Physiology & Behavior xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Physiology & Behavior

journal homepage: www.elsevier.com/locate/phb

Alcohol odor elicits appetitive facial expressions in human neonates prenatally exposed to the drug

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HIGHLIGHTS

- Newborns, prenatally exposed to alcohol, recognize the drug's odor.
- Alcohol odor recognition is evidenced through appetitive facial expressions.
- Maternal levels of alcohol consumption predict the hedonic response to alcohol odor.

ARTICLE INFO

Article history:
Received 29 July 2014
Received in revised form 19 January 2015
Accepted 19 February 2015
Available online xxxx

Keywords:
Fetal ethanol exposure
Human neonates
Ethanol olfactory stimulation
Facial expressions

ABSTRACT

Specific memories arise during prenatal life as a function of fetal processing of chemosensory stimuli present in the amniotic fluid. Preclinical studies indicate that fetal exposure to alcohol modifies subsequent neonatal and infantile responsiveness towards the sensory attributes of the drug. It has been previously demonstrated that 1–2 day-old human neonates recognize ethanol odor as a function of moderate maternal alcohol consumption during gestation. In the present study 7–14 day-old newborns were assessed in terms of behavioral responsiveness to alcohol's chemosensory attributes or to a novel odor (lemon). These newborns were representative of mothers that exhibited infrequent or frequent alcohol drinking patterns during pregnancy. Different clinical assessments indicated that all newborns did not suffer congenital or genetic diseases and that they were completely healthy when behaviorally evaluated. Testing was defined by brief presentations of ethanol or lemon odorants. Two sequences of olfactory stimulation were employed. One sequence included five initial trials defined by ethanol odor stimulation followed by one trial with lemon and five additional trials with the scent of the drug (EtOH–Lem–EtOH). The alternative sequence (Lem–EtOH–Lem) was primarily defined by lemon olfactory exposure. The dependent variables under analysis were duration and frequency of overall body movements and of facial expressions categorized as aversive or appetitive. The main results of this study were as follows: a) at the end of the testing procedure and independent of the sequence of olfactory stimulation, babies born to frequent drinkers exhibited signs of distress as operationalized through higher durations of aversive facial expressions, b) despite this effect, babies born to frequent drinkers relative to newborns delivered by infrequent drinkers exhibited significantly higher frequencies of appetitive facial responses when primarily stimulated with ethanol odor (EtOH–Lem–EtOH sequence) and c) when merging both samples of babies, a positive and significant correlation was found between overall maternal absolute alcohol consumption per month and frequency of appetitive facial expressions elicited by alcohol odor. In conjunction with previous preclinical research, the present results indicate that human prenatal exposure to the drug that yields no evident teratological effects is sufficient to modify the hedonic value of alcohol's chemosensory attributes.

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1. Introduction

From a neuroethological perspective there is consistent evidence that supports the hypothesis of prenatal programming of postnatal specific appetites [1–3]. Depending on the nature of the appetite under consideration, different mechanisms seem to program the developing brain in terms of acceptance or rejection of a given chemosensory stimulus

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http://dx.doi.org/10.1016/j.physbeh.2015.02.031
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Please cite this article as: A.E. Faas, et al., Alcohol odor elicits appetitive facial expressions in human neonates prenatally exposed to the drug, Physiol Behav (2015), http://dx.doi.org/10.1016/j.physbeh.2015.02.031

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that will later define feeding or searching patterns of a palatable substance. For example, both in rats and humans, extracellular dehydration during pregnancy is sufficient to induce a remarkable increase in the offspring's salt appetite that can persist until adulthood [2]. Fetal chemosensory processing of biological and non-biological cues present in the prenatal milieu also exerts profound postnatal changes in terms of how the neonate reacts to these specific stimuli. Human newborns (2–4 days old) evaluated through olfactory orientation tests detect the smell of the amniotic fluid and of a milk formula with which they were fed. Yet, when both odorants are presented in a two-way preference test, the prenatal substrate elicits more behavioral orienting responses than the postnatal olfactory-related feeding substrate [4]. In terms of non-biological chemosensory cues, it has been reported that maternal consumption of anise flavor during pregnancy results in a significant neonatal preference for this odor [5].

Due to the teratogenic properties of ethanol, the link existing between this drug and early development has been primarily analyzed from the perspective of its deleterious morphological and neurobehavioral consequences [6, 7]. Animal research has also emphasized that prenatal exposure to the drug represents a critical factor when considering subsequent ethanol affinity as operationalized through exacerbated drinking, active behavior in search of the drug coupled with heightened sensitivity to its reinforcing effects as well as those centrally exerted by its main metabolite (acetaldehyde) [8–11]. These phenomena have also been reported in human epidemiological studies even when controlling for other variables known to affect ethanol use and abuse (e.g., genetic predisposition as assessed through family history of alcoholism, gender, co-use of other drugs during pregnancy and different environmental factors) [12–16].

A significant fraction of the studies concerning mechanisms through which early alcohol experience drives later ethanol affinity has been conducted through the use of altricial subprimates such as the rat. In this species, acute alcohol contamination of the amniotic fluid during late gestation is sufficient to recruit fetal processing of the drug's olfactory and gustatory cues. This experience, implying a technical approach that avoids fetal alcohol intoxication, is sufficient to promote neonatal and infantile recognition and acceptance of the odor and taste of the drug [17–19]. Furthermore, during developmental stages analogous to the second and third gestational trimester in humans, rats acquire appetitive associative memories comprising salient olfactory stimuli and ethanol's or acetaldehyde's reinforcing effects [11, 20, 21]. Hence, early familiarity with ethanol's sensory cues or the association of these stimuli with the drug's reinforcing effects has been proposed as two congenital mechanisms that determine or modulate subsequent ethanol preference or drinking patterns [8–11].

Relative to possible ethanol-related learning during human fetal development, the literature is scarce; particularly when considering the hedonic component of acquired memories. The prolific work of Mennella and Beauchamp has shown that during lactation babies are capable of processing small concentrations of ethanol in maternal milk and that this experience enhances alcohol odor preferences (e.g.: [22–25]). As stated, when non-toxic substances (e.g. anise) are incorporated in the maternal diet during pregnancy, the hedonic polarity (appetitive versus aversive responding) changes when the neonate is re-exposed to this odorant [5]. To our knowledge, in terms of maternal alcohol ingestion during pregnancy and its impact upon neonatal responding to ethanol odor, only one study has been conducted [26]. Healthy neonates (24–48 h. old) born to moderate or social drinkers were exposed to the scent of the drug or a novel artificial odorant (lemon). When initially exposed to alcohol odor, these babies exhibited significantly higher levels of motor activity relative to age counterparts delivered by mothers who infrequently drank ethanol during gestation. The overall results of this study suggested that intrauterine ethanol experiences promoted behavioral recognition of the scent of the drug without affecting patterns of responsiveness to a novel olfactory cue. These results are analogous to those reported in subprimates prenatally exposed to subthreshold ethanol doses relative to its teratogenic effects [17–19, 27]. Yet, the dependent variables utilized in the human study did not allow examining possible hedonic responsiveness elicited by ethanol's sensory attributes.

Neonatal facial responsiveness to taste and smell seems to function as social cues to communicate emotions [28]. Newborns show expressions of pleasure or grimacing in response to sweet and bitter tastes, respectively [29–33]. In an early study performed by Rosenstein & Oster [34], neonatal facial expressions in response to basic tastes (sour, salty, sweet and bitter) were analyzed using the Baby Facial Action Coding System (BFACS), an adaptation of the FACS technique elaborated by Ekman and Friesen [35]. The authors reported that 2 hour old babies differentiated sour and bitter as well as sweet from non-sweet solutions. It has also been observed that 3-day-old humans mainly exhibit disgust facial reactions when confronted with an odorant judged as aversive by adult raters [36].

The present study was conducted with mothers and babies representative of the same population where we previously observed differential responsiveness to ethanol odor as a function of prenatal drinking patterns [26]. In this opportunity, babies were tested when the mother brought them to the hospital for their first pediatric examination. Hence, one of the questions under analysis was whether older newborns (7–14 days old) relative to the ones originally tested (1-2 days old) [26] still exhibited differential behavioral responding (overall motor activity and facial expressions) to ethanol odor as a function of frequent or infrequent drinking during gestation. Considering that neonates, innately or through prior learned experiences, exhibit specific facial reactions when confronted with certain chemosensory or nociceptive stimuli, we also assessed expressions qualified as appetitive or aversive [26, 34, 35, 37, 38]. Hence, a major goal in the present study was to analyze whether human alcohol experience in utero shapes early alcohol memories characterized by a particular emotional content. Obviously this goal is not independent from the one regarding temporal persistence of differential responding to the smell of the drug as a function of maternal drinking history. It adds alternative modes of expression of possible memories generated in utero through the analysis of specific gestures characterized by either appetitive or aversive emotional contents. It is important to emphasize that in our original study [26] only gross behavioral reactivity was employed as a dependent measure while there was an absence of a more thorough ethological analysis of particular facial expressions that can reveal emotional-related contents of the memories generated during pregnancy. The results will be presented following an analytical sequence that first scrutinizes the gross overall behavioral reactivity to ethanol odor or a novel olfactory cue (lemon). As will be observed and later discussed in detail, the pattern of gross behavioral responsiveness to the smell of the stimuli here employed does not reveal in 7–14 day-old-babies differential action patterns indicative of specific memories linked with maternal drinking habits. The second major block of results is centered in the analyses of facial expressions, either appetitive or aversive, elicited by the odorants under consideration; an experimental approach which was not utilized in younger babies [26]. A first step in this approach implies an inferential analysis of all the behaviors categorized as either appetitive or aversive as a function of maternal drinking patterns and olfactory cues presented to the babies. Subsequently, each particular gesture, being appetitive or aversive, will be analyzed in detail. Finally, correlational analyses will be utilized to examine the strength of the association existing between monthly consumption of ethanol in each particular mother and the magnitude of emotional responsiveness in their corresponding babies.

2. Material and methods

2.1. Assessment of maternal alcohol consumption

Alcohol consumption patterns during pregnancy were assessed through the use of a brief questionnaire that evaluates frequency,

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quantity and the type of beverages consumed [26, 39]. The questionnaire also evaluates the likelihood of alcohol addiction and alcohol health-related problems and has been utilized in different Latin American populations. Two groups of babies were defined in accordance with the consumption profile exhibited by the mothers during pregnancy (frequent vs. infrequent drinkers). Infrequent drinkers drank no more than 25 cm^3 of absolute ethanol per occasion and exhibited less than 4 drinking episodes per month (mean +/− SEM alcohol absolute ingestion per occasion: 9.07 +/− 1.65 g, n = 27). Frequent drinkers drank four or more times per month and within each drinking episode they consumed at least 25 cm^3 of absolute ethanol (mean +/− SEM alcohol ingestion per occasion: 26.68 +/− 2.23 g, n = 16). The category of frequent drinker is analogous to the operational definition of a social drinker as described by Dufour [ ]. In turn, an infrequent drinker is similar to what Dufour describes as a light drinker or an abstemious. None of the mothers that participated in the present study was diagnosed as alcohol dependent.

2.2. Subjects

A total of 46 neonates composed the original sample. As will be later specified, the data corresponding to 3 babies was discarded because of incompletion of the evaluation procedures. The overall gestational and postnatal ages at test of the remaining 43 babies were as follows: 39.44 +/− 1.26 weeks and 9.89 +/− 1.64 days, mean +/− standard deviation; respectively (age range: 7–14 postnatal days). None of these babies had been diagnosed with a genetic or congenital disease (among other congenital pathologies: Fetal Alcohol Syndrome or Fetal Alcohol Spectrum Disorders) nor did they need intensive care treatment or special medical care after vaginal delivery or during the course of the first 7–14 days of postnatal life. Prior to evaluations, the neonatologist judged the babies to be in optimal health conditions. The study was conducted following the ethical guidelines of the American Psychological Association [41], with the signed consent of the mother and was approved by the Ethical Committee of Hospital Universitario de Maternidad y Neonatología, Universidad Nacional de Córdoba and the Council of Research of the Province of Córdoba, Argentina (CONICOR).

2.3. Apparatus, olfactory stimulation and neonatal behavioral evaluation

Babies were breast- or bottle-fed at least 30–120 min prior to the olfactory test. The baby and the mother were taken to a quiet room. Until commencement of the evaluation procedure the mother held the baby in her arms. The infant was then carefully placed in a hospital crib in a supine position wearing a diaper and a light cotton undershirt. A video camera (Panasonic, Omni Movie VHS NV 2000) placed in front of the baby served to record overall body movements and facial expressions. The baby was left undisturbed for 2 min. All evaluations took place when neonates were awake. Baseline motor activity was then recorded during 1 min.

Two sequences of odorant stimulation derived from the smell of alcohol (EtOH) or lemon were defined. Each sequence consisted of 11 consecutive olfactory trials (trial duration: 60 s). The sequence Lem–EtOH–Lem implied the presentation of lemon during trials 1–5 and 7–11 while alcohol was experienced during trial 6. In the alternative sequence (EtOH–Lem–EtOH) the odors were presented in an opposite manner (trials 1–5 and 7–11: alcohol, trial 6: lemon). This test is the same evaluation procedure that we have previously employed in 1–2 day old babies to evaluate possible patterns of alcohol odor recognition as a function of prior gestational exposure to the drug [26]. The procedure was meant to assess possibilities of progressive habituation to odors as a function of sequential repetition of a given olfactory stimulus, dishabituatory effects when presenting a novel odorant as well as spontaneous recovery of the behavioral response following dishabituation. In the original study, the empirical evidence showed that during initial stimulation with ethanol odor, babies born to frequent drinkers showed heightened levels of overall behavioral responding relative to age counterparts delivered by infrequent drinkers. Similar differences were also encountered when ethanol was again presented after a dishabituatory trial defined by lemon odor presentation. The olfactory assessments were performed as follows. During the first 15 s of each trial babies experienced the specific olfactory stimulus. This stimulation was accrued by placing an alcohol or lemon scented cotton swab approximately 2.5 cm away from the nostrils. A trained and blind experimenter, relative to maternal history of alcohol consumption, followed the movements of the baby in order to keep constant the distance between the swab and the nostrils. The cotton swab contained 0.16 g of 96% alcohol (Porta Hnos.) or a similar amount of an alcohol-free lemon extract (Arcor Company, code 0573). Babies born to frequent and infrequent alcohol-drinking mothers (see below) were quasi-randomly assigned to the olfactory assessments under consideration. The intention was to accrue an equivalent representation of maternal history of alcohol consumption across odor assessments (number of babies per group: EtOH–Lem–EtOH test, infrequent drinkers, n = 16, 9 males and 7 females; frequent drinkers, n = 8, 4 males and 4 females; Lem–EtOH–Lem test, infrequent drinkers, n = 12, 7 males and 5 females, frequent drinkers, n = 7; 2 males and 5 females). Three babies originally assigned to the Lem–EtOH–Lem sequence (2 babies born to infrequent drinkers and 1 baby born to a frequent drinker) cried during 3 consecutive trials. These babies were immediately returned to the mother and as previously stated, the corresponding data was not utilized for further statistical analyses.

Duration of overall body activity (hand, arm, foot, leg and torso movements) as well as of head and facial movements (head rotation, mouthing, suckling, tongue protrusion, gaping, smiling, eye blink, brow and nose wrinkling) served as dependent variables. In addition, and according to prior literature [35, 37, 38], three facial expressions were categorized as appetitive (mouthing/suckling, tongue protrusion and smiling). According to the Facial Action Coding System [35] these expressions correspond to the following action units (AUs): AU 18, AU 37 and AU 12; respectively. Four gestures were considered as aversive (gaping: corresponding to a combination of AUs 25–26–27; eye blinking: AU 7E; brow wrinkling: AU 4E and nose wrinkling: AU 9). Frequency of each specific behavior was calculated for each baby and this parameter also served as a dependent variable. A trained experimenter, blind to the sequence of olfactory stimulation and maternal alcohol drinking patterns, recorded duration of overall activity as well as frequencies of the abovementioned facial gestures via a real time computer based program. Preliminary analysis of the data revealed positive and significant correlations across members of the research team relative to the scoring procedures of the dependent variables under consideration (Pearson's correlation coefficient: all r's > .90).

Evaluations took place during the morning (0900–1200 h). Throughout the entire procedure the mother was present in the testing room. The position of the crib in the room did not allow visual contact between the mother and its child.

3. Results

3.1. Maternal intake patterns during pregnancy

Intake patterns of frequent and infrequent drinkers are depicted in Table 1. A two-way ANOVA was employed to analyze absolute grams of alcohol consumed per occasion during pregnancy. The independent variables under consideration were type of drinker (infrequent versus frequent) and sequence of olfactory stimulation employed in their corresponding babies (EtOH–Lem–EtOH or Lem–EtOH–Lem). As can be expected, due to the selection criteria, mothers considered as frequent drinkers drank significantly more alcohol than those defined as infrequent drinkers [$F(1,39) = 11.53$, $p < 0.01$). Neonatal sequence of olfactory stimulation or the interaction of this variable with type of drinker did not exert significant effects. Hence, the distribution of the babies

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Table 1
Alcohol intake patterns of infrequent and frequent drinkers. Data represent means +/− S.E.M.

Alcohol consumption per occasion during pregnancy (grs of 190 proof alcohol)	Infrequent drinker n = 27 (mean +/− SE)	Frequent drinker n = 16 (mean +/− SE)
Overall alcohol intake	9.07 +/− 1.65 grs	26.68 +/− 2.23 grs
Alcohol derived from wine consumption	4.85 +/− 1.49 grs	15.53 +/− 2.07 grs
Alcohol derived from beer consumption	2.98 +/− 0.97 grs	9.03 +/− 1.93 grs
Alcohol derived from liquor consumption	1.24 +/− 0.69 grs	2.12 +/− 1.14 grs

born to frequent or infrequent drinkers across the olfactory tests was similar. Further two-way ANOVAs indicated that the significant main difference in terms of consumption was also observed when considering wine or beer consumption but not when taking into account liquor intake [$F(1,39) = 15.44$, $p < 0.001$, $F(1,39) = 9.00$, $p < 0.01$ and $F(1,39) = 0.51$, $p > 0.10$; respectively]. As can be observed in Table 1, most of the overall absolute ethanol intake was derived from wine and beer intake.

3.2. Birth-related parameters in babies delivered by mothers characterized by differential alcohol drinking patterns

Neonatal and maternal characteristics corresponding to frequent and infrequent drinking categories were also analyzed by two-way ANOVAs where sequence of olfactory stimulation was also included as an independent factor. These tests did not reveal main significant differences or significant interactions when evaluating weight, height and head circumference at birth, gestational age, postpartum age at test, maternal age and parity. The interaction of the factors under consideration was also found to exert non-significant effects in the case of Apgar Scores. Chi square tests revealed a lack of significant differences in Apgar scores at postpartum times 1 and 5 min. when contrasting the frequency of babies in each group that exhibited scores lower than 7 points. This data has been summarized in Table 2.

3.3. Behavioral evaluations

3.3.1. Neonatal overall general activity

Duration and frequency of overall general activity during baseline recordings were similar across maternal alcohol consumption history, olfactory sequence and sex. Appropriate ANOVAs used to process each dependent variable did not reveal main significant effects of the factors under consideration or the interaction between them (all p's > 0.05). Duration of overall baseline motor activity that preceded olfactory assessments as a function of maternal alcohol history was as follows: babies born to frequent drinkers: 27.78 +/− 3.43 and babies born to infrequent drinkers: 25.15 +/− 2.08 s (values represent mean +/− standard errors). In terms of frequencies the values were as follows: babies born to frequent drinkers: 13.53 +/− 1.29 and babies born to infrequent drinkers: 12.17 +/− 0.73 s.

Table 2
Birth-related parameters in babies delivered by mothers characterized by differential alcohol intake during pregnancy.

Neonatal and maternal characteristics	Frequent drinkers (mean +/− SE)	Infrequent drinkers (mean +/− SE)
Body weight (grs)	3.251 +/− 70	3.451 +/− 110
Height (cm)	48.81 +/− 0.52	49.55 +/− 0.38
Head circumference (cm)	34.22 +/− 0.29	34.75 +/− 0.30
Gestational age (weeks)	39.37 +/− 0.38	39.48 +/− 0.21
Apgar score (1 min)	6.70 +/− 0.32	6.73 +/− 0.15
Apgar score (5 min)	8.59 +/− 0.15	8.67 +/− 0.09
Age at test (days)	9.00 +/− 0.30	10.41 +/− 0.30
Maternal age (years)	25.35 +/− 1.67	24.63 +/− 0.99
Parity	2.06 +/− 0.38	1.78 +/− 0.32

When considering the olfactory testing procedures, a first analytical approach consisted in the use of two-way ANOVAs (maternal drinking history × olfactory sequence) to examine the overall duration or frequency of general body movements throughout the testing procedures. The olfactory factor was defined by the odorant that was more prevalent in a given sequence (ethanol in the case of EtOH–Lem–EtOH or lemon in the alternative sequence). These 2 × 2 ANOVAs did not reveal significant main effects or interactions affecting the overall duration or frequency of whole body movements.

Subsequently, via the use of mixed ANOVAs (MANOVAs) we explicitly incorporated the factor "trials" as a repeated measure. The intention was to analyze in more detail possible specific changes that occur within each olfactory sequence as a function of maternal drinking history. In other words the MANOVAs were defined by "maternal drinking history × sequence of olfactory stimulation × trials as repeated measures". In this case as well as in the case of the remaining inferential analyses performed in the present study, significant main effects or interactions were further analyzed via the use of Bonferroni post-hoc tests. This post-hoc approach was chosen to minimize the probability of Type I errors. When considering of overall motor activity, the MANOVA only indicated a main significant effect of trial [$F(10,390) = 1.88$, $p < 0.05$). Durations progressively increased during the olfactory assessments. Bonferroni's post-hoc comparisons showed that the values attained during trial 10 were significantly higher than those recorded in the first trial ($p < 0.05$). In terms of frequencies, no significant main effects or interactions were detected.

3.3.2. Neonatal facial expressions

As stated, four behaviors were scored as aversive (gaping, eye blinking, brow wrinkling and nose wrinkling) while three behaviors were considered as appetitive (mouthing/suckling, tongue protrusion and smiling). Total duration and frequency of each group of behaviors (aversive or appetitive) were first analyzed via a two-way ANOVA defined by maternal drinking history and the sequence of odor presentation where either ethanol or lemon was the most relevant cues (EtOH–Lem–EtOH or Lem–EtOH–Lem; respectively). Following these analyses, each specific behavior was subjected to a MANOVA that took into account maternal drinking history, olfactory sequence of stimulation and trials.

3.3.3. Baseline recordings

As was the case with the duration and frequency of overall motor behavior, baseline parameters (duration and frequency) of the different facial expressions were unaffected by the factors under consideration.

3.3.4. Aversive responding

When considering aversive responding, durations and frequencies were not significantly affected by the two main factors of the mentioned ANOVA (maternal drinking history or olfactory stimulus) or by the interaction between them (all p's > 0.25).

Systematic inferential analysis based on the type of aversive behaviors indicated that the overall duration of gaping was significantly higher relative to the remaining behaviors ($p < 0.001$). Eye blinking lasted longer than nose wrinkling ($p < 0.05$). These differences can be appreciated in Fig. 1. MANOVAs were then performed for the duration of each particular aversive response. The factors defining these inferential analyses were maternal drinking history, olfactory sequence and trials. When considering gaping, trials as well as the interaction between this variable and maternal drinking history were significant [$F(10,390) = 2.44$ and $F(10,390) = 1.99$, both p's < 0.05; respectively]. Bonferroni post-hoc tests showed that babies born to frequent drinkers, during the end of the testing procedure (trial 9) exhibited significantly longer episodes of gaping relative to the ones exhibited at commencement of testing (trial 2); $p < 0.05$. When considering eye blinking duration was also significantly affected by trial and the interaction

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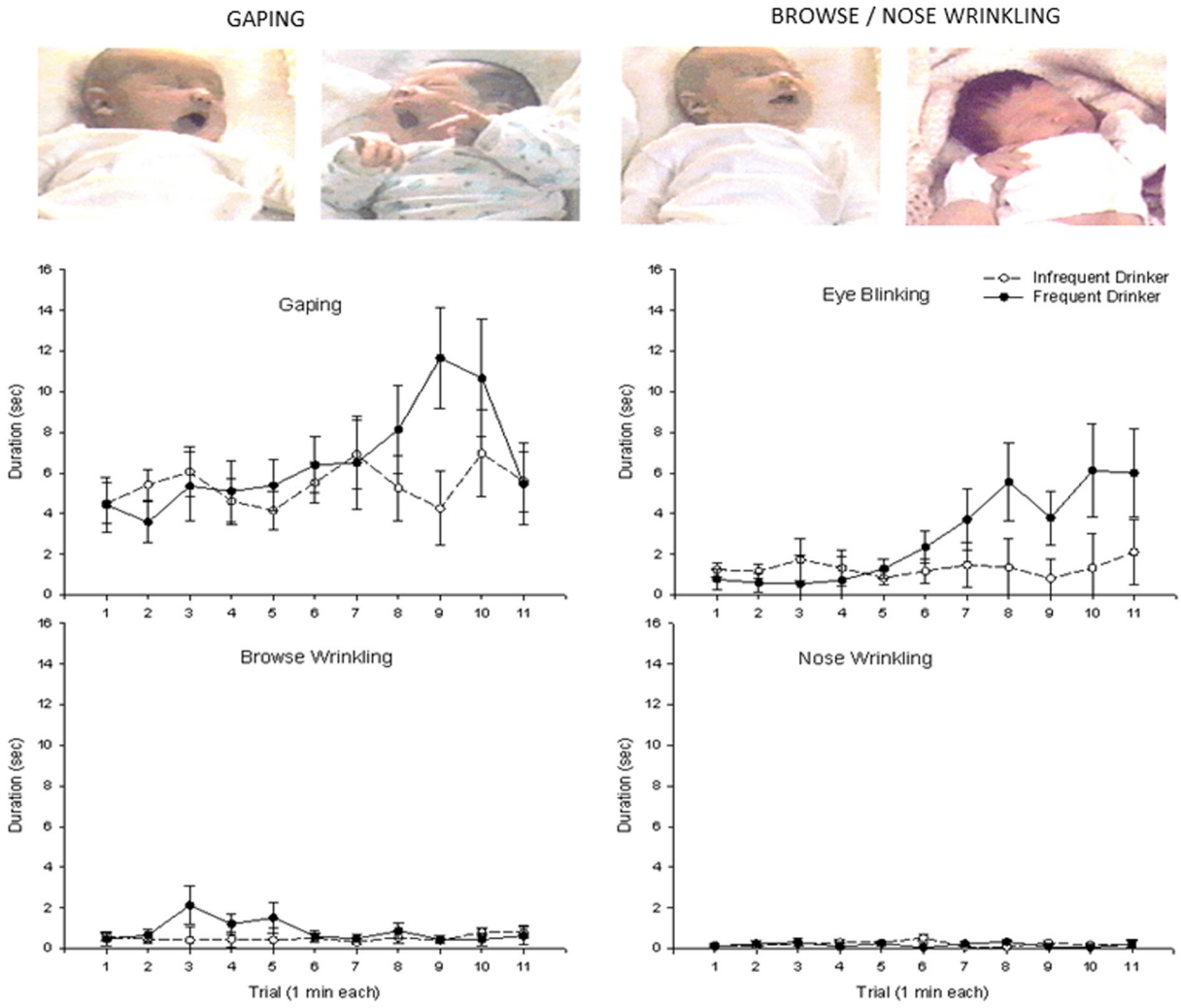

Fig. 1. Neonatal aversive facial expressions: The upper panel presents examples of aversive facial expressions (gaping and browse/nose wrinkling). The bottom panel depicts duration of each aversive facial expression across trials in babies born to mothers that frequently or infrequently drank alcohol during pregnancy. Data has been collapsed across olfactory sequences (EtOH–Lem–EtOH and Lem–EtOH–Lem). In babies born to frequent drinkers, gaping and eye blinking increased during the final trials of both testing sequences (trials 9–10 and trials 10–11; respectively).

comprising this factor and maternal history $F(10,390) = 3244$ and $F(10,390) = 2.64$, both p's < 0.01; respectively]. Post-hoc tests indicated that during the last two trials (10–11), neonates born to frequent drinkers had longer durations of eye blinking when compared with age counterparts (trials 5 and 9) born to infrequent drinkers (p's < 0.05). No significant main effects or interactions were observed in terms of duration of either brow or nose wrinkling. The results concerning each particular aversive expression are illustrated in Fig. 1.

In terms of frequency of aversive responses, preliminary analysis of the data showed a similar profile as the one observed when considering duration; i.e. gaping frequency was significantly higher than the scores recorded for the remaining three behaviors while eye blinking was significantly more frequent than nose wrinkling (all p's < 0.01). Subsequent MANOVAs performed for each specific aversive gesture did not show significant main effects or interactions between the factors under consideration.

3.3.5. Appetitive responding

From a descriptive perspective, within the olfactory sequence where ethanol odor prevailed (EtOH–Lem–EtOH), babies born to frequent drinkers appeared to spend more time showing appetitive facial reactions than newborns born to infrequent drinkers (13.23 +/− 2.10 s and 9.16 +/− 1.16 s; respectively). Yet, the initial two-way ANOVA (maternal drinking patterns × olfactory stimuli) related with the duration of all appetitive behaviors did not show significant main effects or interactions.

In terms of duration, tongue protrusion (liking) was clearly the dependent variable yielding the highest score within the spectrum of appetitive responsiveness (p < 0.0001). Subsequent MANOVAs devoted to the analysis of the duration of each specific appetitive response (tongue protrusion, mouthing/suckling or smiling) also showed no significant main effects or interactions.

The pattern of results related with the frequency of facial expressions markedly differed from what was observed relative to aversive responses or when considering duration of appetitive behaviors. An initial two-way ANOVA (maternal drinking pattern × olfactory stimulus) indicated that the overall frequency of appetitive responses was significantly affected by the interaction between the mentioned factors; $F(1,39) = 7.42$, p < 0.01. Post-hoc tests showed that babies born to frequent drinkers and mainly stimulated with ethanol (EtOH–Lem–EtOH sequence) exhibited significantly higher frequencies of appetitive responses relative to babies born to infrequent drinkers and tested under a similar olfactory sequence (p < 0.025). No differences were encountered when contrasting frequency scores during the olfactory sequence where lemon prevailed. Means +/− SEMs for each group were as follows: Infrequent drinkers — EtOH–Lem–EtOH, 11.20 +/− 1.57; frequent drinkers — EtOH–Lem–EtOH, 19.44 +/− 2.23; infrequent

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drinkers — Lem–EtOH–Lem, 13.59 +/− 1.82; and frequent drinkers — Lem–EtOH–Lem, 10.75 +/− 2.38.

Tongue protrusion was the most frequent appetitive behavior ($p < 0.0001$). The descriptive profile of each appetitive response as a function of test sequence is illustrated in Fig. 2. When considering this specific appetitive behavior, the MANOVA revealed a significant interaction comprising maternal drinking and sequence of odor stimulation, $F(1,39) = 9.29$, $p < 0.005$. No other significant main effects or interactions were observed. Bonferroni post-hoc tests showed that babies born to frequent drinkers and primarily stimulated with ethanol had

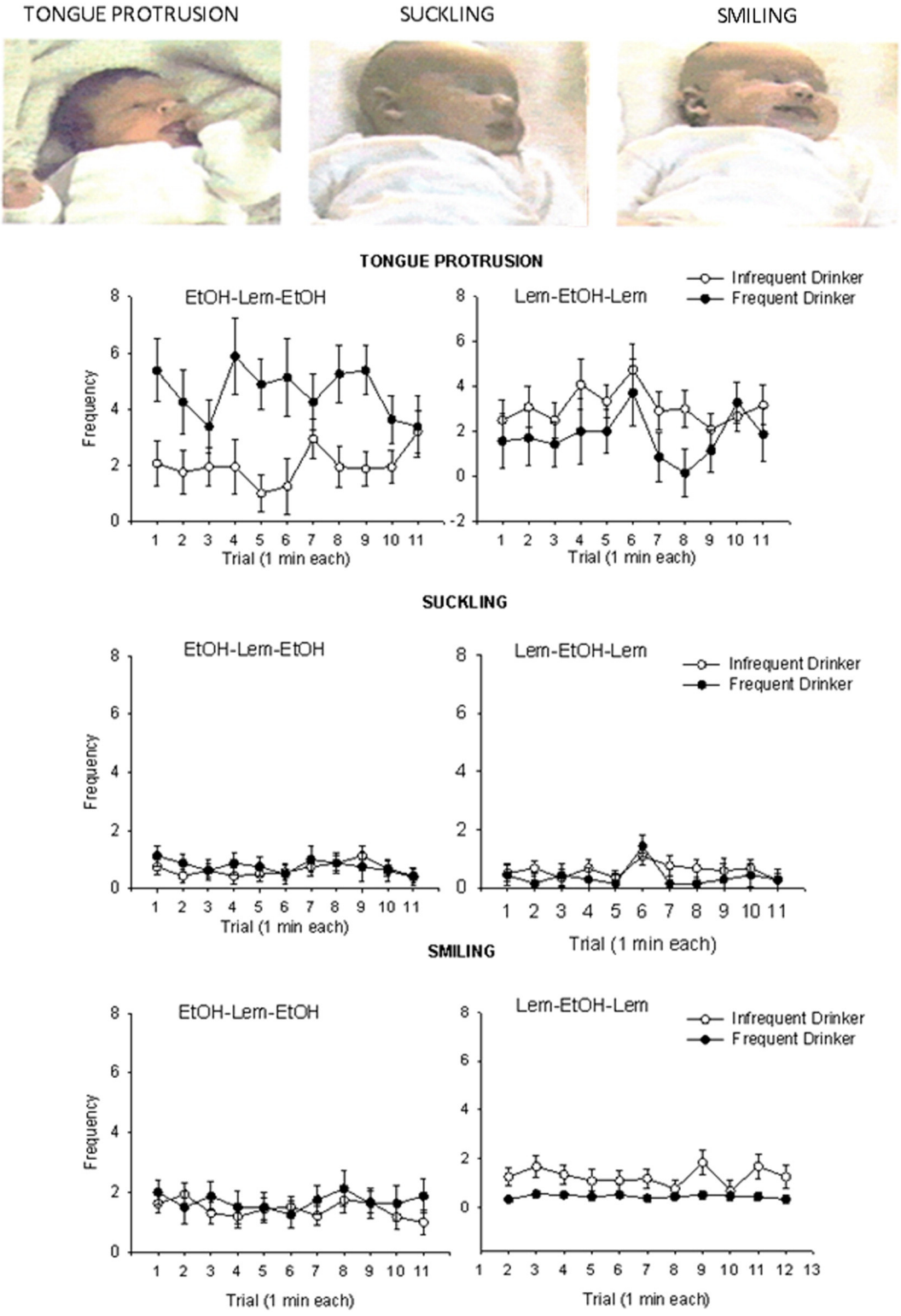

Fig. 2. Neonatal appetitive expressions: The upper panel presents examples of appetitive facial reactions (tongue protrusion, suckling and smiling). Lower panels depict frequency of each specific behavior across testing trials (1–11), olfactory sequence (EtOH–Lem–EtoH or Lem–EtOH–Lem) and maternal drinking habits (infrequent or frequent). Appropriate MANOVAs indicated that in the case of tongue protrusion, babies frequently exposed to ethanol during gestation and tested primarily with ethanol odor (EtOH–Lem–EtOH) exhibited significantly higher levels of this facial expression when compared to newborns delivered by infrequent drinkers.

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significantly higher frequencies relative to those born to infrequent drinkers and tested with a similar olfactory procedure ($p < 0.025$). The overall results in terms of frequencies are illustrated in Fig. 2.

3.3.6. Correlations involving maternal monthly alcohol intake (g/kg) and patterns of neonatal emotional facial expressions

The link existing between maternal drinking patterns and the expression of emotional facial expression was also examined via a correlational approach (Pearson's correlation coefficients with an alpha level set at $p < 0.05$). The variables that were associated were based on the average monthly consumption of each mother (absolute grams of ethanol drank multiplied by the number of drinking episodes) and the overall durations or frequencies of appetitive or aversive responses in each particular test. No significant correlations were observed when considering duration scores. When focusing on facial frequencies, the monthly level of maternal alcohol consumption positively and significantly correlated with appetitive responding when newborns were primarily stimulated with the odor of the drug (EtOH–Lem–EtOH sequence, $r = 0.52$, $df = 22$, $p < 0.05$). A similar significant correlation was observed when just taking into account the most prevalent appetitive expression; i.e. tongue protrusion ($r = 0.42$, $df = 22$, $p < 0.05$). Null effects were obtained when correlating appetitive frequencies and maternal intake patterns when the test was mainly defined by lemon odor. Similarly, aversive responding was not significantly correlated with maternal drinking habits in any of testing conditions (Fig. 3). These non-significant correlations were observed when utilizing overall appetitive or aversive frequencies or when employing each specific behavior.

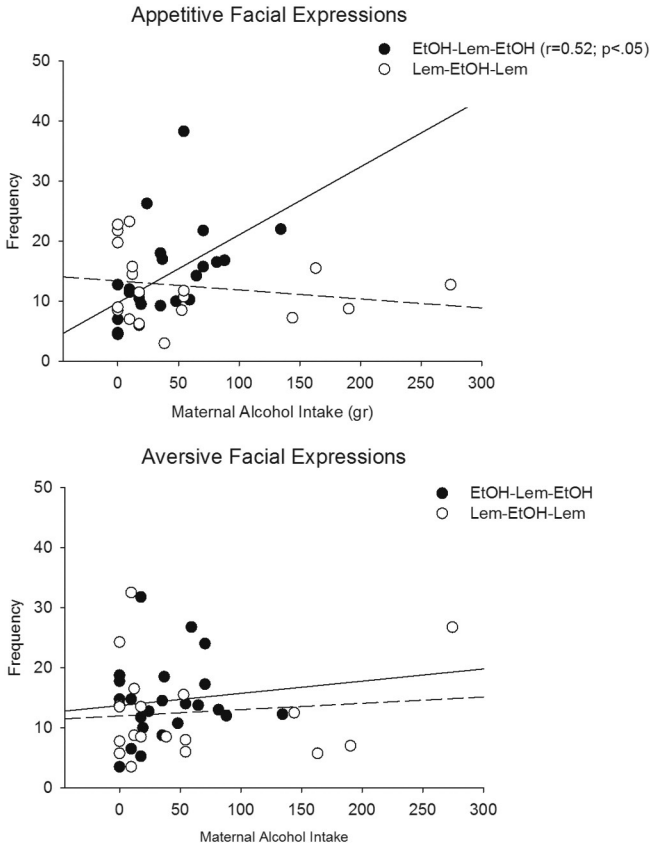

Fig. 3. Correlations between monthly maternal consumption of ethanol and frequency of facial expressions. The upper panel shows frequencies of appetitive facial responses as a function of maternal overall absolute ethanol consumption per month. When the test was mainly defined by ethanol odor (EtOH–Lem–EtOH) a positive and significant correlation was observed ($r = 0.52$, $p < 0.05$). In the case of the testing procedure mainly defined by lemon odor (Lem–EtOH–Lem), the correlation did not achieve significance. The lower panel depicts frequencies of aversive facial responses dependent upon maternal overall monthly ethanol intake. Independent of the nature of the test, no significant correlations were encountered. In both panels black circles refer to individual scores obtained in the EtOH–Lem–EtOH sequence while white circles refer to the alternative olfactory sequence (Lem–EtOH–Lem). Regression lines corresponding to the EtOH–Lem–EtOH sequence are illustrated through solid black lines while those corresponding to the opposite olfactory sequence (Lem–EtOH–Lem) are shown as dashed lines.

4. Discussion

The present study was conducted with 7–14 day-old babies which were vaginally delivered and that according to pre- and perinatal clinical histories and the neonatologists' evaluations were considered as healthy newborns. The mothers and the babies correspond to a similar population as those originally assessed in a previous study aimed at the analysis of possible neonatal (1–2 days old) differential behavioral responsiveness to ethanol odor as a function of moderate or infrequent maternal alcohol drinking during pregnancy [26]. The patterns of ingestion of frequent and infrequent drinkers in the present investigation closely resembled those corresponding to the previous study conducted by Faas et al. [26]. Another similarity between both studies was the use of tests based on ethanol and lemon odorants and the way these stimuli were presented in each olfactory sequence procedure. At both ages overall behavioral activity comprising head and body movements served as a dependent variable but with the older babies employed in the present study we also emphasized the analysis of facial expressions that denote a given emotional component. In newly born babies we observed high levels in the duration of overall body movements as a function of frequent maternal drinking during pregnancy; particularly when ethanol was presented for the first time during the EtOH–Lem–EtOH sequence [26]. During subsequent ethanol trials, this response decreased until reaching baseline levels of activity. Heightened reactivity to the odor of the drug was again detected in babies born to frequent drinkers following the dishabituatory trial where lemon was experienced. Notice that in the present study, there was no clear evidence of possible habituatory or dishabituatory effects when considering either duration or frequency of overall activity in either olfactory procedure. On the contrary, in 7–14 day old babies we observed that the duration of this dependent variable progressively increased as a function of the accumulation of trials and independent of the olfactory characteristics of the test. Apparently, sensitization rather than habituation prevailed at this older age. An explicit comparison of the average overall behavioral duration during testing at 1–2 [26] and 7–14 days of age [present study] shows that the older babies exhibit a 2.0–2.5 fold increase in behavioral duration relative to the younger sample. This age difference may serve to partially explain opposite non-associative learning expressions across ages. Probably, and in order to observe habituatory and dishabituatory effects in older babies it is necessary to increase the levels of exposure to the odorants (e.g. higher duration of odor presentation in each trial, higher number of trials or both) [42]. In other words, maturation of sensory and motor capabilities may pose a problem when intending to assess habituatory processes in older newborns given their relative high levels of behavioral activation and possible heightened attention to other stimuli present within the evaluation context. As suggested by prior literature, babies older than the ones here employed form specific memories of distinctive events that are not only dependent upon certain motor patterns and salient sensory stimuli but also upon redundant information (ambient contextual cues) that are also present during the learning situation [43].

When focusing on the emotional components of facial expressions, three clear differences arose when comparing babies born to infrequent or frequent drinkers. The first significant difference responded to an interaction between the number of trials employed in each olfactory test and the duration of aversive expressions; particularly when considering gaping and eye blinking. Independent from the nature of test and hence, the prevalence of the odor presented in each particular testing procedure (lemon in the Lem–EtOH–Lem sequence and ethanol in the

Please cite this article as: A.E. Faas, et al., Alcohol odor elicits appetitive facial expressions in human neonates prenatally exposed to the drug, Physiol Behav (2015), http://dx.doi.org/10.1016/j.physbeh.2015.02.031

EtOH–Lem–EtOH procedure), babies born to frequent drinkers exhibited higher durations of the two behaviors previously mentioned. This heightened aversive responding was only evident during the last trials of both tests. To a certain extent, this pattern of results seems congruent with an apparent sensitization effect revealed through the analysis of overall body movements. Yet, in the case of aversive responsiveness, the higher durations of gaping and eye blinking were only exhibited by newborns delivered by frequent drinkers. Apparently, the accumulative nature of the olfactory experience generated certain levels of distress in these babies. In addition, it cannot be discarded that, among other factors, the overall duration of the testing procedure, the placement of the infant in a novel context, a continuous supine position of the newborn and the fact of being separated from the mother can generate certain levels of stress. Numerous preclinical and clinical studies indicate that ethanol prenatal exposure results in heightened hypothalamic–pituitary–adrenal responsiveness and behavioral hyperactivity or heightened reactivity to a variety of stressful situations [e.g. 44, 45]. In terms of the effects of moderate ethanol gestational exposure, studies conducted with rhesus monkeys show a marked predisposition towards irritability [46–48]; a behavioral alteration that is also frequently encountered in human babies prenatally exposed to the drug [49, 50]. When merging these considerations, it is possible that disgust reactions in babies born to frequent drinkers can be explained through the predisposition towards irritability conjugated with heightened sensitivity to ambient stressors. Additional studies focusing in a more profound analysis of levels of irritability and patterns of neurohormonal and behavioral responsiveness to relatively mild stressors are needed in order to validate this hypothesis.

The second and third findings of this study relative to facial expressions are related with positive hedonic responsiveness in the test sequence where alcohol odor presentation prevailed (EtOH–Lem–EtOH sequence). In terms of frequencies of appetitive facial expressions, it was clear that sequential stimulation with the odor of the drug elicited higher levels of appetitive responding in babies born to frequent drinkers. Given the difference in the frequencies of the behaviors categorized as appetitive, subsequent inferential processing of the data showed that tongue protrusions (liking) were significantly higher in babies whose mothers were rated as frequent drinkers. This effect was only found when considering the EtOH–Lem–EtOH sequence defined by 10 trials where the smell of the drug was presented and only 1 trial where lemon was inhaled. In the opposite sequence (Lem–EtOH–Lem; 10 trials with lemon and 1 trial with alcohol odor), no differences emerged as a function of maternal drinking habits. The predisposition to exhibit positive hedonic responses to the smell of alcohol due to higher levels of maternal intake during gestation received further support when employing a correlational inferential approach. This approach was defined by merging the data of the two samples of mothers (infrequent and frequent) and calculating for each mother the overall level of absolute ethanol intake per month. These values were then correlated with the number of appetitive or aversive responses exhibited by the offspring in each particular test. When considering aversive facial expressions, non-significant correlations were observed in both olfactory sequences. A similar null result was obtained when evaluating the strength of the association between frequencies of appetitive responses and maternal intake when the test was primarily defined by lemon odor (Lem–EtOH–Lem). When ethanol prevailed (EtOH–Lem–EtOH), the monthly levels of absolute maternal ethanol drinking levels positively and significantly correlated with the frequencies of appetitive facial reactions. This positive correlation was also significant when only considering the most frequently observed appetitive expression (tongue protrusion).

The results in terms of appetitive responding to ethanol odor dependent on heightened levels of maternal consumption of the drug are analogous to what has been reported in prior studies where non-toxic substances were incorporated in the maternal diet during gestation. As stated, neonatal preference for anise odor increases when mothers consume this substance during late gestation [5]. Similarly, human infants exposed to the flavor of carrots in either amniotic fluid or breast milk exhibit fewer aversive facial expressions when re-exposed to this vegetable than babies lacking prior experiences with carrots. In addition, mothers that consumed carrots during late gestation perceived that their infants enjoyed more a carrot-flavored cereal relative to plain cereal [51]. Preclinical research has also shown that brief exposure (10 min) to ethanol in the amniotic fluid (100 mg/ml) in non-intoxicated rat fetuses is sufficient to promote infantile alcohol odor preferences and heightened ingestion of the drug [17]. This brief intrauterine experience with ethanol odor also facilitates appetitive learning and inhibits aversive learning later in life when the olfactory cue is associated with pleasant (sucrose) or unpleasant (peripheral nociception) unconditioned stimuli; respectively [19]. Beyond early familiarization effects with the chemosensory cues of alcohol that appear sufficient to enhance later preference for the drug, other mechanisms should not be discarded when analyzing positive hedonic components of fetal-related ethanol memories. Through the use of animal models related with developmental stages that are equivalent to the second and third gestational trimesters in humans [53, 54], it has been systematically observed that ethanol intoxication as well as acetaldehyde act as appetitive unconditioned stimuli capable of being associated with the drug's sensory properties [8–11, 20, 21, 52]. The acquired associative memory promotes short and long term effects (even during adolescence) upon ethanol affinity [27, 52, 56]. It also generates appetitive responsiveness (e.g. mouthing) to the drug's chemosensory cues [52] and attachment to an artificial nipple under the presence of the odorant that was originally contingent with the state of acute ethanol intoxication [11, 55].

In animals and humans, non-associative and associative memories acquired during fetal, perinatal or early infantile developmental stages can be reactivated or reinstated through re-exposure to relevant components of the original learning situation [57–60]. This is particularly relevant when considering present and past results relative to appetitive fetal learning with ethanol odor. Following the acquisition of alcohol-related fetal memories, different experiences are likely to re-expose the organism to ethanol's chemosensory attributes or the intoxicating effects of the drug. Probably, the most frequent experience is related with the fact that a significant number of mothers that drink during pregnancy continue to do so during breastfeeding [61, 62]. Human babies detect small quantities of the drug in maternal milk (peak levels after maternal consumption of 0.3 g/kg ethanol: 60 mg/dl) [25]. Preclinical studies show that exposure to non-teratogenic alcohol doses during late pregnancy not only promote consumption of milk contaminated with the drug but also that these sequential experiences potentiate later ethanol preference [27]. As stated, the present study was conducted in Argentina. In this and other Latin American countries, during the first weeks of postnatal life, ethanol is frequently employed as an antiseptic agent to avoid infections until the remainder of the umbilical cord is completely removed or as an analgesic drug to alleviate stomach spasms [10, 63]. In these cases, gauze, cloth or cotton soaked with 190 proof alcohol is placed over the infant abdomen. Under these circumstances the baby inhales ethanol and the drug is percutaneously absorbed, leading to relatively high blood alcohol levels. Experimental efforts have been devoted to understand the interactions of pre- and postnatal experiences with the drug. In general terms and even when employing drug treatments capable of generating teratogenic effects, it appears that memories acquired in utero persist during long periods of time and that subsequent brief experiences with alcohol's sensory cues or the drug's intoxicating effects facilitate their expression [27, 56, 64].

The present results, in conjunction with preclinical and epidemiological research indicating heightened alcohol use and abuse derived from intrauterine exposure to even low to moderate ethanol doses, endorse the concept of early life programming of later life disorders [65]. Obviously and as a consequence of this observation, it seems pertinent to still question the existence of safe amounts of prenatal ethanol exposure [8].

Please cite this article as: A.E. Faas, et al., Alcohol odor elicits appetitive facial expressions in human neonates prenatally exposed to the drug, Physiol Behav (2015), http://dx.doi.org/10.1016/j.physbeh.2015.02.031

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Acknowledgments

This work was supported by grants from the Agencia Nacional de Promoción Científica y Tecnológica (FONCyT, PICT 05-254), Consejo de Investigaciones Científicas y Técnicas de la Provincia de Córdoba (PID4059) and Secretaría de Ciencia y Técnica de la Universidad Nacional de Córdoba (PP01), Argentina awarded to Juan C. Molina.

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Please cite this article as: A.E. Faas, et al., Alcohol odor elicits appetitive facial expressions in human neonates prenatally exposed to the drug, Physiol Behav (2015), http://dx.doi.org/10.1016/j.physbeh.2015.02.031