0026-895X/04/6601-85–96$20.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 66:85–96, 2004

Vol. 66, No. 1
3065/1158304
Printed in U.S.A.

Nicotinic Acetylcholine Receptor Subtypes Expression during Rat Retina Development and Their Regulation by Visual Experience

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Received October 29, 2003; accepted March 19, 2004

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

By acting through retinal nicotinic acetylcholine receptors (nAChRs), acetylcholine plays an important role in the development of both the retina and central visual pathways. Ligand binding and immunoprecipitation studies with subunit-specific antibodies showed that the expression of αBungarotoxin (αBgtx) and high-affinity epibatidine (Epi) receptors is regulated developmentally and increases until postnatal day 21 (P21). The increase in Epi receptors is caused by a selective increase in the subtypes containing the α2, α4, α6, β2, and β3 subunits. Immunopurification studies revealed three major populations of Epi receptors on P21: α6* receptors (26%), which contain the α6β3β2, α6α4β3β2, and α6α3/α2β3β2 subtypes; α4(non-α6)* receptors (60%), which contain the α2α4β2 and α4β2 subtypes; and (non-α4/non-α6)* receptors (14%), which contain the α2β2/β4 and α3β2/β4 subtypes. These three populations can be pharmacologically discriminated using αconotoxin MII, which binds the α6* population with high affinity. In situ hybridization showed that the transcripts for all of the subunits are heterogeneously distributed throughout retinal neurons at P21, with α3, α6, and β3 transcripts preferentially concentrated in the ganglion cell layer, α5 in the inner nuclear layer, and α4 and β2 distributed rather homogeneously. To investigate whether nAChR expression is affected by visual experience, we also studied dark-reared P21 rats. Visual deprivation had no effect on the expression of αBgtx receptors or the developmentally regulated Epi receptors containing the α2, α6, and/or β3 subunits but significantly increased the expression of the Epi receptors containing the α4 and β2 subunits. Overall, this study demonstrates that the retina is the rat neural region that expresses the widest array of nAChR subtypes. These receptors have a specific distribution, and their expression is finely regulated during development and by visual experience.

The nicotinic acetylcholine receptors (nAChRs) in vertebrate retina play a role in signaling at the earliest stages of development (long before there is any evidence of synaptic transmission) and also later during neuronal growth and synaptogenesis (Zhou, 2001; Feller, 2002). Neuronal nAChRs are cationic channels whose opening is physiologically controlled by acetylcholine (ACh) neurotransmitter. They form a heterogeneous family of pentameric oligomers made up of combinations of subunits encoded by at least 12 different genes. Although there are many subtypes consisting of different subunits, depending on their phylogenetic, functional, and pharmacological properties (Gotti et al., 1997a; Corringer et al., 2000; Hogg et al., 2003), two main classes have been identified: the αBungarotoxin (αBgtx)-sensitive receptors made up of the α7, α8, α9, and/or α10 subunits, which can form homomeric or heteromeric receptors; and the αBgtx-insensitive receptors consisting of the α2 to α6 and β2 to β4 subunits, which only form heteromeric receptors that bind epibatidine (Epi) with a high affinity. The number of possible receptor subtypes with different pharmacological and func-

This work was supported in part by grant MM05152538 from the Italian Ministero dell'Istruzione, dell'Università e della Ricerca (to F.C. and M.Z.), grant ICS 030.3/RA 0048 from the Italian Ministry of Health, grant HPRN-CT-2002-00258 from the European Research Training Network, Fondo Integrativo Speciale per la Ricerca-Consiglio Nazionale delle Ricerche Neurobiotecnologia 2003, Fondazione Cariplo grant 2002/2010 (to F.C.), and grant RBNE01RHZM 2003 from the Fondo per gli Investimenti della Ricerca di Base (to C.G.).

ABBREVIATIONS: nAChR, neuronal nicotinic acetylcholine receptor; Abs, polyclonal antibodies; ACh, acetylcholine; αBgtx, αBungarotoxin; COOH, COOH peptide; CYT, cytoplasmic peptide; Epi, epibatidine; DR, dark rearing; LR, light rearing; αCntxMII, α-conotoxin MII; INL, inner nuclear layer; GCL, ganglion cell layer; ONL, outer nuclear layer; ANOVA, analysis of variance; KO, knockout; prefix P, postnatal day; RGC, retinal ganglion cell.

tional properties is increased by the fact that more than one type of α or β subunit can participate in forming the receptor pentamer of heteromeric receptors (Lindstrom, 2000).

In adult vertebrate retina, ACh released by the starburst amacrine cells activates a rich array of nAChRs. In situ hybridization and immunolocalization studies, together with Northern blot analyses, have shown that the retina expresses almost all of the nicotinic subunits present in homomeric and heteromeric receptors (Feller, 2002). In particular, α6 and β3 subunits are expressed in a restricted number of neuronal populations, which include catecholaminergic nuclei and visual pathways of the mammalian central nervous system (Le Novère et al., 1996; Champtiaux et al., 2002; Cui et al., 2003).

Biochemical and pharmacological studies using nicotinic ligands and subunit-specific polyclonal antibodies (Abs) have identified the presence of three αBgtx-binding subtypes in chick retina, the homomeric α7 and α8 and the heteromeric α7–α8 subtype (Keyser et al., 1993; Gotti et al., 1994, 1997b). Most of the heteromeric [^{3}H]Epi receptors in chick retina contain the β4 subunit (associated with the α4, α6, and/or β3 subunits) on postnatal day 1 (P1), and both chick αBgtx and heteromeric Epi receptors are developmentally regulated (Vailati et al., 1999, 2000; Barabino et al., 2001). With use of adult rabbit retina, Keyser et al. (2000) have shown that many of the heteromeric receptors contain the β2 structural subunit, which is partially associated with the α3 subunit.

Before phototransduction, spontaneous bursting activity in the developing vertebrate retina (also known as retinal waves) influences the size and complexity of retinal ganglion cell (RGC) dendrites and refines the connections between retinal axons and their thalamic targets (Wong, 1999; Feller, 2002). The role of nAChRs in this activity has been shown clearly by the use of nicotinic antagonists and knockout (KO) mice. Bansal et al. (2000) have shown that the retinal waves present between embryonic day 16 and birth are blocked by nonselective nicotinic antagonists, whereas between P0 and P11, they are blocked by αconotoxin MII (αCntxMII) a nicotinic antagonist believed to be specific for the $\alpha3\beta2^*$ and $\alpha6\beta2^*$ receptors (Cartier et al., 1996, Champtiaux et al., 2002, 2003); between P11 and P14, they are blocked by antagonists of the glutamatergic receptors. Bansal et al. (2000) also showed that mice lacking the α3 or β2 nicotinic subunits have retinal waves with altered spatiotemporal properties.

Further studies have shown that mice lacking the β2 structural subunit (but not those lacking the α4 subunit) have retinofugal projections to the dorsolateral geniculate nucleus and superior colliculus that do not segregate into eye-specific areas, an altered functional organization in the dorsolateral geniculate nucleus, an expanded binocular subfield of the primary cortex, and decreased visual acuity at cortical level (Rossi et al., 2001; Muir-Robinson et al., 2002, Grubb et al., 2003). Taken together, the results of all of these studies indicate that nAChRs containing the β2 subunit are essential for the anatomical and functional development of the visual system in rodents. However, the exact nature of the nAChR subtypes expressed during rat development and adulthood is still unclear.

The aims of the present study were threefold. First, we wished to identify the pattern of nAChR subunit expression during postnatal rat development until adulthood using a combination of ligand binding and immunoprecipitation techniques. Second, we sought to establish the subunit composition of the retina nAChR subtypes, characterize their pharmacological profile, and localize them at cell level by using in situ hybridization techniques on P21 (when nAChRs first reach adult density and subunit expression pattern). Finally, we investigated whether visual experience affects the expression of retinal nAChRs on P21.

Materials and Methods

Animals and Materials. Male pathogen-free Sprague-Dawley rats (Harlan-Nossan, Milan, Italy) were used on P1, P5, P10, P21, P52, or P84. They were kept under standardized temperature, humidity, and lighting conditions (lights on at 8.00 AM and off at 8.00 PM) and had free access to water and food. In the dark-rearing experiments, the rats were kept in total darkness from birth to P21 with free access to food and water and were anesthetized in the dark with chloral hydrate before killing.

All of the animal experimentation was conducted in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). The protease inhibitors, nonradioactive Epi, nicotinic ligands, and Triton X-100 were purchased from Sigma Chemical (St. Louis, MO); the CnBr-activated Sepharose-4BCL, ^{125}I-protein A, and ^{125}I-αBgtx were from Amersham Biosciences UK, Ltd. (Little Chalfont, Buckinghamshire, UK); (±)[^{3}H]Epi (specific activity = 50–66 Ci/mmol) and ^{125}I-Epi (specific activity = 2200 Ci/mmol) were from PerkinElmer Life and Analytical Sciences (Boston, MA); and the reagents for gel electrophoresis were from Bio-Rad (Hercules, CA). αCntxMII was synthesized as described previously (Cartier et al., 1996).

Antibody Production and Characterization. The sequences of polyclonal Abs against the α2, α3, α4, α5, α6, β2, β3, and β4 peptides, which were raised and characterized as described previously (Del Signore et al., 2002; Zoli et al., 2002; Champtiaux et al., 2003), are shown in Table 1. Two different peptides were chosen for almost all of the subunits of the heteromeric receptors: one located in the cytoplasmic loop between M3 and M4 (CYT), which is the most divergent region of the subunits, and the other at the COOH terminal (COOH). Only one Ab was produced for the α2 subunit because the COOH peptide is almost identical with the α4 COOH. For the α6 subunit we used two Abs directed against two separate CYT peptides. The antibodies raised against the peptides were purified on an affinity column made by coupling the corresponding peptide to cyanogen bromide-activated Sepharose-4B according to the manufacturer's instructions.

The specificity and immunoprecipitation capacity of most antibodies has been reported previously (Zoli et al., 2002; Champtiaux et al., 2003). In addition, we tested the immunoprecipitation capacity of the anti-β3 Abs on eye membrane extracts obtained from wild-type and β3 KO animals (Cui et al., 2003). The Abs directed against the CYT and COOH β3 peptides both immunoprecipitated 36% of the [^{3}H]Epi receptors in the extracts obtained from the wild-type animals but did not immunoprecipitate the [^{3}H]Epi receptors in those obtained from the KO animals. The anti-α2 Ab specificity and immunoprecipitation capacity were tested on cell extracts obtained from human embryonic kidney cells transfected with the $\alpha2\beta4$ human subunits (a generous gift from Dr. E. Sher, Eli Lilly & Co. Ltd, Basingstoke, Hampshire, UK). The anti-α2 and β4 Abs, respectively, immunoprecipitated 95 ± 2% and 92 ± 3% of the [^{3}H]Epi–labeled receptors (mean ± S.E.M. of three independent experiments), whereas no specific immunoprecipitation was determined using the Abs directed against the other subunits.

Preparation of Membranes and 2% Triton X-100 Extracts from Eyes and Retinas. The eyes were dissected, immediately frozen in liquid nitrogen, and stored at −80°C for later use. No difference in the binding of the fresh and frozen tissues was observed.

In every experiment, the eyes were homogenized separately in an excess of 50 mM sodium phosphate, pH 7.4, 1 M NaCl, 2 mM EDTA,

2 mM EGTA, and 2 mM phenylmethylsulfonyl fluoride for 2 min in an UltraTurrax homogenizer (IKA Labortecnik, Staufen, Germany). The homogenates were then diluted and centrifuged for 1.5 h at 60,000*g*. The retinas were dissected from frozen eyes, separately homogenized using a Potter homogenizer (Sartorius, Goettingen, Germany), and processed as described for the whole eyes.

The procedures of homogenization, dilution, and centrifugation of the eyes as whole or isolated retinas were performed twice, after which the pellets were collected; rapidly rinsed with 50 mM Tris HCl, pH 7, 120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, and 2 mM phenylmethylsulfonyl fluoride; and then resuspended in the same buffer containing a mixture of 20 μg/ml of each of the following protease inhibitors: leupeptin, bestatin, pepstatin A, and aprotinin. Triton X-100 at a final concentration of 2% was added to the washed membranes, which were extracted for 2 h at 4°C.

The extracts from whole eyes or isolated retinas were then centrifuged for 1.5 h at 60,000*g* and recovered, and an aliquot of the resultant supernatants was collected for protein measurement using the BCA protein assay (Pierce Chemical, Rockford, IL) with bovine serum albumin as the standard.

Binding Assay. β2-, β4-, and α8-containing receptors bind [^{3}H]Epi with picomolar affinity, and α7 receptors bind Epi with nanomolar affinity (Gerzanich et al., 1995). To ensure that the α7 subtype did not contribute to [^{3}H]Epi binding, the binding tissue extract and immunoprecipitation experiments were performed in the presence of 2 μM αBgtx, which specifically binds to the α7 subtype and prevents Epi from binding to the subtypes containing this subunit.

The Triton X-100 extracts of retina at different ages were preincubated with 2 μM αBgtx for 3 h and then labeled with 2 nM [^{3}H]Epi. Tissue extract binding was performed using DE52 ion-exchange resin (Whatman, Maidstone, UK) as described previously (Vailati et al., 1999).

Immunoprecipitation of [^{3}H]Epibatidine-Labeled Receptors by Anti–Subunit-Specific Antibodies. The extracts obtained from the eyes at different ages or from dissected retinas preincubated with 2 μM αBgtx, and labeled with 2 nM [^{3}H]Epi, were incubated overnight with a saturating concentration of affinity purified IgG (20–30 μg; Sigma). The immunoprecipitation was recovered by incubating the samples with beads containing bound anti-rabbit goat IgG (Technogenetics, Trebbano, S.N., Milan, Italy). The level of Ab immunoprecipitation was expressed as the percentage of [^{3}H]Epi–labeled receptors immunoprecipitated by the antibodies (taking the amount present in the Triton X-100 extract solution before immunoprecipitation as 100%) or as femtomole of immunoprecipitated receptors per eye or as femtomole of immunoprecipitated receptors per milligram of protein.

Receptor Subtype Immunopurification and Analysis. The extracts prepared from P21 rat eyes were incubated three times with 5 ml of Sepharose-4B with bound anti-α6 Abs (column A) to remove the α6 subunit-containing receptors (α6* population). This α6* population was eluted from column A by means of incubation with the α6 peptide and was then further incubated with the anti-α4 Abs (column B) to remove the α6 receptors that also contain the α4 subunit (see *Results*). The α6 receptors bound to column B were eluted by competition with the α4 peptide, and those that did not bind the anti-α4 Abs remained in the flow-through of the column and were analyzed.

The flow-through of column A (i.e., the retina extract devoid of α6-containing receptors) was incubated twice with 5 ml of Sepharose-4B with bound anti-β2 (column C) or anti-α4 Abs (column D). The bound receptors were eluted with 0.2 M glycine, pH 2.2, or by means of competition with 100 μM of the corresponding β2 or α4 peptides used for Ab production. The subunit content of the purified receptors was determined by immunoprecipitation using the purified subtypes eluted with the peptides labeled with 2 nM [^{3}H]Epi and the subunit-specific Abs.

Gel Electrophoresis and Western Blotting. SDS-polyacrylamide gel electrophoresis was performed as described previously (Vailati et al., 1999) using 9% acrylamide. The proteins were electrophoretically transferred to nitrocellulose and subsequently probed with affinity-purified antipeptide antibodies. The bound antibodies were detected by means of ^{125}I-protein A.

Pharmacological Experiments on Immunoimmobilized Subtypes. The affinity-purified anti-α6 or anti-β2 Abs were bound to microwells (MaxiSorp; Nalge Nunc International, Naperville, IL) by incubating overnight 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 eye membrane extract containing 20 to 40 fmol ^{125}I-Epi binding sites, which was prepared by sequentially immunodepleting or not the α6-containing receptors. After incubation, the wells were washed, and the presence of immobilized receptors revealed by means of ^{125}I-Epi binding. Binding techniques for immunoimmobilized subtypes and data analysis were as described in Vailati et al. (1999).

In Situ Hybridization. After the analysis of mRNA secondary structure using GCG sequence analysis software version 7.1 (Accelrys, San Diego, CA), oligodeoxynucleotide sequences were chosen in unique regions of the rat nAChR subunit mRNAs. The probe characteristics and specificity controls are reported elsewhere (Zoli et al., 1995; Le Novère et al., 1996). Specificity controls were performed on retina sections and included the demonstration that 1) two or more probes for each mRNA give identical labeling pattern; 2) the labeling disappears when labeled probes are incubated with 50× excess of unlabeled probe; and 3) probes with the same base composition but different sequence do not give the specific labeling pattern. The

TABLE 1

Amino acid sequence of the peptides used to produce nAChR subunit-specific polyclonal antibodies

Amino acid sequence of the peptides used to produce the subunit-specific antibodies. Capital letters indicate the amino acids present in the subunit sequence, whereas the lowercase letters indicate the extra-sequence amino acids introduced to enable specific coupling to carrier protein.

Subunit	Peptide Sequence	Localization	Species
α2	CHPLRLKLSPSYHWLESNVDAEEREV	CYT	Human
α3	TRPTSNEGNAQKPRPLYGAELSNLNC	CYT	Human
α3	CQPLMARDDT	COOH	Rat
α4	PTSSPTSLKARPSQLPVSDQASPC	CYT	Rat
α4	cgPPFLAGMI	COOH	Rat
α5	DRYFTQREEAESGAGPKSRNTLEAALDC	CYT	Rat
α5	cgPVHIGNANK	COOH	Rat
α6 (1)	GVKDPKTHTKRPAKVKFTHRKEPKLLKEC	CYT	Rat
α6 (2)	CHKSSEIAPGKRLSQQPAQWTENSEHPPDV	CYT	Rat
β2	RQREREGAGALFFREAPGADSCT	CYT	Human
β2	cgLHSDHSAPSSK	COOH	Rat
β3	CGKESDTAVRGKVSGKRKQTPASD	CYT	Rat
β3	cgPALKMWIHRFH	COOH	Rat
β4	VSSHTAGLPRDARLRSSGRFREDLQEALEGc	CYT	Rat
β4	cGLPPLFQIHAPSKDS	COOH	Rat

oligonucleotide probes were labeled at the 3′ end using ^{35}S-dATP (Amersham) and terminal deoxynucleotidyl transferase (Roche Diagnostics, Indianapolis, IN) following the specifications of the manufacturer to a specific activity of 100 to 300 KBcq/pmol. The labeled probes were separated from unincorporated ^{35}S-dATP using G50 spin columns (Pharmacia, Peapack, NJ), precipitated in ethanol, and resuspended in distilled water containing 50 mM dithiothreitol.

P21 rat eyes were dissected out, immersed in 4% paraformaldehyde in phosphate-buffered saline overnight, embedded in gelatin, and frozen using crushed dry ice. The eyes were cut at the cryostat (20-μm thick sections) thaw mounted on gelatin-coated slides, and stored at −80°C for 1 to 3 days. The procedure was carried out according to Zoli et al. (1995). Probes were applied at a concentration of 2000 to 3000 Bcq/30 μl/section (corresponding to approximately 15 fmol/section). The slides were exposed for 14 days to ^{3}H Hyperfilm (Amersham) and then to a photographic emulsion (Ilford, Cheshire, United Kingdom) for 2 to 3 months.

Grain counting was performed in the different retinal layers by means of an automatic image analyser (KS300) as described by Zoli et al. (1992) and Pedrazzi et al. (1998). For each labeling, four retinas were analyzed.

Statistical Analysis. Statistical analysis of the expression of [^{3}H]Epi and ^{125}I-αBgtx receptors as well as the subunit content of the expressed [^{3}H]Epi receptors was carried out by one-way analysis of variance (ANOVA), followed by post-hoc Dunnett test. Comparison of nAChR subunit mRNA labeling in different retinal layers was performed by one-way ANOVA followed by Bonferroni test for multiple comparisons.

Results

nAChR Expression during Postnatal Retina Development

[^{3}H]Epibatidine-Binding Receptors. To investigate nAChR expression during postnatal retina development and aging, we performed binding studies using membranes prepared from whole eyes and isolated retinas obtained from the animals on P1, P5, P10, P21, P52, and P84.

We and others (Britto et al., 1992; Keyser et al., 1993; Gerzanich et al., 1995; Gotti et al., 1997b; Barabino et al., 2001) have shown previously that chick retina expresses a high level of αBgtx binding receptors that also bind [^{3}H]Epi receptors with nanomolar affinity. To avoid the contribution of these receptors to [^{3}H]Epi binding, we preincubated the tissue extracts with 2 μM αBgtx and thus only measured the binding of [^{3}H]Epi to αBgtx-insensitive nAChRs.

The expression of [^{3}H]Epi receptors was calculated in femtomole of bound [^{3}H]Epi receptors per eye (mean values ± S.E.M. of four to five experiments) and femtomole of [^{3}H]Epi receptors per milligram of retina protein (mean values ± S.E.M. of three to four experiments). The results are shown in Table 2 and Fig. 1, A and B. When expressed as femtomole per eye, the number of receptors increased almost linearly from P1 to P21 (from 11.1 to 73.6 fmol/eye) and then remained constant from P21 to P84 (71.6 fmol/eye on P52 and 73.0 fmol/eye on P84).

When expressed as femtomole per milligram of retina protein, the number of receptors did not change significantly from P1 to P5, sharply increased from P5 to P21, and then slightly decreased, being always significantly greater than the P1 values from P10 to adulthood (Fig. 1B and Table 2).

^{125}I-αBungarotoxin Binding Receptors. The results expressed as femtomole of ^{125}I-αBgtx receptors per eye (mean values ± S.E.M. of four experiments) are shown in Fig. 1A. As in the case of [^{3}H]Epi, the number increased almost linearly from P1 (16.9 fmol/eye) to P21 (36.6 fmol/eye) and then remained constant until P84 (36.7 fmol/eye). The level was almost constant at each developmental time when expressed as femtomole per milligram of retina protein (Fig. 1B and Table 2).

Subunit Content of Retinal [^{3}H]Epibatidine Receptors. The expression pattern of nAChR subunits during retina postnatal development was determined by quantitative immunoprecipitation experiments using subunit-specific antibodies and [^{3}H]Epi-labeled receptors to quantify the relative contribution of each nicotinic subunit to [^{3}H]Epi binding at each developmental stage. For each subunit (except α2), we used polyclonal Abs directed against two separate peptides. The results, expressed as femtomoles of immunoprecipitated receptors per milligram of retina protein, are the mean values of three to five separate experiments for each subunit at each developmental time (Fig. 2).

By quantifying the number of receptors immunoprecipitated by the specific Ab as the percentage of the total number of [^{3}H]Epi receptors, we also identified the major retinal subtypes present at each developmental time. P1 retina mainly contained the α4 (53.2 ± 4.3%) and β2 subunits (65.8 ± 4.2%), but there were also receptors containing the α3 (23.7 ± 5.0%), α6 (10.6 ± 2.4%), β3 (17.5 ± 4.4%), and β4 (22.7 ± 3.6%) subunits. From P1 to P21, there was an increase in the expression of the receptors containing the α2, α4, α6, β2, and β3 subunits and a decrease in the expression of those containing the α3 and β4 subunits.

By P21, the levels of the α2, α4, α6, β2, and β3 subunits expressed as femtomole per milligram of retina protein had increased by 33, 3.2, 7.3, 4.2, and 5.8 times over their P1 levels, respectively; the level of α3 and α5 increased 1.3 and 2.5 times, respectively, whereas the level of β4 subunit was 0.7 times lower (Fig. 2).

By P21 in addition to β2 (88.6 ± 7.3%) and α4 (56.2 ± 3.5%), which remain the major subunits in the retina, the α2 (23.0 ± 2.9%), α6 (26.3 ± 2.3%), and β3 (35.2 ± 3,8%) subunits were also well represented, whereas the α3 (9.2 ± 0.8%) and β4 (6.3 ± 1.2%) subunits were present in a small minority of Epi receptors.

Purification and Subunit Composition of the Major [^{3}H]Epibatidine Binding Subtypes Expressed on P21. We performed immunopurification experiments using subunit-specific antibodies to establish the subunit assembly of the heteromeric nAChR subtypes in the retina on P21. For these experiments, we only used extracts obtained from

TABLE 2
Expression of nicotinic receptors at different developmental times
Data are presented as means ± S.E.M. (n = 3–5 determinations/time interval).

Day	[^{3}H]Epibatidine Receptors		^{125}I-α-Bungarotoxin receptors	
	fmol/mg of protein	*fmol/eye*	*fmol/mg of protein*	*fmol/eye*
P1	69.7 ± 3.2	11.1 ± 1.9	35.4 ± 5.9	16.9 ± 5.2
P5	71.2 ± 4.7	22.9 ± 2.3	33.7 ± 3.8	22.8 ± 3.6
P10	112.3 ± 7.8*	32.8 ± 3.2*	35.8 ± 5.5	28.7 ± 3.0
P21	201.8 ± 17.2*	73.6 ± 6.2*	44.2 ± 3.8	36.6 ± 7.1
P52	153.3 ± 17.9*	71.6 ± 2.0*	30.5 ± 4.3	37.6 ± 2.6
P84	158.9 ± 13.7*	73.0 ± 3.0*	30.11 ± 3,5	36.7 ± 8.0

* Statistical analysis was carried out by one-way ANOVA, followed by post hoc Dunnett test. Each time point was compared with the previous time point by means of paired sample.

whole eyes because preliminary ligand binding and immunoprecipitation found that, although the specific activity in the isolated retinas was higher (201.8 ± 17.2) than that found in eye membranes (94 ± 7 fmol/mg of protein), both the specific activity of whole-eye extracts (233 ± 24 fmol/mg of protein) and the percentage of subunit-specific immunoprecipitation of [^{3}H]Epi receptors were very similar in whole eye and isolated retina. This indicates that the nAChRs present in the eyes as a whole and labeled by [^{3}H]Epi are only present in the retina, because no binding is detected in the eye membranes deprived of retina.

α6-Containing Receptors. The immunoprecipitation experiments showed that almost all of the receptors in the eluate of column A (see *Materials and Methods*) contained the β2 subunit (90.7 ± 2.4%) and a large majority contained the β3 subunit (71.4 ± 3.0%), whereas the α2, α3, α4, α5, and β4 subunits were present in 18.0 ± 3.0%, 12.9 ± 3.0%, 42.5 ± 3.4%, 3.0 ± 2.0%, and 7.0 ± 4.1% of the receptors, respec-

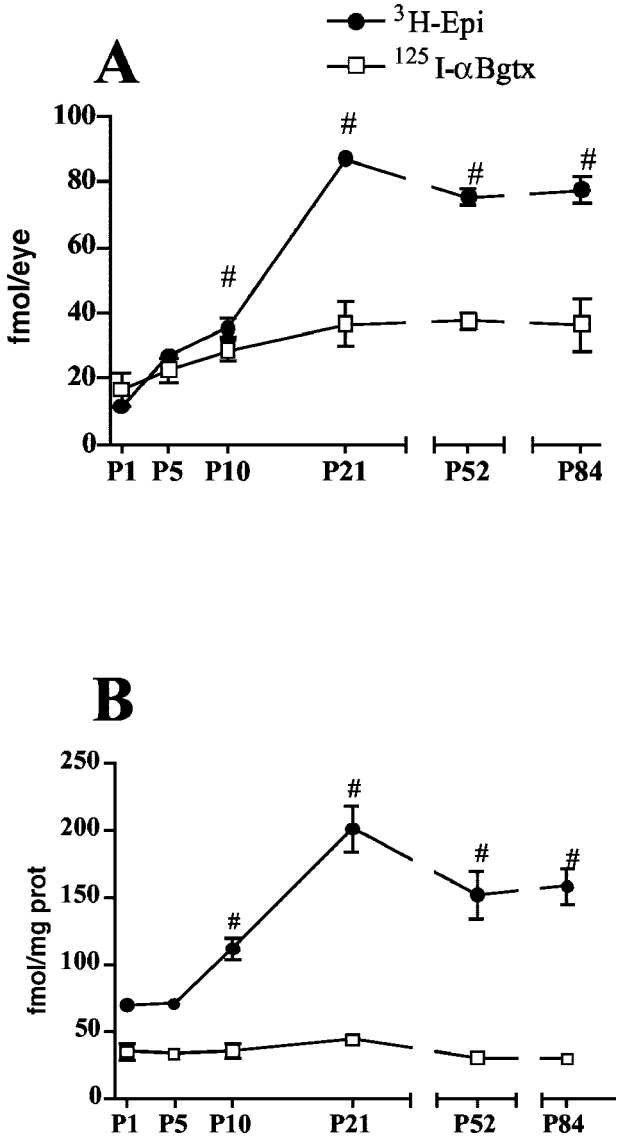


Fig. 1. Developmental changes in ^{125}I-αBgtx (□) and [^{3}H]Epi (●) binding receptors expressed in whole eye membranes (A) and isolated retinas (B). The eyes or isolated retinas were dissected from the animals at the indicated times and frozen, and the membrane homogenates were prepared as described under *Materials and Methods*. At each indicated postnatal day, total binding was performed using 2 nM [^{3}H]Epi or 10 nM ^{125}I-αBgtx and subtracted from the aspecific binding performed in parallel using 2 nM [^{3}H]Epi or 10 nM ^{125}I-αBgtx and 100 nM cold Epi or 1 μM unlabeled αBgtx. For total and aspecific [^{3}H]Epi binding, the eye membranes were always incubated with 2 μM cold αBgtx. The reported values are expressed as femtomoles of specific labeled ^{125}I-αBgtx and [^{3}H]Epi receptor per eye (A) or femtomoles per milligram of retina protein (B) and are the mean values ± S.E.M. of three to five experiments performed in triplicate (unless shown, the S.E.M. is in the range of the symbol). Statistical analysis was carried out by one-way ANOVA followed by post hoc Dunnett's test; #, $P < 0.05$ versus P1 mean value.

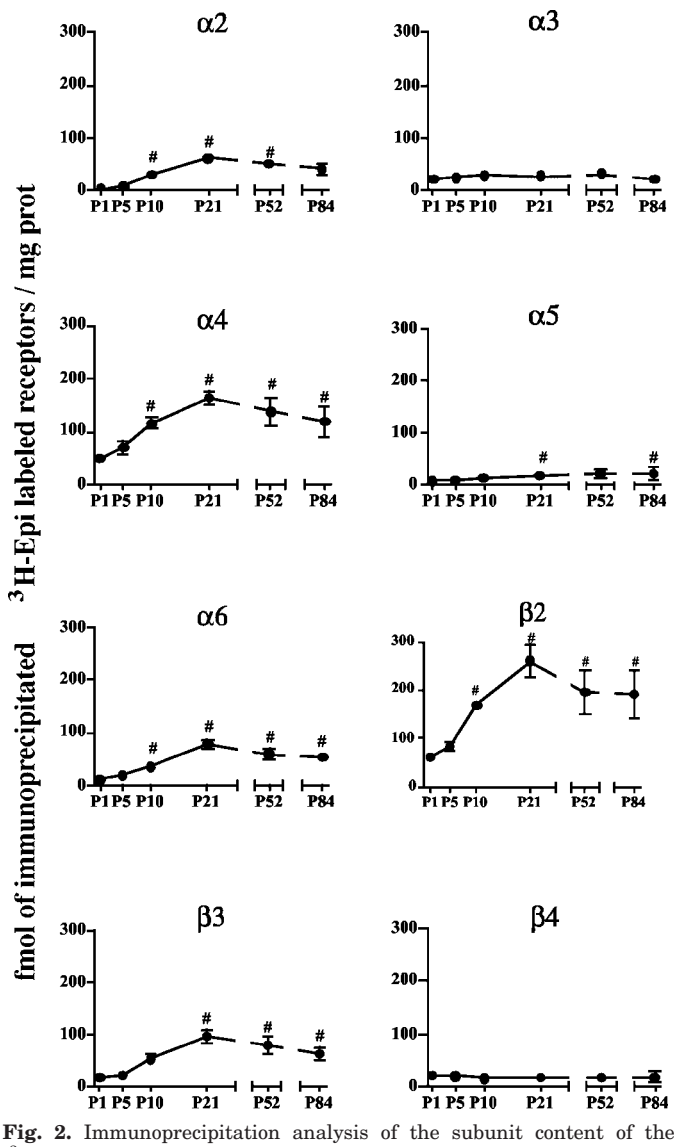


Fig. 2. Immunoprecipitation analysis of the subunit content of the [^{3}H]Epi receptors expressed in retinas. Triton X-100 (2%) extracts were obtained from membranes prepared from retinas dissected from the animals on P1, P5, P10, P21, P52, and P59, preincubated with 2 μM αBgtx, and then labeled with 2 nM [^{3}H]Epi. Immunoprecipitation was carried out as described under *Materials and Methods* using saturating concentrations (20–30 μg) of antisubunit Abs. Two Abs were used for each subunit (except for α2): one directed against a CYT peptide, and the other directed against a COOH peptide. In each experiment, the amount immunoprecipitated by each antibody was subtracted from the value obtained in control samples containing an identical concentration of normal rabbit IgG. The results are expressed as femtomoles of labeled [^{3}H]Epi receptor per milligram of retina protein and are the mean values ± S.E.M. of three to four experiments performed in duplicate (unless shown, the S.E.M. is in the range of the symbol). Statistical analysis was carried out by one-way ANOVA followed by post hoc Dunnett's test; #, $P < 0.05$ versus P1 mean value.

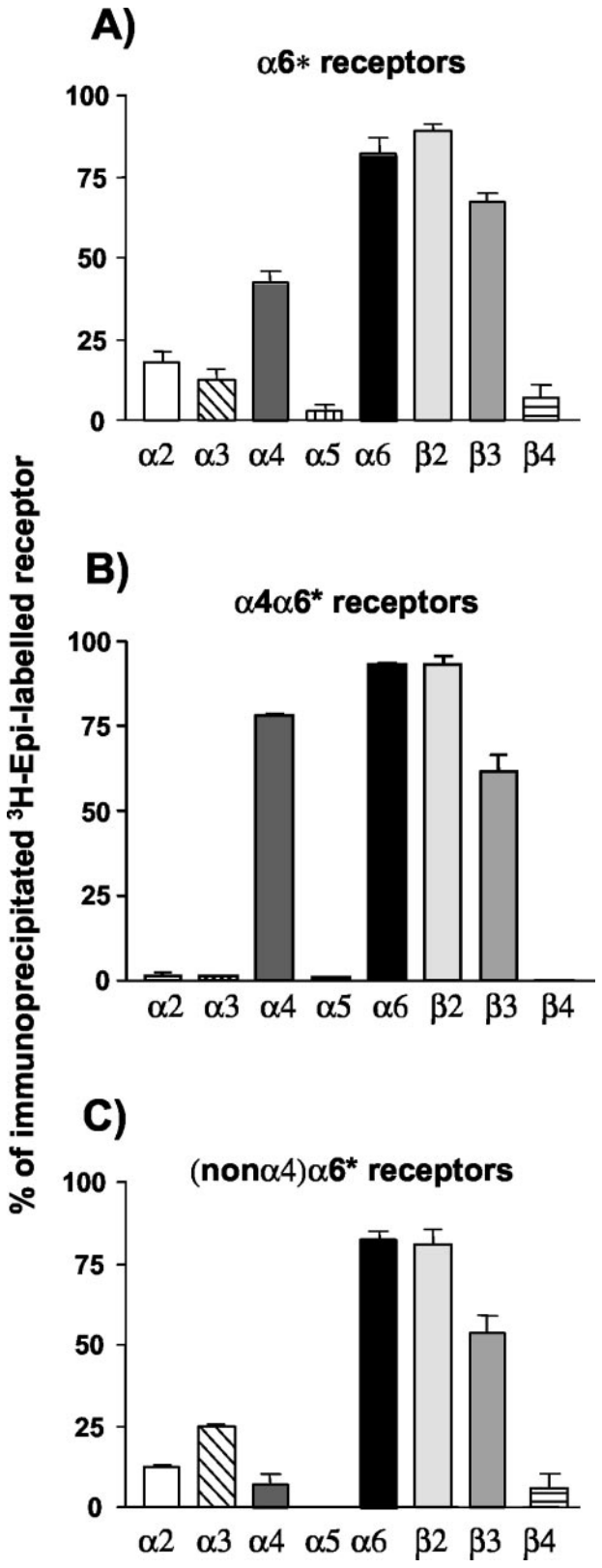


Fig. 3. Immunoprecipitation analysis of the subunit content of the α6* receptors. The extracts prepared from P21 rat eyes were incubated with an affinity column with bound anti-α6 Abs (see *Materials and Methods*) to bind the α6* population, which was eluted from the resin by means of incubation with the α6 peptide, labeled with 2 nM [^{3}H]Epi, and then immunoprecipitated by the indicated subunit-specific Abs (A). The α6* population was further subfractioned by incubation with an affinity resin with bound anti α4 Abs. B and C show the results of the analyses of the eluate (α4α6 receptors) (B) and the flow-through of the column (non-α4α6* receptors) (C) labeled with 2 nM [^{3}H]Epi and immunoprecipitated by the indicated subunit-specific Abs. Immunoprecipitation was carried out as described under *Materials and Methods* using saturating concentrations (20–30 μg) of antisubunit Abs. The amount immunoprecipitated by each antibody was subtracted from the value obtained in control samples containing an identical concentration of normal rabbit IgG, and the results obtained with each Ab are expressed as the percentage of total [^{3}H]Epi binding present in the solution before immunoprecipitation. Each data point is the mean ± S.E.M. of three determinations performed in triplicate.

tively (Fig. 3A). These receptors were defined as the α6* population.

To investigate whether this population can be further subdivided, we subfractioned it by incubating with Sepharose beads with bound anti-α4 Abs (column B) and then recovering by competition with the α4 peptide. The subfractioned α6* receptors were analyzed by immunoprecipitation and found to contain the α6 (92.1 ± 0.9%), α4 (78.6 ± 0.6%), β3 (61.5 ± 5.1%), and β2 (92.9 ± 2.9%) subunits (Fig. 3B). As a further control, we also checked the subunit composition of the flow-through of column B and, as shown in Fig. 3C, found that it contained α6* receptors (82.2 ± 2%) that were devoid of the α4 subunit but contained the β2 (81.2 ± 4.2%), β3 (53.9 ± 5%), α2 (12.8 ± 0.4%), and/or α3 (24.7 ± 1.0%) subunits. These subfractionation and immunoprecipitation studies showed that the rat retina receptor subtypes containing the α6 subunit are α6α4β3β2, α6α2/α3β3β2, and α6β3β2.

(Non-α6)–Containing Receptors. After being immunodepleted of the α6-containing receptors, the membrane extract was incubated twice with Sepharose beads with bound anti-β2 Abs (column C). We defined these β2-containing receptors as the (non-α6)β2* receptor population. The β2 subunit in these receptors was associated with the α4 (71.7 ± 4.4%) and α2 subunits (25.2 ± 5.5%) (Fig. 4A).

To identify whether the α4 and α2 subunits coexist in the same subtype, we performed additional experiments in which the α6-depleted extract was directly incubated with Sepharose beads with bound anti-α4 Abs (column D). The bound receptors eluted by competition with the α4 peptide were analyzed by immunoprecipitation experiments and showed that 90 ± 1.8% of them contained the β2 subunits, 87 ± 5.2% the α4 subunits, and 22 ± 0.6% the α2 subunit. These were therefore defined the α4(non-α6)* receptor population (Fig. 4B).

In the α4(non-α6)* population, coimmunoprecipitation of the α2 and α4 subunits clearly indicated the presence of a subtype containing the α2, α4, and β2 subunits, and a subtype containing the α4 and β2 subunits. We found that 14.1 ± 1.5% of these receptors also contain the β3 subunit, thus indicating that this subunit is present in a minority of retinal receptors without being associated with the α6 subunit.

Moreover, binding and immunoprecipitation analysis of the flow-through of the anti-α4 affinity column (column D) revealed the presence of [^{3}H]Epi receptors that contained the α2 and α3 subunits associated with the β2 (77.8 ± 1.6%) and/or β4 (22.1 ± 2.0%) subunits. This population represented 14% of the total number of [^{3}H]Epi receptors and was defined as the (non-α4/non-α6)* population, i.e., one or more subtypes containing the α2 (40.0 ± 3.0% %), α3 (31.4 ± 2.0%), β2 (77.8 ± 3.1%), and/or β4 (22.1 ± 2.0%) subunits but not the α4 and α6 subunits.

Distribution of nAChR Subunit mRNAs in the Rat Retina at P21. We performed an in situ hybridization study

of the distribution of heteromeric nAChR subunit mRNAs in P21 rat retina. The probes for all of the heteromeric subunit mRNAs (except $\beta4$) gave a positive signal in the retina, each with a specific layer distribution.

Analysis of the in situ hybridization preparations showed that rat retina contains relatively high levels of $\alpha3$, $\alpha5$, $\alpha6$, and $\beta3$ mRNAs, moderate levels of $\beta2$ and $\alpha4$ mRNAs, and relatively low levels of $\alpha2$ mRNA; no specific signal could be detected for $\beta4$ mRNA. Besides differences in the overall intensity of the labeling, the distribution of the different subunit transcripts was heterogeneous (Fig. 5) and subunit-specific.

The quantitative analysis of the preparations (Fig. 6 and Table 3), after correcting labeling intensity for the specific activity of the probes (Le Novère et al., 1996), showed that the rank order for the different subunit mRNAs in the GCL was $\alpha6 > \beta3 > \alpha5 \approx \alpha3 > \beta2 \approx \alpha4 > \alpha2$. However, $\alpha3$ and $\alpha2$ mRNA signals were only detected in scattered cells in the GCL. The rank order in the INL was $\alpha5 \approx \alpha6 > \beta3 \approx \alpha3 > \beta2 \approx \alpha4 > \alpha2$. Again, $\alpha2$ was heterogeneously distributed in the layer, and its amount is therefore underestimated. Finally, the rank order in the ONL was $\alpha5 > \alpha6 > \beta3 \approx \beta2 \approx \alpha3 \approx \alpha4 > \alpha2$. It should be noted that the rank order of the levels of nAChR subunit mRNA and protein (see above) are sharply different. A similar observation was made in the ventral midbrain dopamine neurons (Le Novère et al., 1996; Zoli et al., 2002), which express a pattern of nAChR subunits very similar to that of the retina, suggesting that the efficiency of translation and assembly of the subunits is very diverse and possibly subunit-specific. For instance, in both retina and dopamine neurons, high levels of $\alpha5$, $\alpha6$, and $\beta3$ mRNA correspond to a relatively minor proportion of $\alpha5$-, $\alpha6$-, or $\beta3$-containing receptors, whereas moderate to low levels of $\alpha4$ and $\beta2$ mRNA correspond to a major proportion of $\alpha4$- or $\beta2$-containing receptors.

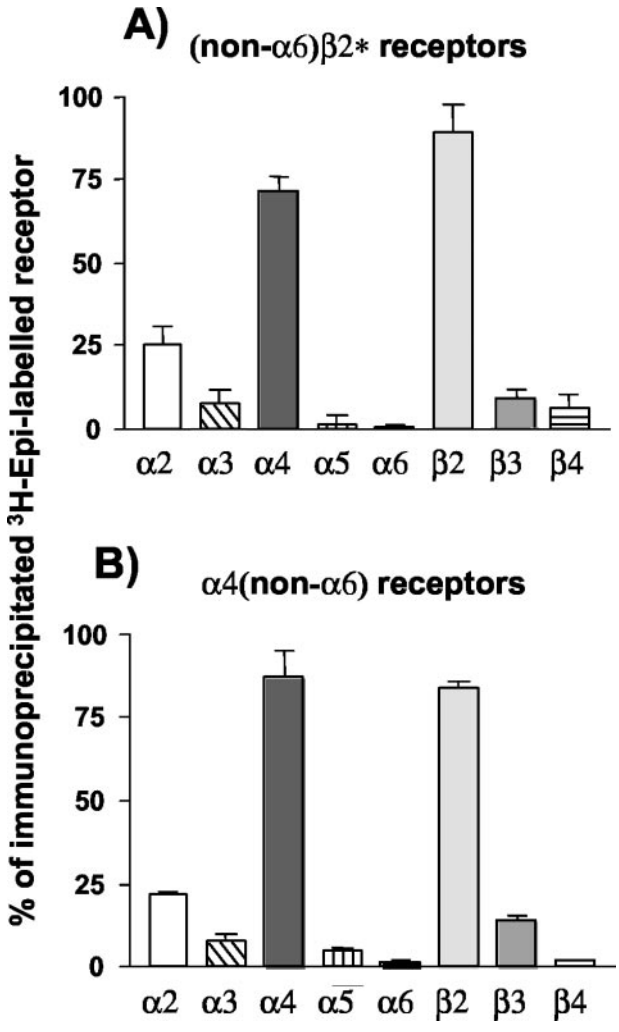


Fig. 4. Immunoprecipitation analysis of the (non-$\alpha6$)$\beta2$* and $\alpha4$(non-$\alpha6$)* populations. The extract devoid of the $\alpha6$-containing receptors was incubated with resin with bound anti-$\beta2$ Abs (A) or anti-$\alpha4$ Abs (B). The bound receptor populations were eluted by the corresponding peptides, labeled with 2 nM [^{3}H]Epi, and immunoprecipitated by the indicated subunit-specific Abs. The results are expressed as described in Fig. 3.

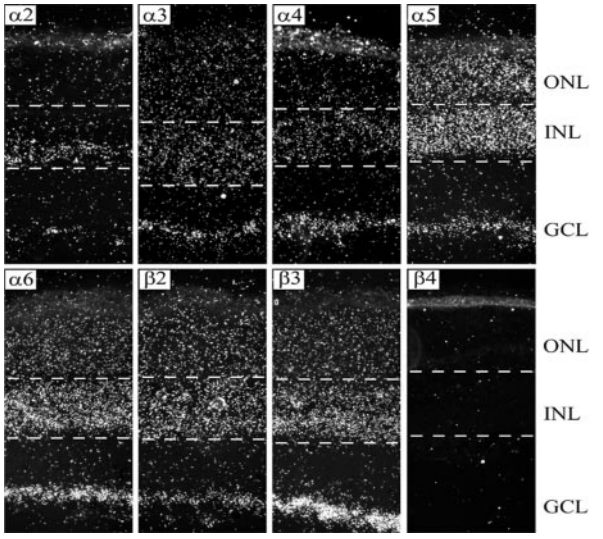


Fig. 5. Distribution of nAChR subunit mRNAs in rat retina at P21. Dark-field microphotographs of emulsion autoradiograms showing $\alpha2$, $\alpha3$, $\alpha4$, $\alpha5$, $\alpha6$, $\beta2$, $\beta3$, and $\beta4$ nAChR subunit mRNAs in the retina of P21 rat. To compare these images with the grain counting results shown in Fig. 6, it needs to be remembered that the quantitation was corrected for background subtraction and specific probe activity. In the images shown here, the relative specific activity was $\alpha2 = 0.16$, $\alpha3 = 0.50$, $\alpha4 = 0.18$, $\alpha5 = 0.42$, $\alpha6 = 1.00$, $\beta2 = 0.25$, $\beta3 = 0.29$, and $\beta4 = 0.38$.

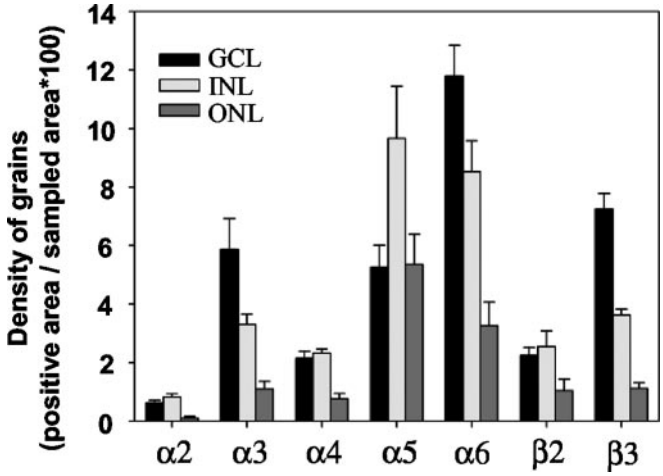


Fig. 6. Density of nAChR subunit mRNAs in the retina of P21 rats. Density of photographic emulsion grains/retinal layer of $\alpha2$, $\alpha3$, $\alpha4$, $\alpha5$, $\alpha6$, $\beta2$, and $\beta3$ nAChR subunit mRNA labeling in the retina of P21 rat. For methodological details, see *Materials and Methods*. Mean values ± S.E.M. ($n = 4$).

Western Blot Analysis of the α6* and (Non-α6)β2* Receptor Populations. The immunopurification analysis described above identified two major populations of heteromeric receptors (those containing and those not containing α6). The subunit composition of the P21 α6* and (non-α6)β2* receptor populations was analyzed by Western blotting using the same subunit-specific Abs as those used for the immunoprecipitation experiments. The results confirmed that the α2, α4, and β2 subunits were present in both populations, but the α6 subunit was only present in the α6* population (Fig. 7). Although present at much higher levels in the α6* population, the β3 subunit was also present in very small amounts in the (non-α6)β2* receptor population. The anti-α2 Ab (lanes 1 and 7) recognized a major peptide with a molecular mass of 60 ± 1 kDa in both populations; the anti-α3 Ab (lanes 2 and 8) faintly recognized a peptide of 51 ± 1 kDa; the anti-α4 (lanes 3 and 9) recognized a single band of 68 kDa in both populations, and the anti-β2 Ab (lanes 5 and 11) recognized a single band of 52 kDa in both populations. The anti-α6 Ab recognized a single band of 57 kDa (lanes 4 and 10) only in the α6* receptor population, and the anti-β3 Ab (lanes 6 and 12) recognized three peptides of 58, 50, and 40 kDa mainly in the α6* receptor population. In agreement with the immunoprecipitation results, the anti-α5 did not recognize any band in either receptor population (data not shown).

Pharmacological Characterization of the α6* and (Non-α6)β2* Receptor Populations. We pharmacologically characterized the nicotinic subtypes in rat retina by performing binding experiments on the immunoimmobilized subtypes as described under *Materials and Methods*. Because the equilibrium binding assays revealed no significant differences in the affinity for [^{3}H]Epi of the α6* and (non-α6)β2* receptor populations [apparent K_d values of, respectively, 10.2 (CV 34%) and 8.7 pM (CV 25%)], competition binding studies were performed using a number of nicotinic ligands. No significant differences were detected for the agonists acetylcholine, nicotine, cytosine, and DMPP or the antagonists dihydro-β-erythroidine and D-tubocurarine (Table 4), but significant differences were observed for αCntxMII, which showed a statistically significant better fit for a two-site model with a high- (K_i = 1.1 nM) and low-affinity site (K_i > 10 μM) when tested on the α6* nAChRs (Fig. 8 and Table 4). We only determined a single low-affinity site for the (non-α6)β2* receptors, with K_i > 10 μM.

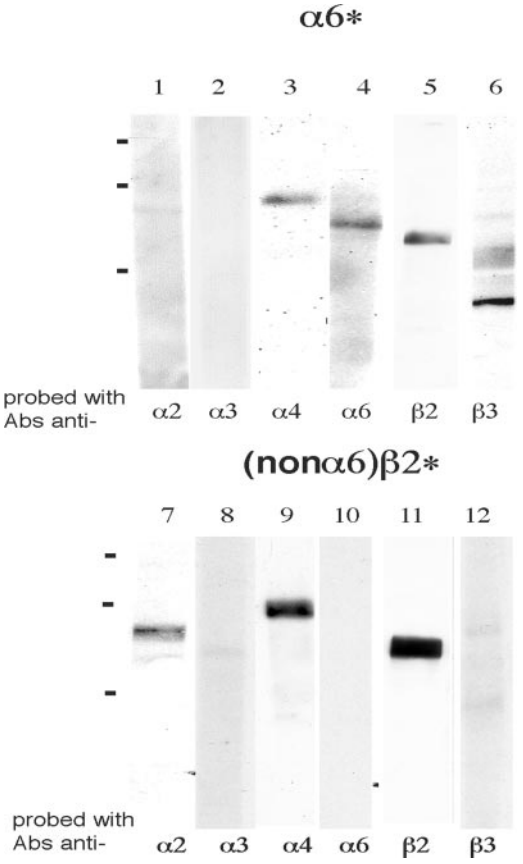


Fig. 7. Western blot analysis of P21 affinity-purified α6* and (non-α6)β2* nAChRs. The receptors were prepared as described under *Materials and Methods*. The eluted receptors were concentrated and separated on 9% acrylamide SDS gels, electrotransferred to nitrocellulose, and then probed with 5 to 10 μg/ml of the indicated Abs. The bound Abs were revealed by means of ^{125}I-protein A. The molecular mass markers (top to bottom) are 97, 66, and 45 kDa.

TABLE 3

Statistics of nAChR mRNA grain counts

Statistical analysis by means of one-way analysis of variance followed by Bonferroni test for multiple comparisons. GCL, $F(6,49) = 31.74$, $p < 0.001$; INL, $F(6,49) = 16.49$, $p < 0.001$; ONL, $F(6,49) = 11.75$; $p < 0.001$. For each pair of nAChR subunit mRNAs, significant differences in grain density in the different retinal layers are indicated in capital letters.

α2							
α3	GCL						
	inl						
	onl						
α4	gcl	GCL					
	inl	inl					
	onl	onl					
α5	GCL	gcl	GCL				
	INL	INL	INL				
	ONL	ONL	ONL				
α6	GCL	GCL	GCL	GCL			
	INL	INL	INL	inl			
	ONL	onl	ONL	onl			
β2	gcl	GCL	gcl	gcl	GCL		
	inl	inl	inl	INL	INL		
	onl	onl	onl	ONL	onl		
β3	GCL	gcl	GCL	gcl	GCL	GCL	
	inl	inl	inl	INL	INL	inl	
	onl	onl	onl	ONL	onl	onl	
	α2	α3	α4	α5	α6	β2	β3

TABLE 4

Affinity of nicotinic agonists and antagonists for immuno-immobilized nAChR subtypes

The K_d and K_i values were derived from the curves of ^{125}I-Epi saturation and competition binding to α 6* or (non-α 6)β 2* immuno-immobilized receptors. The curves obtained from three or four separate experiments were fitted using a nonlinear least squares analysis program. A two-site model was statistically significant (F test) for α -conotoxin MII, whereas the D-tubocurarine and cytisine data better fitted a one-site model. The numbers in parentheses represent the percentage coefficient of variation (CV).

	α6*		(non-α6)β2*	
	pM			
K_d ^{125}I-Epibatidine	10.2	(34)	8.7	(25)
	nM			
K_i				
Cytisine	1.43	(29)	0.25	(42)
Nicotine	5	(33)	3.3	(36)
Acetylcholine	11.3	(37)	11.5	(32)
1,1-Dimethyl-4-phenylpiperazinium	15.7	(40)	24	(43)
Dihydro-β-erythroidine	1012	(36)	1000	(25)
D-Tubocurarine	700	(36)	1650	(27)
α-Conotoxin MII	1.1	(47)		
	>10,000		>10,000	

Dark-Rearing Slightly Affects the Number and Subunit Composition of Rat Retina [^{3}H]Epibatidine Receptors, but Does Not Affect ^{125}I-αBungarotoxin Receptors. Activity-dependent synaptic plasticity is a fundamental feature of the vertebrate central nervous system. To determine whether retinal nAChRs are regulated by visual activity, the expression of ^{125}I-αBgtx and [^{3}H]Epi nicotinic retinal receptors on P21 and the subunit composition of the^{3}H-Epi receptors in dark-reared (DR) rats and rats raised in a diurnal light/dark cycle (LR) were compared.

Radioactive ligand binding studies of the membranes obtained from the eyes of the DR and LR animals showed that the levels of ^{125}I-αBgtx receptors were not significantly different (mean ± S.E.M. of five experiments, DR = 34.4 ± 2.0 fmol/mg of membrane protein, LR = 31.4 ± 2.2 fmol/mg of membrane protein) (Fig. 9A). The DR animals however, had approximately 30% more [^{3}H]Epi receptors (110.2 ± 7.2 and 86.4 ± 5.0 fmol/mg of membrane protein, $P < 0.05$) (Fig. 9B) than the LR animals.

Given the considerable increase in [^{3}H]Epi receptors containing the α2, α4, α6, β2, and β3 subunits on P21, the levels of the subunits contained in 2% Triton X-100 eye extracts from the LR and DR animals were determined. The increase in the number of receptors in the DR rats was statistically significant and associated with an increase in the number of receptors containing the α4 and β2 subunits but not of those containing the α2, α3, α5, α6, β2, and β3 subunits (Fig. 9C).

Discussion

The various nicotinic effects of ACh during retina development may be mediated by the different receptor subtypes that may have a different pattern of signaling (Dmitrieva et al., 2001; Zhou, 2001). Identifying the nAChR subtypes involved in ACh retinal action is thus important for understanding retina circuitry and developing pharmacological tools that modulate specific retinal functions.

In this molecular and pharmacological study, we identified the nAChR subtypes expressed in rat retina and studied their expression at different postnatal developmental stages. Our main findings were that 1) the numbers of αBgtx and Epi nAChRs increased from P1 to P21, when they reached adult levels, with the increase in Epi receptors being caused by selective increases in the receptors containing the α2, α4, α6, β2, and β3 subunits; 2) immunopurification experiments showed that P21 retina contains a relatively wide array of different heteromeric nAChR subtypes, and in situ hybridization experiments showed that nAChR subunit mRNAs have highly heterogeneous distribution patterns throughout the retinal layers; and 3) although visual experience does not markedly alter the developmental expression of nAChRs, there was a selective increase in the expression of the α4β2 subtype in the retina of DR animals.

Because our findings concerning retinal subtype expression and their subunit assembly are derived from ligand binding experiments and the immunoprecipitation of [^{3}H]Epi-labeled receptors with subunit-specific Abs, they critically depend on Ab specificity and efficiency. These parameters were carefully checked by means of previously described immunoprecipitation experiments using tissues obtained from KO animals and purified receptors (Zoli et al., 2002; Champtiaux et al., 2003) but still require previously discussed caveats (Zoli et al., 2002).

In agreement with the results of previous in situ hybridization studies (Zoli et al., 1995) and in accordance with the

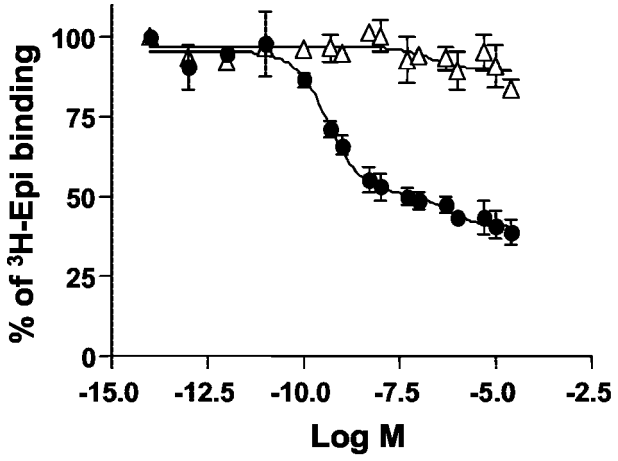


Fig. 8. Inhibition of ^{125}I-Epi binding to native immunoimmobilized α6*(●) and (non-α6)β2* (△) by αCntxMII. The curves were obtained by fitting three to four separate competition experiments using the LIGAND program.

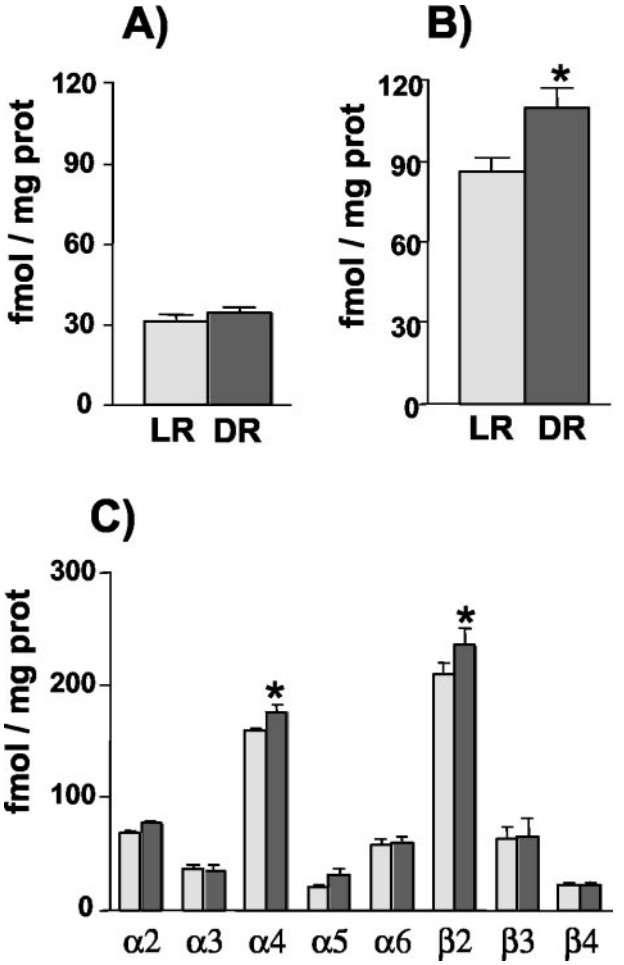


Fig. 9. Effects of dark rearing on nAChR expression. ^{125}I-αBgtx binding (A) and [^{3}H]Epi (B) in eye membranes obtained from LR and DR animals on P21. C, immunoprecipitation of nAChR subunits in 2% Triton X-100 extracts of eye membranes obtained from P21 LR and DR animals. Each value represents the mean ± S.E.M. of three separate experiments. ⋆, $P < 0.05$ (Student's t test).

hypothesis that nAChRs play an important role during the pre- and perinatal retinal development, we found high concentrations of both Epi and αBgtx receptors at birth. However, although the number of both classes of receptors increased during the postnatal period, there were more Epi than αBgtx receptors. Taken from the current hypothesis that homomeric αBgtx-sensitive receptors have five ligand binding sites per receptor and that heteromeric receptors have only two (Le Novère and Changeux, 1995; Corringer et al., 2000), Epi receptors are more expressed than αBgtx receptors at P1 and become largely predominant during postnatal development and adulthood.

As observed in adult rabbit retina (Keyser et al., 2000), we found that the large majority of heteromeric receptors in developing and adult rat retina contain the β2 subunit, although at birth, approximately 20% of receptors were β4* nAChRs. The amount of β4* nAChR then remained constant whereas the amount of β2* nAChRs increased markedly so that by P21, the large majority of retinal receptors contained the β2 subunit. The prevalence of β2* receptors seems to be mammal-specific because we have previously shown that the β4 subunit is the major postnatally expressed structural subunit in chick retina after a developmental shift from β2 to β4 during embryonic development (Vailati et al., 1999, 2003). Besides β2 and α4, which are constantly the most concentrated subunits of the heteromeric receptors, during postnatal development, there is a clear change in the concentration of the other subunits. In the early phase of retinal development (P1), 24% of the receptors contain the α3 and 23% the β4 subunits, but there is a selective increase in the expression of receptors containing the α2, α6, α4, β2, and β3 subunits by P5, which reaches a peak by P21.

Previous pharmacological and KO animal studies have shown that mouse retinal waves depend on heteromeric nAChRs; in particular, β2 KO animals have no nAChR-mediated waves between P0 and P8 (Bansal et al., 2000). This can now be interpreted as a consequence of the fact that the large majority (>80%) of heteromeric nAChRs contain the β2 subunit at all postnatal developmental stages, and therefore, very few nAChRs are left to mediate retinal waves after β2 subunit deletion.

The contribution of α3-containing receptors changes during development: on P1, they represent a substantial fraction of heteromeric nAChRs (24%) and functionally participate in retinal wave activity (Bansal et al., 2000), but although their number (expressed as femtomole per milligram of protein) remains almost constant during development, their relative contribution to the total number of Epi receptors markedly decreases. On the other hand, the receptors containing the α6 subunit are highly up-regulated during development, and their number increases 7.3 times between P1 and P21, when they become largely predominant over α3-containing receptors. Because the amino acid sequence of the α3 subunit is very close to that of the α6 subunit (70% identity), it is likely that most of the retinal receptors in adult vertebrates identified previously as containing α3 on the basis of immunolocalization and immunopurification experiments may actually have contained α6 rather than α3 subunits (Whiting et al., 1991; Keyser et al., 2000).

Another important finding is the considerable developmental increase in the number of α2-containing receptors (more than 33-fold from P1 to P21), which account for 23% of all heteromeric receptors by P21. In addition, α2 mRNA distribution in the retina is unique among nAChR subunits because α2 mRNA labeling was only detected in the GCL and external INL, thus suggesting that α2-containing nAChRs may be expressed selectively in a subpopulation of RGCs. α2-Containing receptors in the retina may play a particular role in the functional and anatomical development of visual systems, as indirectly suggested by the recent finding that β2 KO mice have an altered anatomical and functional visual development, whereas α4 or α6 KO animals do not (Rossi et al., 2001; Champtiaux et al., 2002). It is therefore possible that principal subunits other than α6 and α4 constitute the β2* nAChRs, which are necessary for the normal development of the visual system and/or that subunit heterogeneity plays a role in the functional compensation of the missing α subunits in KO animals.

The immunopurification studies isolated three populations of P21 heteromeric retinal nAChRs: the α6* receptor population (approximately 26% of total Epi binding), the α4(non-α6)* receptor population (approximately 60% of the receptors), and the (non-α4/non-α6)* receptor population (around 14% of receptors). All three populations are heterogeneous, but immunoprecipitation and further immunopurification of the purified receptors allowed for the identification of the subunit composition of most of the different subtypes. A summary of the identified subtypes and their relative percentages of all of the Epi receptors present in rat retina are shown in Table 5.

The pharmacological analysis was limited to the two major retinal populations of α6* and (non-α6)β2* receptors. They have indistinguishable binding affinity for a number of classic nicotinic agonists and antagonists but can be discriminated by using the αCntxMII antagonist, which, as shown previously for striatal α6 receptors purified from rat (Zoli et al., 2002) and wild-type and α4 or α6 KO mice (Champtiaux et al., 2003), binds with nanomolar affinity only to the α6β2 interface and therefore exclusively to the α6* receptor population. Together with the results of previous equilibrium binding experiments showing that αCntxMII binding disappears from the striatum of α6−/− mice (Champtiaux et al., 2002), this clearly indicates that α6* nAChRs are abundantly expressed in the retina.

From the results of pharmacological studies using αCntxMII (Bansal et al., 2000), it has been suggested that the α3β2 subtype is important for retinal wave activity. The high affinity of αCntxMII for the α6 subtype, together with the demonstration of the presence of receptors containing the α6 subunit on P1, suggests that this subtype may also be involved in early-stage retinal wave activity.

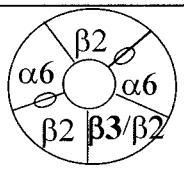
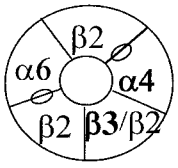
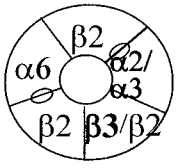
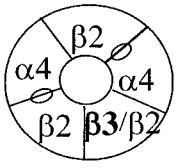
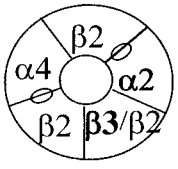
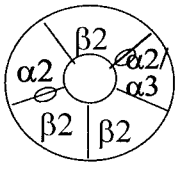
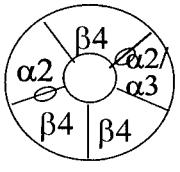
Synaptic plasticity is the ability of neurons to alter the strength of their synaptic connections as a result of activity and experience. This is a common phenomenon in the central nervous system, and it has long been believed that the synaptic plasticity mediated by visual experience only occurs in the cortex and not in the retina or the lateral geniculate nucleus (the two processing centers that relay visual information to the cortex) (Feller, 2002, 2003). Recent studies, however, have demonstrated that the retina also shows morphological and functional alterations induced by visual experience (Tian and Copenhagen, 2003).

We found that visual deprivation does not markedly alter the developmental program of nAChR expression, but it does

TABLE 5

Heteromeric nicotinic receptors in rat retina

Diagram showing the [^{3}H]Epi receptor subtypes present in rat retina, their possible subunit arrangement, and their percentage of the total [^{3}H]Epi receptors present in 2% Triton X-100 retina membrane extracts. The percentage of the α6* population (26%) was obtained by immunoprecipitating the total membrane extract. The percentage of the (non-α4/non-6α) population (14%) was calculated on the basis of [^{3}H]Epi binding and immunoprecipitation on the 2% Triton X-100 extract after the immunodepletion of the α6* and α4(non-α6*) population. The percentage of the α4(non-α6*) population was deduced from the difference. The percentage of each subtype was determined from immunoprecipitation on purified subtypes corrected for the percentage of the population in relation to the total receptor present in the 2% Triton X-100 extract. In defining the retinal nAChR subtypes, we followed the current hypothesis that heteromeric nAChRs have two binding sites per molecule of receptor with at least two subunits bearing the principal amino acid loops for ACh binding interfaces (i.e., α2, α3, α4 or α6 subunits) and two subunits bearing the complementary amino acid loops (i.e, β2 or β4 subunits), whereas the fifth subunit can be either a complementary subunit or a purely structural subunit (α5 or β3 subunits) (Corringer et al., 2001). Based on current experimental and theoretical knowledge concerning the compositional rules of nAChRs, the β3 subunit cannot make a functional receptor in the absence of β2 or β4 subunit; therefore, in the case of the α6β2β3 subtype, we believe that the β3 subunit must always be with the α6 and β2 subunits. In the case of the α6α4β3β2 subtype, the large majority of the [^{3}H]Epi receptors contain these four subunits and we therefore believe that one of the Epi binding sites is at the interface between the α6 and β2 subunits, whereas the other is at the interface between the α4 and β2 subunits and that the β3 subunit is the fifth subunit. The α6α4β3β2 subtype thus theoretically contains one α6, one α4, one β3, and two β2 subunits. The definition of the subunit composition of the α6α2/α3β3β2 subtypes is more complex. All of these receptors must have one binding site at the interface between the α6 and β2 subunits (because they were purified over an anti-α6 Ab), but the other binding site could be at the interface between the β2 and the α3, α2, or α6 subunits. Moreover, 53.9% of the receptors in this receptor population contain the β3 subunit, which may therefore be the fifth subunit in this fraction, whereas the fifth subunit in the remaining receptors may be the β2 subunit.

Population	Subtypes	Subunit Arrangement	Percentage over Total Retinal [^{3}H]Epi Receptors
α6* (26%)	α6β2β3		9
	α6α4β3β2		11
	α6α2/α3β3β2		6
α4(non-α6)* (60%)	α4β2(β3)		47
	α2α4β2(β3)		13
(non-α4/non-α6)* (14%)	α2β2, α3β2		10.8
	α2β4, α3β4		3.2

induce a higher expression of the $\alpha4\beta2$ subtype, which is also one of the earliest expressed subtypes in the retina. We do not know the role of or the reasons for this increased expression and can only hypothesize that it is related to modifications in the RGC dendritic fields, because it has been shown that DR increases the receptive field area of RGCs in turtle, and that this effect is blocked by nicotinic antagonists (Sernagor and Grzywacz, 1966).

It has very recently been reported that the DR abolition of developmental dendritic loss may depend on the activation of nAChRs expressed on third-order retinal neurons, because mice lacking the nAChR $\beta2$ subunit show delayed refinements of RGC dendrites (Bansal et al., 2000; Tian and Copenhagen, 2003). Because RGC dendrites express nAChRs, we speculate that the increased level of nAChRs in DR retina is caused by lack of pruning. These and previous data all together indicate that several nAChRs are present and involved in the developmental shaping of retinal circuits by visual experience in different species.

Acknowledgments

We thank Professor Jean-Pierre Changeux (Pasteur Institute, Paris), Dr. Mariella De Biasi (Baylor College, Huston), and Dr. Stephen F. Heinemann (The Salk Institute for Biological Studies, La Jolla, California) for the generous gift of neuronal tissues from wild-type and knockout mice; Dr. J. Michael McIntosh (University of Utah) for the generous gift of αconotoxin MII; and Dr. Emanuele Sher (Eli Lilly &Co. Ltd., Basingstoke, Hampshire, UK) for the generous gift of membranes of transfected $\alpha2\beta4$ cells.

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