Pharmacology Biochemistry and Behavior, Vol. 64, No. 4, pp. 717–724, 1999

0091-3057/99/$–see front matter

ELSEVIER

PII S0091-3057(99)00149-5

# Sex-Dependent Behavioral Effects of the Neurosteroid Allopregnanolone (3α,5α-THP) in Neonatal and Adult Rats after Postnatal Stress

BETTY ZIMMERBERG, SHARON H. RACKOW AND KAREN P. GEORGE-FRIEDMAN

*Department of Psychology, Bronfman Science Center, Williams College, Williamstown, MA 01267*

ZIMMERBERG, B., S. H. RACKOW AND K. P. GEORGE-FRIEDMAN. *Sex-dependent behavioral effects of the neurosteroid allopregnanolone (3α,5α-THP) in neonatal and adult rats after postnatal stress.* PHARMACOL BIOCHEM BEHAV **64**(4) 717–724, 1999.—The neuroactive steroid allopregnanolone (3a-hydroxy-5a-pregnan-20-one, 3α,5α-THP) has been shown to be involved in the central nervous system's response to stress. This experiment investigated whether response to the neuroactive steroid allopregnanolone, a positive modulator of the $GABA_A$ receptor, would be altered in neonatal or adult rats previously exposed to a chronic stressor–daily maternal separation during the first week of life. Subjects were then tested either as neonates or adults. In neonates, allopregnanolone decreased the number of ultrasonic vocalizations after brief maternal separation. Previously separated subjects vocalized less and were less active than controls, but were not more sensitive to allopregnanolone on either measure. In adulthood, subjects with a prior history of maternal separation had a greater grooming response to a novel environment after a 10-min cold water swim test than nonseparated subjects. Allopregnanolone reduced grooming, but, again, there was no difference due to stress history. A significant effect of gender was noted in the adult subjects—females were largely responsible for the effects reported. These results suggest that early maternal separation stress can produce an habituation response in neonates and a long-term sensitization response to later novel stress in adults. However, because the behavioral effects of allopregnanolone were not differentially influenced by this early stress history, the neuroactive steroid/$GABA_A$ receptor complex may not be the major mediator of these early stress sequela. Results indicating that females were more responsive to allopregnanolone than males are discussed in light of previous findings.


3α,5α-THP Allopreganolone Neurosteroid Stress Ultrasonic vocalization Grooming Sex Maternal separation

INTERACTIONS between the mother and infant dyad are integral to the normal growth and development of preweanling mammalian young. Numerous studies focusing on the rat model have demonstrated that disruption of this bond, through acute or chronic maternal separation, is a profound stressor on the young offspring. During early development, the mother is critical in regulating her infant's physiological homeostasis; therefore, disruption of the mother–infant interaction produces diverse changes in the pup's physiological and behavioral functioning, including alterations in heart rate, circadian rhythms, temperature, levels of various circulating hormones, developmental delays, and immune dysfunction (27,46,47,57).

Researchers have studied a variety of neonatal rat responses to maternal separation, and have focused a great deal of attention on the activation of the hypothalamo–pituitary–adrenal (HPA) axis during this aversive early experience (13,20,30,46). The potentiation of this system produces a variety of physiological changes including abnormal circulating levels of corticotropin releasing hormone (CRH), adrenocorticotropic hormone (ACTH), corticosterone (CORT), and various other hormones and neurotransmitters tied with the stress response. Various studies have found that this complex stress system is regulated by particular critical elements of the mother–infant interaction during development (13,19,20,27, 30,46–49). Although this age period (postnatal days 4–14) is remarkable for its low levels of circulating CORT and hypo-

Requests for reprints should be addressed to Betty Zimmerberg, Department of Psychology, Bronfman Science Center, Williams College, Williamstown, MA 01267.

responsiveness to stressors that are effective in adults (44), maternal deprivation is capable of gradual dysregulation of the HPA axis response (30,46).

It has been well established that the early environmental experiences of neonates can exert a significant influence on their subsequent growth and development, and has also been linked to physiological and behavioral long-term consequences (13,17,20,28,33,57). Chronic maternal separation in early life has been demonstrated to result in changes in the adult animal's behavioral and neuroendocrine stress response mechanisms, including heightened anxiety, increased synthesis of hypothalamic CRH, and decreased HPA axis responsiveness to glucocorticoid negative feedback (28,39,40,48). A variety of studies have also focused on the long-term consequences of maternal deprivation on the CORT response (34,47–49). Early exposure to the elevated levels of CORT have been hypothesized to cause a permanent downregulation of these CORT receptors, thereby altering HPA function in the adult as well as the efficiency of its negative feedback loop (34,49).

One neurotransmitter system that has been found to be highly involved in mediating general stress responses in the modulation of the HPA axis is the GABA system (12). The $GABA_A$ receptor contains specific and distinct binding sites for a variety of substances other than GABA, including barbiturates, benzodiazepines, and neurosteroids, all of which act as allosteric modulators of this receptor complex (3,7,31,35). One such neurosteroid is 3α-hydroxy-5α-pregnan-20-one (3α,5α-THP or allopregnanolone), which is among the most potent positive modulators of the $GABA_A$ receptor complex (2,3). Allopregnanolone is produced endogenously in the brain response to a stressor (42). Allopregnanolone, a metabolite of progesterone (29), has been found to act on the $GABA_A$ receptor by increasing both the open channel duration and frequency of channel opening of this complex (11,37).

Several animal models of stress have indicated that allopregnanolone has significant antianxiolytic properties, including the Vogel conflict (2), plus-maze (4,41), and distress vocalization after maternal separation (55). These vocalizations are emitted in the range of 20–50 kHz, and are, therefore, called ultrasonic vocalizations (USVs). Because rats are born without sight, olfaction, or various self-regulatory abilities, these vocalizations serve the critical function of eliciting maternal retrieval and care (14). USV production has been utilized as an indicator in determining the effectiveness of antianxiety agents such as the benzodiazepines and neurosteroids (6,10, 21,40,50,53,55). Marked increases in vocalizations have been found to parallel HPA activation (19), confirming the stressful nature of the maternal separation experience.

Grooming behavior is an animal model that is highly responsive to even mild stress, such as a novel environment (18,36,43), but it has not yet been tested for the effects of allopregnanolone. Grooming has been hypothesized to function as a displacement activity for the fearful rat, essential for the restoration of homeostasis (25). Therefore, if grooming serves the animal by lowering the anxiety produced by a stressful experience, this behavior may be linked to the anxiolytic action of the neurosteroids such as allopregnanolone. Studies have demonstrated that ICV injections of CRH or ACTH both elicit marked increases in grooming behavior, and this effect can be blocked by a coadministration of the anxiolytic diazepam (9,36). Thus, it is quite possible that allopregnanolone would produce similar behavioral changes in response to stress.

Another aspect of the behavioral effects of allopregnanolone that has also not received much experimental attention is the question of sex differences. Although it is clear that females have higher circulating levels of allopregnanolone as well as higher concentrations in several distinct brain regions than males (23,42,54), there are only a few studies that have directly addressed whether exogenously administered allopregnanolone has a differential effect on males and females. For example, females are more sensitive than males to the ability of allopregnanolone to protect against bicuculline-induced seizures during ethanol withdrawal (8). There were no sex differences, however, reported in allopregnanolone modulation of GABA-activated chloride flux (52).

The goals of this study were therefore multifaceted. We hoped to determine whether, like the HPA axis, the neurosteroid–GABA system might be altered by the early stress experience of chronic postnatal maternal separation. This question was investigated by assessing the neonate's and adult's sensitivity to allopregnanolone's antianxiety effects. This study also investigated whether grooming behavior after an acute stress, a 10-min water swim, would be a sensitive test of allopregnanolone's anxiolytic effects. And finally, we tested both male and female adults to elucidate any sex differences in response to both early stress and neurosteroid administration.

## METHOD

### *Subjects*

Subjects were bred in this laboratory from female and male Long–Evans hooded rats (Harlan–Sprague–Dawley, Indianapolis, IN). Pregnant females, determined by presence of a vaginal plug, were individually housed in plastic cages (45 × 25 × 15 cm) in an isolated nursery on a 0700 to 1900 h light–dark cycle, with the temperature maintained at 23°C. Litters were culled to 12 pups on the day following their birth [postnatal day (PN) 0], six males and six females when possible. On PN 2, litters were randomly assigned to one of two postnatal treatment groups: separated and nonseparated. Half of the litters from both treatment groups were then also randomly designated for neonatal testing at PN 8. The other half of the separated and nonseparated litters were allowed to mature until early adulthood and then tested (PN 70–90). At PN 8 all animals were weighed, and temperatures were recorded. Animals to be tested as adults were weaned at PN 25 into same-sex partner groups of two, and placed into hanging wire cages with unlimited access to water and lab chow. All experimental protocols were approved by the Institutional Animal Care and Use Committee.

### *Procedures*

*Maternal separation PN 2–7: Separated.* At around 0900 h, four male and four female pups were removed from the home nest and placed individually in a partitioned plastic cup. Cups were placed in a circulating, heated water bath, maintained at 30°C. Four pups were left with the dam to ensure continued lactation during the separation period and were never tested. After 7 h of separation the pups were returned to the home cage. Every third day, after the 7-h isolation, pups were weighed and rectal temperature was recorded. To determine rectal temperature, the subject was placed on a flat surface, its tail lifted, and a microthermocouple probe inserted gently into the rectum (Physitemp Instruments, Model I-18). Temperatures were recorded from a digital thermometer (Physitemp

Instruments, Model BAT-12) when the display stabilized, typically, within 5 s.

*Nonseparated.* These litters were left undisturbed, with the exception that every third day pups were weighed and rectal temperature was recorded.

*Neonatal USV test.* On PN 8, dams were removed from the home cage and pups were kept in their huddle. The home cage was placed on a heating pad maintained at 34°C. Ten minutes prior to USV testing, pups were randomly assigned to one of four treatments: ICV injection of 2.5 μg/2 μl allopregnanolone, 5 μg/2 μl allopregnanolone, vehicle, or no injection. Allopregnanolone (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); this cyclodextrin was thus also used for the vehicle injection. Two subjects from each litter were represented in each treatment group (one male and one female when possible). After injections, which were staggered to assure exact pretesting intervals, pups were marked on their dorsal surface and placed back into the huddle until the testing period. Noninjected pups were merely marked.

Ten minutes after injection or control marking, pups were placed individually in the center of a (17 × 20 × 20 cm) polyethylene activity box where USVs and active vs. inactive periods were recorded during a 5-min period. USVs were counted 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 silent electronic counter. After testing, pups were weighed and rectal temperature was recorded.

*Adult grooming test.* During early adulthood, between PN 70 and 90, rats from the two postnatal condition histories (separated and nonseparated) were randomly assigned to one of four treatment groups: intraperitoneal injection of 5 mg/kg allopregnanolone, 10 mg/kg allopregnanolone, vehicle, or no injection. Twenty minutes prior to the behavior testing, the animals were weighed, given the appropriate injection, and then placed in a plastic cage for 10 min. After this time, animals were subjected to a 10-min water swim in a container (38 × 23 × 34 cm) filled with approximately 16 liters of room temperature water (20°C). After the 10-min swim, animals were placed in a plastic observation box for 5 min, and grooming behavior was recorded (18). Grooming behavior was defined as paw licking, face washing, body grooming, genital licking, or tail preening. Both the total number of grooming episodes and the duration of grooming per episode were recorded. An additional group of nonseparated postnatal history littermates were also tested at the same time for grooming behavior 20 min after either allopreganolone, vehicle or no-injection treatments, but without the prior water swim.

### *Statistical Analyses*

Data were analyzed by analysis of variance with postnatal condition (separated or nonseparated), sex, and drug condition (allopregnanolone dose, vehicle, or no injection) as the three independent variables. Significant main effects were subsequently analyzed by Student–Newman–Keuls tests with a $p < 0.05$ criterion for significance. Significant interactions between factors were analyzed by Fisher's Protected LSD post hoc means comparison tests, with a $p < 0.05$ criterion for significance. All analyses were performed using SuperAnova (Abacus Concepts Inc., Berkeley, CA).

## RESULTS

### *Neonatal Testing*

The total number of neonatal subjects tested was 72. The number of subjects in each condition per treatment was 8–10 per cell. Three litters were represented in the nonseparated postnatal treatment group, and five litters were represented in the separated postnatal treatment group. There were no sex effects on any behavioral measures, so results were collapsed across sex.

During the week of separations, there was a significant interaction between postnatal treatment and day on body weight, $F(2, 120) = 24.49$, $ps < 0.0001$. Nonseparated subjects weighed significantly more on PN day 2 and 5 of treatment ($ps < 0.01$) (see Table 1). There was also a significant interaction between postnatal treatment and day on rectal temperature, $F(2, 120) = 46.47$, $ps < 0.0001$. Nonseparated pups had significantly higher temperature on PN day 2 and 5 of treatment ($ps < 0.01$) (see Table 1).

On the day of testing, PN day 8, nonseparated pups weighed significantly more than separated neonates, $F(1, 64) = 138.97$, $p = 0.0001$ (see Table 1). There was no effect of drug condition, and no interaction between postnatal treatment and drug condition, on body weight. There was also no correlation between body weight and the number of USVs emitted. On the day of testing, nonseparated pups exhibited a significantly higher body temperature than separated neonates, $F(1, 64) = 6.94$, $p < 0.02$ (see Table 1). There was no effect of drug condition and no interaction between postnatal treatment and drug condition. There was no correlation between body temperature and the number of USVs emitted.

There were significant main effects of both postnatal treatment and drug condition on the number of USVs emitted by the neonate, $F(1, 64) = 6.40$, $p < 0.02$, and $F(3, 64) = 6.57$, $p < 0.001$, respectively (see Fig. 1). Regardless of drug condition, nonseparated pups vocalized more than separated pups. Regardless of postnatal treatment, pups receiving either dose of allopregnanolone emitted significantly fewer USVs than those neonates who did not receive an injection of allopregnanolone. Post hoc analysis revealed that the control and ve-

TABLE 1

MEAN BODY WEIGHTS AND RECTAL TEMPERATURES (± SEM) OF NEONATAL RATS IN EITHER DAILY MATERNAL SEPARATION OR NONSEPARATION CONDITIONS

| Day and Condition | Body Weight (g) | Rectal Temperature (°C) |
|---|---|---|
| PN 2 | | |
| Separated | 8.14 ± 0.10 | 29.59 ± 0.26 |
| Nonseparated | 8.65 ± 0.15 | 35.21 ± 0.20 |
| PN 5 | | |
| Separated | 11.28 ± 0.13 | 28.89 ± 0.33 |
| Nonseparated | 12.69 ± 0.16 | 34.23 ± 0.28 |
| PN 8 | | |
| Separated | 15.54 ± 0.15 | 30.10 ± 0.32 |
| Nonseparated | 17.84 ± 0.20 | 30.70 ± 0.15 |

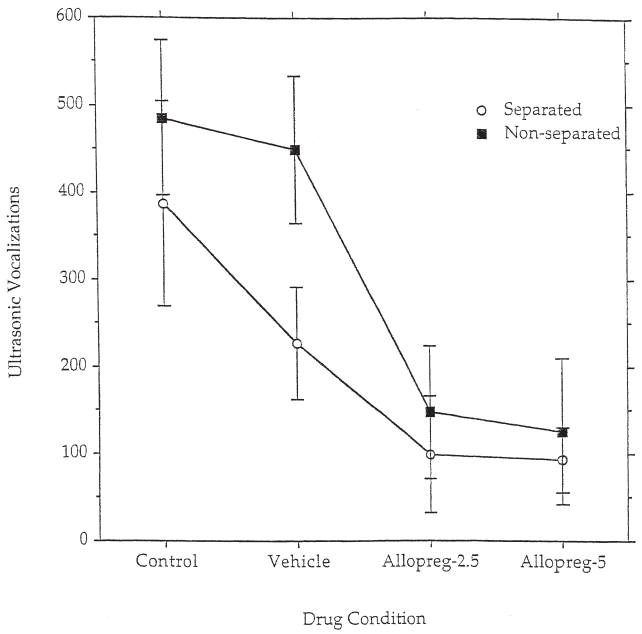


FIG. 1. Mean number of USVs (±SEM) in separated and nonseparated 8-day-old rats after injections of allopregnanolone (2.5 or 5.0 μg), vehicle, or no injection control.

hicle groups did not differ from each other, but did significantly differ from both doses of allopregnanolone ($ps < 0.01$). The two doses of allopregnanolone also did not differ from each other. There was no interaction between postnatal treatment and drug condition.

Analysis revealed a significant effect of drug condition on the neonate's time active, $F(3, 58) = 5.22$, $p < 0.01$ (see Fig. 2). Post hoc analysis revealed that the higher dose of allopregnanolone differed significantly from both control groups as well as the lower dose of allopregnanolone ($ps < 0.02$). The lower dose of allopregnanolone did not differ from either control group, which did not differ from each other. There was no significant effect of postnatal treatment. There was no interaction between postnatal treatment and drug condition. There was no correlation between the number of USVs emitted and the pup's time active.

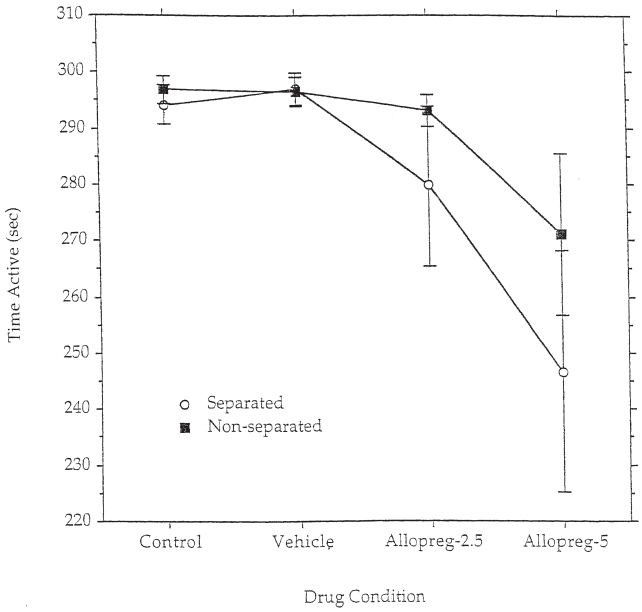


FIG. 2. Mean duration (seconds) of active time (±SEM) in separated and nonseparated 8-day-old rats after injections of allopregnanolone (2.5 or 5.0 μg), vehicle, or no injection control.

*Adult Testing*

The total number of adult subjects tested was 115; *n*s were 6–10 per cell. All adults were tested at 82 ± 1 days of age. Six litters were represented in the nonseparated postnatal treatment group, and seven litters were represented in the separated postnatal treatment group.

There were significant main effects of postnatal treatment and sex on body weight, $F(1, 89) = 6.39$, $p < 0.02$ and $F(1, 89) = 828$, $p < 0.001$, respectively. Female subjects had a mean body weight of 242.67 ± 2.33 g, while male subjects had a mean body weight of 400.1 ± 5.02 g. Postnatally separated subjects weighed a mean of 319.39 ± 12.20 g, whereas postnatally nonseparated subjects weighed a mean of 328.21 ± 12.39 g.

There was a significant main effect of injection condition, $F(3, 77) = 4.33$, $p < 0.01$; allopregnanolone reduced the number of grooming episodes (see Fig. 3). Post hoc analysis revealed that the higher dose of allopregnanolone differed significantly from both control and vehicle groups ($ps < 0.05$), and the lower dose of allopregnanolone differed significantly

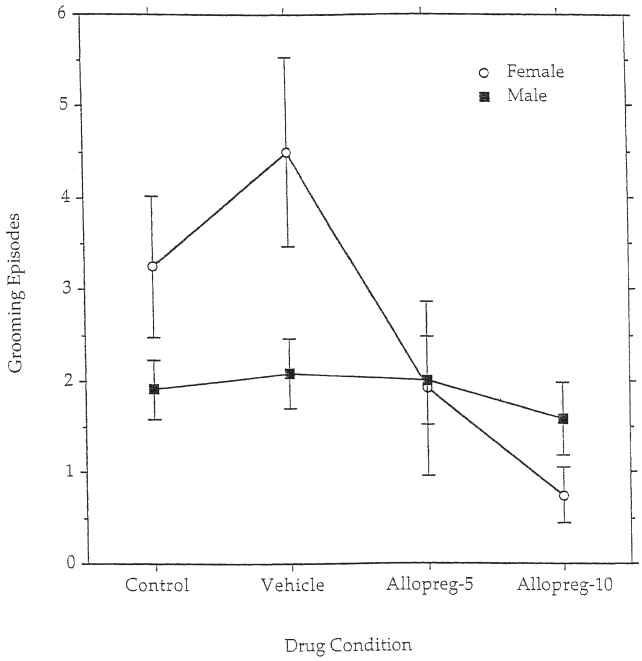


FIG. 3. Mean number of grooming episodes (±SEM) in male and female adult rats after injections of allopregnanolone (5 mg/kg or 10 mg/kg), vehicle, or no injection control tested in a novel environment following a 10 min forced water swim. Data were collapsed across postnatal treatment condition (separated and nonseparated) because there were no significant interactions with that factor.

from the vehicle group ($p < 0.05$). Noninjected and vehicle-injected groups did not differ. However, there was also a significant interaction between sex and injection conditions, $F(3, 77) = 2.79$, $p < 0.05$, qualifying the main effect of injection condition. Females demonstrated a decrease in the number of grooming episodes in response to allopregnanolone, while males did not. Post hoc analysis revealed that among the females, both control groups differed significantly from both doses of allopregnanolone ($ps < 0.05$), but did not differ from each other.

There was also a significant interaction between postnatal treatment and sex on the number of grooming episodes, $F(3, 77) = 5.07$, $p < 0.03$. Females groomed more than males in the separated postnatal treatment group, but not in the nonseparated postnatal treatment group, and only females differed between postnatal treatment groups (see Fig. 4). When duration of grooming per episode was analyzed, there was only a significant main effect of injection condition, $F(3, 77) = 3.22$, $p < 0.03$; allopregnanolone reduced the mean duration per episode at the highest dose (10 mg/kg), regardless of sex or postnatal treatment by more than half; the three other groups did not differ. Finally, there was not any correlation between body weight and grooming behavior.

To determine the effects of the water swim per se on allopregnanolone's modulation of grooming in the novel environment, 22 additional control (nonseparated) subjects were tested without the swim test, and their results compared to those of nonseparated littermates with the swim test. Testing condition did have a significant main effect, $F(1, 54) = 8.59$, $p < 0.005$. Animals that were subjected to the cold-water swim groomed more than animals that did not experience the additional water stressor ($2.20 \pm 0.23$ vs. $0.68 \pm 0.29$ episodes). There was no interaction between injection condition and testing condition; allopregnanolone reduced the number of grooming episodes in both swim and nonswim conditions. There was a main effect of injection condition, $F(3, 54) = 4.40$, $p < 0.01$; allopregnanolone reduced the number of grooming episodes. Post hoc analysis revealed that the higher dose of allopregnanolone differed significantly from both vehicle and control groups ($ps < 0.01$), and the lower dose of allopregnanolone differed significantly from the control group ($p < 0.05$). The two control groups did not differ from each other. Again, when duration of grooming per episode was analyzed, there was a significant main effect of injection condition, $F(3, 54) = 3.40$, $p < 0.03$; allopregnanolone reduced the mean duration per episode at the highest dose (10 mg/kg) regardless of sex or postnatal treatment by 75%; the three other groups did not differ. Regardless of injection condition, testing condition had a significant interaction with sex on the number of grooming episodes, $F(1, 54) = 4.80$, $p < 0.04$. Males groomed significantly more after a water swim than without the swim ($p < 0.001$), while females did not differ by test condition. In addition, males and females only differed in their grooming rate in the nonswim condition ($p < 0.04$), but not in the swim condition.

There was a significant interaction between sex and injection condition on the number of grooming episodes, $F(3, 54) = 3.78$, $p < 0.02$ (see Fig. 5). Among the females, means comparisons revealed that the control groups significantly differed from both allopregnanolone doses ($ps < 0.001$), and the vehicle group differed significantly from the higher dose of allopregnanolone ($p < 0.05$). The two control groups did not

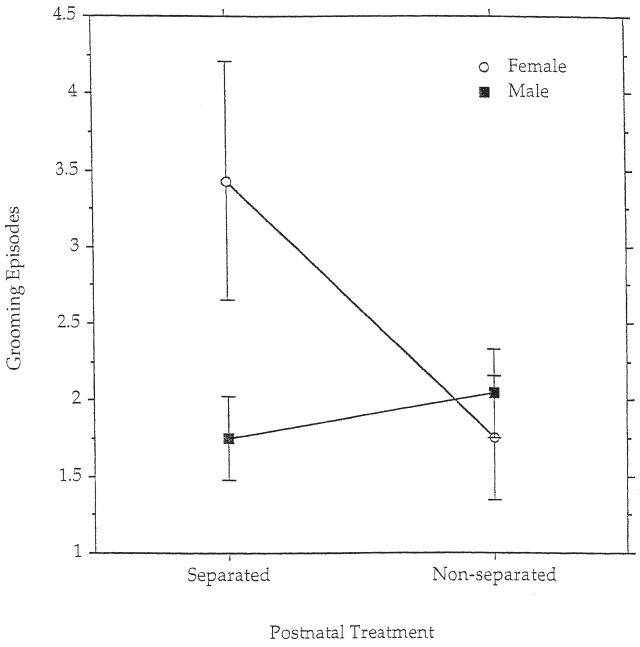


FIG. 4. Mean number of grooming episodes (±SEM) in separated and nonseparated adult rats tested in a novel environment following a 10 min forced swim. Data were collapsed across injection condition (none, vehicle, or allopregnanolone, 5 or 10 mg/kg) because there were no significant interactions with that factor.

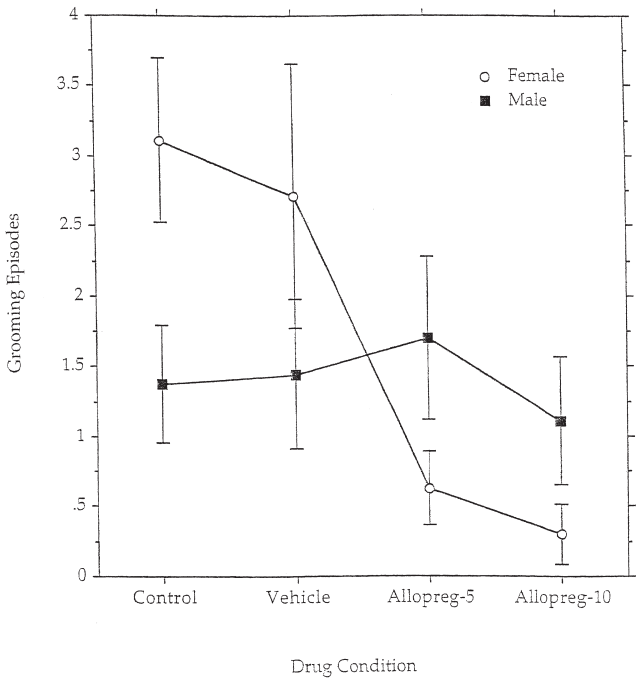


FIG. 5. Mean number of grooming episodes (±SEM) in nonseparated male and female adult rats only after injections of allopregnanolone (5 or 10 mg/kg), vehicle, or no injection control tested in a novel environment. Data were collapsed across testing condition (water swim or no-water swim) because there were no significant interactions with that factor.

differ from each other. Among the males, allopregnanolone did not alter the number of grooming episodes.

## DISCUSSION

The neurosteroid allopregnanolone exhibited anxiolytic effects in both neonatal and adult subjects, demonstrated by a decrease in USV production and a decrease in grooming behavior in a novel environment, either alone or after an additional water stressor. However, among adults, allopregnanolone's effect differed by sex in response to allopregnanolone: whereas females displayed dose-dependent sensitivity to the anxiolytic effects of this neurosteroid, males exhibited no change in grooming behavior. In both age groups, however, there were no interactive effects of a prior maternal separation history; allopregnanolone was anxiolytic in both postnatal treatment history groups. This anxiolytic effect was not secondary to a sedative effect, because the lower dose reduced vocalizations but did not affect activity. Although we had hypothesized that the week of daily maternal separation would have altered the neurosteroid–GABA system so that a differential sensitivity could be observed, there were no interaction effects. In contrast, previous studies have demonstrated differential sensitivity to dopaminergic agents after similar maternal separation paradigms (26,57). It is possible that the measures used in this study were not sensitive to the maternal separation effect, or indeed, that the neurosteroid system is not affected in any long-term way during this period.

Animals exposed to chronic postnatal maternal separation did have a significant subsequent effect on behavior in the two tests, although in adults, the effect of maternal separation was again sex dependent. Maternal separation, a potent stressor (28,34,39,40,49) appears to decrease stress response acutely, but results in a long-term supersensitivity to stress 3 months later. In agreement with these studies, subjects in this study were found to be less responsive to a subsequent separation in the neonatal period when ultrasonic vocalizations were used an index of sensitivity compared to their nonseparated cohorts. This finding is also consistent with that of Goodwin and Barr (14), who found that following chronic maternal separation, neonatal rats demonstrated adaptation to this stressor by producing decreased USV rates as well as activity. Conversely, adult rats subjected to the chronic postnatal maternal separation exhibited an increased sensitivity to a novel stress experience compared to the nonseparated adults. However, it must be noted that females were largely responsible for this effect.

This is the first report that allopregnanolone decreases the number of grooming episodes exhibited by the adult rat in response to a stressful experience. The duration of grooming per episode was also decreased, but only at the higher dose. Grooming is a well-documented behavioral correlate of stress (9,18,25,36). This finding thus supports previous studies that demonstrated the anxiolytic effects of allopregnanolone in adult subjects (2–4,12,38,39). However, the rates of grooming were very low, so there may have been a "basement effect" that prevented us from detecting an allopregnanolone effect in the male subjects. It was interesting that the additional stressor of a water swim test preceding the grooming observation did increase rates of grooming, but only in the males. Previous studies have observed marked increases in serum and brain CORT levels, as well as plasma and brain concentrations of allopregnanolone (1,5,12,16), following exposure to a forced cold-water swim test. Allopregnanolone reduced grooming whether in response to water and novel environment stressors combined, or the novel environment alone. There did not appear to be any influence on this measure from increased levels of circulating neurosteroid, although administration of lower doses might have detected a shift of the dose–response curve to the left in the water swim group.

The postnatally separated neonates weighed significantly less than their nonseparated cohorts during the week of treatment, on the next day when tested for vocalizations, and 3 months later when tested as adults. This surprising long-term effect was true for males and females. In previous studies in this lab, daily maternal separation weight effects were not persistent into adulthood (54,57). However, weight was not correlated with USV production or grooming, so was probably not a casual factor. Similarly, although the postnatally separated neonates also had significantly lower rectal temperatures than the nonseparated pups, temperature did not influence the number of USVs emitted. This finding is supported by an earlier study (21), where body temperature of 2-week-old rats was found to have no relationship with the level of USVs emitted by the pups.

Sex differences in animal models of anxiety behavior have been reported previously. For example, females display less anxiolytic behavior than males on the plus maze test (24,32, 56); however, this sex difference is not consistent across all age groups (22). This sex difference was eliminated by neonatal or pubertal ovariectomy, suggesting a role for circulating hormones both at an organizational and activational level (56). These differences in behavior, however, have not been well correlated with any consistent sex differences in GABA receptor systems. The number of $GABA_A$ receptors as well as benzodiazepine binding sites do not appear to differ by sex or by female hormonal status (45,51,52). Sex differences in grooming behavior have also been reported previously: females were found to have a greater grooming bout duration than males, but no difference in grooming episodes; this sex difference was also only observed in the novel environment condition, and not when a prior swim was added to the test procedure (18).

Differential sex effects were observed in several analyses of the adult results. The females were sensitive to the anxiolytic properties of allopregnanolone, demonstrated by a decrease in grooming episodes, while the males' grooming behavior was not affected by allopregnanolone. Additionally, postnatally separated females exhibited a greater number of grooming episodes compared to nonseparated females and both groups of males, suggesting greater sensitivity to the prior stress. In this laboratory, a similar postnatal maternal separation history led to sexually dimorphic response to a 10-min swim in several neurochemical measures (54). Prior maternal separation in females alone was associated with higher levels of dopamine in the prefrontal cortex compared to nonseparated subjects. Both males and females with a postnatal maternal separation history had higher norepinephrine concentrations in the nucleus accumbens, but not striatum or and hippocampus, compared to nonseparated subjects. It is possible that the increased grooming in the prior stress history females found in this study is a reflection of increased release of dopamine in prefrontal cortex. We have hypothesized that allopregnanolone and dopamine should have a reciprocal relationship (54), because GABA system activation reduces stress activation of the mesolimbocortical dopaminergic pathway (15). If early maternal separation exposes the developing female brain to higher than expected neurosteroid levels, there may be some compensatory response in the number or affinity of GABA receptors such that, as an adult, these fe-

males become hyperresponsive to a stressor as an adult, as seen in both increased grooming and increased dopamine in prefrontal cortex. These indices would be associated theoretically with a decreased release of allopregnanolone or decreased number or affinity of GABA receptors, thus making the females more sensitive to exogenously administered allopregnanolone as reported here. Further studies directly assessing these parameters are needed to answer these questions.

ACKNOWLEDGEMENTS

This research was supported by a grant from the National Institute on Alcohol Abuse and Alcoholism, #R01 AA08605.

## REFERENCES

1. Abel, E. L.: Physiological correlates of the forced swim test in rats. Physiol. Behav. 54:309–317; 1992.
2. Barbaccia, M. L.; Roscetti, G.; Trabucchi, M.; Mostallino, M. C.; Concas, A.; Purdy, R. H.; Biggio, C.: Time-dependent changes in rat brain neuroactive steroid concentrations and $GABA_A$ receptor function after acute stress. Neuroendocrinology 63:166–172; 1996.
3. Biggio, G.; Concas, A.; Mostallino, M. C.; Purdy, R. H.; Trabucchi, M.; Barbaccia, M. L.: Inhibition of GABAergic transmission enhances neurosteroid concentrations in the rat brain. In: Genazzani, A. R.; Petraglia, F.; Purdy, R. H., eds. The brain: Source and target for sex steroid hormones. New York: The Parthenon; 1996:43–62.
4. Bitran, D.; Hilvers, R. J.; Kellogg, C. K.: Anxiolytic effects of 3α-hydroxy-5α[β]-pregnan-20-one: Endogenous metabolites of progesterone that are active at the $GABA_A$ receptor. Brain Res. 561:157–161; 1991.
5. Britton, K. T.; McLeod, S.; Koob, G. F.; Hauger, R.: Pregnane steroid alphaxalone attenuates anxiogenic behavioral effects of corticotropin releasing factor and stress. Pharmacol. Biochem. Behav. 41:399–403; 1992.
6. Carden, S. E.; Hofer, M. A.: Independence of benzodiazepine and opiate action in the suppression of isolation distress in rat pups. Behav. Neurosci. 104:160–166; 1990.
7. Darlison, M. G.; Albrecht, B. E.: $GABA_A$ receptor subtypes: Which, where and why? The Neurosciences 7:115–126; 1995.
8. Devaud, L. L.; Purdy, R. H.; Morrow, A. L.: The neurosteroid; 3a-Hydroxy-5a-pregnan-20-one; protects against bicuculline-induced seizures during ethanol withdrawal in rats. Alcohol. Clin. Exp. Res. 19:350–355; 1995.
9. Dunn, A. J.; Berridge, C. W.; Lai, Y. I.; Yachabach, T. L.; File, S. E.: Excessive grooming behavior in rats and mice induced by corticotropin-releasing factor. In: Colbern, D. L.; Gispen, W. H., eds. Neural mechanisms and biological significance of grooming behavior. New York: The New York Academy of Sciences; 1988:391–393.
10. Gardner, C. R.: Distress vocalization in rat pups a simple screening method for anxiolytic drugs. J. Pharmacol. Methods 14:181–187; 1985.
11. Gee, K. W.; McCauley, L. D.; Lan, N. C.: A putative receptor for neurosteroids on the $GABA_A$ receptor complex: The pharmacological properties and therapeutic potential of epalons. Crit. Rev. Neurobiol. 9:207–227; 1995.
12. Genazzani, A. R.; Salvestroni, C.; Guo, A. L.; Palumbo, M.; Cela, V.; Casarosa, E.; Luisi, M.; Genazzani, A. D.; Petraglia, F.: Neurosteroids and regulation of neurondocrine function. In: Genazzani, A. R.; Petraglia, F.; Purdy, R. H., eds. The brain: Source and target for sex steroid hormones. New York: The Parthenon; 1996:83–91.
13. Gilles, E. E.; Schultz, L.; Baram, T. Z.: Abnormal corticosterone regulation in an immature rat model of continuous chronic stress. Pediatr. Neurol. 15:114–119; 1996.
14. Goodwin, G. A.; Barr, G. A.: Evidence for opioid and nonopioid processes mediating adaptive responses of infant rats that are repeatedly isolated. Dev. Psychobiol. 31:217–227; 1997.
15. Grobin, A. C.; Roth, R. H.; Deutch, A. Y.: Regulation of the prefrontal cortical dopamine system by the neuroactive steroid 3α,21-dihydroxy-5α-pregnane-20-one. Brain Res. 578:351–356; 1992.
16. Guo, A. L.; Petraglia, F.; Criscuolo, M.; Ficarra, G.; Nappi, R. E.; Palumbo, M. A.; Trentini, G. P.; Purdy, R. H.; Genazzani, A. R.: Evidence for a role of neurosteroids in modulation of diurnal changes and acute stress-induced corticosterone secretion in rats. Gynecol. Endocrinol. 9:1–7; 1995.
17. Hall, S. F.: Social deprivation of neonatal, adolescent, and adult rats has distinct neurochemical and behavioral consequences. Crit. Rev. Neurobiol. 12:129–162; 1998.
18. Hannigan, J. H.; Blsnchard, B. A.; Riley, E. P.: Altered grooming responses to stress in rats exposed prenatally to ethanol. Behav. Neural Biol. 47:173–185; 1987.
19. Hennessy, M. B.: Hypothalamic–pituitary–adrenal responses to brief social separation. Neurosci. Biobehav. Rev. 21:11–29; 1997.
20. Hofer, M. A.: On the nature and consequences of early loss. Psychosom. Med. 58:570–581; 1996.
21. Hofer, M. A.; Shair, H.: Ultrasonic vocalization during social interaction and isolation in 2-week-old rats. Dev. Psychobiol. 11:495–504; 1978.
22. Imhof, J. T.; Coelho, Z. M. I.; Schmidtt, M. L.; Morato, G. S.; Carobrez, A. P.: Influence of gender and age on performance of rats in the elevated plus maze apparatus. Behav. Brain Res. 56:177–180; 1993.
23. Janis, G. C.; Devaud, L. L.; Mitsuyama, H.; Morrow, A. L.: Effects of chronic ethanol consumption and withdrawal on the neuroactive steroid, 3a-Hydroxy-5a-pregnan-20-one in male and female rats. Alcohol. Clin. Exp. Res. 22:2055–2061; 1998.
24. Johnston, A. L.; File, S. E.: Sex difference in animal tests of anxiety. Physiol. Behav. 49:245–250; 1990.
25. Kametani, H.: Analysis of age-related changes in stress-induced grooming in the rat: Differential behavioral profile of adaptation to stress. In: Colbern, D. L.; Gispen, W. H., eds. Neural mechanisms and biological significance of grooming behavior. New York: The New York Academy of Sciences; 1988:101–113.
26. Kehoe, P.; Shoemaker, W. J.; Triano, L.; Callahan, M.; Rappolt, G.: Adult rats stressed as neonates show exaggerated behavioral responses to both pharmacological and environmental challenges. Behav. Neurosci. 112:116–125; 1998.
27. Kuhn, C. M.; Pauk, J.; Schanberg, S. M.: Endocrine responses to mother–infant separation in developing rats. Dev. Psychobiol. 23:395–410; 1990.
28. Ladd, C. O.; Owens, M. J.; Nemeroff, C. B.: Persistent changes in corticotropin-releasing factor neuronal systems induced by maternal deprivation. Endocrinology 137:1212–1218; 1996.
29. Lan, N. C.; Gee, K. W.: Neuroactive steroid actions at the $GABA_A$ receptor. Horm. Behav. 28:537–544; 1994.
30. Levine, S.; Huchton, D. M.; Wiener, S. G.; Rosenfeld, P.: Time course of the effect of maternal deprivation on the hypothalamic–pituitary–adrenal axis in the infant rat. Dev. Psychobiol. 24:547–558; 1992.
31. Macdonald, R. L.; Olsen, R. W.: $GABA_A$ receptor channels. In: Cowan, W. M.; Shooter, E. M.; Stevens, C. F.; Thompson, R. F., eds. In: Annual review of neuroscience, vol. 17. New York: Annual Reviews Inc.; 1994:569–602.
32. Marczynski, T. J.; Urbancic, M.: Animal models of chronic anxiety and "fearlessness." Brain Res. Bull. 21:483–490; 1988.
33. Matthews, K.; Wilkinson, L. S.; Robbins, T. W.: Repeated maternal separation of preweanling rats attenuates behavioral responses to primary and conditioned incentives in adulthood. Physiol. Behav. 59:99–107; 1996.
34. Meaney, M. J.; Diorio, J.; Francis, D.; Widdowson, J.; LaPlante, P.; Caldji, C.; Sharma, S.; Seckl, J. R.; Plotsky, P. M.: Early envi-

ronmental regulation of forebrain glucocorticoid receptor gene expression: Implications for adrenocortical responses to stress. Dev. Neurosci. 18:49–72; 1996.
35. Mihic, S. J.; Sanna, E.; Whiting, P. J.; Harris, R. A.: Pharmacology of recombinant $GABA_A$ receptors. In: Biggio, G.; Sanna, E.; Serra, M.; Costa, E., eds. $GABA_A$ receptors and anxiety: From neurobiology to treatment. New York: Raven Press; 1995;17–40.
36. Moody, T. W.; Merali, Z.; Crawley, J. N.: The effects of anxiolytics and other agents on rat grooming behavior. In: Colbern, D. L.; Gispen, W. H., eds. Neural mechanisms and biological significance of grooming behavior. New York: The New York Academy of Sciences; 1988:281–290.
37. Olsen, R. W.; Sapp, D. W.: Neuroactive steroid modulation of $GABA_A$ receptors. In: Biggio, G.; Sanna, E.; Serra, M.; Costa, E., eds. $GABA_A$ receptors and anxiety: From neurobiology to treatment. New York: Raven Press; 1995:57–74.
38. Patchev, V. K.; Hassan, A. H.; Holsboer, F.; Almeida, O. F.: The neurosteroid tetrhydroprogesterone attenuates the endocrine response to stress and exerts glucocorticoid-like effects on vasopressin gene transcription in the rat hypothalamus. Neuropsychopharmacology 15:533–540; 1996.
39. Patchev, V. K.; Holsboer, F.; Almeida, O. F.: Neuroactive steriods and the behavioral and endocrine response to stress. In: Genazzani, A. R.; Petraglia, F.; Purdy, R. H., eds. The brain: Source and target for sex steroid hormones. New York: The Parthenon; 1996:93–101.
40. Patchev, V. K.; Montkowski, A.; Rouskova, D.; Koranyi, L.; Holsboer, F.; Almeida, O. F.: Neonatal treatment of rats with the neuroactive steroid tetrahydrodeoxycorticosterone (THDOC) abolishes the behavioral and neuroendocrine consequences of adverse early life events. J. Clin. Investigat. 99:962–966; 1997.
41. Patchev, V. K.; Shoaib, M.; Holsboer, F.; Almeida, O. F.: The neurosteroid tetrahydroprogesterone counteracts corticotropin-releasing hormone-induced anxiety and alters the release and gene expression of corticotropin-releasing hormone in the rat hypothalamus. Neuroscience 62:265–271; 1994.
42. Purdy, R. H.; Morrow, A. L.; Moore, P. H.; Paul, S. M.: Stress-induced elevations of γ-aminobutyric acid type A receptor-active steroids in the rat brain. Neurobiology 88:4553–4557; 1991.
43. Sachs, B. D.: The development of grooming and its expression in adult animals. In: Colbern, D. L.; Gispen, W. H., eds. Neural mechanisms and biological significance of grooming behavior. New York: The New York Academy of Sciences; 1988:1–17.
44. Schoenfeld, N. M.; Leathem, J. H.; Rabii, J.: Matuaration of the adrenal stress responsiveness in the rat. Neuroendocrinology 31:101–105; 1980.
45. Shepard, R. A.; Nielson, E. B.; Broadhurst, P. L.: Sex and strain differences in benzodiazepine receptor binding in Roman rat strains. Eur. J. Pharmacol. 77:327–330; 1982.
46. Stanton, M. E.; Gutierrez, Y. R.; Levine, S.: Maternal deprivation potentiates pituitary–adrenal stress responses in infant rats. Behav. Neurosci. 102:692–700; 1988.
47. Stanton, M. E.; Levine, S.: Inhibition of infant glucocorticoid stress response: Specific role of maternal cues. Dev. Psychobiol. 23:411–426; 1990.
48. Suchecki, D.; Tufik, S.: Long-term effects of maternal deprivation on the corticosterone response to stress in rats. Am. J. Physiol. 273:R1332–R1338; 1997.
49. Sutano, W.; Rosenfeld, P.; de Kloet, E. R.; Levine, S.: Long-term effects of neonatal maternaldeprivation and ACTH on hippocampal mineralocorticoid and glucocorticoid receptors. Dev. Brain Res. 92:156–163; 1996.
50. Vivian, J. A.; Barros, H. M. T.; Manitiu, A.; Miczek, K. A.: Ultrasonic vocalization in rat pups: Modulation at the g-aminobutyric acida receptor complex and the neurosteroid recognition site. J. Pharmacol. Exp. Ther. 282:318–325; 1997.
51. Wilson, M. A.: Influences of the hormonal milieu on acute and chronic benzodiazepine responses in rats. In: Watson, R. R., ed. Drugs of abuse and neurobiology. Boca Raton, FL: CRC Press; 1992;209–231.
52. Wilson, M. A.; Biscardi, R.: Influence of gender and brain region on neurosteroid modulation of GABA responses in rats. Life Sci. 60:1687–1691; 1997.
53. Winslow, J. T.; Insel, T. R.: The infant rat separation paradigm: A novel test for novel anxiolytics. Trends Pharmacol. Sci. 12:402–404; 1991.
54. Zimmerberg, B.; Brown, R. C.: Prenatal experience and postnatal stress modulate the adult neurosteroid and catecholaminergic stress responses. Int. J. Dev. Neurosci. 16:217–228; 1998.
55. Zimmerberg, B.; Brunelli, S. A.; Hofer, M. A.: Reduction of rat pup ultrasonic vocalizations by the neurosteroid allopregnanolone. Pharmacol. Biochem. Behav. 47:735–738; 1994.
56. Zimmerberg, B.; Farley, M. J.: Sex differences in anxiety behavior in rats: Role of gonadal hormones. Physiol. Behav. 54:1119–1124; 1993.
57. Zimmerberg, B.; Shartrand, A. M.: Temperature-dependent effects of maternal separation on growth, activity, and amphetamine sensitivity in the rat. Dev. Psychobiol. 25:213–226; 1992.