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Heterogeneity and Selective Targeting of Neuronal Nicotinic Acetylcholine Receptor (nAChR) Subtypes Expressed on Retinal Afferents of the Superior Colliculus and Lateral Geniculate Nucleus: Identification of a New Native nAChR Subtype $\alpha3\beta2(\alpha5$ or $\beta3)$ Enriched in Retinocollicular Afferents

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ABSTRACT

The activation of neuronal nicotinic acetylcholine receptors (nAChRs) has been implicated in the activity-dependent development and plasticity of retina and the refinement of retinal projections. Pharmacological and functional studies have also indicated that different presynaptic nAChRs can have a modulatory function in retinotectal synapses. We biochemically and pharmacologically identified the multiple nAChR subtypes expressed on retinal afferents of the superior colliculus (SC) and lateral geniculate nucleus (LGN). We found that the $\alpha6\beta2^*$ and $\alpha4(non\alpha6)\beta2^*$ nAChRs are the major receptor populations expressed in both SC and LGN. In addition, the LGN contains two minor populations of $\alpha2\alpha6\beta2^*$ and $\alpha3\beta2^*$ subtypes, whereas the SC contains a relatively large population of a new native subtype, the $\alpha3\beta2(\alpha5/\beta3)$ nAChR. This subtype binds the α-conotoxin MII with an affinity 50 times lower than that of the native $\alpha6\beta2^*$ subtype. Studies of tissues obtained from eye-enucleated animals allowed the identification of nAChRs expressed by retinal afferents: in SC $\alpha6\beta2^*$, $\alpha4\alpha6\beta2^*$, and $\alpha3\beta2^*$ (approximately 45, 35, and 20%, respectively), in LGN, $\alpha4\alpha6\beta2^*$, $\alpha6\beta2^*$, $\alpha4\beta2^*$, $\alpha2\alpha6\beta2^*$, and $\alpha3\beta2^*$ (approximately 40, 30, 20, 5, and 5%, respectively). In both regions, more than 50% of nAChRs were not expressed by retinal afferents and belonged to the $\alpha4\beta2^*$ (90%) or $\alpha4\alpha5\beta2^*$ (10%) subtypes. Moreover, studies of the SC tissues obtained from wild-type and $\alpha4$, $\alpha6$, and $\beta3$ knockout mice confirmed and extended the data obtained in rat tissue and allowed a comprehensive dissection of the composition of nAChR subtypes present in this retinorecipient area.

Neuronal nicotinic acetylcholine receptors (nAChRs) are highly expressed in the visual system, where they play important roles, not only in the retina itself but also in retinal target tissues (for review, see Feller, 2002). nAChRs are a family of heterogeneous, pentameric receptor complexes consisting of different α and β subunits whose opening is physiologically controlled by the neurotransmitter acetylcholine (for review, see Gotti and Clementi, 2004). So far, 12 different subunits have been identified ($\alpha2$-$\alpha10$, $\beta2$-$\beta4$) whose various

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ABBREVIATIONS: nAChR, neuronal nicotinic acetylcholine receptor; Ko, knockout; LGN, lateral geniculate nucleus; SC, superior colliculus; αBgtx, α-bungarotoxin; WT, wild type; WIN 35,428, 2β-carbomethoxy-3β-(4-fluorophenyl)tropane; Epi, epibatidine; Ab, polyclonal antibody; CYT, cytoplasmic peptide; 6-OHDA, 6-hydroxydopamine; GBR 12935, 1-[2-(diphenylmethoxy)ethyl]-4-(3-phenylpropyl)-piperazine; αCntxMII, α-conotoxin MII; CV, coefficient of variation; *, denotes a receptor subtype that contains those subunits but may contain further unspecified subunits.

combinations give rise to functionally different receptor subtypes with distinct biophysical/pharmacological properties and cellular distributions (Dajas-Bailador and Wonnacott, 2004). Functional heteromeric receptor are thought to be formed by two "true" α subunits (α2, α3, α4, or α6), two "complementary" non-α subunits (β2 or β4), and a fifth "structural" subunit, such as β1 in the muscle receptor (α5, β3, but also β2 or β4) (Corringer et al., 2000).

Several pharmacological studies have shown that the spontaneous retinal activity initially driven by nAChRs is necessary for correct retina morphology and for the refinement of retinothalamic projections during early postnatal development (Penn et al., 1998; Wong, 1999; Firth et al., 2005).

In agreement with these pharmacological results, recent studies have shown that β2 knockout (Ko) mice have retinal waves with altered spatiotemporal properties (Bansal et al., 2000) and retinofugal projections to the lateral geniculate nucleus (LGN) and superior colliculus (SC) do not segregate into eye-specific areas (Rossi et al., 2001). Furthermore, anatomical and functional studies of LGN in β2 Ko mice have revealed normal gross retinotopy but disrupted fine mapping, a loss of retinotopicity in the nasoventral visual axis, and abnormal segregation of on/off cells (Grubb et al., 2003).

These studies clearly demonstrate that β2* nAChRs receptors are necessary for the normal development of the visual system, but the α subunit that coassembles with the β2 subunit to mediate these effects is unknown. The pattern of retinothalamic projection is normal in α4 and α6Ko mice (two subunits highly expressed in the visual system) and in α3Ko mice, whose retinal waves have an altered spatiotemporal characteristics, retinothalamic projections patterns are again normal (for review, see Champtiaux and Changeux, 2002).

We and others have shown recently that nAChRs sensitive and insensitive to α-bungarotoxin (αBgtx) are highly expressed in vertebrate retina (for review, see Feller, 2002). The temporal pattern of expression and subunit composition of the principal subtypes is species-specific and shows increased retinal nAChR heterogeneity and complexity during development and in adulthood (Vailati et al., 1999, 2003; Moretti et al., 2004).

As in many other areas of the CNS, nAChRs are expressed on nerve terminals of the LGN and SC, where they modulate neurotransmitter release. Combined electrophysiological and pharmacological studies have shown that nicotinic agonists acting on different presynaptic nAChR subtypes can produce an increase in GABAergic as well as glutamatergic transmission in the LGN (McMahon et al., 1994a,b; Guo et al., 1998). Moreover, nAChR-mediated ^{86}Rb efflux from SC synaptosomes markedly decreases in β2Ko mice (Marks et al., 2002). These presynaptic receptors are probably transported from retina by means of fast axonal transport, as has been shown in the case of goldfish (Henley et al., 1986).

We used ligand binding, immunoprecipitation, immunopurification, and tissues obtained from eye-enucleated animals and Ko mice to characterize the nAChR subtypes in the LGN and SC at the molecular level and identified those localized presynaptically in the same areas.

Materials and Methods

Animals and Materials

Adult male pathogen-free Sprague-Dawley rats (Harlan-Nossan, Milan, Italy) or wild-type (WT), α6−/− (Champtiaux and Changeux, 2002), α4−/− (Marubio et al., 1999), or β3−/− mice (Cui et al., 2003) were used. All animal experimentation was conducted in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). (±)-[^{3}H]Epibatidine (Epi; specific activity, 66 Ci/mmol) and ^{125}I-α-Bungarotoxin (^{125}I-αBgtx; specific activity, 200–216 Ci/mmol) were purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK). ^{125}I-Epi (specific activity, 2200 Ci/mmol) and [^{3}H]WIN 35,428 (specific activity, 86 Ci/mmol) were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). α-Conotoxin MII (α-CtxMII) was a generous gift of Dr. J. M. McIntosh, University of Utah (Salt Lake City, UT). The nonradioactive αBgtx and the drugs epibatidine, nicotine, and acetylcholine were from Sigma (St. Louis, MO).

Antibody Production and Characterization

We used subunit-specific polyclonal antibodies (Abs) that were produced in rabbit against peptides derived from the C-terminal or intracytoplasmic loop (CYT) regions of the rat, human, or mouse subunit sequences and affinity-purified as described previously. Most of the Abs were the same as those described previously (Zoli et al., 2002; Champtiaux et al., 2003; Moretti et al., 2004; Gotti et al., 2005). In this study, we used the same Ab directed against the COOH peptide (cgPALKMWIHRFH) for the β3 subunit, in both rat and mouse tissues, and Abs directed against the mouse CYT peptide DGTESKGTVRGKFPGKKKQTPTSD for mouse tissues and Abs directed against the rat CYT peptide CGKESDTAVRGKVSGKRKQTPASD for rat tissues.

The affinity-purified antisera were bound to CNBr-activated Sepharose at a concentration of 1 mg/ml, and the columns were used for subtype immunopurification.

Characterization of Antibody Specificity

The anti-α4, α5, α6, β2, and β3 antisera were tested by quantitative immunoprecipitation experiments on [^{3}H]Epi-labeled nAChRs present in 2% Triton X-100 extracts prepared from membranes of total brain of WT and nAChR subunit Ko mice. The α5 Ko mice were obtained from Dr. De Biasi (Baylor College, Houston, TX). Only the receptors labeled with [^{3}H]Epi, a specific nicotinic ligand, were immunoprecipitated, which ensured the specificity of the quantification. Immunoprecipitation of [^{3}H]Epi-labeled receptors present in total brain extracts dropped from 90 ± 2.1 to 1 ± 0.9% (anti-α4 antisera), 6 ± 0.5 to 0% (anti-α5 antiserum), 8 ± 1.2 to 0% (anti-α6 antisera), and 85 ± 1.8 to 2.2 ± 1.2% (anti-β2 antisera) from WT to the respective Ko mice. The anti-α6 and anti-β3 antisera were also tested on [^{3}H]Epi-labeled receptors from striatum, showing a drop from 20 ± 2% to 1.0 ± 0.3% in α6−/− mice and from 19.4 ± 2% to 0.6 ± 0.3% in β3−/− mice.

Using α4β2* nAChRs purified from the rat forebrain the anti-α2, -α4, and -β2 antisera immunoprecipitated 0.2 ± 0.1, 92 ± 2.1, and 89 ± 1.7% of the receptors, respectively, whereas the anti-α3 and -β4 antisera only immunoprecipitated 1 to 2% of the receptors. However, when tested on extracts obtained from rat superior cervical ganglion or pineal gland, the anti-α3 and -β4 antisera, respectively, immunoprecipitated 90 ± 3.2 and 80 ± 3.2% of [^{3}H]Epi-labeled receptors. The anti-α2 antisera were also tested in extracts obtained from postnatal rat retina, where they immunoprecipitated up to 25 ± 3% of the [^{3}H]Epi-labeled receptors (Moretti et al., 2004).

Preparation of Membranes and 2% Triton X-100 Extracts from Lateral Geniculate Nucleus and Superior Colliculus

The tissues obtained from rats or mice were dissected, immediately frozen in liquid nitrogen, and stored at −80°C for later use. In

every experiment, the tissues from LGN (0.25–0.35 g) or SC (0.25–0.35 g) were homogenized in 10 ml of 50 mM sodium phosphate, pH 7.4, 1 M NaCl, 2 mM EDTA, 2 mM EGTA, and 2 mM phenylmethylsulfonyl fluoride with a Potter homogenizer. The homogenates were then diluted and centrifuged for 1.5 h at 60,000*g*.

The procedures of homogenization, dilution, and centrifugation of the total membranes 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 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 BCA protein assay (Pierce, Rockford, IL) with bovine serum albumin as the standard.

Binding Assay and Pharmacological Experiments

[^{3}H]Epi Binding. β2*- and β4*-containing nAChRs bind [^{3}H]Epi with picomolar affinity, and α7 receptors bind it 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.

Binding to membrane homogenate obtained from LGN and SC membranes was performed overnight by incubating aliquots of the membrane with [^{3}H]Epi concentrations ranging from 0.005 to 2.5 nM at 4°C. Nonspecific binding (averaging 5–10% of total binding) was determined in parallel by means of incubation in the presence of 100 nM unlabeled Epi. At the end of the incubation, the samples were filtered on a GFC filter soaked in 0.5% polyethylenimine and washed with 15 ml of 10 mM sodium phosphate, pH 7.4, plus 50 mM NaCl, and the filters counted in a β-counter.

^{125}I-αBgtx Binding. Saturation experiments were performed by incubating LGN and SC membranes overnight with 0.01–10 nM ^{125}I-αBgtx at 20°C. For ^{125}I-αBgtx, 2 mg/ml bovine serum albumin was added to the suspension buffer. Specific radioligand binding was defined as total binding minus nonspecific binding determined in the presence of 1 μM unlabeled αBgtx.

[^{3}H]Epi Binding to Solubilized Receptor. The Triton X-100 extracts 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). We ascertained that 85 ± 2% of [^{3}H]Epi binding could be solubilized from LGN and SC membranes using 2% Triton X-100.

Pharmacological Experiments on Immunoimmobilized Subtypes. The affinity-purified anti-α3, anti-α6, or anti-β2 Abs were bound to microwells (Maxi-Sorp; NUNC A/S, Roskilde, Denmark) 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 SC or LGN membrane extract containing 5 to 10 fmol of ^{125}I-Epi binding sites. After incubation, the wells were washed and the presence of immobilized receptors was revealed by means of ^{125}I-Epi binding.

For saturation experiments, ^{125}I-Epi (2200 Ci/mmol; PerkinElmer Life and Analytical Sciences) was used at concentration ranging from 0.005 to 0.5 nM. For α-conotoxin MII (αCntxMII) and ligand inhibition experiments, the immunoimmobilized receptors were incubated (30 min, room temperature) with various concentrations of toxin or unlabeled nicotinic ligands before adding ^{125}I-Epi at the K_d concentration. Incubation was prolonged overnight at 4°C. Nonspecific binding was measured in the presence of 100 nM unlabeled Epi. For each ligand, data from three to four experiments were analyzed using the LIGAND program (Vailati et al., 1999).

Immunoprecipitation of [^{3}H]Epibatidine-Labeled Receptors by Anti–Subunit-Specific Antibodies. Tissue extracts were obtained from the LGN or SC of rats and WT, α6−/− (Champtiaux and Changeux, 2002), α4−/− (Marubio et al., 1999), or β3−/− (Cui et al., 2003) mice. These extracts were preincubated with 2 μM αBgtx, labeled with 2 nM [^{3}H]Epi, and incubated overnight with a saturating concentration of affinity-purified anti-subunit IgG (20–30 μg; Sigma). 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.

Immunopurification of α6*, α4(nonα6)*, and α3* nAChRs. For each purification experiment the LGN or SC from 30 to 40 animals was dissected and immediately frozen at −70°C before being processed as described above. The membranes (30 ml) were then extracted by addition of 2% Triton X-100 as described above, then centrifuged.

To collect the α6-containing receptors (α6* population), extracts (30 ml) were incubated three times with 5 ml of Sepharose-4B with bound anti-α6 CYT Abs. The α6-depleted flow-through fraction was collected for further processing. The bound α6* population was eluted from column by means of incubation with 100 μM α6 CYT peptide. To collect α4(nonα6)* nAChRs, the α6-depleted flow-through of the α6 column was incubated three further times with 5 ml of anti-α4 CYT Ab derivatized Sepharose-4B. The bound α4(nonα6)* receptors were eluted by competition with a 100 μM concentration of the corresponding α4CYT peptide.

SC α3* receptors were immunopurified by passing 2% Triton X-100 SC extracts three times over 5 ml of Sepharose-4B with bound anti-α3 CYT Abs. This α3* population was eluted from column by means of incubation with 100 μM CYT α3 peptide.

In every case, analysis of the purified, recovered populations' subunit content was performed by immunoprecipitation using subunit-specific Abs, as above, after labeling with 2 nM [^{3}H]Epi.

6-Hydroxydopamine Lesion and Eye Enucleation. Unilateral denervation of dopamine mesotelencephalic pathways was performed by injecting the selective neurotoxin 6-hydroxydopamine (6-OHDA; 10 μg/4 μl) in the medial forebrain bundle (coordinates: anterior, −4 mm; lateral, 1.8 mm; dorsal, −7.5 mm). [^{3}H]WIN 35,428 binding was determined individually in striata from unlesioned and 6OHDA-lesioned rats using a saturating concentration of 100 nM [^{3}H]WIN 35,428 in the presence or absence of 10 μM GBR 12935. 6OHDA-lesioned striata with a decrease of ^{3}H-WIN 35,428 lower than 80% were discarded.

Bilateral enucleation of the eyes was performed to examine the effects of retinal deafferentation on nAChR subtype expression in SC and LGN. Enucleation was performed under deep anesthesia with halothane (Merial Italia, Milan, Italy). The extraocular muscles were cut until reaching the optic nerve. The optic nerve was exposed, cut, and the eye was completely removed. The remained cavity was filled with Gelfoam embedded in Xylocaine and sealed with cyanoacrylamide gel. The enucleated rats were sacrificed 14 or 28 days after the enucleation to allow for degeneration of retinogeniculate and retinocollicular nerve terminals (Lund et al., 1976).

Criteria Used for the Reconstruction of the Subunit Composition of nAChR Subtypes. In the reconstruction of receptor composition, we chose to consider only subunits whose concentration was more than 5% of the overall amount of receptor. This criterion, although arbitrary, was chosen to be safely above the range of variability of false positive subunit concentration in the present experimental conditions as assessed (e.g., using present immunological techniques and antibodies in tissues or reconstituted cellular sys-

tems in which a certain subunit was not present). This criterion was applied to each experiment but the result of the most sensitive experiment prevailed. For instance, the concentration of a subunit might have been below 5% of the total amount of receptors in a certain region, but when an immunopurification for a given subtype is performed in the same region, the subunit might be present in, for example, 20% of the receptor subtype. In this case, the subunit would be considered present in the region even if did not reach the 5% of the overall amount of receptors.

The reconstruction of the subunit composition of nAChRs expressed in visual nerve terminals or other cells present in a given region comes from the combination of three different sources of evidence: 1) the amount of immunoprecipitated subunit in the region, 2) the change of the amount immunoprecipitated subunit after eye enucleation, and 3) the amount of immunoprecipitated subunit after immunopurification of α3*, α6*, or α4(nonα6)* nAChR subtypes. A further caveat that needed to be taken into account was the immunoprecipitation efficiency of the subunit-specific antibody, which was not 100%. In addition, we assumed that a functional heteromeric receptor had two subunits carrying the principal component of the binding site (α2, α3, α4, and α6), two or three subunits carrying the complementary component of the binding site (β2 and β4), and one (or no) structural subunit (α5 and β3) (Corringer et al., 2000). Any revision of these rules; e.g., the fact that some receptors may have three "true" α subunits and two non-α-subunits (Nelson et al., 2003) may require a revision of some of our deductions.

Results

Overall Subunit Composition of Nicotinic Receptors in Rat Superior Colliculus and Lateral Geniculate Nucleus. We first determined the proportion of homomeric (α7*, ^{125}I-Bgtx binding) versus heteromeric nAChRs in homogenates of rat SC and LGN. We found that ^{125}I-αBgtx binding is 72.6 ± 3.0 and 64.9 ± 5.1 fmol/mg of protein in SC and LGN, respectively, whereas [^{3}H]Epi binding (preincubated with 2 μM αBgtx to block binding to α7* nAChRs; see *Materials and Methods*) was 169.2 ± 2.7 and 263.9 ± 10.4 fmol/mg of protein in SC and LG, respectively (Fig. 1).

To determine the overall subunit composition of heteromeric SC and LGN nAChRs, we performed an immunoprecipitation of [^{3}H]Epi-labeled receptors by subunit-specific antisera. In both regions, practically all ^{3}H-epi binding receptors (90.6 ± 0.5% and 88.1 ± 3.3% in SC and LGN, respectively) contained the β2 subunit. α4 (66.3 ± 1.2% and 69.2 ± 2.5%) and α6 (30.6 ± 0.7% and 22.8 ± 1.4%) subunits were the most prevalent α subunits. α3 and α2 subunits were detected at moderate (α3, 10.8 ± 0.6% in SC) or low (α3 and α2, respectively, 4.0 ± 1.0 and 3.5 ± 1% in LGN and α2 2 ± 0.3 in SC) levels. As regards the structural subunits, we found that a rather high percentage of [^{3}H]Epi-labeled receptors contain β3 (28.5 ± 0.5% and 20.2 ± 3.3%), whereas a moderate percentage contained α5 (7.0 ± 0.4% and 6.3 ± 0.6%). β4 subunit content could not be distinguished from background in either region (Fig. 2).

Overall, the immunoprecipitation results show that SC contains large populations of α4β2* and α6β2* nAChRs and a minor population of α3β2* nAChRs, whereas the LGN contains two major (α4β2* and α6β2*) and two minor (α2β2* and α3β2*) nAChR populations.

Subunit Composition of α6β2* Subtypes in Rat Superior Colliculus and Lateral Geniculate Nucleus. To determine the composition of α6β2* receptors, we immunodepleted the SC and LGN extracts of α6* receptors by using an affinity column bearing anti-α6 antisera as described under *Materials and Methods*. Selective α6* nAChR immunodepletion was confirmed by the fact that immunoprecipitated α6-containing [^{3}H]Epi-labeled receptors decreased from 30% in

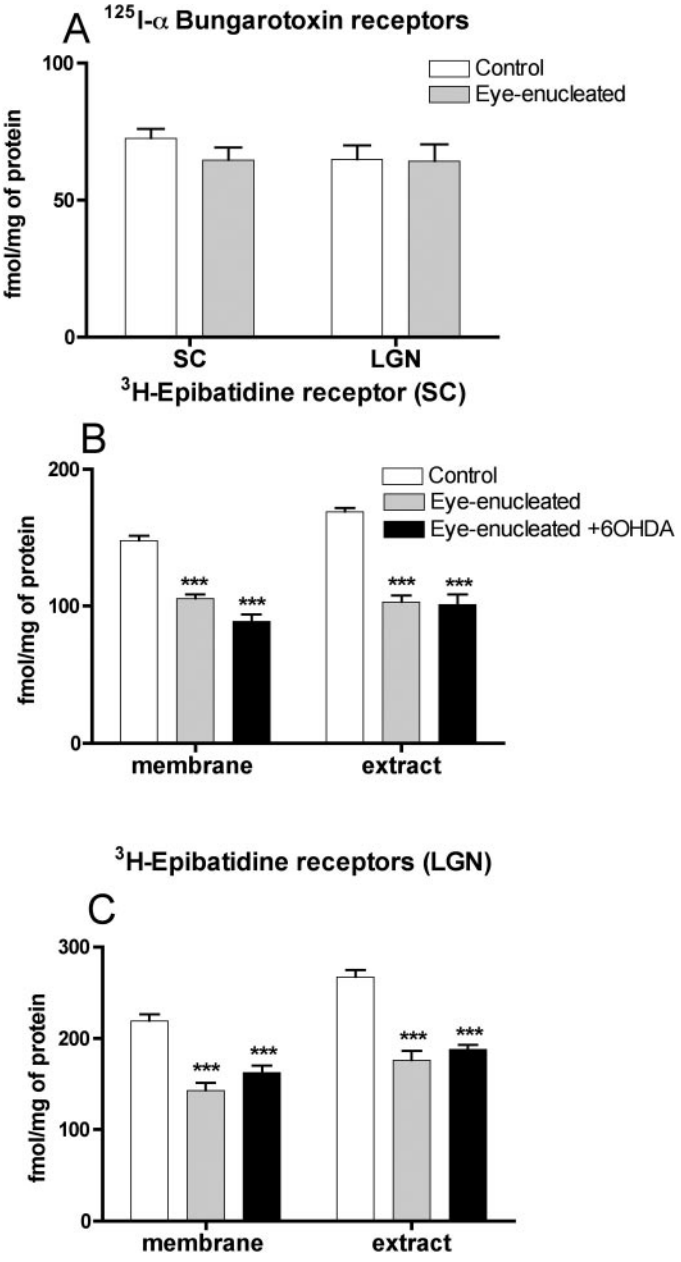


Fig. 1. A, expression of ^{125}I-αBgtx binding nAChRs in SC and LGN membrane from control and eye-enucleated animals. The membrane homogenates were performed as described under *Materials and Methods*. The reported values are expressed as femtomoles of specific labeled ^{125}I-αBgtx binding sites per milligram of protein and are the mean values ± S.E.M. of four to five experiments performed in triplicate per group. B, expression of [^{3}H]Epi binding nAChRs in SC membranes and 2% Triton extracts from control, eye-enucleated, and eye-enucleated + 6-OHDA+treated mice. The membrane homogenates or 2% Triton X-100 extracts were prepared as described under *Materials and Methods*. The reported values are expressed as femtomoles of specific labeled [^{3}H]Epi binding sites per milligram of protein and are the mean values ± S.E.M. of four to five experiments performed in triplicate per group. The statistical analyses were made using Student's paired t test, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. C, expression of [^{3}H]Epi binding nAChRs in LGN membranes and 2% Triton extracts from control, eye-enucleated, and eye-enucleated + 6-OHDA-treated animals. The binding experiments and statistical analysis were performed as described in B.

the total extract to 2% in the flow through of the α6 column. We then eluted α6* receptors from the affinity column with the α6 peptide. The eluted nAChRs were then labeled with [^{3}H]Epi and immunoprecipitated using subunit-specific antisera (Fig. 3, A and C). The results confirmed that α6* receptors contain β2 and not β4 subunit in both SC and LGN. They contain high levels of α4 (40.9 ± 3.1% and 43 ± 0.5% in SC and LGN, respectively) and β3 (65.4 ± 4.3 and 64.7 ± 9.6% in SC and LGN, respectively) subunit. In addition, α2 subunit is associated to a minor portion (18.2 ± 0.7%) of α6* nAChRs of the LGN but not those present in the SC. Finally, α3 antisera did not immunoprecipitate a significant amount of the purified [^{3}H]Epi-labeled α6* receptors in SC or LGN.

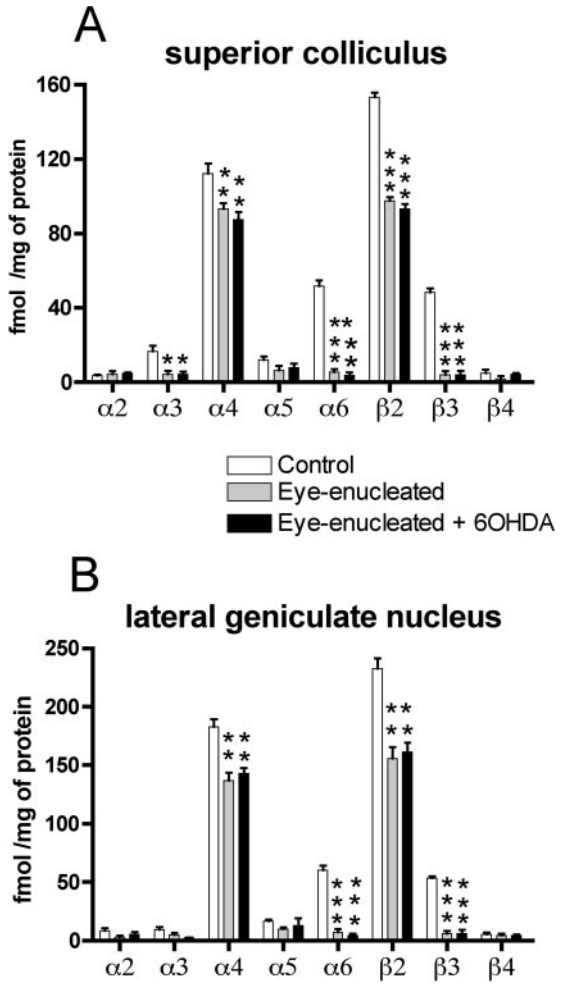


Fig. 2. A, immunoprecipitation analysis of the subunit content of nAChRs labeled with 2 nM [^{3}H]Epi expressed in the SC of control, eye-enucleated, and eye-enucleated + 6-OHDA-treated animals. Immunoprecipitation was carried out as described under *Materials and Methods* using saturating concentrations (20–30 μg) of anti-subunit 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 are expressed as femtomoles of immunoprecipitated labeled [^{3}H]Epi nAChR per milligram of protein. Results are the mean values ± S.E.M. of four to five experiments performed in duplicate per antibody. Statistical analysis was carried by the t test. Each Ab in control animal was compared with the same Ab tested in the eye-enucleated or eye-enucleated + 6-OHDA tissues by means of paired Student's t test, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. B, immunoprecipitation analysis of the subunit content of nAChRs labeled with 2 nM [^{3}H]Epi expressed in the LGN of control, eye-enucleated, and eye-enucleated + 6-OHDA-treated animals. The binding and immunoprecipitation experiments and statistical analysis were performed as described in A.

These data indicate that in both rat SC and LGN, purified α6* receptors are a mixture of two major (α6β2* and α4α6β2*) population of receptors, most of which also contain the β3 subunit. The LGN also seems to contain a minor population of (α2α6β2) nAChRs.

Subunit Composition of α4(nonα6)* Subtypes in Rat Superior Colliculus and Lateral Geniculate Nucleus. As shown above, many α6* nAChRs also contain the α4 subunit. To determine the subunit composition of α4* receptors that do not contain the α6 subunit [α4 (nonα6)*], we immunopurified nAChRs present in the flowthrough of the α6 column on an anti-α4 column (see *Materials and Methods*). Captured α4(nonα6)* nAChRs were then eluted using the α4 peptide and analyzed by immunoprecipitation with subunit-specific antisera. The anti-β2 antiserum immunoprecipitated 82.1 ± 1.3% and 93.8 ± 1.4% of [^{3}H]Epi-labeled receptors in SC and LGN, respectively. Anti-α2, -α3, or -α6 antisera did not immunoprecipitate significant amounts of receptor (respectively, 0 ± 0.2%, 1.3 ± 1% and 2.6 ± 1% in SC and 0 ± 0.2%, 1.3 ± 0.2%, and 2.6 ± 1.0% in LGN). As regards structural subunits, anti-α5 antiserum immunoprecipitated 6.1 ± 2.0% and 11.2 ± 2.6% of [^{3}H]Epi-labeled receptors in SC and LGN, respectively, whereas anti-β3 antiserum immunoprecipitated 1.6 ± 1.0% and 7.1 ± 0.6% of [^{3}H]Epi-labeled receptors in SC and LGN, respectively (Fig. 3, B and D).

These data indicate that in the SC, there is a large population of α4β2* nAChRs, a minority of which also contain α5 (α4α5β2 nAChRs). The situation in LGN is similar, with a large population of α4β2* nAChRs containing a minor populations of α4α5β2 nAChRs. However, LGN also contained a small additional population of α4β3β2 nAChRs.

Subunit Composition of α3* Subtypes in Rat Superior Colliculus. The immunoprecipitation studies (see above) indicated the existence of two large populations of α4β2 * and α6β2* nAChRs in rat superior colliculus, together with a smaller, but significant population of α3β2* nAChRs. To determine the subunit composition of these novel α3* receptors, we immunopurified nAChRs on an anti-α3 column, then eluted them using the α3 peptide. The purified α3* nAChRs were then analyzed by immunoprecipitation (Fig. 4A). The results clearly confirmed that the α3 subtype contains the β2, but not the β4 subunit (79.9 ± 13.3 and 0 ± 0.2% of [^{3}H]Epi-labeled receptors) and no appreciable amounts of the other true α subunits (α2 and α4 antisera immunoprecipitated 3.7 ± 0.2 and 1.5 ± 0.6% of [^{3}H]Epi-labeled receptors, respectively) with the possible exception of α6 (5.0 ± 0.7% of [^{3}H]Epi-labeled receptors). As regards structural subunits, the anti-α5 and anti-β3 antisera, respectively immunoprecipitated 32.4 ± 5.9% and 35.6 ± 0.7% of [^{3}H]Epi-labeled receptors.

These data indicate that purified α3* receptors in the SC are a mixture of three populations (α3α5β2*, α3β3β2*, and α3β2* nAChRs).

Pharmacological Profile of α6β2* and α3β2* Subtypes in the Superior Colliculus. Previous studies have clearly characterized the pharmacological profile of native and heterologously expressed α6β2* and α4β2* nAChRs (Parker et al., 1998; Zoli et al., 2002) but the pharmacology of heterologously expressed α6β2* and α3β2* subtypes seems to be indistinguishable using most current pharmacological tools. This includes α-conotoxin MII (αCntxMII) which is usually considered an α3/α6β2-selective antagonist (for re-

view, see Gotti and Clementi, 2004; Nicke et al., 2004), although derivatives with strong preference for $\alpha6\beta2^*$ over $\alpha3\beta2^*$ nAChRs have recently been described previously (McIntosh et al., 2004). We therefore thought that it would be interesting to compare the pharmacology of native $\alpha6\beta2^*$ and $\alpha3\beta2^*$ nAChRs. To do this, we immunoimmobilized the $\alpha6\beta2^*$ receptors using an anti-$\alpha6$ Ab and compared their pharmacological profile with that of the $\alpha3\beta2^*$ receptors immobilized with an anti-$\alpha3$ Ab.

Equilibrium binding assays revealed no significant differences in the affinity of the $\alpha6\beta2^*$ and $\alpha3\beta2^*$ receptor populations for [^{3}H]Epi [apparent K_d values of 26 pM (CV = 21%) and 35 pM (CV = 40%), respectively]. We then performed competition binding studies using αCntxMII, which showed a statistically significant better fit for a two-site model with a high-affinity site (K_i, 2 nM; CV = 40%) and a low-affinity site (K_i, >10,000 nM) when tested on the $\alpha6\beta2^*$ nAChRs. As already proposed for native $\alpha6\beta2^*$ nAChRs in striatum (Zoli et al., 2002; Champtiaux et al., 2003), the two sites should correspond to the $\alpha6\beta2$ (high affinity) and $\alpha4\beta2$ (low affinity) interfaces present in native nAChRs of the SC. However αCntxMII showed only a single high-affinity site (K_i, 115 nM; CV = 47%) on $\alpha3\beta2^*$ nAChRs. This strongly implies the absence of other true α subunits in these oligomers (Fig. 4B).

Nicotinic Receptor Subtypes Expressed on Retinal Afferents in Rat Superior Colliculus and Lateral Geniculate Nucleus. Retinal afferents constitute a principal input to SC and LGN, but several other afferents or intrinsic neuronal populations probably express nAChRs. To distinguish the nAChR subtypes expressed by retinal afferents from those expressed by other neuronal cells, we performed immunoprecipitation experiments in rats 2 weeks after bilateral eye removal.

The amount of [^{3}H]Epi binding decreased significantly by 39.0 and 31.0% in the SC and LGN, respectively, of the optic denervated rats, whereas no significant change was detected in ^{125}I-αBgtx binding after enucleation (Fig. 1). This demonstrates that the heteromeric nAChRs expressed on retinal afferents constitute less than half of the total amount of heteromeric nAChRs present in these regions, assuming that no compensatory increases occurred in intrinsic or other afferent nAChR-bearing systems (see below). In a similar vein, $\alpha7^*$ nAChRs do not seem to be expressed on retinal afferents.

Immunoprecipitation with subunit-specific antisera of the [^{3}H]Epi-labeled receptors of control and eye-enucleated SC showed the almost complete disappearance of the nAChRs containing the $\alpha6$ (91%) and $\beta3$ (92%) subunits (Fig. 2A) and marked to moderate decreases of the nAChRs containing $\alpha3$ (74%), $\alpha5$ (40%), $\beta2$ (38%), and $\alpha4$ (20%) subunits; the $\alpha2$ and $\beta4$ subunits were not significantly changed. In the LGN, the $\alpha6$ and $\beta3$ subunits were almost completely (90 and 89%, respectively) lost after eye removal, and all of the other subunits except $\beta4$ showed significant decreases: $\alpha2$ (49%), $\alpha3$ (61%), $\alpha5$ (32%), $\beta2$ (32%), and $\alpha4$ (23%) (Fig. 2B). Very

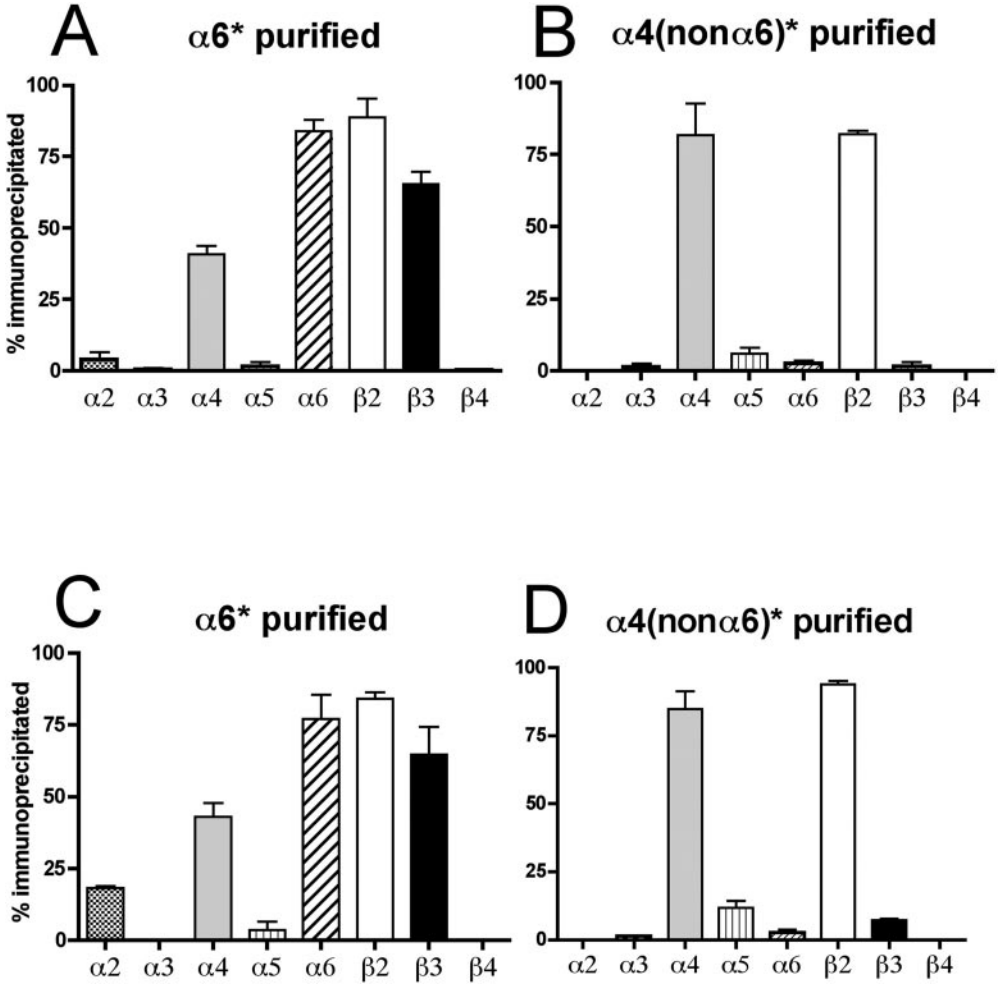


Fig. 3. Immunoprecipitation analysis of the subunit content of purified $\alpha6^*$ and $\alpha4$(non$\alpha6$)* nAChRs. The extracts prepared from control SC (A) or control LGN (C) were incubated on an affinity column with bound anti-$\alpha6$ CYT Abs (see *Materials and Methods*) to bind the $\alpha6^*$ population, which was eluted from the column by means of incubation with the $\alpha6$-CYT peptide. The recovered nAChRs were labeled with 2 nM [^{3}H]Epi and then immunoprecipitated by the indicated subunit-specific Abs. The extract devoid of the $\alpha6^*$ receptors was incubated with resin with bound anti-$\alpha4$ Abs. The bound receptor populations from SC (B) or LGN (D) were eluted by the $\alpha4$ CYT peptide, labeled with 2 nM [^{3}H]Epi, and immunoprecipitated by the indicated subunit-specific Abs. Immunoprecipitation was carried out and expressed as the percentage of [^{3}H]Epi-labeled receptors immunoprecipitated by the antibodies (taking the amount present in the purified receptors before immunoprecipitation as 100%). The results are the mean values ± S.E.M. of three to four immunopurification experiments for each subtype from each tissue

similar quantitative results were obtained in rats sacrificed 28 days after eye enucleation (data not shown), suggesting that no compensatory change in nAChR expression is ongoing in SC and LGN at the time intervals studied.

It was shown previously (Zoli et al., 2002) that terminals of midbrain dopamine neurons express a pattern of nAChR subunits that is very similar to the pattern of subunits expressed by retinal afferents shown here. To assess the possible contribution of dopamine afferents to nAChR subunit expression in SC and LGN, we performed a lesion of midbrain dopamine axons by means of the selective neurotoxin 6-OHDA and studied nAChR subunit expression in the retinorecipient regions. No significant change in the concentration of any nAChR subunit in SC or LGN was observed between eye-enucleated and eye-enucleated + 6-OHDA-lesioned rats (Fig. 2). These findings indicate that midbrain dopamine neurons do not contribute in a substantial manner to nAChR subunit expression in SC or LGN.

These results demonstrate that the $\alpha2$, $\alpha3$, $\alpha6$, and $\beta3$ subunits are almost exclusively expressed on retinal afferents ($\alpha2$ being detected only in the LGN and the other three subunits detected in both SC and LGN). The relative lack of changes in other subunits suggests that nAChR subtypes not expressed on retinal afferents do not compensate for the loss of nAChRs induced by enucleation.

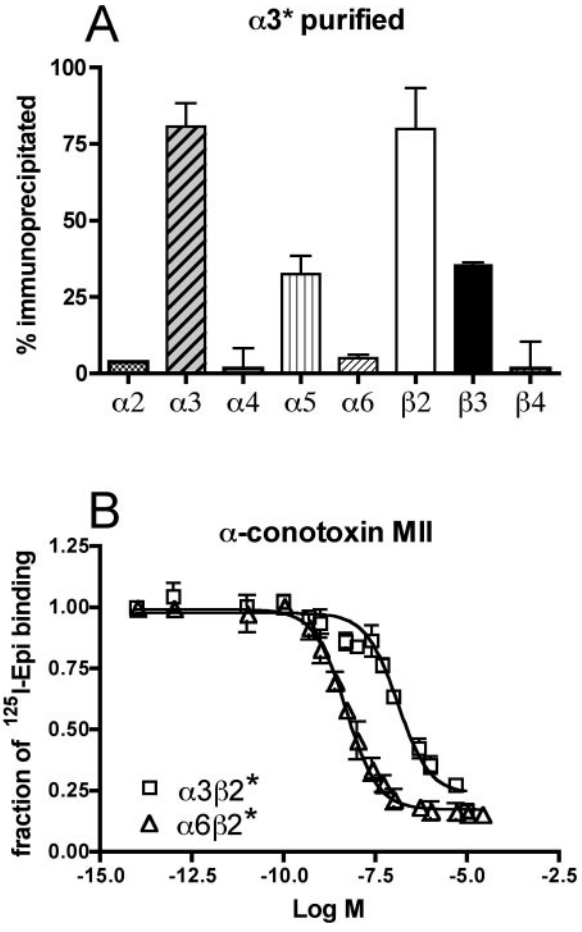


Fig. 4. A, analysis of the subunit content the $\alpha3\beta2$ subtype present in the rat SC. The extracts prepared from rat SC were incubated with an affinity column with bound anti-$\alpha3$ CYT Abs (see *Materials and Methods*) to bind the $\alpha3$* population, which was then eluted from the resin by means of incubation with the $\alpha3$ peptide. B, αCntxMII inhibition of the binding of ^{125}I-Epi to immunoimmobilized $\alpha3$ and $\alpha6$ nAChRs. $\alpha3\beta2$* and $\alpha6$* nAChRs from rat SC were immunoimmobilized using anti-$\alpha3$ or anti-$\alpha6$ Abs, as described under *Materials and Methods*. The inhibition curves were obtained by fitting three separate experiments using the LIGAND program; unless shown, the S.E.M. is in the range of the symbol. In each experiment, each αCntxMII dilution was tested in triplicate. All of the values are expressed in relation to ^{125}I-Epi-specific nAChR binding (considered 100%).

Expression of Nicotinic Receptor Subunits in the Superior Colliculus of$\alpha4$, $\alpha6$, and $\beta3$+/+ and −/− Mice. As a complementary approach to the rat studies, we examined SC nAChR composition using immunoprecipitation with subunit-specific antisera in several nAChR subunit Ko mice and their respective wild-type (WT) control mice. We first assessed nAChR subunit composition in mouse WT SC by immunoprecipitation and found that, when expressed as the percentage of total [^{3}H]Epi-binding, the amount of nAChR subunits was remarkably similar to that observed in rat SC (Table 1).

In $\alpha4-/-$ mice, sites immunoprecipitated by $\alpha4$Abs were reduced to 1% of $\alpha4+/+$ control, demonstrating the specificity of this antiserum. There were also strong reductions in $\alpha5$, $\alpha6$, $\beta2$, and $\beta3$ Ab-reactive subunits (to 34, 48, 26, and 38% of control). $\alpha2$, $\alpha3$, and $\beta4$ Ab-reactive subunits were not significantly altered (Table 2).

In $\alpha6-/-$ mice, the $\alpha6$ Abs failed to immunoprecipitate [^{3}H]Epi binding sites, again confirming the specificity of the antibodies used. The most dramatic effect was seen on sites immunoprecipitated by $\beta3$ Abs, which were reduced to 30% of $\alpha6+/+$ values. $\alpha3$* and $\beta2$* nAChR concentrations were also significantly decreased (to 72 and 69% of control values). Interestingly, expression of $\alpha4$* and $\alpha5$* nAChRs was unaffected by $\alpha6$-null mutation (Table 2).

Finally, $\beta3$-null mutation effectively eliminated $\beta3$-immunoprecipitable sites (reducing them to 2% of $\beta3+/+$ control), demonstrating the specificity of the $\beta3$-directed Abs used. $\alpha6$ and $\beta2$ subunit concentrations in $\beta3-/-$ mice were also significantly decreased (to 26 and 71% of the respective $\beta3+/+$ mean values), but the expression of other nAChR subunits was not affected by $\beta3$-null mutation (Table 2).

Overall, these results unequivocally demonstrate the specificity of $\alpha4$, $\alpha6$, and $\beta3$ antisera and provide some hints to the identification of the nAChR subtypes in mouse SC (see *Discussion*).

Discussion

Despite the fact that many neurochemical, electrophysiological, immunological, and lesion studies have provided ev-

TABLE 1

Expression of nAChR subunits in the superior colliculus of rat and mouse

For each subunit, the values correspond to femtomoles of immunoprecipitate by specific subunit Abs expressed as percentage of the overall femtomoles of [^{3}H]Epi-bound material. Rat values are the mean ± S.E.M. of four immunoprecipitation experiments. Mouse values are the average of mean values obtained in WT $\alpha4$, $\alpha6$, and $\beta3+/+$ mice (three immunoprecipitation experiments).

Subunit	Rat	Mouse
$\alpha2$	2.1 ± 0.3	2.0 ± 1
$\alpha3$	9.8 ± 1.8	10.6 ± 2.1
$\alpha4$	66.3 ± 3.3	61.0 ± 4.5
$\alpha5$	7.1 ± 1.1	3.9 ± 1.1
$\alpha6$	30.6 ± 1.8	30.6 ± 2.9
$\beta2$	90.6 ± 1.5	90.4 ± 5.8
$\beta3$	28.5 ± 1.3	27.7 ± 1.9
$\beta4$	2.8 ± 1.1	2.1 ± 1.2

idence of the presence of presynaptic nAChRs in the vertebrate retinorecipient areas (Henley et al., 1986; Sargent et al., 1989; Britto et al., 1994; Guo et al., 1998; Marks et al., 2002), very little is known about the subunit composition and pharmacology of the major nAChR subtypes in these regions. In the present study, by performing binding studies and immunopurification and immunoprecipitation experiments, we have shown that rat SC and LGN, the two principal retinorecipient areas in mammals, express partially different nAChR subtypes and, by analyzing the tissues of eye-enucleated rats, we have identified which subtypes are selectively expressed by retinal afferents (see Fig. 5). Moreover, because we have determined that rat and mouse SC express a very similar pattern of nAChR subunits, we used tissues from several nAChR subunit Ko mice to confirm and extend the reconstruction of nAChR subtype composition based on rat studies.

Immunoprecipitation data obtained from rats that underwent enucleation show that only 40% (SC) and 30% (LGN) of heteromeric, Epi-binding nAChRs are expressed on the retinal afferents. In both regions, after enucleation, remaining nAChRs included a large majority of $\alpha4\beta2$ nAChR subtype and a small proportion of $\alpha4\alpha5\beta2$ nAChR subtype and αBgtx-binding $\alpha7$ nAChRs. The subunit composition of nAChRs localized on retinal afferents can be deduced by combining the results derived from enucleation experiments and immunopurification data. The lack of effect of enucleation on αBgtx binding suggests that $\alpha7$nAChRs are not present on retinal afferents. As for heteromeric, Epi-binding nAChRs, three major nAChR subtypes were detected on retinocollicular afferents: $\alpha6\beta2$* (approximately 45% of the total nAChRs on retinal afferents), $\alpha4\alpha6\beta2$* (approximately 35%) and $\alpha3\beta2$* (approximately 20%). On retinogeniculate afferents, heteromeric nAChRs were more diverse, including $\alpha4\alpha6\beta2$* (approximately 40%), $\alpha6\beta2$* (approximately 30%), $\alpha4\beta2$* (approximately 20%), $\alpha2\alpha6\beta2$* (approximately 5%), and $\alpha3\beta2$* (approximately 5%) subtypes.

Further complexity is added by the presence of structural subunits, namely $\alpha5$ and $\beta3$ (replacing the * symbol) in these various subtypes. Indeed, immunopurification experiments indicated that most of the $\alpha6\beta2$* and $\alpha4\alpha6\beta2$* nAChRs in both SC and LGN contain the $\beta3$ subunit. In contrast, only a minority of $\alpha4\beta2$* nAChRs actually contained an additional subunit, $\alpha5$ in both the SC and LGN and $\beta3$ in the LGN. As for $\alpha3\beta2$* nAChRs, they represent a mixture of $\alpha3\beta2\beta3$, $\alpha3\beta2\alpha5$, and "pure" $\alpha3\beta2$ nAChRs, in roughly equal proportions.

Immunoprecipitation studies performed using SC extracts from WT as well as $\alpha4$, $\alpha6$, and $\beta3$ Ko mice support the results obtained in rats. It should be noted that the proportion of nAChR subunits in SC is very similar in mice and rats (see Table 1), making reasonable the assumption that the subunit composition of nAChRs is similar in the two species. First, the decrease in the $\alpha6$ subunit observed in $\alpha4$ Ko mice tallies well with the evidence obtained in rat studies that approximately 50% of $\alpha6\beta2$* nAChRs contain the $\alpha4$ subunit. Second, the marked decrease in the concentration of the $\beta3$ subunit in $\alpha4$ and $\alpha6$ Ko confirms that most $\alpha6\beta2$* and $\alpha4\alpha6\beta2$* also contain $\beta3$. Third, a minority of $\alpha4\beta2$* nAChRs and no $\alpha6\beta2$* nAChRs contain $\alpha5$ subunit, whereas a significant proportion of $\alpha3\beta2$* nAChRs probably do, because the concentration of $\alpha5$ is decreased in $\alpha4-/-$ mice and unchanged in $\alpha6-/-$ mice. Finally, although the data obtained in $\beta3$ Ko mice require a complex discussion that is beyond the scope of this article (see Gotti et al., 2005), they are compatible with the idea that $\beta3$ subunit is associated with both $\alpha4\alpha6$* and $\alpha6$* nAChRs.

A major finding of this study is the demonstration of the existence of two novel native nAChRs subtypes in rat: $\alpha2\alpha6\beta2$* and $\alpha3\beta2$*. Although $\alpha2\alpha6\beta2$* nAChRs are scarce (approximately 5% of the LGN heterogeneous subtype population), the $\alpha3\beta2$* subtype is much more abundant, representing an important fraction of both SC and LGN Epi binding sites (up to 11% in the SC). The immunopurification study clearly shows that the $\alpha3$ subunit is separate from the previously identified $\alpha6$* and $\alpha4(non\alpha6)$* nAChRs of the rat SC or LGN because immunopurified $\alpha3$* nAChRs did not contain appreciable amounts of the $\alpha2$, $\alpha4$, or $\alpha6$ subunits. This is also confirmed by data obtained from immunoprecipitation experiments in Ko mice, because the concentration of the $\alpha3$ subunit was not significantly altered in $\alpha4$, $\alpha6$, or $\beta3-/-$ mice. All together these results point to the existence of a previously undescribed $\alpha3\beta2$* nAChR subtype in rodent brain. These receptors are mostly localized on retinocollicular afferents and bind αCntxMII with high nanomolar affinity, approximately 50 times lower than native $\alpha6\beta2$ subtype. In general, $\alpha3$* nAChRs are uncommon in the central nervous system (Whiteaker et al., 2002), and most of them coassemble (Marks et al., 2002) with the $\beta4$ subunit [e.g., those present in the pineal gland, medial habenula, interpeduncular nucleus, area postrema, and dorsal nucleus of the vagus nerve (Zoli et al., 1998)]. Previous pharmacological experiments performed on $\alpha6$ Ko mice (Champtiaux et al., 2002) showed that the deletion of $\alpha6$ subunit leads to the loss of high-affinity αCntxMII binding in most brain areas but leaves some residual αCntxMII binding in the SC. Present

TABLE 2

Expression of nAChR subunits in the superior colliculus of $\alpha4$, $\alpha6$, and $\beta3$ +/+ and −/− mice

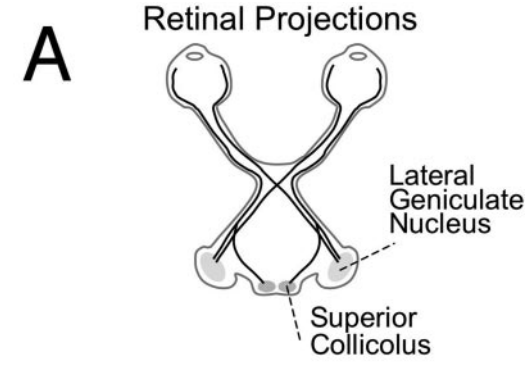
The reported values are expressed as femtomoles of specific labeled [^{3}H]Epi binding sites per milligram of protein and are the mean values ± S.E.M. of three to four experiments performed in triplicate per group, or as percentage of each subunit Ko mean value versus the respective WT mean value.

nAChR subunit	α4+/+	α4−/−	%	α6+/+	α6−/−	%	β3+/+	β3−/−	%
α2	3.4 ± 1.0	2.2 ± 1.5	64.7	4.3 ± 2.5	5.4 ± 3.9	124.9	7.5 ± 3.9	5.1 ± 3.3	68.0
α3	25.7 ± 6.8	22.8 ± 3.9	88.7	24.7 ± 5.4	17.8 ± 3.0	72.1	28.5 ± 3.5	23.9 ± 7.5	83.9
α4	160.4 ± 3.7	2.5 ± 1.9***	1.6	146.9 ± 10.6	147.1 ± 8.2	100.1	150.0 ± 13.3	132.1 ± 22.0	88.1
α5	10.5 ± 2.4	3.6 ± 0.3*	34.2	9.3 ± 3.2	9.2 ± 4.7	98.9	9.4 ± 2.3	8.2 ± 3.1	87.2
α6	80.3 ± 9.7	38.3 ± 6.5*	47.7	88.3 ± 4.4	1.3 ± 0.4***	1.5	60.6 ± 7.5	15.8 ± 1.5***	26.1
β2	216.7 ± 15.9	56.3 ± 7.8***	26.0	242.4 ± 9.7	167.5 ± 10.8**	69.1	219.1 ± 17.3	155.2 ± 16.2*	70.8
β3	70.6 ± 2.2	26.9 ± 2.2***	38.1	72.0 ± 5.7	21.6 ± 2.3***	30.0	65.0 ± 3.5	1.6 ± 0.7***	2.5
β4	7.0 ± 2.2	4.3 ± 1.2	61.4	3.9 ± 1.6	3.1 ± 2.0	79.5	4.7 ± 2.0	0.3 ± 0.2	7.0

Statistical analysis according to unpaired Student t test: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ Ko versus respective WT values.

findings strongly indicate that this lower-affinity αCntxMII binding site corresponds to the α3β2* subtype.

Overall, the data obtained in rat SC and LGN demonstrate that retinocollicular and retinogeniculate axons express a partially different range of nAChR subtypes (Fig. 5B). For example, α4(nonα6)β2 * and α2α6β2* subtypes were detected only in the LGN. Moreover, a comparison of the subtype expression of retinal afferents and retina (Moretti et al., 2004), suggests that some nAChR subtypes are preferentially expressed on retinal axons rather than in the retinal cell body/dendrite compartment. The α3β2* and α6β2* subtypes seem to be preferentially transported to retinal nerve terminals, whereas the α2α4β2* and α4(nonα6)β2 * receptors are minimally or not transported to the axonal compartment. A similar compartmental segregation was previously observed in midbrain dopamine neurons, which express a larger percentage of α6β2* nAChRs on striatal terminals than on midbrain cell bodies (Champtiaux et al., 2003). This suggests that the presence of the α6 subunit preferentially targets this subtype to the axonal compartment. On the other hand, the β3 subunit (which is associated with α6* nAChRs) may perform the targeting role, a hypothesis that is supported by the recent findings in the midbrain and striatum of β3 Ko mice (Gotti et al., 2005). In β3−/− mice, α6* nAChR expression is significantly reduced in both the dopamine cell body (ventral midbrain) and terminal (striatum) regions but more markedly in the latter (76%) than in the former (42%) region. Whatever the reason for the decrease in α6* nAChR expression (e.g., defects in nAChR assembly, degradation, and/or cell trafficking), the much greater decline in striatal terminals than in midbrain cell bodies suggests that the β3 subunit may play a role in targeting nAChRs to the terminal compartment of dopamine neurons.



B

	Retinal afferents		Other Neurons	
	SC(40%)	LGN(30%)	SC(60%)	LGN(70%)
α3β2	20%	5%		
α4α6β2β3	35%	40%		
α6β2β3	43%	30%		
α2α6β2		5%		
α4β2		20%	90%	90%
α4α5β2			10%	10%

Fig. 5. A, schematic representation of the mammalian visual projections. B, diagram showing the [^{3}H]Epi receptor subtypes present in rat retinal afferents to the SC and LGN, their possible subunit arrangement, and their relative amount expressed as percentage of the total [^{3}H]Epi receptors present in 2% Triton X-100 membrane extracts of each region. The value indicated at the top of each column represents the percentage of [^{3}H]Epi receptor expressed on retinal afferent with respect to the overall amount of receptors present in the region.

In conclusion, by combining neurochemical, immunological, and lesion techniques in normal rats and mice as well as mice lacking nAChR subunits, we could reconstruct the composition of the nAChR subtypes expressed on retinal afferents to the SC and LGN, the main retinorecipient regions, and show that they are highly heterogeneous, partially distinct from the subtypes expressed in the retina, and differently addressed to the SC and LGN. Moreover, we identified and characterized pharmacologically α3β2(β3 or α5) receptors, a new native nAChR subtype enriched in the retinocollicular projections

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