AD_____



# USE OF MONOCLONAL ANTIBODIES TO STUDY THE STRUCTURAL BASIS OF THE FUNCTION OF NICOTINIC ACETYLCHOLINE RECEPTORS ON ELECTRIC ORGAN AND MUSCLE, AND TO DETERMINE THE STRUCTURE OF NICOTINIC ACETYLCHOLINE RECEPTORS ON NEURONS

Annual Report

Jon M. Lindstrom

March 16, 1987





Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-86-C-6148

The Salk Institute Receptor Biology Laboratory
San Diego, California 92138-9216

UNCLASSIFIED
SECURITY CLASSIFICATION OF THIS PAGE

AD-A179 650

# REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

| | |
|---|---|
| 1a. REPORT SECURITY CLASSIFICATION<br>Unclassified | 1b. RESTRICTIVE MARKINGS |
| 2a. SECURITY CLASSIFICATION AUTHORITY | 3. DISTRIBUTION/AVAILABILITY OF REPORT<br>Approved for public release; distribution unlimited. |
| 2b. DECLASSIFICATION/DOWNGRADING SCHEDULE | |
| 4. PERFORMING ORGANIZATION REPORT NUMBER(S) | 5. MONITORING ORGANIZATION REPORT NUMBER(S) |
| 6a. NAME OF PERFORMING ORGANIZATION<br>The Salk Institute<br>Receptor Biology Lab. | 6b. OFFICE SYMBOL (If applicable) |
| 6c. ADDRESS (City, State, and ZIP Code)<br>La Jolla, California 92037 | 7a. NAME OF MONITORING ORGANIZATION<br>7b. ADDRESS (City, State, and ZIP Code) |
| 8a. NAME OF FUNDING/SPONSORING ORGANIZATION U.S. Army Medical Research & Devel. Cmd. | 8b. OFFICE SYMBOL (If applicable) |
| 8c. ADDRESS (City, State, and ZIP Code)<br>Fort Deterick<br>Frederick, Maryland 21701-5012 | 9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER<br>DAMD17-86-C-6148 |

10. SOURCE OF FUNDING NUMBERS

| PROGRAM ELEMENT NO. | PROJECT NO. | TASK NO. | WORK UNIT ACCESSION NO. |
|---|---|---|---|
| 61102A | 3M161 102BS11 | EF | 062 |

11. TITLE (Include Security Classification) USE OF MONOCLONAL ANTIBODIES TO STUDY THE STRUCTURAL BASIS OF THE FUNCTION OF NICOTINIC ACETYLCHOLINE RECEPTORS ON ELECTRIC ORGAN & MUSCLE, & TO DETERMINE THE STRUCTURE OF NICOTINIC ACETYLCHOLINE RECEPTORS ON NEURONS.

12. PERSONAL AUTHOR(S)
LINDSTROM, Jon M.

| 13a. TYPE OF REPORT | 13b. TIME COVERED | 14. DATE OF REPORT (Year, Month, Day) | 15. PAGE COUNT |
|---|---|---|---|
| Annual | FROM 2/15/86 TO 2/14/87 | 1987 March 16 | 63 |

16. SUPPLEMENTARY NOTATION

| 17. COSATI CODES | | | 18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number) |
|---|---|---|---|
| FIELD | GROUP | SUB-GROUP | |
| 06 | 01 | | nicotinic acetylcholine receptors of muscle; nicotinic acetylcholine receptors of neurons |
| 06 | 13 | | |

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

The structure of muscle-type nicotinic acetylcholine receptors from Torpedo electric organ was studied. These are composed of four kinds of subunits termed α, β, γ and δ in order of increasing molecular weight. The α subunits form the acetylcholine binding sites. The subunits were shown to be organized around the central cation channel in the order αβαγδ. The acetylcholine binding site was shown to be near the sequence α189-196. The main immunogenic region was shown to be within the sequence α46-127. Epitopes for several monoclonal antibodies to α subunits were mapped, and the transmembrane orientation of these sequences was determined. Keywords: molecular structure, (KT)

Neuronal nicotinic receptors were found to differ from those in muscle by subunit structure, pharmacological properties, and functional role. Nicotinic acetylcholine receptors were immunoaffinity purified from the brains

(continued on reverse)

| 20. DISTRIBUTION/AVAILABILITY OF ABSTRACT | 21. ABSTRACT SECURITY CLASSIFICATION |
|---|---|
| ☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT. ☐ DTIC USERS | Unclassified |

| 22a. NAME OF RESPONSIBLE INDIVIDUAL | 22b. TELEPHONE (Include Area Code) | 22c. OFFICE SYMBOL |
|---|---|---|
| Mrs. Virginia Miller | (301)663-7325 | SGRD-RMI-S |

DD Form 1473, JUN 86 — Previous editions are obsolete. — SECURITY CLASSIFICATION OF THIS PAGE

UNCLASSIFIED

**19. Abstract (cont'd)**

of chickens and rats, and characterized using libraries of subunit-specific monoclonal antibodies raised to these proteins. These receptors have nanomolar affinity for nicotine, but negligible affinity for α-bungarotoxin. Receptors were immunohistologically localized throughout rat brains. Many receptors were found in pre- or extrasynaptic locations. These receptors were composed of two kinds of subunits, termed α and β in order of increasing molecular weight. The β subunits were shown by affinity labeling to form the acetylcholine binding sites, and it was assumed that the α subunits played a structural role. There are at least two, but no more than three, subunits of each kind in a receptor molecule. In adult chicken brains there are equal amounts of two nicotinic receptor subtypes which use the same type of 49,000 apparent molecular weight structural subunits. One subtype uses an acetylcholine-binding subunit of 75,000 apparent molecular weight. In rat brains a single receptor subtype accounts for more than 90% of the high affinity nicotine binding sites. It is composed of structural subunits of apparent molecular weight 51,000 and acetylcholine-binding subunits of apparent molecular weight 79,000.

AD_____

# USE OF MONOCLONAL ANTIBODIES TO STUDY THE STRUCTURAL BASIS OF THE FUNCTION OF NICOTINIC ACETYLCHOLINE RECEPTORS ON ELECTRIC ORGAN AND MUSCLE, AND TO DETERMINE THE STRUCTURE OF NICOTINIC ACETYLCHOLINE RECEPTORS ON NEURONS

Annual Report

Jon M. Lindstrom

March 16, 1987

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-86-C-6148

The Salk Institute Receptor Biology Laboratory
San Diego, California 92138-9216

## Abstract

Structure of nicotinic acetylcholine receptors from the electric organ of Torpedo californica was studied using monoclonal antibodies (mAbs) and synthetic peptides. The subunits of the receptor were shown to be organized in the order $\alpha\beta\alpha\gamma\delta$ like barrel staves around a central cation channel. The acetylcholine binding site on the $\alpha$ subunits was shown to be near the sequence $\alpha$189-196. Antigenic determinants were localized on the sequence of the subunits leading to the demonstration that the transmembrane orientation of the polypeptide chain could not be accounted for by simple models proposed by others on the basis of inspection of the amino acid sequences.

A human cerebellar medulloblastoma cell line, TE671, was found to express acetylcholine receptors antigenically similar to those of human muscle. Investigation was begun of the structure of this receptor and the regulation of its expression.

These studies extend the studies of nicotinic receptors of electric organ and muscle which are the best characterized neurotransmitter receptors.

Acetylcholine receptors with high affinity for nicotine and no affinity for $\alpha$-bungarotoxin were immunoaffinity purified from the brains of chickens and rats. Libraries of mAbs to these receptors aided in their characterization. These neuronal nicotinic receptors were found to have only two kinds of subunits, $\alpha$ and $\beta$. The $\alpha$ subunits shared antigenic determinants with $\alpha$ subunits of receptors from muscle, but affinity labeling experiments showed that the $\beta$ subunits formed the acetylcholine binding site. Two subtypes of receptors were distinguished in chick brains, one composed of $\alpha$ subunits (apparent molecular weight (mw) 49,000) and $\beta$ subunits (mw 59,000) and the other composed of similar or identical $\alpha$ subunits and $\beta'$ subunits (mw 75,000). Nicotinic receptors in chicken and rat ganglionic neurons were found to be closely antigenically related, but to have much lower affinity for nicotine. In rat brains, only receptors of the $\beta'$ subtype were found (mw $\alpha$=51,000, mw $\beta'$=79,000). mAbs to receptors from rat brains identified a nicotinic receptor in human brain. Immunohistochemical localization of receptor in rat brain corresponded to the localization of [$^3$H]nicotine binding sites previously determined by others. Some of these receptors were shown to be presynaptic.

$\alpha$-Bungarotoxin-binding protein affinity purified from rat brain was found to exhibit four bands on gel electrophoresis with apparent molecular weights similar to the subunits of nicotinic receptors from muscle, yet the $\alpha$-bungarotoxin-binding protein of brains of chickens, rats, and humans was found to be antigenically distinct from nicotinic receptors from both muscle and brain.

These studies for the first time are allowing characterization of the structure of purified neuronal nicotinic receptors.

## Foreword

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigator adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, Commission of Life Sciences, National Research Council (DHEW Publication No. (NIH) 86-23, Revised 1985).

The investigator(s) have abided by the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules (April 1982) and the Administrative Practices Supplements.

For the protection of human subjects the investigator(s) have adhered to policies of applicable Federal Law 45CFR46.

## Table of Contents

## Introduction

Acetylcholine receptors are of special interest to the Army because the devastating effects of many chemical warfare agents are mediated through these receptors. Acetylcholine receptors are excessively stimulated and desensitized by acetylcholine after the agents inactivate acetylcholinesterase, and the agents may also have direct effects on the receptors. Inhibition of nicotinic acetylcholine receptors on skeletal muscles results in paralysis and asphyxiation. Effects of inhibition of central nicotinic receptors are less well known. Peripheral and central muscarinic receptors are also affected by many chemical warfare agents.

Although they both respond to acetylcholine, nicotinic and muscarinic acetylcholine receptors are quite distinct in structure and function. Nicotinic acetylcholine receptors have an acetylcholine-regulated cation channel which is an integral part of the receptor molecule and have higher affinity for nicotine than for muscarine and higher affinity for curare than for atropine. Muscarinic acetylcholine receptors act via coupling proteins and second messengers, hence with a greater latency and duration, and have the inverse pharmacological properties. Nicotinic receptors in muscle have the subunit composition $\alpha_2\beta\gamma\delta$.[1,2] All of these subunits exhibit extensive sequence homologies, indicating that they evolved from a single primordial receptor subunit by a process of repeated gene duplication.[2,3] Muscarinic receptors are composed of a single type of subunit unrelated to those of the nicotinic receptor. Two subtypes of muscarinic receptors have been cloned and sequenced and found to exhibit extensive homology, although one contains an additional domain not found in the other.[4,5]

Our studies concern nicotinic acetylcholine receptors. Nicotinic receptors of fish electric organs have been extensively studied because they closely resemble those in skeletal muscle, but are present in much larger amounts.[6] Nicotinic receptors of both electric organs and muscle bind $\alpha$-bungarotoxin at their acetylcholine binding sites with great affinity and specificity. Snake venom toxins have been used for the quantitation, localization, and affinity purification of these receptors. Monoclonal antibodies (mAbs) are now proving to be useful probes for other parts of the molecule.[7] Nicotinic receptors on neurons were found which did not bind $\alpha$-bungarotoxin.[8,9] The small amount of these receptors and lack of a good biochemical probe for them long delayed studies of their structure. A further complication is that on many of the same neurons which contain nicotinic receptors, and on other neurons as well, there are $\alpha$-bungarotoxin binding components which are not acetylcholine-gated cation channels and whose function is unknown.[8,9] There is evidence that these $\alpha$-bungarotoxin binding components are members of the nicotinic receptor gene family.[10] Recently, mAbs have given us a tool for quantitating, localizing, affinity purifying, and characterizing neuronal nicotinic receptors. Our studies during the past year of nicotinic receptors from Torpedo

californica electric organ and chicken, rat, and human brains are summarized below. Continued studies using mAbs, purified receptors and receptor subunits, and molecular genetic techniques should provide increasingly detailed information about the structure and function of these important receptors.

## Progress Report

During the interval between application and initiation of this contract, significant progress was made on several of its goals.

A large number of mAbs were screened for their ability to inhibit Torpedo electric organ acetylcholine receptor function.[11] Most were not able to inhibit function; however, two mAbs directed at the cytoplasmic surface of $\beta$ and $\gamma$ subunits were able to inhibit function.[11] This was confirmed by single channel electrophysiological recording.[12]

Significant progress was made on locating the acetylcholine binding site and the main immunogenic region and other antigenic determinants on the primary structure of Torpedo electric organ acetylcholine receptor $\alpha$ subunits.[13-19] Some groups proposed that the acetylcholine binding site was formed by the sequence $\alpha$127-143.[20-22] However, using mAbs to a synthetic peptide including this sequence, we could show that these antibodies bound to the receptor did not obscure the acetylcholine binding site.[13] These antibodies did compete for binding with the lectin concanavalin A,[13] which is consistent with the proposal that the single site of N-glycosylation on $\alpha$ subunits occurs at $\alpha$141.[20] The main immunogenic region had been proposed by others to be formed by the sequence $\alpha$152-169.[20] We[15,16,23] and others[24] used antibodies to this synthetic peptide to show that it did not form the main immunogenic region. Instead, we were able to map the main immunogenic region to between $\alpha$46 and $\alpha$127.[18] The immunogenicity and antigenicity of the sequence comprising the main immunogenic region are maintained only in the native conformation of the receptor. Other immunogenic sequences which are not conformation-dependent were mapped to the C-terminal third of $\alpha$, $\beta$, and $\delta$ subunits, which is located on the cytoplasmic surface of the receptor.[17,18]

The transmembrane orientation of the subunit polypeptide chains of Torpedo electric organ acetylcholine receptor was studied. Two groups proposed that the "barrel stave" contributed by each receptor subunit to the lining of the cation channel was an amphipathic $\alpha$ helix located between the third and fourth hydrophobic putative transmembrane domain in each subunit.[25,26] We showed that the putative amphipathic transmembrane domain was in fact not a transmembrane domain, but was on the cytoplasmic surface.[17,18] Also, we provided evidence that the putative fourth hydrophobic transmembrane domain may not actually cross the membrane. We mapped the binding sites of anti-$\alpha$ subunit mAbs to sequences throughout the putative amphipathic domain and to the C terminus. By colloidal gold immunoelectron microscopy and other techniques, we showed that all of these mAbs bound to the cytoplasmic surface.[17] Our experiments showed that models derived from inspection of subunit cDNA sequences with either four[3,27,28] or five[25,26] putative transmembrane domains were incorrect, and we provided some evidence,[15] although not

colloidal gold electron microscopy evidence, for additional transmembrane domains. Thus, the complete transmembrane orientation of the subunit polypeptide chains has yet to be determined, but it is not surprising that this is a difficult problem with a large and complex molecule like the receptor.

Substantial progress was made on identifying and purifying neuronal nicotinic receptors which do not bind α-bungarotoxin. These were purified from chicken brain by immunoaffinity chromatography using a mAb to the main immunogenic region of receptors from Electrophorus (mAb 35).[29] We had previously provided evidence that mAb 35 bound to chicken brain neurons and to receptors on chicken ciliary ganglion neurons.[30-34] Antisera to receptors purified from chicken brain blocked the function of receptors in chicken ciliary ganglion neurons.[35] mAbs to these receptors gave us probes which, unlike mAb 35, bound to receptors from rat brains.[36] Receptors immunoisolated from chicken and rat brains were shown to have nanomolar affinity for nicotine.[36]

During the past year, studies have continued on nicotinic receptors from Torpedo electric organ and chicken, rat, and human brains. We have also been studying nicotinic receptors from a human neuronal cell line which appear to be of the muscle type.

Using synthetic peptides[37] corresponding to sequences of α subunits of receptors from Torpedo californica (Table 1), we were able to confirm the recent work of others[38,39] that α-bungarotoxin interacts with denatured α subunits at amino acids within the sequence α189-194 (Figure 1). Some or all of the amino acids which form the acetylcholine binding site are in this region, but high affinity for cholinergic ligands depends on the native conformation of the receptor molecule. Cysteines α192,193 appeared to be critical for binding of α-bungarotoxin, because treatment of the synthetic peptide α172-205 with the disulfide bond reducing agent dithiothreitol followed by the thiol alkylating reagent iodoacetamide prevented binding of α-bungarotoxin.[37]

Epitopes on α subunits of receptors from Torpedo californica recognized by antisera to native receptor and its denatured α subunits were located using $^{125}$I-labeled synthetic peptides as antigens in immunoprecipitation assays[37] (Table 1, Figure 2). Despite screening many small synthetic α subunit peptides for binding to mAbs directed at the main immunogenic region, we could not map this region more precisely.[37] Obviously, the conformation dependence of this region is a problem. A mAb which has high affinity for the main immunogenic region in the intact receptor may not bind detectably to any synthetic receptor peptide if the receptor amino acids critical for mAb binding are not adjacent in the primary sequence of the receptor subunit and are only found adjacent to one another after the polypeptide chain folds into its native conformation. A mAb with high affinity for the native main immunogenic region might bind with low affinity to synthetic receptor peptides if most or all of the receptor amino acids critical for mAb binding were adjacent in

the primary sequence of the receptor subunit; then the ability of the mAb to bind detectably to a synthetic receptor peptide might depend on the conformation assumed by the synthetic peptide and the assay conditions. Immune precipitation of $^{125}I$ peptides might be the least sensitive assay because binding depends on the affinity between a single peptide molecule and a single antibody site. Solid-phase assays using high concentrations of bound peptide might be more sensitive because the avidity of a bivalent antibody able to bind two peptides simultaneously would be much greater than the affinity of either monovalent interaction. If the particular solid phase used (nitrocellulose membrane, plastic microwell, etc.) happened to bind the peptide by amino acids that were critical to recognition by the mAb, then mAb binding would not be detected, even if a peptide containing the proper sequence were used. Thus it is difficult to be certain that a particular synthetic peptide does not contain part or all of the sequence(s) that form(s) the main immunogenic region in the native receptor. Another assay condition may be found which could detect specific low affinity binding of synthetic peptides to some mAbs to the main immunogenic region. It is clear that high affinity binding of these mAbs depends on the native conformation of the receptor and that none of the peptides tested in solution or on blots assumed this native conformation.

When cloned Torpedo electric organ acetylcholine receptor $\alpha$ subunits were expressed in yeast cells, it was found that they bound the main immunogenic region antibody mAb 35 and had moderate affinity for $\alpha$-bungarotoxin.[40,41] In these respects, they resemble the synthetic intermediate of mouse muscle acetylcholine receptor $\alpha$ subunits.[42] Therefore, it is especially interesting that while mAb 35 and $\alpha$-bungarotoxin were found to bind to the extracellular surface of the yeast membrane, as would be expected in the case of mature receptors, mAb 142 was also found to bind to the extracellular surface. mAb 142 binds to the cytoplasmic surface of mature receptors.[17] Thus, it may be that this system will allow us to study the transmembrane orientation of the polypeptide chain in the synthetic intermediate conformations of the subunits.

We are continuing our studies of the antigenic structure and transmembrane orientation of the subunit polypeptide chains of receptors from Torpedo electric organ.

The relative positions of the acetylcholine binding site and the main immunogenic region within the $\alpha$ subunit, and the relative positions of the subunits within the receptor were studied in collaboration with Nigel Unwin of Stanford University.[43] Previously, he showed that incubation of receptor-rich membrane vesicles from the electric organ of Torpedo marmorata at room temperature for weeks led to the formation of tubular crystalline arrays of receptor, and that optical diffraction studies of electron micrographs of these crystalline arrays gave a high resolution picture of the receptor showing rod-like subunits organized around the central cation channel.[44] We showed by purification that if he employed protease

inhibitors, the subunits were not proteolyzed during this prolonged incubation (Figure 3). αBungarotoxin and mAb 35 were used to locate α subunits. αBungarotoxin bound to the top of α subunits, whereas mAb 35 bound to the side. mAb 111 was used to localize β subunits to between the two α subunits. Wheat germ agglutinin (WGA) was shown to bind specifically to δ subunits (Figure 4). The organization of subunits around the cation channel was shown to be αβαγδ (Figure 5).

In collaboration with Nigel Unwin, we have also been attempting to make three-dimensional crystals of affinity-purified receptor from Torpedo electric organ suitable for x-ray crystallography.

Many of our studies during the past year have concerned neuronal nicotinic acetylcholine receptors. We first purified neuronal nicotinic receptors from chicken brains by immunoaffinity chromatography using mAb 35 to the main immunogenic region of receptors from the electric organ of Electrophorus electricus.[29] These purified receptors were then used as immunogen to generate a library of mAbs specific for neuronal nicotinic receptors (Table 2).[45]

Using mAbs to nicotinic receptors purified from chicken brain (Table 2), two subclasses of receptor were found to be present in equal amounts in adult chicken brains (Figure 6).[45] Both have nanomolar binding affinity for L-nicotine. These receptors are composed of two kinds of subunits. We termed these α and β in order of increasing molecular weights, according to the convention used for naming the subunits of receptor from muscle and electric organs. As will be described below, we later determined by affinity labeling that the β subunits form the acetylcholine binding sites.[29,35,36,46] This contrasts with the case of muscle-type acetylcholine receptors, where the lowest molecular weight, α, subunits form the acetylcholine binding sites.[47] To avoid conflicts in nomenclature, now that the ligand-binding subunits have been identified, we try to avoid referring to the subunits of neuronal nicotinic receptors as α and β, but instead usually refer to them as "structural subunits" and "acetylcholine-binding subunits," respectively. The two receptor subtypes present in chicken brain both have similar or identical structural (α) subunits of mw 49,000 (Figure 7). However, one has acetylcholine-binding (β) subunits of mw 59,000, whereas the other has β′ subunits of mw 75,000 (Figure 8). By using mAb 35, the β-type receptors can be identified; by using mAb 285, the β′-type receptors can be identified; and by using mAb 270, which binds to the structural subunits, both types of receptors can be identified. mAb 35 binds only to native receptor. mAb 210, like mAb 35, binds to the main immunogenic region of receptors from muscle. On western blots of receptors from chicken brain, mAb 210 binds to structural subunits of both receptor subtypes; but with native chicken brain nicotinic receptors, mAb 210, like mAb 35, binds only to the β-type receptors. These results are consistent with the idea that the structural subunits are identical in both subtypes of brain

nicotinic receptors, but that in the $\beta'$ subtype the large $\beta'$ acetylcholine-binding subunit obscures the epitope recognized by mAb 35 and mAb 210. Each chicken brain nicotinic receptor molecule contains more than one structural subunit and more than one acetylcholine-binding subunit (Figure 9).

Nicotinic receptors were identified in rat brains, using mAb 270 raised to structural subunits of nicotinic receptors from chicken brain.[48] Nicotinic receptors from rat brain have nanomolar affinity for L-nicotine.[36] mAb 270 bound to >90% of the high affinity nicotine binding components in detergent extracts of rat brain. Nicotinic receptors were immunoaffinity purified from rat brains using mAb 270.[48] Two kinds of subunits were found: structural ($\alpha$) subunits of mw 51,000, and acetylcholine-binding ($\beta$) subunits of mw 79,000 (Figure 10). mAb 286, specific for chicken brain nicotinic receptor acetylcholine-binding subunits,[45] cross-reacted with rat acetylcholine-binding subunits[48] (Figure 11). mAb 270, specific for chicken brain nicotinic receptor structural subunits,[45] cross-reacted with the structural subunits of receptors from rat brain,[48] showing that these subunits were homologous (Figure 11). There was very limited antigenic homology between nicotinic receptors of brain and muscle, and in general antisera and mAbs to receptors from brain did not cross-react with receptors from muscle. Weak cross-reaction of antisera to subunits of receptor from Torpedo electric organ was detected with denatured subunits of receptor from rat brain, but not with native receptor from rat brains (Figure 12). These results suggest that the most conserved epitopes shared by subunits of receptors from electric organ and brain are in hydrophobic sequences which are not accessible on the surface of native receptors.

Nicotinic receptors from both chicken and rat brains can be affinity labeled with 4-(N-maleimido)benzyltrimethylammonium iodide (MBTA) or bromoacetylcholine after treatment with reducing reagents.[29,35,36,46] This is similar to the effects of these reagents on nicotinic receptors from electric organ and muscle, and suggests that neuronal nicotinic receptor acetylcholine binding sites have analogues of cysteines $\alpha$192 and 193, the sites labeled by MBTA in receptors from Torpedo.[47] In neuronal nicotinic receptors from chicken and rat brain, however, the $\beta$ and $\beta'$ subunits are affinity labeled by [$^3$H]MBTA (Figures 13,14).[46] This surprising result suggests that structural features of the primordial nicotinic receptor subunit sorted differently between descendent subunits along the muscle lineage and the neuronal lineage of this gene family. In the muscle lineage, the $\alpha$ subunit retained the acetylcholine binding site which was lost by the higher molecular weight $\beta$, $\gamma$, and $\delta$ subunits. In the neuronal lineage, the $\alpha$ subunit retained some antigenic features shared by $\alpha$ subunits of receptors from muscle, but it is the higher molecular weight $\beta$ subunit which retains the acetylcholine binding site. The observation that the $\beta$ and $\beta'$ receptor subtypes have similar binding affinity for nicotine, even though there is a 16,000 dalton difference in apparent molecular weight between $\beta$ and $\beta'$ subunits, suggests that $\beta$ and

$\beta'$ subunits may have extensive sequence homology except for an additional domain present in $\beta'$.[47] The two subtypes of muscarinic receptors are observed to differ in this way.[4,5]

Subunit stoichiometry of nicotinic receptors from brains of chickens and rats was studied.[29,45,48] Figure 9 shows that each receptor molecule from chicken brain contains more than one structural subunit and more than one acetylcholine-binding subunit. Similar experiments with receptors from rat brain lead to the same conclusion. Table 3 shows that a subunit composition of $\alpha_3\beta_2$ or $\alpha_2\beta_2$ could account for the observed sedimentation behavior of receptors from rat brains. This is also true for receptors from chicken brains. The data are insufficiently precise to conclude more than that the subunit stoichiometry is $\alpha_{n=2-3}\beta_{n=2-3}$. The idea that the stoichiometry is $\alpha_3\beta_2$ is appealing because of the resemblance to the pentagonal subunit arrangement of receptors from Torpedo electric organ (Figure 5), where there are also three subunits which do not bind acetylcholine ($\beta$, $\gamma$, and $\delta$) and two subunits which do bind acetylcholine (two $\alpha$ subunits). However, the idea that the stoichiometry is $\alpha_2\beta_2$ is also appealing because $\alpha\beta\alpha\beta$ is the simplest and most symmetric way to organize at least two copies of two subunit types. Future experiments using other techniques will be required to establish the subunit stoichiometry of neuronal nicotinic receptors. This is a very difficult problem because it requires precise measurements on the very small amounts of receptor available.

mAb 270 identifies the functional acetylcholine receptor in the rat PC12 pheochromocytoma cell line (Figure 15).[49] This nicotinic receptor seems to be a homologue of the receptor in chick ciliary ganglion cells,[31-35] in that both receptors show extensive antigenic similarity to their brain counterparts, both are blocked by MBTA and bromoacetylcholine,[35,50] but both have only micromolar affinity for nicotine.[51,52] Both PC12 cells[8] and ciliary ganglion neurons[9] also contain $\alpha$-bungarotoxin binding components which are not receptors. mAb 270 binds to the structural subunits of receptors from brain.[48] Like mAb 35 on ciliary ganglion neurons,[35] it has no direct effect on receptor function, so, as with mAb 35,[29,36] a more indirect path had to be taken to show that the antibody bound to the receptor.[49] PC12 cells normally produce very little receptor, but treatment with $\beta$ nerve growth factor ($\beta$NGF) causes morphological differentiation and production of more receptor.[53] The amount of mAb 270 binding sites increased in proportion to the amount of functional receptor.[49] Incubation with mAb 270 plus anti-immunoglobulin decreased the amount of functional receptor. This strongly argues that mAb 270 binds the functional nicotinic receptor of PC12 cells. The observations that nicotinic receptors in PC12 cells have micromolar affinity for nicotine[52] whereas nicotinic receptors from rat brains have nanomolar affinity for nicotine[36] show that the acetylcholine-binding subunits of these two receptor subtypes must differ; but the observation that mAb 270, which is specific for structural subunits, binds to both subtypes suggests that these subtypes of nicotinic receptors from rats,

like the two subtypes of nicotinic receptor in chicken brains,[45] might share the same structural subunits.

Several groups are trying to identify cDNAs for subunits of neuronal nicotinic receptors by screening for hybridization at low stringency with cDNAs for subunits of nicotinic receptors from muscle.[54,55] Boulter and coworkers identified a putative nicotinic receptor acetylcholine-binding subunit cDNA termed λPCA48 in a library from PC12 cells.[54] We found that the mRNA in PC12 cells which hybridizes with this cDNA was not induced by βNGF.[49] In muscle, the amount of α subunit is regulated at the transcription level.[56] Thus, this could suggest that λPCA48 did not code for PC12 nicotinic receptor acetylcholine-binding subunits, but instead for a subunit of the α-bungarotoxin binding component whose amount remains constant after treatment with βNGF.[49] Alternatively, this could suggest that λPCA48 may code for the acetylcholine-binding subunit of the receptor in PC12 cells, but that the level of this receptor is not strongly regulated at the level of transcription of this subunit. λPCA48 does not code for subunits of the receptor we have purified from rat brain because the amino-terminal sequences of both subunits of receptor purified from rat brain are different from λPCA48 (unpublished).

We are currently using the λgt11 cloning system[57] with mAbs to neuronal nicotinic receptors to identify subunit cDNAs. Soon these studies should lead to an integration of results with those of laboratories seeking these cDNAs from the low stringency hybridization route.

The nicotinic receptors in rat brain were histochemically localized using $^{125}$I-labeled mAb 270 (Figures 16-20).[49,58] Figures 16 and 17 present an overview of the labeling pattern. The pattern was very similar to that previously reported by Clarke et al.[59] using [$^{3}$H]nicotine and very different from the distribution of α-bungarotoxin binding sites. We confirmed the localization of α-bungarotoxin binding sites reported by Clarke et al.[59] Figure 18 gives an example of the different locations of binding sites for mAb 270 and α-bungarotoxin. It is especially interesting that many nicotinic receptors were found in presynaptic locations, for example, on dorsal root ganglion cells, on central projections of retinal ganglion cells, and on the projection of cells of the medial habenular nucleus to the interpeduncular nucleus (Figures 17-19). Removal of an eye eliminated the otherwise intense labeling of the contralateral superior colliculus, clearly demonstrating that these receptors were transported along retinal ganglion cell axons to the superior colliculus (Figure 18). The observation that many brain nicotinic receptors are apparently in presynaptic locations is consistent with the idea that these receptors may be involved in modulating the release of other transmitters.

The α-bungarotoxin binding component from brain was first reported to consist of a single subunit[60] and later of several,[10] one of which had amino-terminal amino acid sequence homology with

subunits of receptors from muscle.[10] We affinity purified the α-bungarotoxin binding component from rat brain and found four subunits reminiscent of those in receptors from muscle (Figure 10).[48] This component did not cross-react with mAbs to neuronal nicotinic receptors.

mAbs to nicotinic receptors from rat brain permitted identification of a similar nicotinic receptor in extracts of human brains.[61] These nicotinic receptors did not cross-react with autoantibodies to muscle nicotinic receptors from myasthenia gravis patients. The autoantibodies also did not react with α-bungarotoxin binding components from human brain. The autoantibodies reacted very well with receptors from the human neuromedulloblastoma cell line TE671.

We found that cells of the human neuromedulloblastoma cell line TE671 contain functional nicotinic receptors which bind α-bungarotoxin and appear antigenically very similar to those of human muscle.[62] These receptors have been affinity purified in high yield, but their subunit composition is still uncertain due to partial proteolytic degradation (unpublished). These receptors have been found to exist both as monomers and dimers, a case previously only observed with receptors from Torpedo electric organ. We confirmed the observation of Siegel and Lukas[63] that nicotine increases the amount of receptor in these cells. Furthermore, we found that forskolin induces differentiation to a nerve-like morphology and terminates expression of receptor. Expression of muscle-type acetylcholine receptors in a neuroblastoma cell line could be an artefact of transformation. If this were the case, the observation of muscle-type acetylcholine receptors in these cells would have no neurobiological significance in vivo but, nonetheless, would provide for the first time a convenient cell line for studying human muscle acetylcholine receptor pharmacology and function, for purifying significant amounts of receptor, and for cloning receptor subunit cDNAs. Alternatively, the expression of muscle-type nicotinic receptors in these neuroblastoma cells may be neurobiologically significant. This cell line may derive from a developmental intermediate that expresses muscle-type receptors and would normally develop into a cell type that does not express this receptor. This would explain why muscle-type receptors are not found in mature human brains. A final possibility is that this neuroblastoma derives from a cell type which does express muscle-type receptors in mature brains, but this cell type is very infrequent so that the small amount of receptors it contains has escaped detection.

We are continuing our characterization of receptor from TE671 cells and plan to clone cDNAs for at least their α subunits.

In summary, we have achieved substantial success on two broad fronts. We have made substantial progress on the difficult problem of determining the detailed structure of nicotinic receptors of the muscle type. Also, we have made very rapid progress on neuronal nicotinic receptors, for the first time

purifying them, characterizing their structure, and immunohistochemically localizing them. Continued studies using both mAbs and cloned DNA technology should continue this progress during the coming year.

## Materials and Methods

### Synthetic Peptide Studies

Nicotinic acetylcholine receptors were purified from the electric organ of Torpedo californica by affinity chromatography on toxin agarose.[64] The $\alpha$ subunit was purified from receptor by preparative sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Briefly, the preparative gel (5mm) was lightly stained with Coomassie blue and destained. The band corresponding to the $\alpha$ subunit was excised and frozen. The gel containing the $\alpha$ subunit was sonicated in $H_2O$ at 4°C until the gel was dispersed. The suspension was centrifuged and filtered through a 0.22-$\mu$m filter to remove remaining gel particles. The $\alpha$ subunit preparation was then dialyzed versus $H_2O$ and lyophilized.

Peptides corresponding to various segments of the $\alpha$ subunit were synthesized by V. Sarin, J.L. Fox, H.L. Thanh, and J. Rivier using the Merrifield method, as previously described,[15-18,23] or were purchased from Bachem (Los Angeles, CA). These peptides are listed in Table 2. For radioiodination, several of the peptides contained an additional tyrosine at the $NH_2$ or COOH terminus. Peptides were labeled with $^{125}I$ by using chloramine-T to specific activities of $9 \times 10^{16}$ to $3 \times 10^{18}$ cpm/mol.[17,64]

Antisera were raised in rats against both native receptor and $\alpha$ subunits as previously described.[64] Antisera were assayed by incubation overnight with $^{125}I$-$\alpha$-bungarotoxin-labeled receptor (1nM) or $^{125}I$-labeled $\alpha$ subunit in 100$\mu$l of 10mM sodium phosphate (pH 7.5)/100mM NaCl/0.5% Triton X-100/10mM $NaN_3$ buffer. Immune complexes were precipitated with 100$\mu$l of goat anti-rat immunoglobulin, diluted with 1ml of buffer, and centrifuged. Precipitates were washed with 2 x 1mL of buffer and counted for radioactivity, as previously described.[64] The binding of antibodies to 10nM $^{125}I$ peptides was assayed similarly and has been described.[17]

A slot-blot apparatus was used to apply synthetic peptides to a Biodyne immunoaffinity membrane (Pall, East Hills, NY) in 16mM borate, pH 9.0, and 15mM NaCl. Remaining reactive membrane sites were quenched overnight with 5% Carnation dried milk and 50mM Tris, pH 7.5, with 0.01% anti-foam A (quench buffer), and the membrane was rinsed with five changes of buffer. $^{125}I$-$\alpha$-bungarotoxin (5nM) was applied overnight in the quench buffer containing 0.5% Triton X-100. The membrane was washed with buffer and autoradiographed on preflashed Kodak XAR5 film.

### Tubular Crystals of Receptor-Rich Membrane

Crystalline vesicles of tubular morphology (tubes) were prepared from freshly killed and dissected Torpedo marmorata (Marine Station, Arcachon, France), essentially as described[44] except for the addition of 1mM N-ethylmaleimide to the isolation buffer and substitution of 100mM sodium cacodylate, pH 6.8, and

protease inhibitors (0.3μg/ml leupeptin and 1μg/ml pepstatin) for Tris-HCl in the final solutions. In the best preparations, at least 10% of the receptor-rich vesicles developed into tubes after incubating at 17°C over a period of ±4 weeks.

Receptor was purified from these preparations and from the fresh electric organ by affinity chromatography on toxin-agarose.[64] The purified material was denatured in 2% SDS in the presence and absence of 2% β-mercaptoethanol, and subjected to SDS-PAGE. The samples on the gels were either stained with Coomassie blue or electrotransferred to nitrocellulose or diazophenylthioether (DPT) paper.[18]

WGA labeling of the subunits was by the method of Nomoto et al.[65] The nitrocellulose sheet was quenched by three (10-min) washes in 100mM NaCl, 10mM sodium phosphate, pH 7.5 (PBS), containing 0.1% Tween 20 (quench buffer). The nitrocellulose sheet was incubated (1 hour at room temperature) with WGA-biotin (5μg/ml) in the quench buffer using a volume of approximately 8ml/100cm$^2$ nitrocellulose, and then washed for three (10-min) periods in quench buffer. Avidin-peroxidase (5μg/ml) in quench buffer was added to the nitrocellulose sheet (volume ±8ml/100cm$^2$) and incubated for 1 hour at room temperature. The nitrocellulose was washed for 30 min with several changes of PBS. The peroxidase substrate was freshly prepared and consisted of (a) 30mg of α-chloro-napthol in 10ml of cold methanol and (b) 30μl of 30% $H_2O_2$ in 50ml cold PBS. Parts (a) and (b) were mixed and added immediately to the nitrocellulose sheet. Development was stopped by pouring off the substrate and rinsing the sheet in PBS. WGA binding was identified by the appearance of a dark-purple band.

Antibody labeling of receptors, following SDS-PAGE and electrotransfer to nitrocellulose (or DPT paper), was as described.[18] One milliliter of mAb 111 (10nM in quench buffer) was added to a strip of nitrocellulose (or DPT paper) and incubated for 2 hours. After a final washing, the paper strips were then autoradiographed for 6 hours on preflashed[66] Kodak XAR film.

Samples in 5-μl aliquots were applied to freshly glow-discharged carbon support grids, washed with ~1mg/ml cytochrome c and negatively stained with 2% sodium phosphotungstate, pH 7.2. The labeling reagents were reacted with the tubes by application to the grids after the cytochrome c wash at the following concentrations: 4μM for α-bungarotoxin, 1μM for Fab 35 and Fab 11, and 10μM (in the presence of 0.1mM $CaCl_2$) for WGA. Reaction times ranged between 10 and 30 minutes. Prolonged application of WGA caused disordering of the crystals, rendering them unsuitable for further analysis. Fab 111 was the only reagent used that bound to the cytoplasmic portion of the receptor, and hence to the inside of the tubes[18]; the accessibility of these sites may have been facilitated by the presence of holes at or near the extremities of many of the tubes.

The specimens were examined at 100 KV within 1-2 days of preparing the grids, using a Philips EM400 electron microscope equipped with a low dose kit. Micrographs were recorded at a magnification of 33,000X and a total dose of less than 10 electrons/$A^2$. The Kodak SO163 film was developed in undiluted D19 developer for 10 minutes. To minimize variability between images, defocus values were required to be in the range of 12,000-16,000A, estimated from the positions in the optical diffraction patterns of Thon rings.[67]

To identify the positions of the ligands in the crystal lattice, Fourier syntheses were conducted of the difference terms: $F_1(h,k) - F_n(h,k)$, where $F_1(h,k)$ and $F_n(h,k)$ are the averaged Fourier terms obtained for the ligand-bound and native structures, respectively. Data were scaled so that $\epsilon F_1(h,k) = \epsilon F_n(h,k)$; minor adjustments of the relative scales to account for the additional mass of the ligand did not affect the positions of the major difference peaks.

**Purification and Characterization of Chicken Brain Nicotinic Receptors**

Nicotinic receptors from chicken brain were purified by immunoaffinity chromatography using mAb 35 or mAb 270 coupled to Sepharose CL4B (at 10 or 8mg/ml, respectively), as previously described.[29,48] Purified receptor was radioiodinated essentially as previously described.[29] Briefly, receptor from 100 brains (obtained from Pel-Freez Biologicals, P.O. Box 68, Rogers, Arkansas 72757) was purified through two rounds of immunoaffinity chromatography, eluted from the second antibody column in a pH 3.0 buffer containing 0.05% Tween 20 detergent, and then concentrated and desalted to a final volume of approximately 100$\mu$l using a Centricon 30 (Amicon) microconcentrator. The purified receptor, 5-10pmol, was then radioiodinated using the lactoperoxidase-glucose oxidase method (BioRad) and kept at 4°C in 10mM sodium phosphate, pH 7.5, 100mM NaCl (PBS) containing 0.5% Triton X-100, 10mM sodium azide, and 10mg/ml $\beta$-lactoglobulin.

Detergent extracts of Torpedo, rat, bovine, and human brains, and of chick muscle were also prepared as previously described.[29] Human brain was obtained through the courtesy of Dr. Robert Terry.

Female Lewis rats (6-8 weeks old) received intramuscular injections of purified chicken brain receptor, both intact and SDS denatured, emulsified in 100-200$\mu$l of complete Freund's adjuvant (cfa). details of the immunization schedules are given in table 3. the rats were sacrificed and their spleen cells fused with the mouse myeloma cell line s194 15.xxo.bu, using 50% polyethylene glycol 4000 (merck) as previously described in detail.[68] culture supernatants were initially screened by solid-phase immunoassay using immobilized, affinity-purified receptor from chicken brain.[29] Hybridoma supernatants which were positive in this assay, and upon rescreening exhibited binding to

receptors labeled with [$^3$H]nicotine in detergent extracts of chicken brain (see below), and/or binding to subunits on western blots of purified receptor from chicken brain, were selected for recloning. Hybridoma cells were cloned directly in agarose[68] and then grown in bulk in Iscove's medium containing 1% fetal calf serum. Supernatants from mass cultures were concentrated to about 300ml using a Millipore Minitan concentrator and the immunoglobulin fraction isolated by precipitation with 18% sodium sulfate and then dialyzed against PBS containing 10mM $NaN_3$. Immunoglobulin class and subclass were determined by the Ouchterlony technique using anti-rat immunoglobulin subclass antisera (Miles). mAb 270 was purified as previously described[48] and mAb 285 was purified by HPLC on a hydroxylapatite column (BioRad).

Cell culture supernatants were screened by solid-phase immunoassay.[29] Briefly, affinity-purified receptor from chicken brain (10-100fmol quantitated in terms of $^{125}$I-mAb 35 binding sites) in 30$\mu$l of PBS was applied to Millipore Millititer 96-well nitrocellulose plates and incubated overnight at 4$^o$C. The plates were quenched for 30 minutes at room temperature with 50$\mu$l of PBS containing 1% bovine serum albumin and 0.2% Tween 20 (quench buffer), and then 100$\mu$l of culture supernatant was added, followed by incubation overnight at 4$^o$C. Plates were then washed twice with 300$\mu$l of quench buffer and incubated 2 hours at room temperature with 10nM $^{125}$I-goat anti-rat IgG (2-3x10$^{-18}$cpm/mol). After two additional washes, the nitrocellulose disks were punched out and bound radioactivity determined by gamma counting. Nonspecific binding was determined by incubation with control culture supernatant.

Antibody binding to receptor in crude detergent extracts of brain was determined as described previously.[36] Briefly, detergent extract (200-500$\mu$l) was shaken gently for 15 hours at 4$^o$C with 100$\mu$l of culture supernatant, diluted mAb, or serum, and 20-30$\mu$l of a 1:1 slurry of goat anti-rat IgG-Sepharose (8-12.5mg IgG/ml gel). After washing with 2 x 1ml of PBS containing 0.5% Triton X-100, the aliquots were incubated for 15 minutes at room temperature in 20nM [$^3$H]nicotine (DL-[N-methyl $^3$H]nicotine, specific activity 68.6Ci/mmol (obtained from New England Nuclear, Boston, MA), in the same buffer, and then rapidly washed at 4$^o$C with 4 x 1ml of ice cold PBS, 0.5% Triton X-100 by resuspending in the buffer and centrifuging for 20 seconds at 10,000xg in a microfuge. Bound protein was then eluted by incubating the gel for 15 minutes with 100$\mu$l of 2.5% SDS, 5% $\beta$-mercaptoethanol, and then sampling into 5ml of scintillant (5% Biosolve [Beckman], 4% Liquifluor [New England Nuclear] in toluene). Radioactivity was determined by scintillation counting. Specific binding was determined by subtraction of binding in the absence of antibody.

SDS-PAGE and western blotting of protein samples were performed as previously described,[29] with the exception that when probing western blots with antibodies all incubations were

carried out in PBS, 0.5% Triton X-100 buffer which contained 5% (w/v) Carnation milk powder rather than bovine serum albumin.

**Purification of Nicotinic Receptor and α-Bungarotoxin-Binding Protein From Rat Brain**

Rat brains were obtained from Pel-Freez Biologicals. Immunoaffinity purification with mAb 270 and radioiodination of the affinity-purified nicotinic receptor were essentially as previously described for receptor from chicken brain with mAb 35.[29]

The α-bungarotoxin binding protein was purified from detergent extracts from which receptor had previously been removed by immunoaffinity adsorption on mAb 270-Sepharose. Rat brain detergent extract, after incubation with the mAb 270-Sepharose, was passed through a 2-ml column of Naja naja siamensis toxin coupled to Sepharose CL-4B (0.5mg/ml)[64] at 20-30ml/hour flow rate at 4°C. The column was rapidly washed with 50ml PBS containing 0.5% Triton X-100, 50ml 1M sodium chloride, 10mM sodium phosphate, pH 7.5, containing 0.5% Triton X-100, and again with 50ml PBS, 0.5% Triton X-100. Bound protein was eluted by recirculating 1M carbachol in 10mM Na phosphate, pH 6.8, 0.1% Triton X-100 through the affinity column onto 0.5ml hydroxylapatite (BioRad Bio-Gel HTP). The hydroxylapatite was washed with 25ml of 10mM Na phosphate, pH 6.8, 0.1% Triton X-100. Bound protein was rapidly eluted at 22°C with 100mM Na phosphate, pH 6.8, 0.05% Triton X-100, and immediately dialyzed at 4°C against 10mM Na phosphate, pH 7.5, 0.05% Triton X-100.

[$^3$H]Nicotine binding sites in brain detergent extract were determined by gel filtration assay.[36] Antibody binding to [$^3$H]nicotine binding sites was investigated by indirect immobilization of antibodies upon goat anti-rat IgG-Sepharose, as previously described.[36]

α-Bungarotoxin binding sites were determined by DEAE assay[29] using α-bungarotoxin radioiodinated to specific activities of 3-4 x $10^{17}$cpm/mol.

**Affinity Labeling With [$^3$H]MBTA**

Detergent extracts of 70-90 chicken brains (250-350ml, 0.2-0.3nM [$^3$H]nicotine binding sites) or 30-40 rat brains (160-190ml, 0.3-0.5nM [$^3$H]nicotine binding sites) were recirculated for 15 hours at 4°C through 200μl of mAb 35-Sepharose or mAb 285-Sepharose (chicken brain extract) or mAb 270-Sepharose (rat brain extract). mAb 35-Sepharose (200μl) was also gently shaken for 15 hours at 4°C with 50pmol of affinity-purified Torpedo receptor, in 500μl final volume 10mM sodium phosphate, pH 7.5, 100mM NaCl (PBS) containing 0.5% Triton X-100. The affinity gels were divided into two aliquots in 1.5-ml microfuge tubes, washed with 4 x 1ml PBS, 0.5% Triton X-100 by pelleting and resuspending, and then reduced for 40 minutes with 200μl 1mM dithiothreitol (DTT) in the same buffer. A Torpedo receptor control tube was reduced in the presence of 1μM α-bungarotoxin. The aliquots were then rapidly washed with

1ml of PBS, 0.5% Triton X-100, and alkylated for 3 minutes with 1 x $10^{-5}$M [$^3$H]MBTA (specific activity 1900cpm/pmol, a gift from Dr. Mark McNamee) in the presence or absence of 1$\mu$M $\alpha$-bungarotoxin (for Torpedo receptor) or 2mM nicotine (for brain receptors) in 200$\mu$l final volume. The aliquots were rapidly washed with 4 x 1ml PBS containing 0.5% Triton X-100. Bound protein was then eluted from the antibody columns by 5-minute incubations with 2 x 150$\mu$l volumes of 50mM sodium citrate, pH 3.0, 0.1% Triton X-100, and rapidly neutralized with 1M Tris (hydroxymethyl) aminomethane. The eluates were shaken for 2 hours at 4°C with 50$\mu$l of goat anti-rat IgG coupled to Sepharose 4B (to absorb any IgG heavy and light chains which may have been nonspecifically labeled with the [$^3$H]MBTA and subsequently leaked from the column during the elution procedure). The eluate was then lyophilized, resuspended in SDS-PAGE sample buffer and resolved by SDS 10% PAGE, as previously described.[29] The acrylamide gels were then impregnated with Enhance (New England Nuclear), dried, and fluorographed for 4-21 days at -70°C using preflashed Kodak XAR film.

**Studies of PC12 Cells**

PC12J cells were maintained in culture in 175-cm$^2$ plastic flasks in Iscove's medium supplemented with a 10% fetal calf serum and 10% horse serum. For assays, cells were replated on polylysine-coated dishes.

Carbamylcholine-stimulated $^{86}Rb^+$ influx was determined by the method of Catterall.[69] Briefly, cell monolayers were washed with 3ml of assay buffer, with 1ml of assay buffer containing ouabain, and incubated for 30 seconds in ouabain containing buffer with 2$\mu$Ci/ml $^{86}Rb^+$ (from New England Nuclear) and 5mM carbamylcholine. Cell monolayers were washed three times with 3ml assay buffer, scraped off in 2ml of 0.5M NaOH, and radioactivity determined on a scintillation counter at 55% efficiency. Nonspecific uptake of $^{86}Rb^+$ was determined in the absence of carbamylcholine, and subsequently subtracted.

mAb 270 for labeling studies was prepared from rats immunized with immunoaffinity-purified chicken brain nicotinic receptor. It was purified by chromatography on DEAE Tris Acryl (Pharmacia) and radioiodinated by a modified chloramine-T method.[64] Labeling procedures were carried out in culture medium containing 5% horse serum. Cell cultures were washed with 3ml of medium and then incubated for 30 minutes at 37°C in 0.25ml of medium ± 1.0$\mu$M nonradioactive mAb 270. mAb 270 (0.25ml of 20nM $^{125}I$) in culture medium was then added and, after 45 minutes at 37°C, the cell monolayers were washed three times with 3ml of culture medium and scraped off in 2ml of 0.5M NaOH, and radioactivity was determined by gamma counting. Specific binding of $^{125}I$ mAb 270 was determined by subtraction of binding in the presence of 1$\mu$M nonradioactive mAb 270.

α-Bungarotoxin for labeling studies was radioiodinated to an initial specific activity of 3-4 x $10^{17}$cpm/mol.[64] Cultures were washed with 3ml of medium, and then incubated for 30 minutes at 37°C in 0.25 ml medium ± 5mM carbamylcholine. $^{125}$I-α-bungarotoxin (0.25ml of 20nM) was then added, and after a further 45-minute incubation, cell monolayers were washed three times and radioactivity was determined by gamma counting. Specific binding of $^{125}$I-α-bungarotoxin was determined by subtraction of the binding in 5mM carbamylcholine.

**Immunohistochemistry**

Animals were decapitated and the brains removed and frozen with liquid nitrogen. Cryostat sections (20μm) were thaw-mounted onto slides and desiccated at 0-4°C under vacuum overnight. The sections were incubated overnight at 4°C with 4nM $^{125}$I-mAb 270 (specific activity 2-4 x $10^{18}$cpm/mol) in 100mM NaCl, 10mM sodium phosphate buffer (pH 7.5), 10mM $NaN_3$, 10% normal rat serum, and 5% Carnation dried milk. The slides were then transferred to Coplin jars and rinsed five times over 30 minutes with 100mM NaCl, 10mM sodium phosphate buffer (pH 7.5), and 10mM $NaN_3$ at room temperature. They were again rinsed in three changes of buffer over 3 hours on a rocking platform at 4°C, dried at 37°C, mounted on cardboard, overlaid with an 8 x 10-inch sheet of Kodak XAR5 film in a cassette, and autoradiographed at room temperature for 12-36 hours. Virtually no labeling was observed when the sections were coincubated in 400nM cold mAb 270. Adjacent Nicsl-stained sections were used to identify labeled structures.

**Figure 1.** Binding of $^{125}$I-α-bungarotoxin (α-Bgt) to synthetic peptides from Torpedo californica acetylcholine receptor α subunits. Aliquots (25μl) of 1 x $10^{-7}$M synthetic peptide in 16mM borate buffer, pH 9.0, were applied to a Biodyne immunoaffinity membrane via a slot-blot apparatus. Remaining reactive sites were quenched overnight with 5% Carnation dried milk in Tris buffer (see Materials and Methods) and washed with five changes of 0.5% Triton X-100 in phosphate-buffered saline (PBS) + $NaN_3$. $^{125}$I-α-Bgt (5 x $10^{-9}$M) was applied in the Carnation milk quench buffer plus or minus 2 x $10^{-7}$M unlabeled α-Bgt. Antiserum to α subunits followed by $^{125}$I anti-antibody was used to demonstrate that comparable amounts of all peptides were bound to the membrane. A 25-μl aliquot of 2.8 x $10^{-6}$M α185-199 was required to bind α-Bgt, as shown here. Application of α194-215, α172-189, or α127-143 at this same high molar concentration did not result in specific binding of $^{125}$Iα-Bgt. Note that excess unlabeled α-Bgt inhibits all binding of $^{125}$I-labeled α-Bgt to α172-205 and α185-199. Note also that antisera to α subunits do not detect α185-199 bound to the filter. This may be because this short sequence does not contain an epitope or because amino acids critical to the epitope are involved in binding to the filter. Reproduced from reference 37.

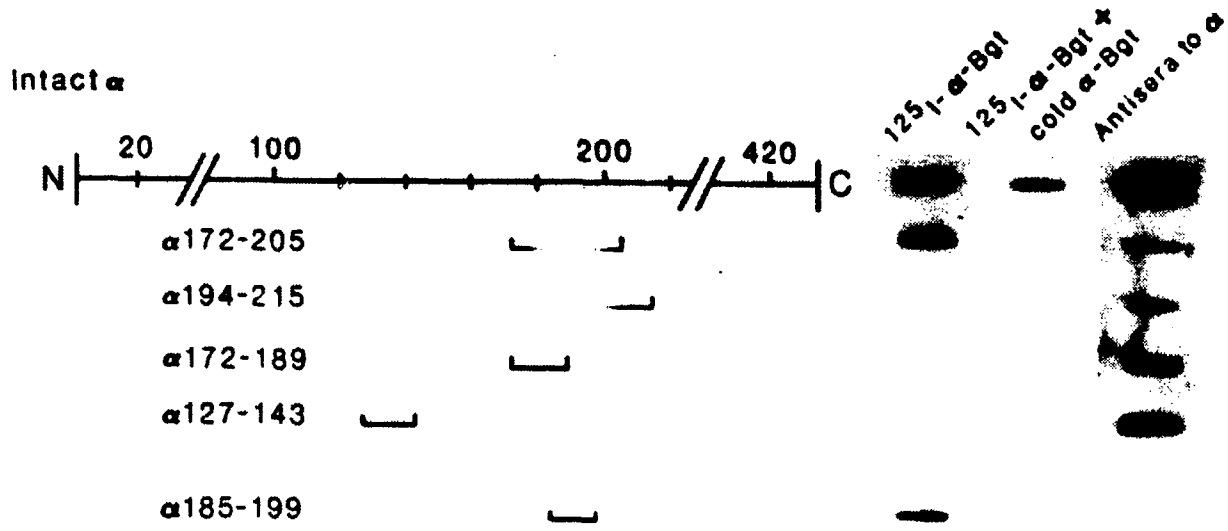


**Figure 2.** Reaction of synthetic peptides with antisera to native Torpedo californica electric organ acetylcholine receptor and to its denatured $\alpha$ subunits. Synthetic peptides corresponding to segments of the $\alpha$ subunit sequence, indicated by the bars, were labeled with $^{125}I$ as described in Materials and Methods. Antisera (5-10$\mu$l) were assayed by reacting with $^{125}I$-peptide (10nM) in 100$\mu$l of 10mM sodium phosphate buffer (pH 7.5), 100$\mu$M NaCl, 0.5% Triton X-100, and 10mM $NaN_3$. The immune complexes were precipitated with 100$\mu$l of goat anti-rat immunoglobulin. Solid black bars below the base line indicate that antisera did not react with these peptides. Panel A shows the reaction of antisera to native receptor with the synthetic peptides. Panel B shows the reaction of antisera to SDS-denatured $\alpha$ subunits. Insert C shows a magnified view of the overlap which occurred with antisera to $\alpha$ subunits versus [Tyr-83]$\alpha$66-83 and [Gly 89,Tyr 90]$\alpha$73-90 and $\alpha$78-93. Reproduced from reference 37.

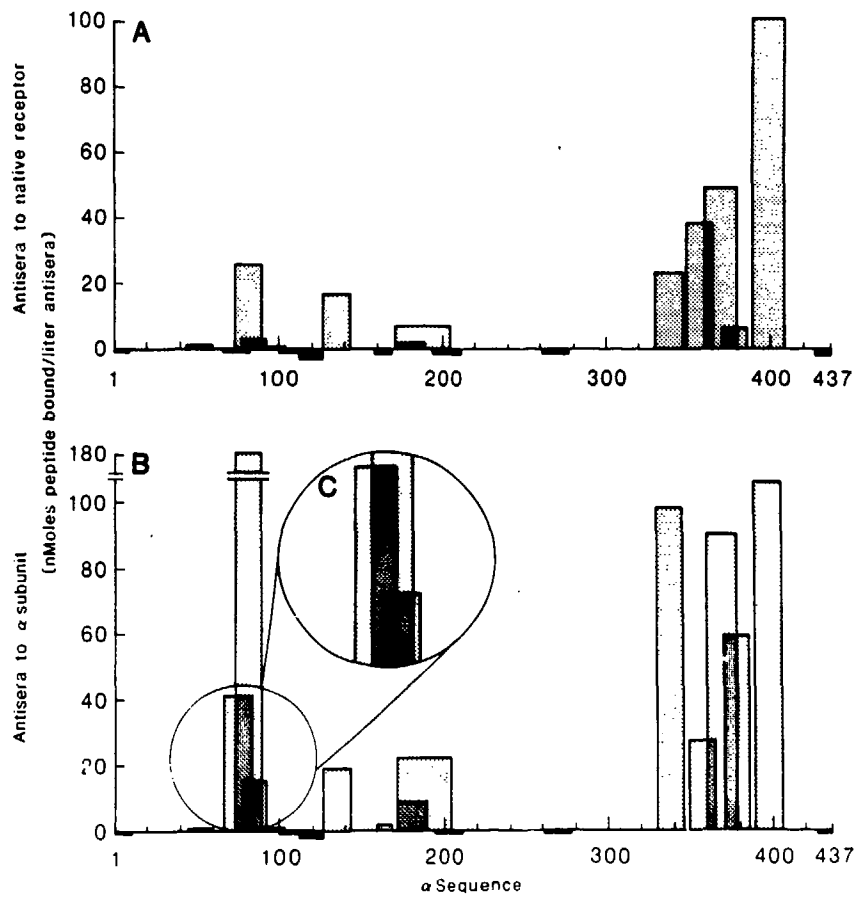


**Figure 3.** Structural integrity of receptors in tubular crystalline arrays. Receptor was affinity purified from fresh Torpedo marmorata electric organ or from receptor-rich membrane vesicles which had been aged and formed tubular crystalline arrays in the presence of protease inhibitors,[43] as described in Materials and Methods. Clearly, no significant proteolysis has occurred, despite the prolonged incubation. Reproduced from reference 43.

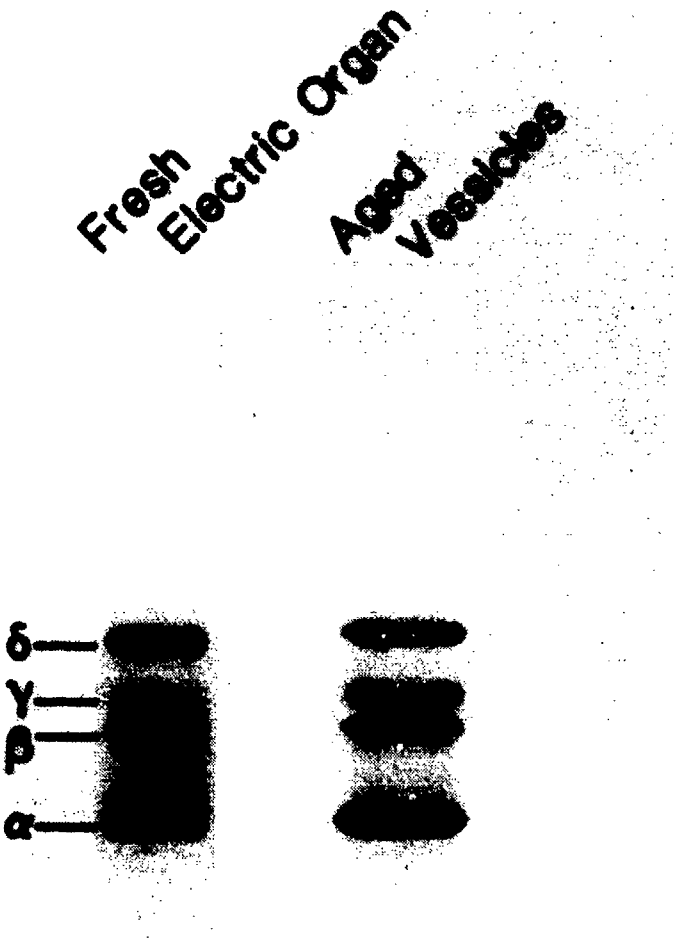


**Figure 4.** Biochemical characterization of purified Torpedo marmorata electric organ acetylcholine receptor and labeling reagents. Receptors purified from fresh electric organ (upper panel) or from aged vesicles (lower panel) were subjected to SDS-PAGE. The samples were stained with Coomassie blue (lanes 1 and 2), or electrotransferred to nitrocellulose and probed with mAb 111 (lane 3) and WGA (lanes 4 and 5), as described in Materials and Methods. Ten micrograms of receptor was electrophoresed on lanes 1 and 2 and 1μg on lanes 3, 4, and 5. The samples in lanes 1, 3, and 5 were reduced with 2% β-mercaptoethanol prior to electrophoresis. The slight apparent cross-reaction of mAb 111 with α subunits of the incubated vesicles is most likely due to the presence of small amounts of a proteolytic fragment of the β subunit, the size of the α subunit. Reproduced from reference 43.

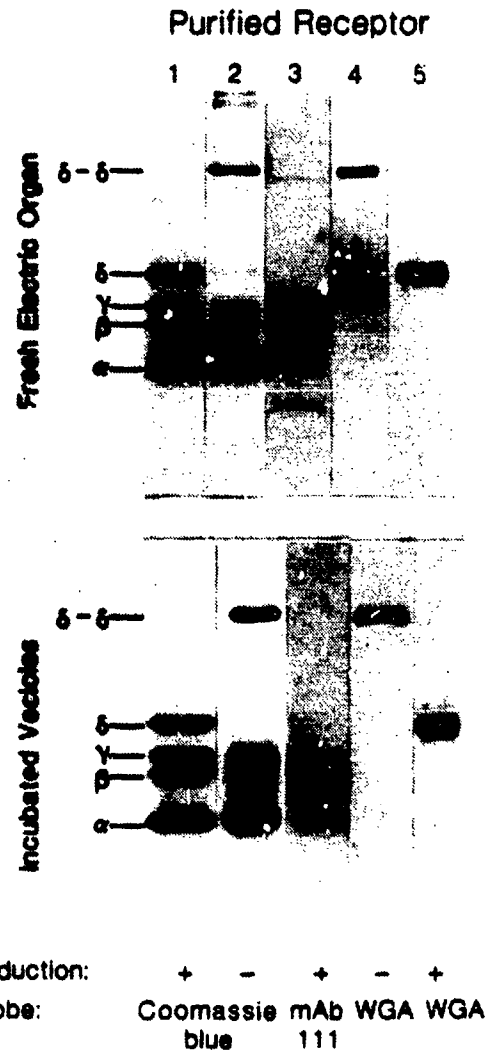


**Figure 5.** Identification of binding sites of $\alpha$-Bgt, Fab 35, WGA, and Fab 111 on receptors from Torpedo marmorata electric organ, as viewed from the synaptic side of the membrane. The Fourier difference maps (top) show positive (continuous contours) and negative (dotted contours) peaks, corresponding, respectively, to exclusion of the stain (i.e. presence of ligand) and accumulation of the stain; the zero contour has been omitted. The statistical difference maps (middle) show contours of increasing "t" values (see Materials and Methods); the outermost contours enclose regions where the probability that the differences are real and not due to chance is >99.9%; stars over the highest "t" values identify the ligand binding sites. The projection maps (bottom) show the positions of the binding sites with respect to individual receptors in the crystal lattice; the sites associated with central receptor are emphasized; the dyad symbol ( ◊ ) relates the pair of receptors tentatively identified previously as the $\delta$ subunit-linked dimer. Reproduced from reference 43.

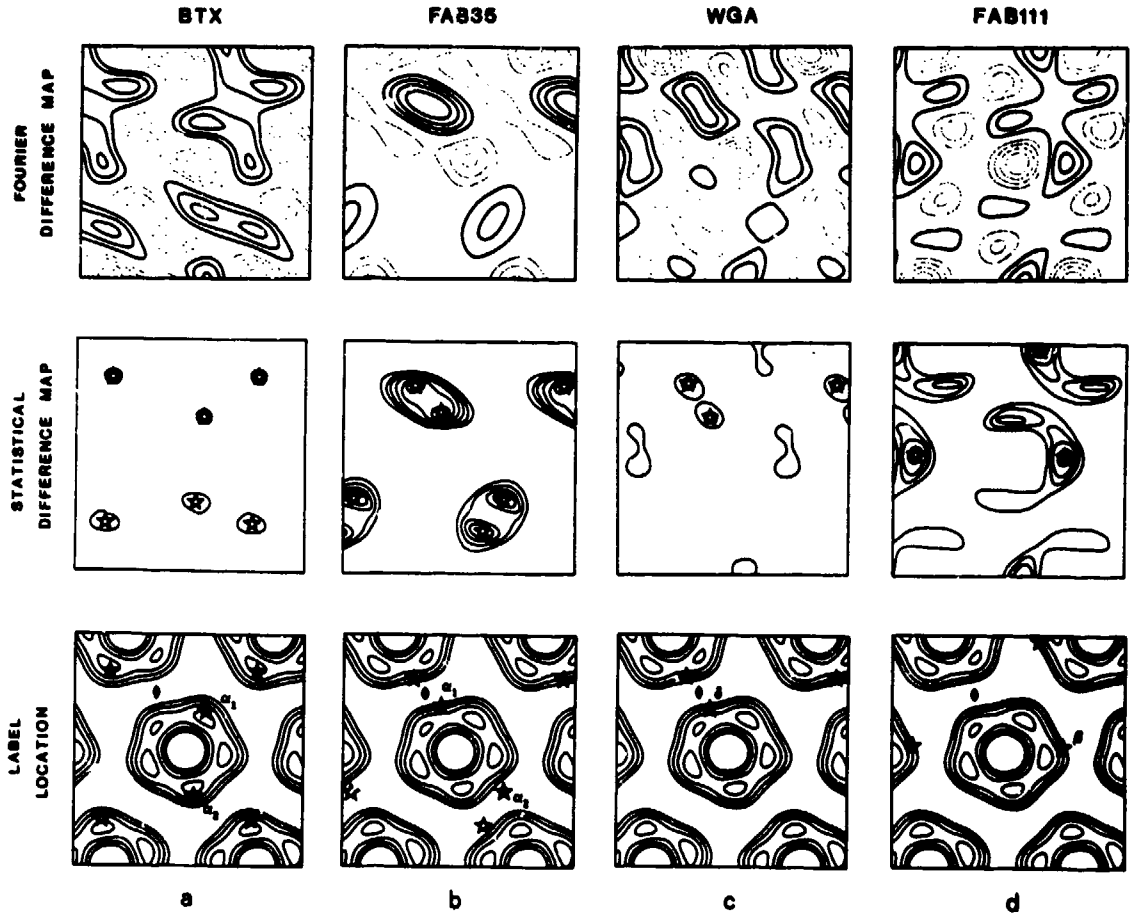


**Figure 6.** Nicotinic receptors in adult chicken brains are about one-half of the $\alpha\beta$ subtype and one-half of the $\alpha\beta'$ subtype. This was determined by depletion of (DL)[$^3$H]nicotine binding sites from detergent extracts of adult chicken brain by mAb 35 ( O ), mAb 285 ( ▲ ), and a combination of mAbs 35 and 285 ( ■ ). Aliquots (300$\mu$l) of chicken brain detergent extract (0.20nM [$^3$H]nicotine binding sites) were gently shaken for 15 hours at 4$^o$C with 20$\mu$l of goat anti-rat IgG-Sepharose and increasing amounts of mAbs. The Sepharose was pelleted and the supernatant assayed for [$^3$H]nicotine binding sites by filtration assay. Triplicate aliquots of 100$\mu$l were incubated for 1 hour at 4$^o$C with 20nM [$^3$H]nicotine and then diluted with 4ml of ice-cold 50mM Tris, pH 7.4, and filtered through Whatman GF/B filters presoaked in 0.3% polyethyleneimine. The filters were washed with 3 x 4ml of the same buffer and bound radioactivity was determined by scintillation counting. Nonspecific binding was determined by incubation in the presence of 1mM nonradioactive nicotine, and has been subtracted. The total (DL)[$^3$H]nicotine binding sites (considered 100%) was determined by incubation with goat anti-rat IgG-Sepharose alone, and all values expressed relative to this. Reproduced from reference 45.

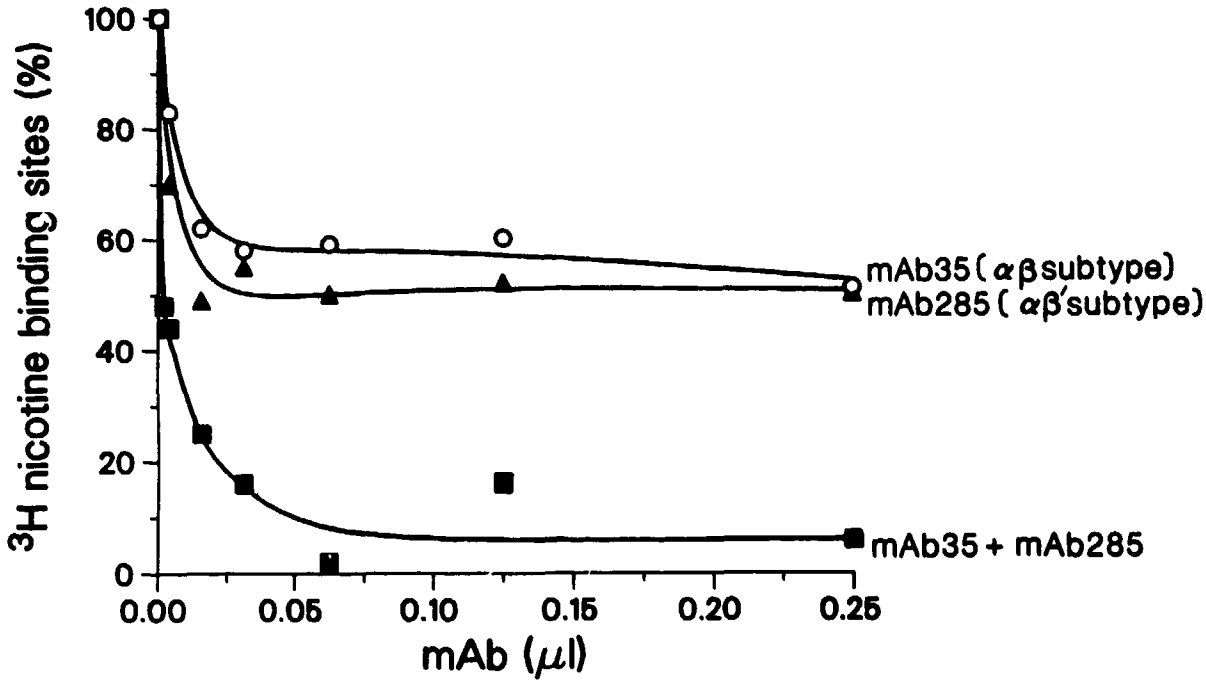


**Figure 7.** The structural subunits of both nicotinic receptor subtypes present in chicken brains are similar or identical. The nearly identical peptide maps obtained from the structural subunits of both receptor subtypes are shown. Receptor was immunoaffinity purified from chicken brains through two rounds of affinity chromatography on mAb 270-Sepharose and radioiodination. Then the two receptor subtypes were isolated using mAb 35-Sepharose and mAb 285-Sepharose, as described in Materials and Methods. The receptor polypeptides were resolved by 10% PAGE in SDS and the subunits located by autoradiography of the wet gel. The polypeptides were eluted from gel slices as described previously[29] and their purity checked by re-electrophoresis. Digestion of isolated structural subunits with Staphylococcus V8 protease and resolution of the fragments by 15% PAGE in SDS have been previously described.[29] The peptide maps were detected by autoradiography. Lanes 1-5 show mAb 35-purified receptor structural subunits. Lanes 6-10 show mAb 285-purified receptor structural subunits. Lanes 1 and 10 used 0.008μg Staphylococcus V8 protease; lanes 2 and 9 used 0.04μg protease; lanes 3 and 8 used 0.2μg protease; lanes 4 and 7 used 1.0μg protease; and lanes 5 and 6 used 5.0μg protease. Reproduced from reference 45.

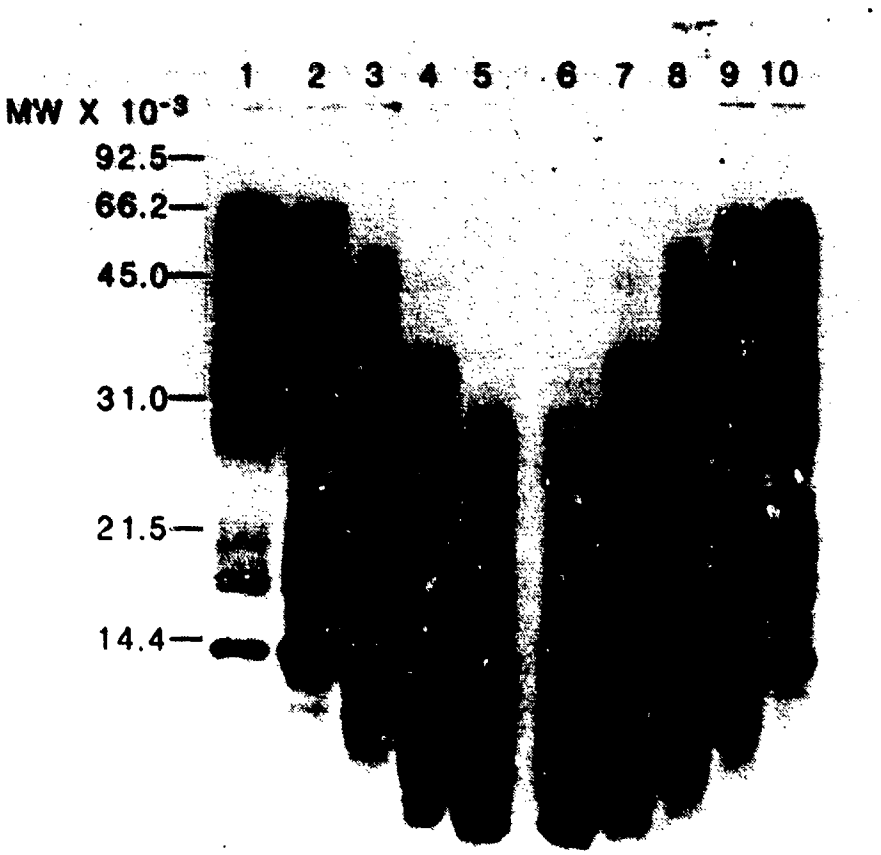
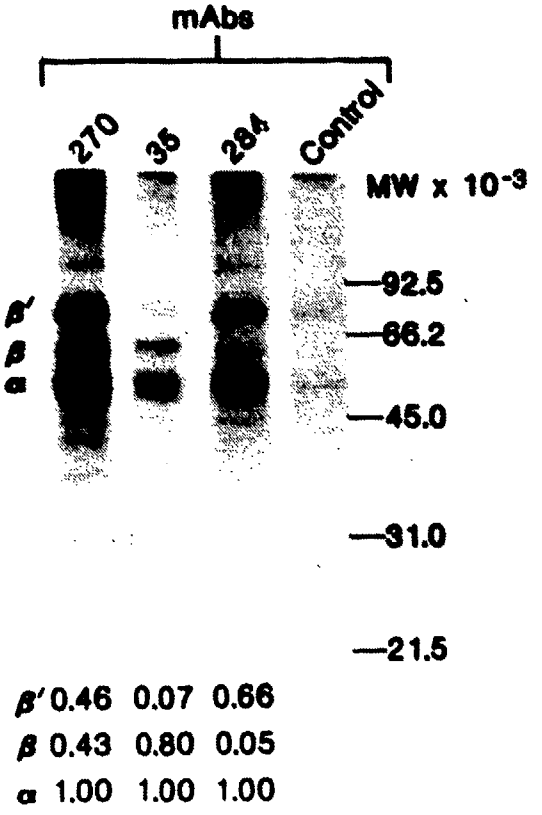


**Figure 8.** Resolution of nicotinic receptor subtypes from chicken brains by immune precipitation with mAbs. Both receptor subtypes were simultaneously affinity purified from chicken brains using mAb 270-Sepharose. The purified receptor preparation containing both subtypes was radioiodinated. Aliquots of 3.5 x $10^6$cpm of the receptors were incubated with mAb 270 to bind both receptor subtypes, with mAb 35 to bind the $\alpha\beta$ subtype, and with mAb 284 to bind the $\alpha\beta'$ subtype, or with normal rat serum (2$\mu$l) as a control and gently shaken for 15 hours at 4°C in 100$\mu$l of PBS, 0.5% Triton X-100 containing 5% (w/v) nonfat milk powder with 20$\mu$l of goat anti-rat IgG-Sepharose to precipitate the immune complexes. The immune precipitates were washed with 4 x 1ml of PBS, 0.5% Triton X-100 by pelleting in a microfuge and resuspending. Then bound protein was eluted with 60$\mu$l of 125mM Tris-HCl, pH 6.8, containing 2.3% (w/v) SDS, 10% (v/v) glycerol, 0.005% (w/v) bromophenol blue. Samples were made 5% (v/v) in $\beta$-mercapto-ethanol. The subunits in the receptor subtypes bound by each mAb were resolved by electrophoresis on 10% acrylamide gels in SDS; the gel was dried and autoradiographed. In order to estimate the subunit ratios in each receptor subtype, the band intensities were quantitated on a scanning densitometer (Hoeffer Scientific Instruments) and the relative intensity of each subunit was expressed as a ratio shown beneath each lane. Reproduced from reference 45.



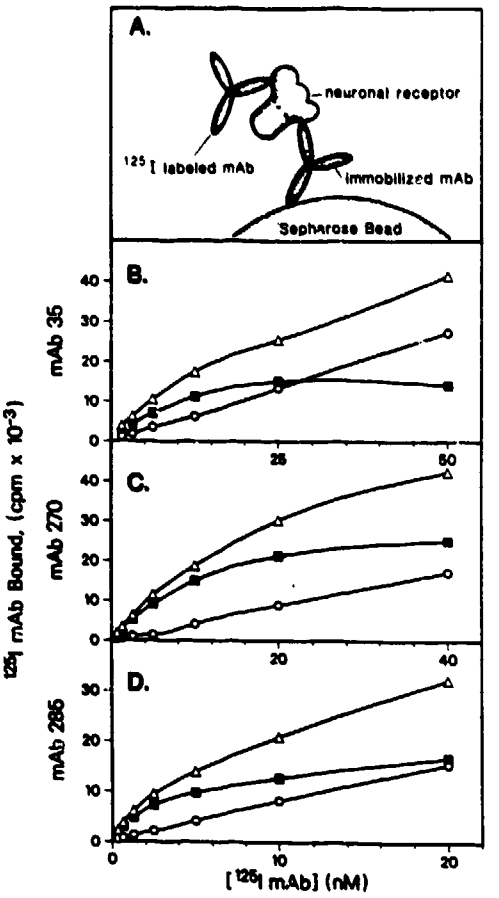



**Figure 9.** More than one copy of the structural subunit and more than one copy of the acetylcholine-binding subunit are present in each chicken neuronal nicotinic receptor macromolecule. (A) Diagrammatic representation of binding assay showing that binding of an $^{125}$I-labeled mAb to a receptor immobilized by another mAb of the same specificity indicates that at least two copies of the subunit type recognized by this mAb must be present in the receptor. (B) Binding of $^{125}$I-mAb 35 (specific for the $\beta$-type acetylcholine-binding subunit) to receptor immobilized upon mAb 35-Sepharose. (C) Binding of $^{125}$I-mAb 270 (specific for the structural subunit type present in both receptor subtypes) to receptor immobilized upon mAb 270-Sepharose. (D) Binding of $^{125}$I-mAb 285 (specific for the $\beta'$-type acetylcholine-binding subunit) to receptor immobilized upon mAb 285-Sepharose. Chicken brain detergent extract (200$\mu$l) was gently shaken for 15 hours at 4°C with 15$\mu$l of a 1:1 slurry of mAb 35-Sepharose, mAb 270-Sepharose, and mAb 285-Sepharose. The aliquots were washed with 1ml PBS, 0.5% Triton X-100 and then incubated for 1 hour at 22°C in 100$\mu$l PBS, 0.5% Triton X-100 containing increasing concentrations of $^{125}$I-labeled mAb 35 (2.2 x $10^{18}$cpm/mol), mAb 270 (0.94 x $10^{18}$cpm/mol) and mAb 285 (3.9 x $10^{18}$cpm/mol), radiolabeled by a modified chloramine-T method. Parallel incubations were carried out in which aliquots were preincubated for 30 minutes with excess nonradioactive mAb (final concentrations: 6$\mu$M mAb 35, 0.25$\mu$M mAb 270, and 0.28$\mu$M mAb 285 before addition of $^{125}$I-labeled mAb). The aliquots were washed with 4 x 1ml PBS and 0.5% Triton X-100 by pelleting in a microfuge and resuspending, and bound radioactivity was determined by gamma counting. Data points are the mean of triplicate incubations. Specific binding ( ■ ) is the difference between binding of $^{125}$I-mAb in the absence of competing nonradioactive mAb ( Δ ) and the binding in the presence of competing nonradioactive mAb ( o ). Reproduced from reference 45.

**Figure 10.** Analysis of affinity-purified rat brain nicotinic receptor and α-Bgt-binding protein by SDS-PAGE. (A) Lanes 1-4 were stained with silver: Lane 1, 0.5μg Torpedo receptor; Lane 2, 3μg rat brain extract; Lane3, ~0.35pmol rat brain receptor; Lane4, ~0.2pmol rat brain α-Bgt-binding protein; (B) $^{125}$I-labeled affinity-purified rat brain nicotinic receptor, detected by autoradiography using Kodak XAR film and Cronex intensifying screens. Positions of the molecular weight standards (low molecular weight standards, BioRad) were resolved on the same gel and are indicated on the left side of the figure. Reproduced from reference 48.

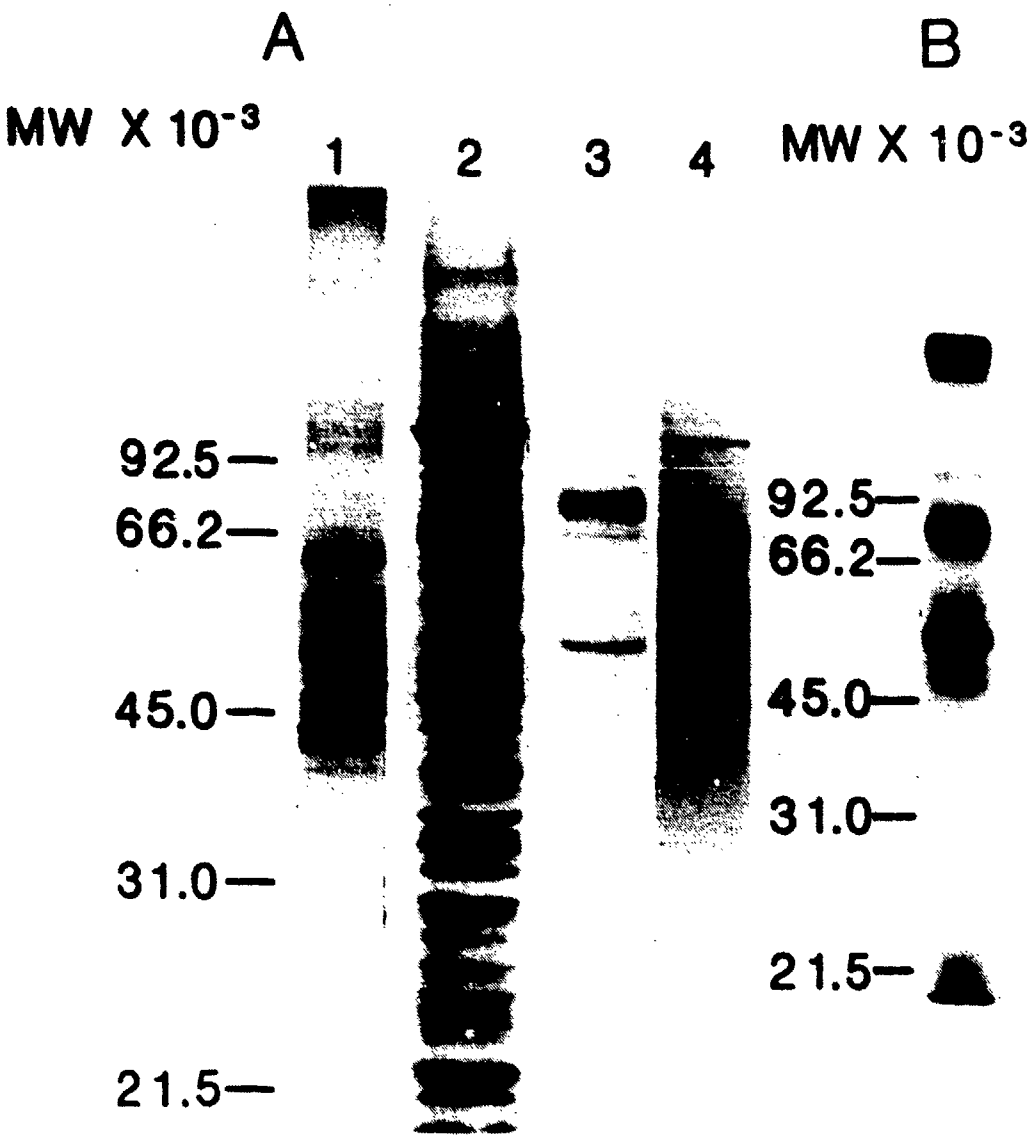


**Figure 11.** Western blots of nicotinic acetylcholine receptors from the brains of chickens and rats. Receptors (approximately 0.5-2.0 pmol in each lane), immunoaffinity purified on mAb 270-Sepharose, were resolved by SDS-PAGE transferred to diazophenylthioether paper and subsequently probed with antibody. Lanes 1 and 3, chicken brain receptor. Lanes 2, 4, 5, 6, rat brain receptor. Lanes 1 and 2 were probed with 50nM mAb 270, and Lanes 3 and 4 were probed with 20nM mAb 286. Lanes 5 and 6 were probed with a 1/500 dilution of antiserum to chicken brain receptor and normal rat serum, respectively. Bound antibody was localized with 0.4nM $^{125}$I-goat anti-rat IgG, and subsequent autoradiography. Reproduced from reference 48.

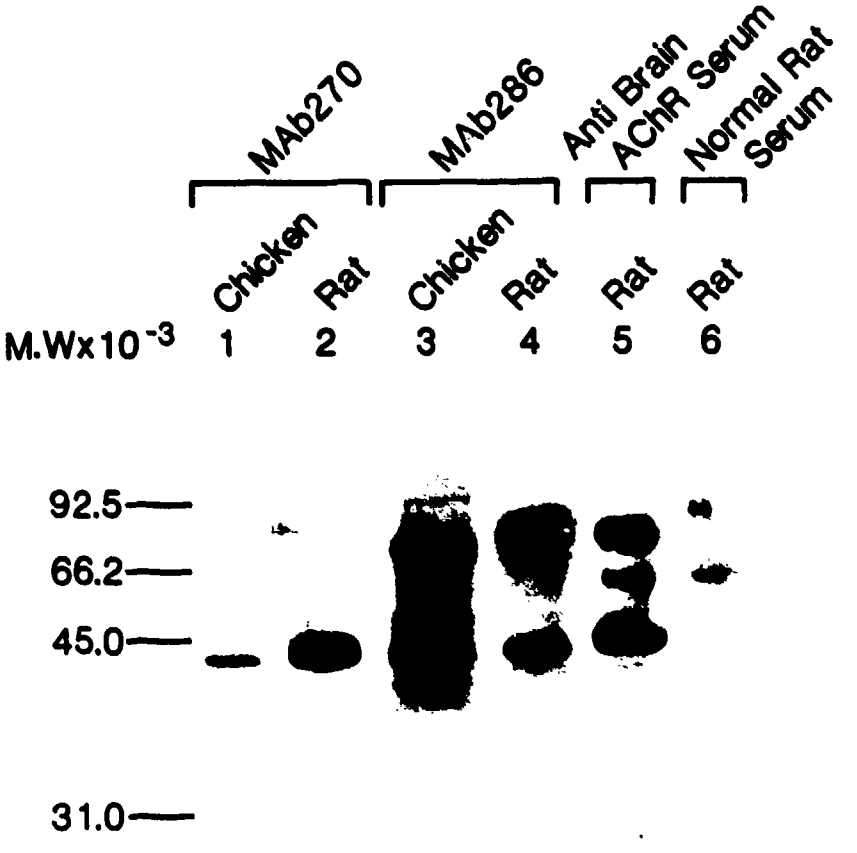


**Figure 12.** Binding to nicotinic receptors from rat brain of antibodies raised against nicotinic receptors from electric organs and chicken brain. (A) Binding to native receptor. Rat brain detergent extract (200µl, 0.44 nM [$^3$H]nicotine binding sites) was gently shaken for 15 hours at 4°C with 20µl of goat anti-rat IgG-Sepharose (10mg/ml gel) and 5µl of antiserum to chicken brain receptor (titer 32µM), or antisera to subunits of receptors from Torpedo electric organ (titers ~22µM), or mAb 270 (titer 5µM), or mAb 35 (titer 490µM), or normal rat serum. [$^3$H]Nicotine binding to each aliquot was then determined. (B) [$^{125}$I] 51,000 $M_r$ subunit, 13,000cpm, and (C) [$^{125}$I] 79,000 $M_r$ subunit, 11,800cpm, were incubated for 15 hours at 4°C with 5µl of antibody in a final volume of 100µl of PBS, 0.5% Triton X-100. The antigen-antibody complexes were precipitated with goat anti-rat IgG, pelleted and washed twice with PBS, 0.5% Triton, and radioactivity was quantitated by gamma counting. 1, normal rat serum; 2, antiserum to chicken brain nicotinic receptor; 3, antiserum to Torpedo electric organ nicotinic receptor $\alpha$ subunits; 4, antiserum to Torpedo $\beta$ subunits; 5, antiserum to Torpedo $\gamma$ subunits; 6, antiserum to Torpedo $\delta$ subunits; 7, mAb 35; 8, mAb 270. Reproduced from reference 48.

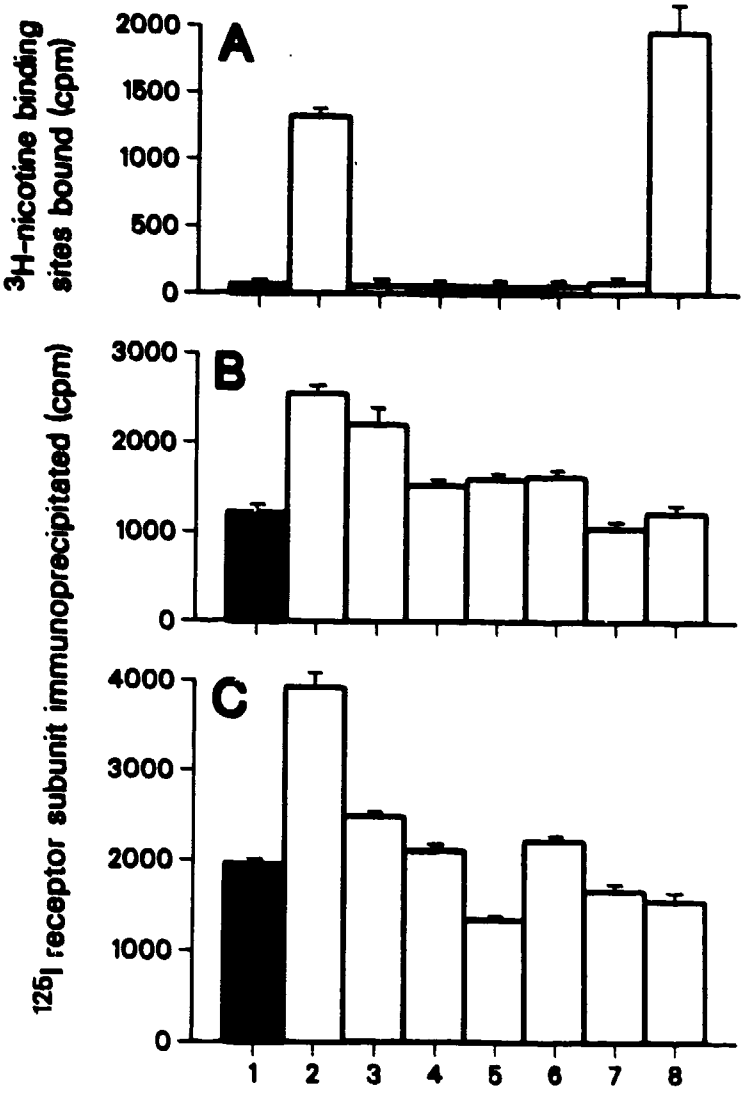


**Figure 13.** Fluorogram of SDS-PAGE of nicotinic receptors from Torpedo electric organ, chicken brain, and rat brain labeled with [$^3$H]MBTA. Labeling procedures and SDS-PAGE were carried out as described in Materials and Methods. Apparent molecular weights were determined by resolving molecular weight standards (BioRad) on the same gel and staining for protein with Coomassie blue. By specific affinity labeling, the higher molecular weight $\beta$ or $\beta'$ subunits of the neuronal receptors are shown to form the acetylcholine binding site which, in receptor from electric organ, is formed from the lowest molecular weight $\alpha$ subunit. Reproduced from reference 46.

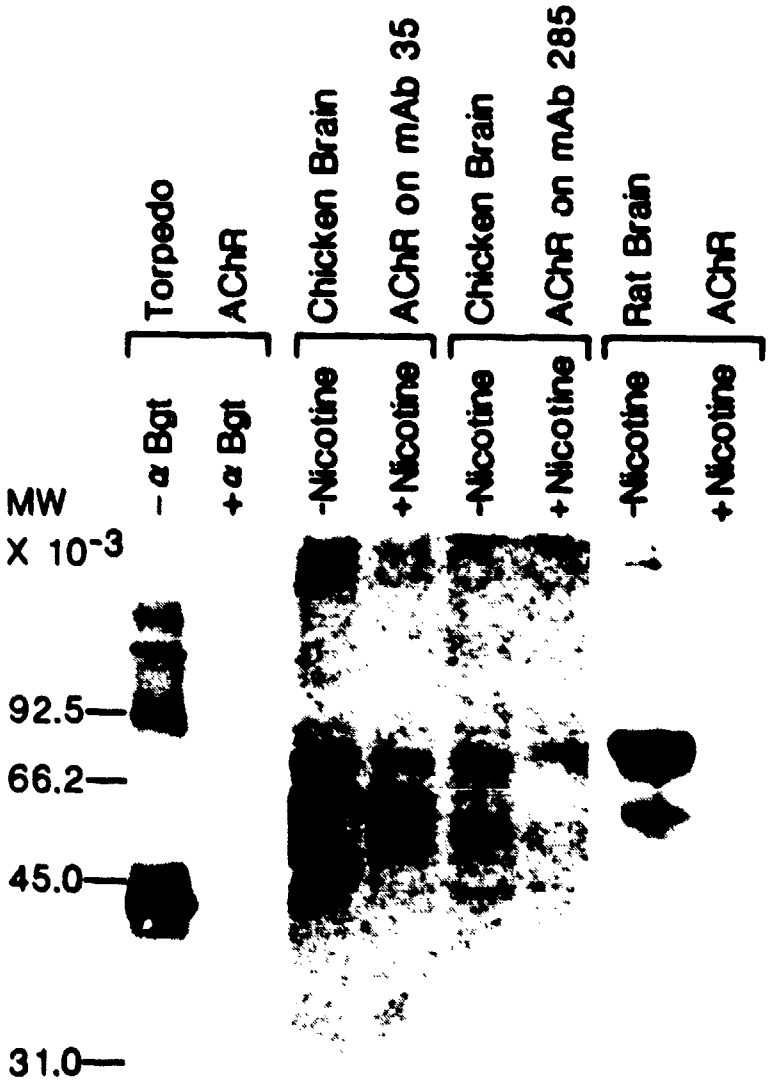


**Figure 14.** Comparison of the subunit structure of nicotinic receptors immunoaffinity purified from brains of chickens and rats. These are autoradiographs of $^{125}$I-labeled receptors resolved into subunits by PAGE in SDS. The data on receptors from chicken brain appeared as part of Figure 3 in reference 45, and the data on receptors from rat brain appeared as part of Figure 1 in reference 48.

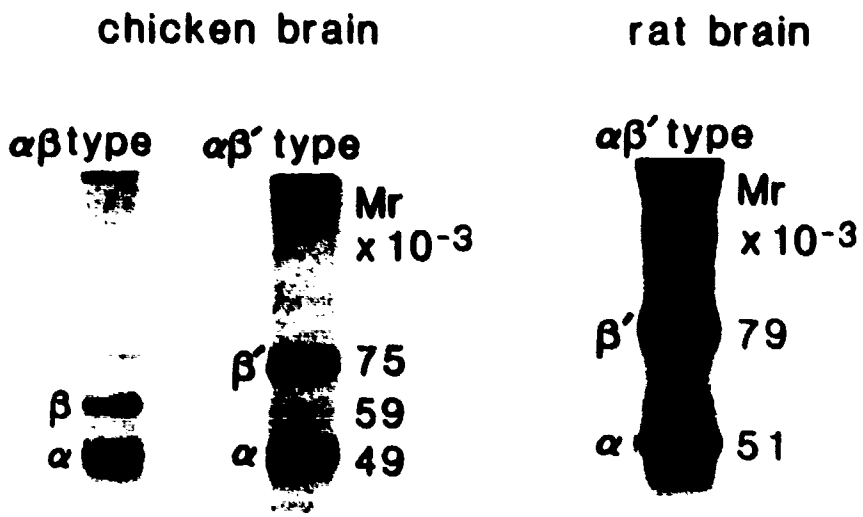


**Figure 15.** mAb 270 binds to a functional nicotinic receptor on PC12 cells. (A) $\beta$ nerve growth factor ($\beta$NGF) induces carbamylcholine-stimulated $^{86}Rb^+$ influx and mAb 270 binding sites, but not $\alpha$-Bgt binding sites in PC12 cells. PC12 cells were plated out on 35-mm dishes and on day 0, medium containing $\beta$NGF (50ng/ml) was added. The carbamylcholine-stimulated $^{86}Rb^+$ influx ( ● ), $^{125}I$-mAb 270 binding sites ( Δ ), and $^{125}I$-$\alpha$-Bgt binding sites ( ■ ) were measured on days 0, 1, 2, and 3. Data are shown as the mean ± standard deviation of triplicate cultures. (B) Modulation of carbamylcholine-stimulated $^{86}Rb^+$ influx by mAb 270. Cell monolayers were cultured for 20 hours in 1ml medium containing 50ng/ml $\beta$NGF. mAb 270 or control mAb 164 (which has the same IgG subclass as mAb 270, IgG2a, but binds only to nicotinic acetylcholine receptors from Torpedo) was then added to a final concentration of 100nM, and after 5 hours, at time 0, 1ml of $\beta$NGF medium containing a 1/80 dilution of goat anti-rat IgG (prepared by 18% sodium sulfate fractionation of immune goat serum) was added. At time 0, 4, 9, and 19 hours, the carbamylcholine-stimulated $^{86}Rb^+$ uptake of mAb 270- ( ● ) and mAb 164- ( o ) treated cells was determined. Results are expressed as mean ± standard deviation of triplicate cultures. (C) Modulation of carbamylcholine-stimulated $^{86}Rb^+$ influx by antisera to nicotinic receptors from chicken brain. Cell monolayers were cultured for 4 days in medium containing 50ng/ml $\beta$-NGF and then for 16 hours in medium containing a 1:25 dilution of normal rat serum, rat serum raised against nicotinic acetylcholine receptors from Torpedo (titer 22$\mu$M), and two rat sera raised against immunoaffinity-purified receptors from chicken brain (titers 32$\mu$M and 96$\mu$M). The carbamylcholine-stimulated $^{86}Rb^+$ influx was then determined as described above. Results are mean ± standard deviation of triplicate cultures and are expressed considering the $^{86}Rb^+$ influx in the presence of normal rat serum (85nmol/30 seconds/8 x $10^5$ cells) as 100%. Reproduced from reference 49.

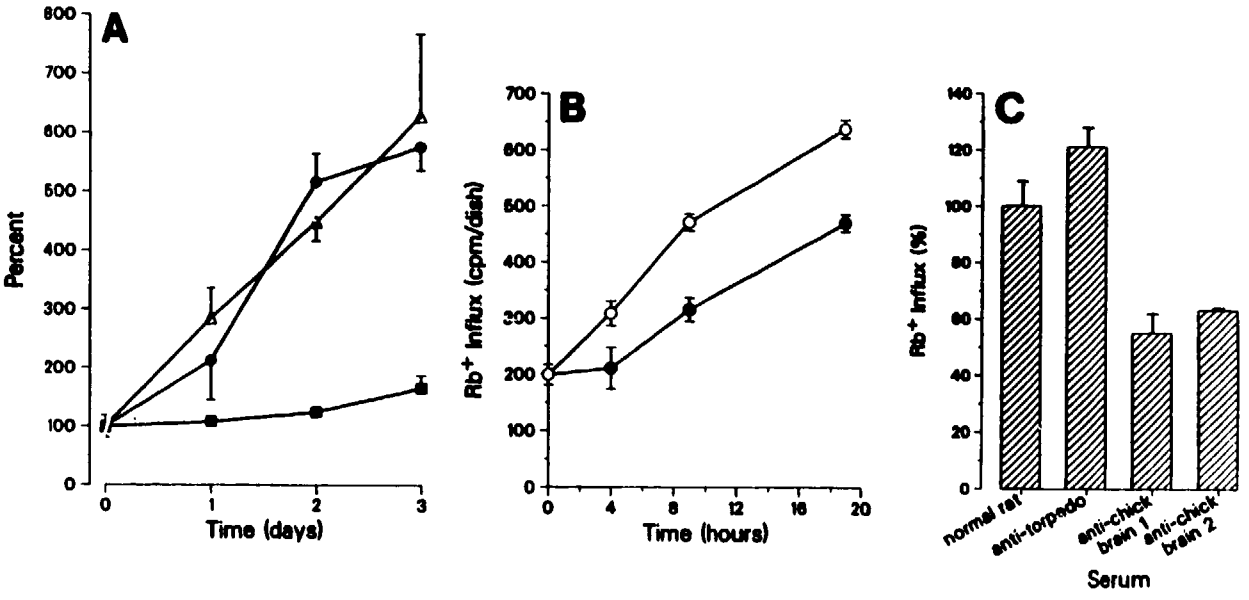


**Figure 16.** Photomicrographs illustrating the distribution of $^{125}$I-mAb 270 binding sites in two horizontal sections through the rat brain and spinal cord. **OBip** - olfactory bulb inner plexiform layer, **CP** - caudoputamen, **TS** - triangular nucleus of the septum, **HIP** - hippocampus and dentate gyrus, **MH** - medial habenula, **THAL** - thalamus, **PRT** - pretectal region, **SC** - superior colliculus, **SUB** - subiculum, **PRE** - presubiculum, **CER** - cerebellar granular layer, **DH** - spinal cord dorsal horn. Reproduced from reference 49.

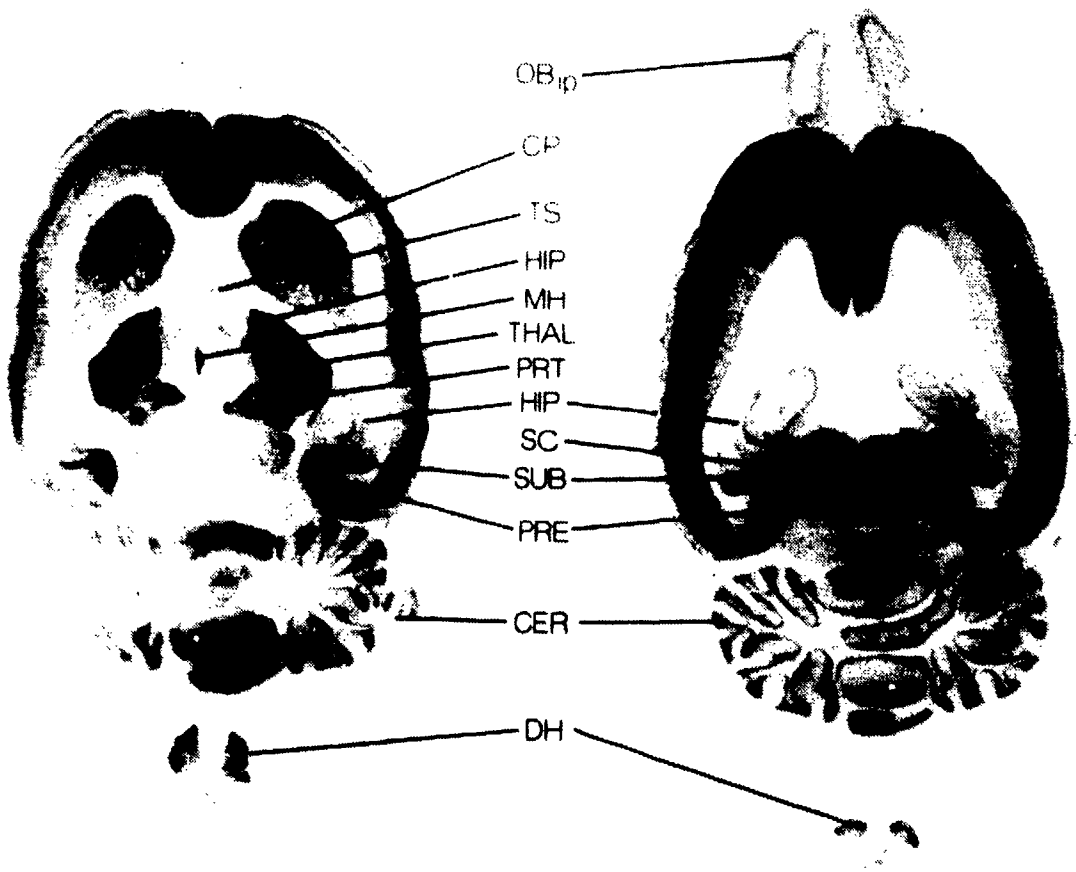


**Figure 17.** The distribution of $^{125}$I-mAb 270 immunolabeling in a rostrocaudal (A-P) series of sections through the rat CNS. Virtually no labeling was observed when the sections were coincubated in 40nM unlabeled mAb 270. Adjacent Nissl-stained sections were used to identify labeled structures. X1.8. Abbreviations of immunolabeled regions: AD, anterodorsal nucleus (n.); AHZ, amygdalohippocampal area (a.); AL, anterior limbic a.; AM, anteromedial n.; AMB, n. ambiguus; AON, anterior olfactory n.; AP, a. postrema; AV, anteroventral n.; BLA(p), basolateral n. amygdala (posterior); BST, bed n. stria terminalis; CA1m-CA3m, molecular layer of Ammon's horn fields; CM, central medial n.; CoA(p), cortical n. amygdala (posterior); CP, caudoputamen; DC, dorsal cochlear n.; DGm, dentate gyrus molecular layer; DMX, dorsal motor n. vagus; DRG, dorsal root ganglion; DTN, dorsal tegmental n.; EC, external cuneate n.; ENT, entorhinal a.; EP, endopiriform n.; FP, frontal pole; GV, trigeminal ganglion; IC, inferior colliculus; IO, inferior olive; IPN, interpeduncular n.; LA, lateral n. amygdala; LD, lateral dorsal n.; LGd,v, dorsal, ventral lateral geniculate n.; LP, lateral posterior n.; MD, mediodorsal n.; MG, medial geniculate n.; MH, medial habenula; MoV, motor n. trigeminal; MR, median raphe; MV, medial vestibular n.; MZ, marginal zone; NC, cuneiform n.; NG, gracile n.; NLOT, n. lateral olfactory tract; NOT, n. optic tract; NTS, n. solitary tract; OP, olivary pretectal n.; OT, olfactory tubercle; PAG, periaqueductal gray; PAR, parasubiculum; PB, parabranchial n.; PF, parafascicular n.; PG, pontine gray; PHA, posterior hypothalamic a.; PIN, pineal; PIR, piriform cortex; PO, posterior complex; PPN, pedunculopontine n.; PRE, presubiculum; PSV, sensory n. trigeminal; PT, parataenial n.; PVTa, paraventricular n. thalamus (anterior); Re, n. reuniens; RF, rhinal fissure; RSP, retrosplenial a.; RT, reticular n.; SC, superior colliculus; SG, substantia gelatinosa; SI, substantia innominata; SNc, compact part, substantia nigra; SpV, spinal n. trigeminal; SUB(m), subiculum (molecular layer); TRN, tegmental reticular n.; TS, triangular n. septum; VA, ventral anterior n.; VH, ventral horn; VM, ventromedial n. thalamus; VP(p), ventral posterior n. (parvicellular); VTA, ventral tegmental a.; VTN, ventral tegmental n.; ZI, zona incerta; ch, optic chiasm; fr, fasciculus retroflexus; gl, granular layer cerebellum; on, optic nerve; ot, optic tract. Reproduced from reference 58.

(see next page)

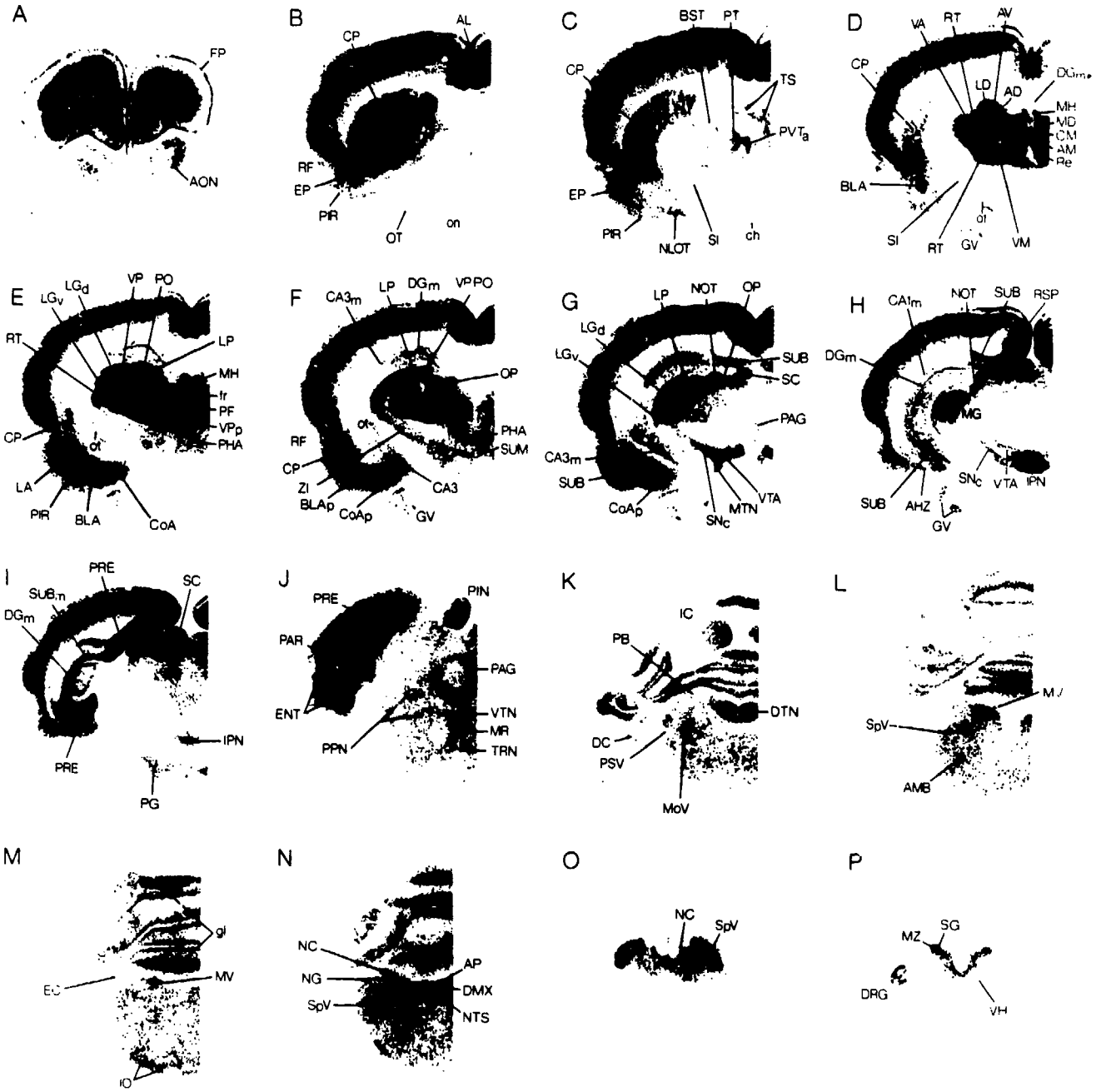

A
FP
AON
B
CP
AL
RF
EP
PIR
OT
on
C
BST
PT
CP
TS
PVTa
EP
PIR
NLOT
SI
ch
D
VA
RT
AV
CP
LD
AD
DGm
MH
MD
CM
AM
Re
BLA
SI
RT
GV
VM
E
LGv
LGd
VP
PO
RT
LP
MH
fr
PF
VPp
PHA
CP
ot
LA
PIR
BLA
CoA
F
CA3m
LP
DGm
VP PO
OP
PHA
SUM
RF
ot
CP
ZI
CA3
BLAp
CoAp
GV
G
LP
NOT
OP
LGd
LGv
SUB
SC
PAG
CA3m
SUB
CoAp
VTA
MTN
SNc
H
CA1m
NOT
SUB
RSP
DGm
MG
SNc
VTA
IPN
SUB
AHZ
GV
I
PRE
SUBm
SC
DGm
IPN
PRE
PG
J
PRE
PIN
PAR
PAG
ENT
VTN
MR
PPN
TRN
K
IC
PB
DTN
DC
PSV
MoV
L
MJ
SpV
AMB
M
gl
EC
MV
IO
N
NC
AP
NG
DMX
SpV
NTS
O
NC
SpV
P
MZ
SG
DRG
VH

**Figure 18.** Effect of left enucleation 3 weeks before sacrifice on $^{125}$I-mAb 270 immunolabeling (top) and $^{125}$I-$\alpha$-bungarotoxin binding (bottom) in adjacent sections of the rat superior colliculus (SC). Note disappearance of mAb 270 labeling in the contralateral SC (SCc), and dense labeling in the ipsilateral SC (SCi) of this slightly asymmetrically cut section. X5. Inset: Dark-field photomicrograph (left) of $^{125}$I-mAb 270 immunolabeling in the ganglion cell layer (g) and inner plexiform layer (ip) of the rat retina. Labeling is particularly dense in the deep part of the ip. Apparent labeling in the outer nuclear layer (on) is artifactual, due to cracks between densely packed cells. Brightfield view (right) of Nissl stain. in, inner nuclear layer; op, outer plexiform layer. X75. Reproduced from reference 58.

**Figure 19.** Photomicrographs showing the indirect immunofluorescence localization of mAb 270 (A,B,D) and mAb 290 (C) in the mouse brain. (**A**) Dorsal (top) and ventral (bottom) lateral geniculate nucleus; compare with Figure 17. (**B**) Olivary pretectal nucleus; compare with Figure 17F. (**C**) Right medial terminal nucleus; compare with Figure 17G. (**D**) Medial habenula with unlabeled lateral habenula and stria medullaris to the left; compare with Figure 17D,E. Because the fasciculus retroflexus and interpeduncular nucleus were clearly labeled (Figure 17E-I) it appears likely that neuronal acetylcholine receptor is synthesized in medial habenular cells[70-72] and undergoes axonal transport to the interpeduncular nucleus. Indirect immunofluorescence was only successful in the mouse, probably because the primary mAbs were raised in rats where background staining was high, and was not sensitive enough to reveal areas moderately or lightly labeled with $^{125}I$ mAbs, such as the cerebral cortex. All micrographs X75. Reproduced from reference 58.

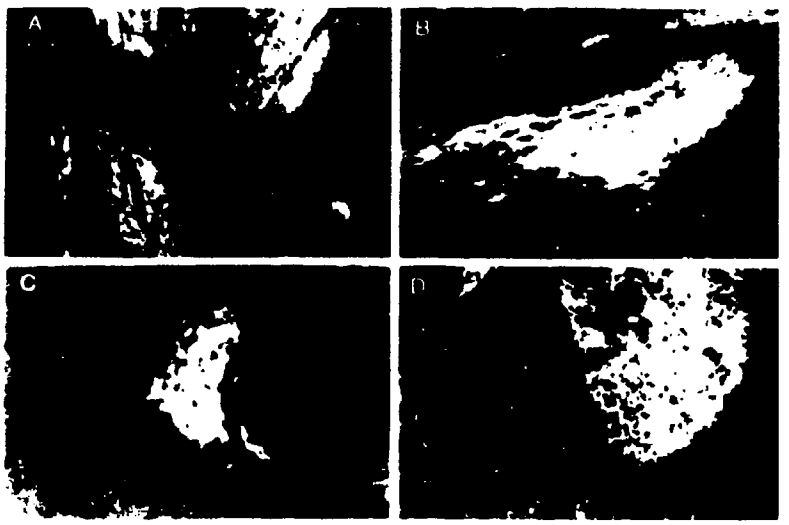


**Figure 20A,B.** Dark-field photomicrographs of $^{125}$I-mAb 270 immunolabeling in the lateral geniculate nucleus on the side ipsilateral (A) and contralateral (B) to enucleation in the rat illustrated in Figure 18; comparable to level F in Figure 17. X30. (C) Dark-field photomicrograph of $^{125}$I-mAb 270 immunolabeled ganglion cells in the trigeminal ganglion (see Figure 17E-G). (D) Nissl-stained section adjacent to C. C,D X100. Reproduced from reference 58.

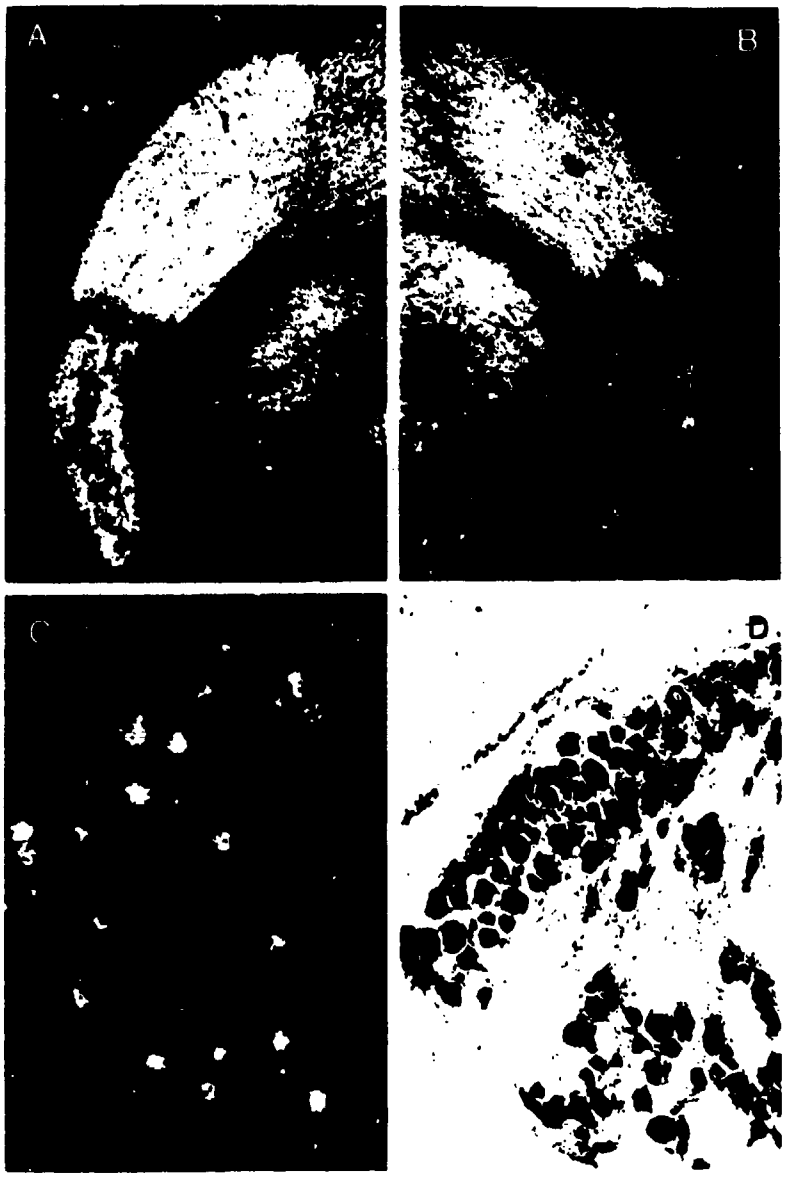


Table 1

Synthetic Peptides Corresponding to Sequences of the Torpedo californica Electric Organ Acetylcholine Receptor α Subunit

| peptide | sequence |
|---|---|
| [Tyr-11]α1–11 | SEHETRLVANY |
| [Tyr-60]α44–60 | DEVNQIVETNVRLRQQY |
| [Tyr-83]α66–83 | RWNPADYGGIKKIRLPSY |
| [Gly-89,Tyr-90]α73–90 | GGIKKIRLPSDDVWLPGY |
| α78–93 | IRLPSDDVWLPDLVLY |
| [Tyr-104]α89–104 | DLVLYNNADGDFAIVY |
| [Tyr-100]α100–116 | YAIVHMTKLLLDYTGKI |
| α112–127 | YTGKIMWTPPAIFKSY |
| | S–S |
| α127–143 | YCEIIVTHFPFDQQNCT |
| [Tyr-170]α159–170 | SPESDRPDLSTY |
| α172–189 | ESGEWVMKDYRGWKHWVY |
| α185–199 | KHWVYYTCCPDTPYL |
| α172–205 | ESGEWVMKDYRGWKHWVYYTCCP-DTPYLDITYHF |
| α194–212 | PDTPYLDITYHFIMQRIPL |
| α261–277 | VELIPSTSSAVPLIGKY |
| [Tyr-347]α330–347 | KRASKEKQENKIFADDIY |
| [Tyr-365]α349–365 | SDISGKQVTGEVIFQTY |
| [Tyr-379]α360–379 | VIFQTPLIKNPDVKSAIEGY |
| [Tyr-386]α371–386 | DVKSAIEGVKYIAEHY |
| [Tyr-409]α389–409 | DEESSNAAEEWKYVAMVIDHY |
| [Tyr-427]α427–437 | YGRLIELSQEG |

## Table 2

### Properties of mAbs To Chicken Brain Nicotinic Acetylcholine Receptors (AChRs)

| mAb | Immunization Schedule[a] | Ig Class[b] | Titer (μM) to AChRs from: Torpedo Electric Organ[c] | Brains[d] Chicken | Rat | Fetal Bovine | Human | Subunit Specificity[e] | AChR Subtype Specificity |
|---|---|---|---|---|---|---|---|---|---|
| 35[f] | electric organ AChR | IgG1 | 28.5 | 0.33 | 0 | 0 | 0 | $\alpha^a$ | $\alpha\beta$ |
| 210[f] | muscle AChR | IgG1 | 31.3 | 0.39 | 0 | 0 | 0 | $\alpha^a$ | $\alpha\beta/\alpha\beta'$ |
| | chicken brain AChR | | | | | | | | |
| 267 | A | IgG2a | 0 | 0 | 0 | 0 | 0 | $\alpha$, $\beta$ | $\alpha\beta/\alpha\beta'$ |
| 268 | A | IgG1/2a[b] | 0 | 0 | 0 | 0 | 0 | $\alpha$ | $\alpha\beta/\alpha\beta'$ |
| 270 | B | IgG2a | 0 | 1.10 | 0.18 | 0 | 0 | $\alpha$ | $\alpha\beta/\alpha\beta'$ |
| 284 | C | IgG2a | 0 | 2.00 | 0 | 0 | 0 | $\beta'$ | $\alpha\beta'$ |
| 285 | C | IgG2a | 0.014 | 1.40 | 0 | 0 | 0 | $\beta'$ | $\alpha\beta'$ |
| 286 | C | IgM | 0 | 0.64 | 0.45 | 0 | 0.13 | $\beta'$ ($\alpha$) | $\alpha\beta/\alpha\beta'$ |
| 287 | C | IgM | 0 | 0 | 0 | 0 | 0 | $\alpha$ | $\alpha\beta/\alpha\beta'$ |
| 289 | D | IgM | 0 | 0.18 | 0 | 0 | 0 | $\beta'$ | $\alpha\beta'$ |

[a] (A) Lewis rats were injected on days 0, 26, 39, 57, and 75 with 10, 30, 30, 20, and 12 pmol of SDS-denatured chicken brain AChR in CFA, and on days 81, 82, and 83 with 21, 21, and 9 pmol AChR in PBS, and then killed on day 85. (B) Lewis rats were injected on days 0, 18, 32, 62, and 84 with 20, 12, 22, 21, and 21 pmol of the SDS-denatured chicken brain AChR in CFA, and on days 94, 95, and 96 with 10 pmol of AChR in PBS, and then killed on day 98. (C) Lewis rats were injected on days 0, 21, and 42 with 15, 22, and 15 pmol of chicken brain AChR (50% of which had been denatured in SDS) in CFA, and on day 70 with 32 pmol AChR in PBS, and then killed on day 73. (D) Lewis rats were injected on days 0, 21, 42, and 98 with 15, 22, 15, and 24 pmol chicken brain AChR (50% of which had been denatured in SDS) in CFA, on day 137 with 25 pmol rat brain AChR (purified on mAb 270) in CFA, and on day 141 with 32 pmol of AChR in PBS, and killed on day 144.

Table 2 cont'd

Properties of mAbs To Chicken Brain Nicotinic Acetylcholine Receptors (AChRs)

[b] Ig class was determined by Ouchterlony double diffusion, using class- and subclass-specific antisera. mAb 268 reacted with both IgG1 and IgG2a subclass-specific antisera.

[c] Titer = μmol $^{125}$I-α-Bgt binding sites per liter mAb.

[d] Titer = μmol [$^{3}$H]nicotine binding sites bound per liter mAb. Binding of mAbs to native AChR in detergent extracts of brain was quantitated by a previously described assay. mAbs 267, 268, and 287 exhibited no detectable binding to native AChR, binding only to AChR which had undergone denaturation due to exposure to low pH (immunoaffinity-purified AChR undergoes some denaturation as it is eluted from the affinity column by a pH 3.0 buffer), or SDS-denatured AChR. None of the mAbs inhibited the binding of [$^{3}$H]nicotine to AChR detergent solubilized from chicken brain.

[e] Subunit specificity was determined by Western blot analysis.

[f] mAb 35 was prepared from rats immunized with AChR from Electrophorus electricus electric organ. mAb 210 was prepared from rats immunized with AChR from fetal bovine muscle and the BC3H1 muscle-like mouse cell line.

[g] mAb 35 has high affinity for the main immunogenic region on α subunits, a conformation-dependent epitope. mAb 35 binds weakly to denatured α subunits of AChR from Electrophorus, but not to denatured α subunits from other species. It competes for binding to native AChRs from muscle and brain with mAb 210, which is specific for the α subunit of these AChRs.

## Table 3

## Subunit Stoichiometry of the Rat Brain Neuronal Nicotinic Acetylcholine Receptor

| Possible stoichiometry | Predicted | | | Observed | | |
|---|---|---|---|---|---|---|
| | Apparent molecular mass[a] | $\alpha/\beta$ per cycle of sequenator[b] | $\alpha/\beta$ from SDS-PAGE | Apparent molecular mass[c] | $\alpha/\beta$ per cycle of sequenator[d] | $\alpha/\beta$ from SDS-PAGE[e] |
| $\alpha_2\beta_2$ | 260000 | 1 | 1 | | | |
| $\alpha_3\beta_2$ | 310000 | 1.5 | 1.5 | 338000 | 1.39 ± 0.44 | 1.29 ± 0.18 |
| $\alpha_3\beta_3$ | 339000 | 0.67 | 0.67 | | | |

[a] Apparent molecular mass was calculated from apparent molecular masses of subunits determined by SDS-PAGE ($\alpha$ mw 51,000 and $\beta$ mw 79,000).

[b] Ratio of moles of $\alpha$ subunit amino acid to $\beta$ subunit amino acid at each cycle of sequencing.

[c] The apparent molecular mass of the acetylcholine receptor was determined from sucrose gradient analysis using $^{125}$I-$\alpha$-Bgt-labeled Torpedo acetylcholine receptor monomers and dimers as standards. The $S_{20,w}$ value for brain acetylcholine receptor was found to be 10.6, which is equivalent to an apparent molecular mass of 338kDa.

[d] The mole ratio (mean ± standard deviation) of $\alpha$:$\beta$ subunit is shown for the first 11 sequencing cycles.

[e] Ratio $\alpha$:$\beta$ of autoradiogram band intensities of radioiodinated rat brain acetylcholine receptor resolved by SDS-PAGE. The value shown is the mean ± standard deviation derived from four analyses. This assumes that $^{125}$I is incorporated at an equal weight ratio in each subunit.

## Literature Cited


1. Lindstrom J., J. Merlie, and B. Yogeeswaran (1979) Biochemical properties of acetylcholine receptor subunits from Torpedo californica. **Biochem. 18, 4465-4470.**

2. Raftery M., M. Hunkapillar, C. Strader, and L. Hood (1980) Acetylcholine receptor: Complex of homologous subunits. **Science 208, 1454-1457.**

3. Noda M., H. Takahashi, T. Tanabe, M. Toyosato, S. Kikyotani, Y. Furutani, T. Hirose, H. Takashima, S. Inayama, T. Miyata, and S. Numa (1983) Structural homology of Torpedo californica acetylcholine receptor subunits. **Nature 302, 528-532.**

4. Kubo T., K. Fukuda, A. Mikami, A. Maeda, H. Takohashi, M. Mishina, T. Haga, K. Haga, A. Ichiyama, K. Kangawa, M. Kojima, H. Matsuo, T. Hirose, and S. Numa (1986) Cloning, sequencing, and expression of complementary DNA encoding the muscarinic acetylcholine receptor. **Nature 323, 411-416.**

5. Kubo T., A. Maeda, K. Sugimoto, I. Akiba, A. Mikami, H. Takahashi, T. Haga, K. Haga, A. Ichiyama, K. Kangawa, H. Matsuo, T. Hirose, and S. Numa (1986) Primary structure of porcine cardiac muscarinic acetylcholine receptor deduced from the cDNA sequence. **FEBS Lett. 209, 367-372.**

6. Anholt R., J. Lindstrom, and M. Montal (1985) The molecular basis of neurotransmission: Structure and function of the nicotinic acetylcholine receptor. Pages 335-401 in: **The Enzymes of Biological Membranes,** Vol. 3, A. Martonosi (Ed.), Plenum Press, New York.

7. Lindstrom J. (1985) Nicotinic acetylcholine receptors: Use of monoclonal antibodies to study synthesis, structure, function, and autoimmune response. Pages 21-57 in: **Monoclonal and Anti-Idiotypic Antibodies: Probes for Receptor Structure and Function,** J. Venter, C. Fraser, and J. Lindstrom (Eds.), Alan R. Liss, Inc., New York.

8. Patrick J. and W. Stallcup (1977) Immunological distinction between acetylcholine receptor and the $\alpha$-bungarotoxin-binding component on sympathetic neurons. **Proc. Natl. Acad. Sci. U.S.A. 74, 4689-4692.**

9. Jacob M. and D. Berg (1983) The ultrastructural localization of $\alpha$-bungarotoxin binding sites in relation to synapses on chick ciliary ganglion neurons. **J. Neurosci. 3, 260-271.**

10. Conti-Tronconi B., S. Dunn, E. Barnard, J. Dolly, F. Lai, N. Ray, and M. Raftery (1985) Brain and muscle nicotinic

acetylcholine receptors are different but homologous proteins. **Proc. Natl. Acad. Sci. U.S.A. 82, 5208-5212.**

11. Wan K. and J. Lindstrom (1985) Effects of monoclonal antibodies on the function of acetylcholine receptors purified from Torpedo californica and reconstituted into vesicles. **Biochem. 24, 1212-1221.**

12. Blatt Y., M. Montal, J. Lindstrom, and M. Montal (1986) Monoclonal antibodies directed against epitopes in the beta and gamma subunits of the Torpedo cholinergic receptor affect channel gating. **J. Neurosci. 6, 481-486.**

13. Criado M., V. Sarin, J. Fox, and J. Lindstrom (1986) Evidence that the acetylcholine binding site is not formed by the sequence α127-143 of the acetylcholine receptor. **Biochem. 25, 2839-2846.**

14. Criado M., V. Sarin, J.L. Fox, and J. Lindstrom (1985) Structural localization of the sequence α235-242 of the nicotinic acetylcholine receptor. **Biochem. Biophys. Res. Commun. 128, 864-871.**

15. Criado M., S. Hochschwender, V. Sarin, J.L. Fox, and J. Lindstrom (1985) Evidence for unpredicted transmembrane domains in acetylcholine receptor subunits. **Proc. Natl. Acad. Sci. U.S.A. 82, 2004-2008.**

16. Criado M. and J. Lindstrom (1985) Antigenicity of the sequence 152-167 in α subunits of Torpedo acetylcholine receptor. **Biochem. J. 231, 247.**

17. Ratnam M., D. Le Nguyen, J. Rivier, P.B. Sargent, and J. Lindstrom (1986) Transmembrane topography of nicotinic acetylcholine receptor: Immunochemical tests contradict theoretical predictions based on hydrophobicity profiles. **Biochem. 25, 2633-2643.**

18. Ratnam M., P.B. Sargent, V. Sarin, J.L. Fox, D. Le Nguyen, J. Rivier, M. Criado, and J. Lindstrom (1986) Location of antigenic determinants on primary sequences of subunits of nicotinic acetylcholine receptor by peptide mapping. **Biochem. 25, 2621-2632.**

19. Ratnam M., W. Gullick, J. Spiess, K. Wan, M. Criado, and J. Lindstrom (1986) Structural heterogeneity of the α subunits of the nicotinic acetylcholine receptor in relation to agonist affinity alkylation and antagonist binding. **Biochem. 25, 4268-4275.**

20. Noda M., H. Takahashi, T. Tanabe, M. Toyosato, Y. Furutani, T. Hirose, M. Asai, S. Inayama, T. Miyata, and S. Numa (1982) Primary structure of α subunit precursor of Torpedo californica acetylcholine receptor deduced from cDNA sequence. **Nature 299, 793-797.**

21. Smart L., H-W. Meyers, R. Hilgenfeld, W. Saenger, and A. Maelicke (1984) A structural model for the ligand-binding sites at the nicotinic acetylcholine receptor. **FEBS Lett. 178, 64-68.**

22. Boulter J., W. Luyten, K. Evans, P. Mason, M. Ballivet, D. Goldman, S. Stengelin, G. Martin, S. Heinemann, and J. Patrick (1985) Isolation of a clone coding for the $\alpha$ subunit of a mouse acetylcholine receptor. **J. Neurosci. 5, 2545-2552.**

23. Lindstrom J., M. Criado, S. Hochschwender, J.L. Fox, and V. Sarin (1984) Immunochemical tests of acetylcholine receptor subunit models. **Nature 311, 573-575.**

24. Juillerat M., T. Barkas, and S. Tzartos (1984) Antigenic sites of the nicotinic acetylcholine receptor cannot be predicted from the hydrophobicity profile. **FEBS Lett. 168, 143-148.**

25. Guy R. (1983) A structural model of the acetylcholine receptor channel based on partition energy and helix packing calculations. **Biophys. J. 45, 249-261.**

26. Finer-Moore J. and R. Stroud (1984) Amphipathic analysis and possible formation of the ion channel in an acetylcholine receptor. **Proc. Natl. Acad. Sci. U.S.A. 81, 155-159.**

27. Claudio T., M. Ballivet, J. Patrick, and S. Heinemann (1983) Torpedo californica acetylcholine receptor 60,000 dalton subunit: Nucleotide sequence of cloned cDNA deduced amino acid sequence, subunit structural predictions. **Proc. Natl. Acad. Sci. U.S.A. 80, 1111-1115.**

28. Devillers-Thiery A., J. Giraudat, M. Bentaboulet, and J-P. Changeux (1983) Complete mRNA coding sequence of the acetylcholine binding $\alpha$ subunit of Torpedo marmorata acetylcholine receptor: A model for the transmembrane organization of the polypeptide chain. **Proc. Natl. Acad. Sci. U.S.A. 80, 2067-2071.**

29. Whiting P.J. and J.M. Lindstrom (1986) Purification and characterization of a nicotinic acetylcholine receptor from chick brain. **Biochem. 25, 2082-2093.**

30. Swanson L., J. Lindstrom, S. Tzartos, L. Schmued, D. O'Leary, and W. Cowan (1983) Immunohistochemical localization of monoclonal antibodies to the nicotinic acetylcholine receptor in the midbrain of the chick. **Proc. Natl. Acad. Sci. U.S.A. 80, 4532-4536.**

31. Jacob M., D. Berg, and J. Lindstrom (1984) A shared antigenic determinant between the Electrophorus acetylcholine receptor and a synaptic component on chick ciliary ganglion neurons. **Proc. Natl. Acad. Sci. U.S.A. 81, 3223-3227.**

32. Smith M., J. Stollberg, D.K. Berg, and J. Lindstrom (1985) Characterization of a component in chick ciliary ganglia that cross-reacts with monoclonal antibodies to muscle and electric organ acetylcholine receptor. J. Neurosci. 5, 2726-2731.

33. Smith M., J. Margiotta, A. Franco, J. Lindstrom, and D. Berg (1986) Cholinergic modulation of an acetylcholine receptor-like antigen on the surface of chick ciliary ganglion neurons in cell culture. J. Neurosci. 6, 946-953.

34. Jacob M., J. Lindstrom, and D. Berg (1986) Surface and intracellular distribution of a putative neuronal nicotinic acetylcholine receptor. J. Cell Biol. 103, 205-214.

35. Stollberg J., P.J. Whiting, J. Lindstrom, and D.K. Berg (1986) Functional blockade of neuronal acetylcholine receptors by antisera to a putative receptor from brain. Brain Res. 378, 179-182.

36. Whiting P. and J. Lindstrom (1986) Pharmacological properties of immunoisolated neuronal nicotinic receptors. J. Neurosci. 6, 3061-3069.

37. Ralston S., V. Sarin, H. Thanh, J. Rivier, J.L. Fox, and J. Lindstrom (1987) Synthetic peptides used to locate the α-bungarotoxin binding site and immunogenic regions on α subunits of the nicotinic acetylcholine receptor. Biochem. 26, 3261-3266.

38. Wilson P., T. Lentz, and E. Hawrot (1985) Determination of the primary amino acid sequence specifying the α-bungarotoxin on the α subunit of the acetylcholine receptor from Torpedo californica. Proc. Natl. Acad. Sci. U.S.A. 82, 8790-8794.

39. Neumann D., D. Barchan, M. Fridkin, and S. Fuchs (1986) Analysis of ligand binding to the synthetic dodecapeptide 185-186 of the acetylcholine receptor α subunit. Proc. Natl. Acad. Sci. U.S.A. 83, 9250-9253.

40. Fujita N., N. Nelson, T. Fox, T. Claudio, J. Lindstrom, H. Reizman, and G. Hess (1986) Biosynthesis of the Torpedo californica acetylcholine receptor α subunit in yeast. Science 231, 1284-1287.

41. Fujita N., M. Sweet, T. Fox, N. Nelson, T. Claudio, J. Lindstrom, and G. Hess (in press) Expression of cDNAs for acetylcholine receptor subunits in yeast cell plasma membrane. Biochem. J.

42. Merlie J.P. and J. Lindstrom (1983) Assembly in vivo of mouse muscle acetylcholine receptor: Identification of an α subunit species which may be an assembly intermediate. Cell 34, 747-757.

43. Kubalek E., S. Ralston, J. Lindstrom, and N. Unwin (1987) Location of subunits within the acetylcholine receptor: Analysis of tubular crystals from Torpedo marmorata. J. Cell Biol. 105, 9-18.

44. Brisson A. and P. Unwin (1985) Quaternary structure of the acetylcholine receptor. Nature 315, 474-477.

45. Whiting P., R. Liu, B.J. Morley, and J. Lindstrom (1987) Structurally different neuronal nicotinic acetylcholine receptor subtypes purified and characterized using monoclonal antibodies. J. Neurosci. 7, 4005-4016.

46. Whiting P.J. and J.M. Lindstrom (1987) Affinity labeling of neuronal acetylcholine receptors localizes the neurotransmitter binding site to the $\beta$ subunits. FEBS Lett. 213, 55-60.

47. Kao P., A. Dwork, R. Kaldany, M. Silver, J. Wideman, S. Stein, and A. Karlin (1984) Identification of the $\alpha$ subunit half cystine specifically labeled by an affinity reagent for the acetylcholine receptor binding site. J. Biol. Chem. 259, 11662-11665.

48. Whiting P.J. and J.M. Lindstrom (1987) Purification and characterization of a nicotinic acetylcholine receptor from rat brain. Proc. Natl. Acad. Sci. U.S.A. 81, 595-599.

49. Whiting P., R. Schoepfer, L. Swanson, D. Simmons, and J. Lindstrom (1987) Functional acetylcholine receptor in PC12 cells reacts with a monoclonal antibody to brain nicotinic receptors. Nature 327, 515-518.

50. Leprince P. (1983) Chemical modification of the nicotinic cholinergic receptor of PC-12 nerve cell line. Biochem. 22, 5551-5556.

51. Halvorsen S. and D. Berg (1986) Identification of a nicotinic acetylcholine receptor on neurons using an $\alpha$ neurotoxin that blocks receptor function. J. Neurosci. 6, 3405-3412.

52. Kemp G. and B. Morley (1986) Ganglionic nAChRs and high affinity nicotinic binding sites are not equivalent. FEBS Lett. 205, 265-268.

53. Mitsuka M. and H. Hatanoka (1984) Increase of carbamylcholine-induced $^{22}Na^{+}$ influx into pheochromocytoma PC12h cells by nerve growth factor. Dev. Brain Res. 12, 255-260.

54. Boulter J., K. Evans, D. Goldman, G. Martin, D. Treco, S. Heinemann, and J. Patrick (1986) Isolation of a cDNA clone coding for a possible neuronal nicotinic acetylcholine receptor $\alpha$ subunit. Nature 319, 368-374.

55. Nef P., C. Oneyser, T. Barkas, and M. Ballivet (1986) Acetylcholine receptor related genes expressed in the nervous system. Pages 417-422 in: **Nicotinic Acetylcholine Receptor Structure and Function**, A. Maelicke (Ed.), NATO ASI Series H3, Springer-Verlag, Berlin.

56. Merlie J., K. Isenberg, S. Russel, and J. Sanes (1984) Denervation supersensitivity in skeletal muscle: Analysis with a cloned cDNA probe. **J. Cell Biol. 99, 332-335.**

57. Young R. and R. Davis (1983) Efficient isolation of genes by using antibody probes. **Proc. Natl. Acad. Sci. U.S.A. 80, 1194-1198.**

58. Swanson L., D. Simmons, P. Whiting, and J. Lindstrom (1987) Immunohistochemical localization of neuronal nicotinic receptors in the rodent central nervous system. **J. Neurosci. 7, 3334-3342.**

59. Clarke P., R. Schwartz, S. Paul, C. Pert, and A. Pert (1985) Nicotinic binding in rat brain: Autoradiographic comparison of [$^{3}$H]acetylcholine, [$^{3}$H]nicotine, and [$^{125}$I] $\alpha$-bungarotoxin. **J. Neurosci. 5, 1307-1315.**

60. Norman R., F. Mehraban, E. Barnard, and O. Dolly (1982) Nicotinic acetylcholine receptor from chick optic lobe. **Proc. Natl. Acad. Sci. U.S.A. 79, 1321-1325.**

61. Whiting P.J., J. Cooper, and J.M. Lindstrom (1987) Antibodies in sera from patients with myasthenia gravis do not bind to nicotinic acetylcholine receptors from human brain. **J. Neuroimmunol. 16, 205-213.**

62. Lindstrom J., M. Criado, M. Ratnam, P. Whiting, S. Ralston, J. Rivier, V. Sarin, and P. Sargent (1987) Using monoclonal antibodies to determine the structures of acetylcholine receptors from electric organs, muscles, and neurons. **Ann. N.Y. Acad. Sci. 505, 208-225.**

63. Siegel H. and R. Lukas (1986) Nicotinic acetylcholine receptor activation increases $\alpha$-bungarotoxin binding site density on the human meduloblastoma cell line TE671. **Neurosci. Soc. Abst. 11.9.**

64. Lindstrom J., B. Einarson, and S. Tzartos (1981) Production and assay of antibodies to acetylcholine receptors. **Meth. Enzymol. 74, 432-460.**

65. Nomoto H., N. Tokahashi, Y. Nagaki, S. Endo, Y. Arata, and K. Hayashi (1986) Carbohydrate structures of acetylcholine receptor from Torpedo californica and distribution of oligosaccharides among the subunits. **Eur. J. Biochem. 157, 233-242.**

66. Bonner W. and R. Laskey (1974) A film detection method for tritium labeled proteins and nucleic acids in polyacrylamide gels. **Eur. J. Biochem. 46, 83-88.**

67. Thon F. (1966) Zur Defokussierungsahaengigkeit des Phasenkontrastes bei der elektronenmikroskopischen Abblildung. **Z. Naturforsch 219, 476-478.**

68. Hochschwender S., L. Langeberg, D. Schneider, and J. Lindstrom (1985) Production of rat X mouse hybridomas for the study of the nicotinic acetylcholine receptor. Pages 223-238 in: **Hybridoma Technology in the Biosciences and Medicine**, T.A. Springer (Ed.), Plenum Press, New York.

69. Catterall W. (1975) Sodium transport by the acetylcholine receptor of cultured muscle cells. **J. Biol. Chem. 250, 1776-1781.**

70. Clarke P., G. Hamill, N. Nadi, D. Jacobowitz, and A. Pert (1986) $^{3}$H-Nicotine and $^{125}$I-$\alpha$-bungarotoxin-labeled nicotinic receptors in the interpeduncular nucleus of rats. II. Effects of habenular deafferentation. **J. Comp. Neurol. 251, 407-413.**

71. Clarke P., D. Hommer, A. Pert, and L. Skirboll (1985) Electrophysiological actions of nicotine on substantia nigra single units. **Br. J. Pharmacol. 85, 827-835.**

72. Goldman D., D. Simmons, L. Swanson, J. Patrick, and S. Heinemann (1986) Mapping of brain areas expressing RNA homologous to two different acetylcholine receptor $\alpha$ subunit cDNAs. **Proc. Natl. Acad. Sci. USA 83, 4076-4080.**

## Distribution List

| | |
|---|---|
| 1 copy | Commander<br>U.S. Army Medical Research and Development Command<br>ATTN: SGRD-RMI-S<br>Fort Detrick, Frederick, MD 21701-5012 |
| 5 copies | Commander<br>U.S. Army Medical Research and Development Command<br>ATTN: SGRD-PLE<br>Fort Detrick, Frederick, MD 21701-5012 |
| 12 copies | Defense Technical Information Center (DTIC)<br>ATTN: DTIC-DDAC<br>Cameron Station<br>Alexandria, VA 22304-6145 |
| 1 copy | Dean<br>School of Medicine<br>Uniformed Services University of the Health Sciences<br>4301 Jones Bridge Road<br>Bethesda, MD 20814-4799 |
| 1 copy | Commandant<br>Academy of Health Sciences, U.S. Army<br>ATTN: AHS-CDM<br>Fort Sam Houston, TX 78234-6100 |