Molecular and Pharmacological Characterization of Native Cortical $\gamma$-Aminobutyric Acid$_A$ Receptors Containing Both $\alpha_1$ and $\alpha_3$ Subunits*

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We have investigated the existence, molecular composition, and benzodiazepine binding properties of native cortical $\alpha_1$-$\alpha_3$ $\gamma$-aminobutyric acid$_A$ (GABA$_A$) receptors using subunit-specific antibodies.

The co-existence of $\alpha_1$ and $\alpha_3$ subunits in native GABA$_A$ receptors was demonstrated by immunoblot analysis of the anti-$\alpha_1$ or anti-$\alpha_3$-immunopurified receptors and by immunoprecipitation experiments of the $[3H]$zolpidem binding activity. Furthermore, immunodepletion experiments indicated that the $\alpha_1$-$\alpha_3$ GABA$_A$ receptors represented 54.7 ± 5.0 and 23.6 ± 3.3% of the $\alpha_2$ and $\alpha_1$ populations, respectively. Therefore, $\alpha_1$ and $\alpha_3$ subunits are associated in the same native GABA$_A$ receptor complex, but, on the other hand, these $\alpha_1$-$\alpha_3$ GABA$_A$ receptors from the cortex constitute a large proportion of the total $\alpha_3$ population and a relatively minor component of the $\alpha_1$ population.

The pharmacological analysis of the $\alpha_1$- or $\alpha_2$-immunopurified receptors demonstrated the presence of two different benzodiazepine binding sites in each receptor population with high (type I binding sites) and low (type II binding sites) affinities for zolpidem and CI 218,872. These results indicate the existence of native GABA$_A$ receptors possessing both $\alpha_1$ and $\alpha_3$ subunits, with $\alpha_1$ and $\alpha_3$ subunits expressing their characteristic benzodiazepine pharmacology.

The molecular characterization of the anti-$\alpha_1$-anti-$\alpha_3$ double-immunopurified receptors demonstrated the presence of stoichiometric amounts of $\alpha_1$ and $\alpha_2$ subunits, associated with $\beta_2\alpha_2$ and $\gamma_2$ subunits. The pharmacological analysis of $\alpha_1$-$\alpha_2$ GABA$_A$ receptors demonstrated that, despite the fact that each $\alpha$ subunit retained its benzodiazepine binding properties, the relative proportion between type I and II binding sites was lower than 51- and 59–61-kDa $[3H]$Ro15-4513-photolabeled peptides was 70:30. Therefore, the $\alpha_1$ subunit is pharmacologically predominant over the $\alpha_3$ subunit. These results indicate the existence of active and nonactive $\alpha$ subunits in the native $\alpha_1$-$\alpha_3$ GABA$_A$ receptors from rat cortex.

The neuropharmacological effects of benzodiazepines are mediated by the benzodiazepine ($\omega$) binding sites associated with the GABA$_A^1$ receptor complex (for reviews, see Refs. 1 and 2). Based on their affinity for different drugs, two different benzodiazepine binding sites have been identified in the central nervous system. Type I (benzodiazepine receptor 1, $\omega_1$) displays high affinity for CI 218,872 (2), $\beta$-carboline derivatives (3), and the imidazopyridine zolpidem (4, 5). Type II (benzodiazepine receptor 2, $\omega_2$) displays low affinity for these compounds. A third benzodiazepine binding site with very low affinity for zolpidem (type $\omega_3$) has also been identified in isolated rat brain membranes (6) and sections (7).

Molecular cloning experiments have demonstrated the existence of five different families of subunits that are components of the GABA$_A$ receptor complex. Most of these families comprise several isoforms: $\alpha_1$-$\alpha_6$, $\beta_1$-$\beta_3$, $\gamma_1$-$\gamma_3$, $\delta$, and $\rho_1$ and $\rho_2$ (for reviews, see Refs. 8 and 9). A minimum of $\alpha$, $\beta$, and $\gamma$ subunits should be co-expressed in transfected cells to resemble all the pharmacological properties of native GABA$_A$ receptors (10). On the other hand, the presence of different $\alpha$ subunits determines the affinity of the different benzodiazepine binding sites. In this sense, the $\alpha_1$-$\beta_1$-$\beta_2$-$\gamma_2$ combination confers type I pharmacology to the recombinant GABA$_A$ receptor (i.e. high affinity for, among others, zolpidem and CI 218,872) (11). Type II properties are conferred by the presence of $\alpha_2$, $\alpha_3$, or $\alpha_6$ subunits (11, 12).

Several approaches have been taken to identify which subunits coexist in the native GABA$_A$ receptor complex. However, the subunit composition of the different native GABA$_A$ receptor complexes remains unsolved. Immunoprecipitations or immunoffinity purifications using anti-$\alpha$ subunit antibodies (anti-$\alpha_1$, -$\alpha_2$, -$\alpha_3$, -$\alpha_5$, and -$\alpha_6$ subunits) indicated that a significant proportion of native receptors are made by the association of two different $\alpha$ subunits (such as $\alpha_1\alpha_2$, $\alpha_1\alpha_3$, $\alpha_1\alpha_5$, or $\alpha_1\alpha_6$) (13–17) in a single receptor complex. However, other authors have indicated the absence of association between different $\alpha$ subunits (18, 19). On the other hand, the pharmacological properties of these GABA$_A$ receptors are also unknown.

In the present article we have addressed these questions by determining the molecular and pharmacological properties of the immunopurified receptors using subunit-specific antibodies to the major $\alpha$ subunits expressed in the rat cerebral cortex, the $\alpha_1$ and $\alpha_3$ subunits.

EXPERIMENTAL PROCEDURES

Materials—$[3H]$Zolpidem (58.0 Ci/mmol), $[^3H]$flumazenil (75.2 Ci/mmol), $[^3H]$Ro15-4513 (24.1 Ci/mmol), and $[^3H]$flunitrazepam (84.0 Ci/mmol).

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† The abbreviations used are: GABA$_A$, $\gamma$-aminobutyric acid$_A$; PBS, phosphate-buffered saline; FMZ, flumazenil; FNZ, flunitrazepam; mAb, monoclonal antibody; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid.
mmol) were from DuPont NEN. Zolpidem was synthesized in the preclinical research department of Synthélabo Recherche. CI 218,872 was from Cynamid. All other benzodiazepines were from Hoffmann-La Roche.

**Antibody Preparation**—Peptides NH2-α3 (amino acids 1–10, pyroglutamyl-GESRQRGQP) and COO-α3 (amino acids 419–428, PQLKAPT-PHQ) were synthesized and coupled to keyhole limpet hemocyanin, via an extra tyrosine located at the COOH or NH2 terminus of α3 or α1 peptides, by Neosystem SA (Strasbourg, France). For immunizations, rabbits (New Zealand White) were subcutaneously injected with 200 μg of coupled peptide emulsified (1:2) in Freund’s complete adjuvant followed 20 days later by a booster injection of conjugate with incomplete adjuvant (1:1). Rabbits were then boosted every 2–3 weeks. The animals were bled 10 days after each booster injection. Development of an immune response was followed by immunoprecipitation of the solubilized receptor.

The antibodies were purified through peptide affinity columns. The α3 and α1 peptides were coupled to adipic acid dihydrazide-agarose (sigma) or CNBr-activated Sepharose 4B (Pharmacia Biotech), respectively, as recommended by the manufacturer. Two ml of anti-α3 or anti-α1 antisera (diluted 1/5 in PBS) were recirculated, overnight at 4°C (fly), 1–2 mg of each purified antibody were absorbed to 0.5 ml of immunoprecipitation. On the other hand, preimmune sera was also washed three times with 1.4 ml of solubilization buffer, and used for IgG-protein A-Sepharose complexes were isolated by centrifugation, using peptides from 2–10 or 1–15 amino acids of the NH2-terminus of protein A-Sepharose in a final volume of 300 μl. After washing with peptide at 150 μl of PBS, the antibodies were eluted with 3 ml of 140 mM NaCl, 1 mM EDTA, 1 mM EGTA, and the fractions (0.5 ml) were neutralized by 1 M Tris, pooled and dialyzed in 1 liter of PBS (overnight at 4°C).

Other antibodies used in this work were the mAb 63-3G1 and anti-γ2 and γ1 antibodies. These two polyclonal antibodies were produced using 5 or 10 or 1–15 amino acids of the NH2-terminus of the γ2 and γ1 subunits, respectively (to be published elsewhere).

For immunobLOTS, the purified antibodies were labeled with digoxigenin as recommended by the manufacturer (Boehringer Mannheim). The digoxigenin incorporated into anti-γ2 or anti-α3 antibodies was determined by enzyme-linked immunosorbent assay or dot blot. Both antibodies displayed a similar activity (not shown).

**Membrane Preparation and Receptor Solubilization**—Membranes from a 3-month-old Wistar rat cerebral cortex were prepared as described elsewhere (6, 20) in presence of protease inhibitors: 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM benzamidine, 50 μM/ml trypsin inhibitor type II-S, and 50 μg/ml bacitracin. The GABAA receptor was solubilized at 4 mg of protein/ml at 4°C for 60 min with 0.5% (v/v) sodium deoxycholate, 0.5% (v/v) CHAPPS, 140 mM NaCl, and 10 mM Tris-HCl, pH 7.5 (solubilization buffer), containing the same protease inhibitors as above. After centrifugation at 100,000 × g for 60 min at 4°C, the supernatant was collected. The recovery of the benzodiazepine binding activity in the solubilized material was estimated to be 80–90% of the 5 nM [3H]FMZ, 10 nM [3H]NFNZ, or 5 nM [3H]zolpidem binding activity found in membranes (also see Ref. 21).

**Immunoprecipitation and Immunopurification**—For immunoprecipitation experiments, the different antisera were adsorbed to a suspension of protein A-Sepharose (10%, w/v, in solubilization buffer; also see Ref. 20). The GABAA receptor was solubilized at 4 mg of protein/ml at 4°C for 60 min with 0.5% (v/v) sodium deoxycholate, 0.5% (w/v) CHAPPS, 140 mM NaCl, and 10 mM Tris-HCl, pH 7.5 (solubilization buffer), containing the same protease inhibitors as above. After centrifugation at 100,000 × g for 60 min at 4°C, the supernatant was collected. The recovery of the benzodiazepine binding activity in the solubilized material was estimated to be 80–90% of the 5 nM [3H]FMZ, 10 nM [3H]NFNZ, or 5 nM [3H]zolpidem binding activity found in membranes (also see Ref. 21).

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Pharmacological Properties of α₁-α₃-containing GABAₐ Receptors

![Image](27904)

**FIG. 1. Specificity of anti-α₁ and anti-α₃ antisera.** A, different amounts of peptides from NH₂ termini (amino acids 1-10) of α₃, α₁, or α₅ subunits or COOH termini of α₁ (amino acids 419-428), α₃ (amino acids 459-465), or α₅ (amino acids 424-433) were blotted and immunostained with anti-α₁ (1/1000 dilution) and anti-α₃ (1/1000 dilution) antisera, respectively. B and C, solubilized receptor (0.1-0.2 pmol of [³H]FMZ binding activity) was immunoprecipitated with 0.5 or 7.5 µl of anti-α₁ or anti-α₃ antisera, respectively, in the presence of increasing concentrations of the peptides specified above. B, COOH-terminal peptides α₁ ( ), α₃ ( ), and α₅ (Δ); C, NH₂-terminal peptides α₁ ( ), α₃ ( ), and α₅ (Δ). No inhibition was observed when anti-α₁ or anti-α₃ antisera were incubated in presence of NH₂-terminal α₁ or COOH-terminal α₃ peptides, respectively (not shown). Results are expressed as percentages from the [³H]FMZ binding activity immunoprecipitated in absence of peptide and are mean ± S.D. (bars) of three independent experiments. D, GABAₐ receptors purified from adult bovine cerebral cortex (1 µg/lane) were subjected to SDS-polyacrylamide gel electrophoresis, blotted, and immunostained with anti-α₁ (1/500 dilution) or anti-α₃ (1/1000 dilution). Both antibodies show reactivity with single peptide bands of 59 and 51 kDa for anti-α₃ and anti-α₁, respectively. E, cortical membranes (75 µg of protein/lane) were processed as above and immunostained with 10 µg/ml of purified anti-α₁ or anti-α₃ antibodies. The mAb 62-3G1 (1/5 dilution), specific for β₁ and β₃ subunits, was included as a reference. The molecular sizes of the subunits are 58 and 60 kDa (α₁), 51 kDa (α₃), and 55–57 kDa (β₁ and β₃).

band of 100 kDa was also observed in some experiments (also see Fig. 2B). The mAb 62-3G1 (specific to β₁ and β₃, M., 55,000–57,000 peptides; Refs. 27 and 31) was included as a control. 5) In the three brain regions studied (cortex, hippocampus, and cerebellum), the percentage of immunoprecipitation by these antibodies is consistent with the level of expression of α₁ or α₃ subunits, determined by in situ hybridization or immunoprecipitation (18, 19, 32–35). As expected, the anti-α₁ antisera immunoprecipitated most of the [³H]FMZ binding activity from the cerebellum (85.3 ± 7.5%), followed by the cortex (71.0 ± 5.3%) and hippocampus (52.2 ± 2.0%). Anti-α₃ antisera immunoprecipitated a low proportion of receptors compared with anti-α₁. The maximal immunoprecipitation was obtained in the cortex (25.8 ± 4.7%), followed by the hippocampus (19.1 ± 3.2%) and cerebellum (9.8 ± 3.5%). In conclusion, by all these criteria both antibodies are specific for their corresponding subunits.

**Association between α₁ and α₃ Subunits**—To determine the presence of α₁ subunits, co-assembled with α₃ subunits in the same receptor complex, we first quantified the [³H]zolpidem binding activity immunoprecipitated by anti-α₃ antiserum. [³H]Zolpidem binds with high affinity to α₃ subunit-containing GABAₐ receptors (type I benzodiazepine binding sites) (11, 12, 21, 22). Therefore, [³H]zolpidem binding activity was used as a marker of the presence of α₁ subunits in the immunoprecipitated receptor (also see Ref. 21). The quantitative immunoprecipitation of [³H]zolpidem binding was tested by two sequential incubations with anti-α₁ or anti-α₃ antibodies. The second incubation yielded 3.3 ± 2.8 and 3.8 ± 1.7% of immunoprecipitation for anti-α₁ and -α₃, respectively, indicating that the immunoprecipitation of the receptor was maximal. As shown in Fig. 2A, anti-α₁ and -α₃ antibodies immunoprecipitated 90.0 ± 5.4 and 26.9 ± 3.6% of the [³H]zolpidem binding activity, respectively. These results demonstrated that, in native GABAₐ receptors, the high affinity [³H]zolpidem (5 nM) binding sites (type I benzodiazepine binding sites) are largely associated with the presence of an α₁ subunit (also see Refs. 21 and 22) and, importantly, that these sites can be immunoprecipitated in association with α₃ subunits.

To ascertain the co-assembling of α₁ and α₃ subunits in the
same receptor complex, the solubilized GABA$\text{A}$ receptor was purified by anti-$\alpha_1$ or anti-$\alpha_3$ immunopurification columns. The results of the purification experiments are shown in Table I. Both immunopurification columns retained the GABA$\text{A}$ receptor complex. The percentage of binding immunoblot absorbed to the columns was similar to that determined by immunoprecipitation experiments. The anti-$\alpha_1$ immunopurification column was efficiently eluted at pH 11.5 (21.3 ± 3.1% of the solubilized receptors or 33.0 ± 1.0% of the immunopurified material). However, no significant benzodiazepine binding could be eluted from anti-$\alpha_3$ columns (0.7 ± 0.3%). Other treatments, such as low pH, did not improve the elution step (not shown). Therefore, to analyze the immunopurified receptor, both immunopurification columns were eluted with SDS, and the purified material was subjected to immunoblot analysis. As shown in Fig. 2B, the presence of $\alpha_3$ subunits (59- and 61-kDa peptides) could be detected in anti-$\alpha_1$-immunopurified receptors, and, on the other hand, the $\alpha_1$ immunoreactivity (51-kDa peptide) was identified in anti-$\alpha_3$-immunopurified receptors.

The co-purification of both $\alpha$ subunits was not due to cross-reaction between the antibodies. As shown in Fig. 2C (lane 2), the anti-$\alpha_1$ antibody produces absolutely no immunoreaction products in Western blots of the anti-$\alpha_3$-immunopurified receptors that have been immunodepleted of the $\alpha_1$ subunits. On the other hand, the anti-$\alpha_3$ antibody immunoreacted with 59–61-kDa peptides (Fig. 2C, lane 3), demonstrating the presence of $\alpha_3$-containing GABA$\text{A}$ receptors. Conversely, no immunoreaction products were produced by the anti-$\alpha_3$ antibody using the $\alpha_1$ immunodepleted and anti-$\alpha_1$-immunopurified receptor as an antigen (Fig. 2C, lanes 5 and 6). Furthermore, in membrane preparations of human embryonic kidney cells, transfected

**Table I**

**Immunopurification of $\alpha_1$- and $\alpha_3$-containing GABA$\text{A}$ receptors from rat cerebral cortex**

The solubilized GABA$\text{A}$ receptor was purified on 0.5-ml immunopurification columns, as described under “Experimental Procedures.” The $[^{3}H]$FMZ (5 nM) binding activity was determined in each fraction. The results are mean ± S.D. of at least three independent experiments.

| $[^{3}H]$FMZ binding | Anti-$\alpha_1$ column | Anti-$\alpha_3$ column |
|-----------------------|-----------------------|-----------------------|
| Solubilized receptor  | 30 ± 6                | 100                   |
| Bound                 | 19.3 ± 1.5             | 64.3 ± 1.5             |
| pH 11.5 elution       | 6.3 ± 0.9              | 21.3 ± 3.1             |
(3H)zolpidem binding activity immunoabsorbed to anti-α3 immunobeads (26.3 ± 1.5 versus 32.5 ± 5.4 and 31.2 ± 3.7%, n = 3, for control and demecolcine- or cytochalasin D-treated membranes, respectively). Furthermore, after treatment with either drug, two (3H)Ro15-4513 photoaffinity-labeled peptides of 51 and 59–61 kDa were immunopurified by anti-α3 columns (not shown). On the other hand, results similar to those shown in Fig. 2A were obtained using purified synaptic membranes as starting material (not shown).

The co-assembling of α1 and α3 in the same receptor complex also could be due to redistribution of subunits during solubilization. This possibility was tested by determining the immunoprecipitation by anti-α3 of the diazepam-insensitive (3H)Ro15-4513 binding sites in solubilized receptors from cerebellar membranes or from the mixture (1:1) of cerebellar plus cortical membranes. The diazepam-insensitive binding sites are associated with the presence of α6 subunits (38, 39), and this subunit is not expressed in the cortex (32, 33, 39). The immunoprecipitation of diazepam-insensitive (3H)Ro15-4513 binding activity by anti-α3 was very low and similar in both solubilized preparations, pure cerebellar membranes, and a mixture of cerebellal and cortical membranes (0.01 ± 0.01 and 0.01 ± 0.01 pmol, n = 2, respectively), thus indicating that no apparent subunit redistribution takes place due to solubilization procedures.

The association between both α subunits was quantified by immunodepletion experiments. In these experiments, a particular α subunit was depleted by two sequential immunoprecipitations with the specific antiserum. After depletion, the remaining GABA_A receptor complex was immunoprecipitated by the other α subunit. As shown in Table II, depletion of α1 subunits produced a significant decrease in the (3H)FMZ binding activity immunoprecipitated by anti-α3 antiserum (0.21 ± 0.04 versus 0.10 ± 0.01 pmol, respectively). Thus, 54.7 ± 5.0% of the benzodiazepine binding activity immunoprecipitated by anti-α3 was depleted by preincubation with the anti-α3 antiserum. On the other hand, most of the (3H)zolpidem immunoprecipitated by the anti-α3 antiserum was depleted by preincubation with the anti-α1 antibody (89.0 ± 7.8%; 0.05 ± 0.01 versus 0.005 ± 0.004 pmol). These results indicated that most, if not all, of the high affinity binding sites immunoprecipitated by the anti-α3 antiserum were due to the presence of an α1 subunit.

Reciprocally, depletion of α3 subunits also affected the immunoprecipitation by the anti-α1 antiserum. As shown in Table II, depletion of α3 subunits produced a decrease in the (3H)FMZ or (3H)zolpidem binding activity immunoprecipitated by anti-α1 (0.70 ± 0.05 or 0.24 ± 0.01 pmol versus 0.54 ± 0.03 or 0.17 ± 0.01 pmol for (3H)FMZ and (3H)zolpidem, respectively). Thus, 20–25% of the α1 population is associated with an α3 subunit in the same receptor complex.
The solubilized receptor was immunosorbed to anti-α3 or anti-α1 antibodies. For saturation or displacement experiments, aliquots of the anti-α1 or anti-α3 immunobeads (0.4–0.6 pmol of [3H]FNZ binding activity/tube) were used. Saturation experiments were done by incubating the immunobeads with five or six different concentrations of [3H]FNZ (1–20 nM) or [3H]zolpidem (1–10 nM). The Scatchard transformation of the data was performed by LIGAND. Displacement experiments were performed by determining the binding activity of 2 nM [3H]FNZ and 13 or 10 different concentrations of zolpidem (ranging from 5 × 10^{-6}–10^{-4} M) or CL 218,872 (ranging from 5 × 10^{-6}–10^{-4} M), respectively. Displacement curves were fit (LIGAND) to a one or two binding site model. Results, mean ± S.D. of three experiments, are expressed in nM.

### Table III

| Immunoaffinity column | Ligand          | nH | One binding site, Kd or Kr | Two binding sites | P(25/15) |
|-----------------------|-----------------|----|----------------------------|-------------------|---------|
| Anti-α3               | [3H]FNZ         | 0.94 ± 0.02 | 3.5 ± 2.0                  |                   |         |
|                       | [3H]Zolpidem    | 0.96 ± 0.03 | 14.5 ± 2.3                 |                   |         |
|                       | Zolpidem        | 0.57 ± 0.05 | 204 ± 68                   | 8.0 ± 7.1         | 392 ± 100 | 0.02   |
|                       | Cl 218,872      | 0.55 ± 0.02 | 1,525 ± 220                | 67.5 ± 9.0        | 3,460 ± 900 | 0.03   |
| Anti-α1               | [3H]FNZ         | 0.94 ± 0.02 | 6.2 ± 1.9                  |                   |         |
|                       | [3H]Zolpidem    | 0.99 ± 0.01 | 13.3 ± 2.8                 |                   |         |
|                       | Zolpidem        | 0.60 ± 0.03 | 88 ± 20                    | 5.0 ± 4.9         | 424 ± 71  | 0.01   |
|                       | Cl 218,872      | 0.58 ± 0.03 | 310 ± 84                   | 39.6 ± 2.4        | 1,840 ± 360 | 0.03   |

**Fig. 3.** Binding activity and molecular composition of the α1-α3 GABA<sub>A</sub> receptor from rat cortex. A, the α1-α3-immunopurified GABA<sub>A</sub> receptors were immunosorbed to anti-α1 (I) or anti-α3 (II) immunobeads, and the [3H]FMZ (5 nM) or [3H]zolpidem (5 nM) binding activity was quantified in pellets and supernatants. The results, expressed as percentages of binding activity retained by the columns, are mean ± S.D. (bars) of three experiments. B, the GABA<sub>A</sub> receptors were immunopurified by anti-α1 and anti-α3 columns in series. The SDS-eluted material was blotted and incubated with 5 μg of purified anti-α1 (lane 1) or anti-α3 (lane 2) antibodies, a 1/5 dilution of the mAb 62-3G1 (specific for β3 and β2/3 subunits; lane 3), or 5 μg of purified anti-γ1 antibody (lane 4). Numbers on the left, Mr values of the immunostained bands.

material was absorbed to anti-α3 immunoaffinity columns. As was mentioned before (see Table I), no significant elution from the anti-α3 immunoaffinity columns could be achieved. Therefore, the GABA<sub>A</sub> receptor retained by the anti-α3 columns was analyzed by binding assays using aliquots of the anti-α3 immunobeads (see Ref. 23) or by immunoblots of the SDS-eluted receptors.

We first quantified the presence of α3 subunits in the anti-α1-immunopurified receptors by testing the percentage of [3H]FMZ and [3H]zolpidem binding activity immunosorbed to anti-α3 columns. Anti-α1 immunobeads were used as control. As shown in Fig. 3A, the second anti-α1 columns immunosorbed 90–95% of the previously anti-α1-immunopurified receptors, whereas anti-α3 columns immunosorbed 20–25% of either [3H]FMZ and [3H]zolpidem binding sites previously immunopurified by anti-α1 columns. This indicated that 20–25% of the α1 population also contained an α3 subunit. These values agree with those calculated by depletion experiments (see Table II).

The presence of both α subunits, together with β2/3 and γ2 subunits, in the α1- and α3-immunopurified receptors was demonstrated by immunoblots. As shown in Fig. 3B, after sequential anti-α1 and anti-α3 immunopurifications, the anti-α1 antibody immunoreacted with a single 51-kDa band, whereas the anti-α3 antibody immunostained a doublet of 59 and 61 kDa. The mAb 62-3G1 and anti-γ1 antibodies immunostained peptides of 55–57 and 47–49 kDa, respectively. The presence of γ2 was not detected (not shown). These results demonstrated that both α1 and α3 subunits, β2/3 and γ2 are co-assembled in the same GABA<sub>A</sub> receptor complex.

The stoichiometry between both α subunits was estimated by densitometric analysis of semiquantitative immunoblots (Fig. 4; also see Ref. 16). For these experiments, a fixed amount of receptor was immunoblotted and incubated with increasing concentrations of both antibodies in combination. After 4 h of incubation, the medium was aspirated and replaced by a new batch of antibodies. The immunoreaction products were quantified by densitometry. As shown in Fig. 4, A and B, at saturating concentrations, both antibodies yielded similar amounts of immunoreaction products. Thus, these results indicated a stoichiometry of approximately 1:1 (α3/α1 ratio, 1.1 ± 0.1, n = 2; Fig. 4B).

Finally, we have tested the pharmacological properties of the α1- and α3-immunopurified GABA<sub>A</sub> receptors by displacement experiments with Cl 218,872 or zolpidem and also by [3H]Ro15-4513 photoaffinity labeling experiments of the double-immunopurified receptor. The results are shown in Table IV and Fig. 5. The displacement experiments of both [3H]FNZ or [3H]FMZ (not shown) binding activity by both zolpidem or Cl 218,872 demonstrated the presence of two different binding sites with high (type I) and low (type II) affinities. The proportion between both binding sites, calculated from displacement ex-
Currently accepted that the benzodiazepine binding properties are co-assembled in a single native GABA A receptor complex. How-
α₁-containing receptors and, reciprocally, the presence of α₁ in anti-α₃-immunopurified receptors. Furthermore, the association between both α subunits was not due to interactions with cytoskeletal elements. Taken together, these results demonstrated the existence of α₁-α₃ GABA_Δ receptors from the rat cortex. Immunodepletion experiments indicated that the α₁-α₃ GABA_Δ receptors constituted a relatively minor proportion of the total α₁-containing GABA_Δ receptors (20–25% of this population) but 50–55% of the α₃ containing GABA_Δ receptors. Thus, in partial agreement with previous reports (13–15), the association between two different α subunits represents a minor population from the total α₁-containing receptors but a high proportion of other α subunits, such as α₃.

The presence of different α subtypes, in combination with β₁–β₃ and γ₂ subunits, determines the benzodiazepine binding properties of recombinant GABA_Δ receptors (10, 11, 12). As mentioned above, the α₁ subunit confers type I benzodiazepine binding properties (high affinity for zolpidem and Cl 218,872), whereas the α₃ subunit confers type II binding properties (low affinity for these ligands). Therefore, if two different α subunits, such as α₁ and α₃, are co-assembled in the same receptor complex, and both α₁ and α₃ subunits are pharmacologically active, two different benzodiazepine binding subtypes should be discriminated in either anti-α₁- and anti-α₃-immunopurified receptors. As shown in Table III, in anti-α₁- and anti-α₃-immunopurified receptors, two different binding sites were identified. The affinities for zolpidem (determined by Scatchard and displacement experiments) or Cl 218,872 (determined by displacement experiments) were similar in both immunopurified receptors and similar to those reported for type I and II benzodiazepine binding sites in cortical membranes (6, 7). Furthermore, the affinities for both ligands corresponded to those reported for recombinant receptors containing α₁ subunits (high affinity binding sites) and α₃ subunits (low affinity binding sites) (11, 12). In consequence, these results suggest the presence of benzodiazepine binding sites in both α₁ and α₃ subunits co-assembled in a single GABA_Δ receptor complex (also see Ref. 17).

To discern whether both α₁ and α₃ subunits, co-assembled in a single complex, display benzodiazepine binding activity, the GABA_Δ receptor was immunopurified by anti-α₁ and anti-α₃ affinity columns in series; therefore, the whole population of the isolated GABA_Δ receptors should contain two different α subunits. It is noteworthy that anti-α₃ immunopurification columns retained 20–25% of the α₁-immunopurified GABA_Δ receptors, corroborating the proportion of α₁ to α₃ GABA_Δ receptors calculated by depletion experiments (compare Fig. 3A and Table II). Immunoblot analysis (Fig. 3B) indicates that α₁ and α₃ subunits are mainly associated with β₂/₃ and γ₂ in the same receptor complex, consistent with previous experiments (21, 22). The β₃ subunits are a relatively minor component of the receptor (41), and, on the other hand, it has been demonstrated that γ₁ is not associated with γ₂-containing GABA_Δ receptors (42). Thus, we propose a molecular composition of α₁, α₃, β₂/₃, and γ₂ for these native GABA_Δ receptor complexes from rat cortex.

A relevant question to ascertain the pharmacological activity of the α subunits, co-assembled in a single native GABA_Δ receptor, is the stoichiometry between both subunits in the complex. Thus, we have estimated the stoichiometry between both α subunits by quantifying the immunoreaction products of anti-α₁ and anti-α₃ antibodies in immunoblots. We are aware that immunoblots are only semiquantitative. However, within the limitations of the technique, the results (Fig. 4) indicated the presence of stoichiometric amounts of each α subunit (ratio 1:1; also see Ref. 16 for discussion). The stoichiometry of γ₂, β₂, and β₃ subunits was not determined.

If both α subunits display benzodiazepine binding sites, the double-immunopurified receptors should display type I and II binding properties in similar proportions, and two peptides should be photoaffinity labeled by [³H]Ro 15-4513 to a similar extent. Indeed, the pharmacological analysis of the α₁-α₃ GABA_Δ receptors indicated the presence of two different benzodiazepine binding sites. Both Cl 218,872 and zolpidem discriminated between two different binding sites with high (type I) and low affinities (type II). The calculated Kᵢ values for either ligand were similar to those of immunopurified α₁ or α₃ receptors (compare Tables III and IV) and to cerebral membranes (6, 7). However, the proportion between both binding sites (70:30 for high and low affinity, respectively) demonstrates that the α₁ subunits are predominantly active over the α₃ subunits. It could be argued that the different proportions between both binding sites, determined by displacement experiments, is due to differences in the Kᵢ values of α₁ and α₃ subunits for the
Benzodiazepine binding sites of the α₁-α3 GABA<sub>A</sub> receptors from rat cortex

![Diagram of benzodiazepine binding sites](image)

**Fig. 6. Model of the α₁-α3 GABA<sub>A</sub> receptor and the benzodiazepine binding sites associated with the different α subunits.**

3H-labeled ligand ([3H]FNZ or [3H]FMZ). However, these results were confirmed by [3H]Ro15-4513 photoaffinity-labeling experiments at three different degrees of saturation. As expected, in the double-immunopurified two subunits, two photolabeled peptides of 51 kDa (corresponding to α₁ subunits) and 59–61 kDa (α₂ subunits) were identified. However, despite the fact that both α subunits are assembled in stoichiometric amounts in the same receptor complex, the proportion between both photolabeled peptides (at all three concentrations) was 70:30 for 51 and 59–61 kDa, respectively (Fig. 5). Thus, α₁ subunits are pharmacologically predominant over the α₂ subunits. It should be noted that [3H]Ro15-4513 photolabeled subunits could also co-exist in the same receptor complex (46, 51). Therefore, the presence and pharmacological activity of two different α subunit subtypes in native receptor complexes, localized in discrete brain areas and/or cellular regions, could influence the functional and pharmacological properties of the GABA<sub>A</sub> receptor. The existence and pharmacological properties of α₁α₂-containing receptors increase the heterogeneity of the native GABA<sub>A</sub> receptor complex in the central nervous system.

In summary, our results demonstrate the existence of cortical GABA<sub>A</sub> receptors containing both α₁ and α₂ subunits in stoichiometric amounts. Furthermore, both α subunits retain their benzodiazepine binding properties. However, the α₁ subunit is pharmacologically predominant over α₂ subunits, indicating the existence of active and nonactive benzodiazepine binding sites associated with these α subunits.

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