0026-895X/03/6306-1329–1337$7.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 63:1329–1337, 2003

Vol. 63, No. 6
2401/1064769
Printed in U.S.A.

Developmental Expression of Heteromeric Nicotinic Receptor Subtypes in Chick Retina

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Received January 13, 2003; accepted February 12, 2003

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

ABSTRACT

Acting through nicotinic acetylcholine receptors (nAChRs), acetylcholine plays an important role in retinal development and the formation of retinal connections to target tissues, but very little is known about the nAChR subtypes expressed in vertebrate retina during neuronal development. We used immunoprecipitation and [^{3}H]epibatidine binding to study the expression of chick retina α-bungarotoxin–insensitive heteromeric nAChRs during development and adulthood, and found that it is strictly developmentally regulated, reaching a peak on postnatal day 1. The increase in [^{3}H]epibatidine receptors is caused mainly by an increase in the receptors containing the α2, α6, β3, and β4 subunits. The contribution of β subunits to [^{3}H]epibatidine receptors significantly changes during development: the β2 subunit is contained in the majority (84%) of receptors on embryonic day (E) 7 but in only 32% on postnatal day (P) 1, whereas the β4-containing receptors increase from 22% to 78% during the same period. Using a sequential immunodepletion procedure, we purified the β2- and β4-containing subtypes and found that they coassemble with α4 and/or α3 on E11, and also with the α2, α6, and β3 on P1. After the immunodepletion of α6-containing receptors, the β2- and β4-containing receptors have a very similar pharmacological profile on P1. Parallel immunoprecipitation experiments in other brain areas showed that the developmentally regulated receptors in optic lobe are those containing the α2, α5, and β2 subunits and those containing the α4 and β2 subunits, whereas the receptors in forebrain-cerebellum contain the α4 and β2 subunits with or without the α5 subunit. These results indicate that there is an increase in receptor heterogeneity and complexity in chick retina during development that is also maintained in adulthood.

Vertebrate retina contains a variety of neurotransmitters involved in retinal development and the formation of retinal connections to target tissues (Daw et al., 1989; Wong, 1999). Acting through nicotinic acetylcholine receptors (nAChRs), acetylcholine (ACh) seems to play an important role in neurite outgrowth, dendritic filopodia motility and remodeling during synaptogenesis, and the development of spontaneous rhythmic activity in retinal ganglion cells (RGCs) during the period in which their connectivity pattern is shaped (Lipton, 1988; Wong et al., 1998, 2000; Sernagor et al., 2000; Wong and Wong, 2001; Feller, 2002). The spontaneous bursting activity of RGCs (called retinal waves) influences the size and complexity of RGC dendrites and is important for refining the connections between retinal axons and their thalamic target (Wong et al., 2000; Wong and Wong, 2001; Feller, 2002). The retinal wave activity of KO mice lacking the α3 or β2 nicotinic subunits has altered spatiotemporal properties, and KO mice lacking the β2 structural subunit have retinofugal projections in the dorsolateral geniculate nucleus and the superior colliculus that do not segregate into eye-specific areas (Bansal et al., 2000; Rossi et al., 2001; Muir-Robinson et al., 2002).

However, the role of nAChRs in dendritic remodeling and the spontaneous activity of RGCs seems to be species-specific and developmentally regulated (Wong et al., 1998, 2000). In the chick, pharmacological experiments with nicotinic antagonists have shown that nicotinic cholinergic transmission is important in driving dendritic filopodia motility and spontaneous activity early in retinal development, but less so as development continues (Wong, 1999; Sernagor et al., 2000; Wong et al., 2000; Wong and Wong, 2001). The influence of ACh on in vivo retinal development probably depends on the nAChR subtype expressed at each stage, but it is not yet clear how many subtypes are expressed or which subtypes are the most important.

This work was supported in part by grants to Francesco Clementi from the Italian Ministero dell'Istruzione, dell'Università e della Ricerca (grant MM05152538), the Italian Ministry of Health (grant ICS 030.3/RA 0048), and the European Research Training Network (grant HPRN-CT-2002-00258).

ABBREVIATIONS: nAChR, neuronal nicotinic acetylcholine receptor; ACh, acetylcholine; RGC, retinal ganglion cell; KO, knock-out; αBgtx, α-bungarotoxin; Epi, epibatidine; E, embryonic day; P, postnatal day; Abs, polyclonal antibodies; COOH, subunit COOH peptide; MG624, *N*,*N*,*N*,-triethyl-2-(4-*trans*-stilbenoxy)ethylammoniam iodide.

Chick neuronal nAChRs are cationic channels whose opening is physiologically controlled by the 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, based on their phylogenetic, functional, and pharmacological properties (Le Novère and Changeux, 1995; Corringer et al., 2000), two main classes have been identified: the α-bungarotoxin (αBgtx)-sensitive receptors made of α7, α8, or α9 subunits, which can form homomeric or heteromeric receptors, and the αBgtx-insensitive receptors made of α2–α6 and β2–β4 subunits, which form heteromeric receptors. In heteromeric receptors, more than one type of α or β subunit can participate in the formation of the receptor pentamer, thus increasing the number of possible receptor subtypes with different pharmacological and functional properties (Lindstrom, 2000).

Previous nicotinic ligands binding and immunolocalization studies have shown that chick retina expresses both classes of nAChRs, which are localized on amacrine, displaced amacrine, ganglion, and bipolar retinal cells (Betz, 1981; Whiting et al., 1991; Britto et al., 1992, 1994; Anand et al., 1993; Keyser et al., 1993; Hamassaki-Britto et al., 1994). Moreover, biochemical and pharmacological studies have identified the presence of three αBgtx binding subtypes in chick retina, the homomeric α7 and α8 subtypes and the heteromeric α7–α8 subtype, all of which have a developmentally regulated expression (Keyser et al., 1993; Gotti et al., 1994, 1997). In situ hybridization and immunolocalization studies, together with Northern blot analyses, have shown that chick retina contains almost all of the nicotinic subunits present in heteromeric receptors (Matter et al., 1990; Whiting et al., 1991; Britto et al., 1992, 1994; Hamassaki-Britto et al., 1994; Hernandez et al., 1995; Fucile et al., 1998). In particular, there is a selective expression of the α6 and β3 subunits, which are only present in catecholaminergic nuclei and retina in the mammalian central nervous system (Le Novère et al., 1996). In previous biochemical, immunological, and pharmacological studies, we have shown that most of the αBgtx-insensitive [^{3}H]Epi receptors in chick retina contain the β4 subunit (associated with the α4, α6, and/or β3 subunits) on postnatal day (P) 1 (Vailati et al., 1999, 2000; Barabino et al., 2001), but nothing is known about their developmental expression.

We used ligand binding and immunoprecipitation experiments to study the expression of the high-affinity [^{3}H]Epi binding receptors in chick retina and the optic tectum (its target tissue), using the forebrain-cerebellum tissue as a further control. Because we found a developmental change in the retinal receptors containing the β2 and β4 subunits, we also immunopurified the subtypes containing these subunits on embryonic day (E) 11 and P1 and studied their subunit coassembly and pharmacology.

Materials and Methods

Materials. The protease inhibitors, the nonradioactive Epi, nicotinic ligands, and Triton X-100 were purchased from Sigma-Aldrich (St. Louis, MO); the CnBr-activated Sepharose 4BCL and ^{125}I-protein A from Amersham Biosciences UK, Ltd. (Buckinghamshire, UK); (±)-[^{3}H]Epi from PerkinElmer Life Sciences (Boston, MA); and the reagents for gel electrophoresis from Bio-Rad Laboratories (Hercules, CA).

Antibody Production and Characterization. The polyclonal antibodies (Abs) against the α2, α3, α4, α5, α6, β2, β3, and β4 peptides were raised and characterized as described previously (Vailati et al., 1999, 2000; Balestra et al., 2000; Barabino et al., 2001). Two different peptides were chosen for all the subunits: one located in the cytoplasmic loop between M3 and M4, which is the most divergent region of the subunits, and the other located at the COOH-terminal (COOH). 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 of the antibodies has been previously reported (Vailati et al., 1999, 2000; Balestra et al., 2000; Barabino et al., 2001), and additional experiments on BOSC 23 cell lines transfected with different nAChR subunits are reported under *Results*.

Preparation of Retina, Chick Optic Lobe, and Forebrain-Cerebellum Triton X-100 Extracts. The embryos, 1-day-old animals, and adult animals were of the *Gallus gallus* strain and obtained from a local hatchery. The embryos were kept in the dark, whereas the chicks were kept under natural lighting conditions. The retina, optic lobe, and forebrain-cerebellum samples were dissected from in ovo chickens on E7, E11, E14, and E18 and from chickens on P1, P35, and P59, 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. In every experiment, the three types of tissue were separately homogenized 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. The homogenates were then diluted and centrifuged for 1.5 h at 60,000*g*.

This procedure of homogenization, dilution, and centrifugation was performed twice, after which the pellets were collected, rapidly rinsed with 50 mM sodium phosphate, 50 mM NaCl, 2 mM EDTA, 2 mM EGTA, and 2 mM phenylmethylsulfonyl fluoride, and then resuspended in the same buffer containing a mixture of 10 μg/ml 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 were then centrifuged for 1.5 h at 60,000*g*, recovered, and an aliquot of the resultant supernatants was collected for protein measurement using the bicinchoninic acid protein assay (Pierce Chemical, Rockford, IL) with bovine serum albumin as the standard.

Binding Assay. The (±)-[^{3}H]Epi with a specific activity of 66.6 Ci/mmol was purchased from PerkinElmer Life Sciences; the nonradioactive Epi was purchased from Sigma/RBI (Natick, MA). Because β2-, β4-, and α8-containing receptors bind [^{3}H]Epi with picomolar affinity and α7 receptors bind it with nanomolar affinity (Gerzanich et al., 1995), the binding tissue extract and immunoprecipitation experiments were performed in the presence of 2 μM αBgtx, which specifically binds to the α7 and α8 subtypes and blocks [^{3}H]Epi binding, to ensure that the α7 and α8 subtypes did not contribute to [^{3}H]Epi binding

The Triton X-100 extracts of retina, optic lobe, and forebrain-cerebellum 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]Epi-Labeled Receptors by Antisubunit-Specific Abs. The extracts obtained from the three tissues at different ages, 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). The immunoprecipitation was recovered by incubating the samples with beads containing bound anti-rabbit goat IgG (Technogenetics, 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 femtomoles of immunoprecipitated receptors per milligram of protein.

Receptor Subtype Immunopurification and Analysis. The extracts prepared from E11 and P1 chick retina were incubated twice with 5 ml of Sepharose-4B and bound anti-β2 Abs to remove the β2 receptors and then twice with 5 ml of Sepharose-4B with bound anti-β4 Abs; 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 β4 peptide 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 chick 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 2% Triton X-100 extract of P1 chick retina was immunodepleted of α6-containing receptors by passing it over a column of Sepharose-4B with bound anti-α6 Abs.

The affinity-purified anti-β2 or anti-β4 Abs were bound to microwells (Maxi-Sorp; Nalge Nunc International, Naperville, IL) 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 it of the α6-containing receptors. After incubation, the wells were washed and the presence of immobilized receptors revealed by means of [^{3}H]Epi binding. The binding techniques for immunoimmobilized subtypes and the data analysis were the same as those described previously (Vailati et al., 1999).

Expression of nAChR Subunits in BOSC 23 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 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (Hyclone Laboratories, 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 Dulbecco's modified Eagle's medium containing 10% fetal calf serum. The cells were collected in ice-cold phosphate-buffered saline (Invitrogen) 36 to 48 h after transfection and stored at −70°C.

Results

Characterization of the Subunit Specificity of the Antibodies. We have previously purified α6-containing receptors from chick retina and α2α5β2 and α4β2 subtypes from the optic lobe on P1 and have shown their subunit composition by means of quantitative immunoprecipitation (see Table 1) and Western blotting. The α2, α5, and β2 Abs had respective immunoprecipitation capacities of 51, 66, and 80% on the α2α5β2 subtype. The Abs directed against the α6 or β4 subunits both had an immunoprecipitation capacity of almost 90% on the α6-purified subtype, whereas the anti-α3 and anti-β3 Abs only immunoprecipitated 42 and 51% of these receptors. This incomplete immunoprecipitation by anti-α3 and anti-β3 Abs may have been caused by the limited presence of the α3 and β3 subunits in the α6β4 receptors or by reduced immunoprecipitation efficiency. To answer this question, we transiently transfected the BOSC cells with the α3β2 or α4β3β2 or α4β4 chick subunits and measured the [^{3}H]Epi-labeled receptors immunoprecipitated in 2% Triton X-100 extract by the same antibodies as those used to immunoprecipitate the native receptors (see Table 1). The studies of transfected cells confirmed the subunit specificity of the Abs and showed that the anti-α3, anti-α4, anti-β2, and anti-β4 Abs had an immunoprecipitation capacity ranging from 80 to 97%, whereas the anti-β3 Ab (although they are subunit-specific, because they do not recognize the other subunits) recognized only 45% of the [^{3}H]Epi receptors in the cells transfected with α4β3β2 subunits. This may be because the anti-β3 Ab has a relatively low capacity or because the β3 subunit is associated with α4 and β2 in only 45% of the receptors in this cell line. On the basis of these results and the previous immunoprecipitation study, we can only conclude that the β3 Ab has an immunoprecipitation capacity of at least 45%.

[^{3}H]Epibatidine-Binding Receptors in Chick Central Nervous System during Development. We and others (Gerzanich et al., 1995; Barabino et al., 2001) have previously shown that chick retina expresses a high level of αBgtx-binding receptors and that these receptors 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 nicotinic receptors. We have previously shown the presence of [^{3}H]Epi-labeled receptors in chick retina on P1 (Vailati et al., 1999; Barabino et al., 2001). To investigate their expression during embryonic development and the aging process, we performed binding studies on 2% Triton X-100 retina extracts obtained from

TABLE 1
Percentage of subunit-specific antibody immunoprecipitation of [^{3}H]Epi-labeled receptors purified from chick tissues or present in transfected cells
The immunoprecipitation was carried out as described under *Materials and Methods* using saturating concentrations of anti-subunit Abs and purified subtypes (α2α5β2 from optic lobe, α6 from retina, and α4α5β2 from forebrain) or 2% Triton X-100 extracts obtained from transfected cells (α3β2, α4β3β2, and α4β4) labeled with 2 nM [^{3}H]Epi. 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 mean ± S.E.M. of three determinations.

Abs	Purified α2α5β2[a]	Purified α6[b]	Purified α4α5β2[a]	Transfected α3β2	Transfected α4β3β2	Transfected α4β4
Anti-α2	51 ± 4	0.2 ± 0.2	3.6 ± 2	0.1 ± 0.2	3 ± 2	2 ± 2
Anti-α3	0.1 ± 0.3	42 ± 7	0.3 ± 0.1	83 ± 5	0.5 ± 0.5	0 ± 0
Anti-α4	2.2 ± 0.7	1 ± 1.3	61 ± 7	2 ± 1	88 ± 4	98 ± 3
Anti-α5	66 ± 3	1.3 ± 0.7	69 ± 7	3 ± 1	0.5 ± 0.1	0.2 ± 0.2
Anti-α6	0.5 ± 0.5	93 ± 5	0.2 ± 0.1	2.5 ± 1.5	3 ± 1.5	0.1 ± 0.2
Anti-β2	80 ± 3	8 ± 1	95 ± 4	85 ± 2.5	88 ± 5.5	2.5 ± 1
Anti-β3	0.4 ± 0.4	51 ± 2	0.3 ± 0.1	1.5 ± 1.5	45 ± 2	3 ± 0.2
Anti-β4	1 ± 0.9	94 ± 6	1.5 ± 0.6	2.5 ± 1	2.5 ± 0.5	97 ± 10

[a] From Balestra et al. (2000).
[b] From Vailati et al. (1999).

chicks on E7, E11, E14, E18, P1, P35, and P59 and compared the expression of the [³H]Epi receptors with that of the receptors present in the optic lobe and forebrain-cerebellum at the same ages (see Fig. 1). The receptor level (mean ± S.E.M. of three experiments) was similar in retina, optic lobe, and forebrain-cerebellum on E7 (41.4 ± 2.9, 46.9 ± 3.2, and 46.9 ± 3.2 fmol/mg of protein, respectively) and E11 (103.2 ± 5.6, 86.9 ± 2.1, and 78.3 ± 2.7 fmol/mg of protein) but subsequently increased much more in the retina and optic lobe (246.6 ± 21.4 and 224.7 ± 2.9 fmol/mg of protein on P1) than in the forebrain-cerebellum (77.5 ± 2 fmol/mg of protein on P1). After birth, the receptor level gradually decreased in every tissue (170.6 ± 33.4 and 149 ± 20.9 fmol/mg of protein on P35 and on P59 in retina; 161.6 ± 17.6 and 140.3 ± 14.9 fmol/mg of protein in optic lobe; and 46.5 ± 5.7 and 46.1 ± 3.4 fmol/mg of protein in forebrain-cerebellum).

Subunit Content of the [³H]Epi Receptors in Retina. To identify whether different subtypes are expressed during embryonic development, at birth and afterward, we performed quantitative immunoprecipitation experiments using subunit-specific antibodies and [³H]Epi-labeled receptors to quantify the relative contribution of each nicotinic subunit to [³H]Epi binding at each developmental stage. For each subunit except α2, we used polyclonal Abs directed against two separate peptides: one located in the cytoplasmic loop and the other in the COOH-terminal region. The reported values are the mean values obtained in three separate experiments for each subunit. On E7, the large majority of retinal receptors contained the α4 and β2 subunits. By E11 (together with the receptors containing the α4 and β2 subunits), there was an increase in the receptors containing the α3 and/or β3 and/or β4 subunits; by E14, there was an increase in the expression of the α6 and/or α2 subunits (see Fig. 2). By P1, the α6, α3, α2, β4, and β3 subunits had increased 70, 9, 26, 16, and 9 times, respectively, over their levels on E7, whereas the increase in the α4, α5, and β2 subunits was much more limited (respectively 3.1, 4.6, and 2.5 times) (see Fig. 3)

Subunit Content of the [³H]Epi Receptors in Optic Lobe and Forebrain-Cerebellum. In parallel experiments, we checked the expression of the subunit receptors present in the optic lobe and found that the α4-containing receptors were already highly expressed on E7 and had increased 2- to 3-fold by P59. The level of the β2-containing receptors was similar to that of the α4-containing receptors on E7 but then increased much more (from 30.57 ± 5.3 on E7 to 188.1 ± 3 fmol/mg of protein on P1). As described previously (Balestra et al., 2000), there was a selective increase in the α2 and α5 subunits from E11 to P1 (from 2 ± 0.4 to 57.5 ± 2.5 fmol/mg of protein for the α2 subunit and from 1.2 ± 1 to 62 ± 1 fmol/mg of protein for the α5), and their level of expression was still high on P35 and P59.

The immunoprecipitation studies of the forebrain-cerebellum only detected the presence of a considerable level of α4 and β2 subunits on E7 (21.5 ± 1 and 30.5 ± 5 fmol/mg of protein) and these levels increased by approximately 2 to 3 times between E7 and P1. We also detected a developmental increase in the α5-containing receptor (from 2.4 to 13 fmol/mg of protein) and, to a much lesser extent, the α2-, α3-, and β4-containing receptors, but we never detected the presence of any α6- or β3-containing receptors at any time.

Change in the Expression of the β2- and β4-Containing Receptors in Retina. The above results show that there is a change in the expression of the β subunits during development and adulthood. As shown in Fig. 2, the level of β2-containing receptors was higher than that of β4-containing receptors on E7, increased until E14, remained constant until P1, and then slightly decreased. The level of the β4-containing receptors increased almost linearly from E7 to P1 and then decreased (although it remained higher than that of the β2-containing receptors). This suggests that the contribution of the β subunits to the expressed subtypes changes during development. On E7, 84% of the [³H]Epi receptors contained the β2 subunit and only 22% the β4 subunit; by P1, however, 78% of the receptors contained the β4 and only 32% the β2 subunit (Fig. 4). To identify the subunits coassembled with the β2 or β4 subunits during early and late embryonic development, we immunopurified the receptors containing the β2 and β4 subunits from 2% Triton X-100 extracts (there was too little material available on E7) on E11 and P1.

We first immunopurified the β2-containing receptors by passing the extracts obtained on E11 or P1 over a column with bound anti-β2 Abs, and then the flowthroughs of these columns (devoid of the β2-containing receptors) were passed over a column with bound anti-β4 Abs. The β2- or β4-bound receptors were recovered by competition with the β2 or β4 peptides, labeled with 2 nM [³H]Epi, and then immunoprecipitated by means of subunit-specific Abs. The results of the quantitative immunoprecipitation studies of the purified receptors are shown in Fig. 5, A and B. On E11, the α and β subunits in the β2-containing receptors were α3 (12.2 ± 0.8%), α4 (55 ± 5%), β3 (28 ± 2%), and β4 (6.7 ± 1.1%), whereas the other subunits were almost absent. On P1, the subunits in the β2-containing receptors were more heterogeneous, with α2, α3, α4, α5, α6, β3, and β4 being present in 28 ± 1.7, 11 ± 1.4, 23 ± 4, 3.6 ± 2, 19.3 ± 2.4, 30 ± 1.7, and 18.7 ± 4% of receptors, respectively (Fig. 5A).

The purified β4-containing receptors mainly contained the α3 (72 ± 5%), α4 (22.5 ± 0.9%), and α6 (11 ± 1%) subunits on E11, whereas their subunit composition on P1 was much more complex, with the anti-α2, anti-α3, anti-α4, anti-α5, anti-α6, and anti-β3 subunit-specific Abs, respectively, im-

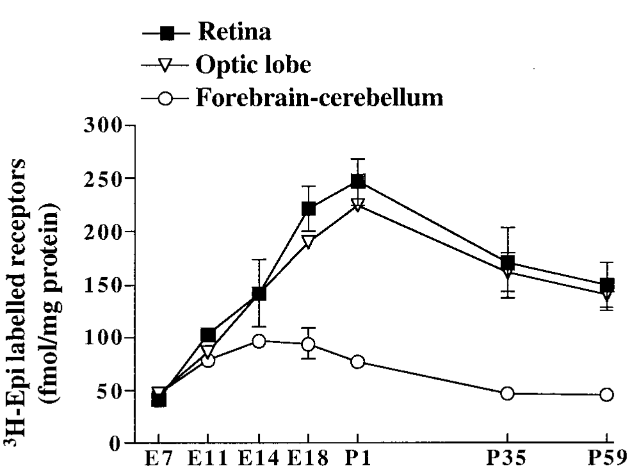

Fig. 1. Developmental changes in [³H]Epi-binding receptors expressed in the retina, optic lobe, and forebrain-cerebellum. The tissues were dissected from the embryos at the indicated times and frozen. Triton X-100 (2%) extracts were prepared as described under *Materials and Methods*, preincubated with 2 μM αBgtx, and then labeled with 2 nM [³H]Epi. The reported values are expressed as femtomoles of labeled [³H]Epi receptor per milligram of protein and are the mean values ± S.E.M. of three to four experiments performed in triplicate.

munoprecipitating 14.7 ± 0.9, 35 ± 2.6, 40 ± 2.6, 5 ± 1, 27 ± 0.9, and 19 ± 4% (Fig. 5B) of the receptors. The subunit compositions of the β2- and β4-containing receptors on P1 were also analyzed on Western blots using the same subunit-specific Abs as those used for the immunoprecipitation and previously tested on Western blots of purified chick subtypes (Vailati et al., 1999, 2000; Balestra et al., 2000; Barabino et al., 2001).

The results confirmed that the α2, α3, α4, and α6 subunits coassemble with the β2 and β4 subunits (Fig. 6). The anti-α2 Ab recognized a major peptide with a molecular mass of 60 ± 1 kDa on β2- and β4-purified subtypes (lanes 1 and 9); the anti-α3 Ab recognized a major band of 56 kDa and a lower band of 54 kDa (lanes 2 and 10); the anti-α4 Ab recognized a major band of 68 kDa (lanes 3 and 11); and the anti-α6 Ab a single band of 57 kDa (lanes 5 and 13). The anti-α5 Ab (lanes 4 and 12) did not recognize any band, thus indicating that the expression of the receptors containing this subunit was too low (as also found by immunoprecipitation). The anti-β2 Abs recognized a peptide with a molecular mass of 54 kDa on the β2- (lane 6) but not on the β4-containing receptors (lane 14), whereas the anti-β3 (lanes 7 and 15) and anti-β4 Abs (lanes 8 and 16) recognized peptides with molecular masses of 55 and 52 kDa. We also used immunoprecipitation experiments to test the purified E11 and P1 receptors for the presence of α7 or α8 subunits coassembled in the β2- or β4-containing receptors, but neither was detectable at either developmental time (Fig. 5).

Pharmacological Characterization of the β2- and β4-Containing Receptors Present on P1. Most studies of the functional role of retinal nAChRs rely on pharmacological experiments performed using nicotinic drugs. To see whether the available nicotinic drugs are selective on the retina subtypes, we pharmacologically characterized the β2- and β4-containing receptors using nicotinic agonists and antagonists. Because we have previously shown (Vailati et al., 1999; Barabino et al., 2001) that the α-conotoxin MII binds to α6-containing receptors with high affinity and selectivity, we

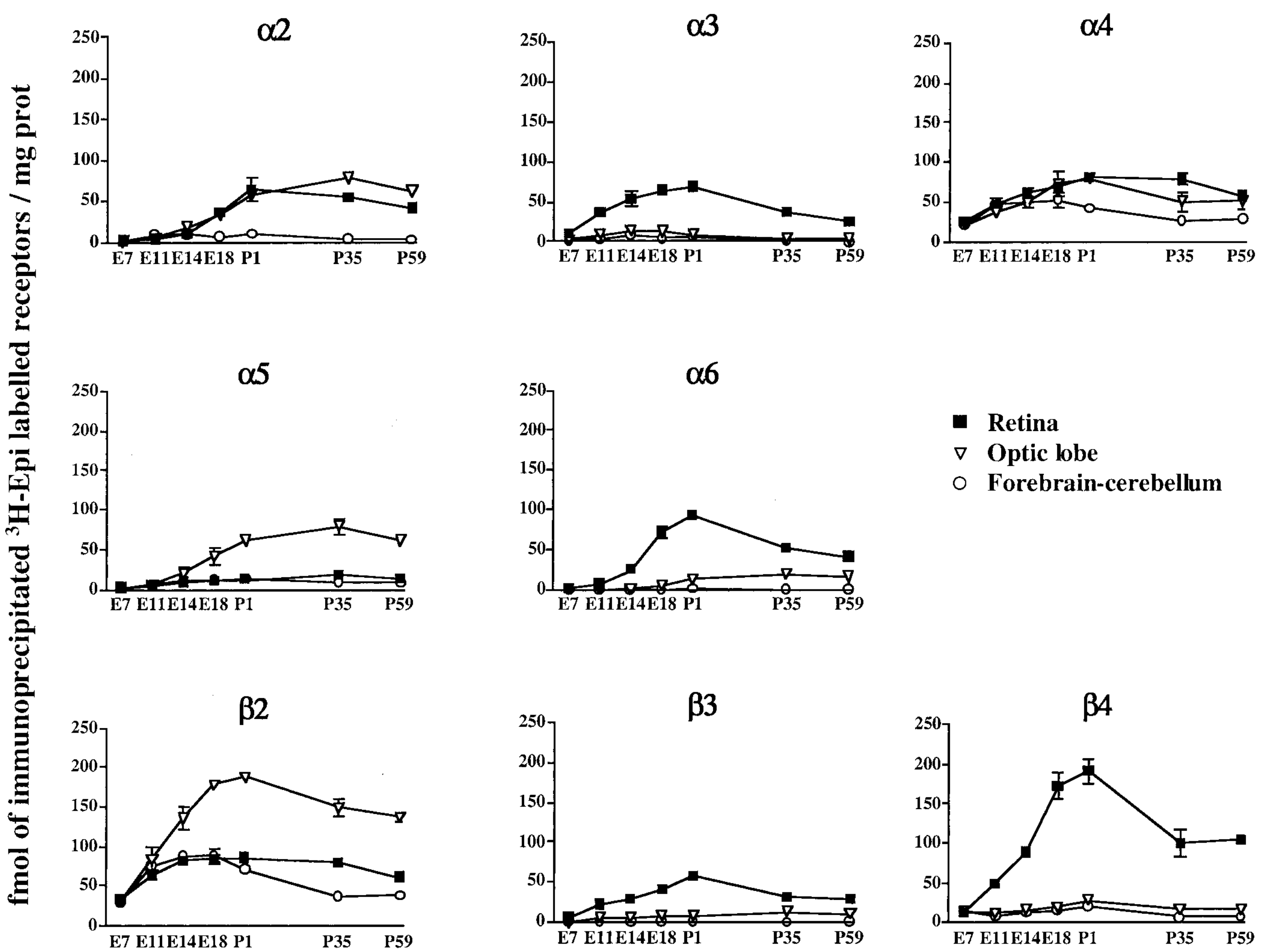

Fig. 2. Immunoprecipitation analysis of the subunit content of the [^{3}H]Epi receptors expressed in the retina, optic lobe, and forebrain-cerebellum. Triton X-100 (2%) extracts were prepared from tissues dissected from the animals on E7, E11, E14, E18, P1, P35, and P59, preincubated with 2 μM αBgtx, 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 α2): one directed against a subunit cytoplasmic peptide and the other 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 protein and are the mean values ± S.E.M. of three experiments performed in duplicate (unless shown, the S.E.M. is in the range of the symbol).

performed the binding experiments on β2- and β4-containing receptors previously depleted of the α6-containing receptors. [^{3}H]Epi binds to the β2- and β4-containing receptors with a high affinity; the K_d values calculated from four separate experiments were 27 pM (coefficient of variation, 20%) and 26 pM (coefficient of variation, 16%) for the β2- and β4-containing receptors, respectively.

The pharmacological profiles of the β2- and β4-containing receptors were characterized by testing the relative efficacy by which cholinergic agonists and antagonists inhibited the binding of 0.05 nM [^{3}H]Epi at equilibrium. The K_i values of the inhibition curves obtained by simultaneously fitting the data of three to four different experiments are shown in Table 2, together with the K_i values of the same drugs for the α6-containing receptors (Vailati et al., 1999). We determined that the rank order of antagonist potency for β2- and β4-containing receptors was identical: dihydro-β-erythroidine > *d*-tubocurarine > MG624 > decamethonium > hexamethonium. The α-conotoxin MII, which was the most potent drug (K_i, 66 nM) in competing for α6 receptors (Table 2), inhibited the binding of β2- and β4-containing receptors only at very high concentrations of >2 μM.

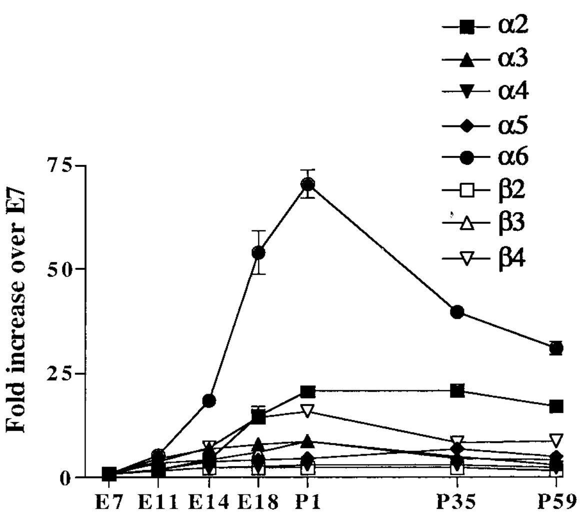

Fig. 3. Developmental changes in the expression of nicotinic receptor subunits in 2% Triton X-100 extract of retina. The receptors preincubated with 2 μM αBgtx and labeled with [^{3}H]Epi were immunoprecipitated at the indicated times as described in the legend to Fig. 2. The reported values are the -fold increases in immunoprecipitation for each subunit at each developmental time normalized to the E7 value expressed as femtomoles per milligram of protein.

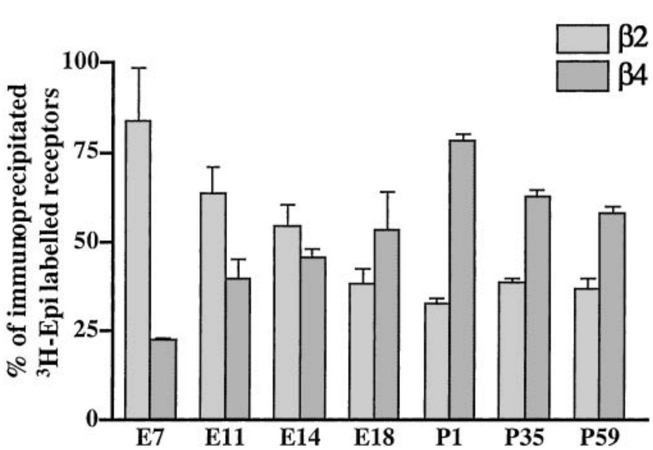

Fig. 4. Changes in β2/β4 subunit expression in chick retina. The level of expression of the β2- and β4-containing receptors in extracts obtained from animals at the indicated times is expressed as the percentage of [^{3}H]Epi-labeled receptors present in the solution before immunoprecipitation. The reported values are the mean ± S.E.M. of three to four separate experiments.

Discussion

ACh is synthesized and released in vertebrate retina by two populations of amacrine cells (one in the inner nuclear layer and the other in the ganglion cell layer) and can regulate many aspects of neuronal development by acting on nAChRs (reviewed in Feller, 2002). The various nicotinic

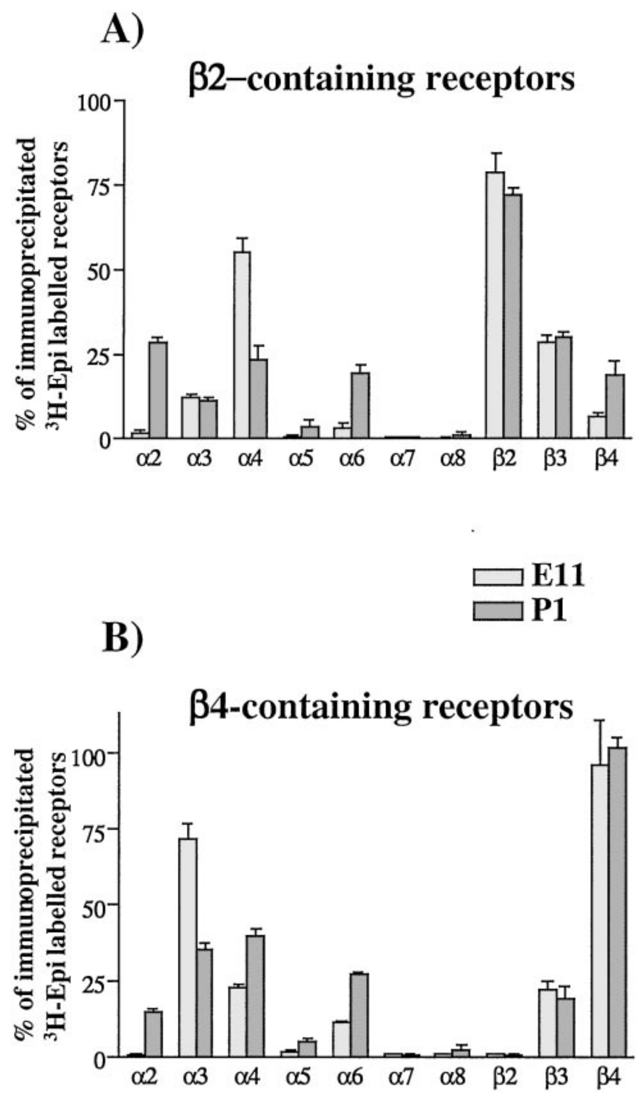

Fig. 5. Immunoprecipitation of purified β2- and β4-containing receptors. The subtypes were purified from tissues obtained from animals on E11 or P1 as described under *Materials and Methods*. After extensive dialysis to remove the β4 or β2 peptides used to elute the receptors from the affinity columns, the receptors were labeled with 2 nM [^{3}H]Epi and immunoprecipitated using saturating concentrations (20–30 μg) of the indicated Abs. 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 control samples containing an identical concentration of normal rabbit IgG. The values are the mean ± S.E.M. of three determinations.

effects of ACh may be mediated by the different receptor subtypes functionally expressed during development (Zhou, 2001), which can activate different signaling pathways (Dmitrieva et al., 2001) or have a different pattern of signaling because of the biophysical properties of the different receptor subtypes (Role and Berg, 1996).

In this molecular and pharmacological study, we identified the major nAChR subtypes expressed in chick retina and studied their expression at different stages of development. Our main findings were that 1) during embryonic development, there is an increase in [^{3}H]Epi binding receptors because of a developmental increase that is particularly prominent for the receptors containing the α2, α6, β3, and β4 subunits; 2) there is a developmental change in β subunit expression, with the large majority of receptors containing the β2 subunit early in development and two thirds containing the β4 subunit by P1; 3) the major subtype early in development is that containing the α4β2 subunits, but subtype expression becomes more heterogeneous by E11, and even more so at hatching; and 4) the α7 or α8 subunits never coassemble with heteromeric subunits in receptors containing the β2 or β4 subunits on E11 or P1.

Our conclusions concerning the subtypes expressed in retina and their subunit coassembly are based on the immunoprecipitation of [^{3}H]Epi-labeled receptors using subunit-specific Abs, and thus critically depend on antibody specificity and efficiency, which were carefully checked in immunoprecipitation experiments on purified receptors and transfected cells.

On the basis of the current hypothesis that homomeric αBgtx-sensitive receptors have five ligand-binding sites per receptor and heteromeric receptors have only two (Le Novère and Changeux, 1995; Corringer et al., 2000), the most abundant class of nAChRs expressed throughout embryonic retinal development are the αBgtx receptors containing the α7 and/or α8 subunits (Gotti et al., 1994). However, heteromeric receptors binding [^{3}H]Epi are also highly expressed during embryonic development and their number increases 6-fold between E7 and P1. The level and temporal expression of retinal [^{3}H]Epi receptors are very similar to those of the optic lobe receptors, which suggests a common receptor regulation in the visual pathway that is not present in other regions (the increase in nAChRs in the forebrain-cerebellum is much more limited and peaks between E14 and E18).

The receptors expressed in the early phase of retinal development (E7) are those containing the α4β2 subunits, and their expression is qualitatively and quantitatively very similar to that of the subtypes present in the optic lobe and forebrain-cerebellum. By E11, there is an increase in the expression of the α3, β3, and β4 subunits, and affinity purification of the β2- and β4-containing receptors at this age shows that the α3 and α4 subunits are present in both, but α4 is associated mainly with the β2 subunit and α3 with the β4

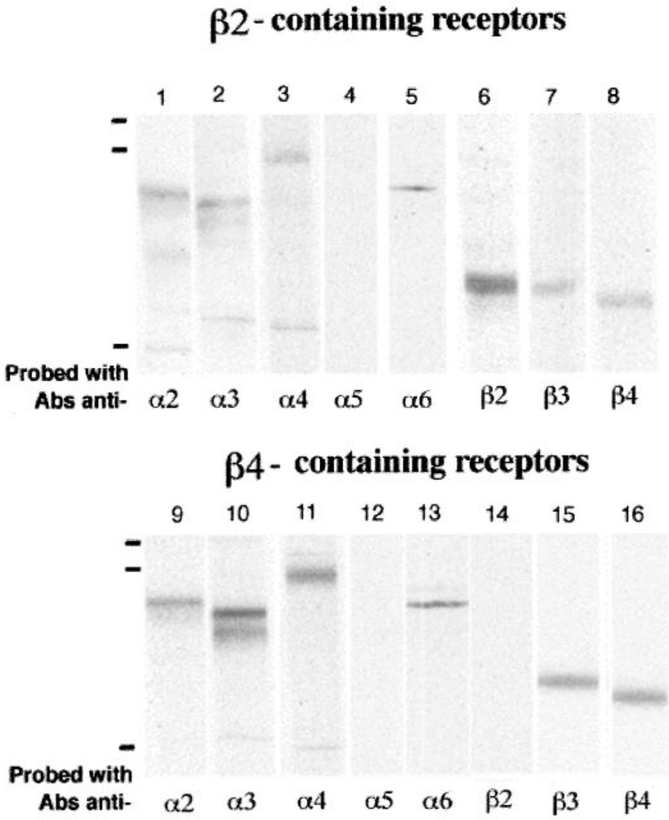

Fig. 6. Western blot analysis of P1 affinity-purified β2- and β4-containing receptors. The receptors were prepared as described in the legend to Fig. 5. The eluted receptors were concentrated and separated on 9% acrylamide SDS gels, electrotransferred to nitrocellulose, and then probed with 5 to 10 μg/ml 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 2

Affinity of cholinergic agonists and antagonists

The K_d and K_i values were derived from [^{3}H]Epi saturation and competition binding studies of immunoimmobilized native β2-, β4-, and α6-containing receptors prepared as described under *Materials and Methods*. The curves obtained from three separate experiments were fitted using a nonlinear least-squares analysis program and the F test as described by Vailati et al. (1999). The numbers in parentheses represent the percentage of coefficient of variation.

	K_i		
	β2-Containing Receptors (α6-Depleted)	β4-Containing Receptors (α6-Depleted)	α6-Containing Receptors[a]
	nM		
Acetylcholine	123 (49)	72 (17)	76 (26)
Nicotine	131 (56)	98 (24)	20 (31)
α-Conotoxin MII	>2000	>2000	66 (24)
Dihydro-β-erythroidine	502 (58)	201 (49)	2800 (13)
d-Tubocurarine	3085 (52)	6900 (25)	7700 (18)
MG624	12,000 (35)	9200 (25)	4520 (26)
Decamethonium	41,000 (24)	38,570 (28)	35,900 (16)
Hexamethonium	990,000 (18)	373,000 (33)	349,000 (18)
[^{3}H]epibatidine (K_d, pM)	27 (20)	26 (16)	35 (18)

[a] From Vailati et al., 1999.

subunit; the $\beta3$ subunit is present in a similar fraction of both types of receptors. After E14, there is a considerable increase in the number of receptors containing the $\alpha6$ and $\alpha2$ subunits that reaches a peak by P1, when both the $\beta2$- and $\beta4$-containing subtypes are heterogeneously associated with the $\alpha2$, $\alpha3$, $\alpha4$, and $\alpha6$ subunits. The results of our immunoprecipitation studies are consistent with those of previous Northern blot analyses showing an increase in $\alpha3$, $\alpha6$, and $\beta3$ mRNAs from the early to late stages of embryonic development and then a decrease in adult animals (Matter et al., 1990; Whiting et al., 1991; Fucile et al., 1998).

The heterogeneity in the subunit composition of the $\beta2$- and $\beta4$-containing receptors at P1 is not revealed by their pharmacological profile, in that both have a very similar affinity and rank order of potency for the tested nicotinic agonists and antagonists. Unlike the heterologously expressed $\beta2$- or $\beta4$-containing rat subtypes (Parker et al., 1998), the presence of more than one type of α and β subunit/receptor may change the pharmacology of native chick retinal subtypes. Another possibility is that the affinities of different subtypes to nicotinic drugs are so close that they cannot be discriminated by binding.

Moreover, our pharmacological experiments showed that the $\beta2$- and $\beta4$-containing receptors devoid of the $\alpha6$-containing receptors have a low affinity for the α-conotoxin MII toxin, thus confirming that the $\alpha6$ subunit is the crucial subunit conferring high affinity for this toxin in chick subtypes, as previously shown (Vailati et al., 1999; Barabino et al., 2001), and also recently demonstrated in $\alpha6$ KO mice by Champtiaux et al. (2002). α-Conotoxin MII is therefore the only available tool capable of discriminating some of the nAChR subtypes in the chick retina.

Another important finding of this study is that there is a developmental increase in the number of $\alpha2$-containing retinal receptors, reach a peak on P1 and decrease only slightly in adulthood. These receptors represent roughly 18% of the heteromeric receptors at P1 and are associated with the $\beta2$ and/or $\beta4$ subunits but never with the $\alpha6$ subunit. The presence of the $\alpha2$ subunit in retina may be important in the functional and anatomical development of visual systems, as also suggested by the recent finding that $\beta2$ KO mice have an altered anatomical and functional visual development, whereas $\alpha4$ or $\alpha6$ KO animals do not (Rossi et al., 2001; Champtiaux et al., 2002). It is therefore possible that subunits other than $\alpha6$ and $\alpha4$ are important for the development of the visual system and/or that subunit heterogeneity plays a role in the functional compensation of α subunits in KO animals.

We found that the major β subunit expressed in chick retina on P1 and in adulthood is the $\beta4$ subunit, whereas Keyser et al. (2000) found that the large majority of heteromeric receptors in adult rabbit retina contain the $\beta2$ subunit and we have found the same preponderance throughout postnatal development and adulthood in rat retina (C. Gotti, M. Moretti, S. Vailati, unpublished results). These results, together with the previous demonstration of the specific expression of the homomeric $\alpha8$ subtype in retina (Keyser et al., 1993; Gotti et al., 1994), demonstrate the species-specific expression of nAChR subtypes in retina.

Comparison of nAChR expression in the retina and optic lobe shows greater developmental expression of $\beta2$-containing receptors in the latter, in which the $\beta2$ subunit is mainly assembled with the $\alpha2$ and $\alpha5$ subunits in the $\alpha2\alpha5\beta2$ subtype and with the $\alpha4$ subunit in the $\alpha4\beta2$ subtype. The expression of the $\alpha2$ and $\alpha5$ subunits remains high on P35 and P59, thus suggesting that this subtype is also selectively expressed in the optic lobe in adult animals. Interestingly, the increase in $\beta2$ subunit expression that we detected at protein level has been previously observed at the mRNA level by Matter et al. (1990), who found that it is absent in eyeless animals and appears when the optic nerve axons are invading the optic tectum and making retino-tectal synapses (peaking at E12).

Unlike Fucile et al. (1998), whose Northern blot studies failed to detect $\alpha6$ mRNA in the optic tectum at any age, we found that there was a small increase in $\alpha6$-containing receptors after E14. It is possible that $\alpha6$-containing receptors are made in the retina and then transported to the tectum starting from E12. Our studies of chick forebrain-cerebellum showed that the largest developmental increase involved the $\alpha4$, $\beta2$, and $\alpha5$ subunits, thus indicating a developmental increase in receptors containing the $\alpha4$, $\beta2$, and the $\alpha4\alpha5\beta2$ subunits. These results are consistent with the earlier findings of Conroy and Berg (1998) in chick brain.

Although we do not know the physiological role of all of the subtypes described here, our results provide a more defined picture of the heteromeric nAChR subtypes expressed in retina during embryonic development, upon hatching, and in adult ages. On the basis of these findings and those of previous studies of αBgtx-sensitive receptors (Keyser et al., 1993; Gotti et al., 1994, 1997), we can conclude that the heteromeric receptors containing the $\alpha3$ and/or $\alpha4$ subunits with the $\beta2$ subunit, and the homomeric receptors containing the $\alpha7$ subunit, are expressed long before there is any evidence of synaptic connections and at later stages of embryonic development, whereas the receptors containing the $\alpha2$, $\alpha6$, $\beta3$, and $\beta4$ subunits, and the αBgtx receptors containing the $\alpha8$ subunit, are present (and may thus play a role) only late in development, when complex functional circuits have been established in the retina itself and the retinal projections to subcortical structures.

Acknowledgments

We thank Dr. Benedetta Barabino for the generous gift of the transfected cells, Dr. J. M. McIntosh for the generous gift of α-conotoxin MII, Dr. Michele Zoli for critically reading the manuscript, and Kevin Smart for help with the manuscript.

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