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Differential Motivational Properties of Ethanol During Early Ontogeny as a Function of Dose and Postadministration Time

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Abstract

While appetitive reinforcement effects of ethanol (EtOH) are easily detected in rat neonates, such phenomena rarely have been observed in older infants. Recently, Molina et al. (2006) reported such effects of ethanol in 14-days olds using a second-order conditioning procedure. Infants also appear to be sensitive to biphasic reinforcement or general motivational effects of ethanol, with appetitive effects seeming to occur early in the state of intoxication and aversive effects predominant during late stages, but tests have been inconclusive. The present study examined the possibility of biphasic motivational effects of ethanol during infancy through the use of second-order conditioning procedures. Preweanling rats (14 days old) experienced intraoral water infusions (conditioned stimulus, CS) either 5-20 or 30-45 min after administration of 0.5 or 2.0 g/kg i.g. ethanol. Pups were then exposed to the CS while over a novel texture (second-order phase). Tests of tactile preference for that texture followed. Locomotive, thermal, hormonal (corticosterone release) and pharmacokinetic patterns likely to underlie the acquisition of ethanol-mediated conditioning were also examined in subsequent experiments. Intraoral CSs paired with either early or late effects of low dose ethanol [0.5 g/kg, blood ethanol concentration (BEC): 40 mg%] became positive second-order reinforcers. Appetitive effects were also exhibited by pups exposed to the CS during commencement of the toxic episode induced by a 2.0 g/kg EtOH dose, 5-20 min after administration of ethanol, whereas aversions emerged when CS presentation occurred 30-45 min postadministration time (BECs: 157 and 200 mg%, respectively). Overall, the results indicate that infants rapidly detect differential motivational properties of ethanol as a function of dose or drug postadministration time. Relatively neutral stimuli associated with these properties are later capable of acting as either positive or aversive reinforcers. Thermal and motor responses that accompany ethanol intoxication do not seem to be directly associated with differential hedonic properties of the drug at this stage of development.

Keywords

Ethanol; Motivational Properties; Infant; Locomotion; Blood ethanol concentration; Thermoregulation

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1. Introduction

Even though expressing different core views, most contemporary theories of drug use and abuse agree that positive, appetitive effects of psychotropics play a major role in shaping drug seeking and consumption in human subjects (e.g., Deadwyler et al., 2004; Koob & Le Moal, 2001; Robinson & Berridge, 2000). In regards to EtOH, this has encouraged behavioral researchers to develop animal models whereby subjects demonstrate preference for the drug in voluntary intake tests, exhibit changes in the probability of execution of behaviors contingent with ethanol's postabsorptive effects or approach discrete stimuli (conditional stimuli, CS) associated with administration of the drug (Cunningham et al., 2000; Koob & Roberts, 1997).

Appetitive properties of ethanol often have been reported when employing genetically selected lines of rats and mice (Bechtholt & Cunningham, 2005; Cicocioppo et al., 1999; Cunningham et al., 2002). However, the search for appetitive effects of ethanol in heterogeneous lines of ethanol-naïve rats has proven troublesome. Most often, aversive hedonic components of this drug have been found in adult and infant animals when EtOH doses equivalent to or above 1 g/kg are paired with taste or tactile cues (Gauvin and Holloway, 1992; Hunt et al., 1991; Lee et al., 1998; Molina et al., 1996; Pueta et al., 2005; Pautassi et al., 2002; 2005b; Sherman et al., 1983). During adulthood, long-term preexposure to the drug and/or concurrent use of other drug reinforcers has been necessary to allow the expression of ethanol-mediated appetitive memories (Bienkowsky et al., 1995; 1996; Marglin et al., 1988). These experimental manipulations are not suitable to be employed in preweanling (infant) animals. Specifically, the brevity of this ontogenetic period in the rat (0-21 days) as well as the likelihood of teratological effects impose serious limitations when assessing ethanol's motivational effects during early ontogeny.

Nonetheless, increasing experimental evidence indicates that early infancy is a period of special sensitivity to ethanol's motivational properties, particularly when employing animals younger than 14 days of age. Exposure to ethanol during late gestation induces an enhanced consumption of ethanol as well as an increased palatability towards the drug later in life (Arias & Chotro, 2005), a result likely to be explained in terms of a conditioned preference acquired to the chemosensory properties of ethanol (Abate et al., 2002; Chotro et al., 2006). When a discrete odorant signals ethanol during late gestation neonates later show enhanced attachment to a surrogate nipple scented with that olfactory stimulus (Abate et al., 2002). Using the surrogate nipple technique, Nizhnikov et al. (2006) have shown not only reinforcing properties of ethanol in neonates but also heightened sensitivity to these effects as a function of prenatal ethanol exposure. Special sensitivity to sensory (Truxell & Spear 2004) and behavioral and physiological (Hunt et al., 1991) consequences of ethanol in young rats may underlie the preceding results (for reviews see Bachmanov et al., 2003; Spear & Molina, 2005).

Evidence for non-aversive motivational effects of ethanol (i.e., appetitive and/or anxiolytic) in infants older than 14 days postnatal (P14) is relatively scarce. Truxell and Spear (2004) have reported that 18-day-old pups will consume ethanol and reach pharmacologically relevant blood ethanol concentrations (BECs) when having the possibility to drink the drug from a warmed floor, though to a lesser degree than 12-day-old pups. It has also been reported that low to moderate doses of ethanol compete with or inhibit the expression of associative learning mediated by various aversive stimuli. Pautassi et al. (2005a) observed that pups avoided a salient odor that had been paired with aversive intraoral stimulation. The expression of this conditioned response was completely inhibited when pups were administered with 0.25 g/kg ethanol during training. These results suggested that young animals are sensitive to non-aversive effects of the drug, including ethanol's anxiolytic

effects. Similar suggestions were derived from a subsequent study (Pautasi et al., 2006). Specifically, pups were exposed to an odor CS paired with and aversive US (citric acid intraoral infusion). One day later pups were briefly re-exposed to the US while experiencing the postabsorptive effects of ethanol (0.5 – 1.25 g/kg) or vehicle. Ethanol exposure significantly reduced the magnitude of the conditioned odor aversion.

Molina et al. (2006) recently reported substantial ethanol-mediated appetitive learning in heterogeneous 14-day-old animals. In this study, no differential intake of water, quinine or sucrose was found after pairing these stimuli (conditioned stimuli, CSs) with early (postadministration time: 5 -10 min) postabsorptive effects of ethanol (0.25, 0.50 or 2.0 g/kg). A completely different profile was observed when ethanol-mediated conditioning was analyzed in terms of the capability of the taste CSs to act as second-order reinforcers when later associated with a neutral stimulus (sandpaper texture). Conditioned preference for the sandpaper surface was observed after pups were given that surface paired with an intraoral tastant, which itself had previously been paired with ethanol. Interestingly, none of the EtOH doses employed by Molina et al (2006) resulted in behaviors indicative of the development of ethanol-mediated conditioned aversions. Also, the latter work made no systematic attempt to examine the mechanisms underlying the acquisition of the observed ethanol-mediated learning.

The procedure employed by Molina et al. (2006) is referred to as second-order conditioning (SOC). The change in behavior derived from a CS₁-US experience is assessed not through direct exposure to the CS₁ alone but rather through the capability of the original CS₁ to transfer behavioral control to a second, different neutral stimulus (CS₂). If the original learning was successful, a substantial conditional response (CR) should now be elicited by the CS₂ (for a comprehensive review, see Rescorla, 1980). This procedure has been useful for detection of otherwise “silent” associations, even in young organisms. Miller et al. (1990) found that although 8 day-old rats avoided an odor previously associated with lithium chloride (LiCl), no signs of this conditioned aversion were observed in 4-day-old animals. Nevertheless, the younger pups were able to express the acquired odor-LiCl association when utilizing a second-order conditioning procedure. Specifically, following initial training pups were subjected to a subsequent pairing of original odor (CS₁) and a novel tactile stimulus (CS₂). When later tested for their preference for this tactile cue, pups avoided the CS₂ texture. In other words, second-order conditioning allowed pups to express their acquired aversion to the CS₁ odor.

One possible reason for the capability of second-order conditioning to reveal acquired associations is this procedure minimizes effects of conditioned responses that might otherwise compete with the target CR. For example, potentially interfering conditioned responses have been observed in tests of conditioned place preference with ethanol as the US (Cunningham & Noble, 1992). Substantial behavioral activation in young animals was also observed by Molina et al. (2006) in assessment of responsiveness to the tastants previously paired with EtOH. While no differences were observed in general locomotion, pups did more wall-climbing when exposed to a flavor previously paired with ethanol. This result suggests that conditioned motor responses compete with the expression of ethanol's motivational properties and might explain part of the failures observed in tests of ethanol-mediated appetitive learning through more conventional first-order conditioning procedures.

As observed with drugs such as cocaine (Ettenberg, 1999; 2004) or amphetamine (Lett, 1988), ethanol seems to exert differential hedonic effects across the time course of the process of intoxication. In other words, the motivational value of the drug changes as a function of the development of the acute state of intoxication. In mice, ethanol exerts appetitive effects when blood ethanol concentrations are rising (Risinger & Cunningham,

1992). Aversive effects of ethanol, on the other hand, are easily detected when pairing neutral taste stimuli with later postabsorptive stages characterized by peak BECs (Chester & Cunningham, 1999). Cunningham & Prather (1992) also observed that the longer the pairing between a distinctive location and postabsorptive effects of EtOH, the smaller the magnitude of the conditioned preference, a result suggesting that the drug's hedonic effects change from appetitive to aversive as a function of the temporal course of the intoxication. In preweanling (infant) rats, we have observed that a tactile stimulus paired with the commencement of the toxic state inhibits the expression of a conditioned taste aversion established through the association between a taste CS and peak blood ethanol levels (Pautassi et al., 2002). This result suggests that the tactile cue acquired non-aversive hedonic properties (appetitive or anxiolytic) that counteract the aversive conditioned taste response. Pautassi et al. (2006) also reported that ethanol's capability to modify the magnitude of an aversive memory changes not only as a function of dose but also as a function of postadministration time intervals. While relatively low ethanol doses (0.5-1.25 g/kg, all BEC's < 70 mg%) inhibit the expression of a conditioned aversion, a higher dose (2.5 g/kg), particularly when resulting in high blood ethanol levels (175 mg%), exerts the opposite effect and inflates expression of the conditioned aversion.

The present study assessed whether infants are sensitive to differential hedonic properties of ethanol as a function of dose (0.5 or 2.0 g/kg) and time course of the acute state of intoxication. The second-order conditioning procedure previously used by Molina et al. (2006) was employed. In the Molina et al. (2006) study, however, the original associative conditioning trial was always conducted during the early stage of the ethanol-induced toxic process (5-10 min postadministration). That is, there were no attempts to analyze whether motivational properties of the drug change as a function of the time course of the toxic process. As already discussed, previous studies (Cunningham & Prather, 1992; Pautassi et al, 2006; Risinger & Cunningham, 1992; Wilson et al, 2004) suggest that early and delayed effects of ethanol differ in their motivational value. Appetitive and/or anxiolytic properties seem to prevail shortly after ethanol administration while aversive effects are more prevalent during later postadministration times. Taking this into account, in the present study we explicitly varied ethanol dose and postadministration time to examine possible changes in the motivational value of the drug during early ontogeny. Specifically, pups were exposed to an intraoral stimulus (Water, CS₁) during either an early or late stage of the toxic process induced by ethanol (postadministration times 5-20' and 30-45', respectively). The water CS was then paired with a tactile cue (Sandpaper, CS₂). Two-way location preference tests, meant to assess the preference for the CS₂ relative to a novel cue, were later conducted. As can be observed, this study not only aims to validate previous research suggesting early sensitivity to ethanol's appetitive effects when assessed by a SOC procedure (Molina et al, 2006) but also intends to analyze changes in EtOH's hedonic properties as a function of the temporal course of the state of intoxication. Ethanol's biphasic effects have been long recognized as important factors mediating ethanol use and abuse (for a classic review, see Pohorecky, 1977).

While the SOC procedure has been useful to analyze reinforcing properties of ethanol in early ontogeny, the behavioral and/or physiological mechanisms associated with the acquisition of these early memories still remain to be identified. Hence, a second experiment analyzed several variables likely to underlie the acquisition, in young animals, of ethanol-mediated second-order learning, including acute and conditioned effects of ethanol upon locomotive and thermal patterns. This set of variables was selected on the basis of previous research indicating their involvement in ethanol-mediated reinforcement (Hunt et al, 1991; Masur et al., 1986).

Prior studies have also suggested a role for corticosterone in modulating reinforcing properties of several substances of abuse, including ethanol. The hypothalamic–adrenal–pituitary (HPA) axis not only is activated by stressful situations (Aguilera et al., 1992) but also by psychotropics like EtOH (Spencer and McEwen, 1997). This activation results in augmented levels of corticosterone, a hormone that has proven to exert reinforcing properties (Piazza et al., 1991). Removal of endogenous corticosterone decreases ethanol self-administration in rats, whereas long term exposure to subcutaneous corticosterone increases consumption of the drug (Fahlke et al., 1995). Moreover, it has been demonstrated that levels of this hormone are modified not only by acute ethanol but also by environmental cues associated with the drug (Seeley et al., 1996). Hence, in the present study plasma corticosterone levels were measured in infants immediately after test. Finally, BECs associated with the ethanol doses employed in the behavioral experiments were assessed.

2. Experiment 1

Preweanling rats express ethanol-mediated taste aversions following only one (Hunt & Spear, 1989) or two (Hunt et al., 1990) conditioning trials. Infants are also able to rapidly acquire and transfer information between neutral and biologically relevant stimuli when employing high order conditioning procedures (e.g. sensory preconditioning, Chen et al., 1993; second-order conditioning, Cheslock et al., 2003, Miller et al., 1990; revaluation of the representation of the unconditioned stimulus, Kraemer et al., 1992, Molina et al., 1996). The use of a procedure akin to second-order conditioning has recently revealed that ethanol exerts positive reinforcing effects quickly after the onset of the toxic process when employing doses between 0.25 and 2.0 g/kg (Molina et al., 2006). As stated, previous studies suggest that the hedonic value of the drug is likely to change during later stages of the intoxication, particularly when relatively higher doses are employed. For instance, Pautassi et al. (2006) observed in 15-day old rats that ethanol's capability (2.5 g/kg) to inhibit a previously acquired aversive memory changes with development of the toxic episode.

In this experiment second-order conditioning procedures were employed to scrutinize the possibility that ethanol exerts differential motivational properties depending on dose and postadministration time. If the drug exerts appetitive and aversive effects associated with early and late postadministration times, respectively, the behavioral control acquired by an intraoral CS experienced during a given stage should vary accordingly. Hence, ethanol-mediated learning was not tested directly in terms of responsiveness to the intraoral CS, but rather as a function of its capability to endow a tactile CS with reinforcing properties. In other words, the aim of this experiment was to assess whether a relatively neutral stimulus would be likely to later act as an appetitive or aversive second-order reinforcer as a function of a preceding association with different ethanol doses, or within each dose with varying postadministration time intervals.

2.1. Material and Methods

2.1.1. Animals—Seventy-one preweanling Sprague Dawley pups (14 days-old at the start of the experiment; body weight range: 29–42 g), representative of 8 litters were used. All pups were born and reared at the Vivarium of the Center for Developmental Psychobiology (Binghamton University, NY, USA). Births were examined daily and the day of parturition was considered as Postnatal Day 0 (PD0). Newborns were always kept with their biological mothers in standard maternity cages (47 × 25 × 20 cm) partially filled with wood shavings. All animals were housed in a temperature-controlled (22°C) vivarium maintained under a 14-hr light / 10 dark cycle (lights on at 0700) with ad libitum access to food (Purina Mills, St. Louis, MO) and water. At P1, litters were culled to 10 pups (5 males and 5 females whenever possible) and were left undisturbed until commencement of the experiment.

Maintenance and experimental procedures were in accordance with the Guide for Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996) and the guidelines indicated by the Binghamton University animal handling review committee.

2.1.2. Experimental Design—The experimental approach was defined by a factorial design with two orthogonal between-group factors: ethanol dose (0.5 or 2.0 g/kg) and conditioning procedure [the conditioned stimulus was presented either during an early (5–20 min; early pairing, EP) or a later (30–45 min; late pairing, LP) ethanol postadministration time, or was explicitly unpaired with ethanol's postabsorptive effects, UP]. Six groups were thus formed. Each group had 11 or 12 animals. In order to avoid overrepresentation of litters within each specific group, no more than 2 animals per litter (one male and one female) were assigned to each particular treatment. Sex was also considered as a factor in the present and following experiments. Yet, since this variable failed to exert significant main effects or interact with the remaining factors in all experiments, data were collapsed across this variable.

2.1.3. Procedures—On PD 14 pups were removed from the maternal cage and placed in pairs in a holding chamber. These chambers were maintained at 35° C by means of a heating pad placed underneath. A polyethylene cannula was then implanted in the pup's cheek, as described elsewhere (i.e., Abate et al., 2000; Pautassi et al., 2002). This procedure has been proven to be minimally stressful to rat pups (Spear et al., 1989). After the cannulation procedure pups were left undisturbed for two hours until commencement of the conditioning session.

Conditioning procedures followed closely those employed by Molina et al. (2006). Pups on P14 were individually placed in a trapezoid-shaped chamber (wall lengths: front = 29 cm, back = 18 cm, sides = 11.5 cm, height = 12.5 cm). The back and side walls of this cage were made of mirrored Plexiglas while the front wall and the floor were lined with clear Plexiglas. The floor of the chamber was covered with cotton. A 10-minute habituation phase was initially conducted in order to familiarize pups with the chambers as well as to facilitate a better discrimination of the forthcoming conditioned stimulus. Immediately following habituation, paired pups were weighed to the nearest 0.01 g (Sartorius, Gottingen, Germany) and intragastrically administered with 0.5 or 2.0 g/kg ethanol. These EtOH doses were achieved by administering 0.015 ml/kg of a 4.2 or 16.8% v/v ethanol solution, respectively (190-proof Ethanol, Pharmaco, Brookfield). Pups were then returned to the holding chambers where they remained until postadministration times 5 (paired early group) or 30 min (paired late group). Conditioning took place after pups were returned to the trapezoid chambers. In these chambers, preweanlings received intraoral pulses of distilled water (conditioned stimulus, CS₁). Fifteen 5-sec pulses (5 uls per pulse) were delivered throughout the conditioning trial (interstimulus interval: 55 sec). To control fluid delivery, the cannulae of the pups were attached to polyethylene tubing (PE50, Clay Adams) connected to an infusion pump (AutoPump, Kashinsky 5/2000, Binghamton, NY). Paired pups were then returned to the holding chambers until completing a two-hour period that began with administration of the corresponding ethanol dose. Pups assigned to the unpaired control groups received the same intraoral water and ethanol as paired subjects, but in this case the water CS was experienced two hours before intragastric ethanol administration. Immature rats consistently fail to exhibit conditioning with long-delay intervals between CSs and biologically relevant stimuli (e.g. Miller et al., 1990; Pepino et al., 1998; Rudy & Cheattle, 1979), and this is in any case a conservative unpaired control. Unpaired pups were returned to the mother after CS exposure. Following intragastric ethanol administration they were deprived from maternal care for two hours. These manipulations allowed maternal deprivation to be equated across paired and unpaired groups.

At PD15 the intraoral cannulae were positioned in the cheek opposite to that employed during PD14. One-hour later pups were placed in the trapezoid chambers. A sandpaper lining was now covering the floor of these cages (second order-conditioning stimulus; CS₂, coarse: 50, Gatorgrit, USA). While in contact with sandpaper, four pulses of the original water CS were delivered through the intraoral cannula (volume per pulse: 5 ul, duration: 5 sec, interstimulus interval: 55 sec). The sandpaper sheets were changed after each animal. A 5 min two-way texture preference test followed 30 min later. This test took place in a clear Plexiglas rectangular chamber (28 × 13 × 15.5 cm). Half of the floor was lined with the sandpaper tactile stimulus (CS₂) that had been previously paired with the water CS₁. The remaining floor surface was covered with the smooth backside of a similar piece of sandpaper. New, clean textures were employed for each individual pup. Texture preference assessments started by placing the animal in the middle section of the apparatus. Time spent over each particular texture of the apparatus was recorded. A subject was considered to be in a particular texture when three paws and the head were over that section. Time spent over each section was recorded during each minute of the test session. Testing was conducted by experimenters blind in regard to treatment conditions.

2.1.4. Data Analysis—The dependent variable under analysis was absolute time spent per minute on each tactile surface. This variable was analyzed by means of a four-way mixed analysis of variance (ANOVA). The between-group factors under consideration were ethanol dose (0.5 and 2.0 g/kg) and conditioning procedures (EP, LP and UP) while the within-subject factors were texture (sandpaper versus smooth) and evaluation bin (1, 2, 3, 4 and 5 minutes). In this, as well as in the following experiments, the loci of significant main effects or interactions were further examined through follow-up ANOVAs and by means of pair-wise post-hoc comparisons (Fisher's Least Mean Significant tests, with an alpha level set at 0.05).

2.1.5. Results—As stated, absolute time spent over each texture during the second-order test was subjected to a 4-way mixed ANOVA (dose × conditioning procedure × texture × bin). The ANOVA yielded significant main effects of texture and bin, $F(1, 65) = 8.37$, $p < 0.05$; $F(4, 260) = 2.63$, $p < 0.05$. The interactions between dose and texture, conditioning and texture and texture and bin also reached significance, $F(1, 65) = 5.52$, $p < 0.05$; $F(2, 65) = 7.07$, $p < 0.005$; $F(4, 260) = 3.38$, $p < 0.05$. More importantly, the four-way interaction between the factors was also significant, $F(8, 260) = 2.21$; $p < 0.05$. Absolute time spent over each texture at test is depicted in Figure 1. To better understand the significant interactions shown by the overall analysis, follow-up ANOVAs (texture × bin) were performed for each of the 6 groups derived from the original experimental design (UP/0.5, UP/2.0, EP/0.5, EP/2.0, LP/0.5, LP/2.0, letters indicate conditioning procedures while numbers allude to ethanol dosage). In the case of Unpaired animals no significant effects were observed. When considering Paired pups that experienced the water CS paired with the early postabsorptive effects of 0.5 g/kg ethanol (EP/0.5) the ANOVA indicated a significant main effect of texture, $F(1,10) = 7.99$, $p < 0.025$. These pups exhibited heightened preference to the tactile cue (sandpaper) paired with the water CS that was originally associated with this low ethanol dose. Similar heightened time spent over sandpaper was observed in groups LP/0.5 and EP/2.0; $F(1, 11) = 6.75$, $p < 0.025$ and $F(1,11) = 15.19$, $p < 0.0025$; respectively. Yet for pups given water infusions as a first-order CS paired with late postabsorptive effects of 2.0 g/kg ethanol and subsequently exposed to the same water infusions paired with sandpaper (group LP/2.0), the results were markedly different. In this group there were indications of an acquired aversion to sandpaper. The ANOVA revealed an interaction between texture and bin, $F(1, 44) = 2.97$, $p < 0.05$. Post-hoc Fisher tests showed that during the first three minutes of the test, these animals avoided sandpaper relative to the

alternative smooth surface. This expression of a second-order conditioned aversion was no longer observed during the last two minutes of evaluation.

In summary, when infants were intraorally stimulated with water under a sober state (Groups UP), later pairings between this liquid and a distinctive texture failed to induce either preference or aversions towards the tactile CS. In other words, regardless of the ethanol dose administered during the first phase of the conditioning, animals assigned to unpaired control conditions spent roughly 50% of the time over the target CS. In sharp contrast, intraoral infusion of water served in the present experiment as an effective second-order reinforcer provided it had been initially paired with either early (5-20 min) or late (30-45 min) postabsorptive consequences of low dose ethanol (0.5 g/kg). These animals exhibited a robust preference towards the sandpaper texture, which appeared to persist during the entire testing procedure. Differential hedonic effects of the drug were evident when a higher ethanol dose (2.0 g/kg) was employed. Positive reinforcing effects of ethanol were observed when the original CS was associated with commencement of the intoxication, whereas a second-order conditioned aversion was evident when this pairing was delayed until a later postadministration time period, a period characterized by much higher BECs (200 mg%, see experiment 3). This aversion was particularly evident during the first minutes of the test; apparently, extinction took place during the later phase of this behavioral assessment.

3. Experiment 2

The previous experiment indicates that infant rats encode differential hedonic effects of ethanol as a function of dose and postadministration time. Specifically, intraoral CSs paired with either early or late effects of low dose ethanol (0.5 g/kg) became effective positive second-order reinforcers. Appetitive effects were also exhibited by pups exposed to the CS during commencement of the toxic episode induced by a higher ethanol dose (2.0 g/kg), whereas a conditioned aversion was developed when the first-order CS was presented during a later postadministration time.

The purpose of the present experiment was to analyze possible mechanisms associated with ethanol-mediated second-order conditioning. To this end, a substantial part of the procedures conducted in Experiment 1 were replicated while recording acute and conditioned effects of the drug upon thermal, motor and hormonal responses. Specifically, first- and second-order conditioning phases similar to those employed in Experiment 1 were conducted. In this experiment vehicle treated infants rather than unpaired controls were employed to adequately determine unconditioned effects of each ethanol dose at each postadministration time. On PD 14 preweanling rats given vehicle (0.0 g/kg ethanol) or ethanol (0.5 or 2.0 g/kg) intragastrically were intraorally stimulated with water (CS) during postadministration times 5-20 or 30-45 min. Twenty-four hours later, pups were exposed to the water CS while placed over a distinctive texture (sandpaper). Locomotive patterns as well as rectal body temperatures were recorded during both phases of the experiment. Corticosterone levels were also measured on PD 15 after pups were exposed to the water CS which, as mentioned, had been previously paired with ethanol. As will be discussed (see General Discussion section) the behavioral, physiological and hormonal variables under consideration seem to be associated with ethanol's motivational properties (Cunningham et al., 1991; Cunningham et al., 1993; Fahlke et al., 1995).

3.1. Material and Methods

3.1.1. Animals—Sixty-six Sprague-Dawley derived pups representative of ten litters born and reared at the Center for Developmental Psychobiology were employed. Genetic, housing

and breeding conditions of these animals replicated those described in Experiment 1. Animals (14-days old at the beginning of the experiment) had a mean body weight of 33.8g.

3.1.2. Experimental Design—A randomized factorial experimental design was employed. Animals were assigned to one of six groups defined as a function of ethanol dose administered during conditioning. (0.0, 0.5 or 2.0 g/kg of the drug) and postadministration time at which intraoral infusions of the water CS took place (Early Paired, EP: 5-20 min or Late Paired: 30-45 min). Each of the groups had a minimum of 10 and a maximum of 12 pups. Precautions were taken to avoid overrepresentation of litter and sex across groups. No more than one male and one female corresponding to each particular litter were assigned to a specific treatment. Due to blood sampling problems eight data points corresponding to corticosterone assessments were unavailable. No more than two data samples for a given group were lost.

3.1.3. Procedures—As in Experiment 1, pups were implanted with intraoral cannulae and left undisturbed for two hours in heated holding chambers (34-35°C). Throughout the experiment (conditioning and testing procedures) ambient room temperature was maintained at 22° C.

The first-order conditioning phase started with a 10 min habituation trial. Pups were individually introduced in clear Plexiglas activity chambers (10 × 10 × 12 cm) lined with cotton. These chambers were equipped with six infrared photo emitters and six infrared photoreceptors. The photo beams crossed the chamber generating a matrix of nine cells. Each activity chamber was in turn connected to a computer. Custom-made software served to analyze the number of beams crossed by each subject every tenth of a second. Data were subsequently grouped using 1-min time intervals.

At the end of the habituation phase animals were weighed to the nearest 0.01 g (Sartorius, Gottingen, Germany) and their temperature was recorded using a Physitemp Temperature Monitor (TH8 Model, Clifton, NJ) equipped with a rectal probe (RET-3, tip diameter: 0.065"). Specifically, the probe was lubricated with mineral oil kept at room temperature and was then inserted 2.5 cms in the rectum. Temperature recordings were obtained 20 s following insertion of the probe. Immediately after temperature recording animals received an intragastric administration of ethanol (0.0, 0.5 or 2.0 g/kg, vehicle: tap water) and returned to the holding chambers. Ethanol doses were achieved by administering 0.015 ml of a 0.0, 4.2 or 16.8 % v/v ethanol solution per gram of body weight.

Pups remained in the heated holding chambers until 5 or 30 minutes postadministration. Pups were then gently placed in the activity chambers and intraorally stimulated with water for 15 minutes, employing the parameters already described in Experimental 1 (15 pulses, 5 ul per pulse, duration: 5 sec, interpulse interval 55 sec). Throughout the conditioning trial locomotive patterns were recorded by means of the activity devices previously described. The PE 50 tubing connecting each subject's cannula to the infusion pump exited from the activity chamber through a hole located in its upper section. This prevented accidental activity counts due to beams breaks caused by the tubing. Following the infusion procedure rectal temperatures were again recorded to estimate ethanol-induced thermoregulatory changes. After completion of water stimulation procedures, oral cannulae were removed and pups were returned to their holding chambers. Two hours following vehicle or ethanol administration procedures, pups were returned to their respective maternity cages.

On PD15 oral cannulae were again implanted in the cheeks of the animals. One hour later they were weighed and rectal temperatures were recorded (baseline measurement). Pups were then immediately placed in the activity chambers that were now lined with sandpaper.

While over the rough surface pups received four intraoral pulses of water (5 μ l, pulse duration: 5 sec, interpulse interval: 55 sec) in each of two consecutive 5-min trials. The intertrial interval was also five minutes. Activity levels were recorded during each trial while rectal temperatures were registered at the end of each trial. It should be noted that, relative to the procedures of Experiment 1, a second 5-min conditioning trial comprising pairings between water pulses (CS₁) and sandpaper (CS₂) was added. The rationale for this was to analyze whether conditioned effects of EtOH likely to mediate transfer of information during the SOC procedure would change as a function of successive presentations of CS₁. It is known that while short second-order conditioning trials allow transfer of information to the CS₂, additional trials can result in extinction of the original memory (Rescorla, 1980). Immediately after the second temperature recording animals were transferred to the sacrifice room. Following decapitation, blood trunk samples were collected. Samples were centrifuged at 6000 rpm to create a plasma phase. The vials containing the plasma phase were stored at -15° C for later analysis. Corticosterone levels were obtained by means of radioimmunoassay of the plasma samples. 3H Kits reagents (MP Biomedicals, Orangeburg, NY) were employed and the values obtained were expressed as ng/ml.

3.1.3. Data Analysis—Rectal temperatures ($^{\circ}$ C), locomotion scores (total number of beams crossed) and plasma corticosterone levels [ng/ml] were considered as dependent variables. Due to different procedural manipulations inherent to each particular day (PD14, first-order conditioning; PD15, presentation of first-order CS) and different time points at which data was collected, separate analysis were conducted for each specific day. Rectal temperatures on PD 14 were analyzed using a 3-way mixed ANOVA. Ethanol Dose (0.0, 0.5 or 2.0 g/kg) and postadministration time (5-20 min or 30-45 min, Early or Late Pairings, respectively) served as between factors. Time of rectal temperature assessment (baseline and postadministration) was included as a within-measure factor. Ethanol dose and postadministration time also served as between factors in the ANOVA employed to analyze thermal recordings during the second-order conditioning phase (PD 15). In this particular instance, the within time factor comprised three repeated measures (baseline and immediately after the first or second trial).

Overall locomotion during baseline and during the first-order conditioning trial (PD 14) were analyzed by means of a 3 (Ethanol dose: 0, 0.5 or 2.0 g/kg) $\times$ 2 (Postadministration time, Early or Late) $\times$ 2 (Recording time: Baseline or during conditioning). Motor scores at PD 15 were scrutinized by means of a 3 (Ethanol dose) $\times$ 2 (Postadministration Time) $\times$ 2 (Infusion trial: 1 or 2 as a within measure factor) ANOVA. Finally, a 3 (EtOH dose) $\times$ 2 (Postadministration Time) ANOVA served to analyze corticosterone plasma levels at PD15.

3.1.5. Results

3.1.5.1 Temperature responsiveness—The analysis on rectal temperatures during PD 14 indicated a significant main effect of time of assessment, $F(1, 60) = 70.55$, $p < 0.0001$. The interaction between this factor and ethanol dose also achieved significance, $F(2, 60) = 11.60$, $p < 0.0001$. Post-hoc tests revealed that all groups exhibited similar rectal temperatures during baseline. In turn, all groups exhibited within-differences between baseline and postadministration recordings. Temperatures decreased following vehicle or ethanol administrations. Nevertheless, the highest ethanol dose (2.0 g/kg) resulted in significantly lower rectal temperatures when compared with the values attained in vehicle treated pups or those exposed to the lowest ethanol dose (0.5 g/kg). This dose effect was similar in both postadministration times (Early or Late). At PD15, time of assessment exerted a significant main effect [$F(2, 120) = 3.85$, $p < 0.05$] that was tempered by a significant interaction with ethanol dose, $F(4, 120) = 3.48$, $p < 0.05$. The locus of this

interaction was analyzed by subsequent post-hoc comparisons. No differences were observed among groups during baseline. As was the case during first-order conditioning, pups originally treated with vehicle showed a significant decrement in rectal temperature relative to baseline values during subsequent recordings. Interestingly, this was not the case in groups that during conditioning experienced the water CS under the effects of ethanol. Further post-hoc comparisons demonstrated that the difference between thermal responsiveness following CS presentations only achieved significance when comparing pups previously treated with vehicle and those administered with the highest ethanol dose (2.0 g/kg). These results are depicted in Figure 2. Since postadministration time at which intraoral infusions of the water CS took place did not exert a significant main effect upon thermal responsiveness nor interacted with the remaining factors, data in Figure 2 has been collapsed across this factor to facilitate visualization of the results.

In summary, thermoregulation was disrupted during conditioning when animals were intragastrically administered with ethanol, particularly when employing a 2.0 g/kg dose which resulted in a clear hypothermic response. Lesser degrees of hypothermia were also encountered in vehicle pups and those treated with 0.5 g/kg ethanol. In these groups it is likely that the temperature of the solution or the fact of being isolated during habituation and conditioning affected thermoregulatory homeostasis. Mild hypothermia was again observed during PD15 when vehicle pups were simply exposed to the water CS. Interestingly, and despite the fact that heightened hypothermia was observed during PD14 when using 2.0 g/kg ethanol, these pups appeared to be as resistant to thermoregulatory disruptions as vehicle controls during the second phase of the experiment. These particular effects of drug treatment upon unconditioned and possible conditioned responses comprising thermoregulation will be analyzed in detail in the General Discussion section.

3.1.5.2. Locomotive patterns—As illustrated in Figure 3, it seems clear that at PD14 motor activity was similar across groups during the habituation phase. This was not the case when activity was scored following drug administration. The highest ethanol dose (2.0 g/kg) resulted in motor activity decrements. These observations were supported by the corresponding ANOVA which indicated a significant main effect of time of assessment and a significant interaction comprising this factor and ethanol dose; $F(1, 60) = 41.19$ and $F(2, 60) = 25.67$, both p 's < 0.001 , respectively. As indicated by post-hoc analysis, animals treated with 2.0 g/kg ethanol showed a significant decrease in locomotive behavior relative to both water-treated controls and pups that had received the lowest ethanol dose (0.5 g/kg). The latter group also showed a lessened pattern of locomotion when compared with vehicle controls ($p = 0.08$).

At PD15, the ANOVA indicated a significant main effect of infusion trial, $F(1, 60) = 15.62$; $p < 0.0005$. Specifically, a decrease in motor activity was observed during the second infusion trial relative to previous one, an effect probably attributable to progressive habituation to the environment or to the water infusions. According to the ANOVA, neither dose of ethanol received during the first-order conditioning phase (PD 14) nor postadministration time of the original associative process (early or late) exerted significant main effects upon motor activity scores registered on PD 15. Also, no significant interaction between these factors was observed. These results have been depicted in Figure 3. Results in this figure are presented after having collapsed data across postadministration time (early or late). As stated, this factor did not exert a significant main effect upon locomotor patterns nor interacted with the remaining factors.

3.1.5.3. Corticosterone levels—In regards to corticosterone levels after exposure to the water CS during PD15, the ANOVA indicated no significant main effects or interactions

between the factors under consideration. Mean levels of corticosterone across groups was 148 ± 8 ng/ml, mean $\pm$ SEM.

In summary, the higher dose of ethanol (2.0 g/kg) exerted significant depressant effects upon locomotor activity when acutely administered on PD 14, regardless of postadministration time. The lower EtOH dose also induced a somewhat depressed pattern of locomotor activity. Specifically, animals administered with 0.5 g/kg ethanol tended to move less than vehicle controls. When pups were reexposed to the water CS on PD 15, locomotive patterns and corticosterone levels were similar across conditioning treatments.

A substantial decrease in rectal temperature was observed after administering a 2.0 g/kg ethanol dose. This hypothermic response was observed at both postadministration time periods. In terms of thermoregulatory process during the second phase of the experiment, pups with no prior ethanol experience showed decrements in rectal temperature while isolated, a response very similar to the one attained during first-order conditioning. Interestingly, pups which exhibited marked hypothermia as an unconditioned response to ethanol during conditioning were not observed to exhibit significant changes in thermoregulation when isolated and exposed to the water CS during the second phase of the experiment. Possible explanations relative to this effect will be presented in the discussion section. The thermoregulatory effects observed in this Experiment failed to vary across trials, a result that suggests that the amount of exposure to CS1 was not sufficient to result in significant levels of extinction of the memory under analysis.

4. Experiment 3

Pharmacological effects of EtOH vary as a function of dose and postadministration time, both variables being regulated by the particular genetic and housing conditions of the organisms under analysis (Cunningham et al., 1993; Bau et al., 2005). In the present experiment we examined blood ethanol concentrations resulting from the ethanol doses and postadministration times employed in Experiments 1 and 2.

4.1. Material and Methods

4.1.1. Animals—Fifty Sprague-Dawley derived rats were employed. These animals were representative of six litters, born and reared at the vivarium of the Center for Developmental Psychobiology. Housing conditions were similar to those described in Experiments 1 and 2. Animals had 14-days of age at the start of the experiment and weighed 27-34 g.

4.1.2. Experimental Design and Procedures—The experimental design comprised two independent factors: ethanol dose and postadministration time. Each of the four groups resulting from this design was composed of 12-13 pups. As was the case in prior experiments, precautions were taken to avoid overrepresentation of litters and sex within each specific group. Animals received either 0.5 or 2.0 g/kg of ethanol. BECs were registered at 12.5 or 37.5 minutes after the corresponding administration, with independent groups of animals being employed at each time point. These time periods were chosen since they are temporally equidistant to the onset and offset of the early and late conditioning phases employed in Experiments 1 and 2. Ethanol doses were achieved by intragastrically administering 0.015 mls of a 4.2 or 16.8 % v/v ethanol solution per gram of body weight, respectively. On PD 14, infant rats were placed in pairs in holding cages maintained at 35°C through the use of heating pads placed beneath them. Pups remained in these cages 120 min and were then randomly assigned to one of four conditions defined by ethanol dose administered and postadministration time when blood collection took place. Trunk blood was obtained through decapitation. Samples were collected using a heparinized capillary tube. They were immediately centrifuged at high speed (6000 rpm; Micro-Haematocrit

Centrifuge, Hawksley & Sons LTD, Sussex, England) and stored at -70°C for later analysis. An AM1 Alcohol Analyzer (Analox Instruments, Lunenburg, MA) was employed to process the samples. Calculation of blood ethanol concentrations was made by oxidating ethanol to acetaldehyde in the presence of ethanol oxidase. The apparatus measures the rate of oxygen required by this process, which is proportional to EtOH concentration, and produces a printout 20-30 seconds after the plasma is injected. All BECs values were expressed as milligrams of ethanol per deciliter of body fluid ($\text{mg/dl} = \text{mg} \%$)

4.1.3. Results

BECs were analyzed by means of a two way ANOVA [EtOH dose (0.5 or 2.0 g/kg) $\times$ postadministration time (12.5 or 37.5 min)]. Significant effects of both main factors were detected, $F(1, 46) = 636.33$; $F(1, 46) = 15.19$; both p 's < 0.0001 ; respectively. The interaction between the factors also achieved significance, $F(1,46) = 15.5$, $p < 0.0005$. As could be expected, post hoc tests indicated that BECs derived from 2.0 g/kg ethanol were significantly higher than those obtained with 0.5 g/kg ethanol. While BECs associated with the 0.5 g/kg dose remained constant across postadministration times, BECs derived from the 2.0 g/kg dose at the latter time period were significantly higher than the ones corresponding to the early phase of the toxic state. These results have been depicted in Figure 4.

5. Discussion

The main experimental finding of the present study is that preweanlings (PD14-15) rats appear to be sensitive to biphasic motivational properties of ethanol. Specifically, second-order conditioned preferences and aversions were established as a function of ethanol dose and postadministration time. More in detail, after intraoral infusion of water (CS) was paired with postabsorptive effects of a relatively low ethanol dose (0.5 g/kg), this CS acted as a second-order appetitive reinforcer when subsequently paired with sandpaper. This resulted in preference for sandpaper. Control animals exposed to water intraoral infusions (CS) explicitly unpaired with ethanol's postabsorptive effects spent equivalent time over sandpaper and the alternative tactile cue. The latter results are in agreement with those originally found by Molina et al (2006). Interestingly, a novel finding of this study is that conditioned tactile preferences were also found in pups originally exposed to the CS while intoxicated with a higher ethanol dose (2.0 g/kg), but only if the CS was presented during the initial phase of the toxic state. In contrast, second-order conditioned aversions emerged during the initial intervals of testing whenever the water CS was originally experienced at a later stage of the intoxication induced by the 2.0 g/kg dose. That is, biphasic motivational effects of the drug are observed as a function of dose and temporal course of the ethanol intoxication. When taking into account the blood ethanol levels associated with dose and postadministration time (Experiment 3; see Figure 4), some preliminary conclusions can be drawn. The motivational value of low dose ethanol remains constant across postadministration times characterized by similar amounts of circulating ethanol (40 mg %). Appetitive hedonic effects seem to be also present after a relatively high dose of ethanol (2.0 g/kg) during the ascending limb with BECs about 160 mg% (postadministration time: 12.5 min). When BECs reached approximately 210 mg% (postadministration time: 37.5 min) aversive rather than appetitive motivational properties of ethanol seem to prevail.

Is it possible that pups in group PL/2.0 spent less time over sandpaper not because of ethanol's aversive effects, but rather due to sedative effects of ethanol that compete with sensory processing of the CS? This possibility seems highly unlikely in light of prior literature. Several studies indicate that, while experiencing the toxic effects induced by EtOH doses equivalent to 2.0 g/kg or above, pups are capable of processing tactile and chemosensory CSs and to associate them with different USs, including unconditioned effects of EtOH (e.g. Molina et al., 1996; Pautassi et al., 2002; 2005b). In this respect,

Pautassi et al (2006) trained pups to avoid an odor CS by pairing it with an intraoral infusion of citric acid (US). Twenty-four hours later animals were re-exposed to the CS under the delayed toxic effects of a 2.5 g/kg dose (postadministration interval 25 – 30 min). Interestingly, when later tested for their odor preference these animals exhibited an enhanced avoidance response. That is, delayed effects of 2.5 g/kg ethanol inflated the aversive value of citric acid. Obviously, this result implies that ethanol intoxication did not preclude perception of the taste US. Reliable odor aversive conditioning promoted by foot-shock is also detected in rat pups under the effects of a 2.0 g/kg ethanol dose (López et al., 1996).

The results obtained in this study replicate the previous finding of Molina et al. (2006) showing positive reinforcing properties of EtOH assessed by means of higher-order conditioning. The motivational profile induced by EtOH is also in accordance with previous experiments showing that the drug, specifically during the early ontogeny of the rat, exerts biphasic hedonic effects when utilizing relatively high doses (Pautassi et al., 2002; 2006). This work also adds to a growing body of experimental evidences indicating that neonates, infants and periadolescent rats rarely develop conditioned aversions when treated with relatively low ethanol doses and, on the contrary, seem to be highly sensitive to either appetitive or negative (antianxiety) reinforcing effects of ethanol (Cheslock, 2001; Fernandez Vidal, 2003; Molina et al., 2006; Nizhnikov, 2006; Pautassi et al., 2005). These effects are observed using procedures that require minimal experience with the drug, a phenomenon rarely encountered in heterogeneous adult animals.

Drug-induced locomotive activity has long been considered as an index of reinforcing properties of substances of abuse such as cocaine and amphetamines (Orsini et al, 2004; Wise & Bozarth, 1987). In mice, increased levels of ethanol-induced activity have been associated with reinforcing properties of this drug (Cunningham et al, 1993, but also see Risinger et al, 1992). In Experiment 2, substantial acute motor depressant effects of ethanol were observed in pups administered with 2.0 g/kg ethanol, independently of postadministration time. Even though not significant in statistical terms, pups treated with 0.5 g/kg ethanol also exhibited a somewhat depressed motor unconditioned response. Despite this motor profile, both EtOH doses induced substantial appetitive learning as assessed through second-order conditioning (Experiment 1). Hence, these results indicate dissociation between ethanol-induced changes in motor activity and reinforcing capabilities of the drug in early infancy. These findings also fit well with previous suggestions that although low doses of ethanol stimulate locomotive activity in mice (Masur et al, 1986), rats typically show a dose-dependent suppression of motor activity (Chuck et al, 2006).

Conditioned motor responses to discrete or contextual cues are likely to be acquired during ethanol intoxication (Cunningham & Noble, 1993) and apparently can mask expression of ethanol-induced reinforcement with first-order conditioning procedures. When focusing on activity patterns elicited by the water CS during the second-order phase (PD 15, dependent variable: locomotion operationalized through number of beams crossed), there were no indications of ethanol-mediated conditioned motor responses (Experiment 2). Although Molina et al. (2006) described motor conditioned responses after conditioning procedures similar to that of the present study, the motor behavior that proved sensitive in that study was wall-climbing. The results of Molina et al. (2006) agreed with the present study in the absence of conditioned motor responding in terms of overall locomotion.

Previous studies indicate that EtOH-induced hypothermia may underlie the capability of the drug to support learned aversions. Drug-mediated taste aversions are disrupted when ethanol-induced hypothermia is attenuated by exposing rats to high ambient temperatures (Cunningham et al., 1988; 1992). This experimental manipulation also interferes with the

acquisition of ethanol-induced place aversion in rats (Cunningham & Niehus, 1988). In young animals the effectiveness of ethanol as an aversive US seems associated with the capability of the drug to decrease body temperature (Hunt et al., 1991). Cunningham (1994) also observed that a stimulus paired with self-administered ethanol acquires the ability to evoke a conditioned increase in body temperature. Interestingly, animals subsequently self-administered more ethanol when re-exposed to the original CS than when this stimulus was absent. This result suggests that the hyperthermic response helped counteract aversive consequences of the drug derived from its hypothermic consequences. In the present study, high-dose ethanol (2.0 g/kg) induced substantial hypothermia soon after its intragastric administration. Thermoregulatory disruptions of pups administered with 0.5 g/kg ethanol were very similar to those encountered in vehicle control animals. A similar dose-response effect in preweanling rats has been previously reported by Hunt et al. (1991). In the present study a decrease in rectal temperature following baseline recordings was observed in vehicle and pups administered with 0.5 g/kg ethanol, even though the magnitude of this decrement was significantly lower than encountered with the highest ethanol dose (Experiment 2). This drop in temperature in vehicle controls is probably due to the administration of water at room temperature (PD 14) and/or to removal of animals from their heated holding cages and placement into chambers kept at room temperature where they remained for a relatively long period of time (PD 14 and 15). Similar and even higher temperature decrements have been previously reported in vehicle-treated young animals (Hunt et al., 1991).

On PD 15, when exposed to intraoral water, control animals again showed a decrease in body temperature relative to initial baseline recordings. This was not the case in ethanol-treated pups, which showed relatively stable rectal temperatures throughout the entire experimental session. This difference in thermal responsiveness after CS delivery only attained significance, however, when contrasting pups previously treated with vehicle and those given the highest ethanol dose (2.0 g/kg). What mechanisms might help explain this differential thermoregulatory response as a function of prior drug treatment? A possibility is that ethanol-treated pups developed conditioned tolerance to disruptive thermoregulatory effects of ethanol. During conditioning, the water CS was presented while pups experienced procedural manipulations and drug effects leading to hypothermia. Under the assumption of conditioned tolerance, subsequent exposure to the CS could elicit opponent thermoregulatory processes in an effort to mitigate the predicted homeostatic disturbance. This hypothesis is supported by several reports indicating that conditioned tolerance develops to several effects of ethanol. For example, ethanol-induced ataxia is significantly attenuated when adult rats suffer the state of intoxication under environmental conditions originally associated with the drug's motor impairing effects (White et al., 2002). This also seems to be the case for ethanol-induced hypothermia. Le et al. (1979) observed that chronic tolerance to ethanol-induced hypothermia was disrupted when subjects were moved to a novel environment prior to drug administration. In this study it was also noted that the context paired with ethanol-induced hypothermia subsequently elicited compensatory conditioned responses (hyperthermia) when animals received vehicle rather than ethanol. Similar results were observed in later studies that incorporated more adequate control conditions to assess Pavlovian associative learning mechanisms (Mansfield & Cunningham, 1980; Melchior, 1990; Tirelli et al., 1992). While in these studies the putative CS was the general environmental context, ethanol-induced hyperthermic conditioned responses have also been observed when utilizing more discrete CSs (Cunningham, 1994). In these studies, repeated administrations of ethanol were performed before assessing conditioned tolerance (e.g., Le et al, 1979) while in the present study this phenomenon was detected after a single ethanol administration. It is necessary to observe that the above stated studies were conducted in adult rodents, organisms that possess fully-developed thermoregulatory capabilities. In infants, self-control of body temperature is still developing (Leon et al,

1986). Hence, we cannot discard the possibility that rates of development of ethanol-mediated thermoregulatory responses vary as a function of maturational processes.

One possible objection to the proposed learned tolerance hypothesis is that the temporal duration of the ethanol-mediated hypothermia was too short to warrant the acquisition of this learned response. This possibility is not supported by the data obtained in Experiment 2 or by prior literature. Specifically, the 2.0 g/kg EtOH caused a significant drop in temperature not only shortly after the onset of the intoxication but also in those pups tested during the late postadministration interval. Temperatures measurements in these animals were conducted 45 minutes after EtOH administration. Hypothermic effects of ethanol (1.6 g/kg) have been observed in 16-day old pups even 120 minutes after administration of the drug (Hunt et al. 1991). Hunt's (1991) results were obtained while employing the same rat strain and similar test conditions as those used in Experiment 2. These findings support the possibility that pups tested in Experiment 2 experienced ethanol-mediated hypothermia for a considerable amount of time.

Yet, there is an alternative non-learning explanation related with the enhanced thermal responsiveness observed during PD 15. Specifically, it could be the case that EtOH administration (2.0 g/kg, PD 14) induced an unconditioned change in thermoregulatory capabilities that caused animals to exhibit hyperthermia when tested on PD 15. This possibility cannot be dismissed since Experiment 2 did not include unpaired control conditions. Unconditioned thermal rebound effects as a function of prior ethanol exposure have been previously reported in two-week old rats (Spiers & Fusco, 1992). It should be noted that the latter study employed a much higher EtOH dose (4 g/kg, i.p.) than the one here employed. In addition, the unconditioned rebound effect reported by Spiers & Fusco (1992) was only observed when pups were severely challenged in terms of thermoregulatory capabilities (i.e., test was conducted at 27.5 °C). This was not the case in the present study.

Is there a relationship between the observed thermoregulatory effects of the drug and the establishment of EtOH-mediated appetitive or aversive effects? Results reported by Hunt et al. (1991) suggest that drug-induced hypothermia is a necessary condition for the acquisition of ethanol-mediated aversions in preweanling rats. In Hunt's study (1991), EtOH-mediated conditioned aversions were only observed when treated pups showed reliable ethanol-induced hypothermia. In the present study, infants treated with 2.0 g/kg during first-order conditioning did show marked hypothermia during early and later phases of the process of intoxication. But second-order conditioned aversions were only observed in those subjects initially exposed to the CS during the later phase of the toxic process, a stage characterized by higher BECs. According to these observations in conjunction with those reported by Hunt et al. (1991), ethanol-induced hypothermia can represent a necessary but not a sufficient factor to promote aversive learning in the developing rat. This statement receives further support when considering that pups undergoing early postabsorptive effects of the 2.0 g/kg ethanol dose expressed conditioned preferences rather than aversions despite the fact that ethanol-induced hypothermia was also evident in these subjects.

Ethanol administration is associated with activation of the HPA axis which results in heightened levels of corticosterone (Spencer & McEwen, 1997). The role of this hormone in ethanol reinforcement, however, is far from clear. For instance, antalarmin, a compound that antagonizes the type 1 corticotropin-releasing factor (CRF1) receptor, has been observed to reduce oral intake of ethanol in rodents, a result not related to possible antianxiety effects of the drug (Lodge & Lawrence, 2003). CRF1 antagonists are also capable of mitigating the enhanced anxiety-like behaviors associated with repeated ethanol withdrawal episodes (Overstreet et al, 2004). On the other hand, neither acquisition nor expression of ethanol-induced conditioned place preference in mice is altered as a function of the administration of

aminoglutethimide, an inhibitor of corticosterone synthesis (Chester & Cunningham, 1998). More recently, it has been observed that knock-out mice for the type 2 corticotropin-releasing factor receptor do not show differences in a wide array of ethanol-related behaviors, including drug intake, ethanol-induced conditioned taste aversions and hypothermia (Sharpe et al., 2006). The results obtained in Experiment 2 seem to agree with this latter set of studies indicating that corticosterone responsiveness is not strongly related with the motivational effects of EtOH. Specifically, it was observed that neither ethanol dose nor postadministration time affected later corticosterone responses to the water CS.

In summary, the results obtained in this study argue in favor of infantile sensitivity to differential hedonic properties of ethanol. Both appetitive and aversive effects of the drug are evident as a function of dose and the time course of the acute state of intoxication. Results also indicate that pulsating water infusions can acquire either appetitive or aversive value as a function of associative learning processes mediated by ethanol. The second-order conditioning procedure seems to be a valuable tool allowing detection of differential motivational properties of ethanol. Further studies are needed to dissect the mechanisms underlying the capability of this second-order preparation to detect ethanol-mediated learning. Independent from these considerations, and in agreement with a growing body of literature (for a review on this matter see Spear & Molina, 2005), the present results indicate that even brief experiences with ethanol during early development are sufficient to affect subsequent responsiveness to the drug itself or to stimuli that predict its postabsorptive effects.

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Figure 1.

Time spent over sandpaper and the novel surface as a function of intraoral water paired with early (5-20 min postadministration) or late (30-45 min postadministration) ethanol dose (0.5 or 2.0 g/kg) and time at test (1-5 min). UP groups, that do not exhibit significant differences between them, have been collapsed across drug treatment and postadministration time. In this study, intraoral water was subsequently paired with sandpaper. Vertical lines illustrate standard error of the means.

Figure 2. Rectal temperatures ($^{\circ}$ C) during postnatal day (PD) 14 and 15 (first- and second-order conditioning phases, respectively) as a function of ethanol dose (0.5, 2.0 or 0.0 g/kg, water) and time point of measurement (PD 14: Baseline and after CS Exposure; PD 15: Baseline and after the first and second CS exposure). To facilitate visualization, data has been collapsed across postadministration time (early or late). This factor failed to significantly affect the pattern of results during both PD 14 and 15 and did not significantly interacted with the remaining factors. Vertical lines illustrate standard error of the means.

Figure 3.

Locomotor activity during postnatal days (PD) 14 and 15 as a function of ethanol dose (0.5, 2.0 or 0.0 g/kg, water) and time point of measurement (PD 14: Baseline and First-order conditioning phase; PD 15: Infusion Trial 1 and 2). To facilitate visualization, data has been collapsed across postadministration time (early or late). This factor did not have a significant effect neither during the habituation and conditioning phases conducted on PD 14 nor on the second-order conditioning phase (PD 15). Also, postadministration time did not significantly interacted with the remaining factors. Vertical lines illustrate standard error of the means.

Figure 4. Blood ethanol concentrations (mg%) as a function of ethanol dose (0.50 or 2.0 g/kg) and postadministration time (12.5 or 37.5 min). Vertical lines illustrate standard errors of the means.