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Prenatal Association between a Chemosensory Cue (Cineole) and Ethanol Postabsortive Effects Modulates Later Operant Responsiveness to Cineole-Flavored Milk in 1 Day Old Rats

March, Samanta Mabel [*a,b], Abate, Paula [a,b] y Molina Juan Carlos [a,b]

[a] Instituto de Investigación Médica Mercedes y Martín Ferreyra – CONICET, Córdoba, Argentina
[b] Facultad de Psicología, Universidad Nacional de Córdoba, Córdoba, Argentina.

Artículo Original

Resumen

Los fetos de rata a término detectan, y asocian entre sí, las propiedades sensoriales y farmacológicas del etanol. En el presente estudio se evaluaron cambios en un aprendizaje operante, en función de las experiencias fetales con etanol. Los tratamientos prenatales fueron: Grupo "Apareado", las hembras fueron administradas con cineol y 15-min después con etanol (2.0 g/kg); Grupo "Larga-Huella", se administró cineol y 6 horas mas tarde, etanol y Grupo "Agua", las hembras recibieron dos administraciones de agua separadas por 15 minutos. Las crías fueron entrenadas en una tarea operante. Se emplearon dos condiciones de aprendizaje (apareado y acoplado). Los sujetos "Apareados" fueron entrenados bajo un esquema de reforzamiento continuo. Los sujetos "Acoplados" recibieron el reforzador de manera independiente de su conducta. Los reforzadores fueron leche o leche saborizada con cineol. Los resultados sugieren que las memorias prenatales relacionadas al alcohol pueden modular las respuestas operantes cuando las crías son re-expuestas a claves predictoras de los efectos tóxicos etílicos.

Palabras claves:
Aprendizaje Fetal; Etanol; Reforzamiento; Condicionamiento

Abstract

Near-term rat fetuses detect and associate ethanol's sensory and postabsortive properties. We aimed to evaluate changes upon operant conditioning in neonates, as a function of prenatal ethanol-mediated associative experiences. Prenatal treatments were: Paired group, dams administered (i.e.) with cineole and 15-min later with EtOH (2.0 g/kg); Long-delay group, dams received cineole 6 hours before ethanol intoxication; Water group, dams received two administrations of water separated by 15 min. On PD1 neonates were trained in an operant task. Two different learning conditions were used. Paired pups were trained under a FR=1 reinforcement schedule. Yoked pups received the reinforcer but, unrelated to their own behavior. Milk or Cineole-flavored milk were the reinforce solutions. Present results support the idea that ethanol-related prenatal memories modulate operant responsiveness when pups are re-exposed to cues that predict toxic consequences of the drug..

Key Words:
Fetal Learning; Ethanol; Reinforcement; Conditioning

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1. Introduction

Associative learning capabilities have been observed before birth. For example, near-term rat fetuses associate an aromatic cue (apple juice) with the aversive effects of a lithium chloride administration (Smotherman, 1982). When low alcohol concentrations are applied near the rostral area of rat fetuses (gestational day 21; GD 21) they detect ethanol's odor and also, are capable of associating this chemosensory stimulation with biologically relevant events, such us mechanical stimulation that mimics labor (Domínguez, Chotro & Molina, 1993; Domínguez, López & Molina, 1999; Molina & Chotro, 1991) and nociceptive stimulation (Chotro & Molina, 1992).

When administering alcohol to pregnant rats, fetuses are exposed to both, ethanol chemosensory properties and postabsortive effects. Ethanol

* Corresponding author: Lic. March, Samanta M.
E-mail: smarch@immf.uncor.edu

concentrations found in fetal blood and amniotic fluid resembles alcohol levels reached in maternal blood (Domínguez, López, Chotro & Molina, 1996). Prenatal ethanol experience through maternal intoxication has been found to modulate postnatal responsiveness to ethanol's odor and to increase alcohol consumption levels in infant rats (Arias & Chotro, 2005a, 2005b; Domínguez, López & Molina, 1998; Pueta, Abate, Spear & Molina, 2005; Pueta, Rovasio, Abate, Spear & Molina, 2011). This increase in alcohol consumption has been hypothesized to result from the association between alcohol's chemosensory properties and its post-absorptive effects (Spear & Molina, 2005). Considering such possibility, Abate and colleagues (2000) developed a methodological strategy in which a non-alcohol chemosensory cue (cineole) was administered to the dam. This chemosensory cue reaches the amniotic fluid and has salient aromatic characteristics (Keller, Hansel & Chandler, 1992) that can be perceived by the fetus. As mentioned, the fetus can potentially associate odorant cues with biologically relevant stimuli. To test the capability of ethanol to act as unconditioned stimuli, the authors induced an alcohol intoxication state explicitly paired with the presence of cineole in the amniotic fluid. It was found that during infancy, these pups responded differentially to cineole-flavored milk compared to infants exposed to these cues but in a non contingently manner (Abate, Pepino, Dominguez, Spear & Molina, 2000). In a follow up study, Abate and collaborators (2002) found that prenatal exposure to cineole in association with ethanol's post-absorptive effects modifies subsequent attachment to a cineole scented surrogate nipple. Pups from the Paired group (animals exposed during late gestation to cineole and ethanol in temporal contingency) displayed higher mean grasp duration compared to Long-Delay (animals exposed to these cues in a non-contingently manner) and Water control groups (Abate, Varlinkaya, Cheslock, Spear & Molina, 2002).

The importance of studying the effects of ethanol exposure during early ontogeny has been already highlighted (Abate, Pueta, Spear & Molina, 2008; Molina, Spear, Spear, Mennella & Lewis, 2007; Spear & Molina, 2005). In accordance with animal studies, preclinical research showed that prenatal exposure to ethanol is linked to the development of alcohol drinking problems during adolescence and adulthood (Alati, et al., 2006; J.S. Baer, Barr, Bookstein, Sampson & Streissguth, 1998; J.S. Baer, Sampson, Barr, Connor & Streissguth, 2003). It is generally accepted that the motivational properties of ethanol play a critical role in the modulation of alcohol drinking and seeking (Cunningham, Fidler & Hill, 2000; Pautassi, Nizhnikov & Spear, 2009). Ethanol's rewarding properties have been extensively studied through the strength of operant responsiveness -i.e. bar pressing- (Samson et al., 2004). However, the direct application of these conditioning paradigms during early ontogeny seems problematic when considering the developmental changes ongoing during the first weeks of postnatal life and the limited behavioral repertoire of neonates in comparison with adult rats. Nevertheless, one-day old pups learn to perform side head movements to receive access to warmth stimulation (Flory, Langley, Pfister & Alberts, 1997). Same age rats develop an instrumental response to gain milk (Johanson & Hall, 1979). Alcohol-contaminated milk has also been found to be reinforcing in older pups [postnatal days –PDs- 3 to 4 and 15 to 16; (Domínguez, Bocco, Chotro, Spear & Molina, 1993)]. Nevertheless, the study conducted by Johanson & Hall (1979) and replicated by Dominguez et al. (1993) involved potentially stressful conditions such as prolonged maternal deprivation and undernourishment. Recently, two studies (Arias, Spear, Molina & Molina, 2007; Bordner, Molina & Spear, 2008) have shown that neonatal forepaw and head movements, rapidly and significantly increase when milk acts as a reinforcer, and the organism is held in a supine position. In both studies, only 15 minutes of training were sufficient to install a rate of sensor presses that was significantly higher than that of yoked controls (pups given reinforcement only when the experimental paired counterpart touched the sensor). Using a similar procedure of operant conditioning, we have found that prenatal exposure to ethanol (during gestational days 17-20) increases self-administration of both alcohol (3% v/v) and alcohol-like (sucrose-quinine) solutions in one day old pups, compared with neonates that lack such prenatal history of alcohol intoxication (March, Abate, Spear & Molina, 2009).

In the present study, the affective value of an associative prenatal memory was challenged by means of an operant conditioning paradigm. We hypothesized that if prenatal experience with cineole paired with alcohol derives in an appetitive memory, a new experience with cineole would be rewarding for neonates. According to this, the present study was conducted to determine if prenatal alcohol-mediated experiences can modulate the pattern of neonatal instrumental responsiveness when neonates are re-exposed to chemosensory cues that predicted ethanol post-absorptive effects before birth.

2. Materials and Methods.

2.1. Subjects

One-day old Wistar rat pups (n=108) derived from 28 dams were used. Animals were born and reared at the Vivarium of the Instituto de Investigaciones Médicas Mercedes y Martín Ferreyra. Temperature was kept at 22-24o C with a 14-hr light/10-hr dark cycle (light onset at 0700 hr). At all times, the animals that compose the present study were maintained and treated in compliance with guidelines for animal care established by the Institute of Laboratory Animal Resources, National Research Council, U.S.A. (1996) (National Institute of Health, 1996).

2.2. Maternal Treatments

During GD 17, dams were randomly assigned to one of three possible treatments. Two groups were defined by the temporal relationship existing between cineole and alcohol administrations. One group of females received an intragastric (i.g.) administration of cineole (eucalyptus essential oil emulsion: 10.87% v/v; volume administered: 0.0015 ml/g) and 15 min later, they were subjected to an i.g. administration of a 2 g/kg alcohol dose (ethanol solution: 16.8 % v/v; volume of administration: 0.015 ml/g). This prenatal manipulation provides close temporal contiguity between cineole as a potential conditioned stimulus (CS) and alcohol as unconditioned stimulus (US; Paired Group). Paired animals received this treatment during GDs 17-20. A second group of females were also exposed to cineole and alcohol at GDs 17-20, but with a 6-hour delay between presentations (Long-delay Control Group). The remaining group of females experienced two i.g. administrations of water with volume and temporal parameters similar to those used for the Paired group (Water Control Group).

2.3. Neonatal Treatments

At PD 1, neonates were intraorally cannulated to allow delivery of fluids directly into the intraoral cavity. This procedure has been extensively described in previous studies (Abate, Pepino, Spear & Molina, 2004; Chotro, Cordoba & Molina, 1991; Dominguez, et al., 1998). Briefly, a polyethylene tubing (Clay Adams, PE10) was heated at one end to form a small flange. The non flanged end of the cannula was positioned on the medial internal surface of the right cheek of the pup and pushed until the flanged end rested on the mouth's mucosa. Cannulation procedures did not last more than 20 sec per pup. Subsequently, pups were placed into a temperature and humidity-controlled incubator (Fábrica Eléctrica Delver, La Plata, Argentina).

Neonatal operant conditioning started one hour later. To avoid overrepresentation, no more than one subject from a given litter was assigned to the same treatment condition (Holson & Pearce, 1992). Before commencement of each session, pups were stimulated in the anogenital area with a cotton swab to induce miction and defecation. Neonates were then weighed (+/- 0.01 g).

At this time, neonates were trained in an operant task. For a more detailed description of conditioning and testing procedures, see (March et al., 2009; Miranda-Morales, Molina, Spear & Abate, 2010). Shortly, pups were fitted with a vest that allowed them to remain in a supine position. The vest was attached to a rounded soft surface placed upon a platform that was kept at 35.5 +/- 0.5º C. Training and evaluation procedures took place in a transparent Plexiglas glove box equipped with a fan system for ventilation and two holes in the front section that allowed the experimenter to access the neonate.

Head and forepaw movements resulted in the activation of a touch sensitive key (Quantum, research group. E11x Evaluation board, Pittsburgh, USA) that triggered an infusion pump. A 4-way syringe infusion pump (Apema, PC11U, Bs.As., Argentina) settled at 36 ml/hour rate was employed. Each press on the sensor performed by postnatal paired pups resulted in one-second activation of the infusion pump that delivered a volume of 10 µl of milk (San Regim, SanCor, Santa Fé, Argentina; 1.5% fat content, with supplement of vitamins A and D) or cineole-flavored milk (milk plus 1% v/v of a 10.87% v/v eucalyptus emulsion). The reinforcement schedule applied in the present study was settled at a fixed rate equals to 1 (FR=1). Each postnatal paired subject was tested with a same sex littermate that acted as yoked control. Yoked pups received the corresponding reinforcer only when the postnatal paired pup touched the sensor key. A 15-min conditioning session was followed an hour later by a 15-min extinction trial. During extinction session, reinforcer was not available. Remaining manipulations and contextual conditions were identical to the ones presented during training.

2.4. Experimental design and data analysis

The present study was defined by: the prenatal treatment received during GDs 17-20 (Paired, Long Delay and Water treatments); the nature of the milk given as a reinforcer at PD1 (Milk or Cineole-flavored milk); and the contingency between emission of a target behavior and the delivery of the corresponding milk solution (Paired and Yoked animals). Neonates were tested in two sessions, each session lasted 15 minutes. During the second session the reinforcer solution was

withheld.

The Analysis of Variance (ANOVA) assumes that each observation of a between-factor design must be completely independent. This is not the case for the data derived from the postnatal paired and yoked conditions. Consequently, whenever conditioning treatment (paired or yoked) was included in an ANOVA it was regarded as a within-measure factor. As a function of these considerations, data was analyzed through a four-way mixed analysis of variance (prenatal treatment x reinforcer solution x learning condition x session). The loci of significant main effects or interactions were further analyzed with Fisher's LSD test (with an alpha value set at .05). A total of 12 different groups composed the present study. Each group was integrated by 8 to 10 individuals.

3. Results

A one way ANOVA with Prenatal Treatment as independent factor was carried to evaluate the percentage of maternal body weight gains (% MBWG) from GDs 17-20. This index was calculated as follows: [(MBW at GD20 - MBW at GD17)/ MBW at GD17 *100]. Prenatal Treatment exerted a significant main effect upon this dependent measure [F (2, 25) =3.98; $p< .05$]. Post-hoc tests revealed that dams from the Long-Delay group had lower levels of weight increments than Water-treated dams ($p< .05$). In addition, no significant differences were found between Paired treated dams and the remaining control conditions.

When comparing the number of pups successfully delivered per litter, maternal manipulations did not produce any apparent detrimental effect (F(2, 25) =.83; $p= .44$). Means and S.D. for each particular group were as follow: Paired dams: 12.1 ± 2.26; Long-Delay dams: 13.3 ± 2.17 and; Water dams: 12 ± 3.2. To evaluate differences in pups' weight at PD 1 we averaged data within each litter. A one-way ANOVA revealed no differences across prenatal treatments upon this dependent measure (F(2, 25) =1.26; $p= .30$). These results are summarized in Table 1.

A change in the motivational value of cineole-flavored milk as a function of prenatal experience with this cue was analyzed. The corresponding ANOVA showed that total number of contacts with the sensor key was significantly affected by the interaction between prenatal treatment, solution given as a reinforcer and learning condition [F(2, 48) =4,6250; $p< .05$]. When focusing on pups from prenatal Paired group, post-hoc test indicated that when cineole-flavored milk was used as a reinforcer, postnatal Paired pups displayed significantly more contacts with the sensor key than its Yoked siblings ($p< .05$), this group also differed from postnatal Paired pups born from Paired dams and trained with milk as a reinforcer ($p<0.025$).

Table 1.
Percentage of maternal body weight gains during GDs 17-20, number of pups per litter, and averaged litters' weight at PD 1, as a function of prenatal treatments during late gestation (GDs 17-20). Data represent mean group values and standard error of the mean.

Prenatal Treatment	Percentage of maternal body weight gains (GDs17-20)	Number of pups per litter	Litter weight on PD1 (g)
Paired	6.38 ±1.30	12.1 ± 2.26	6.87 ± 0,59
Long-Delay	5.01 ± 1.23	13.3 ± 2.18	6.48 ± 0.48
Water	9.94 ±1.30	12 ± 3.20	6.31 ± 1.12

Additionally, subjects from this particular arrangement of experimental conditions (prenatal treatment: Paired / postnatal treatment: Paired / reinforcer solution: cineole-flavored milk) performed more instrumental responses than postnatal paired pups belonging to the prenatal Long-Delay group that were trained with milk or cineole-flavored milk ($p< .05$ and $p< .05$, respectively). These results are summarized in Figure 1.

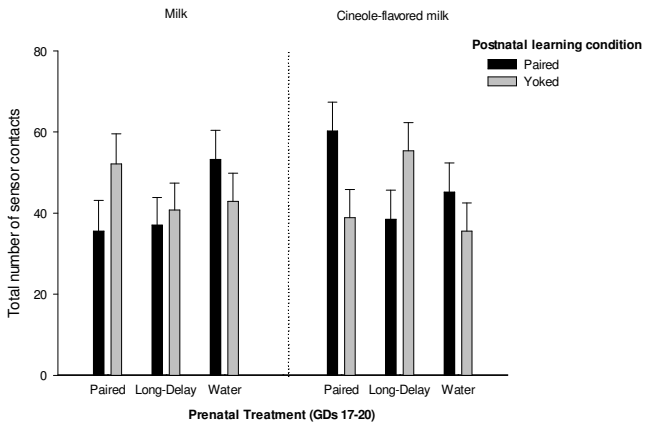

Figure 1. Total number of operant responses (physical contacts with the sensor) as a function of prenatal treatment (Paired, Long-delay and Water), conditioning treatment (Paired or Yoked) and the solution that was intraorally infused (Milk or Cineole-flavored milk). Vertical lines represent standard errors of the means.

Total number of sensor contacts was also different across sessions. The above mentioned ANOVA showed a significant main effect of session [F(1, 48) =31.85; $p< .001$]. Rate of responding was significantly higher when reinforcer solution was available (acquisition session) compared to the level of response when it was withheld

(extinction session). No further interactions were found when analyzing this dependent variable.

A 3-way ANOVA (prenatal treatment x solution x subject) was performed to analyze fluid ingestion during acquisition session. This analysis did not show significant effects or interactions between the factors under analysis.

4. Discussion and Conclusions

Present results confirm and extend the results found by Abate and colleagues (2002). When cineole was prenatally associated with ethanol's post-absorptive effects, pups exhibited heightened operant performance patterns when processing such stimulus, in milk. Present data can be interpreted in the following way: stimuli that are associated with intrauterine ethanol intoxication can modulate the strength of alternative positive reinforcers, in this case milk. It has been observed that prenatal experience with interoceptive properties of ethanol along with chemosensory stimulation heightens later responsiveness to ethanol solution and a sucrose-quinine compound (March, et al., 2009), a solution that mimics ethanol chemosensory properties (Kiefer, 1995; Kiefer, Bice, Orr & Dopp, 1990).

The results of the present experiment are not better explained by arguing a hyperactivity or hyperreactivity effect. These effects have been encountered when high doses of ethanol have been chronically administered during gestation (Abel, 1980; Anandam, Felegi & Stern, 1980; Bond, 1988) or even when low-to moderate ethanol exposure is restricted to late pregnancy (Arias, Molina, Mlewski, Pautassi & Spear, 2008; Chotro & Spear, 1997; Domínguez, et al., 1996). These teratological consequences of the drug imply heightened levels of activity or increased reactivity to environmental stimulation. Both phenomena may in turn, increase the probability of body movements leading to physical contacts with the sensor. In other words, these phenomena may alter the probability of reinforcement due to changes in baseline or sensory-elicited patterns of activity. This argument seems unlikely since neonates from the Long-Delay group did not display the same pattern of instrumental responses. This later group was also exposed to ethanol during GDs 17-20. Additionally, March and collaborators (2009) tested general motor activity, which included stretching; probing; kicking; hindlimb and forelimb movements, and found no evidence of hyperactivity or hypereactivity in 1-day old pups exposed to ethanol during late gestation (GD 17-20).

Using a similar operant conditioning procedure in 1-day old rats, Miranda-Morales et al. (2010) found that the opiate system plays a crucial role in modulating prenatal memories related to ethanol. As found by March et al. (2009), the authors reported that when ethanol solution was utilized as a reinforcer, only paired animals prenatally exposed to ethanol develop a successful operant response. Nevertheless, if the opiate system has been silenced by co-administration of naloxone during GDs 17-20, the effects of a prenatal history of alcohol exposure were no longer observed (Miranda-Morales et al., 2010). Related studies also support the participation of the opiate system in early motivational effects of ethanol. For example, it has been observed that preweanling rats prenatally exposed to ethanol displayed higher levels of ethanol consumption and expressed more ingestive responses in reaction to its taste than non-exposed pups. However, simultaneous prenatal administration of naloxone with ethanol prevented both the increased intake of ethanol and the higher amount of appetitive responses to its taste (Arias & Chotro, 2005a).

The central mechanisms underlying early disposition to consume ethanol are not yet fully understood. Since such early disposition occurs in a period of brain maturation, these ontogenetic shifts may provide a useful tool for understanding the mechanisms underlying alcohol consumption, as well as its positive, anxiolytic and aversive effects (Pautassi et al., 2009).

In summary, the present study brings new evidence indicating that prenatal experiences with alcohol are associated with chemosensory cues present in the fetal milieu. Positive affective attributes of the drug can be processed as early as during the last days of gestation. This experience is sufficient to increase seeking behavior profiles in an instrumental learning task, in rat neonates which have been previously stimulated with chemosensory cues prenatally associated with ethanol intoxication.

The present data adds support to the idea of utilizing operant conditioning techniques in newborn rats. It is possible to affirm that the study of fetal associative memories can be pursued through an operant conditioning paradigm, whenever the target behavior selected and the solution intraorally infused are chosen in accordance with neonatal sensory-motor capabilities.

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