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# Preferential Coassembly of $\alpha$4 and $\delta$ Subunits of the $\gamma$-Aminobutyric Acid$_A$ Receptor in Rat Thalamus

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## ABSTRACT

Pharmacological study of rat thalamic $\gamma$-aminobutyric acid$_A$ (GABA$_A$) receptors revealed the presence of two distinct populations, namely, diazepam-sensitive and diazepam-insensitive [$^3$H]Ro15–4513 binding sites accounting for 94 ± 2% (1339 ± 253 fmol/mg protein) and 6 ± 2% (90 ± 44 fmol/mg protein) of total sites, respectively. Thalamic diazepam-insensitive sites exhibited a pharmacology that was distinct from diazepam-sensitive sites but comparable to that of the $\alpha$4$\beta$3$\gamma$2 subtype of the GABA$_A$ receptor stably expressed in L(tk$^-$) cells. Immunoprecipitation experiments with a specific anti-$\alpha$4-antiserum immunoprecipitated 20 and 7% of total thalamic [$^3$H]muscimol and [$^3$H]Ro15–4513 sites, respectively. Combinatorial immunoprecipitation using antisera against the $\alpha$4, $\gamma$2, and $\delta$ subunit revealed that $\alpha$4$\delta$- and $\alpha$4$\gamma$2-containing receptors account for 13 ± 2 and 8 ± 3% of [$^3$H]muscimol sites from thalamus, respectively. It also indicated that all $\delta$ subunits coexist with an $\alpha$4 subunit in this brain region. In conclusion, our results show that in rat thalamus both $\alpha$4$\beta\gamma$2 and $\alpha$4$\beta\delta$ subtypes are expressed but $\alpha$4$\beta\delta$ is the major $\alpha$4-containing GABA$_A$ receptor population.

$\gamma$-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the mammalian central nervous system. Its effects are mediated largely through the GABA$_A$ receptors, a family of GABA-gated $Cl^-$ ion channels (for reviews, see Sieghart, 1995; McKernan and Whiting, 1996), which are pentameric assemblies of the 14 different subunits cloned to date ($\alpha$1–6, $\beta$1–3, $\gamma$1–3, $\delta$, and $\epsilon$). The combination of $\alpha$ and $\gamma$ subunits has been shown to confer specific functional and pharmacological properties, in particular the affinity and efficacy of compounds at the benzodiazepine binding site. These two subunit types also contribute to the affinity and efficacy of GABA and $Zn^{2+}$ sensitivity of the channel.

Dysfunction of GABAergic neurotransmission has been implicated in neurological disorders such as epilepsy. Studies of temporal lobe epilepsy using different animal models have reported up-regulation of various GABA$_A$ receptor subunit mRNAs and proteins as well as modification of the pharmacological profile of receptors in rat hippocampus. For example, in electrical kindled rat, Clark and coworkers (1994) found increased levels of $\alpha$4, $\beta$1, and $\beta$3 subunit mRNAs in dentate gyrus. Similarly, in kainic acid-induced temporal lobe epilepsy a marked up-regulation of $\alpha$1, $\alpha$2, $\alpha$4, $\alpha$5, $\beta$1, $\beta$3, $\gamma$2, and $\delta$ subunit proteins has been reported in the molecular layer of the rat dentate gyrus (Schwarzer et al., 1997). A recent study in rat (Brooks-Kayal et al., 1998) investigating GABAergic currents and mRNA expression in single dentate granule cells demonstrated profound changes in subunit expression and GABA$_A$ receptor properties after pilocarpine treatment. The most dramatic changes were a 175 and 225% increase in the relative expression of $\alpha$4 and $\delta$ subunit mRNAs, respectively, together with an enhanced sensitivity of GABA$_A$ receptors to block by $Zn^{2+}$. An emerging view from these and other studies (Mahmoudi et al., 1997; Matthews et al., 1998; Smith et al., 1998) is that $\alpha$4 subunit-containing GABA$_A$ receptors are highly plastic and, compared with other subtypes, are rapidly up-regulated in response to changes in neuronal activity.

Biochemical and pharmacological reports have shown that in rat brain some $\alpha$4 receptors bind [$^3$H]Ro15–4513 with high affinity (Benke et al., 1997) whereas others do not (Khan et al., 1996), suggesting the existence of a heterogeneous population of $\alpha$4 subunit-containing GABA$_A$ receptors. In the present study, we have used pharmacological analyses and quantitative immunoprecipitation (Sur et al., 1998) to further characterize $\alpha$4 subunit-containing GABA$_A$ receptors. We have focused our attention on subpopulations of $\alpha$4 subunit-containing receptors present in rat thalamus and hippocampus, brain regions that express high level of $\alpha$4 subunits and are involved in epilepsy (Wisden et al., 1992; Lowenstein, 1996).

## Materials and Methods

[$^3$H]Muscimol (19.1 Ci/mmol) and [$^3$H]Ro 15–4513 (20.9 Ci/mmol) were obtained from DuPont-New England Nuclear (Boston, MA).

**ABBREVIATIONS:** GABA, $\gamma$-aminobutyric acid; DS, diazepam-sensitive; DIS, diazepam-insensitive; TBS, Tris-buffered saline.

Benzodiazepine site ligands were obtained from Sigma (St. Louis, MO) or Research Biologicals, Inc. (Natick, MA).

**Radioligand Binding Studies.** Binding of [$^3$H]Ro15–4513 (8 nM) or [$^3$H]muscimol (40 nM) to thalamic or $\alpha 4\beta 3\gamma 2$ cell membranes was carried out in 10 mM $KH_2PO_4$, 100 mM KCl pH 7.4 in a total volume of 0.5 ml. After incubation at 4°C for 1 h binding assays were terminated by filtration through Whatman GF/B filters, followed by washing three times in 10 mM $KH_2PO_4$, 100 mM KCl pH 7.4, and scintillation counting. Nonspecific binding was determined using 1 mM GABA for [$^3$H]muscimol binding and 40 $\mu$M bretazenil for [$^3$H]Ro15–4513 binding because bretazenil binds to all $\alpha 1$ to $\alpha 6$ subtypes (Sieghart, 1995). Nonlinear regression and statistical analyses were performed with Prism (GraphPad Software, San Diego, CA).

**Generation of $\alpha 4$ Antiserum.** Expression of the $\alpha 4$ subunit putative cytoplasmic loop was carried out as described elsewhere (McKernan et al., 1991). cDNA sequences encoding the domain between TM3 and TM4 (residues Pro332–Pro475 of bovine $\alpha 4$) were engineered into the bacterial expression vector pRSET5a using the polymerase chain reaction. Oligonucleotide primers used were 5′ tttcaggaattccagtgctgagagaaaagcatcctgaaac 3′ (sense, incorporating an *Eco*RI site) and 5′ atccagaagcttgtggagcagagggagtagtagtggc 3′ (antisense, incorporating a *Hin*dIII site), and polymerase chain reaction was performed using bovine brain cDNA as template. The construct was confirmed by DNA sequencing. Polypeptide was expressed in *Escherichia coli* strain BL21 DE3 (lys-S) and using methods described previously (McKernan et al., 1991) purified to 1 mg/ml and emulsified with Freund's complete adjuvant (1:1, v/v). Rabbits were then immunized with 50-$\mu$g aliquots s.c., and boosted monthly for another 2 months with 50 $\mu$g of polypeptide emulsified with Freund's incomplete adjuvant. Rabbits were bled 7 days after each boost and the presence of anti-$\alpha 4$ antibodies was then assayed by Western blot against bacterially expressed $\alpha 1$, 2, 3, 4, 5, and 6 polypeptides as described previously (McKernan et al., 1991; Quirk et al., 1994).

**Generation of $\alpha 4\beta 3\gamma 2$ Cell Line.** cDNAs encoding human $\alpha 4$, $\beta 3$, and $\gamma 2S$ have been described previously (Hadingham et al., 1993a,b; Wafford et al., 1996). The expression of the $\alpha 4$ subunit in oocytes was poor, so the 5′-untranslated region and the signal peptide of the $\alpha 1$ subunit was engineered onto the $\alpha 4$ subunit, which resulted in much higher levels of expression (Wafford et al., 1996). This construct was then used to generate a stable cell line expressing human $\alpha 4\beta 3\gamma 2$ $GABA_A$ receptors by transfection of the individual subunits in the dexamethasone-inducible expression vector pMSG-neo in mouse L(tk$^-$) cells as described previously (Hadingham et al., 1993a). Geneticin-resistant cell colonies were subcloned and assayed for [$^3$H]Ro 15–4513 binding 5 days after the induction of receptor expression. Cells expressing the highest levels of [$^3$H]Ro 15–4513 binding were recloned and the resultant cell line was maintained as described previously (Hadingham et al., 1993a).

**Immunoprecipitation.** Receptors were solubilized from rat brain or from cell lines using 0.5% deoxycholate as described previously (McKernan et al., 1991). Briefly, antiserum (100 $\mu$l) and protein-A beads (100 $\mu$l) were incubated in a total volume of 1 ml of Tris-buffered saline (TBS) for 1 h at room temperature. After three washes with TBS, the antibody-protein A complex was loaded with 0.5% deoxycholate-solubilized receptors (0.4–0.6 ml) from thalamus, hippocampus, or cell line and incubated overnight at 4°C. The beads were then washed three times in TBS/0.1% Tween 20 and resuspended in 10 mM $KH_2PO_4$, 100 mM KCl, pH 7.4. Controls with protein A beads only or anti-$5HT_3$-antibody-protein A beads were used to determine nonspecific immunoprecipitation. Quantitative coimmunoprecipitations were carried out as described by Quirk et al. (1994). The $\gamma 2$- and $\delta$-specific antibodies have been described previously and characterized (Quirk et al., 1994, 1995).

## Results

**[$^3$H]Ro15–4513 Saturation Binding.** Saturation experiments in rat thalamus were performed with [$^3$H]Ro15–4513, a benzodiazepine site radioligand that binds to all $\gamma 2$ subunit-containing $GABA_A$ receptors (i.e., $\alpha 1\beta\gamma 2$, $\alpha 2\beta\gamma 2$, $\alpha 3\beta\gamma 2$, $\alpha 4\beta\gamma 2$, etc.; Sieghart, 1995). The experiments were carried out either in the absence of diazepam to determine the total number of receptors or in the presence of 10 $\mu$M diazepam to reveal the existence of diazepam-insensitive (DIS) [$^3$H]Ro15–4513 sites. The diazepam-sensitive (DS) [$^3$H]Ro15–4513 binding sites were then defined as the difference between total receptors and DIS. As illustrated in Fig. 1, [$^3$H]Ro15–4513 binds to both DS and DIS receptors with a similar affinity ($Kd_{DS}$ = 7.1 ± 0.3 nM; $Kd_{DIS}$ = 7.0 ± 0.7 nM; mean ± S.E.M., $n$ = 2) but with different $B_{max}$ values; DS and DIS sites accounting for 94 ± 2% (1339 ± 253 fmol/mg protein) and 6 ± 2% (90 ± 44 fmol/mg protein) of total sites, respectively.

**[$^3$H]Ro15–4513 Binding Sites Pharmacology.** Displacement of bound [$^3$H]Ro15–4513 from thalamic membrane by various benzodiazepine site ligands also revealed distinct $GABA_A$ receptor populations (Fig. 2A and Table 1). The $\alpha 1$-selective compound, zolpidem, inhibited 67 ± 6% of binding sites with a $K_i$ value of 20 nM, establishing $\alpha 1$-containing receptors as the main $\alpha$ subunit population in the thalamus. Flunitrazepam did not block 11 ± 2% of [$^3$H]Ro15–4513 sites whereas all other tested drugs fully displaced bound radioligand (Fig. 2A and Table 1). Competition experiments (Fig. 2B and Table 1) showed that CGS8216, bretazenil, DMCM, Ro15–1788, and ZK93426 bind to DIS [$^3$H]Ro15–4513 with a reduced affinity.

Binding of [$^3$H]Ro15–4513 and [$^3$H]muscimol to the $\alpha 4\beta 3\gamma 2$ cell line was saturable with $K_d$ values of 3.4 ± 0.6 and 16 ± 6 nM and $B_{max}$ values of 355 ± 96 and 698 ± 98 fmol/mg protein (mean ± S.E.M., $n$ = 3), respectively. The existence of 2-fold (2.1 ± 0.4, $n$ = 3) more [$^3$H]muscimol binding sites than [$^3$H]Ro15–4513 binding sites is consistent with expressed receptors having a $(\alpha 4)_2(\beta 3)_2(\gamma 2)_1$ stoichiometry as already reported for other recombinant $GABA_A$ receptors (Chang et al., 1996; Tretter et al., 1997; Farrar et al., 1999). The affinity of a series of compounds for the binding site labeled by [$^3$H]Ro15–4513 was characterized (Table 1). The $\alpha 4\beta 3\gamma 2$ subtype had low affinity for classical benzodiazepine site ligands such as flunitrazepam, a moderate affinity

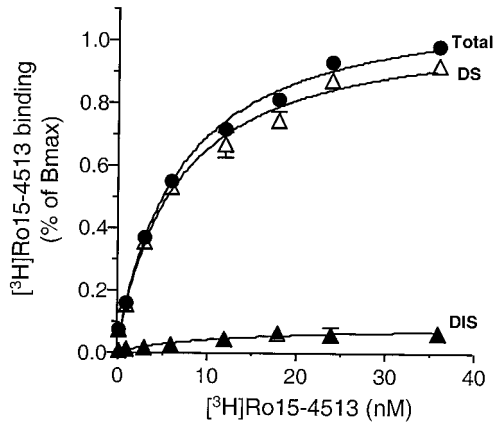


**Fig. 1.** Saturation experiments with [$^3$H]Ro15–4513 in rat thalamus in the presence (▲) or absence (●) of 10 $\mu$M diazepam revealed the existence of DS sites (△: $K_d$ = 7.1 ± 0.3 nM; $B_{max}$ = 1339 ± 253 fmol/mg protein) and DIS sites (▲: $K_d$ = 7.0 ± 0.7 nM; $B_{max}$ = 90 ± 44 fmol/mg protein). The proportion of DIS-[$^3$H]Ro15–4513 sites was 6 ± 2% of total [$^3$H]Ro15–4513 sites. Data are the mean ± S.E.M. of two experiments.

for Ro15–1788 and ZK93426, but retained some affinity for CGS8216 and β-carboline structures such as DMCM.

**Characterization of α4 Antibody.** To further characterize the native rat α4 subunit-containing receptor, an α4-specific antiserum was developed. Immunoblotting data (not shown) indicated that α4 antiserum does not cross-react with bacterially expressed peptides corresponding to the intracellular loop of α1, α2, α3, α5, and α6 $GABA_A$ receptor subunit. The ability of the antiserum to detect native and recombinant receptors was investigated by immunoprecipitating solubilized α4-containing receptors from the rat brain and stable cell line, respectively. As shown in Fig. 3, the antiserum immunoprecipitated essentially all (93 ± 14%) [$^3$H]Ro 15–4513 binding sites solubilized from the cell line. In contrast, α4 antiserum did not precipitate a significant amount of [$^3$H]Ro15–4513 binding from solubilized α1β3γ2, α2β3γ2, α3β3γ2, α5β3γ2, and α6β3γ2 recombinant receptors, which have high-affinity [$^3$H]Ro15–4513 binding sites.

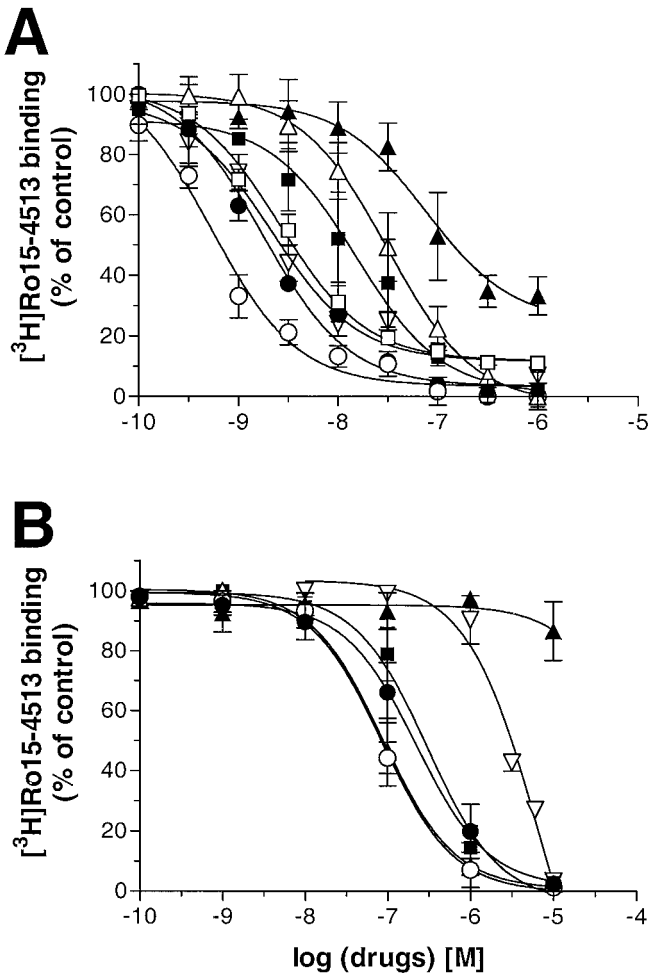


**Fig. 2.** Displacement of [$^3$H]Ro15–4513 (8 nM) bound to rat thalamic membrane in the absence (A) or presence (B) of 10 μM flunitrazepam by various benzodiazepine site ligands. A, note that the α1 receptor-selective drug, zolpidem (▲) inhibited only 66 ± 6% bound radioligand. Similarly, 11 ± 2% of the radiolabeled sites were insensitive to flunitrazepam (□). B, bretazenil (●), CGS8216 (○), DMCM (△), Ro15–1788 (■), and ZK93426 (▽) fully inhibited flunitrazepam-insensitive [$^3$H]Ro15–4513 sites with $K_i$ values (nM) of 59 ± 8, 28 ± 6, 32 ± 7, 119 ± 25, and 1142 ± 133, respectively. Plotted values are the mean ± S.E.M. of three determinations.

When α4 subunit-containing $GABA_A$ receptors were immunoprecipitated from solubilized thalamic membranes, 20 ± 3% ($n$ = 7) and 7 ± 2% ($n$ = 3) of total [$^3$H]muscimol and [$^3$H]Ro15–4513 binding sites were immunoprecipitated, respectively. Interestingly, the proportion of α4-immunoprecipitated [$^3$H]Ro15–4513 sites was not different ($t$ test) from the proportion of DIS [$^3$H]Ro15–4513 sites determined by saturation experiments (see above), suggesting that α4βγ2 subunit-containing receptors represents around one third of the total α4 receptor population in this brain region.

To investigate this further, immunoprecipitation with combinations of α4-, γ2-, and δ-specific antibodies were carried out in rat thalamus. As shown in Fig. 4A, α4 and γ2 antibodies precipitated 22 ± 13 and 52 ± 2% of total [$^3$H]muscimol binding, respectively. Coimmunoprecipitation with both antisera in combination yielded less [$^3$H]muscimol binding than the sum of individual values, indicating the existence of α4βγ2 subtype that accounts for 8 ± 3% of total receptors. This proportion is not different from the quantity of DIS [$^3$H]Ro15–4513 sites determined by saturation experiments (6%) nor from [$^3$H]Ro15–4513 sites immunoprecipitated by α4 antibody (7%). Similar quantitative immunoprecipitation experiments with α4 and δ antibodies (Fig. 4B) showed that δ subunit-containing receptors account for 16 ± 3% of total [$^3$H]muscimol binding sites and revealed the existence of α4βδ receptors (13 ± 2%). Furthermore, they indicated that all δ subunits are present within α4βδ receptor subtype in rat thalamus. To test whether the α4βδ subtype is specific to the thalamus, similar immunoprecipitation experiments were performed in the hippocampus, a region known to express both α4 and δ subunits (Wisden et al., 1992; Schwarzer et al., 1997). As presented in Fig. 4C, α4 and δ antibodies precipitated 13 ± 3% and 13 ± 2% of total [$^3$H]muscimol binding sites, respectively. The α4βδ subtype population accounted for 7 ± 2% of total [$^3$H]muscimol sites or 52 ± 7% and 51 ± 12% of the α4 subunit- and δ subunit-containing receptor population, respectively.

TABLE 1

Comparison of the affinity ($K_i$, nM) of compounds for [$^3$H]Ro15-4513 (8–10 nM) binding sites in rat thalamus in the absence or presence of 10 μM flunitrazepam and to α4β3γ2 receptors stably expressed in L(tk$^-$) cells

Values are the mean ± S.E.M. of two to three determinations. Percentages (%) correspond to the inhibition of [$^3$H]Ro15-4513 binding by 10 μM of the drug.

| | Thalamus (Total Benzodiazepine Receptor Population) | Thalamus (α4-containing $GABA_A$ Receptors) | α4β3γ2 Cells |
|---|---|---|---|
| CGS8216 | 0.30 ± 0.05 | 28 ± 6 | 8.1 ± 1.6 |
| Bretazenil | 0.40 ± 0.05 | 59 ± 8 | 36 ± 10 |
| DMCM | 8.1 ± 2.6 | 32 ± 7 | 11.3 ± 1.4 |
| Ro15-1788 | 3.5 ± 0.4 | 119 ± 25 | 66 ± 9 |
| ZK93426 | 0.79 ± 0.11 | 1142 ± 133 | 1386 ± 795 |
| Flunitrazepam | 2.9 ± 0.2 | n.d. | 12 ± 1% |
| Zolpidem | 20 ± 9 | 12 ± 10% | 4 ± 3% |

## Discussion

The pharmacology of benzodiazepine sites is determined primarily by the α and γ subunits present in the pentameric $GABA_A$ receptor (McKernan et al., 1991; Sieghart, 1995). Here, pharmacological study of rat thalamus revealed the presence of multiple $GABA_A$ receptor subtypes.

Analysis with zolpidem, an α1 subunit benzodiazepine site-selective ligand revealed that α1 subunit-containing $GABA_A$ receptors contribute around two-thirds of total thalamic [$^3$H]Ro15–4513 sites. This is consistent with the predominant expression of α1 mRNA and protein in thalamus (Wisden et al., 1992; Fritschy and Mohler, 1995).

DIS [$^3$H]Ro15–4513 sites were shown to be present in thalamus, where they account for 6 to 11% of total [$^3$H]Ro15–4513 sites in agreement with previous autoradiographic and binding studies (Turner et al., 1991; Benke et al., 1997). The pharmacology of the DIS [$^3$H]Ro15–4513 sites in rat thalamus has been characterized and shown to be similar to the benzodiazepine binding site conferred by α4 in combination with γ2 in recombinant α4β3γ2 receptor. This is in agreement with a report showing the congruence between α4β3γ2 subtype and DIS [$^3$H]Ro15–4513 sites in rat forebrain (Benke et al., 1997).

Quantitative immunoprecipitation with α4- and γ2-specific antisera and [$^3$H]muscimol binding to label all $GABA_A$ receptors showed that α4βγ2 receptors are indeed present in the thalamus, where they represent a minor population (8%) of total $GABA_A$ receptors. Our α4 antibody also specifically immunoprecipitated 7% of total thalamic [$^3$H]Ro15–4513 binding sites. Binding sites measured either by α4γ2 coprecipitation of [$^3$H]muscimol sites (8%), α4 precipitation of [$^3$H]Ro15–4513 sites (7%), or saturation analysis of DIS [$^3$H]Ro15–4513 binding sites (6%) all support the conclusion that they represent the same receptor population and α4γ2-containing receptors in the thalamus account for a relatively minor proportion of total $GABA_A$ receptors.

Our experiments also indicated that α4 subunit-containing

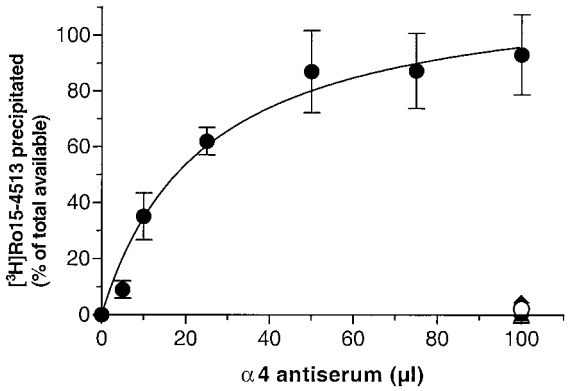


**Fig. 3.** Specific immunoprecipitation of α4 subunit-containing receptors by the anti-α4 antiserum. Saturable immunoprecipitation of [$^3$H]Ro15–4513 binding sites from detergentsolubilized α4β3γ2 cell line is observed. Protein A beads were incubated with increasing volumes of antiserum and $GABA_A$ receptors solubilized from the α4β3γ2 cell line were immunoprecipitated as described in *Materials and Methods*. The α4 antiserum (100 μl) precipitated 93 ± 14% of the total number of [$^3$H]Ro15–4513 sites (mean ± S.E.M.; $n$ = 3). In contrast, no significant amount of [$^3$H]Ro15–4513 binding sites was immunoprecipitated by 100 μl of α4 antibody from α1 (1 ± 1%; mean ± S.E.M.; $n$ = 2), α2 (0.4 ± 0.4%; $n$ = 2), α3 (0%; $n$ = 2), α5 (3 ± 1%; $n$ = 2), or α6 (1 ± 1%; $n$ = 2) subunit-containing receptors.

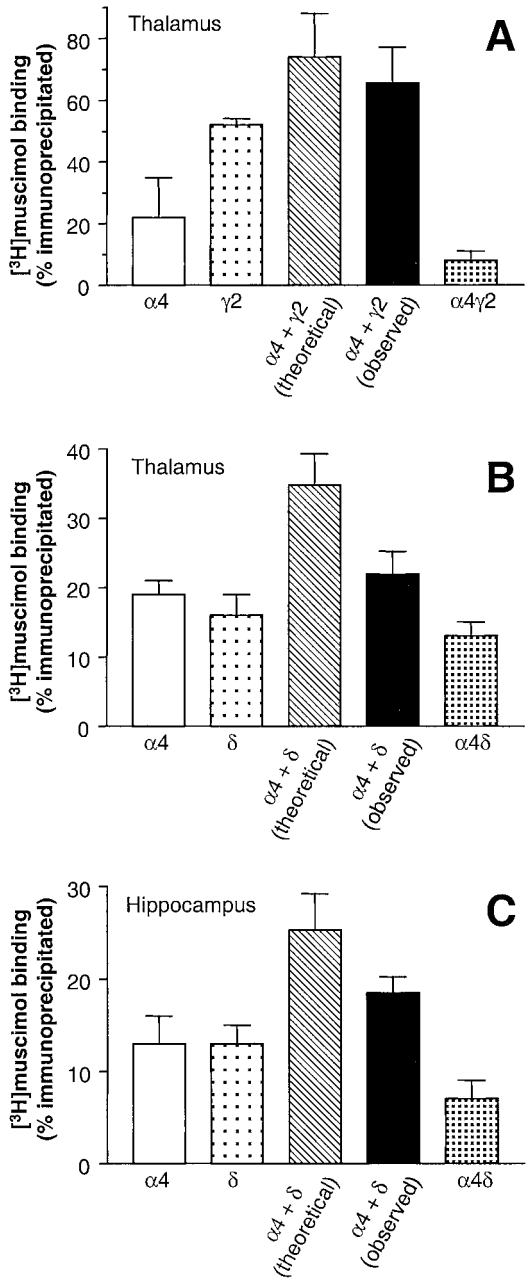


**Fig. 4.** Quantitative immunoprecipitation of [$^3$H]muscimol binding sites from rat thalamus by α4, γ2 (A), and α4, δ (B) antisera and from rat hippocampus (C) by α4, δ. A, $GABA_A$ receptors were solubilized from thalamic membranes and incubated overnight with protein A beads previously incubated with specific antibodies alone or in combination. Data (mean ± S.E.M.; $n$ = 2) showed that α4- and γ2-containing receptors account for 22 ± 13 and 52 ± 3% of the total population, respectively. The theoretical value (α4 + γ2) was higher than the amount of receptors coprecipitated by both α4 and γ2 antibodies. The difference between theoretical and observed values (rightmost column) indicates that 8 ± 3% of total [$^3$H]muscimol binding sites in thalamus originate from α4γ2 receptor subtype. B, similar experiments performed with α4 and δ antibodies indicate that α4, δ, and α4δ subunit-containing receptors represent 19 ± 2%, 16 ± 3%, and 13 ± 2% (mean ± S.E.M.; $n$ = 4) of the total thalamic population, respectively. They also revealed that in rat thalamus all δ subunits coassemble with an α4 subunit. C, quantitative immunoprecipitation from rat hippocampus indicates the presence of α4 and δ subunit-containing receptors that account for 13 ± 3% and 13 ± 2% of total [$^3$H]muscimol binding sites, respectively, as well as a small population (7 ± 2%; rightmost column) of α4δ receptors. Data are the mean ± S.E.M. of three determinations.

receptors account for one-fifth of total thalamic $GABA_A$ receptors, a proportion similar to the 27% reported by Khan and colleagues (1996) using another specific $\alpha4$ antibody. The difference in the amount of $\alpha4$-containing receptors and both $\alpha4\beta\gamma2$ subtype and DIS [$^3$H]Ro15–4513 sites suggested that the $\alpha4$ subunit could be present in another subunit assembly that does not contain a $\gamma$ subunit and [$^3$H]Ro15–4513 binding site. This observation prompted us to investigate the putative coassembly of $\alpha4$ with $\delta$ subunit. Indeed, the thalamus has been shown to be a high $\delta$ subunit-expressing area by both in situ hybridization and immunocytochemistry (Wisden et al., 1992; Fritschy and Mohler, 1995). Coimmunoprecipitation with $\alpha4$ and $\delta$ antisera demonstrated the existence of $\alpha4\beta\delta$ subtype in rat thalamus that accounts for all $\delta$ subunit-containing receptors and around 60 to 70% of the $\alpha4$ population. Concomitantly, the sum of $\alpha4\beta\gamma2$ (8%) and $\alpha4\beta\delta$ (13%) populations measured using [$^3$H]muscimol is roughly equivalent to the total $\alpha4$ population (22%). The $\alpha4\beta\delta$ subtype receptor is also present in rat hippocampus but in contrast to thalamus it accounts for only half of both $\alpha4$ and $\delta$ receptor populations, suggesting the existence of other $\alpha4$ subunit- and $\delta$ subunit-containing $GABA_A$ receptor subtypes. Indeed, Benke and coworkers (1997) have reported the presence of $\alpha4\beta\gamma2$ receptors in rat hippocampus. Future experiments should reveal which $\alpha$ subunit besides $\alpha4$ coassembles with the $\delta$ subunit in hippocampus as well as which $\beta$ subunit is present in thalamic and hippocampal $\alpha4\beta\delta$ receptors.

It should be noted that these binding and immunoprecipitation experiments were performed with membranes that probably contain both surface and intracellularly (i.e., endoplasmic reticulum) located receptors. One cannot exclude the possibility that $\alpha4\beta\gamma2$ isoform represents intracellular, nonfunctional receptors. However, given that this subtype can be expressed in vitro (Wafford et al., 1996; Knoflach et al., 1996) it is probable that at least some of these receptors are localized to the cell surface. Furthermore, the $\alpha4\beta\delta$ subtype is presumed to be functional because $\delta$ subunit knockout mice show epileptic seizures (Olsen et al., 1997), a phenotype probably resulting from the lack of $\alpha4\beta\delta$ receptors in thalamus and/or hippocampus because $\alpha6\beta\delta$ isoform knockout mice have no seizures (Jones et al., 1997). Recent studies on the assembly of $GABA_A$ receptors conclude that the $\gamma$ subunit is the last to be included in the receptor complex, yet it is needed for correct clustering of $GABA_A$ receptors (Gunther et al., 1995; Essrich et al., 1998). Because the $\delta$ subunit substitutes for a $\gamma$ subunit (Quirk et al., 1995) it is more likely that $\alpha$ and $\beta$ subunits are first assembled in the endoplasmic reticulum and then are joined by a $\gamma$ or $\delta$ subunit. If $\alpha4\beta$ dimers expressed in endoplasmic reticulum contribute significantly to total immunoprecipitated [$^3$H]muscimol binding, then the proportion of $\alpha4\beta\delta$ receptors on the surface may be underestimated by this technique.

Receptors that contain the $\alpha6$ subunit in combination with the $\delta$ subunit do not bind benzodiazepine ligands with high affinity (Quirk et al., 1994). Given the qualitatively similar benzodiazepine pharmacology of $\alpha4$- and $\alpha6$-containing $GABA_A$ receptors (Knoflach et al., 1996), it is anticipated that benzodiazepine site ligands will have low affinity for $\alpha4\beta\delta$ receptors. However, this subtype probably has some unique pharmacological properties conferred by the combination of both $\alpha4$ and $\delta$ subunits. Electrophysiological recordings have demonstrated that $\alpha4$ subunit-containing receptors display a higher GABA sensitivity than other $\alpha$ subunit-containing receptors (Knoflach et al., 1996). This effect may even be exacerbated by the presence of a $\delta$ subunit because $\alpha6\beta3\delta$ receptors are more sensitive to GABA ($EC_{50}$ of 0.4 $\mu$M) than those containing a $\gamma2$ subunit ($EC_{50}$ of 2 $\mu$M; Saxena and MacDonald, 1996). In addition to a putative relatively high sensitivity for GABA, $\alpha4\beta\delta$ subtypes might be particularly sensitive to modulation by $Zn^{2+}$. Thus, $\alpha4\beta2\gamma2$ receptors are sensitive to $Zn^{2+}$ despite the presence of a $\gamma2$ subunit (Knoflach et al., 1996) and the presence of a $\delta$ subunit has been shown to increase $Zn^{2+}$ sensitivity to $\alpha1$- or $\alpha6$-containing receptors (Saxena and MacDonald, 1994, 1996). Furthermore, $\delta$-containing receptors exhibit currents of low amplitude, but with a slow rate of desensitization even in the presence of GABA (Saxena and MacDonald, 1994), suggesting that they might be involved in the generation of long-lasting inhibitory postsynaptic potentials and consequently in tonic neuronal inhibition. Such a proposal has received a recent morphological support as Nusser and coworkers (1998) have clearly shown that in rat cerebellum all $\delta$-containing receptors are located at extrasynaptic sites.

Recent reports (Schwarzer et al., 1997; Brooks-Kayal et al., 1998) have shown an up-regulation of both $\alpha4$ and $\delta$ subunit immunoreactivities and mRNA levels in dentate gyrus neurons after chemically induced temporal lobe epilepsy in rat. It is tempting to speculate that an overexpression of $\alpha4\beta\delta$ receptor subtype with its putative long-lasting inhibitory potential and wide nonsynaptic membrane localization (see above) may represent an adaptive change to compensate neuronal hyperexcitability. Future studies are needed to clarify these issues and to establish the involvement of $\alpha4\beta\delta$ receptors in animal seizure models.

In conclusion, our results show that a heterogeneous complement of $GABA_A$ receptors is expressed in rat thalamus and provides evidence for the existence of $\alpha4\beta\delta$ subtype. Although this receptor subtype accounts for 13% of total $GABA_A$ receptors, its pharmacological and anatomical features may confer it a unique role in monitoring both normal and hyperactive neuronal networks.

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