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Benzodiazepine + neuroactive steroid combinations in rats: Anxiolytic-like and discriminative stimulus effects

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Abstract

Rationale: Benzodiazepines are effective anxiolytics, hypnotics, and anticonvulsants but unwanted side effects, including abuse potential, limit their use. A possible strategy to increase the therapeutic index of this drug class is to combine benzodiazepines with neuroactive steroids.

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Participated in research design: Gunter, Platt, Paul, Rowlett

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The results reported in this paper have not been previously presented. The authors have no Conflicts of Interest to disclose.

Objectives: The present study evaluated the extent to which combinations of benzodiazepines (triazolam, clonazepam) and neuroactive steroids (pregnanolone, ganaxolone) induced additive, supra-additive, or infra-additive effects in elevated zero maze and a drug discrimination procedure in rats.

Methods: Male Sprague-Dawley rats (N=7–8/group) were placed into an elevated zero maze apparatus following injections of multiple doses of triazolam and pregnanolone, alone and combined, or clonazepam and pregnanolone, alone and combined. These drugs/drug combinations also were evaluated in rats (N=8) trained to discriminate triazolam (0.1 mg/kg, i.p.) from vehicle. Drug interactions were evaluated using isobolographic and dose-addition analysis.

Results: In the elevated zero maze, all drugs engendered dose-dependent increases in time spent in the open quadrant when administered alone. Triazolam + pregnanolone, as well as clonazepam + ganaxolone combinations produced additive or supra-additive effects depending on the fixed-proportion that was tested. In triazolam discrimination, all drugs engendered dose-dependent increases in triazolam-lever responding. In combination, triazolam + pregnanolone and clonazepam + ganaxolone produced predominantly additive discriminative stimulus effects, except for one fixed proportion of clonazepam + ganaxolone which had supra-additive effects.

Conclusions: Although drug interactions depended on the constituent drugs, the combination tested, and the behavioral endpoint; a combination was identified that would be predicted to result in supra-additive anxiolytic-like effects with predominantly additive discriminative stimulus effects.

Keywords

Benzodiazepine; Neuroactive steroid; $GABA_A$ receptor; Elevated Zero Maze; Drug discrimination; anxiolysis

Introduction

Benzodiazepines are prescribed frequently for the treatment of anxiety disorders because they are highly efficacious in relieving symptoms, reasonably safe, and have a high rate of compliance (Bandelow et al. 2002). Within the past decade, prescriptions of these drugs have increased, with alprazolam (Xanax®) being the most prescribed psychiatric medication in 2011 (IMS 2012). Perhaps in accordance with the rise in numbers of prescriptions is the increase in emergency room visits associated with benzodiazepine use (SAMSHA 2013). This emerging problem may be due to the use of these drugs in a chronic situation that can lead to dependence (Olfson et al. 2015), despite the labeled use of 3–4 weeks. Other side effects associated with their use (e.g., tolerance, sedation, and likelihood of abuse) further compromise their therapeutic usefulness. A strategy for increasing the therapeutic utility of this drug class would be to enhance the anxiolytic effects of the drug and/or minimize the side effects.

Benzodiazepines bind to a unique site on the $GABA_A$ receptor and positively modulate the receptor ionophore, which constitutes a Cl- ion channel. One possible approach to enhance the anxiolytic effects of these drugs is to combine them with another positive modulator of the $GABA_A$ receptor. Neuroactive steroids are positive allosteric modulators of $GABA_A$

receptors synthesized peripherally by the adrenal glands and locally by neurons and glia (Belelli and Lambert 2005). Neuroactive steroids are metabolized by a group of 5-alpha reductases from the parent hormones progesterone and deoxycorticosterone (Lambert et al. 2009). Once generated, these molecules bind to the $GABA_A$ receptor at a unique site and increase the duration of time the ion channel remains open, as well as the frequency of channel openings (Akk et al. 2005; Belelli and Lambert 2005). Neuroactive steroids are involved in phasic inhibition at synaptic receptors and may modulate primarily tonic inhibition of extrasynaptic receptors (Herd et al. 2007).

Neuroactive steroids show effects similar to benzodiazepines in a variety of animal models. Like benzodiazepines, neuroactive steroids increase time spent in the open arms of the elevated plus maze, an ethologically-based model of anxiety in rodents (Rodgers and Johnson 1998), as well as increase suppressed responding in operant-based conflict models of anxiety (Wieland et al. 1995; Brot et al. 1997; Akwa et al. 1999; Vanover et al. 1999a). In drug discrimination procedures, the neuroactive steroid pregnanolone substitutes for the benzodiazepine midazolam in rats (Bai and Gerak 2011) and monkeys (McMahon and France 2005; Gerak and France 2014) suggesting that the two drugs have similar discriminative stimulus effects and have a shared mechanism of action ($GABA_A$ receptors). Pregnanolone also has been shown to serve as a reinforcer in rhesus monkeys trained to self-administer midazolam (Fischer and Rowlett 2011) or the barbiturate methohexital (Rowlett et al. 1999).

To date, relatively few preclinical studies have evaluated the behavioral effects of benzodiazepine and neuroactive steroid combinations using quantitative approaches to assess potential interactions. In that regard, using isobolographic analysis, combinations of pregnanolone and midazolam appear to have additive discriminative stimulus effects in monkeys trained to discriminate midazolam from saline (McMahon and France, 2005) suggesting that no interaction exists between the drugs in that procedure. We have shown previously with dose-addition and isobolographic analysis that combinations of pregnanolone and the benzodiazepine triazolam have supra-additive anti-conflict effects and infra-additive reinforcing effects in monkeys (Fischer and Rowlett 2011). Recently, we evaluated similar combinations in rats trained to press a lever to obtain food pellets (Gunter et al. 2015) and found mostly supra-additive reductions in rates of responding. Together, these findings suggest that the presence and type of interaction observed may vary markedly across behavioral endpoints.

Although tempting to conclude that a specific type of interaction can be assigned to a particular clinically-relevant behavioral effect, the range of conditions that have been evaluated to date have been limited to relatively few procedures and ligands. Therefore, the objectives of the present experiments were to (1) evaluate the extent to which benzodiazepine+neuroactive steroid combinations are supra-additive in a model of anxiolytic-like effects based on a rodent's tendency to avoid open, brightly-lit spaces; (2) evaluate discriminative stimulus effects of the combinations using multiple dose proportions; and (3) include an additional benzodiazepine (clonazepam) and neuroactive steroid (ganaxolone) to comparisons of triazolam and pregnanolone. For all studies, combinations

were analyzed using isobolograms and deviation from additivity was assessed using dose-addition analysis (Tallarida, 2001).

Materials and Methods

Drugs

Triazolam (NIDA, Baltimore, MD) was dissolved in 100% propylene glycol and diluted to a 20% propylene glycol/80% water mixture. Clonazepam (Sigma-Aldrich, St. Louis, MO) was dissolved in 100% propylene glycol and diluted to a 50% propylene glycol/50% water mixture. Pregnanolone [(3α,5β)-3-Hydroxy-pregnan-20-one] and ganaxolone [(3α,5α)-3-Hydroxy-3-methyl-pregnan-20-one] (Tocris Biosciences, Ellisville, MO) were dissolved in 45% (w/v) 2-hydroxypropyl-β-cyclodextrin. Morphine was dissolved in saline. All drugs were administered intraperitoneally in volumes of 1–2 ml/kg.

Elevated Zero Maze

Subjects and Apparatus.

Adult male Sprague Dawley rats (N=7/group), approximately 70 days old (Harlan, Indianapolis, IN) and weighing between 260–300 g, were pair-housed under a 12/12-h light/dark cycle (experiments were conducted during the dark period) with water and food available ad libitum. In order to minimize stress, rats were handled and given sham injections for a week before test sessions were conducted. All animals were maintained and experiments were conducted in accordance with the University of Mississippi Medical Center's Institutional Animal Care and Use Committee and were in accordance with the National Research Council's Guide for Care and Use of Laboratory Animals (8th edition, 2011).

The elevated zero maze consisted of a custom-made Plexiglas circular track with runways that were 10 cm wide. The maze was divided into four alternating quadrants, two of which had 50 cm high walls (closed arms) and two of which had 1 cm walls (open arms). Trials were conducted under low light conditions (i.e., 175–200 lux).

Procedure.

For the single-day session, rats were administered an injection (i.p.) and then placed back in their home cage for 10 minutes. After this pretreatment period had elapsed, rats were placed on one of the two open quadrants of the maze and allowed to explore for 5 minutes (Braun et al., 2011). An overhead video camera and Noldus Ethovision software (Wageningen, The Netherlands) were used to track and record the behavior of the rats. Experimenters were blinded to the drug conditions. Between rats, the maze was cleaned with a 10% ethanol solution. Doses tested were 0.003–0.1 mg/kg triazolam, 0.1–3.0 mg/kg pregnanolone, and the combinations; 0.01–0.3 mg/kg clonazepam, 0.3–10 mg/kg ganaxolone, and the combinations. For all drugs, combinations were administered in fixed proportions based upon the relative potencies of the two component drugs. Each rat was tested only once in this between-groups design, and drugs were tested generally in the order described above (including vehicle tests), with dose varied pseudo-randomly within a drug.

Data Analysis.

Dependent measures included percent time spent in open arms and number of open and closed arm entries. The percent time spent in the open arms was calculated by dividing the time spent in the open arms by the total time spent in the maze. All measures were plotted as a function of dose of drugs alone and drug combinations (mean ±SEM). Dose-response functions were analyzed by one-way analysis of variance (ANOVA) with Dunnett's tests comparing each dose to the saline control. Doses that increased open arm time by 50% (ED_{50}) were estimated using linear regression analysis in cases where the linear ascending portion of the log dose-response function was defined by at least three data points or by linear interpolation in cases where the log dose-response function was defined best by two points. ED_{50}s for drugs alone and drug combinations were averaged to determine means and 95% confidence limits (CL).

Drug Discrimination

Subjects and Apparatus.

Eight adult male Sprague Dawley rats, approximately 70 days old and weighing between 260–300 g at the start of the experiment (Harlan, Indianapolis, IN), were housed individually under a 12/12-h light/dark cycle with water available ad libitum. Rats were maintained at 85% of their free feeding weight for the duration of the study based on a standard growth curve and fed with standard rodent chow (Harlan Teklad, Madison, WI). All animals were maintained and experiments were conducted in accordance with the University of Mississippi Medical Center's Institutional Animal Care and Use Committee and were in accordance with the National Research Council's Guide for Care and Use of Laboratory Animals (8th edition, 2011).

Behavioral tests were conducted during the light phase in operant chambers equipped with two response levers, stimulus lights, and a food pellet dispenser (Med Associates, St. Albans, VT) located within sound attenuating enclosures. Data were collected using a Macintosh computer with custom software and interface that controlled the experiment and recorded data.

Procedure.

All sessions were conducted Sunday through Friday at approximately noon each day. Rats initially were trained to press either lever for a food pellet (45 mg, Bio-Serve, grain-based pellets) under a 1-response, fixed-ratio (FR1) schedule. Once lever pressing was acquired (≥ 50 reinforcers/session, 2 consecutive sessions), discrimination training began. Half of the rats were trained to respond on the right lever after an injection of triazolam (0.1 mg/kg i.p.) and on the left lever after a saline injection (i.p.) while the reverse was true for the remaining rats. Training continued while the FR requirement was increased to the final schedule of FR 10. In all sessions, responses on the incorrect lever before completion of the FR 10 reset the FR requirement.

For each training session, an injection (triazolam or saline) was administered followed by a 10-min pretreatment period in which stimulus lights were off and responding on either lever

had no programmed consequences. Once the pretreatment period had elapsed, a 2.5-min response period ensued during which stimulus lights were illuminated and responding on the correct lever resulted in the delivery of a food pellet. At the conclusion of the session, the lights were turned off and the rat was removed from the chamber and returned to the home cage. Training sessions were conducted on a single alternation schedule (SDSD; S, saline; D, drug) until the discrimination criteria were met (i.e., ≥ 80% of total responses on the injection-appropriate lever for seven of eight consecutive sessions). Training then proceeded on a double-alternation schedule (SSDD).

Drug testing began once all terminal conditions were met (i.e., FR 10, ≥ 80% injection-appropriate responding for seven of eight consecutive sessions). Tests were then inserted into the double alternation sequence (SSTDDT; T, test). During tests, all of the training parameters remained in effect except that completion of FR10 on either lever produced a food pellet. The training drug triazolam was tested first (0.01–0.3 mg/kg) followed by pregnanolone (1–17 mg/kg) and then their combination. Next, clonazepam (0.01–0.3 mg/kg) and ganaxolone (1–10 mg/kg) were tested alone and then in combination. Finally, morphine was tested as a negative control drug (i.e., would not be expected to substitute; 0.1–3 mg/kg). For all drugs, combinations were administered in fixed proportions based upon the relative potencies of the two component drugs. Within a drug or drug combination, doses were tested in a random order once after S training and once after D training.

Data Analysis.

The percentage of triazolam-lever responding was computed for individual rats only when at least one food pellet was earned during the session. The mean percentage of triazolam-lever responding (± SEM) was then calculated for the group at each dose. Doses that engendered ≥ 80% triazolam-lever responding were considered to substitute for the training dose of triazolam. Dose-response functions were analyzed by repeated measures analysis of variance (ANOVA) with Dunnett's tests comparing each dose to the vehicle control. The dose of each drug alone or drug combination required to engender 50% triazolam-lever responding (ED_{50}) was estimated for individual subjects using linear regression analysis as described above. 95% confidence limits (CL) were calculated by averaging the ED_{50} values of all rats. Rates of responding for individual subjects at each dose or dose combination were calculated by dividing the total number of responses (regardless of lever) by the total component duration and normalized as a percent of control. Individual control responding was calculated to be the average response rate during the preceding two training days for each rat and then averaged across subjects.

Isobolographic and Dose-Addition Analysis.

The effects of drug combinations in all procedures were assessed graphically with the use of isobolograms. Isobolograms were constructed by connecting the ED_{50} of the neuroactive steroid (i.e., pregnanolone or ganaxolone) plotted on the y axis with the ED_{50} of the benzodiazepine (i.e., triazolam or clonazepam) plotted on the x axis. The additivity line connects these points and contains the loci of dose pairs that would produce an ED_{50} equal to the ED_{50} of the component drugs when administered alone if the combination is additive. Dose pairs that fall below the additivity line indicate that an ED_{50} was reached with lesser

quantities of the drugs and is suggestive of supra-additivity. In contrast, points representing dose pairs that fall above the additivity line are suggestive of infra-additivity (Tallarida, 2001).

Drug interactions were analyzed statistically by comparing the ED_{50mix} or the total dose of the two drugs in the combination to the predicted additive dose or ED_{50add}. This value is calculated using the following equation: $ED_{50add}=fA+(1-f)B$ where A and B are the ED_{50}s of the two individual drugs alone, f is the fractional multiplier associated with a specific combination mixture and is calculated by f= FP ÷ (FP + RPA), where FP = fixed proportion in a particular mixture and RPA = relative potency ratio = $ED_{50}A \div ED_{50}B$. T-tests between the ED_{50mix} and ED_{50add} determined statistical significance. The interaction index (γ) was also calculated to quantify deviation from additivity (Tallarida, 2002). A value of 1 suggests additivity, while values less than one suggest supra-additivity and greater than 1 suggest infra-additivity. All data were analyzed using Graphpad Prism Version 6.0 for Windows.

Results

Elevated Zero Maze

Figure 1A shows the effects of triazolam and pregnanolone alone on the percent of time spent in the open portions of the maze. Both drugs elicited dose-dependent increases in this measure [triazolam: $F(4,30) = 19.56$, $p < 0.05$; pregnanolone: $F(4,30) = 9.418$, $p < 0.05$]. When compared to saline, 0.01–0.1 mg/kg of triazolam and 1–3 mg/kg of pregnanolone engendered significant increases in the percent of time spent in the open area (Dunnett's test, p's < 0.05). The ED_{50} values (95% confidence interval) of triazolam and pregnanolone were 0.04 mg/kg (0.03–0.06) and 1.2 mg/kg (1.11–1.4), respectively. Rats then were tested with mixtures of triazolam:pregnanolone in the fixed-proportion of 1:10, 1:30, and 1:100 because these ratios result in fractional multipliers of triazolam and pregnanolone ED_{50} values that range from 0–1 (Tallarida, 2001). All drug combinations reliably increased the percent of time spent in the open arms compared to saline [1:10: $F(4,30) = 16.67$, $p < 0.05$; 1:30: $F(3,24) = 8.551$, $p < 0.05$; 1:100: $F(3,24) = 16.59$, $p < 0.05$]. As with triazolam alone, individual combinations were significantly above saline levels (Figure 1B, note that *p<0.05; Dunnett's test). Isobolographic representation of the ED_{50} values of the combinations compared with ED_{50} values of the two drugs alone (Figure 1C) suggested that the 1:30 mixture had supra-additive effects as it was below the line of additivity, while the 1:10 and 1:100 mixtures fell close to the line, suggesting additivity. Dose-addition analysis comparing the experimentally-derived and predicted values of these mixtures confirmed these results (Table 1), indicating that over the range of fixed proportions tested, combining triazolam and pregnanolone resulted in proportion-dependent enhancement that consisted of additive effects or a supra-additive interaction.

Figure 2A shows the dose-response functions for clonazepam and ganaxolone administered alone in the elevated zero maze. Both drugs elicited dose-dependent increases in percent time in the open area [clonazepam: $F(4,30) = 3.515$, $p < 0.05$; ganaxolone: $F(4,30) = 2.865$, $p < 0.05$]. When compared to saline, 0.1–0.3 mg/kg of clonazepam, and 10 mg/kg ganaxolone engendered significant increases in percent open area time (Dunnett's test, p's < 0.05). The ED_{50} values (95% confidence interval) of clonazepam and ganaxolone were 0.06

mg/kg (0.04–0.08) and 3 mg/kg (2.6–3.4), respectively. Rats then were tested with fixedproportion mixtures of 1:10, 1:30, 1:100 clonazepam-ganaxolone because these ratios result in fractional multipliers of clonazepam and ganaxolone ED_{50} values that range from 0–1 (Figure 2B; Tallarida, 2001). Two drug combinations, 1:30 and 1:100, reliably increased the percent of time spent in the open arms compared to saline (1:30: $F(3,24) = 13.17$, $p < 0.05$); 1:100: $F(3,24) = 10.02$, $p < 0.05$; Figure 2B). Isobolographic representation of the ED_{50} values of the combinations compared with ED_{50} values of the two drugs alone (Figure 2C) suggests that the 1:100 mixture had supra-additive effects as it lies below the line of additivity, whereas the 1:10 and 1:30 mixture did not deviate from the line, suggesting additivity. Dose-addition analysis compared the experimentally-derived and predicted values of these mixtures and confirmed these results (Table 1), indicating that as with triazolam +pregnanolone, combining clonazepam and ganaxolone resulted in proportion-dependent enhancement that consisted of additive effects or a supra-additive interaction.

Finally, for all of the drugs alone and drug combinations tested, the number of open and closed arm entries was not significantly different from that observed after saline administration (data not shown). These findings suggest that changes in percent time in open arms reflect an anxiolytic-like effect rather than solely alterations in motor behavior.

Drug discrimination

Once trained and for the duration of the study, individual rats made an average of 98.7% (± 0.45 SEM) responses on the triazolam-associated lever after injections of triazolam and 1.2 % (± 0.77 SEM) responses on the triazolam-lever after injections of saline. Over the course of the study, rates of responding during training sessions were 1.15 (± 0.09 SEM) responses/s after injections of triazolam and 1.14 (± 0.13 SEM) responses/s after saline.

Figure 3 shows mean percent triazolam-lever responding (panel A) and mean response rates (responses/s as a percent of control; panel B) for the training drug triazolam (closed circles). Under test conditions, increasing doses of triazolam engendered dose-dependent increases in the percentage of triazolam-lever responding, i.e., a low dose of triazolam (0.01 mg/kg) engendered little to no responding on the triazolam lever, whereas doses of triazolam ≥0.1 mg/kg elicited virtually exclusive responding on the triazolam lever. As shown in panel B, the average rates of responding was attenuated with increasing doses of triazolam: $F(4,28) = 23.30$, $p < 0.05$. After administration of 0.3 mg/kg triazolam, average rates of responding were significantly lower compared with average rates of responding following vehicle administration (Dunnett's test, $p < 0.05$).

Dose-related increases in the percentage of triazolam-lever responding also were observed after administration of clonazepam, pregnanolone, ganaxolone, but not morphine (Figure 3A), with all drugs (except morphine) engendering ≥ 80% triazolam-lever responding, i.e., full substitution. Triazolam and pregnanolone had ED_{50} values (95% confidence interval) of 0.05 (0.03–0.06) and 4.72 (3.38–6.06), respectively. ED_{50} values for clonazepam and ganaxolone were 0.096 mg/kg (0.077–0.12) and 4.78 mg/kg (3.53–6.02) respectively. Pregnanolone and morphine reduced response rates to 8 and 11% of control rates, respectively, at the highest doses tested [Figure 3B; pregnanolone: $F(5,35) = 29.23$, $p < 0.05$;

morphine: $F(4,28) = 31.80$, $p < 0.05$; Dunnett's test, p's < 0.05]. In contrast, neither clonazepam nor ganaxolone reduced average rates of responding at any dose tested.

In drug combination experiments, rats were tested with fixed-proportion mixtures of 1:30, 1:100, 1:300 triazolam:pregnanolone because these ratios result in fractional multipliers of triazolam and pregnanolone ED_{50} values that range from 0 to 1 (Tallarida, 2001). Figure 4A shows the effects of these mixtures on percent triazolam-lever responding. Increasing the proportion of the neuroactive steroid in the mixture produced dose-dependent leftward shifts in the dose-response function compared to triazolam alone. Isobolographic representation of the percent triazolam-lever responding data (Figure 4B) suggests that the mixtures were additive as the ED_{50} values of the mixtures did not deviate reliably from the line of additivity. Dose-addition analysis comparing the experimentally-derived and predicted values of these mixtures confirmed these results (Table 2). Figure 4C shows the average response rates engendered by the combinations compared to triazolam alone. As with the percent triazolam-lever responding, increasing the proportion of pregnanolone in the drug combination produced dose-dependent leftward shifts in the dose-response functions for rates of responding. In addition, all dose combinations significantly reduced average response rates at the highest doses tested [1:30: $F(4,35) = 31.64$, $p < 0.05$; 1:100: $F(4,35) = 77.59$, $p < 0.05$; 1:300: $F(4,28) = 28.61$, $p < 0.05$; Dunnett's test, p's < 0.05]. Isobolographic representation of the ED_{50} values derived from these data (Figure 4D) suggests that the 1:30 and 1:100 mixtures were additive as the ED_{50} values of the mixtures do not deviate significantly from the line of additivity, while the 1:300 mixture fell below the additivity line and suggests a supra-additive drug interaction. Dose-addition analysis comparing the experimentally-derived and predicted values of these mixtures confirmed these results (Table 2).

The fixed-proportion mixtures of 1:10, 1:30, 1:100 were used for clonazepam-ganaxolone, as these proportions result in fractional multipliers of the ED_{50} values that range from 0 to 1 (Tallarida, 2001). Figure 5A shows the effects of these mixtures on percent triazolam-lever responding. At least one mixture, 1:100, showed a leftward shift in the dose-response function compared with clonazepam alone. Isobolographic representation of the potencies of the drugs alone and combined (Figure 5B) suggests that the 1:10 and 1:30 mixtures produced additive effects as the ED_{50} values of these mixtures do not deviate reliably from the line of additivity. In contrast, the 1:100 mixture falls below the additivity line and suggests a supra-additive drug interaction. Dose-addition analysis comparing the experimentally-derived and predicted values of these mixtures confirmed these results (Table 2). Similar to the results seen when the drugs were administered alone, the clonazepam + ganaxolone combinations did not reduce response rates at doses that engendered triazolam-lever responding (data not shown).

Discussion

The present study investigated the anxiolytic-like and discriminative stimulus effects of benzodiazepine and neuroactive steroid combinations in rats. Triazolam and pregnanolone were selected based on our previous work evaluating the anxiolytic-like and reinforcing effects of these drug combinations in monkeys and effects on food-maintained responding in

rats (Fischer and Rowlett 2011; Gunter et al. 2015). In addition, we expanded our evaluation of benzodiazepine+neuroactive steroid combinations to clonazepam and ganaxolone. Clonazepam has been used widely as a treatment for anxiety as well as seizure disorders, whereas ganaxolone is currently in clinical trials as an adjunct for the treatment of epilepsy (Monaghan et al., 1999; Mula, 2013; Pieribone et al., 2007). A feature of the pairing of triazolam and pregnanolone was based on the relatively short durations of action for these drugs, whereas clonazepam and ganaxolone both have similarly long-acting durations of action in behavioral studies (e.g., Gunter et al. 2015).

Considerable research is available regarding the anxiolytic-like properties of benzodiazepines and neuroactive steroids alone using relevant behavioral models, i.e., elevated plus maze (e.g., Griebel et al. 1998; Nishino et al. 2008; Hogenkamp et al. 2014). We recently evaluated these drugs in combination for anxiolytic-like activity (Fischer and Rowlett, 2011). In this previous study, we used a conflict model in rhesus monkeys and found enhanced anxiolytic-like effects with triazolam + pregnanolone combinations (Fischer and Rowlett, 2011). Interestingly, in that study, pregnanolone (unlike triazolam) did not have the anti-conflict effect characteristic of anxiolytic benzodiazepines. A goal of the present study was to assess combinations of benzodiazepines and neuroactive steroids in an anxiolysis test differing from the operant-based conflict model. The elevated zero maze is a modification of the elevated plus maze, a standard in anxiolytic drug screening (File et al., 2005; Griebel and Holmes, 2013), and is an effective approach for assessing anxiolytic-like effects after administration of benzodiazepines (Braun et al., 2011). In contrast to our previous work, we found anxiolytic-like effects for pregnanolone using the elevated zero maze in rats, and extended this finding to the synthetic neuroactive steroid ganaxolone. Thus, while neuroactive steroids generally appear to have anxiolytic-like properties, it may be that these effects are species and/or assay specific.

In the present study, we found proportion-dependent, supra-additive anxiolytic-like effects for the triazolam and pregnanolone combinations as well as clonazepam and ganaxolone combinations. Other mixtures resulted in no interaction, i.e., the effects were additive. Overall, these findings are similar to results from our previous work, in which pregnanolone shifted the triazolam dose-response function for increased punished responding to the left in a monkey conflict procedure (Fischer and Rowlett 2011). Because isobolographic/dose addition analyses were not conducted by Fischer and Rowlett (2011) due to pregnanolone being ineffective, the present study extends these findings by raising the possibility that at certain fixed proportions, a supra-additive interaction of anxiolysis can be observed for combinations of benzodiazepines and neuroactive steroids.

To date, there are relatively few studies that have investigated the discriminative stimulus effects of benzodiazepine + neuroactive steroid combinations, although more is known about the discriminative stimulus effects of these drugs alone in rats (e.g., Ator 1999; Vanover et al. 1999b; Vanover 2000; Eppolito et al. 2014) and monkeys (e.g., Lelas et al. 1999; McMahon and France 2005; Gerak and France 2014). Our results confirm these previous studies in that pregnanolone engendered >80% triazolam-lever responding, i.e., showed full substitution. Moreover, combinations of the two drugs in multiple fixed-proportions had additive discriminative stimulus effects. Similar results were found using a mixture of the

benzodiazepine midazolam and pregnanolone in rhesus monkeys trained to discriminate midazolam from vehicle (McMahon and France 2005). Collectively, these findings are consistent with the idea that benzodiazepines and neuroactive steroids share discriminative stimulus effects that are mediated by a similar mechanism of action, presumably positive allosteric modulation of the $GABA_A$ receptor, albeit via distinct sites on the receptor.

As expected, clonazepam substituted fully in rats trained to discriminate triazolam from saline (cf. Ator 1999; Sanger et al. 1999). While there are no previous reports of the discriminative stimulus properties of ganaxolone, it is a synthetic derivative of allopregnanolone (Carter et al. 1997; Hogenkamp et al. 2014) which shared discriminative stimulus effects with the benzodiazepine midazolam (Gerak and France 2014). Surprisingly, when combinations of clonazepam and ganaxolone were assessed, a supra-additive effect was observed at the proportion with the highest ratio of ganaxolone to clonazepam. These findings suggest that combining benzodiazepines and neuroactive steroids can result in discriminative stimulus effects greater than predicted from additivity, and although mostly we found additive effects, the presence or absence of an interaction may depend not only on the proportion of drugs in the combination but also on the drugs that are being tested.

Characteristic of drug discrimination procedures, in addition to discriminative stimulus effects we also obtained results on the ability of the drugs and their combinations to alter rates of responding during test sessions. Our previous study explored the effects of benzodiazepine + neuroactive steroid combinations on food-maintained responding under a similar schedule of reinforcement, except only a single lever was active with no corresponding discriminative stimulus contingencies (Gunter et al. 2015). The results of the two studies were, by-and-large, concordant; with the triazolam + pregnanolone combinations having additive and supra-additive rate-reducing effects. However, in the drug discrimination studies clonazepam and ganaxolone had no effects on response rates, either alone or combined. The reason(s) for this discrepancy are unclear, although it may simply reflect procedural differences, i.e., the use of a drug discrimination procedure vs. single-lever food-maintained responding.

In summary, the results of the present and previous studies suggest that the anxiolytic-like effects of benzodiazepine+neuroactive steroid combinations are dependent on both the method used to evaluate anxiolytic-like effects as well as the fixed proportions studied. In general, however, the combination of benzodiazepines and neuroactive steroids resulted in enhanced anxiolytic-like effects that were supra-additive at certain dose ranges. For discriminative-stimulus effects, the extent to which interactions or additive effects depended not only on the fixed proportions used but also on the constituent drugs in the mixtures, suggesting that caution should be taken in generalizing effects of one set of drug combinations to other seemingly related drug combinations. Nevertheless, the findings of this study demonstrated enhanced anxiolytic-like effects of benzodiazepine and neuroactive steroid combinations with an elevated zero-maze procedure in rats that along with our previous work using a conflict procedure in rhesus monkeys, raises the possibility of therapeutic benefit from combining these two drug classes.

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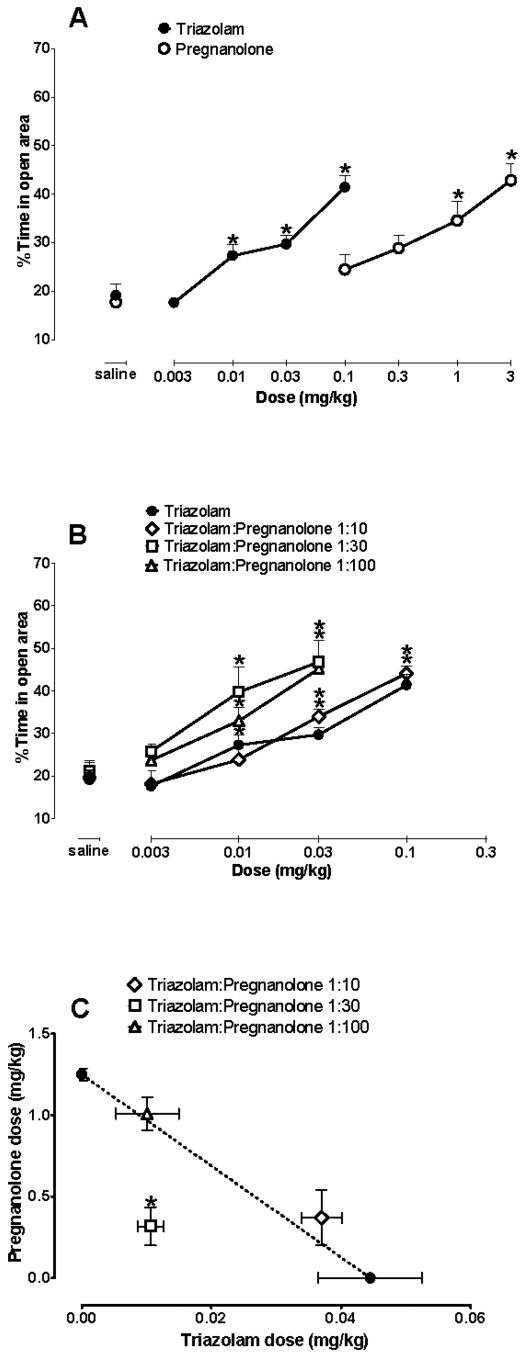

Figure 1.

A, effects of triazolam and pregnanolone alone on percent time spent in the open arms of the elevated zero maze. X-axis, drug dose in mg/kg. Y-axis, time spent in the open arms of the maze as a percent of total session time. B, effects of triazolam and triazolam + pregnanolone mixtures on percent time spent in the open arms of the elevated zero maze. C, isobologram of triazolam + pregnanolone mixtures. X-axis dose of triazolam in mg/kg. Y-axis, dose of pregnanolone in mg/kg. Each data point represents the mean (±SEM) from a group of seven rats.

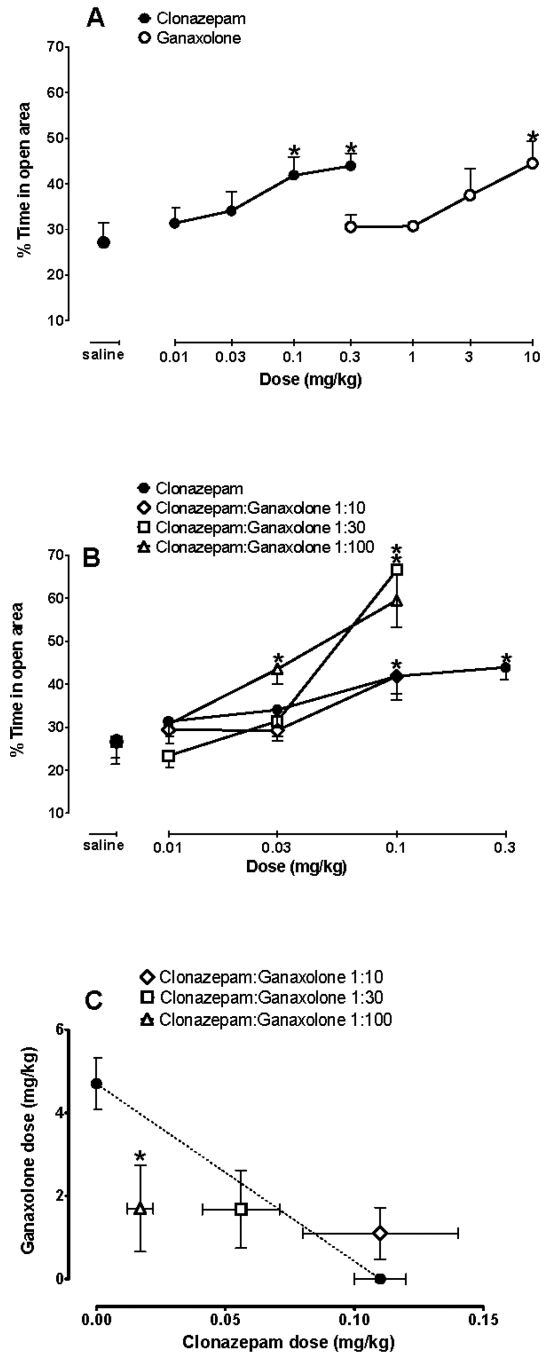

Figure 2.

A, effects of clonazepam and ganaxolone alone on percent time spent in the open arms of the elevated zero maze. X-axis, drug dose in mg/kg. Y-axis, time spent in the open arms of the maze as a percent of total session time. B, effects of clonazepam and clonazepam + ganaxolone mixtures on percent time spent in the open arms of the elevated zero maze. C, isobologram of clonazepam + ganaxolone mixtures. X-axis dose of clonazepam in mg/kg. Y-axis, dose of ganaxolone in mg/kg. Each data point represents the mean (±SEM) from a group of seven rats.

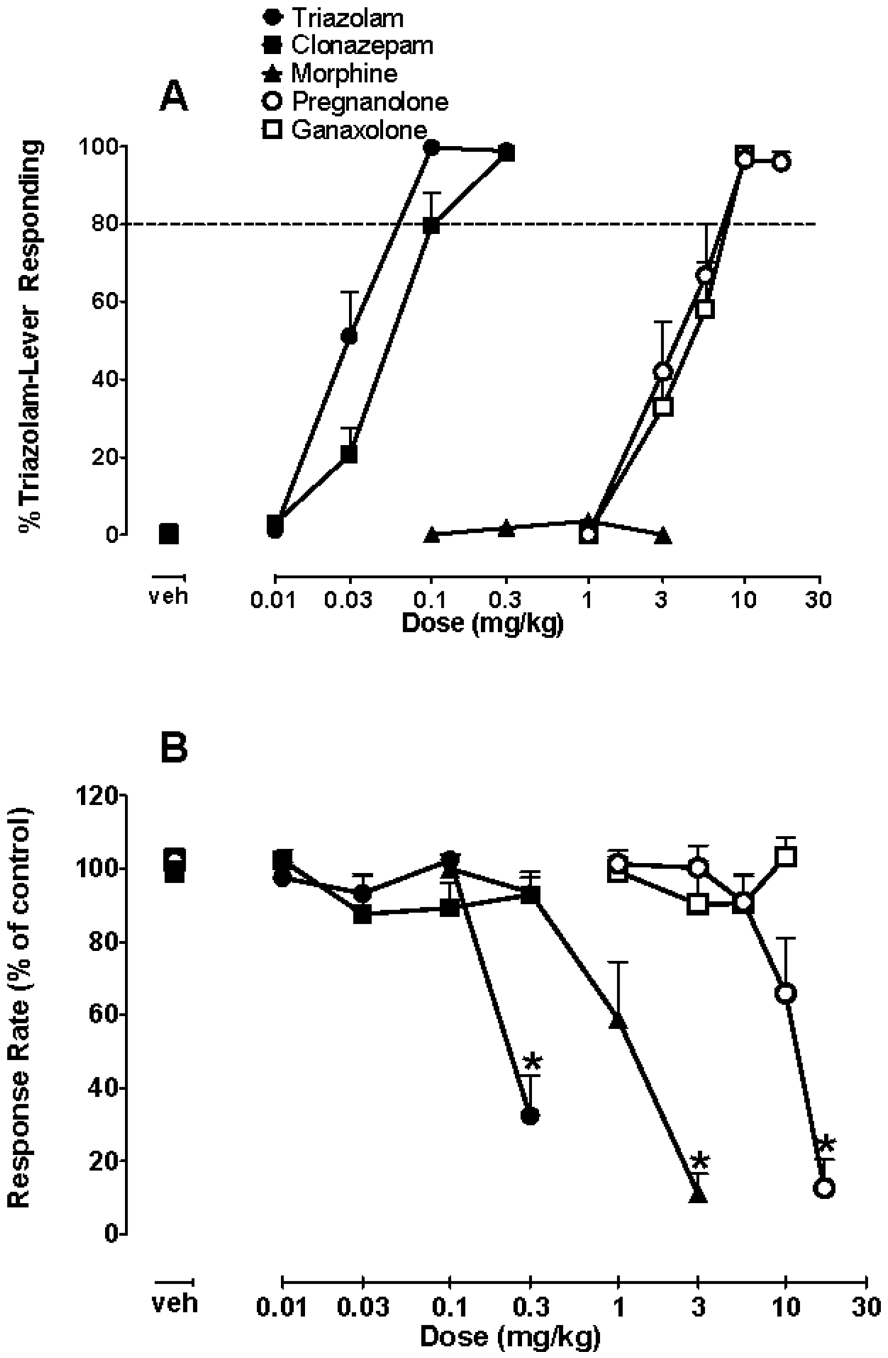

Figure 3.

A, effects of triazolam, pregnanolone, clonazepam, ganaxolone and morphine on percent triazolam-lever responding in rats trained to discriminate triazolam (0.1 mg/kg) from vehicle. X-axis, drug dose in mg/kg. Y-axis, % drug lever responding. Points above "veh" represent vehicle administration. The dotted line at 80% triazolam-lever responses represents threshold levels for full substitution. B, effects of triazolam, pregnanolone, clonazepam, ganaxolone and morphine on response rate as a percent of control. Each data point represents the mean (±SEM) from eight rats.

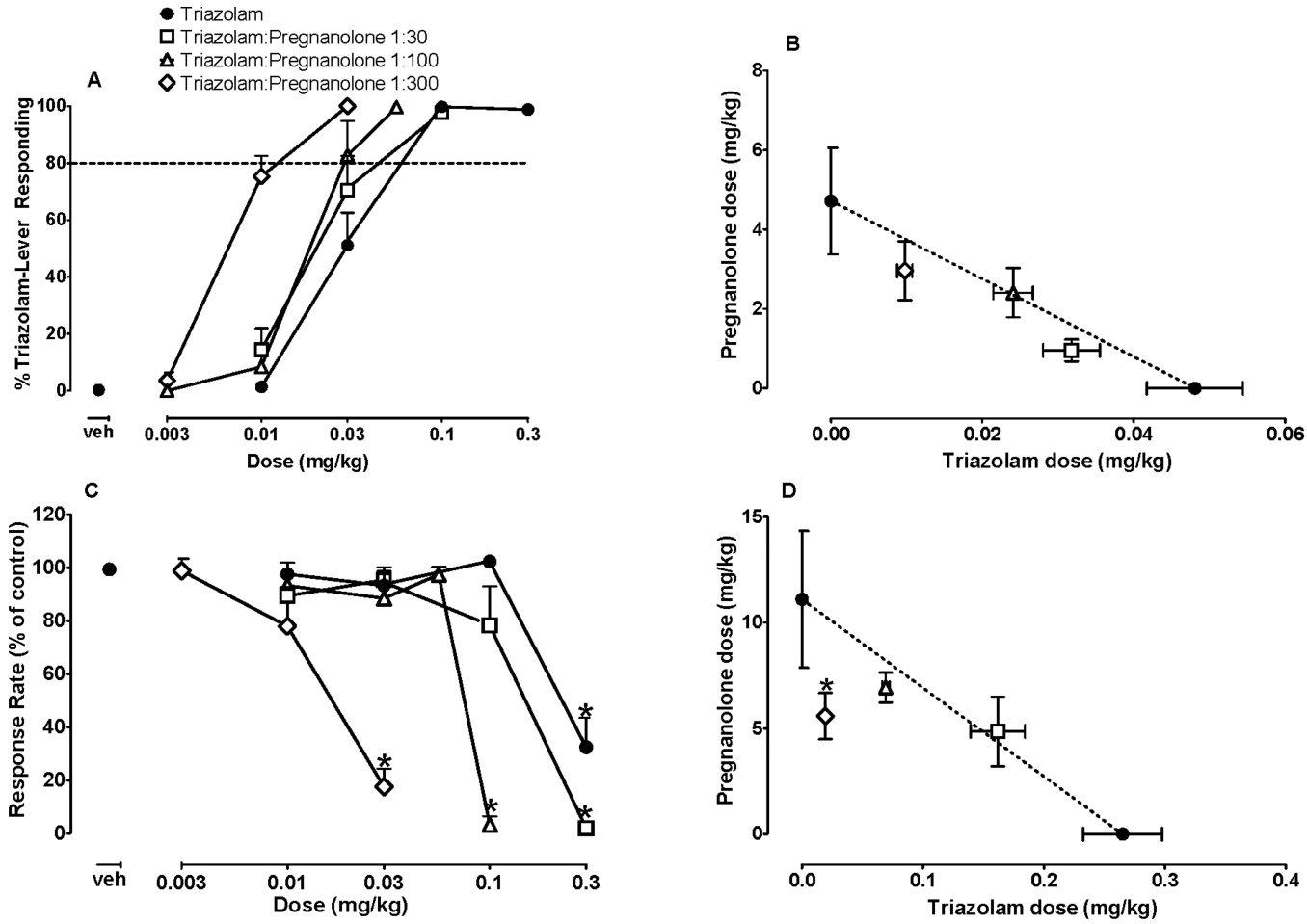

Figure 4.

A, effects of triazolam and triazolam + pregnanolone mixtures on percent triazolam-lever responding. X-axis, drug dose in mg/kg. Y-axis, % triazolam-lever responding. B, isobologram of triazolam + pregnanolone mixtures on triazolam-lever responding. X-axis dose of triazolam in mg/kg. Y-axis, dose of pregnanolone in mg/kg. C, effects of triazolam + pregnanolone mixtures on response rate expressed as a percent of control. D, isobologram of triazolam + pregnanolone mixtures on response rate. X-axis dose of triazolam in mg/kg. Y-axis, dose of pregnanolone in mg/kg. Each data point represents the mean (±SEM) from eight rats

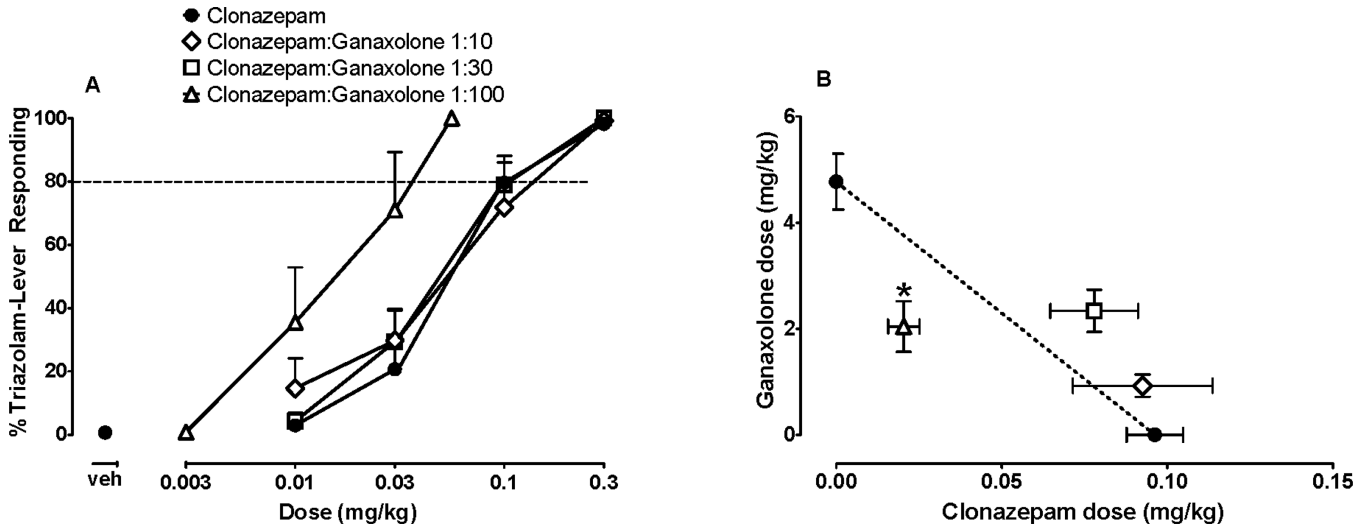

Figure 5.

A, effects of clonazepam and clonazepam + ganaxolone mixtures on percent triazolam-lever responding. X-axis, drug dose in mg/kg. Y-axis, % triazolam-lever responding. B, isobologram of clonazepam + ganaxolone mixtures. X-axis dose of triazolam in mg/kg. Y-axis, dose of pregnanolone in mg/kg. Each data point represents the mean (±SEM) from eight rats

TABLE 1

Experimentally determined (ED_{50}mix) and predicted additive (ED_{50}add) values of triazolam + pregnanolone and clonazepam + ganaxolone mixtures in the elevated zero maze.

Drug combination	ED_{50}mix (95% Confidence Interval)	ED_{50}add (95% Confidence Interval)	Interaction index^
Triazolam/pregnanolone (1:10)	0.41 (0.34–48)	0.36 (0.35–37)	1.12
Triazolam/pregnanolone (1:30)	0.33 (0.21–0.45) *	0.67 (0.64–0.68)	0.49
Triazolam/pregnanolone (1:100)	1.02 (0.95–1.09)	0.99 (0.96–1.01)	1.03
Clonazepam/ganaxolone (1:10)	1.21 (0.52–1.90)	0.98 (0.77–2.73)	1.23
Clonazepam/ganaxolone (1:30)	1.71 (0.72–2.69)	2.00 (1.71–2.30)	0.86
Clonazepam/ganaxolone (1:100)	1.72 (0.65–2.78) *	3.32 (2.47–4.18)	0.52

*experimentally determined value significantly different than the predictive additive value ($p < 0.05$).

^interaction index (i.e. ED_{50}mix/ED_{50}add).

TABLE 2

Experimentally-determined (ED_{50}mix) and predicted additive (ED_{50}add) values of triazolam + pregnanolone and clonazepam + ganaxolone mixtures in the drug discrimination procedure.

Drug combination	ED_{50}mix (95% Confidence Interval)	ED_{50}add (95% Confidence Interval)	Interaction index^
Discriminative Stimulus Effects			
Triazolam/pregnanolone (1:30)	0.99 (0.68–1.29)	1.12 (0.78–1.47)	0.88
Triazolam/pregnanolone (1:100)	2.43 (1.81–3.06)	2.27 (1.71–2.83)	1.07
Triazolam/pregnanolone (1:300)	2.94 (2.2–3.68)	3.61 (2.74–4.48)	0.81
Clonazepam/ganaxolone (1:10)	1.02 (0.45–1.59)	0.86 (0.66–1.06)	1.12
Clonazepam/ganaxolone (1:30)	2.42 (1.41–3.42)	1.83 (1.40–2.25)	1.39
Clonazepam/ganaxolone (1:100)	2.06 (0.88–3.24) *	3.24 (2.46–4.03)	0.62
Rate-Altering Effects			
Triazolam/pregnanolone (1:30) Response rate	5.02 (3.21–6.83)	4.59 (3.22–5.97)	1.09
Triazolam/pregnanolone (1:100) Response rate	7.00 (6.28–7.71)	7.72 (5.79–9.66)	0.91
Triazolam/pregnanolone (1:300) Response rate	5.80 (4.70–6.90) *	9.53 (6.46–12.60)	0.61

* experimentally determined value significantly different than the predictive additive value ($p < 0.05$).

^ interaction index (i.e. ED_{50}mix/ED_{50}add).