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Gestational Cocaine and Ethanol Exposure Alter Spontaneous and Cocaine-induced Behavior in Weanling Rats

PAUL M. KUNKO,[1] MELISA J. WALLACE AND SUSAN E. ROBINSON[2]

Department of Pharmacology and Toxicology, Medical College of Virginia, Virginia Commonwealth University, Richmond, VA 23298-0613

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KUNKO, P. M., M. J. WALLACE AND S. E. ROBINSON. *Gestational cocaine and ethanol exposure alter spontaneous and cocaine-induced behavior in weanling rats.* PHARMACOL BIOCHEM BEHAV **55**(4) 559–564, 1996.—The developmental and behavioral effects of prenatal exposure to cocaine and/or ethanol were examined in rats. Pregnant rats received ethanol (E; 2 g/kg, b.i.d.) orally, cocaine (C; 6 mg/kg/day, IV), or both (C/E) on gestational days 8-20. Controls consisted of pair-fed (PF) and untreated (UNT) groups. Offspring were weighed and examined for developmental markers beginning postnatal day one (PD1). On PD21 pups were individually observed in an open-field following either an injection of cocaine (10 mg/kg, IP), an injection of saline, or no treatment. Drug-treated and PF dams ate less food and gained less weight than the UNT dams. C and E litters had slightly increased mortality rates. Pups from both the C and E groups appeared less sensitive to the locomotor stimulant effect of cocaine. Pups from the E group engaged in significantly less spontaneous stereotypic locomotion than UNT and PF pups, while male pups from the C group exhibited a decrease in spontaneous exploratory behavior. Thus, prenatal exposure to C or E altered spontaneous and/or cocaine-induced behavior in weanling-aged rats, while the C/E combination did not augment either effect. **Copyright © 1996 Elsevier Science Inc.**

Prenatal exposure Cocaine Alcohol Rat Open-field Stereotypy Locomotion Teratology Chronic administration

THE USE of alcohol in combination with cocaine is common within the cocaine abusing population (8). While the teratogenic effects of both cocaine and alcohol have been described, few studies have examined whether they act synergistically or antagonistically, or whether they interact at all. There have been several studies of prenatal cocaine and alcohol (ethanol) effects in animal models. These include reports that the combined chronic administration of cocaine and ethanol has a more adverse effect than either drug administered alone in terms of pregnancy risk (4) and offspring mortality and maturity (5) in rats. Ethanol and cocaine might not always increase fetotoxicity however, as effects due to ethanol are not always compounded by cocaine (23).

The current study examines the effects of prenatal exposure to ethanol and cocaine, alone and in combination, on offspring development and behavior through the weaning period. In this instance, cocaine is administered intravenously as opposed to the more common practice of subcutaneous administration. Intravenous administration produces rapid increases in blood and brain levels of cocaine (10), a phenomenon which mimics cocaine kinetics in humans (11); conversely, subcutaneous administration is characterized by a slower rise and subsequent fall in the levels of cocaine and its metabolites (27). To the best of our knowledge, the teratogenic effects of the combination of intravenous cocaine administration with oral ethanol administration have not been examined. The results from our model suggest that both cocaine and ethanol alter offspring viability and behavior, while the combination of the drugs did not increase their effects.

METHODS

Subjects

Sprague–Dawley "CD" rats were purchased from Harlan Labs (Indianapolis, IN) and housed individually in a tempera-

[1] Present address: Addiction Research Center, NIDA, Baltimore, MD 21224.
[2] To whom requests for reprints should be addressed.

ture and humidity controlled facility fully accredited by the American Association for the Accreditation of Laboratory Animal Care (AAALAC), and the studies were conducted in accordance with the Guide for Care and Use of Laboratory Animals provided by the NIH and adopted by NIDA. All animals were maintained on a 12 h/12 h light/dark cycle with food and water available ad lib. Nulliparous females were placed with male conspecifics for breeding purposes. The detection of a seminal plug beneath the cage indicated copulation and the day the plug was detected was designated day 0 of gestation (GD0). Beginning GD0 each dam was weighed and individually housed in a plastic breeding cage. Body weights, as well as food and water intakes, were recorded daily.

The day of parturition was designated as postnatal day 0 (PD0). Within 24 h of birth each litter was counted, sexed, and weighed. Litters were then culled to 10 pups, with equal numbers of females and males retained when possible, and fostered to untreated dams which had delivered within 24 h of the treated dams. Pups were weighed daily through PD21 and observed for the emergence of several developmental milestones which included righting reflex, negative geotaxic response, vertical screen test, ear opening and eye opening. Treated dams were euthanized and their uteri examined to determine the number of implantation sites.

Dosing Procedure

On GD8 pregnant rats were randomly assigned to one of five treatment groups. Each morning at approximately 0900 h the cocaine/ethanol group (C/E) received ethanol (2.0 g/kg) by oral gavage, followed 10 min later by an intravenous (IV) injection of cocaine HCl (6.0 mg/kg). The cocaine/water group (C) received distilled water by gavage followed by IV cocaine, and the saline/ethanol group (E) received ethanol by gavage followed by IV saline. A second dose of ethanol or water (or sucrose, see below) was administered approximately 6h after the initial administration (1,4,5,6). This brought the total ethanol dose to 4.0 g/kg/day, a subteratogenic dose which produces no gross morphological defects (4,5,23,26). A pair-fed control group (PF) received a sucrose solution, isocaloric to 40% ethanol, by gavage followed by an IV saline injection. The pair-fed group was yoke pair-fed to the C/E group. An additional group of control dams was untreated (UNT).

The ethanol solution was prepared by diluting 95% ethanol with distilled water to produce a final concentration of 40% ethanol by volume, and administered in a volume of 6.3 ml/kg body weight. Cocaine (National Institute on Drug Abuse, NIH, Bethesda, MD) was prepared once per week in a concentration of 1 mg/ml, and the unused portion frozen. Previous studies indicated that 6.0 mg/kg was the highest dose that could be chronically injected via the tail vein without producing seizures (17).

Behavior Measures

Individual pups were placed in an open-field and observed on PD21 as described previously (17). Briefly, the open-field was a 90cm X 90cm square divided into five equal sections (including a circle in the middle). Pups were injected with cocaine HCl (10 mg/kg, ip) or saline, or received no treatment and were immediately placed in the open-field. One female and one male from each litter was assigned to each treatment group. The 30-min sessions (24) were videotaped and were later scored by an observer blind to treatment. During the observation each pup's locomotor path was traced on a schematic of the open-field. Several measures were used to determine the animals' activity levels, the prevelance of stereotypic behaviors, and exploratory behavior. Horizontal locomotion and vertical locomotion were measured as line crossings (movement between sections) and rearing, respectively. Each animal's locomotor pattern was examined for repeated, or stereotypic, patterns using the gamma ($\hat{\gamma}$) statistic (21). The incidence of repeated locomotor patterns in untreated animals is typically 20%, corresponding to a $\hat{\gamma}$ score of 0.200. Stereotypic locomotion is characterized by scores of 0.400 (40%) or more. A subjective activity measure was also used. At five minute intervals each pup was observed for 30 seconds and received an activity rating from a six-point scale. The scale ranged from a score of zero for animals that were asleep or stationary, through levels of increasing activity (1-3), to scores of four and five for animals exhibiting varying levels of stereotypy. A stereotypy index was calculated for each pup by summing the number of stereotypic episodes from both the $\hat{\gamma}$ scores and from the activity rating. For instance, each pup received six behavioral ratings, one every five minutes, during the 30-min observation; when locomotor stereotypy is also determined at five-minute intervals there are 12 possible incidences of stereotypic behavior. A pup with three behavior ratings in the stereotypic range (4's and 5's) and three incidences of locomotor stereotypy ($\hat{\gamma} \geq 0.400$) would receive an index score of six. Exploratory behavior was assessed in pups in the untreated condition by determining the latency to enter all five sections of the open-field. Exploratory behavior was not examined in cocaine- and saline-injected pups due to the confounding nature of the injection procedure. The open-field provided a novel environment as the pups had no pre-exposure or habituation sessions.

Data Analyses

Weight gain, food intake and water intake for the dams were analyzed by two-way analysis of variance (ANOVA) with treatment as a between-subjects factor, and time as a within-subjects factor. Litter variables (e.g. litter size, mortality index) were analyzed by one-way ANOVA. Female and male litter averages were used to analyze pup weight and developmental data. Pup weights were analyzed by three-way ANOVA with prenatal treatment and sex as between-subjects factors and time as a within-subjects factor. The development data were analyzed by two-way ANOVA with prenatal treatment and sex as between-subjects factors. Open-field data were analyzed by four-way ANOVA with prenatal treatment, sex and drug treatment (cocaine, saline, untreated) as between-subjects factors and time as a within-subjects factor. Significant interactions were followed by tests for simple interactions and simple main effects. Post hoc analyses were performed by Fisher's protected least significant difference, which is a multiple comparison test (16), and by linear contrast analyses. Exploratory behavior data was analyzed by contingency table using a Chi-square statistic. Locomotor difference scores were ranked and analyzed by Kruskal-Wallis and Mann-Whitney U tests. Significance levels were set at $p < 0.05$ for omnibus ANOVAs, post hocs, and nonparametric analyses. Significance levels for simple interactions and simple main effects were adjusted according to Dunn's procedure, based on the Bonferroni inequality (16).

RESULTS

Dams

Dams did not differ in weight gain from GD1 through GD8, prior to the treatment period. From GD9 through GD20

there was a significant effect of treatment on weight gain, $F(4, 47) = 13.7$, $p < 0.05$. Post hoc analyses indicated that dams from all the treatment groups, including the pair-fed dams, gained less weight than the untreated dams (Table 1). The interaction of treatment and time was not significant. Food intake also varied due to treatment, $F(3, 38) = 51.2$, $p < 0.05$, as all treated dams consumed less food than the untreated dams (pair-fed dams were not included in the analyses; Table 1), but the treatment by time interaction was not significant. Water intake did not differ among the treatment groups.

Litters

Gestation length, litter size, the ratio of female to male pups, the number of resorptions, and the number of stillbirths did not differ among groups (Table 1). A mortality index score was calculated for each litter as follows: (number of resorptions + number of stillbirths) / number of implantation sites. The calculated mortality scores did vary significantly due to treatment, $F(4, 48) = 2.98$, $p < 0.05$. Litters from C dams and E dams had the highest mortality rates, both of which were greater than the UNT litters. Additionally, the C litters had a higher incidence of mortality than the PF litters. There was a significant difference between female and male litter weights on PD1, $F(1, 96) = 4.8$, $p < 0.05$, but no significant interaction with treatment (Table 1). Subsequently, litter weights were collapsed across sex for analysis through PD21.

Pup Development

Repeated measures analysis of pup weights from PD2 through PD21 failed to reveal differences among treatment groups due to the effect of prenatal treatment, nor was there a significant treatment by time interaction. Additionally, the onset of developmental milestones was not delayed by any of the prenatal treatment conditions (data not shown).

Open-field Behavior

Overall levels of horizontal (lines crossed) and vertical (rearing) locomotion across the 30-min observation session did not vary as a function of prenatal treatment. In addition, there were no significant interactions between prenatal treatment, sex, and the treatment pups received when tested in the open-field.

It appeared that pups from the C and E groups were not as sensitive to the locomotor-activating effects of cocaine, in comparison to the other groups, and that perhaps this effect was masked by different levels of baseline activity. To determine if possible differences in cocaine-induced horizontal locomotion were obscured by changes in baseline horizontal locomotion within each treatment group, a difference score was calculated for each litter. The difference score was derived by averaging the amount of activity of the saline-injected and untreated pups from a litter (horizontal locomotion did not differ between untreated and saline-injected pups), then subtracting this amount from the average amount of activity of the cocaine-injected pups in the same litter. The difference scores were ranked and then analyzed by nonparametric tests. Statistically significant differences were found only during the first 10-min of the observation period, Kruskal-Wallis, $(H) = 12.53$, $p < 0.05$ (Fig. 1). Litters from the C and E prenatal treatment groups had the lowest difference scores, suggesting that these groups were not as responsive to cocaine, though neither group was significantly different than the UNT control ($p \leq 0.1$). The difference score for the E group was lower than the PF controls, Mann-Whitney U test $= -2.00$, $p < 0.05$, and marginally lower than the C/E treatment group, Mann-Whitney U test $= -1.92$, $p = 0.05$. Difference scores for the C group were also lower than the PF control group, Mann-Whitney U test $= -2.58$, $p < 0.05$, and significantly lower than the C/E group, Mann-Whitney U test $= -2.97$, $p < 0.05$.

Pups injected with cocaine in the open-field had the highest

TABLE 1

EFFECTS OF PRENATAL EXPOSURE TO ETHANOL AND/OR COCAINE ON DAM AND LITTER VARIABLES

	Untreated	Pair-fed	Ethanol	Cocaine	Cocaine/ Ethanol
n	10	10	11	10	11
Dams¶					
Weight Gain %	41.4 ± 2.2	23.1 ± 1.9†	23.4 ± 2.2†	26.8 ± 2.2†	22.6 ± 2.5†
Avg. Food Intake (g/kg body wt.)	1232.3 ± 84.7	NA	846.7 ± 54.1†	897.1 ± 24.5†	969.3 ± 114.8†
Avg. Water Intake (g/kg body wt.)	1917.7 ± 75.8	1598.9 ± 88.4	1721.1 ± 70.5	1880.7 ± 97.9	1741.7 ± 85.9
Gestation Length (days)	21.0 ± 0.2	21.0 ± 0.0	21.4 ± 0.2	21.0 ± 0.0	21.1 ± 0.1
Litters§					
Litter Size	13.0 ± 0.8	14.6 ± 0.8	12.8 ± 0.8	12.8 ± 0.5	13.9 ± 0.5
Avg. Pup Weight, PD1 (grams)	7.3 ± 0.3	6.7 ± 0.3	7.0 ± 0.2	7.0 ± 0.3	6.4 ± 0.2
Sex Ratio	1.08 ± 0.26	1.42 ± 0.29	1.06 ± 0.15	1.42 ± 0.34	1.14 ± 0.29
Resorptions	0.3 ± 0.2	0.8 ± 0.3	1.3 ± 0.3	1.5 ± 0.5	1.2 ± 0.5
Stillbirths	0.0 ± 0.0	0.1 ± 0.1	0.2 ± 0.2	0.4 ± 0.2	0.3 ± 0.1
Mortality Index (percent)	2.0 ± 2.0	6.0 ± 2.0	12.0 ± 2.0†	14.0 ± 4.0‡	10.0 ± 3.0

¶Data represent cumulative totals during the treatment period from GD9–20. §Litter averages served as the unit of measurement. Data are represented as the mean ± SEM. † indicates a significant difference from the Untreated group, $p < 0.05$. ‡ indicates a significant difference from both the Untreated and the Pair-fed groups, $p < 0.05$.

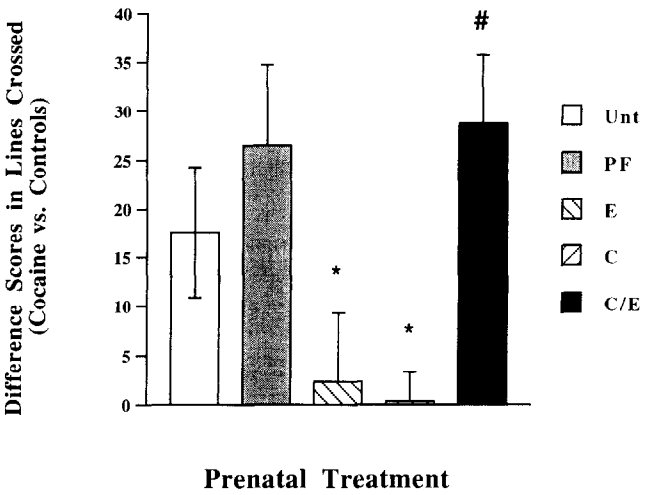

FIG. 1. The effect of prenatal exposure to ethanol and/or cocaine on cocaine-induced horizontal locomotion in the open-field. Data represent difference scores which contrast levels of locomotion for cocaine-treated versus control pups from the same litter. Data are expressed as the mean ± S.E.M. Prenatal exposure groups: UNT = untreated; PF = pair-fed; E = ethanol; C = cocaine; C/E = cocaine/ethanol. * indicates a significant difference from the PF group, $p < 0.05$. # indicates a significant difference from the C group, $p < 0.05$.

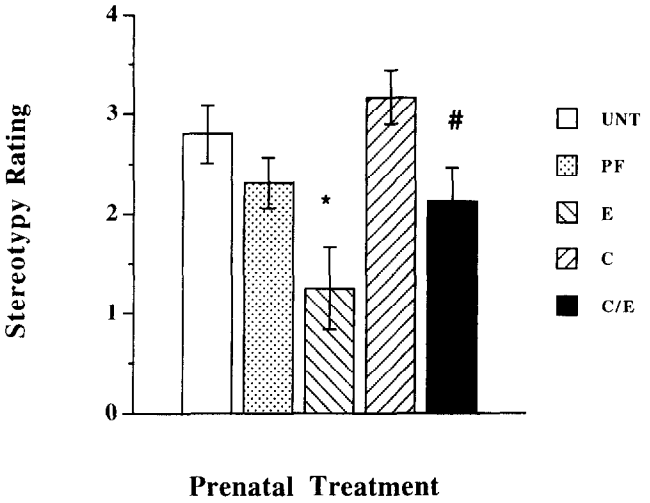

FIG. 2. The effect of prenatal to ethanol and/or cocaine on stereotypy rating in the open field behavior. Data represent stereotypy ratings in untreated pups during the initial 10-min observation period. Data are expressed as means ± S.E.M. Abbreviations are the same as in Fig. 1. * indicates a significant difference from the UNT and PF groups, $p < 0.05$. # indicates a significant difference from the C group only, $p < 0.05$.

$\hat{\gamma}$ scores for stereotypic locomotion. However, the open-field treatment did not interact significantly with either prenatal treatment condition, or sex.

Subjective ratings of stereotypic behavior differed only during the initial 10 minutes of the test session. There was a main effect for drug treatment, $F(2, 168) = 132.9$, $p < 0.05$, as well as a prenatal treatment by drug treatment interaction, $F(8, 168) = 3.1$, $p < 0.05$. Cocaine-injected pups had the highest behavior scores which were significantly higher than both saline-injected and untreated pups, but there were no differences by prenatal treatment condition. There was a significant effect of prenatal treatment on behavior rating in the pups that were untreated during the open-field test. Among the pups in the untreated drug condition the E pups had the lowest average behavior rating, and were significantly lower than the PF and UNT pups (Fig. 2). Pups in the C/E group, while not significantly lower than controls, did differ from the C pups.

Overall stereotypic behavior, as represented by the stereotypy index, was also affected by the open-field drug treatment, $F(2, 168) = 73.0$, $p < 0.05$, as pups injected with cocaine exhibited more stereotypic behavior than both sets of controls (data not shown). However, a significant 3-way interaction and subsequent post hoc tests revealed that the only difference was between PF female and males (female > male). Other apparent differences among cocaine-injected pups (females: PF > C,C/E; males: UNT, C/E > PF), saline-injected females (E > UNT,C), and untreated C pups (female > male) failed to reach statistical significance ($0.045 < p < 0.09$).

Prenatal treatment affected exploratory behavior among pups which were untreated prior to placement in the open-field. Observations were transformed into the percentage of pups in each prenatal treatment group which had entered all sections of the open-field by a given time period. Male pups from the C group were delayed in exploring the open-field, with a significantly decreased percentage (17%) entering all five sections within the first five minutes, in comparison to UNT males (80%), Total Chi-square (1) = 4.41, $p < 0.05$, and to C females (83%), Total Chi-square (1) = 6.20, $p < 0.05$ (data not shown).

DISCUSSION

Prenatal exposure to cocaine alone produced several differences in the offspring. The C treatment produced the highest mortality rate (14%) which was significantly higher than either UNT (2%) or PF (6%) controls. In our previous study we found a similar mortality level following prenatal cocaine exposure (17). Others (5) have reported a postnatal mortality of less than 3% following cocaine exposure but almost 16% following chronic exposure to both cocaine and ethanol, while acute exposure to both drugs appears to have little effect (26). A higher mortality rate in C/E litters might be expected due to the increased toxicity of cocaethylene compared to cocaine (14). The discrepancy between studies of prenatal drug exposure may result from genetic factors, as strain differences are known to affect susceptibility to the effects of cocaine (6), and ethanol (24). Fetal and pup mortality may also be due in part to the stress induced by restraint during the cocaine dosing procedure. At least two other studies reported the use of intravenous cocaine administration without restraint (18,22) but neither of these reported rates of offspring mortality. Thus, whether the difference is due to route of administration or restraint during IV dosing remains to be determined.

Gestational cocaine exposure also produced several differences in open-field behavior. The difference in levels of horizontal locomotion between cocaine-injected and control pups was significantly reduced for C pups, during the initial 10-min of the observation, in comparison to PF pups. The reduction could result from either increased locomotion in the control animals, or a diminished effect of cocaine. Direct comparisons of locomotor activity failed to reveal differences among the prenatal treatment groups for either untreated, saline-injected, or cocaine-injected pups. However, it appears that the C pups were less susceptible to the behavior activating effect of cocaine. Control pups from the C group averaged 22.3 lines

crossed in the first 10-min while the average among all groups was 18.4; the cocaine-injected pups from the C group averaged only 24.8 lines crossed compared to an average of 37.1 lines crossed among all groups. One possible explanation for the reduced horizontal locomotion could be an increase in focused stereotypic behavior which is accompanied by a decrease in horizontal movement. However, the behavior ratings for the C pups treated with cocaine were similar to the other groups and did not indicate high levels of stereotypic behavior. Another possibility would be a lack of sensitivity to the behavior activating effects of cocaine. There are reports of reduced behavior effects in prenatally cocaine-exposed offspring following either a single cocaine challenge (19,20), or repeated cocaine administration (2).

In the current study there was evidence of a sex-dependent decrease in exploratory behavior due to prenatal cocaine exposure. Untreated males from the C litters took longer to explore the open-field than either untreated C females or untreated UNT males. Decreased exploratory behavior, or neophobia, due to prenatal cocaine-exposure has been reported by several investigators (5,12,13,17,25). The decreased exploratory behavior may be due to an effect on the opiate system. Prenatal cocaine has already been reported to increase opiate receptor binding (7), and prenatal stress-induced decreases in exploratory behavior can be negated by the coadministration of the opioid antagonist naltrexone (15).

Despite the change in exploratory behavior, the untreated C pups had the highest behavior rating in the initial 10-min period. However, among the untreated pups, the ratings of the C pups did not differ from the UNT and PF pups, but were significantly higher than either the E pups or the C/E pups. This indicates that the untreated E and C/E pups, all the pups exposed to ethanol, were the least active. This is directly opposite to previous findings which reported the highest levels of spontaneous activity in pups exposed to ethanol, alone and in combination with cocaine (5). The difference may be due to different methodologies; activity in (5) was determined using automated activity boxes, while the activity level in the current study was determined by an observation of open-field behavior.

In general, prenatal ethanol exposure had similar effects to those of cocaine. Weight gain and food intake were decreased in ethanol- and cocaine-treated dams. There was a significant increase in pup mortality associated with either ethanol or cocaine exposure, in comparison with UNT pups. In the open-field, locomotor difference scores between cocaine-injected and control pups were significantly lower for both E and C pups compared to the difference scores for the PF pups. However, not all of the ethanol effects mirrored the effects of cocaine. As stated previously, there was a decrease in stereotypy rating in untreated pups from the E and C/E groups. This effect was seen in the initial 10 minutes of the observation and may be, at least partially, accounted for by a decrease in locomotor activity as both E (15.8 $\pm$ 3.7) and C/E (14.5 $\pm$ 3.7) pups were below average (18.4 $\pm$ 1.7) in the number of lines crossed. The low levels of horizontal locomotion for E and C/E pups were unexpected, as the link between prenatal ethanol exposure and hyperactivity has been well established. A strain-dependent difference in response to neonatal ethanol exposure has been reported (24), suggesting that further studies in genetic susceptibility to prenatal drug exposure are warranted (6).

While prenatal exposure to either cocaine (C) or ethanol (E) significantly altered offspring behavior, it is interesting to note that the combination of the two drugs (C/E) had little effect on the dependent measures used in this study. The C/E pups seldom differed from the PF and UNT controls. The C/E and PF dams gained significantly less weight than the UNT dams, as did the E and C dams, but the incidence of pup mortality in the C/E litters, while increased, did not differ significantly from controls. There was a sex-dependent difference in cocaine-induced stereotypic behavior for the PF group, an effect not seen in the C/E pups or any other group. The failure of the cocaine-ethanol combination to produce behavioral effects consistent with either cocaine or ethanol is difficult to reconcile. Except for the decrease in the open-field behavioral rating scores for untreated pups, which was similar for both C/E and E groups, there were no similarities. Findings of reduced ethanol levels in dam and fetal plasma and brain following chronic cocaine administration suggest that cocaine could reduce the effects of ethanol (9). However, reported behavioral evidence suggests that the combination of the drugs has more detrimental effects than either drug alone (4,5,14), although there have been discrepancies (23). The dose of drug, time of drug exposure, and the teratogenic effect observed as the dependent variable are important factors to consider. In a recent study of neonatal cocaine and ethanol exposure during the brain growth spurt (postnatal days 4-9), ethanol-induced microencephaly was not altered by cocaine, while cocaine-induced mortality was increased by the addition of ethanol (3).

In summary, both cocaine and ethanol produced differences in offspring behavior at doses which decreased dam weights and slightly increased mortality, but did not produce gross morphological defects or developmental delays. Prenatal cocaine exposure attenuated the behavioral effects of a cocaine challenge and decreased exploratory behavior. Prenatal ethanol exposure also attenuated the behavior effects of a cocaine challenge, and when administered alone or in combination with cocaine to dams, decreased the spontaneous activity of their offspring. These results lend further evidence for the role of both ethanol and cocaine in behavioral teratogenesis, and while few effects were seen following the coadministration of ethanol and cocaine in the present study, the deleterious effects of this combination on the developing fetus cannot be ruled out.

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