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Nicotinic Cholinergic Receptors in the Rat Retina: Simple and Mixed Heteromeric Subtypes

Andrea M. Marritt, Brandon C. Cox, Robert P. Yasuda, J. Michael McIntosh, Yingxian Xiao, Barry B. Wolfe, and Kenneth J. Kellar

Department of Pharmacology, Georgetown University School of Medicine, Washington, DC (A.M.M., B.C.C., R.P.Y., Y.X., B.B.W., K.J.K.); and Departments of Biology and Psychiatry, University of Utah, Salt Lake City, Utah (J.M.M.)

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ABSTRACT

Neuronal nicotinic acetylcholine receptors (nAChRs) were measured in the rat retina to determine the heteromeric subtypes. We detected seven nicotinic receptor subunit mRNA transcripts, $\alpha2$–$\alpha4$, $\alpha6$, and $\beta2$–$\beta4$, with RNase protection assays. The density of heteromeric nAChR binding sites is ~3 times higher in the retina than in the cerebral cortex. Moreover, the density of the sites in the retina measured with [^{3}H]epibatidine ([^{3}H]EB) is ~30% higher than with ^{125}I-3-(2(*S*)-azetidinylmethoxy)pyridine (A-85380) and more than twice that measured with [^{3}H]cytisine or [^{3}H](−)nicotine. These data suggest that the retina expresses multiple subtypes of nAChRs, including a large fraction of receptors containing the $\beta2$ subunit and a smaller fraction containing the $\beta4$ subunit. Consistent with this, in binding competition studies, nicotinic ligands fit a model for two affinity classes of binding sites, with the higher affinity sites representing 70 to 80% of the nAChRs in the retina. To determine the specific subtypes of nAChRs in the rat retina, we used subunit-specific antibodies in immunoprecipitation assays. Immunoprecipitation of [^{3}H]EB-labeled nAChRs with antibodies specific to the $\beta2$ and $\beta4$ subunits indicated that ~80% of the receptors contained $\beta2$ subunits and ~25% contained $\beta4$ receptors, consistent with the binding pharmacology results. Sequential immunoprecipitation assays indicated that the rat retina contains multiple subtypes of nAChRs. The majority of the receptors measured seemed to be simple heteromeric subtypes, composed of a single type of α and a single type of β subunit; but a significant fraction are mixed heteromeric subtypes, composed of two or more α and/or β subunits.

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The neuronal circuitry of the mammalian retina involves multiple neurotransmitters and includes acetylcholine (ACh) signaling through neuronal nicotinic acetylcholinergic receptors (nAChRs). These receptors mediate fast excitatory postsynaptic potentials (Lipton et al., 1987) and more subtle presynaptic signals that modulate the release of neurotransmitters, including GABA, glutamate, and possibly dopamine (Neal et al., 2001). Evidence of nAChRs has been found in at least three different cell types in the mammalian retina, including amacrine, bipolar, and ganglion cells, all of which may be stimulated by ACh released from retinal starburst amacrine cells (Masland and Livingstone, 1976; Hutchins and Hollyfield, 1986; Feller et al., 1996; Keyser et al., 2000).

nAChRs exist as subtypes composed of different combinations of α and β subunits. Vertebrate nervous systems express nine α and three β nAChR subunits, and the mammalian retina expresses mRNA transcripts for most of them, including $\alpha2$, $\alpha3$, $\alpha4$, $\alpha6$, $\alpha7$, $\beta2$, $\beta3$, and $\beta4$ (Hoover and Goldman, 1992; Britto et al., 1994; Zoli et al., 1995; Moretti et al., 2004). All of the nAChR subtypes conduct Na^+, K^+, and Ca^{2+}, but the different subtypes have distinguishing biophysical properties such as channel conductances and rates of desensitization and resensitization that could markedly affect signaling in the pathways in which they function. In addition, the receptor subtypes display differences in pharmacological characteristics that in some cases allow subtypes to be distinguished by the affinity and efficacy of certain drugs.

nAChRs mediate signals related to different aspects of retina development and physiology, including those that influence neurite outgrowth from ganglion cells (Lipton et al., 1988) and those that coordinate increases in cytosolic calcium in amacrine and ganglion cells (Wong et al., 1995). In addition, these receptors seem to play a critical role in the proper development of visual pathways. For example, the precise

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ABBREVIATIONS: ACh, acetylcholine; nAChR, neuronal nicotinic acetylcholine receptor; NRS, normal rabbit serum; mAb, monoclonal antibody; EB, epibatidine; A-85380, 3-(2(*S*)-azetidinylmethoxy)pyridine; HFI-55, 3-(5-(((*S*)-azetidin-2-yl)methoxy)pyridin-3-yl)prop-2-ynyl 2-(methylamino)-benzoate.

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projections of the optic nerves from the retinal ganglion cells to their specific targets in the lateral geniculate nuclei and the superior colliculi during development depend on spontaneous bursts of action potentials from the ganglion cells called retinal waves (Meister et al., 1991; Feller et al., 1996). These retinal waves depend on cholinergic transmission from amacrine cells to ganglion cells mediated by nAChRs (Feller et al., 1996; Bansal et al., 2000; Feller, 2002). Moreover, in mice lacking the β2 subunit (β2 knockout mice), retinal waves are absent (Bansal et al., 2000), and the segregation of the axonal projections to eye-specific layers is markedly altered (Hwang et al., 2000).

The presence of several nAChR subunit mRNA transcripts (Hoover and Goldman, 1992; Britto et al., 1994; Zoli et al., 1995) and subunit proteins (Swanson et al., 1987; Britto et al., 1994) suggests the presence of more than one nAChR subtype in the mammalian retina. In fact, Gotti and colleagues (Moretti et al., 2004), using subunit-selective antibodies to immunoprecipitate [^{3}H]epibatidine-labeled receptors, identified multiple nAChR subtypes in the rat retina. The studies reported here also address the question of which heteromeric nAChR subtypes are in rat retina by using sequential immunoprecipitation assays with subunit-specific antibodies to determine the associations of the subunits that comprise putative subtypes. Our data confirm that the rat retina contains multiple nAChR subtypes, including both simple heteromeric subtypes, composed of a single type of α and a single type of β subunit, and mixed heteromeric subtypes, composed of two or more α and/or two or more β subunits; however, some differences from the exact subtypes reported by Moretti et al. (2004) are noted.

Materials and Methods

Materials. Frozen retinas and brains from adult Sprague-Dawley rats were purchased from Zivic-Miller Laboratories (Portersville, PA). [^{3}H]Epibatidine ([^{3}H]EB), ^{125}I-A-85380, [^{3}H](−)nicotine, and [^{3}H]cytisine were purchased or were gifts from PerkinElmer Life and Analytical Sciences (Boston, MA). Dihydro-β-erythroidine was from Sigma/RBI (Natick, MA). An A-85380 analog, HFI-55 was synthesized in collaboration with Drs. John Musachio (National Institute of Mental Health, Bethesda, MD) and Hong Fan (The Johns Hopkins University, Baltimore, MD) and will be described in greater detail in a future article. [α-^{32}P]CTP and [γ-^{32}P]ATP were obtained from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Electrophoresis reagents were purchased from Bio-Rad (Melville, NY). Protein G Sepharose beads were purchased from GE Healthcare. Protein A (Pansorbin) and normal rabbit serum (NRS) were purchased from Calbiochem (San Diego, CA). Nicotine tartrate, cytisine, A85380, and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. Rabbit antisera directed at a bacterially expressed fusion protein containing partial sequences of the cytoplasmic domains of nAChR α2, α4, β3, and β4 subunits were kind gifts from Drs. Scott Rogers and Lorise Gahring (University of Utah, Salt Lake City, UT). These antisera have been described previously (Flores et al., 1992; Rogers et al., 1992). An antibody directed at a peptide sequence of the rat nAChR α3 subunit was affinity-purified from rabbit serum. This antibody has been described previously (Yeh et al., 2001). In addition to these antibodies, a peptide antibody directed at the α6 subunit (R. Yasuda and B. Wolfe, unpublished data) was used. A monoclonal antibody (mAb 270) to the chick β2 subunit was made from hybridoma stocks (American Type Culture Collection, Manassas, VA). This mAb was originally developed and characterized by Whiting and Lindstrom (1987). Table 1 provides references to all of these antibodies and antisera. In some studies, we also measured nAChRs containing α6 and β4 subunits with antibodies that were generously provided by Dr. Cecilia Gotti (University of Milan, Milan, Italy). These antibodies are described in Moretti et al. (2004). For simplicity, in this article we use the term "antibody" to refer to unpurified antisera and to affinity-purified antisera and monoclonal antibody.

RNA Isolation and RNase Protection Assay. Total cellular RNA was isolated using RNA-STAT-60 (Tel Test B, Friendswood, TX). DNA templates for antisense riboprobes were prepared as described previously (Xiao et al., 1998). Antisense riboprobes for the α2–α7 and β2–β4 nAChR subunits were generated from DNA templates using T7 RNA polymerase and [α-^{32}P]CTP. The RNase protection assays were carried out using the RPA II kit (Ambion, Austin, TX). Total RNA (20 μg) from the tissue samples was hybridized overnight at 42°C with the subunit riboprobes and a riboprobe for rat glyceraldehyde-3-phosphate dehydrogenase, which was used as an internal and loading control. After hybridization, nonprotected fragments were digested with a combination of RNase A and RNase T1 for 30 min at 37°C. The numbers of bases of the full-length probes and the protected fragments of the probe, respectively, were as follows: α2, 416 and 332; α3, 306 and 230; α4, 496 and 408; α5, 411 and 380; α6, 462 and 396; α7, 450 and 376; β2, 322 and 263; β3, 430 and 394; β4, 252 and 170; and glyceraldehyde-3-phosphate dehydrogenase, 204 and 135. The protected probe fragments were separated by electrophoresis on a 6% denaturing polyacrylamide gel, and the fragments were visualized by X-ray filming or phosphorimaging.

Receptor Binding. Tissues were homogenized in 50 mM Tris-HCl buffer, pH 7.4 at 24°C, and centrifuged twice at 35,000*g* for 10 min in fresh buffer. The membrane pellets were resuspended in fresh buffer and added to tubes containing [^{3}H]EB, ^{125}I-A-85380, [^{3}H]cytisine, or [^{3}H](−)nicotine with or without competing drugs. Incubations with were carried out in Tris buffer at pH 7.4 for 2 h at 24°C with [^{3}H]EB and ^{125}I-A-85380 or at 4°C in Tris buffer at pH 7.0 for 2 h with [^{3}H]cytisine and [^{3}H](−)nicotine. In assays to assess competition by α-conotoxin MII, the tissues were preincubated in buffer containing a protease inhibitor cocktail (Roche Applied Science, Penzberg, Germany) and then incubated with [^{3}H]EB in the presence of both the protease inhibitors and 0.01 mg/ml bovine serum albumin. Bound receptors were separated from free ligand by vacuum filtration over GF/C glass-fiber filters that were prewet with 0.5% poly-

TABLE 1
Antigenic sequence to which the nAChR subunit-selective antibodies are directed

Subunit	Antigen	Reference
α2	Rat aa371-aa511	Rogers et al., 1992
α3	Rat C terminus CLQPLMARDDT	Yeh et al., 2001
α4	Rat aa461-aa594	Rogers et al., 1992
α5	Rat aa345-aa452	Rogers et al., 1992
α6	Rat IC loop CLDKTKEMDGVKDamide	R. Yasuda and B. Wolfe, unpublished data
β2	mAb270 whole receptor from chick brain	Whiting et al., 1987
β3	Rat aa330-aa464	Rogers et al., 1992
β4	Rat aa328-aa426	Rogers et al., 1992

aa, amino acid.

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ethyleneimine, and the filters were then counted in a liquid scintillation counter. Nonspecific binding was determined in the presence of 300 μM nicotine, and specific binding was defined as the difference between total binding and nonspecific binding.

Immunoprecipitation. Tissue membrane homogenates were prepared as above for binding assays. The receptors were solubilized by incubating the homogenates in 2% Triton X-100 with gentle rotation for 2 h at room temperature. After centrifuging the mixture at 35,000*g* for 10 min, aliquots of the clear supernatant (equivalent to 6 mg of original tissue weight) were added to sample tubes containing [^{3}H]EB and either one of the subunit-specific antibodies at a concentration determined in preliminary studies to be optimal for each or an equivalent volume of NRS. The samples were then rotated overnight at 4°C, after which 50 μl of an ~6% slurry of Pansorbin (source of Protein A) for the polyclonal antisera or a 50% slurry of Protein G Sepharose beads for the mAb was added, and the rotation of the samples was continued for 1 h. The samples were then centrifuged at ~7000*g* for 5 min, and the supernatants were removed and either filtered over GF/B filters wet with 0.5% polyethyleneimine or placed on ice for later use in sequential immunoprecipitation studies. The remaining tissue pellets were washed once with 1.2 ml of 50 mM Tris-HCl buffer, pH 7.0 at 4°C, dissolved in 0.2 ml of 1 N NaOH and then counted in a scintillation counter. After subtracting the number of counts precipitated in tubes containing NRS, the number of [^{3}H]EB-labeled nAChRs immunoprecipitated by each antibody was compared with the total number of labeled receptors in samples of the solubilized retina membranes measured in filtration binding assays.

Sequential Immunoprecipitation. The immunoprecipitation assay provides important information about the presence of any one subunit in an nAChR, but it does not specify the actual receptor subtype, which is defined by its subunit combination. Even the presence of two or more subunits measured independently in the same tissue preparation does not necessarily specify the receptor subtype because the subunits could each be components of different nAChRs. Therefore, to determine the actual receptor subtype based on subunit composition, we used a sequential immunoprecipitation protocol in which the supernatant remaining after immunoprecipitation with the first antibody (here called the clearing antibody), is subjected to a second round of immunoprecipitation with an antibody against a different subunit (here called the capturing antibody). This procedure or a conceptually similar approach has been used successfully to identify the subunit compositions of nAChR subtypes in the rat forebrain (Flores et al., 1992), trigeminal ganglia (Flores et al., 1996), striatum (Zoli et al., 2002), pineal gland (Hernandez et al., 2004), and retina (Moretti et al., 2004), and in chick ciliary ganglia and brain (Conroy et al., 1992; Conroy and Berg, 1995, 1998) and chick retina (Vailati et al., 2003).

For these sequential immunoprecipitation assays, the clear supernatant remaining after immunoprecipitation with the first antibody or NRS was incubated with a different subunit-selective antibody, and the immunoprecipitation steps with Protein A or Protein G were then repeated, as described above. In control studies, the number of solubilized nAChRs labeled by [^{3}H]EB was not decreased when incubated in the absence of an antibody or NRS over the course of the sequential immunoprecipitation procedure.

Statistical Analysis. Data were analyzed using GraphPad Prism 4.0 software package (GraphPad Software, Inc., San Diego, CA). A one-sample *t* test was used in drug binding competition assays to determine whether the Hill coefficients were different from 1 and in immunoprecipitation assays to determine whether residual values were different from 0. The propagation of error method (Bevington, 1969) was used to calculate the S.E.M. for the difference between groups and for comparing the sum of subtypes to the total number of receptors in the retina. Differences between group means were compared using the Student's *t* test or one-way analysis of variance followed by Bonferroni's multiple comparison test.

Results

nAChR Subunit mRNA Expression Levels in the Retina. We detected mRNA transcripts for seven nAChR subunits in the rat retina, which were the following: α2, α3, α4, α6, β2, β3, and β4 subunits (Fig. 1). Each of these has been found in mammalian retina in previous studies using in situ hybridization and/or RNase protection assays (Hoover and Goldman, 1992; Zoli et al., 1995; Moretti et al., 2004). We did not detect α5 or α7 subunit transcripts in the retina with this method, although we did detect them in parallel studies carried out in rat brain and PC12 cells as positive controls (data not shown).

Radioligand Binding to nAChRs in the Retina. Saturation binding studies revealed that the density of nAChRs labeled by [^{3}H]EB is ~3 times higher in the rat retina than in rat cerebral cortex (Fig. 2, inset). [^{3}H]EB labels all known heteromeric nAChR subtypes (Parker et al., 1998; Xiao and Kellar, 2004), whereas other radioligands label subsets of nAChRs. For example, at the concentrations used, ^{125}I-A-85380 has high enough affinity to label all nAChRs composed of α subunits in association with β2 subunits, but not those lacking β2 subunits (Mukhin et al., 2000; Xiao and Kellar, 2004). The affinities of cytisine and (−)nicotine for different nAChR subunit combinations predict that as radioligands they would be even more restrictive, labeling $\alpha4\beta2$ and $\alpha2\beta2$ receptors but probably not $\alpha3\beta2$ receptors (Parker et al., 1998; Xiao and Kellar, 2004).

Saturation binding studies with [^{3}H]EB, ^{125}I-A-85380, [^{3}H]cytisine, and [^{3}H](−)nicotine were carried out to compare the density (B_{max}) of receptors labeled with each radioligand in the retina. As shown in Fig. 2 and Table 2, [^{3}H]EB labeled ~30% more sites than ^{125}I-A-85380 and more than twice the number of sites labeled by either [^{3}H]cytisine or [^{3}H](−)nicotine. The higher density of binding sites measured with [^{3}H]EB suggests that, in addition to the $\alpha4\beta2$ subtype(s), which is thought to be the predominant nAChR subtype in most the of the rat central nervous system, the retina expresses subtypes containing β4 subunits, which would be labeled by [^{3}H]EB but not by the other radioligands. In addition, the data suggest that the retina contains one or more $\alpha3\beta2$* subtypes (where the asterisk indicates the pos-

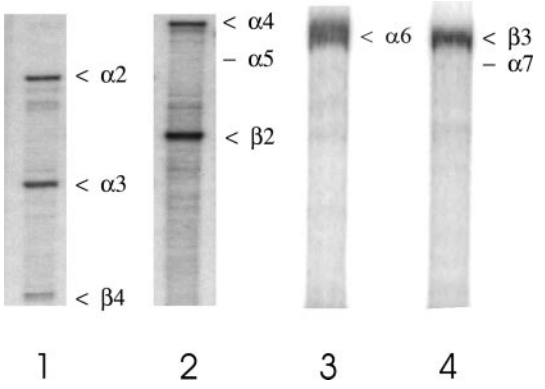

Fig. 1. Expression of nAChR subunit genes in the rat retina. RNase protection assays were carried out as described under *Materials and Methods*. Total RNA from rat retina was hybridized with a combination of ^{32}P-labeled antisense probes corresponding to the nine rat nAChR subunit genes α2, α3, β4 (lane 1); α4, α5, β2 (lane 2); and α6 (lane 3); α7, β3 (lane 4). All mRNA measurements were repeated at least three times with similar results.

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sible presence of additional subunits), which can be labeled by [^{3}H]EB and ^{125}I-A-85380 but not by [^{3}H]cytisine or [^{3}H](−)nicotine.

Despite the ability of [^{3}H]EB to label multiple nAChR subtypes in the retina, curve-fitting and nonlinear regression analysis found that its saturation binding curve fit a model for a single class of binding sites. This probably reflects its relatively similar affinity for the binding sites of all of the known heteromeric nAChR subtypes. Because of this characteristic, [^{3}H]EB does not readily discriminate among these subtypes.

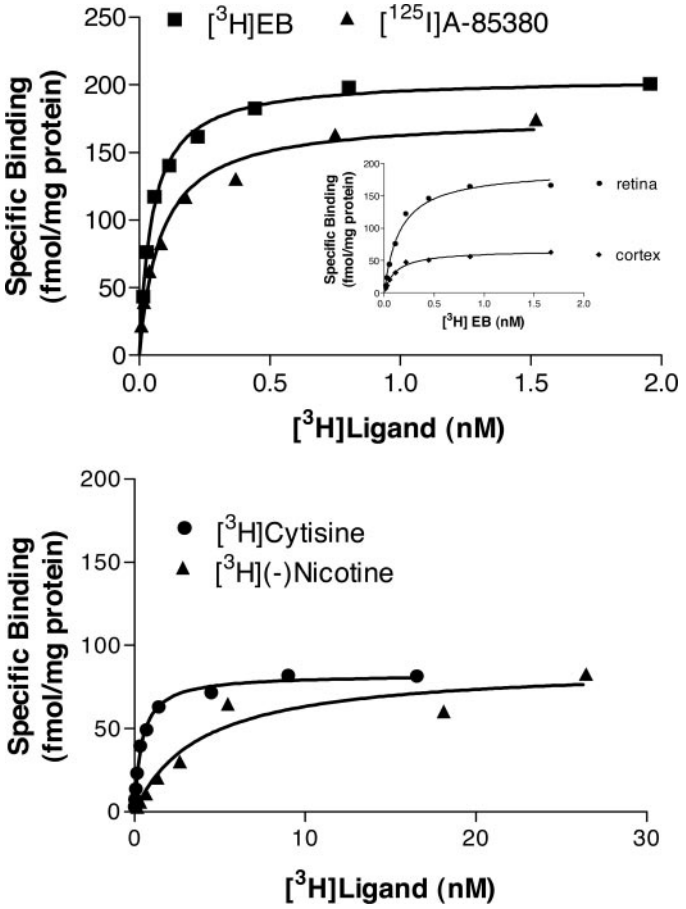

Fig. 2. Saturation binding to nAChRs in rat retina: comparison between four different radiolabeled ligands. Receptor binding sites in retina homogenates were measured using [^{3}H]EB, ^{125}I-A-85380, [^{3}H]cytisine, and [^{3}H](−)nicotine as described under *Materials and Methods*. These curves are representative of two to five independent studies. All of the curves fit best to a model for one binding site, as determined by nonlinear, least-square regression analyses using Prism 3 software. Inset, comparison of [^{3}H]EB binding in rat retina and cerebral cortex. The values for the dissociation constants (K_d) and binding site density (B_{max}) from all of the studies are shown in Table 2.

Drug Competition for [^{3}H]EB Binding Sites in the Retina. Multiple nAChR subtypes in the retina would be consistent with the multiple nAChR subunit mRNA transcripts expressed there. To examine this further, we carried out competition binding assays with several ligands that can discriminate among some of the subtypes. As shown in Fig. 3, all of these nicotinic ligands competed for the sites labeled by [^{3}H]EB in the retina with shallow competition curves, yielding Hill coefficients significantly less than 1. This is consistent with competition for more than one class of nAChR binding sites; in fact, all of these ligands fit a model for two classes of binding sites (Table 3). The competing ligands shown here all have much higher affinity for nAChRs containing β2 subunits than β4 subunits (Parker et al., 1998; Xiao et al., 1998; Xiao and Kellar, 2004); thus, in general, they can discriminate between these two broad classes of subtypes. The higher-affinity binding site for these competing ligands represented ~70 to 80% of the nAChR labeled by [^{3}H]EB in the retina (Table 3), indicating that β2-containing receptors constitute the majority of the nAChRs in the rat retina, which is consistent with the binding site density of ^{125}I-A-85380 compared with [^{3}H]EB (Table 2).

Immunoprecipitation of nAChRs in the Retina. nAChR subtypes are defined by their subunit composition, and although the pharmacological profile can point to possible receptor subtypes or eliminate some, pharmacological studies alone are seldom definitive. Therefore, to begin to determine the nAChR subtypes in the retina, we labeled the receptors with [^{3}H]EB and then carried out immunoprecipitation assays with seven different antibodies, each directed at a specific receptor subunit for which mRNA was detected in the retina.

Before carrying out the immunoprecipitation studies in the retina, we tested the specificity of each of the antibody preparations, which are directed at α2, α3, α4, α6, β2, β3, and β4 subunits. To do this, we determined their ability to immunoprecipitate receptors from rat cerebral cortex, which expresses predominantly $\alpha4\beta2$ nAChRs (Whiting et al., 1987; Flores et al., 1992); pineal gland, which expresses $\alpha3\beta4$ nAChRs virtually exclusively (Hernandez et al., 2004); superior cervical ganglia, which expresses α3-containing receptors (Xu et al., 1999); and superior colliculus and striatum,

TABLE 2

Comparisons of the B_{max} and K_d values for nAChRs in rat retina measured with four radioligands and comparison of values in the retina and cerebral cortex measured with [^{3}H]EB

The B_{max} and K_d values were derived from nonlinear, least-square regression analyses applied to saturation curves, as shown in Fig. 2. Data are the mean ± S.E.M. from 2 to 10 independent saturation curves. One-way analysis of variance followed by Bonferroni's multiple comparison test found that the B_{max} value for [^{3}H]EB is significantly higher than those for [^{125}I]A-85380, [^{3}H]cytisine, and [^{3}H](−)nicotine in the retina.

Ligand	Retina		Cortex	
	K_d	B_{max}	K_d	B_{max}
	nM	*fmol/mg protein*	*nM*	*fmol/mg protein*
[^{3}H]EB	0.092 ± 0.02	204 ± 5.2	0.10 ± 0.0065	64.3 ± 1.2
^{125}I-A85380	0.085 ± 0.02	152 ± 18**		
[^{3}H]Cytisine	0.40 ± 0.017	82.6 ± 1.9***		
[^{3}H](−)Nicotine	4.3 ± 0.49	94.7 ± 6.7***		

** $p < 0.01$.
*** $p < 0.001$.

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which express a significant fraction of receptors containing α6 subunits (Whiteaker et al., 2000b; Champtiaux et al., 2002) and β3 subunit mRNA (Cui et al., 2003). In some cases, we also used human embryonic kidney 293 cells stably transfected with different rat nAChR subunit combinations (Xiao et al., 1998; Xiao and Kellar, 2004). As shown in Table 4, the antibody preparations directed at these seven subunits are all highly effective, as measured by the ability of most of them to immunoprecipitate 80 to 100% of the receptors from tissues containing known predominant receptor subtypes with cognate subunits. Our α6 and β3 antibodies immunoprecipitated 25 and 9% of the receptors in the superior colliculus, respectively, one of the few tissues other than the retina itself (Moretti et al., 2004; see below) that express a relatively high percentage of nAChRs which contain these subunits. Moreover, and just as important, all of the antibodies displayed high specificity, recognizing less than 2% of nAChRs that do not contain their cognate subunits (Table 4).

The antibodies directed at the α2, α3, and α4 subunits each immunoprecipitated ~33% of the [^{3}H]EB-labeled receptors in the retina, whereas our α6 subunit antibody immunoprecipitated ~10% of the receptors (Fig. 4). The antibody directed at the β2 subunit immunoprecipitated ~80% of the receptors in the retina, whereas the antibodies directed at the β3 and β4 subunits immunoprecipitated ~10% and ~25% of the receptors, respectively (Fig. 4).

nAChR Subunit Associations in the Rat Retina. [^{3}H]EB binds to α and β subunit combinations that represent potential heteromeric nAChRs, but it does not bind to α subunits that are not associated with a β subunit partner or vice versa (Xiao et al., 1998; Xiao and Kellar, 2004). Therefore, once the presence of a particular nAChR subunit was established with an antibody in the first immunoprecipitation assay, we determined which subunit(s) it was associated with by carrying out a second immunoprecipitation on the resultant supernatant with a different antibody. The rationale for this sequential immunoprecipitation procedure is that if two (or more) different subunits are components of the same receptor, then initial immunoprecipitation of that receptor with an antibody directed at one subunit (the clearing antibody) will decrease the amount of the receptor available in the sample's remaining supernatant for immunoprecipitation with a subsequent antibody directed at the second subunit (the capturing antibody).

An example of the efficacy of this procedure is shown in Fig. 5. The β2 and β4 antibodies immunoprecipitated, respectively, ~80 and 25% of the [^{3}H]EB-labeled nAChRs in retinal homogenates, and combining both antibodies in one assay tube immunoprecipitated essentially all of the [^{3}H]EB-labeled receptors. Moreover, after an initial immunoprecipitation (clearing) of retinal homogenates with the combination of the β2 and β4 antibodies, subsequent immunoprecipitation of the remaining supernatants by the antibodies directed at α2, α3, and α4 subunits was reduced to nearly background levels, indicating that the clearing procedure with the combined β2 and β4 antibodies removed essentially all of the heteromeric receptors from the remaining supernatant. This is consistent with the long-established concept that all heteromeric nAChRs contain an α subunit in association with a β2 and/or β4 subunit.

Associations with the β2 Subunit. As shown in Fig. 6A, when retina homogenates were first subjected to a control immunoprecipitation with normal rabbit serum (or an irrelevant monoclonal antibody), subsequent incubation with the antibody directed at the β2 subunit immunoprecipitated ~80% of the [^{3}H]EB-labeled nAChRs in the retina. In contrast, when the samples were first subjected to immunoprecipitation with antisera directed at any one of the four α subunits, subsequent incubation with the β2 antibody immunoprecipitated significantly fewer [^{3}H]EB-labeled nAChRs. Specifically, after immunoprecipitation with the α2 antiserum, the β2 antibody immunoprecipitated ~22% fewer receptors from the retina homogenates (i.e., from ~80% of the total nAChRs to ~58%). After immunoprecipitation with the α3 antiserum, the β2 antibody immunoprecipitated ~18% fewer receptors, and after immunoprecipitation with α4 and α6 antisera, the β2 antibody immunoprecipitated, respec-

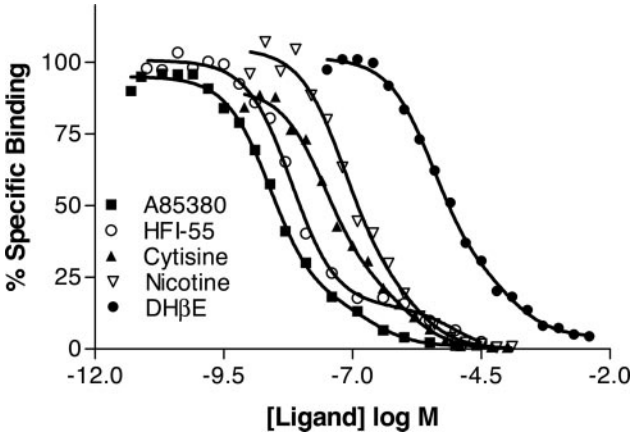

Fig. 3. Competition binding of ligands for nAChRs labeled by [^{3}H]EB in membrane homogenates from rat retina is consistent with multiple nAChR subtypes. Nicotinic ligands competed against 500 pM [^{3}H]EB in binding assays as described under *Materials and Methods*. In all cases, the competition curves were shallow, with Hill coefficients <1, and fit best to a model for two binding sites. Data shown are representative of three to four independent studies. See Table 3 for summary and analyses of data.

TABLE 3

Analyses of ligand competition binding studies in the rat retina

The K_i values, Hill slope, and fraction of sites were derived from nonlinear, least square analyses using Prism software applied to competition binding curves, as shown in Fig. 3. The concentration of [^{3}H]EB used in these studies was 500 pM. The binding of all ligands fit best to a two-site model, with the larger fraction representing the higher-affinity site. In all cases, a one-sample t test indicated that the Hill slopes were <1 ($p < 0.01$). Data are the mean ± S.E.M. of three to four independent experiments.

Ligand	K_i(1)	K_i(2)	Hill Slope	Fraction of K_i(1)	N
	nM	*nM*			
A-85380	0.22 ± 0.02	105 ± 17	0.71 ± 0.04	0.81 ± 0.01	4
HFI-55	0.89 ± 0.2	4348 ± 595	0.48 ± 0.02	0.84 ± 0.001	4
Cytisine	1.8 ± 0.4	398 ± 123	0.63 ± 0.04	0.67 ± 0.04	4
Nicotine	8.6 ± 1	593 ± 107	0.80 ± 0.02	0.72 ± 0.04	4
Dihydro-β-erythroidine	162 ± 55	36,000 ± 6,000	0.65 ± 0.04	0.75 ± 0.02	3

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tively, ~26% and ~8% fewer receptors from the retina homogenates (Fig. 6A).

We then reversed the order of the antibodies—that is, we immunoprecipitated first with the β2 antibody and then carried out the second immunoprecipitation with an antibody directed at one of the α subunits. The results of these studies are shown in Fig. 6B. After clearing with the β2 antibody, the α2, α3, and α4 antisera immunoprecipitated, respectively, ~26, 17, and 26% fewer receptors from the remaining retina homogenates. These reductions in the number of retinal nAChRs after clearing the receptors containing β2 subunits are consistent with the results obtained with the initial order of antibodies (Fig. 6A), which helps to reinforce and confirm the results. The results obtained when the antiserum directed at the α6 subunit was used after clearing the β2-containing receptors were variable, possibly because the number of nAChRs containing α6 subunits was relatively low to begin with and/or the affinity of the α6 antibody for the subunit is low; therefore, the results of assays in which the α6 antibody followed the β2 or β4 antibodies are not included in these studies. The results of the studies in Fig. 6 are consistent with the following division of measurable subunit associations for β2-containing nAChRs in the retina homogenates: α2β2* (~24%), α3β2* (~18%), α4β2*(~26%), and α6β2* (8%).

Associations with the β4 Subunit. Similar studies were carried out to examine the association of each of the α subunits with β4 subunits. As shown in Fig. 7A, when the retina homogenates were first incubated with normal rabbit serum, subsequent incubation with the β4 antiserum immunoprecipitated ~20% of the [^{3}H]EB-labeled nAChRs. Clearing with the α2 or α4 antisera did not significantly decrease the subsequent immunoprecipitation by the β4 antiserum. These data indicate that there are few, if any, retinal nAChR subtypes that include both α2 and β4 subunits or α4 and β4 subunits. In contrast, initial immunoprecipitation with the α3 antibody nearly eliminated subsequent immunoprecipitation of nAChRs by the β4 antiserum. This indicates that nearly all β4 subunits in the retina associate with α3 subunits, forming α3β4* nAChR subtypes. It is interesting to note that initial immunoprecipitation with the α6 antiserum decreased the number of receptors subsequently immunoprecipitated by the β4 antiserum to ~8% of the total nAChRs in the retina (Fig. 7A). This indicates that α6 and β4 subunits are associated in ~11% of the nAChRs in the retina and implies that approximately half of the α3β4* receptors are an α3α6β4* mixed heteromeric subtype.

We then examined the implied subunit associations when the order of antibodies was reversed. Initial immunoprecipitation of the retinal homogenates with the β4 antiserum did not significantly affect the amount of nAChRs subsequently immunoprecipitated by the α2 or α4 antisera (Fig. 7B). In contrast, the amount of retinal receptors immunoprecipitated by the α3 antibody was decreased by approximately half, to ~17% of the total nAChRs (Fig. 7B). This indicates that half, or ~17%, of the total α3-containing nAChRs in the retina are associated with β4 subunits. This value is close to that found when the α3 antibody was used as the clearing antibody (Fig. 7A).

Associations with the β3 Subunit. Previous studies have shown that the α6 and β3 subunits are often found together in the same nAChR (Cui et al., 2003; Salminen et al., 2004; Gotti et al., 2005). Consistent with this possibility in the retina, both α6 and β3 subunits were initially found in ~10% of the retinal nAChRs (Fig. 4). To directly test whether these two subunits are components of the same receptor we carried out a sequential immunoprecipitation study. As shown in Fig. 8A, initial immunoprecipitation of retinal homogenates with our α6 antiserum cleared virtually all of the receptors that could be immunoprecipitated with the β3 antiserum. To determine whether β3 subunits were also associated with α3 subunits, we carried out a similar study with α3 and β3 antibodies. As shown in Fig. 8B, clearing with the α3 antibody eliminated nearly all of the nAChRs that could be immunoprecipitated by the β3 antiserum. We then tested for associations between β3 and β2 subunits and β3 and β4 subunits. Clearing with the β3 antibody indicated that ~9% of the retinal receptors contained both β2 and β3 subunits

TABLE 4

Specificity of the antibodies used in these immunoprecipitation studies

Native tissues and/or stably transfected cell lines that express the defined nAChRs indicated were used to estimate the efficacy and test the specificity of each of the antibodies used in the immunoprecipitation studies described here. All of the antibodies were effective in immunoprecipitating [^{3}H]EB-labeled nAChRs containing their cognate nAChR subunit (as indicated under Positive Tissues), and each displayed a high degree of specificity (as indicated by the low immunoprecipitation under Negative Control Tissues). Data represent the mean ± S.E.M. of 3 to 14 separate experiments.

Antibody	Positive Tissues			Negative Control Tissues		
α2	α2β4 cells	85 ± 5%	n = 4	Pineal gland (α3β4)[a]	<1%	n = 3
				Cerebral cortex (α4β2)[b]	<1%	n = 3
				α3β4 cells	<1%	n = 3
α3	Pineal gland	99 ± 1%	n = 6	α2β4 cells	<1%	n = 3
	Sup cervical ganglia	92 ± 2%	n = 3	α4β2 cells	<1%	n = 3
α4	Cerebral cortex	83 ± 5%	n = 10	Pineal gland	<1%	n = 3
				α3β4 cells	<1%	n = 3
α6	Superior colliculus	25 ± 1%	n = 3	Cerebral cortex	<2%	n = 4
				Pineal gland	<1%	n = 5
				α3β4 cells	<2%	n = 3
β2	Cerebral cortex	94 ± 7%	n = 14	Pineal gland	<2%	n = 4
	α4β2 cells	99 ± 1%	n = 2	α2β4 cells	<2%	n = 3
				α3β4 cells	<2%	n = 3
β4	Pineal gland	100 ± 3%	n = 6	Cerebral cortex	<2%	n = 4
				α4β2 cells	<1%	n = 3
β3	Striatum	8 ± 1%	n = 4	Pineal gland	<1%	n = 3
	Superior colliculus	9 ± 1%	n = 7	Cerebral cortex	<2%	n = 3

[a] Virtually all of the nAChRs in the pineal gland are an α3β4 subtype (Hernandez et al., 2004).
[b] The nAChRs in the cerebral cortex are predominantly an α4β2 subtype (Whitinget al. 1987; Flores et al., 1992).

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(Fig. 8C). In contrast, no association between β3 and β4 subunits was detected (Fig. 8D). Because these two β subunits are not associated with each other but the α6 subunit was found to be associated with both β3 and β4 subunits (Figs. 7 and 8), it suggests that the α6 subunit is a component of more than one nAChR in the rat retina.

Other nAChR Subunit Associations. To test for the presence of other mixed heteromeric nAChRs in the retina, we measured the association between two different α subunits and between the β2 and β4 subunits. As shown in Fig. 9A, clearing retinal homogenates with the α3 antibody did not decrease the amount of receptors subsequently immunoprecipitated by either α2 or α4 antibodies. This indicates that nAChR subtypes in the rat retina do not contain α3 subunits in association with either α2 subunits or α4 subunits. In contrast, clearing with the α4 antibody decreased the receptors immunoprecipitated by the α2 antibody by ~9% (Fig. 9B), indicating that ~9% of the retinal nAChRs contain both α2 and α4 subunits. The α2α4 subunit combination would require a β subunit to bind [^{3}H]EB. The β2 subunit is associated with both the α2 and α4 subunits (Fig. 6), but the β4 subunit does not seem to be associated with either (Fig. 7); therefore, we conclude that the ~9% of the retinal nAChRs that contain α2 and α4 subunits are associated with the β2 subunit to form an α2α4β2 subtype.

Initial immunoprecipitation with the β4 antiserum decreased the amount of receptors subsequently immunoprecipitated by the β2 antibody by ~13% (Fig. 9C), indicating that ~13% of the receptors in the retina contain both β2 and β4 subunits; moreover, these β subunits would have to be associated with at least one α subunit to form a [^{3}H]EB binding site. Again, because the β4 subunit does not seem to be associated with either the α2 or α4 subunits (Fig. 7), and we found no association between β3 and β4 subunits, we conclude that ~13% of the retina nAChRs that contain both β2 and β4 subunits are associated with α3 and/or α6 subunits.

Further Studies of the α6 and β4 Subunits in the Retina. There are some similarities between the results of the study presented here and a previous study by Gotti and

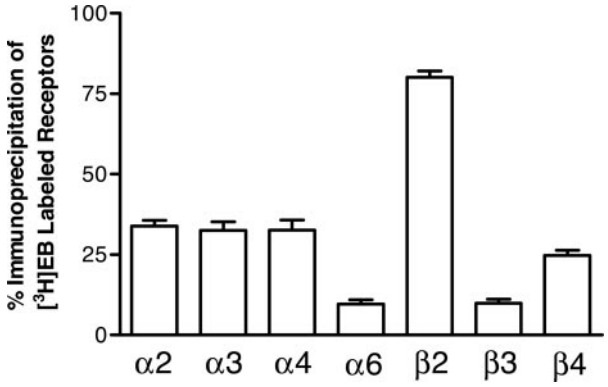

Fig. 4. Immunoprecipitation of nAChRs with antibodies indicates multiple nAChR subunits in the rat retina. Solubilized rat retina homogenates were labeled with 2 nM [^{3}H]EB and were added to tubes containing NRS or one of the subunit-selective antibodies at a previously determined optimal concentration. Protein A (Pansorbin) for the polyclonal antibodies or Protein G Sepharose beads for the monoclonal β2 antibody was used to precipitate the nAChR-antibody complex and after brief centrifugation, the ^{3}H-labeled nAChRs contained in the pellet were counted. The results of the immunoprecipitation studies are expressed as a percentage of the total solubilized nAChRs labeled by [^{3}H]EB in the retina, which was measured in each experiment. The data shown are the mean ± S.E.M. of four to eight separate experiments.

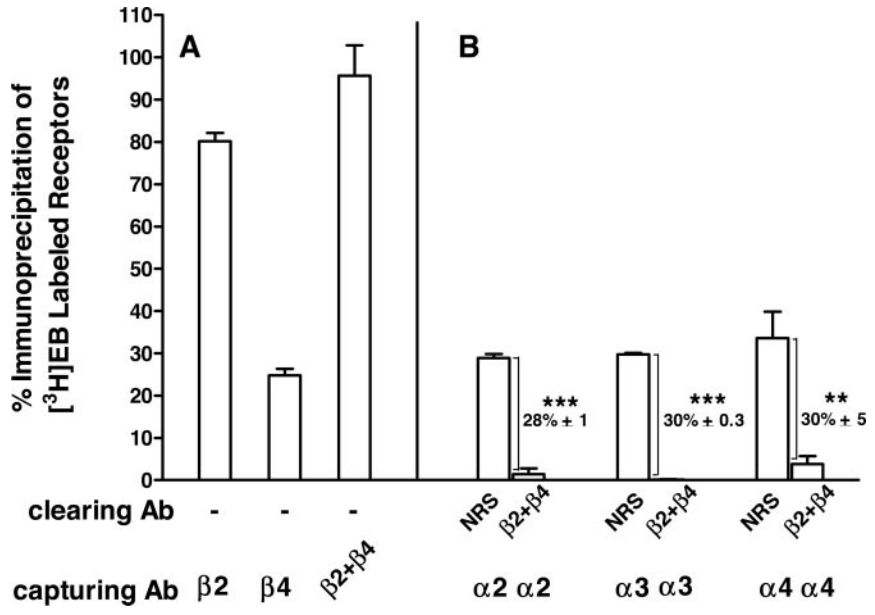

Fig. 5. Sequential immunoprecipitation of nAChRs in the rat retina: demonstration that heteromeric nAChRs contain either a β2 or β4 subunit. A, solubilized retinal homogenates were incubated with antibodies to the β2 and β4 subunits either individually or combined. The β2 and β4 subunits individually immunoprecipitated ~80% and 25% of the receptors, respectively, whereas incubation with the two antibodies combined immunoprecipitated virtually all of the receptors. B, the supernatant resulting from an initial immunoprecipitation with NRS or the combined β2 and β4 subunit antibodies (the clearing antibodies) was subjected to a second immunoprecipitation with antibodies to the α2, α3, or α4 subunits (the capturing antibodies). As shown, after initial immunoprecipitation with NRS, the antibodies to the three α subunits each immunoprecipitated 30 to 35% of the retinal nAChRs from the resultant supernatant; however, after initial immunoprecipitation with the combined antibodies to the β subunits, nearly all of the nAChRs containing these α subunits were removed from the resultant supernatant, and the antibodies directed at the α subunits immunoprecipitated few if any receptors. The percentage of the total number of retinal nAChRs decreased by the initial immunoprecipitation is indicated. Data are the mean ± S.E.M. of three to eight experiments. **, $p < 0.01$; ***, $p < 0.001$ values significantly different from the values after NRS.

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colleagues that also examined nAChRs subtypes in the rat retina (Moretti et al., 2004). For examples, both studies found a large number of subtypes, including several different mixed heteromeric nAChRs; in addition, both studies found that most nAChRs in the rat retina include the $\beta 2$ subunit. However, there are also some differences between the two studies. In particular, in our initial studies, the percentage of rat retinal nAChRs containing $\alpha 6$ subunits was considerably lower, and the percentage containing $\beta 4$ subunits was much higher compared with the results reported previously. To try to resolve the differences related to the percentage of nAChRs that contain $\alpha 6$ and $\beta 4$ subunits, we carried out studies with $\alpha 6$ and $\beta 4$ antibodies generously provided to us by Dr. Cecilia Gotti (University of Milan). We refer to these antibodies here as the "Milan antibodies". As shown in Fig. 10A, using the Milan $\alpha 6$ antibody in our assay system, we immunoprecipitated ~32% of the retinal nAChRs. This is approximately 3 times higher than the results with our $\alpha 6$ antibody and, in fact, is close to the value reported by Moretti et al. (2004). In contrast to this agreement, using the Milan $\beta 4$ antibody we immunoprecipitated ~25% of the retinal nAChRs (Fig. 10A), which is much higher than reported previously (Moretti et al., 2004) but very similar to the value we found with our $\beta 4$ antibody.

In sequential immunoprecipitation studies, clearing with the Milan $\alpha 6$ antibody decreased the retinal nAChRs subsequently immunoprecipitated by the $\beta 2$ antibody by ~25% (Fig. 10B). This agrees closely with the value for $\alpha 6\beta 2^*$ subtypes reported by Moretti et al. (2004) and indicates that most (but probably not all) of the $\alpha 6$ subunits in the rat retina are associated with $\beta 2$ subunits.

To further investigate the $\alpha 6$-containing receptors in the retina, we measured the ability of α-conotoxin MII, which has high affinity for nAChRs with the subunit composition of $\alpha 6\beta 2^*$ (Champtiaux et al., 2002; McIntosh et al., 2004) to compete for retinal nAChRs binding sites labeled with [^{3}H]EB. As shown in Fig. 11, α-conotoxin MII competed with high affinity for ~17% of the retina receptors labeled with [^{3}H]EB. It is interesting that this value is significantly different from the values for the $\alpha 6\beta 2^*$ subunit association measured with either $\alpha 6$ antibody. This suggests that in addition to the $\beta 2$ subunit, one or more other subunits influence the ability of α-conotoxin MII to bind to the receptor. For

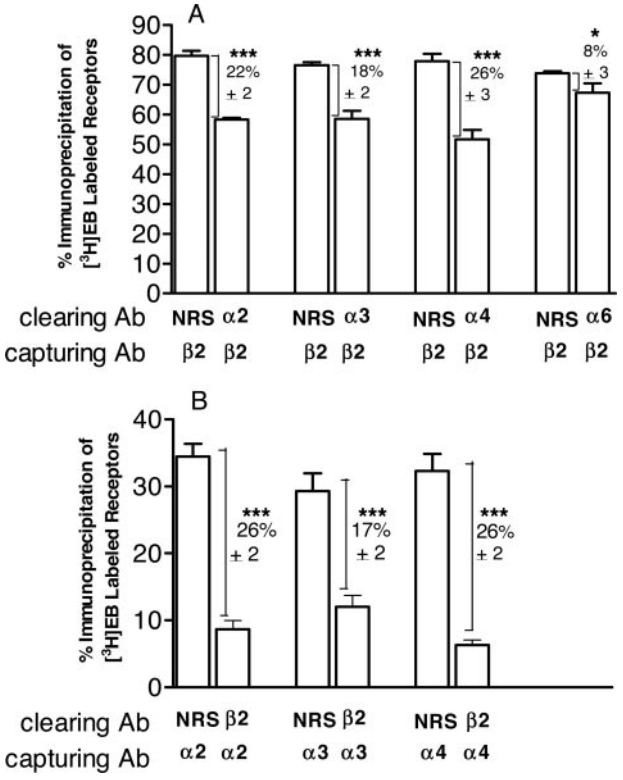

Fig. 6. Sequential immunoprecipitation of retinal nAChRs demonstrates associations of the $\beta 2$ subunit with $\alpha 2$, $\alpha 3$, $\alpha 4$, and $\alpha 6$ subunits. A, retinal homogenates were first immunoprecipitated with NRS or one of the antibodies to the α subunits shown (the clearing antibody), and the resulting supernatants were then subjected to a second immunoprecipitation with the $\beta 2$ antibody (the capturing antibody). B, the sequential immunoprecipitation procedure was carried out in reverse order; that is, the retinal homogenates were first immunoprecipitated with NRS or the $\beta 2$ antibody, and the resulting supernatants were then subjected to a second immunoprecipitation with one of the antibodies to the α subunits. In each case, initial immunoprecipitation with an α subunit antibody decreased the immunoprecipitation by the $\beta 2$ subunit antibody and vice versa (except that the $\beta 2/\alpha 6$ sequence was not done, see text). The percentage of the total number of retinal nAChRs decreased by the initial immunoprecipitation is indicated. Data are the mean ± S.E.M. of three experiments. *, $p < 0.05$; ***, $p < 0.001$, values significantly different from the values after NRS.

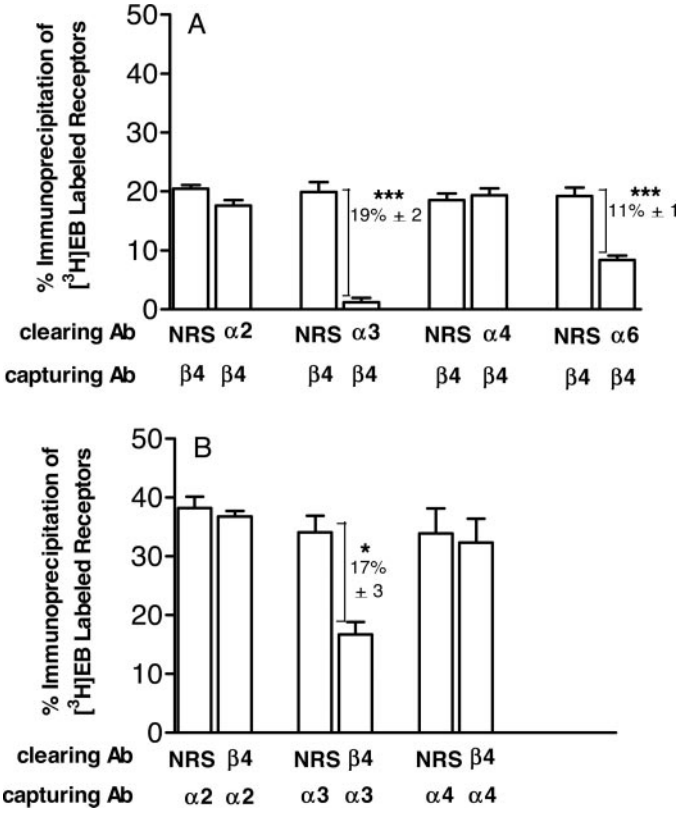

Fig. 7. Sequential immunoprecipitation of retinal nAChRs demonstrates associations of the $\beta 4$ subunit with $\alpha 3$ and $\alpha 6$ subunits. A, retinal homogenates were first immunoprecipitated with NRS or one of the antibodies to the α subunits (the clearing antibody), and the resulting supernatants were then subjected to a second immunoprecipitation with the $\beta 4$ antibody (the capturing antibody). B, the sequential immunoprecipitation procedure was carried out in reverse order; that is, the retinal homogenates were first immunoprecipitated with NRS or the $\beta 4$ antibody, and the resulting supernatants were then subjected a second immunoprecipitation with one of the α subunit antibodies shown. The percentage of the total number of retinal nAChRs decreased by the initial immunoprecipitation is indicated. Data are the mean ± S.E.M. of at least three experiments. *, $p < 0.05$; ***, $p < 0.001$, values significantly different from the values after NRS.

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example, most $\alpha6\beta2^*$ receptors seem to also contain the $\beta3$ subunit (Cui et al., 2003; Gotti et al., 2005; Salminen et al., 2005), and binding of α-conotoxin MII was substantially decreased in the absence of $\beta3$ subunits (Cui et al., 2003; Salminen et al., 2005).

Discussion

Figure 12 summarizes the measured subunit associations that we found in the rat retina and estimates the percentage of seven different nAChR subtypes that can be deduced from these studies. The uncertainty of the measurements of subunit associations in these sequential immunoprecipitation assays was estimated by the propagation of error method, which takes into account the variance for each of the individual measurements from which the percentage of association was derived. From this method, we expressed the percentage of each proposed subtype as a range.

The data indicate that ~80% of the receptors contain $\beta2$ subunits and ~25% contain $\beta4$ subunits. The $\beta2$ and $\beta4$ subunits exert major influences on the pharmacology of nAChRs (Luetje and Patrick, 1991), with most drugs having higher affinity for $\beta2$-containing subtypes (Parker et al., 1998; Xiao and Kellar, 2004). Thus, the division between $\beta2$- and $\beta4$-containing receptors indicated by these immunoprecipitation studies helps to explain three observations about the pharmacology of the nAChRs in the retina. The first is that ^{125}I-A-85380 labeled only ~75% of the receptors labeled by [^{3}H]EB, which probably reflects the much lower affinity of ^{125}I-A-85380 for nAChRs that contain the $\beta4$ subunit and thus its inability to label these receptors (at least at the interface between α and $\beta4$ subunits). The second observation is that [^{3}H]cytisine and [^{3}H](−)nicotine labeled only 40 to 50% of the receptors labeled by [^{3}H]EB in these studies. [^{3}H]EB would be expected to label all heteromeric nAChRs; in contrast, [^{3}H]cytisine and [^{3}H](−)nicotine would be expected to label only the $\alpha4\beta2^*$ and $\alpha2\beta2^*$ subtypes completely, and a

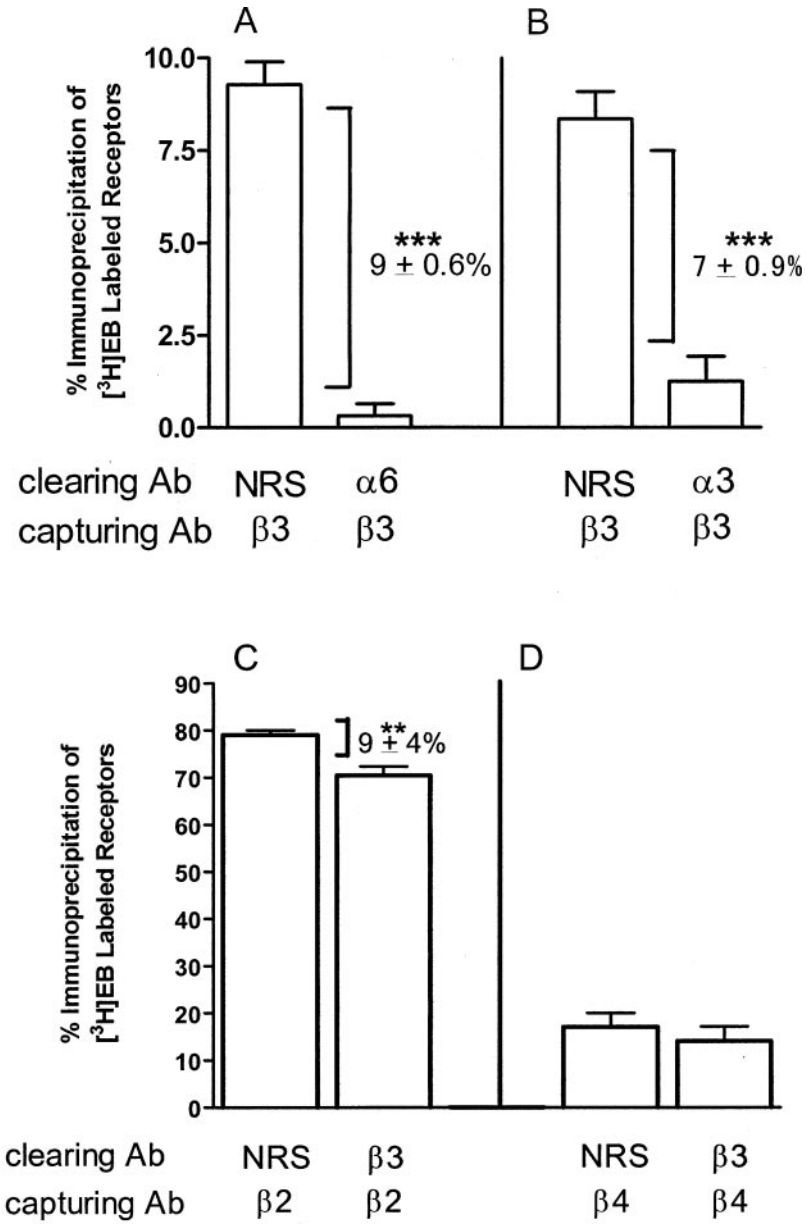

Fig. 8. Sequential immunoprecipitation demonstrates associations of the $\beta3$ subunit with $\alpha6$, $\alpha3$, and $\beta2$ subunits in rat retina. Retinal homogenates were first immunoprecipitated with NRS or $\alpha6$ antibody (A) or $\alpha3$ antibody (B) (the clearing antibody), and the resulting supernatants were then immunoprecipitated with the $\beta3$ antibody (the capturing Ab). In C and D, the homogenates were first immunoprecipitated with NRS or the $\beta3$ antibody, followed by immunoprecipitation with either the $\beta2$ or $\beta4$ antibodies. Associations between $\beta3$ and $\alpha6$, $\beta3$ and $\alpha3$, and $\beta3$ and $\beta2$ subunits were found, but not between $\beta3$ and $\beta4$ subunits. Data are the mean ± S.E.M. from three to eight experiments. **, $p < 0.01$; ***, $p < 0.001$, values significantly different from the values after NRS.

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fraction of the $\alpha6\beta2^*$ subtypes. This is because the affinities of the other subtypes are outside of the radioligand concentration ranges that were used for these studies (Parker et al., 1998; Xiao and Kellar, 2004; Salminen et al., 2005). The third observation is that the binding competition studies with the ligands examined fit a model for two classes of binding sites, with the high-affinity class representing ~75% of the total population of nAChRs (Fig. 3 and Table 3). From the pharmacology of the binding sites of different nAChR subunit combinations heterologously expressed in *Xenopus laevis* oocytes or in mammalian cells, it is probable that the higher-affinity class of binding sites in the retina reflects receptors that contain $\beta2$ subunits and the lower-affinity class reflects receptors that contain $\beta4$ subunits (Parker et al., 1998; Xiao and Kellar, 2004). This would be consistent with the approximate percentages of retinal receptors containing $\beta2$ and $\beta4$ subunits found here by immunoprecipitation, although we do not know into

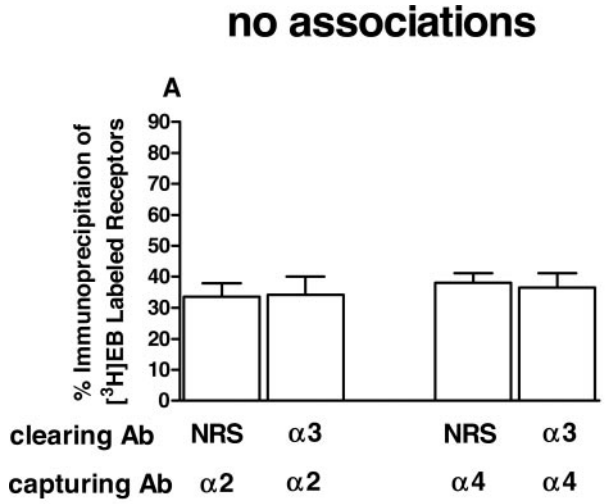

Fig. 9. Sequential immunoprecipitation indicates an association between $\alpha2$ and $\alpha4$ subunits and between $\beta2$ and $\beta4$ subunits in rat retina. Sequential immunoprecipitation studies indicate that $\alpha2$ and $\alpha3$ subunits and $\alpha3$ and $\alpha4$ subunits are not associated (A), ~9% of the retinal receptors contain both $\alpha2$ and $\alpha4$ subunits (B), and ~13% of retinal nAChRs contain both $\beta2$ and $\beta4$ subunits (C). Data are mean ± S.E.M. of three to seven experiments. *, $p < 0.05$; **, $p < 0.01$, values significantly different from the values after NRS.

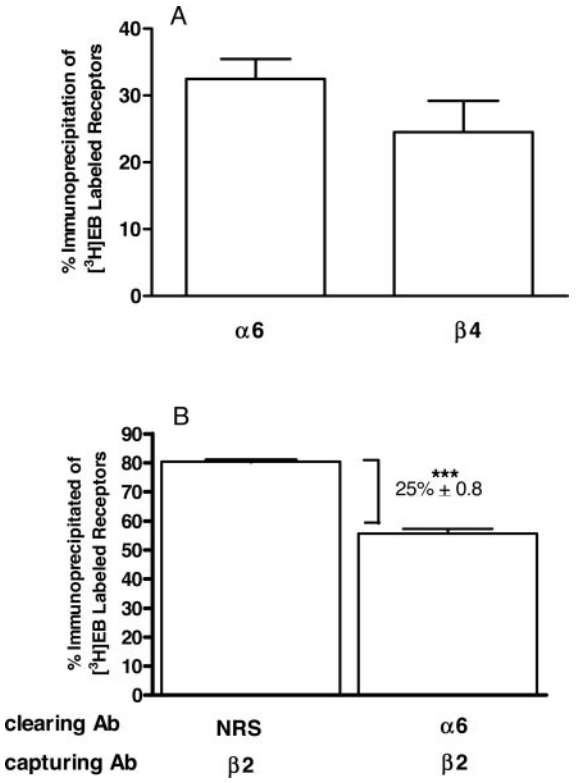

Fig. 10. Immunoprecipitation studies with the Milan $\alpha6$ and $\beta4$ antibodies in rat retina. A, nAChRs in rat retina were immunoprecipitated with $\alpha6$ and $\beta4$ antibodies provided by Dr. Cecilia Gotti's laboratory. B, sequential immunoprecipitation with the Milan $\alpha6$ antibody and the $\beta2$ antibody demonstrates that ~25% of the nAChRs in the rat retina contain both $\alpha6$ and $\beta2$ subunits. Data are the mean ± S.E.M. of three to five experiments. ***, $p < 0.001$, value is significantly different from the value after NRS.

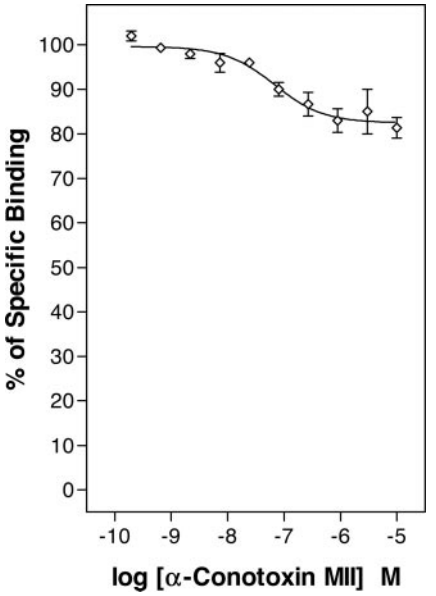

Fig. 11. Inhibition of [^{3}H]EB binding by α-conotoxin MII in rat retina homogenates. Binding assays were carried out as described under *Materials and Methods* using 500 pM [^{3}H]EB. The K_i value for α-conotoxin MII was 9.9 ± 2.5 nM, assuming a K_d value of 90 pM for [^{3}H]EB (Table 2). The maximum inhibition of binding by α-conotoxin MII was 17 ± 2%. Data shown are the mean ± S.E.M. from three experiments.

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which affinity class an nAChR that contains both β2 and β4 subunits would fall. The competition binding studies used here do not allow discrimination among more than two classes of binding sites, but our immunoprecipitation data indicate that the two binding classes each represent more than one nAChR subtype.

Deduced Receptor Subtypes. The measured associations between the α2 and β2 subunits accounted for 20 to 28% of the retinal nAChRs in these studies (Fig. 6). We also found that ~9% of the receptors contain both α2 and α4 subunits (Fig. 9B). Because this receptor would require either a β2 or β4 subunit, and we found no associations between the β4 subunit and either the α2 or α4 subunit (Fig. 7), we assigned the β2 subunit to this receptor to yield an α2α4β2 subtype, which would represent ~9% of the total nAChRs in the retina. The remaining α2-containing receptors are likely to be the simple α2β2 subtype, which would represent ~15% of the retina nAChR receptors.

The associations between the α4 and β2 subunits represented 24 to 29% of the nAChRs in the retina (Fig. 6). Because ~9% of the retinal receptors can be accounted for by the α2α4β2 subtype (see above) and, again, we found no association between the α4 and β4 subunits, the remaining α4β2 subunit association probably represents the simple α4β2 subtype, which would represent 15 to 20% of the nAChRs in the retina.

Approximately 25% of the nAChRs in the rat retina contain β4 subunits; moreover, ~13% of the total nAChRs in the retina seem to be a mixed heteromeric subtype containing both β2 and β4 subunits (Fig. 9C). This receptor would, of course, have to include an α subunit; and because nearly all of the β4 subunits are associated with α3 subunits and approximately half are associated with α6 subunits (Fig. 7A), we propose that 10 to 16% of the retinal receptors are an α3β2β4 subtype and another 10 to 12% are an α3α6β4 subtype. However, it is also possible that some fraction of these two subtypes represent yet a third β4-containing receptor with the more complex composition of α3α6β2β4. Although the present data do not persuasively argue for one possibility over the other, inclusion of the more complex subtype containing four different subunits instead of the two subtypes containing three different subunits would result in a somewhat higher percentage of both α6 and β2 subunits than we measured directly.

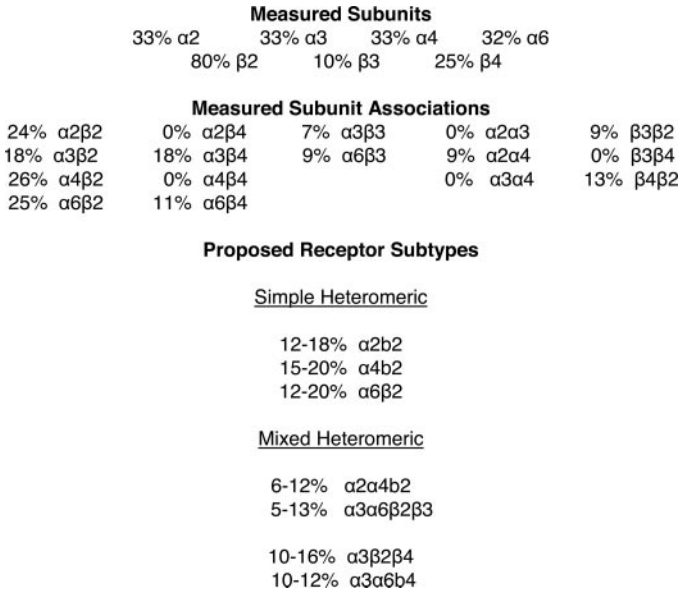

Fig. 12. Summary of measured nAChR subunits, subunit associations, and the proposed simple and mixed heteromeric nAChR subtypes in the rat retina determined from the sequential immunoprecipitation data.

We found β3 subunits in ~10% of the nAChRs in rat retina. These receptors would require another β subunit as well as at least one α subunit (Deneris et al., 1989). The β3 subunit is often found associated with the α6 subunit (Cui et al., 2003; Salminen et al., 2004); consistent with this, nearly all of the β3 subunits we measured in the rat retina seem to be associated with α6 subunits (Fig. 8A). Moreover, we found a similar percentage of β3 subunits associated with α3 and β2 subunits (Fig. 8, B and C); in contrast, we found no association between the β3 and β4 subunits (Fig. 8D). Taken together, these data suggest that the β3 subunit is incorporated in a mixed heteromeric subtype with the subunit composition of α3α6β2β3 that represents 5 to 13% of the retinal receptors.

Using the Milan α6 antibody provided by Dr. Gotti, we measured α6 subunits in 28 to 36% of the nAChRs in the rat retina (Fig. 10A); moreover, using this antibody, we found that ~25% of the retinal receptors are an α6β2* subtype (Fig. 10B). Because 5 to 13% of the retinal receptors seem to be the α3α6β2β3 subtype, we designated the remaining 12 to 20% as an α6β2 subtype.

As noted above, although both our α6 antibody used in our initial studies and the Milan α6 antibody detect α6-containing nAChRs in the retina, the values with the Milan antibody are much higher. This suggests that the Milan antibody intrinsically has higher affinity and/or efficacy. It is also possible, however, that the affinity of our antibody depends on the other subunits with which the α6 subunit is associated. Previous studies found that high-affinity α-conotoxin MII binding to α6-containing receptors was partially dependent on the presence of β3 subunits (Cui et al., 2003; Salminen et al., 2005) and β2 subunits (Whiteaker et al., 2000a). It is interesting, therefore, that α-conotoxin MII competed with high affinity for ~17% of the receptors, a value in between the ~9% of the α3α6β2β3 receptors and the 25% of the receptors in which we measured an association between α6 and β2 subunits. It is not clear how this should be interpreted, but it could suggest that the subunit requirements for high-affinity α-conotoxin MII binding may be somewhat more complex than believed previously.

Moretti et al. (2004) recently used subunit-selective antibodies to analyze the nAChR subtypes in rat retina. Whereas our conclusions about the nAChR subtypes present in the adult rat retina are similar to theirs in several important respects, there are also some notable differences. Among the similarities, for example, both studies found that a large majority of the receptors contain β2 subunits and that the relatively rare α2 and α6 subunits are well represented as α2β2* and α6β2* subtypes. Chief among the differences are that we found a much greater number of β4-containing receptors, but approximately half the number of α4β2* subtypes and approximately one third of the number of receptors containing β3 subunits. Moreover, although both studies found complex subunit associations, representing what we term here as mixed heteromeric subtypes, and there seems to be good agreement on the α2α4β2 subtype, in most cases, the

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specific subunit combinations identified differ. The reasons for these differences are not obvious; both studies used antibodies that seem to be highly selective for their cognate nAChR subunits, although the methods used in the immunoprecipitation procedures are different. Despite these differences, both studies indicate that the mammalian retina expresses an extraordinary variety of nAChR subtypes, including mixed heteromeric subtypes.

Although it is not known how the addition of a second α and/or β subunit to a heteromeric receptor affects the channel and pharmacological properties of nAChRs or their trafficking and the regulation, the presence of mixed heteromeric subtypes is a clear indication of the rich diversity of nAChR subtypes in the mammalian nervous system. The exact roles of these nAChRs in retina physiology and the advantages conferred by the expression of so many different receptor subtypes are not known. However, previous studies in goldfish (Henley et al., 1986), chick, and rat (Swanson et al., 1987) found evidence for axonal transport of at least two different nAChRs from the retina to the brain. Consistent with this, we recently found [^{3}H]EB binding and evidence for several nAChR subunit associations in the optic nerve and tract, which suggest that several different nAChRs are transported down the optic nerve from the retina (Marritt et al., 2003). Moreover, removal of one eye resulted in marked decreases in ^{125}I-EB and ^{125}I-A-85380 binding in the superior colliculus, lateral geniculate nucleus, and pretectal nucleus (Marritt et al., 2003), which is further evidence for the transport of nAChRs down the optic nerve. The physiological roles of the multiple nAChRs in the retina and those transported down the optic nerve axons will require further studies with varied approaches.

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Address correspondence to: Dr. Kenneth J. Kellar, Department of Pharmacology, Georgetown University School of Medicine, Washington, DC 20057. E-mail: kellark@georgetown.edu

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