0026-895X/98/030392-06$3.00/0

MOLECULAR PHARMACOLOGY, **53:**392–401 (1998).

Nicotinic Receptor Subtypes in the Developing Chick Brain: Appearance of a Species Containing the α4, β2, and α5 Gene Products

WILLIAM G. CONROY and DARWIN K. BERG

Department of Biology, University of California, San Diego, San Diego, California 92093-0357

Received August 5, 1997; Accepted November 23, 1997

This paper is available online at http://www.molpharm.org

ABSTRACT

Increasing evidence suggests nicotinic receptors regulate developmental events in the nervous system. We used [^{3}H]epibatidine and ^{125}I-α-bungarotoxin, together with subunit-specific monoclonal antibodies, to distinguish and quantify nicotinic receptor subtypes in developing chick brain. The results show that more than three fourths of the epibatidine-binding receptors at both early and late embryonic stages contain α4 and β2 subunits, representing receptors previously distinguished by high affinity nicotine binding. A fraction of these also contain the α5 gene product, which is consistent with studies on transfected cells showing that the α4, β2, and α5 gene products coassemble to produce epibatidine-binding receptors. A small portion of the receptors contain α3 and β4 subunits, assembled in part with either α4 or β2 subunits. The most abundant nicotinic receptors, however, at both early and late embryonic stages are those having high affinity for α-bungarotoxin rather than epibatidine. Most contain α7 subunits, whereas about half contain α8 subunits as well. The sharpest developmental increase between embryonic days 8 and 17/18 occurs with receptors containing α5 subunits, whereas receptors containing α3 or β4 subunits undergo no specific increase. The three major receptor species (containing α4 and β2 but not α5 subunits; α7 subunits; or α7 and α8 subunits) each increase ≈3-fold during the same period. The results indicate greater receptor complexity than appreciated previously; they provide information about the rules governing subunit assembly in neuronal nicotinic receptors and draw attention to the role of α5 subunits in late development.

AChRs in the vertebrate nervous system are ligand-gated ion channels that depolarize neurons by permitting cations to flow across the plasma membrane. Like their counterparts in vertebrate skeletal muscle, the receptors are thought to be pentameric transmembrane proteins encoded by one or more members of a multigene family (Karlin and Akabas, 1996). It is clear that neuronal AChRs can participate in a variety of functions. Presynaptically, the receptors can modulate neurotransmitter release, whereas postsynaptically, they can generate synaptic currents from both synaptic and perisynaptic locations (Role and Berg, 1996; Zhang *et al*., 1996; Wonnacott, 1997). Some classes of AChRs have a high relative permeability to calcium and can influence calcium-dependent events in neurons, including activation of second messenger cascades.

Neuronal AChRs may play important developmental roles as well. The receptors are expressed early during embryogenesis, as is the enzyme responsible for synthesis of ACh (Zoli *et al*., 1995; Role and Berg, 1996). AChRs can be found on the tips of growing neurites in cell culture and, when activated, can influence the pattern of neurite growth. Presynaptic AChRs also can enhance neurotransmitter release at newly formed neuromuscular synapses in culture (Fu and Liu, 1996), and they may contribute to the early stages of synaptogenesis.

It is not clear which AChR subtypes are likely to be most important during development, or, in fact, how many AChR subtypes exist. Nine genes encoding neuronal AChR subunits (α2–7; β2–4) have been isolated from both chick and rat (Role and Berg, 1996). In addition, α8 has been isolated uniquely from chick, and α9 has been isolated uniquely from rat. Two major AChR subtypes have been identified in brain from both species: one is a receptor with α4 and β2 subunits that binds nicotine with high affinity, and the other is a receptor with α7 subunits that binds α-Bgt with high affinity (Lindstrom, 1996). Receptor analyses with subunit-specific mAbs also have identified other AChR subunit combinations in brain

This work was supported by grants from the National Institutes of Health (NS12601 and NS35469), Muscular Dystrophy Association, Council for Tobacco Research (4191), and Tobacco-Related Disease Research Program.

ABBREVIATIONS: ACh, acetylcholine; AChR, nicotinic acetylcholine receptor; α-Bgt, α-bungarotoxin; DHBE, dihydro-β-erythroidine; HEK, human embryonic kidney; mAb, monoclonal antibody; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PBS-TX, phosphate-buffered saline containing 0.5% (w/v) Triton X-100; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

(Flores *et al*., 1996; Lindstrom, 1996; Forsayeth and Kobrin, 1997).

Recently, the alkaloid epibatidine emerged as a broad-spectrum cholinergic ligand for neuronal AChRs. [^{3}H]Epibatidine seems to bind with high affinity to all AChR species examined to date, except for some that bind α-Bgt (Gerzanich *et al*., 1995; Houghtling *et al*., 1995; Wang *et al*., 1996). As a result, [^{3}H]epibatidine, together with ^{125}I-α-Bgt and subunit-specific mAbs, offers a means of identifying and quantifying additional AChR subtypes in brain. In the current report, we used this strategy to address four questions: Do the major AChR species identified in brain contain other gene products as well? Do individual AChR genes often contribute subunits to different AChR subtypes in the central versus peripheral nervous system? Can new AChR subtypes be detected? Do the relative amounts of individual AChR subtypes change substantially during development? Answers to these questions should provide information about the rules governing subunit assembly in neuronal AChRs and may help identify the receptor species most important at early developmental stages.

Experimental Procedures

mAbs. All subunit-specific mAbs used in the current study have been characterized and described previously (see references in Vernallis *et al*., 1993; Conroy and Berg, 1995). Regarding specificities with chicken neuronal AChR gene products, mAbs 270, 289, 308, 313, and B4–1 both immunoprecipitate and immunoblot selectively the β2, α4, α8, α3, and β4 gene products, respectively, whereas mAbs 318 and 319 do the same with the α7 gene product. mAb 268 selectively immunoblots (but does not immunoprecipitate) the α5 gene product, whereas mAb 35 immunoprecipitates and immunoblots the α5 gene product (it was raised against electric organ AChRs and recognizes the α1 subunit therein). Experiments with transfected cells indicate that mAb 35 also immunoprecipitates receptors with α3 subunits but does not recognize the α4, α7, α8, β2, or β4 gene products (Conroy *et al*., 1992; W. Conroy and D. Berg, unpublished observations). Unless otherwise indicated, the mAbs were diluted from concentrated stocks of hybridoma culture supernatants, or mouse ascites fluid (B4–1 only) for use or were purified using Protein G-Sepharose-4-Fast-Flow (Pharmacia, Piscataway, NJ) or size exclusion chromatography (mAb 289). Purified mAbs 35, 270, 289, 313, and B4–1 and normal IgG were coupled individually to Actigel (Sterogene Bioseparations, Arcadia, CA) at 2–4 mg/ml according to the manufacturer's specifications.

Binding assays. Whole brains were dissected from embryonic day (E) 8–18 chicks and stored frozen at −70° until use. Triton X-100 (Pierce Chemical, Rockford, IL) extracts of the brains were prepared as described previously (Conroy *et al*., 1992) using two to four volumes of 2% (w/v) Triton X-100 extraction buffer/g of tissue. For filter binding assays, 2–4 nM [^{3}H]epibatidine or 5–10 nM ^{125}I-α-Bgt was incubated with 25 μl of brain extract in a total volume of 0.1 ml for 2 hr at room temperature. After the incubation, 4 ml of wash buffer (10 mM Tris, pH 7.5, containing 0.05% Triton X-100) was added, and the solution was filtered immediately through Whatman (Clifton, NJ) GF/B filters presoaked in 0.5% polyethyleneimine. The filters were washed two additional times with 4 ml of wash buffer and counted either by liquid scintillation counting in Ecolite (ICN, Costa Mesa, CA) for [^{3}H]epibatidine or by γ-counting for ^{125}I-α-Bgt. Nonspecific binding was determined by incubation in the presence of 200 μM nicotine. Protein in the extracts was quantified by the BCA Protein Assay (Pierce) using bovine serum albumin as standard.

[^{3}H]Epibatidine binding sites associated with specific AChR subunits were quantified using a modification of a solid-phase immunoprecipitation assay (Conroy and Berg, 1995) and the mAbs 35 (α3, α5), 270 (β2), 289 (α4), 313 (α3), and B4–1 (β4) to tether AChRs. Immulon 2 Removawells (Dynatech Laboratories, Chantilly, VA) were coated with mAb by first incubating the wells overnight at 4° on a shaker with affinity-purified rabbit anti-mouse antibodies (Jackson Immunoresearch, West Grove, PA) at a concentration of 20 μg/ml in PBS (1× = 0.15 M sodium chloride and 0.01 M sodium phosphate, pH 7.4) containing 0.02% azide. The wells then were washed three times with PBS-TX and incubated on a shaker overnight at 4° with 50 μl of anti-AChR mAb diluted in buffer. The wells were rinsed three times with PBS-TX and incubated overnight with the detergent extracts. The extracts were removed, and the wells were washed four times with PBS-TX. [^{3}H]Epibatidine (100–200 μl) was added at 2–4 nM in the presence and absence of 200 μM nicotine. The wells were washed four times with PBS-TX and placed in 5 ml of Ecolite (ICN) for liquid scintillation counting.

When competition binding experiments were performed with [^{3}H]epibatidine, the incubations included the indicated compounds with 1 nM [^{3}H]epibatidine and were carried out for 4 hr at room temperature. Plots of competition curves were generated, and IC_{50} values were determined by a nonlinear least-squares method using Prism (GraphPAD Software, San Diego, CA) assuming a single class of sites in each case. K_D values for epibatidine were determined from saturation binding reactions in which the receptor concentration did not exceed 60 pM. Because ligand depletion may nevertheless have had some effect on the K_D determinations (Kenakin, 1993), the values are considered approximate (apparent K_D values).

Immunodepletions. Extracts were depleted of AChR subtypes by incubation of extracts (0.2 ml) with 40 μl of anti-AChR mAb coupled to Actigel beads. After an overnight incubation, the Actigel beads with bound material were removed by centrifugation. [^{3}H]Epibatidine binding sites then were quantified in the depleted extracts using the filter binding assay.

AChRs also were depleted by incubation of extracts in Immulon 2 Removawells (Dynatech Laboratories) coated with mAbs 270 or 289, as described above for the solid-phase immunoprecipitation assay, to remove AChRs containing β2 and α4 subunits, respectively. After recovery of the depleted extracts from the wells, they were used in a solid-phase assay with [^{3}H]epibatidine to quantify the remaining AChRs. Similarly, a combination of mAbs 318 and 319 was used to deplete AChRs with α7 subunits, and mAb 308 was used to deplete AChRs with α8 subunits. ^{125}I-α-Bgt binding sites remaining in the depleted extracts then were quantified by the filter binding assay. In sequential depletion experiments, recovered extracts from one round of depletion were subjected to a second round of depletion in a new well containing either the same mAb as in the first round or an mAb to another subunit. In all depletion experiments, normal rat IgG was used in place of the anti-AChR mAbs as a negative control.

Immunoprecipitations and immunoblots. AChRs containing α4, β2, and α5 subunits were analyzed by monitoring the coprecipitation of α5 subunits with AChRs containing both α4 and β2 subunits. Similar analyses have been used to show the association of α3, α5, and β4 subunits in ganglia (Vernallis *et al*., 1993; Conroy and Berg, 1995). E8 and E18 brain extracts containing equal amounts of protein (0.7 mg) were incubated on Immulon 2 Removawells (Dynatech Laboratories) coated with mAb 289, as described above for the solid-phase assay, to remove AChRs containing α4 subunits. Removawells coated with normal rat IgM served as the negative control. Extracts recovered from these wells then were incubated overnight with 20 μl of mAb 270-Actigel to bind AChRs containing β2 subunits. The gel was washed three times with PBS-TX and 2 times with PBS-TX containing 1 M NaCl. Bound material was eluted with SDS-PAGE sample buffer, subjected to SDS-PAGE, electroblotted to nitrocellulose, and probed with mAbs 268 for α5 and 289 for α4 as described previously (Vernallis *et al*., 1993; Conroy and Berg, 1995). Horseradish peroxidase coupled to goat anti-rat IgG (Jackson Immunoresearch) was used to detect bound mAbs. Signals were visualized by enhanced chemiluminescence (Amersham, Arlington Heights, IL). Molecular mass markers for the blots included phosphorylase B

(97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), and carbonic anhydrase (31 kDa) (low range; BioRad).

Transfections. Chicken AChR gene constructs were used for the transfections. The expression constructs pCDM8-Ch 23.1 and pCDM8-Ch 26.1 containing the β2 and α4 cDNAs, respectively, under CMV promoters (Whiting *et al*., 1991) were kindly provided by Dr. Paul Whiting (Merk, Sharp, & Dohme Laboratories, Essex, England). An α5 cDNA kindly provided by M. Ballivet (University of Geneva, Geneva) was modified to delete untranslated 3′ and 5′ sequences and to add the α3 leader sequence, which has an efficient Kozak consensus sequence for translation initiation. The modified α5 construct was subcloned into the RSV.An vector (obtained from D. Donahue, University of California, San Diego) under an RSV promoter.

Transfections were performed on HEK 293 cells obtained from J. Wahl (Salk Institute, La Jolla, CA) and grown in Eagle's minimum essential medium supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, and 50 units each of penicillin and streptamycin. The cells were plated at 3.4×10^5/35-mm culture dish and maintained at 37° in 95% air/5% CO_2. After 24 hr, the cells were transfected using calcium phosphate-DNA precipitation in a BES buffer as described previously (Chen and Okayama, 1987). Transfections usually contained 1 μg of α4, 0.2 μg of β2, and/or 0.5 μg of α5 cDNA/dish; pBluescript SK$^-$ DNA (Stratagene, La Jolla, CA) was added as needed to adjust the total to 2 μg in each case. The ratio of α4 to β2 cDNA was chosen to optimize the amount of [^{3}H]epibatidine binding expressed by the cells (see below). The presence of α5 DNA did not significantly alter the amount of [^{3}H]epibatidine binding expressed by cells transfected with α4 and β2 cDNA.

After 18–24 hr of transfection, the cells were washed with fresh medium, incubated an additional 24 hr, and then harvested by scraping in 0.5 ml of solubilization buffer containing 50 mM sodium phosphate, pH 7.4, 1% Triton X-100, and the protease inhibitors iodoacetamide (0.4 mM), benzamidine (5 mM), phosphoramidon (5 μg/ml), soybean trypsin inhibitor (10 μg/ml), leupeptin (10 μg/ml), pepstatin A (20 μg/ml), EDTA (5 mM), EGTA (5 mM), aprotinin (2 μg/ml), and phenylmethylsulfonyl fluoride (1 mM). Insoluble material was removed by centrifugation for 15 min in a microfuge at 4°. [^{3}H]Epibatidine binding sites were quantified using the solid-phase assay. Immunoblot analysis was used to confirm the expression of transfected genes.

Materials. White Leghorn embryonated chick eggs were obtained locally and maintained at 39° in a humidified incubator as described by Conroy *et al*. (1992). mAb 35 was purified and radioiodinated as described by Smith *et al*. (1985). α-Bgt was purchased from Biotoxins (St. Cloud, FL) and radioiodinated to a specific activity of $0.5–0.7 \times 10^{18}$ cpm/mol using chloramine T. [^{3}H]Epibatidine (56.5 Ci/mmol) was a generous gift from DuPont-New England Nuclear (Boston, MA). Unlabeled epibatidine was purchased from Research Biochemicals (Natick, MA). mAbs 268, 270, 289, 308, 313, 318, and 319 were generously supplied by Dr. Jon Lindstrom (University of Pennsylvania, Philadelphia, PA). All compounds were purchased from Sigma Chemical (St. Louis, MO) unless otherwise indicated. Dr. Paul Whiting (Merck, Sharp, & Dohme Laboratories) generously provided the chicken AChR α4 and β2 gene constructs, and Dr. Marc Ballivet (University of Geneva, Geneva, Switzerland) generously provided the chicken AChR α5 cDNA. Dr. Jeffrey Wahl (Salk Institute, La Jolla, CA) provided the HEK 293 cells, and Dr. Daniel Donoghue (University of California, San Diego) provided the RSV.An vector.

Results

Epibatidine-binding AChRs in brain. Filter binding assays were used to measure the total number of epibatidine binding sites present in chick brain extracts. A mean value of 109 ± 28 fmol/mg of protein (mean ± standard error; three experiments) was obtained with 2 nM [^{3}H]epibatidine for extracts prepared from E17/18 chick brain. No additional sites were revealed by increasing the concentration of epibatidine (data not shown), which is consistent with previous studies showing that 2 nM epibatidine is sufficient to saturate epibatidine binding sites on neuronal AChRs (Gerzanich *et al*., 1995; Houghtling *et al*., 1995; Wang *et al*., 1996).

Immunodepletion with subunit-specific mAbs before conducting the filter assays provides a means of identifying AChR gene products making up the epibatidine-binding receptors. Anti-α4 mAb 289 immunodepleted ≈75% of the epibatidine binding capacity of E17/18 brain extracts, whereas anti-β2 mAb 270 immunodepleted ≈85% (Fig. 1A). No significant depletion was caused by either anti-α3 mAb 313 or

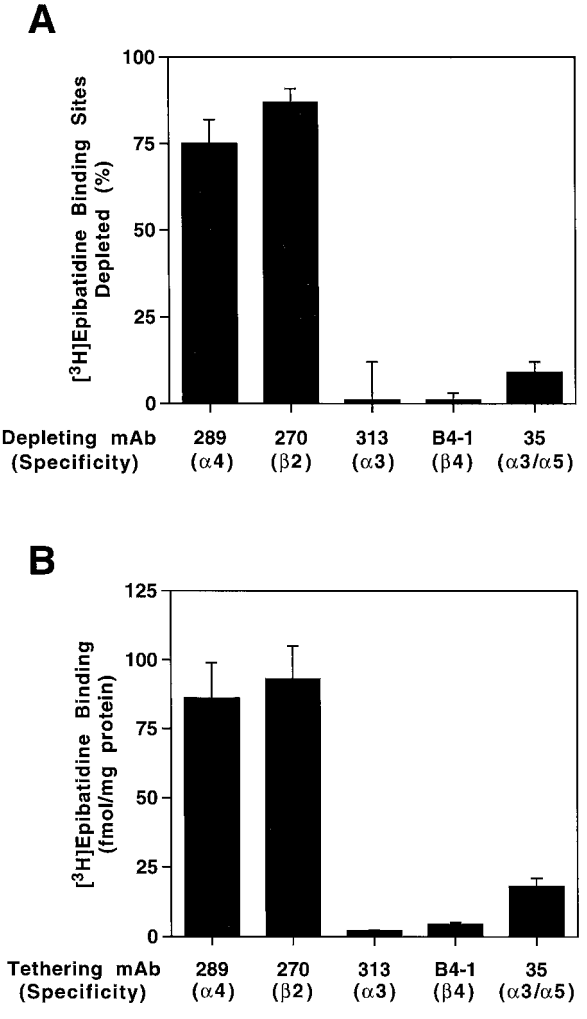

Fig. 1. Brain AChR species identified by [^{3}H]epibatidine binding and subunit-specific mAbs. A, Receptors containing α4 or β2 subunits. Detergent extracts of E17/18 chick brains were incubated with the indicated mAbs (depleting mAb with indicated specificity) to deplete AChRs. The depleted extracts then were assayed for [^{3}H]epibatidine binding in filter binding assays. Normal rat IgG was used as a negative control for the immunodepletions. mAbs to either the α4 or β2 gene products depleted most of the [^{3}H]epibatidine binding sites from chick brain extracts. mAb 35 depleted approximately one tenth of the sites, indicating that a small proportion of the AChRs contain at least one other gene product as well (most likely α5). B, Receptors containing α3 or β4 subunits. Direct binding of [^{3}H]epibatidine to AChRs immunotethered in solid-phase assays with the indicated mAbs (tethering mAb with indicated specificity) confirms that most contain α4 and β2 subunits but also shows that small but significant numbers contain α3 and β4 subunits. Values represent the mean ± standard error from three experiments.

anti-β4 mAb B4–1. mAb 35, which recognizes the neuronal α3 and α5 gene products, immunodepleted ≈10% of the binding. The results are consistent with the major epibatidine-binding AChR species in E17/18-brain containing the α4 and β2 gene products. Few AChRs at this stage contain either the α3 or β4 gene products, but a portion are likely to contain the α5. This latter conclusion comes from the different amounts of immunodepletion achieved by mAbs 313 and 35.

A more sensitive method for identifying subpopulations of brain AChRs capable of binding epibatidine is to use a solid-phase immunoprecipitation assay. In this case, a subunit-specific mAb is used to immunotether all receptors containing a given gene product, and then [^{3}H]epibatidine binding is used to quantify the number of receptors retained by the antibody. By this criterion, many epibatidine-binding receptors in E17/18 brain extracts seem to contain only the α4 and β2 gene products (α4/β2-AChRs; Fig. 1B). The anti-α4 mAb 289 immunoprecipitated 86 ± 13% (three experiments), whereas the anti-β2 mAb 270 immunoprecipitated 93 ± 12% of the total [^{3}H]epibatidine binding measured with the filter assay. mAb 289 is specific under these conditions; it does not, for example, retain in the two-site assay chick ciliary ganglion AChRs that contain the α3, β4, and α5 subunits coassembled (Vernallis *et al.*, 1993) and bind epibatidine in E17/18 extracts (data not shown).

Only small numbers of binding sites were retained by either the anti-α3 mAb 313 or the anti-β4 mAb B4–1 in E17/18 brain extracts (Fig. 1B). mAb 35, in contrast, immunotethered approximately one sixth of the total number of sites. Control experiments performed with E17/18 ciliary ganglion extracts demonstrated that mAb 35 is 2.0 ± 0.1-fold (mean ± standard error; three experiments) more efficient than mAb 313 in the two-site assay at capturing AChRs containing the appropriate subunit (summing two sequential immunoprecipitations for each mAb). Identical experiments performed on E17/18 brain extracts indicated that mAb 35 was able to capture 12.1 ± 4.6-fold (three experiments) more receptors than mAb 35 (again summing two sequential passes with each mAb). The results are consistent with those in Fig. 1B and suggest that substantially more brain AChRs contain α5 subunits than α3 subunits. This, together with the other immunoprecipitation data, is consistent with most of the brain receptors recognized by mAb 35 being α4/β2/α5-AChRs.

Subunits coassembled with the α4 and β2 gene products. The ability of the α4 and β2 gene products to form receptors that bind nicotine with high affinity is well documented (Whiting *et al.*, 1991; Lindstrom, 1996). Less clear is the extent to which the two kinds of gene products are associated with other kinds of subunits in brain AChRs. This was tested by using a combination of immunodepletions and solid-phase assays. One mAb was used to immunodeplete all receptors containing a given gene product, whereas a second mAb was used to immunotether the remaining receptors containing a different gene product. The tethered receptors then were quantified with [^{3}H]epibatidine binding in the solid-phase assay. By comparing the binding values obtained with and without the immunodepletion step, it was possible to assess the proportion of receptors containing both gene products.

When the anti-α4 mAb 289 was used for immunodepletion, approximately three fourths of the epibatidine-binding receptors containing the β2 gene product were removed from the extract (Fig. 2A). In addition, more than half of the receptors containing either the α3 or β4 gene products were removed. More than half of the receptors recognized by mAb 35 also were removed by the depletion with mAb 289. To test the efficiency of the immunodepletion step, the depleted extracts were tested for residual receptors containing the α4 gene product; approximately one fifth of the control value remained. Taking into account the immunodepletion efficiency determined in this manner, the results indicate that most of the brain AChRs containing the β2 gene product and more than half of the receptors containing the α3 or β4 gene products also contain the α4 gene product. The same is likely to be true of receptors containing the α5 gene product and

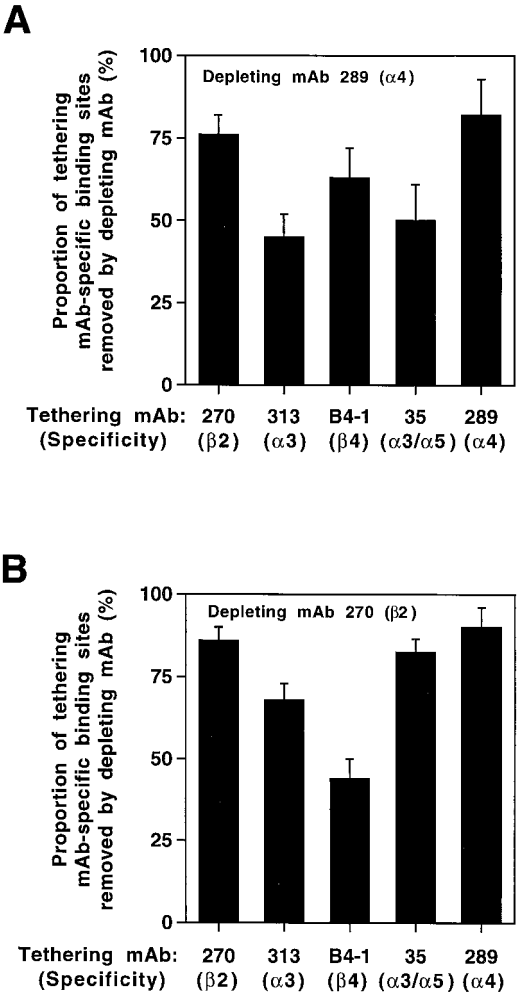

Fig. 2. Sequential immunodepletions to identify the proportion of receptors containing two given gene products coassembled. A, Receptors containing α4 coassembled with other subunits. After immunodepletion with the anti-α4 mAb 289, extracts were analyzed for [^{3}H]epibatidine binding using solid-phase immunoprecipitation assays in which the indicated mAbs (tethering mAb with indicated specificity) were used to immunotether receptors containing the corresponding gene product. The results are expressed as a percentage of the binding obtained in the absence of immunodepletion [i.e., when normal rat IgM was substituted for the IgM mAb 289 (defined as 100%)]. With mAb 289 used for both the depletion and tethering of receptor in the solid-phase assay, the efficiency of depletion was shown to be ≥75%. B, Receptors containing β2 coassembled with other subunits. Experiments were performed as in A except that anti-β2 mAb 270 replaced mAb 289 in the immunodepletion step; efficiency of depletion was ≈85%. Values represent the mean ± standard error from three separate experiments.

recognized by mAb 35, namely, that they also contain the α4 gene product.

Immunodepletion with the anti-β2 mAb 270 removed >80% of the α4- and β2-containing receptors, corroborating the results obtained with mAb 289 (Fig. 2B). Between one half and three fourths of the α3 and β4-containing receptors were removed, as was more than three fourths of the receptors recognized by mAb 35. More than three fourths of the β2-containing receptors also were removed by the immunodepletion, demonstrating the efficiency of the antibody. The results support previous findings indicating that a substantial portion of the α4 and β2 gene products are assembled into α4/β2-AChRs with no other apparent subunits. The results also show, however, that some α4 and β2 subunits combine with additional kinds of subunits to make up a heterogeneous population of epibatidine-binding AChRs in the CNS.

Coassembly of α4, β2, and α5 subunits in transfected cells. The fact that mAb 35 immunoprecipitates many more epibatidine-binding sites from brain extracts than does the α3-specific mAb 313 suggests that the receptors contain α5 subunits instead of α3 subunits. These could represent α4/β2/α5-AChRs and constitute a significant fraction of all brain receptors containing the α4 and β2 gene products at the end of embryogenesis. Recently, the α5 gene product has been shown capable of assembling with α4 and β2 when coexpressed in *Xenopus laevis* oocytes (Ramirez-Latorre *et al.*, 1996). We examined this issue in cells that were not oocytes.

HEK 293 cells were transiently transfected with various combinations of the α4, β2, and α5 cDNAs. Two days later, cell extracts were prepared and examined in solid-phase assays for receptors that could bind [^{3}H]epibatidine and be immunotethered by anti-AChR mAbs. The α4 and β2 gene combination produced a large number of receptors tethered either by anti-α4 mAb 289 or by anti-β2 mAb 270, but none were recognized by mAb 35 (Fig. 3). Inclusion of α5 cDNA in the transfection with α4 and β2 had little effect on the total number of epibatidine-binding receptors tethered by mAbs 289 or 270 but generated a significant number of receptors recognized by mAb 35. Transfection with α5 alone, α5 plus α4, or α5 plus β2 yielded no significant epibatidine binding retained by any of the mAbs. The results clearly indicate that the α5 gene product can combine with the α4 and β2 gene products to produce epibatidine-binding α4/β2/α5-AChRs.

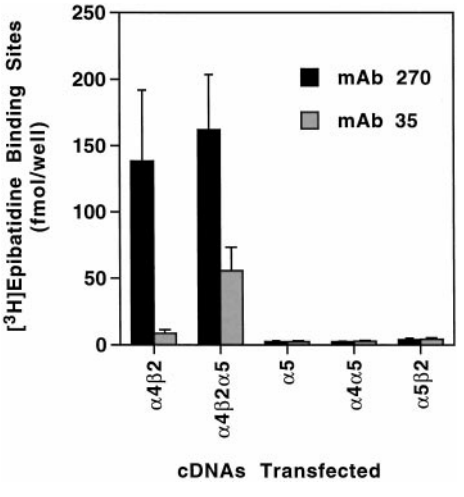

Fig. 3. Coassembly of the α4, β2, and α5 gene products in transfected cells to produce epibatidine-binding AChRs. HEK 293 cells were transiently transfected with the indicated combinations of AChR gene cDNAs and subsequently solubilized and assayed for [^{3}H]epibatidine binding in solid-phase assays using the indicated mAbs to tether receptors. The total amount of epibatidine binding was not influenced by expression of the α5 gene, but only expression of the α5 gene in concert with α4 and β2 produced epibatidine-binding receptors that could be immunoprecipitated by mAb 35. Because mAb 35 recognizes α5 protein but not α4 or β2 protein, the data indicate that the three gene products coassembled.

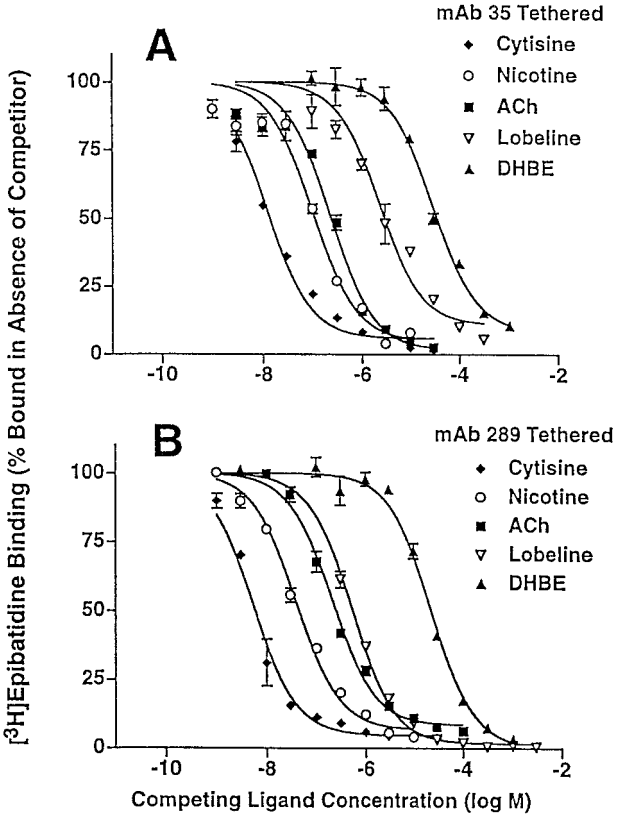

Fig. 4. Binding isotherms showing little difference between receptors containing the α5 subunit and those lacking it. AChRs were solubilized from E17 chick brain and immunotethered either (A) with mAb 35 or (B) with mAb 289 after having first depleted the extract with mAb 35. Competition binding studies were carried out with [^{3}H]epibatidine and the indicated ligands in solid-phase assays. Values represent the mean ± standard error of three determinations for each point from a single experiment. Similar results were obtained in at least two additional experiments. *DHBE*, dihydro-β-erythroidine.

Ligand binding properties of AChR subtypes. In *X. laevis* oocytes, heterologous expression of the chick AChR α4 and β2 genes with and without the α5 gene shows that α4/β2/α5-AChRs have a 100-fold higher EC_{50} value for activation by ACh than that of α4/β2-AChRs (20). In contrast, expression of the human α5 gene in oocytes with the α3 and β4 genes has no effect on the ACh dose-response curve, whereas expression of α5 with the α3 and β2 genes shifts the EC_{50} value to smaller values (Wang *et al.*, 1996).

To examine the possible contribution of α5 subunits to the pharmacology of native AChRs containing the α4 and β2 gene product, we first used mAb 35 to isolate a population of receptors from brain extract that included those with α5 subunits. mAb 289 then was used on the same mAb 35-depleted extracts to isolate a receptor population containing α4 (and presumably β2) but not α5 subunits. Binding assays revealed no significant difference in the affinity of the two

receptor populations for [^{3}H]epibatidine. The presumed $\alpha4/\beta2/\alpha5$-AChRs had an apparent K_D value of 2.8 ± 0.7 pM (five experiments), whereas $\alpha4/\beta2$-AChRs had an apparent K_D value of 5.4 ± 2.6 pM (three experiments). Similar binding assays with $\alpha4/\beta2/\alpha5$-AChRs isolated by mAb 35 from HEK 293 cells transfected with $\alpha4$, $\beta2$, and $\alpha5$ cDNA or with $\alpha4/\beta2$-AChRs isolated by mAb 289 from cells transfected with $\alpha4$ and $\beta2$ cDNA yielded apparent K_D values of 2.3 ± 0.5 pM (seven experiments) and 2.6 ± 0.9 pM (four experiments), respectively. Thus, receptors of known subunit composition from transfected cells corroborated the results with native AChRs in showing no difference in epibatidine affinity that could be ascribed to the presence of $\alpha5$ subunits.

Competition binding studies with [^{3}H]epibatidine were used to compare the binding affinities of receptor subtypes for other nicotinic ligands (Fig. 4). A comparison of IC_{50} values indicated at most a nominal 5-fold difference in affinity between putative $\alpha4/\beta2/\alpha5$- and $\alpha4/\beta2$-AChRs isolated from brain extract as described above (Table 1). The IC_{50} values for $\alpha4/\beta2/\alpha5$- and $\alpha4/\beta2$-AChRs from transfected cells were essentially indistinguishable from their brain counterparts. The results are consistent with the inferred subunit composition of the brain AChR subtypes and indicate little effect of $\alpha5$ subunits on ligand affinity in binding assays.

Changing populations of epibatidine-binding AChRs during development. The relative contributions of individual AChR gene products to epibatidine-binding AChR subtypes in brain were measured during development. The goal was to determine whether different species predominated at early versus late times. Solid-phase assays revealed three patterns. Of the epibatidine-binding AChRs, receptors containing either the $\alpha4$ or the $\beta2$ gene product were the most abundant at all times examined and increased developmentally ≈3-fold between E8 and E17/18 (Fig. 5A). This was over and above increases due to cell growth as reflected in the amount of total protein. Receptors containing either the $\alpha3$ or $\beta4$ gene products were the least abundant at all times examined and showed no developmental increase between E8 and E17/18; the small increase in binding sites represented net growth because no change was apparent when the values were normalized for protein. Most striking was the change in the number of epibatidine-binding receptors recognized by mAb 35. These increased 7-fold between E8 and E17/18 when normalized for protein (Fig. 5B). Because no developmental increase was observed in the number of receptors containing the $\alpha3$ gene product over the same time period, much of the increase is likely to involve receptors with $\alpha5$ subunits.

Immunoblot analysis provides a method for using the available mAbs to confirm the presence of individual gene products in AChRs and determine whether developmental increases in receptors recognized by mAb 35 correlates with increases in receptors containing $\alpha5$ subunits. Brain AChRs were concentrated by immunoprecipitation, eluted, gel electrophoresed, and analyzed on blots probed with subunit-specific mAbs. All five AChR gene products inferred above to be present in brain AChRs were detected on the blots (Fig. 6A). The relative amounts of $\alpha5$ protein assembled with $\alpha4$ and $\beta2$ protein at early and late developmental stages then was assessed by using the anti-$\beta2$ mAb 270 to immunoprecipitate the receptors. The material was eluted, electropho-

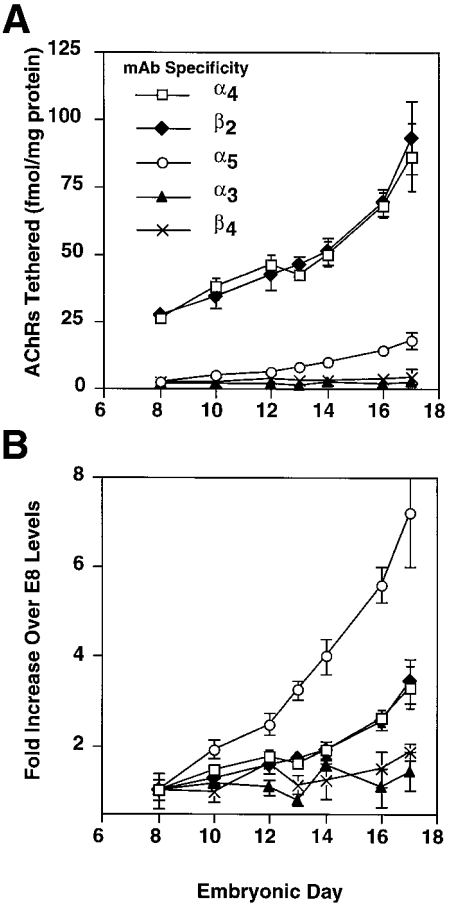

Fig. 5. Developmental changes in the epibatidine-binding AChRs expressed *in vivo*. Whole brains dissected from chick embryos at the indicated times were solubilized and assayed for [^{3}H]epibatidine binding using the solid-phase assay and subunit-specific mAbs to immunotether selected AChR subtypes. Receptors containing $\alpha4$ protein were tethered with mAb 289 (□), $\beta2$ with mAb 270 (♦), $\alpha3$ with mAb 313 (▲), $\beta4$ with mAb B4–1 (×), and the combination of $\alpha3$ and $\alpha5$ (and possibly unknown AChR gene products) with mAb 35 (○). A, Absolute amounts; binding is expressed per mg of protein. B, Relative increases; binding is normalized to that present at E8. Values represent the mean ± standard error from three or four experiments conducted on two or more preparations of extract.

TABLE 1

Ligand binding properties of AChRs immunoisolated from brain and transfected HEK 293 cells

AChRs from brain and HEK 293 cell extracts were immunotethered with subunit-specific mAbs in solid-phase immunoprecipitation assays and subjected to competition binding with [^{3}H]epibatidine and the indicated ligands as described in Fig. 4. Brain mAb 35 AChRs were isolated from brain extracts with mAb 35; brain mAb 289 AChRS were isolated from brain extracts with mAb 289 after depletion with mAb 35; HEK 293 $\alpha4/\beta2/\alpha5$ AChRs were isolated from HEK 293 cells transfected with the $\alpha4$, $\beta2$, and $\alpha5$ genes and using mAb 35; and HEK 293 $\alpha4/\beta2$ AChRs were isolated from HEK 293 cells transfected with the $\alpha4$ and $\beta2$ genes and using mAb 270. Values are mean ± standard error of three or four separate determinations. Few differences exist among them; HEK 293 $\alpha4/\beta2/\alpha5$ AChRs seem to be indistinguishable from brain mAb 35 AChRs, whereas HEK 293 $\alpha4/\beta2$ AChRs are very similar to brain mAb 289 AChRs.

Competing ligand	IC_{50} for competition with [^{3}H]epibatidine			
	Brain mAb 35 AChRs	Brain mAb 289 AChRs	HEK 293 $\alpha4/\beta2/\alpha5$ AChRs	HEK 293 $\alpha4/\beta2$ AChRs
	μM			
ACh	0.31 ± 0.01	0.20 ± 0.08	0.55 ± 0.07	0.15 ± 0.02
Nicotine	0.19 ± 0.06	0.07 ± 0.02	0.22 ± 0.02	0.06 ± 0.01
Cytisine	0.06 ± 0.05	0.05 ± 0.04	0.02 ± 0.01	0.004 ± 0.003
Lobeline	4.1 ± 1.7	1.5 ± 1.0	N.D.	N.D.
DHBE	37.8 ± 4.2	20.2 ± 1.3	38.8 ± 5.5	9.0 ± 3.1

DHBE, dihydro-β-erythroidine.

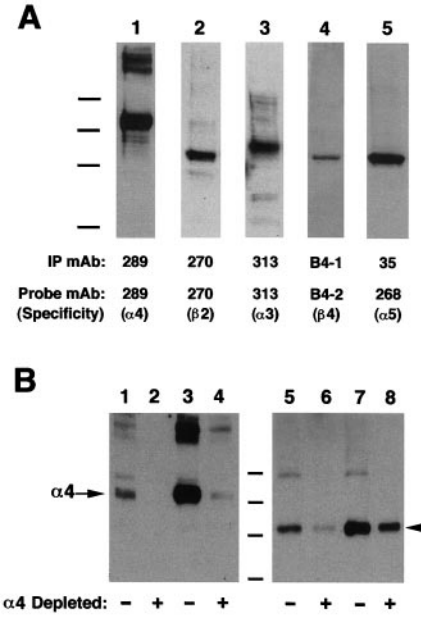

Fig. 6. Immunoblot analysis of brain AChR subtypes probed with subunit-specific mAbs. A, AChR gene products in E18 brain extracts. Receptors were immunopurified with the indicated mAb (*IP mAb*), subjected to SDS-PAGE, electroblotted, and probed with the indicated mAb (*probe mAb* with indicated specificity). *Lane 1*, α4. *Lane 2*, β2. *Lane 3*, α3. *Lane 4*, β4. *Lane 5*, α5 gene product. B, Developmental increase in brain AChRs containing α4, β2, and α5 subunits coassembled. Receptors were immunopurified from E8 (*lanes 1, 2, 5, and 6*) and E17/18 (*lanes 3, 4, 7, and 8*) brain extracts with the anti-β2 mAb 270 (*IP mAb*) before (*lanes 1, 3, 5, and 7*) and after (*lanes 2, 4, 6, and 8*) immunodepletion with the anti-α4 mAb 289 (*depleting mAb*) and then subjected to SDS-PAGE, electroblotted, and probed (*probe mAb*) with either the anti-α4 mAb 289 (*lanes 1–4*) or the anti-α5 mAb 268 (*lanes 5–8*). Almost all of the α5 protein at E8 is coassembled with both α4 and β2 subunits, and the amount increases substantially by E17/18. Some of the α5 protein at the later developmental stage also is found in a species lacking α4 subunits. The large molecular weight material detected with mAb 289 as probe (A, *lane 1*; B, *lanes 1–4*) is aggregated α4 protein. Similar results were obtained in three experiments.

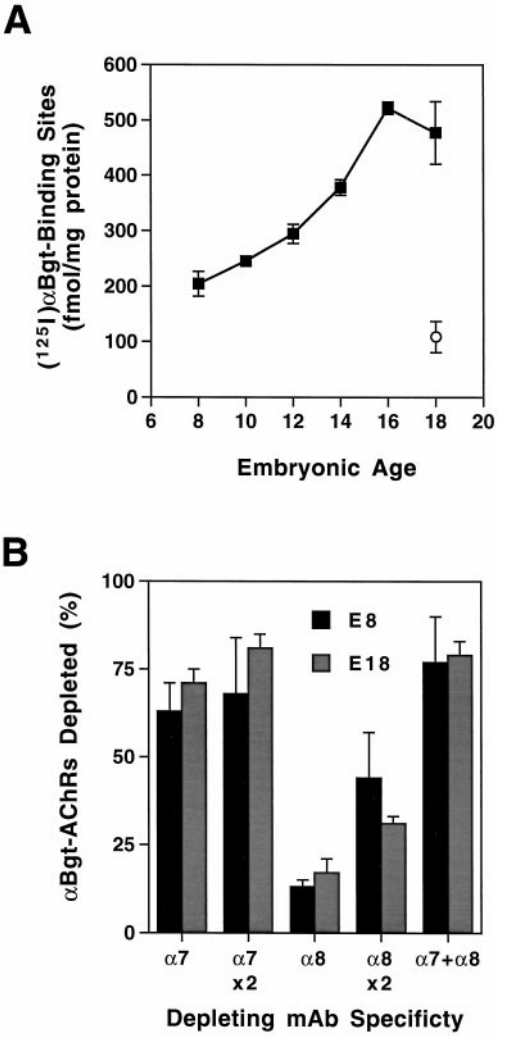

Fig. 7. Developmental changes in the amount of α-Bgt-binding AChRs. A, Total α-Bgt binding sites/mg of protein in brain as a function of embryonic age. Whole-brain extracts were prepared at the indicated times and assayed for ^{125}I-α-Bgt binding sites (■) or, for comparison, [^{3}H]epibatidine binding sites (○) using the filter binding assay. Values were normalized for protein and represent the mean ± standard error from three to seven experiments. B, Proportion of α-Bgt-binding AChRs in brain containing α7 and/or α8 subunits. Brain extracts were prepared from E8 and E17/18 chicks, depleted with either the anti-α7 mAbs 318 and 319 or with the anti-α8 mAb 308, and then assayed for residual ^{125}I-α-Bgt binding sites using the filter binding assay. Sequential immunodepletions either with the same mAbs (*x2*) or with mAbs to α7 and then α8 (*α7 + α8*) were performed to evaluate the efficiency of the immunodepletions. Values represent the mean ± standard error from three or four experiments.

resed, and analyzed on blots probed with mAb 289 to detect α4 protein and mAb 268 to detect α5 protein. Both α4 and α5 protein could be detected, and both increased substantially between E8 and E17/18 (Fig. 6B). Immunodepletion with the anti-α4 mAb 289 before immunoprecipitation with mAb 270 removed large amounts of the α4 and α5 proteins subsequently detected on the blots. The results confirm the conclusions that a portion of the native AChRs containing α4 and β2 subunits also contain α5 subunits and that the amount of this species increases developmentally. Interestingly, a portion of the α5 protein also seems to be associated with a different receptor species late in development because some of the α5 gene product associated with β2 (and immunoprecipitated by the anti-β2 mAb 270) cannot be immunodepleted by the anti-α4 mAb 289 (Fig. 6B).

Developmental changes in the population of brain AChRs that bind α-Bgt. The total number of α-Bgt-binding AChRs detected in brain extracts using ^{125}I-α-Bgt in the filter assay was substantially higher at all stages examined than was the total number of epibatidine-binding AChRs (Fig. 7A). A developmental increase of ≈3-fold occurred in the number of α-Bgt-binding receptors between E8 and E17/18. Immunodepletion with the anti-α7 mAbs 318/319 indicated that most of the α-Bgt-binding receptors at both early and late times in embryonic brain contain the α7 gene product, whereas approximately half also contain the α8 gene product (Fig. 7B). Sequential immunoprecipitations were required to collect all of the receptors containing the α8 gene product, presumably because of the inefficiency of the antibody. A small fraction of the total α-Bgt-binding AChRs at both early and late times may contain gene products other than α7 and α8 gene because some α-Bgt-binding species remained in solution even after repeated immunoprecipitations. A similar fraction of α-Bgt-binding species remained in E17/18 ciliary ganglion extracts after sequential immunoprecipitations in the two-site assay with mAbs 318/319 (data not shown), and a portion of this has been attributed previously to an AChR

species lacking the α7 gene product (Pugh *et al*., 1995). Much of the remainder, however, could represent receptor in which the relevant epitopes are blocked or degraded. No significant change was observed between E8 and E17/18 in the ratios of the α-Bgt-binding species in brain.

Discussion

Epibatidine and α-Bgt together as probes distinguish several major classes of AChRs in brain. Most abundant are receptors binding α-Bgt and containing either α7 or α7 plus α8 subunits. Together they make up at least three fourths of the total α-Bgt-binding species at all developmental stages examined and represent nearly two thirds of the total AChRs distinguished in the combined filter assays. The findings are consistent with earlier reports of abundant α-Bgt-binding AChRs containing α7 and α8 subunits in chick brain (Gotti *et al*., 1994a; Lindstrom, 1996). The current study shows that only a small fraction of the total α-Bgt binding in brain at either early or late developmental times is a candidate for receptor species lacking both the α7 and α8 gene products. Gotti *et al*. (1994b) reported that approximately half of the α-Bgt-binding receptors in chick brain between E7 and E13 could not be immunoprecipitated by antisera against either α7 or α8 peptides. The reason for the discrepancy between the earlier results and those reported here is not clear but may be due to differences in the efficiency of receptor immunoprecipitation in the two studies.

The next most abundant species of brain AChRs is one that binds epibatidine and contains the α4 and β2 gene products. These constitute >80% of the total epibatidine-binding receptors at all developmental stages examined and represent ≈15% of the total brain AChRs detected in filter assays with epibatidine and α-Bgt. The same AChR subtype was identified previously as a major species capable of binding nicotine with high affinity in rat and chick brain (Lindstrom, 1996).

Another AChR species distinguished here in brain is one that binds epibatidine and contains not only the α4 and β2 gene products but also gene products recognized by mAb 35. Previous studies in chick have shown that mAb 35 recognizes the α3 and α5 neuronal AChR gene products but not the α4, α7, α8, β2, or β4 gene products (Conroy *et al*., 1992; Vernallis *et al*., 1993; Lindstrom, 1996). Because the anti-α3 mAb 313 is unable to immunoprecipitate the vast majority of such receptors, they are unlikely to contain α3 subunits. Instead, sequential immunoprecipitations with other mAbs followed by immunoblot analysis with the anti-α5 mAb 268 confirm that a portion of the AChRs in brain containing α4 and β2 subunits also contain α5 subunits and seem to be α4/β2/α5-AChRs.

Heterologous coexpression of α4, β2, and α5 constructs in both *X. laevis* oocytes (Ramirez-Latorre *et al*., 1996) and HEK 293 cells produces α4/β2/α5-AChRs. An electrophysiological comparison of α4/β2- and α4/β2/α5-AChR in oocytes shows that the α5 subunit reduces receptor affinity for ACh as judged by dose-response curves (Ramirez-Latorre *et al*., 1996). Binding studies on receptors from HEK 293 cells, however, reveal no difference between α4/β2- and α4/β2/α5-AChRs in affinity for several nicotinic ligands. A likely reason for the different results is that the electrophysiological analysis measured ligand affinity of nondesensitized receptors, whereas the equilibrium binding studies used for the current report almost certainly measured ligand affinity of desensitized receptors. An alternative possibility is that oocytes and HEK 293 cells influence receptor pharmacology differently through the kinds of post-translational modifications they provide. A similar argument has been advanced to account for differences in the single-channel events seen for AChRs produced by oocytes versus neurons (Sivilotti *et al*., 1997). Consistent with current results, previous researchers (Lindstrom, 1996) have found no difference in the binding affinities of nicotine, cytisine, or epibatidine for chick brain AChRs immunoprecipitated by either mAb 35 or anti-α4 mAbs. The results underscore the need for caution in using pharmacology alone to draw conclusions about subunit composition, but they do not conflict with the conclusion based on immunoprecipitations and immunoblot analysis showing that the α5 gene product coassembles with α4 and β2 in brain.

In situ hybridization analyses demonstrate that α4 and β2 transcripts are widely expressed in both rat and chick brain (Wada *et al*., 1989; Morris *et al*., 1990). Although the distribution of α5 mRNA has not been examined in chick, *in situ* hybridization studies in rat brain show extensive overlap between regions expressing α5 transcripts and those expressing α4 and β2 (Wada *et al*., 1990). Overlap also is found in both rat and chick between brain regions expressing α3 and those expressing α4 and β2 (Wada *et al*., 1989; Morris *et al*., 1990; Lobron *et al*., 1995). The same is true for β4 expression in rat brain (Dineley-Miller *et al*., 1992).

Three remaining neuronal AChR genes in chick, α2, α6, and β3, were not included in the current study because subunit-specific antibodies were not available. Least relevant is β3 because *in situ* hybridization analysis indicates that expression of the gene is largely confined to the retina and trigeminal ganglion in chick (Hernandez *et al*., 1995). Both the α2 and α6 genes are expressed in chick brain (Wada *et al*., 1989; Daubas *et al*., 1990; Gerzanich *et al*., 1997) and may contribute subunits to receptors currently thought to be either α4/β2-AChRs or α4/β2/α5-AChRs. Neither α2 nor α6 is likely to define a large class of entirely separate brain AChRs, however, because most epibatidine-binding receptors can be immunoprecipitated by both anti-α4 and anti-β2 mAbs. The α2 and α6 gene products could, in principle, account for some of the mAb 35 binding to brain AChRs. Preliminary results opposing this for the α2 gene product come from immunoprecipitations with mAb 321, which was raised against an α2-specific peptide sequence (R. Schoepfer, W. Conroy, and J. Lindstrom, unpublished observations); mAb 321 immunoprecipitates only 1–2% of the total epibatidine-binding receptors from E17/18 chick brain extracts (W. Conroy and D. Berg, unpublished observations). The α6 gene product has yet to be tested for mAb 35 binding but is not a likely candidate because it lacks a critical moiety in the domain recognized by the antibody (Saedi *et al*., 1990; Gerzanich *et al*., 1997). Nevertheless, the subunit heterogeneity of AChRs that bind mAb 35 and contain α4 and β2 gene products could be even greater than shown here.

Previous studies indicated that neuronal AChRs can contain more than one kind of α-type subunit, as well as more than one kind of β-type subunit (Conroy *et al*., 1992; Vernallis *et al*., 1993; Conroy and Berg, 1995; Lindstrom, 1996; Ramirez-Latorre *et al*., 1996; Wang *et al*., 1996; Forsayeth and Kobrin, 1997). An individual AChR gene product also can

enter into different subunit associations depending on the partners available. The current findings extend this pattern, showing that AChR gene products common in the peripheral nervous system also are expressed in the central nervous system but largely assemble with different partners in the two cases. Thus, the α5 gene product is assembled with α3, β2, and β4 in autonomic neurons but usually not with α4 (Listerud *et al*., 1991; Vernallis *et al*., 1993; Mandelzys *et al*., 1994; Conroy and Berg, 1995; Flores *et al*., 1996), whereas in brain, α5 subunits are assembled in considerable measure with α4 and β2. Similarly, the α3 and β4 gene products are assembled with α5 and to some extent β2 in autonomic neurons but usually not with α4 (Listerud *et al*., 1991; Vernallis *et al*., 1993; Mandelzys *et al*., 1994; Conroy and Berg, 1995; Flores *et al*., 1996), whereas in brain α3 and β4 are assembled mostly with α4 and β2.

Changing AChR patterns during brain development suggest that receptors containing the α3 and β4 gene products are likely to be most important at early times and less so at later stages because their levels undergo no developmental increase beyond that present at E8. This view is consistent with *in situ* hybridization studies in rat showing that α3 and β4 mRNA appear early in development and then virtually disappear from the brain and spinal cord at later times (Zoli *et al*., 1995). Transcripts for α7 and for α4 and β2 in rat brain undergo more sustained increases throughout development (Broide *et al*., 1995; Ostermann *et al*., 1995; Zoli *et al*., 1995; del Toro *et al*., 1997), which is consistent with the sustained developmental increases in AChRs containing the gene products reported here. An interesting exception is the case of β2 gene expression in chick optic tectum, which is transient and dependent on innervation from the eye (Matter *et al*., 1990).

The largest developmental increase observed in the current study was that of AChRs recognized by mAb 35. They undergo a 7-fold increase between E8 and E17/18 over and above that expected from net growth and represent approximately one sixth of all epibatidine-binding receptors at the end of embryogenesis. Immunoblot analysis confirmed a developmental increase in α5 protein associated with α4 and β2 subunits in such receptors. Some of the increase in epibatidine-binding AChRs recognized by mAb 35 also may represent the appearance of receptors containing α5 subunits coassembled with different AChR gene products, however, because not all of the receptors could be immunoprecipitated by anti-α4 mAbs. In fact, AChRs that bind mAb 35 constitute approximately one half of the receptors capable of high affinity nicotine binding in adult chicken brain and can be immunoprecipitated by the anti-β2 mAb 270 but not by the anti-α4 mAb 285 (Lindstrom, 1996). The subunit composition of this species includes components of 49 and 58 kDa (Whiting and Lindstrom, 1986), with the former being in the size range expected for both the α5 and β2 proteins (Conroy *et al*., 1992).

Differences in subunit composition are likely to influence many aspects of receptor function. Among these are the rates of receptor activation and desensitization, agonist sensitivity, calcium permeability, second messenger regulation, and possibly location on the cell surface. Considered broadly, the fact that a given AChR gene product is assembled with different subunit partners suggests the receptors perform different physiological functions but share a need for the specific features conferred by individual subunits. Identification of unique features contributed by individual subunits is a challenge for subsequent studies.

Acknowledgments

We thank Dr. Jon Lindstrom (University of Pennsylvania, Philadelphia, PA) for generously providing the indicated mAbs and Dr. Paul Whiting (Merk, Sharp, & Dohme Laboratories, Essex, England) and Dr. Marc Ballivet (University of Geneva, Geneva, Switzerland) for generously providing cDNA constructs. Lynn Ogden provided expert technical assistance.

References

Broide RS, O'Connor LT, Smith MA, Smith JAM, and Leslie FM (1995) Developmental expression of α7 neuronal nicotinic receptor messenger RNA in rat sensory cortex and thalamus. *Neuroscience* **67:**83–94.

Chen C and Okayama H (1987) High-efficiency transformation of mammalian cells by plasmid DNA. *Mol Cell Biol* **7:**2745–2752.

Conroy WG and Berg DK (1995) Neurons can maintain multiple classes of nicotinic acetylcholine receptors distinguished by different subunit compositions. *J Biol Chem* **270:**4424–4431.

Conroy WG, Vernallis AB, and Berg DK (1992) The α5 gene product assembles with multiple acetylcholine receptor subunits to form distinctive receptor subtypes in brain. *Neuron* **9:**679–691.

Daubas P, Devillers-Thiery A, Geoffroy B, Martinez S, Bessis S, and Changeux JP (1990) Differential expression of the neuronal acetylcholine receptor α2 subunit gene during chick brain development. *Neuron* **5:**49–60.

del Toro ED, Juiz JM, Smillie FI, Lindstrom J, and Criado M (1997) Expression of α7 neuronal nicotinic receptors during postnatal development of the rat cerebellum. *Dev Brain Res* **98:**125–133.

Dineley-Miller K and Patrick J (1992) Gene transcripts for the nicotinic acetylcholine receptor subunit, *beta*4, are distributed in multiple areas of the rat central nervous system. *Mol Brain Res* **16:**339–344.

Flores CM, DeCamp RM, Kilo S, Rogers SW, and Hargreaves KM (1996) Neuronal nicotinic receptor expression in sensory neurons of the rat trigeminal ganglion: demonstration of $\alpha3\beta4$, a novel subtype in the mammalian nervous system. *J Neurosci* **16:**7892–7901.

Forsayeth JR and Kobrin E (1997) Formation of oligomers containing the β3 and β4 subunits of the rat nicotinic receptor. *J Neurosci* **17:**1531–1538.

Fu W-M and Liu J-J (1997) Regulation of acetylcholine release by presynaptic nicotinic receptors at developing neuromuscular synapses. *Mol Pharmacol* **51:** 390–398.

Gerzanich V, Kuryatov A, Anand R, and Lindstrom J (1997) 'Orphan' α6 nicotinic AChR subunit can form a functional heteromeric acetylcholine receptor. *Mol Pharmacol* **51:**320–327.

Gerzanich V, Peng X, Wang F, Wells G, Anand R, Fletcher S, and Lindstrom J (1995) Comparative pharmacology of epibatidine: a potent agonist for neuronal nicotinic acetylcholine receptors. *Mol Pharmacol* **48:**774–782.

Gotti C, Hanke W, Maury K, Moretti M, Ballivet M, Clementi F, and Bertrand D (1994a) Pharmacology and biophysical properties of α7 and α8 α-bungarotoxin receptor subtypes immunopurified from chick optic lobe. *Eur J Neurosci* **6:**1281–1291.

Gotti C, Moretti M, Longhi R, Briscini L, Balestra B, and Clementi F (1994b) Expression of α-bungarotoxin receptor subtypes in chick central nervous system during development. *J Recept Res* **14:**335–346.

Hernandez M-C, Erkman L, Matter-Sadzinski L, Roztocil T, Ballivet M, and Matter JM (1995) Characterization of the nicotinic acetylcholine receptor β3 gene. *J Biol Chem* **270:**3224–3233.

Houghtling RA, Davila-Garcia MI, and Kellar KJ (1995) Characterization of (±)-[^{3}H]epibatidine binding to nicotinic cholinergic receptors in rat and human brain. *Mol Pharmacol* **48:**280–287.

Karlin A and Akabas M (1996) Toward a structural basis for the function of nicotinic acetylcholine receptors and their cousins. *Neuron* **15:**1231–1244.

Kenakin T (1993) *Pharmacologic Analysis of Drug-Receptor Interaction*, 2nd ed, pp 385–410, Raven Press, New York.

Lindstrom J (1996) Neuronal nicotinic acetylcholine receptors, in *Ion Channels* (Narahashi T, ed), vol 4, pp 377–450, Plenum Press, New York.

Listerud M, Brussaard AB, Devay P, Colman DR, and Role LW (1991) Functional contribution of neuronal nAChR subunits revealed by antisense oligonucleotides. *Science (Washington DC)* **254:**1518–1521.

Lobron Ch, Wevers A, Damgen K, Jeske A, Rontal D, Birtsch Ch, Heinemann S, Reinhardt S, Maelicke A, and Schroder H (1995) Cellular distribution in the rat telencephalon of mRNAs encoding for the α3 and α4 subunits of the nicotinic acetylcholine receptor. *Mol Brain Res* **30:**70–76.

Mandelzys A, Pie P, Deneris ES, and Cooper E (1994) The developmental increase in ACh current densities on rat sympathetic neurons correlates with changes in nicotinic ACh receptor α-subunit gene expression and occurs independent of innervation. *J Neurosci* **14:**2357–2364.

Matter JM, Matter-Sadzinski L, and Ballivet M (1990) Expression of neuronal nicotinic acetylcholine receptor genes in the developing chick visual system. *EMBO J* **9:**1021–1026.

Morris B. J, Hicks AA, Wisden W, Darlison MG, Hunt SP, and Barnard EA (1990) Distinct regional expression of nicotinic acetylcholine receptor genes in chick brain. *Mol Brain Res* **7:**305–315.

Ostermann C-H, Grunwald J, Wevers A, Lorke DE, Reinhardt S, Maelicke A, and Schroder H (1995) Cellular expression of α4 subunit mRNA of the nicotinic acetylcholine receptor in the developing rat telencephalon. *Neurosci Lett* **192:**21–24.

Pugh PC, Corriveau RA, Conroy WG, and Berg DK (1995) A novel subpopulation of neuronal acetylcholine receptors among those binding α-bungarotoxin. *Mol Pharmacol* **47:**717–725.
Ramirez-Latorre J, Yu CR, Qu X, Perin F, Karlin A, and Role L (1996) Functional contributions of α5 subunits to neuronal acetylcholine receptor channels. *Nature (Lond)* **380:**347–351.
Role LW and Berg DK (1996) Nicotinic receptors in the development and modulation of CNS synapses. *Neuron* **16:**1077–1085.
Saedi MS, Anand R, Conroy WG, and Lindstrom J (1990) Determination of amino acids critical to the main immunogenic region of intact acetylcholine receptors by in vitro mutagenesis. *FEBS Lett* **267:**55–59.
Sivilotti LG, McNeil DK, Lewis TM, Nassar MA, Schoepfer R, and Colquhoun D (1997) Recombinant nicotinic receptors, expressed in *Xenopus* oocytes, do not resemble native rat sympathetic ganglion receptors in single-channel behavior. *J Physiol* **500:**123–138.
Smith MA, Stollberg J, Lindstrom JM, and Berg DK (1985) Characterization of a component in chick ciliary ganglia that cross reacts with monoclonal antibodies in muscle and electric organ acetylcholine receptors. *J Neurosci* **5:**2726–2731.
Vernallis AB, Conroy WG, and Berg DK (1993) Neurons assemble acetylcholine receptors with as many as three kinds of subunits while maintaining subunit segregation among receptor subtypes. *Neuron* **10:**451–64.
Wada E, McKinnon D, Heinemann S, Patrick J, and Swanson LW (1990) The distribution of mRNA encoded by a new member of the neuronal nicotinic acetylcholine receptor gene family (α_5) in the rat central nervous system. *Brain Res* **526:**45–53.
Wada E, Wada K, Boulter J, Deneris E, Heinemann S, Patrick J, and Swanson LW (1989) Distribution of *alpha*2, *alpha*3, *alpha*4, and beta2 neuronal nicotinic receptor subunit mRNAs in the central nervous system: a hybridization histochemical study in the rat. *J Comp Neurol* **284:**314–335.
Wang F, Gerzanich V, Wells GB, Anand R, Peng X, Keyser K, and Lindstrom J (1996) Assembly of human neuronal nicotinic receptor α5 subunits with α3, β2, and β4 subunits. *J Biol Chem* **271:**17656–17665.
Whiting P and Lindstrom J (1986) Purification and characterization of a nicotinic acetylcholine receptor from chick brain. *Biochem* **25:**2082–2093.
Whiting P, Schoepfer R, Lindstrom J, and Priestley T (1991) Structural and pharmacological characterization of the major brain nicotinic acetylcholine receptor subtype stably expressed in mouse fibroblasts. *Mol Pharmacol* **40:**463–472.
Wonnacott S (1997) Presynaptic nicotinic ACh receptors. *Trends Neurosci* **20:**92–98.
Zhang Z-W, Coggan JS, and Berg DK (1996) Synaptic currents generated by neuronal acetylcholine receptor sensitive to α-bungarotoxin. *Neuron* **17:**1231–1240.
Zoli M, Le Novere N, Hill JA Jr, and Changeux JP (1995) Developmental regulation of nicotinic ACh receptor subunit mRNAs in the rat central and peripheral nervous systems. *J Neurosci* **15:**1912–1939.

Send reprint requests to: Dr. Darwin K. Berg, Department of Biology, 0357, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0357. E-mail: dberg@ucsd.edu.