POSTNATAL ALCOHOL EXPOSURE INFLUENCES ADOLESCENT OLFACTORY RESPONSES TO THE DRUG

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Abstract

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Abstract

Human studies illustrate that alcohol exposure while breastfeeding produces a memory of the alcohol scent and modifies behavioral responses to the odor of the drug. The memory and modified behavioral response to alcohol odor suggest that the addictive attributes of alcohol may contribute to patterns of use that increases the risk for alcohol abuse later in life. There is a growing body of evidence that demonstrates prenatal alcohol exposure produces a memory and modified behavioral response to alcohol odor that persists into adolescence, and contributes to alcohol abuse. Given that both postnatal and prenatal alcohol exposure has lasting effects on infants, this study investigated whether rats exposed to alcohol while breastfeeding have a prolonged memory and modified behavioral response to alcohol odor. Long-Evans Hooded rat pups were exposed to alcohol during breastfeeding via the dams' liquid diet. Control animals received ad lib access to an isocaloric, iso-nutritive liquid diet after delivery of their litter up to weaning. To control for effects of malnutrition pair-fed animals were given a control liquid diet equivalent in quantity to the amount their matched animal provided with an alcohol diet consumed the day before. When litters reached adolescence, the behavioral

and neurophysiological responses to alcohol odor in a male and female animal from each litter was examined. Relative to controls, animals exposed to alcohol postnatally displayed an altered breathing pattern response to alcohol odor specifically, and an altered breathing pattern and neurophysiological response to novel odorants. The findings of this study builds on the growing body of research that shows the consequences of postnatal alcohol exposure.

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BACKGROUND

Early Postnatal Alcohol Exposure

Early postnatal alcohol exposure is a concern due to growing evidence that shows the detrimental impact alcohol has on the developing nervous system (Goodlett and Lundahl, 1996; Ikonomidou et al., 1999; Pierce et al., 2010). Throughout time, humans have inadvertently exposed infants to alcohol, risking the child's developing nervous system. In underdeveloped countries, exposure typically occurs via skin absorption as parents and doctors treat the umbilical cord with alcohol to prevent infection and advance the detachment of the cord (Backstrand, 2001; Pepino and Mennella, 2004). Infant alcohol exposure also occurs via breastmilk due to the common use of alcohol consumption by mothers to increase milk production (Backstrand et al., 2001; Pepino and Mennella, 2004).

The use of alcohol to increase a mother's milk production is documented to have occurred or to be occurring in multiple countries including Australia, Argentina, Brazil, Canada, Mexico, the US, and Spain (Mennella, 2002; Nascimento et al., 2013; Marchetta et al., 2012; Pepino and Mennella, 2004). For example, a study in Australia conducted by the National Health Survey reported that alcohol consumption increased in lactating Australian women between 1995 and 2001 from 76.6% to 80.9% (Giglia et al., 2008). These authors also reported that, as of 2006, the Netherlands, New Zealand, and the United Kingdom's departments of health "suggested scheduled occasional drinking of low to moderate amounts while lactating." Though the US Department of Health and Human Services suggests that

no alcohol should be consumed while pregnant or lactating, the Center for Disease Control and Prevention reported that 1 in 13 pregnant women in the US consumed alcohol (Giglia et al., 2008; Marchetta et al., 2012). Researchers also found that women who drank during pregnancy were significantly more likely to drink while lactating, supporting the suggestion that the 1 in 13 women who drink during pregnancy in the US are likely to also consume alcohol while breastfeeding (Giglia et al., 2008; Marchetta et al., 2012). In 2002, Mennella published a study that reported the advice health professionals gave to 410 women in the Delaware Valley of Pennsylvania throughout pregnancy and lactation (Mennella and Beauchamp, 2002). Mennella reported that 25% of women claimed that they were encouraged to consume alcohol by health professionals, 54% received no advice, and 21% were advised by health professionals to refrain from alcohol (Mennella and Beauchamp, 2002). Of the 21% that were advised to refrain from drinking, 23% of the women were encouraged to consume alcohol after they began lactating (Mennella and Beauchamp, 2002). It was reported that 20% of breastfeeding women in Canada consume alcohol due to a dearth of information about the impact alcohol in breastmilk has on infants, and because of myths promoting alcohol use to improve the quality of breastmilk, improve the letdown reflex, and promote infant sleep (Popova et al., 2013).

Cultural studies show that there are varying reasons why women drink alcohol while breastfeeding. In Mexico, it is documented that mothers drunk Pulque, an alcoholic beverage believed to provide the mother with the necessary nutrients that were considered “problem micronutrients” in Mexico, and to increase

their yield of milk (Backstrand et al., 2001). Women in Argentina used the alcoholic beverage, Mate, to increase their milk productivity because of a dearth of information from friends, family, and health professionals about alcohol's effect on lactating mothers and their developing infants (Pepino and Mennella, 2004). Breastfeeding Brazilian women were found to consume alcohol moderately despite "national guidelines recommending that alcohol should not be consumed during pre or post-natal care" (Nascimento et al., 2013). Interestingly, no socioeconomic differences were found between Brazilian women who chose to consume alcohol while breastfeeding relative to women who chose not to consume alcohol while breastfeeding (Nascimento et al., 2013). The Brazilian study stated that alcohol "is present in the daily life of the Brazilian population" and "common during pregnancy or lactation" (Nascimento et al., 2013).

Despite claims of alcohol use by mothers to increase their yield of milk, Mennella and Beauchamp, found that alcoholic drinks do not increase milk production (Mennella and Beauchamp, 1993). Importantly, their study concluded that infants who fed from a mother that consumed one alcoholic drink within 4 hours of breastfeeding drank significantly less milk than infants who fed from a mother that did not consume any alcohol prior to their infant's feeding. The consumption difference Mennella and Beauchamp observed between infants that fed from mothers who consumed alcohol and mothers that had not consumed alcohol prior to breastfeeding was recorded from infants that all spent an equivalent time attached to their mother feeding (Mennella and Beauchamp, 1993). It is not clear why the infants in Mennella and Beauchamp's study had not consumed the

same amount of milk since they were attached to their mother's for an equivalent amount of time, and mothers from both groups described similar sensations for milk let down during and immediately after the infant had detached from their breast (Mennella and Beauchamp, 1993). An experiment that used rat pups demonstrated that suckling-induced prolactin and oxytocin release were impeded by alcohol consumption (Subramanian and Abel, 1988; Subramanian, 1999). Since prolactin release stimulates milk production, Subramanian and Abel's findings suggest that milk production is likely inhibited by alcohol (Subramanian and Abel, 1988).

The ingestion of alcohol via breastfeeding is a concern because of evidence from both human and rodent studies that show alcohol is transferred from the lactating mother to her infant through breastmilk (Guerri and Sanchis, 1986; Mennella and Beauchamp, 1991; Pepino et al., 1998). Alcohol passes into a lactating mother's milk resulting in milk alcohol levels comparable to the mother's blood alcohol levels (Lawton, 1985; Guerri and Sanchis, 1986). Breastmilk with traces of alcohol is, in turn, transferred to a feeding infant, resulting in infant blood alcohol levels that are significantly lower than the blood alcohol levels observed in their mother (Lawton, 1985; Guerri and Sanchis, 1986). A rat study demonstrated that preweanlings learned alcohol's orosensory cues via breastmilk exposure, and recognized alcohol's odor 24 hours after exposure (Molina et al., 1989).

Consequences Of Postnatal Alcohol Exposure

Since the brain is not fully developed at birth and continues to develop throughout infancy and into adolescence, exposure to alcohol continues to put the

child at risk for aberrant neuronal development. Past experiments provided evidence that the exposure of a single dose of alcohol or more through a mother's milk resulted in aberrant behavioral and physiological development in animals (Bannoura et al., 1998; Driscoll et al., 1990; Pautassi et al., 2007; Pierce et al., 2010; Ponce et al., 2004; Silveri and Spear, 1998). A major behavioral modification observed in infants after exposure to alcohol in breastmilk is the infant's sleeping pattern. Mennella and Gerrish reported infants who drank breastmilk contaminated with alcohol experienced a shortened time span spent in active sleep relative to infants who consumed breastmilk not contaminated with alcohol (Mennella and Gerrish, 1998).

Active sleep is a main factor needed to establish declarative and procedural memories (Rasch and Gais, 2006). When memory processing is disrupted, learning is hindered which affects a child's overall development. For example, children exposed to alcohol postnatally are put at risk for impaired motor development, since procedural memory is used to encode motor skills. Pierce et al., looked at the cerebellum given its key role in motor skills (Pierce et al., 2010). A reduction of the vesicular glutamate transporter 2 (VGluT2), known to be present on the olivary climbing fibers that signal to the dendrites of Purkinje cells was found in the cerebellum of infant rats postnatally exposed to alcohol (Pierce et al., 2006; Pierce et al., 2010). Results showed a dose dependent effect of alcohol, higher doses of alcohol lead to greater loss of connections between olivary climbing fibers and Purkinje cells (Pierce et al., 2010). Pierce et al., also explored an age dependent relationship between alcohol exposure and observed damage in the cerebellum

(Pierce et al., 2010). Animals exposed to alcohol at younger ages relative to animals exposed to alcohol at only a few days older experienced a significantly higher diminution of olivary climbing fiber and Purkinje cell connections (Pierce et al., 2010). Evidence showed that postnatal alcohol exposure resulted in a decrease of the synaptic VGluT2 components of olivary climbing fibers and a decrease in quantity of Purkinje cells in the cerebellum (Pierce et al., 2010). Goodlett and Lundahl also recorded a negative relationship between dose of alcohol exposure between postnatal day (PD) 4 and PD9, and loss of Purkinje cells in rodents (Goodlett and Lundahl, 1996). In conjunction with Purkinje cell loss, Goodlett and Lundahl also looked at motor performance in rats between PD42 and 44 that were exposed to alcohol between PD4 and PD9, and found significant motor performance impairment across all dose exposures relative to adolescent rats not postnatally exposed to alcohol (Goodlett and Lundahl, 1996). A study by Ponce et al., supported Goodlett and Lundahl's findings with an infant rat study that observed impaired motor performance in an accelerod task in adolescent rats that experienced repeated exposure to alcohol via breastmilk between PD3 and PD13 (Goodlett and Lundahl, 1996; Ponce et al., 2004). Impaired motor performance was also shown by PD19-20 rats on the rotarod test in a study that exposed rats to alcohol on PD2, 4, 6, and 8 (Ewenczyk et al., 2012). Reduced oligodendrocyte gene expression was also shown in the cerebellum of PD30 rats as a result of alcohol exposure on PD2, 4, 6, and 8 (Ewenczyk et al., 2012). Postnatal alcohol exposure was also shown to reduce oligodendrocyte density in the optic nerve (Vasquez et al., 2011).

Optic nerve hypoplasia (ONH) is another condition suspected to manifest as a result of postnatal alcohol exposure. In pursuit of an explanation for ONH as a result of postnatal alcohol exposure, Vasquez et al., used an experimental design that exposed rats to alcohol throughout gestation and lactation (Vasquez et al., 2011). Their research revealed that animals at postnatal day 7, 14, and 21 exposed to alcohol throughout gestation and breastfeeding shared traits observed in subjects with impaired vision including: decreased oligodendrocyte density, decreased rate of myelination, and an increased rate of optic axon loss (Vasquez et al., 2011). This research determined that early alcohol exposure deteriorated cellular optic development via decreased oligodendrocyte density, decreased rate of myelination, and increased the rate of axon loss in the optic nerve.

Declarative memory defects have also been observed in rats exposed to alcohol postnatally. Alcohol exposure throughout gestation and infancy via breastmilk in rats disrupted social recognition in both male and female adult rodents, relative to controls (Kelly et al., 2009). The early alcohol exposure lengthened the time an adult male and female rodent needed to recognize a rat it had previously interacted with (Kelly et al., 2009).

Gestational and lactational alcohol exposure has long-term effects on the development of anxiety behavior. Maia et al., found that rats exposed to alcohol during gestation and while nursing showed significantly more anxiety-like behavior during adolescence than control animals (Maia et al., 2009). Although long-term developmental effects of alcohol result in anxiogenic behavior, the immediate effects of alcohol are known to be anxiolytic (Koob et al., 1998; Koob et al., 2004; Maia et al.,

2009). The activation of γ-aminobutyric acid (GABA) via alcohol exposure was shown to reduce anxiety and increase alcohol consumption relative to animals who were given a GABA antagonist prior to alcohol exposure (Koob et al., 1998; Koob et al., 2004; Rassnick et al., 1993; Wilson et al., 2004). This anxiolytic effect observed in alcohol exposure is believed to be a key contributor to alcohol's motivational features that contribute to repeated alcohol use (Koob et al., 1998; Koob et al., 2004). These motivational features influence later alcohol consumption patterns of abuse (Koob et al., 1998; Koob et al., 2004).

Postnatal Responsiveness To Alcohol As A Consequence Of Postnatal Exposure

As infants construct memories from orosensory cues, physiological modifications occur in the sensory system that can influence later responses. A memory of alcohol permits infants who were exposed to alcohol prior to PD21 to recognize alcohol in adolescence (Pueta et al., 2008; Pueta et al., 2011; Spear and Molina, 2005). An infant's ability to detect alcohol's chemosensory attributes as a consequence of postnatal alcohol exposure in breastmilk has been demonstrated in studies that observed an infant's response to odors presented to the infant while breastfeeding. Mennella et al., presented evidence of learned responses as a consequence of early postnatal exposure in human infants (Mennella et al., 2001). Mennella's investigation implemented carrot juice or water into the diet of mothers in the first two months of breastfeeding (Mennella et al., 2001). Results demonstrated that infants fed from mothers who consumed carrot juice in the first

two months of breastfeeding later exhibited a preference for carrot flavored food (Mennella et al., 2001).

An earlier study also performed by Mennella reported that human infants postnatally exposed to alcohol later responded to alcohol scented toys with a greater mouthing rate in comparison to infants that were not postnatally exposed to alcohol (Mennella and Beauchamp, 1998). In a similar study, researchers showed that rat pups exposed to alcohol via milk later responded to alcohol odor with an increased mouthing rate, and were more willing to ingest alcohol relative to pups not exposed to alcohol (Hunt et al., 1993). With an infant's ability to recognize alcohol associations are made in connection with the memory of alcohol's chemosensory attributes. Alcohol associations formed in infancy are observed to last into adolescence through evidence of early alcohol exposure increasing self-administration of alcohol in adolescence (Pueta et al., 2008; Pueta et al., 2011; Spear and Molina, 2005).

The Role Of Olfactory Plasticity In Altered Postnatal Flavor Perception

Preferences and associations for alcohol made in infancy are developed through the chemosensory attributes of alcohol, which include the perceptual integration of olfaction, taste, and orosomatosensation. In particular, previously reported olfactory studies demonstrated that early postnatal exposure to salient odors increased ingestion of foods and beverages that smelled like the exposed odor after a prolonged absence from the exposed odor (Coopersmith and Leon, 1984; Coyle et al., 2000). This appears to occur by virtue of an enhanced neural response

at the level of the olfactory bulb, that is, increased activation of the glomeruli was observed in response to the previously exposed odor (Coopersmith and Leon, 1984; Coyle et al., 2000). The enhanced neural response was suggested by Coopersmith and Leon to "increase the intensity of the perceived odor" which in the case of alcohol could modify the perception of alcohol's odor/flavor (Coopersmith and Leon, 1984).

In keeping with the general phenomenon outlined above, animal behavior studies found that infants exposed postnatally to alcohol showed modified ingestion and preference for the drug later in life (Pueta et al., 2008; Pueta et al., 2011; Spear and Molina, 2005). These studies reported that rats at PD15 and PD16 exposed to alcohol via breastmilk on PD3, 5, 7, 9, 11, and 13 drank a greater amount of alcohol relative to animals not postnatally exposed to alcohol (Pueta et al., 2008; Pueta et al., 2011; Spear and Molina, 2005). Lui and Weiss also showed that early exposure to alcohol or to contextual cues associated with alcohol directly increased the risk of possible future alcohol abuse (Lui and Weiss, 2002).

The positive variables present during exposure to alcohol via breastmilk and alcohol's physiological positive reinforcements are factors of the increased alcohol use recorded in adolescent rats exposed to alcohol via breastmilk (Pueta et al., 2008; Pueta et al., 2011; Spear and Molina, 2005). Two of the positive reinforcement variables during exposure to alcohol via breastmilk include the presence of a caregiver and the suckling action performed when infants feed (Hunt et al., 1993; Pueta et al., 2011). Simultaneous to the positive reinforcement variables, physiological responses to alcohol develop an orosensory memory and preference

for alcohol as a result of early alcohol exposure. The physiological reinforcement pathway for the conditioned response to alcohol odor is an increase of dopamine in the nucleus accumbens as a result of the release of endogenous opioids in response to alcohol odor (Gianoulakis, 2004; Miranda-Morales et al., 2010).

As suggested by the postnatal odorant exposure studies of Coopersmith and Leon (e.g., 1984) and the more recent studies of prenatal alcohol exposure (Eade et al., 2010; Schaal et al., 2000; Youngentob and Glendinning, 2009) olfactory plasticity plays prominently in this sensory driven effect. For example, Youngentob et al., demonstrated an altered breathing pattern and olfactory epithelium response to alcohol odor that was predictive of an enhanced odor-mediated behavioral response and alcohol intake (Youngentob et al., 2007a; Youngentob et al., 2007b). In this regard olfactory plasticity occurs throughout the life span making it possible for an individual to strengthen an olfactory response to salient stimuli.

Summary of Experiments

The above summary provides the general theoretical basis for the hypotheses to be tested. Prompted by the data demonstrating that chemosensory plasticity serves to focus an animal's attention to presumed salient stimuli, in the present study, we used behavioral and neurophysiologic methods to test whether postnatal alcohol experience (a) altered the odor-mediated behavioral responsiveness to alcohol and/or a novel odorant and, if so, (b) whether such changes correlated with neurophysiologic alterations in the response of the olfactory epithelium in the adolescent animal.

METHODS

EXPERIMENTAL DESIGN

This experiment utilized 30 Long-Evans Hooded female rats and 30 Long-Evans Hooded male rats that were the progeny of 30 Long-Evans Hooded dams. Litters from each dam were culled to 10 pups on PD3. A male and female from each dam was randomly selected for use in the behavioral and electrophysiological recordings at PD35. Pups delivered from a dam assigned to the alcohol treatment were exposed to alcohol through breastmilk. All animals were kept in a 12-hour light/dark cycle with controlled humidity and temperature at SUNY Upstate Medical University. All tests and procedures for this study were executed in accordance with the Institutional Animal Care and Use Committee guidelines.

TREATMENT OF DAMS

Pregnant dams were randomly assigned to either an alcohol (ET), free choice (FC), or pair fed (PF) treatment. On PD2 ET dams were started on a liquid alcohol diet that they had ad libitum access beginning on the day after delivering their litters. The liquid alcohol diet was started at 2.2% alcohol for two days at which point the diet advanced to a 4.5% alcohol mix for one day, and finally the liquid diet progressed to a 6.7% alcohol mix that provided 35% of the animal's daily calories from alcohol, and was provided up to the day weaning occurred (PD21). The 6.7% alcohol mix mimics a moderate consumption of alcohol (Driscoll et al., 1990; Vavrousek-Jakuba et al., 1991).

FC dams were started on a liquid diet that they had ad libitum to the day after delivering their litters, at PD2. PF dams were started on a liquid diet equivalent in calories and volume to the amount that their paired alcohol dam drank the day before, and therefore initiated their liquid diet 2 days after delivery on PD3. To replace the calories obtained from alcohol in the diets of the FC and PF dams an isonutritive liquid diet with maltose/dextrin was used (L10252, Research Diets, NJ). The average total volume of liquid diets consumed by treatment groups is shown in Figure 1. The caloric restriction of PF dams that imitated the caloric intake of ET dams controlled for effects that may have been caused by insufficient nutritional intake since ET dams voluntarily ingest less of their diet than FC dams, shown in Figure 1. Figure 1, shows that ET dams voluntarily ingested less than half of the total volume of their liquid diets relative to FC dams. Given that the diet volume offered to PF dams was equivalent to what an ET dam drank, Figure 1 shows that PF dams drank on average a comparable total volume of their liquid diet relative to ET dams.

TREATMENT OF PUPS

On the day of weaning ET litters were kept with their mother until 9pm at which the dam, a male, and a female pup were euthanized for the collection of trunk blood. Plasma was then separated and stored in an -80 freezer. Plasma samples were sent to NMS labs in Pennsylvania for gas chromatography analysis of blood alcohol levels (BALs).

Figure 1. Average total volume of liquid diet consumed by dams

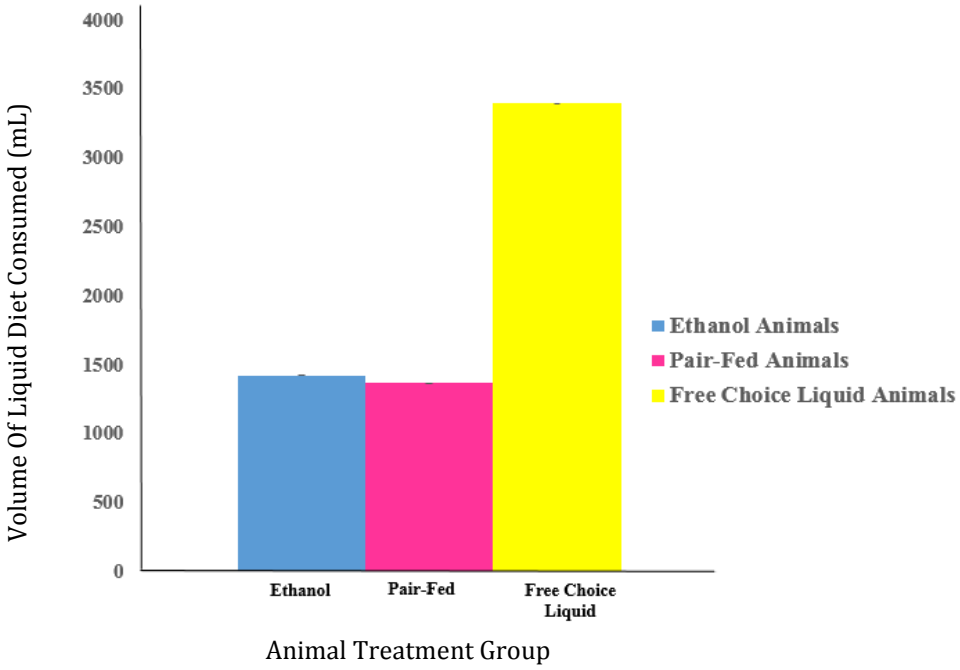

Figure 1. Average total volume of liquid diet consumed by dams. Displays the total volume of liquid diet consumed on average by treatment group (mean ± se) calculated from the data used to calculate the averages.

On the day of testing one female and one male pup from each dam was randomly selected for testing. Each pup selected for testing was weighed prior to testing. Figure 2 shows the average weight of pups by treatment group. The average weight of ET animals at PD35 was 82 grams and the average weight of PF animals at PD35 was 93 grams (Figure 2). FC animals weighed more than ET and PF animals with an average weight of 138 grams at PD35 (Figure 2). All treatments were found to be significantly different from each other. PF and ET animals had a p-value = 0.0172, and the comparison between FC and PF animals as well as FC and ET animals had a p-value < 0.0001 (Figure 2).

BEHAVIOR TEST

Whole body plethysmography was used to collect data representing an animal's breathing pattern response to alcohol odor. A continuous-flow RM-80 Respiration Frequency and Volume Monitoring System (Columbus Instruments, Columbus, Ohio) recorded the fluctuations of an animal's respiration within a chamber as the animal responded to an odorant. The animal was enclosed within a chamber to ensure a constant flow of air in its surroundings, and so that odorants could be presented and expelled from the animal's environment. Our chambers consisted of a Plexiglas cylinder with an internal volume of 1.3L, and a conical input and output to allow rapid entry and removal of each stimulus. An empty testing chamber of equivalent size was included in the set-up as a reference chamber.

The plethysmograph setup utilizes computer directed electronic mass flow

Figure 2. Average animal weights by treatment

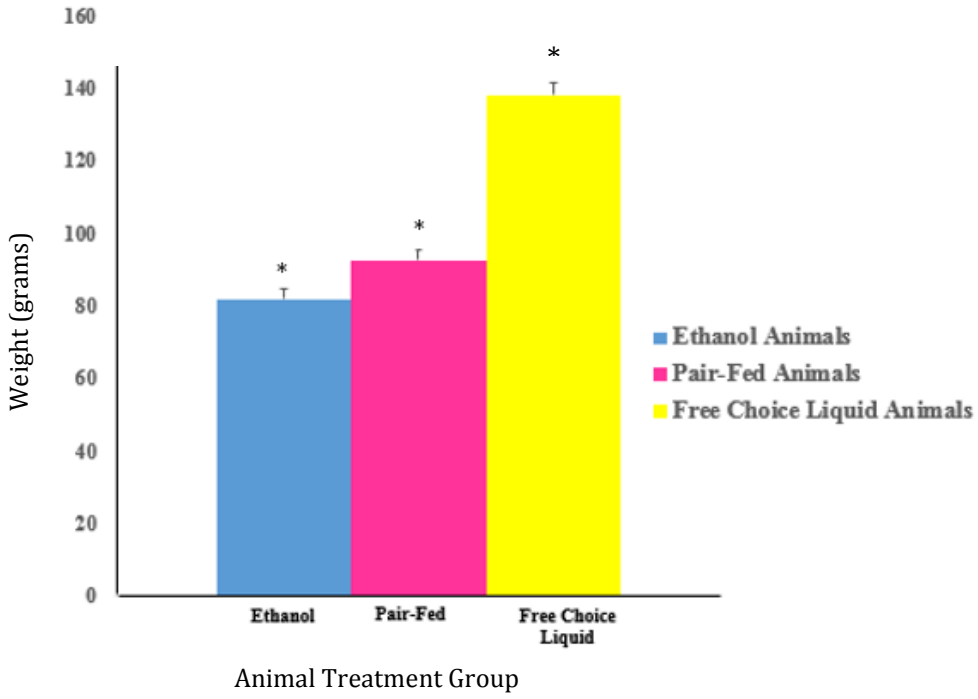

Figure 2. Average animal weights by treatment.
Displays the average weight of animals by treatment group at the time of testing (mean ± se) calculated from the data used to calculate the averages. *=p<0.05; see text for details.

controllers (Teledyne Co, Hampton, Virginia, USA) and a standard flow-dilution olfactometer to generate and present odorants to the animal (Eade and Youngentob, 2009). The computer also collected the data recorded by the RM80 Respiration Frequency and Volume Monitoring System (Youngentob et al., 2007a). Four valves regulating the delivery of stimuli (odorant stimulus valve, prestimulus valve, dummy valve, and back valve) were kept at a constant flow of 400cc/min (Youngentob et al., 2007a). The dummy valve was kept open until the prestimulus valve opened at which point the dummy closed simultaneously with the opening valve (Youngentob et al., 2007a). The prestimulus valve was activated for six seconds after which it closed simultaneously as the stimulus valve opened for six seconds (Youngentob et al., 2007a). This valve activation pattern resulted in a constant total flow delivery to both the test chamber and the reference chamber of 2.0L/min (Youngentob et al., 2007a).

For any given trial animals were enclosed in a chamber within a darkened plexiglas box and first exposed to 40 air trials in order to habituate the animals to the apparatus. After the habituation trials animals were randomly exposed to either air or alcohol odor. The alcohol odor was presented in ascending order of 5 concentrations (.313%, .625%, 1.25%, 2.5%, and 5% of vapor saturation at 20˚C) in 20 trials (10 air trails and 10 alcohol trials) (Middleton et al., 2009; Youngentob et al., 2007a). An exposure occurred via the following pattern: 6sec of air, then a stimulus for 6 seconds, and then an interval for 6 seconds (Youngentob, 2005).

The breathing pattern resulting from an animal's response to every stimulus presentation was recorded and broken down by a computer analysis into 14

respiratory measures. The 14 measures were: sniff frequency; the number of inspiratory and expiratory sniffs; the duration, volume, average flow rate, and peak flow rate of an inspiratory and expiratory sniff; the total inspiratory and expiratory volume; and the total apneic duration (Mantella et al., 2013; Mantella and Youngentob, 2014; Youngentob, 2005). A principal component analysis (PCA) was performed to simplify the 14 single variables into 2 complex variables (factors) that best describe the variance of the data. Then a multivariate linear regression analysis was performed on each PCA Factor to integrate the behavioral responses across all alcohol odorant concentrations for each animal. The effect sizes were then calculated to estimate how much of the total variance of the data could be accounted for by changes in the single fixed variable of a treatment group. Following an animal's completion in the plethysmograph they were euthanized and their nasal epithelium was collected for electrophysiological recordings.

To explore the specificity of the alcohol odor-mediated response, behavioral tests were performed on an additional set of adolescent animals in response to a non-exposure odor. A male and female pup from an additional 6 ET, 6FC, and 6PF dams were utilized to perform an analysis of their breathing pattern response to ethyl acetoacetate odor rather than alcohol odor. Ethyl acetoacetate was chosen as the non-exposure odorant for the behavioral test due to prior research that showed decreased mucosal responses to ethyl acetoacetate as a result of prenatal alcohol exposure (Youngentob et al., 2004). Treatment of these animals was identical to the treatment of the previous animals used for behavioral testing in this study with the exception of the exposure odor in the plethysmograph apparatus. The pups that

were exposed to ethyl acetoacetate in the plethysmograph did not have their nasal epithelium responses to odorants recorded.

NASAL EPITHELIUM RECORDINGS

To collect olfactory epithelium, nasal dissections that split the right nasal cavity in half exposing the olfactory epithelium of the septum and turbinates were performed after each animal completed their behavior test. The analysis order of septums and turbinates as well as male and female animals were randomized. Tissue samples were first placed in a voltage sensitive dye (di-4-ANEPPS) solution and then rinsed in saline before being enclosed in a chamber under a camera with an odorant input and vacuum connection at 200cc/min (Eade et al., 2009). Olfactory epitheliums were oriented in a position within the chamber so that odorants were pulled across the olfactory epithelium from the external naris to the nasopharynx (Eade et al., 2009; Mantella et al., 2013).

Six odorants (0.8% amyl acetate, 0.8% propyl acetate, 4% heptanal, 30% carvone, 33% alcohol, and 45A% ethyl acetoacetate of a vapor saturation at 23˚C) were presented to each tissue sample one at a time in random orders. The 0.8% amyl acetate was used as a standard to correct for tissue responsiveness over time and was therefore exposed to each tissue sample at the start and end of each tissue exposure (Youngentob et al., 1995). The other four non-exposure odorants exposed to tissues during electrophysiological recordings (0.8% propyl acetate, 4% heptanal, 30% carvone, and 45A% ethyl acetoacetate) were used because they previously were shown to produce different olfactory epithelium activity patterns in the rat

that are changed as a result of prominent experiences (Youngentob et al., 1995; Youngentob and Kent, 1995). Tissue responses to odors were recorded using a Sony XCD-V50 1/3" CCD FireWire.B Monochrome camera imaging the tissues on a 640 x 480 pixel array with a depth of 14bits (Eade et al., 2009; Mantella et al., 2013).

Responses from olfactory epithelium were compiled from each pixel of the resultant camera array and each stimulus. Given that previous research show that unique odorants create unique spatial patterns across the olfactory epithelium that initiate odor identification the dissimilarity of spatial activity patterns was measured across maternal feeding experiences (Youngentob et al., 1995; Youngentob and Kent, 1995). Response magnitude and temporal responses were measured to represent spatial patterns (Kent et al., 1995; Mantella et al., 2013; Youngentob et al., 1995; Youngentob and Kent, 1995). The olfactory epithelium's average and peak response heights are representative of the response magnitude. Temporal responses of olfactory epithelium were measured via start, peak, and end times of responses.

Each odorants resultant turbinate and septum responses were combined. Pixel by pixel equilibrated peak responses of individual odors were subtracted from the equilibrated response to the standard control odor (Kent et al., 1995; Mantella et al., 2013; Youngentob et al., 1995; Youngentob and Kent, 1995). Spatial pattern recordings underwent several steps prior to final presentation. First, each pixel of the array was summed. Then, the summed data was divided by the number of pixels in an array. Third, the peak height of the spatially averaged response was

determined. An analysis of variance (ANOVA) was performed on all electrophysiological data.

RESULTS

Prior experiments observed an altered behavioral response to alcohol odor at adolescence in animals exposed to alcohol in-utero relative to FC animals (Eade et al., 2009; Eade et al., 2010). Previous research also reported that the altered behavioral response observed in adolescent animals exposed to alcohol in-utero is associated with a less aversive experience to alcohol and an increased consumption of alcohol in adolescence (Glendinning et al., 2012; Youngentob and Glendinning, 2009). Given the evidence that an altered behavioral response to alcohol odor occurs in adolescence after in-utero alcohol exposure, the behavioral and neurophysiological response to alcohol odor was investigated in PD35 rats that were exposed to alcohol postnatally.

Blood Alcohol Levels Of Dams And Pups On The Day Of Weaning

The BAL analysis of dams resulted in an average BAL of 290mg/dL with a range of 78-433mg/dL (Figure3). The wide range of BALs obtained from dams is possibly due to the sedative effect of alcohol, consequently effecting waking times of the dams and therefore the consumption times and amounts of dams and pups. Previous studies show that pregnant dams given the same alcohol diet given in this study resulted in peak BALs around 150 mg/dL (Miller, 1992; Youngentob et al.,

Figure 3. Blood alcohol levels of dams

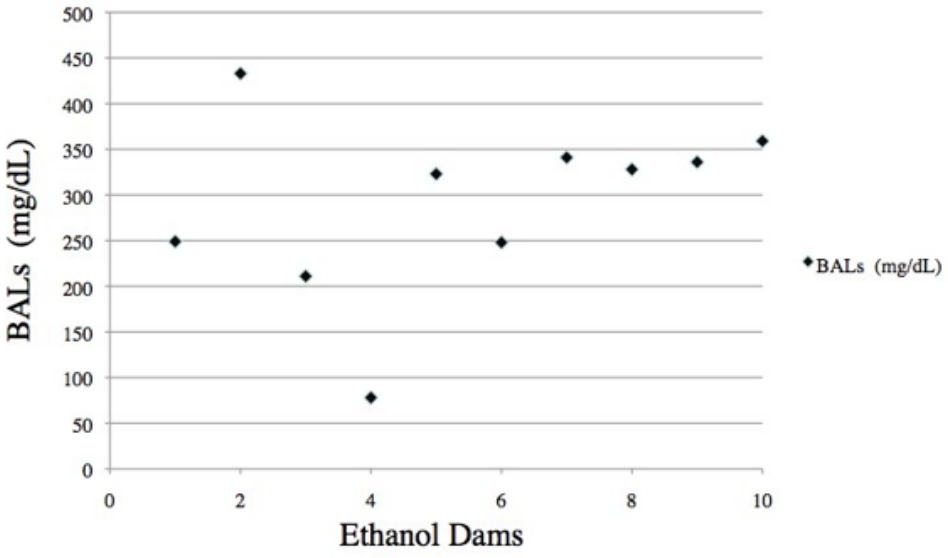

Figure 3. Blood alcohol levels of dams.
Displays the blood alcohol levels of dams at time of weaning. Samples were collected and sent to NMS labs for gas chromatography to determine reported BALs.

2007a). One explanation for our observed higher BALs is malnutrition. To meet the demands of their feeding litters, FC dams consumed on average a total volume of 3,398mLs ± 90mLs of the liquid diet while breastfeeding relative to ET dams that only consumed on average 1,422 ± 38mLs of their liquid diet from the PD2 through weaning (Figure 1).

Based on historical data from gestational alcohol exposure studies, FC dams on average drank 189mLs more of their liquid diets than ET dams for the 10 days gestational exposure dams were on the full strength alcohol liquid diet. In the current study FC dams on average drank 1,414mLs more than ET dams for the first 10 days that ET dams were on the full strength alcohol liquid diet. The large difference in total volume of liquid diet consumed between the FC and ET dams in the current study relative to gestational alcohol exposure studies suggest that ET dams did not imbibe as much nutrients as naturally needed by dams while feeding a litter, and were malnutritioned. Given that "zinc is a cofactor for alcohol dehydrogenase (ADH), the ethanol-metabolizing enzyme" and malnutrition was reported to decrease serum zinc levels it is suggested that ET dams had a decreased serum ADH level that increased their BALs relative to pregnant dams that consume alcohol and are not under the strain of feeding a litter (Murillo-Fuentes et al., 2007; Seyoum and Persaud, 2000).

The BALs in pups were undetected by gas chromatography analysis. The undetected levels of alcohol in pups may be due to very low alcohol levels in the pups as a result of the time samples were collected. Given that rats are nocturnal the 9pm sample collection time could have been prior to the pups feeding after

waking. Confirmation that the ET pups were exposed to alcohol was behaviorally observed while caring for the animals. Several observations that support that the ET pups were exposed to alcohol via their mother's breastmilk include lack of movement when PF and FC pups were very active, decreased alertness relative to PF and FC pups (didn't look around or sniff when cages were handled), and were most likely to be found asleep when PF and FC pups were awake.

Analysis Of Reflexive Sniffing Responses To Alcohol Odor

To test whether or not infant pups exposed to alcohol experienced an altered breathing pattern response to alcohol odor presented during adolescence, the animal's breathing pattern response was recorded while the animal was being exposed to different concentrations of alcohol odor. A computer analysis broke down the breathing pattern responses from each animal for each alcohol concentration presented to the animal. The computer evaluation broke the breathing patterns into the following 14 respiratory variables: sniff frequency; the number of inspiratory and expiratory sniffs; the duration, volume, average flow rate, and peak flow rate of an inspiratory and expiratory sniff; the total inspiratory and expiratory volume; and the total apneic duration (Mantella et al., 2013; Mantella and Youngentob, 2014; Youngentob et al., 2012). Based on the testing paradigm, each animal initially contributed a 14 x 5 response matrix (fourteen measures of reflexive sniffing responses for each of the five concentrations of alcohol odor) to the total data. In order to reduce the high dimensionality of the data, we used PCA to reduce the fourteen measures to *N* orthogonal response vectors (namely, Factors of the PCA

analyses). These Factors, in turn, allowed us to evaluate how the data varied collectively based on variance. In short, the PCA analysis reduced each of the animals' individual 14 x 5 response matrix to an *N* x 5 data matrix (*N* PCA Factor values for each concentration). Historically, two Factors account for essentially all the variance.

To create a behavioral Index for each rat that incorporated the behavioral responses across all concentrations of alcohol odorant tested, multivariate linear regression analyses were performed on each PCA Factor. For each regression analysis the five behavioral response values (one at each concentration) served as the dependent variables and postnatal treatment as the independent variable. The regression results for each PCA Factor provided estimates of the coefficients for each concentration of odorant plus a constant. The behavioral "Index" value calculated from each PCA Factor for an individual animal was the sum of the constant from the regression analysis, plus the corresponding PCA value at each concentration of alcohol odor multiplied by the estimated coefficient. This approach resulted in a set of coordinates that located each animal in a *2*-dimensional behavioral response space. Moreover, the *2*-dimensional response data were used in later analyses of specific main effects (e.g., Mantella and Youngentob, 2014; Youngentob et al., 2012).

Figure 4A shows the two dimensions of the animals' reflexive sniffing index by treatment. Each data point represents a treatment's mean sniffing index in response to alcohol odor. Figure 4A shows that ET animals experienced an altered

Figure 4. The innate odor-mediated behavioral response to alcohol odor as a consequence of postnatal alcohol exposure

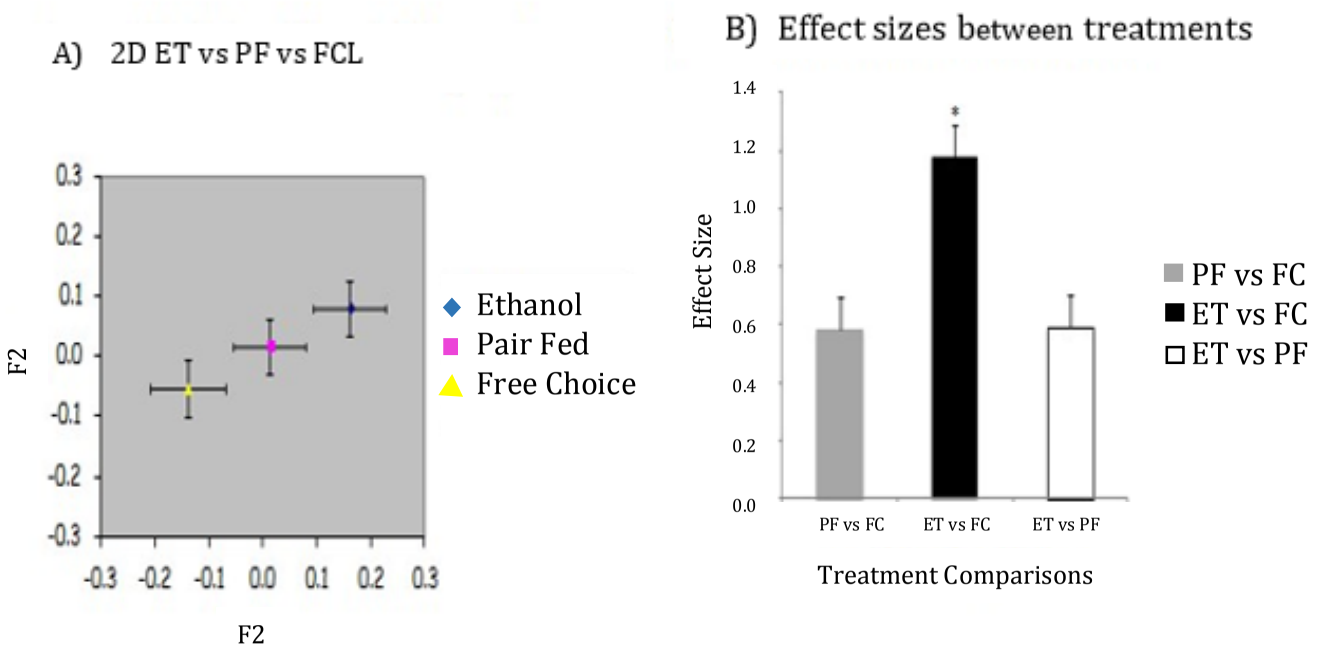

Figure 4. The innate odor-mediated behavioral response to alcohol odor as a consequence of postnatal alcohol exposure. Panel A shows the comparative location of the postnatal alcohol group relative to FC and PF groups in an alcohol odor-mediated behavioral response space. The data points are the adjusted least square mean sniffing indexes (± two-dimensional se) as a function of the three postnatal treatments (Diamond=ET group; Square= PF group; Triangle=FC group). Panel B shows the alcohol response-mediated weighted effect size (mean ± se) calculated from the data illustrated in Panel A. * = $P<0.05$; see text for details

sniffing behavior in response to alcohol odor relative to FC animals. PF animals expressed a sniffing response less altered than ET animals, and a sniffing response more altered relative to FC animals (Figure 4A). These results indicate that lactational exposure to alcohol alters an animal's sniffing response to alcohol odor relative to animals that did not experience postnatal alcohol exposure.

To quantify differences between the three maternal treatment groups a significance test (two-tailed t: $P < 0.05$) was ran on the combined weighted city-block distance of the effect sizes for the two dimensions related to two of the randomized maternal treatments (PF verses FC, ET verses FC, and ET verses PF) (Mantella et al., 2013; Mantella et al., 2014; Youngentob et al., 2012). The city-block distance was based on a weighting scheme that assumed each principal component factor's real effect size was reflected by the excess of its Eigen value above the Kaiser criterion of 1 used in the PCA (Mantella et al., 2013; Mantella et al., 2014; Youngentob et al., 2012). The effect size between PF and FC was .586, a two-tailed t-test found these effect sizes to be insignificant with a P-value of .085 (Figure 4B). A comparison between ET and PF animals revealed an effect size of .597, and an insignificant difference with a P-value of .072 (Figure 4B). The t-test did yield a significant difference between the effect sizes of ET and FC animals, with an effect size of 1.18, and a P-value of .0008 (Figure 4B). The results from Figure 4B indicate that pups exposed to alcohol via lactation experiences have an altered sniffing response to alcohol odor relative to animals not exposed to alcohol in infancy. The insignificant difference between effect sizes of ET and PF animals indicate that infant nutrition may be a factor of the observed behavioral response to alcohol odor.

The PF treatment group was to control for any nutritional effects we observed as a result of ET dams consuming less of their liquid diet relative to FC dams. As shown in Figure 1, FC dams consumed a higher overall volume of their liquid diet relative to ET and PF dams that consumed less of their liquid diet throughout the duration of feeding their litters. The weight of pups at PD35 reflected the overall consumption of dams. As shown in Figure 2, FC animals weighed significantly more than ET and PF animals and PF animals weighed significantly more than ET animals at the time of testing, PD35. The insignificant difference observed between the breathing pattern of ET and PF animals in response to alcohol odor suggest that the decreased nutritional intake of ET and PF dams relative to FC dams (Figure 1) impacted the ET and PF offspring to have an altered breathing pattern response to alcohol odor that is not significantly different. The significantly higher weights of PF animals relative to ET animals at PD35 suggest that the nutritional impact of maternal treatment on pups was different between PF and ET animals (Figure 2).

The difference in dam consumption and decreased weight of animals from dams that consumed less of their liquid diet suggest that ET and PF pups were undernourished relative to FC pups while feeding from their mothers. A rat study showed that neonatal undernourishment resulted in a reduced size of the "olfactory bulb and the individual layers areas" (Frias et al., 2009). The altered olfactory physiology of undernourished neonates suggest that undernourished neonates may also have altered olfactory responses (Frias et al., 2009). The findings of Frias et al., 2009 suggest that the undernourishment of the PF and ET pups in this study could

attribute to the insignificant difference between effect sizes of PF and ET animals breathing pattern in response to alcohol odor.

Analysis Of Reflexive Sniffing Responses To Ethyl Acetoacetate Odor

To address whether or not infant pups exposed to alcohol experienced an altered breathing pattern response to odors other than alcohol in adolescence relative to infant pups that were not exposed to alcohol, the breathing pattern of animals while being exposed to different concentrations of ethyl acetoacetate odor was recorded at PD35. As was done with the breathing pattern data in response to alcohol odor, a computer analysis broke down the breathing pattern responses to each concentration of ethyl acetoacetate odor presented to each animal into 14 respiratory dimensions. The same analyses performed on the 14 respiratory dimensions that resulted for each alcohol concentration each animal was presented with was also performed on the data that resulted from the concentrations of ethyl acetoacetate odor presented to each animal.

Figure 5A shows the two dimensions of the animals' reflexive sniffing index by treatment. Each data point represents a treatment's mean sniffing index in response to ethyl acetoacetate odor. Figure 5A shows that ET animals experienced an altered sniffing behavior in response to ethyl acetoacetate odor relative to FC animals. PF animals experienced a sniffing behavior in response to ethyl acetoacetate odor less altered than ET animals. These results indicate that lactational exposure to alcohol alters an animal's sniffing response to the novel

Figure 5. The innate odor-mediated behavioral response to ethyl acetoacetate odor as a consequence of postnatal alcohol exposure

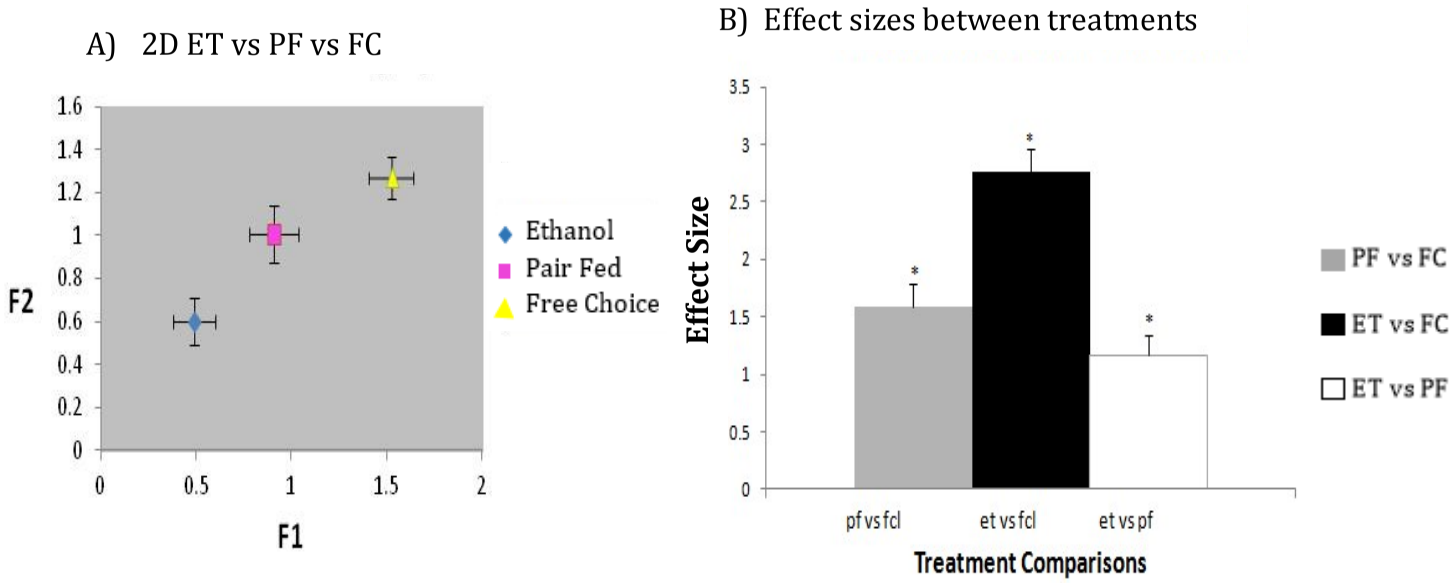

Figure 5. The innate odor-mediated behavioral response to ethyl acetoacetate odor as a consequence of postnatal alcohol exposure. Panel A shows the comparative location of the postnatal alcohol group relative to FC and PF groups in an ethyl acetoacetate odor-mediated behavioral response space. The data points are the adjusted least square mean sniffing indexes (± two-dimensional SE) as a function of the three postnatal treatments (Diamond=ET group; Square= PF group; Triangle=FC group). Panel B shows the alcohol response mediated weighted effect size (mean ± se) calculated from the data illustrated in Panel A. * = $P<0.05$; see text for details

odorant, ethyl acetoacetate, relative to animals that did not experience lactational alcohol exposure.

The effect sizes displayed in Figure 5B show a significant difference between PF and FC animals with a resultant p-value = 0.001, between ET and FC animals with a resultant p-value = 10^{-6}, and between ET and PF animals with a resultant p-value = 0.008. The significant difference observed between ET and PF animals in response to ethyl acetoacetate odor suggest that postnatal alcohol exposure alters behavioral responses to novel odorants. The significant difference observed in behavioral responses to ethyl acetoacetate as a result of maternal treatment in this study was not observed in animals exposed to alcohol prenatally (Youngentob et al., 2007a). The difference observed between animals prenatally and postnatally exposed to alcohol can be attributed to physiological differences in the olfactory system at the time of exposure to alcohol and the method of exposure, fetal absorption verses infant consumption and interactions with their mother.

Analysis Of Olfactory Epithelium Activity In Response To Odors

Peak magnitude of olfactory epithelium in response to odorants was resultant of data recorded for each pixel of an array being summed, the sums being divided by the number of pixels, and the peak height of the spatially averaged response (Mantella et al., 2013). The average peak response of all the animals' olfactory epithelium to five odors (0.8% propyl acetate, 4% heptanal, 30% carvone, 33% alcohol, and 45% ethyl acetoacetate) was compared using an analysis of variance. The resultant P-value ($F [1, 2] = 0.04$; $P = 0.83$) from the test did not yield

any significant differences (Figure 6). Average peak responses to only alcohol (F [1, 2] = 0.33; *P* = 0.56) and to the non-exposure odorants (F [1, 2] = 0.03; *P* = 0.86) also did not show any significant differences dependent on sex or maternal treatment. In previous studies that exposed rats to alcohol prenatally a significant difference of spatial pattern of different odors and response magnitude was observed across olfactory epithelium in response to alcohol odor (Youngentob and Kent, 1995; Youngentob et al., 1995; Youngentob et al., 2007a). The observed difference in olfactory epithelium responses to alcohol odor between adolescent pups exposed to alcohol prenatally and adolescent pups exposed to alcohol postnatally can be attributed to physiological differences in the olfactory system at the time of exposure to alcohol. Prenatal exposure occurs at a time when the olfactory system is still developing and making connections, postnatal exposure occurs at a time when the olfactory system is plastic and whole (Treloar et al., 2010).

Given the expectation from prenatal exposure studies the peak dissimilarity of olfactory epithelium activity patterns of all odors were averaged to increase the power of analysis. Average peak dissimilarity of peak responses was compared across treatment and sex by treatment interaction, shown in Figure 7. An ANOVA was performed on the average dissimilarity of peak responses and reported a significant difference between ET, PF and FC males and females (F [1, 2] = 7.05; *P* = 0.01). Analysis of the average dissimilarity of peak responses for only alcohol odor (F [1, 2] = 0.37; *P* = 0.54) and for the remaining non-exposure odors (F [1, 2] = 7.82; *P* = 0.01) suggest the significance found in pattern dissimilarities to be driven by non-exposure odorants and not alcohol odor. In conclusion the electrophysiology

Figure 6. The consequence of postnatal alcohol exposure on the peak neurophysiological olfactory response

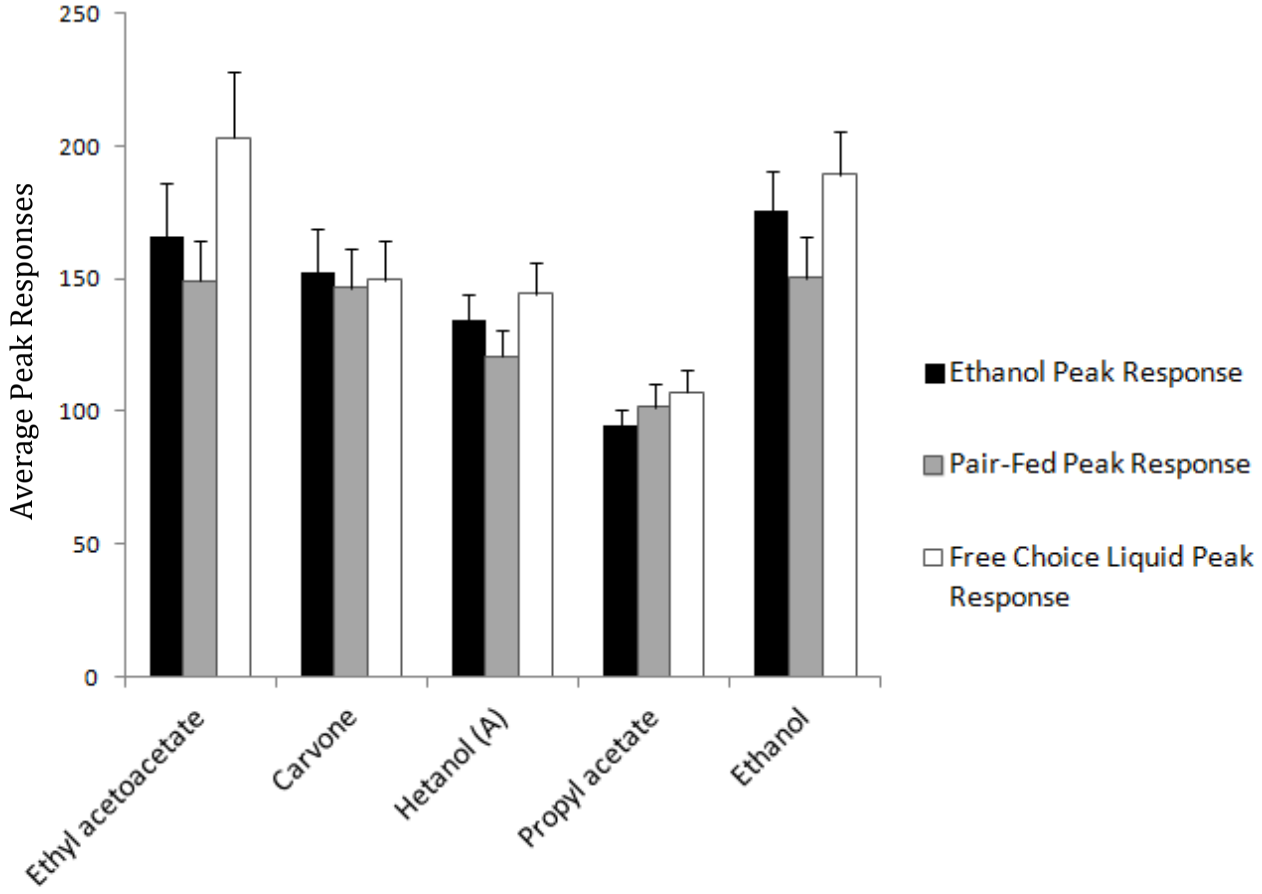

Figure 6. The consequence of postnatal alcohol exposure on the peak neurophysiological olfactory response. Peak mean responses (mean ± SE) of electrophysiological responses to alcohol and novel odorants. Means are representative of treatment groups (ET, PF, or FC).

Figure 7. The average peak dissimilarity of peak neurophysiological olfactory responses compared across treatment and sex as a consequence of postnatal alcohol exposure

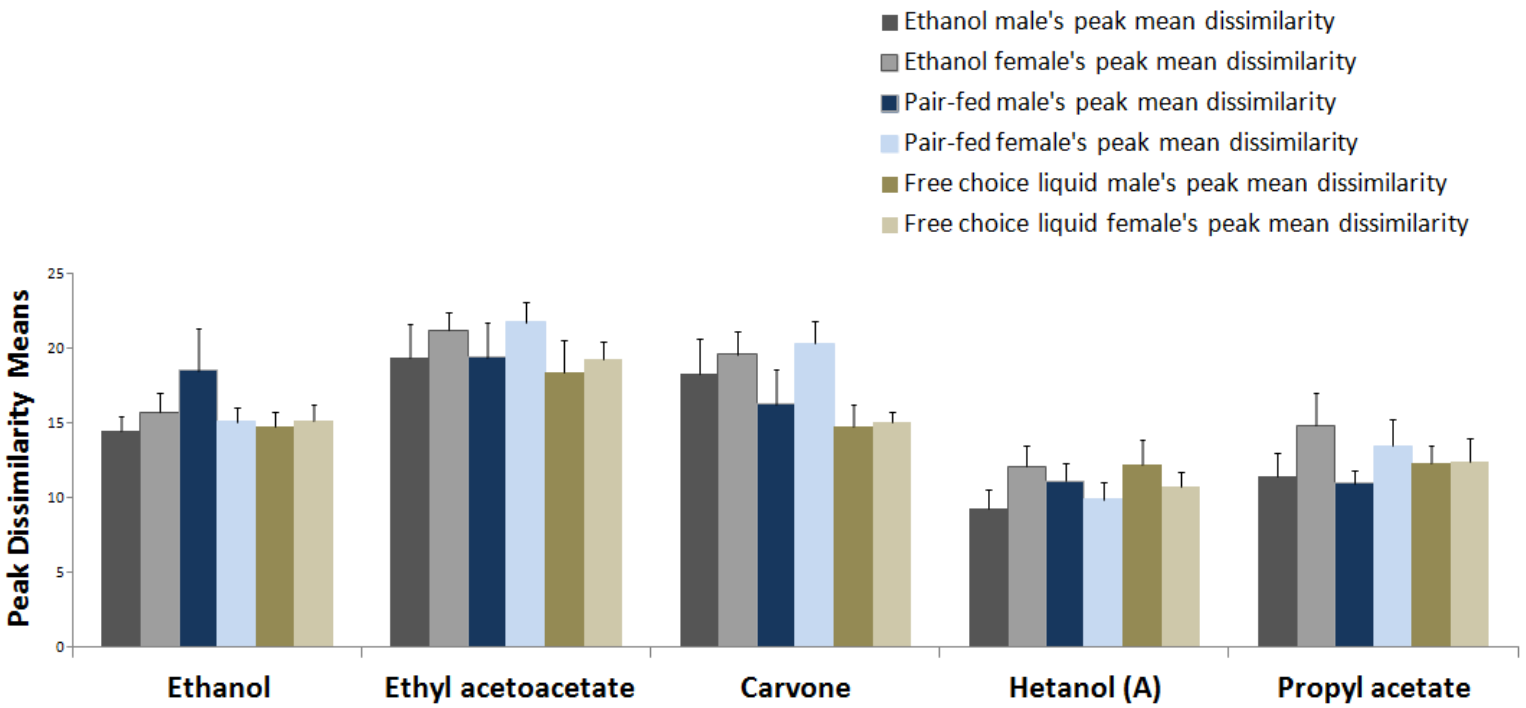

Figure 7. The average peak dissimilarity of peak neurophysiological olfactory responses compared across treatment and sex as a consequence of postnatal alcohol exposure. Peak mean dissimilarities (mean ± se) of electrophysiological responses to alcohol and novel odorants. Means are representative treatment groups (ET, PF, or FC) by sex.

results show that females had a modified olfactory response to non-exposure odors relative to males.

DISCUSSION

Past experiments have documented the ability of an infant to learn chemosensory stimuli to which they are exposed to while nursing, and observed a modified physiological and behavioral response to the learned stimulus (Coopersmith and Leon, 1984; Mennella and Beauchamp, 1991; Mennella and Beauchamp, 1993; Mennella and Beauchamp, 1996; Pepino et al., 1999; Sullivan and Leon, 1986; Sullivan and Wilson, 1991; Woo and Leon, 1991). Like nursing infants, it is well established that fetuses can learn, remember, and react to experienced chemosensory stimuli, suggesting that like fetuses, infants will have modified reactions to learned chemosensory stimuli that persist into adolescence (Eade et al., 2010; Faas et al., 2000; Schaal et al., 2000). Previous work showed that fetal exposure to alcohol altered the neurophysiologic response of the olfactory epithelium to alcohol odor and altered the behavioral response to alcohol odor in adolescence (Youngentob et al., 2007a). The present work indicates that rats exposed to alcohol via breastmilk have a modified behavioral and neurophysiological olfactory response at PD35 relative to adolescent rats not exposed to alcohol postnatally. The conclusion from this study expands on previous work by others examining the manifestation of postnatal alcohol exposure effects

(Mennella and Beauchamp, 1991; Mennella and Beauchamp, 1993; Mennella and Beauchamp, 1996; Pepino et al., 1999).

The behavioral and electrophysiology results from the present study confirmed a behavioral response similarity between prenatal and postnatal alcohol exposure, and introduced an electrophysiology result unique to postnatal alcohol exposure. The behavioral analysis of breathing pattern responses to alcohol confirmed that there was an altered response to alcohol odor in PD35 animals exposed to alcohol postnatally. The results from electrophysiology tests indicated ET female animals experienced an altered neurophysiological response at the level of the olfactory epithelium to odors the rats were not previously exposed to relative to FC and PF animals. These data contradict the findings of prior experiments in which animals were prenatally exposed to alcohol and no neurophysiological differences between treatments or sexes relative to animals not prenatally exposed to alcohol was found (Eade et al., 2010). Since PF animals did not differ in their neurophysiological responses compared to ET animals these results suggest a partial effect from malnutrition in infancy plays a role in the observed altered neurophysiological responses. The significantly lower weights of ET and PF animals at PD35 relative to FC animals at PD35 (Figure 2) and the comparably lower consumption volumes of ET and PF dams relative to FC dams (Figure 1), suggest that the nutritional experience of the ET and PF litters were not as plentiful as the FC litters.

To further investigate the observed altered neurophysiology response to odors the rats were not previously exposed to, the breathing pattern in response to

a novel odorant, ethyl acetoacetate odor, was recorded from an additional 36 rats (18 male and 18 female) at PD35. A male and female from 6 ET dams, 6 PF dams, and 6 FC dams were randomly selected for testing at PD35. The behavioral analysis in response to ethyl acetoacetate odor showed that ET animals had a significantly altered behavioral response to ethyl acetoacetate odor relative to PF and FC animals (Figure 5). The results from this analysis support the primary electrophysiology findings from this study; that is, animals exposed to alcohol in their infant diet experience an altered behavioral and electrophysiology response to novel odorants. The significant difference observed between the breathing pattern responses of ET animals and PF animals in response to ethyl acetoacetate odor suggests that the observed neurophysiological response that was altered in female ET animals is not an effect of malnutrition. This is further evidence that despite undetectable BALs in infancy, ET pups had a unique experience in infancy that altered their response to ethyl acetoacetate odor at PD35. The modified electrophysiology response to novel odorants in ET females was absent in previous studies that exposed animals to alcohol prenatally (Eade et al., 2010; Youngentob et al., 2012). One possibility for the electrophysiological response differences between animals prenatally exposed to alcohol and animals postnatally exposed to alcohol is the stage of brain development at the time of alcohol exposure. For instance, activity patterns in the prefrontal cortex (PFC) are developing at the time of alcohol exposure in animals postnatally exposed to alcohol (Zhang et al., 2004).

Activity of the PFC relays inhibitory signals to the primary olfactory cortex restricting olfactory excitability (Barbas, 2000; Rolls, 2004). Researchers presented

evidence that alcohol reduces the inhibitory signals going from the PFC to the primary olfactory cortex and therefore, heightens olfactory responses (Endevelt-Shapira et al., 2014). Alcohol's effect on the developing PFC signaling is one possibility for the enhanced olfactory response to non-exposure odors observed in the present study. Further experimentation is needed to confirm the physiological cause of the altered olfactory response to non-exposure odors that was observed in ET females from the present study.

Alcohol's impact on the developing PFC offers a possible explanation for the altered response to novel odors observed in animals exposed to alcohol postnatally relative to animals not exposed to alcohol postnatally. However, the activity patterns of the PFC developing at the time of alcohol exposure does not explain why only females displayed an altered electrophysiology response or why the altered response was not observed in response to alcohol odor. One explanation for our observed sex difference comes from studies that reported females perceive odors as more intense than males (Brand and Millot, 2001; Porter and Moore, 1981; Verron and Gaultier, 1976; Wysocki and Gilbert, 1989).

Brand and Millot showed that the more intense experience of odors by females is likely the result of a difference in cognitive style between males and females (Brand and Millot, 2001). Since our experiment did not show a significant difference between males and females in all treatment groups, the data suggest the cognitive style of females that naturally enhance their odor experience may be exaggerated in response to postnatal alcohol exposure. If our observed difference between males and females in electrophysiology responses are driven by cognitive

differences between the sexes, then perhaps cognition is also the reason neither males nor females experienced an altered electrophysiology response to alcohol odor, given that alcohol is toxic.

Despite alcohol's toxic impact on physiology the altered behavioral response to alcohol odor as a result of prenatal exposure is correlated with increased ingestion of alcohol. This suggests that the altered behavioral response in adolescent animals exposed to alcohol postnatally may also lead to increased consumption of alcohol. The memory of alcohol odor displayed in the behavioral data of the present study is likely to be prolonged into adolescence because of animals' inherent use of odors for survival (Hudson, 1999). Infants make associations with odors they learn from their mothers to attach to their mother for feeding, as well as to make preferential choices of foods and liquids as they are weaned from breastmilk and onto a mature diet (Coyle et al., 2000; Allam et al., 2010; Sullivan et al., 2000). The use of learned odors for diet preferences is beneficial to the infant, as normally a mother would consume and smell of odors that were beneficial to their child's health (Hudson, 1999). However, the future health of a child is at risk when an infant forms "good memories of a bad odor" (Sullivan et al., 2000).

A positive association with a negative stimulus often leads to trouble in adolescence as humans and animals are weaned from the guidance of their parents, and begin to make more independent choices based on instinct and memories from their youth. The new-found independence of adolescents is associated with the increased behavior of risk taking. The increased behavior of risk taking increases

the chance a child will start to use drugs and spiral into drug abuse (Spear, 2000). The current study observed behavioral results in postnatally exposed animals that contrast with studies of prenatal alcohol exposure. It is a concern that animals exposed to alcohol via breastmilk may also increase their consumption of alcohol in adolescence, as do prenatally alcohol-exposed animals, and be at a greater risk for alcohol abuse than adolescents that were not exposed to alcohol in infancy (Eade et al., 2010; Youngentob and Glendinning, 2009).

Future studies focused on differences in the olfactory structure and cognitive structures that process olfaction in adolescent males and females, will confirm and advance the mechanistic cause of the electrophysiology difference observed between males and females in the present study. The electrophysiology and behavioral findings of this experiment adds to the evidence that infants exposed to alcohol experience a prolonged effect that lasts into adolescence. This profound effect alcohol has on olfaction in infancy offers insight to the development of alcohol abuse in adolescence.

LIMITATIONS

Given that this was the first postnatal alcohol exposure study of its kind in this lab there were several limitations of the experimental design that should be taken into account for future studies. The BALs of pups would have provided a physiological confirmation that the pups were exposed to alcohol via consumption of alcohol through their mother's milk. To be certain BALs of pups are obtained in

future studies the pattern of activity for ET dams and litters can be closely monitored to determine activity pattern differences between maternal treatments and an appropriate time to sample for pup BALs.

Future study designs should also try to minimize the nutritional differences between the litters from different maternal treatments to minimize the concern of nutrition playing a role in the results. To minimize nutritional difference between maternal treatments future studies can consider shortening the duration dams and litters are exposed to alcohol via a later start date for the liquid diets. Prenatal work in this lab had mothers on the liquid diets for only 15 days from G6-G20 whereas the current study had dams on the liquid diets for 19 days. A four day decrease in postnatal pup exposure to alcohol may decrease the concern that nutrition may impact results. With the mentioned modifications of the postnatal alcohol exposure design BALs should be successfully collected in future studies and the nutritional impact may be reduced for future postnatal alcohol exposure work.

REFERENCES

Abel EL, Dintcheff BA (1978) Effects of prenatal alcohol exposure on growth and development in rats. The Journal Of Pharmacology And Experimental Therapeutics 207: 916-921.

Allam MD, Soussignan R, Patris B, Marlier L, Schaal B (2010) Long-lasting memory for an odor acquired at the mother's breast. Developmental Science 13: 849-863.

Backstrand JR, Allen LH, Martinez E, Pelto GH (2001) Maternal consumption of pulque, a traditional central Mexican alcoholic beverage: relationships to infant growth and development. Public Health Nutr 4: 883-91.

Bannoura MD, Kraebel KS, Spear LP, Spear NE (1998) Effects of preweanling ethanol odor exposure of ethanol preference. Alcohol 15: 213-217.

Barbas H (2000) Complementary roles of prefrontal cortical regions in cognition, memory, and emotion in primates. Adv Neurol 84: 87-110.

Brand G, Millot JL (2001) Sex differences in human olfaction: between evidence and enigma. Q J Exp Psychol B 54: 259-70.

Coopersmith R, Leon M (1984) Enhanced neural response to familiar olfactory cues. Science 24: 849-51.

Coyle S, Arnold HM, Goldberg-Arnold JS, Rubin DC, Hall WG (2000) Olfactory conditioning facilitates diet transition in human infants. Dev Psychobiol 37: 144-52.

Driscoll CD, Streissguth AP, Riley EP (1990) Prenatal alcohol exposure: comparability of effects in humans and animal models. Neurotoxicol Teratol 12: 231-7.

Eade AM, Sheehe PR, Molina JC, Spear NE, Youngentob LM et al. (2009) The consequence of fetal ethanol exposure and adolescent odor re-exposure on the response to ethanol odor In adolescent and adult rats. Behav Brain Funct 5: 3.

Eade AM, Sheehe PR, Youngentob SL (2010) Ontogeny of the enhanced fetal-ethanol-induced behavioral and neurophysiologic olfactory response to ethanol odor. Alcohol Clin Exp Res 34: 206-213.

Endevelt-Shapira Y, Shushan S, Roth Y, Sobel N (2014) Disinhibition of olfaction: human olfactory performance improves following low levels of alcohol. Behav Brain Res 272: 66-74.

Faas AE, Sponton ED, Moya PR, Molina JC (2000) Differential responsiveness to alcohol odor in human neonates: effects of maternal consumption during gestation. Alcohol 22: 7-17.

Frias C, Torrero C, Regalado M, Salas M (2009) Development of mitral cells and olfactory bulb layers in neonatally undernourished rats. Nutr Neurosci 12:96-104.

Gianoulakis C (2004) Endogenous opioids and addiction to alcohol and other drugs of abuse. Curr Top Med Chem 4: 39-50.

Giglia RC, Binns CW (2008) Alcohol, pregnancy and breastfeeding; a comparison of the 1995 and 2001 National Health Survey data. Breastfeed Rev 16: 17-24.

Giglia RC, Binns CW, Alfono HS, Scott JA, Oddy WH (2008) The effect of alcohol intake on breastfeeding duration in Australian women. Acta Paediatr 97: 624-9.

Goodlett CR, Lundahl KR (1996) Temporal determinants of neonatal alcohol induced cerebellar damage and motor performance deficits. Pharmacol Biochem Behav 55: 531-40.

Hudson R (1999) From molecule to mind: the role of experience in shaping olfactory function. J Comp Physiol A 185: 297-304.

Hunt PS, Kraebel KS, Rabine H, Spear LP, Spear NE (1993) Enhanced ethanol intake in preweanling rats following exposure to ethanol in a nursing context. Developmental Psychology 26: 133-153.

Ikonomidou C, Bosch F, Miksa M, Bittiqau P, Vöckler J, et al. (1999) Blockade of NMDA receptors and apoptotic neurodegeneration in the developing brain. Science 283: 70-4.

Kelly SJ, Leggett DC, Cronise K (2009) Sexually dimorphic effects of alcohol exposure during development on the processing of social cues. Alcohol and alcoholism 44: 555-560.

Koob GF (2004) A role for GABA mechanisms in the motivational effects of alcohol. Biochemical Pharmacology 68: 1515-1525.

Koob GF, Roberts AJ, Schulteies G, Parsons LH, Heyser CJ, Hyytia P, Merlo-Pich E, Weiss F (1998) Neurocircuitry targets in ethanol reward and dependence. Alcohol Clin Exp Res. 22:3-9.

Lawton ME (1985) Alcohol in breast milk. Gynecol Oncol 20: 170-6.

Lui X, Weiss F (2002) Reversal of ethanol-seeking behavior by D1 and D2 antagonists in an animal model of relapse: differences in antagonist potency in previously ethanol-dependent vs nondependent rats. J Pharmacol Exp Ther 300: 882-889.

Maia C, Lucena G, Correa P, Serra R, Matos R (2009) Interference of ethanol and methylmercury in the developing central nervous system. Neuro Toxicology 30: 2330.

Mantella NM, Kent PF, Youngentob SL (2013) Fetal nicotine exposure increases preference for nicotine odor in early postnatal and adolescent, but not adult rats. PLoS One 8: e84989.

Mantella NM, Youngentob SL (2014) Prenatal alcohol exposure increases postnatal acceptability of nicotine odor and taste in adolescent rats. PLoS One 9:e102255.

Marchetta CA (2012) Alcohol use and binge drinking among women of childbearing age-United States, 2006-2010. MMWR Mord Mortal Wkly Rep 61: 534.

Mennella JA (1998) Short-term effects of maternal alcohol consumption on lactational performance. Alcohol Clin Exp Res 22: 1389-92.

Mennella JA, Beauchamp GK (1991) The transfer of alcohol to human milk effects on flavor and the infant's behavior. New England Journal of Medicine 325: 981-985.

Mennella JA, Beauchamp GK (1993) Beer, breast feeding, and folklore. J Hum Lact 11: 39-45.

Mennella JA, Beauchamp GK (1996) Developmental changes in the acceptance of protein hydrolysate formula. J Dev Bahav Pediatr 17: 386-91.

Mennella JA, Beauchamp GK (1998) Infants' exploration of scented toys: effects of prior experiences. Chem Senses 23: 11-7.

Mennella JA, Beauchamp GK (2002) Flavor experiences during formula feeding are related to preferences during childhood. Early Hum Dev 68: 71-82.

Mennella JA, Gerrish CJ (1998) Effects of exposure to alcohol in mother's milk on infant sleep. Pediatrics 101: 5.

Mennella JA, Jagnow CP, Beauchamp GK (2001) Prenatal and postnatal flavor learning by human infants. Pediatrics 107: 88.

Miller MW (1992) Effects of alcohol and opiates. Wiley-Liss; New York: Development of the central nervous system.

Miranda-Morales RS, Molina JC, Spear NE, Abate P (2010) Participation of the endogenous opioid system in the acquisition of a prenatal ethanol-related memory: Effects on neonatal and preweanling responsiveness to ethanol. Physiology & Behavior 101: 153-160.

Molina JC, Chotro MG (1989) Acute alcohol intoxication paired with aversive reinforcement ethanol odor as a conditioned reinforcer In rat pups. Behav Neural biol 52: 1-19.

Murillo-Fuentes ML, Artillo R, Ojeda ML, Delgado MJ, Murillo ML, Carreras O (2007) Effects of prenatal or postnatal ethanol consumption on zinc intestinal absorption and excretion in rats. Alcohol & Alcoholism 42: 3-10.

Nascimento A, de Souza A, de Amorim A, Leitao M, Maio R (2013) Alcohol intake in lactating women assisted in a university hospital. Rev Paul Pediatr 31: 198-204.

Pautassi RM, Nizhnikov M, Molina JC, Boehm SL, Spear N (2007) Differential effects of ethanol and midazolam upon the devaluation of an aversive memory in infant rats. Alcohol 41: 421-431.

Pepino MY, Kraebel KS, Lopez MF, Spear NE, Molina JC (1998) Behavioral detection of low concentrations of ethanol in milk in the preweanling rat. Alcohol 15: 337-353.

Pepino MY, Lopez MF, Spear NE, Molina JC (1999) Infant rats respond differently to alcohol after nursing from an alcohol-intoxicated dam.

Pepino MY, Mennella JA (2004) Advice given to women in Argentina about breastfeeding and the use of alcohol. Rev Panam Salu Publica 16: 408-14.

Pierce DR, Cook CC, Hinson JA, Light KE (2006) Are oxidative mechanisms primary in ethanol induced purkinje neuron death of the neonatal rat. Neurosci Lett 29: 130-4.

Pierce DR, Hayer A, Williams DK, Light KE (2010) Developmental alterations in olivary climbing fiber distribution following postnatal ethanol exposure in the rat. Neuroscience 3: 1438-48.

Ponce LF, Pautassi RM, Spear NE, Molina JC (2004) Nursing from an ethanolintoxicated dam induces short- and long-term disruptions in motor performance and enhances later self-administration of the drug. Alcoholism: Clinical and Experimental Research 28: 1039-1049.

Popova S, Lange S, Rehm J (2013) Twenty percent of breastfeeding women in Canada consume alcohol. J Obstet Gynaecol Can 35: 695-696.

Porter RH, Moore JD (1981) Human kin recognition by olfactory cues. Physiol Behav 27: 493-5.

Pueta M, Abate P, Haymal OB, Spear NE, Molina JC (2008) Ethanol exposure during late gestation and nursing in the rat: effects upon maternal care, ethanol metabolism and infantile milk intake. Pharmacol Biochem Behav 91: 21-31.

Pueta M, Rovasio RA, Abate P, Spear NA, Molina JC (2011) Prenatal and postnatal ethanol experiences modulate consumption of the drug in rat pups, without impairment in the granular cell layer of the main olfactory bulb. Physiology & Behavior 102: 63-75.

Rasch BH, Born J, Gais S (2006) Combined blockade of cholinergic receptors shifts the brain stimulus encoding to memory consolidation. J Cogn Neurosci 18: 793-802.

Rassnick S, Pulvirenti L, Koob GF (1993) SDZ-205,152, a novel dopamine receptor agonist, reduces oral ethanol self-administration in rats. Alcohol 10: 127-132

Rolls ET (2004) Convergence of sensory systems in the orbitofrontal cortex in primates and brain design for emotion. Anat Rec A Discov Mol Cell Evol Biol 28: 1212-25.

Schaal B, Marlier L, Soussignan R (2000) Human foetuses learn odours from their pregnant mother's diet. Chem Senses 25: 729-737.

Seyoum G, Persaud TV (1995) Protective influence of zinc against the deleterious effects of ethanol in postimplantation rat embryos in vivo. Experimental and Toxicologic Pathology 47: 75-79.

Silveri MM, Spear LP (1998) Decreased sensitivity to the hypnotic effects of ethanol early in ontogeny. Alcohol Clin Exp Res 22:670-6.

Spear LP (2000) The adolescent brain and age-related behavioral manifestations. Neuroscience and Behavioral Reviews 24: 417-463.

Spear NE, Molina JC (2005) Fetal or infantile exposure to ethanol promotes ethanol ingestion in adolescence and adulthood: a theoretical review. Alcoholism: Clinical and Experimental Research 29: 909-929.

Subramanian MG, Abel EL (1988) Alcohol inhibits suckling-induced prolactin release and milk yield. Alcohol 5: 95-8.

Subramanian MG (1999) Alcohol inhibits suckling induced oxytocin release in the lactating rat. Alcohol 19: 51-55.

Sullivan RM, Landers M, Yeaman B, Wilson DA (2000) Good memories of bad events in infancy. Nature 407: 38-39.

Sullivan RM, Leon M (1986) Early olfactory learning induces an enhanced olfactory bulb response in young rats. Brain Res 392: 278-82.

Sullivan RM, Wilson DA (1991) Neural correlates of conditioned odor avoidance in infant rats. Behavioral Neuroscience 105: 307-312.

Treloar HB, Miller AM, Ray A, Greer CA (2010) "Development of the olfactory system." The Neurobiology of olfaction. Boca Raton: CRC/Taylor & Francis.

Vasquez S, Gallego-Pinazo R, Galbis-Estrada C, Zanon-Moreno V, Garcia-Medina JJ (2011) Combined pre- and postnatal ethanol exposure in rats disturbs the myelination of optic axons. Alcohol and Alcoholism 46: 514-522.

Vavrousek-Jakuba EM, Baker RA, Shoemaker WJ (1991) Effect of ethanol on maternal and offspring characteristics: comparison of three liquid diet formulations fed during gestation. [erratum appears in Alcohol Clin Res 1992 Jun; 16(3):479]. Alcohol Clin Exp Res 15: 129-135.

Verron H, Gaultier C (1976) Olfactory processes and their relational structures. Psychologie Francaise 21: 205-208.

Wilson MA, Burghardt PR, Ford KA, Wilkinson MB, Primeaux SD (2004) Anxiolytic effects of diazepam and ethanol in two behavioral models: comparison of males and females 78: 445-458.

Woo CC, Leon M (1991) Increase in a focal population of juxtaglomerular cells in the olfactory bulb associated with early learning. J Comp Neurol 305: 49-56.

Wysocki CJ, Gilbert AN (1989) National geographic smell survey. Effects of age are heterogenous. Ann N Y Acad Sci 561: 12-28.

Youngentob SL (2005) A method for the rapid automated assessment of olfactory function. Chem Senses 30:219-229.

Youngentob SL, Glendinning JI (2009) Fetal ethanol exposure increases ethanol intake by making it smell and taste better. Proc Natl Acad Sci USA 106: 5359-5364.

Youngentob SL, Kent PF. (1995) Enhancement of odorant-induced mucosal activity patterns in rats trained on an odorant identification task. Brain Res 670:82-88.

Youngentob SL, Kent PF, Mooney SM, Spear NE, Molina JC. (2004) In utero ethanol experience and olfactory plasticity. Alcoholism: Clinical and Experimental Research 28:94A.

Youngentob SL, Kent PF, Sheehe PR, Schwob JE, Tzoumake E (1995) Mucosal inherent activity patterns in the rat: evidence from voltage-sensitive dyes. J Neurophysiol 73: 387-398.

Youngentob SL, Kent PF, Youngentob LM (2012) Gestational naltrexone ameliorates fetal ethanol exposures enhancing effect on the postnatal behavioral and neural response to ethanol. Exp Biol Med (Maywood) 237: 1197-1208.

Youngentob SL, Kent PF, Sheehe PR, Spear NE, Molina JC, Youngentob LM (2007a) Experience-induced fetal plasticity: the effect of gestation ethanol exposure on the behavioral and neurophysiologic olfactory response to ethanol odor in early postnatal and adult rats. Behav Neurosci 121: 1293-1305.

Youngentob SL, Molina JC, Spear NE, Youngentob LM (2007b) The effect of gestational ethanol exposure on voluntary ethanol intake in early postnatal and adult rats. Behav Neurosci 121: 1306-1315.

Youngentob SL, Stern NM, Mozell MM, Leopoid DA, Hornung DE (1986) Effect of airway resistance on perceived odor intensity. Am J Otolaryngol 7: 187-93.

Zhang Z (2004) Maturation of layer V pyramidal neurons in the rat prefrontal cortex: intrinsic properties and synaptic function. J of Neurophysiology 91: 11711182.