JPET Fast Forward. Published on May 16, 2006 as DOI: 10.1124/jpet.106.103168
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Efficacy and the discriminative stimulus effects of negative $GABA_A$ modulators, or inverse agonists, in diazepam-treated rhesus monkeys

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Running Title: Negative $GABA_A$ modulators in monkeys

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Text Pages: 26

Tables: 1

Figures: 4

References: 20

Abstract: 257 words

Introduction: 624 words

Discussion: 1,331 words

Abbreviations: β-CCE, ethyl β-carboline-3-carboxylate; BZ, benzodiazepine; CL, confidence limit; FR, fixed ratio; GABA, γ-aminobutyric acid; Ro 15-4513, ethyl 8-azido-6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5-α]-[1,4]benzodiazepine-3-carboxylate

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ABSTRACT

In benzodiazepine (BZ)-dependent animals, the effects of negative $GABA_A$ modulators at BZ sites are not clearly related to differences in negative efficacy (i.e., inverse agonist activity). A flumazenil discriminative stimulus in diazepam (5.6 mg/kg/day)-treated rhesus monkeys was used to test the hypothesis that the effects of negative $GABA_A$ modulators at BZ sites do not vary as a function of efficacy in BZ-dependent animals. Negative $GABA_A$ modulators varying in efficacy were studied in combination with positive modulators acting at different modulatory sites (BZ, barbiturate, and neuroactive steroid sites). The negative modulators Ro 15-4513 and β-CCE substituted for the flumazenil discriminative stimulus. Acute pretreatment with diazepam (3.2 and 10 mg/kg s.c.; in addition to 5.6 mg/kg/day p.o.), pentobarbital (3.2 and 10 mg/kg), or pregnanolone (1 and 3.2 mg/kg) attenuated the flumazenil discriminative stimulus and also attenuated the flumazenil-like discriminative stimulus effects of Ro 15-4513 and β-CCE. Attenuation of the discriminative stimulus effects of flumazenil, Ro 15-4513 and β-CCE did not systematically vary as a function of efficacy. When compared to their discriminative stimulus effects in untreated monkeys discriminating midazolam, both pregnanolone and pentobarbital were relatively more potent than diazepam in attenuating the discriminative stimulus effects of flumazenil, Ro 15-4513, and β-CCE in diazepam-treated monkeys. These results show that the discriminative stimulus effects of BZ-site neutral and negative modulators are not different in BZ-dependent animals trained to discriminate flumazenil, and extend the results of a previous study showing that positive modulators acting at non-BZ sites are especially potent in attenuating the effects of flumazenil in diazepam-treated monkeys (i.e. diazepam withdrawal).

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INTRODUCTION

The $GABA_A$ receptor complex is a Cl^- channel that consists of multiple distinct subunits where drugs can act at specific binding sites to facilitate or inhibit GABA-mediated Cl^- flux (referred to as positive and negative $GABA_A$ modulators, respectively; Mehta and Ticku, 1999). The consequences of positive and negative $GABA_A$ modulation are different, often resulting in opposite effects. For example, positive modulation can result in anxiolytic, anticonvulsant and respiratory depressant effects, whereas negative modulation can result in anxiogenic, convulsant and respiratory stimulant effects (Corda et al., 1983; Petersen, 1983; Wettstein et al., 1993). Some effects of negative modulators vary as a function of negative efficacy, e.g., increasing negative efficacy results in increasing convulsant and anxiogenic-like effects (Corda et al., 1983; Petersen, 1983). Thus, under some conditions, negative efficacy can be an important determinant of the effects of modulators acting at BZ sites.

Despite the many important therapeutic uses of BZs, one unwanted consequence of their use is dependence that emerges after repeated treatment, evidenced by a withdrawal syndrome that is characterized by anxiety, insomnia and, in some cases, tremor and convulsions (Woods et al., 1992). Neutral modulators at BZ sites, like flumazenil, induce signs and symptoms of withdrawal in BZ-treated animals (Lukas and Griffiths, 1982; Griffiths et al., 1993), thereby providing important drugs for evaluating BZ dependence. In addition to measuring directly observable signs and symptoms of withdrawal, there are other behavioral assays that can be used to examine BZ dependence. For example, sensitivity to the effects of flumazenil on rates of operant responding is enhanced during BZ treatment, perhaps due to the development of dependence (McMahon and France, 2002). Moreover, sensitivity to the discriminative stimulus effects of flumazenil can be enhanced by BZ treatment, and the pharmacologic profile of the

flumazenil discriminative stimulus in BZ-treated animals appears to be predictive of BZ withdrawal (Gerak and France, 1999).

Whereas neutral modulators at BZ sites typically have little or no behavioral activity in untreated animals, negative modulators at BZ sites have anxiogenic-like and convulsant activity that increase as a function of efficacy (Corda et al., 1983; Petersen, 1983); thus, their use in vivo is somewhat limited. In contrast, both neutral and negative modulators at BZ sites can antagonize the behavioral effects of positive modulators at BZ sites and, furthermore, the behavioral effects of neutral and negative modulators can be strikingly similar in BZ-treated animals (Sannerud et al., 1991; Gerak and France, 1999; McMahon and France, 2005). Thus, whereas the behavioral effects of BZ site ligands can be distinguished on the basis of negative efficacy in untreated animals, differences in negative efficacy appear less important for the behavioral effects of BZ site ligands in BZ-dependent animals. To test the hypothesis that the effects of BZ-site negative $GABA_A$ modulators do not vary as a function of efficacy in BZ-dependent animals, negative modulators that have low (Ro 15-4513) and high efficacy (β-CCE), as measured by inhibition of $GABA_A$-mediated Cl^- flux (Mehta and Ticku, 1989), and that substitute for a flumazenil discriminative stimulus in diazepam (5.6 mg/kg/day)-treated monkeys (Gerak and France, 1999), were studied in combination with positive modulators acting at different modulatory sites (BZ, barbiturate, and neuroactive steroid sites) on the $GABA_A$ receptor complex. Studies were conducted with pentobarbital and pregnanolone because a previous study in untreated monkeys indicated that negative modulators at BZ sites attenuate the effects of pregnanolone in an efficacy-dependent manner (i.e., negative efficacy appears to be positively correlated with antagonism of pregnanolone; McMahon and France, 2005). Moreover, positive modulators at non-BZ sites attenuate the discriminative stimulus

effects of flumazenil in diazepam-treated monkeys. Therefore, the importance of efficacy to the behavioral effects of negative modulators in diazepam-treated animals could be reflected by differences in the magnitude of attenuation of the effects of flumazenil, Ro 15-4513, and β-CCE by positive modulators at non-BZ sites.

MATERIALS AND METHODS

Subjects. One female and five adult male rhesus monkeys were housed individually on a 14-h light/10-h dark schedule, were maintained at 95% free-feeding weight (range 8.0-11 kg) with a diet comprising primate chow (Harlan Teklad, High Protein Monkey Diet, Madison, WI), fresh fruit, and peanuts, and were provided water in the home cage. Monkeys received 5.6 mg/kg/day of diazepam, were trained to discriminate flumazenil, and had received $GABA_A$ ligands in previous studies (e.g., McMahon and France, 2005). 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 (Institute of Laboratory Animal Resources on Life Sciences, National Research Council, National Academy of Sciences).

Apparatus. During experimental sessions, monkeys were seated in chairs (Model R001, Primate Products, Miami, FL) that provided restraint at the neck, and were placed in ventilated, sound-attenuating chambers equipped with 2 response levers, stimulus lights, and a food cup to which pellets (BioServ, Frenchtown, NJ) could be delivered from a dispenser. An interface (MedAssociates, St. Albans, VT) connected the chambers to a computer which controlled and recorded experimental events.

Discrimination procedure. Monkeys drank a solution containing diazepam (5.6 mg/kg) 3 hr prior to experimental sessions consisting of multiple 15-min cycles. Each cycle comprised a 10-min timeout period, during which responses had no programmed consequence, followed by a 5-min response period, during which green stimulus lights were illuminated and a fixed ratio (FR) 5 schedule of food presentation was in effect. A maximum of 10 food pellets was available during a cycle; when the maximum number of food pellets was obtained in less than 5 min, the

remainder of the response period was a timeout. The selection of vehicle- and flumazenil-appropriate levers varied among monkeys and remained the same for an individual throughout the study. Responding on the incorrect lever reset the response requirement on the correct lever.

Vehicle training was conducted by administering vehicle or sham injections during the first min of each of no more than eight cycles; responding on the vehicle lever was required in each of these cycles to obtain reinforcers. Flumazenil training was conducted by administering the training dose of flumazenil during the first min of a cycle followed by a vehicle or sham injection during the first min of a second cycle; responding on the flumazenil lever was required in each of these cycles to obtain reinforcers. In some training sessions, 1-6 vehicle-training cycles preceded 2 flumazenil-training cycles. The training dose of flumazenil was 0.32 mg/kg for four monkeys and 0.1 mg/kg for two monkeys. Test sessions were conducted following training sessions in which ≥80% of the total responses occurred on the lever designated correct by the injection administered during the first min of the cycle and fewer than five responses (one FR) occurred on the incorrect lever prior to completion of the first FR on the correct lever. Prior to each test, these criteria had to be satisfied for training sessions during which both flumazenil and vehicle or sham injections were administered. The type of training session preceding test sessions varied non-systematically.

Test sessions were identical to training sessions except that five consecutive responses on either lever resulted in delivery of food. Cumulative flumazenil dose-effect tests were conducted by injecting the appropriate vehicle solution during the first min of the first cycle followed by increasing doses of flumazenil during the first min of subsequent cycles with the cumulative dose increasing by 0.25 or 0.5 log unit per cycle. Similar dose-effect tests were conducted with Ro 15-4513 and β-CCE. On separate occasions, diazepam (3.2 or 10.0 mg/kg), pentobarbital

(3.2 or 10.0 mg/kg), or pregnanolone (1 and 3.2 mg/kg) was injected s.c. 45 min prior to cumulative dose-effect tests with flumazenil, Ro 15-4513 or β-CCE. Test sessions ended when ≥80% of the total responses occurred on the flumazenil-appropriate lever or when animals did not respond within a particular cycle.

Drugs. The vehicle for oral administration of diazepam was fruit punch combined with Suspending agent K (Bio-Serv, Frenchtown, NJ) in a concentration of 1 g suspending agent per liter of fruit punch. Tablets containing 10 mg diazepam (Zenith Laboratories, Inc., Northvale, N.J.) were dissolved in vehicle, mixed in a blender, and administered using a 12 G drinking needle attached to a 60 cc syringe. To obtain a dose of 5.6 mg/kg diazepam, a standard concentration (1 mg/ml) of diazepam was given 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 0.01-0.1 ml/kg: β-CCE, diazepam, Ro 15-4513 and pentobarbital (Sigma, St. Louis, MO); flumazenil (F. Hoffmann LaRoche Ltd., Basel, Switzerland); and pregnanolone (Steraloids, Newport, RI). Diazepam and β-CCE were dissolved in a vehicle comprising 50% ethanol and 50% emulphor and were diluted with saline for lower concentrations. Flumazenil, pentobarbital and Ro 15-4513 were dissolved in a vehicle comprising 40% propylene glycol (Sigma), 50% saline and 10% ethanol. Pregnanolone was dissolved in 45% hydroxypropyl-γ-cyclodextrin (Sigma) in sterile water.

Data analyses. Data from 3-6 monkeys were averaged for each drug combination using a within-subjects design. Drug discrimination data are expressed as the percentage of total responses occurring on the flumazenil lever averaged among monkeys (± S.E.M.) and plotted as a function of dose. The potencies of flumazenil, Ro 15-4513, and β-CCE, alone and in combination with positive modulators, were estimated by simultaneously fitting straight lines to

the individual dose-response data (separately for each positive modulator combination with flumazenil, Ro 15-4513, or β-CCE) by means of GraphPad Prism version 4.02 for Windows (GraphPad Prism version 4.03 for Windows, San Diego, CA), using the following equation: effect = slope * log(dose) + intercept. Straight lines were fitted to the linear portion of dose-effect curves, defined by doses producing 25-75% of the maximum effect, including not more than one dose producing less than 25% of the maximum effect, and not more than one dose producing more than 75% of the maximum effect. Other doses were excluded from the analyses.

The slopes of the three dose-effect curves generated from each drug combination were compared with an F-ratio test using GraphPad. If the slopes were not significantly different, then a common, best-fitting slope was used for further analyses (for detailed examples of this approach, see Kenakin, 1997). For best-fitting models, doses corresponding to the 50% level of the effect (ED_{50}), potency ratios, and their 95% confidence limits (CL) were calculated by parallel line analyses (Tallarida, 2000) of data from individual subjects.

Control response rate represents the average of the five vehicle training sessions before the test. Response rate was calculated as a percentage of the control rate for individual animals, then averaged among subjects (± S.E.M.) and plotted as a function of dose.

RESULTS

Discriminative stimulus and rate effects of flumazenil, Ro 15-4513 and β-CCE. Flumazenil, Ro 15-4513, and β-CCE increased responding on the flumazenil lever in a dose-related manner (Figure 1, top panels, circles); the ED_{50} values (95% CL) were 0.03 (0.02 – 0.05), 0.04 (0.02 – 0.08), and 0.30 (0.20 – 0.44) mg/kg, respectively. The vehicle solutions for flumazenil, Ro 15-4513, and β-CCE occasioned predominantly vehicle-appropriate responding (Figure 1, top panels, circles above V). Flumazenil was significantly more potent than β-CCE, and not Ro 15-4513, as evidenced by potency ratios (95% CL) of 11 (6.3 – 18) and 1.3 (0.6 – 2.9), respectively. Ro 15-4513 appeared to be more potent than β-CCE; however, a test for parallel lines revealed that the dose-effect curves for Ro 15-4513 and β-CCE were not parallel, precluding a comparison of their ED_{50} values. Up to doses occasioning predominantly flumazenil-lever responding, flumazenil, Ro 15-4513 and β-CCE did not systematically alter response rate (Figure 1, bottom panels, circles).

Attenuation of the discriminative stimulus effects of flumazenil, Ro 15-4513 and β-CCE by positive $GABA_A$ modulators. When administered alone, diazepam (3.2 and 10 mg/kg), pentobarbital (3.2 and 10 mg/kg), and pregnanolone (1 and 3.2 mg/kg) occasioned responding predominantly on the vehicle lever (Figures 1, 2, and 3, respectively; top panels, squares, triangles, and inverted triangles above V). Response rate was slightly decreased by acute injection of diazepam, pentobarbital, or pregnanolone (Figures 1, 2, and 3, respectively; bottom panels).

Diazepam (10 mg/kg) significantly attenuated the discriminative stimulus effects of flumazenil (Figure 1, top left panel), as evidenced by a potency ratio (95% CL) of 5.1 (2.5-11); a

smaller dose (3.2 mg/kg) of diazepam did not significantly attenuate the flumazenil discriminative stimulus (Table 1). Likewise, diazepam (10 mg/kg) significantly attenuated the flumazenil-like discriminative stimulus effects of Ro 15-4513 and β-CCE (Figure 1, top middle and right panels, respectively), as evidenced by potency ratios (95% CL) of 8.3 (2.9-24) and 6.9 (3.7-13), respectively. A smaller dose (3.2 mg/kg) of diazepam did not significantly attenuate the flumazenil-like discriminative stimulus effects of Ro 15-4513 and β-CCE (Table 1). In general, response rate was not systematically decreased by diazepam in combination with flumazenil, Ro 15-4513 or β-CCE, although in some monkeys there was a tendency for a decrease in response rate with combinations of diazepam (10 mg/kg) and relatively large doses of flumazenil or Ro 15-4513 (Figure 1, bottom left and middle panels, respectively)

Pentobarbital (10 mg/kg) significantly attenuated the discriminative stimulus effects of flumazenil (Figure 2, top left panel), as evidenced by a potency ratio (95% CL) of 11 (4.1-27); a smaller dose (3.2 mg/kg) of pentobarbital did not significantly attenuate the flumazenil discriminative stimulus (Table 1). Pentobarbital (3.2 and 10 mg/kg) dose-dependently and significantly attenuated the flumazenil-like discriminative stimulus effects of Ro 15-4513 and β-CCE (Figure 2, top middle and right panels, respectively; Table 1). The tendency for pentobarbital to decrease response rate was less evident upon administration of some doses of Ro 15-4513 and β-CCE (Figure 2, bottom middle and right panels, respectively).

Pregnanolone (l and 3.2 mg/kg) dose-dependently and significantly attenuated the discriminative stimulus effects of flumazenil (Figure 3, top left panel), as well as the flumazenil-like discriminative stimulus effects of Ro 15-4513 and β-CCE (Figure 3, top middle and right panels, respectively; Table 1). The tendency for pregnanolone to decrease response rate was less evident upon administration of flumazenil and intermediate dose of Ro 15-4513 (Figure 3,

bottom left and middle, respectively); in contrast, pregnanolone-induced decreases in response rate were enhanced by relatively large doses of Ro 15-4513 and β-CCE (Figure 3, bottom middle and left, respectively).

Figure 4 depicts the magnitude of rightward shift (ordinate) in the flumazenil (left), Ro 15-4513 (middle) and β-CCE dose-effect curves (right) produced by positive modulators plotted as a function of their potency (ED_{50}) to substitute for a midazolam discriminative stimulus in untreated rhesus monkeys (abscissa; from McMahon et al., 2001). A dose equal to 1 represents the ED_{50} of the positive modulator in substituting for midazolam. Doses of pregnanolone and pentobarbital smaller than their respective ED_{50} values in substituting for midazolam shifted the flumazenil, Ro 15-4513, and β-CCE dose-effect functions to the right (Figure 4, squares and diamonds). In contrast, doses of diazepam much larger than the ED_{50} value in substituting for midazolam were required to shift the flumazenil, Ro 15-4513, and β-CCE dose-effect curves to the right (Figure 4, circles). The magnitude of shift from the various positive modulators did not systematically vary as a function of negative efficacy reported for flumazenil (i.e., little to none), Ro 15-4513 (i.e., low) and β-CCE (i.e., intermediate to high), as evidenced by overlapping 95% CL for the potency ratios (Table 1, compare potency ratios and 95% CL within the same row). For example, potency ratios (ED_{50} determined in the presence of positive modulator / control ED_{50}) were 11 (4.1 – 27), 7.2 (3.8 – 13), and 6.4 (3.4 – 12) for flumazenil, Ro 15-4513, and β-CCE, respectively, in combination with 10 mg/kg of pentobarbital.

DISCUSSION

Whereas in untreated animals the behavioral effects of BZ-site negative $GABA_A$ modulators ligands can be differentiated on the basis of efficacy, the relationship between negative efficacy and behavioral activity appears to be less important in BZ-dependent animals. To examine the relationship between negative efficacy and the flumazenil-like effects of BZ site ligands in BZ-dependent monkeys, flumazenil, Ro 15-4513 and β-CCE were combined with diazepam, pentobarbital and pregnanolone in diazepam-treated rhesus monkeys that discriminated flumazenil. Ro 15-4513 and β-CCE substituted for flumazenil, and the discriminative stimulus effects of flumazenil, Ro 15-4513, and β-CCE were attenuated by diazepam, pentobarbital and pregnanolone. The magnitude of attenuation did not systematically vary as a function of negative efficacy, i.e., did not vary among flumazenil, Ro 15-4513 and β-CCE. When compared to their potency in substituting for a midazolam discriminative stimulus in untreated monkeys (McMahon et al., 2001), pentobarbital and pregnanolone were relatively more potent than diazepam in attenuating the discriminative stimulus of flumazenil, Ro 15-4513, and β-CCE. These results show that BZ-site neutral and negative modulators have strikingly similar discriminative stimulus effects in BZ-dependent monkeys, and suggest that negative efficacy is not important for the capacity of these ligands to induce BZ withdrawal. In addition, these results suggest that positive modulators at non-BZ sites are particularly effective in preventing BZ withdrawal.

BZ treatment increases the sensitivity of monkeys to the effects of flumazenil, and the discriminative stimulus effects of flumazenil in BZ-treated animals appear to be related to BZ withdrawal (Gerak and France, 1999; McMahon and France, 2002). The combined effects of flumazenil and BZs are similar in both untreated and BZ-treated animals, with flumazenil

surmountably antagonizing the discriminative stimulus effects of BZs (Lelas et al., 1999), and BZs attenuating the discriminative stimulus effects of flumazenil (i.e., BZ withdrawal). In contrast, although positive modulators at barbiturate and neuroactive steroid sites are qualitatively similar to BZs, flumazenil typically does not attenuate their behavioral effects (Herling and Shannon, 1982; McMahon and France, 2001; 2005), and under some conditions can enhance the behavioral effects of positive modulators acting at non-BZ sites (McMahon and France, 2006), consistent with the view that flumazenil has low positive efficacy under some conditions (e.g., Mehta and Ticku, 1989). The present study demonstrates that positive modulators acting at barbiturate and neuroactive steroid sites attenuate the flumazenil discriminative stimulus (also McMahon et al., 2001). Thus, whereas flumazenil does not attenuate the behavioral effects of positive modulators at non-BZ sites in untreated monkeys, the discriminative stimulus effects of flumazenil in diazepam-treated monkeys are attenuated by positive modulators at non-BZ sites, thereby providing a measure of the interaction between flumazenil and positive modulators at non-BZ sites.

As demonstrated previously (Gerak and France, 1999; McMahon and France, 2005), negative modulators with low (Ro 15-4513) and high efficacy (β-CCE; Mehta and Ticku, 1989) at BZ sites substitute for flumazenil in diazepam-treated monkeys. The present study shows that, in addition to attenuating the flumazenil discriminative stimulus, a BZ-site positive modulator (diazepam) also attenuates the flumazenil-like effects of Ro 15-4513 and β-CCE. Similar results have been obtained in untreated monkeys, i.e., Ro 15-4513 and β-CCE surmountably antagonized the discriminative stimulus effects of diazepam and other BZs in untreated monkeys (Lelas et al., 1999). Like diazepam, a barbiturate (pentobarbital) and a neuroactive steroid (pregnanolone) attenuated the flumazenil-like discriminative stimulus effects

of Ro 15-4513 and β-CCE. In a previous study, pregnanolone substituted for a midazolam discriminative stimulus in rhesus monkeys, and the effects of pregnanolone were attenuated by β-CCE and not by flumazenil or Ro 15-4513 (McMahon and France, 2005); similar results have been obtained with pentobarbital in these monkeys (unpublished observations). Therefore, in otherwise untreated monkeys, negative efficacy appears to be an important determinant of the interaction between negative and positive modulators acting at different sites.

Whereas flumazenil typically has little or no behavioral activity in untreated animals, in BZ-treated animals it has behavioral effects that can resemble the effects of negative modulators in untreated animals (Sannerud et al., 1991). Moreover, negative modulators at BZ sites substitute for a flumazenil discriminative stimulus in BZ-treated animals (present results; Gerak and France, 1999; McMahon and France, 2005). To examine whether induction of BZ withdrawal varies as a function of negative efficacy at BZ sites, this study examined whether positive modulators differentially attenuate the discriminative stimulus effects flumazenil, Ro 15-4513, and β-CCE in BZ-dependent monkeys. The magnitude of attenuation by pentobarbital and pregnanolone did not systematically vary among flumazenil, Ro 15-4513 and β-CCE. Thus, whereas negative efficacy appears to be important for the effects of BZ site ligands in untreated animals (e.g., the effects of pregnanolone are attenuated by β-CCE and not by flumazenil; McMahon and France, 2005), negative efficacy is not important for the flumazenil-like discriminative stimulus effects of BZ site ligands in BZ-dependent monkeys (e.g., the effects of flumazenil and β-CCE are equally attenuated by pregnanolone).

The current study suggests that the capacity of a ligand to induce some indices of BZ withdrawal (i.e., discriminative stimulus effects) does not vary as a function of the efficacy of that ligand at BZ sites. In contrast, efficacy at BZ sites was reportedly an important feature of

the capacity of a ligand to induce other indices (e.g., convulsions) of BZ withdrawal (Martin et al., 1995). While differences in negative efficacy did not appear to be important for discriminative stimulus effects in BZ-dependent animals trained to discriminate flumazenil, modulators that vary in negative efficacy might have differential effects in BZ-dependent animals trained to discriminate a BZ-site negative modulator. However, the feasibility of this approach would likely be limited by adverse effects (e.g., convulsions) that can be induced by negative modulators in BZ-dependent rhesus monkeys (e.g., McMahon and France, 2005).

The relative potency among positive modulators in substituting for the discriminative stimulus effects of midazolam (0.56 mg/kg) in untreated monkeys did not predict their relative potency for attenuating the effects of flumazenil (also McMahon et al., 2001), or the flumazenil-like effects of Ro 15-4513 and β-CCE, in BZ-treated monkeys (Figure 4). For example, diazepam attenuated the effects of flumazenil, Ro 15-4513, and β-CCE at doses larger than the doses of diazepam required to substitute for midazolam. The opposite relationship was obtained with positive modulators at non-BZ sites, i.e., pentobarbital and pregnanolone attenuated the effects of flumazenil, Ro 15-4513, and β-CCE at doses smaller than the doses of pentobarbital and pregnanolone required to substitute for midazolam. In a previous study in rhesus monkeys, diazepam (5.6 mg/kg/day) conferred cross-tolerance to BZs and not pentobarbital or pregnanolone (McMahon and France, 2002), suggesting that the greater potency of non-BZ site ligands to attenuate diazepam withdrawal is due to the development of BZ tolerance without cross-tolerance to non-BZ site ligands. Another explanation for these results is that noncompetitive interactions at the $GABA_A$ receptor complex (e.g., between flumazenil and pregnanolone) more potently attenuate flumazenil than competitive interactions at BZ receptors (e.g., between flumazenil and diazepam).

In BZ-dependent rhesus monkeys, positive modulators acting at different sites on the $GABA_A$ receptor complex attenuated the discriminative stimulus effects of flumazenil, and the flumazenil-like discriminative stimulus effects of Ro 15-4513 and β-CCE. The magnitude of attenuation by positive modulators was the same for flumazenil, Ro 15-4513, and β-CCE (i.e., did not vary as a function of negative efficacy), indicating that differences in negative modulation are not important for the flumazenil-like discriminative stimulus effects of these ligands in BZ-dependent monkeys. Modulatory site was an important determinant of the relative potency of positive modulators to attenuate the discriminative stimulus effects of flumazenil (i.e., BZ withdrawal). When compared to their potency in untreated animals, pentobarbital and pregnanolone were relatively more potent than diazepam in attenuating these discriminative stimulus effects that are related to BZ withdrawal. Whereas efficacy (negative modulation) at BZ sites does not appear to be important for the capacity of drugs to induce BZ withdrawal, the particular site at which a positive modulator acts is one important determinant for the capacity of drugs to attenuate BZ withdrawal, such that positive modulators at non-BZ sites (neuroactive steroids) are particularly effective in attenuating BZ withdrawal.

ACKNOWLEDGMENTS

The authors thank C. Cruz, B. Harrington, M. Hernandez, D. Logan, and R. Luna for providing technical assistance.

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FOOTNOTES

This study was supported by U.S. Public Health Service Grant DA09157 and a Senior Scientist Award to C.P.F. (K05 DA17918).

LEGENDS FOR FIGURES

Figure 1 Attenuation of the discriminative stimulus effects of flumazenil (left), Ro 15-4513 (middle), and β-CCE (right) by diazepam in diazepam-treated monkeys. *Abscissae:* dose in mg/kg body weight; V, vehicle. *Ordinates:* mean (± S.E.M.) percentage of responding on the flumazenil lever (top) and mean (± S.E.M.) response rate expressed as percentage of control (vehicle training days) rates [rate (% of control), bottom].

Figure 2 Attenuation of the discriminative stimulus effects of flumazenil, Ro 15-4513, and β-CCE by pentobarbital in diazepam-treated monkeys. See Figure 1 for other details.

Figure 3 Attenuation of the discriminative stimulus effects of flumazenil, Ro 15-4513, and β-CCE by pregnanolone in diazepam-treated monkeys. Discrimination data for 3.2 mg/kg of pregnanolone in combination with Ro 15-4513 and β-CCE represent data from 2 monkeys and 1 monkey, respectively. See Figure 1 for other details.

Figure 4 Rightward shift in the flumazenil, Ro 15-4513, and β-CCE dose-effect functions elicited by positive $GABA_A$ modulators expressed as a multiple of their midazolam substitution ED_{50}. *Abscissae:* multiple of the ED_{50} of the appropriate positive $GABA_A$ modulator in substituting for the discriminative stimulus effects of midazolam (0.56 mg/kg). *Ordinate:* mean (±S.E.M.) rightward shift in the dose-effect function expressed as ED_{50} following pretreatment with the appropriate positive $GABA_A$ modulator divided by the corresponding control ED_{50}. Error variance represents the 95% CL. Vertical dashed line represents the ED_{50} for

midazolam substitution. ED_{50}s for 3.2 mg/kg of pregnanolone in combination with Ro 15-4513 and β-CCE represent data from 2 monkeys and 1 monkey, respectively.

JPET Fast Forward. Published on May 16, 2006 as DOI: 10.1124/jpet.106.103168
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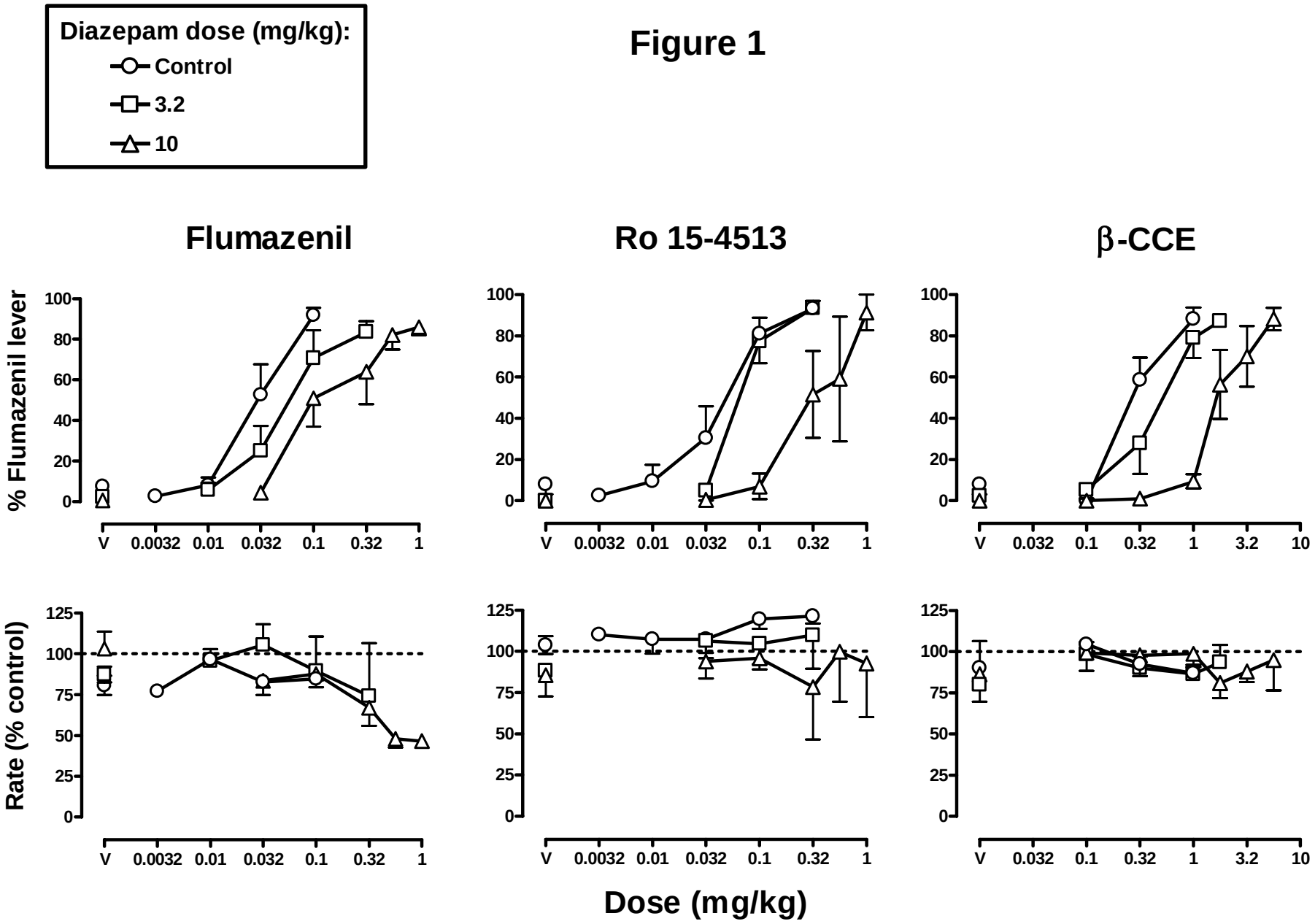
Table 1 Attenuation of the discriminative stimulus effects of flumazenil, Ro 15-4513, and β-CCE (expressed as ratio of ED_{50} following pretreatment with positive modulator / control ED_{50}) by diazepam, pentobarbital and pregnanolone in diazepam-treated monkeys.

Positive modulator (dose in mg/kg)	**Dose ratio (95% CL)**		
	Flumazenil	**Ro 15-4513**	**β-CCE**
Diazepam (3.2)	1.9 (0.9-4.0)	2.0 (0.8-4.6)	1.6 (1.0-2.5)
Diazepam (10)	5.1 (2.5-11)*	8.3 (2.9-24)*	6.9 (3.7-13)*
Pentobarbital (3.2)	2.4 (1.0-5.8)	3.9 (1.6-9.0)*	3.7 (1.6-8.3)*
Pentobarbital (10)	11 (4.1-27)*	7.2 (3.8-13)*	6.4 (3.4-12)*
Pregnanolone (1)	2.6 (1.2-5.4)*	3.8 (1.6-9.1)*	3.9 (2.3-6.7)*
Pregnanolone (3.2)	9.3 (5.1-17)*	12 (2.7-54)* [a]	6.1 (3.1-12)* [b]

*significantly different from 1 (i.e., ED_{50} determined after pretreatment with positive modulator significantly different from the control ED_{50})

[a] data from 2 monkeys
[b] data from 1 monkey



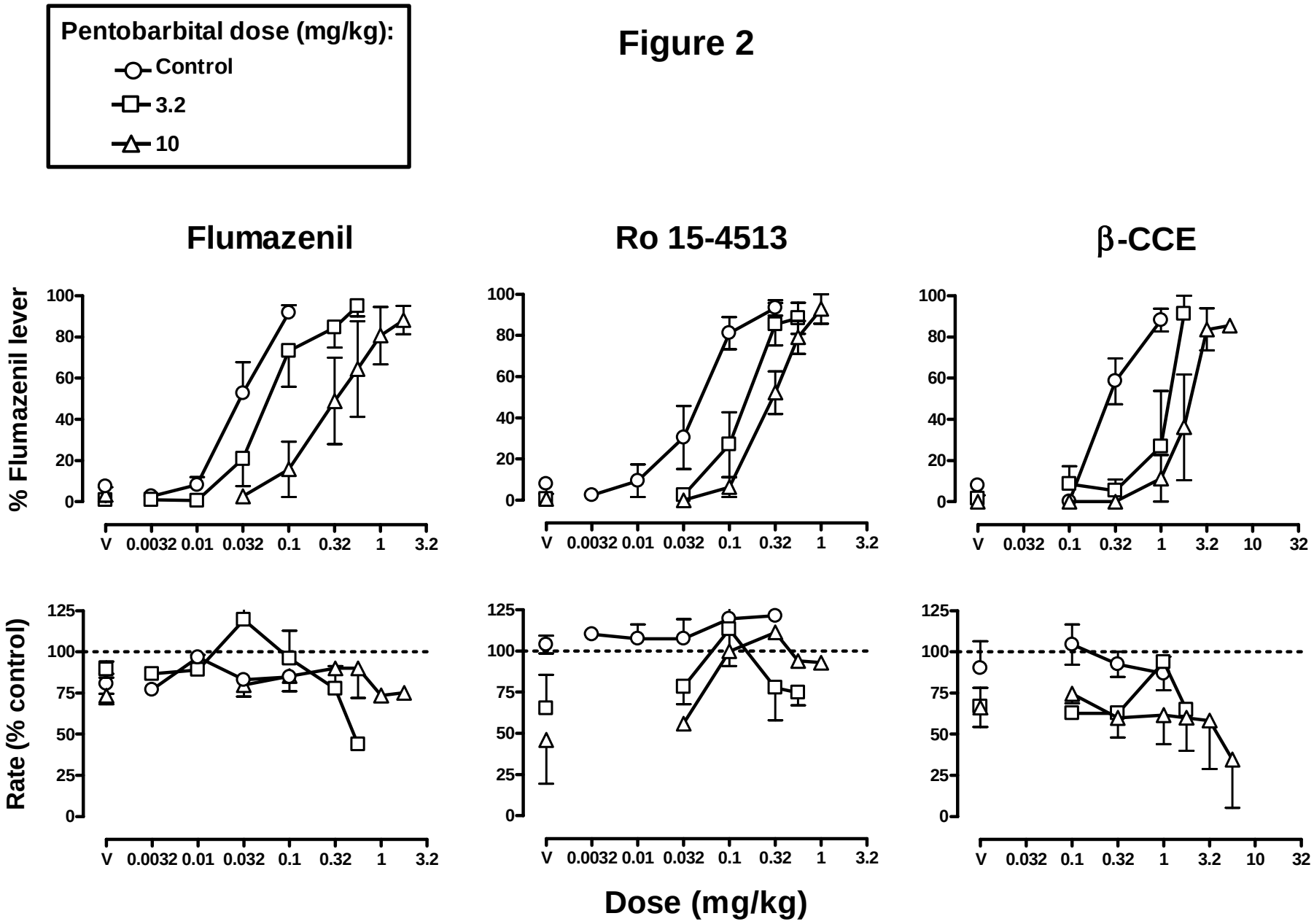


Figure 2

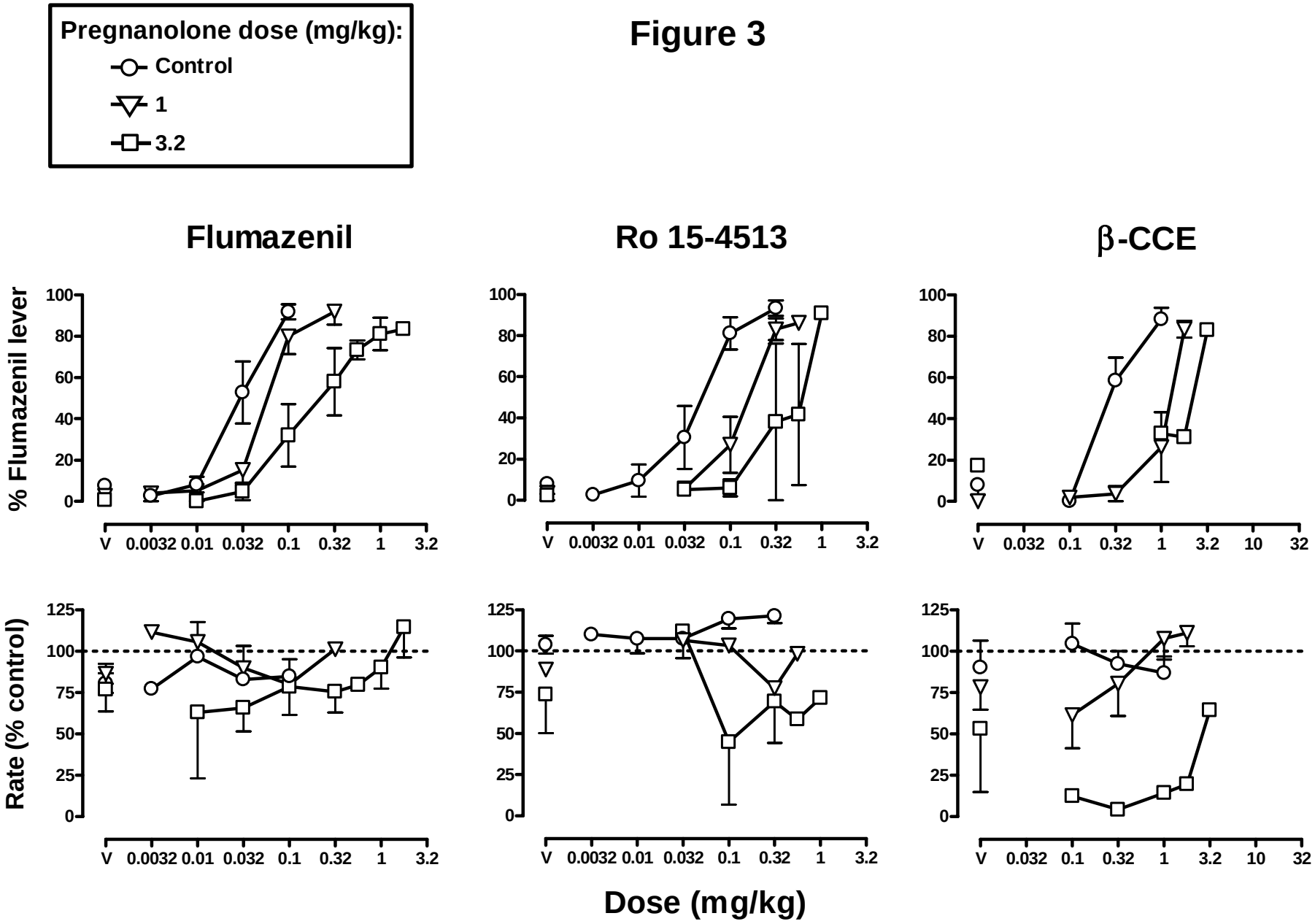


Figure 3

Figure 4