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Using drug combinations to assess potential contributions of non-GABA_A receptors in the discriminative stimulus effects of the neuroactive steroid pregnanolone in rats

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Abstract

Neuroactive steroids are increasingly implicated in the development of depression and anxiety and have been suggested as possible treatments for these disorders. While neuroactive steroids, such as pregnanolone, act primarily at γ -aminobutyric acid (GABA_A) receptors, other mechanisms might contribute to their behavioral effects and could increase their clinical effectiveness, as compared with drugs acting exclusively at GABA_A receptors (e.g., benzodiazepines). The current study examined the role of non-GABA_A receptors, including n-methyl-D-aspartate (NMDA) and serotonin₃ (5-HT₃) receptors, in the discriminative stimulus effects of pregnanolone. Separate groups of rats discriminated either 3.2 mg/kg pregnanolone from vehicle or 0.32 mg/kg of the benzodiazepine midazolam from vehicle while responding under a fixed ratio 10 schedule for food pellets. When administered alone in both groups, pregnanolone and midazolam produced 80% drug-lever responding, the NMDA receptor antagonists dizocilpine and phencyclidine produced 60 and 30% drug-lever responding, respectively, and the 5-HT₃ receptor agonist 1-(m-chlorophenyl)-biguanide (CPBG) and morphine produced <20% drug-lever responding up to doses that markedly decreased response rates. When studied together, neither dizocilpine, phencyclidine, CPBG nor morphine significantly altered the midazolam dose-effect curve in either group. Given that CPBG is without effect, it is unlikely that 5-HT₃ receptors contribute substantially to the discriminative stimulus effects of pregnanolone. Similarities across groups in effects of dizocilpine and phencyclidine suggest that NMDA receptors do not differentially contribute to the effects of pregnanolone. Thus, NMDA and 5-HT₃ receptors are not involved in the discriminative stimulus effects of pregnanolone.

Keywords

neuroactive steroids; pregnanolone; midazolam; drug discrimination; rats

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1 Introduction

The role of neuroactive steroids in a variety of affective disorders is becoming increasingly apparent (Schüle et al 2013). Endogenous neuroactive steroids are synthesized from cholesterol and steroidal precursors, and the 3 α -reduced metabolites of progesterone allopregnanolone and pregnanolone are important products of neurosteroid biosynthesis. A large number of clinical trials have shown that a reduction in circulating levels of these metabolites is associated with anxiety and depressive disorders, and one strategy for treating these conditions is to restore levels of endogenous neuroactive steroids by giving allopregnanolone or pregnanolone exogenously (Schüle et al 2013).

The mechanisms that contribute to these potential therapeutic effects of neuroactive steroids are not entirely clear. It is well established that allopregnanolone and pregnanolone positively modulate γ -aminobutyric acid_A (GABA_A) receptors (Lan and Gee 1994), and actions at these receptors play an important role in their behavioral effects, in general, and in their anxiolytic effects, in particular. Other positive GABA_A modulators, primarily benzodiazepines, have long been used clinically for anxiety. Because of their similar actions on GABA_A receptors, neuroactive steroids produce behavioral effects that are similar to those of benzodiazepines, including anxiolytic effects (Wieland et al. 1997) as well as sedative and anticonvulsant effects (Kokate et al. 1994; Lancel 1999; Reddy and Rogawski 2001; Vanover et al. 1999); however, benzodiazepines are not effective in treating depression, suggesting that an action of neuroactive steroids other than modulation of GABA_A receptors accounts for their antidepressant effects. There are other important differences between neuroactive steroids and benzodiazepines that could provide a clinical advantage for neuroactive steroids. For example, tolerance does not develop to some effects of neuroactive steroids (Kokate et al. 1998; McMahon and France 2002a; Reddy and Rogawski 2000). In contrast, tolerance develops readily to many effects of benzodiazepines (Gonsalves and Gallagher 1987; Löscher et al. 1996; McMahon and France 2002b). Thus, despite similarities in their actions at GABA_A receptors, the effects of neuroactive steroids and benzodiazepines are not identical, suggesting that other mechanisms might be involved in the behavioral effects of neuroactive steroids. While benzodiazepines act exclusively at benzodiazepine sites on GABA_A receptors, neuroactive steroids act at distinct modulatory sites on GABA_A receptors as well as on other receptors, such as N-methyl-D-aspartate (NMDA) and 5-hydroxytryptamine (5-HT₃) receptors (Rupprecht et al. 2001; Dubrovsky 2005), and it might be their actions at these other receptors that account for differences between neuroactive steroids and benzodiazepines.

Drug discrimination is a behavioral procedure that has been used to examine possible differences among positive GABA_A modulators, including neuroactive steroids, benzodiazepines and barbiturates. Drugs acting at any of these distinct sites on GABA_A receptors can be established as discriminative stimuli (e.g., de la Garza and Johanson 1987; Ator et al. 1993; Engel et al. 2001; Bai and Gerak 2011). Regardless of which training drug is used to establish the discrimination, positive GABA_A modulators generally produce drug-lever responding, although some exceptions have been reported. For example, in rats, the benzodiazepine lorazepam produces pentobarbital-lever responding, although pentobarbital does not produce lorazepam-lever responding (Ator and Griffiths 1983; Ator et al. 1993). In

addition, some subjects discriminating midazolam do not respond on the drug lever after administration of barbiturates or neuroactive steroids (Evans and Johanson 1989; Gerak et al. 2008) whereas all subjects discriminating a neuroactive steroid respond on the drug lever after administration of benzodiazepines or barbiturates (Eppolito et al. 2012; Gerak and France 2013). These differences support the notion that the discriminative stimulus effects of positive GABA_A modulators are not identical. In addition, drug discrimination procedures have identified multiple mechanisms of action for another positive GABA_A modulator, ethanol, including actions at GABA_A, NMDA and 5-HT₃ receptors (Grant and Colombo 1993; Bienkowski and Kostowski 1998).

This study used drug discrimination procedures to determine whether actions of pregnanolone at receptors other than GABA_A receptors contribute to its discriminative stimulus effects in rats. That GABA_A receptors have a predominant role in the discriminative stimulus effects of pregnanolone has been established (Bai and Gerak 2011; Eppolito et al. 2012; Gerak and France 2013); however, a role of other receptors has not been clearly determined. Drugs with actions at 5-HT₃ and NMDA receptors have been studied previously in subjects discriminating pregnanolone, and the results have been inconsistent with 5-HT₃ receptor agonists and NMDA receptor antagonists producing pregnanolone-lever responding in some studies (Engel et al. 2001) and not in others (Shannon et al. 2005; Eppolito et al. 2012). The current study extends those findings by determining whether the 5-HT₃ receptor agonist CPBG and the NMDA receptor antagonists phencyclidine and dizocilpine modify the discriminative stimulus effects of pregnanolone or midazolam. Two separate groups of rats were used with one group discriminating pregnanolone from vehicle and the second group discriminating midazolam from vehicle; experimental conditions were identical in the two groups except for the training drug. Because the midazolam discriminative stimulus is pharmacologically selective with only positive GABA_A modulators producing effects similar to those of the training drug (Lelas et al. 1999; Bai and Gerak 2011), drugs acting at other receptors would not be expected to produce midazolam-lever responding or to enhance the effects of either midazolam or pregnanolone (i.e., shift dose-effect curves for positive modulators to the left) in rats discriminating midazolam. In contrast, actions of neuroactive steroids at 5-HT₃ or NMDA receptors might contribute to their discriminative stimulus effects. Under those conditions, drugs acting at those other receptors might produce pregnanolone-lever responding, although the inconsistent results obtained in previous studies suggest that any role of 5-HT₃ or NMDA receptors in the discriminative stimulus effects of pregnanolone might be small, as compared with the role of GABA_A receptors, so that drugs acting at these other receptors do not reliably mimic the effects of pregnanolone. To the extent that these other receptors contribute to the effects of pregnanolone, they would be expected to enhance the discriminative stimulus effects of positive GABA_A modulators in rats discriminating pregnanolone, shifting their dose-effect curves leftward. This enhancement would be especially apparent when drugs acting 5-HT₃ or NMDA receptors are studied in combination with midazolam, which acts only at GABA_A receptors, as compared to the enhancement obtained when those drugs are studied with pregnanolone, which might already have actions at 5-HT₃ or NMDA receptors in addition to its actions at GABA_A receptors.

2. Methods

2.1. Subjects

One group of male Sprague-Dawley rats discriminated pregnanolone from vehicle and the other group discriminated midazolam from vehicle. They received 45-mg grain-based food pellets (Bio Serv, Inc, Frenchtown, NJ) during experimental sessions with rodent chow (Harlan Teklad, Madison, WI) provided in the home cage in sufficient quantities to maintain weights between 320 and 330 g throughout the experiment. Rats had unlimited access to water in the home cage and were housed individually in a humidity- and temperature-controlled vivarium under a 12-h light/dark cycle with experiments conducted during the light cycle. Animals used in these studies were maintained in accordance with the Institutional Animal Care and Use Committee at The University of Texas Health Science Center at San Antonio, and the guidelines of the Committee on Care and Use of Laboratory Animal Resources, National Research Council [Department of Health, Education and Welfare, publication No. (NIH) 85-23, revised 2011].

2.2. Apparatus

Sessions were conducted in chambers enclosed within sound-attenuating cubicles. Each chamber was equipped with a houselight, two response levers, two stimulus lights, a pellet trough, a pellet dispenser and a fan for ventilation (MED Associates, Inc., St. Albans, VT). During sessions, white noise was present in the room to mask extraneous noise. An interface connected the chambers to a computer that controlled experimental events and recorded data using Med-PC/Medstate Notation software (MED Associates, Inc., St. Albans, VT).

2.3. Procedure

One group of 13 rats discriminated 3.2 mg/kg pregnanolone from vehicle and the other group of 11 rats discriminated 0.32 mg/kg midazolam from vehicle while responding under a fixed-ratio 10 schedule of food presentation; other than the training drug, experimental sessions were identical in the two groups. These training doses were selected because previous studies indicated that the potency of pregnanolone and midazolam to produce drug-lever responding would be similar across the two groups with these training doses (Bai and Gerak 2011). Sessions were divided into 15-min cycles and there could be up to 8 cycles in a session. Each cycle began with a 10-min timeout period, during which the chamber was dark and responding had no programmed consequence, and ended with a response period that could last up to 5 min. Response periods were signaled by illumination of the stimulus lights located above the levers. Under these stimulus conditions, 10 responses on the lever designated correct by the injection given during the first minute of the timeout resulted in the delivery of a food pellet; the lever designated correct after an injection of the training drug was counterbalanced across rats. Responses on the incorrect lever reset the response requirement on the correct lever. Lights were extinguished and response periods ended after 5 min or the delivery of 10 pellets, whichever occurred first. When 10 pellets were delivered in less than 5 min, the time remaining between the end of the response period and the end of the cycle was a timeout.

Rats received injections at the beginning of each cycle. There were two types of training sessions. Drug training sessions could begin with the training drug given on the first cycle or with vehicle or sham given for up to 5 cycles followed by administration of the training drug; one cycle that began with a sham injection followed the drug cycle. Responding on the vehicle lever resulted in the delivery of food for cycles that proceeded the drug cycle and responding on the drug lever resulted in delivery of food during the drug cycle and the subsequent sham cycle. During vehicle training sessions, vehicle or sham was administered for up to 8 cycles. The first test session was conducted when the following criteria were satisfied for 5 consecutive or 6 of 7 training sessions: 80% of the total responses were emitted on the correct lever and fewer than 10 responses were emitted on the incorrect lever prior to delivery of the first food pellet. Thereafter, test sessions were conducted every third day as long as the above criteria were satisfied during intervening training sessions.

Test sessions were identical to training sessions except that 10 consecutive responses on either lever resulted in the delivery of food and test compounds were administered. Several different types of tests were conducted to determine whether drugs with actions at receptors other than GABA_A receptors produce drug-lever responding. First, dose-effect curves were determined using a cumulative-dosing procedure. Vehicle was administered on the first cycle followed by increasing doses of the test compound on subsequent cycles with the cumulative dose increasing by 0.25 or 0.5 log units per cycle. Dosing continued until 80% responding occurred on the drug lever or rates were decreased to <20% of control. In both groups, dose-effect curves were determined for the two training drugs, one other positive GABA_A receptor modulator (flunitrazepam), and drugs with primary mechanisms of action at receptors other than GABA_A receptors, including the NMDA receptor antagonists ketamine and phencyclidine, the 5-HT₃ receptor agonist CPBG, and the μ opioid receptor agonist morphine. Because phencyclidine occasioned more than 20% and less than 80% drug-lever responding when it was studied under the cumulative-dosing procedure and because the NMDA receptor antagonist dizocilpine has a slow onset, a second type of test was conducted with these two drugs to determine their time course. A single dose of the test drug was given at the beginning of a session comprising 8 cycles with sham injections given during all cycles after the first. Drugs with actions at receptors other than GABA_A receptors were also studied in combination with midazolam and pregnanolone. For drug combination studies, an injection of the test compound either replaced the vehicle injection given during the first cycle of the session (phencyclidine) or was given 30 min before the start of the session (dizocilpine, morphine and CPBG) with cumulative dose-effect curves for either midazolam or pregnanolone determined on subsequent cycles.

2.4. Drugs

Pregnanolone (5 β -pregnan-3 α -ol-20-one; Steraloids, Inc., Newport, RI) was dissolved in 45% (w/v) 2-hydroxypropyl- β -cyclodextrin. Midazolam hydrochloride (Bedford Laboratories, Bedford, OH) and ketamine hydrochloride (racemate; Fort Dodge Laboratories, Fort Dodge, IA) were purchased as commercially prepared solutions and diluted with sterile 0.9% saline. Flunitrazepam (Sigma-Aldrich Co., St. Louis, MO) was dissolved in a vehicle containing 20% emulphor, 10% ethanol and 70% sterile 0.9% saline. Morphine sulfate and phencyclidine hydrochloride (Research Technology Branch, National

Institute on Drug Abuse, Rockville, MD), along with 1-(m-chlorophenyl)-biguanide hydrochloride (CPBG) and dizocilpine hydrogen maleate (Sigma-Aldrich Co., St. Louis, MO), were dissolved in sterile 0.9% saline. Drugs were administered i.p. typically in a volume of 1 ml/kg body weight. Doses are expressed in the form listed above in mg/kg body weight.

2.5. Data analyses

Control response rates were obtained during training sessions in which rats received only vehicle or sham injections (i.e., no drug) and they satisfied the testing criteria. For individual rats, rates were averaged across cycles within sessions and then averaged across 10 training sessions (mean $\pm$ 1 SEM). Group mean response rates were then obtained by averaging across rats and were compared between the two groups using a t-test. The percentage of responses on the drug-appropriate lever and response rates were plotted as a function of dose or time since drug administration. Discrimination data for an individual rat were not included in analyses when response rate was less than 20% of control. Moreover, discrimination data were not plotted when response rate was less than 20% of control for more than half of the rats tested.

Midazolam and pregnanolone dose-effect curves obtained in the presence or absence of a drug acting at non-GABA_A receptors were compared by simultaneously fitting straight lines to the dose-effect curves for individual rats using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA). Straight lines were fitted to the linear portion of the dose-effect curves which included one data point below 25%, one data point above 75% and all data points in between. To determine the simplest model that best fit the data, slopes of those lines were compared using an F-ratio test. Slopes that were significantly different indicated that the test compound altered the dose-effect curve and required the more complex model to fit the data; when slopes were not different, a simpler model with a common slope was used. Dose-effect curves were further compared by determining whether the data were best fit by a common intercept. Significance was set at $P < 0.05$.

3. Results

When vehicle or sham injections were given, mean control response rates were not significantly different ($t_{22}=0.068$, n.s.) between rats discriminating pregnanolone (0.91 ± 0.05 responses/sec) and those discriminating midazolam (0.92 ± 0.05 responses/sec). Flunitrazepam, midazolam, and pregnanolone dose dependently increased drug-lever responding in both groups (solid symbols, upper panels, Fig 1). Rank order potency was the same with no more than a 2-fold difference in ED₅₀ values for each drug across groups (Table 1). Doses that produced 80% drug-lever responding did not decrease response rates in either group (solid symbols, lower panels, Figures 1); rather, a cumulative dose of 0.178 mg/kg midazolam increased rates to 1.12 ± 0.05 and 1.06 ± 0.09 responses/sec in rats discriminating pregnanolone and midazolam, respectively, and a cumulative dose of 1.78 mg/kg pregnanolone increased rates to 0.97 ± 0.07 and 0.97 ± 0.05 responses/sec in rats discriminating pregnanolone and midazolam, respectively.

Drugs that act at non-GABA_A receptors produced predominantly vehicle-lever responding in both groups up to the dose that markedly decreased response rates (open symbols, Fig 1). On average, morphine, CPBG and ketamine produced less than 20% drug-lever responding at all doses. In contrast, phencyclidine produced a maximum of 31.2% and 40.4% drug-lever responding in rats discriminating pregnanolone and midazolam, respectively. The dose that was needed to produce the effect in rats discriminating pregnanolone (5.6 mg/kg) was slightly larger than the dose needed in rats discriminating midazolam (3.2 mg/kg); these doses only modestly decreased rates to 0.57 and 0.64 responses/sec, respectively, and for each group a dose ¼ log unit larger decreased rates to <0.2 responses/sec. Because phencyclidine occasioned more than 20% drug-lever responding when it was studied under the cumulative-dosing procedure, a time course was also obtained to determine whether greater effects would emerge at a later time. In rats discriminating pregnanolone, drug-lever responding did not exceed 31.2% (i.e., the maximum amount of drug-lever responding obtained using the cumulative-dosing procedure) at doses smaller than 5.6 mg/kg and was 33.3% 120 min after administration of 5.6 mg/kg, a dose that markedly decreased rates (left panels, Fig 2). In rats discriminating midazolam, phencyclidine was slightly more potent with 3.2 mg/kg phencyclidine producing more than 60% drug-lever responding from 60 to 120 min after administration and decreasing rates to 0.30 responses/sec 30 min after administration (right panels, Fig 2). The discriminative stimulus and rate-decreasing effects of 5.6 mg/kg phencyclidine were similar to those of 3.2 mg/kg. A time course for dizocilpine was also obtained due to its slow onset. The potency and onset of dizocilpine was similar in the two groups; 60 min after administration, 0.178 mg/kg produced 67% and 65% drug-lever responding in rats discriminating pregnanolone or midazolam, respectively, with little effect on response rates (Fig 3). A larger dose of dizocilpine (0.32 mg/kg) decreased rates to 0.1 responses/sec in both groups 60 min after administration.

Drugs acting at non-GABA_A receptors were also studied in combination with midazolam and pregnanolone to determine whether they could alter the discriminative stimulus effects of positive GABA_A modulators. Morphine did not significantly shift the midazolam dose-effect curve (upper panels, Fig 4). Straight lines fitted to midazolam dose-effect curves obtained in the presence and absence of 3.2 mg/kg morphine indicated no significant difference between slopes (rats discriminating pregnanolone: $F_{1,18}=0.83$, n.s.; rats discriminating midazolam: $F_{1,35}=0.40$, n.s) or between intercepts (rats discriminating pregnanolone: $F_{1,19}=2.36$, n.s.; rats discriminating midazolam: $F_{1,36}=1.46$, n.s), and, for both groups, the simplest model that could be used to fit the data was one with a common slope and intercept. When given 30 min before sessions, this dose of morphine decreased rates to 0.63 ± 0.17 responses/sec in rats discriminating pregnanolone and to 0.66 ± 0.07 responses/sec in rats discriminating midazolam; up to a dose of 1 mg/kg and 0.32 mg/kg, respectively, midazolam did not further decrease rates (lower panels, Fig 4).

CPBG also did not significantly alter the discriminative stimulus effects of pregnanolone or midazolam in either group (upper panels, Fig 5). Although there was a trend for a shift leftward in the midazolam and pregnanolone dose-effect curves in rats discriminating pregnanolone, comparison of fits indicated no significant difference in slope (CPBG with pregnanolone: $F_{1,21}=0.13$, n.s.; CPBG with midazolam: $F_{2,33}=0.72$, n.s) or intercept (CPBG with pregnanolone: $F_{1,22}=0.80$, n.s.; CPBG with midazolam: $F_{2,35}=0.05$, n.s) for dose-effect

curves determined in the presence or absence of CPBG in rats discriminating pregnanolone and no significant difference in slope (CPBG with pregnanolone: $F_{1,30}=3.00$, n.s.; CPBG with midazolam: $F_{2,36}=0.52$, n.s) or intercept (CPBG with pregnanolone: $F_{1,22}=0.80$, n.s.; CPBG with midazolam: $F_{1,31}=0.01$, n.s) for dose-effect curves determined in the presence or absence of CPBG in rats discriminating midazolam. When given 30 min before sessions, 3.2 mg/kg CPBG modestly decreased response rates to 0.83 ± 0.08 responses/sec in rats discriminating pregnanolone, and rates were not decreased further by midazolam or pregnanolone (lower left panel, Fig 5). CPBG had a greater effect on response rates in rats discriminating midazolam, decreasing rates to 0.63 ± 0.12 responses/sec 30 min after administration of 3.2 mg/kg (points above V, lower right panel, Fig 5). While pregnanolone did not further decrease response rates, midazolam dose dependently enhanced the rate-decreasing effects of CPBG (open circles, lower right panel, Fig 5).

Finally, two drugs with actions at NMDA receptors did not significantly enhance the discriminative stimulus effects of midazolam or pregnanolone in either group. When given immediately before sessions, phencyclidine did not change midazolam or pregnanolone dose-effect curves, regardless of the training drug (upper panels, Fig 6). Comparison of fits indicated no significant difference in slope (phencyclidine with pregnanolone: $F_{2,48}=0.66$, n.s.; phencyclidine with midazolam: $F_{2,35}=0.30$, n.s) or intercept (phencyclidine with pregnanolone: $F_{2,50}=0.05$, n.s.; phencyclidine with midazolam: $F_{2,37}=0.27$, n.s) for dose-effect curves determined in the presence or absence of phencyclidine in rats discriminating pregnanolone and no significant difference in slope (phencyclidine with pregnanolone: $F_{2,33}=1.17$, n.s.; phencyclidine with midazolam: $F_{2,48}=0.38$, n.s) or intercept (phencyclidine with pregnanolone: $F_{2,35}=1.38$, n.s.; phencyclidine with midazolam: $F_{2,50}=1.33$, n.s) for dose-effect curves determined in the presence or absence of phencyclidine in rats discriminating midazolam. Response rates were lower following administration of 3.2 mg/kg phencyclidine in rats discriminating midazolam (0.69 ± 0.08 responses/sec), as compared with rates obtained in rats discriminating pregnanolone (1.01 ± 0.09 responses/sec); rates were decreased when phencyclidine was combined with doses of midazolam and pregnanolone that produced >80% drug-lever responding (lower panels, Fig 6). Effects obtained with dizocilpine were similar, although not identical, to those of phencyclidine. In rats discriminating midazolam, dizocilpine did not significantly alter the midazolam or pregnanolone dose-effect curve (upper right panel, Fig 7), and comparison of fits indicated no significant difference in slope (dizocilpine with pregnanolone: $F_{1,26}=0.01$, n.s.; dizocilpine with midazolam: $F_{2,50}=0.04$, n.s) or intercept (dizocilpine with pregnanolone: $F_{1,27}=2.71$, n.s.; dizocilpine with midazolam: $F_{2,52}=0.09$, n.s). In rats discriminating pregnanolone, there was a trend toward a leftward shift in the midazolam and pregnanolone dose-effect curves by dizocilpine (upper left panel, Fig 7). Dizocilpine did not alter either the slope ($F_{2,50}=2.57$, n.s.) or intercept ($F_{2,52}=0.59$, n.s.) of the midazolam dose-effect curve in rats discriminating pregnanolone, although it significantly altered the slope of the pregnanolone dose-effect curve ($F_{2,42}=3.707$, $p<0.05$). When the more complex model was used to analyze pregnanolone dose-effect curves determined in the absence or presence of dizocilpine, with slope allowed to vary, intercepts were not significantly different ($F_{2,42}=0.55$, n.s.). These doses of dizocilpine, alone or when combined with increasing doses of positive GABA_A modulators, did not markedly alter response rates (lower panels, Fig 7).

4. Discussion

GABA_A receptors mediate, at least in part, the discriminative stimulus effects of benzodiazepines, neuroactive steroids and ethanol. While the discriminative stimulus effects of benzodiazepines are mediated exclusively by GABA_A receptors, other receptors appear to contribute to the effects of ethanol (Grant and Colombo 1993). Similarly, non-GABA_A receptors might contribute to the discriminative stimulus effects of pregnanolone, and this study examined whether 5-HT₃ and NMDA receptors are involved in those effects. CPBG, a 5-HT₃ receptor agonist that has been shown to produce some pregnanolone-lever responding in rats (Engel et al. 2001), did not produce drug-lever responding or enhance the discriminative stimulus effects of midazolam or pregnanolone in rats discriminating either positive GABA_A modulator from vehicle, suggesting that agonism at 5-HT₃ receptors does not contribute to the discriminative stimulus effects of pregnanolone. Although phencyclidine and dizocilpine produced 31 and 71% drug-lever responding, respectively, in rats discriminating pregnanolone, these drugs also produced at least 60% drug-lever responding in rats discriminating midazolam. Because benzodiazepines do not act at NMDA receptors, these similarities in the discriminative stimulus effects of phencyclidine and dizocilpine across training drugs suggest that NMDA receptors do not differentially contribute to the effects of pregnanolone. Moreover, they did not significantly enhance the discriminative stimulus effects of midazolam in either group. Together, these results suggest that 5-HT₃ or NMDA receptors are not involved in the discriminative stimulus effects of pregnanolone.

Using substitution studies, which determine whether drugs mimic the effects of the training drug, along with drug combination studies, which determine whether drugs alter the discriminative stimulus effects of the training drug, is important when examining mechanism of action. Both kinds of studies were used previously to demonstrate similarities in the role of GABA_A receptors in the discriminative stimulus effects of pregnanolone and midazolam. In substitution studies, positive GABA_A modulators produced drug-lever responding and drugs with actions at other receptors produced vehicle-lever responding in rats discriminating either pregnanolone from vehicle or midazolam from vehicle (Bai and Gerak 2011), demonstrating the importance of GABA_A receptors in the discriminative stimulus effects of pregnanolone and midazolam. Drug combination studies further support the role of GABA_A receptors in these effects. For example, flumazenil acts at benzodiazepine sites on GABA_A receptors where it antagonizes the discriminative stimulus effects of benzodiazepines; consequently it does not produce drug-lever responding in subjects discriminating either midazolam from vehicle or pregnanolone from vehicle. Flumazenil shifts benzodiazepine dose-effect curves rightward, and similarities in antagonism of benzodiazepines in subjects discriminating either midazolam from vehicle or pregnanolone from vehicle (Lelas et al. 2000; Bai and Gerak 2011; Gerak and France 2013) confirms the role of GABA_A receptors in the discriminative stimulus effects of benzodiazepines and pregnanolone.

The importance of using both substitution and drug combination studies was evident in the current investigation in which a possible role of non-GABA_A receptors in the discriminative stimulus effects of pregnanolone was examined. Under some conditions, actions of

neuroactive steroids at receptors other than GABA_A receptors have been implicated in the discriminative stimulus effects of pregnanolone, as evidenced by increased pregnanolone-lever responding following administration of 5-HT₃ receptor agonists and NMDA receptor antagonists (Engel et al. 2001). In the current study, agonist actions at 5-HT₃ receptors do not appear to contribute to the discriminative stimulus effects of pregnanolone because the 5-HT₃ receptor agonist CPBG did not produce drug-lever responding or significantly alter the discriminative stimulus effects of pregnanolone or midazolam. Similarly, antagonist actions at NMDA receptors do not seem to play a role in the discriminative stimulus effects of pregnanolone; however, substitution and combination studies in both groups of rats are needed to draw this conclusion. Results of substitution studies alone might suggest a role of NMDA receptors with both phencyclidine and dizocilpine producing more than 60% drug-lever responding in rats discriminating pregnanolone. These results are similar to those obtained in a previous study in which dizocilpine produced 73% drug-lever responding in rats discriminating 5 mg/kg pregnanolone (Engel et al. 2001). However, substitution studies in rats discriminating midazolam and drug combination studies in rats discriminating either pregnanolone or midazolam suggest that NMDA receptors have very little role in the discriminative stimulus effects of pregnanolone. First, phencyclidine and dizocilpine produced at least 60% responding on the drug lever in rats discriminating midazolam, indicating that drug-lever responding produced by NMDA antagonists is not unique to rats discriminating pregnanolone; because midazolam does not act at NMDA receptors, these results suggest that something other than multiple mechanisms of action accounts for drug-lever responding produced by NMDA receptor antagonists. Although dizocilpine or phencyclidine might be acting directly at GABA_A receptors to produce these effects, it seems unlikely given an absence of binding data to indicate an interaction between these drugs and GABA_A receptors as well as drug combination studies showing that flumazenil does not attenuate effects of either dizocilpine or phencyclidine (Clineschmidt, 1982; Moreau et al. 1989; DeNoble et al. 1990). Furthermore, while leftward shifts of the midazolam dose-effect curve in rats discriminating pregnanolone would suggest that both GABA_A and NMDA receptors play a role in the discriminative stimulus effects of pregnanolone, neither phencyclidine nor dizocilpine significantly altered the midazolam dose-effect curve in either group, further supporting the notion that NMDA receptors do not contribute to the discriminative stimulus effects of pregnanolone. In rats discriminating midazolam, administration of a drug acting at receptors other than GABA_A receptors did not alter dose-effect curves for either midazolam or pregnanolone; however, in rats discriminating pregnanolone, there was a trend for small, leftward shifts of the midazolam and pregnanolone dose-effect curves produced by CPBG and dizocilpine and there was a significant change in slope of the pregnanolone dose-effect curve produced by dizocilpine. These apparent trends do not indicate that dose-effect curves are less reliable in rats discriminating pregnanolone, as compared with rats discriminating midazolam, because dose-effects curves are remarkably similar in the presence and absence of phencyclidine (upper left panel, Fig 6). Those trends might reveal exceptionally minor roles of other mechanisms in the discriminative stimulus effects of pregnanolone. Because of the high pharmacological selectivity of drug discrimination procedures, other mechanisms that appear to have minor roles in the current study could have a greater role in other effects of pregnanolone, including potential therapeutic effects.

NMDA antagonists have been shown to produce drug-lever responding under other conditions as well. For example, in rats discriminating the μ opioid receptor agonist fentanyl, ketamine, phencyclidine and dizocilpine increased drug-lever responding, although their maximal effect was less than 80% (Koek et al. 1993). These intermediate effects of NMDA antagonists in rats discriminating fentanyl were not attenuated by an opioid receptor antagonist, indicating that the effects were not mediated by opioid receptors; overall, these effects were determined to be mediated by NMDA receptors and resulted from performance deficits perhaps due to a loss of stimulus control or state dependency (Koek et al. 1993). The same behavioral mechanisms could account for drug-lever responding in rats discriminating either pregnanolone or midazolam, further demonstrating that results of the substitution studies are not indicative of a contribution of NMDA receptors to the discriminative stimulus effects of pregnanolone.

The actions of neuroactive steroids and benzodiazepines on GABA_A receptors are similar, resulting in similar behavioral effects; however, their effects are not identical. Differences can emerge between these drug classes during chronic treatment with tolerance to neuroactive steroids less likely to occur (Reddy and Rogawski 2000; McMahon and France 2002a). Recently, decreased levels of endogenous neuroactive steroids have been implicated in the development of anxiety and depression, and the use of neuroactive steroids to treat these conditions has been proposed (Schüle et al 2013). Given that positive GABA_A modulators are well known for their anxiolytic effects, actions at GABA_A receptors likely account for the role of neuroactive steroids in anxiety; however, other positive GABA_A modulators (e.g., benzodiazepines) are not effective in treating depression, raising the possibility that actions of neuroactive steroids at non-GABA_A mechanism(s) might account for their effects in depression. While neuroactive steroids are thought to act at a variety of ligand-gated ion channels, primarily because they interact with a region of the receptor that is highly conserved (Rupprecht et al. 2001; Dubrovsky 2005), the current study indicates that agonism at 5-HT₃ receptors and antagonism at NMDA receptors contributes to the discriminative stimulus effects of pregnanolone. Other possible mechanisms that might contribute to the behavioral effects of neuroactive steroids and account for their differences from benzodiazepines include changes in neurogenesis, which can be due to actions of neuroactive steroids at translocator protein (18 kDa) (TSPO), neuroprotection, and regulation of function of the HPA axis (Schüle et al 2013). While these data demonstrate that 5-HT₃ and NMDA receptors are not involved in the discriminative stimulus effects of pregnanolone, these discrimination data do not exclude a possible role of other receptors, including 5-HT₃ and NMDA receptors, in the therapeutic or other effects of neuroactive steroids.

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Highlights

- Potential role was examined for non-GABA_A receptors in effects of pregnanolone.
- Drug combinations were studied in rats discriminating pregnanolone or midazolam.
- Neither 5-HT₃ nor NMDA receptors are involved in these effects of pregnanolone.
- Non-GABA_A receptors might contribute to other effects of pregnanolone.

Figure 1.

Discriminative stimulus and rate-decreasing effects of positive GABA_A modulators (solid symbols) and drugs acting at non-GABA_A receptors (open symbols) in rats discriminating pregnanolone (left panels) or midazolam (right panels). Data were obtained using the cumulative-dosing procedure. For rats discriminating pregnanolone, data from 12 rats are shown for flunitrazepam, midazolam and pregnanolone; data from 11 rats are shown for CPBG and ketamine; data from 10 rats are shown for morphine; and data from 9 rats are shown to phencyclidine. For rats discriminating midazolam, data from 11 rats are shown for flunitrazepam, midazolam, and pregnanolone; data from 10 rats are shown for ketamine, morphine and phencyclidine; and data from 9 rats are shown for CPBG. Ordinates: top panel, percentage of total responses emitted on the drug (i.e., pregnanolone or midazolam) lever; bottom panel, average response rate. Abscissa: dose in mg/kg.

Figure 2.

Time course for the discriminative stimulus and rate-decreasing effects of phencyclidine in rats discriminating pregnanolone (left panels) or midazolam (right panels). Data, which were obtained by giving a single injection of drug immediately before the start of the session, are shown for 8 rats for all doses except 5.6 mg/kg in rats discriminating midazolam which was given to only 4 rats; a smaller dose of phencyclidine (3.2 mg/kg) markedly decreased responding for at least 2 cycles in 4 rats and consequently the larger dose was not given to those animals. Ordinates: percentage of total responses emitted on the drug (i.e., pregnanolone or midazolam) lever. Abscissa: time (min) since administration of phencyclidine.

Figure 3.

Time course for the discriminative stimulus and rate-decreasing effects of dizocilpine in rats discriminating pregnanolone (left panels) or midazolam (right panels). Data from 7 rats discriminating pregnanolone and 9 rats discriminating midazolam were obtained by giving a single injection of drug immediately before the start of the session. Ordinates: percentage of total responses emitted on the drug (i.e., pregnanolone or midazolam) lever. Abscissa: time (min) since administration of dizocilpine.

Figure 4.

Discriminative stimulus and rate-decreasing effects of midazolam administered alone (solid circles) and in combination with 3.2 mg/kg morphine (open circles) in 6 rats discriminating pregnanolone (left panels) and in 9 rats discriminating midazolam (right panels). Morphine was given 30 min before the start of a session in which midazolam dose-effect curves were determined using the cumulative-dosing procedure. Ordinates: top panel, percentage of total responses emitted on the drug (i.e., pregnanolone or midazolam) lever; bottom panel, average response rate. Abscissa: dose in mg/kg.

Figure 5.

Discriminative stimulus and rate-decreasing effects of midazolam and pregnanolone administered alone (solid symbols) and in combination with CPBG (open symbols). For rats discriminating pregnanolone (left panels), data from 6 rats are shown for all dose-effect curves. For rats discriminating midazolam (right panels), 7 rats contributed to midazolam dose-effect curves and 8 rats contributed to pregnanolone dose-effect curves. CPBG was given 30 min before the start of a session in which dose-effect curves were determined using the cumulative-dosing procedure. Ordinates: top panel, percentage of total responses emitted on the drug (i.e., pregnanolone or midazolam) lever; bottom panel, average response rate. Abscissa: dose in mg/kg.

Figure 6.

Discriminative stimulus and rate-decreasing effects of midazolam and pregnanolone administered alone (solid symbols) and in combination with phencyclidine (open symbols). For rats discriminating pregnanolone (left panels), 7 rats contributed to midazolam dose-effect curves and 9 rats contributed to pregnanolone dose-effect curves. For rats discriminating midazolam (right panels), 8 rats contributed to midazolam dose-effect curves and 6 rats contributed to pregnanolone dose-effect curves. Phencyclidine was given immediately before the start of a session in which dose-effect curves were determined using the cumulative-dosing procedure. Ordinates: top panel, percentage of total responses emitted on the drug (i.e., pregnanolone or midazolam) lever; bottom panel, average response rate. Abscissa: dose in mg/kg.

Figure 7.

Discriminative stimulus and rate-decreasing effects of midazolam and pregnanolone administered alone (solid symbols) and in combination with dizocilpine (open symbols). For rats discriminating pregnanolone (left panels), 9 rats contributed to midazolam dose-effect curves and 8 rats contributed to pregnanolone dose-effect curves. For rats discriminating midazolam (right panels), 9 rats contributed to midazolam dose-effect curves and 7 rats contributed to pregnanolone dose-effect curves. Dizocilpine was given 30 min before the start of a session in which dose-effect curves were determined using the cumulative-dosing procedure. Ordinates: top panel, percentage of total responses emitted on the drug (i.e., pregnanolone or midazolam) lever; bottom panel, average response rate. Abscissa: dose in mg/kg.

Table 1

ED₅₀ values (mg/kg $\pm$ 1 S.E.M.) for the discriminative stimulus effects of drugs that produced at least 80% responding on the drug lever.

	Pregnanolone discrimination	Midazolam discrimination
Flunitrazepam	0.05 $\pm$ 0.01	0.05 $\pm$ 0.01
Midazolam	0.36 $\pm$ 0.06	0.20 $\pm$ 0.03
Pregnanolone	2.16 $\pm$ 0.26	2.95 $\pm$ 0.35