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Transgenerational Transmission of the Effect of Gestational Ethanol Exposure on Ethanol Use-Related Behavior

Michael E. Nizhnikov*, Daniel O. Popoola*, and Nicole M. Cameron

Southern Connecticut State University (MEN), New Haven, Connecticut; and Center for Development and Behavioral Neuroscience, and Developmental Exposure Alcohol Research Center (DOP, NMC), Binghamton University, Binghamton, New York

Abstract

Background—Prenatal alcohol exposure (PAE) enhances the risk for alcoholism by increasing the propensity to consume alcohol and altering neurophysiological response to alcohol challenge. Transgenerationally transmittable genetic alterations have been implicated in these behavioral changes. To date, transgenerational transmission of PAE-induced behavioral responses to alcohol has never been experimentally investigated. Therefore, we explored the transgenerational transmission of PAE-induced behavioral effects across 3 generations.

Methods—Pregnant Sprague Dawley dams received 1 g/kg ethanol (EtOH) or water daily on gestational days 17 through 20 via gavage, or remained untreated in their home cages. To produce second filial (F2) or F3 generations, similarly treated adult F1 or F2 offspring were mated and left undisturbed through gestation. On postnatal day (PND) 14, male and female F1, F2, and F3 offspring were tested for consumption of 5% (w/v) EtOH (in water), or water. Using the loss of righting reflex (LORR) paradigm on PND 42, F1 and F2 adolescent male offspring were tested for sensitivity to acute EtOH-induced sedation–hypnosis at 3.5 or 4.5 g/kg dose. F3 male adolescents were similarly tested at 3.5 g/kg dose. Blood EtOH concentration (BEC) was measured at waking.

Results—EtOH exposure increased EtOH consumption compared to both water and untreated control groups in all generations. EtOH-treated group F1 and F2 adolescents displayed attenuated LORR duration compared to the water group. No attenuated LORR was observed in the F3 generation. BEC at waking corroborated with the significant LORR duration differences while also revealing differences between untreated control and water groups in F1 and F2 generations.

Conclusions—Our results provide novel behavioral evidence attesting that late gestational moderate EtOH exposure increases EtOH intake across 3 generations and may alter sensitivity to EtOH-induced sedation–hypnosis across 2 generations.

Keywords

Ethanol; Rat; Intake; Infant; Loss of Righting Reflex

Reprint requests: Michael E. Nizhnikov, PhD, Southern Connecticut State University, 501 Crescent Street, New Haven, CT 06515-1355; Tel.: 203-392-8886; Fax: 203-392-6805; nizhnikovm1@southernct.edu.

*Both authors collaborated equally.

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Alcoholism and alcohol use disorders are a major problem worldwide. Exposure to moderate amounts of alcohol prenatally or during early life in humans increases the risk for alcohol abuse in adolescence and adulthood (Alati et al., 2008; Baer et al., 2003). Alati and colleagues (2008) have shown that adolescents of mothers who consumed 3 or more drinks during pregnancy had a significantly greater incidence of alcohol use disorders than individuals whose mothers did not drink. It is estimated that 10 to 15% of women in the United States drink some alcohol during pregnancy (Ponce et al., 2004). These findings suggest that prenatal alcohol exposure (PAE) is a major risk factor for alcohol use disorders later in life. Alcohol is consumed during infancy through maternal milk and in very young children on many occasions (religious, festive, and accidental). Starkman and colleagues (2012) outline early onset of drinking (United States and Argentina). For example, 39% of 8- to 10-year-old children in Pennsylvania had drunk or sipped alcohol.

Studies using rats seem to corroborate the data obtained in humans (Chotro and Arias, 2006; Fabio et al., 2013; Pautassi et al., 2012). Exposure to low and moderate doses of ethanol (EtOH) during late gestation yields enhanced EtOH intake in infancy and adolescence (Fabio et al., 2013; Sommer et al., 2006), alters immediate early gene expression in the brain (Fabio et al., 2013), and significantly broadens the range of EtOH doses that infant rats find positively reinforcing (Mendez and Morales-Mulia, 2008; Nizhnikov et al., 2006a,b). In adults, EtOH exposure can modulate the γ -aminobutyric acid (GABA) system through its receptor subunit expression (Kumar et al., 2012), which in turn may affect EtOH sensitivity (Blednov et al., 2011; Iyer et al., 2011).

Mechanisms behind the effects of PAE and underage drinking need further study, particularly as genetics alone may not account for all of the predisposition for alcoholism (for review, see Starkman et al., 2012). Epigenetic modifications such as DNA methylation may carry on across generations and result in transgenerational phenotype persistence. Epigenetic inheritance induced by environmental factors such as diet (Carone et al., 2010; Ng et al., 2010), stress (Franklin et al., 2010), and EtOH exposure (Finegersh and Homanics, 2014a,b) persists into the second generation and is associated with changes in DNA methylation (Franklin et al., 2010). These results indicate that epigenetic mechanisms could regulate transgenerational transmission of phenotypes. However, to date, no research shows EtOH-induced transgenerational persistence in behavior associated with EtOH use/abuse.

Our laboratories have shown that prenatal exposure to moderate levels of EtOH increases intake of EtOH in infants. Administration of 1.0 g/kg EtOH to dams during gestational day (GD) 17 to 20 results in offspring (F1 generation) that drink more 5.0% EtOH than controls (Nizhnikov et al., 2014). The increase in EtOH intake persists into adolescence (Diaz-Cenzano and Chotro, 2010). Interestingly, EtOH-induced locomotor activation appears not to be affected by this prenatal treatment (Fabio et al., 2013).

Sedation–hypnosis is an important measure of alcohol’s physiological effects. This measure can be assessed through the loss of righting reflex (LORR) test (Majchrowicz, 1975). Specifically, rats are given hypnotic doses of EtOH, and both the time sedated and blood alcohol content at waking are measured. This allows researchers to measure changes in sensitivity to EtOH’s sedative properties. A decrease in sensitivity to the sedative–hypnotic

effects of alcohol is negatively associated with drinking practices and may indicate a higher risk of developing alcoholism in the future (Tabakoff and Hoffman, 1988). Thus, it is important to investigate this measure in an adolescent model as the adolescent human population often abuses large quantities of alcohol (Wechsler et al., 2002).

The current set of experiments is aimed at exploring our central hypothesis that increased EtOH drinking in infants (Nizhnikov et al., 2014) and changed sensitivity to EtOH's sedative–hypnotic effects in adolescence persists across 3 generations even if only the first-generation experiences EtOH in the womb. We used a rat model to study transgenerational transmission (3 generations) of PAE effects by investigating the influence of moderate levels of EtOH (1 g/kg) during GD 17 to 20 on EtOH consumption and sensitivity to EtOH's sedative–hypnotic effects.

MATERIALS AND METHODS

Subjects

Adult Sprague Dawley rats originating from Taconic (Germantown, NY) and bred at Binghamton University (parental generation, F0) were mated (2 females with 1 male) in a standard mating cage (20"×16"×8") to produce the first filial (F1) generation. The day that sperm was detected was designated as GD 0. On GD 17 to 20, pregnant dams received intragastric (i.g.) EtOH, water, or remained in their home cages in the colony room (control). Date of birth was designated as postnatal day (PND) 0.

The EtOH intake test was conducted on PND 14 (total of 221 pups) and all remaining pups were weaned on PND 21. Same sex and same litter weanlings were pair-housed in regular-sized cages (19"×10.5"×8") and left undisturbed until subsequent testing. During adolescence (PND 42), a test for sensitivity to EtOH's sedative–hypnotic effect was conducted on randomly selected sets of male offspring. Some animals were left undisturbed until adulthood (>PND 70) to produce the second and third filial (F2 and F3) generations.

At reproductive maturity, the same mating procedure as conducted in the F0 generation was repeated. However, these subjects were not exposed to any prenatal treatment. Postnatal testing protocols for the F2 and F3 generations were identical to F1. For the F2 and F3 generations, mating was conducted between the animals of the same treatment group (EtOH with EtOH, water with water, and untreated with untreated), and mated pairs did not share parents or grandparents.

Prior to weaning, the colony rooms were maintained at a 10/14 light–dark cycle with lights on at 9 AM, while postweaning holding rooms were maintained at a 12/12 light–dark cycle with lights on at 12 AM, with room temperature at 22°C and humidity at 40%. No environmental enrichment was provided in cages. Food and water were provided ad libitum. All experimental protocols were in accordance with the Institutional Animal Care and Use Committee at Binghamton University.

No animal was ever used more than once. Subjects tested for infant intake or LORR were euthanized immediately after testing. Breeding pairs never experienced any testing or manipulations.

Gestational Treatment

This procedure was performed only on pregnant F0 female rats. From GD 17 to 20, pregnant dams received EtOH (12.6% v/v EtOH solution; volume administered: 0.01 ml/g; dose: 1 g/kg; vehicle tap water) or the corresponding amount of tap water via i.g. administration as previously described (Nizhnikov et al., 2014). An isocaloric fluid was not used in this experiment as all animals were ad lib fed and watered so we did not foresee any differences in total caloric intake through the 4 days of EtOH exposure. Furthermore, previous research using this model used water (vehicle for EtOH) as a control solution. To maintain continuity across studies, we used the same methodology (i.e., Chotro and Arias, 2006; Diaz-Cenzano and Chotro, 2010; Nizhnikov et al., 2006a,b). Intragastric administration was performed by introducing a polyethylene cannula (PE50; Clay Adams, Parsippany, NJ), mounted on a 21-G needle connected to a 10-cc syringe (Becton Dickinson & Co., Rutherford NJ), into the stomach through the oral cavity. Untreated control animals were left undisturbed in the colony room. This procedure occurred between 10 to 11 AM daily and took about 15 to 20 seconds per rat. In accordance with most of the previous literature using this animal model (i.e., Chotro and Arias, 2006; Diaz-Cenzano and Chotro, 2010; Nizhnikov et al., 2006a,b), pregnant dams were not acclimated to the gavage procedure justifying the use of an untreated control group.

Infant EtOH Intake Test

Infant rats (F1, F2, and F3) derived from EtOH, water, or untreated control prenatal treatment groups were tested for intake of EtOH or water on PND 14. The design of this experiment was a 3 (prenatal treatment: EtOH, water, or untreated control) by 2 (sex: male or female) by 2 (fluid: EtOH or water) factorial. Only 1 individual per sex, per litter was subjected to each treatment (water or EtOH). Two male and 2 female pups were randomly selected from each litter 3 hours before testing (9 AM), transferred into a preheated plastic cage with wood shavings, and pair-housed. Intra-oral cheek cannula made from PE10 polyethylene tubing (Clay Adams) was implanted into the anterior portion of the mouth, 2 to 3 mm caudal to the mystacial pad, and flanged on the intra-oral end (3 to 5 seconds per pup) as previously described (Nizhnikov et al., 2014). After a 3-hour waiting period, each pup was voided with cotton wool to induce urination and defecation, weighed, and transferred into the testing area. The testing procedure occurred in a plastic rectangular chamber with white walls and a slightly heated floor lined with paper towels. A syringe (reservoir) mounted on a pump (KD Scientific, Holliston, MA) dispensed the fluid (EtOH 5.0% or water) and delivered it through the intra-oral cheek cannula (total volume: 5.5% of body weight; 15 minutes) connected to the pump by another polyethylene tube. Following the completion of the intake, test pups were removed from the testing chamber, disconnected from the cannula, dried, and weighed again. The percentage weight gain was an indirect measure of volume of fluid consumed and thus was used in computing volume ingested in general as well as g/kg of EtOH ingested and volume of water consumption.

No anesthetic was used during any part of the procedure. This method of infant EtOH intake has been used for over 20 years in a variety of laboratories and has become common practice in infant intake studies (i.e., Arias and Chotro, 2005; Diaz-Cenzano and Chotro, 2010; Fabio et al., 2013; Nizhnikov et al., 2006a,b).

Loss of Righting Reflex

To assess the influence of F1 prenatal EtOH treatment on sensitivity to EtOH's dose-dependent sedative-hypnotic effect in adolescence (PND 42), in 3 generations of male rats, animals were administered 3.5 or 4.5 g/kg of 20% v/v EtOH solution in 0.9% w/v saline (vehicle) intraperitoneally (i.p.). For the F3 generation, the 3.5 g/kg EtOH dose alone was used, as only this dose showed any differences due to prenatal treatment in the previous generations. Only males were chosen to avoid confounds with the estrous cycle of female rats. Future studies will use both sexes for analysis. Adolescence was chosen as testing age as heavy drinking (to a narcoleptic dose) can be seen most often in this age in humans. EtOH administration was performed between 6 to 7 AM. LORR duration was defined as the interval between the loss of ability to flip over onto all 4 limbs when placed in a supine position and the time when this reflex was successfully demonstrated 3 consecutive times within 60 seconds. LORR latency was defined as the time between EtOH administration and the LORR. Animals with a LORR latency period exceeding 5 minutes were excluded from further testing. After demonstrating the LORR, rats were placed in a supine position in a V-shaped (90° angle) trough, where they remained undisturbed until they voluntarily regained their righting reflex. On awakening, trunk blood was collected on ice in an ethylenediaminetetraacetic acid (EDTA)-coated vacutainer (BD, Franklin Lakes, NJ), centrifuged at 4°C and 1,000×g for 15 minutes, and the supernatant (plasma) was collected and stored at -20° C. Blood EtOH concentration (BEC) was analyzed for entire cohorts in 1 sitting, using an AM1 Alcohol Analyzer (Analox Instruments, Lunenburg, MA).

Statistical Analysis

Data were compared between prenatal treatment groups for all experiments. Analysis of juvenile intake (both in ml of solution or in g/kg of EtOH consumed) and weight at that age was conducted using 3-way analyses of variance (ANOVAs) (treatment × generation × fluid) for infant EtOH consumption. From a total of 221 rat pups used in the fluid intake test, 5 animals were removed because their intake was more than 2 standard deviations from the mean (drinking either almost nothing or over 10% of their body weight, almost certainly an equipment error and a clerical error, respectively) and 7 subjects disconnected their tubing from the pump and were removed from analyses. The effect of prenatal treatment on LORR duration and BEC at awakening was first analyzed with 2-way ANOVAs (treatment × generation). Main interactions were further analyzed independently within generations by ANOVAs. A total of 581 animals were used for this experiment, only 11 animals were excluded (2 control, 6 water-treated, and 3 EtOH-treated animals from F2 and F3) because of latency to LORR longer than 5 minutes. No F1 animals were excluded. Data were analyzed using the SPSS v21 statistical analysis program (IBM, Armonk, NY). For all experiments, p -value < 0.05 was considered significant and 0.05 < p < 0.09 was described as approaching significance. When appropriate, Tukey's honest significant difference post hoc tests were also conducted. All data are presented as mean ± SEM.

RESULTS

Experiment 1: Effects of Moderate Levels of Gestational EtOH Exposure on EtOH Consumption Across 3 Generations

Experiment 1 measured the effect of prenatal treatment (EtOH, water, or untreated control) on EtOH or water intake across 3 generations of rat pups. A 4-way ANOVA was conducted and showed no effect of sex or any interaction, $F(1, 173) = 0.099$, $p = 0.75$. Therefore, all data were collapsed across sex. When comparing EtOH and water intake, we must use ml ingested rather than g/kg because water does not lend itself to g/kg analysis. Therefore, the following analysis was performed using this measure (see Fig. 1 and Table 1). It is important to note that Fig. 1 and Table 1 represent the same measure. However, Fig. 1 is collapsed across all generations, while Table 1 shows the intake in ml for all generations separately. The 3-way ANOVA revealed a significant main effect of test fluid, $F(1, 191) = 57.80$, $p < 0.0001$, power = 1.0. Tukey's post hoc tests indicated that all PD 14 rat pups drank more EtOH than water ($p < 0.05$; Fig. 1). We also found a main effect of prenatal treatment, $F(2, 191) = 18.30$, $p < 0.0001$, power = 1.0, as pups from EtOH-exposed groups across all generations drank significantly more fluid (collapsed across EtOH and water) than their water or untreated control counterparts, which did not differ from each other (Fig. 1). Last, a significant test fluid $\times$ prenatal treatment interaction was found, $F(2, 191) = 8.70$, $p < 0.005$, power = 0.978. Due to the significant interaction, we performed a post hoc analysis separating fluid. In other words, we ran separate ANOVAs for EtOH and water consumption depending on prenatal EtOH treatment and F generation. These data can be found in Table 1. The ANOVA for water intake (ml consumed) in EtOH-exposed groups showed no main effect or interaction either when collapsed across generations, $F(2, 90) = 2.58$, $p > 0.05$ (Fig. 1), or when using generation as a factor, $F(2, 84) = 0.40$, $p > 0.05$ (Table 1), indicating that water consumption did not differ due to prenatal treatment. On the other hand, analysis of EtOH intake in terms of ml consumed showed a significant main effect of prenatal treatment either when collapsed across generations, $F(2, 113) = 22.08$, $p < 0.0001$ (Fig. 1), or when using generation as a factor, $F(2, 107) = 15.620$, $p < 0.0001$ (Table 1), but no interaction between prenatal treatment and generation (Table 1). Prenatal EtOH-treated subjects drank more EtOH than either control groups. Most importantly there were no significant interactions with generation.

To further confirm that increased EtOH drinking due to prenatal EtOH exposure persisted across all 3 generations, we performed separate 1-way ANOVAs for each generation. As EtOH is the only test fluid being analyzed and to account for the weight differences between prenatal treatments, all of the following analyses are performed using g/kg EtOH intake as the dependent variable. Specifically, for the F1 generation the ANOVA revealed a significant main effect of prenatal treatment, $F(2, 30) = 6.65$, $p < 0.005$, power = 0.894 (Fig. 2). Intake of water did not differ between any of the groups, $F(2, 25) = 0.375$, $p > 0.65$. For the F2 generation, the ANOVA indicated a main effect of parent exposure, as offspring of prenatally EtOH-treated animals drank more alcohol than either control group, $F(2, 43) = 10.818$, $p < 0.001$, power = 0.992 (Fig. 2). Intake of water did not differ, $F(2, 27) = 1.437$, $p > 0.25$. Finally, the ANOVA analyzing intake in the F3 generation indicated a main effect of grandparent exposure, $F(2, 29) = 5.166$, $p < 0.02$, power = 0.8 (Fig. 2). Once more animals

in the prenatal EtOH line drank more EtOH than either control group, intake of water did not differ, $F(2, 30) = 1.08$, $p > 0.35$.

At PND 14, general effect of prenatal treatment was found on body weight, $F(2, 200) = 8.39$, $p < 0.001$, prior to testing. Pups in the prenatally EtOH-treated line weighed significantly less than untreated pups at birth. A main effect of generation was also found, $F(2, 200) = 30.70$, $p < 0.0001$, with the F2 generation weighing significantly less than either the F1 or F3 generations, which did not differ from each other. There was also a significant prenatal $\times$ generation interaction, $F(4, 200) = 4.217$, $p = 0.0027$. Post hoc analysis showed that there are no differences in weight in the F1 generation. However, the F2 offspring of prenatally EtOH-treated subjects weighed less than either the water or the untreated groups, which did not differ from each other ($p < 0.05$). The F3 generation water and the prenatally EtOH-treated groups weighed less than the untreated controls but did not differ from each other ($p < 0.05$; Table 1).

Experiment 2A: Effects of Gestational EtOH Exposure on Adolescent Sensitivity to EtOH's Sedative Properties Across 3 Filial Generations

Using the LORR test, adolescent (PND 42) male rats were tested for sensitivity to EtOH-induced sedation-hypnosis after i.p. administration of a 3.5 or 4.5 g/kg dose of EtOH.

For the 3.5 g/kg dose challenge, the 2-way ANOVA indicated a main effect of gestational treatment, $F(2, 91) = 5.09$, $p < 0.01$, and filial generation, $F(2, 91) = 3.44$, $p < 0.05$. More importantly, no treatment $\times$ generation interaction was seen, $F(4, 91) = 0.47$, $p = 0.76$. One-way ANOVA per generation at 3.5 g/kg also revealed a main effect of treatment in F1, $F(2, 41) = 5.233$, $p < 0.05$, and F2, $F(2, 30) = 4.133$, $p < 0.05$, but not in the F3, $F(2, 15) = 0.297$, $p > 0.05$, generation (see Fig. 3, top). Post hoc analysis revealed that EtOH-exposed groups had shorter LORR duration compared to water but not untreated controls in F1 and F2 generations ($p < 0.05$). In these generations, water and untreated control groups were not different from each other (see Fig. 3). None of the other comparisons reached significance.

BEC at waking was also analyzed using a 2-way ANOVA and revealed a significant main effect of treatment at the 3.5 g/kg dose, $F(2, 91) = 3.99$, $p < 0.05$. Post hoc analysis revealed that EtOH-treated animals regained their righting reflex at a significantly higher BEC than water prenatally exposed animals ($p < 0.05$). However, there was only a trend for the ethanol-exposed lineage waking at higher BEC than untreated controls ($p = 0.085$). To investigate the effects of treatment on BEC at waking in each generation, 1-way ANOVAs were used. A main effect of treatment at 3.5 g/kg dose was found in F1, $F(2, 41) = 9.611$, $p < 0.001$, and F2, $F(2, 30) = 5.405$, $p < 0.05$, but not in the F3, $F(2, 15) = 0.96$, $p > 0.05$, generations (Fig. 3). In F1 generation, post hoc analysis revealed that EtOH-treated group had higher BEC compared to water-treated and untreated control groups ($p < 0.05$). In the F2 generation, EtOH-treated and untreated control groups had higher BEC than water-treated group ($p < 0.05$).

For the higher EtOH (4.5 g/kg) challenge dose, no significant differences between any groups nor interactions were found (Fig. 4).

DISCUSSION

The present set of experiments demonstrates that moderate EtOH exposure during late gestation (GD 17 to 20) in the F0 dam increases EtOH intake across at least 3 generations. Specifically, intake of EtOH during infancy (PD 14) is significantly greater in all 3 (F1, F2, and F3) generations compared to controls. This effect cannot be due to overall increases in drinking because water intake did not differ between groups. This effect was seen even though the F2 and F3 generations did not have direct exposure to EtOH. Furthermore, prenatal manipulation dose-dependently altered male adolescents' sensitivity to EtOH-induced sedation-hypnosis, both in exposed F1 and their naïve F2 male offspring. However, F3 male sensitivity was not affected by grandparent prenatal exposure.

The present study confirms and extends existing evidence that PAE increases the propensity to consume EtOH during infancy. Moderate PAE of the first generation significantly increased EtOH consumption in all 3 (F1 to F3) generations of infants compared to controls. These effects were specific to EtOH, as there was no significant effect of EtOH exposure on water consumption in any generation. For the F1 generation, these results are in accordance with the majority of previous PAE studies that report increased EtOH consumption accompanied by no significant alterations in water consumption following low-moderate (1 to 2 g/kg EtOH daily on GD 17 to 20) EtOH exposure (Abate et al., 2004; Arias and Chotro, 2005; Nizhnikov et al., 2006a,b). Interestingly, results from Experiment 1 suggest that the observed increase in EtOH drinking persists to the second and third generations, although the mechanisms behind this effect are yet to be fully explored.

We found a decrease in the pup's body weight in EtOH-treated animals, at PND 14 in the F2 and F3 generations. This result could be a major concern. However, all 3 generations (F1 to F3) of the prenatally EtOH-treated line show increased EtOH intake, while there is no significant change in water intake across any treatment in any generation. We do not believe that this weight difference influenced the effect found on intake, as it was calculated in g/kg intake and this measure controls for differences in weight across subjects. Even when analyzing total volume of fluid consumed for EtOH, the smaller EtOH-treated pups drank significantly more in terms of absolute volume of fluid (in ml consumed) than the water-treated and untreated animals (see Table 1). F3 water-treated pups were also smaller than their control counterparts and this effect did not change their EtOH consumption. All groups drank the same amount of water in terms of total volume (ml) consumed. Therefore, the difference in intake was specific to EtOH and not across all fluids. An unexpected result was that the F2 generation pups weighed significantly less than the other generations.

The increase in EtOH consumption following PAE may result from associative learning (Abate et al., 2004). Specifically, the orosensory and the postabsorptive reinforcing effects of EtOH may be associated in utero, creating a preference for the drug later in life (Nizhnikov et al., 2006a,b; Pautassi et al., 2012). While this explanation is logical when considering the increase in EtOH intake for the F1 generation, it cannot account for the persistence of this behavior for the F2 and F3 offspring.

Increased EtOH intake in the F1 generation could be the result of a change in the pharmacological reinforcing properties of EtOH. Prenatal EtOH increases the dose of EtOH rats find reinforcing (Nizhnikov et al., 2006a,b; Pautassi et al., 2012). While intake over a 15-minute period may seem to be a short period, increased appetitive properties of EtOH could exert increased seeking for the drug and result in more drinking (Anway et al., 2005; D'Addario et al., 2013; Finegersh and Homanics, 2014a; Kim et al., 2013; Nizhnikov et al., 2014).

A few candidate systems have been shown to play a role in increase EtOH acceptance in PAE animals. The opioid system is an important regulator of EtOH consumption and we have shown that PAE treatment down-regulates kappa opioid expression in brain regions critically involved in EtOH's rewarding properties such as the nucleus accumbens, the amygdala, and the hippocampus in the F1 generation (Nizhnikov et al., 2014). PAE may also change taste perception of EtOH and increase its acceptance (Díaz-Cenzano et al., 2014; Youngentob and Glendinning, 2009), possibly by reducing the burning sensation of EtOH through down-regulation of capsaicin receptors, resulting in lower aversive responses in the newborn (Glendinning et al., 2012).

Altered sensitivity to EtOH-induced sedative–hypnotic effects in offspring following gestational EtOH exposure is of critical importance as it has been associated with increased risk of abuse (Zucker and Wong, 2005). The present study assessed sensitivity to 2 doses of EtOH (3.5 or 4.5 g/kg) and found that gestational exposure attenuated acute EtOH-induced sedation–hypnosis at 3.5 g/kg but not 4.5 g/kg during adolescence. This occurred when compared to water-treated control group but not untreated animals in the F1 generation (Fig. 3A). This exact pattern of effects was transgenerationally transmitted to the EtOH-naïve F2 generation (Fig. 3B). The relevance of this effect to EtOH's pharmacokinetic properties was assessed by BEC analysis at waking, which revealed the same effects between EtOH-treated and water-treated control animals. F1 and F2 EtOH-treated subjects woke with a higher BEC compared to water-treated groups (see Fig. 3D,E). Interestingly, untreated subjects woke with a lower BEC than EtOH-treated subjects in the F1 and comparable BEC to EtOH-treated subjects in the F2 (see Fig. 3D,E) generation. Although the BEC result for the untreated group in the F1 generation was unexpected, it should be noted that the LORR for this group was slightly higher (although not significantly) than the EtOH group. Thus, the BEC may be a more sensitive marker than LORR to investigate the sensitivity to EtOH. Nevertheless, the difference between the EtOH-treated and water-treated groups supports the fact that the observed attenuated sensitivity in F1 and F2 generations are specifically mediated by prenatal EtOH exposure.

The results from our F1 group corroborate the previously reported attenuation effect to the sensitivity of EtOH (Barbier et al., 2009). Previous work, however, used much longer prenatal EtOH exposure with higher doses. A few others also report altered sensitivity to other low nonsedative doses of EtOH (Becker et al., 1993, 1995). Interestingly, our F2 LORR result suggests a transgenerational effect of prenatal manipulation, which was not carried onto the F3 generation. Although we did not measure other markers of hypothalamic–pituitary–adrenal (HPA) activity, these changes in LORR duration suggest gavage is stressful, as previously suggested (Kelly and Lawrence, 2008). It is important to

note that this suggestion is speculative in nature because, to our knowledge, no study has investigated the effect of gavage on stress response. Nevertheless, we suggest that the alteration in sensitivity to EtOH is possibly in part gavage induced rather than only EtOH induced. It is also possible that prenatal stress increases adolescent sensitivity to acute EtOH, while EtOH protects against this effect. A recent study shows a reversal of stress-induced deficits in cognition and anxiety when alcohol (2 g/kg of 20% v/v EtOH, i.g.) was given post-stress (Gomez et al., 2013). Thus, alcohol when administered during or immediately following a stressful event can provide a stress alleviating effect. Future research will be needed to verify if the EtOH treatment that was given to the dams significantly alleviated the effects of the manipulation in our paradigm.

The effect of prenatal treatment on sensitivity to EtOH's sedative-hypnotic effects is dose dependent. Interestingly, Barbier and colleagues (2009) tested adult animals with a 4.0 g/kg EtOH dose and reported an attenuation of LORR duration similar to what we observed at our lower but not higher dose. This group used a different rat strain, as well as chronic higher-dose prenatal and postnatal EtOH exposure. We propose 2 possible explanations for our dose-dependent effect. First, the HPA axis (Rivier, 1996; Rivier and Lee, 1996) influences EtOH metabolism. Stressors have been shown to alter BEC curves in humans (Sayette et al., 1994). At high doses of EtOH, neurosteroids influence alcohol metabolism via shared metabolic pathways (Helms et al., 2012). Therefore, at our higher dose of EtOH, metabolic rate and other pathways mediating EtOH-induced sedative-hypnosis may be compromised. Additionally, allosteric modulation and kinetic properties of the GABAergic system that principally mediate EtOH's sedative-hypnotic effects have been demonstrated to depend on concentrations of EtOH (Aguayo et al., 2002; Olsen et al., 2007; Wallner et al., 2006) and GABA (Houston et al., 2012). Wallner and colleagues (2006) suggest the existence of 2 EtOH modulation sites on certain GABA_A receptors, each of which are activated at either a lower or higher dose of EtOH. It is possible that the neurophysiological response to the 4.5 g/kg dose of EtOH involves additional factors that override the influence of other mechanisms mediating exposure differences at the lower 3.5 g/kg dose. Further investigation is required to verify these explanations.

Another possible mechanism that may result in altered response to EtOH is treatment-induced variations in maternal care. Low levels of maternal care have been suggested to represent a form of early-life stress and to program offspring HPA axis and GABA_A receptor subunit expression (Caldji et al., 2003; Cameron et al., 2005; Fish et al., 2004). Early-life stress increases EtOH self-administration as well as operant responding for EtOH in adulthood (Cruz et al., 2008; Huot et al., 2001). However, changes in maternal behavior are unlikely to be the cause of alterations in EtOH consumption and LORR duration in our F2 and F3 generations, as our laboratory has shown no differences in maternal behavior following EtOH exposure in the F2 generation (Popoola et al., 2015). Therefore, transgenerational transmission of these behaviors may not be related to maternal care. Furthermore, the 2 tests were not conducted at the same age; thus, the dissociation in the results between the intake test (which was transferred to 3 generations) and LORR (which was only transferred to 2 generations) may be a simple effect of maturation.

In conclusion, the present study provides evidence that moderate levels of gestational EtOH exposure increase EtOH consumption during infancy across a minimum of 3 generations. We also demonstrate that sensitivity to EtOH's sedative–hypnotic effects is altered. Future studies need to directly investigate the neurobiological alterations associated with the transgenerational transmission of gestational EtOH exposure-induced alterations in EtOH use-related behavior.

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Volume of Fluid Ingested Across All Generations

Fig. 1.

Consumption in ml fluid of water or 5% (w/v) ethanol by prenatal treatment across all generations. Data presented as mean $\pm$ SEM. (* $p < 0.05$) (UT—untreated). g/kg intake denoted within each bar. The g/kg intake in the group ingesting water is the theoretical g/kg intake if the fluid were ethanol.

Ethanol intake across 3 generations (F1 – F3)

Fig. 2. Consumption of 5% (w/v) ethanol by F1, F2, and F3 generations of infants (postnatal day 14) measured as g/kg drunk. Data presented as mean $\pm$ SEM. (* $p < 0.05$) (UT—untreated).

Fig. 3.

Top: Loss of righting reflex (LORR) duration for (A) F1, (B) F2, and (C) F3 generations of male adolescents (postnatal day [PND] 42) following acute 3.5 g/kg ethanol (EtOH).

Bottom: Blood EtOH concentration (BEC) at awakening for (D) F1, (E) F2, and (F) F3 generations of male adolescents (PND 42) following acute 3.5 g/kg EtOH. Group size indicated by number within each graph. Data expressed as mean $\pm$ SEM. (# $p < 0.1$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$).

Fig. 4.

Top: Loss of righting reflex (LORR) duration for (A) F1 and (B) F2 generations of male adolescents (postnatal day [PND] 42) following acute 4.5 g/kg ethanol. **Bottom:** Blood ethanol concentration (BEC) at awakening for (C) F1 and (D) F2 generations of male adolescents (PND 42) following acute 4.5 g/kg ethanol. Group size indicated by number within each graph. Data expressed as mean $\pm$ SEM. (* $p < 0.05$).

Table 1Mean $\pm$ SEM of Fluid Consumed and Pup's Weight Before Intake and Solution Ingested

	Prenatal treatment							
	Untreated				Water			
	Pup weight (gm)	Water volume ingested (ml)	Ethanol volume ingested (ml)	Pup weight (gm)	Water volume ingested (ml)	Ethanol volume ingested (ml)	Pup weight (gm)	Water volume ingested (ml)
F1	31.74 (± 0.87)	0.43 (± 0.03)	0.63 (± 0.04)	33.27 (± 1.01)	0.43 (± 0.04)	0.64 (± 0.08)	31.80 (± 0.89)	0.46 (± 0.05)
F2	29.33 (± 0.78)	0.46 (± 0.03)	0.74 (± 0.03)	28.32 (± 0.88)	0.46 (± 0.07)	0.65 (± 0.05)	25.66 [#] (± 0.89)	0.53 (± 0.06)
F3	35.83 (± 0.49)	0.53 (± 0.10)	0.62 (± 0.06)	30.55 [#] (± 0.59)	0.58 (± 0.08)	0.61 (± 0.06)	31.01 [#] (± 0.81)	0.55 (± 0.06)
								0.87 [*] (± 0.10)

The * denotes that ethanol-treated animals consumed significantly more ethanol (ml) than water-treated and untreated animals ($p < 0.0001$).

The # denotes that in the F2 ethanol-treated group, animals are lighter than untreated animals and that in F3 both the water and the ethanol groups were lighter than untreated ($p < 0.05$). SEM is denoted in parenthesis.