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Prenatal Stress Effects Are Partially Ameliorated by Prenatal Administration of the Neurosteroid Allopregnanolone

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ZIMMERBERG, B. AND L. G. BLASKEY. *Prenatal stress effects are partially ameliorated by prenatal administration of the neurosteroid allopregnanolone.* PHARMACOL BIOCHEM BEHAV **59**(4) 819–827, 1998.—This study examined the effects of exposure to prenatal stress on young and adult rats, and whether the concomitant administration of an anxiolytic neurosteroid, allopregnanolone (3-alpha-hydroxy-5 alpha-pregnan-20-one), could ameliorate some of the behavioral dysfunctions associated with prenatal stress. Pregnant dams were assigned to one of five treatment groups on gestational day 14. These groups were exposed to either 1) restraint for 45 min three times daily; 2) a vehicle injection twice daily; 3) 5 mg/kg allopregnanolone twice daily; 4) restraint with allopregnanolone injections; or 5) nonhandled controls. Assays for plasma allopregnanolone concentrations indicated that exogenous allopregnanolone injections significantly raised circulating levels to a comparable degree in gestational day 20 dams and their fetuses. At 7 days of age, however, subjects prenatally exposed to allopregnanolone either alone or with restraint now had lower circulating levels compared to the other groups, suggesting some negative compensatory change. Behavioral results suggested that the effects of prenatal stress on affective behaviors (ultrasonic vocalizations emitted after a brief maternal separation at 7 days of age, and plus-maze behavior at 70 days of age) could be reversed by coadministration of allopregnanolone. When locomotor activity was assessed at 16 and 60 days of age, no comparable reversal effect was observed. In fact, the allopregnanolone groups had results similar to those of the restraint alone group. Thus, for some neuronal systems, allopregnanolone may exert either a direct teratogenic effect or an indirect effect due to neurosteroid-induced behavioral changes in the pregnant dam. © 1998 Elsevier Science Inc.

Allopregnanolone Neurosteroids Neuroactive steroids GABA

ALTHOUGH the effects of prenatal stress in humans has been well documented, the inability to control for levels of stress and other confounding environmental factors as well as the typically retrospective nature of stress reports have necessitated the use of animal models in the study of prenatal stress (10). In pregnant rats, random light and noise stressors administered intermittently caused increases in plasma corticosterone three to four times that of controls (5). Exposure of pregnant rats to immobilization or restraint also produced significant increases in plasma corticosterone (23). Many of the behavioral effects of prenatal stress can be linked to alterations in various neurotransmitters. Included among these changes are increased dopamine turnover rates in the left striatum (8), transient increases (peaking at 16 days) in early development of 5-HT and NE levels in the cerebral cortex, and more long-term changes in 5-HT and NE in the hypothalamus, beginning at day 23 and continuing through adulthood (16). Fameli and co-workers (4) reported decreased dopaminergic and increased serotonergic activity in rats prenatally exposed to ACTH administration. Fride et al. (6) also found lower levels of dopamine in the medial preoptic nucleus of prenatally stressed animals, and Henry et al. (10) report increased D_2 receptor binding and decreased D_3 receptor binding in the nucleus accumbens. In addition, higher levels of β-endorphins in the hypothalamus account for increased HPA axis functioning in prenatally stressed animals, due to their stimulation of CRF secretion (19). Furthermore, prenatal stress reduced the number of benzodiazepine receptors in the hippocampus, which play a role in inhibiting the stress response (6).

Such alterations in these neurotransmitter levels and receptor functioning produce differences in the activity levels and emotional behavior of prenatally stressed animals in addi-

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tion to affecting their adaptability to stressful situations. For example, offspring of pregnant rats exposed to foot shock were found to display greater spontaneous motor activity until postnatal day 10, peaking about a week earlier than control animals (21); while Fride et al. (7) found motor development to be retarded in animals exposed prenatally to light and noise. Hence, both early and retarded maturation of different aspects of motor development have been reported. Additionally, both during development and in adulthood, prenatally stressed animals tend to exhibit greater emotionality, showing increased defecation (24,25) and decreased exploration in novel environments (5,11,25). Also indicative of increased emotionality and heightened responsiveness to stressors, males prenatally exposed to stress performed better than controls on a water-maze task in high stress conditions (23). Feminized male behavior (26), and altered immune functioning (13) are also commonly noted effects of prenatal stress exposure.

In addition to the alterations in behavior produced by prenatal exposure to stress, morphological effects have also been reported. For example, pregnant rats exposed to stress gain less weight in pregnancy, give birth earlier than do controls, and produce smaller numbers of offspring that often weigh less than controls (2,9). Furthermore, morphological differences such as reduced hippocampal weights in prenatally stressed animals have also been noted (23); as well as reduced ano-genital distances in male pups (26).

Although studies of stress response have typically focused attention on the HPA axis, the involvement of both GABA and the recently discovered neurosteroids, endogenous anxiolytic compounds that act at the GABA receptor are also of increasing interest. Stressors activate peripheral benzodiazepine receptors on the outer mitochondrial membrane of glial cells, thus initiating a chain of events by which cholesterol is converted to pregnenolone and pregnenolone is then converted to progesterone. which is then converted to 3-alpha-hydroxylated ring A reduced neurosteroid metabolite commonly known as allopregnanolone (15) . Allopregnanolone acts by binding to an independent site on the GABA receptor, modulating its Cl^- uptake in a way similar to other synthetic neuromodulatory agonists of the receptor, such as diazepam. Two neuroactive steroids, allopregnanolone and 5-alpha-THDOC, are among the most potent known positive modulators of the GABA receptor (15,18). Concentrations of allopregnanolone have been found to increase rapidly (under 5 min) in response to stress in both the cerebral cortex and hypothalamus as well as blood plasma (18). Reaching levels 2.3 times that of baseline, levels of allopregnanolone then remain elevated over normal levels for up to 2 h following a stressful event (18). Purdy et al. (18) hypothesize that such stress-induced increases in allopregnanolone may reduce stress by causing a decreased release of CRF and, thereby, of ACTH and corticosterone. Several studies have already demonstrated the ability of exogenously administered allopregnanolone to reduce anxiety in animal models (3,27,28).

Due to their teratogenic effects (12), the benzodiazepines would not be useful for treatment of anxiety during pregnancy. This experiment was designed to determine whether allopregnanolone administration might ameliorate the deleterious effects of prenatal stress. Because prenatal stress reduces the density of benzodiazepine receptors (6), the positive neuromodulation of $GABA_A$ receptors provided by this neurosteroid might be beneficial. However, due to its similarity in mechanism of action to benzodiazepines, allopregnanolone may, in itself, produce teratogenic effects, and this question has not been examined as yet. Thus, there were five experimental groups in this study: prenatal stress alone, prenatal stress + allopregnanolone, allopregnanolone, vehicle injection control, and a nonhandled control. The "allopregnanolone" group was designed to test for teratogenic effects of drug administration independent of any effects it may have had in ameliorating effects of prenatal stress. "Vehicle injection control" was designed to control for possible independent effects of the vehicle in which the allopregnanolone was suspended; while the "nonhandled control" acted as a point of comparison for the effect of the stress as well as the independent allopregnanolone administration. Outcome measures included weight gain as well as motor and affective behaviors at developmental milestones. In addition, plasma levels of the neurosteroid were assessed before and after parturition.

METHOD

Subjects

After acclimation to the laboratory, female Long–Evans rats (Harlan–Sprague–Dawley, Indianapolis, IN) were placed singly with males for breeding. Detection of a vaginal plug was marked as gestational day 1 (GD 1) and the female weighed and placed in a plastic breeding cage in the nursery. The nursery was maintained on a 12 L:12 D cycle, with lights on at 0700 h with free access to food and water available. Each female was randomly assigned to one of five treatment groups: control, vehicle, prenatal stress, allopregnanalone, or prenatal stress + allopregnanalone. From GD 1–12 pregnant females remained in breeding cages untouched. On GD 13 the pregnant rat was removed from the cage and handled by an experimenter for 5 min to acclimate the dam to future treatment procedures. From GD 14–20, each pregnant rat was weighed in the morning and exposed to treatment. On GD 21 animals were weighed, returned to breeding cages, and the presence of pups checked for several times a day. Presence of pups was marked as postnatal day 0 (PN 0) and on PN 1 pups were weighed and sexed and litters culled to 12 (when possible 6 males and 6 females). Pups were also weighed, sexed, and ear clipped for identification on PN 12 and weighed and weaned on PN 25 in same-sex sibling pairs.

Pups were distibuted across tests as follows: litters were randomly assigned to each treatment group with two males and two females from each litter distributed to the USV, activity, and plus-maze tests, respectively. No fewer than eight males and eight females were tested from each treatment group.

Eleven other dams were bred for analysis of plasma allopregnanolone levels at GD 20 of both dams and fetuses. Dams were assigned randomly to four treatment groups (ns = 3 except for n = 2 for nonhandled control). Drug costs precluded having an allopregnanolone + prenatal stress group.

Apparatus

Restraint. The restraint apparatus consisted of a Plexiglas box (20′ × 18.5′ × 2.5′) elevated 6 inches on stilts running along the short-ended sides of the box. The box itself was divided into two rows of rectangular compartments (8′ × 3′ × 2.5′), with five compartments in each row. The compartments each had individual Plexiglas lids with screws to secure them; and the bottom of the entire box was covered in wire mesh. This apparatus was placed in a larger Plexiglas container measuring 24′ × 16.5′ × 6.5′ with bedding inside it. Lights were suspended above the maze and designed to provide equal distribution of light over all compartments of the restraint apparatus.

Maternal separation test. Ultrasonic vocalizations (USVs) were recorded using a capacitance microphone with a mylar diaphragm and the broadband-countdown circuitry of an S-25 ultrasound detector (Ultra Sound Advice, London). This system responds to the strongest component of the signal within the microphone range of 10–200 kHz and produces an audible signal in earphones worn by an experimenter, who could then count ultrasounds by activating a counter. The behavioral observation box ($18' \times 18' \times 8'$ was constructed of white Plexiglas.

Open-field activity. Activity was assessed in four wooden activity boxes ($2' \times 2' \times 6'$) with wire mesh covers. The number of lateral and longitudinal movements made by the subject as it crossed photodiode beams placed in the base of the box were recorded (Labview 2).

Plus-maze. The plus-maze was made of wood and painted black. It contained two open and two closed arms ($20' \times 4'$) extending from a central platform ($4' \times 4'$) and raised $36'$ from the ground. Lights focused upon the maze maintained the open arms at approximately 200 lx and the closed arms from 0 to 10 lx. A VHS camera was suspended from the ceiling above the maze and connected to a video recorder and monitor.

Procedure

Maternal treatments. Pregnant rats in the prenatal stress groups were restrained under bright lights 200 foot candles bright for 45 min, three times daily from days 14 to 20 of gestation. Restraint sessions occurred at varying times each day between 1000 and 1900 h, and were changed by day alternating among one of seven daily schedules, each rat being exposed to all seven schedules throughout the course of treatment. Allopregnanalone (3-alpha-hydroxy-5-alpha-pregnan-20-one, synthesized by Robert H. Purdy, UCSD, San Diego, CA), was suspended in 20% 2-hydroxypropyl-β-cyclodextrin (Research Biochemicals International, Natick, MA) and was administered intraperitoneally (5 mg/kg, IP) twice daily to pregnant rats in the allopregnanolone group at 0930 and 1430 h from days 14 to 20 of gestation. Pregnant rats in the prenatal stress + allopregnanolone group were administered allopregnanalone twice daily (5 mg/kg, IP) at 0930 and 1430 h in addition to restraint treatments administered for 45 min, three times daily according to the same varying schedule as those animals in the prenatal stress treatment. Vehicle (20% 2-hydroxypropyl-β-cyclodextrin) was administered IP to pregnant rats at 0930 and 1430 h from days 14 to 20 of gestation. Control animals were weighed each morning from gestational days 14–20. Otherwise, these animals were left undisturbed in their cages.

Maternal separation. On PN 7, subjects were randomly selected from the litter and removed from the home cage. No more than four pups from each litter were tested, with two males and two females from each litter when possible. The remaining eight pups were left untouched in the homecage with the mother. The pups removed from the cage were placed in a plastic container containing fresh bedding, removed to an adjacent room, and placed on a heating pad so that a littermate "huddle" temperature of 34°C was maintained. After the pups had been separated from their mother for 20 min, one pup was removed from the litter and taken into the adjacent testing room. For 5 min the pup's number of ultrasonic vocalizations were recorded and behavior monitored. All testing was conducted between 1000 and 1130 h and completed within 30 min of testing the first pup. Following testing, pups were decapitated and trunk bloods collected. Bloods from each sex per each litter were pooled.

Activity testing. At 16 and 60 days of age, two males and two females from each litter, previously randomly assigned and ear punched for identification on PN 12, were removed from their home cages and brought to an adjacent testing room. Subjects were placed in the activity boxes for 15 min, after which they were weighed and returned to their home cages. Between each subject the boxes were wiped clean with a damp sponge to erase any odor traces. All testing was conducted between 1330 and 1500 h. Subjects were returned to their littermates at 16 days of age for repeated testing at 60 days of age.

Plus-maze. Two males and two females previously selected and assigned at PN 12 were tested from each litter. At 65–70 days of age, a subject was removed from its home cage in the colony room and brought into a nearby unfamiliar testing room. The subject was placed in the testing room in a white opaque breeding cage for a 5-min acclimation period without the experimenter present. Following this acclimation period, the experimenter, using alternating hands, placed the subject in the center maze facing either the closed or open arms. The subject's activity was videotaped and later coded for entries into the open and closed arms. Entrance into an arm or the center was determined by the movement of the rat's head and front paws into that arm. Following testing, the rat was removed from the room, weighed, and returned to the home cage. All testing was conducted between 1300 and 1530 h.

Plasma Allopregnanolone Analysis

Blood samples were always collected 2 h after the morning injection. After collecting blood, the samples were centrifuged and then stored at −80°C until ready for later analysis. Allopregnanolone extractions were performed by radioimmunoassay by P. Moore, Southwest Foundation for Biomedical Research, San Antonio, TX. Recovery was achieved by a ^{3}H-allopregnanolone standard and the allopregnanolone purified by high-performance liquid (17).

Data Analysis

All data were analyzed using analysis of variance (ANOVA) with sex and treatment condition as between-subjects factors (SuperAnova, Abacus Concepts, Berkeley, CA). Significant main effects were further analyzed using Fisher's Protected LSD tests ($ps < 0.05$ criteria). Because the same subjects were tested for activity at two ages, postnatal day 16 and postnatal day 60, a repeated measures analysis of variance was used to measure performance across both testing sessions with age as the within-subject factor.

RESULTS

Maternal Weight Gain

Analysis of maternal percent weight gain during the experimental treatment (gestational days 14–20) indicated a main effect of treatment, $F(4, 25) = 4.608$, $p = 0.006$. Table 1 shows mean values for pregnancy weight gain during treatment and overall weight -gain during gestation. Post hoc tests indicated significant differences between control dams and three of the other treatment groups: prenatal stress, prenatal stress + allopregnanolone, and vehicle. No significant treatment difference for overall percent weight gain during pregnancy was obtained.

Birth Characteristics

There was no significant main effect of treatment on birth weight and no significant interaction between sex and treatment.

TABLE 1

MEAN PERCENT WEIGHT GAIN (±SEM) BY TREATMENT GROUP FOR THE TREATMENT AND TOTAL GESTATIONAL PERIOD

Prenatal Treatment	Weight Gain GD 14–21 (%)	Total Gestational Weight Gain (%)
Control	24.1 ± 2.0	51.5 ± 4.6
Vehicle	18.2 ± 2.0	41.9 ± 5.4
Stress	16.9 ± 0.4	42.9 ± 2.5
AP	19.7 ± 1.0	46.6 ± 1.9
AP + Stress	14.8 ± 1.9	34.2 ± 6.0

There was, however, a significant main effect of sex on mean litter birth weight, with males weighing more than females, $F(1, 23) = 21.41$, $p = 0.0001$. The number of pups in each litter (5.69 ± 0.38 females and 5.41 ± 0.34 males) was unaffected by prematurity, sex, or treatment. There were no apparent morphological abnormalities in pups from any treatment group.

Developmental Changes in Weight

Significant differences in weight were found throughout development, with effects becoming less clearly pronounced as the animals reached adulthood. Table 2 shows the mean weights of these animals by condition. On postnatal day 7, treatment significantly affected mean weight, $F(4, 135) = 5.841$, $p = 0.0002$, with controls weighing signficantly more than prenatal stress, prenatal stress + allopregnanolone, and allopregnanolone subjects. The combined prenatal stress + allopregnanolone group also weighed less than the vehicle group. Additionally, on postnatal day 12 there was a main effect of treatment, $F(4, 186) = 5.594$, $p = 0.0003$, as well as a main effect of sex, with males weighing significantly more than females, $F(1, 186) = 5.337$, $p = 0.02$. The prenatal stress + allopregnanolone group weighed significantly less than the vehicle, control, and allopregnanolone groups. The Allopregnanolone group's weight was also significantly less than controls, while prenatally stress animals weighed significantly less than both vehicle and control subjects.

Similar patterns in weight were found throughout young adulthood and into adulthood. On postnatal day 16 a marginally significant main effect of treatment, $F(4, 84) = 2.387$, $p = 0.06$, as well as a main effect of sex, $F(1, 84) = 6.261$, $p = 0.01$, were found. The performance of post hocs indicated that the animals exposed prenatally to the combined prenatal stress + allopregnanolone treatment weighed significantly less than all other treatment groups. The prenatally stressed animals were also found to weigh less than vehicle animals by a marginally significant level ($p = 0.07$). Also, as expected, males weighed more than females across all treatment groups. On postnatal day 25 there was once again a main effect of treatment on weight as well as a main effect of sex on weight. At this age, vehicle animals were found to weigh more than all the other treatment groups. There were no other significant differences among treatment groups. On postnatal day 60 a significant main effect of treatment was again detected, $F(4, 80) = 2.858$, $p = 0.03$, with animals prenatally exposed to vehicle again weighing significantly more than all of the other treatment groups. Furthermore, control animals were found to weigh significantly more than those prenatally exposed to allopregnanolone and marginally more than the combined AP + stress group ($p = 0.08$). No significant differences of treatment on weight were found at 65 days of age. On both days 60 and 65, however, there were significant main effects of sex, $F(4, 80) = 5.892$, $p = 0.0001$; $F(4, 83) = 6.202$, $p = 0.0001$, respectively, with males weighing more than females.

Plasma Analysis

There were significant effects of treatment on plasma levels of allopregnanolone at GD 20 in both the dams, $F(3, 7) = 35.72$, $p = 0.01$, and the pups, $F(3, 6) = 9.241$, $p = 0.01$. Figure 1 shows the mean concentrations of allopregnanolone in blood plasma. Pregnant dams in the allopregnanolone-alone prenatal treatment group exhibited significantly greater levels of the drug in their blood plasma on gestational day 20 than the control, vehicle, and stress groups. Allopregnanolone plasma levels of pups exposed to the prenatal allopregnanolone treatment alone were significantly higher than all of the other groups on gestational day 20.

On postnatal day 7, plasma allopreganolone concentrations were again significantly affected by treatment, $F(3, 27) = 2.763$, $p = 0$ .05, with decreased concentrations in pups exposed prenatally to allopregnanolone compared to both controls. The combined prenatal stress + allopregnanolone group also had significantly lower plasma concentrations of allopregnanolone than both control groups.

Ultrasonic Vocalizations

On postnatal day 7 a significant main effect of treatment was found for the mean number of ultrasonic vocalizations

TABLE 2

MEAN WEIGHTS (±SEM), IN GRAMS, FOR ALL TREATMENT GROUPS ON POSTNATAL DAYS 7, 12, 16, 25, 60, AND 65

Age		Control	Vehicle	Stress	AP	Stress + AP
D7	male and female	16.5 ± .17 ($n = 50$)	16.0 ± .29 ($n = 24$)	15.4 ± .18 ($n = 15$)	15.7 ± .55 ($n = 15$)	14.9 ± .21 ($n = 15$)
D12	male	34.0 ± .95 ($n = 21$)	32.5 ± .78 ($n = 27$)	30.6 ± .48 ($n = 20$)	32.6 ± .52 ($n = 16$)	30.3 ± .59 ($n = 16$)
	female	32.4 ± .94 ($n = 19$)	31.8 ± 1.2 ($n = 18$)	30.2 ± .47 ($n = 20$)	30.58 ± .64 ($n = 23$)	29.3 ± .943 ($n = 16$)
D16	male	41.5 ± .85 ($n = 10$)	42.4 ± .97 ($n = 14$)	40.1 ± 1.1 ($n = 8$)	41.5 ± .71 ($n = 10$)	38.6 ± 1.1 ($n = 6$)
	female	40.2 ± .75 ($n = 10$)	39.7 ± 1.5 ($n = 9$)	38.7 ± 1.2 ($n = 8$)	39.7 ± .61 ($n = 13$)	37.3 ± 2.2 ($n = 6$)
D25	male	80.5 ± 1.4 ($n = 21$)	83.0 ± 1.5 ($n = 27$)	79.4 ± 1.6 ($n = 16$)	79.1 ± 1.9 ($n = 12$)	77.2 ± 1.6 ($n = 15$)
	female	73.9 ± 1.1 ($n = 19$)	77.6 ± 2.0 ($n = 18$)	75.0 ± 1.6 ($n = 16$)	72.8 ± 1.1 ($n = 20$)	72.4 ± 1.8 ($n = 16$)
D60	male	350.6 ± 7.8 ($n = 8$)	361.0 ± 7.3 ($n = 11$)	338.3 ± 9.9 ($n = 10$)	344.1 ± 11.7 ($n = 8$)	336.0 ± 10.1 ($n = 8$)
	female	232.9 ± 5.7 ($n = 8$)	236.7 ± 7.3 ($n = 8$)	218.1 ± 4.3 ($n = 10$)	221.9 ± 3.8 ($n = 11$)	218.1 ± 7.9 ($n = 8$)
D65	male	407.4 ± 8.7 ($n = 11$)	385.1 ± 9.3 ($n = 13$)	380.3 ± 10.0 ($n = 10$)	379.3 ± 9.0 ($n = 8$)	370.5 ± 13.6 ($n = 6$)
	female	243.1 ± 7.5 ($n = 8$)	247.3 ± 6.8 ($n = 9$)	242.8 ± 5.9 ($n = 10$)	255.1 ± 5.1 ($n = 12$)	230.6 ± 11.4 ($n = 6$)

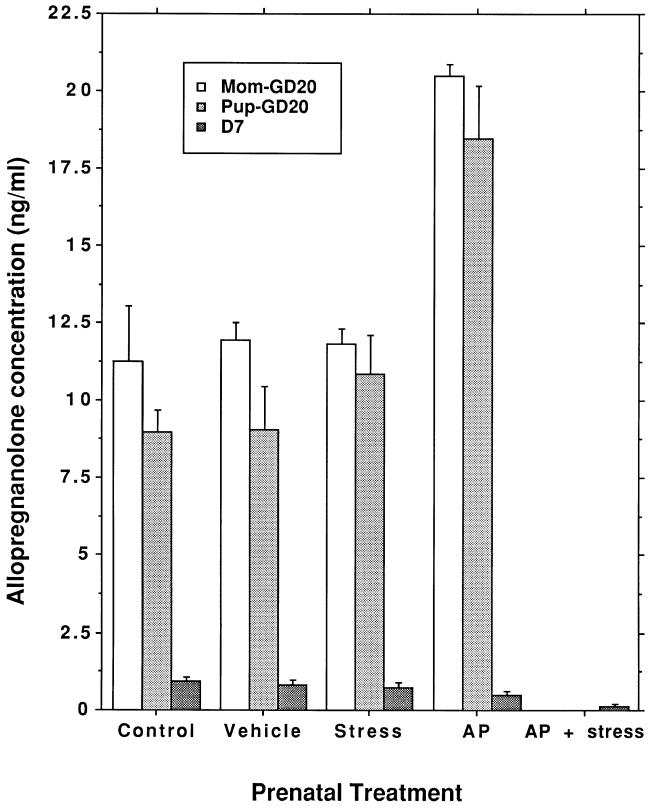

FIG. 1. Mean concentrations (ng/ml) of allopregnanolone (±SEM) in the plasma of pregnant rats and their fetuses at gestational day 20, and offspring pups at postnatal day 7 in five prenatal treatment groups.

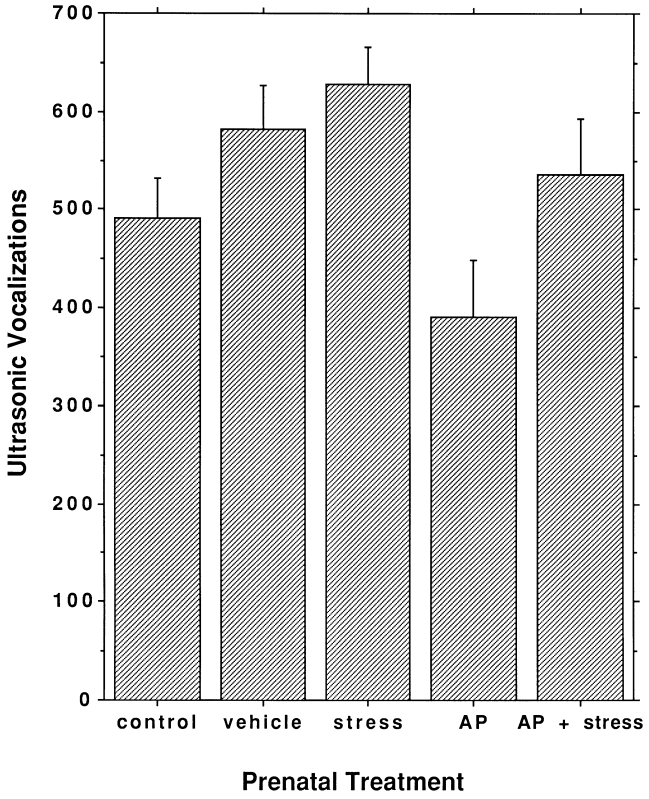

FIG. 2. Mean number of ultrasonic vocalizations (±SEM) at postnatal day 7 in five prenatal treatment groups after a brief maternal separation.

produced by pups during separation, $F(4, 64) = 3.843$, $p = 0.007$ (see Fig. 2). Prenatally stressed animals had a greater number of ultrasonic vocalizations, compared with control and allopregnanolone treated groups. The allopregnanolone prenatal treatment group, on the other hand, had a lower mean number of vocalizations compared to all groups except control animals (only a marginal difference, $p = 0.12$). In addition to changes in ultrasonic vocalizations, a main effect of treatment was found on the amount of time subjects spent inactive during the five minute testing session, $F(4, 55) = 2.882$, $p = 0.03$ (see Fig. 3) There was significantly more inactivity in the vehicle group compared to the other three prenatally treated groups—prenatal stress, prenatal stress + allopregnanolone, and allopregnanolone—while prenatally stressed animals were significantly more active than controls. There were no significant differences between control and vehicle groups.

Activity Testing

The results of the open-field activity tests at both 16 and 60 days of age are shown in Fig. 4. A significant interaction between treatment and age was detected, $F(4, 168) = 3.35$, $p = 0.01$. The combined prenatal stress + allopregnanolone group was less active than controls at 16 days of age but more hyperactive compared to all of the other treatment groups at 60 days of age. Also, the stress treament group was hypoactive compared to controls at 16 days of age, but showed no significant differences at 60 days of age. No main effects of treatment, sex, or an interaction of sex and treatment were found. There was, however, a significant main effect of age, $F(1, 168) = 441.247$, $p = 0.0001$, with animals showing significantly greater activity at 60 days of age (600.17 ± 9.99) than at 16 days of age (205.47 ± 9.36).

A significant effect of activity over the three 5-min testing intervals comprising the 15-min testing period was also found, $F(2, 168) = 37.19$, $p = 0.0001$. Animals during both testing sessions (days 16 and 60) showed habituation to the novelty of the testing environment with decreasing activity in each consecutive period. No significant interaction between period and sex or treatment occurred. However, a significant interaction did occur between period and age, $F(2, 168) = 14.93$, $p = 0.001$, showing that animals habituated more at 60 days of age, decreasing their activity significantly more in each testing period than did animals at 16 days of age.

Plus-Maze

The mean proportion of time spent in the open arms of the plus-maze is shown in Fig. 5. All subjects spent a significantly greater amount of time in the closed compared to the center or closed arms, $F(2, 162) = 1089.875$, $p = 0.0001$. Despite the minimal time spent by subjects in the open arms of the maze, a significant main effect of treatment was found for the percentage of time subjects spent in the open arms, $F(4, 81) = 3.249$, $p = 0.02$. Animals prenatally exposed to vehicle and stress spent significantly less time in the open arms than controls, while the amount of time those prenatally exposed to allopregnanolone spent in the open arms was marginally signifi-

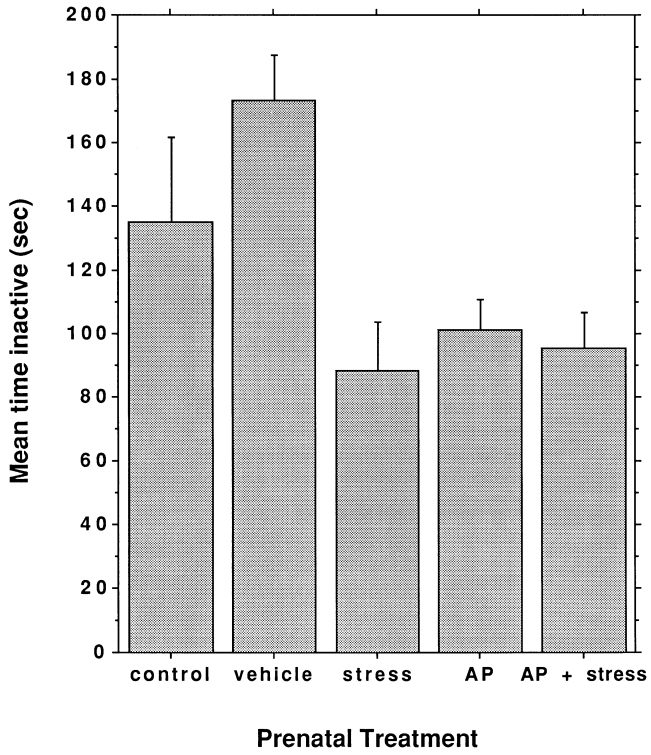

FIG. 3. Mean seconds spent inactive (±SEM) during the 5-min UVS test on postnatal day 7 in five prenatal treatment groups after a brief maternal separation.

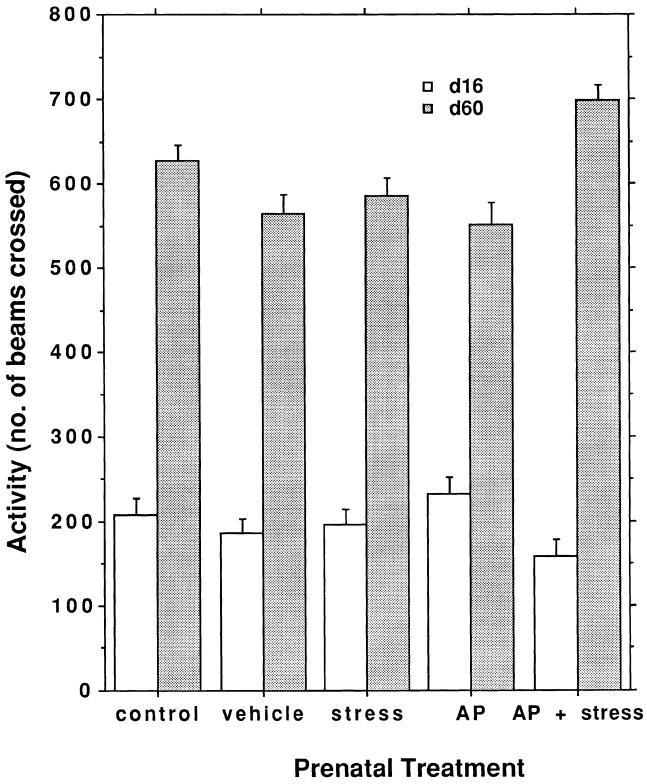

FIG. 4. Mean activity (±SEM) during the 15-min open-field test at postnatal days 16 and 60 in five prenatal treatment groups.

cantly less ($p = 0.09$). Furthermore, the combined allopregnanolone and stress group also spent a significantly greater percentage of time in the open arms than the vehicle, prenatal stress, and allopregnanolone groups. The combined prenatal stress + allopregnanolone group did not, however, differ significantly from the control group. These results were not due to overall activity levels on the maze, as measured by total number of entries into each arm. No significant effects of treatment or sex, or an interaction between the two was found for total activity on the maze. Finally, though there was no interaction between sex and treatment, a marginally significant main effect of sex was discovered, with females spending more time on the open arms than did the males, $F(1, 81) = 2.925$, $p = 0.09$. A significant effect of sex was also found for the percentage of time subjects spent in the center portion of the maze, $F(1, 81) = 5.945$, $p = 0.02$, with males spending more time in the center than did females.

DISCUSSION

The results of this study provide novel information about the effects of administering allopregnanolone to rats during pregnancy, either alone or in combination with prenatal stress. Allopregnanolone when administered by itself during gestation did alter offspring behavior compared to control animals. In conjunction with stress, however, allopregnanolone appeared to return affective behavior towards control levels of functioning. Whether these results reflect an ameliorative effect of neurosteroid administration, or simply that allopregnanolone and prenatal stress have similar mechanisms and thus counterbalance each other at a common site, will need further investigation.

Differences in weight gain for the pregnant dams in the five conditions during the later gestational treatment indirectly indicate that the treatments were likely producing a stress response. Pregnant dams in the prenatal stress, vehicle, and allopregnanolone-alone treatment groups gained less weight than controls, while the combined allopregnanolone and prenatal stress group gained even less weight than the other treatment groups. Dams exposed to any of the treatments appeared to gain more weight on later days of the treatment period, suggesting that they were habituating to the stressors. Furthermore, there were no significant effects of treatment on maternal weight gain over the entire gestational period, indicating some compensation before parturition. In addition, this decrease in gestational weight gain did not affect birth weight. Thus, any behavioral effects resulting from maternal treatment condition were likely not due to any nutritional differences among the groups prenatally, although they may have been affected by postnatal growth retardation. Not only did the offspring not differ in birthweights, but no gross morphological teratologies were observed. Prenatal treatment was also found to have no effect on litter size.

Although no weight differences among treatment groups were discovered at birth, an interesting pattern of postnatal growth was found throughout development. At 7, 12, and 16 (marginally) days of age, pups prenatally exposed to allopregnanolone alone, prenatal stress alone, and prenantal stress and allopregnanolone combined all weighed significantly less than both control and vehicle groups. There were also either marginally significant or significant trends for the combined prenatal stress + allopregnanolone group to weigh less than the allopregnanolone-alone treatment group. As the animals

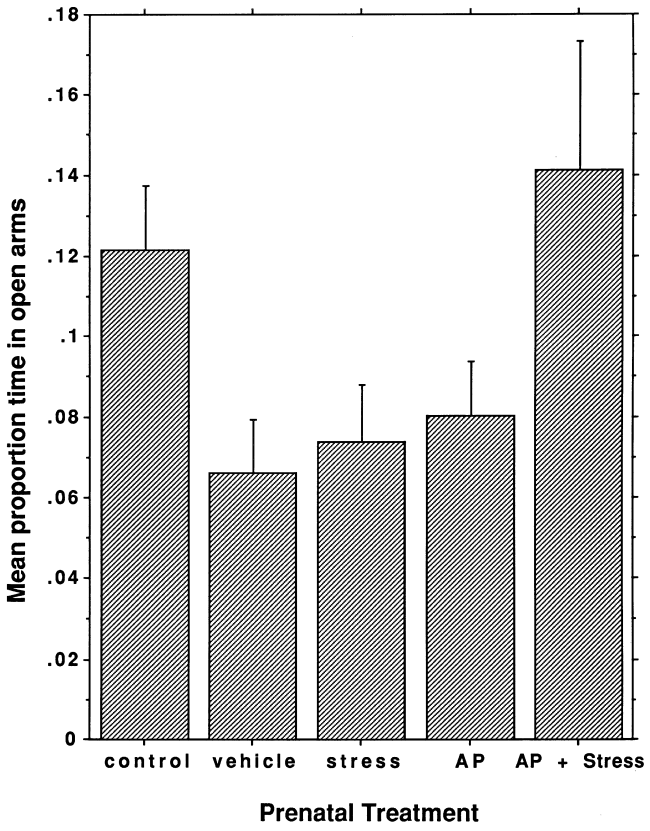

FIG. 5. Mean proportion of time spent (±SEM) in the open arms of the plus-maze in five prenatal treatment groups.

began to mature, these persistent differences in weight appeared to be waning. At 25 and 60 days of age, all groups, including the control group, weighed less than the vehicle-treated animals. The reason for this increase in weight for vehicle animals is unclear. Importantly, however, all of the other prenatal treatment groups did not differ significantly from the other prenatal treatment groups, although a trend still existed for the combined allopregnanolone and stress animals to weigh less than controls. By 65 days of age there were no significant differences in weight among any of the treatment groups.

It is possible that postnatal weight differences among treatment groups before weaning (7, 12, and 16 days) occurred due to impaired maternal behavior. Housing and cost limitations prevented the postnatal behavior of the mother towards her pups from being controlled through crossfostering. Several experimental observations were made of instances of inappropriate maternal care of pups. For example, one allopregnanolone dam was observed to scatter her pups arround the home cage rather than maintaining an appropriate nursing huddle after giving birth. In addition, one vehicle dam did not properly clean her pups after parturtion. However, these observations were not quantified and were noted only for a few litters. Thus, maternal behavior may have been a factor in the altered weight gain in early development.

Another interesting possibility that could be considered as a cause for these weight differences is the inhibition of growth hormone. The stress response has been found to inhibit growth hormone through the action of the pituitary (22). Increased levels of corticosterone have been commonly reported in prenatally stressed animals. Hence, if the HPA axis hyperfunctions under basal conditions in these animals, as has been suggested by Famelli and co-workers (4), growth hormone may be inhibited, thus causing lower weights in the animals prenatally exposed to stress. If injection of allopregnanolone itself also proved to act as a stressor, similar mechanisms would explain the decreased weights in both the allopregnanolone and combined treatment groups.

Plasma levels of allopregnanolone were altered by prenatal treatment in both the pregnant dams, their fetuses, and their week-old offspring. Plasma levels of allopregnanolone in the pregnant rats administered the 10 mg/kg dose of allopregnanolone were highly elevated compared to the pregnant animals in all of the other treatment groups. When measured on gestational day 20, control as well as vehicle and stress groups showed mean concentrations of approximately 11.5 ng/ml compared to allopregnanolone treated animals who showed mean concentrations of 20.5 ng/ml. Levels of allopregnanolone in fetal plasma on day 20 of gestation were also significantly higher than all other treatment groups in drug-treated animals (18.5 ng/ml compared to 9.0 ng/ml in control animals). These results also indicate that plasma levels are comparable in fetal and maternal circulations.

Interestingly, there was a persistent effect of treatment on plasma levels of allopregnanolone in the week-old offspring. Pups who had experienced prenatal administration of allopregnanolone and stress had lower serum neurosteroid levels after a brief maternal separation. There was some suggestion that pups in the prenantal allopregnanolone alone group also had lower levels. These result do indicate that a negative feedback system may be involved in regulating developmental patterns of neurosteroid production.

These results are important because levels in both maternal and fetal blood were significantly elevated above those of the other treatment groups, suggesting that high levels of allpregnanolone were, in fact, present in plasma after its exogenous administration. Furthermore, with levels of the blood almost equal in maternal and fetal blood, it appears that the fetuses were exposed directly to nearly as much allopregnanolone as was their mother. Thus, the effects of allopregnanolone administration on the fetus could be mediated through direct action of the steroid on the fetal brain, rather than solely through maternal mediated factors.

Prenatally stressed animals emitted the greatest number of vocalizations compared to control and allopregnanolone treated animals, while allopregnanolone-treated animals emitted significantly fewer vocalizations than all but the control groups. According to the original hypothesis, such a result suggests an increased stress response to restraint by prenatally exposed animals and a decreased response in allopregnanolone-treated animals. The combined allopregnanolone and prenatal stress-treated animals exhibited a response somewhat like the prenatal stress animals, showing no significant difference from animals prenatallly exposed to stress despite a slightly lower mean number of vocalizations. However, the combined allopregnanolone and prenatal stress group also did not significantly differ from the control group, suggesting that these animals may have performed similarly to controls despite a slightly higher mean number of vocalizations. Also important to note in analyzing the effect the drug had in reversing the prenatal stress, the combined allopregnanolone and prenatal stress group had a significantly greater number of vocalizations than the allopregnanolon-alone group. Therefore, allopregnanolone showed a trend towards reversing some of the

effects of prenatal stress exposure, because animals receiving the combined treatment showed no difference from the control animals.

A "stress-hyporesponsive period" reported by Sapolsky (20) in which corticosterone levels are found not to rise in response to stressors in rat pups until after 14 days of age suggests that some other mechanism produces the stress response. One possibility is that the stress response is mediated during this period by neurosteroids created by diazepam binding inhibitor (DBI) in response to stress. If, as Fride and coworkers (6) suggest, the number of benzodiazepine receptors is reduced in animals prenatally exposed to stress, the efficacy of DBI in producing neurosteroids capable of attenuating the stress response may be diminished. For allopregnanolone-treated animals, on the other hand, sensitivity to DBI may be enhanced through an increased sensitivity of the GABA receptor complex. DBI may, thus, exercise an enhanced ability to combat stress, resulting in a dampened stress response. Following the stress hyporesponsive period when corticosterone production is no longer suppressed it may be that an enhanced responsiveness to CRF in all of these animals eclipses the anxiolytic effects achieved at 7 days old.

The first measure of activity was the amount of time spent inactive during the USV test in 7-day-old subjects. Hyperactivity was observed in the stress, allopregnanolone, and combined groups. These results may reflect an earlier maturation of motor system. Previous studies of prenatal stress (foot shock) have also reported hyperactivity, interpreted as a shift of the peak in arousal to an earlier age mediated by an organizational prenatal effect of corticosterone (21). In contrast to these results, another prenatal stress study reported retarded motor development, although activity levels were not directly assessed (7).

Later tests of activity at older ages largely indicated no differences between any of the prenatal treatment groups. The effect of hyperactivity seems largely to disappear following the early motor maturation evident at 16 days of age and remains the same at 60 days of age. Nevertheless, the combined prenatal stress + allopregnanolone group differed from this general trend, actually showing hypoactivity compared to the control group at 16 days of age and hyperactivity compared to all prenatal treatment groups at 60 days of age. It seems likely that this effect was the result of the increased prenatal stress exposure resulting from the combined treatments. Conflicting findings on prenatal stress exposure report both increased and decreased activity levels (1). Although less severe stress like the allopregnanolone or stress treatments appeared not to influence activity after 7 days of age, a more severe stress may produce results commensurate with previous studies. Although hypoactivity was the initial effect reported for the combined treatment group, the frequent occurrence of overcompensation in organisms as a response to abnormalities in neuronal functioning does not make the change to hyperactivity at 60 days of age surprising.

Just as the combined prenatal stress + allopreganolone group was more active than the other treatment groups at day 60, these animals were also more explorative on the plus-maze. In many ways, plus-maze data produced effects very similar to those of USV data. Like the isolation that produces distress in 7-day-old rat pups, the plus-maze also tests an animal's anxiety level. These two tests assess emotionality, in contrast to the activity test, which traditionally measures spontaneous activity. On the plus-maze, the combined stress and allopregnanolone group spent a significantly greater percentage of time on the open arms of the maze than did the vehicle, allopregnanolone, or stress groups. Importantly, the combined prenatal stress + allopreganolone group did not make more total entries into all of the arms over the testing period than did the other treatment groups. This finding indicates that the increased activity exhibited by the combined treatment group during activity testing at 60 days of age was not responsible for the increased amount of time these animals spent on the open arms.

The control group also proved to spend more time on the open arms than the allopregnanolone or prenatal stress groups by levels only of marginal significance. The control and combined allopregnanolone + prenatal stress group did not differ from each other. In this way, the activity of the combined group appears to have been more "normal" than that of the vehicle, stress, and allopregnanolone groups. This effect can be interpreted in several ways. First, according to the premise that administration of allopregnanolone at sedative doses in this study may have produced an anxiogenic rather than an anxiolytic response, the behavior of the three "anxious" treatment groups can be seen as the commonly reported increased stress responsiveness. If basal levels of corticosterone are raised in these animals and they have a decreased ability to cope with novelty it would make sense for them to spend less time in the open arms than did controls. The behavior of the combined treatment group can then be explained by the more severe nature of their prenatal stress exposure. According to Fameli et al. (4), hyperactivity of the HPA system can lead to its exhaustion in response to challenge. If the animals in the combined treatment group had levels of corticosterone higher than those exposed to the more minor stress, a minor "challenge" or stressor such as that posed by the plus-maze would have produced a stress response of greater magnitude. This response would then result in the exhaustion of the HPA system, a decreased level of corticosterone, and, consequently, a decrease in defensive or anxiogenic behavior such as increased open arm exploration. In this case, such an increase produced an effect that appeared similar to a normal animal's exploratory behavior. Because in a normal situation the animals in the combined treatment group would have exhibited behavior below that of the other prenatal treatment groups, an increase in exploratory behavior resembled that of controls. This group's behavior, therefore, may not be reflective of hyperactivity, but of increased exploration of a novel environment due to decreased emotionality.

Although the expectation was that allopregnanolone would play an anxiolytic role in the modulation of stress, some of these results suggest that it may, in fact, have acted as an anxiogenic agent. Lahti and Bersuhn (14) reported that high doses of phenobarbitol (30 mg/kg) produced anxiogenic rather than anxiolytic effects on rats, as measured by increased levels of corticosteroids. They hypothesized that this effect was probably due to the stressful nature of sedation to rats and found that when extremely large anesthetic doses of phenobarbital were administered to rats, the anxiolytic effects of the drug were restored. In other words, when the rat was unconscious and could no longer sense the sedating effects of the drug, the stressful response to it was abated.

In summary, the pattern that emerges from these results suggests that affective and activity behaviors were differentially affected by the prenatal treatments. In the affective behaviors (distress vocalizations and plus-maze) the hypothesis that allopregnanolone would ameliorate prenatal stress effects was at least partially supprted. The activity results, however, suggest an alternative hypothesis, that the injections of allopregnanolone themsleves were stressful, so that the stress response in the combined prenatal stress + allopregnanolone

group was augmented. Because different neuronal systems subserve these two classes of behaviors, further studies might focus on the mechanism underlying the differential results reported here.

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