0022-3565/97/2803-1284$03.00/0
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Vol. 280, No. 3

Printed in U.S.A.
JPET 280:1284–1295, 1997

Characterization of the Anticonvulsant Properties of Ganaxolone (CCD 1042; 3α-Hydroxy-3β-methyl-5α-pregnan-20-one), a Selective, High-Affinity, Steroid Modulator of the γ-Aminobutyric Acid$_A$ Receptor

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Accepted for publication November 14, 1996

ABSTRACT

Ganaxolone (CCD 1042) is a 3β-methyl-substituted analog of the endogenous neuroactive steroid 3α-hydroxy-5α-pregnan-20-one. Ganaxolone inhibited binding of the γ-aminobutyric acid (GABA)$_A$ receptor-chloride channel ligand *t*-[^{35}S]butylbicyclophosphorothionate (IC_{50} of 80 nM) and enhanced binding of the benzodiazepine site ligand [^{3}H]flunitrazepam (EC_{50} of 125 nM) and the GABA site ligand [^{3}H]muscimol (EC_{50} of 86 nM), consistent with activity as a positive allosteric modulator of the $GABA_A$ receptor. Electrophysiological recordings showed that, whereas nanomolar concentrations of ganaxolone potentiated GABA-evoked chloride currents in *Xenopus* oocytes expressing the human $GABA_A$ receptor subunits $\alpha1\beta1\gamma2_L$, $\alpha2\beta1\gamma2_L$ or $\alpha3\beta1\gamma2_L$, direct activation of chloride flux occurred to a limited extent only at micromolar concentrations. Ganaxolone was effective in nontoxic doses against clonic convulsions induced by s.c. pentylenetetrazol administration in mice and rats (ED_{50} values of 4.3 and 7.8 mg/kg i.p., respectively). Ganaxolone also exhibited potent anticonvulsant activity against seizures induced by s.c. bicuculline (ED_{50} of 4.6 mg/kg i.p.), i.p. TBPS (ED_{50} of 11.7 mg/kg i.p.) and i.p. aminophylline (ED_{50} of 11.5 mg/kg i.p.) in mice. Although ganaxolone effectively blocked tonic seizures induced by maximal electroshock in mice (ED_{50} of 29.7 mg/kg i.p.), it did so only at doses that produced ataxia on the Rotorod (TD_{50} of 33.4 mg/kg i.p.). Conversely, ganaxolone was a potent anticonvulsant against fully kindled stage 5 seizures induced by corneal kindling in rats (ED_{50} of 4.5 mg/kg i.p.), producing these effects at doses well below those that resulted in ataxia (TD_{50} of 14.2 mg/kg i.p.). The seizure threshold, as determined by an increase in the dose of i.v. infused pentylenetetrazol required to induce clonus, was also significantly elevated by nontoxic doses of ganaxolone in mice. In summary, these data indicate that ganaxolone is a high-affinity, stereoselective, positive allosteric modulator of the $GABA_A$ receptor complex that exhibits potent anticonvulsant activity across a range of animal procedures. The profile of anticonvulsant activity obtained for ganaxolone supports clinical evaluation of this drug as an antiepileptic therapy with potential utility in the treatment of generalized absence seizures as well as simple and complex partial seizures.

GABA-containing neurons are the predominant inhibitory neural elements within the brain. Correspondingly, GABA-mediated inhibition plays a critical role in the epileptic process by contributing to the termination of the ictal discharge and limiting the spread of hyperexcitability. Potentiation of GABAergic inhibitory function has therefore served as the rational basis for a number of programs targeted toward the discovery of novel antiepileptic agents (Löscher and Schmidt, 1994; Rogawski and Porter, 1990; Satzinger, 1994).

Numerous strategies for creating antiepileptic agents that act by increasing GABAergic neurotransmission have been used, including the synthesis of direct $GABA_A$ agonists, GABA-transaminase inhibitors, GABA uptake blockers, GABA prodrugs and allosteric modulators of the $GABA_A$ receptor, albeit with mixed success. For example, the direct $GABA_A$ receptor agonist gaboxadol (4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol), which exhibits potent anticonvul-

Received for publication April 22, 1996.

ABBREVIATIONS: CD_{97}, 97% convulsive dose; DMSO, dimethylsulfoxide; E_{max}, maximal extent of potentiation; EC_{10}, 10% effective concentration; GABA, γ-aminobutyric acid; MES, maximal electroshock; 3α,5α-P, 3α-hydroxy-5α-pregnan-20-one; 3α,5β-P, 3α-hydroxy-5β-pregnan-20-one; PI, protective index; PTZ, pentylenetetrazol; TBPS, *t*-butylbicyclophosphorothionate.

sant activity in animal seizure models (Meldrum and Horton, 1980), failed to demonstrate subsequent clinical efficacy in the treatment of partial epilepsy (Dam *et al.*, 1982). Likewise, the GABA prodrug progabide, although demonstrating good activity in preclinical tests (Worms *et al.*, 1982), consistently failed to demonstrate efficacy in ensuing controlled clinical investigations (Dam *et al.*, 1983; Leppik *et al.*, 1987; Schmidt and Utech, 1986). Blockers of neuronal and glial GABA uptake, such as tiagabine and CI-966, are potent anticonvulsants in animal tests (Nielsen *et al.*, 1991; Smith *et al.*, 1995) and, in the case of tiagabine, effective in human complex partial seizures (Mengel, 1994; Schachter, 1995). Unacceptable psychiatric and neurological side effects in humans may, however, limit the utility of at least some members of this class as antiepileptic medications (Sedman *et al.*, 1990). In contrast, the irreversible GABA-transaminase inhibitor vigabatrin exhibits robust anticonvulsant activity in a variety of animal seizure models (Mysolobodsky *et al.*, 1979; Palfreyman *et al.*, 1981) and displays good therapeutic utility in the treatment of complex partial epilepsy, a property that has been confirmed repeatedly in controlled clinical trials (Grant and Heel, 1991; Loiseau *et al.*, 1986). Vigabatrin may also be useful in the treatment of drug-resistant childhood epileptic disorders such as West and Lennox-Gastaut syndromes (Löscher and Schmidt, 1994). Side effects associated with vigabatrin treatment include mood disturbances, dizziness, sedation and, on occasion, acute psychotic episodes in patients with temporal lobe disorders (Aldenkamp *et al*., 1994; Grant and Heel, 1991; Sheth *et al.*, 1996). Among positive allosteric modulators of the $GABA_A$ receptor-Cl^- ionophore, benzodiazepines such as diazepam and clonazepam are used primarily for termination of *status epilepticus* and treatment of myoclonic disorders, respectively (Sato, 1989; Schmidt, 1989). Side effects associated with benzodiazepine treatment include drowsiness and ataxia, as well as other behavioral and personality changes (Sato, 1989). Antiepileptic barbiturates, such as phenobarbital, also exert positive allosteric effects on $GABA_A$ neurotransmission, although some, if not all, barbiturates are capable of directly activating the Cl^- ion channel (Barker and Ransom, 1978; Nicoll and Wojtowicz, 1980). Notwithstanding their utility in the treatment of generalized tonic-clonic and partial seizures, the latter characteristic may account for the preponderance of dose-limiting side effects observed with barbiturate antiepileptic medications (Farwell *et al.*, 1990; Vining *et al.*, 1987).

More recently, a new class of positive allosteric modulators of the $GABA_A$ receptor-Cl^- ionophore, termed neuroactive steroids, has been described (for review, see Gee *et al.*, 1995). Endogenous metabolites of the steroid hormone progesterone, such as allopregnanolone (3α,5α-P) and pregnanolone (3α,5β-P), modulate neuronal function through interaction with a unique nongenomic recognition site on the $GABA_A$ receptor complex that is distinct from the benzodiazepine and barbiturate binding sites (Gee *et al.*, 1988; Majewska *et al.*, 1986; Turner *et al.*, 1989). Electrophysiological and $^{36}Cl^-$ uptake studies have demonstrated that neuroactive steroids exert a positive modulatory effect on GABA-evoked activity (Im *et al.*, 1990; Morrow *et al.*, 1987; Peters *et al.*, 1988). Moreover, modulation of [^{35}S]TBPS and [^{3}H]flunitrazepam binding by neuroactive steroids is predictive of functional activity (Hawkinson *et al.*, 1994). As might be expected, based on their ability to facilitate GABAergic neurotransmission, 3α,5α-P and 3α,5β-P exhibit potent anticonvulsant effects in animal tests (Belelli *et al.*, 1989, 1990; Högskilde *et al.*, 1988; Kokate *et al.*, 1994; Landgren *et al.*, 1987). 3α,5α-P and 3α,5β-P exhibit good therapeutic indices in mice, protecting against PTZ-induced seizures with ED_{50} values of approximately 3.0 mg/kg i.p. and producing locomotor impairment in the Rotorod ataxia test (TD_{50}) at approximately 20.0 mg/kg (Wieland *et al.*, 1995). Endogenous neurosteroids, such as 3α,5α-P and 3α,5β-P, are unsuitable as therapeutic agents, however, because they are readily oxidized at the 3α-position (Phillipps, 1975), resulting in compounds that are inactive at neuronal but potentially active at hormonal steroid receptors (Gee *et al.*, 1988; Harrison *et al.*, 1987; Hawkinson *et al.*, 1994).

Ganaxolone (CCD 1042; 3α-hydroxy-3β-methyl-5α-pregnan-20-one) is a 3β-methylated synthetic analog of the endogenous neuroactive steroid 3α,5α-P (fig. 1). 3β-Substitution, which in part prevents metabolism of the 3α-hydroxy moiety, has been suggested to enhance the bioavailability of pregnane steroids without altering their primary pharmacological properties (Gee *et al.*, 1995). Thus, ganaxolone might be expected to retain the anticonvulsant activity of the endogenous neurosteroid 3α,5α-P while acquiring a pharmacokinetic profile that would be expected to enhance its use as an antiepileptic drug. The present experiments were conducted to describe the *in vitro* modulatory properties of ganaxolone at the $GABA_A$ receptor complex as well as to define its *in vivo* preclinical anticonvulsant profile. Ganaxolone is currently in phase II clinical trials.

Methods

Synthesis

Ganaxolone was manufactured at Pharm-Eco Laboratories (under contract to CoCensys) according to synthetic procedures described elsewhere (Hogenkamp *et al.*, 1997). The other neuroactive steroids used in this study, *i.e.,* 3α,5α-P, 3α-hydroxy-3β-methyl-5β-pregnan-20-one, 3α-methyl-3β-hydroxy-5α-pregnan-20-one and 3α-methyl-3β-hydroxy-5β-pregnan-20-one, were synthesized at CoCensys also using chemical procedures outlined by Hogenkamp *et al.*, 1997.

Radioligand Binding Assays

Membrane preparation. Rat brain cortical membranes were prepared as described previously (Hawkinson *et al.*, 1994). Briefly, cortices were removed rapidly after decapitation of carbon dioxide-anesthetized Sprague-Dawley rats (200–250 g), homogenized in 10 volumes of ice-cold 0.32 M sucrose using a glass/Teflon homogenizer and centrifuged at 1500 $\times$ *g* for 10 min at 4°C. The resultant supernatants were centrifuged at 10,000 $\times$ *g* for 20 min at 4°C to obtain

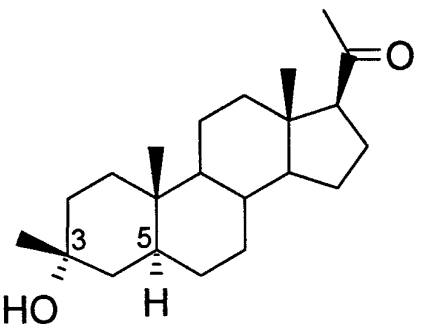

Fig. 1. Structure of ganaxolone, with numbered positions encompassing congeners examined.

the P2 pellets. The P2 pellets were resuspended in 200 mM NaCl/50 mM sodium/potassium phosphate buffer, pH 7.4, and centrifuged at 10,000 × *g* for 10 min at 4°C. This washing procedure was repeated twice, and the pellets were resuspended in 10 volumes of buffer.

[^{35}S]TBPS binding assay. The [^{35}S]TBPS assay was conducted according to methods described previously (Hawkinson *et al.*, 1994). Briefly, aliquots (100 μl) of the membrane suspensions were incubated with 2 nM [^{35}S]TBPS (60–100 Ci/mmol; New England Nuclear) and 5-μl aliquots of test drug (nine concentrations ranging from 1 nM to 10 μM final concentration) dissolved in DMSO (final concentration, 0.5%), in the presence of 5 μM GABA (Sigma Chemical Co.). The incubation was brought to a final volume of 1.0 ml with buffer. Nonspecific binding was determined in the presence of 2 μM unlabeled TBPS (Research Biochemicals International) and ranged from 15 to 25%. After a 90-min incubation at room temperature, the assays were terminated by filtration through glass fiber filters (Schleicher and Schuell no. 32), using a cell harvester (Brandel), and were rinsed three times with ice-cold buffer. Filter-bound radioactivity was measured by liquid scintillation counting.

[^{3}H]Flunitrazepam binding assay. The [^{3}H]flunitrazepam assay was identical to the [^{35}S]TBPS assay except that the membranes were incubated with 1 nM [^{3}H]flunitrazepam (74–84 Ci/mmol; NEN) in the presence of 1 μM GABA. Nonspecific binding was determined in the presence of 1 μM clonazepam (Sigma) and ranged from 2 to 5%.

[^{3}H]Muscimol binding assay. The [^{3}H]muscimol assay was conducted according to methods described previously (Goodnough and Hawkinson, 1995). Briefly, this assay differed from the [^{35}S]TBPS assay in that cortical membranes were extensively washed and preincubated at 37°C to remove endogenous GABA, suspended in Na^{+}-free buffer containing 100 mM KCl and incubated with 5 nM [^{3}H]muscimol (10–20 Ci/mmol; NEN). Nonspecific binding was determined in the presence of 1 mM GABA and ranged from 5 to 10% of total binding.

Non-target receptor binding assays. The effects of ganaxolone on binding at cytosolic steroid receptors (Panlabs ProfilingScreen) and neurotransmitter receptors (NovaScreen) were also determined. The activity of ganaxolone was compared, in each instance, with that of a reference compound with known affinity. Each experiment was replicated three times.

Data analysis. Nonlinear curve-fitting of binding data for each drug averaged over concentration was performed using the following equations: for inhibition, $Y = A + [(B - A)/[1 + (X/IC_{50})^{D}]]$; for enhancement, $Y = A + [(B - A)/[1 + (EC_{50}/X)^{D}]]$, where Y is the percent specifically bound, A is the bottom plateau, B is the top plateau, X is the concentration and D is the Hill coefficient. The concentration of compound that produced 50% inhibition (IC_{50}) or enhancement (EC_{50}) of specific binding was determined using a commercial computer program (Prism v2.0; GraphPad).

Modulation of Cloned Human $GABA_A$ Receptor-mediated Currents

Preparation of *in vitro* transcripts and expression. Human GABA α1, α2, α3, β1 and $\gamma 2_L$ subunit cDNAs were linearized in the PCDM8 vector according to standard protocols (Chen *et al.*, 1994). Capped cRNA transcripts were prepared *in vitro* using T7 RNA polymerase. The integrity of the RNA transcripts was verified by denaturing gel electrophoresis.

Xenopus laevis oocytes were isolated as previously described (Chen *et al.*, 1994). Briefly, after treatment with 2 mg/ml collagenase type I (Boehringer Mannheim) in Ca^{++} -free Barth's saline for 3 hr at room temperature (19–23°C), oocytes (stage V–VI) were injected with 20 to 40 nl of the cRNA transcripts (1 mg/ml) in the cytoplasm or 10 to 20 nl of the cDNA (0.25 mg/ml) in the nucleus, resulting in a total amount of 20 to 40 ng of cRNA or 2.5 to 5 ng of cDNA respectively. To evaluate possible direct activation of $GABA_A$ receptors by ganaxolone and 3α,5α-P, concentration-response curves, in the absence of exogenous GABA, were determined in *Xenopus* oocytes that had been previously injected intranuclearly with a greater amount of the cDNA encoding the human α1, β1 or $\gamma 2_L$ subunits, *i.e.,* 15 to 20 nl of a 0.7 mg/ml solution, resulting in a total amount of 10 to 15 ng of cDNA. This protocol was chosen to result in a high level of $GABA_A$ receptor expression, which aided the determination and quantification of the relatively small steroid-induced currents (see below). Injected oocytes were maintained at 19–20°C in Barth's saline [88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 0.5 mM $Ca(NO_3)_2$, 0.5 mM $CaCl_2$, 1 mM $MgSO_4$; adjusted to pH 7.6 with NaOH], supplemented with gentamicin (100 μg/ml), in individual wells of 96-well microtiter plates (200 μl/well) for up to 12 days.

Electrophysiological recording. Two to 12 days after injection, recordings were performed on such oocytes voltage-clamped at a holding potential of −60 mV, using an Axoclamp 2A (Axon Instruments) amplifier in the twin-electrode voltage-clamp mode. The voltage- and current-passing microelectrodes were filled with 3 M KCl and had resistances of 0.8 to 2 MΩ when measured in the recording solution (frog Ringer solution). The oocytes were continuously superfused with frog Ringer solution [120 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; adjusted to pH 7.4 with NaOH] at a rate of 7 to 10 ml/min. Experiments were conducted at ambient temperature (18–22°C). Membrane current responses were low-pass-filtered at 500 Hz and recorded on magnetic tape or digital audio tape, using an FM tape (Racal Store 4DS) or DAT (Biologic DTR-1204) recorder, and were simultaneously displayed on a Lectromed multitrace two-pen recorder.

The GABA-enhancing properties of the putative allosteric modulators were assessed by using a concentration of GABA that evoked a current approximately equivalent to 10% of the maximal response (EC_{10}). This parameter was determined for each oocyte, for each receptor subunit combination tested. All allosteric modulators were preapplied for 60 to 90 sec before being coapplied with the appropriate GABA concentration. In the construction of the concentration-response relationship, the peak amplitude of the inward current response was the measured parameter. All data were expressed as a percentage of the maximal response to GABA.

Pharmacological procedure. Ganaxolone, 3α,5α-P, propofol (purchased from Aldrich Chemical Co.), GABA and pentobarbital (purchased from Sigma) were prepared daily as concentrated stock solutions of 4 to 10 mM in DMSO or 0.1 mM ethanol (propofol) and were then diluted in frog Ringer solution to the appropriate concentration, within 30 sec of being applied to the oocyte. The final DMSO (0.1–0.75%, v/v) or ethanol (0.1%, v/v) concentration had no effect on GABA-evoked current. All drugs were applied *via* the perfusion system.

Data analysis. The concentrations of steroid producing half of its maximal potentiation of the GABA-evoked response (EC_{50}), E_{max} and the relative Hill coefficient were estimated by fitting the concentration-response function to the following sigmoidal function (Fig P version 6.0c; Biosoft): $I/I_{max} = [A]/[n/[A]^{n} + (EC_{50})^{n}]$, where [A] is the steroid concentration, I_{max} is the inward current evoked by GABA in the presence of a maximally potentiating concentration of the steroid, I is the inward current induced by the steroid, EC_{50} is the concentration of the steroid required to produce half of its own maximal effect and n is the Hill coefficient. Quantitative data are reported as the mean ± S.E.M.

In Vivo Pharmacology

Animals. Male NSA mice weighing between 15 and 20 g and male Sprague-Dawley rats weighing between 200 and 225 g were obtained from Harlan Sprague-Dawley, Inc. Upon arrival they were housed in standard polycarbonate cages (mice, four/cage; rats, two/cage) containing sterilized bedding material (Sani-Chips; P.J. Murray), in a room of constant temperature (23.0 ± 2.5°C), with a 12-hr (lights on from 7:00 A.M. to 7:00 P.M.) light/dark cycle. Food (Teklad LM 485;

Harlan Sprague-Dawley) and water were freely available. Animals were acclimated for at least 4 days before experimentation.

Chemically induced seizures. Seizures were induced by administration of PTZ (85 mg/kg s.c. in mice and 70 mg/kg s.c. in rats; 30-min observation period), bicuculline (2.7 mg/kg s.c. in mice; 30-min observation period), TBPS (0.6 mg/kg i.p. in mice; 10-min observation period), aminophylline (300 mg/kg i.p. in mice; 45-min observation period) or strychnine (1.25 mg/kg s.c. in mice; 30-min observation period). The dose of chemoconvulsant used was previously determined to be the 97% convulsant dose (CD_{97}) for that compound. A clonic seizure was defined as forelimb clonus of ≥3-sec duration. Data were treated quantally.

MES-induced seizures. Seizures were induced by application of current (50 mA, 60 pulses/sec, 0.8-msec pulse width, 1-sec duration, d.c., for mice; 99 mA, 125 pulses/sec, 0.8-msec pulse width, 2-sec duration, d.c., for rats) using a Ugo Basile electroconvulsive treatment device (model 7801). Mice were restrained by gripping the loose skin on their dorsal surface, and saline-coated corneal electrodes were held lightly against the two corneas. Rats were allowed free movement on the bench-top, and ear-clip electrodes were used. Current was applied and animals were observed for a period of up to 30 sec for the occurrence of a tonic hindlimb extensor response. A tonic seizure was defined as a hindlimb extension in excess of 90 degrees from the plane of the body. Results were treated in a quantal manner.

Cornea-kindled seizures. Kindled seizures were induced by twice-daily application of current (8 mA, 60 Hz, 2-sec duration, a.c.) through saline-coated corneal electrodes until stage 5 seizures (rearing and falling) were manifest (Racine, 1972), according to methods described previously (Swinyard *et al.*, 1993). Rats were then stimulated once daily for at least 10 additional days until 10 consecutive stage 5 seizures were evoked. At least 72 hr after the last stimulus, rats having reproducible stage 5 seizures were treated with vehicle or drug before corneal stimulation and the seizure stage was determined. Data were treated quantally; the number of animals in which seizures were reduced to stage 3 or less was used to calculate the ED_{50}.

Seizure threshold. PTZ (0.5%) was infused into the tail vein of freely moving mice at a constant rate (0.37 ml/min), using a catheter (PE20) and calibrated syringe pump (model 44; Harvard Apparatus) (Hint and Richter, 1958; Orlot *et al.*, 1949). The onset of forelimb clonus was used as the endpoint, and the volume of drug solution required to attain the endpoint was recorded.

Rotorod test. The Rotorod test used a custom-built apparatus that consisted of an elevated drum, with a textured surface (diameter, 2.5 cm for mice and 7.62 cm for rats), that rotated at a constant speed (mice, 6 rpm; rats, 8 rpm). The height of the drum from the floor of the test apparatus was approximately 30 cm. After administration of test substance, animals were trained to walk continuously on the drum for a period of 2 min. During testing, animals were given three opportunities to remain on the apparatus continuously for 1 min. Results were treated quantally.

Pharmacological procedure. The drugs used and the forms in which the doses were calculated were as follows: phenytoin, ethosuximide, sodium valproate, (+)-bicuculline, aminophylline hydrate, PTZ, strychnine (purchased from Sigma), TBPS (purchased from Research Biochemicals International) and ganaxolone. PTZ, bicuculline, aminophylline, strychnine and TBPS were dissolved in physiological saline (0.9%). Valproate, phenytoin, ethosuximide and ganaxolone were dissolved in 50% hydroxypropyl-β-cyclodextrin (Amazio)/50% distilled water. Test materials were placed in solution by warming and sonication for 1 to 4 hr. Solutions were prepared on a weight/volume basis on the day of or the evening before use. PTZ and bicuculline were administered s.c.; valproate, phenytoin, ethosuximide, ganaxolone, strychnine, aminophylline and TBPS were administered i.p. or p.o. All drugs were administered in volumes of 200 μl/20 g in mice and 2 ml/kg in rats.

Data analysis. The dose of drug required to produce an anticonvulsant effect (ED_{50}) or motor impairment (TD_{50}) in 50% of animals and its associated 95% confidence limits was calculated by the method of Litchfield and Wilcoxon (1949), using a commercial computer program (PHARM/PCS version 4.2; MicroComputer Specialists). The PI was calculated by dividing the TD_{50} by the ED_{50}.

Results

Modulation of [^{35}S]TBPS binding to rat brain cortex. Ganaxolone inhibited specific binding of [^{35}S]TBPS to rat brain cortical membranes in a concentration-dependent manner, with an IC_{50} of 80 nM, whereas its non-3β-methyl-substituted counterpart 3α,5α-P exhibited an IC_{50} of 51 nM (table 1; fig. 2, top). Although the profiles for inhibition of [^{35}S]TBPS binding were similar for the two compounds (identical maximal inhibition, Hill coefficients of 1.0), 3α,5α-P was 1.6-fold more potent than ganaxolone. Ganaxolone demonstrated stereoselectivity in its interaction (table 2), in that the 3α-methyl,3β-hydroxy epimer exhibited a 37-fold loss of affinity (IC_{50} of 2.9 μM), relative to ganaxolone. Similarly, although the 5β-pregnane isomer of ganaxolone was twice as potent at inhibiting [^{35}S]TBPS binding, the 3α-methyl,3β-hydroxy epimer of the 5β-pregnane isomer was only weakly active in the [^{35}S]TBPS binding assay (IC_{50} of 7.4 μM).

Modulation of [^{3}H]flunitrazepam binding to rat brain cortex. Ganaxolone enhanced [^{3}H]flunitrazepam binding in rat brain cortical membranes with an EC_{50} of 125 nM, an E_{max} of 82% and a Hill coefficient of 1.0 (table 1; fig. 2, middle). Consistent with [^{35}S]TBPS binding activity, the endogenous neuroactive steroid 3α,5α-P was 1.6-fold more potent than ganaxolone at stimulating [^{3}H]flunitrazepam binding. There was no apparent difference, however, in the efficacy of ganaxolone and 3α,5α-P, due to the observed variability in E_{max} values.

Modulation of [^{3}H]muscimol binding to rat brain cortex. Ganaxolone enhanced [^{3}H]muscimol binding with two components in cortical membranes from rat brain, with an EC_{50} for the high-affinity component of 86 nM and an E_{max} of 29%; its non-3β-substituted congener also displayed

TABLE 1
Ganaxolone and 3α,5α-P modulation of radioligand binding at the $GABA_A$ receptor complex

	[^{35}S]TBPS Inhibition			[^{3}H]Flunitrazepam Enhancement			[^{3}H]Muscimol Enhancement[a]		
	IC_{50}	I_{max}	Hill coefficient	EC_{50}	E_{max}	Hill coefficient	EC_{50}	E_{max}	Hill coefficient
	nM	*%*		*nM*	*%*		*nM*	*%*	
Ganaxolone	80 ± 18	94 ± 1	1.0	125 ± 13	82 ± 7	1.0	86 ± 15	29 ± 5	1.0
3α,5α-P	51 ± 5	95 ± 1	1.0	80 ± 5	70 ± 8	1.0	86 ± 9	39 ± 3	1.0

[a] Values listed are for the high-affinity component. From the combined data, the low-affinity component contributed an additional 11% (EC_{50} of 17 μM) or 12% (EC_{50} of 10 μM) enhancement for ganaxolone and 3α,5α-P, respectively.

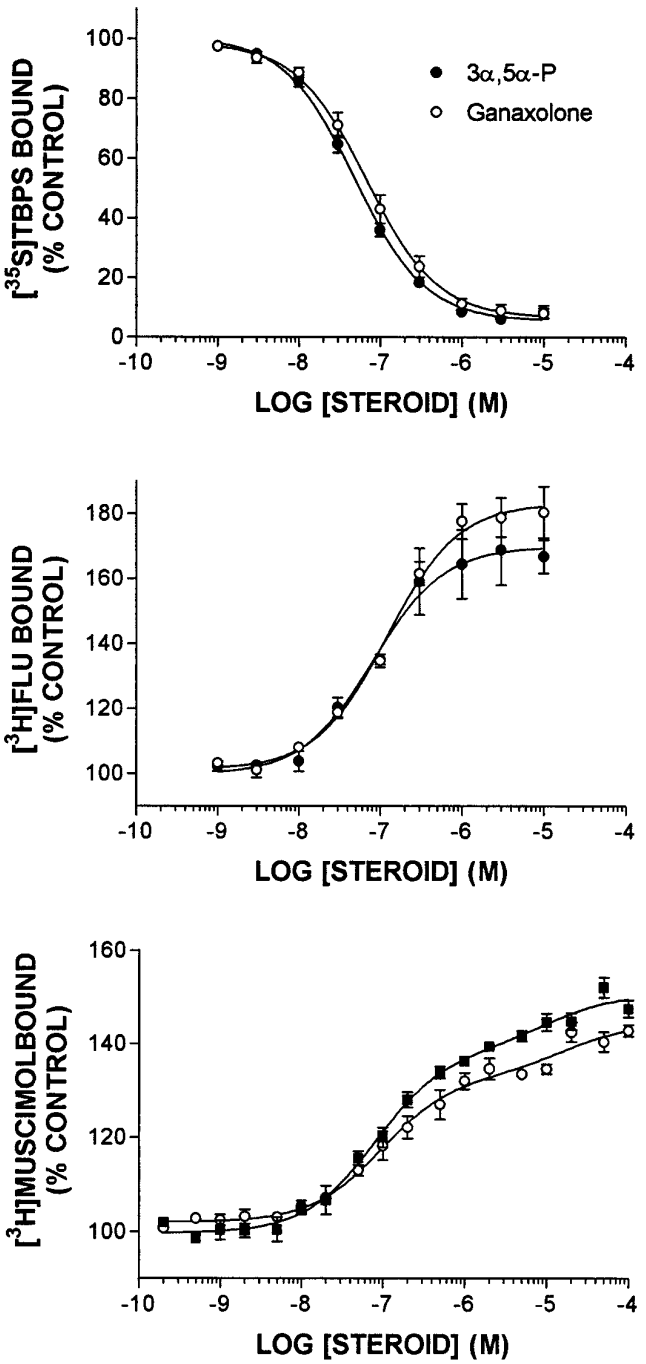

Fig. 2. Concentration-effect curves for inhibition of [^{35}S]TBPS binding (top) or enhancement of [^{3}H]flunitrazepam binding (middle) or [^{3}H]muscimol binding (bottom) by ganaxolone and 3α,5α-P in rat brain cortical membranes. Each point represents the mean ± S.E.M. of at least three independent experiments.

two-component enhancement, as previously reported (Goodnough and Hawkinson, 1995), with identical potency (EC_{50} for the high-affinity component of 86 nM) but greater efficacy (E_{max} of 39%) (table 1; fig. 2, bottom).

Non-target receptor binding. Ganaxolone exhibited negligible inhibition ($IC_{50} > 10$ μM) in radioligand binding assays for a large number of non-target receptors, including cytosolic steroid, excitatory amino acid, inhibitory amino acid, peptide and monoamine receptors (table 3).

TABLE 2
Ganaxolone stereoselective inhibition of [^{35}S]TBPS binding

	Substitution[a]			IC_{50}
	3α	3β	5	
				nM
Ganaxolone	OH	CH_3	α	80 ± 18
	CH_3	OH	α	2948 ± 678
	OH	CH_3	β	37 ± 10
	CH_3	OH	β	7483 ± 556

[a] Refer to structure in figure 1.

TABLE 3
Receptors at which ganaxolone was inactive ($IC_{50} > 10$ μM)

Receptor Class	Receptor	Radioligand
Cytosolic steroid	Estrogen	Estradiol
	Androgen	Mibolerone
	Glucocorticoid	Dexamethasone
	Mineralocorticoid	Aldosterone
	Progesterone	Progesterone
Inhibitory amino acid	$GABA_B$	GABA + isoguvacine
	Glycine	Strychnine
Excitatory amino acid	NMDA-associated glycine	Glycine
	NMDA	CGS 19755
	PCP	TCP
	AMPA	AMPA
	Kainate	Kainate
	Sigma	DTG
Monoamine	DA_1	SCH 23390
	DA_2	Sulpiride
	5-HT_1	5-HT
	5-HT_2	Ketanserin
Adenosine	A_2	NECA
Peptide	A_{II}	Angiotensin
	ANF	ANF
	V_1	Arg-vasopressin
	Bombesin	Bombesin
	CCK	CCK
	EGF	EGF
	Substance K	Neurokinin A
	Neurotensin	Neurotensin
	NGF	NGF
	NPY	NPY
	Somatostatin	Somatostatin
	Substance P	Substance P
	VIP	VIP
Channel Protein	Calcium	ω-Conotoxin
	Calcium	Nifedipine
	Potassium	Apamin
Second messenger	Adenylate cyclase	Forskolin
	IP3	IP3
	Protein kinase	PDBU

Modulation of cloned human $GABA_A$ receptor-mediated currents. Both ganaxolone and 3α,5α-P produced concentration-dependent enhancement of chloride currents evoked by bath application of an EC_{10} concentration of GABA to *X. laevis* oocytes expressing human recombinant $\alpha1\beta1\gamma2_L$ $GABA_A$ receptors (fig. 3, top). Potentiation of GABA-induced current by ganaxolone was evident at 10 nM (12.4 ± 0.3% of the GABA maximal response) (fig. 4) and maximal at 3 μM (68.2 ± 7.9% of GABA). In agreement with binding studies, ganaxolone was ~2.6 fold less potent than 3α,5α-P as a positive allosteric modulator of the GABA-evoked response (EC_{50} of 213 ± 14 nM and 82 ± 7 nM for ganaxolone and 3α,5α-P, respectively) (table 4). The maximal enhancement of

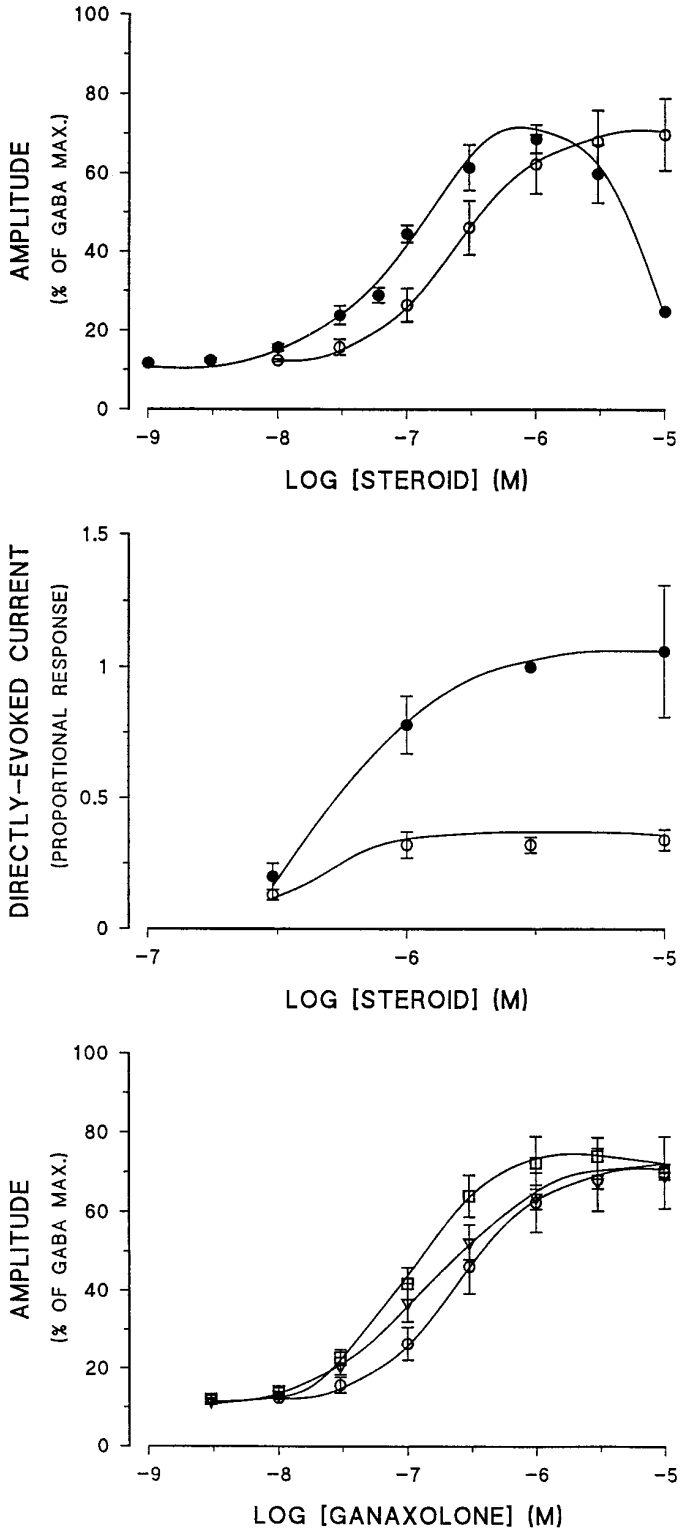

Fig. 3. Top and middle, concentration-response curves for ganaxolone (○) and 3α,5α-P (●) potentiation of GABA-evoked currents (top) or direct activation (middle) of $GABA_A$ receptors (human cloned α1, β1 and $\gamma 2_L$) expressed in *Xenopus* oocytes. Bottom, concentration-response functions for ganaxolone enhancement of GABA-evoked currents recorded from oocytes expressing human recombinant $\alpha 1\beta 1\gamma 2_L$ (○), $\alpha 2\beta 1\gamma 2_L$ (□) or $\alpha 3\beta 1\gamma 2_L$ (▽) $GABA_A$ receptors. Data are expressed as percent potentiation of current elicited by GABA (top and bottom) or response proportional to the current evoked by 3 μM 3α,5α-P (middle). Each point represents the mean ± S.E.M. of four to seven oocytes.

the GABA-evoked current produced by 3 μM ganaxolone (68.2 ± 7.9% of the GABA maximum) was similar to the maximal effect produced by 1 μM 3α,5α-P (68.8 ± 4% of GABA). Concentrations of 3α,5α-P greater than 1 μM resulted, however, in a reduced magnitude of potentiation, giving a "bell-shaped" steroid concentration-response curve (Woodward *et al.*, 1992). In contrast, the magnitude of the potentiation of GABA-evoked currents by ganaxolone was well maintained at a supramaximal concentration (10 μM) of the steroid (fig. 3, top). At relatively high concentrations, neuroactive steroids such as 3α,5α-P, in the absence of GABA, directly activate the $GABA_A$ receptor-channel complex (Lambert *et al.*, 1995). Here, in the absence of GABA, bath application of 1 to 10 μM 3α,5α-P induced a relatively small inward current that was potentiated by the coapplication of flunitrazepam (0.3 μM) and antagonized by picrotoxin (30 μM) (data not shown). Collectively, these observations suggest the inward current to be mediated *via* activation of $GABA_A$ receptors. The magnitude of the maximal current produced by the steroid is only 1% of that produced by a maximally effective concentration of GABA. For comparison, under identical recording conditions, the anesthetics propofol (300 μM) and pentobarbital (2 mM) produced maximal currents of 36.7 ± 6.4% (n = 4) and 28.2 ± 2.1% (n = 3) of the GABA maximum, respectively (data not shown). Relatively high concentrations of ganaxolone (1–10 μM) also evoked an inward current response, but this current was only 32% of the maximal current evoked by 3α,5α-P (fig. 3, middle). The α subtype (α1, α2 or α3) has little or no influence on the GABA receptor-modulatory actions of 3α,5α-P (D. Belelli and C. Hill-Venning, unpublished observations). Here, we investigated the influence of the α subtype ($\alpha 1\beta 1\gamma 2_L$, $\alpha 2\beta 1\gamma 2_L$ and $\alpha 3\beta 1\gamma 2_L$) on the positive allosteric actions of ganaxolone. Ganaxolone produced similar concentration-dependent enhancements of the currents induced by equieffective GABA concentrations (EC_{10}) with all three receptor subunit combinations tested (fig. 3, bottom). The magnitude of the maximal steroid effect was similar across the three human recombinant $GABA_A$ receptor subtypes tested here, although ganaxolone was modestly (~2 fold) more potent at $\alpha 2\beta 1\gamma 2_L$ and $\alpha 3\beta 1\gamma 2_L$ $GABA_A$ receptors, relative to the $\alpha 1\beta 1\gamma 2_L$ receptor subunit combination.

Effect on chemically induced seizures in mice and rats. Dose-response and time-course data for ganaxolone, valproate and ethosuximide protection against clonic seizures induced by s.c. PTZ in mice are presented in figure 5. All compounds exhibited rapid onset of action after i.p. administration (peak effect, 10 min) and displayed similar durations of effect (fig. 5, bottom). Ganaxolone produced potent anticonvulsant effects, with an i.p. ED_{50} of 4.3 mg/kg in mice and 7.8 mg/kg in rats (table 5). Ganaxolone was also active after oral administration in rats, with an ED_{50} of 21.0 mg/kg. Both ganaxolone and valproate produced impairment of motor function at multiples of their effective doses against PTZ. The Rotorod test yielded TD_{50} values of 33.4 and 14.2 mg/kg i.p. for ganaxolone in mice and rats, respectively. PI (TD_{50}/ED_{50}) values for ganaxolone compared favorably with those of valproate (table 5), with indices of 7.8 and 1.8 for ganaxolone after i.p. administration in mice and rats, respectively. Ganaxolone also demonstrated potent anticonvulsant activity against clonic seizures induced by systemic administration of bicuculline, TBPS and aminophylline in mice (table 6).

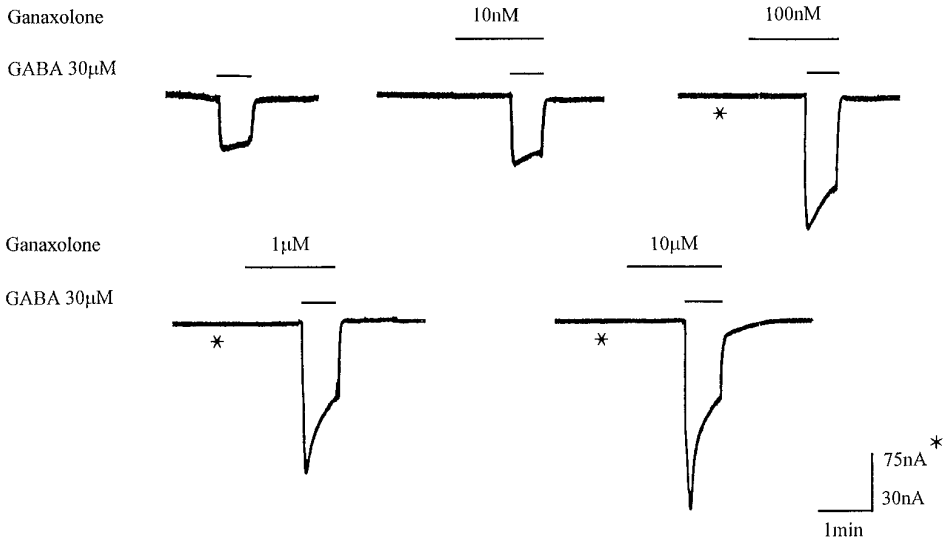

Fig. 4. Sample records illustrating potentiation of GABA-evoked currents by ganaxolone in a *Xenopus* oocyte expressing the human $GABA_A$ $\alpha1\beta1\gamma2_L$ subunit combination. Each trace depicts the response evoked by 30 μM GABA superfused for the period denoted by the bottom line. Application of ganaxolone (top line) enhances the peak inward current response to GABA in a concentration-dependent manner. ⋆, note the change in the vertical calibration bar.

TABLE 4
Ganaxolone potentiation of GABA-evoked currents from human recombinant $GABA_A$ receptors

	$\alpha1\beta1\gamma2_L$	$\alpha2\beta1\gamma2_L$	$\alpha3\beta1\gamma2_L$
EC_{50} (nM)	213 ± 14	94 ± 6	122 ± 9
E_{max} (% of GABA maximum)	70 ± 9	74 ± 5	69 ± 1
Hill coefficient	1.2 ± 0.1	1.3 ± 0.1	1.1 ± 0.1

Notably, ganaxolone displayed potency against bicuculline (ED_{50} of 4.6 mg/kg i.p.) equivalent to that obtained for PTZ, whereas markedly higher doses of valproate and ethosuximide were required to block bicuculline-induced convulsions.

Effect on MES-induced seizures in mice and rats. Dose-response data for ganaxolone, phenytoin and valproate protection against tonic convulsions induced by MES in mice are presented in figure 6. The onset of action against MES after i.p. administration was slower than observed against PTZ, with ganaxolone and valproate exerting peak effects at 30 min after the dose and phenytoin doing so at 60 min (data not shown). Ganaxolone was less potent against MES (ED_{50} of 29.7 mg/kg i.p.) than against PTZ, resulting in a PI of 1.1, as opposed to 1.9 for valproate (table 5). Similar results were obtained in rats (table 5).

Effect on cornea-kindled seizures in rats. Dose-response data for ganaxolone and valproate inhibition of cornea-kindled seizures in rats are presented in figure 7. In animals kindled to stage 5 seizures by daily electrical stimulation of the cornea, i.p. administration of ganaxolone and valproate completely suppressed kindled seizures, *i.e.,* reduced seizure severity scores to 0 (fig. 7, bottom). Ganaxolone exhibited potent anticonvulsant effects against kindled seizures, with an i.p. ED_{50} of 4.5 mg/kg calculated for a reduction of stage 5 seizures to stage 3 or less (fig. 7, top). Rotorod data used to calculate the rat TD_{50} values presented in table 5 are presented in figure 7, top, and illustrate the PI values of 3.2 and 4.1 obtained for ganaxolone and valproate activity, respectively, against kindled seizures.

Effect on seizure threshold in mice. Dose-response curves for ganaxolone and valproate effects on the seizure threshold are presented in figure 8. Ganaxolone administered i.p. increased the dose of i.v. infused PTZ required to produce clonic convulsions in unrestrained mice, in a dose-dependent manner. Valproate also increased the dose required to induce clonic seizures; however, it did so at doses roughly equivalent to those that impaired motor function. In contrast, ganaxolone increased the seizure threshold >200% before attaining a dose sufficient to produce ataxia in the Rotorod test (fig. 8).

Discussion

The present study shows that ganaxolone is a stereoselective, high-affinity, steroid modulator of the $GABA_A$ receptor complex *in vitro*. Moreover, these experiments demonstrate that ganaxolone exerts potent anticonvulsant effects in a broad range of animal seizure models with predictive validity for a number of different human epileptic conditions. The profile of anticonvulsant activity obtained for ganaxolone is similar in many respects to that of the clinically used reference agent valproate, although it differs with respect to its PI for MES. Ganaxolone is superior to valproate, however, in its ability to increase the seizure threshold for i.v. PTZ infusion at nonataxic doses. Most notably, in cornea-kindled rats, ganaxolone and valproate produce a complete abolition of seizure activity.

Although steroid hormones (Craig, 1966; Seyle, 1942), steroid anesthetics (Högskilde *et al.*, 1988; Peterson, 1989) and endogenous neurosteroids (Bellelli *et al.*, 1989; Wieland *et al.*, 1995) have long been known to possess anticonvulsant properties, only recently has the potential clinical use of neuroactive steroids as antiepileptic drugs been seriously considered. Low potency arising from poor oral bioavailability is considered one of the major obstacles to neuroactive steroid drug development (Gee *et al.*, 1995; Kokate *et al.*, 1994). Therefore, ganaxolone, the 3β-methylated analog of the endogenous compound 3α,5α-P, was synthesized (Hogenkamp *et al.*, 1997). Our intent was to increase the therapeutic utility of the molecule by preventing rapid oxidation or conjugation at the 3α-hydroxy position, and the resulting loss of neuronal activity, without altering the fundamental pharmacological properties of the compound. Data from the *in vitro* experiments reported herein support the view that ganaxolone retains positive allosteric modulatory effects at $GABA_A$

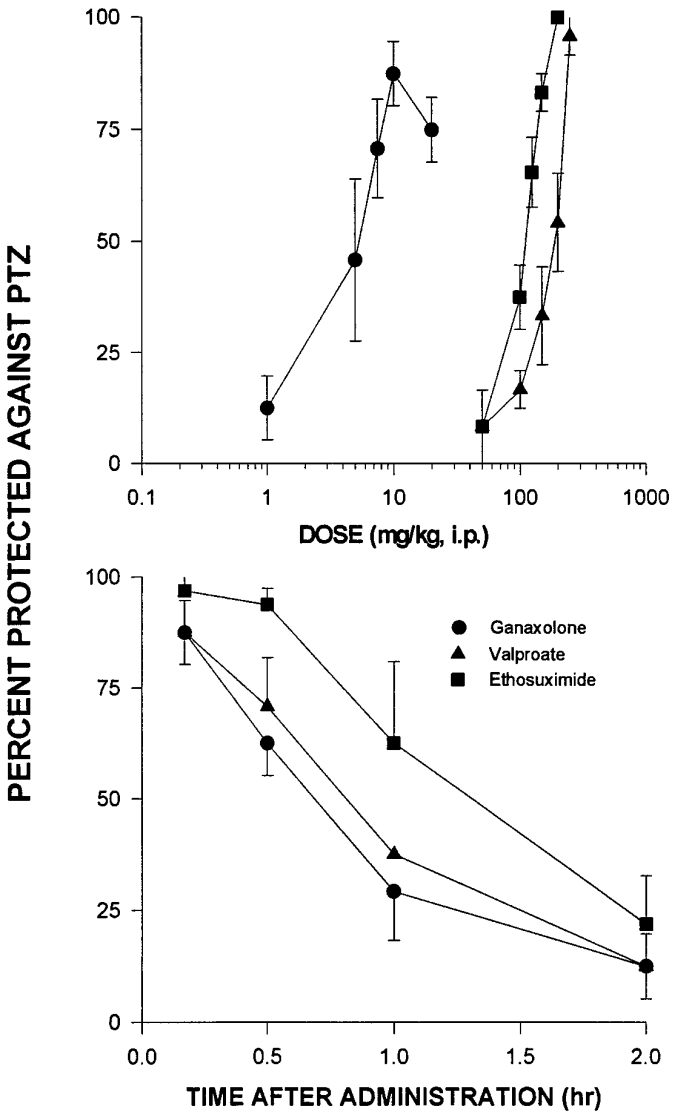

Fig. 5. Dose-response (top) and time-course (bottom) data for ganaxolone, valproate and ethosuximide inhibition of s.c. PTZ-induced seizures in mice after i.p. administration. Dose-effect curves were determined at the time of peak effect for each compound (10 min). Each point represents the mean ± S.E.M. of three independent experiments (n = 8).

receptors similar to those of its endogenous congener 3α,5α-P.

Ganaxolone inhibits specific binding of the $GABA_A$ receptor-chloride channel ligand [^{35}S]TBPS to rat brain cortical membranes in a concentration-dependent manner. Ganaxolone also produces a concentration-related enhancement of specific [^{3}H]flunitrazepam binding to the benzodiazepine modulatory site and [^{3}H]muscimol binding to the GABA recognition site on the $GABA_A$ complex. The potency and efficacy with which ganaxolone produces these effects are similar, in each instance, to those of 3α,5α-P. The effects obtained for 3α,5α-P are consistent with previous reports of neuroactive steroid binding affinity (Gee *et al.*, 1988; Goodnough and Hawkinson, 1995; Harrison *et al.*, 1987; Hawkinson *et al.*, 1994). Consistent with these findings, electrophysiological studies demonstrated that ganaxolone potentiated GABA-evoked currents recorded from human $\alpha1\beta1\gamma2_L$ $GABA_A$ receptor subunits. Ganaxolone is active over a similar concentration range, compared with 3α,5α-P. The effects of ganaxolone and 3α,5α-P differ, however, in that a reduction in the magnitude of the potentiation produced was associated with concentrations of 3α,5α-P of >1 μM, whereas this phenomenon was not evident with supramaximal (>3 μM) concentrations of ganaxolone. This property of 3α,5α-P has been previously observed and has been attributed to an increase of receptor desensitization and/or ion channel blockade by the steroid (Woodward *et al.*, 1992).

At relatively high concentrations, 3α,5α-P has been reported to directly activate the $GABA_A$ receptor channel complex in neuronal tissue (for review, see Hill-Venning *et al.*, 1994). Consistent with these observations, both 3α,5α-P and ganaxolone directly activated the recombinant $GABA_A$ receptors expressed in oocytes. The maximum current induced by these steroids was much less than that evoked by the anesthetics propofol and pentobarbital, however, and amounted to $<1\%$ of the current evoked by a maximally effective concentration of GABA. The limited direct agonist action of neuroactive steroids is not peculiar to recombinant receptors, because we have recently obtained similar data for the $GABA_A$ receptors native to bovine chromaffin cells (J. J. Lambert and D. Belelli, unpublished observations). This property appears to distinguish such steroids from the non-steroidal anesthetics pentobarbital and propofol. Although the magnitude of the direct current elicited by 3α,5α-P is modest, in comparison with that evoked by propofol and pentobarbital, it is approximately 3 times that evoked by ganaxolone. Because the GABA-mimetic action of the barbiturates has been associated with their anesthetic actions (Shulz and MacDonald, 1981), the relatively weak "agonist" effects of ganaxolone may be of advantage in considering its potential therapeutic utility as an antiepileptic agent.

The effects of benzodiazepines at $GABA_A$ receptor isoforms are clearly influenced by subunit composition (for review, see Lüddens *et al.*, 1995). In contrast, neuroactive steroids do not require a strict subunit composition for activity (for review, see Lambert *et al.*, 1995). Consistent with previous results, in the present study the α subtype (α1, α2 or α3) had no influence on the magnitude of the maximal potentiation of the GABA-evoked current produced by ganaxolone, although the steroid did display a modest (2-fold) selectivity for α2- or α3-containing receptors, compared with α1-containing receptors.

The profile of anticonvulsant activity exhibited by ganaxolone in the present experiments, although distinct, is similar in many respects to that of the reference antiepileptic valproate. Ganaxolone is effective against clonic convulsions induced by the CD_{97} dose of systemically administered PTZ in mice and rats. Moreover, it blocks clonic seizures induced by the chemoconvulsants bicuculline, TBPS and aminophylline but not those produced by strychnine in mice. These data are consistent with the demonstrated ability of ganaxolone to facilitate GABAergic neurotransmission (Löscher, 1981a, 1985; Meldrum, 1985) and suggest that this compound may have potential utility in the treatment of absence (*petit mal*) epilepsy (Stone, 1972; Swinyard and Woodhead, 1982). It is interesting to note that valproate, which exhibits these anticonvulsant activities, has been shown to increase whole-brain GABA levels (Löscher, 1981b), as well as to interact with the picrotoxin binding site on the GABA receptor-chlo-

TABLE 5
Anticonvulsant profile of ganaxolone, compared with valproate

	Ganaxolone				Valproate			
	Mice		Rats		Mice		Rats	
	ED_{50} or TD_{50}[a]	PI	ED_{50} or TD_{50}[a]	PI	ED_{50} or TD_{50}[a]	PI	ED_{50} or TD_{50}[a]	PI
	mg/kg		*mg/kg*		*mg/kg*		*mg/kg*	
PTZ, i.p.	4.3 (2.8–6.9)	7.8	7.8 (6.2–8.9)	1.8	159.3 (82–256)	3.2	226.9 (194–254)	2.1
PTZ, p.o.	ND[b]		21.0 (18.1–24.3)	2.3	225.0 (182–329)	6.0	ND	
MES, i.p.	29.7 (25.3–34.8)	1.1	ND		259.2 (231–291)	1.9	ND	
MES, p.o.	ND		58.4 (44.5–76.8)	0.8	316.5 (273–367)	4.3	477.6 (349–655)	
Kindling, i.p.	ND		4.5 (4.0–5.1)	3.2	ND		119.1 (89.7–132.9)[c]	4.1
Rotorod, i.p.	33.4 (30.9–39.4)		14.2 (12.6–15.8)		514.2 (364–712)		485.3 (393–555)	
Rotorod, p.o.	ND		48.3 (38.7–60.3)		1350 (1174–1552)		ND	

[a] ED_{50} and TD_{50} values expressed in mg/kg, with 95% confidence intervals (in parentheses).
[b] ND, not determined.
[c] From Swinyard *et al.* (1993).

TABLE 6
Anticonvulsant effects of ganaxolone and reference antiepileptic drugs against various chemoconvulsants in mice

	Ganaxolone		Valproate		Ethosuximide	
	ED_{50}	PI	ED_{50}	PI	ED_{50}	PI
	mg/kg		*mg/kg*		*mg/kg*	
PTZ	4.3 (2.9–6.9)	7.8	159 (82–256)	3.2	101 (83–114)	3.3
Bicuculline	4.6 (3.2–6.8)	7.3	360[b]	1.4	459[b]	0.7
Aminophylline	11.5 (8.1–16.3)	2.9	146 (108–196)	3.5	195 (156–243)	1.7
Strychnine	>40	<1	293[b]	1.8	>360[b]	0.9
TBPS	11.7 (8.8–15.7)	2.9	212 (147–306)	2.4	272 (203–365)	1.2

[a] ED_{50} values expressed in mg/kg, i.p., with 95% confidence limits (in parentheses).
[b] From Gladding *et al.* (1985).

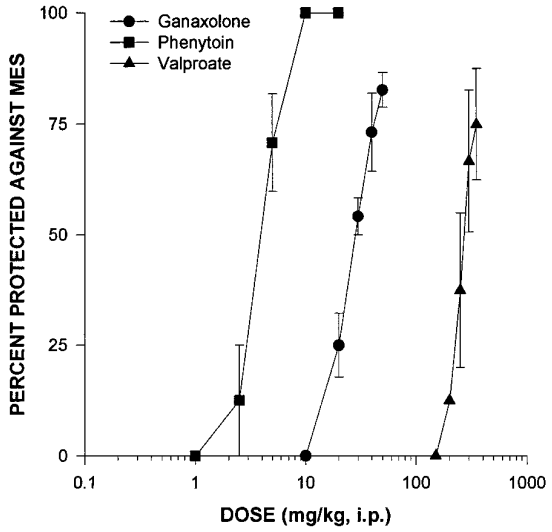

Fig. 6. Dose-response data for ganaxolone, valproate and phenytoin inhibition of MES-induced seizures after i.p. administration in mice. Dose-effect curves were determined at the time of peak effect for each compound (ganaxolone and valproate, 30 min; phenytoin, 60 min). Each point represents the mean ± S.E.M. of three independent experiments (n = 8).

ride ionophore (Ticku and Davis, 1981). Ganaxolone is also effective at blocking tonic seizures induced by MES in mice, although this activity is observed only at doses that produce motor impairment on the Rotorod. Thus, these data suggest that ganaxolone may not be as useful in the management of generalized tonic-clonic convulsions (Swinyard, 1972; Swinyard and Woodhead, 1982).

Of particular significance, ganaxolone exhibits potent anticonvulsant activity toward cornea-kindled seizures in rats. Against fully kindled stage 5 convulsions, ganaxolone is as effective as, and more potent than, valproate at seizure suppression. More importantly, like valproate, ganaxolone completely abolishes the behavioral manifestations of kindling. In contrast, many antiepileptic agents that inhibit cornea-kindled seizures, such as carbamazepine and phenytoin, produce only partial suppression of seizure score even when doses that produce frank ataxia are administered (Swinyard *et al.*, 1993). Among positive allosteric modulators of the $GABA_A$ receptor, these data distinguish the neuroactive steroid ganaxolone from the benzodiazepine diazepam, in that the latter compound does not completely block kindled seizures (Löscher *et al.*, 1986). It should be noted, however, that the latter studies were conducted in amygdala-kindled rats, whereas the present experiments were performed in cornea-kindled rats. Further study of ganaxolone in kindling models of epilepsy is warranted; however, data obtained thus far suggest that ganaxolone may prove to be of some utility in the treatment of complex partial epilepsy in humans (McNamara, 1984; Racine, 1972).

It has often been stated that antiepileptic drugs that block MES-induced tonic extension act by blocking seizure spread, whereas drugs that prevent or delay clonic seizures induced by i.v. infusion of PTZ act by elevating the seizure threshold (Löscher and Schmidt, 1988; Rogawski and Porter, 1990). There are numerous molecular mechanisms through which drugs can block seizure spread and/or elevate seizure threshold. Indeed, attempts to correlate the anticonvulsant profiles of antiepileptic drugs with specific mechanisms of action

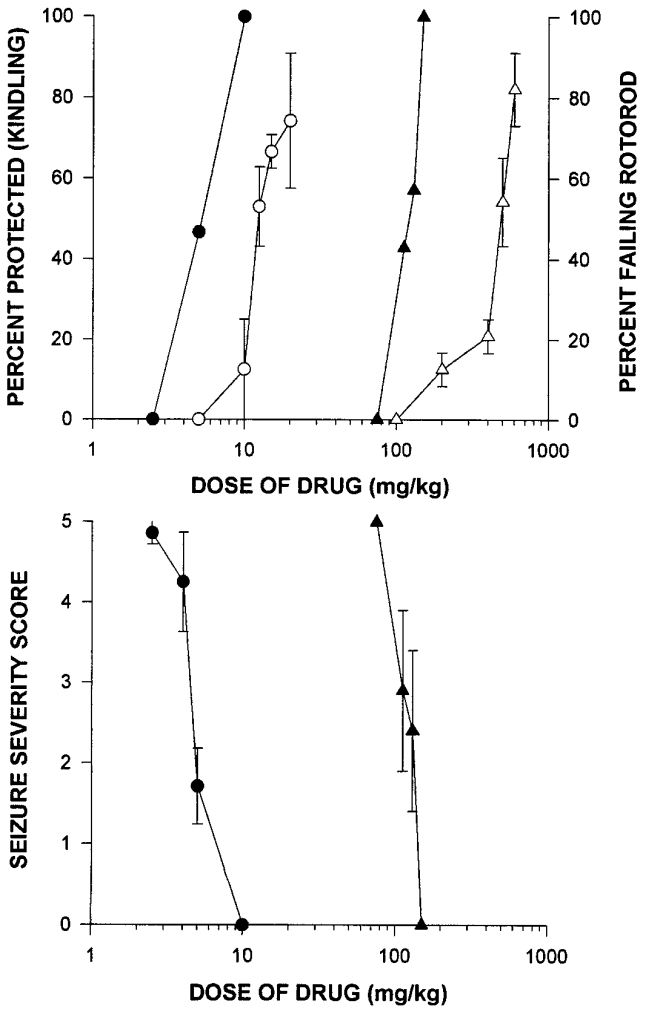

Fig. 7. Dose-response data for ganaxolone (circles) and valproate (triangles) inhibition of cornea-kindled seizures (closed symbols) and deficits in Rotorod performance (open symbols) (top) and effect on seizure severity score (bottom) in rats after i.p. administration. Kindling experiments were conducted once (n = 5–8); dose-effect curves were determined at the time of peak effect (30 min); severity scores are expressed as mean ± S.E.M. Rotorod dose-response data were obtained at the time of peak activity (30 min); each point represents the mean ± S.E.M. of three independent experiments (n = 8).

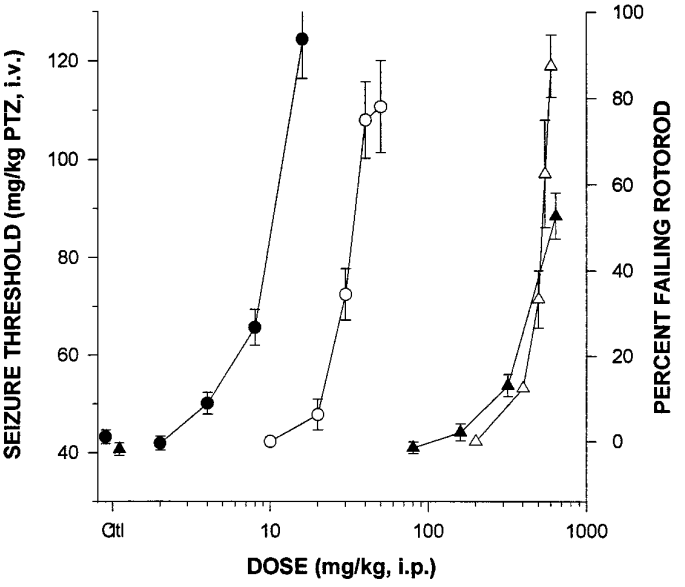

Fig. 8. Dose-response data for ganaxolone (circles) and valproate (triangles) increases in the seizure threshold (closed symbols) and deficits in Rotorod performance (open symbols) after i.p. administration in mice. For seizure threshold data, each point represents the mean ± S.D. of data pooled from two independent experiments (n = 18–20). For Rotorod data, each point represents the mean ± S.E.M. of three independent experiments (n = 8).

reveal certain notable trends. For example, MES-induced tonic extension can be blocked by drugs that inhibit voltage-dependent Na^+ channels, such as phenytoin, carbamazepine, lamotrigine, felbamate and valproate (Macdonald and Kelly, 1995; Rogawski and Porter, 1990; White, 1997), as well as by drugs that block glutamatergic excitation mediated by the *N*-methyl-D-aspartate receptor, such as felbamate (McCabe *et al.*, 1993; Subramaniam *et al.*, 1995; White *et al.*, 1995). In contrast, clonic seizures induced by PTZ can be blocked by drugs that reduce T-type Ca^{++} currents, such as ethosuximide (Coulter *et al.*, 1989), and drugs that enhance $GABA_A$ receptor-mediated inhibitory neurotransmission, such as benzodiazepines, phenobarbital and perhaps valproate and felbamate (Macdonald and Kelly, 1995; Rogawski and Porter, 1990; White, 1997). Thus, it is not surprising that antiepileptic drugs with multiple mechanisms of action, like valproate and felbamate, are effective in both types of seizure tests and display the broadest therapeutic utility. Because ganaxolone exhibits anticonvulsant activity against s.c. PTZ and to a lesser extent against MES, its ability to also increase the i.v. PTZ infusion threshold is significant. Moreover, although valproate increased the dose of i.v. PTZ required to induce clonic seizures, it did so at doses roughly equivalent to those that impaired motor function. In contrast, ganaxolone increased the seizure threshold >200% before attaining a dose sufficient to produce ataxia on the Rotorod. The ability of ganaxolone to both elevate seizure threshold and block s.c. PTZ-induced clonus can be attributed to its modulatory effect on $GABA_A$ neurotransmission. Whether this effect contributes to its ability to block MES-induced tonic extension is not known, but that is likely, because there is at present no experimental evidence to suggest that ganaxolone blocks voltage-dependent Na^+ channels or *N*-methyl-D-aspartate receptors. Nevertheless, the present data suggest that ganaxolone may possess broad therapeutic utility in the treatment of human epilepsy.

The PIs obtained for ganaxolone in the present study deserve comment. Clinically used antiepileptic drugs yield PIs after i.p. administration that range between 2 and 60, depending upon the drug, species and anticonvulsant test used (Löscher and Schmidt, 1988). Although the values obtained for ganaxolone (table 5) do not approximate those for some recently developed compounds, *e.g.,* felbamate (Swinyard *et al.*, 1986), but are closer to those reported for the endogenous neuroactive steroids (Kokate *et al.*, 1994), they do compare favorably with PIs obtained for valproate in s.c. PTZ and corneal kindling procedures and far exceed the separation observed for valproate in the i.v. PTZ test. Valproate is acknowledged to exhibit a much wider separation between its therapeutic effects and dose-limiting side effects clinically than would be predicted on the basis of the animal data, whereas the opposite is true for phenytoin (Löscher and Schmidt, 1994; Rogawski and Porter, 1990). Thus, the pre-

dictive validity of PI values for neuroactive steroids, such as ganaxolone, awaits clinical verification. These numbers are useful, however, for selecting from among neurosteroid drug development candidates and set the standard with which future compounds from this class can be compared.

As stated at the outset, the primary rationale underlying 3β-substitution of the pregnane steroid nucleus was to enhance the oral bioavailability of the neuroactive molecule without altering its fundamental pharmacological profile. Data from *in vitro* experiments clearly indicate that ganaxolone retains positive allosteric modulatory effects at the $GABA_A$ receptor comparable to those of its endogenous non-3β-substituted congener 3α,5α-P. Moreover, *in vivo* studies reveal that ganaxolone administered i.p. possesses anticonvulsant properties comparable to those previously reported for 3α,5α-P (Bellili *et al.*, 1989; Kokate *et al.*, 1994; Wieland *et al.*, 1995). Inasmuch as the present experiments demonstrate the oral anticonvulsant activity of ganaxolone in rats, a pharmacological action not possessed by 3α,5α-P (R. B. Carter and S. Wieland, unpublished observations), our attempt to increase the potential therapeutic utility of a neuroactive steroid through protection of the 3α-hydroxy moiety may be judged successful.

In summary, ganaxolone is a high-affinity, stereoselective, positive allosteric modulator of $GABA_A$ receptors that exhibits potent, broad-spectrum, anticonvulsant activity. The profile of anticonvulsant activity obtained for ganaxolone compares favorably with that of valproate. The present study supports clinical evaluation of ganaxolone as an antiepileptic medication with potential therapeutic utility in the treatment of generalized absence seizures as well as simple and complex partial seizures.

Acknowledgments

The authors thank the following people for their excellent technical assistance: Mian Alauddin for organic synthesis, Manuel Acosta for radioligand binding and Rennolds Ostrom, Silvia Robledo, Michael Suruki and Leah Swanekamp for animal studies.

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