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The effects of prenatal cocaine, post-weaning housing and sex on conditioned place preference in adolescent rats

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Abstract

Rationale—Gestational exposure to cocaine now affects several million people including adolescents and young adults. Whether prenatal drug exposures alter an individual's tendency to take and/or abuse drugs is still a matter of debate.

Objectives—This study sought to answer the question does prenatal exposure to cocaine, in a dose-response fashion, alter the rewarding effects of cocaine using a conditioned place preference (CPP) procedure during adolescence in the rat. Further, we wanted to assess possible sex differences and the role of being raised in an enriched vs impoverished environment.

Methods—Virgin female Sprague-Dawley rats were dosed daily with cocaine at 30mg/kg (C30), 60mg/kg (C60) or vehicle intragastrically prior to mating and throughout gestation. Pups were culled, fostered and on postnatal day (PND) 23 placed into isolation cages or enriched cages with 3 same-sex littermates and stimulus objects. On PND43-47, CPP was determined across a range of cocaine doses.

Results—C30 exposure increased sensitivity to the rewarding effects of cocaine in adolescent males and being raised in an enriched environment further enhanced this effect. Rats exposed to C60 resembled the controls in cocaine CPP. Overall, females were modestly affected by prenatal cocaine and enrichment.

Conclusions—These data support the unique sensitivity of males to the effects of gestational cocaine, that moderate prenatal cocaine doses produce greater effects on developing reward circuits than high doses, and that housing condition interacts with prenatal treatment and sex such that enrichment increases cocaine CPP most in adolescent males prenatally exposed to moderate cocaine doses.

Keywords

prenatal cocaine; sex differences; conditioned place preference; enrichment; isolation housing; reward

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No conflicts of interest.

While cocaine use has gone down in the general population since the early 1990's, a substantial segment of the population still uses the drug. The 2005 Monitoring the Future Study showed that cocaine and crack use has remained steady among 12th graders at about 5% for annual prevalence [2005 MTF 12/19/05 NIDA]. In the Miami Prenatal Cocaine Study which includes 267 women who self-reported cocaine use during pregnancy, 93 admitted to using crack cocaine at least 5 times per day as their "usual dose per day" during at least one full trimester. Children in this cohort have a variety of neurobehavioral problems including deficits on continuous performance tests, impaired sustained attention and other learning disabilities (Morrow et al. 2006). Other clinical studies from multiple centers describe a range of impairments in cognition, information processing, impulse control and spatial learning in exposed offspring (Mayes et al. 2005); (Singer et al. 2004); (Schroder et al. 2004); (Savage et al. 2005); (Jacobson et al. 1996).

Recent reports also show that prenatal cocaine exposure places adolescents at risk for illicit substance use including tobacco, marijuana, alcohol and in some studies, cocaine (Bennett et al. 2007), (Delaney-Black et al. 2011), (Frank et al. 2011), (Minnes et al. 2012) (Richardson et al. 2013). While the studies generally report higher incidences of licit and illicit drug use among males exposed to cocaine prenatally, multiple confounding factors including environmental risk factors and parental drug use were significantly related to adolescent drug use in these studies. Since environmental and genetic factors impact clinical studies, data from preclinical (animal) studies can help to tease out the biological effects of prenatal drug exposure from overall effects of an adverse genetic and/or postnatal environment. However, most animal studies terminate drug exposure at birth, therefore, they only address drug effects on developing brain during a period roughly equivalent to the first half of human brain development (Clancy et al 2007). Several animal studies using subcutaneous (sc) administration have reported that prenatal cocaine exposure increases drug self-administration (Keller et al. 1996) (Kelley et al. 1997); (Hecht et al. 1998). Other studies have found that prenatal cocaine reduced place conditioning to morphine (Estelles et al. 2006b) and dampened conditioned place preference (CPP) to both 2 and 5 mg/kg cocaine training doses Heyser et al (1992). However, Estelles et al. (2006a) found that prenatal cocaine exposure resulted in significant cocaine CPP at 3 and 25mg/kg but not at 50mg/kg. All of these studies used a single dose of cocaine during pregnancy precluding any dose-response examination of the effects of prenatal cocaine on CPP. Kosofsky, on the other hand, has studied prenatal cocaine in a dose-response fashion in the mouse and found long term effects on cocaine reward that can be summarized as increasing the sensitivity to cocaine assessed by operant behavior and reducing the sensitivity to cocaine measured with classical conditioning such as CPP (Rocha et al. 2002; Malanga et al. 2007). These findings can be interpreted as selective effects of prenatal cocaine on descending cortical projections perhaps mediated by altered neural development in cerebral cortex (Lee et al. 2011).

Since environment has been shown to have an impact on substance abuse by adolescents exposed to prenatal cocaine (Delaney-Black et al. 2011; Bennett et al. 2007; Minnes et al. 2012), preclinical studies should be able to confirm or deny the biological basis of this finding. Housing conditions of the rats can be varied to examine the effects of enriched or isolated conditions on cognition, emotion and reward (see review by (Simpson and Kelly 2011). While the conditions can vary from one study to the next, enrichment typically

occurs from postnatal day (PND) 21/22 (weaning) until the behavioral test is conducted. Isolation is generally believed to be stressful for a naturally social animal like the rat (Fox et al. 2006) while housing with novel objects and with peers is considered less stressful. Previous studies have examined the effects of environmental enrichment on drug reward and generally find that enrichment is “protective” against drug taking. Green et al. (2002) reported that enriched rats self administer less amphetamine than isolated rats and have attenuated accumbal extracellular dopamine following cocaine infusion. Others have reported that enrichment produces a rightward shift (reduced sensitivity) in the dose-response curve for cocaine self-administration (Howes et al. 2000; Green et al. 2010). However, several authors have found that enriched rats show greater CPP compared to isolated rats (Bardo et al. 1995; Bowling and Bardo 1994; Green et al. 2010). In fact, Green et al. (2010) examined both cocaine self administration and CPP as a function of post-weaning enrichment and found reduced self administration and increased CPP. One interpretation for this finding is that the enriched condition results in greater reward per dose of cocaine which would result in lower self administration rates. Therefore, most studies have examined adult rats and found that enrichment generally decreases drug self administration (reviewed in (Stairs and Bardo 2009). However, effects of enrichment could certainly be different in adults compared to adolescents and the various enrichment methodologies may contribute to the disparate results previously reported.

We hypothesized that prenatal cocaine would alter the response to environmental enrichment as assessed by conditioned place preference in adolescent male and female rats. We have now conducted CPP assessments on over 900 rats following prenatal exposure to intragastric (IG) cocaine at either 30 or 60 mg/kg and postweaning environmental/social enrichment or isolation.

We found that prenatal cocaine at the lower dose increased cocaine CPP especially in males and that enrichment further enhanced this effect.

Prenatal Treatment Method

Subjects

Sprague-Dawley rats (VAF strain, Charles River Laboratories, Wilmington, MA) were assigned to treatment groups (see prenatal dosing below). Rats were kept under a 12 h light-dark cycle (lights on at 7:00 h) and temperature of 20-22 °C. Females in proestrus were placed with males of the same strain at 4:00 PM. The next morning, rats were checked for sperm by vaginal smear. If sperm was present, that day was designated as gestational day 1 (G1). Pregnant dams were individually housed in plastic cages with bedding. Additional females in proestrus were mated with males on the day the treated females were sperm-positive to become surrogates for the treated pups. On the day of birth (usually G23), designated as postnatal day 1 (PND1), all pups were sexed, weighed and their toes were clipped for identification. Litters were culled to 10 pups (5 males, 5 females) and surrogate fostered to non-treated dams delivering within 2 days of treated rats' delivery. At PND 21 animals underwent ear punching for identification and were separated into same sex cages containing 5 pups until PND 23. On PND 23, rats were housed in one of two conditions: either one rat/cage (isolated environment) or 3 same-sex littermates/cage which also

contained stimulus objects (enriched environment) (see supplement for details of housing conditions). Rats in both housing conditions had access to food and water ad lib. Subjects were weighed at weekly intervals from PND 8 to PND 42 prior to testing. All procedures were approved by the IACUC in accord with the recommendations of the American Association of Laboratory Animal Science.

Prenatal dosing

Females were randomly assigned to receive either 60 mg/kg/day of cocaine, 30 mg/kg/day of cocaine, or vehicle (sterile water). Dosing by daily intragastric (IG) intubations using a 16 gauge straight feeding needle began 1 week after arrival of the rats in the vivarium and continued through mating and up to the day before delivery (G22).

Cocaine HCl (generously supplied by National Institute on Drug Abuse through Research Triangle Institute, Research Triangle Park, NC) was diluted in sterile water at 12mg/ml for intubation and in saline at the dose/ml/kg body weight for intraperitoneal (ip) injection during CPP training (see CPP procedure below).

Pup assignment

Of the 10 pups/litter, subjects were randomly assigned to enriched (3 same sex housed together) or isolated (2/sex) condition. Cocaine training dose was then assigned such that pups within each housing condition were administered one of the 3 core doses (5, 10, and 15mg/kg) with the goal that no two pups from the same sex/housing condition within a litter received the same training dose of cocaine. Pups were then randomly assigned to either saline or the higher or lower training doses as needed to obtain an ascending and descending limb of the doseresponse curve. However, in some cases, more than one pup/litter was in the same final group requiring an analysis that considered the litter as the statistical unit (see below).

CPP Method

Equipment

The apparatus consisted of Plexiglas boxes (42 × 42 × 30 cm) with removable opaque center doors. On one side, the walls were white, the lid was black and white striped, and the floor was smooth. On the other side, the walls were black and white vertical striped, the lid white, and the bottom was rough. There was approximately the same amount of light on both sides of the testing chamber. However, since approximately 75% of the rats preferred the rough side of the chamber on the preconditioning day, the apparatus was inherently biased (see supplemental figure 1 for initial side preferences by group). RCA video cameras were used to record movement of the rat from one side of the chamber to the other on the pre and post conditioning days.

Procedure

Subjects (offspring of prenatal treatment dams) were 44.5±1 day at the start of testing. Since we wanted to confine the CPP training and testing to a well-defined window within mid adolescence, we utilized the twice daily training procedure originally described by Badanich

et al. (2006) and modified by Zakharova et al (2009a). On the first day, each rat was placed in the testing chamber with door removed to allow free movement from one side to the other. The 30 min session was videotaped for analysis of the amount of time spent on each side of the chamber (time in seconds) to determine the side preference for subsequent conditioning days. A biased design was then utilized; i.e., pairing a cocaine dose with the “non-preferred side” and saline with the “preferred side” on day 1. For the next 3 days, the conditioning phase (run with the center door closed), rats were trained in the morning with saline (1ml/kg) on one side of the box (initially the preferred side) and in the afternoon with a dose of cocaine HCl on the other side of the box (initially the non-preferred side). Training doses were either 1.0, 3.0, 5.0, 10, 15, 20, or 30 mg/kg cocaine ip. Each rat received only a single training dose of cocaine. Some rats received saline on both sides of the chamber to assess possible effects of prenatal treatments and housing condition on the natural variation in side preference. Each training session lasted 30 min. and the AM and PM sessions were separated by 3 hours. On the fifth day, CPP testing occurred around midday. Each rat was injected with saline (1ml/kg) and randomly placed in the apparatus with the center door open and allowed to freely explore for 30 min. The session was video taped for analysis of the amount of time spent on each side of the chamber. The dependent measure was calculated by subtracting the time spent on the drug side of the chamber on day 1 (prior to conditioning) from the time spent on that side of the chamber on day 5 (post conditioning).

Since CPP has been shown to vary across the days of the estrous cycle (Mathews and McCormick 2007), on day 5 immediately after testing, all females underwent vaginal lavage for the determination of the phase of the estrous cycle. Fresh smears were microscopically examined under 10X.

Statistical methods

For maternal and litter variables

one way ANOVA was conducted using SYSTAT version 7. For pup weight gain and the CPP scores, mixed linear models were constructed. SAS (SAS Institute, Cary NC) Release 9.2 was used. The change in body weight from day 1 to 42 was analyzed using prenatal treatment, sex and housing condition as fixed factors and litter as a random factor. To account for heterogeneity of variance, residual variance was estimated separately for each of the 2×3×2 study arms. Satterthwaite adjustments were made to the denominator degrees of freedom. Significant interactions were interpreted using simple effects analyses. CPP scores or change from day 1 to day 5 for time spent on the side associated with the drug injection (i.e., post-conditioning minus pre-conditioning) were analyzed as the dependent variable; fixed factors were housing condition, sex, maternal dose, pup training dose; litter was a random factor. Satterthwaite corrections were made to denominator degrees of freedom. Model residuals were inspected for symmetry and for outliers. Scores at each training dose for each prenatal treatment/sex/housing condition were also compared to 0 (no conditioning) using planned *t* tests.

Stage of the estrous cycle was correlated with the CPP score using a Pearson correlation.

Results

Maternal/litter values

This study initially contained 31 control (vehicle intubated) dams, 32 dams receiving 30mg/kg/day cocaine (C30) and 29 dams receiving 60mg/kg/day cocaine (C60). There were deaths among all groups: 1 vehicle (3%), 3 C30 (9%) and 8 C60 (28%). Data from these dams were not included in any further analyses. Maternal weight gain was not affected by treatment (Table 1). Similarly, there were no differences in litter size or in % male pups across the treatment groups (Table 1).

Postnatal growth

For pup weight gain from PND 1 to PND42, there were significant main effects of housing condition and sex but not prenatal treatment. There were significant interactions between housing and sex [$F(1,309)=8.63$, $p=0.003$]; housing and treatment [$F(2,233)=3.71$, $p=0.025$] but not sex by treatment. Simple effects analysis of the housing by sex effect indicated that housing altered weight gain in both sexes with males showing significantly more weight increase in isolated condition than females (Figure 1). Simple effect analysis of the treatment by housing interaction revealed that there was a significant difference between the two housing conditions in each of the 3 treatment groups. The increase in weight gain under isolated conditions was greatest in the C60 condition (Figure 1). Litter did significantly contribute to the variance ($\chi^2_1=170.2$, $p<0.001$) therefore all main effects and interactions incorporate this contribution.

Conditioned Place Preference

Prior to conditioning, a logistic regression model to examine side preference by prenatal treatment, sex and housing condition (using litter as a random variable) found that while prenatal treatment had no influence on initial side preference, isolated males preferred the rough side (87%) significantly more than other males and either group of females (74-76%) (see Supplemental figure 1) $p<0.05$ for all three comparisons.

Following conditioning, scores from training doses of 5, 10 and 15 mg/kg cocaine as well as saline controls were analyzed as core doses administered to all prenatal treatment and housing groups (Figure 2, underlined doses). There was significantly more variability of scores among animals coming from enriched environments than from isolated ones ($\chi^2[1]=11.1$, $p<0.001$), so error variance was estimated separately for these two groups. There was a statistically significant litter effect ($Z=1.83$, $p=0.034$) which suggests that litter contributed to the variance of this analysis, and main effects and interactions take this into consideration. None of the 3- or 4-way interactions among fixed effects approached statistical significance, therefore, the model was refitted without these higher-order terms in order to more efficiently estimate lower-order effects. There was a statistically significant prenatal treatment main effect [$F(2,28)=4.18$; $p=0.0212$]; post-hoc analyses showed that scores of the C30 group differed significantly from those of both C60 ($p=0.025$) and vehicle ($p=0.010$) groups, while scores in the C60 and vehicle groups did not differ significantly from each other. Test dose was significant [$F(3,47)=3.97$, $p=0.0133$]. Post hoc analysis indicated that scores at all doses (5, 10 and 15 mg/kg) were significantly different from

those of the saline controls ($p=0.036, 0.002, 0.016$ respectively). There was also a main effect of sex [$F(1, 397)=4.53; p=0.0339$] and a significant sex-byhousing interaction [$F(1,396)=5.68; p=0.0176$]. Stratified by housing type, there was a significant sex effect for enriched environment animals ($p=0.001$) with males showing greater CPP than females but no sex difference among isolated animals. The effect of housing was marginally nonsignificant for females ($p=0.088$) and males ($p=0.101$)

While the overall analysis assessed prenatal treatment, sex and housing effects against saline controls, the small sample sizes in several of the saline groups (see supplement table 1) presented a problem when examining the effects of individual training doses within prenatal treatment/sex/housing groups. Therefore, the time spent on the drug side post-conditioning minus the pre-conditioning time at each training dose was compared to zero (no conditioning seen) using planned t tests. Examination of the full dose curves (Figure 2) reveals that among males, all groups showed maximal CPP at some training dose of cocaine. C30 isolated males showed a maximal CPP at a training dose of 10mg/kg (as did the C60 isolated males) while isolated males exposed to vehicle prenatally showed a maximal CPP at 20 mg/kg. Enrichment generally increased CPP in males since the C30 males showed highly significant CPP ($p<0.001$) at 5, 10 and 15 mg/kg training doses (dashed line). The enriched C60 males (dotted line) showed a maximal CPP at 20mg/kg. Also, the enriched vehicle exposed males (solid line) showed a significant CPP ($p<0.01$) at 5mg/kg, a dose which did not produce CPP in the isolated male prenatal controls. Those animals receiving saline on both sides of the chamber showed no significant CPP. As seen in Table 2, in males, enrichment lowered the lowest dose of cocaine and/or increased the range of doses showing significant CPP compared to isolation. (Supplemental figure 2 shows the mean (+ sem) pre and post conditioning times from which Figure 2 was derived).

For the females, a very different overall pattern was seen. The curves were generally flatter and significant CPP was seen across a narrow range of doses. For the isolated C30 females, significant CPP was seen at 5, 10 and 15 mg/kg training doses. For the prenatal vehicle controls, CPP was seen at lower doses (1 and 3 mg/kg) and again at the highest training dose tested (15 mg/kg) but not in between. The isolated C60 females did not show significant CPP at any dose ($p>0.05$). Enrichment, on the other hand, both dampened CPP at higher training doses and shifted the curve to the left in the C30 females. C60 females also showed a significant CPP following enrichment (an increase compared to isolated C60 females) and the vehicle treated females generally showed significant CPP in a narrow range of doses (Table 2). Direct comparisons between the effects of enrichment and isolation in control and C30 females (supplement figure 2), show that enrichment generally dampened sensitivity to cocaine but compared to males, the effects of enrichment were subtle. Since CPP has been shown to be affected by the day of estrous (Mathews and McCormick 2007), we examined the relationship between day of estrous and the CPP scores for each animal using a Pearson correlation (Table 3). There were no significant correlations for any prenatal treatment/housing group.

An additional group of 173 offspring from non-treated litters was exposed to identical postnatal treatment (housing, weighing, etc) and examined for CPP as described above. Due to differences in prenatal handling, non-treated litters were analyzed separately from the

treated litters and the results show a different pattern of effects. A mixed linear model analysis for training doses saline, 5, 10 and 15 mg/kg showed no statistically significant main effects or interactions. Planned analyses of differences from zero (*t* tests) show that enrichment increased CPP in both sexes (Figure 3) since males and females showed significant CPP across a wide range of doses (Table 2). However, there were relatively small sample sizes in some cells particularly in the groups receiving the lowest or highest doses (*N*'s ranged from 5 in one case to 14) somewhat limiting the opportunity to conduct a thorough analysis. Those animals receiving saline on both sides of the chamber showed no significant CPP.

Discussion

The results of our study of over 900 rats, bred in our lab, treated both prior to pregnancy and throughout pregnancy, fostered at birth, raised under either isolated or enriched conditions and then tested in the CPP procedure using a range of training doses reveals sex-specific effects of the prenatal treatments and environmental enrichment on cocaine CPP. We selected a bias design to conduct the CPP test (administering the drug in the non-preferred side) since we were interested in avoiding a ceiling effect (that can be seen by administering the drug on the preferred side in a random design) and to maximize the likelihood that we would find CPP in controls (Nomikos and Spyraiki 1988); (Schenk et al. 1985). We also avoided discarding subjects which showed a strong initial side preference as several authors have done in the unbiased design since each experimental subject represented a substantial investment of time and effort. One difficulty with the biased experimental design is that instead of showing an association of the chamber with the rewarding aspects of the drug, the drug may produce an anxiolytic effect increasing the likelihood that the subject would return to the previously aversive chamber. There is no way we can tease out this possibility using the current experimental design. Also, our apparatus is “biased” in that most subjects preferred the rough side of the chamber (data shown in Supplement figure 1) and an analysis of the percentage preferring this side showed that isolated males preferred the rough side significantly more often than all other groups. This difference, however, does not impact our conclusions about the effects of prenatal cocaine since there was no interaction of prenatal treatments with sex and housing conditions for initial side preference (see Cunningham et al. 2003 for complete discussion of apparatus bias). While we report CPP as the difference in time spent on the drug side post-training and the time spent on the same side pre-training, there are other ways of calculating CPP such as comparing day 5 time on drug side with day 5 time on saline side. We chose the day 5-day1 (or post-conditioning minus pre-conditioning) time as the dependent measure to simplify the statistical design and illustrate the change in time spent in a specific chamber due to the drug exposure. One limitation of our method is that only a single “pre-test” assessment was conducted and the time spent on either side necessarily includes habituation to the chambers. Others have utilized multiple exposures prior to conditioning as well as extensive prior handling (e.g. Malanga et al. 2007). Also, the role of novelty seeking on the final test day may be a factor since the pairing of the drug with the context necessarily alters the perception of the context. Testing without the drug may render the drug-associated context more novel than the saline context on the test day (see Bardo & Bevins 2000 for additional discussion). Since prenatal cocaine

has been reported to alter responses to novelty (Morrow et al. 2002), one cannot exclude the possibility that differential responses to the novel aspects of the drug-paired chamber contributed to the results obtained.

Effects of prenatal cocaine

In males, prenatal cocaine at 30mg/kg/day generally increased CPP under both housing conditions (Figure 2). That is, in isolated males, the lowest dose of cocaine producing CPP was a dose of 5mg/kg for the prenatal C30 group while the vehicle control group required a 10mg/kg training dose to show CPP (Table 2). In isolated males, the higher dose of prenatal cocaine, C60, produced CPP which was not different from prenatal controls according to the mixed linear model. Therefore, in males, daily prenatal cocaine exposure at 30mg/kg enhanced cocaine CPP in adolescence while the higher dose of prenatal cocaine produced relatively minor effects. That is, the C30 treatment increased sensitivity to the conditioned rewarding effects of cocaine compared to the C60 and vehicle treatments. In general, these results resemble those of Estelles et al (2006a) in offspring of mice exposed to 25mg/kg G12-18 showing CPP at training doses of 3 and 25 mg/kg cocaine but not the 50mg/kg dose. On the other hand, Heyser et al (1992) found reduced cocaine CPP in adulthood in offspring of rats exposed to 40mg/kg cocaine G8-20. Malanga et al (2007), the only other study to examine prenatal cocaine in a dose-response fashion, also conclude that prenatal cocaine reduces CPP in adult offspring. While all these studies examined prenatal cocaine effects on CPP in adult offspring, none have examined the full range of training doses to reveal the full inverted "U" shaped curve, none have examined adolescents, none have examined the effects of altered housing conditions and none have used IG administration during pregnancy. Therefore, while most of these other studies suggest that prenatal cocaine reduces responsivity to later cocaine administered noncontingently, our study clearly demonstrates that cocaine at 30mg/kg throughout pregnancy increases the intensity of CPP as well as reduces the dose at which the maximal CPP is obtained; i.e., prenatal cocaine increases the sensitivity to cocaine during conditioning (Figure 2). Also most studies examining the effects of prenatal cocaine on self administration found that exposure resulted in increases in contingently-delivered cocaine (Keller et al. 1996); (Hecht et al. 1998); (Rocha et al. 2002); (Malanga et al. 2008). Our studies are in general agreement with these studies. However, we found that administering a high dose of cocaine produced less of an effect on CPP than the lower prenatal dose. C60 males did show an enhanced CPP (compared to controls) but only at training doses of 15 and 20mg/kg following exposure to enriched conditions (Figure 2). Since the previous studies all used sc cocaine administration, they are not directly comparable to our studies which used the IG route. Intragastric administration produces relatively rapid increases and decreases in maternal and fetal plasma cocaine levels compared to the sc route (Dow-Edwards 1990). This high dose produced maternal death in almost 1/3rd of the rats indicating that it was not well tolerated. Other studies in our laboratory have used this dose (e.g. Torres-Reveron and Dow-Edwards 2006) and not experienced this degree of maternal death. However, in the current study, dosing was initiated prior to pregnancy instead of at G8. Dams in the current study received 10-12 additional doses of cocaine which may have produced sensitization and lethal seizures beyond what we normally see when dosing begins on G8. Therefore, since we are clearly delivering a pharmacologically active amount of drug, the reason for the relative lack of an

effect in the C60 prenatal group is not clear. Certainly, there is ample evidence that prenatal cocaine disrupts dopamine (DA) signaling when examined in offspring at multiple ages in rats and mice although the direction of change varies. For example, Keller et al (1994, 1996) and Malanga et al (2009) reported that prenatal cocaine increased basal DA and DA release; Yablonsky-Alter et al. (2005) found decreased basal DA levels and Salvatore et al (2004) found decreased dopamine transporter (DAT) in striatum. On the other hand, Phillips et al (2003) found presynaptic DA function relatively intact. Dose, route of administration, exposure period and age of assessment undoubtedly contribute to the differences in the reported effects of cocaine on DA system dynamics as concluded in a meta-analysis on the topic (Glatt et al. 2000). Presumably, the developing striatum is highly sensitive to cocaine. High dose prenatal cocaine may have hypothetically produced a down-regulation of the DA response element rendering the system unresponsive to subsequent cocaine administration. The low dose may have been insufficient to produce this change. High doses of cocaine may produce relatively greater impairment of uterine blood flow, greater hypoxia and malnutrition than lower doses would (Patel et al 1999). Also, it is unlikely that persistently high fetal levels of cocaine resulted in the development of tolerance since cocaine clears from the fetal circulation within a few hours (Dow-Edwards 1990). A preliminary analysis of the DAT levels in striatum of our animals suggests that there are no differences between DAT levels in the C60 and vehicle groups (Izenwasser, pers communication). Therefore, while the cause of the relative lack of effect of prenatal 60mg/kg cocaine on CPP compared to 30mg/kg is unknown, this finding emphasizes the importance of conducting dose-response studies. Certainly, high doses of psychostimulants administered during early development may have produced qualitatively different effects compared to lower doses due to multiple interacting effects of cocaine.

While CPP is generally considered a measure of the rewarding effects of drugs, the test depends on the formation of associations between the interoceptive cues provided by the drug and the exteroceptive cues provided by the conditioning chamber. Prenatal cocaine exposure has been reported to affect association processes in both animal and human studies (Trksak et al. 2007); (Morrow et al. 2006); (Levin and Seidler 1993); (Singer et al. 2004). Therefore, our diminished CPP following high dose prenatal cocaine may simply reflect a failure to associate the interoceptive cues imparted by cocaine during conditioning with the conditioning chamber. The C30 treatment may have been subthreshold to affect these learning and memory processes. However, the C60 males did show CPP at a similar level as the controls across most training doses and showed robust CPP at high training doses (20mg/kg) particularly if they had been raised in an enriched environment. Therefore, it is likely that the C60 males were able to form associations between cocaine and the conditioning environment; i.e., impaired learning was not responsible for the subtle effects of the C60 treatment.

Alterations in locomotor activity may also confound the results of the CPP testing. That is, failure to move from the initial site of placement could certainly impact on the preference score as we calculate it. However, several studies from this laboratory have examined baseline locomotor activity following prenatal cocaine and found no effect on activity in adolescence (e.g., Torres-Reveron and Dow-Edwards 2006). Therefore, it is unlikely that

effects on locomotor activity substantially influenced the results of the current study. (The animals were tested in a drug-free condition).

Effects of enrichment

Enrichment increased CPP in males, regardless of the prenatal condition and in females, increased CPP in NT groups and decreased CPP variably in all offspring of intubated dams (vehicle controls and cocaine-treated) (Figures 2,3, Table 2 and supplemental figure 3). Therefore, in females, the prenatal dosing/handling determines the response to environmental enrichment (see further discussion below). Enrichment has been shown to enhance cognition (reviewed in Petrosini et al. 2009) and may enhance the association between the interoceptive cues and the context thus increasing the magnitude of CPP at a given training dose (as seen in the C30 and vehicle males, Figure 2; and supplemental figure 3 for comparison of individual training doses). It should be noted, however, that while overall there was a significant interaction between sex and housing condition, the effect of housing was marginally nonsignificant within males and females and thus assessments of housing effects within individual sex and training dose groupings were not conducted. The increase in CPP following enrichment, in general, resembles that reported by several groups studying enrichment using large cages of 8 rats exposed from weaning to adulthood. In particular, a study by Green and coworkers (Green et al. 2010) examined both cocaine CPP and self administration in adult males following post-weaning enrichment (12 rats/cage) and found that CPP at a training dose of 10mg/kg was enhanced while self administration in similarly housed rats was reduced. One interpretation of these data is that the enriched rats found cocaine more rewarding (CPP study) and thus self-administered less cocaine. While this interpretation appears reasonable, a full dose-response profile of CPP was not conducted in their study and isolation may have produced greater CPP at a lower dose (such as 5mg/kg) thus altering their conclusions about the relationship between CPP and self administration. On the other hand, no study has examined CPP and self administration in the same subjects except one study which examined the correlation between single dose amphetamine preference and number of infusions in self administration in adult male rats; they found no significant relationship (Bardo et al 1999). Since drug taking is the most relevant measure of drug abuse, decreased self administration and increased CPP produced by enrichment suggest a lower tendency to abuse drugs like cocaine (Bardo and Bevins 2000). How would this relate to the human findings of increased drug taking in adolescents raised in poor/stressful environments? First, we found that male rats raised in isolation manifested reduced cocaine reward compared to enriched rats which by extension should be associated with increased self-administration as others have found (Green et al. 2010) (Bardo and Bevins 2000). Secondly, since prenatal cocaine (C30) increased CPP in both isolated and enriched rats, either the sensitivity to the rewarding effects of cocaine is increased by the prenatal treatment or the rats were better able to associate the unconditioned cue (cocaine) with the conditioning context. A study of self-administration in rats exposed to IG cocaine throughout pregnancy would clarify this issue.

A study by Zakharova et al. (2009a) in which several training doses were used to examine CPP following enrichment or isolation using parameters similar to the current report found that adding objects and cage mates decreased CPP when tested in adolescent males.

Surprisingly, the effects of enrichment in our non-treated rats (which are in some ways similar to their rats which are shipped from the supplier on PND 21) are opposite theirs. That is, we saw an increase in CPP at 10mg/kg training dose in both sexes of non treated rats following enrichment. While the details of the enrichment procedures, ages and procedures of the CPP studies are similar between the two laboratories, there are some procedural differences. We use CPP chambers with opaque dividers, we use video recording of day 1 and day 5 behaviors to quantify time on each side of the chamber (as opposed to experimenter observation they use) and we inject the rats with saline just prior to the day 5 test to maximize the cocaine-elicited cues. However, perhaps the biggest difference between the Zakharova et al. experiments and ours is the shipment of the rats on PND21 for their study while our rats are all offspring of females mated in our vivarium. Preliminary data collected in our laboratory show that the age of shipping of the rats (PND 14 vs PND 1) reverses the effects of enrichment on CPP in males (Dow-Edwards 2007). For the present study, all of our rats were born in our vivarium from females purchased from the same vendor as Zakharova et al.(2009a). Indeed, Brake and coworkers (2004) have shown that daily handling during the postnatal period results in a dampening of the effects of stress on nucleus accumbens function and that both maternal separation and non-handling during the early postnatal period result in hyperreactivity to novelty, increased sensitivity to cocaine and increased accumbal responses to a mild stressor. In addition, Campbell and Spear (1999) have shown that postnatal handling decreases amphetamine CPP in adulthood. All of our subjects are weighed at weekly intervals and undergo biweekly cage changes, activities which constitute mild handling during the early postnatal period. Rats shipped from the supplier at PND 21 have experienced no handling and the housing of 4 litters in the same cage during the postnatal period. Importantly, shipping on day of weaning may be a significant stressor. Therefore, we must conclude that conditions during the early postnatal period result in very different responses to enrichment, a concept which has broad implications for studies of drug responses in adolescence and perhaps in adulthood as well since the neurochemical alterations in the mesocorticolimbic dopamine system produced by early handling persist to adulthood (Brake et al. 2004). Please see supplemental figure 4 for idealized illustration of the relationships between early experience, enrichment, sex and CPP.

Sex differences

Overall, females showed a reduced magnitude of CPP compared to the males (Figure 2) while the range of doses producing CPP was shifted to the left (females more sensitive) as previously reported in adolescents and adults (Zakharova et al 2009b)(Russo et al 2003) (Table 2). Prenatal cocaine also appeared to produce smaller effects on CPP in females compared to males but female offspring of the prenatal 30mg/kg (C30) dams did show CPP at more training doses than control females (Table 2). The C60 female offspring showed no CPP following isolation rearing and in a very narrow range following enrichment. Certainly the phase of the estrous cycle may have contributed to increased variability in CPP scores since CPP has been reported to be different across days of the cycle (Mathews and McCormick 2007). Estrous cycle also influences cognitive performance (Bowman et al 2003; Shors et al 1998). Therefore, the association of cocaine effects with the context during training in females was undoubtedly influenced by the estrous cycle. However, on the final

test day, there were no significant differences in the phase of the estrous cycle across treatment groups as determined by vaginal smear and no correlation between day of estrous and CPP scores (Table 3). Interestingly, females appear to respond differently than males to enrichment. While in male offspring of both nontreated and vehicle treated (intubation control) dams, enrichment increased CPP at most doses studied, female offspring responded to enrichment differently depending on their prenatal history: offspring of vehicle treated dams showed modest decreases in CPP following enrichment and offspring of nontreated dams showed increases in CPP following enrichment (Figure 4). The most parsimonious explanation for this effect is that the daily intubations of vehicle constitute a stressor which initiates a series of changes in the developing female brain that eventually alters the effects of enrichment on reward circuits. While few groups have studied the effects of prenatal stress in both males and females, Thomas et al (2009) reported that prenatal stress increased cocaine self-administration in males and increased cocaine sensitization in females supporting a differential sensitivity of the brain circuits mediating these two behaviors. However, they did not examine the effects of enrichment. Very early enrichment decreases stress responses in female offspring and has no effect in males (Welberg et al 2006) while social housing in adulthood decreases corticosterone levels in females and increases them in males (Brown & Grunberg 1995). Importantly, prenatal stress induces sex-specific changes in gene expression in prefrontal cortex and hippocampus such that the hippocampi of females showed changes in genes which code for growth factors, an effect related to altered depressive-like behavior (Mychasiuk et al 2011). Others have reported sex-specific alterations in a range of behaviors including stress responses (Bhatnagar et al 2005) (see review by Fernandez-Guasti et al 2012). Therefore, our control data support a selective vulnerability to prenatal stress in females with stress altering the response to enrichment as assessed by cocaine CPP in adolescence. Hypothetically, the relative sparing of females for prenatal cocaine effects (compared to males) must be viewed in the context of an altered basic function of the mesolimbic system initiated by early maternal stress (from the intubations), undoubtedly involving altered gene expression and resulting in a modest increase in cocaine reward in the C30 females compared to the vehicle controls.

Certainly, the majority of clinical studies on the effects of prenatal cocaine exposure cite males as being more sensitive than females. For example, prenatal cocaine is associated with poor inhibitory control in males (Carmody et al. 2011) and reduced cognitive processing efficiency and executive function in boys compared to girls and compared to the non-exposed controls (Bridgett and Mayes 2011). In the mouse, Malanga et al. (2008) found that the reward potentiating effects of cocaine were increased in males following prenatal cocaine exposure while females were not affected. However, another preclinical study where both sexes were examined did not observe sex differences in the effects of prenatal cocaine on CPP (Heyser et al 1992). There is ample evidence that the brain regions responsive to cocaine (especially the mesolimbic dopamine system) undergo different developmental trajectories in females and males with female brains typically developing earlier than male brains (Reisert et al. 1990). It is important to note that while many preclinical studies administer cocaine during the later 1/3 of gestation, the current study administered the drug prior to and throughout gestation. Thus differential exposure would not occur early in

prenatal development but rather impact those events which occur during the postnatal period since drug administration was terminated at parturition.

Previous studies of prenatal cocaine and enrichment

There have been few previous studies on prenatal cocaine and environmental enrichment. Estelles et al (2005) reported that cocaine at 25mg/kg during the last third of pregnancy in the mouse altered the responses to isolation in adult males since group housing increased and isolation decreased social interactions compared to offspring of vehicle-treated controls. Magalhães et al (2006) administered Wistar dams a very high dose of cocaine (60mg/kg) sc, enriched pups during the preweaning period only and examined social behaviors. Neugebauer et al. (2004) examined the effects of 40mg/kg cocaine sc (also a fairly high dose of cocaine) throughout pregnancy on social behavior and nicotine responses in adult female offspring ovariectomized at mid adolescence. While enrichment (after weaning) dampened the effects of prenatal cocaine on play behavior, enrichment increased the effects of prenatal cocaine on DAT function in medial prefrontal cortex when the females were elderly (345 days) (Neugebauer et al. 2004). Their results actually resemble ours since we found modest effects of prenatal cocaine and enrichment in females. Certainly the high dose of cocaine in our study (which would produce similar blood levels to 40mg/kg sc) had very little effect in females (Figure 2). These results emphasize the value of conducting dose-response studies in both sexes to examine the effects of prenatal treatments on the effects of environmental manipulation and drug responsiveness.

Summary and Implications

The results of this study demonstrate first that, compared to controls and cocaine at 60mg/kg/day, gestational cocaine at 30mg/kg/day increases the sensitivity to the conditioned rewarding effects of cocaine as measured by CPP. That is, the dose of cocaine at which CPP is observed is lower in the C30 offspring than in the other groups. Males also show more robust increases in CPP than females following prenatal cocaine. Secondly, environmental enrichment increases CPP in males and in females depending on the drug/stress history of the mothers during pregnancy. Prenatal stress (with or without drug administration) alters the effects of enrichment in females. Therefore, for females, the prenatal stress history (treated vs nontreated) determines the effects of enrichment on CPP.

These results have several implications: First, together with several clinical studies examining the effects of prenatal cocaine on drug taking, prenatal exposure to a moderate amount of cocaine increases the sensitivity of the neural substrates underlying reward to cocaine in adolescents. Cocaine exposed rats can certainly perceive the interoceptive cues generated by cocaine during CPP training and readily associate the place where these cues are perceived. Prenatal cocaine also enhances the effects of environmental enrichment to increase CPP.

Others have reported that enrichment increases CPP and the current data support an enhancement of this effect by prenatal cocaine. Secondly, studies showing decreased drug self-administration in adults following enrichment are usually studying rats which were purchased from suppliers and have unknown prenatal or neonatal stress histories and

presumably little or no post-natal handling. Many studies have documented that postnatal handling, such as used in the current study, has significant effects on responsivity of the mesolimbic dopamine system and could certainly be expected to affect the dose-response relationships for cocaine CPP. Thirdly, the fact that the higher dose of prenatal cocaine produces minimal effects on CPP suggests that studies using high doses of prenatal cocaine may have reported no effects when there actually may have been effects of cocaine exposure on the developing brain if a lower prenatal dose had been studied. Of course, other brain regions (aside from the nucleus accumbens) may be less sensitive to cocaine during fetal development and thus exhibit fewer untoward effects of the high dose prenatal exposure. We previously found that many brain regions show metabolic abnormalities following prenatal 60mg/kg exposures (Dow-Edwards et al 2001) and we found that adolescents exposed to C60 show behavioral alterations following methylphenidate challenge (Torres-Reveron and Dow-Edwards 2006). Lastly, the minor effects of prenatal cocaine on cocaine reward in females contrasts with the enhanced responses of adult females to cocaine. Adolescent females are more sensitive to cocaine in the CPP test than males and adult females (show CPP at lower training doses; Zakharova et al. 2009b), but prenatal exposures and housing conditions have relatively minor effects on overall CPP in females. These results suggest that there are complex interactions between prenatal drug exposures, housing and the development of reward circuits and that these interactions differ in males and females.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Pup body weight change (g) by sex and prenatal treatment for isolated and enriched rats.

Pups derived from 30 vehicle, 29 C-30 and 21 C-60 litters. (mean $\pm$ sem).

Figure 2.

Conditioned place preference (time on drug side on Day 5-Day 1 in sec) in male and female rats at PND49 for several training doses (X axis) Prenatal treatments are:

Vehicle cocaine 30mg/kg/day cocaine 60 mg/kg/day
 —■— veh —▲— c30 —▼— c60

Overall analysis was conducted using mixed linear model comparing saline and C5, C10 and C15 training doses (underlined) which indicated that scores of all training doses were significantly different from saline. Within prenatal treatments, sexes and housing conditions, scores of individual training doses were subjected to t tests (compared to zero) (***) denotes significantly different from 0 at $p < 0.001$, ** denotes significantly different from 0 at $p < 0.01$, * denotes significantly different from 0 at $p < 0.05$, no stars, no difference from 0 = no preference).

Figure 3. CPP in offspring of non-treated dams. Pups were reared under isolated (■□) or enriched (●○) conditions as for the treated offspring and tested under identical conditions. (***) denotes significantly different from 0 at $p < 0.001$ by t test, ** denotes significantly different from 0 at $p < 0.01$, * denotes significantly different from 0 at $p < 0.05$, no stars, no difference from 0 = no preference).

NonTreated vs Vehicle Treated

Figure 4.

CPP in offspring of non-treated (···) or vehicle treated (—) dams. (Data from Figs 2 and 3 re-plotted) Pups were reared under isolated or enriched conditions and tested under identical conditions. Results show that enrichment (●) enhanced CPP in all groups except vehicle treated females compared to isolation housing (■). (***) denotes significantly different from 0 at $p < 0.001$ by t test, ** denotes significantly different from 0 at $p < 0.01$ * denotes significantly different from 0 at $p < 0.05$, no stars, no difference from 0 = no preference).

Table 1

Treatment	N	Weight gain (g)	Litter size	% male pups
Vehicle	30	147.8±4.2	14.1±0.3	49.7±1.8
Cocaine 30 mg/kg	29	149.9±10.4	13.6±0.3	46.9±2.2
Cocaine 60 mg/kg	21	135.6±4.8	14.0±0.4	48.7±2.9

Mean ± sem

No significant treatment-related effects

Table 2
Range of doses producing CPP

	Isolated males	Enriched males
Prenatal treatment		
vehicle	C10-C20	C5-C30
C 30	C5-C15	C5-C15
C 60	C10-C15	C10-C20
Nontreated	C5	C5-C15
	Isolated females	Enriched females
vehicle	C1, C3, C15	C1-C5
C 30	C5-C15	C1-C10
C 60	-----	C3-C5
Nontreated	C5	C3-C10

Doses of cocaine at which CPP was significant (different from 0 by t test) for each prenatal treatment, sex and housing condition.

Table 3
Correlation coefficients (Pearson) for day of estrous cycle with CPP preference. Prenatal treatment and postweaning housing condition

Group	N	Pearson value
All females	471	0.068
Vehicle/isolated	59	0.231
Vehicle/enriched	89	0.046
C30/isolated	52	-0.030
C30/enriched	74	0.166
C60/isolated	38	0.132
C60/enriched	64	0.013
NT/isolated	38	-0.187
NT/enriched	57	-0.067

Each day of estrous, including intermittent phases, was assigned a numerical value (1-6) and correlated with the day 5-day1 score in sec for each rat.