Tolerance to the rate-increasing and not rate-decreasing effects of pregnanolone in rats

Amy K. Eppolito and **Lisa R. Gerak**
Department of Pharmacology, The University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, mail code 7764, San Antonio, TX, USA 78229-3900

Abstract

Chronic treatment with benzodiazepines, which positively modulate $GABA_A$ receptors, can lead to the development of tolerance. Similar effects might also occur during chronic treatment with positive modulators acting at other sites on $GABA_A$ receptors (e.g., neuroactive steroids). In the current study, tolerance and cross tolerance were examined in 7 rats treated daily with the neuroactive steroid pregnanolone (25.6 mg/kg/day) and responding under a fixed ratio 10 schedule of food presentation. Dose-effect curves were determined for positive $GABA_A$ modulators (pregnanolone, flunitrazepam, midazolam, and pentobarbital) as well as other drugs (ketamine and morphine) before, during and after chronic treatment. Initially, daily pregnanolone administration increased responding, although tolerance developed to the rate-increasing effects after 14 weeks; tolerance did not develop to the rate-decreasing effects. The potencies of pregnanolone, midazolam and morphine to decrease responding did not change during treatment, while flunitrazepam was more potent and pentobarbital and ketamine were less potent during treatment as compared to before treatment. After treatment, pregnanolone and midazolam were more potent than before treatment. The development of tolerance to the rate-increasing effects of pregnanolone indicates that neuroadaptations occur during chronic treatment; that tolerance develops to only some effects suggests that the behavioral consequences of these neuroadaptations are limited.

Keywords

neuroactive steroids; scheduled-controlled behavior; benzodiazepines; tolerance; rats

Introduction

Gamma-aminobutyric acid (GABA) is the primary inhibitory neurotransmitter in the mammalian nervous system and is involved in numerous processes including development and organization of the central nervous system (Andang and Lendahl, 2008), endocrine and neuroendocrine responses (Cullinan et al., 2008; Morrow et al., 1995), and regulation of mood and anxiety states (Paul and Purdy, 1992; Smith et al., 2007). Many effects of GABA are mediated by $GABA_A$ receptors, which are ligand-gated chloride channels that have several modulatory sites; benzodiazepines, barbiturates, and neuroactive steroids act at these distinct sites. Positive modulation at these sites enhances the effects of GABA, thereby facilitating chloride conductance (Allan and Harris, 1986; Majewska et al., 1986; Tallman and Gallager, 1985).

Positive $GABA_A$ modulators acting at benzodiazepine or barbiturate sites have long been used therapeutically in the treatment of anxiety, sleep disorders, and seizure disorders.

Corresponding author: Lisa R. Gerak, Phone: 210 567 0106, Fax: 210 567 4303, gerak@uthscsa.edu.

Although benzodiazepines have a larger margin of safety than barbiturates, the use of these drugs can be limited by the development of tolerance and dependence, with benzodiazepine withdrawal symptoms ranging from uncomfortable (increased anxiety and insomnia) to potentially dangerous (seizures). Like benzodiazepines, drugs acting at the neuroactive steroid site, including pregnanolone, positively modulate $GABA_A$ receptors resulting in anxiolytic (i.e. Akwa et al., 1999), sedative (i.e. Lancel et al., 1997) and anticonvulsant (i.e. Kokate et al., 1998; Reddy and Rogawski, 2000a) effects; however, neuroactive steroids appear to differ from benzodiazepines in several important ways. One difference involves effects that occur when positive modulators are administered repeatedly with tolerance developing to benzodiazepines under many different conditions (e.g., McMillan, 1992; Löscher et al., 1996; Gerak et al., 2008; Gerak, 2009) and developing to neuroactive steroids under a much more limited range of conditions. For example, tolerance to the anticonvulsant effects of benzodiazepines is readily apparent (Gonsalves and Gallagher, 1987; Löscher et al., 1996), although tolerance does not develop to the anticonvulsant effects of pregnanolone (Kokate et al., 1998) or another neuroactive steroid, ganaxolone (Reddy and Rogawski, 2000b). Such differences might provide a clinical advantage for neuroactive steroids as compared to benzodiazepines.

There are conditions under which tolerance can develop to neuroactive steroids, although it appears to be selective with tolerance developing differentially depending on the particular neuroactive steroid studied or the dependent variable measured. For example, tolerance develops to the anesthetic (Zhu et al., 2004) and memory-impairing (Türkmen et al., 2006) effects of allopregnanolone and to the effects of minaxolone on locomotor activity (Marshall et al., 1997), but not to the rate-decreasing effects of pregnanolone on schedule-controlled responding (McMahon and France, 2002a). Direct comparisons between different neuroactive steroids are few, and the effects of chronic treatment must be compared across studies and dependent variables; however, one study examined the anesthetic effects of pregnanolone and allopregnanolone and found no evidence for acute tolerance to either neuroactive steroid (Zhu et al., 2001). Taken together, these studies suggest that treatment with neuroactive steroids produces differential tolerance depending upon the treatment dose, species, dependent variable, or route, frequency or duration of administration.

This study systematically increased the dose and frequency of pregnanolone administered chronically in order to examine the development of tolerance to and dependence on a neuroactive steroid. To test whether tolerance developed, response rates and the potency of pregnanolone were compared before, during and after chronic pregnanolone treatment. Cross tolerance to positive $GABA_A$ modulators acting at other sites was examined by comparing potencies of the benzodiazepines midazolam and flunitrazepam and the barbiturate pentobarbital obtained during and after chronic treatment to their potencies obtained before chronic pregnanolone treatment. In addition, the NMDA receptor antagonist ketamine and the mu opioid receptor agonist morphine were also studied before, during and after chronic pregnanolone treatment. Another consequence of chronic treatment is the development of dependence, which is evidenced by the emergence of withdrawal when treatment is discontinued. In studies that did not find evidence of tolerance to neuroactive steroids, discontinuation of treatment resulted in an increased susceptibility to pentylenetetrazol-induced seizures (Reddy and Rogawski, 2000a) and disruption in rates of food-maintained responding (McMahon and France, 2002a), suggesting that dependence can develop during chronic neuroactive steroid treatment in the absence of tolerance. One reliable indicator of withdrawal is suppression of responding maintained by food presentation (Ator et al., 2000; Ator and Griffiths, 2003; Gellert and Sparber 1977). To the extent that the current treatment conditions result in dependence, it would be indicated by disruptions in responding following temporary suspension of chronic pregnanolone treatment.

Methods

Animals

Seven male Sprague-Dawley rats (Harlan, Indianapolis, IN, USA) weighing 250-300 g upon arrival were individually housed in a temperature- and humidity-controlled vivarium on a 14 hour light/10 hour dark cycle. Weights were initially increased and then maintained at 320-330 g for the duration of the study by providing measured amounts of rodent chow daily (Harlan Teklad, Madison, WI, USA). Rats had *ad libitum* access to water in the home cage and grain-based pellets during experimental sessions (BioServ, Inc., Frenchtown, NJ, USA). All experimental procedures were carried out under guidelines provided by the Institutional Animal Care and Use Committee at the University of Texas Health Science Center at San Antonio and in accordance with the Guide for the Care and Use of Laboratory Animals (National Academy of Sciences, 1996).

Apparatus and procedure

Experimental sessions were conducted daily at the same time. White noise was used in the testing room to reduce extraneous noise. Standard operant boxes were contained in ventilated, sound-attenuating chambers (Med Associates, Inc., St. Albans, VT, USA). The operant boxes ($30 \times 24 \times 21$ cm) were constructed of clear polycarbonate and aluminum. One side of the box was a response panel with two levers, two stimulus lights, a pellet dispenser and food cup. The floor of the box was constructed of stainless steel rods. During sessions, rats responded on one of the two levers under a fixed ratio 10 schedule for food. Daily sessions consisted of two to eight 15-minute cycles, each divided into a 10-minute timeout, when responses had no programmed consequence, followed by a 5-minute response period during which time the stimulus light above the active lever was illuminated. Ten consecutive responses on the active lever resulted in the delivery of a single food pellet with a maximum of 10 pellets delivered during each cycle. Responses on the inactive lever reset the count on the active lever to 0. Assignment of the left or right lever as the active lever was counterbalanced among rats. Stimulus lights were extinguished after 5 minutes or the delivery of 10 food pellets, whichever occurred first. Any time remaining between delivery of 10 food pellets and the next cycle was a timeout. Data were collected using MED-PC IV software (Med Associates, Inc., St. Albans, VT, USA).

During training sessions, vehicle or sham injections were given during the first minute of each cycle. Training continued until animals satisfied the following testing criteria: response rates for each cycle within ±20% of the mean for individual sessions and mean response rates for a single session within ±20% of the mean of 10 consecutive sessions, including the current session. Tests were conducted every third session as long as the testing criteria were satisfied during the intervening training sessions. If criteria were not met between tests, additional training sessions were conducted until testing criteria were satisfied for 2 consecutive sessions. To determine the treatment dose of pregnanolone and the interval between pregnanolone administration and sessions, time course studies were conducted prior to chronic treatment by giving pregnanolone on the first cycle, followed by seven sham cycles. Dose-effect curves were determined by administering vehicle on the first cycle followed by increasing doses of the test drug; injections were given during the first minute of each cycle. Cumulative doses increased in 0.25 or 0.50 log unit increments until response rates decreased to less than 10% of control rates. Dose-effect curves were determined for pregnanolone, flunitrazepam, midazolam, pentobarbital, ketamine, and morphine before chronic pregnanolone treatment.

Once dose-effect curves for all six drugs were determined, rats were treated daily with increasing doses of pregnanolone; an outline of the experimental design can be found in

Table 1. Chronic treatment continued for a total of 20 weeks. At the start of chronic treatment, rats received one injection of 5.6 mg/kg of pregnanolone in the home cage one hour before sessions. Because tolerance was not evident after 2 weeks of treatment with a single dose of pregnanolone, the frequency of dosing was increased to twice daily injections of 5.6 mg/kg of pregnanolone (total dose: 11.2 mg/kg/day), given five hours and one hour prior to sessions, for 5 weeks. Thereafter, a third daily injection of 5.6 mg/kg of pregnanolone was added four hours after the second injection (approximately two hours after sessions; total dose: 16.8 mg/kg/day of pregnanolone); this treatment continued for 2 more weeks. Response rates and the potency of pregnanolone determined at this time indicated no development of tolerance; as a result, the treatment dose was further increased. For the remaining 11 weeks of treatment, one dose of 10.0 mg/kg of pregnanolone was given five hours prior to sessions, a second dose of 5.6 mg/kg was given one hour prior to sessions, and a third dose of 10.0 mg/kg was given four hours after the second injection (total dose: 25.6 mg/kg/day of pregnanolone). Throughout the study, dose-effect curves were determined twice weekly, beginning with a pregnanolone dose-effect curve on the fourth day of treatment and a flunitrazepam dose-effect curve on the seventh day of treatment. Thereafter, pregnanolone and flunitrazepam dose-effect curves were determined each week while treatment conditions were changing with the exception of week 7 during which ketamine dose-effect curves were determined twice. The final treatment conditions were established by week 12; for the next 11 weeks, dose-effect curves for other drugs were determined when rats received pregnanolone before the session so that the interval between the last pregnanolone injection and the session was one hour. The third injection of pregnanolone was not given on testing days to avoid adverse effects. To test for the development of dependence as measured by disruptions in rates of food-maintained responding, pregnanolone treatment was temporarily suspended for 25 hours and rats received vehicle instead of pregnanolone for the three consecutive injections that preceded the session; vehicle was administered during the first cycle of sessions. On separate occasions, the vehicle cycle was followed by redetermination of dose-effect curves for each of the 6 drugs. For 2 weeks following termination of chronic pregnanolone treatment, rats received three vehicle injections daily and dose-effect curves for pregnanolone and flunitrazepam were determined. Four weeks after termination of treatment dose-effect curves for all drugs were redetermined.

Drugs

Pregnanolone (5ß-pregnan-3α-ol-20-one; Steraloids, Inc., Newport, RI, USA) was dissolved in 45% (w/v) 2-hydroxypropyl-γ-cyclodextrin (Sigma-Aldrich, St. Louis, MO, USA). Sodium pentobarbital (Sigma-Aldrich, St. Louis, MO, USA) and morphine sulfate (Research Technology Branch, National Institute on Drug Abuse, Rockville MD, USA) were dissolved in 0.9% saline. Ketamine hydrochloride (Fort Dodge Laboratories, Fort Dodge, IA, USA) and midazolam hydrochloride (Bedford Laboratories, Bedford, OH, USA) were purchased in solution and diluted in 0.9% saline. Flunitrazepam (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 20% emulphor, 10% ethanol and 70% saline. All drugs were administered i.p.

Data analyses

Control response rates were determined for each rat prior to chronic treatment by first averaging response rates across cycles within a training session, then across the 10 training sessions that immediately preceded the start of daily pregnanolone treatment. A paired *t*-test was used to determine changes in rates before and after chronic pregnanolone treatment. Time course data were analyzed by two-way repeated measures analysis of variance (ANOVA) using GraphPad 5.01 Software (San Diego, CA, USA, www.graphpad.com) with pregnanolone dose and time since injection as the between-subjects factors. Significant

ANOVAs were followed with Bonferroni's post hoc test. A one-way repeated measures ANOVA was used to compare response rates on the first cycle (vehicle injection) of weekly test sessions before, during and after treatment; post hoc comparisons were made using Dunnett's Multiple Comparison Test to compare response rates on the first cycle of the session during and after treatment to response rates before treatment. Statistical tests were deemed significant when $p < 0.05$.

The dose of drug needed to decrease response rates to 50% of control rates (ED_{50}) was estimated by fitting straight lines to individual dose-response curves for each rat; each line was estimated using one dose that produced response rates greater than or equal to 75% of control rates and one dose that produced response rates less than or equal to 25% of control rates and all doses in between. Potency ratios were calculated for each animal by dividing the ED_{50} values during and after treatment by the ED_{50} value before treatment to determine changes in potency that might indicate the development of tolerance or cross tolerance during chronic treatment. Significant changes in potencies during and after treatment relative to before treatment were detected when the 95% confidence intervals of the potency ratios averaged across rats did not include 1.

Results

Control rates of responding

Prior to chronic treatment, mean control response rates varied across individual rats from 0.45 to 0.89 responses per second, with a group mean ($\pm$SEM) of 0.67 ± 0.06 responses per second. Rates of responding obtained four weeks after treatment (group mean: 0.64 ± 0.05 responses per second) were not significantly different from those obtained before treatment ($t_{(6)} = 0.79$, $p > 0.05$).

Time course studies

The rate-decreasing effects of a single, bolus dose of 5.6 mg/kg of pregnanolone were evident 15 minutes after administration (Fig. 1). A significant time $\times$ dose interaction ($F_{(21,120)} = 4.11$, $p < 0.001$) and a significant main effect of dose ($F_{(3,120)} = 26.97$, $p < 0.001$) were detected. Bonferroni post hoc tests revealed decreased response rates for 60 minutes after 5.6 mg/kg of pregnanolone; response rates were not different from control rates 75 minutes after administration. There was no significant change in rates of responding following administration of 1.0 (data not shown) or 3.2 mg/kg of pregnanolone compared to vehicle.

Daily treatment with pregnanolone

During chronic pregnanolone treatment, response rates on the first cycle of sessions were increased relative to response rates obtained on the first cycle of sessions before chronic treatment ($F_{(16,119)} = 6.85$, $p < 0.001$). This increase was evident on the first day of treatment, resulting in average response rates of 0.92 ± 0.07 responses per second. For 13 weeks, response rates on the first cycle of weekly test sessions were increased relative to rates obtained during the first cycle of the test session immediately before treatment began, regardless of daily pregnanolone dose (compare triangles and circle, Fig. 2); when pregnanolone treatment was suspended for 25 hours before the session (squares, weeks 6 and 12, Fig. 2), rates on the first cycle decreased and were no different from before treatment. Tolerance developed to the rate-increasing effects of pregnanolone by the 14th week of treatment. Thereafter, suspension of treatment for 25 hours before sessions had no effect on response rates (squares, week 18, Fig. 2), and there was no change in rates four weeks after chronic pregnanolone treatment was terminated (diamond, Fig. 2).

Dose-effect curves determined before treatment

Prior to chronic pregnanolone treatment, small doses of pregnanolone and midazolam increased rates of responding and larger doses had no effect or decreased rates (open circles, Fig. 3A and 3C). The highest response rates occurred at a cumulative dose of 5.6 mg/kg of pregnanolone, which increased rates from 0.67 ± 0.06 under control conditions to 1.01 ± 0.05 responses per second. A dose of 0.1 mg/kg of midazolam increased responding to 0.90 ± 0.07 responses per second. Flunitrazepam, pentobarbital, ketamine and morphine only decreased rates (open circles, Fig. 3B, 3D, 3E and 3F).

Dose-effect curves determined during treatment

During chronic treatment, small doses of pregnanolone, midazolam and flunitrazepam increased rates of responding and larger doses had no effect or decreased rates (open triangles, Fig. 3A, 3B and 3C). When pregnanolone treatment was suspended for 25 hours, small doses of pregnanolone and flunitrazepam increased rates of responding and larger doses decreased rates (open squares, Fig. 3A and 3B). Potency ratios with 95% confidence intervals for each drug and treatment condition are listed in Table 2. During pregnanolone treatment, there was no change in the potencies of pregnanolone (Fig. 3A), midazolam (Fig. 3C), or morphine (Fig. 3F) to decrease response rates as compared to their potencies before chronic treatment. The potency of flunitrazepam (Fig. 3B) was increased during chronic pregnanolone treatment; there was a 3-fold shift leftward in the flunitrazepam dose-effect curve during treatment. Pentobarbital (Fig. 3D) and ketamine (Fig. 3E) were significantly less potent in decreasing response rates during chronic treatment; there was a significant 1.5-fold shift rightward in the pentobarbital dose-effect curve and a significant 5-fold shift rightward in the ketamine dose-effect curve. When pregnanolone treatment was suspended for 25 hours, there was a significant 2-fold shift rightward in the midazolam dose-effect curve and a significant 3-fold shift rightward in the ketamine dose-effect curve. The potencies of pregnanolone and morphine to decrease rates were unchanged when treatment was suspended for 25 hours compared to before treatment. The potency of flunitrazepam was increased when pregnanolone treatment was suspended for 25 hours resulting in a 3-fold shift leftward in the flunitrazepam dose-effect curve.

Dose-effect curves determined after treatment

After chronic treatment, pregnanolone and midazolam were significantly more potent in decreasing response rates relative to before treatment as indicated by 2-fold leftward shifts. The potencies of flunitrazepam, pentobarbital, ketamine, and morphine following treatment were not different from before treatment (Table 2).

Discussion

This study examined the development of tolerance and cross tolerance during chronic pregnanolone treatment. Daily treatment with pregnanolone increased responding for several weeks; however, by the 14th week of treatment, tolerance developed to the rate-increasing effects of pregnanolone and response rates were not different from rates before treatment. Larger doses of pregnanolone decreased responding; systematically increasing the daily treatment dose did not shift the rate-decreasing portion of the pregnanolone dose-effect curve rightward, indicating a lack of tolerance to the rate-decreasing effects of pregnanolone. When pregnanolone treatment was suspended for 25 hours, rates of food-maintained responding were not different from rates obtained before chronic treatment, regardless of whether tolerance had developed; that rates were not disrupted suggests that dependence did not develop.

Despite similarities in the acute behavioral effects (e.g. sedation and anticonvulsant effects) of positive GABA$_A$ modulators acting at different sites, the effects of chronic treatment with benzodiazepines or with neuroactive steroids appear to differ widely. Repeated treatment with benzodiazepines leads to the development of tolerance to the anxiolytic (e.g. Fernandes et al., 1999), sedative (e.g. McMillan, 1992), and anticonvulsant effects (e.g. Izzo et al., 2001) in rats and mice. Tolerance also develops to the rate-decreasing effects of benzodiazepines under a variety of conditions (e.g. Cohen and Sanger, 1994; Gerak, 2009; McMahon and France, 2002b). Although, under some conditions, sensitivity to neuroactive steroids decreases with repeated administration, the conditions under which tolerance develops to neuroactive steroids appear to be narrow. For example, tolerance develops to the memory-impairing (Türkmen et al., 2006), anticonvulsant (Członkowska et al., 2001) and anesthetic effects (Zhu et al., 2004; Türkmen et al., 2008) of allopregnanolone; however, tolerance does not develop either to allopregnanolone when its effects on the components of sleep are measured (Damianisch et al., 2001) or to the anticonvulsant effects of pregnanolone (Kokate et al., 1998). In addition, strain differences have been noted in the development of tolerance to the hypothermic effects of allopregnanolone in mice selectively bred for high or low sensitivity to ethanol-induced hypothermia (Palmer et al., 2002). In the current study, tolerance developed to the rate-increasing effects of pregnanolone, and not to its rate-decreasing effects in this or other (McMahon and France, 2002a) studies. Although some of the differences in the development of tolerance to neuroactive steroids noted across studies might be due to the species studied, or route and duration of administration, these explanations cannot account for other inconsistencies, such as the differential development of tolerance observed in the current study. Thus, decreased sensitivity to neuroactive steroids occurs under a limited set of conditions, possibly indicating an important difference between neuroactive steroids and benzodiazepines.

Following acute (Gerak et al., 2008) and chronic treatment (McMahon and France 2002a) with benzodiazepines, cross tolerance to other benzodiazepines develops; however, cross tolerance does not develop to neuroactive steroids (Gerak et al., 2008; Gerak 2009; McMahon and France 2002a, 2002b; McMahon et al., 2007). Although cross tolerance to benzodiazepines has been shown in subjects treated chronically with neuroactive steroids (McMahon and France 2002a; Reddy and Rogawski 2000b), in the current study, chronic pregnanolone treatment did not result in cross tolerance to benzodiazepines and produced a modest 1.5-fold shift to the right in the pentobarbital dose-effect curve. One possible explanation for differential development of cross tolerance is that the neuroadaptations that occur at GABA$_A$ receptors during chronic treatment differ depending on the site of action of the treatment drug. For example, in vitro studies have shown that subunit composition of GABA$_A$ receptors is an important determinant of the affinity of positive modulators (Baburin et al., 2008; Benke et al., 1996; Kohm et al., 2006). Chronic benzodiazepine treatment results in decreased benzodiazepine binding (Wu et al., 1994a) likely due to changes in the relative numbers of α1 and γ2 subunits (Biggio et al., 2003; Primus and Gallager, 1992; Wu et al., 1994b); in contrast, chronic neuroactive steroid treatment alters expression of α4 mRNA (Smith et al., 2007). Thus, the neuroadaptations of GABA$_A$ receptors during chronic benzodiazepine or neuroactive steroid treatment seem to differ. Changes in subunit composition during chronic pregnanolone treatment might also have differentially affected the potencies of the two benzodiazepines in the current study. Whatever the reason for the lack of cross tolerance and the differential change in potencies of flunitrazepam and midazolam, differences in the development of cross tolerance between benzodiazepines and neuroactive steroids suggests that the mechanism by which tolerance develops varies across positive GABA$_A$ modulators.

When chronic benzodiazepine treatment is abruptly terminated, behavioral signs of withdrawal such as increased anxiety, insomnia, muscle tremors and seizures are readily

apparent (Ator and Griffiths, 2003; Woods et al., 1992). Discontinuation of chronic benzodiazepine treatment can also disrupt operant responding for food (Ator et al., 2000) and is considered a sensitive measure of withdrawal, often noted in the absence of other physiological withdrawal signs (Emmett-Oglesby et al., 1990). Given the similarities between benzodiazepines and neuroactive steroids administered acutely, including the ability of neuroactive steroids to reverse withdrawal in diazepam-dependent monkeys (McMahon et al., 2007), it was expected that large doses of pregnanolone given repeatedly would result in the development of dependence and that withdrawal signs following discontinuation of pregnanolone treatment would be similar to those that emerge following termination of chronic benzodiazepine treatment. Despite large doses given repeatedly, pregnanolone treatment failed to result in a disruption of food-maintained responding or any other overt behavioral signs of withdrawal, suggesting that, at least under these conditions, dependence does not develop to pregnanolone.

During pregnanolone treatment, sensitivity to ketamine decreased. There is evidence that NMDA receptors have distinct modulatory sites for neuroactive steroids (Park-Chung et al., 1997), and these sites could play an important role in the changes in sensitivity to ketamine that were observed during chronic pregnanolone treatment. In addition, the decreased potency of ketamine during treatment might be due to compensatory changes occurring at NMDA receptors in response to enhanced GABAergic neurotransmission as a result of daily pregnanolone treatment. Compensatory changes in the glutamatergic system are also evident during chronic benzodiazepine treatment (Izzo et al., 2001). Changes in sensitivity to ketamine were temporary; four weeks after termination of daily pregnanolone treatment the potency of ketamine was no different from before treatment. The apparent cross tolerance to ketamine in addition to tolerance to the rate-increasing effects of pregnanolone provide evidence that complex interactions occur between the GABAergic and glutamatergic neurotransmitter systems during chronic pregnanolone treatment.

After chronic treatment, sensitivity to pregnanolone and midazolam was modestly increased suggesting that $GABA_A$ receptors were still changed long after daily pregnanolone treatment was terminated. It is known that changes in subunit composition of $GABA_A$ receptors can affect the functional properties of the receptor (Baburin et al., 2008) and that drug treatment can alter subunit expression (Yu et al., 1996) and coupling efficiency of the various binding sites on $GABA_A$ receptors (Yu and Ticku, 1995), although it is not known which of the changes in $GABA_A$ receptors account for the development of tolerance to the rate-increasing effects of pregnanolone or which changes persist after termination of treatment. Importantly, the potency of morphine, a drug that does not have actions at $GABA_A$ receptors, was unchanged by chronic pregnanolone treatment further supporting the notion of selective changes at $GABA_A$ receptors.

In summary, daily treatment with pregnanolone resulted in selective tolerance to the rate-increasing but not rate-decreasing effects of pregnanolone. Chronic pregnanolone treatment did not result in cross tolerance to the benzodiazepines flunitrazepam or midazolam, although cross tolerance to ketamine developed, and temporary suspension of treatment failed to result in any behavioral signs of withdrawal. Despite having similar modulatory actions at $GABA_A$ receptors, benzodiazepines and neuroactive steroids appear to differ in the development of tolerance and dependence, suggesting that neuroactive steroids may be an effective therapeutic target for the treatment of insomnia and seizure disorders while minimizing the unwanted effects that occur with repeated benzodiazepine use.

Acknowledgments

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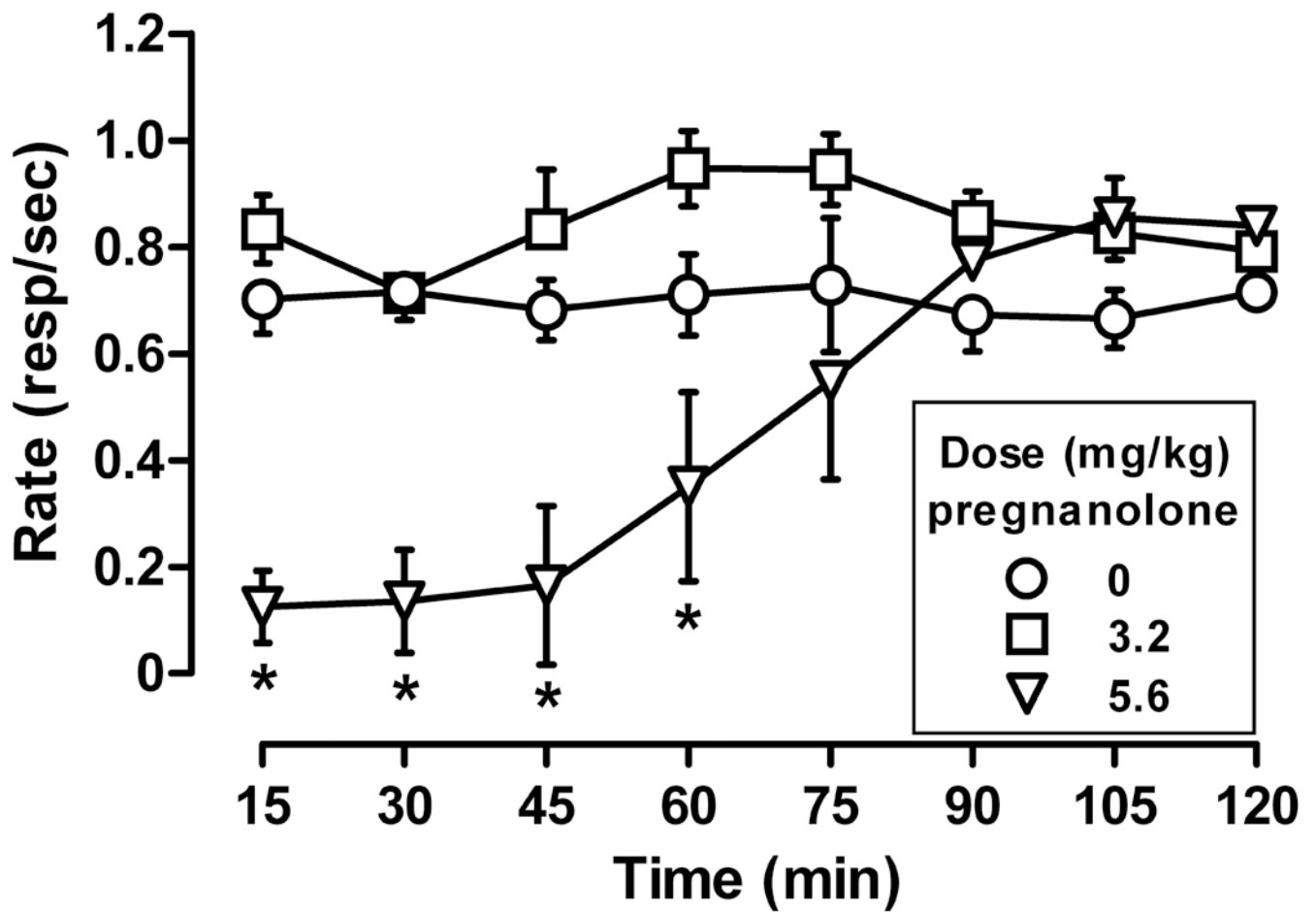
Figure 1.
Time-course for the rate-decreasing effects of pregnanolone. A bolus dose of 5.6 mg/kg of pregnanolone significantly decreases responding for 60 minutes. Abscissa: time since pregnanolone injection in minutes. Ordinate: mean response rates (±SEM) expressed as number of responses per second. * Response rates significantly different from vehicle (open circles; $p < 0.05$).
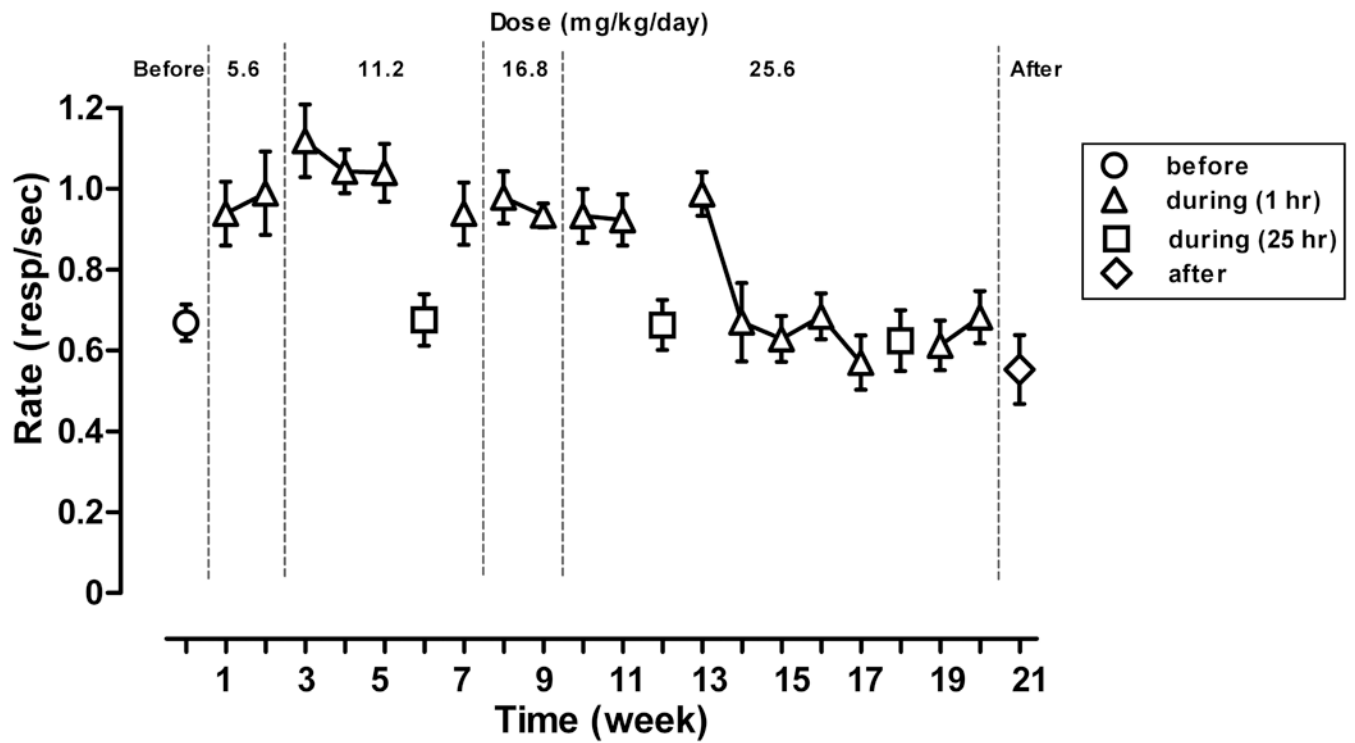
Figure 2.
Response rates on the first cycle of weekly test sessions before (circles), during (triangles: pregnanolone last given 1 hr prior to session; squares: pregnanolone last given 25 hr prior to session), and after (diamonds) chronic pregnanolone treatment. Data points represent rates obtained on the first cycle of a single test session. Abscissa: week of chronic treatment. Ordinate: response rates (±SEM) expressed as number of responses per second.
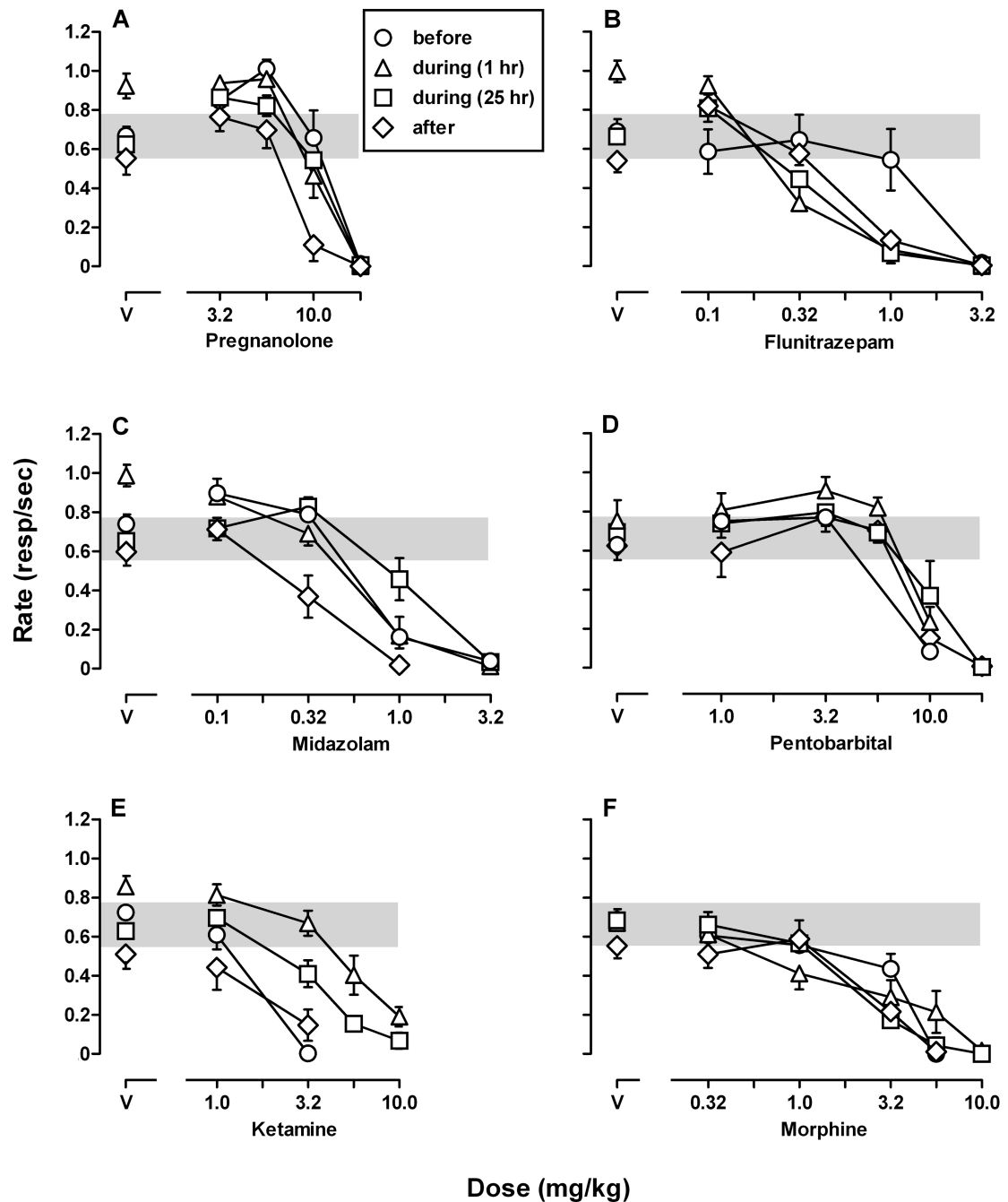
Figure 3.

Cumulative dose-effect curves for pregnanolone (A), flunitrazepam (B), midazolam (C), pentobarbital (D), ketamine (E), and morphine (F) before (circles), during (triangles: pregnanolone last administered 1 hour prior to session; squares: pregnanolone last administered 25 hours prior to session), and after (diamonds) chronic pregnanolone treatment. During chronic treatment, dose-effect curves obtained when pregnanolone was last given 1 hr prior to the session (triangles) were determined during week 11 for pregnanolone (A) and flunitrazepam (B) and during week 12 for midazolam (C) and ketamine (E), before tolerance developed to the rate-increasing effects of pregnanolone (points above V); dose-effect curves for pentobarbital (D) and morphine (F) were

determined during week 16 of treatment after tolerance developed to the rate-increasing effects of pregnanolone. Abscissae: cumulative dose in mg/kg body weight. Ordinates: response rates expressed as number of responses per second. Gray shading indicates the 95% confidence intervals of control response rates prior to chronic treatment. See Table 2 for significant potency ratios.

Table 1

Outline of experimental design detailing tests conducted before, during and after chronic pregnanolone treatment.

	Treatment dose (mg/kg/day)	Week of treatment	Test
Before	0	--	Pregnanolone time course
	0	--	Dose-effect curves for all drugs
During	5.6	1, 2	Pregnanolone and flunitrazepam dose-effect curves[a]
	11.2	3-6	Pregnanolone and flunitrazepam dose-effect curves[a]
	11.2	7	Ketamine dose-effect curves
	16.8	8, 9	Pregnanolone and flunitrazepam dose-effect curves[a]
	25.6	10, 11	Pregnanolone and flunitrazepam dose-effect curves[a]
	25.6	12-20	Dose-effect curves for all drugs
After	0	2 weeks after	Pregnanolone and flunitrazepam dose-effect curves[a]
	0	4 weeks after	Dose-effect curves for all drugs

[a]Pregnanolone dose-effect curves were determined on the fourth day of each week and flunitrazepam dose-effect curves were determined on the seventh day of each week.

Table 2

Mean ED_{50} values and potency ratios with 95% confidence intervals (CI) for the rate-decreasing effects of pregnanolone, flunitrazepam, midazolam, morphine, ketamine, and pentobarbital before, during, and after daily pregnanolone treatment. Dose-effect curves were determined 1 and 25 hours after the previous pregnanolone dose.

	ED_{50} (mg/kg)		Potency Ratio[a]	
	Mean	95% CI	Mean	95% CI
Pregnanolone				
Before	12.95	10.68-15.21		
During				
1 hour	12.01	7.68-16.35	0.97	0.56-1.38
25 hours	11.85	8.73-14.98	0.94	0.67-1.20
After	7.94	5.49-10.39	0.63*	0.43-0.82
Flunitrazepam				
Before	1.72	1.03-2.40		
During				
1 hour	0.58	0.08-1.08	0.49*	0.17-0.82
25 hours	0.55	0.26-0.84	0.33*	0.19-0.47
After	1.63	0.27-3.00	0.92	0.43-1.40
Midazolam				
Before	0.85	0.44-1.26		
During				
1 hour	0.85	0.48-1.22	1.23	0.58-1.88
25 hours	1.45	0.86-2.04	1.96*	1.09-2.82
After	0.37	0.20-0.56	0.55*	0.22-0.88
Pentobarbital				
Before	6.73	6.05-7.41		
During				
1 hour	9.92	7.53-12.31	1.48*	1.14-1.83
25 hours	9.48	5.84-13.11	1.42	0.84-2.00
After	8.79	6.7-10.89	1.32	1.00-1.64
Ketamine				
Before	1.62	1.27-1.97		
During				
1 hour	7.43	4.65-10.22	4.90*	2.43-7.37
25 hours	4.34	2.69-5.99	2.88*	1.48-4.28
After	1.67	1.24-2.11	1.10	0.70-1.50
Morphine				
Before	3.05	2.04-4.06		
During				
1 hour	3.11	0.50-5.72	1.24	-0.05-2.53

	ED$_{50}$ (mg/kg)		Potency Ratio[a]	
	Mean	95% CI	Mean	95% CI
25 hours	2.16	1.50-2.83	0.85	0.39-1.30
After	2.04	1.36-2.72	0.79	0.31-1.28

[a]Potency ratio relative to ED$_{50}$ values before treatment.

*Indicate significant differences from before treatment.