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Pharmacological Properties of Immuno-Isolated Neuronal Nicotinic Receptors

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Recently we immunoaffinity-purified an ACh receptor from chicken brain using a monoclonal antibody raised against receptors from fish electric organ (Whiting and Lindstrom, 1986). This neuronal receptor could be affinity-labeled with ^{3}H-bromoacetylcholine, and antisera to it specifically blocked ACh-induced depolarization of chicken ciliary ganglion cells. Here we show that this neuronal ACh receptor binds ^{3}H-nicotine with high affinity (K_D = 6.61 ± 0.13 nM). ^{3}H-Nicotine binding was blocked by various nicotinic cholinergic ligands but not by α-bungarotoxin or the muscarinic antagonist atropine. Binding was also blocked by affinity labeling the receptor with bromoacetylcholine (after reduction by dithiothreitol). Additionally, we were able to use rat antisera raised against the chicken brain receptor to isolate a component from detergent extracts of rat brain that also bound ^{3}H-nicotine with high affinity (K_D = 1.5 nM). The pharmacology of this putative ACh receptor from rat brain was almost identical to the receptor from chicken brain, and its regional distribution was in good agreement with that of ^{3}H-nicotine binding to rodent brain membranes reported by other workers. Thus, by analogy to the receptor we have purified and characterized from chicken brain, this nicotine-binding component from rat brain is probably a functional mammalian neuronal nicotinic ACh receptor.

Nicotinic acetylcholine receptors (AChRs) from both electric organ and skeletal muscle have been extensively characterized (for reviews, see Popot and Changeux, 1984, or Anholt et al., 1985), primarily because of the availability of a probe, α-bungarotoxin (αBGT), which binds to these AChRs with both great specificity and high affinity. By contrast, little is known about the neuronal AChR, primarily due to lack of a suitable probe. Although αBGT binds to a component of chicken neuronal tissue that can be affinity-labeled with bromoacetylcholine (BAC) (Kemp et al., 1985; Norman et al., 1982) and that has some amino acid sequence homology with muscle AChRs (Conti-Tronconi et al., 1985), its physiological significance is uncertain. αBGT has been shown to block AChR function in neuronal preparations from toads and goldfish (Freeman et al., 1980; Marshall, 1981). However, in chicken and rat neuronal cells, the αBGT binding component has been shown not to be the electrophysiologically significant AChR (Brown and Fumigalli, 1977; Carbonetto et al., 1978; Jacob and Berg, 1983; Kouvelas et al., 1978; Patrick and Stallcup, 1977a, b; Ravdin and Berg, 1979).

Neuronal AChRs have also been investigated using the radiolabeled cholinergic ligands ^{3}H-nicotine (Abood et al., 1980; Clarke et al., 1984; Larsson and Nordberg, 1985; Marks and Collins, 1982; Romano and Goldstein, 1980; Yamada et al., 1985) and ^{3}H-ACh (Reulecke and Hucho, 1985; Schneider et al., 1985; Schwartz et al., 1982). The binding of these ligands to rodent brain membrane preparations was of high affinity and inhibited poorly, if at all, by αBGT. In autoradiographic studies, Clarke and coworkers (1985a, b) were able to demonstrate that ^{3}H-nicotine and ^{3}H-ACh (in the presence of the muscarinic antagonist atropine) bound to rat brain sections with nearly identical patterns. Brain regions rich in binding sites for these ligands were frequently distinct from regions rich in binding sites for ^{125}I-αBGT.

Immunological techniques have also been used to identify neuronal AChRs. Patrick and Stallcup (1977b) showed that although αBGT could not block AChR function in the rat sympathetic neuronal cell line PC12, antiserum to AChRs from *Electrophorus* electric organ could block AChR function. A monoclonal antibody (mAb 35) to AChRs from *Electrophorus* was shown to bind to the lateral spiriform nucleus in chicken brain and its projections to the optic tectum (Lindstrom et al., 1983; Swanson et al., 1983). mAb 35 is directed at the main immunogenic region (MIR), which is located on the extracellular surface of AChR α subunits (Tzartos et al., 1981). Antibodies to the MIR do not inhibit AChR function (Blatt et al., 1986; Lindstrom et al., 1981b). mAb 35 binds to synapses on chicken ciliary ganglion neurons, whereas αBGT binds to sites outside of the synapse (Jacob et al., 1984). The component in ciliary ganglia identified by mAb 35 has biochemical properties expected of an AChR, and its amount can be modulated by treatment of ganglion cultures with cholinergic ligands (Smith et al., 1985, 1986). By immunoaffinity chromatography on mAb 35 coupled to Sepharose, we have purified its binding component from chicken brain (Whiting and Lindstrom, 1986). It is an integral membrane glycoprotein, slightly larger than *Torpedo* AChR monomers, for which 2 subunits have been identified with apparent molecular weights 48,000 and 59,000. The smaller of these subunits binds antibodies that are specific for the α subunit of electric organ and muscle receptors. The component can be affinity-labeled with ^{3}H-BAC but does not bind αBGT. Rats immunized with the purified protein developed high antibody titers. Antisera from these rats specifically blocked the ACh-induced depolarization of chicken ciliary ganglion cells in culture (Stollberg et al., 1986). These results clearly suggest that the neuronal component recognized by mAb 35 is an electrophysiologically active neuronal AChR.

In this study we demonstrate that the chicken neuronal AChR identified by mAb 35 binds ^{3}H-nicotine with high affinity. Furthermore, using antisera raised against purified chicken neuronal AChR, we demonstrate that a homologous protein can be iso-

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lated that is responsible for most or all of the high-affinity ^{3}H-nicotine binding in rat brain. The pharmacology of these 2 neuronal AChRs is described.

Materials and Methods

Antisera and mAbs

mAb 35 was obtained by fusing mouse myeloma cells with spleen cells of rats immunized with purified *Electrophorus* electric organ AChR (Tzartos et al., 1981). mAb 210 was raised against AChRs purified from fetal bovine muscle and the mouse neuroblastoma cell line BC3H1 and then partially denatured with SDS (S. Hochschwender and J. Lindstrom, unpublished observations). mAb 270 was derived from rats immunized with immunoaffinity-purified chicken neuronal AChR. Its preparation and characterization, along with that of other mAbs to neuronal AChRs, will be described in more detail elsewhere. Stocks of mAbs were prepared by ammonium sulfate precipitation of hybridoma culture supernatants. mAb 35 was purified by chromatography on DEAE Affigel Blue (Bio-Rad) (S. Hochschwender and J. Lindstrom, unpublished observations) and was coupled to Sepharose C1-4B by cyanogen bromide activation. mAb 35 was also labeled with ^{125}I to a specific activity of $2–3 \times 10^{18}$ cpm/mol using essentially the same procedure described for αBGT (Lindstrom et al., 1981a, b) and stored at 4°C in PBS (100 mM NaCl, 10 mM phosphate, pH 7.5) containing 10 mg/ml BSA.

Antisera to the affinity-purified chicken neuronal AChR were prepared as described previously (Whiting and Lindstrom, 1986). For coupling to Sepharose C1-4B, the IgG fraction was prepared by ammonium sulfate precipitation of serum and then coupled at 15 mg/ml by cyanogen bromide activation.

Goat anti-rat IgG was affinity-purified using a rat IgG–Sepharose column and then coupled to Sepharose C1-4B by cyanogen bromide activation.

Preparation of brain detergent extracts

Chicken and rat brains were obtained from Pel-Freeze Biologicals and stored at −70°C. Triton X-100 extracts of brain membranes were prepared as described previously (Whiting and Lindstrom, 1986). Regional dissection of rat brains was carried out by Dr. Barbara Morley, and detergent extracts were similarly prepared. mAb 35 binding sites in chicken brain extracts were determined by a DEAE cellulose assay, as described previously (Whiting and Lindstrom, 1986).

Probing of chicken neuronal AChR immunoblots with mAb 210 and mAb 270

Chicken neuronal AChR was immunoaffinity-purified, electrophoretically transferred to diazophenylthioether paper, and subsequently probed with mAbs as has been previously described (Whiting and Lindstrom, 1986).

^{3}H-Nicotine binding to immobilized neuronal AChR

Chicken brain

Chicken brain detergent extract (75–100 ml) was recirculated through 0.25 ml mAb 35–Sepharose at 4°C. After 15 hr the affinity gel was then aliquoted into 1.5 ml plastic microfuge tubes (0.25–0.6 pmol mAb 35 binding sites per tube, as determined from the depletion of ^{125}I-mAb 35 binding sites from the detergent extract). The aliquots were washed by resuspending in 1 ml of PBS and 0.5% Triton X-100 and centrifuging for 20 sec at 10,000 × *g* in a microfuge. The required concentration of ^{3}H-nicotine (DL-*N*-methyl-^{3}H-nicotine; specific activity 68.8 Ci/mmol; New England Nuclear), stored in aliquots at −70°C in a 100-fold molar excess of mercaptoacetic acid (Romano and Goldstein, 1980), was then added, in a final volume of 100 μl PBS 0.5% Triton X-100, and incubated for 15 min at room temperature. The affinity gel was then rapidly washed at 4°C with 4 × 1 ml of cold PBS, 0.5% Triton X-100, as described above, to remove unbound ^{3}H-nicotine. To elute bound protein and ^{3}H-nicotine, 100 μl of 2.5% SDS, 5% β-mercaptoethanol was then added. After 15 min, duplicate 45 μl aliquots were sampled into 5 ml of scintillant—5% Biosolve (Beckman), 4% Liquifluor (New England Nuclear) in toluene—and radioactivity determined by scintillation counting.

The saturability of binding was investigated by varying the concentration of ^{3}H-nicotine between 0.5 and 20 nM. Nonspecific binding was determined in the presence of 1 mM carbachol. Values for K_D and B_{max} were derived by Scatchard analysis.

The ability of cholinergic ligands to inhibit ^{3}H-nicotine binding to immobilized neuronal AChR was investigated by including various concentrations of the ligand in the reaction mix. Cytisine, decamethonium, and hexamethonium were obtained from ICN Pharmaceuticals; nicotine was obtained from Eastman Kodak; mecamylamine was obtained from Merck Sharpe and Dohme; atropine, curare, ACh, and carbachol were obtained from Sigma. The IC_{50} was determined as the concentration of ligand that inhibited 50% of the specific ^{3}H-nicotine binding. The K_I was calculated from $K_I = IC_{50}/(1 + [^3\text{H-nicotine}]/K_D)$.

The inhibition of ^{3}H-nicotine binding by the affinity label BAC was also investigated. Aliquots of immobilized receptor were reduced with 100 μl 0.1–1 mM dithiothreitol (DTT) in PBS, 0.5% Triton X-100 for 40 min at room temperature. The aliquots were then washed with 1 ml PBS, 0.5% Triton X-100 and alkylated for 5 min with 0.001–1.0 μM BAC (prepared by Dr. K. Wan using the method of Chiou and Sastry, 1968) in the same buffer. The affinity gel was then washed with 3 × 1 ml PBS, 0.5% Triton X-100 and then incubated for 10 min with 100 μl of 0.1 mM dithiobis (2-nitrobenzoic acid) (DTNB) in the same buffer. After washing twice more, ^{3}H-nicotine binding was determined as described above. To determine the total ^{3}H-nicotine binding, parallel incubations were carried out omitting the BAC labeling step.

Rat brain

Rat brain extract (200–800 μl) was gently shaken in 1.5 ml microfuge tubes for 4–6 hr at room temperature and then 15 hr at 4°C with 15 μl of anti-chicken neuronal AChR serum and 25 μl goat anti-rat IgG–Sepharose, or 5–50 μl of directly coupled anti-chicken neuronal AChR–Sepharose. Labeling with ^{3}H-nicotine was then carried out as has been described above for chicken neuronal AChR.

Binding of ^{3}H-nicotine in brain detergent extract

Brain detergent extract was first dialyzed against PBS, 0.5% Triton X-100, and aliquots (100 μl) were incubated for 15 min at room temperature with 20 nM ^{3}H-nicotine and then applied to a 1 × 6 cm Sephadex G25 column equilibrated with PBS 0.5% Triton X-100 at 4°C. Following elution with the same buffer, 100 μl fractions were collected and radioactivity determined by scintillation counting as described above. The separation procedure took approximately 3 min. The total radioactivity in the first peak was summated to determine bound ^{3}H-nicotine. The specificity of the binding was demonstrated by inclusion of 1 mM carbachol in the reaction mixture.

Toxin binding

αBGT was radioiodinated as previously described (Lindstrom et al., 1981a) to specific activities of $3–4 \times 10^{17}$ cpm/mol. Toxin binding to solubilized rat and chicken brain was measured by a DEAE ion-exchange assay and was performed as previously described (Whiting and Lindstrom, 1986).

Results

^{3}H-Nicotine binding to immobilized chicken neuronal AChR

Chicken neuronal AChR was immobilized upon mAb 35–Sepharose. This approach was taken both to purify and to concentrate the AChR from the detergent extract in a single step. We have previously used this technique to affinity-label the neuronal AChR with ^{3}H-BAC (Whiting and Lindstrom, 1986). Immobilization on mAb 35 should not affect ligand binding since mAbs to the MIR bind to AChRs without altering their function (Blatt et al., 1986; Lindstrom et al., 1983). ^{3}H-Nicotine was allowed to bind to the AChR for 15 min at room temperature, an incubation time that gave maximal binding (data not shown). Washing of the labeled AChR was done rapidly at 4°C to minimize dissociation of bound ligand. Figure 1*A* demonstrates saturable binding of ^{3}H-nicotine to immobilized chicken neuronal AChR. Negligible binding was observed in the presence of 1 mM carbachol. Similarly, no binding of ^{3}H-nicotine was observed to antibody–Sepharose which did not have immobi-

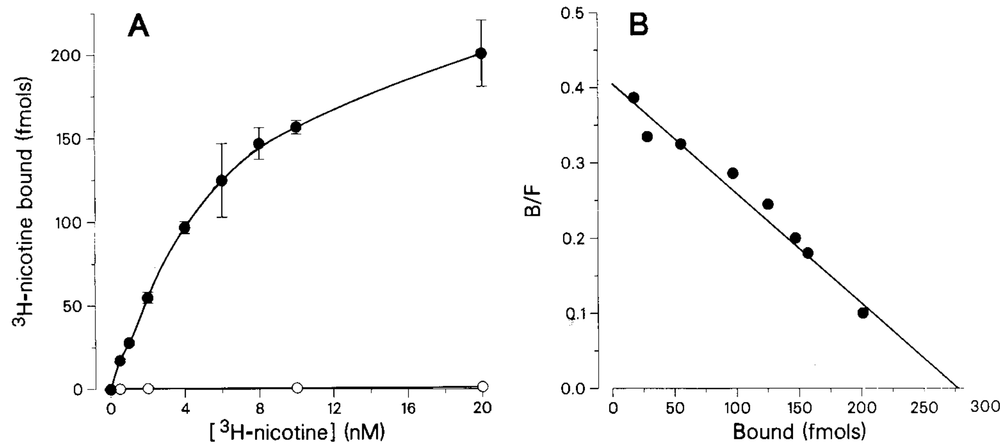

Figure 1. Binding of ^{3}H-nicotine to immobilized chick neuronal AChR. *A*, Chick brain detergent extract (85 ml) was recirculated for 15 hr, 4°C, through 250 μl of mAb 35–Sepharose. Affinity gel was divided into 40 aliquots of 0.54 pmol ^{125}I-mAb 35 binding sites per aliquot, washed with 2 ml PBS, 0.5% Triton X-100 and incubated for 15 min, room temperature, in 100 μl of the same buffer containing various concentrations of ^{3}H-nicotine in the presence or absence of 1 mM carbachol. Aliquots were rapidly washed with 4 × 1 ml ice-cold PBS, 0.5% Triton X-100. Then 100 μl of 5% SDS, 2.5% β-mercaptoethanol was then added, and after 15 min, 45 μl duplicates sampled for scintillation counting and the ^{3}H-nicotine binding per aliquot determined. Each point is the mean ± SD of the values from 3 aliquots. Symbols: ●, binding in the absence of carbachol; ○, binding in the presence of carbachol. *B*, Scatchard plot of data from *A*.

lized neuronal AChR (not shown). Scatchard analysis (Fig. 1*B*) indicated binding with a single affinity, K_D = 6.61 ± 0.13 nM (mean ± SD, 3 experiments). The total nicotine binding to the immobilized AChR, B_{max}, amounted to about half of the amount of mAb 35 binding sites immobilized (0.53 ± 0.02:1 ± 0.12; ratio of ^{3}H-nicotine binding sites to ^{125}I-mAb 35 binding sites, mean ± SD, 3 experiments). This indicates that the specific activity of the immobilized AChR for nicotine binding is very high.

^{3}H-Nicotine binding to immobilized rat brain AChR

mAb 35 and mAb 210 were also able to immobilize some nicotine binding sites from rat brain extracts, but rat antisera raised against the chicken neuronal AChR were far more effective. Figure 2 shows that the IgG fraction of rat anti-chicken neuronal AChR serum, directly coupled to Sepharose, binds the neuronal AChR from rat brain in a concentration-dependent manner. Similar results were obtained when the neuronal AChR was immobilized indirectly with anti-chicken neuronal AChR serum–goat anti-rat IgG–Sepharose. As was found for the chicken brain AChR, ^{3}H-nicotine bound in a saturable manner to immobilized rat neuronal AChR with a single affinity, K_D = 1.5 nM (data not shown).

^{3}H-Nicotine binding to chicken and rat neuronal AChR in detergent extract

The binding of ^{3}H-nicotine to AChR in detergent extracts of brain was investigated to determine whether the neuronal AChR bound by anti-receptor antibodies was the only nicotine binding component, or whether it represented only a small fraction of the total nicotine binding sites. The total number of ^{3}H-nicotine binding sites in both chicken and rat brain extracts was determined before and after incubation with mAb 270 bound to goat anti-rat IgG–Sepharose. This mAb was raised from rats immunized with chicken neuronal AChR and binds neither to chicken skeletal muscle AChR nor to the chicken or rat brain αBGT binding proteins. Its production and characterization will be described in detail elsewhere. Here we demonstrate that this mAb specifically binds to the 48,000 apparent molecular weight subunit of immunoblotted chicken neuronal AChR (Fig. 3, lane 1). As we have previously reported (Whiting and Lindstrom, 1986), this subunit is also bound by mAb 210, which is specific for the α subunit of skeletal muscle AChR (Fig. 3, lane 2), and by antiserum to *Torpedo* AChR α subunits (not shown), and it is therefore considered to be the α subunit analog.

To measure the nicotine binding in crude detergent extract, it was necessary to dialyze the extracts against PBS, 0.5% Triton X-100; we observed little binding of ^{3}H-nicotine to nondialyzed brain extract. Extracts were prepared in a buffer containing 2% Triton X-100, 50 mM Tris, pH 8.8, 1 mM EDTA, 1 mM EGTA, 5 mM iodoacetamide, 5 mM benzamidine, and 2 mM phenyl-

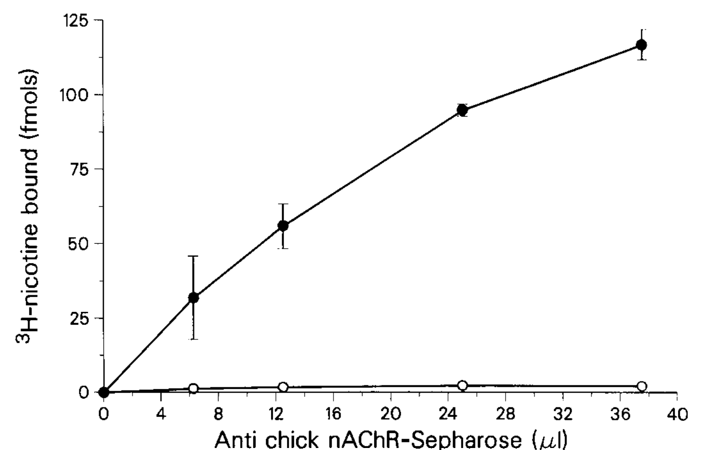

Figure 2. Binding of anti-chick neuronal AChR–Sepharose to the ^{3}H-nicotine binding site from rat brain. Triplicate aliquots of anti-chick neuronal AChR–Sepharose were gently shaken overnight at 4°C with 400 μl of rat brain detergent extract. After washing with 2 × 1 ml of PBS, 0.5% Triton X-100, the aliquots were incubated in 100 μl of 20 nM ^{3}H-nicotine in the same buffer with or without 1 mM carbachol for 15 min at room temperature. Aliquots were then washed at 4°C with 4 × 1 ml PBS, 0.5% Triton X-100 and 100 μl of 5% SDS, 2.5% β-mercaptoethanol was then added; after 15 min, 2 × 45 μl samples were taken for scintillation counting. The ^{3}H-nicotine bound per aliquot was then determined. Symbols: ●, binding in the absence of carbachol; ○, binding in the presence of carbachol. From these data, the concentration of ^{3}H-nicotine binding sites in the rat brain detergent extract was 0.30 nM, or 0.75 pmol/g brain.

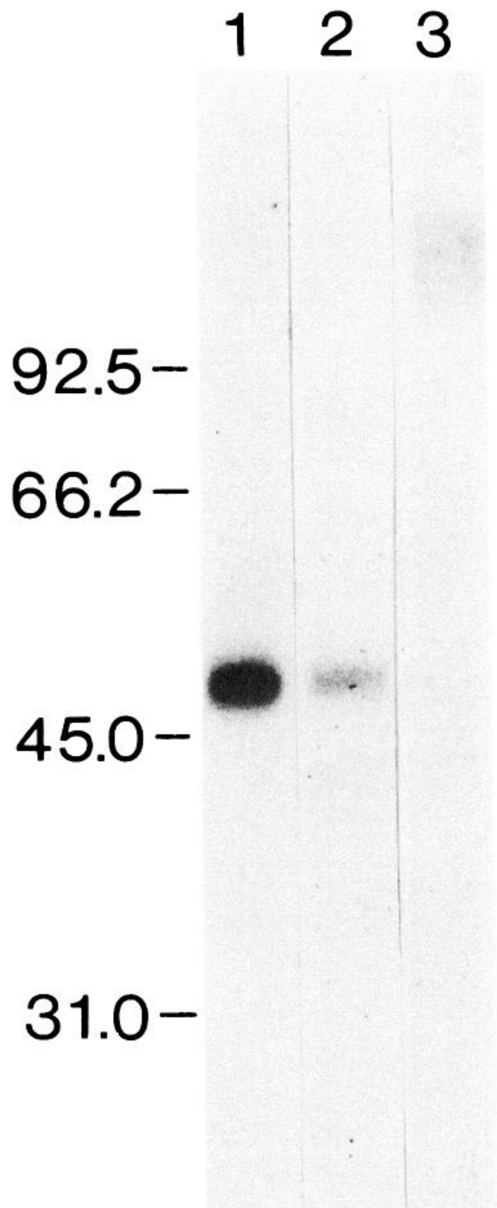

Figure 3. Binding of mAb 210 and mAb 270 to immunoblots of chick neuronal AChR. Affinity-purified chick neuronal AChR (30 pmol ^{125}I-mAb 35 binding sites) was resolved by SDS-PAGE and electrophoretically transferred to activated DPT paper. The paper was cut into 20 strips containing approximately 0.6 pmol neuronal AChR per strip. These strips were then probed with 10 nM mAb 210, undiluted mAb 270 culture supernatant, or control culture supernatant, using previously described techniques (Whiting and Lindstrom, 1986). mAb binding was visualized by incubation with 0.5 nM ^{125}I–goat anti-rat IgG and subsequent autoradiography for 15 hr at −70°C using preflashed Kodak XAR-5 film and an intensifying screen. Apparent molecular weights were determined by resolving molecular-weight standards (Bio-Rad) on the same gel and staining for protein with Coomassie Blue. Lane *1*, mAb 210; lane *2*, mAb 270; lane *3*, control culture supernatant.

methylsulfonyl fluoride. We did not determine what components of the extract or buffer inhibited binding prior to dialysis.

After labeling of detergent extracts, the bound ^{3}H-nicotine was separated from the free by gel filtration, which was done rapidly at 4°C to minimize ligand dissociation. Total binding was then determined by summation of radioactivity in the first peak. Specificity of ^{3}H-nicotine binding measured in this way

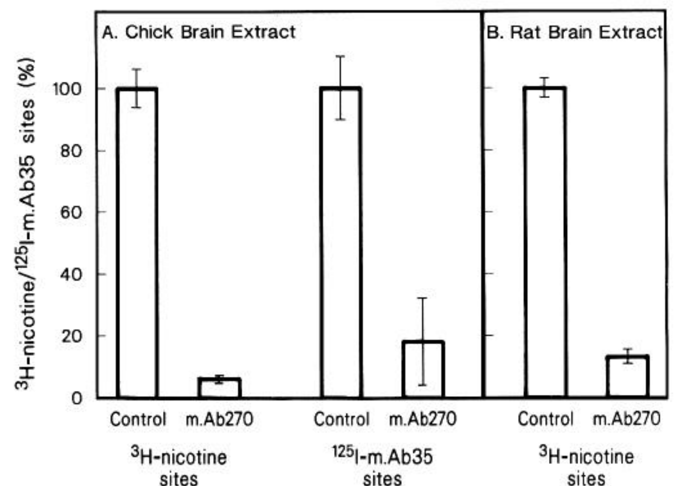

Figure 4. Depletion of ^{3}H-nicotine binding sites from chick and rat brain detergent extract by mAb 270. *A,* Chick brain detergent extract (600 μl) was gently shaken overnight at 4°C, 50 μl of mAb 270 (concentrated approximately 20-fold from culture supernatant by ammonium sulfate precipitation) and 50 μl of goat anti-rat IgG–Sepharose. Triplicate 100 μl aliquots of the supernatants were then incubated for 15 min at room temperature with 20 nM ^{3}H-nicotine, and bound nicotine was then determined by gel filtration, as is described in Materials and Methods. ^{3}H-Nicotine binding was also determined for the nondepleted chick brain extract. Results are expressed considering the ^{3}H-nicotine binding sites in the nondepleted extract as 100% and are the mean ± SD of 3 determinations. ^{125}I-mAb 35 binding sites in the original extract and the depleted extract were determined by DEAE assay (Whiting and Lindstrom, 1986). Results are expressed considering the ^{125}I-mAb 35 binding sites in the nondepleted extract as 100% and are the mean ± SD of quadruplicate determinations. Chicken brain extract had a concentration of 0.21 nM ^{3}H-nicotine binding sites (0.26 pmol/g brain) and 0.36 nM ^{125}I-mAb 35 binding sites (0.45 pmol/g brain). *B,* Rat brain extract (400 μl) was gently shaken overnight, 4°C with 40 μl of mAb 270 and 40 μl of goat anti-rat IgG–Sepharose. Triplicate 100 μl aliquots of the supernatants were then incubated for 15 min, room temperature, with 20 nM ^{3}H-nicotine, and bound nicotine was then determined by gel filtration (see Materials and Methods). ^{3}H-Nicotine binding to nondepleted rat brain extract was also determined. Results are expressed considering the ^{3}H-nicotine binding sites in the nondepleted extract as 100% and are the mean ± SD of triplicate determinations. The rat brain extract had a concentration of 0.45 nM ^{3}H-nicotine binding sites (0.93 pmol/g brain).

was demonstrated by the observation that when 1 mM carbachol was included in the reaction volume, binding was completely inhibited.

Figure 4*A* demonstrates that mAb 270 is able to deplete the majority of both the nicotine binding sites and the mAb 35 binding sites from chicken brain extract, indicating that the neuronal AChR bound by mAb 35 is a major high-affinity nicotine binding site in chicken brain. Figure 4*B* also demonstrates that mAb 270 is able to deplete the majority of the nicotine binding sites from rat brain extract. Ninety-two percent of the nicotine binding sites depleted from the rat brain extract were immobilized upon the mAb 270–goat anti-rat IgG–Sepharose (as determined by incubation with ^{3}H-nicotine), confirming that the depletion of nicotine binding sites was due to their immobilization by the antibody. The ^{125}I-mAb 35 binding sites could not be measured accurately in rat brain extract because mAb 35 exhibits very low cross-reactivity with the rat brain nicotine binding component.

Inhibition of ^{3}H-nicotine binding by cholinergic ligands

The effect of nicotinic and muscarinic ligands on the binding of ^{3}H-nicotine to immobilized chicken and rat neuronal AChRs

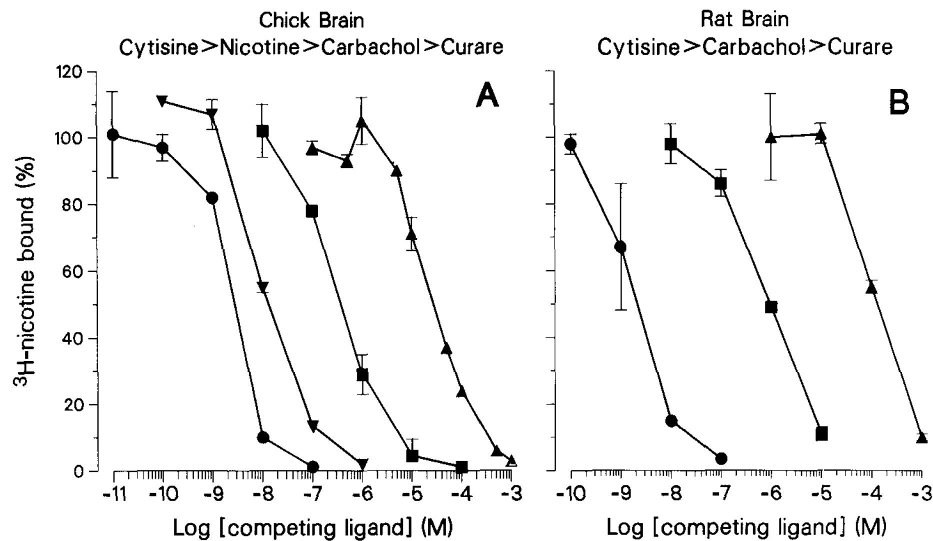

Figure 5. Inhibition of ³H-nicotine binding to immobilized chicken neuronal AChR and rat neuronal AChR by cholinergic ligands. *A*, Chicken neuronal AChR was immobilized upon mAb 35–Sepharose, as described in Materials and Methods, and divided into aliquots containing between 0.5 and 0.6 pmol mAb 35 binding sites. Aliquots were incubated for 15 min at room temperature with 100 μl of 10 nM of ³H-nicotine in the presence or absence of various concentrations of cholinergic ligands. After washing with 4 × 1 ml of ice-cold PBS, 0.5% Triton X-100, the ³H-nicotine bound was determined as described in Materials and Methods. Each point is the mean ± SD of triplicate determinations, and the results are expressed considering ³H-nicotine binding in the absence of competing ligands as 100%. *B*, Aliquots (800 μl) of rat brain detergent extract were incubated for 4 hr at room temperature and then overnight at 4°C with 15 μl of anti-chick neuronal AChR serum and 25 μl of goat anti-rat IgG–Sepharose, and then washed with 2 × 1 ml of PBS, 0.5% Triton X-100. Aliquots were then incubated for 15 min at room temperature with 100 μl of 10 nM ³H-nicotine in the presence or absence of cholinergic ligands, washed with 4 × 1 ml of ice-cold PBS, 0.5% Triton X-100, and bound ³H-nicotine determined as in Materials and Methods. Each point is the mean ± SD of triplicate determinations, and the results are expressed considering ³H-nicotine binding in the absence of competing ligands as 100%. Symbols: ●, cytisine; ▼, nicotine; ■, carbachol; ▲, curare.

was determined to further define their pharmacology. The binding curves for some of these ligands are shown in Figure 5. The IC_{50} and K_I values for all the ligands tested are shown in Table 1. Chicken and rat neuronal AChRs had very similar ligand binding properties, as would be expected for homologous molecules. Both had high affinities for cholinergic agonists and comparatively low affinities for antagonists. αBGT, at concentrations of up to 1 μM, did not exhibit any inhibition of ³H-nicotine binding. Similarly, the IC_{50} for the muscarinic antagonist atropine was greater than 1 mM, confirming the nicotinic pharmacology of this neuronal AChR.

Affinity labeling of the neuronal AChR

BAC is an affinity-labeling reagent that reacts with the AChR only after reduction of a disulfide bond adjacent to the neurotransmitter binding site (Damle et al., 1978). It has been found that BAC is more reactive with 1 of the 2 acetylcholine binding sites of electric organ and muscle AChR (Wolosin et al., 1980). On chicken ciliary ganglion cells BAC irreversibly inhibits activation of the AChR only after reduction with DTT (Stollberg et al., 1984). We have previously shown that the neuronal AChR from chicken brain can be affinity labeled with ³H-BAC (Whiting and Lindstrom, 1986). In the present study we have further investigated the nature of the BAC labeling. Figure 6*A* shows the effect of DTT concentration on the affinity labeling of immobilized chicken neuronal AChR. The curve for inhibition of ³H-nicotine binding was clearly not biphasic. Similarly, when the DTT concentration was constant (1 mM) and the BAC concentration varied, the inhibition curve indicated affinity labeling at a single site, or more than one site of equal reactivity (Fig. 6*B*).

We also investigated whether the rat neuronal AChR could

Table 1. Inhibition of ³H-nicotine binding by cholinergic ligands

	Chicken neuronal AChR		Rat neuronal AChR	
Ligand	IC_{50} (M)	K_I (M)	IC_{50} (M)	K_I (M)
Cytisine	2.8×10^{-9}	1.1×10^{-9}	2.1×10^{-9}	2.7×10^{-10}
Nicotine	1.3×10^{-8}	5.2×10^{-9}	—[a]	—
Acetylcholine	1.4×10^{-8}	5.6×10^{-9}	—[a]	—
Carbachol	3.7×10^{-7}	8.4×10^{-8}	9.5×10^{-7}	1.2×10^{-7}
Curare	2.8×10^{-5}	1.1×10^{-5}	1.3×10^{-4}	1.7×10^{-5}
Decamethonium	2.2×10^{-5}	8.7×10^{-6}	—[a]	—
Hexamethonium	$>1 \times 10^{-3}$	—	—[a]	—
Mecamylamine	$>1 \times 10^{-3}$	—	—[a]	—
αBGT	$>1 \times 10^{-6}$	—	$>1 \times 10^{-6}$	—
Atropine	$>1 \times 10^{-3}$	—	$>1 \times 10^{-3}$	—

[a] Not determined.

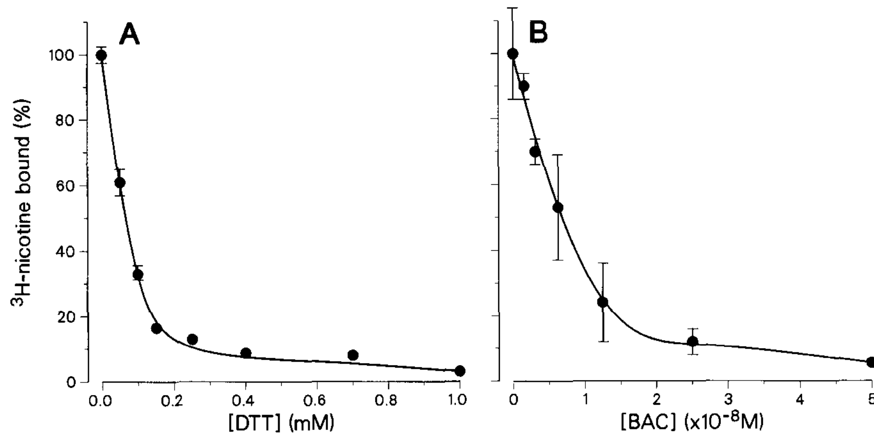

Figure 6. Affinity labeling of chicken neuronal AChR with BAC. *A,* Titration of DTT concentration. Chicken brain detergent extract (80 ml) was recirculated through 250 μl of mAb 35–Sepharose for 15 hr at 4°C, and the affinity gel then divided into 36 aliquots, containing 0.44 pmol ^{125}I-mAb 35 binding sites per aliquot. The aliquots were washed with 2 × 1 ml of PBS, 0.5% Triton and then reduced for 40 min at room temperature, with 150 μl of various concentrations of DTT in the same buffer. After washing with 1 ml PBS, 0.5% Triton (without DTT), the aliquots were resuspended in 70 μl of the same buffer containing 10 μM BAC, incubated for 5 min at room temperature, and then washed with 3 × 1 ml PBS, 0.5% Triton X-100 to remove noncovalently bound BAC. Samples were then incubated for 10 min with 0.1 mM DTNB, in the same buffer, washed again with 2 × 1 ml PBS, 0.5% Triton X-100, and then incubated with 100 μl 10 nM ^{3}H-nicotine for 15 min at room temperature. Bound ^{3}H-nicotine was then determined as in Materials and Methods. A parallel incubation was carried out with the BAC labeling step omitted, the ^{3}H-nicotine binding subsequently determined was considered as 100%, and the binding to the BAC-labeled aliquots expressed relative to this. Nonspecific binding of ^{3}H-nicotine was determined in the presence of 1 mM carbachol and represented less than 2% of the total binding. Each point represents the mean ± SD of triplicate determinations. *B,* Titration of BAC concentration. Chick brain detergent extract (80 ml) was recirculated for 15 hr, 4°C, through 250 μl of mAb 35–Sepharose and the affinity gel divided into 36 aliquots, 0.33 pmol ^{125}I-mAb 35 binding sites per aliquot. The aliquots were then reduced with 1 mM DTT, affinity-labeled with various concentrations of BAC, reoxidized with 0.1 mM DTNB, and then ^{3}H-nicotine binding determined as in *A.* ^{3}H-Nicotine binding to immobilized neuronal AChR that was reduced and reoxidized, but not affinity-labeled with BAC, was considered as 100% and ^{3}H-nicotine binding to BAC-labeled aliquots expressed as a percentage. Each point represents the mean ± SD of triplicate determinations.

be affinity-labeled with BAC. After reduction with 1 mM DTT, affinity labeling with 10 μM BAC, and reoxidation with 0.1 mM DTNB, the ^{3}H-nicotine binding to rat neuronal AChR immobilized upon anti-chicken neuronal AChR–goat anti-rat IgG–Sepharose was reduced to 15 ± 2% (mean ± SD, 3 determinations) of that obtained when AChR was reduced and reoxidized, but not affinity-labeled. Hence, rat neuronal AChR also has a disulfide bridge adjacent to its neurotransmitter binding site(s).

Inhibition of ^{3}H-nicotine binding by anti-chicken neuronal AChR serum

It was of interest to determine whether the anti-chicken neuronal AChR serum used in these studies had antibodies directed to the neurotransmitter binding site. Immobilized chicken neuronal AChR was preincubated with various dilutions of serum, and ^{3}H-nicotine binding then determined. The antiserum significantly inhibited ^{3}H-nicotine binding (Fig. 7), indicating a population of antibodies in the serum directed against the neurotransmitter binding site.

Using a solid-phase immunoassay, we previously determined that the titer of the antiserum used in Figure 6 against chicken neuronal AChR was 66 μM (Whiting and Lindstrom, 1986). From Figure 7 it can be calculated that the titer of the antineurotransmitter binding site antibodies in this serum was 22 nmol ^{3}H-nicotine binding inhibited/liter serum. Because the antineurotransmitter binding site antibodies are such a small proportion (<0.1%) of the total antineuronal AChR activity, they have a negligible effect upon total ^{3}H-nicotine binding determined using antiserum to immobilize the neuronal AChR. Also, the low concentration of antisite antibodies makes it unlikely that they are responsible for the inhibition of AChR function (meaning the depolarization of chick ciliary ganglion neurons by 3 mM carbachol) observed using 1/100 dilutions of this serum (Stollberg et al., 1986). Other antibody specificities, such as those directed against the ion channel, may account for the observed inhibition of function.

Regional distribution of neuronal AChR in rat brain

Rat brains were dissected into 6 different regions and detergent extracts prepared. The neuronal AChRs from each extract were then immobilized on anti-chicken neuronal AChR–Sepharose and ^{3}H-nicotine binding determined (Fig. 8*A*). Simultaneously, the ^{125}I-αBGT binding was determined by DEAE assay (Fig. 8*B*). For whole brain, we determined a value of 0.70 ± 0.07 pmol ^{3}H-nicotine binding sites/g rat brain (mean ± SD, 3 preparations). The concentration of neuronal AChR in the brain regions, determined by ^{3}H-nicotine binding, ranged from 0.23 to 1.05 pmol/g rat brain. It was highest in the superior colliculus, thalamus, and interpeduncular nucleus, and lowest in the cerebellum. ^{125}I-αBGT binding was also highest in the superior colliculus and lowest in the cerebellum, but the other areas of the brain differed in their rank order binding of αBGT compared to nicotine.

Discussion

There is now much biochemical evidence to show that the neuronal component bound by the anti-electric organ AChR antibody mAb 35 is a neuronal AChR. The component from chicken brain binds some antibodies to electric organ and skeletal muscle AChR and does not bind αBGT, but it can be affinity-labeled with ^{3}H-BAC (Whiting and Lindstrom, 1986). Similarly, the mAb 35 binding component from chicken ciliary ganglion cells binds some mAbs to electric organ and skeletal muscle and does not bind αBGT, but it can be modulated with cholinergic ligands (Smith et al., 1986). In addition, antisera to the chicken brain component specifically block the ACh-induced depolarization

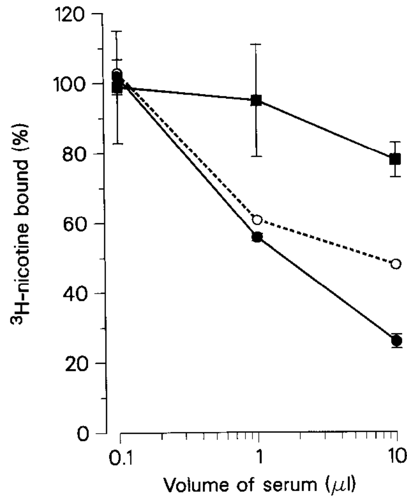

Figure 7. Inhibition of ³H-nicotine binding to immobilized chick neuronal AChR by anti-chick neuronal AChR serum. Chick brain detergent extract (80 ml) was recirculated through 250 μl of mAb 35–Sepharose for 15 hr, 4°C, and then divided into aliquots containing 0.47 pmol ¹²⁵I-mAb 35 binding sites per aliquot. After washing with 2 × 1 ml PBS, 0.5% Triton X-100, the aliquots were incubated for 90 min, room temperature, with periodic agitation in 100 μl of rat anti-chick neuronal AChR serum [anti-chick neuronal AChR titer of 66 μM determined by solid-phase radioimmunoassay (Whiting and Lindstrom, 1986)] or normal rat serum, diluted in the same buffer. ³H-Nicotine (11 μl 100 nM) was then added (to give a final concentration of 10 nM) and after 15 min incubation the ³H-nicotine bound was determined as described in Materials and Methods. The total binding of ³H-nicotine was determined in the absence of any serum, and binding in the presence of serum expressed as a percentage of the total binding. Nonspecific binding, determined in the presence of 1 mM carbachol, was less than 2% of the total binding. Each point is the mean ± SD of triplicate determinations. Symbols: ■, ³H-nicotine binding in the presence of normal rat serum; ●, ³H-nicotine binding in the presence of anti-chicken neuronal AChR serum; ○, specific inhibition (³H-nicotine binding in the presence of anti-chicken neuronal AChR serum after subtraction of the nonspecific inhibition of binding by normal rat serum).

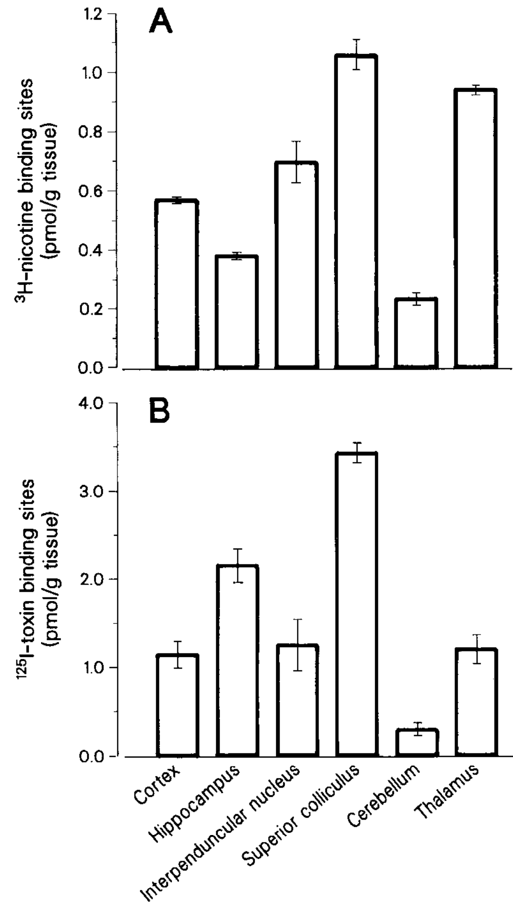

Figure 8. Regional distribution of ³H-nicotine binding sites and ¹²⁵I-αBGT binding sites in rat brain. Detergent extracts of each of the dissected brain regions (pooled from 10–15 rats) were prepared as described in Materials and Methods. *A*, ³H-Nicotine binding. Detergent extracts (180 μl) were gently shaken for 20 hr, 4°C, with 50 μl of anti-chick neuronal AChR–Sepharose. The aliquots were washed with 2 × 1 ml of PBS, 0.5% Triton X-100 and incubated with 100 μl of 20 nM ³H-nicotine for 15 min, room temperature. ³H-Nicotine bound was determined as described in Materials and Methods and expressed as nicotine binding sites/g brain tissue (wet wt). Nonspecific binding was determined by labeling in the presence of 1 mM carbachol and subsequently subtracted. Each column is the mean ± SD of triplicate determinations. *B*, ¹²⁵I-αBGT binding. Toxin binding to each detergent extract was determined by DEAE assay, as described in Materials and Methods and expressed as toxin binding sites/g tissue (wet wt). Nonspecific binding was determined by labeling in the presence of 1 μM nonradioactive αBGT and has been subtracted. Each column is the mean ± SD of quadruplicate determinations.

of cultured chicken ciliary ganglion cells (Stollberg et al., 1986). Here we demonstrate that the chicken brain component binds ³H-nicotine with high affinity. Further, we show that this chicken neuronal AChR and a homologous component from rat brain have a nicotinic cholinergic pharmacology that is essentially identical to that found by other workers who have investigated the binding of ³H-nicotine to rodent brain membranes (Abood et al., 1980; Clarke et al., 1984; Marks and Collins, 1982; Romano and Goldstein, 1980; Yamada et al., 1985).

We detected ³H-nicotine binding by immobilizing the neuronal AChR upon antibody-affinity supports. This both concentrated and purified the neuronal AChR and allowed assays of solubilized AChR to be performed in microfuge tubes, which was both very rapid and convenient. Additionally, the nonspecific binding of ³H-nicotine, which has been a problem when examining binding to membrane preparations, was negligible (Fig. 1, for example). Both chicken and rat neuronal AChRs bound ³H-nicotine with high affinity. Other workers have investigated the binding of this ligand to rodent brain membranes and have reported a wide range of binding affinities, to either a single site (Abood et al., 1980; Clarke et al., 1984; Marks and Collins, 1982) or more than one site (Larsson and Nordberg, 1985; Romano and Goldstein, 1980; Yamada et al., 1985). We found no evidence for more than one binding affinity. However, the heterogeneity of nicotine binding to rodent brain membranes observed by some workers suggested that the neuronal AChR characterized in this report may represent only one of several nicotine binding proteins in the CNS. To address this possibility, we investigated whether an immobilized mAb to the chicken neuronal AChR, mAb 270, could deplete ³H-nicotine binding sites from brain detergent extracts. mAb 270 bound virtually all of the ³H-nicotine binding components (Fig. 4). Thus, the neuronal AChR characterized here represents the major, and perhaps the only, high-affinity nicotine binding component in chicken and rat brain. We cannot discount the possibility that a small population of high-affinity nicotine binding sites (<10% of the total) or a low-affinity nicotine binding component exists that is not bound by anti-neuronal AChR antibodies.

Schneider and coworkers (1985) have recently identified and solubilized a component from chicken brain and optic lobe that binds ³H-ACh with high affinity but does not bind αBGT. As is discussed further below, it is likely that this component is the same as the neuronal AChR bound by mAb 35. However, these

authors reported that the ^{3}H-ACh binding site was present at >1 pmol/g brain. We have consistently found that the mAb 35 binding site is present at 0.2–0.4 pmol/g chicken brain (Lindstrom et al., 1983; Whiting and Lindstrom, 1986) and report here that the ^{3}H-nicotine binding sites are present in very similar quantities, as would be expected if they bind to the same molecule in approximately stoichiometric amounts. The reason for this difference is not currently known, but one possibility is that it represents developmental changes in the expression of neuronal AChRs: Schneider and coworkers used 2-d-old chicks, whereas we have used adult chickens.

In rat brain we have found that there are 0.9 ± 0.03 pmol/g brain of ^{3}H-nicotine binding sites using a gel-filtration assay, and the slightly lower value of 0.7 ± 0.07 pmol/g using anti-chicken neuronal AChR–Sepharose to immobilize the nicotine binding site. This value is lower than the amount of ^{3}H-nicotine and ^{3}H-ACh binding sites in rodent brain determined by other workers, 3–4.5 pmol/g brain (Marks and Collins, 1982; Reulecke and Hucho, 1985; Romano and Goldstein, 1980).

The pharmacology of both the chicken and rat neuronal AChRs was characteristic in having a high affinity for cholinergic agonists and low affinity for cholinergic antagonists (Table 1). As predicted from previous studies (Whiting and Lindstrom, 1986), αBGT showed no inhibition of ^{3}H-nicotine binding to immobilized neuronal AChR at up to micromolar concentrations. In general, the affinities of various cholinergic ligands for the neuronal AChRs are in excellent agreement with the values obtained by other workers for binding to intact brain membranes (Clarke et al., 1984; Marks and Collins, 1982; Romano and Goldstein, 1980; Yamada et al., 1985). The data are also in good agreement with values obtained by Schwartz et al. (1982), who determined the inhibition of ^{3}H-ACh to rodent brain membranes by cholinergic ligands. In a more recent study, these workers have demonstrated autoradiographically that ^{3}H-ACh and ^{3}H-nicotine have almost identical binding patterns on rat brain sections (Clarke et al., 1985a). Similarly, the regional distribution of ^{3}H-nicotine binding sites in rat brain found in this study was in good agreement with both the distribution of membrane-bound nicotine binding sites determined by other workers (Marks and Collins, 1982; Yamada et al., 1985) and with the distribution of membrane-bound ^{3}H-ACh binding sites (Schwartz et al., 1982). Furthermore, the agonist binding site of chicken brain and optic lobe, defined by its high affinity for binding ^{3}H-ACh (Schneider et al., 1985) also has essentially identical pharmacology to the neuronal AChR described here. Thus, it is very likely that the ^{3}H-ACh binding component, the ^{3}H-nicotine binding component, and the neuronal AChR defined here are the same.

As others have previously noted, there is a puzzling discrepancy between (1) the relatively high affinity of the cholinergic agonists compared to their concentration required for stimulation in the CNS (Brown et al., 1983), and (2) the relatively low affinities of antagonists such as hexamethonium and mecamylamine, which act on the CNS nicotinic AChR at relatively low concentrations (Brown et al., 1983; Clarke et al., 1985b). In *Torpedo* electric organ, bound agonist induces conversion of AChR from a low- to high-affinity desensitized form (for review, see Anholt et al., 1985; Popot and Changeux, 1984). One may speculate that the neuronal AChR undergoes an analogous conversion. Additionally, one may consider that some antagonists may also block function by binding in the cation channel. Whether these or other possibilities can account for the above disparities requires further elucidation.

Both the chicken and rat neuronal AChRs could be affinity-labeled with BAC after reduction with DTT, demonstrating the existence of a disulfide bond adjacent to the ACh binding site. This feature is conserved in electric organ and skeletal muscle AChRs (Damle et al., 1978; Wolosin et al., 1980), in the brain αBGT binding protein (Kemp et al., 1985; Norman et al., 1982), as well as in neuronal AChRs. Conservation of this disulfide bond suggests that it has an important structural or functional role in this family of proteins with a shared evolutionary history. We found no evidence that BAC labels the neuronal AChR in a biphasic manner, as is known for electric organ and skeletal muscle AChR due to preferential labeling of 1 of the 2 binding sites (Wolosin et al., 1980). Similarly, the inhibition of ^{3}H-nicotine binding to neuronal AChR by curare (Fig. 5) was clearly indicative of binding with a single affinity. In contrast, it is known that curare binds with a higher affinity to 1 of the 2 neurotransmitter binding sites on electric organ and skeletal muscle AChRs (Neubig and Cohen, 1979; Sine and Taylor, 1981; Whiting et al., 1985). Thus, there is either only 1 neurotransmitter binding site per neuronal AChR molecule, or, more likely, there are 2 or more sites that are indistinguishable by their susceptibility to affinity labeling or by their affinity for certain antagonists. It is interesting to note that the αBGT binding protein from rat brain is also affinity-labeled in a monophasic manner (Kemp et al., 1985), suggesting that this feature has diverged from skeletal muscle AChR but is conserved in the brain αBGT binding protein and the brain AChR described in this report.

Here we have shown that the chicken neuronal AChR bound by mAb 35 has a nicotinic cholinergic pharmacology. Using both a cross-reactive antiserum and a mAb, we have further demonstrated a homologous neuronal AChR exists in rat brain that probably has the same high-affinity agonist binding site as has been identified by previous workers. Since the antisera specifically block ACh-induced depolarization of chicken ciliary ganglion cells (Stollberg et al., 1986), one may speculate that the mammalian neuronal AChR bound by these antisera is also a functional AChR. Furthermore, mAbs to neuronal AChRs are valuable probes that should allow the purification and characterization of the mammalian neuronal AChR, immunohistochemical mapping of the nicotinic cholinergic pathways in the mammalian brain, and molecular genetic studies of the neuronal AChR.

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