

Chick Optic Lobe Contains a Developmentally Regulated $\alpha 2\alpha 5\beta 2$ Nicotinic Receptor Subtype¹

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ABSTRACT

The most widely expressed neuronal nicotinic acetylcholine receptor subtype in chick brain is that containing the $\alpha 4$ and $\beta 2$ subunits. However, immunoprecipitation and localization studies have shown that some brain areas also contain the $\alpha 2$ and/or $\alpha 5$ subunits, whose role in the definition of receptor properties is still intriguing. Using subunit-specific polyclonal antibodies, we found that the optic lobe is the chick central nervous system region that expresses the highest level of $\alpha 2$ -containing receptors. Immunoprecipitation studies of these immunopurified $\alpha 2$ -containing receptors labeled with the nicotinic agonist [³H]epibatidine showed that almost all of them contained the $\beta 2$ subunit and that more than 66% contained the $\alpha 5$ subunit. Western blot analyses of the purified receptors confirmed the presence of the $\alpha 2$, $\alpha 5$, and $\beta 2$ subunits and the

absence of the $\alpha 3$, $\alpha 4$, $\alpha 6$, $\alpha 7$, $\alpha 8$, $\beta 3$, and $\beta 4$ subunits. The $\alpha 2$ -containing receptors are developmentally regulated: their expression increases 25 times from embryonic day 7 to post-hatching day 1 in the optic lobe, compared with an increase of only 5-fold in the forebrain. The $\alpha 2$ -containing optic lobe receptors bind [³H]epibatidine ($K_d = 29$ pM) and a number of other nicotinic agonists with very high affinity and have a pharmacological profile very similar to that of the $\alpha 4\beta 2$ subtype. They form functional cationic channels when reconstituted in lipid bilayers, with pharmacological and biophysical properties different from those of the $\alpha 4\beta 2$ subtype. These channels are activated by nicotinic agonists in a dose-dependent manner and are blocked by the nicotinic antagonist *d*-tubocurarine.

The neuronal nicotinic acetylcholine receptors (nAChRs) are a family of structurally diverse acetylcholine (ACh)-gated cation channels expressed differently in the central and peripheral nervous systems of vertebrates (reviewed in Sargent, 1993; Role and Berg, 1996; Gotti et al., 1997a).

Eleven genes coding for the neuronal nAChR subunits have been cloned so far ($\alpha 2$ – $\alpha 9$, $\beta 2$ – $\beta 4$). At least two classes of receptors (consisting of various subtypes with distinct functional and pharmacological properties) can be generated in heterologous systems: homomeric receptors formed by the expression of a single $\alpha 7$, $\alpha 8$, or $\alpha 9$ subunit and heteromeric receptors formed by pairwise combinations of the $\alpha 2$, $\alpha 3$, $\alpha 4$, or $\alpha 6$ with the $\beta 2$ or $\beta 4$ subunits, or by the coexpression of the $\alpha 5$ or $\beta 3$ subunit with another α - and β -subunit (reviewed in McGehee and Role, 1995; Lindstrom, 2000).

Much of our knowledge of the structure and pharmacology of these receptors comes from studies of heterologously ex-

pressed nAChR subtypes, but recent data have clearly shown that channels with different pharmacological and biophysical properties can be obtained depending on the expression system used (Fucile et al., 1997; Lewis et al., 1997). The results of biochemical and immunological experiments in vertebrate brain and ganglia suggest that native nAChRs may be more complex and heterogeneous than previously thought, and we still do not know the subunit composition and function of a number of nicotinic receptors present in vivo (Vernallis et al., 1993; Conroy and Berg, 1995; Forsayeth and Kobrin, 1997; Vailati et al., 1999).

The major subtype in the brain is the $\alpha 4\beta 2$ subtype, which accounts for most of the high-affinity nicotine binding sites. Its physiological role is not completely clear, although it is mainly located presynaptically, where it can modulate neurotransmitter release (reviewed in Role and Berg, 1996; Wonnacott, 1997). This subtype is also involved in pathology, in that it is up-regulated in the brain of human smokers by means of an adaptive process in response to its desensitization (Dani and Heinemann, 1996; reviewed in Gotti et al.,

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ABBREVIATIONS: nAChR, neuronal nicotinic acetylcholine receptor; α -Bgt, α -bungarotoxin; mAb, monoclonal antibody; SpL, spiriform nucleus; ACh, acetylcholine; Epi, epibatidine; Carb, carbamylcholine; P_o , global open-state probability; Abs, polyclonal antibodies; Cyt, cytoplasmic peptide; DMPP, 1,1-dimethyl-4-phenylpiperazinium.

1997a), and a mutation in the $\alpha 4$ subunit (S247F) produces autosomal dominant nocturnal frontal lobe epilepsy (reviewed in Léna and Changeux, 1997). It has recently been reported that a developmentally regulated $\alpha 4\alpha 5\beta 2$ subtype is also expressed in chick brain (Conroy and Berg, 1998).

Limited chick and mammalian brain areas also have $\alpha 2$ -containing nAChR subtypes that have been well characterized in heterologous systems but not in vivo. The presence of the $\alpha 2$ subunit in chick brain was first reported in the pioneering work of the group of Lindstrom (Whiting and Lindstrom 1986; Whiting et al., 1987), who used monoclonal antibody (mAb) 35 (an mAb directed against the main immunogenic region of muscle AChR that also recognizes the neuronal $\alpha 5$ and $\alpha 3$ subunits) to purify receptors containing the $\alpha 4$, $\alpha 2$, and $\beta 2$ subunits that were later also found to contain the $\alpha 5$ subunit (Conroy et al., 1992).

High levels of the $\alpha 2$ subunit as both mRNA and protein exist in the lateral spiriform nucleus (SpL) of the pretectum, which projects into the optic tectum and also has high levels of the $\alpha 5$, $\alpha 7$, and $\beta 2$ subunits (Daubas et al., 1990; Ullian and Sargent, 1995). Chiappinelli's group has shown that neurons of this nucleus express a heterogeneous family of functional nAChR that are insensitive to α -bungarotoxin (α -Bgt) and κ -bungarotoxin on their somata and/or dendrites (Sorenson and Chiappinelli, 1990; Weaver and Chiappinelli, 1996). They also demonstrated very recently that endogenously released ACh can generate fast excitatory nicotinic transmission in postsynaptic SpL neurons (Nong et al., 1999).

However, the subunit composition and the pharmacological and biophysical properties of native $\alpha 2$ -containing receptors are not yet known.

In this study, we purified the $\alpha 2$ -containing receptors; characterized their subunit composition, ligand-binding properties, and electrophysiological and pharmacological characteristics after reconstitution in lipid bilayers; and compared them with those of the $\alpha 4\beta 2$ subtype.

Experimental Procedures

Antibody Production and Characterization

Both the mAb 270, raised against chicken brain nAChR that recognizes the $\beta 2$ subunit (Whiting et al., 1987), and the mAb 323, directed against the $\alpha 2$ subunit, were generously given by Dr. Lindstrom. mAb 35, which was raised against the muscle-type AChR, recognizes the $\alpha 1$ subunit and cross-reacts with the $\alpha 5$ subunit (Conroy et al., 1992). It was purified from a hybridoma cell line obtained from the American Type Culture Collection (Rockville, MD). The mAb 299 raised against rat brain nAChR was directed against the $\alpha 4$ subunit (Whiting and Lindstrom, 1988), and mAb 313 was raised against the fusion protein containing the putative cytoplasmic of the $\alpha 3$ subunit (Whiting et al., 1991a; both were purchased from Research Biochemicals International (Natick, MA).

The polyclonal antibodies (Abs) against the $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 6$, $\alpha 7$, $\alpha 8$, $\beta 2$, $\beta 3$, and $\beta 4$ peptides were raised and characterized as described by Vailati et al., (1999). In this study, we also used Abs directed against the cytoplasmic peptide (Cyt) LPAEGTTGQYDPPGTRLSTSRC of the $\alpha 2$ subunit. The Abs raised against the peptides were purified on an affinity column made by coupling the corresponding peptide to cyanogen bromide-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions.

The antipeptide serum titers were evaluated by means of enzyme-linked immunosorbent assays and Western blots of the purified subtypes. The serum Abs were specific only for their respective

immunizing peptide in enzyme-linked immunosorbent assay, and each immunoprecipitation and immunolabeling was specifically inhibited only by the peptide used for the immunization.

The affinity-purified Abs were bound to cyanogen bromide-activated Sepharose at a concentration of 1 mg/ml, and the columns were used for immunopurification.

Receptor Subtype Immunopurification

$\alpha 2\alpha 5\beta 2$ and $\alpha 4\beta 2$ Optic Lobe Subtypes. The chick optic lobe and retina extracts were prepared as previously described by Gotti et al., (1994, 1997b). For each experiment, we used 36 g of optic lobe or 150 g of chick eyes. The tissue was homogenized in an excess of 50 mM sodium phosphate, pH 7.4, 1 M NaCl, 2 mM EDTA, 2 mM EGTA, and 2 mM phenylmethylsulfonyl fluoride for 2 min in an ultra-Turrax homogenizer. The homogenate was then diluted and centrifuged for 1.5 h at 60,000g.

This homogenization, dilution, and centrifugation procedure was performed three times, after which the pellets were collected; rapidly rinsed with 50 mM sodium phosphate, 50 mM NaCl, 2 mM EDTA, 2 mM EGTA, and 2 mM phenylmethylsulfonyl fluoride; and then resuspended in the same buffer containing a mixture of a 5 μ g/ml concentration of each of the following protease inhibitors: leupeptin, bestatin, pepstatin A, and aprotinin (Sigma Chemical Co., St. Louis, MO). Triton X-100 at a final concentration of 2% was added to the washed membrane, and the membrane was extracted for 2 h at 4°C. The extract was then centrifuged for 1.5 h at 60,000g and recovered.

To remove the $\alpha 3$ - and $\beta 4$ -containing receptors, the extract was first incubated with 5 ml of Sepharose-4B with bound anti- $\alpha 3$ Abs and then with 5 ml of Sepharose-4B with anti- $\beta 4$ Abs.

The optic lobe extract depleted of the receptors containing the $\alpha 3$ and $\beta 4$ subunits was then incubated twice with Sepharose 4B with bound anti- $\alpha 2$ Cyt Abs. The bound receptors were eluted with 0.2 M glycine (pH 2.2) or a 100 μ M concentration of the corresponding $\alpha 2$ peptide used for Ab production as described by Gotti et al., (1994; $\alpha 2$ -containing receptors).

The flow-through of the anti- $\alpha 2$ Abs column was first incubated with anti- $\alpha 5$ Abs to remove the residual $\alpha 5$ -containing receptor and then with the resin with bound anti- $\beta 2$ Abs. The bound receptors were eluted with 0.2 M glycine (pH 2.2) or with a 100 μ M concentration of the corresponding $\beta 2$ peptide used for Ab production.

In preliminary experiments performed to test the specificity of the Abs, the total extract obtained from optic lobe or retina was incubated with Abs directed against the $\beta 2$ -Cyt or $\beta 4$ -COOH peptides and the bound receptor was eluted with 0.2 M glycine or the corresponding peptides.

Forebrain $\alpha 4\alpha 5\beta 2$ Subtype. When $\alpha 5$ -containing receptors were purified from the chick forebrain, the tissue extract was prepared and immunodepleted of $\alpha 3$ - and $\beta 4$ -containing receptors as described for the optic lobe. The extract was then directly incubated with an affinity resin with bound anti- $\alpha 5$ -COOH Abs, and the receptors were eluted by means of the $\alpha 5$ peptide.

For each subtype purification, recovery was determined by means of [3 H]epibatidine (Epi) binding and quantitative immunoprecipitation of the receptors present in the solution before and after each immunopurification step as previously described (Gotti et al., 1994).

Receptor Immobilization by Subunit-Specific Abs

The affinity-purified anti- $\alpha 2$ or anti- $\beta 2$ Abs were bound to the microwells (Maxi-Sorp; Nunc, Naperville, CT) by means of overnight incubation at 4°C at a concentration of 10 μ g/ml in 50 mM phosphate buffer, pH 7.5. On the next day, the wells were washed to remove the excess unbound Abs and then incubated overnight at 4°C with 200 μ l of 2% Triton X-100 optic lobe membrane extract containing 100 to 200 fmol of [3 H]Epi binding sites prepared as follows. On the wells plated with anti- $\alpha 2$ Abs, the added extract was depleted of $\alpha 3$ - and $\beta 4$ -containing receptors; on the wells coated with anti- $\beta 2$ Abs, it was also depleted of $\alpha 5$ -containing receptors. The immunodepletion of the

extract was performed as described earlier. After overnight incubation with the extract, the wells were washed and the presence of immobilized receptors was revealed by means of [^3H]-Epi binding.

Immunoprecipitation of [^3H]-Epi-Labeled Receptors by Anti-Subunit-Specific Abs during Brain Development

The optic lobes and forebrain plus cerebellum samples were dissected from in ovo chicks on embryonic days 7, 11, 14, and 18 (E7, E11, E14, and E18, respectively) and from 1-day-old chicks (P1); immediately frozen in liquid nitrogen; and stored at -80°C for later use. No differences were observed in the binding properties of the fresh and frozen tissues. At every experiment, the extracts of the two tissues were prepared as described above, preincubated with $2\ \mu\text{M}$ α -Bgt, and then labeled with $2\ \text{nM}$ [^3H]-Epi, and incubated overnight with a saturating concentration of affinity purified IgG ($20\text{--}30\ \mu\text{g}$). Sufficient goat anti-rabbit IgG was added to precipitate all of the immunoglobulins present in the samples and was maintained for 2 h at room temperature. The samples were centrifuged for 15 min in a microcentrifuge ($10,000g$). The pellets were washed twice using wash buffer plus 0.1% Triton X-100 and then counted by means of a beta-counter. The level of Ab immunoprecipitation was expressed as the percentage of [^3H]-Epi-labeled receptors immunoprecipitated by the indicated Abs, taking the amount present in the Triton X-100 extract solution before immunoprecipitation as 100%.

Binding Assay and Pharmacological Experiments

(\pm)-[^3H]-Epi (specific activity, $54.6\ \text{Ci}/\text{mmol}$) was obtained from Amersham International (Buckinghamshire, UK). Nonradioactive Epi was obtained from Research Biochemicals International. Nonradioactive α -Bgt and all of the cholinergic ligands were obtained from Sigma Chemical Co.

Membrane. Preliminary saturation experiments were performed overnight by incubating aliquots of optic lobe membrane with [^3H]-Epi concentrations ranging from 0.005 to $5\ \text{nM}$ at 20°C . Nonspecific binding (averaging $10\text{--}15\%$ of total binding) was determined in parallel by means of incubation in the presence of $100\ \text{nM}$ unlabeled Epi. The binding techniques used for solubilized receptors and for immunoimmobilized subtypes, as well as the data analysis, were the same as those previously described (Vailati et al., 1999).

[^3H]-Epi Binding to Solubilized Receptor. Binding to tissue extracts were performed using DE52 ion-exchange resin (Whatman, Maidstone, UK) as previously described (Vailati et al., 1999).

Like Gerzanich et al. (1995), we found that [^3H]-Epi binds with high affinity to the $\beta 2$ - and $\beta 4$ -containing subtypes (picomolar affinity), but it also binds to the $\alpha 7$ subtypes with a low nanomolar affinity and to the $\alpha 8$ -containing receptor with picomolar affinity. To ensure that the $\alpha 7$ and $\alpha 8$ subtypes did not contribute to [^3H]-Epi binding in tissue extracts, the binding and immunoprecipitation experiments were performed in the presence of $2\ \mu\text{M}$ α -Bgt, which specifically binds to the $\alpha 7$ and $\alpha 8$ subtypes and blocks [^3H]-Epi binding.

Bilayer Formation and Subtype Insertion

The purified subtypes eluted from the corresponding immunoaffinity columns were dialysed, concentrated, and stored at -20°C until use. The purified receptors were incorporated in asolecithin liposomes (Sigma Chemical Co.) by means of dialysis and then fused with preformed bilayers (Gotti et al., 1994, 1997). The current fluctuation traces under different conditions were observed on an oscilloscope and recorded on a computer for later analysis. In our experiments, traces with more than one open state level were rare and are disregarded in the analysis. The integral amplitude histograms were constructed from current fluctuation traces digitized at a sampling rate of $2000\ \text{points}/\text{s}$ and low-pass prefiltered at $1\ \text{kHz}$. Typically, $60\ \text{s}$ of longer stored traces were digitized for one histogram. Two gaussian distributions were fitted (closed and open state) in the histograms (main peaks), and the open channel current at the given

potential was calculated from the distance between the two peaks. Current-voltage curves were constructed from all of the histograms, and the channel conductances were calculated from the linear portion of the curves of the two subtypes. In addition, the global open-state probability (P_o) was calculated from the areas under the peaks of the histograms. Preliminary experiments were performed by adding $1\ \text{mM}$ carbamylcholine (Carb) to the *trans* or *cis* side of the bilayer to identify the orientation of the channels. In the reported experiments, the agonists dissolved in $150\ \text{mM}$ NaCl and $5\ \text{mM}$ Tris-HCl (at the concentrations given under *Results*) were applied to the side of the bilayer in which the channels have been correctly incorporated. The 50% activation value (EC_{50}) was calculated from the plot P_o versus [agonist], as the value of the agonist concentration necessary to obtain a level of activity midway between spontaneous activity and maximum P_o . Each experiment was repeated at least five times, so all of the data in the graphs are given as mean \pm S.D. values. Further details of the experimental procedures have been previously described (Gotti et al., 1994, 1997b).

Materials. The lyophilized α -Bgt, anti-protease inhibitors, asolecithin type IIS, cholinergic ligands, Triton X-100, and anti-rabbit and anti-rat antisera were purchased from Sigma Chemical Co. Nonradioactive Epi was obtained from Research Biochemical International. CnBr-activated Sepharose 4BCL was purchased from Pharmacia. ^{125}I -Protein A and [^3H]-Epi were obtained from Amersham International. The reagents for gel electrophoresis were obtained from Bio-Rad Laboratories (Hercules, CA).

Results

Regional Distribution of [^3H]-Epi Binding Receptors in Chick Central Nervous System

We performed preliminary experiments on tissues obtained from 1-day-old chicks (P1) to determine the presence and amount of high-affinity [^3H]-Epi-labeled receptors in different central nervous system areas. We prepared 2% Triton X-100 extracts from optic lobe, forebrain, cerebellum, and retina and performed binding using $2\ \text{nM}$ [^3H]-Epi in the presence of $2\ \mu\text{M}$ α -Bgt (see *Experimental Procedures*). We found that retina contains the highest level of receptors ($246 \pm 15\ \text{fmol}$ of [^3H]-Epi-labeled receptors/mg protein), closely followed by optic lobe (225 ± 10); there are fewer in the forebrain (107 ± 6) and cerebellum (56 ± 8).

Characterization of Abs against $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\beta 2$, and $\beta 4$ Subunits

Immunoprecipitation Experiments. We produced Abs against the $\alpha 2$ Cyt peptide of the $\alpha 2$ subunit; given that the $\alpha 4$ peptide cgPPWLAGMI has an almost identical sequence as that of the C-terminal $\alpha 2$ peptide cgPPYLAGMI, we tested whether our anti- $\alpha 4$ COOH Abs could also pick up receptors containing the $\alpha 2$ subunits. Immunoprecipitation experiments using anti- $\alpha 2$ Cyt in the retina, optic lobe, and forebrain showed that the anti- $\alpha 2$ Abs immunoprecipitated a substantial number of receptors in retina ($18.8 \pm 3\%$) and in the optic lobe ($31 \pm 2\%$) but only $4.6 \pm 1.1\%$ of the receptors in the forebrain. Because the forebrain had a much lower $\alpha 2$ content than optic lobe, we used our anti-subunit-specific Abs to verify the subunit content of forebrain receptors (see Table 1). With the major exception of the $\alpha 2$ subunit and with some slight differences in the content of the $\alpha 3$, $\alpha 5$, $\alpha 6$, and $\beta 3$ subunits, the subunit content of the receptors in the forebrain is very similar to that of the receptors in the optic lobe (the majority contain the $\beta 2$ and $\alpha 4$ subunits and a minority contain the $\alpha 3$ and $\beta 4$ subunits) but different from that of the

receptors in the retina (see Vailati et al., 1999). The ratio of the immunoprecipitation obtained between the anti- α 4-COOH and anti- α 4 Cyt was relatively higher in the optic lobe (1.91) and retina (1.8) than in the forebrain (1.38), thus suggesting that the anti- α 4-COOH Abs can also recognize the α 2 subunit.

Western Blot Analysis of Abs. We have previously demonstrated that chick optic lobe is highly enriched in nAChRs that bind [3 H]Epi and contain the α 4 and β 2 subunits, whereas the majority of nAChRs of the retina that bind [3 H]Epi contain the β 4 subunit and are very heterogeneous in terms of their α -subunit content (Vailati et al., 1999).

We used anti- β 2 Abs to purify the optic lobe receptors containing the β 2 subunit and anti- β 4 Abs to purify the β 4-containing retina receptors, and we checked the Ab specificity on blots of the immunopurified subtypes. Blots of the receptors purified from chick optic lobe by means of affinity chromatography on anti- β 2-Cyt Abs bound to Sepharose 4B were tested using anti- α 4 (α 4-Cyt and α 4-COOH), anti- α 2 (α 2-Cyt), anti- α 5 (α 5-Cyt and α 5-COOH), and anti- β 2 (β 2-Cyt and β 2-COOH) Abs, as well as mAb 299 (specific for the α 4 subunit) and mAb 323 (specific for the α 2 subunit). The results are shown in Fig. 1, top. The anti- α 4-Cyt Ab recognized only one band of molecular mass 68 kDa (lane 2), which was also recognized by mAb 299 (lane 1); in addition to the 68-kDa band, the anti- α 4-COOH (lane 3) also recognized a band of 59 kDa, the same peptide recognized by the anti- α 2-Cyt Abs (lane 5) and mAb 323 (lane 4). In the case of the anti- α 5 Abs, the α 5-Cyt and α 5-COOH Abs (lanes 6 and 7) recognized the same band of 51 ± 0.5 kDa, whereas the anti- β 2 Abs only recognized a single band of 53.6 ± 0.6 kDa (lanes 9 and 10), which was also recognized by mAb 270 (lane 8).

Using the anti- β 4-COOH Abs, we purified receptors from chick retina and probed them with the anti- α 3 (α 3-COOH and α 3-Cyt) and mAb 313 and the anti- β 4 Abs (β 4-COOH and β 4-Cyt); the results are shown in Fig. 1, bottom. mAb 313

(lane 11), anti- α 3-Cyt (lane 12), and anti- α 3-COOH (lane 13) recognized the same band of 57 kDa, and the anti- β 4-Cyt (lane 14) and anti- β 4-COOH Abs recognized the same band of 54 kDa (lane 15).

All of these kDa values are mean \pm S.E. values calculated in three experiments.

These Western blot analyses demonstrated that except for the anti- α 4 COOH Abs (which recognize both the α 4 the α 2 subunits), our Abs recognize only a single band of the expected molecular mass and that the Abs directed against two different epitopes of the same protein recognize in Western blots peptides of similar molecular mass and a similar number of receptors in immunoprecipitation experiments.

Purification of Chick Optic Lobe and Forebrain Subtypes

To remove the small number of α 3- and β 4-containing receptors, we passed the optic lobe extract on an affinity column with bound anti- α 3 and then on a second column with bound anti- β 4. After this double passage, depletion was monitored by means of immunoprecipitation with Abs specific for both subunits: the anti- β 4 and the anti- α 3 Abs immunoprecipitated, respectively $1.4 \pm 0.2\%$ and $0.5 \pm 0.1\%$ of the [3 H]Epi-binding receptors.

After α 3 and β 4 immunodepletion, the extract was incubated twice with anti- α 2 Abs, and the bound receptors were eluted and analyzed (α 2-containing subtype).

Because the flowthrough of the second anti- α 2 immunoaffinity column still had 5% of the α 5-containing receptors, it

TABLE 1

Percentage of immunoprecipitation in extracts of [3 H]Epi-labeled forebrain and optic lobe receptors by anti-subunit-specific Abs and mAbs

Apart from the anti- α 2 Ab values, the optic lobe immunoprecipitation values are taken from Vailati et al., 1999. The immunoprecipitation was carried out as described in *Materials and Methods* using saturating concentrations of anti-subunit Abs or mAbs. The results are expressed as the percentages of [3 H]Epi-labeled receptors, taking the amount of receptor present in the solution before immunoprecipitation as 100%. The percentage of immunoprecipitation was subtracted from the value obtained in control samples containing an identical concentration of normal rabbit IgG. The values are the mean \pm S.E. of three determinations.

	Forebrain	Optic Lobe
α 2-Cyt	4.6 ± 1	31 ± 2
α 3-COOH	3.6 ± 0.5	8 ± 2
α 3-Cyt	2.6 ± 0.4	6 ± 1
α 4-COOH	83 ± 6	86 ± 2
α 4-Cyt	60 ± 3	45 ± 2
mAb 299	90 ± 5	80 ± 5
α 5-COOH	15.2 ± 1	25 ± 0.9
mAb 35	12.6 ± 0.6	30 ± 1
α 6-COOH	3.6 ± 1	5 ± 1
α 6-Cyt	0.8 ± 0.3	4 ± 0.5
β 2-COOH	85 ± 2	93 ± 5
β 2-Cyt	81 ± 5	89 ± 2
mAb 270	85 ± 3	95 ± 5
β 3-COOH	0.2 ± 0.2	0.6 ± 0.2
β 3-Cyt	1.1 ± 0.2	5 ± 0.4
β 4-COOH	18.5 ± 0.3	11 ± 2
β 4-Cyt	14 ± 1	13 ± 0.5

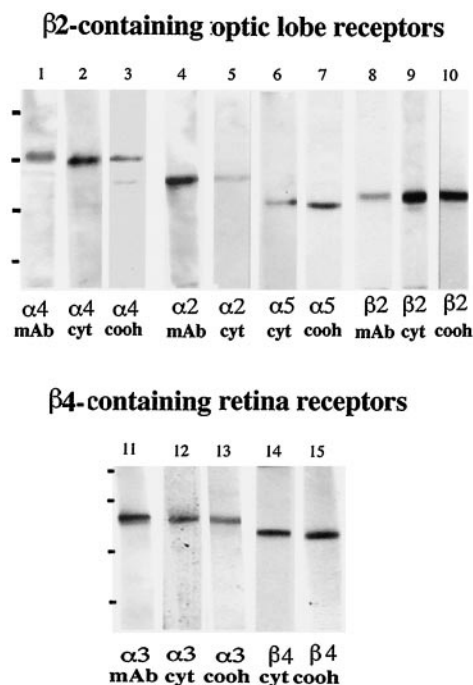


Fig. 1. Western blot analysis of the subunit-specific Abs and mAbs: Top, nAChRs purified from chick optic lobe extract using anti- β 2 Abs coupled to Sepharose 4B. Bottom, nAChRs purified from chick retina extract using anti- β 4 Abs coupled to Sepharose 4B. The immunopurified receptors were separated on 9% acrylamide-SDS gels, electrotransferred to nitrocellulose, and probed with the indicated subunit-specific Abs or mAbs at concentrations of 5 to 10 μ g/ml. The mAbs were anti- α 4 (mAb 299), anti- α 2 (mAb 323), and anti- β 2 (mAb 270). The bound Abs were revealed by means of 125 I-protein A. When the mAbs were used, the blots were incubated for an additional 2 h with anti-rat IgG diluted 1:1000. The molecular weight markers (top to bottom) are 97, 67, 45, and 31 kDa.

was immunodepleted by passing it on a column with bound anti- $\alpha 5$ Abs. The follow-through of this column (devoid of $\alpha 5$ -containing receptors) was passed on an affinity column with bound anti- $\beta 2$ Abs, and the bound receptors were eluted and analyzed (subtype $\alpha 4\beta 2$).

To identify their subunit content, we immunoprecipitated the subtypes eluted from the affinity column by the corresponding anti-subunit Abs. The anti- $\alpha 2$ -Cyt, anti- $\alpha 4$ -Cyt, anti- $\alpha 5$ (anti- $\alpha 5$ -COOH and mAb 35), and anti- $\beta 2$ Abs (anti- $\beta 2$ -COOH and anti- $\beta 2$ -Cyt), respectively, immunoprecipitated (mean \pm S.E.) $51 \pm 4\%$, $2.2 \pm 0.7\%$, $66 \pm 3.2\%$, and $80 \pm 3\%$ of the [^3H]-Epi-labeled $\alpha 2$ -containing receptors (Fig. 2A, left). The same Abs, respectively, immunoprecipitated $2.3 \pm 1\%$, $65 \pm 9\%$, $1.1 \pm 0.6\%$, and $82 \pm 6\%$ of the [^3H]-Epi-labeled $\alpha 4\beta 2$ receptors (Fig. 2A, right).

The immunopurified $\alpha 4\beta 2$ - and $\alpha 2$ -containing subtypes were analyzed on Western blots; as shown in Fig. 3, the $\alpha 2$ -containing receptors (top) contained the $\beta 2$ subunit of 54 ± 1 kDa, the $\alpha 2$ subunit of 59 kDa, and the $\alpha 5$ subunit of 51 kDa, whereas the $\alpha 4\beta 2$ subtype (bottom) contained only the $\beta 2$ subunit of 54 ± 1 kDa and the $\alpha 4$ subunit of 68 kDa.

We also used Western blots and immunoprecipitation experiments to test the purified subtypes for the possible presence of other subunits (e.g., $\alpha 3$, $\alpha 6$, $\alpha 7$, $\alpha 8$, $\beta 3$, and $\beta 4$ subunits) but could not detect any specific immunoprecipitation or labeling using these subunit specific Abs (the immunoprecipitation results are shown in Fig. 2 and the Western blot results are shown in Fig. 3).

Because we could detect the presence of only the $\alpha 2$, $\alpha 5$, and $\beta 2$ subunits in our $\alpha 2$ -containing receptors, we defined it as the $\alpha 2\alpha 5\beta 2$ subtype.

The molecular masses of both the $\alpha 5$ and $\beta 2$ subunits determined by Western blotting corresponded to the expected sizes deduced from their cDNA sequences, whereas the molecular mass of the $\alpha 4$ subunit was slightly lower.

Conroy and Berg (1998) have previously reported that chick brain has an $\alpha 5$ -containing receptor associated with the $\alpha 4$ and $\beta 2$ subunits ($\alpha 4\alpha 5\beta 2$ subtype), so we looked for the presence of this subtype as a control. Because our immunoprecipitation experiments with anti-subunit Abs have shown that the forebrain has a low level of $\alpha 2$ -containing receptors and 10 to 15% of the receptors contain the $\alpha 5$ subunit, we

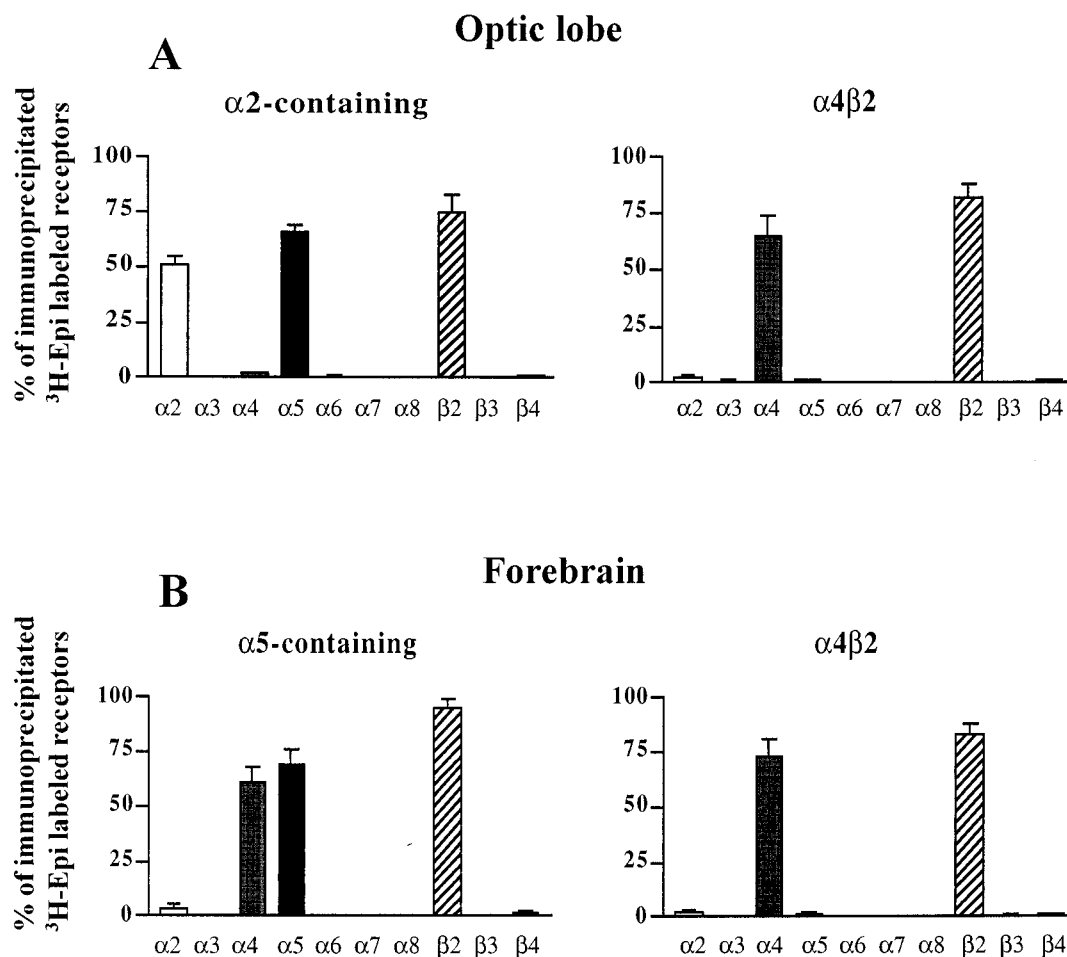


Fig. 2. Immunoprecipitation analysis of the subunit content of the purified subtypes. The $\alpha 2$ -containing and $\alpha 4\beta 2$ subtypes from optic lobe and the $\alpha 5$ -containing and $\alpha 4\beta 2$ subtypes from chick forebrain were purified as described under *Experimental Procedures*. After extensive dialysis to remove the peptides used for the elution of the receptors from the affinity column, the receptors were labeled with 2 nM [^3H]Epi and immunoprecipitated using saturating concentrations (20–30 μg) of anti- $\alpha 2$ -Cyt, anti- $\alpha 3$ -Cyt, anti- $\alpha 4$ -Cyt, anti- $\alpha 5$ (both anti $\alpha 5$ -COOH and mAb35), anti- $\alpha 6$ -Cyt, anti- $\alpha 7$ -Cyt, anti- $\alpha 8$ -Cyt, anti- $\beta 2$ (anti- $\beta 2$ -COOH and anti- $\beta 2$ -Cyt), anti- $\beta 3$ -Cyt, and anti- $\beta 4$ -Cyt. The results are expressed as percentages of the [^3H]Epi-labeled receptors, taking the amount of receptor present in the solution before immunoprecipitation as 100%. The percentage of immunoprecipitation was subtracted from the value obtained in the control samples containing an identical concentration of normal rabbit or rat IgG. The values are the mean \pm S.E. of three determinations.

purified the $\alpha 4\beta 2$ subtype and $\alpha 5$ -containing subtypes and studied their subunit composition. Forebrain extract devoid of the $\alpha 3$ - and $\beta 4$ -containing receptors was passed on the immunoaffinity column with bound anti- $\alpha 5$ Abs, and the flow-through was incubated with anti- $\beta 2$ Abs. The bound $\beta 2$ - and $\alpha 5$ -containing receptors were eluted by competition with $\beta 2$ and $\alpha 5$ peptides, respectively, labeled with [3 H]Epi, and immunoprecipitated with the same Abs used for the characterization of the optic lobe subtypes. The percentage of immunoprecipitation of the forebrain $\alpha 5$ -containing receptors by the anti- $\alpha 2$, anti- $\alpha 4$, anti- $\alpha 5$, and anti- $\beta 2$ Abs was, respectively, 3.6 ± 2 , 61 ± 7 , 69 ± 7 , and $95 \pm 4\%$ (Fig. 2B, left); the same Abs immunoprecipitated, respectively, 2 ± 1 , 73 ± 8 , 1.5 ± 1 , and $83 \pm 5\%$ of the $\alpha 4\beta 2$ receptors. These immunoprecipitation experiments confirm that the $\alpha 4\beta 2$ subtype is present together with an $\alpha 4\alpha 5\beta 2$ subtype (Fig. 2B, right), whereas there was almost no $\alpha 2\alpha 5\beta 2$ subtype.

Ontogeny of $\alpha 2$ - and $\alpha 5$ -Containing Receptors

Given the selective enrichment of the $\alpha 2$ -containing receptors in the optic lobe at P1, we studied their relative contri-

bution to the [3 H]Epi receptors present at different developmental stages in the optic lobe and forebrain-cerebellum by means of immunoprecipitation experiments with subunit-specific Abs.

We first used saturation binding experiments to determine the presence and number of [3 H]Epi-labeled receptors in the membranes and extracts at E18 and P1; the K_d value of the binding was 70 ± 10 pM in all four samples.

Given the small amounts of membrane at E7 and E11, no saturation binding experiments could be carried out, so the number of receptors was determined using [3 H]Epi at saturating concentrations of 2 nM.

Measured as [3 H]Epi binding, the level of the receptors, expressed at E7 and E11, is very similar in both optic lobe and forebrain-cerebellum, but after E11, it increases much more in the optic lobe (from 47 to 225 fmol/mg protein at P1) than in the forebrain-cerebellum (from 47 to 77 fmol/mg protein, with a slight increase at E14; Fig. 4).

Immunoprecipitation experiments with the anti- $\alpha 2$, anti- $\alpha 5$, and anti- $\alpha 3$ Abs showed that there was a selective increase in both the $\alpha 2$ and $\alpha 5$ subunits in the optic lobe after E11; the increase from E7 to P1 was 26-fold for the $\alpha 2$ and 25-fold for the $\alpha 5$ subunit compared with respective increases of 5- and 6-fold for the $\alpha 2$ and $\alpha 5$ in the forebrain. The increase in the $\alpha 3$ subunit was very low: a maximum of 3.5-fold in the optic lobe and 2-fold in the forebrain-cerebellum. Figure 5 shows the number of receptors expressed as fmol of [3 H]Epi-labeled receptors/mg protein immunoprecipitated by the subunit-specific Abs at each stage of development in both tissues; the values are the mean \pm S.E. of three different experiments.

Pharmacological Experiments on $\alpha 4\beta 2$ and $\alpha 2\alpha 5\beta 2$ Subtypes

The pharmacological experiments were carried out using receptors immobilized by the corresponding anti-subunit specific Abs as described under *Experimental Procedures*.

Figure 6 shows the saturation curves of the specific binding of [3 H]Epi to the immunoimmobilized subtypes. The interaction of [3 H]Epi with each subtype was consistent with the presence of a single class of high-affinity binding sites. The K_d values calculated from four separate experiments were 29 pM (CV = 23%) for the $\alpha 2\alpha 5\beta 2$ subtype and 86 pM (CV =

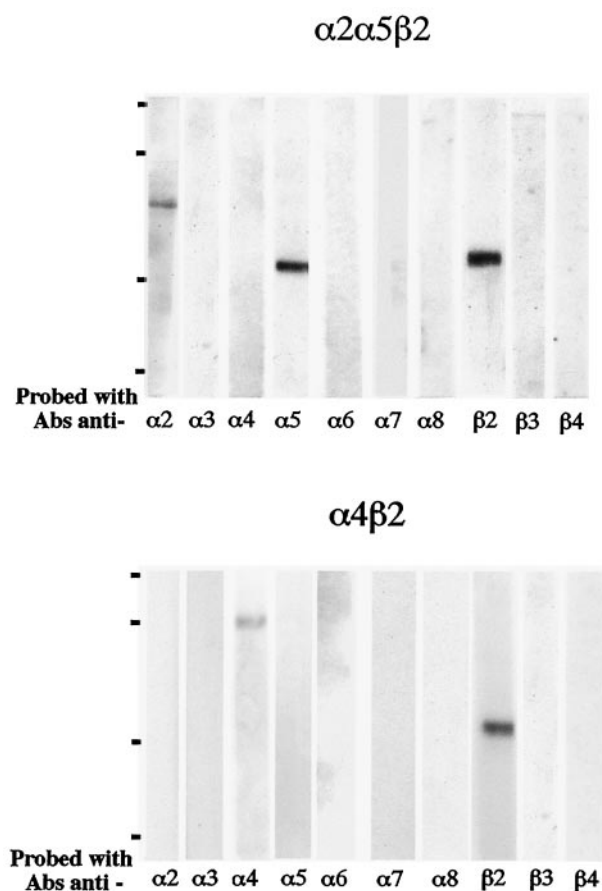


Fig. 3. Western blot analysis of the immunopurified $\alpha 4\beta 2$ and $\alpha 2\alpha 5\beta 2$ subtypes. After immunodepletion of the $\alpha 3$ - and $\beta 4$ -containing receptors, the chick optic lobe extract was incubated twice with anti- $\alpha 2$ Abs bound to Sepharose 4B, and the bound receptors eluted with the $\alpha 2$ peptide (subtype $\alpha 2\alpha 5\beta 2$). The follow-through of the anti- $\alpha 2$ Sepharose column was first completely depleted of $\alpha 5$ -containing receptors and incubated twice with anti- $\beta 2$ Abs (subtype $\alpha 4\beta 2$); the bound receptors were then eluted with the $\beta 2$ peptide. The eluted receptors were concentrated and separated on 9% acrylamide-SDS gel, electrotransferred to nitrocellulose, and the $\alpha 2\alpha 5\beta 2$ (top) and $\alpha 4\beta 2$ (bottom) subtypes probed with the indicated anti-subunit specific Abs as described in Fig. 1. The molecular weight markers (top to bottom) are 97, 67, 45, and 31 kDa.

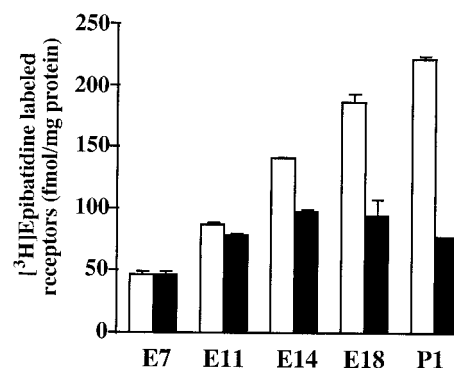


Fig. 4. Expression of [3 H]-Epi receptors (fmol/mg protein) at different developmental stages of the optic lobe and forebrain-cerebellum. The optic lobes (open columns) and forebrain-cerebellum (filled columns) were dissected at the indicated times and frozen. Extracts of 2% Triton X-100 were prepared from the tissues and assayed for [3 H]-Epi binding as described under *Experimental Procedures*. The values represent the mean \pm S.E. from three experiments performed in triplicate.

19%) for the $\alpha 4\beta 2$ subtype. The statistical analysis performed using the LIGAND program did not reveal any significant difference in the K_d value of [^3H]Epi between the two subtypes. Scatchard plots of the data obtained from the saturation curves are shown in Fig. 6; both subtypes had a single class of high-affinity sites.

The pharmacological profiles of the two subtypes were further characterized by testing the relative potencies of various cholinergic agonists and antagonists in competing for the binding of 0.1 nM [^3H]Epi at equilibrium.

Table 2 shows the K_i values obtained from the inhibition curves of cholinergic agonists and antagonists for the binding of [^3H]Epi to the immunoprecipitated subtypes. These values were obtained by simultaneously fitting the data from three or four separate experiments.

The results show that both subtypes are sensitive to the tested agonists and antagonists; the relative potencies of the

agonists in the competition experiments were Epi > cytosine > nicotine > acetylcholine > DMPP > Carb for the $\alpha 2\alpha 5\beta 2$ subtype and Epi > cytosine > nicotine = DMPP > acetylcholine > Carb for the $\alpha 4\beta 2$ subtype. Except for Carb, all of the agonists had relatively low K_i values, whereas all of the antagonists had a lower affinity and higher K_i values (in the micromolar and millimolar ranges). We found that the K_i values of some agonists for the $\alpha 2\alpha 5\beta 2$ subtype were lower than those of the some compounds for the $\alpha 4\beta 2$ subtype. This indicates a slightly higher affinity for $\alpha 2\alpha 5\beta 2$, but because this difference was not statistically significant, we can conclude that the $\alpha 4\beta 2$ and $\alpha 2\alpha 5\beta 2$ subtypes have very similar pharmacological profiles.

Reconstitution of nAChR Subtypes in Lipid Bilayers

To see whether the purified subtypes were able to form functional channels, the $\alpha 2\alpha 5\beta 2$ and $\alpha 4\beta 2$ immunopurified

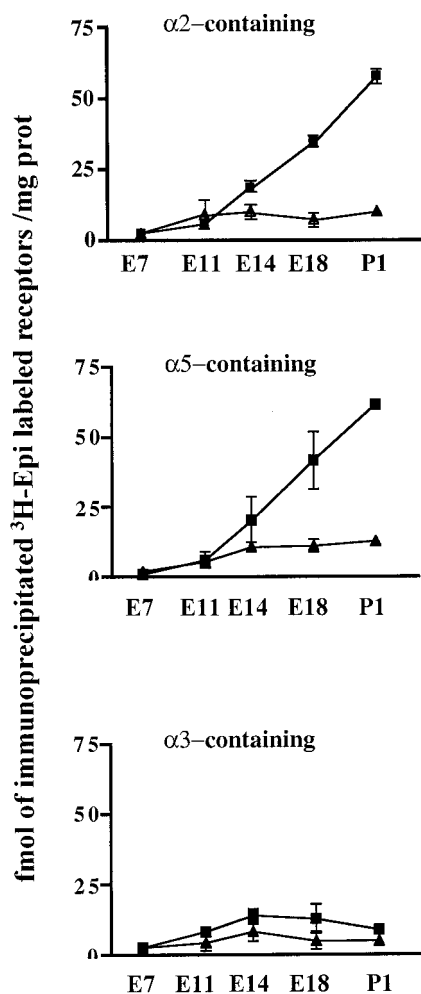


Fig. 5. Developmental changes in the expression of $\alpha 2$ -, $\alpha 5$ -, and $\alpha 3$ -containing receptors in the chick optic lobe and forebrain-cerebellum extracts. The receptors labeled with [^3H]Epi were immunoprecipitated in the optic lobe (■) or forebrain-cerebellum (▲) extracts obtained on embryonic day 7, 11, 14, and 18 (E7, E11, E14, and E18) and 1 day after hatching (P1). The receptors were labeled with 2 nM [^3H]Epi and immunoprecipitated as described under *Experimental Procedures* using saturating concentrations of anti- $\alpha 2$, anti- $\alpha 5$, or anti- $\alpha 3$ Abs. The immunoprecipitation was subtracted from the value obtained in control samples containing an identical concentration of preimmune Abs. The values are the mean \pm S.E. of three experiments, and the results are expressed as fmol of [^3H]Epi-labeled receptors immunoprecipitated/mg protein.

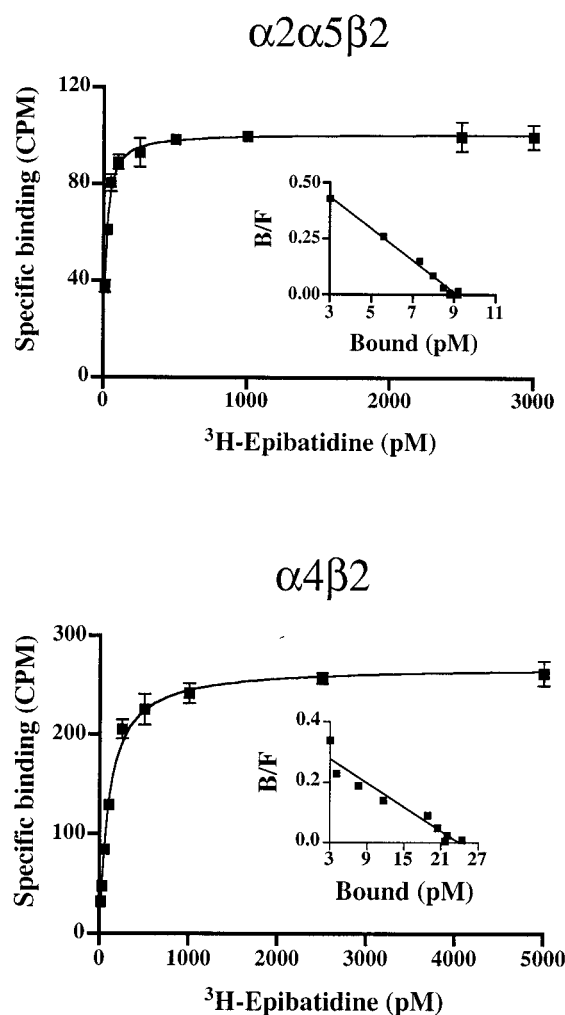


Fig. 6. Saturation curves of specific [^3H]Epi binding to the $\alpha 4\beta 2$ and $\alpha 2\alpha 5\beta 2$ immunoprecipitated subtypes and their Scatchard analyses. The immunoprecipitated subtypes were incubated overnight with the indicated concentrations of [^3H]Epi at 4°C. The specific binding shown is that obtained from a representative experiment and is defined as total binding minus the binding in the presence of 100 nM Epi. The K_d values calculated by simultaneously fitting four separate experiments were 86 pM (CV = 19%) for the $\alpha 4\beta 2$ subtype and 29 pM (CV 23%) for the $\alpha 2\alpha 5\beta 2$ subtype. The Scatchard plots of the saturation curves of the two subtypes show the presence of a single class of high-affinity sites.

receptors were reconstituted in lipid bilayers and their properties were studied after agonist activation.

In our reconstitution experiments, traces with more than one open state level were rare and excluded from the data evaluation. According to a statistical analysis (binomial test), the traces in all of the other cases came from only one active channel with a high probability (>0.9). This is also due to the fact that as a consequence of the incorporation of the AChRs into liposomes under the given conditions, one or no protein molecule is preferentially incorporated into one liposome. The rate of vesicle fusion in the experiments was also quite low, so the fusion of a larger number of liposomes into one bilayer during the time course of a typical experiment is unlikely.

Figure 7A shows current fluctuation traces of the single channel for both subtypes activated with 500 μ M Carb, together with the integral-amplitude and lifetime histograms (open and closed state) of each trace (Fig. 7B).

The $\alpha 2\alpha 5\beta 2$ channel had a much lower probability of being in the open state, possibly because the mean open-state lifetimes of both subtypes were similar at 50 mV and 500 μ M Carb ($T_{\text{open}\alpha 4\beta 2} = 3$ ms, $T_{\text{open}\alpha 2\alpha 5\beta 2} = 4$ ms), whereas the mean closed-state lifetime for the $\alpha 4\beta 2$ subtype was much shorter ($T_{\text{closed}\alpha 4\beta 2} = 4$ ms, $T_{\text{closed}\alpha 2\alpha 5\beta 2} = 40$ ms).

Figure 8A shows the integral P_o of each reconstituted subtype as a function of Carb concentrations. Channel activity could also be induced using the ACh agonist ($EC_{50} = 300$ μ M and 1 mM for the $\alpha 4\beta 2$ and $\alpha 2\alpha 5\beta 2$ subtypes, respectively) and blocked by 50 μ M concentrations of *d*-tubocurarine (Table 3).

The EC_{50} values were determined by plotting the integral P_o for both receptor subtypes as a function of agonist concentrations. The $\alpha 2\alpha 5\beta 2$ subtype had a significantly higher EC_{50} value, so the P_o of the $\alpha 4\beta 2$ subtype at the same agonist concentration was also significantly higher.

Figure 8B shows the current-voltage relationship for both subtypes, which can be fitted by straight lines with main conductances of 38 pS for the $\alpha 2\alpha 5\beta 2$ subtype and 40 pS for the $\alpha 4\beta 2$ subtype. The P_o of the $\alpha 4\beta 2$ subtype was slightly voltage-dependent, whereas that of the $\alpha 2\alpha 5\beta 2$ subtype was not (Fig. 8C).

In addition to the main conductances, both of the receptor

subtypes showed other conductances, all of which were blocked by *d*-tubocurarine; their frequency of occurrence was very low in the $\alpha 4\beta 2$ (less than 1%) and higher (5–10%) in the $\alpha 2\alpha 5\beta 2$ subtype. In the $\alpha 2\alpha 5\beta 2$ subtype, the second most frequent level was one of higher conductance (59 pS). In experiments with different salt solutions, it was found that both receptors formed monovalent cation channels whose permeabilities for sodium and potassium were almost identical.

Discussion

The major finding reported here is that a subtype containing the $\alpha 2$, $\alpha 5$, and $\beta 2$ subunits is selectively present in chick optic lobe. It coexists with the $\alpha 4\beta 2$ subtype and is strictly developmentally regulated because it only appears after E11. In addition to the $\alpha 4\beta 2$ subtype, the forebrain has an $\alpha 5$ -containing subtype, but it contains the $\alpha 4$ and $\beta 2$ subunits and not the $\alpha 2$ subunit.

Conroy and Berg (1998) found that almost all of the $\alpha 5$ -containing receptors present in chick brain at E8 are associated with the $\alpha 4$ and $\beta 2$ subunits, but the fact that some of the receptors containing the $\alpha 5$ subunit cannot be immunodepleted from E18 brain extract using an anti- $\alpha 4$ mAb suggests that some of the $\alpha 5$ -containing receptors can be associated with an α -subunit other than $\alpha 4$ late in brain development.

This study demonstrates that this hypothesized subtype is the $\alpha 2\alpha 5\beta 2$ subtype and that it is specifically enriched in the optic lobe.

Using the anti- $\alpha 2$ Abs, we immunoprecipitated more than 50% of this $\alpha 2\alpha 5\beta 2$ subtype, whereas the anti- $\alpha 4$ Abs immunoprecipitated only 2 to 3%. The same Abs had opposite immunoprecipitation capacities on the $\alpha 4\beta 2$ subtype; the anti- $\alpha 2$ immunoprecipitated only a maximum of 2%, whereas the anti- $\alpha 4$ immunoprecipitated more than 65% of the $\alpha 4\beta 2$ optic lobe receptors. Using the same Abs, we also obtained very similar immunoprecipitation results with the $\alpha 4\beta 2$ subtype purified from the forebrain.

The incomplete immunoprecipitation of the $\alpha 2\alpha 5\beta 2$ subtype by the $\alpha 2$ Abs (a maximum of 60%) may have been due to 1) incomplete dialysis of the peptide used to recover the receptors from the immunoaffinity column, 2) the limited immunoprecipitation capability of the Abs, or 3) proteolysis of the receptors during the long purification processes. These last two possibilities may also account for the incomplete immunoprecipitation (a maximum of 80%) obtained using two different anti- $\alpha 5$ Abs (the anti- $\alpha 5$ COOH and mAb 35) on the $\alpha 4\alpha 5\beta 2$ subtype purified from the forebrain.

During development, the receptors containing the $\alpha 2$ and $\alpha 5$ subunits are strongly regulated in the optic lobe but much less so in the forebrain. It is possible that the $\alpha 2\alpha 5\beta 2$ subtype is mainly expressed after hatching when the chick's visuo-motor system is first used, probably because its expression is activity dependent. Changes in the level of the $\alpha 7$ and $\alpha 8$ subtype nAChR subtypes have also been reported in the chick retina at the time of hatching (Keyser et al., 1993).

We do not yet know whether the increase in the $\alpha 2$ - and $\alpha 5$ -containing receptors is due to a local increase in receptor expression in optic lobe neurons or to their possible transportation from other parts of the brain that are connected to the optic lobe late during development. The chick optic lobe con-

TABLE 2

Affinity of cholinergic agonists and antagonists for immunoimmobilized subtypes

The K_d and K_i values were derived from [3 H]-Epi saturation and competition binding curves to the $\alpha 2\alpha 5\beta 2$ and $\alpha 4\beta 2$ subtypes. The curves obtained from three separate experiments were fitted using a nonlinear least-squares analysis program and the F test (Munson and Rodbard, 1980). The numbers in parentheses represent the percent of CV.

	$\alpha 2\alpha 5\beta 2$	$\alpha 4\beta 2$
K_d (nM)	0.029 (23)	0.086 (19)
K_i (nM)		
Epi	0.032 (18)	0.046 (18)
Cytisine	1 (21)	2.7 (31)
Nicotine	9 (12)	38. (30)
ACh	13.8 (23)	57 (12)
DMPP	32 (14)	40 (15)
Carb	599 (10)	582 (18)
K_i (μ M)		
β -Erythroidine	4.7 (25)	1.1 (35)
<i>d</i> -Tubocurarine	22.2 (21)	19.6 (18)
Decamethonium	25.8 (15)	54.9 (18)
Hexamethonium	1640 (29)	2720 (22)

sists of the optic tectum and a region subjacent to the tectal ventricle that contains a number of isthmus nuclei and probably also the SpL. The stratum griseum centrale of the optic tectum receives the projection from the SpL (Reiner et al., 1982), which expresses developmentally regulated $\alpha 2$ subunit mRNA (Daubas et al., 1990) and immunoreactivity for the $\alpha 2$, $\alpha 5$, $\alpha 7$, and $\beta 2$ subunits late in development (Ullian and Sargent, 1995). After SpL lesions, both the $\alpha 5$ and $\beta 2$ subunits are markedly depleted in the deep layers of the ipsilateral optic tectum (Torrao et al., 1996). Lesion studies have also shown that the superficial layers of the chick optic

tectum have $\beta 2$ -containing receptors transported from retinal ganglion cells (Britto et al., 1994). Because we have found that 18.8% of the chick retina nAChRs contain the $\alpha 2$ subunit, we cannot exclude the possibility that these receptors are transported to the optic tectum. However, additional experiments (e.g., lesion studies, metabolic labeling, and immunoprecipitations) are needed to answer these questions.

We also pharmacologically characterized the optic lobe $\alpha 2\alpha 5\beta 2$ and $\alpha 4\beta 2$ subtypes by binding studies and found that both have a single high-affinity class of receptors that similarly bind agonists with nanomolar affinity and antagonists

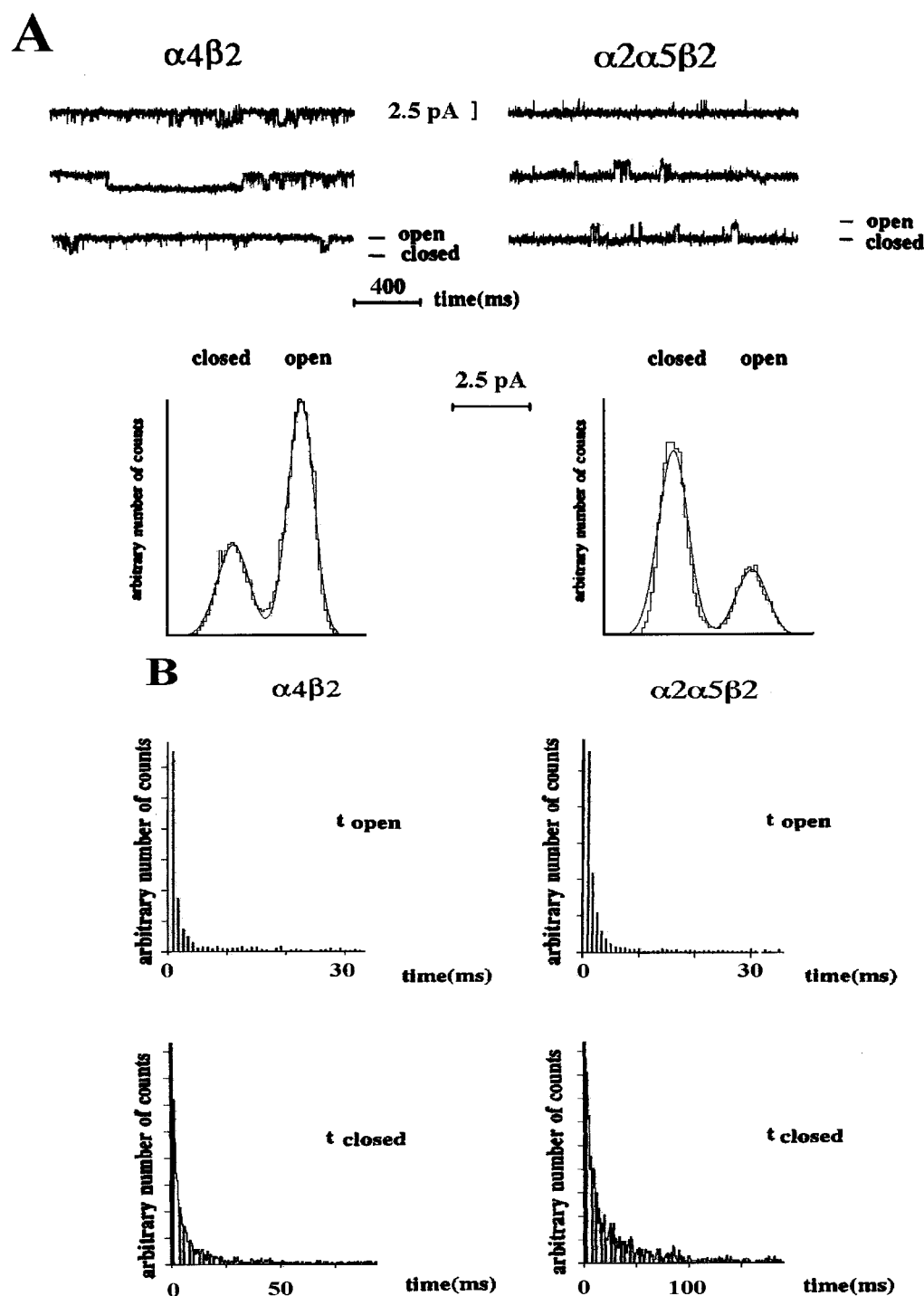


Fig. 7. A, traces of the Carb-activated $\alpha 4\beta 2$ and $\alpha 2\alpha 5\beta 2$ receptor channels, together with their integral amplitude histograms. The applied membrane voltage was 100 mV; the Carb concentration was 500 μ M. The traces were digitized at a sampling rate of 2000 points/s. The $\alpha 4\beta 2$ subtype had well-defined closed and open states, with the open state having a major conductance of 40 pS; the $\alpha 2\alpha 5\beta 2$ subtypes had a major conductance of 38 pS and an additional conductance with a quite low probability of 59 pS. The $\alpha 4\beta 2$ trace clearly shows bursting behavior. The bar in the amplitude histograms represents 2.5 pA. The histograms were taken from 60-s traces (i.e., a total of 120,000 points). B, lifetime histograms of the open and closed states from the fluctuations shown in A. The mean lifetimes of the $\alpha 4\beta 2$ and $\alpha 2\alpha 5\beta 2$ subtypes taken from these histograms (fitted by sums of exponentials, not shown) were, respectively, 3 and 4 ms in the open state and 4 and 40 ms in the closed state.

with lower affinity. There are a number of possible reasons for the absence of a statistical difference in the affinity of the $\alpha 4\beta 2$ and $\alpha 2\alpha 5\beta 2$ subtypes for nicotinic ligands: the β -subunit is the major factor in determining agonist affinity

(Parker et al., 1998) and the same $\beta 2$ subunit is contained in both subtypes. The $\alpha 2$ and $\alpha 4$ subunits are highly homologous in the extracellular N terminus (Sargent, 1993; Gotti et al., 1997a). The presence of the $\alpha 5$ subunit in the subtypes may not change the affinity of [^3H]-Epi binding, as has already been demonstrated in the case of the human $\alpha 3\beta 2$ and $\alpha 3\beta 4$ subtypes expressed in oocytes (Wang et al., 1996) and chick subtypes (Conroy and Berg, 1998).

Like muscle AChRs, neuronal subtypes can coexist in a minimum of three interconvertible states: an active state with a low affinity for agonists, a closed state, and a desensitized closed state in which the receptors are refractory to activation and have a high affinity for agonists (reviewed in Galzi and Changeux, 1995). It is possible that under our equilibrium binding experimental conditions, receptor desensitization may underestimate the pharmacological profiles of the subtypes.

The pharmacological profile of the $\alpha 4\beta 2$ subtype described here is very similar to that previously reported for the same subtype in the chick brain or after expression in heterologous systems (Whiting et al., 1991b; Conroy and Berg, 1998).

Reconstitution experiments in lipid bilayers showed that both receptor subtypes form agonist-activated channels but that these have different gating properties and different efficacy after ligand binding. At the same agonist concentration, the two channels have the very similar open-state but different closed-state lifetimes, with the closed-state lifetime of the $\alpha 2\alpha 5\beta 2$ subtype being 10 times longer. This increase in the closed-state lifetime of the $\alpha 5$ -containing subtype accounts for its different P_o and higher EC_{50} values for nicotinic agonists.

Although our binding studies revealed very similar pharmacological profiles for the two subtypes, the reconstitution experiments found that the EC_{50} values of the nicotinic agonists for the two subtypes were different, with higher values in the case of the $\alpha 2\alpha 5\beta 2$ subtype. We do not know if this is due to the presence of the $\alpha 2$ or $\alpha 5$ subunit or both. As reported by Gerzanich et al., (1998) for the human $\alpha 3$ subtypes, it is possible that the presence of the $\alpha 5$ subunit alters receptor desensitization in such a way as to compete with receptor activation. Another possible explanation for the different results of the binding and electrophysiological experiments is that they may have measured agonist affinities in two different receptor states (i.e., desensitized in the case of

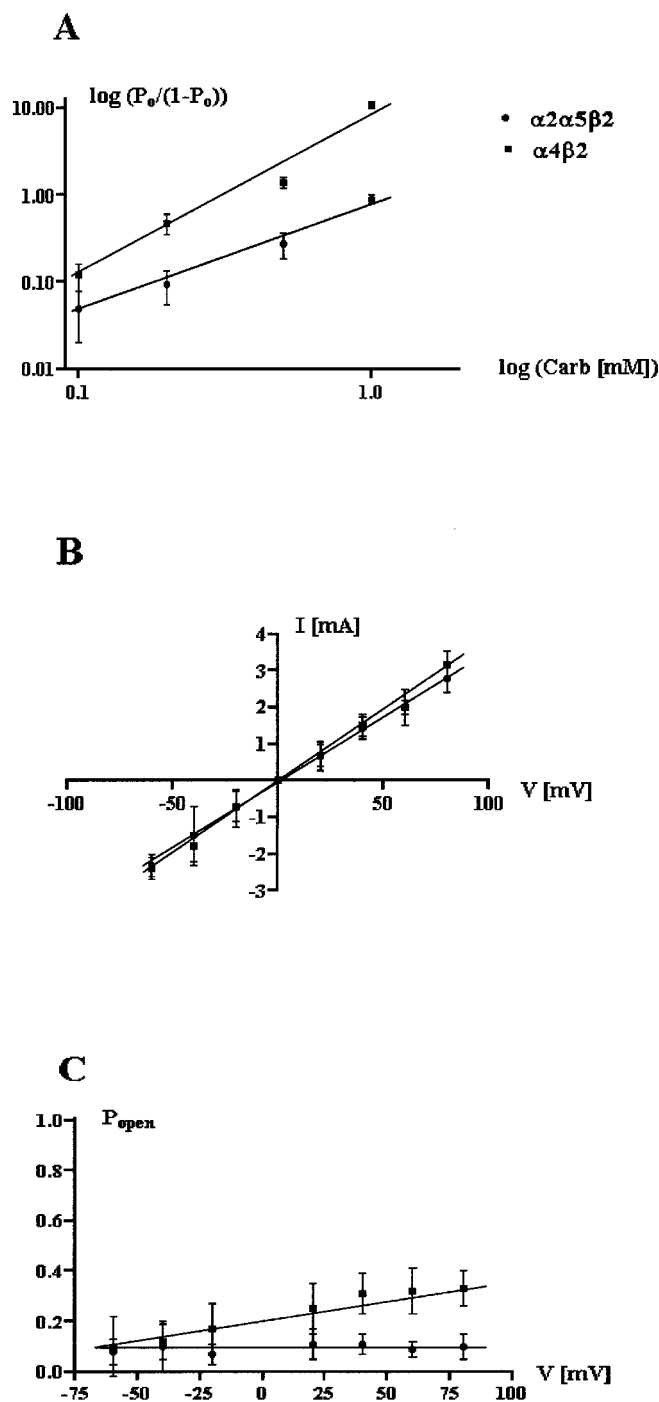


Fig. 8. A, agonist concentration dependence of the P_o of the $\alpha 2\alpha 5\beta 2$ and $\alpha 4\beta 2$ receptor channels reconstituted in planar lipid bilayers. The probability of the two Carb-activated channel types being in the open state (taken from the all-point amplitude histograms) is shown as a function of Carb concentration. All of the values were measured at a membrane potential of 50 mV. B, current-voltage relationships of the channel types shown in A. The data can be fitted between +80 and -60 by straight lines, with conductances of, respectively, 40 and 38 pS for $\alpha 4\beta 2$ and $\alpha 2\alpha 5\beta 2$. C, P_o of the $\alpha 4\beta 2$ and $\alpha 2\alpha 5\beta 2$ channels at 200 μM Carb, plotted as a function of voltage. All of the data are plotted as mean values \pm S. D. from at least five experiments.

TABLE 3

Electrophysiological characteristics of $\alpha 2\alpha 5\beta 2$ and $\alpha 4\beta 2$ subtypes

Channel Properties	$\alpha 2\alpha 5\beta 2$ Subtype	$\alpha 4\beta 2$ Subtype
Conductance (pS) ^a	38	40
Mean lifetime (ms) ^a		
$\tau(o)$	4	3
$\tau(c)$	40	4
Mean open probability (P_o)	0.1	0.43
Ion selectivity	Na \approx K \gg Cl ⁻	Na \approx K \gg Cl ⁻
Agonist profile		
ACh (EC_{50} , μM) ^b	1000	300
Carb (EC_{50} , μM) ^b	>1000	500
Sensitivity to d-tubocurarine	++	++

^a $\tau(o)$, $\tau(c)$, mean open- or closed-state lifetimes.

^a These were determined using a concentration of 500 μM Carb and obtained from three different experiments for each receptor type.

^b The agonist EC_{50} values were calculated from three different experiments for each receptor type and represent the agonist concentrations that give 50% of maximal receptor activation. They were calculated from the plot P_o versus [agonist], as the value of the agonist concentration necessary to obtain a level of activity midway between spontaneous activity and maximal P_o .

binding, closed in the case of electrophysiological experiments).

The response of the $\alpha 2\alpha 5\beta 2$ is voltage-independent, whereas that of the $\alpha 4\beta 2$ subtype is slightly voltage-dependent. In addition to the main conductances of 40 pS for the $\alpha 4\beta 2$ and 38 pS for the $\alpha 2\alpha 5\beta 2$ subtype, both subtypes have other conductances (but these are more frequent in the $\alpha 2\alpha 5\beta 2$ subtype).

The chick $\alpha 4\beta 2$ subtype has also been expressed in heterologous systems: it has a conductance of 24 ± 3 pS in oocytes (Ramirez-Latorre et al., 1996) but two major conductances in BOSC 23 cells, one of which is 22 pS (very similar to that of the oocyte-expressed subtype) and the other is 42 pS. Both of these conductances have brief mean channel open times (1.9 ± 0.4 and 2.8 ± 0.7 , respectively; Ragozzino et al., 1997).

Our bilayer reconstituted $\alpha 4\beta 2$ subtype has a different conductance from that of the oocyte-expressed subtype but very similar conductance and mean channel open time as the 42-pS channel of the BOSC23-expressed $\alpha 4\beta 2$ subtype

We believe that this difference could be due to the heterologous expression systems or the fact that artificial bilayers do not completely mimic the natural environment. Recent data concerning the rat $\alpha 3\beta 4$ subtype expressed in different host cells (Coverton et al., 1994; Lewis et al., 1997) have clearly shown that channels with different pharmacological and biophysical properties can be obtained depending on the expression system used.

Weaver and Chiappinelli (1996) analyzed the nicotinic receptors present in the chick lateral spiriform nucleus by means of single-channel recordings and found channels with multiple classes of conductances: 18% of these channels have a conductance of 40.4 ± 0.5 pS, which is very similar to that determined for our reconstituted subtypes.

Another finding common to these and other reconstituted subtypes (Gotti et al., 1994, 1997b; Vailati et al., 1999) is that they do not show fast desensitization. We think that this could be due to the purification process itself and/or the reconstitution technique and/or the fact that certain regulatory processes important for the function of the channels present in native or heterologous cells do not take place in the reconstituted bilayers.

Nevertheless, the reconstitution studies allow us to conclude that the $\alpha 2\alpha 5\beta 2$ subtype is functional and to describe its most relevant biophysical properties.

The reconstitution studies are based on receptor subtypes that we have shown by Western blotting and immunoprecipitation to contain only the $\alpha 2$, $\alpha 5$, and $\beta 2$ or the $\alpha 4$ and $\beta 2$ subunits. We therefore believe that the reconstituted channels are due to these subunit combinations. However, although highly unlikely, we cannot completely exclude the possibility that not-yet-cloned subunits or subunits that are undetectable by Western blotting and immunoprecipitation may be present.

The possibility of studying the pharmacological and functional properties of purified native receptors of a known subunit composition is a remarkable advantage. Molecular biology has shown considerable differences in the oligomeric nAChR channels that can be generated, and it is therefore very important to be able to demonstrate the subunit composition and properties of the receptors present in brain neurons.

Although we do not know the physiological role of the

$\alpha 2\alpha 5\beta 2$ subtype, we have demonstrated its presence in chick brain and further illustrated the diversity and complexity of the nAChR family.

Acknowledgments

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