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Heterologous Expression of Human $\alpha6\beta4\beta3\alpha5$ Nicotinic Acetylcholine Receptors: Binding Properties Consistent with Their Natural Expression Require Quaternary Subunit Assembly Including the $\alpha5$ Subunit

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ABSTRACT

Heterologous expression and lesioning studies were conducted to identify possible subunit assembly partners in nicotinic acetylcholine receptors (nAChR) containing $\alpha6$ subunits ($\alpha6$* nAChR). SH-EP1 human epithelial cells were transfected with the requisite subunits to achieve stable expression of human $\alpha6\beta2$, $\alpha6\beta4$, $\alpha6\beta2\beta3$, $\alpha6\beta4\beta3$, or $\alpha6\beta4\beta3\alpha5$ nAChR. Cells expressing subunits needed to form $\alpha6\beta4\beta3\alpha5$ nAChR exhibited saturable [^{3}H]epibatidine binding (K_d = 95.9 ± 8.3 pM and B_{max} = 84.5 ± 1.6 fmol/mg of protein). The rank order of binding competition potency (K_i) for prototypical nicotinic compounds was α-conotoxin MII (6 nM) > nicotine (156 nM) ~ methyllycaconitine (200 nM) > α-bungarotoxin (>10 μM), similar to that for nAChR in dopamine neurons displaying a distinctive pharmacology. 6-Hydroxydopamine lesioning studies indicated that $\beta3$ and $\alpha5$ subunits are likely partners of the $\alpha6$ subunits in nAChR expressed in dopaminergic cell bodies. Similar to findings in rodents, quantitative real-time reverse transcription-polymerase chain reactions of human brain indicated that $\alpha6$ subunit mRNA expression was 13-fold higher in the substantia nigra than in the cortex or the rest of the brain. Thus, heterologous expression studies suggest that the human $\alpha5$ subunit makes a critical contribution to $\alpha6\beta4\beta3\alpha5$ nAChR assembly into a ligand-binding form with native $\alpha6$*-nAChR-like pharmacology and of potential physiological and pathophysiological relevance.

Distinct nicotinic acetylcholine receptor (nAChR) subtypes expressed in mesostriatal dopaminergic neurons are involved in modulation of striatal dopamine (DA) release (Wonnacott, 1997; Champtiaux et al., 2002; Luetje, 2004); nAChR containing $\alpha4$ and $\beta2$ subunits ($\alpha4\beta2$* nAChR) or containing $\alpha6$ subunits ($\alpha6$* nAChR) participate directly, and $\alpha7$* nAChR participate indirectly. Whereas the biochemistry of $\alpha4\beta2$* and $\alpha7$* nAChR is well characterized, the subunit composition and functional properties of $\alpha6$* nAChR remain unclear. Suggested roles for $\alpha6$* nAChR in modulation of DA transmission imply their potential importance in locomotion, reward, schizophrenia, and Parkinson's disease (le Novère et al., 1999; for review, see Dani, 2001; Bencherif and Schmitt, 2002; Quik and Kulak, 2002).

Natural expression of the nAChR $\alpha6$ subunit is restricted

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ABBREVIATIONS: nAChR, nicotinic acetylcholine receptor(s); DA, dopamine; αCtxMII, α-conotoxin MII; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; EPI, epibatidine; NIC, nicotine; MLA, methyllycaconitine; PBS, phosphate-buffered saline; A-85380, 3-[2(*S*)-azetidinylmethoxy]pyridine; ABT-418, (*S*)-3-methyl-5-(1-methyl-2-pyrrolidinyl)isoxazole; αBtx, α-bungarotoxin; CYT, cytisine; CAR, carbachol; DHβE, dihydro-β-erythoidine; LOB, lobeline; MEC, mecamylamine; MCC, methylcarbamylcholine; SIB-1508Y, altinicline; GTS-21, 3-[(2,4-dimethoxy)benzylidene]-anabaseine; RT-PCR, reverse transcription polymerase chain reaction; TC-2429, 2-(-3-pyridinyl)-azabicyclo[2,2,2]octane; DMEM, Dulbecco's modified Eagle's medium; 6-OHDA, 6-hydroxydopamine; ANOVA, analysis of variance.

in rat brain but abundant in dopaminergic nuclei of the midbrain (Le Novère et al., 1996; Klink et al., 2001; Azam et al., 2002). In DA neurons, $\alpha6$ is the predominant form of nAChR subunit mRNA that extensively colocalizes with nAChR $\beta3$ subunit mRNA (Le Novère et al., 1996). Immunoprecipitation studies in rats and mutant mice, however, have suggested that $\alpha4\beta2$*, $\alpha6\beta2$*, and $\alpha6\alpha4\beta2$* are the primary nAChR subtypes in rodent DA terminals, with a possible contribution of $\alpha6(\alpha4)\beta2\beta3$, $\alpha6\beta3\beta2$, and $\alpha4\alpha5\beta2$ subunit combinations (Zoli et al., 2002; Champtiaux et al., 2003; Salminen et al., 2004). The subunit composition of $\alpha6$* nAChR expressed by human or monkey DA neurons remains unknown, although there is greater relative expression of $\beta4$ subunit in primates (Quik et al., 2000, 2004). To date, $\alpha6$* nAChR have been identified primarily by sensitivity to α-conotoxin MII (αCtxMII), an $\alpha6$-selective antagonist (Champtiaux et al., 2002). In humans with Parkinson's disease and in monkeys treated with the DA neurotoxin MPTP, αCTxMII sites are lost, confirming their location at DA terminals (Kulak et al., 2002; Quik et al., 2004), and consistent with identity of toxin-binding sites as $\alpha6$* nAChR.

Heterologous expression studies suggest that nAChR $\alpha6$ subunits may coassemble with $\beta2$ or $\beta4$ subunits. Chick or rat nAChR $\alpha6$ subunits form functional channels when coexpressed with human $\beta4$ subunit in *Xenopus* oocytes, but the $\alpha6\beta2$ combination failed to form functional channels (Gerzanich et al., 1997). Chick $\alpha6$ subunits also formed functional receptors when coexpressed with chick $\beta2$ or $\beta4$ subunits in BOSC 23 cells (Fucile et al., 1998). More recently, Kuryatov et al. (2000) reported functional expression of human $\alpha6\beta4$ nAChR in *Xenopus* oocytes, although suggesting $\alpha6\beta2$ nAChR form ligand-binding (nonfunctional) aggregates and that $\beta3$ subunits may facilitate $\alpha6$* nAChR trafficking to the cell membrane. Coexpression of a human $\alpha6/\alpha4$ chimeric subunit with human $\beta4$ subunit in *Xenopus* oocytes and HEK-293 human embryonic kidney cells results in functional receptors with a pharmacological profile representative of wild-type $\alpha6$* nAChR, although expression of wild-type $\alpha6$ subunits with $\beta4$ subunits did not produce ligand-binding and functional $\alpha6$* nAChR (Evans et al., 2003).

The objectives of this study were to heterologously express $\alpha6$* nAChR of different but defined subunit composition to identify possible $\alpha6$ subunit assembly partners and to permit pharmacological comparison to subunit expression in lesioned animals and in human brain. The results provide new insights into structure-activity relationships for $\alpha6$* nAChR that might benefit development of novel specific therapies for central nervous system disorders.

Materials and Methods

Animals and Materials. Three-month-old male Sprague-Dawley rats (Iffa Credo, L'Arbresele, France) were housed 5 per cage (12-h light/dark cycle) with free access to food and water. The animal experimentation has been carried out in accordance with the Declaration of Helsinki and approved by Aventis' local Institutional Animal Care and Use Committee as adopted and promulgated by the U.S. National Institute of Health. [^{3}H]EPI (56.2 Ci/mmol) and [^{3}H]*S*-(−)-NIC ([^{3}H]NIC, 81.5 Ci/mmol) were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). MLA, [^{3}H]MLA, (25.4 Ci/mmol), and αCtxMII were purchased from Tocris Cookson Inc, (Ellisville, MO). Dulbecco's phosphate-buffered saline (PBS) was purchased from Invitrogen Corporation, (Carlsbad, CA). A-85380, ABT-418, αBtx, cytisine (CYT), carbachol (CAR), dihydro-β-erythoidine (DHβE), EPI, leupeptin trifluoroacetate, lobeline (LOB), mecamylamine (MEC), methylcarbamylcholine (MCC), *S*-(−)-NIC, and pepstatin A were purchased from Sigma-Aldrich (St. Louis, MO). Remaining materials were purchased from Sigma-Aldrich or Fisher Scientific Co. (Pittsburgh, PA) unless specified otherwise in the text. (−)-Altinicline (SIB-1508Y) and 3-(2,4)-dimethoxybenzylidine anabaseine (GTS-21) were synthesized at Aventis Pharma SA. 2-(3-Pyridinyl-1-azabicyclo[2,2,2]octane (TC-2429) was synthesized at Targacept, Inc.

cDNA Preparation. Human nAChR subunit cDNA was obtained from RT-PCR of a total brain mRNA library following classical methods. Briefly, the $\alpha6$ subunit cDNA was amplified using hA6N (GACTCTCGAGAGTGGGCTTCTGATGATGT) and hA6C (CTAGCTCGAGGGTTTTAGCAGATGGGGGACTTG) primers containing XhoI restriction enzyme sites, and the desired 1.771-kB band was extracted using a Prep-a-Gene kit (Bio-Rad, Hercules, CA) from samples subjected to electrophoresis on a 1% agarose gel. The cDNA insert was excised with XhoI, blunt-ended with Klenow, and ligated into blunt-ended pcDNA3.1-hygro previously linearized at the multiple cloning site with EcoRV and dephosphorylated to prevent self-ligation. The resulting cDNA was purified using Quantum Prep Freeze 'N Squeeze Gel-Extraction Spin Column (Bio-Rad) and transformed into *Escherichia coli* via heat shock. The $\beta3$ subunit cDNA was amplified with hB3N (CTAGAAGCTTAACCCCCTTTTCCAGTG) and hB3C (GTACGAATTCCGCATTCGGGGTTCGTA) primers containing HindIII and EcoRI restriction sites, respectively. The desired 1.6-kB band was gel-extracted and cloned into pcDNA-neo. The other human nAChR subunits used in this study were subcloned into the indicated expression vectors in a similar fashion using classical methods. All constructs were verified by restriction mapping and complete sequencing of the insert (Genetic Analysis and Technology Core, Tucson, AZ). Subunit cDNA were amplified according to the DNA plasmid Maxiprep protocol (Marligen Biosciences, Inc., Ijamsville, MD). All cDNA-containing inserts were used for transfection either as circular DNA or as linearized plasmids cut with the indicated restriction endonucleases: $\alpha6$, in pcDNA3.1-hygro, cut with FspI; $\beta4$, in pcDNA3.1-zeo, cut with PvuI; $\beta3$, in pcDNA3.1-neo, cut with PvuI; and $\alpha5$, in pEF6 (conferring blasticydin resistance), cut with FspI.

Preparation of Cell Lines. Cells of the SH-EP1 human epithelial line (kindly provided by Dr. June Biedler, Sloan Kettering Institute, New York) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% horse serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (all obtained from Invitrogen, Carlsbad, CA) plus 5% fetal bovine serum (HyClone, Logan, UT) in a humidified atmosphere containing 5% CO_2 in air at 37°C (Lukas, 1986; Lukas et al., 1993).

For stable transfection of cells, either SuperFect (QIAGEN, Valencia, CA) or an electroporation method was used. In brief, for the SuperFect method, 10 μg of DNA dissolved in TE (10 mM Tris-HCl containing 1 mM EDTA) buffer, pH 7.4 (minimum DNA concentration of 0.1 μg/μl), was diluted in supplement-free DMEM to a total volume of 300 μl, then mixed with 60 μl of SuperFect Transfection Reagent. The mixed sample was incubated for 10 min at 22°C, and the growth medium was aspirated. Then, 3 ml of complete, serum-supplemented DMEM was added, each sample was immediately transferred to a 100-mm dish containing cells at 40 to 80% confluence (prewashed with PBS), and cells were incubated for 2 to 3 h under their normal growth conditions. Transfection medium was gently aspirated, and cells were washed 3 to 4 times with PBS. Then, fresh DMEM was added, and cells were incubated for 24 h under their normal growth conditions. The medium was further supplemented with the selection antibiotic. For electroporation, the Bio-Rad Gene Pulsar (model 1652076 with pulse control module model 1652098; 960 μF; 0.20 kV/cm; t = 28–36 ms) was used. Cells (~2 million, exposed to 0.25% trypsin) were harvested and suspended in fresh medium and then centrifuged at 7000 rpm for 4 min. The pellet was resuspended in 800 μl of buffer containing 20 mM HEPES, 87 mM NaCl, 5 mM KCl, 0.7 mM $NaHPO_4$, and 6 mM dextrose, pH 7.05. After the addition of 100 μg of DNA (in TE buffer), the sample was

triturated and transferred into an electroporation cuvette. After electroporation, the sample was allowed to settle for 10 to 15 min, 10 ml of complete DMEM was added, and the sample was mixed and transferred to a 100-mm plate. The transfected cells were incubated for 24 h at 37°C and supplemented with the selection antibiotic. Regardless of the transfection method, cell growth was monitored until ring cloning or the "stab-and-grab" technique (Lukas et al., 2001) was used to isolate single, transfected cell colonies, which were then expanded. The cell lines SH-EP1-hα6β2, SH-EP1-hα6β4, SH-EP1-hα6β2β3, SH-EP1-hα6β4β3, and SH-EP1-hα6β4β3α5 were created by sequential transfection with pcDNA3.1-hygro-hα6, and pcDNA3.1-zeo-hβ2 or pcDNA3.1-zeo-hβ4, and pcDNA3.1-neo-hβ3, and pEF6-hα5.

Reverse Transcription-PCR (RT-PCR). Total mRNA was isolated from cells growing at approximately 80% confluency in a 100-mm culture dish using 2 ml of Trizol reagent (Invitrogen). Prior to RT-PCR, RNA preparations were treated with DNase I (Ambion, Austin, TX) to remove residual genomic DNA contamination. Typically, 40 μg of mRNA was incubated with 4 units of DNase I in a 50-μl reaction at 37°C for 30 min. DNase I was then inactivated by the addition of 5 μl of 25 mM EDTA and incubation at 65°C for 10 min. RT was carried out using 2 μg of DNA-free total RNA, oligo(dT) 12-18 primer, and a Superscript II preamplification system (Invitrogen) in a 20-μl reaction volume. At the end of the RT reaction, reverse transcriptase was deactivated by incubation at 75°C for 10 min, and RNA was removed by adding 1 unit of RNaseH followed by incubation at 37°C for 30 min. Reactions excluding reverse transcriptase were also conducted as RT negative controls. PCR was performed using 1 μl of cDNA preparation, 1 μl of 10 μM each of 5′ and 3′ gene-specific primers, 1 μl of 10 mM dNTP, and 2.5 units of RedTaq (Sigma-Aldrich) in 50-μl reaction volume. Amplification reactions were carried out in a RoboCycler (Stratagene; La Jolla, CA) for 35 cycles at 95°C for 1 min, 55°C for 90 s, and 72°C for 90 s, followed by an additional 4-min extension at 72°C. One-tenth of each RT-PCR product was then resolved on a 1% agarose gel, and sizes of products were determined based on migration relative to mass markers loaded adjacently.

Membrane Preparations for Binding Studies. Cells were harvested in ice-cold PBS, pH 7.4, then homogenized with a polytron (Brinkmann Instruments, Westbury, NY) at setting 6 for 15 s. Combined homogenates (18 ml) were centrifuged at 40,000*g* for 20 min (4°C). The pellet was resuspended in 12 ml of ice-cold PBS and centrifuged again. The final pellet was resuspended in ~10 ml of PBS and contained 1.2 to 1.6 mg/ml of total membrane protein.

Binding Assays. [^{3}H]EPI was used to probe for α6 nAChR binding sites at final radioligand concentrations of 0.01 to 3.0 nM for saturation assays or of 0.5 nM for competition binding assays. Binding was assayed in assay buffer containing: 0.9 mM $CaCl_2$, 2.67 mM KCl, 1.47 mM KH_2PO_4, 0.49 mM $MgCl_2$, 137.93 mM NaCl, and 4.29 mM Na_2HPO_4, pH 7.4 in either 48-well or 96-well plates. Each sample (performed in triplicate at minimum) contained 50 μl of test compound in solution at the desired concentration, 50 μl of 4× [^{3}H]EPI stock solution, and 100 μl of membrane suspension. Incubation was conducted for 2 h at room temperature. Total and nonspecific bindings were measured in the presence of assay buffer or 100 μM nicotine, respectively. For the 48-well assay, binding was terminated by dilution with cold PBS and immediate filtration onto GF/B filters (presoaked in 0.3% EPI) using a 48-sample, semiauto harvester (Brandel Inc., Gaithersburg, MD). After washing three times with ~1 ml of buffer, filters were transferred into scintillation vials filled with 3 ml of scintillation cocktail. Radioactivity was measured after 8 to 12 h using a liquid scintillation analyzer (model Tri-Carb 2200CA; PerkinElmer Life and Analytical Sciences Inc.). Data expressed in DPM were transformed to fentomoles of bound [^{3}H]EPI per milligram of total protein or as a percentage of control [^{3}H]EPI binding (i.e., total-nonspecific). For the 96-well assay, samples were filtered using a 96-sample, semiauto harvester (Brandel Inc.). After washing 3 times with ~350 μl of buffer, the filter plate was dried for 60 min in an oven at 49°C, bottom-sealed, and each well was filled with 40 μl of scintillation cocktail. After 60 min, the filter plate was top-sealed, and radioactivity was measured using a Wallac 1450 Microbeta liquid scintillation counter. Data expressed in CPM were transformed to percentage of control [^{3}H]EPI binding (i.e., total-nonspecific). Competition assays using [^{3}H]NIC binding to rat cortical membranes or [^{3}H]MLA binding to rat hippocampal membranes were performed at 5 nM radioligand concentration. The procedure was identical to that described for [^{3}H]EPI binding but in these assays nonspecific binding was determined in the presence of 10 μM NIC or 10 μM MLA, respectively.

Competition Binding Assays Using αCtxMII. Test samples (triplicates in 48-well plate) containing 100 μl of αCtxMII solution at the desired concentration, 750 μl of the assay buffer (PBS, containing 5 mM EDTA, 5 mM EGTA, 10 mg/l aprotinin, 10 mg/l leupeptin trifluoroacetate, 10 mg/l pepstatin A, and 0.1% (w/v) bovine serum albumin, pH 7.4) and 100 μl of cell membrane suspension were preincubated for 30 min at room temperature. Total and nonspecific bindings were measured in the presence of PBS or 100 μM nicotine, respectively. Next, 50 μl of [^{3}H]EPI was added to all samples, and samples were incubated for an additional 90 min. The rest of procedure was performed as described above.

Rat and Human Tissue Collection and RNA Preparation. Anesthetized rats received microinjections of 8 μg 6-hydroxydopamine (6-OHDA, 10 rats) or saline (5 rats) in the medial forebrain bundle (coordinates for injection: 8.2 mm below the dura, 2 mm lateral to the midline, and 2.1 mm posterior to the bregma). Eight months later, animals were euthanized by CO_2 inhalation, their brains were quickly removed, and the substantia nigra was dissected out. Pooled tissue of each group was divided to allow 2 PCR measurements. Human substantia nigra samples ($n = 2$) were obtained from the brain bank, Department of Neuropathology, King's College, London. Commercial human RNA for total brain and cortex was obtained from BD Biosciences Clontech (Palo Alto, CA) and Biochain (Hayward, CA), respectively. Pooled tissue samples were homogenized in QIAzol Lysis Reagent using the Mixer Mill MM 300 (QIAGEN GmbH, Hilden, Germany) (1 ml /100 mg of tissue) during 2 × 2 min at 20 Hz. Chloroform (200 μl) was added to the homogenate, and samples were sonicated for 20 s and centrifuged at 12,000*g* for 15 min. The aqueous phase (600 μl) was taken to isolate total RNA using RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions, including a DNase treatment. To assess the quality and concentration of the total RNA, 1 μl was directly analyzed on an RNA LabChip Agilent using the 2100 Bioanalyser (Agilent Technologies, Waldbronn, Germany).

Quantitative, Real-Time RT-PCR. Two micrograms of total RNA from each group were reverse-transcribed with oligo(dT)16 (Proligos France SAS, Paris, France) following the Applied Biosystems RT reaction procedure. The final RT reaction mix included template, 1× cDNA first strand synthesis buffer, 5.5 mM $MgCl_2$, 0.5 mM each dNTP, 0.4 U/μl of RNase inhibitor, 2.5 μM oligo(dT), and 1.25 U/μl of Multiscribe reverse transcriptase in a total volume brought to 100 μl with water. Samples were then incubated for 10 min at 25°C, followed by 30 min at 48°C, and then heated at 95°C to denature the enzymes and stop the reaction. Appropriate gene-specific primer sets were designed using Vector NTI software. FastStart DNA Master SYBR Green I mix (containing FastStart *Taq*DNA polymerase, dNTP, $MgCl_2$, and SYBR Green I dye; Roche Diagnostics, Mannheim, Germany) was mixed with 1 μl of cDNA and primers at 0.4 μM in a final volume of 20 μl and amplified according to the manufacturer's instructions. A negative control without cDNA template was run with every assay to assess overall specificity. The PCR was run on the Light Cycler (Roche Diagnostics) as follows: 1 cycle of 95°C for 8 min, followed by 40 cycles of 95°C for 15 s, 65°C for 10 s, and 72°C for 10 s. This was followed by melt curve analysis beginning at 70°C and increasing by 0.1°C/s to 95°C. The amplified transcripts were quantified with the comparative crossing point method (see Roche applied science technical note n° LC13/2001) using the mean of 3 housekeeping genes (β-actin, GAPDH, and PLA-2) as an

internal control to normalize RNA expression. The extent of nigral lesion in 6-OHDA treated animals was assessed by gene expression of the dopamine transporter, which was decreased by more than 90%.

Data Analysis. The equilibrium dissociation constant K_d (mean ± S.E.M.) and the maximum number of binding sites B_{max} (mean ± S.E.M.) in the saturation experiments and the concentration of drug that inhibits specific binding by 50%, IC_{50}, in the competition experiments were determined by nonlinear regression, fitting to both one- and two-site binding models. An F-test determined whether the one-site or two-site model best fit the data. The inhibition constant (K_i, mean and 95% confidence interval) for each drug was calculated from IC_{50} values using the Cheng-Prusoff equation [$K_i = IC_{50}/(1 + \text{ligand}/K_d)$]. Pseudo Hill slope was determined by fitting data to a sigmoidal dose-response equation: % binding = Bottom + (Top−Bottom)/[1 + $10^{(\log IC50\ -X)\cdot n}$], where X is the logarithm of inhibitor concentration and n is the slope. All data analyses were performed using the GraphPAD-Prism (GraphPAD, San Diego, CA).

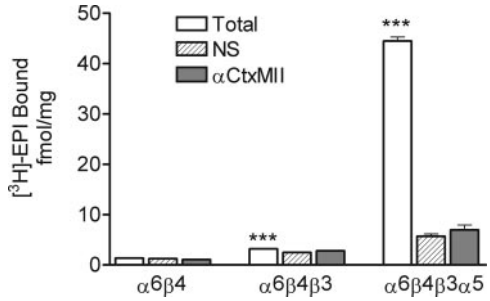

Fig. 1. Density of specific [^{3}H]EPI binding on membranes of SH-EP1-hα6β4, -hα6β4β3, and -α6β4β3α5 cells. Data expressed as fentomoles of bound [^{3}H]EPI per milligram of membrane protein are means ± S.E.M. Each data set was generated from three independent experiments using 0.5 nM [^{3}H]EPI alone (total binding; open bars) or in the presence of 100 μM NIC (NS, nonspecific binding; cross-hatched bars) or 5 μM αCtxMII (shaded bars). Data on SH-EP1-hα6β4β3α5 cells are from cells at passage 37 (level of nAChR expression changes over passages). One-way ANOVA with Dunnett's post hoc test was used for comparison of total binding, NS, and αCtxMII samples. ***, $p < 0.001$.

Results

Expression of Human α6* nAChR by SH-EP1 Human Epithelial Cells. Based on the current understanding of the possible composition(s) of nAChR containing α6 subunits as well as findings from the lesioning studies presented below, we sought to determine whether and how efficiently nAChR could be formed containing: α6 subunits with β2 or β4 subunits as simple, binary complexes; α6 and β2/β4 subunits with β3 subunits thought to be required for formation of αCtxMII-sensitive α6* nAChR; and α6 and both β3 and α5 subunits, shown to be lost in concert after dopaminergic lesions (see below). RT-PCR was used to confirm that the desired human nAChR subunits were stably expressed in SH-EP1 human epithelial cells. Clonal lines were isolated that express the requisite transcripts for generation of nAChR of the following composition: α6β2, α6β4, α6β2β3, α6β4β3, and α6β4β3α5.

Little-to-No Radioligand Binding to Human α6β2-, α6β4-, α6β2β3-, and α6β4β3 nAChR Stably Expressed in SH-EP1 Human Epithelial Cells. The α6β2 and α6β4 subunit combinations stably expressed in SH-EP1 human epithelian cells did not produce any meaningful high affinity binding capacity for [^{3}H]EPI or [^{3}H]MLA. Also, only barely detectable high affinity binding levels were observed for [^{3}H]MLA or [^{3}H]NIC using membranes made from cells transfected with α6β2β3 or α6β4β3 combinations. Although the density of [^{3}H]EPI binding sites on membranes from SH-EP1-hα6β2β3 cells (data not shown) or SH-EP1-hα6β4β3 cells (Fig. 1) reached significance ($p < 0.05$, Student's t test and one-way ANOVA, $F_{4,55} = 5.86$, $p < 0.001$, Dunnett's post hoc, respectively), the levels of expression were very low (0.3–0.8 fmol/mg of protein). Moreover, the binding was not displaceable by 5 μM αCtxMII (a selective antagonist for α6-containing nAChR), at the concentration that exceeded 800-fold its K_i determined for another α6* nAChR (see Table 1).

Pharmacological Characterization of Human α6β4β3α5 nAChR Stably Expressed in SH-EP1 Human Epithelial Cells. Specific, αCtxMII-sensitive [^{3}H]EPI binding was observed with membrane preparations derived from SH-EP1-hα6β4β3α5 cells (Fig. 1). Saturation binding analysis indicated that the binding of [^{3}H]EPI was specific and saturable (Fig. 2). Calculations yielded a K_d value of 95.9 ± 8.3 pM and a B_{max} value of 84.5 ± 1.6 fmol/mg as determined by fitting data to the simplest, one-site model ($R^2 = 0.97$; $F_{2,6} = 0.539$: $p = 0.61$). Competition binding assays using α-CtxMII were conducted to confirm the α6-specificity of the expressed binding sites. αCtxMII exhibited high affinity for this type of nAChR with K_i = 6.15 (4.48–8.45) nM and a pseudo Hill slope value of −1.15 (Fig. 3a and Table 1). Interestingly, TC-2429, a previously described nicotinic agonist that discriminates striatal from thalamic nAChR (Bencherif et al., 1998), exhibited higher affinity than αCtxMII in competition toward [^{3}H]EPI binding, with K_i = 1.75 (1.42–2.14) nM. Compared with A-85380, MLA, and NIC, αCtxMII did not displace completely [^{3}H]EPI (with an estimated 11 ± 2% residual binding refractory to displacement) although it exerted a one-site competition (R^2 = 0.89; $F_{2,109} = 2.06$, $p = 0.13$) similarly to other ligands. SH-EP1-hα6β4β3α5 cell membranes exhibited a unique binding profile in competition assays conducted with 15 chemically different standard nicotinic ligands (Table 1). The rank order of potency for nicotinic ligands in competition with [^{3}H]EPI was TC-2429 (K_i = 1.75 nM) > αCtxMII (K_i = 6.15 nM) ≥ LOB (K_i = 6.39 nM) > A-85380 (K_i = 8.71 nM) > CYT ≈ NIC ≈ MLA (K_i = 156–200 nM) > ABT-418 ≈ MCC ≈ SIB-1508Y ≈ GTS-21 (K_i = 0.97 −1.29 μM) > CAR (K_i = 3.25 μM) > dihydro-β-erythoidine, DHβE ≈ αBtx (K_i > 10 μM). All compounds examined had a slope value near unity (range: −0.80 to −1.15).

The pharmacological profile of α6β3β4α5 nAChR was distinctly different from that of α4β2* nAChR as probed with [^{3}H]NIC on rat cortex membranes, or of α7* nAChR as probed with [^{3}H]MLA on membranes of the rat hippocampus (Fig. 3b and Table 1). Indeed, whereas αCtxMII was more than 500 times more potent at α6β4β3α5 nAChR than at α4β2* nAChR (Table 1 and Fig. 3a), typical α4β2* nAChR agonists such as A-85380, NIC, CYT, ABT-418, MCC, and SIB-1508Y were at least 100 times less potent at α6β4β3α5 nAChR than at α4β2* nAChR. Moreover, the α4β2* nAChR antagonist DHβE did not display any affinity for α6β4β3α5 nAChR. Likewise, αCtxMII was 100 times more potent at α6β4β3α5 nAChR than at α7* nAChR (Table 1 and Fig. 3a), and the α7* nAChR antagonist α-Bgt did not displace [^{3}H]EPI binding to SH-EP1-hα6β4β3α5 cell-derived membranes. MLA, another potent α7* nAChR antagonist, displayed moderate affinity for α6β4β3α5 nAChR (K_i = 200 nM).

α6 nAChR Subunit Gene Expression in Rat and Human Substantia Nigra. Insight into the possible subunit

TABLE 1
Ligand binding affinities [K_i (nM); mean (95% confidence interval); three to four independent experiments] for selected nicotinic agents at human $\alpha6\beta3\beta4\alpha5$ nAChR stably expressed in SH-EP1 cells, at native rat $\alpha4\beta2$* nAChR (cortex) or at $\alpha7$* nAChR (hippocampus)

Compound	$\alpha6\beta3\beta4\alpha5$ [^{3}H]EPI Binding	$\alpha4\beta2$* [^{3}H]NIC Binding	$\alpha7$* [^{3}H]MLA Binding
		nM	
EPI	0.096 ± 0.008[a]	0.07 (0.06–0.08)	15.4 (10.9–21.9)
TC-2429	1.75 (1.42–2.14)	1[b]	12.4 (7.57–20.4)
αCtxMII	6.15 (4.48–8.45)	3210 (2200–4690)	745 (526–1060)
(−)-LOB	6.39 (4.80–8.50)	12.1 (0.96–15.2)	8880 (5660–13660)
A-85380	8.71 (6.51–11.7)	0.09 (0.07–0.11)	54.9 (36.9–81.9)
(−)-NIC	156 (126–193)	0.84 (0.68–1.03)	668 (480–929)
(−)-CYT	176 (135–229)	0.20 (0.17–0.23)	1140 (805–1610)
MLA	200 (160–251)	585 (432–792)	1.39 (1.04–1.85)
ABT-418	803 (617–1040)	4.69 (3.46–6.35)	1970 (1300–2980)
MCC	969 (548–1710)[c]	2.51 (2.00–3.14)	4350 (2750–6880
SIB-1508Y	991 (636–1540)	0.50 (0.39–0.65)	14400 (9540–21600)
GTS-21	1290 (900–1860)	278 (219–353)	596 (391–908)
CAR	3250 (2300–4580)	66.4 (53.6–82.3)	9920 (6380–15400)
DHβE	>10,000[c]	24.6 (16.9–35.8)	7700 (4510–13100)
αBtx	>10,000	>10,000	2.16 (1.56–3.01)

[a] K_d, mean ± S.E.M.
[b] Reported in Bencherif et al., 1998.
[c] Data represent two independent experiments.

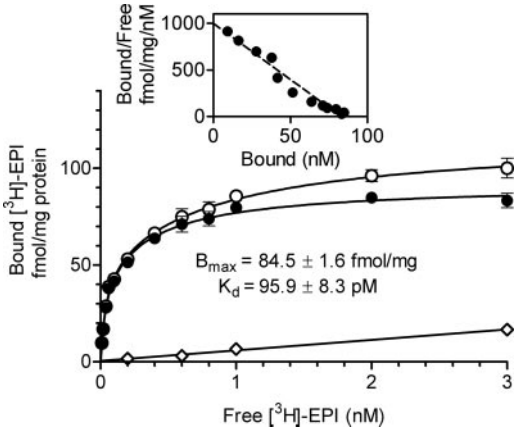

Fig. 2. Saturation [^{3}H]EPI binding to membranes of SH-EP1 cells stably expressing human $\alpha6\beta4\beta3\alpha5$ nAChR. Saturation analysis was conducted using a concentration range of 0.01 to 3.0 nM [^{3}H]EPI. Data expressed as fmol/mg protein are means ± S.E.M. of three independent experiments using cells of passages 34-35. Curves for total (○) and specific binding (●) were generated using nonlinear regression (a one-site model). Linear regression was used to plot nonspecific binding (◇). The inset illustrates Scatchard transformation of the specific binding data.

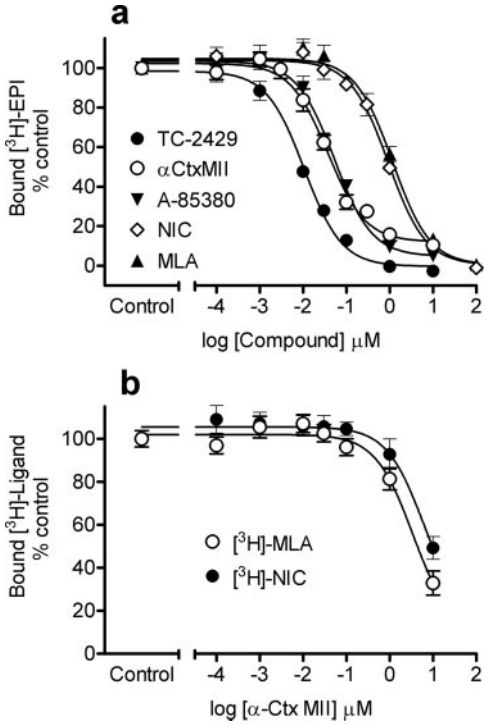

Fig. 3. A, competition of αCtxMII and other nicotinic ligands for [^{3}H]EPI binding to human $\alpha6\beta4\beta3\alpha5$ nAChR. Data expressed as percent of control are means ± S.E.M. of three to five independent experiments. Curves were generated using nonlinear regression (individual data were best fitted to a one-site model) for TC-2429 (●), αCtxMII (○), A-85380 (▼), nicotine (◇), and methyllycaconitine (▲) competing for specific [^{3}H]EPI binding. B, αCtxMII binding to rat $\alpha4\beta2$* or $\alpha7$* nAChR. Data expressed as a percent of control are mean ± S.E.M. of three to four independent experiments for αCtxMII competition toward [^{3}H]MLA binding to $\alpha7$* nAChR (○) or toward [^{3}H]NIC binding to $\alpha4\beta2$* nAChR (●). Curves were generated using nonlinear regression (individual data were best fitted to a one-site model).

composition of $\alpha6$* nAChR was provided from findings when the nigro-striatal pathway in the brain of rats was chemically destroyed using the selective dopaminergic neurotoxin, 6-OHDA. Expression in the substantia nigra of $\alpha6$ subunits and its possible assembly partners ($\alpha4$, $\alpha5$, $\beta2$, $\beta3$, $\beta4$) was quantified eight months later for 6-OHDA-lesioned animals and normalized to levels of message in saline-injected controls. The level of $\alpha6$ expression was decreased in lesioned animals to 19% of controls (Fig. 4a). Whereas mRNA levels for $\alpha5$ subunits were decreased to 44% of controls, $\alpha4$ subunit expression remained high after lesion at 84% of controls. Furthermore, destruction of the nigro-striatal pathway resulted in loss of $\beta3$ subunit expression identical to that for the loss of $\alpha6$ subunit message levels (19% of controls, Fig. 4a), whereas expression of $\beta2$ and $\beta4$ subunits remained unaffected (92 and 107%, respectively) by the lesion. These data suggest that $\beta3$ and $\alpha5$ subunits are likely partners of the $\alpha6$ subunits in nicotinic receptors expressed in dopaminergic cell bodies. Quantitative real-time RT-PCR indicated that $\alpha6$ subunit mRNA expression was 13-fold higher in the human substantia nigra than in the cortex or the rest of the brain (Fig. 4b). These data suggest that nicotinic receptors containing the $\alpha6$ subunit are specifically enriched in this brain region most affected in Parkinson's disease.

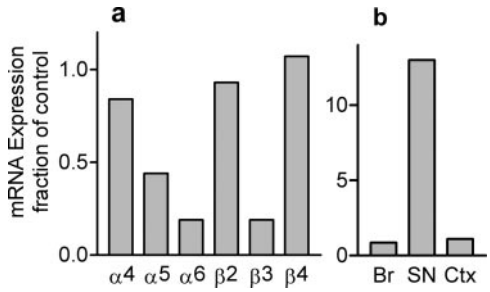

Fig. 4. A, lesion of the nigro-striatal dopaminergic pathway coaffects $\alpha 6$, $\alpha 5$, and $\beta 3$ nAChR subunit gene expression. Rats ($n = 10$) that received microinjections of 8 μg 6-OHDA in the medial forebrain bundle were tested for nAChR subunit gene expression 8 months later. In control animals ($n = 5$), 6-OHDA was substituted with saline. mRNA in pooled nigral samples from each group was measured by real-time RT-PCR (in duplicate). Data shown were normalized as a fraction of saline-injected control. B, $\alpha 6$ nAChR subunit mRNA expression in the human substantia nigra (SN), cortex (Ctx) and total brain (Br). $\alpha 6$ mRNA was analyzed by real-time RT-PCR (in duplicate) in pooled samples of human substantia nigra ($n = 2$) in comparison with the samples of human cortex and total brain. Data shown were normalized with regard to the mean housekeeping mRNA expression levels, as an internal control.

Discussion

Despite a generally high degree of sequence homology across species for individual nAChR subunits, pharmacological differences are apparent for a specific nAChR subtype across species and across host expression systems. This complicates evaluation of nAChR subtype-specific ligands as potential therapeutic agents. Therefore, we have chosen a strategy to generate human nAChR, expressed in mammalian cells, as therapeutically relevant models for study. We focused on a series of SH-EP1 cell lines heterologously expressing the human nAChR $\alpha 6$ subunit in combination with human nAChR $\beta 2$ or $\beta 4$ and with $\beta 3 \pm \alpha 5$ subunits. We have shown that cells expressing $\alpha 6\beta 4\beta 3\alpha 5$ nAChR exhibited significant binding capacity, whereas the expressed $\alpha 6\beta 2$, $\alpha 6\beta 4$, $\alpha 6\beta 2\beta 3$, or $\alpha 6\beta 4\beta 3$ subunit combinations did not. $\alpha 6\beta 4\beta 3\alpha 5$ nAChR displayed high affinity for [^{3}H]EPI and αCtxMII, a selective antagonist for $\alpha 6$* nAChR (Champtiaux et al., 2002). Using standard nicotinic ligands to displace [^{3}H]EPI, we demonstrated that $\alpha 6\beta 4\beta 3\alpha 5$ nAChR exhibits a binding profile that is distinct from that of $\alpha 4\beta 2$* or $\alpha 7$* nAChR. Moreover, findings reported here on concerted 6-OHDA-induced losses in expression of nAChR $\alpha 6$, $\beta 3$, and $\alpha 5$ subunit genes in nigral dopaminergic neurons would be consistent with coassembly of these subunits in native $\alpha 6$* nAChR. Functional studies of expressed $\alpha 6$* nAChR in progress, revealing complexities that have not yet been illuminated, will be published elsewhere.

Until now, there have been no reports on expression of human $\alpha 6$-containing nAChR in mammalian host systems except for expression of human nAChR containing chimeric $\alpha 6/\alpha 4$ and $\beta 4$ nAChR subunits (Evans et al., 2003). Observations that $\alpha 6$ plus $\beta 2$ subunit combinations produced nonfunctional aggregates (Kuryatov et al., 2000; Evans et al., 2003) swayed us from a major effort to express higher order complexes containing those two subunits. Instead, we focused on the key finding that transfection of SH-EP1-h$\alpha 6\beta 4\beta 3$ cells with $\alpha 5$ nAChR subunit cDNA produces an apparently quaternary complex revealing high affinity for nicotinic ligands like EPI and αCtxMII. We found that any of the $\alpha 6$* nAChR not containing $\alpha 5$ subunits, including the $\alpha 6\beta 4$ and $\alpha 6\beta 4\beta 3$ nAChR parents to $\alpha 6\beta 4\beta 3\alpha 5$ nAChR, failed to substantively exhibit ligand binding, although this observation is at variance with results of studies on expression in oocytes, in which $\alpha 6\beta 4$ nAChR or $\alpha 6\beta 4\beta 3$ nAChR exhibited both binding and function (Kuryatov et al., 2000). The requirement for the $\beta 3$ subunit is logical given the overlap in heightened expression of both $\alpha 6$ and $\beta 3$ subunits in dopaminergic pathways (Le Novère et al., 1996; Cui et al., 2003). However, the apparent requirement for both $\beta 3$ and $\alpha 5$ subunits is surprising, in part because both $\beta 3$ and $\alpha 5$ subunits are apparently unable to form functional and ligand-binding, homomeric or simple binary heteromeric nAChR with any other subunits. Either of these subunits can integrate into selected binary complexes containing $\alpha 2/\alpha 3/\alpha 4/\alpha 6$ plus $\beta 2/\beta 4$ subunits to produce ternary or quaternary complexes with distinctive properties (Ramirez-Latorre et al., 1996; Wang et al., 1996; Gerzanich et al., 1998; Groot-Kormelink et al., 2001), but there is no other example of their coparticipation in nAChR formation. Indeed, assuming that neither $\alpha 5$ nor $\beta 3$ subunits form a ligand-binding interface with $\alpha 6$ subunits, and if both indeed are contained in the same assembly, then the only subunits that would qualify for contributing to the binding site are $\alpha 6$ and $\beta 4$, and there would appear to be only one such possible interface in $\alpha 6\beta 4\beta 3\alpha 5$ nAChR. Thus, more work is warranted to assess whether $\alpha 6$ and $\alpha 5$ or $\beta 3$ subunits can form a ligand-binding interface. Another possibility not yet directly addressed is that $\alpha 5$ or $\beta 3$ subunits might not actually be partners in heteropentameric $\alpha 6$* nAChR assemblies but could facilitate assembly of ligand-binding and functional $\alpha 6$* nAChR containing other subunits. Regardless, and consistent with earlier studies, incorporation of the $\alpha 5$ subunit can alter biophysical and pharmacological properties of nAChR when coexpressed with other subunits, and the effect is extreme in that it allows ligand-binding when expressed in cells containing $\alpha 6$, $\beta 4$, and $\beta 3$ subunits.

The density of $\alpha 6\beta 4\beta 3\alpha 5$ nAChR observed in our study (measured as specific radioligand binding) is about 20 to 150 times lower than the expression of human $\alpha 4\beta 2$ nAChR (between 1.6 and 14 pmol/mg of membrane protein) in the same SH-EP1 cell host (Pacheco et al., 2001; Eaton et al., 2003). Expression of $\alpha 6\beta 2$, $\alpha 6\beta 4$, $\alpha 6\beta 2\beta 3$, or $\alpha 6\beta 4\beta 3$ nAChR was below limits of reliable detection despite the fact that expression of mRNA for the transfected subunits was confirmed by RT-PCR. Presently, we do not know why SH-EP1 cells lack the capacity to assemble the latter sets of subunits into binding sites, but lower efficiency of expression of $\alpha 6\beta 4\beta 3\alpha 5$ nAChR compared with $\alpha 4\beta 2$ nAChR is not entirely unexpected.

The assembly partners for nAChR $\alpha 6$ subunits have been under investigation in several other studies. Previous attempts to combine $\alpha 6$ and $\beta 2$ in cell-based expression systems have failed to produce functional receptors (Kuryatov et al., 2000; Evans et al., 2003) even though $\alpha 6$ and $\beta 2$ subunits clearly are possible assembly partners in DA neurons. However, functional $\alpha 6\beta 4$ nAChR and $\alpha 6\beta 4\beta 3$ nAChR have previously been obtained in *Xenopus* oocytes (Gerzanich et al., 1997; Kuryatov et al., 2000). In the chick visual system, $\alpha 6$ mRNA is found in retinal ganglion cells along with $\alpha 3$, $\beta 2$, $\beta 3$, and $\beta 4$ mRNA (Hernandez et al., 1995; Fucile et al., 1998). A prominent subunit combination is thought to be $\alpha 6\beta 4\beta 3$, based on immunopurification studies showing that $\alpha 6$ sub-

unit protein from chick retina assembles with β4. About half of the α6-containing nAChR in retina also contains β3 and/or α3, whereas less than 10% contain β2 and none contain α4 or α5 subunits (Vailati et al., 1999). The presence of the α6β4β3 subunit combination in chick retina was confirmed in a later study (Vailati et al., 2000). Chick α6β4 nAChR has also been heterologously expressed in human BOSC 23 cells (Barabino et al., 2001). Thus, the bulk of evidence suggested to us that expression of human nAChR α6 and β3 subunits in combination with β4 instead of β2 subunit would most probably generate physiologically-relevant α6* nAChR, although a requirement for α5 subunits as we demonstrate here was not carefully addressed in previous studies.

The chick α6β4 nAChR heterologously expressed in BOSC 23 cells (Barabino et al., 2001) exhibits a pharmacological profile similar to that of human α6β3β4α5 nAChR as reported here for some of the ligands, such as αCtxMII, NIC, MLA, CAR, and DHβE. There are notable variations, however, such as CYT and EPI, which exhibited 25- to 30-fold lower affinities for human α6β4β3α5 nAChR than for chick α6β4 nAChR (Barabino et al., 2001). Interestingly, αCtxMII was found to have lower affinity for human α6β3β4α5 nAChR than did EPI or TC-2429, which is a novel nicotinic ligand previously described by our laboratory (Fig. 3a and Table 1; Bencherif et al., 1998). TC-2429 is a potent partial agonist at nAChR mediating DA release from rat striatal synaptosomes (EC_{50} = 2 ± 1 nM; E_{max} = 40% of NIC- or EPI-evoked responses) although it lacks activity as an agonist at α4β2 nAChR, where instead it acts as a competitive antagonist. Those data suggested that some or all of the DA release mediated by TC-2429 may reflect interaction with α6* nAChR. Moreover, the observed rank potency for a broad spectrum of nicotinic ligands in competition with [^{3}H]EPI at α6β4β3α5 nAChR revealed a profile distinctly different from that of α4β2* or α7*nAChR and clearly characteristic of α6* nAChR.

Lesioning studies presented in this report support coexpression of α6, β3, and α5 subunits in rat DA neurons (Charpantier et al., 1998; Elliott et al., 1998; Zoli et al., 2002; Champtiaux et al., 2003). Diverse lines of investigation suggest that rodent DA neurons in the substantia nigra and ventral tegmentum express a mixture of α6β2(±α4)(±β3) nAChR and α4β2 nAChR on terminals and a predominance of α4β2* nAChR over α6* nAChR somatodendritically (Reuben et al., 2000; Zoli et al., 2002; Champtiaux et al., 2003, Salminen et al., 2004). Although immunoprecipitation studies suggested that a small fraction of α6* nAChR contained α5 (12%) and/or β3 (8%) subunits, there were very large reductions in the expression of these subunits in radioligand binding complexes upon DA neuronal lesioning or in α6−/− mice (Le Novère et al., 1996; Champtiaux et al., 2003). However, β4 mRNA is not abundant in rodent dopaminergic cells (Le Novère et al., 1996), and studies on β2-null mutant mice have demonstrated that functional heteromeric nAChR (containing α4 and/or α6 subunits) expressed by dopaminergic neurons contain predominantly the β2 subunit (Grady et al., 2001). Thus, the bulk of natural expression evidence points to involvement of β2 subunits in formation of rodent α6* nAChR.

However, results from our studies and others indicate that heterologously expressed α6β2 or α6β2β3 nAChR fail to properly assemble (Gerzanich et al., 1997; Kuryatov et al., 2000; Evans et al., 2003), making uncertain roles of β2 subunit as a mandatory assembly partner for human α6 subunits. Moreover, the greater relative expression of β4 nAChR subunits in primate DA neurons (Quik et al., 2000) suggests that α6* nAChR in primates may have a different subunit composition from that in rodents. Perhaps some human α6 nAChR, as heterologously expressed in SH-EP1 cells, contain β4, β3, and α5 subunits, whereas their rodent "counterpart" utilizes the β2 instead of the β4 subunit and do not require the α5 subunit for binding and function.

The results from our studies suggest possible differences in the phenotype of α6* nAChR expressed across species. The relative high abundance of α6 mRNA in the substantia nigra from human compared with other brain regions is consistent with a role for α6* nAChR in motoric function observed in animal studies. Whereas it remains to be established what roles in general the α5 subunit plays in human nAChR, the current studies represent initial steps in exploring α6* nAChR in human health and disease.

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