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Development of Tolerance in Mice to the Sedative Effects of the Neuroactive Steroid Minaxolone Following Chronic Exposure

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MARSHALL, F. H., S. C. STRATTON, J. MULLINGS, E. FORD, S. P. WORTON, N. R. OAKLEY AND R. M. HAGAN. *Development of tolerance in mice to the sedative effects of the neuroactive steroid minaxolone following chronic exposure.* PHARMACOL BIOCHEM BEHAV **58**(1) 1–8, 1997.—Minaxolone is a potent ligand for the neurosteroid binding site of the $GABA_A$ receptor. In radioligand binding studies to rat brain membranes, minaxolone caused a 69% increase in [^{3}H]muscimol binding and a 25% increase in [^{3}H]flunitrazepam binding and inhibited the binding of [^{3}H]TBOB with an IC_{50} of 1 μM. In mice, minaxolone (100 mg/kg, orally) had marked sedative effects as indicated by a reduction in locomotor activity. Chronic dosing with minaxolone (100 mg/kg, orally, once daily for 7 days) resulted in a loss of sedative response to an acute dose of the drug, indicating the development of tolerance. Chronic dosing with temazepam (10 mg/kg, orally, once daily for 7 days) resulted in the development of tolerance to an acute dose of temazepam; however, the two drugs did not appear to be cross-tolerant, indicating that they may have a different mechanism of action at the level of the $GABA_A$ receptor. © 1997 Elsevier Science Inc.

Neurosteroid Minaxolone GABA $GABA_A$ receptor Temazepam Tolerance Locomotor activity Mice

THE $GABA_A$ receptor complex is the site of action of a number of hypnotic-anxiolytic drugs, including barbiturates and benzodiazepines. Binding of these drugs to polypeptide subunits of the oligomeric protein results in an allosteric change in the receptor which enhances GABA-mediated chloride ion conductance, thereby augmenting GABA-mediated inhibitory synaptic events. In 1984, Harrison and Simmonds (7) demonstrated that the site of action of the anaesthetic steroids, such as Glaxo's alphaxalone, was the $GABA_A$ receptor. This has led to an interest in the possibility of developing such steroids as novel hypnotic, anxiolytic, and anticonvulsant agents. Steroids which are able to act rapidly to modulate the excitability of neurones have been termed "neurosteroids" in the case of steroids which naturally occur in the nervous system, or "neuroactive steroids" for their synthetic analogues. The barbiturate-like action of neurosteroids initially prompted speculation that they acted at a common binding site; however, both electrophysiologic and receptor binding studies with [^{35}S]*t*-butylbicyclophosphorothionate (TBPS) revealed synergistic but nonadditive effects of barbiturates and neurosteroids (23). In addition, because benzodiazepine antagonists fail to alter the ability of neurosteroids to augment GABA-mediated Cl^- conductance, it can be concluded that neurosteroids act at a site distinct from both barbiturates and benzodiazepines.

A number of highly potent neurosteroids exist, including the A-ring reduced metabolites of progesterone and deoxycorticosterone, 5α-pregnan-3α-*ol*-20-one and 5α-pregnan-3α,21-diol-20-one. The affinity of these compounds for the $GABA_A$ receptor is comparable with the benzodiazepines, and it has been suggested that one or both steroids may act as endogenous anxiolytic-sedative agents under physiologic conditions such as pregnancy (1) and stress (16). In animal models, neu-

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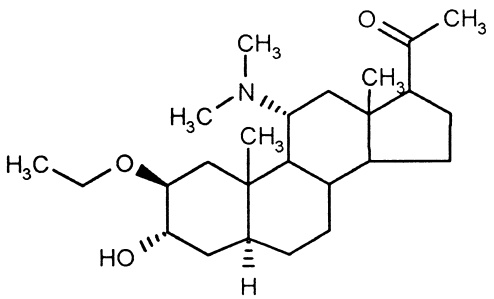

FIG. 1. Chemical structure of minaxolone [(2β,3α,5α,11α)-2-ethoxy-3-hydroxy-11-*N,N*-dimethylamino-pregnane-20-one).

rosteroids have been shown to have a wide spectrum of behavioural actions in addition to their hypnotic and sedative effects; these include anticonvulsant (2), anxiolytic (3), and analgesic effects (10).

Following chronic benzodiazepine administration in both animals and humans, tolerance has been shown to develop to all their pharmacologic effects (5,12). It is now widely accepted that this tolerance appears to be caused by a decrease in the number of benzodiazepine binding sites (14). Binding and functional studies have indicated that chronic benzodiazepine treatment results in a change in the subunit composition of the receptor, particularly those subunits which constitute the benzodiazepine binding site. Chronic benzodiazepine treatment has been shown to decrease levels of mRNA for the α_1 and α_2 subunits in the cerebral cortex of both rats and mice (8,9). Tolerance is also known to develop following chronic barbiturate treatment. Although a large component of this appears to be due to pharmacokinetic parameters, there is some evidence that repeated administration of barbiturates affects receptor subunit mRNA expression in a similar manner to benzodiazepines (22).

In this study we have characterized the action of the neuroactive steroid (2β,3α,5α,11α)-2-ethoxy-3-hydroxy-11-*N,N*-dimethylamino-pregnane-20-one (minaxolone) (Fig. 1) at $GABA_A$ receptors using radioligand binding studies. Given that neurosteroids, like barbiturates and benzodiazepines, act by regulating $GABA_A$ receptor function, we were interested in determining whether chronic treatment with a neuroactive steroid would also lead to the development of tolerance. We used a mouse model of locomotor activity to characterize the sedative effects of minaxolone following acute and chronic administration.

METHOD

Radioligand Binding

Membrane Preparation. For the [^{3}H]muscimol binding assay, forebrains from male Lister Hooded rats (200–250 g) were homogenised in 15 vol. (w/v) of membrane buffer (Tris 50 mM, sucrose 0.32 mM, pH 7.4, 4°C) using a Dounce homogeniser with a Teflon pestle (eight passes at 1000 rpm). The homogenate was centrifuged at 1000 × *g* for 10 min. The supernatant was decanted and centrifuged at 48,000 × *g* for 10 min. The resulting pellet was resuspended, using an Ultra-Turrax homogeniser, in 15 vol. (w/v) of buffer (5 mM Tris, pH 7.4, at 4°C) and allowed to stand for 15 min. The homogenate was then centrifuged for 10 min at 48,000 × *g*. The pellet was then resuspended in membrane buffer and spun again (10 min at 48,000 × *g*). The final pellet was divided into aliquots and frozen at −70°C until required. On the day of the assay aliquots were resuspended in 15 vol. (w/v) of membrane buffer using an Ultra-Turrax homogeniser (8000 rpm) before centrifuging (48,000 × *g* for 10 min). The resulting pellet was again resuspended in membrane buffer and treated as before (48,000 × *g* for 10 min). The pellet was resuspended in 15 vol. of assay buffer (Tris 50 mM, EDTA 5 mM, pH 7.4, at 4°C) and centrifuged (48,000 × *g* for 10 min) twice. Finally the pellet was resuspended in assay buffer (approximately 50 mg/ml wet weight).

For the [^{3}H]flunitrazepam and [^{3}H]TBOB binding assays membranes were prepared according to the methods of Lawrence et al. (11). Briefly, the forebrains of male Lister Hooded rats (200–250 g) were homogenised in ice-cold membrane buffer and centrifuged for 10 min at 1000 × *g*. The supernatant was then spun for 20 min at 9000 × *g*. The supernatant was discarded and the pellet resuspended in assay buffer. This was then centrifuged for 10 min at 14,000 × *g* and the pellet was resuspended in assay buffer.

[^{3}H]Muscimol Binding Assay

Membranes were incubated with [^{3}H]muscimol (20 Ci/mmol; Amersham, Buckinghamshire, UK) (10 nM) in the presence or absence of drugs for 30 min at room temperature. The reaction was terminated by rapid filtration through Whatman GF/B filter paper (Maidstone, Kent, UK) presoaked for 1 h in assay buffer, using a Brandel cell harvester (Gaithersburg, MD) (4 × 1-ml washes with ice-cold assay buffer). The fraction of radioactivity bound was determined using liquid scintillation counting. Nonspecific binding was determined in the presence of GABA (1 mM in the assay), and represented approximately 20% of total binding.

[^{3}H]Flunitrazepam Assay

Membranes were incubated with 1 nM [^{3}H]flunitrazepam (85 Ci/mmol; Amersham), with or without drugs for 60 min at room temperature. The reaction was terminated by rapid filtration through Whatman GF/B filter paper presoaked for 1 h in assay buffer, using a Brandel cell harvester (4 × 1-ml washes with ice-cold assay buffer). The fraction of radioactivity bound was determined using liquid scintillation counting. Nonspecific binding was determined in the presence of diazepam (10 μM), and represented approximately 20% of total binding.

[^{3}H]TBOB Assay

Membranes were incubated with [^{3}H]TBOB (60 Ci/mmol; Amersham) (4–6 nM) in the presence or absence of test drugs for 60 min at room temperature. After this time, the reaction was terminated by rapid filtration through Whatman GF/B filter paper using a Brandel cell harvester (5 × 1-ml washes with ice-cold assay buffer). Bound radioactivity was determined by liquid scintillation counting. Nonspecific binding was assessed by the addition of 1 mM picrotoxinin (0.1 mM in assay), and represented approximately 25% of total binding.

Data Analysis

Receptor binding curves were analysed using ALLFIT. Results are expressed as IC_{50} or EC_{50} values. All binding experiments were performed with triplicate tubes and experiments were repeated three times. Data are means ± SE.

Locomotor Activity Studies

Animals. Male CRH mice (25–28 g) were housed under reverse light (12 L:12 D; lights off at 0900 h) and controlled temperature and humidity conditions (212°C, 45–70% relative humidity) for a minimum of 5 days before the start of each experiment. Therefore, testing took place during their active phase to promote a high baseline of activity. Food and water were available ad lib throughout the housing period.

Apparatus. Locomotor activity (LMA) was monitored in automated activity monitors (AM1051; Benwick Electronics), which recorded animal activity by the interruption of photo beams emitted from the monitor. The photo beams were arranged in two layers: the upper layer recorded rearing activity, and the lower, mobile activity. On the test day, animals were placed into individual Perspex activity monitors (33 × 21 × 19 cm) immediately after being dosed. Tests were performed between 0900 and 1400 h and LMA was recorded for 4 h.

Compounds. Temazepam (Sigma Chemical Co., St. Louis, MO) and minaxolone (Glaxo) were suspended in 5% w/v acacia gum powder in distilled water. Control animals were dosed with the acacia solution. Animals were dosed with a volume of 10 ml/kg in all studies.

Acute Dosing Studies with Temazepam and Minaxolone

Dose–response curves were constructed to temazepam (3, 10, and 30 mg/kg, orally, n = 5–6) and minaxolone (30, 100, and 120 mg/kg, orally, n = 5–14) to establish the onset and duration of sedative effects. Following these experiments, doses for the tolerance studies were selected.

Protocol for Tolerance Studies

Preliminary studies (data not shown) were performed with temazepam to establish optimum study conditions (dosing frequency and duration) for assessing the tolerance liability of minaxolone. The dosing protocol chosen was once-daily treatment (at 0800–0900 h) for 7 days, followed by testing on day 8 immediately after receiving the acute treatment. Studies were composed of three groups of mice (n = 8–10) which received treatments as follows: (a) chronic vehicle, acute vehicle; (b) chronic vehicle, acute drug; (c) chronic drug, acute drug. Temazepam was administered at 10 mg/kg, orally, and minaxolone at 100 mg/kg, orally, throughout these studies.

Cross-Tolerance Studies

Studies were designed to investigate the acute effects of either temazepam or minaxolone following chronic treatment with the other compound. Three groups of mice (n = 7–10) received treatments as follows: (a) chronic vehicle, acute vehicle; (b) chronic vehicle, acute temazepam or chronic vehicle, acute minaxolone; and (c) chronic minaxolone, acute temazepam or chronic temazepam, acute minaxolone. Temazepam was administered at 10 mg/kg, orally, and minaxolone at 100 mg/kg, orally, throughout these studies.

Data Analysis

Data were retrieved as the total number of beam breaks (activity counts) per hour (15 min for dose response studies) for both mobile and rearing behaviour. Means and SE were calculated for each group of mice. Data were submitted to an analysis of variance test, followed by Dunnett's multiple comparison test (for dose–response studies) or Duncan's multiple comparison test (for tolerance studies).

RESULTS

Radioligand Binding

Minaxolone caused a marked dose-dependent potentiation in the binding of [^{3}H]muscimol to rat brain membranes, reaching a maximum of 69 ± 6% (n = 4) over basal (Fig. 2). An EC_{50} of 1.4 ± 0.1 μM (slope = 0.96 ± 0.05) was calculated from the dose–response curves. In comparison, temazepam caused a relatively small increase in [^{3}H]muscimol binding, achieving a potentiation of 24.5 ± 4% at a concentration of 0.1 mM. Minaxolone also potentiated the binding of the benzodiazepine ligand [^{3}H]flunitrazepam, reaching a maximum of 48 ± 2% (n = 3) over basal (Fig. 2). The potency in this assay (0.96 ± 0.1 μM) was similar to that observed in the [^{3}H]muscimol binding assay. Binding of the cage-convulsant [^{3}H]TBOB was completely and dose-dependently inhibited by minaxolone with an IC_{50} of 1.0 ± 0.1 μM (slope = 0.99 ± 0.06, n = 3) (Fig. 2).

Locomotor Activity Studies

Acute dose–response curves. Temazepam (3, 10, and 30 mg/kg, orally) produced a dose-dependent decrease in both rearing (Fig. 3) and mobile activity within the first 30 min of dosing. Maximal effects at all doses were observed within the second time period of 15–30 min. In addition, the duration of action of temazepam demonstrated a dose-dependent relationship. At 30 mg/kg, orally, both rearing and mobile activity were totally attenuated throughout the duration of the experiment. However, recovery of activity was indicated at 210 and 330 min at 3 and 10 mg/kg, orally, respectively. Minaxolone (100 and 120 mg/kg, orally) produced a pronounced decrease in activity within 15 min, which reached a maximum in the 15–30-min period. These effects were short lasting, indicated by the recovery of activity within 60 and 90 min, respectively (Fig. 4).

Tolerance Studies

Significant tolerance to the sedative effects of temazepam developed within 7 days after daily treatment. In animals treated chronically with temazepam, rearing and mobile activity were not reduced by the test dose of temazepam on day 8. In contrast, following chronic vehicle treatment, the test dose of temazepam significantly reduced rearing and mobile activity to 34% [$F(2, 23) = 7.12$, $p < 0.05$] and 48% [$F(2, 23) = 3.83$, $p < 0.05$] of the control responses, respectively, in the first hour (Table 1 and Fig. 5). This profile of action of temazepam was seen throughout the 4-h duration of the experiment (Table 1). Likewise, after chronic minaxolone treatment, the test dose of minaxolone failed to decrease either rearing or mobile behaviour (Table 2 and Fig. 5). As expected,following chronic vehicle treatment, the test dose of minaxolone produced a significant decrease in rearing activity [83%; $F(2, 24) = 5.13$, $p < 0.05$] and mobile activity [73%; $F(2, 24) = 7.81$, $p < 0.05$] compared with control values. The sedative effects of acute minaxolone were only evident in the first hour of the study (Table 2).

Cross-Tolerance Studies

In mice that had received chronic minaxolone treatment, the test dose of temazepam maintained its sedative effect (Table 3 and Fig. 5). The decreases in rearing [63%; $F(2, 27) = 7.3$, $p < 0.01$] and mobile activity [48%; $F(2, 27) = 38.71$, $p < 0.01$] in the first hour were similar to those seen following the

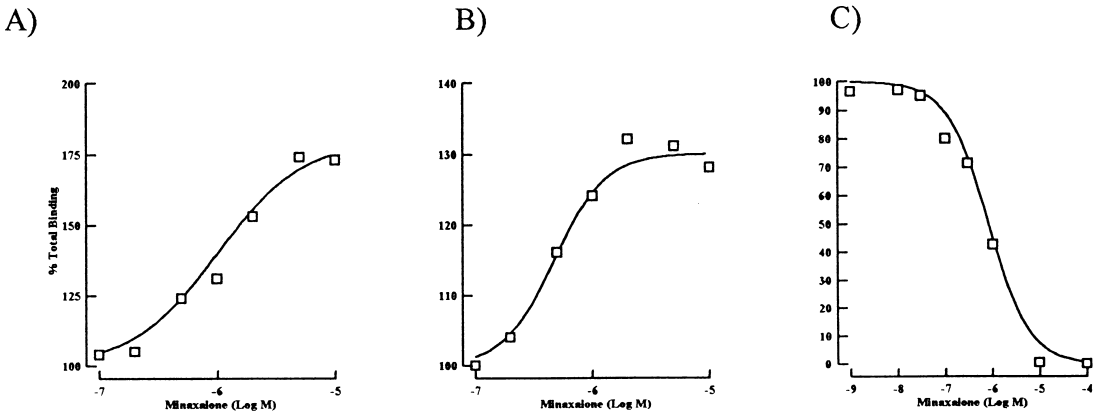

FIG. 2. Effect of minaxolone on the binding of [^{3}H]muscimol (A), [^{3}H]flunitrazepam, (B) and [^{3}H]TBOB (C) to rat brain membranes. Data are taken from single representative experiments.

test dose of temazepam after chronic exposure to vehicle [62%; $F(2, 27) = 7.3$, $p < 0.01$, and 49%; $F(2, 27) = 38.71$, $p < 0.01$ decreases, respectively]. The sedative effect of temazepam was sustained for the remainder of the 4-h experi ment in groups that had been dosed chronically with vehicle or minaxolone (Table 3).

In mice that had received chronic treatment with temazepam, the test dose of minaxolone significantly decreased both rearing and mobile activity in the first hour, by 51% [$F(2, 20) = 19.07$, $p < 0.01$] and 45% [$F(2, 20) = 18.94$, $p < 0.01$], respectively (Table 4 and Fig. 5). The test dose of minaxolone produced a similar degree of sedation in animals that had received chronic vehicle treatment: a 41% [$F(2, 20) = 19.07$, $p < 0.01$] and 34% [$F(2, 20) = 18.94$, $p < 0.01$] decrease in rearing and mobile activity, respectively. As previously observed, the sedative effects of minaxolone were only evident in the initial hour of the study. In the fourth hour, mice that had received minaxolone on the test day were more active than the controls, regardless of their chronic treatment (Table 4). Mobile activity was particularly high in mice that had received chronic vehicle/acute minaxolone compared with the vehicle control group [$F(2, 20) = 10.72$, $p < 0.01$].

DISCUSSION

Minaxolone has been shown in receptor binding studies to be a potent allosteric modulator of $GABA_A$ receptors. Minaxolone enhanced the binding of [^{3}H]muscimol to well-washed freeze–thawed rat brain membranes. The magnitude of effect is similar to that reported for other neuroactive steroids such as alphaxalone (7), which has been shown to act by increasing the proportion of high-affinity muscimol-binding sites. Temazepam also increased [^{3}H]muscimol binding, although the magnitude of effect was considerably lower than that achieved with the neuroactive steroid. Minaxolone also enhanced benzodiazepine binding by 48%. Similar increases in [^{3}H]flunitrazepam binding have been reported for 5α-pregnan-3α-*ol*-20-one and 5α-pregnan-3α,21-diol-20-one, which increased binding by 50–60% (6). In this study, the effects of the neurosteroids were found to be due to an increase in apparent affinity of [^{3}H]flunitrazepam binding. In our studies, the maximum potentiation observed with minaxolone on both [^{3}H]muscimol and [^{3}H]flunitrazepam binding was equal to or greater than a range of other neuroactive steroids tested including alphaxalone, 5α-pregnan-3α-*ol*-20-one and 5α-pregnan-3α,21-diol-20-one (data not shown). This suggests that minaxolone has a high efficacy at the neurosteroid binding site on the $GABA_A$ receptor. Both minaxolone and temazepam inhibited the binding of the cage-convulsant ligand [^{3}H]TBOB. The action of minaxolone on [^{3}H]TBOB binding is likely to be a composite effect of both an allosteric action on the cage-convulsant binding site and a potentiating effect on endogenous GABA, which also inhibits [^{3}H]TBOB binding. Turner

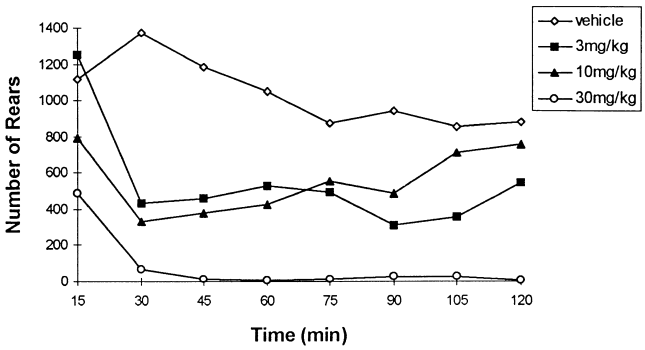

FIG. 3. Effect of temazepam, 3, 10, and 30 mg/kg, orally, on mouse rearing activity for 2 h after drug administration. Data are expressed as mean upper beam breaks (n = 5–6).

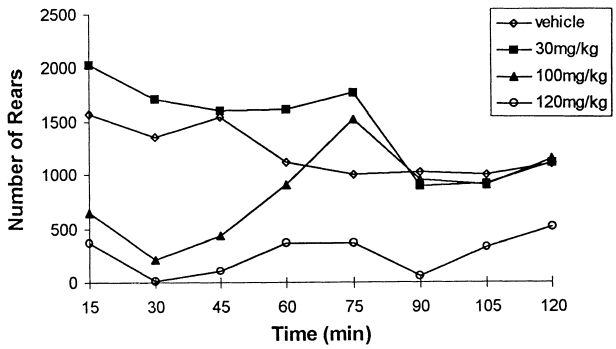

FIG. 4. Effect of minaxolone, 30, 100, and 120 mg/kg, orally, on mouse rearing activity for 2 h after drug administration. Data are expressed as mean upper beam breaks (n = 5–14).

TABLE 1

EFFECT OF CHRONIC TEMAZEPAM TREATMENT ON LOCOMOTOR ACTIVITY IN MICE

Time Period (min)	Activity	Chronic Treatment/Acute Treatment		
		VEH/VEH	VEH/TEM	TEM/TEM
0–60	Mobile	5068 ± 689	2440 ± 622*	5484 ± 1055†
	Rearing	3572 ± 826	1203 ± 536*	5278 ± 824‡
60–120	Mobile	4374 ± 815	1659 ± 470*	3929 ± 661†
	Rearing	4616 ± 710	939 ± 494§	4986 ± 802‡
120–180	Mobile	3199 ± 725	2033 ± 441	3473 ± 670
	Rearing	4125 ± 960	1404 ± 708*	5186 ± 983†
180–240	Mobile	2167 ± 556	2439 ± 714	3007 ± 544
	Rearing	2882 ± 902	1473 ± 581	4332 ± 925

Following 7 days of once-daily treatment with vehicle (VEH) or temazepam, 10 mg/kg, orally (TEM), the effect of temazepam (10 mg/kg orally) on rearing activity and mobile activity was assessed in mice. Data are expressed as beam breaks (mean ± SE, $n = 9$) and were significantly different from VEH/VEH, where $*p < 0.05$, $§p < 0.01$; and from VEH/TEM, where $†p < 0.05$, $‡p < 0.01$.

et al. (23) reported marked differences in the neurosteroid modulation of [^{35}S]TBPS, depending on the extent to which the membranes are washed, and therefore on the concentration of endogenous GABA present in the membranes.

Measurement of locomotor activity in the mouse provides a simple model to assess the sedative properties of drugs such as benzodiazepines and neurosteroids. Decreases in rearing and mobile activity are indicative of an onset of sedation; however, the precise sleep–wake state of the animal cannot be determined without further analysis such as electroencephalography. As expected, in these studies temazepam produced a rapid decrease in activity following oral administration. A dose of 10 mg/kg resulted in a 60–70% decrease in activity within the first 30 min; these effects were maintained for over 5 h. Minaxolone also had a powerful sedative effect in mice; however, compared with temazepam it was less potent, requiring a dose of 100 mg/kg to cause a similar magnitude of effect. This may be due to poor oral bioavailability and rapid metabolism, which is also indicated by its very short duration of action.

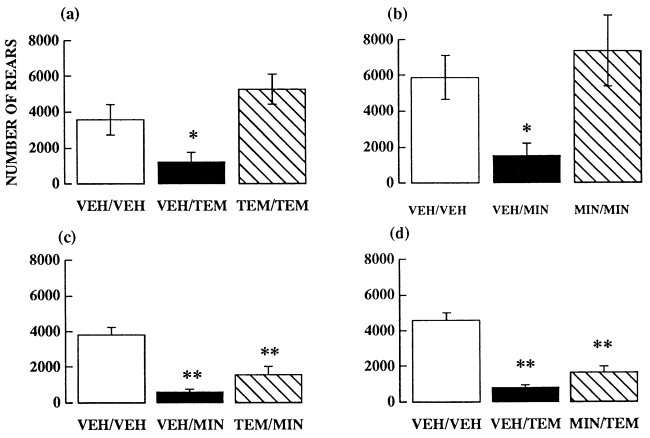

FIG. 5. Tolerance and cross-tolerance studies for the sedative effects of temazepam (TEM) and minaxolone (MIN) after 7 days of chronic dosing: (a) chronic temazepam/acute temazepam; (b) chronic minaxolone/acute minaxolone; (c) chronic temazepam/acute minaxolone, and (d) chronic minaxolone/acute temazepam. Histograms represent total number of rearing counts at the end of the first hour (mean ± SE). Treatments are abbreviated as chronic treatment/acute treatment. Significant differences from the VEH/VEH group: $*p < 0.05$; $**p < 0.01$ ($n = 7$–10).

On recovery from the sedative effects of both minaxolone and temazepam, mice appeared to become hyperactive compared with control animals. This behaviour may be due to the exploration of a novel environment which was prevented at the beginnning of the test period by the fast onset of sedation. Low doses of diazepam have previously been reported to induce hyperactivity by activation of brain dopaminergic systems (21), and it is possible that the effects observed here are mediated by a similar mechanism.

It is well established that chronic dosing with benzodiazepines leads to the development of tolerance to behavioural effects such as sedation and muscle relaxation. For example Miller et al. (14,15) showed that mice receiving lorazepam (1–2 mg/kg per day) became tolerant to decreases in open-field activity and Rotarod performance by day 7 of treatment. Parallel binding studies indicated a reduction in the number of [^{3}H]flunitrazepam binding sites in the cortex. Decreases were also observed in chloride uptake from cortical synaptosomes and in muscimol-induced chloride uptake. The development of tolerance and subsequent dependence may be associated with changes in the subunit composition of the $GABA_A$ receptor, because decreases in the expression of specific α-subunit mRNA have been reported following chronic benzodiazepine administration (8,9). In the present study we also observed marked tolerance to the sedative effects of temazepam (10 mg/kg, orally) after 7 days of daily treatment with the same dose.

Chronic dosing with minaxolone at a dose which produced similar acute sedative effects to temazepam also resulted in the development of tolerance after 7 days of treatment. The magnitude of this effect was very similar to that observed with the benzodiazepine. This is the first report of the development of tolerance to any behavioural effects of neuroactive steroids following chronic dosing. It is not known whether these results reflect an alteration in steroid function at the level of the GABA receptor or whether the tolerance may arise from changed drug pharmacokinetics. However, no induction of liver enzymes have been observed in the rat after repeated

TABLE 2

EFFECT OF CHRONIC MINAXOLONE TREATMENT ON LOCOMOTOR ACTIVITY IN MICE

		Chronic Treatment/Acute Treatment		
Time Period (min)	Activity	VEH/VEH	VEH/MIN	MIN/MIN
0–60	Mobile	7799 ± 1033	2117 ± 543*	10442 ± 2217†
	Rearing	5903 ± 1221	1008 ± 561*	7387 ± 1992‡
60–120	Mobile	5179 ± 1023	4772 ± 1580	8842 ± 1129
	Rearing	5547 ± 1019	1443 ± 520§	8290 ± 1250†
120–180	Mobile	4080 ± 893	10115 ± 2953	8224 ± 1250
	Rearing	4388 ± 868	5536 ± 2398	7964 ± 1332
180–240	Mobile	3620 ± 730	9002 ± 2285*	6060 ± 991
	Rearing	3657 ± 515	5270 ± 1905	7502 ± 1176

Following 7 days of once-daily treatment with vehicle (VEH) or minaxolone, 100 mg/kg, orally (MIN), the effect of minaxolone, 100 mg/kg orally on rearing activity and mobile activity was assessed in mice. Data are expressed as beam breaks (mean ± SE, n = 8–10) and were significantly different from VEH/VEH, where *$p < 0.05$, §$p < 0.01$; and from VEH/MIN, where †$p < 0.01$, ‡$p < 0.05$.

dosing with minaxolone (5 mg/kg, IV, for 7 days; Priestley, S. M., and Pateman, A. J., personal communication). Changes in $GABA_A$-receptor function have been reported in vitro. Chronic exposure of neuronal cultures to 5α-pregnan-3α-*ol*-20-one or 5β-pregnan-3α-*ol*-20-one (4,24) resulted in an uncoupling of the GABA, barbiturate, benzodiazepine, and neurosteroid sites as revealed by receptor-binding studies as well as a downregulation of GABA- and TBPS-binding sites. No alterations were observed in the affinity or number of [^{3}H]flunitrazepam-binding sites. The mechanisms of action of neuroactive steroids at the molecular level to downregulate $GABA_A$-receptor function are not yet known; however, it is possible that like the benzodiazepines, they may cause a change in subunit composition. The possibility of a genomic action of minaxolone cannot be excluded. Intracellular oxidation of some neurosteroids such as 5α-pregnan-3α-*ol*-20-one and 5α-pregnan-3α,21-diol-20-one by the enzyme 5α-dihyroprogesterone 3α-hydroxysteroid oxidoreductase results in the formation of 5α-pregnane-3,20-dione and 5α-pregnane-21-*ol*-3,20-dione, which can regulate gene expression via the progesterone receptor (18). It is not known whether minaxolone or any of its metabolites have progestomimetic effects in vivo.

To provide additional information about the possible mechanisms of action involved in tolerance to benzodiazepines and neurosteroids, we were interested in determining whether cross-tolerance developed between these two classes of drugs. Chronic treatment with the benzodiazepine did not appear to affect the sedative properties of minaxolone, and vica versa, indicating that the drugs are not cross-tolerant. It therefore seem likely that changes in the $GABA_A$ receptor which result from chronic exposure to one drug do not affect the function of the other. These results support existing evidence that neurosteroids act at distinct sites from benzodiazepines, and are consistent with the studies of Yu and Ticku (24) in which chronic exposure of cortical neurones to 5α-pregnan-3α-*ol*-20-one had no effect on benzodiazepine binding. The authors suggest that neurosteroids may decrease the levels of the β-subunit, which would result in a decrease in GABA function while having no effect on α- and γ_2-subunits, which are thought to be required for benzodiazepine binding. Clearly, additional molecular studies examining the expression of subunit mRNA following chronic neurosteroid treament are necessary to confirm this hypothesis.

These studies may have implications for the development

TABLE 3

EFFECT OF CHRONIC MINAXOLONE TREATMENT ON THE HYPNOTIC EFFECT OF TEMAZEPAM IN MICE

		Chronic Treatment/Acute Treatment		
Time Period (min)	Activity	VEH/VEH	VEH/TEM	TEM/TEM
0–60	Mobile	8457 ± 597	2832 ± 390*	3785 ± 440*
	Rearing	4556 ± 415	1727 ± 921*	1666 ± 318*
60–120	Mobile	5313 ± 802	1915 ± 763*	1836 ± 353*
	Rearing	4395 ± 506	1864 ± 925*	1115 ± 321*
120–180	Mobile	3922 ± 436	2550 ± 775	3361 ± 612
	Rearing	3782 ± 446	1801 ± 465†	2608 ± 663
180–204	Mobile	3844 ± 646	3089 ± 847	2341 ± 423
	Rearing	3647 ± 382	2047 ± 535†	1757 ± 434†

Following 7 days of once-daily dosing with vehicle or minaxolone citrate, 100 mg/kg, orally (MIN), the effect of temazepam 10 mg/kg orally (TEM), on rearing activity and mobile activity was assessed in mice. Data are expressed as beam breaks (mean ± SE, n = 10) and are significantly different from VEH/VEH, where *$p < 0.05$, §$p < 0.01$.

TABLE 4

EFFECT OF CHRONIC TEMAZEPAM TREATMENT ON THE HYPNOTIC EFFECT OF MINAXOLONE IN MICE

		Chronic Treatment/Acute Treatment		
Time Period (min)	Activity	VEH/VEH	VEH/MIN	TEM/MIN
0–60	Mobile	7076 ± 587	2141 ± 657‡	2481 ± 740*
	Rearing	3811 ± 435	607 ± 151‡	1544 ± 451*
60–120	Mobile	3717 ± 425	3713 ± 1503	6823 ± 2703
	Rearing	3243 ± 302	932 ± 547	3500 ± 1353
120–180	Mobile	3059 ± 455	6812 ± 2050	6141 ± 1584
	Rearing	3245 ± 359	2876 ± 1410	5593 ± 1415
180–240	Mobile	2360 ± 391	11627 ± 2169‡	6111 ± 1597†
	Rearing	2506 ± 259	4413 ± 723	6221 ± 1718*

Following 7 days of once-daily dosing with vehicle or temazepam, 10 mg/kg, orally (TEM), the effect of minaxolone 100 mg/kg orally (MIN), on rearing and mobile activity was assessed in mice. Data are expressed as beam breaks (mean ± SE, n = 7–9) and are significantly different from VEH/VEH, where *$p < 0.01$, ‡$p < 0.01$ and from VEH/MIN, where †$p < 0.05$.

of neuroactive steroids as therapeutic agents. As well as mediating the anaesthetic properties of neuroactive steroids such as alphaxalone, it has been proposed that the distinct neurosteroid modulatory sites on $GABA_A$ receptors may present attractive drug discovery targets for CNS diseases, including epilepsy, pain, anxiety, or insomnia (13,22). Clearly, the development of effect tolerance after chronic treatment would be undesirable for these indications. Our animal studies are consistent with the idea that receptor-based tolerance to the effects of neuroactive steroids may occur following chronic exposure. However, one clinical study has indicated no tolerance to the sedative properties of althesin, a formulation containing alphaxalone, after 20 days of continuous exposure (17). Moreover, it is possible, as with ligands for the benzodiazepine-binding site (19), that the development or extent of tolerance to a neurosteroid-based drug will vary according to the specific nature of the pharmacologic interaction between the molecule and its cognate receptor. Thus, receptor selectivity or intrinsic activity and the subunit composition of the target $GABA_A$ receptors may be important. Nevertheless, the present findings indicate that, as with other classes of ligands at $GABA_A$ receptors, neuroactive steroid effects have the potential to be limited by tolerance developing with chronic treatment.

REFERENCES

1. Backstrom, T.; Bixo, M.; Hammarback, S.: Ovarian steroid hormones: Effects on mood, behaviour and brain excitability. Acta Obstet. Gynecol. Scand. (Suppl 130):19–24; 1985.
2. Belelli, D.; Bolger, M. B.; Gee, K. W.: Anticonvulsant profile of the progesterone metabolite 5α-pregnan-3α-ol-20-one. Eur. J. Pharmacol. 166:325–329; 1989.
3. Crawley, J. N.; Glowa, J. R.; Majewska, M. D.; Paul, S. M.: Anxiolytic activity of an endogenous adrenal steroid. Brain Res 398; 382–385; 1986.
4. Friedman, L.; Gibbs, T. T.; Farb, D. H.: γ-Aminobutyric $acid_A$ receptor regulation: Chronic treatment with pregnanolone uncouples allosteric interactions between steroid and benzodiazepine recognition sites. Mol. Pharmacol. 44:191–197; 1993.
5. Greenblatt, D. J.; Shader, R. I.: Dependence, tolerance and addiction to benzodiazepines: Clinical and pharmacokinetic considerations. Drug Metab. Rev. 8:13–28; 1978.
6. Harrison, N. L.; Majewska, D.; Harrington, J. W.; Barker, J. L.: Structure-activity relationships for steroid interaction with the gamma-aminobutyric $acid_A$ receptor complex. J. Pharmacol. Exp. Ther. 241:346–350; 1987.
7. Harrison, N. L.; Simmonds, M. A.: Modulation of the $GABA_A$ receptor complex by a steroid anaesthetic. Brain Res. 323:287–292; 1984.
8. Heninger, C.; Saito, N.; Tallman, J. F.; Garrett, K. M.; Vitek, M. P.; Duman, R. S.; Gallager, D. W.: Effects of continuous diazepam administration on $GABA_A$ subunit mRNA in rat brain. J. Mol. Neurosci. 2:101–107; 1990.
9. Kang, I.; Miller, L. G.: Decreased $GABA_A$ receptor subunit mRNA concentrations following chronic lorazepam administration. Br. J. Pharmacol. 103:1285–1287; 1991.
10. Kavaliers, M.; Wiebe, J. P.: Analgesic effects of the progesterone metabolite, 3α-hydroxy-5α-pregnan-20-one. Brain Res. 415:393–398; 1987.
11. Lawrence, L. J. Palmer, C. J. Gee, K. W. Wang, X. Yamamura, H. I and Casida, J. E.: t-[3H]-Butylbicycloorthobenzoate: New radioligand probe for gamma-aminobutyric acid regulated chloride channel ionophore. J. Neuropharmacol. 45:798–804; 1985.
12. Lister, R. G.; File, S. E.; Greenblatt, D. J.: Functional tolerance to lorazepam in the rat. Psychopharmacology 81:292–294; 1984.
13. Majewska, M. D. Neurosteroids—Endogenous bimodal modulators of the GABA-A receptor—Mechanism of action and physiological significance. Prog. Neurobiol. 38, 379–395.
14. Miller, L. G.; Greenblatt, D. J.; Barnhill, J. G.; Shader, R. I.: Chronic benzodiazepine administration. I. Tolerance is associated with benzodiazepine receptor down regulation and decreased γ-aminobutyric acidA receptor function. J. Pharmacol. Exp. Ther. 246:170–176; 1988.
15. Miller, L. G.; Greenblatt, D. J.; Roy, R. B.; Summer, W. R.; Shader, R. I.: Chronic benzodiazepine administration. II. Discontinuation syndrome is associated with upregulation of g-aminobutyric acidA receptor complex binding and function. J. Pharmacol. Exp. Ther. 246:170–176; 1988.
16. Purdy, R. H.; Morrow, A. L.; Moore, P. H.,Jr.; Paul, S. M.: Stress-induced elevations of γ-aminobutyric acid type A receptor-active steroids in the rat brain. Proc. Natl. Acad. Sci. USA 88:4553–4557; 1991.

17. Ramsey, M. A. E.; Savege, T. M.; Simpson, B. R. J.; Goodwin, R.: Controlled sedation with alphaxalone-alphadalone. BMJ 2:656–659; 1974.
18. Rupprecht, R.; Reul, J. M. H. M.; Trapp, T.; van Steensel, B.; Wetzel, C.; Damm, K.; Zieglgansberger, W.; Holsboer, F.: Progesterone receptor-mediated effects of neuroactive steroids. Neuron 11:523–530; 1993.
19. Sanger, D. J; Benavides, J.; Perrault, G.; Morel, E.; Cohen, C.; Joly, D.; Zivkovic, B.: Recent developments in the behavioral pharmacology of benzodiazepine (omega) receptors: Evidence for the functional significance of receptor subtypes. Neurosci. Biobehav. Rev. 18:355–72; 1994.
20. Smith, S.: Female sex steroid hormones: From receptors to networks to performance: Actions on the sensorimotor system. Prog. Neurobiol. 44:55–86; 1994.
21. Sölderpam, B.; Svensson, L.; Hulthe, P.; Johannessen, K.; Engel, J. A.: Evidence for a role for dopamine in the diazepam locomotor stimulating effect. Psychopharmacology 104:97–102; 1991.
22. Tseng, Y. T.; Wellman, S. E.; Ho, I. K.: In situ hybridization evidence of differential modulation by pentobarbital of $GABA_A$ receptor α1- and β3-subunit mRNAs. J. Neurochem. 63:301–309; 1994.
23. Turner, D. M.; Ransom, R. W.; Yang, Jane S.-J.; Olsen, R. W.: Steroid anesthetics and naturally occurring analogs modulate the γ-aminobutyric acid receptor complex at a site distinct from barbiturates. J. Pharmacol. Exp. Ther. 248:960–966; 1989.
24. Yu, R.; Ticku, M. K.: Chronic neurosteroid treatment produces functional heterologous uncoupling at the γ-aminobutyric acid $type_A$/benzodiazepine receptor complex in mammalian cortical neurons. Mol. Pharmacol. 47:603–610; 1995.