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Title Page

Subunit composition of nicotinic receptors in monkey striatum; effect of MPTP and L-dopa treatments

Maryka Quik, Silvia Vailati*, Tanuja Bordia*, Jennifer M. Kulak[1], Hong Fan, J Michael McIntosh, Francesco Clementi and Cecilia Gotti

*The Parkinson's Institute, Sunnyvale, CA, 94089 USA (M.Q., T.B., J.K.); CNR, Institute of Neuroscience, Cellular and Molecular Pharmacology, Dept. Medical Pharmacology, Univ. Milan, Milan, Italy (S.V., F.C., C.G.); Dept. Radiology, Johns Hopkins University School of Medicine, Baltimore, MD 21205(H.F.); Department of Biology and Psychiatry, Univ. Utah, Salt Lake City, UT 84112 USA (J.M.M.). *These authors contributed equally to the work.*

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Running Title Page

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Address correspondence to: Dr. Maryka Quik, The Parkinson's Institute, 1170 Morse Ave, Sunnyvale, CA 94089-1605: Tel. 1-408-542-5601. fax 1-408-734-8522.

E-mail address: mquik@thepi.org.

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ABBREVIATIONS: ANOVA, analysis of variance; L-dopa, L-3,4-dihydroxyphenylalanine; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; nAChR, nicotinic acetylcholine receptor; RTI-121, 3β-(4-Iodophenyl)tropane-2β-carboxylic acid isopropyl ester; *denotes nicotinic receptors containing the indicated α and/or β subunit and also additional undefined subunits.

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ABSTRACT

Nicotinic acetylcholine receptors (nAChRs) represent an important modulator of striatal function both under normal conditions and in pathological states such as Parkinson's disease. Since different nAChR subtypes may have unique functions, immunoprecipitation and ligand binding studies were done to identify their subunit composition. Similar to the rodent, α2, α4, α6, β2 and β3 nAChR subunit-immunoreactivity were identified in monkey striatum. However, distinct from the rodent, the present results also revealed the novel presence of α3 nAChR subunit-immunoreactivity in this same region, but not that for α5 and β4. Relatively high levels of α2 and α3 subunits were also identified in monkey cortex, in addition to α4 and β2. Experiments were next done to determine whether striatal subunit expression was changed with nigrostriatal damage. MPTP treatment decreased α6 and β3 subunit-immunoreactivity by ~80% in parallel with the dopamine transporter suggesting they are predominantly expressed on nigrostriatal dopaminergic projections. In contrast, α3, α4 and β2 subunit-immunoreactivity were decreased ~50%, while α2 was not changed. These data, together with those from dual immunoprecipitation and radioligand binding studies (^{3}H-cytisine, ^{125}I-α-bungarotoxin and ^{125}I-α-conotoxin MII) suggest the following: that α6β2β3, α6α4β2β3 and α3β2* nAChR subtypes are present on dopaminergic terminals; the α4β2 subtype is localized on both dopaminergic and non-dopaminergic neurons; while α2β2* and α7 receptors are localized on non-dopaminergic cells in monkey striatum. Overall, these results suggest that drugs targeting non-α7 nicotinic receptors may be useful in the treatment of disorders characterized by nigrostriatal dopaminergic damage such as Parkinson's disease.

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Introduction

Parkinson's disease is a neurodegenerative disorder characterized by severe movement disability (Olanow, 2004; Samii et al., 2004). Although the underlying cause appears to be a loss of nigrostriatal dopaminergic neurons, other neurotransmitter systems are also affected. This includes the cholinergic system in which declines have been observed in several cholinergic measures, including nicotinic acetylcholine receptors (nAChRs). Binding sites for ^{125}I-epibatidine, ^{3}H-cytisine, ^{3}H-nicotine and ^{125}I-α-conotoxin MII are decreased in Parkinson's disease, with no change in ^{125}I-α-bungarotoxin receptors (Court et al., 2000; Gotti et al., 1997; Quik et al., 2004). These data indicate that receptor subtypes expressing the α4β2 (^{3}H-cytisine and ^{3}H-nicotine binding), and the α3β2 and/or α6β2 (^{125}I-α-conotoxin MII sites) subunits are decreased in Parkinson's disease, while those containing α7 (^{125}I-α-bungarotoxin) are not affected. Studies to identify the other nAChR subunits that comprise these nAChR subtypes are critical for the development of subtype selective agents targeting the receptors deficient in this disorder. However, experiments using antibodies directed to human nAChR subunits have yielded uncertain results (Guan et al., 2002; Martin-Ruiz et al., 2000).

Because animal models represent an excellent first step, studies have been done in both rodents and monkeys to address this question. In rodents, numerous nAChR subunit mRNAs (α2-α7 and β2-β4) have been localized to the substantia nigra (Champtiaux et al., 2002; Le Novere et al., 1996; Marks et al., 1992; Whiteaker et al., 2000; Whiteaker et al., 2002). Moreover, receptor binding and antibody immunoprecipitation studies indicate that these transcripts are expressed with multiple nAChR subtypes present in the striatum, including those expressing α4β2, α4β2α5, α6α2β2β3 and α6β2β3 (Champtiaux et al., 2003; Champtiaux et al., 2002; Klink et al., 2001; Salminen et al., 2004; Whiteaker et al., 2000; Whiteaker et al., 2002;

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Zoli et al., 2002). Nigrostriatal damage, produced by administration of the selective dopaminergic neurotoxins, 6-hydroxydopamine or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), results in losses of both α4* and α6* nAChR populations in rodents (Champtiaux et al., 2003; Quik et al., 2003a; Zoli et al., 2002). Moreover, these receptor losses are associated with functional deficits both at the cellular (Quik et al., 2003a) and behavioral level (le Novere et al., 1999).

Studies to identify the nicotinic receptor subtypes and the effects of nigrostriatal damage and dopamine precursor treatment have also been done in nonhuman primates, a species that bears a close resemblance to man at the genetic, molecular and behavioral level. In addition, monkeys with nigrostriatal damage exhibit symptoms that resemble those in Parkinson's disease, with the motor deficits reversed by the same drug used to treat this disorder. Studies have shown that the α2-α7 and β2-β4 nAChR transcripts are present in monkey substantia nigra (Han et al., 2000; Quik et al., 2000b; Quik et al., 2000a), and that binding sites for ^{125}I-epibatidine, ^{3}H-cytisine, ^{125}I-A85380, ^{125}I-α-conotoxin MII and ^{125}I-α-bungarotoxin are expressed in the striatum and substantia nigra (Han et al., 2003; Kulak et al., 2002a; Kulak et al., 2002b; Quik et al., 2001). Furthermore, there are differential changes in nAChRs after MPTP treatment with a complete loss of ^{125}I-α-conotoxin MII sites and also declines in α-conotoxin MII-resistant ^{125}I-epibatidine sites. Thus, radioligand binding studies suggest that α6β2* and/or α3β2*, as well as α4β2*, nAChRs are present in monkey striatum, with preferential declines in α6β2* and/or α3β2* sites, and smaller losses in α4β2* expressing receptors with nigrostriatal damage. Treatment with L-dopa, the most frequently used therapy for Parkinson's disease, also resulted in changes in nAChRs with a selective loss of a low affinity α-conotoxin MII-sensitive site (Quik et al., 2003b).

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The objective of the present study was to further identify the nAChR subunit composition in monkey striatum and the effect of nigrostriatal damage and L-dopa treatment on the different receptor populations. To approach this, receptor binding studies using nAChR-directed radioligands and immunoprecipitation experiments using subunit-selective antibodies were done in striata from control and treated monkeys.

Materials And Methods

Animals and treatment. Adult squirrel monkeys (*Saimiri sciureus*) weighing 0.5-0.8 kg were purchased from Osage Research Primates (Osage Beach, MO), and quarantined upon arrival. They were housed in a 13 h-11 h light-dark cycle. They had free access to water, and were given food pellets and fruit once daily. All procedures used conform to the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee. MPTP (2 mg/kg subcutaneous) treatment was as previously described (Quik et al., 2000b). To evaluate the behavioral effects of the lesion, animals were rated for parkinsonism using a modified Parkinson rating scale for the squirrel monkey, in which the disability scores ranged from 0 to 20. The composite score was evaluated based on (1) spatial hypokinesia, (2) body bradykinesia, (3) manual dexterity, (4) balance and (5) freezing. A group of unlesioned animals was administered L-dopa (15 mg/kg) in combination with carbidopa by oral gavage twice daily 4 h apart, on a 5-day on, 2-day off schedule for 8 wks.

Animals were euthanized in accordance with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association and conforming to the NIH Guide for the Care and Use of Laboratory Animals. Ketamine hydrochloride (15-20 mg/kg i.m) was administered for sedation, followed by injection of 0.22 ml/kg i.v. euthanasia solution (390 mg sodium pentobarbital and 50 mg phenytoin sodium/ml). When the heart had stopped, the brains

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were rapidly removed, placed in a mold and cut into 6 mm thick blocks. These were frozen in isopentane on dry ice and stored at -80^{o}C. Striatal and cortical tissue was dissected from one-half of the brain and used for the antibody immunoprecipitation studies. The other half of the brain was used for the autoradiographic studies. Sections (20 μm) were cut using a cryostat, thaw mounted onto poly-L-lysine coated slides, air dried and stored at -80^{o}C. For the receptor binding studies, MPTP-treated monkeys were separated into two groups as previously reported (Kulak et al., 2002a; Quik et al., 2001). Monkeys with striatal dopamine transporter levels ~30% of control were defined as moderately lesioned, while those with transporter levels $\leq$ 5% of control were defined as severely lesioned. Only tissue from severely lesioned animals was used for the immunoprecipitation studies.

Antibody production and characterization. The polyclonal antibodies against the human α2, α3, α4, α5, α6, β2, and β4 or β3 monkey nAChR peptide subunits (see Table 1) were produced in rabbit as previously described (Champtiaux et al., 2003; Zoli et al., 2002) and affinity purified. The peptides obtained from monkey or human sequences were located in the putative cytoplasmic loop between M3 and M4. The affinity-purified antisera were bound to CNBr-activated Sepharose at a concentration of 1 mg/ml, and the columns used for subtype immunopurification.

Since no cloned nAChR monkey subunits are available, the specificity of the antibodies produced against the human peptides was tested by quantitative immunoprecipitation experiments using extracts obtained from HEK cells transfected with different combinations of the human α2, α3, α4, α5, α6 and β2 and β4 subunits (a generous gift from Dr E. Sher of Eli Lilly & Co Ltd, UK) or from tissues obtained from wild type and nAChR null mutant mice. Triton X-100 (2%) extracts, labeled with 2 nM ^{3}H-epibatidine, prepared from the transfected

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cells or from tissues obtained from wild type or knockout animals were incubated with the saturating concentrations of the antibodies directed against all the subunits. In these tissues the antibodies only recognized the receptors containing the corresponding subunits. The immunoprecipitation capacity of these antibodies versus the human and rodents subtypes was very high (>80%) (Champtiaux et al., 2003; Moretti et al., 2004; Zoli et al., 2002). The anti-α4, -α6, -β2, and -β3 antibodies were also tested on monkey-purified subtypes (see results) where they also had a very high immunoprecipitation capacity. Binding values ≤6% were at the detection level of the assay, so this value was used as our cut-off for subunit expression.

Preparation of membranes and 2% Triton X-100 extracts from monkey brain. Monkey striatum and cortex, obtained as described above, was separately homogenized in an excess of 50 mM Na phosphate pH 7.4, 1 M NaCl, 2 mM EDTA, 2 mM EGTA and 2 mM phenylmethylsulfonylfluoride (PMSF) for 2 min using an UltraTurrax homogenizer. The homogenates were then diluted and centrifuged for 1.5 h at 60,000*g*. The homogenization, dilution and centrifugation of the indicated tissue was 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 PMSF, and then resuspended in the same buffer containing a mixture of 20 µg/ml of each of the following protease inhibitors: leupeptin, bestatin, pepstatin A and aprotinin. Triton X-100 at a final concentration of 2% was added to the washed membranes, which were extracted for 2 h at 4°C. The extracts from tissues were then centrifuged for 1.5 h at 60,000g, recovered. 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.

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^{3}H-Epibatidine binding assays for immunoprecipitation studies. Membrane binding experiments were performed by incubating membrane homogenates overnight with 2 nM ^{3}H-epibatidine (56 Ci/mmol, Amersham Biosciences, Piscataway, NJ, USA) at 4°C. To prevent binding of ^{3}H-epibatidine to α-bungarotoxin-binding receptors, membranes were preincubated with 2 μM α-bungarotoxin and then with ^{3}H-epibatidine. Specific radioligand binding was defined as total binding minus nonspecific binding determined in the presence of 100 nM cold epibatidine. The 2% Triton X-100 extracts of tissues were preincubated with 2 μM α-bungarotoxin for 3 h, and then labeled with 2 nM ^{3}H-epibatidine. Tissue extract binding was performed using DE52 ion-exchange resin (Whatman, Maidstone, UK) as previously described (Vailati et al., 1999)**.**

Immunoprecipitation of ^{3}H-epibatidine-labeled receptors by anti-subunit-specific antibodies.

Striatal and cortical extracts or purified receptors were preincubated with 2 μM α-bungarotoxin, labeled with 2 nM ^{3}H-epibatidine and incubated overnight with a saturating concentration of affinity purified IgG (20-30 μg, Sigma, St Louis, USA). The immunoprecipitation was recovered by incubating the samples with beads containing bound anti-rabbit goat IgG (Technogenetics, Milan, Italy). The level of antibody immunoprecipitation was expressed as the percentage of ^{3}H-epibatidine-labelled receptors immunoprecipitated by the antibodies (taking the amount present in the 2% Triton X-100 extract solution before immunoprecipitation as 100%) or as fmol immunoprecipitated receptors /mg protein.

For each purification experiment, the 2% Triton X-100 extract obtained from striatal membranes, prepared as described above, was incubated three times with 5 ml of Sepharose-4B bound anti-α6 antibody in order to remove the α6* receptors. The flow-through of the α6

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column was analyzed for the subunit content of the remaining receptors, and then incubated two times with 5 ml of anti-β2 antibody bound to Sepharose–4B. The bound β2* nAChRs were then eluted with the β2 peptide and analyzed for their subunit composition by quantitative immunoprecipitation.

The 2% Triton X-100 cortical extract was incubated with 5 ml of Sepharose-4B bound anti-α4 antibodies in order to remove the α4 receptors. The bound receptors were eluted by competition with 100 μM of the corresponding α6 or α4 peptides used for antiserum production.

Receptor autoradiography

^{125}I-RTI-121 autoradiography. ^{125}I-RTI-121 (3β-(4-^{125}I-iodophenyl)tropane-2β-carboxylic acid isopropyl ester; 2200 Ci/mmol, Perkin Elmer Life Sciences, Boston, MA, USA) was used to measure binding to the dopamine transporter (Quik et al., 2001). Sections were preincubated for 2 x 15 min in 50 mM Tris-HCl buffer pH 7.4, containing 120 mM NaCl and 5 mM KCl. Incubation (2 h) was done in the same buffer plus 0.025% BSA, 1 μM fluoxetine and 50 pM ^{125}I-RTI-121. The sections were washed for 4 x 15 min at 4^{o}C in preincubation buffer, dipped in ice cold water, air dried and placed against Kodak MR film (Perkin Elmer Life Sciences, Boston, MA, USA) for 1-3 d with ^{125}I-microscale standards (Amersham Biosciences, Piscataway, NJ, USA). Nomifensine (100 μM) was used to define nonspecific binding.

^{125}I-Epibatidine autoradiography. ^{125}I-Epibatidine binding to striatal sections was done as previously described (Kulak et al., 2002a; Perry and Kellar, 1995). Briefly, sections were preincubated for 30 min, and then incubated for 40 min at room temperature in 50 mM Tris buffer, pH 7, 120 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, containing 0.015 nM ^{125}I-epibatidine (2200 Ci/mmol, Perkin Elmer Life Sciences, Boston, MA, USA). For competition

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studies, a concentration range of 10 pM to 10 μM α-conotoxin MII was used. Sections were subsequently washed (4°C) for 5 min with buffer (2x), for 10 s in cold H_2O and then air-dried. They were exposed for 2-5 d to Kodak MR film (Perkin Elmer Life Sciences, Boston, MA, USA), together with ^{125}I-standards (Amersham Biosciences, Piscataway, NJ, USA). Nicotine (10 μM) was used to determine nonspecific binding, which was the same as film blank.

^{125}I-A-85380 autoradiography. Preparation of ^{125}I-A85380 (specific activity 1500 Ci/mmol) and binding to brain membranes was done as described (Mukhin et al., 2000). Preincubation was for 20 min in the same buffer used for ^{125}I-epibatidine binding assays, followed by a 40 min incubation in fresh buffer containing ^{125}I-A-85380 (80 pM). Sections were washed in buffer at 4°C for 2 x 5 min, followed by 1 x 10 s in deionized H_2O (4°C). Air dried slides were exposed to Kodak MR film (Perkin Elmer Life Sciences, Boston, MA, USA) for 1-2 d with ^{125}I-standards (Amersham Biosciences, Piscataway, NJ, USA). Nicotine (10 μM) was used to determine nonspecific binding, which was the same as film blank.

^{3}H-Cytisine autoradiography. ^{3}H-cytisine (specific activity 37.5 Ci/mmol, Perkin Elmer Life Sciences, Boston, MA. USA) binding was performed as described (Perry and Kellar, 1995; Sihver et al., 1998). Sections were incubated at room temperature for 60 min in buffer (50 mM Tris, pH 7, 120 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$) plus 2 nM ^{3}H-cytisine. After incubation, sections were washed 2 x 5 min in buffer at 4°C and 1 x 10 s in ice cold H_2O. After drying at room temperature, slides were exposed for 8-12 wk to ^{3}H-sensitive Hyperfilm (Amersham, Piscataway, NJ, USA), along with ^{3}H-standards (American Radiolabeled Chemicals, Inc., St. Louis, MO). Nicotine (10 μM) was used to determine nonspecific binding.

^{125}I-α-Conotoxin MII autoradiography. ^{125}I-α-conotoxin MII (specific activity 2200 Ci/mmol) was synthesized and radiolabeled as described (Whiteaker et al., 2000). For assay

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(Quik et al., 2001; Whiteaker et al., 2000), sections were preincubated at room temperature for 15 min in Binding buffer (144 mM NaCl, 1.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 20 mM Hepes, 0.1% BSA, pH 7.5) plus 1 mM phenylmethylsulfonyl fluoride. This was followed by a 1 h incubation at room temperature in Binding Buffer plus 0.5% BSA, also containing 5 mM EDTA, 5 mM EGTA, and 10 μg/ml each of aprotinin, leupeptin and pepstatin A, and 0.5 nM ^{125}I-α-conotoxin MII. To terminate the assay, slides were rinsed for 30 s in Binding buffer at room temperature followed by 30 s in ice-cold Buffer, 2 x 5 s in 0.1x Buffer (0°C) and two washes in water (0°C). The sections were air dried and exposed to Kodak MR film (Perkin Elmer Life Sciences, Boston, MA, USA) for 2-5 d together with ^{125}I-standards (Amersham Biosciences, Piscataway, NJ, USA). Epibatidine (0.1 μM) was used to determine nonspecific binding.

^{125}I-α-Bungarotoxin autoradiography. Sections were preincubated at room temperature in 50 mM Tris HCl, pH 7 for 30 min (Clarke and Pert, 1985). They were next incubated for 1 h in the same buffer containing 3 nM ^{125}I-α-bungarotoxin (specific activity 128 Ci/mmol, Perkin Elmer Life Sciences, Boston, MA. USA). The sections were then rinsed 4x for 15 min in ice-cold buffer, once in cold water, air-dried and placed against Kodak MR film for 1-2 wk (Perkin Elmer Life Sciences, Boston, MA, USA). Nicotine (100 μM) was used to define nonspecific binding.

Analyses of autoradiographic data. A squirrel monkey (*Saimiri sciureus)* brain atlas was used to identify brain regions, as previously described (Quik et al., 2000a). The optical density values, determined using an ImageQuant system (Molecular Dynamics, Sunnyvale, CA), were assessed by subtracting background from tissue values. This was followed by conversion to fmol/mg tissue using standard curves generated from radioactive standards simultaneously exposed to the films. Sample optical density readings were within the linear range of the film. Receptor binding data for any one animal represents the mean from 1 to 2 sections each, from 2

or more independent experiments.

Competition curves were compared and best-fit to one- and two-site models using GraphPad Prism (San Diego, CA). Statistical analyses were done using one-way ANOVA followed by Newman-Keuls multiple comparison test where $p \leq 0.05$ was considered significant. All values are expressed as the mean $\pm$ S.E.M. of the indicated number of animals.

Results

Characterization of nAChR subunit antibodies. The identification of nAChR subtypes in monkey brain relied on the use of a series of antisera raised against unique amino acid sequences of the different human or monkey subunits. All of the antibodies (except for the anti-β3 antibody, which was not tested) selectively interacted with receptors expressing the appropriate human nAChR subunit in transfected HEK cells. Because of the sequence identity between α3 and α6 subunits, we also tested whether identification of α3* nAChRs (14%) in striatum might be due to cross-reactivity of the anti-α3 antibodies with α6* receptors, however the α3 antibody recognized only 3% of purified α6* receptors. In addition, the immunoprecipitation capacity and specificity of the antibodies was investigated on purified α6* receptors obtained from striatum and on α4* receptors purified from the cortex. We found that the α4, α6, β2 and β3 antibodies had an immunoprecipitation capacity of more than 60%. We did not consider the contribution of subunits to receptor composition that were immunodetected in amounts of 6% or less, and therefore minor nAChR subtypes may have been excluded from the analyses.

NAChR subunit expression in control monkey striatum and cortex. Experiments were first done to quantify the relative contribution of each nicotinic subunit to ^{3}H-epibatidine binding present in the striatum. To approach this, we performed quantitative immunoprecipitation

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experiments using subunit-specific antibodies and ^{3}H-epibatidine labeled receptors. Receptor levels in control monkey striatum were 55.5 ± 4.1 and 69.6 ±5.5 fmol/mg protein in the membrane preparation and 2% Triton extract, respectively. The receptors immunoprecipitated by specific nAChR subunit antibodies (calculated as the percentage of the total number of ^{3}H-epibatidine receptors) were: as follows β2 (91%), α4 (55%), α6 (25%), β3 (18%) α3 (14%) and α2 (12%) (Fig.1A). The α5 and β4 subunit containing receptors fell below the detection limit of the assay (6%). Values represent the mean ± S.E.M. of 6 immunoprecipitation experiments performed in duplicate for each antibody.

A similar approach using ^{125}I-epibatidine-labeled sites was used to identify the major nAChR subtypes in monkey cortex. Receptor levels in control monkey cortex were 41.6 ± 3.6 and 49.9 ± 2.6 fmol/mg protein in the membrane preparation and 2% Triton extract, respectively. Immunoprecipitation studies using crude membrane extracts showed that receptors contained the β2 (96 %), α4 (77%), α2 (21%) and α3 (10%) subunits, while the α5, α6, β3 and β4 subunits were below the level of detection of the assay. Results represent the mean ± S.E.M. of 3 immunoprecipitation experiments performed in duplicate for each antibody (Fig. 1B).

Thus similar to the rodent, the major nicotinic receptor subtypes in monkey cortex contain the α4 and β2 subunits, while in the striatum they contain α4, α6, β2 and β3 subunits. On the other hand, the α2 and α3, but not α5 and β4, subunits are present in monkey striatum and cortex, distinct from rodent brain.

Subunit composition of α6* nAChRs in monkey striatum. Our immunoprecipitation experiments, as well as previous receptors studies, indicate that there is a selective expression of α6* nAChRs in monkey striatum. To identify the subunits that co-assemble with α6, we immunodepleted striatal extract of α6* receptors using an affinity column with a bound anti-α6

antibody. Selective α6* nAChR immunodepletion was confirmed by the fact that immunoprecipitated α6* ^{3}H-epibatidine-labeled receptors decreased from 25% in the total striatal extract to 1% in the flow-through of the α6 column. In addition, α4* receptors were increased (from 58.5 to 71.6%), suggesting that an appreciable portion of the α4 subunit pool is not assembled with the α6 subunit. α2* nAChRs were also substantially increased in the flow through (from 12 to 30%), suggesting they may primarily be associated with non-α6* nAChRs. On the other hand, β3* receptors markedly decreased suggesting a co-localization with α6. β2* nAChRs remained unchanged indicating they are present in the majority of receptor subtypes.

To identify their subunit composition, α6* receptors were eluted from the affinity column with α6 peptide, labeled with ^{3}H-epibatidine and the eluate immunoprecipitated with nAChR subunit specific antisera. As shown in Fig. 1C, the anti-α4, -β2 and -β3 sera immunoprecipitated 47, 100 and 61 % of the purified ^{3}H-epibatidine-labeled receptors, respectively. In contrast, the anti-α2, -α3, -α5 and -β4 sera immunoprecipitated ≤6% (detection limit of the assay) of ^{3}H-epibatidine binding, suggesting they do not co-assemble with α6.

The dual immunoprecipitation data suggest that α6* nAChRs may be composed of α6β2β3 and/or α6α4β2β3 subunits. Additionally, analyses of the α6-affinity column flow-through indicate that α4β2* nAChRs also form major striatal subtypes.

Subunit composition of non-α6* nAChRs in monkey striatum. To identify striatal nAChRs not containing the α6 subunit, we also immunopurified the flow-through of the α6 affinity column using an anti-β2 column. We then eluted the bound receptors with β2 peptide and performed immunoprecipitation studies using subunit specific antisera. The anti α2, α3, and α4 antibodies immunoprecipitated 22.9 ± 3.9%, 20.4 ± 5.6%, and 73.4 ± 2.4% (mean ± S.E.M.,

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n=2) of the ^{3}H-epibatidine-labeled purified β2* receptors, respectively. The other antibodies did not yield any detectable immunoreactive material.

These studies clearly show that, in addition to α6* nAChRs, α4β2* receptors are also present in monkey striatum together with a minor population of α2β2* and α3β2* nAChRs. Because of the low recovery of the α2* and α3* subtypes in the β2 purified receptor preparation, it was not feasible to further investigate their subunit composition.

Subunit composition of α4* nAChRs in monkey cortex. Since α4 is the major acetylcholine binding subunit in cortex, experiments were done to determine with which subunits α4 is co-expressed (Fig. 1D). Cortical extracts were incubated with anti-α4 antibody linked to Sepharose beads. Bound α4* receptors were then eluted with α4 peptide. Immunoprecipitation experiments showed that 95% of these receptors contained the β2 subunit, 17% the α2 subunit and 8% the α3 subunit. Therefore, all α4* receptors most likely couple with β2, while a subpopulation of α4β2* subtypes also contain the α2 and α3 subunits.

Nigrostriatal damage decreases select nAChR subunits in monkey striatum. Studies were next done to determine the effect of nigrostriatal damage on nAChR subunit expression in monkey striatum (Table 2). Animals were lesioned with the selective dopaminergic neurotoxin MPTP and euthanized one month later when the effects of the lesion were maximal. ^{3}H-Epibatidine binding in monkey striatum was significantly ($p< 0.005$) reduced from 55.5 ± 4.1 to 30.0 ± 3.5 fmol/mg (n = 6 experiments) in the membrane preparation and from 69.6 ± 5.5 to 35.2 ±1 in the 2% Triton extract (n = 6 experiments), similar to previous results (Kulak et al., 2002a). Immunoprecipitation of solubilized ^{3}H-epibatidine binding sites using subunit-specific antibodies (Table 2) showed that MPTP-lesioning produced the greatest decline (expressed as % decrease) in α6* (83%) and β3* (86%) subtypes, as well as significant reductions in receptors containing

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α3 (50%) α4 (32%), β2 (48%), but not α2, subunits. Since α6* and β3* nAChRs were decreased in parallel with the dopamine transporter, these subtypes are most likely co-expressed on dopamine terminals. In contrast, receptors expressing the α4 and β2 subunits appear to be present on both dopaminergic and nondopaminergic neurons, whereas α2* subtypes are on nondopaminergic cells. Putative receptor subtypes in striatum thus include α6β2β3, α6α4β2β3, α4β2 and α2β2*.

Consistent with previous results, cortical ^{3}H-epibatidine receptors were unaffected by MPTP-treatment with 41.6 $\pm$ 3.6 and 41.4 $\pm$ 5.8 fmol/mg protein in membranes from controls and MPTP-treated animals, respectively. The 2% Triton extracts were also similar in controls and MPTP treated animals with values of 49.9 $\pm$ 2. 7 and 47.3 $\pm$ 0.8 fmol/mg protein, respectively. Immunoprecipitation studies performed on cortical tissues confirmed that there was no change in the expressed subtypes after MPTP lesioning.

Radioligand binding studies - effect of nigrostriatal damage. Earlier work had shown that receptors labeled with ^{125}I-epibatidine, a ligand that identifies multiple receptor subtypes (α2* through α6*) were reduced with nigrostriatal damage (Kulak et al., 2002a), consistent with the present immunoprecipitation data. Other studies using the more selective radioligand ^{125}I-α-conotoxin MII further demonstrated specific declines with lesioning in α3* and/or α6* nAChRs (Quik et al., 2001). In the present experiments we investigated binding of ^{125}I-α-bungarotoxin to α7 receptors, and ^{3}H-cytisine, which interacts with α4β2* and α2β2*subtypes (Luetje and Patrick, 1991). Autoradiographic studies showed there was a decrease in ^{3}H-cytisine binding in caudate and putamen (Fig. 2A), with no change in ^{125}I-α-bungarotoxin binding (Fig. 2B).

Previous work in rodents had indicated that ^{3}H-cytisine binds to an α4* nAChR (Flores et al., 1992). The present results (Fig. 3A) show that α-conotoxin MII does not compete with ^{3}H-

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cytisine in striatal slices from either control or MPTP-lesioned animals. This observation suggests that ^{3}H-cytisine binds at a similar receptor interface (that is, $\alpha4\beta2$) in monkey striatum. Nicotine completely blocked ^{3}H-cytisine binding in striatum from both control and MPTP-lesioned monkeys (Fig. 3B) demonstrating that the radioligand binds to a receptor with nicotinic characteristics. Previous studies (Quik et al., 2001) had shown that the nAChRs decreased with nigrostriatal damage were α-conotoxin MII-sensitive (that is, $\alpha3^*$ and/or $\alpha6^*$). This work, combined with the present experiments showing that ^{3}H-cytisine binding ($\alpha4^*$) receptors are decreased after MPTP treatment (Fig. 4), suggests these nAChRs may have both an $\alpha4\beta2$ and an $\alpha6\beta2$ interface, that is, $\alpha6\beta2\alpha4\beta2^*$.

L-Dopa treatment decreases nAChRs in monkey striatum. Previous studies had shown that two weeks of L-dopa treatment (15 mg/kg 2x daily every 4 h) reduced striatal ^{125}I-epibatidine sites (Quik et al., 2003b). To determine whether a longer course of treatment might result in a differential decline, we investigated the effect of 8 weeks of administration. Results (Fig. 5A) show that there was a somewhat greater decline in ^{125}I-epibatidine binding (~25%) with similar results obtained using ^{125}I-A85380. No change was observed in ^{125}I-α-conotoxin MII binding sites or ^{125}I-RTI-121 binding to the dopamine transporter.

Competition studies of ^{125}I-epibatidine binding by α-conotoxin MII were then done to determine whether L-dopa treatment had selective effects on different nAChR populations after 8 wk of treatment. Analyses of the inhibition curves demonstrated a biphasic α-conotoxin MII inhibition of striatal ^{125}I-epibatidine binding in control but not L-dopa treated animals. The control data best fit to a two-site competition model with IC_{50} values of 1.78 nM (CI 0.7 to 4.0 nM) and 1.14 μM (CI 0.10 to 9.0 μM), while the data from the treated animals fit best to a one-site competition model with an IC_{50} value of 8.37 nM (CI 2.4 to 28 nM). Thus 8 wk of L-dopa

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treatment led to a selective decrease in low affinity, but not high affinity α-conotoxin MII-sensitive sites consistent with the lack of change in ^{125}I-α-conotoxin MII (Fig 5B).

As an approach to understand the subunit composition of the striatal nAChR sites affected by L-dopa treatment, immunoprecipitation studies were done (Fig. 5C). No significant declines were observed in nAChR subunit-immunoreactivity with L-dopa treatment as compared to controls.

Discussion

Using a combined molecular and pharmacological approach, we investigated nAChR subunit composition in striatum using control and MPTP-lesioned monkeys. The results show that several major populations are present in striatum including α7, α4β2*, α6β2*, α3β2* and α2β2* nAChRs. Detailed analyses of the present data, combined with previous receptor binding and recent functional studies suggest the following: (1) α6β2* nAChRs contain β3 and also, in part, α4 to form α6β2β3 and α4α6β2β3 subtypes; (2) the presence of striatal α4β2 and α2β2* nAChRs; and (3) the existence of a novel α3β2* nAChR population. A detailed rationale for the existence of these subtypes and their localization (Fig. 6) in monkey striatum is discussed below.

Receptor subtypes present in monkey striatum. Our postulated composition of striatal nAChR subtypes is based on the current hypothesis that heteromeric nAChRs have at least two subunits bearing the principal amino acid loops for acetylcholine binding interfaces (α2, α3, α4 or α6 subunits) and two subunits bearing the complementary amino acid loops (β2 or β4 subunits), whereas the fifth subunit can be either a complementary or a purely structural subunit (α5 or β3 subunits).

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(a) Receptor subtypes present on striatal dopaminergic terminals - α6α4β2β3, α6β2β3, α3β2.* Our previous data had shown that nigrostriatal damage leads to a selective decline in striatal nAChRs that bind ^{125}I-α-conotoxin MII, a ligand that interacts at an α3β2* and/or α6β2* interface, with no change in other receptor subtypes (Kulak et al., 2002a; McIntosh et al., 1999; Nicke et al., 2004; Quik et al., 2001). These findings suggested that receptors expressing α6β2 and/or α3β2 subunits are localized to dopaminergic terminals in monkey striatum. The present results show that ^{3}H-cytisine, a ligand that interacts at an α4β2 receptor interface (Flores et al., 1992), binds to monkey striatum and, in addition, that ^{3}H-cytisine binding is reduced with moderate nigrostriatal damage. Previous data using ^{125}I-epibatidine had shown that a moderate lesion decreased only α-conotoxin MII-sensitive nAChRs (Kulak et al., 2002a; Quik et al., 2001). These combined data can most readily be explained by postulating the existence of a receptor subtype with both an α6β2 and also an α4β2 interface, that is, an α6β2α4β2* subtype.

The current antibody experiments support and extend the results from the receptor studies. The dual immunoprecipitation shows that all striatal α6-subunit-immunoreactivity is precipitated by the anti-β2 antibody, suggesting an absolute requirement for an α6β2 interface, in agreement with the ^{125}I-α-conotoxin MII binding data. In addition, the lesion studies show that the α6 and β3 subunit are decreased in parallel after nigrostriatal damage, suggesting they are co-expressed, thus forming an α6β2β3* receptor. The anti-α4, but not the anti-α2 and anti-α3 antibodies, also immunoprecipitated α6* nAChRs, while the α5 and β4 subunits were not detectable in striatum. Altogether, these observations reduce the potential subunit combinations to α6β2β3 and

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α6α4β2β3. These subtypes may both be present in striatum, since the anti-α4 antibody only precipitated a portion of the α6* sites.

The immunoprecipitation data also show that α3 subunit-immunoreactivity is present in striatal extracts. Furthermore, our studies using a purified β2* receptor preparation clearly show that the α3 and β2 subunit co-precipitate. These results provide direct evidence that α-conotoxin MII binds at an α3β2 interface in monkey striatum, as previously suggested (Kulak et al., 2002b; McIntosh et al., 1999; Nicke et al., 2004). Lesion studies show that all α-conotoxin MII-sensitive receptors are lost with nigrostriatal damage, suggesting they are present on striatal dopaminergic terminals. These combined data suggest that α3β2* nicotinic receptors are located on nigrostriatal terminals in monkey brain, together with the α6α4β2β3 and α6β2β3 subtypes.

(b) Receptor subtypes present on dopaminergic and non-dopaminergic striatal neurons - α4β2 and α2β2.* As discussed earlier, results show that 30% of the ^{3}H-cytisine sites (containing an α4β2 interface) are decreased with moderate nigrostriatal damage suggesting they form an α6β2α4β2β3 subtype. The remaining ^{3}H-cytisine binding sites would represent non-α6 α4β2* nAChRs, which may be both pre- and post-synaptic. The presence of this latter population is also confirmed from the results of the dual label β2 immunoprecipitation experiments using the non-α6* receptor preparation. Evidence for a presynaptic localization for a portion of the α4β2* receptors stems from the results of our functional studies showing that ~30% of nicotine-evoked ^{3}H-dopamine release from striatal synaptosomes is resistant to inhibition by α-conotoxin MII (McCallum et al., 2004).

The immunoprecipitation data are consistent with these findings and allow us to speculate as to the remaining composition of the α4β2* sites. They do not appear to contain α3 or α6 since

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they are α-conotoxin MII-resistant. They are also most likely not expressed with the β3 subunit because the lesion studies indicate that β3 is co-expressed with α6. The absence of the α5 and β4 subunits in monkey striatum rules out their presence in the α4β2* pentamer. Thus, the only remaining subunit that can form a receptor with α4β2* receptors is α2, yielding α4β2 and α4α2β2 nAChRs. This finding is supported by our studies using total striatal extracts and a non-α6 containing β2 purified receptor preparation, which showed that a large proportion of β2* receptors contain the α4 subunit, and a minority the α2 subunit.

The α4 and α2 subunits may be present within the same or on distinct nAChR subtypes, allowing for the presence of α4β2 and α2β2* nAChRs. Because α2 is not affected by nigrostriatal damage, the α2β2* receptors are most likely on non-dopaminergic neurons, as in the rodent (Zoli et al., 2002). In summary, dopaminergic terminals exclusively express α4β2 receptors, whereas α4β2 and α2β2* receptors may be expressed on non-dopaminergic neurons.

(c) Receptors present exclusively on non-dopaminergic striatal elements. The ^{125}I-α-bungarotoxin binding studies show that striatal α7 receptor expression is relatively low and unaffected by nigrostriatal damage. These data suggest that these sites are localized on striatal GABAergic and cholinergic neurons, glutamatergic inputs and/or nonneuronal cells (Kaiser and Wonnacott, 2000; Rogers et al., 2001)**.** With respect to number of binding sites per receptor, homomeric α7 nAChR are likely to have 5 acetylcholine sites, while heteromeric receptors with several different α subunits contain at least two binding sites and possibly more depending on the nature of the other α subunits.

L-dopa treatment differentially affects striatal nicotinic receptors. Previous studies had shown that a 2 wk treatment with L-dopa, a commonly used therapy for Parkinson's disease,

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resulted in a ~20% decline in striatal α-conotoxin MII-sensitive ^{125}I-epibatidine sites with no change in ^{125}I-α-conotoxin MII binding (Quik et al., 2003b). Since patients are treated with L-dopa for extended time periods, we next investigated the effect of an 8 wk treatment course. The results show that the decline in striatal α-conotoxin MII-sensitive ^{125}I-epibatidine sites persists with continued L-dopa treatment. The finding that there is no change in binding of ^{125}I-α-conotoxin MII (0.5 nM) to high affinity sites, suggests a preferential loss in low-, but not high-affinity α-conotoxin MII-sensitive receptors. This is supported by the competition data, which best fit to a one-site model after L-dopa treatment, but a two-site model in the control condition.

These data are in apparent contradiction with the immunoprecipitation results, which show no significant difference in nAChR subunit-immunoreactivity in animals treated with L-dopa as compared to control. These results may suggest that L-dopa treatment induces a change/redistribution in composition of α3* and/or α6* nAChR subtypes (as detected in the radioligand binding assays) without affecting the total amount of these two subunit (as measured by the immunoprecipitation assay). Alternatively, or as well, the varying results between the two assays may reflect a greater sensitivity of the autoradiographic binding technique as compared to immunoprecipitation.

Receptor subtypes present in monkey cortex. The major nAChR receptor populations in cortex appear to contain α4β2 subunits, in agreement with previous studies in rodents (Champtiaux et al., 2003; Flores et al., 1992; Zoli et al., 2002). In contrast, α2* nAChRs were also identified in monkey cortex, an observation consistent with the identification of α2 mRNA in monkey brain (Han et al., 2003). Both α4β2 and α4β2α2 nAChRs appear to be present with this latter subtype representing ~16% of the α4β2* cortical receptor population. We also identified α3* receptors in monkey cortex (8%), an observation consistent with recent findings

demonstrating the presence of α3β2* and/or α6β2* nAChRs in human cortex (Amtage et al., 2004; Quik et al., 2004). Neither MPTP-lesioning nor L-DOPA treatments affected cortical nAChRs, as previously shown (Kulak et al., 2002a; Quik et al., 2003b).

Summary. The present results show that several major nAChR populations are present in monkey brain. In cortex, we identified α7 and α4β2 subtypes, and also novel nAChR populations expressing α4β2α2 and α3β2* subunits. In striatum, α7, α4β2, α6β2* (α6β2β3 and α4α6β2β3), and α2β2* subtypes were identified in agreement with rodent studies, as well as the α3β2* subtype that is distinct from rodent brain.

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Footnotes

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[1]Present address: Dept. Pathology, Anatomy, and Cell Biology, Thomas Jefferson University, Philadelphia, PA

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Legends for Figure

Fig. 1. Immunoprecipitation analyses of the subunit composition of ^{3}H-epibatidine receptors expressed in monkey striatum (A) and cortex (B). Triton X-100 (2%) membrane extracts from control monkey striatum or cortex were labeled with 2 nM ^{3}H-epibatidine. Immunoprecipitation was done as described using saturating concentrations (20-30 μg) of anti-subunit antibodies. The amount immunoprecipitated by each antibody was subtracted from the value obtained in control samples containing an identical concentration of normal rabbit IgG. Note the presence of α2, α3, α4, α6, β2 and β3 nAChR subunit-immunoreactivity in monkey striatum, and α2, α3, α4, and β2 nAChR subunit-immunoreactivity in cortex. The remaining subunits were below the detection limit of the assay (< 6%). In (A) and (B) values represent the mean ± S.E.M. of 6 (striatum) and 3 (cortex) separate immunoprecipitation experiments. In each immunoprecipitation experiment, each antibody was tested in duplicate. (C) Dual immunoprecipitation analyses of the subunit composition of striatal α6* nAChRs. Control striatal extracts were loaded onto an anti-α6 affinity column to bind the α6* nAChR population. The receptors were eluted from the resin using α6 peptide, labelled with 2 nM ^{3}H-epibatidine and then immunoprecipitated with the indicated subunit-specific antibodies. Note that all α6* nAChRs have β2 subunits, and that the α6 and α3 subunits do not co-assemble. (D) Dual immunoprecipitation of α4* nAChRs in monkey cortex. Experiments were performed using an anti-α4 affinity column, followed by elution with α4 peptide. Results are expressed as fmol ^{3}H-epibatidine binding/mg protein. Each data point in (C) and (D) is the mean ± SEM of two experiments performed in triplicate.

Fig. 2. Computer-generated autoradiograms of nAChR binding in striatum of control and MPTP-treated monkeys. Note the decline in binding of ^{3}H-cytisine to α4* nAChRs (A) in

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monkey striatum with nigrostriatal damage, but not in ^{125}I-α-bungarotoxin (α-BGT) binding (B) to α7 receptors.

Fig. 3. Competition analyses of ^{3}H-cytisine binding to striatum from control and MPTP-treated animals. (A) ^{3}H-Cytisine binding in the presence of increasing concentration of α-conotoxin MII. The lack of inhibition by α-conotoxin MII suggests that ^{3}H-cytisine binds exclusively to a receptor with an α4β2 interface in monkey striatum. (B) ^{3}H-cytisine binding in the presence of increasing concentrations of nicotine. Each value represents the mean ± S.E.M. from 3 monkeys.

Fig. 4. Nicotinic receptor subunit composition in monkey striatum based on receptor binding and immunoprecipitation data from control and MPTP-lesioned animals. Left column; quantitative analyses of binding using radioligands that label different nAChR subtypes. (A) ^{125}I-α-bungarotoxin (BGT) labeling of α7 nAChRs was similar in striatum from control and lesioned animals. (B) ^{3}H-cytisine binding was partially reduced with MPTP treatment, indicating that subtypes containing at least one α4β2 interface are decreased with nigrostriatal damage. (C) ^{125}I-α-conotoxin MII (CtxMII), a radioligand that binds to nAChRs with an α3β2 and/or α6β2 interface, is completely abolished with nigrostriatal damage (Quik et al., 2001) suggesting that these receptors are primarily localized to nigrostriatal dopaminergic terminals and (D) ^{125}I-epibatidine binds multiple (α2* through α6*) nAChR subtypes and is partially reduced with lesioning (Kulak et al., 2002a). These data, coupled with the immunoprecipitation results (see Fig. 1) showing the presence of the α2, α3, α4, α6, β2 and β3 subunits in control and lesioned striatum, indicate that multiple subtypes are present in monkey striatum, including α7, α4β2, α6α4β2β3, α6β2β3, α2β2*, α3β2*, and possibly others. These are similar to the

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subtypes identified in rodent brain with some differences including (i) the presence of nAChRs expressing α3, but not α5 and β4 subunits in monkey striatum and cortex and (ii) a larger proportion α6* and/ or α3* nAChRs in monkey (50%) compared to rodent (15%) striatum. For (A) and (B), each value represents the mean ± S.E.M of 3 to 7 animals. Significance of difference from control, *** $p< 0.001$. α-CtxMII, α-conotoxin MII.

Fig. 5. Effect of L-dopa treatment on striatal nAChRs. ^{125}I-Epibatidine and ^{125}I-A-85380 binding were significantly decreased in monkey caudate following 8 wk of L-dopa treatment, with no decline in the ^{125}I-α-conotoxin MII and ^{125}I-RTI-121 sites. Values represent the mean ± S.E.M. of 3 to 12 animals. (B) α-Conotoxin MII competition of ^{125}I-epibatidine binding in control and L-dopa treated monkeys. Competition analyses demonstrate the presence of both a high and low affinity α-conotoxin MII sensitive in control striatum (fit best to a two-site model), but only a high affinity component after L-dopa treatment (fit best to a one-site model) consistent with the lack of effect of L-dopa on ^{125}I-α-conotoxin MII binding sites. Values represent the mean ± S.E.M. of 3 to 4 animals. (C) Immunoprecipitation analyses suggest that nAChR subunit-immunoreactivity is similar before and after L-dopa treatment. The results are expressed as % control, and are the mean ± S.E.M. value of 3 experiments done in duplicate. Significance of difference from control, * $p< 0.05$, ** $p< 0.01$.

Fig. 6. Schematic localization of nAChR subtypes on dopaminergic and non-dopaminergic neurons in the primate nigrostriatal pathway. ACh, acetylcholine; DA, dopamine; Glu, glutamate; SN, substantia nigra.

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TABLE 1

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

Capital letters indicate the amino acids present in the subunit sequence, whereas the lowercase letters indicate the extra-sequence amino acids introduced to enable specific coupling to carrier protein.

Subunit	Peptide Sequence	Localization	Species
α2	CHPLRLKLSPSYHWLESNVDAEEREV	CYT	HUMAN
α3	TRPTSNEGNAQKPRPLYGAELSNLNC	CYT	HUMAN
α4	SPSDQLPPQQPLEAEKASPHPSPGP	CYT	HUMAN
α5	DRYFTQKEETESGSGPKSSRNTLEA	CYT	HUMAN
α6	PRGLARRPAKGKLASHGEPRHLKEC	CYT	HUMAN
β2	RQREREGAGALFFREAPGADSCT	CYT	HUMAN
β3	cDRYSFPEKEESQPVVKGKVLKK	CYT	MONKEY
β4	GPDSSPARAFPPSKSCVTKPEATATSPP	CYT	HUMAN

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TABLE 2

Selective declines in nAChR subunit-immunoreactivity in monkey striatum after MPTP treatment

NAChR subunit immunoprecipitation was done as described in the Fig. 1 legend using striatal and cortical extracts prepared from control and MPTP-lesioned monkey brain. Note the similar declines in α6 and β3-subunit-immunoreactivity after MPTP treatment suggesting these 2 subunits are decreased in parallel with the dopamine transporter. Declines were also observed in α3, α4, and β2 subunit-immunoreactivity. Binding in extracts from control and MPTP-lesioned striatum was 69.6 ± 5.55 and 35.2 ±1.0 fmol/mg, respectively, and in cortex 49.9 ± 2.6 and 47.3 0 ± 0.8 fmol/mg, respectively. Values represent the mean ± S.E.M of 6 (striatum control and MPTP-treated) and 3 (cortex control and MPTP-treated) immunoprecipitation experiments. In each immunoprecipitation experiment, each antibody was tested in duplicate. Significance of difference from control, * $p<0.05$; ** $p< 0.01$; *** $p< 0.001$. Not detected (--).

NAChR Subunit	^{3}H-Epibatidine binding (fmol/mg protein)					
	Striatum			Cortex		
	Control	MPTP	% Control	Control	MPTP	% Control
α2	6.62 ± 0.52	6.12 ± 0.54	92	11.83 ± 1.16	11.55 ± 1.45	98
α3	7.55 ± 1.18	3.80 ± 0.62*	50	5.50 ± 1.04	4.01 ± 0.3	73
α4	37.00 ± 4.03	25.01 ± 2.26*	68	39.11 ± 4.04	36.03 ± 0.9	92
α5	--	--		--	--	
α6	19.06 ± 2.90	3.37 ± 0.49***	18	4.12 ± 0.93	3.74 ± 0.64	91
β2	58.38 ± 5.46	30.30 ± 2.14**	52	48.39 ± 4.67	44.50 ± 2.28	92
β3	15.67 ± 2.00	2.15 ± 0.44***	14	2.03 ± 0.56	2.27 ± 0.36	112
β4	--	--		--	--	

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Fig. 1.

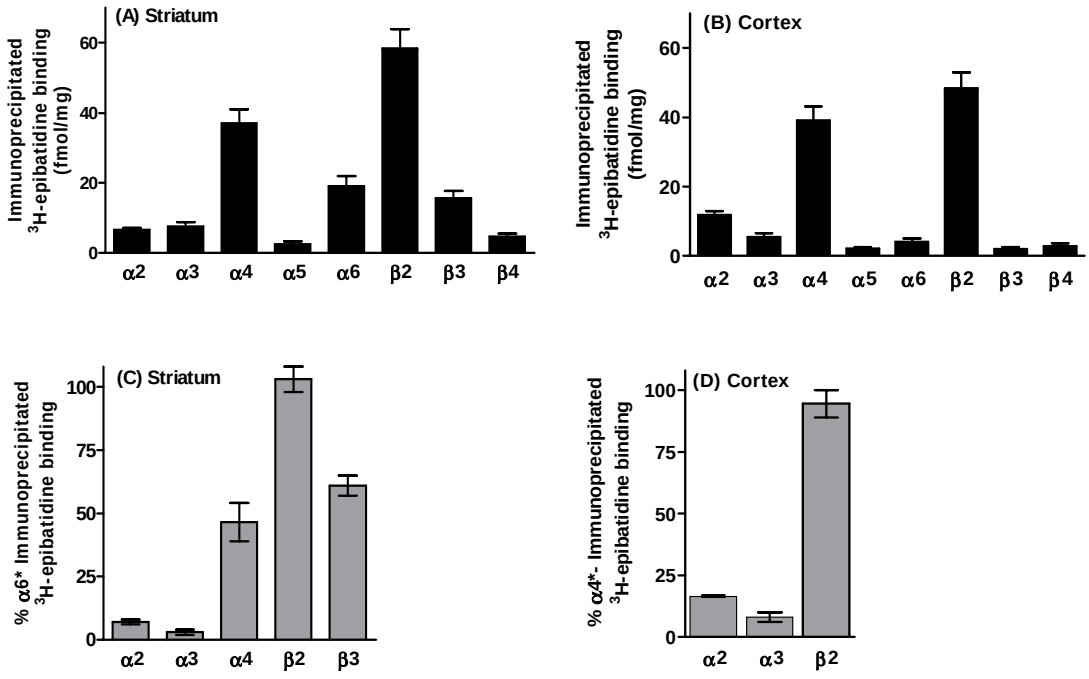

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Fig. 2.

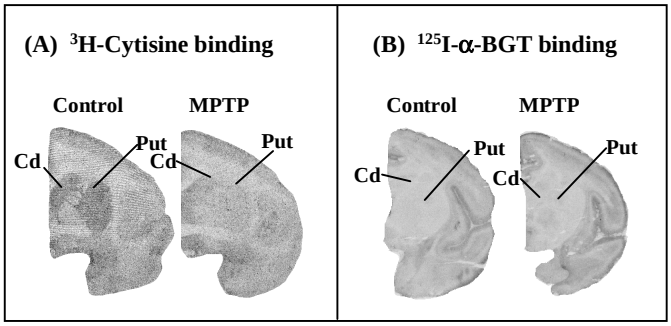

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Fig. 3.

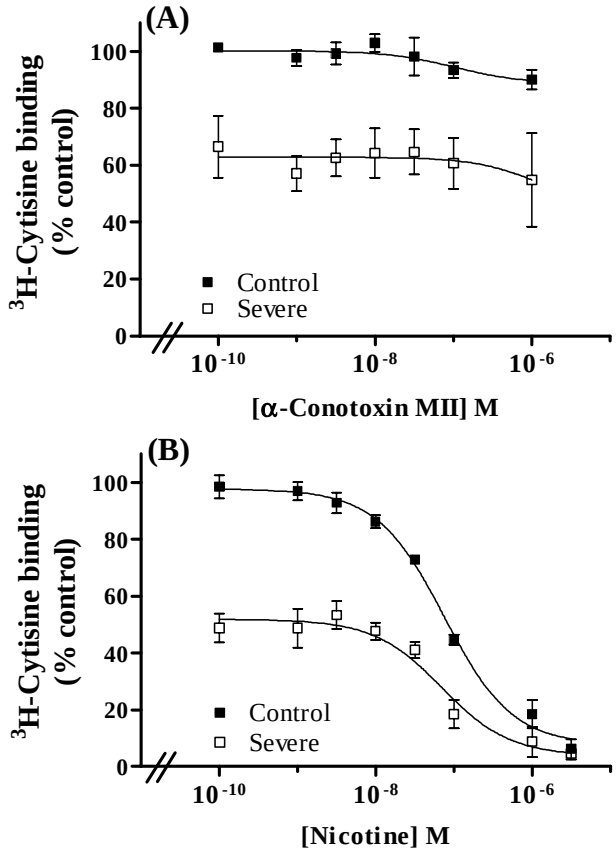

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Fig. 4.

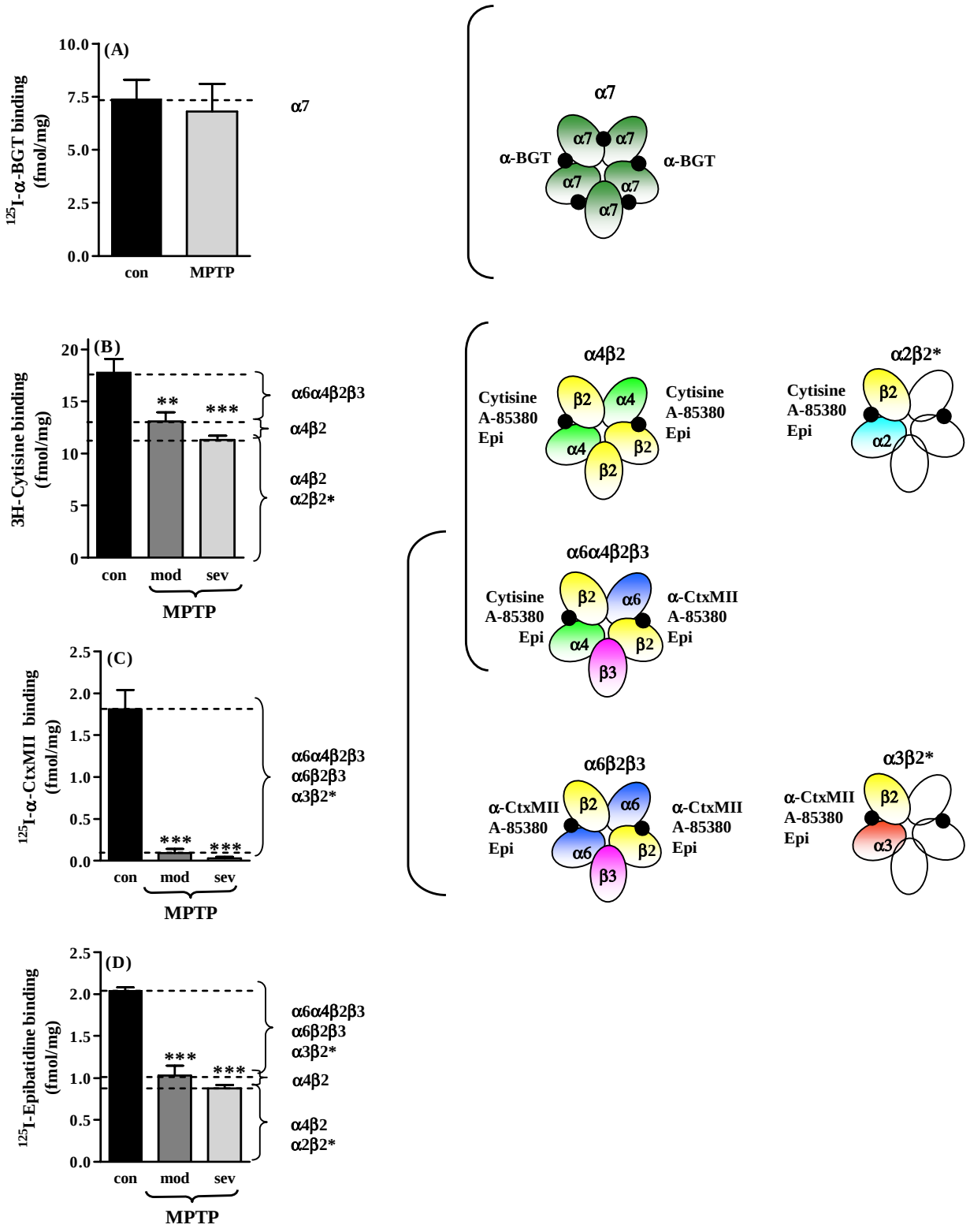

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Fig. 5.

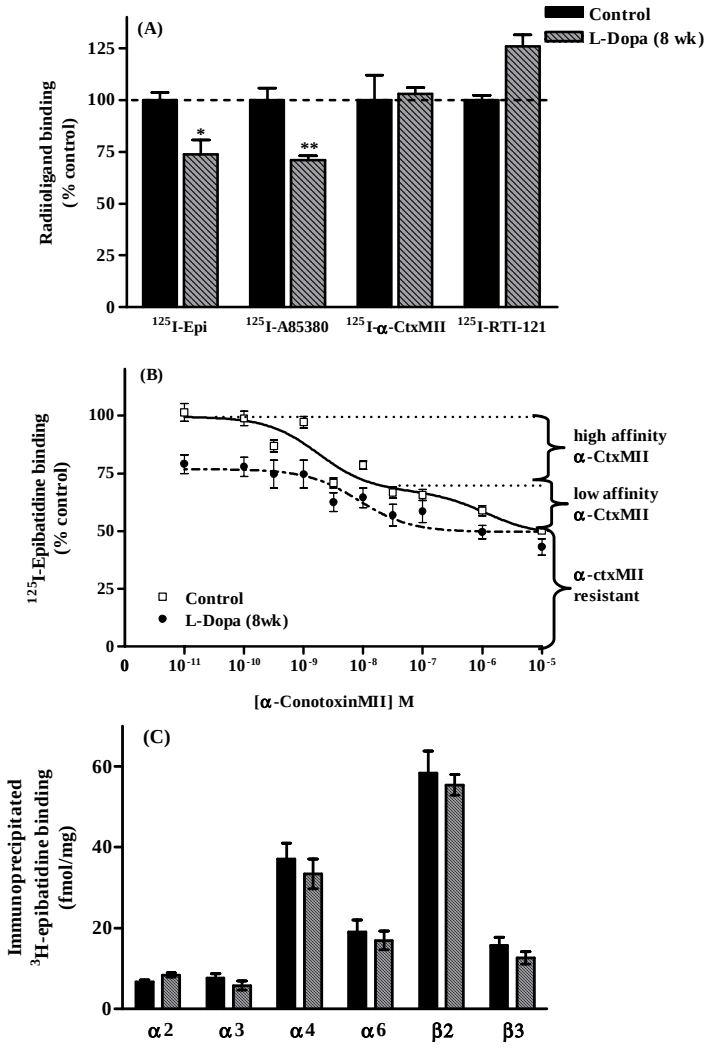

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Fig. 6.