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Impact of Combined Prenatal Ethanol and Prenatal Stress Exposure on Anxiety and Hippocampal-sensitive Learning in Adult Offspring

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Abstract

Background—Prenatal ethanol (EtOH) and prenatal stress have both been independently shown to induce learning deficits and anxiety behavior in adult offspring. However, the interactive effects of these two developmental teratogens on behavioral outcomes have not been systematically evaluated.

Methods—We combined an established moderate prenatal EtOH consumption paradigm where Long-Evans rat dams voluntarily consume either a 0% or 5% EtOH solution in 0.066% saccharin water (resulting in a mean peak maternal serum EtOH concentration of 84 mg/dL) with a novel prenatal stress paradigm. Pregnant rats were exposed to 3% 2,3,5-trimethyl-3-thiazoline (TMT) for 20 minutes a day on Gestational Days 13, 15, 17, and 19. Adult female offspring were evaluated for anxiety-like behavior using an elevated plus maze and hippocampal-sensitive learning using a two-trial trace conditioning task.

Results—TMT exposure produced a three-fold increase in maternal serum corticosterone compared to non-exposed, unhandled controls. Neither prenatal exposure paradigm, either alone or in combination, altered maternal weight gain, ethanol consumption, maternal care of litters, litter size, pup birth weight, or pup weight gain up to weaning. Offspring exposed to prenatal stress displayed significant increases in anxiety-like behavior in the elevated plus maze in terms of open arm entries and time spent on the open arms, with no significant effect of prenatal EtOH exposure and no interaction of the two prenatal exposures. Performance in a two-trial trace conditioning task revealed a significant effect of prenatal ethanol exposure on freezing behavior on the testing day, with no significant effect of prenatal stress exposure and no interaction of the two prenatal exposures.

Conclusions—While each prenatal exposure independently produced different behavioral outcomes, the results indicate that there is no significant interaction of prenatal ethanol and prenatal stress exposures on learning or anxiety at the exposure levels employed in this dual exposure paradigm. Subsequent studies will examine whether similar outcomes occur in male offspring and whether other measures of anxiety or learning are differentially impacted by these prenatal exposure paradigms.

Keywords

Fetal Alcohol Spectrum Disorder; Maternal Stress; Trace Conditioning; Anxiety

INTRODUCTION

It is well established that exposure to ethanol during gestation is harmful to the developing fetus (Jones and Smith, 1973). The severity of the effects, which can range from intellectual and other behavioral deficits to physical dysmorphology, is related to the quantity of ethanol the mother consumed, how often during pregnancy she consumed ethanol, and when during her pregnancy the ethanol was consumed (Kelly et al., 2000; Sood et al., 2001). These effects are termed Fetal Alcohol Spectrum Disorders (FASD), an umbrella phrase which collectively describes the varying ranges of possible effects due to maternal consumption of alcohol during pregnancy. While 12.2% of women report consuming ethanol at some point during their pregnancy (CDC 2002), the vast majority of the 2-5% of US children diagnosed with a FASD are on the less severe end of the spectrum (Green et al., 2009; Kodituwakku, 2009; Willoughby et al., 2008), meaning that their exposure to prenatal ethanol results in mild or subtle changes in intelligence which persist into adulthood in the absence of physical dysmorphology characteristic of Fetal Alcohol Syndrome. In addition to the well-characterized cognitive deficits, children prenatally exposed to alcohol have also been described as having non-cognitive deficits such as altered stress reactivity, heightened anxiety, hyperactivity and attention disorders (Famy et al., 1998; Kodituwakku, 2009; Steinhausen et al., 2003). These behavioral changes can also persist well into adulthood and can exacerbate the cognitive challenges.

While alcohol is a known teratogen capable of causing a range of developmental defects, there are other pregnancy risk factors, such as maternal stress, that have been associated with adverse neurobehavioral outcomes in offspring. Due to the difficulty in defining and characterizing “maternal stress”, the precise epidemiology of prenatal stress is unknown, but the occurrence of anxiety during pregnancy is estimated at 8.5% (Ross and McLean, 2006). Similar to the effects of prenatal alcohol exposure, the effects seen in the offspring of mothers reporting stress or heightened anxiety during their pregnancy is dependent on the amount of stress incurred, the frequency of the stress, and the timing of the stress during their pregnancy (Mychasiuk et al., 2011; O'Connor et al., 2003). In human studies of prenatal stress, altered emotionality, elevated levels of anxiety, attention disorders, and cognitive impairments are consistently reported (Laplante et al., 2004; Martini et al., 2010; O'Connor et al., 2003; Rodriguez and Bohlin, 2005). In one study, 17.6% of respondents self-reported high levels of anxiety and consumed high levels of alcohol (CDC, 2002). Given the overlap in behavioral and cognitive consequences of prenatal ethanol and prenatal stress exposure, it is conceivable that when these two prenatal insults occur together, they may exacerbate the neurobehavioral consequences of each individual insult alone in affected offspring.

To date, there has been limited investigation on the putative synergistic effects of prenatal ethanol and prenatal stress exposure on behavioral outcomes. Using a non-human primate model, Schneider and colleagues have demonstrated that moderate prenatal alcohol exposure in combination with chronic stress during mid to late gestation results in altered stress reactivity and reduced behavioral adaptation to a novel environment in both male and female offspring (Schneider et al., 2004). Additional evidence from this model shows that offspring from mothers who consumed ethanol and were exposed to chronic stress during gestation have an increased ratio of dopamine D2 receptors to dopamine synthesis and this

observation correlated with reduced inhibition in a non-matching to sample task (Roberts et al., 2004).

While the non-human primate studies support the notion of synergistic interactions between prenatal ethanol and prenatal stress, it is not practical to pursue more detailed investigations of the underlying teratogenic mechanisms and the neurobiologic consequences of these interactions using this model. Thus, more detailed studies necessitated the development of a dual prenatal exposure model using rodents. However, the choice of prenatal ethanol and prenatal stress paradigms to employ in the characterization of a dual exposure paradigm warranted careful consideration from the onset. For example, many fetal alcohol researchers utilize methods of ethanol administration in their animal models which induce maternal stress, including intraperitoneal (IP) injection, oral gavage, and liquid diet ethanol administration (Norton and Kotkoskie, 1991), which each require the use of a third group (sham-injected, sham-gavage, or pair-fed, respectively) to control for the stress associated with the ethanol administration. However, the inherent additional stress involved with these models could confound the interpretation of results in a dual exposure paradigm as the dams exposed to both ethanol and stress would essentially be receiving two stressors. For example, Titterness and Christie (2011) recently reported that liquid diet administration produces a two-fold increase in circulating corticosterone level in rat dams compared to dams consuming rat chow ad libitum, which raises concern regarding the ability to systematically assess the effects of ethanol and stress in a dual exposure paradigm. Further, relative to repetitive maternal stress, it was important to utilize a stress procedure which the dams would not adapt to during the course of pregnancy, nor would the stressor adversely affect the level of ethanol consumption compared to non-stress controls.

In the present study, we employed a recently developed voluntary ethanol drinking paradigm (Savage et al., 2010), that minimizes the stress of ethanol administration and reduces the number of animals required for testing, in combination with a predator scent stressor paradigm, that minimizes the dam's habituation to the stressor, to examine the behavioral consequences of prenatal ethanol and prenatal stress exposure. Behavioral outcomes were measured in adult female offspring as they respond to forms of trace conditioning better than males (Dalla et al., 2009) and females demonstrate increased anxiety as a result of prenatal stress compared to males (Matthews and Phillips, 2012; Schulz et al., 2011).

METHODS

Unless indicated in parenthetical text, all materials were obtained from Sigma Chemical Company, St. Louis, MO. All animal care and usage was in compliance with the University of New Mexico Health Sciences Center Institutional Animal Care and Use Committee.

Voluntary Drinking Paradigm

As previously described in Savage *et al* (2010), four-month-old Long-Evan rat breeders (Harlan, Indianapolis, IN) were single-housed in plastic cages at 22° C and kept on a "reverse" 12-hour dark / 12-hour light schedule (lights on from 2100 to 0900 hours) with rat chow and water ad libitum. After acclimation to the animal facility, all female rats were provided 0.066% saccharin in tap water for four hours each day from 1000 to 1400 hours in addition to their ad libitum home cage food and water. On Days 1 & 2, the saccharin water contained 0% ethanol, on Days 3 & 4, the saccharin water contained 2.5% ethanol (v/v). On Day 5 and thereafter, the saccharin water contained 5% ethanol. Daily four-hour ethanol consumption was monitored for two weeks. At the end of two weeks of ethanol consumption, females that drank greater than one standard deviation below the mean of the entire group were removed from the study. The remainder of the females were assigned to either a saccharin (Sacc) control or 5% ethanol drinking (EtOH) group and matched such

that the mean pre-pregnancy ethanol consumption by each group was similar. Subsequently, females were placed with proven male breeders until pregnant, as evidenced by the presence of a vaginal plug. Female rats did not consume ethanol during the breeding procedure. Beginning on Gestational Day (GD) 1, rat dams were provided saccharin water containing either 0% or 5% ethanol for four hours a day in addition to their home cage food and water. The volume of 0% ethanol saccharin water provided to the controls was matched to the mean volume of saccharin water consumed by the ethanol drinking group. Daily four-hour ethanol consumption was recorded for each dam. Once a litter was delivered, the drinking tubes were no longer provided.

Predator Scent Stress Exposure Paradigm

Prior to GD13, one-half of the dams in each of the two drinking groups were randomly assigned to either a control (No Stress) or predator scent exposure (Stress) group. Dams assigned to the No Stress group were left undisturbed in their home cages throughout the maternal stress procedures to minimize elevations in corticosterone levels. At 1800 hours on GDs 13, 15, 17, and 19, dams in the Stress group were transported from the breeding colony room to a nearby treatment room and placed separately in individual cages, which were connected via plastic tubing to an air pump. Three percent (v/v) 2,3,5-Trimethyl-3-thiazoline (TMT, Contech, Victoria, BC, Canada) was pipetted onto a small piece of filter paper and placed inline between the air pump and the cage. Dams were exposed to the scent gently blown into the cage for 20 minutes, and then returned to their home cage. Subsequently they were transported back to the breeding colony room where they were maintained in a separately vented cubicle to reduce scent contamination with the No Stress groups. On GD20, stressed females were returned to their original housing location among the control females. A diagrammatic summary of the dual exposure paradigm is provided as Figure 1.

Rat dams from all four prenatal exposure groups were weighed on GDs 1, 7, 14, and 20. Prior to weighing on postnatal day (PN) 3, a single pup was removed from the nest and place on the opposite side of the cage. The latency for the dam to approach the displaced pup was recorded. Litters were weighed on PN3, 7, 14, and 21. The number of pups per litter was recorded on PN3. Litters were culled to 10 pups (five males and five females optimally) on PN3 and litters with less than 5 pups were not used in the study. Observed neonatal mortality in litters in each of the exposure conditions was less than 0.01% (data not shown). Dams were videotaped for 30 minutes PN7, 8, or 9 in their home cage in the home room between 1000 hours and 1200 hours. Time spent on their nest was recorded. All litters were weaned on PN25-27 into sex- and exposure group-specific pair housing (cages contained 2-3 adult females). All offspring were maintained in group housing conditions until behavioral testing.

Blood Collection, Preparation and Corticosterone Assay

In a separate set of dams at 1830 hours on GDs 13 and 19, pregnant dams were briefly anesthetized with isoflurane. A small nick was made at the base of the tail and 900 μ L of blood collected in gel collection tubes (Ram Scientific, Yonkers, New York). Samples were kept on ice until the collections were complete, then centrifuged at $3500 \times g$ for 5 minutes using a MiniSpin tabletop centrifuge (Eppendorf, Hamburg, Germany). Following centrifugation, plasma was collected and stored at -80° C until analysis. Plasma samples were thawed and maintained at 4° C prior to analysis. Samples were processed using a 125 I-Corticosterone Radioimmunoassay Kit (RIA, Siemens, Los Angeles, CA) according to the manufacturer's instructions. Samples were measured in a gamma counter (Auto-Gamma 5000, Packard Instrument Company, Meriden, CT).

Elevated Plus Maze

Anxiety-like behavior was assessed using an elevated plus maze apparatus similar to that originally described by Pellow et al., (1985). Adult female offspring (12-14 weeks of age) were exposed to used male bedding in their home cage to synchronize their estrus cycles into the diestrous phase 24 hours prior to behavioral testing. Synchronicity was confirmed via vaginal cytology in a separate set of female offspring similarly exposed to male bedding to avoid additional stress effects in behavioral testing. Animals were the same mean age and no difference in behavioral performance was noted between 12 week and 14 week old offspring. Animals were transferred to a testing room beginning at 0900 hours and immediately placed in the maze. The maze consisted of two open arms (50.8 cm long) and two closed arms (50.8 cm long). The open arms had a ledge 1 cm high to prevent the animals from slipping off the edge. The closed arms were surrounded by walls 30.5 cm tall and were covered to provide an enclosed environment. The plus-maze platform was elevated 50.8 cm off above the floor. Animals were allowed to freely move in the maze for five minutes. Their activity was video recorded and open arm entries, closed arm entries, and time spent in the open arms were recorded by an investigator blinded to the experimental condition of the subject.

Two Trial Trace Conditioning (TTTC)

Adult female offspring (12-14 weeks of age) were exposed to used male bedding in their home cage to synchronize their estrus cycles into the diestrous phase 24 hours prior to behavioral testing. Synchronicity was confirmed via vaginal cytology in a separate set of female offspring similarly exposed to male bedding to avoid additional stress effects in behavioral testing. Animals were the same mean age and no difference in behavioral performance was noted between 12 week and 14 week old offspring. On the conditioning day, animals were transferred from their home room to the conditioning room one at a time starting at 0900 hours. The animal was placed in the conditioning chamber, which consisted of a Habitest System (Coulbourn Instruments, Allentown, PA) equipped with a Precision Regulated Shocker and a 12" W × 10" D × 12" H test cage having a metal grid floor and allowed to acclimate to the novel surroundings for three minutes. As illustrated in Figure 2, immediately following the acclimation period, a constant, 30-second, 90 dB tone (the conditioned stimulus, CS) was played. After a 60-second trace interval where no stimulus was present, a two-second, one mA foot shock (the unconditioned stimulus, US) was administered. Ninety seconds later, the tone-trace-foot shock pairing was repeated. One minute following the second foot shock, the animal was moved back to their home cage and home room. The testing phase of the task began 24 hours after the conditioning session. The animals were moved individually from their home cage to a novel room and placed in a clean, plexiglass arena with a video monitoring camera positioned directly above it. The animal was placed in the novel environment for three minutes before the same 30-second, 90 dB tone from the conditioning session was played. Following the tone, the animal remained in the testing cage for three minutes and was then removed back to their home cage and home room. The entire six and a one-half minute testing sessions were recorded and freezing behavior was continuously scored across the session.

Statistical Analysis

Statistical analysis was conducted using SPSS (two-way Repeated Measures ANOVA, maternal ethanol consumption [Figure 3] and maternal stress reactivity [Figure 4]) and SigmaPlot 11.0 (2-way ANOVA, all other analysis). All graphs were assembled in Graphpad Prism 5.

RESULTS

Maternal Ethanol Consumption

Daily four-hour ethanol consumption was measured throughout pregnancy. The weekly consumption data for both of the ethanol-consuming No Stress and Stress groups is presented in Figure 3. A repeated measures two-way ANOVA analysis (stress X week of pregnancy) revealed a main effect of pregnancy week [$F(2,216)=5.63$, $p=0.004$] with no significant difference in ethanol consumption between the No Stress and the Stress exposed dams. Both groups drank significantly more during the second week of gestation compared to the first and third weeks. Overall, the dams in the No Stress group consumed a mean of 2.44 ± 0.09 g EtOH/kg/day while the TMT-exposed consumed 2.39 ± 0.09 g EtOH/kg/day. There was no statistical difference in the blood ethanol concentrations at the end of the four-hour drinking period between the dams exposed to TMT and No Stress dams (data not shown).

Maternal Stress Exposure

In order to monitor the pregnant dams' response to the predator scent, blood samples were analyzed for plasma corticosterone (CORT), a hormone produced in response to stressful stimuli. Samples were measured 30 minutes following the onset of the scent presentation or time-matched for non-stressed controls, following the first presentation of scent on GD 13 and last presentation on GD 19. A repeated measure (with missing measures) two-way ANOVA (Ethanol $\times$ Stress) revealed a significant main effect of Stress on CORT levels [$F(1,26)=134.75$, $p<0.01$], and a significant interaction of Stress and Ethanol [$F(1,26)=6.23$, $p=0.02$]. Post-hoc comparisons revealed a significant difference between the Sacc/Stress group and the EtOH/Stress group on GD 13 ($p=0.02$) with no effect between the two groups on GD 19 ($p>0.05$). There was no significant difference between the CORT levels on GD 13 and those measured on GD 19 for any of the four exposure groups.

Other Exposure Paradigm Data

Table 1 summarizes other data collected on the rat dams and their offspring. Maternal weight gain during pregnancy was not affected by either prenatal exposure paradigm, either alone or in combination. Likewise, the number of live pups born and their body weights on PN 3 and body weight at weaning were not different among the four prenatal treatment groups. Further, the quality of maternal care was not affected, based on measures of the latency to retrieve a displaced pup and the percent of time a dam spent on her nest.

Elevated Plus Maze

A two-way ANOVA analysis (Ethanol X Stress) of behavior on the elevated plus maze using adult females generated from the current model of combined exposure demonstrated a main effect of stress resulting in a reduction of percent of time spent on the open arms [$F(1, 36)=4.39$, $p=0.04$] and the number of open arm entries [$F(1, 36)=4.11$, $p=0.05$], with no effect of ethanol exposure and no interactive effect of the dual exposure. While there was a 47% difference between the Sacc and EtOH groups in the No Stress group in time spent in the open arms, this difference was not statistically significant (unpaired two-tailed t-test, $t=1.214$, $df=17$, $p=0.24$).

Two-Trial Trace Conditioning

We assessed behavioral performance in a hippocampal-sensitive learning task (Raybuck and Lattal, 2011), the Two-Trial Trace Conditioning task (TTTC). A two-way ANOVA (Ethanol X Stress) analysis of the freezing behavior during the three minutes following the tone on the testing day indicated a main effect of Ethanol resulting in a reduction of freezing

behavior [$F(1, 44)=4.88, p=0.03$]. There was no significant effect of Stress, nor an interaction of the dual exposure.

DISCUSSION

The primary objective of this study was to establish a dual exposure prenatal ethanol and prenatal stress paradigm that would allow a systematic examination of the impact of each of these two pregnancy risk factors on behavioral outcomes in the offspring. Operationally, the goal was to overlay a relatively novel predator scent stress paradigm with a previously established moderate prenatal ethanol exposure paradigm (Savage et al., 2010) where each exposure was delivered during the rodent awake cycle, yet in a manner that would minimize potential confounds of the dual exposure paradigm. The predator scent exposure time was scheduled near the awake-phase trough of the diurnal serum corticosterone (CORT) cycle both to maximize its impact on maternal CORT levels as well as to minimize potential alterations in voluntary ethanol consumption. As illustrated in Figure 2, TMT exposure during the third week of gestation did not alter ethanol consumption compared to the No Stress group (Figure 2).

Exposure to TMT elicited a roughly three-fold increase in maternal plasma CORT levels compared to No Stress controls. The elevations in maternal CORT corticosterone reported here are similar to the levels to those reported by Patin et al., (2002) as a result of acute and repeated exposure to cat predator scent on GD 10 or GD 14, as well as the levels reported by Barbazanges et al. (1996) as a result of a 45-minute restraint stress session during the last week of gestation. A small, but statistically significant, increase in the TMT exposure-induced elevation in maternal CORT was observed in the dams also consuming ethanol (Figure 3). However, this interactive effect was not associated with any synergistic effects on the various outcome measures examined in the course of this study.

Other potential confounds which could arise as a result of this dual exposure paradigm and thus create a challenge in interpreting the behavioral outcome data in adult offspring included altered maternal nutritional intake (reviewed in Alamy and Bengelloun, 2012), reduced care of litters (Liu et al., 2000), and the possibility that both prenatal ethanol and prenatal stress exposures can result in reduced litter size (Baker et al., 2008; Patin et al., 2002), or pup growth retardation (Drago et al., 1999; Levitt et al., 1996; Lopez-Tejer et al., 1986). However, no significant differences were observed among the four exposure groups relative to maternal weight gain during pregnancy, maternal care, number of pups per litter or pup weight gain to weaning (Table 1). Further, the lack of differences in the level of maternal care and the amount of weight gained by the pups until weaning mitigated the need to cross-foster the pups in order to avoid these possible confounds. Taken as a whole, this data also implies that each of these prenatal exposure procedures as conducted in this study, either alone or in combination, are relatively moderate in nature as they did not cause some of the gross deviations reported using more severe prenatal ethanol or prenatal stress exposure conditions.

Moderately severe prenatal stress exposure, elicited by the application of eighty 0.5 mA foot-shocks over a two-hour period to the dams every other day throughout gestation, has been shown to induce anxiety-like behavior in the adult female offspring (Estanislau and Morato, 2006). In the present study, we demonstrate that a more moderate prenatal stress procedure can also elicit increased anxiety-like behavior using the elevated plus maze (Figure 5). Further, this difference cannot be attributed to differences in either locomotor or exploratory activity as these measures were not different among the four prenatal exposure groups (data not shown).

The effects of prenatal ethanol exposure on anxiety have mainly been reported in male offspring. Prenatal exposure to relatively high ethanol levels (~350 mg/dL peak maternal serum ethanol concentrations) resulted in a significant reduction in the time spent on the open arms of the elevated plus maze (Dursen et al., 2006). However, this effect was not observed in adult female offspring using a more moderate prenatal ethanol exposure paradigm that produced a 135 mg/dL peak maternal serum ethanol concentration (Osborn et al., 1998). Similarly, our voluntary maternal ethanol consumption paradigm which produces a peak of 80 mg/dL did not affect the time spent on the open arms of the elevated plus maze. Further, the combination of prenatal ethanol exposure with prenatal stress did not increase anxiety-like behavior compared to prenatal stress alone suggesting the level of prenatal ethanol exposure achieved with our voluntary drinking paradigm was possibly too low to exacerbate the effect of moderate prenatal stress on the elevated plus maze.

Previous work utilizing the prenatal ethanol arm of the model presented here demonstrated reduced learning in a contextual delay conditioning task in prenatal ethanol-exposed female offspring (Savage et al., 2010). We replicated this finding using a hippocampal-sensitive two-trial trace conditioning task (Raybuck and Lattal, 2011), where prenatal ethanol-exposed offspring demonstrated significantly less freezing behavior in response to the acoustic stimulus on the testing day (Figure 6). In contrast, prenatal stress exposure did not impair learning in this task. This lack of an effect in female offspring from prenatal stress was also observed by Zagron and Weinstock (2006) who subjected dams to a single 30 minute session of restraint stress each day during the last week of gestation and Salomon et al. (2011) who used a variable stress paradigm during the last week of gestation. These observations are in contrast to findings that more severe levels of prenatal stress do cause learning deficits (Lemaire et al., 2000; Lordi et al., 2000; Wu et al., 2007) suggesting that the impact of maternal stress on learning may, in part, be dependent on the duration, frequency and/or intensity of the stressor events, gender being evaluated, and type of learning being assessed. However, direct comparisons between different prenatal stress studies is difficult because the stress paradigms employed vary widely by stressor type, the duration, frequency and intensity of stressor events, and by the fact that quantifiable markers, such as maternal corticosterone levels following stress exposure, are often not reported. In addition to not affecting two-trace conditioning alone, combining prenatal stress with prenatal ethanol exposure did not exacerbate the effect of prenatal ethanol exposure alone on learning, again suggesting a lack of interaction between these two prenatal insults on a specific behavioral response using the moderate exposure conditions employed in this study.

In summary, the work presented here represents a first effort to systematically characterize the neurobehavioral consequences of a dual prenatal ethanol and prenatal stress exposure paradigm. We observed differential behavioral effects in that moderate prenatal stress produced anxiety-like behavior in offspring but did not affect hippocampal-sensitive learning, whereas moderate prenatal ethanol exposure produced an opposite effect on these two measures. No synergistic interactions were observed at the levels of prenatal exposure employed here, suggesting that separate teratologic mechanisms and brain regions may be involved in mediating prenatal stress versus prenatal ethanol effects. A similar lack of synergistic interaction was recently reported relative to the effects of maternal stress on prenatal ethanol-induced alterations in synaptic plasticity (Titterness and Christie, 2012). Given that higher or more severe levels of prenatal exposure to either ethanol or stress affect both anxiety and learning behavior (Dursen et al., 2006; Lemaire et al., 2000; Lordi et al., 2000; Wu et al., 2007), one alternative explanation for our results is that different behavioral outcomes have differential sensitivities or “exposure thresholds” for producing adverse neurobehavioral outcomes in response to different prenatal insults. Thus, variations in a

“behavioral phenotype” may likely be attributable to varied degrees of exposure to variable combinations of prenatal insults.

A number of different types of follow-up studies employing this dual exposure paradigm are contemplated for future study. For example, one question is whether these behavioral responses to prenatal ethanol and/or prenatal stress are affected by the estrus cycle phase during which the behavioral testing is conducted. The diestrous phase was selected for initial study here as hormone levels remain relatively stable for the duration of the two-day phase, minimizing the potential impact of hormonal variations during the two-day trace conditioning task. Re-assessing these behaviors across different phases of estrous would be of interest since these variations can play a major role in learning and memory performance, as well as anxiety levels in female rats (Marcondes et al., 2001; Warren and Juraska, 1997). Another question is the impact of the dual exposure paradigm on male offspring. Females were employed in this initial study due to the effects of the voluntary maternal drinking paradigm on female offspring performance in a delay fear conditioning task (Savage et al., 2010) as well as a heightened susceptibility of female offspring to anxiety compared to controls (Weinstock, 2011). It would be important to evaluate male offspring to determine whether the dual exposure paradigm produces sex differences in behavioral responses. It is also possible that the male offspring from the combined exposure group would demonstrate interactive effects of prenatal ethanol and prenatal stress, as males have been reported to exhibit learning and memory impairments as a result of prenatal stress, while females are more often subject to increased anxiety behavior (reviewed in Weinstock, 2011). Beyond putative estrus phase- and gender-based differences in behavioral outcomes, it would be of interest to conduct a more systematic investigation of the impact of this dual exposure paradigm on different types of learning as well as other kinds of behavioral responses.

Another avenue for subsequent investigation would be to examine the extent to which manipulations in the design of the dual exposure paradigm would affect behavioral outcomes. For example, it is conceivable that a more severe prenatal stress paradigm may have produced deficits in two-trial trace conditioning and/or a synergistic interaction with the effects of moderate prenatal ethanol exposure on learning. Conversely, higher levels of prenatal ethanol exposure may have produced anxiety-like behavior and/or exacerbated the effects of prenatal stress on anxiety responses. While the voluntary ethanol consumption paradigm employed in the current studies minimizes the potential confound of stress due to the method of ethanol administration, it is limited in that it is not possible to produce markedly higher maternal serum ethanol concentrations. Higher maternal ethanol levels can be achieved with other ethanol administration paradigms, such as oral gavage or ethanol vapor inhalation. However, these administration paradigms are more stressful, thus increasing a potential confound for interpreting ethanol-stress interactions. Another experimental design manipulation to consider would be reversing the order of presentation of the prenatal exposures, having the maternal stress precede or even overlap with the ethanol consumption period, a pattern with perhaps more clinical relevancy. It is possible that the temporal separation of the prenatal exposures employed here contributed to the lack of interactive effects of the dual exposure paradigm on offspring. Again however, this approach would need to be carefully examined to determine whether concurrent exposures affects either ethanol consumption or stress response in a manner that could limit the ability to assess the interactive effects of each factor on offspring outcome measures.

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REFERENCES

- Alamy M, Bengelloun W. Malnutrition and brain development: An analysis of the effects of inadequate diet during different stages of life in rat. *Neurosci Biobehav Rev.* 2012; 36:1463–1480. [PubMed: 22487135]
- Baker S, Chebli M, Rees S, Lemarec N, Godbout R, Bielajew C. Effects of gestational stress: 1. Evaluation of maternal and juvenile offspring behavior. *Brain Res.* 2008; 1213:98–110. [PubMed: 18456246]
- Barbazanges A, Piazza PV, Le Moal M, Maccari S. Maternal glucocorticoid secretion mediates long-term effects of prenatal stress. *J Neurosci.* 1996; 16:3943–3949. [PubMed: 8656288]
- Centers for Disease Control. Alcohol use among women of childbearing age—United States, 1994–1999. *MMWR.* 2002; 51:273–276.
- Dalla C, Papachristos EB, Whetstone AS, Shors TJ. Female rats learn trace memories better than male rats and consequently retain a greater proportion of new neurons in their hippocampi. *Proc Natl Acad Sci U S A.* 2009; 106:2927–2932. [PubMed: 19188598]
- Drago F, Di Leo F, Giardina L. Prenatal stress induces body weight deficit and behavioural alterations in rats: the effect of diazepam. *Eur Neuropsychopharmacol.* 1999; 9:239–245. [PubMed: 10208294]
- Dursun I, Jakubowska-Doru E, Uzbay T. Effects of prenatal exposure to alcohol on activity, anxiety, motor coordination, and memory in young adult Wistar rats. *Pharmacol Biochem Behav.* 2006; 85:345–355. [PubMed: 17049371]
- Estanislau C, Morato S. Behavior ontogeny in the elevated plus-maze: prenatal stress effects. *Int J Dev Neurosci.* 2006; 24:255–262. [PubMed: 16698220]
- Famy C, Streissguth AP, Unis AS. Mental illness in adults with fetal alcohol syndrome or fetal alcohol effects. *Am J Psychiatry.* 1998; 155:552–554. [PubMed: 9546004]
- Green CR, Mihic AM, Nikkel SM, Stade BC, Rasmussen C, Munoz DP, Reynolds JN. Executive function deficits in children with fetal alcohol spectrum disorders (FASD) measured using the Cambridge Neuropsychological Tests Automated Battery (CANTAB). *J Child Psychol Psychiatry.* 2009; 50:688–697. [PubMed: 19175817]
- Jones KL, Smith MJ. Recognition of a fetal alcohol syndrome in early infancy. *Lancet.* 1973; 302:999–1001. [PubMed: 4127281]
- Kelly SJ, Day N, Streissguth AP. Effects of prenatal alcohol exposure on social behavior in humans and other species. *Neurotoxicol Teratol.* 2000; 22:143–149. [PubMed: 10758343]
- Kodituwakku PW. Neurocognitive profile in children with fetal alcohol spectrum disorders. *Dev Disabil Res Rev.* 2009; 15:218–224. [PubMed: 19731385]
- Laplanche DP, Barr RG, Brunet A, Galbaud du Fort G, Meaney ML, Saucier JF, Zelazo PR, King S. Stress during pregnancy affects general intellectual and language functioning in human toddlers. *Pediatr Res.* 2004; 56:400–410. [PubMed: 15240860]
- Lemaire V, Koehl M, Le Moal M, Abrous DN. Prenatal stress produces learning deficits associated with an inhibition of neurogenesis in the hippocampus. *Proc Natl Acad Sci U S A.* 2000; 97:11032–11037. [PubMed: 11005874]
- Levitt NS, Lindsay RS, Holmes MC, Seckl JR. Dexamethasone in the Last Week of Pregnancy Attenuates Hippocampal Glucocorticoid Receptor Gene Expression and Elevates Blood Pressure in the Adult Offspring in the Rat. *Neuroendocrinology.* 1996; 64:412–418. [PubMed: 8990073]
- Liu D, Diorio J, Day JC, Francis DD, Meaney MJ. Maternal care, hippocampal synaptogenesis and cognitive development in rats. *Nat Neurosci.* 2000; 3:799–806. [PubMed: 10903573]
- Lopez-Tejedor D, Ferrer I, Llobera M, Herrera E. Effects of prenatal ethanol exposure on physical growth, sensory reflex maturation and brain development in the rat. *Neuropathol Appl Neurobiol.* 1986; 12:251–260. [PubMed: 3736773]

- Lordi B, Patin V, Protais P, Mellier C, Caston J. Chronic stress in pregnant rats: effects on growth rate, anxiety and memory capabilities of the offspring. *Int J Psychophysiol.* 2000; 37:195–205. [PubMed: 10832006]
- Martini J, Knappe S, Beesdo-Baum K, Lieb R, Wittchen HU. Anxiety disorders before birth and self-perceived distress during pregnancy: associations with maternal depression and obstetric, neonatal and early childhood outcomes. *Early Hum Dev.* 2010; 86:305–310. [PubMed: 20547016]
- Marcondes FK, Miguel KJ, Melo LL, Spadari-Bratfisch RC. Estrous cycle influences the response of female rats in the elevated plus-maze test. *Physiol Behav.* 2001; 74:435–440. [PubMed: 11790402]
- Matthews SG, Phillips DI. Transgenerational inheritance of stress pathology. *Exp Neurol.* 2012; 233:95–101. [PubMed: 21281632]
- Mychasiuk R, Ilnytskyy S, Kovalchuk O, Kolb B, Gibb R. Intensity matters: brain, behaviour and the epigenome of prenatally stressed rats. *Neuroscience.* 2011; 180:105–110. [PubMed: 21335068]
- Norton S, Kotkoskie LA. Basic animal research. *Recent Dev Alcohol.* 1991; 9:95–115. [PubMed: 1758996]
- O'Connor TG, Heron J, Golding J, Glover V, ALSPAC Study Team. Maternal antenatal anxiety and behavioural/emotional problems in children: a test of a programming hypothesis. *J Child Psychol Psychiatry.* 2003; 44:1025–1036. [PubMed: 14531585]
- Osborn JA, Kim CK, Steiger J, Weinberg J. Prenatal ethanol exposure differentially alters behavior in males and females on the elevated plus maze. *Alcohol Clin Exp Res.* 1998; 22:685–696. [PubMed: 9622451]
- Patin V, Lordi B, Vincent A, Thoumas JL, Vaudry H, Caston J. Effects of prenatal stress on maternal behavior in the rat. *Brain Res Dev Brain Res.* 2002; 139:1–8.
- Pellow S, Chopin P, File SE, Briley M. Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. *J Neurosci Methods.* 1985; 14:149–167. [PubMed: 2864480]
- Raybuck JD, Lattal KM. Double dissociation of amygdala and hippocampal contributions to trace and delay fear conditioning. *PLoS One.* 2011; 6:e15982. [PubMed: 21283812]
- Roberts AD, Moore CF, DeJesus OT, Barnhart TE, Larson JA, Mukherjee J, Nickles RJ, Schueller MJ, Shelton SE, Schneider ML. Prenatal stress, moderate fetal alcohol, and dopamine system function in rhesus monkeys. *Neurotoxicol and Teratol.* 2004; 26:169–178.
- Rodriguez A, Bohlin G. Are maternal smoking and stress during pregnancy related to ADHD symptoms in children? *J Child Psychol Psychiatry.* 2005; 46:246–254. [PubMed: 15755301]
- Ross LE, McLean LM. Anxiety disorders during pregnancy and the postpartum period: A systematic review. *J Clin Psychiatry.* 2006; 67:1285–1298. [PubMed: 16965210]
- Salomon S, Bejar C, Schorer-Apelbaum D, Weinstock M. Corticosterone mediates some but not other behavioural changes induced by prenatal stress in rats. *J Neuroendocrinol.* 2011; 23:118–128. [PubMed: 21108672]
- Savage DD, Rosenberg MJ, Wolff CR, Akers KG, El-Emawy A, Staples MC, Varaschin RK, Wright CA, Seidel JL, Caldwell KK, Hamilton DA. Effects of a novel cognition-enhancing agent on fetal ethanol-induced learning deficits. *Alcohol Clin Exp Res.* 2010; 34:1793–1802. [PubMed: 20626729]
- Schneider ML, Moore CF, Kraemer GW. Moderate Level Alcohol During Pregnancy, Prenatal Stress, or Both and Limbic-Hypothalamic-Pituitary-Adrenocortical Axis Response to Stress in Rhesus Monkeys. *Child Dev.* 2004; 75:96–109. [PubMed: 15015677]
- Schulz KM, Pearson JN, Neeley EW, Berger R, Leonard S, Adams CE, Stevens KE. Maternal stress during pregnancy causes sex-specific alterations in offspring memory performance, social interactions, indices of anxiety, and body mass. *Physiol Behav.* 2011; 104:340–347. [PubMed: 21334352]
- Sood B, Delaney-Black V, Covington C, Nordstrom-Klee B, Ager J, Templin T, Janisse J, Martier S, Sokol RJ. Prenatal alcohol exposure and childhood behavior at age 6 to 7 years: I. dose-response effect. *Pediatrics.* 2001; 108:E34. [PubMed: 11483844]
- Steinhausen HC, Willms J, Metzke CW, Spohr HL. Behavioural phenotype in foetal alcohol syndrome and foetal alcohol effects. *Dev Med Child Neurol.* 2003; 45:179–182. [PubMed: 12613774]

- Titterness AK, Christie BR. Prenatal ethanol exposure enhances NMDAR-dependent long-term potentiation in the adolescent female dentate gyrus. *Hippocampus*. 2012; 22:69–81. [PubMed: 21080406]
- Warren SG, Juraska JM. Spatial and nonspatial learning across the rat estrous cycle. *Behav Neurosci*. 1997; 111:259–266. [PubMed: 9106666]
- Weinstock M. Sex-dependent changes induced by prenatal stress in cortical and hippocampal morphology and behaviour in rats: an update. *Stress*. 2011; 14:604–613. [PubMed: 21790452]
- Willoughby KA, Sheard ED, Nash K, Rovet J. Effects of prenatal alcohol exposure on hippocampal volume, verbal learning, and verbal and spatial recall in late childhood. *J Int Neuropsychol Soc*. 2008; 14:1022–1033. [PubMed: 18954482]
- Wu J, Song TB, Li YJ, He KS, Ge L, Wang LR. Prenatal restraint stress impairs learning and memory and hippocampal PKC β 1 expression and translocation in offspring rats. *Brain Res*. 2007; 1141:205–213. [PubMed: 17291462]
- Zargon G, Weinshock M. Maternal adrenal hormone secretion mediates behavioral alterations induced by prenatal stress in male and female rats. *Behav. Brain Res*. 2006; 175:323–328. [PubMed: 17023059]

A. Paradigm Overview **B. Single Day Overview** **Figure 1.**

Schematic diagrams illustrating the design of the prenatal ethanol and prenatal stress exposure paradigms. 1A: Disposition of female rats throughout the exposure paradigms. The concentration of ethanol (EtOH) is increased from 0% up to 5% (v/v) over the first five days and then 5% EtOH consumption measured for two weeks to establish pre-pregnancy drinking levels. After breeding, the dams are allocated into two groups. One group consumes saccharin water and the other consumes 5% EtOH. Beginning on GD13, each drinking group is subdivided with one-half subjected to maternal stress (3% TMT) and the other half left undisturbed in their home cage. Maternal TMT exposure is repeated on GD15, GD17 and GD19. 1B: Diagram of events over a twenty-four hour cycle. Room lights go off at 0900 hours. Drinking tubes are introduced at 1000 hours and removed at 1400 hours. Maternal stress dams are placed in air-tight cages at 1800 hours, subjected to 3% TMT in air for 20 minutes and then returned to their home cage. Room lights go on at 2100 hours.

A. Training Day **B. Testing Day (24 Hours Later)** **Figure 2.**

Diagram illustrating the experimental design of the two-trial trace conditioning paradigm. 2A: Training phase. 2B: The testing phase, which occurred 24 hours following training. Freezing behavior was measured during the three-minute recovery period after the tone.

Figure 3.

Weekly ethanol consumption during pregnancy and the impact of maternal stress on ethanol consumption. Data bars represent the mean $\pm$ S.E.M. ethanol consumed each week in the No Stress group (open bars) and the Stress group during the third week of gestation (filled bars). Asterisk denotes a main effect of gestational week on ethanol consumption (2-way ANOVA; *- $p < 0.05$).

Figure 4.

Impact of 3% TMT exposure on maternal serum corticosterone levels on Gestational Days 13 and 19. Data bars represent the mean $\pm$ S.E.M. corticosterone on GD13 (open bars) and GD19 (filled bars) in rat dams. Asterisks denote a statistically significant main effect of Stress (repeated measure two-way ANOVA; $p < 0.05$). Hatch symbol denotes a significant interaction of Stress and Ethanol (repeated measure two-way ANOVA; $p < 0.05$) and arrow denotes a significant increase in CORT levels in EtOH/Stress as compared to Sacc/Stress on GD 13 only (post-hoc comparison; $p < 0.05$)

Figure 5.

Impact of prenatal stress and/or prenatal ethanol exposure on female adult offspring behavior in the elevated plus maze. 5A: Number of open arm entries. Data bars represent the mean $\pm$ S.E.M. 5B: Percent time spent in the open arms. Data bars represent the mean $\pm$ S.E.M. Asterisks denote a significant main effect of Stress on exploratory behavior in the elevated plus maze (two-way ANOVA; $p < 0.05$.)

Figure 6.

Impact of prenatal stress and/or prenatal ethanol exposure on hippocampal-sensitive learning in the Two-Trial Trace Conditioning task. Data bars represent the mean $\pm$ S.E.M of twelve adult female offspring in each experimental group. Adult females prenatally exposed to ethanol showed reduced freezing on the testing day compared to their Sacc-exposed counterparts, with no impact of Stress. (Ethanol main effect; $p < 0.05$).

Table 1

Summary of the effects of prenatal exposure to ethanol and/or stress on rat dams and their offspring.

	Sacc / No Stress	Sacc / Stress	EtOH / No-Stress	EtOH / Stress
Maternal weight gain (g) during pregnancy	81.2 ± 45.4 ^a (11) ^b	76.5 ± 29.3 (13)	94.6 ± 26.0 (22)	94.8 ± 19.6 (16)
Pup weight on PN 3 (g)	10.6 ± 1.9 (10)	10.5 ± 1.9 (10)	11.3 ± 1.5 (18)	9.6 ± 1.8 (13)
Number of pups on PN 3	10.6 ± 1.9 (10)	10.5 ± 1.9 (10)	11.3 ± 1.5 (18)	9.6 ± 1.8 (13)
Pup retrieval time PN 3 (s)	12.7 ± 10.8 (10)	8.7 ± 5.9 (12)	9.9 ± 7.4 (20)	7.9 ± 6.0 (14)
Time spent on nest PN 7- 9 (%)	49.1 ± 4.2 (11)	58.1 ± 7.6 (9)	49.4 ± 4.4 (16)	60.7 ± 6.3 (9)
Pup weight at weaning PN 25-27(g)	40.9 ± 8.47 (10)	39.7 ± 9.7 (12)	39.8 ± 10.3 (20)	39.5 ± 7.1 (15)

^a - Mean ± standard error of the mean

^b - Sample size