ABSTRACT

The most widely expressed neuronal nicotinic acetylcholine receptor subtype in chick brain is that containing the α4 and β2 subunits. However, immunoprecipitation and localization studies have shown that some brain areas also contain the α2 and/or α5 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 α2-containing receptors. Immunoprecipitation studies of these immunopurified α2-containing receptors labeled with the nicotinic agonist [3H]epibatidine showed that almost all of them contained the β2 subunit and that more than 66% contained the α5 subunit. Western blot analyses of the purified receptors confirmed the presence of the α2, α5, and β2 subunits and the absence of the α3, α4, α6, α7, α8, β3, and β4 subunits. The α2-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 α2-containing optic lobe receptors bind [3H]epibatidine (Kd = 29 pM) and a number of other nicotinic agonists with very high affinity and have a pharmacological profile very similar to that of the α4β2 subtype. They form functional cationic channels when reconstituted in lipid bilayers, with pharmacological and biophysical properties different from those of the α4β2 subtype. These channels are activated by nicotinic agonists in a dose-dependent manner and are blocked by the nicotinic antagonist d-tubocurarine.

MOL 58:300–311, 2000 /1/841787
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MOL 58:300–311, 2000 /1/841787

ABBREVIATIONS: nAChR, neuronal nicotinic acetylcholine receptor; α-β, α-bungarotoxin; mAb, monoclonal antibody; SpL, spirifim nucleus; ACh, acetylcholine; Epi, epibatidine; Carb, carbachol; α, β, and γ, global open-state probability; Cyt, cytoplasmic peptide; DMPP, 1,1-dimethyl-4-phenylpiperazinim.
1997a), and a mutation in the α4 subunit (S247F) produces autosomal dominant nocturnal frontal lobe epilepsy (reviewed in Lena and Changeux, 1997). It has recently been reported that a developmentally regulated α4δ5β2 subtype is also expressed in chick brain (Conroy and Berg, 1998).

Limited chick and mammalian brain areas also have α2-containing nAChR subtypes that have been well characterized in heterologous systems but not in vivo. The presence of the α2 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 α5 and α3 subunits) to purify receptors containing the α4, α2, and β2 subunits that were later also found to contain the α5 subunit (Conroy et al., 1992).

High levels of the α2 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 α5, α7, and β2 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 k-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 α2-containing receptors are not yet known.

In this study, we purified the α2-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 α4β2 subtype.

**Experimental Procedures**

**Antibody Production and Characterization**

Both the mAb 270, raised against chicken brain nAChR that recognizes the β2 subunit (Whiting et al., 1987), and the mAb 323, directed against the α2 subunit, were generously given by Dr. Lindstrom. mAb 35, which was raised against the muscle-type AChR, recognizes the α5 subunit and cross-reacts with the α3 subunit (Whiting et al., 1987), and the mAb 323, prepared and immunodepleted of α5-containing receptors; was purified from the chick forebrain, the tissue extract was prepared as follows. On the wells of [3H]epibatidine (Epi) binding and quantitative immunoprecipitation of the receptors present in the solution before and after each immunoprecipitation and immunolabeling was specifically inhibited only by the peptide used for the immunization.

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

**Receptor Subtype Immunopurification**

α2δ5β2 and α4β2 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 ultraturrax homogenizer. The homogenate was then diluted and centrifuged for 1.5 h at 60,000g.

This homogenization, dilution, and centrifugation procedure was then 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 µg/ml concentration of each of the following protease inhibitors: leupeptin, bestatin, pepstatin A, and aprotonin (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 α3- and β4-containing receptors, the extract was first incubated with 5 ml of Sepharose-4B with bound anti-α3 Abs and then with 5 ml of Sepharose-4B with anti-β4 Abs.

The optic lobe extract depleted of the receptors containing the α3 and β4 subunits was then incubated twice with Sepharose 4B with bound anti-α2 Cyt Abs. The bound receptors were eluted with 0.2 M glycine (pH 2.2) or a 100 µM concentration of the corresponding α2 peptide used for Ab production as described by Gotti et al., (1994; α2-containing receptors).

The flow-through of the anti-α2 Abs column was then incubated with anti-α5 Abs to remove the residual α5-containing receptor and then with the resin with bound anti-β2 Abs. The bound receptors were eluted with 0.2 M glycine (pH 2.2) or with a 100 µM concentration of the corresponding β2 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 β2-Cyt or β4-COOH peptides and the bound receptor was eluted with 0.2 M glycine or the corresponding peptides.

**Forebrain α4δ5β2 Subtype.** When α5-containing receptors were purified from the chick forebrain, the tissue extract was prepared and immunodepleted of α3- and β4-containing receptors as described for the optic lobe. The extract was then directly incubated with an affinity resin with bound anti-α5-COOH Abs, and the receptors were eluted by means of the α5 peptide.

For each subtype purification, recovery was determined by means of [3H]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-α2 or anti-β2 Abs were bound to the microwells (Maxi-Sorp; Nunc, Naperville, CT) by means of overnight incubation at 4°C at a concentration of 10 µg/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 µl of 2% Triton X-100 optic lobe membrane extract containing 100 to 200 fmol of [3H]Epi binding sites prepared as follows. On the wells plated with anti-α2 Abs, the added extract was depleted of α3- and β4-containing receptors; on the wells coated with anti-β2 Abs, it was also depleted of α5-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 $[^{3}H]$-Epi binding.

**Immunoprecipitation of $[^{3}H]$-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 μM α-Bgt, and then labeled with 2 nM $[^{3}H]$Epi, and incubated overnight with a saturating concentration of affinity purified IgG (20–30 μg). 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 (10,000 g). 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 $[^{3}H]$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**

($\pm$) $[^{3}H]$-Epi (specific activity, 54.6 Ci/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 by incubating aliquots of optic lobe membrane with $[^{3}H]$Epi concentrations ranging from 0.005 to 5 nM at 20°C. Nonspecific binding (averaging 10–15% of total binding) was determined in parallel by means of incubation in the presence of 100 nM unlabeled Epi. The binding techniques used for solubilized receptors and for immunomobilized subtypes, as well as the data analysis, were the same as those previously described (Vailati et al., 1999).

$[^{3}H]$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., (1985), we found that $[^{3}H]$Epi binds with high affinity to the β2- and β4-containing subtypes (picomolar affinity), but it also binds to the α7 subtypes with a low nanomolar affinity and to the α8-containing receptor with picomolar affinity. To ensure that the α7 and α8 subtypes did not contribute to $[^{3}H]$Epi binding in tissue extracts, the binding and immunoprecipitation experiments were performed in the presence of 2 μM α-Bgt, which specifically binds to the α7 and α8 subtypes and blocks $[^{3}H]$Epi binding.

**Biayer Formation and Subtype Insertion**

The purified subtypes eluted from the corresponding immunofinity 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 points/s and low-pass prefiltered at 1 kHz. Typically, 60 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 mM carbachol (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 mM NaCl and 5 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 (EC50) 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 ± 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. 125I-Protein A and $[^{3}H]$Epi were obtained from Amersham International. The reagents for gel electrophoresis were obtained from Bio-Rad Laboratories (Hercules, CA).

**Results**

**Regional Distribution of $[^{3}H]$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 $[^{3}H]$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 nM $[^{3}H]$Epi in the presence of 2 μM α-Bgt (see Experimental Procedures). We found that retina contains the highest level of receptors (246 ± 15 fmol of $[^{3}H]$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 α2, α3, α4, α5, β2, and β4 Subunits**

**Immunoprecipitation Experiments.** We produced Abs against the α2 Cyt peptide of the α2 subunit; given that the α4 peptide cgPPWLAGMI has an almost identical sequence as that of the C-terminal α2 Cyt peptide cgPPYLAGMI, we tested whether our anti-α4 COOH Abs could also pick up receptors containing the α2 subunits. Immunoprecipitation experiments using anti-α2 Cyt in the retina, optic lobe, and forebrain showed that the anti-α2 Abs immunoprecipitated a substantial number of receptors in retina (18.8 ± 3%) and in the optic lobe (31 ± 2%) but only 4.6 ± 1.1% of the receptors in the forebrain. Because the forebrain had a much lower α2 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 α2 subunit and with some slight differences in the content of the α3, α5, α6, and β3 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 β2 and α4 subunits and a minority contain the α3 and β4 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-α4-COOH and anti-α4 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-α4-COOH Abs can also recognize the α2 subunit.

**Western Blot Analysis of Abs.** We have previously demonstrated that chick optic lobe is highly enriched in nAChRs that bind [3H]Epi and contain the α4 and β2 subunits, whereas the majority of nAChRs of the retina that bind [3H]Epi contain the β4 subunit and are very heterogeneous in terms of their α-subunit content (Vailati et al., 1999).

We used anti-β2 Abs to purify the optic lobe receptors containing the β2 subunit and anti-β4 Abs to purify the β4-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-β2-Cyt Abs bound to Sepharose 4B were tested using anti-α4 (α4-Cyt and α4-COOH), anti-α2 (α2-Cyt), anti-α5 (α5-Cyt and α5-COOH), and anti-β2 (β2-Cyt and β2-COOH) Abs, as well as mAb 299 (specific for the α4 subunit) and mAb 323 (specific for the α2 subunit). The results are shown in Fig. 1, top. The anti-α4-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-α4-COOH Abs (lane 3) also recognized a band of 59 kDa, the same peptide recognized by the anti-α2-Cyt Abs (lane 5) and mAb 323 (lane 4). In the case of the anti-α5 Abs, the α5-Cyt and α5-COOH Abs (lanes 6 and 7) recognized the same band of 51 ± 0.5 kDa, whereas the anti-β2 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-β4-COOH Abs, we purified receptors from chick retina and probed them with the anti-α3 (α3-COOH and α3-Cyt) and mAb 313 and the anti-β4 Abs (β4-COOH and β4-Cyt); the results are shown in Fig. 1, bottom. mAb 313 (lanes 11), anti-α3-Cyt (lane 12), and anti-α3-COOH (lane 13) recognized the same band of 57 kDa, and the anti-β4-Cyt (lane 14) and anti-β4-COOH Abs recognized the same band of 54 kDa (lane 15).

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

These Western blot analyses demonstrated that except for the anti-α4 COOH Abs (which recognize both the α4 the α2 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 α3- and β4-containing receptors, we passed the optic lobe extract on an affinity column with bound anti-α3 and then on a second column with bound anti-β4. After this double passage, depletion was monitored by means of immunoprecipitation with Abs specific for both subunits: the anti-β4 and the anti-α3 Abs immunoprecipitated, respectively 1.4 ± 0.2% and 0.5 ± 0.1% of the [3H]Epi-binding receptors.

After α3 and β4 immunodepletion, the extract was incubated twice with anti-α2 Abs, and the bound receptors were eluted and analyzed (α2-containing subtype).

Because the flowthrough of the second anti-α2 immunofinity column still had 5% of the α5-containing receptors, it

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**TABLE 1**

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

<table>
<thead>
<tr>
<th>Subunit</th>
<th>Forebrain</th>
<th>Optic Lobe</th>
</tr>
</thead>
<tbody>
<tr>
<td>α2-Cyt</td>
<td>4.6 ± 1</td>
<td>31 ± 2</td>
</tr>
<tr>
<td>α3-COOH</td>
<td>3.6 ± 0.5</td>
<td>8 ± 2</td>
</tr>
<tr>
<td>α3-Cyt</td>
<td>2.6 ± 0.4</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>α4-COOH</td>
<td>83 ± 6</td>
<td>86 ± 2</td>
</tr>
<tr>
<td>α4-Cyt</td>
<td>60 ± 3</td>
<td>45 ± 2</td>
</tr>
<tr>
<td>mAb 299</td>
<td>90 ± 5</td>
<td>80 ± 5</td>
</tr>
<tr>
<td>α5-COOH</td>
<td>15.2 ± 1</td>
<td>25 ± 0.9</td>
</tr>
<tr>
<td>mAb 35</td>
<td>12.6 ± 0.6</td>
<td>30 ± 1</td>
</tr>
<tr>
<td>α6-COOH</td>
<td>3.6 ± 1</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>α6-Cyt</td>
<td>0.8 ± 0.3</td>
<td>4 ± 0.5</td>
</tr>
<tr>
<td>β2-COOH</td>
<td>85 ± 2</td>
<td>93 ± 5</td>
</tr>
<tr>
<td>β2-Cyt</td>
<td>81 ± 5</td>
<td>89 ± 2</td>
</tr>
<tr>
<td>mAb 270</td>
<td>85 ± 3</td>
<td>95 ± 5</td>
</tr>
<tr>
<td>β3-COOH</td>
<td>0.2 ± 0.2</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>β3-Cyt</td>
<td>1.1 ± 0.2</td>
<td>5 ± 0.4</td>
</tr>
<tr>
<td>β4-COOH</td>
<td>18.5 ± 0.3</td>
<td>11 ± 2</td>
</tr>
<tr>
<td>β4-Cyt</td>
<td>14 ± 1</td>
<td>13 ± 0.5</td>
</tr>
</tbody>
</table>

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![Fig. 1. Western blot analysis of the subunit-specific Abs and mAbs](image-url)
was immunodepleted by passing it on a column with bound anti-α5 Abs. The follow-through of this column (devoid of α5-containing receptors) was passed on an affinity column with bound anti-β2 Abs, and the bound receptors were eluted and analyzed (subtype α4β2).

To identify their subunit content, we immunoprecipitated the subtypes eluted from the affinity column by the corresponding anti-subunit Abs. The anti-α2-Cyt, anti-α4-Cyt, anti-α5 (anti-α5-COOH and mAb 35), and anti-β2 Abs (anti-β2-COOH and anti-β2-Cyt), respectively, immunoprecipitated (mean ± S.E.) 51 ± 4%, 2.2 ± 0.7%, 66 ± 3.2%, and 80 ± 3% of the [3H]-Epi-labeled α2-containing receptors (Fig. 2A, left). The same Abs, respectively, immunoprecipitated 2.3 ± 1%, 65 ± 9%, 1.1 ± 0.6%, and 82 ± 6% of the [3H]-Epi-labeled α4β2 receptors (Fig. 2A, right).

The immunopurified α4β2 and α2-containing subtypes were analyzed on Western blots; as shown in Fig. 3, the α2-containing receptors (top) contained the β2 subunit of 54 ± 1 kDa, the α2 subunit of 59 kDa, and the α5 subunit of 51 kDa, whereas the α4β2 subtype (bottom) contained only the β2 subunit of 54 ± 1 kDa and the α4 subunit of 68 kDa.

Fig. 2. Immunoprecipitation analysis of the subunit content of the purified subtypes. The α2-containing and α4β2 subtypes from optic lobe and the α5-containing and α4β2 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-α2-Cyt, anti-α4-Cyt, anti-α5-Cyt, anti-α6-Cyt, anti-α7-Cyt, anti-α8-Cyt, anti-β2 (anti-β2-COOH and anti-β2-Cyt), anti-β3-Cyt, and anti-β4-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 ± S.E. of three determinations.

We also used Western blots and immunoprecipitation experiments to test the purified subtypes for the possible presence of other subunits (e.g., α3, α6, α7, α8, β3, and β4 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 α2, α5, and β2 subunits in our α2-containing receptors, we defined it as the α2α5β2 subtype.

The molecular masses of both the α5 and β2 subunits determined by Western blotting corresponded to the expected sizes deduced from their cDNA sequences, whereas the molecular mass of the α4 subunit was slightly lower.

Conroy and Berg (1998) have previously reported that chick brain has an α5-containing receptor associated with the α4 and β2 subunits (α4α5β2 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 α2-containing receptors and 10 to 15% of the receptors contain the α5 subunit, we
purified the α4β2 subtype and α5-containing subtypes and studied their subunit composition. Forebrain extract devoid of the α3- and β4-containing receptors was passed on the immunoaffinity column with bound anti-α5 Abs, and the flow-through was incubated with anti-β2 Abs. The bound β2- and α5-containing receptors were eluted by competition with β2 and α5 peptides, respectively, labeled with [3H]Epi, and immunoprecipitated with the same Abs used for the characterization of the optic lobe subtypes. The percentage of immunoprecipitation of the forebrain α5-containing receptors by the anti-α2, anti-α4, anti-α5, and anti-β2 Abs was, respectively, 3.6 ± 2, 61 ± 7, 69 ± 7, and 95 ± 4% (Fig. 2B, left); the same Abs immunoprecipitated, respectively, 2 ± 1, 73 ± 8, 1.5 ± 1, and 83 ± 5% of the α4β2 receptors. These immunoprecipitation experiments confirm that the α4β2 subtype is present together with an α2α5β2 subtype (Fig. 2B, right), whereas there was almost no α2α5β2 subtype.

**Ontogeny of α2- and α5-Containing Receptors**

Given the selective enrichment of the α2-containing receptors in the optic lobe at P1, we studied their relative contribution to the [3H]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 [3H]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 [3H]Epi at saturating concentrations of 2 nM.

Measured as [3H]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-α2, anti-α5, and anti-α3 Abs showed that there was a selective increase in both the α2 and α5 subunits in the optic lobe after E11; the increase from E7 to P1 was 26-fold for the α2 and 25-fold for the α5 subunit compared with respective increases of 5- and 6-fold for the α2 and α5 in the forebrain. The increase in the α3 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 [3H]Epi-labeled receptors/mg protein immunoprecipitated by the subunit-specific Abs at each stage of development in both tissues; the values are the mean ± S.E. of three different experiments.

**Pharmacological Experiments on α4β2 and α2α5β2 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 [3H]Epi to the immunoimmobilized subtypes. The interaction of [3H]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 α2α5β2 subtype and 86 pM (CV =...
19%) for the α4β2 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 immunoimmobilized 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 > cytisine > nicotine > acetylcholine > DMPP > Carb for the α2α5β2 subtype and Epi > cytisine > nicotine = DMPP > acetylcholine > Carb for the α4β2 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 α2α5β2 subtype were lower than those of the same compounds for the α4β2 subtype. This indicates a slightly higher affinity for α2α5β2, but because this difference was not statistically significant, we can conclude that the α4β2 and α2α5β2 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 α2α5β2 and α4β2 immunopurified...
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 μM Carb, together with the integral-amplitude and lifetime histograms (open and closed state) of each trace (Fig. 7B).

The α2α5β2 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 μM Carb (T_{o2α5β2} = 3 ms, T_{oα2α5β2} = 4 ms), whereas the mean closed-state lifetime for the α4β2 subtype was much shorter (T_{cα4β2} = 4 ms, T_{cα2α5β2} = 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 μM and 1 mM for the α4β2 and α2α5β2 subtypes, respectively) and blocked by 50 μM 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 α2α5β2 subtype had a significantly higher EC_{50} value, so the P_{o} of the α4β2 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 α2α5β2 subtype and 40 pS for the α4β2 subtype. The P_{o} of the α4β2 subtype was slightly voltage-dependent, whereas that of the α2α5β2 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 α4β2 (less than 1%) and higher (5–10%) in the α2α5β2 subtype. In the α2α5β2 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 α2, α5, and β2 subunits is selectively present in chick optic lobe. It coexists with the α4β2 subtype and is strictly developmentally regulated because it only appears after E11. In addition to the α4β2 subtype, the forebrain has an α5-containing subtype, but it contains the α4 and β2 subunits and not the α2 subtype.

Conroy and Berg (1998) found that almost all of the α5-containing receptors present in chick brain at E8 are associated with the α4 and β2 subunits, but the fact that some of the receptors containing the α5 subunit cannot be immunodepleted from E18 brain extract using an anti-α4 mAb suggests that some of the α5-containing receptors can be associated with an α-subunit other than α4 late in brain development.

This study demonstrates that this hypothesized subtype is the α2α5β2 subtype and that it is specifically enriched in the optic lobe.

Using the anti-α2 Abs, we immunoprecipitated more than 50% of this α2α5β2 subtype, whereas the anti-α4 Abs immunoprecipitated only 2 to 3%. The same Abs had opposite immunoprecipitation capacities on the α4β2 subtype; the anti-α2 immunoprecipitated only a maximum of 2%, whereas the anti-α4 immunoprecipitated more than 65% of the α4β2 optic lobe receptors. Using the same Abs, we also obtained very similar immunoprecipitation results with the α4β2 subtype purified from the forebrain.

The incomplete immunoprecipitation of the α2α5β2 subtype by the α2 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-α5 Abs (the anti-α5 COOH and mAb 35) on the α4α5β2 subtype purified from the forebrain.

During development, the receptors containing the α2 and α5 subunits are strongly regulated in the optic lobe but much less so in the forebrain. It is possible that the α2α5β2 subtype is mainly expressed after hatching when the chick’s visuomotor system is first used, probably because its expression is activity dependent. Changes in the level of the α7 and α8 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 α2- and α5-containing receptors is due to a local increase in receptor expression in optic lobe neurons or to their possible transporation 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

<table>
<thead>
<tr>
<th>Subtype</th>
<th>K_{+} (nM)</th>
<th>K_{-} (nM)</th>
<th>K_{E} (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α2α5β2</td>
<td>0.029 (23)</td>
<td>0.046 (18)</td>
<td>582 (18)</td>
</tr>
<tr>
<td>α4β2</td>
<td>0.086 (19)</td>
<td>0.032 (18)</td>
<td>599 (10)</td>
</tr>
<tr>
<td>Epi</td>
<td>2.7 (31)</td>
<td>5.7 (12)</td>
<td>38 (30)</td>
</tr>
<tr>
<td>Cytisine</td>
<td>25 (35)</td>
<td>40 (15)</td>
<td>13.8 (23)</td>
</tr>
<tr>
<td>Nicotine</td>
<td>25 (35)</td>
<td>38 (30)</td>
<td>9 (12)</td>
</tr>
<tr>
<td>ACh</td>
<td>60 (35)</td>
<td>38 (30)</td>
<td>32 (14)</td>
</tr>
<tr>
<td>DMPP</td>
<td>11 (14)</td>
<td>582 (18)</td>
<td>599 (10)</td>
</tr>
<tr>
<td>Carb</td>
<td>582 (18)</td>
<td>38 (30)</td>
<td>13.8 (23)</td>
</tr>
<tr>
<td>β-erythroidine</td>
<td>1 (21)</td>
<td>38 (30)</td>
<td>9 (12)</td>
</tr>
<tr>
<td>d-Tubocurarine</td>
<td>71.6 (18)</td>
<td>582 (18)</td>
<td>599 (10)</td>
</tr>
<tr>
<td>Hexamethonium</td>
<td>2720 (22)</td>
<td>605 (20)</td>
<td>1640 (29)</td>
</tr>
</tbody>
</table>

The K_{+} and K_{-} values were derived from [3H]-Epi saturation and competition binding curves to the α2α5β2 and α4β2 subtypes. The curves obtained from three separate experiments were fitted using the nonlinear least-squares analysis program and the F test (Munson and Rodbard, 1980). The numbers in parentheses represent the percent of CV.
sists of the optic tectum and a region subjacent to the tectal ventricle that contains a number of isthmic 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 α2 subunit mRNA (Daubas et al., 1990) and immunoreactivity for the α2, α5, α7, and β2 subunits late in development (Ullian and Sargent, 1995). After SpL lesions, both the α5 and β2 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 β2-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 α2 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 α2α5β2 and α4β2 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 α4β2 and α2α5β2 receptor channels, together with their integral amplitude histograms. The applied membrane voltage was 100 mV, the Carb concentration was 500 μM. The traces were digitized at a sampling rate of 2000 points/s. The α4β2 subtype had well-defined closed and open states, with the open state having a major conductance of 40 pS; the α2α5β2 subtypes had a major conductance of 38 pS and an additional conductance with a quite low probability of 59 pS. The α4β2 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 α4β2 and α2α5β2 subtypes taken form 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 α4β2 and α2α5β2 subtypes for nicotinic ligands: the β-subunit is the major factor in determining agonist affinity (Parker et al., 1998) and the same β2 subunit is contained in both subtypes. The α2 and α4 subunits are highly homologous in the extracellular N terminus (Sargent, 1993; Gotti et al., 1997a). The presence of the α5 subunit in the subtypes may not change the affinity of [3H]-Epi binding, as has already been demonstrated in the case of the human α3β2 and α3β4 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 α4β2 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 α2α5β2 subtype being 10 times longer. This increase in the closed-state lifetime of the α5-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 α2α5β2 subtype. We do not know if this is due to the presence of the α2 or α5 subunit or both. As reported by Gerzanich et al., (1998) for the human α3 subtypes, it is possible that the presence of the α5 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

**TABLE 3**
Electrophysiological characteristics of α2α5β2 and α4β2 subtypes

<table>
<thead>
<tr>
<th>Channel Properties</th>
<th>α2α5β2 Subtype</th>
<th>α4β2 Subtype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conductance (pS) a</td>
<td>38</td>
<td>40</td>
</tr>
<tr>
<td>Mean lifetime (ms) b</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>τ(α)</td>
<td>40</td>
<td>4</td>
</tr>
<tr>
<td>Mean open probability (P o) c</td>
<td>0.1</td>
<td>0.43</td>
</tr>
<tr>
<td>Ion selectivity</td>
<td>Na ~ K =&gt; Cl −</td>
<td>Na ~ K =&gt; Cl −</td>
</tr>
<tr>
<td>Agonist profile</td>
<td>ACh (EC 50, μM) d</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Carb (EC 50, μM) d</td>
<td>300</td>
<td>500</td>
</tr>
<tr>
<td>Sensitivity to d-tubocurarine</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

a, b, c, d mean open- or closed-state lifetimes.

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

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 α2α5β2 is voltage-independent, whereas that of the α4β2 subtype is slightly voltage-dependent. In addition to the main conductances of 40 pS for the α4β2 and 38 pS for the α2α5β2 subtype, both subtypes have other conductances (but these are more frequent in the α2α5β2 subtype).

The chick α4β2 subtype has also been expressed in heterologous systems: it has a conductance of 24 ± 5 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 α4β2 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 α4β2 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 expression systems or the fact that artificial bilayers could be due to the purification process itself and/or the fact that certain regulatory processes important for the function of the channels are due to these subunit combinations. However, although we do not know the physiological role of the α2α5β2 subtype, we have demonstrated its presence in chick brain and further illustrated the diversity and complexity of the nAChR family.

Acknowledgments

We thank Kevin Smart and Ida Ruffoni for aid with the manuscript.

References


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