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Evidence that Sedative Effects of Benzodiazepines Involve Unexpected $GABA_A$ Receptor Subtypes: Quantitative Observation Studies in Rhesus Monkeys

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a) **Running title:** Novel sedation measures for benzodiazepines

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a) Nonstandard Abbreviations: βCCT (β-carboline-3-carboxylate-*t*-butyl ester); α1GABA$_A$ receptors (GABA$_A$ receptors containing the α1 subunit); α2GABA$_A$ receptors (GABA$_A$ receptors containing the α2 subunit); α3GABA$_A$ receptors (GABA$_A$ receptors containing the α3 subunit); α5GABA$_A$ receptors (GABA$_A$ receptors containing the α5 subunit); HZ-166 (8-ethynyl-6-(2′-pyridine)-4*H*-2,5,10b-triaza-benzo[*e*]azulene-3-carboxylic acid ethyl ester); MRK-696 (7-cyclobutyl-6-(2-methyl-2*H*-1,2,4-triazol-2-ylmethoxy)-3-(2-flurophenyl)-1,2,4-triazolo(4,3-*b*)pyridazine); TPA023B (6,2′-difluro-5′-(3-(1-hydroxy-1-methylethyl)imidazo(1,2-*b*((1,2,4)triazin-7-yl)(1,1′-biphenyl)-2-carbonitrile);

b) **Recommended Section Assignment:** Behavioral Pharmacology

Abstract

Using non-human primates, we introduced a new set of behavioral categories for observable sedative effects based on pediatric anesthesiology classifications. We examined the effects of alprazolam and diazepam (non-selective benzodiazepines), zolpidem (preferential binding to α1 subunit-containing $GABA_A$ receptors), HZ-166 (8-ethynyl-6-(2′-pyridine)-4*H*-2,5,10b-triaza-benzo[*e*]azulene-3-carboxylic acid ethyl ester; functionally selective with relatively high intrinsic efficacy for α2 and α3 subunit-containing $GABA_A$ receptors), MRK-696 (7-cyclobutyl-6-(2-methyl-2*H*-1,2,4-triazol-2-ylmethoxy)-3-(2-flurophenyl)-1,2,4-triazolo(4,3-*b*)pyridazine; no selectivity but partial intrinsic activity), and TPA023B (6,2′-difluro-5′-(3-(1-hydroxy-1-methylethyl)imidazo(1,2-*b*((1,2,4)triazin-7-yl)(1,1′-biphenyl)-2-carbonitrile; partial intrinsic efficacy and selectivity for α2, α3, α5 subunit-containing $GABA_A$ receptors) using quantitative behavioral observation techniques in rhesus monkeys. We further examined the role of α1 subunit-containing $GABA_A$ receptors in benzodiazepine-induced sedative effects by pretreating animals with the α1 subunit-preferring antagonist β-carboline-3-carboxylate-*t*-butyl ester (βCCT). Increasing doses of alprazolam and diazepam resulted in the emergence of observable ataxia, rest/sleep posture, moderate and deep sedation. In contrast, zolpidem engendered dose-dependent observable ataxia and deep sedation but not rest/sleep posture or moderate sedation, while HZ-166, and TPA023 induced primarily rest/sleep posture. MRK-696 induced rest/sleep posture and observable ataxia. Zolpidem, but no other compounds, significantly increased tactile/oral exploration. The sedative effects engendered by alprazolam, diazepam, and zolpidem generally were attenuated by βCCT pretreatments, whereas rest/sleep posture and suppression of tactile/oral exploration were insensitive to βCCT administration. These data suggest that α2/3-containing $GABA_A$ receptor subtypes unexpectedly may mediate a

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mild form of sedation (rest/sleep posture), whereas α1-containing $GABA_A$ receptors may play a role in moderate/deep sedation.

INTRODUCTION

Benzodiazepines exert their effects by binding to the γ-aminobutyric acid type A ($GABA_A$) receptor. $GABA_A$ receptors are pentameric chloride ion channels consisting primarily of two α, two β, and one γ subunit with multiple subtypes for each subunit. Accumulating evidence suggests that the behavioral effects of benzodiazepines can be attributed to different $GABA_A$ receptor subtypes (Rowlett *et al*, 2005; Rudolph *et al*, 2011; Tan *et al*, 2011). Studies have shown that $GABA_A$ receptors containing the α1 subunit (i.e., $\alpha1GABA_A$ receptors) play a role in the sedative and motor-impairing effects of benzodiazepines (e.g., Rudolph *et al*, 1999; McKernan *et al*, 2000). In contrast, $GABA_A$ receptors containing α2 and/or α3 subunits (i.e., $\alpha2GABA_A$ and/or $\alpha3GABA_A$ receptors) are believed to be involved in the anxiolytic properties of benzodiazepines (e.g., Löw *et al*, 2000; McKernan *et al*, 2000; Rowlett *et al*, 2005) although recent evidence suggests a role for α5 subunit-containing $GABA_A$ receptors ($\alpha5GABA_A$ receptors; Behlke *et al.*, 2016).

While the idea that $\alpha1GABA_A$ receptors mediate the sedative and motoric effects of benzodiazepines has gained considerable acceptance, there are findings in the literature that suggest some aspects of the sedative actions of benzodiazepines might involve other $GABA_A$ receptor subtypes. For example, administration of zolpidem, a benzodiazepine-type ligand that binds preferentially to $\alpha1GABA_A$ receptors, was associated with procumbent posture (essentially a measure of robust sedation and/or anesthesia) in squirrel monkeys that was insensitive to pretreatments with β-carboline-3-carboxylate-*t*-butyl ester (βCCT), an $\alpha1GABA_A$-preferring antagonist (Platt *et al*, 2002). Moreover, in a review of the clinical literature, Skolnick (2012) noted several instances of compounds lacking activity at $\alpha1GABA_A$ receptors but having demonstrable sedative effects in human subjects (see also Atack, 2011; Nickolls *et al.* 2018). A

recent study using transgenic mouse technology may provide a clue to these effects in human subjects, in that mice with point mutations resulting in loss of benzodiazepine sensitivity for all but α3GABA$_A$ receptors showed significant inhibition of locomotor activity when administered diazepam (Behlke *et al.* 2016). These results raise the possibility that subtypes other than α1GABA$_A$ subtypes may play a role in sedative and motoric effects of benzodiazepines.

The incongruent results with respect to GABA$_A$ receptor subtypes and sedative effects of benzodiazepine-type compounds may reflect, in part, differences across both laboratories and species in definitions of sedation. These definitions vary widely across laboratories using non-human animal species, ranging from measures of locomotor activity to operant behavior to observational measures. The extent to which these preclinical measures are reliable measures of the typical sedative metrics used in human clinical studies (e.g., self-report of "dizziness, light headedness, tiredness, drowsiness" and so on) is unclear at present. To begin to address this issue, we adapted definitions of sedation based on levels of arousal as defined by the American Society of Anesthesiologists (ASA), which has published standard definitions for drug-induced levels of sedation in pediatric patients (see American Society of Anesthesiologists, 2002). Arousal was measured by trained observers who assessed the ability of the animal to attend to external stimuli (e.g., the observer, other animals in the room) and consisted of distinct levels adapted from the ASA standards. In addition to levels of sedation, we evaluated the effects of benzodiazepine-type compounds on species-typical behavior, in order to determine the effects of the compounds on ongoing behavior and the ability of the compounds to elicit behaviors potentially indicative of side effects (e.g., observable ataxia).

Using this behavioral method, the current study determined levels of sedation and observable species-typical behaviors in monkeys following administration of alprazolam and

diazepam, non-selective benzodiazepines used commonly to treat anxiety disorders, and zolpidem, a non-benzodiazepine with preferential binding to α1GABA$_A$ receptors. In addition, we included a series of compounds with differing selectivity profiles, including HZ-166, which has functional selectivity for, and relatively high efficacy at α2- and α3GABA$_A$ receptors (Rivas *et al*, 2009; Fischer *et al*, 2011). Additional compounds were MRK-696, which is a non-selective partial positive allosteric modulator, and TPA023B, which is a zero efficacy ligand at α1GABA$_A$ subtypes but a partial modulator at α2-, α3-, and α5GABA$_A$ subtypes (profiles summarized below and in Shinday *et al.*, 2013). We further investigated the involvement of GABA$_A$ receptor subtypes in observable behavioral effects by assessing the effects of alprazolam, diazepam, zolpidem and HZ-166 in the presence of βCCT, an antagonist that binds preferentially to α1GABA$_A$ receptors (Huang *et al*, 2000).

MATERIALS AND METHODS

Subjects and Surgery

Ten adult rhesus monkeys (*Macaca mulatta,* five female and five male, weighing between 6.0 to 9.0 kg) were housed in individual home cages under free-feeding conditions, except for 4 monkeys used in conflict experiments (see below). Water was available *ad libitum*. Rooms were maintained on a 12-hr light/12-hr dark schedule (lights on at 0630 hr). Animals in this study were maintained in accordance with the *Guide for Care and Use of Laboratory Animals* (8th edition, 2011). Research protocols were approved by the Harvard Medical School Institutional Animal Care and Use Committee.

Monkeys were prepared with chronic indwelling venous catheters following the general surgical procedures described by Platt et al. (2011). The external end of the catheter was either fed through a fitted jacket and tether system (Lomir Biomedical, NY, USA), attached to a fluid swivel mounted to the cages. The catheters were flushed daily with heparinized saline (100 U/ml) and the exit site of the catheter was inspected routinely.

Functional Selectivity Profiles and Drug/Compound Information

Two functionally-selective compounds were evaluated: HZ-166 and TPA023B. Based on patch-clamp electrophysiology in cloned $GABA_A$ receptor subtypes, HZ-166 is functionally-selective for, and has relatively high efficacy at α2- and α3$GABA_A$ receptors (Rivas *et al*, 2009; Fischer *et al*, 2011). Using similar approaches, TPA023B was characterized as functionally selective for α2-, α3-, and α5$GABA_A$ receptors, although it considered a partial allosteric modulator at those sites (Atack, 2011). In order to evaluate the role of α1$GABA_A$ receptors in the observable behavioral effects of benzodiazepines, zolpidem and βCCT were assessed, both of

which bind preferentially to α1GABA$_A$ receptors (Huang *et al.* 2000). Comparisons were made with conventional benzodiazepines (alprazolam, diazepam) as well as with a non-selective partial allosteric modulator, MRK-696, the latter of which is structurally-related to and pharmacologically comparable with MRK-409 which progressed into Phase 1 clinical trials (Atack et al., 2011).

All drugs were administered intravenously. The base forms of alprazolam, diazepam, zolpidem, (Sigma-Aldrich, St. Louis, MO, USA), flumazenil (Tocris bioscience, Bristol, UK), HZ-166 and βCCT (both synthesized from the Department of Chemistry and Biochemistry, University of Wisconsin-Milwaukee, Milwaukee, WI, USA) were dissolved in 95% ethanol as required. Solutions were then diluted to the desired concentration using propylene glycol (50%-80%) and sterile water. The base forms of MRK-696 and TPA023B (synthesized at the Merck, Sharp & Dohme Neuroscience Research Centre, Terlings Park, Harlow, UK; Atack 2011) were prepared in solutions of 10% benzoyl ethanol, 50% propylene glycol, and 40% water. All drugs/compounds were filter-sterilized prior to infusion (0.2 μm pore syringe filters).

Behavioral Observation Procedure

Behavioral observations were conducted using the focal animal behavioral scoring system described previously by Novak and colleagues (1992; 1998) which we modified to include benzodiazepine-associated behaviors (Yanagita *et al*, 1980; Weerts and Griffiths, 1998; Platt *et al*, 2002). The observers (6 total) met a 95% inter-observer reliability criterion prior to the experiments and were blind to the drug treatments. A range of 19 species typical behaviors, as well as characteristic drug effects (see Table 1 for all definitions of behaviors), were scored by recording the presence or absence of each behavior in 15 sec. intervals during a 5-min

observation period. Scores were calculated from these data as the number of intervals in which a particular behavior occurred. The maximum possible score for each behavior was 20 for each 5-min observation period.

For sedation measures, we used an assessment strategy based on standards used for anesthesia of pediatric patients (American Society of Anesthesiologists, 2002). We adapted the definitions for use in non-human primates such that when a monkey was observed to have closed eyes, an assessment of the animal's responsiveness to the stimuli was determined. Specifically, the observer presented three stimuli: 1) walked at a normal pace towards the cage, 2) spoke the animal's name, and 3) moved the lock used to secure the door of the cage (monkeys were usually very attentive to unlocking the cage door). If the monkey responded immediately (i.e., opened eyes and oriented to the observer), *rest/sleep posture* was scored. If the monkey attended more slowly (i.e., > 3 seconds following stimuli) and was observed to be assuming an atypical posture that differed from the characteristic rest/sleep posture (e.g., unable to keep an upright posture), the observer scored *moderate sedation*. If the monkey did not open eyes across/throughout the 15-s interval after all three stimuli, the observer noted the loss of ability to respond to external stimuli and made as assessment of *deep sedation*[1], which also was accompanied characteristically by atypical posture as described above. The assessment of sedation was initiated during the 5-min sampling period if the animal presented, at any time during that period, with its eyes closed. The result of this assessment was recorded for each remaining 15-sec interval of the 60-sec epoch unless eyes opened. Afterwards, eyes closing again initiated the

[1] The American Society of Anesthesiologists (2002) equivalents to the monkey levels of sedation are: Rest/sleep posture ≈ "anxiolysis" (calm, patient shows no impairment in ability to respond to verbal or tactile stimulation), moderate sedation ≈ "conscious sedation" (a depression of consciousness, but the patient maintains the ability to respond to verbal or tactile stimulation), and deep sedation ≈ "deep sedation" (patient not easily aroused by verbal stimuli and has only a limited response to tactile stimulation).

assessment. If eyes remained closed, then the assessment was repeated at the beginning of the next 60-sec epoch. Therefore, the maximum score for sedation during a 5-min period was 20 (4 possible scores for 4 x 15-sec intervals per min, 5 min total, i.e., 4 possible scores x 5 min= 20).

Administration of Compounds

Behavioral profiles for the benzodiazepine ligands were determined after monkeys habituated to the presence of observers. Baseline data were obtained following saline or vehicle injections. Subsequently, the effects of a range of doses of alprazolam (0.01-1 mg/kg), diazepam (0.1-10 mg/kg), zolpidem (0.1-10 mg/kg), HZ-166 (1-30 mg/kg) MRK-696 (0.03-3.0 mg/kg), and TPA-023B (0.003-1.0 mg/kg) were evaluated immediately following the i.v. injection (i.e., sampling time of 0 min post-injection) and until 4 hrs post-injection (i.e., sampling times of 7.5, 15, 30, 60, 120 and 240 min) in at least four monkeys per drug (2 females, 2 males). Different doses of each compound were evaluated with at least a 1-day drug free period between tests. Doses were tested in an irregular order, with each compound completed prior to tests with another compound.

After completion of testing of the above compounds alone, the behavioral effects of selected doses of alprazolam, diazepam, and HZ-166 were re-assessed following pretreatment with βCCT (0.1 – 3.0 mg/kg, i.v., immediately before session), an $\alpha 1GABA_A$ receptor-preferring antagonist and flumazenil (0.3 mg/kg, i.v.), a non-selective benzodiazepine receptor antagonist.

Data Analysis

For each subject, scores for each behavior were calculated as the number of 15-s intervals in which the behavior occurred (maximum score = 20). Scores were then cumulated across the

time points (the dependent measure is referred to as "cumulative score"). These data points were averaged across subjects to obtain group means. In order to make statistical determinations, the data were evaluated for departures from normal distribution using the Shapiro-Wilk Normality test (*W*-statistic) in cases in which behaviors occurred above zero (one-sample t-test). For conflict studies, data were collected as averaged responses/s for individual monkeys and then averaged to obtain group means. To determine statistical reliability of treatment effects on each behavior (including conflict data), the effect of dose was determined for each drug by separate repeated measures analyses of variance (ANOVA) with dose or dose and time after injection as the factors. Treatment effects were assessed further with Bonferroni t-tests in which the effects of different doses of each drug were compared to vehicle (statistics performed with SigmaPlot version 13, Build 13.0.0.83).

Potency values were calculated from ascending limbs of dose-response functions by calculating ED_{50} values (i.e., dose that induced a behavioral effect that was 50% of the average maximum). The ED_{50} values were calculated for individual animals by log linear regression using $\log_{10}$ transformed dose and effect calculated as percentage of the average maximum for a treatment condition (ED_{50} values are presented as mean ± SEM). For all statistical analyses, significance levels were set at $p \leq 0.05$.

RESULTS

Summary of Behavioral Effects

Under baseline conditions (i.e., saline pretreatment), monkeys displayed varying degrees of species-typical behaviors, with passive/visual exploration, locomotion, grooming, and tactile/oral exploration being the most commonly observed behaviors (for details, see Supplemental Figure 1, Table 1). In preliminary tests, no significant behavioral differences were observed following saline or vehicle, indicating the propylene glycol-based vehicles had no effects on baseline behaviors (data not shown). None of the behaviors indicative of sedative effects were observed during baseline sessions (e.g., rest/sleep posture, moderate sedation or deep sedation; Supplemental Figure 1).

For concise presentation and analyses of data, we collapsed the time course of each drug by cumulating average scores across the post-injection times. For species-typical behavior, all but nose rub (NRU), fear grimace (FGR), and cage shake (CSH) were present under baseline conditions (Supplemental Figure 1, one-sample t-tests, $p<0.05$). For 8/13 behaviors that occurred, tests for normality were significant ($W\geq0.86$, Shapiro-Wilk test). Additionally, we evaluated departures from normality for sedation measures at the highest effective doses for each drug/compound that induced an effect, and the tests for normality were significant for all cases (data not shown). Given the majority of datasets were normally distributed and the robustness of ANOVA to departures from normality, we used parametric statistics (consistent with our other report using this technique, Rüedi-Bettschen *et al.* 2013).

Of the 19 behaviors quantified after treatments with drugs/compounds, the majority of species-typical behaviors did not change. In contrast, sedation-related behaviors were induced by the compounds. Alterations in behavior varied across the compounds, with alprazolam and

diazepam engendering the most changes in behavior and HZ-166 and TPA-023B the least changes (Supplemental Table 1). Our more detailed analysis (below) focused mainly on sedative measures, as well as a measure that occurred to some degree for all ligands (e.g., tactile/oral stimulation). Note that passive visual was not included in the more detailed analysis, although this behavior was altered by all drugs/compounds. This omission was due primarily because this measure always co-varied with the emergence of other behavioral effects. For example, increases in rest/sleep posture or decreases in tactile/oral exploration were accompanied by decreases in passive visual, reflecting that this category was mutually exclusive with (not independent of) other behaviors.

Measures of Sedation

Results from our new sedation measures for the conventional benzodiazepines are shown in Figure 1. Alprazolam (0.03 and 0.1 mg/kg) and diazepam (≥3.0 mg/kg) induced rest/sleep posture (Bonferroni t-tests, $p < 0.05$ vs. vehicle, Figure 1, top panels). For moderate sedation, higher doses of alprazolam (0.3 and 1 mg/kg) and the 3.0 mg/kg dose of diazepam resulted in significantly higher mean cumulative scores compared with vehicle (Bonferroni t-tests, $p < 0.05$, Figure 1, middle panels). For deep sedation, only the highest doses tested of alprazolam and diazepam (1.0 and 10 mg/kg, respectively) resulted in significantly higher mean cumulative scores compared with vehicle (Bonferroni t-tests, $p<0.05$, Figure 1, bottom panels).

Zolpidem (selective affinity for α1GABA$_A$ receptors), the non-selective partial allosteric modulator MRK-696, and the compounds lacking efficacy at α1GABA$_A$ receptors (HZ-166, TPA-023B) differed from the conventional benzodiazepines with respect to sedation measures. In this regard, a noted difference is that none of these drugs/compounds induced moderate

sedation at any dose tested (data not shown). That is, all doses of zolpidem, HZ-166, MRK-696, and TPA-023B resulted in mean cumulative scores at or near zero for moderate sedation (ANOVA and Bonferroni t-tests, $p>0.05$). Zolpidem did not induce rest/sleep posture at any dose tested (ANOVA and Bonferroni t-tests, $p > 0.05$, Figure 2, top panels). In contrast, HZ-166 (≥3.0 mg/kg), MRK-696 (≥1.0 mg/kg), and TPA-023B (≥0.03 mg/kg) induced significant and dose-dependent rest/sleep posture compared with vehicle (Bonferroni t-tests, $p<0.05$, Figure 2, top panels).

The mean cumulative scores for deep sedation were significantly higher than vehicle for the highest dose of zolpidem (10 mg/kg, Bonferroni t-test, $p < 0.05$, Figure 2, bottom panels). In contrast, no dose of HZ-166, MRK-696, or TPA-023B resulted in mean cumulative scores significantly above vehicle level for this measure (ANOVA and Bonferroni t-tests, $p >0.05$, Figure 2, bottom panels).

Tactile/Oral Exploration and Observable Ataxia

Figure 3 shows the species-typical behavior tactile/oral exploration, and a measure associated with motor impairment (observable ataxia) that were altered significantly by treatment with the two conventional benzodiazepines. Tactile/oral exploration behavior (exploring objects in the environment by hand and/or foot manipulation as well as any contact with environmental objects via the mouth, lips, and/or tongue) was suppressed by the highest doses of alprazolam and diazepam (1.0 mg/kg and 10 mg/kg, respectively; Bonferroni t-tests, $p < 0.05$, Figure 3 top panels). Increases in observable ataxia, defined as "any slip, trip, fall, loss of balance" (Table 1) were induced by both alprazolam and diazepam, with 0.3 mg/kg of alprazolam and 3.0 and 10 mg/kg of diazepam increasing observable ataxia significantly above vehicle levels (Bonferroni t-

tests, $p < 0.05$, Figure 3 bottom panels).

The compounds with selectivity at $GABA_A$ receptor subtypes could be differentiated based on alterations of tactile/oral exploration and observable ataxia (Figure 4). For example, zolpidem was unique in that a lower dose (1.0 mg/kg) significantly *increased* cumulative scores of tactile/oral exploration (Bonferroni t-test, $p < 0.05$ vs. vehicle), an effect not observed with any other drug/compound at any dose (Figure 4, top panels). In contrast, at the highest doses tested, zolpidem (10 mg/kg), HZ-166 (30 mg/kg), MRK-696 (3.0 mg/kg), and TPA-023B (1.0 mg/kg) significantly *decreased* tactile/oral exploration (Bonferroni t-tests, p's < 0.05 vs. vehicle, Figure 4, top panels). Zolpidem induced observable ataxia at a dose of 3.0 mg/kg, an effect also observed for MRK-696 at a dose of 1.0 mg/kg, but no other compounds (significant effects via Bonferroni t-tests, p's < 0.05 vs. vehicle, Figure 4, bottom panels).

Effects of βCCT on Drug-induced Sedation Measures

A range of doses (vehicle, 0.3, 1.0, 3.0 mg/kg, i.v.) of βCCT alone and 0.3 mg/kg, i.v. of flumazenil alone were tested at the beginning of the antagonism studies (N = 4) with no effects observed for any behavior (ANOVA, Bonferroni t-tests comparing each dose with vehicle, p's>0.05; data not shown). Results from sedative measures with the conventional benzodiazepines alprazolam and diazepam are shown in Figure 5. Peak effective doses (in parentheses above panels) for alprazolam and diazepam alone (points above "V"), and with pretreatments of 0.3 mg/kg, i.v., of flumazenil or a range of doses of βCCT (0.3-3.0 mg/kg, i.v.) are shown for the three sedation measures. For rest/sleep posture (Figure 5, top panels), both alprazolam and diazepam engendered average cumulative scores of ~40-50 that were reduced to an average of <10 by 0.3 mg/kg of flumazenil (Bonferroni t-tests, p's<0.05). However, no dose

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of βCCT (0.3-3.0 mg/kg) altered rest/sleep posture for either drug (Bonferroni t-tests, p's<0.05).

While βCCT was largely ineffective in blocking rest/sleep posture engendered by alprazolam and diazepam, dose-dependent attenuation of other sedation measures was evident. In this regard, mean moderate sedation scores induced by peak doses of both alprazolam and diazepam were attenuated significantly by 1.0 and 3.0 mg/kg βCCT, with the higher dose suppressing moderate sedation to the same level as 0.3 mg/kg flumazenil (Bonferroni t-tests, p's<0.05, Figure 5 middle panels). A similar pattern of attenuation was observed with deep sedation: 1.0 and 3.0 mg/kg of βCCT significantly reduced mean deep sedation scores induced by peak doses of alprazolam and diazepam to the same level as 0.3 mg/kg flumazenil (Bonferroni t-tests, p's<0.05, Figure 5 bottom panels).

Based on the studies with βCCT and the two conventional benzodiazepines, we conducted targeted studies in which the highest dose of βCCT evaluated was used as a pretreatment in observation tests with peak effective doses of zolpidem and HZ-166. Rest/sleep posture and deep sedation data for zolpidem and HZ-166 following βCCT or flumazenil pretreatment are summarized in Figure 6 (moderate sedation data are not shown because of the lack of effects in this measure by zolpidem and HZ-166). For HZ-166, pretreatment with flumazenil but not βCCT significantly decreased HZ-166-induced rest/sleep posture at a peak effective dose (Bonferroni t-test, $p < 0.05$ vs. HZ-166 alone, Figure 6 top panel). For zolpidem, pretreatment with both βCCT and flumazenil resulted in a significant attenuation of 10 mg/kg zolpidem-induced deep sedation (Bonferroni t-test, $p < 0.05$ vs. zolpidem alone, Figure 6 bottom panel).

Effects of βCCT on Tactile/Oral Exploration and Observable Ataxia

Concomitant with the sedation measures, antagonism data with tactile/oral exploration

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and observable ataxia were obtained with βCCT and flumazenil and results are summarized in Figures 7 and 8. For Fig. 7, peak effective doses (in parentheses above panels) for vehicle pre-treatments (i.e., alprazolam or diazepam alone; points above "V") and with 0.3 mg/kg, i.v., of flumazenil pretreatment (points above "F") or a range of doses of βCCT (0.3-3.0 mg/kg, i.v.) are shown for the behaviors. For tactile/oral exploration (Figure 7, top panels), both alprazolam and diazepam reduced average cumulative scores, an effect reversed by 0.3 mg/kg of flumazenil (Bonferroni t-tests, p's<0.05 "V" vs. "F"; Figure 7 top panels). However, no dose of βCCT (0.3-3.0 mg/kg) altered tactile/oral exploration for either drug (Bonferroni t-tests, p's<0.05 vs. "V"). While βCCT was largely ineffective in blocking the effects of alprazolam and diazepam on tactile/oral exploration, attenuation of observable ataxia was observed. In this regard, 0.3 mg/kg flumazenil, as well as 3.0 mg/kg (alprazolam) or 1.0 and 3.0 mg/kg (diazepam) of βCCT significantly reduced mean observable ataxia scores induced by peak doses of the two benzodiazepines to control levels (Bonferroni t-tests, p's<0.05 vs. "V", Figure 7 bottom panels).

For Fig. 8, the primary comparisons depicted are significant differences vs. the vehicle condition ("Veh). For zolpidem, the 1.0 mg/kg dose alone induced a significant increase in mean tactile/oral exploration scores, and this increase was blocked by pretreatment with both βCCT and flumazenil (Bonferroni t-tests, $p < 0.05$ vs. "Veh"; Figure 8, left panel). In contrast, a higher dose of zolpidem (10 mg/kg) significantly suppressed tactile/oral exploration, but this suppression unexpectedly was not reversed by pretreatment with βCCT, although it was reversed by pretreatment with flumazenil (Bonferroni t-tests, $p < 0.05$ and $p > 0.05$ vs. "Veh"; Figure 8, middle panel). Similarly, the reductions in tactile/oral exploration induced by HZ-166 were not altered significantly by βCCT but were reversed by flumazenil (Bonferroni t-tests, Figure 8 top right panel).

Potency Comparisons across Procedures

Table 2 shows potencies of all drugs and compounds from the present study, comparing sedative measures and observable ataxia with previously published data from our conflict model (e.g., Rowlett *et al*., 2006) and unpublished data (TPA-023B, MRK-696). The characteristic anti-conflict effect of conventional benzodiazepines is an increase in operant responding suppressed by response-contingent shock presentations at doses lower than those that decrease operant responding without contingent shock. For the behavioral measures shown in Table 2, alprazolam and diazepam had measurable effects for all behaviors, with anxiolytic-like effects and rest/sleep posture occurring at the lowest ED_{50} values, and moderate as well as deep sedation occurring at the highest ED_{50} values. In contrast, zolpidem demonstrated no anti-conflict effects, but reduced operant responding and induced observable ataxia as well as deep sedation only. Strikingly, HZ-166 (functionally selective full modulator of $\alpha2GABA_A$ and $\alpha3GABA_A$ receptors), MRK-696 (non-selective partial agonist), and TPA-023B (functionally selective partial modulator of a $\alpha2GABA_A$, $\alpha3GABA_A$, $\alpha5GABA_A$ receptors) had only anti-conflict effects and rest/sleep posture increases with similar potencies, with only MRK-696 engendering observable ataxia.

DISCUSSION

While clinical research has suggested that even relatively low levels of intrinsic efficacy at α1GABA$_A$ receptors can produce marked sedation in human subjects (Atack et al, 2011; Nickolls et al. 2018), evidence also has emerged that compounds lacking α1GABA$_A$ receptor efficacy can have sedative effects (for review, see Skolnick, 2012). The results from the present study suggest that the differences between preclinical and clinical research on benzodiazepine-associated sedation reflect, at least in part, the methodology used to measure sedative effects pre-clinically. To this end, we used a quantitative observation technique that separates sedation into three distinct functional categories, and found that α1GABA$_A$ receptors are likely responsible for moderate-to-deep sedation whereas α2 and/or α3GABA$_A$ receptors likely underlie a milder sedative effect referred to as "rest/sleep posture".

Novel Sedation Measures and Unexpected Roles for GABA$_A$ Subtypes

The conventional benzodiazepines alprazolam and diazepam showed a distinct profile of sedative effects, consisting of increased rest/sleep posture at the lowest doses, followed by emergence of moderate sedation, and then deep sedation at the highest doses tested. A similar transition from relatively mild to robust sedative effects was observed in squirrel monkeys with the nonselective benzodiazepine triazolam (Platt *et al*, 2002). There are different hypotheses as to why different effects of benzodiazepines emerge in a dose-dependent manner; one possibility is that activation of distinct receptor subtypes underlies this phenomenon. Support for this idea comes from the finding that zolpidem, an α1GABA$_A$-preferring allosteric modulator, engendered only deep sedation (for similar effects in squirrel monkeys, see Platt *et al*, 2002; and for humans, see Evans *et al*, 1990), whereas compounds with functional selectivity at α2GABA$_A$, α3GABA$_A$,

and/or α5GABA$_A$ receptors (Rivas *et al*, 2009; Fischer *et al*, 2010; Di Lio *et al*, 2011) induced rest/sleep posture but not moderate or deep sedation over a wide range of doses. It is important to note that we cannot rule out moderate/deep sedation emerging at doses higher than the ones tested here. However, moderate/deep sedation occurred at doses of the conventional benzodiazepines that were ~10-20-fold higher than doses that engendered rest/sleep posture, whereas for some compounds we were able to evaluate doses ~30-70-fold higher than those that resulted in significant rest/sleep posture. Moreover, available binding site occupancy data in nonhuman primates using positron-emission topography (PET) technology supports the idea that in most cases, the compounds were administered over sufficiently high dose ranges. For example, in PET studies with baboons, i.v. doses of TPA023B were estimated to occupy ~67% of binding sites (averaged across multiple brain regions) at 0.032 mg/kg (Atack *et al.*, 2011), a dose associated with rest/sleep posture in the present study. At a 10-fold higher dose (0.32 mg/kg, i.v.), the estimated occupancy of TPA023B was ≥95%, suggesting that our dose range of up to 1.0 mg/kg, i.v. was sufficient (assuming minimal differences between rhesus monkey and baboon, Atack *et al.*, 2011).

Although the finding of rest/sleep posture without more robust forms of sedation may be attributable to efficacy at α2/3/5GABA$_A$ receptors (either one subtype or combinations of the subtypes) but not α1GABA$_A$ subtypes, a caveat of this conclusion is that the same pattern of sedative effects was observed with MRK-696, a non-selective partial allosteric modulator. The latter finding raises the possibility that the lack of moderate/deep sedation may be due to low intrinsic efficacy, irrespective of subtype selectivity. However, HZ-166 is characterized as a higher efficacy compound at α2/3GABA$_A$ receptors (e.g., Fischer *et al*. 2010) and it did not engender deep sedation over a 30-fold dose range.

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Corroborating evidence for a role of α2 and/or α3GABA$_A$ receptors in rest/sleep posture also comes from our antagonism studies. We found that the α1GABA$_A$ receptor-preferring antagonist βCCT did not alter drug-induced rest/sleep posture, whereas the non-selective benzodiazepine antagonist flumazenil completely blocked this effect. Strikingly, βCCT dose-dependently and completely blocked both moderate and deep sedation induced by alprazolam and diazepam. Perhaps most convincingly, the dose of βCCT that completely blocked alprazolam- and diazepam-induced moderate and deep sedation did not reduce HZ-166-induced rest/sleep posture; however, this dose of βCCT did significantly reduce zolpidem-induced deep sedation. Altogether, these findings provide further evidence that deep and moderate sedation induced by benzodiazepines is mediated by α1GABA$_A$ receptors whereas rest/sleep posture is mediated by receptor subtypes other than α1GABA$_A$ receptors (i.e., α2-, α3- and/or α5GABA$_A$ subtypes).

Although the role (or lack thereof) of α1GABA$_A$ receptors in the present study is relatively unambiguous, our ability to parse behavioral effects among the distinct α2-, α3- and/or α5GABA$_A$ subtypes is unclear. Because HZ-166 resembled TPA023B with respect to behavioral effects, it seems logical that α5GABA$_A$ receptors are not required for engendering rest/sleep posture. However, the relative contribution of α2 vs. α3GABA$_A$ receptors remains to be determined, hampered primarily by the lack of compounds with selectivity for either receptor. We also cannot rule out the potential role of other GABA$_A$ receptor subunit subtypes as key mediators of different levels of sedation. In this regard, anesthetic agents such as etomidate have shown dose-dependent differences in specific components of the anesthetic response that appear to involve distinct subtypes of β-containing GABA$_A$ receptors (for review, see Rudolph and Antkowiak, 2004). However, differences in selectivity for the etomidate-sensitive receptors

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involved in the anesthetic response (β2 vs. β3-subunit containing receptors) have not been reported for the compounds tested in our studies (cf. Atack, 2011).

$GABA_A$ Receptors: Tactile/Oral Exploration and Observable Ataxia

In addition to sedation, changes in tactile/oral exploration and observable ataxia, a measure based on motor coordination, were observed. For alprazolam and diazepam, decreases in tactile/oral exploration, as well as emergence of observable ataxia, correlated with moderate and deep sedation, at first glance suggesting that changes in these behaviors may be due to emergence of the more robust sedative effects (essentially, mutually-exclusive or competing behaviors). Interestingly, MRK-696 induced observable ataxia, likely reflecting its binding and intrinsic efficacy at $\alpha 1GABA_A$ receptors. In this regard, it is interesting to note that the structurally- and pharmacologically-related compound MRK-409 did not produce overt signs of sedation in preclinical species (rotarod performance in mice and operant responding squirrel monkeys), suggesting that the observational measures of ataxia may be a more sensitive indicator of sedation in human subjects (Atack *et al*., 2011). Pretreatment with βCCT reversed observable ataxia induced by alprazolam and diazepam, suggesting a role of the $\alpha 1GABA_A$ receptor subtype in these effects. Surprisingly, a dose of βCCT that was effective in blocking other observable behavioral effects of benzodiazepines and the related compounds was ineffective in reversing decreases in tactile/oral exploration behavior for all drugs/compounds tested. These results suggest that benzodiazepine-induced decreases in tactile/oral exploration might be associated with one or a combination of $\alpha 2/\alpha 3/\alpha 5GABA_A$ receptors. Notably, a robust *increase* in tactile/oral exploration was observed following low-to-intermediate doses of zolpidem, but no other drug or compound. This increase was reversed by pretreatment with βCCT, consistent

with this behavioral increase involving the α1GABA$_A$ receptors subtype.

Comparisons with Anxiolytic-Like Effects

An important feature of our observation studies is the ability to compare with other studies in which the benzodiazepine-type compounds were administered to rhesus monkeys via the i.v. route. Comparison of potencies of alprazolam and diazepam across procedures suggests that rest/sleep posture might occur at doses that overlap with anxiolytic-like effects (the latter more typically associated with relatively lower dose ranges, Jones *et al.* 1994; Lingford-Hughes *et al.* 2005). Consistent with these findings, subject-rated effects of somnolence, drowsiness, fatigue, and tiredness occasionally were reported in humans with TPA-023B (Atack *et al*, 2011). Other compounds with similar functional selectivity profiles have proven to be mildly sedative in human subjects, despite preclinical indicators that sedation should be lacking (e.g., no decrease in locomotor activity in rodents; for review, see Skolnick, 2012; Nickolls *et al.*, 2018). Collectively, these results suggest that although compounds with functional selectivity for α2-, α3-, and/or α5GABA$_A$ receptors can reduce anxiety, these anxiolytic effects might be accompanied by mild sedation mediated by the same receptor subtype(s). It is critical to note that rest/sleep posture is defined by sleep-associated postural elements (e.g., eyes closed) in which the monkeys are easily roused by external stimuli. Therefore, this type of mild sedation may not be detectable in subjects if external stimuli are already present. Under these circumstances, the effects might only be detected by subject-rated effects.

Another behavioral effect occurring over the same dose ranges as anti-conflict effects and rest/sleep posture is inhibition of tactile/oral exploration. The translational relevance of this specific suppression of species-typical behavior is unclear at present. Nevertheless, both

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rest/sleep posture and tactile/oral exploration appear correlated with anti-conflict effects, suggesting that assessment of either (or both) might serve as a "proxy" for the more difficult to obtain anti-conflict effects. Taken together, we propose that the sedation measures used in the present experiment are more sensitive to sedative actions than assessments of motor function (e.g., locomotor activity) and that, combined with changes in other characteristic behavioral effects; this approach can delineate effects associated with $\alpha 2/3/5GABA_A$ receptors vs. $\alpha 1GABA_A$ receptors.

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AUTHORSHIP CONTRIBUTIONS

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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. Dose-dependent sedative behaviors were induced following intravenous injections with the conventional benzodiazepines alprazolam and diazepam (see Table 1 for behavior definitions). The results are shown as mean ± SEM of scores cumulated across the multiple observation periods. *Top panels:* Rest/sleep posture behavior was induced by alprazolam and diazepam; *Middle panels:* Moderate sedation was induced by alprazolam and diazepam; *Bottom panels:* Deep sedation was induced by the highest doses of alprazolam and diazepam. Note that *$p \leq 0.05$, vs. vehicle (V), Bonferroni t-tests, N = 4.

Figure 2. Dose-dependent sedative behaviors differentially induced following intravenous injections of zolpidem and experimental compounds with subtype selectivity. Zolpidem has preferential affinity for α1 subunit-containing $GABA_A$ receptors, whereas the 3 compounds have the following profiles: HZ-166 (selective efficacy for α2 and α3 subunit-containing $GABA_A$ receptors, relatively high efficacy as allosteric modulator), MRK-696 (non-selective partial allosteric modulator), TPA-023B (selective efficacy for α2, α3 and α5 subunit-containing $GABA_A$ receptors, partial allosteric modulator). All other details as in Figure 1. Note the absence of moderate sedation data—these ligands did not induce significant changes in this observable behavior.

Figure 3. Tactile/oral exploration and observable ataxia were altered following intravenous injections with the conventional benzodiazepines alprazolam and diazepam (see Table 1 for definitions). The results are shown as mean ± SEM of scores cumulated across the multiple observation periods. *Top panels:* Tactile/oral exploration was attenuated by alprazolam and

diazepam; *Bottom panels:* Observable ataxia was induced by the highest doses of alprazolam and diazepam. Note that *p ≤ 0.05, vs. vehicle (V), Bonferroni t-tests, N = 4.

Figure 4. Tactile/oral exploration and observable ataxia were altered following intravenous injections with selective compounds. Zolpidem has preferential affinity for α1 subunit-containing $GABA_A$ receptors, whereas the 3 compounds have the following profiles: HZ-166 (selective efficacy for α2 and α3 subunit-containing $GABA_A$ receptors, full efficacy allosteric modulator), MRK-696 (non-selective partial allosteric modulator), TPA-023B (selective efficacy for α2, α3 and α5 subunit-containing $GABA_A$ receptors, partial allosteric modulator), Other details as in Figure 3. *Top panels:* Tactile/oral exploration was dose-dependently enhanced and attenuated by zolpidem (note different scaling on y-axis), but attenuated by all other compounds at the highest dose tested; *Bottom panels:* Observable ataxia was induced by zolpidem and MRK-696 only. Note that *p ≤ 0.05, vs. vehicle (V), Bonferroni t-tests, N = 4.

Figure 5. Differential effects of pre-treatment with βCCT (α1$GABA_A$-preferring antagonist) and flumazenil (non-selective benzodiazepine-site antagonist) on drug-induced sedative behaviors. Data are mean ± SEM of scores cumulated across a test day with multiple observation periods. Multiple doses of βCCT (0.3-3.0 mg/kg, i.v.) and a single dose of flumazenil ("F", 0.3 mg/kg, i.v.) were administered prior to the session in which peak doses of alprazolam or diazepam were administered (benzodiazepine dose depended on individual dose-response function for each behavioral effect). *Top panels:* Rest/sleep posture induced by alprazolam (0.1 mg/kg, data point above V, vehicle) or diazepam (3.0 mg/kg, i.v.) was attenuated by flumazenil (F) but not βCCT; *Middle panels:* Moderate sedation was induced by alprazolam

(0.3 mg/kg, i.v.) or diazepam (3.0 mg/kg, i.v.) and blocked by both flumazenil (0.3 mg/kg, i.v.) and dose-dependently by βCCT (1.0 and 3.0 mg/kg, i.v.); *Bottom panels:* Deep sedation was induced by the highest doses of alprazolam (1.0 mg/kg) or diazepam (10 mg/kg) and attenuated by flumazenil (0.3 mg/kg) and dose-dependently by βCCT (1.0 and 3.0 mg/kg). Note that *p ≤ 0.05, vs. vehicle (V), Bonferroni t-tests, N = 4.

Figure 6. Effects of pre-treatment with βCCT (α1GABA$_A$-preferring antagonist) and flumazenil ("flum", non-selective benzodiazepine-site antagonist) on sedative behaviors induced by selective compounds. *Top panel:* Rest/sleep posture induced by HZ-166 (functionally selective α2/3GABA$_A$ allosteric modulator) was blocked by flumazenil (0.3 mg/kg, i.v.) but not the highest dose of βCCT tested (3.0 mg/kg, i.v.); *Bottom panel:* Deep sedation induced by zolpidem (α1GABA$_A$-preferring allosteric modulator) was blocked by both flumazenil and the highest dose of βCCT tested. Note that *p ≤ 0.05 vs. HZ-166 or zolpidem alone, Bonferroni t-tests, N = 4 monkeys.

Figure 7. Effects of pre-treatment with βCCT (α1GABA$_A$-preferring antagonist) and flumazenil ("F", non-selective benzodiazepine-site antagonist) on attenuation of species-typical behavior and observable ataxia induced by alprazolam and diazepam. Data are mean ± SEM of scores cumulated across a test day with multiple observation periods. Multiple doses of βCCT (0.3-3.0 mg/kg, i.v.) and a single dose of flumazenil (0.3 mg/kg, i.v.) were administered prior to the session in which peak doses of alprazolam or diazepam were administered (benzodiazepine dose depended on individual dose-response function for each behavioral effect). *Top panels:* Tactile/oral exploration attenuated by alprazolam (1.0 mg/kg, data point above vehicle "V") or

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diazepam (10 mg/kg, i.v.) was attenuated by flumazenil (F) but not βCCT, note that *p<0.05 vs. vehicle (V, note that horizontal dashed lines represent levels of behavior without drug treatment; *Bottom panels:* Observable ataxia was induced by alprazolam (0.3 mg/kg) or diazepam (3.0 mg/kg) and attenuated by flumazenil (0.3 mg/kg) and dose-dependently by βCCT (1.0 and 3.0 mg/kg), note that *p ≤ 0.05, vs. vehicle (V), Bonferroni t-tests, N = 4.

Figure 8. Antagonism by βCCT and flumazenil of zolpidem- and HZ-166-induced changes in tactile/oral exploration. Other details as in Figure 7. *Left panel:* Zolpidem ("Zolp") at a lower dose (1.0 mg/kg, i.v.) enhanced tactile/oral exploration that was blocked by both flumazenil ("Flum", 0.3 mg/kg, i.v.) and βCCT (3.0 mg/kg, i.v.); *Center panel:* Zolpidem at a higher dose (10 mg/kg, i.v.) attenuated tactile/oral exploration, which was reversed by flumazenil (0.3 mg/kg, i.v.) but not βCCT (3.0 mg/kg, i.v.); *Right panel:* HZ-166 (30 mg/kg, i.v.) attenuated tactile/oral exploration that was reversed by flumazenil (0.3 mg/kg, i.v.) but not βCCT (3.0 mg/kg, i.v.); Note that *p ≤ 0.05 vs. vehicle (Veh) or "Zolp" condition, Bonferroni t-tests, N = 4 monkeys.

Table 1. Behavioral categories, abbreviations, and definitions.

Behavior	Abbreviation	Brief Description
Species-Typical Behaviors		
Passive Visual	VIS	Animal is standing or sitting motionless with eyes open
Locomotion	LOC	At least two directed steps in the horizontal and/or vertical plane
Self-Groom	GRM	Picking, scraping, spreading or licking of an animal's own hair
Tactile/Oral Exploration	TAC	Any tactile or oral manipulation of the cage or environment
Scratch	SCR	Vigorous strokes of the hair with fingers or toenails
Stereotypy	STY	Any repetitive, ritualized pattern of behavior that serves no obvious function
Forage	FOR	Sweeping and/or picking through wood chip substrate
Vocalization	VOC	Species-typical sounds emitted by monkey (not differentiated into different types)
Threat/Aggress	THR	Multifaceted display involving one or more of the following: Open mouth stare with teeth partially exposed, eyebrows lifted, ears flattened or flapping, rigid body posture, piloerection, attack (e.g., biting, slapping) of inanimate object or other monkey
Yawn	YWN	To open mouth wide and expose teeth
Body Spasm	BSP	An involuntary twitch or shudder of the entire body; also "wet dog" shake
Present	PRE	Posture involving presentation of rump, belly, flank, and/or neck to observer or other monkey
Drink	DRI	Mouth contact to fluid delivery sippers
Nose Rub	NRU	Excessive wiping of nose with hand or arm
Fear Grimace	FGR	Grin-like facial expression involving the retraction of the lips exposing clenched teeth; may be accompanied by flattened ears, stiff, huddled body posture, screech/chattering vocalizations
Lip Smack	LIP	Pursing the lips and moving them together to produce a smacking sound, often accompanied by moaning
Lip Droop	LDR	Bottom lip drooping, showing bottom teeth
Cage Shake	CSH	Any vigorous shaking of the cage that may or may not make noise
Observable Ataxia	ATX	Any slip, trip, fall, loss of balance.
Sedation measures		
Rest/Sleep Posture	RSP	Idiosyncratic posture adopted by monkeys during rest or sleep, easily roused; eyes closed <3 s after stimulus
Moderate Sedation	MSE	Atypical loose-limbed posture (e.g., propped on the cage by the body or a limb), eyes closed, delayed response to external stimuli (> 3 sec)
Deep Sedation	DSE	Atypical loose-limbed posture, eyes closed, does not respond to external stimuli

Table 2. Comparisons of potencies between anxiolytic-like effects (operant-based anti-conflict procedure) and measures of sedation in rhesus monkeys

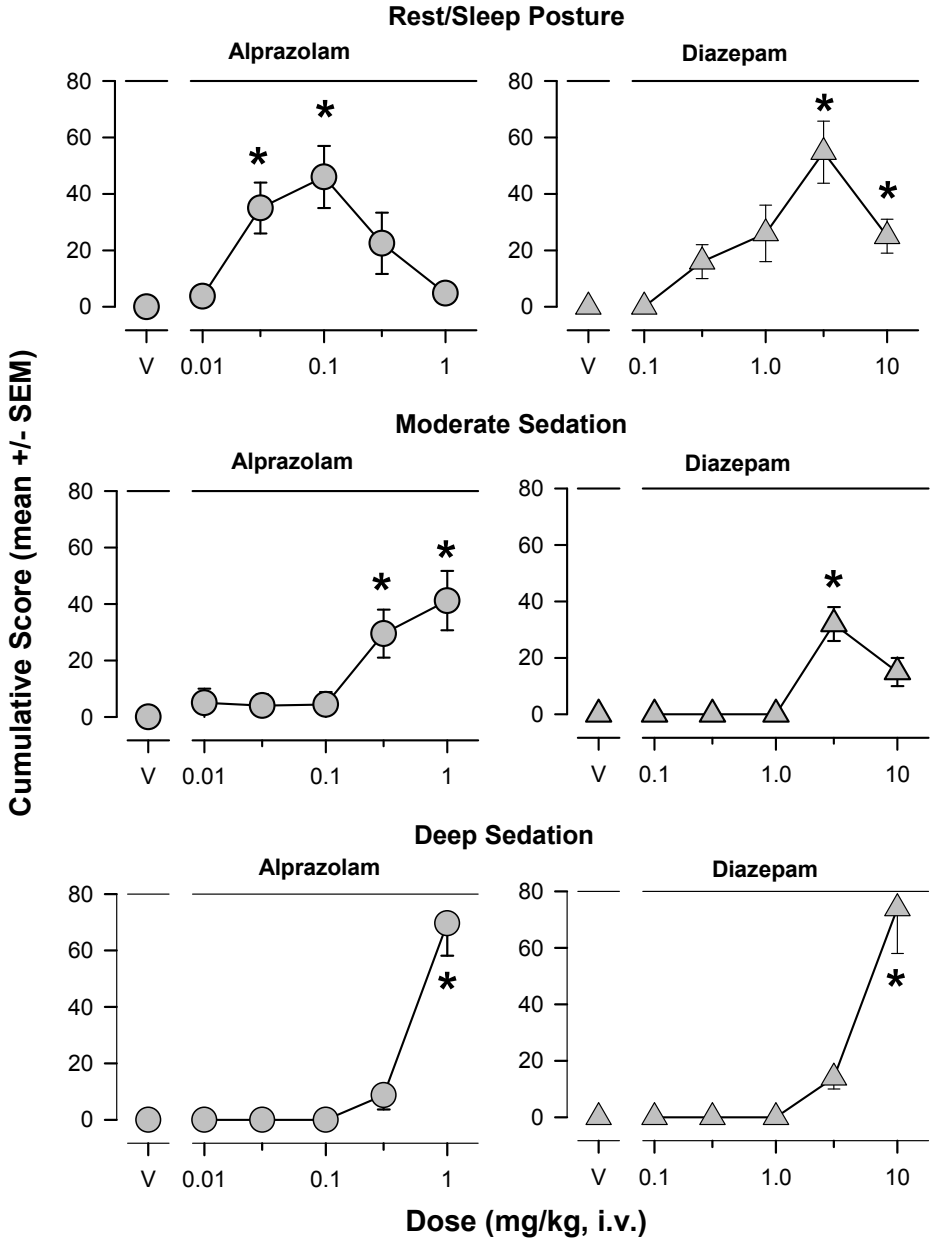
Compound	Anti-Conflict Effects[a]	Rest/Sleep Posture[b]	Observable Ataxia[b]	Operant Responding -Decrease[a]	Moderate Sedation[b]	Deep Sedation[b]
	ED_{50}, mg/kg, i.v. (SEM)					
Alprazolam	0.007 (0.002)	0.028 (0.01)	0.08 (0.03)	0.27 (0.07)	0.31 (0.02)	0.59 (0.11)
Diazepam	0.11 (0.07)	0.71 (0.33)	1.6 (0.11)	1.7 (0.79)	1.8 (0.12)	6.9 (0.91)
Zolpidem	NE	NE	1.1 (0.1)	0.10 (0.03)	NE	5.7 (0.67)
HZ-166	0.80 (0.3)	1.2 (0.15)	NE [30]	NE [30]	NE [30]	NE [30]
MRK-696	0.18 (0.06)	0.27 (0.13)	0.61 (0.28)	NE [3.0]	NE [3.0]	NE [3.0]
TPA-023B	0.031 (0.014)	0.014 (0.003)	NE [1.0]	NE [1.0]	NE [1.0]	NE [1.0]

ED_{50}: Dose engendering 50% of the maximum effect. Numbers in parentheses are SEMs.

NE: no effect [maximum dose tested, mg/kg, i.v.]

References: a. Rowlett et al. (2006), Fischer et al. (2010); unpublished; b. Present study.

Figure 1.



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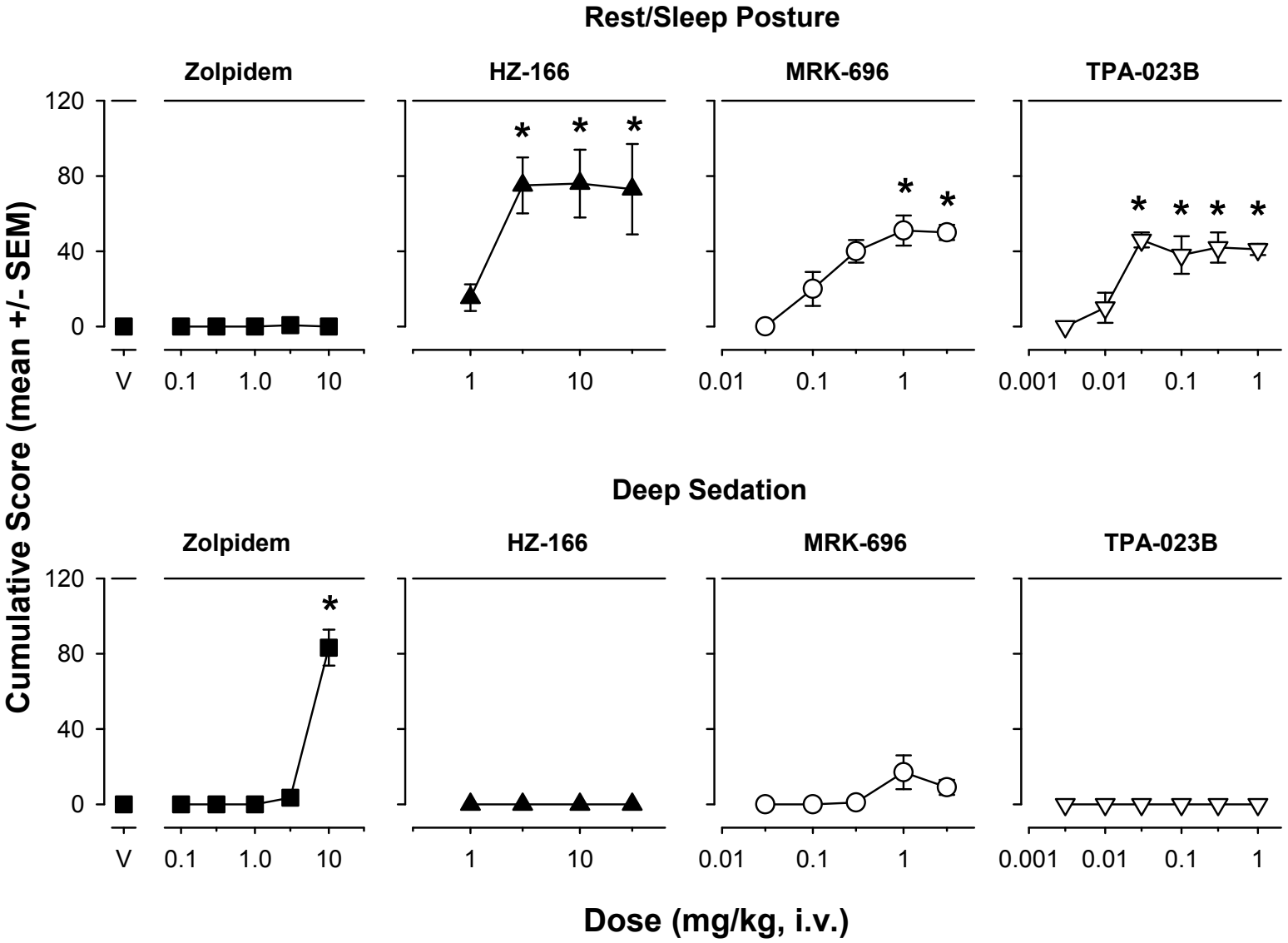

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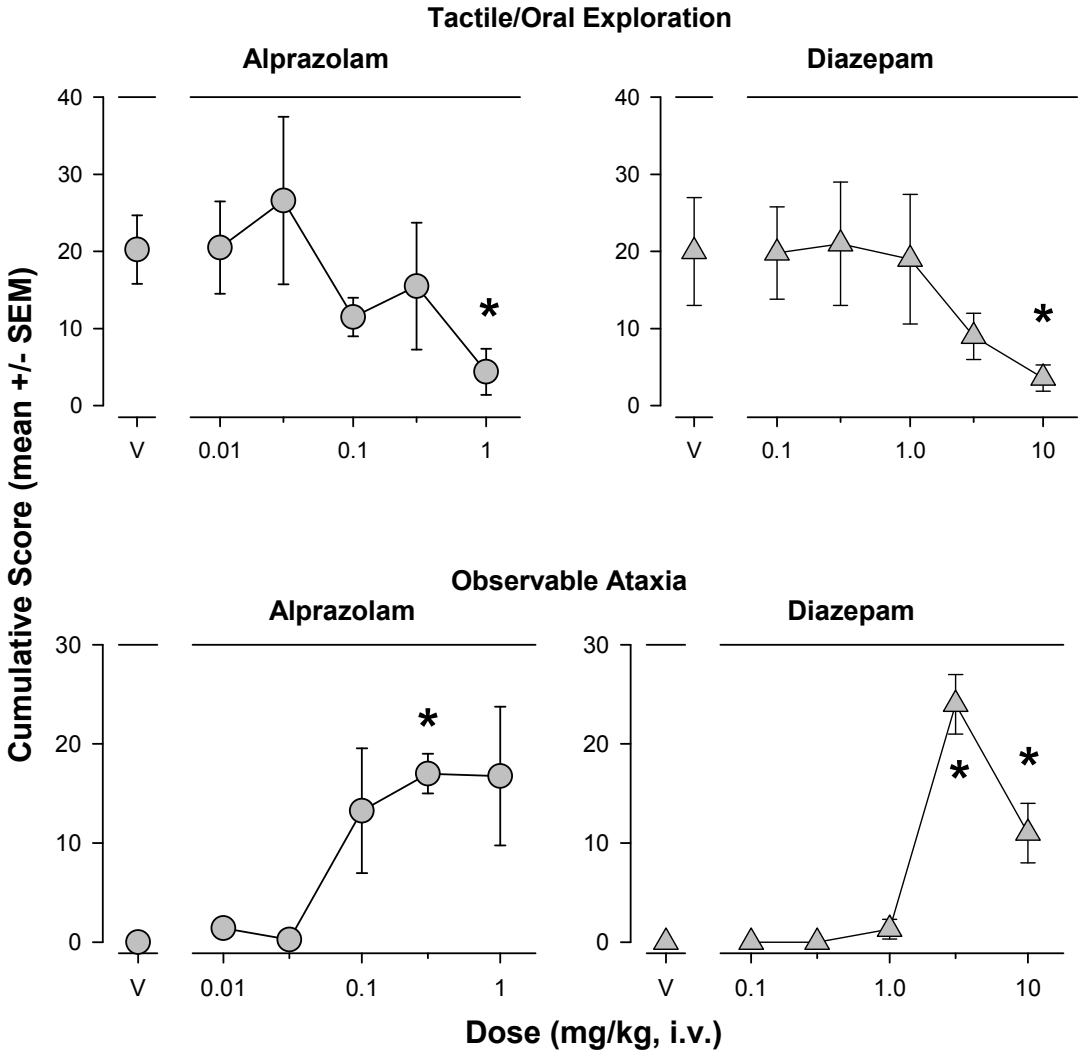

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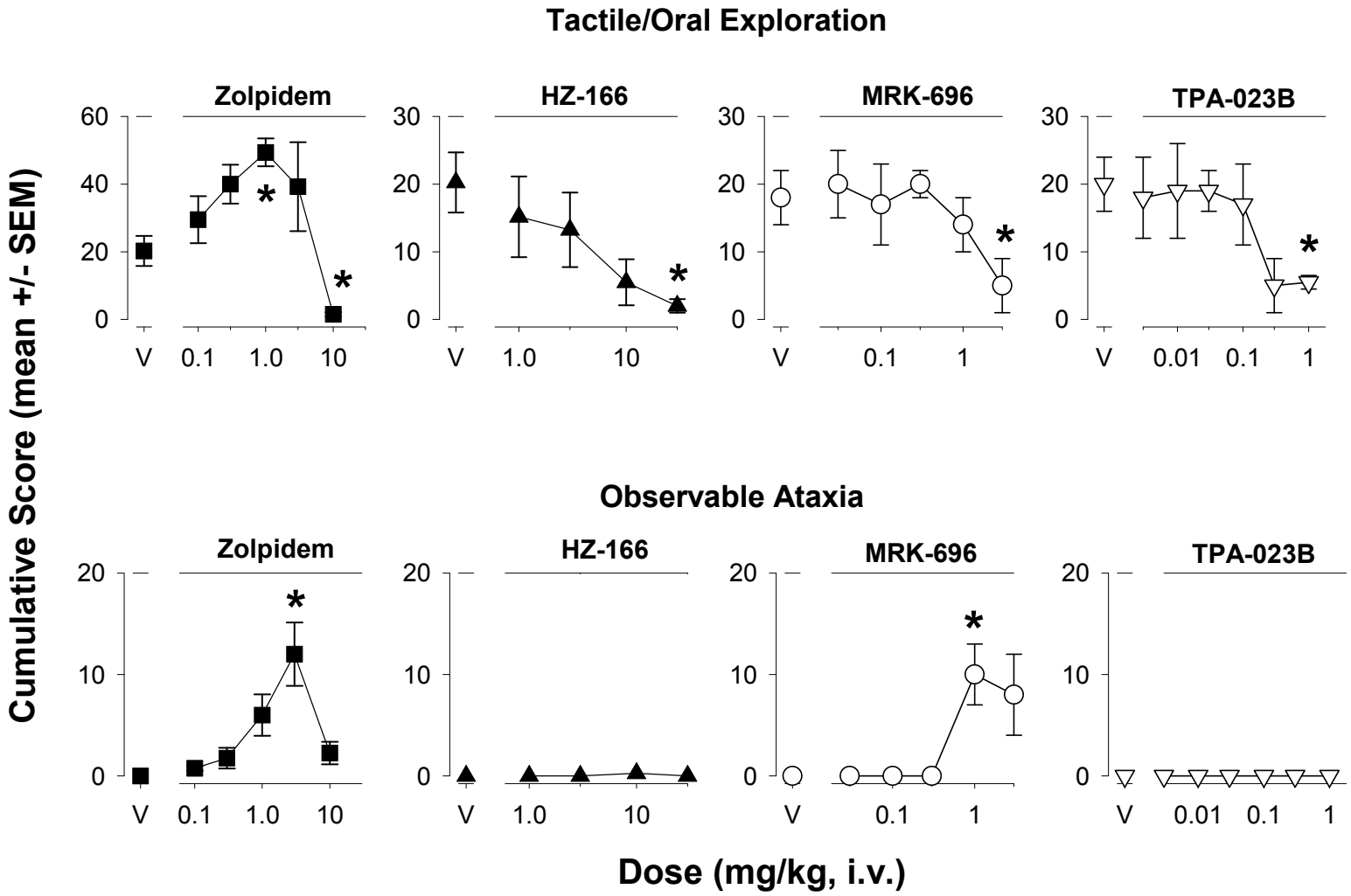

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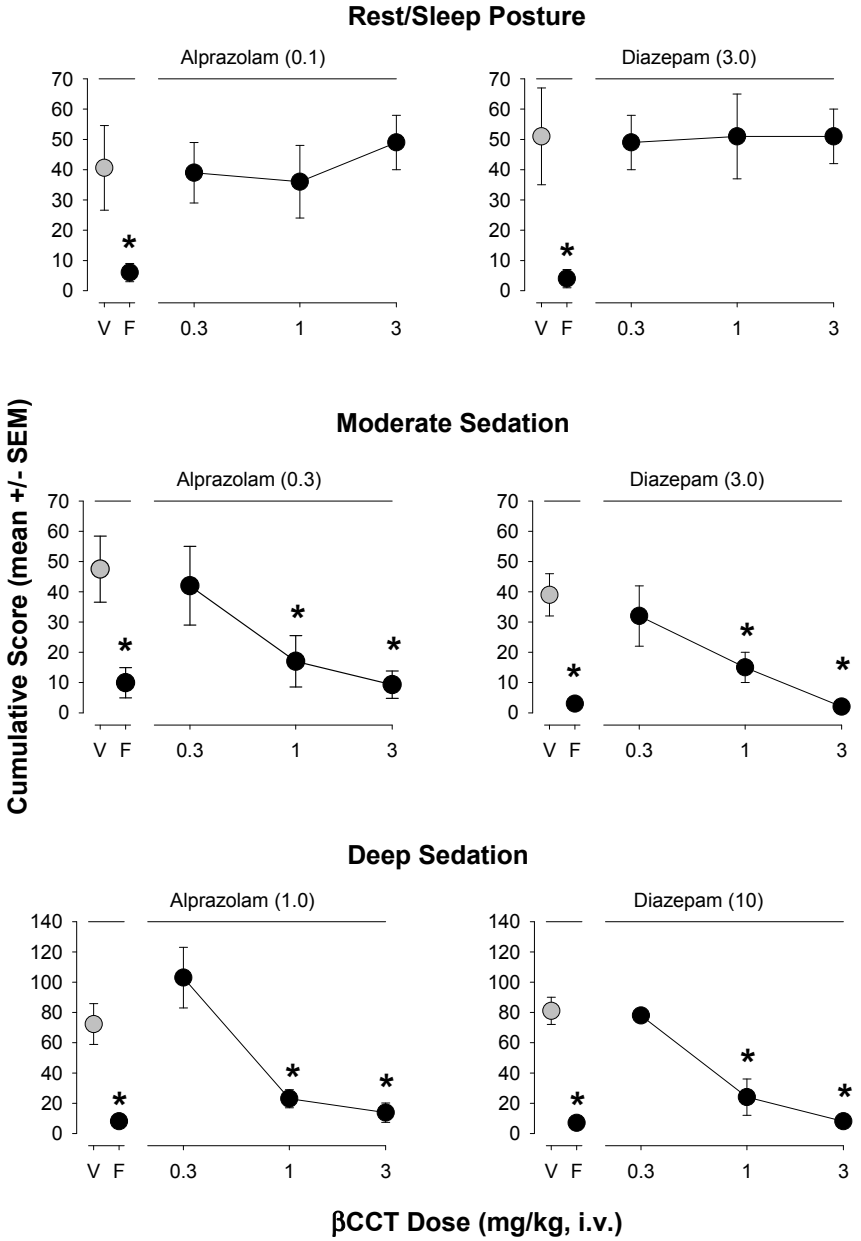

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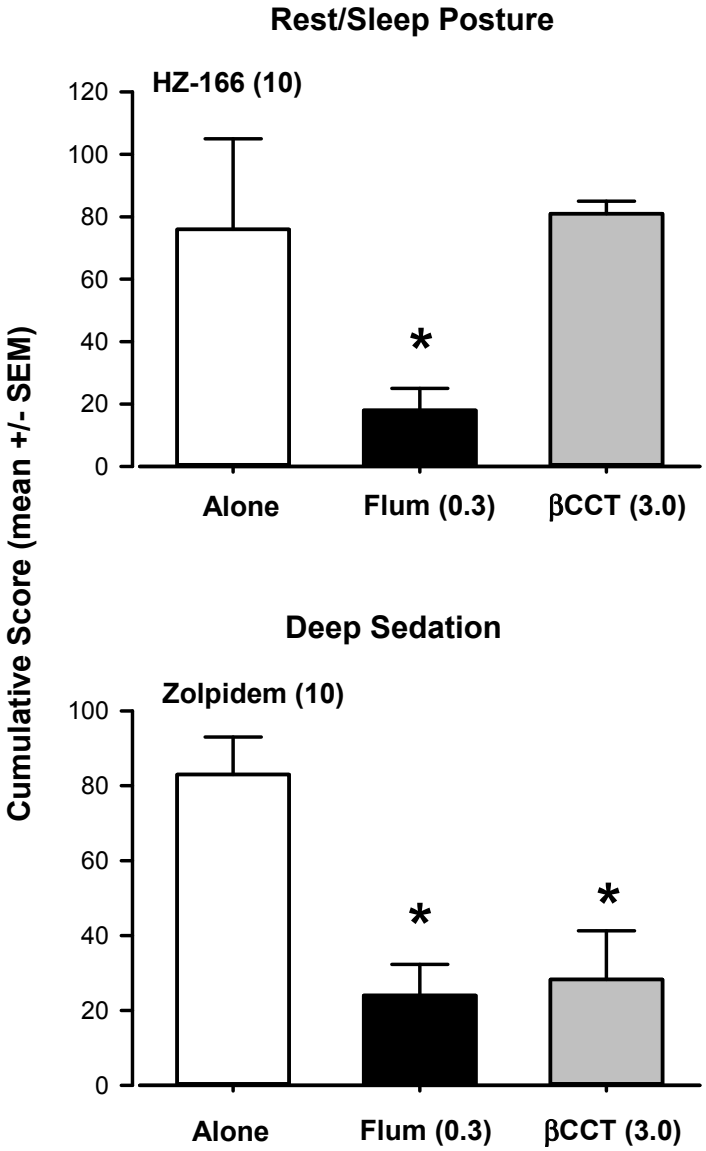

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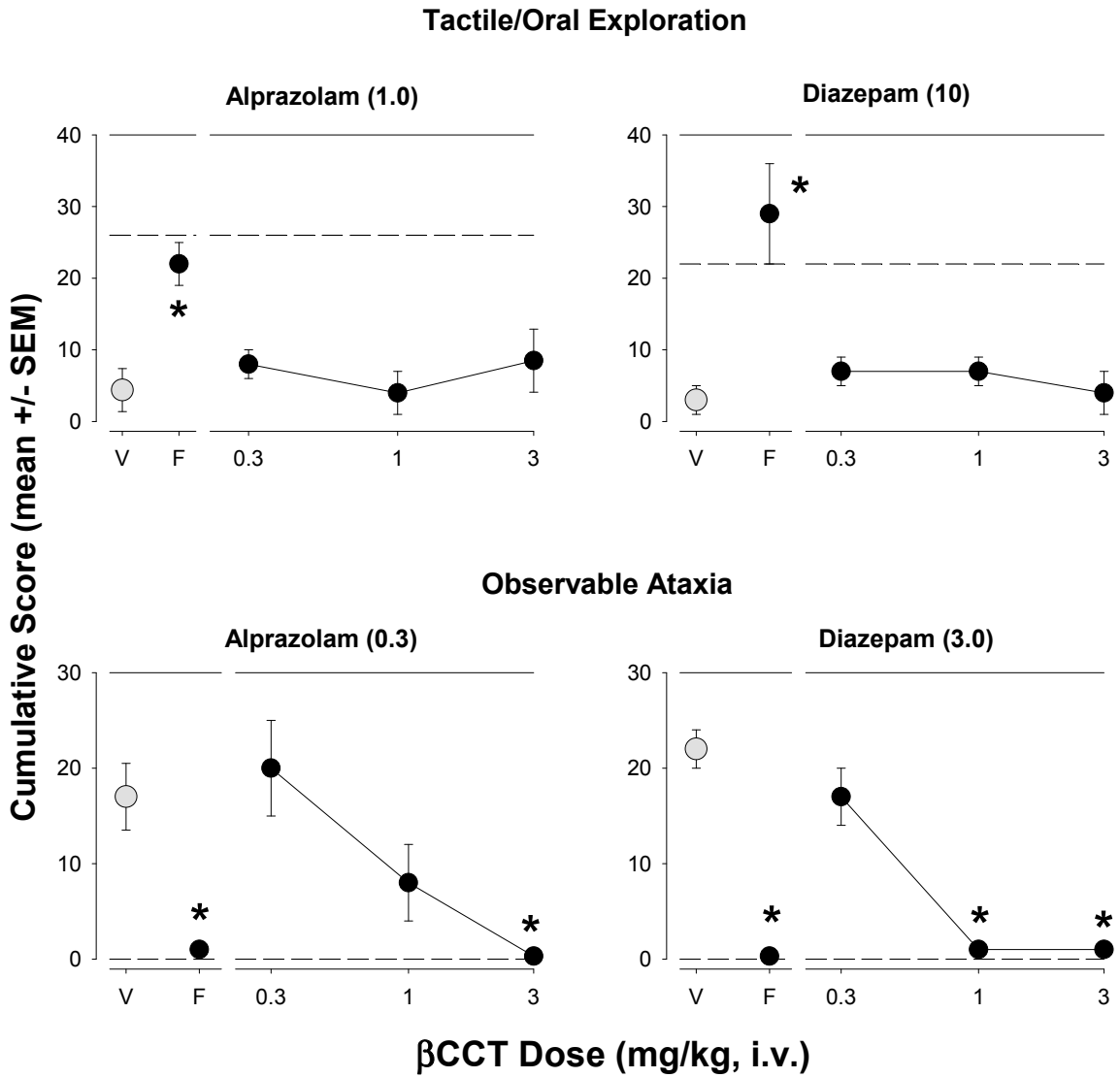

Figure 8.