Lack of Anticonvulsant Tolerance to the Neuroactive Steroid Pregnanolone in Mice

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ABSTRACT

GABA-potentiating neuroactive steroids such as pregnanolone have potent protective effects in the pentylentetrazol seizure test. We sought to determine if tolerance develops to the anticonvulsant activity of pregnanolone with chronic administration. Mice were treated with two daily injections of a 2 × ED₅₀ dose of pregnanolone (25 mg/kg, i.p.) for 7 days. On the day after the chronic treatment protocol, the dose-response relationship for protection in the pentylentetrazol seizure test was obtained. The ED₅₀ value after the chronic treatment protocol was not significantly different from that in naive mice (12 mg/kg), indicating that tolerance does not develop to the anticonvulsant activity of pregnanolone. In subsequent experiments, we extended the chronic treatment protocol to 14 days with three daily injections of pregnanolone (25 mg/kg, i.p.). Again, no tolerance was observed (ED₅₀, 13 mg/kg). The anticonvulsant activity of pregnanolone was well correlated with plasma levels in both the naive and chronically (14 day) treated mice. The estimated plasma concentrations of pregnanolone representing threshold (10%) protection (125–150 ng/ml) and 50% protection (575–700 ng/ml) were similar in naive and chronically treated animals. In both chronically treated and naive animals, plasma levels of pregnanolone declined rapidly (t½, 16–19 min) and there was a corresponding reduction in the anticonvulsant activity. Our results with pregnanolone suggest that tolerance does not develop to the anticonvulsant activity of neuroactive steroids as it does with other GABA potentiating drugs such as benzodiazepines, supporting the potential clinical utility of neuroactive steroids in chronic seizure therapy.

Neuroactive steroids are endogenous metabolites of certain steroid hormones (and their synthetic analogs) that rapidly alter the excitability of neurons by direct actions on membrane ion channels. Modulatory effects of neuroactive steroids have been most extensively characterized at the GABAₐ receptor Cl⁻ channel complex (Majewska, 1992; Paul and Purdy, 1992; Gee et al., 1995). In particular, the progestosterone metabolites pregnanolone (5β-pregn-3α-ol-20-one) and allopregnanolone (5α-pregn-3α-ol-20-one), produce a potent enhancement of GABAₐ receptor responses in vitro (Majewska et al., 1986; Harrison et al., 1987; Gee et al., 1988; Peters et al., 1988) and have powerful anticonvulsant, anxiolytic and sedative activity when administered in vivo (Belelli et al., 1989; Bitran et al., 1991; Hogsikilde et al., 1988; Wieland et al., 1991).

We have recently reported that several structurally related neuroactive steroids including pregnanolone are effective anticonvulsants in the PTZ seizure test in mice (Kokate et al., 1994). The anticonvulsant activity of these steroids was well correlated with their ability to potentiate GABA-activated Cl⁻ currents in hippocampal neurons. Although it is now well established that neuroactive steroids have powerful anticonvulsant activity upon acute administration, there is no information regarding the anticonvulsant effects of neuroactive steroids when administered chronically. Chronic administration of some anticonvulsant drugs leads to a progressive loss in their ability to protect against seizures, a phenomenon referred to as “tolerance.” For example, tolerance develops to the anticonvulsant activity of benzodiazepines, which also enhance GABAₐ receptor activity, thus limiting their clinical utility (Gonsalves and Gallagher, 1987; Haigh and Feely, 1988; Wildin and Pleuvry, 1992; Rundfeldt et al., 1995). Benzodiazepine tolerance occurs upon repeated drug exposure for periods as short as one to 6 days even when drug levels are subtherapeutic between doses (Gonsalves and Gallagher, 1987; Garratt et al., 1988; Haigh and Feely, 1988; Reddy and Kulkarni, 1997). In our study, we sought to determine if the anticonvulsant activity of neuroactive steroids diminishes when they are administered chronically. Using the PTZ seizure test, we compared the anticonvulsant potency of preg-

ABBREVIATIONS: GABA, γ-aminobutyric acid; PTZ, pentylentetrazol; BSTFA, N,O-bis(trimethylsilyl) trifluoroacetamide; TMCS, trimethylchlorosilane; GC-MS, gas chromatography-mass spectrometry.
Pregnanolone in naive or vehicle-treated mice with that obtained in mice exposed for 7 and 14 days to pregnanolone. We also determined pregnanolone plasma levels to verify that the pharmacokinetic properties of the steroid are not altered in the chronically treated animals.

Methods

Animals. Male NIH Swiss mice (25–30 g) were obtained from the National Institutes of Health (NIH) animal program. Animals were allowed to acclimatize with free access to food and water for a 24-hr period before testing. All procedures were carried out under strict compliance with the NIH Guide for the Care and Use of Laboratory Animals under a protocol approved by the NIH Animal Use Committee.

PTZ seizure test. Pregnanolone was evaluated for protective activity against PTZ-induced clonic seizures according to the procedure described by White et al. (1995). In brief, mice received i.p. injections with pregnanolone and 15 min later (or at the specified intervals in the time course studies) received a subcutaneous injection of PTZ (85 mg/kg). Animals were then observed for a 30-min period. Mice failing to show clonic spasms lasting longer than 5 sec were scored as protected.

Motor toxicity test. Pregnanolone was evaluated for motor toxicity using a modification of the horizontal screen test, which determines an animal’s ability to support its own body weight by grasping a grid (Coughenour et al., 1977). Mice were placed on a horizontally oriented grid (consisting of parallel 1.5-mm diameter rods situated 1 cm apart) and the grid was inverted. Animals that fell from the grid within 5 sec were scored as impaired. Control mice never fell from the grid.

Chronic treatment protocols. Figure 1 illustrates the two chronic treatment protocols and indicates the time of anticonvulsant testing and blood collection for determination of plasma pregnanolone levels. The pregnanolone dose, 25 mg/kg (i.p.), was approximately twice the ED₅₀ value determined in an acute dose-response study (see fig. 2). Mice received either two daily injections (8 A.M. and 4 P.M.) of pregnanolone for 7 days or three daily injections (8 A.M., 2 P.M. and 8 P.M.) of pregnanolone for 14 days. The body weight of the animals was not affected in either the 7- or 14-day chronic treatment protocols. Mild hyperactivity (running and jumping) was often observed during the 5- to 10-min period after pregnanolone injection, followed invariably by ataxia and reduced locomotion. On the morning after the chronic treatment period (when pregnanolone plasma levels in the chronic steroid-treated animals were no different from those in naive animals; see “Results”), the mice received injections with pregnanolone at doses ranging between 3 and 50 mg/kg, and 15 min later (or at the indicated intervals in the time course studies) were examined for motor toxicity and then immediately subjected to the PTZ seizure test. To construct dose-effect curves, pregnanolone was tested at several doses spanning the dose producing 50% protection (ED₅₀) or motor toxicity (TD₅₀). At least eight mice were tested at each dose.

Anticonvulsant activity and motor toxicity of pregnanolone. In naive animals, acute administration of pregnanolone (3–50 mg/kg, i.p.) protected mice against PTZ-induced seizures in a dose-dependent fashion (fig. 2). The steroid also produced a dose-dependent impairment in motor function as assessed with the horizontal screen test. The dose-response curve for toxicity was shifted in a parallel fashion to the right from that for seizure protection. The

Pregnanolone solutions. Pregnanolone stock solution were prepared daily in aqueous 45% hydroxypropyl-γ-cyclodextrin (γ-cyclodextrin; Research Biochemicals, Inc., Natick, MA) and stored in the cold. Further dilutions were made immediately before use in 0.9% saline. Pregnanolone solutions for chronic injection contained 15% γ-cyclodextrin. Control animals received injections of the vehicle. All drug solutions were administered in a volume equaling 1% of the animal’s body weight. Pregnanolone was obtained from Sigma Chemical Co. (St. Louis, MO).

Pregnanolone plasma level determinations. Blood was collected in heparinized tubes by cardiac puncture from animals anesthetized with CO₂ gas. The plasma was separated by centrifugation and stored at −20°C. Thawed samples were ultracentrifuged for 5 min, and 50 μl of the supernatant were placed in a 2-ml volumetric flask to which 5 μl of a 10 mg/ml isopregnanolone (5α-pregnan-3β-ol-20-one) solution was added as internal standard. The mixture was extracted with 350 μl butyl chloride by vigorous shaking for 1 min with a vortex mixer. The solution was then centrifuged, and the upper butyl chloride layer was separated into a 2-ml vial. A total of 100 μl of 1% HCl-MeOH solution was added, and the mixture was evaporated to dryness by a gentle stream of nitrogen gas. The residue was dissolved in 50 μl of BSTFA/TMCS and acetonitrile solution (30 μl BSTFA/TMCS + 20 μl acetonitrile) and the sample was sealed under nitrogen. The sample was then heated at 80°C for 15 min and analyzed by GC-MS using multiple-ion detection in the electron impact mode. The chromatography column was a Rtx-1 (OV-1) cross-bonded 100% dimethylpolysiloxane capillary (30 m × 0.32 mm I.D.). The injector temperature was 270°C. A temperature gradient of 220 to 300°C at 20°C/min was used. The sample eluted at 8 min and the internal standard at 8.5 min. Ions were monitored at m/z 300 and 375 for both compounds.

Data analysis. ED₅₀ and TD₅₀ values and their corresponding 95% confidence limits were determined by log-probit analysis. Pregnanolone dose-response data were fitted to the logistic function 100/[1 + (k/x⁰ᵖ)] where x is the pregnanolone dose, k is the ED₅₀ or TD₅₀ and nₑ is an empirical parameter describing the steepness of fit. The significance of differences between potencies (ED₅₀ or TD₅₀ values) were determined using the χ² test (α = 0.05). The time course data were fit to the single exponential function Cₘₐₓₑ⁻Rx where Cₘₐₓ is the plasma concentration at time zero and K is the elimination rate constant. The elimination half-life t½ was calculated as 0.693/K. Numerical values are expressed as the mean ± S.E.M.

Results

Fig. 1. Chronic treatment protocols indicating schedule of dosing with pregnanolone and day of anticonvulsant testing (PTZ seizure test) and blood collection for pregnanolone plasma level determinations. Numbers above bars indicate days in the 1- and 2-wk protocols.
ED$_{50}$ and TD$_{50}$ values in this acute study for seizure protection and motor toxicity are given in table 1.

**Seven-day chronic study.** In the chronic treatment protocols, we used a pregnanolone dose of 25 mg/kg (approximately twice the ED$_{50}$ in naive animals). In the first chronic study, animals were treated with pregnanolone twice daily for 7 days and the dose-response relationships for protection in the PTZ test and for motor impairment were determined. As shown in figure 2, pregnanolone produced comparable dose-dependent protection in the PTZ seizure test in naive animals as it did in those that had received pregnanolone for 7 days. 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 motor impairment were not significantly different in the naive and chronic treatment groups.

**Pregnanolone plasma levels.** Plasma levels of pregnanolone were determined immediately after the PTZ seizure test (i.e., 45 min after dosing with pregnanolone) in both naive and chronically treated mice. As shown in figure 3, pregnanolone plasma levels increased in a dose-dependent fashion in both groups of mice. There were no significant differences in the plasma levels achieved with corresponding doses of pregnanolone in animals selected from the naive and chronic treatment groups. Pregnanolone plasma levels before the challenge dose of pregnanolone were comparable in the naive and chronically treated mice [1.8 ± 0.6 ng/ml (n = 6) and 1.4 ± 0.1 ng/ml (n = 3), respectively].

**Fourteen-day chronic study.** In the second chronic study, pregnanolone or its vehicle were administered three times daily for 14 days (25 mg/kg, i.p.). As is apparent in figure 4, the dose-response curves for protection from PTZ-induced seizures were similar in the chronic pregnanolone-treated and vehicle control animals (table 1). Moreover, similar dose-response relationships were obtained in the vehicle control animals as in the study with naive animals (fig. 2), indicating that repeated handling associated with multiple daily injections does not alter PTZ sensitivity. Finally, there was also no significant difference in the TD$_{50}$ values for motor toxicity in the two groups of mice (table 1).

Figure 4 also shows the plasma levels obtained at various doses of pregnanolone (taken at times of peak activity, i.e., 15 min after pregnanolone administration) in mice randomly selected from the pregnanolone chronic treatment cohort and a group of naive mice. Plasma levels increased in a monotonic fashion with increasing dose in both groups of mice. The increase in pregnanolone plasma levels was well correlated with its protection against PTZ-induced seizures (fig. 4). The plasma levels estimated by interpolation for 50% PTZ-seizure protection (ED$_{50}$ dose) in the naive and chronically treated mice were 700 and 650 ng/ml, respectively. The estimated plasma levels for 50% motor impairment (TD$_{50}$ dose) were also similar in both groups of mice (1500 and 1450 ng/ml, respectively).

**Time course studies.** In another set of experiments, we investigated the time course of seizure protection following a 25 mg/kg, i.p., dose of pregnanolone in naive and 14-day chronic pregnanolone treated animals. Protection was maximal at 15 min and diminished during the 180-min period after the injection in both the naive and chronically treated

### TABLE 1

Anticonvulsant activity and motor toxicity of pregnanolone in untreated and chronically pregnanolone-treated mice

<table>
<thead>
<tr>
<th>Chronic Treatment</th>
<th>PTZ Test (ED$_{50}$, mg/kg, i.p.)</th>
<th>Motor Toxicity (TD$_{50}$, mg/kg, i.p.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (naive)</td>
<td>12.2 (9.5–15.5)</td>
<td>17.6 (14.5–21.3)</td>
</tr>
<tr>
<td>Pregnanolone (7 days)</td>
<td>12.0 (9.7–14.9)</td>
<td>20.5 (16.6–25.4)</td>
</tr>
<tr>
<td>Vehicle</td>
<td>11.3</td>
<td>19.2</td>
</tr>
<tr>
<td>(14 days)</td>
<td>11.3</td>
<td>19.2</td>
</tr>
<tr>
<td>Pregnanolone (14 days)</td>
<td>13.1 (10.4–16.4)</td>
<td>18.7 (14.0–24.8)</td>
</tr>
</tbody>
</table>

Numbers in parentheses are 95% confidence limits. Values were obtained from log-probit fits to the data presented in figures 2 and 4.
mice (figs. 5 and 6). Seizure protection was reduced to 50% at 60 min in the naive group and at 45 min in the chronic group. There was no apparent anticonvulsant effect at 180 min in both groups of mice. Pregnanolone plasma concentrations also exhibited a correspondingly rapid decline in the naive and chronic treatment groups during the initial (t_{1/2}) period after the injection (t_{1/2} values, 18.9 and 16.3 min, respectively). The C_{max} values were 4305 and 3580 ng/ml, respectively, and the calculated K values 2.22 and 2.58 hr^{-1}. The theoretical threshold levels defined as the concentration producing 10% protection were determined by interpolation of the data in figures 5 and 6. These values were 150 and 125 ng/ml, respectively, in the naive and chronically treated mice. On the basis of the time course data, the estimated plasma concentrations of pregnanolone for 50% seizure protection in the naive and chronic treatment groups was 625 and 575 ng/ml, respectively.

Discussion

In this study, we show for the first time that tolerance does not occur to the anticonvulsant activity of the neuroactive steroid pregnanolone when administered intermittently over many days. Thus, the anticonvulsant efficacy and potency of pregnanolone was not diminished by twice daily administration for 7 days or thrice daily administration for 14 days. The dose chosen in these chronic studies was considerably higher than that required to produce anticonvulsant activity. Therefore the dose can be assumed to intermittently activate brain mechanisms associated with seizure protection and the results demonstrate that such activation according to the schedules in the two protocols does not lead to tolerance. In animals treated according to these protocols, tolerance also did not develop to the motor toxicity that occurs with higher doses of the steroid.

Plasma concentrations of the steroid were determined to assess whether chronic treatment results in a modification in the pharmacokinetic properties of the steroid, for example by changes in its absorption, distribution, metabolism or elimination. Based on the results with the 14-day protocol, it is quite apparent that there are no substantial pharmacokinetic changes affecting the disposition of the steroid. Thus, there was no effect of chronic treatment on the relationship between dose (within the range 10–30 mg/kg) and the plasma
levels achieved at 15 min (fig. 4) or in the time course of the plasma levels following a test dose (figs. 5 and 6).

The measurement of plasma levels also allowed us to estimate the plasma concentrations associated with seizure protection and motor toxicity. The degree of seizure protection was closely correlated with steroid plasma concentration, both in the dose-response (fig. 4) and time course studies (figs. 5 and 6). Taking all the available data together, the threshold plasma concentration for seizure protection was in the range of 125 to 150 ng/ml and the estimated plasma concentration producing 50% seizure protection was in the range of 575 to 700 ng/ml. Because pregnanolone is cleared rapidly (τ½, 16–19 min), steroid plasma levels would be expected to fluctuate during the day even with three times daily dosing. Whether tolerance would develop if blood concentrations are maintained at constant levels cannot be determined by the results of our study.

Neuroactive steroids, as with benzodiazepines, are believed to exert their anticonvulsant activity and motor toxicity by potentiating GABA$_\text{A}$ receptor-mediated inhibitory responses in the central nervous system (Kokate et al., 1994). However, with benzodiazepines, profound tolerance to the anticonvulsant and motor impairing (sedative) effects often occurs upon repeated administration. Full tolerance typically occurs within the first 1 to 3 days (Haigh and Feely, 1988) but may require as long as 6 days or more in some protocols (Gonsalves and Gallagher, 1987; Haigh and Feely, 1988; Reddy and Kulkarni, 1997; Garratt et al., 1988; Rundfeldt et al., 1995; Löschler et al., 1996). Such tolerance has been observed even with rapidly metabolized benzodiazepines, indicating that maintained blood levels are not necessary for tolerance induction (Boisse et al., 1990; Perrault et al., 1992). Tolerance to benzodiazepines is of the pharmacodynamic (functional) type because benzodiazepines do not induce their own metabolism or produce other long-term effects that would alter their pharmacokinetic properties. In contrast to the pharmacokinetic (metabolic) tolerance that occurs with many anticonvulsant medications such as carbamazepine (Levy and Wurden, 1995), the pharmacodynamic tolerance to benzodiazepines is difficult to overcome by raising the dose, so that benzodiazepines often exhibit diminished therapeutic efficacy when administered chronically.

Our results indicate that neuroactive steroids such as pregnanolone may not have the tolerance liability of benzo- diazepines and could therefore be of greater utility in chronic seizure therapy. However, because tolerance also failed to occur to the pregnanolone-induced motor impairment, side effects may continue to be a problem even with prolonged therapy. It will be of interest to determine in clinical studies whether the tendency of steroids to produce such side effects limits their clinical utility. In any case, the relatively short duration of action of naturally occurring steroids such as pregnanolone is not optimal for chronic therapy. Recently, however, a synthetic neuroactive steroid ganaxolone (CCD-1042) has entered clinical trials (Carter et al., 1997). Ganaxolone is structurally related to pregnanolone but has an improved pharmacokinetic profile. Whether ganaxolone, as with pregnanolone, has a low liability for tolerance remains to be seen.

Although our study failed to show tolerance to pregnanolone in vivo, several in vitro studies using neurons in tissue culture have reported tolerance of GABA$_\text{A}$ receptors to neuroactive steroids (Friedman et al., 1993; Yu and Ticku, 1995ab). This in vitro form of tolerance is manifest as “uncoupling” of the steroid and benzodiazepine recognition sites defined as diminished allosteric interaction between the two sites in radioligand binding experiments. However, the relevance of uncoupling to pharmacological tolerance in vivo is not yet established. Recently, Smith et al. (1998) reported that withdrawal from physiological levels of progesterone (a precursor for pregnanolone) was associated with increased susceptibility to benzodiazepine receptor inverse agonist-induced seizures (attributed to enhanced desensitization of GABA$_\text{A}$ receptors) and reduced sensitivity to allopregnanolone modulation of GABA activated Cl$^-$ currents. These effects resulted from enhanced α4 GABA$_\text{A}$ receptor subunit expression. The enhanced α4 subunit expression occurred in a delayed fashion during a 24-hr period after withdrawal of the chronic treatment regimen; α4 levels were unchanged during chronic progesterone treatment. Thus, although withdrawal was associated with enhanced brain excitability and reduced sensitivity of at least some GABA$_\text{A}$ receptors to neuroactive steroids, there was no evidence for the development of tolerance to the anticonvulsant activity of the steroids. Interestingly, tolerance may not occur to the anticonvulsant activity of progesterone in patients treated with the hormone for catamenial epilepsy (Herzog, 1995, 1996). Progesterone is believed to produce its anticonvulsant activity via conversion to the neuroactive steroid metabolites pregnanolone and allopregnanolone (Kokate and Rogawski, 1997). Thus the available limited clinical data appear to be consistent with the results of our study in animals.

In summary, the GABA$_\text{A}$ receptor positive modulator pregnanolone does not appear to produce tolerance as is the case with other GABA$_\text{A}$ receptor positive modulators, most notably benzodiazepines. Indeed, a recent study has suggested that coadministration of neuroactive steroids may have utility in preventing the development of benzodiazepine tolerance (Reddy and Kulkarni, 1997). Additionally, our results indicate that pregnanolone does not induce its own metabolism with chronic treatment. If our results with pregnanolone are generalizable to other neuroactive steroids, this class of drugs may have potential therapeutic use in the chronic treatment of seizure disorders as well as other conditions where GABA$_\text{A}$ receptor positive modulators are useful, such as for sedation, muscle relaxation and in the treatment of anxiety disorders.

References


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