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# γ-Aminobutyric Acid$_A$ Receptor Complexes in Rat Frontal Cortex and Spinal Cord Show Differential Responses to Steroid Modulation

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**SUMMARY**

Regional differences in neuroactive steroid modulation of the γ-aminobutyric acid$_A$ receptor-chloride ionophore complex (GBRC), as measured by *t*-butylbicyclophosphoro[$^{35}$S]thionate ([$^{35}$S]TBPS) binding and $^{36}Cl^-$ uptake, were demonstrated in rat spinal cord versus frontal cortex. The rank order of potencies of a series of 5α- and 5β-reduced isomers of 3α-hydroxylated steroids against [$^{35}$S]TBPS binding were different between regions. The differences in rank order of potencies imply the possible existence of heterogeneous populations of GBRC-coupled steroid recognition sites. The relative potencies of selected 5α- and 5β-reduced isomers as potentiators of $^{36}Cl^-$ uptake paralleled their potencies as inhibitors of [$^{35}$S]TBPS binding. Differential sensitivity of the steroid recognition site to the allosteric influence of γ-aminobutyric acid was also demonstrated. It appears that regionally specific responses to GBRC-active steroids do occur, although the functional consequences of these effects await evaluation in appropriate *in vivo* models.

Stringent structure-activity requirements for pregnane steroid modulation of the GBRC in brain supports the existence of a steroid recognition site that is on or intimately associated with the GBRC (1, 2). Recent evidence derived from the recombinantly expressed GBRC argues strongly in favor of a distinct steroid modulatory site on the GBRC (3, 4). This expressed steroid site has structure-activity requirements similar to those reported in brain homogenate studies. Collectively, the evidence for the existence of a high affinity steroid modulatory site on the GBRC is now compelling.

The presence of another allosteric modulatory site of the GABA$_A$ receptor provides a novel locus for the pharmacological manipulation of GABA$_A$ receptor-mediated neurotransmission. Whether activation of the putative steroid site results in pharmacological effects similar to those produced by the BZs or barbiturates remains to be determined. Clearly, regional differences in responses to steroids versus the BZs or the barbiturates could result in qualitative and/or quantitative differences in pharmacological response. For example, regional differences in response may result from a lack of 1:1 correspondence in the distribution of steroid versus BZ sites or a heterogeneous population of steroid sites. It is the latter possibility that is the most intriguing from the point of view of pharmacological specificity. If heterogeneity of steroid sites exists, can ligands with high subtype, and thus pharmacological, specificities be identified? First, the issue of heterogeneity of GBRC-coupled steroid sites must be addressed.

In light of the pharmacological and biochemical evidence (5) for the existence of a heterogeneous population of GBRCs that are structurally distinct, it is reasonable to predict that different subtypes of the GBRC may exist that have different structure-activity requirements for steroid interactions. Such a possibility may underlie the regional differences in the potency of 5α-3αOHDHP as an allosteric modulator of [$^{35}$S]TBPS binding in autoradiographic studies, although this may not be the only explanation (1). Other studies have also implicated steroid site heterogeneity as a potential explanation for the differences in the interactions of 5α-THDOC and 5α-THDOC-21-mesylate with the GBRC (6). In the present study, regional differences in the GBRC response to steroids were assessed. The effects of a series of 5α- and 5β-reduced isomers of GBRC-active steroids, the barbiturate sodium pentobarbital, and the BZ diazepam on [$^{35}$S]TBPS binding and GABA-stimulated $^{36}Cl^-$ uptake in rat spinal cord versus frontal cortex were determined.

## Materials and Methods

**[$^{35}$S]TBPS binding assay.** Brains and spinal cords from male Sprague-Dawley rats (150–200 g; Simonsen Labs) were removed im-

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**ABBREVIATIONS:** GBRC, GABA$_A$ receptor-chloride ionophore complex; GABA, γ-aminobutyric acid; [$^{35}$S]TBPS, *t*-butylbicyclophosphoro[$^{35}$S]thionate; DMSO, dimethylsulfoxide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; 3αOHDHP, pregnane-3α-ol-20-one; BZ, benzodiazepine; THDOC, pregnane-3α,21-diol-20-one.

mediately after sacrifice, and the frontal cortex was dissected over ice. Spinal cords were composed of the tissue posterior to the cerebellum through to the midthoracic region. A $P_2$ homogenate of each region was prepared immediately, as previously described (1), in 50 mM Na/K phosphate buffer (pH 7.4), 200 mM NaCl, and was used in binding assays. Endogenous levels of GABA were reduced by washing $P_2$ homogenates three times in 100 volumes of buffer, using centrifugation (9000 × *g*, 20 min).

One hundred-microliter aliquots (3–4 mg of protein/ml) of the $P_2$ homogenate were incubated with 3 nM [$^{35}$S]TBPS (60–110 Ci/mmol; DuPont/New England Nuclear, Boston, MA) in the presence or absence of various concentrations of diazepam (Sigma Chemical Co., St. Louis, MO), sodium pentobarbital (Sigma), or steroids (Sigma or Steraloids, Wilton, NH). All test drugs were dissolved in DMSO (Sigma) and added to the incubation mixture in 5-μl aliquots. Nonspecific binding was defined as binding in the presence of 2 μM TBPS. Assays were performed in the presence of GABA (Sigma) as indicated. After a 2-hr incubation at 25° (steady state conditions), assays were terminated by filtration through glass fiber filters (no. 32; Schleicher and Schuell, Keene, NH). Filter-bound radioactivity was quantitated by liquid scintillation counting. The data were plotted as log steroid/drug concentration versus percentage of control [$^{35}$S]TBPS bound. Percentage of control binding was defined as specific [$^{35}$S]TBPS binding in the absence of steroid or drug. $IC_{50}$ (concentration at which half-maximal inhibition of control [$^{35}$S]TBPS binding occurs) values were determined by computerized nonlinear regression analysis, using a single independent-site model. The percentage of maximal inhibition was defined as percentage of inhibition at the maximally effective concentration of steroid. Control levels of [$^{35}$S]TBPS binding in frontal cortex and spinal cord were approximately 20 and 60 fmol/mg of protein, respectively.

**$^{36}Cl^-$ uptake studies.** Synaptoneurosomes of spinal cord and frontal cortex were prepared for $^{36}Cl^-$ uptake studies as previously prescribed by Suzdak *et al.* (7). The tissue was homogenized in 10 mM Tris-HEPES (pH 7.5) buffer containing 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM D-glucose, and 1 mM $CaCl_2$ (Sigma). The homogenate was filtered through three layers of nylon mesh (pore size, 160 μm). The filtrate was washed twice by centrifugation at 1000 × *g* for 15 min, and the final pellet was resuspended in ice-cold buffer to yield a protein concentration of ~8 mg/ml.

Aliquots (200 μl) of the synaptoneurosomal preparation were preincubated at 30° for 10 min. Uptake was initiated by the simultaneous addition of steroid or drug in DMSO (2 μl) and 200 μl of $^{36}Cl^-$ (0.55 mCi/mmol, 1.6 μCi/ml; DuPont/New England Nuclear). An equal volume of vehicle (DMSO) was always included in the control assay. The concentration of DMSO used had no effect on basal $^{36}Cl^-$ uptake. The mixture was gently vortexed, and $^{36}Cl^-$ uptake was terminated after 5 sec by the addition of 5 ml of ice-cold buffer containing 100 μM picrotoxin (Sigma), followed immediately by rapid filtration through glass fiber filters (Whatman GF/C) under vacuum. The filters were washed twice with 5 ml of ice-cold buffer, and filter-bound radioactivity was quantified by liquid scintillation counting. Specific uptake was calculated by subtracting the amount of $^{36}Cl^-$ bound to the filters in the absence of synaptoneurosomes from that observed in their presence. Uptake was expressed as nmol of $^{36}Cl^-$/mg of protein.

## Results

**[$^{35}$S]TBPS binding studies.** The effects of the 5α- and 5β-reduced isomers of 3αOHDHP, THDOC, and pregnane-3α,20α-diol (pregnanediol) on [$^{35}$S]TBPS binding in spinal cord and cortex are expressed as $IC_{50}$ values and degree of maximal inhibition in Table 1. It is clear from the data that, under similar conditions, the absolute potencies (i.e., $IC_{50}$) for the stereoisomers of 3αOHDHP and THDOC were greater in frontal cortex than in spinal cord. The possibility that the differences in sensitivity to the modulation of [$^{35}$S]TBPS binding by GABA in spinal cord versus frontal cortex could account for the differences in steroid potency was evaluated by determining the $IC_{50}$ values for GABA inhibition of [$^{35}$S]TBPS binding under similar conditions in the two regions. The $IC_{50}$ values for GABA in spinal cord versus cortex were 7.9 ± 1.4 μM versus 6.1 ± 1.1 μM, respectively. The 1.3-fold difference between these values was not statistically significant (Student's *t* test). Moreover, the degree of efficacy and rank order of potency for modulators of [$^{35}$S]TBPS binding were different between the two regions. It is also interesting to note the trend that 5α-reduced isomers had greater potency than their corresponding 5β-reduced isomers in both regions examined. The rank order of potency in spinal cord was 5α-pregnanediol ≃ 5α-3αOHDHP ≥ 5β-3αOHDHP ≃ 5β-pregnanediol > 5α-THDOC > 5β-THDOC. In contrast, the rank order of potency was 5α-3αOHDHP > 5β-3αOHDHP > 5α-THDOC ≃ 5α-pregnanediol > 5β-THDOC > 5β-pregnanediol for the frontal cortex.

The difference in potency and efficacy between regions was most pronounced with 5β-THDOC (Fig. 1). Under the conditions used, 5β-THDOC was incapable of modulating [$^{35}$S]TBPS binding in spinal cord, whereas it had an $IC_{50}$ of 145 ± 13 nM and maximal inhibition of 65% at 10 μM in frontal cortex. The importance of the orientation of the steroid A-ring (i.e., *cis* versus *trans* fusion) was demonstrated by the ability of 5α-THDOC to produce dose-dependent inhibition of [$^{35}$S]TBPS

TABLE 1

**Allosteric modulation of [$^{35}$S]TBPS binding in spinal cord versus frontal cortex by various steroids**

All $IC_{50}$ values represent the geometric mean ± standard error of five or six independent determinations.

| Steroid | Spinal cord | | Frontal cortex | |
|---|---|---|---|---|
| | $IC_{50}$ | Maximal inhibition | $IC_{50}$ | Maximal inhibition |
| | nM | % | nM | % |
| 5α-3αOHDHP | 155 ± 45 | 68 | 30 ± 4 | 100 |
| 5β-3αOHDHP | 204 ± 94 | 90 | 58 ± 13 | 100 |
| 5α-THDOC | 662 ± 64 | 90 | 77 ± 7 | 100 |
| 5β-THDOC | >10,000 | 0 | 145 ± 13 | 65 |
| 5α-Pregnanediol | 79 ± 35 | 25 | 82 ± 11 | 52 |
| 5β-Pregnanediol | 284 ± 130 | 70 | 303 ± 50 | 100 |

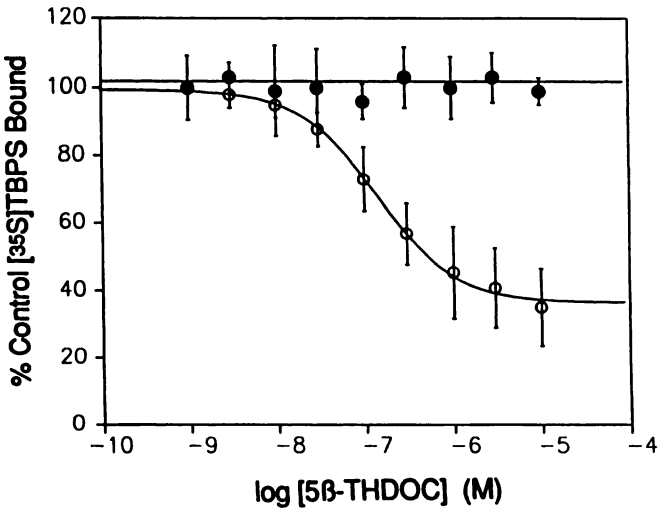


**Fig. 1.** Modulation of 3 nM [$^{35}$S]TBPS binding by 5β-THDOC in rat spinal cord (●) versus frontal cortex (○). Assays were performed in the presence of 5 μM GABA. Each *point* represents the mean ± standard error of three to six independent determinations.

binding in spinal cord under conditions in which 5β-THDOC was inactive (Fig. 2). For comparison, modulation of [$^{35}$S]TBPS binding via the BZ or barbiturate sites was determined under the same conditions in which steroid modulation was measured. The BZ diazepam produced dose-dependent inhibition of [$^{35}$S]TBPS binding, with $IC_{50}$ values of 14.6 ± 6.2 nM and 23.5 ± 7 nM in spinal cord and frontal cortex, respectively (Fig. 3). In this case, diazepam was equipotent in spinal cord and frontal cortex, a feature clearly different from that observed for 5β-THDOC and the other steroids tested. Interestingly, sodium pentobarbital inhibited [$^{35}$S]TBPS binding with $IC_{50}$ values of 81.1 ± 0.7 μM and 30.8 ± 0.4 μM in spinal cord and frontal cortex, respectively. Thus, the steroids and sodium pentobarbital show differential potencies between regions.

Increasing of the GABA concentration has been observed to enhance the potency of 5α-3αOHDHP and 5α-THDOC as modulators of [$^{35}$S]TBPS binding in cortex (8). Using a similar strategy, the GABA concentration was increased from 5 μM to 10 μM and the effect of 5β-THDOC on [$^{35}$S]TBPS binding in spinal cord was assessed. Interestingly, in the presence of 10 μM GABA, 5β-THDOC produced dose-dependent inhibition of [$^{35}$S]TBPS binding, with an $IC_{50}$ of 149 ± 65 nM and a maximal inhibition of 50% (Fig. 4A). Similar GABA dependence was not observed with diazepam in spinal cord, where the $IC_{50}$ values were 14.6 ± 6.2 nM and 9.7 ± 5.2 nM in the presence of 5 and 10 μM GABA, respectively (Fig. 4B).

The question of whether 5β-THDOC interacts with the prototypical GBRC-active steroid 5α-3αOHDHP was addressed by determining the effect of 5β-THDOC on 5α-3αOHDHP modulation of [$^{35}$S]TBPS binding. Under conditions in which 5β-THDOC had no efficacy (i.e., plus 5 μM GABA), this steroid produced an apparent antagonism of 5α-3αOHDHP modulation of [$^{35}$S]TBPS binding (Fig. 5). The 5α-3αOHDHP/[$^{35}$S]TBPS dose-response curve was shifted to the right in the presence of 3 and 10 μM 5β-THDOC, with complete antagonism of up to 30 μM 5α-3αOHDHP under the latter condition.

**$^{36}Cl^-$ uptake studies.** A comparison of the profiles of 5β-THDOC and diazepam modulation of [$^{35}$S]TBPS binding in the presence of 10 μM GABA (see Fig. 4, A versus B) suggested that both compounds should be equally efficacious at concentrations in excess of 100 nM. In addition, the maximal efficacy

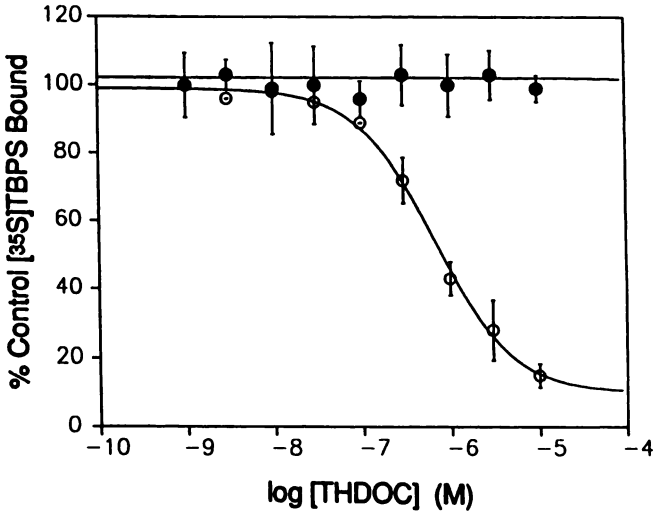


**Fig. 2.** Modulation of 3 nM [$^{35}$S]TBPS binding by 5α-THDOC (○) versus 5β-THDOC (●) in rat spinal cord. Assays were performed in the presence of 5 μM GABA. Each *point* represents the mean ± standard error of three independent determinations.

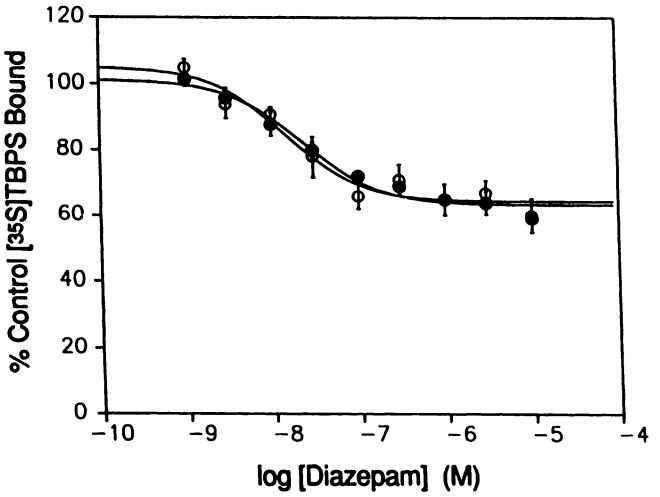


**Fig. 3.** Modulation of 3 nM [$^{35}$S]TBPS binding by diazepam in rat spinal cord (○) versus frontal cortex (●). Assays were performed in the presence of 5 μM GABA. Each *point* represents the mean ± standard error of four to six independent determinations.

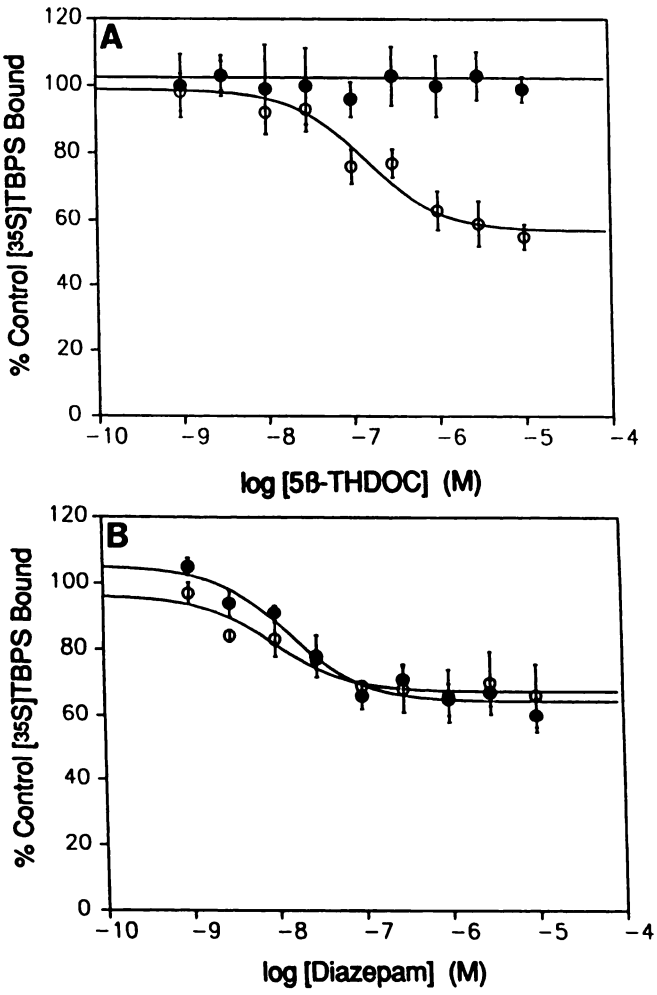


**Fig. 4.** Effect of GABA on 5β-THDOC (A) or diazepam (B) modulation of 3 nM [$^{35}$S]TBPS binding in rat spinal cord. Assays were performed in the presence of 5 μM (●) or 10 μM (○) GABA. Each *point* represents the mean ± standard error of six independent determinations.

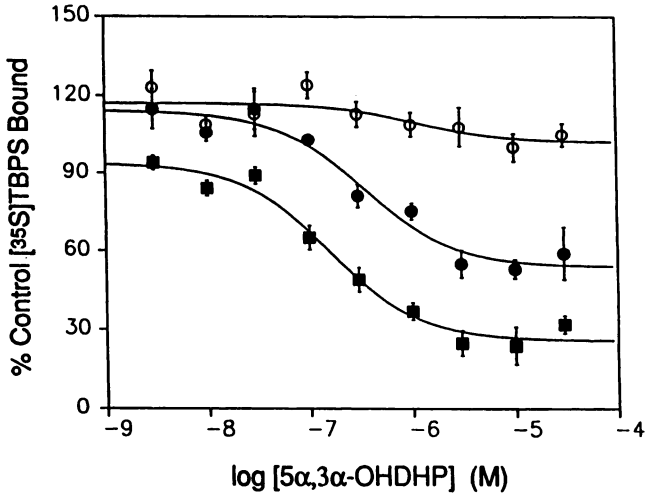


**Fig. 5.** Effect of 0 (○), 3 (●), and 10 μM (■) 5β-THDOC on the dose-dependent inhibition of 3 nM [$^{35}$S]TBPS binding by 5α-3αOHDHP in rat spinal cord. Assays were performed in the presence of 5 μM GABA. Each *point* represents the mean ± standard error of four or five independent determinations.

of 5α-THDOC against [$^{35}$S]TBPS in spinal cord was greater than that of 5β-THDOC. These observations were also observed in assays measuring GABA-stimulated $^{36}Cl^-$ uptake in synaptoneurosomes. Table 2 summarizes the effects of 5α-THDOC, 5β-THDOC, and diazepam on GABA-stimulated $^{36}Cl^-$ uptake in spinal cord and frontal cortex.

Using GABA at 25 μM resulted in a significant 2.3-fold increase in basal $^{36}Cl^-$ uptake in frontal cortex, whereas this concentration did not produce a statistically significant increase in spinal cord (Table 2). 5β-THDOC or diazepam alone at 10 μM had no effect on the basal uptake of $^{36}Cl^-$ in spinal cord (data not shown). In the presence of 10 μM 5β-THDOC or diazepam and 25 μM GABA, a significant increase in uptake over that of basal but not GABA-stimulated uptake was observed in spinal cord. In contrast to 5β-THDOC and diazepam, 10 μM 5α-THDOC in spinal cord produced significant enhancement of uptake above that observed in the presence of 25 μM GABA alone. In the frontal cortex, 1 μM 5β-THDOC failed to produce statistically significant enhancement of GABA-stimulated uptake (data not shown). However, 10 μM 5β-THDOC plus GABA produced a 42% increase in uptake over that of the GABA-stimulated condition. The effect of 10 μM diazepam in cortex was not significantly different from that produced by 10 μM 5β-THDOC. A 90% increase in uptake over the GABA-stimulated condition was produced by 1 μM 5α-THDOC, although this increase was not statistically different from that produced by 10 μM 5β-THDOC.

TABLE 2

**Effect of stereoisomers of THDOC and diazepam on $^{36}Cl^-$ uptake in spinal cord and frontal cortex**

All values represent the mean ± standard error of four to 28 independent determinations.

| Condition | $^{36}Cl^-$ uptake | |
|---|---|---|
| | Spinal cord | Frontal cortex |
| | *nmol/mg of protein* | |
| Basal | 37.4 ± 1.4 | 25.6 ± 1.0 |
| + 25 μM GABA | 41.0 ± 1.4 | 58.3 ± 1.9[a] |
| + 25 μM GABA + 10 μM 5β-THDOC | 44.0 ± 2.0[b] | 83.0 ± 6.5[a] |
| + 25 μM GABA + 10 μM diazepam | 43.2 ± 2.1[c] | 70.7 ± 3.5[f] |
| + 25 μM GABA + 1 μM 5α-THDOC | ND[h] | 111 ± 21[a] |
| + 25 μM GABA + 10 μM 5α-THDOC | 49.8 ± 1.9[d,g] | ND |

[a–d] Significantly different from basal uptake, at [a] $p < 0.001$, [b] $p < 0.01$, [c] $p < 0.05$, or [d] $p < 0.005$, by Student's *t* test.
[a–g] Significantly different from uptake in the presence of 25 μM GABA, at [a] $p < 0.001$, [f] $p < 0.002$, or [g] $p < 0.02$, by Student's *t* test.
[h] ND, not determined.

## Discussion

The allosteric modulation of [$^{35}$S]TBPS binding by GBRC-active ligands provides a convenient method to measure the potential efficacy and potency of these ligands as modulators of the $GABA_A$ receptor-gated chloride channel. [$^{35}$S]TBPS is believed to bind to a site on or near the chloride ionophore, and thus its affinity is sensitive to the conformation (i.e., opened versus closed) of the chloride channel (8, 9). 3α-Hydroxylated pregnane steroids have been shown to be allosteric modulators of [$^{35}$S]TBPS binding in steady state and kinetic studies (1, 10). The present study demonstrates that GBRC-active steroids differentially modulate [$^{35}$S]TBPS binding in a regionally dependent manner. Not only are the absolute potencies different between spinal cord and frontal cortex but the rank order potencies and maximal efficacies also are different. In a functional assay, these differences are also reflected in the ability of the 5-reduced isomers of THDOC to potentiate GABA-stimulated $^{36}Cl^-$ uptake.

In the presence of 5 μM GABA, the potencies of the steroids examined in spinal cord were significantly less than those observed in frontal cortex. The most prominent example of this is that 5β-THDOC was inactive against [$^{35}$S]TBPS in spinal cord under conditions in which it was active in cortex. A possible explanation for this observation is that spinal cord has more residual GABA present or is more sensitive to GABA, relative to the frontal cortex. However, the latter is unlikely because, under the same conditions, GABA is equipotent in both spinal cord and cortex as a inhibitor of [$^{35}$S]TBPS binding. Alternatively, the sensitivity of the steroid site to GABA may account for the regional differences, because we have previously demonstrated that the affinity of steroids for the GBRC is significantly enhanced by GABA (11). This possibility is supported by the observation that 5β-THDOC becomes active against [$^{35}$S]TBPS in the presence of 10 μM GABA. Interestingly, maximal potency and efficacy of diazepam against [$^{35}$S]TBPS is already achieved at 5 μM GABA. The lack of activity of 5β-THDOC in the presence of 5 μM GABA raised the issue of whether the steroid was still interacting with the putative steroid site recognized by the other GBRC-active steroids in spinal cord. Because 5β-THDOC was found to shift the 5α-3αOHDHP/[$^{35}$S]TBPS dose-response curve to the right in the presence of 5 μM GABA, a common site of action through an antagonist-like action of 5β-THDOC is a possible mode of action, although this experiment did not unequivocally establish this mechanism.

The apparent differential sensitivity of 5β-THDOC to the effects of GABA may explain, in a broader application, why some of the GBRC-active steroids evaluated are more potent modulators of [$^{35}$S]TBPS in the cortex than in spinal cord. However, it does not explain the differences in the relative

rank order of potency of the steroids against [$^{35}$S]TBPS in the two regions. Such an observation would, instead, support the possibility that the two regions contain different subtypes of steroid sites (i.e., heterogeneity of steroid sites). Like sodium pentobarbital, some of the steroids can discriminate regional differences, in contrast to the BZ diazepam, which is equipotent in both spinal cord and frontal cortex. However, regional differences have been observed for certain non-BZs that can discriminate BZ receptor subtypes (12). The existence of subtypes of steroid recognition sites is conceivable, in light of the heterogeneity in distribution of the protein subunits that make up the GBRC. Indeed, *in situ* hybridization studies have demonstrated a differential distribution of the mRNAs encoding the various subunits in the mammalian brain (13–16). Differential subunit composition has been suggested to be the basis for apparent BZ receptor heterogeneity (17). Nevertheless, studies involving the expression of cDNA encoding known subunits to reconstitute GBRCs of known composition are necessary to determine the presence of steroid site heterogeneity in a definitive manner.

The functional consequences of differential potency and efficacy of 5$\alpha$- and 5$\beta$-isomers of THDOC as allosteric modulators of [$^{35}$S]TBPS binding were reflected in their effects on $^{36}Cl^-$ uptake. As predicted by [$^{35}$S]TBPS binding, 5$\alpha$-THDOC was found to be more potent and efficacious in enhancing $^{36}Cl^-$ uptake in the frontal cortex than in spinal cord. In a similar comparison, 5$\beta$-THDOC was more efficacious in frontal cortex. Diazepam shows a pattern similar to that of 5$\beta$-THDOC, with greater efficacy in potentiating $^{36}Cl^-$ uptake in cortex versus spinal cord. However, the apparent efficacy of diazepam in modulating [$^{35}$S]TBPS binding was the same in these two regions. The differences in efficacy may be reflective of differences in the density of steroid versus BZ receptors in the two regions. Thus, it is conceivable that cortical function can be selectively affected, with the exclusion of effects on spinal cord, based simply on the dose of steroid. Clearly, GABA content at the receptor site may also play a prominent role. Local levels of GABA may not be sufficient to allow steroids to modulate channel function at certain synapses, thus rendering these neurons insensitive to GBRC-active steroids.

It is tempting to speculate that the identification of steroids that have a regional selectivity similar to that of 5$\beta$-THDOC will result in compounds with selective pharmacological actions. For example, it is thought that the muscle-relaxant effects of the BZs may be mediated in part by the potentiation of GABA in the spinal cord (18). Thus, compounds that have little or no efficacy in potentiating GABA in the spinal cord but retain this feature in cortical regions may possess the desirable pharmacological properties of the BZs (i.e., anticonvulsant and anxiolytic effects) without muscle relaxation. The GBRC-active steroids have already been reported to possess anxiolytic and anticonvulsant properties (19–21). Moreover, at anxiolytic doses of 5$\alpha$-3$\alpha$OHDHP, this steroid will stimulate locomotor activity, which is an effect different from that produced by anxiolytic doses of diazepam under similar conditions (21). The therapeutic potential of this class of steroids would be further enhanced if analogues with limited muscle relaxant and/or sedative potential could be identified. Whether the muscle relaxant and sedative effects can be "designed out," based on selective targeting of steroid site subtypes, remains to be determined. Finally, the *in vivo* consequences of these regionally dependent responses await the evaluation of these steroids in appropriate animal models.

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