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The Interaction of Gestational and Postnatal Ethanol Experience on the Adolescent and Adult Odor-Mediated Responses to Ethanol in Observer and Demonstrator Rats

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Abstract

Background—Gestational ethanol exposure enhances the adolescent reflexive sniffing response to ethanol odor. Postnatal exposures of naïve animals as either an observer (i.e., conspecific) or demonstrator (i.e., intoxicated peer) using a social transmission of food odor preference paradigm also yields enhanced odor-mediated responses. Studies on the interaction of fetal and postnatal exposures using the social transmission paradigm have been limited to the responses of observers. When combined the enhanced response is greater than either form of exposure alone and, in observer females, yields adult persistence. The absence of a male effect is noteworthy given that chemosensory mechanisms are suggested to be an important antecedent factor in the progression of ethanol preference. Observers gain odor information on the breath of the demonstrator through social interaction. Demonstrators experience the pharmacologic properties of ethanol along with retronasal and hematogenic olfaction. Thus, we tested whether augmentation of the fetal-ethanol induced behavioral response with postnatal exposure as a demonstrator differed from that as an observer. We also examined whether re-exposure as a demonstrator yields persistence in both sexes.

Methods—Pregnant dams were fed an ethanol containing or control liquid diet throughout gestation. Progeny received four ethanol or water exposures: one every 48 hours through either intragastric infusion or social interaction with the infused peer beginning on P29. The reflexive behavioral sniffing response to ethanol odor was tested at postnatal (P) day 37 or P90, using whole-body plethysmography.

Results—When tested in either adolescence or adulthood - fetal ethanol exposed adolescent ethanol observers and demonstrators significantly differed in their odor-mediated response to ethanol odor both between themselves and from their respective water controls. Nonetheless, adolescent ethanol re-exposure as a demonstrator, like an observer, enhanced the reflexive sniffing response to ethanol odor at both testing ages by augmenting the known effects of prior fetal ethanol experience. At each age, the magnitude of the enhanced odor response in demonstrators was similar to that of observers. Interestingly, only re-exposure as a demonstrator resulted in persistence of the behavioral response into adulthood in both sexes.

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AUTHORS CONTRIBUTIONS

Both authors were involved in the design of the study, interpretation of the data, and preparation of the manuscript; AME executed the study and analyzed the data. Both authors read and approved the final manuscript.

Conclusions—The method of ethanol re-exposure plays an important role in prolonging the odor-mediated effects of fetal exposure. While ethanol odor specific exposure through social interaction is important, additional factors such as the pairing of retronasal and hematogenic olfaction with ethanol's intoxicating properties appear necessary to achieve persistence in both sexes.

Keywords

Fetal Ethanol Exposure; Olfactory Plasticity; Whole-body Plethysmography; Social Interaction

BACKGROUND

There is strong evidence in the human literature for a cascading pattern of ethanol use that, beginning with fetal exposure (Alati et al., 2006; Baer et al., 1998, 2003; Streissguth, 1998; Yates et al., 1998), results in adolescent (or earlier) intake and ends in long-term patterns of abuse (Streissguth, 1998; Yates et al., 1998). Two fundamental questions arise from these observations. How does fetal exposure alter adolescent ethanol acceptability? How does subsequent re-exposure during adolescence, following the fetal experience, increase adult acceptance?

Recent chemosensory studies extend upon an already rich animal literature demonstrating that fetal ethanol exposure alters early postnatal ethanol seeking behavior and intake (Abate et al., 2008; Chotro et al., 2007; Spear and Molina, 2005). Specifically, these studies demonstrate that chemosensory plasticity is an important antecedent factor contributing, not only, to the early postnatal effect observed by others but also potentially to the progressive pattern outlined in the clinical literature. In a rodent model of gestational ethanol exposure, fetal experience-induced olfactory plasticity altered olfactory system function (i.e., the reflexive behavioral responsivity and peripheral neural response to ethanol odor) and the odor attributes of ethanol that are key for both odor preference behavior and flavor perception, as well as intake (Youngentob et al., 2007a,b; Youngentob and Glendinning, 2009). These developmental effects persisted into the age of adolescence (Eade et al., 2009), peaking at this time and declining through young adulthood (Eade et al., 2010). Importantly, absent ethanol re-exposure, the available data demonstrates that the chemosensory consequence of fetal exposure (both smell and taste) ameliorates by adulthood (Youngentob et al., 2007a,b; Youngentob and Glendinning, 2009).

In keeping with the cascading pattern predicted by the human clinical literature, animal studies show that, relative to fetal exposure alone, combinations of pre- and postnatal exposure can augment later responsiveness to ethanol, in terms of autonomic responding to ethanol odor (Chotro et al., 1996) and ethanol avidity (Honey and Galef, 2003). Similarly, specific adolescent re-exposure to ethanol odor in a biologically relevant setting amplifies the already enhanced odor-mediated behavioral consequence (i.e. reflexive sniffing response to ethanol odor) of prior fetal experience with the drug (Eade et al., 2009). Using a social transmission of food odor preference paradigm (e.g., Galef, 1981; Galef and Wigmore, 1983) in which a conspecific observer animal interacts with an intoxicated peer, Eade and colleagues (Eade et al., 2009) demonstrated an interaction between prenatal ethanol and adolescent ethanol odor exposures in observer rats that had interacted with an intoxicated peer (i.e., demonstrator). When combined, the fetal and adolescent experiences of observer rats produced an enhanced reflexive sniffing response to ethanol odor that was greater than in either form of exposure alone. Nonetheless, the combination of fetal ethanol exposure with adolescent odor re-exposure only yielded adult persistence of this behavioral effect in female observers. No persistence was observed in males. While a female consequence is clinically germane in terms of perpetuating the cycle of fetal exposure, the absence of a male

effect is still noteworthy. It is clear from these data that fetal exposed females are sensitive to the perpetuating consequence of ethanol odor re-exposure, alone. However, if chemosensory mechanisms are important antecedent factors in both the initiation and the progression of ethanol abuse, then it is important to understand the re-exposure conditions that uniformly yield adult persistence in both sexes.

Observers and demonstrators differ in their postnatal experience. Whereas, observers gain odor information on the breath of the demonstrator through social interaction, demonstrators experience the pharmacologic properties of ethanol along with retronasal and hematogenic olfaction (Maruniak et al., 1983a,b; Molina and Chotro, 1989; VanDishoeck and Versteeg, 1957). In this regard, using a social transmission paradigm we recently compared the effects of direct ethanol exposure (i.e., demonstrator) and indirect ethanol odor exposure (observer) on the adolescent response to ethanol odor (Eade and Youngentob, 2009). We found that, in previously ethanol naïve animals, the magnitude of the enhanced behavioral response to ethanol odor was similar for both forms of exposure when tested at P37. Importantly, in contrast to previous studies demonstrating aversive effects of acute ethanol intoxication (Molina and Chotro, 1989; Molina et al, 1989; Hunt et al., 1993), our findings showed that direct infusion of ethanol experienced within the context of the social transmission paradigm led to an enhanced behavioral response to ethanol odor.

Given the foregoing observation that *de novo* ethanol exposure augments the demonstrator response to its odor, the present study tested whether the combination of fetal ethanol experience with adolescent re-exposure as a demonstrator differed from that of observers when tested in adolescence. Moreover, given the potential interaction between the reinforcing properties of ethanol and retronasal/hematogenic olfaction, we tested whether re-exposure as a demonstrator yields adult persistence of the enhanced adolescent odor-mediated response in both sexes.

METHODS

Animal housing, testing, and experimental treatments were conducted in accordance with the guidelines of SUNY Upstate Medical University's Institutional Animal Care and Use Committee.

Maternal Treatment

The present study utilized 288 experimental Long-Evans Hooded rats, the progeny of 36 dams. Using our standard approach, pregnant dams were placed in weight-matched blocks and randomly allocated to one of three dietary treatment groups: ethanol, pair-fed, or free-choice (e.g. Eade et al., 2009; Youngentob et al., 2007a,b). The ethanol dams were fed an *ad-libitum* liquid diet (L10251; Research Diets, New Brunswick, NJ) that provided 35% of daily calories from ethanol from gestational (G) day 11 to 20 (after weaning onto the diet from G6–G10). This diet produces peak blood ethanol levels in the dam of approximately 150mg/dl on the evening of G17 (e.g., Miller, 1992; Youngentob et al., 2007a). Pair-fed dams received an iso-caloric, iso-nutritive liquid diet (L100252; Research Diets, New Brunswick, NJ) equivalent in volume to that consumed by their respective ethanol-fed dams on the prior day. Free-choice dams had continuous access to this same non-ethanol containing liquid diet and water.

Test Subjects

Within 24hrs of birth each experimental litter was surrogate fostered to dams that received lab chow and water throughout gestation. All litters were sexed and culled to a maximum of

10 pups on postnatal (P) day 2, with the restriction that a minimum of 4 male and 4 female pups was present in each experimental litter.

Re-Exposure of Adolescent Animals

Ethanol re-exposure was accomplished using a social transmission of food odor preference paradigm (e.g., see review: Hunt and Hallmark, 2004; Eade et al., 2009). Briefly, as illustrated in Figure 1, eight pups from each litter were randomly distributed into cages of 2 same sex siblings at the time of weaning (P21). This resulted in 4 cages of same sex siblings from each litter. From these 4 cages, one cage of each sex was randomly selected to receive ethanol exposure in adolescence while the other experienced water. Sibling pairs remained housed together for the duration of the experiment with one being randomly selected as the observer and the other as the demonstrator. Each exposure began with an hour-long separation period of the siblings. The first thirty minutes of this period served as acclimation time to social isolation. At the end of this first thirty minute period, demonstrators were intragastrically (i.g.) infused with either a subnarcoleptic dose of ethanol (i.e., 1.5 g/kg) or the equivalent amount of tap water (e.g. Fernandez-Vidal and Molina, 2004; Hunt et al., 2001). The infusion procedure consisted of inserting the free end of a polyethylene cannula (PE-10), attached to a 5cc syringe, through the oral cavity and into the stomach while holding the animal by the scruff of the neck (Chotro et al., 1996). Following infusion, the animals remained separated for an additional thirty minutes (*ibid*). This period allowed for the absorption, distribution, and respiratory elimination of ethanol (Molina & Chotro, 1989; Pohorecky & Brick, 1982). At the end of the one-hour total separation period (i.e. 30 minutes after i.g. infusion occurred in demonstrators), siblings were allowed to interact for 30 minutes (a time period shown in numerous studies to be sufficient for the social transmission of food odor cues, and in particular ethanol odor). In order to allow time for ethanol to clear from the demonstrators, siblings were then separated for 4hrs after which the animals were again reunited for overnight housing (e.g., Fernandez-Vidal and Molina, 2004). Exposure to ethanol, or water control, began on P29 and occurred 4 times, once every 48 hours (i.e., at P29, P31, P33, and P35).

Odor-Mediated Response to Ethanol

Whole-body plethysmography was used to monitor and evaluate each animal's reflexive odor-mediated sniffing response to ethanol. This well-established method (e.g. Youngentob, 2005; Youngentob and Glendinning, 2009; Eade et al., 2010) consists of monitoring small changes in respiration in response to the presentation of air or odorant in an enclosed chamber. Following a 40-trial air-only habituation period, a fixed 6s inter-trial interval schedule was utilized to randomly present air and ethanol odor stimuli in blocks of 20 trials (10 air and 10 ethanol). Each ethanol concentration was presented for one complete block of trials. Animals were tested once, either 48 hours after their final ethanol or water related experience (i.e., on P37) or in adulthood (P90), for their response to air and an ascending series of five ethanol odor concentrations (0.313%, 0.625%, 1.25%, 2.5% and 5% of vapor saturation at 20°C).

An animal's sniffing response represents a complex airflow pattern that varies with the presentation of odorant stimulation (Youngentob et al., 1987; Youngentob, 2005). In order to quantify this behavior, the sniffing response to each stimulus was computer deconstructed into 14 individual response measures: respiratory frequency; number of inspiratory and expiratory sniffs; average duration, volume, flow rate, and peak flow rate of inspiratory and expiratory sniffs; total inspiratory and total expiratory volumes; and the total apneic duration. Previous studies have shown that the 14 individual variables are insufficient at describing complex changes in overall sniffing behavior (e.g., Youngentob et al., 1987; Youngentob et al, 2007a). Therefore, in keeping with our previously established approach

(e.g., Youngentob and Glendinning; Eade et al., 2010) principal components analysis, in conjunction with multivariate multiple regression, was used to reduce the 14 measures into a primary measure of ethanol odor-mediated response behavior for each tested animal within a specific data set. In this regard, the derived 14×5 data matrix for each animal (i.e., 14 response measures $\times$ 5 ethanol odorant concentrations) was reduced to a pair of X and Y coordinates that were used in subsequent multivariate analyses. This 2-dimensional measure, namely, the “Composite Sniffing Index” quantitatively defined each animal’s ethanol odor-mediated response behavior across all concentrations of odorant in a behavioral response space. It must be emphasized that fetal ethanol induced increases in this behavioral index are causally linked to enhanced ethanol intake patterns (Youngentob and Glendinning, 2009). That is, increases in this primary measure are both predictive of and causally associated with increases in ethanol avidity.

RESULTS

Adolescent Behavior

The main objective of the behavioral evaluation was to test the hypothesis that fetal ethanol exposed observer and demonstrator animals differ in their response to ethanol odor as a consequence of their adolescent re-exposure experience. Figure 2 illustrates the mean relative position of observer and demonstrator animals in an ethanol odor-induced behavioral response space (i.e. their 2-dimensional mean Composite Sniffing Index value: $\pm$ 2-dimensional s.e.m.). There is a clear segregation of the two populations. Randomized-blocks MANOVA (multivariate analysis of variance) revealed an overall significant effect of the type of ethanol re-exposure the animal received on the overall behavioral response to ethanol odor when tested at P37 ($F_{2, 14} = 4.42, p < 0.04$). Evidence of a main effect of sex ($F_{2, 14} = 5.01, p < 0.03$) in the absence of a sex by treatment interaction ($F_{2, 14} = 0.41, p > 0.67$) demonstrated a differential response between males and females that was not specific to the mode of ethanol re-exposure.

In a previous study we demonstrated that adolescent ethanol odor observers that had received prior fetal ethanol exposure show an enhanced reflexive sniffing response to ethanol odor relative to the augmentation effect of fetal exposure alone (Eade et al., 2009). This observation notwithstanding, in order to fully interpret the current demonstrator finding it was necessary to determine whether, (1) relative to their fetal ethanol exposed adolescent water controls, ethanol demonstrators, like observers (*ibid*), display an augmentation of the fetal effect and (2) to what extent the magnitude of any observed augmentation differs between observers and demonstrators. With regard to question 1, we performed an exploratory analysis of fetal ethanol exposed demonstrators. That is, relative to their applicable water exposed control, we evaluated whether re-exposure as an ethanol demonstrator, indeed, resulted in an enhanced ethanol odor response. Randomized-blocks MANOVA of the 2-dimensional behavioral response data demonstrated that, in fetal ethanol exposed animals, adolescent ethanol re-exposure as a demonstrator resulted in an enhanced behavioral response to ethanol odor that significantly differed from control exposure ($F_{2, 14} = 11.85$, nominal $p < 0.001$). No evidence of a sex effect ($F_{2, 14} = 1.90$, nominal $p > 0.18$) or sex by treatment interaction ($F_{2, 14} = 0.25$, nominal $p > 0.77$) was observed.

Figure 3 illustrates the magnitude (i.e. the difference between the 2-dimensional mean behavioral index values of adolescent ethanol and water exposed animals) of the enhanced adolescent behavioral response to ethanol odor as a function of the method of ethanol re-exposure (i.e., observers vs. demonstrators). To craft this figure, we calculated a displacement vector between the mean locations, in two dimensions, of each form of adolescent ethanol re-exposure relative to their respective control. This yielded a single-dimensional value, namely, an “Ethanol Re-exposure Effect Size” that represented the

magnitude of the adolescent ethanol re-exposure vs. control effect. Importantly, effect sizes allows for the direct comparison of magnitudes of enhancement using standard *t*-tests (Eade et al., 2010; Eade and Youngentob, 2009). Figure 3 gives the qualitative impression that, relative to their respective controls, exposure as an ethanol demonstrator yielded a larger enhancement as compared to ethanol observers. Nonetheless, we could find no strong statistical evidence for a differential effect (two-tailed $t [15] = 1.46$; nominal $p > 0.1$).

Taken together, the foregoing results present a conundrum similar to that ascribed to a stress-induced effect of the i.g. procedure applied to naïve adolescent ethanol demonstrator exposed animals using the same social transmission paradigm (Eade and Youngentob, 2009). That is, on the one hand, adolescent ethanol observers and demonstrators in the current study are significantly different from each other (Fig. 2). On the other hand, the enhanced behavioral response (relative to their respective controls) was statistically equivalent for both observers and demonstrators (Fig. 3). For such a finding to have occurred implied that the ethanol odor-induced behavioral response of fetal ethanol exposed adolescent water demonstrators was less than that of their respective water observers. To confirm this proposition, exploratory MANOVA showed a significant effect of mode of water exposure in fetal ethanol exposed animals ($F_{2, 14} = 11.12$, nominal $p < 0.002$).

In a previous study we demonstrated that relative to fetal or adolescent odor observer exposure alone, the combined effect of fetal exposure and adolescent re-exposure as an observer enhanced the reflexive behavioral response to ethanol odor in P37 animals (Eade et al., 2009). Importantly, a key feature of these findings was that the enhanced behavioral response was not a consequence of the most proximate adolescent experience but rather it was the combined effect of both exposures. Although parsimony would suggest a similar finding in the present study for adolescent re-exposed demonstrators that had received prior fetal exposure, this proposition required confirmation. The behavioral results demonstrated an unambiguous difference in the response to ethanol odor in the adolescent ethanol demonstrators exposed to ethanol *in utero* as compared to adolescent ethanol demonstrators that were obtained from either pair-fed or free-choice control litters (Randomized-blocks MANOVA: $F_{4, 48} = 3.15$, nominal $p < 0.03$). Further, there was no evidence of a main effect of sex ($F_{2, 24} = 0.37$, nominal $p > 0.69$) or sex by treatment interaction ($F_{4, 48} = 1.16$, nominal $p > 0.33$).

In short, taken together, the constellation of the foregoing results demonstrates an augmentation of the fetal ethanol induced behavioral response to ethanol odor as a result of ethanol re-exposure via both direct (i.e., demonstrator) and indirect (i.e., observer) means.

Adult Behavior

As noted, in a previous study we showed that, when combined, fetal ethanol exposure and adolescent re-exposure as an observer perpetuates the effects of fetal exposure only in female animals (Eade et al., 2009). Thus, the focus of the adult evaluation was to test the proposition that re-exposure as a demonstrator would yield persistence of the enhanced reflexive odor-mediated sniffing response in both males and females. In keeping with the adolescent analysis above we determined whether, on average: (1) a differential effect of mode of ethanol re-exposure (i.e. observer vs. demonstrator) existed in fetal ethanol exposed animals when tested as adults (P90); (2) demonstrators tested as adults, like observers (Eade et al., 2009) differ from their water controls; (3) re-exposure as a demonstrator yields persistence of the enhancement effect in both sexes; (4) the magnitude of the enhanced effect from their respective controls differed between observers and demonstrators; and (5) the observed demonstrator effects were the result of the most proximate experience.

Regarding the first consideration, on average, there was a clear-cut distinction between the two populations in their relative behavioral response to ethanol odor ($F_{2, 14} = 6.41, p < 0.02$) (Fig. 4). There was no evidence of an overall effect of sex ($F_{2, 14} = 0.51, p > 0.60$) or sex by treatment interaction ($F_{2, 14} = 0.70, p > 0.50$). Second, demonstrator animals showed an enhanced behavioral response that significantly differed from their water controls ($F_{1, 15} = 12.00$, nominal $p < 0.004$). Further, there was no evidence of an overall effect of sex ($F_{1, 15} = 1.96$, nominal $p > 0.18$) or sex by treatment interaction ($F_{1, 15} = 1.64$, nominal $p > 0.21$). Third, since previous post-hoc analysis of findings in observers had revealed an effect of sex (Eade et al., 2009) we further investigated the possibility of a sex related difference in demonstrators. Analysis of effect sizes in ethanol re-exposed demonstrator animals revealed no strong evidence for a differential behavioral response of males and females (two-tailed $t [15] = 1.83$; nominal $p > 0.085$). Thus, whereas only female observers show adult persistence of the fetal exposure effect on the enhanced response to ethanol odor both male and female demonstrators manifest the adult enhanced response.

With respect to our fourth question, as illustrated in Figure 5, both modes of ethanol exposure displayed a similar magnitude of an enhanced behavioral response relative to their respective adult controls (two-tailed $t [15] = 0.075$; nominal $p > 0.5$). Interestingly, by contrast to the adolescent findings, the differential effect of mode of water exposure (i.e., observer vs. demonstrator) on the behavioral response to ethanol odor ameliorated by adulthood ($F_{2, 14} = 2.82$, nominal $p < 0.10$). That is, unlike in adolescence, adult tested water observers and demonstrators did not differ.

Finally, evaluation of fetal ethanol, pair-fed and free-choice adolescent demonstrators tested in adulthood revealed a significant effect of prenatal treatment on the adult response to ethanol odor ($F_{4, 48} = 3.86$, nominal $p < 0.009$). There was no evidence of an overall sex effect in demonstrators ($F_{2, 24} = 0.18$, nominal $p > 0.83$) or sex by treatment interaction ($F_{4, 48} = 1.28$, nominal $p > 0.28$).

Taken together, our adult findings demonstrate that secondary adolescent exposure to ethanol augments the effects of prior fetal experience in a mode-dependent manner.

DISCUSSION

The relationship between human fetal ethanol exposure and the cascading pattern of subsequent alcohol abuse is well known. Human studies demonstrate: (a) a predictive association between fetal exposure and the increased probability for adolescent acceptance (Alati et al., 2006; Baer et al., 1998, 2003; Streissguth, 1998; Yates et al., 1998); (b) that fetal exposure is, perhaps, the best predictor of adolescent abuse; and (c) that the earlier the age of ethanol experience the greater the risk of long term abuse (Streissguth, 1998; Yates et al., 1998). Despite these data, little is known regarding the underlying factors contributing to this pattern of progression.

There is a growing body of data from animal studies demonstrating epigenetic chemosensory mechanisms by which maternal patterns of alcohol use are transferred to offspring. Moreover, the ensuing developmental changes in the chemosensory systems involved in the behavioral preference for ethanol odor and the perception of the flavor of ethanol (i.e., smell and taste), in turn, add to the risk of subsequent intake in adolescence, thereby contributing to adult progression. In summary of these studies, postnatal rats exposed to ethanol throughout gestation show an enhanced neural and reflexive sniffing response to ethanol odor and these alterations are specific to the drug (Youngentob et al., 2007a). The behavioral enhancement is causally related to the neural tuning. Importantly, fetal ethanol exposure alters both the smell and taste systems such that the normally aversive

odor and flavor attributes of ethanol become more acceptable, thereby enhancing intake (Youngentob and Glendinning, 2009). Specifically, elevated ethanol intake stemming from fetal exposure is causally linked to the enhanced response to its odor as well as an increased taste-mediated acceptability of ethanol. In this later respect, Youngentob and Glendinning (2009) revealed that fetal exposure reduced the aversion to the known quinine-like taste quality of ethanol (Di Lorenzo et al., 1986; Kiefer and Mahadevan, 1993).

Although the forgoing consequences of fetal exposure are absent in adults (Youngentob et al., 2007a,b; Youngentob and Glendinning, 2009) they persist into adolescence (Middleton et al., 2009; Eade et al., 2009, 2010; Youngentob and Glendinning, 2009). Notably, re-exposure to ethanol odor appears critical, at least in part, for persistence of the initial fetal exposure effects in adulthood. Using a social transmission of food odor preference paradigm in conjunction with fetal ethanol exposures, Eade and colleagues (2009) demonstrated that, relative to fetal ethanol or adolescent odor exposure alone, adolescent odor re-exposure (i.e., odor exposure in observer animals that had received prior fetal exposure) enhanced the behavioral response to ethanol odor in these adolescent animals. Further, the combined exposures yielded persistence of the odor-mediated behavioral effects only in adult females. While such a finding was pertinent in terms of understanding the minimum type of secondary ethanol related experience (i.e., odor re-exposure) necessary to yield persistence in the gender clinically relevant to the continuing cycle of fetal exposure, the absence of a male effect was instructive. That is, although females are susceptible to ethanol odor re-exposure, from a clinical perspective, such a condition is unlikely to occur in a vacuum absent attendant ethanol intake. So what are the re-exposure conditions necessary to yield adult persistence in both sexes?

The results of the present study significantly extend upon this prior work in several important ways. Testing in late adolescence demonstrated that ethanol re-exposure as a demonstrator, like an observer (Eade et al., 2009), yielded an enhanced odor-mediated reflexive response that resulted from the cumulative effect of the combined exposures. Nonetheless, although re-exposure to ethanol by both direct (i.g. infusion) and indirect (odor) means augments the odor-mediated response resulting from fetal drug experience, the magnitude of the enhancement effect, relative to their respective controls, was not different (Fig. 3). This latter finding may seem somewhat surprising given that demonstrators are believed to experience the pharmacologic properties of ethanol along with retronasal and hematogenic olfaction (Eade and Youngentob, 2009; Molina and Chotro, 1989; Maruniak et al., 1983a,b; VanDishoeck and Versteeg, 1957). However, within the context of the standard social transmission paradigm (Galef, 1981; Galef and Wigmore, 1983) it has been suggested there is a devaluation of the enhanced behavioral response to ethanol odor in demonstrators due to the aversive stress effect of the i.g. infusion procedure (Eade and Youngentob, 2009). Such an effect may be exacerbated in the present study by the added consequence of prior fetal exposure. That is, findings in both humans (Haley et al., 2006; Jacobsen et al., 1999) and animals (e.g., Kim et al., 1996; Weinberg, 1988, 1993; Weinberg et al., 1996) demonstrate that fetal ethanol exposure results in an increased responsiveness to stressors. Implicit in this suggestion is the proposition that in contrast to the present results, re-exposure as a demonstrator through more naturalistic means, such as acute voluntary ethanol intake, should result in an enhancement effect greater than that found in observers.

With regard to the above, although the design of our experiment was not specifically intended to inform the foregoing question, exploratory evaluation of the data provided important insight, nonetheless. Recall that, the ethanol odor-induced behavioral response of fetal ethanol exposed adolescent water demonstrators was significantly less than that of the water observers. We propose that the magnitude of the difference (i.e., the effect size) between the two control groups represents the devaluating consequence due to the stress of

intubation in the demonstrators. The value of the calculated effect size in this instance was 2.05 ± 0.41 (mean $\pm$ SEM). For exploratory purposes we used this value to “correct” for the untoward effect of the intubation procedure. As can be seen in Fig. 6, when this correction is applied to the demonstrators the magnitude of the ethanol re-exposure effect size approximately doubles (compared to the uncorrected value) and becomes roughly 3.0 times larger than the effect size of odor exposure alone (observers). It must be emphasized that our manipulation of the data should be viewed with a note of caution. Importantly, however, they highlight that questions regarding the method of demonstrator re-exposure warrants further investigation and that the results of more naturalistic exposures would be highly clinically relevant given the known epidemiologic association between fetal exposure, adolescent drug use and the continued risk of abuse (Streissguth, 1998; Yates et al., 1998).

Our results also establish that, despite the apparent untoward effect of intubation, adolescent re-exposure to ethanol as a demonstrator was sufficient (as compared to a female only effect in observers: Eade et al., 2009) to yield adult persistence of the fetal induced behavioral response in both sexes. The dichotomous result between adult observers and demonstrators deserves attention given our lack of understanding regarding the minimum levels and context of olfactory stimulation needed to sustain the fetal plasticity effect. As we have noted (Eade et al., 2009), it is possible that males and females require differing levels of re-exposure to yield adult persistence. It is also likely that the context and effectiveness of the re-exposure conditions will interact in this process as well. Indeed, adolescent males and females are ontogenetically different physiologically, hormonally and in terms of brain development (see review: Spear, 2000). Thus, differing conditions for minimum stimulation are not entirely surprising.

Regarding the above, recall that observers experience odor on the breath of the demonstrator whereas demonstrators experience the pharmacologic properties of ethanol accompanied by retronasal and hematogenic olfaction. In the present study, ethanol demonstrator animals were administered a 1.5g/kg body weight dose. In both humans (see review: Norberg et al., 2003) and rats (Eade et al., 2009) the same dose of ethanol in g/kg body weight are known to produce higher blood alcohol levels (BALs) in females than in males. As we have previously noted, increased BALs in female demonstrators may provide, relative to their male counterparts, sufficient intensity of ethanol odor on their breath to achieve effective transmission, thereby providing female persistence into adulthood (Eade et al., 2009). By contrast, the multiplicity of consequences stemming from experiencing the reinforcing properties of ethanol along with the attendant chemosensory stimulation appears minimally necessary to yield equivalent persistence in both sexes. Although females may have acquired a higher BAL from ethanol infusion than males, within the general context of the demonstrator's experience, this difference may be moot. That is, in general, both male and female adolescents are known to be less sensitive to many of the negative effects of ethanol such as motor impairment, sedation, and even withdrawal (Spear and Varlinskaya, 2005). Conversely, animals of this age are more sensitive to the positive attributes of the state of ethanol intoxication (Fernandez-Vidal et al., 2003; Philpot et al., 2003) as well as to facilitation of social interactions (Varlinskaya et al., 2001; Varlinskaya and Spear, 2002). Thus, it does not seem unreasonable that, taken together, the interaction of these effects coupled with olfactory stimulation yields a relatively equivalent combined effect in both sexes. Further, it is unknown whether a ceiling effect exists with regard to the impact of the demonstrator re-exposure experience.

CONCLUSIONS

The results of this study illustrate that the method of ethanol exposure plays an important role in the uniform enhancement and preservation of prior fetal ethanol-induced behavioral

responses to ethanol odor. Specifically, re-exposure to ethanol as a demonstrator, like observers, augments the adolescent behavioral response to ethanol odor due to prior fetal experience. However, unlike the effect of ethanol odor exposure obtained by observers, direct ethanol re-exposure in a social setting produced persistence of the enhanced behavioral effect into adulthood in both sexes. As such, our findings have important implications with regard to our understanding of underlying factors contributing to the clinical progression of developing patterns of ethanol abuse.

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Fig. 1.

Adolescent re-exposure model. Each prenatal treatment group provided 4 cages of same sex sibling pairs, 1 cage per sex for each of the adolescent exposure substances (ethanol or water). Within each pair, one animal was allocated as the demonstrator, receiving exposure through intragastric infusion (IG), and the other as the observer, receiving exposure through social interaction (SI).

Fig. 2. Behavioral index values (mean $\pm$ 2-dimensional SEM) for P37 fetal ethanol exposed observers and demonstrators that had been re-exposed to ethanol in adolescence. Ethanol observers and demonstrators responded differently to ethanol odor following adolescent re-exposure to the drug.

Fig. 3. Ethanol re-exposure effect size (mean $\pm$ SEM) of P37 fetal ethanol exposed animals as a function of mode of adolescent treatment. Both types of ethanol re-exposure led to similar magnitudes of behavioral enhancement as compared to their respective water controls.

Fig. 4. Behavioral index values (mean $\pm$ 2-dimensional SEM) for P90 fetal ethanol exposed observers and demonstrators that had been re-exposed to ethanol in adolescence. Ethanol observers and demonstrators differentially responded to ethanol odor when tested in adulthood as a consequence of their adolescent re-exposure experience.

Fig. 5. Ethanol re-exposure effect size (mean $\pm$ SEM) of P90 fetal ethanol exposed animals as a function of mode of adolescent treatment. On average, both modes of adolescent ethanol re-exposure resulted in an enhanced behavioral response to ethanol odor in adulthood.

Fig. 6.

“Corrected” ethanol re-exposure effect size for P37 fetal ethanol exposed demonstrators (Ds - Corrected) as compared with the uncorrected effect sizes of both modes of treatment (i.e. observers and demonstrators (Ds - Uncorrected)). The data are expressed as (mean ± SEM). When corrected for the hypothesized untoward effect of the intubation procedure, the magnitude of ethanol demonstrator re-exposure approximately doubles, making it roughly 3 times larger than the observer (odor exposure) effect.