# Immunological Approaches to Nicotine Receptors

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Nicotine is a drug whose abuse results in approximately 400,000 deaths per year in the United States alone (Surgeon General 1988). It is by many accounts the most addictive drug available without prescription; nearly 40 percent of those currently addicted have tried to stop using the drug (Schellingt 1992). Although many are successful in overcoming their dependence, 80 percent of those who stop using return to the drug within 24 months (Schellingt 1992). New drug replacement protocols are increasing the success rate, but pharmacokinetic problems of dosage, route, and side effects remain. Clearly, new drugs and new protocols that would help overcome addiction to nicotine would be valuable and might reduce the loss of life that results from its use.

Drugs of abuse such as nicotine exert both short-term and long-term effects through their interactions with specific molecular targets such as receptors, ion channels, and transporters. A powerful approach to the study of these drugs has been the identification and study of their specific binding sites and mechanisms of action. This approach has the straight-forward and valid rationale that if one understands the mechanism of action of a drug one can eventually understand and reduce its potential for abuse. In addition, if one understands the binding site one can perhaps design drugs with great receptor specificity and lower abuse potential, which could be used to break dependence.

Early biochemical data supported this idea that the sites of action of drugs were homogeneous. However, the idea of a single class of sites for each drug essentially precluded the idea of designing ligands that might distin-guish between binding sites associated with abuse and binding sites for the same drug that might not be associated with abuse. Although the apparent homogeneity of binding sites seemingly facilitated biochemical and binding studies, it moved the authors' explanations of drug action from the drug and its interactions with its receptor to some more complex, interactive, but ill-defined aspect of the nervous system. The potential for critical analysis of drug function was essentially limited to those few cases in which the drug-binding sites were restricted to one or a few specific

brain nuclei or cell types. Only under these conditions could one associate the actions of a drug with the function of specific central nervous system (CNS) nuclei.

It is now known that the diversity of drug/receptor interactions far exceeds scientists' earlier estimates. The diversity is found at two levels. First, binding sites previously thought to be homogeneous are now known to be heterogenous (for reviews see Hollenberg 1991 and Scholfield et al. 1990). For example, nicotine was once thought to bind to a homogeneous class of binding sites in brain; it is now known that there are many different oligomeric receptors that bind and are activated by nicotine (for a review see Luetje et al. 1990b). Drugs that bind to gamma aminobutyric acid (GABA) receptors in brain extracts are now known to distinguish between dozens of functionally different GABA receptors (Stephenson and Duggan 1991). Second, drugs bind to specific identified receptors that were not previously thought to bear the appropriate binding sites. Curare, a drug that was once thought to bind exclusively to, and be diagnostic for, nicotinic acetylcholine receptors, is now known to have its highest affinities for serotonin (5-HT3) receptors (Andres et al. 1991). Phencyclidines are no longer thought to be specific for any receptor but appear to block a wide variety of ligand-gated ion channels (Amador and Dani 1991). Even glycine is no longer considered specific for glycine receptors, but is known to be required for activation of one class of the glutamate receptors (Johnson and Ascher 1987). Both the diversity of receptor types and the cross-modality of drug/receptor interactions is likely to become more significant as more is learned.

The consequences of drug use are also more diverse than anticipated. It was once thought that drug use produced changes in the number of specific binding sites, changes in the efficacy of the drug inactivation mechanisms, or perhaps a change in the abundance of an endogenous ligand. The diversity of drug receptors now forces a consideration of changes in the actual structure of the receptor molecules or changes in the distribution of these molecules on the surface of a neuron. One must now consider the possibility that drugs of abuse have long-term effects that result from the expression of specific genes that are activated as a consequence of the action of the drug on one of its many different receptors. Dopamine modulation of progesterone-regulated gene expression is an important example because it may tie either abuse potential or drug need to variations in normal physiological parameters such as endocrine function (Power et al. 1991a, 1991b). Finally, the potential for long-term activity-dependent modification of synaptic function and the roles these

mechanisms play both in the adult and during development is appreciated more fully. This offers potential for under-standing and perhaps ameliorating some adverse effects of drugs on the fetus during development.

There have been many elegant studies of nicotine binding sites in the CNS, both in extracts and in brain slices (Baneerjee et al. 1990; Clarke et al. 1985; Lippiello and Fernandes 1986; Marks et al. 1986; Martino-Barrows and Kellar 1987; Pabreza et al. 1991; Raja et al. 1988; Wonnacott 1987). These studies show that both high and low affinity binding sites are abundant, and that they are widely distributed in the brain. A key contribution from these studies is the observation that the number of binding sites changes with chronic exposure to nicotine (Marks and Collins 1983; Norbert et al. 1983; Schwartz and Kellar 1983). In addition, the toxin alpha-bungarotoxin, which played a major role in the impressive progress made on understanding the nicotinic acetyl-choline receptor present at the neuromuscular junction (Fambrough 1976), also binds to brain membrane preparations and to cells known to contain neuronal nicotinic acetylcholine receptors (Brown and Fumagalli 1977; Carbonetto et al. 1978; Duggan et al. 1976; Jacob and Berg 1983; Patrick and Stallcup 1977a, 1977b). Although more is known about this toxin-binding component (Couturier et al. 1990; Schoepfer et al. 1990; Seguela et al. 1992), its role on neurons is still unknown.

Antibodies against the Torpedo nicotinic acetylcholine receptor recognize proteins in the CNS and have been used to determine the distribution of the antigenic determinants in the chicken brain (Lindstrom et al. 1987). They have also been used to purify the molecules that carry the antigenic deter-minants (Whiting and Lindstrom 1986) and one subunit of these oligo-meric molecules binds the affinity labeling reagent MBTA (Whiting and Lindstrom 1987). There is also good evidence in chick ciliary ganglion neurons that at least one of the antibodies recognizes a functional receptor (Smith et al. 1986). It is also the case that the amino terminal sequence of one of the proteins purified on the basis of its immunological cross-reactivity corresponds to the sequence of a functional neuronal nicotinic receptor deduced from an expressible cDNA clone (Whiting et al. 1987).

An alternative approach to understanding nicotine function in the CNS was based on the idea that nucleic acids encoding muscle nicotinic acetyl-choline receptor subunits would hybridize, at low stringency, to nucleic acids encoding neuronal nicotinic acetylcholine

receptor subunits. This work resulted first in a clone encoding an alpha subunit expressed in the PC12 cell line (Boulter et al. 1986) and subsequently in the isolation of clones encoding nine members of the rat neuronal nicotinic acetylcholine receptor subunit gene family (Boutler et al. 1990; Deneris et al. 1987, 1988; Duvoisin et al. 1989; Goldman et al. 1987; Lamar et al. 1990; Wada et al. 1988) and a rat alpha-bungarotoxin binding component (Seguela et al. 1992). Other labs have isolated clones encoding chicken nicotinic receptor subunits (Nef et al. 1987, 1988) and two chicken alpha-bungarotoxin binding components (Couturier et al. 1990; Shoepfer et al. 1990).

Nine clones define nine genes that are included in the neuronal nicotinic acetylcholine receptor gene family on the basis of their similarities in sequence. These genes are expressed in unique but overlapping sets of anatomical loci in the brain. Using in situ hybridization the combination of neuronal nicotinic receptor genes expressed in over 500 brain loci has been determined (Miller and Patrick 1992; Wada et al. 1989, 1990).

Some but not all of these genes encode subunits that participate in the formation of oligomeric nicotine-gated ion channels. Oocyte expression studies have established four functional alpha subunits (alpha2, alpha3, alpha4, and alpha7) and two functional beta subunits (beta2 and beta4) (Boulter et al. 1987; Deneris et al. 1987; Duvoisin et al. 1989). Expression of functional nicotine-gated ion channels in Xenopus oocytes results when DNA encoding beta2 or beta4 is injected in pairwise combination with DNA encoding either alpha2, alpha3, alpha4, or when alpha7 is expressed alone. Other members of the gene family, alpha5, alpha6, alpha8, and beta3, have not yet to been shown to participate in the formation of functional ligand-gated ion channels.

Different combinations of alpha and beta subunits produce receptors with strikingly different responses to agonists and antagonists. Receptor sensitivities to agonists and antagonists are dependent upon the subunit composition of the receptor, and both the alpha and the beta subunits confer pharmacological and electrophysiological characteristics to these receptors (Boulter et al. 1987; Duvoisin et al. 1989; Luetje and Patrick 1991; Luetje et al. 1990a, 1993; Papke et al. 1989). These receptor-specific characteristics are not simply quantitative differences in agonist sensitivity but are qualitative in nature. A ligand that is an agonist on a receptor composed of one combination of subunits can be an antagonist on a receptor composed of another combination of subunits (Leutje and Patrick 1991). There

are also striking quantitative differences. Nicotine differs by about one hundredfold in its ability to activate certain receptor combinations. These ligands, therefore, provide structural backbones for chemical modifications that would enhance their ability to discriminate between receptor subtypes. In principle, the potential to make subtype-specific agonists and antagonists exists. Such drugs, coupled with knowledge of the known anatomical location of expression of individual subunits in the CNS, will provide a strong rational basis for whole-animal experiments designed to dissect out the roles that different nicotinic receptors may play in behavioral responses to nicotine.

The neuronal nicotinic receptors differ from one another and from the muscle-type nicotinic receptor in two additional ways. They have a substantial permeability to calcium ions (Mulle et al. 1992; Seguela et al. 1992; Vernino et al. 1991) and their function is regulated by calcium ions acting on the outside of the cell (Vernino et al. 1991). The hetero-oligomeric receptors comprised of both alpha and beta subunits have permeabilities to calcium that are about five times that of the muscle receptor and about one-fifth that of the N-methyl-d-aspartate (NMDA) receptor. The homooligomeric alpha7 receptor has a permeability to calcium that is greater than that of the NMDA receptor (Seguela et al. 1992). This suggests that these nicotine receptors play an important role in regulating calcium-dependent cytoplasmic processes and may contribute to activity-dependent neurotoxic cell death. Furthermore, the activation of these receptors by nicotine is regulated by external calcium over physiologically relevant concentrations (Vernino et al. 1991). This regulation by external calcium provides yet another mechanism for control of receptor function. Clearly, these studies on the pharmacology, permeability, and regulation of the various nicotine receptors provide an important foundation on which to build scientists' understanding of the cellular basis of the behavioral and addictive effects of nicotine.

Exploitation of the diversity of these receptors requires reagents to identify specific combinations of receptor subunits in the brain, tools to quantitate these receptors in specific brain loci, techniques to localize receptors on the neuronal cell surface, and mechanisms for testing specific hypotheses that might account for the nicotine-induced regulation of nicotine binding sites. The following paragraphs describe progress in expressing the subunits of neuronal nicotinic receptors in bacterial expression systems, the production of antisera, and the selection and testing of subunit-specific antibodies.

Nine cDNA clones that encode subunits of the neuronal nicotinic acetyl-choline receptors expressed in rat brain have been cloned and sequenced. These cDNA clones provided the primary structure for these proteins and produced functional receptors in single-cell expression systems such as the Xenopus oocyte. Although these clones are extremely valuable they do not, in and of themselves, readily provide access to the in vivo function of the proteins they encode. Neither do they provide biochemical quantities of protein or even biochemical access to the proteins expressed in the CNS. Therefore, they must be used in an expression system to generate reason-able quantities of protein corresponding to each of the nine cDNA clones that encode the members of this gene family. The pET expression system has several advantages (Rosenberg et al. 1987; Studier et al. 1990). It is available in three reading frames and the amount of sequence added to the expressed protein can be both known and controlled. Most important, the system is well designed to protect the inserted coding sequences from selective processes such as deletion until synthesis is initiated. The system is also known to produce large quantities of protein.

Choice of the region of the subunit to be used for antibody production is extremely important and involves several considerations. It would obviously be easier to obtain subunit-specific antibodies if nonconserved regions were used. This would also increase the likelihood of obtaining a good immune response as those sequences not conserved among subunits are generally not conserved across species. The disadvantage to this approach is that the obvious nonconserved sequences are in the cyto-plasmic domains of the receptor and are thus not accessible to the anti-bodies applied to the outside of the cell. Antibodies to this domain will not stain living cells. More importantly, antibodies to this domain cannot be used to distinguish between the receptors on the cell surface and the precursor receptors, targeted for the cell surface, which exist in vesicles inside the cell. This is because the cytoplasmic domains of the cell surface receptors and their precursors are in the same topological space (i.e., the cytoplasm). However, antibodies to extracellular domains will stain living cells and can be used to distinguish between surface and precursor receptors. The disadvantage of the extracellular domains is that they are very conserved and it is difficult to obtain subunit-specific antibodies.

Antibodies were made against the extracellular domain of each subunit and peptides were used to generate specificity as described below. This approach allows production of polyclonal antibodies

against the extra-cellular domain of the protein and affinity purification of antibodies that are specific for each subunit. In addition, it also provides tools for a quantitative mechanism for assessing both the specificity of the anti-bodies and the abundance of the antigen in intact tissue.

Figure 1 shows the regions of the subunits used as immunogens and the sequences chosen for peptide synthesis. Figure 2 shows the sequences of the peptides used to select the subunit-specific antibodies from the poly-clonal sera. It is important that the peptides used to acquire specificity be derived from homologous regions of each subunit so the peptides can be used to demonstrate nonreactivity with peptides derived from the other subunits. Peptides corresponding to this specific region of the protein were chosen for several reasons. First, it is extracellular and the specific sequence offers substantial variability between subunits. Second, it is a region known to promote antibody synthesis in the case of the muscle nicotinic receptor (Bellone et al. 1989; Tzartos et al. 1988) and antibodies directed against this sequence are known to react with the native protein (Tzartos and Lindstrom 1980). Each of the indicated constructs except alpha7 were made and checked for proper insertion and reading frame by sequencing through the ligation sites. Bacteria were transformed with the pET vector containing the indicated insert and induced to make protein. Induced bacteria were harvested and the protein purified by extraction of the inclusion bodies out of detergent solutions. The coomassie-stained gel in figure 3 shows the bands corresponding to each subunit except alpha7.

The authors' approach to generation of specific antisera by making anti-bodies first against the protein expressed in bacteria and then by acquiring specificity through affinity purification is not the usual one. Many laboratories have tried to make antibodies against peptides or to make monoclonal antibodies against bacterially expressed protein. The anti-bodies against the peptides frequently recognize the amino or carboxy-terminal sequences and thus fail to recognize the native protein. Although there are many successful applications of each of these approaches, the production of antibodies against the very similar extracellular domains of neurotransmitter receptors has been difficult. The results in figure 4 show the reaction of anti-beta2 antibody with bacterially expressed proteins and the specificity attained following purification with peptide. The affinity-purified antibody has lost its cross-reactivity to beta4 and retained activity on beta2. The authors have obtained the same results with antibodies directed against each of the other subunits.

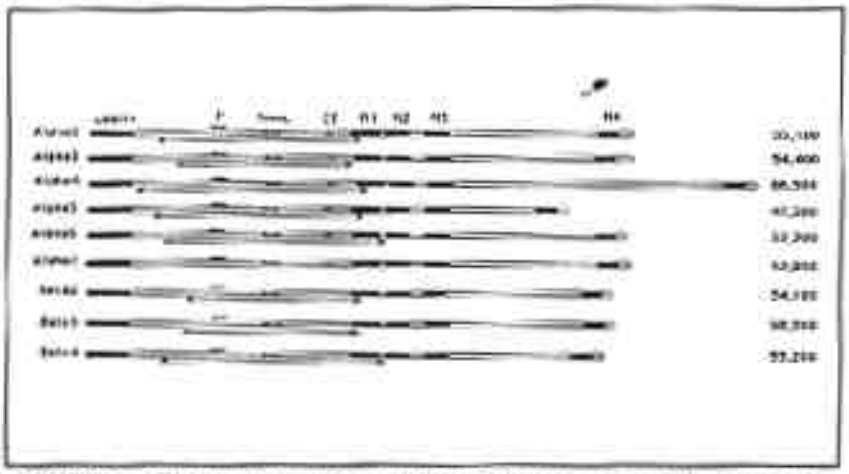

**FIGURE 1.** *This figure represents the coding sequence of the neuronal nicotinic receptor subunits. Characteristic regions of the proteins are indicated. The double arrow shows the region of the extracellular domain chosen for synthesis in the pET bacterial expression system and the dark bar labeled "P" indicates the region corresponding to the peptide used for purification of subunit-specific antibody.*

| | |
|---|---|
| ALPHA2 | DPAEFGNVTSLRVC |
| ALPHA3 | KPSDYQGVEFMRVC |
| ALPHA4 | DPGDYENVTSIRIC |
| ALPHA5 | NPDDYGGIKIIRVC |
| ALPHA6 | DPTEYDGIETLRVC |
| ALPHA7 | NMSEYPGVKNVRFC |
| BETA2 | KPEDFDNMKKVRLC |
| BETA3 | NPEEYGGINSIKVC |
| BETA4 | NSSCYEGVNILRIC |

**FIGURE 2.** *Synthetic peptides corresponding to amino acids 68-81 plus C-terminal cysteine.*

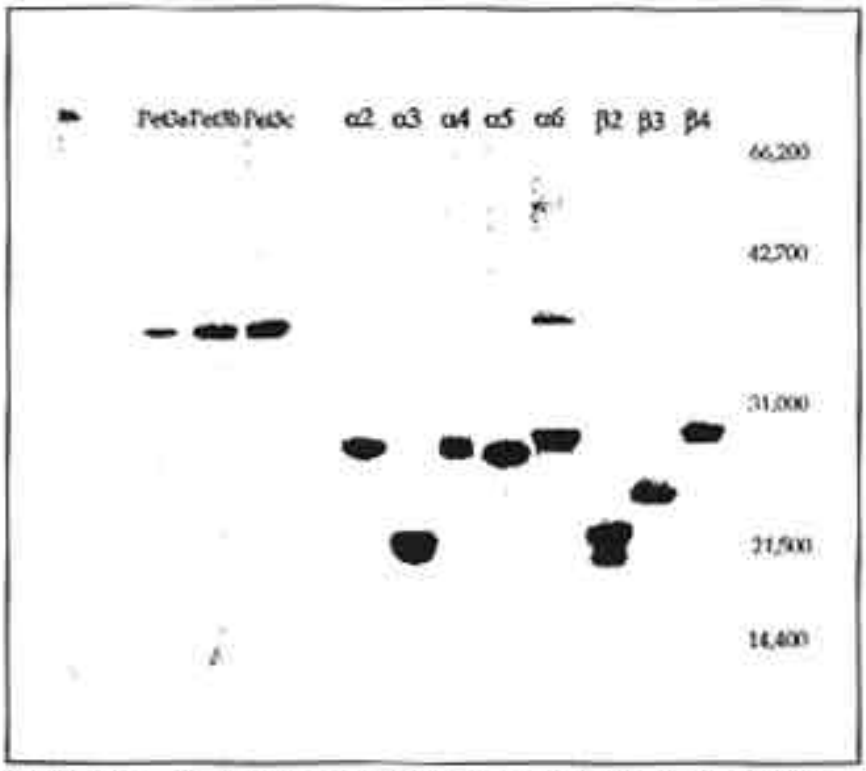


**FIGURE 3.** *Bacterial expression of the amino-terminal extracellular domain of neuronal nAChR's. Bacteria containing the pET vectors and constructs were grown to an $O.D._{600}$ of 0.4 and induced with 1mM IPTG for 3 hr. Bacteria were pelleted, treated with lysozyme (1 mg/mL, 20 min. on ice), sonicated, treated with DNAse I (1 mg/mL, 30 min, 37° C), and sedimented. The insoluble pellet was washed in 1x PBS containing 1 percent deoxycholate, 1 percent b-mercaptoethanol, and 1 percent Triton X-100 and resedimented. The resulting insoluble pellet was washed in the above solution and resuspended in 1x PBS. Proteins were resolved on 15 percent SDS-PAGE using 15 mg of the detergent-insoluble pellet fraction.*

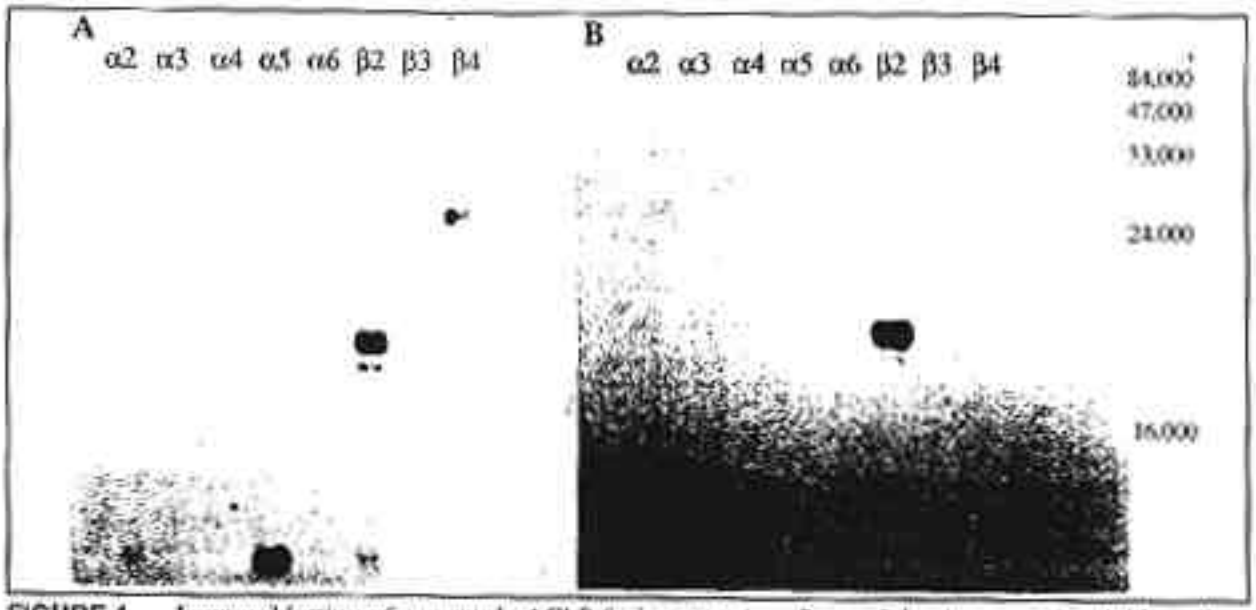


**FIGURE 4.** *Immunoblotting of neuronal nAChR fusion proteins. 2 mg of the detergent-insoluble pellet fraction from induced cultures of E. coli transfected with one of the eight pET constructs was dissolved in SDS PAGE sample buffer, separated by 15 percent SDS-PAGE, and transferred to nitrocellulose. Blots were reacted with (A.) antisera raised against the b2 pET construct or (B.) b2 antisera affinity purified against the b2 peptide. In order to reduce background staining of bacterial proteins and vector-derived proteins, antisera were absorbed against an acetone precipitate of E. coli cultures transfected with the appropriate parental vector and induced for vector-derived protein production.*

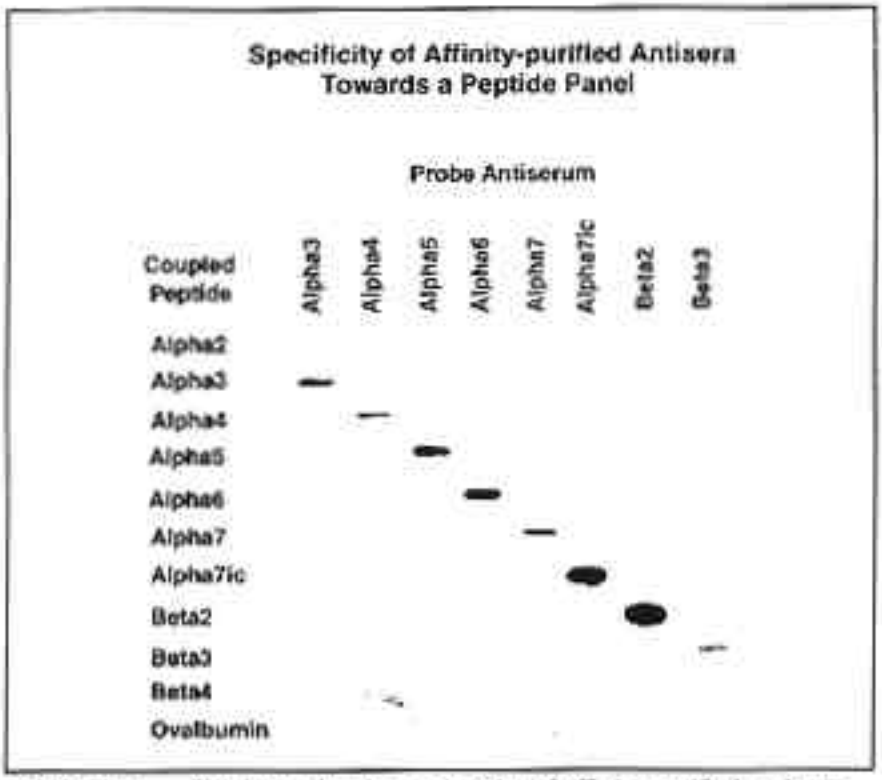


**FIGURE 5.** *Slot-blots showing specificity of affinity-purified antisera toward a peptide panel. Peptides corresponding to amino acid residues 68-81 of the alpha subunit of muscle nAChR were synthesized for each neuronal nAChR subunit and coupled to ovalbumin according to Yoshitake and colleagues (1979). Five ng of each peptide conjugate was slotted onto a nitrocellulose membrane and reacted with antisera purified using the appropriate synthetic peptide. Immunoreactivity was observed only for the corresponding affinity-purified antiserum.*

The authors used affinity chromatography on peptide columns to prepare a subunit-specific antibody for each of the subunits and tested the product on all of the peptides. The data in figure 5 show a slot blot of peptide coupled to ovalbumin and reacted with each of the antibodies (and with an antibody directed against the intracellular domain of alpha7). The results show reactivity with the appropriate peptide and nonreactivity with the inappropriate peptides. These results show that the affinity-purified antibodies distinguish between peptides but do not address the issue of whether they react with native

protein. These antibodies have been used to detect neuronal nicotinic receptors on the surface of transiently expressed COS cells indicating that they do recognize the native protein (Neff et al. 1995).

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