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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[1]

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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 h before sessions, chlordiazepoxide did not produce pregnanolone-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 h 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.

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 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

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it antagonizes the effects of benzodiazepines that have greater efficacy than flumazenil (Lelas et al., 2000; Gerak and France, 2012). Neuroactive steroids and flumazenil act at different binding sites on $GABA_A$ receptors, and 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 h 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 h light and 10 h 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 2–8 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 on 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 $\geq 80\%$ 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 ¼ log unit increments until $\geq 80\%$ 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 (0.01–0.1 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 sessions were finished within 90 min (6 cycles). Two hours after the start of the session, 10 mg/kg chlordiazepoxide was given. The session conducted 22 h 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 h after chlordiazepoxide administration, dose-effect curves for the same drug studied immediately before chlordiazepoxide administration were redetermined; 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 (5β-pregnan-3α-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)