Discriminative Stimulus Effects of Positive GABA\textsubscript{A} Modulators and Other Anxiolytics, Sedatives, and Anticonvulsants in Untreated and Diazepam-Treated Monkeys

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ABSTRACT

Positive GABA\textsubscript{A} modulators and other sedatives, anxiolytics, and anticonvulsants were used to evaluate mechanisms underlying the discriminative stimulus effects of midazolam in untreated monkeys and of flumazenil in monkeys treated with diazepam (5.6 mg/kg/day). Positive GABA\textsubscript{A} modulators at benzodiazepine (e.g., flunitrazepam and abecarnil) and neuroactive steroid sites (e.g., androsterone) substituted for midazolam in all monkeys; the neuroactive steroids dihydroandrosterone and epipregnanolone substituted for midazolam in two of three monkeys. All positive GABA\textsubscript{A} modulators attenuated flumazenil in diazepam-treated monkeys; doses of flunitrazepam and abecarnil larger than doses substituting for midazolam were required to attenuate flumazenil, whereas doses of neuroactive steroids smaller than doses substituting for midazolam attenuated flumazenil. Drugs with mechanisms that do not predominantly involve allosteric modulation of GABA (e.g., buspirone, ketamine, valproic acid, and diphenhydramine) did not substitute for midazolam or flumazenil. However, valproic acid enhanced the midazolam discriminative stimulus and attenuated the flumazenil discriminative stimulus; diphenhydramine attenuated the midazolam discriminative stimulus. These results suggest that drugs not sharing a mechanism of action with benzodiazepines can modulate the behavioral effects of benzodiazepines. In addition, this study demonstrates that endogenous ligands, presumably by acting at neuroactive steroid sites on the GABA\textsubscript{A} receptor complex, share discriminative stimulus effects with benzodiazepines. This study also suggests that positive GABA\textsubscript{A}-modulating neuroactive steroids are especially potent in attenuating behavioral effects that are related to diazepam withdrawal.

Benzodiazepines that positively modulate GABA at the GABA\textsubscript{A} receptor complex are the most commonly prescribed drugs for anxiety, insomnia, and convulsions because they are safe and effective (for review, see Woods et al., 1992). However, long-term daily treatment with benzodiazepines can lead to tolerance and, as evidenced by withdrawal signs that emerge upon discontinuation of treatment, dependence. A variety of approaches have been used to study benzodiazepine tolerance and dependence (e.g., observational procedures; Lukas and Griffiths, 1982), including drug discrimination. For example, the benzodiazepine antagonist flumazenil has been established as a discriminative stimulus in rhesus monkeys treated daily with the benzodiazepine diazepam (Gerak and France, 1999; McMahon et al., 2001). The consequences of chronic benzodiazepine treatment (e.g., tolerance) have been examined by comparing the discriminative stimulus effects of various compounds in diazepam-treated monkeys discriminating flumazenil to the discriminative stimulus effects of the same compounds in untreated monkeys discriminating midazolam (Lelas et al., 1999; McMahon et al., 2001, 2002). Such comparisons might be relevant to benzodiazepine dependence and withdrawal because the flumazenil discriminative stimulus in diazepam-treated monkeys is qualitatively similar to discriminative stimulus effects that emerge when diazepam treatment is temporarily discontinued (Gerak and France, 1999).

In addition to benzodiazepines, some barbiturates and neuroactive steroids positively modulate GABA by acting at nonbenzodiazepine sites on the GABA\textsubscript{A} receptor complex (Gee et al., 1988; Turner et al., 1989). The effects of positive modulators acting at different sites have been shown to be qualitatively similar in untreated and diazepam-treated monkeys, i.e., benzodiazepines, barbiturates, and a neuroactive steroid substituted for midazolam and attenuated flumazenil in diazepam-treated monkeys (McMahon et al., 2001). On the other hand, the potency of positive modulators acting...
Effects of flumazenil in diazepam-treated monkeys. For example, doses of benzodiazepines larger than doses substituting for midazolam in untreated monkeys were required to attenuate flumazenil in diazepam-treated monkeys. The opposite relationship was revealed for nonbenzodiazepine ligands, i.e., doses of barbiturates and a neuroactive steroid smaller than doses substituting for midazolam attenuated flumazenil in diazepam-treated monkeys. One goal of the present study was to determine whether similar differences in relative potency are evident in untreated and diazepam-treated monkeys for other positive modulators acting at benzodiazepine (e.g., flunitrazepam and abecarnil) and neuroactive steroid sites (e.g., androsterone, dihydroandrosterone, and epipregnanolone).

Previous studies have demonstrated that efficacy in positively modulating GABA is an important determinant of discriminative stimulus effects in untreated and diazepam-treated monkeys, i.e., low-efficacy benzodiazepines (e.g., brexarenzil) substituted for flumazenil and not for midazolam (Gerak and France, 1999; Lelas et al., 1999). Thus, another goal of this study was to further evaluate the importance of efficacy as a determinant of discriminative stimulus effects in untreated and diazepam-treated monkeys. Positive modulators chosen for study were reported to have low efficacy at some benzodiazepine receptor subtypes comprising $\alpha_1$, $\alpha_2$, $\alpha_3$, and $\alpha_4$-subunits (e.g., abecarnil; Smith et al., 2001) or low efficacy at neuroactive steroid sites (e.g., dihydroandrosterone and epipregnanolone; Park-Chung et al., 1999).

Thus far, the features of the midazolam and flumazenil discriminative stimulus in untreated and diazepam-treated monkeys, respectively, have been evaluated with GABA$_A$ modulators. A number of other drugs that do not have as a primary mechanism of action modulation of GABA share certain effects with benzodiazepines and other positive GABA$_A$ modulators. For example, buspirone has anxiolytic effects (Uhlenhuth, 1982), ketamine has dissociative-anesthetic effects (Sadove et al., 1971), and diphenhydramine has sedative-hypnotic effects (Sunshine et al., 1978). Other drugs, such as $\gamma$-hydroxybutyrate (GHB; Mamelak et al., 1977) and valproic acid (Bruni and Wilder, 1979) can influence GABA transmission; however, the mechanism of action that mediates the behavioral effects of these compounds is unclear. Thus, this study examined whether these compounds substitute for or modulate the discriminative stimulus effects of midazolam. In addition, the present study examined whether non-GABAergic drugs that modulate various aspects of benzodiazepine withdrawal (e.g., buspirone, File and Andrews, 1991; valproic acid, Harris et al., 2000) substitute for or modulate the discriminative stimulus effects of flumazenil in diazepam-treated monkeys.

### Materials and Methods

#### Subjects

Adult rhesus monkeys (*Macaca mulatta*) discriminating midazolam (four females and one male) or flumazenil (one female and four males) were housed individually on a 14-h light/10-h dark schedule and maintained at 95% free-feeding weight (range 3.8–10.0 kg) with a diet provided in the home cage comprising primate chow (High Protein Monkey Diet; Harlan Teklad, Madison, WI), fresh fruit, peanuts, and water. Monkeys discriminating flumazenil were treated daily with diazepam (5.6 mg/kg p.o.) for at least 1 year before these studies. The animals used in these studies 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 1996 Guide for the Care and Use of Laboratory Animals (Committee on Laboratory Animal Resources, National Research Council, National Academy of Sciences).

#### Apparatus

Monkeys were trained to discriminate midazolam or flumazenil as described previously (Gerak and France, 1999; Lelas et al., 1999). During experimental sessions, monkeys were seated in chairs (model R001; Primate Products, Miami, FL) that provided neck restraint and placed in ventilated, sound-attenuating chambers equipped with two response levers, lights, and a food cup into which pellets could be delivered from a dispenser. For monkeys discriminating midazolam under a schedule of stimulus-shock termination, their feet were placed in shoes containing brass electrodes through which a brief electric stimulus (3 mA, 250 ms) could be delivered from an A/C generator. An interface (MED Associates, St., Albans, VT) connected the chambers to a computer, which controlled and recorded experimental events.

#### Midazolam Discrimination Procedure

Experimental sessions consisted of multiple 15-min cycles, each comprising a 10-min timeout period, during which responses had no programmed consequence. A 5-min response period followed, during which red lights were illuminated, thereby signaling the beginning of the response period in which an electric stimulus could be delivered every 15 s. The correct lever was designated by an injection (saline or midazolam) during the first minute of the cycle; designation of correct levers (e.g., left, saline; right, midazolam) varied among monkeys and remained the same for an individual throughout the study. Ten consecutive responses (fixed ratio 10) on the correct lever extinguished the red lights and postponed delivery of the electric stimulus for 30 s. Responding on the incorrect lever reset the response requirement on the correct lever. Response periods ended after 5 min or after the delivery of four electric stimuli, whichever occurred first.

Saline training comprised administration of saline or sham injections during the first minute of each of no more than eight cycles. Midazolam training sessions comprised administration of midazolam (0.56 mg/kg for three monkeys or 0.32 mg/kg for two monkeys s.c.) during the first minute of a cycle followed by a saline or sham injection during the first minute of a second cycle; midazolam training cycles could be preceded by one to six saline or sham injection cycles. Test sessions were conducted after training sessions in which ≥80% of the total responses occurred on the correct lever and fewer than 10 responses occurred on the incorrect lever before the first injection of the response requirement on the correct lever. Before each test, these criteria had to be satisfied for both midazolam and saline training sessions. Test sessions were identical to training sessions except that 10 consecutive responses on either lever postponed the schedule. Cumulative midazolam dose-effect tests were conducted by injecting saline during the first minute of the first cycle followed by increasing doses (0.25 or 0.5 log unit per cycle) of midazolam during the first minute of subsequent cycles. On separate occasions, single doses of the following drugs were injected s.c. during the first minute of the first cycle followed by saline or sham injections on subsequent cycles: flunitrazepam (0.01–0.1 mg/kg), abecarnil (0.1–1.0 mg/kg), androsterone (10.0–56.0 mg/kg), dihydroandrosterone (32.0 and 100.0 mg/kg), epipregnanolone (32.0 and 100.0 mg/kg), buspirone (0.1–1.0 mg/kg), valproic acid (320.0–1000.0 mg/kg), ketamine (1.0 and 3.2 mg/kg), and GHB (560.0 mg/kg). On separate occasions, single doses of the following drugs were injected s.c. during the first minute of the first cycle followed by cumulative doses of midazolam during subsequent cycles: buspirone (0.1 and 0.32 mg/kg), diphenhydramine (3.2–17.8 mg/kg), valproic acid (320.0–1000.0 mg/kg), GHB (100.0–560.0 mg/kg), and ketamine (1.0 mg/kg). Midazolam dose-effect tests ended when ≥80% of the total responses occurred on the midazolam-appropriate lever or when two electric stimuli were delivered.

#### Flumazenil Discrimination Procedure

Diazepam was given 3 h before experimental sessions. Multiple cycle procedures were
similar to those described above except that the 5-min response period comprised a fixed ratio 5 schedule of food presentation during which a maximum of 10 food pellets (300-mg banana-flavored pellets; Bio-Serv, Frenchtown, NJ) were available. When the maximum number of food pellets was obtained in less than 5 min, the remainder of the response period was a time-out. Vehicle training comprised administration of vehicle or sham injections during the first minute of each of no more than eight cycles. Flumazenil training sessions comprised administration of flumazenil (0.32 mg/kg for three monkeys or 0.1 mg/kg for two monkeys s.c.) during the first minute of a cycle followed by a vehicle or sham injection during the first minute of a second cycle; flumazenil training cycles could be preceded by one to six vehicle or sham injection cycles. Test sessions were conducted after training sessions in which ≥80% of the total responses occurred on the lever designated as correct and fewer than five responses occurred on the incorrect lever before the first completion of the response requirement on the correct lever for all cycles. Before each test, these criteria had to be satisfied for both flumazenil and vehicle training sessions.

Test sessions were identical to training sessions except that five consecutive responses on either lever resulted in delivery of a food pellet. Cumulative flumazenil dose-effect tests were conducted by injecting the flumazenil vehicle solution during the first minute of the first cycle followed by increasing doses (0.25 or 0.5 log units/cycle) of flumazenil during the first minute of subsequent cycles. On separate occasions, single doses of the following drugs were injected s.c. during the first minute of the first cycle followed by cumulative doses of flumazenil during subsequent cycles: flunitrazepam (0.32 and 1.0 mg/kg), androsterone (10.0 and 32.0 mg/kg), dihydroandrosterone (32.0 and 56.0 mg/kg), epipregnanolone (32.0 and 56.0 mg/kg), buspirone (0.1 and 0.32 mg/kg), GHB (1000.0–10000.0 mg/kg), ketamine (0.32 and 1.0 mg/kg), valproic acid (320.0–1000.0 mg/kg), and diphenhydramine (3.2 and 5.6 mg/kg). Flumazenil dose-effect tests were also conducted by injecting vehicle during the first cycle 105 min after administration of abecarnil (3.2 and 5.6 mg/kg); the pre-treatment interval for abecarnil was chosen based on the time at which abecarnil occasioned ≥80% midazolam-lever responding in midazolam-discriminating monkeys. Test sessions ended when ≥80% of the total responses occurred on the flumazenil-appropriate lever or when fewer than four food pellets were delivered in a single cycle.

Drugs. The vehicle for oral administration of diazepam was fruit punch combined with Suspending Agent K (Bio-Serv) in a concentration sufficient to produce a liter of fruit punch. Tablets containing 10 mg of diazepam (Zenith Laboratories, Inc., Northvale, NJ) were dissolved in vehicle, mixed in a blender, and administered using a 12-gauge drinking needle attached to a 60-ml syringe. To obtain a dose of 5.6 mg/kg diazepam, a standard concentration of diazepam (1.0 mg/ml) was administered in a volume adjusted to individual body weights. The diazepam mixture was prepared immediately before administration.

The following drugs were administered s.c. in a volume of 0.01 to 0.1 ml/kg b.wt. expressed in terms of the forms listed below: abecarnil (Dr. D. Stephens, Schering AG, Berlin, Germany); buspirone hydrochloride, diphenhydramine hydrochloride, γ-hydroxybutyric acid sodium salt, and sodium valproate (Sigma-Aldrich, St. Louis, MO); flumazenil (F. Hoffmann LaRoche, Basel, Switzerland); flunitrazepam (Dr. Peter Sorter, F. Hoffmann LaRoche); ketamine hydrochloride (Fort Dodge Laboratories, Fort Dodge, IA); and midazolam hydrochloride (Roche Pharma Inc., Manati, Puerto Rico). Androstenedione, 17β-dihydroandrosterone, and epipregnanolone (Steraloids, Newport, RI) were administered s.c. in a volume of 0.1 to 1.0 ml/kg b.wt. Abecarnil and flunitrazepam were dissolved in a vehicle comprising 50% ethanol and 50% Emulphor. Buspirone, diphenhydramine, GHB, and sodium valproate were dissolved in sterile distilled water. Flumazenil was dissolved in a vehicle comprising 40% propylene glycol (Sigma-Aldrich), 50% saline, and 10% ethanol. Ketamine hydrochloride and midazolam were commercially prepared solutions in concentrations of 5 and 100 mg/ml, respectively, and were subsequently diluted with saline. Androsterone, dihydroandrosterone, and epipregnanolone were dissolved in 45% hydroxypropylcellulose–glycerol 80% water. Flumazenil was dissolved in a vehicle comprising 40% propylene glycol (Sigma-Aldrich), 50% saline, and 10% ethanol. Ketamine hydrochloride and midazolam were commercially prepared solutions in concentrations of 5 and 100 mg/ml, respectively, and were subsequently diluted with saline. Androsterone, dihydroandrosterone, and epipregnanolone were dissolved in 45% hydroxypropylcellulose–glycerol 80% water.

Data Analyses. Drug discrimination data are expressed as the percentage of total responses occurring on the drug-appropriate lever averaged among monkeys (± S.E.M.) and plotted as a function of dose. Substitution for the training drug was defined as ≥80% responding on the drug-appropriate lever. When a test with a given compound was conducted more than once, the determinations were averaged for an individual subject for further analyses. Doses of a compound required to produce 50% drug-appropriate responding (ED50) and the 95% confidence limits (95% CLs) were estimated using linear regression by using more than two appropriate data points, otherwise by interpolation. These values were determined first for individual monkeys and then averaged among all monkeys. Control midazolam or flumazenil ED50 values and 95% CLs were determined periodically throughout the course of these studies, and these values were averaged to obtain an overall mean of the control ED50 values and 95% CLs. ED50 values for midazolam- or flumazenil-appropriate responding after administration of another drug were compared to the overall average of the control ED50 values and 95% CLs. ED50 values were considered to be significantly different from control when the average ED50 value was not within the 95% CL values of the overall average of the control. The magnitude of shift elicited by a given drug was determined from the averaged data. Responding on both levers was divided by the duration of time that both levers were active. Control response rate represents the average of the five saline or vehicle-training sessions immediately preceding a test. Response rate was calculated as a percentage of control rate for individual animals and then averaged among subjects (± S.E.M.) and plotted as a function of dose.

Results

Substitution of Positive GABAA Modulators for Midazolam in Untreated Monkeys. Cumulative doses of midazolam increased midazolam-lever responding in a dose-related manner with a dose of 0.32 mg/kg occasioning ≥80% midazolam-lever responding in all monkeys (Fig. 1, top, closed circles). Administration of saline during the first cycle of these tests occasioned predominantly saline-appropriate responding (data not shown). The largest dose (0.32 mg/kg) of midazolam did not substantially modify response rate (Fig. 1, bottom, closed circles). The range of control midazolam ED50 values was 0.11 to 0.17 mg/kg; the overall average of ED50 values and 95% CLs was 0.15 mg/kg (0.08–0.23) (Table 1).

Single doses of flunitrazepam (0.01–0.1 mg/kg), abecarnil (0.1–1.0 mg/kg), and androsterone (10.0–56.0 mg/kg) were administered in separate tests at the beginning of eight cycles (Figs. 2–4). The two largest doses of flunitrazepam (Fig. 2, top), abecarnil (Fig. 3, top), and androsterone (Fig. 4, top) increased midazolam-lever responding across cycles in a time-related manner. Substitution for midazolam (≥80% midazolam-lever responding) was observed within 30 min for flunitrazepam and androsterone; in contrast, abecarnil had a delayed onset of action (90 min). The largest dose (0.1 mg/kg) of flunitrazepam did not substantially modify response rate (Fig. 2, bottom). Smaller doses of abecarnil and androsterone slightly increased response rate, whereas larger doses of these compounds decreased response rate to 50 to 60% of control (Figs. 3 and 4, bottom, respectively).

Single doses (32.0 and 100.0 mg/kg) of dihydroandrosterone and epipregnanolone, administered in separate tests at the beginning of eight cycles, increased midazolam-lever
responding in a dose- and time-related manner in two of three monkeys (data not shown). In these two monkeys, substitution for midazolam occurred at 15 min for epipregnanolone and 30 min for dihydroandrosterone; thereafter, monkeys responded ≥80% on the midazolam lever for the remainder of the test sessions for both of these compounds.

Epipregnanolone and dihydroandrosterone, each up to a dose of 100.0 mg/kg, occasioned predominantly saline-lever responding in a third monkey. Larger doses of these compounds were not studied due to solubility limitations. In the monkeys for which epipregnanolone substituted for midazolam, response rate was increased in one monkey and decreased in the other by the larger dose (100.0 mg/kg) of epipregnanolone. Dihydroandrosterone did not substantially modify response rate across eight cycles (data not shown).

Dose-effect curves for discriminative stimulus and rate effects were constructed from values observed 30 min after injection of flunitrazepam, androsterone, dihydroandrosterone, and epipregnanolone and 90 min after injection of abecarnil (Fig. 1). The order of potency for midazolam-like discriminative stimulus effects was flunitrazepam > mida-
zolam > abecarnil > androsterone (Table 1). The ED$_{50}$ values and 95% CLs for dihydroandrosterone and epipregnanolone were calculated from the two monkeys for which these compounds occasioned $\geq 80\%$ midazolam-lever responding (Table 1); potency of these two compounds was similar, with both being significantly less potent than other positive modulators.

**Attenuation of Flumazenil Discriminative Stimulus in Diazepam-Treated Monkeys with Positive GABA$_A$ Modulators.** In monkeys treated daily with diazepam, administration of flumazenil dose dependently increased responding on the flumazenil-appropriate lever with a dose of 0.1 mg/kg occasioning $\geq 80\%$ flumazenil-lever responding (Figs. 5–9 and 12, top, closed circles). Administration of the flumazenil vehicle solution during the first cycle of these tests occasioned predominantly vehicle-appropriate responding (Figs. 5–9 and 12, top, closed circles above “V”). The range of control flumazenil ED$_{50}$ values was 0.009 to 0.050 mg/kg; the overall average ED$_{50}$ (95% CL) was 0.031 mg/kg (0.013–0.057) flumazenil (Table 2). Before administration of flumazenil in control tests, response rate was slightly decreased during the first cycle; on average, flumazenil did not substantially modify response rate (Figs. 5–9 and 12, bottom, closed circles).

On separate occasions, single doses of positive GABA$_A$ modulators were administered before flumazenil dose-response tests. Flunitrazepam occasioned primarily vehicle-lever responding and attenuated the flumazenil discriminative stimulus (Fig. 5, top). Doses of 0.32 and 1.0 mg/kg flunitrazepam shifted the flumazenil dose-effect curve to the right as evidenced by 3- and 7-fold increases, respectively, in the ED$_{50}$ value for flumazenil discrimination (Table 2); however, in a third monkey, responding was disrupted by 3.2 mg/kg abecarnil in combination with larger doses ($>0.1$ mg/kg) of flumazenil. A larger dose (5.6 mg/kg) of abecarnil occasioned 57% flumazenil-lever responding during the first cycle in one of three monkeys. The larger dose of abecarnil decreased response rate and, in combination with larger doses of flumazenil, disrupted responding in all three monkeys (Fig. 6, bottom, open triangles); therefore, an ED$_{50}$ value for flumazenil discrimination could not be determined after pretreatment with the larger dose of abecarnil.

Androsterone (Fig. 7, top), dihydroandrosterone (Fig. 8, top) and epipregnanolone (Fig. 9, top) administered before flumazenil dose-response tests occasioned primarily vehicle-lever responding and attenuated the flumazenil discriminative stimulus as evidenced by rightward shifts in the flumazenil dose-effect curve. The ED$_{50}$ value for flumazenil discrimination was increased 2- and 11-fold by 10.0 and 32.0 mg/kg androsterone, respectively; 4- and 10-fold by doses of 32.0 and 56.0 mg/kg dihydroandrosterone, respectively; and...
2- and 3-fold by doses of 32.0 and 56.0 mg/kg epipregnanolone, respectively (Table 2). Before administration of flumazenil, response rate was slightly decreased by larger doses of androsterone and dihydroandrosterone (32.0 and 56.0 mg/kg, respectively); flumazenil attenuated the rate-decreasing effects produced by the combination of diazepam and androsterone or dihydroandrosterone (Figs. 7 and 8, respectively, bottom). Epipregnanolone alone or in combination with flumazenil did not substantially modify response rate (Fig. 9, bottom).

**Effects of Other Anxiolytics, Sedatives, and Anticonvulsants in Untreated Monkeys Discriminating Midazolam.** Single doses (0.1–1.0 mg/kg) of buspirone administered at the beginning of eight cycles occasioned predominantly saline-lever responding (data not shown), except in one monkey for which buspirone (0.32 mg/kg) substituted for midazolam at a single time point (30 min after injection). The group average response on the midazolam lever was 27% at 30 min after injection of 0.32 mg/kg buspirone (data not shown). In the monkey for which buspirone (0.32 mg/kg) substituted for midazolam, single doses (0.1 and 0.32 mg/kg) of buspirone administered before midazolam dose-response tests enhanced the midazolam discriminative stimulus. In this monkey, each dose (0.1 and 0.32 mg/kg) of buspirone shifted the midazolam discrimination dose-effect curve to the left as evidenced by 3-fold decreases in the ED$_{50}$ value for midazolam discrimination. However, when data from this monkey were averaged with data from three other monkeys, buspirone (0.1 and 0.32 mg/kg) did not significantly modify the ED$_{50}$ value for midazolam discrimination (Table 3). The smaller doses (0.1 and 0.32 mg/kg) of buspirone administered before cumulative doses of midazolam did not alter response rate (Table 3, before midazolam); however, buspirone (0.1 and 0.32 mg/kg) in combination with midazolam decreased response rate (Table 3, after midazolam). The largest dose (1.0 mg/kg) of buspirone decreased responding to 36% at 15 min after injection (Table 3, before midazolam). A single dose (560.0 mg/kg) of GHB administered at the beginning of eight cycles occasioned saline-lever responding in two monkeys and a maximum of 70% midazolam-lever responding 75 to 90 min after injection in a third monkey; GHB (560.0 mg/kg) did not systematically alter response rate in any monkey (data not shown). When administered before midazolam, smaller doses (100.0 and 320.0 mg/kg) of GHB...
failed to modify the ED$_{50}$ value for midazolam discrimination (Table 3). In two monkeys, including the monkey that responded on the midazolam lever after GHB alone, GHB (560.0 mg/kg) shifted the midazolam dose-effect curve leftward as evidenced by a significant 5-fold decrease in the ED$_{50}$ value for midazolam discrimination (Table 3). In a third monkey, GHB (560.0 mg/kg) in combination with midazolam (0.32 mg/kg) disrupted responding. Larger doses of GHB in combination with midazolam decreased the group average response rate (Table 3, after midazolam).

A dose of 3.2 and not 1.0 mg/kg ketamine administered at the beginning of eight cycles suppressed responding for 30 min after injection (Table 3, before midazolam); thereafter, response rate was not different from control and monkeys responded predominantly on the saline lever (data not shown). Pretreatment with the smaller dose (1.0 mg/kg) of ketamine, in a separate test before a midazolam dose-response test, did not significantly modify the ED$_{50}$ value for midazolam discrimination (Table 3).

Diphenhydramine (3.2–17.8 mg/kg) administered before midazolam dose-response tests occasioned saline-lever responding and increased response rate (Fig. 10, points above “V”, Table 3, before midazolam). Smaller doses of diphenhydramine (3.2 and 10.0 mg/kg) did not significantly modify the ED$_{50}$ value for midazolam discrimination (Fig. 10, top). However, a larger dose (17.8 mg/kg) of diphenhydramine shifted the midazolam dose-effect curve to the right as evidenced by a significant 3-fold increase in the ED$_{50}$ value for midazolam discrimination (Table 3). Smaller doses of diphenhydramine in combination with midazolam increased response rate (Fig. 10, bottom; Table 3, after midazolam).

Single doses (320.0–1000.0 mg/kg) of valproic acid administered at the beginning of eight cycles occasioned predominantly saline-lever responding and did not substantially modify response rate (data not shown). Valproic acid (320.0–1000.0 mg/kg) administered before midazolam dose-response tests enhanced the midazolam discriminative stimulus as evidenced by leftward shifts in the midazolam dose-effect curve (Fig. 11, top). Doses of 560.0 and 1000.0 mg/kg valproic acid decreased the ED$_{50}$ value for midazolam discrimination by 3- and 5-fold, respectively; a smaller dose of 320.0 mg/kg valproic acid did not significantly modify the ED$_{50}$ value for midazolam discrimination (Table 3). The smaller dose (320.0 mg/kg) of valproic acid in combination with midazolam slightly increased response rate (Fig. 11, bottom); larger
dose-effect curve alone and after pretreatment with various compounds. Positive GABA_A modulators, such as flumazenil (Table 4), partially reversed the rate-decreasing effects produced by the combination of buspirone (0.32 mg/kg) and the daily dose of diazepam in two other monkeys (Table 4, after flumazenil). Buspirone (0.32 mg/kg) did not significantly modify the ED_50 value for flumazenil discrimination in these two monkeys (Table 4). Similarly, single doses (100.0–1000.0 mg/kg) of GHB administered before flumazenil dose-effect tests did not substitute for flumazenil and did not substantially modify response rate; GHB (100.0–1000.0 mg/kg) did not modify the discriminative stimulus effects of flumazenil (Table 4). A larger dose (0.32 mg/kg) of buspirone alone or in combination with flumazenil suppressed responding in two of four monkeys (Table 4, before flumazenil). Flumazenil partially reversed the rate-decreasing effects produced by the combination of buspirone (0.32 mg/kg) and the daily dose of diazepam in two other monkeys (Table 4, after flumazenil); buspirone (0.32 mg/kg) did not significantly modify the ED_50 value for flumazenil discrimination in these two monkeys (Table 4).

A smaller dose (0.32 mg/kg) of ketamine administered before a flumazenil dose-effect test occurred predominantly vehicle-lever responding (data not shown) and did not substantially modify response rate; GHB (100.0–1000.0 mg/kg) did not modify the discriminative stimulus effects of flumazenil (Table 4).

### Table 2

<table>
<thead>
<tr>
<th>Drug/Dose</th>
<th>Flumazenil Discrimination</th>
<th>ED_50 95% CL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.03</td>
<td>0.01–0.06</td>
</tr>
<tr>
<td>Benzdiazepine site</td>
<td>+ Flunitrazepam 0.32 mg/kg</td>
<td>0.09* 0.04–0.15</td>
</tr>
<tr>
<td></td>
<td>1.0 mg/kg</td>
<td>0.21* 0.03–0.51</td>
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<tr>
<td></td>
<td>+ Abecarnil 3.2 mg/kg</td>
<td>0.08* 0.03–0.15</td>
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<tr>
<td></td>
<td>5.6 mg/kg</td>
<td>N.A. N.A.</td>
</tr>
<tr>
<td>Neuroactive steroid site</td>
<td>+ Androsterone 10.0 mg/kg</td>
<td>0.07* 0.02–0.11</td>
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<tr>
<td></td>
<td>32.0 mg/kg</td>
<td>0.33* 0.09–0.69</td>
</tr>
<tr>
<td></td>
<td>+ Dihydroandrosterone 32.0 mg/kg</td>
<td>0.12* 0.02–0.23</td>
</tr>
<tr>
<td></td>
<td>56.0 mg/kg</td>
<td>0.30* 0.10–0.58</td>
</tr>
<tr>
<td></td>
<td>+ Epipregnanolone 32.0 mg/kg</td>
<td>0.07* 0.05–0.09</td>
</tr>
<tr>
<td></td>
<td>56.0 mg/kg</td>
<td>0.09* 0.03–0.16</td>
</tr>
</tbody>
</table>

N.A., not applicable; only one monkey responded ≥80% on the flumazenil lever.

### Table 3

<table>
<thead>
<tr>
<th>Drug/Dose</th>
<th>Midazolam Discrimination</th>
<th>Rate % Control ± S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ED_50 95% CL</td>
<td>Before Midazolam  After Midazolam</td>
</tr>
<tr>
<td>Control</td>
<td>0.15 0.08–0.23</td>
<td>102 ± 6 97 ± 4</td>
</tr>
<tr>
<td>+ Buspirone</td>
<td>0.1 mg/kg 0.13 0.03–0.33</td>
<td>96 ± 16 30 ± 15</td>
</tr>
<tr>
<td></td>
<td>0.32 mg/kg 0.09 0.01–0.22</td>
<td>102 ± 15 45 ± 36</td>
</tr>
<tr>
<td>+ GHB</td>
<td>1.0 mg/kg N.S. N.S.</td>
<td>36 ± 31 N.S.</td>
</tr>
<tr>
<td>100.0 mg/kg</td>
<td>0.22 0.05–0.51</td>
<td>87 ± 11 108 ± 12</td>
</tr>
<tr>
<td>320.0 mg/kg</td>
<td>0.22 0.05–0.49</td>
<td>91 ± 5 87 ± 27</td>
</tr>
<tr>
<td>560.0 mg/kg</td>
<td>0.03* 0.01–0.07</td>
<td>105 ± 9 71 ± 12</td>
</tr>
<tr>
<td>+ Ketamine</td>
<td>1.0 mg/kg 0.12 0.10–0.13</td>
<td>109 ± 8 86 ± 25</td>
</tr>
<tr>
<td>+ Diphenhydramine</td>
<td>3.2 mg/kg N.S. N.S.</td>
<td>0 N.S.</td>
</tr>
</tbody>
</table>

N.S., not studied.

* Significantly different from control (p < 0.05).
two other monkeys (Table 4, before flumazenil). Flumazenil partially reversed the rate-decreasing effects produced by the combination of ketamine (1.0 mg/kg) and the daily dose of diazepam in two monkeys (Table 4, after flumazenil); ketamine (1.0 mg/kg) did not significantly modify the discriminative stimulus effects of flumazenil (Table 4).

Single doses (3.2 and 5.6 mg/kg) of diphenhydramine administered before flumazenil dose-effect tests did not substitute for flumazenil and decreased response rate to 47 and 73% of control, respectively (Fig. 12, bottom; Table 4, after flumazenil).

Discussion

Positive GABA<sub>A</sub> modulators acting at benzodiazepine and neuroactive steroid sites substituted for midazolam in untreated monkeys and attenuated flumazenil in diazepam-treated monkeys. These results suggest that positive modulators acting at benzodiazepine and neuroactive steroid sites had qualitatively similar effects that might include attenuation of diazepam withdrawal. Although positive modulators at different sites had qualitatively similar effects, site of action was an important determinant of potency in attenuating a flumazenil discriminative stimulus in diazepam-treated monkeys, i.e., neuroactive steroids were relatively more potent than benzodiazepines compared with their respective potency in midazolam-discriminating monkeys. Drugs with mechanisms not predominantly involving modulation of GABA did not substitute for midazolam or flumazenil; however, valproic acid enhanced midazolam and attenuated flumazenil. Thus, although these discrimination assays seem to be selective for positive GABA<sub>A</sub> modulation and not other mechanisms underlying anxiolytic, sedative or anticonvulsant activity, some drugs (e.g., valproic acid) have in common with positive GABA<sub>A</sub> modulators the ability to modulate benzodiazepine dependence and withdrawal.
Compounds that modulate GABA with high efficacy at benzodiazepine receptors have been shown to substitute for midazolam and to attenuate flumazenil in diazepam-treated monkeys (Gerak and France, 1999; Lelas et al., 1999; McMahon et al., 2001, 2002). In the current study, flunitrazepam substituted for midazolam and attenuated flumazenil in diazepam-treated monkeys (McMahon et al., 2001). In the current study, androsterone, a neuroactive steroid with efficacy comparable with that of pregnanolone, substituted for midazolam and attenuated flumazenil. Androsterone (ED<sub>50</sub> value of 26.64) was less potent than pregnanolone (ED<sub>50</sub> value of 6.40 mg/kg) in substituting for midazolam, a result consistent with androsterone being less potent than pregnanolone in modulating GABA in vitro (Park-Chung et al., 1999). Dihydroandrosterone and epipregnanolone are neuroactive steroids that have been reported to have approximately 5-fold lower efficacy than other steroids (Pignataro and Fiszer de Plazas, 1997; Park-Chung et al., 1999). Dihydroandrosterone and epipregnanolone, up to a dose of 100 mg/kg, substituted for midazolam in two of three monkeys and attenuated flumazenil in diazepam-treated monkeys. Failure of dihydroandrosterone and epipregnanolone to substitute for midazolam could be related to the limited range of doses that could be studied, to limited bioavailability, or to low efficacy.

A previous study reported that potency of positive GABA<sub>A</sub> modulators in substituting for midazolam in untreated monkeys did not predict potency in attenuating flumazenil in diazepam-treated monkeys (McMahon et al., 2001). Results of the present study confirm and extend these findings to other modulators acting at different sites on the GABA<sub>A</sub> receptor complex. Figure 13 compares the potency of positive modulators in untreated and diazepam-treated monkeys by depicting the magnitude of the rightward shift in the flumazenil dose-effect curve (ordinate) as a function of each dose of each of the modulators, where the dose of the modulator is expressed as a multiple of its ED<sub>50</sub> value in substituting for midazolam (abscissa). A dose of 1 (abscissa) represents the midazolam substitution ED<sub>50</sub> value for the appropriate positive modulator. Benzodiazepine site ligands (Fig. 13, closed symbols) were relatively less potent in diazepam-treated monkeys, shifting the flumazenil dose-effect curve 2- to 7-fold rightward; it is possible that larger doses of benzodiazepine site ligands would shift the flumazenil dose-effect curve further to the right. In contrast, neuroactive steroids (Fig. 13, open symbols) were relatively more potent in diazepam-treated monkeys compared with untreated monkeys. A dose of pregnenolone one-half the ED<sub>50</sub> value in midazolam-discriminating monkeys shifted the flumazenil dose-effect curve more than 20-fold to the right, whereas comparable doses of other neuroactive steroids produced smaller rightward shifts in the flumazenil dose-effect curve. Differences in relative potency among drugs that act at neuroactive steroid sites might be due to differential efficacy at GABA<sub>A</sub> receptor sub-
Collectively, these results with clinical studies showing that buspirone does not attenuate benzodiazepine withdrawal in diazepam-treated monkeys, a result that is consistent with previous studies in rodents (File and Andrews, 1991). However, buspirone did not modify the flumazenil-discriminative stimulus in diazepam-treated monkeys if that attenuation is due to anxiolytic activity.

Valproic acid is used to treat anxiety, seizures, bipolar disorder, and drug abuse (for review, see Davis et al., 2000). Although valproic acid blocks sodium channels and increases GABA levels through inhibition of GABA transaminase, the mechanism responsible for its therapeutic effects is not well understood. Valproic acid, up to a dose of 1000 mg/kg, did not substitute for midazolam or flumazenil; however, valproic acid shifted the midazolam dose-effect curve leftward and the flumazenil dose-effect curve rightward. Valproic acid enhances the anesthetic effects of ethanol and a barbiturate (Hoffman and Habib, 1994), suggesting that valproic acid can modulate effects of some positive GABA_A modulators. The ability of valproic acid to attenuate the flumazenil discriminative stimulus in diazepam-treated monkeys is consistent with some (Harriss et al., 2000) and not other (Rickels et al., 1999) results obtained in benzodiazepine-dependent subjects.

The sedative hypnotics and anesthetics GHB and ketamine did not substitute for or modify the discriminative-stimulus effects of midazolam or flumazenil (Table 3; Gerak and France, 1999; Lelas et al., 1999; Woolverton et al., 1999). Diphenhydramine did not substitute for midazolam or flumazenil, a finding that is consistent with previous studies in monkeys (Spealman, 1985; Evans and Johanson, 1989). However, relatively large doses of diphenhydramine substituted for amphetamine in some nonprimate species, produced hyperactivity and convulsions in monkeys (Evans and Johanson, 1989), and shifted the midazolam dose-effect curve to the right in the present study. Antagonism of midazolam by diphenhydramine is likely due to a functional interaction mediated by histaminic or muscarinic and not benzodiazepine receptors (Kubo et al., 1987). Diphenhydramine did not modify the flumazenil discriminative stimulus in diazepam-treated monkeys, suggesting that diphenhydramine does not exacerbate diazepam withdrawal. Differential effects of diphenhydramine in untreated and diazepam-treated monkeys might suggest that antagonism of the acute effects of benzodiazepines does not necessarily confer flumazenil-like effects in diazepam-treated monkeys.

In summary, the present results demonstrate that the discriminative-stimulus effects of midazolam in untreated monkeys and of flumazenil in diazepam-treated monkeys are mediated by GABA_A modulation and not other mechanisms underlying anxiolytic, sedative, or anticonvulsant activity. These results also suggest that valproic acid and diphenhydramine do not act at the GABA_A receptor complex to modify behavioral effects of benzodiazepines resulting from positive GABA_A modulation. Results with compounds acting at benzodiazepine and neuroactive steroid sites demonstrate that positive GABA_A modulation at different sites results in qualitatively similar effects. However, the greater relative potency of neuroactive steroids in diazepam-treated monkeys, compared with benzodiazepines, indicates that site of action is an important determinant of relative potency under conditions of benzodiazepine treatment that result in tolerance.

**Fig. 13.** Rightward shift in the flumazenil dose-effect function elicited by positive GABA_A modulators expressed as a multiple of the midazolam substitution ED_{50} value. Abscissa: multiple of the ED_{50} value of the appropriate positive GABA_A modulator in substituting for midazolam. Ordinate: mean (±S.E.M.) rightward shift in the flumazenil dose-effect curve expressed as flumazenil ED_{50} value after pretreatment with a dose of positive GABA_A modulator divided by the corresponding control flumazenil ED_{50} value. Vertical dashed line represents the ED_{50} value for midazolam substitution. Data for pregnanolone, diazepam, and midazolam are from McMahon et al. (2001).

Drugs with mechanisms that do not predominantly involve modulation of GABA and that share therapeutic or other effects with benzodiazepines (e.g., buspirone, ketamine, and diphenhydramine) were studied to examine the pharmacological specificity of these discrimination assays and to determine whether these compounds modify the acute and withdrawal-related effects of benzodiazepines. It is not clear to what extent GABA is modulated by other compounds that share therapeutic or other effects and that buspirone does not substantially modify the behavioral effects of benzodiazepines. However, because daily administration of buspirone is typically required for anxiolyis in humans (Goldberg, 1984), daily treatment with buspirone might be required to attenuate the flumazenil discriminative stimulus in diazepam-treated monkeys if that attenuation is due to anxiolytic activity.

Buspirone did not substitute for midazolam or flumazenil, a finding that is consistent with previous studies in monkeys (Spealman, 1985; Evans and Johanson, 1989). However, relatively large doses of diphenhydramine substituted for amphetamine in some nonprimate species, produced hyperactivity and convulsions in monkeys (Evans and Johanson, 1989), and shifted the midazolam dose-effect curve to the right in the present study. Antagonism of midazolam by diphenhydramine is likely due to a functional interaction mediated by histaminic or muscarinic and not benzodiazepine receptors (Kubo et al., 1987). Diphenhydramine did not modify the flumazenil discriminative stimulus in diazepam-treated monkeys, suggesting that diphenhydramine does not exacerbate diazepam withdrawal. Differential effects of diphenhydramine in untreated and diazepam-treated monkeys might suggest that antagonism of the acute effects of benzodiazepines does not necessarily confer flumazenil-like effects in diazepam-treated monkeys.
and dependence. If the flumazenil discrimination assay represents diazepam withdrawal, these results might be relevant to the future development of neuroactive steroids and other drugs (e.g., valproic acid) for modulating benzodiazepine dependence and withdrawal.

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