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Brain and muscle nicotinic acetylcholine receptors are different but homologous proteins

(nicotinic receptor sequence analysis/gene families)

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ABSTRACT **An α-bungarotoxin-binding protein was purified from chick optic lobe and brain by an improved method. Previous and present observations justify its designation as a brain nicotinic acetylcholine receptor (AcChoR). It contains subunits whose apparent molecular weights are somewhat larger than those of subunits of peripheral AcChoRs. The size of the optic lobe AcChoR complex is greater than that of the peripheral receptor when estimated from its sedimentation behavior. Brain AcChoR subunits can be specifically precipitated by a monoclonal antibody directed against chick muscle AcChoR. Amino-terminal amino acid sequence analysis was performed on AcChoR preparations and isolated subunits from the optic lobe and from the rest of the chick brain. The sequences obtained demonstrate that, at least for the lowest molecular weight component, the AcChoRs from different brain areas are identical and they are highly homologous to muscle AcChoR. It is concluded that the brain α-bungarotoxin-binding protein is indeed a nicotinic AcChoR and is encoded by a set of genes that is different from, but strongly related to, that for the muscle AcChoR.**

The nicotinic acetylcholine receptors (AcChoRs) from vertebrate skeletal muscle (1, 2) and from fish electric organ (3) are pseudosymmetric complex proteins, composed of four homologous subunits (α, β, γ, and δ) in the stoichiometry $\alpha_2\beta\gamma\delta$. This homology has been fully quantitated in certain cases by cDNA cloning and total sequencing of subunits from both muscle and electric organ AcChoR (4–7). Each of the four subunit types is specified by a separate gene, but the homologies found between subunits and between species show that these genes belong to a single family.

Some regions of the vertebrate brain contain a nicotinic AcChoR with pharmacological characteristics similar but not identical to those of muscle AcChoR (8, 9). In addition, some brain areas and peripheral ganglia contain high-affinity binding sites for α-bungarotoxin (α-BTX) and similar snake toxins, which are known to bind to peripheral AcChoR. However, the identity of the neuronal toxin-binding component with a nicotinic AcChoR has been disputed (reviews, see refs. 10 and 11). In avian and amphibian optic lobe (10), human medullablastoma cells (12), and some sympathetic ganglionic sites (11, 13–17) there is evidence in favor of this identity, even though α-BTX binding does not always block receptor function. From avian optic lobe (10), where α-BTX blocks AcChoR function, the α-BTX binding protein has been purified (18). This protein displays the ligand-binding characteristics of a nicotinic receptor (2) and it can be covalently labeled by the affinity reagent bromoacetylcholine (BrAcCho) (18) in a manner similar to muscle and electric organ. It also shows distinct immunological crossreactivity with an antiserum to mammalian muscle AcChoR (18) and with some monoclonal antibodies raised against chicken muscle AcChoR (2, 19). Identification of this brain α-BTX-binding protein as a true AcChoR and resolution of the question as to whether it is encoded by genes of the family described above for the nonneuronal AcChoRs requires information on the nature and amino acid sequence of its subunits. We provide here evidence that the central nervous system (CNS) α-BTX-binding protein is a nicotinic AcChoR structurally homologous to peripheral AcChoR.

METHODS AND MATERIALS

Methods and materials not specified were as given by Norman *et al.* (18). Optic lobes (100–200 per preparation) were dissected from 1-day old chicks and used either freshly or after freezing in liquid N_2 and stored at −70°C. The remainder of the brains after optic lobe removal was similarly frozen and stored at −70°C. This part of the chick CNS will be referred to as "brain." Vacuum dialysis was through Spectrapor 25K tubing, prewashed in 2% $NaHCO_3$ for 45 min at 40°C, then in 1% $NaDodSO_4$ at 40°C, and extensively rinsed with water. All water used for samples for sequencing was redistilled in glass.

Preparation of AcChoR. Chick optic lobes or brains were homogenized (glass/Teflon, 10 passes) in 10 vol of buffer 1 (50 mM sodium phosphate, pH 7.0/5 mM EDTA/1 mM EGTA) containing also 1 mM phenylmethylsulfonyl fluoride, bacitracin at 100 μg/ml, soybean trypsin inhibitor at 25 μg/ml, 1 mM benzamidine, and (except where noted) 0.5 mM *N*-ethylmaleimide or 2 mM iodoacetamide. The pellet collected at 100,000 × *g* was homogenized in 4 vol of buffer 1 containing the protease inhibitors less *N*-ethylmaleimide. Triton X-100 was added to a final concentration of 1% and the extract was shaken at 0°C for 1 hr. The supernatant (100,000 × *g*, 45 min) was filtered through glass wool and shaken at 4°C for 3 hr with 2 ml of Sepharose-α-BTX (concentration of coupled toxin ≈ 0.5 mg/ml of gel) per 100 ml of supernatant. The beads were washed rapidly in a column, first with 30 vol of buffer 1 containing 0.2% Triton X-100 and protease inhibitors, then with 100 vol of the latter medium containing 1 M NaCl, and finally with 60 vol of buffer 1/0.2% Triton X-100. The beads were extracted with 1.5 vol of 1 M carbachol in buffer 1/0.2% Triton X-100 with rotation for 12 hr at 4°C, and the extract was combined with a second 2-hr extract (extract a) or with 1.5 vol of 62 mM Tris·HCl, pH 6.8/3% $NaDodSO_4$/5% (vol/vol) 2-mercaptoethanol, rotat-

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Abbreviations: AcCho, acetylcholine; AcChoR, acetylcholine receptor; BrAcCho, bromoacetylcholine; α-BTX, α-bungarotoxin; CNS, central nervous system.

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ing 1 hr at room temperature (extract b). Extract a was dialyzed against several changes of 10 mM sodium phosphate/50 mM NaCl/0.1% Triton X-100, pH 7.4. $NaDodSO_4$ and mercaptoethanol were added to final concentrations of 1.5% and 2.5%, respectively, and the samples were dialyzed extensively against 16.7 mM Tris·HCl/0.05% $NaDoDSO_4$, pH 6.8. Extract b was dialyzed similarly.

Purification of AcChoR Subunits. The peptides present in preparations of purified AcChoR (50–200 pmol of toxin-binding sites) were isolated by $NaDodSO_4$ gel electrophoresis, recovered by electroelution, and electrodesalted as described (20).

The purity and integrity of the isolated subunits were assessed by gel electrophoresis (21). The protein bands were visualized by silver staining (22). The purified subunits were lyophilized and stored frozen.

Amino-Terminal Amino Acid Sequence Analysis. The lyophilized samples were dissolved in glass-distilled water and 30 μl was loaded on a Polybrene-coated glass filter disk in a gas-phase sequencer (Applied Biosystems, Foster City, CA) and submitted to amino-terminal sequence analysis by automated Edman degradation (23). The Polybrene-coated disk had been precycled by 10 cycles of automated Edman degradation. For each analysis 5–20 pmol of protein was used. Phenylthiohydantoin derivatives of amino acids were identified by HPLC in an IBM Cyano column (24). Typical repetitive yields for these analyses were between 93 and 96%.

RESULTS AND DISCUSSION

Purified Optic Lobe and Brain AcChoR. The method used for chick optic lobe and brain AcChoR purification was a modification of a previous method (18) and was designed to minimize AcChoR proteolysis. These modifications were (*i*) solubilization for 1 hr in 1% Triton X-100 instead of 5% Lubrol PX for 2 hr; (*ii*) increase in the phenylmethylsulfonyl fluoride concentration 10-fold to 1 mM and the EDTA 5-fold to 5 mM; (*iii*) reduction of the period allowed for binding of AcChoR to toxin-Sepharose from 18 hr to 3 hr; (*iv*) omission of the lentil lectin-Sepharose treatment and of the recycling previously used during the elution by carbachol. The combined modifications led to some differences (as detailed below) in the subunit pattern and sedimentation behavior from those previously described (18).

The purified α-BTX-binding component from optic lobe showed high-affinity binding of ^{125}I-labeled α-BTX (^{125}I-α-BTX) with specific activities of 4000–6000 nmol/g of protein in different preparations. The component purified from "brain" (see *Methods and Materials*) had similar α-BTX-binding characteristics and specific activity. In sucrose density gradients the optic lobe AcChoR sedimented mostly as a peak with a sedimentation coefficient of 10.5 S (Fig. 1). For the AcChoRs from chick or other muscles (2) this value is about 9 S, and the molecular size of the optic lobe AcChoR is indeed significantly larger, as was shown by hydrodynamic determination with ^2H_2O correction for bound detergent (to be reported in detail elsewhere).

Peptide Composition of Purified CNS AcChoR. Upon $NaDodSO_4$ gel electrophoresis the α-BTX-binding proteins from either chick optic lobe or brain had very similar peptide compositions. Different preparations consistently contained four or five major components, whose molecular weights ranged between 48,000 and 72,000 (Fig. 2). The component of lightest molecular weight (48,000) sometimes migrated in a diffuse form, which was poorly stained by Coomassie blue. This component was also very sensitive to proteolytic degradation, as demonstrated by its progressive disappearance with more lengthy purification procedures. Similar patterns were observed when the specifically bound protein was either

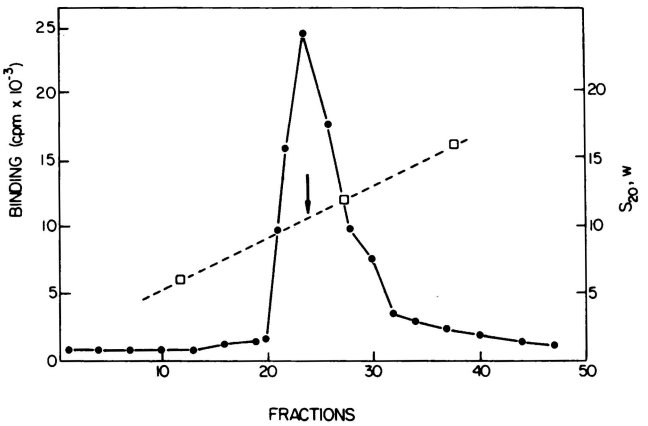

FIG. 1. Separation of the purified receptor by centrifugation in a 5–20% sucrose gradient in 10 mM sodium phosphate, pH 7/50 mM NaCl/0.2% Triton X-100. Each fraction was assayed for the binding of ^{125}I-α-BTX (●). Calibration standards were yeast alcohol dehydrogenase, bovine catalase, and *Escherichia coli* β-galactosidase, with their $s_{20,w}$ values (□) in S units on the right-hand scale. In four such determinations on different preparations, the receptor peak was centered (arrow) at a mean of 10.5 S on the linear plot shown.

stripped from the toxin-Sepharose $NaDodSO_4$ or specifically eluted by carbachol.

Amino-Terminal Amino Acid Sequencing. All the peptides contained in purified optic lobe AcChoR were isolated (Fig. 3) and submitted to amino-terminal amino acid sequencing. The lowest molecular weight component gave a readily

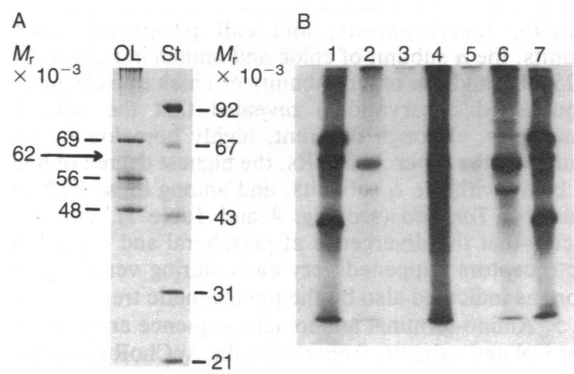

FIG. 2. Analysis of the subunit composition of AcChoR from chick optic lobe by $NaDodSO_4$ gel electrophoresis. (*A*) AcChoR from optic lobe (OL) was eluted from the α-BTX-Sepharose affinity gel with 62 mM Tris·HCl buffer, pH 6.8, containing 3% $NaDodSO_4$ and 5% mercaptoethanol. After electrophoresis the proteins were silver stained. Standard proteins (St) used were phosphorylase *b*, bovine serum albumin, glutamate dehydrogenase, ovalbumin, lactate dehydrogenase, and carbonic anhydrase. (*B*) Autoradiogram of [^{125}I]-iodinated AcChoR. The sample run in track 4 was radioiodinated by using the chloramine-T method while adsorbed to the α-BTX-Sepharose resin; it was then washed and eluted with 62 mM Tris·HCl buffer, pH 6.8, containing 3% $NaDodSO_4$ and 5% mercaptoethanol. Aliquots of material, eluted from the α-BTX affinity gel with carbachol, were labeled with ^{125}I and gel filtered to remove free ^{125}I. Samples were then incubated at 22°C for 2½ hr with nonimmune serum (tracks 3 and 5) or with monoclonal antibody 7B2 (19), raised against chick muscle AcChoR (tracks 2 and 6); after reaction (22°C for 2 hr) with staphylococcal protein A-Sepharose, the resultant precipitates were washed, dissolved in $NaDodSO_4$ buffer (see above), and subjected to electrophoresis. ^{125}I-labeled standard proteins (bovine serum albumin, catalase, ovalbumin, and lactate dehydrogenase) were run in tracks 1 and 7. The monoclonal antibody selectively precipitated polypeptides of M_r 48,000, 56,000, 69,000, and 72,000 (tracks 2 and 6), while negligible radioactivity was precipitated in control samples (tracks 3 and 5).

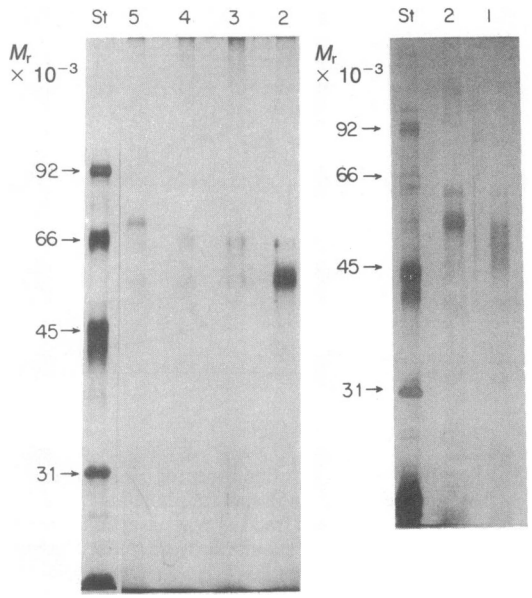

FIG. 3. $NaDodSO_4$ gel electrophoresis of isolated subunits from chick optic lobe AcChoR visualized by silver staining. St, Bio-Rad low molecular weight standards; lanes 1–5, isolated subunits in order of increasing molecular weight. Band 1 (M_r, 48,000) gave the sequence reported in Fig. 4 and band 2 (M_r, 56,000) has previously been shown to be labeled by BrAcCho (18). Bands 3 (M_r, 62,000) and 4 (M_r, 66,000) together with 2 can be precipitated by monoclonal antibody 7B2.

identifiable single sequence, reported in Fig. 4. Comparison of this sequence with the known amino-terminal sequences of *Torpedo*, *Electrophorus*, and calf peripheral AcChoR subunits, the α subunit of chick and human muscle AcChoR (1, 25–28), and the other subunits of chick muscle AcChoR (unpublished observations) revealed that the optic lobe sequence is, although different, highly homologous to the subunits of the other AcChoRs, the highest degree of homology being with the α subunits, and among these with the α subunit of *Torpedo* (see Fig. 4 and Table 1). This would indicate that the divergence of peripheral and central nicotinic receptors happened very early during vertebrate evolution, as indicated also by the phylogenetic tree depicted in Fig. 5. Amino-terminal amino acid sequence analysis of the other isolated subunits from optic lobe AcChoR did not yield any signal above the high background consistently present, indicating that these subunits had blocked amino termini. To determine whether this blockage was caused by the isolation of the subunits from $NaDodSO_4$ gels, intact optic lobe AcChoR preparations were submitted to simultaneous amino-terminal analysis of all the sequences present in such preparations. Only one signal was detected, which corresponded to the sequence reported above for the lightest component of optic lobe AcChoR, and it was therefore concluded that animo-terminus blockage preexisted subunit isolation. Due to the agonizingly low amounts of AcChoR present in the rest of the chick brain the isolation of individual subunits for sequencing was not feasible. Intact brain preparations were submitted to simultaneous amino-terminal analysis as described above. Only one signal was present above a high background, which indicated the presence of other proteins with blocked amino termini. The sequence obtained was identical to that of the lowest molecular weight component of optic lobe AcChoR. These data, together with

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BRAIN   XEFETKLYKELLKNYNPLEXPVAXD
RAY     SEHETRLVANLLENYNKVIRPVEHH
EEL     SEDETRLVKLNFSGYNKVVRPVNHF
CALF    SEHETRLVAKLFEDYNSVVRPVEDH
CHICK   XEHETRLVDDLFRDYSKVVRPVENH
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FIG. 4. Comparison of the amino-terminal amino acid sequence (standard one-letter code) obtained for the M_r 48,000 subunit of brain AcChoR (top) and the amino-terminal sequences of known α subunits of peripheral AcChoR from *Torpedo* electroplax (25), *Electrophorus* electroplax and muscle (20, 26), and calf muscle (1).

Table 1. Percent identity between the amino-terminal sequence obtained for the lightest component of chick brain AcChoR and the amino-terminal segment of subunits from peripheral AcChoRs

Subunit	Chick	Ray	Eel	Calf	Human
α	35	48	43	39	39
β	38	30	39	35	—
γ	—	26	30	—	—
δ	28	32	24	—	—

Sequences are from the following: chick muscle α subunit, ref. 27; chick muscle β and δ subunits, unpublished observations; *Torpedo* ray, ref. 25; *Electrophorus* eel, ref. 26; calf, ref. 1; and human, ref. 28.

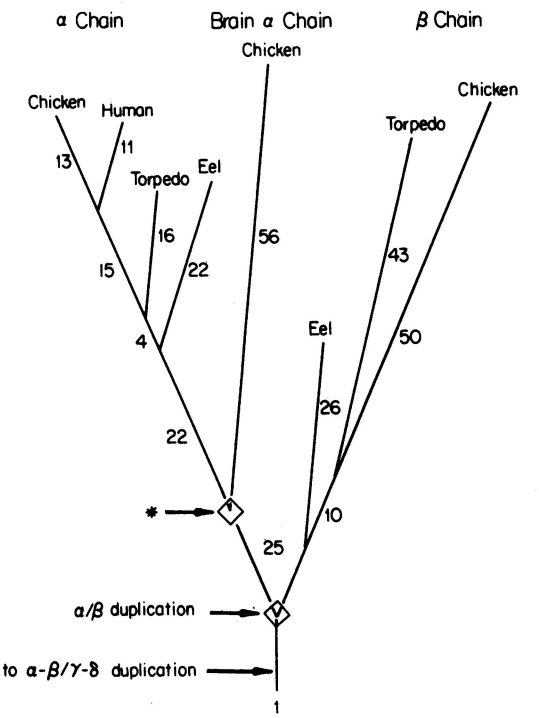

FIG. 5. A phylogenetic tree generated from the amino-terminal sequence data of the known α and β subunits of peripheral AcChoRs (1, 7, 20, 25–27) and chicken brain AcChoR by using the best-fit matrix method.[§] Each branch point represents a nodal or ancestral sequence. The numbers associated with each branch length represent the "accepted point mutations" (PAMs) per 100 amino acid residues that occurred in generating the contemporary AcChoR subunits. The position of the α/β duplication is arbitrarily located along a 25-PAM segment separating the ancestral sequences of modern α and β subunits. The * indicates the divergence of the α subunit of the brain AcChoR from the ancestor of the peripheral α subunits.

[§]Orcutt, B. C. & Dayhoff, M. O. (1975) Matrix Topology Program—MATTOP, NBR Report No. 09810-751101 (Natl. Biomed. Res. Found., Washington, D.C.).

the similar gel patterns and the indistinguishable reactivities (18, 19) to antibodies against muscle AcChoR (both polyclonal and monoclonal), suggest that the AcChoRs from the optic lobe and from other regions of the chick brain are similar if not identical complex proteins.

In the absence of sequencing data, other methods had to be used to identify the other peptides as AcChoR subunits. The peptide of apparent M_r 56,000 has already been shown (18) to be the site of specific alkylation by the affinity reagent BrAcCho and is therefore part of the receptor, since it must contain at least part of one of the binding sites for cholinergic ligands. In the case of peripheral AcChoR and under similar conditions, only the α subunit is labeled by BrAcCho (3). However, since multiple ligand binding sites have been shown to exist, at least on *Torpedo* AcChoR (29–32), and since BrAcCho itself can label other *Torpedo* AcChoR subunits under different labeling conditions (unpublished observations), it is reasonable to conclude that in the case of the highly divergent CNS receptor a different subunit can be more easily labeled by BrAcCho. Likewise ^{125}I-α-BTX can be crosslinked to the optic lobe M_r 56,000 subunit by using dimethyl suberimidate (ref. 18 and unpublished observations). Hence, the M_r 56,000 subunit carries a high-affinity site for AcCho and for α-BTX, as is known for the α subunit of the peripheral AcChoR (3).

Further evidence that other polypeptides are components of the AcChoR was obtained by virtue of their immunoreactivity. For this test, we used a monoclonal antibody, 7B2, raised against chick muscle AcChoR, which has been shown to give distinct crossreaction with the α-BTX complex of the AcChoR purified from chick optic lobe (19). Optic lobe AcChoR was labeled with ^{125}I and immunoprecipitated by antibody 7B2, and the precipitate was extracted in $NaDodSO_4$ solution and analyzed. Three receptor bands were present (Fig. 2*B*), which corresponded to the M_r 48,000, 56,000, and 69,000 subunits observed upon $NaDodSO_4$ gel electrophoresis of noniodinated receptor. Note that this includes the subunit of lowest molecular weight, for which the sequence data of Fig. 4 reveal a correspondence with the α subunit of peripheral AcChoR. Iodination also caused some breakdown of the two higher molecular weight subunits, which appeared weaker upon fluorography of ^{125}I-AcChoR when compared with Coomassie- or silver-stained gels of noniodinated AcChoR (Figs. 1 and 3). A M_r 72,000 polypeptide was precipitated but was faint (Fig. 2*B*); it therefore remains to be established whether this is an AcChoR subunit. The latter statement applies also to the M_r 62,000 polypeptide, which is not readily seen in iodinated or (Fig. 2*A*) silver-stained AcChoR analyses.

The data reported here permit the identification of the M_r 48,000, 56,000, and 69,000 polypeptides as subunits of the central AcChoR. In the absence of amino terminals available for sequencing it was not possible to determine the stoichiometry of the receptor subunits, or to ascertain whether the other two polypeptides present in $NaDodSO_4$ gels of purified CNS receptors, having molecular weights of 62,000 and 72,000, represent one or more contaminants or are AcChoR subunits or different proteolytic products or post-translational modifications of one subunit. We can exclude the possibility that the M_r 48,000 subunit is a breakdown product of any of the others, since the amino terminus is different from theirs (not blocked) and corresponds in sequence to the true amino terminus of the peripheral AcChoR α subunit. Actin, which is found complexed to the calf muscle AcChoR (1) and which migrates to the M_r 45,000–48,000 region and has a blocked amino terminus, was absent here, as shown by comparison with a chicken gizzard actin standard and by the lack of change of the pattern upon the treatment of the AcChoR with anti-actin antibody.

These results establish that the CNS α-BTX-binding protein is indeed a nicotinic receptor similar to the receptors found in muscle and electric organ. Brain and muscle AcChoR from the same species, although homologous, must be encoded by different genes and must have originated from the same ancestral gene. Since the similarity between α subunits of central and peripheral receptors from the same animal is much less than between α subunits of peripheral AcChoRs from different animals (Table 1), it may be concluded that the central and peripheral nicotinic receptors diverged very early during vertebrate evolution (see Fig. 5). These divergences from an ancestral common structure explain well the pharmacological characteristics of these receptors, which only partially overlap. Similarly, the partial structural identity explains why in certain cases polyclonal and monoclonal antibodies raised against peripheral AcChoRs failed to recognize central receptors (9, 33) and antibodies against chick optic lobe receptor did not bind to peripheral AcChoR from chick or from *Torpedo californica* (34). Our data do not exclude the possibility that within the nervous system different nicotinic receptors may exist. This possibility is supported by the following findings: (*i*) neuronal proteins exist that do not bind α-BTX but bind anti-muscle AcChoR antibodies as well as other α-BTX-like snake venom toxins (15, 35, 36) and (*ii*) in chick sympathetic ganglia two nicotinic receptors with slightly different pharmacological specificities exist, both of which bind α-BTX-like toxins, but only one of them is functionally blocked as a consequence of α-BTX binding (13, 16).

It has been reported that antibodies raised against chick optic lobe receptor crossreact not with chick muscle AcChoR but with the receptor from the PC12 neuronal cell line (34). This raises the possibility that neuronal nicotinic receptors are highly conserved proteins and that the genes encoding chick brain AcChoR, whose isolation will be greatly facilitated by the reported sequence data, will permit the isolation of mammalian brain AcChoR genes.

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