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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 and Final Report

Jon M. Lindstrom

September 30, 1989

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
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The Salk Institute Receptor Biology Laboratory
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During the 3 years of this project, extensive progress has been made on studies of the structure of nicotinic acetylcholine receptors from muscles and nerves. This has provided altogether new insights into the structure of neuronal nicotinic receptors. Many important questions remain, but the conceptual foundation, experimental techniques, and reagents are now available to permit rapid further progress, if this project is continued.

Muscle-type receptors from *Torpedo* electric organs were shown to be composed of subunits organized like barrel staves around a central cation channel, in the order alpha, alpha, beta, gamma, delta. Specific amino acid sequences were mapped topographically and to various functional domains. Yeast were found to be a poor expression system for native receptors. *Xenopus* oocytes were found to be a good expression system for small amounts of receptor.

A human cell line was shown to synthesize relatively large amounts of functional receptors composed of alpha, beta, gamma, and delta subunits, and to synthesize nearly equal amounts of unassembled, partially mature alpha subunits. cDNAs for delta and alpha subunits were cloned and sequenced.

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Neuronal receptors from the brains of several species were immunoaffinity purified and shown to be composed of equal numbers (at least 2, but no more than 3) of acetylcholine-binding and structural subunits. Libraries of subunit-specific monoclonal antibodies were constructed. The genes encoding these receptors are members of the nicotinic receptor gene family and ligand-gated ion channel gene superfamily. cDNAs for several subunits were cloned and sequenced, and fragments of these were expressed in bacteria to produce antigens for the preparation of further subunit-specific antibodies. Receptors were histologically localized in several species. Human brain receptors were pharmacologically characterized.

Neuronal α-bungarotoxin-binding proteins were purified, cDNAs for two subunits were cloned and sequenced, and monoclonal antibodies were prepared to native binding protein and bacterially expressed fragments of subunit cDNA. These proteins of unknown function were shown to be members of the ligand-gated ion channel gene superfamily.

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ABSTRACT

Nerve gases produce their lethal effects through direct and indirect effects on acetylcholine receptors. Thus, to improve defenses against these agents it is important to understand the structure of these receptors.

Acetylcholine receptors are also medically important. These receptors additionally serve as models for understanding all other members of the ligand-gated ion channel gene superfamily, which includes receptors for glycine, γ-aminobutyric acid, and probably excitatory amino acids.

During the three years of this project, substantial progress has been made toward understanding the structure of muscle nicotinic acetylcholine receptors. Further, for the first time, many new insights were achieved into the structure of neuronal nicotinic receptors. Nicotinic receptors from both muscles and nerves are part of a gene family of receptors with multiple homologous subunits that form a transmitter-gated ion channel across the cell surface membrane. Neuronal α-bungarotoxin-binding proteins bind cholinergic ligands, but do not appear to behave as acetylcholine-gated ion channels. We have begun to crack the mystery of these proteins by cloning complimentary DNAs (cDNAs) for two of their subunits whose sequences prove that α-bungarotoxin-binding proteins are also members of the ligand-gated ion channel gene superfamily. Many important questions remain, but the conceptual foundation, experimental techniques, and reagents are now available to permit rapid further progress, if funding is available.

Muscle-type nicotinic acetylcholine receptors were shown to consist of subunits organized like barrel staves around a central cation channel, in the order $\alpha\beta\alpha\gamma\delta$. Specific amino acid sequences were mapped to the extracellular or cytoplasmic surface of the receptor, helping to reveal the overall transmembrane orientation of the polypeptide chains in receptor subunits. Specific functional domains such as the acetylcholine binding site and main immunogenic region were mapped to particular sequences of α subunits. These studies provide a baseline of information for understanding not only the structure of muscle nicotinic receptors, but also all other neurotransmitter receptors in the ligand-gated ion channel gene superfamily.

Yeast were investigated as a system in which receptors for acetylcholine and other transmitters might be efficiently expressed in large amounts. However, it was found that although yeast synthesized small amounts of receptor subunits, they did not efficiently assemble them into mature receptors. These studies indicate that for research and practical applications for which large amounts of receptor proteins expressed from cloned cDNAs are desired, expression systems other than the yeast system investigated should be employed.

Xenopus oocytes efficiently express small amounts of receptor subunits. Expression of various combinations of receptor subunits suggest that α subunits mature in conformation on association with γ or δ subunits, and that β subunits can only efficiently assemble with α subunits after the α subunits have associated with γ and δ subunits.

Human muscle nicotinic receptors were found to be synthesized in relatively large amounts by the cell line TE671, which is readily amenable to electrophysiological and pharmacological studies of receptor function. These receptors are composed of α, β, γ, and δ subunits. The cells also synthesize, but do not express on their surface membranes, nearly equal amounts of unassembled α subunits, which have a conformation intermediate between nascent or denatured α subunits and mature α subunits in native receptors. Cloned cDNAs for α and δ subunits were sequenced. These studies establish, for the first time, a practically useful system for studying the functional pharmacology of nicotinic receptors from human muscle. Further, they begin experiments that offer new insights into receptor synthesis and provide tools, for the first time, for further detailed studies of the structure and function of receptors from human muscle.

Neuronal nicotinic receptors were studied in detail at the protein and cDNA levels. These receptors have been immunoaffinity purified from the brains of several species, and libraries of subunit-specific monoclonal antibodies have been prepared. There are several subtypes of these receptors, each formed from at least two, but no more than three, copies of an acetylcholine-binding subunit and an equal number of copies of a structural subunit. These are presumed to be organized like barrel staves around a central cation channel, probably in the order $\alpha\beta\alpha\beta$. Several candidate genes have been reported for neuronal acetylcholine-binding subunits ($\alpha 2$, $\alpha 3$, $\alpha 4$, etc.) and for neuronal structural subunits ($\beta 2$, $\beta 3$, etc.). The sequences of these genes indicate that these subunits and the subunits of nicotinic receptors from muscle are all part of a gene family with many similar structural features. The genes encoding both subunits of the major subtype of neuronal nicotinic receptors in mammalian brains have been identified as $\alpha 4$ for acetylcholine-binding subunits and $\beta 2$ for structural subunits. Another subtype composed of $\alpha 3$ acetylcholine-binding subunits and $\beta 2$ subunits is the major subtype in ganglia and retina. Another subtype which is present in substantial amounts in chicken brains, but in small amounts in mammals, is composed of $\alpha 2$ acetylcholine-binding subunits and $\beta 2$ structural subunits. Identifying the genes encoding subunits has involved the determination of N-terminal amino acid sequences of purified subunits, cloning and sequencing of subunit cDNAs, making subunit-specific monoclonal antibodies to purified receptors, and making antibodies to bacterially expressed peptides from fragments of subunit cDNAs. Receptors have been histologically

localized in brains and retinas of several species, and found to be extensively distributed throughout the brain. Many neuronal nicotinic receptors may be localized on extrasynaptic or presynaptic processes rather than just in postsynaptic membranes, as is the case for receptors in muscle and ganglia. The diversity of neuronal nicotinic receptor subtypes may reflect the diversity of functional roles which they are called upon to perform. These studies, for the first time, purify neuronal nicotinic receptors and define their subunit compositions. This is critical to understanding their detailed structure, understanding their functional roles, and studying their pharmacology. Human neuronal nicotinic acetylcholine receptors have been immunoisolated using subunit-specific monoclonal antibodies, and their pharmacological properties characterized. It will be important to use the cDNA probes now available from other species to identify cDNAs for the subunits of human neuronal nicotinic receptors so that they can be expressed in cell lines and structurally and pharmacologically characterized.

Neuronal α-bungarotoxin-binding proteins are enigmatic, but potentially important, membrane proteins which outnumber neuronal nicotinic receptors. These proteins bind cholinergic ligands, but their endogenous ligand, their functional properties, and their functional roles are unknown. Cloned cDNAs for two subunits of α-bungarotoxin-binding proteins have been sequenced. This reveals that they are members of the ligand-gated ion channel gene family. Antisera and monoclonal antibodies to bacterially expressed fragments of these subunits bind to native α-bungarotoxin-binding proteins, proving the identity of the cDNA clones, and provide evidence that suggests the existence of subtypes of α-bungarotoxin-binding proteins. These experiments, for the first time, provide cDNAs and subunit-specific monoclonal antibodies for α-bungarotoxin-binding proteins, thereby providing the critical tools which can be used to reveal the subunit structure, functional properties, and functional role of this black sheep of the nicotinic receptor gene family.

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, 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 investigators have adhered to policies of applicable Federal Law 45CFR46.

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INTRODUCTION

Significance of Nicotinic Receptors

Although the U.S. and U.S.S.R. are negotiating a ban on chemical weapons, these weapons have recently proliferated to many other countries and have been used by them in their regional conflicts. Terrorists could make such weapons more easily than nuclear weapons and could use them with devastating effects. Thus, the problem of defense against chemical weapons remains a real and difficult problem.

Neurotoxic agents such as sarin, soman, and tabun act primarily by inhibiting acetylcholinesterase. This causes acetylcholine released at nerve endings to persist for long periods at high concentrations, first excessively activating acetylcholine receptors and then inactivating them by desensitization. Some of these agents may also directly impair receptor function. Death can result from paralysis of respiratory muscles. Effects on both acute and chronic brain function may be severe, but are less well characterized.

To understand precisely the effects of neurotoxic agents like sarin and to help devise defenses against them, it is important to understand the structure of acetylcholine receptors. The research described here is quite basic and far removed from the immediate practicalities of defense against chemical warfare. However, because it is basic research, at the cutting edge of its field, it is applicable not only to chemical defense but also to many practical medical problems.

The basic research described here can provide not only conceptual information about receptor properties, but also practical tools for evaluating the effects on receptors of chemical warfare agents, of drugs used to treat the effects of chemical warfare agents or used prophylactically against them, and of other drugs. The best such practical tools would be cell lines that express receptors and permit easy measurement of their pharmacological and electrophysiological properties. The research reported here describes the development of such a cell line for studies of human muscle nicotinic acetylcholine receptors and has provided much information required for making cell lines that express subtypes of human neuronal nicotinic receptors.

Acetylcholine acts on two broad classes of receptors termed "nicotinic" or "muscarinic" because of their pharmacological properties. These are quite distinct in their structure and function. Nicotinic acetylcholine receptors have an integral acetylcholine-gated cation channel which is formed by several homologous subunits organized around the channel like barrel staves. These receptors are involved in nerve signaling at the millisecond time scale. Muscarinic acetylcholine receptors act

via coupling proteins and second messengers, and consequently they are involved in slower signaling that involves longer latencies and longer durations. Nicotinic[1] and muscarinic[2] receptors are not structurally related and are members of two different gene families which, together, are responsible for encoding all neurotransmitter receptors that have been characterized.

The studies reported here are concerned only with nicotinic acetylcholine receptors and another member of the same gene family with similar pharmacological properties, neuronal α-bungarotoxin-binding proteins.

Chemical warfare agents kill through muscle paralysis via their effects on muscle nicotinic receptors. Their effects on the central nervous system may be mediated by nicotinic receptors, which are being shown, in part through the research reported here, to be located throughout the nervous system. The combination of cholinergic drugs used as prophylactics and treatment for these chemical warfare agents surely has direct effects on nicotinic receptors of muscles and nerves and on α-bungarotoxin-binding proteins, but these effects are currently poorly characterized.

Many medical issues involve nicotinic acetylcholine receptors.[1] Short-acting nicotinic receptor antagonists are used as muscle relaxants during surgery. Local anesthetics and perhaps general anesthetics can impair receptor function. Nicotine acts on neuronal nicotinic receptors to produce the pleasure experienced by and addiction characteristic of smokers. Some insecticides (of which nicotine is an example) can act directly on nicotinic receptors, and others also act indirectly, like chemical warfare agents, through inhibition of acetylcholinesterase. Venoms from cobras, kraits, some frogs, and some corals contain toxins that block nicotinic receptor function. Drugs of abuse, such as phencyclidine, can inhibit nicotinic receptor function. Autoantibodies to muscle nicotinic receptors cause the weakness characteristic of myasthenia gravis. Neuronal nicotinic receptor amounts are reduced in Alzheimer's and Parkinson's diseases.

Introduction to Our Studies of the Past Three Years

Our studies have concerned the three known branches of the nicotinic acetylcholine receptor gene family: muscle nicotinic receptors, neuronal nicotinic receptors (of which there are several subtypes), and neuronal α-bungarotoxin-binding proteins (of which there also appear to be subtypes). This gene family is part of a gene superfamily of ligand-gated ion channels which includes receptors for glycine, γ-aminobutyric acid, and, probably, excitatory amino acids[3]; and it is distinct from the gene family which encodes receptors for muscarinic acetylcholine receptors, adrenergic receptors, and rhodopsin,[2] or the gene family which encodes membrane-potential-gated ion channels.[4]

Muscle nicotinic receptors are the best-characterized neurotransmitter receptors and the best-characterized membrane ion channels (for reviews, see references 1,5,6). In addition to their intrinsic significance, they serve as models for the study of other receptors and channels. Nicotinic receptors of fish electric organs have been intensively studied because they closely resemble those in skeletal muscle, but are present in much larger amounts. Nicotinic receptors of electric organs and muscle bind α-bungarotoxin with great affinity and specificity. Snake venom toxins have been used for quantitation, localization, and affinity purification of these receptors. Monoclonal antibodies (mAbs) are proving to be useful probes for other parts of the molecule. Human muscle available from biopsies, amputations, or autopsies contains too little nicotinic receptor to be useful for structural and pharmacological studies. Our recent studies[7] now provide a cell line which produces substantial amounts of human muscle nicotinic receptors which can be studied electrophysiologically and pharmacologically, and whose pharmacological properties are significantly different from the rodent muscle nicotinic receptors previously studied.[8]

Neuronal nicotinic receptors are only beginning to be studied at the molecular level[1] through the use of mAb probes,[9-23] which we have exclusively developed, and cDNA probes,[24-33] to which we have made a significant contribution.[34-37] The roles of neuronal nicotinic receptors in normal, much less pathological, brain function are not well characterized. Whereas muscle nicotinic receptors evolved to their current form by the time of the appearance of marine elasmobranchs like Torpedo californica some 400 million years ago, and their amino acid sequences remain highly conserved between species as distant as Torpedo and human,[38] neuronal nicotinic receptors appear to be evolving rapidly over this same interval, as the central nervous system has rapidly evolved; and several subtypes of neuronal nicotinic receptors have evolved whose sequences and uses differ significantly between species, as will be described later. There are differences in subunit composition between muscle and neuronal nicotinic receptors which our studies have defined in some detail. Neuronal nicotinic receptors, unlike muscle nicotinic receptors, do not bind α-bungarotoxin and have many other pharmacological differences. Neuronal nicotinic receptors typically have much lower affinity for antagonists, some of which appear to act noncompetitively to block the cation channel rather than the acetylcholine binding site.[1] Some subtypes of neuronal nicotinic receptors have very high affinity for nicotine. But, in general, the pharmacological properties of the various subtypes and their functional roles remain to be determined. The genes that encode the subunits of the various subtypes, and thereby critically define the subtypes, are beginning to be determined, and the differential localization of the various subtypes is beginning.

Neuronal α-bungarotoxin-binding proteins outnumber neuronal nicotinic receptors, but their function is not known.[1] They bind

cholinergic ligands, but their endogenous ligand is unknown. They do not appear to be acetylcholine-gated cation channels,[39,40] as are nicotinic receptors from muscle and neurons, yet the sequence of their subunit cDNAs which we have discovered appear to be compatible with this function. Their functional role is unknown, but their widespread distribution in the nervous system[41] suggests that they may plan an important role. Our studies of neuronal α-bungarotoxin-binding proteins have for the first time allowed us to obtain subunit cDNAs which prove that these proteins are members of the ligand-gated ion channel gated superfamily. These studies provide critical tools which, along with subunit-specific mAbs we have developed, should allow us in future studies to characterize the subunit structure of these proteins as we have neuronal nicotinic receptors, and which may permit us to discover the function of these enigmatic proteins.

The basic research to which this contract has contributed has resulted in the publication of 25 manuscripts in well-regarded scientific journals and books, and will result in the publication of several more such articles.

The following section reviews in detail progress during the three years of the contract period.

PROGRESS DURING THE PAST THREE YEARS

Muscle-Type Nicotinic Receptors

Torpedo Electric Organ Acetylcholine Receptors

Receptors in electric organ membranes have been shown by electron microscopy of two-dimensional crystalline arrays of receptors to consist of five rod-like subunits organized around a central cation channel.[42,43] We provided subunit-specific mAb and lectin probes to Nigel Unwin, who used these to identify the individual subunits within the receptors,[44] as shown in Figures 1 and 2. These studies revealed that the subunits are arranged around the channel in the order $\alpha\beta\alpha\gamma\delta$ (Figure 2). Receptors in Torpedo, unlike those in muscles or nerves, are linked into dimers by a disulfide bond between the δ subunits of adjacent receptors.[45] The studies shown in Figure 1 suggest that the acetylcholine binding sites on α subunits, which are labeled by α-bungarotoxin, are located within the α subunit as it is viewed from above, whereas the main immunogenic regions, which are labeled by mAb 35, are located on the side of the α subunits opposite to the lumen of the cation channel.

Amino acids that contribute to the binding site for α-bungarotoxin on α subunits were mapped[46] to within the sequence α189-194, as shown in Figure 3. Using synthetic peptides corresponding to sequences of α subunits (Table 1) we[46] were able to confirm the work of others[47,48] that α-bungarotoxin binds within the sequence α189-194. Cysteines α192,193 are known to be linked by a disulfide bond[49] and known to react specifically with affinity labels for the acetylcholine binding site.[50] These cysteines seem critical for binding of α-bungarotoxin to the peptide, since 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.[46]

Amino acids which contribute to epitopes recognized by antibodies to α subunits of receptors from Torpedo were mapped by immunoprecipitation of ^{125}I-labeled synthetic α subunit peptides,[46] as shown in Figure 4. This method could not detect significant binding to synthetic peptides of mAbs directed at the main immunogenic region, presumably because the immunogenicity of this region depends strongly on the native conformation of the receptor.[51] It did indicate that much of the sequence between α300 and α400 contains prominent epitopes recognized by antibodies to denatured subunits, in contrast to much of the N-terminal part of the subunit. Previously we had demonstrated, using mapped mAbs, that much of the sequence between α300 and α400 in native receptors is part of a loosely structured domain on the surface of the protein exposed to the cytoplasm.[52,53] Much of the N-terminal part of the α subunit is thought to form the extracellular part of the receptor.

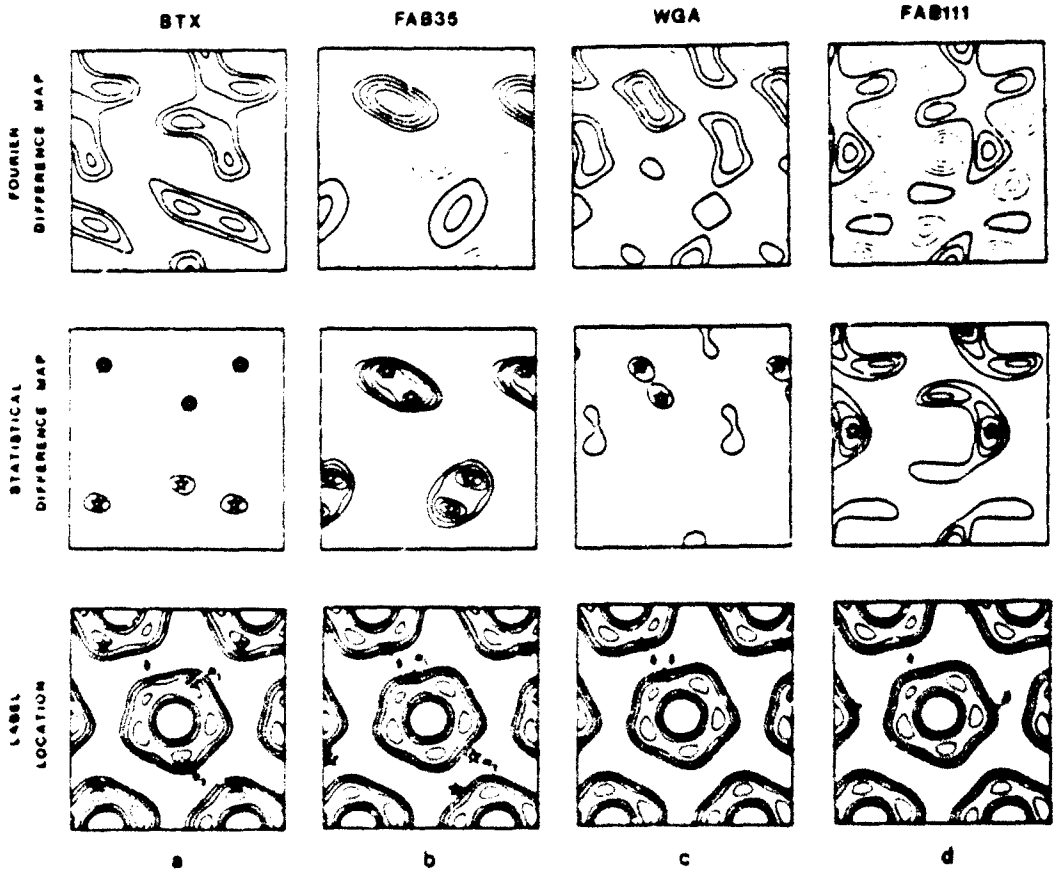

Figure 1. Identification of binding sites for α-bungarotoxin, Fab 35, WGA, and Fab 111 on receptors from Torpedo marmorata electric organ, as viewed from the synaptic side of the membrane. The Fourier difference maps (top) show positive (continuous contours) and negative (dotted contours) peaks, corresponding, 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 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 (θ) relates the pair of receptors tentatively identified previously as the δ subunit-linked dimer. Reproduced from reference 44.

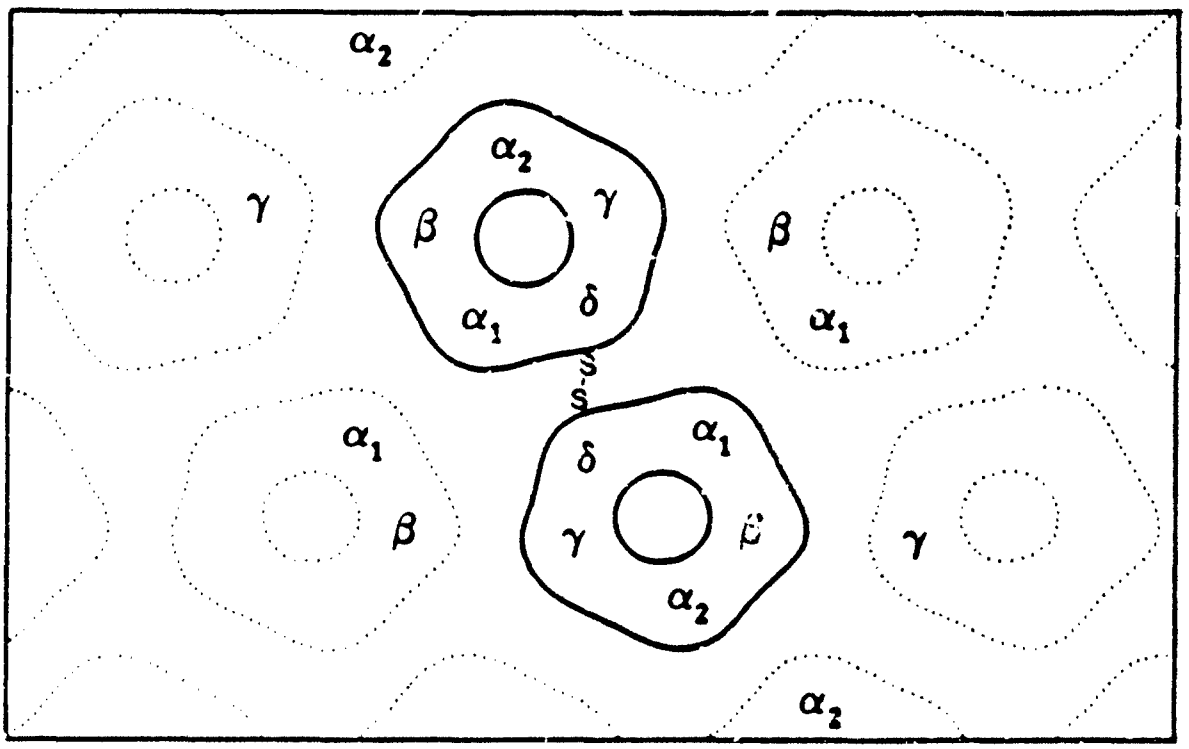

Figure 2. The arrangement of subunits around receptors in Torpedo electric organ determined by the experiments shown in Figure 1 Reproduced from reference 44.

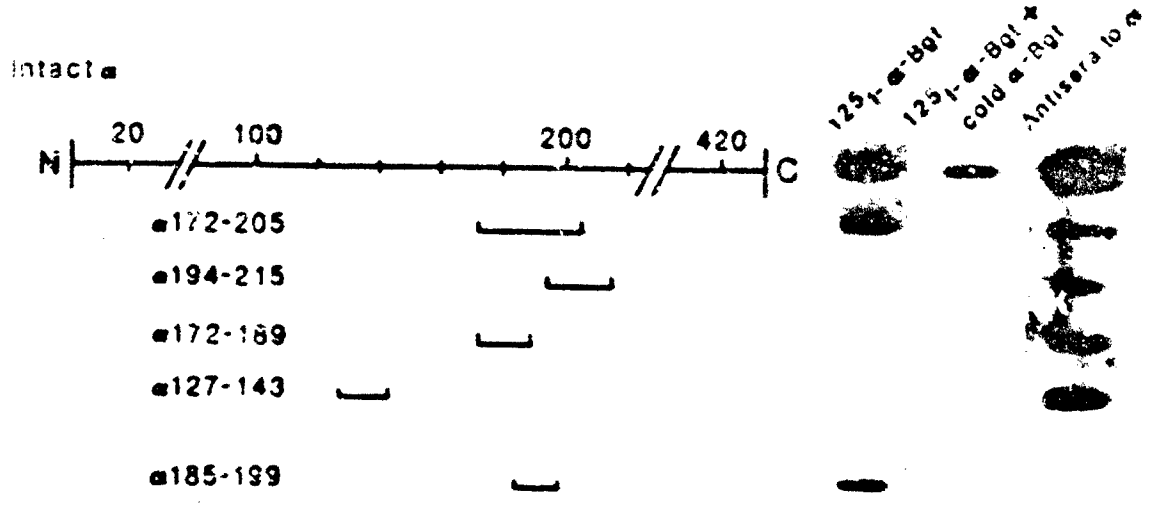

Figure 3. Binding of ^{125}I-α-bungarotoxin (α-Bgt) to synthetic peptides from Torpedo californica acetylcholine receptor α subunits. Aliquots (25 μl) of 1x10^{-7} M synthetic peptide in 16 mM borate buffer, pH 9.0, were applied to a Biodyne immunoaffinity membrane via a slot-blot apparatus. Remaining reactive sites were quenched overnight with 5% Carnation dried milk in Tris buffer (see Methods) and washed with five changes of 0.5% Triton X-100 in PBS + NaN$_3$. ^{125}I-α-bungarotoxin (5x10^{-9} M) was applied in the Carnation milk quench buffer plus or minus 2x10^{-7} M unlabeled α-bungarotoxin. 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 2.8x10^{-6}M α185-199 was required to bind α-bungarotoxin, 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}I-α-bungarotoxin. Note that excess unlabeled α-bungarotoxin inhibits all binding of ^{125}I-labeled α-bungarotoxin 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 46.

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]α[illegible]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	DEESSIAAEEWKYVAMVIDHY
[Tyr-427]α427–437	YGRLIELSQEG

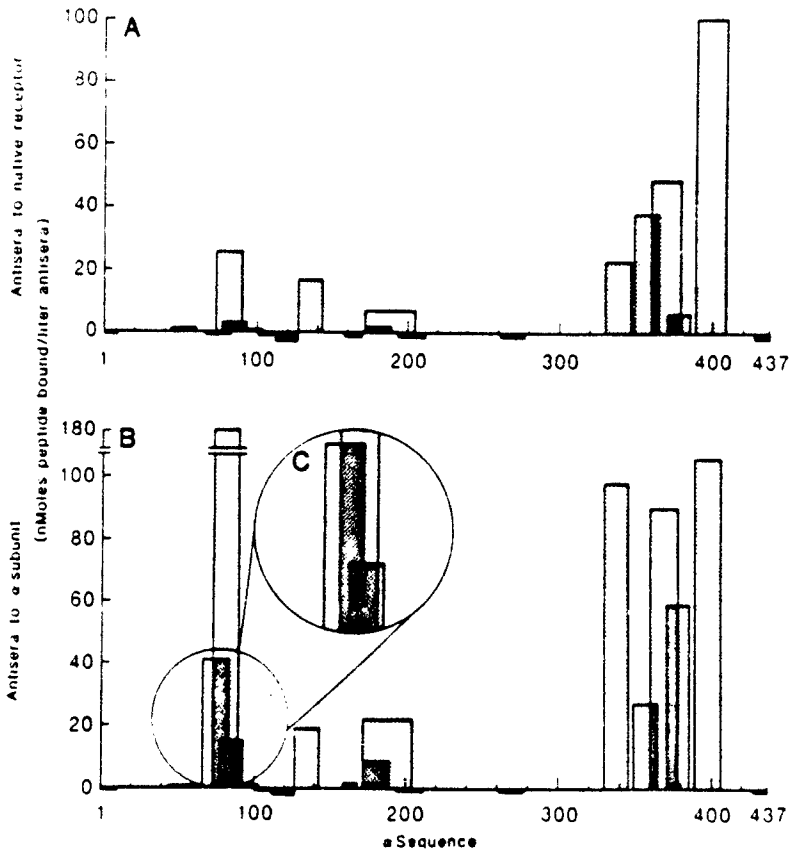

Figure 4. Reaction of synthetic peptides with antisera to native Torpedo californica electric organ acetylcholine receptor and to its denatured α subunits. Synthetic peptides corresponding to segments of the α subunit sequence, indicated by the bars, were labeled with ^{125}I as described in Methods. Antisera (5-10 μl) were assayed by reacting with ^{125}I-peptide (10 nM) in 100 μl of 10 mM sodium phosphate buffer (pH 7.5), 100 μM NaCl, 0.5% Triton X-100, and 10 mM NaN_3. The immune complexes were precipitated with 100 μl 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 α subunits. Insert C shows a magnified view of the overlap which occurred with antisera to α subunits versus [Tyr-83]α66-83 and [Gly 89,Tyr 90]α73-90 and α78-93. Reproduced from reference 46.

Amino acids contributing to epitopes on α subunits were subsequently mapped at much higher resolution using the Geysen synthetic peptide technique,[54,55] as shown in **Figure 5**. The complete sequence of α subunits of receptor from Torpedo was synthesized as a series of 430 overlapping octamers (peptide 1=amino acids 1-8, peptide 2=amino acids 2-9, etc.). The peptides were synthesized on plastic pegs in a format which fit into 96-well microtiter plates to permit assay of antibodies bound to the peptides with peroxidase-labeled anti-antibody. After each assay the bound antibodies were removed and the peptides reused. Both synthesis and assays were monitored by computer to manage the large amount of data involved.

Figure 5 shows typical results. Part A shows the pattern obtained using rabbit antisera raised to α subunits of receptors from Torpedo. There are several prominent epitopes. The pattern is consistent with results we obtained previously by immunoprecipitation of synthetic peptides[52,53] (**Figure 4**). Of course, on native receptor there are epitopes which depend on the native conformation of the receptor, and these will not be detected by this technique. Part B of **Figure 5** shows that using an affinity column with the synthetic peptide α152-167, you can affinity purify antibodies directed at the epitopes in that sequence from rabbit antiserum to α subunits. This validates the specificity of the Geysen approach. This epitope is interesting because all antibodies raised to denatured subunits are thought to bind to the cytoplasmic surface of the receptor.[56] If this sequence were exposed on the cytoplasmic surface, it would mean that there were at least two transmembrane domains prior to the first hydrophobic sequence in α. However, these antibodies are not adsorbed by reasonable excesses of native receptor, and these affinity-purified antibodies do not bind well to native receptor in membranes as detected by electron microscopy with colloidal gold labeling. Also, mAbs to this epitope do not bind to the native receptor. Part C of **Figure 5** shows that rat antisera to α subunits detect the same basic pattern of epitopes detected by rabbit antisera to α subunits. Part D of **Figure 5** shows that we have mAbs directed at several of the prominent epitopes on α subunits. We had previously mapped these mAbs[52,53] to the same epitopes by immunoprecipitation of synthetic peptides, and in some cases by peptide mapping, confirming the Geysen technique. mAb 236 does not bind to native receptor, whereas mAbs 142 and 147 bind to the cytoplasmic surface. These results show that the sequence between α350 and α370 is on the cytoplasmic surface of the native receptor.

The use of the Geysen technique to precisely map the epitopes for subunit-specific mAbs is further illustrated in **Figure 6**.

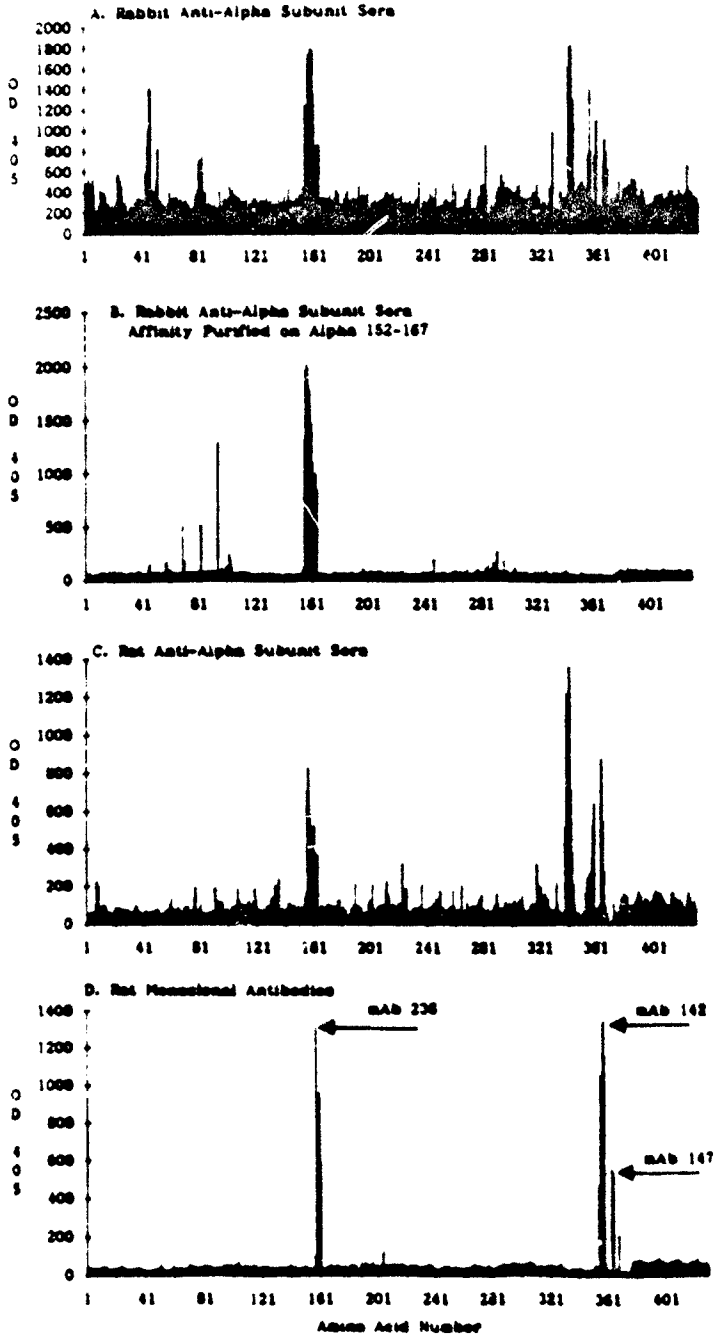

Figure 5. Mapping epitopes on α subunits of receptors from Torpedo using the Geysen synthetic peptide technique. As described in Methods, the indicated antisera, affinity-purified antibodies, and cocktail of mAbs were assayed in solid phase using peroxidase-labeled anti-antibody for binding to 430 overlapping synthetic peptide octamers corresponding to the complete sequence of α subunits from receptors from Torpedo californica. Reproduced from reference 55.

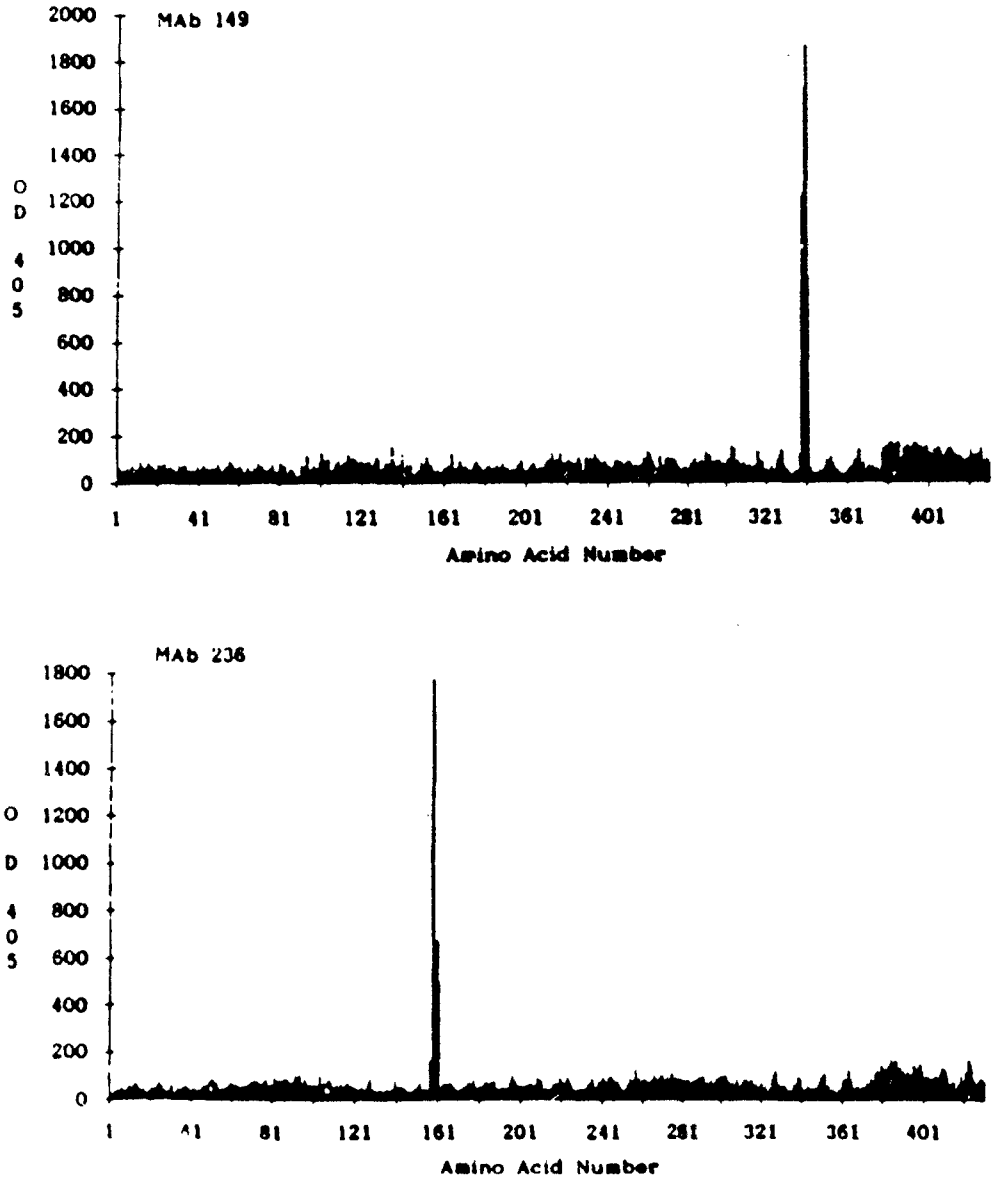

Figure 6. Mapping epitopes on <u>Torpedo</u> acetylcholine receptor α subunits for mAbs by the Geysen synthetic peptide technique.

The mAbs whose epitopes are mapped in Figures 5 and 6, as well as other mapped mAbs, have previously been tested for binding to the extracellular or cytoplasmic surface of acetylcholine receptors in electric organ membranes.[52,53] Precise mapping of their epitopes provides precise information about the transmembrane orientation of specific sequences of the α subunit sequence, which helps to determine its overall topology.

The Geysen technique[54] has advantages and disadvantages. It offers very high resolution epitope mapping using large numbers of synthetic peptides which are relatively quick and easy to synthesize. Several of the mAbs in our library to Torpedo receptor α subunits have been mapped in this way. However, it is expensive in reagents ($4,000-5,000/subunit), and there is no quality control possible on the peptides produced, in contrast to conventionally synthesized peptides that can be purified and characterized. Thus, if an antibody does not bind, it could be because the peptide epitope synthesis did not proceed correctly. Finally, the Geysen technique only detects the binding of antibodies that do not depend absolutely on the native conformation of the receptor and that react with very short synthetic peptides. The α subunit peptides used in Figure 5 did not react detectably with mAbs to the main immunogenic region.

By using another synthetic peptide technique we were able to map the epitope for mAbs to the main immunogenic region. Recently Tzartos and coworkers[57] were able to detect binding of anti-main immunogenic region mAbs to shorter peptides by using an assay in which the peptides were bound to polylysine-coated plastic microwells. We were able to confirm and extend these results, as shown in Figure 7 and summarized in Figure 8. Many mAbs to the main immunogenic region bind to the peptide α68-76. Thus, some of the amino acids that form the main immunogenic region are in this sequence. Some mAbs, such as mAb 35, appear to be absolutely conformation dependent. Presumably these mAbs recognize amino acids that are contiguous only in the native conformation of the receptor, whereas those mAbs that bind to synthetic peptides probably recognize several consecutive amino acids in the peptide as part of their epitope. Curiously, mAbs to the main immunogenic region bind to muscle nicotinic receptors from essentially all species tested (e.g. Torpedo, Electrophorus, Rana, chickens, mice, rats, cattle, and humans), except Xenopus.[58] The mAbs tested also do not bind to the Xenopus sequence α66-76. This establishes the biological significance of the binding assay and suggests that asparagine α68 and aspartate α71 are important contact residues. In the Xenopus peptide[59] α68 is aspartate and α71 is a lysine, altogether nonconservative changes. Removing α68 converts the human sequence α68-76 from one which binds mAb 210, to α69-76, which does not, further demonstrating the importance of asparagine α68. These results permit us to map this important epitope with fairly high resolution. The function of this part of α subunits is unknown; mAbs bound to it do not interfere with ligand binding or channel function. It is a pathologically important epitope in myasthenia gravis.[60] The highly conserved structure of the main immunogenic region suggests that it may be important for an aspect of receptor function yet to be appreciated.

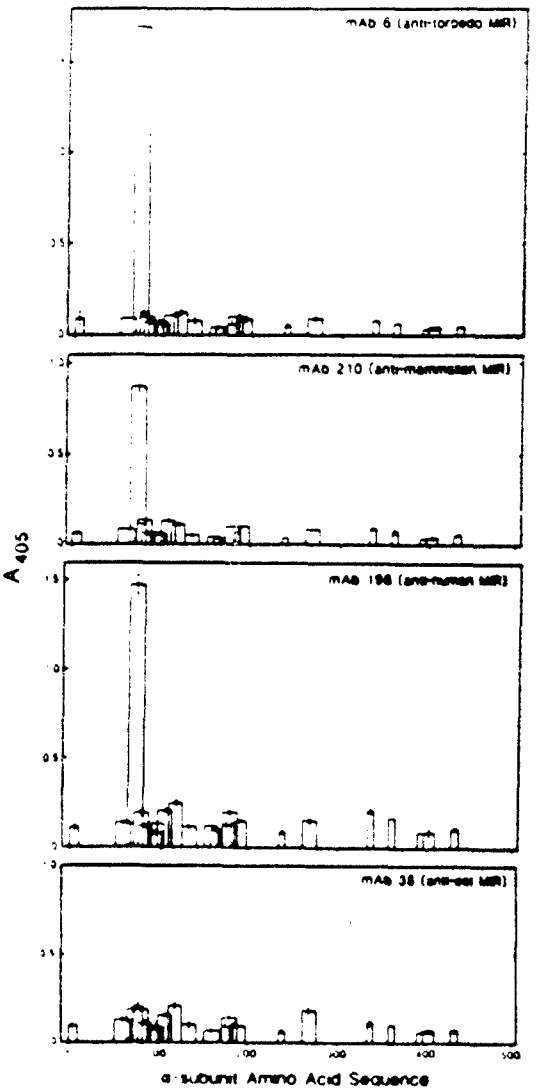

Figure 7. Mapping the main immunogenic region epitope on the sequence of α subunits by an enzyme-linked immunoassay using synthetic Torpedo acetylcholine receptor α subunit peptides coupled to polylysine-coated microwells to detect binding of mAbs to the main immunogenic region. Peptides were glutaraldehyde coupled to polylysine-coated wells. PBS containing 1% bovine serum albumin, 1% ovalbumin, and 0.1% Tween 20 to inhibit nonspecific binding was used to quench and wash the wells and for all subsequent incubations. mAbs at concentrations >1 μM were allowed to bind overnight. After three washes bound mAbs were detected using an anti-rat IgG mAb coupled to peroxidase.

Synthetic peptides that *bind* to MIR mAbs:

α	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	
	R	W	N	P	A	D	Y	G	G	I	K	K	I	R	L	P	S	(tyr 83) *Torpedo* α66-83
	K	W	N	P	D	D	Y	G	G	V	K							Human α66-76
			N	P	D	D	Y	G	G	V	K							Human α68-76

Synthetic peptides that *do not bind* to MIR mAbs:

α	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	
	K	W	D	P	A	K	Y	G	G	V	K							*Xenopus* α66-76
				P	D	D	Y	G	G	V	K							Human α69-76
					D	D	Y	G	G	V	K							" α70-76
						D	Y	G	G	V	K							" α71-76
		W	N	P	D	D			could these have failed									" α67-71
		W	N	P	D	D	Y		for lack of K to glutar-									" α67-72
			N	P	D	D	Y	G	aldehyde link in ELISA?									" α68-73
...																		*Torpedo* α44-60
								G	G	I	K	K	I	R	L	P	S ...	" α73-90
													I	R	L	P	S ...	" α78-93
																		" α1-11
																		" α44-60
																		" α89-104
																		" α100-116
																		" α112-127
																		" α127-143
																		" α159-170
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																		" α194-212
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Figure 6. Summary of the reaction of synthetic peptides with mAb 210 using the enzyme-linked immunoassay described in Figure 7.

Why mAbs to the main immunogenic region should react detectably with synthetic peptides in an ELISA assay, but not with the same synthetic peptides in immunoprecipitation assays, is not entirely clear. In part it is probably because immunoprecipitation assays depend on the microscopic affinity of a single mAb binding site for a single soluble peptide, whereas in an ELISA, avidity greatly increases if peptides on the solid phase are close enough so that both binding sites on a mAb can bind simultaneously. Soluble peptide in huge molar excess is not effective at inhibiting mAb binding to the same peptide on the solid phase. Quantitation of the solid-phase assay using ^{125}I-mAb 210 or ^{125}I-peptide indicates that only 0.2% of the bound peptide can bind mAb 210, but this mAb 210 binding occurs with very high affinity ($K_D=3\times10^{-8}$ M) as compared to the affinity of mAb 210 for native receptor (9.2×10^{-10} M). Attempts to synthesize soluble peptides with high affinity for mAb 210 by synthesizing dimers of α66-76 or dimers of α68-76 joined by a 10-glycine linker have not succeeded in making a soluble multimer with high affinity for mAb 210. Thus, either a peptide has not been synthesized which has the proper conformation to permit bivalent binding by the mAb, or some other mechanism than multivalent binding is responsible for the small amount of high-affinity binding that produces signal in ELISA assays. It may be that in glutaraldehyde cross-linking peptides to polylysine-coated microwells, the reaction product somehow occasionally mimics the native conformation of the main immunogenic region.

A summary analysis of structural features of the α subunits of Torpedo acetylcholine receptors determined using mAbs and synthetic peptides is presented in Figure 9. Structural features are related to the amino acid sequence of the α subunit. The sequence is analyzed theoretically by standard analyses for hydrophobicity, charge, and propensity for forming α helices or β-pleated sheet structures for comparison with the locations of structural features and antibody binding sites. A plot of a Geysen analysis of the binding of rat antisera to denatured α subunits reveals the location of all major epitopes on denatured α subunits. Mapping of epitopes for mAbs in our library reveals that there are mAbs that recognize all major epitopes. In addition, there are mAbs that recognize minor epitopes. These were often prepared using synthetic peptides as immunogens to drive the immune response to poorly immunogenic sequences. Where the transmembrane orientation of the epitope for the mAbs has been determined, it is indicated. This information provides most of the support for determination of the extracellular or cytoplasmic orientation of the polypeptide chain indicated at the top of the figure.

George Hess at Cornell University is investigating the possibility of using yeast cells transformed using cloned cDNAs for receptor subunits to express large amounts of receptor proteins which would otherwise be available only in tiny amounts. If successful, this approach would be very valuable for providing

Figure 9. Summary of the location of epitopes on α subunits for several mAbs which have been mapped. The side of the membrane to which the mAbs bind was determined by electron microscopy and used to determine the transmembrane orientation in the native receptor of the sequence recognized by the mAb.

receptors for biochemical studies, pharmacological characterization, or for use in practical applications that might require large amounts of receptor protein. As a model system, he chose to try to express Torpedo acetylcholine receptors. We have collaborated with him, providing biochemical and immunochemical characterization of the receptor subunits synthesized by the transfected yeast prepared in his laboratory.[61-64]

First, a yeast strain was transformed only with Torpedo receptor α subunits. Small amounts of subunits could be detected in Western blots. Their properties were studied in a sandwich radioimmunoassay. A mAb to α subunits (mAb 173) coated on plastic microwells was used to immobilize detergent-extracted α subunits, whose properties were then characterized by the binding of ^{125}I-labeled ligands (Figure 10). α subunits expressed in yeast were as effective as SDS-denatured purified α subunits or native receptor in binding ^{125}I-mAb 142 (Figure 10A). This mAb binds to the sequence α349-365 on the cytoplasmic surface of α subunits (Figures 5 and 6). This part of the receptor appears very flexible and can achieve similar conformations in both native and denatured receptor. α subunits expressed in yeast were intermediate in affinity for ^{125}I-mAb 236, between denatured α subunits (which had high affinity for this mAb) and native receptor (which had very low affinity) (Figure 10B). mAb 236 was made to the synthetic peptide α152-167 and basically cannot bind to α subunits in their native conformation, but it detects an intermediate conformation in α subunits expressed in yeast. α subunits expressed in yeast bind ^{125}I-mAb 210 with an affinity intermediate between native receptors (which have high affinity) and denatured α subunits (which have low affinity) (Figure 10C). mAb 210 binds to the main immunogenic region of native receptor with high affinity and binds with low affinity, but high specificity, to the synthetic α subunit peptide α66-76 (Figures 7 and 8). α subunits expressed in yeast have much higher affinity for ^{125}I-mAb 35 than do denatured α subunits, but much lower affinity than do native receptors (Figure 10D). mAb 35 binds to the main immunogenic region of native receptor with high affinity, but has no detectable affinity for synthetic α subunit peptides (Figure 7). The results with mAbs 210 and 35 suggest that α subunits in yeast exhibit conformations of their main immunogenic regions intermediate between denatured and native receptors. α subunits expressed in yeast bind ^{125}I-α-bungarotoxin more avidly than do denatured α subunits, but much less avidly than do native receptors (Figure 10E). Carbamylcholine and curare are effective at inhibiting ^{125}I-α-bungarotoxin binding to native receptor, but not to α subunits expressed in yeast or to denatured α subunits (Figure 10F). These results suggest that the acetylcholine binding site of α subunits expressed in yeast, like the main immunogenic region, is in a conformation intermediate between native and denatured. Thus, when all are compared solubilized with nondenaturing detergents, the properties of the expressed α subunits closely resemble those of the intermediates of receptor synthesis detected in mouse BC3H-1 cells[51] and the unassembled α subunits detected in human TE671 cells to be described subsequently.

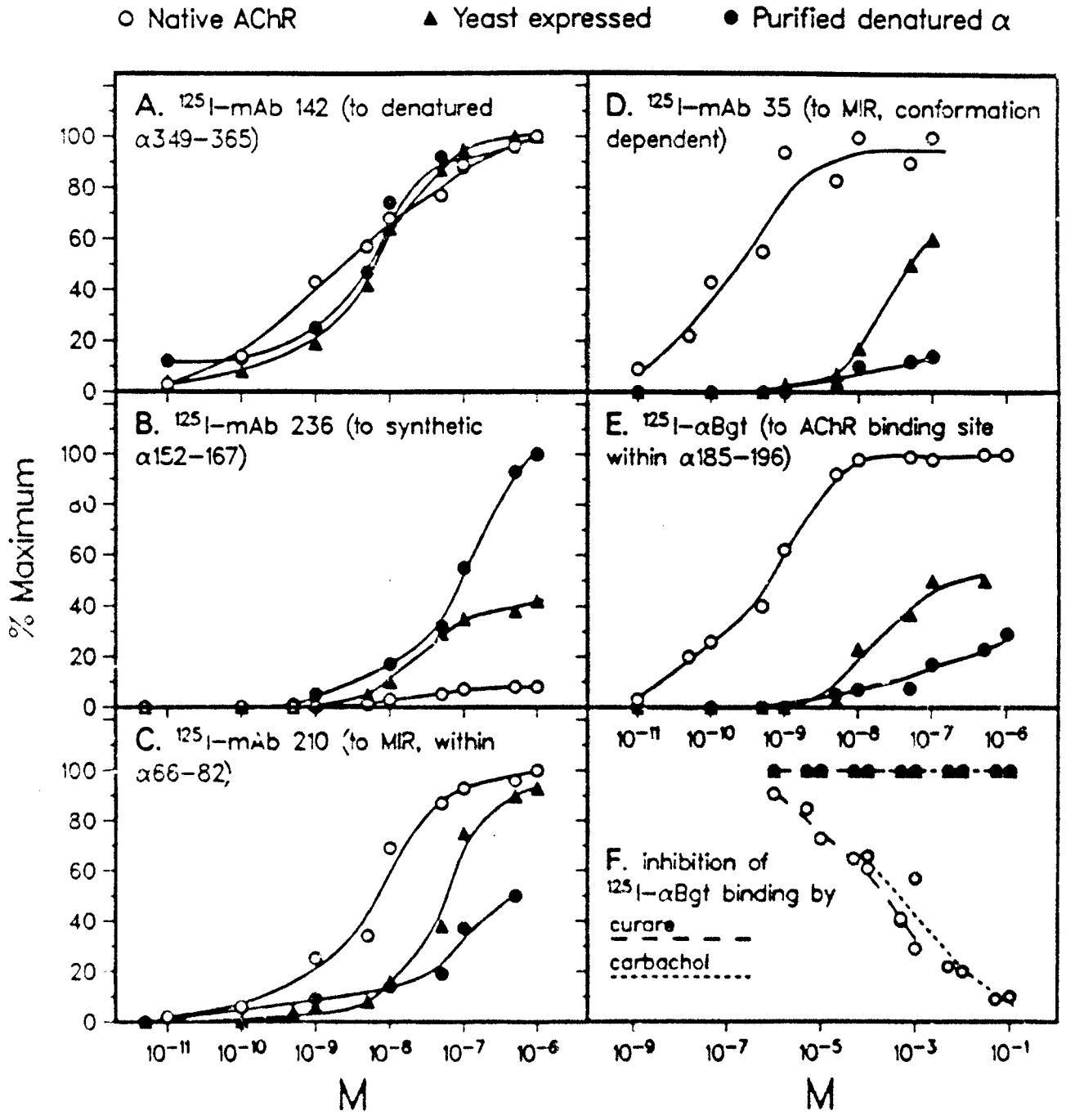


Figure 10. *Properties of Torpedo nicotinic receptor α subunits expressed in yeast cells compared with SDS-denatured α subunits and native receptors by a sandwich radioimmunoassay. Plastic microwells coated with mAb 173 were used to immobilize receptor or subunits and then binding of ^{125}I-labeled ligands was measured.*

A yeast strain was transformed simultaneously with cDNAs for α, β, γ, and δ subunits of receptors from Torpedo electric organ.[64] mRNA extracted from this strain was injected into Xenopus oocytes and shown to code for the synthesis of functional receptors. Thus, transcription of Torpedo receptor subunits in the yeast cells was effective. However, only small amounts of subunit proteins could be detected by Western blots, and no β subunit protein was detected. It was found that by making a chimera substituting most of the α subunit signal sequence for the β signal sequence, and by growing the yeast at 5°C, detectable amounts of β subunits could be made (Figure 11). In any case, the α subunits retained the properties of the synthetic intermediate and did not assemble efficiently with the other subunits to form native receptors. There is clearly a translation or posttranslation problem in yeast which impairs their ability to synthesize large amounts of subunits and properly assemble them into native nicotinic receptors. One problem may be proteolysis of these foreign proteins in the yeast. Another problem may be different properties of the yeast membranes. For example, native receptors and α subunits can be easily solubilized from Torpedo electric organ, BC3H-1 cells, TE671 cells, etc., using 0.5% Triton X-100 (Boehringer Mannheim, Indianapolis, IN). However, solubilization of receptor subunits from yeast required a mixture of detergents: 0.5% Zwittergent 3-14 (Calbiochem, San Diego, CA), 2% Triton X-100, and 0.05% SDS (Sigma, St. Louis, MO). A third problem could be incompatible "chaperonins"[65] required to assist assembly of multisubunit proteins like receptors. Whatever the reason, so far, yeast have not proven to be an effective system for synthesizing receptors from their cDNAs.

Injection of synthetic mRNAs into Xenopus oocytes has been found by several investigators to be an effective way to express small amounts of acetylcholine receptors from their subunit cDNAs.[26,28-30,66]

We used the Xenopus oocyte expression system to express various combinations of Torpedo acetylcholine receptor subunits, as shown in Figure 12. When α subunits were expressed alone or in combination with β subunits, the α subunits did not exhibit substantial affinity for α-bungarotoxin. Other experiments (not shown) using subunit-specific mAbs to locate α and β subunits showed that substantial amounts of the subunits were made, but that they formed amorphous aggregates of many sizes, distributed from the 5S size of single subunits to much larger aggregations. When α subunits were expressed in combination with either γ or δ subunits, the conformation of the α subunit matured so that it not only exhibited affinity for α-bungarotoxin, but also specifically bound the small cholinergic ligand carbamylcholine. Also, the α subunits efficiently bound to γ or δ subunits to form a unique complex of about the size expected for a pair of subunits. Coexpression of α, γ, and δ subunits resulted in a still larger complex nearly the size of mature receptors, as well as some complexes the size of subunit pairs. Then addition of β

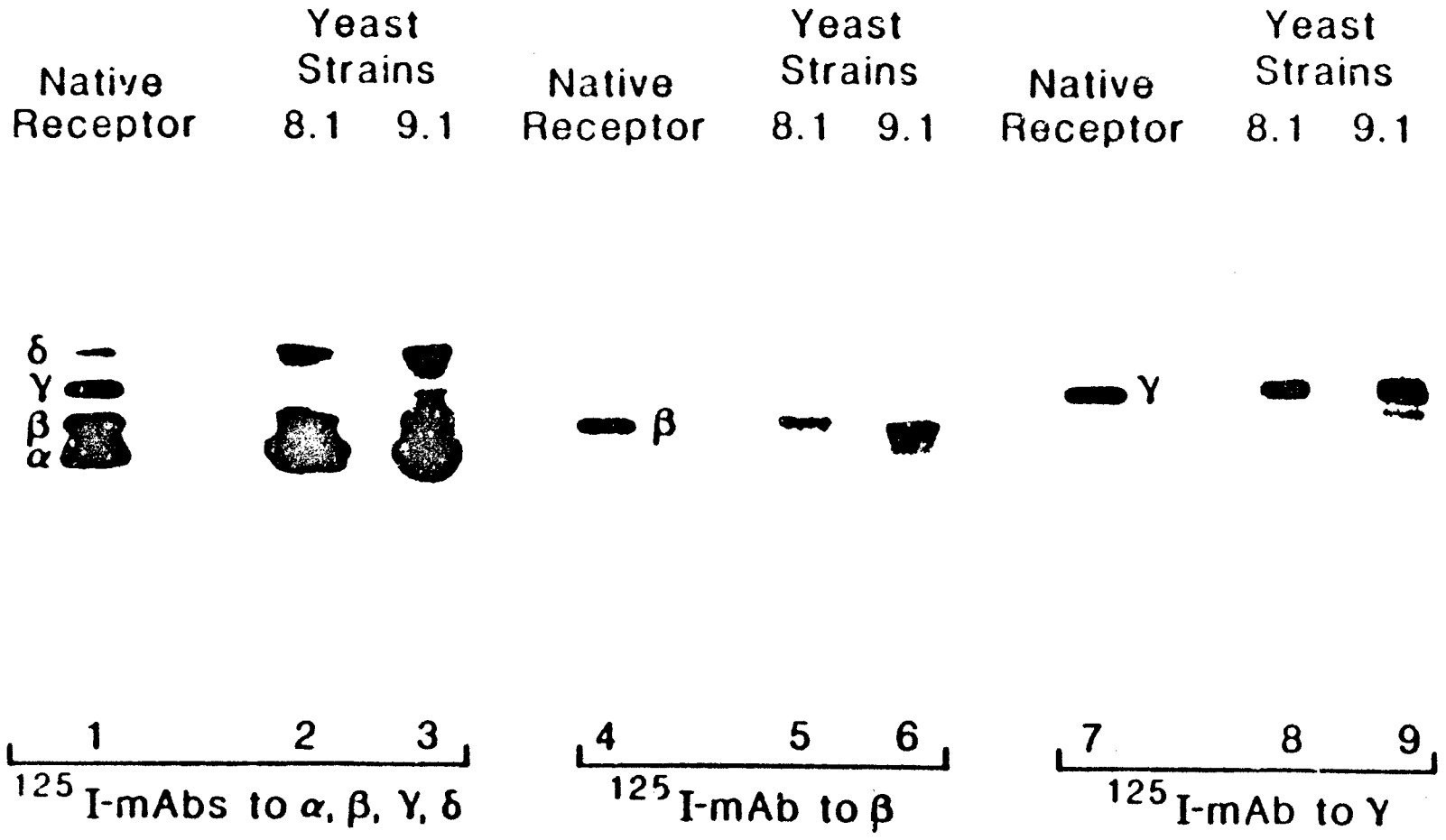


Figure 11. Yeast strains KUJ 8.1 and KUJ 9.1 transformed with Torpedo nicotinic receptor α, γ, and δ subunits and chimeras of most of the r signal sequence with β subunits express receptor subunits when grown at 5°C. Western blots were probed with 1 nM ^{125}I-mAb 142 to α subunits, 3 nM ^{125}I-mAb 111 to β subunits, 5 nM ^{125}I-mAb 168 to γ subunits, and 5 nM ^{125}I-mAb 166 to δ subunits. Autoradiograms were exposed 4-10 hours. Reproduced from reference 64.

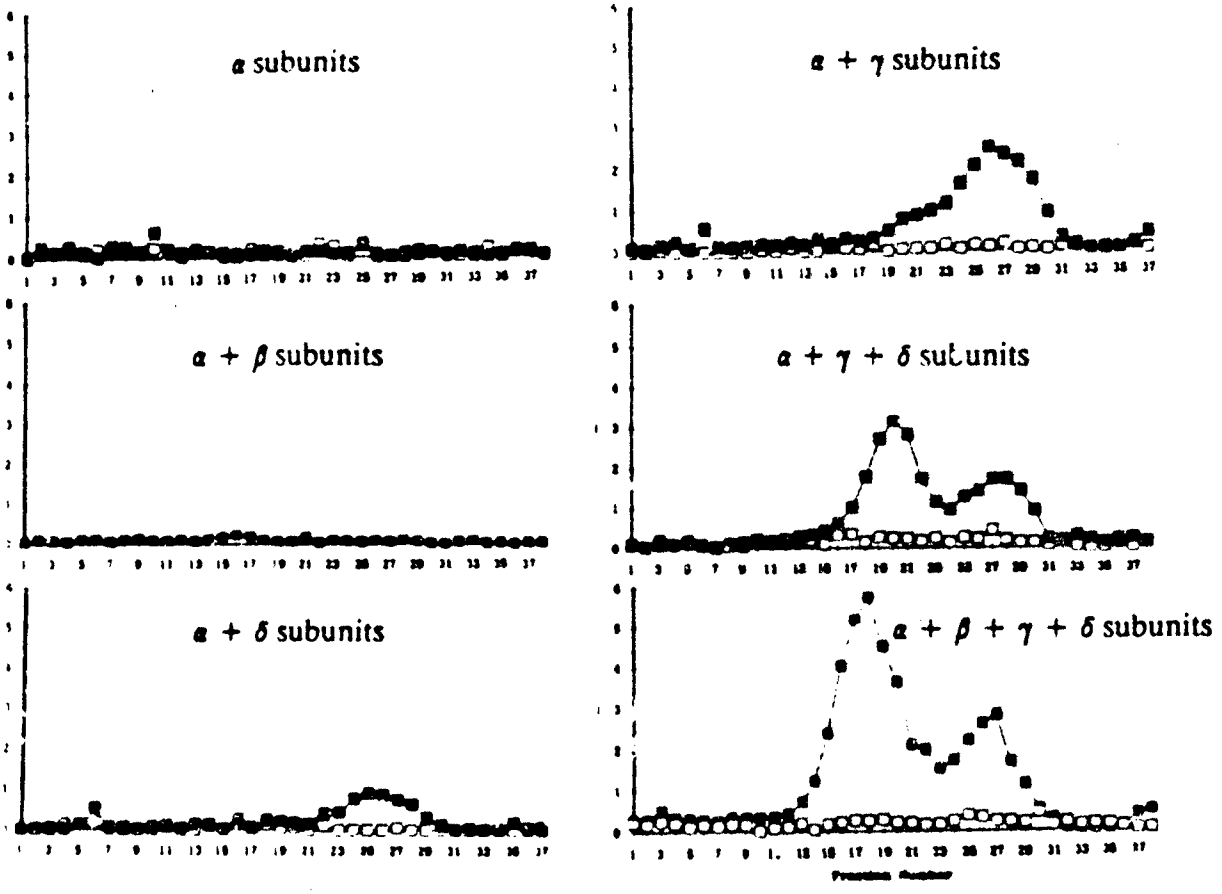


Figure 12. Cholinergic ligand-binding and sedimentation properties of Torpedo acetylcholine receptor subunits expressed in various combinations in Xenopus oocytes. Oocytes were injected with 1.5 ng of mRNA for each Torpedo receptor subunit synthesized in vitro. Two days later Triton X-100 extracts of pools of 10 oocytes were sedimented on 5 ml sucrose gradients at 65,000 rpm in a VTi 65 rotor for 70 minutes. Fractions were collected in plastic microwells coated with mAb 210 (to the main immunogenic region). ^{125}I-α-bungarotoxin at 2 nM plus (□—□) or minus (■—■) $10^{-3}M$ carbamylcholine was added, and the samples were incubated overnight at 4°C on a shaker. Then the microwells were washed three times and ^{125}I measured by γ counting.

subunits resulted in expression of native receptor monomers as well as some unassembled subunit pairs. Receptor dimers which are characteristic of receptors in electric organs were not seen. α subunits can mature in their ligand-binding properties without concerted assembly of all receptor subunits. These results are consistent with and substantially extend those of others[67,68] and suggest, as will be tested by further experimentation, that receptor subunits may assemble first as pairs of α subunits and γ subunits or α subunits and δ subunits, and these pairs may further associate before receptor synthesis is completed by the addition of β subunits to form the circular $\alpha\beta\alpha\gamma\delta$ arrangement of receptor subunits shown in **Figure 2**.

Human Acetylcholine Receptors

We first discovered that the human cell line TE671 expresses muscle-like acetylcholine receptors by observing that autoantibodies from myasthenia gravis patients reacted as well with these receptors as they did with receptors extracted from human legs.[69] Then we found that mAbs which recognized neuronal nicotinic receptors with high affinity for nicotine from human brain did not recognize receptors from TE671.[18] Recently we found that antisera to the α-bungarotoxin-binding protein purified from chicken brain reacted with α-bungarotoxin-binding proteins from human brain, but did not react with receptors from TE671 cells.[7] These results all contradicted the earlier idea that TE671 cells expressed receptors corresponding to the α-bungarotoxin-binding protein from human brain[70,71] and supported the idea that these cells express muscle-type receptors.

We then established that TE671 expresses large numbers of functional muscle-type nicotinic receptors.[7] Others now have similar results.[8] Acetylcholine induced the opening of receptor channels with a conductance of 44-45 pS (**Figure 13**). α-Bungarotoxin blocked receptor activity (**Figure 14**). A majority of the channel openings were brief (65% had a time constant of 0.82 ms) whereas a minority of the openings were more prolonged (35% had a time constant of 3.3 ms). These properties are all consistent with those of muscle nicotinic receptors.

Receptors from TE671 cells were shown to be of muscle type by immunoprecipitation reaction with mAbs raised against receptors purified from human leg muscle, such as mAbs 203 and 207.[7] These receptors were shown to be antigenically of the extrajunctional type by their ability to react with two other mAbs to receptors from human muscle, C_9 and F_8, which have been shown to react only with extrajunctional receptors.

Receptors were affinity purified from TE671 cells[7] by affinity chromatography using α-bungarotoxin (**Table 2**). The specific activity of unpurified receptors from TE671 is 1/20 that of Torpedo electric organ, twice that of mouse BC3H-1 muscle cells, and 27 times that of fetal calf muscle. Pure receptor was obtained at high yield, free from proteolytic degradation.

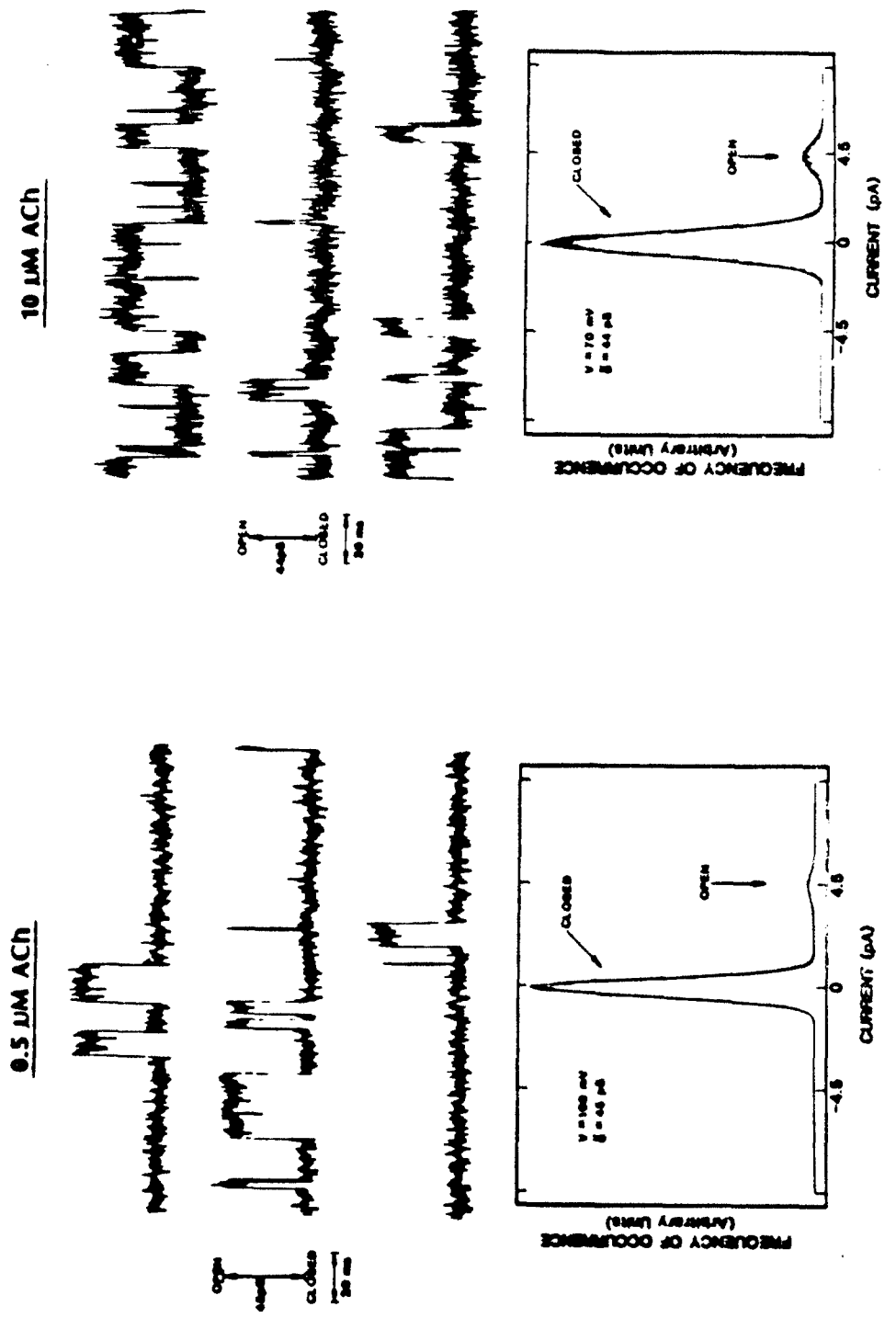


Figure 13. Acetylcholine induces openings of single receptor channels in TE671 cells. Openings were recorded at an applied voltage of 100 mv at 0.5 μM acetylcholine or 70 mv at 10 μM acetylcholine. Reproduced from reference 7.

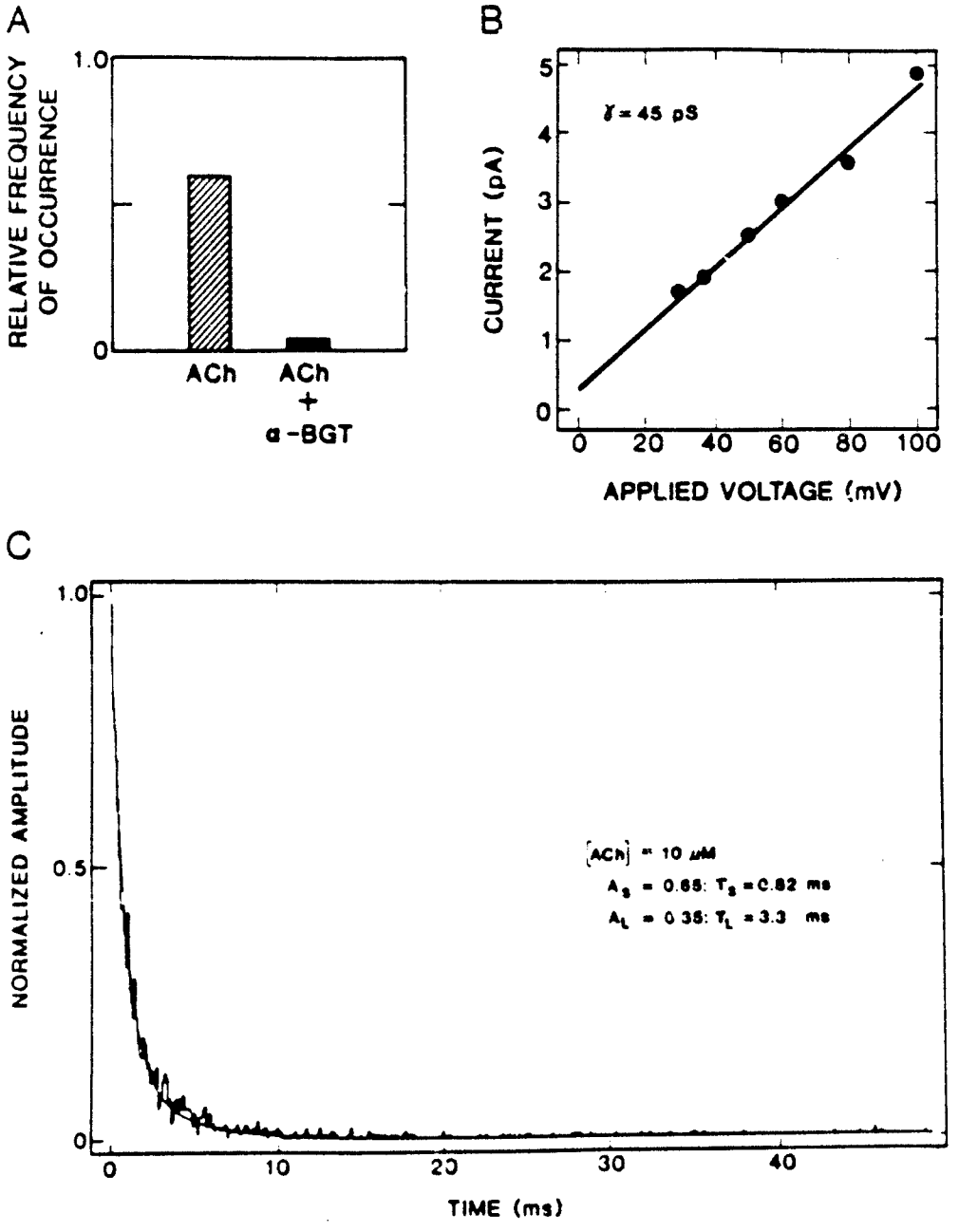


Figure 14. Analysis of single acetylcholine receptor currents in TE671 cells. *A, α-bungarotoxin (0.04-0.15 μM in various experiments) blocks the opening of receptor channels induced by acetylcholine (at 0.5-50 μM). B, The receptor channels behave ohmically with a conductance of 45 pS. C, Receptor channels frequently open for short durations (0.82 ms) and less frequently open for longer durations (3.3 ms) in 10 μM acetylcholine at 100 mv. The data for 2,035 openings (noisy curve) are fit by a sum of two exponentials (smooth curve). Reproduced from reference 7.*

Table 2. Purification of Receptor from 20g (12 roller bottles) of TE671 Cells.

Fraction	Volume (ml)	Protein (mg)	AChR α-Bgt[a] Binding Sites (pmol)	(%)	^{125}I-α-Bgt Specific Activity (pmol/mg protein)
Initial extract	100	570	1,804	100	3.16
Unbound to α-Bgt affinity column	100	380	702	39	
Wash steps	650	180	342	19	
Affinity column eluate	6	0.100	776	43	7,800

[a] α-Bungarotoxin.

Receptors purified from TE671 cells were composed of four kinds of subunits of apparent molecular weights 42,000, 52,500, 55,000, and 62,000 (Figure 15A), which corresponded antigenically to the α, β, γ, and δ subunits of receptor from Torpedo electric organ by Western blotting (Figure 15B). The α subunits were shown to form the acetylcholine binding site by affinity labeling with MBTA (Figure 15C).

TE671 cells contain poly (A^+) mRNAs corresponding to the α, β, γ, and δ subunits of receptors from mouse muscle (Figure 15D).

The sequence of a cDNA for the α subunit of receptor from TE671 is identical to that expected from a human genomic clone[38] and exhibits ≥95% sequence identity with α subunits from murine and bovine muscle (Figures 16 and 17). This unequivocally identifies TE671 α subunits as being of the muscle type. It further confirms the remarkable degree of sequence conservation characteristic of muscle nicotinic receptor α subunits. Many sequences are conserved including a glycosylation site, four hydrophobic domains, and cysteines at 192, 193, which in Torpedo[49,50] are known to be disulfide linked and which are near the acetylcholine binding site and can be affinity labeled by MBTA after reduction of this disulfide bond.

The sequence of a cDNA for the δ subunit of receptor from TE671 is that expected for δ subunits of a muscle nicotinic receptor (Figures 18 and 19). It exhibits ≥90% sequence identity with δ subunits from murine and bovine muscle. The sequences of δ subunits are less well conserved than are those of α subunits. The greatest extent of dissimilarity is in the sequences between amino acids δ398-451. This region is expected to be on the cytoplasmic surface.[52,53] The sequences of δ subunits and α subunits from TE671 exhibit 30% identity, showing that the subunits are homologous, as expected. The δ subunits from TE671 show conservation of four hydrophobic domains, three putative N-glycosylation sites, and three putative phosphorylation sites. The δ subunits of receptors from TE671, like those of muscles from other sources, lack the penultimate C-terminal cysteine through which δ subunits from Torpedo dimerize[45] (Figure 16).

Sucrose gradient analysis of receptors synthesized by TE671 cells revealed the presence of two components that bound α-bungarotoxin (Figure 20). The larger of these two components corresponded to the size of 9S monomers of Torpedo receptors, as expected. The smaller component sedimented at about 5S. It was bound by mAbs to α subunits, but not β, γ, or δ subunits. Although these unassembled α subunits bound α-bungarotoxin, they did not bind carbamylcholine. Although these subunits bound antibodies to the main immunogenic region, such as mAbs 35 and 210, their affinity was much lower than for native receptors. In all these properties the unassembled α subunits in TE671 cells resemble a synthetic intermediate of α subunits previously described in the mouse cell line BC3H-1.[51,72] In TE671 cells the amount of this intermediate is nearly equal to the amount of

mature receptor. The intermediate does not appear on the surface of TE671 cells, as shown by the observations that it is not labeled by ^{125}I-α-bungarotoxin applied to intact cells and is not antigenically modulated by mAb 210 applied to intact cells, in contrast to intact receptor monomers. Although unassembled α subunits of human muscle acetylcholine receptor appear at least as conformationally mature as α subunits of Torpedo acetylcholine receptors expressed in yeast (**Figure 10**), and more mature than Torpedo α subunits expressed in Xenopus oocytes (**Figure 12**), they are still immature, and like α subunits of Torpedo receptors (**Figure 12**), might be expected to acquire affinity for small cholinergic ligands after association with γ or δ subunits.

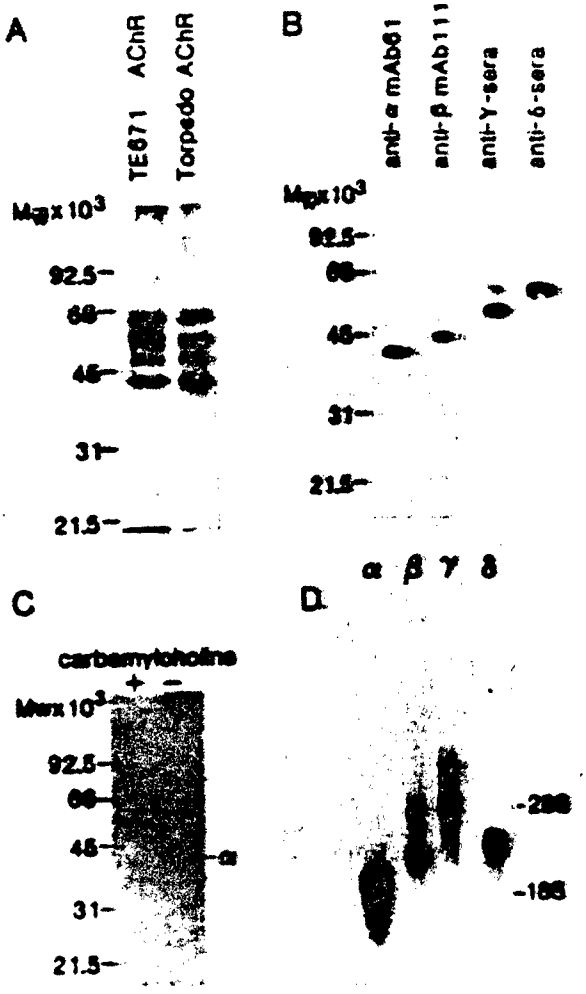


Figure 15. Subunits of receptors from TE671 cells. A, Receptors purified from TE671 and Torpedo electric organ have similar molecular weights as shown by electrophoresis under reducing conditions on acrylamide gel in SDS and staining with Coomassie blue. B, Subunits from TE671 receptors correspond to those of receptor from Torpedo by western blotting. Bound antibodies were localized by autoradiography using ^{125}I-mouse anti-rat IgG. C, Affinity labeling with ^{3}H-MBTA and specific inhibition of labeling by carbamylcholine identify the α subunit of receptor from TE671 as forming the acetylcholine binding sits. D, Poly (A^{+}) mRNAs for the four subunits of receptor from TE671 are detected by high stringency hybridization using cDNAs for mouse muscle receptor α, β, γ, and δ subunits. The cDNA probes used were described by Heinemann et al.[73] Reproduced from reference 7.

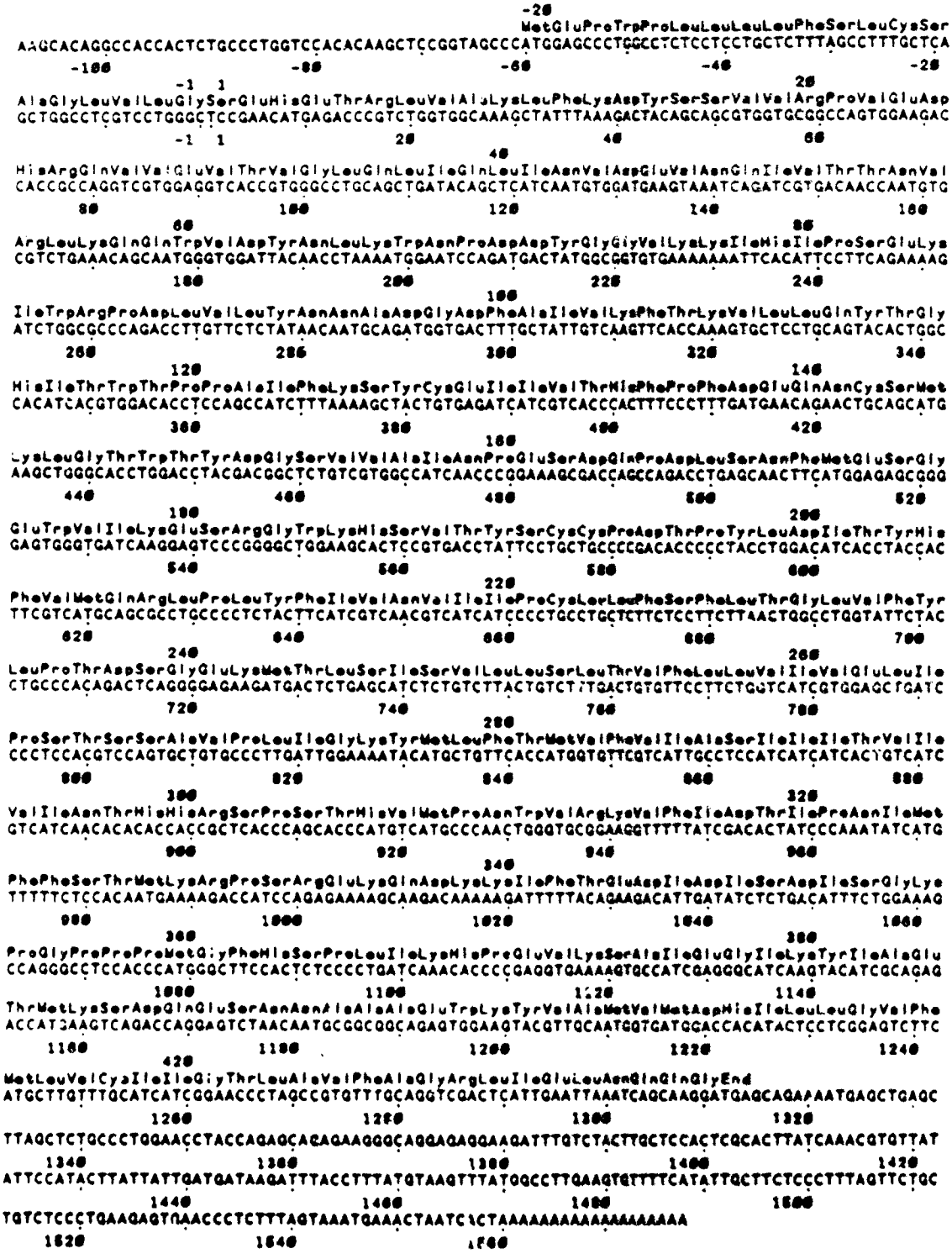


Figure 16. Nucleic acid sequence and deduced amino acid sequence of a cDNA for the α subunits of receptors from TE671 cells. Reproduced from reference 7.

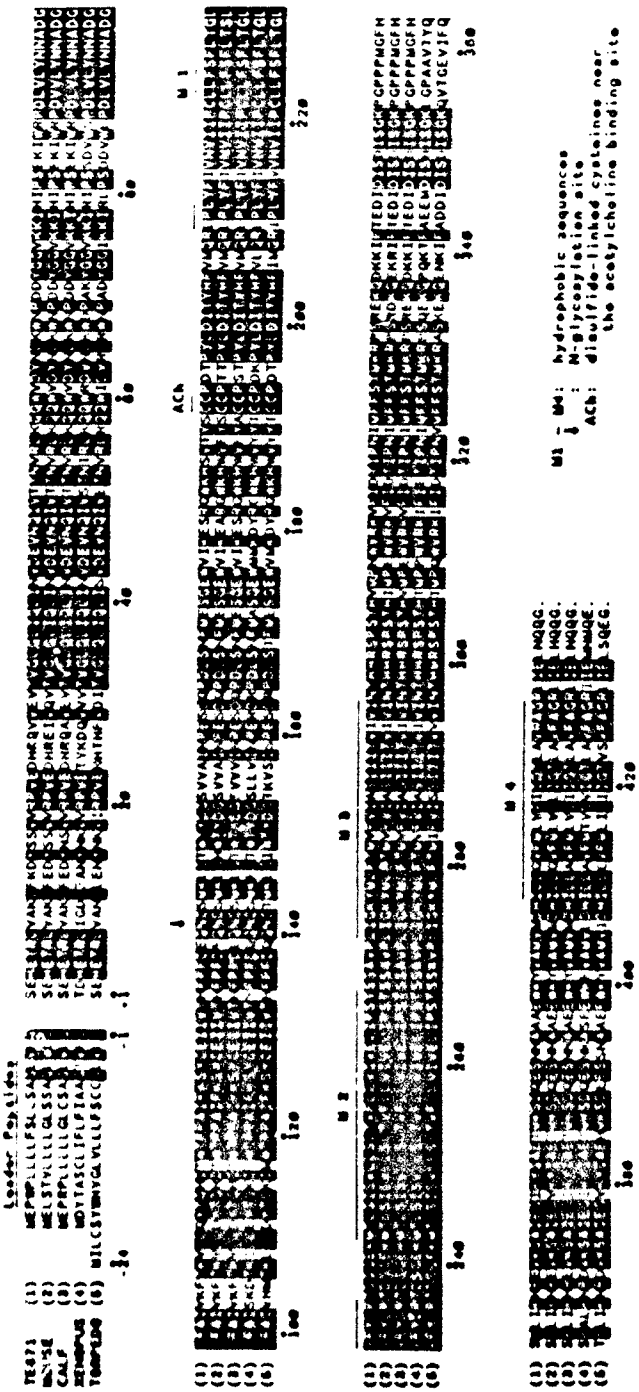


Figure 17. Comparison of the amino acid sequence deduced for α subunits of receptors from TE671 cells with the sequences deduced for α subunits of muscle-type nicotinic receptors from other species. Reproduced from reference 7.

Figure 18. Nucleotide and deduced amino acid sequence of a TE671 cDNA clone coding for the δ subunit of receptor from TE671 cells. Reproduced from reference 7.

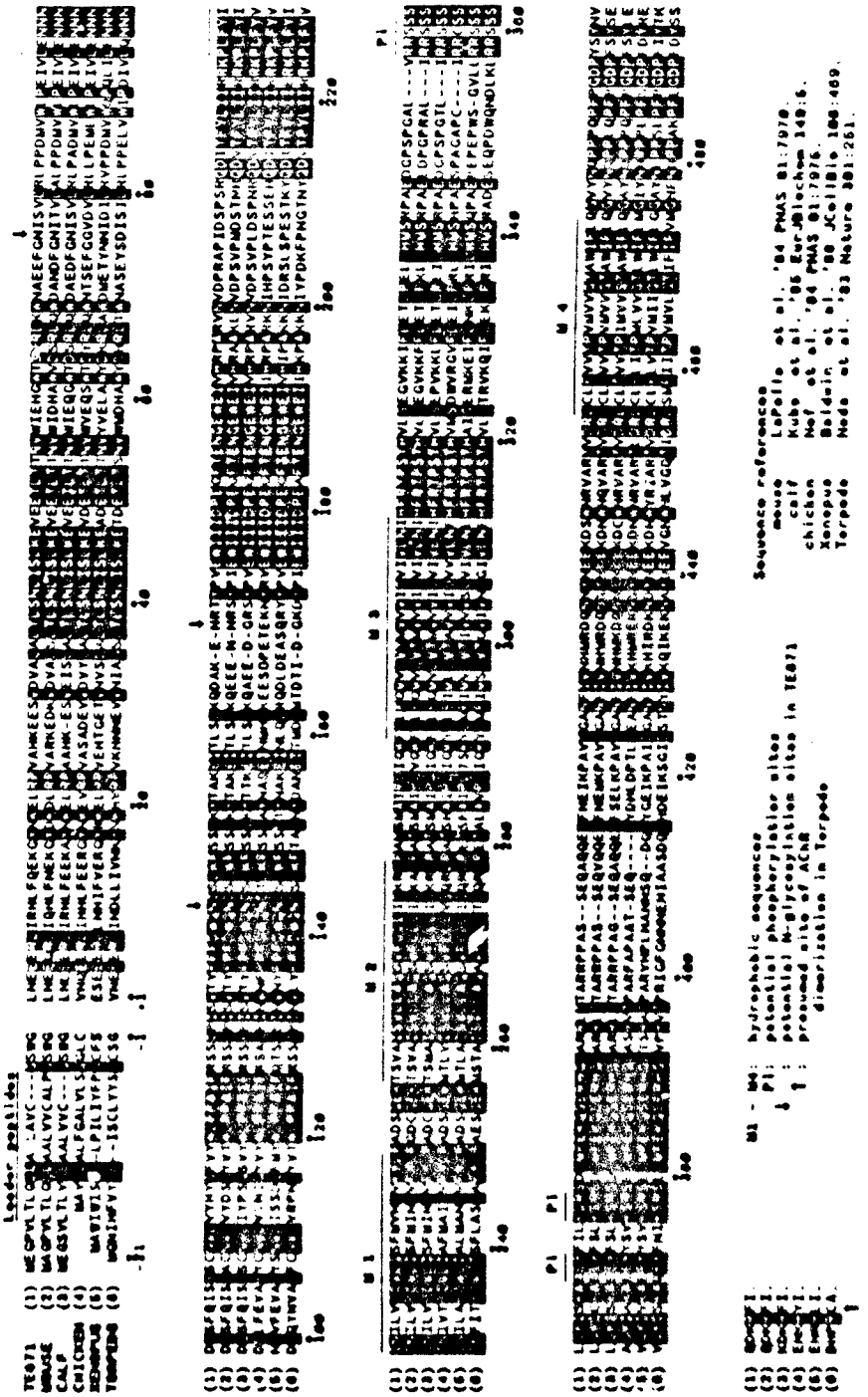


Figure 19. Comparison of the amino acid sequence deduced for δ subunits of receptors from TE671 with the sequences of δ subunits from nicotinic receptors from several species. Reproduced from reference 7.

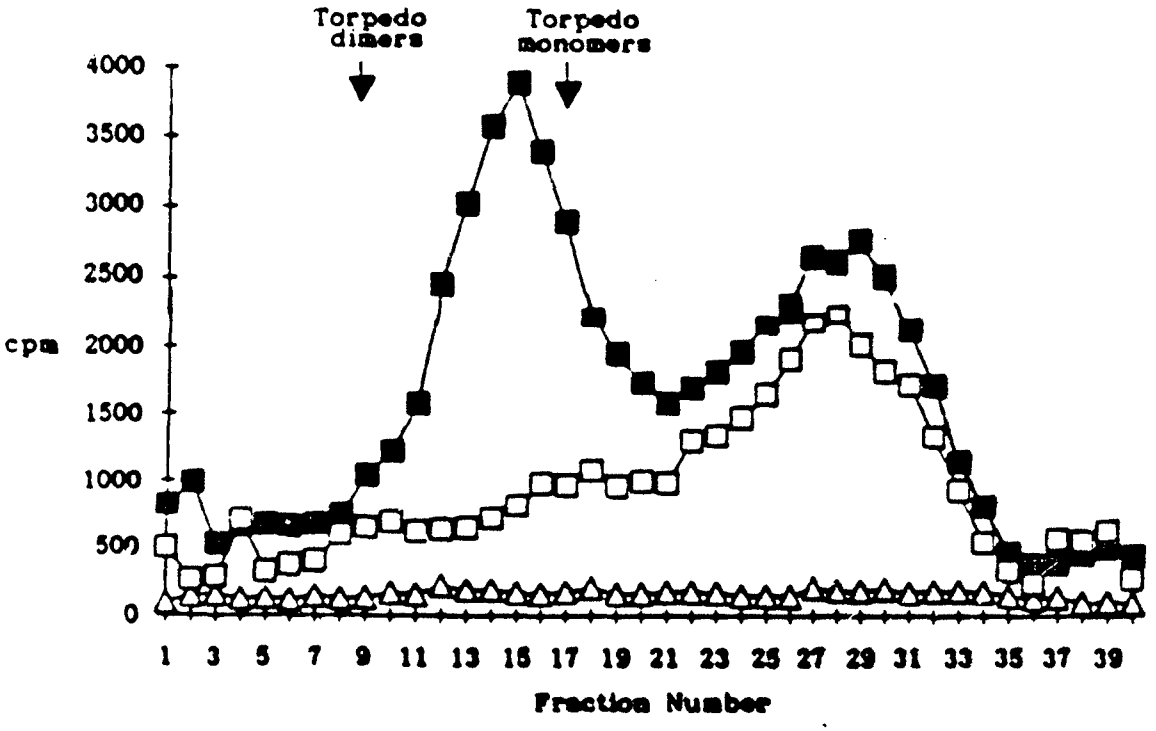


Figure 20. Sucrose gradient density sedimentation of TE671 extracts shows the presence of unassembled α subunits with immature acetylcholine binding sites. ^{125}*I-α-bungarotoxin binding in gradient fractions of TE671 extrac assayed in the absence (■) and presence (Δ) of cold α-bungarotoxin (1 μM) to determine total and nonspecific binding and in the presence of 100 mM carbamylcholine (□) to determine the species which could also recognize small cholinergic ligands. Plastic microwells coated with mAb 210 were used to bind* ^{125}*I-α-bungarotoxin-labeled receptor. The native human acetylcholine receptors are about the size of Torpedo receptor monomers. The smaller (~5S) component consists only of α subunits and is bound by mAbs to α subunits, but not by mAbs to β, γ, or δ subunits. These unassembled α subunits bind α-Bgt, but have negligible affinity for carbamylcholine.*

Neuronal Nicotinic Receptors

Purification and Characterization of Receptor Subtypes

An overview of our biochemical and molecular genetic studies of the structure of neuronal nicotinic receptors and a comparison with muscle nicotinic receptors is presented in **Figure 21**. This uses receptor subtype nomenclature that I hope will become standard, in which the subtype name is determined by the subunit genes and their stoichiometry, in cases for which these are known. Muscle nicotinic receptors are known to exist in two developmental forms, which differ by the substitution of a subunit.[74] It is not known if neuronal nicotinic receptors vary developmentally in this way. Receptors in uninnervated or denervated muscle use γ subunits, whereas receptors at mature neuromuscular junctions use ϵ subunits.[74] Subunits of electric organ nicotinic receptors were initially termed α, β, γ, and δ in order of increasing apparent molecular weight, and this nomenclature has persisted. Later it was discovered, primarily as a result of affinity labeling with MBTA at cysteines α192,193, that the lowest molecular weight α subunits formed the acetylcholine binding sites.[50] All neuronal nicotinic receptors also label with MBTA,[1] but this always reacts with the highest molecular weight subunits.[14,16,19,20] The term "α" for acetylcholine-binding subunits has persisted in the case of neuronal nicotinic receptors. A series of cDNAs containing cysteines homologous to α192,193 of muscle receptors were termed α2, α3, α4, etc., in order of discovery.[29,31,32,35] A series of neuronal nicotinic receptor subunit cDNAs that lack cysteines homologous to α192,193 have been termed β2, β3, etc. Muscle nicotinic receptors bind α-bungarotoxin with high affinity to their acetylcholine binding sites, but neuronal nicotinic receptors do not.[1,11,13-15,19,20] Muscle nicotinic receptors have moderate affinity for nicotine, whereas some, but not all, neuronal nicotinic receptor subtypes have very high affinity for nicotine.[1,11,13-15,19] As shown in **Figure 2**, muscle nicotinic receptors have a pseudopentagonal symmetry and the subunit stoichiometry $\alpha_2\beta\gamma\delta$.[44] We have found that neuronal nicotinic receptors are composed of equal numbers of only two types of subunits,[12,14,19,20] and it is currently uncertain whether there are two or three copies of each subunit, but it is clear that neuronal nicotinic receptors cannot exhibit the pentagonal symmetry of muscle nicotinic receptors. Nicotinic receptors of three subtypes are immunoaffinity purified from chicken brains if a mAb that binds to a kind of structural subunit that is used by several receptor subtypes is used on the affinity column. Two of the three subtypes predominate.[19] One of these predominant subtypes is composed of β2 structural subunits of apparent molecular weight 49kD[35] and α2 acetylcholine-binding subunits of 59kD. The other predominant subtype in chicken brain is composed of β2 structural subunits[35] and α4 acetylcholine-binding subunits of 75kD.[17] Trace amounts of a third subtype composed of β2 structural subunits and α3 acetylcholine-binding subunits of 59kD copurifies from brain, but this subtype predominates in ganglia and retina.[37,75] This ganglionic subtype of receptor has lower affinity for nicotine than do the $\alpha2\beta2$ and $\alpha4\beta2$ subtypes. From

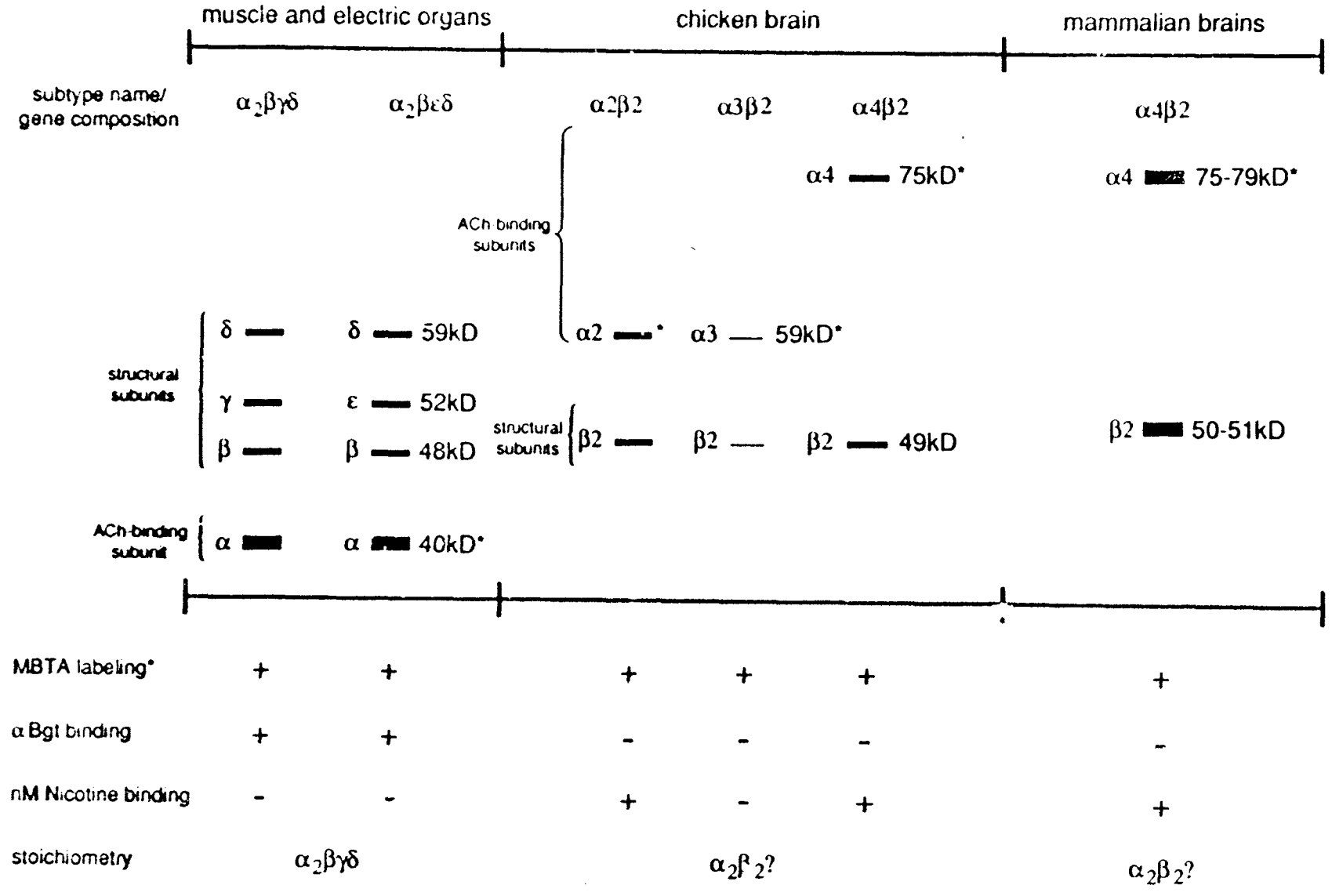


Figure 21. Comparison of nicotinic receptor subtypes purified from muscles and brains.

mammalian brains a single subtype has been immunoaffinity purified,[14,20] but there are probably small amounts of receptors with the same structural subunit in combination with α2, α3, and other acetylcholine-binding subunits.[33] Nicotinic receptors immunoaffinity purified from rat brains using a mAb to structural subunits consist of β2 structural subunits of apparent molecular weight 51kD[35] and α4 acetylcholine-binding subunits of apparent molecular weight 79kD.[17] These account for >90% of the nicotine binding in rat brain.[13] Immunologically and pharmacologically homologous receptors predominate in brains of cattle and humans, and these are thought to be encoded by the same genes.[20] Receptors immunoaffinity purified from bovine brains have structural subunits of apparent molecular weight 50kD and acetylcholine-binding subunits of 75kD.

For clarity in subsequent descriptions of the data, receptor subtypes and subunits will be referred to by the names of the genes that encode them. In fact, this information is hard won and recent. Initially the subunits were characterized by molecular weight, then by MBTA-affinity labeling and by mAb-binding properties. Only recently have N-terminal amino acid sequencing of purified subunits and reaction with antibodies to bacterially expressed unique sequences of subunits permitted association of the subunits of receptors as they occur <u>in vivo</u> with specific cDNAs identified by low stringency hybridization, initially using cDNA probes for subunits of muscle nicotinic receptors.

We began studies of neuronal nicotinic receptors by screening a library of mAbs to muscle nicotinic receptors for mAbs that would cross-react with neuronal nicotinic receptors.[9] We found that mAb 35 to the main immunogenic region reacted with nicotinic receptors in chickens.[9-11] mAb 35 was used to affinity purify α2β2 receptors from chicken brains.[12] These receptors were used as immunogens to raise a library of subunit-specific mAbs.[19] These were used to purify α4β2 receptors from chickens.[19] Later, mAbs to a unique fragment of α3 expressed in bacteria[37] were used on Western blots to demonstrate that receptor preparations purified from chicken brains using mAbs to structural subunits shared by the various subtypes contained small amounts of the α3β2 subtype. mAb 270 to structural subunits was used to purify α4β2 receptors from rat brains.[14] Then these receptors were used as immunogens to raise a library of subunit-specific mAbs for mammalian receptors, one of which was used to purify receptors from bovine brains, and others of which were used to immunoisolate receptors from human brains for pharmacological studies.[20] These mAbs were also used for immunohistochemical studies.[21-23,76] Meanwhile, partial or complete cDNAs for α2, α3, α4, and β2 subunits[35] were isolated from chicken brains, sequenced, and in some cases used to express unique peptide sequences for the preparation of subunit-specific antisera and mAbs.[37] These studies are summarized below.

Although mAb 35 reacts with acetylcholine-binding subunits of muscle nicotinic receptors, it binds to structural subunits of neuronal receptors, as shown in **Figure 22.** In native neuronal receptors from chickens, mAb 35 has substantial affinity only for the $\alpha 2\beta 2$ and $\alpha 3\beta 2$ subtypes.[19,37] mAb 210, also directed at the main immunogenic region, has binding properties similar to mAb 35, though it reacts better with denatured subunits and reacts on Western blots with the denatured structural subunits of both the $\alpha 2\beta 2$ subtype and the $\alpha 4\beta 2$ subtype.[19] In brain there is only a small amount of the $\alpha 3\beta 2$ subtype,[37] as shown in **Figure 23,** and nearly equal amounts of $\alpha 2\beta 2$ and $\alpha 4\beta 2$ subtypes,[19] as shown in **Figure 24.** In ganglia essentially all of the receptors recognized by mAb 35 are of the $\alpha 3\beta 2$ subtype,[37] as shown in **Figure 23.**

Receptors immunoaffinity purified from chicken brains using mAb 35 were used as immunogens to raise the library of subunit-specific mAbs[19] described in **Table 3.** mAb 270 to $\beta 2$ structural subunits from chickens immunoaffinity purified both the $\alpha 3\beta 2$ and the $\alpha 4\beta 2$ receptor subtypes from chicken brains.[19] These two subtypes could be separately purified by using mAb 35 to bind the $\alpha 3\beta 2$ subtype and mAb 284 to bind the $\alpha 4\beta 2$ subtype, as shown in **Figure 25.**

mAb 270 raised against chicken neuronal receptors was used to affinity purify the single $\alpha 4\beta 2$ receptor subtype that predominates in rat brains. This receptor was then used as an immunogen to generate a library of subunit-specific mAbs[20] described in **Table 4.**

mAb 295 raised against receptors from rat brains was then used to immunoaffinity purify the dominant $\alpha 4\beta 2$ receptor subtype from bovine brains,[20] as shown in **Figure 26.** Like the $\alpha 4\beta 2$ receptor subtype in rat brains, the $\alpha 4\beta 2$ receptor in bovine brain accounts for at least 90% of the high-affinity nicotine binding sites present. Purification of receptors from human brains was hampered by autolysis due to prolonged intervals between death and autopsy. The partially proteolyzed receptors retained their ligand-binding properties, as will be described later.

Acetylcholine-binding subunits were identified by affinity labeling with ^{3}H-MBTA,[16,19,20] as shown in **Figures 26** and **27.** This is important because it proves that these subunits form the acetylcholine binding sites. All of these subunits contain a cysteine pair homologous to $\alpha 192,193$ in their cDNA sequence, as described below, and these cysteines are the probable site of MBTA reaction, by homology with the known site of reaction of MBTA on <u>Torpedo</u> α subunits.[50]

cDNAs for chicken neuronal nicotinic receptor subunits were identified by low-stringency hybridization using cDNA clones for neuronal nicotinic receptors from rat brain obtained from Drs. Patrick and Heinemann.[35] $\beta 2$ structural subunit sequences and $\alpha 4$ acetylcholine-binding subunit sequences were highly conserved between chickens and rats, as shown in **Figure 28.** The sequences of all of the chicken neuronal nicotinic receptor subunit cDNAs

studied are compared with the sequence of chicken muscle α subunits in **Figure 29**. Neuronal nicotinic receptor sequences exhibit features characteristic of all nicotinic receptor subunits, such as conserved cysteines at consensus sequences 137 and 151, various short identical sequences, four hydrophobic sequences, and a large putative cytoplasmic domain unique to each subunit. The chicken cDNA sequences determined were essentially identical to genomic sequences determined by Ballivet and coworkers.[31]

Determination of the cDNAs which correspond to the subunits of purified nicotinic receptors was achieved by N-terminal amino acid sequencing in several cases, such as α4β2 receptors from rat and chicken brains, as illustrated in **Figure 30**.[17,35] The identity of the structural subunits in α2β2 receptors was also established by N-terminal sequencing. The identification of α4 genes as encoding the 75kD acetylcholine-binding subunits was further confirmed by showing that antisera and mAb 289 specific for a bacterially expressed sequence corresponding to the putative large cytoplasmic domain (the shaded sequence on α4 in **Figure 29**) bound to this subunit. Antisera and mAbs were prepared to a similar sequence from α3[37] (the shaded sequence on α3 in **Figure 29**); see **Table 5**. These antibodies did not bind the major brain receptor subtypes, but did bind ganglionic receptors, as shown in **Figure 23**. These mAbs to α3 applied to Western blots of receptors purified from chicken brains using mAb 270 to β2 structural subunits revealed a faint band at 59kD, corresponding to the small amounts of α3β2 receptors present in brain, which copurify with the major subtypes. Antisera to the putative large cytoplasmic domain of α2 expressed in bacteria (α2 331-545 in the numbering on **Figure 29**) did not bind well to native α2β2 receptors, but heavily labeled the 59kD acetylcholine-binding subunit of these receptors on Western blots, thereby establishing their identity.

Neuronal nicotinic receptor subunit stoichiometry is distinctly different from that of muscle nicotinic receptors. Each neuronal nicotinic receptor contains more than one copy of its acetylcholine-binding subunit and more than one copy of its structural subunit, as shown by studies like those described in **Figure 31**, which shows that each receptor can bind at least two mAbs specific for each subunit.[19] Each neuronal nicotinic receptor was shown to contain equal numbers of acetylcholine-binding and structural subunits by experiments like those shown in **Figure 32**, in which the relative amounts of subunits are measured using ^{125}I-labeled subunits. The presence of equal numbers of each of two subunit types proves that neuronal nicotinic receptors cannot exhibit the pseudopentagonal symmetry of muscle nicotinic receptors. The size of neuronal nicotinic receptors as determined by sucrose gradient sedimentation experiments like those shown in **Figure 33** indicate that there must be between two and three copies of each subunit, but do not permit critical distinction between these two numbers. Neuronal receptors sediment slightly more rapidly than do monomers of

Torpedo receptors. The relative sedimentation rates are affected by the detergent and lipid environments. The calculated molecular weight of Torpedo receptor monomers, including protein, sugars, and α-bungarotoxin as a label, is 303,366. The calculated molecular weight of two rat α4 subunits plus two β2 subunits plus glycosylation equivalent to Torpedo is 262,820; whereas the calculated molecular weight for three subunits of each is 394,230. Size determination by gradients cannot clearly distinguish between these alternatives. A diagrammatic depiction of some alternate subunit stoichiometries is given in **Figure 34.** The concept that subunit types alternate between acetylcholine-binding and structural around a central channel seems likely by homology with muscle receptors. The idea that there are only two subunits of each kind is appealing for its simplicity. It is consistent with the observation of a Hill coefficient for activation by acetylcholine of 1.5 reported for chicken α4β2 receptors expressed in oocytes, because this approximates the value for muscle receptor (with two acetylcholine-binding subunits) of 1.6-1.7.[30] It is also consistent with the observation that these expressed receptors exhibit a conductance of only 20 pS, approximately half the value exhibited by muscle receptors.[30] This could result from a smaller diameter channel due to a reduced number of "barrel staves" surrounding it.

Developmental changes in the amount of α3, α4, and β2 subunit mRNAs in brain and retina during chicken development were measured by Northern blot analysis, as shown in **Figure 35.** Higher levels of subunit mRNAs were present in embryos than in adults. Similarly, the concentration of receptor protein was higher in embryos. The level of α3 mRNA is quite low in brain, but much more substantial in retina. Within the brain there are changes in the relative amounts of α2β2 and α4β2 receptor subtypes during development.

Pharmacological properties of detergent-solubilized and immunoisolated neuronal nicotinic receptors from several species were measured.[13,19,20] None of the subtypes studied bound α-bungarotoxin. All of the subtypes bound MBTA, but there were differences in reactivity with MBTA and bromoacetylcholine between species, as shown in **Figure 36.** The α2β2 and α4β2 subtypes from chicken brain exhibited similar pharmacological profiles characterized by nM affinity for nicotine and cytosine, and much lower affinity for classic antagonists like curare, as shown in **Table 6.**[19] Similarly, rat α4β2 receptors exhibited a K_I for cytosine of 2.7×10^{-10}M, but a K_I for curare of only 1.7×10^{-5}M.[13] Roughly similar properties were also exhibited by the α4β2 receptors from bovine and human brains, as shown in **Figures 36, 37 and Table 7.**[20]

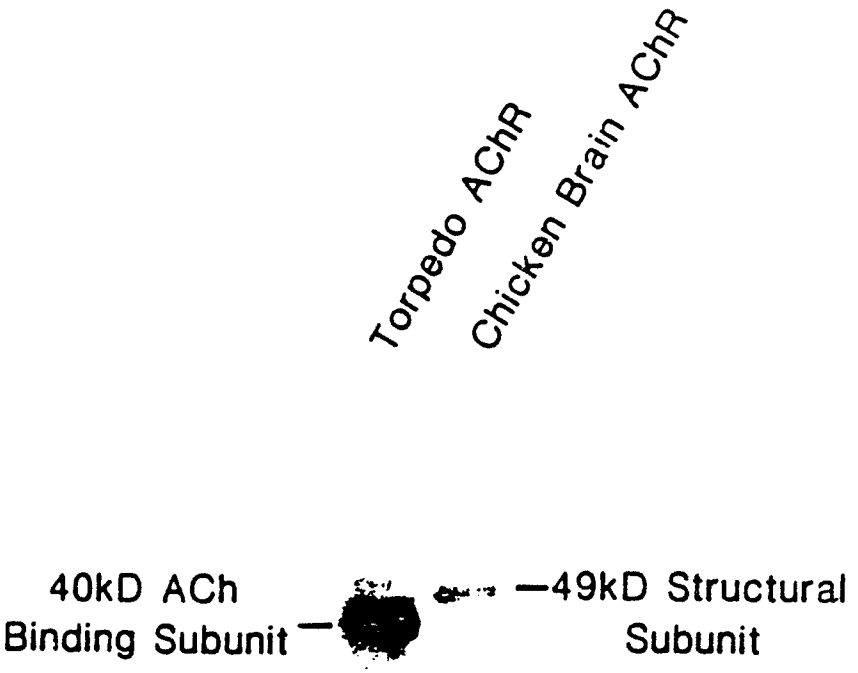


Figure 22. mAb 35 binds to the acetylcholine-binding subunit of Torpedo electric organ receptors, but to the structural subunit of chicken brain receptors. Purified receptors were resolved into their subunits by electrophoresis and the binding of mAb 35 determined by Western blotting, as described in Methods. Reproduced from reference 37.

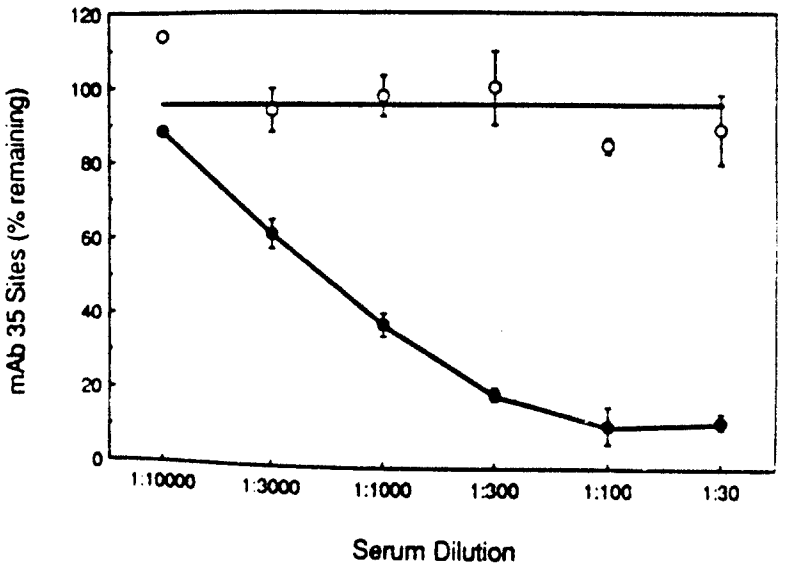


Figure 23. Antisera to α3 bind to receptors in chicken ciliary ganglia, which are labeled by mAb 35, but not to receptors in chicken brains, which are labeled by mAb 35. *Extracts of ganglia (●—●) or brains (O—O) were incubated with antisera to a unique sequence of α3 expressed in bacteria. Then fixed Staph-A cells were added to remove the antibodies and the receptors to which they bound. Receptors remaining in the extract were assayed for binding of ^{125}I-mAb 35, as described in Methods. Reproduced from reference 37.*

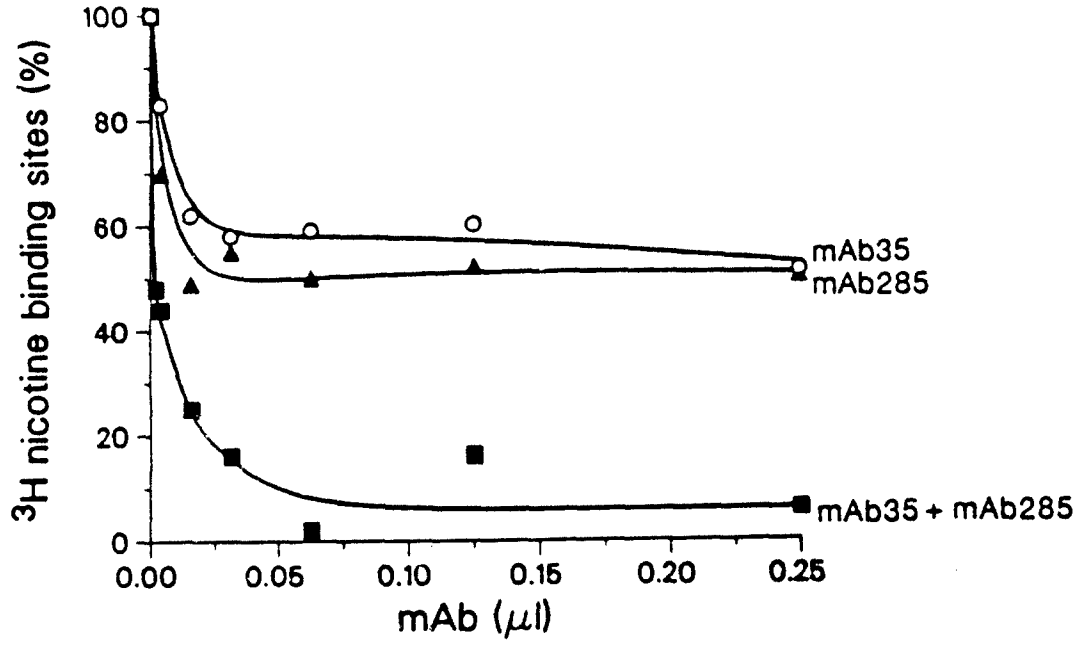


Figure 24. Nicotinic receptors in adult chicken brains are about one-half of the α2β2 subtype and one-half of the α4β2 subtype. This was determined by depletion of (DL)^{3}H-nicotine binding sites from detergent extracts of adult chicken brain by mAb 35 (O), mAb 285 (which is specific for α4) (▲), and a combination of mAbs 35 and 285 (■). Aliquots (300 μl) of chicken brain detergent extract (0.20 nM ^{3}H-nicotine binding sites) were gently shaken for 15 hours at 4°C with 20 μl 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 μl were incubated for 1 hour at 4°C with 20 nM ^{3}H-nicotine and then diluted with 4 ml of ice-cold 50 mM Tris, pH 7.4, and filtered through Whatman GF/B filters presoaked in 0.3% polyethylenimine. The filters were washed with 3x4 ml of the same buffer and bound radioactivity was determined by scintillation counting. Nonspecific binding was determined by incubation in the presence of 1 mM 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 19.

Table 3. Properties of mAbs Raised Against Chicken Brain Nicotinic Acetylcholine Receptors

mAb	Ig Class	Titer (μM) to Receptors from: Torpedo Electric Organ	Brains: Chicken	Rat	Fetal Bovine	Human	Subunit Specificity
267	IgG2a	0	0	0	0	0	$\beta 2$, $\alpha 2$
268	IgG1/2a	0	0	0	0	0	$\beta 2$
270	IgG2a	0	1.10	0.18	0	0	$\beta 2$
284	IgG2a	0	2.00	0	0	0	$\alpha 4$
285	IgG2a	0.014	1.40	0	0	0	$\alpha 4$
286	IgM	0	0.64	0.45	0	0.13	$\beta 2$, $\alpha 4$
287	IgM	0	0	0	0	0	$\beta 2$
289	D	IgM	0	0.18	0	0	$\alpha 4$

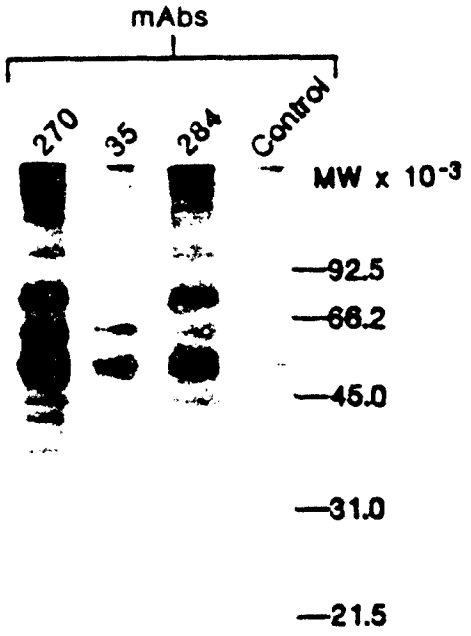


Figure 25. Resolution of nicotinic receptor subtypes from chicken brains by immunoprecipitation with mAbs. Both receptor subtypes were simultaneously affinity purified from chicken brains using mAb 270-Sepharose. The purified receptor preparation containing both subtypes was radioiodinated. Aliquots of $3.5x10^6$ cpm of the receptors were incubated with mAb 270 to bind both receptor subtypes, with mAb 35 to bind the $\alpha3\beta2$ subtype and with mAb 284 to bind the $\alpha2\beta2$ subtype, or with normal rat serum (2 µl) as a control, and gently shaken for 15 hours at 4°C in 100 µl of PBS, 0.5% Triton X-100 containing 5% (w/v) nonfat milk powder with 20 µl of goat anti-rat IgG-Sepharose to precipitate the immune complexes. The immunoprecipitates were washed with 4x1 ml of PBS, 0.5% Triton X-100 by pelleting in a microfuge and resuspending. Then bound protein was eluted with 60 µl of 125 mM 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 β-mercaptoethanol. 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. Reproduced from reference 19.

Table 4. Properties of mAbs Raised Against Rat Brain Nicotinic Receptors

mAb	Titer to Receptors From Rat Brain (μM)	Cross-Reaction With Brain Receptors of			Subunit Specificity	
		Chicken	Cattle	Humans	Western Blot	By Other Criteria
290	26.9	+	+	+	?	β2 Structural
291	1.1	+	-	-	?	β2 Structural
292	9.4	-	-	-	α4 ACh binding	
293	4.4	+	-	+	α4 ACh binding	
294	1.5	-	-	-	?	
295	0.5	+	+	+	?	β2 Structural
297	1.0	+	+	+	?	β2 Structural
298	0.7	+	+	+	?	β2 Structural
299	31.2	+	-	+	α4 ACh binding	α4 ACh binding

Figure 26. Structural characterization of nicotinic receptors immunoaffinity purified from bovine brain using mAb 295. A, Silver-stained SDS acrylamide gels. B, Autoradiogram of ^{125}I-labeled purified receptor resolved on an SDS acrylamide gel. C, Western blot using mAb 268 to the structural subunit of receptor from chicken brain. D, Specific affinity labeling of the acetylcholine-binding subunit with ^{3}H-MBTA after reduction. Reproduced from reference 20.

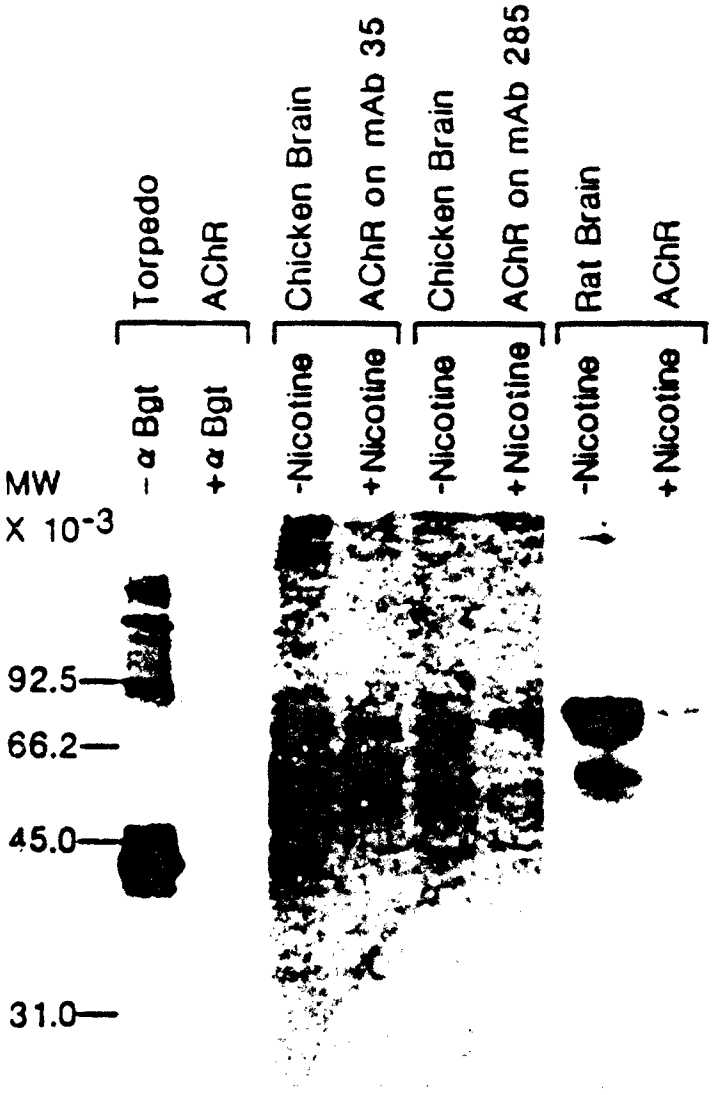


Figure 27. Fluorogram of SDS-PAGE of nicotinic receptors from Torpedo electric organ, chicken brain, and rat brain labeled with ^{3}H-MBTA. Labeling procedures and SDS-PAGE were carried out as described in Methods. Apparent molecular weights were determined by resolving molecular weight standards (BioRad) on the same gel and staining for protein with Coomassie blue. By specific affinity labeling, the higher molecular weight α2 or α4 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 α subunit. Reproduced from reference 16.

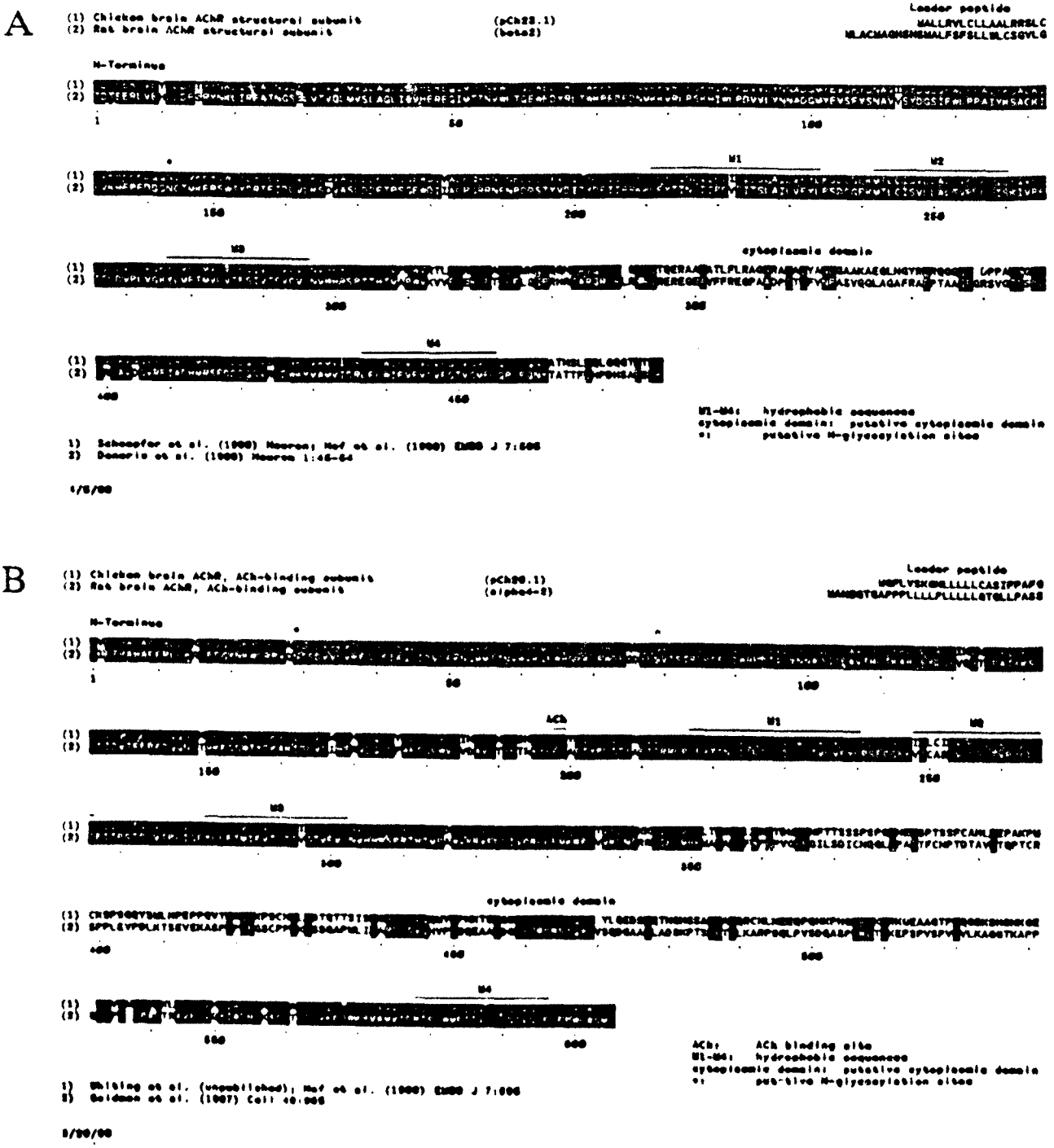


Figure 28. Comparison of β2 structural subunit (A) and α4 acetylcholine-binding subunit (B) sequences of neuronal nicotinic receptors from chickens and rats.

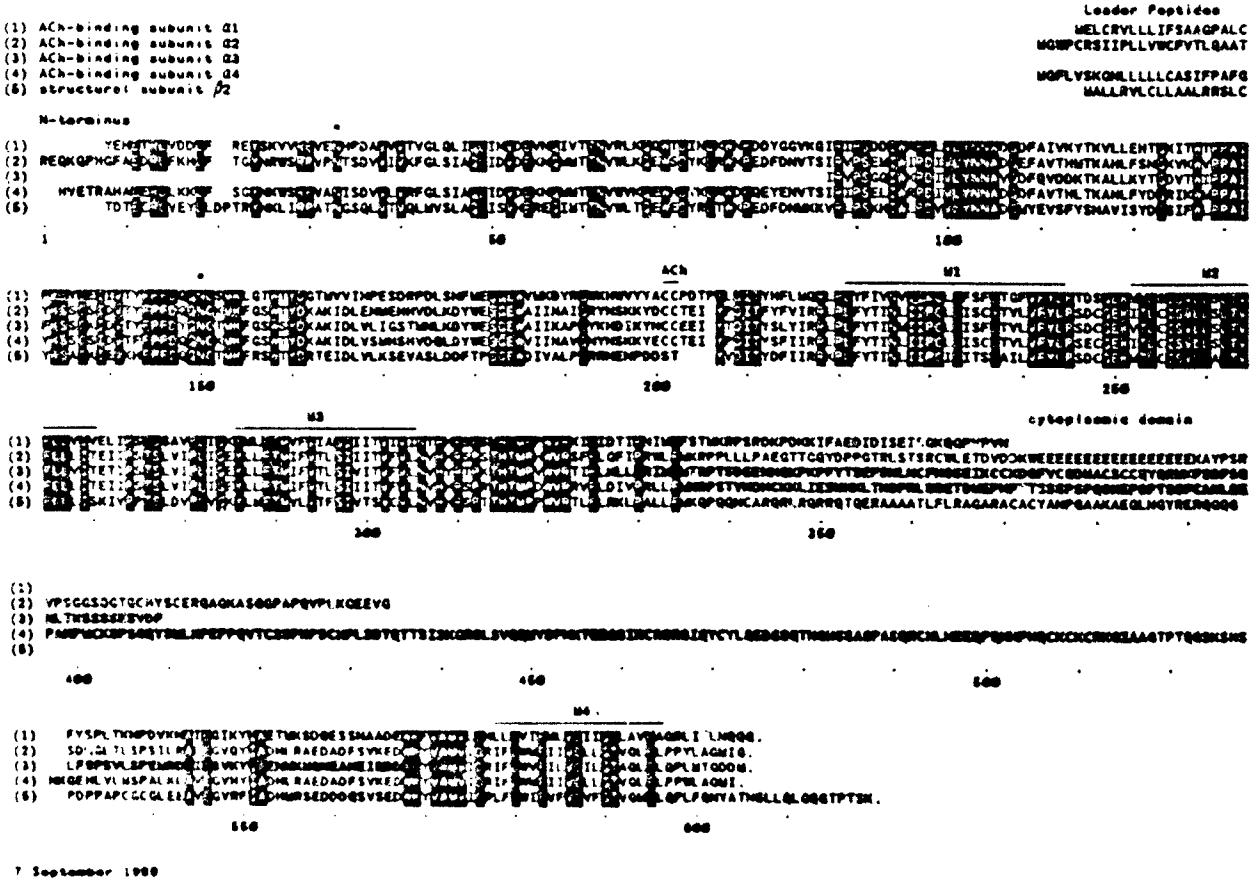


*Figure 29. Comparison of the deduced amino acid sequences of chicken neuronal receptor subunits. Sequences of α1 and α2 are from Nef et al.[31] Sequences of α3 and α4 were determined from clones described here, but are identical to those reported by Nef et al.[31] The sequence of the structural subunit reported here[35] is identical to the sequence termed "non-α" by Nef et al.[31] and is clearly homologous to the rat sequence termed β2 by Deneris et al.[29] Blackened residues are identical in all sequences. Shaded sequences indicate sequences expressed in bacteria to obtain antigens for subunit-specific sera. " * " indicate putative N-glycosylation sites. "ACh" indicates the cysteine pair located near the acetylcholine binding site. "M1-M4" indicate hydrophobic sequences thought to be transmembrane domains.*

Figure 30. Structure and N-terminal sequences of chicken and rat brain nicotinic receptors.

Table 5. Properties of mAbs to a Bacterially Expressed Putative Large Cytoplasmic Domain Fragment of Chicken α3 Acetylcholine-Binding Subunits

mAb	*immunoglobulin subclass*	*binding specificity*
313	IgG2a	native α3 AChR and denatured α3 peptide
314	IgG2a	"
315	IgG2a	"
316	?	only denatured α3 peptide
317	?	"

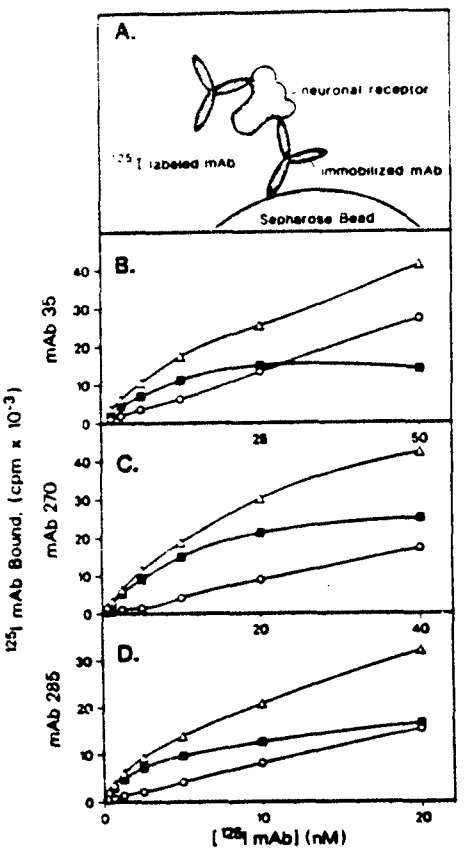


Figure 31. More than one copy of the structural subunit and more than one copy of the acetylcholine-binding subunit are present in each chicken neuronal nicotinic receptor macromolecule. (A) Diagrammatic representation of binding assay showing that binding of an ^{125}I-labeled mAb to a receptor immobilized 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 β2 structural subunit) to receptor immobilized upon mAb 35-Sepharose. (C) Binding of ^{125}I-mAb 270 (also specific for the β2 structural subunit) to receptor immobilized upon mAb 270-Sepharose. (D) Binding of ^{125}I-mAb 285 (specific for the α4 acetylcholine-binding subunit) to receptor immobilized upon mAb 285-Sepharose. Chicken brain detergent extract (200 μl) was gently shaken for 15 hours at 4°C with 15 μl of a 1:1 slurry of mAb 35-Sepharose, mAb 270-Sepharose, and mAb 285-Sepharose. The aliquots were washed with 1 ml PBS, 0.5% Triton X-100 and then incubated for 1 hour at 22°C in 100 μl PBS, 0.5% Triton X-100 containing increasing concentrations of ^{125}I-labeled mAb 35 ($2.2x10^{18}$ cpm/mol), mAb 270 (0.94×10^{18} cpm/mol) and mAb 285 ($3.9x10^{18}$ 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 μM mAb 35, 0.25 μM mAb 270, and 0.28 μM mAb 285 before addition of ^{125}I-labeled mAb). The aliquots were washed with 4x1 ml PBS and 0.5% Triton X-100 by pelleting in a microfuge and resuspending, and bound radioactivity was determined by γ 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 19.

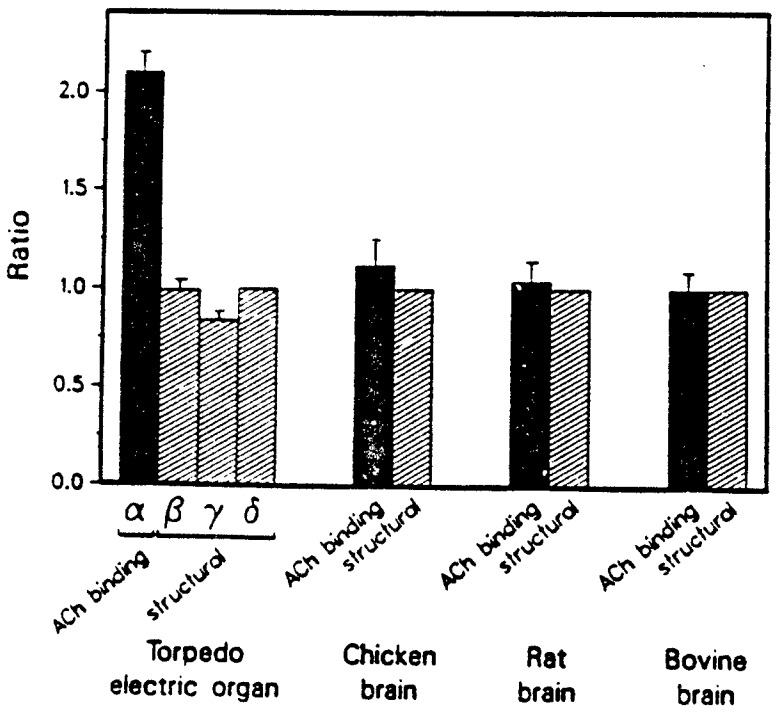


Figure 32. Ratio of subunits in Torpedo electric organ nicotinic receptors and receptors from brains of chickens, rats, and cattle determined by labeling with ^{125}I and determining the relative amount of ^{125}I in each subunit. Purified receptors were denatured in a mixture of 4 M urea and 1% SDS to make all of its tyrosines equally accessible to labeling. After labeling with ^{125}I, the subunits were separated by electrophoresis on acrylamide gels in SDS, located by brief contact autoradiography, and then the subunit bands were cut out of the gel and quantitated by gamma counting. The tyrosine content of each subunit known from the cDNA sequence was used to correct slightly for the difference in tyrosine content in each subunit, and then the relative amounts of the subunits were compared. This method yields the expected stoichiometry for receptor from Torpedo, $\alpha_2\beta\gamma\delta$. Further, it shows that in neuronal nicotinic receptors from chicken, rats, and cattle, there are equal numbers of acetylcholine-binding and structural subunits.

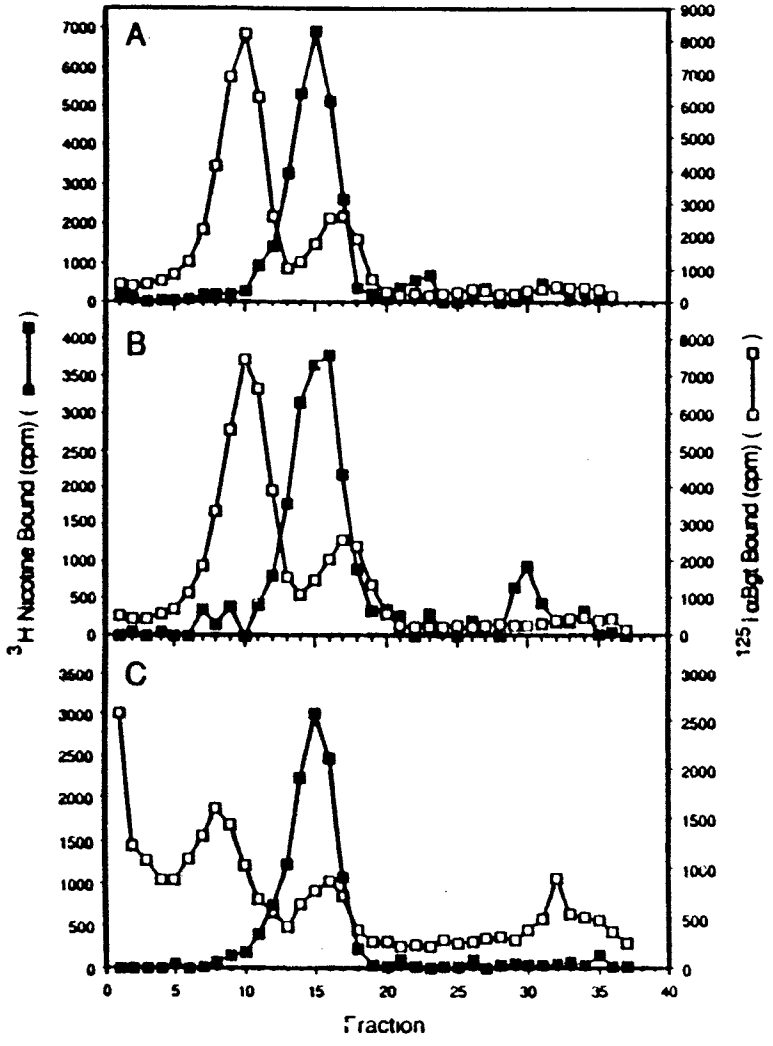


Figure 33. Sucrose gradient sedimentation analysis of neuronal nicotinic receptors. *Rat and chicken brain receptors were sedimented on 5-20% sucrose gradients as described in Methods. Each figure shown is the average of duplicate gradients. Fractions are numbered from the bottom of the gradients. Torpedo electric organ receptors trace labeled with ^{125}I-α-bungarotoxin were included in each gradient as an internal control ([]--[]). Neuronal receptors were quantitated by ^{3}H-L-nicotine binding in an immunoimmobilization assay using mAb 270 coupled to agarose to bind the receptors through their structural subunits (■--■). Nonspecific binding has been subtracted from each immunoassay point. A) Rat brain and Torpedo receptors extracted with 2% Triton X-100. B) Chicken brain and Torpedo receptors extracted with 2% Triton X-100. C) Rat brain and Torpedo receptors extracted with 2% cholate/0.2% asolectin and sedimented on gradients containing 2% cholate/0.2% asolectin.*

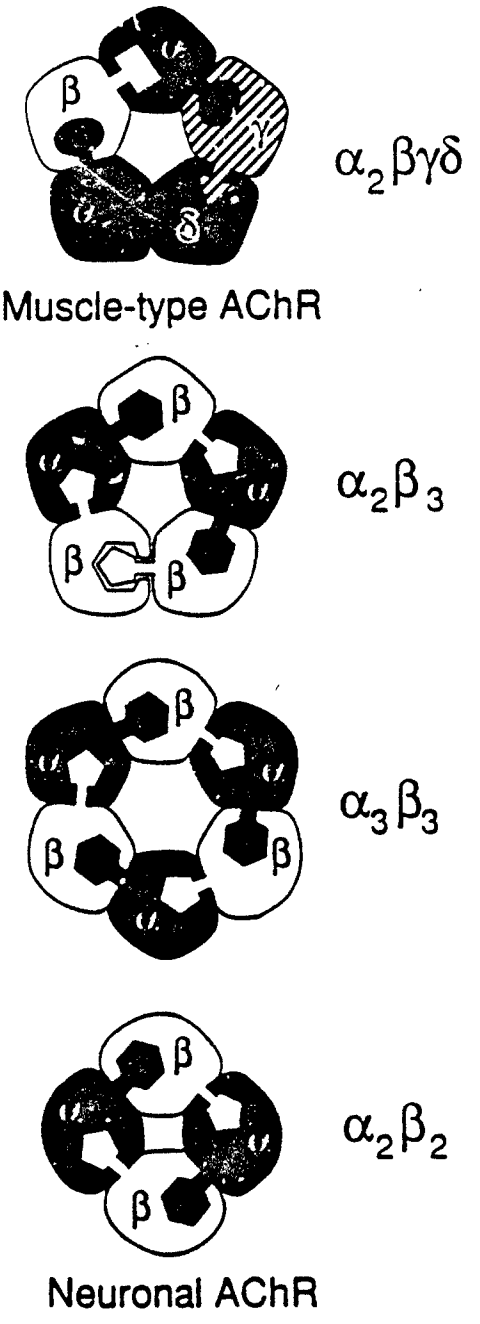


Figure 34. Nicotinic receptor subunit organization. *Diagrammatic end-on views of subunits around the cation channel are shown. The interlocking contacts between each subunit depict conserved (probably hydrophobic) interfaces through which the subunits might assemble to form the channel. Reproduced from reference 1.*

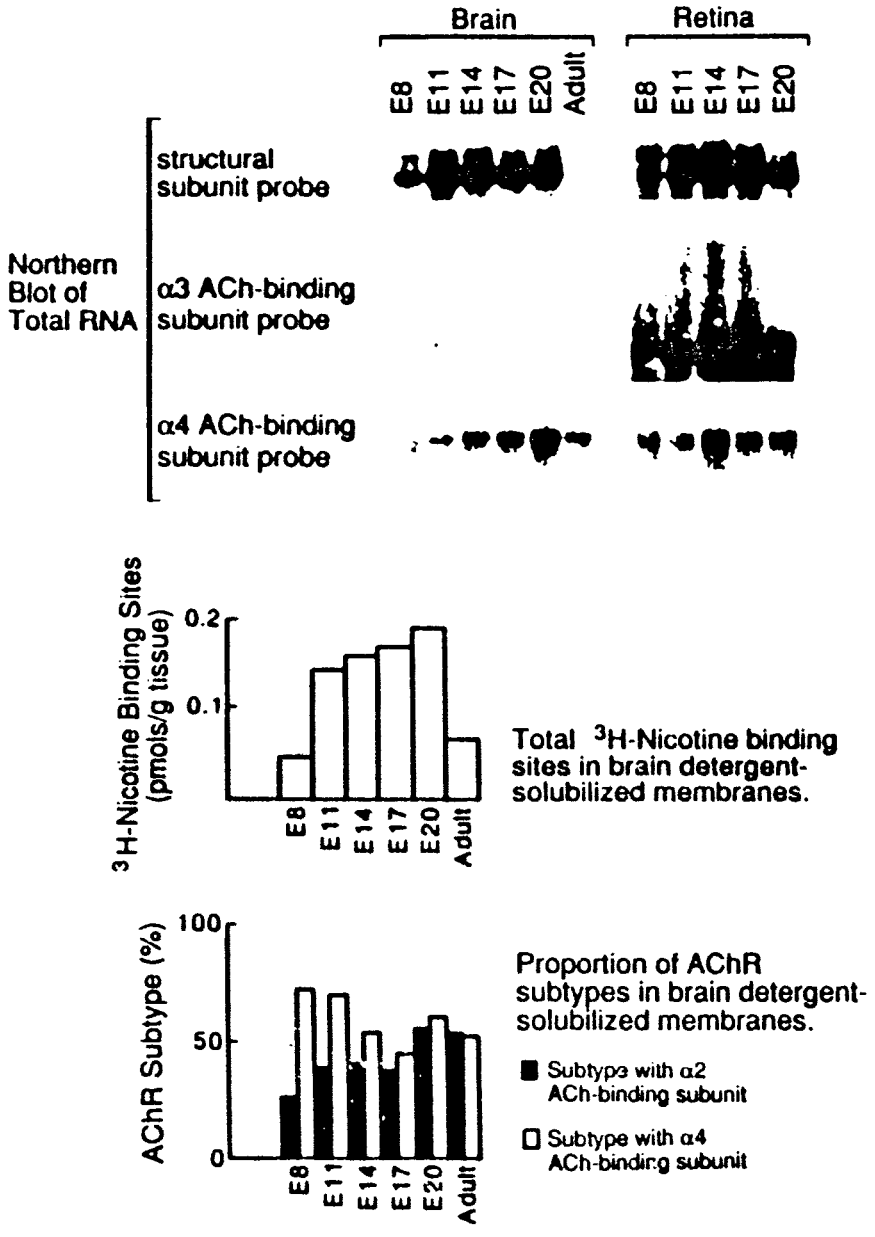


Figure 35. Detection of nicotinic receptor RNAs in developing chicken brain and retina. (1) Northern blot analysis: Total brain and retina RNA (20 µg/lane) was resolved through a formaldehyde-containing agarose gel, transferred to a nylon membrane, and then hybridized at high stringency with each ^{32}P-labeled cDNA probe. Only the relevant part of each blot is shown. (2) Total ^{3}H-nicotine binding sites in detergent extracts of brain were determined by a glass fiber filter binding assay.[13] Detergent extracts were prepared from 4-8 g of brain tissue for each time point. Quadruplicate aliquots (200 µl) of detergent extract were incubated for 1 hour, 4°C, with 20 nM ^{3}H-nicotine, and then diluted with 4 ml of ice-cold 50 mM Tris, pH 7.6, and filtered through Whatman GF/B filters presoaked in 0.3% polyethylenimine. Filters were washed 3 times with 4 ml of the same buffer and bound radioactivity determined by scintillation counting. Nonspecific binding was determined by incubation in the presence of 1 mM nonradioactive nicotine, and has been subtracted. (3) The proportion of receptor subtypes was determined by shaking aliquots of each detergent extract overnight at 4°C with mAb 35-Sepharose (to deplete receptors with the α2 acetylcholire-binding subunit), or mAb 285-Sepharose (to deplete receptors with the α4 acetylcholine-binding subunit), and goat anti-rat IgG Sepharose (which will not deplete any receptors and, therefore, is a control to determine total receptor amount). The Sepharose was pelleted and the ^{3}H-nicotine binding sites in the extracts measured by filter binding assay, as described above. The percent of the total ^{3}H-nicotine binding sites depleted by each mAb (i.e. the percent of that receptor subtype) was determined and expressed as the percent of the total.

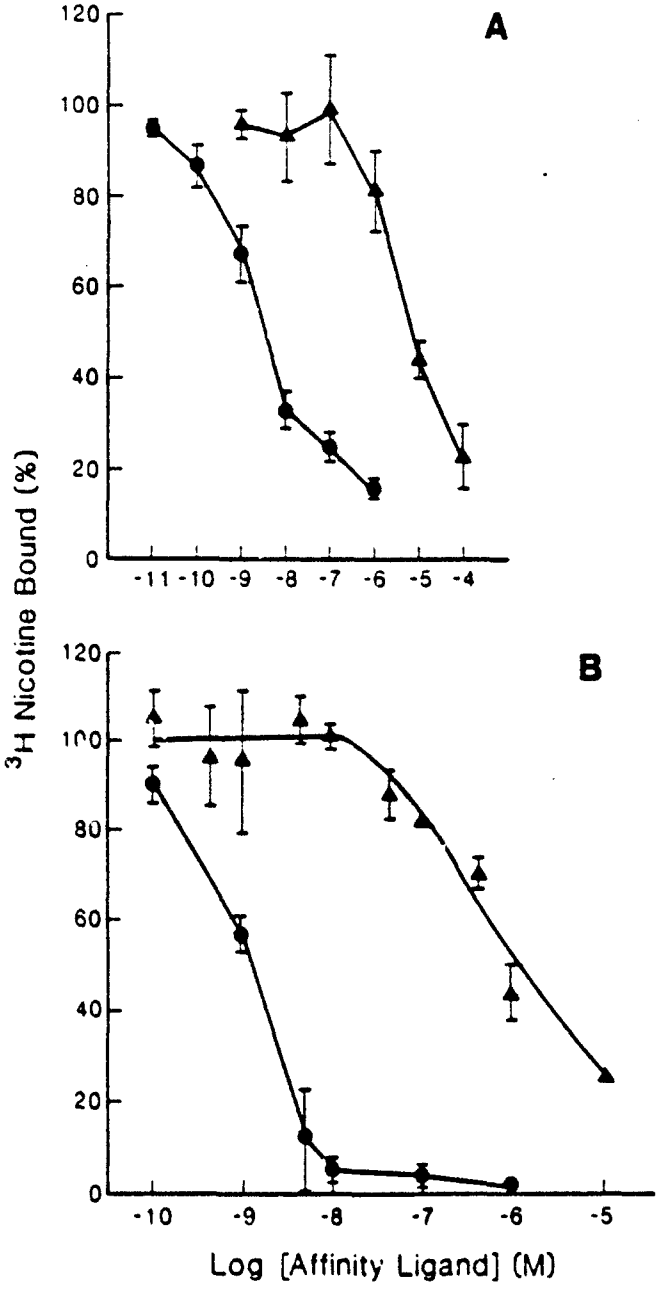


Figure 36. Affinity labeling of nicotine receptors from bovine brain (A) and human brain (B) with bromoacetylcholine (O) and MBTA (Δ).
Bromoacetylcholine was 10^3-fold more effective. Receptors in Triton X-100 extracts were immobilized on mAb 290 coupled to goat anti-rat IgG-Sepharose, reduced with 1 mM dithiothreitol, then labeled with BAC or MBTA. Following re-oxidation with 0.1 mM dithiobis (2-nitrobenzoic acid), binding of 10 nM ^{3}H-nicotine was assayed. Reproduced from reference 20.

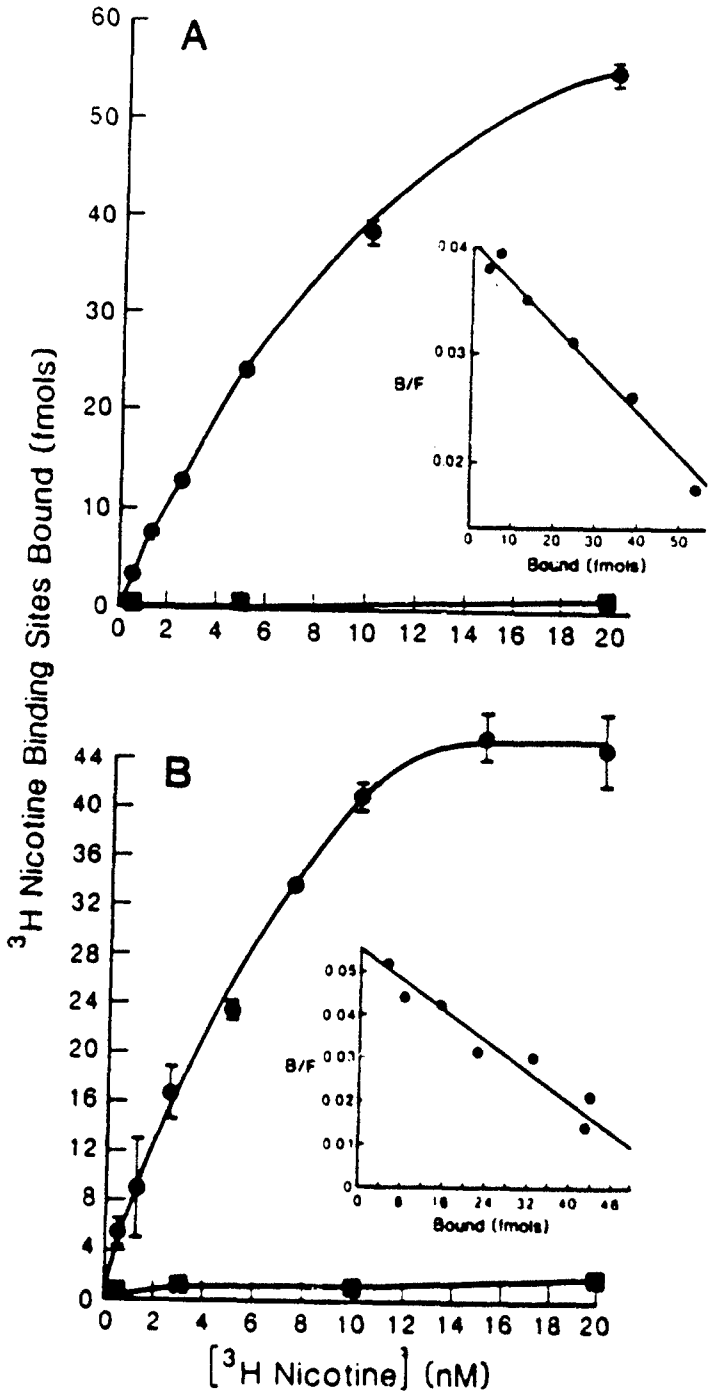


Figure 37. Binding of ^{3}H-nicotine to receptors from bovine brain (A) and human brain (B). Receptors in Triton X-100 extracts were immobilized on mAb 290 coupled to goat anti-rat IgG-Sepharose. Reproduced from reference 20.

*Table 6. Inhibition of (DL)-^{3}H-Nicotine Binding to Chicken Brain Receptor Subtypes by Cholinergic Ligands**

	K_I (M)	
Ligand	mAb 35-Sepharose-immobilized receptor ($\alpha 2\gamma 2$ subtype)	mAb 285-Sepharose-immobilized receptor ($\alpha 4\beta 2$ subtype)
L-Nicotine	1.1×10^{-9}	1.6×10^{-9}
Cytosine	1.1×10^{-9}	1.4×10^{-9}
Carbachol	8.4×10^{-8}	1.4×10^{-7}
Decamethonium	8.7×10^{-6}	3.0×10^{-6}
Curare	1.1×10^{-5}	3.7×10^{-6}
Hexamethonium	$>10^{-3}$	$>10^{-3}$
Mecamylamine	$>10^{-3}$	$>10^{-3}$
αBgt	$>10^{-6}$	$>10^{-6}$
Atropine	$>10^{-3}$	$>10^{-3}$

* Reproduced from reference 19.

*Table 7. Inhibition of (DL)^{3}H-Nicotine Binding to Bovine and Human Brain Nicotinic Receptors**

	K_I (M)	
Ligand	Bovine Brain	Human Brain
Cytosine	4.2×10^{-9}	1.1×10^{-9}
(L)Nicotine	1.6×10^{-8}	6.5×10^{-9}
Acetylcholine	4.3×10^{-8}	2.7×10^{-9}
Carbachol	2.1×10^{-7}	4.1×10^{-7}
Curare	1.9×10^{-5}	4.7×10^{-5}
α-Bungarotoxin	$>10^{-6}$	$>10^{-6}$
Mecamylamine	No data	$>10^{-3}$
Hexamethonium	No data	$>10^{-3}$

* Reproduced from reference 20.

Histological Localization of Receptors

The nicotinic receptors in rat brain were histochemically localized using ^{125}I-labeled mAb 270 (**Figures 38-42**).[76] mAb 270 recognizes structural subunits common to several receptor subtypes,[19] but in rat brains most of the receptors are of the $\alpha4\beta2$ subtype.[14,17,35] Figures 38 and 39 present an overview of the labeling pattern. The pattern was very similar to that previously reported by Clarke et al.[41] 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.[41] **Figure 40** 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 39-41**). 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 40**). The observation that many brain nicotinic receptors are apparently in presynaptic locations[76,77] is consistent with the idea that these receptors may be involved in modulating the release of other transmitters.[78]

Nicotinic receptors were localized autoradiographically in zebra finch brains[21] in a collaborative study with Dr. Thomas Podleski and coworkers at Cornell University, for which we provided the labeled reagents ^{125}I-α-bungarotoxin, ^{125}I-mAb 270, and ^{125}I-mAb 35. These agents were localized throughout brains in substantial detail using frozen sections of unfixed tissue (**Figure 43**). mAbs 35 and 270 both bind to structural subunits, but in chickens mAb 35 binds only to native $\alpha2\beta2$ and $\alpha3\beta2$ subtypes, whereas mAb 270 also binds to $\alpha4\beta2$ subtypes. The binding pattern for mAb 270 and mAb 35 in finch brains was very similar, as expected, though not quite identical. ^{125}I-α-bungarotoxin showed an overlapping but distinct pattern of binding, as expected.

Nicotinic receptors were localized by indirect immunofluorescence and indirect immunoperoxidase labeling of chicken retinas with mAbs 210 and 270.[22] This is a collaborative study with Drs. Kent Keyser and Harvey Karten at the University of California, San Diego. Formalin-fixed tissue was used to permit cell-level resolution, but fixation may alter receptor antigenicity. mAb 270 recognizes the structural subunits of $\alpha2\beta2$, $\alpha3\beta2$, and $\alpha4\beta2$ receptor subtypes. mAb 210 binds strongly only to $\alpha2\beta2$ and $\alpha3\beta2$ brain receptor subtypes. **Figure 35** shows that retina contains a great deal of structural subunit mRNAs and more $\alpha3$- than $\alpha4$-type acetylcholine-binding subunit mRNA. mAb 210 and mAb 270 gave similar labeling patterns in retina, but mAb 210 was more effective. **Figures 44-46** illustrate labeling of retinas with mAb 210. A nearly identical labeling pattern can be

obtained with mAb 315 to $\alpha 3$ subunits, as shown in **Figure 48**, suggesting that most of the receptors in retina localized by mAb 210 are of the $\alpha 3\beta 2$ subtype.

mAbs 210, 270, and 315 labeled cells in the inner nuclear layer and ganglion cell layer of chicken retinas, including ganglion cells, displaced ganglion cells, and amacrine cells. Approximately 12-18% of ganglion cells were labeled. The retina provides a small, relatively accessible, and well-characterized piece of central nervous tissue in which we can begin to work out the techniques and data necessary to understand the functional roles of nicotinic receptor subtypes. This will ultimately involve differentially localizing receptor subtypes at high resolution, localizing both the proteins and the mRNAs which code them (as in **Figures 47 and 48**), and localizing the receptors with respect to other cells, such as those which release acetylcholine (as in **Figure 46**).

Nicotinic receptors were immunolocalized in frog brains at both the light and electron microscopic level[23] in collaborative experiments with Dr. Peter Sargent of the University of California, Riverside. Previously, our library of mAbs to receptors from electric organs were tested for ability to bind to receptors in frog muscle.[58] A subset of 28 of these mAbs was recently found to bind to the optic tectum of Rana pipiens.[23] Most of these were directed against the main immunogenic region. Of these, mAb 22 was used most extensively. This mAb labeled a subset of retinotectal projections (**Figure 49**). In frogs it is unknown what receptor subtype or subtypes are being detected. Since they are detected by mAbs to the main immunogenic region, the mAbs may be binding to structural subunits, perhaps in $\alpha 2\beta 2$ or $\alpha 3\beta 2$ subtypes. Electron microscopy revealed that labeling was associated with extrasynaptic regions (**Figure 50**). As in goldfish,[79] chickens,[22,76] and rats,[76] removal of one retina resulted in the loss of immunoreactivity in the contralateral tectum (**Figure 51**). Labeling was found in the optic tract and associated with retinal ganglion cells. Thus, in all species examined, nicotinic receptors on central processes of retinal ganglion cells are a prominent feature.

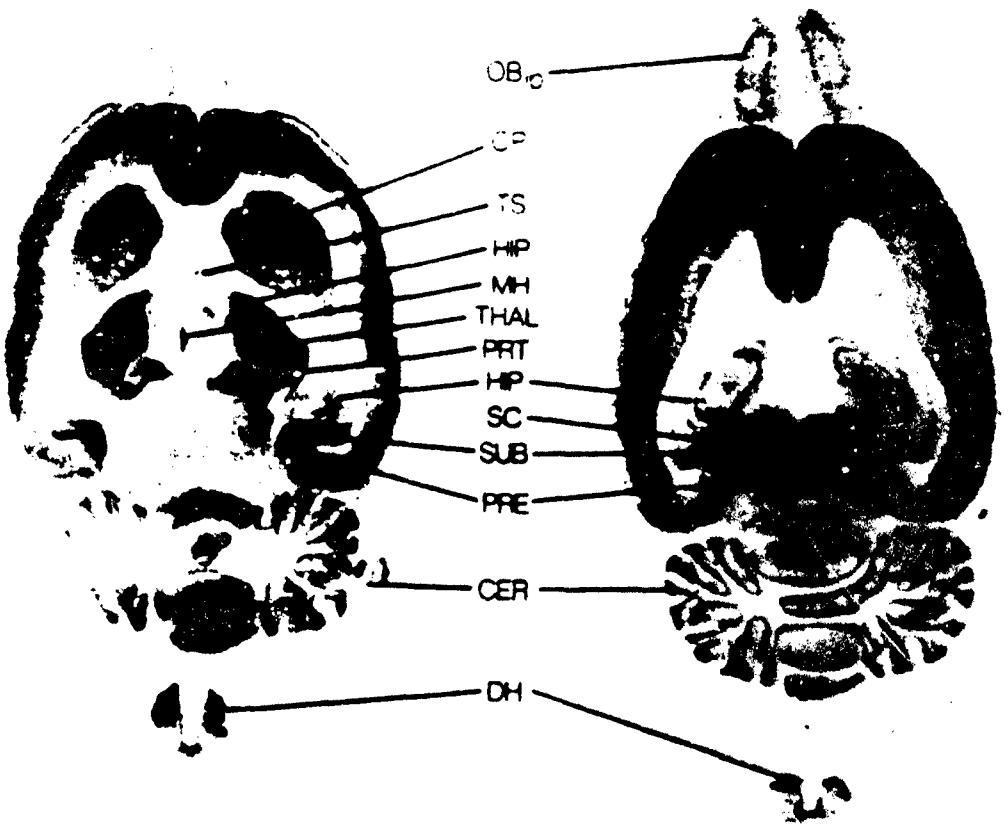


Figure 38. Photomicrographs illustrating the distribution of ^{125}I-mAb 270 binding sites in two horizontal sections through the rat brain and spinal cord. OBip - olfactory bulb inner plexiform layer, CP - caudoputamen, TS - triangular nucleus of the septum, HIP - hippocampus and dentate gyrus, MH - 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 15.

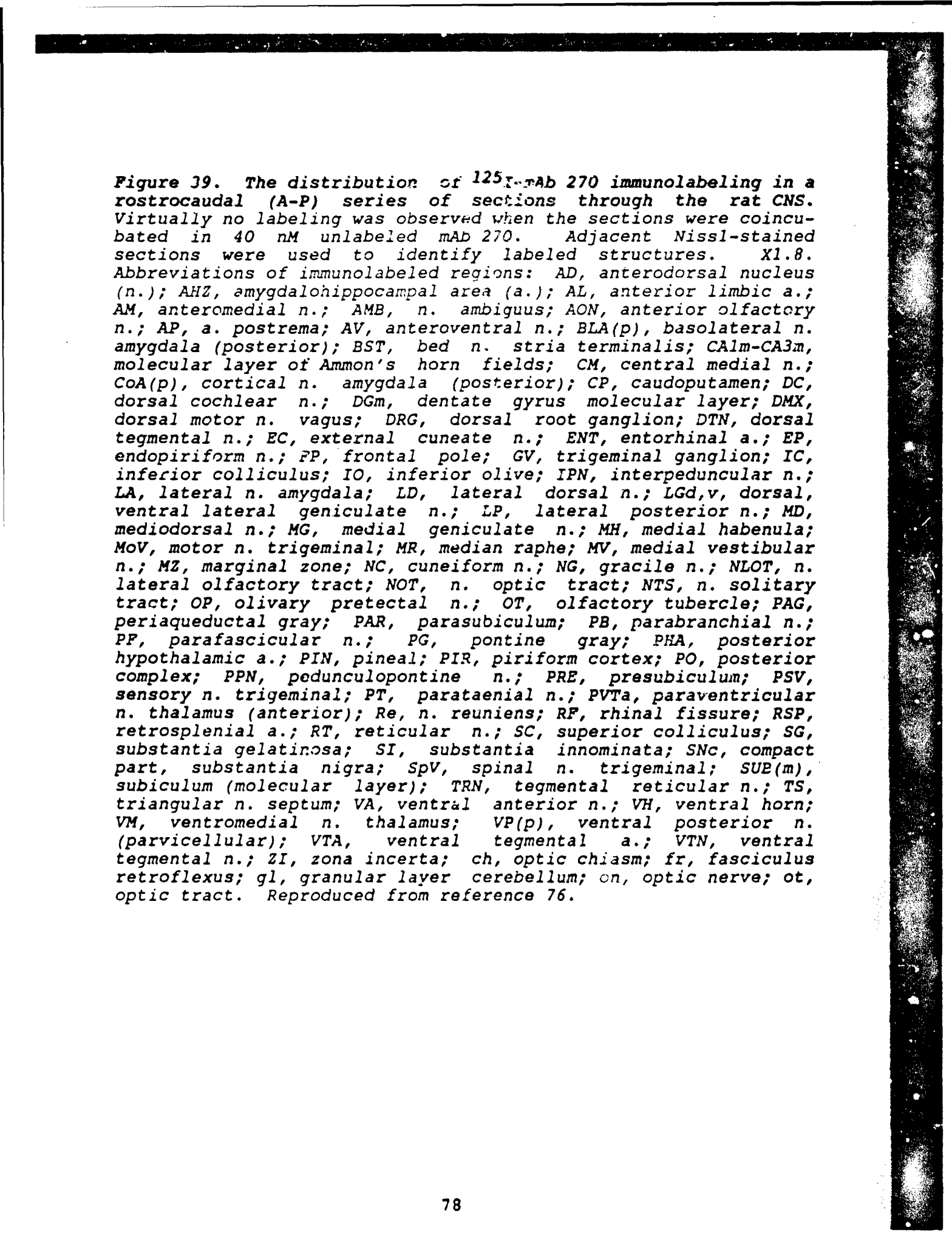
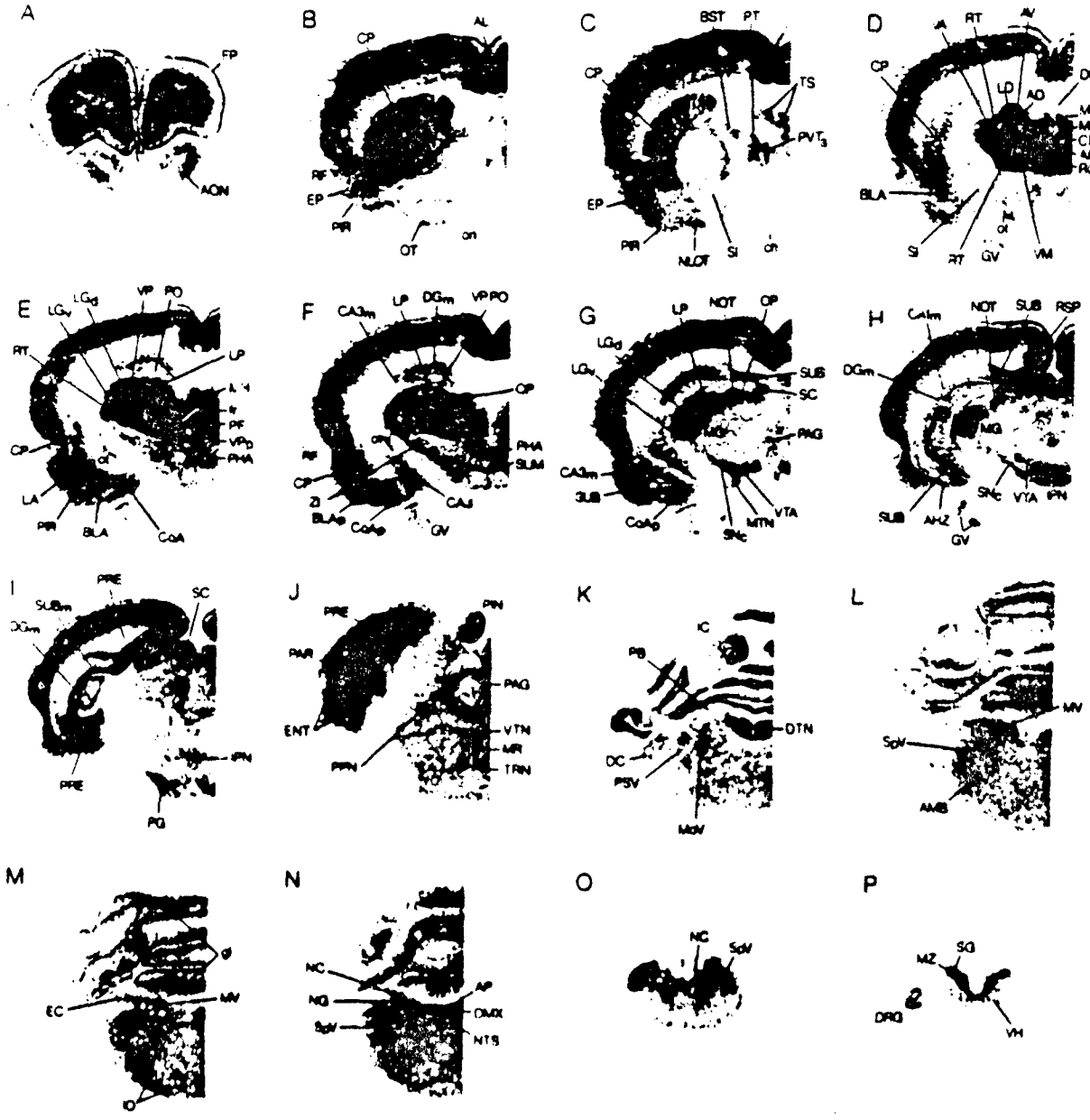


Figure 39. *The distribution of ^{125}I-mAb 270 immunolabeling in a rostrocaudal (A-P) series of sections through the rat CNS. Virtually no labeling was observed when the sections were coincubated in 40 nM unlabeled mAb 270. Adjacent Nissl-stained sections were used to identify labeled structures. X1.8. 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 76.*

Figure 39. The distribution of ^{125}I-mAb 270 immunolabeling in a rostrocaudal (A-P) series of sections through the rat CNS.



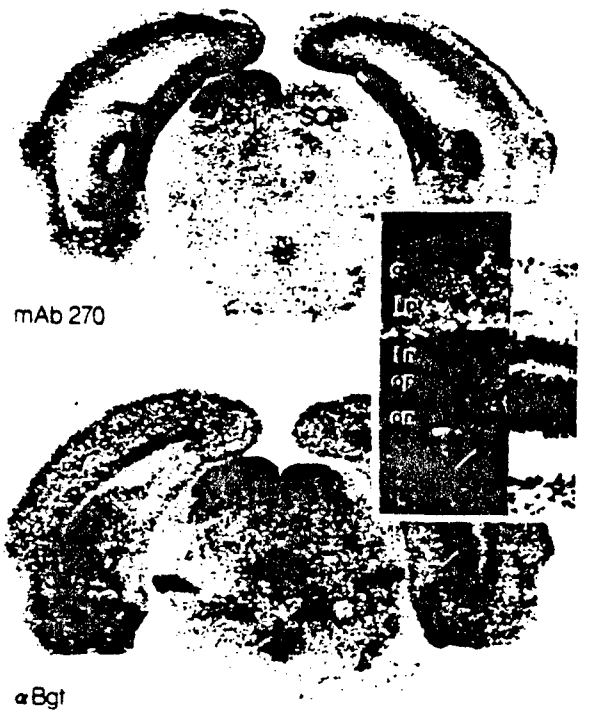


Figure 40. Effect of left enucleation 3 weeks before sacrifice on ^{125}I-mAb 270 immunolabeling (top) and ^{125}I-α-bungarotoxin binding (bottom) in adjacent sections of the rat superior colliculus (SC). Note disappearance of mAb 270 labeling in the contralateral SC (SCc), and dense labeling in the 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 76.

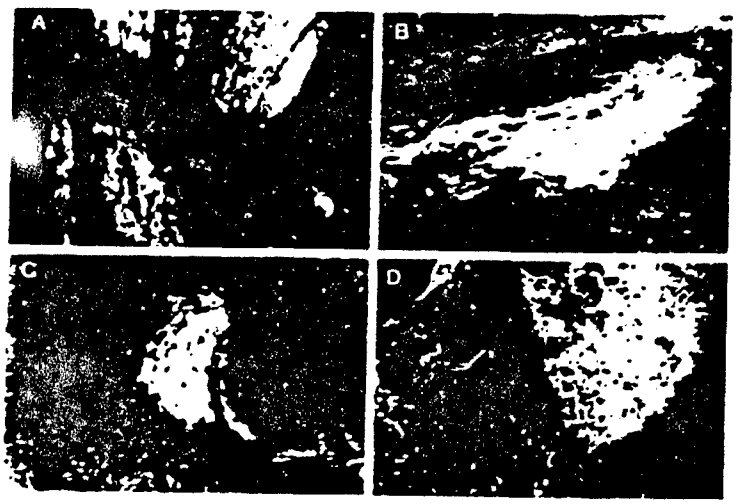


Figure 41. Photomicrographs showing the indirect immunofluorescence localization of mAb 270 (A,B,D) and mAb 290 (C) in the mouse brain. (A) Dorsal (top) and ventral (bottom) lateral geniculate nucleus; compare with Figure 39. (B) Olivary pretectal nucleus; compare with Figure 39F. (C) Right medial terminal nucleus; compare with Figure 39G. (D) Medial habenula with unlabeled lateral habenula and stria medullaris to the left; compare with Figure 39D,E. Because the fasciculus retroflexus and interpeduncular nucleus were clearly labeled (Figure 39E-I) it appears likely that neuronal acetylcholine receptor is synthesized in medial habenular cells 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 76.

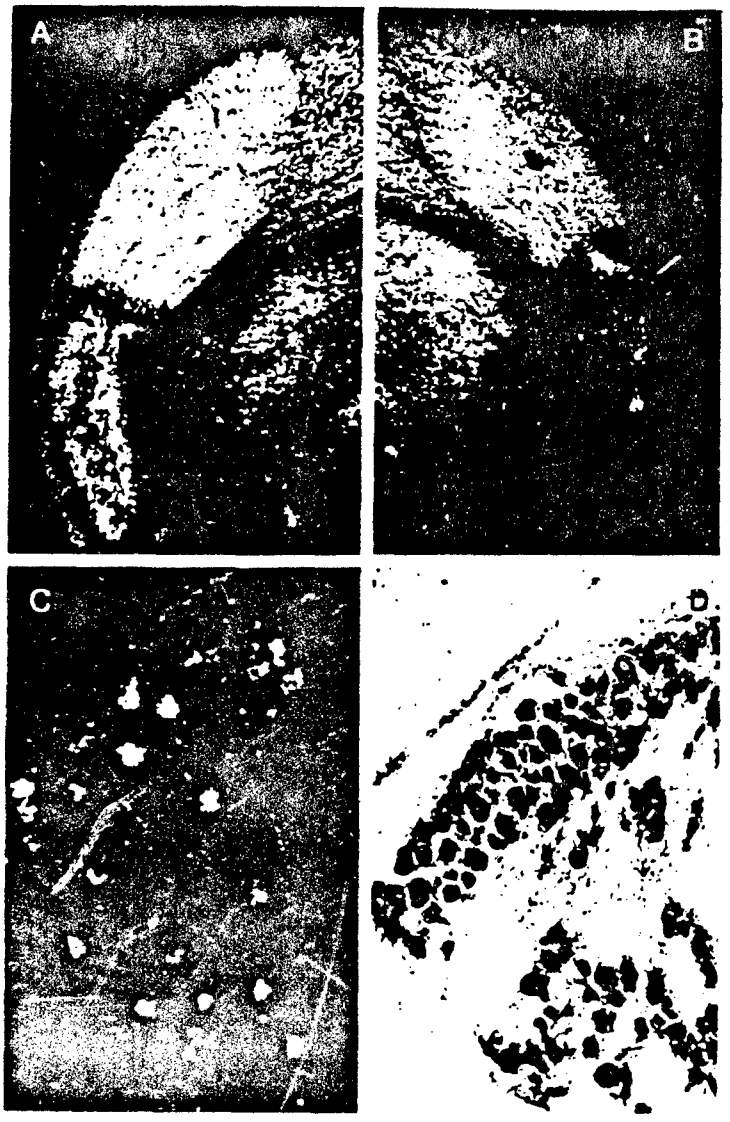
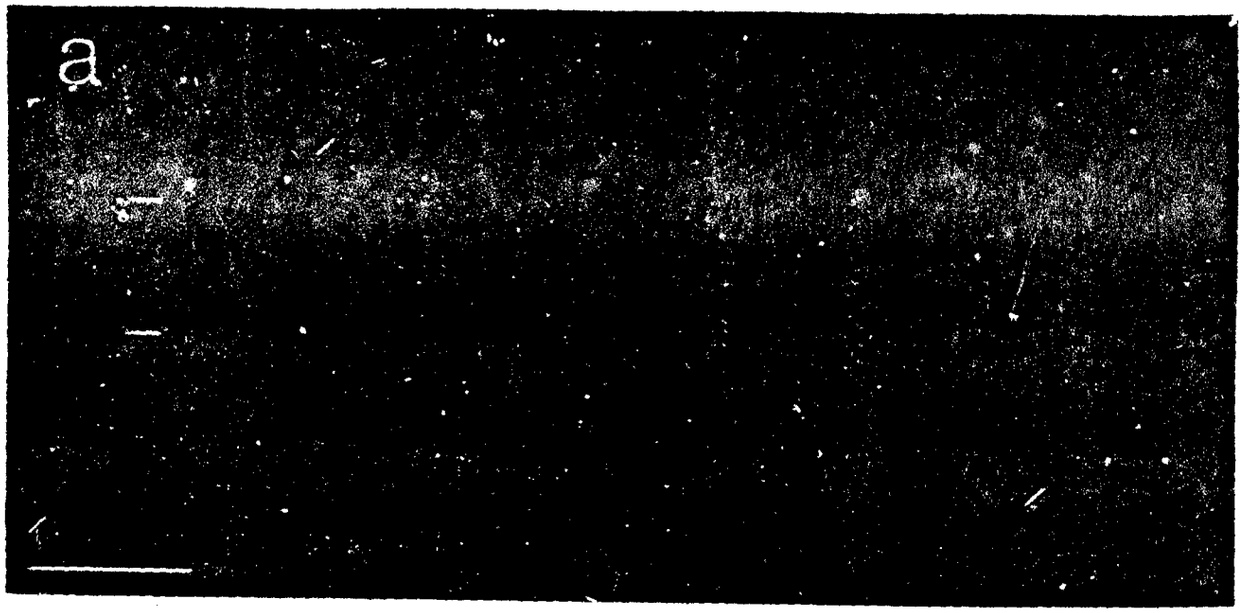


Figure 42. Dark-field photomicrographs of ^{125}I-mAb 270 immunolabeling in the lateral geniculate nucleus on the side ipsilateral (A) and contralateral (B) to enucleation in the rat illustrated in Figure 40; comparable to level F in Figure 39. X30. (C) Dark-field photomicrograph of ^{125}I-mAb 270 immunolabeled ganglion cells in the trigeminal ganglion (see Figure 39E-G). (D) Nissl-stained section adjacent to C. C,D X100. Reproduced from reference 76.

Structure	Relative staining intensity α-bungarotoxin	mAb 35	mAb 270
nucleus accumbens	1	1	1
nucleus basalis	1	1	1
nucleus cerebelli intermedium	2	3	3
nucleus cerebelli medialis	2	3	3
nucleus cuneatus externus	1	1	2
ectostriatum	1	1	1
fasciculus prosencephali lateralis	3	1	2
substantia grisea centralis	2	2	2
nucleus geniculatus lateralis, pars ventralis	2	4	4
hyperstriatum accessorium	4	1	1
hyperstriatum dorsalis	3	3	3
nucleus habenula medialis	2	3	3
hippocampus	3	3	3
hyperstriatum ventralis	2	3	3
nucleus hyperstriatum ventralis, pars caudalis	3	1	1
nucleus intercollicularis	2	1	3
nucleus isthmi, pars magnocellularis	1	3	3
nucleus isthmi, pars parvocellularis	2	2	2
nucleus laminaris	1	3	1
nucleus lentiformis mesencephali	3	3	3
locus ceruleus	3	3	3
lobus parolfactorius	1	1	1
nucleus magnocellularis, anterior neostriatum	3	1	1
nucleus mesencephalicus lateralis, pars dorsalis			
(peripheral shell)	3	3	3
(core region)	1	1	1
nucleus motorius nervi trigemini	3	3	3
neostriatum	2	1	1
nucleus nervi trochlearis	3	3	3
nucleus nervi abducens	3	3	3
nucleus nervi facialis	3	3	3
nucleus motorius dorsalis nervi vagi	3	3	3
nucleus nervi hypoglossi	3	1	1
nucleus olivaris inferior	4	1	2
nucleus nervi occulomotorii	3	3	3
tractus occipitomesencephalicus	1	1	1
paleostriatum augmentatum	2	1	2
nucleus pontis lateralis	1	3	1
nucleus pontis medialis	1	3	1
nucleus medialis hypothalami posterioris	3	1	3
nucleus preopticus medialis	3	1	3
nucleus pretectalis	3	3	3
periventricular organ	4	1	1
nucleus robustus archistriatalis	1	1	1
nucleus reticularis gigantocellularis	1	3	3
nucleus reticularis lateralis	1	3	3
nucleus reticularis pontis caudalis	1	1	3
nucleus reticularis parvocellularis	1	1	2
nucleus rotundus	1	1	1
nucleus tractus solitarii	1	3	3
nucleus semilunaris	3	1	2
nucleus septalis medialis	2	1	2
nucleus subpretectalis	1	3	3
nucleus superficialis parvocellularis	1	2	2
nucleus spiriformis lateralis	1	4	4
torus semicircularis	2	1	2
tectum opticum:			
striatum opticum	3	2	2
stratum griseum & fibrosum superficialis	3	3	3
stratum griseum centrale	2	3	3
stratum album centralis	1	1	1
substantia grisea et fibrosa periventricularis	2	1	2
tractus opticus	1	1	1
nucleus et tractus descendens nervi trigemini	1	3	3
nucleus vestibularis dorsolateralis	2	3	2
nucleus vestibularis lateralis	2	3	2
nucleus vestibularis medialis	3	2	3

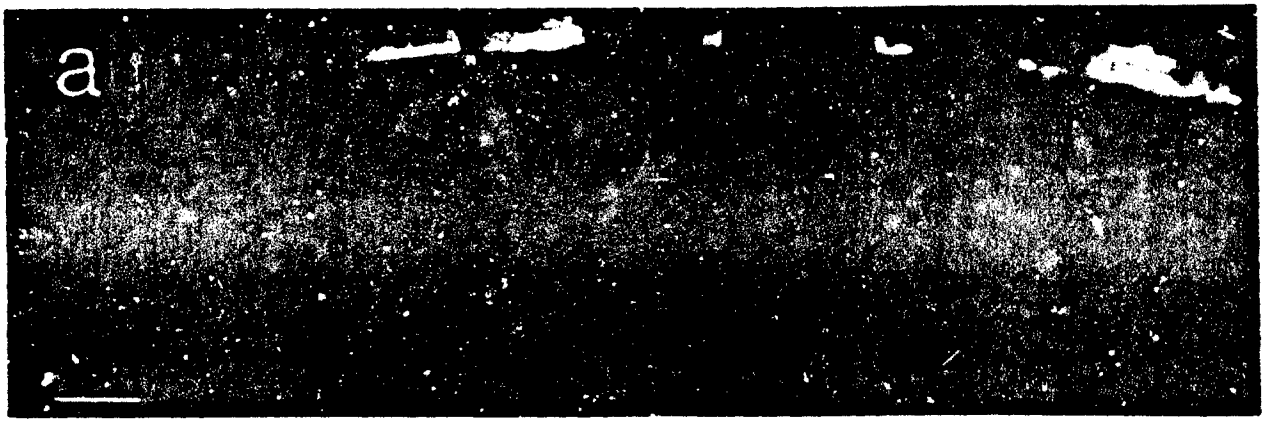
Figure 43. Summary of the pattern of nicotinic ligand labeling in the Zebra finch brain. *Reproduced from reference 21.*

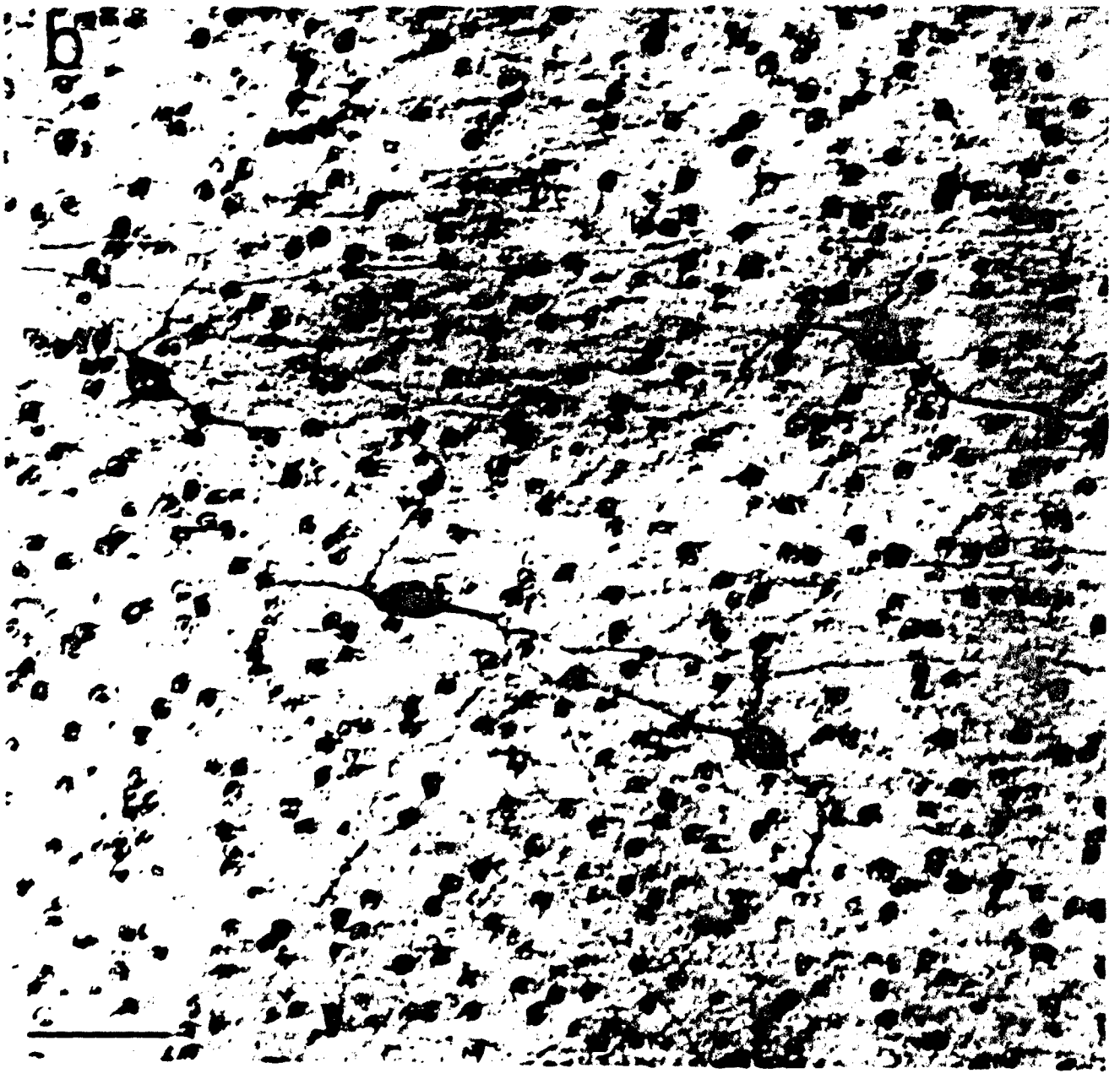

a


b


c

Figure 44. Photomicrographs and a drawing of cross sections of mAb 210-labeled retinas. The top panel (a) illustrates the distribution of immunoreactivity in a 10-μm-thick transverse section labeled with a fluorescein-conjugated secondary antiserum. Labeled somata visible in both the inner nuclear layer (INL) and ganglion cell layer (GCL) gave rise to processes that arborized in two distinct laminae of the inner plexiform layer (IPL). The middle panel (b) illustrates similar features in an avidin-biotin-horseradish-peroxidase-reacted, 20-μm-thick section. Note, however, the large cell visible in the INL. The lower panel (c) is a camera lucida drawing of a section processed as in panel (b). This illustrates that dendrites could be followed from the labeled somata (stippled) in the INL and GCL into two laminae of the IPL. The processes often extended beyond the more proximal band of labeling and terminated in the more distal one. Scale bar = 50 μm in (a); 50 μm in (b); 30 μm in (c). Reproduced from reference 22.


a


b

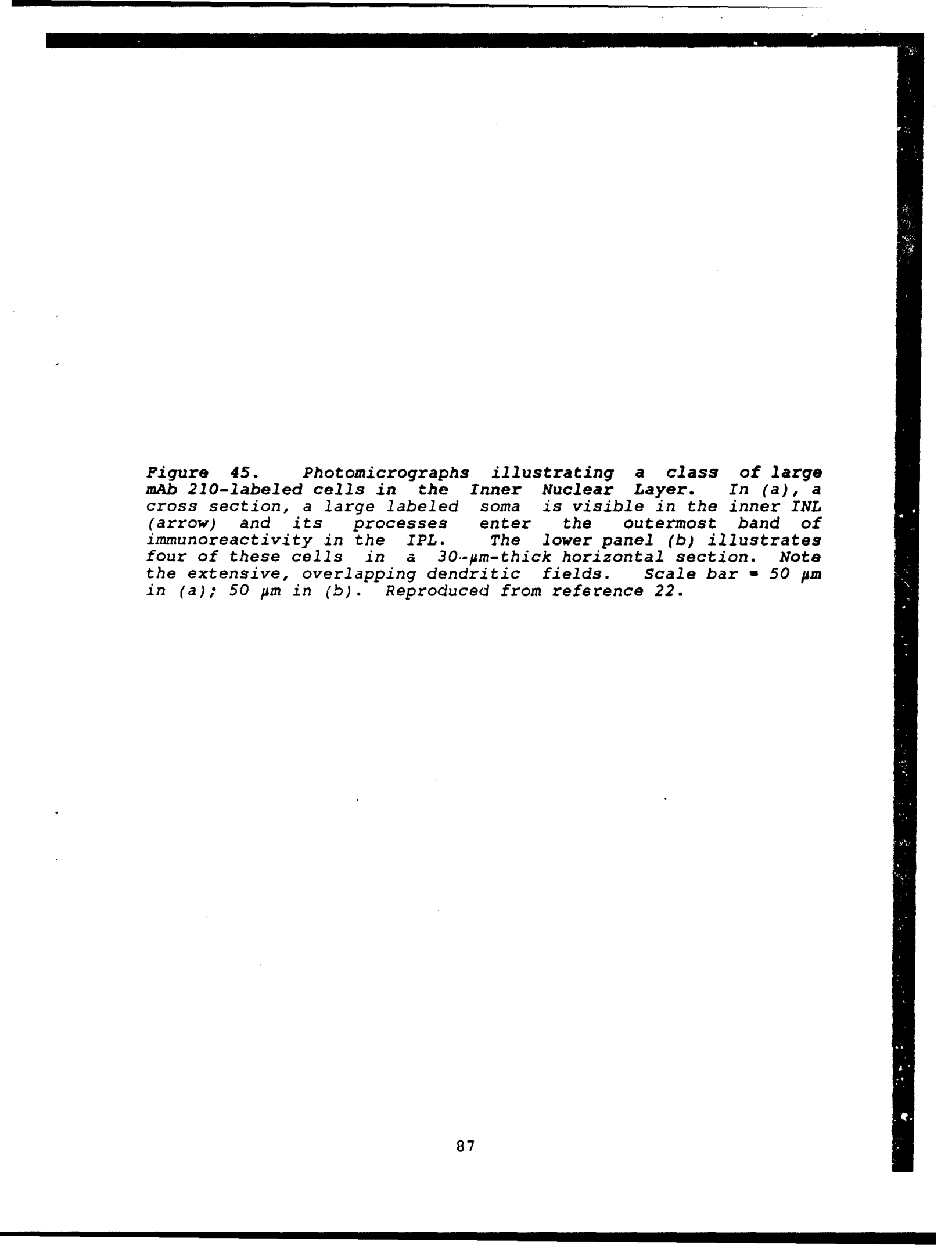


Figure 45. Photomicrographs illustrating a class of large mAb 210-labeled cells in the Inner Nuclear Layer. In (a), a cross section, a large labeled soma is visible in the inner INL (arrow) and its processes enter the outermost band of immunoreactivity in the IPL. The lower panel (b) illustrates four of these cells in a 30-μm-thick horizontal section. Note the extensive, overlapping dendritic fields. Scale bar = 50 μm in (a); 50 μm in (b). Reproduced from reference 22.

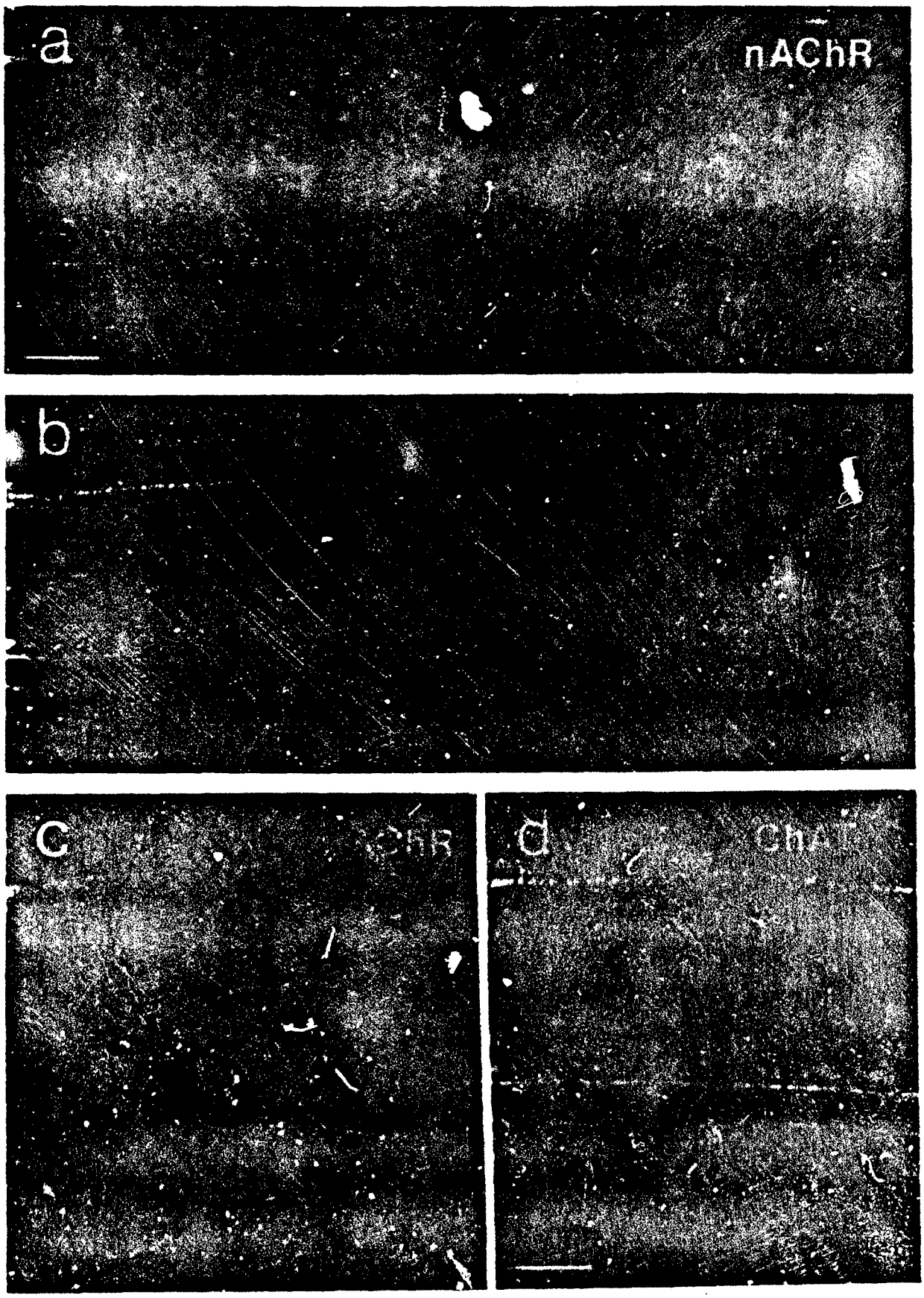

a
nAChR
b
c
d

Figure 46. Fluorescence micrographs of a transverse section of retina illustrating the distribution of mAb 210-positive cells and processes and the corresponding pattern of choline-acetyltransferase (ChAT) immunoreactivity in the same section. These panels illustrate that the patterns of immunoreactivity in the INL, IPL, and GCL were similar (a,b) but that the ChAT-positive cells were smaller and more numerous than receptor-positive cells. Panels (c,d) are contiguous portions of the same section placed side by side to illustrate that the arborization patterns of the two cell types were in register. However, the band of mAb 210-positive immunoreactivity in lamina 2 extended inward more than did the corresponding ChAT-positive band. Scale bar = 50 μm for (a) and (b); 50 μm for (c) and (d). Reproduced from reference 22.

A. in situ hybridization B. monoclonal antibody

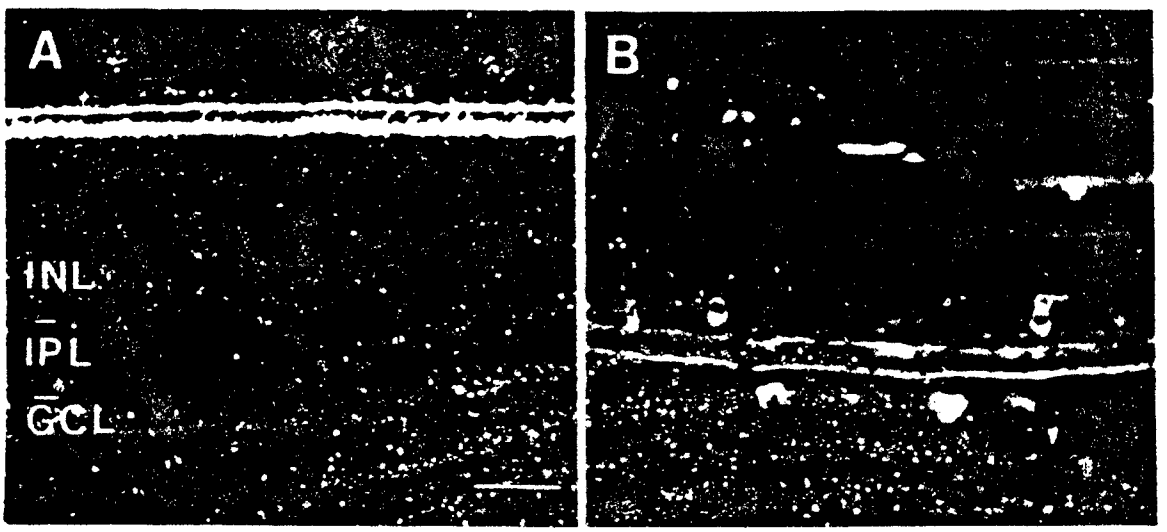


Figure 47. *Localization of the structural subunit in chicken retina. (A) mRNA for the structural subunit is localized using an ^{35}S-labeled anti-sense RNA probe corresponding to the putative cytoplasmic loop (amino acids 306 to 410) of the structural subunit. Cell bodies in the GCL are labeled, as are cells in the INL, whereas dendritic processes in the IPL are not heavily labeled. The pigment layer produces an intense artifactual birefringent double layer at the top of this darkfield micrograph. A control sense cRNA probe gave uniform low background labeling. The scale bar is 40 μ. (B) Structural subunit protein is localized by indirect immunofluorescence using mAb 270. The subunit protein is localized in the cell bodies of ganglion cells and displaced ganglion cells, as well as in the dendritic and axonal processes of these cells, and some others, located in the inner and outer plexiform layers. The pigment layer at the top appears dark in this fluorescence micrograph. The sections in A and B are from different parts of the retina and differ in sizes. Reproduced from reference 36.*

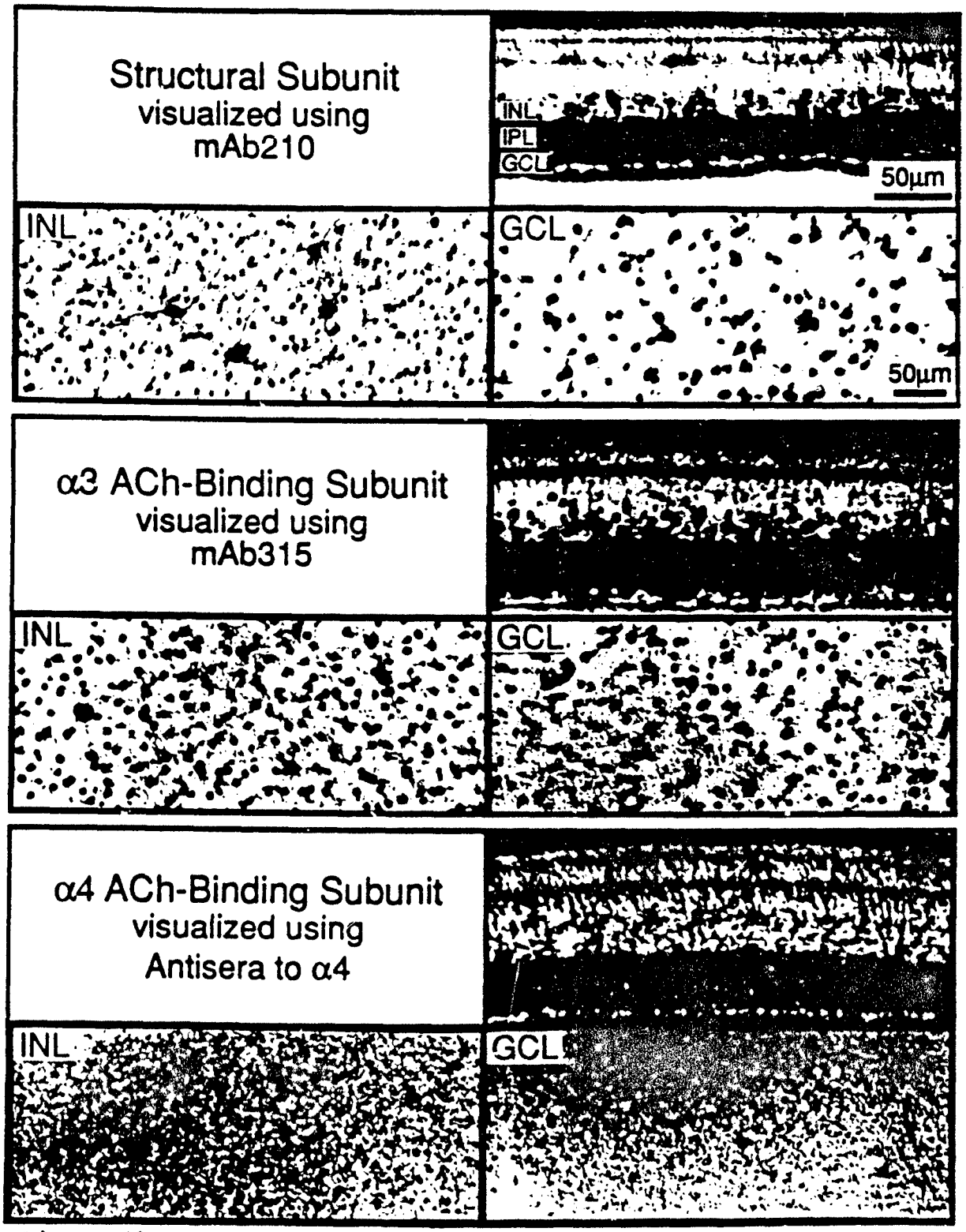


Figure 48. Detection of nicotinic receptor subunits in chicken retina. In the upper right of each panel is a cross-section of retina, in all cases at the same magnification. "INL" indicates the inner nuclear layer. "IPL" indicates the inner plexiform layer. "GCL" indicates the ganglion cell layer. In the lower two halves of each panel are horizontal sections through the INL (on the left) and the GCL (on the right), all at the same magnification. Bound antibodies are visualized using biotinylated goat anti-rat IgG and avidin-labeled peroxidase.

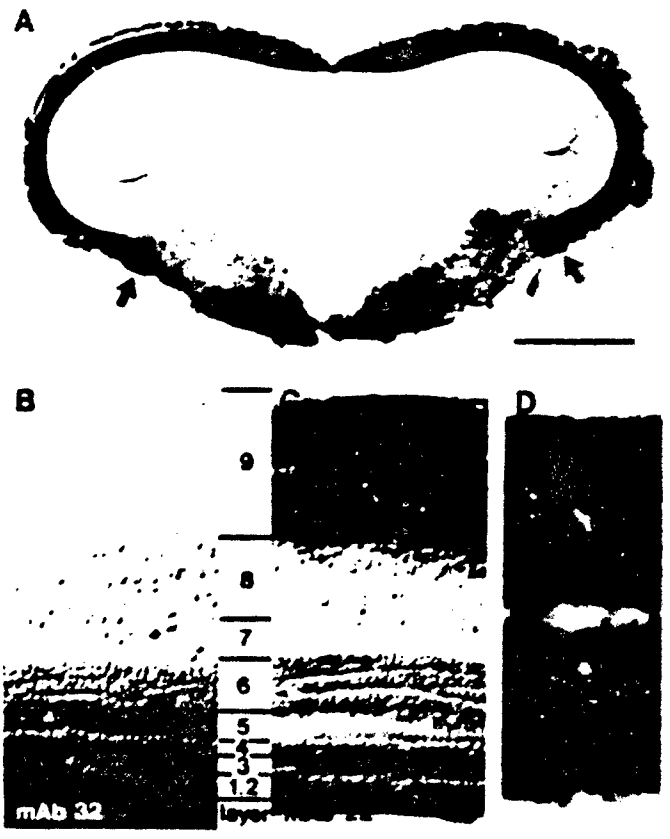


Figure 49. Immunoperoxidase staining of the optic tectum using anti-receptor mAb 22. (A) Low-power, bright-field photomicrograph of a 100 μm Vibratome section from the midbrain of Rana pipiens. Peroxidase staining is found within the superficial parts of the tectum and extends to the lateral optic tracts (arrows). Density at the base of the tectum (between the 2 arrows) represents cobalt staining of myelin tracts and not horseradish peroxidase reaction product. (B) and (C) Higher-power differential interference contrast (Nomarski) photomicrographs after incubating Vibratome sections with the control mAb 32 (B) and the cross-reactive mAb 22 (C) and visualizing mAb binding using the avidin biotinylated-horseradish peroxidase technique. Strong staining is observed after using mAb 22, but not mAb 32 (density at bottom of slices is shadowing due to Nomarski optics). The vertically oriented set of numbers between (B) and (C) refer to the layers of the tectum, extending from the ventricular surface (layer 1) to the pia (layer 9). (D) Banding pattern of stain within the neuropil at high magnification (bright-field optics). The entire field in (D) corresponds to layer 9. Distinct bands of stain are visible. Scale bar (in A): 1 mm in (A), 150 μm in (B,C) and 60 μm in (D). Reproduced from reference 23.

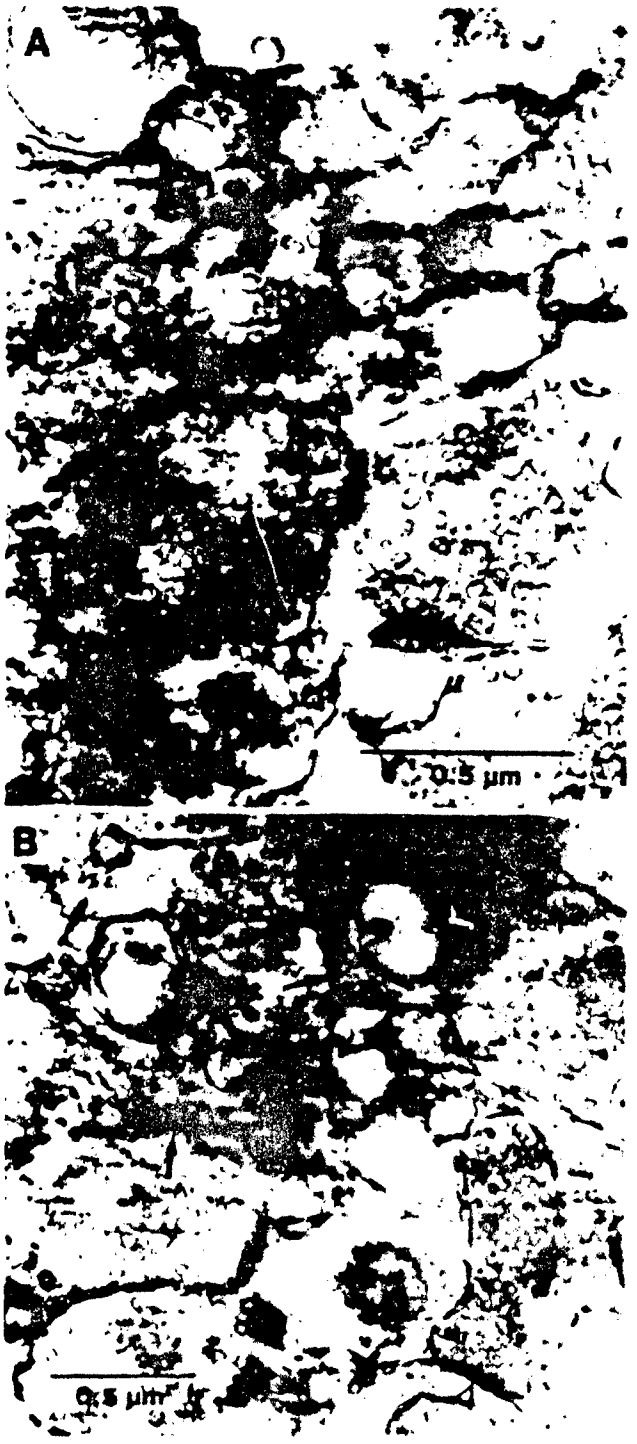


Figure 50. Electron micrographs of the optic neuropil showing extrasynaptic location of receptor-like immunoreactivity. (A) and (B) show two fields taken from the neuropil. Peroxidase stain obtained using mAb 22 and the avidin-biotin technique (arrows) is associated with membranes but not with either the pre- or the postsynaptic membrane at synapses (open arrowheads). Reproduced from reference 23.

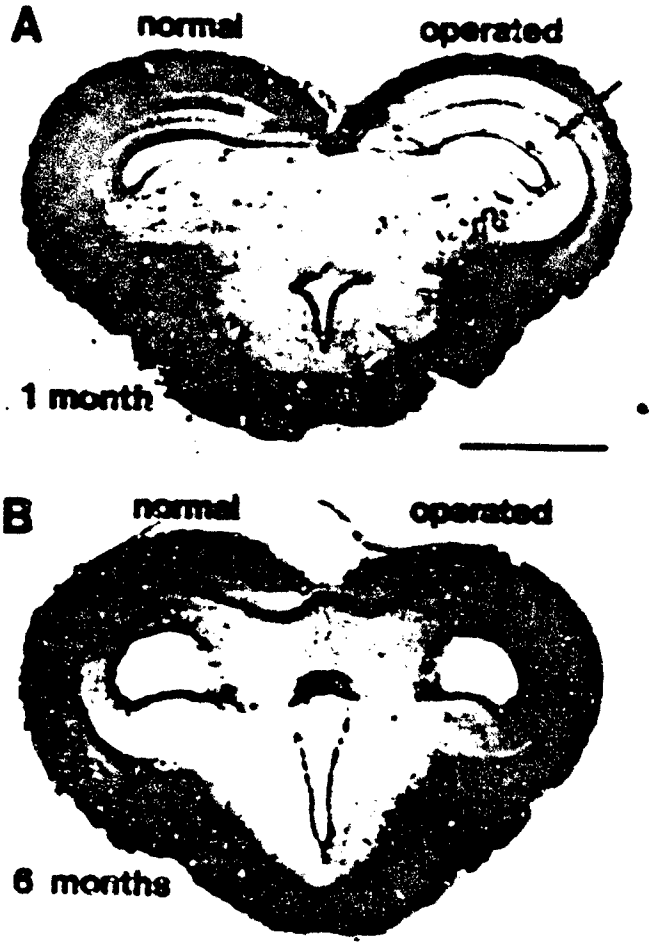


Figure 51. Loss of immunoreactivity following removal of the retina. (A) Removal of one retina results in partial loss of mAb 22 binding in the contralateral tectum 30 days after surgery. The residual staining is located in bands corresponding to projection layers "a," "c," and "e." Staining associated with layer "c" (delineated by the arrows) is most obvious, while that associated with layers "a" and "e" are most evident near the lateral optic tract. Projection layers "a," "c," and "e," which are unmyelinated, survive for long periods after eye enucleation. (B) Removal of the retina 6 months prior to staining resulted in a complete loss of immunoreactivity. Staining at the base of the tectum on both normal and operated sides (see arrowheads in A) corresponds to cobalt staining of myelinated tracts and not to horseradish peroxidase reaction product. Scale bar (in A): 1 mm. Reproduced from reference 23.

Neuronal α-Bungarotoxin-Binding Proteins

The subunit structures of neuronal α-bungarotoxin-binding proteins are not well established. Conti-Tronconi and coworkers were able to obtain limited N-terminal amino acid sequence data from only one subunit of protein affinity purified from chicken brains using α-bungarotoxin.[80] This sequence exhibited similarities to nicotinic receptor sequences, and this data, along with the pharmacological properties of this protein for binding α-bungarotoxin, MBTA, and small cholinergic ligands suggested that these proteins are members of the nicotinic receptor gene family.

Using as a probe a synthetic oligonucleotide whose sequence corresponded to the reported N-terminal amino acid sequence, a cDNA library from chicken brains was screened. This identified a cDNA encoding a virtually identical sequence for what was termed α1 subunits of neuronal α-bungarotoxin-binding protein, as shown in **Figure 52**. The cDNA was termed α because it seemed likely that it encoded an acetylcholine-binding subunit, since the deduced sequence of the subunit included a cysteine pair homologous to the cysteine pair at α192,193 of muscle and neuronal nicotinic receptor acetylcholine-binding subunits (termed α) which react with MBTA. The cDNA initially identified (pCh29-3) was incomplete. As summarized in **Figure 53**, this partial clone was used to rescreen the library, resulting in the identification of a cDNA (pCh31-1) encoding the complete sequence of the closely related neuronal α-bungarotoxin-binding subunit termed α2. This complete sequence was then used to identify a cDNA clone (pCh34-1) which encoded the remainder of the α1 subunit. To prove that the two partial cDNAs for the α1 subunit of the neuronal α-bungarotoxin-binding protein were actually fragments of an mRNA cleaved during cloning at an EcoRI restriction enzyme site, the polymerase chain reaction (PCR) technique was used. Oligonucleotides derived from each of the partial clones were used to prime a PCR reaction on a fresh brain cDNA preparation. This yielded an amplified sequence which overlapped the EcoRI cleavage site, and thereby proved that cDNA clones pCh29-3 and pCh34-1 encode parts of the sequence of a single subunit.

The sequences of α1 and α2 subunits of neuronal α-bungarotoxin-binding proteins from chicken brain are shown in **Figures 54** and **55**, and these sequences are compared with the sequences of acetylcholine-binding subunits of chicken nicotinic receptors in **Figure 56**. The sequences of the α-bungarotoxin-binding proteins clearly reveal that these subunits are members of the nicotinic receptor gene family. Although their degree of sequence identity with other members of this family is rather limited, there is nothing in the sequences of the putative transmembrane channel forming domains M1 and M2 to suggest that these subunits could not be components of a ligand-gated cation channel.

```
                    * *   * * * * * * * * * * * * * * *   * * *   *
cDNA αBgtBP α1      G E F Q R K L Y K E L L K N Y N P L E R P V A N D...
protein             X E F E T K L Y K E L L K N Y N P L E X P V A X D
cDNA αBgtBP α2      G E S Q R R L Y R D L L R N Y N R L E R P V M N D...
                    *     * *   * *   * * *   * *   * *     *
```

Figure 52. Comparison between a sequence encoded by a cDNA for α1 subunits of neuronal α-bungarotoxin-binding protein and the N-terminal protein sequence reported[80] for a subunit of a neuronal α-bungarotoxin-binding protein from chicken brain. Also shown is the similar, but less homologous, sequence encoded by a cDNA for the closely related α2 subunit of α-bungarotoxin-binding protein.

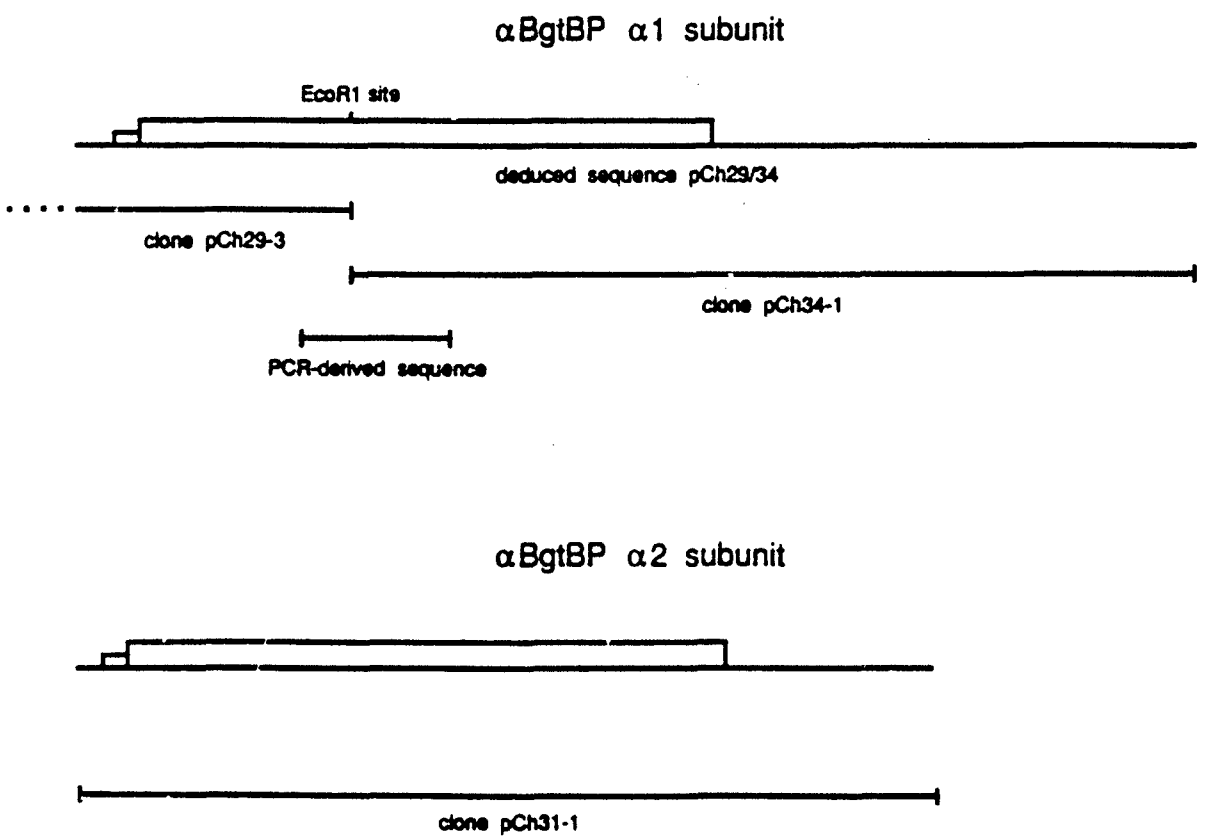


Figure 53. Summary of the cDNA clones used to define the sequences of the α1 and α2 subunits of neuronal α-bungarotoxin-binding proteins.

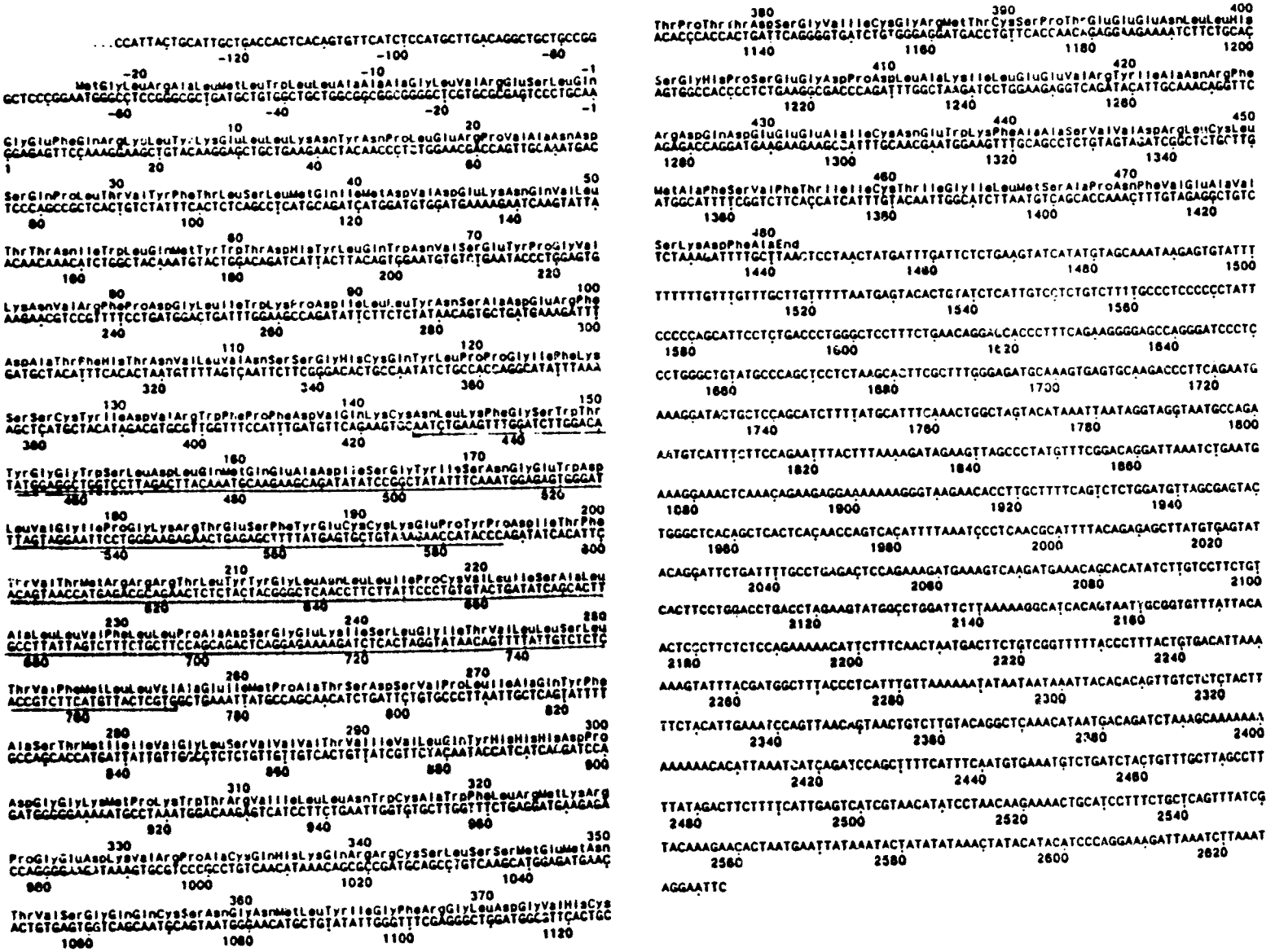


Figure 54. The cDNA and deduced protein sequence for α1 subunits of neuronal α-bungarotoxin-binding protein from chicken brains.

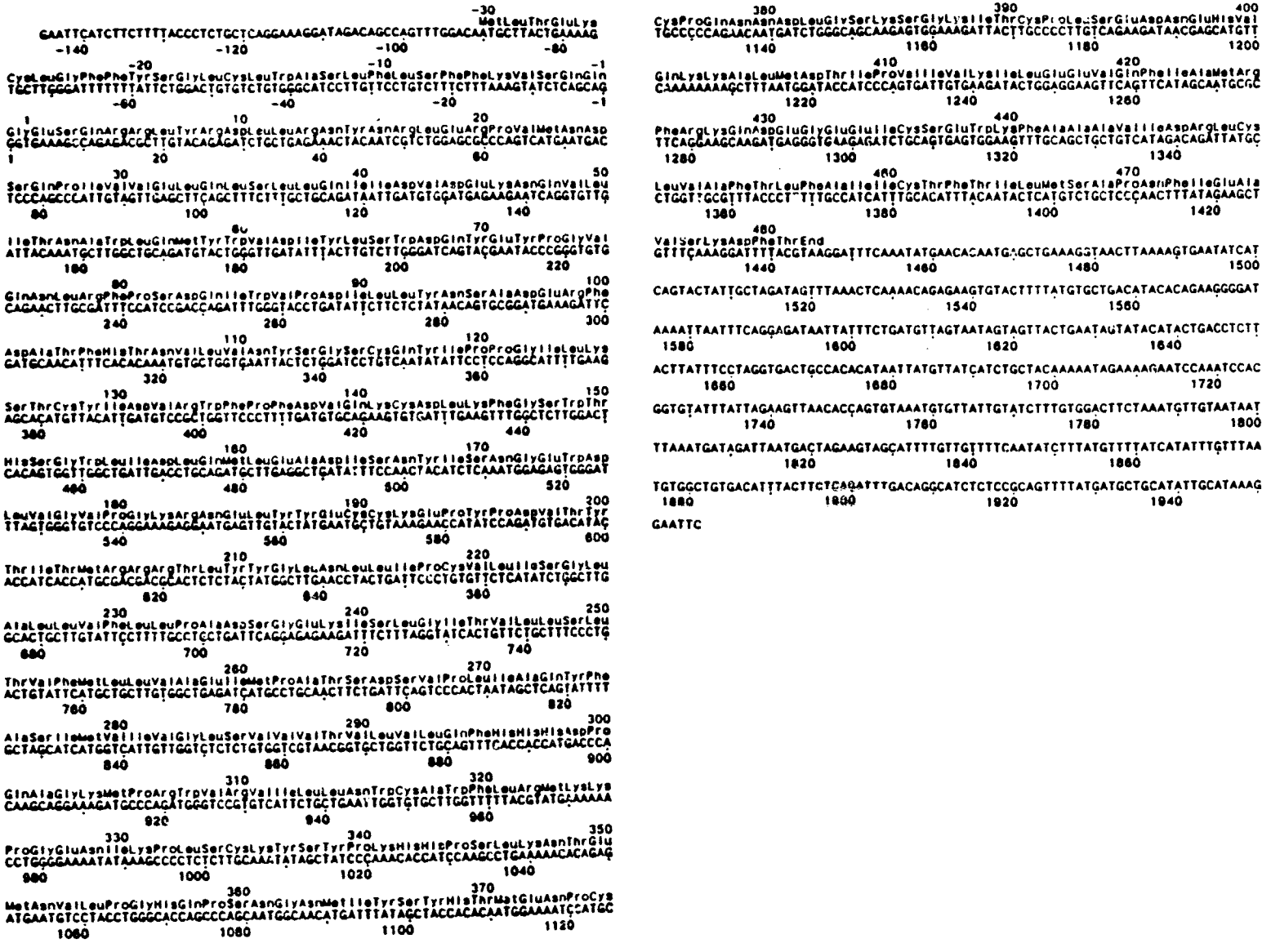


Figure 55. The cDNA and deduced protein sequence for α2 subunits of neuronal α-bungarotoxin-binding subunits from chicken brains.

Figure 56. Comparison of the deduced sequences of the α1 and α2 subunits of neuronal α-bungarotoxin-binding proteins from chicken brain with the deduced sequences of the acetylcholine-binding subunits of nicotinic receptor subtypes from chickens. Lines indicate the extent of bacterially expressed peptides from the α1 subunit (proteins Ch31-6 and Ch31-5) or the α2 subunit (protein Ch34-2) used to raise antibodies.

The cDNAs for the α1 and α2 subunits were proven to encode subunits of neuronal α-bungarotoxin-binding protein by demonstrating that antibodies raised to bacterially expressed unique sequences of these cDNAs bound to authentic neuronal α-bungarotoxin-binding proteins extracted from chicken brains, as shown in **Figure 57.** mAb 306 was prepared against affinity-purified α-bungarotoxin-binding protein. It binds all detectable α-bungarotoxin-binding protein in extracts, but its subunit specificity is unknown, since it binds only to the native protein. Antisera to a bacterially expressed α1 peptide (Ch31-6) corresponding to the large putative cytoplasmic domain of the α1 subunit (see **Figure 56**) bound a maximum of about 75% of the α-bungarotoxin-binding protein, as shown in **Figure 57.** This demonstrates that this subunit is a component of authentic α-bungarotoxin-binding protein, and suggests that it is a component of a major subtype. mAb 308 to a bacterially expressed peptide (Ch34-2) for the corresponding large putative cytoplasmic domain from α2 binds to a maximum of less than 20% of the α-bungarotoxin-binding protein. This indicates that this subunit is also a component of authentic α-bungarotoxin-binding protein, and suggests that it may perhaps encode a subunit of a minor subtype.

Thus, for the first time, subunit cDNA and corresponding antibody probes are available for neuronal α-bungarotoxin-binding proteins, they prove that the protein is a member of the ligand-gated ion channel gene superfamily, and they suggest that there may be subtypes of this protein comparable to those observed with neuronal nicotinic receptors. The sequence data appears consistent with the idea that these subunits could be components of a ligand-gated cation channel. However, there is evidence that these proteins do not function as acetylcholine-gated cation channels.[39,40] The presence of a cysteine pair characteristic of acetylcholine-binding subunits of receptors suggests that these could be the subunits that bind cholinergic ligands. However, the endogenous ligand, if any, is unknown. It has been suggested that the endogenous ligand may be the thymic peptide thymopoietin acting as a hormone of some sort.[81] Clearly, there is much to be learned from the studies which can be done with the tools now available.

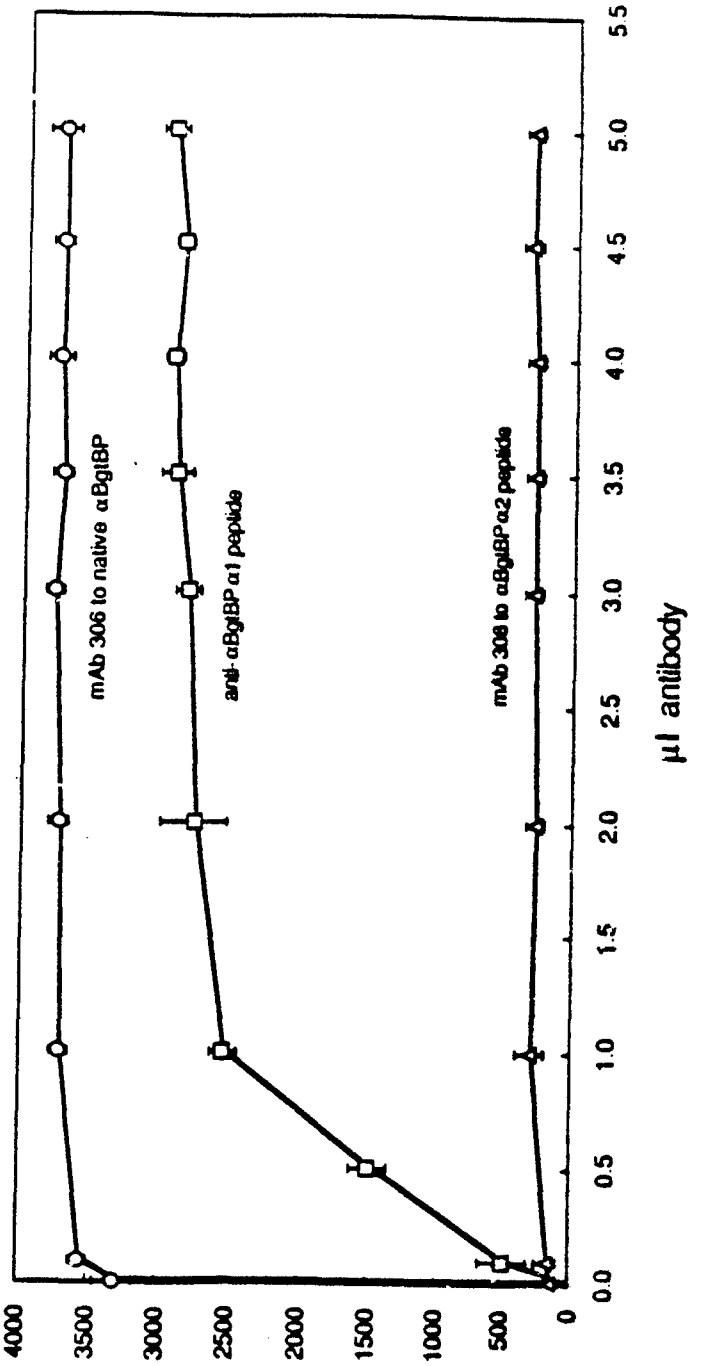


Figure 57. Antibodies to bacterially expressed unique sequences from cDNAs for α1 and α2 subunits bind to authentic α-bungarotoxin-binding protein from chicken brain. Immunoprecipitation of ^{125}I-α-bungarotoxin-labeled binding protein from Triton X-100 extracts of brain is shown. The antiserum is to the α-bungarotoxin-binding protein α1 subunit peptide Ch31-6, which encodes the large putative cytoplasmic domain shown on Figure 56. mAb 308 is to the corresponding peptide Ch34-2 from the α2 subunit.

METHODS

Muscle-Type Nicotinic Receptors

Torpedo Acetylcholine Receptors

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[127] except for the addition of 1 mM N-ethylmaleimide to the isolation buffer and substitution of 100 mM sodium cacodylate, pH 6.8, and protease inhibitors (0.3 μg/ml leupeptin and 1 μg/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.[82] 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.[53]

WGA labeling of the subunits was by the method of Nomoto et al.[83] The nitrocellulose sheet was quenched by three (10-min) washes in 100 mM NaCl, 10 mM sodium phosphate, pH 7.5 (phosphate-buffered saline (PBS)), containing 0.1% Tween 20 (quench buffer). The nitrocellulose sheet was incubated (1 hour at room temperature) with WGA-biotin (5 μg/ml) in the quench buffer using a volume of approximately 8 ml/100 cm^2 nitrocellulose, and then washed for three (10-min) periods in quench buffer. Avidin-peroxidase (5 μg/ml) in quench buffer was added to the nitrocellulose sheet (volume ±8 ml/100 cm^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) 30 mg of α-chloronapthol in 10 ml of cold methanol and (b) 30 μl of 30% H_2O_2 in 50 ml 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.[53] One ml of mAb 111 (10 nM 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[84] Kodak XAR film.

Samples in 5-μl aliquots were applied to freshly glow-discharged carbon support grids, washed with ~1 mg/ml cytochrome c and negatively stained with 2% sodium phosphotungstate, pH 7.2.

Antisera were raised in rats against both native receptor and α subunits as previously described.[82] Antisera were assayed by incubation overnight with ^{125}I-α-bungarotoxin-labeled receptor (1 nM) or ^{125}I-labeled α subunit in 100 μl of 10 mM sodium phosphate (pH 7.5)/100 mM NaCl/0.5% Triton X-100/10 mM NaN_3 buffer. Immune complexes were precipitated with 100 μl of goat anti-rat immunoglobulin, diluted with 1 ml of buffer, and centrifuged. Precipitates were washed with 2 x 1 ml of buffer and counted for radioactivity, as previously described.[82] The binding of antibodies to 10 nM ^{125}I-peptides was assayed similarly and has been described.[52]

A slot-blot apparatus was used to apply synthetic peptides to a Biodyne immunoaffinity membrane (Pall, East Hills, NY) in 16 mM borate, pH 9.0, and 15 mM NaCl. Remaining reactive membrane sites were quenched overnight with 5% Carnation dried milk and 50 mM Tris, pH 7.5, with 0.01% anti-foam A (quench buffer), and the membrane was rinsed with five changes of buffer. ^{125}I-α-bungarotoxin (5 nM) 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.

Geysen Epitope Mapping. The Geysen[54] epitope mapping system was purchased from Cambridge Research Biochemicals (Atlantic Beach, NY) and used in conjunction with an IBM-PC and a Titertek (McLean, VA) MCC/340 Plate Reader, according to the manufacturer's directions. The sequence of Torpedo α subunits reported by Noda et al.[86] was used to synthesize α as a set of overlapping octamers. Bound antibodies were detected with commercial (CalBiochem, San Diego, CA) peroxidase-labeled goat anti-rabbit IgG at 1/10,000 dilution or with homemade peroxidase-labeled affinity purified goat anti-rat IgG or the peroxidase-labeled mouse mAb to rat IgG MAR 18.5.[87]

Mapping the Binding Site of mAbs to the Main Immunogenic Region Using Synthetic Peptides. Some of the synthetic peptides are shown in Table 1.[46] The Torpedo and human α subunit peptides around α66-76 were synthesized using the RaMPS multiple peptide synthesis system (DuPont, Wilmington, DE) according to the manufacturer's directions.

Microtiter plates (Immulon, from Dynatech) were coated with poly-D-lysine HBr (20 μg/ml, 100 μl/well) in 0.1 M $NaHCO_3$ at 4°C overnight. All subsequent treatments were at room temperature. The plates were washed three times with 10 mM K phosphate buffer, pH 7.0. Peptides at 5 μM in this buffer were mixed with an equal volume of 0.25% glutaraldehyde and 10 μl aliquots were added to the wells. After 2 hours the wells were washed three times with buffer and then quenched with 200 μl of 1% bovine serum albumin, 1% ovalbumin, 0.1% Tween 20 in 10 mM Na phosphate, pH 7.5. After shaking for 1 hour, this solution was removed, and mAbs were added at 50 μl/well containing about $1x10^{-10}$mol of mAb in the same buffer. After shaking for a further 2 hours, the wells were washed three times with 200 μl of 0.05% Tween 20 in phosphate-

The labeling reagents were reacted with the tubes by application to the grids after the cytochrome c wash at the following concentrations: 4 μM for α-bungarotoxin, 1 μM for Fab 35 and Fab 11, and 10 μM (in the presence of 0.1 mM $CaCl_2$) 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[53]; 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 100KV 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.[85]

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.

Synthetic Peptide Studies. Nicotinic acetylcholine receptors were purified from the electric organ of Torpedo californica by affinity chromatography on toxin agarose.[82] The α subunit was purified from receptor by preparative SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Briefly, the preparative gel (5 mm) was lightly stained with Coomassie blue and destained. The band corresponding to the α subunit was excised and frozen. The gel containing the α subunit was sonicated in H_2O at 4°C until the gel was dispersed. The suspension was centrifuged and filtered through a 0.22-μm filter to remove remaining gel particles. The α subunit preparation was then dialyzed versus H_2O and lyophilized.

Peptides corresponding to various segments of the α subunit were synthesized by V. Sarin, J.L. Fox, H.L. Thanh, and J. Rivier using the Merrifield method, as previously described, or were purchased from Bachem (Los Angeles, CA). These peptides are listed in Table 1. 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 9×10^{16} to 3×10^{18} cpm/mol.[52,82]

buffered saline. A mouse anti-rat IgG mAb (MAR 18.5) conjugated to horseradish peroxidase was then added at 50 μl/well. After shaking for 1 hour, the wells were washed three times with the 0.05% Tween 20 buffer. Then 150 μl/well of peroxidase substrate solution (20 mg 2,2'-azino-di-[3 ethyl-benzthiazolinsulfonate] in 72 ml phosphate-citrate buffer, pH 4.0, with 22 μl of H_2O_2) was added. After color development for 20 minutes the plate was read in a Titertek Multiskan MCC/340 (Flow Laboratories, McLean, VA).

Expression of Torpedo Nicotinic Receptor Subunits in Yeast. E. coli HB 101 was used to amplify plasmid DNA.[88] E. coli DH5α was used for sequencing purposes. Saccharomyces cerevisiae strain TD4 (Mat a, his4, leu2, ura3, trp1) and strain 8534-8C (Mat α, his4, leu2, ura3) were from B. Tye (Cornell University), and strain TD71.8 (Mat a, his3, leu2, ura3, trp1, lys2) from D. Dawson (Massachusetts General Hospital). The negative control strain for the production of subunit polypeptides in yeast, KUJ6.0, was obtained by mating TD4 with TD71.8.

The full-length β subunit cDNA was isolated as an EcoRI restriction fragment from clone pSS2β.[89] The γ subunit cDNA was obtained as an NcoI-PvuII restriction fragment from clone γ-28.[90] The ends were made flush with Klenow fragment, and EcoRI linkers (#5295I[illegible], Bethesda Research Laboratories, Bethesda, MD) were added according to standard procedures.[88] The EcoRI restriction fragments were inserted individually into the yeast expression vector pMAC561[91] to generate pYTcβ or pYTcγ. Together with pYTcα and pYTcδ, which were obtained previously,[61-63] the vectors were used for the construction of the integrating expression vectors. Plasmids pD41 and pSZ62 containing the yeast selectable markers TRP1 and HIS3, respectively, were obtained from D. Dawson (Massachusetts General Hospital), and pSZ58 containing the LEU2 gene of yeast was from B. Tye (Cornell University). Yeast transformation was performed using the lithium acetate method.[92] For E. coli transformation the $CaCl_2$ procedure was used.[88] Sequence analysis was done according to the manual supplied with the Sequenase sequencing kit (United States Biochemicals, Cleveland, OH). All other DNA manipulations were performed according to standard procedures.[88]

SP6 mRNA transcripts from Torpedo receptor subunit cDNAs were made in vitro as described.[93] Yeast total RNA was prepared, with minor variations, according to Silverman et al.[94] Poly $(A)^+$ RNA was purified from this total RNA by chromatography on oligo (dT)-cellulose.[95]

For the detection of the receptor α, γ, and δ subunits, yeast strains were grown at 30°C or at 5°C in SD medium (Difco, Detroit, MI).[96] For the detection of the β subunit, 5 ml of a preculture were grown in SD medium at 30°C to an optical density measured at 600 nm of >0.6, then diluted into 500 ml fresh medium and grown for an additional 5 hours at 30°C; the cells were then incubated for 5 days at 5°C until the optical density was 0.3. Cells were harvested from 500 ml cultures by centrifugation at

100 g for 20 minutes at 5°C. All further treatments of the cells to obtain the membrane fraction were also carried out at 5°C. The cell pellet was washed twice in 500 ml of 50 mM Tris-HCl, 100 mM NaCl, 100 mM KF, 5 mM EDTA, 5 mM ethylene glycol bis (β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), 1 mM phenylmethylsulfonylfluoride (PMSF), 25 mM NEM, pH 7.5. After freezing the pellet at -80°C for at least 30 minutes, it was suspended in 20 ml of 25 mM Tricine-KOH, 0.6 M sorbitol, 50 mM KF, 1 mM PMSF, 25 mM NEM, 5 mM EDTA, 5 mM EGTA, and 10 μg/ml soybean trypsin inhibitor, pH 7.5. The suspension was sonicated for 5 minutes and then centrifuged at 1100g for 5 minutes. The supernatant was saved and the pellet treated as before in 20 ml of the above buffer. The last supernatant was combined with the previous one and centrifuged at 3000g for 10 minutes. The resulting supernatant was then centrifuged at 300,000g for 30 minutes, to obtain the yeast membrane fraction.

For immunoblot analysis yeast membrane proteins were solubilized in SDS-sample buffer, and proteins were separated by SDS-PAGE using a 10% gel.[97] Proteins were then transferred to Immobilon membranes (Millipore, Bedford, MA) by the method of Matsudaira.[98] The membranes were quenched with 5% (w/v) dried milk in phosphate-buffered saline, 0.5% Triton X-100, pH 7.5, and then labeled with iodinated mAbs against the individual subunits (specific radioactivities $1\text{-}2\times10^8$ cpm/mol). The mAbs were iodinated using a modified chloramine-T procedure.[82] The membranes were washed with phosphate-buffered saline, 0.5% Triton, pH 7.5, and bound ^{125}I-mAbs were visualized by autoradiography.

For solid-phase sandwich assay of mAb and α-bungarotoxin binding to Torpedo nicotinic receptor α subunits expressed in yeast, Immobilon "C" microtiter wells (Dynatech) were coated with 1 μg of mAb 173 in bicarbonate buffer (10 mM $NaHCO_3$, pH 8.8, 50 μl/well) for 4 hours at room temperature. After three washes the wells were quenched for a further 2 hours with 1% bovine serum albumin in the same buffer. Finally the wells were washed three times with 200 μl of 0.5% Triton X-100 in phosphate-buffered saline. Aliquots (50 μl) of crude extracts of yeast membranes, purified receptor, or α subunits in 0.5% Triton X-100 buffer were incubated in the wells overnight at 4°C with shaking. After three washes the ^{125}I-labeled ligand (3×10^{17} - 3×10^{18} cpm/mol) was added in 50 μl of 1% bovine serum albumin, 1% ovalbumin, 0.1% Tween 20, and 10 mM Na phosphate, pH 7.5. After shaking overnight at 4°C, the wells were washed three times and counted in a γ counter.

Expression of Torpedo Acetylcholine Receptors in Xenopus Oocytes. cDNAs for the α, β, γ, and δ subunits of Torpedo acetylcholine receptor were gifts from Dr. Toni Claudio at Yale University. These were expressed in Xenopus oocytes using precisely the technique described in her detailed review.[93]

Human Acetylcholine Receptors

Culturing TE671 Cells. Cultures were grown at 37°C in 90% air 10% CO_2 in Iscove's modified Dulbecco's modified essential medium (DMEM; Irvine Laboratories (Irvine, CA) supplemented with either 10% fetal bovine serum or 5% bovine calf serum. For electrophysiological studies, 10^4 cells were plated per well in a 24-well plate on 12 mm diameter glass cover slips in medium with 10% serum. One day later, serum was reduced to 0.01%, and 2 mM liters of glutamine, 10 μg/ml insulin, and transferrin were added. Electrophysiological studies were done in 115 mM NaCl, 5 mM CsCl, 1 mM $MgCl_2$, 25 mM glucose, 25 mM 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES), pH 7.4, 10 mM tetraethylammonium chloride, and 0.1 mM anthracene-9-carboxylic acid.

Electrical Recordings from TE671 Cells. Single channel current electrical recordings were performed in the laboratory of Dr. Mauricio Montal at the University of California, San Diego according to methods described in detail.[99] Recordings were obtained in both the cell-attached and the excised-patch configurations. The pipettes were fabricated from KOVAR glass (Corning 7052, inner diameter=1.1 mm, 70 mm long) using a vertical pipette puller (David Kopf 700C, Tujunga, CA). The pipettes were coated with Sylgard-180 (Dow Corning) within 40 μm from the tip and fire-polished immediately before use, under 320X magnification. The tip size was adjusted to yield 5-15 Mohms of open pipette resistance when filled and immersed in the buffer described before. The patch pipettes contained the indicated concentration of acetylcholine diluted in the same solution. The cells were observed with an inverted microscope (Nikon-diaphot) using a 40X objective (LWD DL 40XC, Nikon) equipped with Hoffman modulation contrast optics (Modulation Optics, Greenvale, NY). The microscope was mounted on a vibration isolation table (Micro g Technical Mfg. Corp., Waltham, MA).

A commercially available extracellular patch clamp system was used (LM EPC-5, List Electronics, Darmstadt, West Germany and Medical Systems Corp., NY). The headstage of the amplifier was mounted on a hydraulic micromanipulator (MO-103N, Narishige, Japan). The signal output from the clamp was recorded on FM tape (Racal 4DS, Hythe, Southhampton, England, bandwidth DC to 5kHz). All the records were filtered at 2kHz on an 8-pole Bassel low pass filter (Frequency Devices, 9028LPF, Haverhill, MA). The data were digitized at the sampling frequency of 10kHz in an Indec-L-11/73-70 microcomputer system (Indec, Sunnyvale, CA). Conductance levels were discriminated as described previously. Histograms of dwell times in the open state and closed states of the receptor channel were analyzed as described in detail previously.[100-103] The results of at least five different experiments in each condition are presented. All experiments were done at room temperature (22°C).

Preparation of Solubilized TE671 Membrane Extracts. TE671 cell cultures were grown in T-flasks for 6 days and then expanded to 2 liter (850 cm^2) roller bottles in 5% fetal calf serum in Iscove's modified DMEM (Irvine Laboratories) with 2.5 μM dexamethasone. After 10 days in culture, the cells were harvested after aspiration of media by first rinsing with cold PBS, pH 7.5, containing 10 mM iodoacetamide, 10 mM aminobenzamidine, and 1 mM PMSF to remove the excess media, and secondly by shaking in 25 ml per bottle of 50 mM Tris, 150 mM NaCl, 100 mM KF, 5 mM EDTA, 5 mM EGTA, 5 mM iodoacetamide, 5 mM aminobenzamidine, 0.5 mM PMSF, bestatin (10 μg/ml), Trasylol (10 μg/ml), and soybean trypsin inhibitor (10 μg/ml), pH 7.5 (buffer A). The bottles were then rinsed with four volumes of buffer A to remove any remaining cells. The cells were then pelleted by centrifugation at 3000g for 30 minutes. The resulting cell pellet was resuspended in 400 ml of buffer A, homogenized, and centrifuged as described in the previous step. The resulting pellet was then extracted for 30 minutes in four volumes of buffer A with 1% Thesit detergent (Boehringer, Mannheim, Indianapolis, IN) and 0.05% SDS, pH 7.5, centrifuged at 140,000g for 30 minutes, and the clarified supernatant retained.

Purification of the TE671 Nicotinic Receptor. α-Bungarotoxin was first coupled to Sepharose CL-4B at 5.0 mg of protein per ml/ml of gel by a modified procedure of Kohn and Wilchek.[104] The clarified, solubilized TE671 membrane extract (75-100 ml) from, typically, 12 roller bottles was applied to a 20 ml column of Sepharose CL-4B to adsorb any proteins which may nonspecifically adsorb to the affinity matrix. The eluate was then applied to a 1 ml column of α-bungarotoxin-affinity gel and both columns were washed with 150 ml of the extraction buffer. The affinity column was consecutively washed with 200 ml of buffer A containing 1.0 M NaCl, 0.5% Thesit, and 0.05% SDS, pH 7.5, followed by 150 ml of 10 mM Tris, 0.1% Thesit, 1 mM NaN_3, 10 mM KF, 1 mM iodoacetamide, 1 mM aminobenzamidine, 1 mM EDTA, and 1 mM EGTA, pH 7.5 (buffer B). The affinity column was then coupled to a hydroxylapatite (HPT) column (1 ml) and the TE671 receptor eluted onto the HPT column by recirculating through both columns for 12 hours 10 ml of buffer B containing 200 mM carbamylcholine, using a peristaltic pump. After displacement of the bound protein, the HPT column was washed with 200 ml of buffer B and then eluted with 150 mM sodium phosphate, 0.5% Thesit, 1 mM NaN_3, 1 mM PMSF, 1 mM EDTA, 1 mM EGTA, 1 M aminobenzamidine, and 1 mM iodoacetamide at pH 7.5.

Affinity Labeling of TE671 Receptor. TE671 receptor was immobilized on α-bungarotoxin-Sepharose and then affinity labeled with ^{3}H-MBTA (a gift from Dr. Mark McNamee) as previously described.[16]

Electrophoresis. Electrophoresis was conducted on acrylamide slab gels in SDS using the Laemmli[97] discontinuous buffer system. Polyacrylamide gels were silver stained for protein according to the method of Oakley et al.[105] Polyacrylamide gels of radiolabeled protein were autoradiographed for 4-24 hours at -70°C

using preflashed Kodak (Rochester, NY) X-Omat-AR film and an intensifying screen. Autoradiograms were standardized by using Sigma (St. Louis, MO) prestained low molecular weight standards resolved on the same gel. Electrophoretic transfer of proteins from gels to diazophenylthioether (DPT) paper and subsequent probing with antibodies were as described previously.[53] After being probed, bound antibodies were detected by incubation with 0.5 nM ^{125}I-labeled mouse anti-rat IgG (1-3x10^{18}cpm/mol) and autoradiography.

Cloning and Sequencing of TE671 Receptor α Subunit cDNA. cDNA was synthesized by the RNase H method,[106] following the protocol of Watson and Jackson.[107] cDNA >1 kilobase (kb) was ligated into the cloning vector λ-zap (Stratagene, San Diego, CA). Approximately 10^5 recombinants were screened at high stringency with the 430-base pair (bp) PstI fragment of pMARα15, containing the sequences for the N-terminal of the mouse muscle nicotinic receptor α subunit.[108] Positive clones were plaque purified, and inset-bearing plasmids were obtained using a helper phage, following the supplier's protocol. Plasmid DNA was characterized by restriction enzyme digestion followed by agarose gel electrophoresis and Southern blotting.

For DNA sequencing, nested deletions were produced by the EXO III/mung bean protocol (Stratagene) in both orientations. DNA sequencing was performed using a modification of the dideoxynucleotide chain termination method of Sanger et al.[109]

Cloning and Sequencing of TE671 Receptor δ Subunit cDNA. A cDNA library was prepared as previously described.[34] The filters were screened under high stringency with the ~450-bp EcoRI-AvaI fragment of cDNA clone BMD451 (a gift of Dr. Jim Boulter, The Salk Institute) coding for the 114 N-terminal amino acids of the mouse muscle acetylcholine receptor δ subunit. A single positive clone was identified. Plasmid DNA was characterized by restriction enzyme digestion followed by agarose gel electrophoresis and southern blot analysis. From the ~3-kb insert, the 5' ~1860-bp Eco-Ava fragment was subcloned into a second λ-Zap vector. Nested deletions were produced by the Exo III/mung bean protocol provided by Stratagene. DNA sequencing was performed using a modification of the dideoxynucleotide chain termination method of Sanger et al.[109]

Sucrose Gradient Sedimentation Analysis of Receptors from TE671 Cells. The cells were harvested and receptors extracted in 1% Triton X-100 detergent, 50 mM Tris, pH 7.5, 150 mM NaCl, 100 mM KF, 5 mM EDTA, 5 mM EGTA, 5 mM IAA, 5 mM aminobenzamidine, 0.5 mM PMSF, 10 μg/ml bestatin, 10 μg/ml Trasylol, and 10 μg/ml soybean trypsin inhibitor, as previously described. Aliquots of the extract (150 μl) were layered onto 5 ml sucrose gradients (5-20% sucrose wt/wt, in 0.5% Triton X-100, 10 mM Na phosphate buffer, pH 7.5, 100 mM NaCl, 1 mM NaN_3). The gradients were centrifuged in a VTi 65.2 rotor (Beckman, Fullerton, CA) for 67 minutes at 4°C. The gradients were fractionated from the bottom

of the tubes and collected into Immulon "C" Removawells (Dynatech, Chantilly, VA) coated with mAb 210 (1 μg/well, 4 hours, followed by 3 washes). After incubating overnight at 4°C with shaking, the wells were washed three times with 0.5% Triton X-100 in 100 mM NaCl, 1 mM NaN_3, 10 mM Na phosphate buffer, pH 7.5. ^{125}I-α-bungarotoxin was then added at 2 nM in the Triton X-100 buffer, and 100 mM carbamylcholine was added as a competitive inhibitor on some gradients to address affinity for small cholinergic ligands, while unlabeled α-bungarotoxin was added at 1 μM to other gradients to determine the level of nonspecific binding. After overnight incubation the wells were washed three times, and then bound ^{125}I-α-bungarotoxin was determined by γ counting. Receptor from Torpedo electric organ was analyzed simultaneously to provide size markers.

Neuronal Nicotinic Receptors

Purification and Characterization of Receptor Subtypes

Western blot with ^{125}I-mAb 35. Nicotinic receptors affinity purified from Torpedo electric organ on a toxin-affinity column[82] (5 μg) or immunoaffinity purified[19] from chicken brain using mAb 295 were electrophoresed on acrylamide gels in SDS, blotted, and probed overnight at 4°C with ^{125}I-mAb 35 ($5x10^{18}$cpm/mol, 1 nM). After washing, binding of ^{125}I-mAb 35 was visualized by autoradiography.

Binding of ^{125}I-mAb 35. Antiserum to the α3 peptide was tested for its ability to deplete binding sites for mAb 35 from detergent extracts of chick ciliary ganglia and brains. From embryonic day 17-19 (E18) chicks, 40-50 ciliary ganglia or 0.5-0.6 g brains were homogenized in 1 ml or 10 ml, respectively, of 75 mM NaCl, 5 mM Na phosphate buffer, pH 7.4. Membranes were isolated by centrifugation at 15,000g for 15 minutes. The receptors were solubilized in 0.5 ml 5 mM $NaPO_4$/75 mM NaCl/1% Triton X-100, pH 7.4, and cleared from debris by 15 minutes (ganglia) or 30 minutes (brain) centrifugation at 15,000g. Aliquots were incubated with antisera or preimmune sera for 60 minutes at room temperature in a final volume of 60 μl. Control reactions were carried out in the absence of sera. Then the antibodies were absorbed onto fixed Staph-A cells (Bethesda Research Labs) (the pellet from 30 μl of a 10% suspension) for an additional 15 minutes, and cleared by a 15 second centrifugation. Triplicate 30 μl aliquots of supernate were incubated with 2.5 nM ^{125}I-mAb 35 for 45 minutes at room temperature. The amount of ^{125}I-mAb 35 bound to receptor was determined by a DE-52 resin column assay (50 μl resin for ganglia, 150 μl for brain) as previously described.[10]

Purification 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 8 mg/ml, respectively), as previously described.[19] Purified receptor was radioiodinated essentially as previously described.[12] 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 μl using a Centricon 30 (Amicon) microconcentrator. The purified receptor, 5-10 pmol, was then radioiodinated using the lactoperoxidase-glucose oxidase method (BioRad) and kept at 4°C in 10 mM sodium phosphate, pH 7.5, 100 mM NaCl (PBS) containing 0.5% Triton X-100, 10 mM sodium azide, and 10 mg/ml β-lactoglobulin.

Preparation of mAbs to Chicken Brain Nicotinic Receptors. Female Lewis rats (6-8 weeks old) received intramuscular injections of purified chicken brain receptor, both intact and SDS denatured, emulsified in 100-200 μl of complete Freund's adjuvant. The rats were sacrificed and their spleen cells fused with the mouse myeloma cell line s194 15.XX0.BU, using 50% polyethylene glycol 4000 (Merck) as previously described in detail.[110] Culture supernatants were initially screened by solid-phase immunoassay using immobilized, affinity-purified receptor from chicken brain. 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 and then grown in bulk in Iscove's medium containing 1% fetal calf serum. Supernatants from mass cultures were concentrated to about 300 ml using a Millipore Minitan concentrator and the immunoglobulin fraction isolated by precipitation with 18% sodium sulfate and then dialyzed against PBS containing 10 mM 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 and mAb 285 was purified by HPLC on a hydroxylapatite column (BioRad).

Cell culture supernatants were screened by solid-phase immunoassay.[12] Briefly, affinity-purified receptor from chicken brain (10-100 fmol quantitated in terms of ^{125}I-mAb 35 binding sites) in 30 μl of PBS was applied to Millipore Millititer 96-well nitrocellulose plates and incubated overnight at 4°C. The plates were quenched for 30 minutes at room temperature with 50 μl of PBS containing 1% bovine serum albumin and 0.2% Tween 20 (quench buffer), and then 100 μl of culture supernatant was added, followed by incubation overnight at 4°C. Plates were then washed twice with 300 μl of quench buffer and incubated 2 hours at room temperature with 10 nM ^{125}I-goat anti-rat IgG ($2-3\times10^{-18}$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.[13] Briefly, detergent extract (200-500 μl) was shaken gently for 15 hours at 4°C with 100 μl of culture supernatant, diluted mAb, or serum, and 20-30 μl of a 1:1 slurry of goat anti-rat IgG-Sepharose (8-12.5 mg IgG/ml gel). After washing with 2x1 ml of PBS containing 0.5% Triton X-100, the aliquots were incubated for 15 minutes at room temperature in 20 nM ^{3}H-nicotine (DL-[N-methyl]-^{3}H-nicotine, specific activity 68.6 Ci/mmol (obtained from New England Nuclear, Boston, MA), in the same buffer, and then rapidly washed at 4°C with 4x1 ml of ice cold PBS, 0.5% Triton X-100 by resuspending in the buffer and centrifuging for 20 seconds at 10,000g in a microfuge. Bound protein was then eluted by incubating the gel for 15 minutes with 100 μl of 2.5% SDS, 5% β-mercaptoethanol, and then sampling into 5 ml 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,[12] 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.[12]

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 (0.5 mg/ml) at 20-30 ml/hour flow rate at 4°C. The column was rapidly washed with 50 ml PBS containing 0.5% Triton X-100, 50 ml 1 M sodium chloride, 10 mM sodium phosphate, pH 7.5, containing 0.5% Triton X-100, and again with 50 ml PBS, 0.5% Triton X-100. Bound protein was eluted by recirculating 1 M carbachol in 10 mM Na phosphate, pH 6.8, 0.1% Triton X-100 through the affinity column onto 0.5 ml hydroxylapatite (BioRad Bio-Gel HTP). The hydroxylapatite was washed with 25 ml of 10 mM Na phosphate, pH 6.8, 0.1% Triton X-100. Bound protein was rapidly eluted at 22°C with 100 mM Na phosphate, pH 6.8, 0.05% Triton X-100, and immediately dialyzed at 4°C against 10 mM 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.[13] Antibody binding to ^{3}H-nicotine binding sites was investigated by indirect immobiliza-

tion of antibodies upon goat anti-rat IgG-Sepharose, as previously described.[13]

α-Burgarotoxin binding sites were determined by DEAE assay[12] using α-bungarotoxin radioiodinated to specific activities of $3\text{-}4\times10^{17}$ cpm/mol.

Preparation of mAbs to Receptors from Rat Brains. Female Lewis rats (6-8 weeks old) were immunized with immunoaffinity purified rat brain receptors, both intact and denatured in SDS, emulsified in 100-200 μl of complete Freund's adjuvant (CFA). The rat that gave rise to the mAbs described was immunized intraperitoneally on day 1 with 10 pmol of receptor in CFA, on day 17 with 5 pmol of receptor (SDS denatured) in CFA, and on day 31 with 45 pmol of receptor (half of which was SDS denatured). Five days later, the rat was sacrificed and its spleen cells fused with the mouse myeloma cell line S194 15.XX0.BU1, using 50% polyethylene glycol 4000 (Merck), as described in detail elsewhere.[110] Culture supernatants were screened for binding to ^{125}I-labeled rat brain receptor by indirect immunoprecipitation using goat anti-rat IgG. Supernatants that were positive (greater than 2X background binding) upon retesting, and that upon subsequent rescreening exhibited binding to ^{3}H-nicotine-labeled receptors in detergent extracts of chicken brain (see below) were selected for recloning. Hybridoma cells were cloned directly in agarose and then grown in bulk in Iscove's medium containing 1% fetal calf serum. Supernatants from mass cultures were concentrated to ~300 ml using a Millipore (Bedford, MA) Minitan concentrator and the immunoglobulin fraction isolated by precipitation with 18% sodium sulfate and then dialyzed against PBS containing 10 mM sodium azide.

mAb 295 was purified by chromatography upon S-Sepharose (Pharmacia, Piscataway, NJ). Briefly, 7 ml of concentrated mAb 295 was dialyzed against 50 mM morpholine ethanesulfonic acid, pH 6, 5 mM NaCl, and then applied to a 0.5x10 cm column of S-Sepharose. Bound mAb was eluted with the same buffer containing 1 M NaCl; 127 mg of mAb was recovered.

Purification of Nicotinic Receptors from Bovine Brain. Receptors from bovine brain were purified by immunoaffinity chromatography using mAb 295 (see below) coupled to AFC resin (New Brunswick Scientific, Edison, NJ). Briefly, a bovine brain (obtained from a local abattoir and stored at -70°C) was pulverized into small pieces while still frozen and then 150- to 200-g amounts were homogenized and the membranes isolated exactly as previously described.[12] The membranes were then extracted for 2 hours at 4°C in one volume of 2% Triton X-100 in 50 mM Tris, pH 7.2, 1 mM EGTA, 1 mM EDTA, 5 mM iodoacetamide, 5 mM benzamidine, and 2 mM PMSF. The extract was centrifuged at 140,000 x g for 1 hour in a Beckman Ti50.2 rotor and the clear supernatant collected and then gently shaken for 15 hours at 4°C with 3 ml mAb 295-AFC resin (6.7 mg protein/ml resin). The resin was then loaded into a 10-ml column and washed successively with

approximately 200 ml of 10 mM Na phosphate, pH 7.5, 100 mM NaCl (PBS) containing 0.5% Triton X-100, 100 ml of 10 nM Na phosphate, pH 7.5, 1 M NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% Triton X-100, and finally with 20 ml of 20 mM Tris, pH 7.5, 100 mM NaCl, and 0.1% Thesit detergent (CalBiochem, San Diego, CA). Bound receptor was eluted with two-column volumes of AFC elution medium (New Brunswick Scientific) and dialyzed against 4 liters of 20 mM Tris, pH 7.5, 100 mM NaCl, and 0.05% Thesit.

Purified receptor was radioiodinated as previously described[12] by the lactoperoxidase-glucose oxidase method (BioRad, Richmond, CA) and kept at 4°C in PBS, 0.5% Triton X-100, containing 1 mM Na azide and 10 mg/ml β-lactoglobulin.

Human Brain Tissue. Human brain tissue, primarily from patients with Alzheimer's disease, was obtained through the generosity of Dr. Robert Terry at the University of California, San Diego.

^{3}H-Nicotine Binding Assay. Antibody binding to receptors in crude detergent extracts of brain was determined as described previously.[13] Briefly, detergent extract (200-600 µl) was shaken gently for 15 hours at 4°C with 100 µl of culture supernatant or an appropriate amount of mAb, or serum, and 20-30 µl of a 1:1 slurry of goat anti-rat IgG Sepharose (8-12.5 mg IgG/ml gel). After washing with 2x1 ml of PBS containing 0.5% Triton X-100, the aliquots were incubated for 15 minutes at room temperature in 50 µl of 20 nM ^{3}H-nicotine (DL-[N-methyl]-^{3}H-nicotine, specific activity 68.6 Ci/mmol, obtained from New England Nuclear, Boston, MA) in the same buffer, and then rapidly washed at 4°C with 4x1 ml of ice-cold PBS, 0.5% Triton X-100 by resuspending in the buffer and centrifuging for 20 seconds at 10,000g in a microfuge. Bound protein was then eluted by incubating the gel for 15 minutes with 100 µl of 2.5% SDS, 5% β-mercaptoethanol, and then sampling into 5 ml 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.

Purification and N-Terminal Sequencing of Receptor Subunits from Brains of Rats and Chickens. Nicotinic receptors from rat brain were immunoaffinity purified using mAb 270, as has been previously described.[14] Receptors from chicken brain were purified by affinity chromatography upon mAb 270 and the receptor subtype with a 75,000 molecular weight acetylcholine-binding subunit was isolated by a second round of immunoaffinity purification using mAb 299, which is specific for this acetylcholine-binding subunit.[19] Purified receptors (~60 pmol) were resolved into subunits by electrophoresis in a 10% polyacrylamide gel and electroblotted onto quaternary ammonium derivatized glass fiber sheets.[111] Protein bands were located by fluorescent staining, excised, and subjected to gas phase microsequencing upon an Applied Biosystems (Foster City, CA) model 470A protein sequencer.[112]

Cloning and Sequencing Nicotinic Receptor Structural Subunit cDNAs From Chicken Brain. Standard procedures were carried out as described previously.[34] Total cellular brain RNA was purified from day 17 chick embryos (E17) by the guanidinium isothiocyanate/cesium chloride method. Poly (A^+) RNA was obtained through two rounds of oligo(dT) chromatography. cDNA was synthesized by the RNase H method.[107] cDNA > 1 kb was ligated into the cloning vector λ-Zap (Stratagene). Clones were screened with a cocktail of probes for the nicotinic receptor family of the rat: the subcloned EcoRI insert of the λ clone (a gift of Dr. Steve Heinemann, The Salk Institute) harboring the α4-1 gene,[27] the α3 gene[24] (λPCA48, a gift of Dr. Jim Boulter, The Salk Institute), and the insert of clone pR11, which contains fragments of the α2 gene. Low-stringency temperature and salt conditions were used: hybridization at 58°C in 5xSSPE (1xSSPE=0.18 M NaCl, 0.01 M $NaPO_4$, pH 7.4, 1 mM EDTA), followed by washing at 60°C in 5xSSPE and at room temperature in 0.3xSSPE. The filters were also screened with a 30-mer oligonucleotide derived from the short published sequence of γ_2.[113] Hybridization at 42°C in 5xSSPE, 40% formamide was followed by final washings at 60°C in 1xSSPE.

A subset of clones which were positive in both screening revealed overlapping restriction maps. Two of them, clones pCh20.2 and pCh23.1, were analyzed by DNA sequencing. Both strands of pCh20.2 were sequenced throughout. pCh23.1 was sequenced completely in one orientation.

Cloning, Sequencing, and Bacterial Expression of α2, α3, and α4 cDNAs from Chicken Brains. A cDNA library was prepared in the λ Zap cloning vector (Stratagene, San Diego, CA) from day 17 chick embryo (E17) brain RNA, as previously described.[35] Three screening protocols were used: (1) Clones were screened with a cocktail of probes of the rat nicotinic receptor gene family--the insert of clone pR11, which contains fragments of the α2 gene[28]; the α3 gene[24] (λ PCA48, a gift of Dr. Jim Boulter); and the subcloned insert of the λ clone (a gift of Dr. Steve Heinemann), containing the α4-1 gene.[25] Hybridization was performed at 58°C in 5XSSPE (1XSSPE is 180 mM NaCl, 1 mM EDTA, 10 mM $NaPO_4$, pH 7.4), followed by washing at 60°C in 5XSSPE and at room temperature in 0.3XSSPE.[31] (2) clones were screened with pCh20.2,[35] a full-length chicken receptor structural subunit probe. Hybridization was in 50% formamide, 5XSSPE, 42°C, and washing in 1XSSPE, 65°C. (3) Clones were screened with rat α3. Hybridization was in 30% formamide, 5XSSPE, 42°C, and washing in 1XSSPE, 65°C.

Isolated cDNA clones were analyzed by restriction mapping and DNA sequencing by a modified dideoxy chain termination method[109] using Sequenase enzyme (United States Biochemicals).

A system utilizing the λ P_R and P_L promoters was utilized to express in E. coli the putative cytoplasmic loop of the chicken brain receptor α4 acetylcholine-binding subunit.[114] A BsphI-PvuII fragment of the pCh26.1 clone encoding amino acids met335-ala517 was subcloned into the expression vector pJLA602,

which had been digested with BamHI, blunt-ended with Klenow polymerase, and further digested with NcoI. The vector construct was verified by DNA sequencing. Expression of the protein was performed using the E. coli strain DH5α. Cultures were grown in 2XYT medium[88] at 28°C until an A_{600nm} of 0.4-0.5 was reached. The cultures were then shaken at 42°C for 2 hours to induce expression. Cultures were pelleted in a microfuge, resuspended in SDS-polyacrylamide gel electrophoresis sample buffer, boiled, and aliquots sampled for analysis by 15% SDS-polyacrylamide gel electrophoresis and Western blotting, using previously described techniques.[19] For large-scale preparation of expressed protein, 150 ml of induced culture was pelleted by centrifugation at 5000g for 15 minutes. The pellet was washed in 50 ml of 50 mM Tris, pH 8.0, 50 mM NaCl, 5 mM EDTA, and then resuspended by Polytron homogenization in 5 volumes of 10 mM NaCl, 100 mM KF, 5 mM EDTA. 5 mM EGTA, 5 mM iodoacetamide, 5 mM benzamidine, 1 mM PMSF, 2% SDS, then shaken for 1 hour at room temperature. The extract was centrifuged at 140,000g for 1 hour and the supernatant collected, diluted 5 fold in 100 mM NaCl, 10 mM $NaPO_4$, pH 7.5, 0.5% Triton X-100 (PBS Triton) containing 5 mM EDTA and 5 mM EGTA, and then recirculated for 14 hours at 4°C through 3 ml of mAb 289 coupled to AFC gel (New Brunswick Scientific), at a concentration of 8 mg mAb/ml of gel. The affinity column was washed with 50 ml of PBS Triton, 50 ml of 1 M NaCl, 10 mM $NaPO_4$, pH 7.5, 1 mM EDTA, 1 mM EGTA, 0.5% Triton X-100, and the bound protein eluted with 4 column volumes of 50 mM Na Citrate, pH 3.0, 0.1% Triton X-100. The eluate was neutralized and then lyophilized.

A bacterial expression system based on one described by Rosenberg and Studier[115,116] was used to express fragments of chicken α2 subunits, α3 subunits, putative α-bungarotoxin-binding protein subunits, and other receptor subunits. A fragment corresponding to the large putative cytoplasmic loop of α3 leu323-met571 was prepared by partially digesting the α3 clone with HphI and completely digesting with NcoI. The ends were blunt ended with T4 polymerase in the presence of dNTP, and then the fragment was isolated using electrophoresis on agarose. The fragment was subcloned into pBluescript KS (Stratagene) previously linearized with SmaI, yielding the clone pCh35.2, having the BamHI site of the pBluescript KS polylinker next to the 5' end of the α3 gene fragment. A BamHI EcoRI digest of pCh35.2 yielded a suitable fragment for an expression vector. It was cloned into a pET3c-derived vector, yielding clone Ch35.4. Cloning sites were confirmed by DNA sequencing. The expressed protein should have 15 N-terminal amino acids encoded by the vector, 127 amino acids of α3, and another 7 vector amino acids for a deduced molecular weight of 17,000 and a pI of 4.5. For expression, pCh35.4 was transformed into BL21(D3). From a single plaque an overnight culture was started in 3 ml. The next day this was expanded to 150 ml. At OD_{600}=0.8 expression was induced with 3 mM isopropyl-β-D-thioglactopyranoside. After 2 hours the culture was harvested by centrifugation and the pellet was resuspendad in 5 ml of 50 mM Tris, pH 8.0, 10 mM EDTA, 0.5 mM

PMSF, and frozen. An uninduced culture was similarly harvested as a control. Electrophoresis on acrylamide gels in SDS revealed a predominant band corresponding to an approximately 17,000 molecular weight peptide, only in the induced culture. A peptide corresponding to α2 Arg329-Leu493 was prepared similarly.

Expressed protein was purified from inclusion bodies as follows. To the thawed culture was added 15 ml of 50 mM Tris, pH 8.0, 10 mM EDTA, 1% Triton X-100, 0.5 mM PMSF. Sonication for three bursts of 15 seconds completed lysis. After centrifugation for 30 minutes at 20,000 rpm in a Beckman 50.2 Ti rotor at 4°C, the supernatant was discarded. The pellet was extracted with 3 M NaSCN in 100 mM NaCl, 10 mM Na phosphate, pH 7.5, 0.2 mM PMSF, and centrifuged as before. This pellet was extracted with 0.5 M urea in the same buffer and centrifuged again. This pellet was solubilized in 8 M urea, 100 mM NaCl, 10 mM Na phosphate, pH 7.5, 1 mM dithiothreitol. Dialysis against 100 mM NaCl, 10 mM Na phosphate, pH 7.5, resulted in partial precipitation. The resulting material appeared about 90% pure by electrophoresis.

Preparation of mAbs to the Expressed α3 Fragment. A female Lewis rat was immunized subcutaneously in multiple sites five times over 18 weeks with 150-200 μg/injection of the α3 recombinant protein emulsified in 150 μl complete Freund's adjuvant. On each of the four days preceding the fusion, the rat was boosted by intraperitoneal injection with 100 μg of the protein in 100 μl of PBS.

On the day of the fusion, the spleen was removed and spleen cells were fused with Sp2/0 mouse myeloma cells using 50% polyethylene glycol, as previously described.[110] Fusion products were plated out in 11 96-well plates. Eleven days after the fusion, culture supernatants were assayed for binding to protein Ch35-4 by an ELISA described below. Cultures positive in this assay were then assayed for binding to AChR in extracts of retina and ciliary ganglia by an immunoprecipitation assay described below. The final hybridomas retained were cloned three times by limiting dilution. Culture supernatants from these clones were used in the experiments described here.

The ELISA used for initial screening of hybridomas used the bacterially expressed α3 protein fragment bound to plastic microwells and detected bound antibodies by a colorimetric assay using a mAb to rat IgG labeled with horseradish peroxidase. Immulon 1 96-well plates (Dynatech) were coated with 50 μl/well of 20 μg/ml of the α3 protein in 10 mM Na bicarbonate buffer, pH 8.8, overnight at 4°C. The wells were quenched by adding 200 μl 1% ovalbumin, 1% bovine serum albumin, 0.1% Tween-20 in PBS for 60 minutes at room temperature. Then the wells were washed three times with 200 μl of PBS, 0.05% Tween-20. Culture supernatant (50 μl/well) was added. After two hours at room temperature, the wells were washed as before. Then 100 μl were added of MAR18.5 labeled with horseradish peroxidase[110] diluted in 1% ovalbumin, 1% bovine serum albumin, 0.1% Tween-20, PBS. After one hour the

plates were washed as before. Then 150 μl of substrate solution (0.5 mM 2.2 Azino-di-[3 ethyl benzthiazolinsulfonate] diammonium salt, 0.05% H_2O_2, 0.1 M Na_2PO_4, 0.8 M citrate) were added. After one hour optical density at 405 nm was measured on a Titertek ELISA reader, though positive wells were obvious by eye.

The immunoprecipitation assay used to screen hybridomas for their ability to bind to α3 in native receptors used anti-antibody-coated beads to immobilize the test antibodies and allowed the test antibodies to bind receptors from retina extracts. Then receptors remaining in the extracts were assayed using antiserum to the expressed α3 protein coupled to anti-antibody-coated beads to bind the receptors and ^{125}I-mAb 210 to label their structural subunits. Hybridoma supernatants which contained antibodies that could adsorb receptors from the extracts before they were bound by antiserum and labeled with ^{125}I-mAb 210 were scored as positive for native α3 receptor. Culture supernatant (50 μl) was combined with 20 μl of a 1:1 suspension of goat anti-rat antibody coupled to CNBr-activated Sepharose CL4B and with 50 μl of PBS Triton. After shaking for one hour at 4°C, the beads were pelleted by 20 seconds in a microfuge. The supernatant was removed and the beads were washed twice with 1 ml of PBS Triton. A Triton X-100 extract of chick embryonic day 18 retinas was prepared. Aliquots of extract (100 μl) were added to the washed beads. After shaking overnight at 4°C the beads were pelleted, and 85 μl aliquots of the supernatant were removed and added to 10 μl of goat anti-rabbit antibody coupled to CNBr-activated Sepharose CL4B previously incubated with 5 μl of rabbit antiserum to protein Ch35-4. In a total volume of 100 μl, ^{125}I-mAb 210 (specific activity 1-3x10^{18} cpm/mol) was added to a concentration of 2 nM and 5 μl of normal rat serum were added (to saturate any binding sites for rat antibodies like mAb 210 on the goat anti-rabbit antibody-coated beads). After shaking overnight at 4°C, 1 ml of PBS Triton was added and the beads were washed three times. Then ^{125}I-mAb 210 bound to the beads was measured by γ counting. A value for control receptor content in the extract was obtained by adsorbing with beads not exposed to hybridoma supernatant. A blank value subtracted from all samples was derived using samples not exposed to hybridoma supernatant and goat anti-rabbit antibody beads coated with normal rabbit serum instead of rabbit antisera to the bacterially expressed α3 fragment.

Subunit Stoichiometric Determination of Neuronal Nicotinic Receptors. *Torpedo* nicotinic receptors were purified as previously described,[82] with some modifications. Briefly, a high capacity (10 mg toxin/ml Sepharose) *Naja naja* toxin Sepharose column was used to bind the receptor, which had been solubilized in Thesit (Boehringer Mannheim), rather than Triton X-100. Receptor bound to the affinity column was then eluted by recirculation of 1 M carbachol in 25 mM Tris pH 7.5, 1.0% Thesit, through a hydroxylapatite (BioRad, Richmond, CA) column, from which it was subsequently eluted with 50 ml of 150 mM NaP pH 7.5, 1.0% Thesit.

Chicken brain nicotinic receptors were purified as described previously,[19] with some modifications. Membranes were prepared from 150 g of chicken brains and then extracted in 2 volumes of 2% Triton X-100 buffer. The detergent extract was subsequently shaken overnight with 3 ml of mAb 289 (to the α4 acetylcholine-binding subunit) coupled to AFC resin (New Brunswick Scientific, Edison, NJ) at 8 mg/ml. The column was washed first with 100 ml of 10 mM Na phosphate buffer pH 7.5, containing 1 M NaCl, 1 mM EDTA, 1 mM EGTA, and 0.5% Triton X-100; and then washed with 25 ml of 10 mM Na phosphate buffer pH 7.5, containing 100 mM NaCl and 0.1% Thesit. Bound receptor was then eluted with 7 ml of 50 mM Na citrate buffer pH 3.0, containing 0.05% Thesit. After neutralization the eluate was concentrated using a Centricon 30 (Amicon, Danvers, MA) and lyophilized.

Rat brain receptors were purified from 130 g of rat brains using mAb 295-AFC, as previously described for bovine brain receptors,[20] with the modification that before elution of the bound receptor, the affinity column was washed with 25 ml of 10 mM Na phosphate buffer pH 7.5, containing 100 mM NaCl and 0.1% Thesit. Receptors were then eluted with 5 ml of 50 mM Na citrate buffer pH 3.0, containing 0.05% Thesit. The eluate was neutralized, concentrated and lyophilized as above.

Bovine brain receptors were purified as described previously,[20] concentrated, and lyophilized as described above.

Purified receptors were labeled with ^{125}I as follows. To 20-100 pmol of receptor, in 25 μl of 10 mM Na phosphate buffer, pH 7.5, containing 100 mM NaCl, 1% SDS, and 4 M urea, was added (i) 50 μl of 0.4 M Na phosphate buffer, pH 7.5, containing 2% SDS and 8 M urea, (ii) 50 μl Enzymobeads (BioRad), (iii) 2 μl $Na^{125}I$ (1 mCi), (iv) 25 μl 10 mM Na phosphate buffer, pH 7.5, containing 100 mM NaCl, 1% SDS, 4 M urea, and 1% β(D) glucose. The reaction was allowed to proceed for 90 minutes at room temperature before 20 μl of 1 M NaN_3 were added to stop the reaction. The radioiodinated protein was separated from free ^{125}I using a BioRad Econo-Pac 10 DG disposable desalting column. The column was pretreated with 20 mg of β-lactoglobulin (Sigma) to reduce nonspecific binding, and then equilibrated in buffer (10 mM Na phosphate buffer, pH 7.5, containing 100 mM NaCl, 1% SDS, 4 M urea, 10 mg/ml β-lactoglobulin).

Approximately 0.5-10.0 x 10^5 cpm of ^{125}I-labeled receptor were loaded onto single lanes of 10% SDS polyacrylamide gels and resolved by electrophoresis (in SDS). The gels were fixed, dried, and autoradiographed. The dried gels were aligned with the autoradiograms, the pieces of the gels corresponding to the subunit bands excised, and radioactivity quantitated by γ counting. For ^{125}I rat brain receptor the excised subunits contained 2-5 x 10^5 cpm; ^{125}I bovine brain receptor subunits contained 3.5-4 x 10^5 cpm; ^{125}I Torpedo receptor contained 3-7 x 10^5 cpm; and ^{125}I chicken brain receptor contained about 3 x 10^3 cpm per excised band. The radioactivity per subunit was then

normalized for the number of tyrosine residues present in each subunit, as determined from the deduced amino acid sequences: In Torpedo receptor α, β, γ, and δ subunits[117] there are 17, 16, 17, and 19 tyrosine residues, respectively; in the chicken brain receptor structural subunit[35] there are 18 tyrosines, and in the chicken brain acetylcholine-binding subunit[31] there are 21 tyrosines; in the rat $\beta 2$ brain receptor structural subunit there are 15 tyrosines, and in the $\alpha 4$ acetylcholine-binding subunit[25] there are 17. No cDNA sequences are available for bovine brain receptor subunits. However, since they are certainly very homologous to the chicken and rat brain receptor subunit sequences, which have a ratio (acetylcholine-binding subunit : structural subunit) in the number of tyrosines of 1.0 : 0.86 (chicken) and 1 : 0.88 (rat), the normalization value of 0.87 was used for bovine receptors. After normalization of the number of tyrosine residues, the ratio of cpm was determined between subunits within a single lane to eliminate pipetting errors. The means of the ratios of 6-11 lanes were then determined.

Sucrose gradient analysis of neuronal nicotinic receptors was conducted as follows. Chicken and rat brain receptors were extracted from crude membranes for 2 hours, using an equal volume of 2% Triton X-100 in 50 mM Tris, pH 7.5, 1 mM EDTA, 1 mM EGTA, 5 mM p-aminobenzamidine, 5 mM iodoacetamide, and 0.5 mM PMSF. Day 18 embryonic chicken brains were used in these experiments because they have a higher concentration of receptors than adult brain (unpublished observation). Torpedo receptors, which were used as an internal control for each gradient, were similarly extracted with Triton X-100 from crude membranes of Torpedo electric organ. The Torpedo receptors (77 nM) were trace-labeled with ^{125}I-α-bungarotoxin (10 nM) and then diluted into the brain extracts to give ~40,000 cpm ^{125}I-α-bungarotoxin-labeled Torpedo receptor per gradient. Aliquots (450 μl) of the brain extracts containing the ^{125}I-α-bungarotoxin-labeled Torpedo receptors were layered onto 11 ml sucrose gradients (5-20% sucrose wt/wt, in 10 mM Na phosphate buffer, pH 7.5, containing 100 mM NaCl, 1 mM NaN_3, and 0.5% Triton X-100). The gradients were centrifuged for 17 hours at 41,000 rpm in a Beckman SW41 Ti rotor. Thirty drop fractions were subsequently collected from the bottom of the tubes and analyzed by γ counting to determine the sedimentation of the Torpedo receptors. The brain receptors in the gradient fractions were quantitated by ^{3}H-L-nicotine binding using a mAb-immobilization assay. Gradient fractions were shaken overnight at 4°C with 25 μl of a 1:1 slurry of goat anti-rat immunoglobulin-Sepharose and 1 μl of a stock solution of mAb 270. The Sepharose was washed with 2 x 1 ml of 10 mM Na phosphate buffer, pH 7.5, containing 100 mM NaCl and 0.5% Triton X-100 (PBS-Triton), and incubated with 20 nM ^{3}H-L-nicotine for 15 minutes at room temperature. The Sepharose was then washed with 3 x 1 ml of PBS-Triton by repeated centrifugation and resuspension. A solution of 2.5% SDS and 5% 2-mercaptoethanol (100 μl) was added to the Sepharose pellet, the entire contents were then added to a 5 ml T2 5% scintillation cocktail, and the radioactivity was determined by liquid scintillation counting.

Control tubes containing only buffer solutions were included in each assay to determine nonspecific ^{3}H-L-nicotine binding. To determine the extent of contamination of each tube by residual ^{125}I-α-bungarotoxin, the tubes were again analyzed by γ counting after the ^{3}H-L-nicotine binding assay. In no cases did the gradient fractions contain >100 cpm ^{125}I when analyzed at this time, thus indicating minimal contamination by ^{125}I when counting ^{3}H.

Rat brain receptors were also extracted from crude membranes for 2 hours, using an equal volume of 2% cholate and 0.2% asolectin in the same buffers used for extraction in Triton X-100. Likewise, Torpedo receptors were extracted from crude membranes of electric organ with the same detergent-lipid mixture. The Torpedo receptors were again prelabeled with ^{125}I-α-bungarotoxin and diluted into the rat brain extract. Aliquots (450 µl) of this extract were layered onto 11 ml sucrose gradients (5-20% sucrose wt/wt, 10 mM Na phosphate, pH 7.5, 100 mM NaCl, 1 mM NaN_3, 2% cholate, and 0.2% asolectin). The gradients were centrifuged for 17 hours in a SW41 Ti rotor at 41,000 rpm and fractionated by collecting 20 drops/fraction from the bottom of the tubes. The Torpedo and brain receptors in each fraction were quantitated as described above.

Histological Localization of Receptors

Labeling Sections of Chicken and Rat Brains with mAb 270. One-month-old white Leghorn chickens, adult Sprague-Dawley rats, and adult Balb-C mice were used in these experiments. The animals were decapitated and tissue was removed and frozen with liquid nitrogen. Cryostat sections (20 µm thick) were thaw-mounted onto slides and desiccated at 0-4°C under vacuum overnight. For autoradiographic localization, the sections were overlaid with 4 nM ^{125}I-mAb 270 (radioiodinated to a specific activity of 1-2x10^{18}cpm/mol by a modified chloramine-T method)[82] in 100 mM NaCl, 10 mM Na phosphate buffer, pH 7.5, 10 mM NaN_3, 10% normal rat serum, and 5% Carnation dried milk, and were incubated overnight at 4°C. The slides were transferred to Coplin jars and rinsed 5X over 30 minutes with 100 mM NaCl, 10 mM Na phosphate buffer, pH 7.5, and 10 mM NaN_3 at room temperature. The sections were further rinsed in three changes of buffer over 3 hours on a rocking platform at 4°C. They were then dried at 37°C, mounted on cardboard in groups of 20, overlaid with an 8x10-inch sheet of Kodak XAR5. The sections were then postfixed in 10% formalin, dehydrated in ethanol, defatted in xylene, rehydrated and air dried, dipped in Kodak NTB-2 emulsion, exposed for 4 days, and developed as described elsewhere.[118] For indirect immunofluorescence, sections were obtained as described above, and the mAbs were localized with goat anti-rat IgG conjugated with fluorescein isothiocyanate, as described elsewhere.[119]

Autoradiographic Localization of Receptors in Finch Brains. Intact adult male and female zebra finches (Poephila guttata) aged 6-12 months were killed by metofane overdose and

decapitated. Their brains were immediately removed, submerged in ice-cold Tissue-Tek Frozen Embedding Medium (Miles Laboratories, Naperville, IL), and frozen in a -20°C freezer. The blocks were cryostat sectioned at 14 μm and sections were thaw mounted onto subbed slides and stored on ice until a complete brain was sectioned. The sections were freeze-dried in desiccator boxes in an ice-salt bath. Drops of solution containing one of the nicotinic ligands were applied to each set of dried sections at room temperature. Twenty nM ^{125}I-α-bungarotoxin in bovine serum was placed on sections for 30 minutes. The ^{125}I-mAbs were used at a concentration of 2.0 nM with specific activities of 2-5 x 10^{18} cpm/mol. Labeling was done in 10^{-2} M HEPES buffered with Eagle's solution for 2 hours. Specificity of binding was determined in the case of α-bungarotoxin by including 1 mM unlabeled toxin, 1 mM carbamylcholine or 10 mM d-tubocurarine in the incubation mix; all three competitors reduced the binding of α-bungarotoxin to background levels and no regional variation was seen. Inclusion of excess unlabeled mAb 35 blocked the binding of ^{125}I-mAb 35, and unlabeled mAb 270 blocked the binding of ^{125}I-mAb 270. Following incubation, brain sections were washed for 1 hour, fixed in formaldehyde vapor, and applied to LKB (Pharmacia LKB, Piscataway, NJ) Ultrofilm for autoradiography. Following LKB autoradiography, slides incubated with each ligand were selected from each of four male brains and coated with Kodak (Rochester, NY) NTB-2 emulsion, overexposed, developed, and stained with cresyl violet to confirm the neuroanatomical localization of labeled regions.

A brain structure was judged to be labeled if it was visibly more labeled than ectostriatum (E)-a lightly labeled region using all three ligands. For small medullary structures, microscopic examination was necessary to evaluate silver grain density. A structure was judged to be labeled if it contained discernibly higher grain density than white matter in adjacent regions (such as fasciculus longitudinalis medialis and leminiscus medialis), or if silver grains were found clustered around nissl-stained cell nuclei. A qualitative estimate of the relative intensity of labeling in various brain structures was made separately for each ligand. No quantitative comparison of labeling intensity between ligands was made for any brain structure, so comparisons of labeling intensity between ligands are valid only when a structure is heavily labeled by one ligand and lightly by another ligand.

Histochemical Localization of Receptors in Chicken Retinas. A total of 37 white leghorn chicks from 1-14 days old were used in this study. The chicks were killed with an overdose of ketamine and xylazine. The eyes were rapidly removed, the anterior pole and vitreous cut away, and the eye was immersed in ice-cold 0.1%, 1%, or 4% paraformaldehyde in 0.1 M sodium phosphate buffer at pH 7.4. After 15 minutes-24 hours, the tissue was placed in a solution of 30% sucrose in 0.1 M phosphate buffer for at least 12 hours. The eyes were then frozen in embedding medium. Sections, 10 μm thick, were cut on a cryostat

and collected on gelatin-coated glass slides. Alternatively, radial incisions were made in the retina so that it could be flattened. It was then frozen, and 30-μm-thick sections were cut parallel to the vitreal surface on a sliding microtome. Sections cut in this plane will be referred to as "horizontal sections." These sections were processed "free floating" in small vials. In one case, 20-μm-thick sections were cut perpendicular to the vitreal surface on a cryostat and immersed in buffer solution. They were processed in the same fashion as horizontal sections.

The indirect fluorescence and avidin-biotin techniques were used in this study. In some instances a "double level" fluorescence technique was used, which allowed the visualization of two different immunohistochemical markers in the same section through the use of two different fluorophores.

The sections were washed in three changes of phosphate buffer and then incubated with the primary antibodies diluted in phosphate buffer. The mAbs were used at a concentration of 10-20 nM and the antiserum to choline acetyltransferase (ChAT) was used at a dilution of 1:1000. The incubation buffer included 0.3% Triton X-100 and 0.05% NaN_3, and sections were incubated for 12-24 hours at a temperature of 4°C. The tissue was next washed in three changes of buffer and then incubated in either fluorescein- or rhodamine-isothiocyanate (FITC or RITC, respectively) conjugated goat anti-rat IgG (Boehringer Mannheim) diluted 1:100 in buffer. The sections were subsequently washed in several changes of buffer and coverslipped with a mixture of carbonate buffer and glycerine.

As a determination of secondary antiserum specificity, we processed some sections for immunofluorescence, but omitted the primary antibody. Other sections from the same retina were processed normally in parallel. Those sections that had not been exposed to primary antibody exhibited no staining.

Tissue to be double-labeled with antiserum directed against ChAT and nicotinic receptor antibodies was processed according to the procedure used by Erichsen et al.[120] The antibody directed against ChAT was generously supplied by Miles Epstein and Carl Johnson and has been previously characterized.[121] For these double-label studies the antiserum and the antibody were mixed and applied to the tissue for times similar to those stated above. The tissue was then washed in three changes of phosphate buffer, and a mixture of goat anti-rabbit IgG conjugated to FITC and goat anti-rat IgG conjugated to RITC was applied for 1 hour. In some cases the fluorophores were reversed.

Sections to be processed with the avidin-biotin technique were incubated in primary antibody, washed in three changes of phosphate buffer, and placed in a solution of biotinylated goat anti-rat IgG (Vector Laboratories, Burlingame, CA) diluted 1:200 in 0.3% Triton X-100 in buffer for 1 hour. The sections were then washed and incubated with a mixture of biotin and avidin-

bound-horseradish peroxidase diluted 1:100 in phosphate buffer with 0.3% Triton for 1 hour. Following washes in buffer, the tissue was incubated with 0.05% diaminobenzidine in phosphate buffer for 15 minutes. Hydrogen peroxide was then added to make a final concentration of 0.01% and the solution was gently shaken. The reaction was allowed to proceed for 15 minutes. The tissue was then washed in several changes of buffer, cleared, and coverslipped.

The sections were examined with a microscope fitted for epifluorescence microscopy or differential interference microscopy.

Histological Studies of Nicotinic Receptors in Frog Brains. Most of the experiments were done on Rana pipiens (body length of 5.0-7.5 cm, either sex) obtained from Hazen Company (Alburg, VT).

Biotinylated rabbit anti-rat IgG and avidin-biotinylated horseradish peroxidase complex were purchased in kit form (Vectastain) from Vector Laboratories. Fluorescein-goat-anti-rat IgG was obtained from Cappel Laboratories (Organon Teknika, Malvern, PA). All other reagents were purchased from Sigma Chemical Company unless indicated otherwise.

All surgical procedures were performed following anesthesia by immersion of animals in 2 mM tricaine methanesulfonate. The optic nerve was cut after approaching it through the soft palate. The soft palate was then sutured with 6-0 monofiliment nylon thread. Retinas were eviscerated by aspiration following removal of the lens and vitreous body. The eyelid was then sutured shut. The projection of retinal ganglion cells to the tectum was labeled by placing crystals of horseradish peroxidase (Sigma, Type VI) in contact with the central stump of the severed optic nerve and examining the tectum 2-3 days later.

Light microscopic immunocytochemistry was performed on frozen or vibratome sections of tecta of animals fixed by perfusion with periodate-lysine-paraformaldehyde (PLP) containing 2% paraformaldehyde or with 4% acrolein in 90 mM Na phosphate buffer. The avidin biotinylated-peroxidase complex (ABC) method of Hsu et al.[122] was used to visualize primary antibody binding, and peroxidase was revealed colorimetrically by the cobalt-glucose oxidase procedure of Itoh et al.[123]

Animals were perfused through the truncus arteriosus, first, with frog Ringer's containing 2 mM tricaine methanesulfonate until fluid returning to the heart was clear, and subsequently with fixative for 7.5 (acrolein) or 90 (PLP) minutes. The brain with optic nerves was then removed and immersion-fixed for an additional 7.5 (acrolein) or 30 (PLP) minutes. For frozen sections the brain was then equilibrated in 30% sucrose in 90 mM Na phosphate buffer, frozen by immersion in liquid nitrogen, and mounted in Tissue Tek mounting medium (Miles Laboratories) prior to sectioning at 10 μm in a microtome cryostat. Sections were

placed on subbed glass slides and air-dried for 30 minutes before use. For vibratome sections, the brain was embedded in 4% agar and sectioned at 100 μm. In a separate set of experiments, retinas were vibratome-sectioned after being removed from the sclera of an anesthetized frog and immersion fixed in PLP for 30 minutes.

Vibratome sections of tissue were processed "free-floating" according to following schedule (all steps were done at room temperature unless indicated otherwise). Frozen sections were processed mounted and for shorter times (1 hour for antibody incubations, 30 minutes for washes).

1. Preincubate vibratome sections in frog Ringer's containing 3% normal rabbit serum and 0.025% saponin (RRS) for 30 minutes.

2. Incubate sections in primary antibody diluted to a titer of 10-200 nM in RRS for 16 hours at 4°C.

3. Wash in RRS for 2-3 hours, changing the solution every 20 minutes.

4. Incubate the sections in biotinylated rabbit anti-rat IgG diluted 1:200 in RRS for 6 hours.

5. Wash in RRS for 2-3 hours, changing the solution every 20 minutes.

6. Incubate sections in avidin-HRP, diluted 1:100 in RRS, for 16 hours at 4°C.

7. Wash in RRS for 1 hour, changing the solution every 20 minutes.

8. Wash in Ringer's for 1 hour, changing the solution every 20 minutes.

9. Fix the sections in 1% glutaraldehyde in 60 mM sodium phosphate buffer, pH 7.2, for 1 hour.

10. Rinse in Ringer's, 15 minutes.

11. Preincubate the sections in a solution containing diaminobenzidine, nickel ammonium sulfate, cobalt chloride, and β-D-glucose in phosphate buffer (final concentrations: diaminobenzidine, 0.5 mg/ml; nickel ammonium sulfate, 0.025%; cobalt chloride, 0.025%; β-D-glucose, 2 mg/ml) for 15 minutes.

12. Incubate the sections in a solution identical to the preincubation solutions, but containing in addition 0.2 mg/ml ammonium chloride and 0.003 mg/ml glucose oxidase (Sigma, Type II) until there is adequate staining intensity.

13. Rinse the sections in Ringer's for 15 minutes.

14. Mount the sections in 90% glycerol/10% Ringer's.

In a few instances immunoperoxidase experiments were performed on only one side of the tectum to compare the pattern of staining with the pattern of the retinotectal projection (the retinotectal projection was almost completely crossed). Horseradish peroxidase crystals were placed in contact with the central cut end of the left optic nerve in an anesthetized frog. Two to three days later the animal was reanesthetized and perfused with fixative. The tectum was then serially sectioned, with the plane of the sections being as close as possible to coronal. Each section was then split in the mid-saggital plane and each right half-section was set aside. The left half-sections were reacted for receptor (through step 10, above), and both right and left halves were reacted for horseradish peroxidase. The matching sections were then reassembled photographically, thus producing a reconstituted tectal slice in which the retinal projection appeared on the right half and the receptor immunoreactivity appeared on the left half.

The procedure for performing immunocytochemical staining for analysis in the electron microscope was somewhat similar to that for light microscopy. Only vibratome sections were used, and saponin was omitted to preserve adequate fine structure. Electron microscopic analysis was performed after cutting thin sections orthogonal to the plane of the vibratome section. The heaviest peroxidase stain was found at the surface of the vibratome section, but the tissue was poorly preserved. The best results were obtained 1-3 μm into the section, where peroxidase staining was still strong and where tissue preservation was adequate.

Electron microscopic analysis was performed on sections treated as above (steps 1-13) and postfixed in 1% OsO_4 in 90 mM sodium phosphate buffer, pH 7.2, dehydrated and embedded in Epon/Araldite. Sections having a silver interference color (70-80 nm) were cut on an ultramicrotome and examined without grid staining on an electron microscope. Contrast was sometimes enhanced by adding tannic acid to the glutaraldehyde (step 9, above) to a final concentration of 0.3%.

In a few experiments vibratome sections of tectum were incubated in concentrated solutions of the tracer ferritin or 6 nm colloidal gold-protein A. Cationized ferritin was used at 20 mg/ml in Ringer's. Colloidal gold-protein A was prepared according to the technique of Muehlpfordt,[124] conjugated to protein A and purified after Slot and Geuze,[125] and used at an OD @ 525 nm of 1.0 in Ringer's. Tissue incubated for 1 hour with tracer was fixed in 1% glutaraldehyde in 60 mM Na phosphate without tracer washout. The purpose of these experiments was to learn the extent to which these reagents penetrated the vibratome slice and the cell profiles within its interior.

Neuronal α-Bungarotoxin-Binding Proteins

Cloning of cDNAs for Subunits of α-Bungarotoxin-Binding Proteins

a cDNA library from embryonic day 18 (E18) chicken brains[35] was screened with the 47-mer oligonucleotide designed on the chemically determined N-terminal protein sequence[80] of one subunit of chicken brain α-bungarotoxin-binding protein, basically following codon usage frequencies.

Stringency conditions were 30% formamide, 5xSSPE (1xSSPE is 0.18 M NaCl, 0.01 M $NaPO_4$, pH 7.4, and 1 mM EDTA) at 42°C for hybridization, followed by final washings at 55°C, 1xSSPE (clone pCh29-1 was identified in this way). Using pCh29-1 or a fragment thereof (i.e. pCh29-3) as a probe, pCh31-1 was isolated, and subsequently, using pCh31-1 fragments as probes, pCh34-1. The nucleotide sequences were completely determined in both strands. By deduced amino acid sequence homology to pCh31-1 and by common EcoRI sites (nucleotides 533-539, **Figure 54**) it is likely that pCh29-3 codes for the N-terminal and pCh34-1 for the C-terminal part of the α1 subunit. As apparently all cDNA EcoRI sites in this library were cleaved, the contingency of pCh29-3 and pCh34-1 was demonstrated by the polymerase chain reaction (PCR) technique. About 300 ng of E17 chicken brain first and second strand cDNA was subjected to 35 cycles (1 minute, 92.5°C; 2 minutes, 55°C; 1 minute 30 second, 72°C on an Ericomp Temperature cycler) using Taq polymerase (Perkin Elmer) with the supplier's recommended buffer. The 5' primer (24-mer) sequence was derived from pCh29-3, the 3' primer (24-mer) sequence from pCh34-1. Plasmids containing the specific reaction product were sequenced (underlined in **Figure 54**), proving to be identical between the primers used to pCh29-1 and pCh34-1 spliced together at the EcoRI site.

Antibodies to Bacterially Expressed Peptide Fragments of α1 and α2 Subunits of Neuronal α-Bungarotoxin-Binding Protein.

Fragments of pCh31-1 and pCh34-1 were subcloned into a T7 promoter-based bacterial expression system described by Rosenberg et al.[115,116] ( ). At least the cloning sites were sequenced for all constructs. The deduced protein sequences of the recombinant proteins are:

protein Ch31-5 (encoding the large putative cytoplasmic domain α2, **see Figure 56**):
MASMTGGQQMGRIKLRIPWYQLQFHHHDPQAGKMPRWVRVILLNWCAWFLRMKKPGENIKPLSCK
YSYPKHHPSLKNTEMNVLPGHQPSNGNMIYSYHTMENPCCPQNNDLGSKSGKITCPLSEDNEHVQ
KKALMDTIPVIVKILEEVQFIAMRFRKQDEGEEIRLLTKPERKLSWLLPPLSNN

protein Ch31-6 (encoding a smaller, unique sequence of α2, **see Figure 56**):
MASMTGGQQMGRDPSSRSAGENIKPLSCKYSYPKHHPSLKNTEMNVLPGHQPSNGNMIYSYHTME
NPCCPQNNDLGSKSGKITCPLSEDNEHVQKKALMDTIPVIVNSKLDPAANKARKEAELAAATAEQ

protein Ch34-2 (encoding a small, unique sequence of α1, see **Figure 56**):

MASMTGGQQMGRDPSSRSAGEDKVRPACQHKQRRCSLSSMEMNTVSGQQCSNGNMLYIGFRGLDG
VHCTPTTDSGVICGRMTCSPTEEENLLHSGHPSEGDPDLANSKLDPAANKARKEAELAAATAEQ

The underlined sequences are genuine to the deduced parent plasmid sequences, and the additional amino acids are vector encoded.

Protein Ch31-5 was obtained as inclusion bodies isolated by differential centrifugation. Impurities were successively extracted with 1 M NaCl, 0.5% Triton X-100, and 3 M KSCN, and then the inclusion bodies were solubilized in 8 M urea. After removing the urea by dialysis, the partially soluble protein was more than 50% pure, as judged by Coomassie-stained SDS-PAGE. Lewis rats were immunized repeatedly with approximately 100 μg of protein in complete Freund's adjuvant.

Protein Ch34-2 was not found to form inclusion bodies. Therefore, SDS-PAGE-purified preparations were used for immunization of Lewis rats.

Protein Ch31-6 was not purified for use as an immunogen, but only used as an antigen on Western blots, as described below.

Spleen Cell Fusion

The rat with the highest titer to protein Ch31-5 was immunized intraperitoneally, five days before the fusion, with 100 μg of protein Ch31-5 in PBS. On the day of the fusion the rat was killed and its spleen cells fused with Sp2/0 mouse myeloma cells using polyethylene glycol.[110] Nine days after the fusion, the culture supernatants were screened in a radioimmunoassay for antibodies which bound ^{125}I-α-bungarotoxin-labeled α-bungarotoxin-binding protein in extracts of embryonic chick brain (see protocol below). Positive cultures were cloned twice by limiting dilution and then expanded to large cultures. These five hybridomas were designated as mAbs 308-312. Culture media was collected, concentrated by ultrafiltration and ammonium sulfate precipitation, and dialyzed against PBS containing 10 mM NaN_3.

Radioimmunoassay of Antibodies to α-Bungarotoxin-Binding Proteins

Triton X-100 extracts of E18 chick brains were prepared. Antisera or mAb stocks were diluted in PBS and incubated with 40 μl chick brain extracts containing 4 μl normal rat serum and 2 nM ^{125}I-α-bungarotoxin (specific activity 2-5x10^{17} cpm/mol) in a total volume of 100 μl. After overnight incubation at 5°C, 100 μl of goat anti-rat immunoglobulin was added and incubated for another hour. PBS-Triton X-100 was added (1 ml) and the immune complexes pelleted and washed twice with PBS-Triton. ^{125}I-α-bungarotoxin in the pellet was determined by γ counting. Nonspecific and background counts were determined using preimmune serum and were subtracted from all data. The total amount of α-

bungarotoxin-binding protein extracts was determined by incubating 40 μl extract with 2 nM ^{125}I-α-bungarotoxin in a total volume of 100 μl. After overnight incubation at 5°C, 4 ml 10 mM Tris, 0.05% Triton X-100, pH 7.5, was added and the mixture rapidly filtered through Whatman GF/B filters pretreated with 0.3% polyethylenimine.[126] The filters were washed three times with 4 ml of the same buffer and counted. Nonspecific binding was determined in the presence of 1 mM carbamylcholine.

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