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Acute tolerance to chlordiazepoxide qualitatively changes the interaction between flumazenil and pregnanolone and not the interaction between flumazenil and midazolam in rhesus monkeys discriminating midazolam

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Abstract

Benzodiazepines and neuroactive steroids act at distinct binding sites on γ -aminobutyric acid_A (GABA_A) receptors where they positively modulate GABA, resulting in similar acute behavioral effects. Tolerance to benzodiazepines can develop with repeated treatment; however, cross tolerance to neuroactive steroids does not develop, perhaps due to conformational changes in *benzodiazepine, and not neuroactive steroid, binding sites*. Three monkeys discriminated 0.178 mg/kg midazolam while responding under a fixed-ratio 10 schedule of stimulus-shock termination. On separate occasions, dose-effect curves for midazolam and pregnanolone were determined when monkeys had not received chlordiazepoxide and when they received 10 mg/kg chlordiazepoxide 46 hours earlier; for some tests, flumazenil was given before determination of dose-effect curves. Midazolam and pregnanolone produced 80% midazolam-lever responding. When administered 46 hr before sessions, chlordiazepoxide produced primarily saline-lever responding; under those treatment conditions, midazolam dose-effect curves were shifted 2.8-fold rightward and pregnanolone dose-effect curves were not changed. Flumazenil antagonized midazolam; Schild (linear) analyses yielded slopes that were not different from unity and pA_2 values of 7.46 when monkeys had not received chlordiazepoxide and 7.44 when they received chlordiazepoxide 46 hr earlier. Flumazenil did not alter the effects of pregnanolone in chlordiazepoxide-treated monkeys. Thus, interactions between flumazenil and midazolam were not qualitatively or quantitatively changed *in monkeys acutely tolerant to chlordiazepoxide*, suggesting that mechanisms other than alterations of benzodiazepine binding sites account for the development of acute tolerance.

Keywords

Benzodiazepines; Neuroactive steroids; Tolerance; Rhesus monkeys

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1. Introduction

Benzodiazepines positively modulate γ -aminobutyric acid (GABA) through specific binding sites on GABA_A receptors. Tolerance can develop to benzodiazepines, although the mechanism that *mediates* tolerance to their behavioral effects is not known. Repeated benzodiazepine treatment can change GABA_A receptors in a manner that might contribute to the development of tolerance. For example, long-term benzodiazepine treatment can uncouple benzodiazepine sites from GABA sites (Bateson, 2002), alter subunit composition of GABA_A receptors (Uusi-Oukari and Korpi, 2010), and cause changes in the tertiary conformation of GABA_A receptors that could impact benzodiazepine sites and consequently alter the effects of drugs.

Neuroactive steroids act at distinct sites on GABA_A receptors. Positive modulation by neuroactive steroids results in behavioral effects that are similar to those of benzodiazepines (Vanover et al., 1999; Gasior et al., 2000), although the effects of benzodiazepines and neuroactive steroids are not identical. Tolerance readily develops to benzodiazepines (McMahon and France, 2002b; Löscher et al., 1996), and not to those of neuroactive steroids (McMahon and France 2002a; Kokate et al. 1998). Moreover, *in benzodiazepine-tolerant subjects*, cross tolerance to neuroactive steroids is not evident (McMahon and France, 2002b; Gerak, 2009), indicating that changes in GABA_A receptors induced by benzodiazepine treatment do not alter the effects of neuroactive steroids.

Studying drugs in combination can provide insight into their mechanism of action. Flumazenil acts at benzodiazepine sites with limited ability to positively modulate GABA_A receptors, and *it antagonizes the effects of benzodiazepines that have greater efficacy than flumazenil* (Lelas et al., 2000; Gerak and France, 2012). Because neuroactive steroids and flumazenil act at different binding sites on GABA_A receptors, flumazenil does not antagonize the effects of neuroactive steroids. Instead, the limited positive efficacy of flumazenil enhances the effects of neuroactive steroids (Gerak and France, 2011, 2012). During chronic benzodiazepine treatment, interactions between flumazenil and either benzodiazepines or neuroactive steroids might change, which could be an indicator of conformational changes in benzodiazepine sites.

The present study examined whether interactions between flumazenil and the benzodiazepine midazolam or the neuroactive steroid pregnanolone changed as a consequence of benzodiazepine tolerance. *Single injections of long-lasting benzodiazepines, such as chlordiazepoxide, decrease the potency of midazolam (acute tolerance;* Gerak et al., 2008); therefore, chlordiazepoxide was used to generate acute benzodiazepine tolerance in the current study. On separate occasions, midazolam and pregnanolone dose-effect curves were determined alone and after administration of flumazenil when monkeys did not receive chlordiazepoxide and when they received a single injection of chlordiazepoxide 46 hr earlier. Quantitative pharmacological analyses were used to evaluate antagonism by flumazenil, including linear (i.e., Schild analyses) and nonlinear regression. Schild analyses have been applied previously when flumazenil was studied with benzodiazepines (Lelas et al., 2000; Gerak and France, 2012). *In contrast, nonlinear regression has not been used extensively, although it is useful in estimating the apparent affinity of flumazenil when dose-effect curves obtained in the absence of antagonist are more variable* (Lew and Angus, 1995; Kroboth et al., 1993).

2. Materials and methods

2.1. Subjects

Three adult female rhesus monkeys (subjects HE, NI, and JA) weighed between 7.5 and 9.3 kg. They were housed individually in a temperature-controlled room which was maintained on a 14 hr light and 10 hr dark cycle. Monkeys received a diet of primate chow (Harlan Teklad, High Protein Monkey Diet, Madison, Wisconsin, USA) and fruit provided daily after the experimental session. Water was freely available in the home cages. Two monkeys (HE and NI) were trained previously to discriminate midazolam while responding under a schedule of stimulus-shock termination (Gerak and France, 2011, 2012). Prior to this study, the third monkey (JA) discriminated midazolam while responding under a schedule of food presentation (Bai et al., 2011); the training dose was decreased and the schedule changed to stimulus-shock termination before this study. 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, Texas, USA, and with the 1996 Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources on Life Science, National Research Council, National Academy of Sciences).

2.2. Apparatus

During daily experimental sessions, subjects were seated in primate chairs (Primate Products, Miami, FL) and placed in sound-attenuating chambers equipped with two stimulus lights and two levers. Feet were placed in shoes that were mounted on the front of chairs and contained brass electrodes connected to a shock generator (250 ms, 3 mA). Experiments were controlled and data recorded using software running on a Windows-based computer (MED-PC IV, Med Associates Inc., East Fairfield, VT, USA).

2.3. Procedure

Monkeys discriminated 0.178 mg/kg midazolam while responding under a fixed-ratio 10 schedule of stimulus-shock termination. Sessions comprised 2 to 8 cycles with each cycle lasting 15 min. During the 10-min timeout, lights were extinguished and responding had no programmed consequence; during the subsequent 5-min response period, lights were illuminated red and shock was scheduled to occur every 15 s. Monkeys could extinguish lights and postpone the shock schedule for 30 s by responding 10 consecutive times of the lever designated correct by an injection given at the beginning of the cycle; responding on the *incorrect* lever reset the response requirement *on the correct lever*. Response periods ended when 5 min elapsed or 4 shocks were delivered, whichever occurred first.

Monkeys received injections at the beginning of each cycle. *Training and test sessions, as well as the initial testing criteria, have been described previously (Gerak and France 2012). For these studies, test sessions were conducted when monkeys satisfied the following criteria for at least 2 consecutive sessions: fewer than 10 responses emitted on the incorrect lever prior the completion of the first fixed-ratio on the correct lever, and 80% of the total session responses emitted on the correct lever.* During the first cycle of test sessions, monkeys received vehicle or flumazenil; on subsequent cycles, increasing doses midazolam or pregnanolone were administered using a cumulative dosing procedure. Dosing continued in $\frac{1}{4}$ log unit increments until 80% of the total responses during a cycle were emitted on the midazolam lever. A within-subject design was employed, and each monkey was tested under all experimental conditions.

In monkeys that had not received chlordiazepoxide, midazolam dose-effect curves were determined alone and in combination with flumazenil (0.01 to 0.1 mg/kg). On other occasions, a single dose of chlordiazepoxide was administered to produce acute tolerance

(Gerak et al., 2008). Tests began with determination of a dose-effect curve for either midazolam or pregnanolone; these dose-effect curves were complete within 90 min (6 cycles). Two hours after the start of the session, 10 mg/kg chlordiazepoxide was given. The session conducted 22 hr after chlordiazepoxide administration comprised 2 cycles in which saline and sham injections were given and responding on either lever extinguished lights and postponed shock. During the session that occurred 46 hr after chlordiazepoxide administration, midazolam or pregnanolone dose-effect curves were redetermined, depending on which drug was studied immediately before chlordiazepoxide administration; either vehicle or flumazenil was given on the first cycle with increasing doses of the positive modulator given during subsequent cycles. Under those treatment conditions, chlordiazepoxide shifted midazolam dose-effect curves rightward; to determine *the duration of cross tolerance*, on one occasion, midazolam dose-effect curves were generated 4 and 6 days after chlordiazepoxide administration. Because midazolam dose-effect curves were not shifted 6 days later, relative to control dose-effect curves, injections of chlordiazepoxide were separated by at least 7 days.

2.4. Drugs

Midazolam hydrochloride (Bedford Laboratories, Bedford, OH) was purchased as a commercially available solution and diluted with 0.9% saline. Pregnanolone (5 β -pregnan-3 α -ol-20-one; Research Technology Branch, National Institute on Drug Abuse, Rockville, MD) was dissolved in 45% (w/v) hydroxypropyl- β -cyclodextrin. Flumazenil (Enzo Life Sciences, Farmingdale, NY) was dissolved in 10% ethanol, 40% propylene glycol, and 50% sterile water. Chlordiazepoxide hydrochloride (Sigma-Aldrich Co., St Louis, Missouri, USA) was dissolved in sterile water. Drug concentrations were adjusted so that injection volumes ranged from 0.2 to 1.6 ml. Vehicle was administered in a volume of 0.3 ml. Drugs were administered s.c. in the back.

2.5. Data Analyses

The percentage of total responses emitted on the drug lever during response periods was plotted as a function of dose of midazolam or pregnanolone. Control response rates were determined in the absence of drug by averaging rates across cycles for training sessions in which only saline or sham injections were given and monkeys satisfied the testing criteria during all cycles. Control rates for each monkey were obtained by averaging the means of 5 training sessions. Group mean response rates represent the average (± 1 *S.E.M.*) of control rates across monkeys.

To compare dose-effect curves of midazolam and pregnanolone across treatment conditions, straight lines were fitted to dose-effect curves for individual monkeys. Only data on the linear portion of the dose-effect curve were used in the analyses, including the largest dose that produced 20% responding on the midazolam lever, the smallest dose that produced 80% responding on the midazolam lever and all doses in between. For one monkey, only two data points comprised the linear portion of each pregnanolone dose-effect curve; because variance for the slope of the regression line could not be determined, pregnanolone dose-effect curves in the absence of treatment were averaged for each monkey and analyzed for differences across monkeys. Slopes were compared using an F-ratio test. When slopes were not significantly different, a common slope *was used in the analyses*; the dose that produced 50% responding on the midazolam lever (i.e., ED₅₀ values) could then be estimated. Potency ratios were calculated by dividing ED₅₀ values obtained 46 hr after chlordiazepoxide by ED₅₀ values obtained immediately before chlordiazepoxide. A significant change in potency as a result of chlordiazepoxide administration was detected when the 95% confidence intervals of the potency ratios averaged across monkeys did not include 1.

Dose ratios were also used to construct Schild plots (Arunlakshana and Schild, 1959). For these analyses, dose ratios were determined by dividing ED_{50} values for midazolam obtained in the presence of each dose of flumazenil by ED_{50} values for midazolam obtained in the absence of flumazenil when monkeys had not received chlordiazepoxide and when they received 10 mg/kg chlordiazepoxide 46 hr earlier. $\log(\text{dose ratio}-1)$ was calculated for individual monkeys, averaged among monkeys, and plotted as a function of flumazenil dose ($-\log[\text{mol/kg}]$). Straight lines were fitted to each Schild plot using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA) and the following equation: $\log(\text{dose ratio}-1) = -\log(\text{molar dose of flumazenil}) \times \text{slope} + \text{intercept}$. Schild plots obtained when monkeys had not received chlordiazepoxide and when they received it 46 hr earlier were compared using an F-ratio test (Kenakin, 1997; Koek et al., 2000). If the calculated F value was not significant, then the data were best fit by the simpler model in which slope was constrained to unity and apparent pA_2 values were then compared using an F-ratio test; otherwise, the more complex model best fit the data and slopes were allowed to vary with apparent pA_2 values determined by linear regression of Schild plots.

Apparent affinity of flumazenil was also estimated with a second method (Lew and Angus, 1995) using nonlinear regression, rather than linear regression which is used in Schild analyses. This method might improve accuracy over Schild analyses, particularly under conditions in which the agonist dose-effect curve determined in the absence of antagonist varies. The pED_{50} of midazolam for each monkey was plotted as function of flumazenil dose and analyzed using nonlinear regression to fit the following equation:

$$pED_{50} = -\log(\text{flumazenil dose} + 10^{-pK_b}) - \log c. \quad 1)$$

To determine whether the interaction between drugs was competitive, an F-ratio test compared the goodness of fit of the previous equation with the following equations:

$$pED_{50} = -\log(\text{flumazenil dose}^n + 10^{-pK_b}) - \log c. \quad 2)$$

$$pED_{50} = (-\log \text{flumazenil dose} (1 + n \cdot \text{flumazenil dose} / 10^{-pK_b}) + 10^{-pK_b}) - \log c. \quad 3)$$

The parameter $\log c$ represented the logarithm of the fractional receptor occupancy needed to produce 50% of the maximal effect; increases in this parameter in chlordiazepoxide-treated monkeys provided another indicator of the development of tolerance. Data were displayed using a Clark Plot in which pED_{50} of midazolam was plotted as function of the $\log(\text{flumazenil dose in mol/kg} + K_b)$. When antagonism was competitive, the Clark Plot yielded a straight line with a slope that was not different from unity. Apparent pK_b values obtained in monkeys that had not received chlordiazepoxide were compared to those obtained in chlordiazepoxide-treated monkeys using an F-ratio test. If pK_b values were not significantly different, then a common value best fit the data and $\log c$ values could be compared using an F-ratio test.

3. Results

After an injection of saline, monkeys responded primarily on the saline lever (data not shown) and mean rates of responding (± 1 *S.E.M.*) were 2.84 ± 0.35 responses/s. Midazolam dose dependently increased responding on the midazolam lever; in untreated monkeys, the mean ED_{50} value (± 1 *S.E.M.*) was 0.07 ± 0.01 mg/kg (open circles, top panel, *Fig. 1*). Midazolam control dose-effect curves did not significantly change in individual monkeys

over the course of the experiment (HE: $F_{8,2}=5.61$, n.s.; NI: $F_{8,1}=33.26$, n.s.; JA: $F_{8,2}=2.74$, n.s.; *data not shown*); for example, for one redetermination of the midazolam dose-effect curve, the mean ED_{50} value (± 1 S.E.M.) was 0.07 ± 0.02 mg/kg (open circles, bottom panel, Fig. 1). Flumazenil dose-dependently antagonized the discriminative stimulus effects of midazolam in monkeys that had not received chlordiazepoxide. Slopes of midazolam dose-effect curves determined alone or after flumazenil administration were not significantly different (HE: $F_{3,1}=3.81$, n.s.; NI: $F_{3,2}=1.17$, n.s.; JA: $F_{3,1}=0.91$, n.s.). Doses of 0.01, 0.032, and 0.1 mg/kg flumazenil shifted the midazolam dose-effect curve 2.0-, 4.1- and 11.3-fold rightward (upper panel, Fig. 1).

When monkeys received 10 mg/kg chlordiazepoxide 22 or 46 hr before and saline immediately before sessions, they responded primarily on the saline lever (*data not shown*). Midazolam dose-effect curves determined 46 hr after chlordiazepoxide were shifted 2.8-fold rightward and the 95% confidence interval (2.0, 3.6) did not include 1 (compare open and closed circles, bottom panel, Fig. 1), indicating the development of acute benzodiazepine tolerance. In these benzodiazepine-tolerant monkeys, flumazenil dose dependently antagonized the discriminative stimulus effects of midazolam, *with 0.032 and 0.1 mg/kg flumazenil shifting the dose-effect curve 4.0-, and 10.9-fold rightward* (bottom panel, Fig. 1); *slopes of midazolam dose-effect curves determined alone or after flumazenil administration were not significantly different* (HE: $F_{4,3}=1.45$, n.s.; NI: $F_{4,2}=2.64$, n.s.; JA: $F_{4,5}=1.67$, n.s.).

Pregnanolone dose dependently increased responding on the midazolam lever; in monkeys that had not received chlordiazepoxide, the ED_{50} value (± 1 S.E.M.) was 3.66 ± 1.42 mg/kg (open circles, Fig. 2). In one monkey (JA), all pregnanolone dose-effect curves were described by two data points precluding single animal analyses, and a group analysis was employed. In the absence of other treatment, neither slopes nor intercepts of pregnanolone dose-effect curves changed significantly over the course of the experiment ($F_{6,19}=0.46$, n.s.; *data not shown*). Pregnanolone dose-effect curves were not different when determined immediately before and 46 hr after 10 mg/kg chlordiazepoxide (Fig. 2). In chlordiazepoxide-treated monkeys, flumazenil (0.032–0.32 mg/kg) did not alter the slope ($F_{4,25}=0.54$, n.s.) or the intercept ($F_{4,30}=0.06$, n.s.) of pregnanolone dose-effect curves (Fig. 2).

Schild plots were constructed from data obtained when midazolam was studied in combination with flumazenil in monkeys that had not received chlordiazepoxide (control) and in those that received chlordiazepoxide 46 hr earlier (chlordiazepoxide-treated). Straight lines were fitted to the data and showed that chlordiazepoxide did not alter the slope or intercept of the Schild plot, as compared with those obtained in the absence of chlordiazepoxide administration (Fig. 3). The common slope was not different from unity, and the apparent pA_2 value that was obtained when the common slope was constrained to unity was 7.46. When slopes were not constrained, pA_2 values were not significantly different *between control and chlordiazepoxide-treated monkeys* (Table 1).

The apparent affinity of flumazenil estimated using nonlinear regression yielded apparent pK_b values (95% confidence intervals) of 7.47 (7.33, 7.62) when monkeys had not received chlordiazepoxide and 7.48 (7.11, 7.84) when monkeys received chlordiazepoxide 46 hr earlier ($F_{1,20}=0.01$; n.s.; Fig. 4 and Table 1). Comparison of the goodness of fit of equation 1 versus equations 2 and 3 indicated that the data were best fit by the simpler model (control: equation 1 vs 2: $F_{1,9}=0.15$, n.s.; equation 1 vs 3: $F_{1,9}=0.21$, n.s.; chlordiazepoxide-treated: equation 1 vs 2: $F_{1,9}=0.96$, n.s.; equation 1 vs 3: $F_{1,9}=0.60$, n.s.). An F-ratio test indicated that $\log c$ was significantly increased in chlordiazepoxide-treated monkeys ($F_{1,22}=36.93$, $p<0.01$), indicating *that occupancy needed to produce midazolam-lever*

responding was greater in chlordiazepoxide-treated monkeys, as compared to monkeys that had not received chlordiazepoxide (Table 1).

4. Discussion

Chronic treatment with benzodiazepines can alter GABA_A receptors in a number of ways. In vitro and ex vivo studies have demonstrated changes in subunit composition of GABA_A receptors and uncoupling of benzodiazepine binding sites from GABA binding sites as a consequence of chronic benzodiazepine treatment (Bateson, 2002; Uusi-Oukari and Korpi, 2010). In addition, repeated administration of benzodiazepines can result in the development of tolerance to their behavioral effects; *however, it is unknown whether tolerance is the result of these changes in GABA_A receptors or other factors. Because conditions that produce tolerance to benzodiazepines do not produce cross tolerance to other positive GABA_A modulators* (McMahon and France, 2002b; Gerak, 2009), *any changes in GABA_A receptors contributing to benzodiazepine tolerance would not alter other sites on GABA_A receptors. For example, repeated administration of benzodiazepines might change the tertiary conformation of GABA_A receptors in a manner that impacts only benzodiazepine sites and, therefore, selectively alters effects of drugs acting at benzodiazepine sites. To the extent that conformational changes contribute to benzodiazepine tolerance, interactions between drugs would be expected to differ between non-tolerant and benzodiazepine-tolerant subjects.* In the current study, interactions between flumazenil, which acts at benzodiazepine binding sites, and either benzodiazepines or neuroactive steroids were compared across treatment conditions using quantitative pharmacological analyses.

Tolerance to benzodiazepines has been demonstrated after just a single administration of a benzodiazepine. For example, in monkeys discriminating midazolam, acute tolerance to benzodiazepines is evident 24 hr after administration of 32 mg/kg chlordiazepoxide; under those treatment conditions, the potency of pregnanolone is increased, suggesting that chlordiazepoxide or its metabolites are not completely eliminated within 24 hr (Gerak et al., 2008). In the current study, a smaller dose (10 mg/kg) and a longer interval between chlordiazepoxide administration and tests for the development of acute tolerance were used to increase the likelihood that chlordiazepoxide and its metabolites have been eliminated prior to testing for the development of tolerance. Under these treatment conditions, midazolam dose-effect curves were shifted 2.8-fold rightward and there was no change in the pregnanolone dose-effect curve, indicating that tolerance developed to benzodiazepines and not neuroactive steroids and that chlordiazepoxide and its metabolites are no longer present at the time of testing.

One possible mechanism underlying the development of benzodiazepine tolerance is a conformational change in GABA_A receptors that impacts benzodiazepine binding sites. Although binding studies indicate that the affinity of drugs acting at benzodiazepine sites do not change as a consequence of tolerance (Rosenberg and Chiu, 1979; Zanotti et al., 1996), these assays that are conducted under isolated conditions might not predict effects obtained in vivo. In the current study, a functional assay was used to determine the apparent affinity of flumazenil, which was compared across treatment conditions using two different analyses (Arunlakshana and Schild, 1959; Lew and Angus, 1995). Results of linear and nonlinear analyses were consistent with a simple, competitive, reversible interaction between flumazenil and midazolam in non-tolerant and benzodiazepine-tolerant monkeys; apparent affinity estimates for flumazenil (pA_2 and pK_B values) were not significantly different and were within the range of values obtained previously for flumazenil in non-tolerant monkeys (Lelas et al., 1999, 2000; Gerak and France, 2012). Together, results of these analyses suggest that the conformation of benzodiazepine sites does not change in benzodiazepine

tolerance, and that other mechanisms account for the development to tolerance to benzodiazepines.

Schild analyses have been used extensively in non-tolerant monkeys discriminating midazolam, and similar apparent affinity estimates have been obtained for flumazenil across a variety of positive modulators acting at benzodiazepine sites (Lelas et al., 1999, 2000; Gerak and France, 2012). *A second method using nonlinear regression has not been used previously to estimate the apparent affinity of flumazenil; this method improves accuracy of estimating apparent affinity when variability of control dose-effect curves increases* (Lew and Angus, 1995). *In both non-tolerant and benzodiazepine tolerant monkeys, slopes and apparent affinity estimates for flumazenil were remarkably similar across the two analyses.* Nonlinear regression analyses also provide a measure of receptor occupancy (log c, Table 1) needed to produce 50% effect; those results indicated that greater receptor occupancy was needed in benzodiazepine-tolerant monkeys, as compared to non-tolerant monkeys, to produce 50% responding on the midazolam lever. *While nonlinear regression can accommodate data that is more variable, these results suggest that under the conditions used in this study, Schild analyses is adequate for estimating apparent affinity of flumazenil.*

Although tolerance develops readily to benzodiazepines, cross tolerance often does not develop to neuroactive steroids (Gerak et al., 2008; Gerak, 2009; McMahon and France, 2002b), and in the current study, the potency of pregnanolone did not change in benzodiazepine-tolerant monkeys, indicating that cross tolerance did not develop. Flumazenil acts at benzodiazepine binding sites and has some positive efficacy (Dantzer and Péro, 1982). In non-tolerant monkeys, it enhances the discriminative stimulus effects of pregnanolone, shifting the dose-effect curve slightly leftward (Gerak and France, 2011, 2012), *presumably because the positive efficacy of flumazenil acting at one binding site adds to the positive efficacy of pregnanolone acting at a distinct site.* In contrast, flumazenil did not shift the pregnanolone dose-effect curve in chlordiazepoxide-treated monkeys, suggesting that cross tolerance developed to flumazenil.

While chronic benzodiazepine treatment produces a number of changes in GABA_A receptors in vitro, mechanisms that account for the development of tolerance to the behavioral effects of benzodiazepines are not well established. *The orderly data obtained in this study provides a convincing demonstration that benzodiazepine binding sites are not markedly altered in benzodiazepine-tolerant monkeys* and, therefore, do not account for the development of tolerance to the discriminative stimulus effects of benzodiazepines. *Repeated benzodiazepine treatment also uncouples benzodiazepine and GABA binding sites and changes subunit composition; these effects might contribute to benzodiazepine tolerance and they warrant further investigation. In addition to changes in GABA_A receptors, other receptor systems (e.g., glutamatergic) are known to be altered during chronic benzodiazepine administration* (Vinkers and Olivier, 2012) *and could play a role in the development of tolerance to benzodiazepines.*

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Fig. 1.

Discriminative-stimulus effects of midazolam alone and in combination with flumazenil in monkeys that had not received chlordiazepoxide (control; open symbols) and in monkeys that received chlordiazepoxide 46 hr earlier (chlordiazepoxide-treated; filled symbols). Each point represents the mean of 3 monkeys. *Two different determinations of the midazolam dose-effect curve obtained in monkeys that did not receive other drugs are shown in this figure (open circles, top and bottom panel); straight lines fitted to these midazolam dose-effect curves were not statistically different.* Abscissae, dose in milligrams per kilogram body weight; Ordinate, mean (± 1 S.E.M.) percentage of total responding that occurred on the midazolam lever.

Fig. 2.

Discriminative-stimulus effects of pregnanolone alone and in combination with flumazenil in monkeys that received chlordiazepoxide 46 hr earlier (chlordiazepoxide-treated). Open circles represent the effects of pregnanolone in the absence of other treatment. Each point represents the mean of 3 monkeys. Abscissae, dose in milligrams per kilogram body weight; Ordinate, mean (± 1 S.E.M.) percentage of total responding that occurred on the midazolam lever.

Fig. 3.

Schild plots for flumazenil studied with midazolam in monkeys that had not received chlordiazepoxide (control; open symbols) and in monkeys that received chlordiazepoxide 46 hr earlier (chlordiazepoxide-treated; filled symbols). Raw data are shown in *Fig. 1*. Abscissa, negative log of the molar dose of flumazenil. Ordinate, log (dose ratio-1).

Fig. 4.

Left panel: Effect of flumazenil on pED_{50} values for midazolam in monkeys that had not received chlordiazepoxide (control; open symbols) and in monkeys that received chlordiazepoxide 46 hr earlier (chlordiazepoxide-treated; filled symbols). Apparent pK_b values and $\log c$ were estimated by fitting equation 2 to the data. Abscissa, dose of flumazenil in moles per kilogram. Ordinate, mean (± 1 S.E.M.) negative logarithm of midazolam ED_{50} in moles per kilogram. Right panel: Clark plot of the effects of flumazenil on midazolam pED_{50} values in non-tolerant and benzodiazepine-tolerant monkeys. Abscissa, logarithm of the dose of flumazenil in molar per kilograms plus K_b ; Ordinate, mean (± 1 S.E.M.) of the negative logarithm of midazolam ED_{50} in moles per kilograms.

Slopes of Schild plots, apparent pA_2 values, apparent pK_b and log c values (95% C.I.) of flumazenil in monkeys that had not received chlordiazepoxide (control) and in monkeys that received chlordiazepoxide 46 hr earlier (chlordiazepoxide-treated). Apparent pA_2 values were determined when slopes were constrained to unity or allowed to vary.

Table 1

	Linear analyses			Non-linear analyses		
	Slope	pA_2 (unconstrained)	pA_2 (constrained)	pK_b	log c	
Control	-1.04 (-1.31, -0.76)	7.70 (7.34, 7.61)	7.46 (7.35, 7.56)	7.47 (7.33, 7.62)	0.78 (0.69, 0.84)	
Chlordiazepoxide-treated	-1.07 (-1.40, -0.75)	7.41 (7.28, 7.62)	7.44 (7.32, 7.56)	7.48 (7.11, 7.84)	1.14 (0.93, 1.35)	