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Correlation of Neuroactive Steroid Modulation of [^{35}S]*t*-Butylbicyclophosphorothionate and [^{3}H]Flunitrazepam Binding and γ-Aminobutyric Acid$_A$ Receptor Function

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SUMMARY

Neuroactive steroids, including endogenously occurring metabolites of progesterone and deoxycorticosterone as well as their synthetic derivatives, are positive allosteric modulators of the γ-aminobutyric acid (GABA)$_A$ receptor complex. They inhibit the binding of [^{35}S]*t*-butylbicyclophosphorothionate ([^{35}S]TBPS), enhance the binding of [^{3}H]flunitrazepam, and potentiate GABA-evoked chloride currents and agonist-stimulated $^{36}Cl^-$ uptake. The structure-activity relationship for 31 neuroactive steroids and related compounds was explored by examining their relative ability to inhibit [^{35}S]TBPS binding in rat brain cortical membranes. A free 3α-hydroxy group is necessary for high potency inhibition. Whereas hydroxylation in the 21-position and subsequent esterification maintain activity, 11α- or 12α-hydroxylation greatly reduces activity. The rank order of potency for 17-position substitutions in the 5α-reduced series is 17β-acetyl > 17β-cyano > 17β-methoxycarbonyl > 17α-acetyl > 17-one ≥ 17-oxime ≥ 17α-cyano. Introduction of a double bond between the 9- and 11-positions reduces potency, whereas a double bond in the 4-position reduces the maximal extent of inhibition. Comparing the activities of these neuroactive steroids and related compounds in the [^{35}S]TBPS and [^{3}H]flunitrazepam assays, there is a strong correlation between potency (r = 0.90, n = 17) and magnitude of modulation (r = 0.95, n = 31), indicating that the neuroactive steroid binding site is similarly coupled to the TBPS and benzodiazepine sites in rat cortex. However, there is a weaker correlation (r = 0.74–0.78, n = 31) between the degree of modulation in either binding assay and potentiation of muscimol-stimulated $^{36}Cl^-$ uptake in rat cortical synaptoneurosomes. Using an electrophysiological approach, stronger correlations (r = 0.89–0.94, n = 15) were observed between the magnitude of modulation in the binding assays and potentiation of GABA-evoked chloride currents in *Xenopus* oocytes expressing human $\alpha1\beta1\gamma2L$ receptor complexes. Thus, neuroactive steroid modulation of [^{35}S]TBPS and [^{3}H]flunitrazepam binding is predictive of functional activity at the GABA$_A$ receptor complex.

Neuroactive steroids are steroid hormone metabolites and their synthetic derivatives that modulate brain function by nongenomic mechanisms (1). Certain neuroactive steroids are positive allosteric modulators of the GABA$_A$ receptor complex. 3α,5α-P (Fig. 1) is the 3α-hydroxylated, 5α-reduced metabolite of progesterone and can be considered the prototype for this class of modulators, which have a binding site distinct from those for other classes of GABA$_A$ receptor ligands, such as the benzodiazepines and barbiturates (2–4).

Neuroactive steroids allosterically inhibit the binding of [^{35}S]TBPS, which binds to a site associated with the chloride channel portion of the receptor complex, and enhance the binding of [^{3}H]flunitrazepam to the benzodiazepine site (2, 5). Neuroactive steroids have also been shown, by both $^{36}Cl^-$ uptake and electrophysiological techniques, to modulate the function of the receptor complex (3, 5–11). However, the relative coupling of neuroactive steroids with the TBPS and benzodiazepine sites is not known. Moreover, the relationship between the activity of neuroactive steroids in radioligand binding assays and that in functional assays has not been addressed.

A group of 31 neuroactive steroids and related compounds that were previously examined as modulators of $^{36}Cl^-$ uptake (11) were selected for further evaluation. The present study demonstrates that for these compounds there is a strong correlation between potency and the magnitude of modulation of the binding of [^{35}S]TBPS and [^{3}H]flunitrazepam, indicating that neuroactive steroids are similarly coupled to TBPS and

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ABBREVIATIONS: GABA, γ-aminobutyric acid; TBPS, *t*-butylbicyclophosphorothionate; DMSO, dimethylsulfoxide; 3α,5α-P, 3α-hydroxy-5α-pregnan-20-one; SAR, structure-activity relationship; THDOC, tetrahydrodeoxycorticosterone; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

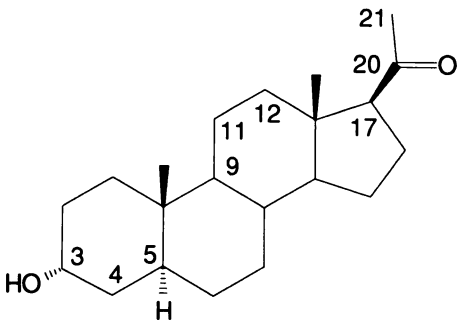

Fig. 1. Structure of 3α,5α-P (1) with numbered positions encompassing the structural modifications examined.

benzodiazepine sites in rat cortex. Although there is not a strong correlation between the degree of modulation of radioligand binding by neuroactive steroids and potentiation of muscimol-stimulated $^{36}Cl^-$ uptake, there is a good correlation between binding and GABA-evoked currents, indicating that neuroactive steroid modulation of the binding of [^{35}S]TBPS and [^{3}H]flunitrazepam predicts their functional activity at the $GABA_A$ receptor complex. A preliminary report of part of this work has been presented (12).

Materials and Methods

Radioligand Binding Assays

Membrane preparation. Rat brain cortical membranes were prepared as described previously (2). Briefly, cortices were rapidly removed 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 × *g* for 10 min at 4°. The resultant supernatants were centrifuged at 10,000 × *g* for 20 min at 4°, to obtain the P_2 pellets. The P_2 pellets were resuspended in 200 mM NaCl, 50 mM sodium/potassium phosphate, pH 7.4, and centrifuged at 10,000 × *g* for 10 min at 4°. This washing procedure was repeated twice and the pellets were resuspended in 10 volumes of buffer.

[^{35}S]TBPS binding assay. 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) 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 the filters were rinsed three times with ice-cold buffer. Filter-bound radioactivity was measured by liquid scintillation counting.

TABLE 1

Activities of 31 neuroactive steroids and related compounds in radioligand binding and $^{36}Cl^-$ uptake assays

Compounds (1 nm to 10 μM) were incubated with rat brain cortical P_2 membranes and 2 nM [^{35}S]TBPS plus 5 μM GABA (TBPS assay) or 1 nM [^{3}H]flunitrazepam plus 1 μM GABA (flunitrazepam assay).The values listed for percentage inhibition, percentage enhancement, and potentiation of muscimol-stimulated chloride uptake (Cl^- uptake) were obtained with 1 μM steroid. IC_{50}, EC_{50}, percentage inhibition, and percentage enhancement values are expressed as means ± standard errors of at least three independent experiments. The concentration-response curves for all active compounds were fit with Hill coefficients of 1.0, except for compounds **7** (1.6 ± 0.2) and **22** (1.4 ± 0.1) in the [^{35}S]TBPS assay and **2** (0.84 ± 0.04) and **16** (1.7 ± 0.2) in the [^{3}H]flunitrazepam assay. Cl^- uptake data are for the steroid-induced potentiation of 3 μM muscimol-stimulated $^{36}Cl^-$ uptake by rat cortical synaptoneurosomes in 5 sec (from Ref. 11).

No.	Compound	TBPS		Flunitrazepam		Cl^- uptake
		IC_{50}	Inhibition	EC_{50}	Enhancement	
		nM	%	*nM*	%	*nmol/mg*
1	3α,5α-P	37 ± 3	87 ± 1	101 ± 11	58 ± 8	10.9
2	3α-Hydroxypregn-4-en-20-one	50 ± 6	61 ± 4	178 ± 40	53 ± 3	10.4
3	5α-THDOC	69 ± 13	90 ± 3	334 ± 67	43 ± 4	13.1
4	21-Acetoxy-3α-hydroxy-5α-pregnan-20-one	76 ± 6	91 ± 3	148 ± 35	47 ± 6	12.2
5	17β-Cyano-3α-hydroxy-5α-androstane	81 ± 15	88 ± 2	122 ± 6	61 ± 4	13.0
6	3α-Hydroxy-21-mesyloxy-5α-pregnan-20-one	100 ± 20	89 ± 2	166 ± 33	46 ± 4	7.9
7	3α,20α-Dihydroxy-5α-pregnane	126 ± 16	42 ± 5	104 ± 12	27 ± 3	1.97
8	5α-Pregnane-3,20-dione	168 ± 16	14 ± 2	>10,000	0 ± 1	2.63
9	21-Acetoxy-3α-hydroxy-5β-pregnan-20-one	168 ± 22	43 ± 8	306 ± 56	33 ± 1	10.3
10	5β-THDOC	176 ± 17	48 ± 3	322 ± 52	35 ± 3	7.7
11	20,20-Ethylenedioxy-3α-hydroxy-5α-pregnane	191 ± 46	71 ± 4	355 ± 69	43 ± 2	0.16
12	3α-Hydroxy-5α-pregn-9(11)-en-20-one	259 ± 67	77 ± 6	293 ± 59	42 ± 2	1.23
13	3α-Hydroxy-17β-methoxycarbonyl-5α-androstane	318 ± 52	55 ± 4	499 ± 18	38 ± 3	3.6
14	21-Acetoxy-3α-hydroxy-5α-pregn-9(11)-en-20-one	363 ± 48	30 ± 5	561 ± 170	11 ± 1	0.34
15	20,20-Ethylenedioxy-3α-hydroxy-5β-pregnane	928 ± 180	46 ± 5	5,130 ± 870	15 ± 4	−0.96
16	3α-Hydroxy-5α,17α-pregnan-20-one	1,310 ± 260	47 ± 8	980 ± 170	17 ± 4	1.12
17	3α-Acetoxy-5α-pregnan-20-one	1,510 ± 250	25 ± 4	>10,000	7 ± 1	2.57
18	3α,21-Dihydroxy-5α-pregn-9(11)-en-20-one	1,770 ± 200	41 ± 3	2,090 ± 400	15 ± 1	1.40
19	3α-Hydroxy-5α-androstan-17-one	2,430 ± 390	35 ± 5	2,680 ± 650	16 ± 1	7.0
20	3α-Hydroxy-5α-androstan-17-one oxime	4,990 ± 850	17 ± 2	>10,000	3 ± 4	−1.91
21	3β-Hydroxypregn-4-en-20-one	6,040 ± 560	15 ± 2	>10,000	3 ± 2	1.43
22	17α-Cyano-3α-hydroxy-5α-androstane	6,340 ± 430	5 ± 3	>10,000	−0.2 ± 0.3	−2.10
23	3α-Acetoxy-21-hydroxy-5α-pregnan-20-one	7,250 ± 110	8 ± 2	>10,000	0 ± 2	0.48
24	3α,11α-Dihydroxy-5β-pregnan-20-one	10,200 ± 990	9 ± 2	>10,000	5 ± 1	0.98
25	3α,11α-Dihydroxy-5α-pregnan-20-one	11,200 ± 2,260	7 ± 3	>10,000	−2 ± 5	0.70
26	3α,21-Diacetoxy-5α-pregnan-20-one	>10,000	3 ± 2	>10,000	−2 ± 1	1.37
27	5α-Pregnane-3,20-dione 3-oxime	>10,000	3 ± 2	>10,000	1.80 ± 0.03	−3.26
28	3α-Hydroxy-5β-androstan-17-one	>10,000	2 ± 3	>10,000	2 ± 3	4.9
29	21-Hydroxy-5α-pregnane-3,20-dione	>10,000	3 ± 1	>10,000	2 ± 1	−1.95
30	3α,12α-Dihydroxy-5β-pregnan-20-one	>10,000	2 ± 1	>10,000	0 ± 1	−2.58
31	Pregn-4-ene-3,20-dione (progesterone)	>10,000	4 ± 1	>10,000	0.6 ± 0.4	−1.80

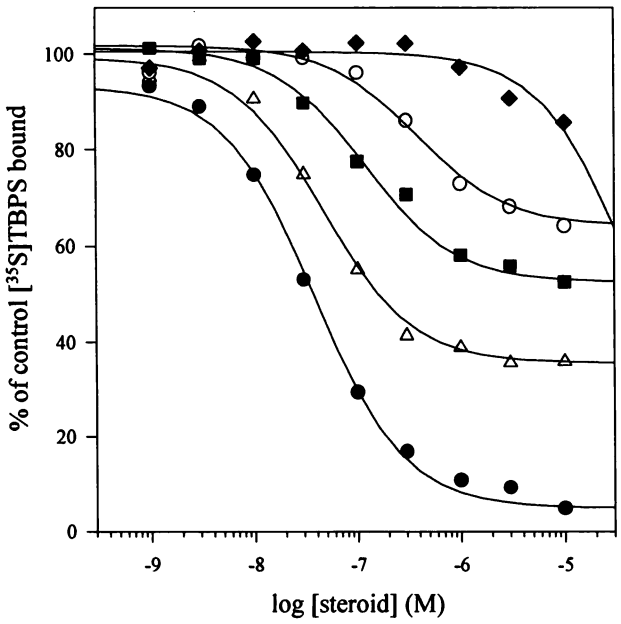

Fig. 2. Concentration-effect curves for inhibition of [^{35}S]TBPS binding by representative neuroactive steroids in rat brain cortical membranes. Compounds plotted are **1** (●), **2** (△), **9** (■), **14** (○), and **27** (♦). The data for each compound represent at least three independent experiments. Neuroactive steroids (1 nM to 10 μM) were incubated with P_2 membranes and 2 nM [^{35}S]TBPS in the presence of 5 μM GABA.

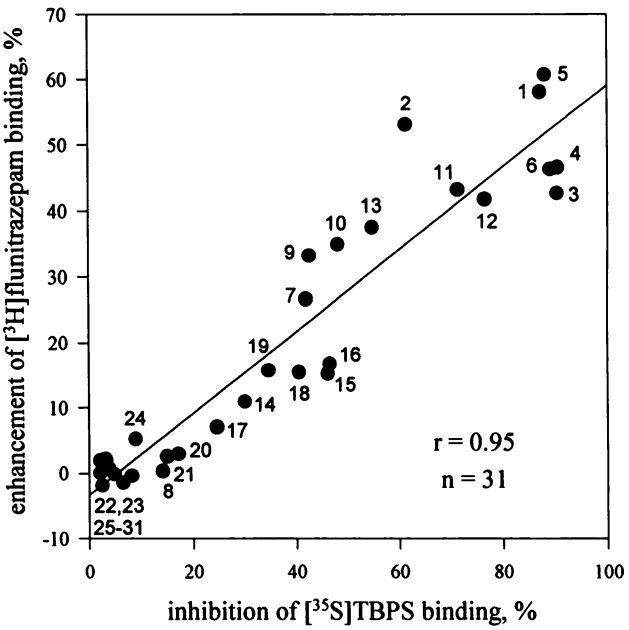

Fig. 4. Magnitude of neuroactive steroid (1 μM) modulation of [^{35}S]TBPS and [^{3}H]flunitrazepam binding in rat brain cortical membranes. Values for inhibition of [^{35}S]TBPS binding and enhancement of [^{3}H]flunitrazepam binding are from Table 1. The equation describing the regression line is $y = 0.625x - 3.35$. The slope is significantly different from 0 ($p < 0.0001$).

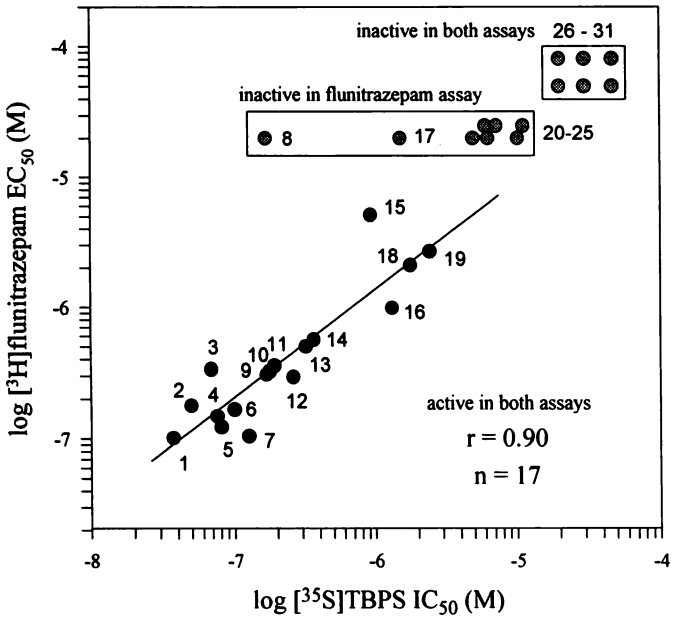

Fig. 3. Potency of neuroactive steroids in the [^{35}S]TBPS and [^{3}H]-flunitrazepam binding assays in rat brain cortical membranes. [^{35}S]TBPS IC_{50} and [^{3}H]flunitrazepam EC_{50} values are from Table 1. The equation describing the regression line is $y = 0.823x - 0.92$. The slope is significantly different from 0 ($p < 0.0001$).

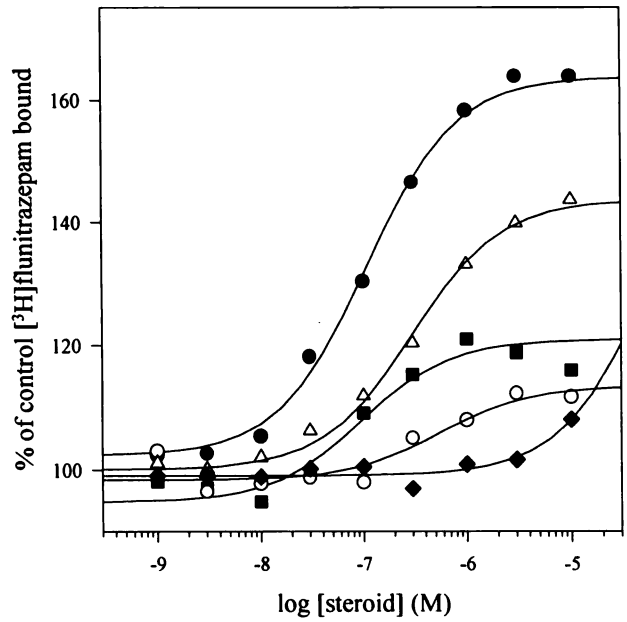

Fig. 5. Concentration-effect curves for enhancement of [^{3}H]flunitrazepam binding by representative neuroactive steroids in rat brain cortical membranes. Compounds plotted are **1** (●), **9** (△), **7** (■), **14** (○), and **27** (♦). The data for each compound represent at least three independent experiments. Neuroactive steroids (1 nM to 10 μM) were incubated with P_2 membranes and 1 nM [^{3}H]flunitrazepam in the presence of 1 μM GABA.

[^{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. The test drugs were added in 5 μl of 2-methoxyethanol or DMSO (final concentration, 0.5%). Nonspecific binding was determined in the presence of 1 μM clonazepam (Sigma) and ranged from 2 to 5%.

In Vitro Transcription of cDNA

Capped transcripts were synthesized from human GABA$_A$ receptor α1, β1, and γ2L subunit cDNAs (13). The cDNA from plasmids containing the human α1, β1, or γ2L subunit was linearized at the 3′ end insert by restriction endonuclease. The linearized cDNAs were transcribed using T7 RNA polymerase. Each RNA was verified as

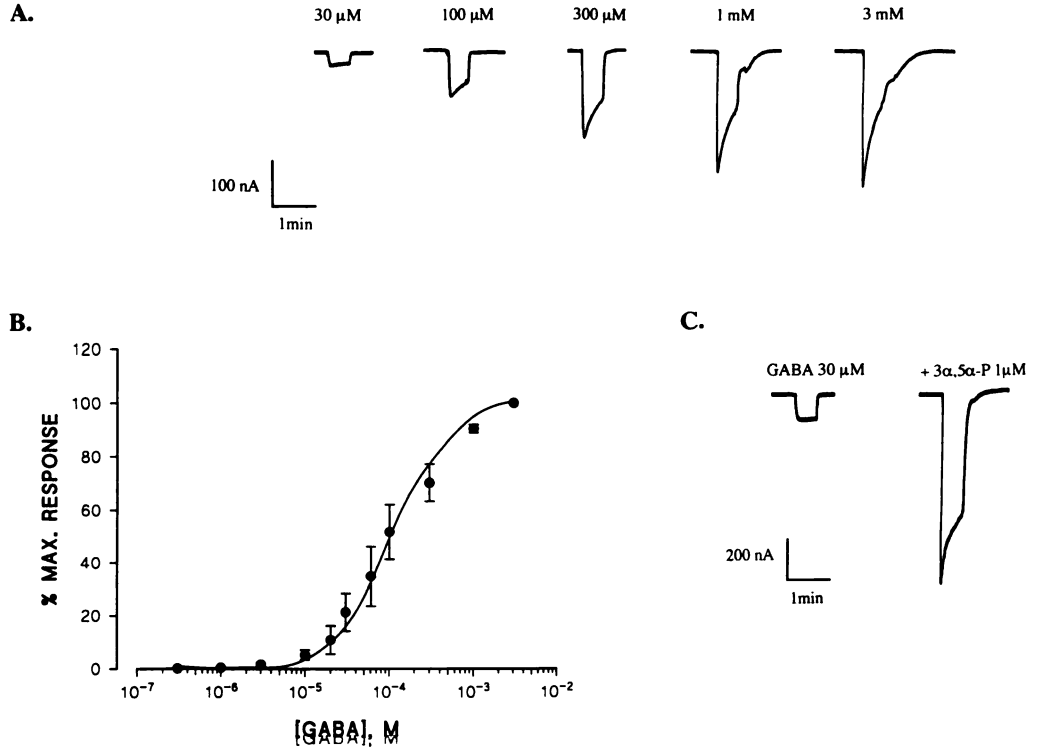

Fig. 6. GABA-evoked currents in *Xenopus* oocytes expressing human $\alpha1\beta1\gamma2L$ receptor complexes and potentiation by 3α,5α-P (compound **1**). A, GABA (30 μM to 3 mM)-evoked currents recorded from a single oocyte voltage-clamped at −60 mV. Note that, for GABA concentrations of ≥100 μM, a clear fading of the response in the continued presence of agonist is evident. B, GABA concentration-response relationship. Responses are expressed as a percentage of the maximal response to 3 mM GABA. Each point is the mean of data obtained from four or five oocytes. *Vertical lines* indicate standard errors where they exceed the size of the symbols. C, GABA (30 μM)-evoked currents recorded in the absence and presence of 1 μM 3α,5α-P. Note the large enhancement of the GABA current by the steroid and how the GABA current fades in amplitude in the presence of the steroid. Both records are taken from the same oocyte voltage-clamped at −60 mV.

giving a single band of the predicted size after electrophoresis on 1% agarose/formaldehyde gels.

Oocyte Injection and Electrophysiology

The cRNA transcripts (50 nl of 0.5–0.9 mg/ml cRNA) were injected into *Xenopus laevis* oocytes (stage VI) that had been defolliculated using the collagenase digestion method [3 hr at 18–23° with 2 mg/ml collagenase type A (Boehringer Mannheim) in Barth's saline with Ca^{2+} salts omitted]. Injected oocytes were individually maintained in 96-well plates (200 μl/well of normal Barth's saline supplemented with 100 mg/ml gentamicin) for up to 9 days at 19–20°.

Currents were recorded from *Xenopus* oocytes voltage-clamped at a holding potential of −60 mV, using an Axoclamp 2A (Axon Instruments) voltage-clamp amplifier in the two-electrode voltage-clamp mode. The voltage-sensing and current-passing microelectrodes were filled with 3 M KCl and had resistances of 0.6–2 MΩ when measured in standard extracellular saline. The oocytes were continuously superfused with frog Ringer solution (120 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 5 mM HEPES, pH 7.4), at a rate of 7–10 ml/min, at room temperature (17–21°). Steroids (10 mM) in DMSO were diluted in Ringer solution to the appropriate concentration. The steroids were preapplied for 2 min before being coapplied together with the appropriate concentration of GABA. Preliminary experiments established that the enhancement of GABA-evoked currents by active steroids was maximal with a preincubation time of ≥20 sec. The final DMSO concentrations (0.1–0.25%, v/v) had no effect on GABA-evoked responses. All drugs were applied via the perfusion system. Membrane current responses were low-pass filtered at 300 Hz, recorded on magnetic tape using an FM tape recorder (Racal Store 4DS), and simultaneously displayed on a Lectromed Multitrace two-pen recorder. The peak amplitude of such currents was determined either manually from the chart recorder or by replaying taped recordings into a Tektronix 5110 digital oscilloscope with a 5D10 waveform digitization unit and measuring the digitized signal with electronic cursors.

Drugs

The syntheses of the neuroactive steroids and related compounds have been described (11). Progesterone (**31**) (Table 1) was obtained commercially from Steraloids, as were samples of compounds **2**, **3**, **10**, and **25** used in some assays.

Data Analysis

Nonlinear curve fitting of the overall binding data for each drug averaged for each concentration was done using the Solver function in Excel (Microsoft), using the following sigmoidal equations: inhibition, $Y \equiv A \pm [(B - A)/[1 \pm (X/IC_{50})^D]]$; enhancement, $Y \equiv A \pm [(B - A)/[1 \pm (EC_{50}/X)^D]]$, where Y is the percentage specifically bound, A is the bottom plateau, B is the top plateau, X is the concentration, and D is the Hill coefficient. For [^{35}S]TBPS experiments, the data were fit to a partial ($A \geq 0$) instead of a full ($A \equiv 0$) inhibition model if the sum of squares was significantly smaller by F test. For both binding assays, Hill coefficients were allowed to vary from unity if the sum of squares was significantly smaller by F test. The concentration of test compound producing 50% inhibition (IC_{50}) or enhancement (EC_{50}) of specific binding was determined for the individual experiments with the same model used for the overall data, and then the means ± standard errors of the individual experiments were calculated. Linear regression analysis for correlation plots was done using InPlot (GraphPad). In electrophysiological experiments, the GABA EC_{50} and Hill coefficient were estimated with a sigmoidal function using Fig P version 6.0 C (Biosoft).

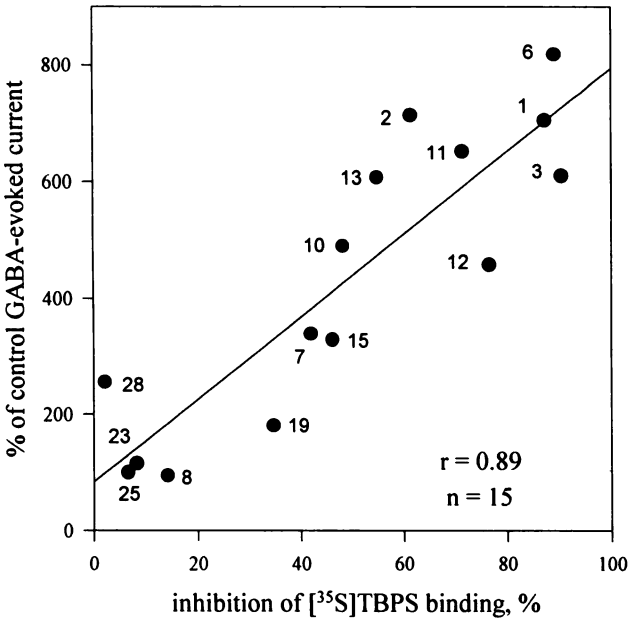

Fig. 7. Correlation between neuroactive steroid (1 μM) inhibition of [^{35}S]TBPS binding in rat brain cortical membranes and potentiation of GABA-evoked currents in *Xenopus* oocytes expressing α1β1γ2L receptor complexes. Values for inhibition of [^{35}S]TBPS binding and potentiation of chloride current (percentage of control) are from Tables 1 and 2, respectively. The equation describing the regression line is $y = 7.10x + 84.5$. The slope is significantly different from 0 ($p < 0.0001$). The regression for percentage inhibition of TBPS binding and percentage of control chloride current, using 300 nM steroid, gives a correlation coefficient of 0.93 ($n = 15$). The regression for percentage enhancement of flunitrazepam binding and percentage of control chloride current, using 300 nM and 1 μM steroid, gives correlation coefficients of 0.93 and 0.94, respectively ($n = 15$).

Results

SAR of neuroactive steroids and related compounds based on the [^{35}S]TBPS assay. The potencies and percentage inhibition values for 31 neuroactive steroids and related compounds as modulators of [^{35}S]TBPS binding to rat brain cortical membranes are shown in Table 1. The most potent compound in this series is the endogenous progesterone metabolite 3α,5α-P (**1**) (Fig. 1), with an IC_{50} of 37 nM. All active compounds possess a free 3α-hydroxy group, which has previously been shown to be necessary for activity (2). 3β-Hydroxy epimers (**21**), 3α-hydroxy esters (**17**, **23**, and **26**), and 3-keto or oxime derivatives (**27**, **29**, and **31**) are weakly active to virtually inactive. The exception is 5α-pregnane-3,20-dione (**8**), which has relatively high potency but very low inhibition (14% at 1 μM). Hydroxylation in the 11α- or 12α-positions markedly reduces activity (**24**, **25**, and **30**). The rank order of potency of 17 substitutions in the 5α-reduced series is 17β-acetyl (**1**) > 17β-cyano (**5**) > 17β-methoxycarbonyl (**13**) > 17-one (**19**) ≥ 17-oxime (**20**). In the 5β series, the 17-one derivative (**28**) is essentially inactive. Epimerization at the 17-position reduces activity 35- and 78-fold for the acetyl (**16**) and cyano (**22**) compounds, respectively. Conversion of the 20-ketone to the ethylenedioxy derivative (**11**) results in a 5-fold loss in activity, with the corresponding compound in the 5β-series (**15**) being even less active. Introduction of a double bond between the 9- and 11-positions of compounds **1** and **3** reduces potency 7- and 26-fold (**12** and **18**, respectively). In general, 21-substitution results in compounds that maintain good activitities, e.g., 21-hydroxyl (**3**, 5α-THDOC; **10**, 5β-THDOC), acetate (**4** and **9**), and mesylate (**6**).

In addition to alterations in potency, certain modifications result in compounds with limited efficacy inhibition. In the 5α series, reduction of the 20-ketone to the 20α-hydroxy-containing pregnanediol (**7**) results in a limited efficacy inhibitor, as reported previously (2). Introduction of a double bond between the 4- and 5-positions (**2**) reduces efficacy with little influence on potency. Although introduction of a double bond between the 9- and 11-positions (**12**) or substitution at the 21-position with an acetoxy group (**4**) does not greatly alter efficacy, both modifications in the same compound (**14**) result in a limited efficacy inhibitor. In the 5β series, the 21-hydroxylated com-

TABLE 2

Activities of 15 neuroactive steroids and related compounds in radioligand binding and electrophysiological assays

Compounds were incubated with rat brain cortical P_2 membranes and 2 nM [^{35}S]TBPS plus 5 μM GABA (TBPS assay) or 1 nM [^{3}H]flunitrazepam plus 1 μM GABA (flunitrazepam assay). The values listed for percentage inhibition of TBPS binding and percentage enhancement of flunitrazepam binding were obtained with 300 nM steroid. The current produced by 20–50 μM GABA (approximate EC_{10}) in the presence of 300 nM or 1 μM steroid is expressed as a percentage of the current produced by GABA alone (chloride current), recorded from *Xenopus* oocytes expressing the human α1β1γ2L subunit combination. Percentage TBPS binding inhibition, percentage flunitrazepam binding enhancement, and chloride current values are expressed as means ± standard errors of at least three independent experiments.

Compound no.	TBPS inhibition, 300 nM	Flunitrazepam enhancement, 300 nM	Chloride current	
			300 nM	1 μM
	%	%	% of control	
1	81 ± 2	47 ± 10	654 ± 45	706 ± 42
2	59 ± 2	40 ± 4	439 ± 94	714 ± 114
3	81 ± 4	30 ± 4	457 ± 21	610 ± 38
6	77 ± 3	34 ± 4	597 ± 159	818 ± 124
7	36 ± 4	21 ± 4	272 ± 17	340 ± 46
8	9 ± 1	2 ± 3	89 ± 4	95 ± 3
10	37 ± 2	21.8 ± 0.3	289 ± 4	489 ± 14
11	53 ± 4	27 ± 2	472 ± 71	652 ± 48
12	52 ± 5	27 ± 2	288 ± 17	458 ± 53
13	38 ± 4	20 ± 1	291 ± 21	607 ± 89
15	20 ± 3	4.6 ± 0.4	158 ± 9	329 ± 16
19	13 ± 4	4 ± 2	127 ± 4	181 ± 18
23	2 ± 1	0 ± 1	105 ± 3	116 ± 1
25	3 ± 2	−0.2 ± 0.4	100[a]	100[a]
28	−4 ± 2	−1 ± 1	209 ± 14	255 ± 17

[a] Single determination.

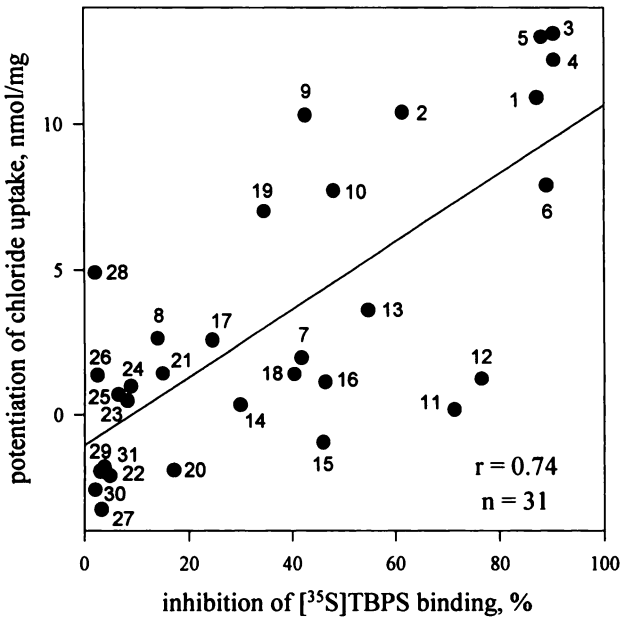

Fig. 8. Correlation between neuroactive steroid (1 μM) inhibition of [^{35}S]TBPS binding in rat brain cortical membranes and potentiation of muscimol-stimulated $^{36}Cl^-$ uptake in rat brain cortical synaptoneurosomes. Values for inhibition of [^{35}S]TBPS binding and potentiation of muscimol-stimulated $^{36}Cl^-$ uptake are from Table 1. The equation describing the regression line is $y = 0.117x - 1.05$. The slope is significantly different from 0 ($p < 0.0001$). The regression for percentage enhancement of flunitrazepam binding and potentiation of muscimol-stimulated $^{36}Cl^-$ uptake, with 1 μM steroid, gives a correlation coefficient of 0.78 ($n = 31$). Chloride uptake data are from Ref. 11.

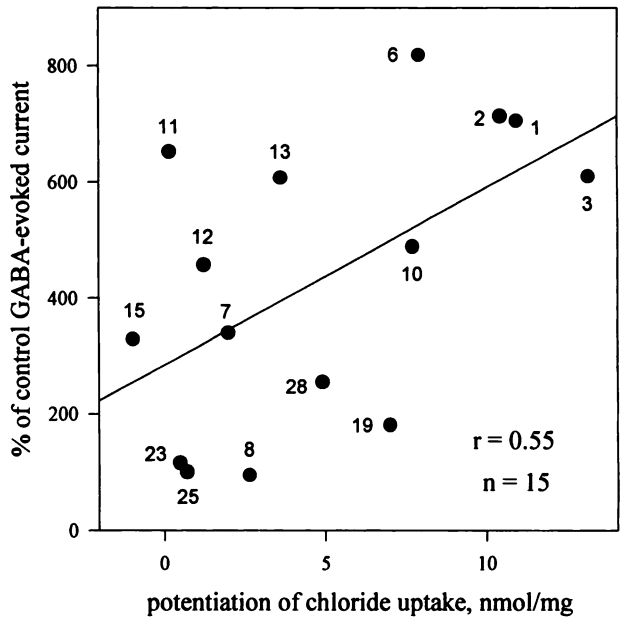

Fig. 9. Correlation between neuroactive steroid (1 μM) potentiation of muscimol-stimulated $^{36}Cl^-$ uptake in rat brain cortical synaptoneurosomes and GABA-evoked current in *Xenopus* oocytes expressing $\alpha1\beta1\gamma2l$ receptor complexes. Values for potentiation of $^{36}Cl^-$ uptake and chloride current (percentage of control), with 1 μM steroid, are from Tables 1 and 2, respectively. The equation describing the regression line is $y = 9.94x + 0.493$. The slope is significantly different from 0 ($p = 0.033$). The regressions for chloride uptake and percentage inhibition of TBPS binding or percentage enhancement of flunitrazepam binding for the same set of 15 compounds (1 μM) give correlation coefficients of 0.51 ($p = 0.052$) and 0.59 ($p = 0.021$), respectively.

pound (**10**) and its acetate derivative (**9**) display limited efficacy inhibition, as shown previously for compound **10** in rat cortex (**8**). None of the compounds examined displayed two-component inhibition, as observed for 3α-hydroxy-5β-pregnan-20-one, the 5β-isomer of 3α,5α-P (**1**) (14). Concentration-effect curves for inhibition of [^{35}S]TBPS binding by full efficacy (**1**), limited efficacy (**2**, **9**, and **14**), and low potency (**27**) compounds are shown in Fig. 2.

Correlation of [^{35}S]TBPS and [^{3}H]flunitrazepam assays. The efficacy of enhancement by neuroactive steroids of [^{3}H]flunitrazepam binding is dependent on the GABA concentration; increasing the GABA concentration from 1 to 5 μM decreases the maximal efficacy for 3α,5α-P (**1**) 1.6-fold, with little change in the EC_{50} (data not shown). For this reason, a lower concentration of GABA was used in the [^{3}H]flunitrazepam assay (1 μM) than in the [^{35}S]TBPS assay (5 μM). Despite these methodological differences, the relative potency and magnitude of modulation for neuroactive steroids in the two assays can be compared.

The inhibitory potencies of neuroactive steroids in the [^{35}S]TBPS assay correlate well with their potencies for enhancing [^{3}H]flunitrazepam binding in rat cortical membranes (Fig. 3; Table 1). For the 17 compounds active in both assays, there is a good correlation ($r = 0.90$) between [^{35}S]TBPS IC_{50} and [^{3}H]flunitrazepam EC_{50} values, although the low slope value of the regression line (0.82) indicates that compounds with high potency in the [^{35}S]TBPS assay are in general less potent in the [^{3}H]flunitrazepam assay, whereas compounds with intermediate potency are approximately equipotent in the two assays. Seven compounds (**17** and **20–25**) with low potency (IC_{50} of 2–11 μM) in the [^{35}S]TBPS assay were essentially inactive in the [^{3}H]flunitrazepam assay, as was compound **8**, a very low efficacy inhibitor of [^{35}S]TBPS binding. Six compounds (**26–31**) were inactive in both assays. For 31 neuroactive steroids and related compounds at 1 μM, the percentage inhibition of [^{35}S]TBPS binding correlates well ($r = 0.95$) with the percentage enhancement of [^{3}H]flunitrazepam binding in rat cortical membranes (Fig. 4; Table 1). However, the absolute magnitude of the modulation is less in the [^{3}H]flunitrazepam assay than in the [^{35}S]TBPS assay under the conditions used. Concentration-effect curves for enhancement of [^{3}H]flunitrazepam binding by full efficacy (**1**), limited efficacy (**7**, **9**, and **14**), and low potency (**27**) compounds are shown in Fig. 5.

Neuroactive steroid potentiation of GABA-evoked currents in *Xenopus* oocytes expressing human $\alpha1\beta1\gamma2L$ receptor complexes. Functional modulation of $GABA_A$ receptors by neuroactive steroids was examined electrophysiologically in *Xenopus* oocytes expressing human $\alpha1\beta1\gamma2L$ receptor complexes. This subunit combination was selected because its pharmacology has been well studied, a majority of native receptor complexes are thought to contain α, β, and γ subunits, and the α1 variant is well represented in rat cortex (15), the tissue selected for binding and $^{36}Cl^-$ uptake studies. All *Xenopus laevis* oocytes preinjected with cRNA encoding the human α1, β1 and γ2L subunits responded to bath-applied GABA (1 μM to 3 mM) with a concentration-dependent inward current at a holding potential of −60 mV. For GABA concentrations of ≤50 μM, the current was well maintained throughout the period of agonist application (Fig. 6A). However, for GABA concentrations of >50 μM, the current amplitude faded in the continued presence of agonist, presumably reflecting receptor desensitization (Fig.

6A). Analysis of the GABA concentration-response curve revealed an EC_{50} for GABA of 110 ± 16 μM (n = 5), with a Hill coefficient of 1.20 ± 0.03 (n = 5) (Fig. 6B). A similar apparent affinity for GABA has been reported in oocytes expressing rat cortex poly(A)$^+$ RNA (16).

The magnitude of the enhancement of the GABA current by positive allosteric modulators is critically dependent upon the concentration of GABA used, relative to the maximal concentration (16, 17). Hence, for each oocyte the concentration of GABA (20–50 μM) giving a response of approximately 10% of the maximum was determined and the influence of the steroid on the current produced by this concentration of GABA was then assessed. Such control currents did not exhibit desensitization (see above) and achieved 90% of peak amplitude, with a mean time of 3.4 ± 0.3 sec (n = 10). In control experiments, flunitrazepam (30 nM) enhanced the GABA-evoked current to 205 ± 7% of control (n = 6) (data not shown), suggesting the successful expression of the γ2L subunit (18).

3α,5α-P (300 nM and 1 μM) produced enhancement of the peak amplitude of the GABA-evoked current to 654 ± 45% of control (n = 8) and 706 ± 42% of control (n = 10), respectively (Fig. 6C). In agreement with previous observations in the presence of this steroid, the GABA current faded, perhaps reflecting an action of the steroid to enhance GABA$_A$ receptor desensitization (16) (Fig. 6C). We previously reported that some steroids in the absence of GABA can directly activate the GABA receptor complex (e.g., 3, 7). Here, the preincubation with 300 nM or 1 μM 3α,5α-P induced a small inward current (never greater than 1% of the maximum GABA current) in oocytes expressing relatively large currents in response to GABA. Because such currents were blocked by picrotoxin (10 μM) and enhanced by flunitrazepam (30 nM), they presumably reflect the activation of the GABA$_A$ receptor by the steroid (data not shown). Due to their small magnitude, such direct effects had little influence on the enhancement of the GABA current amplitude by 3α,5α-P and were not taken into account. None of the steroids tested produced a direct current greater than 1% of the maximum GABA current. Interestingly, under identical recording conditions, at relatively high concentrations the barbiturate pentobarbital and the general anesthetic propofol, in the absence of GABA, induced relatively large inward currents (~30% of the GABA maximum).[2] Because 3α,5α-P also produced relatively small inward currents (compared with those induced by GABA and these anesthetics) in bovine chromaffin cells, recorded with whole-cell clamp techniques,[3] the direct effects of neuroactive steroids appear to be modest, in comparison with those of anesthetic barbiturates and propofol.

Correlation of radioligand binding and functional assays. There is a good correlation for 15 neuroactive steroids and related compounds between enhancement of the peak amplitude of the GABA-evoked current in oocytes expressing human α1β1γ2L receptor complexes and inhibition of [^{35}S]-TBPS (r = 0.93 and 0.89) or enhancement of [^{3}H]flunitrazepam (r = 0.93 and 0.94) binding to cortical membranes, at 300 nM and 1 μM steroid, respectively (Fig. 7; Table 2). Using chloride uptake as an indicator of functional modulation, there is a weaker correlation for 31 neuroactive steroids and related compounds between potentiation of muscimol-stimulated $^{36}Cl^-$ uptake in cortical synaptoneurosomes (data from Ref. 11) and enhancement of [^{3}H]flunitrazepam (r = 0.78) or inhibition of [^{35}S]TBPS (r = 0.74) binding to cortical membranes (Fig. 8; Table 1). For the set of 15 compounds examined electrophysiologically, there is a poor to nonsignificant correlation between activity in the chloride uptake assay and that in the [^{35}S]-TBPS (r = 0.51, p = 0.052), [^{3}H]flunitrazepam (r = 0.59, p = 0.021), or electrophysiological (r = 0.55, p = 0.033) assays, at 1 μM steroid (Fig. 9). Thus, for this particular set of 15 compounds, there are particularly poor correlations between the chloride uptake assay and the other assays. For these 15 compounds, the correlation between binding and electrophysiology as a measure of functional modulation is much better than that for chloride uptake.

Discussion

The inhibition of [^{35}S]TBPS binding has been used to define the SAR for neuroactive steroids that are positive allosteric modulators of the GABA$_A$ receptor complex (2, 19, 20). Those earlier studies demonstrated that 3α-hydroxylation is a crucial determinant of activity for neuroactive steroids. The present study confirms this observation and expands our understanding of the neuroactive steroid SAR. GABA (5 μM) is included in the [^{35}S]TBPS assay because the potency of neuroactive steroids as inhibitors of binding is increased in the presence of GABA (2, 19) and also because certain compounds (e.g., 3α,20α-dihydroxy-5α-pregnane, compound **7**) are virtually inactive in the absence of added GABA (21).

Epimerization, esterification, or oxidation of the 3α-hydroxy group greatly reduces activity. Hydroxylation at the 11α-position (**24** and **25**) or the 12α-position (**30**) greatly reduces activity, whereas the 21-hydroxy-containing, 3α-hydroxylated, 5α- and 5β-reduced metabolites of deoxycorticosterone, i.e., 5α-THDOC (**3**) and 5β-THDOC (**10**), have high potency, as reported previously (8, 19). The high potencies of THDOC acetate (**4** and **9**) and mesylate (**6**) esters suggest that modification of the 21-position is well tolerated by the neuroactive steroid receptor binding site. Unfortunately, the potential for ester hydrolysis *in vitro* complicates this conclusion.

The 17β-acetyl group present in pregnan-20-ones confers high potency; however, certain other 17β-substitutions also result in active compounds. In particular, the 17β-cyano compound (**5**) is a potent inhibitor of [^{35}S]TBPS binding, consistent with its high activity in functional assays (11, 22). Additional examples showing the lack of requirement of the 20-keto group for activity are the 20α-hydroxy (**7**) and 20-ethylenedioxy (**11**) derivatives. Epimerization at the 17-position greatly reduces potency, indicating the stereospecificity at this position, in addition to that established for the hydroxy group at the 3-position (19).

As previously shown for 3α,20α-dihydroxy-5α-pregnane (**7**) and 5β-THDOC (**10**), certain neuroactive steroids display limited efficacy inhibition of [^{35}S]TBPS binding (2, 8). For example, the 4-ene (**2**) and 9(11)-ene-21-acetoxy (**14**) derivatives of 3α,5α-P (**1**), as well as the 21-acetate of 5β-THDOC (**9**), exhibit limited efficacy in inhibition. Again, possible ester hydrolysis *in vitro* is a complicating factor for compound **9**. In the case of 3α,20α-dihydroxy-5α-pregnane (**7**), limited efficacy inhibition of [^{35}S]TBPS binding has been ascribed to true partial agonism at the neuroactive steroid binding site (6), although recent

[2] D. Belelli, C. Hill-Venning, and J. J. Lambert, unpublished observations.
[3] J. J. Lambert and C. Hill-Venning, unpublished observations.

findings suggest that this neuroactive steroid may have selectivity for a subclass of neuroactive steroid receptors (21).

The unexpected concentration-dependent, but very low efficacy, inhibition of [^{35}S]TBPS binding by the 3,20-dione (**8**) is not due to 3α-hydroxylation *in vitro* to form 3α,5α-P (**1**).[4] Although 5α-pregnane-3,20-dione (**8**) was considered inactive in the chloride uptake assay in rat brain (11) and was found to be without effect on GABA-stimulated currents in oocytes expressing $\alpha1\beta1\gamma2$L receptor complexes, the 5β-isomer 5β-pregnane-3,20-dione was reported to weakly potentiate GABA-induced currents in bovine chromaffin cells (7). Nevertheless, considering the inactivity of 5α-pregnane-3,20-dione (**8**) in the [^{3}H]flunitrazepam assay and the weak activity of all other 3-keto derivatives examined, the significance of the very limited inhibition of [^{35}S]TBPS binding by this compound is unknown.

The strong correlations for neuroactive steroid potency (r = 0.90, n = 17) and magnitude of modulation (r = 0.95, n = 31) in the [^{35}S]TBPS and [^{3}H]flunitrazepam assays indicate that the neuroactive steroid binding site is similarly coupled to the chloride channel and benzodiazepine sites present in rat cortex. Moreover, this finding suggests that the neuroactive steroid, TBPS, and flunitrazepam binding sites may be located on similar populations of $GABA_A$ receptor complexes present in rat cortex. This interpretation is surprising, considering the molecular heterogeneity of subunits present in this region (15) and the obligatory role of the γ subunit for benzodiazepine (18) but not neuroactive steroid (8, 23, 24) or TBPS (25, 26) binding. More likely, the majority of compounds examined may not distinguish different populations of $GABA_A$ receptors bound by [^{35}S]TBPS and [^{3}H]flunitrazepam. The relatively higher potencies of both strongly and weakly active neuroactive steroids obtained with [^{35}S]TBPS, relative to [^{3}H]flunitrazepam, and the greater magnitudes of modulation in the [^{35}S]TBPS assay suggest that [^{35}S]TBPS may be a better reporter radioligand to allosterically monitor the neuroactive steroid binding site.

In contrast to the strong correlation for the radioligand binding assays, there is a weaker correlation (r = 0.74–0.78) between the efficacy of 31 neuroactive steroids and related compounds in either binding assay in rat cortical membranes and the previously reported muscimol-stimulated $^{36}Cl^-$ uptake in cortical synaptoneurosomes. This weaker correlation could be due to 1) methodological differences between the binding and chloride uptake assays, including tissue preparation, incubation time and medium, and/or GABA agonist used, 2) limitations of the muscimol-stimulated $^{36}Cl^-$ uptake technique, in that the magnitude of the modulatory effect depends on the chloride ion distribution across the synaptoneurosomal membrane, the resting membrane potential, and degree of agonist-induced desensitization, or 3) real discrepancies between neuroactive steroid activity in radioligand binding and functional assays. However, the good correlation (r = 0.89–0.94) between the activity of 15 neuroactive steroids and related compounds in either binding assay in rat brain cortical membranes and potentiation of GABA-evoked chloride currents in *Xenopus* oocytes expressing $\alpha1\beta1\gamma2$L receptor complexes indicates that the binding assays are good predictors of functional modulation of the $GABA_A$ receptor. This close correspondence between neuroactive steroid modulation of radioligand binding and potentiation of GABA-stimulated currents is surprising, considering not only methodological differences but also the diversity of subunit expression in cortex (15), compared with recombinant expression in oocytes. These data again suggest that the majority of compounds examined do not appear to possess receptor subtype selectivity. Detailed evaluation of the selectivity of neuroactive steroids for $GABA_A$ receptor subtypes is currently being conducted.

Although there is not a good correlation between inhibition of [^{35}S]TBPS binding or enhancement of [^{3}H]flunitrazepam binding and potentiation of muscimol-stimulated $^{36}Cl^-$ uptake, there is a good correlation between the binding assay results and GABA-evoked currents, indicating that neuroactive steroid modulation of the binding of these radioligands reflects functional activity at the $GABA_A$ receptor. Thus, [^{35}S]TBPS and [^{3}H]flunitrazepam are reliable probes to explore neuroactive steroid SAR and mode of action.

[4] D. B. Goodnough, personal communication.

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