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SEX-SPECIFIC EFFECTS OF DEVELOPMENTAL ALCOHOL EXPOSURE ON COCAINE-INDUCED PLACE PREFERENCE IN ADULTHOOD

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Abstract

Fetal Alcohol Syndrome (FAS) is associated with high rates of drug addiction in adulthood. One possible basis for increased drug use in this population is altered sensitivity to drug-associated contexts. This experiment utilized a rat model of FASD to examine behavioral and neural changes in the processing of drug cues in adulthood. Alcohol was given by intragastric intubation to pregnant rats throughout gestation and to rat pups during the early postnatal period (ET group). Controls consisted of a non-treated group (NC) and a pair-fed group given the intubation procedure without alcohol (IC). On postnatal day (PD) 90, rats from all treatment groups were given saline, 0.3 mg/kg, 3.0 mg/kg, or 10.0 mg/kg cocaine pairings with a specific context in the conditioned place preference (CPP) paradigm. While control animals of both sexes showed cocaine CPP at the 3.0 and 10.0 mg/kg doses, ET females also showed cocaine CPP at 0.3 mg/kg. This was accompanied by a decrease in c-Fos/GAD₆₇ cells in the nucleus accumbens (NAc) shell and GAD₆₇-only cells in the NAc shell and PFC at this 0.3 mg/kg dose. ET males failed to show cocaine CPP at the 3.0 mg/kg dose. This was associated with an increase in c-Fos only-labeled cells in the NAc core and PFC at this 3.0 mg/kg dose. These results suggest that developmental alcohol exposure has a sexually-dimorphic effect on cocaine's conditioning effects in adulthood and the NAc.

Keywords

Fetal alcohol; place preference; cocaine; nucleus accumbens; prefrontal cortex; addiction

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1.0 Introduction

1.1 Fetal alcohol Spectrum Disorders and Drug Addiction

Fetal Alcohol Spectrum Disorder (FASD) is the result of exposure to alcohol during gestation with fetal alcohol syndrome (FAS) at the most severe end of this spectrum. These disorders have been considered a clinical syndrome since the early 1970s [1]. While primary FASD disabilities include small birth weight and cognitive impairment, secondary disabilities include high rates of drug addiction in clinical populations: 30% of adolescent males and 40% of adolescent women with FASD have reported drug problems during their teenage years [2]. Alarming, the lifetime rate of substance abuse in individuals with FAS has been estimated to be approximately 54.5% [3]. This makes substance abuse disorders in adults with FASD nearly five times that of the general population [4] and in FAS individuals to be 21 times the general population [3].

Although many environmental factors influence the development of substance abuse disorders in FASD populations, animal models of FASD suggest that alterations in underlying neurocircuitry may influence drug sensitivity across development [5,6]. In particular, attention has been drawn to impairments in the dopaminergic projections from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) and prefrontal cortex (PFC) as dopaminergic activity in these regions is a point of convergence of all drugs of abuse [7]. Animal models of FASD have demonstrated that developmental alcohol exposure produces many alterations in these pathways, including a reduction of dopamine (DA) binding sites and metabolites in cell body and terminal regions of midbrain DA neurons [8], smaller cell bodies and delayed dendritic growth in DA neurons [9], and reductions in spontaneous firing of DA neurons [10]. In addition, GABAergic interneurons tightly regulate dopaminergic activity within this pathway, and some studies have also found alterations in GABAergic populations following developmental exposure to alcohol [11,12]. As such, alterations in GABAergic activity within this pathway may produce effects on substance abuse behaviors, particularly due to their critical roles in conditioning.

As conditioning factors influence the addiction process during both the initial and long-term stages of the disease [13,14], it may be that high rates of addiction in FASD populations result from altered sensitivity to contexts and cues associated with drug effects through conditioning. Most studies that have examined the impact of alcohol exposure during development on general classical conditioning suggest impairments in conditioning. Trace and delay eye blink conditioning are reduced in both children [15–17] and rodents [18,19] exposed to alcohol during development. Similarly, alcohol exposure during development produces deficits in flavored taste aversion [20] and trace fear conditioning [21,22] in rodents. Contrary to conditioning impairments frequently evidenced in FASD models, drugs of abuse may represent a unique case where associations between conditioned stimuli (e.g. drug and context) are augmented rather than impaired due to the unique impact of drugs on neural circuitry. Given the high rates of addiction in the FAS population and the critical role of the context surrounding drug administration [23] in drug addiction/relapse, an investigation of context conditioning to drugs of abuse may illuminate the mechanisms underlying the high rates of addiction in the FAS population.

1.2 Rationale for the Study and Hypotheses

Stimulants yield strong contextual conditioning in rodents and the response to stimulants is enhanced in animals exposed to alcohol during development [6,24]. As phasic firing of VTA dopaminergic neurons is sufficient to instate conditioned place preference in rodents [25], part of the effect of stimulants on contextual conditioning may be linked to their agonist effects on dopaminergic networks. Since the dopaminergic path between the VTA and NAc is altered by alcohol exposure during development and this pathway is impacted by stimulants, this study investigated the effects of cocaine exposure on conditioned place preference using a rat model of FAS. Both sexes were included in the study as there are sexually dimorphic effects of alcohol during development [26–28].

It was hypothesized that conditioning to contextual cues associated with cocaine will be enhanced in alcohol-exposed offspring. This sensitivity to cocaine was hypothesized to be greater in alcohol-exposed females, reflecting clinical distinctions in FASD populations which suggest that females exhibit higher rates of substance abuse problems than males [4]. Expression of the Fos gene as measured by immunoreactivity was utilized to determine NAc, PFC, and VTA activity in response to drug-paired contextual cues. As inhibitory interneurons play critical regulatory roles in activity in these brain regions, cells were also labeled with a marker for inhibitory GABAergic neurons, glutamic acid decarboxylase 67 (GAD₆₇). Activation, as measured by c-Fos expression, of the NAc, PFC, and VTA, in response to the presentation of drug-related cues, were hypothesized to be significantly greater in alcohol-exposed animals compared to control animals. To reflect decreased inhibition of the NAc, PFC, and VTA, it was expected that alcohol-exposed animals would have a significant decrease in Fos/GAD₆₇-labeled neurons in these regions compared to control animals.

2.0 Materials and Methods

2.1 Subjects

Long-Evans rats were housed in the animal colony of the University of South Carolina, Department of Psychology. The colony was maintained at 22°C with a 12-hour light-dark cycle, which begins at 0800 hr. Female Long Evans rats were housed overnight with breeder Long Evans males. Vaginal smears were conducted the following morning to check for the presence of sperm. The first day on which sperm is detected was designated Gestational Day (GD) 1. On GD 1, dams were singly housed in polypropylene cages with wood shavings and assigned to one of the following treatment groups: ET (ethanol treated), IC (intubated control), and NC (non-treated control). One male and one female from each litter was assigned to each condition; additional animals from each litter were assigned to other, non-related, experiments. In order to obtain a statistical power of 0.80 and a medium effect size (0.5, Cohen's *d*), eight subjects within each cell for each experiment were needed [29].

2.2 Treatment

Daily alcohol administration to dams occurred on GD 1 through GD 22 in the first half of the light cycle. ET dams were weighed and received daily intra-gastric intubations of alcohol (4.5 g/kg) in 20 ml/kg distilled water. Intubations consisted of insertion of a stainless steel

gavage tube down the esophagus of the rat and injection of the alcohol dose directly into the stomach. Preceding the intubation, the tube was dipped in corn oil to provide lubrication. ET dams were given free access to water and rat chow which was weighed daily to monitor food intake. An iso-caloric maltose-dextrin solution in a volume of 20 ml/kg was administered to IC dams every day during gestation (GD 1–22) through intra-gastric intubation. IC dams were pair-fed to an alcohol-treated dam matched for body weight on GD 1 such that the IC dam was given the same weight of rat pellets eaten by the ET dam on a particular GD. NC dams were weighed daily and did not receive any other treatments. The day of birth was designated postnatal day 1 (PD 1). Neither the dams nor the pups received any treatment on this day.

Litters were culled to 10 pups (5 male and 5 female) whenever possible. On PD 2 through PD 10, pups from all groups were removed from the nest one at a time, weighed, marked with a nontoxic marker for identification, and intubated if they were in the IC or ET groups. All pup intubations were performed using PE10 *Intramedic* tubing dipped in corn oil for lubrication, and were conducted in the first half of the light cycle. During an intra-gastric feeding, ET pups received a 3.0 g/kg dose of alcohol in a volume of 27.8 ml/kg milk solution. Two hours after the alcohol administration, alcohol-treated pups were intubated a second time with the milk solution only (27.8 ml/kg) to compensate for any reduction in milk intake due to intoxication of the pups. The milk solution was formulated to simulate dam's milk [30]. The IC pups received the same procedure (two intubations) as the ET except no solutions were administered. The postnatal procedure duration was approximately 2 min for each pup, and every effort was made to reduce the time of separation between the pup and the dam. To avoid litter effects, only one pup from each litter was assigned to each condition for CPP testing. All pups were weaned at PD 21 and housed in same-sex pairs.

2.3 Blood Alcohol Concentrations (BACs)

The doses of alcohol used in this study (4.5 g/kg for dams and 3.0 g/kg for pups) were selected to give equivalent peak blood alcohol concentrations (BACs) of between 300–400 mg/dl in dams and pups, at three and two hours respectively [31,32]. These doses were selected for two reasons: 1) high doses are critical to produce reproducible reductions in brain growth [33], and 2) this model aims to represent the high BACs which would likely have been experienced by children with fetal alcohol syndrome, the most severe form of FASD [34]. Three hours after intubations on GD 20, 10 µl of blood was collected from ET and IC dams from the tail vein. The blood samples from the ET dams were processed for determination of peak BACs. No blood was taken from NC dams. Two hours after the first intubations on PD 10, 10 µl of blood was collected via tail-clip from ET and IC pups. The blood samples from the ET pups were then processed for determination of peak BACs. No blood was taken from NC pups. Blood alcohol concentrations from ET dams and pups were determined using an enzymatic procedure as described [35].

2.4 Cocaine-Induced Conditioned Place Preference (CPP)

The CPP experimental apparatus was in a windowless room lit by standard fluorescent lighting. All testing was performed in the late morning and early afternoon. The experimental apparatus was constructed of PVC tubing and Plexiglas and contained three

distinctive interconnected chambers. The two circular chambers were mounted on a rectangular base (58.42 cm × 106 cm). The two outside chambers were circular and identical in size (45.08 cm circumference) but differed in color and flooring. One chamber had alternating black and white striped walls and a black floor. The second chamber had solid gray interior and floor. There was a middle chamber that was used to connect the two circular chambers (27.3 cm × 8.255 cm) that had a solid white interior. This chamber also contained barriers with doors that allow access to both chambers. Seventy-percent isopropyl ethanol was used to clean the chamber in between each rat on all testing days with several minutes allowed for the ethanol to evaporate before any testing commenced.

Animals in the three groups (ET, NC, and IC) were tested in a conditioned place preference test that began on PD 90. This age reflects the onset of adulthood and was chosen in order to make sure that no behavioral variations were due to pubertal changes as opposed to drug manipulations. The rats were assigned to one of four conditions. One condition consisted of saline injections only paired with either chamber. The other three conditions consisted of pairing of one of three cocaine doses (0.3, 3.0 or 10.0 mg/kg) with the non-preferred chamber and pairing a saline injection with the preferred chamber. Cocaine hydrochloride was dissolved in 0.9% saline and injected into the intraperitoneal cavity (i.p.) in a dose of 0.3, 3.0, or 10.0 mg/kg in a volume of 1 ml/kg. An equal number of males and females were assigned to each condition and no more than one rat of each sex from a litter was assigned to a condition.

The CCP procedure consisted of three time periods. During the first period (PD 90), the barriers that confined the animal to the middle compartment were opened to allow free access to all chambers. Rats were placed, drug-free, in the apparatus and allowed to roam freely. This habituation (pre-conditioning) exposure consisted of one 15-min session. The purpose of this period was to allow the rat to become familiar with the apparatus. During this period, time spent in each chamber was recorded and the chamber in which the rat spends the most time was assigned as the rat's preferred side.

The second period began on PD 91 and continued daily until PD 96. This period involved the drug-compartment pairings. Doors were closed to allow access to a particular chamber only. Cocaine-paired rats were given cocaine in the non-preferred side and saline in the preferred chamber. The constant for place preference at preconditioning was identified via the following formula:

$$SP_{\text{pre-conditioning}} = t_{\text{sideA}} - t_{\text{sideB}}$$

where $SP_{\text{preconditioning}}$ refers to the preferred side at the preconditioning timepoint, t_{sideA} and t_{sideB} refer to the time spent in either side of the apparatus. Rats in the saline only condition received saline pairings with both preferred and non-preferred sides. Body weights were taken daily. A rat was given the appropriate injections (0.3, 3.0, or 10 mg/kg of cocaine or saline, i.p.) and was immediately placed in the chamber. Conditioning time consisted of one 20-min session daily and there was one pairing daily for six consecutive days. Over these six days, each of the cocaine-paired rats received a total of three cocaine pairings in the non-preferred chamber and three saline pairings in the preferred chamber beginning with a

cocaine pairing and alternating with saline. In contrast, the rats in the saline only condition received three saline pairings in each chamber.

The third period was on PD 97. On this day, the post-conditioning side preference of the rats was determined. The rats were placed in the central chamber in the absence of drug treatment (cocaine or saline), and the sliding doors were removed and the rat was allowed access to all chambers for 15 min. The position of the animal was monitored by an Ethovision automated detection system (Noldus).

An increase in place preference was measured as a significant percent increase in time spent in the non-preferred side after conditioning. Time in each compartment was converted into two preference constants for both pre- and post-conditioning periods. The post-conditioning constant was identified using the following formula:

$$SP_{\text{post-conditioning}} = t_{\text{drug}} - t_{\text{nc}}$$

where $SP_{\text{post-conditioning}}$ refers to the preferred side after drug conditioning, t_{drug} refers to the side where drug was administered, and t_{nc} refers to the side where drug was not presented and hence no conditioning took place. Percent increase was then determined by subtracting the pre-conditioning constant from the post-conditioning constant, dividing by total time in the chamber, and then multiplying by 100:

$$\text{Percent Increase} = \frac{SP_{\text{post-conditioning}} - SP_{\text{pre-conditioning}}}{900 \text{ seconds}} \times 100$$

Significant positive values represented a conditioned place *preference* induced by cocaine, while significant negative values represented a conditioned place *aversion* induced by cocaine.

2.5 Immunoreactivity in the NAc, VTA, and PFC

The double-labeled c-Fos and GAD₆₇ immunocytochemistry procedure was based on previously published methods [36]. After the final test session (third period) of the CPP procedure, rats were isolated to the non-preferred (and thus cocaine-paired for three of the conditions) side for 60 min to allow for maximal expression of the c-Fos gene [37]. Immediately after the end of this period, rats were anesthetized with sodium pentobarbital and perfused transcardially with 100 ml of saline followed by 500 ml of an ice-cold 4% paraformaldehyde in 0.1 M PBS (pH 7.4) solution. Brains were then removed, post-fixed in 4% paraformaldehyde for 12–15 h, and placed in phosphate-buffered saline (PBS) containing 30% sucrose for 48–72 h.

The next day brains were rapidly frozen and 30 μm sections were cut through the NAc, PFC, and VTA. Sections were cut in the coronal plane using rotary cryostat (Microm HM505E) at -25°C . Areas were determined by using atlas coordinates [38] and each area was sectioned from the most rostral to most caudal extents. Every third section was collected from each area for sampling. Sections were rinsed in 0.3% H_2O_2 for 15 min, followed by several rinses

in tris-buffered saline (TBS). Next, sections were incubated for 20–30 minutes in a TBS solution containing 0.3% Triton X-100 (PBST) and 2% normal donkey serum (TBS+). Sections were then placed in polyclonal rabbit anti-Fos primary antibody (1:10,000; Calbiochem). The primary antibody was prepared in TBS+ and sections were incubated in the primary antiserum overnight at 20°C with constant agitation. Following rinsing in TBS, sections were incubated for 1.5 h at room temperature in biotin-SP-conjugated affini-pure donkey anti-rabbit IgG diluted (1:1000) also prepared in TBS+ solution. After several more rinses in TBS, the sections were incubated in horseradish peroxidase-conjugated streptavidin (1:1600) prepared in TBS + Triton X-100 (no serum) at 20°C for 1 h. Following further rinsing in TBS, the sections were developed in a 1:1 dilution of nickel-enhanced diaminobenzidine (DAB) tetrahydrochloride (purple/black reaction product) for 1.5 min. This reaction was terminated by multiple (4–5) rinses in TBS.

After multiple rinses in TBS, GAD₆₇ immunolabeling began immediately. Sections were rinsed in 0.3% H₂O₂ for 15 min, followed by several rinses in TBS. Sections were then incubated for 20–30 min in TBS+. The GAD₆₇ primary antibody (monoclonal anti-glutamic acid decarboxylase 67) was added (1:2000; Sigma) in a TBS+ solution and incubated overnight at 20°C with constant agitation. After several rinses in TBS, sections were incubated an unlabeled affini-pure donkey anti-mouse IgG (1:200) secondary for 2 h at 20°C. Following several rinses in TBS, sections were then incubated in mouse peroxidase-anti-peroxidase tertiary (1:250) for 1.5 h at 20°C. Next, sections were rinsed several times in TBS. The sections were then developed in a 1:2 dilution of nonmetal-enhanced DAB (light amber reaction product) for 1.5 min. This reaction was terminated by multiple (4–5) rinses in TBS. Finally, sections were then mounted onto gelatin-coated slides, dried, and dehydrated (95% and then 100% EtOH for 2 min each) before coverslipping.

Once coverslipped, the two most intermediate sections from each area were assessed for number of immunoreactive neurons using 10× magnification. Double-labelling was assessed using 10× magnification where the overlay between the nuclear (c-Fos) and cytosolic (GAD₆₇) stains are readily visible (Figure 1). Specific brain structures were defined using surrounding structures as markers including the cingulate cortex (for the PFC), caudate putamen, (for the NAc), and mammillary peduncle (for the VTA). Imaging software (Adobe Photoshop CS2) was used to digitize the slide images. For cell counting, images were counted manually with a counter by a blind observer at 10× magnification in 0.1225 mm² blocks. Every attempt was made to count from identical areas within each structure from each slide.

2.6 Data Analyses

The significance level was set at $\alpha = .05$ for all analyses unless made more stringent with Bonferroni corrections as indicated below.

Body weight data was evaluated using an analysis of variance (ANOVA). Dam weight was analyzed using a 3 × 4 mixed ANOVA with treatment group as the between-subjects factor and gestational day (GD) 5, 10, 15, and 20 as the within-subjects factor. Pup weight data were separated by sex and analyzed using a 3 × 9 mixed ANOVA. Group was the between-subjects factors while postnatal day (PD) 2–10 was the within-subjects factor. Pup weights

on PD 21, 30, 60, and 90 were also separated by sex and analyzed using 3×4 mixed ANOVA with group as the between factors and PD as the within-subjects factor. Tukey's *post-hoc* analyses were used to analyze main effects. All weight-related data with significant interactions were analyzed using simple main effects to describe the interaction. Bonferroni corrections were used to analyze simple main effects. Repeated measures values that violated the assumption of sphericity (based on the results of Mauchly's sphericity test) were adjusted using the Greenhouse-Geisser correction.

Blood alcohol concentrations (BACs) were analyzed using a t-test to determine differences between dam and pups. Sex differences in pup BACs were also analyzed using an independent samples t-test.

Data from the CPP experiment were analyzed using a 3 (Group) $\times$ 4 (conditions; 0.3, 3.0, 10 mg/kg or saline only) between-subjects ANOVA. Males and females were analyzed separately. The dependent measure was percent increase in the non-preferred side (see section 2.4).

Data on overall activity of the rats before and after conditioning in the CPP design was analyzed using a 3 (Group) $\times$ 2 (time periods: pre- and post-conditioning) mixed ANOVA with group as the between-subjects factor and period as the within-subjects factor. Males and females were analyzed separately. Tukey post-hoc analyses were used to analyze main effects. Significant interactions were analyzed using simple main effects to describe the interaction. Bonferroni corrections were used to analyze simple main effects. Repeated measures values that violated the assumption of sphericity were adjusted using the Greenhouse-Geisser correction.

Immunoreactivity was assessed only in groups that exhibited statistically significant differences in behavior. Data from IC and NC groups were combined as a *control* group compared to the ET group. Data were analyzed using a one-way ANOVA for each brain area with Group (ET vs. Control) as the between-subjects factor. Males and females were analyzed separately as determined a priori. The dependent measure was the number of c-Fos, GAD₆₇, or double labeled-immunoreactive neurons.

3.0 Results

3.1 Body Weights and BACs

Within-subjects analysis of the dam body weights showed a main effect of GD on bodyweight [$F(3, 222) = 1014.9, p < .05$]; there was no group $\times$ GD interaction (Table 1). The main effect of GD was due to normal weight gain during pregnancy.

Analysis of PD 2–10 weights of male offspring revealed a group $\times$ PD interaction [$F(16, 840) = 6.0, p < .05$] and a significant main effect of PD, [$F(8, 840) = 2561.9, p < .05$]. Analysis of simple main effects showed a significant group difference on all PDs. Post-hoc analyses showed that ET group weighed significantly less than IC and NC on PDs 2–10 ($p < .05$), and the interaction suggests that ET males did not gain weight at the same rate as control groups. The main effect of PD was due to weight gain across days. Within-subjects

analysis of PD 21–90 weights showed a significant main effect of PD, [$F(3, 276) = 12313.2$, $p < .05$] due to weight gain across days. There was no group $\times$ PD interaction. Between-subjects analysis did not reveal a significant difference among the groups (Table 2).

Within-subjects analysis of PD 2–10 weights of the female offspring revealed a group $\times$ PD interaction [$F(16, 856) = 8.4$, $p < .05$] and a significant main effect of PD, [$F(8, 856) = 2621.3$, $p < .05$]. Analysis of simple main effects showed a significant group difference on all PDs. Post-hoc analyses showed that the ET group weighed significantly less compared to IC and NC on PDs 2–10 ($p < .05$). The interaction suggests that the ET group did not gain weight at the same rate as control groups. The main effect of PD was due to weight gain across days. Within-subjects analysis of PD21–90 weights showed a significant main effect of PD [$F(3, 282) = 6285.7$, $p < .05$] due to weight gain across days. There was no significant group $\times$ PD interaction. Between-subjects analysis did not reveal a significant difference among the groups.

The BACs of dams and pup were significantly different [$t(83) = -2.7$, $p = .05$]; specifically, pups had lower BAC levels than dams. There were no significant differences between male and female pup BACs. The BACs in mg/dl with the standard errors of the means (SEMs) for the dams, male pups and female pups were 445.1 ± 33.3 , 359.5 ± 30.1 and 375.9 ± 19.3 respectively.

3.2 Conditioned Place Preference

Between-subjects ANOVA on the percent increase in time on the non-preferred side in male subjects revealed a significant group $\times$ dose interaction [$F(6,84) = 3.0$, $p < .05$]. Analysis of simple main effects showed a significant group difference at the 3.0 mg/kg dose [$F(2, 21) = 11.0$, $p < .05$]. *Post-hoc* analyses revealed that ET male rats were significantly different from IC and NC male rats in the 3.0 dose condition (p 's $< .05$). A significant main effect of dose [$F(3, 84) = 13.7$, $p < .05$] was also found. *Post-hoc* analyses indicated that the means for the medium (3.0 mg/kg) and high (10.0 mg/kg) cocaine doses were significantly different from those of the 0.3 mg/kg and saline doses ($p < .05$). These data are shown in Figure 2. There were no changes in overall activity among the groups (see Tables 3 and 4).

Between-subjects ANOVA on the percent increase in time spent on the non-preferred side in females showed a significant group $\times$ dose interaction [$F(6,82) = 2.3$, $p < .05$]. Analysis of simple main effects showed a significant group difference at the 0.3 mg/kg dose [$F(2,20) = 14.3$, $p < .05$]. *Post-hoc* analyses revealed that ET rats were significantly different from IC and NC rats ($p < .05$). There was also a significant main effect of dose [$F(3, 82) = 8.0$, $p < .05$]. *Post-hoc* analyses indicated that the means for the medium (3.0 mg/kg) and high (10.0 mg/kg) cocaine doses were significantly different from those of the 0.3 mg/kg and saline doses ($p < .05$). These data are shown in Figure 3. It is important to note that these data reflect increases in preferences in non-preferred sides, which is relative to initial side preference. To determine if the ethanol-induced changes were due to alterations in overall activity, females that received the 0.3 mg/kg cocaine treatment and males that received the 3.0 mg/kg treatment were analyzed for differences in total distance moved. These analyses revealed that there were no changes in overall activity among the groups for either sex (see Table 3).

3.3 Immunoreactivity in the NAc, PFC, and VTA

Immunoreactivity for males was assessed at the 3.0 mg/kg dose as this dose produced statistically different CPP behaviors between ET versus NC and IC controls. One-way ANOVA revealed that ET male animals had significantly more Fos labeled neurons in the selected areas counted of the NAc core and PFC than controls [$F(1,10) = 9.2, p < .05$ and $F(1,10) = 19.4, p < .05$, respectively]. These data are shown in Figure 4. There were no other significant differences found in the remaining brain regions. Separate analyses of the data from control rats (IC vs. NC) revealed no significant differences in any brain region or label (data not shown).

Immunoreactivity for females were assessed at the 0.3 mg/kg dose as at this dose ET females exhibited statistically different CPP behaviors versus NC and IC controls. One-way ANOVA revealed that ET females had fewer double-labeled neurons in the NAc shell than controls [$F(1,10) = 5.1, p < .05$]. Also, ET female subjects had significantly fewer GAD₆₇-only labeled neurons in this region than controls [$F(1,10) = 5.1, p < .05$]. Finally, ET females showed significantly fewer GAD₆₇-only neurons in the PFC [$F(1,10) = 7.9, p < .05$]. These data are shown in Figure 5. There were no other significant differences found in the remaining brain regions. Separate analyses of the data from control rats (IC vs. NC) revealed no significant differences in any brain region or label (data not shown).

4.0 Discussion

4.1 Summary of results

This study is the first to show that alcohol exposure during development can result in sexually dimorphic changes in sensitivity to cocaine-associated cues as measured by conditioned place preference, in adulthood. Not only did this study examine the developmental impact of alcohol exposure on cocaine CPP as a function of sex but also as a function of cocaine dose. Results suggest that sexual-dimorphism in cocaine-induced CPP varies by dose, which provides important methodological and theoretical considerations for the field. Specifically, alcohol-exposed females showed increased sensitivity to cocaine cues as shown by their cocaine-induced CPP at the lowest dose (0.3 mg/kg), a dose that did not produce an increase in preference in the control groups. Alcohol-exposed males were less sensitive in that they failed to show a cocaine-induced CPP at the medium dose (3.0 mg/kg), a dose shown to produce an increase in preference in both control groups. These effects cannot be explained by changes in overall activity in either of the sexes since none were detected.

The behavioral data were accompanied by differential neuronal activation in the sexes in certain brain areas shown to be involved in the processing of drug cues. In the pictured areas, alcohol-exposed females showed a significant decrease in neurons labeled with GAD₆₇-only and both GAD₆₇ and c-Fos in the NAc shell compared to controls. They also showed a significant decrease in neurons labeled for GAD₆₇ in the PFC. Alcohol-exposed males showed a significant increase in c-Fos-only labeled neurons in the NAc core and PFC. Non-biased stereological analysis will need to be performed in future studies to verify that these data reflect changes in total number of GABAergic neurons in these regions. In addition, it is

important to consider the potential effects of handling on c-Fos expression. This is a concern for all behavioral studies as differences in handling have been shown to differentially influence c-Fos expression [39]. While all animals in these experiments were gently handled by the same personnel, it is possible that ethanol-exposed rats are differentially sensitive to handling effects compared to intubated and non-stressed controls. Several studies have addressed this issue in part. For example, Weinberg, Kim, & Yu [40] demonstrated that early handling attenuates the several adverse effects of prenatal ethanol exposure in both males and females. While this study on handling did not examine c-Fos specifically, other studies which have examined a more robust experience-dependent c-Fos activator (e.g. social experience) in FASD models. Hamilton et al. [41] demonstrated that prenatal ethanol exposure did not differentially impact social activity-dependent increases in c-Fos in the prefrontal cortex relative to controls. Similarly, Lawrence et al. [42] used a three-trimester model of alcohol exposure to examine the effects of c-Fos expression in the nucleus accumbens in response to play behavior. In this study, perinatal ethanol exposure decreased c-Fos expression relative to controls in males; ET females exhibited increased c-Fos expression relative to IC but not NC controls. Collectively, these data suggest that handling would attenuate differences in c-Fos expression in ethanol-treated rats relative to controls. In contrast, the current study demonstrated increased c-Fos expression in ethanol-exposed males and females relative to both IC and NC controls. Therefore, we believe the potential effects of handling do not invalidate the effect of a cocaine-induced CPP in this study, although this will need to be confirmed with future studies.

In sum, these data suggest that developmental alcohol exposure can result in both developmentally and sexually dimorphic changes in sensitivity to drug cues in adulthood and that these changes reflect altered neuronal activation in the PFC and NAc of alcohol-exposed animals.

4.2 ET females' behavioral sensitization to the lowest dose of cocaine is correlated with a decrease in Fos-labelled GABAergic neurons in the PFC and NAc

The sensitization of CPP to the low dose of cocaine observed in alcohol-exposed females was accompanied by a significant decrease in active GABAergic (labeled with Fos/GAD₆₇) and inactive GABAergic cells (labeled with GAD₆₇ only) in the NAc shell after exposure to the drug context as well as decreased GABAergic cells in the PFC. These data suggest that females developmentally exposed to alcohol may be particularly sensitive to decreased inhibitory control in the NAc shell and PFC in response to drug cues associated with the low dose of cocaine (0.3 mg/kg).

4.2.1 PFC modulation of behavior in ET females—Several lines of evidence suggest that GABAergic activity in the PFC is a critical contributing factor to the pathology associated with substance abuse: 1) activation of GABAergic cells in the PFC increases in response to cocaine-induced CPP [43], 2) decreased inhibitory control of the PFC contributes to drug-seeking behaviors in cocaine addicts [44], and 3) rescuing hypo-activity in the PFC via optogenetics prevents cocaine seeking in rats [45]. Interestingly, while ET females exhibited decreases in GABAergic interneurons in the PFC, there were no shifts in total number of active interneurons as evidence by double-labeling, suggesting that a

disproportionate number of total GABAergic neurons in the PFC of ET females are active during CPP relative to controls. This suggests that sensitivity of GABAergic neurons to CPP in ET rats could similarly be responsible, at least in part, for the increase sensitivity to drug-induced CPP at low doses. However, as the total number of activated neurons in the PFC remains unchanged in ET females relative to controls, shifts in GABAergic activity could also simply be a compensatory mechanism for decreases in this cell population. Alternatively, the decrease in GABAergic interneurons in the PFC may play a more subtle role in down-stream effects on the NAc and VTA that cannot be fully evidenced by shifts in Fos staining.

4.2.2 NAc modulation of behavior in ET females—The GABAergic interneurons in the PFC regulate glutamatergic afferents which project back to both the NAc and VTA with high levels of target specificity [46]. For example, the core and shell regions of the NAc receive differential limbic and cortical input from the medial and orbital PFC: the medial PFC projects to both of these structures, while PFC orbital projections terminate primarily in the NAc core [47]. As ET females exhibited increased Fos activity in the NAc core despite no differences in cell number of GABAergic neurons or double-labeling of Fos with GAD₆₇ in this region, this could be partially due to disinhibition from cortical afferents.

Anatomical specificity between the NAc and shell is paralleled by functional specificity in relation to CPP. While the NAc has also long been associated with substance abuse and drug-seeking behaviors, there are opposing views within the literature on the exact functions of the NAc shell and core as they relate to drug addiction. Di Chiara [48] suggests that abused drugs and non-drug reinforcers share the ability to stimulate DA transmission in the NAc shell, with little activation of the NAc core. Additionally, Bassareo and Di Chiara [49] suggest that the NAc shell has a prominent role in stimulus-reward learning and the NAc core is involved in the expression of motivation toward a reward, probably due to its role in motor behavior. These functional specificity of these sub-regions of the NAc are supported by the fact that administration of amphetamine directly into the NAc core decreases CPP whereas administration of amphetamine into the NAc shell enhances CPP [50]. Interestingly, enhanced CPP at the lowest dose of cocaine in ET females was paralleled by both decreased number of GABAergic interneurons as well as decreased activation of those GABAergic interneurons in the NAc shell. As both cocaine and amphetamine have been demonstrated to suppress GABAergic-mediated post-synaptic potentials [51], then it is logical to assume that alterations in GABAergic activity in the NAc shell likely plays a role in variations in CPP in ET females in the current experiment. In essence, developmental alcohol exposure may alter the NAc shell such that drug-stimuli associations become enhanced; this results in increased sensitivity to drug cues.

4.3 ET males' decreased sensitivity to the medium dose of cocaine is correlated with an increase in Fos-labelled neurons in the PFC and NAc core

ET males showed decreased sensitivity to the increased place-preference inducing effects of cocaine at the medium dose of cocaine (3.0 mg/kg); this effect was accompanied by a significant increase in Fos-only labeled neurons in the NAc core and PFC but not the NAc shell. Thus, it's possible that the lack of a cocaine CPP displayed in this group partially

results from shifts in excitatory and inhibitory activity in these regions. However, confirmation of this hypothesis will require more specific examination of neuronal populations in these areas, as while cocaine exposure increased Fos expression in both males and females, the sex-dependent effects on behavior were divergent. It is likely that different neuronal populations within the NAc core are differentially activated in males and females in this condition, thereby contributing to both the increase in Fos expression and divergent behavioral phenotypes.

4.3.1 PFC modulation of behavior in ET males—One possible scenario for ET males is that their failure to express cocaine CPP at the medium dose may result from overactivity of the PFC which, in turn, drives activity of the NAc core [36]. Rats not exposed to alcohol during development (i.e. Miller & Marhsall, 2004) that have developed a cocaine CPP show attenuated PFC output. Thus, increased PFC output, possibly driving NAc core activity, may underlie the lack of cocaine CPP shown in ET males.

It may also be that ET males failed to exhibit cocaine CPP at the low and medium doses due to alcohol-induced learning and/or perceptual deficits. Indeed difficulties in learning [52] and perceptual ability [53] are apparent in the clinical FASD literature. These views, however, are overshadowed by the fact that ET males did exhibit a cocaine-induced CPP at the highest cocaine dose-indicating the learning or perceptual deficits were not apparent in this experimental group.

4.3.2 NAc modulation of behavior in ET males—Fos immunoreactivity in ET males provides further support for the idea that the NAc shell, rather than the NAc core, is critically involved in the formation of associations between distinct drug states and particular stimuli. This is evident in the results showing that ET females were more sensitive to cocaine's place preference-inducing effects and this effect accompanied a decrease in activity of GABAergic neurons of the NAc shell. ET males, however, failed to show cocaine CPP at the low and medium doses, and this effect was accompanied by a similar increased Fos activity in the NAc core but opposing effects at the NAc shell where ET males exhibit no differences in GABAergic cell number and activation. Thus, these data indicate a double-dissociation between the NAc shell and core suggesting that the shell is exclusively activated by drug cues.

4.4 Sex differences in FASD

In the current experiment, developmental alcohol exposure had a sexually dimorphic effect on sensitivity to drug cues in adulthood. A considerable amount of research using FASD animal models has shown sex differences in sensitivity to the various effects of abused drugs in the offspring including alcohol [5], amphetamine [54], and benzodiazepine [55].

According to Weinberg, Zimmerberg, & Sonderegger [56], there are two possible mechanisms for the variety of physical and behavioral sex differences that are seen after developmental drug exposure. First, these discrepancies may reflect sex-related differences in brain structures, neuron number, and connectivity among these areas. Sex differences in particular brain structures certainly occur in the current data. It has already been stated that there is a difference in PFC cortex morphology between typical male and female rats, and

shown not to impair memory [61]. This suggests that acamprosate may alter the influence of drug cues without affecting the mechanisms by which they are formed. Thus, acamprosate may be an appropriate treatment for FASD individuals who suffer from drug addiction by reducing the impact of drug cues on additive processes.

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Highlights

- Developmental alcohol exposure results in sexually dimorphic changes to drug cues
- Alcohol-exposed females exhibit CPP at lower doses of cocaine than controls
- Data suggest decreases in GABAergic neurons in the NAc shell and PFC in ET-females

Figure 1. Example of c-Fos and GAD₆₇ double-labeled immunohistochemistry

This example of immunohistochemistry highlights c-Fos (yellow arrow), GAD₆₇ (white arrow), and double-labeling of c-Fos and GAD₆₇ (red arrows). This image is at 20× magnification with the scale bar representing 100 μm. GAD₆₇ was developed using brown DAB, and c-Fos was labeled using blue/black nickel-cobalt based DAB. Co-localization is evident as GAD₆₇ is cytosolic and c-Fos is nuclear.

Figure 2. Percent increase in the non-preferred side after cocaine conditioning trials in males
 ET males failed to show a place preference induced by cocaine at the medium (3.0 mg/kg) cocaine dose, indicated by a star. ET, ethanol-treated; IC, intubated control; NC, non-treated control. $n = 8-10$ per group. Error bars represent standard error of the mean (SEM). *: significantly different from controls at $p < .05$.

Figure 3. Percent increase in the non-preferred side after cocaine conditioning trials in females
 ET females showed a place preference induced by cocaine at the lowest (0.3 mg/kg) dose, indicated by a star. ET, ethanol-treated; IC, intubated control; NC, non-treated control. $n = 8-10$ per group. Error bars represent SEM. significantly different from controls at $p < .05$.

Figure 4. Mean number of immunoreactive cells in the NAc, PFC and VTA of males in 0.1225 mm² blocks

Data reflect differences in the number of immunoreactive cells in ET, IC, and NC males at the 3.0 mg/kg cocaine dose when returned to the conditioning apparatus in the absence of a drug-pairing. Therefore c-Fos labeling reflects responses to the conditioning chamber and not to cocaine. At this dose, ET males had significantly more c-Fos labeled cells in the NAc core, the NAc shell, and PFC compared to controls. There was not a difference in double or Gad₆₇ labelled cells. *n* = 6 per group. ET, ethanol-treated; IC, intubated control; NC, non-treated control. Error bars represent the SEM. *: significantly different from controls at *p* < .05.

Figure 5. Mean number of immunoreactive cells in the NAc, PFC and VTA of females in 0.1225 mm² blocks

Data reflect differences in the number of immunoreactive cells in ET, IC, and NC females at the 0.3 mg/kg cocaine dose when returned to the conditioning apparatus in the absence of a drug-pairing. Therefore, c-Fos labeling reflects responses to the conditioning box and not cocaine. At this dose, ET animals had significantly increased c-Fos labeling in the NAc core. GAD₆₇-only labeled cells were reduced in the PFC and NAc shell, and this reduction of GAD₆₇-only labeled cells was accompanied by a decrease in Fos/GAD₆₇ double-labeled cells in the NAc shell. $n = 6$ per group. ET, ethanol-treated; IC, intubated control; NC, non-treated control. Error bars represent the SEM. *: significantly different from controls at $p < .05$.

Table 1

Dam Bodyweight (grams) by Gestational Day (GD)

Condition	GD 5	GD 10	GD 15	GD 20
NC	268.2 ± 3.5	282.4 ± 3.4	301.2 ± 3.1	338.3 ± 4.2
IC	255.5 ± 4.0	267.0 ± 3.9	285.1 ± 4.2	323.8 ± 5.0
ET	258.1 ± 3.3	270.2 ± 3.1	287.4 ± 3.5	323.7 ± 4.8

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Table 2

Rat Pup Bodyweight (grams) by Postnatal Day (PD)

Condition	Sex	PD 2	PD 10	PD 21	PD 30	PD 60	PD 90
NC	Male	7.4 ± 0.1	19.8 ± 0.1	45.2 ± 1.4	89.5 ± 2.4	303.7 ± 4.9	403.5 ± 6.5
	Female	7.0 ± 0.1	19.2 ± 0.2	43.4 ± 1.1	83.2 ± 2.1	208.8 ± 3.3	250.6 ± 4.1
IC	Male	6.9 ± 0.1	19.4 ± 0.1	43.1 ± 1.1	89.1 ± 2.3	310.9 ± 4.5	412.8 ± 5.5
	Female	6.5 ± 0.1	18.2 ± 0.2	41.0 ± 0.9	82.4 ± 1.8	208.6 ± 3.6	249.0 ± 5.3
ET	Male	6.3 ± 0.1	16.8 ± 0.2	43.2 ± 1.2	86.5 ± 1.7	299.9 ± 3.7	402.7 ± 4.6
	Female	5.9 ± 0.1	16.2 ± 0.3	41.9 ± 1.6	79.6 ± 3.6	198.5 ± 3.2	240.6 ± 4.2

Table 3

Time (seconds) in Non-Preferred Side in Males

Condition	Test Day	Saline	0.3 mg/kg	3.0 mg/kg	10 mg/kg
NC	Pre-Conditioning	276.9 ± 44.8	284.3 ± 24.5	334.5 ± 16.0	252.3 ± 19.5
	Post-Conditioning	225.1 ± 54.3	222.7 ± 41.0	531.1 ± 38.5	383.1 ± 71.3
IC	Pre-Conditioning	272.0 ± 57.0	287.3 ± 26.4	323.8 ± 17.9	280.9 ± 28.1
	Post-Conditioning	309 ± 61.7	257.3 ± 45.1	555.8 ± 46.0	437.1 ± 60.4
ET	Pre-Conditioning	264.9 ± 42.4	244.1 ± 3.1	324.5 ± 18.9	281.0 ± 33.9
	Post-Conditioning	250.1 ± 22.3	219.8 ± 7.7	265.7 ± 49.3	409.1 ± 51.5

Table 4

Time (seconds) in Non-Preferred Side in Females

Condition	Test Day	Saline	0.3 mg/kg	3.0 mg/kg	10 mg/kg
NC	Pre-Conditioning	233.2 ± 44.5	313.4 ± 23.4	254.9 ± 25.2	311.1 ± 20.4
	Post-Conditioning	290.0 ± 35.4	293.0 ± 24.9	366.4 ± 53.0	461.1 ± 24.6
IC	Pre-Conditioning	323.5 ± 23.6	284.4 ± 37.9	294.5 ± 7.7	330.7 ± 17.7
	Post-Conditioning	286.6 ± 37.1	301.5 ± 44.7	403.2 ± 20.2	465.1 ± 34.5
ET	Pre-Conditioning	319.8 ± 17.5	271.9 ± 32.0	269.5 ± 29.0	341.0 ± 12.2
	Post-Conditioning	290.3 ± 24.7	371.8 ± 41.1	396.0 ± 28.2	434.8 ± 24.3