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Prenatal Alcohol Exposure Influences the Effects of Neuroactive Steroids on Separation-Induced Ultrasonic Vocalizations in Rat Pups

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ZIMMERBERG, G B. AND B. C. McDONALD. *Prenatal Alcohol Exposure Influences the Effects of Neuroactive Steroids on Separation-Induced Ultrasonic Vocalizations in Rat Pups.* PHARMACOL BIOCHEM BEHAV **55**(4) 541–547, 1996.—Fetal alcohol exposure has been reported to be associated with hyper-responsiveness to stress. Using a maternal separation paradigm, this study examined whether prenatal alcohol exposure affected sensitivity to neurosteroid modulation of stress. We have shown that the neuroactive steroid allopregnanolone reduces ultrasonic vocalizations (USVs) after brief maternal separation in week-old rat pups. Prenatal alcohol exposure, however, resulted in reduced sensitivity to this neurosteroid. In this study's first experiment, the behavioral effects of pregnenolone sulfate, a neurosteroid with reportedly opposite modulatory effects on the $GABA_A$ receptor, were characterized. Pregnenolone sulfate had a triphasic effect on the production of ultrasonic vocalizations and on open field activity. Blockade of conversion of pregnenolone sulfate to allopregnanolone via the 5α-reductase inhibitor 4-MA also blocked the drug-related reduction in USVs. but not the higher-dose augmentation. The enzyme inhibitor alone had no significant effects on USV production, nor did progesterone. These results suggest that the neuroactive steroid pregnenolone sulfate may play an independent role in the stress response after maternal separation as well as being a precursor for the anxiolytic neurosteroid allopregnanolone. In the second experiment, prenatal alcohol exposure was found to eliminate both the low dose USV-reducing effect and the higher dose USV-increasing effect. These results support previous results demonstrating that prenatal alcohol exposure may cause an altered sensitivity to the neuromodulatory effects of neurosteroids. **Copyright © 1996 Elsevier Science Inc.**

Pregnenolone sulfate 5-pregnen-3b-ol-20-one Alcohol neuroactive steroids ultrasonic vocalizations
Maternal separation Fetal alcohol syndrome

WITH increasing awareness of the interactions between stress and cognitive functioning, the role of stress in mediating neurobehavioral teratological outcome requires examination. Animal models have shown that prenatal exposure alcohol affects the stress response mediated by the developing hypothalamic-pituitary-adrenal (HPA) axis (28,29). As adults, alcohol-exposed offspring appear to have a greater HPA activation than control offspring in response to stress (22,27). This hyper-responsiveness is sex-dependent; although these results have typically been demonstrated only in female offspring, under certain stress conditions only alcohol-exposed males show a high corticosterone level for a much longer and more sustained period of time, as compared to pair-fed and control male subjects (30).

The role of the newly-characterized non-genomic behavioral effects of neuroactive steroids in the stress response has recently come under investigation. Some of these "neuroactive" steroids, most notably allopregnanolone (3α-hydroxy-5α-pregnan-20-one) and pregnenolone sulfate (5-pregnen-3β-ol-20-one), have been shown to have a high affinity, selective binding site on the γ-aminobutyric acid ($GABA_A$) receptor (8,23,25). Neuroactive steroids are synthesized in glial cells from cholesterol; this metabolism may be regulated by the "peripheral" (e.g., mitochondrial) benzodiazepine receptor (4,5). While allopregnanolone is a metabolite of progesterone, pregnenolone sulfate is a precursor. Allopregnanolone positively modulates the $GABA_A$ receptor, by increasing the dura-

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tion of GABA-stimulated Cl- channel openings (9,10,14,23). In contrast, pregnenolone sulfate has been reported to negatively-modulate the $GABA_A$ receptor, by reducing the frequency of Cl- channel opening (20) and inhibiting GABA-activated transport of Cl- into synaptoneurosomes and cultured neurons (14,15). Therefore, pregnenolone sulfate, as an "excitatory" neuroactive steroid, should have behavioral effects that are opposite those of allopregnanolone.

Allopregnanolone reduced stress or anxiety-related behaviors in rodents in several different tests at a variety of ages. Allopregnanolone increased the number of open arm entries on the elevated plus maze test in adult female rats (1). The anxiolytic effects of allopregnanolone were also demonstrated in adult male mice using the lick-suppression and light-dark transition tests (31). Recently, this laboratory has demonstrated that allopregnanolone is also anxiolytic in neonatal rat pups, as measured by the reduction in ultrasonic vocalizations after maternal separation (35). In contrast, pregnenolone sulfate has been shown to be anxiogenic in adult mice on the plus maze (19). In addition, pregnenolone sulfate has been found to increase convulsant potency of NMDA in mice (11), and to attenuate barbiturate-induced hypnosis in rats (12).

Opposite effects for these two neuroactive steroids have been also been demonstrated with cognitive tasks. Pregnenolone sulfate enhanced memory performance when infused into the nucleus basalis magnocellularis of rats (17) and blocked a deficit in passive avoidance response caused by the NMDA receptor antagonist CPP (16). Pregnenolone sulfate has also been reported to enhance learning in the Morris water maze task (7). In contrast, post-training administration of allopregnanolone reduced retention in an conditioned odor task in rat pups (36).

Determining whether neurosteroid modulation of the stress response is altered after prenatal alcohol exposure is important in understanding functional deficits in children with Fetal Alcohol Exposure. We have recently reported that alcohol-exposed offspring displayed a dose-dependent shift to the right in their maternal separation USV response to allopregnanolone (36). This decreased sensitivity may indicate that prenatal alcohol exposure causes a decrease in the density of the GABA receptors involved in stress response, resulting in a reduction in the sensitivity to this neurosteroid. The present study investigated whether pregnenolone sulfate, a neurosteroid with a contrasting neurochemical profile, would also show a different dose response pattern in alcohol-exposed subjects. Prior to this study, however, the first experiment characterized the dose-related behavioral response pattern in a model of separation distress in week old rat pups. In addition, the effects of blocking further synthesis of other neurosteroids with the 5α-reductase inhibitor 4-MA after pregnenolone sulfate administration, as well as the effects of the intermediate steroid progesterone alone, were examined.

EXPERIMENT ONE

Methods

Subjects. Subjects were 421 seven-day-old Long-Evans hooded rats (Harlan Sprague Dawley, Indianapolis, IN) bred in our laboratory. Pups were weighed and litters culled to twelve pups on postnatal day one (PN 1), with equal numbers of male and female pups when possible. Pups were then left undisturbed with their dams until PN 7, when they were tested. Each treatment group had a minimum of 9 subjects. There were never more than one male and one female litter representatives in any one treatment group. Pups were housed in an isolated nursery on a 12 h light-dark cycle, lights on at 0700 h.

Apparatus. 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 silent electronic counter. The activity box (18" × 18" × 8") was constructed of white Plexiglas and the bottom was divided with black tape into nine equal squares. Pregnenolone sulfate and progesterone were dissolved in the vehicle 45% 2-hydroxypropyl-β-cyclodextrin (Research Biochemicals International, Natick, MA) and sonicated (Fisher Scientific 550 Sonic Dismembrator) for 30 min. 4-MA (17β-*N*,*N*-Diethylcarbamoyl-4-methyl-4-aza-5α-androstan-3-one) was a gift from Merck Sharp and Dohme Research Labs (Rahway, NJ).

Procedure. On PN 7, pups were taken from their dams to an adjacent room, and placed with their litter mates in a small plastic tub with bedding from the home cage. Pups were then taken to an adjacent testing room, and the tubs were placed on a heating pad so that a litter mate "huddle" temperature of 34° C was maintained. Pups were then left undisturbed for 20 min. After 20 min, one pup at a time was randomly selected and assigned to one of the treatment conditions.

All injections were unilateral intracerebroventricular (ICV), a method commonly used in unanesthetized seven-day-old rat pups because the skull is not yet calcified and landmarks are easily visible through the skin (32). Injections were made using a 30 ga. needle Hamilton syringe (Cole Parmer, Chicago, IL) with a 1 cm needle with a 22° angle sharpened and beveled tip. Pups in the control group were given no injection. Pups in the sham group had the syringe inserted 2 mm into the right or left lateral ventricle, but received no solution. Pups in the vehicle group were similarly injected with 2 μl of 45% 2-hydroxypropyl-b-cyclodextrin. Pregnenolone sulfate doses ranged from 1.25 μg to 160 μg/2 μl. Progesterone doses tested were from 0.625 to 100 μg/2 μl. The 5α-reductase inhibitor 4-MA was administered either alone or in combination with 2.5 or 20 μg/2 μl pregnenolone sulfate at 10 to 40 μg/2 μl. After sham, vehicle or drug injections, subjects were returned to their litter mates for 15 min prior to testing.

For testing, pups were taken to another adjacent testing room maintained at 22–23°C, placed in the center square of the activity box, and their ultrasonic vocalizations (USVs) were counted for 6 min. During this time, the experimenter also counted how many squares of the activity box the pup entered, by noting the number of times a pup moved at least half of its body over one of the lines on the bottom of the box. When a pup had completed testing, it was returned to its litter mates while the rest of the litter was tested. After testing for an entire litter was completed, pups were weighed. Each litter was tested within two and a half hours. The order of testing of various doses and drugs were randomly varied to avoid any separation time confounds.

Another group of subjects were tested similarly, but had their rectal temperatures assessed at three time points: immediately before injection, immediately before testing (15 min after injection) when removed from the huddle and taken off the heating pad, and immediately after testing (21 min after injection). Subjects were randomly assigned to one of five groups: procedural control with no injection; vehicle injection

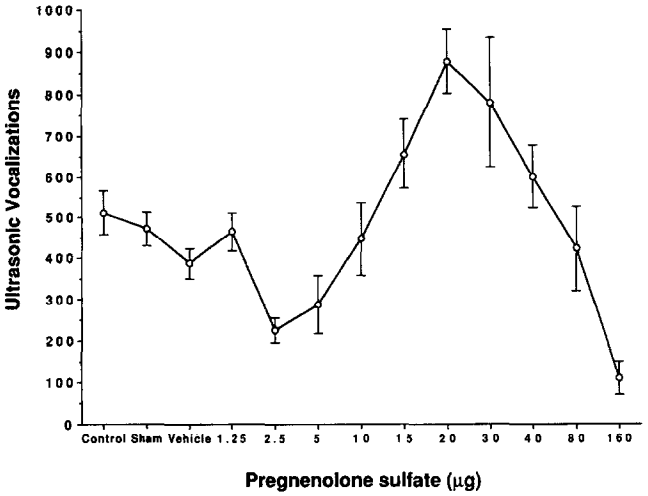

FIG. 1. The mean number of ultrasonic vocalizations (± SEM) following maternal separation during a six minute test period for seven day old rat pups receiving no injection (Control), an i.c.v. injection of Vehicle, or i.c.v. injections of pregnenolone sulfate 15 min prior to testing.

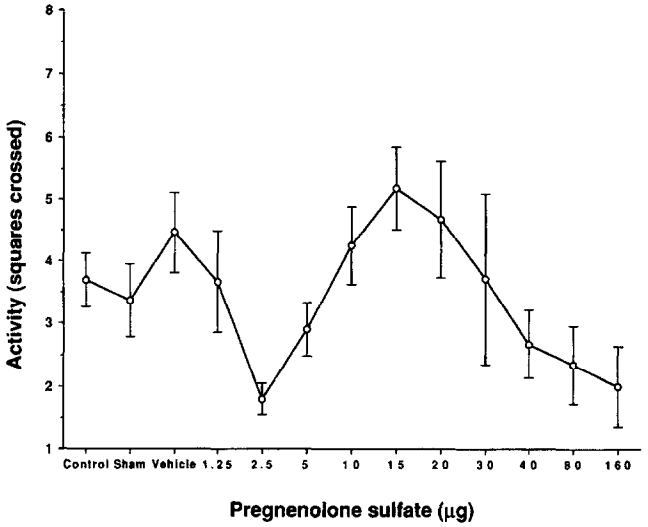

FIG. 2. The mean number of activity counts (± SEM) following maternal separation during a six minute test period for seven day old rat pups receiving no injection (Control), an i.c.v. injection of Vehicle, or i.c.v. injections of pregnenolone sulfate 15 min prior to testing.

(45% 2-hydroxypropyl-β-cyclodextrin); or one of three doses of pregnenolone sulfate (2.5, 15, or 160 μg/2 μl). To determine rectal temperature, the subject was placed on a flat surface, its tail gently lifted, and a micro-thermocouple probe inserted gently into the rectum (Physiotemp Instruments, Model I-18). Temperatures were recorded from a digital thermometer (Physitemp Instruments, Model BAT-12) when the display stabilized, typically within 5 s.

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, p's $< .05$.

RESULTS

Pregnenolone sulfate first decreased and then increased production of USVs after brief maternal separation, $F(12, 182) = 8.54, p < 0.001$ (see Fig. 1). The mean number of USVs after the 2.5 μg dose differed from those of the control, sham, vehicle, 1.25, and 15–40 μg dose groups. The mean number of USVs after the 5.0 μg dose differed from control, sham and 15–40 μg dose groups. The mean number of USVs after the 20 μg doses also differed significantly from the control, sham, vehicle, 10, 15 , and 40–160 μg doses; the 30 μg dose had a similar pattern but did not differ from the 40 μg dose group. Finally, the mean number of USVs after the 160 μg dose differed from all other groups except the 2.5 and 5.0 μg dose groups. The behavioral effects seen at the highest dose are may have been due to non-specific competing behavior and not a reduction in stress response: the subjects were circling, tremoring, and vocalizing audibly at this dose. In addition, the mean number of USVs of females was significantly higher than that of males, with means (± SEM) of 497.2 ± 31.2 for females and 395.1 ± 28.4 for males, $F(1, 182) = 4.32, p < 0.05$. There was no significant interaction of Sex and Dose.

Pregnenolone sulfate also had a similar pattern of alterations in open field activity, $F(12, 182) = 2.93, p < 0.01$ (see Fig. 2). The mean number of squares crossed for the 2.5 μg dose differed from those of the control, sham, vehicle, 1.25, 10–20 μg doses groups. The mean number of squares crossed for the 15 μg dose also differed significantly from the control, sham, 5, and 40–160 0 μg doses groups. The highest dose also differed significantly from the vehicle injection group. No significant effects of Sex or interaction of Sex and Dose were detected.

In the second part of this experiment, a two-way ANOVA tested the effects of the enzyme inhibitor 4-MA both alone and in combination with pregnenolone sulfate. There was a significant main effect of 4-MA dose condition, $F(3, 117) = 3.80, p = 0.01$ (see Fig. 3). All three doses of 4-MA resulted in significantly higher USVs than the vehicle injection. Post-hoc means comparison tests revealed that the number of USVs

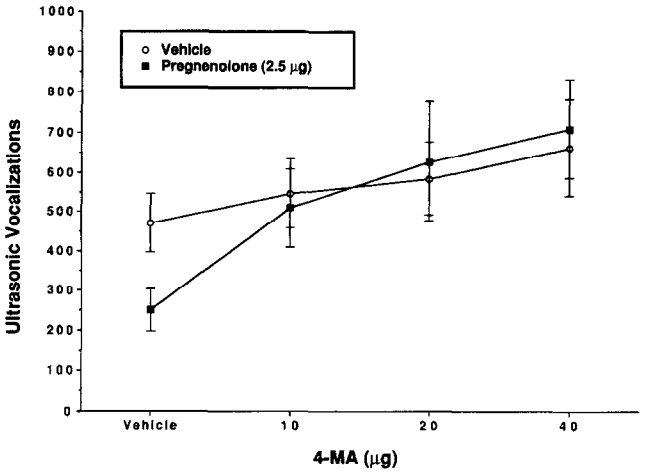

FIG. 3. The mean number of ultrasonic vocalizations (± SEM) following maternal separation during a six minute test period for seven day old rat pups receiving i.c.v. injections of the 5α-reductase inhibitor 4-MA either alone or in combination with 2.5 μg/2 μl pregnenolone sulfate 15 min prior to testing.

produced after the 2.5 μg pregnenolone sulfate dose and vehicle injection was significantly lower than the three combined pregnenolone sulfate and 4-MA doses (p's < 0.05). When 20 μg of 4-MA was given in combination with 20 μg of pregnenolone sulfate, there were no significant differences between these two dose conditions (mean USVs of 644.5 ± 78.6 20 μg of pregnenolone sulfate with 4-MA compared to 661.8 ± 86.3 without 4-MA compared to 472.0 ± 74.1 for vehicle). In the final study in this experiment, no significant differences in USVs were detected among the eight doses of progesterone tested compared to non-injected, sham injected or vehicle injected controls. Pregenolone sulfate had no effect on rectal temperatures either 15 or 20 min after injection (there was no interaction between Condition and Time, $p = 0.4$). However, there was a significant effect of time alone, $F(2, 34) = 20.80$, $p < 0.001$, with the third reading differing significantly from the first and second readings, which did not differ from each other. Mean rectal temperatures at 0, 15 and 21 min were 34.77 ± 0.17, 34.59 ± 0.16 and 33.54 ± 0.53, respectively. There was no relationship between body temperature at the end of testing and the number of USVs in any condition or dose.

DISCUSSION

Pregnenolone sulfate was demonstrated to have an effect on the production of USVs in our stress paradigm of brief maternal separation. A triphasic dose-response curve was detected; the initial decrease in USVs seen at the lowest doses may have been due to conversion of pregnenolone sulfate to allopregnanolone, since this effect disappeared after the conversion was blocked by 4-MA. In addition, blocking the 5α-reductase enzyme by itself led to an increase in USVs. The intermediate step in allopregnanolone synthesis, progesterone, alone had no behavioral effects on USV production.

Changes in body temperature due to maternal separation was probably not a confounding effect in this experiment. In a separate group of subjects, pregnenolone sulfate was found to have no effect on rectal temperature, and, within treatment groups, no relationship was detected between the rate of USV production and rectal temeprature. Since the subjects were kept in a litter huddle and on a heating pad, it is not surprising that rectal temperatures did not change over the 15 min waiting period. Although mean rectal temperatures did drop 1.04°C during the 5 min testing session, there was no differential effect of drug condtion, even at a very high dose (160 μg/2μl). These results parallel those of the previous USV study with allopregnanolone (35), in which no effect of that neurosteroid on body temperature could be detected.

Since prenatal alcohol exposure differentially affected the dose response to allopregnanolone, the next experiment repeated the above experiment using three prenatal treatment groups (alcohol-exposed, pair-fed, and standard lab chow controls) to determine whether a differential dose response would also be apparent after pregnenolone sulfate administration.

EXPERIMENT TWO

Methods

Subjects. Subjects were 113 seven-day-old Long-Evans hooded rats bred in our laboratory from 34 dams (Harlan Sprague Dawley, Indianapolis, IN). After acclimation to the laboratory, females were placed individually with a male in the late afternoon, and the bedding under their cages examined for the presence of a vaginal plug the next morning (gestational day 1; GD 1). If a plug was detected, the female was weighed, individually housed in a standard plastic breeding cage in an isolated room maintained on a 12:12 light/dark cycle, with lights on at 6:00 AM. Each pregnant female was randomly assigned to one of the three prenatal treatment groups: control, pair-fed, or alcohol. Females in the control group had continuous access to standard laboratory rat chow pellets and water throughout their pregnancies. Pregnant females in the other two groups were treated identically to the control groups on GD 1 to 5. Starting on GD 6, pregnant females in the alcohol treatment condition were given a liquid diet containing 6.7% ethanol (Bioserv Liquid Diet F1265, Bioserv, Inc., Frenchtown, NJ). This diet provided 35% of the total caloric content as ethanol. In the nutritional control group ("pair-fed"), pregnant females on GD 6 began receiving a similar liquid diet (Bioserv Liquid Diet F1264), except that the ethanol was replaced isocalorically with maltose-dextrin mixture. A pair-feeding procedure was utilized to control for caloric intake. Each female in the pair-fed group was fed the mean amount consumed by the alcohol group females calculated over the previous years' breeding histories on a ml/kg body weight basis, for each specific day of pregnancy. Thus, pair-fed dams received the same relative volume of diet (ml/kg) and thus the same number of calories on a body weight basis, the only difference being the presence or absence of alcohol. Diets were presented at 1700 h. On GD 20, liquid diets were replaced by continuous access to lab chow and water, and the breeding cages were checked several times daily for births.

Twenty-four hours following parturition (PN 1), dams were removed from the nest, and the litters were weighed, sexed and culled to twelve pups, with equal numbers of male and female pups when possible. Pups were then left undisturbed with their dams until PN 7, when they were tested.

Apparatus and Procedure. In this experiment, only USV production was assessed. All testing was conducted as described in Experiment One. Doses of pregnenolone sulfate injected i.c.v. were 2.5, 10, 20 and 40 μg/2μl. Again, the order of testing of various doses and drugs were randomly varied to avoid any separation time confounds. All data were analyzed using analysis of variance (ANOVA) with prenatal condition, sex and treatment condition as between-subjects factors (SuperAnova, Abacus Concepts, Berkeley, CA). Significant main effects were further analyzed using Fisher's Protected LSD test, p's $< .05$, and significant interactions by post-hoc means comparison F tests (SuperAnova).

Results

The mean daily alcohol consumption by the alcohol dams was 12.14 ± 0.58 g/kg/day. There was a significant effect of prenatal treatment on percent gestational weight gain, $F(2, 31) = 9.34$, $p < .001$ (24.7% ± 1.92, 35.57% ± 2.83 and 46.16% ± 4.35 for the alcohol, pair-fed and lab chow groups, respectively). All three groups differed significantly from each other. There was no effect of prenatal treatment on the total number of pups born, but birth weights did differ significantly by prenatal treatment, $F(2, 31) = 5.21$, $p = 0.01$. Mean birth weights were 6.38 g ± 0.11, 6.98 g ± 0.09 and 7.17 g ± 0.16 for the alcohol, pair-fed and lab chow control pups, respectively. The alcohol pups weighed significantly less than the pair-fed and control pups, who did not differ from each other. There was no longer a significant effect of prenatal treatment on the body weights of the subjects tested at PN 7. Mean body weights at PN 7 were 15.69 g ± 0.24, 16.17 g ± 0.38 and

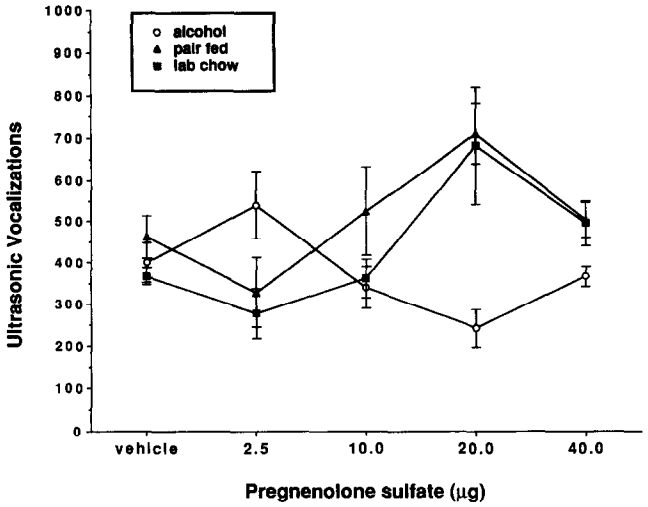

FIG. 4. The mean number of ultrasonic vocalizations (± SEM) following maternal separation during a six minute test period for three prenatal treatment groups (alcohol, pair-fed and lab chow control) receiving an i.c.v. injection of Vehicle or i.c.v. injections of pregnenolone sulfate 15 min prior to testing.

15.38 g ± 0.48, for the alcohol, pair-fed and lab chow control pups, respectively.

There was a significant interaction between Prenatal Condition and Treatment Condition on the production of USVs, $F(8, 83) = 2.91$, $p < 0.01$ (see Fig. 4). Subjects from the prenatal alcohol exposure group had a significantly greater mean number of USVs after the 2.5 μg dose of pregnenolone sulfate than either pair-fed or lab chow control subjects, and a significantly lower mean number of USVs after the 20 μg dose. However, in the prenatal alcohol exposure group, the number of USVs at these doses did not differ from the effects of vehicle injection alone.

In this smaller group of subjects compared to the first experiment, there was no significant effect of sex alone, although the mean number of USVs was again greater in females (455.5 ± 219.9) compared to males (402.8 ± 181.6). Sex did not interact with any other factor.

DISCUSSION

Pregnenolone sulfate has a multiphasic dose response curve for both USVs and activity. At low doses it caused a decrease in vocalization response to maternal separation. This result is similar to that seen after administration of the neurosteroid allopregnanolone (35). Since pregnenolone sulfate is further synthesized into progesterone and then to allopregnanolone, this low dose effect might be mediated by either of these two steroids. We directly addressed that hypothesis: progesterone does not appear to be a likely candidate, since it had no effect on USVs alone. Blocking conversion of pregnenolone sulfate to allopregnanolone, however, did reverse the anxiolytic effect; indeed, there was a significant increase in vocalizations after the low dose and enzyme inhibitor combination. However, the anxiogenic effects at the higher doses were not reversed by the enzyme inhibitor, suggesting that pregnenolone sulfate is the active neuromodulator for that effect. The activity measure also demonstrated a similar dose-response pattern. Multiphasic pregnenolone sulfate dose-response curves, with only some doses causing significant effects, have been reported in mice in both open field activity (18) and plus maze tests (19). The reduction in USVs seen at the highest doses were probably due to non-specific competing behaviors: the subjects were circling, tremoring, and vocalizing audibly at 80 and 160 μg.

There are several possible explanations for the multiphasic dose-response curves seen with pregnenolone sulfate which will require further study. The effects may be due to the steroid's complex effects at its multiple binding sites on the $GABA_A$ receptor (13,24). Pregnenolone sulfate binds to several populations of sites at the $GABA_A$ receptor, and these sites are distinct from the picrotoxin recognition sites at the same receptor (13). Pregnenolone sulfate also interacts with the NMDA receptor, serving as a positive allosteric modulator of that receptor's activity (3,34). Pregnenolone sulfate acts to augment NMDA mediated increases in calcium levels in hippocampal neurons, suggesting that its excitatory properties may be due in part to its action at this receptor (6). Since functional antagonists of the NMDA receptor reduce the number of USVs produced by rat pups (33), some complex interactions of neurosteroids at the NMDA receptor might explain these results.

This is the first report of sex differences in USVs (females males). There may be strain differences, or the relatively large number of subjects may also account for the detection of a sex difference in this study compared to studies in other laboratories. A previous study has found that manipulating the number of male or female pups in a litter will affect the duration and amplitude of USVs at some days of age during the first few weeks of life; the primary finding was that male pups with female litter mates are more "vigorous" in their USV production (21). In this study, equal number of males and females were present in most litters, and only the quantity, not quality of sound was assessed.

The second experiment utilized the information garnered in Experiment One to test the hypothesis that prenatal alcohol exposure would alter the behavioral sensitivity to neurosteroid administration. Prenatal alcohol exposure did affect the dose-response pattern in the production of USVs. The low dose effect is comparable to the results of our previous study (36); prenatal alcohol shifted the allopregnanolone dose-response curve to the right, suggesting fewer $GABA_A$ receptor binding sites. Since this low dose effect was the one eliminated by 4-MA in Experiment One, future in vitro studies on allopregnanolone binding sites in various brain regions in prenatal alcohol-exposed offspring might detect both the developmental pattern and regional specificity of this neurobehavioral teratological effect. Only one preliminary in vitro study to date has explored the effects of neurosteroids on GABA function after prenatal alcohol exposure. In adult offspring, prenatal alcohol exposure eliminated the increase in GABA-mediated Cl- influx after alphaxalone (a positive neurosteroid modulator) in the medial frontal cortex, but not in the cerebellum, and increased GABA-mediated Cl- influx in the hippocampus (26). Only a few studies have been conducted on the effects of prenatal alcohol exposure on developing GABA systems; these studies only examined GABA concentration and found either no effect of prenatal alcohol exposure or an increase in neurotransmitter levels (2). If an alcohol-related increase in GABA concentration in the perinatal period was paralleled by a compensatory decrease in receptor density, this might explain the decreased sensitivity to allopregnanolone and pregnenolone sulfate because the number of steroid binding sites would be diminished as well.

Prenatal alcohol exposure also had an effect on pregneno-

lone's increase in USV production at mid-range doses. In the previous in vitro study, the only brain region differentially affected by prenatal alcohol exposure was the medial frontal cortex; pregnenolone's suppression of GABA-mediated Cl- influx was attenuated in alcohol-exposed adult offspring (26). Although there was not a significant increase ($p = 0.10$) in the rate of USV production after the lowest dose tested, it is possible that the dose-response curve was shifted to the left, suggesting greater sensitivity to pregnenolone's anxiogenic effects. This effect may be mediated by a different subpopulation of $GABA_A$ receptors, $GABA_A$ receptors in different brain regions, or a pregnenolone sulfate binding site on an entirely different receptor such as the NMDA receptor.

Although not significant, there was some suggestion that regardless of drug treatment, pair-fed subjects vocalized more than control subjects, although they did not show a differential drug response compared to controls. The previous allopregnanolone study did find significantly greater production of USVs in pair-fed subjects (36). Other neurobehavioral teratology studies using this liquid diet paradigm for alcohol administration have also reported pair-fed effects, particularly when testing offspring stress response (e.g. 29,30). Observations of the dams during the diet administration typically report that the dams receiving the alcohol diet sip their diet slowly over the 24 h period, while the pair-fed dams tend to drink their non-alcohol diet rapidly and fully when administered. The alcohol may provide some reduction of the stress associated with the liquid diet administration and altered circadian rhythms of feeding that is not available to the pair-fed group. The increased number of USVs in the pair-fed group relative to lab chow and alcohol subjects in both of these studies suggests that pair-fed subjects have an elevated baseline level of distress after maternal separation.

In conclusion, the first experiment has added evidence to the growing literature that non-genomic effects of steroids are involved in the stress response in the central nervous system. Production of USVs after brief maternal stress provides a sensitive and simple animal model to determine stress effects in young animals after teratological insult. As in mice, pregnenolone sulfate can be either anxiolytic or anxiogenic, depending on the dose, in rats pups at one week of age. The second experiment determined that prenatal alcohol exposure altered the behavioral response to pregnenolone sulfate in addition to the previous demonstration of altered response to allopregnanolone (36). The role of neurosteroids in cognitive functioning is just beginning to be examined (7,16,17,36). More study is needed to determine the role of neurosteroids in stress-related cognitive deficits. The results of these studies may therefore help to better understand why children exposed to alcohol prenatally differ in their stress responsiveness across their lifespan as well as in their cognitive functioning in stress-related tasks.

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