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# Antisera against an acetylcholine receptor α3 fusion protein bind to ganglionic but not to brain nicotinic acetylcholine receptors

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Neuronal nicotinic acetylcholine receptor (AChR) subtypes have been defined pharmacologically, immunologically, and by DNA cloning, but the correlations between these approaches are incomplete. Vertebrate neuronal AChRs that have been isolated are composed of structural subunits and ACh-binding subunits. A single kind of subunit can be used in more than one AChR subtype. Monoclonal antibody (mAb) 35 binds to structural subunits of subtypes of AChRs from both chicken brain and ganglia. By using antisera to a unique sequence of α3 ACh-binding subunits expressed in bacteria, we show that ganglionic AChRs contain α3 ACh-binding subunits, whereas the brain AChR subtype that binds mAb 35 does not. Subunit-specific antisera raised against recombinant proteins should be a valuable approach for identifying the subunit composition of receptors in multigene, multisubunit families.

Acetylcholine receptor, subtype; Antisera; Ganglion; Brain; Bacteria, expression in; Recombinant protein

## 1. INTRODUCTION

Subtypes of neuronal nicotinic acetylcholine receptors (AChRs) have been revealed by several independent methods. Ganglionic- and brain-type AChRs have been traditionally distinguished by pharmacological profiles [1]. Recently, more powerful methods have been used. Monoclonal antibodies (mAbs) raised against purified AChRs and cDNA clones permit far more detailed structural studies ([2–13]; reviewed in [14,15]). These two complementary approaches show that nicotinic AChRs belong to the ligand-gated cation channel family, thus all are assumed to present in the same basic pattern of several subunits assembled around a central ion channel. The primary sequences of the subunits show well-conserved stretches of amino acids separated by less-conserved domains [5,12]. To date, at least seven [2–5,10–12,16] vertebrate genes considered to code for neuronal nicotinic AChRs have been identified. The number of possible AChR subtypes exceeds all previous expectations, thus requiring methods to associate genes with AChR subunits, and subunits with AChR subtypes identified by biochemical and pharmacological approaches.

Previously, we reported the purification and characterization of two AChR subtypes from chicken brains [6,7,17,18]. Together they account for >90% of AChRs in chicken brains which bind nicotine with high affinity. One subtype corresponds to the single predominant subtype of AChR in mammalian brains which accounts for >90% of brain high-affinity nicotine binding sites [17,19]. This subtype is composed of 75 kDa ACh-binding subunits encoded by a gene termed α4 and by 49 kDa structural subunits termed β2

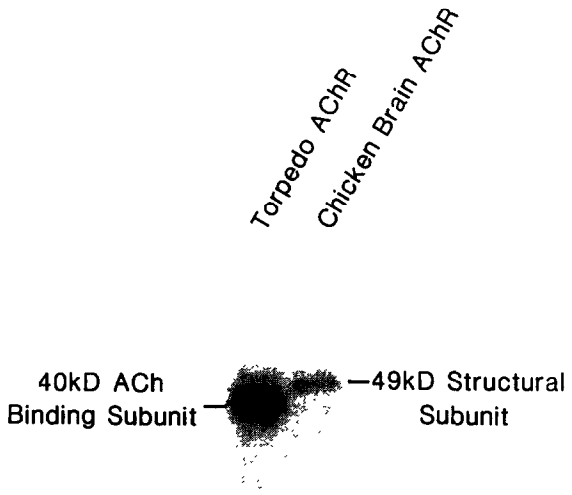


Fig.1. mAb 35 binds to the ACh-binding subunit of *Torpedo* electric organ AChRs, but not to the structural subunit of chicken brain AChRs. Purified AChRs were resolved into their subunits by electrophoresis and the binding of mAb 35 was determined by Western blotting, as described in section 2.

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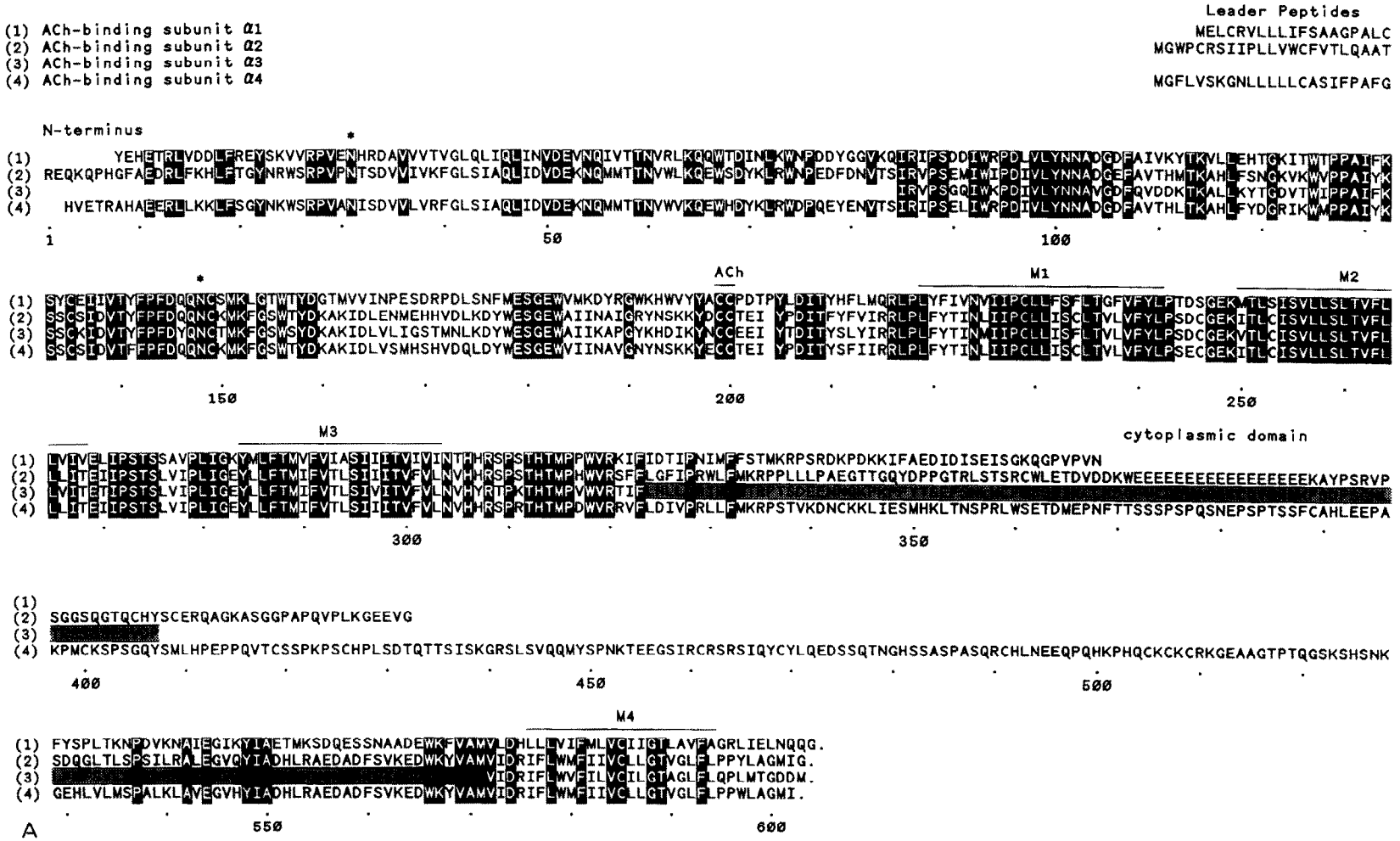
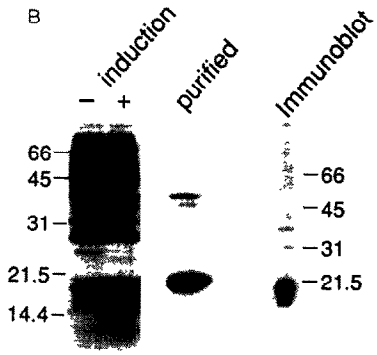



Fig.2. Expression of an $\alpha 3$-specific fragment. (A) Comparison of protein sequences from chicken ACh-binding subunits. Sequences from: muscle $\alpha 1$ [4], neuronal $\alpha 2$, $\alpha 3$, $\alpha 4$ [4,5]. M1–4, hydrophobic segments; *, putative *N*-glycosylation sites; ACh (at position 199–200), putative main ACh binding sites. Conserved sequences are shown in reversed video. The unique stretch of $\alpha 3$ used for expression (position 324–571) is shaded. (B) Bacterially expressed $\alpha 3$ fragment. Coomassie-blue-stained total bacterial lysates with (+) or without (−) induction with IPTG are resolved on SDS-PAGE. The induced culture shows a predominant protCh35-4 band ($M_r = 18\,800$). Purified: protCh35-4 after purification from inclusion bodies. Immunoblot; Western blot of an IPTG-induced total bacterial lysate probed with a 1:400 dilution of antiserum to protCh35-4. Bound antisera was visualized by $^{125}$I-protein A and autoradiography.




or non-$\alpha$, as demonstrated by N-terminal amino acid analysis of purified subunits [5,15,20]. The second subtype of AChR purified from chicken brains is composed of the same structural subunits, but different ACh-binding subunits, with molecular masses of 59 kDa, coded for by a gene yet to be determined [5,7]. These two chicken brain AChR subtypes can be further differentiated by the binding of mAb 35 which was raised to muscle-type AChRs from electric organs and binds to the main immunogenic region on the ACh-binding $\alpha$ subunits of muscle AChRs [21]. mAb 35 binds with high affinity to the AChR subtype with 59 kDa ACh-binding subunits, but with considerably lower affinity to the AChR subtype with 75 kDa subunits coded by $\alpha$4 [6,7]. AChRs on chicken ciliary ganglia (CG) also avidly bind mAb 35 [22–24]. These ganglionic AChRs differ from those in brain by their lower affinity for nicotine [1,25] and higher affinity for neuronal bungarotoxin (Bgt) [24–26].

Here we use antibodies to help determine the genes encoding the subunits of some of these AChR subtypes. mAb 35 is shown to bind to the structural subunits shared by several subtypes of neuronal AChRs. Antiserum to a bacterially expressed, unique sequence of $\alpha$3 ACh-binding subunits is shown to bind AChRs of the subtype found in CG, but not to either of the predominant AChR subtypes in chicken brain.

## 2. MATERIALS AND METHODS

### 2.1. *Western blot with mAb 35*

AChRs affinity purified from *Torpedo* electric organ on a toxin affinity column (5 $\mu$g) [27] or immunoaffinity purified from chicken brain using mAb 295 [7] were electrophoresed on acrylamide gels in SDS, blotted as previously described [7], and probed overnight at 4°C with $^{125}$I-mAb 35 ($5 \times 10^{18}$ cpm/mol, 1nM). After washing, binding of $^{125}$I-mAb 35 was visualized by autoradiography as previously described [7].

### 2.2. *Subunit-specific antiserum*

Standard DNA cloning technology [28] was used to subclone the ~380 bp fragment obtained by a partial *Hph*I/(complete) *Nco*I digestion of pCh35-1 into a pET3c-derived expression vector [29,30], yielding clone pCh35-4. Cloning sites were confirmed by DNA sequencing. pCh35-1 is a partial cDNA clone (Whiting, P. et al., submitted) coding for a chicken $\alpha$3 gene essentially identical to the genomic sequence published by Nef et al. [4]. The 380 bp *Hph*I/*Nco* fragment codes for the peptide Leu315-Met441 (numbered as in [4]) and the deduced complete sequence of the recombinant protein protCh35-4 is: MASMTGGQQMGRIPLLNLLPRIMFMTRPTSDEENNQKPKPF-YTSEFSNLNCFNSSEIKCCKDGFVCQDMACSCCQYQRMKFS-DFSGNLTRSSSSESVDPLFSFSVLSPEMRDAIESVKYIAENMK-MQNEAKEIQDDWKYVAMGLQEFEA (with $\alpha$3 sequences underlined). The additional amino acids are encoded by vector sequences. For expression, pCh35-4 was transformed into BL21(DE3)pLysS. BL21(DE3)pLysS is a variant of BL21(DE3) [31] allowing better expression of toxic genes (Rosenberg A., personal communication). Single colonies were grown to $OD_{600}$ ~ 0.6–1.0 in 50 ml 2×YT, 100 $\mu$M ampicillin and then induced with ~0.5 mM isopropyl-$\beta$-D-thiogalactopyranoside (IPTG). The bacteria were harvested 2–3 h later by centrifugation and the pellet resuspended in 50 mM Tris, 10 mM EDTA, 0.5 mM PMSF, and subsequently lysed by freezing and thawing followed by 3×30 s sonication (Branson 200). After centrifugation for 20 min at 20000 rpm in a Ti50.2 rotor at 4°C, the recombinant protein was found in the pellet, indicating that it was in inclusion bodies. To remove impurities, this pellet was subsequently extracted first by vortexing with 15 ml of 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 0.1 mM PMSF, 1% Triton; then with the same buffer, replacing Triton with 1 M NaCl; and finally replacing the NaCl with 3 M NaSCN. The resulting pellet containing protCh35-4 was dissolved in 10 ml of 8 M urea and 1 mM dithiothreitol (DTT) in phosphate-buffered saline (PBS), and then dialyzed against PBS, 1 mM DTT, 5% cholate, containing urea in step-wise reduced concentration to zero. Rabbit antisera were obtained by subcutaneous immunization with ~200 $\mu$g recombinant protein in complete Freund's adjuvant over a period of 12 weeks.

### 2.3. *Binding of $^{125}$I-mAb 35 and $^{125}$I-$\alpha$Bgt*

Antiserum to the $\alpha$3 peptide was tested for its ability to deplete binding sites for mAb 35 and $\alpha$Bgt from detergent extracts of chick ciliary ganglia and brains. From embryonic day 17–19 (E18) chicks, 40–50 CG or 0.5–0.6 g brains were homogenized in 1 ml or 10 ml, respectively, of 75 mM NaCl, 5 mM Na phosphate buffer, pH 7.4. Membranes were isolated by centrifugation at 15 000×*g* for 15 min. The AChRs were solubilized in 0.5 ml 5 mM $NaPO_4$/75 mM NaCl/1% Triton X-100, pH 7.4, and cleared from debris by 15 min (CG) or 30 min (brain) centrifugation at 15 000×*g*. Aliquots were incubated with antisera or preimmune sera for 60 min at room temperature (RT) in a final volume of 60 $\mu$l. Control reactions were carried out in the absence of sera. Then the antibodies were absorbed onto fixed Staph-A cells (BRL) (the pellet from 30 $\mu$l of a 10% suspension) for an additional 15 min, and cleared by a 15 s centrifugation. Triplicate 30-$\mu$l aliquots of supernate were incubated with 2.5 nM $^{125}$I.mAb 35 for 45 min at RT. The amount of $^{125}$I-mAb 35 bound to AChR was determined by a DE-52 resin column assay (50 $\mu$l resin for CG, 150 $\mu$l for brain) as previously described [6,23]. In separate experiments, triplicate 30-$\mu$l aliquots of extracts were similarly labeled with 10 nM $^{125}$I-$\alpha$Bgt, and bound $^{125}$I-$\alpha$Bgt was measured using a microfiltration assay as previously described [23]. Non-specific binding was determined in the presence of a 100-fold excess of unlabeled mAb 35 or $\alpha$Bgt and was substracted from the total in all cases.

### 2.4. *[$^3$H]Nicotine binding*

Binding of [$^3$H]nicotine to AChRs bound to antibodies was measured basically as previously described [17]. Triplicate 200-$\mu$l aliquots of Triton X-100 extracts of E18 chick brain were incubated at 4°C overnight with 10 $\mu$l diluted antisera and 30 $\mu$l goat anti-rat IgG-coupled Sepharose. The Sepharose pellet was washed twice with PBS, 0.5% Triton by repeated centrifugation. The Sepharose-antibody-AChR complex was then incubated for 15 min in 20 nM [$^3$H]nicotine at RT, followed by three rapid washes with PBS/0.5% Triton X-100 at 4°C. Bound [$^3$H]nicotine in the Sepharose pellet was measured by scintillation counting.

## 3. RESULTS

### 3.1. *Identification of the AChR subunit recognized by mAb 35*

mAb 210, like mAb 35, binds to the main immunogenic region on $\alpha$1 subunits of muscle-type AChRs. Previously, we demonstrated that mAb 210 binds to the structural subunits of both major subtypes of chicken brains AChRs [6,7]. mAb 210 has much higher affinity for native than denatured muscle-type AChRs, but binds specifically to the synthetic *Torpedo* electric organ AChR $\alpha$1 peptide $\alpha$68–76 (M. Das et al., unpublished), a sequence also recognized by several other mAbs to the main immunogenic region [32]. Like mAb 35, mAb 210 binds to the subtype of chicken brain AChR whose ACh-binding subunit has not been determined [7], and it binds to AChRs from CG ([25]; Halvorsen, S. and Berg, D., submitted). The association of $\alpha$4 ACh-binding subunits with structural subunits greatly reduces the affinity of mAb 210 for native chicken brain AChRs of the subtype with $\alpha$4 ACh-binding subunits [7].

mAb 35 is even more dependent on the native conformation of the main immunogenic region of AChRs than is mAb 210 [21,33]. Nonetheless, by using high specific activity $^{125}$I-mAb 35 to label Western blots it was possible to demonstrate specific labeling of denatured subunits (fig.1). Like mAb 210, it labeled $\alpha$1 ACh-binding subunits of electric organ AChRs, but not structural subunits of neuronal AChRs. Thus, an antigenic feature of ACh-binding subunits of muscle-type AChRs is conserved in structural subunits of neuronal AChRs from some species. The demonstration that mAb 35 binds to $\beta$2 structural subunits used by several AChR subtypes may explain the ability of this mAb to bind to AChR subtypes that differ in their ACh-binding subunits.

### 3.2. *Expression of an $\alpha$3 fragment in bacteria*

The sequence between the hydrophobic segments M3 and M4 of AChR subunit genes is highly variable in length and amino acid sequence (fig.2A). This sequence is known to be the cytoplasmic surface of electric organ AChR subunits [34,35], appears loosely organized by electron microscopy [31], and is recognized by many mAbs that bind to both native AChR and its denatured peptides [34,35]. Most subunit-specific mAbs bind in this region [34,35]. Therefore, the sequence between M3 and M4 was used for raising antibodies to the $\alpha$3 subunit.

An $\alpha$3-specific cDNA sequence encoding this putative cytoplasmic domain was cloned into a bacterial expression vector. Only induced bacterial cultures showed a predominant protein (protCh35-4) of apparent molecular mass of 18.8 kDa when analyzed on Coomassie-stained SDS-PAGE gels, which compares well with the calculated molecular mass of 17 kDa (fig.2B). It was found that all of the induced protCh35-4 was present in inclusion bodies which pellet after centrifugation of the bacterial lysate. Subsequent extraction solubilized some contaminating proteins (not shown). After final solubilization of the inclusion bodies in urea plus DTT and low 'renaturation' through removal of urea by dialysis, most of protCh35-4 was still soluble. The resulting recombinant protein was >90% pure as judged by SDS-PAGE (fig.2B). Approximately 15 mg of protCh35-4 was obtained from a 50 ml culture.

### 3.3. *Antisera to the $\alpha$3 peptide bind to AChRs from ganglia but not from brain*

Antisera raised to the purified bacterially expressed $\alpha$3 subunit peptide fragment were highly specific for the $\alpha$3 peptide as shown by Western blots in which only the $\alpha$3 peptide, but no other bacterial protein in a crude extract, was heavily labeled with antibodies (fig.2B).

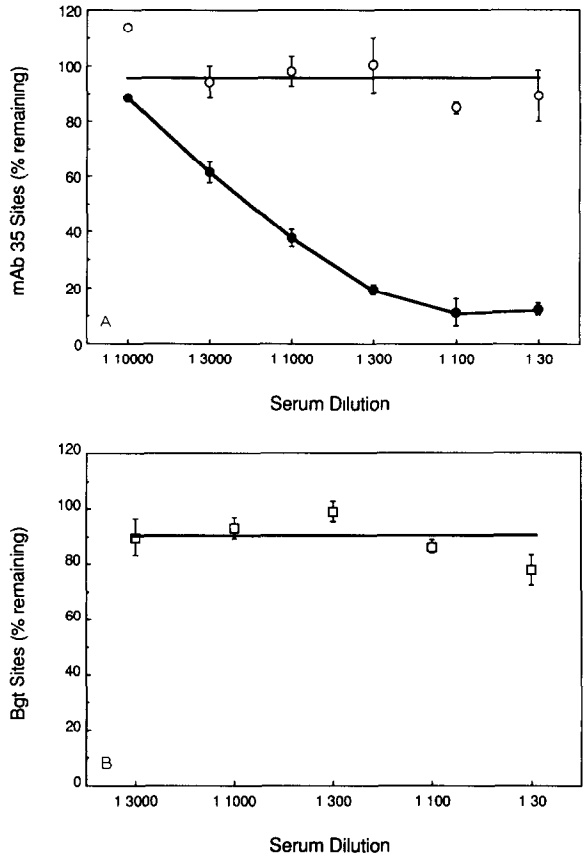



Fig.3. (A) Depletion of CG mAb 35 binding sites by antisera to the $\alpha$3 peptide. Chicken CG extracts (●—●) or brain extracts (○—○) were incubated with antisera to $\alpha$3 and fixed Staph A cells, then assayed for binding of $^{125}$I-mAb 35 as described in section 2. Substitution of antiserum with preimmune sera at 1/300 or 1/30 dilutions resulted in no depletion of $^{125}$I-mAb 35 binding sites. Values indicate the mean of two experiments ± SE, except values at 1/10 000, which are from a single experiment. (B) CG $\alpha$Bgt binding sites (□—□) are not recognized by antisera to the $\alpha$3 peptide. Experiments were carried out as in A, except that $^{125}$I-$\alpha$Bgt binding was measured. Values indicate the mean of two experiments ± SE.

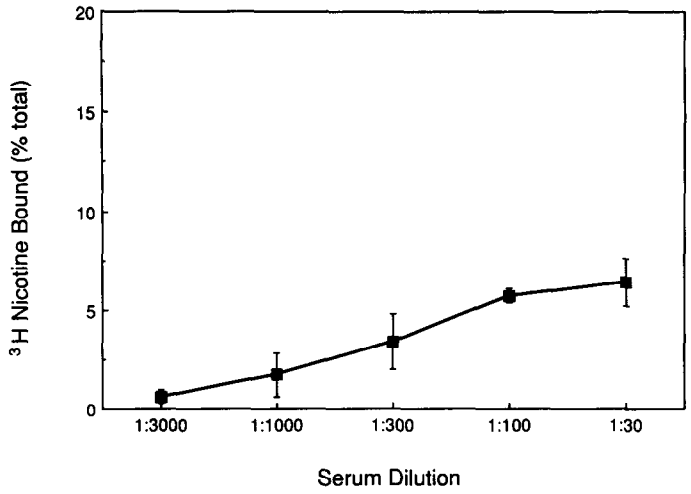



Fig.4 [$^3$]Nicotine-labeled brain AChRs are not efficiently bound by antisera to the $\alpha$3 peptide. mAb 270 was used to determine total [$^3$H]nicotine binding sites in the extract. Values are the mean of triplicate determinations ± SE.

Antisera to the $\alpha$3 peptide were tested for their ability to bind AChRs from chicken CG extracts and brain extracts. AChRs in this assay were measured as mAb 35 binding sites. Greater than 90% of CG AChRs were recognized by the antiserum, while no significant fraction of brain AChRs were (fig.3A). Thus, the ACh-binding subunit of CG AChRs is likely to be encoded by the $\alpha$3 gene, whereas the ACh-binding subunit of the brain AChR subtype bound by mAb 35 is not.

Chicken brain AChRs that bind mAb 35 have high affinity for nicotine [17], whereas CG AChRs do not [24]. Antisera to the $\alpha$3 peptide were also tested for binding to brain high-affinity nicotinic binding sites. Antisera to $\alpha$3 bound less than 7% of high-affinity nicotinic AChRs at antisera concentrations, which bound to virtually all CG AChRs (fig.4). These results using [$^3$H]nicotine-labeled AChRs, together with those in fig.3 using $^{125}$I-mAb 35-labeled AChRs, indicate that AChR subtypes containing $\alpha$3 ACh-binding subunits: (i) probably do not have high affinity for nicotine; (ii) comprise the major AChR subtype in CG; and (iii) are a minor AChR subtype in brain.

### 3.4. *Antisera to the $\alpha$3 peptide do not bind to CG $\alpha$Bgt-binding proteins ($\alpha$BgtBPs)*

In addition to nicotinic AChRs, $\alpha$BgtBPs are found in CG [22,36]. Although neither their exact biochemical structure nor their function is known, they do bind ACh and are considered to be members of the ligand-gated ion channel family [37]. Antisera to the AChR $\alpha$3 peptide do not bind CG $\alpha$BgtBPs.

## 4. DISCUSSION

The growing number of genes considered to code for subunits of nicotinic AChRs requires methods to determine the proteins encoded by the genes and to determine which subunits comprise various AChR subtypes. Due to the multisubunit nature of AChRs, the complexity of this problem is significantly increased by the large number of possible permutations of different subunits in different subtypes. Identical problems exist for $GABA_A$ receptors and probably also for glycine receptors, which are also members of the ligand-gated ion channel superfamily [38–41]. In some cases, it is possible to purify enough protein to chemically determine partial amino acid sequences and thereby identify the cDNA clones encoding the subunits [5,20]. This might be the first approach for identifying a major receptor subtype, but it will be almost impossible for minor subtypes. Here, we took an alternative approach, starting from the available DNA sequences for a putative AChR subunit, by expressing a unique $\alpha$3 gene fragment in bacteria and then using antisera against the recombinant protein as a biochemical probe for the native AChR protein. In contrast to using chemically synthesized peptides as immunogens, recombinant proteins do not require chemical synthesis techniques or machines that may not be available in the laboratory, and they permit expression of relatively long peptides which are difficult to synthesize chemically and that are more likely to contain a desirable epitope. Recombinant proteins from the putative large cytoplasmic domain of subunits in the AChR gene family described here and elsewhere ([49], Schoepfer, R. et al., unpublished; Whiting, P. et al., submitted) have proven to be excellent immunogens, having a significant fraction of antibodies that recognize native subunits.

Others have used the *Xenopus* oocyte and other in vivo expression systems to address the problem of receptor subtypes [42–44]. However, functional expression of combinations of ligant-gated ion channel subunits, which are considered not to exist in vivo, can be obtained in in vitro expression systems [45,46].

Thus, the interpretation of data from in vitro systems has to be seen in context with biochemical data from in vivo systems.

Here we show that mAb 35, like mAb 210 to the same epitope [6] and mAb 270 to another epitope [7], recognize structural subunits that are used by several AChR subtypes. mAbs 35 and 210 also recognize a putative structural subunit of CG AChRs (Halvorsen, S. and Berg, D., submitted). The type of ACh-binding subunit associated with the structural subunit may affect the ability of mAbs to bind to the structural subunit. The structural subunits used by the two prominent subtypes of AChRs from chicken brain are identical by N-terminal sequence [5] and peptide maps [7], but antibodies to the highly conformation-dependent main immunogenic region, such as mAbs 35 and 210, do not bind with high affinity to native AChRs using $\alpha 4$ ACh-binding subunits [7]. However, mAb 270, which binds to another epitope on the extracellular surface of AChR structural subunits, binds well to both AChR subtypes [7].

Here we show that antisera to a unique $\alpha 3$ peptide bind to CG AChRs. This provides strong evidence for an $\alpha 3$-encoded subunit as part of CG AChRs. This is in agreement with the expression of high levels of $\alpha 3$ mRNA in CG, while no $\alpha 2$ and very little $\alpha 4$ message is detectable [47]. Recently we have found that antisera to $\alpha 3$ binds specifically to the ACh-binding subunits of AChRs purified from CG [S. Halvorsen and D. Berg, submitted].

We assume that the rat $\alpha 3$ gene encodes a rat ganglionic AChR as it is expressed in PC12 cells[10]. We cannot test this with the antiserum described here, as the cytoplasmic loop used for obtaining the antiserum is not conserved between chicken and rat, while the rest of the gene is highly conserved. However, the $\alpha 3$ gene is expressed in PC12 at high levels, and when expressed in oocytes in combination with $\beta 2$ structural subunits gives rise to AChRs which, like those in PC12 cells, are blocked by neuronal Bgt [42]. Assuming that $\alpha 3$ codes for AChRs on PC12 cells, our previous observation [48] that the NGF-induced increase of AChRs on PC12 cells is not reflected by increased $\alpha 3$ mRNA amount can now only be interpreted to mean that under these conditions the amount of AChR on the surface of the cells does not increase in proportion to the amount of $\alpha 3$ mRNA.

Because $\alpha 3$ mRNA is also expressed in chicken brain in low levels [4,47, and P. Whiting et al., submitted], we also expect low levels of $\alpha 3$-encoded protein. This low level of $\alpha 3$-containing AChRs in brain might be too low to be detected by depletion of mAb 35 sites by antisera to the $\alpha 3$ peptide (fig.3A). Alternatively, $\alpha 3$ in brain might be associated with a different subunit than in CG, and these may not bind mAb 35. But $\alpha 3$ is not part of the brain AChR that binds mAb 35, has high affinity for nicotine, and is composed of a $\beta 2$-encoded structural subunit together with an as yet unidentified 59 kDa ACh-binding subunit[5,7]. A likely candidate for the ACh-binding subunit of this AChR is $\alpha 2$ (Schoepfer, R. et al., unpublished). The minimal binding of antisera to $\alpha 3$ to nicotine binding sites might reflect an as yet unidentified subtype, or the detection of low-affinity nicotine binding sites on $\alpha 3$ using 20 nM nicotine, or a minimal cross-reactivity to other subunits due to a few conserved amino acids with other genes. mAbs to the $\alpha 3$ subunit (now being prepared) will serve as more specific probes for answering these questions.

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