Formation of Oligomers Containing the β3 and β4 Subunits of the Rat Nicotinic Receptor

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The role of the β3 and β4 subunits of the nicotinic acetylcholine receptor in brain is still unclear. We investigated nicotinic receptor structure with antibodies directed against unique regions of the β3 and β4 subunits of the rat nicotinic acetylcholine receptor. Anti-β4 detected a single band of 66 kDa in most regions of the brain that was strongest in striatum and cerebellum. The 60 kDa β3 subunit was detected primarily in striatum and cerebellum, and faintly in hippocampus. Immunoprecipitation experiments established that the two subunits were coassembled in the cerebellum along with the β2 subunit. Antibodies against the α4, β2, β3, and β4 subunits immunoprecipitated ~75% of the bungarotoxin-insensitive nicotinic receptor from cerebellar extracts as determined by nicotine-dependent acetylcholine binding. Transfection of COS cells with cDNAs for these four subunits induced expression of a high affinity nicotinic receptor. Omission of only a single subunit from the transfection affected either the B_{max} or the apparent K_D of the receptor. Our data suggest that the β3 subunit functions as a structural entity that links a relatively unstable α4β2 heterodimer to a more stable α4β4 heterodimer. The agonist-binding site formed by α4β2 has a much greater affinity than does that formed by α4β4. In this respect, nicotinic receptors that contain the β3 subunit are structurally homologous to the muscle nicotinic receptor.

Key words: nicotine; acetylcholine; receptor; antibody; β3; β4; subunit

Nicotinic acetylcholine receptors (nAChR) expressed in the CNS are members of a superfamily of ligand-gated ion channels that also include the muscle-type nAChR, $GABA_A$, glycine, and 5-HT_3 receptors (Barnard et al., 1987; Egebjerg et al., 1991; Maricq et al., 1991; Moriyoshi et al., 1991; Monyer et al., 1992). Nine different rat neuronal nAChR subunit cDNAs (α2, α3, α4, α5, α6, α7, β2, β3, and β4) have been cloned (for review, see Sargent, 1993). Most of the receptors in the family are thought to contain two or more different subunits, but the precise combinations of these subunits that exist *in vivo* are largely unknown. Previous work has indicated that the major high affinity nicotinic receptor in rat brain is composed of α4 and β2 subunits (Nakayama et al., 1991; Flores et al., 1992; Marks et al., 1992). More recent evidence indicates that deletion of the β2 subunit gene results in the loss of high affinity nicotine-binding sites from mouse brain (Picciotto et al., 1995). Nicotinic receptors have also been reconstituted in *Xenopus* oocytes. Thus, the β2 and β4 subunits can combine with various α subunits to form functional receptors, permitting the formation of many types of receptor with unique pharmacological characteristics (Luetje and Patrick, 1991). Much less is known about the β3 subunit. It does not express any channel activity in oocytes in combination with any other single subunit (Deneris et al., 1989), nor has it been demonstrated at the protein level in the CNS. Both the β3 and β4 subunits seem, by *in situ* hybridization, to have a more restricted distribution than does the β2 subunit (Deneris et al., 1989; Duvoisin et al., 1989; Dineley-Miller and Patrick, 1992; Willoughby et al., 1993). Recent work has suggested that the β4 subunit is more widely expressed in the CNS than previously thought (Dineley-Miller and Patrick, 1992). However, little is known about the types of oligomers in which it occurs. The goal of this study, therefore, was to determine which regions of rat brain contain these two subunits and whether they are assembled into nAChR oligomers.

To establish the role of these two subunits in nAChR structure, we prepared antibodies against unique cytoplasmic domains of each subunit. We found that, in the striatum and in the cerebellum, both subunits overlap in their expression. Immunoprecipitation of extracts of rat cerebellum and transfected COS cells confirmed that these two subunits coassemble with the α4 and β2 subunits to create a hetero-oligomeric receptor. Thus, our data indicate that the β3 and β4 subunits coassemble *in vivo* with the α4 and β2 subunits to form a novel type of nicotinic receptor.

MATERIALS AND METHODS

Production of antibodies. Antibodies against the cytoplasmic loop region between M3 and M4 in the β3 subunit and β4 subunit were generated similarly. The appropriate sequences of each subunit cDNA were amplified by PCR containing restriction sites compatible with the reading frame of the vector, pFLAG (Kodak-IBI). After subcloning into the vector, each clone was sequenced to verify the fidelity of the sequence. The *Escherichia coli* strain, DH5α, transformed with these plasmids, was induced by addition of 0.5 mM isopropylthiogalactoside to express the fusion protein that, at its N-terminal, carried the FLAG epitope. Bacteria were harvested by centrifugation at 3500 × *g* for 10 min at 10°C and resuspended in 10 ml of extraction buffer A (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 25 mg/ml lysozyme, and 50 μg/ml NaN_3)/ml pellet, and incubated until lysis was apparent. Then 0.1 volume of extraction buffer B was added (1.5 M NaCl, 0.1 M $CaCl_2$, 0.1 M $MgCl_2$, 20 μg/ml DNase1, and 50 μg/ml ovomucoid trypsin inhibitor) and was incubated at room temperature until viscosity was sharply reduced. This mixture was centrifuged at 18,000 × *g* for 60 min at 10°C. The pellet was then extracted in TE containing 25 mM octylglucoside, 1 mM PMSF, 1 mM leupeptin, and 1 mM aprotinin and centrifuged at 3700 × *g* for 10 min at 4°C, and the supernatant applied to an affinity column to which was attached a mono-

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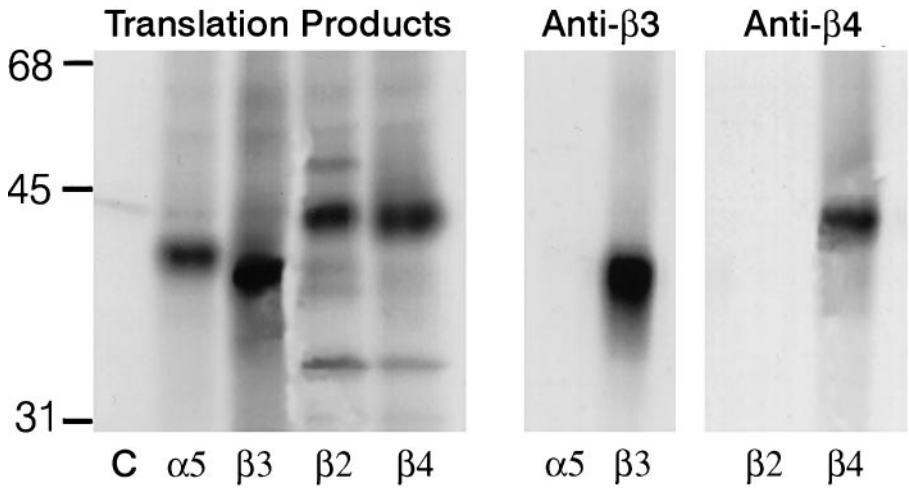

Figure 1. *In vitro* translation of nAChR subunits. The β3, α5, β2, or β4 subunit cDNAs were translated *in vitro* for 2 hr at 30°C. A fraction of each translation reaction was extracted in SDS-PAGE buffer and aliquots separated on a 7.5% gel (*left panel*). A control reaction that lacked any added DNA is marked *C*. The rest of each reaction was then immunoprecipitated with either anti-β3 (*middle panel*) or anti-β4 (*right panel*). The immunoprecipitates were then detected by SDS-PAGE fluorography at −70°C. Because no membranes were present, the apparent molecular weights of the subunits are not representative of the native subunits *in vivo*.

clonal antibody (mAb) directed against the FLAG epitope. After washing of the column, the bound material was eluted with 0.1 M glycine, pH 3.0, with 1 mM octylglucoside. After adjusting the pH to 8.0 with 1 M Tris-HCl, pH 10, the OD_{280} peak was pooled in each case, and a small sample was analyzed by SDS-PAGE and Western blotted with anti-FLAG antibody. Bands of 28 and 24 kDa were observed for the β3 and β4 subunit fusion proteins, respectively. Fifty micrograms of each antigen was injected into rabbits as a 1:1 emulsion with Freund's complete adjuvant. Subsequent boosts were with the same amount of protein mixed with incomplete adjuvant. Antisera were titered by Western blot against several quantities of antigen and serial dilutions of antiserum. To achieve the highest possible level of specificity, the sera were further purified by adsorption to sepharose, to which had been attached synthetic peptides unique to the cytoplasmic domain of either the β3 or the β4 subunit. The β3 subunit-specific peptide had the sequence: NH_2-DGKESDTAVRGK. For the β4 subunit, the following peptides were used: (1) NH_2-KSAVSSHTAGLPR-DAR; and (2) NH_2-HPSQLHLATADT. After applying crude serum to the columns, that were then washed extensively with PBS, the antibodies were eluted with 0.1 M glycine, pH 3.0. After quickly neutralizing to pH 7.4 with 1 M Tris base, the eluates were dialyzed against PBS containing NaN_3 (0.02%). Usually, the concentration of antibody was 1.5–2.0 μM and was stored in small aliquots at −70°C.

Experiments with brain tissue. Cerebellar synaptosomes and Lubrol extracts were prepared from frozen rat brains (PelFreez) as described by Nakayama et al. (1991). Regional dissections of rat brain were accomplished by cutting the outermost sections of a fresh ice-cold rat brain away from the center. The brain was laid on its side, and a scalpel was used to cut away the cortex, followed by the striatum. The hippocampus and thalamic region were pooled and are referred to simply as hippocampus. The cerebellum was then dissected away from the medulla oblongata. All these pieces were weighed and diluted with a 10-fold weight of PBS containing PMSF and iodoacetamide, both at 1 mM. The tissue was then rapidly dispersed by sonication. Protein concentration for each fraction was established by a BioRad reagent. Five milligrams of protein was then boiled in 1 ml of SDS-PAGE buffer and stored at −20°C until use. Aliquots (250 μg) were separated on a 7.5% SDS-PAGE with prestained markers (Life Technologies, Gaithersburg, MD). They were then electrotransferred to PVDF membrane and blotted with subunit specific antibodies as described below. Immunoprecipitation and Western blot of detergent extracts of cerebella were performed by extracting aliquots of 5 mg of crude synaptosomal protein in 1.5% Lubrol, 30 mM Tris, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, and 5 mM iodoacetamide for 30 min at 4°C, followed by removal of insoluble material by centrifugation at 100,00 × *g* for 60 min at 4°C. The supernatant solution was incubated for 2–16 hr with the appropriate antibody at a concentration of 50–100 nM at 4°C. Then Protein G (mAbs) or Protein A (polyclonals) agarose beads were added and mixed for 2 hr. After washing the beads three times in extraction buffer, they were boiled in SDS-PAGE buffer and separated on a 7.5% SDS-polyacrylamide gel. After transfer to PVDF membrane, subunits were detected by incubation with primary antibody (5–10 nM for β4 and 1:1000 dilution for β3) and then with an AP-linked chemiluminescent reagent system (Clontech, Palo Alto, CA).

Immunodepletion of acetylcholine binding activity was performed on Lubrol extracts of synaptosomes by incubating extracts with either non-immune serum or with the appropriate antibody for 2 hr at 4°C and then for another 2 hr with Protein G (mAbs) or Protein A (polyclonals) agarose beads. Atropine (1 μM), α-bungarotoxin (10 nM; Sigma, St. Louis, MO) and κ-bungarotoxin (10 nM; Miami Biotoxins, Miami, FL) were included to block binding to muscarinic, α7 type, and ganglionic (α3) receptors, respectively. The precipitated receptors were removed by centrifugation and the supernatant solution assayed for residual binding of [^{3}H]acetylcholine. Briefly, aliquots were diluted fivefold in PBS containing 20 nM [^{3}H]acetylcholine (Schwartz et al., 1982) and eserine (0.1 mM). Nicotine (10 mM) was included in some replicates to determine nonspecific binding. After incubation for 40 min on ice, the solutions were diluted 10-fold in ice-cold wash buffer (50 mM Tris-HCl, pH 7.4, and 0.1% Triton X-100) and rapidly filtered on GF/F filters presoaked in 0.3% PEI. The filters were washed three times with wash buffer and then counted in a liquid scintillation counter.

In vitro *translations, transfections, and binding assays.* Subunit cDNAs were translated *in vitro* with a coupled transcription-translation system (Promega, Madison, WI). In this protocol, 1 μg of plasmid, encoding either the α5, β2, β3, or β4 subunit cDNAs under the control of the bacteriophage SP6 or T7 promoter, was added to 50 μl of reticulocyte lysate containing 4 μCi of [^{35}S]methionine. After 2 hr at 30°C, 5 μl aliquots were boiled in an equal volume of SDS-PAGE buffer to determine total products. The rest of the reaction was diluted in 1% Triton buffer and immunoprecipitated with the appropriate antibody (Forsayeth et al., 1992).

Transfection of COS cells was performed by an adenovirus-DEAE dextran method (Forsayeth and Garcia, 1994). Briefly, cells were plated 2 hr before transfection to ~80% confluence. Then a mixture of replication-deficient human adenovirus (empirically determined amount), 80 μg/ml DEAE-dextran, and 1 μg/ml plasmid DNA, in serum-free medium was added for 2 hr at 37°C. The cells were then washed in PBS/10% DMSO and incubated for a further 2 d. The cDNAs for the α4, β2, and β4 subunits were encoded within the expression plasmid pCDLSRα296 (Takebe et al., 1988). The β3 and α5 subunit cDNAs were encoded within the plasmid PMT23, a trivially modified PMT2 (Dorner et al., 1988). Metabolic labeling of transfected cells was performed exactly as described previously (Forsayeth et al., 1992). Assay of acetylcholine binding activity in the cells was performed by passing cell pellets suspended in PBS through a 19 gauge needle six times to lyse the cells. Aliquots were incubated in 20 nM [^{3}H]ACh for 40 min on ice in the presence of various concentrations of nicotine. This mixture was then filtered as described above.

RESULTS

Specificity of antibodies against the β3 and β4 subunits

Because the β3 and β4 subunits are quite homologous in their cytoplasmic loop regions to those of the α5 and β2 subunits, respectively, we first determined whether the antibodies cross-reacted with these subunits. The four subunits were translated *in vitro* in the presence of [^{35}S]methionine and immunoprecipitated

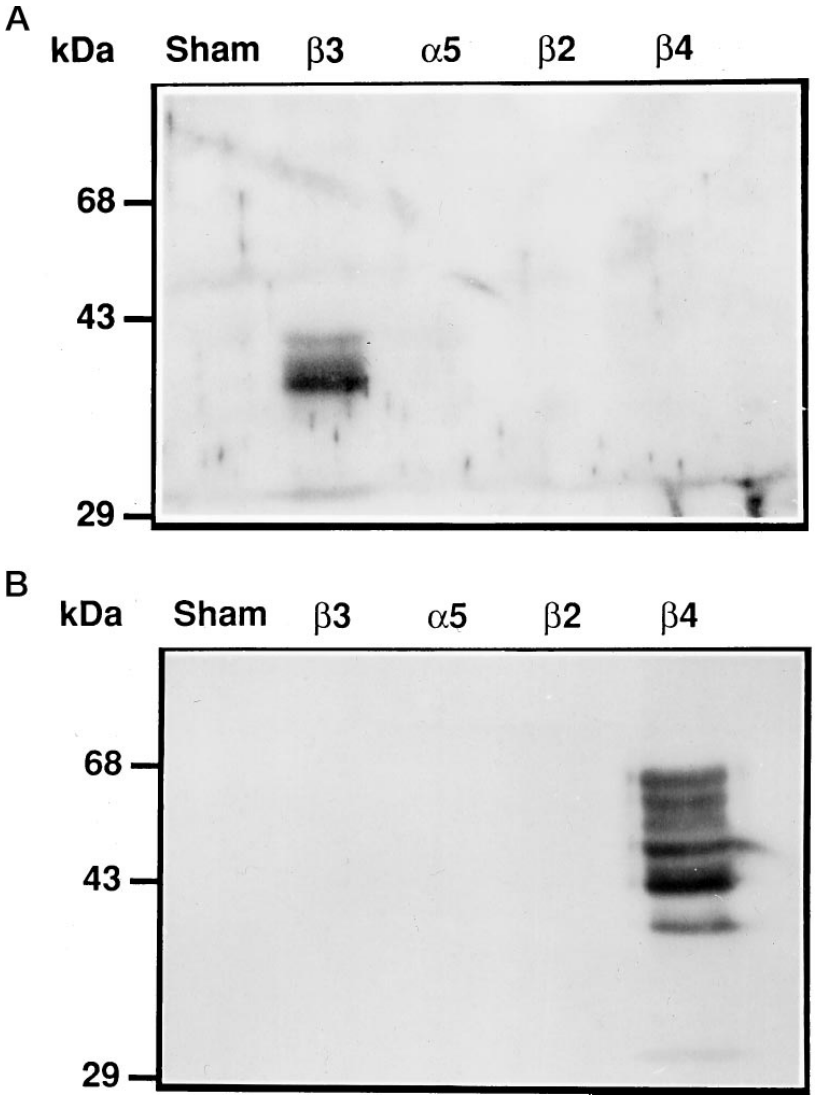

Figure 2. Western blot of nAChR subunits expressed in COS cells. COS cells were transfected separately with one of the indicated subunit cDNAs. Two days after transfection, the cells were extracted in SDS-PAGE buffer and an aliquot (1/50 of a 60 mm dish) was separated on a 7.5% nonreducing PAG and transferred to a PVDF membrane. The membranes were blotted with β3 (*A*) or β4 (*B*). Exposure time was 0.5 min.

with either affinity-purified anti-β3 or anti-β4 (Fig. 1). A sample of each translation reaction was also electrophoresed to determine the presence of the appropriate subunit. Because pancreatic membranes were not included, the mobilities of the subunits represent those of the unglycosylated primary translation products. In the case of the anti-β3 immunoprecipitation, the antibody precipitated only the β3 and not the α5 subunit. Similarly, anti-β4 precipitated the β4 subunit, and did not precipitate the β2 subunit. In other experiments (data not shown), we found that the crude β4 antiserum precipitated the β2 and β4 subunits equally well. The specificity of these antibodies was demonstrated further in transfected COS cells. The cells were transfected with each of the subunits indicated, extracted in SDS buffer, and Western blotted with the appropriate antibody (Fig. 2). In each case, the antibody detected only the subunit against which it had been raised. The β3 subunit can be identified as several bands between 43 and 35 kDa (Fig. 2*A*), and is characteristic of the proteolyzed subunit that accumulates when it is not stabilized by assembly. Similarly, in transfected COS cells, the β4 subunit migrated as a series of fragments with a maximum molecular weight of 67 kDa and a minimum of ~35 kDa (Fig. 2*B*).

A second consideration was to establish the specificity of the anti-β2 subunit specific antibody, mAb270, because its possible cross-reactivity with the β3 and β4 subunits has never been directly assessed to our knowledge. COS cells were transfected with cDNAs encoding the β3, α5, β2, and β4 subunits, then pulse-labeled for 10 min with [^{35}S]methionine. Detergent extracts of the cells were immunoprecipitated with mAb270. Fluorography of the dried gel revealed that mAb270 immunoprecipitated only the 55 kDa β2 subunit (Fig. 3). In contrast to the B3 and B4 subunits, the β2 subunit is remarkably stable and no proteolytic fragments are visible. The affinity-purified anti-β3 and anti-β4 antibodies will be referred to subsequently as B3 and B4, respectively. Taking this experiment, together with those described above, we conclude that mAb270, β3, and β4 react specifically with their cognate subunits.

We used these antibody preparations to study the distribution of the β3 and β4 subunits in rat brain. Regions of rat brain were dissected that were composed primarily of either cerebral cortex, striatum, hippocampus, cerebellum, or medulla. As shown in Figure 4*A*, B3 detected a single 60 kDa protein relatively strongly in striatum and cerebellum, but weakly in the hippocampal and cerebral cortex fraction. Expression of the β3 subunit in multiple areas of the brain was surprising because *in situ* hybridization experiments have suggested a very restricted pattern of expression for this subunit. This result may be explicable in terms both of

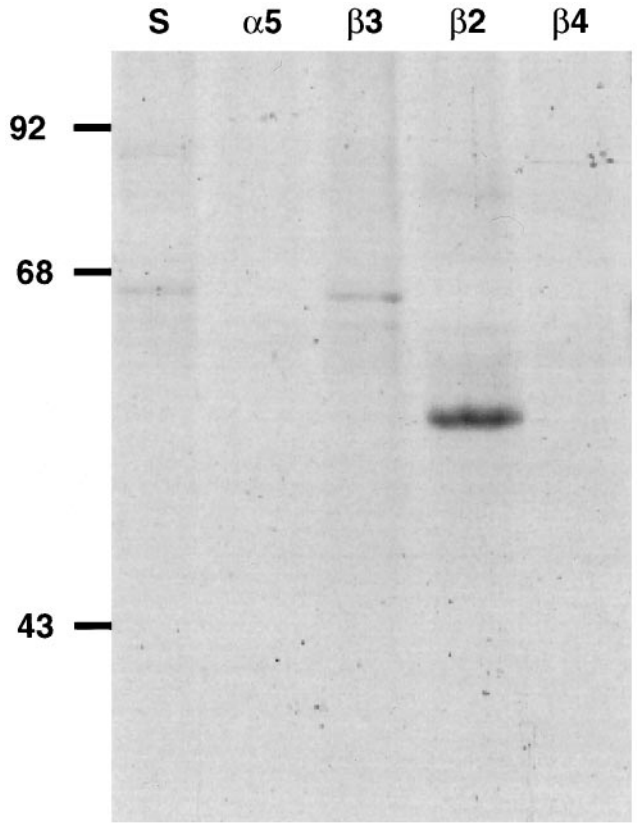

Figure 3. Immunoprecipitation of nAChR subunits by mAb270 (anti-β2 antibody). COS cells were transfected separately with one of the indicated subunit cDNAs. Two days after transfection, the cells were metabolically labeled with [^{35}S]methionine for 15 min, extracted in Triton buffer, and immunoprecipitated with mAb270 (see Materials and Methods). The precipitates were separated on a 7.5% PAGE that was then dried and flurographed with X-ray film for 1 d at −70°C.

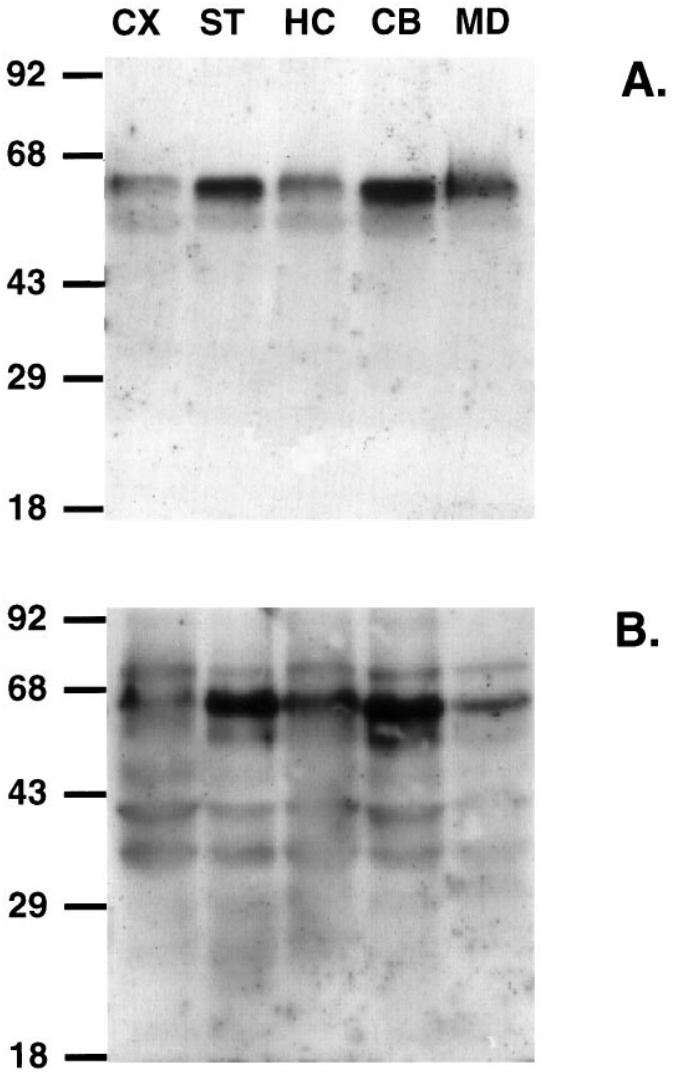

Figure 4. Western blot of rat brain by B3 and B4 antibodies. Rat brain was dissected to separate out the following regions: cerebral cortex (*CX*), striatum (*ST*), hippocampus (*HC*), cerebellum (*CB*), and medulla oblongata (*MD*). Each lane contained 0.25 mg of protein extracted in SDS-PAGE buffer and separated on a 7.5% gel. The proteins were transferred to PVDF membrane and blotted with β3 (*top panel*) or β4 (*bottom panel*) as described in Materials and Methods. The exposure time was ~3 min.

translational efficiency, stability of the subunit protein when it is assembled into a receptor and the sensitivity of immunodetection relative to that of *in situ* hybridization. Similarly, B4 detected a single protein of 66 kDa in all regions of the brain with the highest levels in striatum and cerebellum (Fig. 4*B*). This is consistent with the *in situ* study of Dineley-Miller and Patrick (1992), indicating a broad distribution of β4 subunit mRNA in rat brain. These experiments also indicated that B3 and B4 are specific for the β3 and β4 subunits and do not seems to cross-react with any other brain protein. Moreover, the data showed that both subunits are expressed relatively strongly in cerebellum. Therefore, we investigated this brain region more intensively.

Coassembly of the β3 and β4 subunits in cerebellum

Because the α4 and β2 subunits are both broadly distributed in the mammalian brain and have been detected in Northern blots of chick cerebellum (Nef et al., 1988), we examined whether these two subunits were coassembled with the β3 and β4 subunits. Because the colocalization of the β3 and β4 subunits in rat cerebellum corresponds well with the immunocytochemical distribution of the β2 subunit (Hill, 1993), we used a mAb directed against the β2 subunit to immunoprecipitate receptor from detergent extracts of synaptosomes made from rat cerebellum. The anti-β2 and control immunoprecipitates were separated on a polyacrylamide gel and then Western blotted with either B3 or B4 (Fig. 5). In the case of the B3 blot, a prominent band of 60 kDa was seen in the immune but not the control lane (Fig. 5*A*). The B4 antibody detected a specific band of ~66 kDa (Fig. 5*B*). It should be noted that these molecular weights are in close agreement with those obtained by direct blot of rat brain (Fig. 4). These observations suggested that the β3 and β4 subunits participate with the β2 subunit in the formation of nicotinic receptors in cerebellum.

We then investigated whether nicotinic receptor binding activity could be immunoprecipitated with antibodies against specific subunits from cerebellar extracts. A complicating factor in this experiment was that a large amount of [^{3}H]ACh binding in detergent extracts of the synaptosomes was attributable to bungarotoxin-sensitive receptors. Hence, both α-bungarotoxin and κ-bungarotoxin (1 μM each) were included in the immunoprecipitation reaction. This requirement was determined empirically in preliminary experiments (not shown, but see Luetje et al., 1990). From these experiments we determined that the β3 subunit could not coassemble with the α3 or the α7 subunit, but did

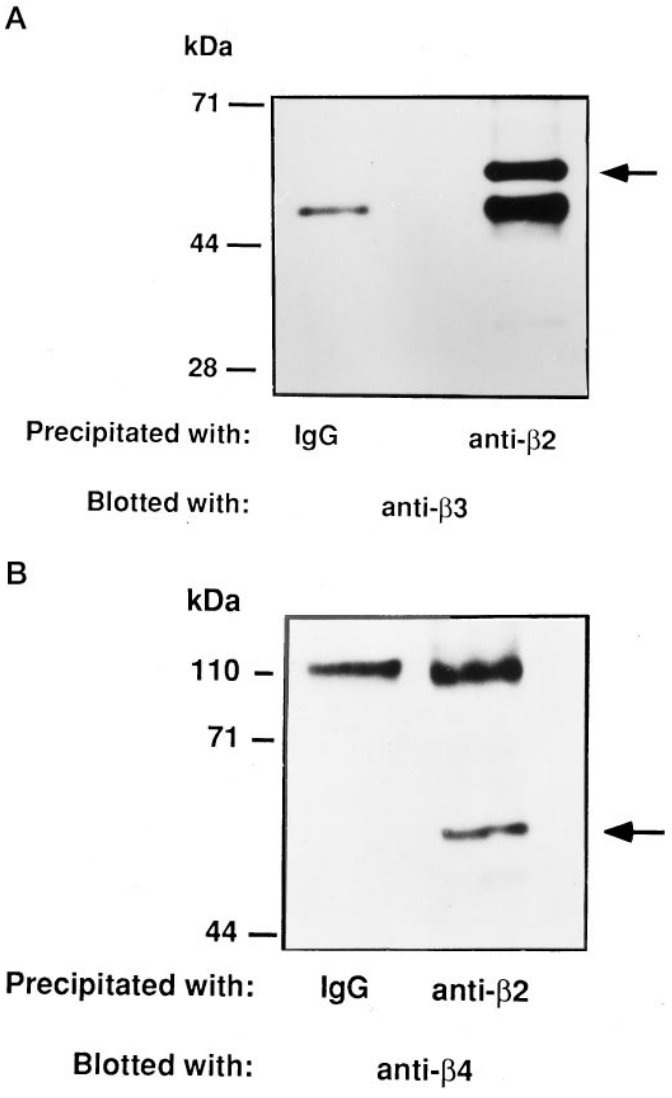

Figure 5. Immunoprecipitation of β3 and β4 subunits from cerebellum by anti-β2 subunit antibody. Crude synaptosomes (5 mg of protein) from rat cerebellum were extracted in 1.5% Lubrol and centrifuged at 100,000 × *g*, and the supernatant was precipitated with either mAb270, an anti-β2 subunit antibody, or nonimmune rat IgG, both at 100 nM. The immunoprecipitates were separated on a 7.5% gel and transferred to a PVDF membrane. They were then blotted with β3 (*A*) or β4 (*B*). The exposure time was ~0.5 min. The bands common to both lanes seem to be derived from the precipitating antibody.

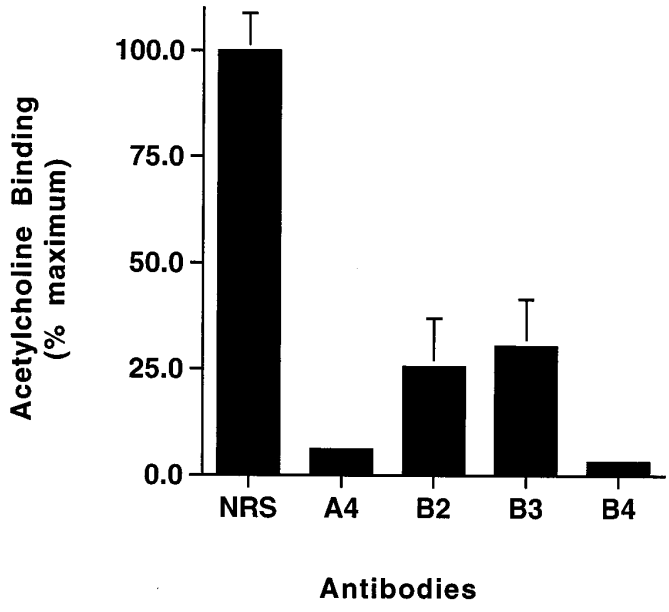

Figure 6. Immunodepletion of nAChR from detergent extracts of rat cerebellum. Lubrol extracts of rat cerebellum were incubated overnight at 4°C in the presence of 0.1 mM eserine, 10 μM atropine, 10 nM α-bungarotoxin, 10 nM κ-bungarotoxin, and the antibodies indicated. *NRS*, Normal rabbit serum; *A4*, anti-α4 subunit antibody (mAb299); *B2*, anti-β2 subunit antibody (mAb270); *B3*, anti-β3 subunit antiserum; *B4*, affinity-purified anti-β4 subunit antibody. NRS and β3 were used at a 1:100 dilution; α4, B2, and β4 were used at 100 nM. The primary antibodies were precipitated with protein G Sepharose, and the supernatant was assayed for residual acetylcholine binding. Nicotine (1 mM) was included in some replicates to determine nonspecific binding. Data are mean ± SEM of three determinations and are expressed as a percentage of the amount of ligand binding obtained in the presence of nonimmune serum. The control binding was ~600 dpm of [^{3}H]ACh specifically bound.

coassemble with the α4 subunit under the conditions described below. Because the α4 subunit is bungarotoxin insensitive, it seemed reasonable to include these two reagents to reveal the remaining ligand binding activity. Also, binding of [^{3}H]ACh to muscarinic receptors was blocked with 10 μM atropine. As shown in Figure 6, all four antibodies were able to precipitate at least 75% of the bungarotoxin-insensitive acetylcholine binding, demonstrating that the four subunits are likely part of a nicotinic receptor that seems to be relatively low in abundance because it can be demonstrated only when the far more abundant (at least 10-fold) toxin-binding receptors are first blocked. One possibility was that this binding activity was a result of the presence of a receptor with the composition α4β2β3β4. If this were so, then it should be possible to reconstitute this type of oligomer in transfected cells by cotransfecting cDNAs for the α4, β2, β3, and β4 subunits.

Reconstitution of nicotinic receptors in COS cells

Reconstitution of nicotinic receptors in transiently transfected COS cells has been used considerably to study assembly of the muscle nicotinic receptor (Gu et al., 1990, 1991; Forsayeth et al., 1992). Thus, COS cells were transfected with various combinations of subunits, and acetylcholine-binding activity was measured in the cells. As shown in Figure 7*A*, cotransfection of COS cells with the α4 and β3 subunits gave no ligand-binding activity, consistent with previous observations that this combination does not form functional channels when expressed in oocytes (Deneris et al., 1989). In contrast, coexpression of the α4 subunit with either the β2 or β4 subunit resulted in significant ligand-binding activity. However, the β4 subunit always directed higher levels of binding than did the β2 subunit, averaging a 10-fold difference in many replicates of this experiment. When all four subunits were coexpressed in COS cells, little change in the amount of ligand-binding activity was observed. Competitive binding experiments revealed a complex series of effects of the three β subunits on ligand-binding activity (Fig. 7*B*). When all four subunits [α4, β2, β3, and β4 (*open circles*)] were coexpressed in COS cells, nicotine competed with [^{3}H]ACh for binding with high affinity (apparent K_i, ~3 nM). Omission of only the β4 subunit (α4β2β3, *filled squares*) reduced the amount of ligand binding drastically to about the same level as obtained with α4 and β2 alone, and slightly altered the shape of the displacement curve, increasing the ap-

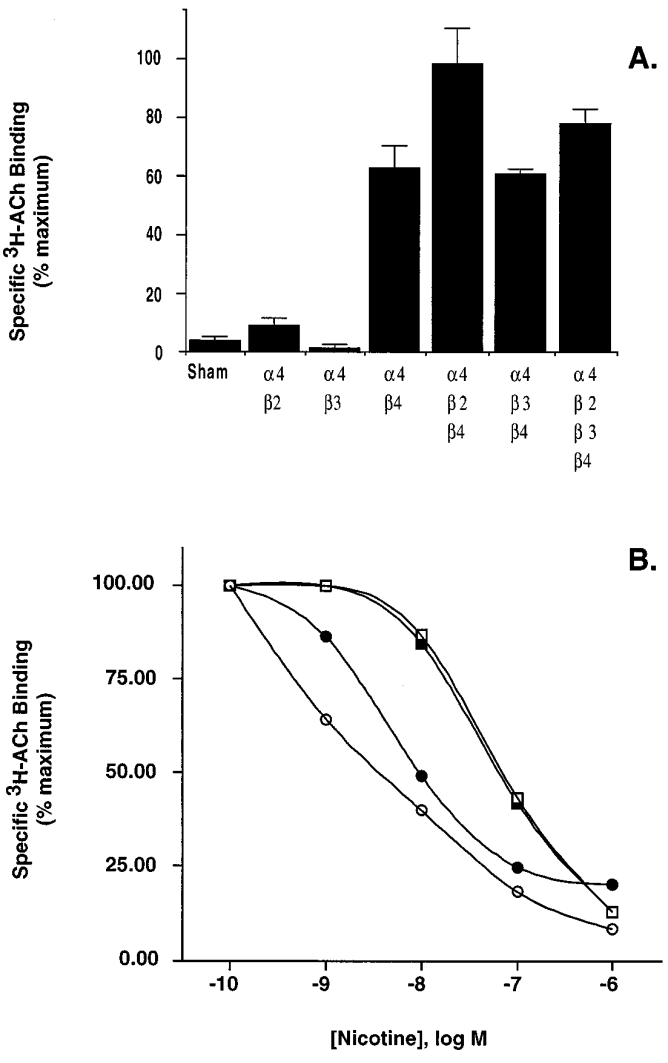

Figure 7. Expression of complex mixtures of subunits in COS cells. *A*, COS cells were transfected with the indicated combinations of subunit cDNAs in roughly equimolar concentration. After 2 d of incubation, the cells were extracted in detergent and assayed for [^{3}H]ACh binding (see Materials and Methods). Nicotine (10 mM) was used to determine nonspecific binding. Data are mean ± SEM of three determinations and are expressed as a percentage of the maximum binding activity obtained with the $\alpha4\beta2\beta4$ combination. *B*, COS cells were transfected with different combinations of subunit cDNAs indicated by the following symbols: $\alpha4\beta2\beta3\beta4$ (*open circle*), $\alpha4\beta2\beta4$ (*filled circle*), $\alpha4\beta2\beta3$ (*filled square*), or $\alpha4\beta3\beta4$ (*open square*). Two days after transfection, the cells were extracted in detergent and incubated with 20 nM [^{3}H]ACh and the indicated concentrations of nicotine. Nonspecific binding, determined in the presence of 1 mM nicotine, was subtracted from the total binding obtained in each case. Data are mean ± SEM of three determinations. The errors are smaller than the size of the symbol. To provide better comparison, the data are expressed as a percentage of the maximum binding obtained in the absence of nicotine. Those combinations lacking β4 subunit were always ~10-fold lower in maximum binding than those that include the subunit. The amount of receptor obtained was as follows: $\alpha4\beta2\beta3\beta4$, $\alpha4\beta2\beta4$, or $\alpha4\beta3\beta4$: 333 ± 35 fmol/10 cm dish; $\alpha4\beta2\beta3$: 40 ± 5 fmol/10 cm dish.

parent K_i to ~10 nM. A more dramatic shift was seen when only the β3 subunit was omitted ($\alpha4\beta2\beta4$, *open squares*). In that case, the apparent K_i was increased to ~100 nM, but the B_{max} was not appreciably affected. A virtually identical effect was obtained by omitting only the β2 subunit ($\alpha4\beta3\beta3$, *filled circles*). The low

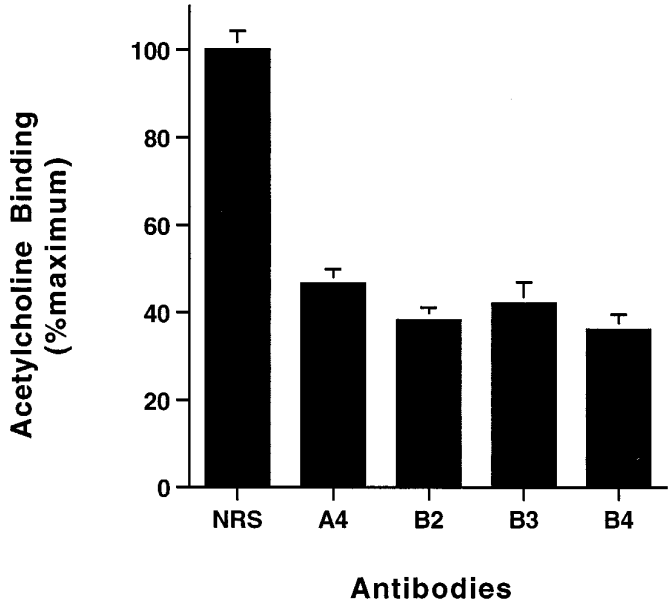

Figure 8. Immunodepletion of nAChR from transfected COS cells. COS cells were transfected with cDNAs for all four subunits (α4, β2, β3, and β4). Two days after transfection, aliquots of detergent extracts of the cells were immunoprecipitated with the indicated antibodies (see legend to Fig. 3). The remaining acetylcholine binding activity in the supernatant was then determined. Nicotine (1 mM) was included in some replicates to determine nonspecific binding. Data are mean ± SEM of three determinations and are expressed as a percentage of the amount of ligand binding obtained in the presence of nonimmune serum.

affinity-binding curves are indistinguishable from those obtained when only the α4 and β4 subunits are coexpressed (data not shown).

To establish that the β3 subunit was in fact coassembling into a ligand-binding oligomer, detergent extracts of COS cells transfected with all four subunits were depleted of [^{3}H]ACh-binding activity with subunit-specific antibodies (Fig. 8). This experiment showed that antibodies against each of the subunits precipitated the majority of the ligand-binding activity from cell extracts. The depletion observed is lower than seen in the cerebellar extracts (Fig. 6), presumably because overexpression of nAChRs frequently results in the formation of ligand-binding intermediates that contain differing stoichiometric mixtures of subunits (Gu et al., 1991). We conclude that the effect of the various β subunits on ligand-binding affinity and abundance is mediated by participation of the α4, β2, β3, and β4 subunits in a single oligomer. Considering these results, together with those obtained with cerebellar extracts (Figs. 5, 6), it is reasonable to conclude that a receptor of composition, $\alpha4\beta2\beta3\beta4$, exists in rat cerebellum.

DISCUSSION

Since its identification by molecular cloning, there has been little progress on the role of the β3 subunit in nicotinic receptor structure. It has not been possible to establish a functional role for it in oocyte recording experiments, because it does not alter any observable parameter in electrophysiological assay in the presence of other subunits. Moreover, the subunit has not been demonstrated to form part of a nicotinic receptor in brain. The present study demonstrates for the first time that the nicotinic β3 subunit is present in brain as part of a receptor. This conclusion is based on three major lines of evidence.

First, our antibodies detected the β3 and β4 subunits in rat

brain in a region-dependent manner, and revealed an interesting distribution for both subunits, not easily predicted by *in situ* hybridization, perhaps explained by differing stabilities of receptor subtypes. We found that the two areas with the most significant levels of β3 and β4 subunits were the striatum and the cerebellum. The presence of these subunits in the cerebellum was surprising because this region of the brain has a relative paucity of nicotinic receptors, although nAChRs are present both in Purkinje cells (Garza et al., 1987a,b) and in granule cells (Didier et al., 1995). However, little is known about their subunit composition.

Second, the β3 and β4 subunits were detected in immunoprecipitates with anti-β2 subunit specific antibody and was the first indication that perhaps all three subunits coexisted in a single oligomer. To establish that the cerebellum contained the β2, β3, and β4 subunits as part of a receptor that binds acetylcholine in a nicotine-dependent manner, we performed immunodepletion experiments with antibodies against the three β subunits and with an anti-α4 subunit antibody. The α4 subunit was considered a likely partner in view of its general codistribution with the β2 subunit (Nef et al., 1988). Also, our transfection experiments indicated that the α4 subunit was the only α subunit that could form a receptor with all three subunits (data not shown). Although antibodies against the α4, β2, β3, and β4 subunits depleted extracts of at least 75% of the ligand-binding activity from cerebellar extracts, this effect was observable only when much of the ligand-binding activity was suppressed by addition of α-bungarotoxin and κ-bungarotoxin to the extract. On this basis, we concluded that the cerebellum contains a low abundance of a receptor composed of the α4, β2, β3, and β4 subunits. The results underscore the possibility that certain subtypes of nicotinic receptor may represent a very small proportion of the population of nicotinic receptors in a given tissue, and may explain why these types of receptor have been undetected until now.

The third major line of evidence pointing to a complex oligomer that contains these four subunits comes from experiments in which COS cells were transfected with various combinations of subunits. We have found that binary concentrations of subunits (α4β2 or α4β4) give widely different abundance in COS cells; the β4 subunit directs about a 10-fold higher level of binding activity than does the β2 subunit. However, the β2 subunit shifts the apparent K_i to the left relative to the β4 subunit curve. Thus, two kinds of receptors can be formed: one with a high affinity for agonist but low abundance (α4β2), and another with low affinity but high abundance (α4β4). In separate experiments (data not shown), we have found that these combinations of subunits direct the appearance of nicotine-sensitive channels on the cell surface. Expression of complex mixtures of subunits revealed an interplay between these two moieties that depended on the presence of the β3 subunit.

Transfection of COS cells with all four subunits (α4β2β3β4) and immunoprecipitation of ligand-binding activity showed that all four subunits coassembled in these cells. To establish the role of each subunit in this receptor, they were omitted from transfections in which all the other subunits were present. Omission of β subunits changed one of two parameters: either the abundance (B_{max}) of receptor in the cell or the affinity (K_i) of the acetylcholine binding activity, depending on whether the α4β2 or α4β4 type binding sites are present in the oligomer. When all four subunits are present (α4β2β3β4), an abundance of the β4 type is seen, but receptor affinity is of the β2 type. Omission of only the β3 subunit causes a reversion to a simple α4β4 type of curve. This suggests that the β3 subunit is able to upregulate the high affinity α4β2 binding site by linking it to the more stable α4β4 heterodimer. When the β4 subunit is omitted, the receptor abundance and ligand affinity is similar to that obtained with just α4 and β2. From these experiments, we advance the following hypothesis. Expression of all four subunits directs formation of a complex receptor that contains a high affinity nicotine-binding site (α4β2) and a low affinity binding site (α4β4). These two heterodimeric binding sites are linked together by the β3 subunit. Because the α4β2 combination is much less stable than is the α4β4 combination, omission of the β3 subunit leads effectively to elimination of α4β2 as a significant component and is replaced by the considerably more stable α4β4. Similarly, elimination of the β4 subunit, leaving only the α4, β2, and β3 subunits, results in accumulation of the high affinity but low abundance α4β2 site. These experiments do not, of course, rule out the possibility that the β3 subunit can form oligomers with only two other subunits (e.g., α4 and β2), but it is clear that no substantial effect of the β3 subunit can be seen unless all four subunits are present.

In some respects, the interaction of the four different subunits (α4, β2, β3, and β4) is reminiscent of the nAChR in muscle and of a ganglionic α3 receptor (α3α5β4). Thus, the β2 and β4 subunits combine with the α4 subunit to create agonist-binding sites of differing binding characteristics, as do the γ, ε, and δ subunits with the α1 subunit in the muscle nAChR. To carry the analogy somewhat further, the β3 subunit seems to function like the β1 subunit; that is, it functions as a linker subunit joining two nonequivalent ligand-binding heterodimers into a pentamer, but is incapable of inducing the formation of ligand binding in an α subunit. This type of subunit has been referred to as "conditional" (Gu et al., 1991). Thus, the β1 and β3 subunits can properly assemble into an oligomer only when they interact with an appropriate heterodimer. Similarly, because the β2, β4, γ, ε, and δ subunits all induce ligand binding in an α subunit, they too could be thought of as members of a single functional class.

Work by Vernallis et al. (1993) has indicated that, in ganglionic neurons, the α3, α5, and β4 subunits can combine to form complex oligomers. Functional assays in oocytes suggest that the α5 subunit when part of an α4α5β2 oligomer increases the response of the channel to nicotine and desensitizes more rapidly (Ramirez-Latorre et al., 1996). In view of the homology between the α5 and β3 subunits, it seems likely that the β3 subunit may have a similar role. It is important to note, however, that the α5 subunit does not seem to be able to substitute for the β3 subunit in transfected COS cells (J. Forsayeth, unpublished data). Nevertheless, the functional differences reported by Ramirez-Latorre et al. make *in vivo* receptor structure an important question. In addition to its possible effects on channel function, it is possible that the β3 subunit confers other properties on the receptor unrelated to channel function but more related to synaptic localization analogous to what has been observed for certain NMDA receptor (Ehlers et al., 1995; Kornau et al., 1995) and glycine receptor subunits (Meyer et al., 1995).

Our identification of a novel subtype of nAChR in the cerebellum suggests that this receptor may play a role in modulating the inhibitory output of the cerebellum and thus influence motor function. It is clear that nicotine not only influences motor function (Collins et al., 1988; Marks et al., 1992) but also acts to inhibit the depressive effects of ethanol on motor coordination (Dar et al., 1994). It should be noted that Purkinje cells also seem to express α7-type receptors (Garza et al., 1987b), and nicotinic receptors are also present on granule cells (Didier et al., 1995); hence, the effects of nicotine in this tissue may be complex and not

attributable to only one type. The fact that the β3 subunit has been detected in various brain regions by us and in retina by others (Hernandez et al., 1995) suggests that β3/β4 receptors might exist in these areas as well. Further experiments must focus on understanding the neurobiological function of these complex nicotinic receptors.

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