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An $\alpha4\beta4$ Nicotinic Receptor Subtype Is Present in Chick Retina: Identification, Characterization and Pharmacological Comparison with the Transfected $\alpha4\beta4$ and $\alpha6\beta4$ Subtypes

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ABSTRACT

Retina from 1-day-old chicks is a valuable tissue model for studying neuronal nicotinic receptors because it expresses a large number of the developmentally regulated high affinity [^{3}H]epibatidine labeled nicotinic receptors. Most of these receptors contain the $\beta4$ subunit associated with different α subunits. Using a sequential immunodepletion procedure with anti-$\alpha6$, anti-$\beta3$, anti-$\beta2$, and anti-$\beta4$ antibodies, we purified an $\alpha4\beta4$ nicotinic receptor subtype that accounts for approximately 20 to 25% of the high affinity [^{3}H]epibatidine labeled receptors present in retina at that developmental time. Immunoprecipitation and Western blotting experiments confirmed that the purified subtype contains only the $\alpha4$ and $\beta4$ subunits. This receptor binds a number of agonists and the antagonist dihydro-β-erythroidine with nanomolar affinity, whereas it has micromolar affinity for the α-conotoxin MII and methyllycaconitine toxins and other nicotinic antagonists. Comparison of the pharmacological profile of this purified native subtype with that of the same subtype transiently expressed in human BOSC23 cells showed that they have very similar rank orders and absolute *K*i values for several nicotinic drugs. Finally, because chick retina expresses an $\alpha6\beta4$-containing subtype with a high affinity for the α-conotoxin MII, we used native and transfected $\alpha4\beta4$ and $\alpha6\beta4$ subtypes to investigate the relative contributions of the α and β subunits to this binding, and found that the $\alpha6$ subunit determines the high affinity for this toxin.

Acetylcholine (ACh) binds to two major subclasses of cholinergic receptors in the central nervous system (CNS): the muscarinic and neuronal nicotinic ACh receptors (nAChRs), which mediate not only between-neuron communications but also the long-lasting modifications that occur during development. ACh acts on muscarinic ACh receptors to regulate cell proliferation and on nAChRs to regulate neurite outgrowth and pathfinding by neuronal growth cones (reviewed in Role and Berg, 1996; Zoli, 2000).

nAChRs are cationic channels whose opening is controlled by ACh. They are mainly involved in fast synaptic transmission in the autonomic nervous system (Berg et al., 2000), but also have regulatory functions in the CNS. Brain nAChRs are predominantly localized at presynaptic sites, where they influence the activity of various neurotransmission systems by regulating the release of specific neurotransmitters, such as ACh, dopamine, norepinephrine, serotonin, γ-aminobutyric acid, and glutamate (reviewed in Wonnacott, 1997; MacDermott et al., 1999).

nAChRs include a variety of subtypes, a heterogeneity that is attributable mainly to the diversity of the genes encoding the receptor subunits. Twelve vertebrate genes coding for nAChR subunits have so far been cloned ($\alpha2$-$\alpha10$ and $\beta2$-$\beta4$), and a number of subtypes with different pharmacological and functional properties can be generated from the homopentameric or heteropentameric assembly of these subunits in heterologous systems. The homomeric channels can be obtained by the expression of $\alpha7$, $\alpha8$, or $\alpha9$ subunits, whereas the heteromeric channels come from the coexpression of different combinations of $\alpha2$, $\alpha3$, $\alpha4$, or $\alpha6$ and $\beta2$ or $\beta4$ in presence or absence of $\alpha5$ or $\beta3$ subunits (reviewed in McGe-

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ABBREVIATIONS: ACh, acetylcholine; CNS, central nervous system; nAChR, neuronal nicotinic acetylcholine receptor; COOH, subunit COOH peptide; CYT, subunit cytoplasmic peptide; Ab, polyclonal antibody; PMSF, phenylmethylsulfonyl fluoride; Epi, epibatidine; MII, α-conotoxin MII; αBgtx, α-bungarotoxin; Carb, carbamylcholine; Cyt, cytisine; DMPP, 1,1-dimethyl-4-phenylpiperazinium; Nic, nicotine; MLA, methyllycaconitine; DHβE, dihydro-β-erythroidine; *d*-TC, *d*-tubocurarine; Hex, hexamethonium; Dec, decamethonium; CV, coefficient of variation.

hee and Role, 1995; Gotti et al.1997; Clementi et al., 2000; Lindstrom, 2000).

The pharmacological and functional properties of nAChR subtypes are mainly determined by the pentameric arrangements of their subunits, although post-translational events, transport to different membrane regions, and/or binding to linker proteins can also affect their function. The fact that more than one type of α or β subunit can coassemble in a single pentameric receptor greatly increases the number of possible receptor subtypes present in the nervous system, but not all of these potential subtypes are actually expressed because some still unknown mechanisms prevent the formation of some possible subunit combinations (reviewed in Lindstrom, 2000).

Given that the effects of ACh on neuronal development and functions after the establishment of synaptic contacts depend on the nAChR subtype expressed at each stage, it is very important to identify and investigate the properties of the subtypes expressed in the nervous systems.

$\alpha7$ and $\alpha4\beta2$ are the predominant subtypes expressed in vertebrate brain, whereas the $\alpha3\beta4$ subtype predominates in the autonomic nervous system (Gotti et al., 1997; Lindstrom, 2000). However, subtypes containing the $\alpha2$, $\alpha5$, $\alpha6$, $\beta3$, and $\beta4$ subunits can be found in more limited CNS regions (Forsayeth and Kobrin, 1997; Lindstrom, 2000). The presence of these minor subtypes is also suggested by studies on knockout animals: functional and ligand binding studies of animals lacking the $\beta2$ subunit suggest that $\beta4$-containing receptors are also present in restricted areas associated with an $\alpha3$, $\alpha2$, or $\alpha4$ subunit (Picciotto et al., 1995; Zoli et al., 1998).

Because selective ligands for specific nAChR isoforms are still scarce, our group has devised an alternative approach toward identifying and characterizing the native subtypes present in the chick nervous system by preparing a series of antibodies (Abs) that specifically recognize all of the known subunits. Using this approach, we have recently been able to identify several new subtypes: $\alpha6$- and $\beta3$-containing receptors in retina (Vailati et al., 1999, 2000), and the $\alpha2\alpha5\beta2$ subtype in chick optic lobe (Balestra et al., 2000).

In this study, we used a sequential immunodepletion procedure to identify the presence of an $\alpha4\beta4$ subtype in chick retina, and then characterized its subunit composition and pharmacological profile and compared it with that of the transfected $\alpha4\beta4$ subtype.

Furthermore, to study the relative contribution of the α or β subunits to the pharmacological profiles of the subtypes, we compared the pharmacological properties of the transfected chick $\alpha4\beta4$ and $\alpha6\beta4$ subtypes.

Experimental Procedures

Antibody Production and Characterization. The polyclonal Abs against the $\alpha2$, $\alpha3$, $\alpha4$, $\alpha5$, $\alpha6$ $\alpha7$, $\alpha8$, $\beta2$, $\beta3$, and $\beta4$ chick peptides were raised and characterized as described by Vailati et al. (1999, 2000) and Balestra et al. (2000). For most of the subunits, two different peptides were chosen: one located in the cytoplasmic loop between M3 and M4 (CYT), and the other located at the COOH terminal (COOH). Each anti-peptide Ab was affinity purified by incubating the serum with an affinity resin made by coupling the corresponding peptide to CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions. The monoclonal Ab 299 raised against rat brain nAChR and directed against the $\alpha4$ subunit (Whiting and Lindstrom, 1988) was purchased from RBI. The affinity-purified Abs were bound to CNBr-activated Sepharose at a concentration of 1 mg/ml, and the columns used for immunopurification.

Receptor Subtype Immunopurification

The $\alpha4\beta4$ Subtype. The retina extracts were prepared as previously described by Vailati et al. (1999); every experiment involved the use of 150 g of chick eyes. The tissue was homogenized in an excess of 50 mM sodium phosphate, pH 7.4, 1 M NaCl, 2 mM EDTA, 2 mM EGTA, and 2 mM PMSF for 2 min in an ultraTurrax homogenizer. The homogenate was then diluted and centrifuged for 1.5 h at 60,000*g*.

This homogenization, dilution, and centrifugation procedure was performed three times, and then the pellets were collected, rapidly rinsed with 50 mM sodium phosphate, 50 mM NaCl, 2 mM EDTA, 2 mM EGTA, and 2 mM PMSF, and then resuspended in the same buffer containing a mixture of 10 μg/ml of each of the following protease inhibitors: leupeptin, bestatin, pepstatin A, and aprotinin (Sigma). Triton X-100 at a final concentration of 2% was added to the washed membrane, and the membrane extracted for 2 h at 4°C. The extract was then centrifuged for 1.5 h at 60,000*g* and recovered.

The extract was incubated twice with 5 ml of Sepharose-4B with bound anti-$\alpha6$ Abs to remove the $\alpha6$ receptors, and then twice with 5 ml of Sepharose-4B with anti-$\beta3$ Abs to remove the residual $\beta3$-containing receptors. The flow-through of the $\beta3$ column was reincubated with 5 ml of Sepharose-4B with anti-$\beta2$ Abs, and the resulting $\beta2$ flow-through incubated with 5 ml of Sepharose-4B with anti-$\beta4$ Abs; the bound receptors were eluted with 0.2 M glycine, pH 2.2, or by competition with 100 μM the corresponding $\beta4$ peptide used for Ab production.

Receptor Immobilization by Subunit-Specific Antibodies.

The affinity-purified anti-$\alpha4$ or anti-$\beta4$ Abs were bound to microwells (Maxi-Sorp; Nunc, Wiesbaden, Germany) by means of overnight incubation at 4°C at a concentration of 10 μg/ml in 50 mM phosphate buffer, pH 7.5. On the following day, the wells were washed to remove the excess of unbound Abs, and then incubated overnight at 4°C with 200 μl of 2% Triton X-100 retina membrane extract containing 50 to 100 fmol of [^{3}H]Epi binding sites, which was prepared by sequentially immunodepleting the extract with the anti-$\alpha6$, anti-$\beta3$, and anti-$\beta2$ Abs as described above. After incubation, the wells were washed and the presence of immobilized receptors revealed by means of [^{3}H]Epi binding.

Immunoprecipitation of [^{3}H]Epi-Labeled Receptors by Anti-Subunit Specific Abs during Retina Development.

The subunit content of the purified and transfected $\alpha4\beta4$ subtypes was determined by immunoprecipitation using chick subunit-specific Abs as described previously (Vailati et al., 1999, 2000; Balestra et al., 2000).

The eyes and retinas were dissected from in ovo chicks on embryonic days 7, 11, 14, and 18 (E7, E11, E14, and E18) and from 1-day-old chicks (P1), immediately frozen in liquid nitrogen and stored at −80°C for later use. No differences in the binding properties of the fresh and frozen tissues were observed. For every experiment, the extracts of the tissues were prepared as described above, labeled with 2 nM [^{3}H]Epi, and incubated overnight with a saturating concentration of affinity-purified IgG (20 to 30 μg). The immunoprecipitation was recovered by incubation of the samples with beads with bound goat anti-rabbit IgG (Tecnogenetics, Milau, Italy). The level of Ab immunoprecipitation was expressed as the percentage of [^{3}H]Epi-labeled receptors immunoprecipitated by the indicated antibodies, taking the amount present in the Triton X-100 extract solution before immunoprecipitation as 100%.

Binding Assay and Pharmacological Experiments

(±)-[^{3}H]Epi with a specific activity of 66.6 Ci/mmol was purchased from PerkinElmer Life Science Products (Boston, MA); nonradioac-

tive Epi was from RBI/Sigma (Natick, MA). The drugs MG624 and F3 have been synthesized in our laboratory according to Gotti et al. (1998). α-Conotoxin MII (MII) was a generous gift from M. M.; nonradioactive α-bungarotoxin (αBgtx) and the drugs cytisine (Cyt), ACh, carbamylcholine (Carb), 1,1-dimethyl-4-phenylpiperazinium (DMPP), nicotine (Nic), methyllycaconitine (MLA), dihydro-β-erythroidine (DHβE), MII, *d*-Tubocurarine (*d*-TC), hexamethonium (Hex), and decamethonium (Dec) were from Sigma.

Membrane. Binding to membrane homogenate obtained from BOSC 23 cells transfected with the α4β4 and α6β4 subunits were performed overnight by incubating aliquots of the membrane with [^{3}H]Epi concentrations ranging from 0.005 to 5 nM at 4°C. Nonspecific binding (averaging 5 to 10% of total binding) was determined in parallel by means of incubation in the presence of 100 to 250 nM unlabeled Epi. A final concentration of 10 μg/ml of the protease inhibitors leupeptin, bestatin, pepstatin A, aprotinin, and 2 mM PMSF was added to the incubation mixture to block possible proteolysis during the long incubation time of the assays. At the end of the incubation, the samples were centrifuged and washed once with 10 sodium phosphate, pH 7.4, plus 50 mM NaCl, the pellet was dissolved with 2N NaOH, and the filters counted in a β-counter.

[^{3}H]Epibatidine Binding to Solubilized Receptor. Tissue extract binding was performed using DE52 ion-exchange resin (Whatman, Maidstone, UK) as previously described (Vailati et al., 1999). The binding techniques for immunoimobilized subtype as well as the data analysis were the same as those described previously (Vailati et al., 1999).

cDNA and Expression Vectors

The cDNAs encoding chick neuronal nAChR α4, α6, and β4 subunits cloned in the SV40-based expression vector Flip (Couturier et al., 1990; Nef et al., 1998) were kindly provided by Dr. Marc Ballivet (University of Geneva, Switzerland).

Expression of nACHR Subunits in BOSC23 Cells

Transient transfections of the nAChR subunits were carried out in the retroviral packaging cell line BOSC 23, as described previously (Ragozzino et al., 1997). The cells were grown in Dulbecco's modified Eagle's medium (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (Hyclone, Logan, UT). The subunit cDNAs were added in equivalent amounts (8 μg each per 100-mm dish). Between 8 and 12 h after transfection, the cells were washed twice and fed again with DME- containing 10% fetal calf serum. The cells were collected in ice-cold phosphate-buffered saline (Life Technologies) 36 to 48 h after transfection, and stored at −70°C.

Materials

The protease inhibitors, cholinergic ligands, Triton X-100, and anti-rabbit and anti-rat antisera were purchased from Sigma, the nonradioactive Epi from RBI/Sigma, CnBr-activated Sepharose 4BCL and ^{125}I-Protein A from Amersham Pharmacia Biotech, (±)[^{3}H]Epi from PerkinElmer Life Sciences, and the reagents for gel electrophoresis from Bio-Rad Laboratories (Hercules, CA).

Results

Epibatidine Binding Receptors in the Retina during Development. We have reported previously (Vailati et al., 1999) that there is a high level of expression of [^{3}H]Epi-labeled receptors in 1-day-old chick retina. To investigate the developmental expression of these receptors, we performed binding studies using 2 nM [^{3}H]Epi and 2% retina extracts obtained from chicks on E7, E11, E14, E18, and P1, and detected 50.3 ± 2, 162 ± 9, 220.7 ± 39, 264 ± 14, and 278 ± 21 fmol/mg of protein (mean ± S.E.M. of three experiments), respectively. If the binding to the same extracts was performed in the presence of 1 μM cold αBgtx, it decreased to 41.4 ± 2.9, 105.7 ± 7, 155 ± 19, 199.9 ± 21, and 204.3 ± 16 fmol/mg of protein, respectively (Fig. 1).

This reduction in the number of [^{3}H]Epi-labeled receptors indicates that αBgtx-sensitive receptors make a contribution at all developmental stages. This was also proved by immunoprecipitation studies performed on E18 and P1 extracts not incubated with αBgtx, from which all of the additional [^{3}H]Epi binding was immunoprecipitated by anti-α7 and/or -α8 Abs. The anti-β2 and -β4 Abs immunoprecipitated the same amounts of ^{3}H-labeled receptors regardless of the presence of αBgtx. These results are consistent with the data previously reported by Gerzanich et al. (1995), who showed that [^{3}H]Epi has pico- and nanomolar affinity for chick α8 and α7 αBgtx receptors.

[^{3}H]Epi-labeled receptors are present as early as E7; because their number increases by approximately 5- to 5.5-fold from E7 to P1, we performed the following experiments using P1 retina.

***α4β4* Subtype Identification.** We have shown previously that the [^{3}H]Epi binding receptors present in P1 chick retina are a heterogeneous population: the majority contain the β4 subunit, but there is a subpopulation that also contains the β2 subunit with or without the β4 subunit. Furthermore, they are also very heterogeneous in terms of their α subunit content (Vailati et al., 1999). Using anti-α6 subunit-specific Abs, we immunodepleted the large majority of α6-containing receptors. The flow-through of the α6 affinity column still had receptors containing the β3, β2, and β4 subunits, and so we used anti-β3 and anti-β2 Abs in sequence, to immunodeplete the retina extract of the residual β3 (Vailati et al. 2000) and β2-containing receptors. Further immunoprecipitation of the flow-through obtained from the sequential columns confirmed the almost total depletion of receptors containing the α6, β3, and β2 subunits (see Table 1), and indicated the presence of 25% of ^{3}H-labeled receptors that were immunoreactive to the α4 and β4 subunits.

Subunit Composition of the *α4β4* Subtype. The retina extract obtained after immunodepletion with the anti-α6,

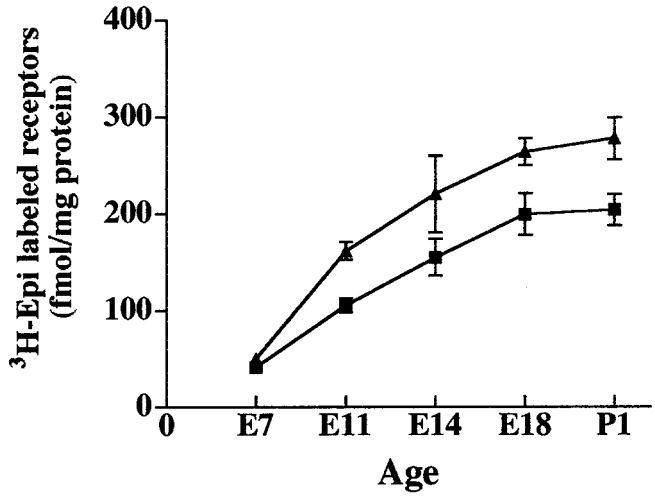

Fig. 1. Developmental changes in [^{3}H]epibatidine-binding receptors expressed in the retina. The retinas were dissected from the embryos at the indicated times and frozen. Triton X-100 extracts (2%) were prepared from the tissues and assayed for [^{3}H]Epi binding, which was performed in the absence (▲) and presence (■) of 1 μM αBgtx. Data are mean values ± S.E.M. from three experiments performed in triplicate.

-β3, and -β2 Abs was incubated with anti-β4 Abs bound to Sepharose, and the bound receptors were eluted by competition with the β4 peptide or glycine pH 2.2.

To identify the subunit content of the immunopurified β4 subtype, we used immunoprecipitation to analyze the receptor eluted from the affinity column by the corresponding β4 peptide. The β4 and α4 Abs immunoprecipitated the vast majority of [^{3}H]Epi-labeled receptors, with 79 ± 6, 63 ± 2, and 66 ± 4% of the receptors being immunoprecipitated by the anti-α4-COOH, anti-α4-CYT, and monoclonal Ab 299, respectively. The anti-β4-COOH and anti-β4-CYT immunoprecipitated 85 ± 3% and 77 ± 1% of the labeled receptors. These results indicate that almost all of the purified receptors contain both the β4 and α4 subunits. Because the anti-α2, -α3, -α5, -α6, -α7, -α8, -β2 and -β3 Abs immunoprecipitated these receptors to only a very limited extent, we do not think that these subunits are coassembled with the α4 and β4 subunits in the immunopurified receptor (Fig. 2, top).

As a further control of the Ab specificity and subunit content of the native subtype, we compared the immunoprecipitation studies of the purified α4β4 subtype with those performed on BOSC23 cells transfected with the chick α4 and β4 subunits. The 2% Triton extract obtained from the transfected cells was labeled by [^{3}H]Epi and immunoprecipitated using the same Abs as those used to characterize the native subtype. Apart from a higher recovery with the α4 (98 ± 3 and 84 ± 7% with the anti-α4 COOH and CYT) and β4 Abs (96 ± 3 and 89 ± 9% with the anti-β4 COOH and CYT), the results were qualitatively very similar (Fig. 2, lower part).

The subunit composition of the purified native α4β4 receptors was also analyzed on Western blots using the same Abs as those used for the immunoprecipitation experiments (see Fig. 3). The anti-α4 Abs recognized a peptide of 68 ± 0.9 kDa (anti-α4, lane 3) and the anti-β4 Abs recognized a single band of 53 ± 0.5 kDa (anti-β4 CYT, lane 8; anti-β4 COOH, lane 9) We also tested the purified α4β4 receptors for the possible presence of α2 (lane 1), α3 (lane 2), α5 (lane 4), α6 (lane 5), β2 (lane 6), and β3 subunits (lane 7) but could not detect any labeling using subunit-specific Abs. This was due to a lack of proteins because the same Abs were able to recognize the subunits in the purified α6α3β3β4 and α2α5β2 subtypes (Gotti et al., 1994a, Vailati et al., 1999; Balestra et al., 2000).

Pharmacological Profile of the Native α4β4 Subtype and Comparison with the Transfected Subtype. The pharmacological experiments were all carried out on receptors immobilized by the corresponding anti-β4-CYT specific Abs as described under *Experimental Procedures*. The α4β4 receptors bind [^{3}H]Epi with high affinity; the K_d value calculated from 10 separate experiments was 11 pM (CV, 17%).

Figure 4 shows a typical saturation curve of the total and aspecific binding of [^{3}H]Epi to the immunoimmobilized subtype. The interaction of [^{3}H]Epi with the α4β4 receptors was consistent with the presence of a single class of high-affinity

TABLE 1

Percentage of immunoprecipitation of chick retina extracts labeled with [^{3}H]Epi before and after immunodepletion with anti-α6 -β3 and -β2 Abs by anti-subunit chick specific Abs.

The immunoprecipitation was carried out as described under *Experimental Procedures* using saturating concentrations of anti-subunit Abs. The results are expressed as the percentages of [^{3}H]Epi labeled receptors, taking the amount of receptor present in the solution before immunoprecipitation as 100%. The percentage of immunoprecipitation was subtracted from the value obtained in control samples containing an identical concentration of normal rabbit IgG. The values are the means ± S.E.M. of five determinations.

Abs Anti-	Retina Extract	after α6, β3 and β2
α2	18 ± 2	3.7 ± 0.5
α3	29 ± 1.4	8 ± 0.7
α4	42 ± 6	26 ± 3
α5	10 ± 3	1 ± 0.2
α6	37 ± 2.3	1.5 ± 0.5
β2	30 ± 4	4.3 ± 1.5
β3	26 ± 3	2.6 ± 0.2
β4	74 ± 4	33 ± 3

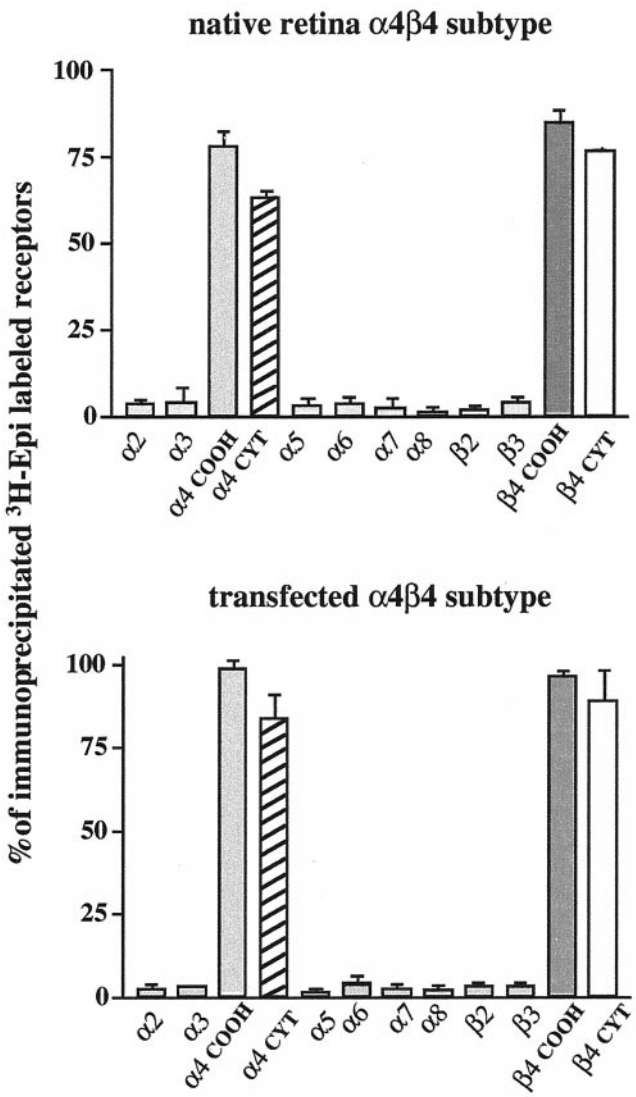

Fig. 2. Immunoprecipitation analysis of the subunit content of the native and transfected α4β4 subtypes. Top, the α4β4 retina subtype was purified as described under *Experimental Procedures*. After extensive dialysis to remove the β4 peptide used for the elution of the receptors from the affinity column, the receptors were labeled with 2 nM [^{3}H]Epi and immunoprecipitated using saturating concentrations (20–30 μg) of anti-α2-CYT, anti-α3-CYT, anti-α4-COOH, anti-α4-CYT, anti α5-COOH, anti-α6-CYT, anti-α7-CYT, anti-α8-CYT, anti-β3-CYT, anti-β4-COOH, and anti-β4-CYT. Bottom, the BOSC23 cells were transiently transfected with chick α4 and β4 cDNA as described under *Experimental Procedures*. A 2% Triton X-100 extract was prepared from the cells and the receptors were labeled with 2 nM [^{3}H]Epi and immunoprecipitated with the same Abs as in the top. The results are expressed as percentages of the [^{3}H]Epi-labeled receptors, taking the amount of receptor present in the solution before immunoprecipitation as 100%. The percentage of immunoprecipitation was subtracted from the value obtained in the control samples containing an identical concentration of normal rabbit. Mean values ± S.E.M. of three determinations performed in triplicate.

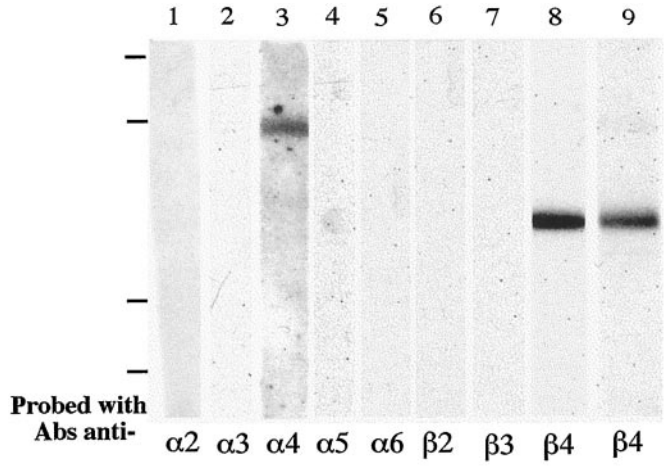

Fig. 3. Western blot analysis of the immunopurified $\alpha4\beta4$ subtype. The receptors bound to the $\beta4$ Abs were eluted by incubation with 100 μM $\beta4$ peptide, concentrated, and separated on 9% acrylamide SDS gel, electrotransferred to nitrocellulose, and probed with the indicated anti-subunit specific Abs. The molecular mass markers (top to bottom) are 97 kDa, 67 kDa, 45 kDa, and 31 kDa.

binding sites; it is also indicated by the Scatchard plot of the saturation curve.

Figure 5 shows the inhibition curves of cholinergic agonists (top) and antagonists (bottom) for the binding of [^{3}H]Epi to the immunoimmobilized subtype in the presence of 0.25 nM [^{3}H]Epi at equilibrium. The inhibition curves for all of the ligands best fitted one class of binding sites; the K_i values of the inhibition curves shown in Table 2 were obtained by simultaneously fitting the data from three separate experiments.

The relative efficacies of the agonists in the competition experiments were Epi $\gg$ Cyt > Nic > DMPP > ACh > Carb: all but Carb had relatively low K_i values (in the low nanomolar range). The rank order of antagonist potencies was DHβE > F3 > MII > MLA > MG624 > Dec > *d*-TC > Hex.

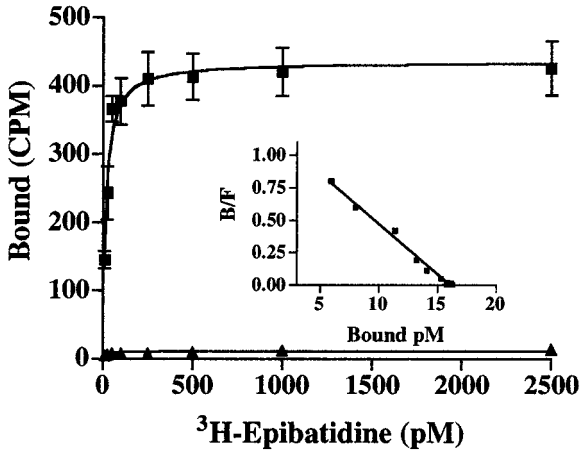

Fig. 4. Saturation curve of [^{3}H]Epi binding to immunoimmobilized native retina $\alpha4\beta4$ receptors, and its Scatchard analysis (insert). The immunoimmobilized receptors were incubated overnight at 4°C with the indicated concentrations of [^{3}H]Epi to measure total binding, and also in the presence of 100 nM Epi to measure aspecific binding. The total (■) and aspecific binding (▲) shown is that obtained from a representative experiment; the K_d value of 11 pM [coefficient of variation (CV) = 17%] was calculated by simultaneously fitting 10 separate experiments. The Scatchard plot of the saturation curve shows the presence of a single class of high-affinity sites.

DHβE was the most potent antagonist, followed by F3, a compound that has nanomolar affinity for the chick $\alpha7$ subtype and has recently been found to block the native nAChRs expressed on the surface of rat chromaffin cells competitively and reversibly (Di Angelantonio et al., 2000).

To exclude possible interference by the immunoimmobilizing Abs on the pharmacology of the $\beta4$ receptors, we also tested the binding of [^{3}H]Epi and ACh in receptors immunoimmobilized on the anti-$\beta4$ COOH Abs, and found that the results were qualitatively and quantitatively the same.

We also compared the pharmacological profiles of the native subtype with that of the corresponding transfected $\alpha4\beta4$ subtype. The BOSC 23 $\alpha4\beta4$ transfected cell line expresses a single class of high-affinity [^{3}H]Epi binding sites, with a K_d of

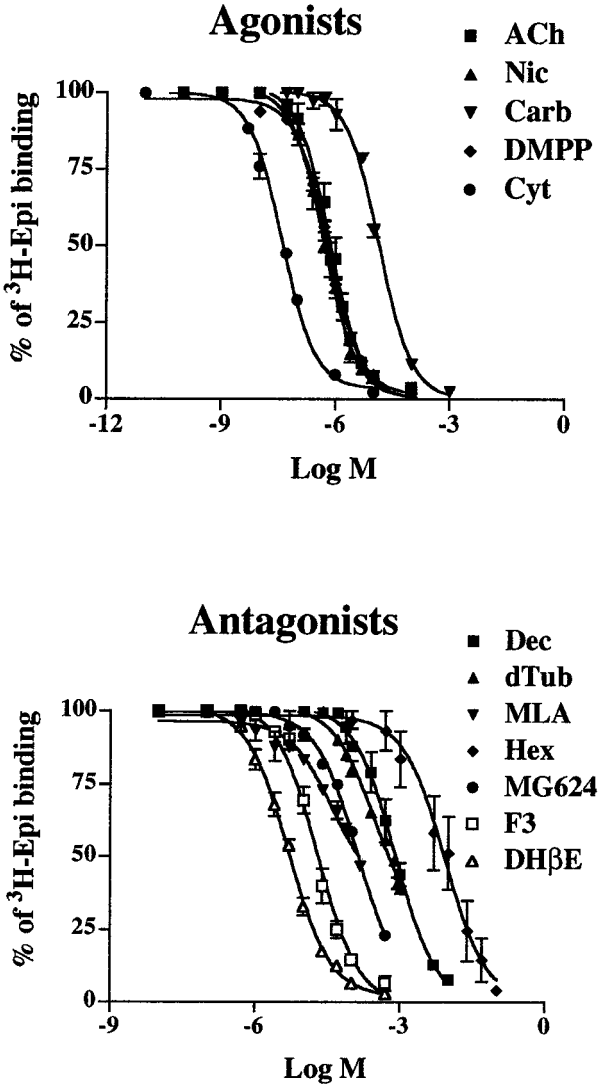

Fig. 5. Inhibition by nicotinic agonists and antagonists of [^{3}H]Epi binding to native immunoimmobilized retina $\alpha4\beta4$ receptors. The receptors immunoimmobilized on the anti-$\beta4$ CYT Abs (as described under *Experimental Procedures*) were preincubated for 30 min at 20°C with the indicated concentrations of nicotinic ligands; [^{3}H]Epi was then added at a final concentration of 250 pM, and the mixture left overnight at 4°C. The curves were obtained by fitting three separate experiments using the LIGAND program (Munson and Rodbard, 1980). In each experiment, each dilution of the drug was tested in triplicate. All of the values are expressed in relation to [^{3}H]Epi specific binding to the receptors (considered as 100%).

18.3 pM (CV, 15%) and a B_{max} (mean ± S.E.M.) of 691 ± 204 fmol/mg of protein. Pharmacological experiments performed on cell membranes obtained from transfected cells incubated for the same time and with the same ligand concentrations as those used for the native immunoimmobilized receptors gave an almost identical profile in terms of the rank order and absolute values of the agonists (Epi ≫ Cyt > Nic > Ach > DMPP > Carb); the pharmacological profile of the antagonists was also very similar, with a rank order of DHβE > F3 > MII > MG624 > MLA > *d*-TC > Dec > Hex. We found a maximum 3-fold difference in the K_i values of *d*-TC, Dec, and Hex in the native and transfected cells. These experiments performed on receptors with a known subunit composition taken from transfected cells corroborated the results with native receptors (Table 2).

Pharmacology of the Transfected $\alpha6\beta4$ Chick Subtype. To study the role of the $\alpha4$ and $\beta4$ subunits in the definition of the pharmacological profile of the subtype, we characterized the profile of the transfected chick $\alpha6\beta4$ subtype (which has the same $\beta4$ subunit but a different α subunit) and compared it with that of the tranfected $\alpha4\beta4$ subype.

The binding of [^{3}H]Epi to transfected $\alpha6\beta4$ cells also determined a single class of [^{3}H]Epi high-affinity sites, with a K_d value of 30 pM (CV, 18%) and a B_{max} value of 74 ± 34 fmol/mg of protein. The order of agonist potency was Epi ≫ Cyt > DMPP > ACh > Nic> Carb and that of the antagonists was MII ≫ MLA > F3 > MG624 > Dh βE > *d*-TC > Dec > Hex (Table 2).

The $\alpha6\beta4$ receptors had high nanomolar affinity for all of the nicotinic agonists (except Carb) and the antagonists MII, MLA, F3 and MG624 and micromolar affinity for the antagonists Dhβ E, *d*-TC, Dec, and Hex. We have previously characterized the pharmacology of the native $\alpha6\beta4$-containing receptors expressed in chick retina (see Table 2), which is very similar to that of the transfected subtype. The only major difference was that MII, MLA, MG624, and F3 had 15.3, 5.4, 10.2, and 6-fold higher affinity, respectively, for the transfected than for the native subtype.

Comparison of the pharmacological profile of the transfected $\alpha6\beta4$ and $\alpha4\beta4$ subtypes shows that the major difference in K_i values is for the agonist DMPP and the antagonists MII, MLA, and *d*-TC, all of which had a higher affinity for the $\alpha6\beta4$ subtype. The main pharmacological difference between the native and transfected $\alpha6\beta4$ and $\alpha4\beta4$ subtypes is the much higher affinity of the $\alpha6\beta4$ subtype for the MII toxin.

Figure 6 shows the inhibition curves of MII on the membranes of the transfected $\alpha6\beta4$ and $\alpha4\beta4$ cells, and on the immunonimmobilized native $\alpha6\beta4$-containing and $\alpha4\beta4$ retina subtypes. In agreement with our previously reported results for the native $\alpha6$-containing subtype (K_i = 66 nM), we found that MII had a high affinity for the $\alpha6\beta4$ (K_i = 4.3 nM). Parallel pharmacological experiments performed on the transfected $\alpha4\beta4$ subtype (K_i = 1750 nM) suggest that the high affinity for the $\alpha6\beta4$ subtype is attributable mainly to the $\alpha6$ subunit.

TABLE 2

Pharmacological characterization of native and transfected chick subtypes

The K_d and K_i values were derived from the [^{3}H]Epi saturation and competition binding curves to the immunoimmobilized native $\alpha4\beta4$- and $\alpha6\beta4$-containing subtypes and to homogenates of BOSC 23 cells transfected with the chick $\alpha4\beta4$ and $\alpha6\beta4$ subtypes. The curves obtained from three separate experiments were fitted using a nonlinear least squares analysis program and the F test according to Munson and Rodboard (1980). The numbers in brackets represent the percentage of CV.

Ligand	K_i			
	Native $\alpha4\beta4$	Transfected $\alpha4\beta4$	Transfected $\alpha6\beta4$	Native[a] $\alpha6\beta4$-
	nM			
Cyt	2.9 (31)	1.57 (38)	7 (41)	11 (36)
Nic	29 (19)	18.5 (35)	68 (31)	20 (31)
DMPP	42 (23)	93 (18)	9 (28)	31 (37)
ACh	59 (30)	63 (22)	55 (33)	76 (26)
Carb	791 (21)	1,310 (41)	1,090 (33)	975 (29)
MII	<2,000	1,750 (26)	4.3 (36)	66 (24)
MLA	2,516 (18)	3,020 (31)	247 (25)	1,350 (25)
DHβE	136 (15)	242 (17)	1,200 (18)	2,800 (13)
MG624	4,066 (14)	2,840 (23)	440 (32)	4,520 (26)
d-TC	20,072 (15)	46,900 (25)	2,894 (28)	7,700 (26)
Dec	18,970 (14)	59,000 (11)	21,820 (27)	35,900 (16)
F3	609 (15)	527 (30)	264 (26)	1,600 (26)
Exa	295,300 (43)	878,000 (27)	821,000 (21)	349,000 (18)
[^{3}H]Epi Kd (pM)	11 (17)	18.3 (15)	30 (18)	35 (18)

[a] Taken from Vailati et al (1999).

Discussion

The pharmacological characteristics of chick, rat, and human $\alpha4\beta4$ subtypes have been studied previously by electrophysiological and binding studies on receptors expressed in heterologous systems (Luetje and Patrick, 1991; Chavez-Noriega et al., 1997; Ragozzino et al., 1997; Stauderman et al., 1998), but this is the first biochemical and immunological demonstration of its presence in vertebrate CNS.

The results described here, together with those of our previous studies, indicate that 70 to 75% of the αBgtx-insensitive [^{3}H]Epi-labeled receptors in chick retina contain the $\beta4$ subunit, which is the predominant retina subunit at P1. This subunit it is coassembled with the $\alpha6$ subunit in 30 to 35% of the receptors (Vailati et al., 1999), with $\beta3$ in 5 to 10% of the receptors (Vailati, 2000), with $\beta2$ in 10 to 15%, and with $\alpha4$ in 20 to 25%.

No direct evidence exists for the physiological role of these subtypes, but they might be involved in fine tuning the spon-

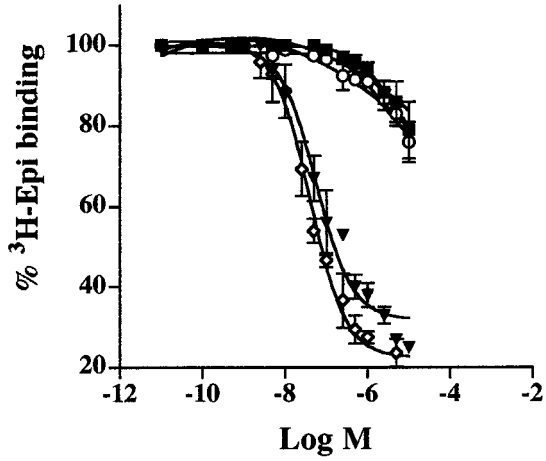

Fig. 6. MII toxin inhibition of the [^{3}H]Epi binding to native and transfected $\beta4$ containing subtypes. The native immunoimmobilized retina $\alpha6\beta4$-containing (▼) and $\alpha4\beta4$ receptors (■), as well as the cell homogenates of the human BOSC23 cells transfected with the $\alpha4\beta4$ (○) or $\alpha6\beta4$ (◇), were preincubated with the indicated concentration of MII toxin for 30 min; a final concentration of 250 pM [^{3}H]Epi was added and left overnight. The results are expressed as in Fig. 5.

taneous activity required for the development of circuits in the retina and/or the formation of the appropriate retinatectum connections. This role of nAChRs in the retina circuits is also suggested by the recent findings of altered spontaneous activity patterns in the retina of mice lacking the β2 and/or β4 nicotinic subunits (Bansal et al., 2000).

We have shown that both the αBgtx-sensitive and -insensitive receptors in the retinas of 1-day-old chicks contribute to the high affinity of [^{3}H]Epi binding. The αBgtx-sensitive receptors are those that contain the α7 and/or α8 subunits, whereas the αBgtx-insensitive receptors include multiple subtypes, 25% of which contain the α4 and β4 subunits. These [^{3}H]Epi-labeled receptors increase by 5- to 6-fold during retina development. At E7, most of the αBgtx-insensitive [^{3}H]Epi binding is caused by receptors containing the α4, α3, and β2 subunits (S.V., M.M., and C.G., unpublished observations) whereas a large number of receptors also contain the β3, β4, and α6 subunits at P1. We (Gotti et al., 1994b) and others (Keyser et al., 1993) have previously shown that there is also a developmental increase in chick retina αBgtx binding receptors, which correlates with an increase in the number of receptors containing the α8 subunit.

Having established that P1 was the developmental time with the largest increase in [^{3}H]Epi-labeled receptors insensitive to αBgtx, we used a series of immunodepletetion procedures to purify the native α4β4 subtype from the retina of 1-day-old chicks.

We analyzed the subunit composition of the purified α4β4 subtype by means of Western blot and immunoprecipitation experiments using Abs directed against all of the known chick nicotinic subunits. The blots of the purified subtypes were recognized only by the Abs directed against the α4 and β4 subunits. These results were confirmed in the immunoprecipitation studies in which only the anti-α4 and β4 Abs immunoprecipitated more than 63% of the immunopurified [^{3}H]Epi labeled receptors. To control the specificity of our immunoprecipitation studies, we performed the same immunoprecipitation experiment on 2% Triton extracts obtained from α4β4 transfected BOSC 23 cells. We obtained the same qualitative results with a higher recovery (more than 83% of the [^{3}H]Epi-labeled receptors were immunoprecipitated), which suggests that the lower recovery of the purified receptors is probably caused by partial proteolysis during the long purification processes. The absence of immunoprecipitation with the other Abs in the native receptors is caused by the lack of subunits, because the same Abs were able to immunoprecipitate the receptors containing the corresponding subunits in control experiments.

Binding studies of the α4β4 subtype showed no difference in the affinity of the native and transfected subtypes for a number of nicotinic ligands: both had nanomolar affinity for the agonists and the DHβE antagonist, and micromolar affinity for the toxins MII and MLA.

The highest agonist affinity was for Epi followed by Cyt, and the K_i values of ACh, Epi, and Cyt were very similar to those reported in the oocyte-transfected rat α4β4 subtype (Parker et al., 1998). The chick α4β4 subtype has a higher affinity for DhβE than the native α4β2 subtype (Balestra et al., 2000), which is in agreement with the results obtained in electrophysiological experiments using oocyte-expressed rat (Harvey et al., 1996) and human subtypes (Chavez-Noriega et al., 1997).

Comparison of the pharmacological profile of the native α4β4 subtype with that of the α6β4 subtype also present in chick retina shows that the two subtypes have different K_i values for the toxin MII, DHβE, *d*-TC, and F3. Because our purified α6β4-containing receptors make up a heterogeneous population in which 40 to 50% also have an additional α3 and/or β3 subunit, we investigated the affinity of these and other nicotinic ligands in the transfected α6β4 chick subtype. The pharmacological profile of the α6β4 subtype was similar but not identical to that reported previously for the native subtype: it has a high affinity for agonists, micromolar affinity for DHβE, and an even higher affinity for the toxins MII (K_i = 4.5 nM) and MLA (K_i = 247 nM) and for the oxystilbene derivatives F3 (K_i = 264 nM) and MG624 (K_i = 440 nM). The high affinity for the MLA toxin is in agreement with the electrophysiological results obtained by Fucile et al. (1998), who found that 10 μM MLA is able to block the ACh-induced current in the same transfected subtype.

The pharmacological properties of the transfected α4β4 subtype reflect those of the native receptors, but the transfected and native α6β4 subtypes differ in terms of the absolute K_i values of some antagonists, thus suggesting that the presence of the α3 and/or β3 subunit may play a role in the definition of antagonist affinity in the native α6β4 subtype. The binding affinity of agonists and antagonists depends on both the α and β subunits (Parker et al., 1998). The greatest difference occurs as a consequence of changing the β subunit, but differences are also seen when the α subunit is changed. In the present study, we found that the K_i values of the MII toxin for the transfected α6β4 and α4β4 subtypes (which differ only in terms of the α subunit), are more than 400 times different. This result allows us to conclude that the MII toxin has a high affinity for the chick α6β4 subtype (K_i = 4.5 nM), and that this high affinity is mainly caused by the α6 subunit because both the native and transfected α4β4 subtypes have only micromolar affinity for MII.

The results obtained in binding studies are in agreement with the very recent finding by Kuryatov et al. (2000) that MII toxin not only inhibits the ACh-induced currents in the α3β2 oocyte-expressed subtype (as also reported previously by Cartier et al., 1996) but also potently inhibits both the chimeric α6/α3 and α6/α4 receptors containing either β2 or β4 subunits.

The high affinity of MII toxin on α6-containing receptors could be very important for dissecting the role of this subtype in brain function and, in particular, for improving our understanding of the addictive properties of nicotine. It has been suggested that the behavioral effects of nicotine depend on dopamine (Di Chiara, 2000), and mRNA for the α6 subunit is in dopaminergic nuclei projecting to the striatum (Le Novère et al., 1996) and MII toxin partially blocks the dopamine release from striatal synaptosomes (Kulak et al., 1997).

It is difficult to attribute specific functional roles to the α4β4 subtype in the CNS because its presence has only been demonstrated in chick retina and could be species-specific. Furthermore, studies performed in KO mice suggest that, if present, it is only a minor subtype: ligand binding and electrophysiological studies in β_2 KO animals (Zoli et al., 1998) have suggested that α4β4 receptors could be present in the interpeduncular nucleus and medial habenula, but the results of later binding studies of α4 KO animals (Marubio et al., 1999) make this possibility very unlikely.

It has recently been found that cocaine, a drug of abuse that primarily blocks the dopamine and serotonin transporters, also affects the heterologously expressed $\alpha4\beta4$ rat subtype at concentrations compatible with those present in the serum of cocaine users (Francis et al., 2000). If this is proven true for the native subtype, a new pharmacological tool will be available for the study of this subtype in vivo.

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