Pergamon

Pharmacology Biochemistry and Behavior, Vol. 47, No. 3, pp. 735–738, 1994

0091-3057/94 $6.00 + .00

0091-3057(93)E0031-X

RAPID COMMUNICATION

Reduction of Rat Pup Ultrasonic Vocalizations by the Neuroactive Steroid Allopregnanolone

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Received 13 September 1993

ZIMMERBERG, B., S. A. BRUNELLI AND M. A. HOFER. *Reduction of rat pup ultrasonic vocalizations by the neuroactive steroid allopregnanolone.* PHARMACOL BIOCHEM BEHAV **47**(3) 735–738, 1994.—Several of the recently characterized neuroactive steroids have been proposed to have anxiolytic effects in behavioral models when subjects were tested as adults. In this experiment, the effects on infant subjects were examined using the isolation distress model of anxiety. The production of ultrasonic vocalizations in week-old rat pups after maternal separation was assessed after ICV injections of vehicle or allopregnanolone (1.25–5 μg), or sham injections. Subjects were also observed for activity and behavioral responses and tested on three measures of sedation. Allopregnanolone caused a dose-dependent decrease in ultrasonic vocalizations, with increasing motor incoordination, ataxia, and turning at the higher doses. Sex differences were not observed for any measure. These results suggest the $GABA_A$ receptor binding site for neuroactive steroids is behaviorally active in neonates as well as in adults, and that the anxiolytic effects of the neuroactive steroids at this site may be dissociable from their sedative effects at low doses.

Allopregnanolone 3α-Hydroxy-5α-pregnan-20-one GABA neuroactive steroids Anxiety
Ultrasonic vocalizations Maternal separation Isolation distress Rats

THE behavioral effects of allopregnanolone (3α-hydroxy-5α-pregnan-20-one), a neuroactive steroid metabolite of progesterone, have been recently well characterized in several behavioral models of anxiety. For example, allopregnanolone reduced anxiety behavior in rats using the elevated plus maze test (2) and in mice using either the lick-suppression or the light–dark transitions test (21). A similar steroid, alphaxalone, was also found to have anxiolytic properties in both the elevated plus maze and in a conflict test (4). The mechanism of action for these anxiolytic effects of allopregnanolone appears to be its positive modulation of a binding site on the $GABA_A$ receptor which is distinct from both the benzodiazepine and barbiturate sites and which increases the duration of the GABA-stimulated Cl^- channel opening (13,14,17). The anxiolytic effects of allopregnanolone can be blocked by GABA receptor anatgonists (2,21). Recent studies demonstrating that swim stress increases brain levels of allopregnanolone (18) and that the anxiolytic effect of progesterone on the elevated plus maze is paralleled by a dose-dependent increase in allopregnanolone in the rat cortex as well as increased GABA-stimulated Cl^- flux in cortical synaptoneurosomes (3) further strengthen the evidence for central mediation of the behavioral effects of allopregnanolone at the $GABA_A$ receptor.

All of these studies, however, have used adult subjects. In this study, we examined the behavioral effects of allopregnanolone in neonatal subjects to determine whether an anxiolytic effect could be detected at one week of age. The test employed here, ultrasonic vocalization production after ma-

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ternal separation [e.g., (10)], is a well-characterized model for testing anti-anxiety agents in young rats [e.g., (16,22)]. For example, several laboratories have demonstrated that benzodiazepines reduce ultrasound production in neonatal rats after separation from their dam and littermates (5,9,11). Benzodiazepine antagonists reverse this ultrasound suppression, and anxiogenic agents, such as pentylenetetrazol, increase ultrasound production (11). Benzodiazepines also inhibit ultrasound production at doses lower than those that produce muscle relaxation and sedation in a tail-holding stress test in neonatal rat pups (8). Thus we assessed activity and motor coordination in addition to ultrasound production to determine whether the effects of allopregnanolone on these behaviors were dissociable. We also evaluated whether there was a sex difference in behavioral response to this neuroactive steroid, since previous studies have detected sex differences in adult subjects with similarly acting agents (6,12,19) and we have reported a sex difference in anxiety behavior on the elevated plus maze (23).

METHODS

Subjects

Subjects (total n = 109) were seven-day-old Wistar rat pups bred in the New York State Psychiatric Institute. Litters were culled to nine pups within 72 h of birth, and subjects were housed with their dams in clear plastic breeding cages in an isolated nursery on a reversed 12-h light–dark cycle and left undisturbed until the day of testing.

Procedure

Twenty minutes before the start of testing, the dam was removed from the home cage and the litter was brought to a nearby room in its home cage and placed on a thermally controlled heating pad maintained at 36.5°C. A subject was then randomly chosen from the litter and randomly assigned to an injection condition, so that order of testing would not be a confounding factor in this experiment. Injection group subjects were administered a 2-μl ICV injection of either vehicle (45% 2-hydroxypropyl-β-cyclodextrin; RBI, Natick, MA) or allopregnanolone (RBI). Allopregnanolone was dissolved in the inert vehicle by sonication for 15 min. Sham subjects received the same handling but no injection. Direct ICV injections are commonly used in unanesthetized seven-day-old rat pups because the skull is not yet calcified and landmarks are easily visible through the skin (22). Thus there were five groups in this experiment, with the following number of subjects in each group: sham (n = 20), vehicle (n = 27), 1.25 μg allopregnanolone (n = 21), 2.5 μg allopregnanolone (n = 22), and 5.0 μg allopregnanolone (n = 19), each group having approximately equal numbers of male and female subjects.

Fifteen minutes after the ICV injection or the noninjection control procedure the pup was brought to a nearby testing room maintained at 22–23°C and placed in a 17 × 20 × 20-cm polyethylene box marked into six equal "squares." Each pup was tested only one time. For the next 6 min the subject's behavior was observed and ultrasounds 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. Activity was monitored by counting the number of squares crossed. After the ultrasound test, the subject's rectal temperature was measured. To determine rectal temperature, the subject was placed on a flat surface, its tail gently 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.

After the ultrasound test, subjects received three standard motor coordination tests to assess the sedative effects of allopregnanolone: righting, negative geotaxis, and cliff avoidance. In the righting test, the subject was placed in a small chamber on 1 in. of hardwood bedding material, held for 1 s on its dorsal surface, and released. The latency for complete righting on all four paws was noted. A subject's failure to right by 3 min was assigned a 180-s cutoff value. In the second test, negative geotaxis, the subject was placed on a 60° inclined wooden plane facing down, and the latency to make a complete 180° turn up the ramp was recorded. A subject's failure to complete the turn by 3 min was assigned a 180-s cutoff value. For the final test, cliff avoidance, the subject was placed with both front paws over the edge of a wooden ledge 8 in. above the counter; the latency for the subject to completely withdraw both paws was recorded. A subject's failure to withdraw by 15 s was assigned a 15-s cutoff value. At the completion of these tests the subject was weighed and returned to its home cage. All testing of any one litter was completed within a 2-h period.

Data Analysis

Data were analyzed by analysis of variance (ANOVA) with sex and condition as between-group factors (SuperAnova, Abacus Concepts, Berkeley, CA). Significant main effects were further analyzed using Newman-Keuls tests. Tests of motor coordination, which had ceiling limits, were analyzed by the nonparametric Kruskal-Wallis test. Chi-square tests were used in tests among differences in proportions.

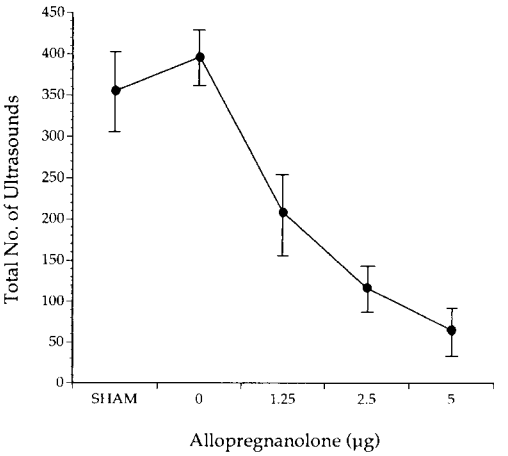

FIG. 1. The mean total number of ultrasonic vocalizations (± SEM) in a 6-min test after either a sham injection or an injection of vehicle (0), 1.25 μg, 2.5 μg, or 5 μg of allopregnanolone in seven-day-old rat pups.

TABLE 1

MEAN ACTIVITY AND MOTOR COORDINATION MEASURES (±SEM) AFTER INTRACEREBROVENTRICULAR ADMINISTRATION OF ALLOPREGNANOLONE IN SEVEN-DAY-OLD RATS

Condition	Squares Crossed	Righting (s)	Negative Geotaxis (s)	Cliff Avoidance (s)
Sham	0.1 ± 0.1	22.1 ± 13.4	127.2 ± 17.8	11.4 ± 1.7
Vehicle (0)	0.3 ± 0.1	14.7 ± 7.6	109.8 ± 14.1	8.2 ± 1.1
1.25 μg	0.2 ± 0.1	20.8 ± 11.7	125.6 ± 14.6	4.5 ± 0.8*
2.5 μg	3.2 ± 0.6†	11.6 ± 8.5	99.7 ± 20.2	5.4 ± 1.1*
5.0 μg	2.7 ± 0.6†	27.8 ± 12.7	103.4 ± 17.8	5.1 ± 1.1*

*Significantly different from sham condition ($ps < 0.05$).
†Significantly different from sham, vehicle, and 1.25-μg conditions ($ps < 0.05$).

RESULTS

ICV injections of allopregnanolone significantly reduced the total number of ultrasounds, $F(4, 99) = 8.62, p < 0.001$, as seen in Fig. 1. All three doses of the neuroactive steroid were significantly different from both the vehicle and sham conditions ($ps < 0.05$), which did not differ from each other. Sex was also analyzed as both a main effect and interacting factor, but there was no effect of sex or any interaction between sex and condition on ultrasound production. The ultrasounds that were produced at the higher doses were often fainter than normal ultrasounds.

Measures of activity and motor coordination are shown in Table 1. There was a significant effect of condition on mean activity, as measured by the number of squares crossed, $F(4, 99) = 13.60, p < 0.001$. The sham, vehicle, and 1.25-μg dose of allopregnanolone all significantly differed from the two higher doses of allopregnanolone ($ps < 0.05$), which did not differ from each other. However, the increased number of squares crossed does not represent an increase in typical forward ambulation. At these two higher doses subjects were observed to have poor motor coordination; they would more often roll over into a new square or back into it than enter a new square by forward movements. Walking backwards, rolling over, turning within a square, continuous lateral head movements, head shakes, and tremors were all observed at these two higher doses. At the lower dose (1.25 μg), chewing, sniffing, turning, and continuous lateral head movements were most often observed, although less frequently than at the higher doses. Control subjects from both groups expressed little activity and infrequently exhibited sniffing, chewing, and small head movements.

On the measures of motor coordination, only cliff avoidance was significantly affected by condition, $H(4) = 12.35$, $p < 0.02$. Subjects receiving either of the three doses of allopregnanolone had shorter latencies to withdraw from the cliff compared to subjects in the sham condition ($ps < 0.05$), but not when compared to those in the vehicle condition. The vehicle and sham groups did not differ from each other. As in the activity measure, this shorter latency did not appear to represent an actual improvement in motor coordination by the drug, but rather an increased expression of backwards movements. Condition also did not affect the proportion of subjects who fell off the cliff or slid down the ramp before the ceiling time limit was reached.

Mean body weights were 16.26 ± 0.25 g for females and 17.17 ± 0.28 g for males; there was no significant effect of injection condition on body weight; males were heavier than females, $F(1, 99) = 4.39, p < 0.04$. Mean rectal temperatures after testing were 32.19 ± 0.14°C for females and 31.81 ± 0.16°C for males. There was also no significant effect of injection condition on rectal temperature, but females did have a significantly higher rectal temperature than males, $F(1, 78) = 4.74, p < 0.05$.

DISCUSSION

Allopregnanolone was found to reduce the production of ultrasound vocalizations in rat pups during a 6-min maternal separation test. Thus this neuroactive steroid has anxiolytic properties in young as well as adult subjects in established animal models of anxiety (2,21). Concurrent studies in another laboratory (20) have also examined the effects of allopregnanolone administered SC on ultrasound production in seven-day-old rat pups, in a 2-min test. At 1–30 mg/kg, allopregnanolone dose-dependently decreased ultrasonic vocalizations, activity, and body temperature. Our results suggest that the decreases in body temperature and activity seen in that laboratory's study are mediated by peripheral mechanisms, since they are not observed with our central administration of low doses of this drug. Similarly, although the subjects were clearly ataxic and at the highest dose sometimes even were unable to maintain an upright position, their level of arousal was increased, as seen by the expression of turning, continuous head movements, body shakes, and tremors. Thus general sedation was not observed at these doses with this route of administration, although ultrasound production was decreased dose-dependently.

We did not detect any sex difference in behavioral response to allopregnanolone; previous studies with this drug have not addressed this issue. Sex differences have been noted for agents with binding sites on the $GABA_A$ receptor. For example, benzodiazepines and barbiturates produce greater hypothermia in females than in males (19); the nonendogenous neuroactive steroid alphaxalone causes greater sedation in females than in males (6); and pentylenetetrazol induces seizures at a lower threshold in males than in females (12). An in vitro study of muscimol, benzodiazepine, and steroid binding sites on the $GABA_A$ receptor did not reveal any sex differences (12). In another study, however, females in estrus did differ from males and from females in diestrus in inhibition of t-butylbicyclophosphorothionate (TBPS, a ligand for the neuroactive steroid binding site) binding at the GABA receptor in the rat cortex, although males and females did not differ in

allopregnanolone's inhibition of TBPS binding in several other brain regions (7). It is not known whether there are any sex differences in allopregnanolone binding in one-week-old crats, but it is not unusual for sex differences in neural function to be "organized" at an early age and only expressed after puberty (1). Although sex differences in quantity of ultrasonic vocalizations in rat pups have not been generally noted in previous studies, qualitative differences in the acoustic features of calls have been reported to be sex-dependent, particularly in males housed with either only males or only females (15).

In summary, the results of this study suggest that the neuroactive binding site detected in the adult rat brain is behaviorally active at one week of age. Further study is necessary to determine whether sex differences in neuroactive steroid modulation of the $GABA_A$ receptor emerge later in development, and which brain regions are critical in mediating the anxiolytic effects.

ACKNOWLEDGEMENTS

This research was supported by a grant from the National Institute on Alcohol Abuse and Alcoholism, #R01 AA08605 (B.Z.) and by grants from the National Institute of Mental Health, #5K05 MH38632 and 5R37 MH40430 (M.A.H.). We thank Jenny Masmela for her excellent technical assistance, Susan E. Carden for her helpful discussions, and James T. Winslow for his assistance in learning the ICV injection technique.

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