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Differential Behavioral Effects of the Neuroactive Steroid Allopregnanolone on Neonatal Rats Prenatally Exposed to Alcohol[1]

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ZIMMERBERG, B., P. C. DRUCKER AND J. M. WEIDER. *Differential behavioral effects of the neuroactive steroid allopregnanolone on neonatal rats prenatally exposed to alcohol.* PHARMACOL BIOCHEM BEHAV **51**(2/3) 463–468, 1995.—The effects of prenatal alcohol exposure on the behavioral response to the neuroactive steroid allopregnanolone (3α-hydroxy-5α-pregnan-20-one) were investigated in neonatal rats. Two behaviors were assessed: retention of an odor conditioning task and production of ultrasonic vocalizations after brief maternal separation. Subjects from one of the three prenatal conditions (lab chow, alcohol, or pair-fed) received either no injection or an ICV injection of vehicle or one of three doses (1.25–5.0 μg) of allopregnanolone either 20 min prior to or immediately after training in an appetitive odor association paradigm. Retention was assessed 1 h later in a two-choice odor preference chamber. Posttraining injections of allopregnanolone caused a dose-dependent impairment in retention in the odor task, but there was no differential sensitivity to allopregnanolone in the alcohol-exposed offspring. All pretraining injections, including the vehicle, resulted in impairments in retention on the task, suggesting an impairment due to stress but not due to allopregnanolone. Allopregnanolone also reduced ultrasonic vocalizations after brief maternal separation in all subjects in a second experiment, but alcohol-exposed offspring displayed a dose-dependent shift to the right in their anxiolytic response to this neurosteroid. This decreased sensitivity suggests that prenatal alcohol exposure may cause a decrease in the density or affinity of the GABA receptors involved in stress response, but not cognitive processes, at this age.

Allopregnanolone 3α-Hydroxy-5α-pregnan-20-one GABA Neuroactive steroids Neurosteroid Ultrasonic vocalizations Maternal separation Isolation distress Odor conditioning Rats

ALTHOUGH there is now an extensive literature confirming the deleterious behavioral effects of prenatal alcohol exposure using animal models, the neural substrates for these behavioral dysfunctions are still under investigation. For example, alcohol-exposed offspring show learning deficits in spatial tasks (5,39), active avoidance (1), and passive avoidance (25). Learning deficits can be demonstrated in very young animals; using both odor-aversion and odor-appetitive conditioning paradigms, 10- and 3-day-old rat pups prenatally exposed to alcohol exhibited deficits in conditioning (3). Prenatal alcohol exposure also leads to developmental alterations in stress responses mediated by the hypothalamic-pituitary-adrenal (HPA) axis. During the first week of life, alcohol-exposed offspring display a hyporesponsiveness to stressors (32). As adults, alcohol-exposed offspring appear to have a greater HPA activation than control offspring in response to stress (22,29,34). In addition, there may be sex differences in alcohol-related birth effects on the HPA axis response to stress postpuberty (31,33).

To understand the relationship between learning deficits and altered stress responses following prenatal alcohol exposure, the present study investigated whether prenatal alcohol exposure alters the behavioral responses to allopregnanolone, a representative of a newly characterized class of stress-related neuromodulators known as neuroactive steroids or neurosteroids. Over 50 years ago, Seyle reported on the sedative-anesthetic activity of progesterone and deoxycorticosterone and identified their ring A-reduced metabolites as potent sedative-hypnotic agents (26). Neuroactive steroids bind to a unique site on the $GABA_A$ (γ-aminobutyric acid) receptor and

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either positively or negatively modulate the receptor's affinity for its ligand (15,16,24). Allopregnanolone (3α-hydroxy-5α-pregnan-20-one) is a metabolite of progesterone that is produced both in neuronal glial cells and peripheral tissues, and has been shown to enhance GABA-stimulated Cl^- uptake in rat cortical synaptoneurosomes (21).

Behavioral studies in adult animals have shown anxiolytic (4,36) and analgesic (20) effects following administration of allopregnanolone. A similar neurosteroid, alphaxalone, was also found to have anxiolytic properties in both the elevated plus maze and in a conflict test (6). Neurosteroids have also been implicated in modulating cognitive processes (17). Pregnenolone sulfate enhanced cognitive processes in rats in a two-trial recognition task when administered immediately following the acquisition trial (18). Additionally, precursors of androgenic and estrogenic steroids, dehydroepiandrosterone (DHEA) and its sulfate (DHEAS), enhanced memory retention in mice using an active avoidance task (9,10).

To determine whether alcohol-exposed offspring are differentially responsive to allopregnanolone, two behavioral paradigms were used, odor conditioning and distress vocalizations. In the first experiment, an odor conditioning paradigm was used in which the continuous exposure to a novel odor was paired with an appetitive reward (milk) and then subjects were tested for retention in a two-odor choice preference chamber (28). The effects of both pre- and posttraining injections of allopreganolone on odor conditioning retention in 6-day-old rat pups were examined in prenatal alcohol-exposed and control rat pups.

Ultrasonic vocalization (USV) production after maternal separation is a well-characterized model for testing antianxiety agents in young rats [e.g., (23,37)]. For example, several laboratories have demonstrated that benzodiazepines reduce ultrasound production in neonatal rats after separation from their dam and littermates (7,11,14). Benzodiazepine antagonists reverse this USV suppression (37), and anxiogenic agents, such as pentylenetetrazol, increase USV production (13). It was originally thought that the vocalizations were only distress calls that pups used to elicit retrieval from the dam, but further research demonstrated that USVs are also emitted in response to stressful stimulation such as isolation, cold temperatures, or physical handling (13,27). We have recently demonstrated that ICV administration of allopregnanolone resulted in a dose-dependent decrease in ultrasonic vocalizations in week-old rat pups following a brief maternal separation (38). Thus, the second experiment was conducted to determine whether prenatal alcohol exposure would produce a differential sensitivity to allopregnanolone's anxiolytic effect at this age.

METHOD

Experiment 1: Odor Conditioning Study

Subjects. Subjects (n = 180) were offspring generated from Long-Evans rats (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 was 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 12L : 12D cycle, with lights on at 0600 h. 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 1-5. 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 amount consumed by a "yoked" alcohol group female on a ml/kg body weight basis, for each specific day of pregnancy. Thus, each yoked pair 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 (postnatal 0; PN 0). Twenty-four hours following parturition (PN 1), dams were removed from the nest, and the litters were weighed and sexed. Litters were randomly culled to 11 offspring per litter.

Litters were then left undisturbed until PN 6. Subjects were randomly selected and asssigned to an experimental group. One group received exposure to the CS only (CS, conditioning control group). Another group underwent the appetitive conditioning procedure with no drug injection (control group). Other groups experienced the appetitive conditioning procedure either 20 min after or immediately prior to an intracerebroventricular (ICV) injection of vehicle or drug. Doses of 1.25, 2.5, or 5.0 μg of allopregnanolone (RBI, Natick, MA) or vehicle (45% 2-hydroxypropyl-β-cyclodextrin, RBI) were directly injected into the lateral ventricle in a 2-μl volume. This procedure is commonly used in unanesthetized week old rat pups because the skull is not yet calcified and landmarks are easily visible through the skin (37). In each experimental group, n = 6 per prenatal treatment; however, only a maximum of two subjects from the same litter, a male and female, were in any one condition.

Procedure. On PN 6, pups were removed from the nest and placed in a plastic container with home cage bedding. The container was then placed in a warm water bath (32°C) for a 6-h food-deprivation period. During this time, pups were implanted with an intraoral cannula (12), allowing for a 1-h minimum recovery period prior to conditioning. The cannula was made from an 8-cm piece of PE-10 tubing. A small flange was formed at one end by gently heating the end of the tubing over a flame until it began to blister, at which point it was pressed flat on a smooth surface.

The cannula was inserted using an 8-cm piece of curved piano wire. The wire was inserted into the ventral surface of the jaw between the angles of the mandible, and up through the digastric muscle and the tongue. The tip of the wire was pulled out through the mouth, and the lubricated (sesame oil, Sigma, St. Louis, MO), nonflanged end of the cannula was friction-fitted over the wire. The wire was then pulled back through the tongue and out of the jaw. The flanged end was pulled flat against the tongue. No anesthetic was used as this procedure was relatively short, lasting approximately 30 s. Just prior to training, subjects were voided by gently rubbing the anogenital region with a warm, moist cotton swab. This was done to ensure that the pup's bladder was not full and therefore uncomfortable during training.

Training on the appetitive odor association task (28) was conducted in a separate room where the temperature was maintained at 32°C. Pups were placed individually in 1-l glass beakers placed in a tray of water on top of a heating pad, thus maintaining a constant temperature of 32 ± 5°C inside the training chamber. The conditioned stimulus (CS) was a novel odor, citral (2.5 μl; Sigma). The CS was suspended on a folded absorbent tissue by a wire hook halfway into the beaker. Pups were continuously exposed to the CS odor for a 10-min period during which they received an infusion of milk (commercial Half and Half) for 5 s every minute via PE-50 tubing attached to the syringe from the infusion pump to the subject's cannula. Pups were trained two at a time, and the odor pads were changed and discarded after every subject. Following the 10-min training period, pups were returned to the water bath. As a conditioning control, subjects in the CS-alone group were treated identically, except that the tubing was not attached to their cannula so they did not receive milk during the training session.

One hour following training, subjects were given an odor preference retention test. The test consisted of a two-odor choice between the CS odor (citral) and clean wood shavings. The testing was performed in another room to ensure no odor contamination. The test apparatus was a clear, Plexiglas box (30 × 18.5 × 13 cm) with a wire grid floor covering (1 × 1 cm), suspended approximately 1 cm off the floor of the chamber. A 2-cm neutral zone running the width of the box divided the testing chamber into two sections. The entire chamber rested on a warm heating pad. Thirty minutes prior to testing, 5 μl of citral was placed on half of a Kim-wipe. The Kim-wipe was placed on one side of the testing chamber and left to equilibrate for 0.5 h. Immediately before testing, clean wood shavings were sprinkled over the side without the citral pad.

Pups were retrieved from the water bath and brought into the testing room. Pups were tested two at a time in separate chambers by two different experimenters blind to the subjects' conditions. A trial began when the pup was placed lengthwise in the neutral zone. Time was recorded when the pup's nose and forepaws were out of the "zone." Pups were tested for four 1-min trials with a 1-min intertrial interval in which the pup was placed on the warm heating pad. The direction in which the pup was placed in the neutral zone was counterbalanced across trials to control for a turning preference. After the testing, the pups were weighed and returned to the water bath. The retention score was defined as the percent time spent over the citral odor compared to total time over both sides.

Experiment 2: Ultrasonic Vocalization Study

Subjects. Subjects (N = 96) were bred in the laboratory as described in Experiment 1. Subjects (n = 6 per prenatal treatment per condition) were administered ICV injections as described above, with all injections 20 min prior to testing (38).

Procedure. On PN 6, pups were removed from the nest and placed in small containers containing home cage bedding. These containers were placed in the water bath as described in Experiment 1 for at least 15 min. Following the separation period, pups were randomly selected for one of five conditions: no injection, vehicle injection, or allopregnanolone (1.25–5.0 μg; an additional 10.0-μg dose was added for the alcohol-exposed pups). Following the injection the pup was returned to the water bath where it remained for 20 min.

Subjects were tested individually and taken to a nearby testing room maintained at 22–23°C. The pup was placed in a clear Plexiglas cage (30 × 18.5 × 13 cm) and for 6 min the pups' USVs were counted using a capacitance microphone with a mylar diaphragm and the broad-band 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 computer-assisted counter program (LabTimer). Following the 6-min testing period the pup was returned to the water bath. Once all the subjects had been tested, they were weighed and returned to the nest. No more than two pups from each litter were used in each condition.

RESULTS

Experiment 1: Odor Conditioning Study

Developmental data. Analyses on maternal and pup characteristics were performed to determine if the prenatal treatment was effective. There was no significant difference between groups for maternal percent weight gain or total number of pups born per litter in each prenatal condition. The mean daily alcohol consumption by the alcohol dams was 12.20 ± 0.39 g/kg/day. Mean birth weights were 6.49 ± 0.23, 7.29 ± 0.14, and 7.01 ± 0.22 for the alcohol, pair-fed, and lab chow control pups, respectively. Birth weights differed significantly by prenatal treatment, $F(2, 34) = 3.82$, $p < 0.05$, with post hoc tests (Fisher's LSD tests) revealing that the alcohol pups weighed significantly less than the pair-fed pups ($p = 0.01$), and marginally significantly less than the control pups ($p = 0.08$), which did not differ from the pair-fed pups. There was also a significant main effect of prenatal condition, $F(2, 181) = 21.91$, $p < 0.0001$, on the weight of the subjects tested at postnatal day 6. Post hoc tests (Fisher's LSD tests) revealed that alcohol pups weighed significantly less than both control and pair-fed pups ($ps < 0.001$), and pair-fed pups also weighed significantly more than the control pups ($p < 0.005$). Mean body weights were 12.29 ± 0.25, 14.13 ± 0.16, and 13.22 ± 0.16, for the alcohol, pair-fed, and lab chow control pups, respectively.

Posttraining injection. Data are presented for both male and female subjects because there were no effects of sex. For pups receiving an injection immediately posttraining, there was no main effect for prenatal condition and no interaction effect between prenatal condition and drug treatment. There was, however, a significant main effect of drug treatment on percent time spent over the CS, $F(5, 94) = 3.65$, $p < 0.005$ (Fig. 1). Post hoc tests (Fisher's LSD tests) revealed that pups receiving exposure to the CS-alone spent less time over the CS in the testing chamber than pups that received vehicle, 1.25 and 2.5 μg injections ($ps < 0.05$). In addition, pups in the 5.0-μg treatment group spent less time over the CS than both control pups and pups that received 1.25 and 2.5 μg injections of allopregnanolone ($ps < 0.05$).

Pretraining injection. For pups receiving drug injections 20 min prior to training, there was also no significant main effect for prenatal condition and no interaction effect of prenatal condition by drug treatment. A significant main effect of drug treatment was present, $F(5, 94) = 2.48$, $p = 0.04$ (Fig. 2). Subsequent post hoc tests (Fisher's LSD) revealed that pups receiving exposure to the CS-alone spent significantly less time over the CS in the testing chamber compared to control pups ($p < 0.05$). Additionally, pups in the control condition spent significantly more time over the CS than pups in the 1.25, 2.5, 5.0 μg, and vehicle treatment groups ($ps < 0.05$).

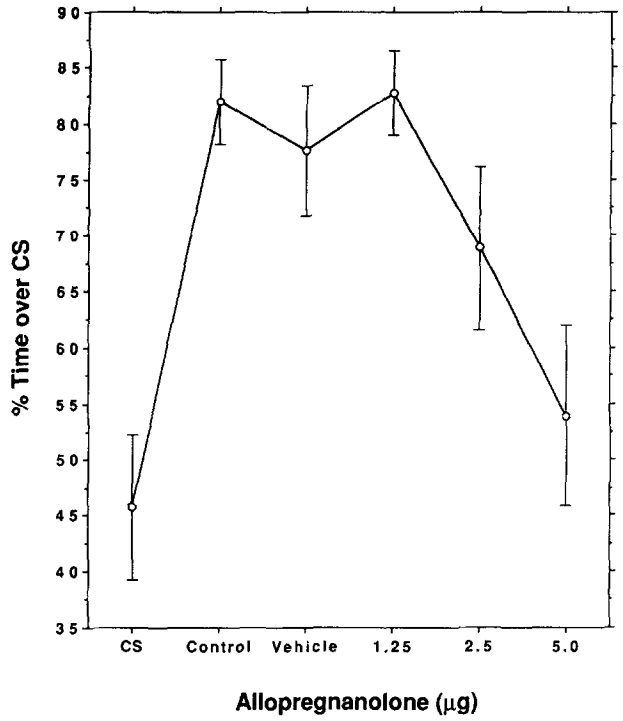

FIG. 1. The mean percent time (± SEM) spent over the conditioned stimulus (CS) odor in a two-choice odor preference test for subjects receiving no injection (control), an ICV injection of vehicle, or ICV injections of allopregnanolone immediately following training on an apppetitive odor association task. The CS group refers to subjects that were exposed to the conditioned stimulus without pairing to the appetitive reward.

Experiment 2: Ultrasonic Vocalization Study

Developmental data. Data were again analyzed and presented for male and female subjects combined because there were no effects of sex. There was no significant difference between groups for maternal percent weight gain or total number of pups born per litter in each prenatal condition. The mean daily alcohol consumption by the alcohol dams was 14.79 ± 0.43 g/kg/day. Mean birth weights were 6.35 ± 0.37, 6.62 ± 0.26, and 7.55 ± 0.17 for the alcohol, pair-fed, and lab chow control pups, respectively. Birth weights differed significantly by prenatal treatment, $F(2, 25) = 6.62$, $p < 0.005$, with post hoc tests (Fisher's LSD tests) revealing that the alcohol pups weighed significantly less than the control pups ($p < 0.01$) but not less than pair-fed pups, whereas pair-fed pups weighed significantly less than the control pups ($p < 0.05$). There was also a significant weight difference between the prenatal treatment groups on day 6, $F(2, 80) = 10.93$, $p = 0.001$. Mean body weights taken immediately after testing were 13.9 ± 0.25, 16.1 ± 0.28, and 15.2 ± 0.27 for the alcohol, pair-fed, and lab chow control groups, respectively. As in Experiment 1, further post hoc tests (Fisher's LSD tests) revealed that the alcohol pups weighed significantly less than both control and pair-fed pups ($ps < 0.05$). In addition, control pups weighed significantly less than pair-fed pups ($p < 0.05$).

Vocalizations. There was a significant interaction effect between prenatal condition and drug treatment group on the mean number of ultrasonic vocalizations, $F(8, 80) = 3.58$, $p < 0.002$ (Fig. 3). In addition, there were significant main effects for both prenatal condition, $F(2, 80) = 7.70$, $p < 0.001$, and drug treatment, $F(5, 80) = 18.58$, $p = 0.0001$, on the number of USVs, which were qualified by the significant interaction between these two factors. Subsequent post hoc means comparisons tests ($ps < 0.05$) revealed that, for alcohol-exposed subjects, there was a shift to the right in the dose-response curve, indicating less sensitivity to the drug. Whereas subjects in the two control groups had lower USVs after 5.0 μg of allopregnanolone, alcohol-exposed subjects did not exhibit significantly decreased USVs until they were administered a 10.0-μg dose.

Pair-fed subjects that received no injection at all, or that received the 1.25-μg injection, vocalized significantly more than their counterpart lab chow subjects. This difference was not statistically significant at the 2.5- and 5.0-μg doses or the vehicle injection. In addition, the pair-fed subjects vocalized significantly more than the alcohol-exposed subjects in the 1.25-μg treatment group. The number of vocalizations for alcohol subjects in the 5.0-μg treatment group was significantly higher ($p < 0.05$) than both pair-fed and lab chow, which did not differ from each other. The number of vocalizations for the pups that received vehicle injections did not differ from that of the pups that received no injection at all.

DISCUSSION

Allopregnanolone, when administered immediately post-training, caused a dose-dependent impairment in memory and

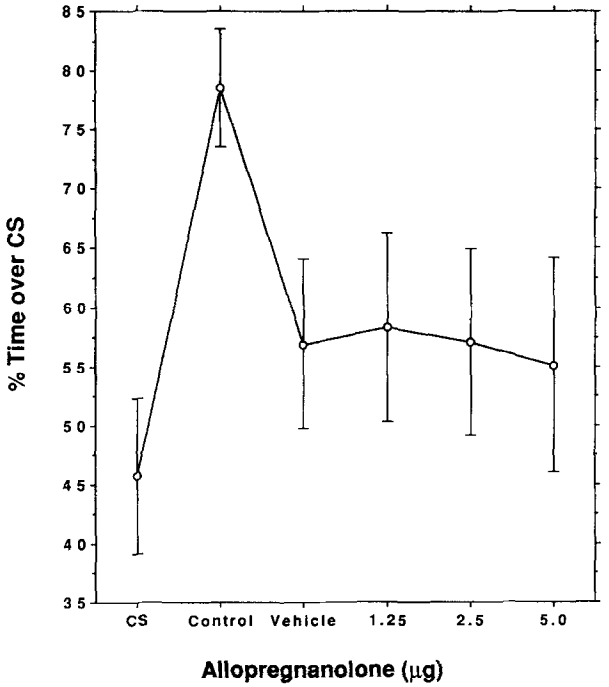

FIG. 2. The mean percent time (± SEM) spent over the conditioned stimulus (CS) odor in a two-choice odor preference test for subjects receiving no injection (control), an ICV injection of vehicle, or ICV injections of allopregnanolone for subjects receiving injections 20 min prior to training on an apppetitive odor association task. The CS group refers to subjects that were exposed to the conditioned stimulus without pairing to the appetitive reward.

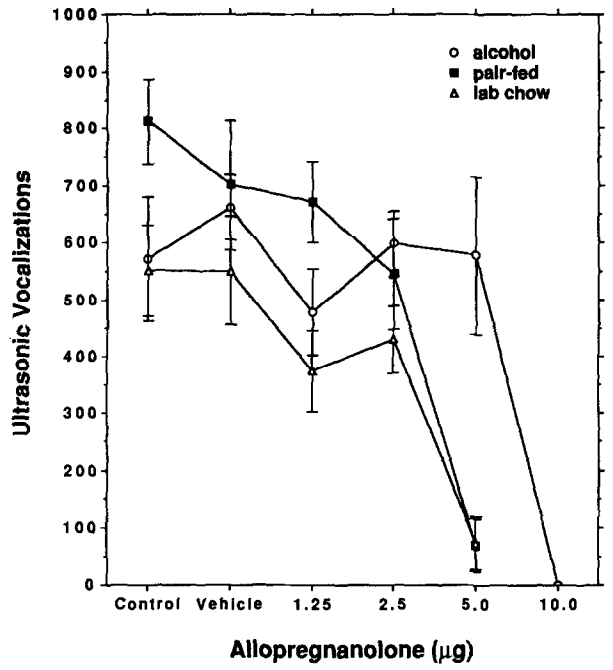

FIG. 3. The mean number of ultrasonic vocalizations (± SEM) following maternal separation during a 6-min test period for three prenatal treatment groups (alcohol, pair-fed, and lab chow control) receiving no injection (control), an ICV injection of vehicle, or ICV injections of allopregnanolone 20 min prior to testing.

learning in 6-day-old rat pups. Injections 20 min prior to training did not disrupt retention. Conditioning was demonstrated in the control group, as seen in the significant decrease in the amount of time subjects receiving the CS-only spent over the CS in the two choice odor preference test compared to controls. However, there was no apparent differential sensitivity caused by prenatal alcohol exposure in this odor conditioning paradigm. Additionally, we failed to replicate the findings of Barron and her colleagues, in which 3-day-old rat pups prenatally exposed to alcohol exhibited deficits in a similar, but not identical, odor associative learning task as compared to controls (3).

Central benzodiazepine receptor agonists as well as inverse agonists have been shown to affect learning and memory by modulating the $GABA_A$ receptors (19). Anxiogenic agents such as picrotoxin, bicuculline, and flumazenil, which inhibit GABA binding, enhance memory acquisition whereas anxiolytic drugs like muscimol and benzodiazepines, which enhance GABA binding, impair memory (19). Thus, the retention impairment seen here with allopreganolone administered posttraining parallels the previous results for drugs acting on the $GABA_A$ receptor complex. This finding also supports other research demonstrating that neurosteroids that enhance GABA binding (e.g., allopregnanolone) impair cognitive processes when administered after learning (18), whereas neurosteroids that diminish GABA binding enhance retention (9,10).

Pretraining injections overall resulted in impairment in retention in the odor conditioning task, which could be attributed to the ICV injection procedure alone, because the vehicle group did not differ from the drug groups. Other laboratories have demonstrated persistent effects of injections; for example, Antelman and his colleagues demonstrated that one injection blocked multiple actions of diazepam for up to a month (2).

In the second experiment, a differential sensitivity to allopregnanolone's behavioral effects was observed. Alcohol-exposed offspring displayed a dose-response shift to the right in their anxiolytic response to allopregnanolone as measured by USVs after brief maternal separation. The effect of allopregnanolone to reduce USVs in the control pups replicated previous work (38). The differential sensitivity of week-old pups prenatally exposed to alcohol suggests that further research into the effects of alcohol on the development of neurosteroid binding sites on the $GABA_A$ receptor is warranted. Other systems that are involved in stress response show a delayed development shortly after birth, and a hyperresponsiveness in adulthood (22,29,31-34). It is possible that the effects of prenatal alcohol exposure on the neurosteroid system involved in stress response follow a similar pattern. 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 (8). 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 because the number of steroid binding sites would be diminished as well.

Because pair-fed subjects responded like neither alcohol nor control subjects in the USV test, we suggest that the methodology currently used for alcohol liquid diet administration in this laboratory and many others may be problematic. It is possible that the feeding procedures cause a prenatal maternal stress. 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 between feedings, whereas the pair-fed dams tend to drink their nonalcohol diet rapidly and fully when administered. The alcohol may provide some reduction of the stress associated with the liquid diet administration and timed feedings that is not available to the pair-fed group. Rats that were exposed to a prenatal stress have demonstrated an increased anxiety in a novel environment (30) as well as greater emotionality in novel situations (35). The increased number of USVs in the pair-fed group relative to lab chow and alcohol subjects in Experiment 2 suggests that pair-fed subjects show an elevated baseline level of distress after maternal separation.

In summary, these experiments have demonstrated that the neuroactive steroid allopregnanolone is behaviorally active in week-old rat pups in both conditioning and stress response tests. It is interesting that prenatal alcohol exposure caused a differential sensitivity to this neuroactive steroid in the ultrasonic vocalization test but not the odor association retention test. These results suggest that neural systems underlying these two behaviors are distinct, and probably have different developmental patterns. This differential sensitivity can thus be used as a guide to focus more closely on the neurochemical substrates of alcohol's behavioral teratology.

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