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ORIGINAL INVESTIGATION

Benzodiazepine and 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.
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 an elevated zero maze and a drug discrimination procedure in rats.
Methods Male Sprague-Dawley rats ($N = 7$/group) were placed into an elevated zero maze apparatus following injections of multiple doses of triazolam and pregnanolone, alone and combined, or clonazepam and ganaxolone, 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 and pregnanolone, as well as clonazepam and 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 and pregnanolone and clonazepam and ganaxolone produced predominantly additive discriminative stimulus effects, except for one fixed proportion of clonazepam and 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 thought to exert therapeutic and unwanted side effects via binding to a unique site on the $GABA_A$ receptor and positively modulating the ability of GABA to increase Cl- influx. One possible approach to enhance the therapeutic 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 by neurons and glia (Belelli and Lambert 2005) and 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

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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 also act to modulate tonic inhibition via extrasynaptic receptors (Herd et al. 2007).

In behavioral studies, neuroactive steroids in general show effects similar to benzodiazepines. In this respect, neuroactive steroids increase time spent in the open arms of the elevated plus maze, a model of anxiolysis in rodents (Rodgers and Johnson 1998), as well as increase punished 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 (Rowlett et al. 1999; Fischer and Rowlett 2011).

To date, relatively few preclinical studies have evaluated the behavioral effects of benzodiazepine and neuroactive steroid combinations using quantitative approaches to assess potential interactions. Using isobolographic analysis, combinations of pregnanolone and midazolam appear to have additive 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 using 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 and 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) for comparisons with triazolam and pregnanolone. 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). 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 (Sigma-Aldrich, St. Louis) was dissolved in 100 % propylene glycol and diluted to a 20 % propylene glycol/80 % distilled water mixture. Clonazepam (Sigma-Aldrich, St. Louis, MO) was dissolved in 100 % propylene glycol and diluted to a 50 % propylene glycol/50 % distilled 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/distilled water. Morphine was dissolved in saline (0.9 %). All solutions used for dissolving drugs served as the appropriate vehicle for vehicle control tests in the behavioral studies. All drugs were administered intraperitoneally in volumes of 1–2 ml/kg. For the drug combinations, the drugs (in solution) were combined and injected simultaneously in a single syringe. Dose ranges were based on our previous report with these drugs (excluding morphine) that included combination experiments (Gunter et al. 2015).

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 dark conditions maintained during transport from cages to testing rooms) with water and food available ad libitum. Each rat was used only once in the elevated zero maze study. 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 (eighth 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 min. 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 min (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 (including its vehicle), 0.1–3.0 mg/kg pregnanolone (including vehicle), and the combinations; vehicle and 0.01–0.3 mg/kg clonazepam, vehicle and 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-group design, and drugs were tested generally in the order described above (including vehicle tests), with dose varied pseudo-randomly within a drug and across animals.

Data analysis

Dependent measures included percentage of time spent in open arms and number of open and closed arm entries. The percentage of 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 vehicle 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 (CLs).

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 (provided by the vendor, Harlan Co.) 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 (eighth edition, 2011).

Behavioral tests were conducted during the light phase in customized operant chambers (Gerbrands Corporation; 19 cm *h* x 23.5 cm *w* x 22 cm *l*) equipped with two levers (Gerbrands) capable of delivering food pellet reinforcers. A single white stimulus light was mounted above each lever. Food pellets (45 mg; Bio-Serve, Frenchtown, New Jersey, USA) were delivered by a pellet dispenser (Gerbrands) into a food tray equidistant and below the two levers. The two levers were mounted 12.5 cm apart, center to center; 10 cm above the floor. A minimal downward force of 0.3 N was required to activate each lever. Extraneous noise was diminished by enclosing each chamber in an insulated box and by operating a ventilation fan mounted on the outside of the box. 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 one-response, fixed-ratio (FR1) schedule. Once lever pressing was acquired (≥50 reinforcers/session, two 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 FR10. In all sessions, responses on the incorrect lever before completion of the FR10 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 (vehicle, 0.01–0.3 mg/kg) followed by pregnanolone (vehicle, 1–17 mg/kg) and then their combination. Next, clonazepam (vehicle, 0.01–0.3 mg/kg) and ganaxolone (vehicle, 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; vehicle, 0.1–3 mg/kg). For all drugs, combinations were administered in fixed proportions based upon the relative potencies of the two component drugs (see Isobolographic and dose addition analysis section). 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 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. Ninety-five percent CLs 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 = $ED_{50}A / ED_{50}B$. *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 percentage of time spent in the open portions of the maze. Administration of both drugs resulted in 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 resulted in significant increases in the percentage of time spent in the open area (Dunnett's test, $ps < 0.05$). The ED_{50} values (95 % confidence interval) of triazolam and pregnanolone were 0.044 mg/kg (0.029–0.057) 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 to 1 (Tallarida 2001). As also shown in Fig. 1a, all drug combinations reliably increased the percentage 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 (Fig. 1a, note that $*p < 0.05$; Dunnett's test). Isobolographic representation of the ED_{50}

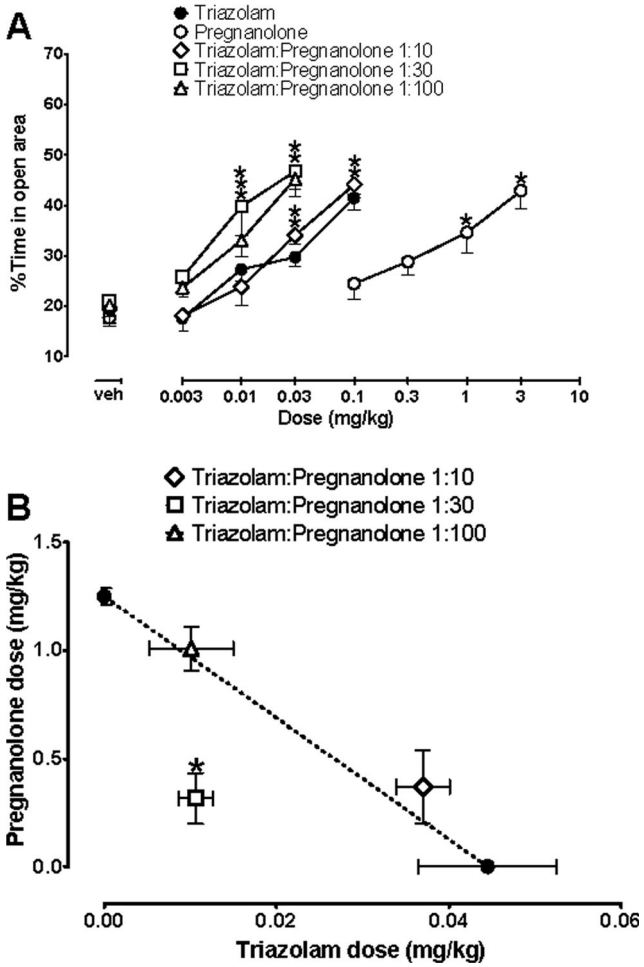

Fig. 1 Effects of triazolam and pregnanolone, alone and combined, in the elevated zero maze in adult male Sprague-Dawley rats ($N = 7$ per group). **a** Percentage of time spent in the open arms (mean ± SEM) following i.p. injections of vehicles, triazolam, pregnanolone, and the combinations of the latter two (expressed as fixed proportions). Note that for **a**, $*p < 0.05$ vs. vehicle control group (Dunnett's tests) and "veh" represents vehicle control (see Drugs section). **b** Isobologram of triazolam and pregnanolone mixtures. *Filled symbols* represent ED_{50} values for the corresponding drug on each axis. Each data point represents the mean (±SEM) from separate groups of rats. Note that for **b**, $*p < 0.05$ compared with the line of additivity (*t* tests)

values of the combinations compared with ED_{50} values of the two drugs alone (Fig. 1b) 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 resulted in dose-dependent increases in the percentage of 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 resulted in significant increases in percentage open area time (Dunnett's test, $ps < 0.05$). The ED_{50} values (95 % confidence interval) of clonazepam and ganaxolone were 0.11 mg/kg (0.089–0.131) and 4.7 mg/kg (4.1–5.3), respectively. Rats then were tested with fixed-proportion mixtures of 1:10, 1:30, and 1:100 clonazepam/ganaxolone (Fig. 2a). Two drug combinations, 1:30 and 1:100, reliably increased the percentage of time spent in the open arms compared to saline [1:30: $F(3,24) = 13.17$, $p < 0.05$); 1:100: $F(3,24) = 10.02$, $p < 0.05$]. Isobolographic representation of the ED_{50} values of the combinations compared with ED_{50} values of the two drugs alone (Fig. 2b) 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 and 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 percentage of time in open arms reflected an anxiolytic-like effect rather than solely alterations in motor behavior. It is also noteworthy that in pilot experiments, doses higher than the ones tested here (triazolam, 0.3 mg/kg; pregnanolone, 10 mg/kg; clonazepam, 1.0 mg/kg; ganaxolone, 18 mg/kg) engendered profound sedation, resulting in no movement on the maze apparatus.

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 (Fig. 3a) and mean response rates (responses/s as a percent of control; Fig. 3b) 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

Table 1 Experimentally determined (ED_{50mix}) and predicted additive (ED_{50add}) values of triazolam plus pregnanolone and clonazepam plus ganaxolone combinations in rats

Drug combination	Elevated zero maze			Drug discrimination (%triazolam-lever responding)			Drug discrimination (response rate)		
	ED_{50mix} (95 % CI)	ED_{50add} (95 % CI)	Interaction index[b]	ED_{50mix} (95 % CI)	ED_{50add} (95 % CI)	Interaction index[b]	ED_{50mix} (95 % CI)	ED_{50add} (95 % CI)	Interaction index[b]
Triazolam plus pregnanolone (1:10)	0.41 (0.34–48)	0.36 (0.35–37)	1.12	–	–	–	–	–	–
Triazolam plus pregnanolone (1:30)	0.33[a] (0.21–0.45)	0.67 (0.64–0.68)	0.49	0.99 (0.68–1.29)	1.12 (0.78–1.47)	0.88	5.02 (3.21–6.83)	4.59 (3.22–5.97)	1.09
Triazolam plus pregnanolone (1:100)	1.02 (0.95–1.09)	0.99 (0.96–1.01)	1.03	2.43 (1.81–3.06)	2.27 (1.71–2.83)	1.07	7.00 (6.28–7.71)	7.72 (5.79–9.66)	0.91
Triazolam plus pregnanolone (1:300)	–	–	–	2.94 (2.2–3.68)	3.61 (2.74–4.48)	0.81	5.80[a] (4.70–6.90)	9.53 (6.46–12.60)	0.61
Clonazepam plus ganaxolone (1:10)	1.21 (0.52–1.90)	0.98 (0.77–2.73)	1.23	1.02 (0.45–1.59)	0.86 (0.66–1.06)	1.12	NE	NE	NE
Clonazepam plus ganaxolone (1:30)	1.71 (0.72–2.69)	2.00 (1.71–2.30)	0.86	2.42 (1.41–3.42)	1.83 (1.40–2.25)	1.39	NE	NE	NE
Clonazepam plus ganaxolone (1:100)	1.72[a] (0.65–2.78)	3.32 (2.47–4.18)	0.52	2.06[a] (0.88–3.24)*	3.24 (2.46–4.03)	0.62	NE	NE	NE

NE no effect, – combination not tested

[a] Experimentally determined value significantly different from the predicted additive value ($p < 0.05$).

[b] Interaction index = (ED_{50mix}/ED_{50add})

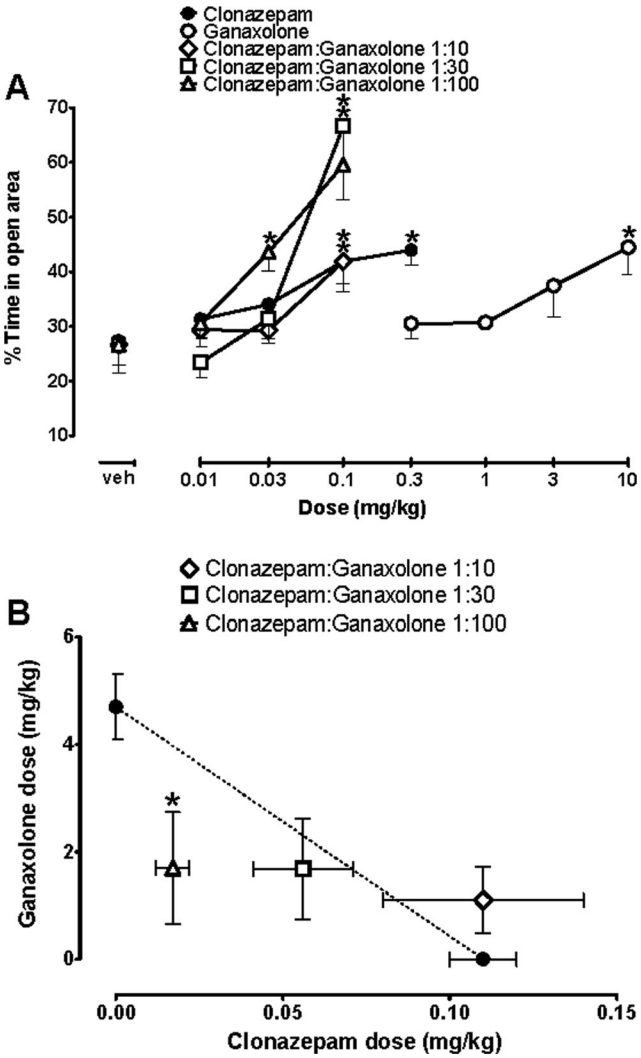

Fig. 2 Effects of clonazepam and ganaxolone, alone and combined, in the elevated zero maze in adult male Sprague-Dawley rats ($N = 7$ per group). Other details as described for Fig. 1

triazolam lever, whereas doses of triazolam ≥0.1 mg/kg elicited virtually exclusive responding on the triazolam lever. As shown in Fig. 3b, 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 (Fig. 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

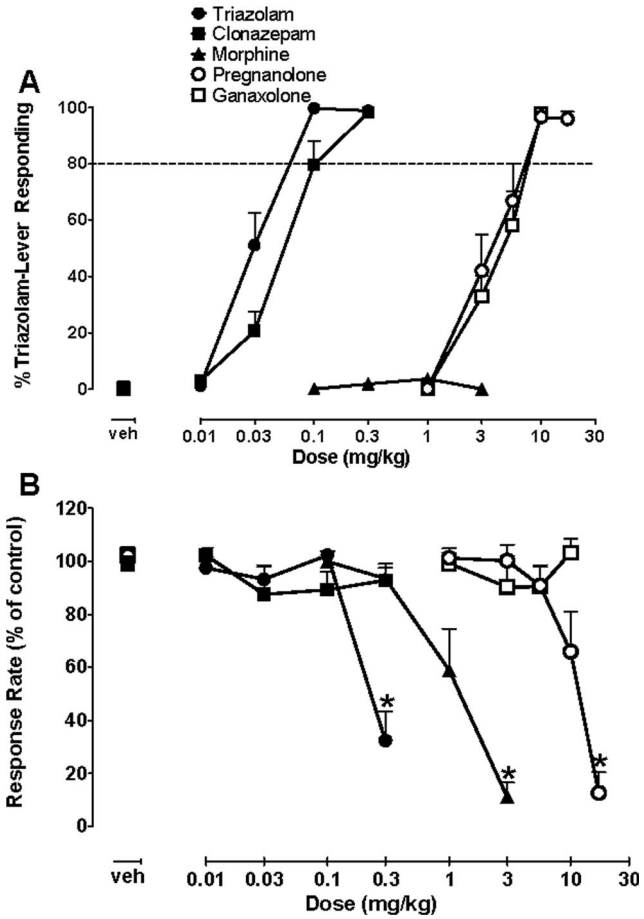

Fig. 3 Effects of triazolam, pregnanolone, clonazepam, ganaxolone, and morphine on percentage of triazolam-lever responding in rats trained to discriminate triazolam (0.1 mg/kg) from vehicle ($N = 8$). For **a**, note that the *X*-axis: drug dose in mg/kg; *Y*-axis: percentage of drug lever responding. Points above "veh" represent effects following vehicle administration (see Drugs section for specific vehicles). The *dotted line* at 80 % triazolam-lever responding represents the level of substitution considered to be "full substitution." **b** Effects of triazolam, pregnanolone, clonazepam, ganaxolone, and morphine on response rate as a percent of control. Note that $*p < 0.05$ vs. vehicle control, Dunnett's tests. All data were expressed as mean ± SEM

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 [Fig. 3b; pregnanolone: $F(5,35) = 29.23$, $p < 0.05$; morphine: $F(4,28) = 31.80$, $p < 0.05$; Dunnett's test, $ps < 0.05$]. In contrast, neither clonazepam nor ganaxolone reduced average rates of responding over the dose range tested.

In drug combination experiments, rats were tested with fixed-proportion mixtures of 1:30, 1:100, and 1:300 triazolam/pregnanolone. Figure 4a shows the effects of these mixtures on the percentage of 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 percentage of triazolam-lever responding data (Fig. 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 1). Figure 4c shows the average response rates engendered by the combinations compared to triazolam alone. As with the percentage of 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, $ps < 0.05$]. Isobolographic representation of the ED_{50} values derived from these data (Fig. 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 1).

The fixed-proportion mixtures of 1:10, 1:30, and 1:100 were used for clonazepam and ganaxolone. 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 (Fig. 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 1). Similar to the results seen when the drugs were administered alone, the clonazepam and 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 and neuroactive steroid combinations to clonazepam and

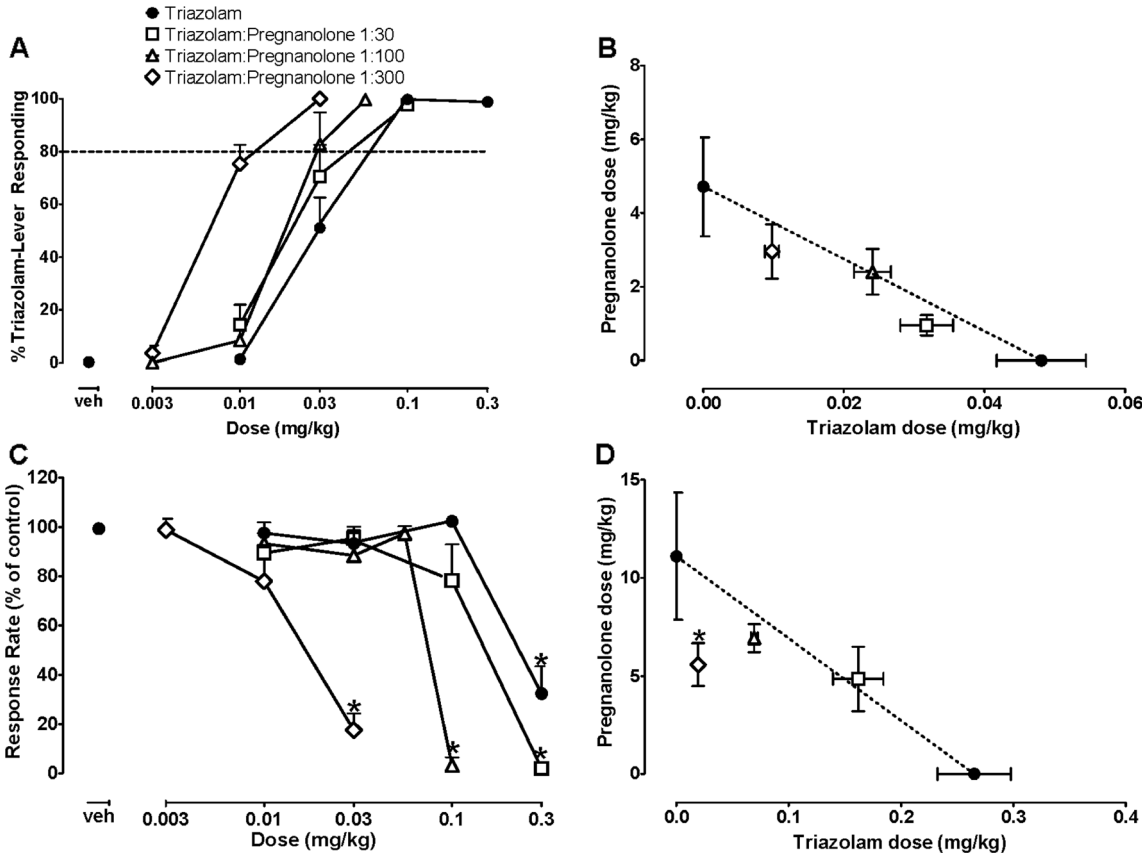

Fig. 4 Effects of triazolam alone and triazolam plus pregnanolone combinations on percentage of triazolam-lever responding and rates of responding. For **a**, *X*-axis, drug dose in mg/kg. *Y*-axis, percentage of triazolam-lever responding (mean ± SEM). For **b**, isobologram of triazolam and pregnanolone combinations. *X*-axis is the dose of triazolam in mg/kg, and *Y*-axis is the dose of pregnanolone in mg/kg. For **c**, effects of triazolam and pregnanolone mixtures on response rate expressed as a percent of control (saline sessions). Note that $^{*}p < 0.05$ vs. vehicle control, Dunnett's tests. For **d**, isobologram of triazolam and pregnanolone mixtures on response rate. *X*-axis is dose of triazolam in mg/kg; *Y*-axis is dose of pregnanolone in mg/kg. Each data point represents the mean (±SEM) from eight rats. Note that "veh" represents drug/drug combination vehicles (see Drugs section for description)

ganaxolone, both of which have clinical relevance as potential anxiolytics and/or anticonvulsants (Monaghan et al. 1999; Mula 2013; Pieribone et al. 2007).

Considerable research is available regarding the anxiolytic-like properties of benzodiazepines and neuroactive steroids alone using relevant behavioral models (e.g., Griebel et al.

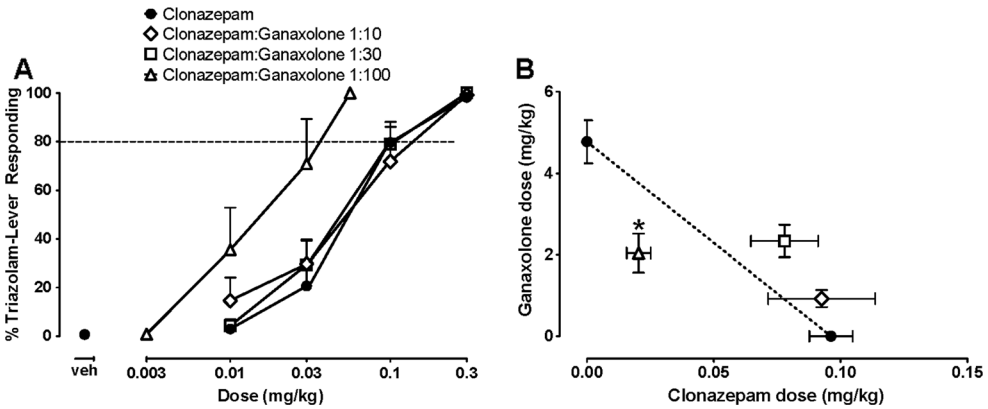

Fig. 5 Effects of clonazepam and clonazepam plus ganaxolone mixtures on percentage of triazolam-lever responding. For **a**, *X*-axis is drug dose in mg/kg; *Y*-axis is the percentage of triazolam-lever responding (mean ± SEM). **b** The isobologram of clonazepam and ganaxolone mixtures. *X*-axis is dose of triazolam in mg/kg; *Y*-axis is dose of pregnanolone in mg/kg. Each data point represents the mean (±SEM) from eight rats. Note that $^{*}p < 0.05$ vs. line of additivity (*t* tests). Also note that response rate data are not shown due to no statistical significance being observed. The abbreviation "veh" indicates drug/drug combination vehicles (see Drugs section for details)

1998; Nishino et al. 2008; Hogenkamp et al. 2014). We have 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 and pregnanolone combinations (Fischer and Rowlett 2011). Interestingly, in that study, pregnanolone (unlike triazolam) did not have the anticonflict effect characteristic of anxiolytic benzodiazepines. A goal of the present study was to assess effects of benzodiazepines and neuroactive steroids, alone and in combination, 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 and 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 are consistent with 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). Ganaxolone 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) in addition to triazolam in the present study. 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 further suggest that the presence or absence of an interaction may depend not only on the proportion of drugs in the combination and the procedure used 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 and 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 and pregnanolone combinations having additive and supra-additive rate-reducing effects. However, in the present study, clonazepam and ganaxolone had no effects on response rates, either alone or combined. The reason(s) for this discrepancy is (are) unclear, although it may simply reflect procedural differences, i.e., the use of a drug discrimination procedure vs. single-lever food-maintained responding; the dose range chosen for study.

In summary, the results of the present and previous studies suggest that the anxiolytic-like effects of benzodiazepine and 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 were observed 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 supra-additive 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, suggest therapeutic benefit from combining these two drug classes. This supra-additive interaction is dissociated from discriminative stimulus effects of the combinations, which are primarily additive; as well as reinforcing effects for which triazolam and pregnanolone were infra-additive (Fischer and Rowlett 2011). Collectively, these results raise the possibility that combinations of benzodiazepines and neuroactive steroids can be identified for which anxiolytic effects are supra-additive yet abuse liability is either minimally enhanced or not altered at all. If this pattern of effects continues to be observed, then combined benzodiazepines and neuroactive steroids might reflect a novel approach to anxiolytic drug discovery.

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

Compliance with ethical standards

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

Conducted experiments: Gunter, Jones

Performed data analysis: Gunter, Jones

Wrote or contributed to the writing of the manuscript: Gunter, Jones, Platt, Paul, Rowlett

Conflict of interest The authors declare that they have no conflicts of interest.

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