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Discriminative stimulus effects of pregnanolone in rhesus monkeys

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Abstract

Rationale—Neuroactive steroids and benzodiazepines can positively modulate GABA by acting at distinct binding sites on synaptic GABA_A receptors. Although these receptors are thought to mediate the behavioral effects of both benzodiazepines and neuroactive steroids, other receptors (e.g., extrasynaptic GABA_A, NMDA, σ_1 , or 5-HT₃ receptors) might contribute to the effects of neuroactive steroids, resulting in differences among positive modulators.

Objective—The current study established the neuroactive steroid pregnanolone as a discriminative stimulus to determine whether actions in addition to positive modulation of synaptic GABA_A receptors might contribute to its discriminative stimulus effects.

Methods—Four rhesus monkeys discriminated 5.6 mg/kg pregnanolone while responding under a fixed-ratio 10 schedule of stimulus-shock termination.

Results—Positive modulators acting at benzodiazepine, barbiturate, or neuroactive steroid sites produced 80% pregnanolone-lever responding, whereas drugs acting primarily at receptors other than synaptic GABA_A receptors, such as extrasynaptic GABA_A, NMDA, σ_1 , and 5-HT₃ receptors, produced vehicle-lever responding. Flumazenil antagonized the benzodiazepines midazolam and flunitrazepam, with Schild analyses yielding slopes that did not deviate from unity and pA₂ values of 7.39 and 7.32, respectively. Flumazenil did not alter the discriminative stimulus effects of pregnanolone.

Conclusion—While these results do not exclude the possibility that pregnanolone acts at receptors other than synaptic GABA_A receptors, they indicate a primary if not exclusive role of synaptic GABA_A receptors in its discriminative stimulus effects. Reported differences in the chronic effects of benzodiazepines and neuroactive steroids are not due to differences in their actions at synaptic GABA_A receptors.

Keywords

pregnanolone; benzodiazepines; drug discrimination; rhesus monkeys

Drugs acting at GABA_A receptors have been used effectively to treat several disorders, such as anxiety and insomnia. Their clinical use is often limited by adverse effects, and one common strategy for reducing unwanted effects without altering therapeutic effects is to target the subset of receptors mediating the desired effect. This approach might be particularly useful for drugs acting at GABA_A receptors due to their diversity. The GABA_A receptor complex comprises 5 protein subunits, and there are multiple subtypes of each subunit; while thousands of different GABA_A receptors are possible, fewer than 20 exist in mammalian brain (McKernan and Whiting 1996; Wafford 2005; Korpi and Sinkkonen

2006). In addition to the GABA binding site, GABA_A receptors have several modulatory sites, and the presence or absence of particular modulatory sites depends on subunit composition. Most therapeutics whose effects are mediated by GABA_A receptors act at modulatory sites, including benzodiazepines and barbiturates which are positive GABA_A modulators acting at distinct sites. Another therapeutic target on GABA_A receptors is the neuroactive steroid site. Positive modulators acting at neuroactive steroid sites produce effects similar to those produced by benzodiazepines, including anxiolytic (Wieland et al. 1997), sedative (Lancel 1999; Vanover et al. 1999) and anticonvulsant effects (Kokate et al. 1994; Reddy and Rogawski 2001). While similar, the effects of positive modulators acting at different sites are not identical. For example, less tolerance and dependence develop with neuroactive steroids, as compared to benzodiazepines (Eppolito and Gerak 2010; Kokate et al. 1998; Reddy and Rogawski 2000). Thus, neuroactive steroids might retain the therapeutic effects of benzodiazepines while limiting their adverse effects, particularly tolerance and dependence.

Several factors could account for differences in the behavioral effects of benzodiazepines and neuroactive steroids. One possibility is the subunit composition of GABA_A receptors, which determines the presence of particular binding sites and selectivity for drugs acting at those sites. Benzodiazepine sites exist only on GABA_A receptors that contain two α subunits ($\alpha 1$, $\alpha 2$, $\alpha 3$, or $\alpha 5$), two β subunits, and one $\gamma 2$ subunit (Olsen and Sieghart 2009) whereas neuroactive steroid sites are thought to be present on most GABA_A receptors (Hosie et al. 2006). GABA_A receptors containing $\alpha 1\beta\gamma 2$ are the most abundant in mammalian brain, can interact with benzodiazepines and neuroactive steroids, and are located in synapses (Olsen and Sieghart 2009; Olsen et al. 2007). Other GABA_A receptors contain $\alpha 4$ or $\alpha 6$ along with δ subunits; these receptors are extrasynaptic and can be modulated by neuroactive steroids but not benzodiazepines, which could contribute to differences in their behavioral effects. Another difference between benzodiazepines and neuroactive steroids is that benzodiazepines primarily act at GABA_A receptors whereas neuroactive steroids might act at other receptors in addition to their actions at GABA_A receptors, including N-methyl-D-aspartate (NMDA), 5-hydroxytryptamine (5-HT₃), and sigma (σ_1) receptors (Maurice et al. 2001; Rupprecht et al. 2001; Dubrovsky 2005).

Although differences in behavioral effects of benzodiazepines and neuroactive steroids are primarily observed during chronic treatment, their effects are not always identical following acute administration, and differences in the acute behavioral effects have been detected using drug discrimination. In general, positive modulators produce drug-lever responding regardless of whether the training drug acts at benzodiazepine, barbiturate, or neuroactive steroid binding sites e.g., (de la Garza and Johanson 1987; Ator et al. 1993; Rowlett et al. 1999; McMahon et al. 2001; Engel et al. 2001). Collectively, those results suggest that the pharmacology of neuroactive steroids largely overlaps with that of benzodiazepines and barbiturates; however, differences in discriminative stimulus effects imply that mechanisms contributing to these effects are not identical for benzodiazepines and neuroactive steroids. For example, in rats discriminating the benzodiazepine lorazepam, neuroactive steroids do not produce lorazepam-lever responding (Ator et al. 1993). Similarly, in pigeons or rhesus monkeys discriminating the benzodiazepine midazolam, neuroactive steroids produce midazolam-lever responding in some, but not all, individuals (Evans and Johanson 1989; Gerak et al. 2008a), suggesting that the discriminative stimulus effects of benzodiazepines and neuroactive steroids are not identical. It is interesting to note that in rats discriminating midazolam, neuroactive steroids produce 80% midazolam-lever responding in all subjects (Bai and Gerak 2011). Barbiturates also seem to be more likely to produce midazolam-lever responding in rats (Ator 1990; 1999; Garcha et al. 1985; Woudenberg and Slangen 1989; but see also Bai and Gerak 2011), as compared to squirrel monkeys (Spealman 1985), baboons (Ator 2003), rhesus monkeys (Gerak et al. 2008a), and pigeons (Evans and Johanson 1989).

Taken together, these results indicate that effects obtained with positive GABA_A modulators acting at different sites are not identical and effects obtained in one species do not necessarily predict effects obtained in others.

The neuroactive steroid pregnanolone has been established as a discriminative stimulus in rodents. Benzodiazepines produce 80% pregnanolone-lever responding in rats (Vanover 1997; 2000; Engel et al. 2001; Gerak et al. 2008b) or mice (Shannon et al. 2005a; b); however, it appears as though differences among positive GABA_A modulators are not as likely to be detected in rodents, as compared to other species. Thus, it is important to establish a pregnanolone discriminative stimulus in other species to examine the generality of results obtained in rodents.

The current study established pregnanolone as a discriminative stimulus in rhesus monkeys and compared results to those reported in monkeys discriminating midazolam to examine whether the effects of pregnanolone are due to actions at synaptic GABA_A receptors or whether other mechanisms play a role. The involvement of GABA_A receptors in the discriminative stimulus effects of pregnanolone was examined by determining whether positive modulators acting at synaptic GABA_A receptors as well as drugs acting at other receptors, including extrasynaptic GABA_A (gaboxadol), NMDA (ketamine, dizocilpine), σ_1 (nallylnormetazocine), and 5-HT₃ [1-(m-chlorophenyl)-biguanide (CPBG)] receptors, produced effects similar to those of pregnanolone. Similarities in the discriminative stimulus effects of benzodiazepines and neuroactive steroids would support a predominant role of synaptic GABA_A receptors, and this hypothesis was tested further using drug combination studies with flumazenil, which acts at benzodiazepine sites and attenuates the effects of benzodiazepines. In monkeys discriminating midazolam, flumazenil shifted dose-effect curves rightward (Lelas et al. 1999; 2000; Gerak and France 2012); in the same monkeys, flumazenil enhanced the midazolam-like effects of neuroactive steroids, shifting dose-effect curves leftward (McMahon and France 2005; Gerak and France 2011; 2012), and reflecting the limited positive modulatory actions of flumazenil at benzodiazepine sites on GABA_A receptors (Dantzer and Prio 1982). To the extent that the same synaptic GABA_A receptors that mediate the effects of benzodiazepines in monkeys discriminating midazolam also mediate the effects of neuroactive steroids in monkeys discriminating pregnanolone, then effects obtained in the current study should be similar to those reported in monkeys discriminating midazolam.

Methods

Subjects

Four experimentally nave male rhesus monkeys were between 4.5 and 5.75 years old when the experiment began. They were housed individually on a 14 hr light and 10 hr dark cycle with free access to water in their home cage. Monkeys received primate chow (Harlan Teklad, High Protein Monkey Diet, Madison, WI), fresh fruit and peanuts daily. Over the course of the study, which lasted at least 2 years, their weights increased from 4.5–6.9 kg to 8.5–11 kg. Monkeys were maintained in accordance with the Institutional Animal Care and Use Committee, The University of Texas Health Science Center at San Antonio, and with the 2011 Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources 2011).

Apparatus

During experimental sessions, monkeys were seated in chairs (Primate Products, Miami, FL), which were placed in ventilated, sound-attenuating chambers. White noise was present in each chamber. Two stimulus lights and two levers were mounted on the chamber wall

within easy reach of seated monkeys. The feet of the monkeys were placed in shoes mounted on the front of the chair. Shoes contained brass electrodes to which brief (250 msec, 3mA) electric shock could be delivered from an a.c. shock generator located outside of chambers. Experimental events were controlled and data were recorded by a computer that used MED-PC IV software (MED Associates, Inc., St. Albans, VT) and was connected to the chambers by a commercially available interface (MED Associates, Inc., St. Albans, VT).

Procedure

Four monkeys discriminated 5.6 mg/kg pregnanolone while responding under a fixed-ratio 10 schedule of stimulus-shock termination. Because there were no previous studies in which monkeys discriminated pregnanolone, the initial dose and temporal conditions used to establish stimulus control were similar but not identical across monkeys, although eventually conditions were the same for all monkeys. For example, a training dose of 3.2 mg/kg was used initially for 2 monkeys; after 100 training sessions, adequate stimulus control had been established only in one monkey. Consequently, the training dose was increased to 5.6 mg/kg in both monkeys and training began with 5.6 mg/kg in the 2 monkeys that were trained later. These slight variations in training did not appear to alter the pregnanolone discriminative stimulus, as results were indistinguishable across subjects.

Sessions were conducted 7 days per week, lasted up to 2 hr, and were divided into 15-min cycles, beginning with 10-min timeout periods, when chambers were dark and responding had no programmed consequence, and ending with 5-min response periods, when red stimulus lights were illuminated and the schedule of stimulus-shock termination was in effect. In the presence of red lights, 10 consecutive responses on the lever designated correct by an injection administered during the first minute of the cycle extinguished lights and postponed the shock schedule for 30 sec. Responses on the incorrect lever reset the response requirement on the correct lever. The lever designated correct after administration of pregnanolone was counterbalanced across monkeys. When the response requirement was not satisfied within 15 sec of illumination of lights, brief electric shock was delivered and continued to be delivered every 15 sec until the response requirement was satisfied or the cycle ended either because four shocks were delivered or 15 min elapsed since the beginning of the cycle.

For some training sessions, 5.6 mg/kg pregnanolone was given in the first cycle followed by a sham injection given in the next cycle; responding on the pregnanolone lever postponed shock during both cycles because the discriminative stimulus effects of 5.6 mg/kg pregnanolone last at least 2 hr in rhesus monkeys (unpublished observation). Other training sessions comprised 2–8 cycles in which vehicle or sham injections were administered and responding on the vehicle lever postponed shock during all cycles. Still other training sessions began with up to 6 cycles in which vehicle or sham was given, followed by a cycle in which 5.6 mg/kg pregnanolone was given and one subsequent sham cycle. Stimulus control was considered adequate to begin testing when the following criteria were satisfied for 5 consecutive or 6 of 7 training sessions: 0% responding on the correct lever and fewer than 10 responses on the incorrect lever prior to completion of the first fixed ratio on the correct lever. Thereafter, test sessions were conducted every third day as long as the testing criteria were satisfied during intervening training sessions; otherwise training continued until the criteria were satisfied for 2 consecutive sessions.

Test sessions were identical to training sessions except that 10 consecutive responses on either lever postponed shock and test compounds were administered. To determine whether the discriminative stimulus effects of rugs from different pharmacological classes were similar to those of pregnanolone, dose-effect curves were determined using an acute

(gaboxadol and dizocilpine) or cumulative (all other drugs) dosing procedure. For cumulative dosing, vehicle was given on the first cycle and a test compound was given beginning on the second cycle with the cumulative dose increasing by $\frac{1}{4}$ log unit per cycle. Dosing continued until monkeys responded $\geq 80\%$ on the pregnanolone lever, response rates decreased sufficiently to result in the delivery of shock, or solubility of drug precluded further increases in dose. The cumulative dosing procedure could not be used with gaboxadol and dizocilpine due to their slow onset of action; consequently, a single dose was given on the first cycle of a session comprising 6 cycles with sham injections given during each cycle after the first. Different doses were given during separate test sessions, beginning with an ineffective dose and increasing in $\frac{1}{4}$ log unit increments up to a dose that markedly decreased responding in at least 2 monkeys. At large doses, response rates were decreased sufficiently to result in the delivery of shock; when shock was delivered in two consecutive cycles, the session was stopped and the effects of drug at later time points were not determined. The ability of flumazenil to alter the discriminative stimulus effects of benzodiazepines and neuroactive steroids was determined by giving a single dose of flumazenil (0.01–0.32 mg/kg) on the first cycle and cumulative doses of a test compound on subsequent cycles, up to doses that produced $\geq 80\%$ responding on the pregnanolone lever.

Drugs

Pregnanolone (5 β -pregnan-3 α -ol-20-one; Research Technology Branch, National Institute on Drug Abuse, Rockville, MD) and allopregnanolone (5 α -pregnan-3 α -ol-20-one; Bujno Synthesis, Warsaw, Poland) were dissolved in 45% (w/v) 2-hydroxypropyl- β -cyclodextrin. Midazolam hydrochloride (Hospira Inc, Lake Forest, IL) and ketamine hydrochloride (Butler Animal Health Supply, Galway, Ireland) were purchased as commercially prepared solutions and diluted with sterile water. Flunitrazepam (Research Technology Branch, National Institute on Drug Abuse, Rockville, MD) was dissolved in a vehicle containing 20% emulphor, 10% ethanol and 70% sterile water. Pentobarbital sodium, gaboxadol hydrochloride, 1-(m-chlorophenyl)-biguanide hydrochloride (CPBG), dizocilpine hydrogen maleate, and chlorpromazine hydrochloride (Sigma-Aldrich Co., St. Louis, MO), along with n-allylnormetazocine hydrochloride (Research Technology Branch, National Institute on Drug Abuse, Rockville, MD), were dissolved in sterile water. Haloperidol (Sigma-Aldrich Co., St. Louis, MO) was dissolved in water containing a small amount of lactic acid; once dissolved, the pH of the solution was increased to ≥ 5 with 5M NaOH. Flumazenil (Enzo Life Sciences, Farmingdale, NY) was dissolved in a vehicle comprising 10% ethanol, 40% propylene glycol, and 50% sterile water. Doses are expressed in terms of the forms listed above in mg/kg body weight. Drugs were administered s.c. in volumes ranging from 0.3–2 ml.

Data Analyses

Discrimination data, expressed as a percentage of total responses emitted on the pregnanolone lever, and response rates, expressed as responses/sec, were averaged across monkeys (± 1 SEM) and plotted as a function of dose or time. Mean control response rates were obtained by averaging rates across cycles for training sessions in which monkeys received only vehicle or sham injections and satisfied the testing criteria during each cycle. Control rates represent the average of the means for 5 training sessions. During test sessions, cumulative dosing for an individual monkey stopped when that individual responded at least 80% on the pregnanolone lever; in cases where larger doses were studied in other monkeys, a value of 100% pregnanolone-lever responding was entered for that individual at those larger doses and used in the group analyses (Gerak and France 2012). With the exception of CPBG, which was determined once in 3 monkeys, all tests were determined twice in 4 monkeys; data were averaged for individuals before the group mean was obtained.

To determine whether flumazenil altered the discriminative stimulus effects of benzodiazepines or neuroactive steroids, ED₅₀ values obtained in the presence and absence of flumazenil were compared. First, straight lines were fitted to individual dose-effect curves for each monkey using one dose that produced not more than 25% responding on the drug lever, one dose that produced at least 75% responding on the drug lever, and all doses in between. Then, ED₅₀ values were calculated using linear regression or interpolation, depending on the number of data points available. For individuals, potency ratios were obtained by dividing ED₅₀ values determined with flumazenil by ED₅₀ values determined without flumazenil. A significant change in the potency of a benzodiazepine or neuroactive steroid was detected when the 95% confidence intervals of the potency ratios averaged among monkeys did not include 1. When flumazenil antagonized the discriminative stimulus effects of a positive modulator, dose ratios were also used to construct Schild plots using the method of Arunlakshana and Schild (1959) and GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA). For individuals, log (dose ratio-1) was calculated; the mean among monkeys was then plotted as a function of flumazenil dose (-log [mol/kg]) and straight lines were fitted. An F-ratio test was used to compare Schild plots; if the calculated F value was not significant, then the data were best fit when slopes were constrained to unity, rather than being allowed to vary, and apparent pA₂ values were compared using an F-ratio test (Kenakin 1993; Koek et al. 2000). When slopes were not constrained, apparent pA₂ values (95% CI) were determined using linear regression. Significance was set at P<0.05.

Results

On average, 174 sessions (range: 112–234 sessions, beginning with the first session in which monkeys received pregnanolone) were required for monkeys to satisfy the testing criteria. After stimulus control was established, the mean response rate (± 1 SEM) was 2.42 ± 0.41 responses/sec in vehicle training sessions and 2.56 ± 0.16 responses/s in pregnanolone (5.6 mg/kg) training sessions; rates were not significantly changed over the course of the experiment (data not shown). During test sessions, administration of vehicle resulted in responding on the vehicle lever and no change in rates (points above V, Figure 1). Pregnanolone dose dependently increased responding on the pregnanolone lever with 3.2 mg/kg producing, on average, 98% drug-lever responding (circles, upper panel, Figure 1). The mean ED₅₀ value (mean ± 1 S.E.M.) was 1.57 ± 0.27 mg/kg. Pregnanolone (3.2 mg/kg) modestly increased response rates.

Other positive GABA_A modulators produced 80% pregnanolone-lever responding (upper panel, Figure 1). The benzodiazepines midazolam and flunitrazepam were the most potent with ED₅₀ values for discriminative stimulus effects of 0.28 ± 0.05 mg/kg and 0.11 ± 0.01 mg/kg, respectively. The neuroactive steroid allopregnanolone (ED₅₀ = 0.82 ± 0.16 mg/kg) was 2-fold more potent than pregnanolone whereas the barbiturate pentobarbital (ED₅₀ = 6.98 ± 0.52 mg/kg) was the least potent. These positive modulators did not markedly alter response rates (lower panel, Figure 1).

The pharmacological selectivity of the pregnanolone discriminative stimulus was further tested with drugs whose primary mechanisms of action are not at synaptic GABA_A receptors. The dopamine receptor antagonists haloperidol and chlorpromazine, the NMDA receptor antagonist ketamine, and the σ receptor agonist n-allylnormetazocine produced predominantly vehicle-lever responding (upper panel, Figure 2) up to doses that decreased response rates (lower panel, Figure 2). The 5-HT₃ receptor agonist CPBG also produced predominantly vehicle-lever responding, although response rates were not decreased up to 56 mg/kg, the largest dose that could be studied due to solubility. In addition, neither the extrasynaptic GABA_A receptor agonist gaboxadol nor the NMDA receptor antagonist

dizocilpine produced >20% pregnanolone-lever responding at any dose (upper panel, Figure 3), and the largest doses of gaboxadol and dizocilpine eliminated responding in 2 and 4 monkeys, respectively (lower panels, Figure 3).

Flumazenil antagonized the discriminative stimulus effects of midazolam and flunitrazepam (Figure 4). A small dose of flumazenil (0.0178 mg/kg) did not significantly change the potency of midazolam; the mean potency ratio for midazolam (95% CI) was 2.63 (0.84, 4.42). Larger doses of flumazenil significantly attenuated the discriminative stimulus effects of both midazolam and flunitrazepam. The mean potency ratio obtained for midazolam in combination with 0.032 mg/kg of flumazenil was 3.90 (2.39, 5.40) and the mean potency ratio for flunitrazepam with the same dose of flumazenil was 3.09 (1.97, 4.21). Straight lines fitted to Schild plots (Figure 5) indicated that slopes [$F(1,24)=0.14$, n.s.] and intercepts [$F(1,26)=1.373$, n.s.] were not significantly different, and the common slope was not different from unity [$F(1,25)=0.252$, n.s.]. Thus, the data were best fit by the simplest model with a common slope and a common intercept. When the common slope was constrained to unity, the apparent pA_2 value was 7.36. When analyzed independently, the unconstrained slope of each Schild plot was not significantly different from unity yielding apparent pA_2 values that were also not significantly different (Table 1).

A dose of 0.1 mg/kg of flumazenil, which shifted the midazolam and flunitrazepam dose-effect curves significantly rightward (Figures 4 and 5), did not alter the discriminative stimulus effects of pregnanolone (Figure 6). A mean potency ratio of 1.08 (0.63, 1.53) was obtained when pregnanolone was studied with 0.1 mg/kg of flumazenil. Despite a trend for a leftward shift in the pregnanolone dose-effect curve, a larger dose of flumazenil (0.32 mg/kg) did not significantly alter the dose-effect curve of either pregnanolone (mean potency ratio=0.89 [0.46, 1.31]) or allopregnanolone (mean potency ratio=1.00 [0.51, 1.48]; Figure 6).

Discussion

Benzodiazepines and neuroactive steroids can positively modulate GABA, although they act at distinct sites on synaptic $GABA_A$ receptors. Both increase Cl^- influx and their acute behavioral effects are similar e.g., (Ator et al. 1993; Engel et al. 2001; McMahon and France 2005; Bai and Gerak 2011), although not identical (e.g., development of tolerance to benzodiazepines and not neuroactive steroids; Gonsalves and Gallagher 1987; Löscher et al. 1996; Kokate et al. 1998; Reddy and Rogawski 2000; McMahon and France 2002a; b). The current study established pregnanolone as a discriminative stimulus in monkeys to determine whether actions at receptors other than $GABA_A$ receptors might contribute to the discriminative stimulus effects of pregnanolone, as has been shown previously in rats. Although the current data do not exclude the possibility that pregnanolone has actions at receptors other than synaptic $GABA_A$ receptors, other receptors do not play a role in the effects of pregnanolone when it is trained as a discriminative stimulus in rhesus monkeys.

Positive $GABA_A$ modulators acting at different sites on $GABA_A$ receptors produced pregnanolone-lever responding in monkeys, despite differences in pharmacokinetics and solubility. Because benzodiazepines act at synaptic $GABA_A$ receptors, and not at either extrasynaptic $GABA_A$ or other (non-GABA) receptors, their substitution for pregnanolone suggests that synaptic $GABA_A$ receptors mediate the pregnanolone discriminative stimulus in monkeys. Gaboxadol did not produce pregnanolone-lever responding, further suggesting little or no role for extrasynaptic $GABA_A$ receptors in the discriminative stimulus effects of pregnanolone. Receptors that have been implicated in the behavioral effects of neuroactive steroids (Maurice et al. 2001; Rupprecht et al. 2001; Dubrovsky 2005), including discriminative stimulus effects of pregnanolone (Engel et al. 2001; Shannon et al. 2005b),

do not seem to have a prominent role in the pregnanolone discriminative stimulus in monkeys. That is, drugs with actions at NMDA or σ_1 receptors did not produce pregnanolone-lever responding up to doses that decreased rates. While CPBG produced only vehicle-lever responding, suggesting that 5-HT₃ receptors also do not contribute to the discriminative stimulus effects of pregnanolone, it did not decrease response rates up to the largest dose that could be studied. Thus, in monkeys, positive GABA_A modulators shared discriminative stimulus effects with pregnanolone and drugs acting at other receptors did not; these results, along with those of other studies (Vanover 1997; 2000; Engel et al. 2001; Shannon et al. 2005a; b; Eppolito et al. 2012), support a primary role of synaptic GABA_A receptors in the discriminative stimulus effects of pregnanolone.

This role of synaptic GABA_A receptors is further supported by drug combination studies. Flumazenil, which acts at benzodiazepine sites and has very low efficacy, antagonized the discriminative stimulus effects of benzodiazepines in monkeys discriminating either midazolam or pregnanolone. When shifts in dose-effect curves were compared using Schild analyses, the slope of the straight line fitting these data was not different from unity, suggesting a simple, competitive and reversible interaction. The potency of flumazenil, estimated using pA₂ values, was similar in monkeys discriminating pregnanolone and those discriminating midazolam, where pA₂ values for flumazenil ranged from 7.41 to 7.69 (Lelas et al. 1999; 2000; Gerak and France 2012). Similarity pA₂ values suggests that the same population of synaptic GABA_A receptors mediates the discriminative stimulus effects of midazolam (Lelas et al. 2000; Gerak and France 2012) and those of pregnanolone (current study) in rhesus monkeys.

Competition between flumazenil and benzodiazepines for benzodiazepine sites results in attenuation of the effects of benzodiazepines (current study; Lelas et al. 1999; 2000; Gerak and France 2012). In contrast, neuroactive steroids act a distinct site (i.e., the interaction between flumazenil and neuroactive steroids is not competitive); consequently, flumazenil would not be expected to antagonize the discriminative stimulus effects of pregnanolone. In monkeys discriminating pregnanolone, doses of flumazenil that shifted benzodiazepine dose-effect curves rightward did not shift the pregnanolone dose-effect curve. Similarly, flumazenil also did not attenuate the effects of pregnanolone in monkeys discriminating midazolam (Gerak and France 2011; 2012). However, in those same animals, flumazenil enhanced the effects of neuroactive steroids, shifting dose-effect curves significantly leftward (Gerak and France 2011; 2012; McMahon and France 2005) due to its low positive modulatory effects at benzodiazepine sites (Dantzer and Prio 1982); if the effects of flumazenil are the same in the two groups, then a similar increase in the potency of neuroactive steroids would be expected in monkeys discriminating pregnanolone. In the current study, flumazenil did not enhance the discriminative stimulus effects of neuroactive steroids. This small difference between the two procedures does not appear to reflect weaker stimulus control for one discriminative stimulus. Time to acquisition of stimulus control cannot be easily compared across groups because training conditions were slightly different across monkeys discriminating pregnanolone early in training; however, the two discriminations were equally robust and stable over time with both groups tested with the same frequency. These results also indicate that the differences in training across monkeys discriminating pregnanolone did not have lingering effects. In addition, the potency and effectiveness of benzodiazepines and pregnanolone were similar in the two groups; midazolam and flunitrazepam were equipotent with pregnanolone 20-fold less potent. Drugs acting primarily at non-GABA_A receptors did not produce responding on either the pregnanolone or midazolam lever. Antagonism of the discriminative stimulus effects of benzodiazepines by flumazenil was similar, with comparable pA₂ values indicating that the same population of GABA_A receptors is involved. Thus, the pregnanolone and midazolam discriminative stimuli appear to be very similar despite the ability of flumazenil to enhance

the discriminative stimulus effects of pregnanolone in monkeys discriminating midazolam and not in those discriminating pregnanolone.

One possible explanation for differential effects of flumazenil across the two groups involves actions of pregnanolone at extrasynaptic GABA_A receptors. Similarities in the effects of benzodiazepines and neuroactive steroids demonstrate the importance of synaptic GABA_A receptors in the pregnanolone discriminative stimulus, although extrasynaptic GABA_A receptors might still play a role. Some extrasynaptic GABA_A receptors contain $\alpha 4$ or $\alpha 6$ subunits. Flumazenil, but not benzodiazepines, binds to GABA_A receptors containing these α subunits; however, the manner in which flumazenil interacts with these receptors (positive, neutral or negative modulation) is not known. The ability of flumazenil to enhance the discriminative stimulus effects of neuroactive steroids in monkeys discriminating midazolam and not in monkeys discriminating pregnanolone could be due to interactions between flumazenil and neuroactive steroids at extrasynaptic GABA_A receptors. If so, actions at extrasynaptic GABA_A receptors might account for differences between benzodiazepines and neuroactive steroids.

The current study established pregnanolone as a discriminative stimulus in rhesus monkeys, which has not been reported previously, and demonstrated the importance of synaptic GABA_A receptors in the discriminative stimulus effects of pregnanolone by showing that benzodiazepines and neuroactive steroids produce remarkably similar effects in monkeys discriminating pregnanolone and those discriminating midazolam. Such similarities in the behavioral effects of neuroactive steroids and benzodiazepines support the therapeutic use of neuroactive steroids, either in addition to or in place of benzodiazepines (Morrow 2007). The qualitative difference in the interaction between flumazenil and pregnanolone depending on the training drug might be indicative of a subtle difference in the behavioral effects of benzodiazepines and neuroactive steroids, which could provide a therapeutic advantage by reducing the incidence of adverse effects (e.g., tolerance).

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Figure 1.

Discriminative stimulus effects of positive GABA_A modulators and their effects on response rate in monkeys discriminating pregnanolone. Each curve was determined twice in 4 monkeys. Abscissae: cumulative dose in milligrams per kilogram body weight. Data above V represent the effects of vehicle 15 min thereafter. Ordinate, upper panel: mean ($\pm$ 1 SEM) percentage of total responding that occurred on the pregnanolone-appropriate lever; lower panel: rate (responses/sec $\pm$ 1 SEM).

Figure 2.

Discriminative stimulus and rate-decreasing effects of drugs whose primary mechanism of action is not at GABA_A receptors in monkeys discriminating pregnanolone. Each curve was determined twice in 4 monkeys. Abscissae: cumulative dose in milligrams per kilogram body weight. Data above V represent the effects of vehicle given on the first cycle with cumulative doses of drug given every 15 min thereafter. Ordinate, upper panel: mean ($\pm$ 1 SEM) percentage of total responding that occurred on the pregnanolone-appropriate lever; lower panel: rate (responses/sec $\pm$ 1 SEM).

Figure 3.

Discriminative stimulus and rate-decreasing effects of gaboxadol and dizocilpine in monkeys discriminating pregnanolone. Because of the long onset of these drugs, the effects of each dose of gaboxadol and dizocilpine were monitored over 90-min sessions with each dose studied twice in 4 monkeys. Abscissae: time (min) since administration of gaboxadol (left panels) or dizocilpine (right panels). Ordinate, upper panel: mean (± 1 SEM) percentage of total responding that occurred on the pregnanolone-appropriate lever; lower panel: rate (responses/sec ± 1 SEM).

Figure 4. Discriminative stimulus effects of midazolam (upper panel) and flunitrazepam (lower panel) given alone (circles) or after various doses of flumazenil in monkeys discriminating pregnanolone. Each curve was determined twice in 4 monkeys. Abscissae: cumulative dose in milligrams per kilogram body weight; data above F represent the effects of flumazenil given on the first cycle with cumulative doses of the benzodiazepine given every 15 min thereafter. Ordinates: mean (± 1 SEM) percentage of total responding that occurred on the pregnanolone-appropriate lever.

Figure 5.

Schild plot for flumazenil studied with midazolam and flunitrazepam. Raw data are shown in Figure 4 and symbols match those used in Figure 4. Abscissa: negative log of the molar dose of flumazenil. Ordinate: $\log(\text{dose-ratio} - 1)$.

Figure 6.

Discriminative stimulus effects of pregnanolone (upper panel) and allopregnanolone (lower panel) given alone (circles) or after various doses of flumazenil in monkeys discriminating pregnanolone. Each curve was determined twice in 4 monkeys. Abscissa: cumulative dose in milligrams per kilogram body weight; data above F represent the effects of flumazenil given on the first cycle with cumulative doses of the neuroactive steroid given every 15 min thereafter. Ordinate: mean (± 1 SEM) percentage of total responding that occurred on the pregnanolone-appropriate lever.

Table 1

Slopes of Schild plots and pA_2 values (95% confidence limits) for flumazenil with midazolam and with flunitrazepam determined with constrained and unconstrained slopes.

	Slope (unconstrained)	pA_2 (unconstrained)	pA_2 (constrained)
Midazolam	-1.03 (-1.40, -0.66)	7.37 (7.21, 7.69)	7.39 (7.30, 7.49)
Flunitrazepam	-1.14 (-1.57, -0.71)	7.25 (7.09, 7.58)	7.32 (7.24, 7.40)