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Inhibition of Nitric Oxide Synthesis With L-NAME Suppresses Isolation-Induced Ultrasounds in Rat Pups

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CAMPBELL, J. O., J. A. FOGARTY AND L. P. SPEAR. *Inhibition of nitric oxide synthesis with L-NAME suppresses isolation-induced ultrasounds in rat pups.* PHARMACOL BIOCHEM BEHAV **63**(1) 45–53, 1999.—The present experiments examined the impact of manipulating the NO system on production of isolation-induced ultrasonic vocalizations (USVs) in 10- and 11-day-old rat pups. Pups were tested under both high- and low-baseline USV emission; the latter was accomplished by pretest administration of cocaine, a drug known to suppress USVs. Treatment with 10, 50, or 100 mg/kg (but not 1 mg/kg) of the nitric oxide synthase (NOS) inhibitor L-nitro-arginine methyl ester (L-NAME) significantly attenuated USV production, as did injection of 10 mg/kg cocaine; combined treatment with both drugs did not result in greater suppression, perhaps due to a floor effect. Although cocaine increased locomotor activity, treatment with L- or D-NAME alone did not alter activity levels. Exposure to L-NAME induced some hypothermia, although these alterations in body temperature were not systematically related to the drug-induced suppression of USVs. Alterations in USV production by L-NAME were not evident after pretreatment with the less active isomer D-NAME, evidence supporting the importance of NO synthesis inhibition per se in the marked L-NAME–induced suppression of USVs in isolated infant rats. © 1999 Elsevier Science Inc.

Ultrasonic vocalizations Rat pups Anxiolysis Nitric oxide L-NAME

NITRIC OXIDE (NO) and its pathways have been implicated in many physiological and CNS functions, ranging from the hypertension and immune dysfunction associated with chronic renal failure (8) to stressor responsivity (7,32), the development of rapid tolerance to ethanol (22), and the development and expression of behavioral sensitization to cocaine (15). In investigations of the functional effects of NO, the neurotransmitter itself is not administered due to its short half-life. Rather, the effects of interfering with the synthesis of NO are typically assessed. NO is synthesized from the amino acid L-arginine by nitric oxide synthase (NOS); N^G-nitro-L-arginine methyl ester (L-NAME) and L-N^G-nitro arginine (L-NOARG) are effective in inhibiting NOS.

Although most psychopharmacological research examining NOS inhibitors has assessed adult animals, there are reports that inhibiting NO synthesis can have behavioral consequences early in life as well. For example, pretreatment with NOS inhibitors significantly impairs avoidance learning and memory retrieval in week-old rat pups (24), and has been reported to induce amnesia for a passive avoidance task in day-old chicks if the inhibitor is present at the time of training (18). These findings are reminiscent of those reported in adult animals where NOS inhibition has been demonstrated to impair learning (11), although these effects are not unbiquitous, with other reports of enhanced acquisition (10) or no significant effects on learning after inhibitor exposure (2).

The nitric oxide pathway has also been implicated in the regulation of anxiety, at least in adulthood. In these assessments, the elevated plus-maze has often been used to index anxiety, with greater levels of anxiety reflected by lower amounts of time spent on open relative to enclosed arms of a +-shaped apparatus (28). For instance, increases in open-arm time induced by nitrous oxide (7) or chlordiazepoxide (29) have been reported to be reversed by pretreatment with the NOS inhibitor L-NOARG. Although these findings are consistent with the hypothesis that NO activation might have anx-

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iolytic consequences, other studies found evidence for potential anxiolytic effects following inhibition of NO synthesis. Several groups have observed increases in exploratory activity in the plus-maze and time spent on the open arms following treatment with L-NAME (13,14,32), effects that appear to be related to inhibition of NO as they are not evident following treatment with L-arginine or D-NAME (the less active isomer of L-NAME) (13). Thus, although there are a number of reports that manipulating NO synthesis alters anxiety as indexed in adults in the elevated plus-maze, the directionality of this effect is disputed, and may depend on baseline levels of anxiolysis.

Although the NO pathway has been implicated in learning processes in young animals (18,24), it is not yet known whether NO is involved in neural processes regulating anxiety early in life. When testing anxiolytic/anxiogenic effects early in ontogeny, testing with an elevated plus-maze is not feasible because young rat pups exhibit only low levels of exploratory locomotion, and their eyes do not open until near the end of the second week of life. As an age-appropriate alternative, isolation-induced ultrasonic vocalizations (USVs) have been used. Rat pups in an age range from approximately 2 to 17 days postnatal emit USVs at high rates when isolated from the dam and littermates in unfamiliar surroundings (26). Some researchers have suggested that USVs are a by-product of laryngeal braking associated with thermogenic processes activated to warm the isolated pup (4). Yet others have suggested that these isolation-induced USVs may be used by pups as a distress call, promoting orientation, approach, and retrieval behavior in lactating dams (1). Pharmacologically, isolation-induced USVs are attenuated by anxiolytic compounds such as diazepam, and are increased by a number of anxiogenic compounds (19). In addition, Hofer and Shair (16) found that providing an isolated pup with an anesthetized dam or littermate also markedly suppressed or eliminated USVs, and that this reduction in rate of calling was seen even when introduction of the anesthetized dam actually cooled the test chamber (17). Thus, production of USVs may be multiply determined, with these vocalizations being sensitive to pharmacological and experimental manipulations that influence the emotional state of the preweanling rat.

The current experiments were designed to test potential alterations in rates of ultrasound production after NOS inhibition in preweanling rats. Because it was not clear whether this manipulation would increase or decrease rate of USV production, testing of isolated pups was conducted under conditions of both high and low baseline USV emission; the latter was accomplished by pretest administration of cocaine, a drug known to produce robust suppression of isolation-induced USVs (20). The specificity of the effects seen were verified by administering D-NAME, the less active isomer of the NOS inhibitor L-NAME [e.g., (13)]. Body temperatures were also monitored to determine whether potential drug-induced alterations in thermoregulatory activities may have contributed to any observed effects of the drugs on USVs.

EXPERIMENT I

In this experiment ultrasounds were measured after treatment with one of four doses of L-NAME (0, 1, 10, or 100 mg/kg) in combination with either saline or 10 mg/kg cocaine HCl. Given that others have found behavioral effects of the NO precursor in neonatal rat pups (24), we also examined the effects of the NO precursor L-arginine on USVs. Although L-arginine has been reported to reverse some effects of L-NAME [e.g., (14)], these data are difficult to interpret. given recent evidence that metabolites of L-arginine are themselves effective inhibitors of NOS (8). Hence, because of the problematic interpretation of any combined effects of L-NAME and L-arginine, we chose to examine the effects of the two compounds separately, and to use the less active isomer of L-NAME (i.e., D-NAME) to examine the issue of specificity (see Experiment II).

Method

Subjects were 80 Sprague–Dawley-derived (Charles River, VAF, Wilmington, MA) rat pups. Litters were culled to 8 to 10 pups on Postnatal Day 1 (P1). Whenever possible, an equal number of male and female pups were maintained in a litter. Throughout the preweanling period home shavings were cleaned twice weekly, and food and water were available ad lib. Pups were tested at 10–11 days of age, with half the pups in each litter being tested on P10 and the remaining pups tested on P11. Testing took place in a cylindrical incubator (20 cm diameter, height 26 cm). An air heater in the ceiling of the incubator allowed for maintenance of the ambient temperature at 25 ± 1°C. A small fan served to circulate air and provide background noise. The floor of the chamber was demarcated into four equally sized quadrants to allow scoring of locomotor behavior. A bat detector, model S25 (Ultrasound Advice, UK), suspended 10 cm above the chamber floor, was used to detect ultrasounds in the frequency range between 35 and 45 kHz. An Apple II computer interfaced with the detector registered the number of USVs emitted by the pup.

On test day the entire litter was removed from the home cage, and pups were marked, weighed, and placed in a clean cage lined with pine shavings. A heating pad under the holding cage maintained the surface of the shavings at 32 ± 1°C. The injection regimen in this experiment consisted of three injections given over a 2-h period prior to testing. All injections were given IP in a volume of 5 cc/kg body weight, with a 0.9% NaCl solution being used as the control and vehicle solutions. The first injection, given 2 h before testing, was one of four doses of the NOS inhibitor (0, 1, 10, or 100 mg/kg L-NAME). Twenty minutes before the behavioral test pups were given an injection of precursor (0 or 50 mg/kg L-arginine). Finally, a cocaine challenge injection was administered 15 min before testing (0 or 10 mg/kg cocaine HCl). Ten treatments were established using certain chosen combinations of the above drugs at their various doses (see Table 1). Four or five pups per sex were tested in each treatment condition, with no more than

TABLE 1
DRUG COMBINATIONS USED TO CREATE 10 TREATMENT CONDITIONS IN EXPERIMENT I

Injection 1	Injection 2	Injection 3
LN100	Saline	Cocaine
LN100	Saline	Saline
LN10	Saline	Cocaine
LN10	Saline	Saline
LN1	Saline	Cocaine
LN1	Saline	Saline
Saline	L-arginine	Cocaine
Saline	L-arginine	Saline
Saline	Saline	Cocaine
Saline	Saline	Saline

one pup/sex/litter being placed into any of these treatment conditions.

At time of testing, an individual pup was removed from the holding cage and its temperature was taken by insertion of a rectal probe (TH-8) (Physitemp, Clifton, NJ) 1.0–1.2 cm beyond the anal orifice. The pup was then immediately placed in the test incubator for a 5-min test session, during which the number of matrix crossings and USVs emitted each minute was recorded. At the conclusion of the test session, rectal temperatures were again determined prior to returning the pup to the holding chamber. Injection solutions were coded daily so that the experimenter could remain blind to pup treatments. The apparatus was wiped clean with a 0.5% acetic acid solution between pups. Using this procedure allowed four pups to be injected and tested over approximately 4 h. After testing four pups from a given litter on P10, the litter was reunited with the dam and sire, and the procedure was conducted with four other pups in the litter on P11.

The data were analyzed by analyses of variance (ANOVAs). Separate 2 (gender) × 4 (inhibitor dose) × 2 (cocaine dose) × 5 (min) ANOVAs with repeated measures on the last variable were used to analyze effect of inhibitor and cocaine on USV production and activity. Pretest and posttest temperatures were analyzed as a repeated measure using a 2 (gender) × 4 (inhibitor dose) × 2 (cocaine dose) × 2 (pretest vs. posttest temperature) mixed factorial ANOVA. For the analysis of the precursor data, 2 (gender) × 2 (precursor dose) × 2 (cocaine dose) × 5 (min) ANOVAs with repeated measures on the last variable were used to analyze the USV and activity dependent measures, with body temperatures being analyzed by a 2 (gender) × 2 (precursor dose) × 2 (cocaine dose) × 2 (pretest vs. posttest temperature) repeated-measure ANOVA. Significant ANOVA results were further examined by analyses of simple effects and Newman–Keuls post hocs.

Preliminary assessments were conducted to determine whether testing pups in each litter across 2 days influenced the dependent variables; for these analyses, data were collapsed across variables observed to be nonsignificant in the above ANOVAs [e.g., (23)], given the reduction in power associated with inclusion of test day as an additional variable. To further ensure that observed effects were not related to test day, Experiment II was designed such that all pups in a given litter were tested on a single day.

Results

The two highest doses of L-NAME (10 and 100 mg/kg) effectively inhibited ultrasounds. These same inhibitor doses lowered pretest and posttest body temperature. Cocaine potently suppressed ultrasounds and increased locomotor activity, as expected. L-NAME alone did not alter activity levels, although the lowest dose of L-NAME interacted with cocaine to produce activity levels that were higher than those of animals given cocaine in combination with the highest dose of L-NAME. The NO precursor L-arginine did not influence ultrasound production, nor were there any effects of test day on locomotor activity or USV production. Although pretest temperatures were not affected by test day, pups tested on the first day had lower posttest temperatures compared to those tested on Day 2.

L-NAME data: Ultrasounds. For ultrasounds there was a significant interaction of inhibitor dose × cocaine dose, $F(3, 48) = 3.05$, $p \leq 0.05$. Simple effects revealed that while cocaine decreased ultrasounds regardless of prior treatment, animals challenged with saline rather than cocaine only showed an attenuation in number of ultrasounds if they had been pretreated with either of the two high doses of L-NAME (see Fig. 1). Rates of USVs following pretreatment with these two high doses of L-NAME did not differ significantly from rates observed in animals given cocaine. There was also a significant interaction between cocaine dose and min of testing, $F(4, 192) = 3.16$, $p \leq 0.05$, with animals not injected with cocaine showing an increase in USV calling from the first 2 min to the last 3 min, while animals given cocaine exhibited a consistent suppression of USVs throughout the test session (see Table 2).

Activity. The ANOVA of the activity data revealed a significant interaction between inhibitor dose and whether the animals received a cocaine injection, $F(3, 48) = 3.0$, $p \leq 0.05$ (see Fig. 2). The inhibitor alone did not alter activity levels. As expected, cocaine caused an increase in activity. The enhanced activity was greater when cocaine was given in combination with the lowest dose of inhibitor (L-NAME 1 mg/kg) than when cocaine was given alone in combination with the highest dose of L-NAME (100 mg/kg).

Temperature. Analysis of pretest and posttest temperatures as a repeated measure revealed significant interactions between inhibitor dose and gender, $F(3, 46) = 3.24$, $p \leq 0.05$, and between inhibitor dose and test phase (pretest vs. posttest), $F(3, 46) = 2.99$, $p \leq 0.05$ (see Fig. 3). Post hoc analyses conducted on data collapsed across test phase to examine the interaction between inhibitor dose and gender (see Fig. 3, top) revealed that males given the high dose of L-NAME exhibited significantly lower temperatures than all other groups. Temperatures of females were not affected significantly by treatment with the inhibitor. Post hoc analyses performed on data collapsed across gender to determine the source of the interaction between inhibitor dose and test phase (Fig. 3 bottom) revealed that temperatures were notably lower posttest than pretest, with all doses of L-NAME significantly decreasing pretest temperatures, but only the two highest doses decreasing posttest temperatures. Although the effects of L-NAME on body temperature were in the opposite direction from what might be predicted if the suppression of USVs by L-NAME were a result of drug-induced temperature alter-

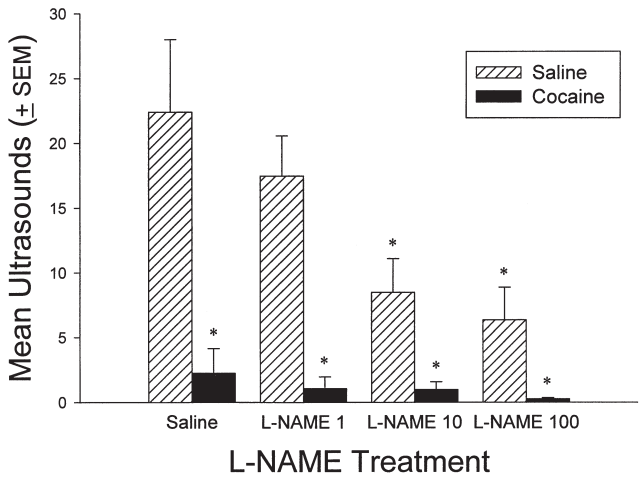

FIG. 1. Mean number of ultrasounds emitted by P10-P11 pups during 5 min of social isolation in Experiment I. Data are collapsed across gender and minute. *Indicates a significant decrease from saline/saline group ($p \leq 0.05$). Cocaine and the two high doses of L-NAME (10 and 100 mg/kg) significantly suppressed ultrasound production compared to saline treatment. Error bars represent SEMs.

TABLE 2
MEAN NUMBER OF ULTRASOUNDS EMITTED BY PUPS TREATED WITH COCAINE OR SALINE DURING THE 5-MIN ISOLATION TEST OF EXPERIMENT I

	Minute				
Drug	1	2	3	4	5
Saline	10.3(3.4)	11.1(2.2)	15.3(2.5)*	14.7(2.1)*	14.5(2.4)*
Cocaine	1.7(1.0)	1.5(0.8)	1.0(0.5)	0.9(0.5)	0.8(0.3)

*Indicates a significant increase from first minute. Rates of calling were higher for saline-treated animals at every minute. Data are collapsed across gender and L-NAME treatment.

ations [see (4) for discussion], correlations were conducted to compare these two dependent measures. For these comparisons, correlations were conducted between pretest body temperatures and mean USV production, as well as posttest body temperatures and USVs. Neither of these correlations were significant ($r = 0.19$, $p > .05$; $r = 0.26$, $p > 0.05$, respectively).

L-Arginine data. There were no significant main effects or interactions in the analysis of potential effects of the precursor L-arginine on rate of ultrasound production. The only significant effect in the analysis of the L-arginine activity data was a main effect of min, $F(4, 100) = 2.68$, $p \leq 0.05$, with animals showing an increase in activity over the first 3 min, followed by a drop in activity during the last 2 min (data not shown). Analyses of the temperature data for animals treated with L-arginine revealed only a significant interaction between gender and cocaine dose, $F(1, 24) = 5.18$, $p \leq 0.05$, and between L-arginine dose and cocaine dose, $F(1, 24) = 5.18$, $p \leq 0.05$, although posthoc analyses of these interactions failed to reveal any significant group differences.

Analyses across test day. The ANOVAs conducted on data collapsed across gender to examine the potential influence of test day on USV production revealed no main effect or interactions involving test day for either the inhibitor or precursor data analyses.

Analyzing the activity data collapsed across gender for effects of test day revealed no significant main effects or interactions involving test day in the analysis of the precursor data. However, the analogous ANOVA for the inhibitor data revealed a significant interaction between inhibitor dose and test day, $F(3, 32) = 3.61$, $p \leq 0.05$. Regardless of test day, L-NAME failed to produce significant differences in activity compared to treatment with saline. However, for animals tested on Day 1, those given the high dose of L-NAME (100 mg/kg) were less active (2.03 ± 0.55) than those given 1 mg/kg L-NAME (3.4 ± 1.1); this effect was not evident on Day 2.

The only effect involving test day in the ANOVA performed on pre- and posttest body temperatures for the inhibitor data was a significant interaction between test day and test phase, $F(1, 30) = 7.87$, $p \leq 0.01$. Although pretest temperatures were not affected by whether animals were tested on Day 1 or Day 2 ($35.7 \pm .17$°C; $35.3 \pm .13$°C, respectively), posttest temperatures were lower in animals tested on Day 1 (32.1 ± 0.16°C) compared to those tested on Day 2 (32.4 ± 0.18°C). This test day effect was not evident in the analysis of

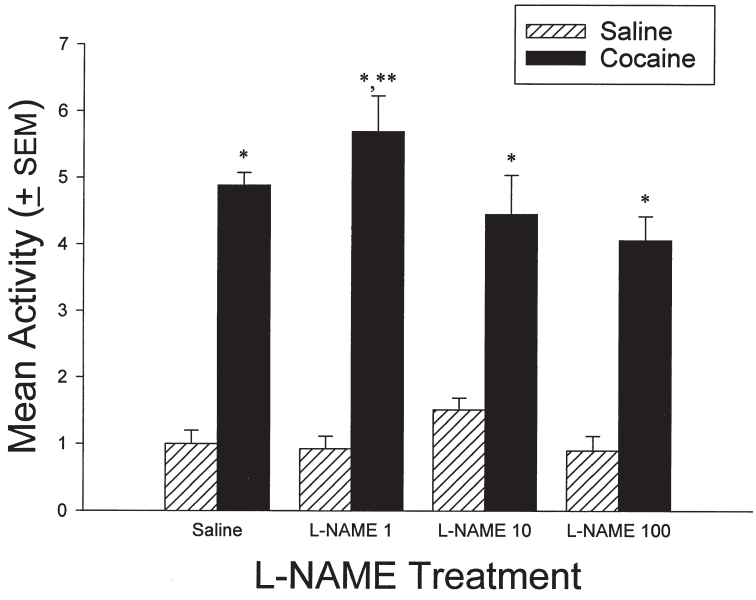

FIG. 2. Mean activity scores for pups during 5 min of social isolation in Experiment I as a function of drug treatment. Data are collapsed across gender and minute. *Indicates significant increases in activity after cocaine compared to similarly treated saline animals ($p \leq 0.05$). *Indicates greater activity after cocaine in combination with the low dose of L-NAME compared to animals given cocaine in combination with high dose of L-NAME ($p \leq .05$). Error bars represent SEMS.

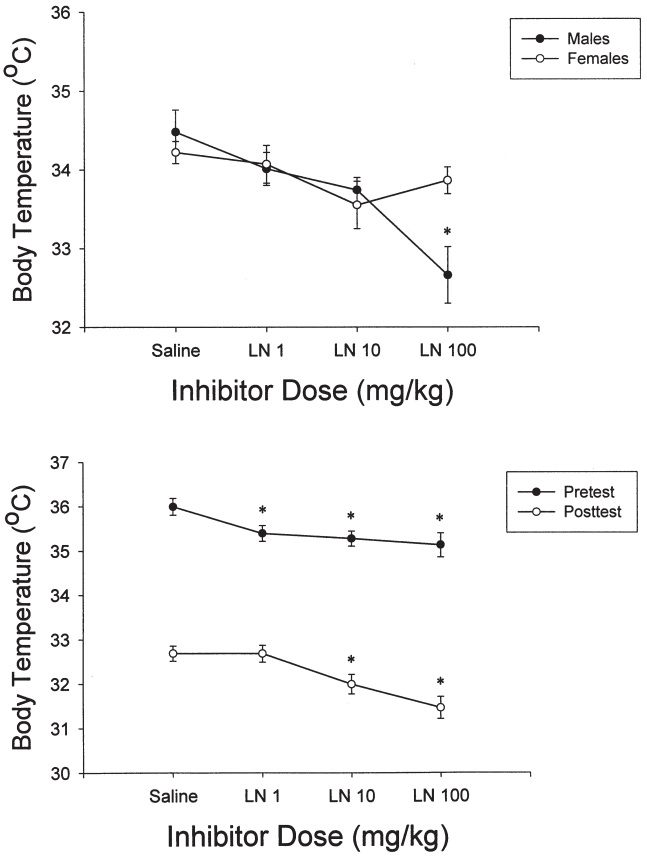

FIG. 3. Effects on body temperature in Experiment I. Top: effect of L-NAME treatment on mean body temperatures for males and females. Data are collapsed across cocaine treatment and test phase (pretest versus posttest). *Indicates that males given the high dose of L-NAME had lower temperatures than all other groups ($p \leq 0.05$). Posthoc comparisons revealed no other group differences. Bottom: effect of L-NAME treatment on pretest and posttest body temperatures. Data are collapsed across cocaine treatment and gender. *Indicates significantly lower body temperatures compared to their saline-treated counterparts examined at the same test phase (pre- or posttest). Temperatures of all groups of animals were lower at posttest relative to pretest ($p \leq 0.05$). Error bars represent SEMs.

the precursor data that revealed no main effects or interactions involving test day.

DISCUSSION

The results of this experiment revealed that NOS inhibition dose dependently decreased USV production in isolated rat pups. As expected from previous research (20), cocaine also suppressed ultrasounds. Although both drugs similarly suppressed USVs, they differed in their effects on locomotor activity; L-NAME given by itself had no effect on the activity level of pups during testing, while cocaine robustly increased locomotion, as had been previously reported (30).

The doses of L-NAME that effectively suppressed ultrasounds also caused decreases in both pretest and posttest body temperatures. However, these differences were in the opposite direction from what would be predicted if L-NAME–induced alterations in temperature were the driving force behind the changes in rate of USV production. That is, if USVs were simply a by-product of thermogenesis (4), the lower temperatures of animals treated with the high and medium doses of L-NAME might be expected to elicit greater rates of ultrasounding relative to warmer saline-treated pups. Alternatively, however, it is possible that the pharmacologically induced suppression of body temperature reflected a general sedative effect or some other physiological response that indirectly suppressed USVs. However, correlations between both pretest and posttest body temperatures and USV production were not significant, and the activity data revealed no apparent sedative actions of L-NAME.

Taken together, this pattern of findings supports the suggestion that the L-NAME–induced suppression of ultrasounds is not simply related to drug-induced alterations in locomotor activity or body temperature, and may possibly reflect an anxiolytic effect of NOS inhibition. These findings are consistent with some previous reports showing that administration of NOS inhibitors causes behavioral changes suggestive of anxiolysis in adults (13,14). In contrast, administration of the NO precursor L-arginine did not affect any of the dependent variables studied, data reminiscent of the finding that L-arginine given alone had no significant effect on anxiety measures in adults tested on the elevated plus maze (13). How these data relate to studies of learning that have revealed alterations in behavior after L-arginine exposure in young animals is unclear (24).

EXPERIMENT II

The second experiment was undertaken to address two issues. First, by comparing L-NAME with its less active isomer, D-NAME, this study examined whether the L-NAME–induced suppression of USVs was related to inhibition of NOS synthesis per se, or whether the effect was due to some nonspecific effect of the compound. Secondly, the experimental procedure was changed to determine whether the effects of NOS inhibition on USV production could be replicated using a procedure that did not require multiple days for the testing of each litter or separation of pups from the home nest for as long a period as that used in the first experiment. Cocaine was not used in this experiment because Experiment I showed that both cocaine and the inhibitor suppressed USVs, with no additive or synergistic effects between the two drugs on rate of ultrasound production.

Method

Subjects were 78 Sprague–Dawley derived (Charles River, VAF, Wilmington, MA) rat pups tested at P11, with all pups in a litter tested on a single day. The testing apparatus and procedure were the same as in the first experiment except for the injection regimen, which consisted of a single IP injection of saline, L-NAME (50 or 100 mg/kg/5 cc), or D-NAME (50 or 100 mg/kg/5 cc) given 1 h prior to testing. No more than one pup/sex/litter was placed into each of these five test groups; with seven to eight subjects per sex being examined in each test condition.

Separate 2 (gender) × 3 (inhibitor dose) × 5 (min) ANOVAs with repeated measures on the last variable were used to analyze effect of the active inhibitor (L-NAME) and its less active isomer (D-NAME) on ultrasound production and activity levels. Effects of the drugs on body temperature were analyzed by separate 2 (gender) × 3 (inhibitor dose) × 2 (pretest vs. posttest temperature) mixed ANOVAs for each of the two isomers. Significant results were followed by Newman–Keuls posthoc analyses.

Results

The two doses of L-NAME studied in the present experiment both effectively suppressed ultrasound production in isolated pups, while no such suppression was seen after treatment with D-NAME. Neither compound had any effect on locomotor activity or on body temperature. As in the first experiment, body temperature decreased significantly during testing. A sex difference in rate of ultrasounding was obtained, with males calling more than females.

Ultrasounds. Analyses of the effects of L-NAME on ultrasound production revealed a suppression of ultrasounds after treatment with L-NAME, $F(2, 41) = 3.60$, $p \leq 0.05$ (see Fig. 4), with animals given either 50 or 100 mg/kg L-NAME exhibiting fewer USVs than pups treated with saline. There was a significant gender $\times$ min interaction, $F(4, 164) = 3.24$, $p \leq 0.05$, in this analysis. Post hoc analyses conducted on the data collapsed across dose revealed that males had significantly higher rates of calling compared to females during the final 2 min of the test session (see Fig. 4, insert). Treatment with D-NAME did not significantly influence USV rate (see Fig. 5). As with the L-NAME groups, there was a significant gender $\times$ min interaction, $F(4, 160) = 3.68$, $p \leq 0.01$, with males having significantly higher rates of calling than females in the last 3 min (see Fig. 5, insert).

Activity. For analyses of both L-NAME and D-NAME treatments, the only significant effect was a main effect of min [L-NAME, $F(4, 164) = 4.48$, $p \leq 0.01$; D-NAME, $F(4, 160) = 6.17$, $p \leq 0.01$]. In both analyses the pattern was a decrease in activity across time, with locomotion in the last 2 min being below that of the first (data not shown).

Temperature. For analyses of both L-NAME and D-NAME data, the only significant effect on temperature was a main effect of test phase, with temperatures being lower following the 5-min isolation test compared to pretest temperatures. In the L-NAME analyses the drop in temperature was from a pretest mean of 35.36 ($\pm$0.24)°C to 32.05 ($\pm$0.17)°C posttest. Comparable data in the D-NAME analysis was a pretest to posttest decline from 35.69 ($\pm$0.21)°C to 32.54 ($\pm$0.15)°C.

Discussion

This experiment replicated the L-NAME–induced suppression of ultrasounds seen in Experiment I. The effect was evident without disturbing the litter on more than 1 test day, and after a shorter treatment-to-test interval than used in the

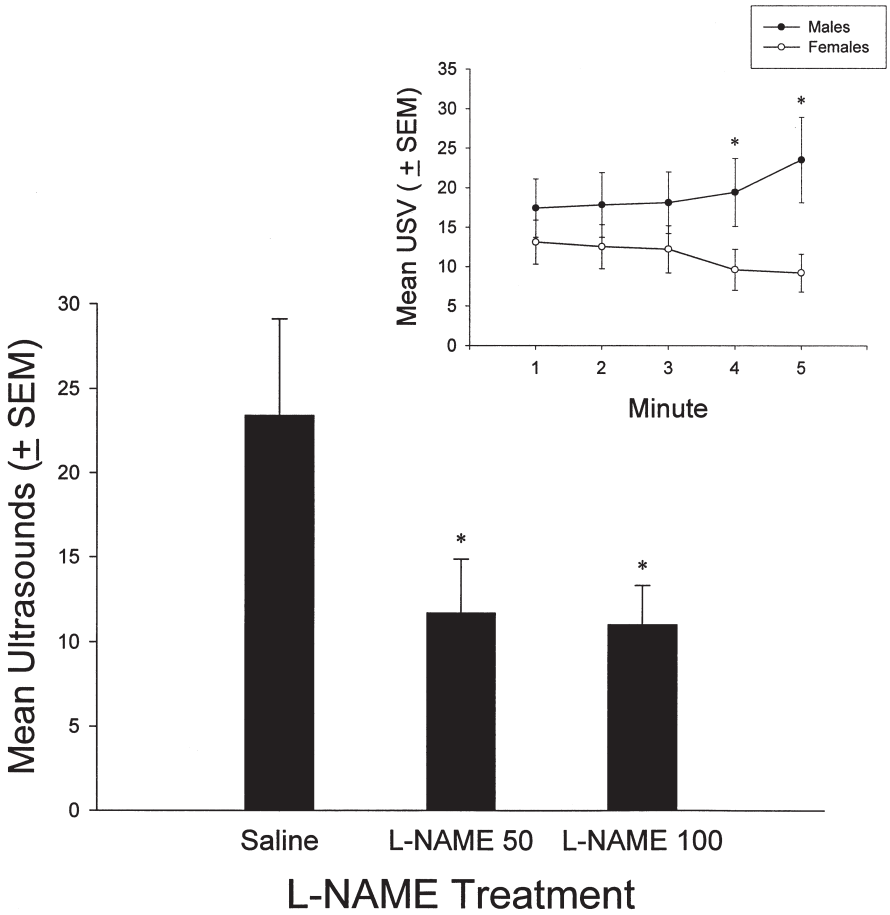

FIG. 4. Mean number of ultrasounds emitted by pups following L-NAME treatment in Experiment II. Data are collapsed across gender and minute. *Indicates a significant suppression of ultrasounds compared to saline-treated animals ($p \leq 0.05$). Insert shows mean USVs emitted by males and females during each minute of the 5-min test. Data are collapsed across drug treatment. *Indicates greater rate of USV production by males compared to females for the last 2 min of the test ($p \leq 0.05$).

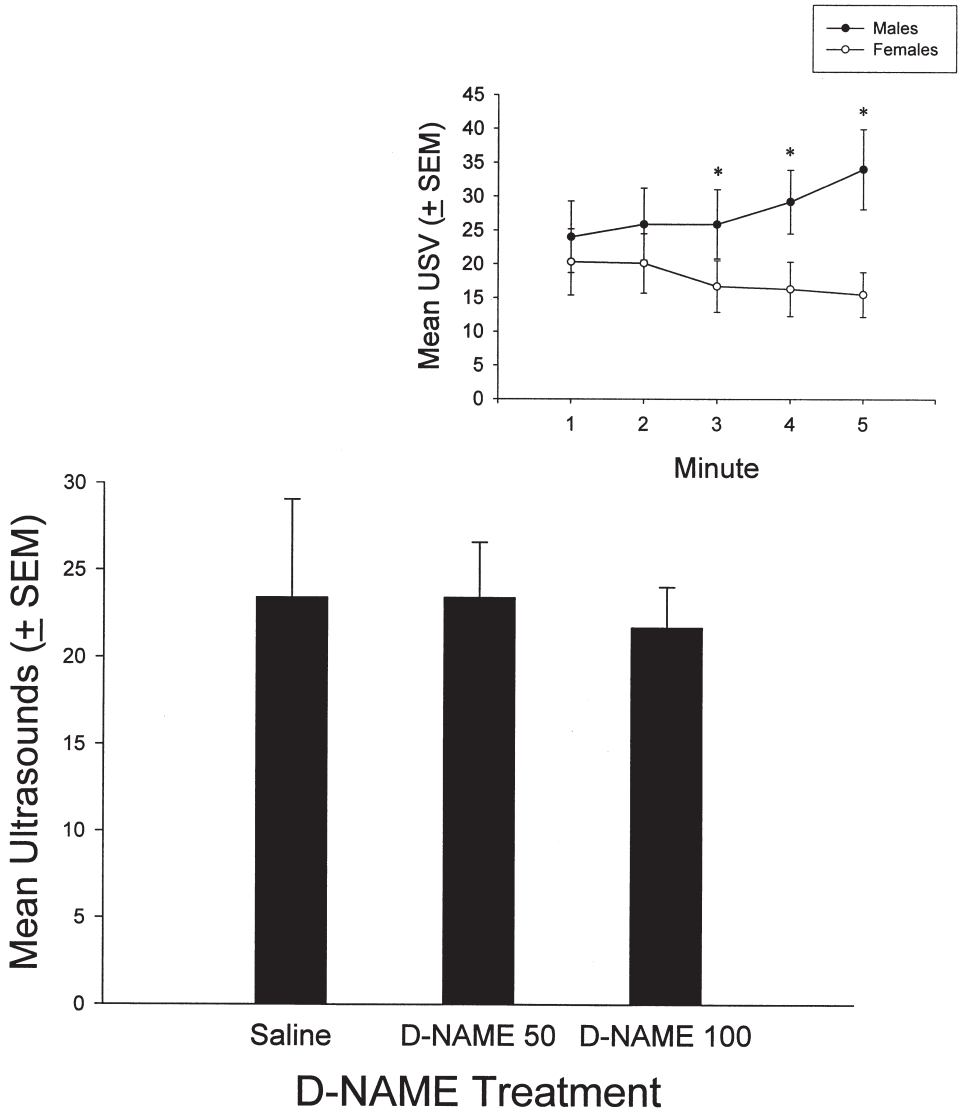

FIG. 5. Mean number of ultrasounds emitted by pups following D-NAME treatment in Experiment II. Data are collapsed across gender and minute. There was no effect of D-NAME on USV production. Insert shows mean USVs emitted by males and females during each minute of the test. Data are collapsed across drug treatment. *Indicates greater rate of USV production by males compared to females for the last 3 min of the test ($p \leq 0.05$).

first experiment (1 h in this study vs. 2 h in Experiment I). This L-NAME–induced suppression in ultrasounds again was seen in the absence of any drug-induced alteration in locomotor activity. As in Experiment I, rectal temperatures dropped during the test session, although under the testing circumstances of Experiment II animals given L-NAME did not vary significantly from saline controls in the magnitude of this decline. These data further buttress the conclusion that the L-NAME–induced suppression of USVs is not related to drug-induced alterations in body temperature. The suppression of ultrasound production by L-NAME in this second experiment seemingly reflects a specific effect of L-NAME on NOS, given that the less active isomer (D-NAME) had no effect on rate of ultrasounding.

A sex difference was seen in USVs in Experiment II, with males ultrasounding more than females in the latter minutes of testing. Greater rates of USV production by males compared to females has been reported in the past (6,25), although no such sex differences were seen in Experiment I of the present study. The source of these disparate sex effects between experiments is unknown, but may be a result of the different durations of isolation used in the two studies or the increase in power associated with the use of larger sample sizes per sex in Experiment II.

GENERAL DISCUSSION

The two experiments reported here were designed to test whether inhibition of NO synthesis produces alterations in rate of ultrasound production in preweanling rat pups. Experiment I revealed that L-NAME treatment markedly sup-

pressed isolation-induced ultrasonic vocalizations. These findings were replicated and extended in Experiment II, with this study showing that the effect was isomer specific and, hence, presumably related to NOS inhibition, and was also evident following less litter perturbation and a shorter time interval between treatment and testing (1 h vs. 2 h).

The lack of consistent L-NAME effects on temperature across experiments is especially important, because body temperature is known to affect ultrasound production in rat pups during the first 2 weeks of life (4). In Experiment I, animals treated with USV-suppressing doses of L-NAME (10 and 100 mg/kg) had lower pretest and posttest temperatures compared to pups given saline. It is unlikely that this effect of L-NAME on body temperature was the cause of the different rates of ultrasounding in the first experiment. No effect of L-NAME on body temperature was seen following the shorter treatment-to-test interval used in Experiment II, although a pronounced L-NAME–induced suppression of USVs was also seen in this study. Moreover, the lower pretest temperatures after L-NAME exposure would actually be expected to cause an increase in rate of ultrasounding if the calls were part of a physiological response to a lowered core temperature [e.g., (4)]. It is possible that the lower temperatures in L-NAME–treated animals following testing was somehow a result of a drug-induced suppression of USVs, but this does not explain why USV production was lower after L-NAME. No evidence was obtained that the suppression of USVs might be related to a general sedative effect, given that the inhibitor had no effect on locomotor activity.

Together, these data provide converging evidence that the suppression of USVs caused by L-NAME is not a function of any drug-induced alterations in body temperature or activity. It should be noted, however, that manipulations of the NO system are known to affect other physiological systems including blood pressure (BP) (27), and alterations in BP have recently been reported to coincide with changes in rate of USV production in rat pups (5). Thus, it is possible that drug-induced alterations in BP could have contributed to observed L-NAME–induced alterations in USVs, a possibility that cannot be addressed directly from the present experiments, because BP was not measured and the injections were given peripherally and not centrally. However, the lack of any consistent effects of L-NAME on body temperature in these experiments lends support to the argument that the lower rates of calling after L-NAME exposure are not simply a response of the organism to drug-induced changes in metabolic regulation.

There is both anatomical and behavioral evidence to suggest that the NO system develops early in ontogeny, and is functionally active in young animals. Studies examining the neuroanatomical development of the NO system have shown that NOS is present from embryonic day 10 (21), and that the distribution of NADPH-d activity (a neuronal marker for NOS) is adult-like in the first few postnatal weeks in the rat (31). Behaviorally, impaired learning has been reported in both developing (24) and adult animals (11) after NOS inhibition. The current study extends the effects of L-NAME inhibition in young animals to include suppression of ultrasounds. Although adult animals do emit ultrasounds in certain test situations (3), to our knowledge modulation of USV production by NOS inhibition has not been examined in adulthood.

The attenuation in USV production in preweanlings by L-NAME observed in the current study may reflect an anxiolytic effect of L-NAME in preweanlings. However, the use of isolation-induced ultrasounds as a measure of anxiety in infant rat pups must be qualified, given some pharmacological evidence problematic for an anxiety model of ultrasound production. For instance, although anxiolytic drugs generally suppress, and anxiogenic drugs increase, isolation-induced USVs in infant rats (19), cocaine is an exception to this generality. As shown here and elsewhere (20), cocaine suppresses ultrasounds; yet, at least in adults, cocaine produces alterations consistent with an anxiogenic profile, as assessed using a defensive withdrawal paradigm (33), the elevated plus-maze (9), and potentiation of the avoidance of an initially aversive environment (12). Whether cocaine has anxiogenic properties in preweanlings, however, has not been determined, although Kehoe and Boylan (20) interpreted the suppression of USVs after cocaine exposure as a quelling of the stress of isolation produced by the reinforcing properties of cocaine.

Given this apparent pharmacological anomaly and other evidence showing multiple determinants of USV production in infancy [e.g., (3,4)], interpreting drug-induced alterations in isolation-induced USVs as reflecting alterations in anxiety must be done with caution. Thus, although these data can be interpreted to support the hypothesis that as in adults NOS inhibition has anxiolytic effects in preweanling rats, this hypothesis remains speculative at present. What can be concluded from the current data is that inhibition of NOS with L-NAME effectively suppresses isolation-induced ultrasound production in 10- and 11-day-old rat pups, an isomer-specific effect that does not appear to be mediated indirectly through nonspecific effects of the drug on body temperature or general activity.

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