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Reinforcing effects of the neurosteroid allopregnanolone in rats

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Abstract

The $GABA_A$ receptor positive modulator allopregnanolone (3α-hydroxy-5α-pregnan-20-one) is a potent neurosteroid with behavioral and biochemical characteristics similar to ethanol, barbiturates, and benzodiazepines. This suggests that neurosteroids may provide an alternative class of sedative/hypnotic, anticonvulsant, and anxiolytic pharmacotherapies. However, there is evidence from animal models that neurosteroids may be susceptible to abuse by humans. Thus, the present study evaluated the reinforcing effects of orally administered allopregnanolone in rats. In the first experiment, male Long–Evans rats ($n=9$) were allowed to voluntarily consume a 50-μg/ml allopregnanolone (50A) solution or water in an unlimited-access two-bottle choice procedure for 10 days. Subsequently, the same animals were trained to lever-press to receive a 50A solution in daily 30-min operant sessions using a sucrose substitution procedure. In the two-bottle choice procedure, rats consumed significantly more allopregnanolone than water, suggesting that allopregnanolone was serving as a reinforcer. In the operant self-administration procedure, allopregnanolone did not maintain levels of responding that were different from water, suggesting that allopregnanolone did not function as a reinforcer in this procedure. These results suggest that orally administered allopregnanolone possesses reinforcing properties; however, additional studies are necessary to determine whether operant oral self-administration will be a viable index of allopregnanolone's reinforcing effects.

Keywords: Neurosteroids; Self-administration; Operant responding; Two-bottle choice; Reinforcement; $GABA_A$ receptors; Rat

1. Introduction

Neurosteroids are derivatives of steroid hormones that can be synthesized in the brain without the aid of peripheral sources (see Mellon, 1994, for review). Of all endogenously occurring neurosteroids, the progesterone metabolite allopregnanolone (3α-hydroxy-5α-pregnan-20-one) is the most potent known positive modulator of $GABA_A$ receptors (e.g., Gasior et al., 1999; Lambert et al., 1995; Paul and Purdy, 1992). In vitro, allopregnanolone enhances GABA-stimulated chloride flux at nanomolar concentrations (Morrow et al., 1987) and interacts with known modulatory sites on $GABA_A$ receptors in a noncompetitive manner (Belelli et al., 1990; Gee et al., 1988). Allopregnanolone is found in plasma and brain of both males and females, and endogenous steroid levels can increase to pharmacologically relevant concentrations under a number of conditions (Barbaccia et al., 1994, 1996; Paul and Purdy, 1992; Purdy et al., 1991). Thus, allopregnanolone may represent a physiologically significant endogenous neuromodulator at $GABA_A$ receptors.

Like allopregnanolone, ethanol, benzodiazepines, and barbiturates are also positive modulators of $GABA_A$ receptors (e.g., Grobin et al., 1998), although there appears to be a separate binding site for neurosteroids (Gee et al., 1988, 1995). Perhaps because of comparable receptor mechanisms, some of the behavioral effects of these compounds, including their anxiolytic, sedative, and anticonvulsant properties, have also been found to be similar (e.g., Gasior et al., 1999). The behavioral and biochemical similarities between these compounds suggest that neurosteroids may provide an alternative class of sedative/hypnotic, anticonvulsant, and anxiolytic pharmacotherapies.

However, like these other drugs, neurosteroids may also be susceptible to abuse by humans. Drug discrimination studies have demonstrated that allopregnanolone and pregnanolone (3α-hydroxy-5β-pregnan-20-one) can substitute for drugs with abuse liability, including ethanol, pentobar-

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bital, and diazepam (Ator et al., 1993; Bowen et al., 1999; Grant et al., 1996, 1997; Rowlett et al., 1999). Additional studies have shown that pregnanolone, which has a very similar structure and pharmacological profile to that of allopregnanolone, can be trained as a discriminative stimulus in rats (Engel et al., 2001; Vanover, 1997, 2000). Together, these studies suggest that neurosteroids have discriminative stimulus effects that are similar to drugs with abuse potential.

Recently, our laboratory has demonstrated that systemic administration of allopregnanolone produced a dose-dependent increase in conditioned place preference in male mice (Finn et al., 1997), indicating that allopregnanolone possesses rewarding effects. In a more direct evaluation of reinforcing effects, Rowlett et al. (1999) demonstrated that pregnanolone was self-administered intravenously by rhesus monkeys. Thus, although neurosteroids may provide a promising new class of therapeutics, a thorough evaluation of their potential for abuse is necessary. The purpose of the current study was to examine whether orally administered allopregnanolone functioned as a reinforcer in rats. Two methods for evaluating a drug's reinforcing effects were employed. First, rats were tested in a two-bottle choice paradigm to determine whether they would demonstrate a preference for an allopregnanolone solution over water in the homecage. Subsequently, the same animals were trained to operantly self-administer an allopregnanolone solution using a sucrose substitution method of initiation (Samson, 1986). This procedure was used to establish high levels of operant responding with sucrose as the available reinforcer; a high initial rate of responding would theoretically allow pharmacologically relevant (and possibly reinforcing) doses of allopregnanolone to be self-administered during the substitution procedure. Following sucrose substitution, levels of allopregnanolone self-administration were compared to that of the established reinforcer, sucrose, and to the vehicle, water.

2. Method

2.1. Animals

Nine male Long–Evans rats (Harlan Sprague–Dawley, Indianapolis, IN), weighing 200–250 g at the beginning of the study, were individually housed in shoebox-type Plexiglas cages in a temperature-, humidity-, and light-controlled environment under a 12-h light/dark cycle. Food and water were available ad libitum throughout the study, except for temporary water restriction ($\sim$15 h) as indicated during the operant self-administration experiment. Body weights were monitored by weighing twice a week. All procedures were carried out in accordance with the United States Public Health Service–National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee of Oregon Health & Science University.

2.2. Drugs

Allopregnanolone was custom synthesized by and purchased from Dr. R.H. Purdy. Allopregnanolone was dissolved in 0.5% v/v ethanol and diluted with tap water to the desired allopregnanolone concentration. No additional ethanol was added. Preliminary studies from our laboratory indicated that rodents exhibited no preference for a 0.5% v/v ethanol solution versus water (D.A. Finn, unpublished observations); therefore, water was used as the vehicle solution in all experiments. Solutions were stored in the refrigerator and stirred daily prior to use. Fresh solutions were prepared every week.

2.3. Two-bottle choice consumption

After acclimating to the homecage environment for 1 week, rats were presented with fluids in two 50-ml graduated cylinders placed on the stainless steel cagetops of their homecages. To prevent food-associated establishment of tube preferences, food was distributed equally in association with both drinking tubes. For the first 2 days, water was available from both tubes. Then, one water tube was replaced with a tube containing a 50-μg/ml solution of allopregnanolone (50A) and 24-h fluid consumption from both tubes was measured at 1600 h daily. Consumption was measured for 10 days and was terminated when stable levels of fluid intake had been reached ($<$20% variability from the mean of the last 3 days, with no trends of increases or decreases in total fluid consumption). To control for side preferences, left–right positions of the tubes were switched daily for the first 2 days and subsequently alternated every other day. Allopregnanolone and water tubes placed on one empty cage allowed for the measurement of leakage and evaporation from the tubes. Average volume depleted from these tubes was subtracted from the 24-h drinking volumes for each rat.

2.4. Operant self-administration

Plexiglas operant chambers (30 $\times$ 25 $\times$ 30 cm $W \times D \times H$, model ENV-008, Med Associates, St. Albans, VT) were located inside sound-attenuating wooden chambers equipped with fans for air circulation. Each chamber contained a houselight and a liquid-delivery dipper system located in a square recess. One retractable response lever with an associated stimulus light above the lever was located to the right of the dipper. The lever was extended and the houselight and stimulus light were illuminated at the beginning of the session. Upon completion of an operant response, the lever retracted, the stimulus light extinguished, and the dipper arm raised up until the dipper cup was flush with a hole in the bottom of the recess. The dipper arm was raised for 5 s, allowing the rats to lick fluids from a 0.1-ml dipper cup. Equipment was controlled and data recorded via computer and associated Med Associates software.

At the end of the two-bottle choice experiment, rats were given 4 days of access to water only. They were then water restricted for 15 h to facilitate initiation of operant training. In the operant chamber, rats were shaped to press the lever to receive a 10% w/v sucrose solution (10S) on a fixed-ratio (FR) 1 schedule in daily 30-min sessions, 5 to 6 days a week. Water restriction ceased upon acquisition of this task (generally 1–5 days). The ratio was then gradually increased to FR4. When stable responding (<20% variability from the mean of three consecutive sessions, with no trends of increases or decreases in responding) was achieved in all rats, animals were given the opportunity to self-administer 50A using an adaptation of the sucrose substitution procedure of Samson (1986). After availability of the 10S solution, the reinforcer solution was changed every one to four sessions to gradually introduce increasing concentrations of allopregnanolone into the sucrose solution as follows: 10S/10A, 10S/25A, and 10S/50A. Then, the sucrose concentration was gradually reduced, beginning with 5S/50A, followed by 2S/50A, until a final concentration of 50A alone was available for self-administration.

50A was available for at least five consecutive sessions. When responding was stable, water was substituted for the 50A solution for at least five consecutive sessions. Then, to compare responding maintained by 10S and 10S/50A, 10S was again made available for self-administration, followed by 10S/50A, then by 10S again, for at least five sessions each.

2.5. Data analysis

The primary dependent variables for the two-bottle choice experiment were volume of allopregnanolone and water consumed (ml), total allopregnanolone intake (mg/kg/24 h), and preference ratio. Preference ratio was calculated by dividing the volume of allopregnanolone consumed by the total volume of allopregnanolone plus water consumed. Preference for allopregnanolone was interpreted to have occurred when allopregnanolone consumption was greater than 50% of the total fluid intake. For the operant self-administration experiment, the primary dependent variables were total responses and total allopregnanolone intake (mg/kg/30 min). Data were analyzed by one-way repeated measures ANOVA, with Newman–Keuls post hoc comparisons when appropriate. Significance was set at $P<.05$. All data are expressed as the mean ± S.E.M.

3. Results

3.1. Two-bottle choice consumption

Over the 10 days of this experiment, body weights increased to a mean ± S.E.M. of 346 ± 7 g (range 319–390 g). Mean 24-h consumption of water and 50A solution are shown in Fig. 1. For the first 3 days, animals drank approximately equal volumes of both solutions. From Days 4–10, however, rats consistently consumed greater volumes of allopregnanolone compared to water, with consumption of the 50A solution significantly greater than water intake on Days 5–10 [F's(1,9) ≥ 11.89, P's < .01; Fig. 1]. Fluid consumption stabilized toward the end of the experiment; over the last 3 days of access, animals drank a mean of 24.2 ± 1.8 ml of allopregnanolone and 5.2 ± 1.7 ml of water. While daily allopregnanolone consumption increased, water intake decreased in a similar manner, indicating that the rats were regulating their daily fluid consumption. Total 24-h fluid intake remained extremely stable throughout the 10-day testing period, with mean consumption over the last 3 days of 29.2 ± 1.2 ml. Rats also exhibited significant preference for the 50A solution [$F(9,81) = 6.58$, $P<.0001$], with a mean preference ratio of 0.83 ± 0.06 over the last 3 days of access (Fig. 2A). Mean dose of allopregnanolone consumed ranged from 2.44 ± 0.37 to 3.75 ± 0.19 mg/kg across the 10 days, with a mean dose of 3.49 ± 0.24 mg/kg consumed over the last 3 days of access (Fig. 2B).

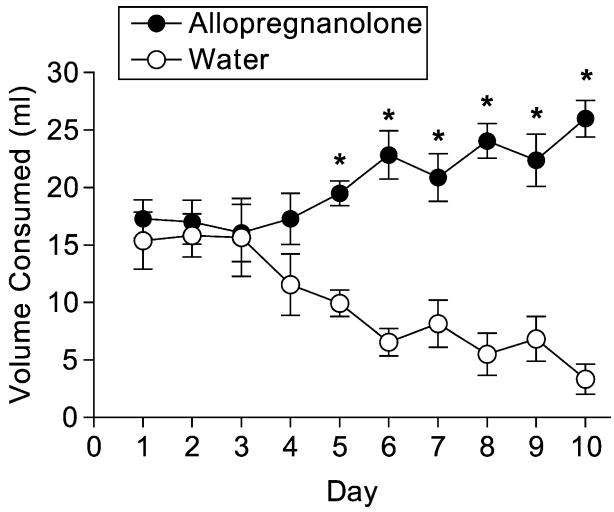

Fig. 1. Volume of 50A solution (closed circles) or water (open circles) consumed during 10 days of 24-h access in a two-bottle choice paradigm. Values represent the means ± S.E.M. for nine rats. * $P<.001$ versus water consumption on each day of access, Newman–Keuls post hoc test.

3.2. Operant self-administration

At the end of this 5-month experiment, body weights averaged 535 ± 25 g (range 454–688 g). Following 15-h water restriction, four rats were successfully shaped to lever-press (FR1) to receive 10S after one 2-h training session. The remaining five rats were shaped after two to five sessions and additional water restriction. All subjects achieved stable 10S-maintained responding under the FR4 schedule within 9–10 sessions. One animal displayed consistent operant performance through the substitution procedure, but never achieved stable levels of responding once sucrose was eliminated. Thus, its data were not included in the analysis.

Fig. 3 depicts the operant performance under the FR4 schedule throughout the sucrose substitution procedure.

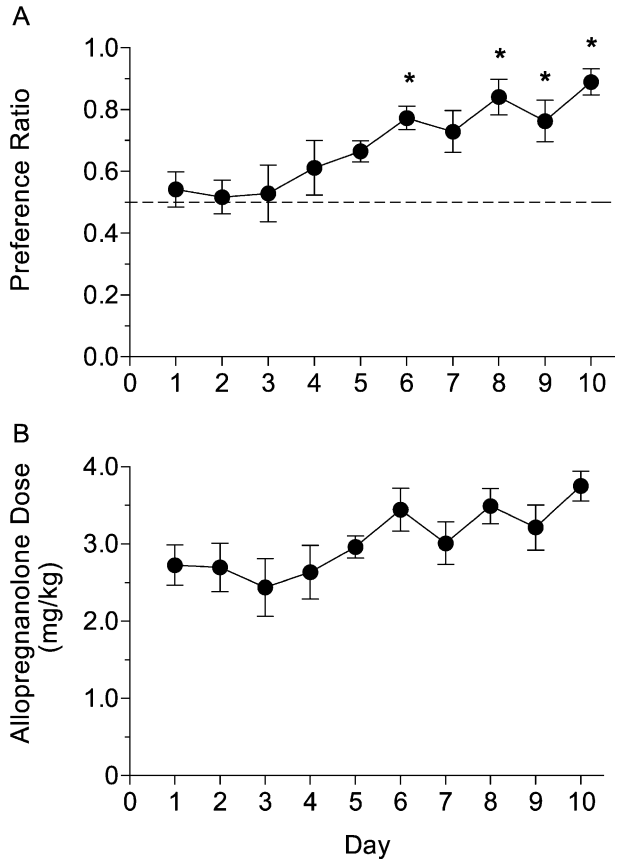

Fig. 2. Preference ratio (A) and allopregnanolone dose consumed (B) in a two-bottle choice paradigm. Rats displayed preference for allopregnanolone on Days 4–10. Values represent the means ± S.E.M. for the rats depicted in Fig. 1. * $P < .05$ versus Day 3, Newman–Keuls post hoc test.

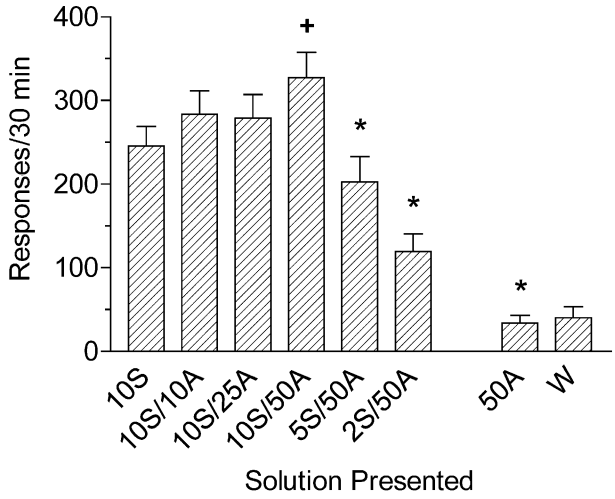

Fig. 3. Total responses maintained by sucrose or sucrose/allopregnanolone solutions under an FR4 operant schedule, during 30-min sessions. "S" indicates percentage of sucrose, while "A" indicates μg/ml of allopregnanolone in the solution. "W" indicates water vehicle. Solutions were made available in the order indicated. Each bar represents the mean ± S.E.M. of the last 2 days that a particular solution was available for self-administration for eight rats. 10S/25A was available for one session and was not included in the statistical analysis. * $P < .01$ versus 10S/50A, Newman–Keuls post hoc test; $^{+}P < .05$ versus 10S, ANOVA.

Each solution, except for 50A, was available for one to four sessions in each animal. As allopregnanolone was added to the solution, there was a trend for an increase in the number of responses made during the sessions [$F(2,23) = 2.39$, $P = .12$]. Statistical comparisons of 10S and 10S/50A revealed a significantly greater level of responding maintained by 10S/50A [$F(1,15) = 4.79$, $P < .05$]. However, as sucrose was gradually removed from the solution, responding decreased significantly [$F(3,31) = 33.56$, $P < .0001$; Fig. 3]. Post hoc comparisons indicated that responding maintained by each subsequent sucrose/allopregnanolone solution was significantly lower than 10S/50A-maintained responding (P's $< .01$).

The 50A solution was available for self-administration for 9–17 sessions across animals. Initial variability in responding during the first several sessions gradually diminished, with stability attained at very low levels of responding (Fig. 3). Responding maintained by the 50A solution was significantly lower than that maintained by the preceding 2S/50A solution ($P < .01$). Next, water was made available for self-administration. Responding maintained by water was not different from 50A-maintained responding, indicating that allopregnanolone did not function as a reinforcer under these conditions. Dose of allopregnanolone consumed during the 30-min session varied according to allopregnanolone concentration and response rate. The maximal allopregnanolone dose consumed (i.e., 0.95 mg/kg) occurred when the 10S/50A solution was available (Table 1). Observation of the animals during allopregnanolone availability did not reveal any overt signs of intoxication.

When allopregnanolone was added to the 10S solution, responding maintained by 10S/50A was significantly greater than that maintained by 10S alone. Thus, it was of interest to examine whether this may have been due to an additive reinforcing effect of allopregnanolone. Therefore, at the end of the experiment, both solutions were again made available for self-administration, with 10S presented both before (10S1) and after (10S2) the 10S/50A solution. This time, there were no differences in responding maintained by the two solutions [$F(2,23) = 0.11$, $P = .89$]. Both 10S and 10S/50A solutions maintained high levels of

Table 1
Mean allopregnanolone intake during presentation of each allopregnanolone solution in 30-min operant sessions

Solution presented in dipper	Allopregnanolone intake (mg/kg)
10S/10A	0.17 ± 0.02
10S/25A	0.40 ± 0.04
10S/50A	0.95 ± 0.08
5S/50A	0.58 ± 0.09
2S/50A	0.34 ± 0.06
50A	0.09 ± 0.02

Solutions were presented in the sequence depicted during the sucrose substitution procedure. Values presented are from the last 2 days of availability, except for 10S/25A, which was available for one session in most rats. Data represent the mean ± S.E.M. for eight rats.

responding that were comparable to responding maintained by 10S/50A the first time it was tested [10S1: 341.4 ± 36.5; 10S/50A: 336.8 ± 38.2; 10S2: 316.7 ± 45.0 responses/30 min; compare to Fig. 3].

4. Discussion

In the present study, rats displayed a strong preference for a 50A solution in an unlimited-access two-bottle choice paradigm. Additionally, the fact that intake of allopregnanolone was significantly greater than that of water suggests that allopregnanolone was serving as a positive reinforcer under these conditions. However, when measured in a limited-access, operant self-administration paradigm after the establishment of high levels of sucrose-maintained responding, the 50A solution failed to maintain self-administration behavior in the same animals. These data suggest that allopregnanolone possesses reinforcing effects; however, demonstration of these effects may be influenced by the paradigm used or the method of testing.

The development of preferential consumption of allopregnanolone in the two-bottle choice experiment may have been due to its pharmacological properties. The fact that the animals continued to exhibit preference for the allopregnanolone-containing drinking tube, even though it did not consistently remain on the same side of the cage, indicates that the rats were specifically tracking the allopregnanolone solution. The present results extend the findings of a recent study showing that the neurosteroid pregnanolone, which is structurally and pharmacologically similar to allopregnanolone, maintained intravenous self-administration in rhesus monkeys (Rowlett et al., 1999), indicating that it was functioning as a reinforcer. The current data are also consistent with the results of a study showing that allopregnanolone was rewarding in mice, as measured by conditioned place preference (Finn et al., 1997). Although there is one report in the literature of a conditioned place aversion produced by allopregnanolone in rats (Beauchamp et al., 2000), a number of methodological differences, such as dose and mode of administration (intracerebroventricular versus intraperitoneal), could explain the disparate findings between place conditioning studies. Finally, further support for allopregnanolone reinforcement is provided by two-bottle choice studies from our laboratory indicating that mice voluntarily consumed anxiolytic doses of allopregnanolone (2.1–3.7 mg/kg; Finn et al., submitted). As it has been hypothesized that a drug's anxiolytic properties may contribute to its reinforcing effects (see discussion in Grant, 1995), it is possible that allopregnanolone may have been voluntarily consumed in the present study due, in part, to its reinforcing properties.

In addition to evidence that neurosteroids possess reinforcing effects, a number of studies have indicated that neurosteroids have discriminative stimulus effects that are similar to drugs with abuse potential (Ator et al., 1993; Bowen et al., 1999; Grant et al., 1996, 1997; Rowlett et al., 1999). It is also possible that chemosensory properties (such as the taste and smell of the solution) could have contributed to the development of allopregnanolone preference in the present study. While this explanation cannot be ruled out at the present time, the majority of existing behavioral data to date provide support for the idea that both allopregnanolone and pregnanolone have reinforcing properties as well as discriminative stimulus effects that are similar to drugs with abuse liability. Thus, neurosteroids may also have some potential for abuse.

In contrast to the results from the unlimited-access two-bottle choice experiment, allopregnanolone did not function as a reinforcer in a limited-access operant self-administration procedure. This suggests that demonstration of allopregnanolone's reinforcing effects may depend on a number of factors, including the length of drug access, the concentration of the allopregnanolone solution, or the unit dose consumed upon availability of the reinforcer. Thus, a longer session duration or higher allopregnanolone concentration may be necessary for a reinforcing dose of allopregnanolone to be self-administered. Indeed, the maximum dose of 50A consumed in the 30-min operant session was 0.09 ± 0.02 mg/kg (see Table 1), far below the doses previously shown to be pharmacologically active in rats (e.g., Bowen et al., 1999; Devaud et al., 1999; Janak et al., 1998). Unfortunately, solubility limits precluded the use of a higher concentration of allopregnanolone in the present operant study.

Another limiting factor in the current operant study may have been the low oral bioavailability of allopregnanolone. Although allopregnanolone's oral bioavailability has not been published, the behavioral potency and plasma concentration of the structurally related neurosteroid pregnanolone have been shown to be extremely low following oral administration (Edgar et al., 1997). The fact that pregnanolone has been shown to function as a reinforcer when self-administered intravenously during periods of limited access (Rowlett et al., 1999) suggests that pharmacokinetic factors, such as route of administration, may play an important role in determining the reinforcing effects of neurosteroids.

It is possible that allopregnanolone is simply a weak reinforcer; therefore, its reinforcing effects may not have been strong enough to maintain self-administration when compared to sucrose in the present operant procedure. The fact that decreases in operant responding corresponded to reductions in sucrose concentration in the solutions available for self-administration (see Fig. 3) suggests that sucrose, not allopregnanolone, was maintaining self-administration throughout the substitution procedure. It would be useful in future operant studies to evaluate how orally administered allopregnanolone compares to other known pharmacological reinforcers.

Although allopregnanolone did not function as a reinforcer in the current operant paradigm, this finding could have been due to methodological issues unrelated to reinforcement. The length of the sucrose substitution procedure,

the sequential nature of the two experiments, and the relatively advanced age and weight of the rats may all have contributed to the lack of operant self-administration in the present study. For example, the unit allopregnanolone dose in a single 0.1-ml reinforcer presentation would be twice as great in a 250- versus a 500-g rat (i.e., 0.02 versus 0.01 mg allopregnanolone/reinforcer, respectively). Additionally, different behavioral and neurochemical effects in older versus younger rats have been observed with other relatively weak oral reinforcers, such as alcohol (e.g., Baird et al., 1998; Woods et al., 1996). Thus, the disparate results between the present two-bottle choice versus operant experiments may not necessarily be due to a differential sensitivity of the two procedures to allopregnanolone reinforcement. Further investigation of operant allopregnanolone self-administration in younger rats, as well as in those who do not have a prior experimental history, are necessary to fully answer the question of whether allopregnanolone will function as a reinforcer in an oral operant self-administration paradigm.

Recently, Samson et al. (1998, 1999) have developed a new procedure for evaluating self-administration of ethanol in which rats are given extended (i.e., 20 min) access to ethanol following completion of an operant requirement (i.e., "sipper" method). Compared to the traditional "dipper" method like the one used in the present study, in which subjects made four responses to receive 5 s of access to 0.1 ml of fluid, the rate of ethanol intake was increased and higher blood ethanol concentrations were achieved with the "sipper" method (Samson et al., 1998, 1999). These findings suggest that a longer period of drug access may allow reinforcing doses of allopregnanolone to be self-administered in an operant procedure.

Analysis of fluid consumption during the sucrose substitution procedure revealed that intake of 10S/50A was greater than that of 10S alone, raising the possibility that allopregnanolone's reinforcing effects may have contributed to the effectiveness of the solution as a reinforcer. This result is similar to that observed in studies of ethanol self-administration, in which sucrose/ethanol solutions were more effective reinforcers than sucrose alone (Files et al., 1995; Heyman, 1993; Samson et al., 1996). However, in the present study, reexposing the animals to the 10S and 10S/50A solutions several months after their initial exposure revealed no differences in responding. When 10S was presented at the beginning of the study (i.e., before 10S/50A), the animals were still acquiring the self-administration behavior. This might suggest that the initial finding of a difference in responding maintained by 10S and 10S/50A was due to differences in levels of operant training. Alternatively, it is possible that the rats may have reached a maximal level of responding when the two solutions were retested at the end of the study (i.e., a ceiling effect), which would have obscured any potential differences in responding maintained by 10S versus 10S/50A. Future studies that examine stable allopregnanolone-maintained responding during the maintenance phase of self-administration or that employ concurrent or progressive ratio schedules of responding are necessary to better evaluate differences in reinforcing strength between solutions.

Although the mechanisms mediating the reinforcing effects of allopregnanolone have not yet been elucidated, the $GABA_A$ receptor system is a logical candidate for examination, given allopregnanolone's known mechanism of action in vitro (Belelli et al., 1990; Gee et al., 1988). In vivo, drug discrimination studies have shown that benzodiazepines, barbiturates, ethanol, and other neurosteroids completely substitute for the $GABA_A$ receptor positive modulator pregnanolone, suggesting that this receptor is involved in the pregnanolone discriminative cue (Engel et al., 2001; Vanover, 1997, 2000). Additionally, antagonism of NMDA receptors and activation of 5-HT_3 and σ_1 receptors also contribute to pregnanolone's discriminative stimulus effects (Engel et al., 2001), suggesting that the discriminative cue of neurosteroids is heterogeneous. These data raise the possibility that several receptor systems may also be involved in allopregnanolone's reinforcing effects. In conclusion, the present data suggest that allopregnanolone possesses reinforcing properties; however, additional investigation of allopregnanolone self-administration is needed to more fully understand the mechanisms underlying the potential of neurosteroids for abuse.

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