0026-895X/93/010087-06$03.00/0

MOLECULAR PHARMACOLOGY, 44:87-92

Regional γ-Aminobutyric Acid Sensitivity of *t*-Butylbicyclophosphoro[^{35}S]thionate Binding Depends on γ-Aminobutyric Acid$_A$ Receptor α Subunit

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Received January 25, 1993; Accepted April 29, 1993

SUMMARY

γ-Aminobutyric acid (GABA) modulates the convulsant binding site on GABA$_A$ receptors labeled by *t*-butylbicyclophosphoro[^{35}S]thionate ([^{35}S]TBPS). The modulation varies between different brain regions, reflecting the molecular heterogeneity of the GABA$_A$ receptors. In rat brain cryostat sections, the main sensitivity difference to GABA between brain regions was observed within the cerebellum. [^{35}S]TBPS binding in the granule cell layer was more sensitive to GABA than was that in the molecular layer and was detected only after blockade of the GABA agonist sites by the specific GABA$_A$ antagonists SR 95531, RU 5135, and bicuculline. This indicates that the [^{35}S]TBPS binding sites in cerebellar granule cells were blocked by endogenous GABA. In contrast, the internal rim of the granule cell layer had a small amount of binding that was largely insensitive to 50 μM GABA. The molecular basis for the sensitivity difference could be traced to the α subunits of the GABA$_A$ receptor. Expression in human embryonic kidney 293 cells of α6β2γ2 receptors produced [^{35}S]TBPS binding sites that were about 10-fold more sensitive to inhibition by GABA than were those inherent to α1β2γ2 receptors. Coexpression of α6 and β2 subunits produced [^{35}S]TBPS binding sites that were largely insensitive to GABA inhibition, resembling in their pharmacological profile the sites in the internal granule cell layer. Furthermore, the differences between α6β2 and α6β2γ2 receptors stress the importance of the γ2 subunit for the proper pharmacological fingerprint of the rest of the granule cell layer. The neurosteroid 5α-pregnan-3α-ol-20-one affected the binding in both α1β2γ2 and α6β2γ2 receptors, but inhibition was greater in α6-containing than in α1-containing receptors, suggesting differential coupling of both GABA and neurosteroid sites with the convulsant site. These data might serve as a platform for additional studies to assess the amino acid residues in the two α subunits that are critically involved in the allosteric interactions between the GABA$_A$ agonist/antagonist or neurosteroid domains and the convulsant site.

GABA is the main inhibitory neurotransmitter in the mammalian central nervous system. Its actions are mediated by GABA$_B$ receptors that are associated with GTP-binding proteins (1) and by GABA$_A$ receptors that have an integral anion channel (2, 3).

GABA$_A$ receptors are affected by a variety of important drugs, including BZ, barbiturates, steroids, convulsants, and ethanol. The cloning of more than 12 GABA$_A$ receptor subunits has given a structural basis to the pharmacological heterogeneity of this receptor system with respect to the diverse central BZ binding sites. A functional GABA$_A$ receptor modulated by BZ ligands consists of a minimum of three different subunits (α, β, and γ) (4) in an unknown stoichiometry. In receptor complexes containing the γ2 variant, it is largely the α subunit variant that determines the BZ receptor pharmacology. The α1 variant confers BZ type I specificity and the α6 variant a BZ agonist-insensitive specificity (5, 6). The γ subunits, especially γ2, are required for BZ modulation (4, 7). Early expression studies suggested that the α subunits are important for differential GABA sensitivity of Cl^- flux (8). However, these studies were performed in αxβ1 receptors without the γ2 subunit, the presence of which affects the overall characteristics of the Cl^- channels (9). No consistent differences have been reported up to now for GABA$_A$ receptors containing the various β variants (10, 11).

We have used the modulation by GABA of convulsant binding (with [^{35}S]TBPS as a ligand) to probe the heterogeneity of the GABA$_A$ receptors in rat brain sections. We have correlated the *in vivo* pharmacological fingerprints with the properties of recombinant receptors expressed *in vitro* from various subunit cDNAs selected on the basis of their regional expression (12, 13). In the present report, a greater GABA sensitivity of [^{35}S]TBPS binding in the rat cerebellar granule cell layer (14) is

This study was supported by the Alexander von Humboldt Foundation, the Academy of Finland, and the German Science Foundation (SFB 317).

ABBREVIATIONS: GABA, γ-aminobutyric acid; BZ, benzodiazepine(s); TBPS, *t*-butylbicyclophosphorothionate; SR 95531, 2′-(3′-carboxy-2′,3′-propyl)-3-amino-6-*p*-methoxyphenylpyrazinium bromide; RU 5135, 3α-hydroxy-11-oxo-16-imino-17-aza-5β-androstane; 3α-OH-DHP, 5α-pregnan-3α-ol-20-one.

described. Furthermore, we provide evidence that this property is inherent to α6 subunit-containing receptors that are expressed only in the cerebellar granule cells.

Materials and Methods

Autoradiography. The procedure, modified from the report of Olsen *et al.* (15), was carried out as described (14). Briefly, 14-μm horizontal cryostat sections from adult male Sprague-Dawley rats were preincubated for 30 min in 50 mM Tris·HCl, pH 7.4, in an ice-water bath. Incubation with 6 nM [^{35}S]TBPS (New England Nuclear) for 90 min at room temperature (22°) was performed in the same buffer supplemented with 120 mM NaCl. Effects of various concentrations of GABA (Serva) and the $GABA_A$ antagonists SR 95531 (Research Biochemicals), RU 5135 (Roussel Uclaf), and bicuculline methiodide (Sigma) were tested. After incubation, the sections were washed three times for 15 sec in ice-cold incubation buffer, dipped into distilled water, air-dried at room temperature, and exposed to Kodak X-OMAT-AR film or to Hyperfilm-βmax (Amersham) for 3 days. Picrotoxinin (10 μM; Sigma) reduced the signal to background levels (data not shown). Autoradiographs were photographed as positive images.

Transfection of 293 cells. Human embryonic kidney 293 cells were transfected (16) with rat α1-, α6-, β2-, and γ2-encoding cDNAs, subcloned individually into eukaryotic expression vectors (4, 6, 17). Transfection of about 4×10^6 cells that had been plated 2–3 days earlier in a 15-cm tissue culture dish was optimized by varying the ratio of the cDNAs for the α, β, and γ subunits.[1] The ratio used in the present transfections, i.e., 1 (=5 μg):5:0.1 for the α:β:γ subunits, yielded the highest binding levels for [^{35}S]TBPS, [^{3}H]muscimol, and [^{3}H]Ro 15–4513. Different amounts of subunit cDNAs in the transfection do not necessarily indicate that the resulting recombinant receptor subunits assemble at the same ratio. The medium was changed 20 hr after transfection, and 48 hr after transfection the cells were washed once and then harvested into phosphate-buffered saline. The cell pellets were homogenized in 50 mM Tris-citrate buffer, pH 7.4, with a Polytron, followed by two centrifugation-resuspension cycles, and were used for ligand binding or were centrifuged and stored at −80°.

Ligand binding to recombinant receptors. Fresh or frozen membranes were used with similar results. Frozen membranes were thawed, resuspended, and centrifuged once before final resuspension in 50 mM Tris-citrate to give a protein concentration of 100–240 μg/ml (Bio-Rad protein assay kit with bovine serum albumin as standard), in a total volume of 0.5 ml/assay tube. All samples were homogenized briefly with a Polytron just before they were added to the binding mixture. After incubation of duplicate samples for defined times, bound and free ligand were separated by rapid filtration of the membranes onto Schleicher & Schuell glass fiber filters (no. 52), using a 24-filter manifold at reduced pressure. The samples were rinsed twice with 5 ml of ice-cold 10 mM Tris·HCl, pH 7.4. The air-dried filters were immersed in 4 ml of Packard Ultima Gold scintillation fluid, and radioactivity was determined in a Beckmann liquid scintillation counter.

[^{35}S]TBPS binding was determined during a 90-min incubation at 22° in 50 mM Tris-citrate supplemented with 200 mM NaCl. Nonspecific binding was defined in the presence of 10 μM picrotoxinin (Sigma). [^{35}S]TBPS was used at a 6 nM concentration and was diluted with unlabeled TBPS in saturation experiments to cover a range from 6 to 200 nM. In some experiments, GABA ranging from 1 nM to 100 μM and 3α-OH-DHP (Sigma) at 100 nM and 10 μM concentrations were added to the incubation mixtuie.

[^{3}H]Muscimol and [^{3}H]Ro 15–4513 (both from New England Nuclear) binding was determined in 50 mM Tris-citrate buffer after 60 min at 0°. GABA at 100 μM and flumazenil at 10 μM defined the nonspecific binding for the radioligands at 6 nM each. In saturation experiments, the concentration of the radioligands ranged from 0.5 to 32 nM. Displacement of [^{3}H]muscimol binding by GABA was studied using GABA concentrations ranging from 3 to 300 nM.

Dissociation of [^{35}S]TBPS from its recognition sites was initiated by 1 μM GABA after equilibrium conditions had been reached (3 hr at 22°). Six 500-μl samples were withdrawn between 2 and 90 min after the start of the dissociation, filtered individually onto Schleicher & Schuell no. 52 glass fiber circles on a Millipore filtration manifold, and washed twice with 5 ml of ice-cold 10 mM Tris·HCl, pH 7.4. No differences in the binding levels were seen between 3-hr and 4.5-hr incubations (data not shown).

Calculation of the binding parameters and statistics. The Inplot program (GraphPad Software, San Diego, CA) was used to calculate the best-fitting values for the parameters of saturation isotherms (K_d and B_{max}), displacement curves (IC_{50} and K_i), and dissociation time curves ($t_{½}$ and maximal displacement). Statistical significance of the differences between the means of various groups was assessed by analysis of variance or Student's *t* test using the GraphPad Instat program. The number of experiments refers to the number of both independent transfections and binding assays.

Results

GABA and $GABA_A$ antagonists affected the picrotoxinin-sensitive [^{35}S]TBPS binding in rat brain sections. Their effects were different in the cerebellar granule cell layer, compared with other brain areas such as the molecular layer of the cerebellar cortex (Figs. 1 and 2). Without added GABA, the molecular layer was labeled, whereas the granule cell layer was essentially free of specific [^{35}S]TBPS binding (Figs. 1 and 2). Conversely, the granule cell layer was heavily labeled when residual GABA was displaced from its receptor by the specific $GABA_A$ antagonists SR 95531 (Fig. 1), RU 5135 (Fig. 2), and bicuculline (14). Whereas higher GABA concentrations (≥50 μM) entirely abolished the binding (except in the internal rim of the granule cell layer) (Fig. 2), the GABA antagonists generally slightly decreased the binding (Fig. 1). GABA and its antagonists reciprocally competed with each others' actions (Figs. 1 and 2). All brain regions other than the cerebellar granule cell layer behaved similarly (Fig. 1), indicating that the $GABA_A$ receptors of the granule cell layer are exceptional in the brain, in that their [^{35}S]TBPS binding exhibits the highest sensitivity to the inhibitory effect of GABA.

To mimic the pharmacological fingerprint of the cerebellar cortex, recombinant $GABA_A$ receptors were created from subunit-specific cDNAs in human embryonic kidney 293 cell membranes. Only the $GABA_A$ receptor α6 subunit is unique to the granule cells (6); the other subunits, namely α1, β2, β3, γ2, and δ subunits (12, 13), are expressed elsewhere in the brain as well. Therefore, we compared in our expression system $GABA_A$ receptors containing either the most abundant α1 subunit or the cerebellum-specific α6 subunit. Fig. 3 shows that α6β2γ2 receptors are at least 10 times more sensitive to the GABA inhibition of [^{35}S]TBPS binding than are the α1β2γ2 receptors. In contrast, the enhancement of binding by low GABA concentrations was greater in α1-containing than in α6-containing receptors. Coexpression of both α subunits with the β2 and γ2 subunits yielded [^{35}S]TBPS binding data with characteristics averaging the results from the single α subunit-containing receptors (Fig. 3; Table 1). To study the importance of the γ2 subunit in the cross-talk between the GABA and TBPS recognition sites, we measured [^{35}S]TBPS binding to 293 cell membranes expressing single subunits or their combinations without the γ2 subunit. Homooligomeric α subunit receptors did not bind [^{35}S]TBPS (data not shown). α1β2 and α6β2

[1] H. Lüddens, Ligand binding to heterologous $GABA_A$ receptors depends on the ratio of the subunit encoding plasmid vectors, manuscript in preparation.

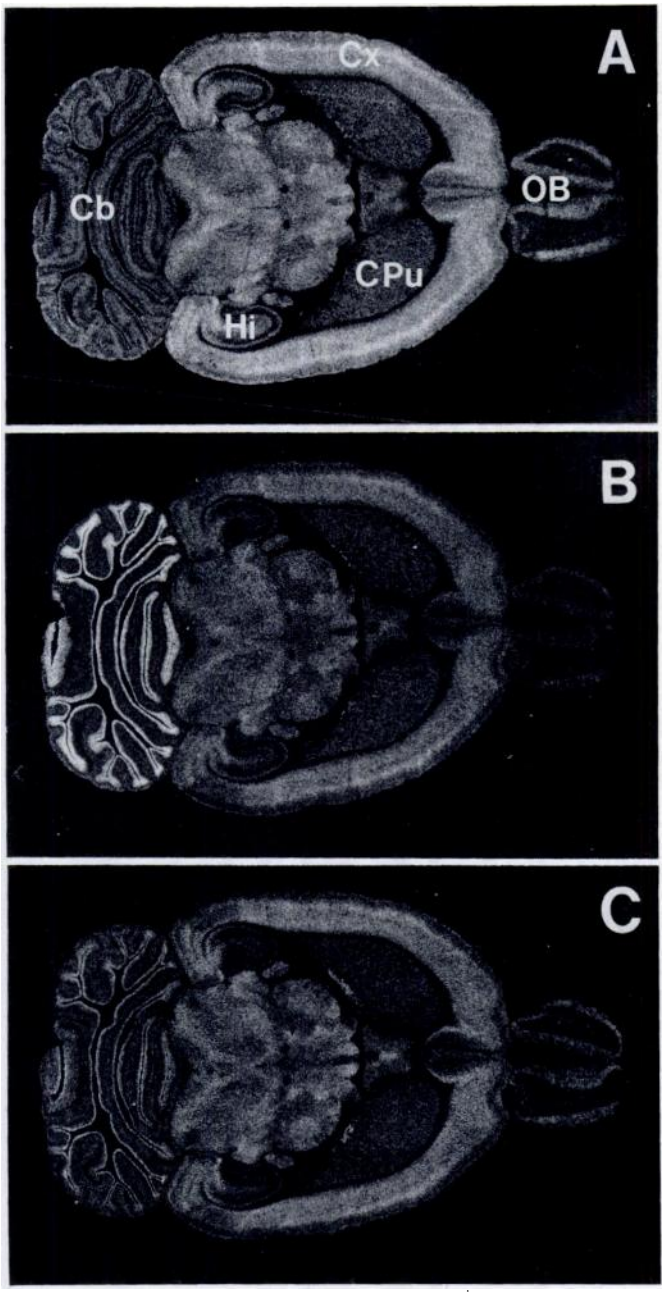

Fig. 1. Autoradiographic images of [^{35}S]TBPS binding to rat brain horizontal sections, as influenced by the $GABA_A$ antagonist SR 95531 and GABA. A, Basal binding, indicating a wide distribution of binding sites throughout the brain; B, 50 μM SR 95531 slightly decreased the binding in most brain regions, while revealing binding sites in the cerebellar granule cell layer; C, 50 μM GABA decreased the effect of the antagonist on binding in the cerebellum and other brain regions. *Cb*, cerebellum; *Cx*, cerebral cortex; *Hi*, hippocampus; *OB*, olfactory bulb; *CPu*, caudate putamen (striatum).

receptors bound [^{35}S]TBPS but showed features that did not fit the fingerprint in brain sections, i.e., 1) $\alpha 6\beta 2$-containing receptors were resistant to inhibition by 10 μM GABA (Fig. 5B) and 2) the $\alpha x\beta 2$ receptors did not bind [^{3}H]Ro 15–4513 (data not shown), although heavy labeling by this BZ receptor ligand is a characteristic feature of the cerebellar granule cells (18, 19).

Because GABA antagonists were used to reveal the granule cell layer binding of [^{35}S]TBPS, the enhanced binding might

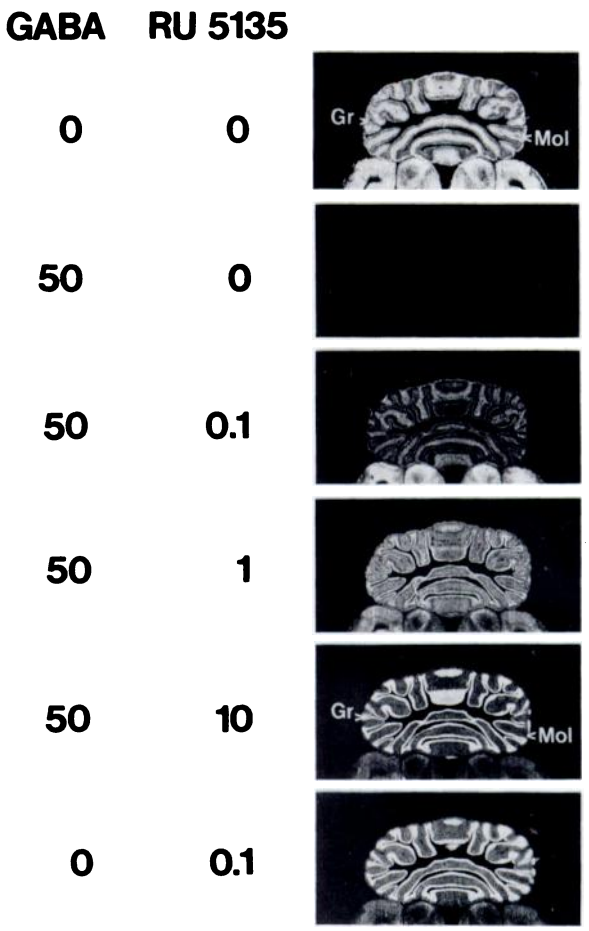

Fig. 2. Autoradiographic images of [^{35}S]TBPS binding to rat cerebellum, as influenced by GABA and the $GABA_A$ antagonist RU 5135. All binding, except for that to the internal surface of the granule cell layer, was abolished by 50 μM GABA. GABA inhibition was completely reversed by RU 5135 (given in μM). RU 5135 at 10 μM in the presence of added GABA and 0.1 μM RU 3135 without exogenous GABA revealed binding in the granule cell layer, thus producing a negative image of the basal binding (*top*). *Mol*, molecular layer; *Gr*, granule cell layer.

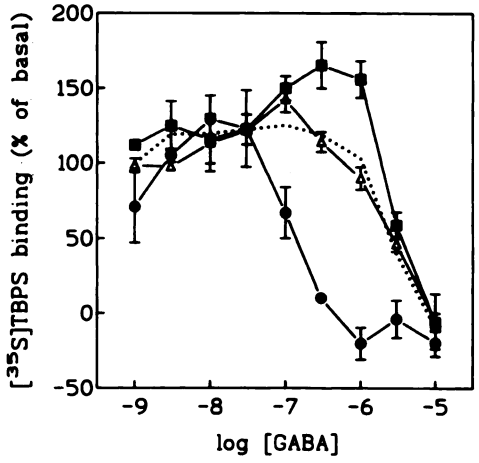

Fig. 3. Effect of GABA on [^{35}S]TBPS binding to recombinant $GABA_A$ receptors consisting of $\alpha 6\beta 2\gamma 2$ (●), $\alpha 1\beta 2\gamma 2$ (■), or $\alpha 1\alpha 6\beta 2\gamma 2$ (Δ) subunits. · · · · ·, Sum of 0.7 × the binding by $\alpha 1\beta 2\gamma 2$ receptors + 0.3 × the binding by $\alpha 6\beta 2\gamma 2$ receptors. Values are means ± standard errors of four to seven experiments.

TABLE 1

Binding characteristics of $GABA_A$ receptors consisting of $\alpha 1$, $\alpha 6$, $\beta 2$, and $\gamma 2$ subunits

All values are given as mean ± standard error (with the number of independent determinations given in parentheses).

Ligand/parameters	Receptor subunit combination		
	$\alpha 1\beta 2\gamma 2$	$\alpha 1\alpha 6\beta 2\gamma 2$	$\alpha 6\beta 2\gamma 2$
[^{3}H]Muscimol			
K_d (nM)	8.1 ± 2.4 (6)	ND[a]	6.8 ± 2.2
B_{max} (pmol/mg of protein)	0.9 ± 0.1 (6)	ND	1.5 ± 0.4
Binding at 6 nM (pmol/mg of protein)[b]	0.23 ± 0.03[c] (3)	0.36 ± 0.05[c]	1.7 ± 0.1
IC_{50} for GABA inhibition (nM)[b]	37 ± 5 (3)	27 ± 1	27 ± 2
K_i for GABA inhibition (nM)[b]	27 ± 3 (3)	ND	14 ± 1
[^{3}H]Ro 15-4513			
K_d (nM)	3.9 ± 0.4 (6)	ND	6.9 ± 1.5
B_{max} (pmol/mg of protein)	1.7 ± 0.4 (6)	ND	1.5 ± 0.5
Binding at 6 nM (pmol/mg of protein)[b]	1.3 ± 0.1[c] (5)	1.1 ± 0.1[c]	2.9 ± 0.2
Binding with 10 μM diazepam (% of total)[b]	1.0 ± 0.7[c] (5)	29 ± 1[c]	99 ± 1
[^{35}S]TBPS			
K_d (nM)	41 ± 7 (3)	ND	46 ± 7
B_{max} (pmol/mg of protein)	0.7 ± 0.1 (3)	ND	0.5 ± 0.1
$t_{1/2}$ for dissociation by GABA (min)[b]	2.8 ± 0.7[d] (4)	ND	1.2 ± 0.3
Maximal displacement (%)[b]	87 ± 6[c] (4)	ND	104 ± 7

[a] ND, not determined.
[b] Assays were carried out using sets of transfected membranes different from the ones used in saturation assays.
[c] Analysis of variance followed by t test using Bonferroni correction, $p \leq 0.001$, compared with $\alpha 6\beta 2\gamma 2$.
[d] $p \leq 0.05$.

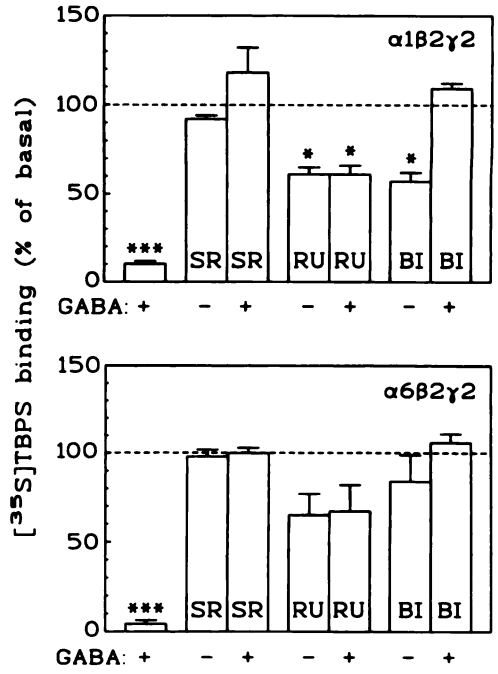

Fig. 4. Effects of the $GABA_A$ antagonists SR 95531 (*SR*) (10 μM), RU 5135 (*RU*) (10 μM), and bicuculline (*BI*) (50 μM) on [^{35}S]TBPS binding to recombinant $GABA_A$ receptors consisting of $\alpha 1\beta 2\gamma 2$ and $\alpha 6\beta 2\gamma 2$ subunits. GABA (10 μM with $\alpha 1\beta 2\gamma 2$ receptors and 1 μM with $\alpha 6\beta 2\gamma 2$ receptors) abolished the binding. The antagonists all blocked GABA inhibition without enhancing binding on their own. The values are means ± standard errors for three experiments. Significance of the differences from the basal binding (Student's t test): *, $p < 0.05$; ***, $p < 0.001$.

have been due to the antagonists themselves, rather than their blockade of endogenous GABA effects. This seems unlikely, however, because in both $\alpha 1\beta 2\gamma 2$ and $\alpha 6\beta 2\gamma 2$ receptors none of the antagonists (SR 95531, RU 5135, and bicuculline) at concentrations used in the autoradiographic experiments enhanced the binding, and they actually decreased it in some cases (Fig. 4). All antagonists abolished the GABA inhibition.

The K_d values for [^{3}H]muscimol and [^{3}H]Ro 15–4513 binding were similar for $\alpha 1\beta 2\gamma 2$ and $\alpha 6\beta 2\gamma 2$ receptors. The receptors did not differ in the displacement of [^{3}H]muscimol binding by GABA (Table 1). The receptors were either fully sensitive ($\alpha 1$) or insensitive ($\alpha 6$) to the BZ agonist diazepam, as has been shown previously (6). No difference was found in the K_d of [^{35}S]TBPS binding after a 90-min incubation. However, a small but significant difference was observed in the dissociation of [^{35}S]TBPS from its binding sites induced by 1 μM GABA (Table 1), indicating a difference in the coupling between the agonist and the [^{35}S]TBPS recognition sites.

The neurosteroid 3α-OH-DHP enhanced the effects of GABA in both $\alpha 1\beta 2\gamma 2$ and $\alpha 6\beta 2\gamma 2$ receptors (Fig. 5A). However, the action of the neurosteroid without added GABA was more pronounced in the $\alpha 6$-containing than in the $\alpha 1$-containing receptors (Fig. 6). The neurosteroid had potent effects on $\alpha x\beta 2$ combinations, giving rise to almost complete inhibition of [^{35}S]TBPS binding by GABA even in the $\alpha 6\beta 2$ dual combination (Fig. 5B). Thus, in the presence of 3α-OH-DHP, GABA inhibited the binding to the $\alpha 1\beta 2$ and $\alpha 6\beta 2$ receptors with similar potency.

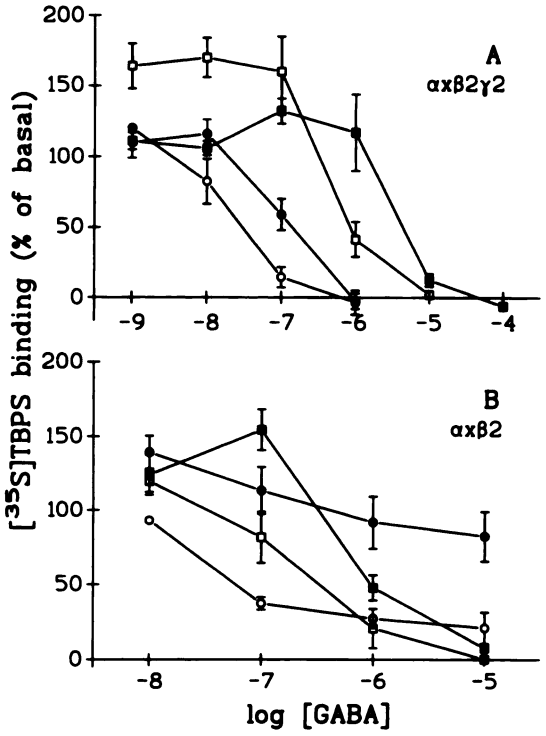

Fig. 5. Effects of GABA and 3α-OH-DHP on [^{35}S]TBPS binding to recombinant $GABA_A$ receptors consisting of $\alpha 6\beta 2\gamma 2$ (*circles*) or $\alpha 1\beta 2\gamma 2$ (*squares*) subunits (A) or $\alpha 6\beta 2$ (*circles*) or $\alpha 1\beta 2$ (*squares*) subunits (B). *Open symbols*, binding in the presence of 100 nM 3α-OH-DHP. *Closed symbols*, binding in the absence of 3α-OH-DHP. Values are means ± standard errors for three or four experiments.

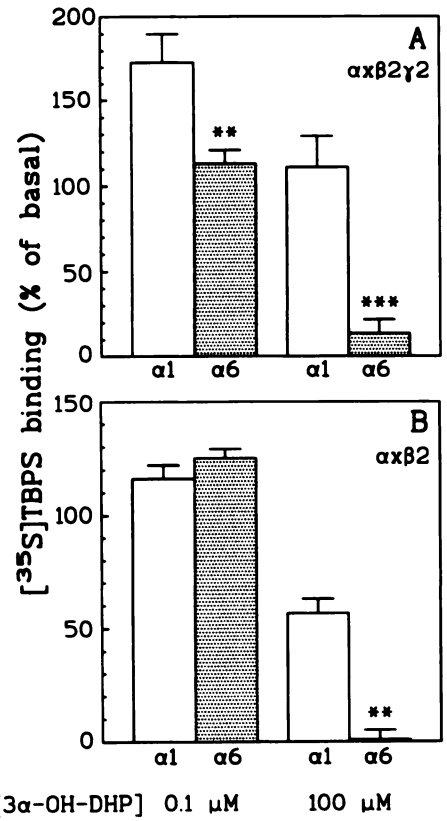

Fig. 6. Effect of 3α-OH-DHP on [35S]TBPS binding to $GABA_A$ receptors containing α1β2γ2 (*α1*) or α6β2γ2 (*α6*) subunits (A) or α1β2 (*α1*) or α6β2 (*α6*) subunits (B). *Bars*, means ± standard errors for three to seven experiments. Significance of the differences from the α1-containing receptors (Student's *t* test): **, $p < 0.01$; ***, $p < 0.001$.

Discussion

Some properties of $GABA_A$ receptor-associated ionophores can be conveniently studied using the 35S-labeled cage convulsant [35S]TBPS as a ligand. [35S]TBPS binding to $GABA_A$ receptors is stimulated or inhibited by GABA, depending on experimental conditions (20–22). The effects of GABA on [35S]TBPS binding can, in turn, be modulated by other ligands, most prominently by the positive and negative allosteric modulators within the group of BZ receptor ligands, barbiturates, and neurosteroids (20, 23, 24). In the present study, pre-equilibrium binding conditions (90-min incubation at 22°) (20) were used to detect any differential GABA modulation of [35S]TBPS binding in rat brain sections. As demonstrated recently (14), the brain region with the highest variation in GABA sensitivity of [35S]TBPS binding is the cerebellum. The cerebellar granule cell layer is far more sensitive to the residual endogenous GABA concentration than is the molecular layer. Consequently, $GABA_A$ antagonists, although slightly inhibitory on their own (Fig. 4), enhanced the [35S]TBPS binding in the granule cell layer and slightly diminished it in the molecular layer and most other brain regions by blocking the effects of endogenous GABA.

In the present study, we demonstrate that the heterogeneity in the GABA modulation of [35S]TBPS binding can be simulated *in vitro* by varying the α subunits in recombinant $GABA_A$ receptors. The α6 subunit-containing receptors, restricted to the cerebellar granule cell layer (6), are >10 times more sensitive to the negative allosteric modulation by GABA than are α1 subunit-containing receptors. Because the α1β2 and α6β2 combinations did not produce the same difference in sensitivity as the αxβ2γ2 combinations, it is likely that the majority of the native $GABA_A$ receptors in granule cells are composed of αxβ2γ2. Although the nature of the β subunit has not been positively established, the abundance of the β2 subunit makes it the most likely candidate for the αxβxγ2 complexes. This is further supported by the fact that the granule cell layer is densely labeled by the imidazobenzodiazepine [3H]Ro 15–4513, the binding of which requires the assembly of the three subunits (α1 or α6 together with β2 and γ2) within the same molecular complex (4, 6). This interpretation does not require assembly with the δ subunit (25), the role of which in $GABA_A$ receptor pharmacology and physiology is still unclear.

Furthermore, we also confirmed the finding made by Edgar and Schwartz (21), who detected some GABA-insensitive [35S]TBPS binding in the internal granule cell layer. GABA at 50 μM decreased the binding in the external granule cell and molecular layers to background levels, while leaving some binding in the internal rim of the granule cell layer. The subunit combination responsible for this binding could be α6β2, because this combination produced [35S]TBPS binding that was not inhibited by GABA, although a number of other combinations are conceivable.

Because the granule cells of the cerebellum express both α6 and α1 subunits, it is possible that a fraction coassembles in single molecular complex in the form of α1α6β2γ2, besides the α1β2γ2 and α6β2γ2 forms. Indeed, this has been suggested by immunoprecipitation of [3H]Ro 15–4513 binding sites from rat brain membranes using an α1 subunit-specific antiserum (26). In the present study, we simultaneously expressed the α1, α6, β2, and γ2 subunits in a heterologous system. About 70% of the resulting BZ receptors resembled the α1β2γ2 type of receptors and 30% of [3H]Ro 15–4513 binding was insensitive to 10 μM diazepam (Table 1), the typical feature of α6β2γ2-containing receptors (6). A theoretical curve constructed from the experimental data (0.7 × binding by α1β2γ2 receptors + 0.3 × binding by α6β2γ2 receptors) was in good agreement with the experimental data from the cotransfection of the α1, α6, β2, and γ2 subunits (Fig. 3). This would indicate that almost all of the $GABA_A$ receptors formed had the subunit combination of either α1β2γ2 or α6β2γ2, as has been suggested previously for rat brain membranes (26).

The sensitivity difference between the α1- and α6-containing [35S]TBPS receptors was not restricted to the effect of the natural transmitter but extended to at least one of the modulators, the neurosteroid 3α-OH-DHP. The α6-containing recombinant receptors were more sensitive to the inhibition of [35S]TBPS binding by 3α-OH-DHP than were the α1-containing receptors, suggesting a fundamental increase in the sensitivity of their convulsant site to inhibition. Autoradiographic experiments by Sapp *et al.* (27) support this conclusion, because the modulation of [35S]TBPS binding by steroids was most pronounced in the cerebellar granule cell layer. Interestingly, the double combinations α1β2 and α6β2 showed a reversed GABA sensitivity, compared with the triple combinations. In fact, the α6β2-containing receptors were largely insensitive to GABA, but the addition of 3α-OH-DHP reestablished the former rank order of GABA sensitivity. The data suggest that the neurosteroid and GABA act synergistically but at different sites on $GABA_A$ receptors. The restoration of the inhibitory effect of GABA by 3α-OH-DHP in α6β2 receptors might pro-

vide a convenient tool to investigate the structural requirements for the coupling of the convulsant site to the GABA and neurosteroid binding sites.

The differential GABA sensitivity seems to be due to differences in allosteric coupling mechanisms and not in the binding sites for [^{35}S]TBPS and GABA. This is indicated by the fact that the K_d of [^{35}S]TBPS and [^{3}H]muscimol binding and the K_i for GABA displacement of [^{3}H]muscimol binding were not statistically different between the $\alpha1\beta2\gamma2$ and $\alpha6\beta2\gamma2$ receptors. Although the off-times of [^{35}S]TBPS binding induced by 1 μM GABA differed between these two subunit combinations, the difference was only 2-fold, compared with the 10-fold difference in their GABA sensitivity. It should be noted that the EC_{50} concentrations for GABA-induced whole-cell currents in 293 cells are about 0.3 μM and 5 μM for the $\alpha6\beta2\gamma2$ and $\alpha1\beta2\gamma2$ receptors, respectively (28), which agrees with the inhibitory effects of GABA on [^{35}S]TBPS binding at high nanomolar and low micromolar concentrations, respectively. Therefore, we suggest that [^{35}S]TBPS binding data are a predictive measure for the regulation of the $GABA_A$ receptor-associated ionophore.

In conclusion, the present data indicate that a population of $GABA_A$ receptors in the cerebellar granule cells are highly sensitive to GABA, compared with $GABA_A$ receptors found elsewhere in the brain. Furthermore, this receptor population is most likely composed of the $\alpha6$, $\beta2$, and $\gamma2$ subunits.

Acknowledgments

The authors wish to thank Sabine Grünewald and Heike Musolff for cell culture and Peter H. Seeburg for valuable comments on the manuscript.

References

1. Bormann, J. Electrophysiology of $GABA_A$ and $GABA_B$ receptor subtypes. *Trends Neurosci.* **11**:112–116 (1988).
2. Olsen, R. W., and A. J. Tobin. Molecular biology of $GABA_A$ receptors. *FASEB J.* **4**:1469–1480 (1990).
3. Wisden, W., and P. H. Seeburg. $GABA_A$ receptor channels: from subunits to functional entities. *Curr. Opin. Biol.* **2**:263–269 (1992).
4. Pritchett, D. B., H. Sontheimer, B. D. Shivers, S. Ymer, H. Kettenmann, P. R. Schofield, and P. H. Seeburg. Importance of a novel $GABA_A$ receptor subunit for benzodiazepine pharmacology. *Nature (Lond.)* **338**:582–585 (1989).
5. Pritchett, D. B., H. Lüddens, and P. H. Seeburg. Type I and type II $GABA_A$-benzodiazepine receptors produced in transfected cells. *Science (Washington D. C.)* **245**:1389–1392 (1989).
6. Lüddens, H., D. B. Pritchett, M. Köhler, I. Killisch, K. Keinänen, H. Monyer, R. Sprengel, and P. H. Seeburg. Cerebellar $GABA_A$ receptor selective for a behavioural alcohol antagonist. *Nature (Lond.)* **346**:648–651 (1990).
7. Herb, A., W. Wisden, H. Lüddens, G. Puia, S. Vicini, and P. H. Seeburg. The third γ subunit of the γ-aminobutyric acid type A receptor family. *Proc. Natl. Acad. Sci. USA* **89**:1433–1437 (1992).
8. Levitan, E. S., P. R. Schofield, D. R. Burt, L. M. Rhee, W. Wisden, M. Köhler, N. Fujita, H. F. Rodriquez, A. Stephenson, M. G. Darlison, E. A. Barnard, and P. H. Seeburg. Structural and functional basis for $GABA_A$ receptor heterogeneity. *Nature (Lond.)* **335**:76–79 (1988).
9. Verdoorn, T. A., A. Draguhn, S. Ymer, P. H. Seeburg, and B. Sakmann. Functional properties of recombinant rat $GABA_A$ receptors depend upon subunit composition. *Neuron* **4**:919–928 (1990).
10. Sigel, E., R. Baur, G. Trube, H. Möhler, and P. Malherbe. The effect of subunit composition of rat brain $GABA_A$ receptors on channel function. *Neuron* **5**:703–711 (1990).
11. Puia, G., I. Ducic, S. Vicini, and E. Costa. Molecular mechanisms of the partial allosteric modulatory effects of bretazenil at γ-aminobutyric acid type A receptor. *Proc. Natl. Acad. Sci. USA* **89**:3620–3624 (1992).
12. Laurie, D. J., P. H. Seeburg, and W. Wisden. The distribution of 13 $GABA_A$ receptor subunit mRNAs in the rat brain. II. Olfactory bulb and cerebellum. *J. Neurosci.* **12**:1063–1076 (1992).
13. Wisden, W., D. J. Laurie, H. Monyer, and P. H. Seeburg. The distribution of 13 $GABA_A$ receptor subunit mRNAs in the rat brain. I. Telencephalon, diencephalon, mesencephalon. *J. Neurosci.* **12**:1040–1062 (1992).
14. Korpi, E. R., H. Lüddens, and P. H. Seeburg. $GABA_A$ antagonists reveal binding sites for [^{35}S]TBPS in cerebellar granular cell layer. *Eur. J. Pharmacol.* **211**:427–428 (1992).
15. Olsen, R. W., R. T. McCabe, and J. K. Wamsley. $GABA_A$ receptor subtypes: autoradiographic comparison of GABA, benzodiazepine, and convulsant binding sites in the rat central nervous system. *J. Chem. Neuroanat.* **3**:59–76 (1990).
16. Chen, C., and H. Okayama. High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.* **7**:2745–2752 (1987).
17. Ymer, S., P. R. Schofield, A. Draguhn, P. Werner, M. Köhler, and P. H. Seeburg. $GABA_A$ receptor β subunit heterogeneity: functional expression of cloned cDNAs. *EMBO J.* **8**:1665–1670 (1989).
18. Sieghart, W., A. Eichinger, J. G. Richards, and H. Möhler. Photoaffinity labeling of benzodiazepine receptor proteins with the partial inverse agonist [^{3}H]Ro 15–4513: a biochemical and autoradiographic study. *J. Neurochem.* **48**:46–52 (1987).
19. Malminiemi, O., and E. R. Korpi. Diazepam-insensitive [^{3}H]Ro 15–4513 binding in intact cultured cerebellar granule cells. *Eur. J. Pharmacol.* **169**:53–60 (1989).
20. Maksay, G., and M. Simonyi. Kinetic regulation of convulsant (TBPS) binding by GABAergic agents. *Mol. Pharmacol.* **30**:321–328 (1986).
21. Edgar, P. P., and R. D. Schwartz. Localization and characterization of ^{35}S-*t*-butylbicyclophosphorothionate binding in rat brain: an autoradiographic study. *J. Neurosci.* **10**:603–612 (1990).
22. Lloyd, G. K., G. Danielou, and F. Thuret. The activity of zolpidem and other hypnotics within the γ-aminobutyric acid ($GABA_A$) receptor supramolecular complex, as determined by ^{35}S-*t*-butylbicyclophosphorothionate (^{35}S-TBPS) binding to rat cerebral cortex membranes. *J. Pharmacol. Exp. Ther.* **255**:690–696 (1990).
23. Supavilai, P., and M. Karobath. Differential modulation of [^{35}S]TBPS binding by the occupancy of benzodiazepine receptors with its ligands. *Eur. J. Pharmacol.* **91**:145–146 (1983).
24. Majevska, M. D., N. L. Harrison, R. D. Schwartz, J. L. Barker, and S. M. Paul. Steroid hormone metabolites are barbiturate-like modulators of GABA receptor. *Science (Washington D. C.)* **232**:1004–1007 (1986).
25. Shivers, B. D., I. Killisch, R. Sprengel, H. Sontheimer, M. Köhler, P. R. Schofield, and P. H. Seeburg. Two novel $GABA_A$ receptor subunits exist in distinct neuronal subpopulations. *Neuron* **3**:327–337 (1989).
26. Lüddens, H., I. Killisch, and P. H. Seeburg. More than one *alpha* variant may exist in a $GABA_A$ benzodiazepine receptor complex. *J. Recept. Res.* **11**:535–551 (1991).
27. Sapp, D. W., U. Witte, D. M. Turner, B. Longoni, N. Kokka, and R. W. Olsen. Regional variation in steroid anesthetic modulation of [^{35}S]TBPS binding to γ-aminobutyric $acid_A$ receptors in rat brain. *J. Pharmacol. Exp. Ther.* **262**:801–808 (1992).
28. Kleingoor, C., H. A. Wieland, E. R. Korpi, P. H. Seeburg, and H. Kettenmann. Current potentiation by diazepam but not GABA sensitivity is determined by a single histidine residue. *NeuroReport*, **4**: 187–190 (1993).

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