Chronic Treatment with the Neuroactive Steroid Ganaxolone in the Rat Induces Anticonvulsant Tolerance to Diazepam but Not to Itself

DOODIPALA S. REDDY and MICHAEL A. ROGAWSKI

Neuronal Excitability Section, Epilepsy Research Branch, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, Maryland

Accepted for publication August 15, 2000 This paper is available online at http://www.jpet.org

ABSTRACT

Ganaxolone (3α-hydroxy-3β-methyl-5α-pregnan-20-one), an orally active synthetic analog of the neuroactive steroid allopregnanolone, is a positive allosteric modulator of GABAA receptors with anticonvulsant properties. We sought to determine whether tolerance occurs to the anticonvulsant activity of ganaxolone in the pentylentetrazol seizure test and whether there is cross-tolerance with diazepam. Rats were treated with two daily injections of a 2 × ED50 dose of ganaxolone for 3- and 7-day treatments. The estimated equilibrium plasma concentrations of ganaxolone were unchanged after 7-day chronic ganaxolone treatment. The estimated equilibrium plasma concentrations of ganaxolone associated with threshold (750–950 ng/ml) and 50% seizure protection (1215–1295 ng/ml) were similar in naive and chronically treated rats. We conclude that there is no tolerance to the anticonvulsant activity of ganaxolone nor is there cross-tolerance to ganaxolone when tolerance develops to diazepam. However, there is cross-tolerance to diazepam with chronic ganaxolone treatment.

Ganaxolone (CCD 1042; 3α-hydroxy-3β-methyl-5α-pregnan-20-one), the synthetic 3β-methyl analog of the natural neurosteroid allopregnanolone, is a potent positive allosteric modulator of GABA_A receptors (Carter et al., 1997) and is an effective anticonvulsant in the pentylentetrazol (PTZ) seizure test as well as in other animal models used in evaluation of antiepileptic drugs (Carter et al., 1997; Gasior et al., 1997, 2000). Unlike allopregnanolone, which has a short duration of action, ganaxolone is orally active and adequate blood levels can be maintained in human subjects with two or three times daily dosing (Monaghan et al., 1997, 1999). In addition, although ganaxolone is extensively metabolized, the potentially hormonally active 3-keto derivative is not formed. Consequently, it has been suggested that ganaxolone could be of value in epilepsy therapy (Monaghan et al., 1997). However, for ganaxolone to be useful clinically, its anticonvulsant activity must be maintained with chronic dosing. We previously demonstrated that anticonvulsant tolerance does not develop to the GABA_A receptor modulating neuroactive steroid pregnanolone with intermittent chronic dosing (Kokate et al., 1998). However, because of its longer duration of action, ganaxolone might have greater liability for tolerance. Therefore, in the present study we examined whether tolerance develops to the protective activity of ganaxolone against PTZ seizures. For comparison, we carried out a parallel experiment with diazepam that is also effective acutely in the PTZ seizure test and is well known to be susceptible to tolerance (Haigh and Feely, 1988), and we also investigated the possibility of cross-tolerance. Unexpectedly, we observed that chronic treatment with ganaxolone induced dramatic cross-tolerance to diazepam without itself exhibiting tolerance.

Materials and Methods

Animals. Normally cycling female 45- to 55-day-old (200–250 g) Sprague-Dawley rats (Taconic Farms, Germantown, NY) were housed in groups of four under a 12/12-h light/dark cycle in an environmentally controlled animal facility. Rats were allowed to acclimatize with free access to food and water for a 24-h period before use. Chronic treatments and testing were performed at random times during the estrous cycle to minimize the effects of any cyclical

Received for publication June 13, 2000.

ABBREVIATIONS: GABA, γ-aminobutyric acid; PTZ, pentylentetrazol; AUC, area under the curve.
changes in endogenous neurosteroids (Finn and Gee, 1993; Palumbo et al., 1995). All procedures were performed in strict compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals under a protocol approved by the National Institutes of Health Animal Use Committee.

**PTZ Seizure Test.** Ganaxolone and diazepam were evaluated for protective activity against PTZ-induced clonic seizures according to the procedure of White et al. (1995). Rats were injected with the test drug and 15 min later (or at the specified intervals in the time course studies) received a s.c. injection of PTZ (90 mg/kg). Animals were observed for a 30-min period. Rats failing to show clonic spasms lasting longer than 5 s were scored as protected.

**Rotarod Motor Toxicity Test.** Ganaxolone and diazepam were evaluated for motor toxicity in an accelerating Rotarod test (Jones and Roberts, 1968). Rats were acclimatized to the Rotarod (Ugo Basile, Milan, Italy) for 2 min at 5 rpm, 30 min before the start of the experiment. Rats that successfully remained on the Rotarod for more than 2 min were selected for drug testing (initial speed 5 rpm, increasing 5 rpm/30 s). After administration of the test drug, rats were given three successive opportunities to remain on the Rotarod continuously for 2 min. An animal was scored as toxic if it fell from the Rotarod three or more times in the 2-min period.

**Chronic Ganaxolone Treatment Protocols.** Ganaxolone was administered subcutaneously twice daily (at 10:00 AM and 5:00 PM) for 3 or 7 consecutive days at a dose of 7 mg/kg (approximately twice the ED50 value determined in a dose-response study in naive animals; Fig. 1). Mild to moderate sedation and ataxia were observed after each injection of ganaxolone. Treated animals gained weight at the same rate as control animals. On the morning after the 3- or 7-day chronic treatment period (at 10:00 AM), each rat received an injection of ganaxolone (0.6–15 mg/kg s.c.) or diazepam (0.5–7.5 mg/kg i.p.), and 15 min (ganaxolone) or 30 min (diazepam) later (or at the indicated intervals in the time course studies) received an injection of PTZ or were examined in the Rotarod motor toxicity test. Separate groups of animals were used for the seizure and Rotarod tests.

**Chronic Diazepam Treatment Protocol.** Diazepam was administered intraperitoneally twice daily (at 10:00 AM and 5:00 PM) for 7 consecutive days at a dose of 4 mg/kg (approximately twice the ED50 value determined in a dose-response study; Fig. 5). Moderate sedation and ataxia were observed after each injection of diazepam. Diazepam-treated animals gained weight slightly more slowly than controls or the chronic ganaxolone-treated animals. At the end of the 7-day treatment period, these animals weighed ~5% less than controls. On the morning after the chronic treatment period, each rat received an injection of ganaxolone (0.6–15 mg/kg s.c.) or diazepam (0.5–7.5 mg/kg i.p.), and 15 min (ganaxolone) or 30 min (diazepam) later (or at the indicated intervals in the time course studies) received an injection of PTZ or were examined in the Rotarod motor toxicity test.

**Ganaxolone Plasma Level Determinations.** Animals were anesthetized with CO2 gas and ~2 ml of carotid blood was collected in heparinized tubes. The plasma was separated by centrifugation at 12,000g for 10 min and stored at ~20°C in 10-ml glass tubes containing 7.5% EDTA solution (68 μl). The concentration of ganaxolone was analyzed by liquid chromatography-mass spectroscopy using a Hewlett-Packard liquid chromatograph (analytical column Genesis C18, 4 μm, 3 × 30 mm; Jones Chromatography, Lakewood, CO) and a Micromass Quattro II mass spectrometer. Briefly, a 0.2-ml plasma sample was added to a tube containing evaporated internal standard (epiallopregnanolone). The steroid and internal standard were extracted with 4 ml of hexane. Each sample was analyzed using the Apel ionization technique under acidic conditions. A standard curve was plotted using pure ganaxolone in methanol mixed with 0.2 ml of blank rat plasma.

**Drugs.** Ganaxolone was dissolved in aqueous 45% hydroxypropyl-β-cyclodextrin (β-cyclodextrin; Research Biochemicals International, Natick, MA) to prepare a stock solution that was stored in the cold.

**Data Analysis.** To construct dose-effect curves, each drug was tested at several doses spanning the dose producing 50% seizure protection (ED50) or motor toxicity (TD50). Each group consisted of six to eight rats. ED50 and TD50 values with 95% confidence limits were determined by log-probit analysis using the Litchfield and Wilcoxon procedure (PHARM/PCS, version 4.2; Microcomputer Specialists, Philadelphia, PA). Dose-response data were fit to the logistic function 100/[1 + (D50/x)m] where x is the dose administered, D50 is either the ED50 or TD50, and m is an empirical parameter describing the steepness of fit. When appropriate, the m values were determined simultaneously using ALLFIT 2.7 (DeLean et al., 1978). The significance of differences between the dose-response curves was determined using the Litchfield-Wilcoxon F test.

A two-compartment model was used to describe the plasma concentration-time profile of ganaxolone after subcutaneous administration and to obtain estimates of the kinetic values. In a two-compartment model, plasma ganaxolone concentration C0 is given as follows:

\[
C_p = \frac{[FDK/V_d(K_s - K_p)]}{K_s - K_p} \left( e^{-K_p t} - e^{-K_d t} \right)
\]

(1)

where \(K_s\) is the first-order absorption rate constant, \(K_p\) is the first-order elimination rate constant, \(V_d\) is the apparent volume of distribution, and \(FD\) is the amount of drug absorbed where \(F\) is the fraction absorbed and \(D\) is the dose administered. At the end of the absorption phase (when \(e^{-K_p t}\) approaches zero), the plasma concentration-time curve simplifies to the following equation:

\[
C_p' = \frac{[FDK/V_d(K_s - K_p)]}{K_s - K_p} e^{-K_d t}
\]

(2)

Further dilutions were made immediately before use in 15% β-cyclodextrin. Diazepam (Elkins-Sinn, Cherry Hill, NJ) was dissolved in sterile isotonic saline. The diazepam solution contained a maximum of 20% propylene glycol and 5% ethanol. By itself, β-cyclodextrin at concentrations as high as 45% failed to affect PTZ seizures. Drug solutions were administered s.c. or i.p. in a volume equaling 1% of the animal’s body weight. Ganaxolone was a gift of CoCensys (Irvine, CA). Epiallopregnanolone (3β-hydroxy-5α-pregnan-20-one) was from Steraloids (Newport, RI).
where \( C_p \) (dotted curves in Fig. 5, A and B) indicates the convolution of the plasma concentration, \( K_a \), and \( [\text{FDK}(V_d (K_{e} - K_{s})) \) are determined by fitting the plasma concentration-time curve for times during the falling phase of the curve. \( K_{e} \) is then determined by fitting the difference of eq. 1 and eq. 2 to a plot of \( C_p - C_{s} \) versus time (Tallarida and Murray, 1987). \( V_d/F \) was estimated as \( D/(\text{AUC} \times K_{s}) \), where area under the curve (AUC) was determined using the trapezoidal rule. The elimination half-life \( t_{1/2} \) was calculated as \( 0.7/K_{e} \).

Numerical values are expressed as the mean ± S.E.M. Statistical differences among mean plasma ganaxolone levels were analyzed by one-way analysis of variance followed by Student’s t test. In all tests, the criterion for statistical significance was \( P < .05 \).

**Results**

**Anticonvulsant Activity and Motor Toxicity of Ganaxolone.** In naive rats, subcutaneous injection of ganaxolone (0.6–15 mg/kg) protected against PTZ-induced seizures in a dose-dependent manner (Fig. 1). Ganaxolone also produced an impairment in motor function as assessed with the accelerating Rotarod test. The dose-response curve for motor toxicity was shifted in a parallel manner to the right from that for seizure protection. The ED\(_{50}\) and TD\(_{50}\) values for seizure protection and motor impairment are given in Table 1.

**Three-Day Chronic Ganaxolone Study.** Two chronic treatment studies were carried out. In both studies, ganaxolone was administered subcutaneously twice daily at a dose of 7 mg/kg (twice the ED\(_{50}\) in naive animals). In the first chronic study, the treatment protocol was 3 days. The dose-response relationships for protection in the PTZ test and for motor toxicity determined on the day after the chronic treatment protocol are shown in Fig. 1. Ganaxolone produced comparable dose-dependent protection in the PTZ seizure test in naive animals as it did in those that had received ganaxolone for 3 days. Although the curve for ganaxolone-treated rats is shifted slightly toward left of the control curve, there was no significant difference in the ED\(_{50}\) values for seizure protection in the two groups (Table 1). Similarly the TD\(_{50}\) values for ataxia in the Rotarod test were not significantly different in the naive and chronic treatment groups.

**Seven-Day Chronic Ganaxolone Study.** In the second chronic study, ganaxolone (7 mg/kg s.c.) or its vehicle (45% β-cyclodextrin) were administered twice daily for 7 days. As shown in Fig. 2, the dose-response curves for protection from PTZ-induced seizures on the day after the chronic treatment period were similar in the animals treated with vehicle and ganaxolone. As in the 3-day study, there was a slight leftward shift of the curve in the chronic ganaxolone group, but the ED\(_{50}\) values were not significantly different from control (Table 1). In contrast, there was a slight rightward shift in the dose-response curves for motor impairment in the chronic ganaxolone group (Fig. 2), but the TD\(_{50}\) values were not significantly different (Table 1). In addition, there was a close correspondence between the dose-response relationships in the vehicle control and naive animals (Fig. 1), indicating that repeated handling associated with multiple daily injections does not alter the anticonvulsant activity or motor toxicity of ganaxolone.

**Ganaxolone Plasma Levels: Relationship to Seizure Protection.** Plasma levels of ganaxolone were determined 15 min after injection with various doses of ganaxolone (1.25–10 mg/kg) in naive and 7-day chronically ganaxolone-treated rats. As shown in Fig. 3, ganaxolone plasma levels increased in a dose-dependent manner with increasing ganaxolone dose. There were no significant differences in the plasma levels achieved with corresponding doses of ganaxolone in animals from the naive and chronic treatment groups.

In a different series of experiments, we determined the time course of seizure protection and the corresponding ganaxolone plasma levels after a 7-mg/kg s.c. dose of ganaxolone in naive and 7-day chronically ganaxolone-treated animals. In both groups, protection was maximal at the first time point tested (15 min) and diminished during the 180-min period after the injection (Fig. 4, A and B). The estimated times associated with 50% seizure protection in the naive and chronic animals were 80 and 90 min, respectively. By 150 and 120 min in the naive and chronic groups, respectively, no seizure protection was evident. In contrast to the monotonic fall in the degree of seizure protection, ganaxolone plasma levels rose during the initial 30 to 60 min after injection and

---

**TABLE 1**

Anticonvulsant activity and motor toxicity of a challenge dose of ganaxolone or diazepam in naive and chronically ganaxolone- or diazepam-treated rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Duration</th>
<th>Challenge Drug</th>
<th>PTZ Test (ED(_{50}))</th>
<th>Rotarod Test (TD(_{50}))</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>days</strong></td>
<td></td>
<td>mg/kg</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Naive</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>Ganaxolone</td>
<td>3.5 (2.1–5.8)</td>
<td>5.6 (3.5–8.9)</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>Diazepam</td>
<td>3.8 (2.4–6.1)</td>
<td>9.8 (7.1–13.7)</td>
</tr>
<tr>
<td><strong>Ganaxolone</strong></td>
<td>7</td>
<td>Ganaxolone</td>
<td>2.8 (1.5–5.0)</td>
<td>5.2 (3.2–8.3)</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>Diazepam</td>
<td>2.6 (1.6–4.5)</td>
<td>8.7 (6.6–11.6)</td>
</tr>
<tr>
<td><strong>Ganaxolone</strong></td>
<td>7</td>
<td>Ganaxolone</td>
<td>4.0* (3.0–4.6)</td>
<td>N.D.</td>
</tr>
<tr>
<td><strong>Naive</strong></td>
<td></td>
<td>Diazepam</td>
<td>1.9 (1.4–2.4)</td>
<td>1.7 (1.2–2.4)</td>
</tr>
<tr>
<td><strong>Diazepam</strong></td>
<td>7</td>
<td>Diazepam</td>
<td>3.7* (3.0–4.6)</td>
<td>N.D.</td>
</tr>
<tr>
<td><strong>Diazepam</strong></td>
<td>7</td>
<td>Ganaxolone</td>
<td>2.9 (1.9–4.5)</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

N.D., not determined.

* \( P < .05 \) vs. naive diazepam-challenged control group (Litchfield and Wilcoxon \( y^2 \) test).
then declined with a time course that followed the fall in seizure protection. Plasma ganaxolone concentrations associated with threshold (10%) and 50% seizure protection were determined using the time course data after steady-state equilibrium had been achieved (falling phase of plasma concentration curves). In the naive and chronically treated rats, the threshold protection levels were 750 and 950 ng/ml, respectively, and the 50% seizure levels were 1215 and 1265 ng/ml, indicating that there is no marked change in ganaxolone sensitivity in the chronically treated animals.

The pharmacokinetic values in naive and chronically ganaxolone-treated animals are shown in Table 2. Although there was a marginal increase in $t_{1/2}$ in the chronic group, none of the pharmacokinetic measures in the chronic group were significantly different from the naive group.

**Seven-Day Chronic Diazepam Study.** To assess whether there is cross-tolerance to ganaxolone in chronically diazepam-treated rats, diazepam (4 mg/kg i.p.) was administered twice daily for 7 days and the animals were challenged on the day after the chronic treatment period with ganaxolone or, for comparison, diazepam. As shown in Fig. 5A, the dose-response curve for diazepam seizure protection in the chronically diazepam-treated rats was significantly shifted toward the right in a parallel manner from that of a naive group, indicating the development of tolerance. The tolerance is reflected in a significantly greater anticonvulsant ED$_{50}$ value for diazepam in the chronically treated animals than in the naive animals (Table 1). In contrast, there was no significant shift in the dose-response curves for ganaxolone in naive and chronically diazepam-treated animals (Fig. 5B), indicating that there is no cross-tolerance to ganaxolone in animals tolerant to diazepam.

**Tolerance to Diazepam after Chronic Ganaxolone Treatment.** To determine whether there is cross-tolerance to diazepam in chronically ganaxolone-treated animals, rats received twice daily injections of ganaxolone for 7 days and were challenged on the day after the chronic treatment protocol with diazepam. There was a significant parallel rightward shift in the diazepam dose-response curve comparable to that observed with chronic diazepam treatment (Fig. 5A), indicating that there is cross-tolerance to diazepam with chronic ganaxolone treatment. In the chronic ganaxolone-treated animals, the diazepam ED$_{50}$ for seizure protection was more than twice the value in control animals (Table 1).

**Discussion**

Repeated treatment with ganaxolone at twice its ED$_{50}$ dose for protection against PTZ seizures was not associated with tolerance to the anticonvulsant activity of the steroid for as long as 7 days of chronic treatment. In addition, tolerance did not develop to the motor toxicity that occurs with higher doses of ganaxolone. In contrast, a similar regimen of chronic...
diazepam treatment resulted in a near doubling of the ED_{50} value for seizure protection, reflecting the well recognized tolerance liability of benzodiazepines. Interestingly, chronic ganaxolone induced cross-tolerance to the anticonvulsant activity of diazepam that was comparable in magnitude to that produced by chronic diazepam itself. This confirms the adequacy of the dosing regimen for ganaxolone, and suggests that neurosteroids have a lower propensity for tolerance than do benzodiazepines.

Determinations of plasma ganaxolone concentrations demonstrated that chronic treatment with ganaxolone does not lead to persistent changes in the pharmacokinetic properties of the steroid. At early times after ganaxolone dosing (<30–60 min), there was a dissociation between the extent of seizure protection and plasma ganaxolone levels. However, at later times, the time course of seizure protection was well correlated with the ganaxolone plasma concentration kinetics. The dissociation between the pharmacodynamic effect and serum plasma levels at early times could be due to rapid entry of the steroid into the brain before distribution to less well perfused tissues (Pratt, 1990). The relatively large apparent volume of distribution of the steroid (Table 2), which

### TABLE 2

Pharmacokinetic values for ganaxolone in naive and chronically ganaxolone-treated rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Naive</th>
<th>Chronic</th>
</tr>
</thead>
<tbody>
<tr>
<td>K_{a} (h^{-1})</td>
<td>1.89 ± 0.58</td>
<td>1.38 ± 0.85</td>
</tr>
<tr>
<td>K_{e} (h^{-1})</td>
<td>0.60 ± 0.03</td>
<td>0.40 ± 0.10</td>
</tr>
<tr>
<td>C_{p} (t = 0) (mg/l)</td>
<td>2.23 ± 0.20</td>
<td>2.12 ± 0.23</td>
</tr>
<tr>
<td>C_{max} (mg/l)</td>
<td>1.50 ± 0.00</td>
<td>1.46 ± 0.03</td>
</tr>
<tr>
<td>( t_{\frac{1}{2}} ) (h)</td>
<td>1.3 ± 0.1</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td>( T_{\frac{1}{2}} ) (h)</td>
<td>0.75 ± 0.15</td>
<td>0.75 ± 0.15</td>
</tr>
<tr>
<td>AUC (0–\infty) (mg-h/l)</td>
<td>3.39 ± 0.12</td>
<td>3.52 ± 0.99</td>
</tr>
<tr>
<td>V_{d}/F (l/kg)</td>
<td>3.19 ± 0.51</td>
<td>3.87 ± 0.31</td>
</tr>
</tbody>
</table>

K_{a} and K_{e}, absorption and elimination rate constants; C_{p} (t = 0), convolution of the serum concentration at time 0; C_{max}, maximum serum concentration at time t_{\frac{1}{2}}; V_{d}, apparent volume of distribution; F, fraction absorbed into plasma.

(assuming \( F = 1 \) is ~6- to 7-fold greater than the value expected for uniform distribution in body water (0.6 l/kg), indicates that ganaxolone is more concentrated in the tissues than in the plasma compartment and is consistent with this idea. Alternatively, the dissociation could be due to acute tolerance of the type that is well recognized to occur with benzodiazepines (Ellinwood et al., 1983; Kroboth et al., 1993). In this case, the effect site concentrations early after dosing would have relatively greater anticonvulsant actions than larger concentrations at later times. However, if this type of tolerance occurs, it would necessarily be short-lived because there was no reduction in activity of a ganaxolone challenge dose administered 15 h after the last ganaxolone injection in the chronic treatment protocol. This rapid and brief tolerance would be distinct from the persistent tolerance that the present study was designed to assess. More extensive pharmacokinetic-pharmacodynamic investigations are required to exclude the existence of such a phenomenon.

The close correspondence between the time course of seizure protection and the ganaxolone plasma levels during the falling phase of the ganaxolone plasma concentration time course allows an assessment of the steady-state plasma levels conferring seizure protection. On the basis of the data in the time course studies (Fig. 4), the estimated threshold plasma concentration for seizure protection is in the range of 750 to 950 ng/ml (2.2–2.8 \( \mu \)M) and the estimated plasma concentration producing 50% seizure protection is in the range of 1215 to 1295 ng/ml (3.6–3.9 \( \mu \)M). These concentrations can be compared with the concentrations of ganaxolone that produce 50% and maximal potentiation of recombinant GABA_{A} receptor responses, 0.1 to 0.2 \( \mu \)M, and 3 \( \mu \)M, respectively (Carter et al., 1997). Because equilibrium between the plasma and effect-site compartment is apparently not achieved 15 min after subcutaneous ganaxolone administration, a similar analysis using the dose-response data (Fig. 3) would not be meaningful.

Ganaxolone has a short half-life (\( t_{1/2} \), 1.3–1.9 h) and its

![Fig. 5. A, dose-response curves for anticonvulsant activity of diazepam in naive rats and in rats that had received two daily injection of diazepam (4 mg/kg i.p.) or ganaxolone (7 mg/kg s.c.) for 7 days. On the morning after the chronic treatment period, animals were challenged with diazepam doses of 0.5 to 7.5 mg/kg. B, dose-response curves for anticonvulsant activity of ganaxolone in naive rats and in rats that had received two daily injections of diazepam (4 mg/kg i.p.) for 7 days. On the morning after the chronic treatment period, animals were challenged with ganaxolone doses of 0.6 to 10 mg/kg. In A and B, each point represents data from six to eight rats. The curves indicate logistic fits to the data points; the ED_{50} and TD_{50} values are given in Table 1.](image-url)
plasma levels would be expected to fluctuate substantially during the day even with the twice daily dosing regimen used here. Whether tolerance would develop if plasma concentrations are maintained at a more constant level remains to be determined. However, such constant plasma levels are apparently not required for diazepam cross-tolerance. Moreover, we note that there are only minor differences in the terminal half-lives of ganaxolone (1.3 h; present study) and diazepam (1.5 h; Lösch and Schwark, 1985) when the drugs are administered parenterally. Although diazepam is known to produce several long-lasting metabolites, the only metabolite detected by Lösch and Schwark (1985) was nordiazepam (N-desmethyldiazepam), which was eliminated with a shorter half-life than that of the parent drug. In our chronic treatment protocols, the doses of ganaxolone and diazepam used were an equal multiple (2-fold) of doses that produce equivalent degrees of peak seizure protection. Therefore, the difference between ganaxolone and diazepam in the extent of tolerance development is not likely to be due to dose-related differences in the magnitude of the pharmacodynamic action of the drugs or the duration of effect at the anticonvulsant target site.

Neuroactive steroids exert their anticonvulsant activity and motor toxicity by potentiating GABAA receptor-mediated inhibitory responses in the brain (Kokate et al., 1994). The present study and a previous report from our laboratory (Kokate et al., 1998) indicate that neuroactive steroids maintain their anticonvulsant activity when administered chronically in vivo. Our results are consistent with two recent clinical studies in women with epilepsy (Herzog, 1986, 1995) that demonstrated a lack of diminution in the anticonvulsant activity of chronically administered progesterone, which produces anticonvulsant effects via conversion to the neurosteroid allopregnanolone (Kokate et al., 1999). Similarly, tolerance has not been observed to the anxiolytic and sedative effects of the synthetic neuroactive steroids alphaxalone and 3β-ethenyl-3α-hydroxy-5α-pregn-20-on (Ramsey et al., 1974; Wieland et al., 1997). However, it has been reported that tolerance does occur to the sedative effects of the neuroactive steroid minaxolone (Marshall et al., 1997). Moreover, chronic treatment of brain neurons in vitro has been associated with altered sensitivity of GABAA receptors to modulation by neuroactive steroids (Friedman et al., 1993; Yu and Ticku, 1995a,b; Yu et al., 1996). Therefore, the present study should not be interpreted as indicating that tolerance to neuroactive steroids cannot occur under any circumstance.

The failure of ganaxolone to exhibit tolerance in the present study highlights the differences in tolerance liability between neuroactive steroids and other GABAA receptor positive modulating agents, most notably benzodiazepines. Tolerance typically occurs to the sedative and anticonvulsant effects of benzodiazepines when dosed chronically for 6 days or more (Gonsalves and Gallagher, 1987; Rundfeldt et al., 1995; Lösch and Schwark, 1985; Reddy and Kulkarni, 1997; Haigh and Feely, 1998). This was confirmed in the present study where the anticonvulsant potency of diazepam was reduced by 49% after 7 days of chronic treatment. Plasma concentrations of diazepam do not decrease during prolonged treatment (Lösch and Schwark, 1985). Thus, the tolerance that occurs with chronic benzodiazepine treatment is of the pharmacodynamic type and is likely related to altered sensitivity of GABAA receptors (Guentert, 1984; Gallagher et al., 1991).

In the present study, we observed that chronic treatment with ganaxolone decreases the anticonvulsant potency of diazepam. Whether this cross-tolerance occurs by the same or a different mechanism from that of benzodiazepine tolerance remains to be determined. However, as in benzodiazepine tolerance, the effect is unlikely to be due to pharmacokinetic factors. In general, benzodiazepines exhibit low susceptibility to pharmacokinetic interactions with steroids (Schmidt, 1989; Kirkwood et al., 1991). The main metabolic route for ganaxolone is 16β-hydroxylation by CYP3A4 (R. B. Carter, personal communication). Although diazepam is also a substrate for CYP3A4 (Yang et al., 1998; Kenworthy et al., 1999; Dresser et al., 2000), there is no evidence for microsomal enzyme induction by ganaxolone, and thus it would be unlikely for chronic ganaxolone treatment to affect diazepam metabolism. In any case, C3-hydroxylation of diazepam by CYP3A4 leads to the production of the active metabolite temazepam so that changes in the relative abundance of this (and other active metabolites such as nordiazepam) would not be expected to cause substantial changes in overall effector activity.

Although in vivo cross-tolerance between neuroactive steroids and benzodiazepines has not previously been reported, chronic in vitro exposure to GABAA receptor positive modulating agents, including neuroactive steroids, barbiturates, and ethanol can cause reduced benzodiazepine sensitivity (Buck and Harris, 1990; Roca et al., 1990; Friedman et al., 1996). Moreover, fluctuations in neurosteroid levels during the estrous and menstrual cycles may be associated with alterations in benzodiazepine responsiveness (Bitran and Dowd, 1996; Sundström et al., 1997). In addition, reduced benzodiazepine sensitivity has been associated with withdrawal from chronic neurosteroid exposure. Thus, after neurosteroid withdrawal, GABAA receptor currents have diminished benzodiazepine sensitivity (Costa et al., 1995; Follesa et al., 2000) and benzodiazepines exhibit reduced sedative and anticonvulsant actions (Smith et al., 1998a,b; Reddy and Rogawski, 2000).

The present study does not address the underlying basis for diazepam cross-tolerance in ganaxolone-treated animals. It is interesting to note, however, that there are not major differences in neurosteroid sensitivity among the principal GABAA receptor subtypes (McKernan and Whiting, 1996), although certain less abundant forms may have reduced steroid responsiveness (Puia et al., 1990; Lambert et al., 1999). In particular, there are only modest differences in ganaxolone sensitivity among GABAA receptors composed of various α-subunits (Carter et al., 1997). In contrast, the α- and γ-subunits profoundly influence benzodiazepine sensitivity (Barnard et al., 1998). Therefore, it is possible that the development of tolerance to diazepam and not ganaxolone results from a relative increase in the expression of benzodiazepine-insensitive but neuroactive steroid-sensitive subunits or a decrease in the expression of subunits conferring benzodiazepine sensitivity (Holton et al., 1996; Impagnatiello et al., 1996, 1997). In fact, chronic neurosteroid exposure and withdrawal have been associated with increased expression of the benzodiazepine-insensitive, neurosteroid sensitive α4γ2 subunit (Smith et al., 1998a,b; Follesa et al., 2000) or with a reduction in the γ2-subunit (Concas et al., 1998; Follesa et al., 2000) that confers benzodiazepine sensitivity but is not ab-
solutely required for neurosteroid sensitivity (Puia et al., 1996; Shingai et al., 1991; Maitre and Reynolds, 1998).

In conclusion, our results indicate that tolerance does not develop to the anticonvulsant activity of ganaxolone when dosed repeatedly over the course of up to 1 week. In contrast, there is marked tolerance to diazepam administered according to a similar regimen. Furthermore, chronic treatment with ganaxolone does not lead to significant changes in its pharmacokinetic properties. Neurosteroids may therefore avoid the problem of tolerance that severely limits the usefulness of benzodiazepines in long-term therapy. Interestingly, chronic ganaxolone treatment led to cross-tolerance of diazepam. Apart from demonstrating the adequacy of the ganaxolone chronic treatment regimen, this observation suggests that chronic neurosteroid exposure is associated with changes in GABA<sub>A</sub> receptors that, although not reflected in altered neuroactive steroid sensitivity, could have important functional and pharmacological consequences. Indeed, a variety of clinical conditions have been linked to fluctuations in endogenous progestereone-derived neurosteroids occurring at menarche, during the menstrual cycle, in pregnancy, at menopause, and under stressful circumstances (Herzog, 1999; Monteleone et al., 2000). If persistent neurosteroid exposure leads to reduced benzodiazepine sensitivity, benzo- diazepines may not be optimal therapeutic agents in such clinical situations. Whether neuroactive steroids such as ganaxolone will prove to be superior remains to be determined.

References


Buck RJ and Harris RA (1990) Benzodiazepine agonist and inverse agonist actions on GABA<sub>B</sub> receptor-oplotactivated chloride channels. II. Chronic effects of ethanol. J Pharmacol Exp Ther 258:73–129.


Holt RA, Bateson AN and Martin IL (1996) Chronic treatment with diazepam or abecarnil differentially effects the expression of GABA<sub>A</sub> receptor subunit mRNAs in the rat cortex. Neuropharmacology 35:1457–1463.


Smith SS, Gong QH, Hsu FC, Markowitz RS, ffrench-Mullen JMH and Li X (1998a) GABA A receptor a4-subunit suppression prevents withdrawal properties of an endogenous steroid. *Nature (Lond)* **392**:826–830.


Send reprint requests to: Michael A. Rogawski, M.D., Ph.D., Epilepsy Research Branch, National Institute of Neurological Disorders and Stroke, National Institutes of Health, 10 Center Dr. Room 5N-250 MSC 1408, Bethesda, MD 20892-1408. E-mail: rogawski@nih.gov