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Prenatal exposure to cocaine alters the development of conditioned place-preference to cocaine in adult mice

C.J. Malanga *, Martina Pejchal, Barry E. Kosofsky [1]

Laboratory of Molecular and Developmental Neuroscience, Department of Neurology, Massachusetts General Hospital, Boston, MA 02129, United States

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Abstract

As addiction is increasingly formulated as a developmental disorder, identifying how early developmental exposures influence later responses to drugs of abuse is important to our understanding of substance abuse neurobiology. We have previously identified behavioral changes in adult mice following gestational exposure to cocaine that differ when assessed with methods employing contingent and non-contingent drug administration. We sought to clarify this distinction using a Pavlovian behavioral measure, conditioned place-preference. Adult mice exposed to cocaine *in utero* (40 or 20 mg/kg/day), vehicle and pair-fed controls were place-conditioned to either cocaine (5 mg/kg or 20 mg/kg, i.p.) or saline injections. The development of conditioned place-preference to cocaine was impaired in mice exposed to cocaine *in utero*, and was abolished by fetal malnutrition. A context-specific place-aversion to vehicle but not cocaine injection was observed in prenatally cocaine-exposed mice. Locomotor behavior did not differ among prenatal treatment groups. We conclude that early developmental exposure to cocaine may diminish the subsequent rewarding effects of cocaine in adulthood measured with classical conditioning techniques, and that this is not due to changes in locomotor behavior. Sensitivity to acute stress is also altered by prenatal cocaine exposure, consistent with earlier findings in this model.

Keywords: *In utero*; Gestation; Psychostimulant; Craving; Place-conditioning; CPP

1. Introduction

Prenatal exposure to drugs of abuse, both legal and illicit, affects over 800,000 infants, or approximately 20% of all live-births, each year in the United States (National Institute on Drug Abuse, 1995). An estimated 45,000 of those infants are exposed to cocaine at least once during their gestation, and the cost to the American public in special educational expenses alone for these children is estimated to exceed $350 million annually (Lester et al., 1998). Long-term follow-up of cocaine-exposed infants has demonstrated that these children have persistent deficits in multiple developmental domains (Bandstra et al., 2002; Griffith et al., 1994; Singer et al., 2002) as well as in their regulation of both affect and attention (Jacobson et al., 1996; Leech et al., 1999; Richardson et al., 1997). An emerging question is whether early developmental drug exposure can increase the liability for addictive behaviors later in life. While prenatal exposure to alcohol (Alati et al., 2006; Baer et al., 2003) and to tobacco (Al Mamun et al., 2006; Cornelius et al., 2000; Kandel et al., 1994) has been shown in clinical studies to be associated with an increased risk of alcohol and nicotine abuse, respectively, in adolescents and young adults, similar studies on the rate of adolescent or adult drug abuse in cocaine-exposed children have not yet been published.

Preclinical behavioral measures such as drug self-administration (Koob et al., 1994), intracranial self-stimulation (Kornetsky and Bain, 1992), and conditioned place-preference (Bardo and Bevins, 2000; Tzschentke, 1998) are useful in the investigation of the rewarding effects of drugs of abuse. Gestational exposure to drugs of abuse has been associated with alterations in reward perception and motivated behavior in adulthood, including altered rewarding potency of drugs of

* Corresponding author. Current address: Department of Neurology, University of North Carolina at Chapel Hill, 3114 Bioinformatics Bldg., CB 7025, Chapel Hill, NC 27599-7025, United States. Tel.: +1 919 966 1683; fax: +1 919 843 4576.
E-mail addresses: malangacj@neurology.unc.edu (C.J. Malanga), bar2009@med.cornell.edu (B.E. Kosofsky).
[1] Current address: Department of Pediatrics, Weill Medical College of Cornell University, The New York Presbyterian Hospital 525 East 68th Street, New York, NY 10021, United States. Tel.: +1 212 746 3278; fax: +1 212 746 8137.

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abuse (reviewed in Malanga and Kosofsky, 2003). Increased responsiveness in two operant behavioral models, intravenous self-administration of cocaine (Keller et al., 1996; Rocha et al., 2002) and potentiation of rewarding electrical self-stimulation by cocaine (Lin and Kellogg, 1996; Malanga et al., in press), have been demonstrated in adult animals following early developmental exposure to cocaine. Conversely, chronic non-contingent administration of cocaine to adult animals exposed to cocaine *in utero* results in less behavioral sensitization than in gestational controls (Crozatier et al., 2003; Guerriero et al., 2005) and reduced conditioned place-preference both to morphine (Estelles et al., 2006b) and to high-doses of cocaine has been reported in adult animals exposed to cocaine *in utero* (Estelles et al., 2006a).

While there is a significant literature on the effects of gestational exposure to drugs of abuse on the development of dopaminergic neural systems, studies employing behavioral measures of reward or reinforcement in adult animals following prenatal exposure to cocaine are comparatively limited. Furthermore, an apparent dissociation exists between our own findings on the subsequent potency of cocaine administered in a behaviorally non-contingent manner (*i.e.*, locomotor sensitization) compared to the effects of cocaine on operant behavior after prenatal cocaine exposure. We have chosen to explore this distinction further using a classical Pavlovian behavioral method, conditioned place-preference testing, and hypothesize that exposure to cocaine *in utero* exerts distinctly different effects on the subsequent effects of cocaine on instrumental *versus* classically-conditioned behaviors in adulthood. We further hypothesize that the development of conditioned place-preference to cocaine in adult mice will be decreased by prenatal exposure to cocaine, similar to the decreased development of locomotor sensitization to cocaine that we observe in adult mice exposed to cocaine *in utero*. We present here the results of a series of experiments using a three-chamber apparatus and 4 days of conditioning with either cocaine or saline injections in adult mice that were exposed to cocaine *in utero*.

2. Materials and methods

2.1. Animal care and handling

All experimental animal procedures were carried out in accordance with the NIH *Guide to the Care and Use of Laboratory Animals* and the Society for Neuroscience *Policy on the Use of Animals in Neuroscience Research*, and were approved by the Subcommittee on Research Animal Care (SRAC) at the Massachusetts General Hospital.

2.2. In utero cocaine exposure

Mice were exposed to cocaine *in utero* as previously described in detail (Wilkins et al., 1998a). Briefly, adult timed-pregnant white Swiss–Webster dams were purchased (Taconic Labs) for all studies, allowed access to food and water *ad libitum*, and housed on a 12-h (7:00 AM light–7:00 PM dark) cycle. On embryonic day 6 (E6), dams of comparable weight were segregated into four experimental groups: two that received cocaine (40 mg/kg/day, COC40; or 20 mg/kg/day, COC20), one that received saline with food available *ad libitum* (SAL), and one that received no cocaine but was pair-fed to the COC40 dam (SPF40). In our protocol, chronic high-dose (40 mg/kg/day) cocaine administration is known to cause anorexia in pregnant dams; a pair-fed gestational group was therefore employed to control for the effects of maternal malnutrition on intrauterine growth and development of offspring. While an untreated control group was not employed in these studies, Estelles et al. (2006a) have shown in mice that saline-injected and uninjected dams and litters do not differ across measures of maternal food intake or weight gain, gestational length, litter number or pup weights. On E7 all pregnant dams were placed on a liquid diet (BioServ, #F1259SP). COC40, COC20 and SAL dams were allowed free access to the diet from E7 until parturition. All dams were weighed and diet consumption was recorded daily for the COC and SAL dams from E7 through term (E18–19). SPF40 dams were purchased with a one-day gestational lag behind the other three groups, and were provided with the same volume of liquid chow consumed on the prior day (*i.e.*, on the same gestational day) by a paired COC40 dam. From E7 through parturition (E18–19) all dams received twice-daily (7:00 AM and 7:00 PM) injections: COC40 and COC20 dams received 20 mg/kg and 10 mg/kg of cocaine HCl, respectively, dissolved in sterile normal saline; SAL and SPF40 received an equivalent volume of sterile saline. All injections were subcutaneous (s.c.) and injection sites were rotated with each administration. At birth (postnatal day zero, P0) each litter was fostered to an untreated, non-drug exposed surrogate black Swiss–Webster dam that had given birth within the preceding 24–72 h. A total of 75 litters were generated to provide offspring for these experiments. Pups were weaned and group-housed (4 mice per cage) by gender on P21.

2.3. Conditioned place-preference (CPP)

Our CPP protocol consisted of a 13-day conditioning regimen. On day 1, mice were separated and individually housed, in order to compare CPP data to findings from our i.v. cocaine self-administration (Rocha et al., 2002) and intracranial self-stimulation studies (Malanga et al., in press). Meta-analysis of multiple studies showed that single housing does not affect the development of cocaine CPP in rats (Bardo et al., 1995), and cocaine CPP has been demonstrated in singly-housed C57Bl/6J (Szumlinski et al., 2002) and 129/Sv mice (Brabant et al., 2007). On days 2–4 each mouse was handled for 5 min daily, and on days 5–7 mice were handled for 5 min daily and injected with saline vehicle in their home cage.

The conditioning apparatus is a three-compartment mouse CPP chamber (MedAssociates, Georgia, VT) consisting of a central grey compartment (7.2 cm × 12.7 cm; smooth plastic floor) with black (3.2 mm bar floor) and white (6.35 mm wire mesh floor) compartments (16.8 cm × 12.7 cm) on either side, separated by guillotine doors. Luminous intensity was measured on both sides (black and white) with a photographic light meter and equalized with adjustable interior lighting. On day 8, mice

were placed in the central grey compartment and allowed free access to all three chambers for 900 s (15 min; pre-conditioning). Duration of time spent in each chamber and total distance traveled during the test period were measured and recorded by an automated infra-red detection system. Mice that spent more than 500 s on either side or more than 400 s in the central grey compartment were excluded from further study. For all subsequent experiments, conditioning trials were counter-balanced by initial bias defined as the chamber (black/bar floor or white/mesh floor) in which the animal spent the greater amount of time in the pre-conditioning trial on day 8: mice were assigned alternately to receive all subsequent conditioning injections in either their initially preferred or non-preferred compartment. Pre-conditioning test data from all mice in aggregate and among all mice grouped by prenatal exposure were not analyzed until after the completion of all experiments. Analysis of individual animal data on the day of the pre-conditioning test was used to determine the chamber in which each mouse spent more time compared to the other side; counterbalancing by initial bias was therefore based on the preference of each animal demonstrated by its own behavior in the pre-conditioning test.

On days 9–12, mice were given two daily conditioning sessions (10 AM/3 PM). In the morning conditioning session, mice were injected with saline vehicle (i.p.) and confined to either the white or the black compartment for 30 min. In the afternoon conditioning session, mice were injected either with saline, cocaine HCl 5 mg/kg i.p., or cocaine HCl 20 mg/kg i.p. and confined to the compartment opposite from the morning session for 30 min. To control for cumulative cocaine exposure, control mice given saline vehicle injections in the afternoon conditioning session were administered cocaine (5 mg/kg i.p.) in their home cage 2 h after the completion of the afternoon session. A total of eight conditioning sessions, four with cocaine and four with saline in the opposite chamber, were administered to each mouse. On day 13, mice were again placed in the central grey compartment and allowed free access to all three chambers for 900 s (15 min; post-conditioning). For the pre- and post-conditioning sessions, total distance traveled and time spent in each compartment were measured. A total of 121 adult (P60–180) male mice divided among the four prenatal treatment groups were examined.

2.4. Statistical analysis

Parametric data were analyzed with Student–Newman–Keuls to assess prenatal effects on parturient dams and litters. Locomotor data were analyzed with 4×4 (conditioning treatment × prenatal treatment) two-way ANOVA; data on initial chamber preference were analyzed with 3×4 (chamber × prenatal treatment) two-way ANOVA for examination of effects of prenatal treatment on initial chamber bias. After inspection of initial pre-conditioning test data, the development of conditioned place-preference was calculated with both within-chamber and between-chamber metrics to control for possible influences of apparatus bias (Cunningham et al., 2003). In the within-chamber method, the dependent variable was calculated for each animal as time spent in the chamber after conditioning minus the time spent in the same chamber in the pre-test; in the between-chamber method, the dependent variable was calculated for each animal as the difference between the times spent in the drug-paired and saline-paired chambers during the post-conditioning test. Conditioning data computed with both metrics were analyzed with 3×4 (cocaine dose × prenatal treatment) two-way ANOVA for examination of effects on place-preference; in all cases simple pairwise comparisons between drug-paired chambers pre- and post-conditioning or between drug-paired and saline vehicle-paired chambers are reported when significant. In order to avoid oversampling bias, or "litter effects" of prenatal treatment in multiparous species (Holson and Pearce, 1992), only one mouse per conditioning treatment (saline, cocaine 5 mg/kg, cocaine 20 mg/kg) from each exposed litter was used.

3. Results

3.1. Dam and litter parameters

The growth parameters of the dams and their litters used in these experiments are summarized in Table 1. As previously published, the prenatal cocaine exposure regimen we employ did not significantly affect pup viability or the gender distribution of the offspring (Wilkins et al., 1998a,b). While food intake was only significantly different from saline controls (SAL) in high-dose cocaine (COC40) dams, both high- and low-dose cocaine (COC20) dams gained significantly less weight during gestation than SAL control dams. In this series of litters, pair-feeding (SPF40) increased the length of gestation by less than 1 day, and both SPF40 and COC40 dams had fewer pups per litter.

3.2. Pre-conditioning test results

Post-hoc analysis of data from the pre-conditioning tests (3×4 ANOVA; chamber color × prenatal treatment) revealed a

Table 1
Summary of maternal and litter parametric data

	COC40	COC20	SPF40	SAL
Total # of litters	20	17	17	21
% Gestational weight gain in dams, E8–E17	33.2±2.5*	50.3±2.7*	20.5±1.8*	59.5±2.4
Total food intake by dams (g), E8–E16	131.7±6.8*	165.7±6.9	N/A	180.5±15.8
Gestational length (days)	18.7±0.1	18.5±0.1	19.1±0.1*	18.5±0.1
Litter size at P0	10.7±0.4*	13.1±0.4	9.4±0.3*	12.5±0.4

All values are means±S.E.M. * = $P<0.01$ *vs.* SAL controls (Student–Newman–Keuls).

Table 2
Summary of initial apparatus bias by chamber color

	n	White	Grey	Black
COC40	29	340±13 s (158–475)	219±11 (131–388)	343±9 (215–427)
COC20	30	339±7 (276–414)	217±8 (149–339)	345±8 (246–472)
SPF40	28	360±14 (211–492)	204±9 (107–288)	336±13 (202–437)
SAL	34	363±12 (229–482)	203±7 (108–282)	336±10 (188–417)

Values are means±S.E.M.; values in parentheses represent the range of times spent in each chamber in the pre-conditioning test. Total pre-conditioning test session=900 s.

significant effect of chamber color ($F_{(2,363)}=223$, $P<0.01$) but not prenatal treatment ($F_{(3,363)}=0.002$, $P=1.00$) or an interaction of color and prenatal treatment ($F_{(6,363)}=1.20$, $P=0.30$) on the distribution of time spent in the three chambers of the apparatus: all mice spent significantly less time in the grey chamber than either the black or white chambers ($P<0.01$ grey *vs.* black; $P<0.01$ grey *vs.* white) and no significant differences were observed between time spent in the black and white compartments (white *vs.* black: $P=0.15$) among mice from all prenatal treatment groups (Table 2; Fig. 2, Pre). In addition, no significant differences were evident among mice from all four prenatal treatment groups for time spent in any one compartment (White: $F_{(3,121)}=1.20$, $P=0.31$; Black: $F_{(3,121)}=0.12$, $P=0.95$; Grey: $F_{(3,121)}=0.92$, $P=0.43$). These data suggest that the three-chamber testing apparatus employed in these experiments is intrinsically unbiased (for an extended discussion of apparatus bias in place-conditioning, see (Cunningham et al., 2003)).

There was a tendency observed among the mice not exposed to cocaine *in utero* (*i.e.*, SPF40 and SAL) to spend more time in the white chamber and less time in the black chamber compared to the cocaine-exposed groups (COC40 and COC20; Table 2). When analyzed directly, grouping mice by the presence or absence of any prenatal cocaine exposure (3×2 ANOVA; chamber color×prenatal cocaine exposure [COC40+COC20 *vs.* SPF40+SAL]), a significant main effect of chamber color ($F_{(2,363)}=228$, $P<0.01$) and a significant interaction of prenatal cocaine exposure and chamber color ($F_{(2,363)}=3.64$, $P<0.05$), but no main effect of prenatal cocaine exposure ($F_{(2,363)}<0.01$, $P=0.97$), on pre-conditioning test times were observed. Similar analysis grouping mice by prenatal malnutrition (COC40+SPF40 *vs.* COC20+SAL) again showed significant effects of chamber color ($F_{(2,363)}=224$, $P<0.01$) but neither prenatal malnutrition nor an interaction of malnutrition with chamber color ($F_{(2,363)}<0.01$, $P=0.97$ and $F_{(2,363)}=0.03$, $P=0.97$, respectively) on pre-conditioning test times.

3.3. Post-conditioning test results

The development of conditioned place-preference was assessed by two methods: 1) comparison of pre- and post-test times within the conditioning chamber; and 2) comparison of post-test times between the two chambers. In the first method (Post–Pre), preference was calculated as the time spent in the chamber after conditioning with either cocaine or saline minus the time spent in the same chamber in the pre-test prior to conditioning (Fig. 1A). Using this within-chamber metric, a significant overall main effect of cocaine dose ($F_{(2,121)}=15.54$, $P<0.01$) but not prenatal treatment ($F_{(3,121)}=2.04$, $P=0.11$) was observed. The interaction of cocaine dose with prenatal treatment approached but did not attain statistical significance ($F_{(6,121)}=2.03$, $P=0.07$). In the second method (Drug–Saline), preference was calculated as the difference between the amount of time spent in the cocaine-paired chamber and the saline-paired chamber during the post-conditioning test (Fig. 1B). Using this between-chamber metric, a significant overall main effect of cocaine dose ($F_{(2,121)}=17.24$, $P<0.01$) but neither prenatal treatment ($F_{(3,121)}=2.36$, $P=0.08$) nor a significant interaction of cocaine dose with prenatal treatment ($F_{(6,121)}=1.21$, $P=0.31$) were observed.

Despite the assignment of mice to chambers for conditioning based on initial preference and not on color, when accounted for in the statistical design (4×3×2 ANOVA; prenatal treatment×cocaine dose×conditioning chamber color) a significant main effect of the color of the chamber in which cocaine conditioning

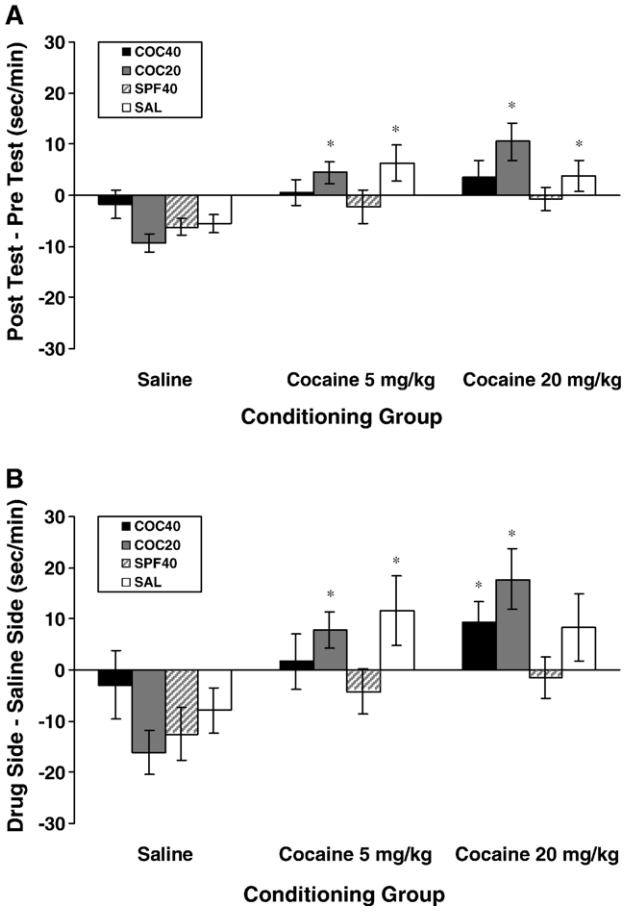

Fig. 1. Time spent in the drug-paired compartment of the apparatus during the 15 min post-test following conditioning. In A., values are plotted as time spent in the drug injection-paired compartment during the post-conditioning test minus time spent in the same compartment during the pre-conditioning test in seconds per minute; in B., values are plotted as time spent in the drug injection-paired compartment minus time spent in the saline-paired compartment during the post-conditioning test in seconds per minute. All values are means±S.E.M.; $n=9$–14 mice per prenatal treatment in each conditioning group. Expressed in this manner, positive values suggest a rewarding effect and negative values suggest an aversive effect of conditioning treatment in both figures. * = $P<0.05$ *vs.* saline. See Results for details.

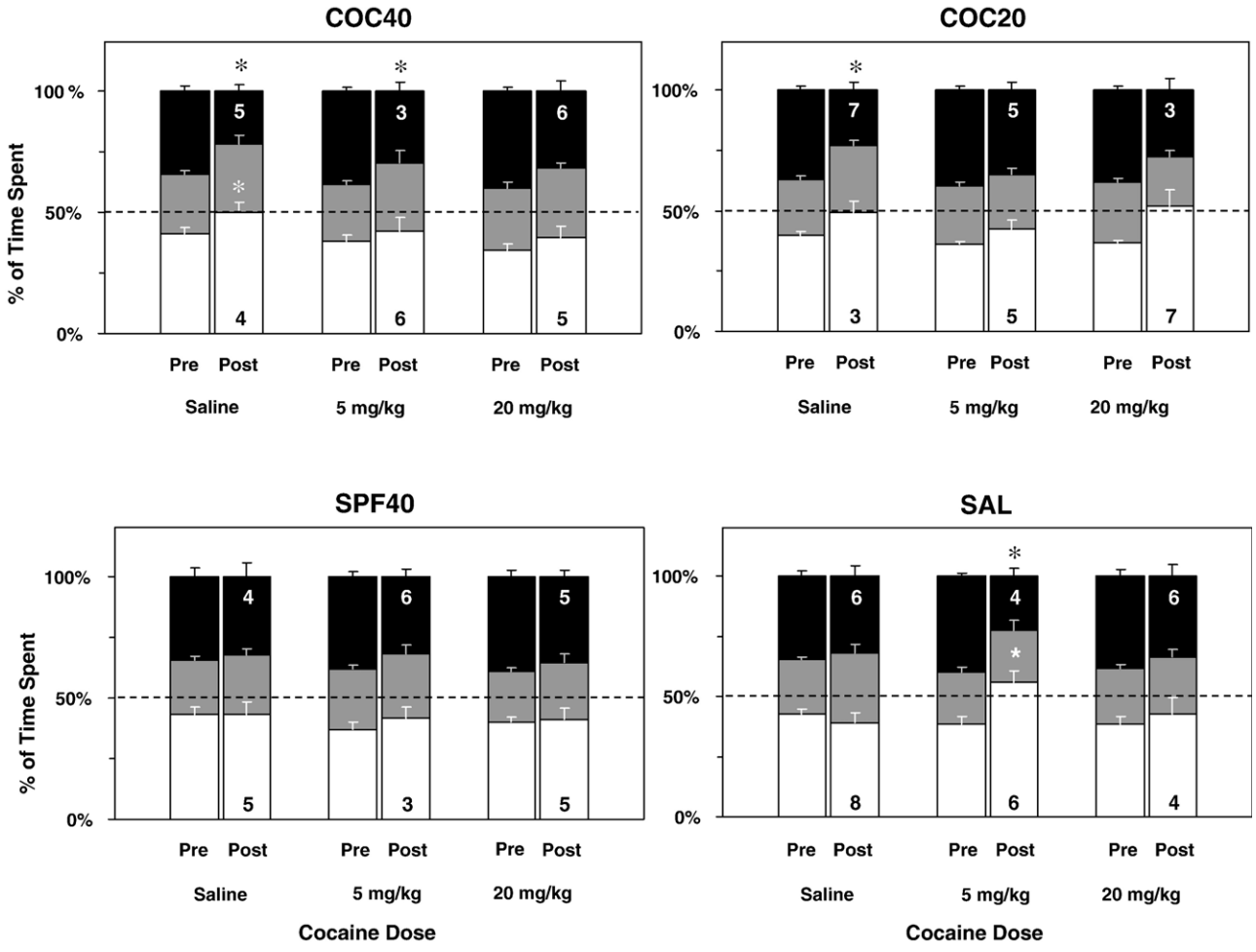

Fig. 2. Percent of time spent in each chamber of the apparatus during the 15 min pre- and post-conditioning tests. Values are plotted as percent of the total 900 s testing session spent in each compartment, independent of the compartment in which mice received conditioning injections. All values are means+S.E.M.; *n* values are illustrated in the compartment in which the mice were conditioned (*i.e.*, for COC40, saline, $n=9$ mice). * = $P<0.05$ *vs.* pre-conditioning. See Results for details.

occurred on time spent in the conditioning chamber calculated with both metrics was observed (Post–Pre: $F_{(1,121)}=23.10$, $P<0.01$; Drug–Saline: $F_{(1,121)}=35.61$, $P<0.01$) as well as a significant main effect of cocaine dose (Post–Pre: $F_{(2,121)}=15.35$, $P<0.01$; Drug–Saline: $F_{(2,121)}=17.60$, $P<0.01$). For the data measured with the Drug–Saline metric, a main effect of prenatal treatment approached but did not attain statistical significance ($F_{(3,121)}=2.50$, $P=0.06$). Significant interactions between prenatal treatment and either chamber color or cocaine dose were not observed. The proportion of the total time of both pre- and post-conditioning test sessions spent in each compartment, independent of the color of the conditioning chamber, was analyzed and is summarized in Fig. 2. After saline conditioning both COC40 and COC20 mice spent a significantly lesser proportion of the post-conditioning test session in the black chamber and COC40 mice spent a significantly greater proportion in the white chamber, regardless of the chamber in which conditioning occurred, whereas there was no difference in the proportion of time spent in each of the three chambers after saline conditioning in the SPF40 or SAL mice. After conditioning with 5 mg/kg of cocaine, SAL mice spent a significantly greater proportion in the white chamber, and both COC40 and SAL mice, but neither COC20 nor SPF40 mice, spent a significantly lesser proportion of the test session in the black chamber. No significant changes in the proportion of the post-test spent in the central grey chamber were observed in any mice after any conditioning regimen, and no significant changes in the percentage of the post-test spent in any of the three chambers were observed after conditioning with 20 mg/kg of cocaine.

As shown in Fig. 2, because mice were assigned to conditioning chambers based on initial preference and not by chamber color, *n* values in several instances were as low as 3 per chamber per condition for each prenatal treatment group (*e.g.*, COC40, 5 mg/kg, black chamber). Preference scores were therefore not calculated based on color of the chamber in which conditioning occurred. However, the apparent context-dependency of the results was further explored by direct comparison of time spent in each of the two conditioning chambers, as shown in Fig. 3. A significant main effect of cocaine conditioning dose (White: $F_{(3,242)}=4.58$, $P<0.01$; Black: $F_{(3,242)}=14.73$; $P<0.01$) as well as a significant interaction of prenatal treatment with cocaine conditioning dose (White: $F_{(9,242)}=2.39$, $P<0.02$; Black: $F_{(9,242)}=2.36$; $P<0.02$) was observed, although no significant main effect of prenatal treatment on time spent in

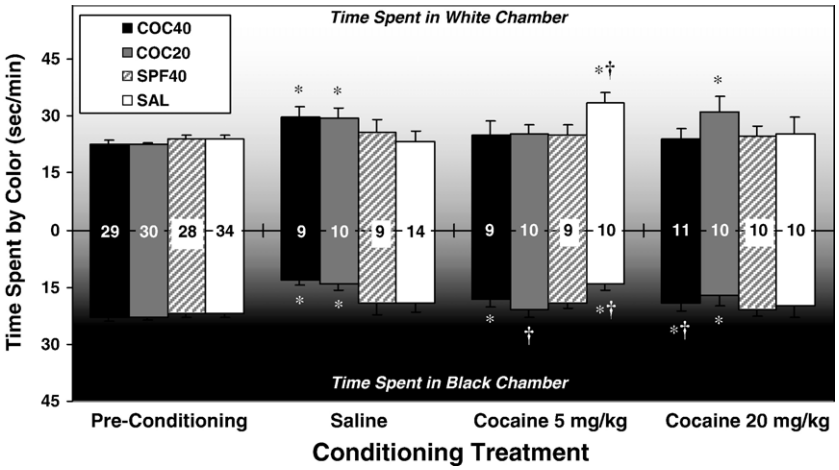

Fig. 3. Time spent in the conditioning compartments of the apparatus during the 15 min pre-test and post-tests, expressed in seconds per minute. All values are means±S.E.M.; *n* values are illustrated for each group. * = $P<0.05$ *vs.* pre-conditioning; † = $P<0.05$ *vs.* saline. See Results for details.

either compartment was seen (White: $F_{(3,242)}=1.04$, $P=0.37$; Black: $F_{(3,242)}=1.30$; $P=0.28$). Pairwise comparisons indicated that COC40 and COC20 mice spent significantly more time in the white chamber and significantly less time in the black chamber after saline conditioning, and COC40 mice spent significantly less time in the black chamber after any conditioning regimen.

3.4. Locomotor behavior

In the 15 min pre-test prior to drug or saline conditioning no differences were observed in spontaneous locomotor behavior, measured as total distance traveled, among the prenatal treatment groups (COC40=2996±96 cm; COC20=2960±95; SPF40=2872±98; SAL=2859±93; $F_{(3,242)}=0.75$, $P=0.53$). In the injection-free 15 min post-conditioning tests after both saline and cocaine conditioning, spontaneous locomotion was significantly decreased in all groups ($F_{(3,242)}=28.72$, $P<0.01$); however, there was no significant main effect of cocaine dose on post-conditioning locomotor behavior and post-test locomotor changes did not differ significantly between prenatal treatment groups ($F_{(3,242)}=1.14$, $P=0.34$).

4. Discussion

In summary, we find that 1. Prenatal cocaine exposure reduces the development of conditioned place-preference to cocaine in adult male mice; 2. Prenatal cocaine exposure is associated with alterations in behavioral responses to acute stress in a context-dependent manner; 3. Prenatal malnutrition in the absence of cocaine exposure profoundly impairs the development of cocaine CPP; and 4. Basal locomotor activity does not differ in adult male mice following prenatal cocaine exposure.

In CPP studies, the dependent variable used to quantify preference can rely solely on post-test behavior or can be determined relative to a pre-conditioning test: the results of these two methods of calculation may or may not differ when animals are assigned to conditioning environments in a biased fashion (Cunningham et al., 2003), as in our present study. In examining our data calculated with either within-chamber (*i.e.*, post-test minus pre-test) or between-chamber post-test metrics, the effect of prenatal treatment on the development of cocaine CPP is not statistically significant in the overall 4×3 ANOVA; however, one-way ANOVA of the effect of cocaine dose on each prenatal treatment group alone clearly demonstrates different patterns of behavior when mice are conditioned with cocaine compared to saline injections. Meta-analysis of multiple studies has shown that the development of conditioned place-preference to cocaine, unlike the opiates, more closely resembles a threshold effect rather than a dose-dependent one (Bardo et al., 1995) and further suggests that the development of CPP to the psychostimulants cocaine and amphetamine follows an inverted U-shaped dose–response relationship with reversal of effects at doses higher than 10 mg/kg of cocaine. Our data are consistent with this interpretation. Calculated with either metric, our data demonstrate conditioned place-preference in COC20 and SAL mice, but not in COC40 mice, to 5 mg/kg of cocaine (Fig. 1A and B). In the between-chamber analysis (Fig. 1B) we no longer observe cocaine CPP at 20 mg/kg in the SAL control mice, but do observe CPP in the COC40 and COC20 mice, suggesting that the inverted U-shaped dose–response curve may be shifted to the right by prenatal cocaine exposure. We conclude from these data that the development of conditioned place-preference to cocaine in adult mice is reduced following prenatal exposure to 40 mg/kg/day of cocaine.

In rats, a prenatal cocaine exposure regimen similar to ours has been shown to interfere with the development of CPP to cocaine in adult animals employing a similar three-chamber (white/grey/black) apparatus and a similar four conditioning trial design (Heyser et al., 1992a). Recently, it has also been shown that prenatal cocaine exposure is associated with decreased development of CPP to both morphine (Estelles et al., 2006b) and to high-dose (50 mg/kg) but not low-dose (3, 25 mg/kg) cocaine (Estelles et al., 2006a) in adult mice. The studies of Estelles et al. differ from ours in two important respects, one related to the gestational cocaine exposure of the mice and one related to the CPP method employed. First, the duration of gestational cocaine exposure is shorter (E12 through term, compared to E7 through term), and the

cocaine-exposed pups are not cross-fostered to unexposed dams. It is known that gestational cocaine exposure profoundly impacts maternal behavior, which in turn strongly influences post-weanling behavior in exposed offspring (Goodwin et al., 1992; Heyser et al., 1992b; Johns et al., 2005; Lubin et al., 2001). Second, in our study, assignment of mice to conditioning chambers alternated by initial chamber preference (half in preferred/half in non-preferred), while in the study by Estelles et al. mice were randomly assigned to the two chambers without reference to the pre-conditioning test data. However, their subsequent *post-hoc* analysis demonstrated no difference in the time spent between the two compartments in the pre-conditioning test; their CPP apparatus was therefore unbiased. Despite these methodological differences, our conclusions that prenatal cocaine exposure results in decreased development of CPP to cocaine in adult mice are in general agreement.

The role of apparatus bias and its relationship to experimental design in place-conditioning has been elegantly treated in a recent review (Cunningham et al., 2003). While the unconditioned mice in our study demonstrated no statistically significant preference for either compartment in aggregate, suggesting that the testing apparatus is intrinsically *unbiased*, our study was designed in a *biased* fashion; that is, half of the mice were assigned to receive the unconditioned stimulus (UCS, *i.e.*, cocaine or saline vehicle injections) in their initially preferred and half in their initially non-preferred compartment, defined as the chamber in which they spent the greater amount of time during the 15 min pre-test. In conditioned place-preference studies, the conditioned stimulus (CS) consists of specific environmental cues, such as wall color and floor texture, which in and of themselves can significantly affect the behavior of the animals and the development of CPP (Cunningham et al., 2003). In agreement with our present findings, other investigators have also found that drug-naïve adult male Swiss–Webster mice demonstrate a small but consistent preference for the white over the black compartment in place-conditioning experiments (Itzhak and Martin, 2000; Itzhak et al., 1998; Martin and Itzhak, 2000), although the effect size does not usually attain statistical significance. We also found that vehicle and nutritional control Swiss–Webster mice tended to prefer the white chamber, while mice exposed to cocaine *in utero* had a tendency to spend equal amounts of time in both chambers in pre-conditioning trials (Table 2). When grouped by prenatal cocaine exposure regardless of dose (40 or 20 mg/kg/day), this difference attained statistical significance. Gestationally cocaine-exposed mice also developed an enhanced relative aversion to the same CS (black, bar floor) that was initially non-preferred by control mice following pairing with a UCS consisting of saline vehicle injections alone (Figs. 2 and 3). One interpretation of these data is that prenatal cocaine exposure changes sensitivity of offspring to the stress of intraperitoneal injection, such that injections paired with one CS (black, bar floor) exposes an enhanced natural aversion to that environment. These data are consistent with, while not directly comparable to, our locomotor studies (Guerriero et al., 2005) in which COC40 and COC20 mice continued to exhibit greater spontaneous locomotion and habituated less than SAL and SPF40 controls after three consecutive daily i.p. saline injections. We conclude that behavior after saline injection, in this case the development of a context-specific aversion, differentiates COC40 and COC20 from SPF40 and SAL control mice.

Male Swiss–Webster mice develop conditioned place-preference to cocaine administered in the black, initially non-preferred compartment compared to saline injections (Itzhak and Martin, 2000; Itzhak et al., 1998) and have been shown to develop CPP to [LEU]enkephalin when administered in an initially non-preferred environment but an aversion to the opioid peptide when administered in an initially preferred environment (Heinrichs and Martinez, 1986). However, cocaine CPP has also been demonstrated in male Swiss–Webster mice using a counterbalanced experimental design (Itzhak and Martin, 2002). Although there is no difference between spontaneous exploration of the two environments by cocaine-exposed mice prior to conditioning in our data (Table 2) the stress of intraperitoneal injection in the black/bar floor chamber, the innately non-preferred environment in control mice, is apparently enhanced in mice exposed to cocaine *in utero*. This response of COC40 and COC20 mice to saline vehicle injections (see Figs. 2 and 3) suggests the possibility that the effects of acute cocaine administration in cocaine-exposed adult offspring may be related to alleviation of a negative hedonic state created by the stress of intraperitoneal injection in a non-preferred environment rather than to the development of a preference for the rewarding property of cocaine, or an appetitive response *per se*. This interpretation assumes that prenatal cocaine exposure increases the sensitivity of exposed offspring to the effects of acute stress compared to controls, a presumption supported by data from both footshock-induced freezing (Molina et al., 1994; Planeta et al., 2001) and Porsolt forced-swim test (Bilitzke and Church, 1992; Molina et al., 1994) experiments.

It is generally appreciated that the use of pair-feeding, while a conceptually important control in teratological studies, nevertheless presents a stressor to the pair-fed dam and her litters (Spear et al., 1998). While this procedure controls for total caloric intake and the anorexic effects of cocaine, and while all dams are stressed by daily injections, it creates the additional confound of the stress of hunger in addition to daily injections, as the COC40 dams consume fewer calories voluntarily and the SPF40 dams are additionally stressed by caloric restriction. Dissociation of effects in nutritional controls should therefore be interpreted cautiously. We find that SPF40 mice do not develop a preference to a cocaine-paired environment. This lack of effect is not due to generalized behavioral suppression, as the locomotor activity of SPF40 mice is not significantly different than either SAL or cocaine-exposed mice. It has been shown that prenatal malnutrition independently impairs spatial learning in adulthood, and that this impairment is associated with deficits in long-term but not short-term memory (Galler and Tonkiss, 1998). It is therefore possible that failure to associate the non-contingent delivery of a reward (UCS) with a specific set of environmental cues (CS) could result in impaired place-conditioning to cocaine. The significance of our findings in SPF40 mice is unclear; however, given that we do observe the development of CPP to cocaine in COC40 offspring at 20 mg/kg, we conclude that prenatal malnutrition does not fully account for the reduction in

cocaine potency we observe in gestationally cocaine-exposed adult mice. The larger question of the role of prenatal stress, including malnutrition, in the sensitivity to drugs of abuse in adulthood is a topic of active investigation. Maternal stress has been shown to increase amphetamine self-administration in adult offspring (Deminiere et al., 1992), but to our knowledge no studies have been published that directly examine the effect of prenatal stress on the subsequent development of conditioned place-preference to any drugs of abuse in affected offspring.

Previous work from our laboratory (Guerriero et al., 2005) demonstrated that prenatal cocaine exposure reduced the development of locomotor sensitization to cocaine in adult mice. In that study spontaneous locomotor activity before and after the first vehicle injection did not differ among prenatal treatment groups, similar to results previously reported by other laboratories (Sobrian et al., 1990), while cocaine-exposed mice demonstrated significantly impaired habituation of spontaneous activity following saline injection over three consecutive days. Our current data show that neither spontaneous pre-conditioning locomotor activity nor spontaneous locomotor activity after 4 days of saline conditioning differ among prenatal treatment groups, although in our procedure both pre- and post-test locomotion are measured without the acute stressor of vehicle injection or the acute effects of cocaine. Thus, while the activity data presented here are not directly comparable, our current findings are consistent with our prior study in that initial spontaneous activity does not differ after prenatal cocaine exposure. We conclude from these data that changes in overall locomotor activity do not contribute to the differences we observe in the development of cocaine CPP between prenatal treatment groups.

While within-chamber comparison of time spent before and after conditioning treatments is commonly employed, it is not the exclusive measure of the development of conditioned place-preference. Other metrics, particularly the comparison of time spent in the UCS+ and UCS− chambers after conditioning (Andersen et al., 2002; Carlezon et al., 1998; Cervo et al., 1996; Heinrichs and Martinez, 1986) are often used to measure the magnitude of CPP (reviewed in Bardo et al., 1995; Cunningham et al., 2003). Our analysis of these data underscores the importance of the metric used to measure preference for the CS. We observe quantitatively but not qualitatively different results when assessing the development of conditioned preference by the difference between time spent in the UCS+ compartment before and after conditioning *versus* assessment by time spent in the UCS+ and UCS− compartments after conditioning (Fig. 1). Also, as seen in our data, analysis by environmental characteristics (*i.e.*, chamber color, floor texture) independent of initial preference may reveal effects on place-conditioning between treatment groups. Even in the absence of a clear apparatus bias, such analysis may inform studies comparing different strains of animals or may reveal subtle effects of other complex treatment regimens preceding the drug conditioning study.

The neurobehavioral teratology literature remains controversial regarding the effects of prenatal exposure to drugs of abuse on later vulnerability to drug-seeking or drug-consuming behaviors: gestational exposure has been associated with increased, decreased, or unchanged behavioral sensitivity of exposed offspring to the effects of both contingent and non-contingent administration of drugs of abuse in adulthood (reviewed in Malanga and Kosofsky, 2003). It is appreciated that operantly and classically-conditioned behaviors rely upon different but interconnected sets of neural circuits (Berridge and Robinson, 2003), and significantly different effects of contingent *versus* non-contingent exposure to drugs of abuse on individual elements of those circuits have been well-documented (Jacobs et al., 2003). It is likely that gestational exposure to drugs of abuse, which from the fetal perspective is always behaviorally non-contingent, may affect discrete elements of these developing neural systems at a cellular level in a manner that may result in different or even opposite changes in learned behaviors later in development. While we have previously reported increases in cocaine sensitivity in adult mice following prenatal cocaine exposure with operant intravenous self-administration (Rocha et al., 2002), and operant intracranial self-stimulation (Malanga et al., in press), our current data are consistent with those from our prior investigation that, like this study, employed a classical, Pavlovian conditioning method and in which we found decreased locomotor sensitization to chronic behaviorally non-contingent cocaine administration in gestationally cocaine-exposed mice compared to controls (Guerriero et al., 2005).

Our findings suggest that adult mice exposed to cocaine *in utero* may be more sensitive to acute stress than non-exposed controls; however, prenatal cocaine exposure may also decrease the rewarding potency of cocaine that may allow animals to overcome aversion to a stressor in classically-conditioned models. Examination of the pharmacological responses of gestationally cocaine-exposed animals in other behavioral procedures that are more specific to drug effects on negative hedonic states, such as fear-potentiated startle (Ralph-Williams et al., 2003), black-white box testing (Crawley and Goodwin, 1980; Griebel et al., 2000), or the forced-swim test (Carlezon et al., 2003) will be more informative regarding their responsiveness to acute stress. One consistently emerging conclusion from our investigations, however, is that among the behavioral methods most commonly used to investigate the rewarding properties of drugs of abuse, early developmental exposure to cocaine appears to increase the potency of cocaine in adulthood when tested with operant procedures such as self-administration and intracranial self-stimulation, and to diminish cocaine potency in procedures employing non-contingent administration and classical conditioning, such as locomotor sensitization and conditioned place-preference. This series of preclinical findings may inform the design of future clinical studies in drug-exposed children regarding both their response to environmental stressors and their later liability for drug-abusing behavior.

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