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The effects of prenatal cocaine exposure and genotype on the ultrasonic calls of infant mice

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Abstract

Estimates are that as many as 44,000 humans are exposed to cocaine in utero per year. In this study we examined the effects of prenatal cocaine exposure on one aspect of the mother–infant relationship in mice, infant ultrasonic calls. We mated C57BL/10J female mice with males of three different inbred strains (producing pups of three different F_1 genotypes). We injected those females, subcutaneously, with saline or 20 mg/kg of cocaine hydrochloride on days 7–17 of gestation. That dosage did not compromise mother or pup viability, weight, or gestation length. On postnatal days 2–4, we recorded and measured the calls of pups while they were separated from their nest and slightly chilled. The results indicate changes in the ultrasonic calls as a function of cocaine and genotype. Overall, cocaine reduced the number of calls and increased the beginning pitch of calls. Pups of one genotype, a C57BL/10J × SJL/J hybrid were unaffected by cocaine exposure. The effects of cocaine, though reliable, were small, explaining only 1–2% of the total sum of squares. The size of the effect is in part due to the differential effect of cocaine on different genotypes.

Keywords: Prenatal cocaine; Ultrasonic calls; Mother–infant interactions; Mice; C57BL/10J mice; SJL/J mice; Genotype by drug interaction

1. Introduction

1.1. The effects of prenatal cocaine exposure

Though recent reports suggest that cocaine use has declined nationwide, the drug is still used by thousands of people. Recent national statistics indicate that substance abuse is declining in adult men, but that trend is not seen in young women and usage in that group increased slightly [20,25]. The results of the National Pregnancy and Health Survey of women who delivered babies born between October 1992 and August 1993 in the United States were used to estimate the numbers of women who used illicit drugs, including cocaine, during pregnancy. Those estimates are that of the 4,000,000 women who deliver live born children annually, approximately 5.5% or 220,000 women will have used some illicit drug during pregnancy. Approximately 20% of this illicit drug use will be with cocaine [26]. This means that approximately 1.1% of 4,000,000 or 44,000 women per year are projected to be delivering babies that have been prenatally exposed to cocaine.

In initial studies, it appeared that prenatal cocaine exposure produced significant effects on newborn and infant humans. Both physical and behavioral consequences were observed in the exposed offspring. For example, the exposed fetuses showed serious physical consequences including neurologic problems, growth retardation, and microcephaly [12]. Behavioral consequences have been grouped into disorders of attention, arousal, affect, and action [22]. Early studies showed disorders of all categories including: the depression of interactive behavior, sleep pattern disturbance, tremor, lowered spontaneous activity, catatonic-like states, increased irritability, aggressive behavior, and altered organizational responses to environmental stimuli [3,4,21,29,33,37]. Recently, however, the initial rush to judgment has been questioned as better-controlled studies have yielded much subtler effects. Perhaps, as suggested by Spear et al. [35], the developing nervous system is remarkably plastic and can compensate to early assaults in early life. There may be a cost of such compensation in later life, however.

The precise nature of the effects and the mechanisms of effects in humans are uncertain. Consequently, it has proven

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helpful to develop animal models in which the effects of prenatal cocaine on the offspring and its mechanism of action can be studied without the confounds associated with research on humans, for example: malnutrition, other drugs, and ill health. Changes in mother–pup interactions [32], open field activity and cognitive function [19,34], and changes in aggressive behavior in rats [18] and mice [13] have all been demonstrated using animal models of prenatal cocaine exposure.

Although it is clear that prenatal cocaine exerts effects on the exposed offspring, the effects appear to be small. The small effects could be produced in at least two ways. It may be that cocaine produces small and consistent effects across individuals that result in small mean differences between drug-exposed and control groups. It is also possible that the effects of the drug are inconsistent — large in some individuals but small in others. In that case, averaging across individuals, the prenatal effects of cocaine would also be small. One factor that might contribute to large individual differences in the action of prenatal cocaine is genotype. Genotype has been shown to play an important role in the response of an animal to a drug treatment (for example, see Ref. [7]) and recent behavioral and neurochemical studies using animal models have begun to clarify the role that genes play in the etiology of individual differences in susceptibility to cocaine administration. For example, one laboratory [23] showed that genetic factors influence changes in sensitivity to the convulsant properties of cocaine following chronic treatment. In four inbred strains of mice (BALB/cJ, DBA/2J, C57BL/10J, and SJL/J) only the SJL/J strain animals were highly resistant to convulsions induced by acute injections of cocaine and after chronic exposure only the SJL/J animals showed no tolerance to chronic administration of the drug.

To date, most animal studies of the behavioral effects of prenatal cocaine have involved sensory-motor development, attention, learning, and other behaviors that are measured in individual animals. These behaviors fall in the categories of attention and action described by Lester [22]. Few studies have examined the role of cocaine on behaviors enriched by the addition of another animal, social behaviors. Social behaviors fit into the arousal and especially affect categories described by Lester. The importance of social behaviors cannot be underestimated for as forcefully argued by Scott "...almost all behavior that is exhibited by the members of highly social species...is expressed within social relationships" [31, pp. 327–328].

1.2. The role of ultrasonic calls in the mother–infant relationship of mice

In mice, auditory signals produced by pups play an important role in the regulation of the mother–infant relationship [11]. The roles of three such signals have been studied. Low frequency calls (below 10 kHz) are sometimes called "wriggling calls," and are emitted while pups struggle in the nest and push to attach to teats. Mothers respond by licking the pups and by nest building [9]. Broad-spectrum vocalizations (4–40 kHz), perceived as squeaks by humans, include both sonic and ultrasonic components and are elicited by rough parental handling [11]. Pure ultrasonic calls (above 20 kHz) are produced when pups are isolated [11], chilled [15,16], or receive tactile stimulation that might correspond to falling [27]. Ultrasonic calls produced by pups reliably elicit maternal retrieval [5,11] and mothers search for pups when the cues available are from actual pups or from virtual pups, that is, calls produced electronically [10].

Individual mice vary in the number of ultrasonic calls they emit per unit of time (rate of calling) and calls vary in length and frequency characteristics. These differences may alter maternal responses [1]. The rate of calling may be the important characteristic in eliciting search and retrieval by adults and call length and call frequency characteristics may carry information about individual identity and the age and sex of the caller [14]. Call length and frequency characteristics must be within certain limits for the calls to be effective in eliciting searching and retrieval [8,19].

Ultrasonic call characteristics have been shown to change as a function of age, genotype, and experimental treatment. For example, the rate of calling follows a shallow inverted U-shaped function across ages from birth through about 12 days of age [16,30]. Calls become shorter and call frequency characteristics increase across those same ages [16,30]. As shown early on [1] and more recently [14,15], pups of different inbred strains of mice differ in the rate, length, and frequency characteristics of calls they produce. One investigator [27,28] has shown that cooling and tactile stimulation produce different patterns of call characteristics across ages.

1.3. Statement of the problem

There are still numbers of newborn human infants likely exposed to cocaine in utero. It remains important to catalog and characterize the effects of prenatal cocaine exposure on the behavior of infants, especially social behaviors. The effects of cocaine on the behavior of infants and thus the mother–infant relationship can be modeled in the mouse. Since genotype is known to modulate the effects of numerous pharmacological agents, it may modulate the effects of prenatal cocaine on a social behavior of infant mice.

The objectives of the current study are twofold. First, we set out to examine the influence of cocaine on a central component of the mother–infant relationship in mice, the ultrasonic calls of infant mice in their early postnatal days. Second, we set out to examine the role of genotype as a factor modulating the effects of cocaine on the behavior of young mice.

2. Methods

2.1. Subjects

The subjects of this study were 317 male and female mouse pups from 48 litters. The pups were the F_1 progeny of a cross between C57BL/10J females and a male from one of three inbred strains: BALB/cJ, DBA/2J, or SJL/J. We chose these four strains for their divergent origins [36] that may increase the generality of any findings and because we have an extensive database on the ultrasonic calls of these strains [14–16]. One half of the litters was exposed to cocaine prenatally.

All mice were born and raised in the colony rooms of the animal facility of the Department of Biology of William Paterson University and were maintained in transparent colony cages with stainless steel tops. The cages were $30 \times 20 \times 15$ cm in dimension. All mice were fed a diet of Agway RMH 3000 animal chow. Food and tap water were available at all times. The colony was maintained on a 12:12 h, light/dark cycle with lights on at 0800 h. The animal facilities at William Paterson are maintained to the standards for animal care issued by the USDA and NIH.

2.2. Procedures for breeding and injecting

The breeding and injection procedures were the same as those reported by Hahn et al. [13] and are summarized below. Pairs of mice, a C57BL/10J female and a male of either the BALB/cJ, DBA/2J, or SJL/J strain, were placed in a standard cage late in the afternoon and remained there until the next morning (a total time of about 17 h). At that time, females were weighed, checked for the presence of a vaginal plug and placed alone in a standard colony cage. One week later, they were weighed again and when weight gain confirmed pregnancy, the females were randomly assigned to the control (saline) or drug (cocaine) conditions. Injections of 20 mg/kg of cocaine hydrochloride or saline were administered daily by subcutaneous injection starting on Day 7 (Day 0 of gestation was the morning after pairing with a male) of gestation and continuing through Day 17. In order to maintain the concentration of cocaine across animals of different weights, the cocaine was injected in a volume that varied as a function of body weight (injection volume in microliters = two times the body weight in grams). The injection site was systematically varied over the entire back of each animal to minimize local tissue necrosis in animals of the drug-treated group. This procedure followed an earlier protocol from our laboratory [2] that was based on extensive testing of various dosages of cocaine, and it models a pattern of repeated drug use in humans. Further, this protocol (without the systematic under nutrition for all animals, associated with pair feeding) has consistently resulted in no differences in weight gain in mothers, differences in gestational length, litter sizes, or pup weights when comparing control and drug-treated females. Pregnant females were checked daily for a new litter at 0830 h. After birth and through the days of testing, pups remained with their birth mothers. Only litters of four to eight pups were employed in this study.

2.3. Ultrasound recording and analysis equipment

We recorded the ultrasonic vocalizations of mouse pups using a Bruel and Kjaer (B & K) Type 4135, 1/4 in. (6.4 mm) microphone, a B & K Type 2619T preamplifier, a B & K Type 2606 measuring amplifier, and a Teac instrumentation tape recorder. Using high quality videocassette tapes and a recording speed of 76 cm/s, we obtained a frequency response on taping of 150 Hz to 150 kHz.

We measured the characteristics of ultrasonic vocalizations using a Kay Elemetrics 5500 Digital Sonagraph sound spectrum analyzer. Playback of tapes at 9.5 cm/s (1/8 recording speed) allowed analysis within the frequency capacity of the Kay Sonagraph.

2.4. Ultrasound recording procedure

We followed the basic recording procedures that we have used previously [15,16]. At about 0830h each day, the cage of each breeding female was checked for the birth of a new litter (the day the litter was found = 0 days of age). The pups were counted and the litters were culled to eight pups when necessary. Only litters of four to eight pups were used in this study and pups remained with their mother throughout the study period.

At the time of testing, the entire litter was removed from the nest and placed into a 250-ml plastic beaker at air temperature (about 21°C). One at a time, the pups were removed from the beaker without regard to order, identified, and placed into the recording chamber. After recording on the first day, each pup was marked for permanent identification.

Each pup was placed individually on a cotton pad inside an aluminum weighing dish atop about 100 ml of ice in a 250-ml beaker. The surface of the cotton pad was maintained at between 10°C and 11°C. The beaker was placed in a dark, sound-attenuated chamber where the air temperature was about 21°C, and the B & K microphone was located above the beaker about 5 cm away. Recording began immediately and continued for 20 s (about 1500 cm of tape). Each pup was recorded individually in that cool, isolated environment on ages 2–4 days. By always using two experimenters, the out of nest time was minimized for all pups. The time of testing for an entire litter, which included the identification and sound recording of each pup, varied from about 2 min (four pup litters) to about 4 min (eight pup litters). During that litter testing time, each pup spent the majority of its time with its littermates in the 250-ml beaker, about 5 s in the hand of an experimenter and 20 s on the cotton pad in the recording chamber. The cotton pad and aluminum dish were changed after each litter tested. After we had recorded each pup, the entire litter was

returned as a unit to the nest of the home cage. All recordings were carried out between 1000 and 1500 h.

2.5. Sonagraphic analysis

We analyzed the calls of each mouse with two tape passes. On the first pass, we counted the total number of ultrasonic vocalizations by identifying and counting the calls as they scrolled by on the screen of the Sonagraph. On the second pass, we "froze" each call and measured the frequency and length characteristics of the first five calls. To obtain a value for each mouse on each day, we averaged the values of five calls. In the rare cases when there were fewer than five calls, we averaged those present.

The characteristics we measured were:

1. rate of calling (calls/second for 20 s),
2. length of calls,
3. beginning frequency of calls,
4. ending frequency of calls,
5. highest frequency of calls, and
6. lowest frequency of calls.

3. Results

3.1. Mother and pup health

In order to assess the basic health of mothers and their pups treated with cocaine, we compared them to saline controls, using the following four indices: maternal weight gain during pregnancy, the gestational age of the litters born, the number of pups born per litter, and the weight of entire litters at birth. The means and standard errors are shown in Table 1. An inspection of the table indicates that the cocaine treatment produced no differences in maternal or pup health and viability. A 2×3 (condition $\times$ F_1 genotype) analysis of variance confirmed the appearance, as with the P level set at .05, we found no significant differences for any of the indices as a function of the control/cocaine grouping.

3.2. Ultrasonic calls

The data analysis strategy we adopted for ultrasonic calls was a complete examination including the effects of days, sex, drug treatment, and genotype on all call characteristics. This analysis included a description of the between and within litter sums of squares, appropriate in an examination of the effects of a drug and because the characteristics of the calls may allow for individual, sex, or age recognition by an adult mouse. The final set of analyses are in line with current thinking that the use of litter means provides the best test of a substance in a study of behavioral teratology.

Fig. 1 illustrates a sample set of results, the mean rates of calling of infant mice as a function of their genotype and drug treatment condition on Days 2, 3, and 4 of age. Male and female pups were pooled for this figure. The data of Fig. 1 show mean rates of calling that appear to differ as a function of genotype and treatment at each of the three ages tested. BALB/cJ and DBA/2J F_1 pups exhibit a similar rate of calling and both appear to produce more calls than SJL/J F_1 pups. Cocaine treatment appears to suppress the rate of ultrasonic calling in BALB/cJ and DBA/2J F_1 groups but not SJL/J F_1 pups. Table 2 contains the means and standard errors of the mean (S.E.M.) for all the measures of ultrasonic calls. Substantial differences are apparent among the F_1 pups of the three genotypes. SJL F_1 pups make fewer calls, the calls are higher in frequency and shorter than those of the BALB or DBA F_1 pups. Cocaine appears to increase the frequency characteristics of calls in the BALB and DBA F_1 pups and may lower the frequency characteristics of calls produced by SJL F_1 pups. Cocaine does not appear to alter the length of calls in any systematic manner.

To test these apparent differences, we completed a $2 \times 3 \times 2$ (condition $\times$ F_1 genotype $\times$ sex) repeated over

Table 1
Means, standard errors, and ranges of mother and pup characteristics following control or prenatal cocaine treatment

	BALB/c F_1		DBA/2 F_1		SJL F_1	
Health measure	Saline, $n=9$	Cocaine, $n=7$	Saline, $n=10$	Cocaine, $n=11$	Saline, $n=5$	Cocaine, $n=6$
Maternal weight gain (g)	19.05	19.73	17.02	16.24	18.06	17.83
	1.10	0.50	1.01	0.31	1.11	1.17
	15.3–24.3	18.1–21.7	12.1–22.3	14.5–17.7	15.7–21.7	13.8–20.7
Gestational age (days)	19.0	18.4	18.4	18.3	18.4	18.7
	0.0	0.2	0.2	0.1	0.2	0.2
	19	18–19	18–19	18–19	18–19	18–19
Pups per litter[a]	7.4	8.6	6.7	6.4	6.8	6.3
	0.5	0.5	0.7	0.3	1.0	0.7
	6–10	7–11	4–11	5–8	5–8	4–8
Weight of entire litter[a] (g)	11.58	11.53	9.57	9.22	10.33	9.89
	0.76	0.45	0.94	0.33	1.40	0.76
	9.1–15.7	9.9–13.3	6.0–15.7	7.4–11.2	8.1–11.5	7.5–12.3

The number of litters of each F1 type and condition is listed (total litters = 48).

[a] Prior to culling litters to eight pups maximum.

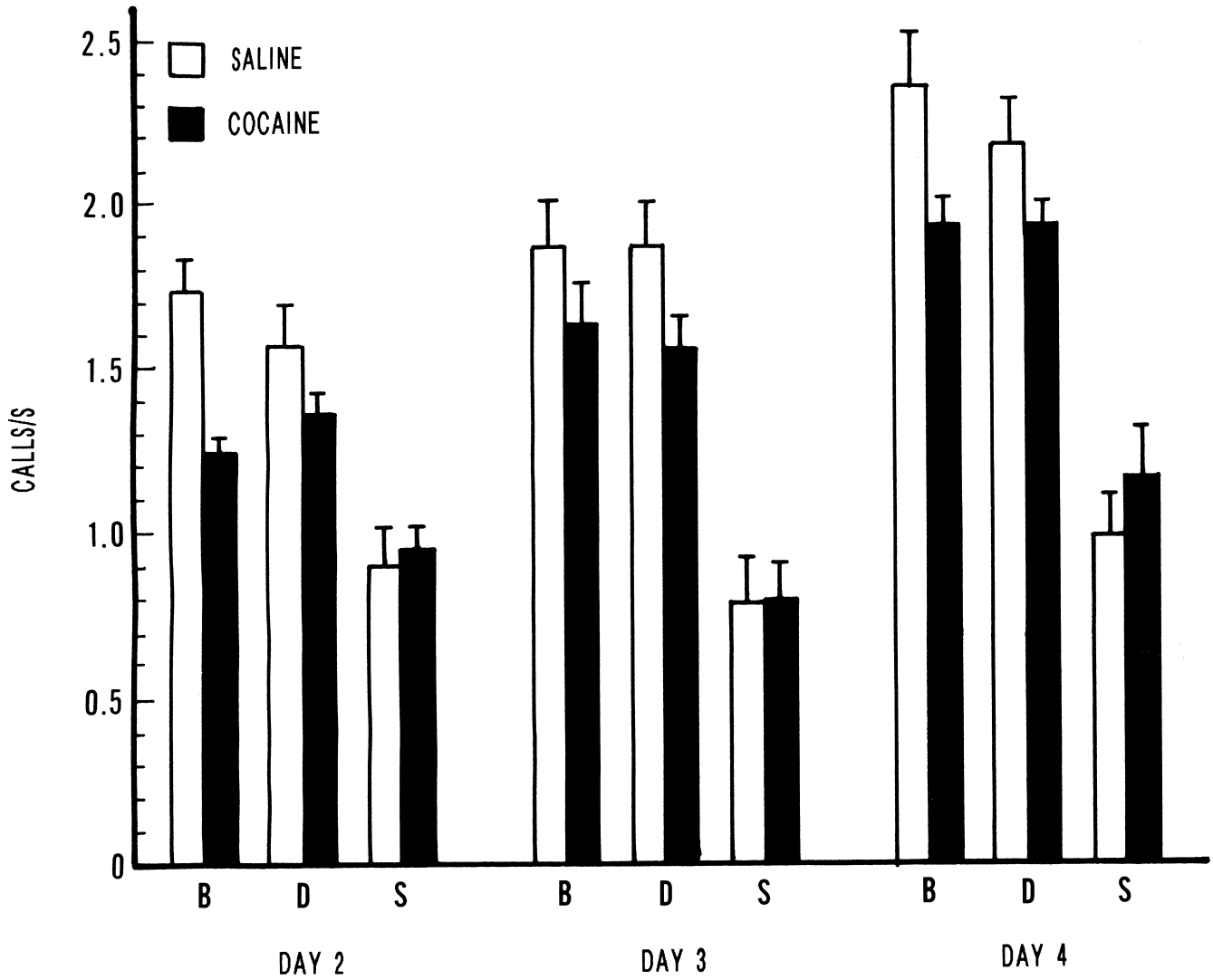

Fig. 1. Mean rates of calling (±S.E.M.) of infant mice at ages 2–4 days as a function of drug treatment and genotype. The calls of males and females are pooled.

three ages (repeated measure) analysis of variance on each call characteristic. That analysis revealed no sex effects nor effects of sex interacting with any of the other factors on any of the call characteristics. Not unexpectedly, the repeated measure, age, produced a significant effect on rate ($F=19.57$, $df=2/258$, $P<.0001$), beginning frequency ($F=31.98$, $df=2/212$, $P<.0001$), ending frequency ($F=3.05$, $df=2/212$, $P<.05$), highest frequency ($F=80.03$, $df=2/212$, $P<.0001$), and lowest frequency ($F=4.69$, $df=2/212$, $P<.02$). Call length was not affected by age ($F=1.94$, $df=2/212$, $P>.05$). Given the significant effect of age on all variables except call length, we completed the between subjects analyses on each age separately. The results of those analyses using the model: condition, genotype, and the interaction between the two are shown in Table 3. The total degrees of freedom for each analysis is the total number of pups measured at that age, minus one. An overview of the table indicates that the model containing condition, genotype, and the interaction explained only a modest portion of the total variance in call characteristics of pups. R^2's ranged between .096 and .185.

The classification genotype produced a significant difference in every call characteristic at each age. Of the between litter effects, genotype accounted for the majority of the total sums of squares in each case. Classifying pups by condition produced a significant difference in two call characteristics: the rate of calling at ages 2, 3, and 4, and the beginning frequency of calls at age 2 days. Condition and genotype interacted on the measures: beginning, highest, and lowest frequency in pups aged 2 days, but not on the rate of calling that might have been expected after looking at Fig. 1. In each case, the percent of the total sum of squares attributable to cocaine or the interaction of genotype and condition was less than 5%. As shown by the means in Table 2, the interactions between genotype and condition were primarily due to pups of SJL fathers

Table 2
Means and S.E.M. of pup ultrasound characteristics

		Day 2		Day 3		Day 4	
Measure	Genotype of father	Saline	Cocaine	Saline	Cocaine	Saline	Cocaine
Rate (calls/s)	BALB	1.72 ± 0.12	1.27 ± 0.12	1.87 ± 0.14	1.63 ± 0.14	2.35 ± 0.16	1.91 ± 0.16
	DBA	1.57 ± 0.12	1.33 ± 0.09	1.87 ± 0.12	1.55 ± 0.10	2.19 ± 0.13	1.91 ± 0.11
	SJL	0.90 ± 0.14	0.97 ± 0.11	0.79 ± 0.15	0.80 ± 0.11	0.99 ± 0.14	1.17 ± 0.15
Length (ms)	BALB	57.1 ± 2.40	52.7 ± 2.23	52.7 ± 2.05	53.5 ± 2.21	56.6 ± 2.58	55.0 ± 1.87
	DBA	54.2 ± 2.04	58.9 ± 2.16	53.7 ± 1.86	50.1 ± 1.51	50.0 ± 1.69	55.0 ± 2.02
	SJL	44.1 ± 2.55	44.7 ± 2.16	40.3 ± 3.12	38.0 ± 2.45	40.6 ± 2.10	41.9 ± 2.28
Beg frequency (kHz)	BALB	62.4 ± 1.05	66.5 ± 0.93	61.4 ± 0.86	63.2 ± 0.90	60.2 ± 0.97	52.5 ± 0.90
	DBA	66.1 ± 0.88	68.0 ± 0.80	64.1 ± 0.76	64.8 ± 0.73	61.6 ± 0.69	61.2 ± 0.86
	SJL	73.3 ± 1.20	71.2 ± 0.72	69.5 ± 1.44	68.8 ± 1.16	66.6 ± 1.73	67.3 ± 1.23
End frequency (kHz)	BALB	53.5 ± 1.44	58.1 ± 1.46	54.9 ± 1.16	55.3 ± 1.15	53.9 ± 1.34	52.5 ± 1.07
	DBA	58.4 ± 1.25	58.7 ± 1.17	56.7 ± 1.04	57.6 ± 1.05	57.7 ± 0.87	56.5 ± 1.02
	SJL	66.6 ± 1.75	64.0 ± 1.32	63.8 ± 1.82	63.6 ± 1.44	61.2 ± 1.71	61.6 ± 1.38
High frequency (kHz)	BALB	70.8 ± 0.63	72.6 ± 0.52	69.4 ± 0.59	70.3 ± 0.41	66.4 ± 0.68	67.2 ± 0.55
	DBA	74.7 ± 0.41	75.3 ± 0.46	72.6 ± 0.46	73.4 ± 0.46	71.0 ± 0.51	71.0 ± 0.47
	SJL	76.7 ± 0.92	73.9 ± 0.62	72.9 ± 1.03	72.1 ± 1.07	71.0 ± 1.33	70.1 ± 1.10
Low frequency (kHz)	BALB	53.2 ± 1.43	58.1 ± 1.46	54.3 ± 1.14	54.9 ± 1.17	53.0 ± 1.32	52.2 ± 1.06
	DBA	58.0 ± 1.25	58.5 ± 1.16	55.9 ± 1.03	57.1 ± 1.02	56.5 ± 0.82	55.5 ± 1.02
	SJL	66.5 ± 1.75	63.8 ± 1.33	63.8 ± 1.82	63.4 ± 1.42	60.8 ± 1.72	61.3 ± 1.38

Pups are grouped by age, condition, and genotype (sexes are pooled).

who reversed the effects of cocaine compared to the other two genotypes.

This first set of analyses demonstrated strong genetic effects, very modest effects of cocaine, and on some measures an interaction between genotype and prenatal cocaine exposure. However, the model accounted for only a small portion of the total variance. In order to gain a better understanding of the sources of variation in call characteristics we carried out an additional set of analyses. We completed a set of analyses similar to that shown in Table 3, however, adding the factor "litter" (within condition and strain). These analyses showed that the factor litter was significant beyond the $P < .05$ on 14 of the 18 total call characteristics examined at the three ages (six characteris-

Table 3
Results of analysis of variance using the model: condition, genotype, and their interaction on each day measured (ages 2, 3, and 4) on each call characteristic

Measure	Effect	Day 2 [P value of effect (% sum of squares[a]) and R^2 of the model]	Day 3 [P value of effect (% sum of squares[a]) and R^2 of the model]	Day 4 [P value of effect (% sum of squares[a]) and R^2 of the model]
Rate	Condition	.007 (2.2)	.019 (1.5)	.026 (1.4)
	Genotype	.0001 (6.2)	.0001 (14.5)	.0001 (14.8)
	C × G	.138	.491	.122
	Model R^2	.096	.164	.175
Length	Condition	.884	.191	.418
	Genotype	.0001 (8.1)	.0001 (12.0)	.0001 (12.8)
	C × G	.101	.593	.248
	Model R^2	.096	.129	.139
Beginning frequency	Condition	.046 (2.4)	.118	.883
	Genotype	.0001 (12.4)	.0001 (12.4)	.0001 (13.9)
	C × G	.015 (2.5)	.482	.877
	Model R^2	.191	.135	.140
Ending frequency	Condition	.132	.340	.400
	Genotype	.0001 (10.3)	.0001 (11.8)	.0001 (13.1)
	C × G	.062	.915	.748
	Model R^2	.129	.122	.135
Highest frequency	Condition	.400	.194	.899
	Genotype	.0001 (14.1)	.0001 (10.7)	.0001 (14.4)
	C × G	.0009 (4.2)	.421	.578
	Model R^2	.185	.117	.135
Lowest frequency	Condition	.096	.246	.622
	Genotype	.0001 (10.5)	.0001 (13.0)	.0001 (14.0)
	C × G	.049 (1.9)	.838	.828
	Model R^2	.133	.135	.142

[a] Indicated only where the effect reaches statistical significance.

Table 4
Partition of sums of squares into between cells, within condition and genotype, and within cell for animals at 2 days of age

	Source of sums of squares	
Call variable	$SS_{between\ litter}$ (within condition and genotype)	$SS_{within\ litter}$
Rate	18.7	71.7
Length	22.0	68.3
Beginning frequency	19.5	61.4
Ending frequency	15.5	71.6
Highest frequency	25.2	56.3
Lowest frequency	16.4	70.3

tics × three ages). The exceptions were ending and low frequencies on Day 2, and call length and highest frequency on Day 4. These analyses also allowed us to calculate the sums of squares within litters overall, and between litter within each condition and genotype grouping. Table 4 presents the within litters sum of squares and between litters sum of squares (within each condition and genotype) as a percentage of the total sum of squares. The data presented are for age 2 days only since the results on the other 2 days were very similar. As shown in Table 4 and confirmed by analysis of variance, there is considerable variability between litters that is not explained by the groupings, condition, and genotype. The percent of sum of squares within litters, however, is much larger by comparison and depending on the call measure is a little over two, to four and one half times larger than the sum of squares between litters.

Noting the difference between litters within condition and genotype groupings, we decided on two final analysis steps. Since the litters in the experiment ranged from four to eight pups, litter size was a possible contributor to the between litter variation within condition and genotype. In order to examine this idea, we grouped litters by size, establishing three categories of size: four to six pups, seven pups, or eight pups.

Table 5 details the numbers of litters in each of the size categories and the means and standard errors of the mean for litters grouped by condition, genotype, and litter size on the measure, rate of calling. An examination of the means seems to indicate that within the DBA F_1 pups, litter size, and the rate of calling are linearly related as the mean calls increase from low to high for litters of four to six, seven, and eight pups, respectively, on each day and in both conditions. That linear trend does not appear in either of the other genotypes. This examination of litter size and its potential influence on call characteristics is ad hoc and meant to be exploratory rather than definitive since we did not systematically vary litter size across condition and genotype.

The final analysis presented in Table 6 shows the results of a complete set of analyses using a model that includes: condition, genotype, and litter size as well as the interactions between those main effects. These analyses were completed collapsing on sex and individuals within each litter and thus with a total degrees of freedom equaling the number of litters minus one. An overview of Table 6 indicates that the model accounts for a much greater proportion of the variance, the model R^2's ranged between .344 and .689, when compared to the analyses presented in Table 3. This is predictable in light of the large individual differences in call characteristics seen between pups within litters.

The results of these analyses presented in Table 6 are similar to those seen in Table 3. First, genotype makes a strong contribution to each call characteristic, at each age. The percentage of the total sum of squares associated with genotype ranges from 21.6 to 43.5. Prenatal cocaine exposure has a significant but small impact on two call characteristics (rate and beginning frequency) at age 2 days. Litter size has an impact on two measures, the rate of calling and the highest frequency of calls. Only one interaction

Table 5
Mean rate of calling for litters of different sizes within condition and genotype

		Mean ± S.E.M. for rate of calling				Mean ± S.E.M. for rate of calling			
		Days of age				Days of age			
Genotype	Saline	2	3	4	Cocaine	2	3	4	Total litters
BALB/cJ F_1	4–6 = 3	1.28 ± 0.99	2.18 ± 1.11	1.85 ± 1.21	4–6 = 0	–	–	–	
	7 = 1	1.44 ± 0.73	1.66 ± 0.60	1.49 ± 0.79	7 = 1	1.65 ± 1.26	1.76 ± 0.99	1.26 ± 0.73	
	8 = 4	2.10 ± 0.73	1.74 ± 1.08	2.76 ± 0.93	8 = 6	1.22 ± 0.82	1.62 ± 1.02	2.01 ± 1.19	
	Total = 8				Total = 7				15
DBA/2J F_1	4–6 = 5	1.01 ± 0.82	1.46 ± 1.13	1.88 ± 1.04	4–6 = 7	1.11 ± 0.71	1.41 ± 0.81	1.64 ± 0.82	
	7 = 3	1.76 ± 0.91	2.02 ± 0.83	2.12 ± 0.91	7 = 2	1.50 ± 0.67	1.65 ± 0.61	1.90 ± 0.52	
	8 = 3	1.92 ± 0.90	2.12 ± 0.90	2.57 ± 1.12	8 = 2	1.64 ± 0.81	1.81 ± 0.92	2.40 ± 0.79	
	Total = 11				Total = 11				22
SJL/J F_1	4–6 = 3	0.63 ± 0.70	0.62 ± 0.52	1.01 ± 0.86	4–6 = 3	0.87 ± 0.66	0.74 ± 0.76	1.03 ± 0.88	
	7 = 1	1.24 ± 0.57	0.22 ± 0.35	0.71 ± 0.47	7 = 1	0.96 ± 0.68	0.54 ± 0.53	0.86 ± 0.60	
	8 = 1	1.13 ± 0.97	1.61 ± 0.99	1.15 ± 0.89	8 = 2	1.06 ± 0.68	0.97 ± 0.67	1.44 ± 1.00	
	Total = 5				Total = 6				11
Total litters	24				24				48

Table 6
Results of analysis of variance on litter means of the listed call characteristics using the model: condition, genotype, litter size, and all interactions

Measure	Effect	Day 2, [P value of effect, (% sum of squares)[a] and R^2 of the model]	Day 3 [P value of effect, (% sum of squares)[a] and R^2 of the model]	Day 4 [P value of effect, (% sum of squares)[a] and R^2 of the model]
Rate	Condition	.021 (5.3)	.147	.097
	Genotype	.0001 (21.6)	.0001 (35.0)	.0001 (35.5)
	C × G	.109	.747	.541
	Litter size	.0001 (31.1)	.304	.004 (16.8)
	C × L	.078	.986	.961
	G × L	.768	.116	.710
	Model R^2	.689	.533	.605
Length	Condition	.839	.302	.348
	Genotype	.0043 (24.7)	.0001 (39.0)	.0001 (43.5)
	C × G	.479	.743	.437
	Litter size	.614	.338	.494
	C × L	.995	.914	.735
	G × L	.664	.850	.591
	Model R^2	.344	.474	.552
Beginning frequency	Condition	.027 (6.7)	.381	.899
	Genotype	.0001 (36.5)	.0004 (30.6)	.0001 (39.3)
	C × G	.075	.587	.857
	Litter size	.292	.107	.152
	C × L	.843	.852	.577
	G × L	.501	.405	.602
	Model R^2	.579	.477	.520
Ending frequency	Condition	.160	.496	.306
	Genotype	.0001 (35.1)	.0003 (33.2)	.0002 (34.6)
	C × G	.126	.955	.755
	Litter size	.587	.169	.069
	C × L	.980	.465	.265
	G × L	.475	.817	.604
	Model R^2	.511	.451	.539
Highest frequency	Condition	.533	.297	.780
	Genotype	.0001 (26.6)	.0001 (28.0)	.0002 (38.4)
	C × G	.006 (12.8)	.455	.599
	Litter size	.003 (15.2)	.0004 (23.0)	.466
	C × L	.631	.803	.787
	G × L	.191	.356	.519
	Model R^2	.631	.601	.492
Lowest frequency	Condition	.136	.424	.472
	Genotype	.0001 (34.4)	.0001 (36.1)	.0002 (23.1)
	C × G	.117	.913	.842
	Litter size	.573	.200	.075
	C × L	.973	.587	.313
	G × L	.504	.736	.553
	Model R^2	.510	.474	.530

[a] Indicated only where the effect reaches statistical significance.

between main effects was observed, that between condition and genotype on the measure, highest frequency of a call, at age 2 days.

4. Discussion

In summary, we studied the ultrasonic calls of infant mice on days of age 2–4. These mice were the F_1 hybrids of C57BL/10J females and a male of the BALB/cJ, DBA/2J, or SJL/J inbred strain. During pregnancy, one half of the females was dosed with 20 mg/kg of cocaine and the other half with saline for 11 days, Days 7 through 17 following conception. This cocaine dosage and injection regime did not alter maternal weight gain, the number of pups produced, the gestation length, pup viability, or pup weight. This result is in line with previous studies [2,24] that showed that the threshold for perinatal effects in the mothers and fetuses was between 20 and 40 mg/kg in prenatally exposed C57BL mice (C57BL/6J in Middaugh et al. [24] and C57BL/10J in our study). Thus, the effects we observe are attributable to the direct effects of cocaine on pups and not the indirect effects of malnutrition or a shortened gestation. After birth, pups remained with their birth mothers and on days of age 2–4 we recorded the ultrasonic calls the pups produced for 20 s in an isolated and chilled environment. Cocaine reduced the rate of calling at ages 2–4 days and raised the beginning frequency of calls at age 2.

These effects were small (1–2% of the total sums of squares) but significant using full subjects analyses. Changes in ultrasonic calls as a result of cocaine exposure were shown by two of the F_1 hybrids pups, those with BALB/cJ and DBA/2J fathers. A second set of analyses using litter means rather than all subjects confirmed some of the effects seen in the full subjects analyses. In these analyses, cocaine exposure caused changes in ultrasonic calls only in 2-day-old pups. Cocaine reliably reduced the rate of calling and reliably increased the beginning frequency of calls. Again, these effects of cocaine exposure were seen in the BALB/cJ and DBA/2J F_1 pups but not in the SJL/J F_1 pups.

While the comparability of human cries and ultrasonic calls produced by mice could be challenged, a comparison is useful. We are aware of only one study of the effects of prenatal cocaine exposure on the crying of newborn human infants [6]. In that study, cocaine-exposed infants produced fewer cries and more short cries than neonates not exposed to cocaine. The results of our study are in partial agreement as prenatal cocaine exposure reduced the rate of calling of two of the three genotypes of mice we tested.

The small size of the effects of prenatal cocaine that we show here is consistent with findings in other laboratories (see review, Ref. [35]). The authors of that review suggest that the effects of cocaine are small in young animals because of the ability of the developing nervous system to compensate for the effects of cocaine. They hypothesize that such compensations in early life will have a cost, however, and that cost is a loss in the ability to deal with environmental stresses later in life. Others [22] have a similar position, agreeing that the effects of prenatal cocaine exposure are small. They characterize such effects as subtle, however. Subtle because of the small size of some of the effects and because larger effects are sometimes observed, but in specific abilities that might escape notice.

With respect to the ultrasonic calls of infant mice, we have shown evidence for two additional or perhaps, competing ideas. First, small effects may in part be the case because some genotypes are affected by prenatal cocaine and others are not. The small effect size we observe in the rate of calling, for example, occurred in part because two of the genotypes we employed exhibited reduced calling rates when exposed to cocaine, while the third was unaffected. Secondly, we observed reliable differences between litters within the treatment and genotype groupings and substantial variation within litters overall. These individual differences were responsible for a large portion of the total sums of squares. Thus, the small effect sizes seen in the literature could well be caused by gene × treatment interactions and factors operating within and between litters to increase individual differences with respect to the effects of prenatal cocaine exposure.

We can be more specific about the gene × environment interaction we observed. The F_1 progeny of the SJL males were not affected by the prenatal cocaine while the offspring of the DBA and BALB mice both showed significant changes in their rates of calling. This finding is in line with previous studies that have shown that in a comparison of adults of four inbred strains of mice: BALB/cJ, DBA/2J, C57BL/10J, and SJL/J mice, the SJL/J strain animals alone were resistant to convulsions produced by acute injections of cocaine [23]. A recent study investigating the effects of cocaine on operant responding for food also depicted mice of the SJL/J strain as being significantly different from mice of several other strains including those of the BALB/cJ, DBA/2J, C57BL/10J, and a cross of the C57BL/10J × SJL/J strains [17]. These authors found that the level of cocaine required to reduce operant conditioning in SJL/J mice overlapped with the dosage necessary to induce seizure activity. To the best of our knowledge, there have been no biochemical studies that directly compared the brains of SJL/J mice with other strains particularly on the three neurotransmitters known to be affected by cocaine: serotonin, norepinephrine, and dopamine. However, differences in the neurochemistry of SJL/J mice might underlie the differences in behavioral response to cocaine, compared to mice of other genotypes.

Finally, we suggest that the roles of genotype and litter with respect to prenatal cocaine and perhaps other drugs or ages of exposure cannot be understood by averaging over them or placing them in the error term. Rather, an understanding of such factors will be facilitated when they are treated as explicit experimental variables.

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References

[1] Bell RW, Nitschke W, Zachman T. Ultrasounds in three inbred strains of young mice. Behav Biol 1972;7:805–14.
[2] Benno RH, Hofsess S, Vallarta J, Hahn ME. The effects of prenatal cocaine upon morphological development, early behavior and mother–pup interactions in inbred mice. Behav Genet 1994;24:506.
[3] Brazelton TB. What we can learn from the status of the newborn. In: Kilbey MM, Asghar K, editors. Methodological issues in controlled studies on effects of prenatal exposure to drug abuse. NIDA Res Monogr 114. Rockville, MD: National Institute on Drug Abuse, 1991. pp. 93–105.
[4] Chasnoff IJ. Perinatal effects of cocaine. Contemp Obstet Gynecol 1987;29:163–8.
[5] Cohen-Salmon C, Carlier M, Roubertoux P, Jouhaneau J, Semal C, Paillette M. Differences in patterns of pup care in mice: V. Pup ultrasonic emissions and pup care behavior. Physiol Behav 1985;35:167–74.
[6] Corwin MJ, Lester BM, Sepkoski C, McLaughlin S, Kayne H, Golub H. Effects of in utero cocaine exposure on newborn acoustical cry characteristics. Pediatrics 1992;89:1199–2004.
[7] Dudek BC, Phillips TJ, Hahn ME. Genetic analyses of the biphasic nature of the alcohol dose–response curve. Alcohol: Clin Exp Res 1991;15:262–9.

[8] Ehret G. Categorical perception of mouse-pup ultrasounds in the temporal domain. Anim Behav 1992;43:409–16.
[9] Ehret G, Bernecker C. Low-frequency sound communication by mouse pups (*Mus musculus*): wriggling calls release maternal behaviour. Anim Behav 1986;34:821–30.
[10] Ehret G, Haack B. Ultrasonic recognition in house mice: key stimulus configuration and recognition mechanism. J Comp Physiol 1982;148: 245–51.
[11] Haack B, Markl H, Ehret G. Sound communication between parents and offspring. In: Willott JF, editor. The auditory psychobiology of the mouse. Springfield, IL: Charles C. Thomas, 1983. pp. 57–97.
[12] Hadeed AJ, Siegel SR. Maternal cocaine use during pregnancy: effects on the newborn infant. Pediatrics 1989;84:205–10.
[13] Hahn ME, Benno R, Caldwell HM, Schanz N. Effects of prenatal cocaine and genotype on intermale agonistic behavior in *Mus musculus*. Aggressive Behav 1997;23:183–96.
[14] Hahn ME, Hewitt JK, Adams M, Tully T. Genetic influences on ultrasonic vocalizations in young mice. Behav Genet 1987;17: 155–66.
[15] Hahn ME, Hewitt JK, Schanz N, Karkowski L, Henry A. Genetic and developmental influences on infant mouse ultrasonic calling: I. A diallel analysis of the calls of 3-day olds. Behav Genet 1997;27:133–43.
[16] Hahn ME, Karkowski L, Weinreb L, Henry A, Schanz N. Genetic and developmental influences on infant mouse ultrasonic calling: II. Developmental patterns in the calls of mice 2–12 days of age. Behav Genet 1998;28:315–25.
[17] Heyser CJ, McDonald JS, Beauchamp V, Koob GF, Gold LH. The effects of cocaine on operant responding for food in several strains of mice. Psychopharmacology 1997;132:202–8.
[18] Johns JM, Means MJ, Bass EW, Means LW, Zimmerman LI, McMillen BA. Prenatal exposure to cocaine: effects on aggression in Sprague–Dawley rats. Dev Psychobiol 1994;27:227–39.
[19] Johns JM, Means MJ, Means LW, McMillen BA. Prenatal exposure to cocaine: I. Effects on gestation, development and activity in Sprague–Dawley rats. Neurotoxicol Teratol 1992;14:337–42.
[20] Johnston LD, O'Malley PM, Bachman JG. National survey results on drug use from the monitoring the future study: 1975–1993, vol. 2: College students and young adults. Rockville, MD: National Institute on Drug Abuse, 1994.
[21] Kosofsky B. The effect of cocaine on developing human brain. In: Kilbey MM, Asghar K, editors. Methodological issues in controlled studies on effects of prenatal exposure to drug abuse. NIDA Res Monogr 114. Rockville, MD: National Institute on Drug Abuse, 1991. pp. 128–43.
[22] Lester B. The maternal lifestyles study. In: Harvey JA, Kosofsky BE, editors. Cocaine: effects on the developing brain. New York: New York Academy of Sciences, 1998. pp. 296–305.
[23] Marley RJ, Witkin JM, Goldberg SR. Genetic factors influence changes in the sensitivity to the convulsant properties of cocaine following chronic treatment. Brain Res 1991;542:1–7.
[24] Middaugh LD, Boggan WO, Bingel SA, Patrick KS, Xu W. A murine model of prenatal cocaine exposure: effects on the mother and fetus. Pharmacol, Biochem Behav 1996;55:565–74.
[25] National Center for Health Statistics. Health, United States, 1995. Hyattsville, MD: US Public Health Service, 1994 (Table 65).
[26] National Institutes of Health, Department of Health and Human Services. National Pregnancy and Health Survey. A National Institute on Drug Abuse Report. Washington, DC: Government Printing Office, 1995.
[27] Okon EE. The ultrasonic responses of albino mouse pups to tactile stimuli. J Zool (London) 1970;162:485–92.
[28] Okon EE. The effect of environmental temperature on the production of ultrasound by isolated, non-handled, albino mouse pups. J Zool (London) 1970;162:71–83.
[29] Rosenak D, Diamant YZ, Yaffe H, Hornstein E. Cocaine: maternal use during pregnancy and its effect on the mother, the fetus and the infant. Obstet Gynecol Surv 1990;45:348–59.
[30] Sales GD, Smith JC. Comparative studies of the ultrasonic calls of infant murid rodents. Dev Psychobiol 1978;11:595–619.
[31] Scott JP. Social genetics. Behav Genet 1977;7:327–46.
[32] Sobrian SK, Burton LE, Robinson NL, Ashe WK, Hutchinson J, Stokes DL, Turner LM. Neurobehavioral and immunological effects of prenatal cocaine exposure in rat. Pharmacol, Biochem Behav 1990;35:617–29.
[33] Spear LP. Neurobehavioral consequences of gestational cocaine exposure: a comparative analysis. In: Rovee-Collier C, Lipsitt LP, editors. Advances in infant research, vol. 9. Norwood, NJ: Ablex, 1995. pp. 55–105.
[34] Spear LP. Alternations in cognitive function following prenatal cocaine exposure: studies in an animal model. In: Lewis M, Bendersky M, editors. Mothers, babies and cocaine: the role of toxins in development. Hillsdale, NJ: Lawrence Erlbaum Associates, 1995. pp. 207–27.
[35] Spear LP, Campbell J, Snyder K, Silveri M, Katovic N. Animal behavior models. Increased sensitivity to stressor and other environmental experiences after prenatal cocaine exposure. In: Harvey JA, Kosofsky BE, editors. Cocaine: effects on the developing brain. New York: New York Academy of Sciences, 1998. pp. 76–88.
[36] Staats J. Nomenclature. In: Green E, editor. Biology of the laboratory mouse. 2nd ed. New York: McGraw-Hill Book, 1966. pp. 45–50.
[37] Yudofsky SC, Silver JM, Hales RE. Cocaine and aggressive behavior: neurobiological and clinical perspectives. Bull Menninger Clin 1993; 57:218–26.