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A Subtype of Nicotinic Cholinergic Receptor in Rat Brain Is Composed of $\alpha 4$ and $\beta 2$ Subunits and Is Up-regulated by Chronic Nicotine Treatment

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SUMMARY

The subunit composition and pharmacological regulation of rat neuronal nicotinic cholinergic receptors were assessed. Specific immunoprecipitation was determined in solubilized rat brain homogenates using [^{3}H]cytisine, a high affinity agonist at nicotinic receptors, in conjunction with polyclonal antisera generated against nonhomologous domains of the various subunits comprising this receptor class. In all brain regions tested, only antisera generated against the $\alpha 4$ and $\beta 2$ subunits were able to immunoprecipitate specifically receptors labeled by [^{3}H]cytisine. Thus, these sera were further characterized in order to validate and optimize their use in the immunoprecipitation protocol. Preincubation of solubilized receptors from rat forebrain with antisera generated against the $\alpha 2$, $\alpha 3$, $\alpha 5$, $\beta 3$, or $\beta 4$ subunits did not decrease the amount of precipitable $\alpha 4$ or $\beta 2$ subunit. On the other hand, when either anti-$\alpha 4$ or anti-$\beta 2$ serum was used to immunoprecipitate solubilized receptors from rat forebrain, the supernatants contained little if any remaining receptors that could be specifically precipitated by either antibody. Because these antisera do not cross-react, the data indicate that $\alpha 4$ and $\beta 2$ subunits are associated with each other in at least one neuronal nicotinic receptor subtype that has high affinity for agonists. Moreover, these results imply that all $\alpha 4$ subunits that are labeled by [^{3}H]cytisine are coupled to $\beta 2$ subunits. We also present evidence that the $\alpha 4/\beta 2$ subtype characterized in this report is significantly increased in the cortex of rats chronically treated with nicotine.

Compared with the detailed information available regarding the structure, function, and regulation of nicotinic cholinergic receptors in muscle and electric tissue (for review, see Ref. 1), much less is known about neuronal nicotinic receptors found in ganglia and in the CNS. Low stringency hybridization of rat brain cDNA libraries with probes generated from the cloned muscle $\alpha 1$ subunit has led to the cloning of at least four α ($\alpha 2$–5) and three β ($\beta 2$–4) neuronal-type subunits, as well as the determination of the CNS distribution of their respective transcripts (2–9). Moreover, pairwise injection into *Xenopus* oocytes of the mRNA encoding either the $\alpha 2$, $\alpha 3$, or $\alpha 4$ subunit in combination with that encoding either the $\beta 2$ or $\beta 4$ subunit leads to the formation of functional receptors responsive to both nicotine and acetylcholine (5, 8–10).

Like the muscle receptor, the agonist recognition site of the neuronal receptors is located on the α subunit, as defined by paired cysteine residues at or near positions 192 and 193. However, in contrast to the muscle receptor, which is assembled from four different protein subunits arranged as the pentamer $\alpha_2\beta\gamma\delta$ (or ϵ), available evidence suggests that the neuronal receptor is composed of α and β subunits only (11). But, neither the specific subunit composition nor the overall subunit stoichiometry of these receptors in mammalian brain is presently known.

Chronic treatment with nicotine leads to increased nicotinic receptor binding sites in brains of rats and mice (12–15). However, it has not been determined which subtypes, as defined by their subunit composition, are subject to such up-regulation.

Recently, two tools have become available that have helped to address these questions directly. The first is [^{3}H]cytisine, an agonist radioligand at neuronal nicotinic receptors that exhibits high affinity ($K_d \approx 1$ nM), a slow dissociation rate, and low nonspecific binding (16); the second is a panel of polyclonal antisera that were generated against nonhomologous regions of the cloned neuronal subunits. Thus, the purpose of the present studies was to use these two tools in an immunoprecipitation protocol that would directly evaluate the subunit composition and regional distribution of receptors with high affinity for nicotinic agonists in rat brain. Additionally, these experiments

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ABBREVIATIONS: CNS, central nervous system; NRS, normal rabbit serum.

sought to determine which subtypes are up-regulated by repeated injections of nicotine.

Materials and Methods

Drugs and reagents. Nicotine bitartrate dihydrate (ICN Pharmaceuticals, Plainview, NY) was dissolved in isotonic saline for injection or in assay buffer to define nonspecific binding (see below). PANSORBIN cells (Calbiochem, San Diego, CA) were stripped (17) and prepared as a 10% suspension in Tris-buffered saline. [^{3}H]Cytisine (42 Ci/mmol) was synthesized by Dr. Ernest Do (DuPont-New England Nuclear Products, Boston, MA) and was determined to be >98% pure by high performance liquid chromatography. Triton X-100 (Sigma Chemical Co., St. Louis, MO) was used at a final concentration of 2%. Sodium deoxycholate (Sigma) was made as a 3% solution in 0.1 N NaOH (Fisher Scientific, Fair Lawn, NJ). Molecular weight markers were obtained from Bio-Rad (Richmond, CA).

Antibody production. cDNA clones encoding subunits of neuronal nicotinic acetylcholine receptor subunits were used for constructs. Portions of the cDNA encoding a region from the putative cytoplasmic domain to the carboxyl terminus of subunits α4–1 (amino acids 460–594) (3) and β2 (amino acids 394–503) (4) were removed by restriction digest and subcloned into the appropriate pATH vector (*trp*E bacterial overproduction system) (18). The host strain was *Escherichia coli* strain HB101.

Overexpression of fusion proteins was achieved essentially as described by Dieckmann and Tzagoloff (18). The bacterial colonies containing recombinant pATH plasmids were grown overnight in M9 salts, 1% casamino acids, 100 μg/ml ampicillin, 10 μg/ml thiamine, 20 μg/ml tryptophan (stock dissolved in dimethyl sulfoxide), at 37°. These cultures were diluted 1/50 in the aforementioned medium minus tryptophan and were allowed to continue for approximately 2 hr at 37° before addition of freshly prepared 3-β-indoleacrylic acid (100× stock solution in absolute ethanol) to a final concentration of 50 μg/ml. Cultures were then continued for 3 hr. Cells were harvested by centrifugation and lysed for 2 hr by addition to one fifth the original culture volume of ice-cold lysis buffer consisting of 50 mM Tris (pH 7.5), 5 mM EDTA, and 3 mg/ml lysozyme. For every 20 ml of this mixture, 1.4 ml of 5 M NaCl and 1.5 ml of 10% Nonidet P-40 were added, and the solution was left to stand on ice for at least 30 min. DNA was sheared by sonication, the insoluble fraction was collected by centrifugation, and the pellet was washed twice with 20 ml of 10 mM Tris, 1 mM EDTA (pH 7.5). Overproduced protein typically represented >80% of the protein recovered. Overproduced proteins were fractionated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Protein bands were visualized by soaking the gel in ice-cold 0.25 M KCl for 1 hr and were then cut from the gel. The gel fragments were macerated, emulsified in complete Freund's adjuvant, and injected subcutaneously into young New Zealand white rabbits. Each rabbit received 200–400 μg of protein. Three weeks later, rabbits were boosted with 200–400 μg of antigen as described above, except that emulsification was performed in incomplete Freund's adjuvant. Serum was collected 12 days later. Subsequent boosts were administered at 1-month intervals.

Western analysis. Overproduced proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose filters (19). Filters were then blocked in phosphate-buffered saline containing 5% nonfat dry milk (Blotto; Carnation), for 1 hr at room temperature. To each blot was added rabbit serum (either complete or adsorbed with proteins from bacteria containing the pATH 1 vector) in Blotto (1/3000). After 14 hr at 4°, blots were washed in three changes of Blotto. Goat anti-rabbit alkaline phosphatase-conjugated second antibody was added in Blotto (1/1000) for 2 hr at room temperature. Blots were subsequently rinsed in developing buffer consisting of 50 mM sodium glycinate (pH 9.5) and 1 mM $MgCl_2$. Blots were then visualized in developing buffer containing 0.1 mg/ml *p*-nitroblue tetrazolium chloride and 0.05 mg/ml 5-bromo-4-chloro-3-indolyl phosphate. The reaction was stopped with phosphate-buffered saline containing 1 mM EDTA.

Immunoprecipitation protocol. Brain tissue from adult male Sprague-Dawley rats was suspended in 50 mM Tris-HCl buffer (pH 7.0 at room temperature) containing 120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 2.5 mM $CaCl_2$, homogenized for 10 sec at setting 6 with a Brinkmann Polytron, and washed twice by centrifugation at 48,000 × *g*. Unless otherwise indicated, all ensuing incubations were performed at 4°. The tissue pellet was rehomogenized in fresh buffer and incubated with the indicated concentration of [^{3}H]cytisine, in the presence or absence of 100 μM nicotine, for 75 min. The tissue was then solubilized by the addition of Triton X-100 (2% final concentration) under gentle stirring for 2 hr. The mixture was then centrifuged at 48,000 × *g* for 30 min. Aliquots of the clear supernatant, equivalent to 20 mg of original tissue weight (unless otherwise indicated), were added in triplicate to microcentrifuge tubes containing either polyclonal antisera directed against the specific subunits, their respective preimmune sera, or NRS. The sera were used at the dilutions indicated in the text or figure legends. After a 2–4-hr incubation, 100 μl of stripped PANSORBIN (10%) were added to each of the tubes, which were then gently rotated for 1 hr. The PANSORBIN-antibody-receptor-[^{3}H]cytisine complex was then precipitated by centrifugation at 14,000 × *g* for 30 sec. The resulting pellet was washed on the surface with 1 ml of Tris-EDTA buffer, dissolved in 100 μl of 0.1 N NaOH, 3% deoxycholate, and counted in a liquid scintillation counter at 33% efficiency.

Animals. Male Sprague-Dawley rats, weighing 250–350 g, were group-housed in a temperature- and light-controlled room (23°; lights on from 7:00 a.m. to 7:00 p.m.), with food and water *ad libitum*. For the pharmacological studies, rats were injected subcutaneously twice daily for 10 days with nicotine bitartrate dihydrate (2.0 mg/kg) or isotonic saline. Animals were decapitated 18 hr after their last injection, and their brains were dissected, frozen on dry ice, and stored at −70° until assayed.

Analysis of data. Specific immunoprecipitation (dpm/mg of tissue) was defined to be the difference between total immunoprecipitation and nonspecific immunoprecipitation, as determined with preimmune sera or NRS or the inclusion of 100 μM nicotine throughout the assay. Statistical comparisons for two-sample testing were determined by a one-tailed Student's *t* test for grouped data. Effects were considered statistically significant at $p \leq 0.05$.

Results

Antibody characterization. We first validated the antisera and optimized the assay conditions for their use in the immunoprecipitation of neuronal nicotinic receptors. Labeling and solubilization of rat brain homogenates were achieved as described, and each of the antibodies (i.e., anti-α2, -α3, -α4, -α5, -β2, -β3, and -β4) was tested for its ability to immunoprecipitate bound solubilized receptors. Although each of these antisera was capable of detecting its respective overproduced subunit by enzyme-linked immunosorbent assay and Western blot,[1] only antisera generated against α4 and β2 subunits were able to immunoprecipitate [^{3}H]cytisine-bound receptor specifically. Moreover, qualitatively similar data were obtained using two other agonist radioligands, [^{3}H]nicotine and *N*-[^{3}H]methylcarbamylcholine (data not shown), indicating that the results reported here are not restricted to [^{3}H]cytisine. Thus, anti-α4 and anti-β2 sera were characterized further.

The time dependence of incubation with anti-α4 and anti-β2 sera in precipitating solubilized receptors at 4° and 25° is shown in Fig. 1. Maximum immunoprecipitation was obtained between 2 and 4 hr for both antisera at both temperatures. Moreover, immunoprecipitation with both antisera remained relatively stable for up to 24 hr of incubation at 4°. However, immuno-

[1] S. W. Rogers, unpublished observations.

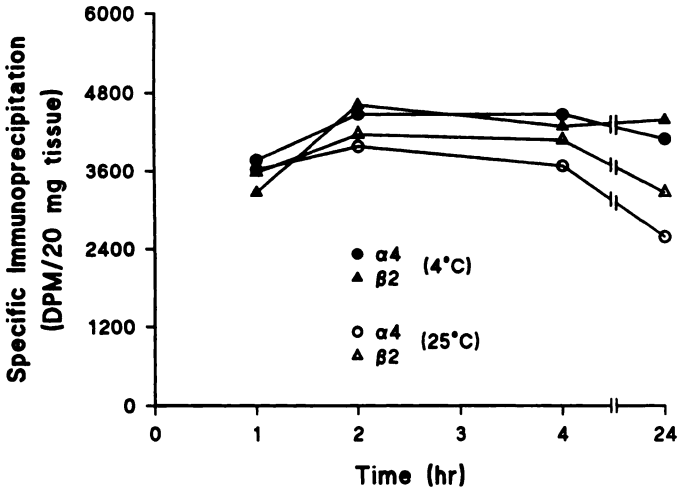

Fig. 1. Time and temperature dependence of the immunoprecipitation of solubilized neuronal nicotinic receptors by anti-α4 and anti-β2 sera. Aliquots of [^{3}H]cytisine-bound solubilized rat forebrain, equivalent to 20 mg of original weight, were incubated with either anti-α4 serum (*circles*) or anti-β2 serum (*triangles*) at 4° (*closed symbols*) or 25° (*open symbols*), for the indicated lengths of time. Nonspecific immunoprecipitation was determined with NRS. Data are expressed in dpm/20 mg of tissue and are representative of an experiment performed three times.

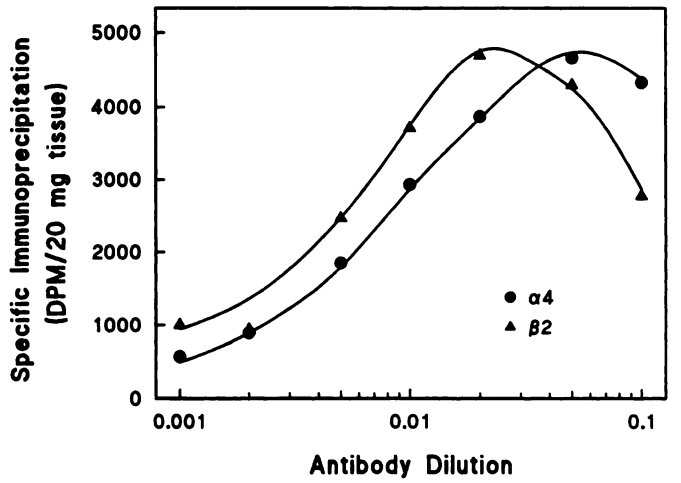

Fig. 2. Anti-α4 and anti-β2 sera concentration dependence of the immunoprecipitation of solubilized neuronal nicotinic receptors. Aliquots of [^{3}H]cytisine-bound solubilized rat forebrain, equivalent to 20 mg of original weight, were incubated for 4 hr at 4° with either anti-α4 serum (●) or anti-β2 serum (▲) at the indicated dilutions. Nonspecific immunoprecipitation was determined with NRS. Data are expressed in dpm/20 mg of tissue and are representative of an experiment performed six times.

precipitation by both antisera was reduced by 24 hr at 25°. Based on these results, all subsequent incubations were conducted for 2–4 hr at 4°.

Fig. 2 shows immunoprecipitation data for both sera as a function of antibody dilution, ranging from 1/10 to 1/1000. Immunoprecipitation with anti-α4 and anti-β2 sera was maximal at dilutions of 1/20 and 1/50, respectively, and declined thereafter. Based on these data, a dilution of 1/20 for anti-α4 and 1/50 for anti-β2 was used in succeeding studies.

Nonspecific immunoprecipitation was assessed three separate ways: 1) using anti-α4 or anti-β2 serum in the presence of 100 μM nicotine, 2) using preimmune sera, or 3) using NRS. Fig. 3 shows total immunoprecipitation of [^{3}H]cytisine-bound receptor by anti-α4 and anti-β2 sera, their respective preim-

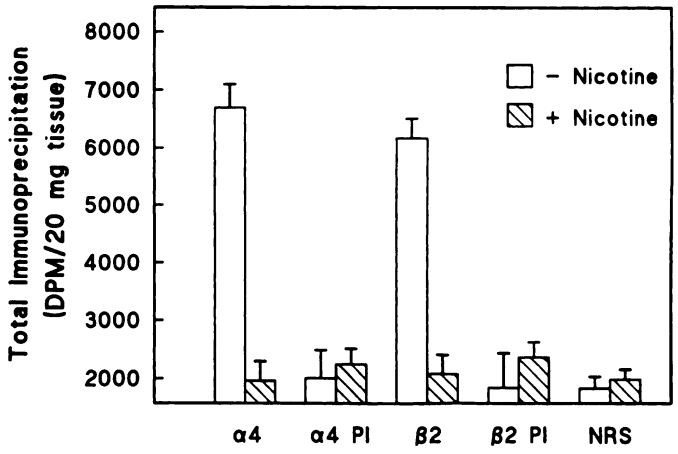

Fig. 3. Determination of nonspecific immunoprecipitation of solubilized neuronal nicotinic receptors. Rat forebrain homogenates were incubated with [^{3}H]cytisine, in the absence (□) or presence (▨) of 100 μM nicotine bitartrate dihydrate, and then solubilized. Aliquots of tissue, equivalent to 20 mg of original weight, were incubated for 4 hr at 4° with either anti-α4 serum, anti-β2 serum, their respective preimmune sera (*PI*), or NRS. Data from three experiments are expressed as the mean ± standard error, in dpm/20 mg of tissue.

mune sera, and NRS, each in the absence and presence of 100 μM nicotine. These data demonstrate that the total immunoprecipitation of solubilized receptor by anti-α4 or anti-β2 sera in the presence of 100 μM nicotine does not differ significantly from that obtained with either preimmune sera or NRS, in the presence or absence of nicotine. Nonspecific immunoprecipitation by any of these methods typically represented <35% of the total at a [^{3}H]cytisine concentration of approximately 10 nM. For practical considerations, precipitation of solubilized receptor with NRS at the same dilution as the specific antisera was selected as the routine method to determine nonspecific immunoprecipitation. It should be noted, however, that in the context of these experiments immunoprecipitation refers to radioactive counts and, therefore, is dependent upon antibody recognition of subunit that is associated with radioligand either directly or indirectly (i.e., through subunit attachment).

The data presented in Fig. 4 depict immunoprecipitation by anti-α4 and anti-β2 sera of solubilized rat forebrain homogenates over a range of original tissue weights equivalent to 10–50 mg. It can be seen that immunoprecipitation by both antibodies is linear up to approximately 40 mg of tissue, before beginning to plateau. Thus, we routinely used 20 mg of tissue in experiments.

Specificity of the antisera was tested using Western blot analysis. In Fig. 5, a Coomassie blue-stained gel and alkaline phosphatase-stained Western blot of insoluble protein obtained from *E. coli* containing either the *trp*E, α4, or β2 fusion contruct are shown in the *upper left* and *right*, respectively. As shown in Fig. 5, *lower left*, after preadsorption of antibodies specific to *trp*E and bacterial protein, anti-α4 serum recognized α4 fusion protein and its degradation products (Fig. 5, *lower left, lane 2*) but not β2 fusion protein (Fig. 5, *lower left, lane 3*); similarly, *trp*E-adsorbed anti-β2 serum detected β2 fusion protein and its degradation products (Fig. 5, *lower right, lane 3*) but not α4 fusion protein (Fig. 5, *lower right, lane 2*). Although immunoreactivity to a bacterial protein was not removed in anti-β2 serum (Fig. 5, *lower right, arrow*), essentially no immunoreactivity to *trp*E remained in either serum (Fig. 5, *lower left* and

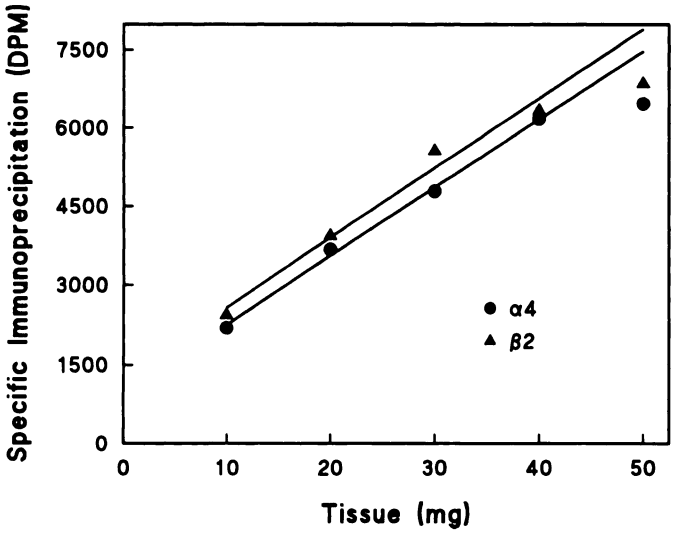

Fig. 4. Immunoprecipitation of solubilized neuronal nicotinic receptors by anti-α4 or anti-β2 serum as a function of tissue amount. Aliquots of [^{3}H] cytisine-bound solubilized rat forebrain, equivalent to 10–50 mg, were incubated for 4 hr at 4° with either anti-α4 serum (●) or anti-β2 serum (▲). Nonspecific immunoprecipitation was determined with NRS. Data are expressed in dpm and are representative of an experiment performed three times.

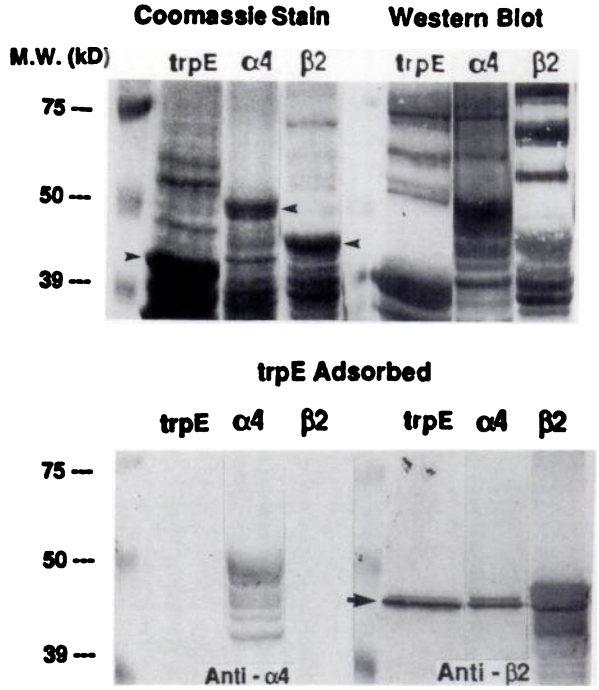

Fig. 5. Specificity of anti-α4 and anti-β2 sera in the immunoprecipitation of neuronal nicotinic receptors. *Upper left*, Coomassie blue-stained gel of ≈50 μg of insoluble protein from *E. coli* strain HB101 containing either the parent pATH 1 plasmid (*trpE*) or a fusion construct containing a portion of the cDNA encoding the putative cytoplasmic portion of either the α4 or β2 neuronal nicotinic acetylcholine receptor subunit (*arrowheads*). *Upper right*, Western blot of an identical gel visualized using alkaline phosphatase staining after incubation with immune serum diluted 1/3000. In the lower Western blots, antibodies specific to *trpE* and bacterial protein were removed from the α4 (*lower left*) and β2 (*lower right*) immune serum, and the remaining immunoreactivity was visualized. Immunoreactivity to a bacterial protein was not removed in anti-β2 serum (*arrow*). Lower molecular weight bands are degradation products of the fusion protein and vary between protein preparations. Prestained molecular weight markers are indicated.

right, lane 1). Thus, cross-reactivity between the overproduced regions of α4 and β2 was not detected in either serum. This procedure has also been used to demonstrate that no cross-reactivity is observed between these antisera and bacterially overproduced proteins containing the corresponding regions of the α2, α3, α5, β3, and β4 nicotinic acetylcholine receptor subunits.[1]

Properties and distribution. In [^{3}H]cytisine saturation assays, the amount of labeled receptor immunoprecipitated with either anti-α4 or anti-β2 serum was saturable (Fig. 6) and fit a model for a single class of binding sites (Table 1). The B_{max} values generated by anti-α4 and anti-β2 sera were 2.8 and 2.7 fmol/mg of original tissue weight, respectively, and the K_d value for both antisera was approximately 1 nM (Table 1). Importantly, the B_{max} and K_d values were similar using either of the two antisera and were in good accordance with conventional homogenate binding assays (16), suggesting near-quantitative solubilization efficiency.

The distribution of receptors containing α4 and β2 subunits was assessed in nine CNS regions. Fig. 7 shows that in each region tested immunoprecipitation by α4 and β2 antisera was approximately equal, with expression being highest in thalamus, intermediate in striatum, cortex, and colliculi, and lowest in hippocampus, brainstem, cerebellum, hypothalamus, and spinal cord.

Subunit composition. To determine whether α4 and β2 subunits are associated in forming neuronal nicotinic receptors, a double-immunoprecipitation protocol was used. [^{3}H]Cytisine-bound, solubilized forebrain homogenates were immunoprecipitated (i.e., precleared) with one antibody, and the resulting supernatant was then incubated with a second antibody. The rationale for these studies was that, if one antibody could decrease the specific immunoprecipitation by a second antibody, it would imply an association between the two subunits against which the two antibodies were directed.

Fig. 8 demonstrates that preclearing solubilized fractions with anti-α4 serum not only depleted essentially all specific immunoprecipitation of α4 subunit but also almost completely eliminated that of β2 subunit as well; similarly, when solubilized fractions were precleared with anti-β2 serum, specific immunoprecipitation of both subunits was nearly abolished. On the other hand, preclearing of solubilized fractions with anti-α2, -α3, -α5, -β3, or -β4 sera did not decrease specifically immunoprecipitable α4 or β2 subunits (Fig. 8).

Pharmacological regulation. Specific immunoprecipitation of receptors containing α4 and β2 subunits was determined in cerebral cortex of rats injected twice daily for 10 days with nicotine bitartrate dihydrate (2.0 mg/kg) or isotonic saline. Fig. 9 demonstrates that specific immunoprecipitation of both subunits was significantly increased in the cortex of animals treated with nicotine, compared with saline-treated controls.

Discussion

We have examined the ability of polyclonal antisera directed against four different α and three different β subunits of rat neuronal nicotinic receptors to specifically immunoprecipitate [^{3}H]cytisine-labeled receptors from solubilized rat brain homogenates. Our data indicate that, of the seven antisera tested throughout the rat CNS, only anti-α4 and anti-β2 sera immunoprecipitated specifically bound [^{3}H]cytisine. Furthermore,

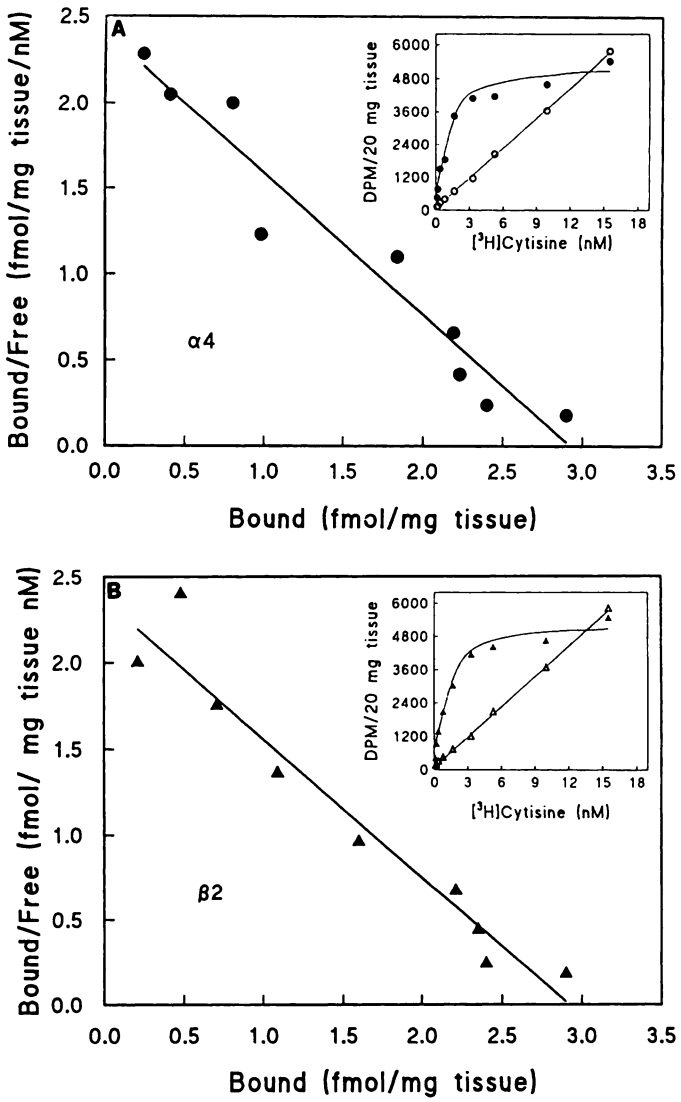

Fig. 6. Saturation analysis of [^{3}H]cytisine binding to rat forebrain homogenates immunoprecipitated by anti-α4 and anti-β2 sera. Rat forebrain homogenates were incubated with 0.15–15 nM [^{3}H]cytisine and then solubilized. Aliquots of tissue, equivalent to 20 mg of original weight, were incubated for 4 hr at 4° with antisera. Nonspecific immunoprecipitation was determined with NRS. Scatchard plots depict specific immunoprecipitation by anti-α4 serum (A) or anti-β2 serum (B); *insets*, saturation curves depict specific (*closed symbols*) and nonspecific (*open symbols*) immunoprecipitation of [^{3}H]cytisine-bound receptor. Data are expressed in dpm/20 mg of tissue and are representative of an experiment performed eight times (see Table 1).

TABLE 1

[^{3}H]Cytisine bnding values for rat forebrain nicotinic receptors immunoprecipitated with anti-α4 and anti-β2 sera

Data represent the mean ± standard error of eight separate experiments, for which representative Scatchard plots and saturation curves are shown in Fig. 6.

	K_d	B_{max}	n_H
	nM	fmol/mg of tissue	
α4	1.2 ± 0.2	2.8 ± 0.2	0.9 ± 0.04
β2	1.1 ± 0.2	2.7 ± 0.4	0.9 ± 0.02

whereas preincubation with either anti-α4 or anti-β2 serum could almost completely deplete solubilized tissue aliquots of both α4 and β2 subunits, specific immunoprecipitation of these subunits was unaffected by preincubation with any of the other

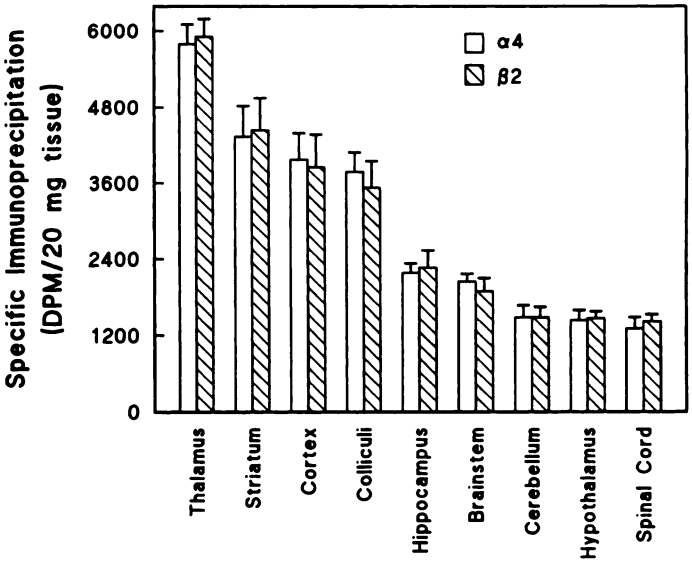

Fig. 7. Regional distribution of neuronal nicotinic receptors containing α4 and β2 subunits. Homogenates from the indicated rat CNS regions were incubated with [^{3}H]cytisine and solubilized. Aliquots of solubilized tissue, equivalent to 20 mg of original weight, were incubated for 4 hr at 4° with either anti-α4 serum (□) or anti-β2 serum (▩). Nonspecific immunoprecipitation was determined with NRS. Data from four experiments are expressed as the mean ± standard error, in dpm/20 mg of tissue.

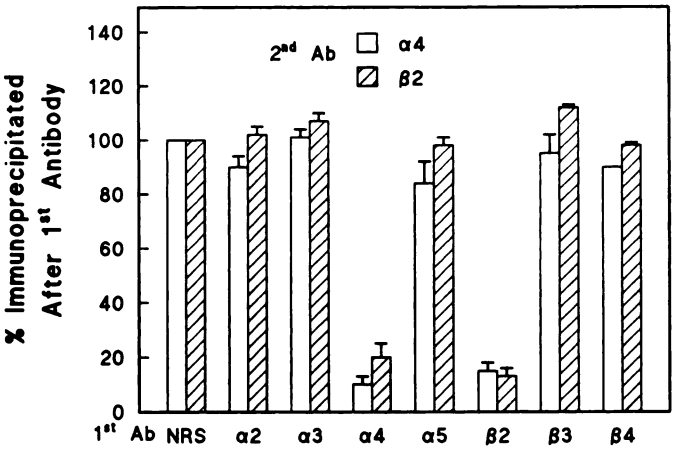

Fig. 8. Association of α4 and β2 subunits in neuronal nicotinic receptors. Aliquots of [^{3}H]cytisine-bound solubilized rat forebrain, equivalent to 20 mg of original weight, were incubated for 4 hr at 4° with either NRS, anti-α2, -α3, -α4, -α5, -β2, -β3, or -β4 serum (*1st Ab*). After precipitation with stripped PANSORBIN, supernatants were incubated for an additional 4 hr with a second antibody (*2nd Ab*), either anti-α4 serum (□) or anti-β2 serum (▩). Nonspecific immunoprecipitation was determined with NRS. Data from four experiments are expressed as the mean ± standard error in dpm/20 mg of tissue.

antisera. Taken together, the data strongly suggest that in the rat CNS α4 and β2 subunits are associated in forming the predominant, and possibly the only, subtype of neuronal nicotinic receptor with high affinity for agonists. Consistent with this conclusion, we found that the density and regional distribution profile exhibited by α4 and β2 subunits immunoprecipitated in nine CNS areas are similar to those generated by conventional nicotinic agonist binding assays in rat brain homogentes. In addition, these studies demonstrate that the neuronal nicotinic receptor subtype composed of α4 and β2 sub-

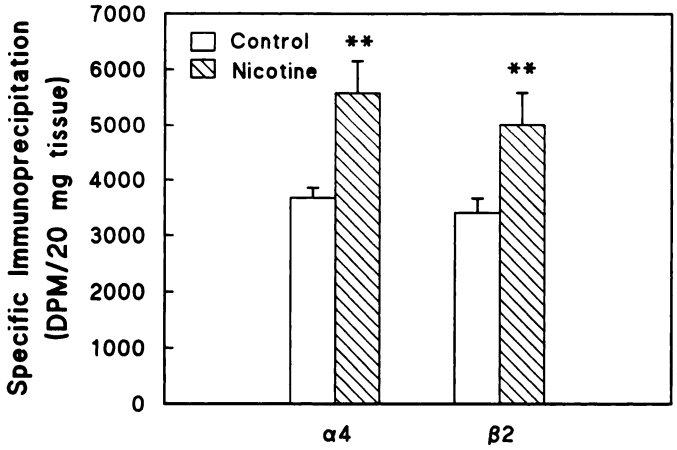

Fig. 9. Up-regulation by chronic nicotine treatment of neuronal nicotinic receptors containing $\alpha 4$ and $\beta 2$ subunits. Adult male Sprague-Dawley rats were injected subcutaneously twice daily for 10 days with nicotine bitartrate dihydrate (2.0 mg/kg) or vehicle (isotonic saline). Eighteen hours after the last injection, rats were sacrificed and the brains were dissected, frozen on dry ice, and stored at −70°. Aliquots of [^{3}H]cytisine-bound, solubilized, cerebral cortex, equivalent to 20 mg of original weight, were incubated for 4 hr at 4° with either anti-$\alpha 4$ or anti-$\beta 2$ serum. Nonspecific immunoprecipitation was determined with NRS. Data are expressed as the mean ± standard error, in dpm/20 mg of cortex, from control (□) and nicotine-treated (▨) rats (n = 4/group). **, Differs significantly from control value, $p \leq 0.01$.

units is significantly increased in the cerebral cortex of rats chronically treated with nicotine.

Monoclonal antibodies have been developed against various subunits of nicotinic receptors from both chicken and rat brain (20, 21). One of these antibodies, monoclonal antibody 270, is specific for the $\beta 2$ subunit and can be used to immunoprecipitate >90% of the high affinity nicotinic agonist binding sites in rat brain (11, 21, 22). Based on amino-terminal sequencing, the α subunit associated with this monoclonal antibody 270 precipitant displays identity, over a region of 17 amino acids, with a sequence predicted for the $\alpha 4$ subunit (23). Taken together, these data also suggest that, in rat brain, $\alpha 4$ and $\beta 2$ subunits make up the predominant subtype of nicotinic receptor that has been detected by agonist binding to date.

In the present studies, the failure of antisera directed against subunits other than $\alpha 4$ and $\beta 2$ to immunoprecipitate [^{3}H] cytisine-bound receptors implies either that these subunits are not expressed in the rat CNS or that, if they are expressed, they are not constituents of receptors with high affinity for agonists. We favor the latter hypothesis for several reasons. First, *in situ* hybridization histochemistry has revealed that the mRNA transcripts encoding these undetected α and β subunits are expressed throughout the rat CNS (4, 7, 24). Second, PC12 cells, which express mRNA encoding the $\alpha 3$ subunit (2), contain functional receptors with low affinity for nicotine and acetylcholine (25, 26), suggesting that at least this putative agonist binding subunit (i.e., $\alpha 3$) exhibits low affinity for agonists. Finally, consistent with this correlation, whole-cell recordings from *Xenopus* oocytes injected with mRNA encoding $\alpha 3$ in combination with that encoding either $\beta 2$ or $\beta 4$ exhibited a decreased functional sensitivity to agonist, compared with $\alpha 4$ in combination with $\beta 2$ or $\beta 4$ (9, 10).

It could be argued that the quaternary structure of other potential subtypes sterically hinders antibody recognition of the epitopes for the undetected subunits. This seems unlikely, however, because antisera directed against the $\alpha 4$ and $\beta 2$ subunits do not suffer from such constraints; furthermore, all of the antisera were generated against epitopes contained in the same carboxyl-terminal cytoplasmic domain of each of the subunits. It should also be noted that anti-$\alpha 2$, anti-$\beta 2$ (27), and anti-$\alpha 3$[1] sera have been shown to immunoprecipitate their respective ^{35}S-labeled proteins from transfected rat fibroblasts.

One of the most intriguing features of neuronal nicotinic receptors is their regulation by acute and chronic exposure to nicotine. For examples, an acute injection of nicotine desensitizes the receptor for hours (28, 29), whereas chronic nicotine administration can abolish receptor function for more than 7 days (30). Thus, it is feasible that the long term desensitization or inactivation of receptor function after chronic nicotine treatment could lead to the increase in nicotinic binding sites previously cited. The data presented in this report demonstrate that the nicotinic receptor subtype composed of $\alpha 4$ and $\beta 2$ subunits is up-regulated in response to chronic nicotine treatment. Moreover, the magnitude of this increase (40–50%) is similar to that reported for conventional homogenate binding assays (12–15).

It should be noted that the CNS distribution of the subunits comprising the various nicotinic receptors and the mRNA encoding them may differ. Such differences would likely result from axonal transport of the translated subunits to brain regions distant from the cell bodies expressing their respective transcripts. In addition, relative amounts of specific subunit mRNAs and the protein subunits they encode may not be correlated, due to the potential for differential translational efficiency and/or stability of individual subunits or the receptors they comprise.

As the number of cloned candidate nicotinic receptor subunits increases, so does the potential number of subtypes. Therefore, it is significant that the present data indicate that the high affinity nicotinic receptor in rat brain is composed of only one type of α and one type of β subunit, i.e., $\alpha 4$ and $\beta 2$. If this structural motif is exhibited across the other subtypes of this receptor family, then the total number of receptor subtypes possible is exponentially reduced. It will be particularly interesting and important to determine the subunit composition, brain region distribution, pharmacological characteristics, and regulation of the other potential subtypes of this receptor class. These other subtypes apparently do not have the high affinity (low nanomolar range) for agonists displayed by the $\alpha 4/\beta 2$ subtype; thus, different methods will be required to characterize them. As we gain a more detailed understanding of the subunit composition and stoichiometry of neuronal nicotinic receptors, it should become possible to subtype them, as has been done for other receptor families.

References

1. Hucho, F. The nicotinic acetylcholine receptor and its ion channel. *Eur. J. Biochem.* **158**:211–226 (1986).
2. Boulter, J., K. Evans, D. Goldman, G. Martin, D. Treco, S. Heinemann, and J. Patrick. Isolation of a cDNA clone coding for a possible neural nicotinic acetylcholine receptor α-subunit. *Nature (Lond.)* **319**:368–374 (1986).
3. Goldman, D., E. Deneris, W. Luyten, A. Kochhar, J. Patrick, and S. Heinemann. Members of a nicotinic acetylcholine receptor gene family are expressed in different regions of the mammalian central nervous system. *Cell* **48**:965–973 (1987).
4. Deneris, E., J. Connolly, J. Boulter, E. Wada, K. Wada, L. W. Swanson, J. Patrick, and S. Heinemann. Primary structure and expression of $\beta 2$: a novel subunit of neuronal nicotinic acetylcholine receptor. *Neuron* **1**:45–54 (1988).
5. Wada, K., M. Ballivet, J. Boulter, J. Connolly, E. Wada, E. S. Deneris, L. W. Swanson, S. Heinemann, and J. Patrick. Functional expression of a new

pharmacological subtype of brain nicotinic acetylcholine receptor. *Science (Washington D. C.)* **240**:330–334 (1988).
6. Deneris, E. S., J. Boulter, L. W. Swanson, J. Patrick, and S. Heinemann. β3: a new member of nicotinic acetylcholine receptor gene family is expressed in brain. *J. Biol. Chem.* **264**:6268–6272 (1989).
7. Boulter, J., A. O'Shea-Greenfield, R. M. Duvoisin, J. G. Connolly, E. Wada, A. Jensen, P. D. Gardner, M. Ballivet, E. S. Deneris, D. McKinnon, S. Heinemann, and J. Patrick. α3, α5 and β4: three members of the rat neuronal nicotinic acetylcholine receptor-related gene family form a gene cluster. *J. Biol. Chem.* **265**:4472–4482 (1990).
8. Boulter, J., J. Connolly, E. Deneris, D. Goldman, S. Heinemann, and J. Patrick. Functional expression of two neuronal nicotinic acetylcholine receptors from cDNA clones identifies a gene family. *Proc. Natl. Acad. Sci. USA* **84**:7763–7767 (1987).
9. Duvoisin, R. M., E. S. Deneris, J. Patrick, and S. Heinemann. The functional diversity of the neuronal nicotinic acetylcholine receptors is increased by a novel subunit: β4. *Neuron* **3**:487–496 (1989).
10. Papke, R. L., J. Boulter, J. Patrick, and S. Heinemann. Single-channel currents of rat neuronal nicotinic acetylcholine receptors expressed in *Xenopus* oocytes. *Neuron* **3**:589–596 (1989).
11. Whiting, P., and J. Lindstrom. Purification and characterization of a nicotinic acetylcholine receptor from rat brain. *Proc. Natl. Acad. Sci. USA* **84**:595–599 (1987).
12. Schwartz, R. D., and K. J. Kellar. Nicotinic cholinergic receptor binding sites in brain: *in vivo* regulation. *Science (Washington D. C.)* **220**:214–216 (1983).
13. Marks, M. J., J. B. Burch, and A. C. Collins. Effects of chronic nicotine infusion on tolerance and cholinergic receptors. *J. Pharmacol. Exp. Ther.* **226**:817–825 (1983).
14. Schwartz, R. D., and K. J. Kellar. *In vivo* regulation of [^{3}H]acetylcholine recognition sites in brain by nicotinic cholinergic drugs. *J. Neurochem.* **45**:427–433 (1985).
15. Marks, M. J., J. A. Stitzel, and A. C. Collins. Time course study of the effects of chronic nicotine infusion on drug response and brain receptors. *J. Pharmacol. Exp. Ther.* **235**:619–628 (1985).
16. Pabreza, L. A., S. Dhawan, and K. J. Kellar. [^{3}H]Cytisine binding to nicotinic cholinergic receptors in brain. *Mol. Pharmacol.* **39**:9–12 (1991).
17. Luthin, G. R., J. Harkness, R. P. Artymyshyn, and B. B. Wolfe. Antibodies to a synthetic peptide can be used to distinguish between muscarinic acetylcholine receptor binding sites in brain and heart. *Mol. Pharmacol.* **34**:327–333 (1988).
18. Dieckmann, C. L., and A. Tzagoloff. Assembly of mitochondrial membrane system. *J. Biol. Chem.* **260**:1513–1520 (1985).
19. Kyhse-Andersen, J. Electroblotting of multiple gels: a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. *J. Biochem. Biophys. Methods* **10**:203–209 (1984).
20. Whiting, P. J., and J. M. Lindstrom. Purification and characterization of a nicotinic acetylcholine receptor from chick brain. *Biochemistry* **25**:2082–2093 (1986).
21. Whiting, P. J., R. S. Liu, B. J. Morley, and J. Lindstrom. Structurally different neuronal nicotinic acetylcholine receptor subtypes purified and characterized using monoclonal antibodies. *J. Neurosci.* **7**:4005–4016 (1987).
22. Whiting, P., and J. Lindstrom. Pharmacological properties of immunoisolated neuronal nicotinic receptors. *J. Neurosci.* **6**:3061–3069 (1986).
23. Whiting, P., F. Esch, S. Shimasaki, and J. Lindstrom. Neuronal nicotinic acetylcholine receptor β-subunit is coded for by the cDNA clone α4. *FEBS Lett.* **219**:459–463 (1987).
24. Wada, E., K. Wada, J. Boulter, E. Deneris, S. Heinemann, J. Patrick, and L. W. Swanson. Distribution of *alpha2*, *alpha3*, *alpha4* and *beta2* neuronal nicotinic receptor subunit mRNAs in the central nervous system: a hybridization histochemical study in the rat. *J. Comp. Neurol.* **284**:314–335 (1989).
25. Whiting, P. J., R. Schoepfer, L. W. Swanson, D. M. Simmons, and J. M. Lindstrom. Functional acetylcholine receptor in PC12 cells reacts with a monoclonal antibody to brain nicotinic receptors. *Nature (Lond.)* **327**:515–518 (1987).
26. Kemp, G., and B. J. Morley. Ganglionic nAChRs and high-affinity nicotinic binding sites are not equivalent. *FEBS Lett.* **205**:265–268 (1986).
27. Rogers, S. W., L. C. Gahring, R. L. Papke, and S. Heinemann. Identification of cultured cells expressing ligand-gated cationic channels. *Protein Expression Purification*, **2**:108–116 (1991).
28. Sharp, B. M., and S. H. Beyer. Rapid desensitization of the acute stimulatory effects of nicotine on rat plasma adrenocorticotropin and prolactin. *J. Pharmacol. Exp. Ther.* **238**:486–491 (1986).
29. Hulihan-Giblin, B. A., M. D. Lumpkin, and K. J. Kellar. Acute effects of nicotine on prolactin release in the rat: agonist and antagonist effects of a single injection of nicotine. *J. Pharmacol. Exp. Ther.* **252**:15–20 (1990).
30. Hulihan-Giblin, B. A., M. D. Lumpkin, and K. J. Kellar. Effects of chronic administration of nicotine on prolactin release in the rat: inactivation of prolactin response by repeated injections of nicotine. *J. Pharmacol. Exp. Ther.* **252**:21–25 (1990).

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