Molecular Studies of the Neuronal Nicotinic Acetylcholine Receptor Family

Jon Lindstrom, Ralf Schoepfer, and Paul Whiting

Receptor Biology Laboratory
The Salk Institute for Biological Studies
PO Box 85800
San Diego, CA 92138

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Abstract

Nicotinic acetylcholine receptors on neurons are part of a gene family that includes nicotinic acetylcholine receptors on skeletal muscles and neuronal αbungarotoxin-binding proteins that in many species, unlike receptors, do not have an acetylcholine-regulated cation channel. This gene superfamily of ligand-gated receptors also includes receptors for glycine and gamma-aminobutyric acid. Rapid progress on neuronal nicotinic recep-

tors has recently been possible using monoclonal antibodies as probes for receptor proteins and cDNAs as probes for receptor genes. These studies are the primary focus of this review, although other aspects of these receptors are also considered.

In birds and mammals, there are subtypes of neuronal nicotinic receptors. All of these receptors differ from nicotinic receptors of muscle pharmacologically (none bind αbungarotoxin, and some have very high affinity for nicotine), structurally (having only two types of subunits rather than four), and, in some cases, in functional role (some are located presynaptically). However, there are amino acid sequence homologies between the subunits of these receptors that suggest the location of important functional domains.

Sequence homologies also suggest that the subunits of the proteins of this family all evolved from a common ancestral protein subunit. The ligand-gated ion channel characteristic of this superfamily is formed from multiple copies of homologous subunits. Conserved domains responsible for strong stereospecific association of the subunits are probably a fundamental organizing principle of the superfamily. Whereas the structure of muscle-type nicotinic receptors appears to have been established by the time of elasmobranchs and has evolved quite conservatively since then, the evolution of neuronal-type nicotinic receptors appears to be in more rapid flux.

Certainly, the studies of these receptors are in rapid flux, with the availability of monoclonal antibody probes for localizing, purifying, and characterizing the proteins, and cDNA probes for determining sequences, localizing mRNAs, expressing functional receptors, and studying genetic regulation.

The role of nicotinic receptors in neuromuscular transmission is well understood, but the role of nicotinic receptors in brain function is not. The current deluge of data using antibodies and cDNAs is beginning to come together nicely to describe the structure of these receptors. Soon, these techniques may combine with others to better reveal the functional roles of neuronal nicotinic receptors.

Index Entries: Acetylcholine receptor; nicotinic receptors; neuronal receptors

Nomenclature

ACh	Acetylcholine
AChR	Aceylcholine receptor
BAC	Bromoacetylcholine
αBgt	αBungarotoxin
cDNA	Complementary DNA
DTT	Dithiothreitol
GABA	Gamma amino butyric acid
mAb	Monoclonal antibody
MBTA	4-(*N*-Maleimido)-benzyltrimethylammonium iodide
MIR	Main immunogenic region
mw	Molecular weight
βNGF	Nerve growth factor, β subunit

Introduction

Nicotinic acetylcholine receptors (AChRs) on neurons have been much less well characterized than have AChRs from striated muscle and electric organs. Now, with the use of monoclonal antibody (mAb) and complementary DNA (cDNA) probes derived from studies of AChRs on electric organs, rapid progress is being made on identifying and characterizing neuronal nicotinic AChR proteins. The primary focus of this paper will be to review the studies using these probes, but in order to put these studies in proper perspective and to help anticipate the future of such studies, we will also briefly review studies of neuronal nicotinic AChRs by other approaches. Evolution of the structural features of AChRs and other members of this gene superfamily will be considered in some detail.

Until recently, studies of neuronal nicotinic AChRs were hampered by lack of suitable molecular probes. Also, there were no neural systems as convenient as muscle for studying their electrophysiology, and there was no neural system that provided as much AChR as electric organs for studying their biochemistry. αBungarotoxin (αBgt) and cobra toxin were tremendous boons to studies of muscle-type AChRs because they bound with great affinity and specificity to the ACh-binding sites of muscle-type nicotinic AChRs and could be used as histological labels, affinity column ligands, and probes for the ACh-binding site of the purified AChR. Of course, these toxins were soon investigated as probes for neuronal nicotinic AChRs, but this led to two sorts of problems. It was immediately clear that in many neuronal systems there were binding sites for αBgt (e.g., Hunt and Schmidt, 1978; Wang et al., 1978). However, in later studies it became clear that in at least some of these systems these binding sites were not AChRs (in the sense of ACh-regulated cation channels) (Patrick and Stallcup, 1977a,b; Carbonetto et al., 1978). Individual neurons could be found that had both AChRs and αBgt-binding proteins (Patrick and Stallcup, 1977a,b; Jacob and Berg, 1983; Smith et al., 1986). Data from studies of various species confused things further. For example, vertebrates have nicotinic AChRs at their neuromuscular junctions that bind αBgt, but nicotinic AChRs in their brains and ganglia that do not bind αBgt. Vertebrates also have αBgt-binding proteins in their brains and ganglia that in most cases are probably not AChRs. Insects, however, have glutamate receptors at their neuromuscular junctions, but their central ganglia contain very high concentrations of AChRs that bind αBgt (Breer et al., 1985).

Toxins from *Bungarus multicinctus* venom were found that, unlike αBgt, did block neuronal AChRs, but these were present in small amounts, had lower affinity, and were initially difficult to iodinate in active form (Ravdin and Berg, 1979; Chiappinelli, 1985).

Cholinergic agonists were also used as probes for neuronal AChRs. The very high affinity of some putative neuronal AChRs for tritium-labeled ACh and nicotine permitted autoradiographic localization of these sites in brain distinct from αBgt-binding sites (Clarke et al., 1985b). However, this sort of autoradiography was more time consuming and less precise than immunohistochemical localization, and some ganglionic neuronal AChRs had too low affinity to be detected with these ligands (Kemp and Morley, 1986). Use of cholinergic analogs as affinity ligands for purifying neuronal AChRs was investigated (Abood et al., 1983), but did not lead to a clear breakthrough in purification and characterization.

Affinity labeling reagents (such as bromoacetylcholine (BAC) and 4-(*N*-maleimido)-benzyltrimethylammonium iodide (MBTA) (Karlin, 1980), initially developed for studies of the ACh-binding site of AChRs from electric organ, react with neuronal AChRs (Leprince, 1983, 1986; Whiting and Lindstrom, 1987a) and

neuronal αBgt-binding proteins (Lukas and Bennett, 1980; Norman et al., 1982) and have been useful in identifying ACh-binding subunits.

Rapid progress on molecular studies of neuronal nicotinic AChRs has recently become possible as a result of probes developed through studies of muscle-typed AChRs. In both the cases of mAb and cDNA probes, the path to probes for neuronal nicotinic AChRs started with biochemical studies of electric organ AChRs affinity purified using snake venom toxins and depended on structural homologies between AChRs from nerve and muscle. For example, a mAb raised to AChRs from electric eels (Tzartos et al., 1981) was found to bind to AChRs on chicken neurons (Swanson et al., 1983; Jacob et al., 1984; Smith et al., 1985, 1986), and then used to affinity purify these AChRs (Whiting and Lindstrom, 1986a). This mAb was used to show that it identified functional AChRs on chicken ganglionic neurons that had low affinity for nicotine (Smith et al., 1985, 1986; Halvorsen and Berg, 1986, 1987), and AChRs from brain with high affinity for nicotine (Whiting and Lindstrom, 1986b; Stollberg et al., 1986). Other mAbs made to the AChRs purified from chicken brain were also used in the characterization of this AChR, to discover another subtype of AChR in chicken brain (Whiting et al., 1987d), and to localize (Swanson et al., 1987), purify, and characterize AChRs from rat brain (Whiting and Lindstrom, 1987b). Still other mAbs made to the AChRs from rat brain were used to identify, purify, and characterize AChRS from bovine and human brains (Whiting et al., 1987c; Whiting and Lindstrom, unpublished).

While these studies with mAbs were going on, a parallel approach was being taken using cDNAs. Purification of AChRs from the electric organs of *Torpedo* permitted *N*-terminal amino acid sequence analysis (Raftery et al., 1980) that permitted the synthesis of probes for identifying cDNAs for *Torpedo* AChR subunits (Noda et al., 1983b). These cDNAs were used, in turn, to identify corresponding cDNAs for subunits of AChR from skeletal muscle (Noda et al 1983a; Boulter et al 1985). Then cDNAs for muscle AChR α subunits were used to identify (by low stringency hybridization) corresponding subunit cDNAs in libraries prepared from neuronal sources (Boulter et al., 1986; Goldman et al., 1987). In some cases, it has been possible to use *N*-terminal amino acid sequence analysis of the subunits of immunoaffinity purified AChR to identify the cDNAs to which they correspond (Whiting et al., 1987b).

It is difficult to prove that a cholinergic ligand-binding site, purified protein, or the protein that could be coded for by a cDNA really is part or all of a functional AChR. In striated muscle it has been clearly established that the AChR is an intrinsic protein of the postsynaptic membrane that responds to ACh released from the motor neuron by transiently opening a cation channel through the AChR molecule. But even in this case, our understanding is incomplete. For example, it was recently discovered that γ subunits are characteristic of extrajunctional muscle AChRs, and are replaced by ε subunits in junctional muscle AChRs (Mishina et al., 1986). Still other alternate subunits may exist in the case of muscle AChRs. Ganglia and brains are more anatomically complex than muscle, so establishing precise localization, studying electrophysiological properties, and determining functional role is more difficult. There is the known complication of αBgt-binding proteins that are not AChRs, but perhaps further complexities are as yet unappreciated. For example, in chicken, there should be at least eighteen AChR subunit homologs: α, β, γ, δ, and ε subunits of AChRs from muscle, α and β subunits of AChRs from ganglia, α, β, and β' subunits of AChRs from brain, perhaps four subunits of the αBgt-binding protein, α and β subunits of glycine receptors, α and β subunits

of GABA receptors, and probably subunits of other ligand-gated receptors with intrinsic ion channels. This broad gene family includes even more distant cousins than the αBgt-binding protein (whose endogenous ligand and channel activity are unknown); receptors for GABA and glycine do not bind ACh and their channels are anion-specific, yet their structure shows basic homology to that of AChR subunits, clearly indicating that they evolved from a common ancestor (Grenningloh et al., 1987; Schofield et al., 1987; Barnard et al., 1987). The various techniques used to test whether the proteins and cDNAs proposed to be constituents of AChRs are in fact parts of ACh-regulated cation channels will be discussed as the results of studies with mAbs and cDNA probes are reviewed.

The basic assumption, now validated, of attempts to study neuronal nicotinic AChRs with mAb and cDNA probes initially made using muscle-type AChR is that there are fundamental structural homologies between the AChR molecules from muscle and nerve. Exactly how homologous these structures are will be reviewed in detail below, but the differences are more than trivial. Neuronal nicotinic AChRs differ pharmacologically, antingenically, in subunit composition, and in other respects from muscle-type nicotinic AChRs.

In order to best appreciate the similarities and differences between AChRs from muscles and neurons, we will briefly review the structure of muscle-type AChRs before beginning the body of the review on neuronal AChRs. The muscle-like AChRs of the electric organ of *Torpedo californica* are composed of two α subunits (which contain the ACh-binding sites, both of which must be liganded to efficiently trigger opening of the cation channel) and one each of β, γ, and δ subunits (Reynolds and Karlin, 1978; Lindstrom et al., 1979; Raftery et al., 1980). The apparent molecular weights (mws) of these subunits are α = 40,000, β = 49,000, γ = 57,000, and δ = 64,000, whereas their actual protein mws (not including carbohydrate or lipid) are 50,116, 53,681, 56,279, and 57,565 (Noda et al., 1983b). The subunits are oriented like barrel staves around the central cation channel (Kistler et al., 1982; Brisson and Unwin, 1985) in the order αβαγδ (Hamilton et al., 1985; Kubalek et al., 1987). All of the subunits contribute to the lining of the channel (Giraudat et al., 1986; Hucho et al., 1986), and several may contribute to its kinetics and selectivity (Wan et al., 1985; Sakmann et al., 1985; Mishina et al., 1986; Imoto et al., 1986). All of the AChR subunits have sequence homologies throughout their length, which suggests that they evolved via duplication of a primordial gene (perhaps from an AChR with a single kind of subunit), and also suggests that the structure of all of the subunits is basically homologous (Raftery et al., 1980; Noda et al., 1983a). Evolution of muscle-type AChRs has been quite conservative; there is 80% sequence homology between α subunits of AChRs of the marine elasmobranch *Torpedo californica* and human muscle, and about 55% homology of the other subunits between these species (Noda et al., 1983c; Takai et al., 1984; Tanabe et al., 1984; Kubo et al., 1985). Although both AChRs from *Torpedo* and humans have a main immunogenic region (MIR) (Tzartos et al., 1982, 1983), the specificity of antibody binding makes them much more antigenically distinct than their sequence homologies would suggest; there is 2% or less immunological crossreaction (Lindstrom et al., 1978). All of the subunits are glycosylated (Nomoto et al., 1986) and extend back-and-forth across the membrane several times. Their N-termini are probably on the extracellular surface (Anderson and Blobel, 1983), but are not accessible to antibodies (Ratnam and Lindstrom, 1984; Neumann et al., 1985), and their C-termini are accessible to antibodies on the cytoplasmic surface (Lindstrom et al., 1984; Young et al., 1985; Ratnam et al., 1986a,b). A

disulfide-linked loop between cysteines at positions corresponding to 128 and 142 of α with an *N*-glycosylation site at 141 is probably common to the extracellular surface of all of the subunits (Noda et al., 1983a; Mishina et al., 1985; Criado et al., 1986). The extracellular surface may be rather conformationally constrained, not easily recognized by antibodies to denatured subunits, and more resistant to proteases whereas the cytoplasmic surface may be less constrained and more extended, easily recognized by antibodies to denatured subunits, and susceptible to proteases (Klymkowksy et al., 1980; Ratnam et al., 1986b,c; Ralston et al., 1987; Kordossi and Tzartos, 1987). The α subunit is unique in having disulfide-linked adjacent cysteines at positions α192 and 193 (Kao and Karlin, 1986; Criado et al., 1986). These are near the ACh-binding site, as shown by affinity labeling and αBgt-binding experiments (Kao et al, 1984; Neumann et al., 1986; Gershoni et al., 1983; Ralston et al., 1987). The ACh-binding is on the upper extracellular surface of α subunits (Kubalek et al., 1987). The MIR is formed by residues between α46 and 127 and is located on the side of the extracellular surface of α subunits (Kubalek et al., 1987). Each subunit has hydrophobic sequences corresponding to amino acids 210–236 (termed M1), 243–267 (M2), 273–296 (M3), and 409–428 (M4) of the α subunit (Noda et al., 1983a). Some of these sequences may form transmembrane domains (Claudio et al., 1983; Noda et al., 1983a; Devillers-Thiery et al., 1983), though probably not all (Ratnam et al., 1986c). There may also be other transmembrane domains (Guy, 1983; Finer-Moore and Stroud, 1984; Criado et al., 1985a,b), but there is evidence that a proposed amphipathic transmembrane domain C-terminal of M3 is actually on the cytoplasmic surface (Ratnam et al., 1986a,b). Regions between M2 and M3 on δ subunits affect conductance of the AChR (Imoto et al., 1986), and on γ and ε subunits, and perhaps α and β also, affect conductance (Mishina et al., 1986). In addition to glycosylation, AChRs are subject to other posttranslational modifications including acylation (Olson et al., 1984) and phosphorylation at several sites by different kinases (Huganir et al., 1986; Safran et al., 1986; Smith et al., 1987). Muscle AChRs desensitize on prolonged exposure to agonists (that is, they assume a conformation with a closed channel and much higher than normal affinity for agonists) and the rate of desensitization can be greatly enhanced by phosphorylation at sites on the cytoplasmic surface (Huganir et al., 1986). The physiological significance of desensitization is unclear in the case of neuromuscular transmission, in which activation of the muscle by the nerve is usually assured by a large safety factor; whereas in neuronal systems, where signal integration is important, it is easier to imagine a physiological role for a process that modulates ACh sensitivity. At mature neuromuscular junctions, AChRs are located at the crests of folds in the postsynaptic membrane in close proximity to active zones on the nerve ending (Salpeter and Harris, 1983). They are anchored through nonAChR proteins such as a 43,000 mw protein on the cytoplasmic surface (Sealock et al., 1984). Before innervation or after denervation, an extrajunctional form of AChR is found in larger amounts (Fambrough et al., 1979). The amount of AChR is regulated in part by frequency of activation (Lomo and Rosenthal, 1972; Drachman et al., 1982) and in part by specific trophic factors (Laufer and Changeux, 1987), with regulation at the transcriptional level being important (Merlie et al., 1984; Evans et al., 1987). There is evidence that extrajunctional AChRs have γ subunits, whereas junctional AChRs have ε subunits (Mishina et al., 1986). There may also be other subunit differences, posttranslational modifications, or differences in AChR environment that further differentiate between junctional and extrajunctional AChRs. Extrajunctional AChRs turnover much more rapidly, have

longer channel open time, lower conductance, and lower curare affinity than junctional AChRs (reviewed in Fambrough, 1979).

Nicotinic Functional Effects

In the past, nicotine was used as an insecticide; its peak usage was in 1944 when 1.2 million lb were used in the US (Metcalf, 1948). In recent years its use has declined, primarily as a result of its nonselective toxicity. Nicotine proved to be an effective insecticide because in insects, in contrast to vertebrates, nicotinic AChRs are the predominant neuronal transmitter receptor. However, because this review is primarily concerned with molecular studies of neuronal nicotinic AChRs, and the AChR of invertebrates (with the exception of locust) are poorly understood at the molecular level, these systems will be reviewed only briefly.

Aplysia neurons have three different AChRs, one that results in an increased sodium ion conductance (not blocked by αBgt), one that results in an increased potassium ion conductance (not blocked by αBgt), and a third that results in an increased chloride ion conductance (blocked by αBgt) (Kehoe et al., 1976; Ono and Salvaterra, 1981).

Electrophysiological techniques have demonstrated the existence of a nicotinic AChR in cockroach giant interneurons. Depolarization in response to ACh could be blocked by αBgt (Sattelle et al., 1983).

Recently, the techniques of protein biochemistry and molecular biology have been utilized to demonstrate the presence of functional AChRs in locust neurons. Frog oocytes injected with mRNA from insect tissue expressed functional nicotinic AChRs on their surface (Breer and Benke, 1986). AChR purified from locust nerve tissue using an αBgt affinity column consisted of a single 60,000 mw subunit (Breer et al., 1985; *see below*). This was reconstituted into lipid bilayers and shown to exhibit ACh-gated ion channels which were blocked by αBgt (Hanke and Breer, 1986, 1987). The single channel properties appeared to be very similar to those of muscle AChRs. It is possible that this AChR is a homopolymer homologous to the primordial AChR from which other members of the family evolved.

In the frog sympathetic ganglion and optic tectum there are ACh-gated ion channels that are apparently blocked by αBgt (Marshall, 1981; Freeman et al., 1980). Marshall (1981) located both bound αBgt and antibodies to *Torpedo* AChRs to patches directly beneath synaptic boutons. AChRs are also found in Renshaw cells of the frog spinal cord, but these are not blocked by αBgt (Miledi and Szczepaniak, 1975). Retinotectal synaptic transmission in the goldfish and toad was thought to be nicotinic in nature (Freeman et al., 1980), but recent evidence suggests that this may not in fact be the case (Langdon and Freeman, 1987).

Chick sympathetic ganglia have both AChRs and αBgt-binding sites (Greene et al., 1973), but αBgt fails to block AChR function (Carbonetto et al., 1978; Kouvelas et al., 1978). Similarly, chick ciliary ganglia have both AChRs and αBgt-binding sites, and again αBgt fails to block AChR function (Chiappinelli and Zigmond, 1978; Ravdin and Berg, 1979). However, another toxin from *Bungarus multicinctus* venom, variously known as 3.1 toxin, kappa Bgt, or toxin F, and for the purposes of this review termed "neuronal Bgt," has been shown to block the function of these AChRs (Ravdin and Berg, 1979; Chiappinelli, 1983; Loring et al., 1984). The function of chick ciliary ganglia AChRs is also blocked by affinity labeling with BAC after reduction with DTT, indicating that cysteine residues homologous to Cys 192, 193 of *Torpedo* AChR α subunits are conserved (Stollberg et al., 1986). As will be evident below, chick ciliary ganglia have become a model system for studying neuronal nicotinic AChRs.

Another model system for studying neuronal nicotinic AChRs is the rat pheochromocytoma cell line PC12 (Greene and Tischler, 1976). Like sympathetic neurons, this cell line responds to βNGF by extension of neuron-like processes. These cells have functional nicotinic AChRs that are induced by culture of cells in βNGF (Dichter et al., 1977). PC12 cells also have αBgt-binding sites, but αBgt fails to block AChR function (Patrick and Stallcup, 1977a,b). However, polyclonal antisera to AChRs purified from *Electrophorus* electric organ did block PC12 AChR function (Patrick and Stallcup, 1977b), which was perhaps the first evidence that electric organ and neuronal AChRs were structurally related and that antibodies would be useful probes for neuronal AChRs. Further evidence for structural homology was the observation that AChRs on PC12 cells could be affinity labeled with MBTA or BAC after reduction with DTT, indicating that, like the AChRs of chick ciliary ganglia, PC12 AChRs also have conserved cysteine residues homologous to Cys 192, 193 of *Torpedo* AChR α subunits (Leprince, 1983). Activation of this AChR causes release of catecholamines (Mizobe and Livett, 1983), enkephalins (Eiden et al., 1984), and dopamine (Baizer and Weiner, 1985) from PC12 cells, suggesting a role for these AChRs on similar cells in vivo. More recent studies have shown that nicotinic AChR stimulation leads to transcription of the *c-fos* proto-oncogene and one or more actin genes (Greenberg et al., 1986) and the proenkephalin A gene (Kley et al., 1987), suggesting an important role for these neuronal AChRs in the function of this "model" nerve cell.

In a systematic pharmacological study, Curtis and Ryall (1966) demonstrated the existence of nicotinic AChRs in the Renshaw cells of the cat spinal cord. Later it was demonstrated that αBgt failed to block the excitation of these cells by ACh (Duggan et al., 1976). Other studies have also utilized electrophysiological techniques to demonstrate the existence of nicotinic AChRs in various regions of the mammalian brain, including the brain stem (Bradley and Dray, 1972), interpeduncular nucleus (Brown et al., 1983), substantia nigra (Clarke et al., 1985b), hypothalamus (Cobbett et al., 1986), locus ceruleus (Egan and North, 1986), medial habenula (McCormick and Prince, 1987), and the hippocampus and brainstem (Aracova et al., 1987). Again, where tested, αBgt failed to block any response to applied ACh.

Another approach to studying the function of nicotinic AChRs in the central nervous system is perfusion of either brain slices or purified synaptosomes. Gioguieff-Chesselet et al. (1979) demonstrated nicotine-evoked release of [^{3}H]dopamine from rat brain striatal slices. Similarly, Yoshida et al. (1980) demonstrated nicotine-evoked release of [^{3}H]noradrenaline from hypothalamic synaptosomes, and Wonnacott and coworkers demonstrated nicotine-evoked release of [^{3}H]dopamine (Mills and Wonnacott, 1984) and [^{3}H]GABA (Wonnacott et al., 1987) from hippocampal synaptosomes. These perfusion studies all suggest the presence of presynaptic nicotinic AChRs that modulate the release of other neurotransmitters from nerve terminals. This assay system readily lends itself to the study of the function of the central nicotinic AChRs in their native membranes, and should prove useful in the future.

There is evidence for the presence of functional nicotinic AChRs on retinal ganglion cells (Ikeda and Sheardown, 1982; Lipton et al., 1987). Electrophysiological studies of isolated rat retinal ganglion cells demonstrated that ACh and nicotine evoked depolarization that was blocked by curare, but not by aBgt or atropine (Lipton et al., 1987). This study indicated that the single-channel properties of these neuronal AChRs are very similar to those of muscle AChRs, suggesting that basic properties of the ion channel have been conserved. As is discussed below, the function of these neuronal nicotinic AChRs, like muscle AChRs, is blocked by the noncompetitive channel blocker

histrionicotoxin (Rapier et al., 1987), further indicating structural and functional homologies between muscle and nerve AChRs. More single-channel studies are required, however, to precisely define the electrophysiological properties of these neuronal-type AChRs. Such studies are difficult *in situ*, and perhaps the most detailed studies will be done in the future using the techniques of molecular biology, to express neuronal AChR cDNAs in oocytes and/or cell lines to form functional AChRs.

Acetylcholine and Nicotine as Probes for Neuronal Nicotinic Receptors

Initial ligand binding studies investigated the binding of the muscarinic agonist [^{3}H]muscarine to aqueous preparations of housefly heads (Eldefrawi and O'Brien, 1973). This binding site was proposed to be a 'mixed' site, i.e., both nicotinic and muscarinic. It did not bind αBgt (Mansour et al., 1977). The nature of this site was obscure, especially because it was located in the soluble fraction, rather than the membrane fraction, where AChRs are expected. Later studies detected an αBgt-binding site in membrane preparations of insect nervous tissue, and more recently this αBgt-binding protein has been purified and characterized (*see below*). As discussed above, the electrophysiological studies carried out on insect neurons and upon purified, reconstituted locust αBgt-binding proteins suggest that the αBgt-binding protein is a functional nicotinic AChR in insect neurons.

As will be discussed below, [^{125}I]αBgt has been the radioligand most extensively used to study goldfish brain AChRs. Recently, however, Henley and Oswald (1987) have demonstrated the existence of a high affinity [^{3}H]nicotine-binding site (K_D = 3 n*M*) in goldfish brain. Interestingly, data suggested that two subtypes of nicotine-binding site exist, only one of which binds αBgt. This binding may be an evolutionary intermediate between the αBgt-binding protein and the [^{3}H]nicotine-binding protein found in avian and mammalian brain.

There have been fewer studies investigating the binding of [^{3}H]nicotine and [^{3}H]ACh in avian brains. Schneider and coworkers (1985) demonstrated the existence of a high affinity [^{3}H]ACh-binding protein in chick optic lobe that could be physically separated from the [^{125}I]αBgt-binding protein in this tissue. Similarly, in the chicken brain, the high affinity [^{3}H]nicotine-binding site and [^{125}I]αBgt-binding site are located on different proteins (Whiting and Lindstrom, 1986b, *see below*).

Studies reporting the binding of [^{3}H]nicotine to mammalian brain preparations were first carried out over ten years ago (Schiefer and Eldefrawi, 1974). Since 1980, when high specific activity [^{3}H]nicotine became commercially available, there has been a plethora of detailed studies describing binding of [^{3}H]nicotine to sites in brain. This has been recently reviewed in detail by Wonnacott (1987). Most binding studies have been carried out on rodent brain membrane preparations (*see* Romano and Goldstein, 1980; Marks and Collins, 1982; Yamada et al., 1985; Martino-Barrows and Kellar, 1987; Lippiello et al., 1987) and indicate saturable, high affinity binding (K_D values of 2–60 n*M*) to one or more sites. Differences in binding affinities probably reflect different protocols for membrane preparation and binding assays. The inclusion of protease inhibitors in membrane preparations changes binding plots from a curve to a straight line (Lippiello and Fernandes, 1986), suggesting that proteolytic nicking of AChRs may account for some of the multiple binding sites previously observed. Similar K_D values for [^{3}H]nicotine-binding have been observed to binding sites detergent solubilized from brain tissue (Sugiyama et al., 1986; Whiting and Lindstrom,

1986b). The binding of [^{3}H]nicotine to brain is stereospecific; (–)nicotine is 12–60 times stronger in competing for binding with the radioligand than is (+)nicotine. The pharmacology of the [^{3}H]nicotine-binding sites characteristically have relatively higher affinities for cholinergic agonists (cytisine, ACh, carbamylcholine, lobeline, anatoxin-a) and lower affinities for antagonists (*d*-tubocurare, hexamethonium, mecamylamine). At μ*M* concentrations, αBgt does not inhibit [^{3}H]nicotine-binding .

Recent studies have demonstrated the existence of [^{3}H]nicotine-binding sites in bovine and human brain (Shimahama et al., 1985; Flynn and Mash, 1986; Whiting et al., 1987c; Whiting and Lindstrom, 1988). These sites have essentially the same pharmacology as those found in rodent brain.

[^{3}H]ACh has also been used to investigate nicotinic AChRs in the rat brain (Schwartz et al., 1982). This radioligand has been less extensively used than [^{3}H]nicotine because of its labile nature. Pharmacological studies (Schwartz et al., 1982; Martino-Barrows and Kellar, 1987), autoradiographic studies (*see below*), and biochemical studies (*see below*) all indicate that the site recognized by [^{3}H]nicotine and [^{3}H]ACh in the brain are the same.

One of the more perplexing aspects that has arisen during the study of these brain AChRs is the discrepancy between the pharmacological and the electrophysiological data. Why do antagonists such as hexamethonium and mecamylamine have such low affinity as competitive inhibitors (m*M*) when μ*M* concentrations are sufficient to block the ACh response (*see*, e.g., Brown et al., 1983; Egan and North, 1986; Lipton et al., 1986; and McCormick and Prince, 1987)? The answer may be that many antagonists also act as noncompetitive channel blockers. Additionally, why do agonists have n*M* affinities for the AChR when μ*M* concentrations are required for their activation? It may be that a high affinity desensitized state of the AChR is observed, similar to the phenomenon observed in *Torpedo* electric organ, where prolonged exposure to agonists causes conversion of AChRs to a high affinity closed channel form (*see* Popot and Changeux, 1984).

Radioligand binding has also been used to investigate the regional distribution of putative nicotinic AChRs. Some studies have determined the binding of [^{3}H]nicotine to dissected regions of brain (*see* Marks and Collins, 1982; Yamada et al., 1985; Whiting and Lindstrom, 1986b; and Martino-Barrows and Kellar, 1987). There is some variation between these studies, probably reflecting the problems of accurately dissecting out brain areas. Much more accurate location of [^{3}H]nicotine and [^{3}H]ACh-binding sites in brain was achieved by labeling of brain thin sections and subsequent autoradiography (Clarke et al., 1985b; London et al., 1985). This technique allowed detailed analysis of the anatomical distribution of radioligand binding sites. The most intense labeling was observed in the interpeduncular nucleus, superior colliculus, medial habenula, and the thalamus. Additionally, Clarke et al. (1985b) demonstrated that the distribution of [^{3}H]nicotine- and [^{3}H]ACh-binding sites was essentially identical, and very different from the distribution of [^{125}I]αBgt-binding sites. This was, perhaps, the first convincing evidence demonstrating that the high affinity agonist site in brain is different from the αBgt-binding site. More recently, mAbs to AChRs proved even more useful tools for determining the distribution of these nicotinic AChRs in both the avian and mammalian central nervous system (Swanson et al., 1987).

As is discussed in more detail below, biochemical studies have demonstrated that the rodent brain [^{3}H]nicotine-binding site and [^{125}I]αBgt-binding site are on different proteins (Whiting and Lindstrom, 1986a,b; 1987b; Wonnacott, 1986).

Attempts to demonstrate high affinity binding of [^{3}H]ACh (Kemp and Morley, 1986) or

[^{3}H]nicotine (Whiting et al., 1987) to PC12 cells have failed, indicating that the AChRs on these cells do not exhibit high affinity ligand binding. AChRs on chick ciliary ganglia also exhibit only μM affinity for ACh and nicotine, as determined by inhibition of [^{125}I]neuronal Bgt-binding to these cells (Halvorsen and Berg, 1986). Thus, these ganglionic-type nicotinic AChRs are clearly somewhat different from the central AChRs, which exhibit high affinity binding for both nicotine and ACh. The limited structural data available for ganglionic-type AChRs also suggests that they have a similar, but obviously distinct, structure from brain AChRs, as will be discussed in more detail below.

αBungarotoxin as a Probe for Neuronal Nicotinic Receptors

As has been discussed above, there is very good evidence the αBgt binds to the physiologically functional nicotinic AChR of the insect nervous system. In both *Drosophila* (Ordai, 1978) and locust (Filbin et al., 1983; Breer et al., 1984), the αBgt-binding protein exhibits a nicotinic cholinergic pharmacology with nM affinity for [^{125}I]αBgt and μM affinity for nicotine and ACh. Structural characterization has been limited to the locust αBgt-binding protein, and the results have been somewhat controversial. Lunt and colleagues (Filbin et al., 1983) affinity purified the AChR from locust brain using αBgt coupled to Sepharose and found three major polypeptides of apparent mws 60,000, 41,000, and 25,000. The largest of these polypeptides was affinity labeled with [^{3}H]MBTA after reduction with DTT, indicating that it contains the ACh-binding site and residues homologous to cysteines 192, 193 of the α subunit of AChRs from electric organ. Breer and his coworkers (1985) have also reported the purification and structural characterization of the AChR from locust brain. The native macromolecule had a sedimentation constant of about 10S, about the same size as the αBgt-binding proteins and AChRs of the vertebrate nervous system. SDS-PAGE analysis of the αBgt-binding protein affinity purified upon αBgt-Sepharose revealed only a single polypeptide, apparent mw 65,000 that on western blots was bound by a mAb to AChRs from electric organ, indicating conservation of an antigenic determinant. These data led Breer and his coworkers to propose that the insect αBgt-binding protein is an oligomeric structure composed of four similar or identical subunits. The conflicting data of Lunt and coworkers and Breer and his colleagues regarding the subunit structure of the insect αBgt-binding component might be explained by proteolytic degradation of the protein during the purification procedure. It is possible that the smaller 41,000 and 25,000 apparent mw subunits observed by Lunt and colleagues are proteolytic products of the 60,000 apparent mw subunit. Alternatively, the 41,000 and 25,000 mw proteins could be contaminants. Breer and his colleagues (Hanke and Breer, 1986) have been able to reconstitute purified locust αBgt-binding protein into lipid bilayers and obtain functional ACh-regulated ion channels, arguing that the subunit pattern they observe is correct. Nevertheless, it should be noted that when *Torpedo* electric organ AChR is proteolytically degraded to appear as only a single polypeptide by SDS-PAGE analysis, it can still act as a functional ACh-regulated cation channel (Lindstrom et al., 1980). Breer and his colleagues have also detected functional AChRs and immunologically identified a 60,000 mw band in oocytes injected with locust ganglion mRNA (Breer and Benke, 1986). Expression of AChRs in oocytes injected with cloned mRNA for the 60,000 mw protein, which are equivalent in function to those in native membranes, would be unequivocal proof that the AChR consisted of only a single kind of subunit.

The goldfish brain contains sites with high affinity (n*M*) for [^{125}I]αBgt and a nicotinic cholinergic pharmacology (Oswald and Freeman, 1979; Henley and Oswald, 1987). The αBgt-binding protein has a sedimentation constant of 11.45 S (Oswald and Freeman, 1979) and is thus very similar in size to the αBgt-binding protein and AChR of the mammalian and avian nervous system. As will be discussed in more detail below, recent studies have demonstrated that several mAbs prepared to AChRs from electric organ exhibit significant cross-reactivity with the αBgt-binding protein from goldfish brain. These mAbs have proven to be valuable tools in the study of the structure and synthesis of this protein (Henley et al., 1986a,b).

High concentrations of [^{125}I]αBgt-binding sites in the chick optic lobe were first found by Wang and Schmidt (1976). As in the mammalian brain, the chicken brain (Wang et al., 1978; Schneider et al., 1985) and chicken retina (Betz, 1981), the αBgt-binding protein has a nicotinic cholinergic pharmacology with n*M* affinity for αBgt and μ*M* affinity for agonists ACh and nicotine. Sucrose gradient centrifugation analyses indicated that the αBgt-binding protein from chick retina (Betz, 1981) and optic lobe (Norman et al., 1982; Conti-Tronconi et al., 1985) is about 10–11 S in size and, thus, of similar size or slightly larger than the AChRs from electrtic organ, muscle, and brain.

As with the αBgt-binding protein from rodent brain, antibodies prepared to AChRs from electric organ and muscle have been used in an attempt to demonstrate structural homologies between electric organ and muscle AChRs and the brain αBgt-binding protein. Betz (1981) demonstrated immunoprecipitation of the detergent-solubilized [^{125}I]αBgt-binding sites from chick retina by antisera to electric organ AChR. Barnard and coworkers (Norman et al., 1982) were able to demonstrate slight cross-reactivity of an antiserum to cat muscle AChRs with the chicken brain and optic lobe αBgt-binding protein, but found no cross-reactivity of antisera prepared to chick muscle AChR or electric organ AChR. In later studies (Mehraban et al., 1984; Conti-Tronconi et al., 1985), it was reported that a mAb raised to AChRs from chick muscle immunoprecipitated the αBgt-binding protein from chick optic lobe. We have tested the binding of antisera and over 100 mAbs prepared to both native and denatured subunits of electric organ and muscle AChR and were unable to demonstrate any cross-reactivity with [^{125}I]αBgt-binding protein from chicken brain (Whiting and Lindstrom, 1986a,b, and unpublished results). Together, these data indicate that there is very limited antigenic homology between electric organ and muscle AChRs and the neuronal αBgt-binding protein.

The first structural studies of the avian αBgt-binding protein investigated the polypeptide species that could be covalently crosslinked to [^{125}I]αBgt (Betz et al., 1982). They identified a major labeled component of apparent mw 55,000 (after subtraction of mw 8000 for one covalently attached αBgt molecule), together with several other minor components. Subsequent attempts by Betz and coworkers to affinity purify the αBgt-binding protein from chick optic lobe using αBgt-Sepharose were not very successful, but suggested a major polypeptide of 57,000 mw (Betz and Pfeiffer, 1984). The structure of the αBgt-binding protein from chick optic lobe has been investigated by Barnard and coworkers (Norman et al., 1982). A single polypeptide was identified, of an apparent mw 54,000. This polypeptide could be specifically labeled with [^{3}H]BAC after reduction with DTT, which indicates that amino acid residues homologous to cysteines 192, 193 of α subunits from electric organ AChR have been conserved. In a subsequent study by Barnard and coworkers (Conti-Tronconi et al., 1985), three to four other polypeptides were visualized by SDS-PAGE of apparent mws 48,000–72,000. The amino-

terminal amino acid sequence of the 48,000 mw subunit was found to show considerable homology to subunits of AChRs from electric organ and muscle. This was irrefutable evidence that the αBgt-binding protein of brain is a member of the nicotinic AChR gene family.

[^{125}I]αBgt was the first probe used to investigate putative nicotinic AChRs in the vertebrate nervous system, primarily because this ligand was relatively conveniently radiolabeled, and also because it was assumed that if central nicotinic AChRs were homologous to muscle AChRs, then they should bind αBgt. However, as discussed above, there is now a large body of evidence suggesting that in avian and mammalian nervous systems the αBgt-binding component is not a functional AChR. There have been many studies of the pharmacology of this binding site, and these have been reviewed extensively elsewhere (Oswald and Freeman, 1980; Morley and Kemp, 1981; Chiappinelli, 1985) and will only be discussed briefly here.

The αBgt-binding protein of rodents (Marks and Collins, 1982; Lukas, 1986; Marks et al., 1986; Meeker et al., 1986) has a nicotinic cholinergic pharmacology, with n*M* affinity for αBgt, and μ*M* affinity for agonists ACh and nicotine. This was in obvious contrast to the "agonist" binding site, which, as discussed above, exhibits n*M* binding for ACh and nicotine but does not bind αBgt. As has been discussed earlier, the distribution of [^{125}I]αBgt-binding sites in brain is clearly different from the distribution of [^{3}H]nicotine-binding sites and the αBgtbinding protein is a different protein from the [^{3}H]nicotine-binding protein. Polyclonal antisera to both electric organ and muscle AChRs have been used as probes to investigate antigenic determinants shared with the αBgt-binding protein from rat brain. Block and Billiar (1979) reported that an antisera to electric organ AChR immunoprecipitated [^{125}I]αBgt-binding sites from detergent extracts of rat brain, but the cross-reaction was barely detectable. In contrast, both Morley et al. (1983) and Mills and Wonnacott (1984) reported that antisera to electric organ AChR did not immunoprecipitate the [^{125}I]αBgt-binding protein from rat brain extracts. The latter authors did, however, demonstrate immunoprecipitation of [^{125}I]αBgt labeled protein from rat brain extracts by an antiserum to rat muscle AChR. These studies suggest that there is probably limited antigenic homology, and thus limited structural/amino acid sequence homology, between electric organ and muscle AChRs and the αBgt-binding protein from brain.

Several groups have attempted to purify and characterize the αBgt-binding protein. An early attempt to purify the αBgt-binding protein from mouse brain by affinity chromatography upon an α toxin purified from *Naja naja kaouthia* coupled to Sepharose was reported by Seto and coworkers (1981). They observed a single polypeptide by SDS polyacrylamide gel electrophoresis, apparent mw 52,000. However, these workers detergent-solubilized the protein from whole brain rather than membrane preparations and, thus, proteolysis was probably not controlled. In a more recent study, where proteolysis was more adequately controlled, Kemp and coworkers (1985) affinity purified the αBgt-binding protein from rat brain upon α-toxin coupled to Sepharose. The purified protein consisted of three polypeptides, apparent mws 49,000, 53,500 and 55,000. The largest of these polypeptides could be labeled with [^{3}H]MBTA after reduction with DTT. This confirmed the previous observations (Lukas and Bennett, 1980) that affinity labeling of rat brain membranes with MBTA and BAC after reduction with DTT resulted in inhibition of [^{125}I]αBgt-binding (*see below*). It further suggests that this polypeptide contains amino acid residues homologous to cysteines 192, 193 of the α subunit of *Torpedo* electric organ AChR. We have recently purified the αBgt-binding protein from rat brain by affinity chromatography

on αBgt-Sepharose and identified four polypeptides, apparent mws 44,700, 52,300, 56,000, and 65,200 (Whiting and Lindstrom, 1987b). Sucrose gradient centrifugation analysis of the detergent-solubilized native αBgt-binding protein indicated that it was about 10 S in size, slightly larger than electric organ AChR monomers. Clearly, more data is required before the structure of the mammalian αBgt-binding protein is finally resolved.

There have been fewer studies conducted on the nature of αBgt-binding protein of ganglion cells, but the data available suggest that it is very similar or identical to the αBgt-binding protein in the central nervous system. The αBgt-binding proteins of chick sympathetic neurons (Greene et al., 1973), rat PC12 cells (Patrick and Stallcup, 1977a; Lukas, 1986), chick ciliary ganglion neurons (Messing and Kim, 1981), and bovine chromaffin cells (Quik et al., 1986) have a pharmacology essentially identical to that of the central αBgt-binding protein, with affinities for [^{125}I]αBgt in the n*M* range, and affinities for nicotine and ACh in the μ*M* range.

The αBgt-binding sites on chick ciliary ganglion neurons have been found to be primarily restricted to the extrasynaptic cell processes (Jacob and Berg, 1983; Loring et al., 1985). Little or no αBgt was bound at synapses, supporting the argument that this αBgt-binding protein is not a neuronal nicotinic AChR.

Structural studies on these ganglionic αBgt-binding proteins have been limited. The αBgt-binding protein from PC12 cell sediments at 10.5 S (Patrick and Stallcup, 1977a) and is thus essentially the same size as the αBgt-binding protein from brain. Similarly, the cross-reactivity of antisera to AChRs from electric organ with the ganglionic αBgt-binding component is minimal (Lukas, 1986) or undetectable (Patrick and Stallcup, 1977b). Additionally, the αBgt-binding protein of PC12 cells can be affinity labeled by MBTA and BAC after reduction with DTT (Leprince, 1983) as will be discussed more fully below, suggesting that, like αBgt-binding proteins in the central nervous system, they have amino acid residues homologous to cysteines 192, 193 of the α subunits of AChRs from electric organ.

Affinity Labels as Probes for Neuronal Nicotinic Receptors

As discussed in the Introduction, the affinity labels MBTA and BAC have proven to be useful probes for the ACh-binding site of muscle and electric organ AChRs (Damle et al., 1978; Damle and Karlin, 1978; Kao et al., 1984; Kao and Karlin, 1986). After reduction of a disulfide bond between cysteines 192 and 193 of the α subunits of AChRs from *Torpedo*, MBTA specifically labels cysteine 192 and, to a lesser degree, 193. One of the two α subunits in this AChR is easier to reduce and alkylate than the other, probably as a result of different conformations of the two α subunits; but alkylation of only one is sufficient to block AChR function, because activation depends on simultaneous liganding of both ACh-binding sites in the AChR. Similar approaches have been taken to study both neuronal AChRs and αBgt-binding proteins, and, as will be reviewed, they have allowed determination of the ACh-binding subunit of these proteins, and demonstrated that cysteines homologous to cysteines 192, 193 of the α subunits of electric organ and muscle are conserved in these neuronal proteins.

Lukas and Bennett (1980) demonstrated that both MBTA and BAC were equally effective in blocking [^{125}I]αBgt-binding to rat brain membranes.

Leprince (1983) measured the inhibition of carbachol-stimulated $^{86}Rb^+$ influx to investigate affinity labeling of the PC12 AChR by MBTA and BAC. Again, total inhibition of $^{86}Rb^+$ influx could be achieved, but lower concentrations of MBTA than BAC were required.

We have investigated affinity labeling of immuno-isolated brain AChRs with MBTA and BAC by measuring the inhibition of [^{3}H]nicotine binding. For AChRs from brains of chickens (Whiting and Lindstrom, 1986b, 1987a), rats (Whiting and Lindstrom, 1987b), cattle, and humans (Whiting and Lindstrom, unpublished data), MBTA and BAC could both completely block [^{3}H]nicotine-binding, but much lower concentrations of BAC than MBTA were required.

It is interesting to note the different efficacies of the affinity labeling reagents on different molecules; for the αBgt-binding protein, MBTA and BAC are equally effective; for ganglionic AChRs, MBTA is the more effective affinity label; and for brain AChRs, BAC is more effective. These results are consistent with other pharmacological differences between AChRs from brain and ganglia. BAC is an agonist, whereas MBTA is an antagonist; brain AChRs have much higher affinities for agonists than antagonists, whereas ganglionic AChRs have much lower affinities for agonists, as discussed above.

It is also interesting to note that affinity labeling of the brain αBgt-binding protein, ganglionic AChR, or brain AChR gives a monophasic concentration curve, indicating either labeling at a single site, or labeling at more than one site with the same affinity. This is in contrast to affinity labeling of *Torpedo* electric organ or muscle AChRs, where the concentration dependence is biphasic (Wolosin et al., 1980; Ratnam et al., 1986a), indicating that one of the two ACh-binding sites per AChR monomer is more susceptible to affinity labeling. If true, this could indicate that the ACh-binding subunits in neuronal AChRs have a more symmetric environment than the ACh-binding subunits in muscle-type AChRs, one of which is bordered by β and γ subunits, and the other of which is bordered by β and δ subunits (Kubalek et al., 1987).

Neuronal Bungarotoxin as a Probe for Neuronal Nicotinic Receptors

Neuronal Bgt, variously referred to as kappa Bgt (Chiappinelli and Zigmond, 1978), 3.1 toxin (Ravdin and Berg, 1979), or Toxin F (Loring et al., 1984), is a minor component of the venom from *Bungarus multicinctus* which, in contrast to αBgt, specifically blocks the functional nicotinic AChRs on chick ciliary ganglia at concentrations as low as 10 n*M*. Neuronal Bgt may be a trace contaminant of αBgt preparations and cause spurious inhibition of neuronal AChR function when αBgt preparations are used at high concentrations. The amino acid sequence of neuronal Bgt has been determined (Grant and Chiappinelli, 1985; Loring et al., 1986) and found to exhibit considerable sequence homology to αBgt. Under physiological conditions the toxin appears to exist as a dimer (Chiappinelli, 1983; Loring et al., 1984; Halvorsen and Berg, 1986). [^{125}I]neuronal Bgt bound to the AChR of chick ciliary ganglia with high affinity (K_D = 5–6 n*M*) and was displaced by μ*M* concentrations of ACh and nicotine, but not by αBgt, which is consistent with the physiological properties of this AChR (Halvorsen and Berg, 1986). In addition, [^{125}I]-neuronal Bgt binding is inhibited by pretreatment of ganglia with BAC after reduction with DTT (Chiappinelli, 1983). This is consistent with our observations that treatment of ganglia with BAC after reduction with DTT specifically and irreversibly inhibits AChR function (Stollberg et al., 1986). Using [^{125}I]neuronal Bgt, Loring and Zigmond (1987) localized a large proportion of the neuronal Bgt-binding sites to synaptic regions, as would be predicted if this toxin were a probe for a functional nicotinic AChR, and is in obvious contrast to the extrasynaptic location of the αBgt-binding protein (Jacob and Berg, 1983; Loring et al., 1985).

Approaches using neuronal Bgt as a probe for neuronal AChRs have been complemented by approaches using mAbs, and will be discussed in detail below. Additionally, recent studies have extended the use of neuronal Bgt to bovine chromaffin cells. Higgins and Berg (1987) demonstrated that this toxin blocked nicotine-induced catecholamine release from cultured bovine chromaffin cells. Thus, neuronal Bgt is a useful probe for characterization of mammalian ganglionic-type nicotinic AChRs.

Other Toxins as Probes for Neuronal Nicotinic Receptors

Several other naturally occurring components from both animal and plant sources have been shown to be effective in blocking neuronal nicotinic AChR function.

In addition to neuronal Bgt, several other centrally acting snake toxins have been identified. Three toxins, Bgt II-S1 (Quik and Lamarca, 1982), P-4 Bgt (Saiani et al., 1984), and P15 toxin (Gotti et al., 1985) have been purified from *Bungaricus multicinctus* venom and shown to inhibit nicotinic AChR function in mammalian ganglionic neurons at n*M* concentrations. These three toxins, which are probably identical, have phospholipase A activity. Their mechanism of action is not clear, but both direct AChR blockade (Gotti et al., 1985) and more subtle mechanisms involving phospholipase A activity (Quik, 1987) may be important.

Neosurugatoxin, isolated from the Japanese ivory mollusk, is an antagonist with high affinity (IC_{50} = 70 n*M*) for the [^{3}H]nicotine-binding site of rat brain (Yamada et al., 1985). It does not inhibit binding of [^{125}I]αBgt to brain or muscle (Rapier et al., 1985). At low concentrations (50 n*M*), this toxin specifically inhibited the dimethylphenyl piperazinium (a nicotinic agonist)-evoked dopamine release from rat striatal synaptosomes, strongly suggesting that the [^{3}H]nicotine-binding site of rat brain is a functional AChR, and that neosurugatoxin may prove to be a useful high affinity antagonist for these central AChRs.

Anatoxin-a, isolated from the blue-green algae *Anabaena flosaquae,* is a very potent agonist at AChRs of muscle (Swanson et al., 1986) which rapidly causes desensitization. It has recently been shown also to bind with high (n*M*) affinity to the [^{3}H]nicotine-binding site of rat brain (Zhang et al., 1987). Anatoxin-a is a potent inhibitor of [^{3}H]nicotine-binding to AChRs immuno-isolated from rat brain (Whiting, Lindstrom, and Albuquerque, unpublished).

Lophotoxin, isolated from gorgonian coral, is an unusual, slow-acting, irreversible inhibitor of AChRs from muscle that lacks an amine group and lacks obvious structural homology with ACh, yet blocks αBgt-binding (Culver et al., 1984). Recently it has been shown that lophotoxin also blocks nicotinic AChRs of chick and rat autonomic ganglia (Sorenson et al., 1987).

The properties of both anatoxin-a and lophotoxin clearly demonstrate that many of the features of the ACh-binding site of muscle AChR are conserved in the ACh-binding sites of both the central and ganglionic nicotinic AChRs.

Wonnacott and coworkers (1987) have used the frog toxin histrionicotoxin to show similarities between the cation channels of muscle and neuronal AChRs. Histrionicotoxin is a noncompetitive inhibitor that effectively blocks the ion channel of skeletal muscle AChRs (Albuquerque et al., 1973). Similarly, histrionicotoxin does not inhibit [^{3}H]nicotine-binding in rat brain, but does cause a 50% inhibition of nicotine-evoked release of dopamine from striatal synaptosomes at a concentration of 5 μ*M*, a value very similar to that obtained in frog sartorius muscle. These pharmacological data are consistent with the limited electrophysiological data available (Lipton et al., 1986; Aracova et al.) in suggesting that central nicotinic AChRs have cation channel properties similar to those of muscle-type AChRs

Monoclonal Antibodies as Probes for Neuronal Nicotinic Receptors

In the hope that structural homology between muscle-type AChRs of fish electric organs and vertebrate neuronal AChRs might be detectable with mAbs, and that these mAbs might provide better biochemical probes for these AChRs than the available toxins and small ligands, libraries of mAbs to muscle-type AChRs were screened for binding to brains of several species. Putative AChRs in the central nervous systems of locusts (Breer et al., 1985), goldfish (Henley et al., 1986a,b), frogs (Sargent and Lindstrom, unpublished), and chickens (Swanson et al., 1983) were found to crossreact with some of these mAbs. Crossreaction was also detected in ganglia from chickens (Smith et al., 1985, 1986) and frogs (Sargent and Lindstrom, unpublished).

Three of seventeen mAbs to AChRs from *Torpedo* electric organ were found to bind to AChRs from locusts by ELISA assays (Fels et al., 1983). Two of these mAbs competed with αBgt and small cholinergic ligands for binding to the AChR, while the third reacted outside the ACh-binding site. Antisera to AChRs purified from locusts were used to localize them immunohistochemically (Breer et al., 1985) and to detect only the single 65,000 mw polypeptide expected from oocytes injected with locust mRNA (Breer and Benke, 1986). The single polypeptide in purified locust AChR crossreacted with a mAb to *Torpedo* AChR (Breer et al., 1985).

Five mAbs to AChR were found to label neuronal tissue in *Drosophila* (Chase et al., 1987). Four of these mAbs were specific for α subunits (two for the MIR) and one was specific for β. A different pattern of sites was labeled by the MIR mAbs, and each of the other mAbs; thus, at least four different proteins were crossreacting. None of these appeared to bind αBgt. It is possible that some or all crossreactivity observed was spurious and low affinity. Alternatively, various members of the AChR superfamily may have crossreacted. It is especially curious that none appeared to bind αBgt, since an αBgt-binding protein is present, and in locusts (Fels et al., 1983) some anti-*Torpedo* AChR mAbs crossreact with the AChR that in that species binds αBgt (Breer et al., 1985). It should be remembered that locusts and fruit flies are actually quite distantly related, so that flies might have an αBgt-binding or nicotine-binding protein that is not an AChR, whereas locusts might have AChRs that bind αBgt. It is also important to note that when crossreactions with mAbs or crosshybridizations with cDNAs are detected, the burden of proof for establishing the significance of the observation is always on the observer.

Six of 112 mAbs to AChRs from electric organ were found to bind to an αBgt-binding AChR from goldfish brain (Henley et al., 1986a,b). Synthesis of this AChR in the retina and transport along the axons of retinal ganglion cells to presynaptic sites in the optic tectum was demonstrated by injection of [^{35}S]methionine in the eye, followed by immune precipitation from detergent extracts of the optic tectum with mAb 47 to the MIR of AChRs from *Electrophorus* (Henley et al., 1986b). The AChR bound by mAb 47 binds both αBgt and nicotine with n*M* affinity (Henley and Oswald, 1987). Goldfish brain also contains a protein bound by mAb 172 which binds nicotine with n*M* affinity, but does not bind αBgt (Henley, Lindstrom, and Oswald, unpublished). Thus, there appear to be subtypes of neuronal AChRs in goldfish brain with limited structural homology to muscle-type AChRs; these are distinguishable pharmacologically and immunologically; and at least some are presynaptic.

Twenty-eight of 38 mAbs to AChRs from electric organ were also found to bind to putative AChRs in frog optic tectum (Sargent et al., in preparation). All of the 38 mAbs bound

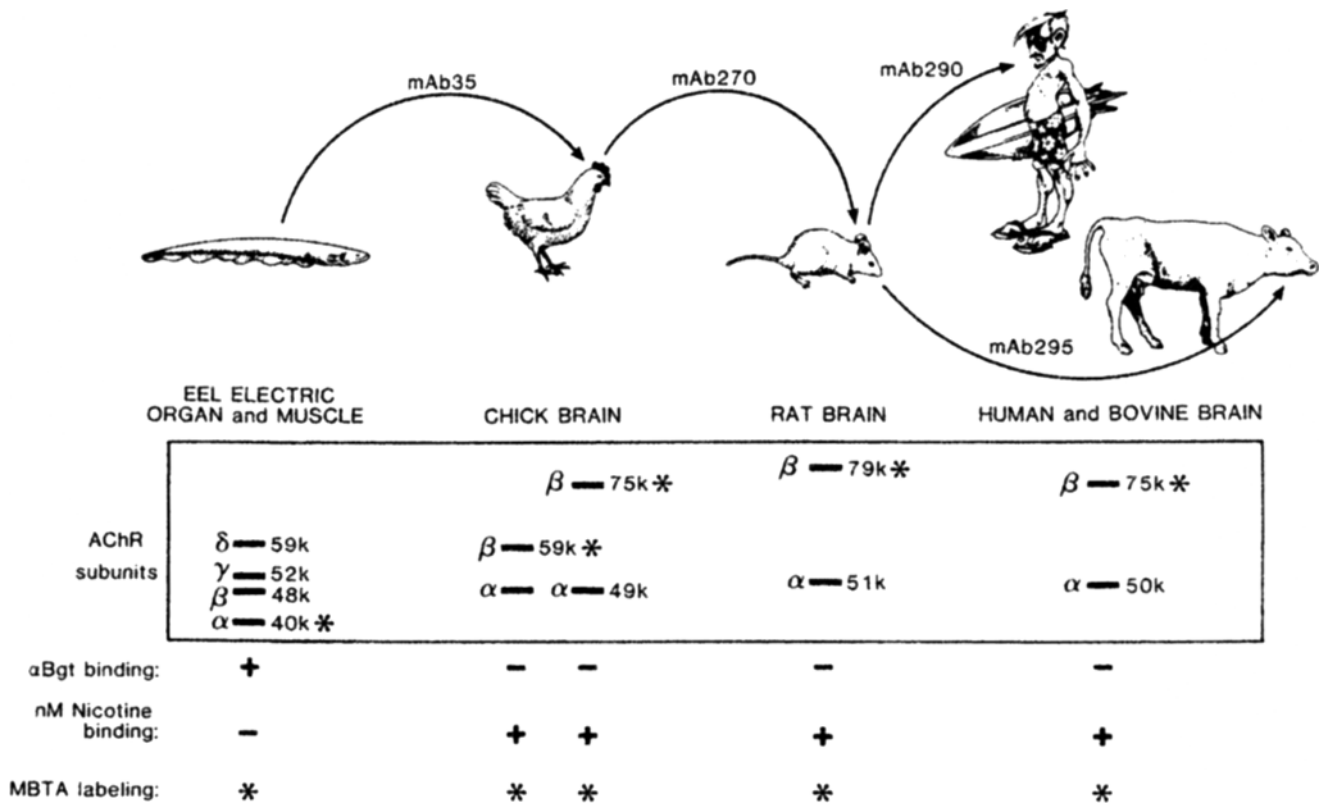


Fig. 1. Characterization of neuronal nicotinic AChRs by bootstrapping between species with mAbs.

strongly to AChRs in frog muscle, including mAbs specific for α, β, γ, and δ subunits (Sargent et al., 1984). Only mAbs to the MIR bound strongly to neurons in the tectum (Sargent et al., in preparation). Unlike the case of goldfish, these mAbs did not bind to an αBgt-binding protein. However, as in the case of goldfish, these putative AChRs were present in retinal ganglion cells, optic tract, and optic tectum, suggesting axonal transport to a presynaptic location after synthesis in the retina. By electron microscopy, AChRs were localized to extrasynaptic regions, rather than to the immediate presynaptic membrane or to obvious postsynaptic sites on the retinal ganglion axons. AChRs in such a location might be involved in a paracrine rather than a synaptic role. This result is similar to others in chickens and rats, described below, where AChRs are synthesized by retinal ganglion cells and transported along their axons. A subset of the mAbs that bound to tectal neurons, all of which were specific for the MIR, bind to AChRs on frog cardiac ganglia. Again, this result is similar to others in chickens, rats, and cattle, described below, where neuronal nicotinic AChRs in the brain and peripheral ganglia are found to be similar but distinguishable immunologically and pharmacologically.

The αBgt-binding protein from chicken brain was reported to be identified by some mAbs to AChRs from electric organ and muscles (Norman et al., 1982; Wonnacott et al., 1982; Mehraban et al., 1984).

In 1983, we reported that mAbs to the MIR intensely labeled the lateral spiriform nucleus of chicken brains and the projections of this nucleus to the optic tectum (Swanson et al.,

1983). This nucleus does not bind αBgt, which suggests the possibility that such antibodies, like mAb 35, might be labeling neuronal nicotinic AChRs which did not bind αBgt.

mAb 35 labels chick ciliary ganglion neurons. These cells are known to contain both AChRs (which do not bind αBgt) and αBgt-binding proteins (which are not AChRs) (Jacob and Berg, 1983). mAb 35 bound with a K_D of 0.85 n*M* (Smith et al., 1985) to a single component, not the αBgt-binding protein, which sedimented at 10 S in detergent extracts of both chicken brains and ciliary ganglia (Lindstrom et al., 1983). Antibodies to the MIR do not directly impair AChR function (Wan and Lindstrom, 1985; Blatt et al., 1986), so more indirect arguments were required to show that mAb 35 in fact labeled the physiologically significant AChR in these neurons. Neuronal Bgt down-regulated both the amount of functional AChRs on cultured ganglionic neurons and the number of binding sites for mAb 35, further suggesting that mAb 35 labeled functional AChRs (Smith et al., 1986). Conversely, mAb 35 could down-regulate the number of neuronal Bgt-binding sites (Halvorsen and Berg, 1987). Later, similar experiments in bovine adrenal chromaffin cells gave similar results, and it was further shown that chronic exposure to carbamycholine down-regulated both the amount of functional AChR and mAb 35 binding sites (Higgins and Berg, 1987). Peroxidase-labeled mAb 35 was shown by electron microscopy to bind the synapses on ciliary ganglion neurons (where AChRs are expected) (Jacob et al., 1984), whereas peroxidase-labeled αBgt bound to pseudodendrites on these cells (Jacob and Berg, 1983). Experiments in which ciliary ganglion AChRs were affinity labeled with neuronal Bgt and then immunoprecipitated with mAb 35 clearly showed that the functional AChR in ciliary ganglia was recognized by mAb 35 (Halvorsen and Berg, 1987).

The protein in detergent extracts of chicken brain immunoisolated by mAb 35 could bind nicotine and ACh with n*M* affinity (Whiting and Lindstrom, 1986b). The AChR on ciliary ganglia has only μ*M* affinity for these ligands (Halvorson and Berg, 1986), so it was evident that, despite their antigenic similarity, the ciliary neuron AChR and the brain AChR differed, at least near their ACh-binding sites. The observation that antisera to the immunoaffinity purified brain protein specifically inhibited the function of ciliary neuron AChRs strongly suggested that the brain nicotinic binding protein identified by mAb 35 was also a functional AChR (Stollberg et al., 1986). This was further supported by the observation that mAbs raised against this purified AChR bound to the functional AChR in rat PC12 pheochromocytoma cells (Whiting et al., 1987a), as well as to AChRs with n*M* affinity for nicotine and ACh extracted from rat brains (Whiting and Lindstrom, 1986b). Neither the antisera to denatured AChRs purified from chicken brain (Stollberg et al., 1986) nor seven of eight mAbs to this protein (Whiting et al., 1987d) reacted with AChR from muscle.

In chicken ciliary ganglion cells, a large intracellular pool of AChRs (comprising about two-thirds of the total) was identified both by immunoelectron microscopy (Jacob et al., 1986) and [^{125}I]mAb 35 labeling of cultures (Stollberg and Berg, 1987). In culture, only about 5% of the internal AChRs ever appear on the surface. These have a half-life of about 22 h. In addition to assembled 10 S AChRs, some smaller components in the 5–9 S range were identified. Transit time to the surface of assembled AChRs was 2–3 h. All of these features of AChR metabolism are similar to features of AChR metabolism in cultured muscle cells (Merlie et al., 1983a). The effects of depolarization (Smith et al., 1985), agonists (Halvorsen and Berg, 1986), and phosphorylation on AChR amount are especially interesting as potentially physio-

logically significant mechanisms of neuronal integration. We have observed that these effectors can have opposite effects, depending on the neuron studied (Whiting and Lindstrom, unpublished).

mAb 35 did not bind to AChRs from brains of rats, cattle, or humans. However, some mAbs raised to AChRs purified from chicken brain using mAb 35 did crossreact with AChRs in rat brains. In turn, mAbs raised to AChRs from rat brain purified using mAb 270 were used to study AChRs from bovine and human brains. These results are summarized in Fig. 1, and reviewed in more detail below.

Immunoaffinity purification of AChRs from chicken brain using mAb 35 revealed a protein apparently composed of only two kinds of subunits, apparent mws 49,000 and 59,000 (Whiting and Lindstrom, 1986a). By convention with the naming of subunits of muscle AChR, we named the lowest mw subunit α and the other β. The observations that both antisera to α subunits of *Torpedo* AChR and mAb 210 (raised to mammalian AChR and specific for the MIR on α subunits) reacted with the 49,000 mw subunits of chicken AChRs on western blots, showed that the α subunits of AChRs from muscle and neurons shared antigenic determinants. We had expected four kinds of subunits by homology with AChRs of muscle, and worried that some subunits might have been lost to proteolysis. Proteolysis of β, γ, and δ subunits is a serious problem during the purification of muscle-type AChRs, and mild proteolysis can make them appear to consist only of α subunits (Lindstrom et al., 1980). The observations that some mAbs recognize both subunits (Whiting et al., 1987d) and that the *N*-terminal amino acid sequences of both subunits are homologous to those of muscle-type AChR subunits (Whiting et al., 1987b and unpublished) indicate that the α and β subunits of neuronal nicotinic AChRs, and the subunits of muscle-type AChRs, derive from a common ancestor. The observation that AChR immobilized on mAb35-Sepharose could bind [^{125}I]mAb 35 shows that there is more than one α subunit per AChR (Whiting and Lindstrom, 1986a). The further observation that AChR immobilized on the β-specific mAb 285 could bind [^{125}I]mAb 285 also shows that there is more than one β subunit per AChR (Whiting et al., 1987d). Two to three of each subunit would account for the 10 S size (approximately 300,000 mw) of these AChRs on sucrose gradients and leave no room for additional subunits. This suggests that this AChR was purified intact and that it contains only two kinds of subunits.

Subtypes of neuronal nicotinic AChRs in chicken brains were discovered using mAbs which had been prepared from rats immunized with AChRs purified from chicken brains using mAb 35 (Whiting et al., 1987d). It was already known that neuronal AChRs in ciliary ganglia and brain, both of which were bound by mAb35, differed pharmacologically because those from brain had n*M* affinity for ACh (Whiting and Lindstrom, 1986b) whereas those in ganglia had μ*M* affinity (Halvorsen and Berg, 1986). Then, it was found that in chicken brains there were equal amounts of two types of AChR, both with n*M* affinity for ACh and pharmacologically indistinguishable, but differing in their β subunits (Whiting et al., 1987d). mAb 270 raised to AChRs purified using mAb 35, binds to α subunits on western blots. It binds equally well to both AChR subtypes, because the 49,000 mw α subunits of both subtypes appear to be very similar or identical as shown by mAb crossactivity, mw, and peptide maps. mAb 285 binds to β' subunits of 75,000 apparent mw, and binds only to the AChR subtype containing this subunit. mAb 35 binds preferentially to the AChR subtype containing β subunits of 59,000 apparent mw. mAb 210 is an α-specific mAb to mammalian AChR which, like mAb 35, binds to the MIR (Tzartos et al., 1987). On western blots, mAb 210 binds to the α subunits of both

AChR subtypes, αβ and αβ', yet like mAb 35 it binds to only the αβ subtype of native AChR (Whiting et al., 1987d). This suggests that the larger β' subunit may obscure the MIR on α subunits in the native αβ' AChR subtype, or cause a conformational change that alters it.

The existence of AChR subtypes in avian brains has been confirmed by immunohistochemistry. mAb 270 binds to regions of the chicken brain labeled by mAb 35 and also to additional areas (Swanson et al., 1987). This is also true of Zebra finch brains (Watson et al., in preparation). mAb 210 binds to several cell types in chicken retina including a substantial fraction of the ganglion cells (Keyser et al., in preparation). As yet, there has not been a detailed differential or high resolution localization of the AChR subtypes in avian brain, so it is unclear whether the subtypes are differentially associated with presynaptic or postsynaptic, junctional or extrajunctional, or other functional roles. It is known that the relative amounts of the subtypes changes during development (Whiting et al., 1987c).

The ACh-binding site of neuronal nicotinic AChRs was localized to their β subunits by affinity labeling (Whiting and Lindstrom, 1987a). After reduction with DTT, AChR function on ciliary ganglion cells (Stollberg et al., 1986), or PC12 cells (Leprince, 1983) is irreversibly blocked by treatment with MBTA or BAC, presumably by reaction at analogs of cysteines α192, 193 of muscle-type AChRs (Kao et al., 1984). Similarly, after reduction, MBTA and BAC permanently inhibit nicotine binding to brain AChRs immunoisolated with mAbs (Whiting and Lindstrom, 1986b, 1987a). [^{3}H]MBTA labels β and β' subunits of AChRs from chicken brain, and this is specifically inhibited by nicotine (Whiting and Lindstrom, 1987a). This is surprising because it suggests that β subunits contain homologs of cysteines 192, 193 characteristic of α subunits of muscle-type AChRs, even though α subunits of neuronal AChR share antigenic determinants with α subunits of muscle-type AChRs. This suggests that the primoridal subunit from which these subunits evolved contained both α antigenic determinants and cysteines 192, 193 and that in neuronal AChR α subunits, cysteines 192, 193 were lost (just as they were in β, γ, and δ subunits of muscle-type AChRs) whereas in neuronal AChR β subunits, the α antigenic determinants were lost (just as they were in β, γ, and δ subunits of muscle-type AChRs). Since β and β' subunits have essentially identical ligand binding properties (Whiting et al., 1987c), it is likely that the part of their extracellular structure around their ACh-binding sites is identical. It also seems likely that β and β' are homologous except for one or two stretches formed by the 16,000 extra mw of β'. This pattern of segmental homology is observed in the cDNAs for two muscarinic AChR subtypes (Kubo et al., 1986) and for some putative neuronal nicotinic AChRs (Goldman et al., 1987). It is interesting that in the case of the GABA receptor, another member of the AChR super gene family composed of two kinds of subunits α (53,000 mw) and β (57,000 mw), it is the higher molecular weight β subunit that contains the transmitter-binding site (Schofield et al., 1987).

AChRs on chicken ciliary ganglion neurons photoaffinity labeled with [^{125}I]neuronal Bgt can be immunoprecipitated with mAb 35 (Halvorson and Berg, 1987). The apparent mw of the ACh-binding subunit is 59,000. This is identical to the mw of the β subunit of AChRs from chicken brain. The observation of about two binding sites for mAb 35 per neuronal Bgt toxin-binding site would be consistent data on brain AChRs (Whiting et al., 1987b,d), which suggest a subunit stoichiometry of $\alpha_3\beta_2$ or $\alpha_2\beta_2$.

mAb 270 reacts with rat brain nicotine AChRs. Additionally, mAb 270 binds to the functional AChR in PC12 cells (Whiting et al., 1987a). An undifferentiated PC12 cell has ~1000 mAb 270 binding sites and ~16,000 αBgt-binding sites. After culture of PC12 cells with βNGF the amount of mAb 270, binding increases five- to sevenfold, whereas the amount

of αBgt-binding sites remains essentially constant. The carbamylcholine-induced $^{86}Rb^+$ influx increases in parallel with the number of mAb 270 binding sites, suggesting that mAb 270 binds to functional AChRs. Further confirmation is provided by the observation that treatment of PC12 cells with mAb 270 plus anti-immunoglobulin decreases the amount of functional AChRs, apparently by the same process of antigenic modulation by which antibodies to AChRs on muscle cells crosslink and promote the internalization and destruction of AChRs on muscle cells.

mAb 270 reacts with >90% of the high affinity nicotine binding sites in detergent extracts of rat brains (Whiting and Lindstrom, 1987b). The pattern of binding of [^{125}I]mAb 270 to sections of rat brain (Swanson et al. 1987) closely resembles the pattern of binding of [^{3}H]nicotine and [^{3}H]ACh (Clarke et al., 1985b). PC12 cell AChRs (Kemp and Morley, 1986) like chicken ciliary ganglion AChRs (Halvorsen and Berg, 1986) do not have n*M* affinity for nicotine. Thus, in both species there are similar but pharmacologically distinct AChRs in peripheral and central neurons.

Immunohistochemical studies suggest that some of the AChRs in rat brain labeled by mAb 270 are presynaptic (Swanson et al., 1987). Labeling is observed in the dorsal (sensory) part of the spinal cord, cranial nerve nuclei, and in the retina, optic tract, and superior colliculus. Removal of an eye eliminated labelling in the contralateral superior colliculus, and reduced labeling in the geniculate nuclei and other regions receiving visual input, clearly demonstrating that AChRs were transported along axons from retinal ganglion cells to the nerve endings. There is evidence that nicotinic AChRs in the superior colliculus effect neuronal activity, because treatment of rats with inhibitors of acetylcholinesterase (to increase the effect of endogenous ACh) and atropine (to block muscarinic AChRs) dramatically increases the incorporation of [^{3}H]2-deoxyglucose in the superior colliculus (Pazdernik et al., 1982; Dam and London, 1983) in a pattern identical to that observed by labeling with [^{3}H]ACh (Clarke et al., 1985) or [^{125}I]mAb 270 (Swanson et al., 1987). Treatment with nicotine similarly increases deoxyglucose incorporation in the superior colliculus (London et al., 1985; Grunwald et al., 1987). There is other evidence for presynaptic nicotinic AChRs in areas like the medial habenula and interpeduncular nucleus (Clarke et al., 1986) where mAb 270 binding is intense. mAb 270 also binds to large areas of caudate putamen and cortex where the transynaptic orientation of the AChR is not apparent (Swanson et al., 1987).

AChRs were immunoaffinity purified from rat brains using mAb 270 (Whiting and Lindstrom, 1987b). α Subunits of 51,000 mw were found which crossreact with antisera to *Torpedo* AChR α subunits and mAb 270. Subunits of 79,000 mw were found that were designated β' because they were similar in mw to the β' subunits of chicken brain, and because they crossreact with mAb 286 to chicken brain AChRs, which binds to β' subunits of chicken brain AChRs. Also, mAb 299 to AChRs from rat brain binds to the β' subunits of AChRs from both rats and chickens (Whiting and Lindstrom, unpublished). Finally, β' subunits of rat brain AChRs are specifically labeled with [^{3}H]MBTA (Whiting and Lindstrom, 1987a). Thus, the AChR purified from rat brain corresponds to the αβ' subtype of AChR from chicken brain. It seems unlikely that there are significant amounts of the αβ type AChR in rat brain, because mAb 270 binds >90% of the high affinity nicotine binding sites from extracts of rat brain. There could be ganglionic type AChRs with low affinity for nicotine, but it seems unlikely that there are very many because only the two types of subunits are observed in AChRs purified using mAb 270,

and because the histological pattern of [^{125}I]mAb 270 binding (Swanson et al., 1987) corresponds to the pattern of high affinity [^{3}H]nicotine-binding (Clarke et al., 1985b). The ganglionic-type AChR in rat PC12 cells has been partially purified and labeled with [^{3}H]MBTA, revealing a labeled band at 52,000 mw (Leprince, 1986). This is smaller than β or β' subunits, but there is the possibility that it could derive from a larger peptide by proteolysis.

After purification of AChRs from rat brains, αBgt-binding proteins were purified from the same detergent extracts (Whiting and Lindstrom, 1987b). These were found to consist of four types of subunits, which in apparent mws resembled those of AChRs from *Torpedo*. Initial reports of purification of the αBgt-binding protein, described a single subunit (Norman et al., 1982) but a later report using more protease inhibitors described three-to-five subunits (Conti-Tronconi et al., 1985), which suggests that this structure is quite labile to proteolysis. The ability to identify four distinct polypeptides of those mws suggests that proteolysis was well controlled and that the AChR purified from these extracts was also intact. Further data is required, however, to unequivocally demonstrate that all four peptides are part of the αBgt-binding protein.

The subunit stoichiometry of AChRs from rat brain, like that of AChRs from chicken brain, includes more than one subunit of each type (Whiting et al., 1987b). Rat AChR immobilized on mAb 270 can bind [^{125}I]mAb 270, showing that there is more than one α subunit. The ratio of α/β subunits in scans of polyacrylamide gels and the ratio of α amino acids/β amino acids on each cycle of the sequenator are consistent with a stoichiometry of $\alpha_3\beta_2$. This would account for the apparent size of these AChRs judged from sucrose gradients. It would also preserve symmetry with the two ACh-binding and three-structural subunit structure of muscle-type AChRs. However, an $\alpha_2\beta_2$ subunit stoichiometry is simpler and more internally symmetric. Absolutely determining stoichiometry is very difficult, and one can only be certain of $\alpha_{n=2-3}\beta_{n=2-3}$.

AChRs were also purified from the brains of cattle by using mAb 295 raised to AChRs from rat brains (Whiting and Lindstrom, unpublished). mAb 270 did not crossreact with bovine AChRs. Bovine AChRs were, however, quite similar to those purified from rat brains using mAb 270. α Subunits of mw 50,000 and β' subunits of mw 75,000 were observed. The β' subunits were labeled with MBTA. These AChRs also exhibited n*M* affinity for nicotine.

AChRs in bovine adrenal chromaffin cells are bound by mAb 35 (Higgins and Berg, 1987). mAb 35 does not bind to AChRs from bovine brain or to rat pheochromocytoma cells (Whiting and Lindstrom, unpublished), demonstrating an immunological as well as a pharmacological distinction between AChRs of brain and adrenal cortex. mAb 35 recognizes a discontinuous epitope, so it does not react well with denatured α subunits from AChRs of any species but its immunogen, *Electrophorus* (Tzartos et al., 1981). It is quite conformation-sensitive and does not bind to nascent α subunits of muscle AChR, but will bind after a conformational maturation that occurs before subunit assembly (Merlie and Lindstrom, 1983). It recognizes only the αβ subtype of chicken brain neuronal AChRs, even though the αβ' subtype has essentially identical α subunits (Whiting et al., 1987d). All these data are consistent with the concepts that the amino acids from separate parts of the α subunit primary sequence come together in the native conformation to form the epitope that is bound by mAb 35; this forms a distinct feature on the extracellular surface of α subunits that has been fairly well conserved in shape over evolution; but an amino acid substitution or slight conformational change in these amino

acids can easily make a profound difference in the ability of mAb 35 to bind.

AChRs with n*M* affinity for nicotine can be identified in extracts of human brains using mAb 290 raised to AChRs purified from rat brains (Whiting et al., 1987c; Whiting and Lindstrom, 1988). mAb 290 does not bind to AChRs from human muscle. Similarly, autoantibodies to muscle AChRs from patients with myasthenia gravis do not bind to human neuronal nicotinic AChRs. Purification of intact human neuronal AChRs was not possible because of autolysis of the tissue available, but it seems likely that these AChRs are structurally very similar to those of rats and cattle.

Anti-idiotype mAbs to antibodies to a nicotine analog were recently used to identify a putative nicotinic AChR in rat brain (Abood et al., 1987). The identity of this protein is unclear. It does not correspond to the AChR purified using mAb 270 (Whiting and Lindstrom, 1987b) because an anti-idiotype mAb did not bind to these AChRs (Whiting, Lindstrom, Bjucke, unpublished).

The human medulloblastoma cell line TE671 contains functional AChRs that bind αBgt (Syapin et al., 1982). These are of the muscle type and are well recognized by mAbs and autoantibodies to human muscle AChRs (Lindstrom et al., 1987), but not recognized by mAbs to neuronal nicotinic AChRs (Whiting et al., 1987c). AChRs purified from TE671 cells are composed of four kinds of subunits that correspond immunologically to α,β,γ, and δ subunits of AChR from electric organ (Luther, Schoepfer, and Lindstrom, unpublished). The cDNA for the α subunits of AChRs from TE671 cells has been cloned (Schoepfer et al., 1988) and found to be identical in sequence to that predicted by the genomic clone for human muscle AChR α subunit identified by Noda et al. (1983c). The AChRs in TE671 cells do not correspond to the αBgt-binding proteins in human brain, because the αBgt-binding protein also is not bound by antibodies to human muscle AChRs (Whiting et al., 1987c). This cell line may derive from immortalization of a neuron that expresses muscle-type AChRs transiently during a developmental stage, but not as a mature neuron.

cDNAs as Probes for Neuronal Nicotinic Receptors

In addition to the approaches that characterize the protein, recombinant DNA technology also makes it possible to identify genes and determine their DNA sequence. From the nucleotide sequence, evolutionary trees and the primary structure of the protein can be deduced. *In situ* hybridization, expression systems, and in vitro mutagenesis, in combination with functional and biochemical assays, tell us even more about structure and function (Claudio, 1986).

Different approaches have been used successfully to clone receptor genes: (a) functional assays, involving hybrid selection, expression, and identification with antibodies (Ballivet et al., 1982; Merlie et al., 1983c; Lubbert et al., 1987), or gene transfer and fluorescent-activated cell sorting (Radeke et al., 1987); (b) expression cloning using antibodies for detection (Weinberger et al., 1985); (c) oligonucleotide screening based on microsequencing the purified protein (Noda et al., 1982); (d) crosshybridizing at low stringency with a DNA probe of a related gene—the probe being either a long fragment (Yu et al., 1986) or an oligonucleotide (Buonnanno et al., 1986).

All published approaches to identifying the putative genes of the neuronal nicotinic AChR used long DNA probes derived from *Torpedo* electric organ or muscle nicotinic AChR genes. Results of these studies are given in Fig. 2.

The sequence of the *Torpedo* electric organ AChR subunit genes has been shown to have several characteristic features (Popot and Changeux, 1984). All four proteins have four domains, M1–M4, consisting of ~25 hydrophobic amino acids. M1–M3, in particular, are located at similar positions in the genes and are highly conserved. Two extracellular cysteines at positions approximating 128 and 142 of muscle AChR α subunits are found in all subunits. The same gene family with an additional fifth member (Takai et al., 1985), all displaying homology in M1–M3, is present in muscle AChRs.

Subunit	Probe	cDNA Size [kb]	mRNA Size [kb]	Deduced Protein Size [kd]	M1,M2, M3,M4	AA Identity to Mouse Muscle α^{20}	Cysteines 192,193	Cysteines 128,142	Asn 141	Asn 24
				Torpedo Californica Electric Organ AChR						
α	Oligo to AA 13-18 AA 25-129[1]	2.0	2.3	50	+	80%	+	+	+	–
β	Oligo to AA 13-17 AA 46-50[2]	1.7	2.0	54	+	42%	–	+	+	–
γ	Anti-Torp antisera[3,4]	2.0	2.1	57	+	36%	–	+	+	–
	Oligo to AA 1-5 AA 13-17[4]	2.1	2.3	57	+	36%	–	+	+	–
δ	Oligo to AA 17-21 AA 25-30[2]	2.2	6.0	58	+	36%	–	+	+	–
				Mouse Muscle AChR						
α	mAb 61[5,7]	0.7[a]	2.0							
	Chick muscle α[8]	1.7	2.0	49	+	100%	+	+	+	–
β	Oligo to conserved region AA 134-142[9]	2.1	2.4	54	+	59% with Torp β[a]	–	+	+	–
γ	Torpedo γ[10]	1.7	2.0	56	+	33%	–	+	+	–
	Human muscle γ[11]	1.9	2.0	56	+	33%	–	+	+	–
δ	Torpedo γ[12]	1.9	3.3		+	36%	–	+	+	–

Fig. 2. cDNA studies of the ligand-gated receptor superfamily.

Subunit	Probe	cDNA Size [kb]	mRNA Size [kb]	Deduced Protein Size [kd]	M1,M2, M3,M4	AA Identity to Mouse Muscle α^{20}	Cysteines 192,193	Cysteines 128,142	Asn 141	Asn 24
				Chicken Optic Lobe						
δ VC	Chick muscle α^{13}	0.9[a]	2.3		+					
				Chicken Brain and Genomic						
α2	?[14]						+			
γ2	?[14]						-			
				Rat PC12						
α3 (λPCA48)	Mouse muscle α^{15}	1.7	2.0	55	+	51%	+	+	+	+
				Rat Brain AChR						
α4	α3 and mouse muscle α^{16}	2.0[a]		67	+		+	+	+	+
β2 (=γ2?)	$\alpha 3^{21}$	2.2			+	~50% with α3, α4	-			
Subunit	Probe	cDNA Size [kb]	mRNA Size [kb]	Deduced Protein Size [kd]	M1,M2, M3,M4	AA Identity to Mouse Muscle α^{20}	Cysteines 192,193	Cysteines 128,142	Asn 141	Asn 24
				Drosophila CNS						
ARD	Torpedo γ^{17}	2.0	3.2	60	+	46% with Torp α^{17}	-	+	-	+
				Rat Spinal Cord Glycine Receptor						
α	Oligo to AA 150-156 AA 150-161[18]	1.4[a] (overlapping clones)	9.0 5.0 2.0	48	+	21% with ARD[18]	-	+	-	-

Fig. 2 (*continued*)

	Bovine Brain $GABA_A$ Receptor									
α	3 Oligos to internal protein sequences[19]	2.4	49	+	18.5% with bovine α AChR[19]	-	+	-	-
β	Oligo to internal protein sequences[19]	3.0	51	+	15% with bovine α AChR[19]	-	+	+	-

[1]Noda et al., 1982; [2]Noda et al., 1983b; [3]Ballivet et al., 1982; [4]Claudio et al., 1983; [5]Noda et al., 1983a; [6]Merlie et al., 1983c; [7]Sebbane et al., 1983; [8]Boulter et al., 1985; [9]Buonanno et al., 1986; [10]Yu et al., 1986; [11]Boulter et al., 1986b; [12]LaPolla et al., 1984; [13]Barnard et al., 1986; [14]Nef et al., 1986; [15]Boulter et al., 1986a; [16]Goldman et al., 1987; [17]Hermans-Borgmeyer et al., 1986; [18]Grenningloh et al., 1987; [19]Schofield et al., 1987; [20]Claudio, 1986b, [21]Boulter et al., 1987.

* Differential hybridization and hybrid selected in vitro translation.

a Clone does not code for entire protein.

Fig. 2 (*continued*)

Knowing that there are similar but different nicotinic AChRs on neurons, the hypothesis that related genes, perhaps in combination with muscle genes, may code for neuronal AChRs, can be tested. Isolating genes of the nicotinic AChR family has been performed in several insect and mammalian species. Cloning attempts in insects like *Drosophila* are favored compared to attempts in mammals, by a genome that is ~10 times smaller. In addition, the concentration of AChRs in insect brain is about 10 times higher (Breer et al., 1986).

Screening a cDNA library from *Drosophila* heads was conducted with probes derived from the γ subunit of AChRs from *Torpedo californica*, permitting the isolation of overlapping clones for a gene termed ARD (Hermans-Borgmeyer et al., 1986; Gundelfinger et al., 1986). The deduced protein sequence showed characteristics of the nicotinic AChR gene family: a hydrophobic leader sequence; four hydrophobic regions corresponding to M1–M4; two cysteines corresponding to Cys 128, Cys 148; and an overall amino acid identity of about 40% to *Torpedo* AChR subunits. A putative *N*-glycosylation site was present at an Asn 24 analog position. However, this clone lacked cysteines corresponding to those at 192, 193 of the ACh-binding subunits of AChRs from electric organ (Kao et al., 1984) and brain (Goldman et al., 1987; Whiting and Lindstrom, 1987a). Because the AChR from locusts can be affinity labeled by MBTA and BAC after reduction by DTT (Filbin et al., 1983), it seems likely that AChR from *Drosophila* might have cysteines at about positions 192, 193. Insect AChR has been reported to consist of a single kind of subunit (Breer et al., 1985). If this is true, it could be that ARD does not code for an AChR subunit, but instead for a subunit of another receptor in the AChR superfamily (Grenningloh et al., 1987; Schofield et al., 1987). Alternatively, it may be that insect AChRs have more than one kind of subunit (Filbin et al., 1983) and ARD codes for one of these. Expression of functional AChR from a single cDNA or from a necessary combination of cDNAs could resolve this question. A 3.2 kb mRNA species that hybridizes with ARD accumulates during the later developmental stages of the *Drosophila* embryo, the period of greatest differentiation in its nervous system, but it has not yet been proven that ARD clones are of neuronal origin. Neuromuscular transmission in insects is mediated by glutamate. Since the AChR gene superfamily has recently been found to include receptors for glycine (Grenningloh et al., 1987) and GABA (Schofield et al., 1987) it increases the possibil-

ity that ARD may code for the subunit of another ligand-gated channel, such as the glutamate receptor.

By analysis of genomic clones and cDNA clones still containing introns, Gundelfinger and coworkers (1986) identified three introns in the ARD gene. Splicing sites of at least two introns were shown to correspond exactly to those in vertebrate muscle AChR subunit genes (Nef et al., 1984; Noda et al., 1983; Shibahara et al., 1985), whereas one splicing site was shifted by 9–15 nucleotides with respect to the homologous vertebrate gene. The overall organization of the ARD gene revealed fewer exons than vertebrate muscle AChR genes.

Barnard and coworkers (Barnard et al., 1986) used a 677 bp Bgl II fragment of a chick muscle α AChR clone containing M1–M3, in addition to a large part of the extracellular domain, for screening a 1-d-old chick optic lobe cDNA library. This region of chicken brain is enriched in both AChRs that do not bind αBgt (Swanson et al., 1983, 1987) and in αBgt-binding protein (Barnard et al., 1986). Two overlapping clones were isolated out of 600,000 recombinants. The larger one (λ VC12) coded for a segment of a protein that extends from approximately the middle of M1 through the poly-A signal, having the typical homology in M1–M4 and the highest identity to the α subunit of the chick muscle AChR. This partial clone does not contain the regions coding for a cysteine pair that might be near the ACh-binding site. It is not evident whether this cDNA codes for a subunit of the AChR, the αBgt-binding protein, or some other member of the AChR superfamily.

Northern blot analysis of 1-d-old chick optic lobe RNA at high stringency using a probe coding for presumably intracellular sequences reveals one band at 2.3 kb (Barnard et al., 1986). Genomic Southern blots indicate the presence of one single gene. When the optic lobe cDNA clone containing M1–M3 sequences was used as a probe for screening a genomic library at high stringency, a homologous gene, the chicken analog to the rat gene α3 (Boulter et al., 1986) *(see below)* was isolated. It showed a high degree of conservation of M1–M3.

Ballivet and coworkers (Nef et al., 1986) used DNA probes >200 bp, including the coding region for amino acids 210–300 of chicken muscle AChR subunits, to screen chick genomic and brain cDNA librarys at stringencies about 40°C below Tm. Under these conditions, they found three genes: α2, α3, and γ2. (The muscle genes were called α1, β1, γ1, and δ1.) All three genes were expressed in brain (Nef et al., 1986; Boulter, et al., 1987), whereas apparently none of them were expressed in muscle. The limited data available show a >70% nucleotide identity of α2 and γ2 in M1 compared to muscle genes. α2 and α3 were named "α" because of the presence of Cys 192, 193, unique to the α subunit of the muscle nicotinic AChR. They have tentatively named the gene that lacks these cysteines γ2, because of its marginally higher similarity to γ1 as compared to β1 or δ1.

Heinemann and coworkers (Boulter et al., 1986) screened a cDNA library of the PC12 cell line, which is known to express a functional nicotinic AChR as well as a αBgt-binding protein (Patrick and Stallcup, 1977a,b). They used a cDNA fragment of the muscle α subunit (Boulter et al., 1985) coding for the *N*-terminal part of the protein including the ACh-binding site and some amino acids of the M1 region. At low stringency, hybridization to Northern blots of PC12 mRNA the probe detected an mRNA species of 2.0 kb and occasionally 3.5 kb, which did not hybridize at high stringency. Screening 10^6 plaques yielded three overlapping clones. The deduced amino acid sequence of the longest clone, λPCA48, revealed the hydrophobic stretches M1–M4 and an overall homology of 42% amino acid identity with mouse muscle α. The deduced mw of 54,780

was 5500 higher than the deduced mw of α subunits from muscle AChR. The additional 37 amino acids were located in the cytoplasmic part between M3 and M4. The four extracellular cysteines—Cys 128, 142, 192, and 193—were present in the same positions as in muscle α. This gene was considered a member of the nicotinic AChR gene family and is referred to as α3 (Nef et al., 1986. Goldman et al., 1987). Using α3 as a probe in Northern blots, a strong hybridization signal was present on PC12 RNA at 2 and 3.5 kb, whereas hardly any signal was visible on RNA preparations from several parts of rat brain, adrenals, and muscle. S1 protection analysis (Goldman et al., 1986) revealed the expression of α3 RNA in the habenula. Genomic restriction fragment analysis indicated the existence of other related genes when α3 is used as a probe at low stringency.

By screening of rat hypothalamus and hippocampus cDNA libraries with mouse muscle α- and α3-derived probes, the same group identified another gene, α4, which belongs to the AChR-gene family based on the presence of M1–M4 and Cys 128,142 equivalents (Goldman et al., 1987). Additionally, the presence of Cys 192, 193 might classify the protein as a ligand binding subunit. A potential glycosylation site at Asn 24 in α4 has not been found in any muscle nicotinic AChR gene, but has been found in α3 and ARD. Two forms of cDNA α4 were found, α4-1 and α4-2, that differed in their 3' untranslated region. The deduced proteins were different in their two *C*-terminal amino acids, and α4-2 coded for one additional amino acid. The clone α4-2 lacked the first 129 amino acids encoded by α4-1, which is also not a full-length clone, missing an initiating methionine, but having a hydrophobic leader sequence. DNA sequence analysis did not allow the unambiguous determination of the *N*-terminus of the mature protein. S1 analysis with a 3' fragment of α4-1 revealed three different fragments, indicating an as yet unidentified third subtype, in addition to α4-1 and α4-2.

A novel neuronal AChR cDNA closne was isolated from a PC12 library using the α3 cDNA as a probe. This cDNA clone, β2, has regions M1–M4, but it lacks cysteines at positions 192 and 193 (Boulter et al., 1987). It was proposed that β2 coded for a structural subunit of neuronal AChRs. Several lines of evidence suggest that this is the case (*see below*).

In situ hybridization (Cox et al., 1984), with [H^3] and [S^{35}] probes, followed by washings and autoradiography, is a technique that can detect the distribution of RNA expression in tissue. In neural tissue, the RNA location indicates the neuronal cell body, whereas the functional protein might be located somewhere else because of axonal transport.

Using a full length α3 probe on mouse and brain sections, the medial habenula showed very strong hybridization (Boulter et al., 1986; Goldman et al., 1986). Labeling was also seen in the hippocampus, the dentate gyrus, neocortex, and parts of substantia nigra and thalamus. Using muscle α as a probe, the hybridization pattern was different, mainly lacking the signal in the medial habenula. These results were obtained at reduced stringency with probes, including M1–M4. As there is now considerable evidence for a large gene family whose members share a high degree of nucleotide identity in the region M1–M4, it seems likely that at least parts of the hybridization signal under the conditions used in these studies is obtained by crosshybridization with other members of this family, perhaps including subunits of the αBgt-binding protein or receptors for GABA. Up to now, extensive brain cDNA library screening has not yet revealed a muscle α clone, supporting the idea that muscle AChR is not expressed in brain and that the total signal is caused by the presence of other genes. The strong signal of α3 in the medial habenula is at least partially a result of the expression of the α3 gene, as demonstrated by S1 mapping experiments (Goldman et al., 1986).

A full length α4 probe hybridizes at high stringency to the necortex, many thalamic nuclei, medial habenula, ventral tegmental area, parts of the substantia nigra, medial geniculate nuclei, and throughout the hypothalamus (Goldman et al., 1987). The observed pattern

for this α4 probe is different from the pattern for α3, indicating that these two genes code for subunits of different proteins.

Because of the inherent problem of crosshybridization of gene probes with other members of the gene family, these preliminary studies have to be completed with specific probes, such as probes derived from the 3' or 5' part of the untranslated regions, or oligonucleotides. This would also speak to the expression of RNA subtypes by differential splicing. We have noticed that in rat brain there appears to be a lot of AChR mRNA still containing introns (Schoepfer, Whiting, and Lindstrom, unpublished). If nuclear intron–exon splicing were a significant mechanism regulating AChR mRNA levels in the endoplasmic reticulum, then incompletely spliced AChR mRNA might be detectable in the nuclei of cells that were not expressing AChR protein.

A major task in the coming years will be to find out which gene codes for which protein and what the function of this protein is. One approach to accomplish this will be expression in *Xenopus* oocytes (Sumikawa et al., 1981). Expression in oocytes has been extremely useful for studies of muscle-type AChRs (Mishina et al., 1984; Sakmann et al., 1985; Imoto et al., 1986) and is now also being used for neuronal AChRs (Boulter et al, 1987) and other members of the AChR gene family (Schofield et al., 1987). Co-expression of α3 or α4 with β2 results in functional AChRs that are not blocked by αBgt, but are blocked by neuronal Bgt (Boulter et al., 1987).

Expression systems in bacteria (Barkas et al., 1987; Gershoni et al., 1987) or yeast (Fujita et al., 1986) have the advantage of expressing large quantities of proteins such as α subunits of AChR from *Torpedo* or fragments of α subunits, and the disadvantage that the α subunits expressed lack native conformations. In transfected fibroblasts, expression of α subunits from mouse muscle in nearly native conformation has been possible (Blount and Merlie, 1987). Several subunits from *Torpedo* AChR have been stably introduced into mammalian cells (Claudio, 1986, 1987). Similar experiments will no doubt soon be done with neuronal AChR subunits.

Making antibodies to proteins expressed from cDNAs or making antibodies to synthetic peptide sequences deduced from cDNA sequences should provide further tools for determining the physiological significance of the proteins they code, although a severe limitation of this approach is that antibodies to synthetic peptides from many parts of the AChR do not bind to the native protein (Ralston et al., 1987).

Comparison of Results Using Cholinergic Ligands, Antibodies, and cDNAs

It has recently become possible to start correlating the large amounts of data obtained by the mAb and cDNA approaches. We have determined the amino-terminal amino acid sequence of the ACh-binding subunit from rat brain AChR (Whiting et al., 1987b) and found that it corresponds to residues 26–43 of the deduced amino acid sequence of the cDNA α4 (Goldman et al., 1987). Similarly, the amino-terminal sequence of the structural subu;nit of this AChR (Whiting, Esch, Shimasaki, and Lindstrom, unpublished) corresponds to the first 11 amino acids of the coding region of the cDNA β2 (Deneris et al., 1987 and personal communication). Thus, in the case of the major micotinic AChR type in rat brain, the results using mAbs and cDNAs are perfectly sonsistent. Corresponding ACh-binding and structural subunits are observed. Only these two kinds of subunits compose the AChR in vivo (Whiting and Lindstrom, 1987b) and these two subunits form functional AChRs in oocytes (Boulter et al., 1987).The observation that the

same rat structural subunit cDNA expressed with two different ACh-binding subunit cDNAs produces functional AChRs in oocytes led to the suggestion that the various neuronal AChR subtypes may share a common structural subunit in vivo (Boulter et al., 1987). The observations that the two subtypes of AChR in chicken brain share a common structural subunit and that a mAb to this subunit crossreacts with AChR in ganglia is consistent with this possibility (Whiting et al., 1987d).

There is a confusion in the nomenclature. The two rationales by which the same protein came to be referred to both as β' and α4 are both logical, but now that this subunit and its homologs are known to contain the ACh-binding sites of these AChRs, it is clearer to simply refer to these subunits as "ACh-binding subunits." The α subunit can be referred to as a "structural subunit." As in the case of muscle-type AChRs, both subunits probably contribute "barrel staves" to the lining of the cation channel. Similarly, in the case of the GABA receptor, another member of the AChR superfamily, it is the 57,000 mw β subunit that contains the GABA-binding site rather than the 53,000 mw α subunit (Schofield et al., 1987). Here too, the β subunit could be referred to as the "GABA-binding subunit" and the α subunit as a "structural subunit."

The identification of the PC12 cDNA λPCA48, also termed α3, is less clear. The presence of cysteines 192, 193 suggest that α3 could code for the ACh-binding subunit of this ganglionic AChR (Boulter et al., 1986). The observation that co-expression in oocytes of the cDNAs α3 and β2 produces functional AChRs that are blocked by neuronal Bgt but not αBgt is strong evidence that α3 codes for the ACh-binding subunit of ganglionic AChRs (Boulter et al., 1987). But, since it was obtained by probing a cDNA library from a cell with ten times as many αBgt-binding sites as mAb 270 binding sites (Whiting et al., 1987a), there is a good chance that a muscle α subunit cDNA probe might also hybridize with one of the four subunits of the αBgt-binding protein. βNGF treatment of PC12 cells increases the amount of functional AChR and amount of mAb 270 binding without increasing the amount of αBgt-binding sites or the amount of mRNA that hybridizes with α3 (Whiting et al., 1987a). Since the amount of AChR in muscle is substantially regulated by the amount of transcription of α subunits (Merlie et al., 1984; Evans et al., 1987), this could suggest that α3 does not code for the ACh-binding subunit of the AChR, but instead codes for a subunit of the αBgt-binding protein. Knowledge of *N*-terminal sequences of the subunits of the rat αBgt-binding protein could help resolve this question. Another possibility is that the AChR structural subunit recognized by mAb 270 is transcriptionally regulated, whereas the ACh-binding subunit is present at a constant level. In the case of muscle, for example, it has been reported that the amount of β subunit mRNA increases after denervation much less than the amount of α, γ, or β subunit mRNA (Evans et al., 1987).

In the near future, one can expect that all of subunits of immunoaffinity purified AChRs will be associated with specific cDNAs. *N*-terminal identity is insufficient evidence for establishing complete identity, since for example cDNA α4 has been shown to exhibit C-terminal alternate transcription (Goldman et al., 1987).

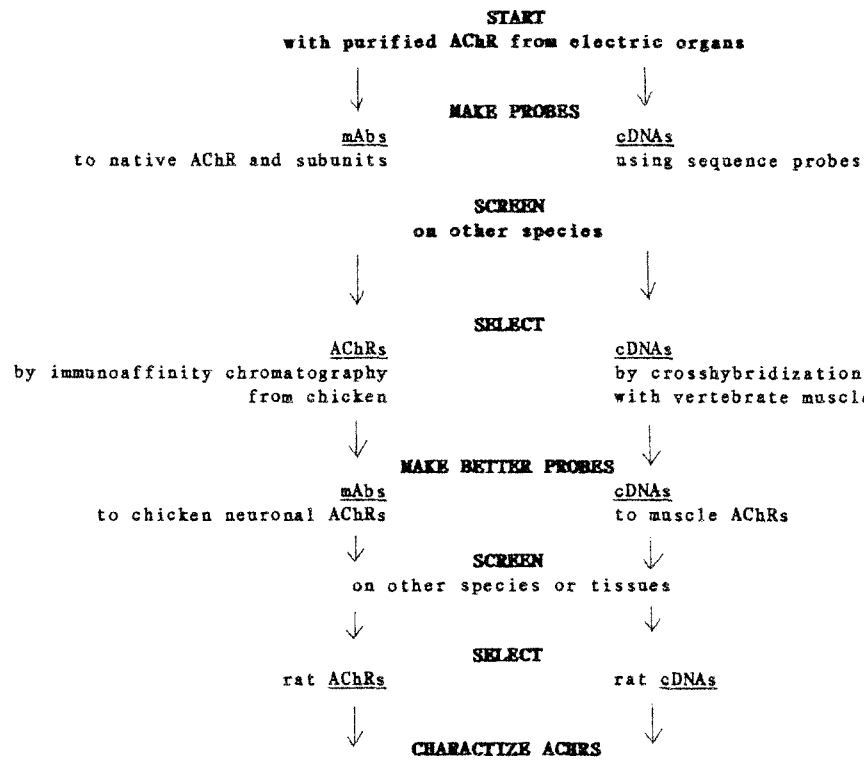
Expression of AChR cDNAs (Boulter et al., 1987) is a very elegant approach. However, it is difficult to determine from this data alone whether the subunit combinations which function in oocytes are associated in vivo. For example, it is known that AChRs with altered properties can be expressed by injection of oocytes with combinations of muscle-type AChRs which occur at different developmental stages (Mishina et al., 1986), or with some combinations of subunits from different species (Sakmann et al., 1985; Imoto et al., 1986), or even very inefficiently by deletion of a subunit

that is normally present (Mishina et al., 1984; Kurosaki et al., 1987). The b2 cDNA even forms functional AChRs when co-expressed with α, γ, and δ cDNAs of muscle AChR (Deneris et al., 1987). However, even with these reservations, in vitro expression of neuronal AChR function can be very useful because the endogenous AChRs may be difficult to study electrophysiologically, and because it permits expression of the products of in vitro mutagenesis.

There is excellent correlation between rat brain regions which exhibit high affinity for [^{3}H]nicotine and [^{3}H]ACh (Clarke et al., 1985b) and those that exhibit high affinity for [^{125}I]mAb 270 (Swanson et al., 1987). This is consistent with the observation that mAb 270 can immune precipitate >90% of the high affinity nicotine binding sites in detergent extracts of rat brain (Whiting and Lindstrom, 1987b). It is known that mAb 270 can also bind to rat ganglionic AChRs that have lower affinity for nicotine (Whiting et al., 1987a). Since there are not extensive brain regions bound by [^{125}I]mAb 270 that are not also bound by [^{3}H]nicotine (Swanson et al., 1987), it seems unlikely that large amounts of ganglionic type AChRs are expressed in brain. This is also consistent with the observation of a simple, two-subunit pattern in AChRs immunoaffinity purified from rat brain with mAb 270 (Whiting and Lindstrom, 1987b).

Correlation of *in situ* hybridization of putative AChR subunit mRNAs with cDNAs (Boulter et al., 1986; Goldman et al., 1986, 1987) with localization of putative AChR proteins by [^{3}H]ligand binding (Clarke et al., 1985b) or [^{125}I]mAb binding (Swanson et al., 1987) is much more difficult because the mRNAs would be expected to be in the cell body, whereas the proteins would be both in the cell body and could be transported to the ends of distant axons and dendrites. The observations of AChR synthesis in rat retina and axonal transport to tectum or superior colliculus (Swanson et al., 1987), where both [^{3}H]ligand binding (Clarke et al., 1985b) and [^{125}I]mAb binding (Swanson et al., 1987) are intense, but cDNA hybridization is not observed (Goldman et al., 1986j, 1987) is a specific example. Another problem is that binding of cholinergic ligands or mAbs probes a small region of a protein formed by 6–10 amino acids which is either present or absent in a recognizable conformation in various AChR homologs, whereas *in situ* hybridization with a digest of a whole subunit cDNA containing many sequences that are highly conserved between many members of the AChR gene family as well as unique sequences may result in some degree of hybridization with several AChR homologs or αBgt-binding components. Solutions to this problem of crosshybridization are to hybridize with a short unique sequence from a coding region or a 3' untranslated region. A further complication is that although patterns of binding of [^{3}H]ACh and [^{125}I]αBgt are distinct, they overlap (Clarke et al., 1985b), and cells are known that express both AChRs and αBgt-binding proteins (Smith et al., 1986). The hybridization pattern of α3 (λPCA48) for putative ganglionic-type AChRs was initially reported to correlate with the pattern expected for [^{3}H]nicotine-binding sites over the regions examined (Boulter et al., 1986), though, in fact, intense labeling of hippocampal regions in a pattern characteristic of [^{125}I] αBgt-binding (Clarke et al., 1985b) was also observed. Later, it was reported (Goldman, 1987) that the pattern of α3 hybridization differed from the pattern of hybridization with cDNA α4, which codes the ACh-binding subunit of the high affinity AChR purified using mAb 270. Ultimately, when localization of AChR mRNAs and proteins can be precisely correlated, it will provide a clear picture of sites of AChR synthesis versus sites of AChR function.

Figure 3 compares the mAb and cDNA approaches to studies of neuronal nicotinic AChRs to the present and slightly into the



Location	Immunohistochemistry.	In situ hybridization.
Structure	Protein sequence. Subunit structure and stoichiometry. Transmembrane orientation. Posttranslational modifications. Clone cDNAs using mAbs	Nucleic acid sequence. Genomic structure Make mAbs using synthetic or expressed peptides.
Function	Ligand binding of immunoisolated AChRs. mAbs to effect function in vivo.	Electrophysiology and pharmacology of AChRs expressed in oocytes. In vitro mutagenesis.
Synthesis	Translation and assembly.	Transcriptional regulation.
Disease	Changes in AChR amount. Autoantibodies.	Genetic variants.

Fig. 3. mAb and cDNA approaches to studying neuronal nicotinic AChRs.

future, emphasizing the parallel and complementary natures of these approaches.

It is interesting to compare the relative advantages and disadvantages of the various approaches taken to studying neuronal nicotinic AChRs. Pharmacological, immunological, and cDNA approaches to the AChR molecule are all necessary and complementary. Pharmacology provides the definition of the AChR, gives clues to its functional role, and provides knowledge of affinities and localization by autoradiography. Affinity labeling reagents have proven especially valuable in identifying subunits that contain ACh-binding sites. Cholinergic ligand affinity columns effective for purification of these AChRs have not been developed. The toxins available are useful, but lower in affinity and amount than αBgt, so part of their role has been superceded by mAbs. mAbs have proven highly specific for location, purification, and characterization of neuronal AChRs. Thus far, the mAbs available, unlike snake toxins, do not block function. However, neuronal Bgt and mAb 35 have been shown to identify the same AChR on ganglionic neurons. A disadvantage of immunoaffinity purification is that elution from an immunoaffinity column cannot be done competitively and therefore denatures much of the purified AChR. However, the observation that the mAbs do not block ligand binding permits pharmacological studies on AChRs immunoisolated from detergnt extracts of brain. The antibodies have not proven very effective at selecting cDNAs in the λgt11 expression cloning system (Schoepfer, Whiting, and Lindstrom, unpublished; Grenningloh and Betz, personal communication). Expression cloning of subunits of the AChR gene family may be difficult because the proteins may not be expressed in a form readily accessible to antibodies or because, as membrane proteins, they are toxic to the bacteria. Small fragments of the *N*-terminal 200 residues of α subunits are produced by bacteria in large amounts as inclusion bodies (Barkas et al., 1987; Luyten, Heinemann, and Lindstrom, unpublished). cDNAs under low stringency hybridization conditions have proven very effective for detecting AChR subunit homologs. cDNA sequencing is much easier than protein sequencing. Expression of mRNAs from cloned cDNAs in oocytes offers a way to study the electrophysiological properties of neuronal AChRs and to correlate this with function by in vitro mutagenesis. *In situ* hybridization for localization of AChRs has several ambiguities as compared to immunohistological and ligand autoradiographic techniques. cDNA technology is not ideal for determining subunit composition or stoichiometry. But cDNA techniques are extremely powerful and can be very useful in conjunction with biochemical, immunochemical, pharmacological, electrophysiological, histological, and other approaches.

Functional Roles of Neuronal Nicotinic Receptors

AChRs at neuromuscular junctions respond to ACh released from the nerve ending. The overall effect of chemical transmission at this synapse is to act as an amplifier of the small currents required for conductance along the small nerve fiber to the much larger currents required for conductance along the large muscle fiber. It is so important that the muscle fire every time the nerve does, that a large safety factor is built into neuromuscular transmission. Because of its strategic importance, neuromuscular transmission is the target of many poisons, both natural (e.g., curare, αBgt, histrionicotixin, lophotoxin, anatoxin) and human made (e.g., nerve gas).

AChRs in ciliary ganglia (Berg et al., 1985) or adrenal chromaffin cells (Higgins and Berg, 1987) appear to have a functional role basically similar to that of the postsynaptic AChRs in

skeletal muscle, although in the case of adrenal chromaffin cells or PC12 cells the end result of AChR-mediated depolarization is release of other transmitters rather than contraction, as in muscle. Ciliary ganglion neurons contain several transmitters and may have much in common with the complexities of central synaptic transmission (Reiner, 1987).

The observation that AChRs are made by retinal ganglion cells and transported in large amounts to the tectum or superior colliculus poses a more complex problem (Henley et al., 1986b; Swanson et al., 1987; Sargent et al., in preparation). AChRs on axon terminals would not be expected to have a postsynaptic role. There is also evidence that nicotinic AChRs in several other brain areas may be presynaptic and modulate the release of other transmitters (Clarke et al., 1986; Rapier et al., 1985; Wonnacott, 1987). Detailed study of such systems with immunoelectron microscopic localization of these AChRs may prove quite informative. The observation of AChRs on sensory neurons is especially puzzling (Swanson et al., 1987). Is this an artifact of immunological crossreaction, sloppy gene regulation or protein transport, or an unanticipated but interesting phenomenon? AChRs in the superior colliculus can be excessively stimulated by endogenous ACh when ACh esterase is inhibited, and this results in increased glucose utilization in the superior colliculus (Dam and London, 1983; London et al., 1985; Pazdernik et al., 1982). Exogenous nicotine has the same effect (Greenwald et al., 1987). These experiments show that the presynaptic AChRs in the superior colliculus are active, though the source of ACh, ultrastructural localization of the AChRs, and determination of which cells are activated by what transmitters remains to be clarified. The source of ACh may be axons from the nucleus isthmi (Desan et al., 1987). The nicotinic AChRs on rat retinal ganglion cells behave electrophysiologically as ACh-gated channels, as expected (Lipton et al., 1986). Curiously, activation of ganglion AChRs in retinal cultures by endogenous ACh was found to reduce neurite outgrowth (Frosch et al., 1986). AChR activation in both muscle (Merlie et al., 1984; Evans et al., 1987) and PC12 cells (Greenberg et al., 1986) has been shown to influence gene regulation. One wonders if there might not be sites in the nervous system at which AChRs function neither postsynaptically nor presynaptically in transmitting electrical signals on the millisecond timescale in association with an immediately juxtaposed nerve ending releasing ACh; but instead (or also) respond to ACh released less focally as a modulator of nerve excitability or growth, in a plastic developmental situation like optical dominance (Imamura and Kasamatsu, 1986). Neuromuscular transmission is very clearly understood, but in the brain it has not even been demonstrated with equal certainty that ACh is the natural ligand of all of these AChRs (Sershen et al., 1984; Perry et al., 1987).

Much more work will be required to define the functions of brain nicotinic AChRs at the molecular, cellular, and systemic levels. Immunological localization of AChRs in many regions of the brain, especially visual, other sensory, and motor regions suggest a wide range of effects of these AChRs (Swanson et al., 1987). In particular, immunological localization on dopamine-containing cells is relevant to numerous studies, indicating that nicotinic AChRs affect dopamine release (De Belleroche et al., 1971; Baizer and Weiner, 1985). Localization in the pineal gland is relevant to evidence for nicotinic effects on circadian rhythms (Keefe et al., 1986). Immunological localization of AChRs in a ventrolateral chemosensitive zone of the medulla which regulates blood pressure is relevant to evidence for a nicotinic role in spontaneous hypertension (Yamada et al., 1987).

Evolution of the Nicotinic AChR Gene Family

The nicotinic AChR superfamily as expressed in a chicken includes at least eighteen genes, including the α, β, γ, and δ subunits of muscle AChRs plus ε and perhaps other subunits that distinguish between junctional and extrajunctional AChRs, α, β, and β' genes for brain AChRs (assuming that the α subunits of both AChR forms are identical), α and β genes for ganglionic AChRs (at least the β gene must differ from brain), perhaps four subunits of the neuronal αBgt-binding protein, two subunits of the glycine receptor, two subunits of one subtype of the GABA receptor (and perhaps other subtype subunits), and probably subunits of other ligand-gated channels as well as pseudogenes. In fact, there could also be more AChR subtype genes, and the products of the recognized genes could also differ as a result of alternate transcription. In addition, there could be related genes for proteins that do not retain ligand-gated channel activity and are not easily recognized biochemically, but might be detected by low stringency DNA hybridization.

The nicotinic AChR superfamily does not include muscarinic AChRs that are members of a gene family that includes adrenergic receptors, rhodopsin, and other membrane proteins that interact with G proteins (Dixon et al., 1986; Kubo et al., 1986). Neither does the AChR superfamily include the potential-regulated sodium channel mediating the action potential (Noda et al., 1984). The membrane-potential regulated channels for sodium (Noda et al., 1984), potassium (Tempel et al., 1987), and calcium (Tanabe et al., 1987) form a gene family characterized by the presence of a similar putative transmembrane voltage-sensitive domain.

Other receptor gene families may share common structural features determined by their effector rather than their transmitter; for example, the muscarinic AChR, adrenergic receptor, rhodopsin family shares a common interaction with G proteins, whereas the steroid receptor gene family shares a DNA-binding domain (Arizza et al., 1987), and the protein growth factor receptor gene family shares a protein kinase domain (Ulrich et al., 1985; Downward et al., 1984). The effector domains may be more conserved and form the basis of gene families in part because the effector domain involves a region of multipoint interaction between one protein and another conserved macromolecule (G protein, DNA, tyrosine kinase substrate protein and so on). The specificity of the transmitter-binding site can be changed between an array of smaller molecule transmitters or hormones by the mutation of only a relatively few residues, and the same transmitter can be bound in different ways by unrelated proteins (e.g., by muscarinic or nicotinic AChRs) and still trigger the effector, but the effector must be very conserved if it is to interact with another conserved macromolecular system.

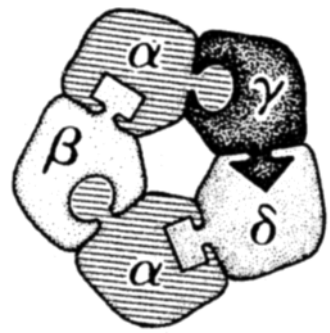
There is recent evidence that suggests that both glycine receptors (Grenningloh et al., 1987) and GABA receptors (Schofield et al., 1987) exhibit sequence homologies with AChR subunits, even though glycine and GABA receptors have anion channels, whereas AChRs have cation channels. Thus, the neurotransmitter receptors with intrinsic ion channels may form a superfamily, just as other receptors with a common effector form superfamilies. In this case, the effector (the ion channel) is intrinsic to the receptor. Conservation of the basic ion channel may be because evolution of the channel involved interaction of several macromolecular subunits; but the ionic specificity of the channel could be altered from cations to anions by changing the charge of only a few residues near the lumen of the channel, and the transmitter specificity could be altered by

changing only a few residues in the ligand-binding site.

The ACh-binding subunit of AChRs from muscle and brain have disulfide-linked cysteines at positions 192, 193 near their ACh-binding site (Kao et al., 1986; Goldman et al., 1987; Whiting and Lindstrom, 1987a). The GABA-binding subunit of its receptor (Schofield et al., 1987), and the glycine-binding subunit of its receptor (Grenningloh et al., 1987) lack these cysteines. The susceptibility of the αBgt-binding protein to affinity labeling by MBTA and BAC after reduction by DTT (Norman et al., 1982; Kemp et al., 1985) suggests that it has this pair of cysteines, as does the insect AChR (Filbin et al., 1983). These cysteines may distinguish the ACh-binding subunit lineage of the AChR super-family.

All subunits of receptors in the AChR super-family have cysteines at about positions 128 and 142 (Noda et al., 1983b; Goldman et al., 1987; Grenningloh et al., 1987; Schofield et al., 1987). This conserved sequence allows for good probe sequences for identifying other members of the gene family (Buonanno et al., 1986). Although part of this sequence is on the extracellular surface, it is partially buried or highly conformationally constrained because anti-peptide antibodies to this sequence do not bind especially well to the native AChR (Criado et al., 1986). Even though this sequence is conserved rather well between subunits of electric organ AChR, mAbs are so specific that most anti-peptide mAbs to this sequence are subunit-specific (Criado et al., 1986). The cysteines at about 128 and 142 are probably linked by a disulfide bond and adjacent to a site of glycosylation on the extracellular surface at asparagine 141 (Mishina et al., 1985; Criado et al., 1986). Disruption of this disulfide bond in α subunits of electric organ AChRs inhibits assembly of γ and especially α subunits with complexes of α and β subunits (Mishina et al., 1985). A disulfide-linked loop between 128 and 142 may be involved in subunit interactions.

All subunits of receptors in the AChR superfamily have four hydrophobic sequences termed M1, M2, M3, and M4 (Noda et al., 1983b; Goldman et al., 1987; Grenningloh et al., 1987; Schofield et al., 1987). These conserved regions are probably excellent regions for choosing probe sequences to identify other members of the gene family by low stringency hybridization. These regions are probably not good sites for making anti-peptide antibody probes because these sequences are probably buried within the protein. All are frequently cited as being probable transmembrane domains, often quite uncritically. There is experimental evidence from the location of binding sites for channel blockers in M2 (Giraudat et al., 1986; Hucho et al., 1986). The expression of AChRs with altered ion transport after alteration of the sequence of M2 and the region between M2 and M3 (Imoto et al., 1986), and immunological detection of sequences between M1 and M2 on the cytoplasmic surface (Criado et al., 1985) suggest that at least M2 may be a transmembrane domain associated with the ion channel. However, it is important to remember that the original rationale for identifying hydrophobic sequences in proteins as putative transmembrane α helices was to explain how to get a single polypeptide chain through the hydrophobic center of a lipid bilayer formed by the oily interactions of lipid fatty acid side chains (Kyte and Dolittle, 1982). This is a compelling rationale when considering a protein with a single transmembrane domain. However, in a macromolecule the size of an AChR, it seems likely that most of these twenty or so hydrophobic domains are in fact interacting with other parts of the interior of the protein, rather than surrounded by lipid, and some may be partially exposed to the surface. It is interesting that there is now evidence from in vitro mutagenesis experiments that the ligand binding site of the adrenergic receptor is in fact formed by residues initially assigned to a putative transmembrane α helix (Strader et al., 1987).

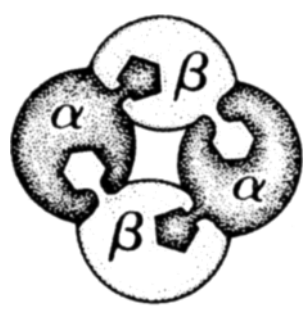


Muscle-type AChR

$\alpha_2\beta\gamma\delta$

Neuronal AChR

$\alpha_3\beta_2$



Neuronal AChR

$\alpha_2\beta_2$

Each subunit in electric organ AChR must be strongly bound to at least two others. Subunits are thought to be oriented like barrel staves around the cation channel in the order αβαγδ (Hamilton et al., 1985; Kubalek et al., 1987). AChR subunits are strongly hydrophobically associated and can only be dissociated by the denaturing detergent SDS; not by extremes of pH or ionic strength, as can the more loosely associated subunits of many soluble proteins. Each β subunit might be thought of as having a key that it inserts in an a subunit lock, while another α subunit inserts its key in the β subunit lock. Similar lock-and-key interactions between α, γ, δ, and α would complete assembly of an AChR. The pattern of keys and locks would assure that the subunits assembled in the right order and conformation. This is illustrated in Fig. 4.

The rigid requirements of these locks and keys are probably reflected in the hour or more required to assemble an intact AChR from its subunits (Merlie et al., 1983) after the 15–30 min required for the conformational maturation of the individual subunits (Merlie and Lindstrom, 1983; Blount and Merlie, 1987); the

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Fig. 4 (left). Illustration of specific association of AChR subunits through conserved "lock" and "key" domains. all of the subunits have a basically similar structure and have conserved interfaces through which they specifically interact that are depicted as interlocking domains. The conserved shapes of the subunits determine the number which can associate to form a functional AChR. In muscle-type AChRs, the subunits are organized around the central cation channel in the order αβαγδ (Kubalek et al., 1987). In neuronal nicotinic AChRs there is some evidence that a similar stoichiometry of two ACh-binding subunits and three structural subunits may be conserved (Whiting et al., 1987b,d). However, it is difficult to determine stoichiometry precisely, and a simpler αβαβ structure may actually be the case. Both stoichiometries for neuronal AChRs are illustrated. It has been argued that the stoichiometry of GABA receptors is $\alpha_2\beta_2$ (Mamalaki et al., 1987).

conformational changes of α subunits that occur after assembly with the other subunits (Blount and Merlie, 1987); and the necessity for all subunit types to be present to efficiently assemble functional AChRs. (Mishina et al., 1984). The conserved nature of these locks and keys is probably reflected in the observation that poorly functional electric organ AChRs can be inefficiently assembled in the absence of one structural subunit (either β, γ, or δ) (Kurosaki et al., 1987). Their conserved nature may also be reflected in the observation that these hydrophobic domains, especially M1, M2, and M3, are highly conserved in sequence.

It seems extremely unlikely that protein quences would need to be highly conserved to interact with the amorphous oily interior of a lipid bilayer. But it seems very likely that protein sequences of these domains would have to be strongly conserved to insure the proper lock and key interactions at protein interfaces between subunits that result in the formation of an ion channel from multiple subunits, which is the hallmark of the AChR superfamily. Thus, some of these hydrophobic sequences are very likely not a helices extending from one side of the membrane to the other through the oily center of the bilayer, but instead are more complexly conformed sites of interaction between subunits. There is experimental evidence that M4 is not a transmembrane domain (Ratnam et al., 1986a,b). The evolutionary difficulty of arranging these complex interactions that are the organizing principle of the ion channel formation is probably the basis of the observed conservation of these sequences and the reason for the evolution of this gene family.

An amphipathic α helical transmembrane domain proposed to form the barrel stave contributed by each subunit to the lining of the ion channel by some groups (Guy, 1983; Finer-Moore and Stroud, 1984) is not conserved in members of the AChR gene family (Goldman et al., 1987; Grenningloh, 1987; Schofield et al., 1987). This is consistent with direct experimental evidence that in electric organ AChRs this putative transmembrane domain is actually on the cytoplasmic surface (Ratnam et al., 1986a,b) and that in vitro mutagenesis that alters or deletes this sequence still permits the formation of partially functional AChRs (Tobimatsu et al., 1987).

Members of the AChR superfamily characteristically exhibit long sequences C-terminal of M3 which are very poorly conserved (Noda et al., 1983b; Boulter et al., 1986; Goldman et al., 1987; Grenningloh et al., 1987; Schofield et al., 987). In electric organ AChRs there is evidence that this region is on the cytoplasmic surface, not rigidly conformationally constrained, and rather extended in conformation (capable of being bound simultaneously by several antipeptide mAbs bound to adjacent sequences) (La Rochelle et al., 1985; Ratnam et al., 1986b,c; Kordossi and Tzartos, 1987). This region may contain sites for phosphorylation (Huganir et al., 1986; Safran et al., 1986) involved in regulation of desensitization (Huganir et al., 1986), turnover, or other properties. This region might also contain sites for interaction with organizing elements like the 43,000 mw protein associated with muscle-type AChRs (Sealock et al., 1984) or cytoskeletal elements involved in the intracellular transport of AChRs or their localization at particular post- or presynaptic locations. The β and β' subunits of the AChR subtypes of chicken brain (Whiting et al., 1987d) might be expected to contain diverse segments in this region. This loose cytoplasmic domain appears to allow considerable variability and exist free of the conserved restraints that define the ligand-gated ion channel features of this gene family. This is a good region for making subunit-specific mAb and cDNA probes. In electric organ AChR, this part of all of the subunits is rather highly immunogenic (Ratnam et al., 1986a,b; Ralston et al., 1987; Souroujon et al.,

1986), perhaps in part because it is flexible and on the surface, and in part because interspecies sequence conservation is low in this region.

It is interesting that *Aplysia* contain a nicotinic AChR that, like GABA, regulates an inhibitory chloride-channel (Kehoe et al., 1976; Ono and Salvaterra, 1981). This suggests that it may be possible for an AChR to change its channel specificity from cations to anions by a relatively minor change in the sequences controlling the ion specificity of the channels, perhaps some polar groups around the channel orifice. Evidence that GABA and glycine receptors which have chloride channels are members of the AChR superfamily confirms this possibility (Greenningloh et al., 1987; Schofield et al., 1987).

Realization that AChRs and GABA receptors have related structures provides an interesting perspective on their ligand binding properties. GABA receptors having anion channels are inhibitory, whereas AChRs having cation channels are excitatory. GABA receptors and neuronal AChRs both have only two kinds of subunits (Mamalaki et al., 1987; Whiting et al., 1987b). GABA receptors are activated at the GABA binding site by the agonist muscimol and inhibited by the antagonist bicuculline (Olsen, 1985). Similarly, there are AChR agonists like anatoxin and antagonists like curare. The anion channel of the GABA receptor is inhibited by picrotoxin, which acts as a convulsant, and this binding site and the others on the receptor interact allosterically (Olsen, 1985). Similarly, the cation channels of AChRs are inhibited by binding of histrionicotoxin, phencyclidine, and local anesthetics, which inhibit transmission through a site that interacts allosterically with the ACh-binding site (Changeux et al., 1986). The inhibitory action of GABA is enhanced by two classes of central nervous system depressant drugs, the benzodiazepines and barbiturates, which interact allosterically with the other binding sites (Olsen, 1985). AChRs have also been reported to interact with opiates (Oswald et al., 1984) and barbiturates (Dodson et al., 1987). Thus, both receptors are affected by many drugs and toxins acting at several functionally important parts of the proteins other than the transmitter binding site. In some cases these sites may be quite specific, whereas in other cases, perhaps in the case of general anesthetics (Evers et al., 1987; Tas et al., 1987) function may be disturbed by lower affinity interactions at multiple sites

Snake venom toxins for AChRs started as digestive enzymes, but few members of this gene family retain enzymatic activity, and these proteins now function as cholinergic antagonists (Chiappenelli, 1985). Snake venom toxins no longer interact with their original ligand (or substrate). This is also true of some members of the AChR gene family. The αBgt-binding protein probably evolved from an AChR, but its current function is unknown. A primordial AChR homopolymer may have evolved from a membrane channel or transport protein in the surface of a protozoan or metazoan which was regulated by a ligand other than ACh, or perhaps by phosphorylation, or other modification in the absence of a specific ligand. This polymeric channel may have evolved from a monomeric intrinsic membrane protein not necessarily involved in transport.

The evolution of this gene family looks even more complex when many species are considered. AChRs in the species covered in this review are summarized in Fig. 5. It is also evident that the AChRs indicated in Fig. 5 also share a common ancestor with receptors for GABA and glycine (Grenningloh et al., 1987; Schofield et al., 1987). It may be that the primordial AChR homopolymer has been preserved in insects (Breer et al., 1985). This neuronal AChR binds αBgt. The presence of the muscle-type AChR in elasmobranchs like *Torpedo*, suggests that the subunit structure of muscle-type AChRs was established early in

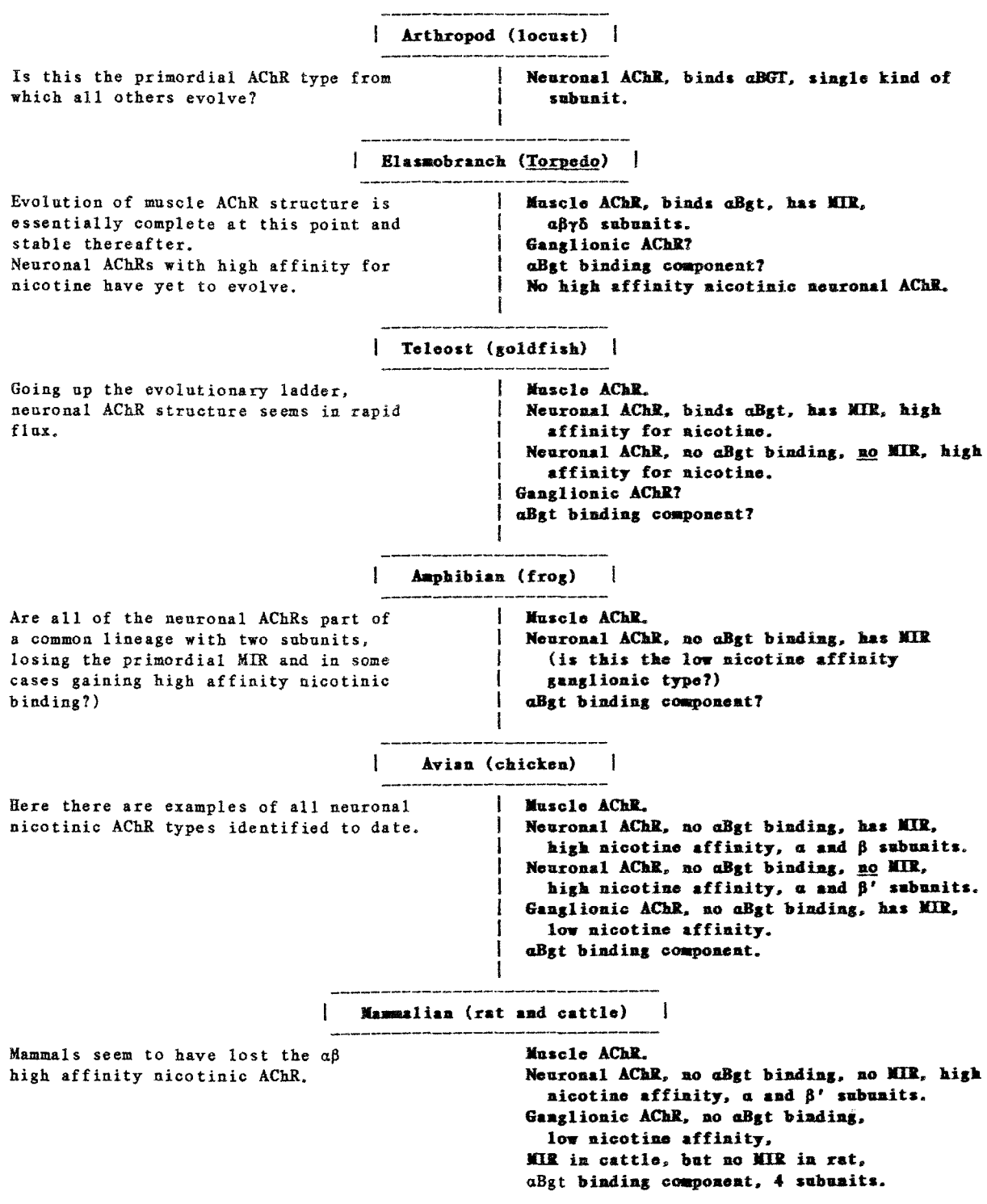

Fig. 5. Evolution of members of the AChR gene family.

evolution. It has changed relatively little in the succeeding 400 million years (Noda et al., 1983b). No high affinity nicotine-binding sites were detected in *Torpedo* brain extracts (Whiting et al., 1987d). But by the time teleosts evolved, both neuronal AChRs that bind αBgt and those with high affinity for nicotine that do not bind αBgt, are found (Henley et al., 1986a,b; Henley and Oswald, 1987). The observation of two brain AChR subtypes in chicken brain (Whiting et al., 1987d), but only one in mammals (Whiting and Lindstrom, 1987b), further gives the impression of rather rapid evolution of neuronal nicotinic AChRs as compared to muscle AChRs. The functional roles of neuronal nicotinic AChRs may be in similarly rapid flux.

Medical Significance

Nicotinic AChRs of muscle are the object of an antibody-mediated auto immune response in myasthenia gravis (Lindstrom, 1985). These autoantibodies do not crossreact with the AChRs or αBgt-binding proteins of human brain (Whiting et al., 1987c). Autoantibodies to brain AChRs were not detected in the sera of patients with Guillain Barre syndrome, amyotrophic lateral sclerosis, multiple sclerosis, or Lambert-Eaton myasthenic syndrome.

The most prevalent known medical involvement of neuronal nicotinic AChRs is their probable role in the behavioral effects and addiction to smoking. The precise mechanisms of nicotine's behavioral effects are not known (Benowitz, 1986; Aceto and Martin, 1982). The evidence for presynaptic nicotinic effects on dopamine release and the involvement of dopamine in pleasure-driven behavior suggest that such a mechanism might be among those involved (De Belleroche et al., 1979; Balfour, 1982).

Alzheimer's disease is a dementia characterized by senile plaques and neurofibrillary tangles. Parallel decrease in [^{3}H]nicotine- and [^{3}H]ACh-binding sites has been found in brains from Alzheimer patients compared to age-matched controls (Shimohama et al., 1986; Flynn and Mash, 1986; Perry et al., 1987). The significance of this alteration in the development of the disease is unclear (Collerton, 1986). However, it is especially interesting to note characteristic degeneration of the optic nerve in these patients (Hinton et al., 1986) in light of the large amount of AChR in retinal ganglion cells (Henley et al., 1986a,b; Swanson et al., 1987; Kaiser et al., in preparation; Sargent et al., in preparation).

Biochemically, Parkinson's disease is characterized by degeneration of dopaminergic neurons in the *substantia nigra.* In addition, a cholinergic involvement is well established. There is autoradiographic evidence for the presence of [^{3}H]nicotine-binding sites on nigrostriatal dopaminergic neurons (Clarke et al., 1985b), and electrophysiological results suggest that nicotine stimulates the dopaminergic cell bodies in the substantia nigra (Clarke et al., 1985a). AChRs have also been localized in these regions using mAbs (Swanson et al., 1987). [^{3}H]nicotine-binding sites were found to decrease in the hippocampus in patients with Parkinson's diesease (Perry et al., 1987). Patients with Down's syndrome also show a decrease in binding sites in the hippocampus (Perry et al., 1987). Presynaptic nicotinic AChRs have long been thought to be involved in release of dopamine (De Belleroche et al., 1979; Balfour, 1982). Cholinergic involvement in Parkinson's disease may involve such a mechanism. Recently, attempts have been made to treat Parkinson's disease by transplanting to the brain adrenal tissue that normally releases catecholamines when nicotinic AChRs on the cells are stimulated. If the trans-

planted cells are regulated by surrounding neurons, it may also be through nicotinic AChRs.

Recently, Yamada et al. (1987) reported a decrease in the density of [^{3}H]nicotine-binding sites in brains of spontaneously hypertensive rats. This biochemical evidence suggests that a cholinergic mechanism may be involved in spontaneous hypertension. This is consistent with the immunological localization of AChRs in the region of the medulla regulating blood pressure (Swanson et al., 1987).

Cholinergic mechanisms may be involved in depression (Dilsaver, 1986), but it is thought that this primarily involves muscarinic AChRs.

In the future, many more medically significant aspects of neuronal nicotinic AChRs may be discovered, because it has not been long since mAb and cDNA probes became available for these AChRs, and the significance of these AChRs in the normal functioning of the brain is not yet clear. It was only when molecular studies of muscle AChRs became possible that the medical significance of muscle AChRs in myasthenia gravis was discovered (Patrick and Lindstrom, 1973; Lindstrom et al., 1976; Lindstrom, 1985).

Future Directions

In the immediate future, it seems likely that the AChR protein subunits and cDNAs that have been identified will be sorted out and correlated and the ambiguities of nomenclature clarified. Localization of AChRs with mAbs will be done at higher resolution in more tissues and species. mAbs will be used in determining the antigenic structure and transmembrane orientation of AChR subunit polypeptide chains, as they have been used in the case of AChRs from muscle and electric organ (Ratnam et al., 1986a,b). mAbs can be used in the case of cultured neurons as they were in the case of cultured muscle cells to study the synthesis and assembly of AChRs (Merlie and Lindstrom, 1983; Blount and Merlie, 1987). The function of neuronal AChRs expressed in oocytes will be studied at the single channel level and in vitro mutagenesis will be used to study the role of various functional domains, as was the case for muscle-type AChRs (Mishina et al., 1984, 1985, 1986).

In the not too distant future, it may be possible to genetically engineer receptor mosaics using ligand-binding domains and channel domains from different members of the superfamily of neurotransmitter receptors with intrinsic ion channels. In vitro point mutations offer even more subtlety. In the not too distant future, X-ray crystallographic determination of the structure of electric organ AChRs should be achieved. The basic concepts gained from this and other studies of the structure and function of the AChR protein should also prove to be to a substantial degree applicable to other members to this superfamily.

Determining the functional roles of neuronal nicotinic AChRs in various neuronal systems is a difficult problem that may not immediately be helped greatly by a ground swell of information about the AChR molecule. However having a better understanding of the AChR molecule and having good probes for it is likely to both focus attention on these problems and facilitate answering them.

The avian and mammalian αBgt-binding protein and other more obscure distant cousins in the AChR superfamily may become much more amenable to study, and determination of their function when mAbs and cDNAs are available for their subunits. These probes should become available from the logical continuation of experiments in progress. It is quite interesting to consider what the function of these proteins might be. Do avian and mammalian αBgt-binding proteins have an endogenous ligand? If so, this may not be ACh, since they are often not located at synapses and do not open cation channels in response to ACh. Their ability to bind ACh might simply be a remanant of their evolution. They might

bind a circulating or local hormone. Alternatively, they may not have an endogenous small ligand, but serve as a link between cytoskeletal and extracellular elements, or some other function. Do αBgt-binding proteins have an ion channel? They do not have an ACh-gated cation channel. They could have a cation or anion channel regulated by another ligand. Alternatively, their channel might be specific for uncharged small molecules, or simply inactive. Current data provides much room for provocative speculation. Future experiments should provide interesting results.

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