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Interactions Between Ethanol Experiences During Late Gestation and Nursing: Effects upon Infantile and Maternal Responsiveness to Ethanol

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Responsiveness to ethanol is markedly affected by fetal or infantile experiences with the drug. Yet, there is minimal information available relative to the interaction of these experiences. This study focused on such interaction and on the impact of ethanol intoxication on maternal care. Water or subnarcoleptic doses of ethanol were administered to pregnant rats during late gestation (2.0 g/kg) and/or while nursing (2.5 g/kg). Infantile intake of a low concentrated ethanol solution (0.22% v/v) was assessed during postpartum days (PPDs) 15 and 16. Following the first intake test, infantile intake was explicitly paired with water or varying ethanol doses (0.5, 1.0, or 2.0 g/kg) to assess possible associative learning comprising ethanol’s sensory and unconditioned properties. The interaction between ethanol pre- and postnatal treatment resulted in heightened ethanol reactivity as assessed through intake scores, particularly during PPD 16. Maternal treatments failed to affect associative learning mediated by ethanol. Ethanol was also found to disrupt both maternal retrieval and crouching latencies. This effect was markedly reduced when females had experienced ethanol during gestation, a phenomenon indicative of tolerance. Sequential experience with ethanol during gestation and nursing facilitates subsequent responsiveness to minimal ethanol concentrations, without affecting the sensitivity to the unconditioned effects of the drug as evaluated through associative learning procedures.

Early experience seems vitally important in the modulation of subsequent processes that allow adaptation to environmental demands. Behaviors controlled by particular chemosensory stimuli, such as nipple attachment and suckling, can be influenced by intrauterine experiences that lead to the formation of specific memories (Pedersen & Blass, 1982; Smotherman, 1982; Stickord, Kimble, & Smotherman, 1982; Varendi, Porter, & Winberg, 1996). From a functional perspective, it has been suggested that prenatal sensory experience may form the basis for kin recognition, promote mother–infant bonding, facilitate learning, and mediate essential postpartum behaviors (Ronca & Alberts, 1995).

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During late gestation, rat fetuses can acquire information about alcohol leading to the establishment of memories that can be evoked or reactivated during subsequent stages of development (Bachmanov et al., 2003; Spear & Molina, 2001). When pregnant females are administered with alcohol, ethanol concentrations in the amniotic fluid and in fetal blood are similar to those encountered in the dam’s blood (Domínguez et al., 1996). Fetal exposure to alcohol induces postpartum changes with respect to the drug's chemosensory attributes. These changes have been observed in neonates and infants following acute administration of alcohol in the amniotic fluid while avoiding fetal intoxication (Chotro, Cordoba, & Molina, 1991; Chotro & Molina, 1990; Molina, Chotro, & Domínguez, 1995; Molina & Chotro, 1991). Furthermore, maternal intoxication with subnarcoleptic alcohol doses during gestational days (GDs) 17 to 20, also leads short and long-term effects related to the capacity of neonates and preweanlings to detect alcohol odor and/or taste (Domínguez, Lopez, & Molina, 1998).

During lactation, infant rats have the opportunity to encode chemosensory information present in maternal milk derived from substances that compose maternal diets (Bronstein & Crockett, 1976; Galef & Sherry, 1973; Mennella, 1995, 1997; Mennella & Beauchamp, 1993). Alcohol maternal intoxication within the nursing context provides information to the progeny that results in the establishment of alcohol-related memories. Pups sense alcohol concentrations present in maternal milk equivalent to 175 mg/dl (Molina et al., 2000; Pepino et al., 1998, 1999; Pepino, Spear, & Molina, 2001). Interestingly, the nursing infant not only acquires information about alcohol present in the milk, but also seems to perceive the deleterious pharmacological effects of the drug upon maternal care. The infant appears to associate these events and later aversively reacts to the chemosensory attributes of ethanol, as a function of the coexistence of the drug's sensory and pharmacological effects within the nursing environment (Pepino et al., 1999, 2001).

A recent study indicated that maternal behaviors are uniformly disrupted when the dam is treated with alcohol during the first two weeks of the lactation period (Pepino et al., 2002). When receiving the first subnarcoleptic alcohol dose yielding blood alcohol levels (BALs) approximately equivalent to 175 mg/dl, dams exhibited pronounced disruptions in a variety of maternal behaviors (e.g., latency to retrieve pups, latency to adopt the crouching posture facilitating nipple attachment, time spent out of the nest, and time spent licking the pups). However, repetitive administrations of this alcohol dose leads to a progressive resistance to the deleterious effects of the drug upon the different behavioral components of maternal care. Pharmacokinetic studies indicated that the nursing dam develops metabolic tolerance to alcohol as a function of chronic exposure to the drug (Pepino et al., 2002).

While a considerable research effort has been devoted to the analysis of how fetal or infantile experience with alcohol affect later alcohol seeking and/or intake behaviors, few studies have analyzed the possible interaction between alcohol experience derived from maternal intoxication during late gestation and during the nursing period. Some years ago we learned that postpartum exposure (PPD 12) to a mild alcohol dose or interaction with a sibling given this dose facilitated the expression of cardiac and behavioral responsiveness to alcohol odor four days later, but only if the pups had also been exposed to ethanol prenatally (Chotro et
al., 1996). The possibility of pre- and postpartum interactions between alcohol experiences leading to specific patterns of responsiveness to the drug has also been addressed through the use of first-order conditioning procedures. Tactile stimulation provided by the mother during and after parturition has been described as a biologically relevant stimulus for the immature rat (Leon et al., 1987; Ronca & Alberts, 1994) Olfactory learning dependent upon the contingency between alcohol chemosensory cues present in the amniotic fluid and unconditioned stimuli related to birth induction and neonatal recovery was observed in a recent study (Domínguez, Lopez, & Molina, 1999). Neonatal reexposure to alcohol cues paired with similar tactile stimulation potentiates the conditioned response under analysis (Domínguez et al., 1999).

The goal of the present study was to analyze possible interactions between early experience comprising the perception of the drug in the amniotic fluid and in the nursing context. One specific goal was to address whether these experiences modify intake patterns of small concentrations of the drug in a novel vehicle such as water. It is important to note that prior studies have consistently shown that fetal as well as preweanling learning derived from exposure to the chemosensory attributes of the drug do not generalize to basic tastants (e.g., quinine or sucrose solutions), to fluids such as water or milk, or to other artificial or biological odorants (e.g., amniotic fluid and lemon scents; Chotro & Molina, 1990; Dominguez et al., 1998; Lopez & Molina, 1999; Molina & Chotro, 1991; Pepino et al., 1998, 2004).

In this study, pregnant rats were given ethanol during late gestation. After birth, pups suckled from dams subjected to a subnarcoleptic ethanol dose. Infantile alcohol consumption tests were conducted using a low alcohol concentration. Alcohol dosage parameters were selected taking into account prior observations indicative of absence of obvious teratological effects of the drug such as malformations, anomalous development of the placenta or the umbilical cord, altered development of different central nervous system structures, or behavioral abnormalities (Abate et al., 2000; Domínguez et al., 1996, 1998).

As is the case in adult animals (Córdoba et al., 1990; Cunningham, Niehus, & Noble, 1993; Cunningham, Hawks, & Niehus, 1988; Eckardt, 1975), near-term fetuses as well as infant rats are sensitive to alcohol's unconditioned properties capable of being associated with olfactory, gustatory, and tactile cues (Abate et al., 2000; Hunt et al., 1990, 1993; Molina et al., 1996). A second specific goal of the present study is related to this phenomenon. We specifically assessed whether intrauterine and/or nursing experiences with alcohol have an impact upon infantile associative learning mediated by the postabsorptive effects of the drug. After an intake evaluation pups representative of each litter received an ethanol administration to establish an associative process between chemosensory cues derived from alcohol infusions during the first intake test, and unconditioned properties of the drug.

Taking into account that alcohol-derived disruptions of maternal care can also affect subsequent infantile reactivity to the drug (Pepino et al., 2002), an additional aim was to evaluate maternal behaviors during the nursing period as a function of ethanol exposure during late gestation and/or nursing. This third goal is intimately related to the possible development of maternal ethanol tolerance as a function of repetitive administrations of the drug.
In summary, this study assesses the impact of alcohol administration during late gestation and nursing on: (1) responsiveness of the offspring to ethanol’s chemosensory attributes, (2) infantile sensitivity to the postabsorptive effects of the drug evaluated through conditioning procedures, and (3) changes in the structure of the maternal behavioral repertoire as a function of sequential alcohol administrations.

Method

Subjects

Wistar-derived rats, representative of 39 litters, were tested. These animals were born and reared at the vivarium of the Instituto Ferreyra, under temperature (22-24 °C) and artificial light (on from 08:00-20:00 h) controlled conditions. Unless specified, maternal enriched lab chow (Cargill, Buenos Aires, Argentina) and water (delivered through automatic dispenser valves) were available ad libitum. Vaginal smears of adult female rats were microscopically analyzed on a daily basis. On the day of proestrus, females (prepregnancy weight 200-300 g) were housed during the dark cycle with males (three females per male). Vaginal smears were checked the following morning (10:00-12:00 h), and the presence of sperm was considered as index of fecundity. The day of the sperm detection was considered as GD 0. Births were checked daily (10:00-12:00 h) and the day of parturition was considered as PPD 0. During PPD 1, each litter was randomly culled to eight pups (four males and four females whenever possible). Pregnant females or litters were individually placed in standard maternity cages filled with wood shavings.

At all times, animals utilized in this study were maintained and treated with guidelines for animal care established by the National Institutes of Health (1986).

Maternal Treatment

Prenatal Treatment. From GD 17 to 20, pregnant females were weighed and intragastrically intubated with either 0.015 ml/g of a 16.8% v/v ethanol (EtOH) solution (vehicle: room temperature tap water; ethanol dose: 2.0 g/kg; Prenatal Ethanol Group; n = 20) or a similar volume of water (Prenatal Water Group; n = 19). The ethanol dose and the days of administration were selected on the basis of prior studies demonstrating fetal chemosensory processing of the drug under similar experimental circumstances and general lack of deleterious effects of ethanol upon different morphological and behavioral parameters (Domínguez et al., 1996, 1998; Molina et al., 1995). Intragastric intubations were performed employing a polyethylene cannulae (PE 50; Clay Adams, Parsippany, New Jersey, U.S.A.) attached to a disposable 5-ml syringe. The free end of the cannula was carefully eased into the stomach of the dam. The whole administration procedure took less than 20 s per animal, and was accompanied by little indication of stress.

Postpartum Treatment. During PPDs 3, 5, 7, 9, 11, and 13, half of the mothers representative of each prenatal treatment were intragastrically administered a 2.5 g/kg ethanol dose (Postpartum Ethanol Group; n = 19). The alcohol dose was achieved by administering 0.015 ml of a 21% v/v ethanol solution per gram of maternal body weight. The remaining dams were administered with an equivalent volume of tap water (Postpartum Water Group; n = 20). Intragastric administrations were performed employing similar procedures as those utilized during gestation. In prior experiments, when using similar maternal alcohol treatment, it has been observed that the peak ethanol concentration found in milk (and in maternal blood) is approximately equivalent to 175 mg/dl. When utilizing these administration parameters, we have not observed inhibition of the milk ejection reflex (Pepino et al., 1998). Indeed, higher BALs (250 mg/dl or more) are necessary to block milk-ejection processes induced by nipple suckling (Cobo & Quintero, 1969; Fuchs, 1969).

Infantile Intake Test and Postpartum Conditioning Procedures

During PPDs 15 and 16, 302 pups representative of the different litters, were intraorally cannulated with polyethylene tubing (length, 5 cm; PE10, Clay Adams, Parsippany, New Jersey, U.S.A.). The intraoral cannulation procedure has been extensively described in previous studies (Domínguez et al., 1993, 1996; Hunt et al., 1993; Pepino et al., 1998, 1999). Briefly, a flanged end of the cannulae (external diameter, 1.2 mm) was shaped by exposure to a heat source. A short dental
needle (30GA Monoject, Sherwood Medical, Munchen, Germany) was attached to the nonflanged end and positioned in the middle portion of the internal mucosa of the pup’s left cheek. The needle was inserted through the cheek, and the cannula was pulled through the tissue until the flanged end rested on the mouth’s mucosa. This cannulation procedure did not last more than 20 s per subject. As demonstrated by prior studies, pups rapidly recover from this minor surgical intervention (Molina et al., 1987; Spear et al., 1989). Pups at this developmental stage are able to control ingestion of fluids delivered via these polyethylene devices (Abate, Spear & Molina, 2001; Domínguez et al., 1998; Pepino et al., 1998). The technique is also sensitive in terms of evaluating learning experience comprising the association between a given tastant and different unconditioned stimuli (Hoffman et al., 1987; Domínguez et al., 1994; Pautassi et al., 2002).

Following the cannulation procedure, pups were left without maternal care for 2 h. During this time period they remained with the corresponding littersmates in clean plastic cages. The use of heating pads placed beneath these chambers allowed to keep ambient temperature at 32 °C. Immediately before commencement of the intake tests, all pups were stimulated in the anogenital area with a cotton swab to induce miction and defecation. Preweanlings were then weighed (=±0.01g) and this value was taken as the preinfusion body weight. Each pup was placed in individual Plexiglas chambers (15 x 7 x 15 cm) lined with a cotton floor, and the cannula was attached to a 10-way peristaltic infusion pump (Manostat Cassette Pump, Standard Model, Barnant Co., Barrington, Illinois, U.S.A.). An ethanol solution equivalent to 0.22% v/v alcohol in water (this solution equals the administration of 0.026 g/kg ethanol dose), was delivered into the infant’s mouth during 10 min using a pulsatile mode of administration (3 s on, 10 s off). The rate of infusion was adjusted for each individual rat in order to infuse a total amount of fluid that was equivalent to 1.83% of the pup’s preinfusion body weight (=±0.01 g). At the end of each intake session, pups were removed and weighed again (postinfusion body weight). Percentage of consumption scores was then calculated: 100x [(postinfusion body weight - preinfusion body weight) / ml infused].

On PPD 15, immediately after the alcohol intake test, pups were quasirandomly assigned to one of the following four conditioning treatments: infants were intubated with water or they received an i.g. administration of 0.015 ml per gram of body weight of a 4.2% v/v, 8.4% v/v, or a 16.8% v/v of ethanol solution (alcohol doses: 0.5, 1.0, or 2.0 g/kg alcohol, respectively). According to a recent study, these doses result in peak blood alcohol levels equivalent to 42, 97 and 204 mg/dl, respectively (Pautassi et al., 2004). The quasirandom distribution was applied to allow equivalent representation of litters across drug treatments. As previously demonstrated (Abate et al., 2001; Hunt et al., 1990; Pautassi et al., 2002), infants are sensitive to ethanol’s postabsorptive effects as evidenced by associative learning paradigms. Pairing a salient gustatory cue with ethanol doses higher than 1.0 g/kg normally results in the establishment of excitatory pavlovian conditioning. In the present study, the comparison between intake scores at both days of test served to analyze whether the association between a low concentrated ethanol solution and different intragastrically administered ethanol doses were sufficient to establish conditioned responses to this solution.

As mentioned, in order to avoid litter overrepresentation, no more than two pups from a given litter were included in a particular treatment (one male and one female, whenever possible). Gender of pups failed to exert main effect and/or did not interact with the remaining factors under consideration. For this reason, the average intake scores corresponding to pups representative of the same litter and exposed to similar drug treatment during PPD 15 served as the unit of analysis concerning ethanol consumption patterns. Each of the sixteen groups defined by the corresponding factorial design (Prenatal treatment: EtOH or Water; Postpartum treatment: EtOH or Water and Infantile drug treatment: Water, 0.5, 1.0 or 2.0 g/kg alcohol) was composed of 9-10 average litter scores.

**Behavioral Evaluation of Nursing Females**

During nursing, 15 min after dams were administrated either EtOH or water, the nest was disarranged and pups were scattered to different sections of the home cage (Pepino et al., 2002). Dams were placed back with their corresponding litter and were videotaped for 2 h. To this aim, the standard wire aluminum cover of each maternity cage was replaced with an acrylic transparent cover to allow better video recording of the dam/pup dyad. Trained observers, blinded to maternal treatment, processed maternal behavior. Latencies to retrieve all pups and to adopt the upright crouching posture (kyphotic position, a posture that facilitates nipple attachment by the young) were scored during PPDs 3 and 13. These postpartum days correspond to the beginning and the end of each maternal drug treatment (Water or Alcohol). Mixed analyses of variance (ANOVAs) were conducted to determine how each behavior (retrieval and crouching) varied as a function of drug treatments during
gestation and lactation. Latencies to perform these behaviors during PPDs 3 and 13 served as repeated measures. The data processed in relation to crouching and retrieval behaviors was derived from a total of 29 litters (Water-Water, n = 6; Water-EtOH, n = 8; EtOH-Water, n = 8 and EtOH-EtOH, n = 7). The videotapes corresponding to the remaining 10 litters were damaged and could not be scored.

Results

**Maternal Weight During Pre- and Postpartum Treatment**

Maternal weights during GDs 17-20 and PPDs 3, 5, 7, 9, 11, and 13 were analyzed through a three-way mixed analysis of variance (ANOVA) where pre- and postpartum treatments served as independent variables and days of drug treatment as repeated measures (see Table 1). The ANOVA indicated a significant main effect of day, as well as significant interactions between prenatal treatment and days, and between postnatal treatment and days; $F(9, 315) = 554.90, F(9, 315) = 4.79,$ and $F(9, 315) = 3.52,$ all $p < 0.001,$ respectively. Follow-up ANOVAs indicated that maternal body weights at commencement of pre- (GD17) and postnatal treatment (PPD3) did not differ across groups. Additional ANOVAs took into account absolute body weight increases between GDs 17 and 20 and PPDs 3-13. Maternal body weight increases were significantly lower in dams treated with ethanol during gestation relative to those administered with water; $F(1, 35) = 21.20, p < 0.001.$ This significant prenatal effect was also attained when focusing on the nursing period; $F(1, 35) = 15.11, p < 0.001.$ Furthermore, dams subjected to ethanol treatment during this period gained significantly less weight than corresponding controls; $F(1, 35) = 9.42, p < 0.005.$ No significant interaction between pre- and postnatal treatment was observed.

**Infantile Ethanol Consumption Patterns**

Infantile body weights at PPD 15 were significantly affected by maternal treatment during nursing. Indeed, a two-way ANOVA (Prenatal x Postpartum Treatment) revealed a main significant effect of postpartum treatment. Pups that suckled from a dam subjected to a 2.5 g/kg ethanol dose during PPDs 3-13 exhibited significantly less body weight at PPD 15 than did pups reared by water-treated dams, $F(1, 35) = 20.70, p < 0.001.$ This significant prenatal effect was also attained when focusing on the nursing period; $F(1, 35) = 15.11, p < 0.001.$ Furthermore, dams subjected to ethanol treatment during this period gained significantly less weight than corresponding controls; $F(1, 35) = 9.42, p < 0.005.$ No significant interaction between pre- and postnatal treatment was observed.

Infantile consumption of a 0.22% v/v alcohol solution was examined with a four-way mixed ANOVA Prenatal Treatment (Water, EtOH) x Postpartum Treatment (Water, EtOH) x Alcohol Dosage at PPD 15 (0.0, 0.5, 1.0, 2.0 g/kg) x Days of Test (PPDs 15, 16). The ANOVA showed a significant main effect of days of test, $F(1, 136)= 25.11; p < 0.001.$ The following interactions also reached significance: infantile alcohol dosage x days of test, $F(3, 136)= 3.56; p < 0.025,$ and prenatal treatments x postpartum treatments x days of test, $F(1, 136)= 4.97; p < 0.05.$ The posthoc tests showed that alcohol intake scores during PPD 15 did not differ across groups. Independently from pre- and postpartum treatments, infants that were intragastrically administered with water or with the lower alcohol dose (0.5 g/kg) exhibited significantly higher consumption scores during PPD 16 rela-
tive to the previous intake test (PPD 15). This heightened intake pattern was not observed whenever preweanlings were subjected to the explicit association between sensory properties of alcohol, derived from the first intake test, and i.g. intubations of 1.0 or 2.0 g/kg alcohol doses.

Table 1
Maternal Body Weights (g) During Gestation and Across Postpartum Days.

<table>
<thead>
<tr>
<th>Maternal Treatment</th>
<th>GD 17</th>
<th>GD 18</th>
<th>GD 19</th>
<th>GD 20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water-Water (n = 10)</td>
<td>274.3±7.0 g</td>
<td>287.9±6.4 g</td>
<td>296.6±6.8 g</td>
<td>307.1±7.7 g</td>
</tr>
<tr>
<td></td>
<td>(5.1±0.8 g)</td>
<td>(8.2±0.7 g)</td>
<td>(12.3±0.8 g)</td>
<td></td>
</tr>
<tr>
<td>Water-EtOH (n = 9)</td>
<td>288.1±11.5 g</td>
<td>299.2±12.8 g</td>
<td>306.7±13.3 g</td>
<td>319.2±11.9 g</td>
</tr>
<tr>
<td></td>
<td>(3.8±0.6 g)</td>
<td>(6.4±1.0 g)</td>
<td>(11.0±2.0 g)</td>
<td></td>
</tr>
<tr>
<td>EtOH-Water (n = 10)</td>
<td>287.8±7.8 g</td>
<td>292.0±7.5 g</td>
<td>294.7±7.4 g</td>
<td>302.4±7.6 g</td>
</tr>
<tr>
<td></td>
<td>(1.5±0.5 g)</td>
<td>(2.5±0.6 g)</td>
<td>(5.2±0.6 g)</td>
<td></td>
</tr>
<tr>
<td>EtOH-EtOH (n = 10)</td>
<td>277.4±4.3 g</td>
<td>285.2±7.3 g</td>
<td>292.6±6.5 g</td>
<td>294.2±6.9 g</td>
</tr>
<tr>
<td></td>
<td>(2.7±1.6 g)</td>
<td>(5.4±1.3 g)</td>
<td>(6.0±1.5 g)</td>
<td></td>
</tr>
</tbody>
</table>

Note. Values between parenthesis represent changes in body weights relative to those observed during GD 17 or PPD 3. All values represent mean ±/ standard error of the mean.

Of major importance for the present study was the significant interaction between pre- and postnatal treatment and testing days. Posthoc analyses revealed that during the first test day (PPD 15) all independent groups drank similar amounts of alcohol. After this initial experience, pups reared by dams treated with alcohol during late gestation and the nursing period showed significantly higher alcohol intake scores when compared with infants derived from dams that never experienced the drug or from females that were only exposed to alcohol during either gestation or nursing.

In order to depict these results, Figure 1 has been arranged using percent-
age intake scores for all independent groups and their corresponding repeated measures. As can be observed in Figure 2A and 2B, percent intake scores have been illustrated as a function of the significant interactions "infantile alcohol dosage x day" and "prenatal treatment x postnatal treatment x day," respectively. In addition, to better appreciate the magnitude of the effects attained during PPD 16 relative to the ones accrued during PPD 15, Figure 3 depicts mean individual difference scores in alcohol consumption (PPD 16 – PPD 15 values) as a function of alcohol dosage applied after the initial intake session (Figure 3A) and as a function of prenatal and postnatal treatment (Figure 3B). As can be expected, a three-way ANOVA (Prenatal Treatment x Postnatal Treatment x Infantile Alcohol Dosage at PPD 15) showed that these difference scores significantly varied as a function of the alcohol dose administered at PPD 15, $F(3, 136) = 3.56, p < 0.025$ (pups treated with 1 or 2 g/kg ethanol had significantly lower scores than pups treated with water) and as a function of the interaction between pre- and postnatal treatments, $F(1, 136) = 4.97, p < 0.05$ (pups exposed to fetal and nursing experiences with ethanol showed higher scores relative to the remaining experimental conditions).

Table 2

<table>
<thead>
<tr>
<th>Maternal Treatment</th>
<th>PPD 15</th>
<th>PPD 16</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water-Water</td>
<td>25.84±0.7</td>
<td>25.77±0.9</td>
</tr>
<tr>
<td>Water-EtOH</td>
<td>23.01±0.6</td>
<td>25.86±0.9</td>
</tr>
<tr>
<td>EtOH-Water</td>
<td>26.52±0.8</td>
<td>25.52±0.7</td>
</tr>
<tr>
<td>EtOH-EtOH</td>
<td>23.38±0.5</td>
<td>25.04±0.8</td>
</tr>
</tbody>
</table>

Note. All values represent mean +/- standard error of the mean.

Figure 1. Percentage of consumption levels (ml %) during postpartum days (PPDs) 15 and 16, as a function of pre- and postnatal manipulations (Water-Water, Water-EtOH, EtOH-Water or EtOH-EtOH) and ethanol dose received during PPD 15 (0.0, 0.5, 1.0 or 2.0 g/kg). Vertical lines illustrate standard errors of the means.
These results can be summarized as follows: (1) pups ingested higher amounts of the alcohol solution during the second day of test, a result that is congruent with prior studies indicating heightened alcohol consumption as a function of preexposure to the drug's sensory attributes (Molina et al., 1984). (2) This preexposure effect was inhibited whenever initial ingestion was explicitly paired with alcohol doses (1.0 or 2.0 g/kg) known to mediate conditioned aversions in preweanlings (Abate et al., 2001; Molina et al., 1996; Pautassi et al., 2002). (3) Information encoded during pre- and postnatal exposure to alcohol seemed to facilitate subsequent responsiveness and perhaps sensitivity to a small concentration of alcohol, even if presented in a different vehicle (water, rather than amniotic fluid or milk) than that in which it was previously experienced.

**Maternal Latency to Retrieve the Pups**

Maternal latency to retrieve all pups was analyzed through a three-way mixed ANOVA (Prenatal Treatment x Postpartum Treatment x Days of Assessment). The analysis showed significant main effects of prenatal and postpartum treatments as well as of day of evaluation, $F(1, 25) = 6.89, p < 0.025; F(1, 25) = 9.89, p < 0.005; F(1, 25) = 9.03, p < 0.01$, respectively. The interactions between pre- and postpartum manipulation, and postpartum manipulation and day of evaluation also achieved significance, $F(1, 25) = 8.69, p < 0.01$ and $F(1, 25) = 5.69, p < 0.025$, respectively. Newman-Keuls posthoc tests indicated that latency to retrieve pups was significantly higher in those mothers that only received ethanol during nursing (Water-EtOH) relative to the remaining treatment conditions. Mothers exposed to ethanol during gestation and during nursing (EtOH-EtOH) had similar retrieval latencies as dams that had never experienced the drug (Water-Water) or only suffered its effects during pregnancy (EtOH-Water). In addition, dams postnatally treated with ethanol, exhibited better performance scores (lower latencies) during PPD 13 relative to the ones recorded during PPD 3. During PPD 13 these scores were not significantly different from those recorded in water-treated dams during the course of the nursing period. These results are depicted in Figure 4.

**Latency to Exhibit Maternal Crouching Behavior**

From a descriptive perspective, the pattern of results attained when focusing on crouching behavior was similar to the one observed while evaluating retrieval latencies (Figure 5). Nevertheless, the corresponding three-way mixed ANOVA (Prenatal Treatment x Postpartum Treatment x Days of Evaluation) showed that crouching performance was only significantly affected by postpartum treatment and day of evaluation, $F(1, 25) = 7.56, p < 0.25; F(1, 25) = 4.67, p < 0.05$, respectively. Dams intoxicated with ethanol during lactation showed higher latencies to exhibit a crouching posture when compared with sober dams. When considering postpartum day of treatment, latencies to adopt the kyphotic posture were significantly lower at PPD 13 when compared with latencies during PPD 3. Prenatal treatment as well as the interactions between the factors under analysis failed to reach significant levels.
Figure 2. Percentage of consumption levels (ml %) during PPDs 15 and 16 as a function of ethanol dose received at PPD 15 (A) and as a function of pre- and postnatal experiences (Water-Water, Water-EtOH, EtOH-Water or EtOH-EtOH) (B). Vertical lines illustrate standard errors of the means.

In summary, the present results confirm prior observations relative to the disruptive effects of ethanol intoxication within the nursing context upon different behaviors related to maternal care (Pepino et al., 2002). Furthermore, these results indicate that prenatal treatment was not sufficient to significantly alter crouching behavior or the ability of the dam to retrieve the pups. On the contrary, ethanol administration during late gestation seemed to modify the consequences of the drug when administering ethanol to the nursing dam, particularly when focusing on retrieval latencies. This effect seems to indicate a progressive development of tolerance to ethanol in the dams. The possibility of tolerance development also emerges when taking into account that during PPD 13 (following six ethanol administrations) dams no longer exhibited abnormal retrieval latencies when exposed to ethanol as they did during PPD 3. When taking into account crouching behavior, prenatal ethanol administration or sequential postnatal exposure to the drug failed
to attenuate the disruptive effects of the drug while dams were in contact with their pups.

**Figure 3.** Difference of percentage of consumption levels (PPD16 - PPD 15) expressed as a function of ethanol dose received during PPD 15 (A), and as a function of pre- and postpartum treatment (Water-Water, Water-EtOH, EtOH-Water, or EtOH-EtOH) (B). Vertical lines illustrate standard errors of the means.

**Discussion**

The present results show that pre- and early postpartum ethanol experiences, derived from maternal alcohol intoxication, interact in terms of promoting ingestion of a low concentrated ethanol solution. Through this interaction animals were capable of sensing minimal concentrations of ethanol in a vehicle different from the one corresponding to each specific ontogenetic experience (amniotic fluid or milk). Taken as a whole, these results suggest that sequential experiences with ethanol during early development can act as a critical factor in the persistence of effects that are derived from pre- (e.g., Dominguez et al., 1993, 1998) and postnatal experiences with alcohol (e.g., Pepino et al., 1998, 1999).
When contrasting the present results with prior literature concerned with short- and long-term effects of early ethanol experiences, a common denominator emerges relative to the expression and/or persistence of these effects. Early sequential exposures to ethanol’s chemosensory cues appear to facilitate the effects of the original experience with the drug. For example, heightened behavioral reactivity to ethanol compounded with milk is observable in pups that had not only experienced the drug via interactions with an intoxicated dam but also had sensed it through intraoral infusions (Pepino et al., 1999). Chotro et al. (1996) also reported that behavioral and autonomic responding to ethanol odor are dependent upon the interaction between ethanol prenatal experiences and a single postnatal exposure to intoxicated peers which probably provide ethanol sensory information through nonmetabolic elimination of the drug via alveolar excretion. Furthermore, conditioned ethanol olfactory responses established in utero through the association of ethanol and an activating tactile stimuli, are strongly potentiated whenever
followed by a single neonatal conditioning trial that also involves ethanol and tactile stimulation. Interestingly, this single trial is not sufficient per se to establish conditioned motor responses elicited by the odor of the drug. To our knowledge, the present study represents the first empirical evidence indicating changes in responsiveness to low ethanol solutions as a function of the interaction between fetal and nursing experiences involving ethanol. Both ontogenetic experiences were necessary to induce heightened ethanol intake. Yet, the expression of this interaction required additional experience through intraoral infusions of the ethanol solution during PPD 15. It is conceivable that this latter exposure to ethanol reactivates memories concerning a similar sensory cue originally processed in the amniotic fluid and in maternal milk. It is important to note that the intensity and/or the duration of this apparent reactivating stimuli can be critical in modulating the magnitude of subsequent responsiveness to the drug. As stated, prenatal effects derived from maternal ethanol intoxication not only imply sensory exposure to the drug, but are also modulated by the association between these cues and ethanol's unconditioned properties (Abate et al., 2000, 2001, 2004; Chotro & Arias, 2003). Effects derived from the interaction with an intoxicated mother also appear to be established by associative mechanisms where infantile perception of ethanol odor and/or taste is paired with changes in the behavioral and physiological status of the dam (Molina et al., 2000; Pepino et al., 2002). Hence, it is conceivable that later experiences with ethanol as a conditioned sensory cue, relative to the ones occurring in utero or in the nursing context, could also promote extinction. This effect has been reported in infants that experienced ethanol as a conditioned stimulus during antenatal life and were later exposed to ambient ethanol odor during a considerable amount of time (Domínguez et al., 1993).

Maturational processes allowing memory expression and/or handling procedures during PPD 15 represent factors that can also explain why the combined prior ethanol treatments were only observed at PPD 16. We have consistently observed that ethanol-related effects established either prenatally or during lactation are evoked at PPD 15 or even at earlier ages (Domínguez et al., 1998; Pepino et al., 2004). Yet, this heightened ethanol intake due to prior fetal or infantile experiences with the drug has been observed when using considerably higher ethanol concentrations (range: 5–10% v/v ethanol) relative to the one here employed (0.22% v/v). When using ethanol concentrations similar to those found in milk delivered by an intoxicated dam (0.22% v/v), the expression of specific ethanol memories has been observed after pups are habituated to intraoral infusion procedures performed with water or milk vehicles which later contained the drug (Pepino et al., 1998; 1999). It can be argued that under the present experimental circumstances, the interaction between fetal and infantile ethanol experiences were only evident when animals were first habituated to different sources of stimulation (intraoral infusion, handling, etc.), that can compete against infantile perception of minimal amounts of ethanol.

In agreement with previous studies (Abate et al., 2001; Pautassi et al., 2002) infantile experiences comprising the explicit association between chemosensory stimuli and ethanol's postabsorptive consequences, apparently results in excitatory aversive conditioning. A single conditioning trial, where a 0.22% v/v ethanol solution served as the conditioned stimulus and intragastric ethanol doses equivalent to 1.0 or 2.0 g/kg served as unconditioned stimulus, was sufficient to
impede subsequent heightened intake patterns of the solution as those observed in pups treated with vehicle alone or a mild ethanol dose (0.5 g/kg). Prenatal and/or nursing experiences with ethanol failed to modulate sensitivity to ethanol's unconditioned properties. We have previously reported that a 1.0 g/kg ethanol dose presented during late gestation does not alter postnatal expression of tolerance or sensitization to ethanol as assessed through neonatal psychomotor activity or infantile associative learning mediated by the drug's unconditioned properties (Abate et al., 2001). Ethanol nursing treatments similar to the ones here employed have also failed to modify infantile and juvenile acute sensitivity or development of rapid tolerance to ethanol when evaluated through a motor coordination task (Ponce et al., 2004). According to the present results, the addition of late prenatal and early postnatal experiences with ethanol do not seem to promote differential processing of ethanol as an unconditioned stimulus using associative learning procedures. It is prudent to avoid generalizing this observation when considering alternative (not aversive) hedonic effects of ethanol. Recent studies suggest that perinatal, infant and periadolescent rats are not only sensitive to ethanol's aversive consequences, but also capable of encoding positive and negative (antianxiety) reinforcing effects of the drug. These reinforcing effects have been noticed during early ontogeny whenever ethanol doses that yield relatively low blood ethanol levels are used (Abate et al., 2002; Cheslock, et al., 2000, 2001; Fernández-Vidal, Spear, & Molina, 2003; Varlinskaya & Spear, 2002). Better understanding of what is specifically encoded about ethanol's postabsorptive effects during different stages of development constitutes a prerequisite for further examination of how early ethanol experience modulates later responsiveness to its differential unconditioned properties.

Ethanol administration during late pregnancy was not sufficient to affect maternal care as evaluated through pup retrieval and crouching behavior. This in itself is an important finding; it strongly argues against the possibility that changes in ethanol responsiveness originating in late gestation are primarily determined by the effects of ethanol on maternal care (e.g., Chotro & Arias, 2003; Dominguez et al., 1998). In this respect, rats deprived of maternal care for brief periods of time during the first two postpartum weeks show heightened anxiety-like behavior and increased stress-mediated neuroendocrine reactivity (e.g., oversecretion of corticosterone in response to mild stressors such as an air puff or handling; Huot et al., 2001; Kalinechek et al., 2002; Levine, 2000; Sanchez, Ladd, & Plotsky, 2001). This stress-related susceptibility predisposes the organism to seek and self-administer ethanol due to its antianxiety effects (Huot et al., 2001; Fahlke et al., 2000). One could argue that, in this study, ethanol concentrations employed during testing were too small to recruit pharmacological effects following consumption and, therefore, that it is inappropriate to speculate about the relationship between maternal care and ethanol consumption in the offspring. Nevertheless, it cannot be discarded that due to prior experience with ethanol's sensory and postabsorptive effects, the developing organism could react to the sensory component of the drug due to its signaling properties of a specific emotional state. Even when accepting this possibility, the lack of disruptive effects of prenatal ethanol treatment upon maternal behavior argues against a direct link between this treatment, altered maternal care, and proclivity of the offspring to seek and consume ethanol. Beyond these considerations, it is important to note that blood alcohol levels were not
measured following infantile intake tests. Even when assuming that pups ingested the total amount of the infused ethanol solution (1.83 % of the individual body weight), the maximum ethanol dose that would be attained is equivalent to 0.03 g/kg. This dose has been calculated as a function of ethanol’s specific weight, the concentration of ethanol in the solution (0.22% v/v) and, obviously, the weight of the infant. In prior studies (Pautassi et al., 2004), we have observed that a 0.25 g/kg dose (i.g.) results in peak blood alcohol levels equivalent to 11 mg/dl. Hence, it is unlikely that the overall absolute ethanol consumption derived from the intake test yields pharmacologically relevant effects.

As was the case in prior studies, maternal ethanol administration during the nursing period strongly disrupted behaviors such as pup retrieval and crouching (Pepino et al., 1999, 2002). These behavioral disruptions seem to be perceived by the nursing infant and can play a significant role in the content of the memory comprising ethanol’s sensory attributes and/or affect the predisposition of juveniles to seek and ingest the drug (Pepino et al., 2004; Ponce et al., 2004). These ethanol-related disruptions were partially attenuated when females were administered with ethanol during late gestation. In particular, retrieval behavior during PPD 3 in dams exposed to ethanol that had also experienced the drug during pregnancy was practically indistinguishable from retrieval patterns exhibited by control females. Tolerance as a function of repeated prenatal ethanol administrations is likely to explain this phenomenon which has also been reported following chronic ethanol administration during nursing (Pepino et al., 2002). The fact that ethanol administration during pregnancy modify maternal behavioral profiles during nursing caused by ethanol can imply an interaction of the pup/dam dyad that facilitates social and/or nutritional transmission of ethanol-related cues.

Experimental as well as epidemiological research supports a critical role of early ontogenetic experience with ethanol in the structure of ethanol use and abuse patterns (Baer et al., 1998, 2003; Molina et al., 1999; Spear & Molina, 2001; Yates et al., 1998). The human literature endorses the likelihood of a continuing sequence of ethanol-related experience across early periods of development. Longitudinal studies in humans as well as research based on animal models appear now necessary to better understand the relative weight of specific ethanol-related experiences and their possible interaction in the persistence and magnitude of the effects. The present study argues in favor of synergism between late gestational and early postnatal ethanol experiences resulting in heightened intake of a fluid solution containing minimal amounts of the drug (Pepino et al., 1998; 1999).

References


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