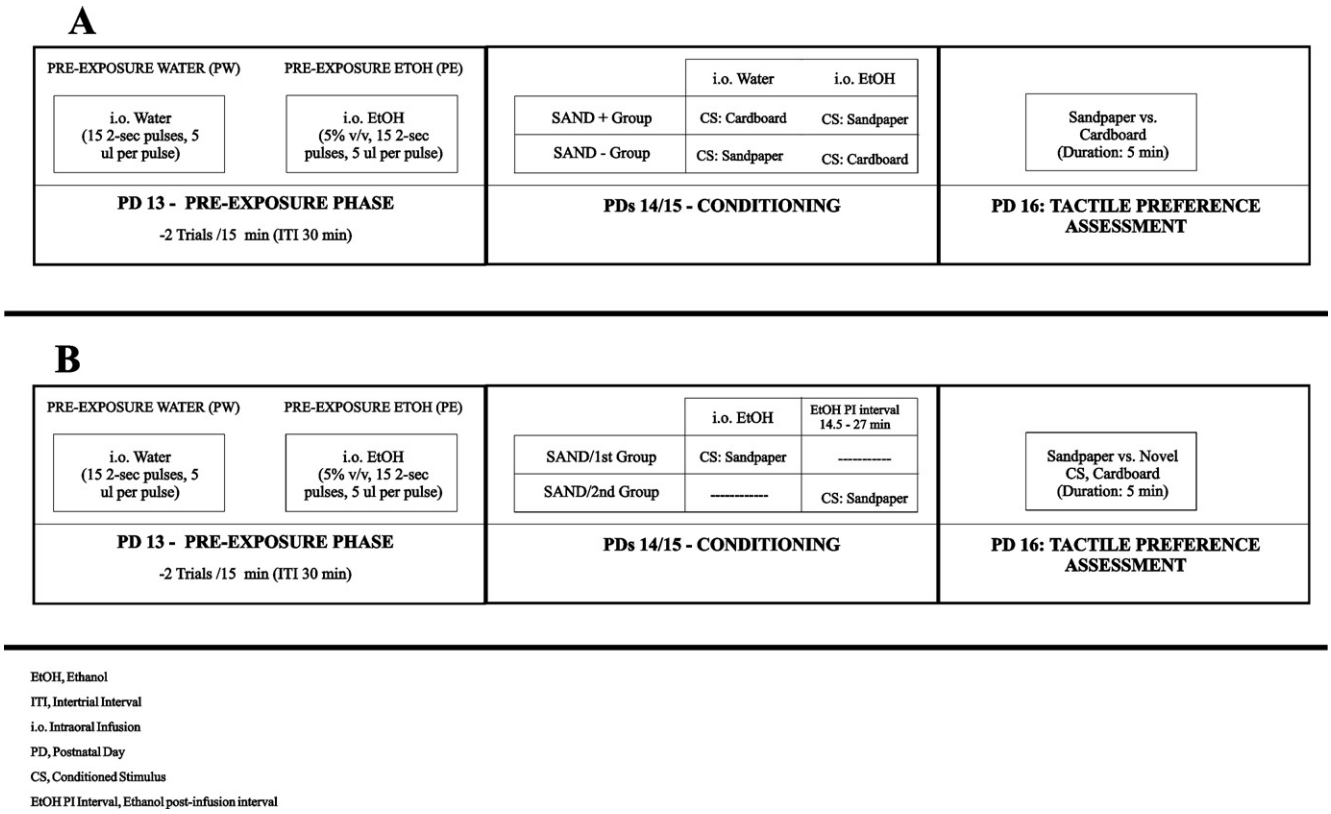

Fig. 2. A. Methods for the analysis of motivational properties of intraorally delivered ethanol in infant rats (Experiment 1). Pre-exposure, postnatal day 13, PD 13: pups were intraorally infused with water or ethanol (5%, v/v). Conditioning, PDs 14–15: pups in the SAND+ condition were exposed to a tactile salient stimulus (sandpaper) while receiving intraoral EtOH infusion and to a smooth cardboard surface when infused with water. Pups in SAND− condition received water infusion paired with sandpaper and oral EtOH paired with the smooth surface. Tactile assessment, PD 16: time spent over the sandpaper CS was recorded in a 2-way tactile preference assessment (sandpaper vs. smooth cardboard). B. Methods for the analysis of motivational properties of the delayed, post-ingestive effects of intraorally delivered ethanol in infant rats (Experiment 2). Pre-exposure, postnatal day 13, PD 13: pups were intraorally infused with water or ethanol (5%, v/v). Conditioning, PDs 14–15: pups in the SAND/1st group were exposed to a tactile CS (sandpaper) while receiving intraoral ethanol infusion and were then placed in chambers lined with pine shavings. Pups in SAND/2nd condition were given ethanol infusions while over pine shavings and then exposed to a tactile CS (sandpaper) during the ethanol post-infusion interval 14–27.5 min. Experiment 2 also included 2 CS-only conditions (not shown in the figure). Pups in these control groups were exposed to sandpaper either during the first or second phase of the conditioning trials but did not receive intraoral infusions at any phase of the Experiment. Tactile assessment, PD 16: time spent over the sandpaper CS was recorded in a 2-way tactile preference assessment (sandpaper vs. novel tactile CS, smooth cardboard).

other hand, pups that had received water in contiguity with the smooth stimulus were introduced in a sandpaper-covered cage (Group SAND+). During this second phase (20 min), animals received 65 intraoral pulses of EtOH (5%, 5 µl each, pulse duration: 1.8 s, interinfusion interval: 15 s in average). Total volume administered to the pups during each trial was 325 µl. The infusion schedule was chosen after reviewing the pattern of EtOH self-administration found by Pautassi et al. (2006). Body weights were registered after the second infusion procedure to determine ethanol intake scores.

A tactile preference test (duration: 5 min; sandpaper vs. smooth surface) was conducted on PD 16.

3.1.3. Data analysis

In this, as well as in the following Experiment, Tactile preference scores were expressed as (a) total number of second spent over the sandpaper section of the apparatus and (b) percent time spent on sandpaper. The latter index was calculated as follows: [(total time spent over sandpaper × 100)/(total time spent over sandpaper + total time spent over smooth].

Preliminary analysis of total and percent time spent on sandpaper showed no main significant effects of gender or interaction with other factors. Similarly sex was never found to affect water or ethanol consumption scores. Hence, descriptive and inferential analysis of the data was performed by collapsing across gender.

The main dependent variable under analysis was total amount of time (seconds and percent time) spent on the sandpaper CS during the 5-minute location preference test conducted on PD 16. When using this particular type of assessment (see Fidler et al., 2004), conditioning is indicated by the difference between SAND+ and SAND− groups. Location aversions would be indicated if the SAND+ subgroup exhibits significantly less time spent over sandpaper than the SAND− group. A reverse pattern would be an index of pups having acquired a preference towards the sandpaper. Hence, absolute and percent time spent over sandpaper were analyzed by means of a 2 × 2 ANOVA [pre-exposure treatment (intraoral EtOH or distilled water) × conditioning group (SAND+ or SAND−)].

Consumption of water and EtOH across conditioning was analyzed by means of 4-way mixed ANOVA that considered pre-exposure and conditioning treatment as between-subjects factors. Solution infused (water and EtOH) and day (PD 14 and PD 15) served as within-measures factors. Consumption scores were expressed in µl and derived from the weight measurements conducted before and after each conditioning session. In this as well in the subsequent experiment, the loci of significant main effects or interactions were further examined by means of post-hoc comparisons (Duncan's multiple range tests, type I error set at .05).

3.2. Results and discussion

SAND+ animals spent less time on the sandpaper section of the testing cage than those in the SAND− condition, indicating the development of a conditioned aversion towards the texture (Fig. 3). This pattern was not affected by the nature of the pre-exposure manipulation. The corresponding ANOVA yielded a significant main effect of conditioning group [$F(1, 46) = 6.04$; $p < 0.05$]. In other words, pre-weanlings exhibited avoidance of a texture previously paired with intraoral EtOH stimulation. No main effect or significant interaction comprising pre-exposure treatment was observed.

The ANOVA for percent time spent on the sandpaper CS yielded a similar pattern of results. That is, a significant main effect of conditioning group was found [$F(1, 46) = 5.86$; $p < 0.005$]. Percent time spent over sandpaper was significantly lower when this CS was previously associated with intraoral EtOH infusion. Percent preference scores, in terms of mean and standard error values for each condition were as follows: PE/SAND− = 61.96 ± 5.51, PE/SAND+ = 50.82 ± 7.75, PW/SAND− = 63.49 ± 5.53, PW/SAND+ = 43.27 ± 7.36.

Pups, regardless of pre-exposure treatment and conditioning subgroup, consumed more EtOH than water across conditioning. The ANOVA detected a significant main effect of solution, $F(1, 42) = 10.44$, $p < 0.005$, an effect that failed to interact with the remaining factors. Overall means and standard errors for ethanol and water intake scores (µl) across conditioning were as follows: Water: PD 14 = 242.60 ± 10.0, PD 15 = 236.30 ± 30.3; Ethanol: PD 14 = 294.35 ± 5.0, PD 15 = 288.70 ± 10.3. Mean and standard error values for ethanol consumption in grams per kilogram (g/kg) were as follows: PD 14 = 0.36 ± 0.01, PD 15 = 0.33 ± 0.01

In summary, animals exhibited avoidance of the texture CS paired with intraoral EtOH delivery. This result supports the hypothesis guiding the experiment. Orosensory properties of oral EtOH, possibly coupled with manipulations required to intraorally deliver tastants (Pautassi et al., 2005) seem to exert aversive hedonic effects in pre-weanlings. This result could help explain the negative results previously found when trying to develop models aimed to promote operant self-administration of EtOH in pre-weanling rats (Pautassi et al., 2006; Ponce et al., 2006,

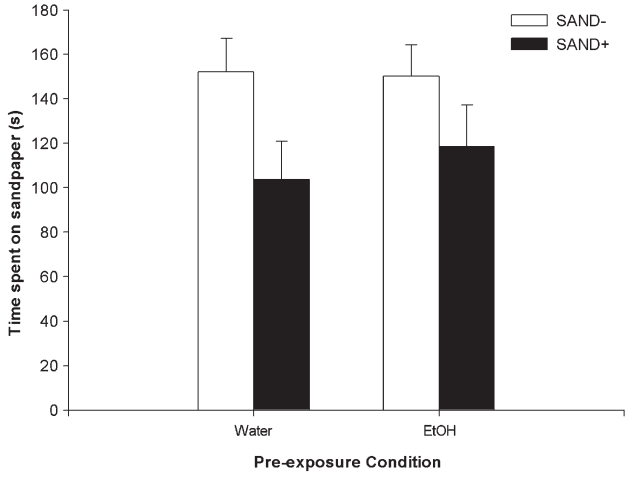

Fig. 3. Time spent on sandpaper (conditioned stimulus) as a function of conditioning procedures [sandpaper paired with ethanol (5% v/v) or water intraoral infusions, SAND+ and SAND−, respectively] and treatment received during pre-exposure (intraoral infusions of ethanol, 5% v/v, or its vehicle, water). Vertical bars represent the standard error of the mean (S.E.M.).

Domínguez et al., 1993). Pre-exposure manipulations were not effective in attenuating the conditioned avoidance response.

4. Experiment 2

Experiment 1 indicated that pups developed a conditioned aversion when intraoral EtOH was paired with a salient texture stimulus. Interestingly, these results neither reject nor support the possibility of post-absorptive effects of oral EtOH exerting appetitive effects in infants. It could be the case that animals perceive orosensory EtOH as aversive but can also perceive its delayed pharmacological effects as appetitive (Pautassi et al., 2006; Ponce et al., 2006). Experiment 2 tested the latter hypothesis by assessing preference patterns to a stimulus paired with the delayed, post-absorptive effects of intraorally delivered EtOH.

On PD 13 pups were pre-exposed to the orosensory properties of EtOH or its vehicle. On PD's 14–15 animals were given one daily conditioning trial. In contrast to Experiment 1, conditioning did not include pairings of a texture and water infusions. Experiment 1 intended to analyze motivational effects of intraorally delivered EtOH during drug administration. Previous evidence suggests that the intraoral infusion method possess some inherent aversive effects (Pautassi et al., 2005). Hence, in Experiment 1 we equated pups in terms of intraoral manipulations. That is, to assess motivational properties of i.o. EtOH it was necessary to include pairings between an alternative texture and intraoral infusions of a relatively neutral fluid. On the other hand, Experiment 2 was specifically aimed at assessing whether a tactile cue paired with post-absorptive EtOH would acquire appetitive motivational properties. Inclusion of pairings between intraoral water and an alternative texture would have been problematic in this Experiment. First, confounding effects due to generalization across textures can represent a critical problem when trying to understand the specific effects of the contingency existing between the CS paired with ethanol's post-ingestive consequences. Also, if intraoral infusion exerts some aversive effects in itself (Pautassi et al., 2005), we cannot discard that the CS paired with this stimulus may acquire negative hedonic valence. Hence, at test it would be very difficult to determine if acceptance of a given texture (e.g. the one paired with ethanol's post-absorptive effects) reflects a conditioned preference or if animals are simply avoiding the alternative texture paired with intraoral liquid delivery. Hence, Experiment 2 did not use the SAND+/SAND− design described in Experiment 1. Rather, pups were exposed to the sandpaper CS while intraorally infused with EtOH or during a later EtOH post-infusion interval. Tactile preferences (sandpaper vs. backside of a sandpaper sheet) were assessed on PD 16. In the present experiment pups were not exposed to the alternative cue during conditioning. This is likely to influence expression of the learning since the back of the sandpaper was a novel cue. Hence, to properly assess the expression of potential location preferences, isolated basal control conditions were included in the present Experiment. Animals in these basic control conditions had neither pre-exposure on PD 13 nor EtOH infusions on PD's 14–15. Yet, they had similar sandpaper exposure as animals in the remaining experimental conditions.

4.1. Materials and methods

4.1.1. Experimental design

A 2 (pre-exposure treatment: EtOH or water) × 2 (conditioning procedure) × 2 (volume of EtOH infusion) factorial design with two isolated control conditions was employed. During pre-exposure, pups were infused with EtOH or with water. The second factor was timing of exposure to the sandpaper CS during conditioning. Pups experienced sandpaper during either the first or second phase of each daily conditioning trial. Also, pups were given 5 or 10 μl of EtOH in each intraoral pulse. Animals in the isolated, CS-only control conditions were exposed to sandpaper either during the first or second phase of the conditioning trials. These control groups did not receive intraoral infusions at any phase of the Experiment. The 8 groups derived from the design were composed by 8–11 pups; isolated control groups had 9–10 animals. Each condition included a balanced number of male and female subjects.

4.1.2. Procedures

The procedure was divided into 3 stages, as depicted in Fig. 2B. On PD 13, animals were pre-exposed to i.o. EtOH or Water, as previously described (see Section 2.2.). Daily conditioning sessions were then conducted (PDs 14 and 15). Pups representative of both pre-exposure conditions were implanted with intraoral cannulae and remained in warmed holding cages for 60 min. Each daily conditioning trial was divided in two consecutive phases, defined by the presence or absence of intraoral EtOH stimulation and the type of tactile stimuli presented to the pups.

First conditioning phase: Pups were introduced in clear Plexiglas boxes (15 × 7 × 14 cm). For half of the pups (Group SAND/1st), these boxes were lined with sandpaper. The remaining pups (Group SAND/2nd) were placed in boxes devoid of any salient new texture (lining: clean pine shavings). Then, stimulation with an EtOH solution (5% v/v) began. During 12.5 min pups received 50 intraoral EtOH pulses (duration: 1.8 s, average interval between infusions: 15 s). Pups received either 5 or 10 μl of EtOH in each pulse. Employment of the higher volume was chosen so as to deliver an EtOH dose approximately equivalent to 0.5 g/kg. Post-administration effects of this EtOH dose have been observed to exert appetitive effects in developing animals (Fernandez-Vidal et al., 2003; Molina et al., 2006, 2007). Hence, total volume of EtOH infused was either 250 or 500 μl. Taking into account the mean weight of the animals employed, subjects infused with 5 μl of EtOH per pulse should have received a maximal EtOH dose equivalent to 0.33 g/kg, while 0.66 g/kg represent the maximal EtOH dose when utilizing 10 μl per intraoral infusion pulse.

Second conditioning phase: Two minutes after completion of the intraoral EtOH procedure, pups were transferred to individual Plexiglas containers (15 × 7 × 14 cm), where they remained for 12.5 min. Those animals that had been exposed to the rough sandpaper texture during the first phase (Group SAND/1st) were kept in standard pine-shavings floored cages. On the other hand, those that had not been exposed to any particular texture while infused with EtOH were now kept in

sandpaper-lined containers (Group SAND/2nd). No additional manipulations were conducted during this 12.5 min session.

Two isolated CS-only groups were also employed. Two animals of each litter were randomly assigned to these conditions. They remained untreated during PD 13. On PD 14 and 15 they were cannulated, separated in pairs in holding cages and later exposed to the sandpaper texture during either the first (Group CS-only 1) or second phase of the conditioning trial (Group CS-only 2). Ethanol infusion was absent in these groups.

Reactivity towards the CS employed during conditioning (sandpaper) was assessed on PD 16 by means of a two-way location preference test (sandpaper vs. the smooth side of the sandpaper sheet).

Procedural differences existed between Experiments 1 and 2. In the present experiment, pups remained alone for 14.5 min after infusion termination. According to extensive unpublished studies conducted in our lab using pups of similar age as those here employed, this isolation period results in weight loss due to defecation and miction, rendering weight measurements unreliable in terms of an intake index. Hence, body weight gains were not registered in Experiment 2. This precluded obtaining a measure of ethanol intake. Also, while Experiment 1 employed a 20 min conditioning trial, both conditioning phases of Experiment 2 lasted 12.5 min. The infusion time (i.e., conditioning phase 1) was shortened with the aim of delivering the desired EtOH dose in a relatively brief period of time. In turn, phase 2 was aimed at pairing the tactile CS with the initial, early ethanol post-absorptive interval, when blood ethanol concentrations are still rising. It has been suggested that during the onset of the state of intoxication ethanol may be exerting appetitive unconditional effects, while aversive effects are likely to emerge at later stages (Risinger and Cunningham, 1992; Pautassi et al., 2002). Hence, duration of phase 2 was meant to restrict conditioning to a temporal window where appetitive effects of the drug are more likely to be encountered.

4.1.3. Data analysis

The dependent variable was time spent over the sandpaper section at test (absolute and percent scores). Preliminary analysis indicated that gender did not affect tactile preferences, leading to data being collapsed across this condition. Also, the two CS-only isolated control groups did not differ in terms of texture preferences as a function of timing of exposure to sandpaper during conditioning, [$F(1, 17)=0.46$; $F(1, 17)=1.57$; both p's >0.20; absolute and relative scores, respectively]. They were thus combined in a single condition. Tactile preferences were then assessed by a three-way ANOVA with these between-group factors: pre-exposure treatment (EtOH or water), conditioning group (SAND/1st or SAND/2nd) and volume of EtOH infusion per pulse (5 or 10 µl). The CS-only Group was included in the ANOVA model as an isolated control condition. Consequently, this group was taken into account in the calculation of the error sums of squares, enhancing the fitness and predictive value of the statistical model. When justified by a priori hypothesis, planned comparisons comprising the CS-only group were also conducted.

4.2. Results and discussion

The ANOVA for absolute time spent on sandpaper yielded a significant interaction between pre-exposure treatment and conditioning procedure, $F(1, 89)=7.12$; $p<0.01$. Post-hoc analysis revealed that sandpaper preferences were not affected by conditioning treatment in water pre-exposed pups. As can be observed in Fig. 4, regardless of whether sandpaper predicted the EtOH intraoral infusion or its post-absorptive consequences, pups pre-exposed to only water (PW) spent approximately 125 s on sandpaper. This value was very similar to the one observed in the CS-only control condition (120+/−9.3 s). A different pattern was observed in ethanol-pre-exposed animals (PE). Among pups pre-exposed to ethanol during PD 13, those given pairings between sandpaper and post-absorptive effects of EtOH (Group PE SAND/2nd) spent significantly more time on sandpaper than animals given pairings of the rough texture and intraoral infusion of EtOH (Group PE SAND/1st). Animals in the PE SAND/2nd condition also showed a trend ($p=0.07$) for greater sandpaper preference than those given similar pairings of sandpaper and post-absorptive EtOH but treated with water during pre-exposure (Group PW SAND/2nd). Interestingly, as indicated by a planned comparison, the group PE SAND/2nd exhibited heightened absolute sandpaper preference than the CS-only group, $F(1, 89)=4.00$, $p<0.05$.

Volume of infusion was not observed to exert a main significant effect or to significantly interact with any of the remaining variables.

Similar results were obtained when analyzing percent time spent on the sandpaper CS. The ANOVA yielded a main effect of conditioning, an effect that was tempered by a significant

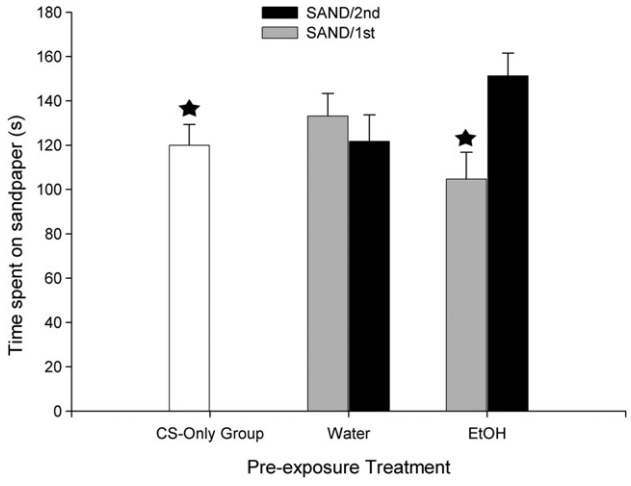

Fig. 4. Time spent on sandpaper (conditioned stimulus) as a function of conditioning procedures [sandpaper paired with acute intraoral infusion of ethanol (5% v/v) or with a later post-ingestive interval, SAND/1st and SAND/2nd, respectively] and treatment received during pre-exposure (intraoral infusions of ethanol, 5% v/v or its vehicle, water). An independent, CS-only condition is represented in the isolated gray bar. To facilitate data visualization, data has been collapsed across volume of EtOH infusion (5 or 10 µl). The latter factor did not affect sandpaper preference scores on PD 16 nor significantly interacted with the remaining factors. Asterisks indicate significant differences from the SAND/2nd group pre-exposed to EtOH ($p<0.05$).Vertical bars represent the standard error of the mean (S.E.M.).

conditioning × pre-exposure interaction, $F(1, 89)=5.23$; $F(1, 89)=5.98$; both p's <0.05. Post-hoc tests indicated that pups in group PE SAND/2nd spent significantly more time on sandpaper than did the remaining experimental conditions. A planned comparison also revealed significantly greater predilection for sandpaper in the PE SAND/2nd group than in the CS-only group [$F(1, 89)=5.51$, $p<0.05$]. Overall means and standard errors for percent time preference across volume of infusion were as follows: PE/SAND/1st = 44.92 ± 4.51, PE/SAND/2nd = 64.30 ± 3.52, PW/SAND/1st = 52.95 ± 3.13, PW/SAND/2nd = 52.33 ± 4.79, CS-only Group = 52.36 ± 3.84

These results indicate expression of an EtOH-mediated conditioned tactile preference in animals pre-exposed to EtOH and later given pairings of sandpaper and post-absorptive effects of the drug (Group PE SAND/2nd). Given prior exposure to ethanol, the delayed pharmacological effects of EtOH seem to endow an initially neutral stimulus with appetitive properties.

In summary, delayed post-absorptive effects of intraoral EtOH exerted appetitive effects, provided that pre-exposure to ethanol preceded conditioning. Specifically, divergent texture-preference profiles were observed as a function of pre-exposure manipulations. While pre-conditioning water failed to alter performance at test, ethanol-pre-exposed animals given pairings of sandpaper and post-absorptive EtOH exhibited preference for sandpaper, even when compared with animals given only non-reinforced exposure to the target CS.

5. General discussion

Previous work suggests that intraorally delivered EtOH may exert both aversive and appetitive hedonic effects in infant rats (Domínguez et al., 1993; Pautassi et al., 2006; Ponce et al., 2006). Employing similar intraoral infusion parameters as those previously cited, the present study analyzed this phenomenon through Pavlovian conditioning procedures aimed at determining the hedonic value of orosensory and post-ingestive effects of ethanol. Pups showed conditioned locational avoidance when confronted with a texture CS previously associated with intraoral EtOH (Experiment 1). On the contrary, infants exhibited conditioned locational preferences towards a texture that originally signaled the drug's post-ingestive effects (Experiment 2).

Aversive orosensory effects of EtOH have been suggested as a major obstacle (a "taste barrier") for the development of animal models aimed at analyzing reinforcing properties of oral EtOH (Kiefer and Dopp, 1989; Samson et al., 1988). In rats, EtOH oral consumption markedly decreases as concentration of the drug reaches approximately 6% (Kiefer et al., 1987; Ponce et al., 2004). Kiefer et al. (2005) observed substantial aversive orofacial reactions in adult rats intraorally stimulated with ethanol (10% v/v). Aversive reactivity decreased in animals given extensive prior experience with i.o. ethanol (also see Kamback, 1973). To our knowledge, no previous research has provided evidence of these apparent aversive sensory properties supporting motivational learning, particularly in young animals. Experiment 1 provides direct evidence of the aversiveness of intraorally delivered EtOH in pre-weanlings. Exposure to oral ethanol endowed an initially neutral exteroceptive CS with an aversive hedonic value.

This phenomenon might underlie the failure of pre-weanling pups to exhibit substantial operant responding when reinforced with intraoral EtOH (Domínguez et al., 1993; Pautassi et al., 2006; Ponce et al., 2006). It should be noticed that intraoral liquid delivery in itself has some inherent aversive properties (e.g., liquid temperature or pressure, unusual source of liquid stimulation; Pautassi et al., 2005) that could have interacted with the orosensory effects of EtOH in terms of mediating aversive conditioning. Experiment 1 included appropriate control groups to safely draw a conclusion about the specificity of this aversive effect relative to EtOH's orosensory properties. Both tactile CSs were made contingent with intraoral infusion delivery. Yet, the tactile conditioned avoidance was expressed when the CSs predicted intraoral ethanol (GRID+ condition). That is, stimulation with ethanol's orosensory properties seems to represent a sufficient factor for the establishment of an aversive conditioned response.

Interestingly, pups avoided the EtOH-related texture even when EtOH's consumption scores during conditioning were higher than those obtained with water. This result adds to previous evidence (Petrov et al., 2001; Samson and Czachowski, 2003) suggesting that affinity for ethanol ingestion and sensitivity for ethanol reinforcement might be governed by different mechanisms. Indeed, a surprisingly weak relationship between ethanol intake and ethanol reinforcement has been observed in animal models (Files et al., 1997; 1998). Divergence of ethanol consumption and reinforcement had also been observed in newborn rats (Petrov et al., 2001) but not in pre-weanlings rats such as those employed in the present experiment.

The possibility that delayed post-absorptive effects of EtOH exerting positive reinforcing consequences was tested in Experiment 2. Provided pre-exposure to oral ethanol had taken place, pups given pairings of a texture CS and delayed EtOH effects exhibited an enhanced level of preference towards this CS. That is, prior exposure to EtOH facilitated subsequent expression of ethanol-mediated appetitive learning. This result is consistent with previous research suggesting that an initial acclimation to the taste and odor of the drug is needed to allow expression of positive unconditional effects of EtOH (Reid et al., 1985). It is possible that pups experienced pharmacological effects of the drug during pre-exposure. Total consumption of EtOH delivered during pre-exposure could have induced a maximum EtOH dose equivalent to 0.19 g/kg. Motivational effects of EtOH have been recently found in two-week old rats when employing doses as low as 0.25 g/kg (Molina et al., 2006). This raises the possibility that central effects of the drug may have been implicated in the pre-exposure effect (Experiment 2).

To our knowledge, this is one of the few studies reporting ethanol-mediated first-order conditioned preferences in heterogeneous rats. Infant and adults usually express conditioned aversions when stimuli are paired with ethanol (Pautassi et al., 2002). The expression of conditioned tactile preferences in prior studies has required prolonged pre-exposure to the pharmacological properties of EtOH (twenty days or more; Bienkowski et al., 1995; Reid et al., 1985). As stated, experience with the post-

absorptive effects of EtOH during pre-exposure could have facilitated subsequent acquisition of the appetitive learning obtained in the present study. Yet, an important difference with these previous studies (Bienkowski et al., 1995; Reid et al., 1985) is that, in Experiment 2, ethanol-mediated conditioned preferences emerged after only a single and brief episode implying contact with EtOH's sensory and post-ingestive effects.

In Experiment 2, pups given pairings of intraoral EtOH infusions and sandpaper (Groups Sand/1st) did not exhibit significant differences when compared with the CS-only control condition. This profile is different from the one found in Experiment 1. In that experiment, the contiguity between intraoral EtOH and a given texture endowed the tactile CS with aversive properties. Procedural differences between experiments could help explain this apparent discrepancy. First, magnitude of the US was greater in Experiment 1, both in terms of intensity and duration. Perhaps more importantly, Experiment 1 employed a differential conditioning procedure. That is, a tactile stimulus (CS+) predicted intraoral infusion of EtOH while the alternative texture (CS−) signaled the absence of this event. This strategy is known to facilitate, particularly in young rats, acquisition of aversive memories otherwise not detected through simple excitatory conditioning procedures that only utilize a CS+ (Kucharski et al., 1985; Kucharski and Spear, 1984). Miller et al. (1989) found that exposure to a CS− (an odor not paired with footshock) was necessary for conditioning of the CS+ (an alternative odor paired with footshock) in 12 days-old rats. Conditioning was not expressed when using only the CS+.

In summary, it was found that pups avoided a tactile CS when this cue predicted EtOH's orosensory properties. Yet, EtOH pre-exposed pups displayed conditioned location preferences when the CS signaled post-ingestive effects of EtOH. The apparent sensory aversive component might compete with the drug's positive reinforcing effects. Despite this observation, it is necessary to remark that a minimal prior experience with EtOH's sensory or post-ingestive effects was sufficient to facilitate later appetitive learning mediated by post-ingestive ethanol. In other words, although intraoral ethanol may act as an aversive US, a brief pre-exposure to the drug allows later expression of appetitive learning mediated by post-absorptive ethanol's consequences.

Ethanol is a complex drug, particularly when administered via the oral route. As described, affinity for the drug can be determined or modulated by differential hedonic contributions of ethanol's orosensory (olfactory, gustatory or trigeminal stimulation) and post-absorptive effects (Bachmanov et al., 2003). This observation implies that the relative contribution of these factors should not be neglected when examining ethanol acceptance or seeking patterns; phenomena that not necessarily are determined by similar mechanisms (Files et al., 1997).

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