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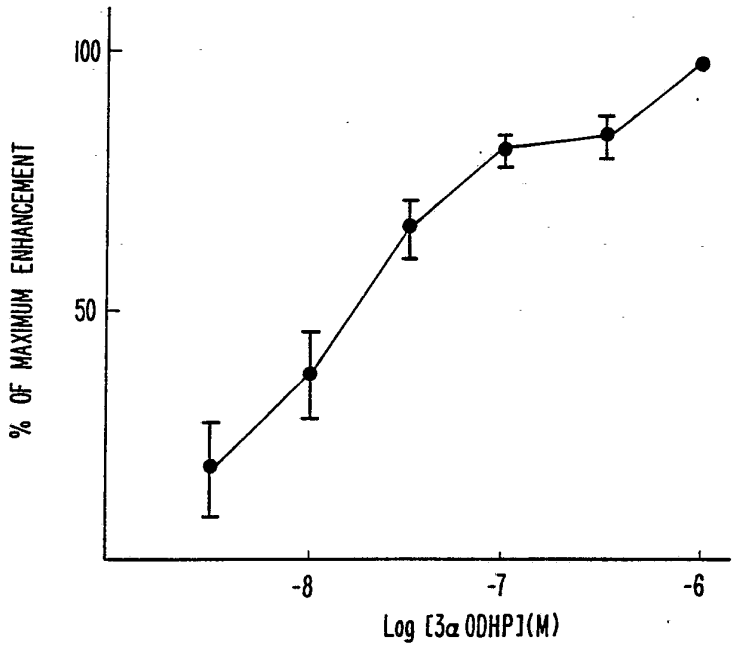

(57) Abstract

There is disclosed a $GABA_A$ receptor-chloride ionophore complex (GRC), where GABA represents gamma-aminobutyric acid, having a novel binding site, the GNR, independent of other known sites on the GRC. There is also disclosed a method for screening for agonists of the GNR by forming an expressed GRC by co-expression cDNA encoding the GRC subunits in cells and determining the ability of a steroid to modulate allosterically benzodiazepine binding to the expressed receptor complex. The figure shows the enhancement of the binding of a benzodiazepine analog to the GRC as a function of the concentration of a steroid modulator which binds to the GNR.

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DESCRIPTION

Novel $GABA_A$ Receptor With Steroid Binding Sites

Reference to Related Applications

This is a continuation-in-part of co-pending application Serial No. 07/517,194 filed May 1, 1990, which, in turn, is a continuation-in-part of co-pending application Serial No. 379,047 filed July 13, 1989, which in turn is a continuation-in-part of application Serial No. 089,362, filed August 25, 1987, now abandoned. The disclosure of application Serial No. 517,194 and application Serial No. 379,047 are expressly incorporated herein by reference.

Background of the Invention

Previous studies have shown that certain pregnane steroids, particularly the 3α-hydroxylated-5α-reduced metabolites of progesterone, have rapid and profound effects on brain excitability. In recent investigations, evidence has indicated that the brain excitability effects may be mediated by the $GABA_A$ receptor-chloride ionophore complex (GRC) where GABA represents γ-aminobutyric acid; the pregnane steroids modulate the GRC through a novel site independent of other known sites on the GRC.

Barbiturates and benzodiazepines, both classes of therapeutically useful drugs, are known to be able to modulate allosterically the GRC. It is believed that there are sites on certain GRCs for barbiturates and the benzodiazepines. The present invention, in part, defines a new receptor site on the GRC that is specific for steroids. Further, it relates to a method for determining whether other receptor sites exist on the GRC, as well as a method for determining what molecules bind to any newly identified site.

General Description of the Invention

The present invention relates to a GRC bearing a steroid recognition or binding site for the opening of the chloride channel in the GRC. We have discovered that this $GABA_A$ associated neurosteroid receptor (GNR) exists, is independent from other known sites on the GRC, and has high affinity for its agonists. We have been able to prepare a GRC having fewer than the number of subunits found in the naturally-occurring receptor but having nevertheless a GNR. Our invention relates to a GRC having fewer than all the subunits of the naturally occurring receptor but at least the alpha (α) and beta (β) subunits, and, in some instances, also the gamma (γ) subunit. Our investigations, discussed in detail below, have shown also that it is not necessary for the receptor to have all of the α, β, and γ subunits for the binding site to be present in order for the site to be active. The GNR may, depending on the binding agent used, reside on the $\alpha\beta$ subunit combination.

The present invention also relates to a method for determining the existence of the GNR in the GRC, as well as any other recognition sites present on that complex. Allosteric modulatory assays are described for determining the ability of compounds to bind to the complex at new or at previously known sites. Competitive assays can also be used for this purpose.

The present invention also provides a means for screening for GNR-agonist drugs effective in treating stress, anxiety, insomnia, post natal depression (PND), pre-menstrual syndrome (PMS), nervous disorders such as depression, and seizures. It also provides a means for treating the above named conditions through the administration of agonists to the GNR.

Additionally, the present invention provides a means for screening for drugs that bind to GNRs with different subtype specificities.

Recent studies suggest that metabolites of progesterone, particularly 3α-hydroxy-5α-pregnan-20-one (3α-OH-DHP), are potent modulators of the $GABA_A$ receptor complex; Majewska et al., "Steroid Hormone Metabolites Are Barbiturate-like Modulators Of The GABA Receptor," Science 232, 1004 (1986); Gee et al., "Gamma-aminobutyric acid-dependent Modulation Of The Chloride Ionophore By Steroids In Rat Brain," Eur. J. Pharmacol. 136, 419 (1987). It was thought originally that this steroid modulation was mediated by the barbiturate site on the receptor complex; Majewska et al., supra; Harrison et al., "Structure-activity Relationships For Steroid Interaction With The Gamma-aminobutyric acid-A Receptor Complex," J. Pharmacol. Exp. Ther. 241, 346 (1987). However, subsequent experiments demonstrated that these steroids do not interact at the barbiturate site, but rather act on a novel site associated with the receptor complex; Gee et al., "Steroid Modulation Of The Chloride Ionophore In Rat Brain: Structure-activity Requirements, Regional Dependence and Mechanism Of Action," J. Pharmacol. Exp. Ther. 246, 803 (1988); U.S. patent application Serial No. 089,362, filed August 25, 1987, now abandoned; Peters et al., "Modulation Of The $GABA_A$ Receptor By Depressant Barbiturates And Pregnane Steroids," Br. J. Pharmacol. 94, 1257 (1988).

Recent cloning efforts reveal that the $GABA_A$ receptor consists of at least 3 subunits, α, ß, and γ; Schofield et al., "Sequence And Functional Expression Of The $GABA_A$ Receptor Shows A Ligand-gated Receptor Super-family," Nature 328, 221 (1987); Pritchett et al., "Importance Of A Novel $GABA_A$ Receptor Subunit For Benzodiazepine Pharmacology," Nature 338, 582 (1989). In addition, variants of the α, ß and γ subunits also exist; Levitan et al., "Structural And Functional Basis For $GABA_A$ Receptor Heterogeneity," Nature 335, 76 (1988); Ymer et al., "$GABA_A$

Receptor ß Subunit Heterogeneity: Functional Expression Of Cloned cDNAs," EMBO J. 8, 1665 (1989). These subunit variants may represent functionally different GRC subtypes. Experiments involving the transient expression of the cDNAs encoding these GRC subunits into mammalian cells demonstrated the reconstitution of the GABA and barbiturate sites in the α and ß subunit combination, whereas the benzodiazepine (BZ) site was detected only when all three subunits were transfected; see Pritchett et al., supra.

To test the hypothesis that a steroid site exists on the GRC, the cDNAs encoding human $GABA_A$ receptor α_1, α_2 or α_3, plus $ß_1$ and γ_2 subunits were co-expressed in human embryonic kidney 293 cells and the ability of steroids to allosterically modulate benzodiazepine and $GABA_A$ binding and to inhibit the direct binding of [^{3}H]3α-OH-DHP to the expressed receptor complex was determined.

This invention demonstrates that the GNR has the same structure activity relationships as have been previously demonstrated in brain homogenate. Gee et al. (1988), supra. Allosteric modulation of $GABA_A$ binding through interactions at the GNR are unique from modulation through interactions at the other known receptor sites on the GRC. This invention therefore demonstrates the existence of the GNR as an independent site through confirmation of the physical independence of the GNR from other receptor sites on the GRC. This newly discovered site has high affinity for its ligand, in contrast to other sites on the GRC.

A heterogenous population of GNRs has been demonstrated in cortex and spinal cord based on variations in activity of GNR agonists at these two locations. Gee et al., "The $GABA_A$ Receptor Complex in Rat Frontal Cortex and Spinal Cord Show Differential Responses to Steroid Modulation," Molecular Pharm. in press. This heterogeneity is reflected in the different protein subunit subtype composition of the GNR, and may be

associated with different functions in the CNS and different effects on the body.

Further, a method for determining what molecules bind to any newly identified steroid site is presented, using, among other things, allosteric modulatory assays and competitive binding assays. The specificity of compounds identified by this invention is described, using both in vitro and in vivo data. These compounds, through their interaction with the steroid receptor and its sub-types described herein, have anxiolytic, anti-convulsant, sedative/hypnotic, PMS and PND relieving, and mood disorder relieving effects when administered in vivo.

Description of the Drawings

The present invention may be better understood and its advantages appreciated by those skilled in the art by referring to the accompanying drawings wherein:

FIG. 1 shows a graph of the percent of maximum enhancement of [^{3}H]FLU binding in the presence of GABA plotted against the log of the amount of 3α-OH-DHP;

FIG. 2 is a bar graph of the time to onset of myoclonus vs. different concentrations of steroid compounds useful in the present invention; 3α-OH-AND stands for 3α-hydroxy-androstane, PREG-SO_4 stands for pregnenolone sulfate, and PREG stands for pregnenolone;

FIG. 3 is a plot of the time course of anticonvulsant activity for various prodrugs and synthetic derivatives of 3α-OH-DHP;

FIG. 4 is a plot of oral anti-convulsant activity of neuroactive steroid prodrug and direct acting molecules;

FIG. 5 is a graph of the number of transitions occurring within ten minutes of administration of 3α-OH-DHP at various doses;

FIG. 6 is a graph of the number of transitions occurring within ten minutes of administration of 3α,21-dihydroxy-5α-pregnan-20-one (5α-THDOC) at various doses;

FIG. 7 is a graph of the number of transitions occurring within ten minutes of administration of Diazepam at various doses;

FIG. 8 is a graph comparing the number of transitions within ten minutes of administration of carrier (ß-Cyclodextrin), 3ß-hydroxy-5α-pregnan-20-one (3ß-OH-DHP) AND 3α-OH-DHP;

FIG. 9 is a set of graphs showing the enhancement of the number of transitions caused by 3α-OH-DHP and Diazepam, and the effect of the experimental drug CGS-8216 on their enhancement;

FIG. 10 shows the percent increase in punished responding caused by 3α-OH-DHP as compared to the increase caused by the positive control, Chlordiazepoxide;

FIG. 11 is a plot showing the effect of progesterone metabolites and promogesterone (R5020) on [^{3}H]-R5020 binding to the progesterone receptor in rat uterus;

FIG. 12 shows differential responses of expressed human GRCs with various α subunits in combination with $ß_1$ and γ_2 subunits to 3α-OH-DHP;

FIG. 13 shows [^{3}H]-flunitrazepam ([^{3}H]-FLU) binding in response to 3α-OH-DHP in P_2 homogenate from cerebellum and spinal cord; and

FIG. 14 shows responses to 3α-OH-DHP in receptors composed of αß subunits.

Detailed Description of the Invention

The present invention utilizes bioengineering techniques to search for the hypothesized steroid site on the GRC separate from any known sites on the GRC such as the barbiturate and BZ sites. In this invention, cDNAs encoding human GRC α_1, α_2 or α_3, plus $ß_1$ and γ_2 subunits were expressed in eukaryotic cell cultures such as cultures of mammalian cells. Additional studies were done on complexes containing α_1, α_2, or α_3 with $ß_1$. Following transfection and expression, binding assays were conducted with and without steroids to determine whether the

steroids can allosterically modulate binding to the benzodiazepine and GABA sites on the expressed receptor complex and to determine the ability of [^{3}H]3α-OH-DHP to label directly the GNR in a stereospecific manner. [^{3}H]-FLU binds to the BZ site on the GRC and can be used for monitoring that BZ site, while [^{3}H]-muscimol binding labels the $GABA_A$ site on the GRC.

Once receptor sites, such as the GNR, are elucidated by this invention, these receptors can be used for screening pharmaceuticals which will have various physiological effects upon administration to patients. For example, the steroid sites of this invention can be used to screen for agonist compounds with anxiolytic, anti-convulsant, hypnotic, sleep inducing, anti-depressant, anti-PND, anti-PMS, and other effects.

Mammalian cells were selected for the cloning steps for their ability to take up vectors coding for the selected DNAs as well as to express the proteins in a functional manner. In this case, expression of the genes coding for the receptor subunits must assemble on the cell surfaces in a manner that mimics their natural configuration in cells in which the genes are normally expressed, i.e. neurons. One non-limiting example of such a cell type is human embryonic kidney cells, used in the Example below.

Numerous methods are available for transferring genes into cultured cells. For example, genes can be inserted into vectors such as plasmids, cosmids or retroviruses and transferred into the cells by known methods. Calcium phosphate precipitation, cell-cell fusion, electroporation, liposomes, lipofection and a variety of other methods are known in the field and can be implemented.

The following working examples provide one method for implementing the invention described herein.

Expression of the GNR

Various subunits of the GRC have been cloned (Pritchett et al., supra) and the cDNAs were used for the following experiments.

Example 1

Expression of $GABA_A$ Receptor Complexes

Human embryonic kidney 293 cells were seeded on 10-cm dishes the day before transfection. The cells were transfected with a total of 20 to about 30 μg of DNA per dish using a modified high efficiency $CaPO_4$ precipitation method described previously (Pritchett et al., supra). DNA used for the transfection was a mixture of equal amounts of cloned cDNA encoding the human $GABA_A$ α_1, α_2 or α_3, β_1 and γ_2 subunits constructed individually in an expression vector (Pritchett et al., supra). Forty-eight hours after transfection, the cells were harvested and washed twice with phosphate buffered saline (PBS) pH 7.2 and frozen or immediately used in [^{3}H]flunitrazepam ([^{3}H]FLU) binding assays.

BZ Site

Flunitrazepam is a BZ which binds to the BZ site on the GRC. Therefore, monitoring of [^{3}H]-FLU binding allows monitoring of the BZ site. The following experiments studied the allosteric modulatory effects on the BZ site of steroid binding at the GNR.

Example 2

[^{3}H]FLU Binding Assay

The transfected cells of Example 1 were homogenized in 10 mM potassium phosphate buffer, pH 7.2, and washed once by centrifugation. The cell membrane pellet (100,000 x g) was homogenized in a mixture of 10 mM potassium phosphate, pH 7.2, and 100 mM potassium chloride. Aliquots (100 μl) of cell membrane were incubated with 2 nM of [^{3}H]FLU (75 - 90 Ci/mmol, duPont,

New England Nuclear) in the presence of GABA (Sigma Chem. Co., St. Louis, MO) with or without steroids (Steraloids, Wilton, NH). All assays were brought to a final volume of 1 ml with 50 mM Na/phosphate buffer and 200 mM NaCl. Non-specific binding was defined as binding in the presence of 1 μM clonazepam (Sigma Chem. Co.). Assays were terminated after a 90 minute incubation at 25°C by rapid filtration through Schleicher and Schuell No. 32 glass fiber filters. The filters were washed twice with 5 ml of ice-cold PBS. Filter-bound radioactivity was determined by liquid scintillation spectrophotometry.

The ability of steroids to modulate allosterically [^{3}H]FLU binding to the expressed BZ receptor on the GRC was used to demonstrate the presence of a specific steroid recognition site, the GNR, on the expressed GRC that is functionally coupled to the BZ site. Since [^{3}H]FLU binding was detected only when the γ_2 subunit was included in the transfection (Pritchett et al., supra), and thus the BZ site was only present when the γ_2 subunit was included, α_1 (or α_2 or α_3), β_1 and γ_2 subunits were co-expressed transiently in all of the experiments performed in this study.

Consistent with previous findings, the enhancement of [^{3}H]FLU binding by GABA was more pronounced when the α_3 subunit was transfected; Pritchett et al., Type I and Type II $GABA_A$-benzodiazepine Receptors Produced In Transfected Cells, Science, 245, 1389 (1989). GABA at 1 μM enhanced [^{3}H]FLU binding and 3α-OH-DHP potentiated this enhancement; see Table 3A infra. Interestingly, at μM concentrations, 3α-OH-DHP alone also enhances [^{3}H]FLU binding although to a lesser extent than that observed in the presence of GABA; see Table 3A. Direct activation of chloride conductance by 3α-OH-DHP in the absence of GABA has also been observed in electrophysiologic studies; see Harrison et al., supra. No qualitative differences in the potentiation of [^{3}H]FLU binding were observed when the α_2 or the α_3 subunit was substituted for the α_1 subunit

(Table 3A). This observation suggests that the steroid binding site is also associated with various BZ receptor subtypes.

Figure 1 (Fig. 1) shows the potentiation of [^{3}H]FLU binding by 3α-OH-DHP in the presence of 1 μM GABA and various concentrations of 3α-OH-DHP. Each point on the graph represents an average of 6 separate experiments; the bars represent the SEM. [^{3}H]FLU binding in the absence of steroid was defined as control. The percentage of maximum enhancement was defined as a percent of the enhancement produced by 1 μM of 3α-OH-DHP which varied between 57% and 195% above control in these experiments.

The ability of several steroids to potentiate GABA stimulation of [^{3}]FLU binding is illustrated in Table 3B. Consistent with that observed in rat brain P_2 homogenates (Gee et al., 1988, supra), 3α-hydroxylated steroids such as tetrahydrodeoxycorticosterone (THDOC) and 3α-hydroxy, 5α-pregnan-20-one(3α,5ß-OH-DHP), were capable of potentiating GABA enhancement of [^{3}H]FLU binding. In contrast, 3ß-hydroxy-5α-pregnan-20-one (3ß-OH-5α-DHP) and progesterone have relatively low potencies. Thus, the stereoselectivity and the structure-activity requirements for the expressed steroid site are similar to that observed in the brain.

The dose-dependent potentiation of [^{3}H]FLU binding by 3α-OH-DHP was carried out in the presence of 1 μM of GABA in each experiment. The EC_{50} (concentration at which half-maximal enhancement is observed) for 3α-OH-DHP ranged from 53 to 340 nM, depending on the subunit composition. These values closely approximate those observed in rat brain homogenate under the similar experimental conditions (i.e., plus 1 μM GABA) (Gee et al., 1988, supra; U.S. patent application Serial No. 089,362, filed August 25, 1987, now abandoned). Thus, the existence of a specific steroid recognition site, the GNR, that is part of the expressed GRC, which is functionally coupled to the BZ and GABA receptors, has been demonstrated.

GABA$_A$ SITE

Muscimol is a specific GABA$_A$ agonist, which binds to the GABA$_A$ site on the GRC resulting in the opening of the Cl-channel. It has been shown in rat brain homogenate that steroids are capable of modulating (i.e., enhancing) [^{3}H]-muscimol binding with specificities similar to that observed for the enhancement of [^{3}H]FLU binding. Therefore, we studied the binding of muscimol in the receptors expressed from cDNA.

The experiments involving the expression of the GABA$_A$ receptor subunit cDNAs indicate that only the α and β subunits are needed to reconstruct the muscimol binding site. [^{3}H]-muscimol binding was performed under conditions similar to that for [^{3}H]FLU binding except 10 μM (+)bicuculline was used to define nonspecific binding. We tested for the possible existence of the steroid site on the complex composed of the α and β subunits. Our results demonstrate that a 3α-OH-DHP is capable of modulating (enhancing) [^{3}H]muscimol binding whereas its 3β-isomer has no activity. Furthermore, 5α-pregnan-3α,20α-diol appears to have limited efficacy similar to that demonstrated in in vivo and in vitro assays using brain homogenate preparations. The data suggest 1) the steroid recognition site resides on the α,β subunits and 2) 5α-pregnan-3α,20α-diol behaves like a partial agonist at this steroid site.

TABLE 1

[^{3}H] Muscimol Binding

Steroid	% of Enhancement	
	Subunits	
	$\alpha_2 + \beta_1$	$\alpha_3 + \beta_1$
3α-OH-DHP	389	801
5α-pregnan-3α,20α-diol	121	295
3β-OH-DHP	0	0

Direct Labeling of the GNR

3α-OH-DHP is a direct agonist at the GNR. [^{3}H]3α-OH-DHP was used in the following experiments to directly bind to and label the GNR.

Example 3

[^{3}H]3α-OH-DHP Binding Assay

The transfected cells of Example 1 were used to determine the presence of specific [^{3}H]3α-OH-DHP binding in a manner similar to that described for [^{3}H]FLU binding in Example 2. [^{3}H]3α-OH-DHP (2 nM) was used to label the steroid site. Nonspecific binding was defined as binding in the presence of 3 μM of the pharmacologically active stereoisomer, 3α-OH-DHP.

Binding to detergent solubilized steroid sites was determined by solubilizing the P_2 fraction from rat cerebral cortex under conditions described in Gee et al., Modulation Of The Chloride Ionophore By Benzodiazepine Receptor Ligands: Influence Of GABA And Ligand Efficacy, Mol. Pharmacol. 30:218-255 (1986). Ammonium sulfate (20%) was added to the soluble fraction and the resultant precipitate was pelleted by centrifugation at 100,000 x g. The pellet was washed three times in 50 mM Na/K phosphate buffer (pH 7.4) and 200 mM NaCl by centrifugation; the final pellet was resuspended in the buffer as a 10% (original wet wt./vol) homogenate. One hundred μl aliquots of the homogenate were incubated with 5 nM [^{3}H]3α-OH-DHP and buffer in a total reaction volume of 1 ml for 60 min. at 25°C.

Conditions for defining nonspecific and stereospecific binding were the same as those described for the expressed receptor. Incubations were terminated by rapid filtration through glass fiber filters (Whatman GF/C) that had been presoaked for 3 hours in 0.3% polyethyleneimine and 1% Triton X-100. Preparation of filters and quantification of filter bound radioactivity were performed as described in Example 2.

TABLE 2

[^{3}H]3α-OH-DHP Bound (counts/min)

Condition	Rat Cortical Membranes	Expressed Receptor
Total bound	2526	1121
+3 μM 3α-OH-DHP	1569	896
+3 μM 3ß-OH-DHP	2557	1262

The results demonstrate that the binding of [^{3}H]3α-OH-DHP can be specifically displaced by 3α-OH-DHP but not by the pharmacologically inactive stereoisomer 3ß-OH-DHP. The presence of a steroid recognition site on the GRC has been confirmed by direct binding.

TABLE 3

The effect of 3α-OH-DHP and other steroids on GABA enhancement of [^{3}H]FLU binding to the expressed $GABA_A$ r ceptor complex.

A				B	
% potentiation of [^{3}H]FLU binding				Steroid (3×10^{-7} M)	% of potentiation [^{3}H]FLU binding in the presence of 1 μM GABA
Modulator Subunit Composition					
	$\alpha1+\beta_1+\gamma_2$	$\alpha_2+\beta_1+\gamma_2$	$\alpha_3+\beta_1+\gamma_2$		
GABA (1μM)	38	87	250	3α-OH-DHP	74
				5ß,3α-OH-DHP	47
GABA (1μM)+ 3α-OH-DHP (1μm)	89	140	320	THDOC	48
				5α,3ß-OH-DHP	3
3α-OH-DHP (1μm)	52	63	112	Progesterone	8

A: Control binding is defined as 2 nM [^{3}]FLU binding in the absence of GABA. The data are the average of two independent transfection experiments. % Potentiation is defined as the % above control binding.

B: Control binding is defined as 2 nM [^{3}H]FLU binding in the presence of GABA (1 μM). The data represent the average of two independent transfection experiments using cDNA encoding the α_1, β_1 and γ_2 subunits.

Based on the knowledge provided herein on the structural requirements of the expressed GNR, classes of compounds that will act as agonists to the GNR are herein identified. Steroids that are active on the GNR include the steroids disclosed in patent application Serial No. 379,049 filed July 13, 1989, as well as Serial No. 521,724, filed May 10, 1990, and Serial No. unknown, filed August 13, 1991. Other active steroids include the steroids disclosed in U.S. Patent Nos. 3,822,297; 3,822,298; 3,869,451; 3,875,148; 3,880,896; 3,882,151; 3,883,569; 3,891,631; 3,933,799; 3,943,124; 3,952,031; 3,953,429; 3,959,260; 3,969,345; 3,980,681; 3,983,111; 3,989,686; 3,998,829; 4,192,875; and 4,197,296 to Glaxo, which patents are expressly incorporated by reference herein.

Two other patents also disclose pregnane-type steroids that can bind to the novel receptor of this invention. Those patents are U.S. 4,029,777 (Schering) and U.S. 4,424,159 (Ciba-Geigy), which patents are expressly incorporated by reference herein.

Anti-Convulsant Activity

Experiments were performed to determine the physiological relevance of the interactions of GNR-active steroid compounds by measuring the ability of the compounds to modulate TBPS-induced convulsions in mice.

Example 4

Modulation of TBPS-Induced Convulsions

Swiss-Webster mice were injected with various doses of the test compounds, as indicated in FIG. 2, 10 minutes prior to the injection of TBPS. The time to onset of myoclonus (presence of forelimb clonic activity) induced by TBPS was determined by observing each mouse for a period of 45 minutes. Significant differences between the time to onset in control mice vs. steroid-treated mice were determined by Student's t-test. The relative rank order potency and efficacy of these steroids in vivo were well correlated with those values determined in vitro.

The anti-convulsant and toxicological profiles of 3α-OH-DHP were further determined. In the anti-convulsant screen, mice were injected with various doses of 3α-OH-DHP or vehicle (dimethyl-sulfoxide) 10 minutes prior to the administration of the following chemical convulsants: metrazol (85 mg/kg); (+)bicuculline (2.7 mg/kg); picrotoxin (3.15 mg/kg); strychnine (1.25 mg/kg); or vehicle (0.9% saline). Immediately after the injection of convulsant or vehicle, the mice were observed for a period of 30 to 45 minutes. The number of animals with tonic and/or clonic convulsions was recorded. In the maximal electroshock test, 50 mA of current at 60 Hz was delivered through corneal electrodes for 200 msec to induce tonic seizure. The ability of 3α-OH-DHP to abolish the tonic component was defined as the endpoint. Sedative potential was determined by a rotorod test 10 minutes after the injection of 3α-OH-DHP where the number of mice staying on a rotating (6 rpm) rod for ≥1 minute in each of 3 trials was determined. The ED_{50} (the dose at which the half-maximal effect occurs) was determined for each screen. The acute LD_{50} (the dose that is lethal to one half of the animals tested) was determined by counting survivors 48 hours after the administration of 3α-OH-DHP.

The results are presented in Table 4, infra, and demonstrate that 3α-OH-DHP, in comparison to other

clinically useful anti-convulsants, is highly effective with a profile similar to that of the BZ clonazepam. The sedative liability at anticonvulsant doses is low as shown by comparing the ED_{50} values for the rotorod test and (+)bicuculline-induced seizures. The therapeutic index (ratio of LD_{50} to ED_{50}) for 3α-OH-DHP is >122 when based on the ED_{50} against (+)bicuculline-induced seizures, thus indicating very low toxicity. These observations demonstrate the therapeutic utility of these compounds as modulators of brain excitability, which is in correspondence with their high affinity interaction with the GRC in vitro.

TABLE 4

Anticonvulsant and acute toxicological profile of 3α-OH-DHP and those of selected clinically useful anticonvulsants in mice.

	ED_{50} (mg/Kg)						
Compound	RR	MES	MTZ	BIC	PICRO	STR	LD_{50}
3α-OH-DHP[(a)]	30	28.6	4.9	12.3	10.2	>300	--
5α-THDOC[(a)]	22.9	26.7	8.1	17.8	5.6	>300	--
3α-OH-DHP[(b)*]	40-100	>300	18.8	4.1	31.7	>300	>500
Clonazepam*	0.184	93	0.009	0.0086	0.043	NP	>6000
Pheno-barbital*	69	22	13	38	28	95	265
Phenytoin*	65	10	NP	NP	NP	**	230
Progabide***	-	75	30	30	105	75	3000
Valproate*	426	272	149	360	387	293	1105

The abbreviations are RR (Rotorod); MES (maximal electroshock); MTZ (metrazol); BIC (bicuculline); PICRO (picrotoxin); STR (strychnine); NP (no protection).

(a)Dissolved in 20% hydroxypropyl-ß-cyclodextrin in water. The route of administration for steroids and convulsants was i.p. and s.c., respectively.

(b)Dissolved in DMSO. Data taken from Belelli et al., 1989. ED_{50} values include the 95% confidence limits.

*Anticonvulsant data are from Swinyard & Woodhead, General principles: experimental detection, quantification and evaluation of anticonvulsants, in Antiepileptic Drugs, D.M. Woodbury, J.K. Penry, and C.E. Pippenger, eds., p. 111, (Raven Press, New York), 1982.

**Maximum protection of 50% at 55-100 mg/kg.

***The chemical convulsants in the progabide studies were administered i.v., all data from Worms et al., Gamma-aminobutyric acid (GABA) receptor stimulation. I. Neuropharmacological profiles of progabide (SL 76002) and SL 75102, with emphasis on their anticonvulsant spectra, Journal of Pharmacology and Experimental Therapeutics 220: 660-671, 1982.

Further study of anti-convulsant activity of prodrugs that upon metabolic activation become agonists and bind to the GNR was done over a time course with modifications of the basic compound 3α-OH-DHP.

Example 5

Metrazol-Induced Seizures

Adult male CF1 mice (20-30g) were used in these studies. Anti-convulsant activities were assessed as previously described by Swinyard and Woodhead (1982) supra. Percent protection against metrazol-induced seizures was plotted against time after administration of the compound. Mice were injected with metrazole (85 mg/kg s.c.) at various times after administration of the compound (3α-OH-DHP at 30 mg/kg, 3α-acetyl-5α-pregnan-20-one (3α-AC-DHP), 3α-propyl-5α-pregnan-20-one (3α-PR-DHP), and 3α-butyryl-5α-pregnan-20-one (3α-BU-DHP) all at 60 mg/kg). Ten to twelve mice were used per dose of test drug.

Mice were injected (i.p.) with the compound dissolved in DMSO or 2-hydroxypropyl-ß-cyclodextrin, or with vehicle alone (for DMSO, 5 μL/g body weight), at various times prior to the administration (s.c.) of a CD_{97} (dose at which 97% of the animals have seizures from Swinyard and

Woodhead, 1982) dose of metrazol (85 mg/kg) or vehicle (0.9% saline, 5 μL/g body weight). Immediately after the injection of the convulsant or vehicle, the mice were observed for a period of 30-45 min. The number of animals with tonic and/or clonic convulsions was recorded.

The ability of the steroid to abolish the tonic-clonic component was defined as the endpoint. Sedative potential was determined by a rotorod test where the number of mice staying on a rotating (6 rpm) rod for ≥ 1 min. in each of three trials was determined. The acute LD_{50} was determined by counting survivors 48 h. after the administration (i.p.) of the anti-convulsant compound. All median effective doses were determined by the method of Litchfield and Wilcoxon (1949).

Figure 3 shows the results of these experiments. Modification of the basic compound 3α-OH-DHP at the 3α position with an acetate, propionate or butyrate group increased the time of protection provided by the compound. Thus, compounds that bind to the GNR can be modified to provide anti-convulsant activity over a period of time, with varying degrees of protection.

The effects of oral administration of the GNR agonists on anti-convulsive activity was also studied. Figure 4 shows oral anticonvulsant activity of a GNR agonist prodrug and a direct acting GNR agonist.

Example 6

Oral Administration

All studies were done using non-fasted CF1 mice (Charles River) during daylight hours (0600-1700). The molecules were administered orally in a vehicle containing 0.35% hydroxypropyl cellulose and 4% Tween 80 in 0.9% NaCl (micronizing solution). The molecules were placed in a glass mill jar with glass beads and micronized for 48 hrs prior to administration. This procedure results in drug particles approximately 7-35 μmeters in size as determined by video microscopy. The anticonvulsant steroids, 3α-isobutyryloxy-5α-pregnan-20-one (100 mg/kg) (•) and 3α-

hydroxy-3ß-methyl-5α-pregnan-20-one (10 mg/kg) (o) were given by oral feeding tube. The chemical convulsant metrazol (85 mg/kg; subcutaneous) was administered at various times following the anticonvulsant steroid.

Results are expressed as the percentage of animals which did not show signs of myoclonus (i.e., percent protected) within 30 min of administration of convulsant agent (Fig. 4).

As can be seen from Fig. 4, there is extensive anti-convulsant activity over a useful period of time when either a prodrug or a direct acting anti-convulsant neuroactive steroid is administered orally. This is an important feature when such drugs are to be used therapeutically.

Anxiolytic Effects

The GNR is useful in screening for and selecting pharmaceuticals effective in treating anxiety. The following experiments demonstrate that the progesterone metabolite and GNR agonist 3α-OH-DHP is an effective anxiolytic in three animal models of human anxiety that measure the behavioral effects of anxiolytic compounds. In addition, they show that the anxiolytic effects of 3α-OH-DHP were mediated through a mechanism that is separate from that of the BZs.

The light/dark transition test (Crawley and Goodwin, 1980) is based on the observation that rodents naturally tend to explore novel environments, but open, brightly lit arenas are aversive to the rodents and inhibit exploratory behavior (Christmas and Maxwell, 1970; File, 1980). A variety of clinically established anxiolytics including diazepam, and clonazepam, as well as pentobarbital, have been shown to increase the number of transitions between the light box and the dark box, whereas nonanxiolytic drugs do not demonstrate this behavioral effect (Blumstein and Crawley, 1983; Crawley, 1981; Crawley and Davis, 1982; Crawley and Goodwin, 1980; Crawley et al., 1984).

Similar to the light/dark transition test, the open-field test measures the antagonism between the tendency to explore a novel environment and the tendency to remain still in an aversive environment (brightly lit arena). BZs have been shown to increase ambulation in open-field arenas (Davies and Steinberg, 1984; Hughes and Greig, 1975; Sansone, 1979; Bruhwyler et al., 1990; Bruhwyler, 1990). The open-field test provides a simple, non-punished assessment of the potential anxiolytic properties of novel compounds.

Finally, 3α-OH-DHP was tested for anxiolytic properties in a Vogel conflict paradigm. The ability of BZs to attenuate the suppression of behavior by punishment in conflict paradigms is well established (Gellar et al., 1962; Vogel et al., 1971). The Vogel test provides classical behavioral pharmacological support to the two "exploratory" models of anxiety in testing the anxiolytic effects of the GNR agonists 3α-OH-DHP and 5α-THDOC. In addition, CGS-8216, a BZ antagonist, has been shown to block diazepam's anxiolytic effects in the light/dark transition test (Crawley et al., 1984). To further demonstrate, in vitro, the uniqueness of the steroid site, we attempted to block the anxiolytic effect of 3α-OH-DHP with CGS-8216 in the light/dark transition test.

Example 7

Anxiolytic Tests

Male N.I.H. Swiss-Webster mice (Harlan) weighing 15-20 g were used in all experiments. The mice were housed 4/cage in polyethylene cages with sawdust bedding. The colony room was environmentally controlled (22°C) with a 12 hr. light/dark cycle (0600-1800 hrs.). The mice had free access to food and water. The experiments were run from 0700-1500 hrs. and groups were counterbalanced for time of day effects.

a) Light/Dark Transitions

The method used was previously described by Crawley and Goodwin (1980). The apparatus included a large box (26 x 33 x 24 cm) connected to a small box (15 x 22 x 14 cm) through an opening (5 x 6 cm). The large box was brightly lit with a standard 100 W light bulb, while the small box was kept dark. Following pretreatment with a test drug, mice were placed in the center of the large box and the number of transitions between the large and small boxes was counted for 10 min. Drug pretreatment times were as follows: diazepam (30 min.); 3α-OH-DHP (10 min.); and 5α-THDOC (10 min.). During the antagonist studies, CGS-8216 was administered 30 min. prior to the test drug.

b) Open-Field Activity

As a secondary measure of anxiolytic effects, naive mice were placed in the center of a large, brightly lit plexiglass box (42 x 42 x 30.5 cm) and the total distance traveled was measured during a 10 min. test period. Anxiolytics have been shown to increase the amount of "exploring" or locomotor activity in a novel environment (Treit, 1985; Lister, 1990). The Digiscan Activity Monitor (Omnitech Electronics, Columbus, OH) includes 16 photobeams that surround the box. The activity monitor is linked to a computer through a Digiscan Analyzer (Omnitech Electronics) and the data is analyzed using the Integrated Lab Animal Monitoring System (Omnitech Electronics). The mice were administered drugs as described for light/dark transitions.

For evaluation of a drug's effects on general activity, mice were first habituated to the open-field apparatus for 15 min. The following day, mice were pretreated with a test drug and placed in the center of the activity chambers. The total distance traveled was measured for 10 min.

c) Vogel Paradigm

An Anxio-Monitor (Omnitech Electronics, Columbus, OH) was used for measuring lick suppression. The testing

chamber consisted of a clear plexiglass box (29 cm x 29 cm x 23 cm) with a metal drinking tube located 2.5 cm from the floor and extending 2 cm into the box. The shock was applied through the drinking spout and was controlled by the Anxio-Monitor. The number of licks was counted and displayed by the Anxio-Monitor. The reinforcer was 0.1 M sucrose.

The conflict test procedure described by Vogel et al. (1980) was employed. This procedure is a modification of the original lick suppression test first described by Vogel et al. (1971). After 24 hr. of water deprivation, mice were allowed to explore the test apparatus and drink without punishment for 10 min. or 100 licks. The following day (48 hr. after the start of water deprivation), mice were pretreated with 3α-OH-DHP (20 mg/kg), chlordiazepoxide (CDP; 10 mg/kg) or vehicle and subsequently placed in the conflict apparatus. Mice were allowed unpunished access to the drinking tube for 20 licks, thereafter, every 10th lick was punished with 0.1 mA shock. The test duration was 5 min.

The steroids 3α-OH-DHP, 3ß-OH-DHP, and 5α-THDOC were synthesized as described in U.S. Patent Application Serial No. unknown filed August 13, 1991, which is incorporated herein by reference. 2-Hydroxypropyl ß-cyclodextrin (ß-cyclodextrin) is available from Aldrich (Milwaukee, WI). Diazepam and chlordiazepoxide are available from Sigma, Co. (St. Louis, MO). CGS-8216 was obtained from Ciba-Geigy (Summit, NJ). All drugs were dissolved in 20% ß-cyclodextrin in water and sonicated overnight. All drugs were administered intraperitoneally in a volume of 100 μL/20 g. CGS-8216 was administered subcutaneously in a volume of 100 μL/20 g.

Dose-response curves for 3α-OH-DHP, 5α-THDOC and diazepam generated in the light/dark transition test were run over several days. The vehicle control data were analyzed across test days using a 1-way analysis of variance (ANOVA). Because the vehicle data were not

significantly different across days, the control data were collapsed for each test drug. The dose-response curves were then analyzed using a 1-way ANOVA, followed by Dunnett's t-test for individual comparisons between doses and control. The open-field and habituated locomotor data were analyzed using ANOVA followed by Dunnett's t-test. 3α-OH-DHP and CDP were tested on separate days in the lick-suppression test. The control groups were significantly different, therefore the data was analyzed using a Student's t-test (2-tailed) and for comparison is graphically displayed as percent of corresponding control. All data is expressed as the mean ± S.E.M.

The GNR agonist 3α-OH-DHP produced anxiolytic effects as seen by an increase in the number of transitions in the light/dark paradigm (Fig. 5). 3α-OH-DHP produced a significant dose-dependent response ($F(4,63)=21.5$; $p=.0001$). The number of transitions was increased by 3α-OH-DHP significantly ($p<.01$) at 10, 20, and 40 mg/kg. 3α-OH-DHP reached maximal effect at a dose of 20 mg/kg with an average of 70.2 ±4.3 transitions in a 10 min. period. The highest dose tested, 40 mg/kg, started a trend towards a reduction in the number of transitions. Several compounds have been shown to produce an inverted U-shaped dose-response curve in the light/dark transition paradigm (Crawley et al., 1986).

The deoxycorticosteroid metabolite and GNR agonist 5α-THDOC produced a significant ($F(4,54)=10.0$; $p=.0001$) dose-related effect in the light/dark transition test (Fig. 6). 5α-THDOC, at a dose of 20 mg/kg was significantly different from vehicle ($p<.01$). Although 5α-THDOC did produce a greater number of transitions at 10 mg/kg compared with vehicle (49.4 ±2.0 vs. 35.2 ±2.0), the difference did not reach significance ($p<.06$). At the highest dose tested, 5α-THDOC (40 mg/kg), produced a significant decrease ($p<.05$) in the number of transitions as compared with vehicle control.

Diazepam's effects on light/dark transitions is shown in Fig. 7. Diazepam produced a significant ($F(5,72)=31.6$; $p=.0001$) inverted U-shaped dose-response curve. Diazepam was significantly ($p<.01$) different from controls at 1.0, 5.0, 10, and 20 mg/kg. Diazepam's maximal response was at 10 mg/kg with 86.4 ± 5.4 transitions. Though significant at 20 mg/kg, diazepam's effects were diminished as compared with the effects at 10 mg/kg. These results are similar to the inverted U-shaped curves seen with the two GNR agonists, 3α-OH-DHP and 5α-THDOC.

As shown in Fig. 8, the diastereomer of 3α-OH-DHP, 3ß-OH-DHP (20 mg/kg), did not produce an anxiolytic effect in the light/dark transition paradigm. In the same experiment, 3α-OH-DHP (20 mg/kg) did produce a significant ($p<.01$) increase in the number of light/dark transitions over those produced by the carrier alone (ß-cyclodextrin). These results demonstrate the stereo-specificity of the anxiolytic effects of the steroid 3α-OH-DHP, and thus the stereo-specificity of the GNR.

The specific BZ antagonist CGS-8216 (10 mg/kg) was unable to block the anxiolytic effect of 3α-OH-DHP (Fig. 9A). 3α-OH-DHP (10 mg/kg) produced significant ($p<.01$) increases alone and in the presence of CGS-8216 (Fig. 9A). However, CGS-8216 was able to block the anxiolytic effect of diazepam (Fig. 9B). Diazepam (1.0 mg/kg) alone produced a significant ($p<.01$) increase in transitions as compared to control (Fig. 9B). CGS-8216 did not demonstrate any intrinsic activity and was not significantly different from vehicle control ($p>.4$). These results demonstrate that the anxiolytic effects of the steroid 3α-OH-DHP are through a separate neural mechanism than that of the BZs.

When placed in a novel, brightly lit open-field, mice demonstrate a low level of activity (i.e., exploration, locomotion, etc.), whereas anxiolytics increase the amount of activity in a novel environment (Lister, 1990). As shown in Table 5, there was a significant drug effect on

open-field activity ($F(4,44)=18.05$; $p=.0001$). Specifically, the GNR agonists 3α-OH-DHP and 5α-THDOC produced significant ($p<.01$) increases in activity as compared with control. In addition, diazepam produced a significant ($p<.01$) increase in activity as compared with ß-cyclodextrin vehicle control. Consistent with the light-dark transition paradigm, 3ß-OH-DHP did not show any effect in the open-field test.

TABLE 5

Effects of Steroids and Diazepam on Open-Field Activity

DRUG	DOSE (mg/kg)	TOTAL DISTANCE (cm)
ß-Cyclodextrin		2004.6 ± 134.6
3ß-OH-DHP	20	1979.8 ± 174.5
3α-OH-DHP	20	5344.9 ± 754.5**
5α-THDOC	20	7328.4 ± 769.5**
DIAZEPAM	10	4817.7 ± 528.4**

Mice were pretreated 10 min or 30 min (diazepam) prior to being placed in the center of the open-field apparatus. Total distance travelled was measured for 10 min (see Methods for details). Each group consisted of 9-10 mice.

**p(.01: significantly different from the B-cyclodextrin vehicle control according to Dunnett's t-test.

3α-OH-DHP also produced a significant increase in locomotor activity ($p<.01$) in mice that were acclimated to the test chambers. 3α-OH-DHP (20 mg/kg) treated mice traveled a total distance of 5694.7 ±608.4 cm compared with controls 2061.2 ±157.7 cm. Diazepam (10 mg/kg) had no effect on locomotor activity (2258.0 ±897.7 cm) under these experimental conditions.

Administration of 3α-OH-DHP (20 mg/kg) disinhibited punished-induced suppression of drinking (Fig. 10). 3α-OH-DHP produced a significant 235.6% increase in the

number of shocks received during the 5 min. test session as compared with controls (13.9 ±2.1 vs. 5.9 ±0.54; t= -3.98; p=.01). In comparison, CDP (10 mg/kg) produced a 197.4% increase in punished responding as compared with controls (6.14 ±1.14 vs. 3.11 ±0.68; t= -2.4; p=.03).

Other Effects

The agonists of the GNR on the GRC will also be effective in treating other conditions affected by the opening and closing of the chloride channel on this receptor complex. Agonist BZs and barbiturates that bind at the GRC induce the hypnotic state and sleep. A correlation is shown by this invention between the modulation of $GABA_A$ binding by BZs and barbiturates and the modulation of $GABA_A$ binding by agonists at the GNR. Additionally, in whole animals, some compounds now known to be agonists of the GNR have been shown to induce the hypnotic state and sleep. Therefore it is shown by this invention that GNR agonists will likewise induce the hypnotic state and/or sleep.

Likewise, BZs and barbiturates are routinely used in the treatment of mood disorders and acute anxiety attacks such as are caused by panic disorders. Their mode of action is through modulation of the $GABA_A$ receptor. Lloyd, K.G. and Morselli, P.L., "Psychopharmacology of GABAergic Drugs," in Psychopharmacology: The Third Generation of Progress, pages 183-195, H.Y. Meltzer, ed., Raven Press, N.Y., 1987). Accordingly, the data presented here demonstrate that agonists of the GNR are useful in the treatment of mood disorders such as depression and acute anxiety attacks such as are caused by panic disorders.

Receptor Specificity

It has also been demonstrated that agonists of the novel GNR lack hormonal side effects by the lack of affinity of these compounds for the progesterone receptor (FIG. 11). The data plotted in FIG. 11 were obtained by

performing assays in accordance with the receptor binding assay described in Gee et al. (1988), supra, incorporated herein by reference, to determine the effect of GNR agonists and the progestin R5020 on the binding of [^{3}H]R5020 to the progesterone receptor in rat uterus. All points on the plot of FIG. 11 represent the mean of triplicate determinations. The following compounds are those listed in FIG. 11: 5α-pregnan-3α-ol-20-one (5ß,3α-OH-DHP), 5α-pregnan-3α,21-diol-20-one (5α-THDOC), and 5ß-pregnan-3α,20α-diol (5BETA).

The hormonal activity of agonists of the novel steroid receptor was further studied through testing their potential estrogenic, progestinic, mineralocorticoid and glucocorticoid activities. These activities were analyzed by monitoring the ability of the drugs to inhibit binding of the steroid hormones to their respective hormone receptors. The results are shown in Tables 6-9. They are expressed as percent inhibition of ^{3}H-ligand binding to the various steroid hormone receptors for the drugs at 10^{-6} and 10^{-5} M. Control values are represented by the binding in the absence of drugs.

Example 8

Mineralocorticoid Receptor

In Table 6, rats were adrenalectomized 3 days prior to sacrifice. To isolate the mineralocorticoid receptor, brain cytosol fractions were prepared as follows. Brains from male Sprague-Dawley rats were removed immediately following sacrifice and the cerebral cortices dissected over ice. A P_2 homogenate was prepared as previously described (Gee et al., "Modulation of the chloride ionophore by benzodiazepine receptor ligands: influence of gamma-aminobutyric acid and ligand efficacy," Molecular Pharmacology, 30, 218 (1986)). Briefly, the cortices were gently homogenized in 0.32 M sucrose followed by centrifugation at 1000 x g for 10 minutes. The supernatant was collected and centrifuged at 9000 x g for 20 minutes. The

resultant P_2 pellet was suspended as a 10% (original wet weight/volume) suspension in 50 mM Na/K phosphate buffer (pH 7.4) 200 mM NaCl to form the homogenate.

The drugs were incubated with 3 nM of ^{3}H-aldosterone (the specific ligand for the mineralocorticoid receptor) in the presence of the selective type II agonist RU28362 (0.5 μM) which blocks ^{3}H-aldosterone binding to the type II (glucocorticoid) receptors. The specific binding was 1756 cpm/fraction.

TABLE 6

Inhibition of ^{3}H-Aldosterone Binding to Hippocampal Cytosol Mineralocorticoid Receptors

COMPETITOR (10^{-6} M)	% of INHIBITION
Aldosterone	95.5
5α-pregnan-3α,21-diol-20-one	76.7
5ß-pregnan-3α,21-diol-20-one	13.8
5α-pregnan-3α,ol-20-one	0
5ß-pregnan-3α,ol-20-one	0
5α-pregnan-3α,20α-diol	0
5ß-pregnan-3α,20α-diol	0
5α-pregnan-3α,20-diol-20-dimethyl	0
5α-pregnan-3α-ol-3ß-methyl-20-one	3.2
5α-pregnan-3ß,20-trimethyl-3α,20-diol	0

Example 9

Glucocorticoid Receptor

For Table 7, brain cytosol fractions were prepared as for Table 6, and the drugs were incubated with 3 nM of ^{3}H-dexamethasone (the specific ligand for the glucocorticoid receptor). The specific binding was 1174 cpm/fraction.

TABLE 7

Inhibition of ^{3}H-Dexamethasone Binding to Glucocorticoid Receptors

COMPETITOR (10^{-6} M)	% of INHIBITION
Dexamethasone	100
5α-pregnan-3α,21-diol-20-one	29.5
5β-pregnan-3α,21-diol-20-one	8.2
5α-pregnan-3α,ol-20-one	8.7
5β-pregnan-3α,ol-20-one	5.9
5α-pregnan-3α,20α-diol	2.6
5β-pregnan-3α,20α-diol	1.4
5α-pregnan-20-dimethyl-3α,20-diol	2.6
5α-pregnan-3α-ol-3β-methyl-20-one	0.6

Example 10

Estrogen Receptor

Table 8 shows the inhibition of ^{3}H-estradiol (the specific ligand for the estrogen receptor) binding to bovine uteri cytosol, prepared as previously described. Two concentrations of ^{3}H-estradiol; (A) 0.15 nM and (B) 0.25 nM, were incubated with the cytosol in the presence of the drugs. The specific binding for ^{3}H-estradiol at 0.15 nM and 0.25 nM was 1241 cpm/fraction and 1951 cpm/fraction, respectively.

TABLE 8

Inhibition of ^{3}H-Estradiol Binding to the Bovine Uteral Estrogen Receptors

COMPETITOR	% of INHIBITION COMPETITOR AT 10^{-6} M		COMPETITOR AT 10^{-5} M	
	A[(a)]	B[(b)]	A	B
5α-pregnan-3α,ol-20-one	0	0	0	3
5α-pregnan-3α,21-diol-20-one	2	4	23	23
5α-pregnan-3α,20α-diol	0	0	7	13
5α-pregnan-3α-ol-3ß-methyl-20-one	0	0	2	6
5ß-pregnan-3α,21-diol-20-one	0	4	3	7
5α-pregnan-3ß,20-trimethyl-3α,20-diol	0	4	3	7
5ß-pregnan-3α,20α-diol	8	0	0	0
5ß-pregnan-3α,ol-20-one	0	0	5	0
5α-pregnan-20-dimethyl-3α,20-diol	0	0	5	5

Example 11

Progesterone Receptor

For the data presented in Table 9, bovine uteri cytosol was isolated as they were for Table 7 and used for following binding to progesterone receptors by following the inhibition of ^{3}H-progesterone, the natural ligand. Two ^{3}H-progesterone concentrations, (A) 0.15 nM and (B) 0.25 nM were incubated with the cytosol in the presence of the drugs. The specific binding for ^{3}H-progesterone at 0.15 nM and 0.25 nM was 2893 cpm/fraction and 4222 cpm/fraction, respectively. This data reinforces our findings shown in Fig. 11 that indicates that GNR agonists have no activity at the progesterone receptor.

TABLE 9

Inhibition of ^{3}H-Progesterone Binding to the Bovine Uteral Progesterone Receptors

COMPETITOR	% of INHIBITION COMPETITOR AT 10^{-6} M		COMPETITOR AT 10^{-5} M	
	A[(a)]	B[(b)]	A	B
5α-pregnan-3α,ol-20-one	14	2	41	40
5α-pregnan-3α,21-diol-20-one	13	5	35	28
5α-pregnan-3α,20α-diol	6	1	2	3
5α-pregnan-3α-ol-3β-methyl-20-one	4	2	10	5
5β-pregnan-3α,21-diol-20-one	6	2	19	10
5α-pregnan-3β,20-trimethyl-3α,20-diol	8	0	5	0
5β-pregnan-3α,20α-diol	0	0	1	1
5β-pregnan-3α,ol-20-one	9	1	17	13
5α-pregnan-20-dimethyl-3α,20-diol	0	0	0	0

To further evaluate the selectivity of agonists of the novel steroid receptor to that receptor, 3α-OH-3β-methyl-5α-pregnan-20-one was tested against a series of radioligands associated with a variety of known receptors or recognition sites. These studies were carried out according to the NovaScreen® (Nova Pharmaceuticals, Baltimore, MD) protocol. The results are shown in Table 10.

TABLE 10

Effect of 3α-OH-3ß-Methyo-5α-Pregnan-20-One on Various Receptors and Proteins

RECEPTOR/ SELECTIVITY	PERCENT INHIBITION (Average N=3) 10^{-7}M	10^{-5}M
Adenosine		
Adenosine	0.97±0.67	0.80±0.92
Amino Acids		
Excitatory		
Quisqualate	-10.73±3.33	-7.10±3.46
Kainate	-3.17±2.73	-3.43±2.55
MK-801	14.80±2.16	14.27±1.9
NMDA	3.60±7.71	13.47±2.52
PCP	-2.47±3.06	-6.37±6.76
Glycine	8.47±4.63	3.60±2.16
Sigma	-10.33±0.87	-2.20±4.42
Inhibitory		
Glycine	-17.10±3.46	-17.90±1.77
$GABA_A$	-6.33±2.19	-8.20±3.70
$GABA_B$	-7.27±1.14	-2.00±4.80
Benzodiazepine	-16.43±2.19	-14.90±2.98
Biogenic Amines		
Dopamine 1	5.57±8.54	11.87±5.17
Dopamine 2	-1.67±2.22	0.97±1.72
Serotonin 1	-2.57±12.15	-17.77±2.37
Serotonin 2	-3.93±3.20	-3.03±0.87
Peptides		
Angiotensin	2.30±1.99	4.63±5.28
Arg-Vasopressin V1	-4.17±3.59	-6.10±2.28
Bombesin	-13.50±1.54	-5.70±3.93
CCK Central	-10.80±1.88	-10.77±2.86
CCK Peripheral	-2.77±1.54	-2.93±1.45
Substance K	-1.57±5.82	8.40±4.29
Substance P	9.50±9.36	15.23±15.61
Neuropeptide Y	12.47±4.07	7.57±3.88
Neurotensin	8.83±5.62	4.90±3.04
Somatostatin	-5.43±2.75	-3.93±3.07
VIP	1.50±2.95	0.93±4.15
Channel Proteins		
Calcium (w-conotoxin)	-2.03±1.77	-0.83±1.37
Calcium (nifedipine)	0.43±2.41	-0.03±1.42
Chloride	7.70±2.40	37.50±5.31
Potassium	-4.97±3.87	1.97±4.45

RECEPTOR/ SELECTIVITY	10^{-7}M	10^{-5}M
Peptide Factors		
ANF (rat)	10.13±5.13	9.20±5.29
EGF	13.73±1.68	13.50±1.92
NGF	7.47±8.08	16.70±15.45
Second Messenger Systems		
Adenylate Cyclase		
Forskolin	2.10±0.37	1.33±1.44
Protein Kinase C		
Phorbol Ester	-2.43±0.37	-4.83±1.48
Inositol Triphosphate	7.97±2.66	13.57±6.22

Note that the NovaScreen assay in Table 10 for $GABA_A$ and BZ binding sites was not performed as were the other $GABA_A$ and BZ studies reported here: No exogenous GABA was provided in the NovaScreen assay. Therefore, positive cooperativity properties were not tested in this data, and the results on the BZ binding sites were not positive. Likewise, while the chloride channel proteins showed positive inhibition, the effects were minimized due to the absence of GABA.

The results of these experiments clearly show that the agonists of the novel GNR do not have a strong affinity for any of the above tested receptors and proteins. In addition, they will not have predicted hormonal side-effects which would result from hormonal steroid receptor binding.

RECEPTOR SUBTYPES

Subtypes of the subunits (α_{1-6}, β_{1-3}, γ_{1-2}, etc.) of the steroid receptor described herein occur in a variety of combinations in vivo. Studies have shown that the spinal cord may be devoid of the α_1 subunit, whereas the cerebellum is rich in α_1 subunits. (Vicini S., (1991) Pharmacologic significance of the structural heterogeneity of the $GABA_A$ receptor-chloride ion channel complex,

Neuropsychopharmacology 4, 9-15). Regionally selective expression of receptor subunit subtypes may account for differential responsiveness of tissues to GABAergic inhibition and neurosteroid modulatory effects.

Likewise, the subunits of the GNR described herein can be expressed in a variety of combinations. Experiments were performed to analyze binding of GNR agonists to various combinations of the expressed subunits. The results demonstrate that there is a differential response to agonists as a function of the different subtypes.

Example 12

Binding to Different Subtypes

a) Expression of $GABA_A$ receptor complexes

Previous transfection procedures were followed (Pritchett et al, (1989) Type I and type II $GABA_A$-receptors produced in transfected cells, Science 245, 1389-1392) except that cells were seeded for 36 hours instead of 24 hours prior to transfection. The human embryonic kidney 293 cells were transfected with a total of 20 μg of DNA per dish using a modified high efficiency calcium phosphate precipitation method described previously (Chen and Okayama, (1987) High efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol 7, 2745-2752). DNA used for the transfection was a mixture of equal amounts of cloned cDNA encoding the human $GABA_A$ α_1, α_2 or α_3, β_1 and γ_2-subunits constructed individually in an expression vector (Chen and Okayama, supra). At 48 h post-transfection, the cells were harvested and washed twice with phosphate buffered saline (PBS) pH 7.2 and frozen or immediately used in the binding assays.

b) [^{3}H]FLU and [^{3}H]Muscimol Binding Assays

The transfected cells or rat (male Sprague-Dawley, 150-200g) brain tissue from selected regions were homogenized in 50 mM potassium phosphate buffer pH 7.2 and washed once by centrifugation. The cell membrane

pellet(10,000 x g) was homogenized in a mixture of 10 mM potassium phosphate, pH 7.2 and 100 mM NaCl. 10 μl aliquots of the cell membrane preparation were incubated with 2nM of [^{3}H]FLU (75-90 Ci/mmol, DuPont, New England Nuclear) in the presence of 1 μM of GABA (Sigma Chem. Co., St. Louis, MO) or with 30 nM[^{3}H]muscimol (20.0 ci/mmol, DuPont, New England Nuclear) and with or without steroids (Steraloids, Wilton, NH). All assays were brought to a final volume of 1 ml with 50 mM sodium phosphate buffer and 100 mM NaCl. Nonspecific binding was defined as binding in the presence of 1 μM clonazepam (Sigma Chemical Co.) and 10 μM GABA in [^{3}H]FLU and [^{3}H]muscimol binding assays, respectively. Assays were terminated after a 90 min ([^{3}H]FLU binding) or 60 min ([^{3}H]muscimol binding) incubation at 25°C by rapid filtration through Schleicher and Schuell (Keene, NH) No. 32 glass fiber filters. The filters were washed twice with 5 ml of ice-cold PBS. Filter-bound radioactivity was quantified by liquid scintillation spectrophotometry. Half-maximal enhancement of binding was determined by nonlinear regression of dose-response curves using the equation: $Y = [Ymax^* X/(X + EC_{50})] + control$ where: Y = % of control binding, X = log [3α-OH-DHP]. Control = 100%, EC_{50} = concentration of 3α-OH-DHP required to produce half-maximal enhancement.

Differential responses to 3α-OH-DHP of expressed human GRCs with various α subunits in combination with β_1 and γ_2 subunits are shown in Figure 12. Maximal enhancement and EC_{50} values are for [^{3}H]FLU (2nM) binding to $\alpha_1\beta_1\gamma_2$-(●, E_{max} =66%, EC_{50} = 340 nM), $\alpha_2\beta_1\gamma_2$-(○, E_{max}=81%, EC_{50} = 53 nM) and $\alpha_3\beta_1\gamma_2$ -(■, E_{max} =208%, EC_{50} =226 nM) transfected cell membranes in the presence of 1 μM GABA and various concentrations of 3α-OH-DHP. Each point represents the mean of 4-6 independent transfection experiments with the bars representing the SEM. [^{3}H]FLU binding in the absence of 3α-OH-DHP is defined as 100% binding. The control binding values for $\alpha_1\beta_1\gamma_2$, $\alpha_2\beta_1\gamma_2$ and

$\alpha_3\beta_1\gamma_2$ ranged from 10-39, 23-87, and 5-33 fmoles/mg protein, respectively.

The steroid clearly enhanced [^{3}H]FLU binding to each of the expressed receptor subtypes. However, assembled receptors with the α_3 subunit consistently gave a much greater maximal response to steroid stimulation than those composed of other α subunits. Using the $\alpha_3\beta_1\gamma_2$ receptor complex, 3α-OH-DHP, produced a 200% enhancement of [^{3}H]FLU binding, whereas $\alpha_1\beta_1\gamma_2$ and $\alpha_2\beta_1\gamma_2$ complexes showed less than 100% enhancement above the control. A similar differential response of the GRC to GABA with various α subunits has also been demonstrated previously (Pritchett et al, supra).

The GABA concentration (1μM) used in the present study did not produce a significant differential enhancement when compared to binding in the absence of GABA (Pritchett et al, supra) and was used in the control conditions in the present study. The percentage enhancement by the steroid remained constant regardless of the level of control binding which varied among different transfection experiments.

Although a greater stimulation of BZ binding by GABA has been reported in brain membranes from young animals (Mallorga et al, (1980) Ontogenetic changes in GABA modulation of brain benzodiazepine binding, Neuropharmacology 19, 405-408) or from tissue in which the postsynaptic GRCs had been destroyed (Lo et al, (1983) Differential localization of type I and type II benzodiazepine binding sites in substantia nigra, Nature 306, 57-60), differential responses in tissues to GNR agonists have not been reported. Therefore we measured [^{3}H]FLU binding in response to 3α-OH-DHP in P_2 homogenate from cerebellum and spinal cord (Figure 13).

Fig. 13 shows potentiation of [^{3}H]FLU binding by 3α-OH-DHP in P_2 homogenates of rat spinal cord (●, E_{max} =51%, EC_{50} = 152 nM) and cerebellum (O, E_{max} =28%, EC_{50} = 42 nM). The assays were performed in the presence of 5μM GABA and

various concentrations of 3α-OH-DHP. Each point represents the mean of 3 experiments ± S.E.M. [^{3}H]FLU binding in the absence of 3α-OH-DHP is defined as 100% binding.

These two regions were chosen because in situ studies suggest that the $GABA_A$ receptors in cerebellum and spinal cord are differentially rich in or devoid of the α_1 subunit, respectively (Vicini S., supra; Memo et al, supra).

Under the same experimental conditions, [^{3}H]FLU binding in the spinal cord showed a greater response to 3α-OH-DHP than cerebellum. However, the differences in response between the two tissues were not as great as those observed in transfected cells, perhaps because the subunit distributions in these tissues are not entirely homogenous.

In previous electrophysiological studies on GRCs formed by co-expression of αß subunits only, differential responses to GABA were also observed (Pritchett et al, supra). However, different observations were made when receptor complexes composed of αßγ subunits were studied. It was found that the $\alpha_2\beta_1$ receptor complex was more sensitive to GABA than the $\alpha_1\beta_1$ and $\alpha_3\beta_1$ subunit combinations (Levitan et al, (1988) Structural and functional basis for $GABA_A$ receptor heterogeneity, Nature 335, 76-79) whereas the $\alpha_3\beta_1\gamma_2$ receptor complex was more sensitive to GABA than the $\alpha_1\beta_1\gamma_2$ and $\alpha_2\beta_1\gamma_2$ complexes. Differences in the pharmacological and electrophysiological properties of receptors composed of two or three subunits may contribute to these observations.

Most recently, GRC subtypes expressed in Xenopus oocytes were also differentially modulated by 3α-OH-DHP dependent upon subunit composition (Shingai et al, (1991) Effects of subunit types of the cloned $GABA_A$ receptor on the response to a neurosteroid, Eur.J.Pharmacol.Mol. Pharmacol.Sec. 206, 77-80). The subunit dependence was different from that observed in the present study.

Although this difference is difficult to reconcile, it may be related to differences in mechanisms by which cooperative binding interactions are mediated versus how GABA-activated chloride currents are potentiated in the Xenopus oocyte.

In the present study, we also examined the responses to 3α-OH-DHP in receptors composed of αß subunits (Fig. 14) using a similar allosteric modulation binding assay. Fig. 14 shows potentiation of [^{3}H]muscimol binding by 3α-OH-DHP in transfected cell membranes. [^{3}H]muscimol (30 nM) binding to $\alpha_1\beta_1$-(●, E_{max} =19%, EC_{50} = 11 μM), $\alpha_2\beta_1$-(○, E_{max} =82%, EC_{50} =463 nM) and $\alpha_3\beta_1$-(■, E_{max} =191%, EC_{50} = 366 nM) transfected cell membranes in the presence of various concentrations of 3α-OH-DHP. Each point represents the mean of 4-6 independent transfection experiments with bars representing the SEM. [^{3}H]muscimol binding in the absence of 3α-OH-DHP is defined as 100% binding. The control binding values for $\alpha_1\beta_1$, $\alpha_2\beta_1$ and $\alpha_3\beta_1$ ranged from 28 - 101, 39 - 112, and 21 - 130 fmol/mg protein, respectively.

Consistent with that observed with the αßγ receptor complex, $\alpha_3\beta_1$ showed a greater enhancement of [^{3}H]muscimol binding by 3α-OH-DHP than those composed of $\alpha_1\beta_1$ or $\alpha_2\beta_1$ subunits, although the difference from $\alpha_2\beta_1$ is less pronounced, thus suggesting that site of interaction with the steroid is probably similar in both αßγ or αß receptor complexes. It is not known whether or not differential responses to GNR agonists will occur when the receptor is composed of a single subunit (homooligomer).

The rank order of activity of several steroids was examined in the expressed $\alpha_1\beta_1\gamma_2$, $\alpha_2\beta_1\gamma_2$ and $\alpha_3\beta_1\gamma_2$ receptors. At 1μM steroid, 3α-OH-DHP produced the greatest enhancement of [^{3}H]FLU binding in all three types of receptor complexes whereas 3ß-OH-DHP and progesterone produced minimal effects (Table 11). The 5-ß configuration of the stereoisomers is less effective when compared to their

corresponding 5-α isomers. Interestingly, the differential responses are more pronounced in complexes composed of α_1 and α_3 than those with the α_2 subunit.

Since the distribution of the α subunit variants is heterogenous in the CNS and variants appear to respond differentially to these GNR agonists, it is possible then that the magnitude of the responses to GNR agonists may vary regionally. Upon further characterization, this property may allow for the design of more subtype selective compounds directed at this novel steroid recognition site.

TABLE 11

Relative Activity Of Various Steroids In Potentiating [^{3}H]FLU Binding To The Expressed $GABA_A$ Receptor Complexes In The Presence Of 1μM GABA.

Steroid	Relative Activity[1]		
	$\alpha_1\beta_1\gamma_2$	$\alpha_2\beta_1\gamma_2$	$\alpha_3\beta_1\gamma_2$
3α-OH-5α-DHP	100	100	100
5α-THDOC	65.2 ± 4.4	88.4 ± 2.6*	79.5 ± 3.4**
3α-OH-5β-DHP	46 ± 8.3	85.2 ± 3.2**	56.6 ± 4.8
5β-THDOC	28.8 ± 4.3	63.5 ± 6.3	26.8 ± 2.6
Alphaxalone	46.3 ± 8.7	52.3 ± 4.3	41.5 ± 4.8
3β-OH-DHP	0	4 ± 1.7	0
Progesterone	0	3.8 ± 1.9	0

1 Potentiation of [^{3}H]FLU binding to 3α-OH-5α-DHP is defined as 100% activity. In each experiment, the effect of 3α-OH-DHP was assayed as the positive control compound. Relative activity was defined as the ratio of the enhancement of [^{3}H]FLU binding in the presence of the indicated steroids to that induced by 3α-OH-5α-DHP in the same experiment. All steroid concentrations were 1μM. The data represent the mean of 4-8 independent transfection experiments ± S.E.M.

*,** Significantly different from 3α-OH-5α-DHP at * $P<0.02$ and ** $P<0.01$ by Student's paired t-test.

DELIVERY

The GNR agonists of this invention are prepared for delivery to an individual in conventional dosage unit forms by incorporating a GNR agonist or a mixture of GNR agonists with a nontoxic pharmaceutical carrier according to accepted procedures in a nontoxic amount sufficient to produce the desired pharmacodynamic activity in a subject, animal or human. Preferably, the composition contains the active ingredient in an active, but nontoxic amount, selected from about 5 mg to about 500 mg of active ingredient per dosage unit. This quantity depends on the affinity of the compound for the GNR, the specific biological activity desired and the condition of the patient. Desirable objects of this invention are the treatment of stress, anxiety, PMS, PND, and seizures such as those caused by epilepsy to ameliorate or prevent the attacks of anxiety, muscle tension, and depression common with patients suffering from these central nervous system abnormalities. Additional desirable objects of this invention are to prevent insomnia and produce hypnotic activity.

The pharmaceutical carrier employed may be, for example, either a solid, liquid, or time release (see e.g. Remington's Pharmaceutical Sciences, 14th Edition, 1970). Representative solid carriers are lactose, terra alba, sucrose, talc, gelatin, agar, pectin, acacia, magnesium stearate, stearic acid, microcrystalline cellulose, polymer hydrogels and the like. Typical liquid carriers are propylene glycol, aqueous solutions of ß-cyclodextrins, syrup, peanut oil, and olive oil and the like emulsions. Similarly, the carrier or diluent may include any time-delay material well known to the art, such as glycerol monostearate or glycerol distearate alone or with wax, microcapsules, microspheres, liposomes, and/or hydrogels.

A wide variety of pharmaceutical forms can be employed. Thus, when using a solid carrier, the

preparation can be plain milled micronized, in oil, tableted, placed in a hard gelatin or enteric-coated capsule in micronized powder or pellet form, or in the form of a troche, lozenge, or suppository. When using a liquid carrier, the preparation can be in the form of a liquid, such as an ampule, or as an aqueous or nonaqueous liquid suspension. Liquid dosage forms also need pharmaceutically acceptable preservatives and the like. In addition, because of the low doses that will be required as based on the data disclosed herein, nasal spray, sublingual administration and timed release skin patches are also suitable pharmaceutical forms for topical administration.

The method of producing anxiolytic, anticonvulsant, mood altering (such as anti-depressant) or hypnotic activity, in accordance with this invention, comprises administering to a subject in need of such activity an agonist at the GNR, usually prepared in a composition as described above with a pharmaceutical carrier, in a nontoxic amount sufficient to produce said activity.

During menses, the levels of excreted metabolites vary approximately fourfold (Rosciszewska, et al., op. cit.). Therefore, therapy for controlling symptoms involves maintaining the patient at a higher level of GNR active progesterone metabolite (i.e., GNR agonists) than normal in the premenstrual state of PMS patients. Plasma levels of active and major metabolites are monitored during pre-menses and post-menses of the patient. The amount of the GNR agonists administered, either singly or as mixtures thereof, are thus calculated to supplement the levels of GNR active progesterone metabolites during the premenses state.

The route of administration may be any route that effectively transports the active compound to the GRCs that are to be stimulated. Administration may be carried out parenterally, enterally, rectally, intravaginally, intradermally, sublingually, or nasally; the oral and

dermal routes are preferred. For example, one dose in a skin patch may supply the active ingredient to the patient for a period of up to one week.

While the preferred embodiments have been described and illustrated, various substitutions and modifications may be made thereto without departing from the scope of the invention. Accordingly, it is to be understood that the present invention has been described by way of illustration and not limitation.

Claims

1. Agonists of the GNR on the GRC.

2. A method of treating anxiety in a mammal which comprises administering an effective amount of an agonist of the GNR on the GRC.

3. The method of claim 2 wherein anxiety is generalized anxiety disorder.

4. The method of claim 2 wherein anxiety is panic disorder.

5. A method of treating seizures in a mammal which comprises administering an effective amount of an agonist of the GNR on the GRC.

6. A method of treating mood disorders in a mammal which comprises administering an effective amount of an agonist of the GNR on the GRC.

7. The method of claim 6 wherein the mood disorder is depression.

8. A method of treating PMS in a mammal which comprises administering an effective amount of an agonist of the GNR on the GRC.

9. A method of treating PND in a mammal which comprises administering an effective amount of an agonist of the GNR on the GRC.

10. A method of treating insomnia in a mammal which comprises administering an effective amount of an agonist of the GNR on the GRC.

11. A method for screening for drugs effective in treating a disease selected from the group consisting of anxiety, convulsions, mood disorders, PMS, PND, and insomnia comprising screening for agonists of the GNR on the GRC.

12. The method of claim 11 wherein the screening is by a competitive assay.

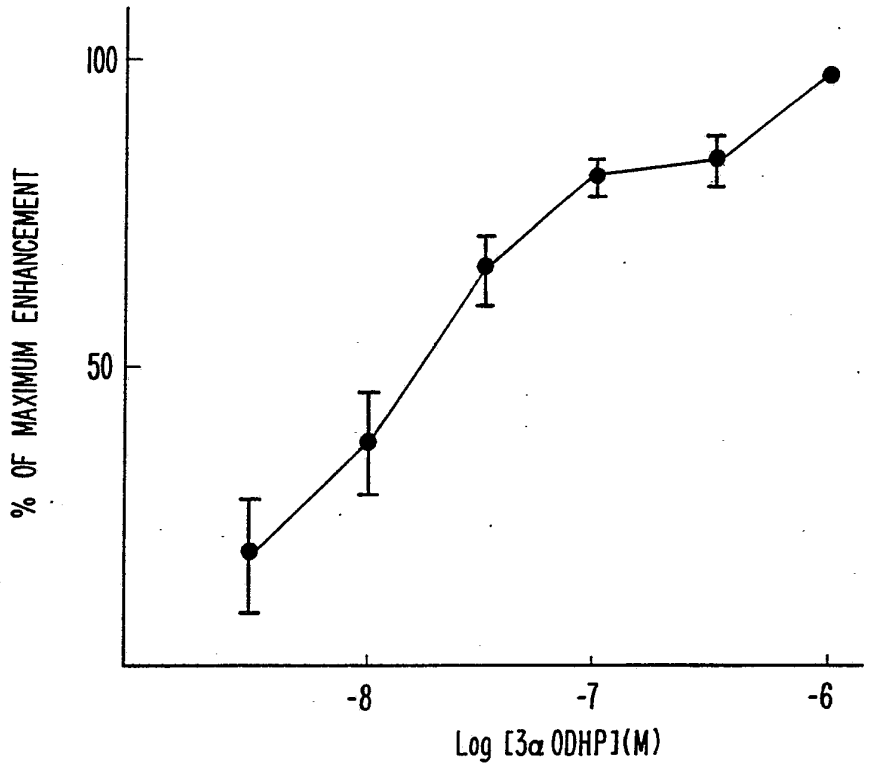
13. The method of claim 11 wherein the screening is by allosteric modulatory assay.

14. A method for screening for drugs that bind to GNRs with different subtype specificity comprising expressing cDNA encoding GRC subtypes into cells to form an expressed GNR subtype and screening for agonists of that subtype.

15. The method of claim 14 wherein the screening is by a competitive assay.

16. The method of claim 14 wherein the screening is by allosteric modulatory assay.

FIG. 1.



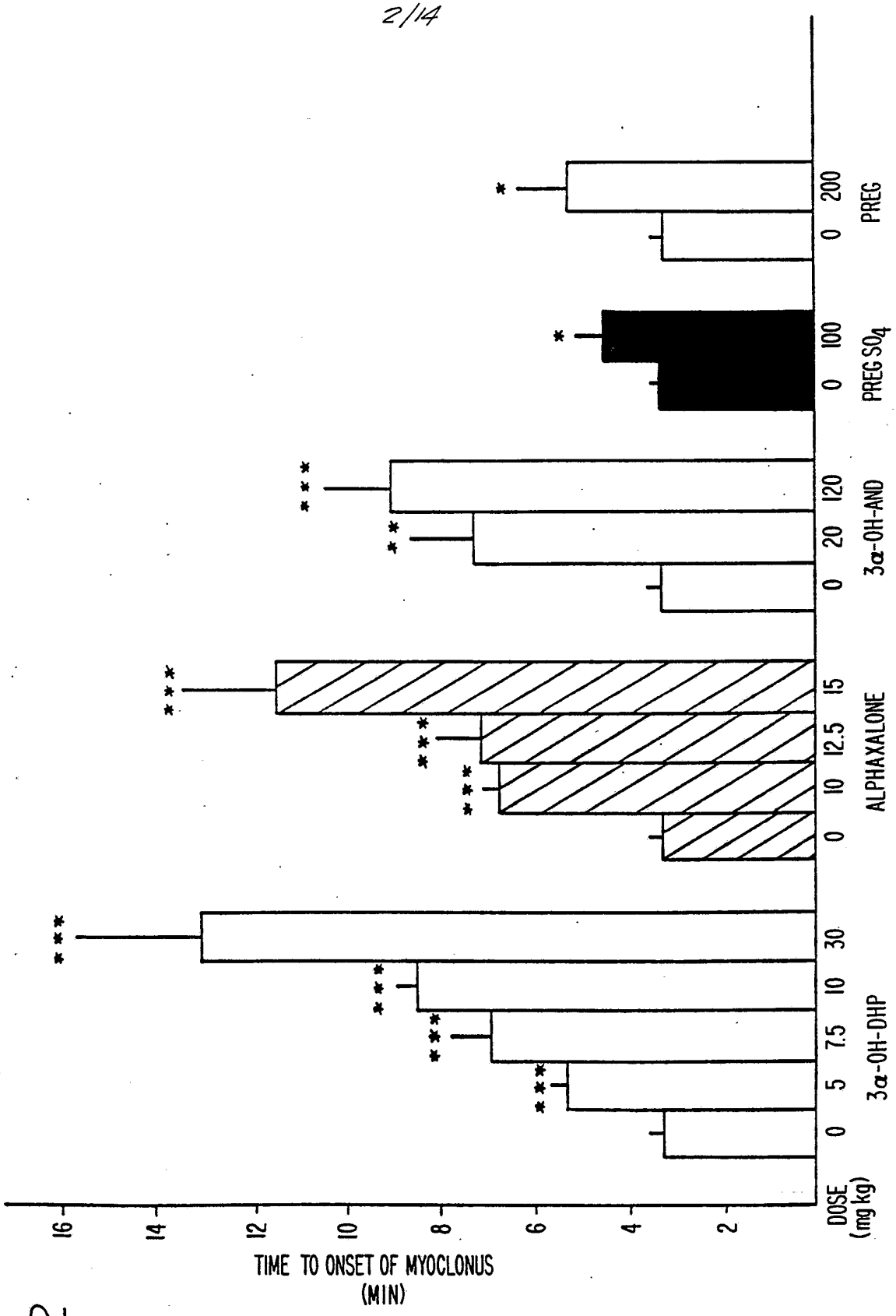

TIME TO ONSET OF MYOCLONUS
(MIN)
16
14
12
10
8
6
4
2
DOSE
(mg kg)
0 5 7.5 10 30
3α-OH-DHP
0 10 12.5 15
ALPHAXALONE
0 20 120
3α-OH-AND
0 100
PREG SO4
0 200
PREG

FIG. 2

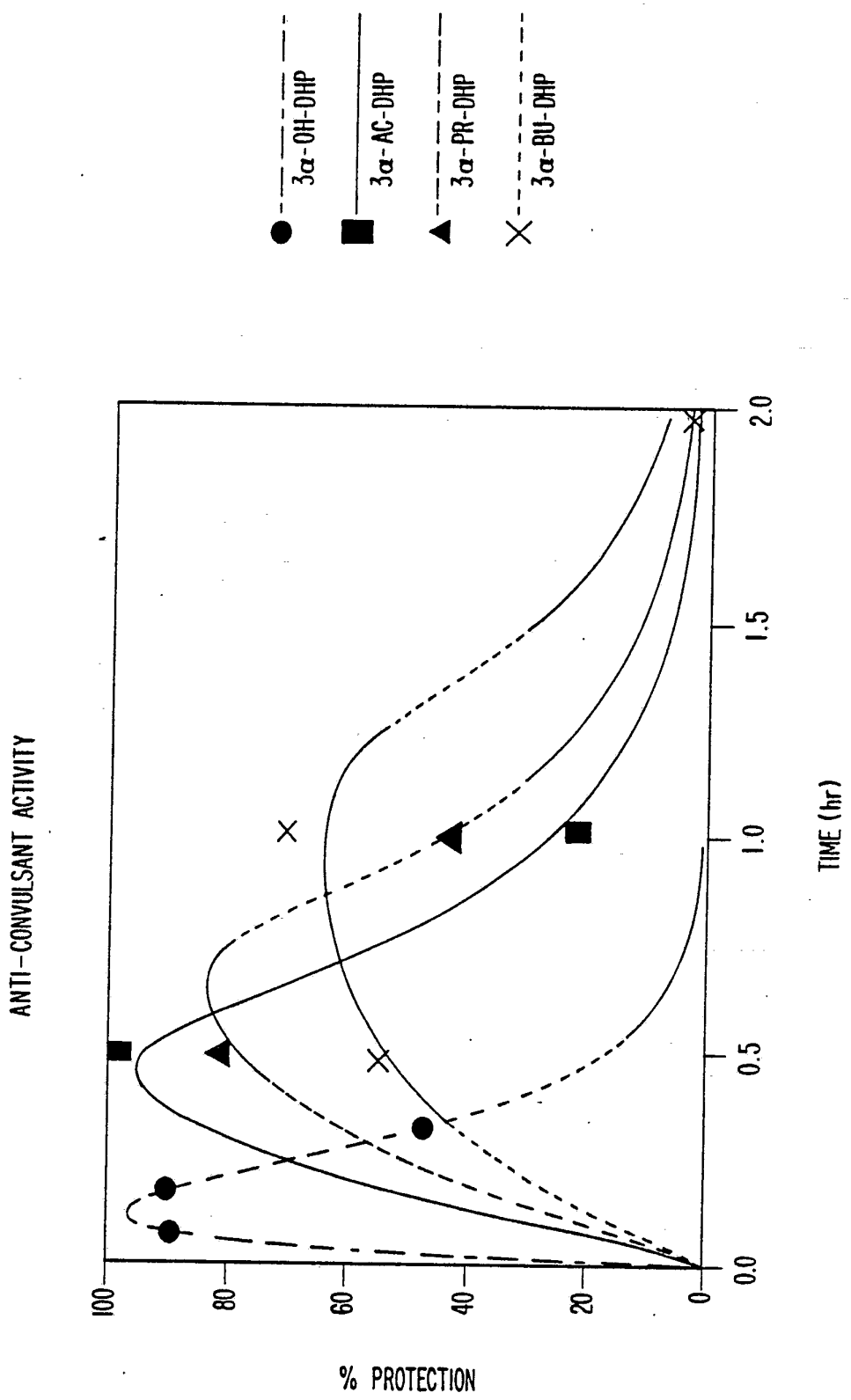

3α-OH-DHP
3α-AC-DHP
3α-PR-DHP
3α-BU-DHP
ANTI-CONVULSANT ACTIVITY
% PROTECTION
TIME (hr)
0
20
40
60
80
100
0.0
0.5
1.0
1.5
2.0

FIG. 3

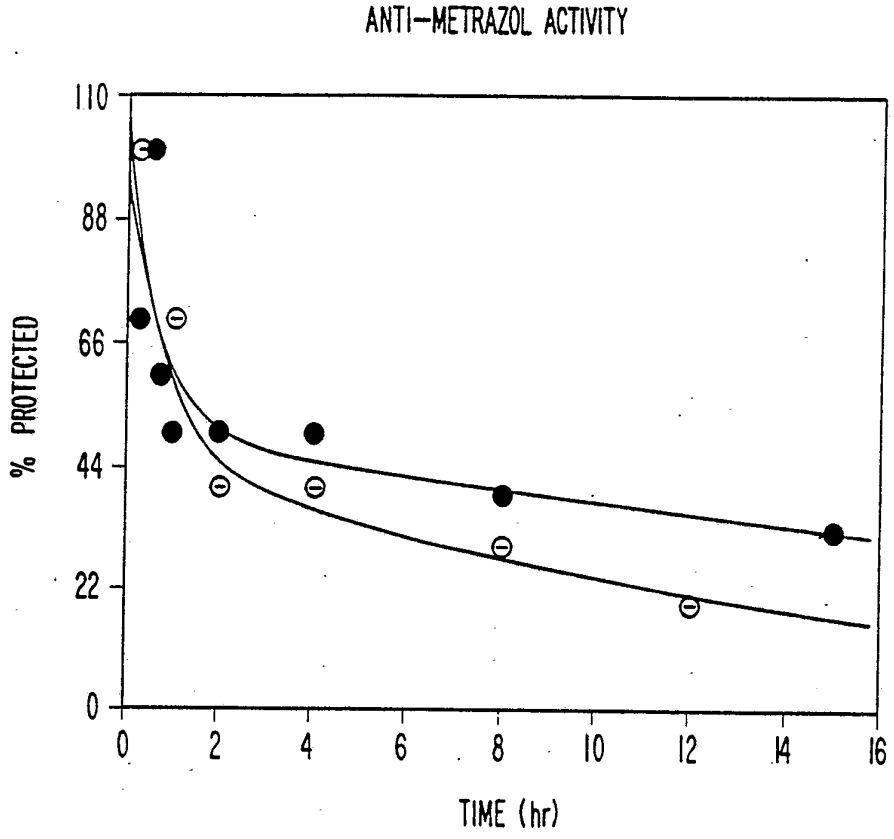

ANTI-METRAZOL ACTIVITY
% PROTECTED
110
88
66
44
22
0
0
2
4
6
8
10
12
14
16
TIME (hr)

FIG. 4

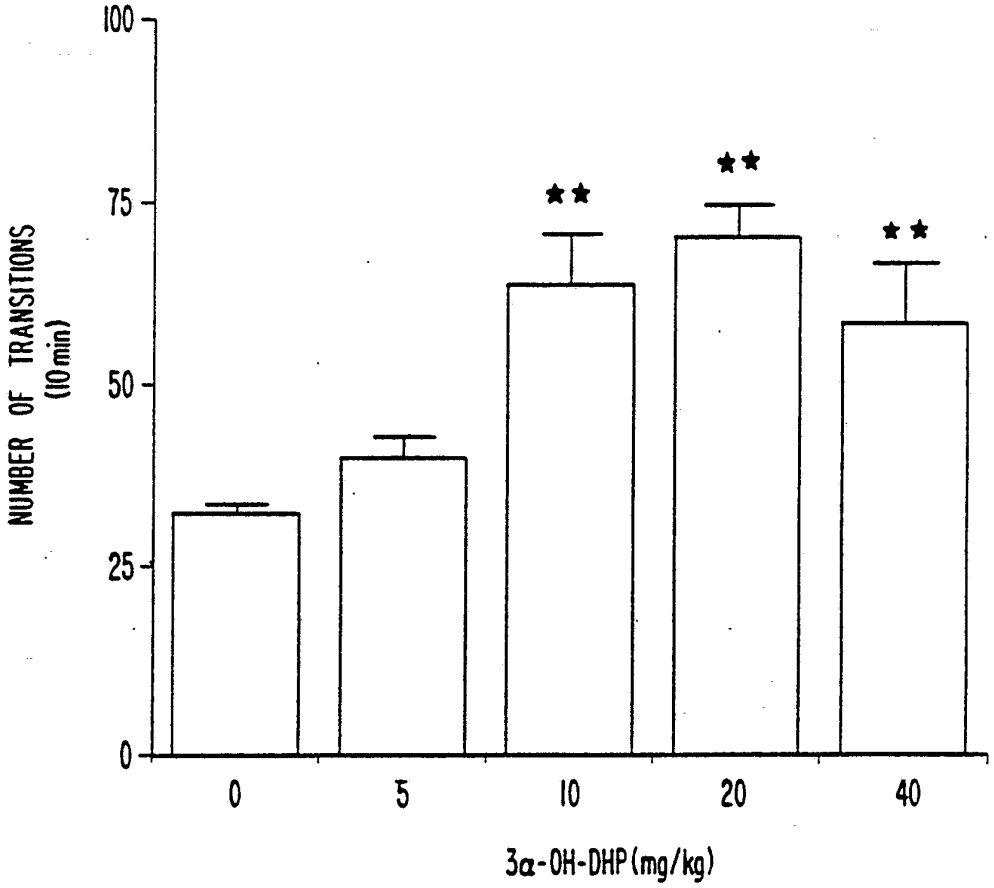

100
75
50
25
0
NUMBER OF TRANSITIONS (10 min)
**
**
**
0
5
10
20
40
3α-OH-DHP (mg/kg)

FIG. 5

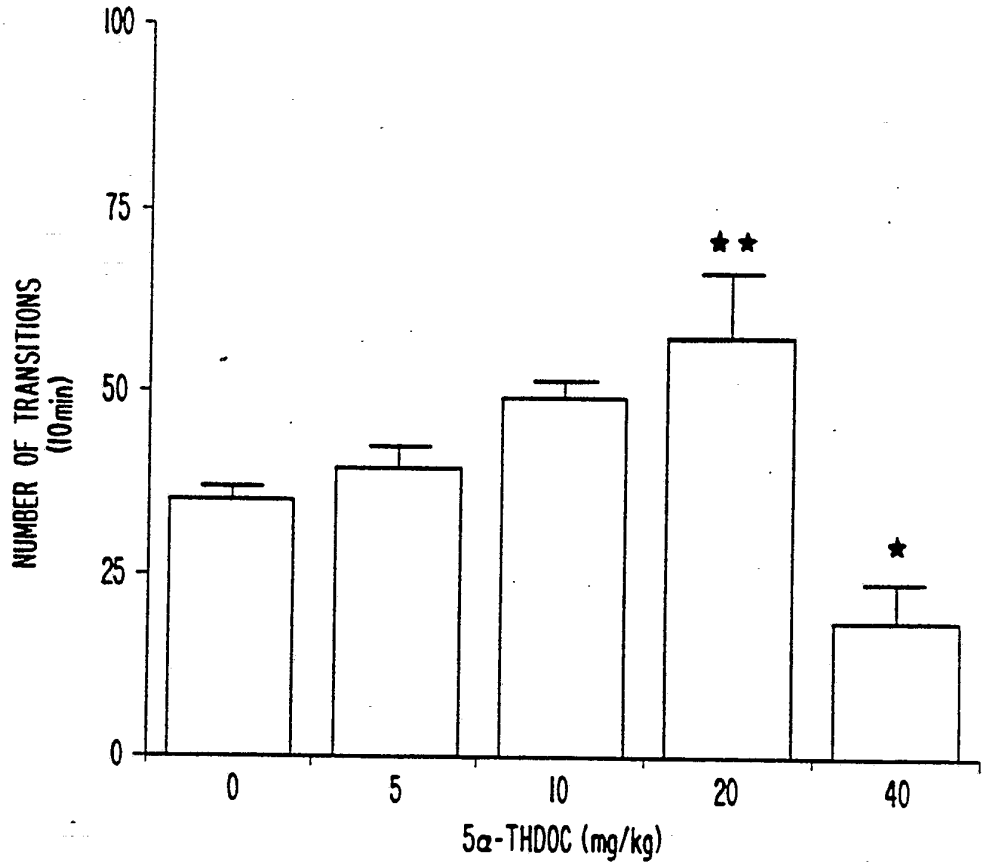

100
75
50
25
0
NUMBER OF TRANSITIONS (10min)
**
*
0
5
10
20
40
5α-THDOC (mg/kg)

FIG. 6

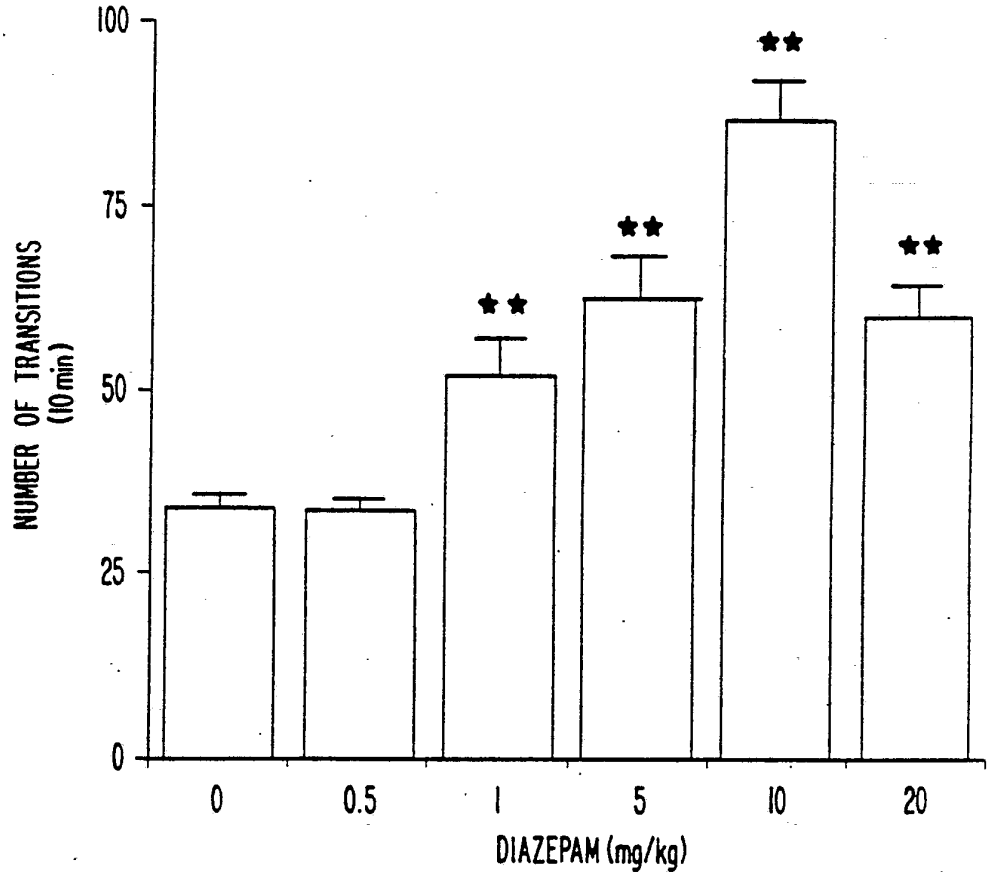

100
75
50
25
0
NUMBER OF TRANSITIONS (10 min)
**
**
**
**
0
0.5
1
5
10
20
DIAZEPAM (mg/kg)

FIG. 7

FIG. 8

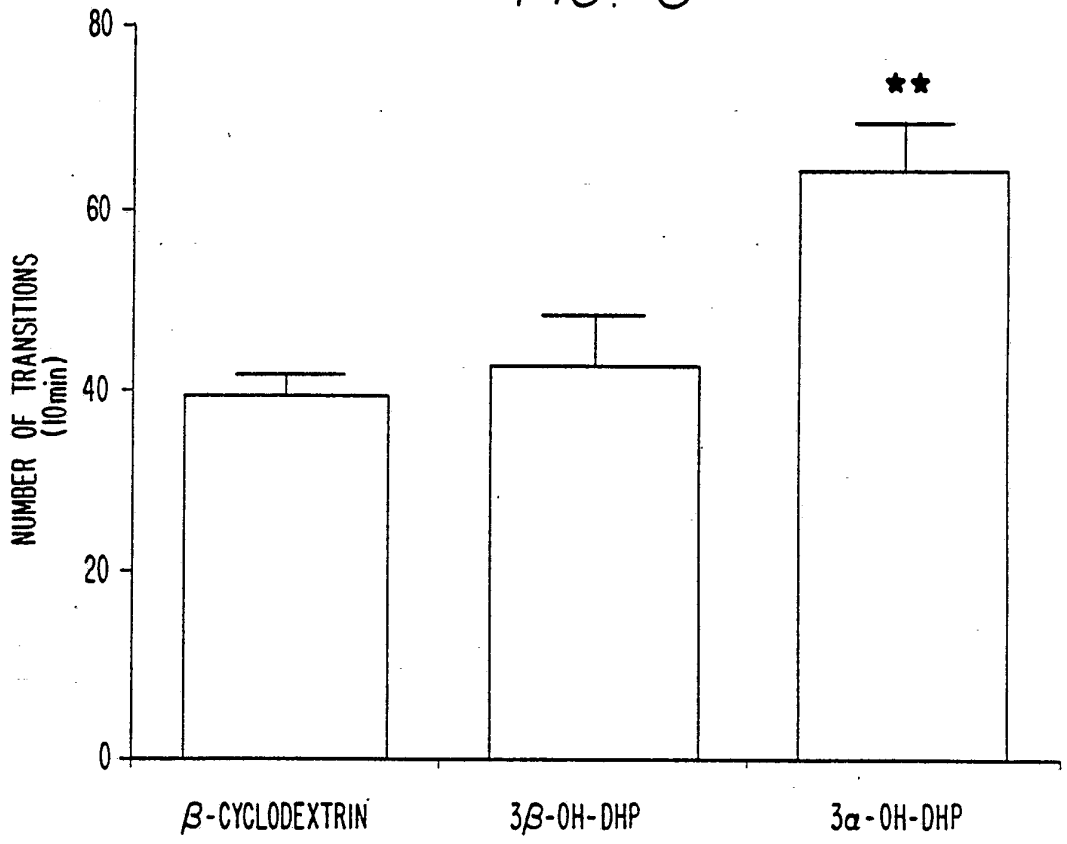

80
60
40
20
0
NUMBER OF TRANSITIONS (10min)
**
β-CYCLODEXTRIN
3β-OH-DHP
3α-OH-DHP

FIG. 9A

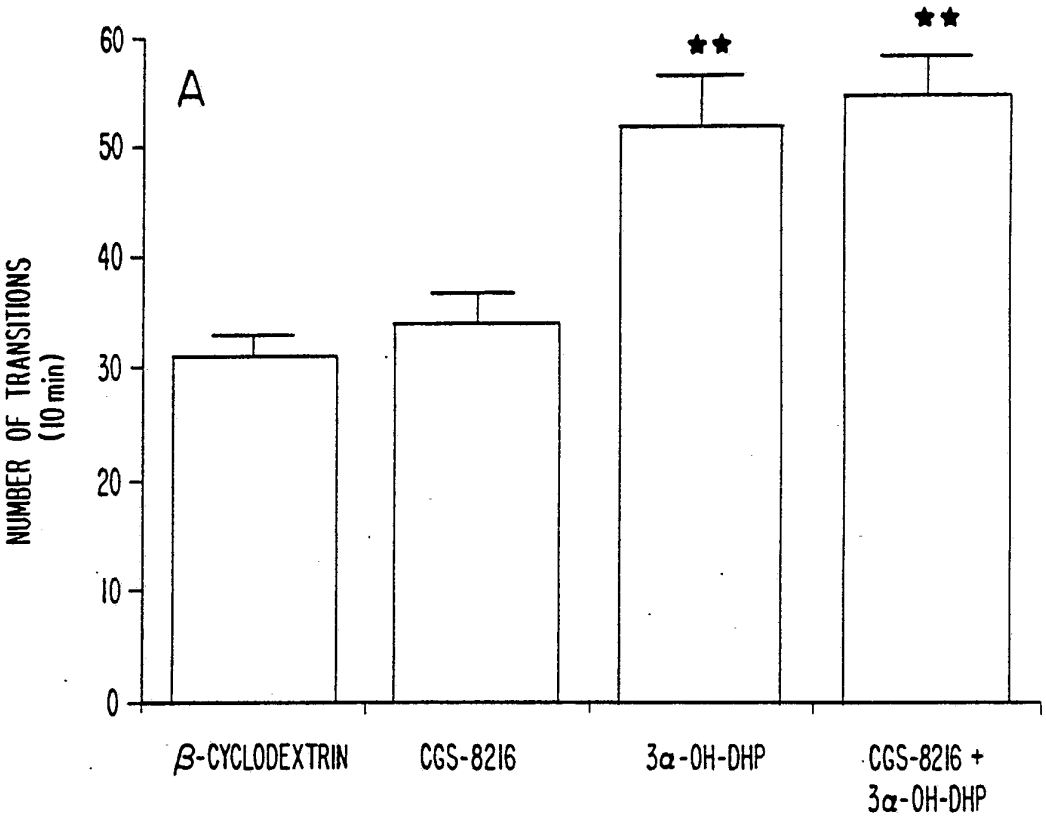
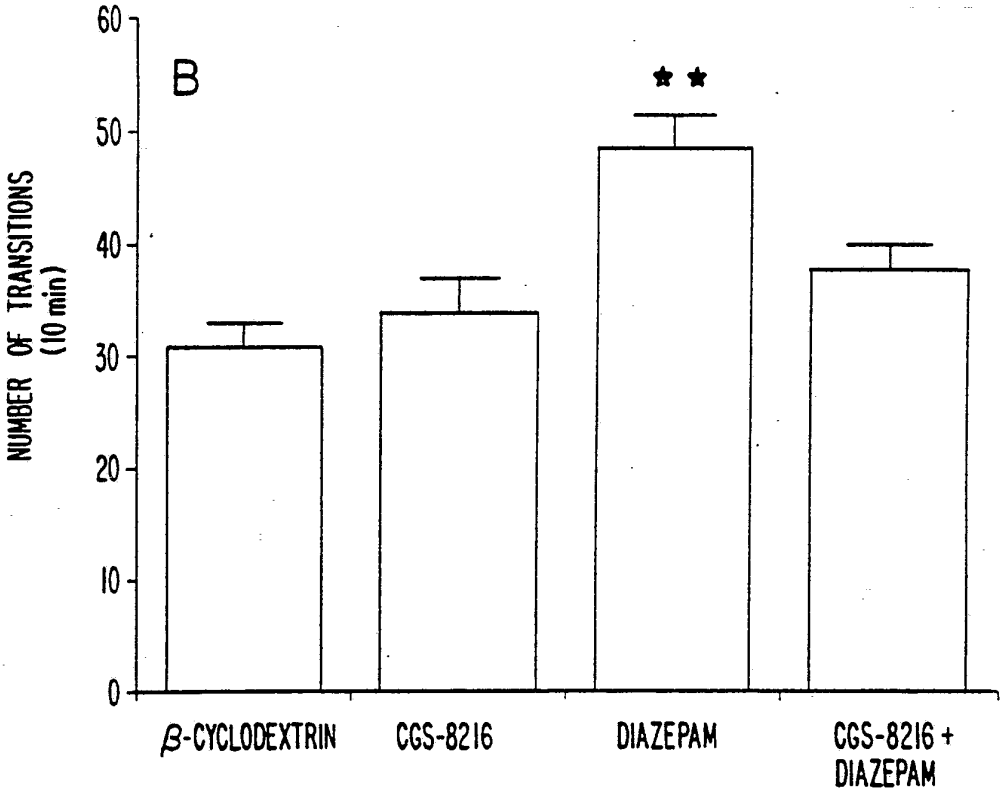

A
NUMBER OF TRANSITIONS (10 min)
60
50
40
30
20
10
0
**
**
β-CYCLODEXTRIN
CGS-8216
3α-OH-DHP
CGS-8216 + 3α-OH-DHP

FIG. 9B


B
NUMBER OF TRANSITIONS (10 min)
60
50
40
30
20
10
0
**
β-CYCLODEXTRIN
CGS-8216
DIAZEPAM
CGS-8216 + DIAZEPAM

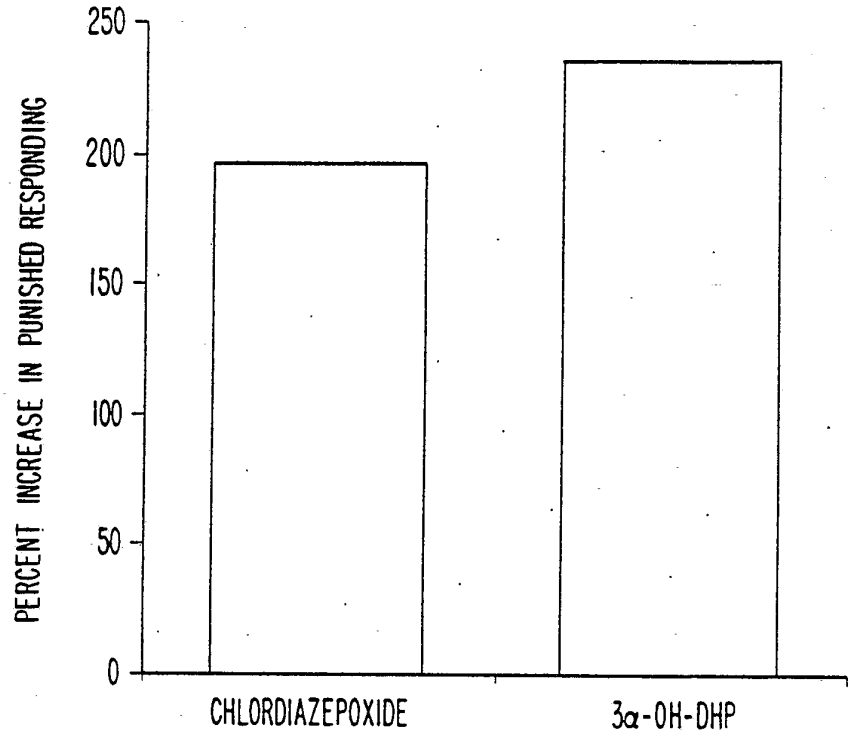

PERCENT INCREASE IN PUNISHED RESPONDING
250
200
150
100
50
0
CHLORDIAZEPOXIDE
3α-OH-DHP

FIG. 10

FIG. 11

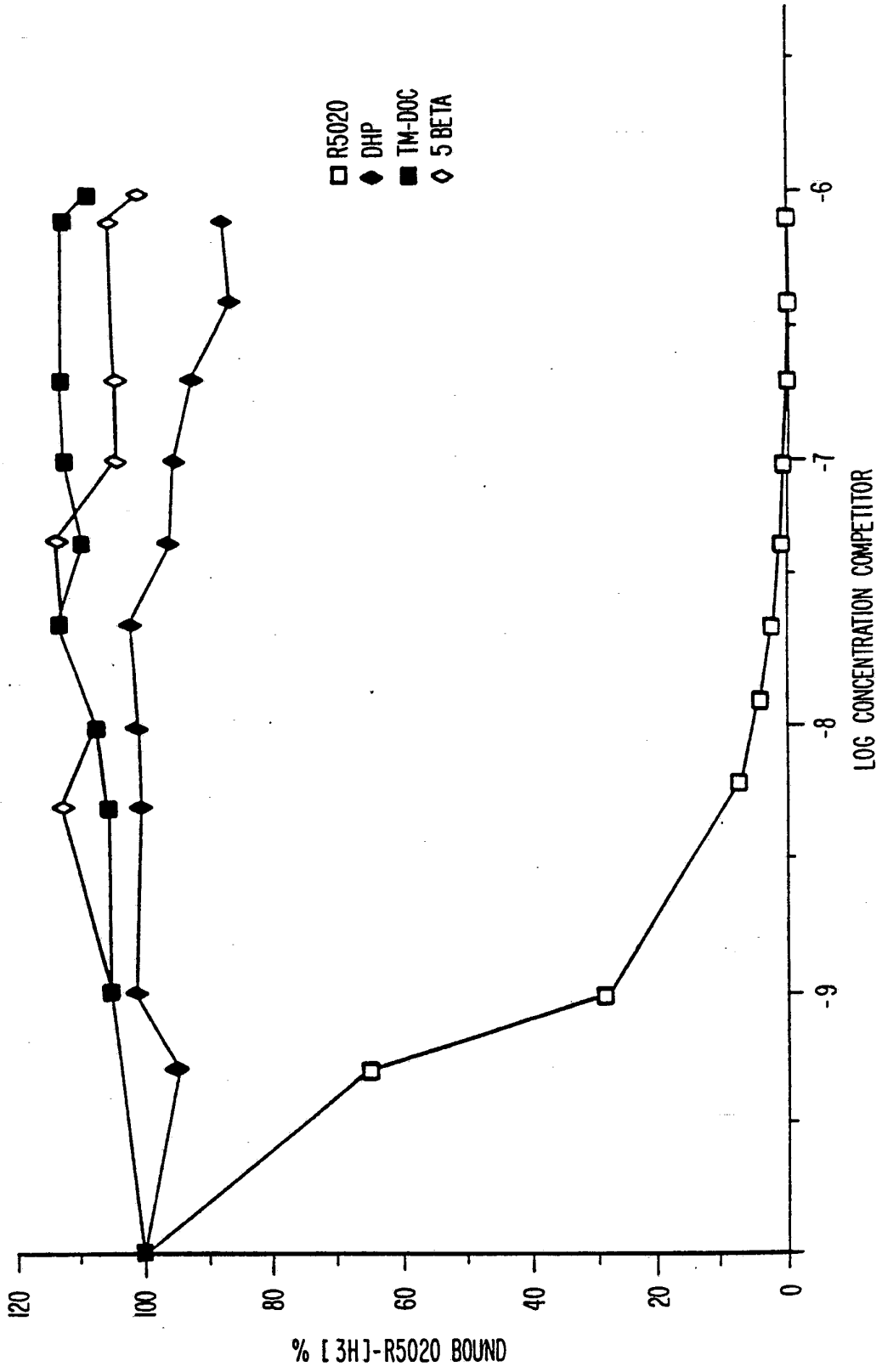

R5020
DHP
TM-DOC
5 BETA
120
100
80
60
40
20
0
% [3H]-R5020 BOUND
-6
-7
-8
-9
LOG CONCENTRATION COMPETITOR

FIG. 12

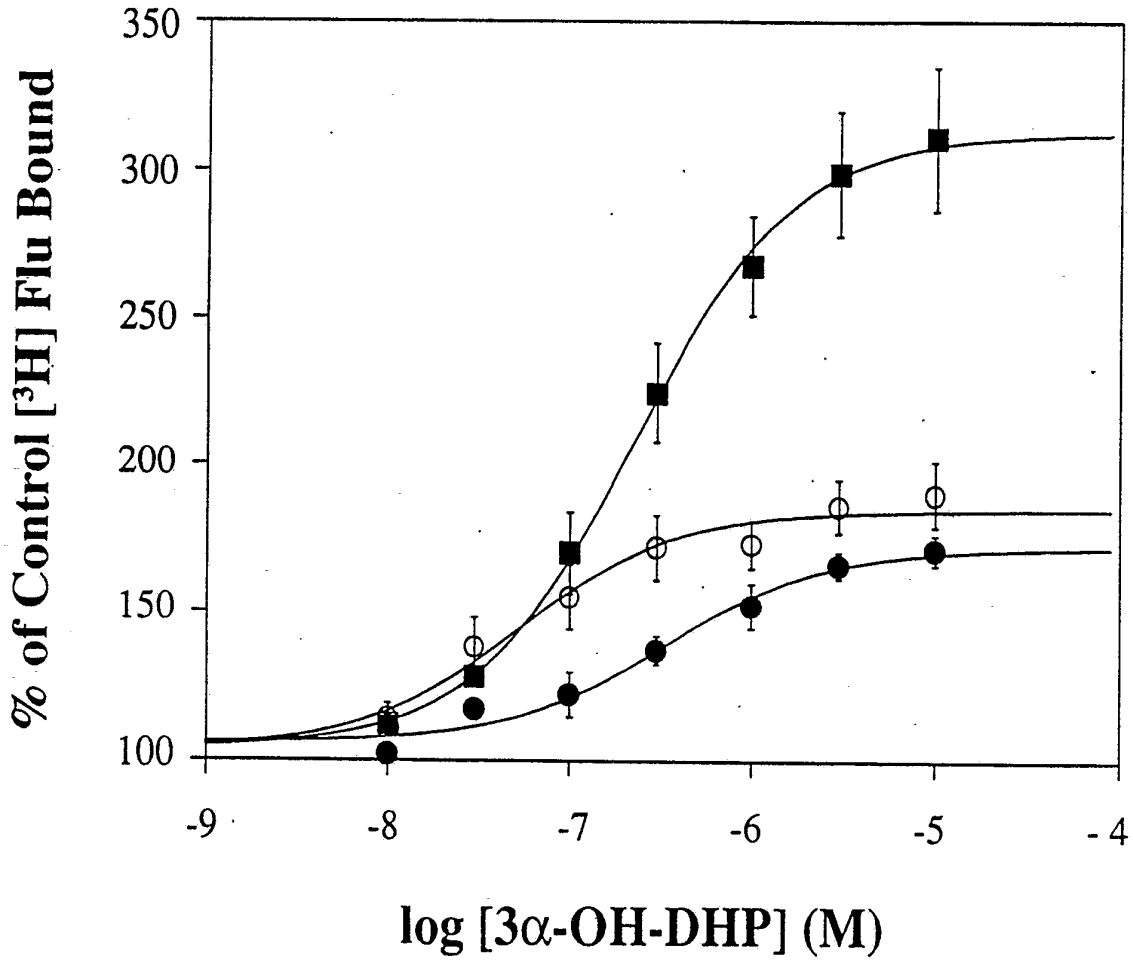

% of Control [3H] Flu Bound
350
300
250
200
150
100
-9
-8
-7
-6
-5
-4
log [3α-OH-DHP] (M)

FIG. 13

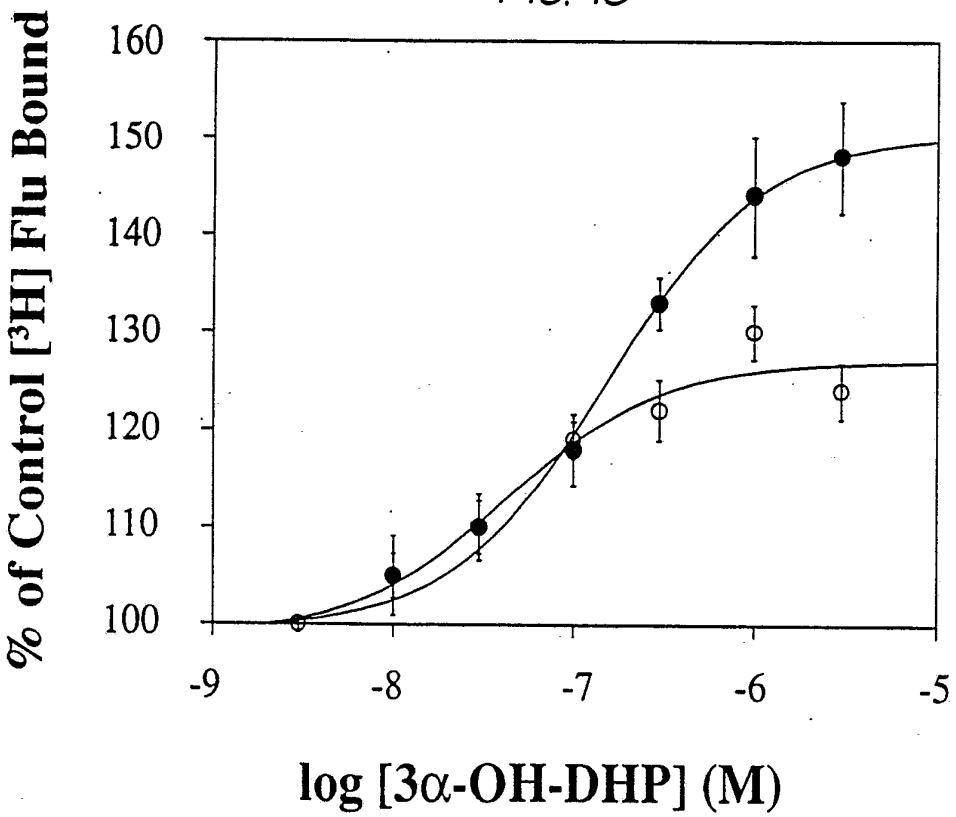
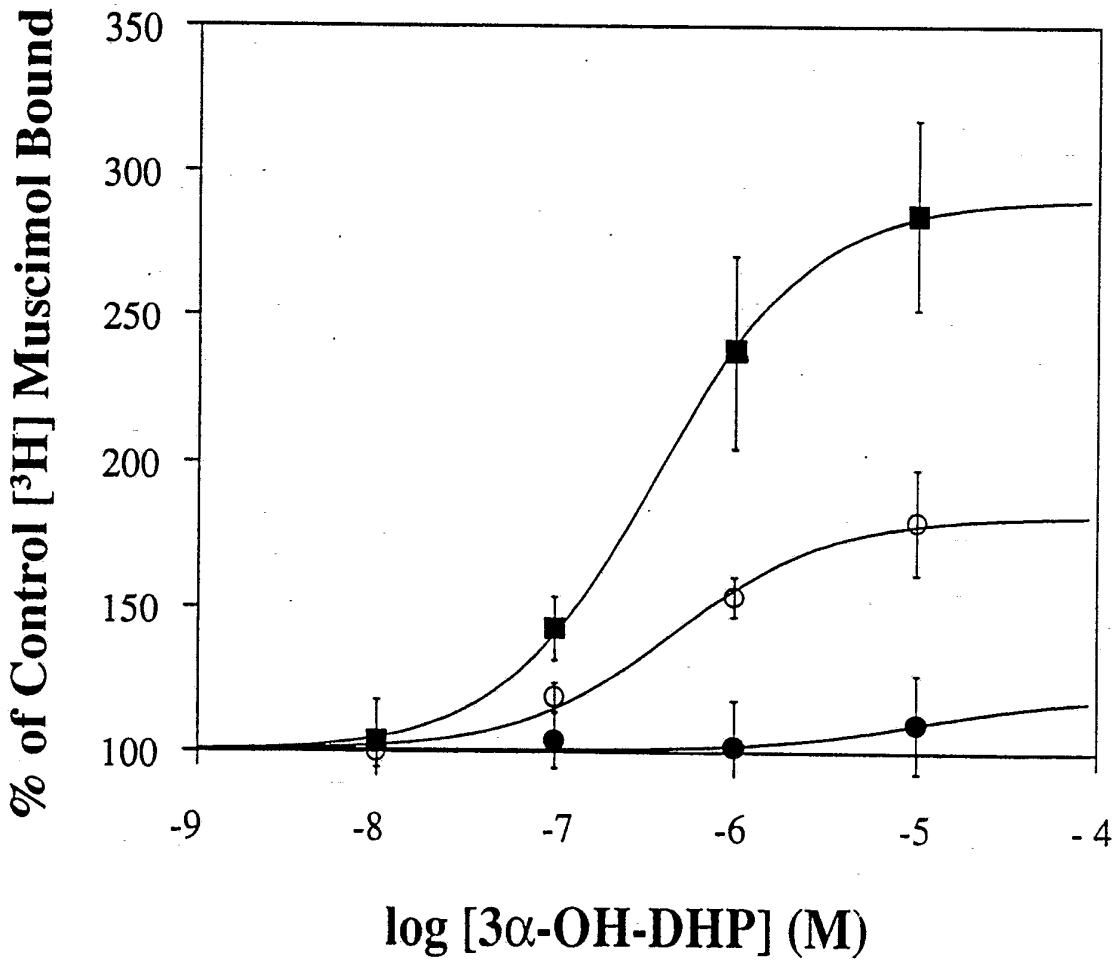

% of Control [3H] Flu Bound
160
150
140
130
120
110
100
-9
-8
-7
-6
-5
log [3α-OH-DHP] (M)

FIG. 14


% of Control [3H] Muscimol Bound
350
300
250
200
150
100
-9
-8
-7
-6
-5
- 4
log [3α-OH-DHP] (M)

INTERNATIONAL SEARCH REPORT

ternational application No.
PCT/US92/07613

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 31/56, 31/57, 37/02; C07K 15/00; C12Q 1/25, 1/68; G01N 33/53
US CL :424/570; 435/6, 7.1, 7.21; 514/12, 169, 177, 182; 530/350
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/570; 435/6, 7.1, 7.21; 514/12, 169, 177, 182; 530/350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	CIBA Foundation Symposium, Volume 153, issued 1990, M. D. Majewska, "Steroid regulation of the $GABA_A$ receptor: ligand binding, chloride transport and behaviour", pages 83-106, especially the abstract and pages 93-95.	1-9 10-16
X Y	Journal of Pharmacology and Experimental Therapeutics, Volume 246, Number 2, issued 1988, K. W. Gee et al., "Steroid Modulation of the Chloride Ionophore in Rat Brain: Structure-Activity Requirements, Regional Dependence and Mechanism of Action", pages 803-812, especially the abstract and page 812, column 1.	1-4, 8 5-7, 9-16
X Y	Trends in Pharmacological Sciences, Volume 11, issued November 1990, W. Haefely et al., "Novel anxioloytics that act as partial agonists at benzodiazepine receptors", pages 452-456; see the entire document.	1-5 6-16

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 30 NOVEMBER 1992	Date of mailing of the international search report 04 DEC 1992
Name and mailing address of the ISA/ Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer DAVID L. FITZGERALD Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/07613

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	Journal of Pharmacology and Experimental Therapeutics, Volume 241, Number 1, issued 1987, N. L. Harrison et al., "Structure-Activity Relationships for Steroid Interaction with the gamma-Aminobutyric Acid$_A$ Receptor Complex", pages 346-353, especially the abstract and page 352, column 2.	1-4 5-16
X Y	Science, Volume 232, issued 23 May 1986, M. D. Majewska et al., "Steroid Hormone Metabolites Are Barbiturate-Like Modulators of the GABA Receptor", pages 1004-1007, especially the abstract.	1 2-16
X Y A	Neuroscience and Behavioral Reviews, Vol. 14, issued 1990, D. Belelli et al., "Anticonvulsant Steroids and the GABA/Benzodiazepine Receptor-Chloride Ionophore Complex", pages 315-322, especially the abstract and pages 317-319.	1, 5, 10, 11 12-16 2-4, 6-9
Y	European Journal of Pharmacology - Molecular Pharmacology Section, Volume 188, issued 1990, N. C. Lan et al., "A steroid recognition site is functionally coupled to an expressed GABA$_A$-benzodiazepine receptor", pages 403-406, especially pages 403-404.	1-16
A	Science, Volume 245, issued 25 September 1989, D. B. Pritchett et al., "Type I and Type II GABA$_A$-Benzodiazepine Receptors Produced in Transfected Cells", pages 1389-1392.	1-16
A	Nature, Volume 328, issued 16 July 1987, P. R. Schofield et al., "Sequence and functional expression of the GABA$_A$ receptor shows a ligand-gated receptor super-family", pages 221-227.	1-16
A	European Journal of Pharmacology, Volume 136, issued 1987, K. W. Gee et al., "GABA-dependent modulation of the Cl^- ionophore by steroids in rat brain", pages 419-423.	1-16
A	Molecular Neurobiology, Volume 2, issued 1988, K. W. Gee, "Steroid Modulation of the GABA/Benzodiazepine Receptor-Linked Chloride Ionophore", pages 291-317.	1-16
A,T	Progress in Neurobiology, Volume 38, issued 1992, M. D. Majewska, "Neurosteroids: endogenous bimodal modulators of the GABA$_A$ receptor. Mechanism of action and physiological significance", pages 379-395.	1-16

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

US PTO-APS; Medline
Search terms: GABA receptor; seizure, anxiety, panic, mood disorder, depression; premenstrual/PMS, postnatal.

SciSearch
Searched citations of: GEE KW, 1987, V136, P419, EUR J PHARMACOL; HARRISON NL, 1987, V241, P346, J PHARMACOL EXP.