Anticonflict and Reinforcing Effects of Triazolam + Pregnanolone Combinations in Rhesus Monkeys

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ABSTRACT

Combinations of positive modulators of benzodiazepine and neuroactive steroid sites on GABA_A receptors have been shown to act in an additive or supra-additive manner depending on the endpoint under study, but they have not been assessed on experimentally induced conflict or drug self-administration. The present study examined the interactive effects of the benzodiazepine triazolam and the neuroactive steroid pregnanolone in a rhesus monkey conflict procedure (a model of anxiolysis) and on a progressive-ratio schedule of drug self-administration (a model of abuse potential). Both triazolam and pregnanolone decreased rates of nonsuppressed responding, whereas only triazolam consistently increased rates of suppressed responding (i.e., had an anticonflict effect). Fixed-ratio

mixtures of triazolam and pregnanolone also decreased rates of nonsuppressed responding and did so in an additive manner. In contrast, mixtures of triazolam and pregnanolone produced either additive or supra-additive rate-increasing effects on suppressed responding, depending on the proportion of drugs in the mixture. Both triazolam and pregnanolone were self-administered significantly, and triazolam and pregnanolone mixtures had either proportion-dependent additive or infra-additive reinforcing effects. These results suggest that combinations of triazolam and pregnanolone may have enhanced anxiolytic effects with reduced behavioral disruption and abuse potential compared with either drug alone.

Introduction

The GABA_A receptor complex is a pentameric assembly of proteins structured around a chloride ion pore. Depending on protein configuration, GABA_A receptors may include benzodiazepine and/or neuroactive steroid binding sites (for review see Rudolph et al., 2001; Lambert et al., 2009). Pharmacological modulators of GABA_A receptors exert their effects by binding to these sites, enhancing the ability of GABA to increase chloride conductance. Positive allosteric modulation of either the benzodiazepine or steroid binding sites results in various neurochemical and behavioral effects, many of which have therapeutic implications.

Benzodiazepines and other positive allosteric modulators with action at the benzodiazepine site of the GABA_A receptor are among the most commonly used pharmacotherapies for the treatment of anxiety- and sleep-related disorders, because of their therapeutic effectiveness and favorable side-effect profile. Their clinical utility is compromised, however, by the occurrence of other characteristic effects including daytime drowsiness, impairment of motor coordination, cognitive impairment, and reinforcing effects that are thought to

contribute to their potential for abuse. These clinical observations are in concordance with animal models used to predict the therapeutic and side effects of benzodiazepines. As examples, these compounds produce anxiolytic-like effects in rodents and primates and their presentation maintains behavior in animal models of drug self-administration (for review see Licata and Rowlett, 2008).

Preclinical studies also suggest that positive modulators with affinity for the neuroactive steroid site of the GABA_A receptor produce behavioral effects similar to those observed after benzodiazepine administration. For example, these compounds can produce anxiolytic-like effects in rodent ethological models (Wieland et al., 1995; Vivian et al., 1997; Rodgers and Johnson, 1998) and conflict models (Wieland et al., 1995; Vanover et al., 1999), although there are limited published reports on this effect in nonhuman primates (Vanover et al., 2000). Furthermore, assays of drug self-administration have been used to demonstrate that neuroactive steroids maintain responding above vehicle levels in both rodents (Sinnott et al., 2002) and nonhuman primates (Rowlett et al., 1999).

Interaction studies also suggest that drugs acting at benzodiazepine and neuroactive steroid sites, when administered concurrently, can produce effects that are either additive or deviate from additivity. It is noteworthy that these effects can depend on the experimental endpoint under study. As examples, isobolographic and/or dose addition

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analysis have been used to demonstrate that benzodiazepines and neuroactive steroids interact additively when assessed on benzodiazepine drug discrimination (McMahon and France, 2005), but in a supra-additive manner when assessed on measures of anesthetic action (Norberg et al., 1999). Clearly, in addition to improving our understanding of GABA_A receptor pharmacology, the assessment of benzodiazepine-neuroactive steroid interactions across behavioral endpoints related to anxiety and drug abuse may have important clinical implications.

The purpose of the current study was to assess the behavioral effects of the benzodiazepine triazolam and the neuroactive steroid pregnanolone, both alone and in combination. Triazolam and pregnanolone were chosen as representative of benzodiazepine-type compounds and neuroactive steroids, respectively, with relatively short durations of action (e.g., Puia et al., 1990; Ducić et al., 1993; Carl et al., 1994; Lelas et al., 2001). The anxiolytic-like and response rate-reducing effects of each drug administered alone were first assessed in a rhesus monkey conflict procedure. The conflict procedure provides measures of both anxiolytic-like effects (increases in suppressed responding) and decreases in operant behavior (rate-reducing effects). Next, the reinforcing effects of triazolam and pregnanolone were assessed using a progressive-ratio schedule of drug self-administration, which is thought to provide a measure of the relative reinforcing strength of drugs (Stafford et al., 1998). Finally, to distinguish an additive effect from an infra- or supra-additive interaction, drugs were administered in combination, assessed across each endpoint, and analyzed with dose-addition analysis (Tallarida, 2000).

Materials and Methods

Animals. Subjects were six adult male rhesus monkeys (*Macaca mulatta*) that were housed individually and maintained on a 12-h lights-on/12-h lights-off cycle, with water available continuously. Monkeys in the conflict studies were maintained at 90 to 95% of their free-feeding weights, and monkeys in the self-administration studies were not food-restricted. Monkeys were prepared with a chronic indwelling venous catheter according to the procedures described by Platt et al. (2005). Animals in this study were maintained in accordance with the *Guide for Care and Use of Laboratory Animals* (National Research Council, Department of Health, Education and Welfare Publication NIH-85-23, revised 1996).

Drugs. Triazolam (8-chloro-6-[2-chlorophenyl]-1-methyl-4H-1,2,4-triazolo[4,3-a][1,4]benzodiazepine) was purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in 50% propylene glycol/50% sterile water. Pregnanolone (3 α -hydroxy-5 β -pregnan-20-one) was purchased from Tocris Biosciences (Ellisville, MO) and dissolved initially in 45% (w/v) 2-hydroxypropyl- β -cyclodextrin. Dilutions of pregnanolone were made in 50% propylene glycol/50% sterile water.

Conflict Procedure. Three rhesus monkeys were trained as described in detail by Rowlett et al. (2006). A daily session consisted of four cycles, each preceded by a 10-min timeout period in which all lights in the chamber were off and responding had no programmed consequences. Each cycle consisted of two components. The first component was signaled by red stimulus lights and consisted of a fixed-ratio 18 schedule of food pellet delivery (Bio-Serv Inc., Frenchtown, NJ) followed by a 10-s timeout. The second component, signaled by green stimulus lights, consisted of the fixed-ratio 18 schedule of food delivery combined with a fixed-ratio 20 schedule of foot shock delivery (1.0–3.0 mA, adjusted for each monkey based on individual performance, 0.25-s duration). Both components were 5 min in duration or ended after the monkey obtained five food pellets

or received three foot shocks, whichever occurred first. Test sessions were conducted once or twice per week when monkeys reached stable performance, as described by Rowlett et al. (2006).

During test sessions, intravenous injections of vehicle or compound were administered in the fifth minute of each timeout. In successive cycles, increasing doses of the test compound were administered using a cumulative dosing procedure. The dependent measure was the average rates of responding (responses/s), calculated by dividing responses by time during components 1 and 2, excluding responding during timeouts or reinforcer delivery.

Self-Administration Procedure. Three rhesus monkeys were trained to self-administer the benzodiazepine midazolam (0.03 mg/kg/infusion) under a progressive-ratio schedule of intravenous drug injection (Rowlett et al., 2005; Rowlett and Lelas, 2007). At the beginning of a daily session, a set of two white stimulus lights above a response lever was illuminated. Upon completion of a response requirement, the white lights were extinguished and a set of two red stimulus lights was illuminated for 1 s, coinciding with a 1-s infusion. Each trial ended with either an injection or the expiration of a 30-min limited hold. Trials were separated by a 30-min timeout period, during which all lights were extinguished and responding had no programmed consequences.

Experimental sessions consisted of five components made up of four trials each. The response requirement remained constant for each of the four trials within a component and doubled during each successive component. The session ended when a monkey self-administered a maximum of 20 injections or when the response requirement was not completed for two consecutive trials. The progressive-ratio schedule consisted of a sequence of response requirements: 40, 80, 160, 320, and 640 responses per injection. Once performance was stable under these conditions (no increasing or decreasing trend in the number of injections/session for three consecutive sessions), midazolam or saline was available on alternating days. Test sessions (T) were added to the alternating sequence of midazolam (M) and saline (S) sessions according to the following sequence: MTSMTSTMST, etc. During test sessions, either triazolam, pregnanolone, or a mixture of triazolam and pregnanolone in the same syringe was available.

The primary dependent measure in the self-administration procedure was the average number of injections per session. Break points were also determined for individual monkeys under each test condition. A break point, defined as the highest response requirement completed during a test session, was used to calculate the maximum break point, which was the highest break point maintained by individual subjects for a compound, irrespective of dose.

Dose-Effect Analysis. The dose of each drug mixture required to produce a 50% decrease in rates of nonsuppressed responding, an increase to 50% in rates of suppressed responding, or an increase to 50% of the maximum effect in drug self-administration was derived using log-linear regression when at least three data points were available on the linear portion of the dose-effect curve or by log-linear interpolation when only two data points (one above and one below 50%) were available. These values were obtained by converting the maximum suppression of the rates of nonsuppressed responding, the maximum increase in the rates of suppressed responding, or the maximum increase in drug self-administration to 100% for individual monkeys. Individual ED₅₀ values were converted to their log values for calculation of means and 95% confidence limits and then converted back to linear values for presentation.

Individual doses on each dose-effect curve were evaluated by conducting a priori Bonferroni *t* tests, comparing individual doses to vehicle injection with an α -level set at $p \leq 0.05$. Bonferroni *t* tests were also used to evaluate differences in maximum break point values between each drug or drug mixture. To evaluate the relationship between the triazolam and pregnanolone dose-effect curves, a parallel line analysis was conducted as described by Tallarida (2000).

Isobolographic and Dose-Addition Analysis. The effects of triazolam + pregnanolone mixtures were assessed graphically with the

use of isobolograms. In the present study, isobolograms were constructed by connecting the ED_{50} of pregnanolone alone plotted on the ordinate with the ED_{50} of triazolam plotted on the abscissa. 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 pregnanolone or triazolam administered alone if the combination is additive. Dose pairs that fall below or to the left of the additivity line suggest an ED_{50} was reached with lesser quantities of the drugs, suggestive of supra-additivity. In contrast, experimental points representing dose pairs that fall above or to the right of the line are suggestive of infra-additivity. Theoretical additive dose pairs (a, b) are described by the equation $[(a/A) + (b/B)] = 1$, where A is defined as the ED_{50} for pregnanolone and B is defined as the ED_{50} for triazolam, both referring to the potency each drug when administered alone.

Drug interactions were analyzed statistically by comparing the experimentally determined ED_{50} values for each mixture (Z_{mix}) with predicted additive ED_{50} values (Z_{add}) as described by Tallarida (2000). Z_{mix} was defined as the total drug dose (i.e., dose triazolam + dose pregnanolone) that produced a 50% decrease in rates of non-suppressed responding, an increase to 50% in the mean rates of suppressed responding, or an increase to 50% of the maximum effect in drug self-administration. Across all endpoints, the mean experimentally determined ED_{50} values (Z_{mix}) and predicted additive ED_{50} values (Z_{add}) for each mixture were compared with a paired t test. An interaction index (γ) was also calculated to quantify deviation from additivity for each mixture (Tallarida, 2002). From this calculation, a γ value of 1 suggested additivity, γ values that approached 0 suggested a greater degree of supra-additivity, and γ values that increased from 1 suggested a greater degree of infra-additivity.

Results

Conflict Procedure

Triazolam and Pregnanolone Alone. Figure 1, left shows the dose-dependent effects of triazolam and pregnanolone on the fixed-ratio schedule of food pellet delivery (non-suppressed responding). Relative to response rates after vehicle administration (~ 1 response/s) both triazolam and pregnanolone produced dose-dependent decreases in average rates of nonsuppressed responding, resulting in ED_{50} values (95% confidence interval) of 0.015 (0.013–0.018) and 0.88 (0.44–1.7) mg/kg for triazolam and pregnanolone, respectively. A statistical test for parallelism revealed that the triazolam dose-effect curve was parallel to the dose-effect curve for pregnanolone ($p < 0.05$). When assessed over time on nonsuppressed responding, the maximally effective doses of 0.03 mg/kg triazolam and 3.0 mg/kg pregnanolone decreased average rates of responding to less than 0.5 responses/s for 20 min, and average rates of responding gradually returned

to near control rates for both drugs within 80 min (data not shown). These data suggest that triazolam and pregnanolone have a similar duration of action. Subsequent fixed-ratio mixtures of 1:30, 1:100, and 1:300 triazolam/pregnanolone were chosen for combination testing, because these ratios result in fractional multipliers of the individual drug ED_{50} values that span the range from 0 to 1 (see Tallarida, 2000).

Figure 1, right also shows the effects of triazolam and pregnanolone on the concurrent schedule of food delivery and electric shock presentation (suppressed responding). After vehicle administration, rates of responding were similar to those observed during training sessions (i.e., less than 0.1 response/s). Triazolam increased the mean rates of suppressed responding compared with vehicle, resulting in an ED_{50} value of 0.0038 (0.0023–0.0065) mg/kg. Across the dose range tested, pregnanolone did not increase average rates of suppressed responding significantly.

Triazolam and Pregnanolone Mixtures. The response rate-decreasing effects of triazolam and triazolam + pregnanolone mixtures are shown in Fig. 2, top. Each drug mixture produced dose-dependent decreases in average response rates. Relative to triazolam administered alone, addition of pregnanolone produced concentration-dependent leftward shifts in the triazolam dose-effect curve. The isobolographic presentation of the effects of these drug combinations is shown in Fig. 2, bottom. This graph suggests that fixed-ratio mixtures 1:30, 1:100, and 1:300 triazolam/pregnanolone produced additive effects because these ED_{50} values (isobols) fell close to the line of additivity. Statistical comparison of experimentally determined ED_{50} values (Z_{mix}) and predicted additive ED_{50} values (Z_{add}) confirmed these findings (i.e., $Z_{add} = Z_{mix}$) (Table 1).

Figure 3 shows the anticonflict effects of triazolam and triazolam + pregnanolone mixtures. Each drug mixture produced dose-dependent increases in average response rates. Relative to triazolam administered alone, addition of pregnanolone produced concentration-dependent leftward shifts in the triazolam dose-effect curve. A statistical comparison confirmed an additive interaction for the 1:30 mixture, because the experimentally determined ED_{50} value (Z_{mix}) was similar to the predicted additive ED_{50} value (Z_{add}) (Table 1). In contrast, statistical comparisons suggested supra-additive effects, because the experimentally determined ED_{50} values (Z_{mix}) for the 1:100 and 1:300 mixtures were significantly less than the predicted additive ED_{50} values (Z_{add}) (Table 1).

Fig. 1. Effects of triazolam and pregnanolone in the conflict procedure. Left, dose-effect curves of triazolam and pregnanolone on nonsuppressed responding. Right, dose-effect curves of triazolam and pregnanolone on suppressed responding. Abscissae, cumulative intravenous dose of triazolam or pregnanolone in milligram/kilogram. Ordinates, response rate as responses/second. Each data point represents the mean ($\pm$ S.E.M.) from three monkeys. Points above V represent data after vehicle administration.

Fig. 2. Effects of triazolam and triazolam + pregnanolone mixtures on nonsuppressed responding. Top, dose-effect curves of triazolam alone or as part of a mixture. Abscissa, cumulative intravenous dose of triazolam in milligram/kilogram. Ordinate, response rate as responses/second. Points above V represent data after vehicle administration. Bottom, isobologram of triazolam + pregnanolone mixtures. Abscissa, ED₅₀ value for triazolam in milligram/kilogram. Ordinate, ED₅₀ value for pregnanolone in milligram/kilogram. Each data point represents the mean ($\pm$ S.E.M.) from three monkeys.

Drug Self-Administration

Triazolam and Pregnanolone Alone. Figure 4 shows the reinforcing effects of triazolam and pregnanolone under the progressive-ratio schedule of drug self-administration. When vehicle was available, the mean ($\pm$ S.E.M.) number of injections/session ranged from 1.7 ± 1.2 to 3.5 ± 0.58 injections/session. Both drugs produced dose-dependent increases

in the number of injections self-administered per session, and the resulting ED₅₀ values for triazolam and pregnanolone were 0.00041 (0.00014–0.0012) and 0.023 (0.010–0.051) mg/kg/injection, respectively. The mean ($\pm$ S.E.M.) maximum break point values were 213 ± 53 and 266 ± 53 for triazolam and pregnanolone, respectively, and these values were not statistically different. A statistical test for parallelism revealed that the triazolam dose-effect curve was parallel to the dose-effect curve for pregnanolone ($p < 0.05$).

Triazolam and Pregnanolone Mixtures. Figure 5 shows the reinforcing effects of triazolam and triazolam + pregnanolone mixtures on progressive-ratio drug self-administration. Each drug mixture produced dose-dependent increases in the average number of injections/session, and the mean maximum break point values were 213 ± 53 , 240 ± 80 , and 320 ± 0 for fixed-ratio mixtures of 1:30, 1:100, and 1:300 triazolam/pregnanolone, respectively. These break point values were not statistically different from each other.

On drug self-administration, the addition of pregnanolone did not shift the triazolam dose-effect curve significantly relative to triazolam alone (Fig. 5, top). Isobolographic presentation of the triazolam + pregnanolone mixture (Fig. 5, bottom) suggested that the mixture with the lowest proportion of pregnanolone relative to triazolam (i.e., 1:30 triazolam/pregnanolone) produced additive effects, because these ED₅₀ values fell close to line of additivity. Statistical comparison of the experimentally determined potency (Z_{mix}) and predicted additive potency (Z_{add}) confirms this finding (i.e., $Z_{\text{add}} = Z_{\text{mix}}$) (Table 1). In contrast, graphical presentation of the 1:100 triazolam + pregnanolone mixture suggested infra-additive effects, as these ED₅₀ values fell above the line of additivity. Statistical comparison determined that the experimentally determined ED₅₀ values (Z_{mix}) for this mixture were significantly greater than the predicted additive ED₅₀ values (Z_{add}) (Table 1). Graphical presentation of 1:300 triazolam/pregnanolone mixture suggested a trend toward infra-additivity, because its isobol fell above the additivity line; however, comparison of Z_{add} and Z_{mix} values demonstrated that the experimentally determined potency and the predicted additive potency were not statistically different ($p = 0.07$; Table 1).

Discussion

The present study assessed the effects of triazolam and pregnanolone in a conflict procedure and a progressive-ratio procedure

TABLE 1

Predicted additive potency (Z_{add}) and experimentally determined potency (Z_{mix}) of triazolam + pregnanolone mixtures on nonsuppressed responding, suppressed responding, and drug self-administration

Drug Combination	Z_{add} ($\pm$ 95% Confidence Limit)	Z_{mix} ($\pm$ 95% Confidence Limit)	$\gamma^{\dagger}$
Nonsuppressed responding			
Triazolam/pregnanolone (1:30)	0.31 (0.16–0.61)	0.42 (0.31–0.57)	1.3
Triazolam/pregnanolone (1:100)	0.56 (0.29–1.1)	0.55 (0.53–0.56)	0.97
Triazolam/pregnanolone (1:300)	0.74 (0.37–1.5)	0.63 (0.51–0.77)	0.85
Suppressed responding			
Triazolam/pregnanolone (1:30)	0.12 (0.070–0.20)	0.099 (0.036–0.27)	0.83
Triazolam/pregnanolone (1:100)	0.39 (0.23–0.66)	0.14 (0.11–0.19)*	0.36
Triazolam/pregnanolone (1:300)	1.2 (0.68–2.0)	0.18 (0.089–0.37)*	0.16
Drug self-administration			
Triazolam/pregnanolone (1:30)	0.0082 (0.0036–0.018)	0.014 (0.0088–0.022)	1.7
Triazolam/pregnanolone (1:100)	0.015 (0.0066–0.033)	0.048 (0.038–0.060)*	3.2
Triazolam/pregnanolone (1:300)	0.019 (0.0086–0.043)	0.082 (0.044–0.15)	4.2

* An experimentally determined potency significantly different from the predicted additive potency ($p < 0.05$).

$\dagger \gamma$ interaction index (i.e. $Z_{\text{mix}} \div Z_{\text{add}}$).

Fig. 3. Dose-effect curves of triazolam alone or as part of a mixture on suppressed responding. Abscissa, cumulative intravenous dose of triazolam in milligram/kilogram. Ordinate, response rate as responses/second. Each data point represents the mean ($\pm$ S.E.M.) from three monkeys. Points above V represent data after vehicle administration.

Fig. 4. Effects of triazolam and pregnanolone on drug self-administration. Abscissa, intravenous dose of triazolam or pregnanolone in milligram/kilogram. Ordinate, drug self-administration as injections/session. Each data point represents the mean ($\pm$ S.E.M.) from three monkeys. Points above V represent data after vehicle self-administration.

of drug self-administration, both alone and in combination. When administered alone, both triazolam and pregnanolone produced dose-dependent decreases in nonsuppressed responding and maintained drug self-administration, but only triazolam was effective in increasing rates of suppressed responding. The main finding from these experiments is that mixtures of triazolam and pregnanolone produced supra-additive effects on suppressed responding, whereas these same drug mixtures produced additive effects on nonsuppressed responding. Furthermore, when assessed on drug self-administration, mixtures of triazolam and pregnanolone produced additive or infra-additive effects. Together, these data demonstrate that the interactive effects of triazolam and pregnanolone depend uniquely on their relative concentrations and the experimental endpoint under study. These data also raise the possibility that adding a neuroactive steroid to a benzodiazepine may enhance its anxiolytic effects without a concomitant enhancement of reinforcing properties.

Procedures that assess the effects of drugs on experimentally induced conflict are often used to assess the potential

Fig. 5. Effects of triazolam and triazolam + pregnanolone mixtures on drug self-administration. Top, dose-effect curves of triazolam alone or as part of a mixture. Abscissa, intravenous dose of triazolam or pregnanolone in milligram/kilogram. Ordinate, drug self-administration as injections/session. Points above V represent data after vehicle administration. Bottom, isobologram of triazolam + pregnanolone mixtures. Abscissa, ED_{50} value for triazolam in milligram/kilogram. Ordinate, ED_{50} value for pregnanolone in milligram/kilogram. Each data point represents the mean ($\pm$ S.E.M.) from three monkeys. Asterisk represents an experimentally determined potency significantly different from the predicted additive potency ($p < 0.05$).

anxiolytic effects of these drugs in humans (Geller and Seifter, 1962; Speelman, 1979; Kleven and Koek, 1999; Rowlett et al., 2006). In the conflict procedure used in the current study, strong positive correlations between the potency of benzodiazepines to increase suppressed responding and therapeutic doses in humans have been reported (Rowlett et al., 2006). Therefore, this model may be particularly predictive of the anxiolytic potency of benzodiazepines, other positive GABA_A receptor modulators, and novel drug combinations. In the present study, triazolam produced a characteristic increase in suppressed operant responding similar to our previous findings with triazolam (Licata et al., 2005) and other clinically available benzodiazepines (Rowlett et al., 2006). Specifically, triazolam increased suppressed responding to rates comparable with those observed under the non-suppressed condition. In contrast, pregnanolone failed to produce an anticonflict effect. This finding is surprising given previously reported findings. For example, pregnanolone has been shown to increase suppressed operant responding in rodent conflict procedures (Wieland et al., 1995; Brot et al., 1997). Similar effects have been observed after synthetic

neuroactive steroid administration in rodents (Britton et al., 1991; Wieland et al., 1997) and squirrel monkeys (Vanover et al., 2000), suggesting that under certain conditions activation of the steroid site on GABA_A receptors is sufficient to produce an anticonflict effect. To our knowledge, the present study is the first to assess the anxiolytic-like effects of pregnanolone in rhesus monkeys, and although the reason for the discrepancy between these previously reported findings and the results reported here are unclear, they may reflect the species differences between the studies. However, this discrepancy may also be caused by other procedural differences, including the nature of the reinforcer or shock delivery or the operant schedule maintaining behavior, among other factors.

The present study is also the first to assess the interactive effects of a benzodiazepine and a neuroactive steroid on experimentally induced conflict. Based on a dose-additive model, it is expected that pregnanolone would not contribute to the effects of triazolam when the two drugs are administered in combination. In contrast, the addition of pregnanolone produced proportion-dependent leftward shifts in the triazolam dose-effect curve, resulting in either additive or supra-additive effects on suppressed responding, depending on their relative concentrations. This is in contrast to the effects observed on nonsuppressed responding. Here, consistent with previous reports (e.g., McMahon and France, 2002a,b), both triazolam and pregnanolone produced dose-dependent decreases in nonsuppressed schedule-controlled responding when administered alone. When administered in combination, triazolam + pregnanolone mixtures also produced decreases in nonsuppressed responding and did so in an additive manner. These divergent behavioral effects that result from the same drug mixtures provide evidence suggesting that the interactive effects of triazolam and pregnanolone depend on the behavioral endpoint under study.

Benzodiazepines are effective anxiolytics; however, their use is constrained by reinforcing properties and subsequent potential for abuse. Assays of drug self-administration are thought to provide a measure of a drug's reinforcing effects, and experimental procedures that use progressive-ratio schedules of reinforcement provide a measure a drug's relative reinforcing effectiveness. In the present study, triazolam engendered operant behavior and subsequent maximum break point values under a progressive-ratio schedule of reinforcement similar to that observed with other conventional benzodiazepines under similar conditions (e.g., Rowlett et al., 2005; Rowlett and Lelas, 2007; Licata and Rowlett, 2011). Self-administration of pregnanolone under a fixed-ratio schedule of reinforcement was reported by Rowlett et al. (1999), consistent with this neuroactive steroid having reinforcing properties. The present study is the first to assess pregnanolone self-administration under a progressive-ratio schedule of reinforcement and suggests that a neuroactive steroid can have a similar reinforcing strength relative to a benzodiazepine, because self-administration of both triazolam and pregnanolone resulted in similar maximum break point values.

When assessed on drug self-administration, mixtures of triazolam and pregnanolone also engendered self-administration at levels greater than saline, suggesting that each mixture also functions as a positive reinforcer. Furthermore, these mixtures were self-administered at maximum break point levels similar to triazolam and pregnanolone alone,

suggesting that triazolam + pregnanolone mixtures have a similar reinforcing strength as their component parts. An interesting finding from the present study is that these drug mixtures produced either mixture-dependent additive or infra-additive reinforcing effects. The detection of an infra-additive effect suggests an antagonistic interaction between two drugs, because the potency of the drug mixture required to maintain self-administration is less than would be predicted based on an additive interaction. This finding provides additional evidence suggesting that the interactive effects of triazolam and pregnanolone are endpoint-dependent.

The behavioral selectivity of triazolam + pregnanolone interactions suggests that there is corresponding selectivity across the receptor mechanisms and/or neural circuits mediating each behavior, rather than a general enhancement of all behavioral effects. The results from these studies not only highlight the importance of assessing drug interactions across endpoints, but also have clear clinical implications. Specifically, the data from the present study suggest that it might be possible to develop benzodiazepine + neuroactive steroid mixtures that interact in a supra-additive manner specifically at receptor-mediated systems that underlie the targeted behavior of anxiolysis. The finding that these same drug mixtures can produce infra-additive reinforcing effects increases the attractiveness of combination therapy.

Across each of the assays, triazolam + pregnanolone mixtures were also assessed using three different proportions, because deviation from additivity often depends on the relative proportions of the drugs under study (Gessner and Cabana, 1970; Tallarida, 2000). In agreement with these findings, the nature of the interactive effects of triazolam and pregnanolone on suppressed responding and drug self-administration depended on the proportion of pregnanolone in each mixture. For example, mixtures of triazolam and pregnanolone produced additive effects in each assay when assessed at a 1:30 ratio, whereas these same drugs produced effects that deviate from additivity when assessed at a 1:100 ratio. Additional support for proportion-dependent effects arises when the degree of deviation from additivity (γ) is measured (i.e., the interaction index; Tallarida, 2002). For example, on suppressed responding, a greater degree of supra-additivity is observed as the proportion of pregnanolone in the mixture is increased. Furthermore, a greater degree of infra-additivity is observed as the proportion of pregnanolone in the mixture is increased on drug self-administration. Together, these data suggest that the interactive effects of a benzodiazepine and neuroactive steroid are not only a property of the drugs under study, but also depend on their relative concentrations.

If neuroactive steroids and benzodiazepines interact in a supra-additive manner on endpoints related to anxiolysis while producing infra-additive effects on drug reinforcement, combination treatment might be useful for the management of anxiety. The successful clinical use of drug combinations to improve therapeutic benefit (e.g., acetaminophen combined with an opioid for the treatment of pain) supports this notion. However, although the present study provides some preliminary evidence in support of future studies assessing the interactive effects of benzodiazepine + neuroactive steroid mixtures, additional characterization of benzodiazepine + neuroactive steroid interactions is necessary to determine their interactive effects after chronic administration and on other behavioral endpoints. Future studies should focus on

behavioral endpoints related to the unwanted effects of benzodiazepines (e.g., sedation) and neuroactive steroids (e.g., respiratory depression). Furthermore, these studies will benefit from the inclusion of female subjects, as the behavioral effects of neuroactive steroids (and benzodiazepines) may vary as a function of endogenous neuroactive steroid levels that fluctuate throughout the menstrual cycle. Finally, it is important to note that although the present study assessed interactions between representative drugs from each class, it will be necessary to determine whether these interactions extend to other benzodiazepine + neuroactive steroid mixtures.

Authorship Contributions

Participated in research design: Fischer and Rowlett.

Conducted experiments: Fischer.

Performed data analysis: Fischer.

Wrote or contributed to the writing of the manuscript: Fischer and Rowlett.

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