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Gestational Ethanol and Nicotine Exposure: Effects on Maternal Behavior, Oxytocin, and Offspring Ethanol Intake in the Rat

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Abstract

Alcohol consumption and smoking during pregnancy is common, despite the known adverse effects of these drugs on fetal development. Though studies on the effects of each drug separately are published, little is known about the effect of concurrent use of alcohol and nicotine in humans or in preclinical models. In this report, we examined the impact of continuous gestational exposure to both ethanol via liquid diet and nicotine via an osmotic minipump on maternal behavior, offspring ethanol intake, and oxytocin levels in a rat model. Dams were tested for the onset of maternal behavior with litters of unexposed surrogate pups and then killed to examine oxytocin levels within specific brain regions. Drug-exposed offspring reared by surrogate dams were tested for ethanol intake at either adolescence or adulthood, and oxytocin levels were measured in relevant brain regions after behavioral tests. Dams exhibited minor deficits in maternal care, which were associated with lower oxytocin levels in both the ventral tegmental and medial preoptic areas compared to control dams. Prenatal exposure altered sex-specific ethanol intake, with differential effects at adolescence and adulthood. Oxytocin system changes were also apparent in the ventral tegmental and medial preoptic regions of drug-exposed adolescent and adult offspring. These results suggest that dam treatment with ethanol and nicotine can somewhat negatively affect the early rearing environment, and that prenatal exposure to both of these drugs results in drinking behavior differing from what would be expected from either drug alone. Oxytocin's possible involvement in the mediation of these effects is highlighted.

Keywords

Prenatal Alcohol; Prenatal Nicotine; Maternal Behavior; Oxytocin; Alcohol Drinking; Development

1. INTRODUCTION

Concurrent use and abuse of both ethanol and nicotine in clinical populations is well documented [16,54], and although there are many reports on the effects of ethanol or nicotine alone, few studies have systematically studied their combined effects on behavior or physiology. Of particular concern are women who drink and smoke during pregnancy. Despite a concerted public health effort, at least 12% of women report ethanol consumption during pregnancy [9,54], while 13–17 percent of pregnant women smoke [22,54] with 50% of women

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of childbearing age reporting drinking or smoking on a regular basis [54]. At least 1% of children born in the United States show symptoms of fetal ethanol exposure (FAE) [45] and babies prenatally exposed to either ethanol or nicotine manifest low birth weight and neurological deficits that are maintained throughout development [21,24,49]. This data, in conjunction with the fact that half of pregnancies are unplanned [17], demonstrates a situation in which the risk of inadvertent fetal drug exposure during the first trimester greatly increases.

It is important to note that the detrimental effects of gestational drug consumption on the developing fetus are not limited to the effects of *in utero* drug exposure, but also manifest in the parental care they receive, which may be altered in drug abusing women. Women who abuse drugs during pregnancy are more likely to be violent and aggressive [23,77,86] and children who live with substance abusing parents are 2.7 times more likely to be abused and 4.2 times more likely to be neglected [1]. Subsequently, children raised in these environments are more likely to develop emotional disturbances [15], have difficulty in school [25], and are more likely to abuse drugs as adults [15]. The majority of cases implicate ethanol in conjunction with other drugs as the precipitating factor, while fewer than 10% indicate ethanol alone as the primary factor [1]. This demonstrates the need to investigate how maternal polydrug use affects maternal behavior.

Studying the effects of combined drug use on specific behaviors in clinical populations poses many problems. In order to better control for intervening variables associated with clinical studies, rat models of drug treatment and prenatal exposure to drugs of abuse have proven useful and have reported interesting findings that may be helpful for elucidating potential problems and mechanisms associated with drug use. Rat pups require consistent care, including licking/grooming, nest building, and crouching/nursing, all of which have been well characterized in rodent models. Though it is likely that drug use would not solely be confined to gestational use in the clinical population, previous work has indicated that maternal behavior can be significantly altered by drug use during this critical period. Any effect on maternal behavior may have lasting effects on stress reactivity [18] and social behaviors [59], including the maternal behavior later performed by these offspring [26].

Gestational ethanol treatment alone has not been shown to affect the onset of maternal behavior, but has been shown to decrease maternal aggression [43,48,85], vastly unlike gestational treatment with some other drugs including cocaine, which significantly disrupts early maternal behavior and protective behavior of the young [28,29]. Conversely, postpartum ethanol treatment decreases the amount of time spent in the nest, nursing, crouching, and licking the pups [48,64] suggesting time of insult is important for ethanol treatment. Unlike ethanol, studies examining the effects of nicotine alone on maternal behavior are relatively sparse, with only one report showing no alteration in crouching [78]; although interestingly, human studies have shown a decreased likelihood of breastfeeding in smoking mothers [38]. The proportion of women who use the two drugs in conjunction dictates the need for further studies to examine how the combined drug use affects pup-directed maternal behavior.

While the effects of prenatal exposure to ethanol alone are well established in both the fetal ethanol syndrome literature (for reviews see [21,52]) and as a factor in later ethanol preference of exposed offspring [11], less is known about prenatal nicotine exposure alone and nothing following prenatal exposure to both drugs as far as preference for ethanol in later life. It is important to examine drug preference in both adolescence and adulthood, as adolescence is a period of rapid brain growth and maturation, as well as a time when most humans will experience their first voluntary drinking opportunities (for a review see [72]). Adolescents also react differently than adults to the socially facilitating [79] and physiological effects of ethanol [66], possibly related to reports demonstrating that adolescents drink more grams per kilogram compared to adults [76] and are less sensitive to the acute withdrawal affects, or 'hangover',

as demonstrated by tests of social anxiety [80]. Since the adolescent brain and behavior differ radically from adults, especially in females who have not yet started to menstruate, it is important to investigate sex as well as prenatal exposure effects on ethanol consumption, both during adolescence and early adulthood to determine the time point at which offspring are most sensitive to prenatal effects.

In the present study, we have investigated the effects of gestational exposure to both ethanol and nicotine on the onset of maternal behavior, and determined if prenatal exposure to these drugs impacts ethanol preference and consumption during either adolescence or early adulthood in male and female offspring. Additionally, we examined oxytocin levels in relevant brain regions of the dams and offspring following behavioral testing since oxytocin has been associated with altered levels of maternal behavior [61] and with both drug reward and tolerance to other drugs of abuse [35,68]. It was hypothesized that the initiation of maternal behavior on postpartum day (PPD) one would be disrupted compared to control treatment dams, and that if maternal behavior was disrupted, that oxytocin levels would also be altered by gestational treatment. We also hypothesized that prenatal exposure to these drugs would increase ethanol preference and if so then oxytocin levels would be altered in brain regions associated with social or reward behavior circuitry compared to control offspring.

2. METHODS

2.1. Subjects

Virgin Sprague-Dawley rats (Charles River, Raleigh, N.C.) were group housed until breeding with *ad libitum* access to water and food for a two week habituation period. During breeding, each female rat was housed with a single breeder male until the presence of a sperm plug or a vaginal smear confirmed pregnancy. The day pregnancy was confirmed, designated as gestational day (GD) zero, the females were weighed and then singly housed. Dams were then randomly assigned to one of two treatment conditions, or assigned as a surrogate (pup-providing) dam. Singly housed females were housed in a reverse light cycle for the first 7 days (lights out at 0900) and then switched to a normal light cycle (lights on at 0700), so that the females gave birth during the daylight hours [26,42,46]. All subjects were weighed every five days to monitor health and pregnancy.

2.2. Treatment

Females were assigned to either a combined ethanol/nicotine treatment (E/N) or a control group. On GD zero, dams were given *ad libitum* access to water and rat pellets. All dams retained *ad libitum* access to water until labor (approximately GD21). On GDs one through four, the pellet diet was replaced with 100mL control liquid diet (LD82, Shake and Pour: Bio-Serv, Frenchtown, NJ).

2.2.1. Ethanol Treatment—Treatment dams received 100mL ethanol liquid diet (LD82, Shake and Pour-Ethanol: Bio-Serv, Frenchtown, NJ), while the control dams continued to receive control liquid diet (LD82, Shake and Pour-Control: Bio-Serv, Frenchtown, NJ) nutritionally balanced with the ethanol diet. E/N dams were introduced to the ethanol liquid diet on GD five when they received diet containing 17.5% ethanol-derived calories (EDC). From GD six through 20, dams received 35% EDC liquid diet. On GD 21 immediately following birth, all dams were switched to pellet diet as pilot work indicated pup survival was lowered if dams remained on the ethanol diet. To control for the anorectic effects of ethanol and nicotine, control rats were food yoked to the mean liquid diet consumption by E/N dams from GD eight through 21. All dams had *ad libitum* access to water throughout gestation to prevent dehydration.

2.2.2. Nicotine Treatment—On GD four, at approximately 0930, dams were surgically implanted with an osmotic minipump (Alzet: 2ML4, Durect, Cupertino, CA) containing bacteriostatic water (Abbott Diagnostics, Abbott Park, IL) and either nicotine hydrogen tartrate (Sigma Chemical, St. Louis, MO) or sodium bitartrate (Fischer Scientific, Pittsburgh, PA), which administered nicotine continuously until dam sacrifice at a dose of approximately 3–6 mg/kg/day. It is important to note that this design resulted in nicotine administration on PPD 1, which was unfortunately the result of the active duration of commercially available osmotic pumps. In order to cease nicotine treatment simultaneously with ethanol treatment, surgery would be required to remove the pump, providing a potentially dangerous and powerful confound to our studies. However, nicotine administration alone has not been shown to alter maternal behavior notably (see Introduction), thus it is unlikely that this played a significant role in the results we report. Animals were anesthetized with ether, and a small incision was made in the left rear flank of the dam, and then a subcutaneous pocket was created using sterile forceps. The pump was inserted into the pocket, and the wound was sealed with wound clips. Bupivacaine (Fischer Scientific, Pittsburgh, PA) was administered to the wound site and animals were allowed to recover for 30 minutes under heat lamps until returning to their home cage. Animals were monitored for three days post-surgery and topical antibiotic ointment (Fougera bacitracin zinc and polymycin B sulfate, Melville, NY) was applied daily. Wound clips were checked daily until removal on GD 14.

2.3. Blood Ethanol Concentration

Blood ethanol concentration (BEC) was measured for all dams (both ethanol/nicotine-exposed and control groups) on GD 15 at approximately 0715 (immediately following the dark cycle) using blood samples collected from the tail, and following maternal behavior testing on PPD one using trunk blood samples collected following sacrifice. On GD 15, tails were cleaned and sterile single blade razors were used to make a small tail-tip nick for blood collection. Blood samples were stored in heparin at 4°C until measurement. Tail blood (6µl) and standards (6µl; 0–300mg%) were mixed with 375ml of distilled water and 0.5g NaCl in 12×75mm borosilicate glass culture tubes. The tubes were capped and then heated to 55° C for 10-min in a water bath, at which point 1.5ml of headspace gas was removed with a plastic 3.0ml syringe and injected directly into an SRI 8610C gas chromatograph (Torrance, CA) equipped with an external syringe adapter and 1.0ml external loading loop. The oven temperature was isothermal at 140°C and contained a Hayesep D column and a flame ionization detector. Hydrogen gas, carrier gas (also hydrogen), and internal air generator flow rates were 13.3, 25, and 250ml/min respectively. Peak retention time was 2-min and the areas under the curve were analyzed with SRI PeakSimple software for Windows running on a Dell Inspiron 3500 laptop computer. E/N treatment resulted in average BECs of 95.0mg% (0.09 blood alcohol concentration) on GD 15, while PPD one BECs were found to be 0.0mg%.

2.4. Cross Fostering

On the day of parturition (PPD one), pups were removed from the dam, weighed, counted, and sex determined before being culled to litters of 4 females and 4 males. E/N and control litters were culled thirty minutes prior to maternal behavior testing and at that time cross-fostered to an unexposed surrogate dam. Pups remained with surrogate dams until weaning at postnatal day (PND) 21, when they were separated into sex-specific group housing until behavioral testing. E/N and control dams received unexposed pups from surrogate dams born within 24 hours of the treatment dam's delivery.

2.5. Maternal Behavior Testing

Maternal behavior test procedures on PPD one have been previously described [26]. Test dams and their unexposed foster litters were brought in the dam's home cage to an enclosed 400 ×

460cm behavioral observation room, where dams were removed from their cage and weighed, and their pups removed. Dams were placed back in their home cages without pups, and the cages were placed in a 61cm high × 41cm wide × 51cm deep dimly lit testing cubicle, designed to reduce environmental distractions, for a 30-min habituation period. Pups were placed in a warm plastic cage lined with paper towels on top of the dam's testing chamber. After habituation, nesting material (ten 2.5cm strips of paper towel) was placed at the back of the cage and each dam's foster litter was placed in the front of her cage. Videotaping with a Panasonic VHS (AG188U) recorder with low light sensitivity began as soon as the pups were placed in the cage and continued for 30 min. The frequency, duration, and latency of behaviors were later scored by two independent observers. The behaviors observed included **pup retrieval** (scored when dams retrieve the first two and again when all 8 pups were retrieved to rear of cage); **touch/sniff** (dam touches pup with her forepaws nose or sniffs them); **lick pup** (dam licks pup); **nest-building** (manipulating paper towel strips with mouth or paws); **self grooming** (dam grooms herself with paws or tongue); **rest away from pups** (dam lies down, away from pups), **lay on pups** (dam is completely flat on top of pups), **crouching** (nursing posture, high dorsal arch with front legs splayed and pups underneath the dam), and **other** (any behavior other than those listed). Immediately following maternal behavioral recording dams were sacrificed for brain dissection and later oxytocin assay.

Changes in oxytocin levels have been most strongly linked to alterations in the onset of maternal behavior, rather than the maintenance of established maternal behavior. As discussed in the introduction, the behavioral patterns observed in a single 30 minute test immediately following parturition have proven to be highly correlated with alterations in the oxytocin system resulting from antagonist administration, brain lesion, and even gestational cocaine treatment. Thus, it is highly likely that such correlations would exist in the current ethanol/nicotine model.

2.6. Offspring Ethanol Preference

One male and one female from each E/N and control litter were chosen to undergo preference testing beginning on PND 30, and a separate male and female were chosen to undergo preference testing beginning on PND 60. The procedures for each test period are detailed below and illustrated in Figure 1. For all preference testing, 95% ethanol (Aaper Ethanol, Shelbyville, KY) or saccharin salt (Acros Organics, New Jersey) was diluted with tap water solutions were measured into 100mL plastic graduated cylinders with rubber stoppers and stainless steel sipper tubes (Fisher Scientific). Cylinders were inverted on the cage front with sippers crossed and cylinder location side was alternated daily to control for side preferences. Volumes were collected for the amount of ethanol and water consumed on each day, and comparisons were made between groups and between sexes, including measures of total overall liquid consumption, grams of water consumed per 100 grams of animal weight per day, grams of ethanol consumed per kilogram of animal weight per day, and the percentage of total liquid consumed per day (both ethanol and water) that was ethanol (also referred to as Preference).

2.6.1. Postnatal Day 30 Adolescent Preference Test—Sweet preference was not examined in adolescents. We have conducted saccharin preference tests in other pilot studies in adolescent populations following prenatal exposure to the same doses of nicotine and ethanol used in the present study. In these studies we found no differences between control and ethanol nicotine exposed offspring on this measure, but in retrospect it would have been preferable to conduct this test in these offspring as well. Additionally, to our knowledge there have been no reports documenting the similarities between lower concentrations of ethanol and saccharin during adolescence, so a test for sweet preference was only conducted during adulthood. Acquisition: One male and one female from each of the control and treatment litters were singly housed on PND 29, and two inverted 100mL graduated cylinders containing tap water were placed on the front on the cage to habituate the subjects to bottle placement. Beginning on PND

30 and continuing for four days, offspring were simultaneously presented with one 100mL graduated cylinder filled with tap water and another filled with a 10% ethanol solution, side by side, in a choice task. **Forced Consumption:** Beginning on PND 35, a single cylinder with 10% ethanol was presented for three days as the sole fluid source. **Two-Bottle Choice:** Beginning on PND 38, a five day two-bottle choice (water and ethanol as done in the acquisition phase) was again presented to the subjects. Following completion of adolescent testing (PND 43), offspring were decapitated and the whole ventral tegmental area (VTA), medial pre-optic area (MPOA), hippocampus, and amygdala were removed from brain for later oxytocin radioimmunoassay.

2.6.2. Postnatal Day 60 Adult Preference Test—Sweet Preference: A separate male and female from each of the treatment and control litters were singly housed beginning on PND 59. On PND 60, a single day two-bottle saccharin preference test using tap water and a 0.1% saccharin solution was initiated to assess sweet preference. **Acquisition:** Following the saccharin test, on PND 61, a two-bottle choice (ethanol/water) was employed using ascending ethanol concentrations over a 5 day period (2%, 4%, 6%, 8%, and 10%). **Forced Consumption:** On PND 66, the bottle containing water was removed, and the rats were allowed access to only 10% ethanol solution for three consecutive days. **Two-bottle choice:** Beginning on PND 69, subjects were again given the two-bottle choice between water and 10% ethanol for an additional 5 consecutive days. Following adult preference testing (PND 74), offspring were decapitated and the same brain regions as in the adolescents were removed for a later oxytocin radioimmunoassay.

2.7. Tissue Collection

Animals were killed within an hour of test completion (PPD one for dams, and PND 43 or 74 for the offspring). Subjects were decapitated and whole brain regions dissected out on ice immediately. Brain dissection procedures have been previously described [27]. Brains were coronally sectioned from the ventral side rostral to the optic chiasm (approximately A7100 μ m, according to [31]) and just caudal to the optic chiasm (approximately A5800 μ m) to define the preoptic-anterior hypothalamic area. Vertical cuts inferior to the lines of lateral ventricles and a horizontal cut ventral to the anterior commissure were made to produce a block section of the medial preoptic area. The brains were sectioned once again just caudal to the tuber cinereum (approximately A3800 μ m) and slightly above the cerebellum and the amygdala was removed in this section, the ventral tegmental area was dissected from the caudal section by making dorsoventral cuts medial to the optic tracts with a dorsal cut at the ventral extent of the central gray, and the whole hippocampus was then removed from the caudal remainder of the brain. After extraction, tissue was weighed and placed into tubes on dry ice. The brain areas were then stored at -80°C until needed for radioimmunoassay (RIA) testing.

2.8. Oxytocin Radioimmunoassay

Brain region tissues were homogenized in cold buffer (19 mM monobasic sodium phosphate, 81 mM dibasic sodium phosphate, 0.05M NaCl, 0.1% BSA, 0.1% Triton 100, 0.1% sodium azide, pH 7.4) and centrifuged at 3,000 g for 30 min. Oxytocin immunoreactive content was assayed in the supernatant according to a protocol from Peninsula Labs (Belmont, CA). Samples and standards (1.0 – 128.0 pg) were incubated in duplicate for 16 to 24 hr at 4°C with rabbit antioxytocin serum. They were then incubated for 16 to 24 hr at 4°C with ^{125}I -Oxytocin after which time normal rabbit serum and goat anti-rabbit IgG serum were added and incubated 90 min at room temperature. The ^{125}I -Oxytocin bound to the antibody complex was separated from free by a 90-min centrifugation at 4°C . The radioactivity in the pellet was measured using a LKB ClinGamma counter (PerkinElmer Wizard 1470-005), which calculates the picogram content of oxytocin in each sample from the standard curve. The intra-assay coefficient of

variance (CV) was 4.05%, and inter-assay CV was 8.95%. The sensitivity of the assay was approximately 0.5 pg/tube.

2.9. Data Analysis

Videotaped maternal behavior sessions were scored by two independent observers, blind to treatment condition, with inter-and intra-reliability set at 90–100% concurrence for frequency and latency, and 80% or better agreement for duration of behaviors displayed by the dam. An in-house computer program calculated the frequency, duration, latency, and sequence of all relevant behaviors displayed by the dams. Behaviors not displayed by the dams were assigned the highest possible latency (1800s for 30-min test).

Statistical analyses were performed using Systat and SAS statistical programming software and all values of significance were set $p \leq 0.05$. Gestational variables, including blood ethanol levels, gestational length, dam gestational weight gain, number of pups per litter, number of still-born pups, birth litter weight, birth litter sex ratio, and culled/cross-fostered litter weight were analyzed by Analysis of Variance (ANOVA). Log linear models for count data (frequency) were used to examine between group differences in maternal behavior on PPD one, while weighted additive models for time of event best fit the duration dataset for this behavioral test, with weights inversely proportional to the within-cell variance estimate. Latency data were analyzed using the Cox Proportional Hazard model, a semi-parametric survival analysis procedure that examines the time to an event. As the offspring preference test was a complex design, offspring data were analyzed using repeated measures additive models to examine between group differences (prenatal exposure condition and sex) within each phase as well as over all the repeated days of testing. Measures examined included total overall liquid consumption (water consumed plus ethanol consumed), grams of ethanol consumed per kilogram animal weight per day (denoted as g/kg EtOH), grams of water consumed per 100 grams animal weight per day (denoted as g/100g H₂O), and the percentage of total liquid consumed that was ethanol (also referred to as Preference). Since water consumption is considerably higher than ethanol consumption in these animals, g/100g H₂O is reported, while g/kg EtOH are reported to maintain consistent scales of results and improve the clarity of the results. Results are presented first for prenatal exposure condition-dependant effects, then for sex-dependant effects, and lastly for effects of the interaction between sex and prenatal exposure condition for each age tested (adolescent and adult). Oxytocin levels (pg/mg tissue) for each brain region examined were analyzed using ANOVA, with Tukey post-hoc tests to examine significant group differences.

3. RESULTS

3.1. Original Dams

3.1.1. Gestational Results—Table 1 presents the means $\pm$ SEM for gestational measures. There were no between-group differences in the number of pups per litter, number of stillborn pups, gestational length, or sex ratio. E/N dams gained less weight than controls during pregnancy ($p \leq 0.01$) and E/N birth litters weighed less than control litters ($p \leq 0.01$). There were no differences in the culled litter pup weights received from surrogate dams in either group.

3.1.2. Maternal behavior—No differences were observed in latency or duration of any task; however, as shown in Figure 2, E/N dams touched/sniffed pups less frequently ($\chi^2=3.55$, $p \leq 0.05$), left the nest and rested away from pups more often ($\chi^2=10.09$, $p \leq 0.01$), and returned to the nest less often after leaving it than did controls ($\chi^2=5.50$, $p \leq 0.05$).

3.2. Offspring

3.2.1. Postnatal Weight Gain—There were no differences in weight at the beginning of either the adolescent or adult testing periods (PND 30/60) resulting from the prenatal exposure condition of the offspring. Not surprisingly, in both E/N-exposed and controls, males weighed more than females at both PND 30 [$F(1,41)=5.2$, $p\leq 0.05$] and PND 60 [$F(1,31)=165.4$, $p\leq 0.01$]. Following the final adult testing phase (PND 73), there was a significant treatment by sex interaction, indicating that E/N males weighed less than control males [$F(1,30)=11.2$, $p\leq 0.01$].

3.2.2. Adolescent Ethanol Preference Testing

Effects of Prenatal Ethanol/Nicotine Exposure: As shown in Figure 3, there were no differences between groups seen in the acquisition phase of testing (PND30-34), or the forced consumption phase (PND 35-37) due to prenatal exposure condition. The only effect of prenatal exposure was seen on the first day of two-bottle choice (PND 38), in which control animals drank more ethanol ($\chi^2=7.10$, $p\leq 0.01$) and exhibited a higher preference for ethanol compared to E/N animals ($\chi^2=7.35$, $p\leq 0.01$), regardless of sex. However, the amount of water consumed was not different between these groups on this particular test day. There were no effects of prenatal exposure condition seen during the remainder of the two-bottle choice phase (PND 39-42).

Effects of Sex: Analysis of data from the acquisition phase revealed the robust sex effect seen in Figure 4, with females drinking more g/kg ethanol than males ($\chi^2=13.48$, $p\leq 0.05$) and demonstrating a higher preference for ethanol than males ($\chi^2=11.40$, $p\leq 0.01$). There were no effects of sex alone seen in the forced consumption phase. During the two-bottle choice phase, there were significant sex effects, as males in both groups drank more total liquid than females ($\chi^2=10.45$, $p\leq 0.01$), however, there was no solely sex-based differences in ethanol consumption during this phase.

Interaction between Prenatal Ethanol/Nicotine Exposure and Sex: During the acquisition period there was a significant interaction between sex and prenatal exposure, with control females consuming less water per body weight compared to control males ($\chi^2=4.01$, $p\leq 0.05$), while E/N females did not exhibit any difference in water consumption compared to males. There was also a significant interaction during the forced consumption phase, when only ethanol was available (no water), with E/N females consuming more g/kg ethanol compared to E/N males ($\chi^2=3.7$, $p\leq 0.05$). Interestingly, this effect continued into the two-bottle choice phase, where E/N females continued to consumed more g/kg ethanol than E/N males ($\chi^2=4.78$, $p\leq 0.05$).

3.2.3. Adult Saccharin Preference Testing—A single day of saccharin testing on PND 60 revealed no effect of prenatal exposure condition on saccharin preference, but there was a significant sex effect, with females in both control and treatment groups consuming more saccharin solution (ml/kg/day) compared to males [$F(1,30)= 5.857$, $p\leq 0.05$].

3.2.4. Adult Ethanol Preference Testing

Effects of Prenatal Ethanol/Nicotine Exposure: There were no effects of prenatal exposure alone on any measurement examined during any phase of testing (see Figure 5).

Effects of Sex: There were no differences in ethanol or water consumption between males and females seen during the acquisition (PND 61-65), forced consumption (PND 66-68), or two-bottle choice phases (PND 69-73).

Interaction between Prenatal Ethanol/Nicotine Exposure and Sex: E/N-exposed females drank less total liquid (water and ethanol) than both E/N-exposed males ($\chi^2=14.46$, $p\leq 0.01$) and control females ($\chi^2=6.46$, $p\leq 0.01$) during the acquisition phase. Additionally, within the control group, control females exhibited a higher preference for ethanol ($\chi^2=3.58$, $p\leq 0.01$) and higher g/kg ethanol consumption than control males ($\chi^2=6.06$, $p\leq 0.05$) during the acquisition phase. Control females also consumed more ethanol than control males ($\chi^2=4.58$, $p\leq 0.05$) during the two-bottle choice phase while drinking less water ($\chi^2=5.68$, $p\leq 0.05$), resulting in higher preference ($\chi^2=7.50$, $p\leq 0.01$). Within the E/N group, no differences were observed in ethanol consumption or preference at any phase.

3.3. Oxytocin Radioimmunoassay

3.3.1. Postpartum Day One Dams—E/N-exposed dams had lower levels of oxytocin in both the MPOA [$F(1,20)=4.49$, $p\leq 0.05$], and the VTA [$F(1,22)=5.82$, $p\leq 0.03$] compared to control dams (see Figure 7), with no differences in oxytocin levels in the amygdala or hippocampus.

3.3.2. Postnatal Day 30—As seen in Figure 8, adolescent females in both groups had higher oxytocin levels in the MPOA [$F(1,41)=5.68$, $p\leq 0.03$], and lower levels in VTA [$F(1,38)=15.04$, $p\leq 0.01$] compared to males. In addition, regardless of sex, E/N-exposed offspring had higher oxytocin levels in the VTA [$F(1,38)=5.12$, $p\leq 0.03$] compared to control offspring.

3.3.3. Postnatal Day 60—As was seen in the adolescents, Figure 9 indicates that there was an overall sex effect such that adult females across treatment conditions also had higher oxytocin levels in the MPOA [$F(1,29)=6.08$, $p\leq 0.05$], and lower levels in the VTA than males [$F(1,27)=18.246$, $p\leq 0.01$]. In general, control offspring of both sexes had higher levels of oxytocin compared to E/N-exposed offspring in the VTA [$F(1,27)=14.784$, $p\leq 0.01$]. There was also a significant treatment by sex interaction [$F(1,27)=7.69$, $p\leq 0.01$] in adulthood. Control males had higher levels of oxytocin in the VTA than control females ($p\leq 0.01$), E/N males ($p\leq 0.01$), and E/N females ($p\leq 0.01$). There were no effects in the other brain regions of interest.

4. DISCUSSION

We found that combined treatment with ethanol and nicotine throughout gestation results in minor deficits in maternal behavior onset, with ethanol/nicotine-exposed dams (E/N) less likely to be in the nest, more likely to rest away from their surrogate pups, and touch/sniff those pups less frequently on PPD one. There was no impact on retrieval, crouching, or licking behaviors, which are most essential for pup survival since they offer the opportunity for pups to feed and eliminate waste. This replicates findings that indicated the capacity to meet the basic physical demands of the pups is not altered by gestational ethanol alone [44,78], unlike the effect of gestational cocaine on these behaviors [26,28–30,41,81–84,88]. These results raise questions about whether alterations in non-pup directed behaviors, such as spending less time in contact with pups, can impact important aspects of development other than basic survival. The dam's presence in the nest is important because separation from the dam is stressful for pups, and multiple periods of separation can alter stress reactivity [39], anxiety and depressive-like behaviors [50], as well as drug seeking behaviors [51] in pups well into adulthood.

The effects we report here are similar to, though more moderate than, reports from previous studies following postpartum ethanol treatment alone, which more strongly impair maternal behaviors [48,64] compared to gestational ethanol alone, which has a lesser affect on maternal behaviors in the rat [43,85]. Maternal behavior following gestational and early postnatal nicotine treatment has not been thoroughly examined in a preclinical model, but in clinical populations, smoking alone has been shown to decrease breastfeeding, potentially through

nicotine's inhibition of prolactin release, though no effect has been observed in plasma oxytocin levels in response to nicotine [3,5]. Given the paucity of data regarding the effects of nicotine in isolation on maternal behavior, it is impossible to draw any conclusions at this time. As our study employed both drugs, this exposure paradigm, specifically with respect to ethanol, may have resulted in behavioral tolerance to some of the effects of these drugs at the time of testing.

Ethanol/nicotine-exposed dams were exposed to forced ethanol consumption for 14 days, long enough to induce dependence and create withdrawal symptoms in virgin females [13]. Though ethanol withdrawal can cause anxiety and decreases in locomotor activity [55], withdrawal during the postpartum period has not been studied. Ethanol/nicotine-exposed dams are without ethanol access for approximately 24–36 hours prior to birth and testing. It is possible that this period was sufficient for ethanol withdrawal to pass. Additionally, high levels of central oxytocin, such as those seen immediately following parturition [61], can relieve acute withdrawal symptoms [75] and may have minimized the effects of withdrawal in the current study. Although no direct measurement of withdrawal was performed, the behaviors displayed by ethanol/nicotine-exposed dams were not indicative of withdrawal states. Animals displayed normal locomotor and exploratory behavior during the behavioral test, and no animal displayed abnormal behaviors such as 'wet-dog shakes'. This lack of apparent severe withdrawal may also have been precipitated in part by the continuous presence of nicotine, which has been shown to alleviate many of the symptoms of ethanol withdrawal [36]. Since there is very little impact of the experimental manipulation in the current study, the effect of withdrawal, if any, appears to be minimal.

The behavioral alterations we observed in the E/N-exposed dams are considered primarily a result of gestational drug treatment rather than any pup behavioral deficits as all dams were tested with surrogate unexposed pups. This is an important strength of this study, since prenatal exposure to ethanol can alter pup behaviors that play an important role in the maternal care these pups receive [4]. The only gestational difference we found was lower weight gain in the E/N-exposed dams during the 21-day gestational period. Due to the anorectic effects of nicotine and perhaps lower liquid intake of ethanol, the pair-feeding paradigm was used to assure that control dams consumed only the amount of food consumed by the E/N dams, thus it is unlikely that these effects were due to differences in caloric intake. It is more likely that this difference is due to the significantly different litter birth weights and slightly different number of pups. Ethanol and nicotine have been independently shown to decrease birth weight in both animal and human models of prenatal exposure [21,24,49], which would result in decreased gestational weight gain in these mothers. Furthermore, E/N-exposed offspring tended to gain less weight during the postpartum period, perhaps indicating that despite the intact crouching displayed by these surrogate dams, the pups may not have been receiving an adequate milk supply, due to poor solicitation of maternal care, which was not examined directly in this study.

Ethanol, a known positive modulator of GABAergic signaling, also has effects on glutamatergic, dopaminergic, serotonergic, and opioid systems, as well as several hypothalamic peptides [32]. While nicotine acts primarily at nicotinic acetylcholine receptors, it can influence dopaminergic signaling as well [32]. Dopamine, serotonin, norepinephrine, corticotrophin releasing hormone (CRH), and opioids, along with oxytocin, have all demonstrated important roles in the initiation and maintenance of maternal behavior [53]. The onset of maternal behavior in the immediate postpartum period is precipitated by an increase in the expression and binding of oxytocin [58,62,63]. It is possible that the interactions between ethanol and nicotine at these target systems could disrupt maternal behavior, potentially through oxytocinergic mechanisms, since it has been shown that gestational cocaine alters the receptor number and affinity of receptors. It is interesting that the behavioral results found in the present study differ in the magnitude and type of those seen following gestational cocaine treatment [26,28–30,41,81–84,88], indicating that not all drugs of abuse effect maternal

behavior similarly. Yet, despite these behavioral differences, there were reductions in oxytocin levels seen in the MPOA and VTA of E/N-exposed dams that are similar to some of those seen in cocaine-exposed dams [27].

Deficits in oxytocin in the MPOA have been most strongly associated with disrupted crouching behavior [19,60], and though we did not see a significant effect on crouching in the E/N dams, lower levels of oxytocin have also been associated with changes in other non-pup directed maternal behaviors, as was found in the present study. Had we examined maternal behavior later in the postpartum period, it is possible that we may have seen other pup-directed maternal behaviors affected by this treatment. Though it is unknown how ethanol affects central release of oxytocin, it has been shown to decrease suckling-induced release of oxytocin into the peripheral bloodstream [73]. The direct action of nicotine on centrally released oxytocin or oxytocin receptor binding has not yet been determined and will be an interesting study for comparison.

Our finding that E/N treatment results in lower oxytocin levels in the VTA may suggest that E/N dams found pup contact less rewarding, though we did not directly test this theory. The VTA plays a prominent role in the dopaminergic-mesolimbic reward circuitry, and oxytocin has been shown to have an antagonistic effect on the dopamine systems associated with drug-seeking behaviors [34]. Parvocellular projections from the oxytocin neurons in the paraventricular nucleus terminate in both the VTA and nucleus accumbens, and it is possible that the aspects of pup stimuli and the interactions with pups that are normally rewarding [37] are mediated through these projections. Dopamine neurotransmission plays a role in specific aspects of maternal behavior [7], and the rapid rise in oxytocin in the VTA may modulate which cues are the most rewarding during the early postpartum period. If this system has been altered due to prolonged drug exposure, it may not respond properly to cues that would normally induce and maintain maternal behavior. Both ethanol and nicotine can act to increase dopamine release from VTA neurons into the nucleus accumbens when given acutely [12, 87], an effect which does not appear to attenuate over repeated or chronic administrations of the drugs [12,65]. Though studies indicate the two may interact synergistically [20], and when given in combination, chronic exposure to these drugs may activate this system differentially.

With regard to the next generation ethanol preference, we did not see an overall increase in ethanol preference in the prenatal E/N exposure group as hypothesized (see Table 2). However, we did observe that prenatal exposure to these drugs altered sex specific differences in ethanol consumption. At PND 30, a period in the rat similar to early adolescence in humans, control animals did not exhibit a sex difference in ethanol consumption overall, but animals prenatally exposed to E/N did, with females consuming more ethanol than males. This effect also appears to be phase-dependent. During the acquisition phase of two-bottle choice testing, both control and E/N females drank more than males, while during the forced consumption and subsequent two-bottle choice period, only E/N females drank more. Ethanol consumption in E/N-exposed males drops to almost nothing following forced ethanol consumption, a shift that is not apparent in other groups. Interestingly, the control subjects in both groups preferred ethanol on the first day following the end of forced choice phase, potentially indicating a slight relative aversion to ethanol in E/N offspring at this time.

The few studies that have investigated sex differences in drinking behavior during adolescence in the rat have shown that females typically drink more than males [14,76]. Though prenatal exposure to ethanol has previously been associated with increased ethanol drinking behavior in adolescence (for review see [14]), it is possible that the forced ethanol consumption period used in the current study is more stressful, and adolescent E/N-exposed males react differently to that stress than females, thus resulting in a greater taste aversion to ethanol.

During early adulthood (PND60) control females consumed more ethanol than control males, a sex effect that is seen in most ethanol consumption studies and in Sprague-Dawley rats in particular [2,8]. Interestingly, in the present study we found that prenatal exposure to ethanol combined with nicotine eliminated the typical sex difference at this age. Some literature suggests that prenatal exposure to ethanol or nicotine, especially in the late gestational period, “feminizes” male behavior. The presence of high levels of brain estradiol is important for masculinization of brains that occurs during this period, and prenatal exposure to either ethanol or nicotine has been shown to prevent this surge [40,47]. This may be related to adult sex effects, since oxytocin is strongly regulated by estrogen [53].

It has been reported that animals tested during the adolescent period will also consume higher amounts of ethanol and exhibit greater preference compared to adults [76]. We could not directly compare the drinking patterns between adolescents and adults since the testing paradigms were different; however, the paradigm difference seems to have affected the ethanol intake primarily during the acquisition phase of testing, where adults consumed more than adolescents. The study designs used for adolescents and adults were dissimilar, although our original intent was to keep the study design consistent throughout both ages, with ethanol administered at a fixed 10% volume. However, after examining the data from the adolescents using this paradigm, the design was altered in an effort to increase consumption. Effects seen during the acquisition phase were not apparent during the forced consumption or two-bottle choice phases, where although most adults drank less than the adolescents did, this difference was not large and may not be biologically relevant. Other studies examining adolescent drinking compared to adult drinking do not employ a forced ethanol phase, which can dramatically affect behavioral responses [76]. The forced consumption phase was included to ensure that each rat would consume enough ethanol to experience its pharmacological effects. We originally thought that this would overcome any barriers to consumption, such as its taste, the age of the animal, or the rat strain used; however, ethanol consumption in most groups was considerably lower following the forced consumption phase, especially in adult animals. Future studies should consider alternatives to this phase.

A common finding at PND 30 and PND 60 was that males with higher levels of oxytocin in the VTA consumed the least ethanol, while females with the highest levels of oxytocin in the VTA drank the most within their gender. Males also had consistently lower levels of oxytocin in the MPOA than did females. These results raise the possibility that oxytocin changes in the VTA are related to increased or decreased drinking in a sex-specific manner, which would be a major finding and perhaps have some clinical relevance for both alcohol research as well as for other dopaminergic reward associated drugs of abuse. Altered oxytocin system dynamics in brain regions associated with the reward system, including the VTA, nucleus accumbens, hippocampus, and amygdala, indicate a possible mechanism for the control of tolerance; however, oxytocin involvement in the behavioral intake of ethanol has not yet been investigated. Higher levels of oxytocin in cerebrospinal fluid have been associated with inhibitions in the development of tolerance to many drugs of abuse, including opiates [70], cocaine [67,69], and ethanol [74]. Systemic and ICV injection of oxytocin can also inhibit the development of physiological and behavioral tolerance induced by cocaine, heroin and ethanol in rats (for review see [35]). It is unknown if ethanol or nicotine effect more rapid changes in oxytocin system dynamics, such as receptors levels, but given the similarities in ethanol and nicotine’s targets, an interaction is likely.

High oxytocin levels in the VTA could also affect drinking behavior through the inhibition of dopaminergic signaling as discussed above. This may be explained by oxytocin’s modulatory role of dopaminergic transmission. Oxytocin pretreatment can attenuate dopamine utilization in the nucleus accumbens in response to cocaine [6]. Chronic oxytocin injections are also able to inhibit the stimulated release of dopamine in the basal forebrain [33]. It is possible that

oxytocin receptor activation may be interfering with D2 receptors in the VTA, potentially leading to fewer incidences of burst firing within VTA projections to the nucleus accumbens, which have been shown to play an important role in the development of addiction [56]. The finding may be one of the most important for the future elucidation of oxytocin's role in the rewarding effects of a number of drugs of abuse.

It is important to note that Sprague-Dawley rats, though well established for the study of maternal behaviors and ethanol choice behaviors, do not spontaneously consume substantial quantities of ethanol. Based on similar ethanol liquid diet studies, we were expecting to see values around 130–150 mg% per deciliter [71]; however, in our study, dams had lower than anticipated BECs. It is possible that these dams did not consume as much diet as those in other studies, though this is hard to determine from the literature. Acute nicotine can lower peak BECs in both adult female [57] and neonatal rats [10], although the effects of chronic nicotine are unknown. Future studies using methods and strains that induce higher levels of ethanol consumption may inform us better of any other effects of prenatal exposure to ethanol and nicotine on ethanol drinking. Additionally, if the interaction of a stimulant (nicotine) and depressant (ethanol) result in fewer behavioral or biological consequences than found with either drug alone, the mechanism of this interaction becomes very interesting in terms of potential interventions and our understanding of why different drugs are often co-administered by users.

Exposure to ethanol and nicotine during gestation affects the time spent in contact with pups and decreased oxytocin levels in both the MPOA and VTA of rat dams. Additionally, the effect of prenatal exposure to both ethanol and nicotine does not appear to increase drinking preference during either adolescence or early adulthood, though it does differentially affect normal sex differences in consumption, possibly involved with alterations in oxytocin interactions with the dopamine-reward system. This becomes particularly important when considering that early exposure to ethanol can affect responses to ethanol at distinct phases of development, and that adolescent drinking can lead to higher levels of adult drinking. The data we present here suggests the need for further research into questions of how the physiology of these drug interactions may affect important behaviors in this and other models.

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Figure 1.

Timelines for ethanol preference tests taking place on postnatal days 30 and 60. The test on postnatal day 30 (A) consists of three phases: the acquisition of the task procedure, the forced consumption period, and the 2-bottle choice task. On postnatal day 60 (B), there are four phases: a saccharin preference test, the acquisition of the task procedure, the forced consumption period, and the 2-bottle choice task.

Figure 2.

The impact of combined gestational treatment with ethanol and nicotine (E/N) on the frequency of touch/sniff, in nest, and rest away behaviors compared to vehicle-exposed dams tested on postpartum day 1. The asterisks indicate that E/N dams touched their surrogate pups less, were less often in the nest, and rested away from those pups more often than vehicle-exposed dams at statistically significant levels ($p \leq 0.05$).

Figure 3.

The effect of prenatal exposure to ethanol and nicotine (E/N) on postnatal day 30 ethanol preference testing. The test consisted of three phases: the acquisition of the task procedure, the forced consumption period, and the 2-bottle choice task. Asterisks indicate that on postnatal day 38, the first day of two-bottle choice, (A) control females consumed more ethanol and exhibited a higher preference for ethanol compared to E/N females ($p \leq 0.01$), but (B) showed no difference in water consumption. Similarly, (C) vehicle males consumed more ethanol and exhibited a higher preference for ethanol compared to E/N males ($p \leq 0.01$), but also showed (D) no difference in water consumption.

Figure 4.

The effect of sex on postnatal day 30 ethanol preference testing. The test consisted of three phases: the acquisition of the task procedure, the forced consumption period, and the 2-bottle choice task. Asterisks indicate that females drank more g/kg ethanol than males ($p \leq 0.05$) during the acquisition phase (A and C), however there were no effects of sex on water consumption (B and D).

Figure 5.

The effect of prenatal exposure to ethanol and nicotine (E/N) on postnatal day 60 ethanol preference testing. The test consisted of four phases: a saccharin preference test (not pictured) the acquisition of the task procedure, the forced consumption period, and the 2-bottle choice task. There were no differences in g/kg ethanol consumption (A and C) or water consumption (B and D) due to prenatal exposure condition seen in any phase of testing.

Figure 6.

The effect of sex on postnatal day 60 ethanol preference testing. The test consisted of four phases: a saccharin preference test (not pictured) the acquisition of the task procedure, the forced consumption period, and the 2-bottle choice task. Asterisks indicate that (C) control females exhibited higher g/kg ethanol consumption than control males ($p \leq 0.05$) during the acquisition phase, and consumed more g/kg ethanol than control males ($p \leq 0.05$) during the two-bottle choice phase, while (D) drinking less water per day ($p \leq 0.05$).

Figure 7.

The effect of gestational treatment with ethanol and nicotine (E/N) on oxytocin levels following a maternal behavior test on postpartum day one. E/N-exposed animals displayed lower levels of oxytocin in the medial preoptic area (MPOA, $p \leq 0.05$) and ventral tegmental area (VTA, $p \leq 0.05$) than vehicle-exposed animals, as denoted by the asterisks, but had no difference in levels within the amygdala (AMY) or hippocampus (HIPP).

Figure 8.

The effect of prenatal ethanol and nicotine (E/N) or vehicle on oxytocin levels in both males and females following an alcohol preference task on postnatal day 30. Regardless of prenatal exposure condition, adolescent females showed higher oxytocin levels in the medial preoptic area (MPOA, $p \leq 0.03$), but lower levels in the ventral tegmental area (VTA, $p \leq 0.01$) compared to males. In addition, E/N-exposed offspring had higher oxytocin levels in the VTA ($p \leq 0.03$) compared to controls. There were no significant differences between the treatments or sexes in the amygdala (AMY) or hippocampus (HIPP).

Figure 9.

The effect of prenatal ethanol and nicotine (E/N) or vehicle on oxytocin levels in both males and females following an alcohol preference task on postnatal day 60. Control males had higher levels of oxytocin in the ventral tegmental area (VTA) than control females ($p \leq 0.01$), E/N males ($p \leq 0.01$), and E/N females ($p \leq 0.01$). Additionally, regardless of treatment condition, females had higher oxytocin levels than males in the medial preoptic area (MPOA, $p \leq 0.05$), but lower levels in the VTA ($p \leq 0.01$). Regardless of sex, control offspring had higher levels of oxytocin compared to E/N-exposed offspring ($p \leq 0.01$) in the VTA as well. There were no sex or treatment effects in either the amygdala (AMY) or hippocampus (HIPP).

Table 1

Maternal and Birthing Outcomes (mean $\pm$ SEM)

	Number of Dams	Gestational Length	Maternal Weight Gain(g)	Number of Stillborn Pups	Number of Live Pups	Number of Male Pups	Number of Female Pups	Whole Litter Weight(g)
E/N ^d	20	20.7 $\pm$ 0.6	95.4 $\pm$ 23.9 *	0.85 $\pm$ 2.3	13.3 $\pm$ 3.3	6.8 $\pm$ 1.9	6.6 $\pm$ 2.4	40.4 $\pm$ 3.2 *
Vehicle	24	20.5 $\pm$ 0.7	157.0 $\pm$ 9.1	0.46 $\pm$ 0.8	14.5 $\pm$ 2.3	6.9 $\pm$ 2.6	7.6 $\pm$ 2.5	50.1 $\pm$ 3.0

^dTreatment with ethanol and nicotine throughout gestation

* Different from vehicle group, $p \leq 0.05$

Table 2
Ethanol Preference in Adolescence and Adulthood (% total fluid consumption, mean $\pm$ SEM)

	Adolescence ^a		Adulthood ^b	
	Acquisition ^c	Two-Bottle Choice ^d	Acquisition ^e	Two-Bottle Choice ^d
E/N ^e	3.2 $\pm$ 0.6	2.4 $\pm$ 0.9	53.9 $\pm$ 3.2	7.2 $\pm$ 3.6
Vehicle	7.7 $\pm$ 1.5 *	8.6 $\pm$ 3.6	55.9 $\pm$ 7.9	5.0 $\pm$ 1.5
	3.0 $\pm$ 1.4 *	5.3 $\pm$ 2.3	44.9 $\pm$ 3.1	9.1 $\pm$ 4.3
	7.7 $\pm$ 1.7 *	8.7 $\pm$ 3.6	54.6 $\pm$ 4.1	8.8 $\pm$ 3.7

^a Testing beginning postnatal day 30 and continuing for 14 days
^b Testing beginning postnatal day 60 and continuing for 14 days
^c Both ethanol and water available, first five days of testing
^d Following forced ethanol exposure, both ethanol and water available, last five days of testing
^e Treatment with ethanol and nicotine throughout gestation
* Difference from Males. $p \leq 0.01$